Variable genotypes at the cpDNA marker locus *trnDT* in spontaneous rejuvenation of the species complex around the European black poplar (*Populus nigra* L.) and its relatives collected in Germany

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Abstract

Young poplars of the *Aigeiros-Tacamahca*-complex in spontaneous rejuvenation in Germany that grew along shores, mainly in gravel mines, were sampled and cultivated *ex situ*. The leaf phenotype was assessed. The cpDNA marker locus *trnDT* was investigated which is suitable for the genetic assignment of the chloroplast DNA to a poplar species. The results of Heinze (1998), who investigated the locus *trnDT* in poplars, were chosen as a reference to account for potential methodological differences between the present fragment length analysis results and that obtained with classic gel electrophoresis.

The assignment of each individual to a leaf phenotype category allowed a basic differentiation of *P.-nigra*– or *P.-deltoides*-like and other leaf shapes. At the cpDNA marker locus *trnDT*, fragment lengths referring to the two variants described by Heinze (1998) or referring to at least one more variant were found. The third genotype is of intermediate length between the two that were described by Heinze (1998) and, very likely, the related fragments (approx. 1,065 base pairs = bp long) are specific for balsam poplar species. Quantitatively, the *P.-nigra*-related genotype was the most important in the present collection. It is shown that phenotypically *Aigeiros*-like rejuvenation does predominantly, but not in all individuals, carried the related *trnDT* marker fragments specific for *P. nigra*.

The seed cloud of the *Aigeiros-Tacamahaca*-complex resulting in the present rejuvenation sample trees was predominantly based on diaspores of *P. nigra* or of intra-specific *Aigeiros*-hybrids. However, the section *Tacamahaca* has contributed to the seed cloud. The results suggest that there must be significant gene flow from alien or hybrid poplars in Germany. Further genetic investigations with microsatellite markers are carried out in the present poplar collective for more detailed quantification of the taxonomic composition.

Keywords: *Genetic introgression, spontaneous hybridization, short rotation coppice, invasive species*

Zusammenfassung

Unterschiedliche Genotypen am cpDNA Genort *trnDT* in spontan aufgelaufener Verjüngung des Artkomplexes um die Europäische Schwarz-Pappel und deren Verwandte in Deutschland

Pappeln (Artkomplex um *P. nigra*) aus spontan aufgelaufener Verjüngung in Deutschland wurden beprobt und *ex situ* kultiviert. Der Blattform-Phänotyp wurde erfasst. Der cpDNA-Marker-Genort *trnDT* wurde analysiert, der für die genetische Zuordnung der cpDNA zu einer Pappelart geeignet ist. Die Analyse-Ergebnisse von Heinze (1998) am Genort *trnDT* in Pappeln wurden als Referenz genutzt, um bei der Interpretation der Fragmentlängen-Ergebnisse mögliche methodisch bedingte Fragmentlängenunterschiede zur klassischen Gelelektrophorese zu beachten.

Es erfolgte mit Hilfe des Blattform-Phänotyps eine grobe Unterscheidung von *P.-nigra*- bzw. *P.-deltoides*-artigen Pappeln sowie Arten mit anderen Blattformen. Am cpDNA Genort *trnDT* wurden Fragmentlängen verzeichnet, die 2 von Heinze (1998) beschriebenen Varianten oder einem 3. Genotyp zuzuordnen waren. Dieser 3. Genotyp weist eine zu den 2 anderen intermediäre Fragmentlänge auf. Vermutlich sind diese ca. 1.065 bp langen Fragmente den Balsam-Pappeln zuzuordnen. Das *P.-nigra*-spezifische Fragment kam am häufigsten vor. Die phänotypisch *Aigeiros*-artige Verjüngung trug vorwiegend ein *P.-nigra*-spezifisches Markerfragment am Genort *trnDT*.

Die Samenwolke, die die Verjüngung hervorbrachte, basierte vorwiegend auf Diasporen der Art *P. nigra* oder intra-spezifischen Hybriden. Daneben hat die Sekt. *Tacamahaca* zur Samenwolke beigetragen. Die Ergebnisse legen einen von nicht-heimischen Pappeln oder Hybriden ausgehenden Genfluss in Deutschland nahe.

Schlüsselwörter: *Genetische Introgression, spontane Hybridisierung, Kurzumtriebsplantage, invasive Art*

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1 Introduction

Poplar species (*Populus* spp.) comprise some of the fastest growing tree species of the northern hemisphere, some of which are able to form hybrid swarms among native stands and with cultivated (hybrid) forest stands, shelter belts, alleys or ornamentals. Even species of two different taxonomic poplar sections, the black and the balsam poplars (*Populus* spp. sections *Aigeiros* Duby and *Tacamahaca* Spach), are able to hybridize interspecifically under certain natural conditions. Due to the hybrid vigor, the inter-specific F1 hybrid offspring frequently shows extraordinary fast growth and partially also superior yield. For at least 100 years, the inter- and intra-sectional compatibility was utilized for hybridization breeding followed by clonal propagation of elite clones (Henry, 1914; Houtzagers, 1937; Schreiner and Stout, 1934). There are hundreds of selected clones for both ornamental use and forest or, respectively, agricultural wood production. But only some of them have become very widespread and are now rather overrepresented genotypes in the diaspore clouds.

The European black poplar *Populus nigra* L. is the only species of the *Aigeiros* section native to Europe, Near East and North Africa (Isebrands and Richardson, 2013). In Germany, it is threatened due to the loss of its natural alluvial habitats, especially of sand or gravel banks that allow for the successful germination and seedling survival (Schmitt et al., 1996) and due to genetic introgression of foreign gene material. However, *P. nigra* has intensively been used in many European states that possess big rivers on their territories (van Dam and Bordács, 2002), for hybridization-breeding and for landscaping (e.g. selected forms like the Lombardy poplar, *P. nigra* cv. '*italica*'). In addition, *P. nigra* is a flagship wild species of the riparian ecosystems in floodplains and along shores of European rivers, like the Danube, the Rhine or the Elbe in Germany (Franke et al., 1997; Schmidt and Huber, 2010; Tröber and Wolf, 2015). Especially in floodplain landscapes, hybrid poplar clones have been cultivated (e.g. Böcker and Koltzenburg, 1996) for producing lightweight wood for several historical or rather modern uses, mainly for veneer production, fruit and cheese packaging, pulp and paper, wood composites, matches or carved shoes, matrices and vats. Some European regions, e.g. at the Upper and at the Lower Rhine, partially gather their specific landscape character from monumental poplar rows. During the last 40 years, short rotation tree cropping with poplars has become a major research focus, either for developing wood-based bio-energy supply chains or for establishing bio-based value chains in European bio-economy.

In particular the riparian regions where both the native black poplars and the hybrid clone plantations grow neighbored, the ability of *P. nigra* to spontaneously hybridize bears the risk for genetic introgression into the native *P. nigra* genepool. In the worst case, this genepool can become totally altered by the gene flow from non-native or hybrid individuals. Therefore, the legal framework for the use of non-native or hybrid poplar trees in forests, short rotation coppices (SRC), landscaping or shelterbelts, fosters the protection of the native black poplar genepool.

The potential and the actual extent of genetic introgression into the European genepool of *P. nigra* are still debated (Csencsics and Holderegger, 2016; van Dam and Bordács, 2002). In Germany, recent investigations revealed that there are still considerable numbers of old growth black poplar trees in some regions but rather scarce rejuvenation (e.g. Tautenhahn et al., 2007).

In the FastWOOD project, several partners had the objective to select individuals from native stands and rejuvenation. For the present work, it was initially intended to collect and cultivate wild growing hybrid or potentially native juvenile individuals for future crossbreeding. Later, genetic investigations were carried out in the collection to assess how important the alien diaspores have been for the origination of the sampled wild rejuvenation. Based on a microsatellite approach (Liesebach et al., 2010a; Liesebach et al., 2010b; Rathmacher et al., 2009; Wypukol et al., 2008), collected individuals had to be identified which had originated from unintentionally released propagation material of yet existing cultivars that had to be excluded from the new collection.

For the present work, the DNA (cpDNA) marker *trnDT* was used. It allows the distinction between cpDNA of the two black poplar species *P. nigra* and *P. deltoides* (Heinze, 1998). Within in the present poplar collection, the leaf phenotype was recorded. For the present collection, it was the objective to assess how frequent individuals that exhibit a *P.-nigra*-like leaf phenotype do not carry the *P.-nigra*-specific *trnDT* marker fragment.

2 Material and Methods

2.1 Sampling rejuvenation, ex situ cultivation and leaf phenotype

In Germany, sites which are suitable for poplar seed germination and subsequent rejuvenation establishment are scarce (Schmitt et al., 1996; Tautenhahn et al., 2007), and they often belong to conservation areas in near-natural floodplains. As the initial objective of the present work was scion sampling for *ex situ* cultivation, which can result in severe impact on smaller sampled poplar seedlings, we had to avoid conservation areas. Therefore, poplar rejuvenation, apparently belonging to the sections *Aigeiros* or *Tacamahaca*, was sampled only on anthropogenic wetland biotopes like in actively working gravel mines. Using publicly available google satellite images, open mineral soil areas that comprise shore lines with water bodies were identified in five German federal states (14 sampling sites, Figure 1, Table 1). After contacting the land owners, sampling was carried out in the late winter or early spring seasons between 2008 and 2015. Mainly, rejuvenation which had survived the seedling stage was found along the shores of artificial lakes or, only partially, in river bank reinforcements. A visual assessment of the site and of the individuals growing in succession stands of poplars and willows was carried out to avoid sampling of root suckers growing of unintentionally transplanted roots. No rubble

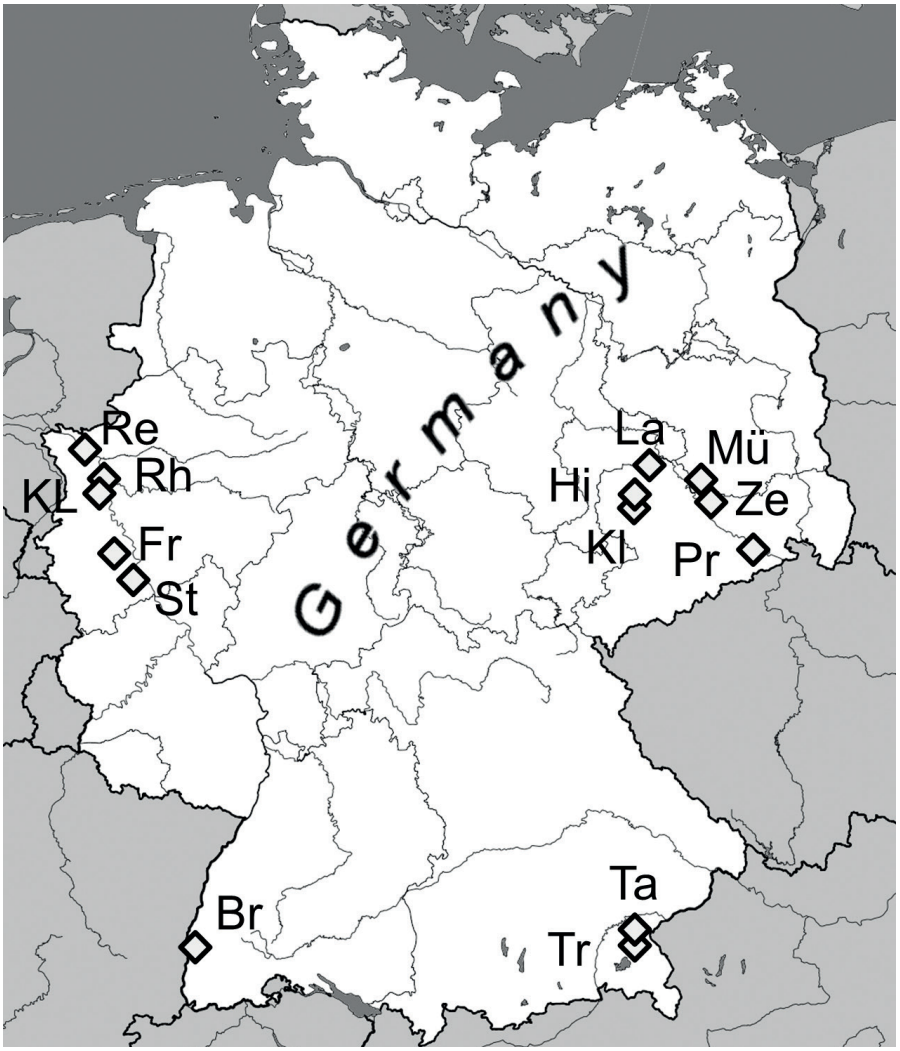


Figure 1
Sampling sites, given with abbreviations as explained in Table 1. Map file originally published by NordNordWest, download from <https://commons. wikimedia.org> (under the license described at <https://creativecommons.org/ licenses /by-sa/3.0/deed.en>), image was modified.

fields or other ruderal sites bearing the risk for anthropogenic root deposition were sampled. If possible, some young trees were torn out to check whether the root system originated from displaced roots or branches. Such rejuvenation was not sampled. Only individuals that (most likely) originated from seed disposal and germination were collected. The height of sampled individuals was between approximately 0.5 m and several meters corresponding with the late seedling or with the sapling stage, respectively. The spatial distribution and number of young poplars was very variable within and among the collection sites. Also, the collection sites varied substantially in their area. Therefore, no equal sample size per site and no equal sampling systematics were applied. In case of more abundant rejuvenation, only dominant individuals were sampled that were spatially distributed over the rejuvenation stand.

One top shoot per individual was cut off for cultivation *ex situ* in the experimental garden at the Department of Forest Sciences of the TU Dresden in Tharandt, Germany. Cuttings of approximately 20 cm length were planted in QuickPot nursery trays (0.65 l pot volume, HerkuPlast Kubern GmbH). All individuals were grown in a substrate mixture consisting of 2 parts by volume of sand and one part of the planting substrate ED73 (Einheitserde Werkverband e.V., distributed by Hermann Meyer KG). Older plants of the collection were cut back once or twice after several years. During cultivation, 28 genotypes died back, and only 143 of the formerly collected 171 genotypes were sampled for the present genetic and leaf analyses (Table 1).

In the summer 2016, leaves were visually assessed and the shape of mature leaves of the genotypes was assigned to leaf phenotype categories described in the taxonomic identification key of Roloff et al. (2014) (Table 2). Three to four fully developed, healthy and non-chlorotic leaves were sampled per genotype from positions between 50 to 75 % of plant height, approximately. At that time, the shoots of the potted plants in the genotype collection were in their 2nd or 3rd year of growth and approximately 0.5 to 1 m high. The

Table 1
Sampling sites, given with the federal state in Germany (BW Baden-Württemberg, BY Bavaria, BRB Brandenburg, NRW North-Rhine Westphalia, SN Saxony), and with the closest river plain or stream plain that the site is closely situated to

Sampling site			Total no.			No. of genotypes per cpDNA class		
Municipality	Federal state	Closest river or stream	collected	dying	cpDNA sampled	< 900 bp	1,000- 1,100 bp	> 1,100 bp
Breisach (Br)	BW	(Upper) Rhine	5	3	2	2	-	-
Tacherting (Ta)	BY	Alz	13	2	11	11	-	-
Trostberg (Tr)	BY	Alz	19	3	16	5	10	1
Mühlberg (Mü)	BRB	Elbe	4	1	3	3	-	-
Frechen (Fr)	NRW	Erft	6	1	5	-	3	2
Straßfeld (St)	NRW	Erft	9	-	9	1	8	-
Kamp Lintfort (KL)	NRW	(Lower) Rhine	10	-	10	5	4	1
Rees (Re)	NRW	(Lower) Rhine	12	1	11	7	1	3
Rheinberg (Rh)	NRW	(Lower) Rhine	43	9	34	19	13	2
Pratzschwitz (Pr)	SN	Elbe, Wesenitz	9	1	8	1	4	3
Zeithain (Ze)	SN	Elbe	14	1	13	11	2	-
Laußig (La)	SN	Mulde	9	1	8	6	-	2
Kleinpösna (Kl)	SN	Parthe	9	1	8	5	-	3
Hirschfeld (Hi)	SN	Parthe	5	-	5	4	1	-
		Total	**171**	**28**	**143**	**80**	**46**	**17**

Total numbers; i.e. the number of collected genotypes; the number of genotypes that died in cultivation; the number of successfully ex situ sampled genotypes for the present analysis; as well as the number of genotypes assigned to cpDNA-genotype classes carrying the trnDT marker fragment with the estimated length of approximately 865 bp (here < 900 bp, referring to *P. nigra*), or approximately 1,065 bp (1,000-1,100 bp, likely referring to the section *Tacamahaca*) or approximately 1,135 bp (> 1,100 bp, referring to *P. deltoides*), Heinze (1998), see Figure 2.

plants were all equally suppressed in growth due to the small pot volume.

2.2 DNA extraction, analysis of the cpDNA locus trnDT

The sampled leaves of each collected genotype were used for DNA extraction. In addition, to provide reference cpDNA samples with known taxonomic identity, eight trees were sampled in the Saxon State Arboretum (Tharandt Forest Botanic Garden of the TU Dresden, Tharandt, Germany), or material was received from the Northwest German Forest Research Institute (NW-FVA, Hannoversch Münden, Germany). Among the 8 reference genotypes were 4 *P.nigra* (2 from the arboretum, 1 from the NW-FVA (NW9-487D, cv. 'Rheinaue') and 1 natural monument from Dresden, Germany), 2 *P. trichocarpa* (cv.'Brühl 8', and one from the arboretum), 1 *P. maximowiczii* and 1 *P. deltoides* (both from the arboretum). All samples were frozen until further laboratory processing. For one extraction sample, 100 mg of healthy leaf material was weighed and milled for 3 min at f = 30 Hz with a vibration mill, type MM200 (Retsch GmbH, Haan, Germany). The DNeasy Plant Mini Kit was used and the related handbook protocol of the manufacturer (QIAGEN GmbH, Hilden, Germany) was followed. Extraction success was tested on agarose gels (1.5 % gel, (150 ml TBE solution + 2.25 g agarose); V_{sample} = 3.0 µl (2.0 µl of template DNA solution + 1.0 µl of 6X DNA Loading Dye, ThermoScientific); run for 60 min at 120 V in a midi electrophoresis unit; staining with ethidium bromide). The PCR was carried out using the fprimer *trnD* (ACC AAT TGA ACT ACAATC CC) and the rprimer *trnT* (CTA CCA CTG AGT TAA AAG GG) as described in Heinze (1998), based on the work of Demesure et al. (1995). The fragment length estimates given by Heinze (1998) are approximately 850 bp (*P. nigra*) and approximately 1,050 bp (*P. deltoides*). For the present work, slightly different fragment length values were expected, because a capillary unit was used for fragment length estimation (described below) instead of classical gel electrophoresis which was used by Heinze (1998). This cpDNA marker locus was chosen because it detects fragment length polymorphisms between *P. nigra* and *P. deltoides* with the PCR step only, without applying a subsequent restriction enzyme digestion. The same simple and very effective method was successfully applied by Ziegenhagen et al. (2008) and by Bialozyt et al. (2012). Another length polymorphism between *P. nigra* and *P. deltoides* (psbA-matK) amounts to 6 bp only (Schroeder et al., 2017). Several SNPs to distinguish *P. nigra* and *P. deltoides* are located within fragments of identical length (Schroeder et al., 2012) and need more laboratory efforts.

The PCR was carried out with a C1000TM cycler (Bio-Rad Laboratories GmbH, Munich, Germany) in 25 µl reaction volume (0.5 µl of template DNA solution (20 ng/µl) and 0.5 µl of each primer solution were taken up in a master mix consisting of 10.0 µl H20, 12.5 µl TopTaq Master Mix and 1.0 µl MgCL2 solution, both Qiagen GmbH, Hilden, Germany). The initial denaturation was carried out for 3.0 min at 94 °C,

followed by 35 cycles of 30 sec denaturation at 94 °C, 30 sec annealing at 57 °C, 60 sec of elongation at 72 °C, and followed by a final elongation of 600 sec at 72 °C (final cooling step at 10 °C). The success of the PCR was tested on agarose gels (same gel and run conditions as described above for extraction products, but 5 µl of PCR products were loaded).

The final fragment length estimation was carried out with the parallel capillary electrophoresis instrument 'FragmentAnalyzer™' and the related software package PROSize 2.0 (Advanced Analytical Technologies GmbH, Heidelberg, Germany). The dsDNA Reagent Kit, 35 bp – 5,000 bp, DNF915 was used (Advanced Analytical Technologies GmbH), and the separation run time was 80 min. To assess the accuracy of this technique, little information is available. One possible true length of 961 bp for the *trnD-trnT* PCR fragment is given for *P. trichocarpa* (clone Nisqually1, Tuskan et al. 2006, GenBank Accession EF489041.1). The respective fragment length for a *P. nigra* sample amounts to 819 bp (GenBank Accession KX377117.1). For *P. deltoides*, comparable chloroplast sequence data are not yet available. The mean deviation of the fragment length estimations with the FragmentAnalyzer™ is approximately 5 % to 10 % (referring to approximately ± 50 to ± 100 bp ranging uncertainty for length estimates of 1,000 bp long fragments) in the laboratory where the present investigation was carried out (Figure 3).

2.3 Data processing and statistics

Data graphs were generated and statistical tests were performed with SPSS Statistics for Windows version 25.0.0 (IBM, Armonk, NY, USA). It was tested whether there are significantly different fragment length (FL) estimates (FragmentAnalyzer™) for the *trnDT* amplicon across the subgroups which exhibited different leaf phenotypes. Two subgroups of the factor variable 'leaf phenotype' comprised only two individuals (oval, elliptic). These subgroups, in total 4 of 143 individuals, were excluded. Hence, five subgroups were considered for testing quadrangular, triangular, triangular-oval (all *Aigeiros*-like), ovate or ovate-lanceolate (both *Tacamahaca*-like). The subgroups had unequal sample size (n = 11 to 69, Table 2, last column), and the assumptions of normality of FL data within, and of homogeneity of FL variances among subgroups were clearly violated. Therefore, the FL difference was tested pairwise across the leaf phenotype subgroups with non-parametric MannWhitney Utests ($\alpha = 0.05$).

3 Results

The genetic investigation of the collected 143 poplar individuals at the cpDNA locus *trnDT* revealed three maxima in the distribution of fragment length (FL) values which were estimated for the amplicons by means of capillary electrophoresis (FragmentAnalyzerTM). The histogram (Figure 2) suggested the existence of three different FL classes at the locus *trnDT*. This is at least one FL class more than described in the work of Heinze (1998). The first FL class comprised all genotypes with fragment length values shorter than 900 bp, the second class all samples with values between 1,000 and 1,100 bp, and the third class all with values longer than 1,100 bp. The mean FL values per class were 865 bp (standard error ± 1.1), 1,065 bp (± 2.4), and 1,135 bp (± 3.0) (given as $\bar{x}$ in Figure 2).

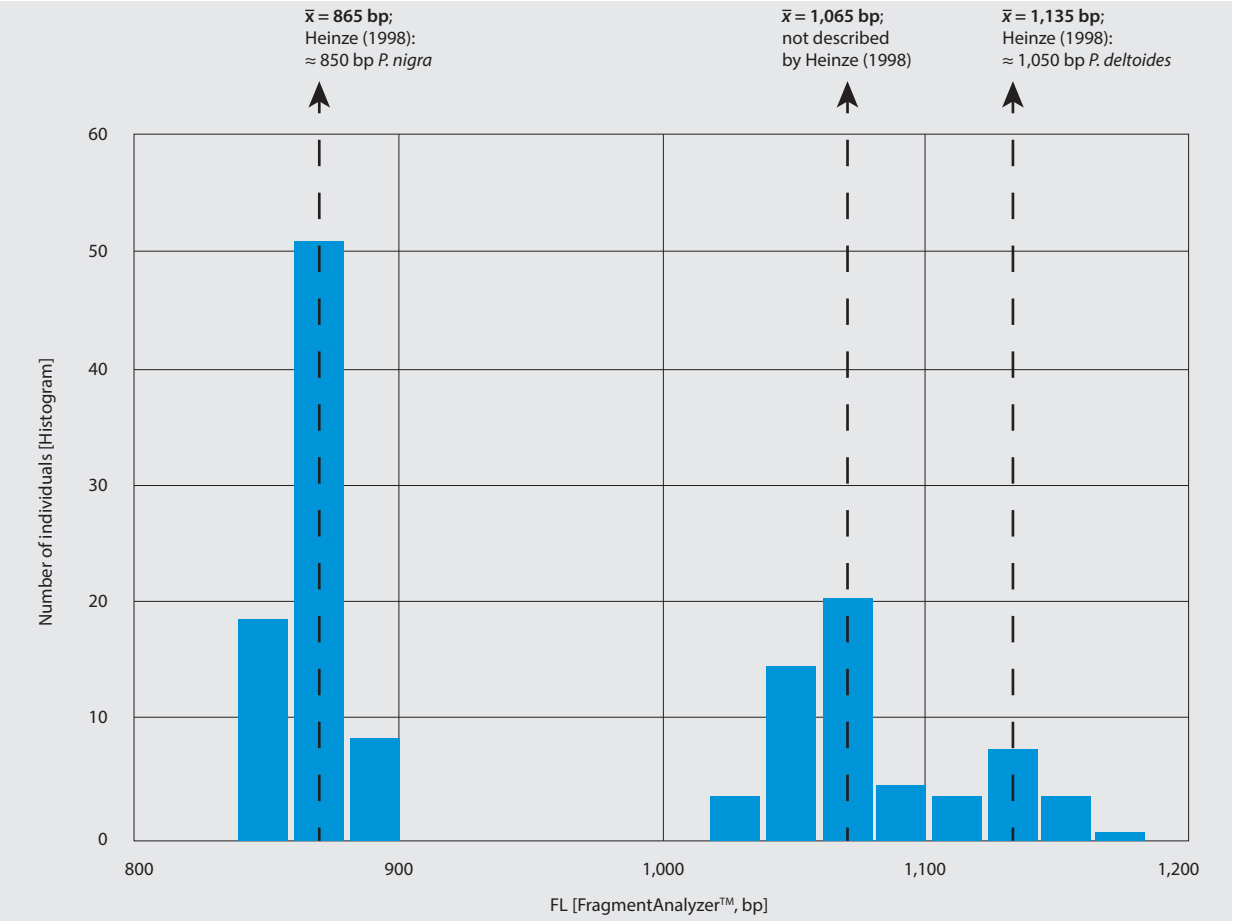
The reference genotypes which were sampled in the Saxon State Arboretum had the analysis results shown in Figure 3. The *trnDT* amplicons of the four *P. nigra* reference

Table 2

Leaf phenotypes found in the *ex situ* cultivated poplar (*Populus* spp.) collection, separately counted in cpDNA-genotype classes carrying the *trnDT* marker fragment with the estimated lengths of < 900 bp (assigned to *P. nigra*), 1,000-1,100 bp (likely referring to the section *Tacamahaca*) or > 1,100 bp (assigned to *P. deltoides*)

	Leaf phenotype	Number of individuals per cpDNA-genotype class			Total
		< 900 bp	1,000-1,100 bp	> 1,100 bp	
Aigeiros - like	quadrangular [a]	10	–	1	**11**
	triangular [a]	11	1	2	**14**
	triangular-oval [a]	**57**	2	**10**	**69**
Tacamahaca - like	oval	–	1	1	**2**
	ovate [b]	1	**30**	2	**33**
	ovate-lanceolate [b]	1	10	1	**12**
	elliptic	–	2	–	**2**
	Total	**80**	**46**	**17**	**143**

[a, b] Subgroups a and b leaf phenotypes have significantly different fragment length estimates at the cpDNA marker locus *trnDT* (*p*-values in Table 3, subgroups oval and elliptic were not included in testing due to minor sub-sample size).



Dashed lines represent the arithmetic means for the three FL classes which were defined on the basis of the histogram: < 900 bp, 1,000 -1,100 bp, > 1,100 bp. Two of the present measured FL classes correspond to the *trnDT* variants specific for *P. nigra* or for *P. deltoides* described by Heinze (1998).

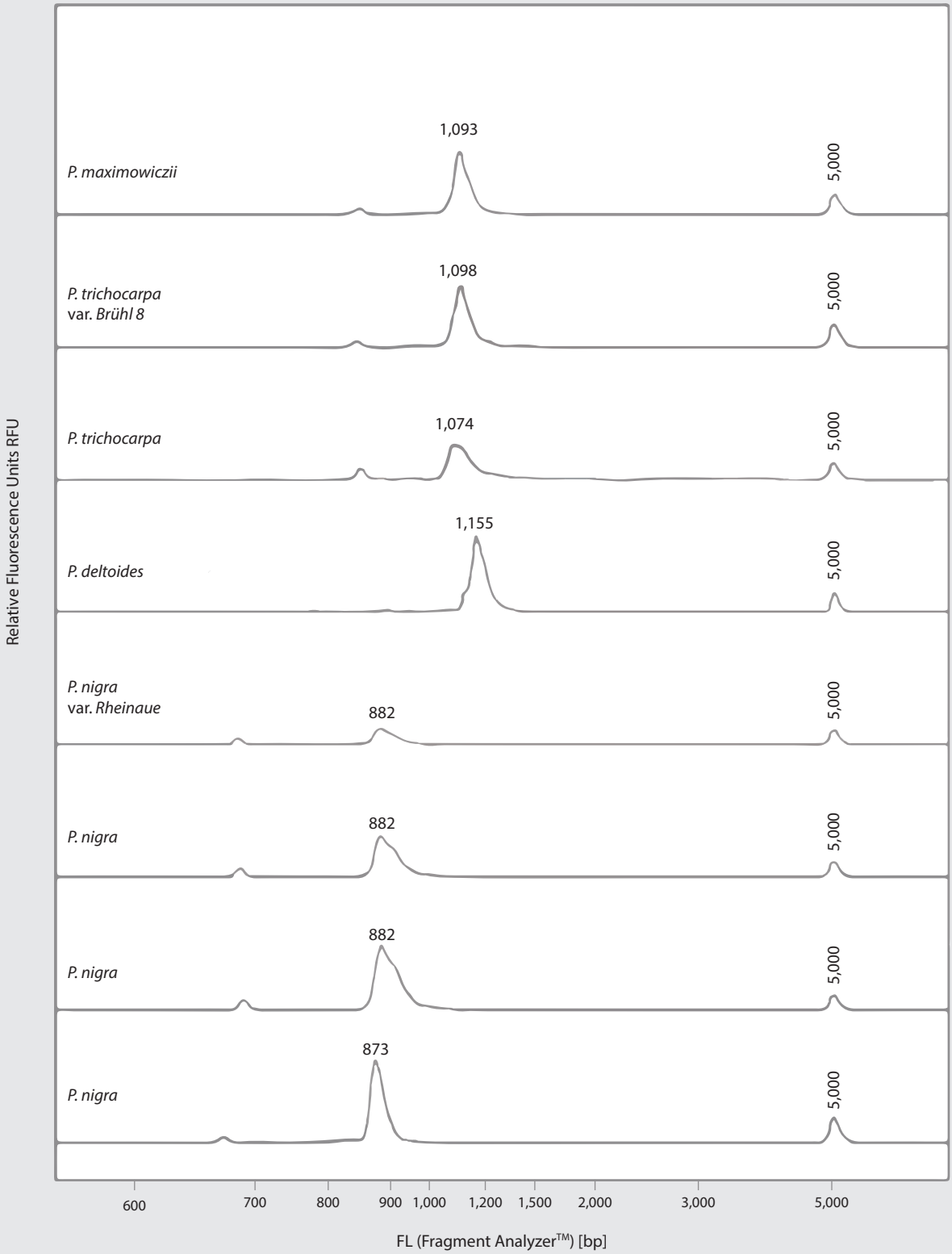
Figure 2
Histogram showing the number of genotypes per class of the fragment length (FL) in base pairs [bp] which was obtained with the FragmentAnalyzer™ (Advanced Analytical).

genotypes were estimated to be 873 or 882 bp long, that of the *P. deltoides* reference genotype was 1,155 bp long, and those of the three balsam poplars were 1,093 (*P. maximowiczii*), 1,074 or 1,098 bp long (*P. trichocarpa*), respectively. Both estimates given by Heinze (1998), that for the *trnDT* fragment specific for *P. nigra* and that specific for *P. deltoides*, were shorter than the respective mean fragment length values for the FL classes in the present work (850 bp vs. 865 bp for *P. nigra* and 1,050 bp vs. 1,135 bp for *P. deltoides*).

Seven leaf phenotypes (following the classification system of Roloff et al., 2014), were observed in the present poplar collection: quadrangular, triangular, triangular-oval, oval, ovate, ovate-lanceolate and elliptic leaf shapes (Table 2). 69 of the 143 investigated individuals carried triangular-ovate leaves. Together with the triangular (n = 14) and the quadrangular (n = 11) phenotypes, these three long-petiole phenotypes are often taxonomically assigned to the black poplar species *P. nigra* and *P. deltoides* (section *Aigeiros*). As it turned out, the majority of these *Aigeiros*-like leaf phenotypes were associated with the *trnDT* fragment length of approximately 865 bp (n = 78) or, to a smaller extent, with the FL of 1,135 bp (n = 13). Only three of the phenotypically *Aigeiros*-like individuals were genotypes with the FL of approximately 1,065 bp. However, their FL estimates and their class assignment may be false due to the measurement uncertainty described in the Material and Methods chapter. One of the three individuals had a FL estimate very close to the 1,100 bp class boundary, and its *trnDT* variant possibly belongs rather to *P. deltoides*.

The mean fragment length of 1,065 bp was, in turn, mainly associated with leaf phenotypes that are often taxonomically assigned to balsam poplar species (oval, ovate or elliptic leaves with rather short petioles). The majority of the individuals showing balsam-poplar-like (*Tacamahaca*-like) leaves exhibited the ovate leaf phenotype. 30 of 33 individuals with an ovate leaf phenotype as well as 10 of 12 individuals with ovate-lanceolate leaves belonged to the genotypes with the approximately 1,065 bp long fragment.

The Mann-Whitney U-tests, which were performed pairwise across the leaf phenotype subgroups to test for significant FL differences, were not significant between the three *Aigeiros*-like leaf phenotypes and not between the two major *Tacamahaca*-like leaf phenotypes (p-values in Table 3). Instead, the FL difference was significant for all



The graph (modified) was generated with the software package PROSize 2.0 for fragment lengths (FL, [bp base pairs]) that were obtained with the capillary unit FragmentAnalyzer™ (both Advanced Analytical Technologies). Each lane refers to the cpDNA of one reference tree which belong to the collections of the Saxon State Arboretum or the Northwest German Forest Research Institute (NW-FVA). Grey numbers indicate the length of internal standard fragments added to each of the measured PCR products for the FragmentAnalyzer™ run.

Figure 3
Peak view of poplar (*Populus* spp.) PCR amplicons, cpDNA locus *trnDT*

tested pairs of one *Aigeiros*-like and one *Tacamahaca*-like leaf phenotype.

In total, 80 genotypes carrying the approximately 865 bp long fragment (cpDNA assigned to *P. nigra*), 46 having a fragment length of approximately 1,065 bp (i.e. cpDNA assigned to a balsam poplar species) and 17 genotypes with the approximately 1,135 bp long fragment (cpDNA assigned to *P. deltoides*) were found. At all 14 sampling sites, either the *trnDT* genotype class carrying the approximately 865 bp long fragments or that class having a fragment length of approximately 1,065 bp dominated over the respective other two classes (Table 1). At no sampling site, the genotype class with the approximately 1,135 bp long fragments (cpDNA assigned to *P. deltoides*) was dominating. Only two sampling sites (Breisach (n = 2) and Tacherting (n = 11)) did not reveal other than the 865 bp genotypes.

Table 3

Significances (*p*-values) resulting from pairwise Mann-Whitney U Tests for testing differences of fragment length estimates (FragmentAnalyzer™) across subgroups of poplars showing different leaf phenotypes (α = 0.05) (Table 2)

p-values pairwise Mann-Whitney U-tests	quadrangular	triangular	triangular-oval	ovate	ovate-lanceolate
Quadrangular		0.467	0.413	< 0.001	0.001
Triangular	–		0.995	< 0.001	0.003
Triangular-oval	–	–		< 0.001	0.001
Oval	–	–	–		0.331
Ovate-lanceolate	–	–	–	–	

4 Discussion

The present investigation focused on the genetic composition of spontaneously occurring natural rejuvenation of black or balsam poplars or their intra- and inter-specific hybrids (*Populus* spp., sections *Aigeiros* and *Tacamahaca*) on anthropogenic wet sites in Germany. The cpDNA and the leaf morphological investigations have, separately from each other, revealed that there are rather large proportions of foreign gene material that have contributed to the establishment of the sampled rejuvenation. However, the present collection of genotypes does not congruently reflect the proportion of foreign gene material *in situ* at the collection sites. Furthermore, several collected genotypes were lost during the *ex situ* cultivation. Hence, no exact quantification of the genetic admixture or of the different species' contribution at the collection sites can be done on the basis of the present results.

The investigation of the cpDNA locus *trnDT* was subject to a method-based uncertainty in fragment length (FL) measurements. As the present method (FragmentAnalyzer™) had an accuracy of ± 5 % to ± 2 %, the obtained FL values are only estimates of the respective true FL which corresponds to genotype class. As a consequence, the present capillary gel-electrophoresis method and also the classic gel electrophoresis method used by Heinze (1998) are not able to allow totally safe distinction between two fragments of almost equal true lengths. Nonetheless, the three maxima in the present stochastic distribution of the FL measurements revealed, that there are at least three different classes in the poplar collection at the locus *trnDT* (see histogram, Figure 2).

The FL estimates for the four *P. nigra* reference trees (873 or 882 bp) allowed the assignment of the first class of fragment lengths (< 900 bp, mean approximately FL of 865 bp) to the 850 bp variant which was described to be specific for *P. nigra* (Heinze 1998). Due to the large length difference, the FL class of the approximately 865 bp long fragments was well separated from the two other FL classes (1,000-1,100 bp; > 1,100 bp).

The marker fragment of the *P. deltoides* reference tree had an estimated length of 1,155 bp. Therefore, the FL class with approximately 1,135 bp long fragments was assigned to the 1,050 bp variant which was described to be specific for *P. deltoides* by Heinze (1998). The third FL class with the intermediate FL of approximately 1,065 bp (1,000-1,100 bp) refers to at least one additional genotype which was not described by Heinze (1998). The existence of at least one other class is supported by the present FL estimates for the reference trees which belong to the two balsam poplar (section *Tacamahaca*) species *P. maximowiczii* (1,093 bp) and *P. trichocarpa* (1,074 or 1,098 bp). Given the methodological measurement uncertainty in addition to the within-species variability, it is not possible to distinguish among the two balsam poplar species. In addition, the approximate length of the balsam poplar variants is relatively close to the fragment length of the *P. deltoides* genotypes. Presumably, the ranges of FL estimations of the balsam poplar and *P. deltoides* classes have overlapped. Hence, the present boundary between the present two FL classes at 1,100 bp is inevitable somewhat arbitrary and needs more efforts for determining correct FLs by sequencing of reference samples. The number of reference trees with assured taxonomic assignment needs to be increased beyond the low number of reference trees in the present work. In addition, chloroplast SNP markers (Schroeder et al. 2017; Schroeder and Fladung, 2015) can be added to achieve higher precision in the taxonomic assignment of the female line in collected wild samples of *Populus* spp.

It must be noted that the present results do only allow the identification of the species origin of the chloroplasts which are only inherited by the female poplar parent to the generatively produced offspring (White et al., 2007). Nothing can be said about the specific genetic identity of the investigated individuals. E.g., a female parent that inherits a *trnDT* fragment of approximately 865 bp length is not necessarily a pure *P. nigra* tree. For example, it can be a hybrid of the second generation with *P. deltoides*, or with other species.

The present results for the locus *trnDT* suggest that not only species or intra-specific hybrids of the section *Aigeiros* (e.g. all *P.* × *canadensis* clones) but also the section *Tacamahaca* can

currently have considerable impact on the diaspore cloud in parts of Germany. This is also supported by literature. Balsam poplar clones like *P. trichocarpa* cvs. 'Muhle Larsen' or 'Fritzi Pauley', *P. maximowiczii* × *P. trichocarpa* cv. 'Androscoggin' and other were cultivated mainly during the second half of the 20th century (Grosscurth, 1983; Hoffmann et al., 1977; Jestaedt ,1978; Joachim, 1957; van den Broeck et al., 2005; Weisgerber, 1983). Now, balsam poplars or their hybrids can be found as mature or old, flowering stands in German forests, shelterbelts and urban groves.

Interestingly, 78 of the 94 individuals which exhibited *Aigeiros*-like leaf phenotypes carried the *P. nigra*-specific *trnDT* genotype (approximately 865 bp, Table 2). Only 13 carried the *P. deltoides* variant. The remaining 3 individuals had *trnDT* fragment lengths that were assigned to the cpDNA of balsam poplars. However, this assignment can be false due to the fragment length measurement uncertainties described above.

The intra-specific hybrids of the two *Aigeiros* species *P. nigra* and *P. deltoides*, *P.* × *canadensis* (syn. *P.* × *euramericana*), were intensively used throughout Europe and the hybrids are found in spontaneous rejuvenation (van Dam and Bordács, 2002). For this intra-specific *Aigeiros* hybridization, *P. deltoides* was chosen as the seed parent (♀) and *P. nigra* as the pollinator (♂) (Ziegenhagen et al., 2008). Likely, the present observation of the quantitatively superior cpDNA marker genotype of *P. nigra* (Table 2) is due to the unilateral pollination incompatibility of *P. nigra* against *P. deltoides*' pollen or due to a more general preference of all black poplar species for the pollen of their own species (Rajora, 1989). However, it was reported that the second generation hybrids or back-cross-hybrids between *P. nigra* and *P. deltoides* seem to reduce the incompatibility, and a stable genetic introgression into the *P. nigra* genepool has to be expected in naturally reproducing sub-populations (Pospíšková and Šálková, 2006; Ziegenhagen et al., 2008). The same effect should be expected for the hybridization with balsam poplar species.

The genetic diversity of the European black poplar in Germany is potentially also threatened by the anthropogenic overrepresentation of a few *P. nigra* clone cultivars (e.g. Lombardy poplar, *Populus nigra* cv. *italica*). But the investigation of the cpDNA marker locus *trnDT* cannot provide information about the contribution of over-represented genotypes to the rejuvenation sample. Therefore, the present poplar collection will be subject to further investigations with microsatellite markers that allow a more detailed assessment of the genetic identity and of the extent of introgression.

To conclude, the genetic composition of German wild poplar rejuvenation is subject to ongoing genetic introgression. It initiated rather long ago by the anthropogenic use of introduced poplar species (of the sections *Aigeiros* and *Tacamahaca*) and of their artificial hybrids.

For future conservation strategies and for practical implications, it has to be considered that many of the existing mature hybrid poplar stands cannot simply be felled everywhere in Germany or Europe and that they will continue providing diaspores. Therefore, conservational cloning of valuable autochthonous *P. nigra* and planting them in the vicinity of other autochthonous individuals can be a strategy to increase the contribution of autochthonous gene material to the diaspore clouds of *P. nigra* and its relatives in the near future.

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