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SSR and SNP Markers for The Identification of Clones, Hybrids and Species Within The Genus *Populus*

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Abstract

Several poplar species within a section, but also between sections, are cross-compatible, thus a high

number of interspecies-hybrids occur naturally or have been artificially produced during the last 100 years. Very often, systematically kept records on the production or vegetative propagation of poplar hybrids and/or clones have not been available to date. Hence the origin of the poplar plant material used for the generation of hybrids or clones is not quite clear in many cases, thus making the differentiation between the clones a difficult task. Therefore, genetic markers are needed to clearly identify and differentiate the species and hybrids in the genus *Populus*, including both identification of existing clones and the breeding of new ones. One aspect of this

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study is therefore to develop molecular markers for the identification and differentiation of species, hybrids, and clones of the genus *Populus*.

Key words: Populus, clones, hybrids, species, SSRs, SNPs.

Introduction

The genus *Populus* comprises more than 29 species, but this number is not quite clear, because of numerous hybrids sometimes declared as species (ECKENWALDER, 1996). Many of the species are of high scientific and economic importance (LICHT et al., 2005; VANDE WALLE et al., 2007), e.g., for biomass production in short rotation coppices (SRC). The genome of *Populus trichocarpa* is one of the first in the plant kingdom that has been totally sequenced.

In plant systematics, *Populus* species are classified in six different sections (Populus, Tacamahaca, Aigeiros, Abaso, Turanga and Leucoides (ECKENWALDER, 1996). Many of the poplar species within a section, but also between sections, are cross-compatible to each other. Thus, a high number of interspecies-hybrids occur naturally or have been used as breeding material (STETTLER et al., 1996). For the use in SRCs in particular, interspecies-hybrids are well suited because of their superior growth and advanced resistance parameters.

Decades of breeding work caused multiple crosses and backcrosses in the clones used today. Therefore, the identification of the parent's origin of the clones is needed. Furthermore, species identification before generating of new clones is essential for future poplar breeding.

So, the aim of this study is to describe the development of molecular markers for the identification and differentiation of species, hybrids, and clones within the genus *Populus*.

SSRs

Overview

Microsatellites or SSRs (simple sequence repeats) are repeating sequences of two to six basepairs of DNA (CHAMBERS and MACAVOY, 2000). SSRs are typically neutral and co-dominant inherited and often occur in noncoding DNA regions. The mutation process in microsatellites differs from a "classical" point mutation (which leads to a single substitution of bases). In microsatellites the mutation process is a slippage replication, so-called 'slipped-strand mispairing' (LEVINSON and GUTMAN, 1987). This means adding or subtracting single repeats. Microsatellites are born from regions of 'cryptic simplicity', i.e., regions in which variants of simple repetitive DNA sequence motifs are already over represented (TAUTZ et al., 1986).

The number of repetitions of the sequence of a microsatellite varies between individuals, within populations or sometimes between species. Thus, they are highly polymorphic. That's the reason why they are extremely useful for population genetics analysis and differentiation or identification of clones, respectively. For example, microsatellite markers are used for parentage analysis and to address questions concerning the degree of relatedness of individuals or populations. They can be used to analyse genetic structure of populations, to assess demographic history (e.g., to look for bottlenecks) and gene flow between populations. Last but not least, SSR marker analyses provide data suitable for phylogeographic studies (SELKOE and TOONEN, 2006).

SSRs in poplar

The highly polymorphic SSRs are excellent markers for clone and hybrid identification, in particular in different poplar species like *P. tremuloides* and *P. deltoides*, according to DAYANANDAN et al. (1998), RAHMAN et al. (2000), RAHMAN and RAJORA (2002), RAJORA and RAHMAN (2003), VAN DER SHOOT et al. (2000). SSR polymorphism may also occur even between closely related individuals. For example, it was possible to use SSR markers developed in *Populus tremuloides* (DAYANANDAN et al., 1998) for differentiation of individuals in other poplar species such as *P. deltoides* and *P. nigra*.

In relation to the publication of the complete genome sequence of *P. trichocarpa*, a very high number of new SSR markers have been identified (http://www.ornl.gov/sci/ipgc/ssr_resource.htm; TUSKAN et al., 2004). Transferability of the *P. trichocarpa* SSR marker to other species of the section Tacamahaca or Ageiros seems very high, however, for species of the section Populus, transferability was very low compared to the number of tested SSR primer pairs (PAKULL et al., 2009). For fine mapping of the sex locus in *P. tremuloides*, a total of 90 primer pairs amplifying SSR-loci were developed based on the *P. trichocarpa* (scaffold 19) genomic sequence and tested for amplification and heterozygosity/segregation in both parents and in four F1-individuals. Of the primer pairs tested, about 60% amplified either in none or only in one of the parents (PAKULL et al., unpublished data). Only 21.1% were polymorphic and could be tested in all individuals of the mapping population.

For the identification and management of hybrid poplar accessions, BEKKAOUI et al. (2003) classified different genotypes of the Prairie Farm Rehabilitation Administration (PFRA) breeding programs. In this study it was possible to show that two clones, foreseen to include in a breeding program, have similar DNA profiles with nine SSR markers, suggesting that these clones are identical. This observation was important because unnecessary duplication of the two accessions in breeding programs could be prevented.

Further, SSR markers have been successfully employed for analysis of natural hybridization events between cultivated poplars and their wild relatives (VAN DEN BROECK et al., 2005). Evidence has been provided that introgressive hybridization and gene flow from domesticated species into their wild relatives can have a profound effect on the persistence and evolution of wild populations. In this study, examples of natural and anthropogenic hybridization were given and possible consequences of anthropogenic hybridization are discussed in order to finally suggest conservation strategies.

In another study, SMULDERS et al. (2008) have analyzed different black poplar seedlings and young trees

that occurred spontaneously along the Rhine River and its tributaries in the Netherlands. Only a few native *P. nigra* L. populations could be detected in combination with many planted *P.* x *canadensis* trees. The results clearly indicate that nearly half of the sampled trees were not pure *P. nigra* but progeny of natural hybridisation that had colonised the Rhine River banks. Furthermore, the taxonomic status of 208 adult *Populus nigra* and *P.* x *canadensis* trees in the population and 140 young poplars along the riverbank was monitored (ZIEGENHAGEN et al., 2008). From the analysed young poplars, almost 20 percent were found to exhibit at least one of the two *P. deltoides* or *P.* x *canadensis* diagnostic alleles or genotypes, respectively. Finally, in analyses of short-distance gene flow in *Populus nigra* L., RATHMACHER et al. (2010) presented evidence for small-scale spatial genetic structures and raised implications for in situ conservation measures. And ten nuclear microsatellite markers have been tested for identification of poplar clones comprised of eleven species belonging to three sections (LIESEBACH et al., 2010).

Identification of Populus clones using SSRs

In our study, we tested 290 microsatellites markers developed for *P. trichocarpa* (http://www.ornl.gov/sci/ipgc/ssr_resource.htm) in the five species *P. deltoides*, *P. nigra*, *P. tremula*, *P. tremuloides*, *P. alba* for presence of PCR amplification products. After the first screening round, 65 microsatellites remained which were then tested for reproducible polymorphisms within the five species mentioned above and *P. trichocarpa*. These results and further technical criteria (e.g., use of several SSRs for multiplexing) resulted in a set of twelve SSR markers (*Table 1*) usable for reproducible amplifications in six *Populus* species.

The twelve SSR markers have been tested in a proof-of-principle in 297 individuals belonging to 54 clones of the three species of the section Populus (*P. tremula*, *P. tremuloides*, *P. alba*) to validate the identification of clones grown in the arboretum of the Institute for Forest Genetics (Grosshansdorf, Germany). In 29 out of the 54 clones, more than one ramet was analyzed. Astonishingly, in 15 of the 29 clones investigated, individuals classified so far as ramets showed different SSR alleles, thus, these individuals are not members of the same clone.

Table 1. – Characteristics of the twelve used microsatellite markers for 297 individuals from six *Populus* species.

Locus	Motif	Size range	number alleles
GCPM1831	(AG)$_x$	189-217	9
PMGC0433	(GA)$_x$	178-208	11
PMGC2679	(GA)$_x$	92-112	7
PMGC2163	(GA)$_x$	190-226	14
PMGC2550	(GA)$_x$	134-156	11
WPMS16	(GCT)$_x$	153-189	10
GCPM3362	(CT)$_x$	208-252	10
GCPM1608	(TA)$_x$	149-191	12
GCPM1532	(AA)$_x$	202-258	13
GCPM0354	(GT)$_x$	154-256	9
PMGC2826	(GA)$_x$	201-233	14
PMGC2607	(GA)$_x$	124-150	9

In a second investigation, for four *Populus* clones grown in *in vitro* culture the origin was unclear. A set of ten different individuals grown in the greenhouse was considered to be the donor individuals for the *in vitro* grown clones. By employing six different SSR markers, the four in vitro grown clones could clearly associate to four individuals belonging to the mentioned set.

Taken together, these investigations revealed the problems arisen from long term "human-use" or breeding activities in different *Populus* clones, and thus demonstrate the power of SSR markers for their use in identification of clones in the first instance of the section Populus.

SNPs

Overview

A SNP (Single Nucleotide Polymorphism) is defined as the sequence substitution of a single base with occurrence of 1% per population to be designated as polymorphic. SNPs are mutation-induced or originated through reading errors during DNA replication. They are the most common form of genome variation (JONES et al., 2009). The frequency of SNPs throughout the genome is different, e.g., for trees, one SNP per 60 to 540 basepairs is described (ZHANG and ZHANG, 2005). In contrast to SSRs, SNPs occur as well in coding as in non-coding regions of the DNA. SNPs in coding regions do not inevitably lead to changes within the phenotype. The position of a SNP within the coding sequence for an amino acid is decisive, because only a substitution at the first or second position within a triplet could cause a different amino acid resulting in a variation of the protein.

Improvement in sequencing technology during the last few years has simplified the search for SNPs throughout the genomes and increased the possibilities of applications for SNPs. Although SNPs are clearly less variable compared to SSRs, they are a valuable tool for gene mapping and population studies (XING et al., 2005), because of their high frequency and their regular occurrence within the genome. Consequently, SNPs were used during the last decade for construction of gene maps (CHO et al., 1999) and for population genetics analyses (GARCIA-GIL et al., 2003).

SNPs in poplar

Only few publications describe SNP detection within forest tree species, and especially within the genus *Populus* (INGVARSSON, 2005; GILCHRIST et al., 2006; DEGEN and FLADUNG, 2008; FLADUNG and BUSCHBOM, 2009). Analyses of SNP polymorphisms in two different *Populus* species resulted in a nucleotide variation from 7.7 SNPs per 1000 basepairs for *P. trichocarpa* (GILCHRIST et al., 2006) to 16.6 SNPs per 1000 bp for *P. tremula* (INGVARSSON, 2005). Comparable frequencies have been found for other forest tree species (*Pinus*, *Eucalyptus*, *Chamaecyparis*) (ZHANG and ZHANG, 2005). GILCHRIST et al. (2006) reported 63 novel SNPs within 8191 bp in nine genes of *P. trichocarpa*.

Within the gene coding for the polyphenoloxidase (PPO) (CONSTABEL et al., 2000), 66 SNPs were detected

Figure 1. – SNP within the barcoding sequence *trn*H-*psb*A, useful for differentiation of *Populus alba* and *P. tremuloides*.

in a 1689 bp fragment allowing the differentiation of *Populus* species (DEGEN and FLADUNG, 2008). Other genes as leafy, GA20-Oxidase or CAD-like could also be usable for differentiation of species and hybrids (FLADUNG and BUSCHBOM, 2009).

Interestingly, when comparing the "consensus sequences" of the five *Populus* species investigated in FLADUNG and BUSCHBOM (2009), and the one *P. trichocarpa* databank sequence, different "levels" of SNPs can be distinguished. In most of the cases, a "two-nucleotide SNP" occurs, which means that in one or more of the *Populus* species one nucleotide is substituted by another one. Sometimes, a "species-specific" SNP is observed, meaning the presence of the same SNP in all members of one species (forming a "consensus sequence"). For instance for the PPO gene, at position 342 all investigated individuals of *Populus* species reveal a "T" but *P. deltoides* an "A".

Also very often, a "two-" or a "three-species-SNP" occurs, i.e., in *PPO* at position 391, *P. tremula* and *P. alba* harbour an "A" and the other four *Populus* species a "G". An example for a "three-species-SNP" or "section-specific-SNP" can be found at position 459 of the *PPO* where the three species of the section Populus carry a "C" and the other three an "A", respectively. Rarely also "three-nucleotide SNPs" can be detected where at the same position in the gene three nucleotides occur in the different *Populus* species, i.e., in the *PPO* at position 911, a "C" in *P. trichocarpa* and *P. deltoides*, an "A" in *P. nigra* and a "G" in the three species of the section Populus.

Sometimes, indels (insertions/deletions) could be detected either in exons or introns of the genes (FLADUNG and BUSCHBOM, 2009). In the exon of the *TB1* gene as well as in the introns of the *LFY* and *CAD*-like genes, for instance, such indels could be identified.

These indels were present/absent in the "consensus sequences" of the respective *Populus* species and could also be used to distinguish the different species. In the analyses by GILCHRIST et al. (2006) the indels couldn't be detected because only members of the species *P. trichocarpa* were included in the analyses.

Identification of Populus species using SNPs

For identification of SNPs within the genus *Populus*, we used both barcoding primers which have already been used for molecular differentiation of several species (e.g., HOLLINGSWORTH et al., 2009; CHASE et al., 2005), and newly designed primers using the chloroplast genome information of *Populus trichocarpa*. Altogether we tested 42 primer combinations from 16 chloroplast encoding genes in six *Populus* species (*P. trichocarpa*, *P. tremula*, *P. tremuloides*, *P. alba*, *P. deltoides* and *P. nigra*). Some of these primer combinations have also been used for *P. maximowiczii*.

Until now we detected at least one SNP for each of the seven species. For three SNPs we apply PCR-RFLPs, because there are restriction sites for enzymes within the SNPs. Using this method we can positively identify three species of the section Populus (*P. tremula*, *P. tremuloides*, *P. alba*). Identification of the other four species requires sequencing of the appropriate fragment. In addition twelve primer combinations within the *PPO* have been tested. Using the sequence of two each, about 400 bp long fragments facilitate the identification of all seven species by means of one or more SNPs.

Identification of Populus clones using sequences

Two clones used for breeding (Brauna11 and Biehla11) were used for testing of the applicability of the markers. Due to historical events it is probable that the same clone may be used under two different names. For the test we used five different individuals to either the one or the other clone: one individual named Brauna11 from the arboretum of the Institute of Forest Genetics (Grosshansdorf, Schleswig-Holstein, Germany), two individuals named Biehla11 from the arboretum of the Institute of Forest Genetics (Waldsieversdorf, Brandenburg, Germany), and two individuals from the "Staatsbetrieb Sachsenforst" (Graupa, Saxony, Germany) also named Brauna11. The five individuals have been
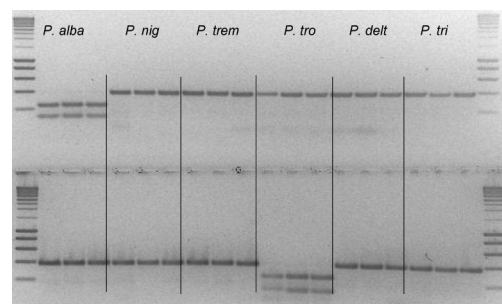

Figure 2. – Example for the result of cutting the barcoding sequence *trn*H-*psb*A with the restriction enzyme *Alw*26I (above) (*P. alba* is cut into two fragments) and *Dra*I (below) (*P. tremuloides* is cut). Nig = *P. nigra*, trem = *P. tremula*, tro = *P. tremuloides*, delt = *P. deltoides* and tri = *P. trichocarpa*.

A	chloroplast	nuclear 1
Parental	♀: A x ♂: T	♀: AA x ♂: TT
F1-generation	A	AT

B	chloroplast	nuclear 1
Parental	♀: A x ♂: T	♀: AT x ♂: TT
F1-generation	A	AT, TT

C	chloroplast	nuclear 1
F1-generation	♀: A x ♂: T	♀: AT x ♂: AT
F2-generation	A	AA, AT, TT

D	chloroplast	nuclear 1	nuclear 2
F1-generation	♀: A x ♂: T	♀: AT x ♂: AT	♀: GC x ♂: GC
F2-generation	A	**AA**, AT, TT	**GC**, GG, CC

Figure 3. – Identification of hybrids/generations by means of SNPs within the chloroplast genome combined with nuclear SNPs. a: Both parents are homozygote, b: One parent is heterozygote, c: The F1-hybrids were crossed, d: An additional nuclear SNP allows detection of F2-hybrids. Further explanations within the text.

sequenced using the above described 400 bp fragment of the *PPO*. Four of the five trees showed identical *PPO* sequences. The sequence of one individual with Biehla11 affiliation differed in two bases from the other four trees. Thus, we could demonstrate that the three individuals named with "Brauna11" and one with "Biehla11" show the identical partial *PPO* sequence, but the second individual named with "Biehla11" is genetically different.

A second example deals with the recognition of hybrids. As already shown in the pilot study by DEGEN and FLADUNG (2008) the *PPO* gene was used to distinguish between different *Populus* species, thus interspecies hybrids could easily be recognized because they were heterozygous in the species-specific loci. The same strategy was followed to characterize two single trees growing in the arboretum of the Institute of Forest Genetics (Grosshansdorf, Schleswig-Holstein, Germany). The first tree revealed heterozygous loci at six different positions within a 600 bp long *PPO*-fragment, with nucleotides characteristic for *P. tremula* and *P. tremuloides*. Thus, this tree seems to be a F1-hybrid between the two aspen species. The other tree showed a more complex pattern: *PPO* sequence between 380 and 405 bp (counting starts with 1 meaning the "A" in the start codon ATG) was similar to *P. tremula*, between 411 and 673 bp similar to *P. tremula* and *P. tremuloides*, between 783 and 914 similar to *P. alba*, and between 1017 and 1095 again similar to both *P. tremula* and *P. tremuloides*. Thus, this second tree is clearly not a F1-interspecies hybrid but it could possibly be a Fx-hybrid with participation of the three species tremula, *P. tremuloides* and *P. alba*. Sequence analyses of further genes could possibly unravel species participation in Fx-hybrids.

Identification of Populus hybrids and generations using SNPs

A further task is the detection of hybrids within the genus *Populus*. By means of SNPs in the chloroplast genome the maternal part of a crossbreed can be identified, whereas SNPs in the nuclear genome allow identification of both crossing partners.

In *Figure 3 (a-d)* an example is given for different levels of hybridisation and the markers needed for identification of the parents of the hybrid: a) The most simple case to identify is a F1-hybrid resulted from two pure species. Employing only one nuclear SNP marker is sufficient to identify both parents. Using an additional chloroplast SNP marker allows also assignment of the mother of the crossbreed. b) If one parent is already a hybrid then only the combination of one nuclear and one chloroplast marker enables us to determine both crossing parents, given that the hybrid is homozygote for the nuclear marker. c) The combination of one nuclear and one chloroplast SNP marker is also sufficient in two of three cases for identification of the F2-hybrid (AT and TT for the nuclear marker). d) Detection of the third F2-hybrid (AA in *Fig. 3c*) requires a second nuclear SNP marker. For this marker the F2-hybrid has to be heterozygote.

The number of needed markers for identification of different hybrids (or generations) beyond the F2-hybridisation will be tested and validated using "saturation curves."

Discussion

The development of molecular markers for the identification and differentiation of species, hybrids, and clones within the genus *Populus* turned out to be more difficult than expected. The molecular methods themselves are totally uncomplicated. But for the purpose of developing molecular markers, reference material is needed, namely pure species to test the species-specificity of the newly developed markers. An optimal sample design for such a purpose would be up to 25 individuals from all over the distribution area of each species. Thus, we tried to receive material from colleagues all over the world and from botanical gardens. Of course our sample

size comprises not at all the whole distribution area, but both the number of samples and expected genetic diversity were sufficiently high to get started. The problem often was that some of this reference material was neither the species nor hybrid declared. Most often we revealed that the declared pure species we received were not a pure species, but already hybrids after some generations, and therefore not only difficult to identify, but also not usable for validating of the markers. We faced the problem described in the introduction: after generations of breeding activities it is of course not clear which species have been used. Thus, the solution may be to harvest material from natural populations. *P. nigra* is the only species for which we have material available from natural populations from different countries. And for this species the markers are convincingly validated. The future purpose is to get further material from natural populations from all other involved *Populus* species.

Acknowledgements

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A DNA Method to Verify The Integrity of Timber Supply Chains; Confirming The Legal Sourcing of Merbau Timber From Logging Concession to Sawmill

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Abstract

Several methods are employed by the timber industry to try to restrict the flow of products from illegal or unsustainable sources into timber supply chains. The most commonly applied are systems of log marking and associated documentation that accompany the logs. However this system is open to falsification, particularly between the logging concession and the timber mill, where the majority of illegally logged timber enters the supply chain. This paper describes the development of a methodology to track a unique genetic fingerprint for single logs of merbau, *Intsia palembanica* (Leguminosae), a high-value Indonesian timber species, from logging concession to the mill, where the DNA profile of individual logs is difficult or impossible to falsify. We find that whilst the ability to extract DNA and amplify a PCR product from logs decreases slightly between forest concession (59.2%) and mill (41.9%) samples, that overall enough samples worked across the 14 microsatellite markers to provide an exact genotype match between forest and sawmill samples for 27 out of 32 logs. Furthermore for these 27 samples, the probability that an illegal log with an exact genotype match to forest samples had been substituted was very low (less than 10^{-5}) for 18 samples, was low (between 10^{-2} and 10^{-4}) for 7 samples and was moderate (10^{-1}) for 2 samples. Improvements to DNA extraction and amplification success are recommended to improve this protocol, and there was a negative correlation between locus size and amplification success but a positive correlation with allele number. However, overall we propose that this methodology is now suitable for broad-scale industry application to track legally harvested timber and check for illegal substitutions along supply chains.

Key words: genetic fingerprinting, illegal logging, merbau, microsatellites, timber certification.

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