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A novel set of polymorphic chloroplast microsatellite markers for northern red oak (*Q. rubra* L.)

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Abstract

Oaks are model species due to their importance in various ecosystems, worldwide distribution, high economic value and emerging genomic resources. As such, knowledge on population differentiation across their distribution range is of high importance for sustainable forest management. As in most angiosperms, chloroplasts are maternally (via seeds) inherited in oak species and acorns are dispersed over comparably short distances. Consequently, chloroplast markers reveal comparatively high differentiation between populations, making them highly viable for the analysis of historic migration patterns and the certification of reproductive material. Despite the existence of various white oak (section: *Quercus*) chloroplast markers, red oak (section: *Lobatae*) chloroplast markers remain limited. Northern red oak is one of the most important North American oak species and a widespread non-native plantation tree species in European forests. We took advantage of chloroplast genomes of *Q. rubra* L. and related oak chloroplast genomes to develop a set of robust and easy-to-use chloroplast microsatellite primers for northern red oak. Furthermore, we tested transferability of those novel red oak primer pairs to *Q. robur* L. and *Q. petraea* Matt. Liebl. The new set of fifteen polymorphic chloroplast microsatellite markers revealed three additional northern red oak haplotypes after screening 80 northern red oak individuals representing seven haplotypes, identified based on formerly available markers. Therefore, they provide a higher resolution of haplotypes as compared to currently available markers.

Introduction

Northern red oak is one of the most important North American lumber species. It ranges from the peninsula of Nova Scotia to Minnesota and south to eastern Oklahoma, Alabama, and North Carolina (Sander, 1990). By now, northern red oak has become widespread in European forests with notable populations in Germany (Schmitz, 2014), and various other countries (Nicolescu *et al.*, 2020).

Previous studies identified northern red oak haplotypes using PCR-RFLP (Aldrich *et al.*, 2003; Magni *et al.*, 2005; Feng *et al.*, 2008; Birchenko *et al.*, 2009; Pettenkofer *et al.*, 2020) and cpSSR markers (Zhang *et al.*, 2015; Pettenkofer *et al.*, 2019; Götz *et al.*, 2020), which were originally developed for angiosperms (Weising and Gardner, 1999) or white oak species (Deguilloux *et al.*, 2002; Zhang *et al.*, 2015; Pettenkofer *et al.*, 2020). Chloroplast markers are commonly used for the certification of reproductive material due to their maternal inheritance (via seeds) and strong phylogeographic signature (Petit *et al.*, 2002; Chmielewski *et al.*, 2015). Here, we introduce fifteen novel and easy-to-score polymorphic cpSSRs for *Q. rubra* chloroplast haplotype identification. We also tested the transferability of those primers to the closely related species *Q. ellipsoidalis* E. J. Hill and the white oak species *Q. robur* L. and *Q. petraea* (Matt.) Liebl. (section *Quercus*).

Experimental

Chloroplast genome

The Northern red oak chloroplast genome was first completely sequenced in 2014 (Alexander and Woeste, 2014) and again in 2019 (Pang *et al.*, 2019). Chloroplast genome sequences of additional oak species from several subsections were also included in the sequence alignment (online Supplementary Table S1). All sequences were downloaded from the NCBI genomic database (Sayers *et al.*, 2020) in November 2021.

Plant material and haplotypes

Northern red oak DNA was obtained from previously conducted red oak studies with known haplotypes based on cpSSR and cpCAPS (PCR-RFLP) analyses (Pettenkofer *et al.*, 2020; online

Table 1. Primer sequences and fragment size range for allele binning of novel polymorphic primer pairs

Marker	Primer sequence (5′–3′)	Fragment size range in base pairs (bp)[a]	Polymorphic in
QRcp01 F	CGTTTAATTAGATCGGGTAATCGT	235–245	*Q. rubra, Q. petraea, Q.robur*
QRcp01 R	GCAGCATGTCGTATCAATGTGG		
QRcp02 F	AGTTTCTGTTTTCCTTGCTTGATTT	185–195	*Q. rubra, Q. petraea, Q.robur*
QRcp02 R	CGAATCCCTCTCTTTCCGCT		
QRcp07 F	ACCTTTCGGGGAGTGATTGG	200–210	*Q. rubra, Q. petraea, Q.robur*
QRcp07 R	TCCCTCTTCCCAGATTTCCA		
QRcp11 F	CGAATGGAGGCCCTTATTTTCA	355–365	*Q. rubra*
QRcp11 R	CCACTTCCGAATGGTATGCT		
QRcp13 F	TACCAAACGATTGGGATGCT	245–255	*Q. rubra, Q. petraea, Q.robur*
QRcp13 R	AGAAATGCATGAAAGAGCCCC		
QRcp14 F	GGGCCCTCTCTTTATACCTCT	162–172	*Q. rubra*
QRcp14 R	TCGCTTTCGAGCCCTTACTT		
QRcp17 F	CCGCGAGACCAGAAAGGG	175–190	*Q. rubra, Q. petraea, Q.robur*
QRcp17 R	TCCCTCTTCCCAGATTTCCA		
QRcp24 F	AACGAGTCACACACTAAGCA	196–210	*Q. rubra, Q. petraea, Q.robur*
QRcp24 R	AGCCTAAGCCTTTATTTGACTTG		
QRcp28 F	TAGATCGTTCTGCAAAGCCC	185–195	*Q. rubra, Q. petraea*
QRcp28 R	TCGTTGGACAACCACTATGTCT		
QRcp29 F	GGCAATTGCGATGGCTTCTT	196–205[b]	*Q. rubra, Q. petraea, Q.robur*
QRcp29 R	TTCGGGGGAAACCACATCAC		
QRcp30 F	TACCAAACGATTGGGATGCT	245–255	*Q. rubra, Q. petraea, Q.robur*
QRcp30 R	AGAAATGCATGAAAGAGCCCC		
QRcp31 F	CGGAGGACTCTTCTGAACAAACA	130–140	*Q. rubra*
QRcp31 R	ATCCAATGCTGAATCGATGACCT		
QRcp33 F[c]	CCAAGGAGAAGATGCGGGTT	180–195	*Q. rubra, Q. petraea, Q.robur*
QRcp33 R[c]	GGTGGGCAGGAGGAAAAGAA		
QRcp34 F[c]	CTTCTTTTCCTCCTGCCCACC	95–110	*Q. rubra*
QRcp34 R[c]	ATGGAGGTTTGACTCCGGTAA		
QRcp41 F	GAAGCACGAACCAACCCCT	100–110	*Q. rubra, Q. petraea, Q.robur*
QRcp41 R	TTGCTCGAAAGGAATGCGGT		

Primer sequences of monomorphic markers are reported in online Supplementary Table S3.
[a]Including M13 primer appendix. Fragment sizes will be shorter if labelled primers without M13 tail (online Supplementary Table S3) are used.
[b]Additional white oak allele: 188.
[c]Comparatively poor amplification during PCR.

Supplementary Table S2), *Q. ellipsoidalis* DNA from a previous study (Lind and Gailing, 2013), *Q. robur* samples with known chloroplast haplotypes (Burger *et al.*, 2021 and unpublished data), and sessile oak samples from three geographic regions were used for transferability tests.

Motif detection and primer design

A red oak chloroplast genome (Alexander and Woeste, 2014) was browsed for repetitive motives (minimum six repetitions for mononucleotide, and three for longer repeat motives) using the tandem repeats finder online application (Benson, 1999). An alignment of *Quercus* chloroplast genome sequences was performed using the Qiagen CLC Genomics Workbench (Hilden, Germany). All detected candidate loci were visually verified for the variability of the potential SSR, revealing 44 promising candidates. Primer design was done using the NCBI Primer blast online application (Ye *et al.*, 2012) with all available chloroplast reference genomes of *Quercus rubra* (taxid:3512). Target fragment size range was set from 70 to 500 bp, melting temperature (T_m) between 57 and 63°C with differences ⩽3°C between both primers and at least two mismatches to unintended targets within the last five base pairs of the 3′ end. All primer pairs were modified with an appendix (Forward primer: 5′-CACGACGTTGTAAACGAC-3′; reverse primer: 5′-GTTTCTT-3′). This enabled the use of HEX (Sigma Aldrich; St. Louis, MO, USA) or 6-FAM™ (Sigma Aldrich;

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Table 2. Origin and novel haplotypes (H_N) of northern red oak populations of previously defined haplotypes (H_P, following Götz *et al.*, 2020)

H_P[1]	H_N	Origin
A.1	A.1_1	Brandenburg, Germany[2]
A.1	A.1_1	Ontario, Canada[3,a]
A.1	A.1_2	Wisconsin, USA[4]
A.1	A.1_2	Tennessee, USA[3,a]
A.2	A.2	NRW, Germany[1]
A.2	A.2	BW, Germany[2]
A.2	A.2	Michigan, USA[5]
A.3	A.3_1	NRW, Germany[1]
A.3	A.3_2, A.3_3	Ontario, Canada[1,a]
A.3	A.3_3	Tennessee, USA[1,a]
A.4	A.4	Michigan, USA[5]
A.5	A.5	Lower Saxony, Germany[2]
A.5	A.5	NRW, Germany[2]
B.1	B.1	Michigan, USA[5]
B.3	B.3	Brandenburg, Germany[2]
B.3	B.3	NRW, Germany[1]
B.3	B.3	Michigan, USA[2]
B.3	B.3	BW, Germany[2]

Alleles of each haplotype are reported in online Supplementary Table S4.
[1]Götz *et al.* (2020), [2] Pettenkofer *et al.* (2019), [3] Liesebach and Schneck (2011), [4] Lind-Riehl *et al.* (2014), [5] Lind and Gailing (2013).
[a]Taken from a German provenance trial.

St. Louis, MO, USA) labelled universal M13 primers for economic reasons (Schuelke, 2000).

Primer testing

For primer testing, PCRs were conducted following the touchdown PCR protocol and reagent mix of Götz *et al.* (2020) in volumes of 14 µl, each using 1 µl of genomic DNA (5–10 ng), 1 µl labelled M13 forward primer (5 µM), 0.2 µl of the designed forward primer (5 µM), and 0.5 µl reverse primer (5 µM). Primer pairs producing distinguishable fragment sizes were multiplexed (Table 1). Electrophoretic separation for allele scoring of 1:150 diluted PCR products was done using an ABI Genetic Analyzer 3130xl (Applied Biosystems, Foster City, USA) with the GeneScan™ 500 ROX™ internal size standard (Applied Biosystems, Foster City, USA) using GeneMapper V 3.7 (Applied Biosystems, Foster City, USA).

Initial tests were conducted for eight samples of four different chloroplast haplotypes (Pettenkofer *et al.*, 2020). Polymorphic markers were subsequently multiplexed in 80 northern red oak samples of seven different haplotypes (Table 2, online Supplementary Table S2), and eight white oak samples with two and three previously defined haplotypes, respectively (online Supplementary Tables S2 and S4). Three of the fifteen polymorphic markers (QRcp01, QRcp07, QRcp17) exhibited various alleles within previously defined widely distributed red oak chloroplast haplotypes A.1 and A.3 (Götz *et al.*, 2020), revealing sub-haplotypes A.1_1, A.1_2, A.3_1, A.3_2, and A.3_3 (Table 2, online Supplementary Table S4).

Discussion

Since our novel primer pairs enabled the distinction of additional haplotypes within two common northern red oak haplotypes of the native range, they provide a powerful tool for further differentiation between northern red oak chloroplast haplotypes. Reproductive material for the establishment of forest plantations, such as for northern red oak, is increasingly demanded due to climate change. However, certified plantations within Europe are rather limited (Steiner, 2012). German red oak probably originated from a limited geographic range within the species' natural distribution (Pettenkofer *et al.*, 2019; Götz *et al.*, 2020). Additional chloroplast markers will be valuable tools to identify novel and rare haplotypes with a restricted native range as a prerequisite to narrow down the geographic origin of reproductive material and potential admixture. German red oak population haplotypes, for example, consist predominantly of haplotype A.1 (Pettenkofer *et al.*, 2019; Götz *et al.*, 2020). Our sample test set included samples with haplotype A.1 from different states of Germany (Brandenburg), Canada (Ontario), and the USA (Wisconsin, Tennessee). Interestingly, we observed an association between populations´ geographic origin and novel sub-haplotypes. Sub-haplotype A.1_1 was observed in all individuals from Germany and Ontario. Wisconsin and Tennessee populations, on the other hand, consisted of samples of sub-haplotype A.1_2. This result provides further evidence for the capability of our marker set to narrow down the origin of European red oak and supports the assumption of a geographically restricted origin of German *Q. rubra* stands (Pettenkofer *et al.*, 2019; Götz *et al.*, 2020).

All fifteen polymorphic red oak markers amplified in the closely related species *Q. ellipsoidalis*, but were monomorphic in the tested samples. Additional alleles might be revealed in future *Q. ellipsoidalis* investigations. All primer pairs amplified in both white oak species and ten primer pairs were polymorphic (Table 1, online Supplementary Table S3). We observed comparable variation within white oak and red oak samples (32 versus 31 total alleles for all polymorphic markers), even though white oak samples were underrepresented (16 versus 80 samples). This result supports the observation of higher variation in white oak haplotypes as compared to red oaks (Birchenko *et al.*, 2009). However, we observed no differentiation within previously defined white oak haplotypes (online Supplementary Fig. S3). Our new cpSSR markers could be used as a fast and effective method to define chloroplast haplotypes in North American red oaks as well as in European white oaks and might potentially differentiate white oak haplotypes in larger datasets.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S1479262122000156.

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