Working Papers of the Finnish Forest Research Institute 77

http://www.metla.fi/julkaisut/workingpapers/2008/mwp077.htm
ISBN 978-951-40-2093-3 (PDF)
ISSN 1795-150X

Marketing of forest reproductive material: the use of microsatellites for identification of registered tree clones in Finland

Leena Koivuranta, Kari Leinonen and Pertti Pulkkinen

METLA www.metla.fi

Working Papers of the Finnish Forest Research Institute publishes preliminary research results and conference proceedings.

The papers published in the series are not peer-reviewed.

The papers are published in pdf format on the Internet only.

http://www.metla.fi/julkaisut/workingpapers/
ISSN 1795-150X

Office
Post Box 18
FI-01301 Vantaa, Finland
tel. +358 10 2111
fax +358 10 211 2101
e-mail julkaisutoimitus@metla.fi

Publisher
Finnish Forest Research Institute
Post Box 18
FI-01301 Vantaa, Finland
tel. +358 10 2111
fax +358 10 211 2101
e-mail info@metla.fi
http://www.metla.fi/

Authors
Koivuranta, Leena, Leinonen, Kari & Pulkkinen, Pertti

Title
Marketing of forest reproductive material: the use of microsatellites for identification of registered tree clones in Finland

Year	Pages	ISBN	ISSN
2008	19	ISBN 978-951-40-2093-3 (PDF)	1795-150X

Unit / Research programme / Projects
Finnish Forest Research Institute, Vantaa Research Unit / Functioning of forest ecosystems and use of forest resources in changing climate, MIL/ Project 3439 Forests 2050

Accepted by
Jari Varjo, Director of Research Unit, 1 May 2008

Abstract
According to the current legislation on the marketing of forest reproductive material, tree clones marketed in the EU must have certain traits which make them identifiable, and these traits must have been accepted and registered by an official body. Due to this obligation, there is a need for reliable, functional and practicable methods for specifying these distinctive characters.

We have developed a clone identification method for European and hybrid aspens and curly birch, based on nuclear microsatellites, which can be used for determining the distinctive characters mentioned in the directive. For aspens, we have used 18 loci, of which nine were developed earlier for P. tremuloides and nine for P. nigra. For curly birch (Betula pendula var. carelica), we have used seven loci developed for B. pendula and three loci developed for B. platyphylla var. japonica. Most of the aspen clones were easily identifiable using only part of the loci. In the case of curly birch, however, two clones could not be separated from each other despite the relatively high number and polymorphism of the loci, which suggests that these two clones were actually samples from the same clone. These kinds of mistakes further emphasise the urgent need for a reliable clone identification method.

Keywords
Betula pendula var. carelica, curly birch, European aspen, hybrid aspen, Populus tremula x P. tremuloides

Available at
http://www.metla.fi/julkaisut/workingpapers/2008/mwp077.htm

Replaces

Is replaced by

Contact information
Pertti Pulkkinen, Finnish Forest Research Institute, Haapastensyrjä Tree Breeding Station, Haapastensyrjäntie 34, FI-12600 Läyliäinen, Finland. E-mail pertti.pulkkinen@metla.fi

Other information

Contents

1 Introduction

According to Council Directive 1999/105/EC (2000) on the marketing of forest reproductive material within the community, clones derived through vegetative propagation should be identifiable on the basis of distinctive characters, and these traits must have been accepted and registered by an official body. The characters must also have been approved and registered by the authorities of each member state. Tree clones can be produced and marketed under the categories "qualified" and "tested". The clones derived from basic material phenotypically selected at the individual level can be registered in the category "qualified", and those possessing a superiority that has been demonstrated by comparative testing can be registered in the category "tested". The comparative field trials required to test the superiority of the clones have already been established for most of the registered clones. However, the field trials are still too young for analysis. Thus, for instance, all the clones registered in Finland belong to the category "qualified".

In Finland there are 71 registered tree clones, of which 3 clones are European aspen (*Populus tremula*), 34 hybrid aspen (*P. tremula x P. tremuloides*) and 31 curly birch (*Betula pendula* var. *carelica*). There are also approx. 50 hybrid aspen clones in field tests, which will probably be subsequently registered (N. Stenvall, pers. com.). The same registered aspen clones are also in use in Estonia (H. Tullus, pers. com.). European and especially hybrid aspen are being planted in increasing numbers in Fennoscandia and the Baltic countries to provide raw material for the manufacture of fine paper (Holm 2004). Aspens have light, long fibres (Ranua 2002), and they grow fast (Yu and Pulkkinen 2002), which makes them a desirable raw material for the paper industry. Curly birch has a delicate grain patterning in the wood, and it is primarily used in the manufacture of furniture and decorative articles (Kosonen 2004). It has the highest commercial price of all the Nordic tree species (Hagqvist 2004).

Clone identification has traditionally been performed using morphological markers, such as stem shape, bark colour and patterning, branch angle, leaf shape, and spring and autumn foliage colour (Barnes 1969, UPOV 1981). However, in practise these methods have proved to be unreliable and their power of resolution insufficient (Cheliak and Pitel 1984, Rogstad et al. 1991). The variation in morphological markers is not always caused by genetic factors, because the morphology of a tree is also dependent e.g. on the habitat, age of the tree and year of observation. Morphological clone identification methods are also often subjective and relative (Suvanto and Latva-Karjanmaa 2005).

More reliable methods that have been applied in the clone identification of different tree species, especially Populus, are molecular methods, such as allozymes (e.g. Cheliak and Pitel 1984) and DNA-based methods, especially RAPDs (Lin et al. 1994, Rajora and Rahman 2003), AFLPs (Arens et al. 1998, Fossati et al. 2005) and microsatellites (Rajora and Rahman 2003, Suvanto and Latva-Karjanmaa 2005). These methods are independent of the age and habitat type of the tree. Allozymes, however, often have insufficient variation (Lin et al. 1994), which makes it difficult to identify closely related clones. The drawback of RAPDs and AFLPs is that they are dominant markers, although the great number of bands outweighs this drawback. The banding profile of AFLPs may vary depending on the DNA extraction method used (Benjak et al. 2006). AFLPs can also give ambiguous results, and it has been suggested that they are not suitable for an identification database (Fossati et al. 2005). In contrast, microsatellites are variable, codominant and neutral markers, technically reliable and the results are more robust

across laboratories, making microsatellites suitable for clone identification even in closely related clones.

Hybrid aspen clones that are used for plantations in Finland and Estonia have been crossed using a limited number of parent individuals, and this may have led to low genetic variation in their gene pool. In Latvia, for instance, the variation may be even more limited because the hybrid aspen clones used in commercial plantations have been crossed using only one *P. tremuloides* male (Aris Janssons, pers. com.). This necessitates the use of sufficiently variable markers for clone identification. In an earlier study, most of the registered hybrid aspen clones were separated from each other using nine microsatellite loci, although the clones were not identifiable on the basis of morphological markers (Alanen 2003). Therefore, microsatellites seem to be suitable markers for aspens and probably also for curly birch, since microsatellites have recently been developed for several birch species (Wu et al. 2002, Ogyu et al. 2003, Kulju et al. 2004).

The purpose of this study was to develop a reliable and practical microsatellite method for identifying aspen and curly birch clones. The method also had to be applicable for the identification of forest reproductive material in different stages of clone production, as required by the EU directive.

2 Material and methods

The material for the DNA extractions was collected from one aspen and two curly birch field tests. Four curly birch samples were also collected from a nursery (61°29'N, 26°51'E). The aspen trial was located in a field in southern Finland (60°36'N, 24°36'E). The trees had been planted in 1998, and they were therefore seven years old at the time of sampling. The curly birch field tests were situated on a *Vaccinium myrtillus* type forest site (Cajander 1926) and had been planted in 2002 and 2003 using one-year-old plants. The curly birch trials were situated in south eastern Finland (61°30'N, 26°51'E and 61°33'N, 23°45'E).

The material consisted of the European and hybrid aspen as well as curly birch clones registered by the Finnish Food Safety Authority Evira (Table 1), with the following exceptions. Two registered European aspen, nine hybrid aspen and eight curly birch clones were not available for the study, because they were not included in the field tests. This was due to problems in propagating these clones, or because the clones had not yet been registered at the time when the trials were established. Some of the curly birch clones had also been discarded because of the poor quality of the curly-grained wood. Instead of the missing registered clones, we sampled one European aspen clone that is currently under field testing and may subsequently be added to the register (Table 1).

Table 1. The clones used in the study, their species and mother tree numbers.

Clone	Species	Mother tree
05-99-8	Hybrid aspen	E10467
C05-99-9	Hybrid aspen	E10468
C05-99-10	Hybrid aspen	E8115
C05-99-11	Hybrid aspen	E10469
C05-99-12	Hybrid aspen	E10470
C05-99-13	Hybrid aspen	E10471
C05-99-14	Hybrid aspen	E10492
C05-99-15	Hybrid aspen	E10475
C05-99-16	Hybrid aspen	E10476
C05-99-17	Hybrid aspen	E10477
C05-99-18	Hybrid aspen	E10489
C05-99-19	Hybrid aspen	E10490
C05-99-20	Hybrid aspen	E10491
C05-99-21	Hybrid aspen	E10473
C05-99-22	Hybrid aspen	E10479
C05-99-23	Hybrid aspen	E10480
C05-99-24	Hybrid aspen	E10481
C05-99-25	Hybrid aspen	E10482
C05-99-26	Hybrid aspen	E10485
C05-99-27	Hybrid aspen	E10478
C05-99-28	Hybrid aspen	E10474
C05-99-30	Hybrid aspen	E10488
C05-99-31	European aspen	E10484
C05-99-32	Hybrid aspen	E10472
C05-99-33	Hybrid aspen	E10486
C05-99-34	Hybrid aspen	E10487
147	European aspen	E1214 x E293
C05-98-2	Curly birch	E3648
C05-98-3	Curly birch	E8274
C05-01-35	Curly birch	E8301
C05-01-36	Curly birch	E8303
C05-01-37	Curly birch	E8304
C05-01-38	Curly birch	E8306
C05-01-40	Curly birch	E8309
C05-01-41	Curly birch	E8278
C05-01-42	Curly birch	E8315
C05-01-43	Curly birch	E10046
C05-01-44	Curly birch	E10493
C05-01-45	Curly birch	E10754
C05-01-50	Curly birch	E8279
C06-01-47	Curly birch	E10434
C06-01-48	Curly birch	E10524
C06-02-51	Curly birch	E10401
C06-02-52	Curly birch	E10402
C06-02-53	Curly birch	E10403
C06-02-55	Curly birch	E10380
C06-04-58	Curly birch	E10872
C06-05-69	Curly birch	E11283
C06-05-71	Curly birch	E11286
C06-05-70	Curly birch	E11285

The DNA was isolated from fresh or refrigerated leaves or buds with a Qiagen DNeasy Plant Mini Kit according to the instructions of the manufacturer. For identification of aspen clones we used nine loci originally developed for *P. tremuloides* and nine loci developed for *P. nigra*

(Table 2). Some of the primers had to be redesigned (for new primer sequences, see Suvanto and Latva-Karjanmaa 2005). The curly birch clones were genotyped using seven loci developed for B. pendula and three loci developed for *B. platyphylla* var. *japonica* (Table 2).

Table 2. The microsatellite loci used in the study, the repeat type, number of alleles found in the study data, studied species, observed (Het obs) and expected (Het exp) heterozygosities and polymorphic information content (PIC) of the loci.

Locus	Repeat	No of alleles	Species	Het obs	Het exp	PIC
PTR1	$(GGT)_5N_{45}(AGG)_9$	6	aspens	0.444	0.387	0.379
PTR2	$(TGG)_8$	8	aspens	0.778	0.792	0.779
PTR3	$(TC)_{11}$	10	aspens	0.778	0.707	0.692
PTR4	$(TC)_{17}$	6	aspens	0.630	0.695	0.707
PTR5	$(TG)_7$	2	aspens	0	0.073	0.077
PTR6	$(AT)_8$	3	aspens	0.185	0.297	0.298
PTR8	$(A)_{11}(CT)_8$	7	aspens	0.481	0.762	0.745
PTR12	$(AAAG)_3A_6N_7(AAAG)_2$	2	aspens	0.037	0.037	0.039
PTR14	$(TGG)_5$	6	aspens	0.556	0.516	0.499
WPMS05	$(GT)_{27}$	9	aspens	0.815	0.804	0.792
WPMS08	$(GT)_{25}$	6	aspens	0.852	0.693	0.678
WPMS10	$(GT)_{23}$	6	aspens	0.185	0.802	0.783
WPMS12	$(GT)_{19}$	9	aspens	0.500	0.845	0.827
WPMS14	$(CGT)_{28}$	11	aspens	0.926	0.830	0.811
WPMS15	$(CCT)_{14}$	5	aspens	0.148	0.646	0.628
WPMS16	$(GTC)_8(ATCCTC)_5$	11	aspens	0.741	0.853	0.814
WPMS18	$(GTG)_{13}$	7	aspens	0.556	0.814	0.797
WPMS20	$(TTCTGG)_8$	3	aspens	0.148	0.458	0.469
Bp04	$(GT)_{12}…(GA)_5$	9	Curly birch	0.556	0.856	0.834
Bp15	$T_9(GT)_{13}$	9	Curly birch	0.353	0.831	0.717
BpTA	$A_6TA_8…(TA)_{13}$	3	Curly birch	0.158	0.323	0.308
L1.10	$(GA)_4AA(GA)_{10}$	15	Curly birch	0.900	0.846	0.816
L2.3	$(AG)_{16}$	4	Curly birch	0.250	0.345	0.336
L2.7	$(TC)_8(TA)_8(TG)_{11}TT(TG)_3$	13	Curly birch	0.783	0.868	0.291
L3.4	$(GTAT)_3(GT)_5$	7	Curly birch	0.647	0.717	0.693
L5.4	$(TC)_{26}$	7	Curly birch	0.826	0.682	0.671
L5.5	$C_{12}CTCC(CT)_7TT(CT)_5$	12	Curly birch	0.348	0.858	0.845
L7.3	$(GT)_{18}(GA)_{14}$	5	Curly birch	0.696	0.750	0.723

The PCR reactions of the aspen samples were carried out as described in Suvanto and Latva-Karjanmaa (2005). The forward primers were labelled either with 6FAM (loci PTR4, PTR12, PTR14, WPMS05, WPMS10, WPMS12 and WPMS15), HEX (PTR1, PTR2, PTR5, PTR6, WPMS08, WPMS18 and WPMS20) or NED (PTR3, PTR8, WPMS14 and WPMS16). The reaction buffer contained 750mM Tris-HCl (pH 8.8 at 25°C), 200mM (NH4)2SO4 and 0.1% Tween 20. We used the PCR programmes described in Rahman et al. (2000) for loci PTR2 – PTR14, van der Schoot et al. (2000) for loci WPMS05 –WPMS12, and Smulders et al. (2001) for loci WPMS14-WPMS20. However, we mainly used 5°C lower annealing temperatures than those reported by van der Schoot et al. (2000) and Smulders et al. (2001), except for loci WPMS10 and WPMS18, where the annealing temperatures were 5°C higher and 3°C lower than the temperatures given in the references, respectively. The aspen microsatellites were amplified separately and combined later for fragment separation with ABI 377 Sequencer (Applied Biosystems, California, U.S.A.) using GeneScan-400HD[ROX] size standard (Applied Biosystems, California, U.S.A.), and the gel analysis was performed with GeneScan 3.1.2 and Genotyper 2.5 software (Applied Biosystems, California, U.S.A.).

DNA amplification for the curly birch samples was performed in 15-µl reactions by multiplexing two loci to the same reaction. The multiplexed locus pairs were: Bp15 and BpTA, Bp04 and L3.4, L2.7 and L5.4, L1.10 and L2.3 and finally L5.5 and L7.3. Forward primers were labelled with either IRD700 (Bp15, L2.3, L2.7, L5.4 and L5.5) or IRD800 (BpTA, Bp04, L1.10, L3.4 and L7.3). The reaction volume (15 µl) consisted of 1 µl DNA (the final concentration in the reaction mix varied approximately between 0.7 and 13 ng/µl), 1 x reaction buffer (75mM Tris-HCl (pH 8.0), 50mM KCl, 1mM EDTA, 0.1% Triton X-100 and 50% Glycerol (v/v)), 2 mM MgCl2, 0.4 µl of all forward and reverse primers (i.e. 4 x 0.4 µl, the end concentration of all primers in the reaction mix being 0.7 pmol/µl), 0.06 µl dNTP (end concentration 0.1 mM) and 0.04 µl DNA polymerase (Biotools, concentration 0.2 U in the reaction mix) using MJ Research PTC-100 Programmable Thermal Controller. The PCR programme first had a denaturation step of four minutes in 94°C, followed by a 30 times repeated denaturation, annealing and DNA elongation step (94°C 1min, 57ºC 1min 15s, 72°C 2min 30s), and finally a 10 minute elongation in 72°C. The amplification products were separated with DNA Sequencer Longread IR 4200 (Li-Cor Inc., Nebraska, U.S.A.) using IRDyeTM700 size standard (Li-Cor Inc., Nebraska, U.S.A.), and the gel analysis was performed using Saga Generation 2 software (Li-Cor Inc., Nebraska, U.S.A.).

Expected and observed heterozygoties of the loci were counted using Microsatellite analyzer (MSA) software version 3.15 (Dieringer & Schlötterer 2002). The power of clone identification was estimated using a measure developed by Parks and Werth (1993), which measures the probability that sampled trees would, by chance, have a similar genotype even though they are in fact different clones:

$$P_{gen} = \left(\prod_{i=1}^{N} piqi \right) 2^{h},$$

where piqi is the product of the two allele frequencies in each locus of the genotype, and h is the number of heterozygous loci within each multilocus genotype. Another measure, polymorphic information content (PIC) of the loci (Botstein et al. 1980), was used for analysing the value of the markers to detect polymorphism. It can be obtained from the following formula:

$$\text{PIC} = 1 - \sum_{i=1}^{k} \text{pi2},$$

where piis the frequency of the ith allele and k is the total number of alleles for that locus.

3 Results

The used loci varied in their usefulness for clone identification. All the aspen and curly birch clones, except for clones C06-01-47 and C06-02-53, could be identified with the loci used in this study. The aspen genotypes are presented in Table 3, and the curly birch genotypes in Table 4.

The number of alleles / locus in the aspen data varied between 2 and 11 and in the birch data between 3 and 15 (Table 2). The Pgen index was, in all cases, smaller than 1 x 10-10, which shows that the loci used were sufficiently powerful for clone identification. We also calculated the Pgen index using only the five most variable loci; for aspen these were WPMS14, WPMS16, PTR3, WPMS05 and WPMS12, and for birch L1.10, L2.7, L5.5, Bp04 and Bp15. With these loci the Pgen values in aspen were always between 7.5 x 10-10 and 3.5 x 10-5 and in birch (when counting only those individuals where all five loci could be amplified) between 1.2 x 10-10 and 3.2 x 10-7. The PIC values varied between 0.039 and 0.824 in aspen and between 0.291 and 0.845 in curly birch. The means were 0.601 and 0.623 for aspen and curly birch, respectively. Almost all aspen loci, except PTR1, PTR5, PTR6 and PTR12, had high PICs. In curly birch loci L5.5, Bp04, L1.10, L7.3, Bp15, L3.4 and L5.4 showed the highest PIC values.

The differences between observed and expected heterozygoties (Table 2) in part of the loci, showing mostly heterozygote deficiency, indicate that there may be problems in the amplification of some loci. Aspen loci PTR3 and PTR8 showed stuttering (a slip of the DNA polymerase in the DNA elongation stage), which made the recognition of the correct allele size sometimes difficult.

Table 3. The genotypes of the aspen clones. The numbers represent different allele sizes. It was not possible to obtain a genotype of clone 147 in locus WPMS12.

Clone	PTR1	PTR2	PTR3	PTR4	PTR5	PTR6	PTR8	PTR12	PTR14	WPMS05	WPMS08	WPMS10	WPMS12	WPMS14	WPMS15	WPMS16	WPMS18	WPMS20
C05-99-8	251/260	216/216	216/226	104/108	150/150	110/110	138144	254/254	196/196	302/302	212/232	222/222	148/158	212/233	183/183	168/186	225/225	174/174
C05-99-9	251/251	207/210	216/232	102/108	150/150	110/110	134/144	254/254	196/196	302/304	212/225	222/254	158/158	212/233	168/168	174/186	222/228	182/182
C05-99-10	251/251	207/210	216/232	102/102	150/150	110/110	134/144	254/254	196/199	302/302	212/225	252/252	150/158	212/224	186/186	183/183	225/231	180/182
C05-99-11	251/251	210/216	216/232	106/128	150/150	114/114	140/146	254/254	196/196	302/302	212/225	228/228	148/160	215/218	183/183	168/174	216/222	182/182
C05-99-12	251/260	210/213	216/232	102/104	150/150	110/120	134/144	254/254	196/199	302/302	212/225	222/252	158/158	212/224	168/168	186/186	225/225	180/182
C05-99-13	251/275	213/219	230/236	102/106	150/150	110/110	140/140	254/254	193/196	262/278	212/232	254/254	148/148	200/215	183/183	168/174	225/225	182/182
C05-99-14	251/251	213/216	216/236	102/106	150/150	110/110	138/146	254/254	196/196	294/302	212/232	222/222	146/158	206/233	183/183	183/183	231/231	182/182
C05-99-15	251/263	213/219	216/236	104/106	150/150	110/110	138/138	254/254	157/196	262/278	212/232	222/222	162/162	210/215	183/183	165/171	213/222	182/182
C05-99-16	251/275	210/213	216/236	102/106	150/150	110/110	138/138	254/254	157/196	262/278	212/232	222/222	148/160	212/212	183/183	177/183	213/231	182/182
C05-99-17	251/275	213/219	216/236	102/104	150/150	110/110	138/138	254/254	157/193	262/278	212/232	222/222	160/162	212/215	183/183	165/177	213/228	182/182
C05-99-18	251/275	213/219	218/230	102/106	150/150	110/110	138/138	254/254	196/196	262/278	212/232	254/254	160/162	212/215	183/186	165/177	213/231	180/182
C05-99-19	251/251	216/234	216/236	106/110	150/150	110/110	140/140	254/254	196/196	282/302	212/232	252/252	146/158	212/233	183/186	186/186	228/231	180/180
C05-99-20	251/251	216/234	216/236	102/106	150/150	110/110	140/146	254/254	196/196	282/294	228/228	252/252	146/160	212/233	183/186	186/186	225/225	180/182
C05-99-21	251/269	210/213	186/186	106/106	150/150	110/110	138/142	254/254	193/196	302/302	232/232	218/218	146/146	212/233	183/183	183/189	219/228	182/182
C05-99-22	251/260	210/210	216/216	104/104	150/150	110/110	138/138	254/254	193/196	262/302	212/225	218/252	172/172	212/233	166/166	165/183	222/222	182/182
C05-99-23	251/251	207/210	216/216	102/106	150/150	110/110	142/146	254/254	190/196	262/294	212/232	254/254	158/158	212/227	186/186	180/186	222/222	182/182
C05-99-24	251/251	210/213	216/216	106/106	150/150	110/110	138/144	254/254	196/196	262/294	212/232	218/218	172/172	212/230	186/186	156/186	222/231	182/182
C05-99-25	251/251	207/210	216/236	102/106	142/142	110/114	140/140	254/254	196/196	262/276	212/232	218/218	158/158	206/233	183/183	165/183	213/219	182/182
C05-99-26	251/260	210/210	216/216	106/106	150/150	110/110	138/138	254/254	196/196	262/302	212/225	218/250	172/172	212/233	166/166	165/183	222/222	182/182
C05-99-27	251/251	213/216	222/232	108/108	150/150	110/110	138/138	254/254	193/196	266/278	206/212	252/252	160/162	212/233	183/186	177/183	222/225	182/182
C05-99-28	251/251	210/210	216/224	102/102	150/150	110/120	136/136	254/254	196/202	262/276	232/232	218/218	154/154	206/233	166/166	165/189	216/231	182/182
C05-99-30	251/251	219/222	216/232	102/106	150/150	110/110	138/138	254/254	196/199	270/302	212/232	228/228	158/160	218/221	183/183	168/186	222/225	182/182
C05-99-31	251/251	210/210	216/216	106/106	150/150	110/120	138/140	254/266	196/202	294/302	218/232	252/252	146/146	218/224	183/183	162/165	225/225	180/180
C05-99-32	251/251	210/216	216/232	106/106	150/150	120/120	142/142	254/254	196/199	276/294	225/232	254/254	158/158	218/218	183/183	156/183	225/228	174/174
C05-99-33	239/251	204/210	216/232	102/106	150/150	110/114	138/146	254/254	196/196	262/302	212/212	222/222	158/164	215/233	172/172	186/186	219/219	182/182
C05-99-34	251/263	207/213	216/232	102/108	150/150	110/110	140/140	254/254	196/196	262/276	212/225	222/250	150/160	215/221	172/172	156/186	222/222	180/180
147	251/251	213/213	214/218	106/106	150/150	110/110	136/138	254/254	193/202	278/294	225/232	252/252	-	215/233	183/183	165/165	222/222	180/180

Working Papers of the Finnish Forest Research Institute 77
http://www.metla.fi/julkaisut/workingpapers/2008/mwp077.htm

Table 4. The genotypes of the curly birch clones. Some samples could not be amplified using all the loci (-). Empty cells indicate that the clone was not analysed using that particular locus.

Clone	Bp04	Bp15	BpTA	L1.10	L2.3	L2.7	L3.4	L5.4	L5.5	L7.3
C05-98-2	160/160	-	142/142	176/180	196/208	156/160	250/262	239/253	136/136	198/220
C05-98-3	142/148	116/116	140/144	176/180	208/214	188/196	262/262	239/259	124/130	200/200
C05-01-35	150/150	102/102	140/144	170/176	196/196	158/180	266/266	251/253	134/134	190/198
C05-01-36	142/160	102/118	140/140	-	-	166/172	262/262	239/239	134/134	198/200
C05-01-37	164/164	102/102	140/140	182/188	196/196	158/162	262/266	239/243	146/146	190/190
C05-01-38	144/160	102/118	140/140	172/176	196/208	154/166	250/262	239/241	134/134	190/200
C05-01-40	160/166	102/102	140/140	176/176	196/196	166/172	264/264	241/243	132/150	198/200
C05-01-41	142/142	-	140/144	176/190	196/196	174/178	262/268	241/253	134/144	190/198
C05-01-42	142/142	100/104	140/140	178/188	196/196	162/178	250/272	239/241	132/132	200/202
C05-01-43		100/104	140/140	176/176	196/196	158/162		239/243	130/130	198/198
C05-01-44	142/158	116/116	140/140	184/204	196/196	178/178	250/262	239/241	132/132	198/220
C05-01-45	144/160	92/98	140/140	176/184	196/196	160/166	262/262	239/253	122/146	190/220
C05-01-50	150/150			202/214	196/208	158/178	250/272	239/241	120/130	198/198
C06-01-47	144/152	100/100	140/140	170/176	196/196	158/178	262/262	239/243	142/142	198/200
C06-01-48	-	108/108	140/140	-	-	158/162	-	239/241	132/138	200/200
C06-02-51		102/112	140/140	176/182	208/208	162/172		239/253	134/134	198/202
C06-02-52		100/100	144/144	-	-	160/160		239/241	132/132	198/198
C06-02-53	144/152	100/100	140/140	170/176	196/196	158/178		239/243	142/142	198/200
C06-02-55		100/100	140/140	190/196	196/196	158/158	250/262	239/239	130/130	190/198
C06-04-58	144/144	118/118	140/140	176/196	196/196	158/178	262/268	239/239	120/136	190/200
C06-05-69	158/160			182/190	196/218	152/152	248/262	239/243	130/134	190/200
C06-05-71	148/160			194/200	196/196	158/158		239/239	134/134	198/200
C06-05-70	160/160			176/184	196/196	158/162	262/268	239/249	120/132	200/202

4 Discussion

The used microsatellite loci suited well for clone identification. All studied clones except two curly birch clones could be identified. Due to the relatively high degree of polymorphism and the large number of conducted loci, we consider it very unlikely that the two genotypically similar curly birch samples would in fact belong to the same clone. The probability of such an occurrence is about 1 x 10-10, which is so low that the number of curly birch individuals in the world would very likely not be enough to find such a case. Thus we conclude that the two similar curly birch clones are members of the same clone. The error in their separation into different clones may have occurred in a number of steps of the study, which include the sampling from original trees, micropropagation, establishment of the field trial, sampling and DNA extraction. Such findings especially emphasize the urgent need for reliable and practical clone identification methods.

Our Pgen values were much smaller than the equivalent values reported in previous studies. Easton (1997) used five allozyme loci for clone identification in European aspen, and his Pgen values varied between 2 x 10-4 and 3 x 10-2. The probability to obtain a similar banding pattern in different clones was reported to be between 10-4 and 10-3 in the studies of Rogstad et al. (1991) in *P. tremuloides* using the M13 repeat probe and Sigurdsson et al. (1995) in *P. trichocarpa* using RAPDs. With microsatellites, Fossati et al. (2005) found in their study on *P. x canadensis* clones that the probability to obtain a similar genotype in all six SSR loci was 7.5 x 10-9. A previous study on European aspen (Suvanto & Latva-Karjanmaa 2005) had Pgen values < 5.0 x 10-4. We did not find any clone identification studies on birch species in the literature, and our study is also probably the first in which curly birch clones have been genotyped using genetic markers.

Four same loci, namely WPMS14, WPMS16, WPMS18 and WPMS20 that we used in our study, have been used for clone identification in *P. deltoides* and *P. x canadensis* (Fossati et al. 2005). The PIC values in our study were mostly higher than the values with *P. deltoides* and lower than the values with *P. x canadensis* in the study of Fossati et al. This is understandable, since these loci were developed for *P. nigra*, which is the other parent of *P. x canadensis*. The fact that our PIC values in two loci (WPMS16 and WPMS18) were actually higher than the values for *P. x canadensis* shows that these loci are polymorphic enough to be used for clone identification in European and hybrid aspen as well. Since 14 out of 18 used aspen loci and 7 out of 10 birch loci had high PICs, these loci have adequately high polymorphism for clone identification.

The deficiency of heterozygotes in some loci, which was indicated by a smaller observed heterozygosity compared to the respective expected heterozygosity, can be due to problems in fragment amplification. This can be caused by null alleles, i.e. alleles that do not amplify, because PCR conditions are not suitable, or because the area, where the primers anneal, is not complementary to the primer sequences (Selkoe & Toonen 2006). This can be the case, when the microsatellites have been developed to a different species than to which they are applied. In our study, there was much heterozygote deficiency in aspen loci PTR8, WPMS10, WPMS12, WPMS15, WPMS18 and WPMS 20, and in birch loci Bp04, Bp15, BpTA and L5.5. Therefore it is recommendable to use other loci for clone identification.

Stuttering caused some problems in genotyping concerning two loci (PTR3 and PTR8). As stuttering is usually locus specific, it is often easy to learn to identify the right band patterning.

Sometimes stuttering can also be diminished e.g. by adding BSA (bovine serum albumin) to the reaction mix or by reducing the dNTP concentration. In our case these methods did however not remove the stuttering completely. Since a large number of microsatellites have been developed for different Populus species (Dayanandan et al. 1998, Rahman et al. 2000, van der Schoot et al. 2000, Smulders et al. 2001, Tuskan et al. 2004, IPGC 2006), and many of them amplify well across species (e.g. Tuskan et al. 2004), it is therefore expedient to choose the most easily readable, sufficiently polymorphic loci.

Another problem was the poor amplification of some of the birch loci (Bp04, Bp15, L3.4 and especially L1.10 and L3.4). Although we repeated the analysis several times, we could not get the genotype of these loci from all the curly birch samples. The unsuccessful amplification could have been caused by the low quality of the extracted DNA, which may have inhibited the PCR reactions. Compared to aspen samples, birch leaves were more difficult to process: they were "sticky" (Annukka Korpijaakko, pers. com.), which could have been due to the presence of secondary metabolites and / or polysaccharides that complicate DNA extraction (Csaikl et al. 1998). However, the DNA used in microsatellite PCRs does not usually have to be very pure (Csaikl et al. 1998). Another reason for the poor amplification could have been null alleles. Although not as many microsatellites have been developed for Betula as for Populus, it is probably possible to choose enough loci to enable reliable clone identification in birches as well.

Although we did not have all the registered Finnish aspen and curly birch clones in our data, we were able to identify all the commercially propagated clones. Identification of all registered clones is also very likely due to the high number and excessive polymorphism of the used loci. Moreover, the use of missing clones in forest reproductive material in the future is unlikely, due mainly to their poor propagation ability or poor quality of the curly-grained wood.

On the basis of our results, we recommend that loci PTR2, PTR4, WPMS05 and WPMS12, WPMS14 and WPMS16 would be used in clone identification for European and hybrid aspen. For curly birch the situation is not so clear, since many otherwise multivariable loci had problems during amplification. However, it seems that at least loci L1.10 and L7.3 are very useful for clone identification. It has to be noted that when the genetic variation of the material is unknown, it is important to test several marker loci and later on select the most variable and reliable ones.

5 Conclusions

We conclude that, for aspens and curly birch, microsatellites offer a reliable and practical clone identification method with sufficient resolution power, which is needed due to the Council Directive 1999/105/EC (2000) on the marketing of forest reproductive material within the community. Microsatellites can be used for trees of different ages, growing in variable habitats, and the analysis can be performed using only one leaf or bud. Compared to morphological identification, the microsatellite-based method is more objective. The clone identification method based on microsatellites could diminish errors in genotyping the clones and thus help to regulate the trading of forest reproductive material. This method could also provide a European-wide certification, thus lightening the bureaucracy. The method would be useful not only for the authorities enforcing the law, but also for the companies producing the forest material and, at the end of the chain, the forest owners themselves.

In order to diminish the unavoidable errors in sampling and processing of the plant material and analysing the microsatellites, the clone identification method should be standardised over Europe. The best way of doing this would be to concentrate the identification process in a limited number of laboratories in Europe, where the analysis could be performed routinely. This would not only diminish the error caused by analysing samples in different places, but would also lower the costs.

Acknowledgements

We thank Annukka Korpijaakko and Toshka Nyman for helping in the DNA extractions and microsatellite runs. Hiski Aro and Raimo Jaatinen collected the plant material. The comments of Niina Stenvall and Saila Varis helped to improve the manuscript. This work was financially supported by the Finnish Ministry of Forestry and Agriculture.

References

Alanen H (2003) Haapakloonien tunnistaminen morfologisten ja fenologisten ominaispiirteiden ja DNA-merkkigeenien avulla. MSc thesis, Department of Forest Ecology, University of Helsinki, Finland. Pp 84 + 17. (In Finnish).

Arens P, Coops H, Jansen J, Vosman B (1998) Molecular genetic analysis of black poplar (Populus nigra L.) along Dutch rivers. Mol Ecol 7: 11-18. doi: 10.1046/j1365-294X.1998.00316.x

Barnes B (1969) Natural variation and delineation of clones of Populus tremuloides and P. grandidentata in northern Lower Michigan. Silvae Genet 18: 130-142.

Benjak A, Kondradi J, Blaich R, Forneck A (2006) Different DNA extraction methods can cause different AFLP profiles in grapevine (Vitis vinifera L.). Vitis 45: 15-21.

Botstein D, White RL, Skolnick M, Davis RW (1980) Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am J Hum Genet 32: 314-331.

Cajander A (1926) The theory of forest types. Acta Forestalia Fennica 29(3).

Cheliak W, Pitel J (1984) Electrophoretic identification of clones in trembling aspen. Can J For Res 14: 740-743.

Council Directive 1999/105/EC (2000) On the marketing of forest reproductive material. OJ L11: 17-40.

Csaikl U, Bastian H, Brettschneider R, Gauch S, Meir A, Schauerte M, Scholtz F, Sperisen C, Vornam B, Ziegenhagen B (1998) Comparative analysis of different DNA extraction protocols: A fast, universal maxi-preparation of high quality plant DNA for genetic evaluation and phylogenetic studies. Plant Mol Biol Rep 16: 69-86. doi: 10.1023/A:1007428009556

Dayanandan S, Rajora O, Bawa K (1998) Isolation and characterization of microsatellites in trembling aspen (Populus tremuloides). Theor Appl Genet 96: 950-956. doi: 10.1007/s001220050825

Dieringer D, Schlötterer C (2002) Microsatellite analyzer (MSA): a platform independent analysis tool for large microsatellite data sets. Mol Ecol Notes 3: 167-169.

Easton E (1997) Genetic variation and conservation of the native aspen (Populus tremula L.) resource in Scotland. PhD thesis, University of Edinburgh, UK.

Fossati T, Zapelli I, Bisoffi S, Micheletti A, Vietto L, Sala F, Castiglione S (2005) Genetic relationships and clonal identity in a collection of commercially relevant poplar cultivars assessed by AFLP and SSR. Tree Genetics & Genomes 1: 11-19. doi: 10.1007/s11295-004-0002-9

Hagqvist R (2004) Cultivation. In: Kosonen M (ed) Curly birch. Metsälehti Publishers, Otava Book Printing Ltd, Keuruu, Finland, pp 52-60.

Holm S (2004) Haavan viljely Suomessa ja Virossa. Metsätieteen aikakauskirja 1/2004: 117-118. (In Finnish).

IPGC (2006) SSR resource. The international Populus Genome Consortium, U.S.A. Available in: http://www.ornl.gov/sci/ipgc/ssr_resource.htm. Cited 31 Aug 2006

Kosonen M (2004) Uses of curly birch from tools and implements to corporate gifts. In: Kosonen M (ed) Curly birch. Metsälehti Publishers, Otava Book Printing Ltd, Keuruu, Finland, pp. 151-163.

Kulju K, Pekkinen M, Varvio S (2004) Twenty-three microsatellite primer pairs for Betula pendula (Betulaceae). Mol Ecol Notes 4: 471-473. doi: 10.1111/j.1471-8286.2004.00704.x

Lin D, Hubbes M, Zsuffa L (1994) Differentiation of poplar and willow clones using RAPD fingerprints. Tree Physiol 14: 1097-1105.

Ogyu K, Tsuda Y, Sugaya T, Yoshimaru H, Ide Y (2003) Identification and characterization of microsatellite loci in Betula maximowicziana Regel. Mol Ecol Notes 3: 268-269. doi: 10.1046/j.1471-8286.2003.00419.x

Parks J, Werth C (1993) A study of spatiel features of clones in a population of bracken fern, Pteridium aquilinum (Dennstaedtiaceae). Am J Bot 80: 537-544.

Rahman M, Dayanandan S, Rajora O (2000) Microsatellite DNA markers in Populus tremuloides. Genome 43: 293-297.

Rajora, O, Rahman M. (2003) Microsatellite DNA and RAPD fingerprinting, identification and genetic relationships of hybrid poplar (Populus x canadiensis) cultivars. Theor Appl Genet 106: 470-477. doi: 10.1007/s00122-002-1082-2

Ranua J (2002) Industrial use of aspen fibres. In: Pulkkinen P, Tigerstedt PMA, Viirros R (eds) Aspen in papermaking. University of Helsinki, Department of Applied Biology, Publication No 5, Helsinki, Finland, pp. 1–4.

Rogstad S, Nybom H, Schaal B (1991) The tetrapod "DNA fingerprinting" M 13 repeat probe reveals genetic diversity and clonal growth in quaking aspen (Populus tremuloides, Salicaceae). Plant Syst Evol 175: 115-123. doi: 10.1007/BF00937841

Schoot J van der, Pospíšková M, Vosman B, Smulders M (2000) Development and characterization of microsatellite markers in black poplar (Populus nigra L.). Theor Appl Genet 101: 317-322. doi: 10.1007/s001220051485

Selkoe K, Toonen R (2006) Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. Ecol Lett 9: 615-629. doi: 10.1111/j.1461-0248.2006.00889.x

Sigurdsson V, Anamthawat-Jónsson K, Sigurgeirsson A (1995) DNA fingerprinting of Populus trichocarpa clones using RAPD markers. New Forests 10: 197-206. doi: 10.1007/BF00027923

Smulders M, Schoot J van der, Arens P, Vosman B (2001) Trinucleotide repeat microsatellite markers for black poplar (Populus nigra L.). Mol Ecol Notes 1: 188-190. doi: 10.1046/j.1471-8278.2001.00071.x

Suvanto L, Latva-Karjanmaa T (2005) Clone identification and clonal structure of the European aspen (Populus tremula). Mol Ecol 14: 2851-2860. doi: 10.111/j.1365-294X.2005.02634x

Tuskan G, Gunter L, Yang Z, Yin T, Sewell M, DiFazio S (2004) Characterization of microsatellites revealed by genomic sequencing of Populus trichocarpa. Can J For Res 34: 85-93. doi: 10.1139/X03-283

UPOV (1981) Guidelines for the conduct of tests for distinctness, homogeneity and stability – Populus L. International Union for the Protection of New Varieties of Plants, Geneva, Switzerland. Available in: http://www.upov.int/en/publications/tg-rom/tg021/tg_21_7.pdf. Cited 31 Aug 2006

Wu B, Lian C, Hogetsu T (2002) Development of microsatellite markers in white birch (Betula platyphylla var. japonica). Mol Ecol Notes 2: 413-415. doi: 10.1046/j.1471-8278.2002.00260.x

Yu Q, Pulkkinen P (2003) Genotype-environment interaction and stability in growth of hybrid aspen clones. For Ecol Manage 173: 25–35. doi: 10.1016/S0378-1127(01)00819-2