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ORIGINAL PAPER

Allelic ladders and reference genotypes for a rigorous standardization of poplar microsatellite data

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Abstract A correct identification of members of the poplar hybrid complex *Populus* × *canadensis* is essential in breeding programs and studies in introgressive gene flow. Molecular marker protocols have been developed for this purpose. However, due to missing standards, these techniques have so far not been suited to the transfer of results between different laboratories. We present here a powerful system of nuclear microsatellite DNA (nSSR) fingerprints, standardized by allelic ladders and reference genotypes. Seven nSSR loci provided fingerprints of 65 commercial poplar clones. Their alleles were used to construct allelic ladders. Thus, a first standardized register of poplar clones is now available. All procedures were optimized according to simplified DNA extraction protocols, multiplexed PCR and electrophoresis procedures. Corresponding data originating from two different electrophoretic platforms in different laboratories were congruent when the allelic ladder was used. Unambiguous differentiation of the clones was based on a very low probability of identity (PI) of 1.95×10^{-8}. Our results revealed discrepancies between clone denotations and genetic fingerprints. This suggests that, potentially, members of the clone collection could have been mixed up, thus confirming the demand for rigorous standards. The protocol presented can be exploited in a manifold way, e.g. to enlarge the present clonal molecular data base, or to use it for purposes of certification and control. Furthermore, the allelic ladders are recommended for use in poplar population genetic studies across different laboratories. The allelic ladders and single sample reference genotypes can be obtained on demand.

Keywords Poplar · Clone · Certification · SSR · Allelic ladder

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Introduction

Members of the genus *Populus* (Salicaceae) are major suppliers of industrial wood worldwide. They are fast-growing, and many poplar species are suitable for clonal forestry. For breeding purposes, one advantage of poplar over any other tree species is that both physical and molecular genetic maps are available from which links between phenotypic traits and genes can be deduced (Cervera et al. 2001; Gaudet et al. 2008; Sims et al. 2006; Taylor 2002).

Through controlled hybridization (Taylor 2002), breeders are able to combine the favorable traits of different parental species in one cultivar. Simple and cost-effective vegetative propagation of cultivars can be exploited for clonal distribution. Clonal plantations exhibit fast growth as well as homogeneity of size and wood quality. They are thus easily processed in industry (Fossati et al. 2005; Zsuffa 1975). Plantations of hybrid poplar have since long been appreciated as an alternative resource for wood production due to increasing demands for renewable energy (Bekkaoui et al. 2003; Sims et al. 2006; Zsuffa 1975; Rajora and Rahman 2003). Recently, the conversion of abandoned agricultural areas to woodland has begun to be

promoted by the European Community (De-Lucas et al. 2008).

More than 90% of all cultivated poplars are assumed to belong to the interspecific hybrid *P.* × *canadensis* Moench [syn. *Populus* × *euramericana* (Dode) Guinier] and their parental species *P. deltoides* Marsh. and *P. nigra* L. (FAO 1979). Consequently, many poplar breeding programs have focused on *P. deltoides* × *P. nigra* controlled crosses which has led to a large number of cultivars and clones.

The proper identification of the highly productive cultivars ensures the correct assignment between the declared and the true identity of a clone. Such interest, shown by poplar breeders, growers and industry, is legitimate and needs to be supported (Fossati et al. 2005; De-Lucas et al. 2008). In addition, European regulations such as the German Law on Forest Reproductive Material (Forstvermehrungsgutgesetz), demand certificates of origin and clonal identity (FoVG 2006).

The traditional method adopted by the International Poplar Commission for identification, registration and certification of poplar clones is based on a total of 64 morphological, phenological and floral characteristics (UPOV 1981).

Phenotypic methods of identification are not always satisfactory because of the instability of morphological characters caused by environmental and management factors as well as by the age of the tree and its state of health (De-Lucas et al. 2008). Individual descriptions are of limited use if two or more similar clones cannot be observed at the same time and in the same environment (Fossati et al. 2005). On the whole, this method for clonal identification is difficult, ambiguous, time-consuming and subjective (Rajora and Rahman 2003). Currently, molecular markers are used for differentiation and identification purposes. These are allozyme polymorphism (Rajora and Dancik 1992) or DNA markers such as RAPDs (Castiglione et al. 1993; Rajora and Rahman 2003), AFLPs (Fossati et al. 2005; Cervera et al. 1996, 2005; Zhou et al. 2005) or nuclear simple sequence repeats (nSSR, syn. nuclear microsatellite markers) (Rajora and Rahman 2003; De-Lucas et al. 2008; Fossati et al. 2005). The latter are the markers of choice. They are reproducible, codominant, highly polymorphic, and if an adequate number of loci is combined, they allow the unambiguous identification of each individual (Jones and Ardren 2003; Gerber et al. 2000; Dayanandan et al. 1998; Smulders et al. 1997; Jones et al. 1997). SSR markers were isolated, characterized and have already been used for clone identification and the assessment of their genetic relationship in poplars (Fossati et al. 2005; Dayanandan et al. 1998; Bekkaoui et al. 2003; Rajora and Rahman 2003; De-Lucas et al. 2008). Because of the common problem of reproducibility of absolute DNA fragment lengths in different electrophoretic platforms and settings (de Valk et al. 2007), these studies did not present any unambiguously transferable results so far.

The aim of our research was to enable a fast, but reliable and transferable identification of commercial poplar clones belonging to the hybrid complex of *P.* × *canadensis*. For that purpose, seven loci SSR genetic fingerprints were used for composing allelic ladders and establishing a first standardized clone register consisting of 65 different clones.

Materials and methods

DNA extraction

Leaves of 91 trees representing commercially relevant poplar clones of different species (*P.* × *canadensis*, *P. deltoides*, *P. nigra*, *P. trichocarpa* Torr. and Grey and *P. nigra* × *P. maximoviczii* Henry, see Table 1) were kindly provided by the holders of four German clone collections. The plant material was dried for 24 h at 36°C. Approximately 0.5 cm^2 of leaf material was homogenized using a Retsch shaking mill (Retsch, Hilden, Germany), according to the protocol described by Ziegenhagen et al. (1993).

The DNA was extracted in two different ways: the first subset of samples was extracted according to the ATMAB procedure (Dumolin-Lapegue et al. 1997). The DNA concentration was measured using the BioPhotometer (Eppendorf, Hamburg, Germany). The quality of the extracted DNA was estimated by calculating the 260:280 OD ratios and by checking the suitability of the DNA as a template in the PCR procedures.

The second subset of samples was extracted using a more time and cost-effective method of DNA extraction following Jump et al. (2003). Deviant from that protocol, homogenized leaf tissue was mixed with 300 µl of 0.5 M NaOH + 2% Tween 20.

Optimization of PCR and genotyping procedures

Populus DNA was analyzed at seven highly polymorphic nSSR loci: WPMS05 and WPMS09 were described by van der Schoot et al. (2000), WPMS14, WPMS18, WPMS20 were taken from Smulders et al. (2001). Loci PMGC14 and PMGC2163 were selected from the IPGC (International Populus Genome Consortium) SSR resource (http://www.ornl.gov/sci/ipgc/ssr_resouce.htm). All markers are completely unlinked (Cervera et al. 2001; Gaudet et al. 2008).

For optimization purposes, the seven loci were subjected to multiplex PCR reactions comparable to studies in *Quercus* and *Pinus* (Dzialuk et al. 2005; Dzialuk and Burczyk 2004). Three groups of marker loci were formed according to three different panels of annealing temperatures. The marker loci PMGC14 and WPMS05, further referred to as the Temp50 panel, were amplified at annealing temperature of 50°C. The marker loci PMGC2163, WPMS09 and

Table 1 Species affiliation, clonal groups (=samples with congruent multilocus genotypes), clone collection of origin (Ori 1–4), licensure of clone variety in Germany (cat. D) and allelic letter code for 91 poplar clones

Species/ clonal groups	Official name of variety FAO-register	Reference Accession name/No.	Ori	cat. D	WPMS05		WPMS09		WPMS14		WPMS18		WPMS20		PMGC14		PMGC2163	
N/1	Blom	Blom10	4	×	G	L	J	J	C	S	C	F	E	F	H	M	J	S
	Not registered	Neunburg	2		G	L	J	J	C	S	C	F	E	F	H	M	J	S
D × N/2	Brabantica	Brabantica	1		M	R	A	K	I	K	A	D	D	F	B	F	A	R
	Brabantica	Brabantica	3		M	R	A	K	I	K	A	D	D	F	B	F	A	R
D × N/3	Baden	Baden (30/58)	1		G	N	A	O	J	K	A	I	D	F	A	H	A	W
	Drömling	Drömling	1	×	G	N	A	O	J	K	A	I	D	F	A	H	A	W
D × N/4	Dorskamp	Dorschkamp	4		G	N	A	J	P	S	A	I	D	E	A	M	A	J
	Grandis	Grandis9	4	×	G	N	A	J	P	S	A	I	D	E	A	M	A	J
D × N/5	Flachslanden	Flachslanden	3	×	N	X	A	J	K	S	A	I	D	E	B	H	A	J
	Flachslanden	Flachslanden	1	×	N	X	A	J	K	S	A	I	D	E	B	H	A	J
	Forndorf	Forndorf	1		N	X	A	J	K	S	A	I	D	E	B	H	A	J
D × N/6	Grandis	Grandis	1	×	L	M	A	Z_5	I	K	A	E	D	D	A	F	A	R
	Grandis	Grandis	3	×	L	M	A	Z_5	I	K	A	E	D	D	A	F	A	R
D × N/7	Gelrica	Gelrica	2	×	N	N	A	M	F	K	A	F	D	I	B	F	A	S
	Löns	Löns3	4	×	N	N	A	M	F	K	A	F	D	I	B	F	A	S
D × N/8	Löns	Löns	1	×	N	N	A	Z_5	K	Q	A	I	D	D	B	E	A	R
	Löns	Löns	3	×	N	N	A	Z_5	K	Q	A	I	D	D	B	E	A	R
D × N/9	Marilandica	Marilandica	1	×	N	R	A	N	I	K	A	F	D	D	A	F	A	R
	Marilandica	Marilandica	2	×	N	R	A	N	I	K	A	F	D	D	A	F	A	R
D × N/10	Robusta	Robusta	1	×	G	N	A	J	P	S	A	I	D	E	A	M	A	J
	Robusta	Robusta	3	×	G	N	A	J	P	S	A	I	D	E	A	M	A	J
	Robusta	Robusta	2	×	G	N	A	J	P	S	A	I	D	E	A	M	A	J
	Zeeland	Robusta Zeeland (231/54)	1		G	N	A	J	P	S	A	I	D	E	A	M	A	J
D × N/11	Selys	Selys	1		M	M	A	N	I	K	A	F	D	F	B	E	A	H
	Serotina	Serotina	1		M	M	A	N	I	K	A	F	D	F	B	E	A	H
D	Not registered	4/45 (1)	2		N	N	A	A	K	L	A	A	D	D	A	B	A	A
D	Marquette	Marquette	1	×	N	N	A	A	K	N	A	A	D	D	B	B	A	A
D	Alcinde	Alcinde	2		M	N	A	A	K	O	A	A	D	D	B	B	A	A
N	Not registered	**Harvard**	**2**		**R**	**T**	**H**	**J**	**I**	**K**	**F**	**F**	**E**	**F**	**D**	**E**	**H**	**R**
D	Harvard	**Harvard**	**1**		**N**	**T**	**A**	**A**	**K**	**N**	**A**	**A**	**A**	**D**	**B**	**B**	**A**	**A**
D × N	Not registered	**Harvard19**	**4**		**M**	**R**	**A**	**K**	**I**	**K**	**A**	**D**	**D**	**F**	**B**	**F**	**A**	**R**
N	Not registered	Plantierensis (101/49)	1		G	X	J	N	J	S	I	I	F	F	H	M	J	J
N	Italica	Italica	2		G	X	J	N	J	S	F	I	E	F	H	M	J	W
N	Not registered	Pyramidalis (134/49)	1		N	N	J	N	D	I	D	F	G	G	H	H	H	W
N	Blanquillo de Bucos	Blanquillo de Bucos	1		N	X	K	N	J	J	D	F	D	F	H	M	J	S
N	Not registered	Erlbach	2		M	P	J	J	F	T	D	J	F	G	E	H	K	S
N	Not registered	Erlbach17	4		L	R	J	N	K	K	D	F	D	G	D	H	L	S
N	Vereecken	Vereecken	2		L	X	J	Z_5	K	S	C	I	F	F	F	M	U	W
T[a]	Fritzi Pauley	Fritzi Pauley	2	×	L	L	Z	Z	F	M	C	C	F	F	E	E	B	B
N × M[a]	Rochester	Rochester	2		M	M	E	S	E	K	B	B	D	D	C	M	Q	Q
D × N	Not registered	Karolina (79/54)	1		L	M	A	N	K	T	A	J	D	H	B	G	A	V
D × N	Allenstein	Allenstein	1	×	N	X	A	O	P	S	A	I	D	F	A	M	A	W
D × N	Bietigheim	Bietigheim	1		G	N	A	O	Q	S	A	I	D	F	B	H	A	J

Table 1 continued

Species/ clonal groups	Official name of variety FAO-register	Reference Accession name/No.	Ori	cat. D	WPMS05		WPMS09		WPMS14		WPMS18		WPMS20		PMGC14		PMGC2163	
D × N	Blanc du Poitou	Blanc du Poitou	2	×	N	N	A	J	I	Q	A	D	D	F	B	F	A	T
D × N	Büchig	Büchig	1	×	G	N	A	J	P	S	A	F	D	F	A	H	A	J
D × N	Carpaccio	Carpaccio	1		N	X	A	J	J	M	A	F	D	F	B	H	A	J
D × N	Dolomiten	Dolomiten	1	×	G	P	A	J	J	Q	A	I	D	F	A	M	A	J
D × N	Dorskamp	Dorskamp	2		G	G	A	Z	K	P	A	F	D	G	A	E	A	H
D × N	Drömling	Drömling14	4	×	P	X	A	N	Q	S	A	F	D	E	A	M	A	J
D × N	Drömling	Drömling	3	×	G	N	A	N	P	S	A	F	D	E	B	H	A	J
D × N	Eckhof	Eckhof	1		G	N	A	N	J	K	A	I	D	F	A	H	A	W
D × N	Florence	Florence	1		D	J	A	I	D	I	A	F	D	E	B	H	A	N
D × N	Not registered	Floßgrün	1		N	Y	A	H	I	I	A	F	D	D	B	H	A	W
D × N	Gelrica	Gelrica B12	1	×	L	M	A	Z_5	I	K	A	J	D	F	B	H	A	S
D × N	Gelrica	Gelrica2	4	×	N	N	A	Z_5	K	Q	A	I	D	D	B	E	A	R
D × N	Not registered	Goldgrund	1		G	N	A	J	J	K	A	I	D	F	A	M	A	W
D × N	Guardi	Guardi	1		N	N	A	J	M	S	A	D	D	D	B	H	A	R
D × N	Harff	Harff	1	×	G	N	A	N	Q	S	A	F	D	E	A	H	A	J
D × N	Harff	Harff	3	×	G	N	A	N	Q	S	A	F	D	E	B	H	A	J
D × N	Heidemij	Heidemij	3	×	Q	X	A	N	I	P	A	F	F	F	B	F	A	J
D × N	Heidemij	Heidemij	2	×	Q	X	A	O	J	P	A	F	F	F	B	M	A	J
D × N	I-154	I-154 Casale	1		G	P	A	J	K	S	A	I	D	E	A	M	A	J
D × N	I-214	I-214	2	×	P	R	A	H	D	I	A	D	D	E	A	G	A	S
D × N	I-214	I-214 Casale	1	×	R	R	A	H	D	I	A	D	D	E	B	H	A	S
D × N	I-262	I-262 Casale	1		P	X	A	J	Q	S	A	I	D	F	A	M	A	W
D × N	I-45/51	I-45/51 Casale	1		N	N	A	H	I	Q	A	K	D	F	A	H	A	S
D × N	I-455	I-455 Casale	1		P	X	A	J	Q	S	A	I	D	F	B	H	A	W
D × N	I-476	I-476 Casale	1		M	M	A	H	I	Q	A	K	D	F	B	H	A	S
D × N	I-488	I-488 Casale	1		G	M	A	J	J	Q	A	I	D	F	A	E	A	J
D × N	Not registered	I-92/40 Casale	1		G	P	A	Z_1	D	Q	A	H	D	F	B	G	A	H
D × N	Jacometti-78-b	Jacometti-78-b	1	×	G	M	A	H	I	I	A	H	D	E	B	H	A	W
D × N	Not registered	Kastenwörth	1		M	R	A	J	K	N	A	D	D	G	B	D	A	R
D × N	Lampertheim	Lampertheim	1	×	Q	X	A	J	P	S	A	I	F	F	A	H	A	J
D × N	Not registered	Lampertheim Findl. (56/55)	1		G	N	A	N	P	S	A	I	E	E	A	M	A	J
D × N	Leipzig	Leipzig	1		M	R	A	Q	F	K	A	E	D	F	B	G	A	R
D × N	Lingenfeld	Lingenfeld	1	×	Q	X	A	J	P	S	A	F	E	E	A	H	A	J
D × N	Marilandica	Marilandica	3	×	N	N	A	Z_5	K	Q	A	I	D	D	B	F	A	R
D × N	Neupotz	Neupotz	1	×	G	M	A	N	K	S	A	I	D	E	B	H	A	W
D × N	Neupotz	Neupotz	3	×	G	M	A	N	K	S	A	I	D	E	A	H	A	W
D × N	Ostia	Ostia	1	×	M	X	A	J	I	Q	A	D	D	D	A	F	A	W
D × N	Régénéré	Regenerata Kew (73/56)	1		M	N	A	J	I	Q	A	E	D	D	B	H	A	T
D × N	Rintheim	Rintheim	1	×	L	M	A	K	I	Q	A	D	D	D	A	D	A	I
D × N	Serotina	Serotina	2		L	L	A	J	I	K	A	F	D	F	B	E	A	H
D × N	Not registered	Speyer 02 (49/59)	1		G	M	A	N	K	S	A	F	D	F	A	M	A	J
D × N	Not registered	Sprengen (55/58)	1		N	X	A	J	Q	S	A	F	D	E	B	M	A	W
D × N	Tannenhoeft	Tannenhöft	1	×	G	N	A	J	J	P	A	F	D	F	A	M	A	J
D × N	Tardif de Champagne	Tardif de Champagne	1	×	N	N	A	Q	G	K	A	F	D	F	B	H	A	R

Table 1 continued

Species/ clonal groups	Official name of variety FAO-register	Reference Accession name/No.	Ori	cat. D	WPMS05		WPMS09		WPMS14		WPMS18		WPMS20		PMGC14		PMGC2163	
D × N	Tardif de Champagne	Tardif de Champagne	2	×	N	N	A	Q	G	K	A	F	D	F	A	G	A	R
D × N	Virginie de Frignicourt	Virginie de Frignicourt	1		M	M	A	J	I	Q	A	E	D	D	B	F	A	T
D × N	Virginie de Nancy	Virginie de Nancy	1		G	N	A	N	P	S	A	I	F	F	A	M	A	J
D × N	Zürich	Zürich	1		M	X	A	J	J	Q	A	D	D	D	B	H	A	W

Clones of the name "Harvard" are highlighted in bold. According to literature, "Harvard" is part of *P. deltoides* and "Blom" is part of *P. trichocarpa* (Fossati et al. 2005)

D = *P. deltoides*, N = *P. nigra*, T = *P. trichocarpa*, D×N = *P* × *canadensis,* N×M = *P. nigra* × *P. maximowiczii*

[a] Species affiliation according to Fossati et al. (2005)

WPMS18, further referred to as the Temp55 panel, were amplified at annealing temperature of 55°C. The marker loci WPMS14 and WPMS20, further referred to as the Temp60 panel, were amplified at annealing temperature of 60°C. Primers were labeled with different fluorescent colors (FAM, HEX and TAMR) in order to distinguish amplification products in the following automated multiplex electrophoresis.

PCR was performed in a volume of 13 µl containing 10 ng of genomic DNA [extraction following Dumolin-Lapegue et al. (1997)] or preferably 1 µl of DNA extract [extraction following Jump et al. (2003)], 2.4 µM of all amplification primers [2.9 µM (WPMS05) and 2.15 µM (PMGC14) for the Temp50 panel], 0.2 mM of each dNTP, 1.75 mM $MgCl_2$ (2.5 mM for the Temp55 panel), and 0.2 U of Promega GoTaq DNA polymerase (Promega, Madison, WI, USA) in 1× Green GoTaq Flexi Buffer (Promega, Madison, WI, USA). Thermocycling was performed in a T1 thermocycler (Biometra, Göttingen, Germany) following the protocol described in Dayanandan et al. (1998): after an initial denaturation of 1 min at 94°C, five cycles were performed for a duration of 1 min each at 94°C (denaturation), annealing temperature depending on the marker panel and 72°C (extension), followed by 30 cycles for a duration of 30 sec each. To check the success of amplification, several PCR products were controlled. Six microliters each were run on 1% agarose gel and then stained with GelRed (Biotum, Hayward, CA, USA).

Automated multiplex capillary electrophoresis

For automated multiplex capillary electrophoresis, 2 µl Temp55 panel PCR were treated separately while each 1 µl of Temp50 and Temp60 panels were merged. This was possible, as the DNA fragments of locus WPMS05 were expected to be far longer than the fragments expected from the other three loci present in the PCR cocktail. Hence, both primers of the panel Temp50 (WPMS05 and PMGC14) were labeled with the same fluorescent color. By this means, four loci could be genotyped in only one step of electrophoresis.

Each cocktail of PCR products was mixed with 7.75 µl of distilled water and 0.25 µl of ET-ROX 400 marker (GE Healthcare, Diegem, Belgium) each. Following denaturation of the samples for 1 min at 95°C and rapid cooling to 4°C they were injected onto a MegaBACE 500 equipped with a 96 capillary array. Injection and running parameters were performed according to the instructions of the manufacturer (GE Healthcare). The fragment size of each PCR product was estimated using Genetic Profiler 2.2 software (GE Healthcare).

Validation of the system

In order to rate the discriminatory power of the multilocus allelic information of the commercial clones, several parameters were calculated; the number of alleles as well as the observed heterozygosity (H_o) and the probability of identity (PI) for each locus and combined loci. All parameters were calculated using the software package GenAlEx 6 (Peakall and Smouse 2006).

Composing allelic ladders

Allelic ladders were composed by merging the PCR products of samples displaying one out of all the alleles occurring in the clone collection. If one allele did not occur in a homozygous status in the clone collection, a homozygous sample of *P. nigra* (own unpublished data) was taken whenever possible. This procedure was used for each

locus. The volume of PCR product taken for the ladder depended on the intensity of amplification as seen in the peaks of the MegaBACE fluorogram. Identification and scoring of the alleles was standardized by a one-letter code (allele A, B, C, etc.). Due to significant stutter peaks of WPMS05 PCR products, the construction of an allelic ladder failed. Instead heterozygous single sample reference genotypes were selected with main peak distances of at least six base pairs (bp). In electrophoresis, each of them had to be analyzed in a single lane.

The allelic ladders were run on the MegaBACE 500 and in a second laboratory on a LI-COR 4300 DNA Analysis System together with samples of known DNA fragment length. Both PCR products of single samples and the allelic ladders were reamplified using 1 µl of PCR product directly as a DNA template in the standard PCR protocol. For the transfer to the LI-COR-system, all markers were labeled with the LI-COR infra-red dye 700. As this system only allows the detection of one fluorescent color, amplified fragments of multiplex PCR reactions can not be distinguished by different label colors but only by substantial differences in bp length. Therefore, multiple PCR reactions could only be performed for the Temp50 panel, as expected fragments of WPMS05 and PMGC14 differed in length of at least 50 bp. All other PCR reactions had to be conducted separately for each locus and consequently amplified fragments had to be run separately in electrophoresis as well.

Preparation of samples and running parameters were performed according to manufacturer's recommendations (LI-COR). The fragment size of each PCR product was estimated using the Saga^{GT} 3.3 genotyping software (LI-COR).

Results

DNA extraction

DNA solutions of both extraction methods produced congruent PCR products. When applied on the same sample, the results were reproducible between experiments. The DNA extraction of dried and powdered wood was also successful (own unpublished data).

PCR procedure and sizing of amplification products

The optimized amplification protocol of PCR reactions and MegaBACE 500 runs constantly yielded clearly identifiable peaks of different colors and sizes. A significant portion of stutter peaks only occurred in the amplification products of WPMS05 but did not hinder the identification of the prominent peak in each sample. With the help of species-specific allele information (Fossati et al. 2003; Bekkaoui et al. 2003; Khasa et al. 2005) species affiliation according to *P. nigra*, *P. deltoides* and *P.* × *canadensis* could be assigned.

Diversity estimates

All SSR markers showed high allelic diversity and observed heterozygosity in the studied clones. Among the 91 samples, 65 separate multilocus genotypes could be obtained. The number of alleles ranged from seven alleles of WPMS20 up to 17 alleles of WPMS14, with an average of 12.14 alleles per locus (Table 3). The observed heterozygosity ranged from 0.67 to 0.96 (average 0.87) with WPMS20 displaying the lowest and WPMS14 the highest allelic diversity values.

Probability of identity (PI)

The probability of two unrelated individuals displaying the same multilocus genotypes by chance is represented by PI (Taberlet and Luikart 1999; Waits et al. 2001). Single locus PI ranged from 0.03 (PMGC2163) to 0.19 (WPMS20). With all seven loci considered, the combined PI was calculated as 1.95×10^{-8} (Table 3).

However, multilocus genotypes of samples originating from different collections but being declared the same clone identity often differed (Table 1). Most strikingly, the three samples of the clone "Harvard" turned out to belong to *P. nigra* and *P. deltoides* as well as to *P.* × *canadensis*. These samples originate from three different clone collections. Additionally, several clone samples with different names surprised by showing identical genetic fingerprints, such as with "Gelrica" and "Löns3". Both labels represent nationally licensed clones in Germany. Clone "Blom" [in fact *P. trichocarpa* (Fossati et al. 2005)] showed typical patterns of *P. nigra* which were identical to clone "Neunburg". Congruent genetic data of all samples was shown by three clone identities ["Brabantica", "Flachslanden" (two trees each) and "Robusta" (four trees)].

Reference genotypes and allelic ladders

Reference genotypes with allele sizes and letter codes of WPMS05 are displayed in Table 4. One reference genotype (ref 2) displays three main peaks.

The combined fluorograms and letter codes of the allelic ladders for loci WPMS09, 14, 18, 20 and PMGC14 and 2163 are displayed in Fig. 1. Not every allele occurring in the clone collection was added to the ladder, e.g. allele "E" in the ladder for PMGC14 (Fig. 1e).

Due to differences in DNA fragment concentration, the peaks show varying intensity. The allelic ladders enabled standardization of genotyping. Allele lengths of the

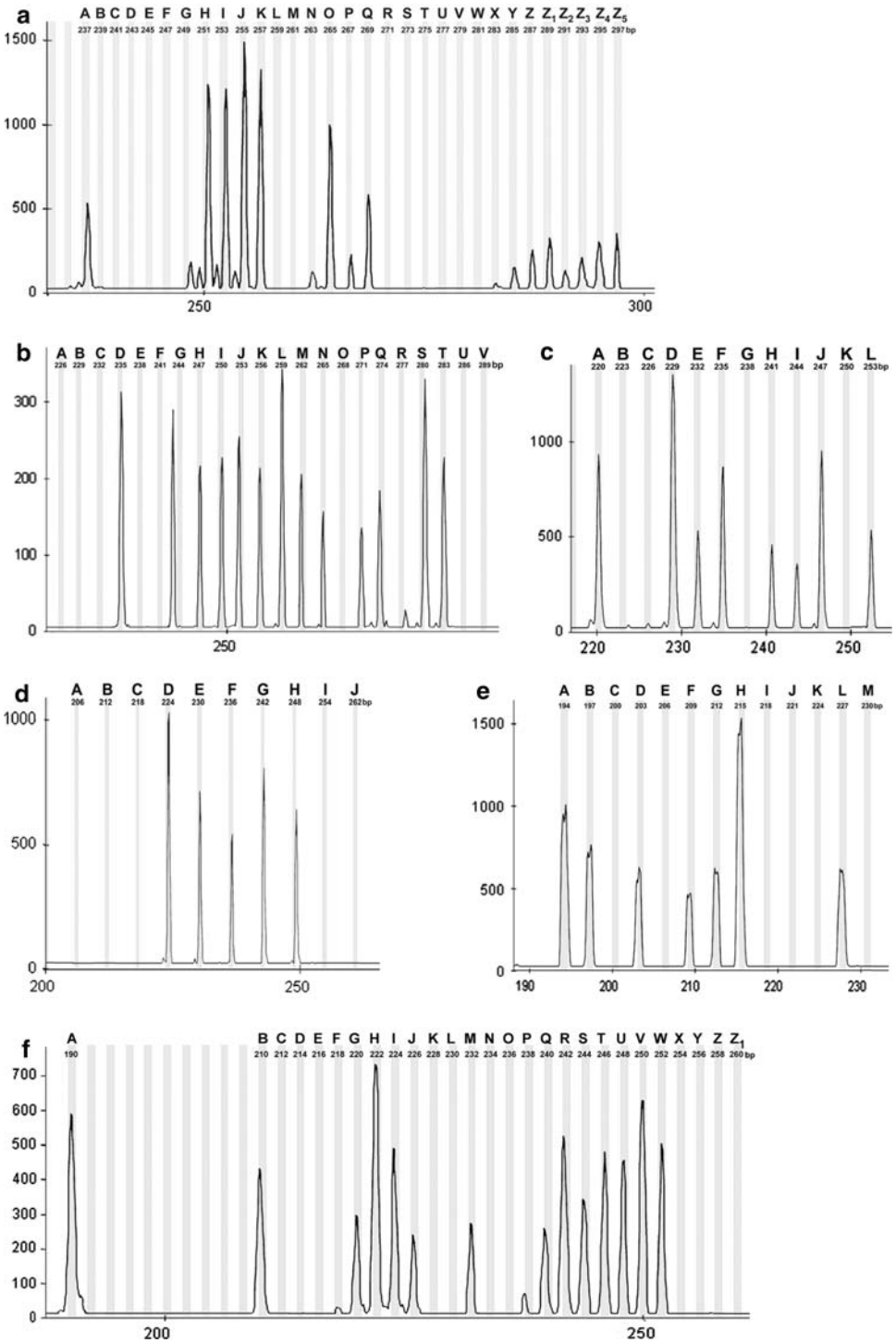

Fig. 1 Allelic ladders of SSR loci. *X*-axis: fragment length in base pairs (bp) (MegaBACE data); *Y*-axis: relative intensity of the fluorescent signal. *Letters* are assigned to allele length in ascending order. **a** WPMS09, **b** WPMS 14, **c** WPMS18, **d** WPMS20, **e** PMGC14, **f** PMGC2163. Species-specific alleles for *P. deltoides* are alleles A [WPMS09, WPMS18, PMGC2163 (Fossati et al. 2003; Khasa et al. 2005)] and alleles A and B [PMGC14 (Fossati et al. 2003)], respectively

examined poplar clones could be transferred to the allelic letter code (Table 1).

By reamplifying the allelic ladders, the signals of single fragments declined with the increase in fragment size. Due to this obstacle, the DNA of long fragments from single sample PCR products in particular needs to be added at regular intervals to the allelic ladder. Reamplification of single sample PCR products proved to be entirely unproblematic. Due to the increasing intensity of stutter peaks during reamplification of WPMS05 single sample PCR products, original DNA extracts had to be used when refilling reference samples.

Without the usage of allelic ladders, corresponding allele sizes of the same samples run on LI-COR and on MegaBACE, respectively, showed differences of up to 4 bp. The corresponding data was congruent when the

Table 2 Allele sizes of *P.deltoides* specific alleles (Fossati et al. 2003; Khasa et al. 2005; Bekkaoui et al. 2003) of the three loci WPMS09, 18 and PMGC14 as obtained in different laboratory settings and their differences in base pairs (bp)

	WPMS09	WPMS18	PMGC14	
LI-COR	233	218	195	198
MegaBACE	237	220	194	197
ABI-PRISM 310	232	215	190	193
Difference LI-Cor/MegaBACE	4	2	1	1
Difference LI-Cor/ABI-PRISM 310	1	3	5	5
Difference MegaBACE/ABI-PRISM 310	5	5	4	4

Data of ABI-PRISM 310 originating from De-Lucas et al. (2008)

letter code of the allelic ladders was applied. Control samples could clearly be assigned. These results were compared to published data from De-Lucas et al. (2008) (Table 2).

Discussion

Our aim was to present a poplar genotyping procedure, which is fast and effective but at the same time reliable, meeting the requirements of rigorous standardization across different laboratories. We report here on the construction of the first allelic ladders available for a forest tree.

Our DNA extraction according to Jump et al. (2003), using dried tissue instead of frozen samples, enables easy sample taking. Whenever possible, multiplexing PCR reactions minimize the effort with regards to time and financial means. Subsequent multiplexing of different PCR products during electrophoresis additionally speeds up genetic fingerprinting. It offers a fast and cheap genotyping protocol with reliable results and the opportunity of large sample throughput (de Valk et al. 2007). In our study, seven loci genotypes could be obtained by just three PCR reactions and two steps of electrophoresis using the MegaBACE system.

The analysis of genetic parameters shows that estimates of diversity are comparable to other studies dealing with the identification of commercial polar clones (De-Lucas et al. 2008; Fossati et al. 2005). In these studies, locus WPMS20 was also found to display the smallest number of six alleles while locus WPMS14 displayed the largest number of alleles, 18 (De-Lucas et al. 2008) or 15 alleles, respectively (Fossati et al. 2005). Observed heterozygosity was high in the present study (0.67–0.96) indicating that a huge amount of information is displayed in samples which are heterozygous at multiple loci. This could be due to the fact that analyzed clones displayed hybrid effects of an elevated level of heterozygosity, typical in crop plant breeding (Stark et al. 2006). Other studies revealed H_o of a clone collection ranging from 0.53 to 0.89 (De-Lucas et al. 2008).

Low rates of PI are required for an unambiguous individual identification. The time and cost limitations of studies require a trade-off between best necessary power of discrimination and a minimum number of loci. In the study of De-Lucas et al. (2008), the combined PI of 12 loci revealed a value of 1.18×10^{-9}. The usage of seven loci in our study results in a PI differing in only one decimal power. Regarding three loci of the same study (De-Lucas et al. 2008), PI resulted in 2.4×10^{-4}. This level was considered sufficient for significant identification of the 28

Table 3 Genetic parameters of nSSR loci combined in three marker panels for the batch of 91 trees of commercial relevant poplar clones

Marker panel	Locus	N_a	Allelic size range in bp (MegaBACE data)	H_o	PI
Temp50	WPMS05	13	266–308	0.76	0.05
	PMGC14	9	193–229	0.95	0.06
Temp55	WPMS09	14	237–297	0.91	0.10
	WPMS18	10	220–253	0.92	0.11
	PMGC2163	15	190–252	0.93	0.09
Temp60	WPMS14	17	232–283	0.96	0.03
	WPMS20	7	206–254	0.67	0.19
Combined PI for all loci		–	–	–	1.95×10^{-8}
	Mean	12.14	–	0.87	0.09

N_a Number of alleles, *clone coll* clone collection, H_o observed heterozygosity, *PI* probability of identity

Table 4 Featured alleles of reference genotypes (ref 1–5) for locus WPMS05

Size (MegaBACE data)	Letter	Reference genotype
252	A	
260	B	
262	C	
264	D	ref 1
266	E	
268	F	
272	G	ref 2
274	H	
276	I	ref 3
278	J	ref 1
280	K	
282	L	ref 3/ref 5
284	M	ref 2
286	N	ref 4
288	O	
290	P	ref 2
292	Q	
294	R	ref 4
296	S	
298	T	
300	U	
302	V	
304	W	
306	X	ref 5
308	Y	
310	Z	

poplar clones in their study. Therefore, the marker combination of the present study represents a useful trade-off between high information content and simple application.

The question arises as to whether the markers as such are stable enough with regard to somatic mutations. Despite the significance and stability of SSR markers, somatic mutations may occur especially in old clonal lineages (Thomas 2002). This is important when several poplar clones have been propagated for some 250 years (Rajora and Rahman 2003). Mutation rates for microsatellite loci in plants have been estimated to fall within the range of 10^{-2}–10^{-3} for nuclear-encoded loci with tri- and dinucleotide repeats (Kovalchuk et al. 2000; Udupa and Baum 2001). However, mutations are spontaneous and non-directional. Clones mismatching in their SSR genotypes due to somatic mutations may also have changed their morphological traits (Franks et al. 2002). A formerly productive clone may (through somatic mutation) actually show weak features, although clonal propagation was performed properly and mislabeling can be excluded. Samples carrying the same label but differing in only one allele length may be results of somatic mutations. A molecular genetic fingerprint can therefore even be useful for detecting the stability of an established clone.

The most substantial evidence for the need of standardized molecular clone identification techniques are the diverging genotypes of the samples labeled "Harvard". In this case, even declared species affiliation was incorrect for at least two of the three samples. Accurate multilocus genotypes representing one distinct clone identity were impossible to achieve because mislabeling could not be retraced.

Furthermore, it was not retraceable, as to whether identical genetic fingerprints of different samples in our results represented true clonal duplicates or just mislabeled trees of one clone in only one specific collection. Clonal group 1 ("Blom"/"Neunburg") clearly demonstrates discrepancies between clone denotations and genetic fingerprints: according to the literature, clone "Blom" belongs to species *P. trichocarpa* (Fossati et al. 2005). However, allelic patterns of locus WPMS18 can be used to attest both samples belonging to *P. nigra*, as this locus amplifies for species *P. nigra* and *P. deltoides* but not for *P. trichocarpa* (Smulders et al. 2001). Species affiliation to *P. deltoides* can be excluded as species-specific alleles did not occur (Fossati et al. 2003). Our results suggest that many trees in clone collections could have been mislabeled and are currently merchandised under the wrong labels. Evidence is given for poplars in Spain (De-Lucas et al. 2008) as well as for apple varieties in Germany (Mosch et al. 2008).

The only reliable way to prevent mislabeling is the use of allelic ladders or reference genotypes. In our study, this method ensured matching results from the determination of correct allele sizes across two laboratories. Without molecular standards, variations in sizing were unpredictable. Consequently, data could not be adjusted by a fixed correction scheme. According to (De-Lucas et al. 2008), variation of allele sizes in different laboratory settings is negligible. Our comparison of the lengths of *P. deltoides* specific alleles of WPMS09, 18 and PMGC14 run in different settings illustrates the opposite: our findings show that differences can range between 2 and 4 bp. A comparison to De-Lucas et al. (2008) even yielded differences up to 5 bp.

Conclusions and perspective

Concerning nationally licensed clone varieties, congruent samples with different names are not desired: either varieties are synonymous or certification errors have occurred. In both cases, commercial damage is predetermined. Our

molecular certification protocol can be used in solving these problems. Identities can now be clearly recognized by a significant seven loci SSR marker assisted genetic fingerprint. Now, duplications among previously registered poplar clones, labeling errors and evolved genetic deviation from the originally established clone variety can be recorded. Technically, all procedures were optimized to the effect that the volume of information obtained per sample was maximized while the effort in terms of costs and time was minimized. With the help of allelic ladders and reference genotypes, clone identities of the hybrid complex *P.* × *canadensis* can be assigned independently of the research facility. The standardization protocol presented may, therefore, contribute to the establishment of new certification systems in the European Community. In this way, legitimate interests of poplar breeders, growers and industries can be protected efficiently.

Future investigations need to clearly identify specific commercial clone identities. These outcomes will then need to be backed up by reference samples and should be compared to the present allelic table. In the case of congruent information, it will be possible to confirm allelic code and variety name for use as adjustment data in the future. Subsequently, trees of unknown identity can be assigned to the "true" specific clone identity. Depending on national interests, different clones or hybrid crossings may be commonly used in other countries. However, additional alleles of new varieties or different poplar species yet missing in the presented list of clones can easily be included in order to enlarge the allelic ladders or to offer additional reference genotypes, respectively.

In addition to the registration of elite clones, the protocol presented can also be used for other applications such as the identification of putative parents, the study of biodiversity, geneflow studies and the verification of crosses. Since species-specific alleles are available for four out of seven loci, species affiliation and hybrid status concerning *P. nigra* and *P. deltoides* can be easily diagnosed (Fossati et al. 2003; Khasa et al. 2005; Bekkaoui et al. 2003).

The allelic ladders and single sample reference genotypes can be obtained on demand at http://www.picme.at. Postal address: Repository centre/PICME; Austrian Research Centers GmbH-ARC; 2444 Seibersdorf, Austria and Silvia.fluch@arcs.ac.at.

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