Development of 34 New Microsatellite Markers from *Actinidia arguta*: Intra- and Interspecies Genetic Analysis

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ABSTRACT The present study investigated the isolation and characterization of 34 polymorphic microsatellite markers developed from *Actinidia arguta* (Sieb. and Zucc.) Planch. ex Miq. var. *arguta*. These markers produced 349 alleles in eight *Actinidia* species, with an average of 10.3 alleles per locus. Observed heterozygosity ranged from 0.50 to 0.87 (mean = 0.72), and polymorphism information content ranged from 0.37 to 0.88 (mean = 0.69). The phylogenetic relationship obtained using microsatellite markers showed minor clustering and population differences among species while 38 *A. arguta* accessions fell into two subgroups. These newly developed polymorphic microsatellite markers will be very useful in sustainable genetic conservation, marker-assisted breeding, and classification of the *Actinidia* genus.

Keywords Microsatellites, *Actinidia arguta*, Genetic diversity

INTRODUCTION

The genus *Actinidia* comprises 76 species and about 120 taxa of perennial, dioecious, viny, and deciduous fruit trees. *Actinidia* species have a wide range of distribution in eastern Asia that includes tropical equatorial and temperate regions (Ferguson and Huang 2007). Four of the 76 species have been domesticated as fruit crops: *A. deliciosa*, *A. chinensis*, *A. arguta*, and *A. eriantha* (Cui et al. 2002; Ferguson 1990). *A. deliciosa* (A. Chev.) C. F. Liang and A. R. Ferguson var. *deliciosa* is a green-fleshed kiwifruit, and *A. chinensis* Planch. var. *chinensis* is yellow-fleshed. Kiwifruit production has increased remarkably since the introduction of *A. deliciosa* seeds to New Zealand in the early 1900s, reaching more than one million tons in 2000 (Huang and Ferguson 2001).

Four *Actinidia* species, *A. arguta*, *A. kolomikta*, *A.*

polygama, and *A. rufa*, are found in Korea (Cui et al. 2002; Ferguson 1990; Kim et al. 2003). *A. arguta* (Sieb. and Zucc.) Planch. ex Miq. var. *arguta* grows in the wild in mountainous and hilly terrain throughout Korea; it is tolerant to freezing and is resistant to various harmful insects. Because the exterior of the fruit is soft, smooth, and leathery, it can be eaten whole without peeling (Williams et al. 2003). Given its richness in various minerals and vitamin C, this fruit is commonly used in soft drinks and jams. It is also used as an anodyne, diuretic, antifebrile, and thirst-quencher in home remedies (Lee et al. 2004). Despite these beneficial traits and uses, commercial cultivars of *A. arguta* have not been available, and most plants are growing in the wild without much improvement. Although several attempts have been made to introduce the genes from *A. arguta* into *A. deliciosa* (Kim et al. 2008; Lee et al. 2004), the hybrids have not yet been widely accepted.

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The *A. arguta* accessions have not been subjected to genetic diversity analysis. For the conservation and sustainable use of wild accessions, it is important to understand the genetic variation, genetic structure, and mating systems of natural populations. Given their high levels of polymorphism with codominance and their excellent reproducibility, microsatellite or simple-sequence repeat (SSR) markers have been preferred for use in the analysis of molecular diversity in conservation biology and molecular ecology (Park et al. 2009). Although microsatellite markers have been developed in the *Actinidia* species, this development has been restricted to the commercial species *A. chinensis* and *A. deliciosa* and has not included *A. arguta* (Huang et al. 1998; Korkovelos et al. 2003). Huang et al (1998) demonstrated that 40 microsatellite markers derived from the genomic library of *A. chinensis* were successful in the genomic DNA of *A. arguta*. Another study (Fraser et al. 2000) found that four of nine microsatellite markers derived from the genomic library of *A. deliciosa* were successful in the polymerase chain reaction (PCR) amplification of *A. arguta* accessions. The same group of researchers subsequently tested cross-species amplification among 21 *Actinidia* species with 20 expressed sequence tag (EST)-derived microsatellite markers (Fraser et al. 2005). Three of these markers failed in amplification, and three others were inconsistent in the amplification of *A. arguta* (4x) accessions.

In an effort to preserve the genetic diversity of *A. arguta* in Korea, we collected wild accessions throughout the country and stored the seeds in a short-term preservation facility. The seeds were screened for genetic redundancy before they were placed in a long-term storage facility. Because microsatellite markers are preferred for genetic diversity analysis, we developed microsatellite markers from the *A. arguta* genomic library. This report describes the new set of markers for genetic diversity analysis in *Actinidia* species and possible markers for sex determination in *A. arguta*.

MATERIALS AND METHODS

Plant materials and genomic DNA extraction

A total of 91 accessions from eight *Actinidia* species

were used in this study (Table 1). The young leaves were acquired from Namhae Sub-Station of National Institute of Horticultural & Herbal Science which conserved diverse accessions of *Actinidia* species in experimental field. Total genomic DNA was extracted from grinded power of young leaves by liquid nitrogen using the Plant DNAzol® Reagent (Invitrogen, Carlsbad, CA, USA), following the supplier's protocols. The extracted DNA was quantified using an ultraviolet-visible (UV-Vis) spectrophotometer (ND-1000; NanoDrop, Wilmington, DE, USA).

Construction of an SSR motif-enriched library

A modified biotin-streptavidin capture method was used to construct an SSR motif-enriched library of *A. arguta* (Sieb. and Zucc) Planch. ex Miq. var. *arguta* genomic DNA (Kwon et al. 2005). Genomic DNA samples (10 µg) were digested with seven blunt-end-producing restriction enzymes (*EcoRV*, *DraI*, *SmaI*, *PvuI*, *AluI*, *HaeIII*, *RsaI*). The fully digested DNA samples were pooled and size-fractionated on 1.4% agarose gels. DNA fragments of 300 bp to 1,500 bp were eluted from the gels and were purified using a gel extraction kit (Qiagen, Hilden, Germany). Approximately 1 µg of DNA fragments was ligated with 1 µg of the double-stranded adaptor (AP11/AP12). The adaptor was prepared by mixing equal molar amounts of oligonucleotides AP11 (5'-CTC TTG CTT AGA TCT GGA CTA-3') and AP12 (5'-TAG TCC AGA TCT AAG CAA GAG CAC A-3'), heated to 94°C, then cooled to 25°C over a period of 4–5 h. Preamplification of adaptor-ligated DNA fragments was performed for 15 cycles of PCR in a 50-µl reaction volume with a single primer (AP11) and an annealing temperature of 56°C. The preamplified products were hybridized with a mixture of long (40–45 nucleotides) biotin-labeled repeat probes. Hybridization was performed for 2 h at 65°C in a reaction mixture (50 µl) that included 6× standard sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS), ~100 ng of preamplified product, and 300 ng of each biotin-labeled oligo: (GA)₂₀, (CA)₂₀, (AGC)₁₅, (GGC)₁₅, (AAG)₁₅, (AAC)₁₅, and (AGG)₁₅. The hybridized DNA fragments were captured with 400 µg of streptavidin-coated magnetic beads (Promega, Madison, WI, USA) by incubating the mixture at 65°C with gentle agitation for 30 min. The beads were separated from the liquid using a

magnetic stand (Promega) and were washed five times in 300 µl 6× SSC/0.1% SDS (1× SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0) at room temperature with gentle agitation. After stringent washing, all samples were briefly washed in 5× SSC to remove the SDS, and DNA fragments were eluted with 50 µl of dH₂O at 90°C for 5

min. Final elutions (5 µl) were amplified for 15 cycles of PCR using the AP11 primer. After checking the gel, the amplified DNA products were cloned into pGEM-T Easy Vector (Promega) and were transformed into *Escherichia coli* cells through electroporation. Recombinant colonies were identified by blue or white colony selection on

Table 1. List of *Actinidia* species used in this study.

Taxon	Number of accessions	Variety				Geographic location
<i>A. arguta</i> (Sieb. and Zucc.) Planch. ex Miq. var. <i>arguta</i>	40	63. K5-1-22	73. K5-3-6	83. K5-4-12	93. K5-10-1	Korea
		64. K5-2-3	74. K5-3-7	84. K5-5-1	94. K5-10-2	
		65. K5-2-7	75. K5-3-8	85. K5-5-8	95. K5-10-3	
		66. K5-2-8	76. K5-3-12	86. K5-5-12	96. K5-10-5	
		67. K5-2-10	77. K5-3-13	87. K5-5-14	97. K5-10-8	
		68. K5-2-13	78. K5-3-18	88. K5-6-10	98. K5-11-1	
		69. K5-2-16	79. K5-4-3	89. K5-6-12	99. K5-11-2	
		70. K5-2-18	80. K5-4-7	90. K5-9-1	100. K5-11-3	
		71. K5-2-22	81. K5-4-8	91. K5-9-4	101. K5-11-8	
		72. K5-3-3	82. K5-4-10	92. K5-9-8	102. K5-12-6	
<i>A. chinensis</i> Planch. var. <i>chinensis</i>	13	14. SKK3	20. SKK10	104. Red-Kiwi	106. Jesi-Gold	Korea
		16. SKK5	21. SKK11,	105. Hort16A		
		17. SKK7 (Gracies)	23. SKK13,	108. Yeo-San-Hyang		
		19. SKK9 (Chin Mei)	62. Jeju-Gold	115. Guem-Goi		
<i>A. chinensis</i> Planch. var. <i>chinensis</i>	6	12. SKK1	30. S13			China
		13. SKK2	15. SKK4	18. SKK8 (Tomuri)	117. SKK12-2	
<i>A. deliciosa</i> (A. Chev.) C.F. Liang and A.R. Ferguson var. <i>deliciosa</i>	25	1. Abbott	7. Hyang-Rok	11. Yellow Queen (First Emperor)	45. SKK 60	New Zealand, Japan, China
		2. Bruno	8. Golden King	38. SKK22 (Jinkui)	47. SKK80 (Red Princess)	
		3. Hayward	9. Golden Yellow	40. SKK33 (Wha-Pyung 2 Ho)	116. Jin-Mi	
		4. Monty	10. Sensation Apple	42. SKK43 (Seo-Hyang)		
		5. Matua	37. SKK18 (Chieftain)	43. SKK44	110. M51-2	
		6. Tomuri	39. SKK23	46. SKK 61		
		34. Sun-WuKong	41. SKK34 (Mi-Lyang 2 Ho)	109. M51-1		
<i>A. eriantha</i> Benth.	3	50. S10	51. S20	103. Bi-Dan		China, Korea
<i>A. arguta</i> var. <i>purpurea</i> (Rehd.) C.F. Liang	1		24. S3			China
<i>A. kolomikta</i> (Maxim. and Rupr.) Maxim.	1		25. S4			China
<i>A. macrosperma</i> C.F. Liang	1		27. S7			China
<i>A. rufa</i> (Sieb. and Zucc.) Planch. ex Miq.	1		61. Seom-Darae			Korea

lysogeny broth (LB) plates containing ampicillin, 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal), and isopropyl β -D-1-thiogalactopyranoside (IPTG).

DNA sequencing and SSR primer design

A total of 762 white colonies were randomly selected from the enriched library. Plasmid DNA was purified with the QIAprep Spin Miniprep Kit (Qiagen), and nucleotide sequencing was performed using an ABI 3100 DNA sequencer with a BigDye terminator kit (Applied Biosystems, Foster City, CA, USA). SSR motif elucidation and primer design were performed using the ARGOS program (Kim 2004).

PCR amplification

The “M13 tail at its 5’ end” PCR method was used to measure the size of the PCR products (Schuelke 2000). PCR amplification was performed in a total volume of 20 μ l containing 2 μ l of genomic DNA (10 ng/ μ l), 0.2 μ l of the specific primer (10 pmol/ μ l), 0.4 μ l of M13 universal primer (10 pmol/ μ l), 0.6 μ l of normal reverse primer, 2.0 μ l of 10 $\times$ PCR buffer (Takara, Tokyo, Japan), 1.6 μ l of deoxynucleoside triphosphate (dNTP; 2.5 mM), and 0.2 μ l of Taq polymerase (5 unit/ μ l; Takara). The PCR amplification conditions were as follows: 94°C for 3 min, followed by 30–33 cycles at 94°C (30 s), 50–55°C (45 s), 72°C (45 s), then 15 cycles at 94°C (30 s), 53°C (45 s), and 72°C (45 s), and final extension at 72°C for 20 min. PCR was performed in PTC-220 thermocyclers (MJ Research, Waltham, MA, USA). The PCR products of three microsatellites were mixed together in a ratio of 6-FAM:HEX:NED (fluorescent dyes) = 1:3:4, which varied depending on the amplification intensity for individual markers as determined on an ABI PRISM 3130xl genetic analyzer (Applied Biosystems). PCR products labeled with HEX and NED were added in greater amounts, and those labeled with FAM were added in lesser amounts to match the different signal intensities of these fluorescent dyes. The 1.5 μ l mixed PCR product was combined with 9.2 μ l of Hi-Di formamide and 0.3 μ l of an internal Genescan-500 ROX (6-carbon-X-rhodamine) molecular size standard (35–500 bp). The samples were denatured at 94°C for 3 min and were analyzed with the

ABI PRISM 3130xl. Base-pair molecular weights of the microsatellite products were estimated with Genescan software (ver. 3.7; Applied Biosystems) using the local Southern method. The individual fragments were assigned as alleles of the appropriate microsatellite loci with Genotyper software (ver. 3.7, Applied Biosystems).

Data analysis

The PowerMarker version 3.25 (Liu and Muse 2005) and GenAlEx version 6.1 (Peakall and Smouse 2006) programs were used to measure variability at each locus, observed heterozygosity (H_o), gene diversity/expected heterozygosity (H_e), and polymorphism information content (PIC). The number of alleles (A) and PIC values were calculated with the equation of $PIC = 1 - \sum P_i^2 - \sum 2 P_i^2 P_j^2$ where $\sum P_i^2$ is the sum of each squared i th haplotype frequency (Botstein et al. 1980). A phylogenetic dendrogram was constructed using the unweighted pair-group method with arithmetic averages (UPGMA) with PowerMarker (Liu and Muse 2005) and MEGA4 (Tamura et al. 2007) software.

Intra- and interpopulational levels of genetic variation identified by the cluster analysis were estimated from allelic frequencies using analysis of molecular variance (AMOVA) (Weir 1996; Weir and Cockerham 1984). Pairwise estimates of the correlation of alleles within subpopulations (F_{ST}) were calculated for subpopulations using an AMOVA approach in GenAlEx (Peakall and Smouse 2006).

RESULTS

Development of SSR markers from *A. arguta*

A total of 762 clones were randomly picked from the primary transformation plates and were sequenced (Table 2). After excluding redundant clones ($n = 35$) and truncated clones with SSR motifs near their ends ($n = 162$), 565 clones were analyzed for primer designing. Of these, 140 clones were suitable for designing primer pairs for PCR amplification of the SSR motifs. Clones containing <12 nucleotides in the SSR motifs were not included in the

primer design. Dinucleotide repeat-motif-containing clones were predominant (86.1%) among the 565 clones, with 864 dinucleotides, 87 trinucleotides (8.7%), and 53 tetranucleotides or more (5.8%) clones (Table 3).

Variation among *Actinidia* accessions

SSR variations were sought using the 140 selected primer pairs with a core collection of 91 *Actinidia* accessions representing diverse regions and species (Table 1). This

collection is maintained at the National Institute of Horticultural & Herbal Science (RDA, Korea). Intra- and interspecies accession polymorphisms were present in 34 primer pairs (Supplementary Table 1). The number of repeating units among these polymorphic SSR loci varied from 6 to 23, and the length of the repeat region varied from 12 bp in GB-AA-017 [(CT)₆] to 174 bp in GB-AA-065 [(GA)₁₁(GAA)₂₃] (Table 4). Whereas 26 (82.6%) polymorphic loci contained di-nucleotide repeating motifs, only three

Table 2. Efficiency of the procedure adopted for the identification of microsatellite markers in *A. arguta*.

Procedural step	Number (percentage)
Sequenced clones	762
Redundant clones	35 (4.6%)
Unique clones	727 (95.4%)
SSR clones	565 (74.1%)
Truncated clones (5' or 3' end)	162 (21.3%)
Fewer than 12 nucleotides	263 (34.5%)
Primer pairs designed	140 (18.4%)
Polymorphic loci	34 (24.3%) ²⁾

²⁾percentage based on the designed primer pairs.

Table 3. Frequency and type of di-, tri- and $\geq$ tetra-nucleotide repeats isolated from the *A. arguta* microsatellite-enriched library.

Repeat unit	Repeat class	Number of microsatellite loci		Polymorphic loci	
		Number	Percentage	Number	Percentage
Di-nucleotide	AC/CA/TG/GT	238	23.7%	8	17.4%
	AG/GA/TC/CT	603	60.1%	30	65.2%
	AT/TA	22	2.2%		
	CG/GC	1	0.1%		
Total		864	86.1%		
Tri-nucleotide	AAC/ACA/CAA	15	1.5%		
	AAG/AGA/GAA	44	4.4%	1	2.2%
	AAT/ATA/TAA	4	0.4%		
	ACC/CCA/CAC	3	0.3%		
	AGC/CGA/GAC	4	0.4%		
	AGG/GGA/GAG	14	1.4%	2	4.3%
	ATC/TCA/CAT	1	0.1%		
	CCG/CGC/GCC	2	0.2%		
Total		87	8.7%		
$\geq$ Tetra-nucleotide		53	5.8%	5	10.9%
Total repeat motifs		1004		46	

Table 4. Characteristics of 34 polymorphic microsatellite loci for *Actinidia* species.

Marker	GeneBank accession	Repeat motif	Primer sequence	Size (bp)	Ta (°C)	A	MAF	He	Ho	PIC
GB-AA-005	FJ647762	(CT) ₁₇	F: AGTTGTGCATCCAAAGGC R: CAGTGGGGTGAAGAACGA	192-218	57	12	0.32	0.73	0.78	0.76
GB-AA-012	FJ647763	(TG) ₁₃	F: TCACAACACTCATTTTCGGC R: ATCCGCTTCCTTAGCTGC	154-176	58	10	0.49	0.47	0.71	0.68
GB-AA-015	FJ647764	(AG) ₁₉	F: CCTGGTCGTTTCAGGGAAT R: ATGGCATTGTGCTTG	267-293	58	14	0.24	0.89	0.83	0.83
GB-AA-017	FJ647765	(CT) ₆	F: AAAGTGTGAGCACGTGACAA R: TGAGAGAGAGAGGTGGCG	170-182	58	2	0.54	0.00	0.50	0.37
GB-AA-018	FJ647766	(CA) ₈ , (GGA) ₄	F: ACCATGGCACAGATGGAA R: TCCAGTGCCTTTTAAAGCC	147-171	58	11	0.23	0.75	0.81	0.80
GB-AA-024	FJ647767	(GA) ₁₉	F: AGGAGACCCAACAGGAACA R: AATTCGGGTCACCACACA	155-181	58	12	0.40	0.49	0.75	0.74
GB-AA-054	FJ647768	(GA) ₁₅	F: ACCAAAAACACCTGCCT R: TGAACCCGTATTCGCATC	186-214	58	15	0.23	0.87	0.85	0.84
GB-AA-065	FJ647769	(GA) ₁₁ (GAA) ₂₃	F: ATTGAAGCCCCATTGAG R: CCAAGGAGGGCATTAGG	212-238	58	11	0.43	0.60	0.74	0.72
GB-AA-069	FJ647770	(GGGA) ₆ (GA) ₈	F: CGTTCTCCTTCGACCCTT R: CCGTTACCTTGTCGAATCC	196-258	58	11	0.43	0.67	0.74	0.71
GB-AA-080	FJ647771	(GA) ₁₅	F: CCAATCAACAAGATGCACG R: TGGGAGGTTGAAACTGGA	179-213	58	11	0.26	1.00	0.82	0.80
GB-AA-084	FJ647772	(GA) ₁₅	F: CATTGGAACCAACGCAAT R: AGTCGGAGCTGGGAGAAG	233-259	58	12	0.22	0.96	0.83	0.82
GB-AA-088	FJ647773	(CT) ₇	F: TCTGTTTGTTCACCA R: GGTTGAGTTCCATTCCCG	177-197	57	8	0.45	0.56	0.69	0.67
GB-AA-091	FJ647774	(CT) ₁₉	F: TGACTTAAGGGCGACCAA R: GGAAATCGCTCATGGACA	201-223	58	8	0.38	0.62	0.74	0.69
GB-AA-094	FJ647775	(AC) ₁₅	F: ACAGGGGAACATCAGTGC R: GTGGGATATAACCGGGGA	208-220	57	6	0.38	0.41	0.68	0.63
GB-AA-096	FJ647776	(GA) ₆ , (GA) ₃	F: TTGGTACACAAGACGCCC R: GAAATTTCTTACCCGCC	229-235	58	3	0.52	0.01	0.50	0.39
GB-AA-302	FJ647777	(AG) ₁₃	F: TCGGTGATAAATGGCAGG R: GGCTCCTTGACAGCACAG	269-311	58	11	0.43	0.31	0.74	0.72
GB-AA-303	FJ647778	(CT) ₁₅	F: TGGGGCATTATGCCTAT R: TCCAACCTTCTTGGCTCA	156-258	57	12	0.33	0.35	0.75	0.73
GB-AA-304	FJ647779	(AG) ₁₁	F: CAGTCCACAAAATGGGTCA R: GGTGAACACCCCAAAAGT	202-364	58	13	0.41	0.15	0.67	0.64
GB-AA-308	FJ647780	(CCCT) ₄ , (GGGT) ₂ , (GGGT) ₂	F: TAGTGCTGCGAAGGAGGA R: ATTGCACCATTCTGTGGC	265-304	58	8	0.32	0.36	0.77	0.75
GB-AA-331	FJ647781	(GA) ₁₅ , (CAGA) ₄	F: CCATCTTTTGTGCTTTG R: TTGTTGGTATCATGCCCC	259-303	57	13	0.28	0.59	0.83	0.80
GB-AA-333	FJ647782	(TG) ₁₂	F: AAGTTCATTCCACGCACG R: ATTGCATTGAGCCGCTA	242-276	58	6	0.49	1.00	0.67	0.61
GB-AA-337	FJ647783	(GA) ₁₀ , (AC) ₆	F: TTCTCTGCGCGTTCTCTC R: AGCCTCAACCAAGAAGGG	290-332	58	10	0.36	0.53	0.74	0.72
GB-AA-340	FJ647784	(CCT) ₅	F: AAGGAATTCGCCCTCAAA R: GGCTGACAAGAAGCGATG	287-296	58	4	0.65	0.68	0.49	0.45

Table 4. Continued.

Marker	GeneBank accession	Repeat motif	Primer sequence	Size (bp)	Ta (°C)	A	MAF	He	Ho	PIC
GB-AA-342	FJ647785	(GA) ₂ , (GA) ₄	F: AAAATTCCAATTCCCCCA R: CATTTCGGAATCCCCTTT	261-293	57	13	0.35	0.59	0.81	0.80
GB-AA-343	FJ647786	(AG) ₁₄	F: TGCTTCTTCGGTCATGCT R: CACCATTTCGAACCCAGA	232-262	58	5	0.44	0.67	0.70	0.64
GB-AA-356	FJ647787	(AC) ₁₂	F: CCCGACTTCCAAGTCTCC R: CTCCGGATGCCCTTTATC	251-287	58	16	0.25	0.70	0.85	0.83
GB-AA-366	FJ647788	(GA) ₇	F: CAGCTCCAAGGGCTATGA R: TGTTCCTACTACCGCAAC	241-247	57	5	0.51	0.09	0.53	0.43
GB-AA-369	FJ647789	(CT) ₁₆ (CA) ₁₇	F: TGATCCACAACGTCATCAA R: GGGCACGCTAGACACACC	185-215	58	13	0.32	0.31	0.76	0.74
GB-AA-370	FJ647790	(GA) ₁₆	F: GGGAATTGGTGAGTGGGT R: ATAGCCCAAACCGTTGGT	229-273	58	16	0.57	0.77	0.61	0.63
GB-AA-372	FJ647791	(TC) ₁₆	F: GGAATTCGGTCACCTTC R: CATCCAAAAACCTCGG	149-215	57	10	0.51	0.59	0.71	0.67
GB-AA-374	FJ647792	(CT) ₁₇	F: GAACGAATCAGGAATCGAAA R: TGAAGTGTAATAAAAGACTTCGCA	153-171	58	9	0.32	0.68	0.78	0.74
GB-AA-380	FJ647793	(CT) ₁₆	F: GGCAAACCTACACCTCA R: TTTTCTCGTCTCTCGTGA	296-326	57	9	0.57	0.02	0.56	0.52
GB-AA-393	FJ647794	(GA) ₇	F: CAAGCAGTGAAGATGCTTACC R: CAGCTCAGGGGTCGACTA	118-216	57	20	0.22	0.68	0.87	0.88
GB-AA-398	FJ647795	(GA) ₁₁	F: CGGGAATGTGAAATCCTTT R: CCAATTGCTTGGGAGTGA	233-267	57	10	0.40	0.65	0.75	0.72
Mean						10.3	0.39	0.55	0.72	0.69

*Loci with significant deviations from Hardy-Weinberg equilibrium.

Ta, annealing temperature; MAF, major allele frequency; A, number of alleles; He, expected heterozygosity; Ho, observed heterozygosity; PIC, polymorphism information content.

(6.5%) and five (10.9%) polymorphic loci had tri- and tetranucleotides or greater repeating motifs, respectively (Table 3). With these 34 SSRs, a total of 349 alleles were detected among the *Actinidia* species accessions, resulting in an average of 10.3 alleles per locus (Table 4). The GB-AA-393 primer pair produced the highest number of alleles ($n = 20$) and also had a high PIC value (PIC = 0.88), whereas GB-AA-017 produced only two alleles (PIC = 0.37). The major allele frequency (MAF) ranged from 0.22 to 0.65, with a mean value of 0.39. The observed heterozygosity (H_o) ranged from 0.50 to 0.87 (mean = 0.72), and the expected heterozygosity (H_e) ranged from 0.00 to 1.00 (mean = 0.55). Considering three species (*A. arguta*, *A. chinensis*, *A. deliciosa*) individually, *A. deliciosa* showed the most variation (PIC = 0.44) and *A. chinensis* (PIC = 0.41) showed the least variation in all diversity denominators (data not shown).

Intra- and interspecies relationships obtained from SSR profiles

Proportions of shared alleles were used to calculate genetic distances between all pairwise combinations among the 91 *Actinidia* accessions used in this study. A phylogenetic dendrogram based on the SSR profiles clearly shows two major groups at a genetic distance of 0.45 (Fig. 1). The first group (GI) comprised *A. chinensis*, *A. deliciosa*, *A. eriantha*, *A. kolomikta*, *A. macrosperma*, and *A. rufa*. The accessions of the four latter species (*A. eriantha*, *A. kolomikta*, *A. macrosperma*, and *A. rufa*) did not form a subspecific clade, but were intermixed in each GI clade. The GII clade included *A. arguta* was clearly distinguished from first group (GI) and formed two major subclades at a genetic distance of 0.25.

Fig. 1. Phylogenetic dendrogram of the varieties and species in *Actinidia* based on SSR amplification profiles.

DISCUSSION

In this study, 762 DNA sequences of *A. arguta* were retrieved from a microsatellite-enriched library, but only 140 (18.4%) were used to design the SSR primers. The majority of DNA sequences (81.6%) were not suitable for primer design; 55.8% had SSRs at the 5' or 3' end and fewer than 12 nucleotides in the SSR motifs; and 4.6% were duplicate or redundant DNA sequences (Table 2). This result was consistent with those reported for *Sorghum bicolor* and cassava (*Manihot esculenta* Crantz), wherein 70% and 45% of the clones, respectively, had SSRs too close to the cloning sites at the 5' or 3' end (Mba et al. 2001; Taramino et al. 1997). Similarly, redundant DNA sequences consisting of the same SSR locus or showing more than 95% similarity in flanking sequences were found in 20% of cassava accessions (Mba et al. 2001), 16% of perennial

ryegrass (*Lolium perenne* L.) accessions (Jones et al. 2001) and 2.2% in avocado (*Persea americana* Mill.) accessions (Ashworth et al. 2004). Most redundancies were due to cloning, locus duplication, or allelism and were from the same microsatellite enrichment library. Ashkenazi et al (2001) also reported that some conserved potato (*Solanum phureja* Juz. & Buk.) DNA sequences flanking microsatellite regions were too short to permit the design of an appropriate primer. Earlier studies reported that the AT/TA repeat was the most frequent type of SSR in plants, followed by the AG/GA/TC/CT repeat (Danin-Poleg et al. 2001; Wang et al. 1994; Yu et al. 1999). In this study, the most frequent type of microsatellite repeat was AG/GA/TC/CT (60.1%), followed by AC/CA/TG/GT (23.7%), AAG/AGA/GAA (4.4%), and AT/TA (2.2%; Table 3). However, the frequency of a microsatellite repeat may vary with species and genus. For instance, Ashworth et al (2004) reported that AG and

ATG were the most frequent repeats in avocado.

The SSR genetic diversity observed among *Actinidia* accessions in our study can be compared to the diversities among *A. chinensis* and *A. deliciosa* that have been measured using other SSR sets (Korkovelos et al. 2008). The average numbers of alleles and PIC in our analysis were 10.3 and 0.69, respectively. Similarly, Korkovelos et al. (2008) reported averages of 7.8 ± 3.2 alleles and $\text{PIC} = 0.739 \pm 0.158$. These values are higher than those detected in cucumber (*Cucumis*; average 2.4 alleles/locus, $\text{PIC} = 0.28$) and common buckwheat (*Fagopyrum*; average 5.9 alleles/locus, $\text{PIC} = 0.48$) (Danin-Poleg et al. 2001; Ma et al. 2009). Thus, the SSR markers among 91 *Actinidia* accessions in the present study were effective for assessing genetic diversity and for understanding population structure.

The species relationship derived from our cluster analysis is consistent with other reports (Huang et al. 2002; Li et al. 2002). In particular, the clustering results confirmed the previously established close relationship between *A. deliciosa* and *A. chinensis* (Cipriani et al. 1998; Huang et al. 1997; Testolin and Ferguson 1997), revealing *A. chinensis* as a progenitor of *A. deliciosa* (Cipriani et al. 1998). Others have considered *A. arguta* to be promising for the development of cultivars (Ferguson 1999; Nishiyama 2007). These major species were unambiguously classified into two groups in the present study. However, *A. kolomikta* and *A. macrosperma* were included in Group I in our analysis. This result contrasts with a previous analysis using RAPD markers that placed these species with *A. arguta* (Huang et al. 2002). This result also contrasts with a study using *matK* gene and internal transcribed spacer (ITS) sequences that found a lesser genetic distance between these species and *A. arguta* than with other groups (Li et al. 2002). The morphological features of *A. kolomikta* and *A. macrosperma* are also more similar to those of species in the *A. arguta* group (Ferguson 1990) than to those of other groups. The grouping result may be due to the limited amplification of transferability in SSR markers. Although most *Actinidia* species were successfully amplified with all 34 SSR markers, the amplifications of transferability in *A. kolomikta* and *A. macrosperma* were 80% and 57%, respectively. The transferability of SSRs to other *Actinida* species showed average value of 77%

ranged from 57% (*A. macrosperma*) to 90% (*A. chinensis*). A little higher transferability than other genus such as *Prunus* (mean=59%) and *Allium* (mean=59%) reveals that genetic distances are relatively low between tested *Actinidia* species (Lee et al. 2011; Wang et al. 2012).

These newly developed polymorphic microsatellite markers will be very useful for selection in marker-assisted breeding, genetic conservation, and classification of the *Actinidia* genus. The sex-related markers identified in our study are also of major importance to kiwifruit breeders and the produce industry because they allow the distinction of female plants at the seedling stage and have great potential utilization in marker-assisted selection in this species.

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