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Research Article

Development of Novel Polymorphic EST-SSR Markers from the Cranberry Fruit Transcriptome

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Background: Cranberry (*Vaccinium macrocarpon* Ait.) has high developmental prospects and great research value. Cranberry has a narrow genetic base, however, its morphological characteristics are not easily distinguishable. Besides, traditional breeding methods are limited, and breeding progress on cranberry cultivars has been slow.

Objective: The objective of this study was to assess polymorphic EST-SSR markers developed from a cranberry fruit transcriptomic sequencing library to provide candidate EST-SSR sequences for future research on stress resistance breeding of cranberry.

Materials and Methods: Thirteen cranberry accessions were used for EST-SSR analysis, and 16 accessions of other *Vaccinium* species were used to test primer transferability. Genomic DNA was extracted from young leaves of 6-year-old cranberry plants and subjected to PCR amplification. A binary matrix was established and analyzed in NTSYS-pc v.2.10e for calculation of the genetic similarity of cranberry cultivars and construction of a cluster dendrogram.

Results: A total of 47 stress-resistance-related primer pairs were designed, of which 7 pairs showed polymorphism. The average number of effective alleles was 1.844, and the average expected heterozygosity was 0.455. The average transfer rate was 63.39%. Genetic similarity coefficients ranged from 0.28 to 1.00, with an average of 0.76. UPGMA clustering divided the 13 cranberry accessions into four groups at a genetic similarity of 0.74.

Conclusions: The seven polymorphic EST-SSR markers were able to reveal genetic relationships among 13 cranberry accessions and can be used for future research on stress resistance breeding of cranberry.

Keywords: Cranberry; EST-SSR markers; Genetic diversity; Transferability

1. Background

Cranberry (*Vaccinium macrocarpon* Ait.) is a perennial, evergreen, fruit-bearing shrub in the family Ericaceae. Native to North America, cranberry prefers strongly acidic, and humid soil and has strong resistance to cold. Within the genus, *Vaccinium*, cranberry, lingonberry (*V. vitis-idaea* L.), and blueberry (*Vaccinium* L.) have been successfully domesticated and are currently the most promising small berries. Cranberry fruit contains a variety of vitamins and organic acids in addition to abundant proanthocyanidins, anthocyanins, and flavonoids. The fruit has cancer-preventing and anti-inflammatory effects. Because it is extremely sour, fresh cranberry fruit is mostly processed into fruit juice, jam, fruit wine, and other products (1-4).

The growing importance of cranberry and its products has created a demand for new cultivars. Although the genetic base of cranberry is relatively narrow (5), however, morphological characteristics (including fruit size and stem and leaf features) are not easily distinguishable among cultivars (6); also, traditional breeding methods are limited. Consequently, breeding progress in cranberry has been slow. Although most cranberries were selected from the Americas 100 to 150 years ago, 'Early Black', 'Howes', 'McFarlin' and 'Searles' are the only cultivars in broad production (7). The use of molecular markers to reveal cranberry genetic relationships and diversity is therefore particularly important for future genetic mapping, gene localization, and innovations in cranberry.

As an effective tool for analyzing the diversity of germplasm resources, molecular marker technology is currently the most convenient method for the determination of genetic relationships and the study of species origins and evolution (8). Simple sequence repeat (SSR) loci, or microsatellite markers, can be detected in both coding and non-coding regions of a gene. The advantage of SSR markers is that they are co-dominant markers conforming to Mendelian inheritance with good site-specificity, high levels of polymorphism, and abundance. SSRs are widely distributed in the genome (9-10). Because of these characteristics, SSRs are important molecular markers for genetic mapping, gene localization, and the determination of genetic relationships. Expressed sequence tags (ESTs), which are located in gene transcriptional regions, can be used to more easily pinpoint functional genes controlling important agronomic traits and thus directly identify meaningful phenotypes. EST-SSR markers are therefore a preferable tool for genetic analysis. Schlautman *et al.* (2010) (11) identified 29 cranberry genotypes based on 697 polymorphic EST-SSR loci. Using a combination of inter-simple sequence repeat (ISSR), EST-SSR, and EST-PCR markers, Dong *et al.* (2015) (12) revealed the genetic diversity and inter-relationships of 5 cranberry cultivars and 102 wild cranberry clones collected from four Canadian provinces.

2. Objectives

Taking into account the current availability of cranberry germplasm resources and the actual needs of breeding projects, we used a cranberry fruit transcriptome library (13) to construct a novel cranberry EST-SSR molecular marker system. The stress-resistance-related EST-SSR molecular markers uncovered in this study should be useful for future research on stress resistance breeding of cranberry.

3. Materials and Methods

3.1. Plant Materials and DNA Extraction

Genomic DNA was extracted from young leaves of 6-year-old cranberry plants by the modified cetyltrimethylammonium bromide method (14). 13 cranberry accessions were used for EST-SSR analysis, and 16 accessions of other *Vaccinium* species were used to test primer transferability (**Table 1**). The integrity of the extracted DNA was checked by 1% agarose gel

Table 1. Information of 13 cranberry accessions used for EST-SSR analysis and 16 *Vaccinium* spp. accessions used for transferability analysis

Family	No.	Accessions	Origin
V. macrocarpon Ait.	1	Pilgrim	America
	2	Stankavich	America
	3	Howes	America
	4	Bergman	America
	5	WSU108	America
	6	Brewer	America
	7	Bain Fav.No.1	America
	8	Bain11	America
	9	LeMunyon	America
	10	Hollister Red	America
	11	Mathewes	America
	12	Bain6	America
	13	Washington	America
V. vitis-idaea L.	14	Sunna	Sweden
	15	Unknown	
Vaccinium spp.	16	Blomidon	America
	17	Fundy	Canada
	18	Chignecto	Canada
	19	Emil	Unknown
	20	Northland	America
	21	Bluecrop	America
	22	Patriot	America
	23	Coville	America
	24	Elliot	America
	25	Dorrow	America
	26	Jersey	America
	27	Duke	America
	28	Reka	New Zealand
	29	Puru	New Zealand

electrophoresis. DNA concentration and purity were assessed on an ultramicro UV spectrophotometer.

3.2. Acquisition of Transcriptomic ESTs and Mining for SSR Loci

We used MISA software (15) to search for SSR loci in a cranberry fruit transcriptome library. The following search criteria were used: sequence length³ 150 bp, and the number of mono-, di-, tri-, tetra-, penta-, and hexanucleotide repetitions equal to at least 1, 2, 3, 4, 5, and 6, respectively. The minimum repeat units were 12 repeats for mono-, 6 repeats for di-, 5 repeats for tri- and tetra-, 4 repeats for penta- and hexanucleotide.

3.3. Gene Ontology (GO) Functional Annotation

Gene Ontology (GO) functional annotation was performed using BLASTx (*E*-value < 0.00001) against GenBank (http://www.ncbi.nlm.nih.gov/) NR database. GO annotations obtained were further analyzed using Blast2GO (16). Sequences in the GO database related to "response to stimulus" were selected as candidate EST-SSRs.

3.4. Design of EST-SSR Primers and Microsatellite Amplification

Primers to amplify the selected EST-SSRs were designed using Primer 3.0 according to the following criteria (17): primer length = 18–28 bp, amplification product length = 80–300 bp, annealing temperature = 51–58 °C, and a difference in annealing temperature between forward and reverse primers < 2 °C. PCR amplifications were performed in 20-µL reaction volumes consisting of 12.9 µL ddH2O, 2.5 µL of 10× PCR buffer (containing Mg^{2+}), 2.0 µL dNTPs (10 mM), 0.5 µL genomic DNA (100 ng.µL-1), 1 µL each of forward and reverse primers (10 mM) and 0.1 µL *Taq* polymerase (5 $U.\mu L^{-1}$). PCR cycling conditions, which were based on Rowland *et al.* (18), were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of 94 °C denaturations for 40 s, annealing for 40 s at the optimal temperature (**Table 2**) and extension at 72°C for 40 s, with a final extension at 72 °C for 10 min. The PCR amplification products were detected by 10% non-denaturing polyacrylamide

Table 2. Information and amplification conditions of 7 pairs of polymorphic primer

No.	Primer name	Repeat motif	Primer sequences (5'-3' Forward-Reverse)	Annealing temperature (°C)	Expected size (bp)	Number of alleles	Size range of alleles	Putative linked function
1	CL2216.Contig1	(TGG)5	F: 5'-CCTAGGAGTATGTTTGCAGAGGA-3' R: 5'-AATCCTCCAAACTACAAGGCAAC-3'	55	148	2	147-207	Chaperone protein dnaJ [*Medicago truncatula*]
2	CL2216.Contig4	(TGG)5	F: 5'-AATCCTCCAAACTACAAGGCAA-3' R: 5'-CCTAGGAGTATGTTTGCAGAGGA-3'	55	148	2	145-200	Chaperone protein dnaJ [*M. truncatula*]
3	CL2296.Contig2	(TC)6	F: 5'-CAATGGTGATAACTCCCTCTTTG-3' R: 5'-ACATCTGAAGAAATAGCCCAGGT-3'	55	115	3	190-1000	chaperonin CPN60-2, mitochondrial isoform 1 [*V. vinifera*]
4	CL3473.Contig1	(TCT)6	F: 5'-TCTTCTCAACTTCCGTGTCTCTC-3' R: 5'-GTCCCGTACAGTAAATCAACCAG-3'	52	112	2	112-198	beta-amylase 1 [*Nicotiana langsdorffiix N.sanderae*]
5	CL3889.Contig2	(GAA)7	F: 5'-TTTTCTTCCTCTCTGCAATTCTG-3' R: 5'-GGTCTGTTCATCGGAGGCT-3'	52	155	3	155-336	DEAD-box ATP-dependent RNA helicase 38-like [*S. lycopersicum*]
6	CL993.Contig1	(TC)6	F: 5'-GTCAGCTCACATTTCCAAACCTT-3' R: 5'-ATTCATCTGATTTTGTTGGGTTG-3'	54	122	3	98-277	Spotted leaf protein, putative [*R. communis*]
7	CU15788	(AG)6	F: 5'-TCAATCATCACAATCTACACTCCC-3' R: 5'-CGATTGATGATGGTACGAATTTT-3'	52	129	3	127-182	syntaxin-related protein Nt-syr [*N.tabacum*]

gel electrophoresis and visualized by silver staining using an Alpha gel-imaging instrument.

3.5. Data Analysis

The size marker of DNA bands was a 20 bp DNA ladder (Dye Plus). Bands having the same migration distance as the target fragment size were recorded as 1, and non-amplified bands were recorded as 0. The resulting binary matrix was analyzed in NTSYS-pc v.2.10e to determine cranberry cultivar genetic similarities and construct a cluster dendrogram. Using the EST-SSR data, we calculated effective allele number (*Ne*), expected heterozygosity (*He*), Shannon's information index (*I*), and polymorphism information content (*PIC*) values in GenAlex 6.5.

4. Results

4.1. GO Analysis of Unigenes Containing SSR Loci

In this study, 1,462 unigenes containing SSR loci, identified from 57,331 unigenes in a cranberry fruit transcriptome library, were subjected to GO functional annotation. A total of 640 unigene sequences were successfully annotated, of which 462 were assigned to two or more functional categories. Unigenes with functional annotations were divided into three major GO categories—biological processes (452; 30.92%), molecular functions (447; 30.57%) and cellular components (437; 29.89%)—and 35 sub-categories. At the sub-category level, the largest proportion of unigenes was assigned to cell parts (49.22%), followed by binding reaction (46.25%), catalytic reaction (44.06%), metabolic process (35.47%), and cellular process (31.72%). Besides, 167 (26.09%) of unigenes were putatively related to response to stimulus (**Fig**. **1**).

4.2. Polymorphism Analysis

EST-SSR sequences annotated with the GO biological process term "response to stimulus" were selected and used to design primers. Using design criteria previously described, 47 pairs of resistance-related primers were designed to amplify 238 SSR loci. The 47 primer pairs were tested using 13 cranberry accessions; 31 and 7 pairs were found to be monomorphic and polymorphic, respectively (**Table 2**), while 9 pairs yielded no amplification bands (**Fig. 2**). A total of 38 primer pairs generated fragments of the expected size. The effective amplification rate was 80.85%, and the polymorphism ratio was 18.42%. Seven pairs of polymorphic SSR primers amplified a total of 72 alleles, with 7 to 12 alleles amplified per locus. On average, 10.29 alleles were detected per primer pair (**Table 3**).

4.3. Genetic Diversity Analysis

A statistical summary of the seven polymorphic primer pairs is given in **Table 3**. Band frequencies ranged between 0.538 and 0.923, with an average of 0.791. The most frequent bands were CL3889. Contig1 and CU15788, and the least frequent was CL993. *Ne* ranged from 1.669 to 1.997, with an average of 1.844; *I* varied from 0.590 to 0.692, with an average of 0.647, and *He* ranged from 0.401 to 0.499, with an average of 0.455. Among the seven polymorphic primer pairs, CL2216.Contig1 had the highest values of *Ne*, *I,* and *He*, and CL3889.Contig2 and CU15788 had the lowest. *PIC* values ranged from 0.0408 to 0.469, with an average of 0.224. The primers with the highest and lowest *PIC* values were CL3889.Contig1 and CU15788, respectively.

4.4. Cluster Analysis

The genetic similarity of the 13 cranberry cultivars was analyzed using the seven polymorphic EST-SSR markers. Genetic similarity coefficients ranged between 0.28 and 1.00, with an average of 0.76. At a genetic similarity of 0.74, cluster analysis divided the 13 cranberry accessions into four groups (**Fig. 3**): I) 'Pilgrim', 'Stankavich', 'LeMunyon' and 'Bain6'; II) 'Bergman', 'Hollister Red', 'Washington', 'Bain Fav. No.1' and 'Bain11'; III) 'WSU108', 'Mathews' and 'Brewer' and IV) 'Howes', the latter with a genetic similarity coefficient to the rest of the accessions of 0.57. Similarity coefficients between 'Hollister Red' and 'Washington' and between 'WSU108' and 'Mathews' were 1.

4.5. Primer Transferability Analysis

The transfer rate of EST-SSR markers depends on the degree of genetic or evolutionary tightness of the species studied (19). Analysis of the seven pairs of polymorphic primers in different *Vaccinium* species yielded an average transfer rate of 63.39%, which indicates that the primers were highly transferable. This level is consistent with the 30% to 100% transfer rate previously reported for EST-SSR markers in *Vaccinium* (20). As shown in **Figure 4**, transfer rates were 100% for the two lingonberry cultivars and the half-high blueberry cultivar, 67.86% for the four lowbush blueberry cultivars, and 46.03% for the nine

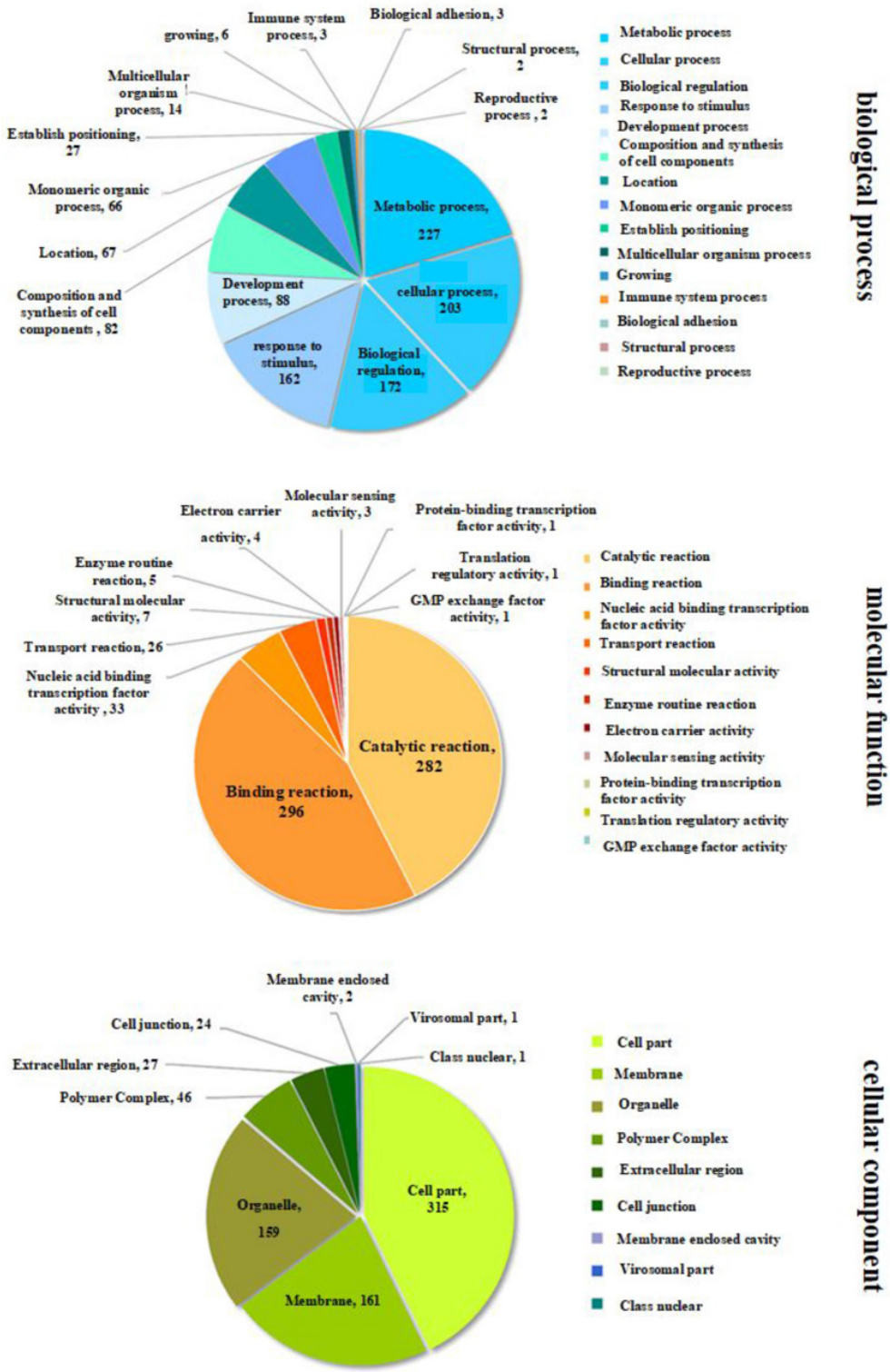

Figure 1. GO functional analysis of 640 Unigenes of transcriptome data from cranberry fruit

highbush blueberry cultivars. The primers used for detection in this study also exhibited polymorphisms in the different *Vaccinium* species and were able to amplify bands of the same fragment size as in cranberry. These results reveal the fragment homology of these primers and suggest that the tested *Vaccinium* plants (highbush, lowbush, and half-high blueberries and lingonberry) have the same resistance gene as cranberry.

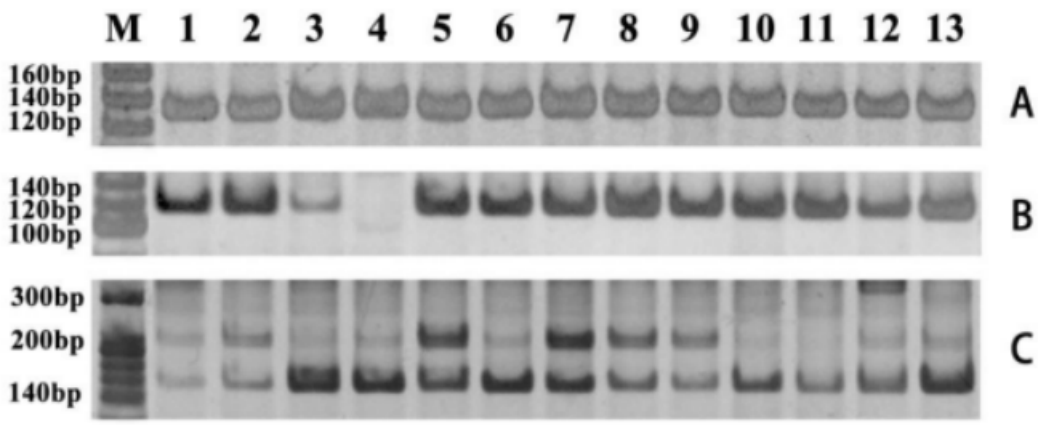

Figure 2. SSR allelic differences among 13 cranberry accessions
Note: Mis 20 bp DNA ladder (Dye plus); 1-13 are accessions in Table l; A is a single product with monomorphic amplification (primer CU19108); Bis a single product with polymorphism amplification (primer CL3473.Contigl); C is category II polymorphic product and the polymorphism is high (primer CL2216.Contigl).

Table 3. Analysis results based on 7 polymorphism pairs of primers

Name	Band Frequency	No. of alleles	*Ne*	*I*	*He*	*PIC*
CL2216.Contig1	0.769	10	1.997	0.692	0.499	0.117
CL2216.Contig 4	0.692	9	1.976	0.687	0.494	0.080
CL2296.Contig2	0.846	11	1.911	0.670	0.477	0.375
CL3473.Contig1	0.846	11	1.911	0.670	0.477	0.117
CL3889.Contig2	0.923	12	1.669	0.590	0.401	0.469
CL993.Contig1	0.538	7	1.772	0.627	0.436	0.375
CU15788	0.923	12	1.669	0.590	0.401	0.0408
average value	0.791	10.29	1.844	0.647	0.455	0.224

Note: *Ne* = No. of Effective Alleles; *I* = Shannon's Information Index; *He* = Expected Heterozygosity; *PIC* = Polymorphism Information Content

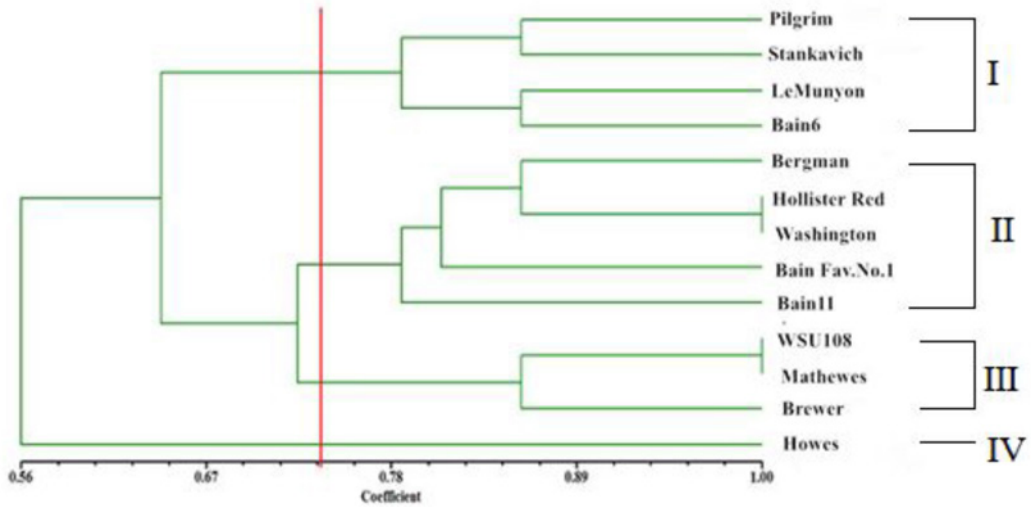

Figure 3. UPGMA-clustering results of 13 cranberry cultivars

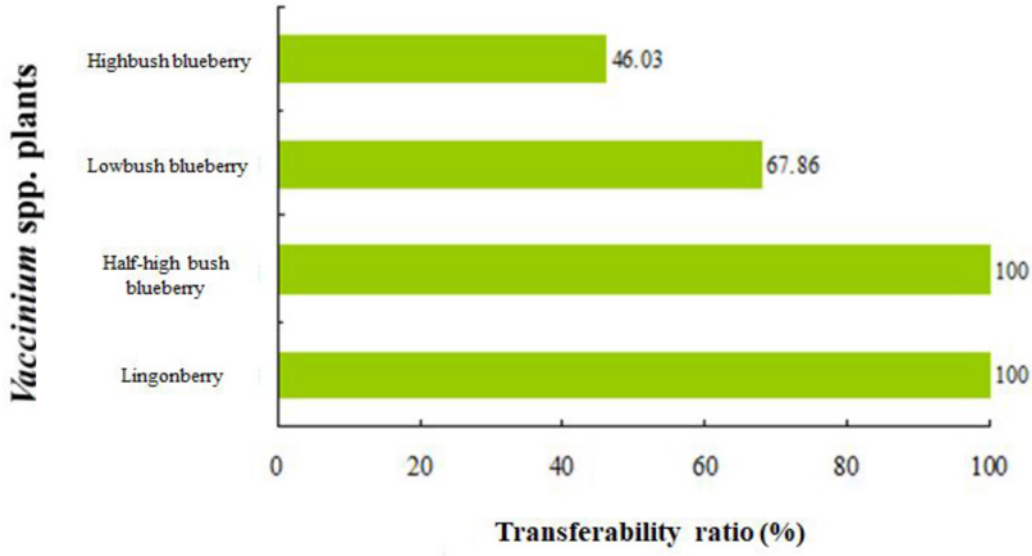

Figure 4. Transferability results of polymorphic primer among 4 plants from *Vaccinium* spp.

5. Discussion

Polymorphic markers are important tools for studying population inheritance at the genomic level (21). The effective amplification rates observed in our study are within the proposed 60% to 90% range of Saha *et al.* (2004) (22). EST-SSR markers have relatively high amplification rates because the sequence data used for primer design come from a relatively highly conserved transcribed region rather than a randomized whole-genome library. A higher effective amplification rate is one of the criteria used for the development of high-quality EST-SSR markers. The polymorphism rate obtained with the seven pairs of polymorphic primers in this study, 18.42%, was lower than that reported in bayberry (26.5%) by Zhang *et al.* (2015) (23). An average of 10.29 alleles per primer was detected using the seven polymorphic pairs, which were higher than the values of 8.16 and 4.8 respectively observed in lowbush blueberry by Boches *et al.* (2010) (20) and Bell *et al.* (2008) (24).

The results of our genetic diversity analysis uncovered a mean *Ne* of 1.844 and a mean *He* of 0.455 for the cranberry EST-SSR polymorphic markers developed in this study. These values are lower than those found in species of *Vaccinium* by Liu *et al*. (2014) (25), and *V. macrocarpon* by Schlautman *et al*. (2015) (11) and indicate that the genetic diversity and heterozygosity of cranberry were low in this study. The average *PIC* value was 0.224. *PIC* reflects the allelic diversity of one locus, and, according to published criteria (26) (high *PIC* > 0.5, intermediate *PIC* = 0.25–0.5, and low *PIC* < 0.25), the observed *PIC* further confirmed that the genetic diversity of cranberry was low in this study. This result is consistent with results reported by Brudererle *et al.* (1996) (27) and Stewart *et al*. (2010) (28) using RAPD markers, who concluded that the mainly clonal reproduction of cranberry is an adaptation to a barren, adverse environment (29).

According to the cluster analysis, similarity coefficients among most cultivars in this study were more than 0.71; in other words, most of the cranberries were closely related or had the same geographical origins, the cranberry cultivars exhibited little variation, and the genetic base was narrow. Group IV consisted solely of 'Howes', which indicates that 'Howes' is distantly related to the other cultivars, perhaps because it was selected from wild cranberry species. At the other extreme, 'Hollister Red' and 'Washington', which have the same parents, had a similarity coefficient of 1. The seven polymorphic EST-SSR markers were, therefore, able to fully distinguish the 13 cranberry accessions and can thus be used to reveal cranberry relationships.

The seven pairs of resistance-related EST-SSR sequences in this study were highly transferable to four other *Vaccinium* species, which indicates that different *Vaccinium* species have homologous genes and share resistance traits. The development of EST-SSR markers thus has a high application value for constructing candidate gene maps, identifying superior cultivars, locating resistance genes, and establishing evolutionary relationships of cranberry.

In conclusion, the seven polymorphic EST-SSR markers developed in this study, which could be used to determine the genetic relationships of 13 cranberry

accessions, should be useful for future research on stress resistance breeding of cranberry.

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