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Original article

Development and validation of polymorphic EST-SSR markers for genetic diversity analysis in *Actinidia arguta*

Haiyue Sun, Jiahui Wang, Li Chen, Jian Xu and Yadong Li[a]

Engineering Center of Genetic Breeding and Innovative Utilization of Small Fruits of Jilin Province, College of Horticulture, Jilin Agricultural University, Changchun 130118, P.R. China

Summary

Introduction – **Hardy kiwifruit (*Actinidia arguta*) is the hardiest species in the *Actinidia* genus and has been recognized as having great development potential in the fruit industry.** *Materials and methods* – **In total, 132,593 *Actinidia* expressed sequence tags (ESTs) were downloaded from the NCBI dbEST database. Among the consensus sequences, 5,755 (4.34%) were found to contain 6,413 SSRs. Dinucleotide, trinucleotide and hexanucleotide repeats respectively accounted for 64.70%, 14.14% and 10.73% of the 6,413 SSRs. Out of 199 newly designed EST-SSR primers, 141 primer pairs successfully generated expected bands and 110 primer pairs showed polymorphism.** *Results and discussion* – **The allele number ranged from 1 to 6 with an average of 2.35 and the PIC value varied from 0.50 to 0.94 with a mean of 0.70. Cluster analysis classified 36 accessions into seven sub-clusters comprising 16, 13, 1, 2, 2, 1 and 1 accessions.** *Conclusion* – **The EST-SSR markers developed in the present study could be useful to accelerate breeding programs in *A. arguta.***

Keywords

China, hardy kiwifruit, *Actinidia arguta*, genetic diversity, molecular markers

Significance of this study

What is already known on this subject?

- Hardy kiwifruit is a fruit tree with great commercial potential. EST-SSR markers can analyze genetic diversity and phylogenetic relationships in plants.

What are the new findings?

- A set of 110 polymorphic EST-SSR markers was developed to characterize *A. arguta* germplasm resources; 36 accessions were clustered into 7 sub-clusters by these markers.

What is the expected impact on horticulture?

- An effective EST-SSR marker system will identify *A. arguta* germplasm resources at the molecular level and promote *A. arguta* breeding.

Introduction

Kiwifruits (*Actinidia* spp.) are economically important fruit trees and lianas that are extraordinarily genetically and morphologically diverse (Ferguson and Huang, 2007). Approximately 55 climbing species are distributed in China (Liang, 1983). Among them, hardy or mini kiwifruit (*A. arguta*) is mainly grown across Northeastern China. *A. arguta* is a perennial dioecious liana and the hardiest species in the *Actinidia* genus, surviving winter temperatures as low as -40 °C. In contrast to other *Actinidia* species, the green-fleshed fruits of *A. arguta* have edible skins. In recent years, *A. arguta* has received increasing attention, particularly for its high nutrient contents (Zhang *et al.*, 2003; Liu *et al.*, 2010; Zuo *et al.*, 2012; Leontowicz *et al.*, 2016). Because *A. arguta* fruit is rich in nutritious compounds, it is a good material for soft drinks and jams. It is also used as an analgesic, diuretic and thirst-quencher (Liang, 1983).

Although kiwifruits are commercially cultivated in approximately 24 countries (FAO, 2016), superior *A. arguta* cultivars are relatively rare and most germplasm resources are directly obtained from the wild without improvement (Ferguson and Huang, 2007; Latocha, 2007). Thus, it is essential to carry out genetic analysis of natural populations to select core germplasms from wild *A. arguta.* Morphological characterization has been traditionally used to study the genetic variation of *A. arguta,* but it is a time-consuming, laborious and even ambiguous process due to the effects of environmental factors and plant developmental stages on trait expression. The advantageous characteristics of simple sequence repeat (SSR) markers, such as their stable reproducibility, high number of alleles, codominant inheritance and good chromosomal coverage, facilitate their low-cost, rapid application to investigate the origins, evolution and genetic diversity of plants (Tautz and Renz, 1984; King, 1994; Kashi *et al.*, 1997; Röder *et al.*, 1998a, b; Kalia *et al.*, 2011). Compared with genomic SSRs (gSSRs), expressed sequence tag (EST)-SSRs show less polymorphism, but are better conserved across species (Caudrado and Schwarzacher, 1998). Thus, they reflect gene diversity directly and are easily transferred across related species. Because of the development of sequencing technologies, massive EST sequences are available from public sequence databases. EST-SSR markers have become an efficient and fast tool for estimating genetic diversity, analyzing population structure, and mapping functional genes. Recently, some SSR markers have been developed for *Actinidia* species (Fraser *et al.*, 2004; Korkovelos *et al.*, 2008; Man *et al.*, 2011; Kwon *et al.*, 2013; Lai *et al.*, 2015; Guo *et al.*, 2017), but the number of effective SSR markers is still limited.

In the present study, we surveyed the characteristics of SSRs in *Actinidia* EST sequences from public databases, and developed new EST-SSR markers that can be used in genetic diversity analysis of *A. arguta* germplasm resources. The ad-

[a] Corresponding author: blueberryli@163.com.

ditional markers should facilitate genetic selection of *A. arguta* germplasm resources in future breeding programs.

Materials and methods

Plant material

A sample of 36 *A. arguta* strains were grown at the experimental base of the Institute of Special Animal and Plant Sciences of the Chinese Academy of Agricultural Science (Table 1).

Genomic DNA extraction

Genomic DNA was extracted from fresh leaves of female plants following the modified CTAB method (Doyle *et al.*, 1987). The DNA quality and concentration were checked on a 0.8% agarose gel and a NanoPhotometer instrument, respectively (Implen, Germany). The DNA concentration of all samples was adjusted to 100 ng μL^{-1} for PCR amplification.

Mining of EST data and identification of SSR loci

Actinidia EST sequences were downloaded from the National Center for Biotechnology Information (NCBI) dbEST database. The CAP3 assembler software was used to remove redundant EST sequences (Huang *et al.*, 1999). The software SSRIT (http://www.gramene.org/db/markers/ssrtool) was used to mine SSRs. The selection criteria for SSR motifs were as follows: dinucleotide, at least 7 repetitions; trinucleotide, at least 5 repetitions; tetranucleotide, at least 4 repetitions; pentanucleotide and hexanucleotide, at least 3 replications. Complementary repeat sequences were classified as the same repeat type; for example, AG and GA repeats (Crowhurst *et al.*, 2008).

Primer design and PCR amplification

The PRIMER 5.0 software was used to design primer pairs for SSRs with at least 50-bp flanking regions. The following parameters were used to screen primers: PCR amplification product length, 150–400 bp; primer size, 18–24 bp; GC content, 40–60%, optimum 50%; T_m, 40–65 °C; and T_m difference between forward and reverse primer, less than 5 °C. These criteria were relaxed in some cases. Primers were commercially synthesized by Genewiz Inc., Suzhou, China.

Four *A. arguta* accessions, namely '8134', '9701', 'T9-4-1' and '13-3-1', were selected to test the primer suitability by PCR amplification. Each PCR reaction was mixed in a total volume of 20 µL, including 40 ng genomic DNA, 2 µL 10× *Ex Taq* Buffer (Mg^{2+} plus), 0.4 µL dNTP mixture (each 2.5 mM), 0.8 µL primers (10 µM, forward and reverse), and 0.5 U *Ex Taq* polymerase (TaKaRa). The PCR reactions were carried out on a Veriti™ Thermal Cycler (Applied Biosystems, USA) with the conditions: 94 °C for 2 min for initial denaturation, followed by 35 cycles of 95 °C for 30 s, optimal T_m for 30 s (range 42–65 °C), and 72 °C for 30 s, and then 72 °C for 5 min for final extension. Amplified products were resolved on 6% native polyacrylamide gels. The gels were visualized by silver staining and photographed using Image System (UVP, USA).

Data analysis

EST-SSR amplification bands were recorded as presence "1" or absence "0" and scored by allele size. The NTSYS-pc version 2.10e software (Applied Biostatistics, Setauket, NY, USA) was used for data analysis. Dendrograms and genetic similarity coefficients were generated from the binary data matrix by the UPGMA method. The polymorphism information content (PIC) value of each primer was based on the formula $PIC = 1 - \Sigma Pi^2$, where Pi is the frequency of the i^{th} allele.

Results and discussion

Characterization of SSR loci in *Actinidia* ESTs

In total, 132,593 *Actinidia* ESTs were downloaded from NCBI dbEST and assembled into 27,101 consensus sequences comprising 14,697 contigs and 12,404 singlets. Of these consensus sequences, 5,755 (4.34%) were found to contain 6,413 SSR loci. Among the consensus sequences containing SSR loci, 4,260 sequences contained only one SSR locus, 785 sequences contained two SSR loci and 177 sequences contained three or more. Consequently, 21.2% of these consen-

Table 1. Accessions of *Actinidia arguta* used for diversity analysis.

No.	Accessions	Types	Places or origin	No.	Accessions	Types	Places of origin
1	Feng Lv	Cultivated	Ji'an, Jilin	19	6-4-3	Wild	Zuojia, Jilin
2	Kui Lv	Cultivated	Ji'an, Jilin	20	11-10-1	Wild	Zuojia, Jilin
3	8134	Breeding line	Zuojia, Jilin	21	11-10-2	Wild	Zuojia, Jilin
4	8401 (Jia Lv)	Breeding line	Huanren, Liaoning	22	13-6-2	Wild	Zuojia, Jilin
5	9701	Breeding line	Zuojia, Jilin	23	18-5-1	Wild	Zuojia, Jilin
6	63-8 (Xin Lv)	Cultivated	Zuojia, Jilin	24	2-2-3	Wild	Zuojia, Jilin
7	T 4-5-2	Wild	Zuojia, Jilin	25	4-10-2	Wild	Zuojia, Jilin
8	T 5-3-1	Wild	Zuojia, Jilin	26	4-1-3	Wild	Zuojia, Jilin
9	T 5-5-3	Wild	Zuojia, Jilin	27	6-2-3	Wild	Zuojia, Jilin
10	T 6-4-1	Wild	Zuojia, Jilin	28	8-3-1	Wild	Zuojia, Jilin
11	T 8-3-3	Wild	Zuojia, Jilin	29	10-1-1	Wild	Zuojia, Jilin
12	T 9-2-2	Wild	Zuojia, Jilin	30	10-7-3	Wild	Zuojia, Jilin
13	T 9-3-3	Wild	Zuojia, Jilin	31	10-8-1	Wild	Zuojia, Jilin
14	T 9-4-1	Wild	Zuojia, Jilin	32	12-1-1	Wild	Zuojia, Jilin
15	T 9-8-1	Wild	Zuojia, Jilin	33	12-5-1	Wild	Zuojia, Jilin
16	T 9-8-3	Wild	Zuojia, Jilin	34	12-6-1	Wild	Zuojia, Jilin
17	S 8-3-2	Wild	Zuojia, Jilin	35	14-6-2	Wild	Zuojia, Jilin
18	S 9-2-1	Wild	Zuojia, Jilin	36	13-3-1	Wild	Zuojia, Jilin

Table 2. Frequency of different types of repeat motif among *Actinidia* EST-SSRs.

Repeat types	Repeat motifs	Number	Total number of each repeat type	Frequency (%)
Dinucleotide	AC/CA/GT/TG	175	4,149	4.22
	AG/GA/CT/TC	3,694		89.03
	AT/TA	276		6.65
	CG/GC	4		0.10
Trinucleotide	AAC/ACA/CAA/GTT/TGT/TTG	55	907	6.06
	AAG/AGA/GAA/CTT/TCT/TTC	241		26.57
	AAT/ATA/TAA/TTA/TAT/ATT	36		3.97
	ACC/CAC/CCA/GGT/GTG/TGG	197		21.72
	ACG/CGA/GAC/CGT/GTC/TCG	49		5.40
	ACT/CTA/TAC/AGT/TAG/GTA	12		1.32
	AGC/CAG/GCA/TGC/CTG/GCT	78		8.60
	AGG/GGA/GAG/TCC/CTC/CCT	96		10.58
	ATC/CAT/TCA/GAT/ATG/TGA	61		6.73
	CCG/CGC/GCC/GGC/GCG/CGG	82		9.04
	AAAC/CAAA/GTTT/TTTG	9		3.77
	AAAG/CTTT/TCTT/TTCT/TTTC	20		8.37
Tetranucleotide	AAAT/AATA/ATAA/TAAA/ATTT/TATT/TTAT/TTTA	65	239	27.20
	AATT/TTAA/ATAT/TAAT	4		1.67
	ACAG/AGAC/TGTC/TCTG	9		3.77
	ACTC/ATCC/CTCA/TCCA/CATC/GTAG	8		3.35
	AGAT/ATAG/GATA/ATCT/CTAT/TATC/TCTA	51		21.34
	AGCG/CGAG/GCGA/GAGC/CTCG/CTGC	9		3.77
	AGCT/CAGT/CGAT/CTAG/GATC/GCTA/GTCA/TAGC/TCGA	17		7.11
	AGGA/TCCT/CTCT/TCTC/CTTC	8		3.35
	ATAC/CATA/TATG	8		3.35
	ATGT/GATT/TTGA/TACA	6		2.51
	CACC/CCCA/TTGT	3		1.26
	CACG/CTGG	2		0.84
	CCAA/TGGT	4		1.67
	CCTC/CTCC/TCCC/GAGG	11		4.60
	CGCC	2		0.84
	GCTT/TGCT	3		1.26
	AAAAC/AAACA/AACAA/CAAAA/GTTTT/TGTTT/TTTGT/TTGTT/TTTTG	32		7.44
	AAAAG/AAAGA/AGAAA/CTTTT/TCTTT/TTCTT/TTTCT/TTTTC	52		12.09
	AAAAT/AAATA/ATAAA/TAAAA/ATTTT/TATTT/TTATT/TTTAT/TTTTA	74		17.21
Pentanucleotide	AAACC/AACCA/ACCAA/CCAAA/GGTTT/TGGTT/TTGGT/TTTGG/GTTTG/TGTGT	22	430	5.12
	AAATC/AATCA/ATAAC/CAATA/TAAAC/GATTT/TTGAT/TTTGA/ATTTG/GTTTA	18		4.19
	AAATG/TGAAA/AAGAT/GAAAT/TTTCA/TTCAT/ATTCT/ATTTC/CTTTA	10		2.33
	AAATT/TAAAT/AATAT/ATATA/TAATA/TATAA/TTTAA/TATAT/TTATA	11		2.56
	AACCC/CAACC/CCCAA/CACCA/CACAC/GGGTT/TTGGG/GTGGT/GTTGG	11		2.56
	AACTG/AATCG/AATGC/ATCGA/ATAGC/ACTAG/AGATC/TCGAA/GATTC/CTGAA/TCTGA/CTTAG/ATGCT	17		3.95
	AAGCA/AGCAA/AAGAC/CAAAG/GAAAC/TCGTT/TTCTG/TGTTC/TCTGT/TTTGC/TTTCG/GCTTT	13		3.02
	AAGGA/AAGAG/AGAAG/GAAGA/AGAGA/TCTTC/TCTCT/TTCCC/TTCTC	22		5.12
	AAGGG/GGAAG/GAGGA/GAGAG/CTCTC/CCTCT/CCTTC/CTCCT/TCCTC/CTTTC	25		5.81
	AAGGT/AAGTG/ATGGA/GAAGT/TGAAG/TCCAT/TTCAC	7		1.63

Repeat types	Repeat motifs	Number	Total number of each repeat type	Frequency (%)
	AATTC/ATCTA/TGAAT/TGTAA/TTGAA/AGATT	9	430	2.09
	ACACG/AGCAC/CAGAC/CCAAG/CGACA/GAACC/CGGTT/ CGTTG/TGGTC/TTGGC/TTCGG	11		2.56
	ACCCG/AGCCC/CCCGA/GACCC/CACGC/CAGCC/GGGCT/ GGGTC/TGGGC/GTCGG/GGTCG	13		3.02
	AGAGC/GCTCT/CGTCT/CTGCT/TCTGC/TCTCG	8		1.86
	AGGCC/AGCCG/CGGCA/CGGAC/GGCAC/GCCGT/CCGTG/ GTCCG	12		2.79
	AGTCC/ATCCG/CCGAT/GATCC/CGCAT/CGTAC/CTGAC/GGACT/ CGAGT/CTGAG/GATCG/GAGCT	18		4.19
	AGTTG/ATGTG/GATTG/TGATG/CAATC/TAACC/CACAT	9		2.09
	CATCC/CCCAT/CTACC/GAGGT/GTGGA	6		1.40
	CCCCA/TGGGG/CCACC/GTGGG	9		2.09
	CCCCT/TCCCC	2		0.47
	CCCGC/GCCCC/GGGCG	9		2.09
	CCGGG/CGGGC/GCCCG/CGCCG/GC GCC	6		1.40
	GCGAG/TCGCC/CTGCC/GCCCT	4		0.93
Hexanucleotide	AAAAAC/AAAACA/AACAAA/ACAAAA/CAAAAA/GTTTTT/TGTTTT/ TTGTTT/TTTGTT/TTTTGT/TTTTTG	25	688	3.63
	AAAAAG/AAAAGA/AAAGAA/AAGAAA/GAAAAA/CTTTTT/TCTTTT/ TTCTTT/TTTCTT/TTTTCT/TTTTTC	24		3.49
	AAAAAT/AAAATA/AAATAA/AATAAA/ATAAAA/ATTTTT/TAAAAA/ TATTTT/TTTTAT/TTTTTA	27		3.92
	AAAACC/AAACCA/ACCAAA/CAAAAC/CACAAA/GGTTTT/ TGTGTT/TTGGTT/TTTTGG	10		1.45
	AAAATC/AACAAT/AATAAC/ATCAAA/ATTGTT/CAAAAT/TGTATT/ TTGTTA/TTTTGA	10		1.45
	AAAATT/AATTTT/ATATAA/ATATTT/ATTAAA	6		0.87
	AAACCC/AACACC/ACACCA/CAACCA/CCAAAC/CCACAA/ CCCAAA/GGAAGA/GGAGAA/GGGTTT/GGTGTT/GGTTTG/ GTGGTT/GTGTTG/GTTGTG/TGGGTT/TGGTGT/TGTTGG/ TTGGGT	32		4.65
	AAACCG/AACCAG/AAGCCA/AGCAAC/CAACAG/CAGACA/ CAGCAA/GAACCA/GCAACA/GTTTGC/TCGGTT/TGCTGT/ TGGTTC/TTGCTG/TTTGGC	21		3.05
	AAAGGA/AAGGAA/AGAGAA/AGGAAA/CTTTCT/CTTTTC/ GGAAAA/TCCTTT/TCTCTT/TTCCTT/TTTCCT	13		1.89
	AAATGA/ATGAAA/GAAAAT/TGAAAA/ TTTCAT	5		0.73
	AACCCT/ACCACT/ACCCAT/ACCTCA/CATCAC/CCAACT/ CCAATC/CCCAAT/CCCTAA/CCTAAC/CTACCA/CTCCAA/ GGGTTA/GTATGG/GTGATG/TAGGGT/TCCACA/TGGTGA/ TTGGAG	37		5.38
	AAGAGG/AAGGAG/AGAAGG/AGAGGA/AGGAAG/AGGGAA/ CTTCCT/GAAGGA/GAGGAA/TCTCCT/TCTTCC/TTCTCC	17		2.47
	AAGATG/AATGGA/AGATAG/AGATGA/ATCTCT/CATCTT/CTTTCA/ GAAATG/GAAGAT/GATGAA/TACTTC/TCTATC/TCTCTA/TTCCAT	19		2.76
	AAGCCC/ACCACG/ACCAGC/AGCCCA/CACACG/CAGCAC/ CAGCCA/CCAACG/CCCAAG/GCACCA/GGGTCT/GGGTTC/ GGTCGT/GTGGCT/TCTGGG/TGGGTC/TGGTGC/TTGGGC	23		3.34
	AATACT/ATACAT/ATATTG/ATTCAA/GATTAT/TACAAT/TATATG/ TCAAAT/TTGTAA	10		1.45
	AATAGC/CTGTAT/GAAATC/GAATCA/GATCAA/GTTTCA/TCAGAA/ TCAGTT/TCGATT/TGATTC/TGTTCA/TTCGTA	14		2.03
	AATGGG/ACTCCT/AGTGGA/ATGGGA/CCATTC/CTACTC/ CTCCAT/CTCTCA/GAGAGT/GAGGAT/GATGAG/GGAGAT/ GGTGAA/GTGAAG/TCACCT/TCATCC/ T CTCAC/TGAAGG/TGAGAG	23		3.34

Repeat types	Repeat motifs	Number	Total number of each repeat type	Frequency (%)
	AATGTG/AATTCC/ATACCT/CCAATT/CT AACT/CTAATC/GTGATA/ TCACAT/TCCATA/TCTCAA/TGAGAT/TGGATA	16		2.33
	ACCCAC/ACCCCA/CAACCC/CACCCA/CCCACA/CCCCAA	10	688	1.45
	ACCCTC/CACCCT/CACCTC/CATCCC/CCATCC/CCCCAT/ CCCTCA/CCTCCA/GAGGTG/GATGGG/GGAGGT/GGGTGA/ GTAGGG	18		2.62
	ACGAAA/AGACAA/CAGAAA/GTTTCT/GTTTTC/TTGTTC/TTTCGT/ TTTTGC	14		2.03
	ACGAAG/AGAAGC/AGAGAC/AGCAAG/AGCAGA/AGGACA/ CAGAGA/CAGGAA/CCTGTT/CGAAGA/CTCTGT/GAACAG/ GAAGCA/GACAAG/GACGAA/GGAAAC/TCGTCT/TCTCTG/ TCTGTC/TCTTCG/TTCGTC	32		4.65
	ACGCCC/CACCGC/CCAGCC/CCCAGC/CCCCAG/CCGCCA/ CGCCAC/CGGTGG/GCCACC/GCCCCA/GGCGGT/GGTGGC/ TGGCGG	23		3.34
	ACGCCG/CCGGCA/CGACGC/CGCGAC/CGTGGC/GCCGAC/ GCCGCA/GCGTCG/GGCGCT/TCGGCG	11		1.60
	ACGGCG/AGGCCG/CACGGG/CCCGTG/CCGGCT/CCGTGC/ CGACGG/CGCCGT/CGTGCC/GCAGCG/GCCCGT/GCCGTC/ GCGACG/GGAGCC/GGCCAG/GGCTCC/TCGCCG	21		3.05
	AGAGCG/AGAGGC/AGCAGG/CAAGGG/CAGGAG/CCCTGT/ CCTCTG/CCTGCT/ CCTTGC/CGCTTC/CGTCCT/CGTCTC/ CTCCTG/CTCGCT/CTCTCG/GACGAG/GAGAGC/GAGGAC/ GCTCCT/GCTTCC/GGAGAC/TCGCTC/TCTCCG/TCTCGC	32		4.65
	AGAGGG/CCCTCT/CCTCTC/CTCCCT/CTCTCC/GAGAGG/ GAGGGA/GGAGAG/GGGAGA/TCCCCT/TCCCTC/TCTCCC	37		5.38
	AGATAT/ATATAG/ATATCT/ATCTAT/ATGATA/TATATC/TATCTA/ TCTATA/TTTCAA	12		1.74
	AGCTCC/CACTCG/CATCCG/CATCGC/CCCTAG/CCGATC/ CCGTAC/CGACTC/CG ATGG/CGCCAT/CTCCGA/CTGGAG/ GACCTC/GAGCTG/GCACCT/GCCCAT/GCCTCA/GCTCCA/ GGAGCT/GGCATG/GGCGAT/GGGATC/GTCAGG/TCACCG/ TGACGG	27		3.92
	AGGTTC/ATTCGG/CAACTG/CAGGTT/CATGAC/CGATCA/ GATGCT/GGATTC/GTGATC/TAGCCA/TCATGG/TCTGAG/ TGGTCA	14		2.03
	ATATAT	1		0.15
	ATCGGA/ATCTCG/ATGGCA/ATTCCG/CAATGG/CAGTAT/ CATCGT/CCGATT/CCTGTA/CGATTC/CTGTAC/GACGAT/ GATAGC/GATCGA/GCGAAT/GCTACT/GCT GAA/GTCACT/ GTCATC/TCATCG/TCCGAT/TCGAGA/TCGGAA/TGCTAC/ TGGAAC/TTCGAC	29		4.22
	ATCGTA/ATGCAT/GAATTC/GATTCA/GCATTA/TTCAGA/TGAATC/ TTGAAC	10		1.45
	ATGGCC/CAGGCT/CGAGCT/CTGAGC/GACCGT/GAGTCC/ GCCGAT/GGACTC/GGCACT/GTCGAC/TCACGG/TGAGCC/ TGCCAG	14		2.03
	CAACGG/CACAGG/CAGAGC/CGGCAA/GCAAGC/GCCGAA/ GCGAAC/TCCGTG	10		1.45
	CACATA/CCAAAT/CCATAA/CTACAA/GGATTT/TGGATT/TGGTTA/ TTATGG/TTTGGA	11		1.60
	CACCCC/CCACCC/CCCACC/CCCCAC/CCCCCA/GGGTGG/ GTGGGG	12		1.74
	CCCCCT/CCCTCC/CCTCCC	3		0.44
	CCCGCT/CCGCCT/CGCCTC/CTCCGC/GAGGCG/GCCTCC/ GCGGAG/GGAGGC/GTCCCC/TCCGCC	12		1.74
	CCCGCC	1		0.15
	GCCCGC	1		0.15
	GCCTGT	1		0.15

sus sequences contained at least one SSR locus. With regards to SSR types, dinucleotides represented the highest proportion of SSRs, followed by trinucleotides and hexanucleotides (Table 2; Figure 1A). AG/CT (89.03%) was the most abundant dinucleotide repeat and was the predominant type among all SSR motifs, followed by AT/AT (6.65%) and AC/GT (4.22%) (Table 2). The most frequent trinucleotide repeat was AAG/CTT (26.57%), followed by ACC/GGT (21.72%) and AGG/CCT (10.58%) (Table 2). The most common tetranucleotide motif was AAAT/ATTT (6.69%). Among pentanucleotide repeats, the motif AAAAT/ATTTT appeared 22 times; the hexanucleotide repeat CTCTCC/GGAGAG was found 12 times. The lengths of repeated SSR motifs ranged from 14 to 138 bp. The majority of SSR motifs were 18 bp long, followed by 14 and 16 bp (Figure 1B). The SSR motifs were repeated 3 to 69 times. The most common number of repeats was 7, followed by 3 and 8 (Figure 1C).

In total, 6,413 SSR motifs (4.84%) were found among 132,593 ESTs. Compared with previously reported SSR frequencies, the SSR frequency observed in *Actinidia* was moderate. It was lower than those previously reported in coffee (18.5%), wheat (7.41%), and grape (5.47%), but was higher than in flax (3.5%), barley (2.8%), and another study of grape (2.5%) (Scott *et al.*, 2000; Peng *et al.*, 2005; Varshney *et al.*, 2006; Aggarwal *et al.*, 2007; Cloutier *et al.*, 2009; Kayesh *et al.*, 2014). The SSR frequency in *Actinidia* ESTs suggests that SSR motifs are relatively prevalent in the *Actinidia* genome.

Among the *Actinidia* EST-SSR motifs, dinucleotides (64.70%) and trinucleotides (14.14%) were the predominant types of repeat motif, collectively accounting for 78.84% of EST-SSRs. High proportions of dinucleotides have also been observed in *Jatropha curcas*, morning glory, pear, apricot and peach, rubber and cassava (Jung *et al.*, 2005; Feng *et al.*, 2009; Raji *et al.*, 2009; Yadav *et al.*, 2011; Ly *et al.*, 2012; Zhou *et al.*, 2016). Many factors affect the frequency, abundance and types of SSR motifs found in different plant species, such as SSR search tools, SSR selection criteria, EST database size, and species differences (Varshney *et al.*, 2005; Aggarwal *et al.*, 2007). In our study, AG/CT (89.03%) was the most abundant repeat type. This suggests that AG/CT nucleotide repeats represent the predominant SSR motif type in *Actinidia.* Similar findings have been reported in apple, citrus, date palm, and strawberry (Newcomb *et al.*, 2006; Palmieri *et al.*, 2007; Bombarely *et al.*, 2010; Zhao *et al.*, 2012). This is probably because the highest frequency amino acids alanine and leucine can be encoded by AG/CT. Consistent with the rarity of GC repeats in plants, the frequency of the CG/CG motif was very low (number = 4; 0.10%) in *Actinidia*. Similar findings have also been reported in apple, apricot, date palm and peach (Jung *et al.*, 2005; Newcomb *et al.*, 2006; Zhao *et al.*, 2012).

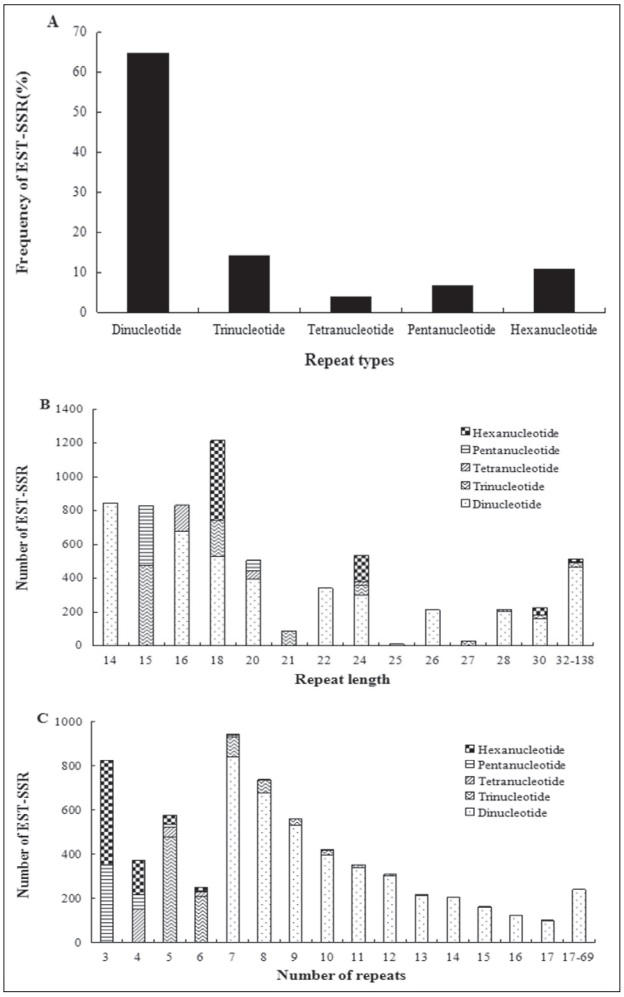

FIGURE 1. Characterizations of EST-SSR repeat motifs in hardy kiwifruit (*Actinidia arguta*). A) Frequency distribution of EST-SSR according to repeat types. B) Frequency distribution of EST-SSR according to repeat length. C) Frequency of different SSR types according to repeat number.

Development and validation of EST-SSR markers

The newly designed primers were developed and verified by PCR amplification using selected primers on 36 *A. arguta* accessions. Among the 199 tested primer pairs, 141 (70.85%) generated amplification bands of the expected size. Among these 141 pairs, 110 were polymorphic and 31 were monomorphic. Of the remaining 57 primer pairs, 23 failed to amplify DNA, while 35 yielded amplicon lengths that were larger or smaller than expected because of small introns present in the target amplicon, unfavorable primer locations or problems during sequencing (Nicot *et al.*, 2004). Genotyping of the 110 polymorphic EST-SSR primers with the 36 *A. arguta* accessions uncovered 331 alleles. The allele number ranged from 1 to 6 with an average of 2.35 and the PIC value varied from 0.50 to 0.94 with a mean of 0.70 (Table 3).

Our successful primer design rate was higher than that published by Man *et al.* (2011) for five accessions of *A. arguta* in Jilin (57.1%) and five accessions in Sichuan (44.9%), but lower than that obtained for *A. chinensis* (93.5%; Fraser *et al.*, 2004). Of the 141 primers, 110 (78.01%) showed polymorphisms. The range of EST-SSR alleles found in the present study, 1 to 6, is similar to the 2 to 6 in the Jilin population and 2 to 4 in the Sichuan population reported by Man *et al.* (2011); however, all of these are much lower than the 9 to 39 published by Zhen *et al.* (2004) for gSSRs in *Actinidia*. These results reveal that EST-SSR markers are less polymorphic than gSSRs. The high average PIC value (0.70) we obtained demonstrates the efficiency and feasibility of *A. arguta* EST-SSR marker development using *Actinidia* ESTs in genetic studies.

Genetic diversity analysis in *A. arguta* accessions

The utility of the novel markers was further assessed by genetic diversity analysis of the *A. arguta* collection. The average genetic similarity coefficient was 0.68 (range 0.58–0.95). The UPGMA analysis classified all 36 accessions into

Table 3. Characterization of 110 polymorphic EST-SSRs in hardy kiwifruit (*Actinidia arguta*).

No.	Primer name	Repeat motif	Primer sequences	Annealing temperature (°C)	Expected size (bp)	Allele numbers
1	A002	(GGAAG)3	F:CCGCACGAGGGTTACATC R:ACAGAGGCTTGGTGGTTG	50	243	5
2	A006	(GGATTC)3	F:AAGGAGAACCGGAGGATT R:TCATAACCGAACGCAGAG	52	216	2
3	A007	(AAAT)6	F:TTATGACTTGGATTCCTT R:GTAGGCTATGTTTGGATG	46	174	3
4	A009	(GA)10	F:TCTTCGTTGCCTGACATT R:GTCCGTTCTCGTCAATAGTT	46	352	4
5	A010	(ACACG)3	F:ATGTCTGAAGAAGGGTGG R:CTTCGGAGGAATACTTGC	48	362	2
6	A012	(TGGTGT)3	F:TGGCTTACCAACTGCTTC R:CCACCTTCATTACTCCTCC	52	366	3
7	A014	(TC)24	F:CACCTGATTTAGCACGAA R:GAATGATAGCCGAACAAC	50	167	4
8	A016	(TTCT)4	F:TTGAAGAGGCTTGTTTGT R:GATTTGAGGGAGAATAGAG	48	354	3
9	A017	(AG)10	F:TACGCAGTTTACTTCCTCTT R:CATTCGGCACCACTTCTA	50	160	3
10	A022	(CTCC)5	F:GCTTCCAACCACCATACA R:CCTTAGGCTACTACCATCTT	48	278	3
11	A023	(TC)18	F:CAGAGCATACAGAGGGAA R:TGACTGGAGTGAAGGAGG	50	173	3
12	A026	(GGAGAG)3	F:AAATCGGACCACAACAGC R:TTCGCACAACCATTCACA	54	353	2
13	A027	(TC)9	F:TTCCCTTACACCGACCAA R:GTAGGAGCCGAGCCAGAT	52	255	4
14	A028	(AG)8	F:ATGAACACGGTGAGTAGCG R:TGAGGAAGAGGAGGAAGG	54	397	3
15	A029	(CT)11	F:CTCATCCACCAGCCTATT R:GTCCTTCCTGACCCTCTT	46	293	3
16	A030	(CT)7	F:ACTGCTTCCTGGTTGCTT R:CTGTTGCCTTTCTTCACG	52	267	2
17	A032	(CT)8	F:GGCGAATACAGAGGTTAG R:TTGCCTTCTCGTTCATAG	48	146	2
18	A033	(GA)15	F:AACTGGACGGTCACGATT R:TCCTCAACCACTGGCTCT	44	407	6
19	A034	(CCT)5	F:CCTCAACATCCTCCAGAC R:GTAATGCCTCAGAACACG	46	474	2
20	A042	(GGCTCC)3	F:CACCTGCTTTGATTATGG R:CAGCCTTGACAATGAACT	50	378	2
21	A043	(TC)8	F:CCCATAGCCAACAACATC R:CTTGACGCCTCTGAACAC	44	168	2
22	A046	(GGCACT)4	F:TGCTCCAGGGACCTTACT R:CTCATTCTCGGCAACCAT	54	259	3
23	A047	(CT)12	F:CGTTATCTCCTTCGCCTCT R:GCCTCTGACTCTAATACCG	48	295	2
24	A048	(CT)11	F:CACCTCTATCTTCAGCCACC R:CGTCGCCTTTGTAGTAGCC	58	389	2
25	A051	(CT)11	F:GAAGACGACAACGCAAAG R:ATCCTGGACATCCTCACC	46	254	2
26	A052	(CCG)5	F:CTGGTGGCTCTATCCATCTT R:CGTGGTCGTAGTATTTCTTCTC	56	367	2

No.	Primer name	Repeat motif	Primer sequences	Annealing temperature (°C)	Expected size (bp)	Allele numbers
27	A053	(GGT)6	F:TGGTGGAATGAATGATGG R:TTGCTGTTGCTGCTGGTG	56	265	3
28	A054	(TC)9	F:GCTAATCATCAGCGACAG R:ACAACAGCATCTTCACCC	48	311	3
29	A055	(CT)8 (CA)7	F:TCCATTCCGCCCATCCTT R:GTCCGACATTCTTGTGGTTCTG	54	143	3
30	A059	(CT)9	F:AAGTGGTTCCGCTCTGGT R:ATGGTCACATCGTCGTCA	54	163	5
31	A060	(CCCGCT)4	F:AGCCATCAACAGCATCTC R:TATTCCACCGCTCTTTCT	44	256	3
32	A061	(CT)12 (AC)8	F:ATCTCCATCATCTCCACCTT R:CAACGCCATCATTGTCCC	56	146	3
33	A062	(CACCA)3	F:TTCATCGGCTCATTGGTT R:CGTAAGAGGACAGGGTTCG	48	295	2
34	A063	(CTT)5	F:GGAGTGAACGCCGCTTAT R:GCCGCTACAGTTCTAAGGTG	52	311	3
35	A064	(GCA)5	F:CCGCAGTTTCATCCTCAT R:AGACTACACGCAACGCATC	56	412	2
36	A068	(CAC)5 (CAA)9	F:CCTGGACACTATGTTGCT R:GATGATGTTGTGGTCCTCT	46	368	2
37	A069	(TC)15	F:GAAGCAACTCGCTACTAAG R:AGCCTGTAACAACCCATT	50	138	4
38	A071	(CT)10	F:ACACCTCAACACCTCCAC R:TATCGCCTCCAGTTCGTC	48	374	2
39	A072	(GGTTTG)6	F:TTGGAACTGAAGGAGGTT R:TCGCCATAGACAAGACAT	48	229	3
40	A073	(CA)9	F:CTCTTCAATCCAGGTCAC R:GCAGCAATAGACAGCCAC	50	226	3
41	A075	(CT)12	F:TCCGATGTTACGCTGAAT R:CCGTAGGACGATGGTGAT	44	299	2
42	A076	(CT)8	F:TCGGCAATCTCGCACAAT R:CGGAGCACTCAAGGCACAT	58	327	2
43	A078	(GA)9	F:TTGGTCCTGCTTTATCTC R:GGTAGCGAACTCAACTCC	44	222	2
44	A080	(TC)16	F:TTGCTCCAGTTTCCTTCT R:TGCTATGGCACTCTATCC	52	187	2
45	A081	(CT)14	F:AATACTTCTCCACCATCTTCC R:ACGACTATGACGGCGACT	50	427	2
46	A085	(AAACC)3	F:TGGTGCTCTTCGTACAGG R:TTGAGTGGGACATTAGGC	50	202	2
47	A086	(ATAG)5 (TATC)6	F:TTCGTCAACGCTCCATCT R:CTCGCTCATCTGCTGCTT	53	309	3
48	A087	(TC)8	F:CAAACCTCAACTCCGTCAT R:TATCCCTTCCAGCAACAC	43	428	2
49	A089	(TC)7	F:GAACCGACACCACCAATA R:ATAGGCGTAAGGGACAGA	46	267	4
50	A092	(AT)7	F:TGGACGAATCAGATAACC R:ACAAGAGTGAGAAGAGGG	48	303	2
51	A093	(CCTCTC)3	F:TCGCCCTATCATTGTCGC R:GTCCCTTATCCATCCATTCT	46	408	3
52	A094		F:CCATTGCCACTACCAGAC R:CCTTCATCCTCCAACTCC	50	280	2

No.	Primer name	Repeat motif	Primer sequences	Annealing temperature (°C)	Expected size (bp)	Allele numbers
53	A095	(AG)12	F:GAAAGTAAGCATAGGCAGAG R:CGAGGAGGTGGTAGGTGT	51	406	2
54	A096	(CT)8	F:AACCACAACCGTAATCGC R:CATCCGCATCGTTATCCC	50	295	3
55	A100	(GTTGG)4	F:AAGTGTTGAGGGTCGTGG R:TTGTGGACTGAGGGCATT	44	194	3
56	A101	(CT)15	F:ACTGCTCGTATCTTGTGGA R:GCATCATAAACTGGTGGG	44	285	4
57	A102	(CT)14	F:AACCCGTTTCCTGACTCCC R:CCGTCCACAAGCCTCGTAT	49	331	2
58	A103	(TC)18	F:GAGCATACAGAGGGAAGA R:ACTGGAGTGAAGGAGGGT	49	169	4
59	A104	(TC)10	F:TTACATCTGCCATAACTCA R:TTCCCATACTCCAACATC	46	207	4
60	A105	(TC)7	F:CCTCATTCATTACCACCTCTT R:ACACCGCTTCCGTCCATA	46	158	4
61	A106	(GCCCG)4	F:TCCGTTCAATCAATACTCCG R:TGCCCTTCACTTTCACATC	49	163	4
62	A107	(GAC)7	F:CGGTTCAGACGGTAGATG R:CCAAGGGACGGACAAGGT	46	174	4
63	A108	(AG)9	F: TCTTCAGATTCAACCCAA R: CCAACATTCAGAGCCAGT	47	233	3
64	A109	(CTG)5	F: TGTTTGTCTTTGGCTTTCC R: GTCCACGGCTTGTTATGC	50	273	4
65	A110	(TC)7 (GCC)6	F:TCTTCGTATGGCACAGCA R:TAGGGTCTCATCCAAGTCAA	46	349	2
66	A115	(TA)10 (AG)9	F:GGCGGAGATACGAGGAGCA R:CAGGCATGGATTGTGGTGTT	50	165	3
67	A119	(CT)9	F:AAATCTCCGACGACCCTC R:TTCCTGCTATCTTCTGACAACT	49	359	2
68	A120	(CT)7 (GA)8	F:CTACTTATCCGCTCACCC R:GATTTGCGAAGACCGTTG	51	243	2
69	A121	(TCT)5	F:TCGTCGTCGTACAATGCC R:AAACCGTCCTCCGTCAAG	52	245	3
70	A123	(TC)8	F:CACCTGCTACACCGACAT R:CCACAGCCGAAACCATAC	49	175	3
71	A124	(CT)7	F:ATACCGTGGACTTCATTC R:ACATACCTTCAACGCTTC	48	194	3
72	A125	(TTTAT)4	F:GTTTATTACACGCCTTCG R:TGACAGCCCTCAACTACG	50	170	3
73	A128	(CT)10	F:AGCCTTCCTCTTCCGTCTC R:CCATCTTCGGTACATCGTC	50	359	2
74	A129	(CAA)5	F:TTACATACGCCCAGAAGG R:CTTGAGGAGCCAGATGAC	48	320	2
75	A130	(CCAATC)3 (AACCCT)5	F:AAGAATGAACAACGGTGG C R:GAGGGAAGTGGCGAAGGA	53	323	3
76	A132	(GCCTCC)3	F:ACCTCGCCGACGACTTAC R:TCCCGTTCTGTCCTTGCT	53	385	2
77	A134	(CCT)5	F:ATCTGATTGGACAGGGTG R:TAACTTCATCGCACTCCA	46	183	2
78	A135	(TC)10	F:TCTTTCTCCGCTACTCTGT R:CTCCATGAACTCCACCAT	48	225	3

No.	Primer name	Repeat motif	Primer sequences	Annealing temperature (°C)	Expected size (bp)	Allele numbers
79	A136	(GA)13	F:GGGAGGAAGAAGGTGGAG R:TGGTATCGCTGGAGGATC	48	209	3
80	A138	(CT)15	F:CCCTATACCCACTCAATCA R:TTCCACCTTCTTTCCATC	45	165	3
81	A139	(TC)21 (CGA)5	F:CTCAAAGTTACCGCCACC R:AAGCAGCATCACATCATCG	50	312	2
82	A140	(CT)9 (CCA)6	F:CCGTACTCCCACTTCACA R:GACGATGCGGTATCCAAC	50	355	3
83	A141	(CCT)8	F:CTACCTTGTCTCGCTCCAC R:TGAGGACTTGGATAAGGGAT	49	316	3
84	A142	(TTTGA)3	F:TTCCCGAAGTAGGTATCCG R:CGCCCATTTACACCAACG	54	283	2
85	A143	(GA)13	F:AAGAAAGATTCGCTGTAGGC R:GGATGAGGAGGTTGTGGG	52	210	3
86	A146	(TC)10	F:CATAGACCGCCGTAAACA R:AGAGTGAAAGCGAGTGA GA	49	260	3
87	A150	(TC)21	F:GGCATCTGTAATCTTCTTCC R:GCAACTGTTTGTCCACGA	46	243	2
88	A151	(ACACCA)4	F:CAGTGGACGAAAGTGAGG R:GTGGCATCTAAGGAAGAGTAAG	48	148	3
89	A152	(GAC)7	F:GACGAAGGAGGAGAAGAC R:GATTGCGGACCTACTAAC	48	149	3
90	A153	(AT)8 (AATA)5	F:AGAAACCAAGTCCGTGAC R:CATAGAGGGAGCAACAGC	50	390	2
91	A155	(TC)12	F:TTCTCCGACTGAACCCTG R:GGTCGCTCTGATAGTCCTT	50	260	3
92	A158	(GA)7	F:AGTCCTCTTTAGCCTTCATCTT R:ATACCTCGTCGCCCTTCT	51	201	2
93	A160	(GA)10	F:CGAAATCACTTCTCATCC R:GCACCGTAACGACTAACT	48	299	3
94	A162	(CT)12	F:ATACCGTGGACTTCATTC R:CAGTTACAGGAACCCATT	46	355	3
95	A164	(AG)10	F:CGCCGCTGAGTAGGGTTT R:CCGCCTTCTGCTTCTGTGAT	51	283	3
96	A166	(CCG)5	F:TTTCTTCTCCCACCCTCT R:ACTCGTCGTGTTGGCTCT	44	273	3
97	A167	(TA)12	F:ATGGCTTGACTGAGACTTG R:TTCATCGTCGGTTGTAGC	47	188	3
98	A169	(AG)10	F:ATCAGACGCCCTCATTCG GA R:TCCACCACCTGTCAAACT	51	145	2
99	A173	(AG)12	F:TTGGGTGGAGATTAGGTT R:TCAACGACTCCTGGTAAG	46	219	2
100	A181	(AG)16	F:TTGCTTGCCTCACCACTT R:ATCAACACGGTTCCCACT	47	270	2
101	A182	(CT)14	F:ACTTCGCATTTCTCCAGC R:ACAGCAATATCCTCCACC	50	227	2
102	A185	(CCCTAG)3	F:TCCCTCTTCTCAACAACC R:TACCCTGAAATGACACCACT	49	205	2
103	A186	(AG)12	F:CTCCCTCGTAGCAGTTCA R:CTCCAGCGATTCCTTCAC	46	167	2
104	A191	(AG)11	F:CCGCATCATAAGCCCAATC R:TTGTCCACCGCCCACTTC	54	193	2

No.	Primer name	Repeat motif	Primer sequences	Annealing temperature (°C)	Expected size (bp)	Allele numbers
105	A193	(TCT)5	F:ATACCCATCATCGGACAC R:CTTCAAGCCCATTACCAC	49	189	2
106	A198	(ATG)5	F:CTTCATTTAGGGCACTTC R:CTTCATCACCAGATACCG	46	363	4
107	A199	(TTC)5	F:TCCCTTTCCTCTTCGTCC R:TAGTAGCAGCAGCGGTAG	51	208	3
108	A202	(GCG)6	F:CAGGAGGAAGATGTTGGAG R:ATGGAGGTAAGGGAAGAGG	50	274	3
109	A204	(GTG)5	F:TTGGGATGATGACGGTATG R:GACTCGTTCTCCGCAATCT	52	219	2
110	A205	(CT)9	F:CCGTCGGCAAACAAACAG R:GAGCGAAGAATCGGAATG	52	392	2

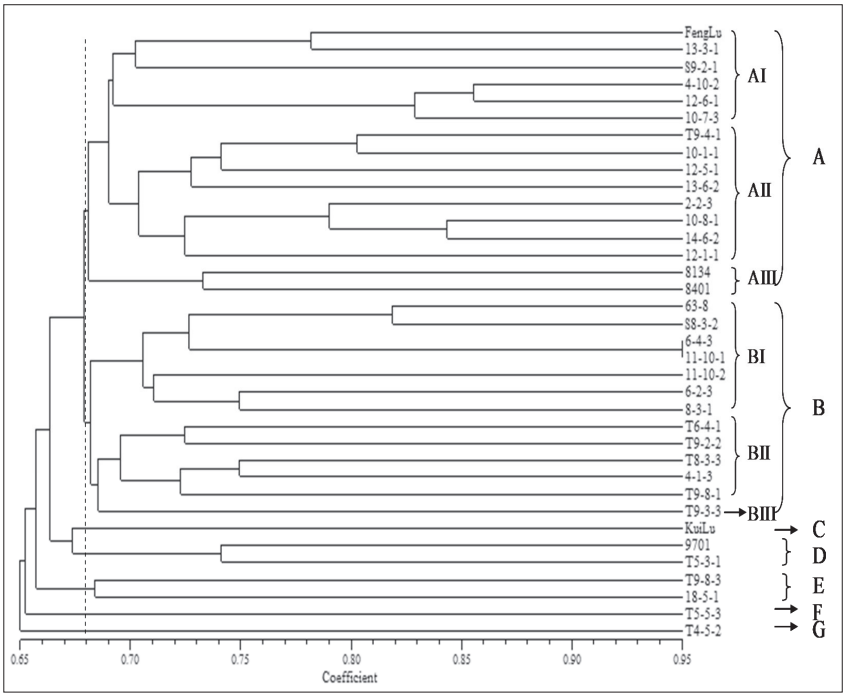

Figure 2. Genetic relationship among 36 accessions of hardy kiwifruit (*Actinidia arguta*).

seven main clusters (Figure 2). The *A. arguta* germplasm presented abundant polymorphism based on molecular markers. The largest number of accessions was placed in cluster A (16; 44.44%), followed by cluster B with 13 accessions (36.11%). The remaining seven accessions (19.44%) were distributed across clusters C, D, E, F and G. The main cluster, A, was further divided into subclusters A_I, A_{II} and A_{III}. Subcluster A_I consisted of six accessions, including 'Feng Lv', which is characterized by very good yielding ability (12,378 kg ha^{-1}), and '12-6-1', which has high vitamin C, sugar and soluble solid contents. Subcluster A_{II} contained eight accessions, one of which was the large-fruited '14-6-2'. Notably, subcluster A_{III} comprised two large-fruited accessions: '8134' (average weight 17.5 g) and '8401' (average weight 19.3 g). Cluster B was also divided into three subclusters. Cluster C only contained one accession, the large-fruited 'Kui Lv' (average weight 18.1 g), while cluster D consisted of the large-fruited accessions '9701' (average weight 17.3 g) and 'T5-3-1'. Therefore, the EST-SSR primers developed from *Actinidia* ESTs could be used for studying genetic diversity in *A. arguta*.

The highest similarity coefficient (0.95) was observed between accessions '6-4-3' and '11-10-1' based on the clustering analysis. Genetic similarities may partly reflect geographic distances and genetic relationships among accessions. In a RAPD study of *Actinidia* (Huang *et al.*, 2002), many species derived from adjoining areas were clustered into the same subgroups in accordance with their known distributions. In our study, however, pedigree information was not very clear because all test accessions were directly or indirectly from wild germplasms. Some accessions showed a high degree of similarity, such as '6-4-3' and '11-10-1', '4-10-2' and '12-6-1', and '10-8-1' and '14-6-2', coinciding with their adjacent collection area. The average similarity (0.76) among the 36 accessions was relatively low. The results thus reveal that the genetic background of *A. arguta* is relatively broad, similar to a previous report on *Actinidia* (Korkovelos *et al.*, 2008). Because most *A. arguta* cultivars mainly come from wild collections, breeders of *A. arguta* need to use appropriate markers to select desirable characteristics to increase breeding efficiency.

Conclusion

The types, distribution, and frequency of SSR motifs have been investigated in *Actinidia* ESTs. About 110 polymorphic EST-SSR markers have been developed and used to study the genetic diversity in *A. arguta.* These newly developed markers are now available to construct an effective EST-SSR marker system and accelerate breeding programs in *A. arguta.*

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