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Experimental evidence for selection against hybrids between two interfertile red oak species

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Abstract

Reproductive isolation between related oak species within one taxonomic section is incomplete. Even though pre- and postzygotic isolation mechanisms have been described for interfertile oak species, natural hybridization is common in contact zones between related oaks. The apparent restriction of interspecific hybrids between ecologically divergent species to intermediate environments in contact zones suggests postzygotic isolation via environmental selection against hybrids in parental environments. Overrepresentation of hybrids in seeds as compared to adult trees provides additional indirect evidence for selection against hybrids.

Here, we used genetic assignment analyses in progeny obtained from a sympatric stand of *Quercus rubra* and *Quercus ellipsoidalis*, two interfertile species with different adaptations to drought, to characterize the number of hybrids and "pure" species in the non-germinated acorns and in seedlings. The occurrence of 43.6 % F_1 hybrids and introgressive forms among the non-germinated acorns and their very low frequency in the seedlings (9.3 %) is to our knowledge the first direct evidence for early selection against hybrids in oaks possibly as result of genetic incompatibilities. Additionally, we found a signature of positive selection on EST-SSR PIE200 in *Q. rubra* which needs further confirmation. These results contribute to our understanding of reproductive isolation and divergence between interfertile oak species with different ecological adaptations.

Keywords: *oaks, Quercus rubra, Q. ellipsoidalis, reproductive isolation, microsatellites, EST-SSRs*

Introduction

Controlled crosses between oak species of the same taxonomic section revealed cross-fertility for many species pairs, but also partial incompatibility for some crosses (Cottam et al., 1982; Abadie et al., 2012). Postzygotic barriers were found to be less pronounced than prezygotic barriers when pre- and postzygotic isolation traits (e.g. pollen tube growth and seed production) were compared in intra- and interspecific crosses between two interfertile European white oaks, *Quercus petraea* (Matt.) Liebl. and *Quercus robur* L. (Abadie et al., 2012). Despite mechanisms of reproductive isolation, natural hybridization between closely related oak species is common in natural populations where interfertile oaks come into close contact (e.g., Curtu et al., 2007; Owusu et al., 2015). While prezygotic isolation was observed in interspecific experimental crosses, for example between *Q. petraea* and *Q. robur* (Steinhoff, 1993; Kleinschmit and Kleinschmit, 2000; Abadie et al., 2012; Lepais et al., 2013), incompatibility was comparatively low between F_1 hybrids and either of the two parental species (Olrik and Kjaer, 2007). Similar results were found for seeds from F_1 hybrids between two Californian white oaks, *Quercus lobata* Née and *Quercus douglasii* Hook. & Arn., which were fathered by both parental species (Abraham et al., 2011). Also, selection against hybrids has been proposed as a potential postzygotic isolation mechanism to explain the maintenance of species integrity in oaks despite interspecific gene flow (Lexer et al., 2006). Indeed, hybrids in natural populations are not randomly distributed but are restricted to intermediate environments suggesting postzygotic environmental selection against hybrids in parental environments (Curtu et al., 2007; De Heredia et al., 2009;

Owusu et al., 2015; Khodwekar and Gailing, 2017). Also, gene flow analyses suggested an overrepresentation of hybrids in the seeds as compared to the adult tree generation in agreement with the hypothesis of selection against hybrids from the seed to the adult tree stage (Curtu et al., 2007; Curtu et al., 2009). Finally, a comparison of intra- and interspecific crosses in *Q. petraea* and *Q. robur* showed better performance of progeny from intraspecific (natural) crosses for some but not for all viability traits (Abadie et al., 2012). Lower germination rate in interspecific than in open pollinated (intraspecific) progeny suggested early postzygotic selection potentially as result of genetic incompatibilities (Abadie et al., 2012). However, direct evidence for selection against hybrids and introgressive forms, which are derived from backcrosses of F_1 hybrids with one of the parental species, has not been demonstrated experimentally in the same random sample.

Quercus rubra L. and *Q. ellipsoidalis* E. J. Hill are two interfertile red oak species (section *Lobatae*) with different adaptations to drought. *Quercus rubra* is a mesophytic species and prefers richer soils with a higher organic matter content while *Q. ellipsoidalis* occurs on very dry sandy outwash plains (Abrams, 1990; Sander, 1990; Abrams, 1992). The species are stratified along a soil water gradient and hybrids and introgressive forms are found in the contact zones between both species on intermediate soil types (Lind-Riehl et al., 2014; Khodwekar and Gailing, 2017). Genetic assignment and gene flow analyses revealed historic and contemporary gene flow between both species where they grow in close proximity (Lind and Gailing, 2013; Lind-Riehl et al., 2014; Zhang et al., 2015; Khodwekar and Gailing, 2017).

To test the hypothesis of early selection against hybrids, we collected seeds in a sympatric stand on the Upper Peninsula of Michigan at the northern distribution limit of both species. Seeds were planted in a greenhouse and the frequency of hybrids and introgressive forms was estimated in non-germinated acorns as well as in seedlings using genetic assignment analysis at microsatellite markers.

Materials and Methods

Plant Material
A total of 677 mature acorns were collected in fall of 2014 from 12 seed trees in a sympatric *Q. rubra* / *Q. ellipsoidalis* mixed stand in the Baraga Plains region (46°67'67.46"N, 88°53'49.41"W) on the Upper Peninsula of Michigan. In this mixed stand, both morphological species occur next to each other on Rubicon sand in about equal proportions in association with *Pinus banksiana* Lamb. and *Acer rubrum* L. (Khodwekar and Gailing, 2017). Leaf morphological differences between genetically assigned species were less pronounced and phenotypic intermediate individuals were more frequent in this sympatric stand than in neighbouring "pure" *Q. rubra* and *Q. ellipsoidalis* stands (Gailing et al., 2018). Seeds were collected from a group of adjacent seed-bearing trees with intermediate

phenotypes. Genetic assignment analyses of the seed trees and the single tree progenies, including adult *Q. rubra* and *Q. ellipsoidalis* trees from neighbouring "pure" stands as reference (Lind and Gailing, 2013), suggested the presence of both species and hybrids/introgressive forms among the seed parents (see below, Supplementary Table 1). Seeds were kept at 4°C in a refrigerator for about 4 months and then at the end of February, 2015 planted and grown in 0.5 gallon pots (ca. 1.89 liter) filled with Sunshine 1 mix potting soil (Sun Grow Horticulture, Canada, 70-80 % Canadian Sphagnum peat moss, perlite, dolomite limestone, Gypsum and wetting agent) in a greenhouse at 20°C. In June 2015 non-germinated acorns and leaves sampled from young seedlings were collected for DNA isolations.

DNA isolation
Genomic DNA was isolated from leaves (ca. 1cm^2) using the DNeasy96 Plant Kit (Qiagen, Hilden, Germany) and DNA from embryos was isolated with the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA quality and quantity was checked using 0.8 % agarose gels stained with GelRed Nucleic Acid stain (Phenix Research Products, Candler, USA). The DNAl-1000-100 ladder was used as size standard (Life Technologies, Carlsbad, California, USA).

Marker analyses
Out of 23 EST-SSRs adapted for use in *Q. rubra* and *Q. ellipsoidalis* populations (Khodwekar and Gailing, 2017), 11 EST-SSRs were selected for further analyses based on reliable amplification in both leaf and embryo samples (Table 1). PCRs were performed in a 15 µl mix with 3 µl HOT FIREPol Mastermix (Solis BioDyne, Tartu, Estonia) which contains 10 mM of $MgCl_2$, 0.6 units of HOTFIREpol Taq polymerase, and 2 mM of each dNTP, 2 µl of dye labelled forward primer (5 µM), 2 µl reverse primer (5 µM), 6 µl deionized water and 2 µl DNA (ca. 2 ng). PCRs were run in the Peltier Thermal Cycler GeneAmp PCR system 2700. The PCR profile consisted of a denaturation at 94°C for 15 min and 35 cycles at 94°C for 45 sec, at T_a (Table 1) for 45 sec, at 72°C for 45 sec and a final extension at 72°C for 20 min. Fragments were separated on a ABI Prism Genetic Analyzer 3730 (Applied Biosystems) together with the internal size standard Gene-ScanTM LIZ-500 (Applied Biosystems). Fragment sizes and genotypes were determined after careful visual inspection with the program GeneMarker V2.63 (SoftGenetics, State College, Pennsylvannia, USA).

Table 1
Microsatellite marker information

Locus	Repeat motif	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	T_a (C°)	Size range (in base pairs)	Linkage group	Functional annotation
FIR013	$(CAG)_5$	6-FAM-CGGGGAGGTTGATGAGTATT	AACACTGTCACCCCCATAGC	56	133-144	2	constans-1
FIR030	$(AG)_7$	6-FAM-GGACATATTTATCTAGGAGACGAGGT	ATGTCCCATAGCACAGAGCA	57	157-183	7	nadh dehydrogenase
FIR035	$(AT)_k$	NED-GCTAAGGTTCCGTGTTCCAA	GGCCAGCAACTAAACCAAGA	56	146-152	5	chaperone protein dnaj
FIR053	$(GTG)_7$	NED-AGTTTCCCCACATTTGTTGC	TACCATGCACCAAGCAATTC	59	136-150	5	glutaredoxin c9
FIR104	$(GGT)_7$	VIC-TTAACTCGGTTTGCGACTCA	AGCACGTGACTCGACCTGTA	59	203-224	11	r2r3-myb transcription factor
PIE040	$(TTC)_k$	NED-GTGAGAGAGAGAGAGACAAAGAAGAAAAA	AAATTCTCCGCCACATTGAG	59	155-174	11	basic leucine zipper transcription factor-like protein
PIE125	$(GGAAGC)_3$	PET-AATACAAATCGCAGGAGGTG	CTAACCCATCGTTCATGGAG	57	146-162	6	unknown
PIE200	$(CAA)_5$	6-FAM-ACACATGTGCCAAAACTGC	TCGATGATGTGGTTGTTGATG	56	107-119	not assigned	zinc finger a20 and an1 domain-containing stress-associated protein 5-like
VIT057	$(AACTCG)_5$	VIC-TCAGCAAAATCCCAACTTTGT	ACACTTCGCTGTTCCTCGAT	57	128-153	9	ap2 erf domain-containing transcription factor
VIT081	$(CAT)_8$	AATTCAAACCCAGCCAACTG	TCCTCTGGATGCTCCATCA	56	108-112	not assigned	proline-rich protein
VIT107	$(TA)_{13}$	NED-TGATCACAGATTGGAGCTTAACA	CCCCCACTTAGGAAAGAAGC	59	124-142	3	light-harvesting complex i protein lhca2

EST-SSRs were originally developed in *Quercus robur* (Durand et al., 2010) and adapted for use in *Querucs rubra* and *Q. ellipsoidalis* (Lind-Riehl et al., 2014)

analyses. No obvious signs of infection were visible on the non-germinating seeds. Only 11 of the 23 markers tested, generated reproducible and easy-to-interpret allelic patterns in acorn samples which were further used for the comparison of genetic structures in seedlings and non-germinated acorns.

Table 2
Germination rate of *Q. rubra*, *Q. ellipsoidalis* and hybrids as determined by genetic assignment analysis of non-germinated acorns and seedlings

	Q. rubra	Q. ellipsoidalis	Q. rubra-introgressive	Q. ellipsoidalis-introgressive	F1-hybrid	overall
# Seedlings	24	73	0	9	1	107
# Non-germinated acorns	49	8	21	14	9	101
# Total	73	81	21	23	10	208
Germination rate (%)	32.9	90.1	0	39.1	10.0	51.4

Data analyses

The genetic variation parameters number of alleles per locus (N_a), observed heterozygosity (H_o), expected heterozygosity (H_e) and the inbreeding coefficient (F_{IS}) were determined in GenAlEx v. 6.502 (Peakall and Smouse, 2012). Genetic assignment analyses were conducted with the Bayesian approach implemented in the program STRUCTURE (Pritchard et al., 2000) to assign acorn and seedling samples to species and putative hybrids. For this purpose, selected seed parents were chosen as reference samples. Genetic assignment analyses were also conducted for the 12 seed parents together with 76 *Q. rubra* and 47 *Q. ellipsoidalis* adult trees from neighbouring stands of the same region as reference (Lind and Gailing, 2013). The admixture model with correlated allele frequencies and no prior species information was used. Five independent runs with a burn-in period of 30,000 iterations followed by 10^6 iterations for each value of K (K = 1 to 4) were applied to determine the value of K. According to Lind and Gailing (2013) hybrids and introgressive forms were defined as having a proportion of ancestry < 0.9 in one of the two species (between 0.4 and 0.6 for hybrids, between 0.61 and 0.89 in one species for introgressive forms). Species were defined as having a proportion of ancestry of ≥ 0.9 in one of the two species.

Survival was scored until May 6, 2016 and the frequency of species, hybrids and introgressive forms among surviving seedlings was calculated for different time points and compared between seedlings and non-germinated acorns.

Genetic variation in non-germinated acorns ($H_o = 0.465$, $H_e = 0.464$) and in seedlings ($H_o = 0.440$, $H_e = 0.467$) was very similar (Supplementary Table 2). However, the level of genetic variation at PIE200 in seedlings ($H_e = 0.067$, $H_o = 0.066$) and acorns ($H_e = 0.547$, $H_o = 0.488$) showed pronounced differences. Also, after genetic assignment (see below) *Q. rubra* seedlings ($H_o = 0.100$, $H_e = 0.096$) showed a much lower variation than non-germinated *Q. rubra* acorns ($H_o = 0.636$, $H_e = 0.538$) at PIE200 (Table 3). As result, genetic differentiation between *Q. rubra* seedling and acorn cohorts is high at PIE200 ($F_{ST} = 0.128$) as compared to the mean differentiation across markers ($F_{ST} = 0.028$). The variation in *Q. rubra* seedlings at PIE200 ($H_o = 0.100$, $H_e = 0.096$) is somewhat higher than the variation observed in *Q. ellipsoidalis* seedlings ($H_o = 0.058$, $H_e = 0.057$) and very similar as in non-germinated acorns of *Q. ellipsoidalis* ($H_o = 0.111$, $H_e = 0.105$) (Supplementary Table 3). The low genetic variation at PIE200 in *Q. rubra* seedlings as compared to non-germinated *Q. rubra* acorns from the same sample could be a signature of positive selection and merits further analysis. PIE200 was annotated as A20/AN1-zinc-finger domain –containing-stress-associated protein 5-like (Lind-Riehl et al., 2014). The members of the A20/AN1-zinc-finger domain –containing protein family are involved in abiotic stress responses in other species (Kang et al., 2011; Paul and Kumar, 2015).

Results and Discussion

The germination rate was comparatively low, only 107 out of 675 acorns (15.9 %) germinated (Table 2). For 101 of the non-germinated acorns and for all 107 seedlings, DNA of sufficient quality and quantity could be isolated for further DNA marker

Table 3
Genetic variation of genetically assigned *Q. rubra* samples

	Locus	N	N_a	H_o	H_e	F
Q. rubra seedlings	FIR013	25	1	0.000	0.000	-
	FIR030	25	5	0.480	0.451	-0.064
	FIR035	25	2	0.480	0.493	0.026
	FIR053	25	5	0.680	0.606	-0.123
	FIR104	25	3	0.120	0.114	-0.049
	PIE040	25	4	0.560	0.582	0.037
	PIE125	24	4	0.833	0.719	-0.159
	PIE200	20	3	0.100	0.096	-0.039
	VIT057	24	3	0.500	0.424	-0.178
	VIT081	25	2	0.080	0.077	-0.042
	VIT107	24	4	0.708	0.553	-0.281
	Mean	24.3	3.3	0.413	0.374	-0.087
Q. rubra acorns	FIR013	48	2	0.021	0.021	-0.011
	FIR030	39	6	0.462	0.526	0.123
	FIR035	48	2	0.396	0.364	-0.086
	FIR053	45	4	0.756	0.628	-0.203
	FIR104	45	4	0.222	0.204	-0.087
	PIE040	37	4	0.541	0.627	0.138
	PIE125	42	4	0.881	0.719	-0.225
	PIE200	44	4	0.636	0.538	-0.182
	VIT057	44	3	0.432	0.514	0.159
	VIT081	48	2	0.042	0.041	-0.021
	VIT107	36	4	0.639	0.601	-0.064
	Mean	43.3	3.5	0.457	0.435	-0.042

$F = 1 - H_o/H_e$

Proportion of ancestry of the seed parents (Supplementary Figure 2) and their offspring was strongly correlated (r = 0.81, P >0.001). Genetic assignment analyses also revealed a much lower germination rate in *Q. rubra* than in *Q. ellipsoidalis* while the seedling mortality was higher in *Q. ellipsoidalis* (7 out of 73) than in *Q. rubra* (1 out of 24) (Figure 1, Table 2). A higher seedling mortality and slower growth of *Q. ellipsoidalis* seedlings was also found in a *Q. rubra* / *Q. ellipsoidalis* common garden seedling trial (Gailing, 2013). However, a lower germination rate of *Q. rubra* as compared to *Q. ellipsoidalis* acorns was not found in earlier studies (pers. observation; Gailing, 2013) and needs further validation under controlled growth chamber conditions. In the genetic admixture analysis, 73 out of the 107 seedlings were genetically assigned to *Q. ellipsoidalis*, 9 to *Q. ellipsoidalis* introgressive forms and only 24 to *Q. rubra*. Out of the 101 non-germinated acorns the majority was assigned to *Q. rubra* or *Q. rubra* introgressive forms (49 + 21= 70) and only 8 and 14 acorns were assigned to *Q. ellipsoidalis* and *Q. ellipsoidalis* introgressive forms, respectively (Figure 1, Table 2). The very low frequency of hybrids and introgressive forms in the seedling cohort (10 out of 107), but the assignment of nearly half (44 out of 101) of the acorns to F[1] hybrids (n = 9) or introgressive forms (14 *Q. rubra* introgressive forms, 21 *Q. ellipsoidalis* introgressive forms) is a strong indication of early selection against hybrids and introgressive forms. Specifically, only one F[1] hybrid and no *Q. rubra* introgressive form were found in the seedling cohort. This early selection may be the result of

genetic incompatibilities (intrinsic barriers) rather than of environmental selection (extrinsic barriers) (Rieseberg and Willis, 2007).
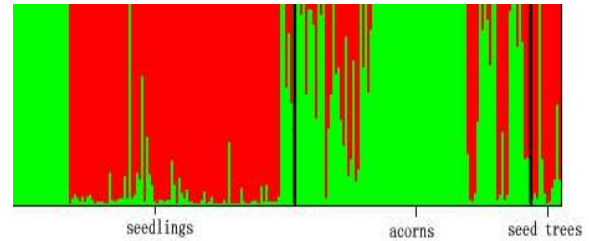

Figure 1
Genetic assignment analysis of seedlings and non-germinated acorns in STRUCTURE. Green: *Q. rubra*. Red: *Q. ellipsoidalis.*

These results are in accordance with a low number of F[1] hybrids and introgressive forms in the sympatric adult population from which the acorns were collected (4.2 % F[1] hybrids, 5.2 % *Q. rubra* introgressive forms, 7.3 % *Q. ellipsoidalis* introgressive forms) using the same program settings and definitions of hybrids/introgressive forms (Khodwekar and Gailing, 2017).

However, the frequency of F[1] hybrids and introgressive forms varies among regions where both species grow in close proximity on different contrasting soil types (Lind-Riehl et al., 2014; Lind-Riehl and Gailing, 2017). For example, in some regions the frequency of F[1] hybrids among adult trees was as high as 10 % and reached 23 % for introgressive forms (Lind-Riehl and Gailing, 2017) suggesting less pronounced early postzygotic selection and possibly a dependency of selection at later seedling stages on soil and other environmental factors. Also, the restriction of oak hybrids and introgressive forms in sympatric stands to contact zones between species in different microenvironments for both American red oak (Owusu et al., 2015; Khodwekar and Gailing, 2017) and European white oak species (Curtu et al., 2007; De Heredia et al., 2009) suggests environment-driven selection against hybrids in parental environments. In line with these results Dodd and Afzal-Rafii (2004) found that the proportion of hybrids and introgressive forms in Californian red oaks was correlated with climate variables and to a lesser degree with geographic distance. Other indirect evidence for postzygotic selection against hybrids originated from comparisons of genetic assignment analyses of adult trees and paternity analyses in single tree pedigree of the same sympatric stands. Thus, the proportion of hybrids and introgressive forms in a sympatric *Q. petraea*, *Q. robur*, *Quercus pubescens* Willd. and *Quercus frainetto* Ten. European white oak stand was considerably lower in the adult tree generation (20.1 %) (Curtu et al., 2007) than in the seeds as inferred from paternity analyses (35.9 %) (Curtu et al., 2009).

Conclusions

To our knowledge, this is the first study in oaks providing direct evidence for early selection against hybrids by comparing their frequency in seedlings and non-germinated acorns from the same random sample. Also, the pronounced genetic differentiation between seedlings and non-germinated acorns of *Q. rubra* and the very low variation in the seedlings at one marker (PIE200) suggests positive selection which merits further analyses. Finally, reciprocal transplant experiments of species and hybrids in sympatric stands with a higher number of hybrids are needed to test for environmental selection against hybrids at later seedling stages.

Acknowledgements

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