First evidence for bidirectional gene flow between the co-occurring red oak species *Quercus imbricaria* Michx. and *Quercus coccinea* Münchh.

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Abstract

Oak species are generally interfertile within taxonomic sections, even if species' distribution ranges are not overlapping. Despite interspecific gene flow in natural populations, oak species form genetically distinct clusters and maintain ecological adaptations. However, especially in contact zones between species and in sympatric stands, hybrids and introgressive forms are comparatively frequent. Here, we describe for the first time hybrid forms between the related red oaks *Quercus imbricaria* Michx. and *Quercus coccinea* Münchh. based on leaf morphological assessment and genetic assignment analyses corroborating bidirectional gene flow between both species. Additionally, we observed introgression for 20 % of the individuals identified by morphologic traits as 'pure taxa' *Q. imbricaria, Q. coccinea, Quercus rubra* L. and *Quercus velutina* Lam. One individual, morphologically identified as intermediate between *Q. imbricaria* and *Q. rubra*, was genetically assigned to *Q. imbricaria* possibly as result of trait introgression by repeated backcrossing of a *Q. imbricaria* x *Q. rubra* hybrid with *Q. imbricaria*. Our results confirm frequent introgression between red oak species in sympatric stands with the potential to increase the genetic variation and adaptive potential within species.

Keywords: hybridization, introgression, oak, microsatellites, genetics

Introduction

Hybridization and introgression are considered major forces of evolution through the transfer of potentially adaptive alleles (Arnold, 2004; Arnold, 2016) increasing the adaptive potential to cope with rapid environmental changes (Brauer et al., 2023; Leroy et al., 2020). On the other hand, hybridization between genetically distant taxa can also result in reduced fitness in the offspring (Whitlock et al., 2013). Species boundaries in oaks including North American red oaks (section/group *Lobatae*, Denk et al., 2017) are morphologically and genetically not clear-cut as result of interspecific gene flow and high phenotypic variation (Gailing et al., 2012; Hipp & Weber, 2008; Khodwekar & Gailing, 2017; Lazic et al., 2021). Species within section *Lobatae* are distinguished based on multiple traits, such as bark, terminal buds, leaf characteristics (e.g., petioles, blade shape, vestiture), and acorns (e.g. cupule morphology) (Jensen, 1997; Hipp & Weber, 2008; Gailing et al., 2012). However, even in the face of interspecific gene flow, oak species generally form genetically distinct clusters with specific ecological adaptations (Curtu et al., 2007; Hipp et al., 2020). Species identity is maintained by different pre- and post-zygotic isolation mechanisms (Gailing & Zhang, 2018; Khodwekar & Gailing, 2017; Lepais et al., 2009). Still, hybrids and introgressive forms are frequently observed in the contact zones between species in intermediate environments (Jensen et al., 2009; Khodwekar & Gailing, 2017). Thus, about 25 % of hybrids and introgressive forms (F_2 and later generation hybrids) were found based on genetic assignment analysis at nuclear microsatellite and genome-wide amplified fragment length polymorphism (AFLP) markers in the contact zones between the two closely related red oak species *Q. velutina* Lam. and *Quercus ellipsoidalis* E.J. Hill in the Great Lakes region of the United States (Sullivan et al., 2016). Likewise, a high rate of hybrids and introgressed individuals was revealed by morphological traits and nuclear SSR markers in a mixed oak stand in contact zones of various taxa (section *Quercus*) at the xeric limit of temperate continental forests (Pintér et al 2025).

In the present study, we used a similar set of species-discriminating nuclear microsatellite markers (Sullivan et al. 2016) to distinguish between the co-occurring red oak species

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Quercus imbricaria Michx., *Quercus coccinea* Münchh., *Quercus rubra* L. and *Q. velutina* and to assess interspecific hybridization based on genetic assignment analysis. Specifically, we applied informative nuclear microsatellite markers to corroborate hybrid forms between *Q. imbricaria* and *Q. coccinea* that were detected for the first time based on leaf morphological assessments (Brandenburg, submitted). These putative hybrid forms reveal remarkable leaf morphological variation ranging from elliptic, non-dissected leaves characteristic for *Q. imbricaria* to serrated/dissected leaves typical for *Q. coccinea*. While *Q. rubra, Q. coccinea* and *Q. velutina* are very closely related based on genome-wide genetic variation (Hipp et al., 2020) and known to hybridize (Owusu et al., 2015; Sullivan et al., 2016), *Q. imbricaria* groups in a related sister clade (Hipp et al., 2020) and no hybrids between *Q. imbricaria* and *Q. coccinea* have yet been described. Based on previous studies we hypothesized that within a contact zone (sympatric population) of various red oak taxa hybrids and introgressed individuals (morphologically intermediate trees) are co-occurring with trees representing the parent taxa ('pure species'). The intermediate forms, classified by morphologic traits, were supposed to be genetically admixed of *Q. imbricaria* and *Q. coccinea*. To test our hypotheses we applied genetic assignment analyses using species-discriminating microsatellites markers identified in an earlier study (Sullivan et al. 2016).

Material and Methods

Plant material

Leaf material was collected from two putative *Q. imbricaria* x *Q. coccinea* hybrids (West Hybrid, WH; East Hybrid, EH) in oak-hickory woods dominated by *Q. coccinea* and *Q. velutina* and from a putative *Q. rubra* x *Q. imbricaria* hydrid (Putative Hybrid, PH) on a lower-mid slope along the SW edge of mature oak-hickory woods dominated by *Q. coccinea* and *Q. velutina* with *Q. rubra* present, though rather uncommon in the general area (Figure 1). As reference, five samples from each *Q. coccinea, Q. imbricaria, Q. rubra* and *Q. velutina* were sampled from the same location close to the village Oak Hill (Jackson County, Ohio) at an altitude of 260 m above sea level (a.s.l.) (Table 1, Figure 2, 3). A detailed description on the location and characteristics of each sample is given in Appendix 1. Herbarium accession numbers can be found in Appendix 2. Samples were dried in silica gel (Roth, Karlsruhe, Germany) and shipped to the University of Göttingen, Germany, for genetic analyses. Identification of species and putative hybrids was based on leaf, acorn, twig and bud characteristics (Hipp & Weber, 2008). Illustrations and ample morphological descriptions of the prospective parents of the hybrid forms, i.e., *Quercus coccinea, Q. imbricaria, Q. rubra*, and *Q. velutina* are given in Stein et al. (2003). Some of the leaves on the hybrid trees at the Ohio study site were nearly unlobed and with entire margins, indicating clearly that *Q. imbricaria* was one of the parents. Well known are several hybrids of this species, notably *Q.* × *leana* (*Q. imbricaria* × *Q. velutina*) and *Q.* × *runcinata* (*Q. imbricaria* × *Q. rubra*). Descriptions of these known hybrids with relationships-both to their parents and to one another-are found in Tucker and Ebinger (2001) and in Wilhelm and Rericha (2017). In comparison to these named hybrids, the newly discovered Ohio hybrid trees under investigation presented more slender twigs; and with late-season leaves that were proportionately smaller and largely glabrous on the abaxial surface. This made *Q. coccinea* a plausible choice for the other parent, confirmation of which might be provided by molecular analysis.

DNA extraction and genotyping

DNA was extracted from dried leaf material (about 1 cm^2 per sample) ground to fine powder with a Retsch mill (Retsch, Haan, Germany) using the DNeasy 96 Plant Kit (Qiagen, Hilden, Germany). DNA was diluted 1:10 before PCR.

Genotyping was performed at seven random genomic simple sequence repeats (SSRs) developed for *Q. rubra* (Aldrich et al., 2002; Sullivan et al., 2013) and adapted to other red oaks such as *Q. coccinea* and *Q. velutina* (Owusu et al., 2015; Sullivan et al., 2016), and at 15 gene-based EST-SSRs originally developed for *Q. petraea* (Durand et al., 2010) and adapted to red oaks (section *Lobatae*) (Lind-Riehl et al., 2014; Sullivan et al., 2013; Sullivan et al., 2016) (Table 2).

Microsatellites were amplified in four multiplexes following (Burger & Gailing, 2022) (Appendix 3). PCR reactions were run with dye labelled forward primers (6-FAM, ROX, NED, and HEX) in a Biometra TOne Thermocycler (Analytik Jena, Jena, Germany) with the following program: initial denaturation at 95°C for 15 min followed by 10 touch down cycles (94°C for 1 min, 60°C minus 1°C per cycle for 1 min, 72°C for 1 min), 25 cycles at 94°C for 1 min, 50°C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 20 min. PCR products were separated on an ABI 3130xl using GeneScan500Liz as internal size standard (Applied Biosystems / Hitachi, ThermoFisher Scientific, Waltham Massachusetts, USA).

Data analysis

The admixture model with correlated allele frequencies was used in STRUCTURE v. 2.3.4 (Pritchard et al., 2000) to identify species, putative hybrids and introgressive forms. We performed 10 independent runs for K =1 to 7 with a burn-in length of 50,000 and a run length of 100,000 iterations for each value of K. The most likely number of K was determined with StructureSelector (Li & Liu, 2018) and the summation and graphical representation of the STRUCTURE results was conducted with CLUMPAK (Kopelman et al., 2015). Individuals with proportions of ancestry (Q-values) in one cluster of ≥ 0.90 are considered as pure species. Individuals are defined as hybrids and introgressive forms if they have proportions of ancestry between 0.11 and 0.89 (Lind & Gailing, 2013). First generation hybrids are defined as having a proportion of ancestry of 0.4 to 0.6 in one of two clusters (Lind & Gailing, 2013).

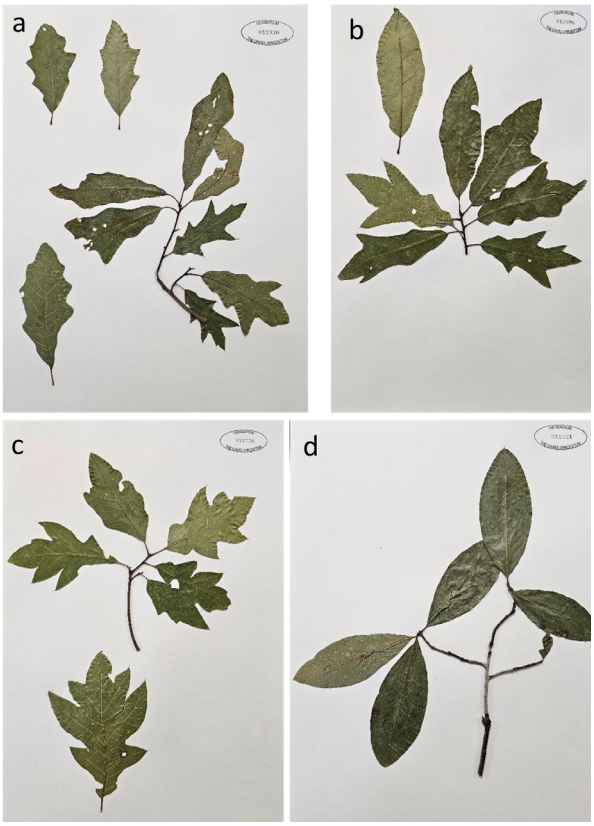

Figure 1. Leaf samples of morphotypes *Q. imbricaria* x *Q. coccinea* (a: East Hybrid, EH, b: West Hybrid, WH), *Quercus rubra* x *Quercus imbricaria* (c: Putative Hybrid, PH), *Quercus imbricaria* (d: QI-1). See also Table 1. Photos: D. Berube.

Table 1.
Geographic coordinates and diameter at breast height (DBH) [C = *Quercus coccinea*; I = *Quercus imbricaria*; R = *Quercus rubra*, V = *Quercus velutina*; WH = *Q. imbricaria* x *Q. coccinea*; EH = *Q. imbricaria* x *Q. coccinea*; PH = *Q. rubra* x *Q. imbricaria*].

Sample	Longitude	Latitude	DBH in cm
QC-1	-82.59228	38.81848	15.88
QC-2	-82.58552	38.81482	n.a.
QC-3	-82.58589	38.81467	n.a.
QC-4	-82.58289	38.81585	n.a.
QC-5	-82.58569	38.81475	n.a.
QI-1	-82,59248	38.81853	n.a.
QI-2	-82.59253	38.81871	13.46
QI-3	-82.59253	38.81871	23.5
QI-4	-82.59261	38.81878	13.56
QI-5	-82.59261	38.81878	22.53
QR-1	-82.59245	38.81876	n.a.
QR-2	-82.59240	38.81851	10.78
QR-3	-82.58480	38.81497	19.69
QR-4	-82.59288	38.80809	n.a.
QR-5	-82.59262	38.81867	12.32
QV-1	-82.58401	38.81479	51.38
QV-2	-82.58424	38.81486	8.51
QV-3	-82.58465	38.81490	n.a.
QV-4	-82.58502	38.81499	20.83
QV-5	-82.58553	38.81478	21.84
WH	-82.59247	38.81862	10.03
EH	-82.59235	38.81853	6.86
PH	-82.59235	38.81853	9.22

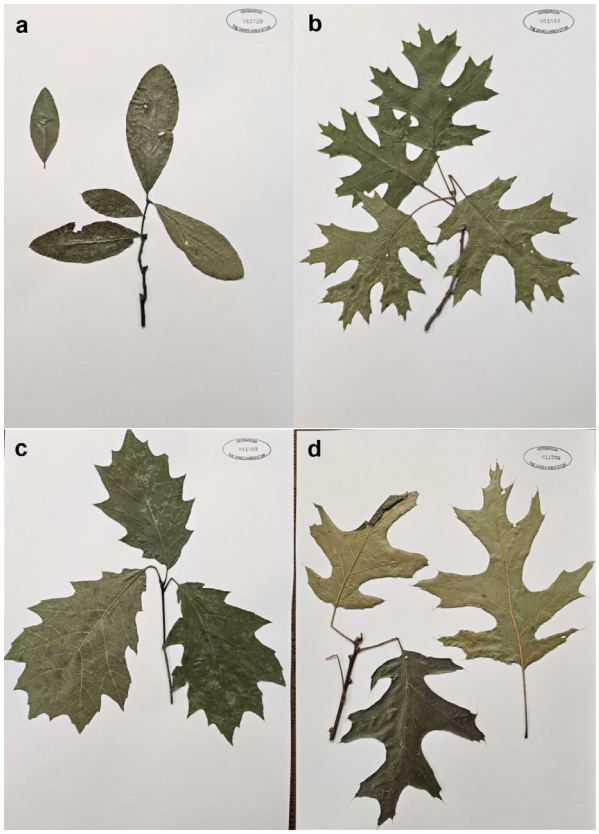

Figure 2. Leaf samples of *Quercus imbricaria* (a), *Quercus coccinea* (b), *Quercus rubra* (c) and *Quercus velutina* (d). Photos: D. Berube.

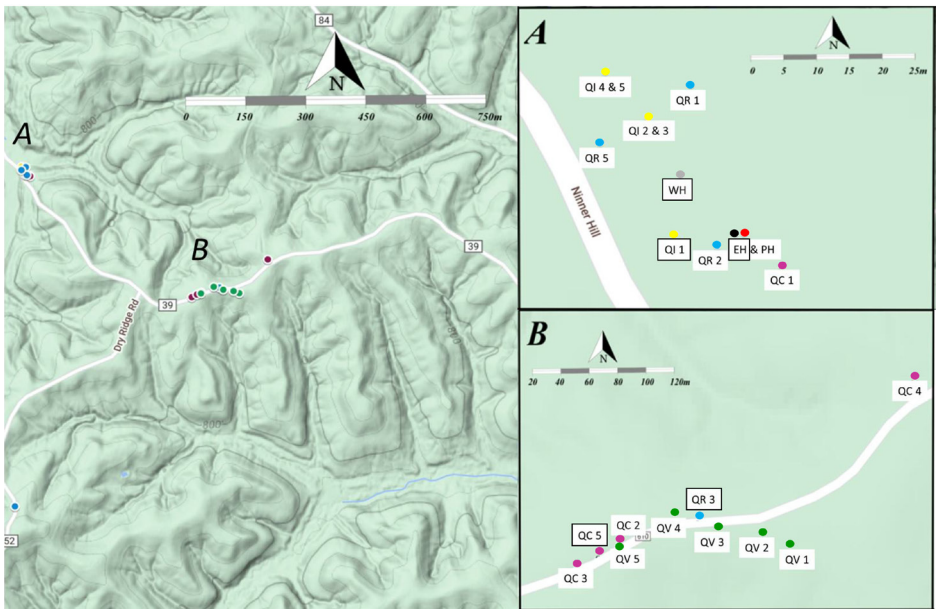

Figure 3.
Sampling location south of Oak Hill. Trees were sampled in two neighboring sites (A, B). Purple: *Quercus coccinea* (QC), yellow: *Quercus imbricaria* (QI), blue: *Quercus rubra* (QR), green: *Quercus velutina* (QV), *Q. imbricaria* x *Q. coccinea* (black: EH, grey: WH), red: *Q. rubra* x *Q. imbricaria* (PH). Genetically validated introgressive forms are surrounded by a black frame (see Table 3 and Figure 4).

Table 2.
Genomic SSRs and EST-SSRs. (*: genomic SSRs (Sullivan et al., 2013), **: genomic SSRs (Aldrich et al., 2002), ***: EST-SSRs (Durand et al., 2010))

Primer name	Repeat motif	Forward primer (5'-3')	Reverse Primer (5'-3')	Size range (in bp)	Annotation
1P10*	$(TG)_{12}GCC(TG)_3$	atttctgagggtgtcg	taggccaaggaccagaga	237-265	-
2P24*	$(CA)_{14}$	gcaagagatcacacacaaactagc	ctttgggttcaccaaacagc	136–164	-
3A05*	$(CA)_{11}(CT)_2$	aacgtgacctctctcacagc	agtgctggagtgctcatgg	138–160	-
3D15*	$(CA)_{15}$	ggtggtggcagatacactgg	gactcagacaaccaacttcagg	208–236	-
quru-GA-M04**	$(ATG)_{14}$	cttacccaccccaccacc	tgtgttctcatcagagactttcg	183-210	-
quru-GA-0C11**	$(CGG)_6$	gtccagcttctcgcagactt	tctcagctggcaggtcatta	174-250	-
quru-GA-2F05**	$(AAG)_6$	catcgacaaggtcaaggaca	caggcctaaacaaggggaga	285-341	-
FIR013***	$(GAG)_5$	cggggaggttgatgagtatt	aacactgtcacccccatagc	133-144	COL2 (constans-like 2)
FIR024***	$(CCT)_6$	cgcttctcctcatcctcaag	ctcaaaaggcacgattctcc	214–229	DNA gyrase subunit A
FIR028***	$(TC)_8$	ggaagagtgttcggaaagca	ccagctcctccacaatagca	201-237	tropinone reductase, putative
FIR031***	$(TC)_7$	acgagtccaacggaagttgt	cacaacttcacaaggcaagg	139–174	5-Adenysulfate reductase 3
FIR035***	$(AT)_6$	gctaaggttccgtgttccaa	ggccagcaactaaaccaaga	146-152	heat shock protein binding
FIR043***	$(TC)_9$	ttctccatttcacacgcttc	acgacatcgttttggagctt	114-146	Proliferation-associated protein 2G4
FIR053***	$(GTG)_7$	agtttccccacatttgttgc	taccatgcaccaagcaattc	126–150	Glutaredoxin c9
FIR104***	$(GGT)_7$	ttaactcggtttgcgactca	agcacgtgactcgacctgta	203-224	R2r3-myb transcription factor
GOT011***	$(TC)_{11}$	ccccaccgtctactctcaaa	gcgttcaccacgtccataat	162-212	pyridoxal biosynthesis protein PDX1
GOT021***	$(AT)_{13}$	agaaagttccagggaaagca	cttcgtccccagttgaatgt	95–101	Histidine kinase 4-like
GOT037***	$(CT)_{11}$	ccatccttttcattctttcca	tgttgttgttgctgttgtcg	239–265	5-Nucleotidase sure-like
GOT040***	$(GA)_{11}$	aaggcactcgtcgctttcta	accgatttgaagctcgagaa	234–254	40s ribosomal protein s16
PIE125***	$(GGAAGC)_3$	aatacaaatcgcaggaggtg	ctaacccatcgttcatggag	147-162	Dnaj-like protein
PIE200***	$(CAA)_5$	acaacatgtgccaaaactgc	tcgatgatgtggttgttgat	107-119	CAPAN Induced stolon tip protein
POR016***	$(GGT)_6$	agcaacagcagagccaaaat	cagcggctttgaggtaattc	110-126	LEIMA Protein antigen LmSTI1

Results

Both putative *Q. imbricaria* x *Q. coccinea* hybrids (EH, WH) display high leaf morphological variation with oval to elliptic, non-dissected leaves typical of *Q. imbricaria* and serrated/dissected leaves showing a closer resemblance to *Q. coccinea* (Figure 1, 2). Based on genetic assignment analysis, EH is identified as an introgressive form between *Q. imbricaria* and *Q. coccinea* (Figure 4, Table 3). The higher proportion of ancestry in *Q. imbricaria* (Q-value: 0.722) than in *Q. coccinea* (Q-value: 0.248) suggests its origin from a backcross of an F_1 *Q. imbricaria* x *Q. coccinea* hybrid with *Q. imbricaria*. On the other hand, WH shows a higher proportion of ancestry in *Q. coccinea* (Q-value: 0.749) as compared to *Q. imbricaria* (Q-value: 0.204) indicating its origin from a backcross of *Q. imbricaria* x *Q. coccinea* with *Q. coccinea*. The observation of backcrosses of F_1 hybrids with both parental species suggest bi-directional introgression. The putative *Q. rubra* x *Q. imbricaria* hybrid (PH) displays non-dissected leaves resembling *Q. imbricaria*, but also dissected leaves characteristic for *Q. rubra* saplings. However, based on genetic assignment analysis it is identified as *Q. imbricaria* (Q-value: 0.938) with no evidence of recent gene flow from *Q. rubra*. On the other hand, the sapling QI-1 (ca. 1.5 m tall) with typical *Q. imbricaria* leaves, is genetically assigned to *Q. imbricaria* (Q-value: 0.553), *Q. velutina* (Q-value: 0.252), *Q. coccinea* (Q-value: 0.101) and *Q. rubra* (Q-value: 0.094) pointing to introgression from *Q. velutina*, *Q. coccinea* and *Q. rubra* into *Q. imbricaria*. While there are *Q. imbricaria*, *Q. coccinea* and *Q. rubra* trees directly adjacent to QI-1 (Figure 3, plot A), *Q. velutina* adult trees seem to be several hundred meters apart (Figure 3, plot B). Evidence of introgression was also found in plot B dominated by *Q. coccinea* and *Q. velutina*, for QC-5 with a noticeable contribution from *Q. velutina* (Q-value: 0.111) and for QV-2 and QR-3.

The remaining four individuals of each *Q. imbricaria, Q. coccinea, Q. rubra* and *Q. velutina* were confirmed as "pure" species based on genetic assignment analysis (Table 3).

While morphologically intermediate forms were confirmed as introgressive forms in two cases (EH, WH), in one example (PH) intermediate leaf morphologies were found in a genetically identified *Q. imbricaria* tree. On the other hand, interspecific introgression is not reflected in intermediate leaf morphology in QI-1.

Table 3
Proportions of ancestry based on the STRUCTURE analysis for K=4 (EH, WH = *Q. imbricaria* x *Q. coccinea*); PH = *Q. rubra* x Q. *imbricaria*; QC = *Quercus coccinea*; QI = *Quercus imbricaria*; QR = *Quercus rubra*; QV = *Quercus velutina*). Proportions of ancestry ≥ 0.10 are highlighted. Individuals with proportions of ancestry in one cluster of ≥ 0.90 are considered as pure species. Proportion of ancestry between 0.11 and 0.89 is indicative interspecies hybridization and introgression (see Lind and Gailing 2013).

Individual	Cluster 1 (QC)	Cluster 2 (QI)	Cluster 3 (QR)	Cluster 4 (QV)
EH	**0.248**	**0.722**	0.018	0.013
PH	0.037	**0.938**	0.013	0.012
WH	**0.749**	**0.204**	0.035	0.012
QC-1	**0.958**	0.011	0.007	0.024
QC-2	**0.975**	0.011	0.007	0.007
QC-3	**0.946**	0.013	0.014	0.027
QC-4	**0.941**	0.01	0.016	0.034
QC-5	**0.798**	0.043	0.048	**0.111**
QI-1	**0.101**	**0.553**	0.094	**0.252**
QI-2	0.005	**0.973**	0.008	0.014
QI-3	0.013	**0.961**	0.011	0.015
QI-4	0.012	**0.946**	0.026	0.015
QI-5	0.005	**0.982**	0.008	0.005
QR-1	0.007	0.008	**0.977**	0.008
QR-2	0.005	0.007	**0.982**	0.006
QR-3	0.084	0.009	**0.874**	0.033
QR-4	0.002	0.007	**0.968**	0.005
QR-5	0.015	0.018	**0.955**	0.012
QV-1	0.012	0.005	0.008	**0.975**
QV-2	0.029	0.074	0.018	**0.879**
QV-3	0.015	0.009	0.008	**0.968**
QV-4	0.010	0.017	0.007	**0.967**
QV-5	0.014	0.017	0.011	**0.959**

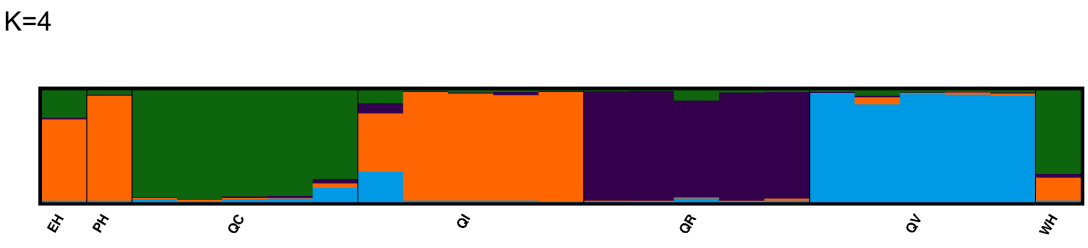

Figure 4
Structure plot for K = 4. *Quercus coccinea* (QC), *Quercus imbricaria* (QI), *Quercus rubra* (QR), *Quercus velutina* (QV), morphotype *Quercus imbricaria* x *Quercus coccinea* (EH, WH), morphotype *Quercus rubra* x *Q. imbricaria* (PH).

Discussion

Genetic assignment analysis of the three putative hybrids and five reference samples of each *Q. imbricaria, Q. coccinea, Q. rubra* and *Q. velutina* from one sympatric forest stand revealed genetic admixture between all four species. Using morphological characterization and genetic assignment, we identified for the first time hybrid forms between *Q. imbricaria* and *Q. coccinea*. Admixture analyses revealed that *Q. imbricaria* x *Q. coccinea* F_1 hybrids can backcross with each parental species to form introgressive forms. Additionally, one admixed individual with shared ancestry of *Q. imbricaria* (Q-value: 0.553), *Q. velutina* (Q-value: 0.252), *Q. coccinea* (Q-value: 0.101) and *Q. rubra* (Q-value: 0.094) was observed in plot A where species co-occur in close proximity. Among the five individuals per morphological species, one individual of each species (20 %) showed admixed ancestry. These results clearly demonstrate frequent gene flow between species, but also a predominance of non-admixed individuals suggesting crossing barriers between related oak species (Curtu et al., 2009; Gailing & Curtu, 2014). Similarly, genetic assignment analyses at nSSRs and AFLPs revealed considerable levels of introgression in the contact zone of the four red oak species *Q. ellipsoidalis, Q. coccinea, Q. velutina* and *Q. rubra* (Owusu et al., 2015; Sullivan et al., 2016). Notably, about 20 % of morphologically identified *Q. velutina* revealed introgression of *Q. ellipsoidalis* and 30 % of *Q. ellipsoidalis* trees revealed mixed ancestry with *Q. velutina* (Sullivan et al., 2016). Thus, the results of the present study align with observations in other sympatric red oak stands and corroborate incomplete crossing barriers for oak species of one taxonomic section. They also confirm earlier results of mixed ancestry of individuals assigned to species based on leaf morphology (Lind & Gailing, 2013; Sullivan et al., 2016). Thus, even larger portions of introgressed regions as observed in QI-1 (*Q. imbricaria* morphotype) apparently do not affect the phenotypic expression of leaf traits. However, unscored traits may well have been affected. On the other hand, considerable leaf morphological variation was found in a non-admixed *Q. imbricaria* individual (Fig. 1c, putative hybrid between *Q. rubra* and *Q. imbricaria* based on leaf shape, but genetically assigned to *Q. imbricaria*), suggesting introgression of specific leaf traits (dissected leaves) into *Q. imbricaria* by repeated backcrosses. However, the number of markers used in the present study was too low to identify small blocks of introgressed genomic regions.

Our results substantiate considerable interspecific gene flow between related red oak species, some of which were not known to form hybrids (*Q. imbricaria* and *Q. coccinea*). From a conservation perspective, interspecific gene flow can be advantageous to increase the genetic variation within species for *in situ* conservation and their adaptive potential in the face of changing environmental conditions (Fady et al., 2016; Cannon & Petit, 2020; Chan et al., 2019; Leroy et al., 2020; Pintér et al., 2025).

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