Development of 32 EST-SSR Markers for Abies firma (Pinaceae) and Their Transferability to Related Species

Authors: Uchiyama, Kentaro, Fujii, Sayaka, Ishizuka, Wataru, Goto, Susumu, and Tsumura, Yoshihiko

Source: Applications in Plant Sciences, 1(2)

Published By: Botanical Society of America

URL: https://doi.org/10.3732/apps.1200464

Applications in Plant Sciences 2013 1(2): 1200464

PRIMER NOTE

DEVELOPMENT OF 32 EST-SSR MARKERS FOR *ABIES FIRMA* (PINACEAE) AND THEIR TRANSFERABILITY TO RELATED SPECIES[1]

KENTARO UCHIYAMA[2], SAYAKA FUJII[3,4], WATARU ISHIZUKA[5], SUSUMU GOTO[6], AND YOSHIHIKO TSUMURA[2,3,7]

[2]Department of Forest Genetics, Forestry and Forest Products Research Institute, Tsukuba, Ibaraki 305-8687, Japan; [3]Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan; [4]Ministry of Environment, Godochosha No. 5, Kasumigaseki 1-2-2, Chiyoda-ku, Tokyo 100-8975, Japan; [5]Department of General Systems Studies, Graduate School of Arts and Sciences, University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan; and [6]University of Tokyo Forests, Graduate School of Agricultural and Life Sciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

- *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 warm-temperate 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 well-conserved 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,

[1]Manuscript received 31 August 2012; revision accepted 3 October 2012.

The authors are grateful to S. Ueno, T. Ujino-Ihara, T. Yasui, Y. Kawamata, A. Hisamatsu, and other members of the Department of Forest Genetics at the Forestry and Forest Products Research Institute (FFPRI) for their support.

[7]Author for correspondence: ytsumu@affrc.go.jp

doi:10.3732/apps.1200464

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	F: TCTGAGTGCTAACCTGTGGACTGC R: CGGGAGTATGAGGAGTTGTTGACTC	$(CTG)_5$	184	no	FX334335	no hit	—	—
As_c14033	F: GACCACACAATTCAAATGATTGCC R: GTAATGAGCTGGAAGCTGGTCTCC	$(AG)_6$	151–156	yes	FX334336	no hit	—	—
As_c14394	F: GTATGTTGCCTCTGTTTTGATGGC R: AGCCTGCCACATCTCTCAATATCC	$(TGC)_5$	103–111	yes	FX334337	no hit	—	—
As_c14606	F: TGTTATTTCGGGTGGAGTTTTTGG R: CCTCAGACCAACCAAAAGAGAGGA	$(TAA)_5$	294–296	yes	FX334334	unknown [*Picea sitchensis*]	ABK21196.1	5.25E-21
As_c23058	F: AACGTTTTGGATCGACTCCATGTT R: GTAACAGCTGAACTACCAGCCACG	$(TGC)_5$	230	no	FX334338	no hit	—	—
As_c28104	F: CGAGGAAGAAGCCAAGTTATCAGG R: CACAGTTAAAAAGGCGGCCTACAG	$(ATA)_5$	153–181	yes	FX334339	no hit	—	—
As_c28696	F: TAAGCAAGGACAGCTTGCATACCC R: TCTTGTACGCACAACCCTGTCAAT	$(TA)_8$	234	no	FX334340	no hit	—	—
As_c32410	F: CTGAGCACGTGAGGAAGCAAAAT R: TGGGAGATAGCCTCATTAGGTTGC	$(AT)_6$	117–123	yes	FX334341	no hit	—	—
As_c35493	F: AAGGACCTGGTCAAAAAGCATTCA R: CCGGTGTTACATAACCAGGACCAT	$(AAG)_6$	288	no	FX334332	heat shock protein [*Picea mariana*]	AAC32131.1	8.52E-15
As_rep_c49	F: GACGAAGATCAGTACAAGGCACGA R: GCGATCCTTCAATTTGTCCTTCTC	$(AGGAGA)_7$	257–284	yes	FX334333	no hit	—	—
As_rep_c66	F: GTTGGGGTCGTGAAGAGGACACT R: GGCATCGTAGCCATAACTGTAGCC	$(GTG)_6$	251–284	yes	FX334318	unknown [*Picea sitchensis*]	ABK22207.1	1.18E-29
As_rep_c4656	F: TCCTCGTCGTGTTCTACTCCCTCT R: ACAAATCCAACAATGTCGACAGGA	$(CTC)_5$	228–251	yes	FX334319	putative syntaxin 1A [*Tanystylum orbiculare*]	ABV81823.1	4.35E-21
As_rep_c5215	F: GATTCTGATCATGATAGGGGCAGG R: TCTCCCTTGTGGCTTTCTTCTTTG	$(AG)_6$	247	no	FX334320	RNA-binding protein, putative [*Ricinus communis*]	XP_002532972.1	4.19E-08
As_rep_c5432	F: TGGGTGAAGAGAGAACCAGAAAGG R: TCCAATGCGACATAATGATTCCAC	$(ATG)_5$	225	no	FX334321	unknown [*Zea mays*]	ACL54598.1	3.92E-73
As_rep_c5928	F: GGTCTCGAGTTCGAGGACAAAGAA R: TGCAAAGTGTGCTTTCTACAAGCC	$(AGG)_5$	164	no	FX334322	60S ribosomal protein L44 [*Elaeis guineensis*]	ACF06522.1	3.32E-41
As_rep_c7912	F: TAGAGGAAATGCTTGCTCGTCTCG R: AGGACTTCCTCTGCAAATCCACAC	$(GAA)_6$	294–299	yes	FX334323	PREDICTED: uncharacterized protein LOC100267326 [*Vitis vinifera*]	XP_002285773.2	5.34E-13
As_rep_c10703	F: GCAGCTGCATCAGTCGCTAAGG R: GCCTTCAAGCAATCCAACTTCACT	$(GCA)_5$	152	no	FX334342	no hit	—	—
As_rep_c10904	F: TCCATGTCATTTATGGAGCACCTG R: CCAATCCAACAGAACATAAATGCAG	$(CAAT)_5$	125	no	FX334324	dormancy/auxin associated-like protein, partial [*Picea sitchensis*]	ADP94920.1	8.93E-15
As_rep_c11017	F: GTTTCATTCGCTGTTACGATGTTGA R: GGAACTTGTCTAAGATTCCGCCAT	$(AT)_6$	234–246	yes	FX334343	no hit	—	—
As_rep_c11401	F: CGGCAACACAGACAGAAGAAAGAA R: GGGGATACCTCACATCCACTCAAC	$(GAA)_5$	151	no	FX334344	no hit	—	—

TABLE 1. Continued.

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_rep_c12415	F: ACTCCTCCTCCTGGCCTTAAATTG	$(TA)_{10}$	285	no	FX334345	no hit	—	—
	R: GTGGATTCTTCTCTTCCTGGATCG							
As_rep_c12939	F: TCCCAATAGAATTTGGGGGATAGC	$(TTC)_5$	233	no	FX334346	no hit	—	—
	R: CTTAGAAGAAGCAGCAGCTCAGCC							
As_rep_c13048	F: ATGCACAAGGGCCAGAAGTTAGAG	$(TGA)_5$	267	no	FX334325	unknown [*Picea sitchensis*]	ABK24403.1	8.16E-60
	R: TCATGTTTGCTTCCTCTGCATCTC							
As_rep_c13359	F: CGGCTTCCTGCTATTACTGTTGCT	$(GCAACG)_5$	210–235	yes	FX334326	unknown [*Picea sitchensis*]	ADE76551.1	2.41E-39
	R: CATCATGTGATCGTGGTCCTCAC							
As_rep_c14053	F: TAATATGAGACAGCCTTCGGGCTT	$(AT)_{10}$	85	no	FX334347	no hit	—	—
	R: CTCCAGGTTACCATCCTTTGGTTG							
As_rep_c14410	F: ACTGAACTGAGGCACCGGAATTAG	$(CT)_7$	152	no	FX334348	no hit	—	—
	R: AGAGGAGTAGAGAGTGTGGGACG							
As_rep_c16096	F: CATCCTTTCGGTGCCTATTATTCG	$(AGA)_5$	200–203	yes	FX334327	unknown [*Picea sitchensis*]	ABK25258.1	4.11E-06
	R: AACTCTGGTAGAAGAAGCGCAGGA							
As_rep_c17556	F: GTGAGACAGTTGCCCCTTTCAGTT	$(CAG)_6$	242–256	yes	FX334328	predicted protein [*Populus trichocarpa*]	XP_002332355.1	3.79E-35
	R: TAAGCTTTCGGAGGCGTTGTATGT							
As_rep_c18764	F: TGTATTCTTAGAGCCTGTGCAGCAA	$(ATAAG)_5$	257	no	FX334349	no hit	—	—
	R: TAAAGGAGGAAATGGCACGTGAAC							
As_rep_c27580	F: TCCAAAGGTGGAAGAGAAGCAATC	$(CTT)_5$	230	no	FX334329	unknown [*Picea sitchensis*]	ABK25146.1	1.81E-13
	R: CTTTGGAGAAAGCCTCATGGAGAA							
As_rep_c32446	F: CAATTGAAGATGTGCGAAAGTTGC	$(CTG)_5$	258–265	yes	FX334330	unknown [*Picea sitchensis*]	ADE75915.1	9.35E-20
	R: CTGCTTGCCCCTACATTCACATTT							
As_rep_c33168	F: TCAACAACGTCGTCAGTGTATAGTCG	$(ATC)_7$	86	no	FX334331	unknown [*Picea sitchensis*]	ADE75720.1	4.16E-22
	R: CGGATGATGCCATACTTCGGTTAT							

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

	A. firma					*A. homolepis*					*A. veitchii*						
Locus	*N*	*A*	H_o	H_e	F_{IS}	*N*	*A*	H_o	H_e	F_{IS}	*N*	*A*	H_o	H_e	F_{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_{IS} = fixation index; H_e = expected heterozygosity; H_o = observed heterozygosity; *N* = number of individuals genotyped.

*Monomorphic in this population but polymorphic in other populations.

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_o), expected heterozygosity (H_e), and fixation index (F_{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 populations of *A. veitchii*.

A ranged from one to three and H_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_{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.

LITERATURE CITED

CHAISSON, M. J., AND P. A. PEVZNER. 2008. Short read fragment assembly of bacterial genomes. *Genome Research* 18: 324–330.

CHEVREUX, B., T. PFISTERER, B. DRESCHER, A. J. DRIESEL, W. E. G. MÜLLER, T. WETTER, AND S. SUHAI. 2004. Using the miraEST assembler for reliable and automated mRNA transcript assembly and SNP detection in sequenced ESTs. *Genome Research* 14: 1147–1159.

FARJON, A. 1990. Pinaceae. Drawings and descriptions of the genera *Abies*, *Cedrus*, *Pseudolarix*, *Keteleeria*, *Nothotsuga*, *Tsuga*, *Cathaya*, *Pseudotsuga*, *Larix* and *Picea*. Koeltz Scientific Books, Königstein, Germany.

GOUDET, J. 2001. FSTAT; a program to estimate and test gene diversities and fixation indices version 2.9.3. Website http://www2.unil.ch/popgen/softwares/fstat.htm [accessed 19 December 2012].

KONDO, H., T. TAHIRA, H. HAYASHI, K. OSHIMA, AND K. HAYASHI. 2000. Microsatellite genotyping of post-PCR fluorescently labeled markers. *BioTechniques* 29: 868–873.

ROZEN, S., AND H. SKALETSKY. 2000. Primer3 on the WWW for general users and for biologist programmers. *In* S. Misener and S. A. Krawetz [eds.], Methods in molecular biology, vol. 132: Bioinformatics methods and protocols, 365–386. Humana Press, Totowa, New Jersey, USA.

SAITO, Y., K. FUJIHIRA, M. SUZUKI, S. SATOMI, M. YONEMICHI, AND Y. IDE. 2005. Allozyme variation in natural populations of *Abies firma* in University Forest in Chiba, University Forests, The University of Tokyo and in and around the Kanto Area. *Bulletin of the Tokyo University Forests* 113: 1–10.

SUZUKI, K. 1992. Fluctuation of momi (*Abies firma*) dead standing trees and change of annual ring width at Mt. Ohyama and the around areas in Kanagawa Prefecture. *Bulletin of the Kanagawa Prefecture Forest Experiment Station* 19: 23–42.

Thiel, T., W. Michalek, R. Varshney, and A. Graner. 2003. Exploiting EST databases for the development and characterization of gene-derived SSR-markers in barley (*Hordeum vulgare* L.). *Theoretical and Applied Genetics* 106: 411–422.

Tsumura, Y., and Y. Suyama. 1998. Differentiation of mitochondrial DNA polymophisms in populations of five Japanese *Abies* species. *Evolution; International Journal of Organic Evolution* 52: 1031–1042.

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