Development of novel EST-SSR markers derived from Japanese pear (*Pyrus pyrifolia*)

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We developed 73 novel expressed sequence tag (EST)—simple sequence repeat (SSR) markers based on ESTs derived from 11 different cDNA libraries of the Japanese pear (*Pyrus pyrifolia* Nakai) cultivar ‘Housui’, comprising 30, 36, two, one, and four SSRs with repeat motifs of di-, tri-, tetra-, penta-, and hexa-nucleotides, respectively. Forty-eight EST sequences showed significant homology to *Arabidopsis thaliana* genes. The linkage groups and positions of 57 loci generated from 53 SSR markers covering all 17 linkage groups, were identified. These EST-SSRs will be useful for high-throughput genotyping of marker-assisted selection. The numbers of alleles and the observed and expected heterozygosities at single-locus EST-SSR markers in ten Japanese and ten European pears suggest high polymorphism and heterozygosity of the EST-SSRs. Di-nucleotide EST-SSRs showed two to three times the polymorphism and heterozygosity of tri-nucleotide EST-SSRs. Some of the EST-SSRs proved transferable to other species in the Rosaceae, paving the way for the development of markers for the same phenotypic traits among species of the Rosaceae.

Key Words: expressed sequence tag, genome mapping, Japanese pear, simple sequence repeat.

Introduction

Pears (*Pyrus* spp.), which belong to the subfamily Maloideae in the family Rosaceae, are one of the most important fruits grown in Europe and Asia for the past 2000 to 3000 years. Pears are grown commercially in all the temperate regions in more than 50 countries (Bell 1990, Bell *et al.* 1996). The Japanese pear (*Pyrus pyrifolia* Nakai), the European pear (*P. communis* L.), and Chinese pears (*P. bretschneideri* Rehd. and *P. ussuriensis* Maxim.) are the major species grown commercially. The Japanese pear is grown in East Asia, and the European pear in Europe, North America, and temperate regions of the southern hemisphere (Bell 1990).

Molecular markers combined with high-density genetic linkage maps are useful for fundamental and applied genetic researches and for breeding programs. Simple sequence repeat (SSR) markers provide a reliable method for constructing genetic maps and evaluating genetic diversity because of their co-dominant inheritance and the abundance of alleles per locus (Weber and May 1989). More than 100 SSR markers have been developed from genomic sequences of Japanese and European pears (Yamamoto *et al.* 2002a, 2002b, 2002c, Sawamura *et al.* 2004, Fernandez-Fernandez *et al.* 2006, Inoue *et al.* 2007). SSR markers developed in pear were used as anchor loci for reference genetic linkage maps of pear (Yamamoto *et al.* 2007). Additionally, SSR markers developed in of apple (*Malus × domestica* Borkh.; also

Maloideae) have been successfully applied to pears (Guilford *et al.* 1997, Gianfranceschi *et al.* 1998, Liebhard *et al.* 2002, Silfverberg-Dilworth *et al.* 2006). Transferability of SSR markers within the Maloideae was investigated (Yamamoto *et al.* 2001, Liebhard *et al.* 2002). Apple SSR markers were used for constructing genetic linkage maps in pears and co-linearity between pear and apple was identified (Dondini *et al.* 2004, Pierantoni *et al.* 2004, Yamamoto *et al.* 2007). However, not enough SSR markers have been developed to allow marker-assisted breeding in pear, and very few functional genes have been used as selection markers.

Several sequencing projects have generated more than 250,000 expressed sequence tags (ESTs) in apple over the past few years (Newcomb *et al.* 2006, Naik *et al.* 2006). SSRs associated with these sequences constitute a large resource of markers which have remained largely unexploited. Furthermore, since EST-based SSRs are located around conserved coding regions, they are often more transferable between species (Decroocq *et al.* 2003) than genomic SSRs. The Genome Database for Rosaceae (GDR, <http://www.bioinfo.wsu.edu/gdr/>, 5 May, 2009) lists 359,001 EST sequences, including 250,907 in *Malus*, 83,751 in *Prunus*, 18,729 in *Fragaria*, and 5,284 in *Rosa*, but only 330 EST sequences and 271 unigenes of *Pyrus*. Therefore, it is necessary to obtain many more EST sequences in pears to provide a source of functional markers such as SSRs and single nucleotide polymorphisms (SNPs). EST-derived markers developed for candidate genes may be suitable for relatively high-throughput genotyping for marker-assisted selection (MAS) (Celton *et al.* 2009).

Here, we developed EST-SSR markers from more than

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1,000 EST sequences with motifs of di-, tri-, tetra-, penta-, and hexa-nucleotide repeats, which were collected from 11 cDNA libraries of the Japanese pear cultivar 'Housui' (syn. 'Hosui'). We established 73 EST-SSR markers, characterized their polymorphism, heterozygosity, and map position, and examined their transferability in apple and *Prunus*.

Materials and Methods

Plant materials and DNA isolation

For construction of cDNA libraries we used the Japanese pear (*Pyrus pyrifolia* Nakai) cultivar 'Housui' (syn. 'Hosui'). For evaluation of EST-SSR markers we used ten Japanese pear cultivars: 'Atago', 'Choju', 'Chojuro', 'Housui', 'Ishiiwase', 'Kinchaku', 'Nanseichabo', 'Niitaka', 'Nijisseiki', and 'Okusankichi'. To examine cross species amplification of the EST-SSR markers we used ten European pear (*Pyrus communis* L.) cultivars: 'Bartlett', 'Brandy Wine', 'Conference', 'Flemish Beauty', 'Margeritt Marilla', 'La France', 'Passe Crassane', 'Perrie Corneir', 'Precose', and 'Winter Nelis'. To test transferability across genera we used apple (*Malus × domestica* Borkh.) cultivars 'Fuji' and 'Sansa', almond (*Prunus dulcis* Mill.) cultivar 'Texas', and peach (*Prunus persica* (L) Batsch.) cultivar 'Earlygold'. 'Texas' and 'Earlygold' were previously used for the *Prunus* reference genetic linkage map (Dirlewanger *et al.* 2004, Howad *et al.* 2005).

Plant samples were maintained at the National Institute of Fruit Tree Science (NIFTS) (Tsukuba and Morioka, Japan). Young leaves were collected and stored at -80°C for DNA extraction. Genomic DNAs were isolated from leaves with G2 Buffer and Genomic-tip 20/G anion-exchange tips (Qiagen, Germany) (Yamamoto *et al.* 2006). Genomic DNAs of 'Texas' and 'Earlygold' were kindly provided by Dr. P. Arús (CSIC-IRTA, Spain).

Construction of cDNA libraries and EST analysis

We constructed 11 cDNA libraries of 'Housui': two from leaves (buds and fully expanded), three from flowers (buds, balloon stage, and open), and six from fruit tissues: at 2–3, 6–7, 11–12, 15–16, 20–21 (mature fruits), and 23–24 (over-mature fruits) week after full bloom (Nishitani *et al.* 2009). Total RNA was extracted by the Hot-Borate method (Wan and Wilkins 1994). Poly(A)+ RNA was purified with a FastTrack 2.0 kit (Invitrogen, USA). The cDNA libraries were constructed with a ZAP-cDNA Gigapack III Gold Cloning kit (Stratagene, USA) or a Creator SMART cDNA Library Construction kit (Clontech, USA). Colonies were manually picked, and plasmids were purified and sequenced with a BigDye Terminator v. 3.1 Cycle Sequencing kit (Applied Biosystems, USA) according to the manufacturer's instructions. Unincorporated nucleotides were removed by column chromatography on a Sepharose G50 column (Amersham Pharmacia, UK) from Multiscreen 96-well plates (Millipore, USA) before sequencing with an ABI Prism 3130xl sequencer (Applied Biosystems, USA). EST se-

quences were processed to remove the vector and unreliable sequences (quality value <20) by using the Pregap4 software (Bonfield *et al.* 1995), and sequences longer than 200 bp were processed for contig assembly with the Gap4 software (Bonfield *et al.* 1995).

From 28,704 cDNA clones sequenced by single-pass sequencing from the 5' end we obtained 23,755 sequences longer than 200 bp. After the removal of redundant clones (Nishitani *et al.* 2009), contig assembly of raw sequences produced 10,350 unigenes (non-redundant sequences). Functional categorization of the non-redundant sequences was based on the GoSlim gene ontology (Berardini *et al.* 2004) of their homologs in *Arabidopsis*. Homology search for *Arabidopsis* genes was performed by BLASTX.

EST-SSR development

SSR sequences among the 10,350 unigenes were searched by using the Tandem Repeats Finder software (Benson 1999) with alignment parameters set to match = 2, mismatch = 7, and indel = 7. We found 1,097 ESTs with repeat sequences, comprising 507 di-nucleotide, 369 tri-nucleotide (or hexa-nucleotide), 72 tetra-nucleotide, and 149 penta-nucleotide motifs. We considered 12 or more repeat nucleotides as criteria of SSRs; viz., six di-nucleotides, four tri-nucleotides, three tetra-nucleotides, three penta-nucleotides, and two hexa-nucleotides.

Primer pairs were designed with the Primer3 Web interface (Rozen and Skaletsky 2000, <http://frodo.wi.mit.edu/primer3/input.htm>). General primer picking conditions used a primer size of 20–25 bp (optimum 23 bp), a primer T_m of 57 – 67°C (optimum 63°C), a maximum T_m difference of 1°C , a primer GC content of 50–60% (optimum 55%), and a product size range of 100–300 bp.

SSR analysis

SSR amplification was performed in a 10- μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl_2 , 0.001% gelatin, 0.2 mM each dNTP, 5 pmol each forward primer (fluorescently labeled with Fam/Vic/Ned) and reverse primer (unlabelled), 5 ng genomic DNA, and 0.5 units Taq polymerase (Invitrogen, USA). DNA was amplified in 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. When clear amplification was not obtained at 55°C annealing temperature, we tried 50 and 60°C . The PCR products were separated and detected in a PRISM 3100 DNA sequencer (Applied Biosystems, USA). The size of the amplified bands was determined against an internal standard DNA (400HD-Rox, Applied Biosystems, USA) by the GeneScan software (Applied Biosystems, USA).

Mapping of SSR markers

Two populations were used for genome mapping of the EST-SSRs: 63 F_1 individuals from an interspecific cross between European pear 'Bartlett' and Japanese pear 'Housui', and 55 F_1 s from an interspecific cross between Japanese pear 'Shinsei' and 282-12 ('Housui' $\times$ European pear 'La

France') (Yamamoto *et al.* 2004, 2007, Terakami *et al.* 2009). Genetic linkage maps of 'Bartlett' and 'Housui' were constructed by adapting the double pseudo-testcross strategy (Grattapaglia and Sederoff 1994) because of the self-incompatibility of pear (Kikuchi 1929). A genetic linkage map of 'La France' was constructed by a BC₁ strategy by scoring La France alleles in 282-12.

Pear genomic SSRs (Fernandez-Fernandez *et al.* 2006, Inoue *et al.* 2007) and apple SSRs (Silfverberg-Dilworth *et al.* 2006) were tried and newly mapped in addition to the previous reports (Yamamoto *et al.* 2007, Terakami *et al.* 2009). We used the three genetic linkage maps of 'Bartlett', 'Housui', and 'La France' for identification of linkage groups (LGs) and positions of EST-SSRs. JoinMap v. 3.0 (Van Ooijen and Voorrips 2001) was used to construct the genetic linkage maps, and the Kosambi's mapping function was used to convert recombination units into genetic distances. The mapping analysis used a minimum LOD score of 5.0.

Data analysis

Using the CERVUS v. 2.0 software (Marshall *et al.* 1998), we estimated the observed heterozygosity (H_O) and expected heterozygosity (H_E) at single-locus EST-SSR markers in the ten Japanese and ten European pears. H_O was calculated as the ratio of the heterozygous genotypes scored at each locus. H_E was calculated using an unbiased formula from allele frequencies as $1 - \sum p_i^2$ ($1 \leq i \leq m$), where m is the number of alleles at the target locus and p_i is the allele frequency of the i^{th} allele at the target locus.

Results

Development of EST-SSR markers

Out of 98 EST sequences tested, 73 were successfully established as SSR markers; these amplified fragments from 'Housui' with the target size, and gave clear and stable amplification in all pear cultivars. The EST-SSR markers comprised 30 di-nucleotide, 36 tri-nucleotide, two tetra-nucleotide, one penta-nucleotide, and four hexa-nucleotide motifs (Table 1). The remaining 25 sequences generated no or unstable amplification, or large bands possibly due to the existence of introns, and were excluded from further analysis.

Characteristics of EST-SSR markers

Forty-eight EST-SSR sequences showed significant homology to *Arabidopsis thaliana* genes (Table 2). Marker TsuENH001 showed high homology to a ferredoxin-NADP(+) reductase gene of *A. thaliana*, and TsuENH091 was identified as a putative serine/threonine protein kinase gene. Putative gene functions were identified for more than 60% of the sequences.

We evaluated the number of alleles, H_O , and H_E at single-locus EST-SSR markers. Markers, TsuENH008 and TsuENH016 showed multiple fragments only in European pears, perhaps because they originated from multiple loci

(Table 1) and so were excluded from the data analysis of European pears. Likewise, marker TsuENH012 showed multiple fragments only in Japanese pears, and so was excluded from the data analysis of Japanese pears. EST-SSRs with di-nucleotide motifs showed two to six alleles in Japanese pears and one to eight in European pears (Table 3). EST-SSRs with tri-nucleotide motifs showed one to four alleles in Japanese pears and one to six in European pears, which is less than half, on average, of the number among di-nucleotide EST-SSRs. Values of H_O and H_E among tri-nucleotide EST-SSRs were one-third of those among di-nucleotide EST-SSRs, indicating that di-nucleotide EST-SSRs generated much more heterozygosity than tri-nucleotide EST-SSRs.

Mapping of EST-SSR markers

Fifty-three SSR markers generated 57 loci which were identified in at least one linkage maps (Fig. 1). All 30 di-nucleotide EST-SSRs were mapped, but only 20 out of 40 tri-nucleotide (or hexa-nucleotide) EST-SSRs were mapped. Two EST-SSRs with tetra-nucleotide motifs and one with penta-nucleotide motifs were mapped.

Out of the 53 EST-SSRs, 49 markers showing one segregating locus generated 49 mapped loci (Fig. 1). Four multiple-locus EST-SSRs had two segregating loci which were mapped in different linkage groups (Fig. 1): TsuENH004, LGs 4 and 12; TsuENH011, LGs 6 and 14; TsuENH042, LGs 13 and 16; and TsuENH054, LGs 13 and 16. The 57 EST-SSR loci were distributed across all 17 LGs.

Transferability to related genera

Out of 61 pear EST-SSRs, 51 (83%) generated clear amplification in apple, comprising 21 di-nucleotide, 29 tri-nucleotide (or hexa-nucleotide), and one penta-nucleotide EST-SSRs showing transferability. But only 17 (<28%) generated clear amplification in almond or peach, showing limited transferability across subfamilies. No distinct differences in transferability across genera were observed between di-nucleotide and tri-nucleotide EST-SSRs.

Discussion

Until now, few EST-SSR markers have been reported in pears. Here, we established 73 EST-SSR markers from 10,350 unigenes collected from the Japanese pear 'Housui'. Since fewer than 400 pear EST sequences were available in the public databases, we tested many candidates for developing EST-SSR and SNP markers. We identified the genome positions of many EST-SSR sequences showing significant homology to *A. thaliana* genes. These will be useful as functional markers that are transferable between different genetic backgrounds, and will be suitable for relatively high-throughput genotyping for MAS (Celton *et al.* 2009, Chagné *et al.* 2008, Newcomb *et al.* 2006). They will be also useful for genetic diversity studies and association genetics.

The EST-SSRs showed high polymorphism, identified as

Table 1. Characteristics of 73 SSR markers derived from pear ESTs

SSR marker	DDBJ accession nos.	Forward primer (5'-3')	Reverse primer (5'-3')	Repeat motifs ^a	Motif type	Target size (bp) ^c	T _a (°C)	Single/ multilocus ^b	The origin of isolated libraries ^c
TsuENH001	AB450689	AAAGACGGCATTGACTGGATAGA	gtttctGATGCAAGAGACTTTTCGCCTATCT	(TC) _{16.5}	di	196	60	single	NLE2
TsuENH002	AB450690	CAGCAGGAAACACAGAAAACAG	gtttctATATCGAGCAATCAAGGAAGCAG	(GA) _{12.5}	di	116	55	single	NLE2
TsuENH003	AB450691	CAGAAATTCCTGCGAGGCTCA	gtttctCGTCGAGCAGAGAGAGAAATC	(TC) _{20.5}	di	121	55	single	NLE2
TsuENH004	AB450692	CGCAATTAAGTCTGGCTTCTTC	gtttctGAATGGCAGAGAGATTGATGG	(AC) _{9.5}	di	157	55	multi	NFR4, NLE2
TsuENH005	AB450693	GAGATTTCCCTCCCTCTCTCT	gtttctCTGAAAGCGATCTAGATGGATGA	(TC) _{20.5}	di	160	60	multi	NLE2
TsuENH006	AB450694	ATCAGAGGCTACTCCAATGGTGA	gtttctTGTTAAAGACAGAAAGCCCTTG	(GA) _{17.5}	di	134	55	single	NLE2
TsuENH007	AB450695	ATTCATTGCAACCGACTACCGATT	gtttctAGGGAGGAGATTAAAGTGGCGTAG	(CT) ₁₆	di	179	55	single	NLE2
TsuENH008	AB450696	CTGAGGTCTCATTCGGGTATCT	gtttctCCTCTCTGCTTTCTTCTTCAAG	(TC) ₁₅	di	156	55	s/m	NFW2
TsuENH009	AB450697	CCTCAGCCTTGATCATGCAC	gtttctGGAAAGTTGTGCTAGCAAGAAAGC	(CT) _{11.5} C(CT) ₁₀	di	157	55	single	NFW2
TsuENH011	AB450698	CGCTTATCCGTAAACTTCA	gtttctCAACGACAGTTTCAATAGGA	(AG) ₁₄	di	180	55	multi	NFR1
TsuENH012	AB450699	GAAGAAAGCAACCCATTTAGCTG	gtttctGAGTGAGAAAACGAAAGAAACG	(CT) _{7.5} A(CT) ₃	di	133	55	m/s	NFR1
TsuENH014	AB450700	ATTCCTACACTCAAAATCTTAT	gtttctTTTGTCTCTGCAATTTTAAAG	(AG) _{5.5} A(AG) ₃ A(AG) _{7.5}	di	101	50	single	NFR1
TsuENH016	AB450701	TCATTTTCATGGACTCTCAATCTCC	gtttctCGAGGAGTCTGCTGCGTCT	(GA) _{10.5}	di	140	60	s/m	NFR1
TsuENH017	AB450702	ACTTCAAGTAGCCAACTATCAG	gtttctGGCACTCTGTTTCTTATCAAC	(GA) ₁₆	di	188	60	single	NFR1, 2, 3
TsuENH018	AB450703	GTAAAGGGCGCAAAATGAAACAT	gtttctGAAAACGTTTGGTGCAATATCAGAC	(AAAT) ₃	tetra	167	55	multi	NFR2
TsuENH019	AB450704	CTCCGCTCACTCTCAGAAATTT	gtttctAAATGCTTGAAGTTGGTTGAGGTTT	(TC) _{20.5}	di	125	55	single	NFR2
TsuENH022	AB450705	CCAATTTCAGAAAGTTTACATAGGG	gtttctGGCCAAAGTGCAATGAGATGTAGT	(TC) _{20.5}	di	107	60	single	NFR5
TsuENH023	AB450706	TCTTCTTACAGGTCACAAACAGC	gtttctCTATGCGCTGCTTCTTGTGATGC	(AG) ₉	di	150	55	single	NFR3, NLE2
TsuENH025	AB450707	CACCTCCGTAAACCCCTCATAT	gtttctCTTACCCCAATCGAATCAAAAC	(CT) _{10.5}	di	200	55	single	NFR1-6, NFW2, 3, NLE2
TsuENH026	AB450708	ACTCCCAATCTGCCTTACAAACA	gtttctCGGTTGAGTGACCTCTTTCATTT	(CT) _{16.5}	di	165	55	single	NLE2
TsuENH028	AB450709	CAAAACAGAGGTATTTTGA	gtttctGATGGCGACTTTGATG	(ACAT) ₃ (AC) ₃ (ACAT) ₆	tetra	104	55	multi	NFW2
TsuENH029	AB450710	GGAAAGTTGTGTAGCAAAAGAACG	gtttctGCCTGTTTCCACCTTATGCTCACT	(CT) ₂₃	di	195	55	single	NFW2
TsuENH031	AB450711	CATTTCTTAGCTCCCTCCAGTT	gtttctGAAACCTTCTCTCTTCCCATCACTT	(TC) _{15.5}	di	131	55	single	NFR1
TsuENH032	AB450712	AACTGGAGGGAGCTAAGGAATG	gtttctCATCAAAACAAACTAGCCGAACC	(CT) ₁₇	di	135	55	single	NFR1
TsuENH033	AB450713	CCTGAGGTTATTGACCCAAAAGA	gtttctGGTGATACCTCACTCAGTTGGAAA	(TC) _{15.5}	di	162	55	single	NFR1
TsuENH034	AB450714	GCCCCCAATATTTCCTCATAT	gtttctTTGGGTGTTGAGTCAACGTGAG	(TA) ₁₃	di	198	55	single	NFR1
TsuENH035	AB450715	TTCTTCTGTGTGTGCTATGCTG	gtttctTTGGGGAGTTCTCACTATCATC	(AG) ₁₇	di	111	55	single	NFR1
TsuENH036	AB450716	TCCCTGCCAAACATTTTCTCTAC	gtttctTTCTCGCCAAAGAGGTATCATAA	(CT) ₁₇	di	146	55	single	NFR1
TsuENH040	AB450717	CATATTAGCCAGAGCTGCAGAT	gtttctGGCTCGAGAGTTCGAAAATTACA	(TA) ₁₂	di	193	55	single	NFR1, 5
TsuENH042	AB450718	AAAGCCGTACATTAGGCAAAAC	gtttctAAAAACTAGAACAGGCCACACAG	(TC) _{20.5}	di	145	55	multi	NLE2
TsuENH043	AB450719	CATCTGCGTCCGGCAAAAC	gtttctGCCCCCTGATATTCGGGAT	(TC) ₁₄	di	196	55	single	NLE2
TsuENH044	AB450720	GACAAATGGCTAAATACCTCTCTCG	gtttctGGCGACGAAAGTTGTGTAGATTA	(CAATC) ₅	penta	167	50	single	NLE2
TsuENH045	AB450721	ACTCAAGATTGGTCCCAAGAGCTG	gtttctCGGTACGGTCTCTCTGTTTTA	(CT) ₂₀	di	178	55	single	NLE2

^a motifs and target size refer to sequenced alleles.^b s/m and m/s denote single locus for Japanese pear/multi locus for European pear, respectively.^c NFR1, 2, 3, 4, 5, 6 were libraries from fruits of 2-3, 6-7, 11-12, 15-16, 20-21 (mature fruits), 23-24 (overmature fruits) week after full bloom, respectively. NFW1, NFW2, NFW3, NLE1 and NLE2 were libraries from flower bud, flower (balloon stage), open flower, leaf bud and leaf, respectively.

Table 1. (Continued)

SSR marker	DDBJ accession nos.	Forward primer (5'-3')	Reverse primer (5'-3')	Repeat motifs ^a	Motif type	Target size (bp) ^b	T _a (°C)	Single/ multilocus ^c	The origin of isolated libraries ^d
TsuENH046	AB450722	GGTCATCACCCACTTAAAAACCA	gtttttGTGCCCTGAAGTAATTGAGATGG	(TTC) ₅	tri	145	55	single	NFW1, NLE2
TsuENH048	AB450723	TGTCAGATCACCTTCACTTCCAA	gtttttGAGAACCCACGAGGTTGAACCT	(CAC) ₄	tri	158	50	pres single	NLE2
TsuENH049	AB450724	CATCAGCCTACGACACATACAC	gtttttGATTACGGCAACACGACACAGAT	(GCA) ₄	tri	184	55	multi	NLE2
TsuENH051	AB450725	GAAAGTTGGTTGAGAGGTTGAAGG	gtttttGGGGAACAACTAGAGACACAAA	(TCA) ₄	tri	112	55	pres single	NFW2, NLE2
TsuENH052	AB450726	CCAGCAGCCTCTTAATCAATA	gtttttAGTTGATGCTCTGCCAAGTCC	(CCA) ₅ , (CCT) ₅	tri	163	55	pres single	NLE2
TsuENH054	AB450727	CACCAACACAACTCAGTGGTA	gtttttGGATGCAAGAAACATCCATCAGT	(CAG) ₇	tri	191	55	s/m	NFW2
TsuENH056	AB450728	CAAGGTAAGGACATCACAGAGC	gtttttTCTCCTTTCTCTCGACCTCT	(CAC) ₄	tri	172	55	multi	NFW2
TsuENH057	AB450729	TGGTGTGATCTGCCATAGTTG	gtttttTCTCACTAAAGCTAACCCACCA	(TTC) ₅	tri	142	50	pre single	NFW2
TsuENH058	AB450730	AGAAAGAGGATAAGAAAGAGATGG	gtttttGTAAACGAAAGGAAACAGGACTTG	(GCCGCT) ₃	hexa	175	55	m/s	NFR6, NFW1, 2
TsuENH059	AB450731	GTGGACAGCAGTGACTCTACAA	gtttttCATGATAATGTGGCACCGTGTAT	(TGA) ₆	tri	109	55	pres single	NFW2
TsuENH060	AB450732	ATCTCCACCAAGAAACCTTACC	gtttttGGGGGTGAGGAATAGATGTAAACC	(ACC) ₅	tri	172	55	multi	NFW1, 2, NLE1
TsuENH062	AB450733	ACTCAGATCGTACGCAGAACAAA	gtttttCGATAAAGATCGATAATCTCATGCG	(CTG) ₆ (CCT) ₄	tri	199	55	single	NFW2, NLE1
TsuENH063	AB450734	CTAACGAACAGAAAGGAAACGA	gtttttAAATTTGGATGAGCTTGGTCTTGTG	(AGC) ₅	tri	189	55	single	NFR1
TsuENH064	AB450735	CTCCAAAACCATGTCCAGACC	gtttttCCGCTCTGGCTCTCTCTCTGTTTC	(CCT) ₄	tri	198	55	single	NFR1
TsuENH066	AB450736	GTGGTGGAGGTGCGTATTGAC	gtttttCGAGGAAAGTCCGACTCGT	(CCA) ₄	tri	194	55	pres single	NFR1
TsuENH067	AB450737	AAACTGAAACCCATTTCTCTGTC	gtttttATTCTTCTCCCAACGACAGAA	(GCT) ₄	tri	176	55	single	NFR1, NFR4
TsuENH068	AB450738	CCAAATTTCTCTCTCTCTCTGTT	gtttttTACAGTTATTTGCCGAAGCCAAAG	(ATG) ₄	tri	177	55	pres single	NFR2
TsuENH069	AB450739	TCTGCCACGTAAGCAGTAGTGA	gtttttCAATCACCATCTGGAGGGTTTAG	(TTG) ₃ (TTC) ₄ (TTG) ₂	tri	162	55	multi	NFR1, 2, 3, 4, 5, 6, NFW1, 3, NLE1, 2
TsuENH071	AB450740	GAATGATGAAGGCTGTGAGTT	gtttttGTATAGCGGGGGCAAGAAC	(CTTCTCT) ₃	hexa	199	55	single	NFR2
TsuENH072	AB450741	CTCGGGGAGAAATTTCAAAGAAATC	gtttttTCTCATTAATGGATGAACCCACCAC	(CCA) ₄	tri	124	55	pres single	NFR2
TsuENH074	AB450742	CAATCCATCATGTTGGTCTCTCTC	gtttttGTCCCCCACTGTGTAATCTTTC	(CTT) ₅	tri	183	55	single	NFR5
TsuENH076	AB450743	CATTAATACGCTGCTGTTCTGCG	gtttttACTTGAATTTGGGTAGGGATGTT	(ACG) ₆	tri	187	55	pres single	NLE2
TsuENH077	AB450744	CAACACCACATATATAGGCATCG	gtttttACAGAAATTAACAACCGTCCCTTG	(CCN) ₁₂	tri	176	55	pres single	NLE2
TsuENH079	AB450745	CGCGCTCTTTGGGAGAAAGT	gtttttGCAATGCTCTTTTACAGACGCTAC	(CCA) ₆	tri	192	55	single	NLE2
TsuENH080	AB450746	TGCTACCCCCAGCAGCATGAT	gtttttGCGATGTTGGATGAGAAGGTAGAA	(NCC) ₁₀	tri	131	55	single	NFW2, 3
TsuENH081	AB450747	GTCTCTCTCTCTCTCTCTCTCTCT	gtttttCCACCCCTGCTCAAAATCAGAGTA	(CGA) ₄	tri	191	55	pres single	NFR1, 4
TsuENH082	AB450748	CACCAGTACTCTGGAGGGTTTC	gtttttGTGCTCTCTGCAACATTTCTCC	(GCC) ₄	tri	108	55	pres single	NFR1, 3, 4, 6, NFW1, 2, NLE2
TsuENH083	AB450749	ACTCTCCGCAAAACAATGTCGTA	gtttttGTGAGAGTTTGAGGAGGAGAGC	(ACC) ₆	tri	154	55	single	NFR1
TsuENH085	AB450750	AAAAATGGCAGGAGAGAAAGAGG	gtttttCAAGTTGTCAGTGTCTCAGAAAG	(GCA) ₄	tri	105	55	pres single	NFR1, NFW2
TsuENH086	AB450751	CTCTGTCTGCTTCGATTTCTGCT	gtttttGTCCACGTTCCACCATTTTTCAGT	(AAG) ₆	tri	172	55	single	NFR1
TsuENH087	AB450752	CGCAGATGAAATCGGAAATCTTA	gtttttGTCTTCAACTTCAGCGCTGTTT	(TCC) ₄	tri	194	55	single	NFR1, NFW2
TsuENH089	AB450753	TTCACCTGCCCTTTTACGTAATGC	gtttttCCCCGACAACTCTGTAGAGAATCA	(CCACCG) ₂ (CCA) ₄	tri/hexa	160	55	pres single	NFR1, NFW2
TsuENH090	AB450754	ATCCGAAGTACTGTTCCCTCTCC	gtttttGTGATGTGGACTCTCCGCTTT	(CCT) ₅	tri	171	55	pres single	NFR1
TsuENH091	AB450755	ATAGTGGAGGAGCAACAAATCC	gtttttGAGGAAGAACTGTGCTCTCAGC	(TCT) ₄	tri	194	55	pres single	NFR2, 3, NFW1
TsuENH092	AB450756	CCATTTGATTTCCGATCTCTCTCT	gtttttGTGAAGGAAATAACGAAGCTGA	(ATG) ₄	tri	135	55	pres single	NFR2, 4, 6, NLE1
TsuENH093	AB450757	GTGGAGATTTTCCGAGTCAAAATG	gtttttAAATAAGACTGCTGAGGGAAATCCA	(TGC) ₄	tri	157	55	multi	NFR5
TsuENH094	AB450758	GCCCGAATAGTCCCAACAC	gtttttTCTCTCACTCGCTGCCATATTTA	(TAA) ₃ (GGA) ₃	tri	285	55	multi	NFR5
TsuENH096	AB450759	AGTGAAGAGAGAGGCGCTTGTT	gtttttGCTCTTCTGCTGCTCTCGAAATG	(GAG) ₇	tri	165	55	single	NFR5
TsuENH097	AB450760	CTGACACCCACTACGATTCAGA	gtttttAAACGAGCTTGTGTCGGAATACA	(CTC) ₇	tri	162	55	single	NFR4, NLE2
TsuENH098	AB450761	AAGACTGCAGCGGATCATAGTTC	gtttttCTCACTGCTTTGAAATCCAACT	(GCCACC) ₂ (GCC) ₂	tri/hexa	136	55	pres single	NLE2

^a motifs and target size refer to sequenced alleles.^b s/m and m/s denote single locus for Japanese pear/multi locus for European pear/multi locus for Japanese pear, respectively.^c pres single denotes "presumably single locus", which shows 1 or 2 amplified bands for all cultivars and whose locus is not identified in genetic linkage maps.^d NFR1, 2, 3, 4, 5, 6 were libraries from fruits of 2-3, 6-7, 11-12, 15-16, 20-21 (mature fruits), 23-24 (overmature fruits) week after full bloom, respectively. NFW1, NFW2, NFW3, NLE1 and NLE2 were libraries from flower bud, flower (balloon stage), open flower, leaf bud and leaf, respectively.^e T_a indicates annealing temperature.

Table 2. SSR sequences showing significant homology to *Arabidopsis thaliana* genes

SSR marker	Homologues of <i>Arabidopsis</i> gene name	ID nos.	E-value
TsuENH001	Ferredoxin-NADP(+) reductase, putative	At5g66190	3E-37
TsuENH002	WRKY family transcription factor	At1g13960	2E-14
TsuENH003	ADP-ribosylation factor, putative	At5g17060	6E-68
TsuENH004	Amino acid permease, putative (AUX1)	At2g38120	3E-21
TsuENH008	Smr (Small MutS Related) domain-containing	At2g26280	1E-11
TsuENH011	Expressed protein contains Pfam profile	At3g18215	6E-63
TsuENH012	Phosphoesterase family protein low	At3g48610	2E-82
TsuENH014	Phosphoglycerate/bisphosphoglycerate	At1g08940	3E-71
TsuENH017	Auxin-responsive AUX/IAA family protein	At4g29080	2E-31
TsuENH019	Tetratricopeptide repeat (TPR)-containing	At4g22670	1E-20
TsuENH022	Expressed protein	At3g03280	9E-09
TsuENH023	TATA box-binding protein	At5g22330	7E-64
TsuENH025	S-adenosylmethionine synthetase 1 (SAM1)	At1g02500	7E-16
TsuENH026	Expansin, putative (EXP5)	At3g29030	7E-23
TsuENH029	Squamosa promoter-binding protein-like	At3g15270	4E-31
TsuENH031	Dof-type zinc finger protein	At5g62940	1E-40
TsuENH032	Dof-type zinc finger protein	At5g62940	1E-40
TsuENH033	Developmental protein SEPALLATA1 / floral	At5g15800	1E-46
TsuENH034	WRKY family transcription factor	At4g31550	7E-45
TsuENH036	ABC transporter family protein	At3g25620	3E-52
TsuENH040	Leucine-rich repeat transmembrane protein	At1g09970	1E-23
TsuENH043	Succinyl-CoA ligase	At2g20420	3E-72
TsuENH044	Ubiquitin-conjugating enzyme 20 (UBC20)	At1g50490	8E-27
TsuENH048	4-hydroxyphenylpyruvate dioxygenase (HPD)	At1g06570	4E-30
TsuENH049	Expressed protein	At5g62090	5E-12
TsuENH051	60S ribosomal protein L5 similar to 60S	At3g25520	2E-20
TsuENH054	NHL repeat-containing protein	At1g70280	2E-33
TsuENH056	60S acidic ribosomal protein P2 (RPP2A)	At2g27720	4E-12
TsuENH057	Smr (Small MutS Related)	At2g26280	1E-11
TsuENH063	Expressed protein contains Pfam domain	At4g11860	2E-69
TsuENH064	Thioredoxin family protein	At3g16110	3E-29
TsuENH067	60S acidic ribosomal protein P0 (RPP0C)	At3g11250	2E-43
TsuENH068	GCIP-interacting family protein	At2g16860	8E-21
TsuENH071	Embryo-specific protein-related	At5g62200	3E-51
TsuENH074	Plastocyanin-like domain-containing	At1g08500	5E-43
TsuENH076	DEAD/DEAH box helicase, putative (RH10)	At5g60990	1E-38
TsuENH079	Proline-rich extensin-like family protein	At1g21310	8E-12
TsuENH080	C2 domain-containing protein	At3g16510	4E-11
TsuENH081	Thioredoxin family protein	At5g60640	8E-46
TsuENH082	Adenosylhomocysteinase, putative	At3g23810	3E-99
TsuENH083	DEAD/DEAH box helicase, putative	At2g47330	8E-11
TsuENH085	40S ribosomal protein S12 (RPS12C)	At2g32060	4E-48
TsuENH087	Cysteine protease inhibitor, putative	At3g12490	4E-63
TsuENH090	Expressed protein	At2g46000	1E-40
TsuENH091	Serine/threonine protein kinase, putative	At5g63650	6E-49
TsuENH092	High mobility group protein beta2	At1g20696	4E-30
TsuENH096	Heterotrimeric G protein gamma-subunit	At3g63420	9E-14
TsuENH097	Hydroxyacylglutathione hydrolase, putative	At1g53580	8E-61

high observed and expected heterozygosities. In our previous study, 13 pear genomic SSRs with di-nucleotide motifs showed $H_O=0.69$ and $H_E=0.71$ (Yamamoto *et al.* 2002a), values similar to those of the di-nucleotide EST-SSRs reported here. Inoue *et al.* (2007) reported that pear genomic SSRs with di-nucleotide motifs showed values of $H_O=0.54$ and $H_E=0.52$ among 17 Japanese pears. Fernandez-Fernandez *et al.* (2006) reported that genomic SSRs derived from Euro-

pean pear gave high of $H_O=0.67$ and $H_E=0.83$ among 21 *Pyrus* accessions. These results suggest that EST-SSRs with di-nucleotide motifs have high heterozygosity, similar to genomic SSRs.

We found higher polymorphism among EST-SSRs with di-nucleotide repeat motifs than among those with tri-nucleotide repeats. Higher polymorphism was found among di- and tetra-nucleotide repeats than tri-nucleotide repeats in

Table 3. Characteristics of EST-SSRs applied for Japanese and European pears and related genera

SSR marker	Motif type	Japanese pear			European pear			Amplification in ^b	
		No. of alleles	H_E^a	H_O^a	No. of alleles	H_E^a	H_O^a	<i>Malus</i>	<i>Prunus</i>
TsuENH001	di	4	0.68	0.70	5	0.64	0.50	+	+
TsuENH002	di	3	0.46	0.60	3	0.63	0.80	+	+
TsuENH003	di	3	0.51	0.50	6	0.79	1.00	–	–
TsuENH006	di	3	0.64	0.70	2	0.10	0.10	+	–
TsuENH007	di	5	0.59	0.70	1	0	0	+	–
TsuENH008	di	6	0.70	0.40	(multi)			+	–
TsuENH009	di	6	0.79	1.00	8	0.84	0.90	–	–
TsuENH012	di	(multi)			3	0.61	0.90	+	–
TsuENH014	di	2	0.42	0.60	3	0.46	0.60	–	–
TsuENH016	di	4	0.47	0.50	(multi)			+	+
TsuENH017	di	4	0.41	0.20	3	0.64	0.80	+	–
TsuENH019	di	3	0.58	0.70	4	0.73	0.90	+	+
TsuENH022	di	5	0.68	0.70	3	0.41	0.50	+	–
TsuENH023	di	3	0.52	0.40	4	0.53	0.70	+	–
TsuENH025	di	3	0.54	0.70	6	0.73	0.90	+	+
TsuENH026	di	6	0.78	0.70	7	0.83	1.00	–	–
TsuENH029	di	6	0.79	1.00	8	0.84	0.90	–	–
TsuENH031	di	4	0.68	0.60	7	0.82	0.80	+	+
TsuENH032	di	6	0.80	0.80	5	0.78	0.60	+	+
TsuENH033	di	5	0.79	0.90	6	0.80	0.90	+	–
TsuENH034	di	4	0.70	0.80	7	0.83	0.40	+	–
TsuENH035	di	5	0.53	0.60	6	0.75	1.00	+	–
TsuENH036	di	5	0.72	0.90	4	0.41	0.50	+	–
TsuENH040	di	4	0.68	0.90	4	0.63	1.00	+	+
TsuENH043	di	4	0.75	0.50	4	0.65	0.70	+	+
TsuENH045	di	5	0.71	0.70	4	0.71	0.40	+	–
(average)		(4.32)	(0.63)	(0.67)	(4.71)	(0.63)	(0.70)		
TsuENH046	tri	2	0.18	0.20	4	0.62	0.60	+	–
TsuENH048	tri	3	0.49	0.50	1	0	0	+	+
TsuENH051	tri	2	0.10	0.10	1	0	0	+	–
TsuENH052	tri	2	0.42	0.40	3	0.51	0.30	+	–
TsuENH054	tri	3	0.27	0.30	(multi)			+	–
TsuENH057	tri	2	0.18	0.00	(multi)			–	–
TsuENH059	tri	1	0	0	1	0	0	+	–
TsuENH062	tri	2	0.50	0.70	5	0.68	0.90	+	–
TsuENH063	tri	2	0.50	0.70	2	0.10	0.10	+	–
TsuENH064	tri	2	0.50	1.00	5	0.68	1.00	+	–
TsuENH066	tri	2	0.26	0.30	1	0	0	+	–
TsuENH068	tri	2	0.10	0.10	2	0.38	0.50	+	–
TsuENH071	tri	1	0	0	1	0	0	–	–
TsuENH072	tri	1	0	0	1	0	0	+	+
TsuENH074	tri	4	0.68	0.90	1	0	0	+	–
TsuENH076	tri	3	0.64	1.00	4	0.65	0.70	+	+
TsuENH077	tri	1	0	0	2	0.32	0.40	+	–
TsuENH079	tri	2	0.18	0.20	2	0.10	0.10	+	–
TsuENH080	tri	3	0.27	0.20	3	0.34	0.40	+	–
TsuENH081	tri	1	0	0	1	0	0	+	–
TsuENH082	tri	1	0	0	1	0	0	+	+
TsuENH083	tri	3	0.59	0.60	3	0.61	0.50	+	–
TsuENH085	tri	1	0	0	1	0	0	+	–
TsuENH086	tri	3	0.58	0.80	4	0.62	0.40	–	–
TsuENH087	tri	3	0.40	0.40	1	0	0	+	–
TsuENH090	tri	2	0.10	0.10	2	0.10	0.10	+	–
TsuENH091	tri	1	0	0	1	0	0	+	+
TsuENH092	tri	1	0	0	1	0	0	–	–
TsuENH096	tri	1	0	0	3	0.67	0.90	+	–
TsuENH097	tri	1	0	0	3	0.54	0.50	–	–
TsuENH058	hexa	(multi)			2	0.10	0.10	+	+
TsuENH067	hexa	1	0	0	2	0.42	0.60	+	+
TsuENH089	hexa	2	0.10	0.10	6	0.55	0.40	+	–
TsuENH098	hexa	1	0	0	1	0	0	+	–
(average)		(1.88)	(0.21)	(0.26)	(2.22)	(0.25)	(0.27)		
TsuENH044	penta	4	0.58	0.70	3	0.54	0.70	+	–

^a H_E and H_O denote expected and observed heterozygosities, respectively.^b Cross genus amplification in *Malus* and *Prunus* is denoted as follows; '+', good amplification; '–', no amplification.

Fig. 1. Genetic linkage maps of 'Bartlett', 'La France', and 'Housui'. Linkage groups are designated Ba1–17 for 'Bartlett', La1–17 for 'La France' and Ho1–17 for 'Housui'. Pear EST-SSR loci are underlined; pear genomic SSR loci are italicized.

apple (Celton *et al.* 2009), because di- and tetra-nucleotide repeats were usually found at higher frequency in 3' and 5' untranslated regions of genes, whereas tri-nucleotides were preferentially found in translated regions (Morgante *et al.* 2002). This uneven distribution of SSR motifs may be a result of mutation pressure and positive selection for specific single amino acid stretches. Yet although tri-nucleotide EST-SSRs may show low polymorphism, they can provide more precise allele identification, because tri-nucleotide SSRs generally give fewer "stutter bands" than di-nucleotide SSRs (Diwan and Cregan 1997).

Integrated reference genetic linkage maps based on SSR and AFLP markers have been constructed in pears (Yamamoto *et al.* 2004, 2007, Terakami *et al.* 2009). Here, the distribution of the 57 new EST-SSR loci across all 17 LGs, could improve pear reference genetic linkage maps: Although more than 100 pear genomic SSR loci have been mapped, genomic SSRs remain sparse in several regions. We found some EST-SSR loci in such sparse regions, including LG 6, the bottom of LG 1, and the bottom of LG 15. In apple, 36% of EST-SSRs were mapped, a lower ratio than that of genomic SSRs, and some EST-SSRs could be mapped in regions where genomic SSRs are sparse, such as LG 1 (Celton *et al.* 2009).

More than 80% of the pear EST-SSR markers could be transferred from pear to apple, which belongs to the same subfamily (Maloideae). The transferability and positional conservation of SSR markers suggest a syntenic relationship between pear and apple (Yamamoto *et al.* 2004, 2007, Pierantoni *et al.* 2004, Celton *et al.* 2009). Our new pear EST-SSR markers pave the way for the development of markers for the same phenotypic traits between pear and apple.

Fewer than 30% of pear EST-SSR markers were transferable to almond and peach, in the subfamily Prunoideae. Liebhard *et al.* (2002) reported that among 15 (AG)_n microsatellite markers developed in apple, only one amplified scorable bands in the Prunoideae. About half of 40 (AG)_n peach microsatellites gave amplification in both *Prunus* and either *Malus* or *Fragaria* (Dirlewanger *et al.* 2002). Nishitani *et al.* (2007) reported that 40% to 51% of peach tri-nucleotide SSR markers amplified fragments in *Malus*, *Pyrus*, *Fragaria*, and *Rosa*. Although the transferability of pear EST-SSRs to other subfamilies is not high, functional EST markers in pear will lead to the development of universal gene-specific markers in mapping and syntenic studies within the Rosaceae.

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