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PRIMER NOTE

DEVELOPMENT OF 32 EST-SSR MARKERS FOR ABIES FIRMA (PINACEAE) AND THEIR TRANSFERABILITY TO RELATED SPECIES¹

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- *Premise of the study:* We developed simple sequence repeat (SSR) markers from expressed sequence tags (ESTs) for *Abies firma*, a conifer endemic in Japan, to facilitate evaluation of the population genetic structure in this species.
- *Methods and Results:* We designed primers for 153 EST-SSRs identified from 486 322 ESTs from *A. sachalinensis* ESTs, and tested 96 of them for PCR amplification. Thirty-two primers provided clear amplification, and 14 of those 32 displayed clear polymorphic patterns in multiple populations of *A. firma* and in two closely related species. The number of alleles per locus and mean expected heterozygosity ranged from one to six and 0 to 0.476, respectively.
- Conclusions: The EST-SSR markers developed in this study may be useful for phylogeography and population genetic studies of A. firma. Successful amplifications were obtained for two other Abies species, suggesting that these markers may also be useful for similar applications in other fir species.

Key words: Abies; cross-amplification; expressed sequence tag; microsatellite; Pinaceae; pyrosequencing.

In the family Pinaceae, Abies is the genus with the second highest number of species. Approximately 40 species are widely distributed in the northern hemisphere in regions ranging from temperate to subarctic zones. Four of the five species that grow in the Japanese archipelago are endemic to Japan. Abies firma Siebold & Zucc. is a major tree species occurring only in warmtemperate forests in Japan. This species is frequently found in mixed forest along with species such as Tsuga sieboldii Carrière and Fagus crenata Blume, but it sporadically forms pure stands at the late succession stage (Farjon, 1990). In recent years, the area covered by A. firma forest has been significantly reduced by logging and exploitation. Moreover, since the early 1960s, forest decline and tree dieback in A. firma forests in many areas of Japan have been observed as a consequence of environmental stress factors such as air pollution (Suzuki, 1992). For effective genetic conservation of these forests, it is necessary to understand the phylogeographic pattern and the genetic diversity within and among A. firma populations. Population genetic studies to date have relied on allozyme markers (Saito et al., 2005) and mitochondrial DNA markers (Tsumura and Suyama, 1998), and have not made use of microsatellites.

Microsatellite markers are recognized as versatile molecular tools for inferring genetic structure and gene flow. In recent years, expressed sequence tag (EST)-based markers have been increasingly used in studies of genetic variation because large numbers of polymorphic markers can be developed with relative ease using EST data and markers of this type are less susceptible to null alleles than are anonymous simple sequence repeats (SSRs). Moreover, because ESTs correspond to coding DNA, the flanking sequences of EST-SSRs are located in wellconserved regions across phylogenetically related species, making them markers of choice for comparative mapping and relevant functional and positional candidate genes to study their colocation with quantitative trait loci. In the work described here, we developed EST-SSR markers for A. firma from published expressed sequence data, and evaluated the extent of the polymorphism that they exhibit and their potential for transfer to two other closely related Japanese Abies species (A. homolepis Siebold & Zucc. and A. veitchii Lindl.).

METHODS AND RESULTS

A total of 486 322 *A. sachalinensis* F. Schmidt (a species related to *A. firma*) ESTs were downloaded from the National Center for Biotechnology Information (NCBI) database and used for PCR primer design. First, polyA and adapter sequences were removed from the cDNA sequences using the program Cross_match (http://bozeman.mbt.washington.edu/phrap.docs/phrap.html) and the TIGR SeqClean sequence trimming pipeline (http://compbio.dfci.harvard.edu/tgi/software/). EST sequences were then assembled de novo using MIRA (Chevreux et al., 2004), resulting in a total of 38 953 contigs (hereafter referred to as unigenes). Using the resultant unigene library, PCR amplicon primers were designed using MISA (Thiel et al., 2003) and Primer3 (Rozen and Skaletsky,

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TABLE 1. Characteristics of the 32 EST-SSR primers used for Abies firma.

Locus	Primer sequences (5′–3′)	Repeat motif	Size range (bp)	Polymorphism	GenBank accession no.	BLAST top hit description [organism]	BLAST top hit accession no.	E-value
As_c10422	l	(CTG) ₅	184	ou	FX334335	no hit	I	
As_c14033		(AG) ₆	151–156	yes	FX334336	no hit	I	
As_c14394		(TGC) ₅	103-111	yes	FX334337	no hit	I	I
As_c14606	K: AGCCIGCCACATCICICAAIAICC F: TGTTATTTCGGGTGGAGTTTTTGG	(TAA) ₅	294–296	yes	FX334334	unknown [Picea sitchensis]	ABK21196.1	5.25E-21
As_c23058		(TGC) ₅	230	no	FX334338	no hit	I	I
As_c28104		$(ATA)_5$	153–181	yes	FX334339	no hit	I	1
As_c28696	K: CACAGITAAAAGGCGGCCIACAG F: TAAGCAAGGACAGCTIGCATACCC	$(TA)_8$	234	no	FX334340	no hit	I	I
As_c32410	N. ICIIGIAGCACAACCCIGICAAI F: CIGAGCACGTGAGGAAGCAAAAT D. HEEGACATAACCTTATATATACCTTTEC	(AT) ₆	117–123	yes	FX334341	no hit	I	
As_c35493		(AAG) ₆	288	ou	FX334332	heat shock protein	AAC32131.1	8.52E-15
As_rep_c49		$(AGGAGA)_7$	257–284	yes	FX334333	no hit	I	I
As_rep_c66		(GTG) ₆	251–284	yes	FX334318	unknown [Picea sitchensis]	ABK22207.1	1.18E-29
As_rep_c4656	F: TCCTCGTCGTGTTCTACTCCTCT R: ACAAATCCAACAATGTCGACAGGA	(CTC) ₅	228–251	yes	FX334319	putative syntaxin 1A [Tanystylum orbiculare]	ABV81823.1	4.35E-21
As_rep_c5215		$(AG)_6$	247	no	FX334320	RNA-binding protein, putative [Ricinus communis]	XP_002532972.1	4.19E-08
As_rep_c5432		(ATG) ₅	225	no	FX334321	unknown [Żea mays]	ACL54598.1	3.92E-73
As_rep_c5928		$(AGG)_5$	164	no	FX334322	60S ribosomal protein L44 [Flaeis guineensis]	ACF06522.1	3.32E-41
As_rep_c7912		(GAA) ₆	294–299	yes	FX334323	PREDICTED: uncharacterized protein LOC100267326	XP_002285773.2	5.34E-13
As_rep_c10703	F: GCAGCTGCATCAGTCGCTAAGG	(GCA) ₅	152	no	FX334342	no hit	I	I
As_rep_c10904		$(CAAT)_5$	125	no	FX334324	dormancy/auxin associated-like protein, partial Piroa sirchansis	ADP94920.1	8.93E-15
As_rep_c11017	F: GITTCATTCGCTGTTACGATGTTGAR: GGAACTTGTCTAAGATTCCGCAT	$(AT)_6$	234–246	yes	FX334343	no hit	I	I
As_rep_c11401		(GAA) ₅	151	ou	FX334344	no hit	I	

Table 1. Continued.

o. description [organism] no hit no hit unknown [Picea sitchensis] no hit no hit no hit no hit no hit unknown [Picea sitchensis]						GenBank	BLAST top hit	BLAST top hit	
F. ACTCCTCCTCCTGGCCTTAAATTG (TA) 0 285	Locus	Primer sequences $(5'-3')$	Repeat motif	Size range (bp)	Polymorphism	accession no.	description [organism]	accession no.	E-value
R. GTGGATTCTTCTCTCTGGATCG F. TCCCAAGGACAGCACCAGCACCAGCACAGCACCAGCAGCACCAGCAG	As_rep_c12415	F: ACTCCTCCTGGCCTTAAATTG	$(TA)_{10}$	285	no	FX334345	no hit	1	
F: TCCCAATAGAATTGGGGGATAGC (TTC) ₃ 233 no FX334346 no hit R: CTAGACAAGGAGCCAGCTGAGCC (TGA) ₃ 267 no FX334325 unknown [Picea sitchensis] R: TCAACCACAAGGGCCAGAAGTTAGGC (TGA) ₀ 85 no FX334326 unknown [Picea sitchensis] F: CGCTTCCTGCTATTACTGCTTCC (AT) ₀ 85 no FX33434 no hit R: CATCATGCTACCTGCGGCTT (AT) ₀ 85 no FX33434 no hit R: CATCATGCTACGCGCCTTTGGGCAC (AGA) ₂ 200-203 yes FX33434 no hit R: ACTCAACTGGGCACGCAGGAC (AGA) ₂ 200-203 yes FX33438 no hit R: ACTCCTGGTGCCTTTCAGTT (AGA) ₂ 242-256 yes FX33432 unknown [Picea sitchensis] R: ACTCGTGCCTTCGGGCACGAGAACGCAGCACAACGCACAACACGCACAACA		R: GIGGATICITCICITCCIGGAICG							
R: CTTAGAAGAAGCACCAGCTCAGCC TCAA 267 no FX334325 unknown [Picea sitchensis] F: ATGCACAGGCCAGAGTTRACAG (TGA) ₅ 210-235 yes FX334326 unknown [Picea sitchensis] F: CGGCTTCCTTCCTTCTCTCTCTC (AT) ₁₀ 85 no FX334347 no hit F: CACAGGTTACCTTCTTGGTTG (TT) ₇ 152 no FX334348 no hit F: AACAGGCACCGGAATTAG (AGA) ₅ 200-203 yes FX334348 no hit F: ACTCAACTTGGTGGCACGGAGGA (AGA) ₅ 200-203 yes FX334348 no hit F: ACTCAACTTGGTAGAGAGCGCAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGA	As_rep_c12939	F: TCCCAATAGAATTTGGGGGATAGC	$(TTC)_5$	233	no	FX334346	no hit	l	
F: ATGCACAAGGGCCAAAGTTAGAG (TGA) ₃ 267 no FX334325 unknown [Picea sitchensis] R: TCATCTTTGCTTCCTTGCTTTGCTT (GCAACG) ₃ 210-235 yes FX334325 unknown [Picea sitchensis] F: CACCTTCTGGTGTCTTTAGTTT (AT) ₁₀ 85 no FX334347 no hit F: TAGTAATTGAGACAGCTTTGGTTG (CT) ₇ 152 no FX334347 no hit F: ACTCAGGTTACCTTTTGGTTG (AGA) ₅ 200-203 yes FX334348 no hit F: ACTCAGGTAGCAGGACG (AGA) ₅ 200-203 yes FX334327 unknown [Picea sitchensis] F: ACTCTTGGTAGAAGAAGCGCAGGA (ATAAG) ₅ 242-256 yes FX334329 predicted protein [Populus richocarpal] F: TGTAATTCTTAGGAGCGTGGAACGCAGAAGCGCAGCAAGCA		R: CITAGAAGAAGCAGCAGCICAGCC							
R: TCATGTTTGCTTCCTGCATCT (GCAACG) ₅ 210-235 yes FX334326 unknown [Picea sitchensis] F: CGGCTTCTGCTTCCTCTCCTCC (AT) ₁₀ 85 no FX33434 no bit F: CGCTTCTGGACGCTTCGGAATTAG (CT) ₇ 152 no FX33434 no bit F: ACTGAACTGGAGGACGCGGAGG (AGA) ₅ 200-203 yes FX33432 unknown [Picea sitchensis] F: ACTGAACTGGAGGACGCAGGAG (AGA) ₅ 242-256 yes FX33432 unknown [Picea sitchensis] F: ACTGAACTGGAGGAGCGTGTAGTT (CAG) ₆ 242-256 yes FX33432 unknown [Picea sitchensis] F: TAAGGTTGCTGTAGTT (CTG) ₆ 242-256 yes FX33432 unknown [Picea sitchensis] F: TGAATTCTTAGAGCCTGTGCAGAA (CTG) ₆ 257 no FX33439 unknown [Picea sitchensis] F: TGAATTGAAGGAGGAGGAGAGGAGAGGAGAGGAGAGGA	As_rep_c13048	F: ATGCACAAGGCCCAGAAGTTAGAG	$(TGA)_5$	267	no	FX334325	unknown [Picea sitchensis]	ABK24403.1	8.16E-60
F: CGGCTTCCTGCTATTACTGTTGCT (GCAACG) ₅ 210-235 yes FX33436 unknown [Picea sitchensis] R: CATCATGTGTTCCTCTCAC (AT) ₁₀ 85 no FX33437 no hit R: CATCAGGTTGCTGGGGTT (CT) ₇ 152 no FX33434 no hit F: ACTGAACTGGGACTTGGGGACG (AGA) ₅ 200-203 yes FX33434 no hit F: ACTGACTTGGTGCCTATTATTCG (AGA) ₅ 200-203 yes FX33438 no hit F: ACTGCTTTGGTGCCTATTATTCG (AGA) ₅ 242-256 yes FX334328 predicted protein [Populus F: TGGAGACTGTGGAGGCCTTGTATTGT (ATAG) ₅ 257 no FX334349 no hit F: TGTATTCTTAGAGGCTGTGAACT (CTT) ₅ 258-265 yes FX334329 unknown [Picea sitchensis] F: TGTAAAGGTGCGAAAGTGGGAAAGTGGGAAAGTGGCAAAGTGGGAAAGTGGAAAGTGGAAAGTGGAAAGTGGAAAGTGGGAAAGTGGGAAAGTGGAAAGTGGG		R: TCATGITIGCITCCICTGCAICIC							
R: CATCATGRACCTCAC (AT) ₁₀ 85 no FX334347 no bit R: CTCAGGRACCTCGGGCTT (CT) ₇ 152 no FX334348 no bit R: ACTCAGGACTGGGGACG (AGA) ₅ 200-203 yes FX334348 no bit R: ACTCTTGGTGCCTATTATTCG (AGA) ₅ 242-256 yes FX334328 predicted protein [Populus R: AACTCTGGTGCAGCAAG (ATAAG) ₅ 242-256 yes FX334328 predicted protein [Populus R: TAAGCTTCGAGGCGTGCAAG (ATAAG) ₅ 257 no FX334349 no bit R: TAAGCTTCGAGGAAGCAAGCAAC (CTT) ₅ 230 no FX334349 unknown [Picea sitchensis] R: TCAAAGGAGAAGCAATGAGAAA (CTG) ₅ 258-265 yes FX334339 unknown [Picea sitchensis] R: CTTGGAGAAGCTGCAATGAGAAA (ATC) ₇ 86 no FX334331 unknown [Picea sitchensis] R: CTGAACACTGCCCTAACGAGAA (ATC) ₇ 86 no FX334331 unknown [Picea sitchensis]	As_rep_c13359	F: CGGCTTCCTGCTATTACTGTTGCT	(GCAACG) ₅	210–235	yes	FX334326	unknown [Picea sitchensis]	ADE76551.1	2.41E-39
F: TAATATGAGACAGCCTTCGGGCTT (AT) ₁₀ 85 no FX33434 no hit R: CTCCAGGTTACCATCCTTGGTG (CT) ₇ 152 no FX334348 no hit F: ACTCAACTGGCGCACCGAATTACG (AGA) ₅ 200-203 yes FX33432 no hit F: CATCCTTCGGTGCCCTTTCAGTT (AGA) ₆ 242-256 yes FX33432 nuknown [Picea sitchensis] R: TAAGCTTTCGGAGGCGTTGTATGT (ATAAG) ₆ 242-256 yes FX334349 no hit R: TAAGCTTTCGGAGCGTTGTATGT (ATAAG) ₆ 257 no FX334349 no hit R: TAAGCTTTCGGAGCAAGCACTGAGAA (CTT) ₅ 230 no FX334329 unknown [Picea sitchensis] R: TTGGAGAAGCCTATCGAGAA (CTG) ₈ 258-265 yes FX334330 unknown [Picea sitchensis] R: CTTTGGAGAAGCTGTATCACATTT (ATC) ₇ 86 no FX334331 unknown [Picea sitchensis] R: CTGCTTGCCCCTACATTCGGTATT (ATC) ₇ 86 no FX334331 unknown [Picea sitchensis]		R: CATCATGTGATCGTGGTCCTCAC							
R:CTCCAGGTTACCATCCTTGGTTGI52noFX334348no hitF:ACTGAACTGGGGACGGAATTAG R:AGA)5200-203yesFX334327unknown [Picea sitchensis]F:CATCCTTTCGGTGCCTATTATTCG R:AGA)6242-256yesFX334328predicted protein [Populus trichocarpa]R:TAAGCTTTCGGAGCGTTGTATGT R:ATAAGCATAAGCATAAGCATAAGCATAAGCF:TGTATTCTTAGAGCAAGTGCAAC CTTCGAAAGGAGAAGCAAGCAATC R:ATAAGCAGGAAATGCACGTGAAC TCCAAAGGTGGAAAGCCTCATGGAAAC CTCTCAAAGGTGGAAAGCCTCATGGAAAC CTCTCAAAGGTGGAAAGCCTCATGGAAAA CTCTCAAAGGTGGAAAGCCTCATGGAAAA CTCTCAAAGGTGGAAAGCCTCATGGAAAA CTCTTGCAAAGGTGGAAAGCCTCATGGAAAA CTCTTGCAAAAGCTGAAAGTTCACATTT R:TCCAAAGGTGGAAAGCTGAAAGCTGAAAAAGCTGAAAAAAAA	As_rep_c14053	F: TAATATGAGACAGCCTTCGGGCTT	$(AT)_{10}$	85	no	FX334347	no hit		
F: ACTGAACTGAGGCACCGGAATTAG (CT) ₇ 152 no FX334348 no hit R: AGAGGACTGGAGGCACCGGAACTAGG (AGA) ₅ 200-203 yes FX334327 unknown [Picea sitchensis] F: CATCCTTTCGGTGCTATTATTCG (AGA) ₅ 242-256 yes FX334328 predicted protein [Populus R: AAAGCTTTCGGAGGCTTGTATGT (ATAAG) ₅ 257 no FX334349 no hit F: TGTATTCTTAGGAGCAACTGGAAC (CTT) ₅ 230 no FX334349 no hit R: TGAAGGAGGAATGGCAACTGAAC (CTT) ₅ 230 no FX334349 unknown [Picea sitchensis] R: CTTTGCAGGAAAGGCAAGGAAAGCCTCATGCAGAA (CTG) ₅ 258-265 yes FX334330 unknown [Picea sitchensis] R: CTTGCAGGCTCACTGCAGTTCATTCCATTTT (ATC) ₇ 86 no FX334331 unknown [Picea sitchensis] R: TGAACAACGTGGTAATTCACATTT F: TGAACAACGTGGTAATTCGGTTAT ATC) ₇ 86 no FX334331 unknown [Picea sitchensis]		R: CICCAGGITACCAICCITIGGIIG							
R: AGAGGAGTAGGAGGAGG (AGA) _s 200–203 yes FX334327 unknown [Picea sitchensis] R: AACTCTTCGGTAGAAGAAGCCAGGA (CAG) _s 242–256 yes FX334328 predicted protein [Populus nichocarpa] F: GTGACACAGTTGCCCTTCTAGGA (ATAAG) _s 257 no FX334349 no hit F: TGAAGGAGAAGGAGAAGAAGCATCA (CTT) _s 230 no FX334329 unknown [Picea sitchensis] R: CTTGCAAGGAGAAGAAGCATCA (CTT) _s 258–265 yes FX334330 unknown [Picea sitchensis] R: CTTGCAAGAGTGCAATCACTTCACATTT (CTG) _s 258–265 yes FX334330 unknown [Picea sitchensis] R: CTGCTTGCACGTCAATTCACATTT (ATC) _s 86 no FX334331 unknown [Picea sitchensis]	As_rep_c14410	F: ACTGAACTGAGGCACCGGAATTAG	$(CT)_7$	152	no	FX334348	no hit	I	
F: CATCCTTTCGGTGCAGAAGCCCAGGA R: AACTCTGGTAGAAGAGCCAGGA F: GTCAACACTTCGGTAGAAGAGCCCAGGA F: GTCAACACTTCGCTAGAAGAGCCCAGGA F: GTCAACACTTCGCTCCCTTCAGTT CAG) CATAAG CAG) CATAAG CATAAGAAGCAGAAAGCCATCAAGAAGCAATC CATAAGCAGAAAGCACACAACATCAAGAAGCAAAC CACAAAGCTGCACACACAACAACAACAACAACAACAACAACAACAACAA		R: AGAGGAGTAGAGAGTGTGGGGACG							
R: AACTCTGGTAGAAGAGCCAGGA (CAG) ₆ 242–256 yes FX334328 predicted protein [Populus F: GTGAGACAGTTGCCCTTTCAGTT (ATAAG) ₅ 257 no FX334349 no hit F: TGTATTCTTAGAGCCTGCACA (CTT) ₅ 230 no FX334329 unknown [Picea sitchensis] F: TCCAAAGGTGGAAGAGAAACCATGCAAC (CTT) ₅ 258–265 yes FX334330 unknown [Picea sitchensis] F: CATTGCAGAAGTGTATAGTCC (ATC) ₇ 86 no FX334331 unknown [Picea sitchensis] F: TCAACAACGTGATGTATAGTCC (ATC) ₇ 86 no FX334331 unknown [Picea sitchensis]	As_rep_c16096	F: CATCCTTTCGGTGCCTATTATTCG	$(AGA)_5$	200–203	yes	FX334327	unknown [Picea sitchensis]	ABK25258.1	4.11E-06
F: GTGAGACAGTTGCCCCTTTCAGTT (CAG) ₆ 242–256 yes FX334328 predicted protein [Populus Inichocarpa] R: TAAGCTTTCGGAGGCGTTGTATGT F: TGTATTCTTAGGAAGCGTTGCAACA F: TGTATTCTTAGGAAATGGCACGTGAAC F: TCCAAAGGTGAAATGGCACGTAGAAC F: CTTTGGAAAAGGCACACTATCAACA F: CAATTGAAAAGCAACACATTCC F: CAATTGAAAAGCACATTCCATTCAACAACTTCCATTCAACAATTCCATTCAACAA		R: AACTCTGGTAGAAGAAGCGCAGGA							
R: TAAGCTTTCGGAGGCGTTGTATGTATAAG)s257noFX334349no hitF: TGTATTCTTAGGACAATGCCACGTGAAC(CTT)s230noFX334329unknown [Picea sitchensis]R: CTTGGAGAAGCCTCATGGAGAA(CTG)s258-265yesFX334330unknown [Picea sitchensis]F: CAATTGACAAGTTGCCCTACATGTGC(CTG)s258-265yesFX334330unknown [Picea sitchensis]R: CTGCTTGCCCTACATTGACAATTT(ATC),86noFX334331unknown [Picea sitchensis]F: TCAACAACGTCGTCAGTTATGTCG(ATC),86noFX334331unknown [Picea sitchensis]	As_rep_c17556	F: GIGAGACAGIIGCCCCIIICAGII	(CAG) ₆	242–256	yes	FX334328	predicted protein [Populus	XP_002332355.1	3.79E-35
F: TGTATTCTTAGAGCCTGTGCAGCAA R: TAAAGGAGGAAATGGCACGTGAAC F: TCCAAAGGAACAAGCAACC F: TCCAAAGGTGGAACCATCATGGAGAA F: CTTTGGAGAAAGCCTCATGGAGAA F: CTTTGGAGAAAGCTTCATGAGAA F: CAATTGAAGATGTGCGAAAGTTGC R: CTGCTTGCAAGTTGCAAAGTTGC R: CTGCTTGCAAAGTTGCAAAGTTGC ATC) R: CGGATGATAAGTTGCAAAAGTTGCAAAAGTTGCAAAAGTTGCAAAAGTTGCAAAAAAAA		R: TAAGCTTTCGGAGGCGTTGTATGT					trichocarpa]		
R: TAAAGGAGGAAATGGCACGTGAAC(CTT)s230noFX334329unknown [Picea sitchensis]F: TCCAAAGGTGGAAGCATCATGGAGAA(CTG)s258–265yesFX334330unknown [Picea sitchensis]F: CAATTGAAGATGTGCGAAAGTTGC(CTG)s258–265yesFX334330unknown [Picea sitchensis]F: TCAACAACGTCGTCAATTAGTGG(ATC),86noFX334331unknown [Picea sitchensis]F: TCAACAACGTCGTCAGTTATTAGTGG(ATC),86noFX334331unknown [Picea sitchensis]	As_rep_c18764	F: TGTATTCTTAGAGCCTGTGCAGCAA	$(ATAAG)_5$	257	no	FX334349	no hit	I	1
F: TCCAAAGGTGGAAGCAATC (CTT) ₅ 230 no FX334329 unknown [<i>Picea sitchensis</i>] R: CTTTGGAGAAAGCCTCATGGAGAA F: CAATTGAAGATGTGCGAAAGTTGC R: CTGCTTGCAGAAGTTTCACATTT F: CTGCTTGCAGAAGTTTCACATTT F: TCAACAACGTCGTCAGTTATAGTCG ATC) ₇ 86 no FX334331 unknown [<i>Picea sitchensis</i>] F: CGGATGATGATGCTCGGTTAT F: CGATGATGATGTTCGGTTAT F: CGATGATGATGTTCGGTTAT F: CGGATGATGTTCGGTTAT F: CGGATGATGTTTCGGTTAT F: CGGATGATGTTCGGTTAT F: CGGATGATGTTCGGTTAT F: CGGATGATGTTCGGTTAT F: CGGATGATGTTCGGTTAT F: CGGATGATGTTCGGTTAT F: CGGATGATGTTTTCGGTTAT F: CGGATGATGTTTTCGTTTTTTTTTTTTTTTTTTTTTTTT		R: TAAAGGAGGAAATGGCACGTGAAC							
R: CTTTGGAGAAAGCCTCATGGAGAA F: CAATTGAAGATGTGCGAAAGTTGC R: CTGCTTGCCCCTACATTCACATTT F: CTGCTTGCCCCTACATTTACACATTT F: TCAACAACGTCGTCAGTTATAGTCG R: CTGCTTGCCCCTACATTTAGTCG F: TCAACAACGTCGTCAGTTATAGTCG R: CGGATGATGCTCAGTTATTAGTCG R: CGGATGATGCTCAGTTATTAGTCG R: CGGATGATGCTCAGTTATTAGTCG R: CTGCTTGCAGTATTAGTCG RICHARD R: CGGATGATACTTCGGTTAT R: CGGATGATAGTTCGGTTAT R: CGGATGATAGTTCGGTTATTAGTCG R: CTGTTGAGTAGTCGGTTATAGTCG RICHARD R: CTTTGAGAGAGGTGGTATAGTCGGTTAGTGTTAGTGTTAGTGTTAGTGTTAGTGTTAGTGTTAGTT	As_rep_c27580	F: TCCAAAGGTGGAAGAAGCAATC	$(CTT)_5$	230	no	FX334329	unknown [Picea sitchensis]	ABK25146.1	1.81E-13
F: CAATTGAAGATGTGCGAAAGTTGC (CTG) _s 258–265 yes FX334330 unknown [<i>Picea sitchensis</i>] R: CTGCTTGCCCCTACATTCACATTT F: TCAACAACGTCGTCAGTGTATAGTCG (ATC) ₇ 86 no FX334331 unknown [<i>Picea sitchensis</i>] R: CGGATGATGCTTGGTTAT		R: CTTTGGAGAAAGCCTCATGGAGAA							
R: CTGCTTGCCCCTACATTT F: TCAACAACGTCGTCAGTGTATAGTCG (ATC), 86 no FX334331 unknown [Picea sitchensis] R: CGGATGATGCCATACTTCGGTTAT	As_rep_c32446	F: CAATTGAAGATGTGCGAAAGTTGC	$(CTG)_5$	258–265	yes	FX334330	unknown [Picea sitchensis]	ADE75915.1	9.35E-20
F: TCAACAACGTCGTCAGTGTATAGTCG $(ATC)_7$ 86 no FX334331 unknown [Picea sitchensis] R: CGGATGATGCCATACTTCGGTTAT		R: CIGCIIGCCCCIACAIICACAIII							
R: CGGATGATGCCATACTTCGGTTAT	As_rep_c33168	F: TCAACAACGTCGTCAGTGTATAGTCG	$(ATC)_7$	98	no	FX334331	unknown [Picea sitchensis]	ADE75720.1	4.16E-22
		R: CGGATGATGCCATACTTCGGTTAT							

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Table 2. Characteristics of the 14 polymorphic EST-SSR markers used for three Abies species.

			A. fi	rma				A. hom	olepis				A. vei	tchii			
Locus	N	A	$H_{\rm o}$	H_{e}	$F_{ m IS}$	N	A	$H_{\rm o}$	H_{e}	$F_{ m IS}$	N	A	$H_{\rm o}$	H_{e}	$F_{ m IS}$	Size range (bp)	Total A
As_c14033	18	2	0.333	0.284	-0.172	22	1*	0.000	0.000	_	24	2	0.375	0.361	-0.040	151-156	3
As_c14394	17	1*	0.000	0.000	_	22	2	0.273	0.240	-0.135	24	2	0.042	0.042	0.000	103-111	3
As_c14606	17	1	0.000	0.000	_	22	1*	0.000	0.000	_	22	2	0.227	0.431	0.472	294-296	2
As_c28104	20	3	0.300	0.267	-0.123	24	2	0.042	0.042	0.000	22	1*	0.000	0.000	_	153-181	3
As_c32410	20	3	0.150	0.145	-0.036	23	2	0.087	0.085	-0.023	22	1*	0.000	0.000	_	117-123	3
As_rep_c49	20	2	0.100	0.097	-0.027	24	3	0.458	0.368	-0.246	24	4	0.417	0.476	0.124	257-284	6
As_rep_c66	20	2	0.150	0.142	-0.056	22	1	0.000	0.000	_	22	2	0.136	0.130	-0.050	251-284	3
As_rep_c4656	20	1*	0.000	0.000	_	22	1*	0.000	0.000	_	22	2	0.364	0.476	0.236	228-251	2
As_rep_c7912	20	1	0.000	0.000	_	24	1	0.000	0.000	_	24	1*	0.000	0.000	_	294-299	2
As_rep_c11017	18	1*	0.000	0.000	_	24	1*	0.000	0.000	_	22	1*	0.000	0.000	_	234-246	1
As_rep_c13359	20	2	0.050	0.050	0.000	24	2	0.083	0.082	-0.022	24	1*	0.000	0.000	_	210-235	3
As_rep_c16096	19	2	0.105	0.102	-0.029	22	2	0.045	0.045	0.000	22	1*	0.000	0.000	_	200-203	2
As_rep_c17556	19	1*	0.000	0.000	_	24	1	0.000	0.000	_	22	1*	0.000	0.000	_	242-256	1
As_rep_c32446	19	1	0.000	0.000	_	22	1*	0.000	0.000	_	22	1*	0.000	0.000	_	258-265	1

Note: A = number of alleles per locus; $F_{\text{IS}} = \text{fixation index}$; $H_{\text{e}} = \text{expected heterozygosity}$; $H_{\text{o}} = \text{observed heterozygosity}$; N = number of individuals genotyped.

2000), after trimming low quality regions using the qualityTrimmer command in the Euler-SR package (Chaisson and Pevzner, 2008). The criteria applied to identify microsatellite loci were at least six dinucleotide repeat units, or five tri- to hexanucleotide repeat units. To eliminate redundancy (i.e., multiple sets of primers for the same locus), all assembled sequences containing microsatellites were subjected to a BLAST search against the NCBI nonredundant (nr) protein database using the BLASTX algorithm with an E-value cutoff of 1.0E-3. A total of 153 EST-SSR primer pairs bordering sequence regions with more than four di- to hexanucleotide repeats were designed. Ninety-six of the 153 primers, for nonredundant loci with large numbers of repeats, were selected for further evaluation. For each primer pair, genomic DNA from one individual of A. firma was used to check PCR amplification. The PCR reaction was carried out following the standard protocol supplied with the QIAGEN Multiplex PCR Kit (QIAGEN, Hilden, Germany), in a final volume of 10 μL, which contained approximately 5 ng of DNA, 5 µL of 2× Multiplex PCR Master Mix, and 0.2 µM of each primer. The PCR thermal profile involved denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 55°C for 1 min, 72°C for 1 min, and a final 7-min extension step at 72°C. PCR products were labeled with ChromaTide Alexa Fluor 488-5-dUTP (Invitrogen, Carlsbad, California, USA) according to Kondo et al. (2000), and loaded onto an automated sequencer (ABI Prism 3100 Genetic Analyzer; Applied Biosystems, Carlsbad, California, USA) to determine fragment lengths, which were analyzed using GENOTYPER software (Applied Biosystems). Thirty-two loci exhibited clear PCR amplification with fragment sizes ranging from 50 to 500 bp (Table 1). The polymorphism of these fragments was evaluated using eight individuals of each of three Abies species (A. firma, A. homolepis, and A. veitchii) sampled across the species' geographical range. Fourteen of the 32 loci were polymorphic and provided clear fragment patterns. The genetic variation at these 14 loci was evaluated using 20 individuals from the A. firma population. Information about the populations sampled is provided in Appendix 1, and specimen vouchers were deposited in the Forestry and Forest Products Research Institute herbarium. To characterize each EST-SSR marker, the following four genetic diversity statistics were calculated using FSTAT 2.9.3 (Goudet, 2001): number of alleles per locus (A), observed heterozygosity (H_0), expected heterozygosity $(H_{\rm e})$, and fixation index $(F_{\rm IS})$. In addition, the significance of Hardy–Weinberg equilibrium and genotypic equilibrium were tested by 1000 randomizations with adjustment of the resulting P values by sequential Bonferroni correction, using FSTAT 2.9.3. Cross-amplification was conducted on one population each for two Abies species (Table 2, Appendix 1) following the protocol described above. Of the 14 polymorphic loci, As_rep_c4656, As_rep_c32446, As_c14394, As_rep_c11017, and As_rep_c17556 were not polymorphic in this population, but they were polymorphic in other populations (data not shown). As_c14606 was also monomorphic in A. firma but polymorphic in A. veitchii. As_rep_ c7912 was monomorphic in all three species but polymorphic in other popula-

A ranged from one to three and $H_{\rm e}$ ranged from 0 to 0.284. The results of cross-species amplification showed that all 14 loci were amplified successfully

in A. homolepis and A. veitchii. The total number of alleles ranged from one to six. Analysis of the 14 polymorphic loci indicated no significant deviation in $F_{\rm IS}$ or genotype disequilibrium among locus pairs for any of the three species.

CONCLUSIONS

The EST-SSR markers described here will be useful for future genetic studies of *A. firma*. Interspecific amplification of these markers also shows their potential for use in closely related species. These markers may therefore provide a tool for understanding population demography, population structure, gene flow, and mating systems in *Abies* species.

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^{*}Monomorphic in this population but polymorphic in other populations.

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APPENDIX 1. Information about the populations of three Abies species sampled in this study.

Species	Locality	Geographic coordinates	Accession no.
A. firma	Onzui, Shiso City, Hyogo Prefecture, Japan	35.249°N, 134.523°E	TF-K11-0098
A. homolepis	Yamanaka, Yamanaka-ko Village, Minami Tsuru County, Yamanashi Prefecture, Japan	35.438°N, 138.885°E	TWTw20773
A. veitchii	Yamanaka, Yamanaka-ko Village, Minami Tsuru County, Yamanashi Prefecture, Japan	35.442°N, 138.902°E	TWTw20818

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