Identification of Molecular Markers for Selected Wood Properties of Norway Spruce *Picea abies* L. (Karst.) II. Extractives Content

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Abstract

We describe the development of a SCAR-marker linked to low extractives content of Norway Spruce (*Picea abies* L [Karst.]) derived from AFLPs. In these analyses 57 different primer enzyme combinations were used in a bulked segregant analysis approach comparing individuals with high and low extractives content. A total of 14 polymorphic AFLP markers were detected between the pools. Five markers were selected for further analyses to verify their linkage to extractives content based on individuals used for pool constitution. One AFLP marker, found to be significant linked to low extractives content was converted into a SCAR marker for further validation. For this marker, a monomorphic band was obtained by using sets of nested primers or restriction site specific primers (RSS) which include the AFLP-restriction recognition site. The separation of the marker from unlinked size homologous marker-alleles was realized by a SSCP-approach. Validation of the marker on different full-sib families confirmed the usability to separate the classes for low and high extractives content of *Picea abies*.

Key words: Bulked segregant analysis, AFLP, Molecular markers, Marker assisted selection (MAS), Wood properties, Marker conversion, SCAR marker.

Introduction

Extractives are low-molecular weight lipophilic components in wood consisting mainly of waxes, triglycerides, steryl esters, sterols, free long chain fatty acids and resin acids (FENGEL and WEGENER, 1989). The deposits of fibre-associated wood extractives in combination with inorganic salts and additives (technically referred to as pitch) interact with the runnability of paper-making machinery. This may result in reducing strength and brightness of pulp (ALLEN, 1980).

Different methods have been developed to remove wood extractives prior to the pulping process including by adding additives (alum, talc, dispersants or lipolytic enzymes), seasoning of logs or chips, or using pitch degrading fungi (BLANCHETTE et al., 1990; GUTIÉRREZ et al., 1999) and bacteria (YU et al., 1999).

To meet industrial demands, a marker-based distinction of trees containing different amounts of extractives is expected to be a great gain to offer optimised material for the different industrial branches. Well selected basic raw material with low extractives content will reduce main costs for removing undesired compounds in paper industry, whereas wood with high extractives content on the other hand allows the establishment of durable wood products with respect to lignocellulose-degrading microorganisms.

For an improvement of selected wood characteristics, many endeavours have been undertaken to increase the understanding about molecular basis of wood properties (MCKAY et al., 1995; ZHANG et al., 1997; WU et al., 1999). A number of genetic maps have been currently established for different forest tree species (PLOMION et al., 1995; PAGLIA et al., 1998; REMINGTON et al., 1999; ARCADE et al., 2000; THAMARUS et al., 2000, RITTER et al., 2002). In addition, several quantitative trait loci (QTLs) influencing important traits such as growth, form and phenology in *Populus trichocarpa* x *deltoides* (BRADSHAW et al., 1995), growth height in *Pinus pinaster* (PLOMION et al., 1996), frost tolerance in *Eucalyptus nitens* (BYRNE et al., 1997), height and diameter in *Pinus sylvestris* (LERCETEAU et al., 2000) or monoterpene composition in *Eucalyptus grandis* (SHEPARD et al., 1999) have been reported. However, there is only fragmentary knowledge concerning the genomic region controlling extractives content in tree species.

Considering the economical relevance of extractives mentioned above, a more detailed knowledge about genes, controlling and influencing extractives content, and their relation to other important traits will be required. Resulting from more basic research it may be possible to manage this trait by marker assisted selection (pyramiding) or by genetic engineering (BISHOP-HURLEY et al., 2001; LÓPEZ et al., 2000; CLAPHAM et al., 2000; SEDEROFF, 1999).

In general, the major obstacle for improving wood properties in trees, is their long generation time. Current breeding practice is mainly focussing on traits which are assessed at the end of rotation cycles. For traits of economical value, early selection of individual trees is efficient half-way through rotation. Tree breeding is even more difficult due to the changes which occur during the transition from juvenile to mature wood.

Methods to improve the accuracy of early selection at the individual level would be of considerable value for increasing the genetic gain per time unit. For this purpose a promising method is the indirect and non-destructive identification of genotypes, using a diagnostic system based on molecular markers, cosegregating with the trait of interest. Selection based on molecular

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markers, will have an enormous impact on cost reduction for breeding programs, and will be of prime importance for paper and other forest products industry, by guaranteeing highest and equal paper and wood quality products.

Despite the identification of QTLs for different wood properties, the conversion of QTL-markers into PCR-based markers and their usefulness for marker assisted selection approaches are until now only published for crop species (OBERHAGEMANN et al., 1999; SHAN et al., 1999; MEKSEM et al., 2001) and no report considering this topic in coniferous species was found.

In this paper, we report for Norway spruce, representing a main coniferous species in Europe, the use of bulked segregant analysis (MICHELMORE et al., 1991) in combination with AFLP marker technology (VOS et al., 1995) to identify informative molecular markers with respect to extractives content of wood. One molecular marker for low extractives content was identified and converted into a SCAR marker. This marker was validated in unrelated Norway spruce families.

Materials and Methods

Plant Material

The plant material was provided by SkogForsk, Uppsala (Sweden) in frame of the EU funded project GENIALITY (http://www.skogforsk.se/geniality). A total of 210 individuals was tested, representing 29 different Norway spruce fullsib families from the Swedish stand Karlsbol (S22F58241A; lat. 59°6', long. 13°6', alt. 80m) which was established in 1958.

Estimation of extractives content

Extractives content was estimated by the BioComposite Centre (University of Wales, Bangor) using the following method: wood disks were chipped and dried at low temperature in a laboratory kiln. The chips were milled and sieved (40 µm). Duplicates of approximately 7 g were extracted for eight hours employing a Soxhlets extraction apparatus and 400 ml toluene/IMS/acetone (4:1:1). The heating rate of the equipment provided at least 50 solvent cycles during the extraction period. The solvent containing the extractives was filtered and evaporated to dryness on a steam bath. All samples were finally dried in an oven at 75°C (overnight incubation). Total extractives content was determined as weight ratio between toluene/IMS/acetone soluble components, and non-extracted oven dried wood.

AFLP analyses

DNA was extracted from spruce needles according to a protocol which will be published elsewhere (Markussen in preparation). AFLP fingerprints were generated with respect to the protocol which has been published by VOS et al. (1995). Following modifications were done: A total of 250 ng genomic DNA was double-digested with *Eco*RI and *Mse*I, *Pst*I and *Mse*I or *Hind*III and *Mse*I (4-5U of each), respectively for 2-2.5 h at 37°C in 1 x restriction buffer OPA (One-Phor-all; Amersham-Pharmacia, Freiburg, Germany) using a final volume of 50 µl. After controlling complete digestion by gel electrophoresis, 10 µl of a ligation mix [50 pmol *Mse*I adapter, 5 pmol *Eco*RI, *Pst*I or *Hind*III adapter, 10 mM ATP, 1 x OPA buffer and 2.5 U T4 DNA ligase] was added and the samples were incubated for 3 h at 37°C.

A pre-amplification reaction was performed in a 50 µl reaction, containing 5 µl of template DNA (from ligation mentioned above), 150 ng of *Mse*I, *Eco*RI, *Pst*I and *Hind*III primers with one nucleotide extension, 0.25 mM dNTPs, 1 x PCR buffer, 1.2 mM $MgCl_2$ and 1 U *Taq* Polymerase (Eurogentec, Seraing, Belgium; Silverstar Taq and buffer). Samples were run on a TGradient cycler (Biometra, Göttingen, Germany) using the following cycling parameters: initial denaturation step 94°C (1 min), 19 cycles of 94°C (30s), 60°C (30 s), 72°C (1 min) and a final extension 72°C (5 min). The pre-amplified DNA was diluted (1:5) and equal aliquots of this DNA from each individual selected for the different bulks was mixed and used for selective amplifications. Selective amplifications were performed in a 20 µl reaction containing 5 µl of the diluted template DNA, 10 ng *Eco*RI, *Pst*I, *Hind*III primer (Cy5-labeled, MWG-Biotech, Ebersberg, Germany) and 50 ng MseI primer each having three selective nucleotides, 1 x PCR buffer, 1.2 mM $MgCl_2$ and 1 U *Taq* Polymerase and buffer (Eurogentec, Seraing, Belgium). Samples were run on a TGradient cycler using the following conditions: initial denaturation step 94°C (5 min); 1x 94°C (30 s), 65°C (30 s), 72°C (1 min); 2 x 94°C (30 s), 64°C (30 s), 72°C (1 min); 2 x 94°C (30 s), 62°C (30 s), 72°C (1 min); 2 x 94°C (30 s), 58°C (30 s), 72°C (1 min); 23 x 94°C (30 s), 56°C (30 s), 72°C (1 min) and a final extension 72°C (5 min). 8.5 µl reaction products were resolved on high-resolution polyacrylamide gels on the automatic sequencer ALFexpress II (Amersham-Pharamcia, Freiburg, Germany) and fragments were detected and analysed by using the Fragment Analyser software (Vers. 1.03; Amersham-Pharamcia, Freiburg, Germany). For each primer enzyme combination (PEC), the bulks were compared on polymorphic fragments, reflecting putative markers linked with high or low extractives content. In the following, each individual used for bulk constitution (high and low extractives content), was analysed separately on presence or absence of putative markers. The significance level for correlation of these markers to high or low extractives content was estimated by a two-sided Fisher's exact test.

Conversion of AFLP into SCAR marker

One specific AFLP fragment was isolated from a high resolution polyacrylamide gel (Amersham-Pharmacia, Freiburg, Germany) and purified by using the QIAEX II-Kit (QIAGEN, Hilden, Germany) according to the instructions of the manufacturer. The fragment was re-amplified by using the unlabeled specific AFLP primer pair of the corresponding selective amplification in a final volume of 50 µl. A 10 µl sample of the PCR product was electrophoresed on a 2% agarose gel for size estimation. The concentration of the PCR product was determined and an aliquot was cloned, using the TOPO TA vector system (Invitrogen) according to the protocol of the manufacturer. Ninty six colonies were chosen from

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the transformation, and transferred to a microtiter plate containing 100 µl LB-media with 50 µg/ml of ampicillin and cultured overnight. An one µl aliquot of the culture was amplified by PCR using the M13 forward- and reverse-sequencing primer and the product screened on 1.4% agarose gels for size homology to the corresponding AFLP fragment. Ten clones were selected and sequenced using the BIG-Dye Terminator Sequencing-Kit (PE Applied Biosystems, Foster City, USA) according to the instructions of the manufacturer. Primers for marker amplification were designed using the Primer 3 program (www.genome. wi.mit. edu/ cgi/ cgi-bin/primer/ primer3_www.cgi). PCR reactions were performed in a 25 µl reaction containing: 25 ng template DNA, 25 pg of each primer, 1 x Williams-buffer modified (WILLIAMS et al., 1991) (10mM Tris-HCl pH 8.3, 50 mM KCl, 2.0 mM $MgCl_2$, 0.001% gelatine) and 1U *Taq* Polymerase (Eurogentec, Seraing, Belgium). Samples were run on a TGradient cycler using the following cycling parameters: initial denaturation step 94°C (3 min); 4x 94°C (1 min), 59°C (1 min), 72°C (1 min); 36 x 94°C (30 s), Tm (30 s); 72°C (30 s); 1 x 94°C (30 s), 59°C (30 s) and 72°C (5 min). PCR-products were electrophoresed on a 2% ethidium-bromide stained agarose-gel. For restriction of PCR-products, 1U restriction enzyme per 25 µl PCR-reaction was used.

SSCP-Analyses

For SSCP-analyses (single strand conformation polymorphism) (PLOMION et al., 1999), PCR was performed by using two Cy5-labeled primers under PCR conditions as described above. The PCR-product was diluted five times with deionised water. 5 µl PCR-product was added to 8 µl loading dye (95% formamide, 0.5 mM EDTA, 0.1 mg/ml fuchsin red), denaturated for 5 min at 95°C and stored on ice for 10 min. An aliquot of 8.5 µl reaction product was resolved on high-resolution polyacrylamide gels using the automatic sequencer ALFexpress II (Amersham-Pharamcia, Freiburg, Germany) under following running conditions: 350V, 40mA, 25W, 15°C for 600 min. Fragments were detected and analysed by using the Fragment Analyser software (Vers. 1.03; Amersham-Pharamcia, Freiburg, Germany).

Database comparison and statistics

Sequence of the fragment was compared to the MAFF DNA Bank (http://www.dna.affrc.go.jp/) by BLAST N- and BLAST X-search. Analysis of Variance of extractive contents in relation to marker classes was carried out by SAS software Vers. 6.1 particularly using Proc ANOVA and the LSD test for the comparison of means.

Results

AFLP analyses

Four DNA-pools were applied to carry out genotyping of the most extreme individuals of Norway Spruce which represent different full-sib families. Two different pairs of pools with 11 individuals each were tested for low and high extractives content, respectively. Their mean values for high extractives content and low extractives content were 3.16% and 1.10% oven dry weight of wood, respectively. In total, 57 different primer enzyme combinations (PECs) (*Eco*RI/*Mse*I, *Pst*I/*Mse*I or *Hind*III/*Mse*I, all 3bp extension) were used for identifying pool specific amplification products. Bands were selected, which were present in each pair of samples and absent in the other pair. A total of 14 polymorphic bands were identified as putatively correlated to low or high extractives content. Five polymorphic bands, showing strongest band intensities and no other nearby bands, were selected for further analysis (*Table 1*). To confirm the polymorphisms observed in the DNA pools, each individual, used for pool construction was analysed separately for presence or absence of the polymorphic AFLP-fragments. One polymorphic fragment (150bp) was selected for conversion into a SCAR marker since this band showed a significant correlation with low extractives content, as estimated by a two side Fisher's exact test (*Table 1*, **bold** *italics*).

Characterization and validation of SCAR marker

The conversion of the selected polymorphic AFLP fragment into a SCAR marker was initiated from the bulked DNA. Following reamplification and sequencing of the band, no significant sequence differences were observed for the different clones, suggesting sequence similaritiy

Table 1. – Results of the two-sided Fisher-exact-test performed for the estimation of marker linkage to extractives content. The marker K29-150 (bold) was selected as significantly correlated to extractives content. a: primer-sequences specific for adaptor: P = *Pst*I; M = *Mse*I + 3bp exten-sion. b: specific fragment size in base pairs. c: Marker presence correlated to LE = low extractives content, HE = high extractives content. d: number of individuals with high extractives content as determined out of 22 resp. 38 individuals from the full sib families. e: number of individuals with low extractives content as determined out of 22 resp. 36 individuals from the full sib families. f: number of deviations from expectation on presence or absence of fragments out of 44 individuals.

PEC [a]	Size [b]	Character [c]	High extr. [d]	Low extr. [e]	Deviation events % [f]	p-value
K4 PCCA/MAGG	175	LE	6 (22)	13 (22)	15 (44) / 34.1	0.269
K4 PCCA/MAGG	275	HE	11 (22)	6 (22)	17 (44) / 40.9	0.394
K9 PCCA/MCAG	175	LE	6 (38)	12 (36)	16 (44) / 36.4	0.272
K29 PCCA/MAAC	**150**	**LE**	**4 (22)**	**18 (22)**	**8 (44) / 18.2**	**< 0.05**
K35 PCCA/MCCC	480	LE	10 (22)	13 (22)	19 (44) / 43.2	0.797

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Table 2. – Marker sequence similarities to sequences in public databases. Marker sequence available under Acc. No. AM049318.

Blast similarity	Organism	Accession number	Positive (% amino acids)	E-value
Dihydroflavonol 4-reductase	*Ipomea purpurea*	AB011667	34/36 (94%)	1e-05
Dihydroflavonol 4-reductase	*Malus domestica*	AF1172668	28/33 (84%)	1e-03
Dihydroflavonol 4-reductase	*Rosa hybrida*	ROZD4R	29/36 (80%)	7e-04
Cinnamoyl-CoA reductase	*Pinus taeda*	AY064169	27/35 (77%)	4e-03
Cinnamoyl-CoA reductase	*Populus tremuloides*	AF217958	26/35 (74%)	3e-02
Cinnamyl alcohol dehydrogenase	*Malus domestica*	AF053084	30/34 (87%)	1e-05
Cinnamyl alcohol dehydrogenase	*Eucalypthus gunnii*	EGRNACAD1	29/36 (80%)	4e-05

Table 3. – Sequences of primers used for marker amplification. A. Sequences of nested primers for the amplification of a size reduced marker. B. primer sequences for marker amplification including the recognition site for the specific restriction enzymes (RSS = restriction site specific, TTAA = *Mse*I; CTGCAG = *Pst*I, marked as bold). Tm = melting temperature.

	primer	primer sequence (5´-3´)	size in bp	Tm
A	K29F	CAA CCC AGT TAT GGT GTT GGT	100	58
	K29R	TGG GAC ATT GAA TTC TCT TTG G		
B	K29-RSS F	**CTG CAG** CCA CAC TTT TAG AAC GC	130	59
	K29-RSS R	**TTA A**AA CAT GGG ACA TTG AAT C		

between the different individuals. Inspecting public databases, significant homologies were found to a number of genes which encode for Dihydroflavonol 4-reductase (DFR), Cinnamoyl-CoA reductase (CCR) and Cinnamyl alcohol dehydrogenase (CAD) (*Table 2*).

Initially, a set of nested primers used for amplification (*Table 3A*), produced a monomorphic band for all individuals, regardless of marker linkage to low or high extractives content. Also a restriction site specific (RSS) primer set, including *Pst*I or *Mse*I restriction recognition sites was used (*Table 3B*), resulting again in an amplification of a monomorphic fragment for all individuals.

By using the sets of nested primers, investigation was expanded by proving the fragment amplification within different coniferous species. Here, the fragment was amplified as a size identical fragment for all *Pinus* species used within the analyses (*Figure 1*).

A restriction approach of PCR-products obtained by using the RSS-primers (*Table 3B*) was performed for separation of the marker-allele in linkage to low extractives content. Eighteen different restriction enzymes, adjusted to defined restriction recognition sites within the PCR product, selected based on sequence information, were tested. By following this strategy, separation of the expected marker was impossible. Due to the amount of size identical fragments (marker-alleles), a SSCP-analyses has been carried out for separation of the right marker-allele based on most extreme individuals for extractives content at first. By performance of the SSCP-analyses, the identification of the marker was feasible as presented in *Figure 2*.

In total, 210 progenies (29 full-sib families) were analysed by a SSCP-analyses with respect to the marker-allele for low extractives content. Based on mean extractives content data (BioComposite Centre, Wales), an ANOVA (linear additive model) and a LSD (least significance difference post-hoc test) was performed to estimate the separability of extractives content by the associated marker. According to the result in *Table 4*, we

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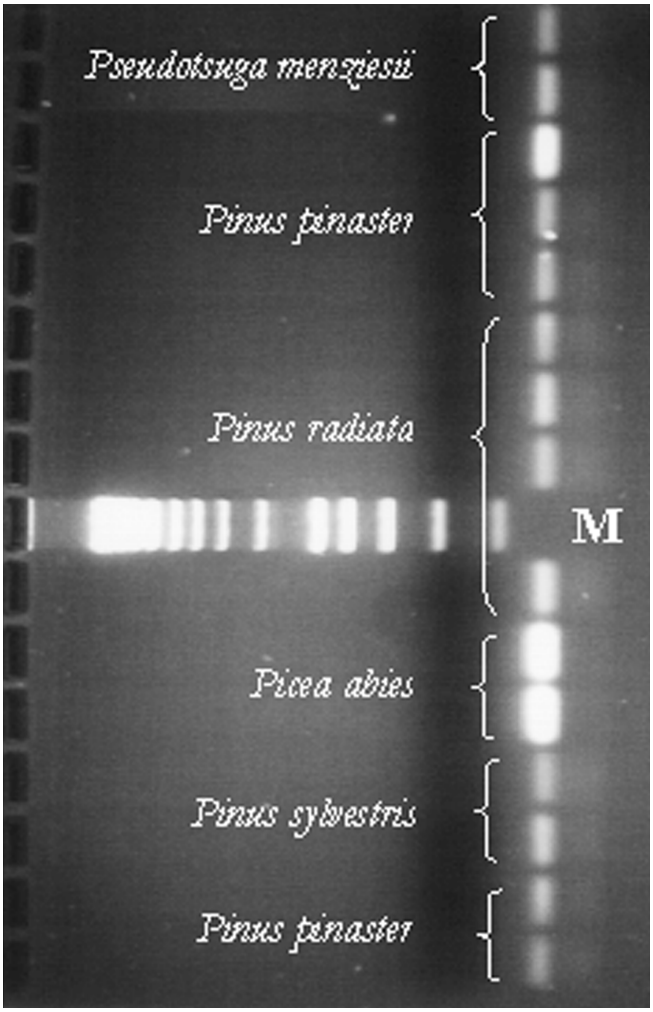

Figure 1. – PCR-product amplified by using nested primers of *Tab. 3A*. A monomorphic band of 100 bp was obtained for different *Pinus* species. M = molecular weight marker.

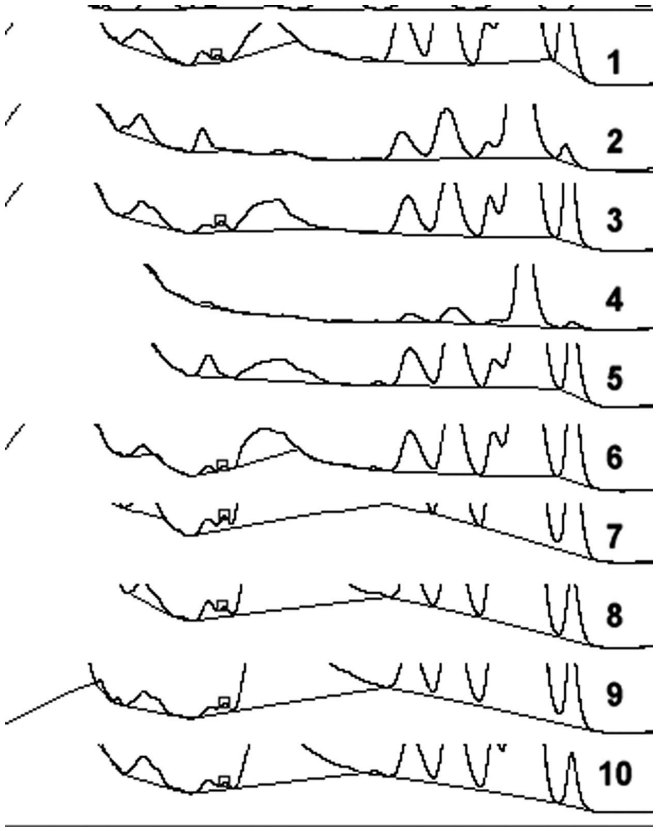

Figure 2. – Result of SSCP-analyses. The marker which is linked to low extractives content are marked by a square. Lane 1–5 representing individuals with high extractives content, 6–10 are individuals with low extractives content.

infer, that this marker is applicable for the selection on high or low extractives content of Norway Spruce. The phenotypical variances, which is explainable by the marker, is 9.35% (*Table 4*).

Discussion

An obstacle for an efficient forest tree breeding is, that wood properties can be often estimated only at rotation age, because most of them are only adequately expressed at the phenotypic level after the tree has produced several growth rings and it will be additionally influenced by environmental factors changes which occur during the development from juvenile to mature wood. Furthermore, the majority of wood properties can be only precisely estimated by destructive methods. The availability of molecular markers correlated with specific trait expression represent an elegant tool to overcome this problem.

Different reports have been published for crop species about molecular markers for selected traits, which were identified by bulked segregant analysis (BSA; MICHELMORE et al., 1991) in combination with AFLP (NEGI et al., 2000; DONG et al., 2000; HAYES et al., 2000). For tree species, the BSA was used to identify RAPDs linked to *Melampsora* resistance in poplar (VILLAR et al., 1996), white pine blister rust fungus resistance of sugar pine

Table 4. – Results of a LSD post-hoc test based on an ANOVA for mean extractives content related to presence or absence of marker K29-150. M0 = mean extractives content (% oven dry weight of wood) of class for marker absence; M1= mean extractives content (% oven dry weight of wood) of class for marker presence. Mean Diff = mean difference between the classes for marker presence or absence. LSD is the least significant difference (95% confidence limits) between the classes. r^2 percentage of phenotypic variation explained by the marker.

	M0	M1	Mean Diff.	LSD	p-value	r^2
K29-150	2.43	1.68	0.75	0.26	< 0.05	9.35

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(HARKINS et al., 1998), or quantitative traits (GRATTAPAGLIA et al., 1996). To our knowledge, only one report is available so far, describing the utilisation of BSA in combination with AFLP for the identification of markers linked to wood density (MARKUSSEN et al., 2003)

Here, we describe the development of a molecular marker for low extractives content of Norway spruce (*Picea abies*), based on BSA-AFLP combination, and its conversion into a SCAR marker. By using SSCP-analyses of PCR-products, the marker linked to low extractives content was confirmed as significant.

The marker offers an opportunity for the first time, to select Norway spruce seedlings on high or low extractives content. Furthermore, the marker is also useful as an indirect predictor for lignin content since a positive correlation between lignin and extractives content was identified for the individuals used within these analyses (Hannrup, pers. comm.). Thus, the establishment of productive populations is also possible when other traits than extractives content are considered.

All AFLP-markers identified here as putatively correlated to extractives content, originate from primer enzyme combinations *Pst*I/*Mse*I (*Tab. 1*). *Pst*I is well known to recognize undermethylated genomic regions and therefore putatively expressed genes (KNOX and ELLIS, 2001). This notion was supported by marker sequence comparisons to public databases. A similarity of 94% to Dihydroflavonol 4-reductase (DFR), 87% to Cinnamyl alcohol dehydrogenase (CAD) and 77% to Cinnamoyl CoA-reductase (CCR) was identified (*Table 2*). DFR belongs to a superfamily which include β-hydroxysteroid dehydrogenases / dihydroflavonol 4-reductases and CAD II of *Eucalypthus gunnii.* CAD II is completely different from CAD I of *E. gunnii* (BAKER and BLASCO, 1992) and, responsible for synthesis of flavan 3,4-diols, a precursor for catechins and condensed tannins (BAVAGE et al., 1997).

Although a high sequence homology was identified for the marker presented here and CAD I of *E. gunnii,* which is different to CAD II and also different to DFR of *E. gunnii* (BAKER and BLASCO, 1992), it is not excludable, that homologies exist between DFR and CAD I/II for *Picea abies*.

Sequence homologies found between the marker and CAD or CCR genes are not inconsistent, because CAD I most closely resembles CCR, the antecedent enzyme in the lignin biosynthesis pathway (CHASE, 1999), and responsible for cinnamoyl CoA synthesis, a precursor for the extractive pinosylvin (CELIMENE at al., 1999; MORIATA et al., 2001). This interrelation will be also emphasized by the correlation found between extractives and lignin content for the individuals used within the analyses (Hannrup, pers. comm.).

No homologies were identified between the marker and CAD 2, CAD 7 or CAD 8 of *Picea abies* (SCHUBERT et al., 1998), which suggest, that the marker may represent a part of a new CAD gene of *P. abies*. To light up the character and functionality of this marker (or the putative gene), which may help to close an information gap within the lignin, stilbene and flavonoid pathway of *P. abies*, it is intended to perform a PCR-walk (SIEBERT et al., 1995; DEVIC et al., 1997) or a tail-PCR (LIU et al., 1995).

The amplification of monomorphic PCR products for AFLP derived markers, also described by DE JONG et al. (1997) and MEKSEM et al. (2001), indicates the existence of conserved homoplasic loci dispersed within the genome. The knowledge about the complexity of coniferous genomes is small, but comparative mapping studies point out the existence of orthologs (same locus in two species), paralogs (genes duplicated in the same species) (DEVEY et al., 1996) and the existence of complex gene families (KINLAW and NEALE, 1997).

To allocate the character of the marker presented here (ortholog/paralog), different coniferous species were analysed for marker presence. Identified for *Picea abies*, the marker was also generated as a size identical fragment in different *Pinus* species (*Pinus pinaster*, *Pinus sylvestris*, *Pinus radiata*, *Fig. 1*). Therefore, the marker might be speculated as orthologous among different conifers, and detailed information about sequence identities between species will be obtained by direct sequence comparisons in the near future.

The differentiation of the marker-allele which is in linkage to low extractives content was a compelling consequence, to make it useable for MAS. Different options like restriction of the PCR-products, direct sequence comparisons or SSCP-analysis (PLOMION et al., 1999) are conceivable methods for such a differentiation approach.

The reason for amplification of monomorphic fragments by usage of nested or restriction site specific (RSS) primer may be the nature of AFLP markers. In general, internal sets of primers are mainly used for amplifications of converted markers (SHAN et al., 1999; MEKSEM et al., 2001), but AFLP fragments resulted definitively from nucleotide difference specific to AFLP primers. Despite the usage of RSS primers, the amplification of a monomorphic fragment was irrevocable. As crucial reason, we assume a high mismatch rate for the *Mse*I sequence (TTAA) within the restriction site specific primer.

Although the marker was detectable by SSCP-analyses, the drawback in this context is, that SSCP-detection is not routinely usable for tree breeders. Therefore, the identification of new molecular markers for extractives content will be an important task for the future in order, to increase the amount of routinely usable markers for MAS. Next a clarification of sequence identities between PCR fragments within and among species, which is of interest not only for synteny studies, it is intended to identify additional markers for extractives content which is prerequisite for a more efficient selection on low or high extractives content.

It is not appreciable, whether the presence or absence of the marker within a genomic background will be a sufficient indicator, that genes which are in marker linkage are definitively switched during life span, resulting in a variation of extractives content. Before usage of the marker described here for MAS, a vast validation on marker presence/absence in different genetic backgrounds and correlation analyses are undoubtedly required.

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