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DEVELOPMENT AND CHARACTERIZATION OF MICROSATELLITE MARKERS FOR CENTRAL AMERICAN *BEGONIA* SECT. *GIREOUDIA* (*BEGONIACEAE*)¹

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- **Premise of the study:** Transcriptome sequence data were used to design microsatellite primers for two widespread Central American *Begonia* species, *B. heracleifolia* and *B. nelumbifolia*, to investigate population structure and hybridization.
- **Methods and Results:** The transcriptome from vegetative meristem tissue from the related *B. plebeja* was mined for microsatellite loci, and 31 primer pairs amplified in the target species. Fifteen primer pairs were combined in two multiplex PCR reactions, which amplified an average of four alleles per locus.
- **Conclusions:** The markers developed will be a valuable genetic resource for medium-throughput genotyping of Central American species of *Begonia* sect. *Gireoudia*. A subset of these markers have perfect sequence matches to Asian *B. venusta*, and are promising for studies in other *Begonia* sections.

Key words: *Begonia heracleifolia*; *Begonia nelumbifolia*; Begoniaceae; hybridization; microsatellite primers; transcriptome sequences.

Begonia L. is a diverse tropical genus with over 1500 species. Evolutionary research has focused on the early-diverging African species (e.g., Hughes and Hollingsworth, 2008) and the more derived Asian species (e.g., Thomas et al., 2011), with the American species largely overlooked. The most recent common ancestor of Central American *Begonia* is likely to be relatively recent (Miocene; Dewitte et al., 2011), and subsequent speciation has resulted in high species richness (total c. 690 species; Goodall-Copstake et al., 2010). Population studies of Central American *Begonia* species will shed light on the evolution of species richness in a morphologically diverse group of neotropical herbs; but to date, studies have been limited by the availability of suitable nuclear markers to complement plastid microsatellite markers (Twyford et al., 2013).

In this study, we describe the development of nuclear microsatellite markers to study gene flow within and between Central American *Begonia* species. This requires markers that amplify over a broad phylogenetic scope, which can then be cross-amplified in divergent species.

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METHODS AND RESULTS

Microsatellite markers were designed from the transcriptome sequence of vegetative meristem tissue from *B. plebeja* Liebm., a related species from *Begonia* sect. *Gireoudia* (European Nucleotide Archive Sequence Read Archive accession number: ERP001195; Brennan et al., 2012). The QDD bioinformatic pipeline (Megléczy et al., 2010), which integrates microsatellite detection, a redundancy check to avoid amplifying multiple PCR products, and designs primers, was used according to Lepais and Bacles (2011). A FASTA file of the *B. plebeja* transcriptome sequence assembly was analyzed in QDD version 1.3 using default parameters: selecting only primers that amplify a PCR product between 90 and 320 bp in length, with a repeat motif of 2–6 bp repeats, and a minimum length of four repeat units. To make microsatellite amplification in other species more likely, primers were excluded if they did not have a perfect BLAST match to the transcriptome of *B. conchifolia* A. Dietr. (sect. *Gireoudia*; Brennan et al., 2012). Reads from which the primers were designed were BLAST searched against the *Arabidopsis* Information Resource (TAIR) database (<http://www.arabidopsis.org>) to investigate the putative function of each locus.

Thirty-one primer pairs detected in QDD were tested for amplification in *B. heracleifolia* Cham. & Schldtl. and *B. nelumbifolia* Cham. & Schldtl. These species were chosen because they are two of the most widespread *Begonia* species in a genus of mostly rare endemics (Hughes and Hollingsworth, 2008). The species are known to hybridize (Burt-Utley, 1985), facilitating studies of species boundaries. Primer amplification was tested in seven individuals of the two species (Appendix 1). A subset of polymorphic markers that amplified reliably in both species was then tested for multiplex compatibility by mixing equimolar ratios of each primer. The PCR multiplexes were then tested on a population of each species (20 individuals) to estimate the genetic diversity of the markers. The primer sequences were BLAST searched against the transcriptome sequence of the divergent Asian species *B. venusta* King (sect. *Platycentrum*) to test for likely cross-amplification of primers in other *Begonia* species.

Approximately 15 mg of silica-dried leaf material was extracted using DNeasy 96-sample kit (QIAGEN, Germantown, Maryland, USA). To overcome an

TABLE 1. Characterization of nuclear microsatellites for Central American *Begonia* species.

Locus	Primer sequences (5'-3') ^a	Multiplex ^b	Fluorescent dye	T _m (°C)	Repeat motif ^c	A		Allele sizes (bp) ^d	Putative function ^e	E-value
						her	nel			
Multiplexed loci										
B14329	F: M13-CAACCAACAATGGCAGCTT R: CATGGAGATAATGGAGCTGG	1	FAM	59	(GGA) ₆	4	2	89–104	immunoglobulin E-set superfamily protein	2E-13
B13043	F: M13-CGACATCCCAACCAACCTG R: TTGATAGATGGAAGGTCCG	1	FAM	60	(TC) ₅	1	2	173–179	—	—
BC432	F: M13-AAATCCGATGGATTGACA R: TGAATAAACACACAAACAAGACA	1	FAM	60	(TG) ₅	1	1	261–263	endotrans glucosylase/hydrolase	5E-18
BC344	F: M13-GAGGGAGGTCCTTGTGTAG R: CCGTCTACGTTGCATCATC	1	VIC	60	(GCA) ₅	1	1	105–108	chitinase-like protein	3E-07
BI6278	F: M13-TGTAGTGTGTGTAGTACGAACTTTG R: CAGATGGTCCGGAGATTTG	1	VIC	59	(TCC) ₇	1	3	238–253	DOF zinc finger protein	3E-25
BI5347	F: M13-TCAGTCATTTCTTAATCAGACC R: CTCATCATTTCCAAGCGATTTT	1	VIC	59	(CTT) ₆	2	1	171–183	unknown gene	0.000002
BC552	F: M13-TGCTGAGATGGAACCTGG R: TAGTGAAGGATCCGAATG	1	NED	60	(GT) ₅	2	2	271–273	—	—
BI3348	F: M13-ACTTGTTCCTCGTTGGAGC R: CTGACCCAGTGGATTTAC	1	PET	60	(CT) ₆	3	3	279–283	—	—
BI06534	F: M13-CGTGTCTGCTTAACCCCT R: AGATACAGCCAACCGGATTC	1	PET	59	(TC) ₆	6	2	97–107	sterol 4-alpha-methyl-oxidase 2-1	7E-57
BI7112	F: M13-ATCCAAATGCAACCTCTCGG R: GTGCATTAGATCCCGTGGT	2	FAM	60	(TCC) ₆	2	2	109–115	—	—
BI3820	F: M13-AGGACAGTTTGTGACGGCTA R: GAAGCTTTTGGTCTCTCTGTGA	2	FAM	59	(CTT) ₇	5	2	158–176	LOB domain-containing protein	2E-39
BI1134	F: M13-ATCAGTCACTCCCTATCCCTCT R: TGCAATCTCCTTCGGTCTTT	2	VIC	60	(CT) ₆	4	2	306–314	—	—
BI4004	F: M13-TCAGGAAATATTCGATTGGGA R: GCATTCCTGTGTACAATGC	2	VIC	59	(AT) ₅	2	3	155–169	O-fucosyltransferase family protein	1E-32
BI362	F: M13-CCTTCACTGCGCTGAACAAC R: GAGGGAAATATATGCGGA	2	NED	60	(ATG) ₆	4	4	147–159	Acyl-CoA N-acyltransferases (NAT) superfamily protein	1.00E-45
BC332	F: M13-GAACCAAGGATCAAGGGTTCA R: AAACATGATTTTCCATCCAA	2	PET	59	(TCA) ₅	4	2	188–200	ATPase	1.00E-122
Additional loci tested										
BC672	F: M13-CCTTGATCGAAGAAACCG R: AAAGCCAGCTCCTTCCTGTA			60	(CTT) ₈	3	1	152–158	cellulose-synthase-like C12	2E-57
BI4477	F: M13-GGATCCTCTGCTTTGCTG R: GCGGAGACCAGAAGAAAGTT			60	(CT) ₉	4	2	111–119	—	—
BI06604	F: M13-ATTTTCCACAGAAGACCC R: GCGAAGCCCGAGTATATC			59	(AT) ₈	6	1	111–127	—	—
BI6294	F: M13-TGCTGGTCTGATCTTTAATCA R: TGGGTCTTGGTACTCTTTCC			59	(AT) ₁₀	1 ^M	1 ^M	148	catalytic LigB subunit of aromatic ring-opening dioxygenase family	3E-13
BI6701	F: M13-AGAATCCCACCTCAGTGCAC R: GAGATGATGAGGGTTACAGC			60	(GA) ₆	1 ^M	1 ^M	195	—	—
BI05710	F: M13-GAAAGTTTGGAGGAGCC R: TGAAGAGATCAGAAGGTACA			60	(GAA) ₇	3	1	178–184	—	—
BI4848	F: M13-CGACGCTCTCAAGAAGAA R: GAGCTTTGAATTCGCTACG			59	(AG) ₆	4	2	71–74	arabinogalactan protein	6E-07
BC402	F: M13-TTACTCGAGCTAGAAGCCG R: AGGGCTTGGAGGCTAGAGG			60	(AT) ₅	1 ^M	1 ^M	92	bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	3E-09
BC932	F: M13-GTAGTCCATCAGTCCGCCAT R: GAGTGATGAGGGCAGAGG			60	(GA) ₅	2	1	660–662†	cysteine proteinase superfamily protein	0.000001

TABLE 1. Continued.

Locus	Primer sequences (5'-3') ^a	Multiplex ^b	Fluorescent dye	T _m (°C)	Repeat motif ^c	A		Allele sizes (bp) ^d	Putative function ^e	E-value
						her	nel			
BI3069	F: M13-AACCACAGTAATCATCCGGC R: TGTCGGTAACTGTGGTAA			60	(CA) ₅	1	1	184–192	—	—
BI3377	F: M13-AACACAAATACACCCGGAC R: GAAGAGATGATATGACGAA			60	(AGG) ₅	MP	MP	—	—	—
BI5174	F: M13-GTCGACGGTTGTCTAGGA R: GGAATCAGAGTCTGGCTC			60	(CTT) ₅	1	1	118–121	stromal cell-derived factor 2-like protein precursor	8E-07
BC42	F: M13-GGTATGCAGTTCTGGTGGT R: ACTGGTTGCTACTACTGCCG			59	(TGG) ₆	3	2	147–173	—	—
BI6984	F: M13-GAAGGGTTCTTGGTCTCA R: TTGTCAATTCTCACCAGACACA			59	(TC) ₆	3	2	148–164	—	—
BI7247	F: M13-CTCTTATCCGGCTCAAAGC R: AGCGGAGAAGTCGAAACAG			60	(AG) ₆	1 ^M	1 ^M	135	—	—
BC312	F: M13-ATTTTCTTCTGGCAACGATG R: ATCGGAATCTGAGCCCTGAA			60	(GA) ₅	2	1	178–180	—	—

Note: A = number of alleles per locus; her = *B. heracleifolia*; MP = multiple PCR products amplified; nel = *B. nelumbiifolia*; T_m = primer melting temperature when amplified individually.

^aM13 sequence is: CACGACGTTGTAACCGAC.

^bMultiplex to which the primer was assigned.

^cRepeat motif in *B. plebeja*.

^dThe observed range of PCR product sizes excluding the M13 motif.

^ePutative function in *Arabidopsis*.

^MMonomorphic in all individuals tested.

† Large product size assumed to be caused by an intron.

unknown PCR inhibitor that coelutes with DNA extractions in *Begonia*, extractions were diluted 100-fold with Millipore dH₂O to a final DNA concentration of ~0.1–1.5 µg/mL. PCR reactions were performed using the M13-tailed primer method (Schuelke, 2000) in a final reaction volume of 10 µL containing: 0.5 µL of 1 mM M13-tailed forward primer (Invitrogen, Grand Island, New York, USA), 1 µL reverse primer (1 mM), 1 µL of 1 mM M13 fluorescently modified primer (6-FAM, VIC, NED, PET), 0.25 µL bovine serum albumin (BSA, 0.4%), 1 µL of 10× reaction buffer, 1 µL of 2 mM dNTPs, 0.6 µL of 25 mM MgCl₂, 0.05 µL BIOTAQ polymerase (Bioline, London, United Kingdom), 1 µL dilute DNA template, and made up to the final volume using dH₂O. PCR cycles consisted of an initial denaturation of 1 min at 95°C, followed by 40 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 57°C, and extension for 1 min at 72°C. Five microliters of each PCR product labeled with the four fluorescent dye colors was pooled and diluted 2× in Millipore dH₂O, and the GeneScan 500 LIZ internal size standard (Applied Biosystems, Foster City, California, USA) was added prior to fragment analysis on the ABI 3730xl analyzer (Applied Biosystems; analysis was performed at GenePool, University of Edinburgh, Edinburgh, United Kingdom). Fluorescent traces were analyzed automatically with manual editing using GeneMapper version 4.0 (Applied Biosystems).

A total of 136 primer pairs were located in the *B. plebeja* transcriptome using the QDD bioinformatic pipeline (Appendix 2). All 31 of the subset of primers tested for amplification yielded a PCR product (Table 1). Sixteen loci had a significant (<E-5) BLAST match in the TAIR database (Table 1). Of these loci, four loci were monomorphic (BI6701, BC402, BI6294, and BI7247) and one amplified multiple PCR products (BI3377). Two PCR multiplex reactions were designed to amplify a total of 15 polymorphic loci (Table 1). All loci were polymorphic in at least one of the populations tested, and showed moderate genetic diversity, with the number of alleles per species ranging from one to five and the expected within-population heterozygosity between 0 and 0.75 (Table 2). Twenty-one of the 62 primers (34%) had perfect BLAST matches in the transcriptome of the divergent *B. venusta*, including both the forward and reverse primers for loci BI3348, BC932, and BC552.

CONCLUSIONS

We have described the development of nuclear microsatellite primers that amplify in two divergent Central American *Begonia* species. Some of the primers have exact BLAST matches in the transcriptome of the Southeast Asian species *B. venusta* and, therefore, may be transferable more widely across the genus. The transferability of markers is important for the study of natural hybrids, and the development of a

TABLE 2. Genetic diversity in population samples of *Begonia heracleifolia* and *B. nelumbiifolia*.

Locus	<i>B. heracleifolia</i>			<i>B. nelumbiifolia</i>			A _t
	A	H _o	H _e	A	H _o	H _e	
BEI4329	3	0.400	0.524	3	0.500	0.537	5
BEI03043	4	0.000	0.444	3	0.500	0.630	4
BEC432	2	0.100	0.097	2	0.000	0.097	3
BEC344	1	—	—	2	0.000	0.097	2
BEI6278	1	—	—	3	0.353	0.668	4
BEI5347	3	0.300	0.449	1	—	—	4
BEC552	1	—	—	3	0.050	0.229	3
BEI3348	4	0.579	0.604	4	0.500	0.665	5
BEI06534	5	0.500	0.750	4	0.105	0.201	7
BEI7112	2	0.400	0.467	3	0.278	0.522	4
BEI3820	5	0.600	0.623	2	0.000	0.108	6
BEC134	4	0.611	0.732	3	0.050	0.145	5
BEI04004	2	0.059	0.059	3	0.188	0.623	4
BIC362	2	0.050	0.050	2	0.000	0.097	2
BEC332	4	0.250	0.483	3	0.154	0.495	5
Mean	3.333	0.321	0.440	2.857	0.191	0.365	4
SD	1.155	0.228	0.246	0.663	0.199	0.243	1.327

Note: A = number of alleles per locus; A_t = total alleles observed in the two species; H_e = expected heterozygosity; H_o = observed heterozygosity.

multiplexed assay of 15 loci should enable accurate assignment to hybrid classes (e.g., F1, backcross). Future studies will use these loci to estimate the genetic structure of populations, the frequency of hybrids, and the extent of introgression in hybrid swarms.

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APPENDIX 1. Information on Mexican *Begonia* voucher specimens deposited in the herbarium at the Royal Botanic Garden Edinburgh (E). Information presented: taxon, collection number, collection locality, GPS coordinates.

Begonia heracleifolia Cham. & Schldl.: AT48, San Andrés Tuxtla, 18.47850, -95.17802; AT244, Agua Azul, 17.22117, -92.11073; AT375, Ocozocoautla, 16.90533, -93.45153; AT505, Berriozábal, 16.86693, -93.32781; AT819, Santa María Jacatepec, 17.85819, -96.21853; AT922, Motzorongo, 18.66953, -96.78714; AT1080, Santa María Xanabi, 15.98808, -96.11061.

Begonia nelumbifolia Cham. & Schldl.: AT28, Los Tuxtlas, 18.59026, -95.07876; AT125, San Andrés Tuxtla, 18.50660, -95.16607; AT619, Huatusco, 19.20367, -96.74256; AT683, Josaa, 16.01419, -96.11289; AT771, Los Cantiles, 17.74356, -96.32803; AT958, Motzorongo, 18.66953, -96.78714; AT1029, San Jeronimo Zochina, 17.22117, -95.23547.

APPENDIX 2. Microsatellite loci in the transcriptome of *Begonia plebeja*.

Locus	Forward primer sequence (5'–3')	Reverse primer sequence (5'–3')	Repeat motif
BC134*	ATCAGCTCACTCCCTATCCTCT	TGCAATCTCCTTCGGTTCTT	(CT) ₆
BC192	AAGTCAAACCTGTTGACCCG	ATCCTCATCGGATTCGTCAT	(GAT) ₉
BC232	TGGAAATGCTGTCGTTGAAT	ATGGGAGAAAAGGCAAAGCA	(TCT) ₈
BC312*	ATTTCTTCTGCGAACGATG	ATCGGAACCTCTGAGCCTGAA	(GA) ₅
BC332*	GAACACAGAAGTCAAGGGTTCA	AAACATGATTTTCTCATCCAA	(TCA) ₅
BC344*	GAGGGAGGGTCCCTTGTTAG	CCGTCTTACGTTGCATCATC	(GCA) ₅
BC362*	CTTACCTCGCCTGAACAAC	GAGGCGAAATATATGCGGA	(ATG) ₆
BC42*	GAAGGGGTTTCTTGGTCTCA	TTGTCAATCTCACCAGACACA	(TG) ₆
BC402*	TTACTCGAGCTAGAAGCCGC	AGGGCTTGGAGAGCTAGAGG	(AT) ₅
BC432*	AAACTCCGATGGATTCAGCA	TTGAAATAAACACACAAACAAAGACA	(TG) ₅
BC532	TCATTCGGCTTCTATGCTCC	CGTCATCGTCAATATCATCCTC	(TGA) ₆
BC552*	TGCTGAGATGGAAACTGCG	TAGTCGAAGGGATCCGAATG	(TG) ₅
BC602	GCAAAGCAGGTAACCTTTAGCC	ACTCACGGAACCTTGGCAAC	(CAG) ₅
BC632	CATAGCGCTCAGCTTGCTC	GAGATCTTATACGAGCTACTGGATAGT	(TC) ₉
BC643	GGAGGAGCTCGGTCATTAGA	AACCACCGGTACCTCATTT	(CT) ₆
BC652	TTTCGTCATGAAGAAAGGC	TCCAGGGAACCTCATCACTC	(GAA) ₅
BC672*	CCTTGATCGAGAAAGAACCG	AAAGCGAGCTCCTTCTGTA	(GAA) ₈
BC692	AACATGGCCGTCAGTGTCC	CAGGCAGACAAAGAAGATCC	(AG) ₁₁
BC752	GGCAGATTTTACTGGGACGA	CGCCCATCTATCTGTATCCAA	(TTC) ₅
BC762	CAACTCTGCAAATGCAAGGA	ACCCATGACAGCATGAACAA	(CT) ₅
BC932*	GTAGTCCATCAGTCCGCGCAT	GAGTGATGAAGGCGAAGAGG	(GA) ₅
BI0537	CAGATCAACCTCTTCTCTGC	ATCGAAAACCCATTTGACTGC	(CCT) ₆
BI1195	TGCTGCAGAACTTTAGCCA	CGGTGATTAAGAAGAGCAAGAA	(GA) ₁₁
BI1430	CACAATTCGTGAAAACACGG	TTCTGCATGATGTTGGCTTT	(GA) ₅

APPENDIX 2. Continued.

Locus	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Repeat motif
BI1733	GTTCCACCACTCCAATGGCTT	CGAGTTTGCCTTCGAATCTC	(GCCACA) ₅
BI1816	GTTTTGCGGTTGAGTTTGGT	CAAATGAATCTTCTCATCCAGTG	(GAT) ₇
BI1937	TCATCATCGCAGCAGAAGAC	CGAAGCTGGGAGTGAGTTTC	(GGA) ₆
BI1948	CAAAACTGGCTTTGCAGACA	CACGGGCACTTTCAATTTCT	(TA) ₅
BI2413	GAATGAAGAGCGAATCGACG	CAGAGCTCCGGAATCTCATC	(AGA) ₅
BI2675	TTCCATTTACTCTCAGCCGC	CGTTCTCTTCGAGGACTTG	(GA) ₇
BI2875	CCCAATCTCCCTGTCTATCG	AAGCTGACGAAGCTCTTCCA	(TC) ₅
BI2935	TGGAAGAAGGTCTCCATATAAGTCA	CATGTTTTCTTCGCCATTC	(CAC) ₉
BI2946	ATTTGAAGCCATTGGGTCTG	AAGACGGGAAAGGGTGAGAG	(TC) ₆
BI2961	TCGCAAAAAGAAGAAATCACAAA	TCCTCCGGCACAATAATCTC	(GAA) ₆
BI2967	GGTGGCTTGACGGTGAGAT	TCGATTTCTCAAAATGCCATCA	(GAA) ₅
BI2994	GATTTCCGTGGAGGAAACAA	AAACATCACAGAGCACAACA	(CT) ₅
BI3043*	CGACATCCAACCAACCTG	TTGATAGATGGAGGGTTCGC	(TC) ₅
BI3069*	AACCACAGTAATCATCCGGC	TGTCCGGTAACTGTGGTGAA	(CA) ₅
BI3131	ACATTTGTGTTCAATGGCGAA	GAGCTCATGCAATGCTTCAA	(GAA) ₆
BI3233	TATGAAGGACGTGGGAGGAG	GGGAATCAGAAGCCAATCAA	(GA) ₅
BI3234	AAACAAGGAACGCTCAATCC	GCTCGAGTTGGCTTCATTTTC	(AG) ₅
BI3286	CCTATGATGATAGCGTCCGA	AGGCCGACATTTCTTTCCCT	(CT) ₁₀
BI3301	GCATGGAGATTGCCGAGATTT	CTATTGCTCAGCGGAGAGG	(GAA) ₅
BI3348*	ACTTGTTTCTCGTTGGGAGC	CTGCAGCCAGTGGATTTAC	(CT) ₆
BI3377*	AACACAATCATCAGCCGGAC	ACGAAGGAGATGATTATGACGAA	(AGG) ₅
BI3384	ATAATTTGGGCTAGGGTTCCG	GCTTTTGGTTGCTTCAGAGG	(TC) ₅
BI3403	TGTAGGAACAACGGTTAGCG	CGTAGAGACGATTTCCCTTAGCC	(GAA) ₆
BI3519	TTCAGAGCGCTTTTGGTTTT	ACGCACATGCCCCTTCTTCT	(TA) ₆
BI3553	TCTGAAATAGCACCCGCTTCC	TTTCTTCGATGACGCACTG	(AAG) ₅
BI3600	CATTATTTCCCTGTCGGGACG	TGCTGAAAAGTTGCAGGAAA	(TGTT) ₅
BI3727	CCTCCACCAGATTTGCTTAAA	AACAGAAACATTTGCCGGTG	(TC) ₁₂
BI3741	GCAACACAGCTCCTCTTCTG	GGTCGGAATCGTCGAGTAAA	(CT) ₇
BI3820*	AGGACCAGTTTTCGACGGCTA	GAAGCTTTTGCTCTTCTGTTGA	(CTT) ₇
BI3865	ACCTCACTCAACCCGCATAG	TTCAGCATCTGTTGCAGGAC	(CT) ₅
BI3970	TGTGTTCACTCAATTTCTGCCA	TCCTTCACCTGAGACGACAA	(TC) ₅
BI4004*	TCAGGAAATATTCGATTGGGA	GCATTCCTCTGTGTACAATGC	(AT) ₅
BI4013	AAGCCAAGATACCCCAAAGG	CGCTTTGCTCTTCTTCTTG	(AG) ₅
BI4021	TGTGTTGCCCTGCAAGTAGA	GGAAACCTTTTCAGAGCTCCA	(AG) ₅
BI4028	GTCTTCTCCCATCCTGTTGAA	GGGCTTTGGAAACATCTCCT	(CT) ₁₅
BI4031	TCTTCGCTCTAAAGGCTTGC	AAATTTTCGCCAAACATGGAG	(TC) ₅
BI4088	GGTTTCGAGATATGGCCTCA	TTGGCAATTTATCCCTCCTC	(GGC) ₅
BI4128	AAGACAACGCCATTTCCAAAC	AGGGACGACCCGGAAGTAGAG	(CT) ₅
BI4166	CGGGACAATGTTAAGCGAT	CAATAAAGAACTTCCGGCGA	(TG) ₅
BI4175	GGCGATCAAAGGGTGATTTA	CGATTAGCCTCTTCTCGACG	(AG) ₅
BI4233	ATGCAGACGTAATCGAAGGC	CAAGTTGGTTGGCAAAGACA	(AG) ₁₂
BI4279	GGGAGGAAGAGGAAGAAGCA	TCAGATTTCAGCGTCATCAGAA	(AGG) ₆
BI4329*	CAACCAACAATGGCAGCTT	TCATGGAGATAATGGAGCTGG	(GGA) ₆
BI4360	CCGCAGATCCTCCATTAGAA	TTATGTCCCAAACCTCCGCTC	(TGT) ₅
BI4477*	GGATCTCCTCTGCTTTGCTG	GGCGAGACCAGAGAAAAGTT	(CT) ₉
BI4594	CCAGAATCGTGGTCACTTCC	CGTGAATCGAAACTTCTCCC	(TC) ₉
BI4600	GCTATGGGAAGTTGCTTGGA	AGCTTTTCCCTCCCTTTCTGG	(AGA) ₇
BI4641	GCCACAGTTTTAGCTGTGCTAT	CTGCAACCACGAGGAGTTTA	(CT) ₅
BI4721	ACTACCCTCCCAAGGCTGTT	GGCCAGAGTCAAACCTCAA	(TC) ₈
BI4740	AGGCACCCTCCCAAAGTAAT	GCCTGTATCTGAAATGGCA	(GA) ₇
BI4746	GTCCGAGTCAGCGAGGGA	TGATCCTATGCACCTCGTGGT	(AG) ₅
BI4779	CGAAGGAGGAAGAGACGATG	TGGCACTATAATTTCCAAGCTCC	(AACG) ₅
BI4793	CAGTCCCCGCACTAATCTTC	GAAAGACCAGCTTCGTTTGC	(GA) ₅
BI4804	TCGCTGATGATTTGTTTGGA	AGAATGCCGACGAAATTTGAG	(TCT) ₁₀
BI4848*	CGACGCCTCTCAAAGAAGAA	GAGCTTTGAATTTTCGCTACG	(AG) ₆
BI4899	CCCATTTGCTTCCAAAACAT	GAGTCGAGGAGCAGCACTCT	(GAA) ₅
BI4987	AGTGAAAACCTTTGGCACCAC	ACCCTTTTCTTATCCACGG	(GAG) ₅
BI5091	TGCTTTCCAGGTTTCATAGGG	GGCAAGCTTGGAACTTTTGT	(AGA) ₇
BI5107	CGCGTTTACATGGCTGAAT	CGATTGAAAACCTTGAAGATGA	(AT) ₅
BI5115	AGACCAGTACCCGAACAATC	TCCGTCGTTTCTAACCCTTC	(TC) ₅
BI5162	CTCTGAAACTCGCTCATCCC	GCTCTTTCCGTCCTCATTTGC	(AGG) ₅
BI5174*	CTCGCAGGGTTTGTCTAGGA	GGAAATCAGAGTGTGGCTC	(CTT) ₅
BI5285	GGTCAAATGGGTAACATGCC	CTGGTTATCATCGCTGCTA	(GGT) ₅
BI5317	GCCCTCAAGTTCCCTCCATCT	GGGACCGTCGATTATTCTCA	(AT) ₅
BI5325	TTCCGGACTGAAAGAAATGG	CGTGAGTGGAGTGGTATTTG	(TC) ₅
BI5347*	TCAGTCCATTTTCTTAATCAGACC	CTCTATCATTTCCAAGCGATTTT	(CTT) ₆
BI5377	ATCCTTCTCCTATCCACCGC	GGGAGACGGTGAACCTCTGA	(TC) ₅
BI5414	GCAAAGCAAAGCTGAAAACC	GGCCAGTCTACCTGCAATA	(AT) ₅
BI5423	GCTTCCAATGATGCAACCTT	GAGAAGCCGAGGAGACTTA	(AG) ₅

APPENDIX 2. Continued.

Locus	Forward primer sequence (5'–3')	Reverse primer sequence (5'–3')	Repeat motif
BI5561	GTTGACTCGTCTCGTCTCC	GTCGTTTCTGCCGATTCTTC	(CTT) ₅
BI5588	CAGCTGGTTGAGAAACGTGA	AATCATATCGCCGATCAAGG	(TC) ₅
BI5593	ACTCCAATTAGGTGCGTGG	AGATAACGAAGCAAAGCGGA	(AG) ₉
BI5638	GCTTCTTCGTCTCTTCTTCC	TTACGGCTCCAGATTCTGCT	(TCT) ₇
BI5668	TATGGGTCCGGATATGGAAA	AGGAAGAGCTCGAAGAAGCC	(GCG) ₅
BI5710*	GAAAGTTTTGGAGGAAGCCC	TGGAAGAGATCAGAAGGTACA	(GAA) ₇
BI5800	CGCCTCCATATCTCGTAAA	GGAAGGTGATGTTGTTGCT	(TCT) ₅
BI5813	CGGTAGATTGAATGGGGAGA	AGCATCGCTCAAGTTGCTCT	(AG) ₅
BI6067	CAGCTTGGAAAATCAGACCC	AGGGGCGTAAGCATAAAGGT	(TA) ₅
BI6141	GTCGCCATGACGATAAGGTT	TCTGACCTGAAGATGGACC	(AG) ₁₀
BI6227	GACGCGACGAAGATAAGGTA	ATACACGGAGGAAGCATA	(TCT) ₅
BI6278*	TGTAGTTGTTGTAGTAGCAGAACTTTG	CAGATGGGTCCGAGATTTTG	(TCC) ₇
BI6294*	TGCTGGTCTGAATCTTAAATCA	TGGGGTCTGGTACTCTTTCC	(AT) ₁₀
BI6299	CATCGCTCTATGAAGCTGCTACT	CCTGAGACCCTGCTATTCCA	(AT) ₅
BI6399	CTGTATCATATCCCATCACA	CAGTGAACATGCGAGGTCA	(TC) ₅
BI6422	TTTGATGGAGAAGATTAGTGAGAAGA	AGGCGGAATACCTTGTCTTT	(TTC) ₅
BI6423	ATATTGGACATGCCAGCACA	CATGAAACAAGACTCTGGAGAA	(AG) ₅
BI6469	TCTAGGCGCCAAAAGAAA	CTCCCTCATCACTTGCAGAT	(GA) ₁₃
BI6534*	CGTTGCTCTGCTCTAACCTT	AGATACGGCAACCGGATTC	(TC) ₆
BI6535	AAAGGGGAAAGCAAGGAAA	GGGATGGATGGCTGATTAAA	(GAA) ₇
BI6561	CTTCTGAGACTCGTACCGGC	TAGCTCGGTTCAAACACCC	(GTG) ₅
BI6581	TTGCTTTTCCTTTCTCATCCA	CCGATCCAGCTCTATCAGC	(TTC) ₆
BI6604*	ATTTTTCACAGAAAGAGCC	GGCAGAAATCGAGTATATC	(TA) ₈
BI6605	TCAAAGCTTCGTTCCCATTC	GGAAAGCGTCAGAGTTGAGG	(TTC) ₅
BI6701*	AGAATCCCCACTCACTGCAC	GAGATGATGAGGGTTCAGGC	(GA) ₆
BI6717	GATCTCGGGGATTTGGATTT	ACTGCCATAGCCTCCATCAC	(GTG) ₅
BI6761	TGTTCTTCGGCTCTCCACTT	ACATGCTCTTCTGGCTTGT	(TC) ₅
BI6776	CCAAACAGCAAAACTCTTCG	GTTTTGTGGAAGGGTGGCTA	(AG) ₅
BI6828	TCGTCTCCTTCTTCGTCTCC	GGTCGTCTGCTGATTCTTC	(CTC) ₅
BI6849	CCTCAGATCCAGAGGAAGGG	GCGCCTTTTCTTTAAGTCC	(TA) ₆
BI6886	TCTTCTCACGGCTCTCCATT	TGGAAATCAAGGAAAGCACC	(CTT) ₅
BI6901	CGAACTGGAAGAAGACTACAATCA	GCTGCAGCACGGAGTTTGTAG	(AG) ₈
BI6984*	GTATGCAAAGGAGAGCCGAG	TTGTCAATTCTCACCAGACACA	(TC) ₆
BI7015	TGGTCCAGATTATGATCAGCC	TCTTCTCCGATTCCGATCAC	(GAA) ₅
BI7023	TAAAGGCGGTGACACAGAGA	CCTTTCGTCTGCAAATGGAT	(GAA) ₅
BI7036	TTGAGCAGGCTTCCAAACTT	ATTGCAAGGAAGAACCGGC	(CTT) ₅
BI7059	CTCCCTCCGACCTCCATAAC	TAGCCTTCTGCGGAGTGTTT	(CT) ₅
BI7085	ACTCGGAATATCTCCGAAA	CACCTCTTACAGTCTGCTCC	(GA) ₅
BI7112*	ATCCAATGTCAACCTCTCGG	GTGCATTAGAGTCCCGTGGT	(TTC) ₆
BI7149	CGGAGAATCGAACCTCTGAT	CCCTGAACGATGGAACTCAT	(CT) ₅
BI7165	AATGAGCACGAACCTGCTTT	GAGGAATTTGGACCGTCTGA	(AG) ₅
BI7247*	CTCTTATTCCCGCTCAAAGC	AGCGGAGAAGTCGAAAACAG	(AG) ₆
BI7287	TTGGGGACAACAAATGATGA	CAGTGCTTCTTTAACAAACGCTT	(TGA) ₅

* Indicates markers tested for amplification and polymorphism.