Article

Chloroplast Haplotypes of Northern Red Oak (*Quercus rubra* L.) Stands in Germany Suggest Their Origin from Northeastern Canada

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Abstract: Northern red oak (*Quercus rubra* L.) is one of the most important foreign tree species in Germany and considered as a major candidate for prospective sustainable forestry in the face of climate change. Therefore, *Q. rubra* was subject of many previous studies on its growth traits and attempts to infer the origin of various populations of this species using nuclear and chloroplast DNA markers. However, the exact geographic origin of German red oak stands has still not been identified. Its native range widely extends over North America, and the species can tolerate a broad range of environmental conditions. We genotyped individual trees in 85 populations distributed in Germany and North America using five chloroplast microsatellite and three novel chloroplast CAPS markers, resulting in the identification of 29 haplotypes. The new marker set enabled the identification of several new red oak haplotypes with restricted geographic origin. Some very rare haplotypes helped us narrow down the origin of *Q. rubra* stands in Germany, especially some stands from North Rhine-Westphalia, to the northern part of the species' natural distribution area including the Peninsula of Nova Scotia, where the most similar haplotype composition was observed, compared to distinct German stands.

Keywords: northern red oak; chloroplast; microsatellite; CAPS; German red oak origin

1. Introduction

Northern red oak (*Quercus rubra* L., section *Lobatae*) is a forest tree species native to North America, where it occurs from the east coast of Nova Scotia, Canada, and Virginia in the East to Minnesota and Oklahoma in the West. Southern offshoots reach Georgia and South Carolina and almost completely include Alabama [1]. The species was introduced to Europe in the late 17th century [2] and has become the most important foreign deciduous tree species in Germany considering the large forest area [3,4].

Q. rubra has a short rotation period in forestry compared to the native white oaks *Q. robur* L. and *Q. petraea* (Matt.) Liebl. and, therefore, has a higher productivity than European oak species. Harvested wood meets good quality standards. Its firewood or fiber properties are similar to that of white oak wood [5]. While its productivity is higher than that of European oak species, its lumber quality is inferior compared to native oaks. For instance, *Q. rubra* wood is missing thyloses [6], which slow down rotting. Therefore, it is not used for outdoor structures or barrel production. The species is considered as invasive in some European countries [7–9] because of its abundant spreading, ability to grow in less fertile soil [8], and in a wide climatic range [4]. However, this is unlikely the case in Germany, because shade tolerance is one of the major factors of successful invasion in German forests [10] and *Q. rubra* cannot compete with the local predominant tree species *Fagus sylvatica* L. [11,12]. Moreover, northern red oak from section *Lobatae* does not hybridize with native German white oaks representing section *Quercus* [4].

Despite the increasing importance of northern red oak cultivation in Europe, little is known about its origin. Additional northern red oak seed orchards have to be established to meet the growing demand for high quality reproductive material [13]. Therefore, it would be beneficial to identify possible sources of genetic material in the native range of the species. In this study, we aim to infer the origin of *Q. rubra* stands in Germany by using chloroplast (cp) haplotypes based on five chloroplast simple sequence repeat (cpSSR) and three chloroplast cleaved amplified polymorphic sequence (cpCAPS) markers. Since chloroplasts are maternally inherited in oaks [14] and thus do not undergo recombination, chloroplast haplotypes are highly suitable to draw conclusions about the geographic origin of populations and recolonization routes after the last glacial maximum [15–20]. Pettenkofer et al. [21] used five cpSSR markers to study the origin of *Q. rubra* and concluded that German stands originated from a limited geographic area, possibly located in the northern part of the species' native distribution range. Using additional haplotypes in red oak stands in Germany and comparing them to reference populations from the native distribution area, we aim to narrow down the geographic origin of German populations. For this purpose, we expanded the sample set by 22 new populations and added three new informative cpCAPS markers. These novel cpCAPS markers were designed for *Q. rubra* based on comparative plastome genome sequencing [22], which enabled identification of additional haplotypes.

We hypothesize that (1) introduction of *Q. rubra* to Europe from a restricted native geographic range caused a founder effect reflected in decreased haplotype diversity in German stands, and (2) identification of additional haplotypes using cpCAPS markers would help us narrow down the geographic origin of most German stands to the northeastern part of the natural distribution range.

2. Materials and Methods

2.1. Samples

In total, 1143 different trees were studied, including 592 samples from 44 planted stands in Germany (8–50 per stand, Table 1) and 551 samples from 37 reference naturally grown populations in North America (2–52 per population, Table 1). The sample set included 40 German stands and three North American populations studied in Pettenkofer et al. [21,23], and 16 natural populations from North America—six studied in Lind & Gailing [24], two studied in Lind-Riehl et al. [25], and eight studied in Liesebach & Schneck [3], which all have been genotyped at five cpSSR markers previously [21]. Additionally, four German planted red oak stands, including two stands, Diergardt 1 and 2, which originated from Virginia, and eight Canadian natural populations, were sampled. Furthermore, samples were collected from a provenance trial located in Schleswig-Holstein that was established in 1990 using acorns collected from single trees in different locations in North America (Table 1). Leaves were collected from randomly selected trees within each stand, maintaining a distance of at least 10 m between single trees. In provenance trials, a minimum distance of 10 m was kept between sampled trees and the border of the respective stand. Quality parameters, such as trunk curvature or branching were not taken into account. The experimental plots in the provenance trial consist of descendants of single mother trees each. From each plot, beginning with

the Northwestern corner, only the tree in the third column/third row was sampled to exclude border effects, provide reproducibility and avoid the sampling of half-siblings from the same seed parent.

Table 1. Name, location, and sample size (*N*) of red oak populations.

#	Population	Country	State	N	Longtitude	Latitude
1	Achern, Großweiher [1]	Germany	Baden Wuerttemberg	10	8°02′15.7″ E	48°39′15.2″ N
2	Oberkirchen, Renchen [1]	Germany	Baden Wuerttemberg	10	7°59′55.3″ E	48°34′23.0″ N
3	Schutterwald 1 [1]	Germany	Baden Wuerttemberg	10	7°52′04.3″ E	48°27′31.4″ N
4	Schutterwald 2 [1]	Germany	Baden Wuerttemberg	10	7°51′33.1″ E	48°27′12.5″ N
5	Schutterwald 3 [1]	Germany	Baden Wuerttemberg	10	7°51′42.6″ E	48°27′36.3″ N
6	Schutterwald 4 [1]	Germany	Baden Wuerttemberg	10	7°51′34.9″ E	48°27′10.8″ N
7	Baruth [1]	Germany	Brandenburg	10	13°22′17.7″ E	51°59′20.2″ N
8	Großbeeren [1]	Germany	Brandenburg	10	13°21′05.9″ E	52°21′49.2″ N
9	Güterfelde 1 [1]	Germany	Brandenburg	10	13°04′46.2″ E	52°27′18.2″ N
10	Güterfelde 2 [1]	Germany	Brandenburg	9	13°00′15.5″ E	52°24′55.7″ N
11	Güterfelde 3 [1]	Germany	Brandenburg	10	13°00′21.9″ E	52°23′00.8″ N
12	Lehnin [1]	Germany	Brandenburg	9	12°27′53.1″ E	52°24′58.7″ N
13	Rathenow, Kater [1]	Germany	Brandenburg	8	12°13′49.4″ E	52°33′14.4″ N
14	Beerbusch [1]	Germany	Lower Saxony	10	9°49′50.0″ E	52°24′22.3″ N
15	Dassel [1]	Germany	Lower Saxony	10	9°35′20.4″ E	51°46′47.0″ N
16	Diensthoop [1]	Germany	Lower Saxony	10	9°22′00.3″ E	52°56′46.5″ N
17	Hepstedt FLA 8 [1]	Germany	Lower Saxony	10	9°11′02.0″ E	53°10′49.7″ N
18	Hepstedt FLA 10 [1]	Germany	Lower Saxony	10	9°11′11.0″ E	53°14′59.0″ N
19	Luhne [1]	Germany	Lower Saxony	10	9°22′12.5″ E	53°08′25.8″ N
20	Spange FLA 2 [1]	Germany	Lower Saxony	10	9°15′43.7″ E	53°00′09.8″ N
21	Spange FLA 3 [1]	Germany	Lower Saxony	10	9°15′43.7″ E	53°00′09.8″ N
22	Thörenwald [1]	Germany	Lower Saxony	10	9°21′14.8″ E	53°20′36.7″ N
23	Zienitz [1]	Germany	Lower Saxony	10	10°51′16.2″ E	53°06′00.9″ N
24	Diergardt 1 ("Bornheimer Roteiche")	Germany	North Rhine-Westphalia (originally from Virginia, USA)	50	7°00′23.2″ E	50°46′52.2″ N
25	Diergardt 2 ("Bornheimer Roteiche")	Germany	North Rhine-Westphalia (originally from Virginia, USA)	50	7°00′22.9″ E	50°46′57.6″ N
26	Fander Ville 1	Germany	North Rhine-Westphalia	49	6°51′09.9″ E	50°49′42.5″ N
37	Fander Ville 2	Germany	North Rhine-Westphalia	49	6°52′29.3″ E	50°47′25.2″ N
27	Leucht FLA 14 [1]	Germany	North Rhine-Westphalia	10	6°29′13.0″ E	51°32′43.4″ N
28	Leucht FLA 15 [1]	Germany	North Rhine-Westphalia	10	6°30′24.7″ E	51°32′16.8″ N
29	Leucht FLA 16 [1]	Germany	North Rhine-Westphalia	10	6°30′29.7″ E	51°33′30.4″ N
30	Leucht FLA 18 [1]	Germany	North Rhine-Westphalia	10	6°29′27.6″ E	51°32′14.7″ N
31	Leucht FLA 19 [1]	Germany	North Rhine-Westphalia	10	6°29′11.8″ E	51°32′39.2″ N
32	Leucht FLA 20 [1]	Germany	North Rhine-Westphalia	10	6°29′52.9″ E	51°32′22.1″ N
33	NRW 11 [1]	Germany	North Rhine-Westphalia	10	6°48′08.2″ E	51°02′20.8″ N
34	NRW 17 [1]	Germany	North Rhine-Westphalia	10	6°30′38.0″ E	51°32′18.7″ N
35	Schwalm-Nette FLA 12 [1]	Germany	North Rhine-Westphalia	10	6°11′41.0″ E	51°09′00.1″ N
36	Schwalm-Nette FLA 13 [1]	Germany	North Rhine-Westphalia	10	6°10′54.2″ E	51°09′49.9″ N
38	Holzland [1]	Germany	Thuringia	10	11°38′24.8″ E	50°46′31.8″ N
39	Leina [1]	Germany	Thuringia	10	12°32′37.1″ E	50°58′23.1″ N
40	Stößwitz 1 [1]	Germany	Thuringia	9	11°43′29.4″ E	50°46′00.9″ N
41	Stößwitz 2 [1]	Germany	Thuringia	10	11°43′27.1″ E	50°45′51.9″ N
42	Treben 1 [1]	Germany	Thuringia	10	12°25′21.5″ E	51°03′47.3″ N
43	Treben 2 [1]	Germany	Thuringia	9	12°30′18.9″ E	51°03′30.5″ N
44	Volkenroda [1]	Germany	Thuringia	10	10°40′00.7″ E	51°17′59.8″ N
45	Chattahoochee [4]	USA	Georgia	5	84°13′54.2″ W	34°50′31.5″ N
46	Rabun 121	USA	Georgia	4	83°25′21.8″ W	34°49′20.5″ N
47	Dekalb Auburn	USA	Indiana	4	85°03′33.7″ W	41°22′48.5″ N
48	Madison, Anderson [4]	USA	Indiana	6	85°38′18.7″ W	40°05′53.2″ N
49	Steuben Angola	USA	Indiana	7	84°59′26.8″ W	41°37′57.9″ N
50	Brockway Mountain [2]	USA	Michigan	8	87°54′59.2″ W	47°27′57.5″ N
51	Chippewah County [4]	USA	Michigan	6	84°25′54.1″ W	46°26′59.1″ N
52	Ford Forestry Center A [2]	USA	Michigan	5	88°36′06.0″ W	46°39′09.0″ N
53	Ford Forestry Center B [2]	USA	Michigan	5	88°31′27.0″ W	46°40′27.0″ N

Table 1. *Cont.*

#	Population	Country	State	N	Longtitude	Latitude
54	Huron Mountain Reserve Ives Hill [2]	USA	Michigan	8	87°50′42.8″ W	46°51′12.9″ N
55	Huron Mountain Reserve Lily Pond [2]	USA	Michigan	8	87°49′48.8″ W	46°50′59.8″ N
56	Keweenaw County	USA	Michigan	8	87°54′59.2″ W	47°27′57.5″ N
57	Calumet Township	USA	Michigan	8	88°25′36.3″ W	47°15′14.3″ N
58	Michigan Tech Trails [2]	USA	Michigan	8	88°32′51″W	47°06′02″N
59	Porcupine	USA	Michigan	8	89°37′27.4″ W	46°49′01.5″ N
60	Bennet Gap Pisgah	USA	North Carolina	8	82°46′11.6″ W	35°19′21.4″ N
61	Clay, Nanthahala, Tusquitee [4]	USA	North Carolina	7	83°45′47.7″ W	35°05′03.0″ N
62	Sullivan Elizabethton	USA	Tennessee	9	82°12′07.5″ W	36°20′53.6″ N
63	Sullivan, Cherokee, Watauga [4]	USA	Tennessee	5	81°48′16.4″ W	36°12′40.8″ N
64	Chequamegon National Forest [3]	USA	Wisconsin	8	91°02′08.0″ W	46°42′54.0″ N
65	Nicolet National Forest [3]	USA	Wisconsin	8	88°23′17″W	45°20′53″N
66	Crafts Cove	Canada	New Brunswick	31	65°59′42.7″ W	45°44′19.7″ N
67	Maitland Bridge	Canada	Nova Scotia	50	65°16′15.1″ W	44°31′15.1″ N
68	Nine Mile Woods	Canada	Nova Scotia	49	65°15′34.8″ W	44°29′26.3″ N
69	Oak Lake	Canada	Nova Scotia	52	65°24′21.9″ W	44°31′02.6″ N
70	Wentzells Lake	Canada	Nova Scotia	40	64°38′41.0″ W	44°29′22.8″ N
71	Constance Bay [4]	Canada	Ontario	6	76°04′29.4″ W	45°29′27.4″ N
72	Elgin Aylmer	Canada	Ontario	2	80°59′17.6″ W	42°46′31.9″ N
73	Hastings Carlow	Canada	Ontario	2	77°37′31.3″ W	45°08′29.4″ N
74	McConnel Road	Canada	Ontario	51	79°09′26.1″ W	46°39′00.5″ N
75	Niagara Point Abino	Canada	Ontario	2	79°05′52.6″ W	42°51′42.9″ N
76	Niagara Ridgeway	Canada	Ontario	5	79°03′21.1″ W	42°52′49.0″ N
77	Phelps	Canada	Ontario	45	79 06 21.8 W	46 22 30.1 N
78	Renfrew, Chalk River [4]	Canada	Ontario	6	77°26′40.7″ W	46°01′22.4″ N
79	Charlottetown East Royalty	Canada	Prince Edward Island	50	63°06′38.5″ W	46°16′06.2″ N
80	Gatineau, Plaines de Kazabazua [4]	Canada	Québec	6	75°24′32.6″ W	45°34′54.9″ N
81	Plaines de Kazabazua 2	Canada	Québec	11	75°41′21.3″ W	45°27′50.5″ N

[1] sampled in [21,23], [2] in [24], [3] in [25], [4] in [3].

2.2. DNA Isolation

The DNeasy™ 96 Plant Kit (Qiagen; Hilden, Germany) was used to extract DNA from a 1 cm^2 large piece cut from each fresh or dried leaf sample according to the manufacturer's manual.

2.3. Chloroplast Microsatellites

The newly collected populations from Canada and Germany were genotyped at five cpSSR markers. Two angiosperm cpSSR markers (*ccmp2* and *ccmp4*, [26]), as well as three oak-specific ones (*ucd4*, *udt1*, and, *udt4*) developed by Deguilloux et al. [27] were used. A multiplex PCR was run for *ccmp2* and *ccmp4* with 1 μL genomic DNA sample, 1.5 μL PCR buffer containing 0.8 M TRIS-HCl and 0.2 M $(NH_4)_2SO_4$, 4.8 μL ddH$_2$O, 1.5 μL (25 mM) MgCl$_2$, 1μL dNTPs (2.5 mM of each dNTP), 1 μL of each primer (forward and reverse for each, *ccmp2* and *ccmp4*; 5 picomole/μL) and, 0.2 μL (5 U/μL) HOT FIREPol® Taq polymerase (Solis BioDyne; Tartu, Estonia). After initial denaturation for 15 min at 95 °C, 37 cycles with 1 min denaturation (94 °C), 1 min annealing (53 °C), and 1 min of elongation (72 °C) were conducted, which were finally followed by 20 min of elongation terminating the procedure.

Markers *ucd4*, *udt1*, and *udt4* were amplified using a touchdown PCR with the following parameters: 15 min denaturation at 95 °C, followed by eight cycles of 1 min denaturation (94 °C), 1 min annealing (53 °C, reduced by 1 °C per cycle) and 1 min of elongation (72 °C). Subsequently, 25 cycles with 1 min denaturation (94 °C), 1 min annealing (45 °C) and 1 min elongation (72 °C) were initiated and finally followed by 20 min of elongation. For each primer pair, 1 μL per primer (forward and

reverse; 5 picomole/μL), 1 μL genomic DNA sample, 1.5 μL PCR buffer containing 0.8 M TRIS-HCl and 0.2 M $(NH_4)_2SO_4$, 6.8 μL ddH$_2$O, 1.5 μL (25 mM) MgCl$_2$, 1μL dNTPs (2.5 mM of each dNTP), and, 0.2 μL (5 U/μL) HOT FIREPol® Taq polymerase (Solis BioDyne; Tartu, Estonia) were mixed before undergoing PCR.

Forward primers were labelled with the fluorescent dye 6-FAM™ (Sigma Aldrich; St. Louis, MO, USA). All PCRs were run in a Biometra Tprofessional thermocycler (Jena, Germany). For fragment separation, we used an ABI Genetic Analyzer 3130xl (Applied Biosystems, Foster City, CA, USA). PCR products were diluted 1:650 for *ccmp* and 1:600 for *ucd* and *udt* markers. GeneMapper software v3.7 (Applied Biosystems, Foster City, CA, USA) was used to score the fragment sizes.

2.4. Chloroplast CAPS Markers

In addition to the five cpSSR markers, three cpCAPS markers (*4.1*, *5.2*, and *17.1*) were used to define different haplotypes. Each sample was amplified with the specific cpCAPS primer pairs (Table S1) and digested as follows: *4.1* with *Hae*III, *5.2* with *Aci*I, and *17.1* with *Apa*I (all endonucleases were from Thermo Scientific™; Waltham, MA, USA), as described in Pettenkofer et al. [22]. PCRs were done for each primer pair individually with similar settings as for the *ccmp* cpSSR primers, except for a longer elongation phase in all 37 cycles for *4.1* and *17.1* (90 s instead of 60 s). The vials contained a mixture as described for *ucd4*, *udt1*, and *udt4*. After PCR, digestion was done by mixing 8 μL of PCR product with 3 U of enzyme and 2 μL of the associated buffer. All reactions were incubated at 37 °C overnight.

Fragment separation was done by agarose gel electrophoresis for about 60 min. For *4.1*/*Hae*III and *5.2*/*Aci*I we used 2.5% agarose gels at 3 V/cm and a 100 bp GeneRuler DNA Ladder (Thermo Scientific™; Waltham, MA, USA), for *17.1*/*Apa*I 2% agarose gels, 4 V/cm and a 1kb GeneRuler as DNA Ladder (Thermo Scientific™; Waltham, MA, USA). *4.1*/*Hae*III had three alleles: a single fragment (uncut), two fragments (cut) and three fragments (cut twice), while *5.2*/*Aci*I and *17.1*/*Apa*I had two alleles each (cut and uncut, respectively). Scoring was performed by visual observation (Figure S1). The well-defined DNA fragments were accompanied by faint bands, which exhibited consistent patterns. Since the summarized size of the strong bands added up to the size of the uncut PCR product, the faint bands were not considered for the analysis.

2.5. Chloroplast Haplotypes, Haplotype Diversity and Inter-Population Differentiation

Haplotypes were identified by combining all eight markers (Table 2). For detecting differences between markers and managing the dataset, we used Microsoft Excel 2016 (Redmond, WA, USA).

Table 2. Marker composition of chloroplast simple sequence repeat (cpSSR) and chloroplast cleaved amplified polymorphic sequences (cpCACPS), and occurrence of *Q. rubra* chloroplast haplotypes.

Haplotype	cpSSR Alleles, bp					cpCAPS Alleles			Occurence		Number
	ccmp2	ccmp4	ucd4	udt1	udt4	4.1/HaeIII	5.2/AciI	17.1/ApaI	Germany	North America	
A.1	228	116	99	86	145	cut	uncut	uncut	+	+	690
A.2	228	116	99	87	145	cut	uncut	uncut	+	+	126
A.3	228	116	99	85	145	cut	uncut	uncut	+	+	49
A.4	228	116	99	85	145	cut twice	uncut	uncut		+	10
A.5	228	116	99	86	145	uncut	uncut	uncut	+	+	39
A.6	228	116	99	86	145	cut twice	uncut	uncut		+	1
A.7	228	116	97	85	146	cut	uncut	uncut		+	4
A.8	228	116	99	85	146	cut twice	uncut	uncut		+	1
A.9	228	116	99	85	146	cut	uncut	uncut		+	1
A.10	228	116	96	86	145	cut	uncut	uncut	+		1
A.11	228	116	96	86	146	cut	uncut	uncut	+		1
A.12	226	117	99	86	145	cut	cut	uncut		+	1
A.13	228	116	98	84	146	cut	uncut	uncut		+	1
A.14	228	116	99	84	145	cut	uncut	uncut		+	1
A.15	228	116	97	84	146	cut	uncut	uncut		+	1

Table 2. *Cont.*

Haplotype	cpSSR Alleles, bp					cpCAPS Alleles			Occurence		Number
	ccmp2	*ccmp4*	*ucd4*	*udt1*	*udt4*	*4.1/HaeIII*	*5.2/AciI*	*17.1/ApaI*	*Germany*	*North America*	
A.16	228	116	99	86	145	cut	uncut	cut		+	7
A.17	228	116	97	86	146	cut	uncut	uncut	+		4
A.18	228	117	99	86	145	cut	uncut	uncut	+		1
A.19	228	116	99	85	145	cut	cut	uncut		+	1
B.1	227	115	98	85	146	cut	uncut	uncut	+	+	9
B.2	227	115	98	84	146	uncut	uncut	uncut		+	1
B.3	227	115	98	85	146	uncut	uncut	uncut	+	+	167
B.4	227	115	98	85	145	uncut	uncut	uncut	+		1
B.5	227	115	99	85	146	uncut	uncut	uncut	+	+	6
B.6	226	115	99	85	145	uncut	uncut	uncut		+	1
C.1	226	117	97	85	146	cut	cut	uncut		+	4
C.2	226	117	97	84	146	cut	cut	uncut		+	6
C.3	226	117	96	85	146	cut	cut	uncut	+		1
C.4	226	118	98	85	146	cut	cut	uncut		+	7

Haplotype diversity (H_T), average expected within-population haplotypic diversity (H_S) and genetic differentiation among populations (G_{ST} & R_{ST}) were calculated using the software PermutCpSSR_1.2.1 [28,29] and 1000 permutations.

2.6. Minimum Spanning Tree

A minimum spanning tree (Figure 1) was created using Microsoft Office 2016 (Redmond, WA, USA) by comparing different haplotypes at each marker with Microsoft Excel and sorting them according to their similarity. Consequently, the haplotypes were linked with the most similar one in the dataset and visualized using Microsoft Publisher. Linkages were not always additive. Thus, if a haplotype 1 (H1) differed from a haplotype 2 (H2) at two positions, and H2 differed from haplotype 3 (H3) at three positions, H1 and H3 do not necessarily differ at five positions. The length of the links between two haplotypes was sometimes adjusted to fit the graph. Furthermore, base pair differences between cpSSR marker alleles were not considered (Table 2). For better visualization, three haplotype groups were assembled by grouping similar haplotypes, which differ from each other in less than three markers. Haplotypes thus compiled, were highlighted in different colors (blue, purple, and orange).
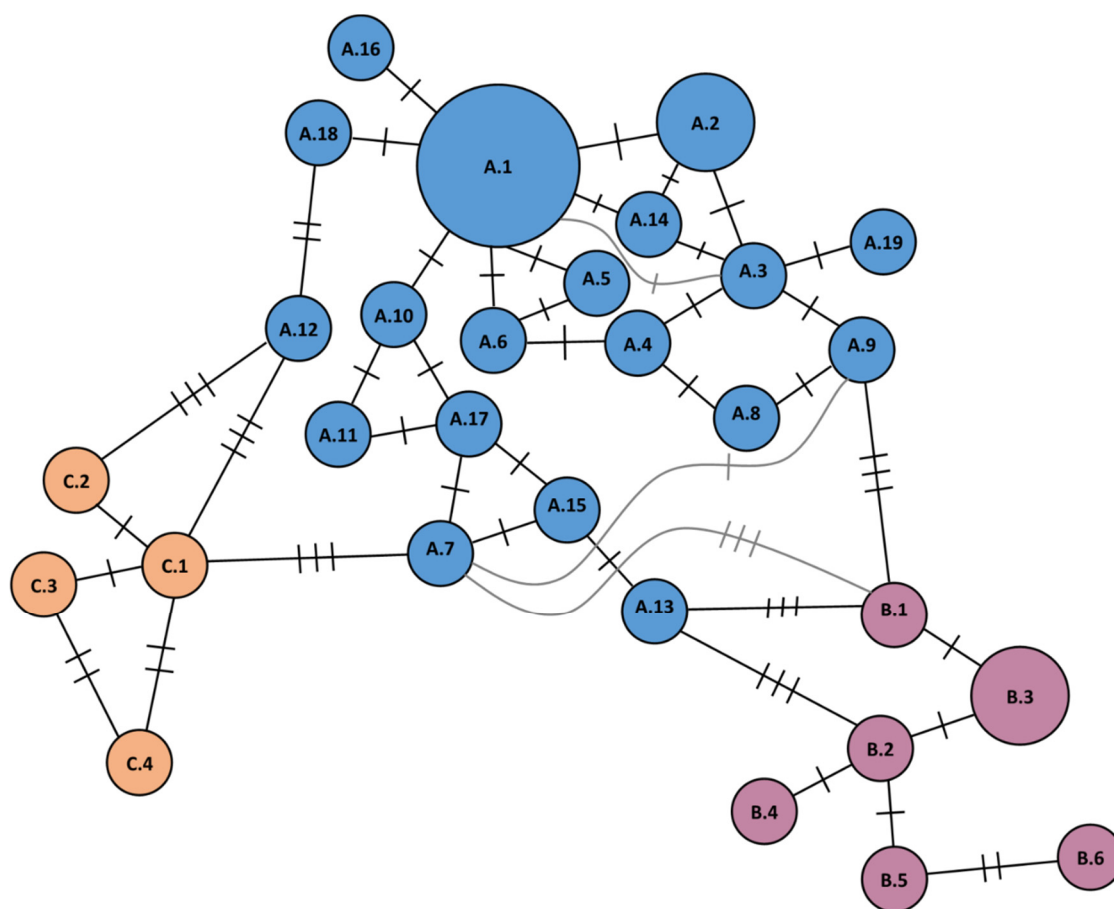
Figure 1. Minimum spanning tree of *Q. rubra* haplotypes. Each haplotype is linked to the others by the minimum number of marker differences necessary. Each crossbar represents one mutation separating haplotypes. The area of the circle reflects sample sizes, where the largest circle represents the most common haplotype A.1 found in 690 trees, and the second largest circle represents two haplotypes A.2 and B.3 found in 126 and 167 trees, respectively (see also Table 2).

3. Results

3.1. Minimum Spanning Tree

Related haplotypes within each cluster are usually separated by differences in only one marker, with the exception of three haplotypes that show two differences instead. The majority of the samples was assigned to haplotypes A.1, A.2, and B.3 with 690, 126, and 167 samples, respectively.

3.2. Haplotype Diversity

Sixteen of the 29 haplotypes (Figure 1, Table 2) (A.4, A.6–A.9, A.12–A.16, A.19, B.2, B.6, C.1, C.2, and C.4) occurred only in North America and six exclusively in Germany (A.10, A.11, A.17, A.18, B.4, and C.3).

Seven haplotypes (A.1, A.2, A.3, A.5, B.1, B.3, and B.5) occurred in both North America and Germany. Haplotypes A.6, A.8–A.15, A.18, A.19, B.2, B.4, B.6, and C.3 were solely observed in single trees, while haplotypes A.4, A.7, A.16, A.17, B.1, B.5, C.1, C.2, and C4 were observed in four and more (up to ten) trees. A.1–A.3, A.5, and B.3 were observed in more than 35 trees, with A.1 being the most common haplotype observed in 690 trees (Table 2, Figures 2 and 3).

A.1 was observed in every single stand within Germany, but only in ~65% of the North American populations (Figures 2 and 3). Haplotype B.3 occurs in 30 German stands (~68% of German stands), while in North America it is only present in seven populations (~19% of American populations),

mostly observed at the northern edge of the distribution range (Nova Scotia, Ontario and northern Michigan) (Figures 2 and 3). The distribution of A.2 shows a similar pattern. It was found in 41% of the German stands, distributed over all federal states but occurs only in five (~13.5%) of the North American populations in the Great Lakes region of Michigan and Canada (Figures 2 and 3).
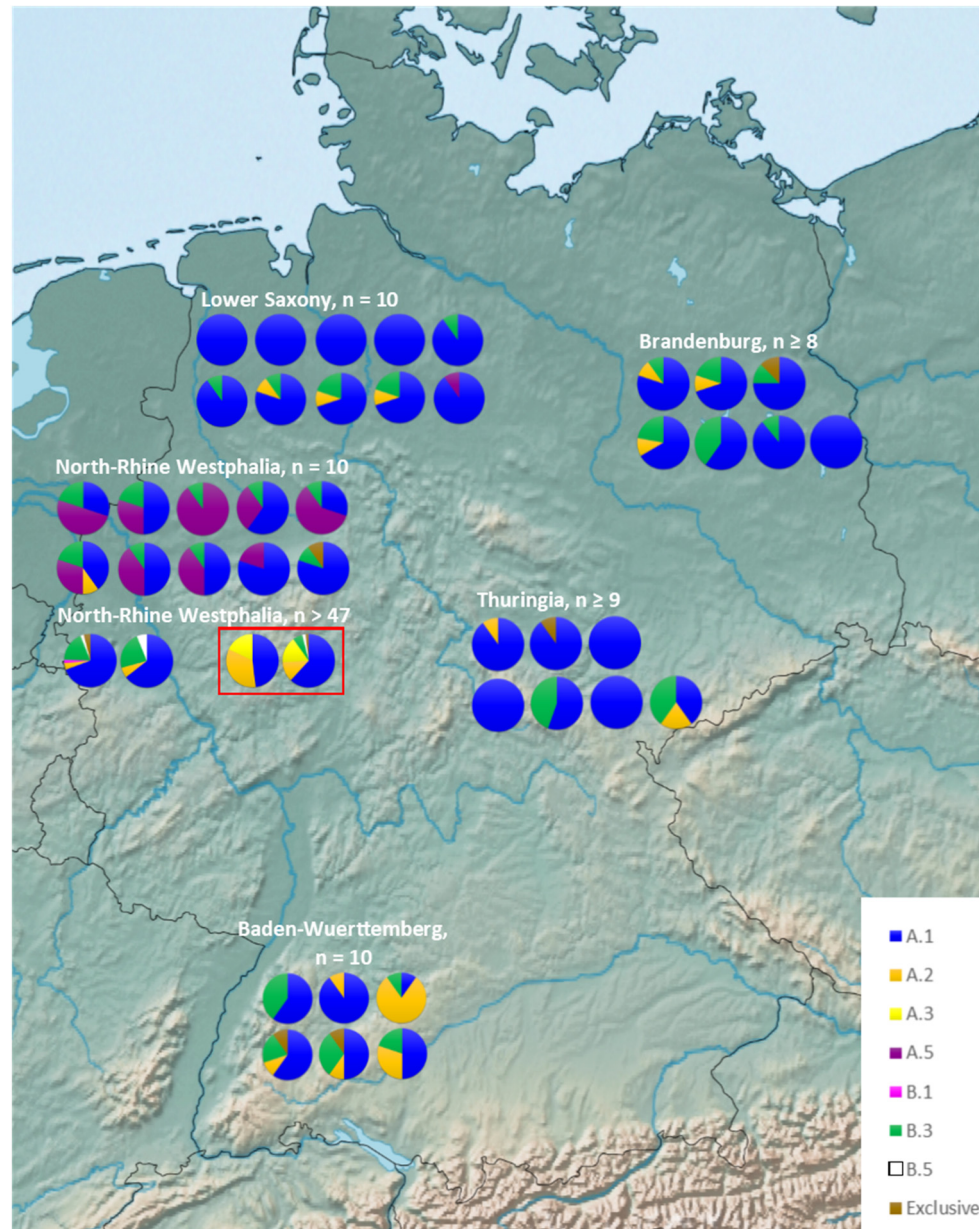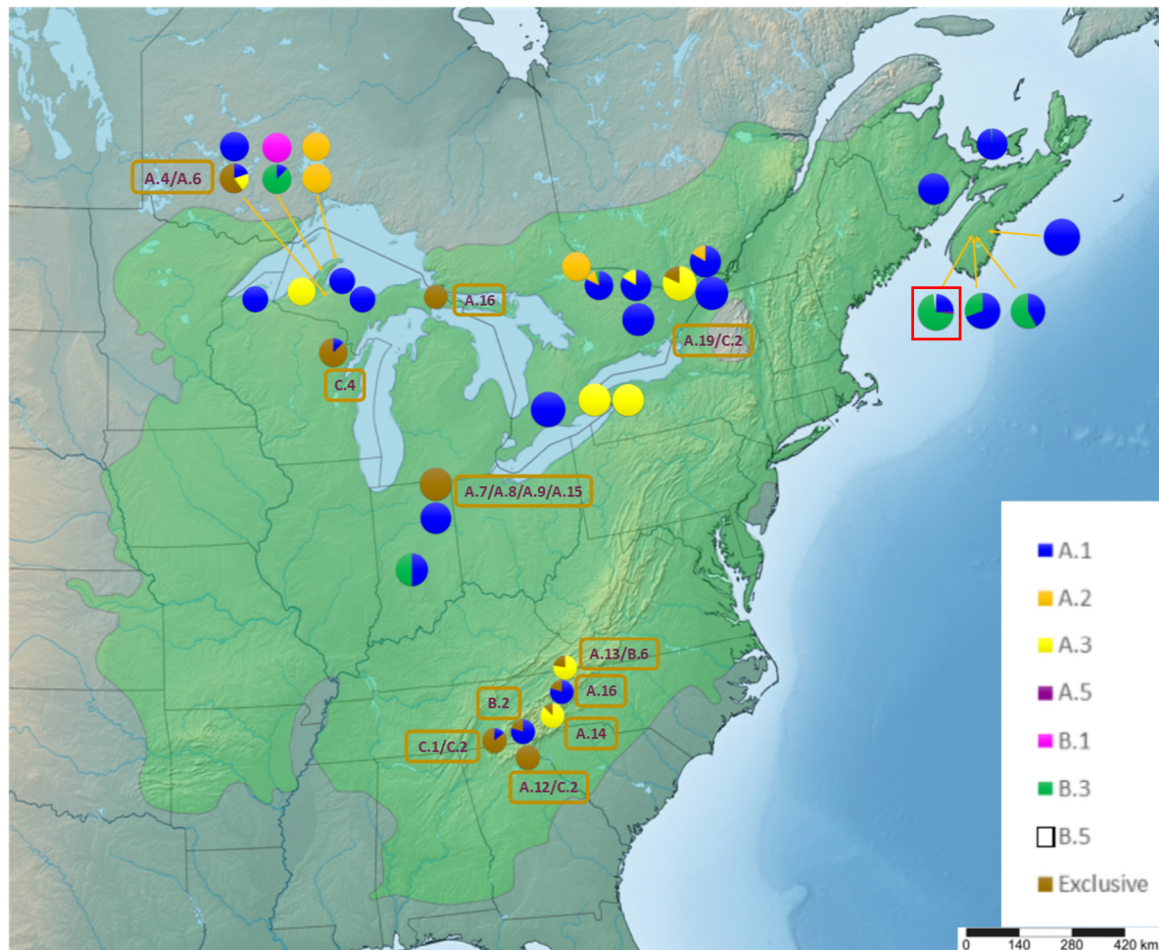

Figure 2. Haplotype distribution of *Q. rubra* in Germany. For better visualization, haplotypes that were exclusive to Germany and did not occur in North America (brown) are pooled together (see Table 2). Those provenances in North Rhine-Westphalia that originated from Virginia ("Sonderherkunft Bornheim") are depicted by a red frame. "Exclusive" haplotype in those provenances is haplotype C.3.

Haplotype A.5 is widespread in North Rhine-Westphalia (64% of NRW stands) and present once in Lower Saxony (Germany). Thus, overall, it is present in 23% of German stands (Figure 2). Nevertheless, it could only be found in a single individual in the Nine Mile Woods population from Nova Scotia in North America (Figure 3). One of the rare haplotypes, B.5, was also observed in the same Nine Miles Woods population from Nova Scotia (Table 1) and in three of the German stands in North Rhine-Westphalia (Figures 2 and 3). Haplotype A.3 was detected in the Appalachian Mountains

and the Great Lakes region around Michigan but does not occur in northeastern populations of New Brunswick, Prince Edward Island, and Nova Scotia (Figure 3). In Germany, A.3 was only observed in two stands Diergardt 1 and 2 ("Bornheimer Roteiche"), with documented origin from Virginia, USA.

Figure 3. Distribution of *Q. rubra* chloroplast haplotypes in North America. The native red oak range is highlighted in green. "Exclusive" are haplotypes that were found only in North America, but not in Germany. For better visualization, they were highlighted by brown color and pooled together in pie charts, but their individual names are listed within the brown frames placed next to the respective population. A5 and B.5 were represented by a single tree each in one population of Nova Scotia highlighted by the red frame.

3.3. Haplotype Structure and Distribution

The 29 defined haplotypes (Figure 1) are assumed to be closely related within their assigned major clusters or haplogroups: A (blue), B (purple), and C (orange). Most of them are related to another haplotype differing at only one marker. Nevertheless, haplotypes A.12, B.6, and C.4 each differed from other haplotypes within their clusters by two mutations instead of one. Therefore, it is not certain whether they belong to their haplogroups.

The majority of defined haplotypes, including the most common haplotype A.1 observed in 690 trees, formed cluster or haplogroup A. Haplotype A.1 is followed by A.2 observed in 126 trees. In total, there were 940 trees in cluster A (~82.2% of all trees). Most of the remaining trees belonged to cluster or haplogroup B (~16.2% of all trees) with B.3 being the most frequent haplotype observed in 185 trees. Cluster C shows minor occurrences (~1.7% of all trees) with less than 10 trees per each haplotype and a total of 19 trees. All three clusters are present in Germany (Figure 2) and the native range (Figure 3). However, haplotypes in cluster C are mostly located near the Great Lakes

region of Michigan and the southern parts of the Appalachian Mountains (Figure S2). Additionally, one population in eastern Ontario holds a small number of haplotypes from cluster C, but it was not observed at the northeastern edge of the distribution area in Canada. Haplotype C.3 occurred only in a single tree in German stand Diergardt 2, which originated presumably from Virginia, USA ("Bornheimer Roteiche"). Haplogroup A is characterized by an allele with fragment size of 228 bp at *ccmp2*, while typical for cluster B was an allele with fragment size of 115 bp at *ccmp4*, and cluster C was characterized by an allele with usually a single cut at *5.2*/*Aci*I and by an allele with fragment size of 226 bp at *ccmp2* and an allele with fragment sizes of 117 or 118 bp at *ccmp4* (see Table 2). The haplotypes from clusters A and B were widespread over the native range and Germany. Cluster C contained only haplotypes exclusive to one of the continents. Haplotypes A.6, A.8-A.15, A.18, A.19, B.2, B.4, B.6, and C.3 occurred only in single trees. A.7 was found in multiple trees, but only in a northern population of Indiana.

Haplotype A.1 was predominant in Germany and was present in all sampled stands (Figure 2). Haplotype B.3 was also common and present in 68% of all German stands, followed by A.2 present in 41% of stands. The distribution of haplotype A.5 was remarkable; it was present in 86% of NRW stands, but was observed only once in Lower Saxony. Haplotypes that were exclusive to Germany were distributed evenly over the federal states. B.5 can only be observed in the stands of NRW with larger sample size of 49–50 trees. These were also stands with the largest diversity containing up to six different haplotypes, probably due to their sample size. By contrast, the smaller stands with 8–10 trees contained a maximum of four different haplotypes (Figure 2). Haplotype A.3 was observed only in the two German stands Diergardt 1 and 2 ("Bornheimer Roteiche") in the provenances that originated presumably from Virginia, USA. Furthermore, one of these stands contained haplotype C.3, which is the only haplotype of the C cluster observed in Germany (Figure S3) and was not observed in North America.

A.1 was the most common haplotype and observed in 65% of the North American populations (Figure 3), followed by A.3, B.3, and A.2 observed in 21.6%, 18.9%, and 13.5% of the North American populations, respectively. The 16 different populations that have haplotypes exclusive to North America are spread mainly from the Northwest to the South of the natural range, with the exception of one population near Ottawa. Six populations with large sample sizes of 30-50 trees located in Nova Scotia and on Prince Edward Island contained four different haplotypes in total, while six smaller populations in the Appalachian Mountains with sample sizes of 4-9 trees contained together 10 haplotypes.

3.4. Haplotype Diversity and Inter-Population Differentiation

Total haplotype diversity in Germany ($H_T = 0.516 \pm 0.047$) was lower than in North America ($H_T = 0.754 \pm 0.059$). However, haplotype diversity within German stands ($H_S = 0.385 \pm 0.037$) was higher than in North American populations ($H_S = 0.218 \pm 0.042$). Genetic differentiation was higher in North America ($G_{ST} = 0.710 \pm 0.054$, $R_{ST} = 0.717 \pm 0.039$), compared with German stands ($G_{ST} = 0.254 \pm 0.068$, $R_{ST} = 0.095 \pm 0.031$). Total haplotype diversity decreased from the South ($H_T = 0.882 \pm 0.040$, Figure S4) and the Northwest ($H_T = 0.860 \pm 0.073$, Figure S4) towards the Northeast ($H_T = 0.439 \pm 0.124$, Figure S4) in the native distribution area. Since some populations had higher sample sizes for North American and German stands, we tested whether there was an influence of the computed parameters by reducing the sample size of German and North American stands with more than eleven sampled trees to an amount of equal-sized randomly taken samples and recalculated the computed values. Resulting values were similar to the values of the entire dataset (Table S3).

4. Discussion

In this study, we successfully detected several new chloroplast haplotypes in German and North American red oaks, which enabled us to validate our hypothesis of a founder effect in German red oak reflected in decreased haplotype diversity compared to the native range. Furthermore, we found evidence for an introduction of the species from various North American populations from the

northeastern part of the natural distribution range displayed by the presence of many haplotypes from the northern Great Lakes Region and the northeastern part of the natural range in German stands. This distribution pattern led us to the conclusion that North Rhine-Westphalian stands might originate from the Peninsula of Nova Scotia at the northeastern edge of the native distribution area.

4.1. Haplotype Diversity and Distribution

Q. rubra exhibits comparatively low intraspecific chloroplast variation [17,30], and Pettenkofer et al. [21] detected thirteen different red oak haplotypes in 62 stands based on five chloroplast SSR markers. Our results demonstrate that the resolution of both haplotype diversity and inter-population genetic differentiation increased with the use of additional chloroplast markers (cpCAPS) and the inclusion of more German stands and North American red oak populations. Pettenkofer et al. [21] observed a relatively low overall haplotype diversity ($H_T = 0.337 \pm 0.046$) of red oak in Germany and a relatively low genetic differentiation among stands ($G_{ST} = 0.137 \pm 0.067$). Our study with additional cpCAPS markers (Table S2) and red oak populations revealed overall higher levels of chloroplast haplotype diversity in German stands as well as overall higher estimates of differentiation among German stands. In particular, stands from North Rhine-Westphalia could now be differentiated from other stands in Germany (Figure 2). Similarly, a finer haplotype resolution and the analysis of additional populations revealed higher levels of the total haplotype diversity in North American populations in comparison to that reported in Pettenkofer et al. [21] ($H_T = 0.652 \pm 0.096$). Genetic differentiation among the North American populations reported in Pettenkofer et al. [21] was high ($G_{ST} = 0.729 \pm 0.086$) and it was similar to that we observed in this study. While the R_{ST} value in North America was not significantly different from the observed G_{ST} value, in Germany we observed a significantly ($p = 0.014$) lower value for R_{ST} than for G_{ST}. This finding is a result of the German haplotype composition that often differs only by a single nucleotide motif in the chloroplast SSR markers and was already observed in Pettenkofer et al. [21].

Due to the predominance of haplotypes A.1 and B.3, total haplotype diversity and genetic differentiation of northeastern North American populations (Figure S4) were low ($H_T = 0.439 \pm 0.124$, $G_{ST} = 0.465 \pm 0.095$), compared with the Appalachian region (Figure S4) in the South ($H_T = 0.882 \pm 0.040$, $G_{ST} = 0.503 \pm 0.052$) or the Great Lakes Region of Michigan and Wisconsin (Figure S4) in the West ($H_T = 0.860 \pm 0.073$, $G_{ST} = 0.864 \pm 0.087$), where a higher number of haplotypes were observed. The reduced number of haplotypes in the Canadian populations, despite their large sample size, may be a result of postglacial migration from the south or a margin effect, which reduces occurring haplotypes to adapted ones. Haplotype diversity within populations was lowest for the western region ($H_S = 0.117 \pm 0.077$, Figure S4) as a result of fixation of many populations on one haplotype. The Northeastern populations had an intermediate level of haplotype diversity ($H_S = 0.235 \pm 0.100$), while the southern populations showed the highest haplotype diversity within populations ($H_S = 0.439 \pm 0.056$) and a high number of different haplotypes.

These findings are in agreement with previous studies that found a continuous trend of increasing genetic variation from the Northeast towards the Southeast for nuclear DNA markers (SNPs) [31]. Furthermore, Merceron et al. [31] identified three different genetic clusters (G1, G2, and G3) in native red oak based on the examination of 73 native and 38 European populations at 69 SNP markers. The G2 cluster represented the southern region from North Carolina and Alabama to Indiana, while the G1 cluster represented mainly the Northeast of the native distribution area. The trees in the third cluster G3 were more or less equally distributed across the species range [31]. Dispersion of the trees in the northeastern cluster G1 follows well the distribution of haplotypes A.2 and B.3 in our study. The haplotype A.3 is associated well with the G2 cluster in the South. The clusters based on the nuclear DNA markers [31] seem to be in agreement with the clusters based on the cpDNA haplotypes observed in this study.

The lower haplotype diversity in German stands compared to North American populations might indicate a founder effect due to introduction of a relatively small number of trees from a

limited range in North America. Since German red oak chloroplast haplotype diversity is still high, we consider multiple introductions, possibly at different times and probably from different stands within the Northeastern parts of the native range. Consequently, German stands showed a higher genetic variation within stands than North American populations, which were often fixed on one haplotype. Haplotype diversity was especially high in North Rhine-Westphalia ($H_S = 0.532 \pm 0.043$) and Baden Wuerttemberg ($H_S = 0.526 \pm 0.082$) stands compared to stands in other German federal states ($H_S = 0.296 \pm 0.050$). These results suggest plausible multiple introductions or mixtures of reproductive material from several separated geographic areas. The low differentiation among stands of Germany compared to the differentiation among North American populations indicates that German stands do not fully represent the haplotype diversity contained in North American populations. This can be explained by a genetic bottleneck during the introduction of the species to Europe. Pettenkofer et al. [21] also mentioned a possible increase in variation within stands due to admixture and multiple introductions of different North American provenances in Southern Germany. Our findings support this conclusion, especially for the federal states North Rhine-Westphalia and Baden-Wuerttemberg.

4.2. Haplotype Distribution and German Red Oak Origin

The number of haplotypes on the North American continent (16 haplotypes) that are completely absent in Germany exceeds by far the number of exclusively German haplotypes (6 haplotypes). Moreover, haplotype A.2 was frequent in many German stands, but rarely present in North American populations, where it is restricted to the Great Lakes region of Upper Michigan and Canada. Finally, the absence in Germany of diverse haplotypes distributed from the Northwestern to Southern areas of the native range points to an origin of most German red oaks from the Northeast of North America (Figure 3, brown frames) [21].

A.3, which is frequent in the center of the native range, is not only rare in German stands, but occurs only in those two stands that supposedly originated from Virginia, USA [32]. One of those two stands from Virginia also contains a single tree with haplotype C.3, which is the only haplotype from the C cluster in Germany. Cluster C is absent in the northeastern edge of the natural range, as well as in other German stands supporting a separate origin of these two stands.

It is unlikely that German private haplotypes are completely novel and cannot be found in North America. It is more likely that rare haplotypes were not found in our sampled trees because of their low frequency and restricted geographic distribution, which caused failure to detect them in the natural range of the species. Thus, additional and more comprehensive collections of populations in North America would be desirable. Rare haplotypes that are exclusive to Germany, such as A.10 or B.4 could then also be of interest to trace the origin of *Q. rubra*. Their identification in a restricted geographic area in North America could further delineate the origin of German stands. Private German haplotype A.18 was found only in a single tree in the stand "Schutterwald 3"and differed from haplotype A.1 only by one mutation (Table 1) suggesting the possibility of a recent mutation.

4.3. Red Oak Stands in North Rhine-Westphalia Might Have Originated from Nova Scotia

The presence of haplotypes B.5 and A.5 only in several stands in North Rhine-Westphalia, Germany, with their simultaneous absence in nearly all other German stands and in most populations of North America suggests a distinct origin of these stands from a very limited geographic area within the natural distribution range. In particular, B.5 and A.5 were observed with low frequency in a Canadian stand on the Peninsula of Nova Scotia. It is possible that A.5 and B.5 haplotypes were not found in other areas of the natural range due to the smaller sample size of North American populations compared to the northeastern Canadian populations. However, the southern parts of Nova Scotia exhibit good geographical exposition for seaborne trade with Boston Harbor, which was assumed to be one of the main transshipment points for northern red oak introduction to Germany [33]. Our inference is consistent with the settlement history of the Nova Scotia area from where the red oak populations were sampled. According to Library and Archives Canada [34] over 2000 German settler arrived in

Nova Scotia in 1750-1752, and in 1753, some of these settlers established the town of Lunenburg. Later, the town of New Germany was established. The sampled Nova Scotia red oak populations are from the New Germany and Lunenburg area. The German settlers may have introduced red oak from Nova Scotia to Germany. Consequently, some North Rhine-Westphalian stands may have originated from the southern parts of Nova Scotia. Since seed traders do not necessarily take genetic variety into account while harvesting acorns, it is plausible that descendants from a limited number of trees resulted in widespread dissemination of single haplotypes that may be rare in the region of origin. This way, anthropogenic influence could create novel haplotype compositions as observed in North Rhine-Westphalian stands.

Haplotype A.5 was identified as a new haplotype based on the analysis of the new cpCAPS markers highlighting the importance of whole plastome sequencing of different provenances for the identification of rare and diagnostic genetic variants. The large sample size of four German stands provided a much better resolution of chloroplast haplotype diversity because of the discovery of several new haplotypes. While the most common haplotype A.1 showed similar shares within the single stands, and therefore was comparable to the chloroplast haplotype based on the cpSSR in Pettenkofer et al. [21], rarer haplotypes with restricted geographic range in North America such as B.1 and B.5 were especially useful.

5. Conclusions

We were able to provide additional evidence that German *Q. rubra* stands originated from the Northern edge of the native distribution area [21,23]. Similar observations were made for French red oak stands [31]. The low haplotype diversity in Germany as compared to North America supports the introduction of *Q. rubra* to Germany from a limited geographic range. Furthermore, our data suggest an introduction of *Q. rubra* from different restricted geographic areas within the natural distribution area and helped us narrow down the putative origin of most German stands in North Rhine-Westphalia to the Peninsula of Nova Scotia in the northeast of the native range. Additional high coverage sampling and cpDNA genotyping in the native range could provide further evidence for the origin of introduced red oak stands.

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