Characterization of a Suite of 40 EST-derived Microsatellite Markers For Use in Sitka Spruce (*Picea sitchensis* (Bong.) Carr)

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Abstract

This paper describes 40 novel, data-mined, polymorphic microsatellite loci for use in a QTL association study in Sitka spruce. Publicly available EST sequences of *Picea* in Genbank were searched *in silico* for simple sequence repeat (SSR) motifs, principally dinucleotide microsatellites, and PCR primers were designed to flank these regions. PCR amplification was carried out in the progeny of a full-sib family to test simple Mendelian inheritance. For further characterization, the amplification products of Sitka spruce material from unrelated trees were assessed to determine the potential of these loci for population genetic studies. These polymorphic markers therefore represent a valuable tool-kit both for establishing a molecular map of this species and for *Picea* genetic population studies.

Key words: Picea sitchensis, microsatellites, genetic map, marker aided selection

Introduction

Sitka spruce [*Picea sitchensis* (Bong.) Carr] is the primary commercial forestry species in the UK. Traditional tree breeding programmes have been successful in improving economically important traits but these are slow and expensive. A quantative trait loci (QTL) association study has been initiated by Forest Research, UK, to accelerate genetic improvement in Sitka spruce. QTL studies aim to associate performance in the field with DNA markers assessed in the laboratory and offer the possibility, in the future, of selecting trees with particular desirable traits without the need for expensive and protracted progeny trials.

Comprehensive field trials comprising 4,500 Sitka spruce clones have been set up, involving complete replication over three climatically contrasting sites. The 4,500 clones are sub-divided into 1,500 clones from each of three families about which prior knowledge of parental genetic quality is known. Each family consists of parents with contrasting phenotypes for the following economically important traits; wood density, straightness and branching. The trials were planted in spring 2005 in North Scotland (57.1302N, 3.5684W; Accumulated Temperature (AT) over 5°C (monthly mean over 30 years) = 1100), South Wales (51.6938N, 4.8157W; AT = 1450) and South England (50.7706N, 4.7807W; AT = 1800). This will enable the robustness of marker/trait association to be tested in three families both within, and between, three sites.

The recent advances in the process of genetic mapping and QTL association in spruce has been greatly assisted by the public release of expressed sequence tagged (EST) information, which has enabled DNA markers to be discovered with relative ease and reduced cost (see UJINO-IHARA *et al.,* 2005 for details of spruce and pine EST databases). Highly polymorphic, co-dominant microsatellite markers are the markers of choice for genetic mapping studies as they not only allow the fusion of male and female maps in a pseudo-test cross mapping strategy, but also are likely to show a degree of transferability across species within a genus. (LIEWLAKSANEEYANAWIN *et al.,* 2004; RUNGIS *et al.,* 2004). Ultimately, this will enable the map position of putative QTLs or candidate genes for a particular trait to be compared across species.

SSR discovery from genomic libraries has proved problematic in conifers, with a low return for effort. (RAJORA *et al.,* 2001; HODGETTS *et al.,* 2001). This is deemed to be because of the large size and repetitive nature of the conifer genome.

It was generally considered that EST derived microsatellite markers are less variable than genomic microsatellites (gSSRs) due to their position in coding regions and the assumption that they are therefore under a degree of selection pressure. Recent results in *Picea*, however, demonstrated only slightly lower levels of polymorphism in EST derived microsatellites (RUNGIS *et al.,* 2004). Furthermore, EST derived microsatellites exhibit a lower number of null alleles than gSSRs (RUNGIS *et al.,* 2004) making them more useful for population genetic studies. These considerations, in addition to their relative ease of discovery, make them high utility markers. In addition, by focussing efforts on dinucleotide repeats, which were shown to be a more polymorphic motif than tri- or tetra-nucleotides in Sitka spruce (RUNGIS *et al.,* 2004), the possibility of finding useful polymorphic loci for the three mapping populations was maximised.

Materials and Methods

Picea EST sequences from *P. glauca*, *P. sitchensis* and *P. sitchensis* x *P. engelmannii* publicly available in Genbank in November 2005 were downloaded and processed with the CAP3 software program (HUANG and MADAN (1999); http://deepc2.psi.iastate.edu/aat/cap/cap.html)

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Table 1. – Primer pairs developed from *Picea* ESTs for amplification of SSR markers. Locus name, Genbank accession number, allele size range, SSR motif and best protein match are presented.

Locus	Accession	Primer pair	Allele size range	Motif	Best protein match
SS18	40766244	F:*M13-GTGCCGTGAACCACATTAAC R: GCGTACATGAAAACCTGCAT	248-282	$(A)_{19}(TA)_6$	unknown
SS19	40767122	F: M13-GAGAAAACCTGGCCTCTAGAAA R: GGGACCATCTCCTAGACACG	218-226	$(AT)_6$	NP_910052 putative Hsp70 binding protein [*Oryza sat*iva (japonica cultivar-group)].
SS20	40775728	F: M13-TAACCGATGCCTCTCACAGC R: TGATGCATCCATCCTTGTTG	196-212	$(AT)_6$	AAB70558 putative protein tyrosine phosphatase homologue [*Homo sapiens*].
SS21	40779303	F: M13-TGCTGTGCCTGCCTATACAT R: TGCATTACAAGCACCTCAGA	180-226	$(A)_8(CA)_5(TA)_{10}$	CAA31594 D-34 Lea protein [*Gossypium hirsutum*].
SS22	49016032	F: M13-CACTCCAAGCAAGAATCTCCA R: GACATCTCAGGCGATTCCAT	246-250	$(AT)_5(CT)(AT)(AT)_3$	AAZ21700 NADH Dehydrogenase I Chain C [*Candidatus Pelagibacter* ubique]
SS23	49017151	F: M13-GCTCCTTCAGATCAGCGAAC R: AGAGGCAAGTTCAGGGGATT	196-210	$(AT)_7(T)_5$	YP_224083 L [Taro vein chlorosis virus].
SS24	49024553	F: M13-CCTTAACCCCTACTGTCAATGC R: CGGCTTGTGGGAGTAACAAG	258-282	$(TA)_4$ $(A)_3$ $(TA)_4$	XP_670126 hypothetical protein PB400116.00.0 [*Plasmodium berghei* strain]
SS25	49037755	F: M13-AACATCCACCCATTCAAAGC R: GTGCATGCGAATCCCTAAAC	218-264	$(AT)_{13}$	ABA96147 expressed protein [*Oryza sativa* (japonica cultivar-group)].
SS26	49041358	F: M13-AGGTTGGAAACCCTGTGATG R: ATTCGGATTGCGGTTCTGTA	198-218	$(TA)_8$	BAD79321 hypothetical protein [*Synechococcus elongatus* PCC 6301].
SS27	49041823	F: M13-TGACGTTATTAATGGCGTTTG R: TTTGAGGGGAGATCTTGTGG	176-204	$(TA)_{10}$	YP_073752 U11 [Human herpesvirus 7].
SS28	49043234	F: M13-GCTGAGGCAATGATGTCAAC R: GACATCTGTTCATTGCTCTACTTG	256-284	$(TA)_{17}$	ABA93984 oxidoreductase, short chain dehydrogenase/reductase family [[*Oryza sativa* (japonica cultivar-group)].
SS29	49043836	F: M13-CAAGGCACATGCTTCTGTCA R: AAGATGGCCTTTGCGTCATA	254-286	$(TA)_{12}$	GSU0558 hypothetical protein [*Geobacter sulfurreducens* PCA].
SS30	49044204	F: M13-TGAGTTTCCCCAAACTCTATTCC R: GGTGTTGTTTAAATGATTTGGAAG	270-294	$(TA)_9$	unknown
SS31	49045444	F: M13-CGGAGTCTTGGTGGAACATT R: GGAGCAATCCCTCTTCTTCC	144-190	$(TA)_{11}$	unknown
SS32	49045765	F: M13-CTGAAGCAAACACGACAAGC R: CCACATGCCTGCACTATCAT	242-262	$(TA)_4(A)(TA)_9$ $(CA)_4(TA)_6$	NP_849973 metal ion binding [*Arabidopsis thaliana*].
SS33	49046524	F: M13-ACCTCAAGGGGCTACACTGA R: TAACGAGCCTGGTGTGTTTG	188-194	$(TA)_8$	unknown
SS34	49047398	F: M13-TTGCCCTGAGTAGGGTACAA R: AGCATTTGCTTGTTTCGTGA	260-262	$(TA)_3(TG)_3(TC)(TA)_4$	XP_640606 hypothetical protein DDB0204634 [*Dictyostelium discoideum*].
SS35	49047601	F: M13-TCCAATCCAAAACCGAAAAC R: GGCCTGTGTCTTTTCCATGT	262-268	$(TA)_8$	AAL39230 GH10154p [*Drosophila melanogaster*].
SS36	49047619	F: M13-GTGTTCGAATCCCAGGAAGA R: TGCCCTGTGCGATGTTATAG	232-238	$(GA)_8$	XP_684896 similar to LReO_3 [*Danio rerio*].
SS37	49047962	F: M13-GGCCCCAAGATTGAAGAAGT R: TTTGGAATGCAATGCAGTTTAC	224-226	$(TA)_5$	XP_480282 putative RGH1 *Oryza sativa* (japonica cultivar-group)
SS38	49048259	F: M13-GAACGCAAGATGGGACACTT R: TGACCGTCTCTGTCCTTGTC	206-221	$(GGA)_6$	CAJ08228 calpain-like cysteine peptidase, putative; cysteine peptidas
SS39	49048405	F: M13-CTGTCCATCATGAACCCTGA R: CGAAGGAAAGGAATTCCACA	224-258	$(TA)_{10}$	XP_473723 OSJNBa0086O06.23 [*Oryza sativa* (japonica cultivar-group)].
SS40	49048492	F: M13-TCCCTCCTATACGGGAATGT R: GCGTACGTTAGTCGATTGGAA	202-204	$(CT)_6$	NP_196009 unknown protein [*Arabidopsis thaliana*].
SS41	49048842	F: M13-GCCAGAATGGTTTTCACGAG R: ATATCCTTGGGAGCGACCTT	274-280	$(TA)_6(AA)(TA)_4$	BAD87127 receptor protein kinase-like [*Oryza sativa* (japonica cultivar-group)].
SS42	49049101	F: M13-TCGACCCTCTAACGTCTTGC R: TGAGAATCATCCCGAACTCC	230-248	$(TA)_6$	EAA04656 ENSANGP00000009427 [*Anopheles gambiae* str. PEST].
SS43	49049495	F: M13-ACAATGAGGATGAGGGCTTG R: CCCCTTTCGAACTGTGTCAT	266-298	$(TA)_{16}$	CAB46056 putative protein [*Arabidopsis thaliana*].
SS44	49049559	F: M13-CAAACGGAAGTCGAACCATT R: ATTTGTTTTCGGGTCTGCTG	222-226	$(A)_{10}$	unknown
SS45	49135131	F: M13-AACACACGAGGGGATTGAAC R: ACCTGGCTTTCGGTATTGTG	216-236	$(AT)_8$	unknown
SS46	49137541	F: M13-GACAGGCACCCAGAACTGAT R: CTGGAATTTACAATGCACAGGA	232-288	$(AT)_{13}$	unknown
SS47	69437955	F: M13-AGGCTCACAGCTCCGTCTTA R: CCCAAGTGCCCTTGAATATG	242-302	$(AT)_{14}$	CAJ4359 Snakin-like cysteine rich protein [*Phaseolus vulgaris*].
SS48	70256787	F: M13-TCAAATCCAATCACGTACAACA R: CAACTTGAAGCCCCACTTGT	256-278	$(AT)_8$	AAB66560 anthocyanidin synthase [*Callistephus chinensis*].
SS49	70258880	F: M13-CAGTGCACGTCCAAACAAGT R: TGGCTGTTTGCTCATATTGC	266-292	$(CT)_{16}$	unknown
SS50	70260028	F: M13-TCAGAAGACGCAAACAATCCT R: TTCTTAGGAGGCAGCGGTTA	164-178	$(AT)_{10}$	AAC05682 germin-like protein [*Oryza sativa*].
SS51	70285403	F: M13-CAAAACACAACTTGCCCACA R: TGGAACACTTGGAACTCCATT	176-220	$(TA)_{11}$	NP_565937 hypothetical protein [*Arabidopsis thaliana*].
SS52	70297371	F: M13-AAGAGTCGCTCAAGGGCATA R: CATCTGAGAGGTCGCCATTT	196-218	$(TA)_{10}$	AAT80888 chloroplast chaperonin 21 [*Vitis vinifera*].
SS53	70318999	F: M13-CCGACGGTAGTTCCTTTCAA R: CCGGTGCTAAATCCTTGTGT	198-218	$(TA)_8$	AAL13436 anaphase promoting complex subunit 11 [*Arabidopsis thaliana*]
SS54	70346839	F: M13-AAGCATGATCAGTGGATAGCA R: AATTGAGGCAATTGGTGAGG	220-240	$(GA)_{10}$	unknown
SS55	70349470	F: M13-GAATTTAATGGCAACCATACCC R: TGTTTTGACTCGGATCTCTCTC	160-178	$(TA)_{12}$	XP_474343 OSJNBa0064G10.8 [*Oryza sativa* (japonica cultivar-group)].
SS56	70350346	F: M13-GAAAATCGCCGAAAGATCAC R: GCCAGCATTCACTTGACAGA	248-264	$(TA)_8$	CAA70101 L-lactate dehydrogenase [*Lycopersicon esculentum*].
SS57	70355804	F: M13-CATCGTACAGCCAATCTCCA R: GAGGTCGTCCCGACAGTAGT	126-140	$(TA)_{10}$	unknown

* M13 tail: 5'-AGGGTTTTCCCAGTCACGACGTT-3'

which aligns over-lapping sequences into "contigs". This "contiging" process allows any SSR length polymorphisms from different accessions to be readily highlighted *in silico.* Such polymorphic sequences were chosen preferentially for primer design, although some unique sequences containing a microsatellite motif were also used. The microsatellite repeat regions were identified by searching the CAP3 output files using the MREPS program (KOLPAKOV *et al.,* 2003); http://bioinfo.lifl.fr/mreps/) which identifies repeat motifs. Primer sequences were then designed around the repeats using the PRIMER 3 program (ROZEN and SKALETSKY, 2000); http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (*Table 1*). Primers were ordered from MWG Biotech, Germany. Forward primers were tailed with a M13 sequence which meant a considerable reduction in costs when screening primers.

DNA was extracted by freezing four finely chopped needles per sample in liquid nitrogen and grinding with two stainless steel balls (4 mm) in a 2 ml Eppendorf tube using a Retsch MM300 mixer-mill. Once the needles were reduced to a fine powder, DNA was extracted

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using a Qiagen DNeasy kit (http://Qiagen.com) following the manufacturer's instructions.

PCR was carried out in a 10 µl total volume containing 25–50 ng genomic DNA. The reaction mix consisted of 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, 2mM $MgSO_4$, 0.1% Triton X-100 at pH 8.8 (New England Biolabs, UK) and 0.2 mM of each dNTP, 0.3 mM of M13-tailed forward primer, 0.3 mM of reverse primer, 0.05 mM of M13 oligo, labelled with either 700 or 800 fluorescent dye (MWG Biotech, Germany) and 1.0U *Taq* polymerase (New England Biolabs, UK). The PCR protocol consisted of an initial denaturation step of 3 mins at 94 °C, followed by 10 cycles of: 94 °C for 30 secs, 57 °C for 1 min and 72 °C for 30 secs. This was followed by 28 cycles of: 94 °C for 30 secs, 55 °C for 1 min then 72 °C for 30 secs. A final elongation step of 6 mins at 72 °C was then carried out.

The amplification products were electrophoresed through a 6% denaturing polyacrylamide gel on a Licor IR sequencer.

The number of alleles, effective number of alleles, observed and expected heterozygosities and inbreeding coefficient (F) were calculated using the POPGENE program (YEH and BOYLE, 1997; http://www.ualberta.ca/~fyeh). The g-square test and the chi-square test were also applied to these data to test for Hardy-Weinberg Equilibrium.

Table 2. – Characterization and statistics of SSR markers in *P. sitchensis* in the progeny of a full-sib cross and in a set of unrelated samples.

	Full-sib cross							Breeding population			
Locus	Maternal genotype	Paternal genotype	Progeny genotypes	Expected ratio	Observed ratio	Chi square	No.of Trees	Observed Hetero-zygosity	Expected Hetero-zygosity	Effective Number of Alleles	F
SS18	lm	ll	ll:lm	1:1	94:93	0.0	20	0.800	0.873	6.723	0.060
SS19	ef	eg	ee:ef:eg:fg	1:1:1:1	44:39:42:40	0.4	21	0.476	0.617	2.513	0.209
SS20	nn	np	nn:np	1:1	60:90	6.0	21	0.667	0.637	2.641	-0.073
SS21	ab	cd	ac:ad:bc:bd	1:1:1:1	31:37:41:35	1.4	21	0.857	0.920	9.800	0.046
SS22	-*	-	-	-	-	-	21	0.238	0.220	1.273	-0.111
SS23	-	-	-	-	-	-	21	0.429	0.418	1.690	-0.050
SS24	ab	cd	ac:ad:bc:bd	1:1:1:1	46:53:35:51	4.2	19	1.000	0.799	4.513	-0.285
SS25	ab	cd	ac:ad:bc:bd	1:1:1:1	34:48:32:49	6.0	20	0.950	0.958	15.094	-0.017
SS26	ef	eg	ee:ef:eg:fg	1:1:1:1	39:54:41:47	3.0	21	0.667	0.826	5.158	0.173
SS27	lm	ll	ll:lm	1:1	97:92	0.1	20	0.500	0.615	2.500	0.167
SS28	ab	cd	ac:ad:bc:bd	1:1:1:1	49:52:42:39	2.4	20	0.700	0.897	8.000	0.200
SS29	ab	cd	ac:ad:bc:bd	1:1:1:1	43:43:46:53	1.4	19	0.895	0.922	9.757	0.003
SS30	ab	cd	ac:ad:bc:bd	1:1:1:1	39:44:50:47	1.5	18	0.667	0.724	3.375	0.053
SS31	ab	cd	ac:ad:bc:bd	1:1:1:1	46:49:47:49	0.1	20	0.900	0.917	9.412	-0.007
SS32	lm	ll	ll:lm	1:1	78:73	0.2	21	1.000	0.858	6.168	-0.194
SS33	nn	np	nn:np	1:1	90:101	0.6	21	0.524	0.610	2.471	0.120
SS34	lm	ll	ll:lm	1:1	41:37	0.2	21	0.429	0.438	1.747	-0.003
SS35	nn	np	nn:np	1:1	99:87	0.8	19	0.421	0.679	2.947	0.363
SS36	lm	ll	ll:lm	1:1	75:107	5.6	21	0.524	0.554	2.178	0.031
SS37	-	-	-	-	-	-	21	0.286	0.418	1.690	0.300
SS38	ab	cd	ac:ad:bc:bd	1:1:1:1	37:35:34:43	1.3	20	0.600	0.664	2.837	0.073
SS39	ab	cd	ac:ad:bc:bd	1:1:1:1	18:20:23:21	0.6	20	0.600	0.794	4.420	0.225
SS40	lm	ll	ll:lm	1:1	97:89	0.3	18	0.167	0.322	1.456	0.468
SS41	-	-	-	-	-	-	20	0.450	0.580	2.299	0.204
SS42	ab	cd	ac:ad:bc:bd	1:1:1:1	46:45:51:49	0.5	19	0.579	0.619	2.516	0.039
SS43	ab	cd	ac:ad:bc:bd	1:1:1:1	35:41:44:55	4.8	20	0.700	0.915	9.302	0.216
SS44	lm	ll	ll:lm	1:1	87:94	0.3	21	0.286	0.441	1.757	0.337
SS45	ab	cd	ac:ad:bc:bd	1:1:1:1	48:38:35:48	3.2	21	0.857	0.836	5.444	-0.050
SS46	lm	ll	ll:lm	1:1	78:83	0.2	20	0.700	0.683	2.996	-0.051
SS47	ab	cd	ac:ad:bc:bd	1:1:1:1	26:37:33:32	1.9	20	1.000	0.922	9.877	-0.113
SS48	-	-	-	-	-	-	21	0.571	0.677	2.950	0.136
SS49	ab	cd	ac:ad:bc:bd	1:1:1:1	41:40:46:39	0.7	20	0.750	0.886	7.339	0.132
SS50	lm	ll	ll:lm	1:1	28:20	0.7	20	0.700	0.651	2.740	-0.102
SS51	nn	np	nn:np	1:1	68:82	1.3	21	0.857	0.899	8.167	0.023
SS52	ef	eg	ee:ef:eg:fg	1:1:1:1	49:39:34:49	4.0	21	0.667	0.841	5.582	0.188
SS53	ab	cd	ac:ad:bc:bd	1:1:1:1	16:21:24:23	0.6	19	0.579	0.787	4.272	0.244
SS54	-	-	-	-	-	-	21	0.333	0.292	1.398	-0.171
SS55	ab	cd	ac:ad:bc:bd	1:1:1:1	46:41:42:38	0.8	20	0.650	0.755	3.792	0.117
SS56	nn	np	nn:np	1:1	96:89	0.3	20	0.600	0.623	2.548	0.012
SS57	ab	cd	ac:ad:bc:bd	1:1:1:1	27:20:28:19	0.4	21	0.762	0.820	5.011	0.048
Mean							20	0.633	0.699	4.659	
S.D.								0.220	0.196	3.183	

* A hyphen (-) indicates that the controlled cross was not polymorphic for that particular locus. Nomenclature for parental genotypes is adopted from JoinMap software package (http://www.kyazma.nl).

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The Mendelian segregation of the loci was examined by applying the chi-square test to data from the full-sib progeny.

The best fit protein was investigated by carrying out a blastx search in the NCBI website using default parameters.

All the markers presented in this paper have been checked against previously published microsatellite based EST markers in *Picea* to ensure there was no duplication.

Results and Discussion

Table 1 reports the PCR primer pairs used to amplify each locus, and the allele size range, as recorded from a panel of trees selected from the FR Sitka spruce breeding population situated at the Glencorse clone bank, near Edinburgh, UK (see LEE, 2001 for details). Variation in both perfect and imperfect repeat motifs was investigated. The most common class of data-mined dinucleotide repeat was AT, and this is in agreement with the findings of RUNGIS *et al.* (2004). The best protein match for each sequence was explored via the blastx function in Genbank, and the accessions showed matches from several kingdoms. The segregation ratios of all the loci were in accordance with a simple co-dominant mode of inheritance (*Table 2*). None of the loci deviated significantly from expected Mendelian segregations. The average number of alleles per locus in the panel of unrelated trees was 7.6, with a range of 2–16 alleles. This highlights that EST derived microsatellites are a good source of polymorphic markers. Indeed, RUNGIS *et al.* (2004) reported only a slightly higher average number of alleles in Sitka spruce gSSRs when compared to EST derived microsatellites (5.76/5.00). Observed heterozygosity varied considerably between loci, ranging from 0.17 to 1.00 (*Table 2*) with some evidence of null alleles based on F values and from the segregation data in the full-sib family. Within one of the full-sib family, six loci were monomorphic and 17 loci displayed four alleles and were therefore fully informative in this cross (*Table 2*). Forest Research aim to carry out a similar study in Sitka spruce to that of DEVEY *et al.* (2004), who explored the association between 92 co-dominant markers and economically important traits in *Pinus radiata.* A number of microsatellite markers are available in the literature for *Picea*, derived both from genomic and EST libraries. Sixteen markers specifically developed for Sitka spruce are published (VAN DE VEN and MCNICOL, 1996; A'HARA and COTTRELL, 2004). In addition, a number of other studies within *Picea* report successful amplification in Sitka spruce, demonstrating the transferability of microsatellite markers (HODGETTS *et al.,* 2001; RAJORA *et al.,* 2001; RUNGIS *et al.,* 2004). However, to ensure a good framework map of Sitka spruce with a high degree of transferability across species this suite of 40 further co-dominant microsatellite markers is a significant addition to the *Picea* genetic mapping effort.

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