Single Nucleotide Polymorphisms, Indels, and Simple Sequence Repeats for Onion Cultivar Identification

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ABSTRACT. The commercial production of onion (*Allium cepa* L.) inbreds, hybrids, and open-pollinated (OP) cultivars would benefit from a robust set of molecular markers that confidently distinguish among elite germplasms. Large-scale DNA sequencing has revealed that single nucleotide polymorphisms (SNPs), short insertion-deletion (indel) events, and simple sequence repeats (SSRs) are relatively abundant classes of codominant DNA markers. We identified 398 SNPs, indels, and SSRs among 35 elite onion populations and observed that all populations could be distinguished. Phylogenetic analyses of simple-matching and Jaccard's coefficients for SSRs produced essentially identical trees and relationships were consistent with known pedigrees and previous marker evaluations. The SSRs revealed that elite germplasms from specific companies or breeding programs were often closely related. In contrast, phylogenetic analyses of SNPs and indels did not reveal clear relationships among elite onion populations and there was no agreement among trees generated using SNPs and indels vs. SSRs. This discrepancy was likely due to SNPs and indels occurring among amplicons from duplicated regions (paralogs) of the onion genome. Nevertheless, these PCR-based markers will be useful in the quality control of inbred, hybrid, and OP onion seed lots.

Commercial onion production is based on open-pollinated and hybrid cultivars. Occasional mistakes at field planting and harvest or mislabeling of seed require grow outs in the appropriate environments to identify specific seed lots. These grow-outs are costly because they can cause significant delays in the movement of seed to market. The onion seed industry would benefit from a robust set of codominant, easily scorable molecular markers for cultivar identification. However onion possesses two main challenges to the development of molecular markers. The first is the huge nuclear genome of onion, more than 36 and 6 times larger than rice (*Oryza sativa* L.) or maize (*Zea mays* L.), respectively (Arumuganathan and Earle, 1991). This enormous amount of DNA causes some PCR-based marker systems, such as randomly amplified polymorphic DNAs (RAPDs) and amplified fragment length polymorphisms (AFLPs), to produce too many fragments that may not resolve well in electrophoretic gels (Bradeen and Havey, 1995; Gökçe, 2001). This problem can be reduced by

using primers with additional selective nucleotides (Heusden et al., 2000). The second challenge is that onion inbreds and OP populations often retain relatively high levels of heterozygosity (King et al., 1998); hybrids are obviously highly heterozygous. This relatively high heterozygosity with low levels of allelic diversity among onion populations (King et al., 1998) causes molecular markers, especially dominant ones, to appear monomorphic among hybrid or OP cultivars. Codominant restriction fragment length polymorphisms (RFLPs) exist among elite onion populations (Bark and Havey, 1995; King et al., 1998), but their identification is too expensive and time-consuming to routinely use for cultivar identification. Although codominant SSRs have been identified in onion (Fischer and Bachmann, 2000), relatively complex PCR reaction conditions were difficult to use.

Large-scale sequencing of expressed sequences and genomic regions in onion revealed that SNPs and SSRs are relatively abundant classes of codominant DNA markers (Kuhl et al., 2004). SNPs are polymorphic nucleotides at orthologous positions in the genome. SSRs exist as different numbers of short nucleotide repeats. In this report, we identified SNPs, small insertions or deletions (indels), and SSRs in genomic regions highly similar to expressed sequence tags (ESTs) of onion (Kuhl et al., 2004; Martin et al., in press) and assessed their usefulness to distinguish among elite onion inbreds and populations.

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Materials and Methods

We evaluated 25 commercial populations, five each from five seed companies [European company A (A1 to A5), European company B (B1 to B5), a U.S. dehydrating company (D1 to D5), a U.S. company (US1 to US5), and a Japanese company (J1 to J5)], and inbreds from the USDA (B1794B, B1828B, B7728B, MSU826B, MSU611-1B, MSU2399B, and MSU8155B), Wisconsin (W202B and W420B), and Polish (P) onion-breeding programs. These populations were classified by the individual companies or breeding programs according to their day-length response for bulbing [long (LD), intermediate (ID), vs. short (SD) day] and their relative storage abilities. Populations A1 through A5; B2, B3, and B5; J1 through J5; US1 and US2; and the USDA, UW, and Polish inbreds were classified as LD with relatively long storage ability. A1 through A5, B2, and B3 are European Rijnsburger types. US3 is a LD Spanish type (grown primarily in the northwestern United States) population with relatively short storage ability. B1 and US5 are short-day and B4 and US4 are ID populations with relatively poor to intermediate storage abilities. The dehydrating populations were classified as SD (D1), LD (D5), or ID (D2, D3, and D4). USDA inbreds B1794B and B1828B were included because their pedigrees were reported in release notices and they trace back to crosses between a pair of publicly developed onion inbreds. B1794B was released as an F_3M_5 population from the cross of MSU8155B with MSU826B (M = massing generation in which three to five full-sib plants are intercrossed; F or S = self pollination of single plant). B1828B was reported as an $F_2M_2SM_3$ population from the cross of MSU826B with MSU611-1B. DNAs were isolated from at least 50 seedlings of each population by CsCl-banding as previously described (Bark and Havey, 1995). Ten onion ESTs were selected that carried genetically mapped SNPs and indels in genomic amplicons revealed using nested oligonucleotide

primers (Table 1) (Kuhl et al., 2004; Martin et al., in press). PCR conditions were described by Martin et al. (in press). PCR reactions were run on 1%-agarose gels, stained with EtBr, and single amplicons from each onion accession were cut out of the gel, purified through columns (Qiagen, Valencia, Calif.), ligated into the pGEM-T vector (Promega, Madison, Wis.), and transformed into competent bacteria following the manufacturers' protocols. At least five individual colonies from each gel-isolated fragment were sequenced as previously described (Lilly and Havey, 2001). Sequences were aligned using Sequencher (Genecodes, Ann Arbor, Mich.) to reveal SNPs and indels. We required SNPs or indels to be present in at least two amplicons from at least two different onion populations to avoid scoring amplification or sequencing errors as polymorphisms. The 10 onion cDNAs carrying SNPs or indels were hybridized to DNA-gel blots (Bark and Havey, 1995) of onion doubled haploid DNA (gift of Seminis Seed Co., Woodland, Calif.) to estimate relative copy numbers. For SSRs, onion ESTs carrying SSR-like regions were selected using the MISA program (Thiel et al., 2003) and primer pairs designed as described by Kuhl et al. (2004). Forward primer from each pair was labelled fluorescently (5' FAM) or one primer from each pair was tailed with the ABI-forward sequence 5'-GAC-GTTGTAAAACGACGGCC (Xin et al., 2004) and a universal fluorescently labelled primer (5' FAM) of the same tail sequence was synthesized (Table 2). PCR conditions with the two primers were an initial denaturation at 95 °C for 4 min followed by 28 cycles at 94 °C for 30 s, 58 °C for 45 s, and 72 °C for 1 min 20 s, and the final elongation step was at 72 °C for 8 min. The three-primer PCR protocol is described by Schuelke (2000), timing conditions were the same as for two primer protocol except that annealing temperature was lowered to 55 °C and 35 cycles were completed. PCR reactions were diluted with 4-fold with water, 3 µL of reaction was mixed with 10 µL of formamide and ROX-labelled molecular marker (Chimerx, Milwaukee). Fragments were

Table 1. Sequences of nested oligonucleotide primers producing single amplicons carrying polymorphic indels or single nucleotide polymorphisms among elite onion populations.

Onion EST [†]	Primer sequences [‡]		Amplicon size (bp)	
	Dir. [§]	Outside		Inside
ACAAJ79	F	CGCATAGAACTGACACCACTTTTCCTCG	TGACACCACTTTTCCTCGTTTG	682
	R	TCCAGGAAGGATCAATGTCACACAAAAT	CTTAAAGTCTTCTTCGCTTGGC	
ACABX01	F	CTGCTGAGTGCATACATCAAAAAAC	CATTCTCACAGTGTTCTTTTGCGG	495
	R	GATACAGCACCTCTCATCAGACCAGCCC	ATTGGATAAGAAAGAAAGAGGGAA	
ACACU34	R	GAACCAGGTGGTCCATATAGAAGTATGC	CCACTTCATCCAACCTATCTTCATCC	854
	F	GAAGAACCTAAGATTAGGATGAATAAGG	GTCAGACTTGGTGATGTTGTATCTGTCC	
ACADT92	F	CCCAGATATTTGAGCTTTGTAAAAGGTG	ATGATCTACCCTCAATGTTTCTCGTGA	632
	R	CTCTGGCATAATTTCAACATATTGATCC	GTGGTTCCCAGAATCCTCAATGCCA	
ACAED83	F	TTTGGTGATGCTAGTACCGAAGACTCTG	AAGATTACGTCCACGTTCCGCATAC	596
	R	ACCCTAAGCCTAGTACATGCCATGATTT	GTTCTTCCTCTGATCACCTTGCAG	
ACADJ12/ACAFK13	F	AAAGATTGGGCTGTTTGGTGGTGCTG	TTCTCTGTCTTTGCTGGAGTTGGTGA	598
	R	ATGAAAAGGCTGGCTCAAGAAACGCT	TGTCAAATCGTCAGCAGGCACATAAA	
ACAAX07	F	GGGACGACATCGCCCCTGTACATGAG	TGAGTTGGTGATTTCAGCCACGGAGA	394
	R	TCTAGAGCACACCGATGTCTCCGTC	GCATGTGCCCCGTGCTATATTTGTTG	
ACAET81	F	GAAGGAACCTCCGTTCTAGTGCATG	TGCTAACATTGCACATGGGAACCTCTT	436
	R	ATCATGCTCATGGTGAAAAGGAGC	ACAACAGCATCAAAGCACCAGCAG	
ACAGT43/ACAIC61	F	GAGTATGTTAAGGCCATCAAGGAGG	TATCGATGTTTGGTCCATGGAGAG	689
	R	AGCGGGAGTTCTCAATTGTGATCAC	GTCGATGATTGAGTGGATGATGTCTG	
ACAHO42	F	GGTTAGAATCACGAGGTCATAAATACAT	GAGAAACACATTCCAGACTTGC	746
	R	AACCATTCAACTGTAATCTTCTTTATCA	ATGGTTTCAAACGCTGAAGTAA	

[‡]Primer sequences were reported by Martin et al. (in press) and are included here for convenience only.

[†]EST = expressed sequence tag.

[§]Direction listed as forward (F) and reverse (R).

separated on an automatic sequencing machine (ABI3700) and revealed using the Genescan (ABI, Foster City, Calif.) software. A total of 398 polymorphisms were revealed among the 35 onion DNAs. All polymorphisms were scored as presence (1) vs. absence (0) of a specific state. NTSYS-pc version 2.02i (Exeter Software, Setauket, N.Y.) was used to calculate similarity matrices using the simple-matching (the presence and absence of fragments in two populations are both scored as matches) and Jaccard's (only presence of fragments in two populations scored as matches) coefficients. Relationships among populations were assessed using NTSYS-pc and the unweighted pair group method with arithmetic mean (UPGMA) and neighbor joining (NJ) analyses (Rohlf, 1993).

Results and Discussion

We evaluated 35 elite commercial and public onion populations for 10 genomic regions carrying SNPs or indels and for 58 SSRs to reveal totals of 327 SNPs, 34 indels, and 37 SSRs among the onion populations (Tables 1 and 2). We observed that all populations could be distinguished. Both UPGMA and NJ analyses of simple-matching and Jaccard's coefficients for SSRs produced essentially identical trees (Fig. 1) and estimates of genetic relationships were consistent with known pedigrees and previous marker evaluations. Using RFLPs, we previously demonstrated that the LD storage onions are a derived group relative to onion populations grown under shorter days (Bark and Havey, 1995). Phylogenetic analyses of SSR markers were consistent with a similar genetic background for U.S. and Japanese LD storage populations, consistent with the introduction of storage germplasm

from the United States to Japan (Goldman et al., 2000). In addition, these markers revealed that elite germplasms from specific companies or breeding programs were often closely related, likely due to the company's predominance in a specific market class. Publicly released USDA inbreds were all closely related (Fig. 1). European LD storage populations were also closely related, but distinct from U.S. and Japanese storage populations. US3, a Spanish-type population, was placed more closely to LD storage than ID or SD populations. ID and LD populations of dehydrator onions were closely related, but clearly separated from D1, a SD dehydrator population. ID and SD populations with relatively short storage abilities comprised the last well defined group of germplasms based on SSRs.

The SNP markers did not reveal clear relationships among elite onion populations and there was no agreement among trees generated using SNPs and SSRs (figure not shown). We compared trees based on SNPs or indels in exons vs. introns and observed no greater resolution of relationships among the elite populations. This discrepancy between relationships estimated using SSRs and SNPs/indels could be due to the amplification of genomic fragments from duplicated regions of the onion genome (paralogs). We selected onion ESTs showing significant similarities to single positions in the rice genome and designed nested PCR primers to amplify from these regions (Kuhl et al., 2004). In the segregating family from BYG15-23 × AC43, single genomic amplicons were produced by the nested primer sets and SNPs or indels in these genomic amplicons segregated as single loci (Martin et al., in press). In this study, we also observed single amplicons from the onion populations; however sequencing of these genomic amplicons revealed numerous SNPs and indels not observed in

Table 2. Sequences of oligonucleotide primers and nature of repeats for polymorphic simple sequence repeats identified among elite onion populations.

SSR ^z	Fragment sizes (bp)	Onion EST ^y	Forward ^w	Reverse ^w	Type of repeat
ACM004	203, 206, 213	ACS31-JM4.F01	TCGTTCTTTAGAACACGTTAGGAA	TGTCGGCGGATATAGTGACA	(CAA)4
ACM006	220, 223, 226, 232	ACB62-JM6.C10	GCAGTTCTCCCTTTGTAAAATCA	GTGATGGATGAGTGGATGGA ^w	(CTC)7
ACM013	183, 186, 192	ACAHX21	CAACCTCGAAGAACTCACCG ^w	GCGAATCTTGTTTTGGGAA	(TCC)9
ACM017	265, 268	ACAAM70	CCTTCTCCCCATTCTCTTCC ^w	CATCGTCCTCGTCCTCATC	(CAT)5
ACM018	275, 278	ACAAN60	GGGGAATGGTGGAGAATAGA	AACAGAGGCAAGAGGAGCG	(CTT)6
ACM024	120, 123, 126, 131, 134, 137, 140	ACABS25	CCCCATTTTCTTCATTTTCTCA	TGCTGTTGCTGTTGTTGTTG	(GCA)10-NNN-(GCA)4(ACA)4
ACM031	205, 207, 209	ACAAD80	CCAAAGCCGACCTCCTCT ^w	CGTGGGAAGACCAAGGGT	(AC)6
ACM045	264, 267, 269, 271	ACAAW78	AAAACGAAGCAACAAACAAAA	CGACGAAGGTCATAAGTAGGC ^w	(TC)6
ACM066	184, 186	ACADR40	CTCCCCGCAACCAGTAATAA	GCTTGGGTTTTGTTTCTCCA	(TA)5-NNN-(CT)7
ACM068	277, 281	ACADD43	CGAAGGTGAAGGTGTACGGT ^w	CAAATGGCTGCAATAAGCAA	(TA)6
ACM071	162, 166, 167, 170	ACABO63	TCTCATTTCACTTTCTACCTATCC	CTGACATTGCTCGACTGGA ^w	(AG)10
ACM078	282, 288	ACACJ44	CGCAGAATCTCGTCCTTTTT ^w	AATGGTTTGGAGGTCAGTCG	(TCG)7

continued next page

Table 2. Continued.

SSR ^z	Fragment sizes (bp)	Onion EST ^y	Forward ^x	Reverse ^x	Type of repeat
ACM082	200, 203, 206, 209	ACACY23	CACCGTTCCTCAGCTCACTT	AGAGGGACGAAATGAAAGCA	(TCT)13
ACM091	177, 183	ACAFQ28	TCTCCTCCTCTAACCAGCCA	GGTGCTCCAGTTGAGCTTTC	(TCT)10
ACM093	134, 137	ACAHV10	GCCAACAGTTTTTCGTAAGTTGA	ATTCTCTTCGGCTTTCGTGA	(CCA)7
ACM094	110, 114	ACABX11	GATGATGGCGAAGACACAGA	AAAAACGGCTTAGGAATTTAACG	(TGG)5
ACM097	194, 197	ACAHU83	TTCTCAGCTGCAATGGTGAC	ACGTGCTTGGGCATTATTTTC	(AAG)4
ACM099	183, 186	ACABR66	CATTTCTCCCTTCTGCTTTGTT	TTTGGATTGTGAGTTTGCA	(ATC)6
ACM101	209, 212, 215, 221	ACAH088	CCTTTGCTAACCAAATCCGA	CTTGTTGAGAAGGAGGACGC	(TCC)5
ACM102	151, 154, 160	ACAPL43	TGGATTTGTGAACAACCGAA	GATGCAGGCAGTGTTTTGAA	(CAA)7
ACM105	151, 154	ACAGL15	CAAGTGGAGCGGGTATTTGT	GAGGCACAACCTCCTCTTCG	(ATG)5
ACM112	177, 180	ACADA50	TTCCCAACAAACGTTTCATCA	GTGAAGGGAGAGCAGTGGAG	(TCC)5
ACM115	239, 242	ACAGH25	TCCATCTATGCATCTGCCAC	CTATTCTTCCACTGGGGCAA ^w	(CAC)6
ACM119	252, 255, 261, 265	ACAFQ04	TTTCAGCAACATAGTATTGCGTC	TCTTCGGGATTGGTATGGAG ^w	(AAT)8
ACM121	163, 166, 172	ACAFQ44	GCAAACCTCATATAGTGCCGC ^w	GAACCGATTCTACGAGCAGC	(TAT)5
ACM124	220, 228, 245	ACAAB30	GCAAACCTGAATCCCTTCCA ^w	AACCCGTTAAGAGGAGGGAA	(AAAG)5
ACM132	200, 202, 204, 206, 208, 212, 220, 224, 228	ACABH94	ATGGGGCCTGGTAAGTTTTT	TGCACACCGTTTCCATTTTA	(ACAT)14AC(CATG)4
ACM133	191, 193, 196, 198	ACACL08	CCACATGGATGAAAAACACAA ^w	CGCTGGTAGCTGAAGCAAAT	(CA)8(CG)6
ACM134	192, 200, 201, 204	ACACT16	ACACACACAAGAGGGAAGGG	CACACACCCACACACATCAA ^w	(GA)8
ACM138	235, 239, 247, 264, 268	ACAEM68	ACGGTTTGATGCACAAGATG	CCAACCAACAGTTGATACTGC ^w	(CTGC)11
ACM146	213, 216	ACAHX41	ATGTCCCAATTCGACCAGAG	CGTTACGGCTGAGAACTTCC	(ACA)5
ACM147	264, 267	ACAIE32	CACTTTCCCGTCTAATCGACA ^w	TTCCCACAATCAAAACACCA	(CTC)5
ACM151	264, 266	ACAGO25	TGTCAGACAAGCAACTCCTCC	AGGTGAGGCTTAGATGGGGT ^w	(ACA)5
ACM152	229, 241, 244	ACAFD18	TCCCAAGAGTCCAAGAATGG ^w	TGTTCTCCCTTAAACAGTGCAA	(CAG)8
ACM169	259, 261, 267	ACADN53	ACTTTCCCCCTCCAACATTC ^w	TAGCACAAGGAGGGTCGAGT	(TA)30
ACM170	227, 236	ACADV27	TTCTGCAATGAAAAACATTGA	ATCCAACCTGAGTCGGCAATC ^w	(TTC)6
ACM187	228, 260, 262, 264	ACAHZ42	GTA CTGCGGCAGTGGAGGTA	GGAGCTGTCCAAATGCTAGG ^w	(GT)6

^zSSR = simple sequence repeat.^yEST = expressed sequence tag.^x, ^w indicates primer tailed with the sequence 5'-GACGTTGTAAAACGACGGCC. Some of these primer sequences were reported by Martin et al. (in press) and are included here for convenience only.

Fig. 1. Neighbor joining analyses of Jaccard's similarities for simple sequence repeats among populations of onion (listed on left). Designations of major nodes are shown with arrows on right. LD, ID, and SD indicate long-day, intermediate-day, and short-day response for bulbing, respectively. Spanish refers to U.S. populations of Spanish-type onions.

the BYG15-23 $\times$ AC43 family, which would be expected if the elite populations were more genetically diverse than BYG15-23 and AC43 are to each other. We hybridized the onion cDNAs revealing SNPs and indels to double haploid DNA of onion and observed that some ESTs were duplicated in the onion genome (Fig. 2). As a result, amplicons produced from duplicated regions (paralogs) may accumulate SNPs or indels at different rates and this sequence variability did not reveal relationships consistent with other marker classes, such as RFLPs or SSRs. In contrast, the SSRs were located within coding regions and often were allelic (Martin et al., in press).

The utility of these molecular markers for quality control of

onion seed production can be illustrated using the USDA inbreds B1794B and B1828B. Inbred B1794B was reported to be an F_3M_5 population from the cross of MSU8155B with MSU826B. Inbred B1828B was reported as an $F_2M_2SM_3$ population from the cross of MSU826B with MSU611-1B. We compared the genotypes of the parental and progeny inbreds to see if any polymorphisms were present in the progeny inbreds that were lacking on both parents. For B1794B, all fragments present in this inbred were also present in one or both of the parental inbreds. However for B1828B, we observed six SSR fragments [ACM024 (134 and 137 bp), ACM071 (162 bp), ACM082 (209 bp), ACM134 (200 bp), and ACM151 (264 bp)] present in this inbred that were

Fig. 2. Representative autoradiograms revealing relative copy numbers of genomic amplicons of onion carrying SNPs or indels, corresponding to onion expressed sequence tags ACAAJ79 and ACABX04. DNAs were from two (A and B) doubled haploid populations of onion. Restriction enzymes are listed above the DNA names. The left-most lane is the size marker with sizes of fragments in kilobases shown on left.

absent from both parental inbreds. The most likely explanation is that the pedigree of B1828B was not correctly reported in the release notice.

It is our intention that these PCR-based SSR, SNP, and indel markers aid in the quality control of inbred and hybrid seed lots. However we caution about use of these markers to demonstrate identity among specific inbreds or hybrids. Even if two populations were identical for all markers developed in this study, one could always argue that evaluation of additional markers may reveal a polymorphism.

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