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Fast and cheap identification of elite aspen clones in the North-West of Russia using ISSR markers

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Abstract

In 2001–2006, several experimental aspen plantations were established in the North-West of Russia **(**Leningrad region). Three *in vitro* propagated elite aspen (*Populus tremula* L.) clones from the Kostroma Forest Research Station were used as the planting stock for plantations. The planting plans of some experimental plantations were lost, which made it impossible to identify the genetic lineages. 13-years old unknown aspen clones demonstrated prominent growth rates, and reliably overtook natural aspen coppice. ISSR markers were used for fast and cheap restoring of the missing planting plan of the experimental aspen plantation under study; as a result, progenies of three elite aspen clones were recognized. The best fast-growing and stem rot resistant aspen clones was identified and called "Kostroma".

Key words

aspen, elite clones, molecular markers, ISSR, clonal plantations

Introduction

Short rotation forest plantations could serve as an alternative timber source for the fast-developing wood processing industry (pulp and paper) to prevent deforestation of the Russian North. Currently, timber industry companies extensively exploit natural forests that are mostly coniferous species forming the nature ecosystems in the North of Russia. As a consequence, the natural forest areas around the woodworking enterprises are rapidly reducing. Since the companies begin to face the problem of diminishing nearby timber sources, and, in addition, transport expenses are rather high, they show interest in some alternative sources of timber supply – fast-growing short-rotation forest plantations. Aspen (*Populus tremula* L.) is one of the most common tree species in the Russian North-West forests. Since aspen shows high growth rate all over the country, this species is suitable for the propagation in forest plantations. The main problem for aspen is heart rot, which is a fungal disease caused by polypore fungus *Phellinus tremulae* (Bondartsev) Bondartsev & P.N. Borisov. At present, almost all aspen trunks in mature native tree stands have central stem rot.

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In Russia, the first steps on aspen breeding were taken in the 1940s (Yablokov 1963; Bagaev 1990; Ivannikov 1952). It was in this period that a forest plot with fast-growing and stem rot resistant aspen trees was discovered in the natural forests of the Kostroma region (Central Russia). The research staff of the Kostroma Forest Research Station supervised by A.S. Yablokov and S.N. Bagaev selected the best and elite clones of aspen. The collected and replanted aspen clones were distinguished by high rates of growth and higher resistance to stem rot.

In 1999–2000, the Saint-Petersburg Forestry Research Institute (SPbFRI) received some twigs of different Kostroma aspen clones. The buds of those twigs were introduced *in vitro* culture and microshoots were obtained. The plants propagated from the microshoots were used to establish several experimental plantations of the aspen clones in 2001–2006 in the Leningrad region. Special attention was given to the most promising Kostroma clone 35, which was maintained at the SPbFRI *in vitro* germplasm collection under the name line f.11. The plantations generated from aspen line f.11 are well documented: the line was propagated and planted in 2006 in the Leningrad region (Gatchina forest district) on two experimental plots – Plot 1 (square 68, site 3; 59,3167 N 30,0735 E) and Plot 3 (square 15, site 4; 59,4059 N 30,1354 E). 12 years of tree growth monitoring on the experimental plots evidenced of an accelerated growth rate of the plantations created with ramets of clone 35 (line f.11) (Zhigunov et al. 2014).

The other Kostroma clones (in all, three lines) were propagated *in vitro* culture and planted in 2004 by the employees on the experimental plot 6 of Gatchina forest district (square 49, site 4; 59,3469 N 30,1909 E). Shortly after that, this forestry enterprise was closed, and the planting plan of the aspen clones was lost, and it is still unknown. The recovery of the aspen planting plan would be of great value for breeding purposes since some trees on experimental plot 6 demonstrate unexpectedly high growth rates (Fig. 1).

The application of genetic markers is a very important tool for the identification of clones in poplar and aspen breeding since morphological traits are insufficient for that purpose for closely related material (Liesebach et al., 2009). To identify different aspen clones in the experimental plot 6, we employed one of the simplest molecular marker methods known as inter-simple sequence repeats, ISSRs (Zietkewicz et al. 1994). The method is based on PCR with a single 15–20 bp primer targeted microsatellite locus with subsequent electrophoresis separation of amplificated fragments. ISSR primers often have anchors (a short, 1–4 bp, random degenerated sequence at the 3' or 5' end). An anchor makes primers more selective and reproducible. Since microsatellites widely occur in genomes and evolve very rapidly due to their structure, the ISSR method allows revealing polymorphism even in related intraspecific groups. ISSR analysis is a more sensitive and reliable method than RAPD and it is less laborious than AFLP (Souframanien et al. 2004; Jianming et al. 2006).

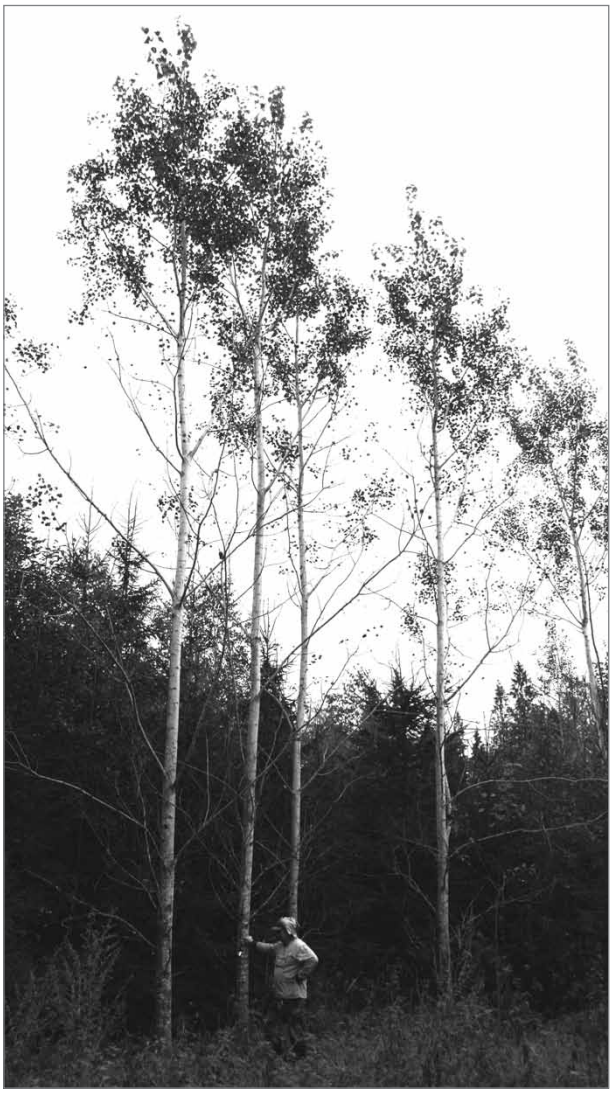

Figure 1. Plot 6 in the experimental aspen plantation (59°19,874' N, 30°10,567' E): the highest trees at age of 13

Each ISSR primer investigates many genetic loci, so that there is no need to use many primers for genotyping. For example, Prevost *et al.* (1999) reported successful identification of 34 genotypes of potato cultivars with just two ISSR primers. To reduce the cost of genotyping of extremely large plantations of forest species, ISSR markers could be the best choice.

Material and methods

Plant material and ISSR analysis

In molecular analysis, we included four elite Kostroma aspen clones from *in vitro* collection of SPbFRI and their assumed clone progeny from experimental plot 6. DNA was extracted from fresh leaves using a modified CTAB method (Bousquet et al. 1990).

For the analysis, we applied one tetranucleotide ISSR primer without anchor and seven anchored ISSR primers (Tab. 1). Selected primers proved to have high efficiency in revealing the intraspecific polymorphism in different plant species (Tsumura et al. 1996; Godwin et al. 1997; Kochieva et al. 2002; Younis et al. 2008).

ISSR-PCR was performed in 15 μL volume containing 1×buffer for Taq-polymerase (pH 8.6, 2.5 mM Mg^{2+}), 200 μmol dNTPs, 0.5 μmol of ISSR primer, 0.6 unit of Taq-polymerase (Dialat, Russia), and 30–60 ng of the template DNA. The PCR reaction was carried out in thermocycler GeneAmp PCR system 9700 (Applied Biosystems) with the following program: initial denaturation at 94°C for 5 min, 39 cycles of denaturation at 94°C for 30 s, primer annealing for 45 s (annealing temperature for each primer is given in Table 1), elongation at 72°C for 1 min), and the final elongation at 72°C for 10 min.

PCR products were separated by electrophoresis in 2% agarose gel in 0.5% TBE buffer and then stained with ethidium bromide.

For primer testing, we used four different aspen clones from *in vitro* collection of SPbFRI; each clone was presented by two replications. We were looking for a polymorphic and reproducible amplification profiles. Besides, the profiles should not produce too many bands of overlapping sizes, compromising the accuracy of the fragment analysis. Clone aspens from experimental plot 6 were examined with ISSR primer, which is the most suitable for these conditions.

ISSR bands for each progeny tree were scored as present (1) or absent (0). The genetic distances between all clones were calculated using the DARwin 6.0.0 software (http://darwin.cirad.fr/, accessed on 19 September 2018) according to the algorithm "Simple matching". In addition, the hierarchical cluster analysis was performed using the unweighted pair group method (UPGMA) and a dendrogram was constructed.

Measurement of growth traits

Tree height was measured for all aspen clones from experimental plot 6 every year for seven years (starting with 7-year-old trees, except the twelfth year). As control, each year we measured the nearby natural aspen coppice (in all, 60 trees) of the same age as the aspen clones. Annual height measures of natural aspen and genetics groups of clone aspen were comprised by the Student's t-test.

Results

ISSR analysis

Of the eight primers examined, only HB12, M12C and M10 fitted the requirements (Fig. 2). Among them, HB12 and M14 distinguished two groups, while M12C

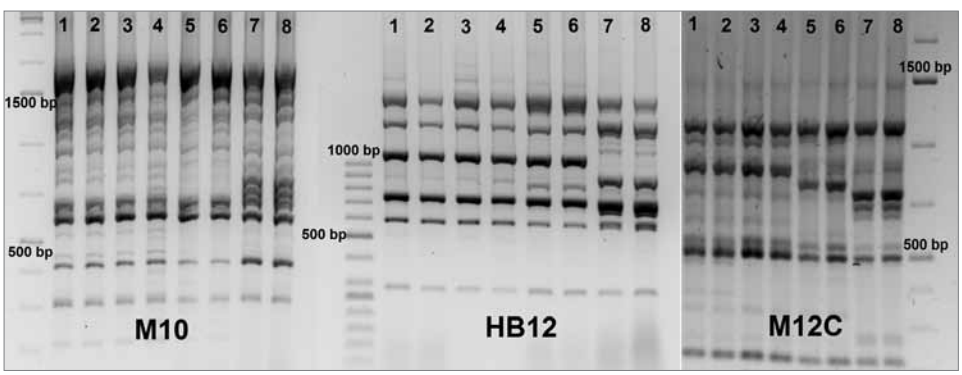

Figure 2. The best DNA profiles generated with ISSR primers for four aspen genotypes propagated *in vitro*. Each genotype was presented in the analysis by two ramets (lines on gels: 1,2; 3,4; 5,6; 7 and 8)

discriminated the three groups in the same material. Moreover, M12C allowed to analyse 20 loci, 15 of them were polymorphic (Tab. 1). Nonetheless, ISSR profile of M12C was clear and easy-reading, instead of M2 – another high-polymorphic ISSR. We concluded that the analysis with M12C ISSR marker would be enough to separate the different clones in the experimental aspen plantation. Thus, 65 unknown aspen trees from the experimental plot 6 were genotyped with M12C.

Table 1. Characterization of ISSR primers tested for aspen clone identification efficiency

Primer	Sequence 5'–3'	Annealing temperature, °C	Total fragments	Polymorphic fragments
HB12	$(CAC)_3GC$	43	15	10 (67%)
M2	$(AC)_8YG$	54	24	18 (75%)
M10	$(CA)_6RG$	44	18	4 (22%)
M12C	$(CA)_6RC$	44	20	15 (75%)
M11G	$(CA)_6G$	40	22	9 (40%)
M13T	$(AGC)_4T$	45	20	11 (55%)
M13C	$(AGC)_4C$	48	16	10 (62%)
M14	$(GACA)_4$	50	13	4 (31%)

Y = C or T, R = A or G.

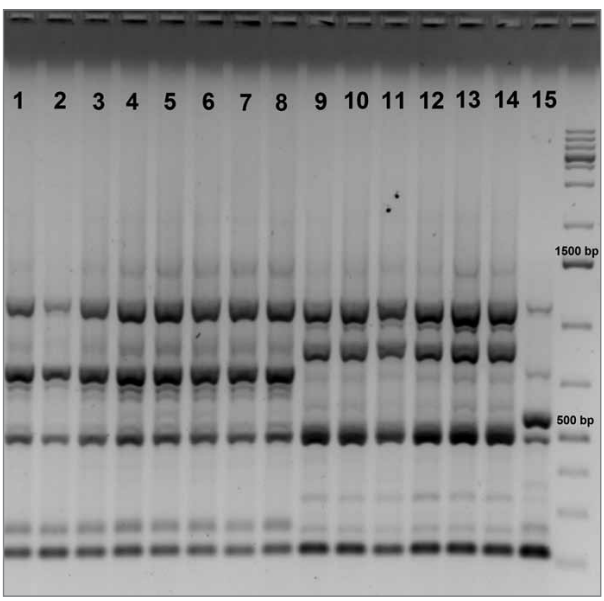

Figure 3. Three types of DNA profiles obtained with M12C primer corresponding to the three different aspen clones growing on the experimental plot 6 (lines 1–8 - Group 1, lines 9–14 - Group 3, line 15 - Group 2)

M12C distinguished the three groups of genetically identical aspen in the experimental plot 6 by generating three clearly recognizable DNA profiles (Fig. 3).

The genetic distances were calculated using the polymorphic loci identified by M12C. The dendrogram (UPGMA) in Figure 4 reflects three groups probably corersponding with progenies of three different clones. Revealed genetic groups combined 34, 6 and 24 trees, correspondingly. Tree 9 didn't belong to any group.

Growth dynamic of elite aspen clones

According to the results of ISSR analysis, we allocated trees to the three groups (Fig. 5). When trees were 7–9 years old, all three groups were reliably growing worse than the natural aspen coppice, which was taken as a control (Tab. 2). Then, the growth rates didn't have any difference. However, since the twelfth age, the first and second groups have overtaken the natural aspen coppice.

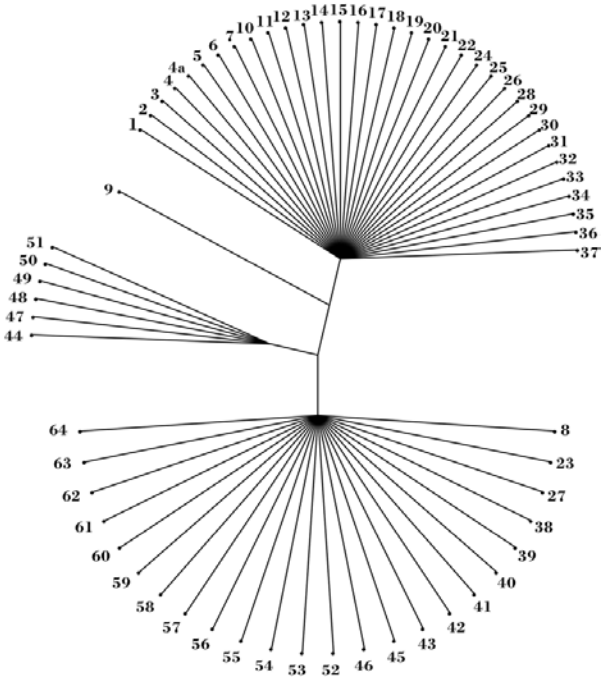

Figure 4. Unrooted UPGMA dendrogram based on the bands generated by M12C marker. Trees are divided into three groups, corresponding to the three parent genotypes

Table 2. Comparison growth dynamics of two groups of aspen clones and natural aspen coppice with Student's t-test

Group	Age, year	*H*±6 (m)	t-value	p
1	7	5.3±0.34	2.670	0.003**
2		4.8±0.14	2.771	0.000**
natural aspen		5.1±0.29	–	–
1	8	6.7±0.49	2.028	0.027*
2		6.3±0.49	2.262	0.016*
natural aspen		7.9±0.28	–	–
1	9	7.3±0.53	2.086	0.223
2		6.6±0.39	3.499	0.012*
natural aspen		8.0±0.16	–	–
1	10	9.1±0.63	2.056	0.593
2		8.6±0.44	2.228	0.138
natural aspen		9.5±0.33	–	–
1	11	9,6±0.94	2.101	0.941
2		9,7±0.59	2.571	0.977
natural aspen		9.7±0.23	–	–
1	13	13.8±0.46	2.771	0.006**
2		13.6±0.28	2.819	0.003**
natural aspen		12.0±0.39	–	–

t – value and the significance level were calculated using Student's t-test for independent samples (* $p < 0.05$; ** $p < 0.01$).

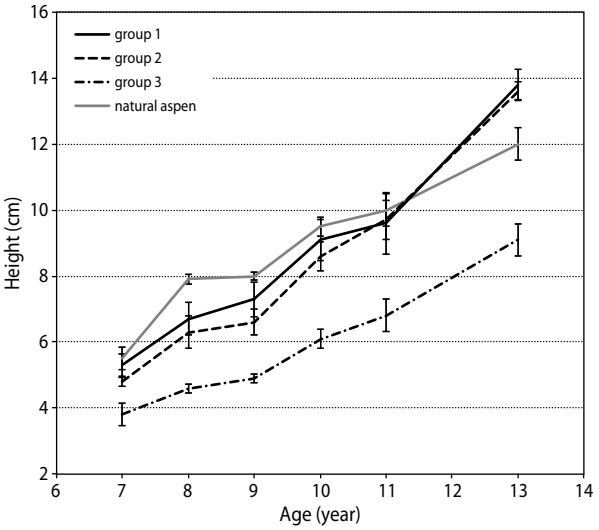

Figure 5. Growth dynamics of different aspen clones on the experimental plot 6. Natural aspen coppice is taken as a control.

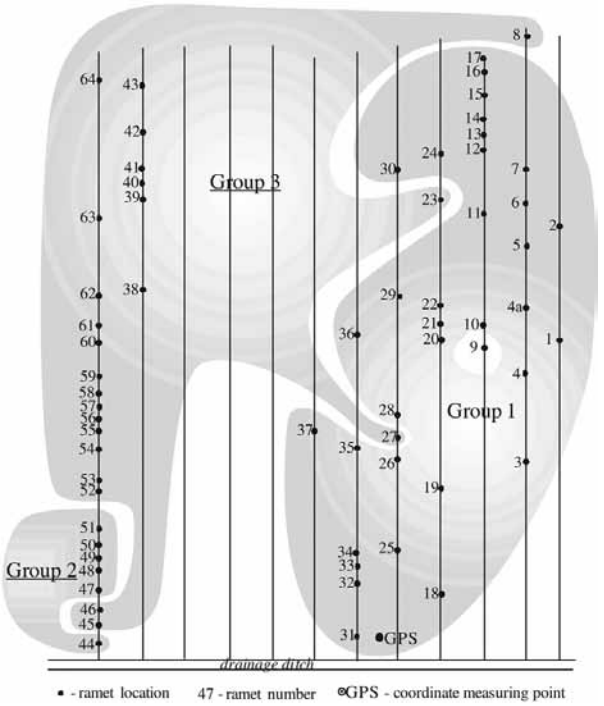

Figure 6. Allocation of aspen genotypes to the three ancestral clones (Group 1, 2, 3) on the experimental plot 6. Tree 9 differs from the established three groups

Discussion

In some cases (Labra et al. 2006; Lopez-Aljorna et al. 2007), ISSR markers were used for fingerprinting or revealing genetic variability on different plant species, including poplars (Gao et al. 2006; Jianming et al. 2006). ISSR markers are a powerful instrument for genotyping. This method has made possible to recognize aspen genotypes and restore the missing planting plan of the experimental plantation without large costs of money and time. Moreover, in our case, only one high-polymorphic ISSR marker was enough to reliably distinguish parent's genotypes. Spatial distribution of the revealed genetic groups on experimental plot 6 is shown in Figure 6.

It is easy to see that aspen groups 1 and 2 had similar growth rates (Fig. 5). Besides, trees of group 1 keep an apical dominance despite the lack of side shading. This makes it possible to grow this genotype in plantations with low density. The best trees from group 1 reached the heights of 15–16 m and diameters of 16–17 cm at the age of 13 (Fig. 1) and reliably excel trees from control (Tab. 2). These are very good

parameters for aspen growing in the climatic conditions of the North-West of Russia. Moreover, there are much more alive clones from the first group (34 trees) as compared to the clones from the second group (6 trees). Of course, it can be randomness, but it can also imply better vitality of the first group genotype. Thus, we decided to mark out the most promising clone line (group 1) and called it "Kostroma".

Conclusions

Good-quality aspen clone "Kostroma" was identified in the experimental plot with a polymorphic ISSR marker. Due to high growth rates and stem rot resistance, this clone is strongly recommended for plantation cultivation in the North-West of Russia and in other regions with the same conditions.

Acknowledgements

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