Genetic Diversity Analysis and Single-nucleotide Polymorphism Marker Development in Cultivated Bulb Onion Based on Expressed Sequence Tag–Simple Sequence Repeat Markers

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ABSTRACT. Bulb onion (*Allium cepa* L.) is a globally significant crop, but the structure of genetic variation within and among populations is poorly understood. We broadly surveyed genetic variation in a cultivated onion germplasm using simple sequence repeat (SSR) markers and sequenced regions flanking expressed sequence tag (EST)-SSRs to develop single-nucleotide polymorphism (SNP) markers. Samples from 89 inbred and open-pollinated (OP) bulb onion populations of wide geographical adaptation and four related *Allium* L. accessions were genotyped with 56 EST-SSR and four genomic SSR markers. Multivariate analysis of genetic distances among populations resolved long-day, short-day, and Indian populations. EST-SSR markers frequently revealed two major alleles at high frequency in OP populations. The median proportion of single-locus polymorphic loci was 0.70 in OP and landrace populations compared with 0.43 in inbred lines. Resequencing of 24 marker amplicons revealed additional SNPs in 17 (68%) and five SNP assays were developed from these, suggesting that resequencing of EST markers can readily provide SNP markers for purity testing of inbreds and other applications in *Allium* genetics.

Bulb onion is one of the most widely cultivated vegetables but is poorly characterized at the genetic level. It is a biennial, outcrossing diploid that exhibits severe inbreeding depression (Havey, 1993) and is traditionally propagated from seed produced by insect crosspollination of large populations. Increasingly, commercial breeding is focused on F₁ hybrids based on cytoplasmic male sterility, which necessitates inbreeding to fix nuclear restorer loci (Berninger, 1965; Jones and Clarke, 1943). Shallot (*Allium cepa* Aggregatum group) is a morphologically distinctive but closely related horticultural type that is mostly propagated asexually, although fertile types can freely intercross with bulb onion (Rabinowitch and Kamenetsky, 2002). As a result of a lack of wild relatives, poor representation in genetic resource collections (Cross, 1998; Kik, 2008), and the expense of asexual or seed maintenance of *A. cepa* germplasm, it is desirable to better understand the population structure of global resources to better target maintenance and exploitation. A more detailed understanding of genetic variation within and among populations is also required to develop marker-based

methods for testing purity and authenticity of commercial inbreds and hybrids.

Studies of genetic diversity in onion have been hampered by a lack of portable codominant molecular markers (McCallum, 2007). Although a variety of molecular marker methods have been successfully used to resolve questions of genetic diversity and relatedness to species level in *Allium* (Klaas and Friesen, 2002), identifying robust and informative markers within *A. cepa* has proved much more challenging. Dominant randomly applied polymorphic DNA markers have been used in limited studies of *A. cepa* diversity (Dennequin et al., 1997; Tanikawa et al., 2002), but a more detailed evaluation by Bradeen and Havey (1995a) showed that identification of reliable, heritable polymorphisms is very challenging in onion. Isozyme (Cramer and Havey, 1999; Rouamba et al., 2001) and restriction fragment length polymorphism (RFLP) (Bark and Havey, 1995) markers have been used but are limited, respectively, by low polymorphism (Peffley and Orozco-Castillo, 1987) and the unusually large genome size typical of *Allium* species (Bennett and Leitch, 1995). Fischer and Bachmann (2000) reported development of a set of genomic dinucleotide simple sequence repeat (SSR) markers from onion. As a result of complex amplification requirements, these markers have not proved sufficiently portable to enable wider use in mapping and diversity studies, although Masuzaki et al. (2006a) reported optimization and chromosomal allocation of a subset of these. More recently, SSR and single-nucleotide polymorphism

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(SNP) markers have been developed from onion expressed sequence tag (EST) resources (Kuhl et al., 2004; Martin et al., 2005) and proved to be readily reproducible for mapping (McCallum et al., 2006a, 2006b) and cultivar discrimination (Jakse et al., 2005).

The goal of this study was to perform a broad survey of genetic variation within and among cultivated onion populations and assess potential of EST-derived markers for genetic identity testing. To permit a sufficiently broad survey and ensure relevance for seedlot identity testing, we estimated allele numbers in bulked samples, an approach previously used in similar surveys of maize (*Zea mays* L.) diversity. This revealed high levels of within-population heterozygosity and limited EST-SSR size polymorphism in cultivated onion. The results

suggest that discrimination of inbred onion lines with SNP markers will be feasible and that such markers can be readily developed from onion EST resources.

Methods and Materials

PLANT MATERIALS. A set of 82 bulb onion populations was selected to represent the broadest possible range of germplasms relevant to modern genetics and breeding, including mapping population parents, inbred and open-pollinated (OP) populations widely exploited by breeders and landraces. A supplementary table containing full details of this material is available online at www.ASHS.org. Seven doubled haploid (DH) bulb onion lines were obtained from Cornell University (Alan et al., 2004). Four

Table 1. Simple sequence repeat (SSR) primer sets developed from onion sequence resources for genetic diversity analysis of onion and *Allium* relatives.

Primer set	Type ^z	Genbank accession no.	Forward primer sequence Reverse primer sequence	Expected size (bp)
ACM004	EST-SSR	BI095629	TCGTTCTTTAGAACACGTTAGG GTCGGCGGATATAGTGACA	214
ACM018	EST-SSR	CF438995	GGGGAATGGTGGAGAATAGA AACAGAGGCAAGAGGAGCG	300
ACM046	EST-SSR	CF439398	TCCTCGTCACCACCACAG CTGAAAGGGAGTAGCGGAG	280
ACM050	EST-SSR	CF438491	GGTTCTCTGTTTGGGACA CCGTTTCGGCTACCTTGTAT	277
ACM065	EST-SSR	CF449328	GCTCTGATGGAGGATGGTTC CTTGCCATCTTTGTCGGT	197
ACM068	EST-SSR	CF450463	GAAGGTGAAGGTGTACGGT CAAATGGCTGCAATAAGCAA	261
ACM183	EST-SSR	CF443106	GATGATGGTGATGGCATTGA GTTTGCAGGCTCCATTGATT	213
ACM187	EST-SSR	CF446468	GTAATCGGGCAGTGAGGTA GGAGCTGTCCAAATGCTAGG	246
ACM227	EST-SSR	CF439865	AGCAGCTCATTTCAGCAAAA GAGGTTCGGAGAAGGAGGAGT	198
ACM238	EST-SSR	CF443464	TGATAGCCAGTTGATTGCGA TTCCCCAGTACACACCTTCC	261
ACM240	EST-SSR	CF444554	GTGCAACTCCAAGAGAAGGG AATATAAAGGCGTTGGCCTG	194
ACM243	EST-SSR	CF445129	ATCAGGAGGTTTCGAGGAAA TCATTTCGTATCATTGTCGGT	141
ACM295	EST-SSR	CF437320	AGATCCGTCCCATGAAACT GATCCGCTTCTGAAATCTCG	146
ACM300	EST-SSR	CF448987	AGGTGCAGTTTCGTGGTAGG TTAGCCCCTGGTAAGTGTGG	154
ACM301	EST-SSR	CF434545	TGGAGTCAAAAGGTGGTGAA TTCTTGATTCTCCGTTTGG	109
ACM303	gSSR	DQ273270	CGATGATTATAGATATTTTCGTTATGG CATTTCATAATCTCACAGTGAAGG	115
ACM315	EST-SSR	ES449741	TTTTCTCCACCTTCAGCCAG AGAAATGGTGGTCGAGGATG	269
ACM316	EST-SSR	ES449430	TGGATCATAAAAAGCGGCAT GAAGAGGCTTTTTCCGACT	204
ACM318	EST-SSR	ES449367	TCCTCCTTCCAAACCACATC GATCAGAAACAGCAGCGTC	243
ACM326	EST-SSR	CF437364	AAACCAGCAACAACCAATG AAAATTGGAGAGCAGGCAAA	200

^zEST-SSR and gSSR denote SSR markers developed, respectively, from expressed sequence tag (EST) and genomic sequence resources, respectively.

Table 2. Allele sizes and observed proportion of polymorphic bulb onion populations (P_p) revealed by simple sequence repeat (SSR) markers in a genetic diversity analysis of onion and *Allium* relatives.^z

Primer set	Repeat motif	P_p	<i>A. fistulosum</i> allele sizes (bp)	<i>A. roylei</i> allele sizes (bp)	Shallot allele sizes (bp)	Bulb onion allele sizes (bp)
ACM004	(CAA) ₄	0.90	NULL	230	230	227, 230, 236
ACM006	(CTC) ₇	0.44	218, 221	NULL	224, 228	221, 224, 227, 233
ACM018	(CTT) ₆	0.84	300	303	300, 303	300, 303
ACM024	(GCA) ₁₀ N ₃₃ (GCA) ₄ (ACA) ₄	0.85	NULL	140	146	140, 143, 146, 148, 151, 154, 157, 160, 163, 169
ACM031	(TA) ₅	0.60	NULL	NULL	NULL	210, 212, 214, 216
ACM033	(CAT) ₅	0.87	272	272	272	272, 275
ACM045	(TC) ₆	0.84	NULL	NULL	226	226, 269, 271, 273, 275
ACM046	(TCC) ₉	0.51	287	290	287, 299	293, 296, 299, 302
ACM061	(AGC) ₆	0.63	210	207	207, 215	215, 218
ACM063	(TA) ₆	0.50	251	NULL	251	249, 251, 253
ACM066	(TA) ₅ N ₅₀ (CT) ₇	0.17	NULL	NULL	209	209, 211
ACM068	(TA) ₆	0.48	284	289	281, 285	281, 285
ACM071	(AG) ₁₀		172	167	170	166, 170, 172, 174
ACM078	(TCG) ₇	0.14	NULL	NULL	292	286, 292
ACM091	(TG) ₆	0.68	206	209	203, 206	197, 200, 203, 206, 209, 212
ACM093	(CCA) ₇	0.59	NULL	NULL	156	156, 159
ACM094	(TGG) ₅	0.27	NULL	NULL	NULL	136, 139
ACM099	(ATC) ₆	0.91	NULL	NULL	206	203, 206, 209
ACM101	(TCC) ₅	0.98	230	233, 242	230, 236, 239	227, 230, 233, 236, 239, 242, 245, 248
ACM112	(TCC) ₅	0.89	NULL	209	205	203, 205
ACM115	(CAC) ₆	0.84	241	241	241, 246	243, 246
ACM119	(AAT) ₈	0.57	245	244	244, 249	241, 250, 259
ACM121	(TAT) ₅	0.81	165	168	165, 168	168, 171
ACM124	(AAAG) ₅	0.80	221	224	221, 226	224, 226, 228, 233, 250
ACM125	(GTAT) ₅	0.93	NULL	NULL	220	216, 220, 224
ACM132	(ACAT) ₁₄ AC(CATG) ₄	0.91	NULL	NULL	228	220, 224, 226, 228, 230, 232, 236
ACM133	(CA) ₈ (CG) ₆	0.74	186	NULL	NULL	196, 198, 200, 202
ACM134	(GA) ₈	0.74	198	NULL	206, 210	198, 200, 206, 210, 212
ACM138	(CTGC) ₁₁	0.70	252	252	250	242, 247, 250, 252, 255, 259, 262, 270, 274, 278, 286
ACM146	(ACA) ₅	0.91	233	233	233, 239, 242	239, 242
ACM147	(CTC) ₅	0.79	NULL	NULL	270	267, 270, 273
ACM151	(ACA) ₅	0.48	NULL	NULL	267	267, 269
ACM154	(AGA) ₁₀	0.84	NULL	NULL	239	239, 242, 245, 248, 251
ACM157	(AAT) ₆		276, 279, 280	261, 264, 276	270, 276, 279, 280	261, 264, 267, 270, 273, 276, 277, 279, 280
ACM168	(TG) ₆	0.09	248	262	262	220, 262, 285
ACM169	(TA) ₃₀	0.80	NULL	NULL	263	263, 265, 269, 271
ACM170	(TTC) ₆	0.46	NULL	NULL	241	231, 241, 244

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Table 2. Continued.

Primer set	Repeat motif	P_p	<i>A. fistulosum</i> allele sizes (bp)	<i>A. roylei</i> allele sizes (bp)	Shallot allele sizes (bp)	Bulb onion allele sizes (bp)
ACM177	(ATC) ₁₄	0.68	NULL	NULL	268	253, 256, 259, 262, 265, 268, 271, 274, 277
ACM179	(TCA) ₅	0.29	336	338	334	349, 350, 353
ACM180	(ATG) ₄ N ₂₀ (AAT) ₆	0.74	NULL	253	253	244, 250, 253
ACM187	(GT) ₆	0.85	225	262	225	225, 227, 228, 260, 262
ACM221	(AC) ₇	0.22	182	184, 186	180	188, 190
ACM227	(CAG) ₇	0.06	213	207	213	204, 213, 221
ACM229	(CAG) ₈	0.81	292	295	292, 295	290, 292, 295, 301
ACM235	(TTTG) ₄	0.18	316	292	296, 300	292, 296, 300, 304
ACM243 [#]	(TGA) ₆	0.00	162	165	162	159
ACM251	(ATTC) ₄	0.73	193	187	193	187, 193, 198
ACM266	(ACC) ₆	0.68	314	310	314	308, 314
ACM271	(CAC) ₅	0.70	462	471	458, 462	458, 462
ACM282	(TCC) ₅	0.65	215, 218	NULL	215, 218	212, 215, 218
ACM300	(GCA) ₇	0.12	177	174	170, 177	170, 174, 177
ACM301	(TAG) ₅		133	133	130, 133	130, 133, 136
ACM303	(AC) ₈	0.48	NULL	128	126	126, 133, 143, 145
ACM315 [#]	(CTC) ₅	0.00	295	281	292, 295	292
ACM316 [#]	(ATGT) ₄	0.00	226	NULL	226, 230	230
ACM318 [#]	(CTC) ₄ N ₄₁ (TTC) ₄	0.00	263	263	263, 266	266
ACM326 [#]	(CTT) ₄	0.00	NULL	224	224, 227	224
AMS12	(CA) ₂₅	0.48	NULL	NULL	256, 287, 289	256, 258, 283, 285, 287, 289, 291, 293, 294, 296, 298, 300
AMS17	(CA) ₇ TG	0.89	NULL	NULL	285	258, 262, 271, 273, 274, 279, 281, 283, 285, 287, 289, 291, 293
AMS21	(CA) ₂₁ (TA) ₃	0.85	249	279	249, 270	249, 258, 260, 262, 264, 266, 268, 270, 273, 277, 279, 281, 283, 285, 287, 289, 291, 292, 294, 297, 298, 300, 302

[#]Values of P_p are omitted for multilocus markers and do not include doubled haploid lines. Hash symbol ([#]) denotes loci monomorphic for bulb onion but polymorphic between onion and shallot. NULL denotes failure to amplify with a given sample.

related *Allium* outgroups were provided by single accessions of *A. fistulosum* L. (Ohara et al., 2005), *A. roylei* Stern (van der Meer and de Vries, 1990), shallot (*A. cepa* L. aggregatum group), and the amphidiploid between *A. cepa* and *A. fistulosum* 'Beltsville Bunching' (Jones and Clarke, 1942). DNA samples used were either isolated in earlier studies (Bark and Havey, 1995; Bradeen and Havey, 1995b; Havey, 2000; Leite et al., 1999) or purified from bulked tissue of at least 25 seedlings as described previously (McCallum et al., 2006a).

cDNA LIBRARY DEVELOPMENT. Immature, unopened flower buds were pooled in Dec. 2004 from multiple genotypes that included maintainer and restorer lines in nitrogen and sulfur cytoplasm. Total RNA was isolated as described previously (McCallum et al., 2002) and a nonnormalized cDNA library was constructed in pCMV.SPORT 6.1 (Invitrogen, Carlsbad, CA). Random clones (672) were purified using NucleoSpin 96 Flash Kit (Macherey-Nagel, Duren, Germany) and sequenced from the 5' end using Big Dye version 3.0 chemistry (Applied Biosystems, Foster City, CA). Trimmed reads were submitted to the Genbank EST division (accession numbers ES449250 to ES449826).

MARKER ANALYSIS. EST-SSR primer sets were described previously (Kuhl et al., 2004; Martin et al., 2005; McCallum et al., 2006a, 2006b) or designed as described previously (Kuhl et al., 2004) from floral EST and other onion EST resources (Table 1). Genomic SSR primer sets were those originally reported by Fischer and Bachmann (2000) and chromosomally assigned by Masuzaki et al. (2006b). Polymerase chain reaction (PCR) amplification and analysis of SSR products was performed as described previously (Martin et al., 2005) with the following modifications. Primer ratios were modified from the method of Schuelke (2000) to use marker tailed forward plus reverse primers at 0.5 μ M and fluorescently labeled universal primer at 0.2 μ M. A "PIG-tail" sequence (GTTTCTT) was added to the 5' end of reverse primers to minimize variability in nontemplated adenylation of amplicons (Brownstein et al., 1996).

Chromatograms were analyzed using visual and quantitative tools provided in GeneMarker (version 1.42; SoftGenetics, State College, PA). Peak interpretation was guided by reference to prior segregation data from mapping populations (Martin et al., 2005; McCallum et al., 2006a) and DH controls. Analyses were restricted to measures of allele presence judged on conservative criteria. Principal coordinates analysis (PCO) was performed in Genstat (Payne et al., 2006) on the similarity matrix calculated from 289 peaks revealed by 60 primer sets using the Jaccard coefficient. A total of 51 primer sets (48 EST-SSRs, three genomic SSRs), which yielded profiles of quality 1 or 2, as defined by Leigh et al. (2003), which could be reliably scored as single loci, were used to estimate population heterozygosity as the proportion of polymorphic loci [P_i (Berg and Hamrick, 1997)].

RESEQUENCING OF MARKER LOCI. A small subset of two to four templates was selected for resequencing, including a DH line and one or more populations exhibiting high frequencies of an alternate size allele. PCR products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions and cloned into pGEM-T Easy Vector (Promega, Madison, WI). DNA for sequencing was amplified directly from at least eight white colonies from each template using the Templiphi Amplification Kit (Amersham Biosciences, Piscataway, NJ).

Sequencing was performed with M13 forward and reverse primers using a Big Dye version 3.0 cycle sequencing kit (Applied Biosystems) and analyzed on an ABI3100 Genetic Analyser (Applied Biosystems). Sequence comparison and SNP detection was carried out using SeqScape version 2.1 (Applied Biosystems) using Genbank sequences of ESTs used in original primer design as reference sequences.

SINGLE-NUCLEOTIDE POLYMORPHISM ASSAY DESIGN. Restriction polymorphisms were identified using BlastDigester (Ilic et al., 2004) and if suitable polymorphisms could not be

Fig. 1. Distribution of proportion of polymorphic populations (P_p) revealed by 48 single-locus expressed sequence tag-simple sequence repeat (EST-SSR) markers among 82 bulb onion populations plotted by observed allele number and repeat unit.

Fig. 2. Distribution of observed proportion of polymorphic loci (P_i) for 89 bulb onion accessions (seven doubled haploid, 12 inbred, 25 landrace, and 45 open-pollinated) evaluated at 51 simple sequence repeat (SSR) loci. Triangles denote medians and arrowhead denotes a doubled haploid line believed to have been contaminated during seed production.

identified, potential for engineering these was identified using dCAPS finder 2.1 (Neff et al., 2002). Cleaved amplified polymorphic sequence or derived cleaved amplified polymorphic sequence primer sets for assaying the polymorphisms were designed using Primer 3 software (Rozen and Skaletsky, 2000). Ability of assays to reveal polymorphisms within bulb onion was confirmed by genotyping a small subset of surveyed germplasm, including a DH control.

Results and Discussion

DISTRIBUTION OF POLYMORPHISM WITHIN AND AMONG BULB ONION. We screened a total of 76 EST-SSR and 12 genomic SSR markers for ability to reveal polymorphism in this set of accessions and used data from 56 EST-SSR and four genomic SSR primer sets, which revealed reproducible patterns. Presence of DH lines in the panel greatly aided interpretation of chromatograms, because visual evaluation of aligned chromatograms suggested a high frequency of heterozygosity for most markers. As observed during earlier mapping studies (Martin et al., 2005), EST-SSR markers designed using standard criteria performed well across onion germplasm under uniform PCR conditions. Amplicons of similar size were amplified in the related species *A. roylei* and *A. fistulosum* by 33 and 32 of these markers, respectively, confirming their value for interspecific and comparative studies. By contrast, dinucleotide genomic SSRs (gSSRs) required optimization of conditions and amplified rarely in allied *Allium* species. Chromatograms of gSSRs revealed much higher levels of size polymorphism than EST-SSR but were more challenging to interpret in population bulk samples as a result of high levels of stutter such that only three were selected for estimation of locus heterozygosity. EST-SSR primer sets revealed an average of 3.59 alleles (range, one to 10 alleles), similar to the distribution observed in durum wheat (*Triticum aestivum* L.) EST-SSRs by Wang et al. (2007) with di- and trinucleotide SSRs revealing similar ranges of polymorphism (Table 2; Fig. 1). Five primer sets were monomorphic in bulb onion but revealed polymorphism between bulb onion and *Allium* outgroups.

Population heterozygosity in the bulb onion accessions (excluding DH lines) was conservatively estimated as P_i revealed by 51 of the 60 markers (219 alleles), which revealed variation in bulb onion that

could be confidently interpreted as representing alleles at one locus. Because we expect that alleles with frequencies below 5% may not be detected in a bulk PCR, this estimate of heterozygosity is conservative but directly relevant to PCR-based discrimination and authenticity testing of bulked seed samples. The heterozygosity detected by markers in the 82 non-DH bulb onion populations, expressed as the proportion of heterozygous populations (P_p), shows a bimodal distribution (histogram in Fig. 1). The markers that detected polymorphism in less than 30% of populations surveyed were characterized by rare informative alleles, whereas the majority were characterized by several alleles at high frequency across the populations. Bulb onion populations exhibited a wide range of heterozygosity with P_i ranging from 0.12 to 0.84 (Fig. 2). Median P_i in landrace and OP or hybrid populations was 0.7 compared with 0.43 in the sample of inbred lines. The range of P_i observed in the noninbred populations is at the high end of the range observed in isozyme studies of outcrossing species (Berg and Hamrick, 1997;

Fig. 3. Principal coordinates (PCO) ordination of Jaccard similarity matrix for 89 bulb onion and four related *Allium* accessions calculated from all 289 polymorphic peaks revealed by 60 simple sequence repeat (SSR) markers. Common words in cultivar names were abbreviated as follows: R = red, W = white, Y = yellow, G = globe, S = sweet. Triangles denote Cornell University doubled haploid lines and dots denote unnamed accessions from USDA-ARS germplasm collections with PI numbers.

Hamrick and Godt, 1989). The wide range of P_i observed in the inbred lines is consistent with the fact that inbred development in onion can involve a wide range of bottlenecks, ranging from limited mass pollinations of three to 20 plants to one or more cycles of self-pollination. The narrower range of P_i in OP cultivars and breeding populations compared with landraces may reflect the success of modern plant breeding in increasing heterozygosity through intercrossing and selection for vigor. The DH line DH2178 showed heterozygosity at a few loci, which is believed to have arisen from crosscontamination in seed production (M. Mutschler, personal communication).

These observations of high heterozygosity and low allele number confirm the previous report of Bark and Havey (1995), who commonly observed two alleles using RFLP and together suggest that allelic diversity in onion is lower than in maize. Similar genotyping of bulked samples has been widely used and well proven in maize (Reif et al., 2005), but more detailed analysis of allele frequencies within populations is required to provide a more quantitative picture of heterozygosity in breeding and landrace onion germplasm.

MULTIVARIATE ANALYSIS OF MARKER DATA. The complete data set comprising presence/absence data for 289 peaks scored

Table 3. DNA sequence polymorphisms detected in regions flanking simple sequence repeats (SSRs) amplified by onion expressed sequence tag (EST)-SSR markers.^z

Primer set	Reference sequence Genbank accession	Polymorphisms
ACM006	BQ580184	G61A, C97A, T99C, G126C, C159T, C175T
ACM018	CF438995	T124C, G184A
ACM091	CF440522	A129T, T188A, C189A, C190T
ACM093	CF445996	C58T
ACM112	CF450008	T76A
ACM121	CF441947	C5T, T61A, T154A, C156T, T159C
ACM146	CF446333	G23A, C24G, C142T, C147T
ACM147	CF444705	A71C, T105C, C126G, T134C, G247A, T248A
ACM151	CF440337	T22G, C86G, C135T
ACM170	CF437581	T162C, T48C
ACM183	CF443106	G105A, G114A
ACM227	CF439865	A33G, G51A, G99A
ACM235	CF441946	C51T, A52G, C196T, A199T, G201C, A210T, A211T, A212T, A225G, C227T, A232G
ACM243	CF445129	T43C
ACM266	CF451915	A20G, C22T, C24T, C25A, A26T, C33G, C35T, C72T, T115C, C128T, C153T
ACM271	CF452664	G27T, C60A
ACM303	DQ273270	C104678G, G104648T

^zNo polymorphisms were identified in regions flanking SSRs in the markers ACM033, ACM066, ACM094, ACM115, ACM154, ACM229, and ACM245.

on all 60 markers was subjected to multivariate analysis of the Jaccard similarity matrix. PCO was used for ordination and exploration of the similarity matrix (Fig. 3). The first PCO explaining 15% of the variability was highly correlated ($r = -0.95$) with P_i , indicating that this component represents differences in heterozygosity. The second PCO (7% of variation) provides resolution of populations congruent with their known geographical origins. In particular, long- and intermediate-day types originating from temperate growing regions are resolved from short-day (SD) tropical types. However, the most notable feature of this ordination is the clear resolution of a group of Indian cultivars and landraces from the main grouping of SD populations. This grouping may represent populations more closely related to ancestral bulb onion types from the center of origin. The survey of *A. cepa* by Fischer and Bachmann (2000), based on genomic SSRs, also revealed groupings related to geographical origin but focused on shallot and landrace onion germplasm, making comparison with our findings difficult.

Table 4. Cleaved amplified polymorphic sequence (CAPS) and derived CAPS (dCAPS) markers developed from single-nucleotide polymorphisms (SNPs) in regions flanking simple sequence repeats (SSRs) in onion expressed sequence tag (EST)-SSR markers.

Assay primer Set	Parent primer set	Reference sequence Genbank accession no.	Target polymorphism	Forward and reverse primer sequences	Type
ACM006	—	BQ580184	G126C	See Kuhl et al. (2004)	TaqI CAPS
ACM018	—	CF438995	T124C	See Table 1	TaqI CAPS
ACP014	ACM183	CF443106	G105A	GACGACAACAATGATAATATAGATACTCG AATTGTTTGCAGGCTCCATT	XhoI dCAPS
ACP015	ACM093	CF445996	C58T	TACTATACCGGCCACCACGA TATTCTCTTCGGCTTTCGTGA	MboI dCAPS
ACP017	ACM227	CF439865	G99A	ACCTTCCCAACAGCACGC GAGGTCGAGAAGGAGGAGT	HhaI dCAPS

Fig. 4. Single-nucleotide polymorphisms (SNPs) revealed in a set of onion lines by cleaved amplified polymorphic sequence (CAPS) assays developed by resequencing onion expressed sequence tag-simple sequence repeat (EST-SSR) markers. Approximate migration of DNA size standards in basepairs indicated on right.

DEVELOPMENT OF SINGLE-NUCLEOTIDE POLYMORPHISM MARKERS BY EXPRESSED SEQUENCE TAG-SIMPLE SEQUENCE REPEAT RESEQUENCING. After observations of relatively low size allelic diversity revealed by EST-SSR markers, we conducted resequencing to determine whether these might provide a source of biallelic SNP markers more suitable for commercial and research purposes. Additional polymorphisms were identified in 17 of 24 loci sequenced [68% (Table 3)] suggesting that more general mutation scanning of 5' UTR regions of onion genes may provide more polymorphic markers than sizing alone. From these, five polymorphic SNP assays were developed (Table 4; Fig. 4), suggesting that the existing onion EST resources can readily provide many more such markers if properly exploited. Although the levels of diversity observed in noninbred populations would make marker-based purity and authenticity testing using such markers inefficient, the higher levels of fixation observed among inbred lines (Fig. 2) suggests that testing inbreds and their derived hybrids with such single-locus markers derived from highly heterozygous loci would be quite practical. To improve the efficiency of such an approach, key inbreds could also be subjected to marker-aided inbreeding at one or more loci, as proposed by Tsukazaki et al. (2006) for *A. fistulosum*. Bunching onion appears to be more tolerant of inbreeding than onion, and therefore some validation that inbreeding did not compromise fitness would be required.

These observations have implications for conservation and exploitation of genetic diversity in onion. In particular, the observation of high allelic diversity in OP populations confirms that these are key reservoirs of diversity in onion worthy of conservation. The lower values of heterozygosity observed in inbreds illustrate the potential for erosion of diversity associated with hybrid breeding. The observation that Indian populations form a genetically distinctive group suggests that this region may have potential to provide novel germplasm resources to broaden the base for breeding and genetic studies in bulb onion.

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Supplemental Table 1. Populations of bulb onion and allied *Allium* species surveyed in this study.

Description	Source	Country of origin	Accession ID no. ^z	Type	Reference or URL
Roxa de Barreira	Agroflora	Brazil		Open-pollinated	Leite et al. (1999)
Baia Performe	Agroflora	Brazil		Open-pollinated	Leite et al. (1999)
Crioula	Agroflora,	Brazil		Open-pollinated	Leite et al. (1999)
Texas Grano 438	Asgrow Seed Co.	USA		Open-pollinated	http://www.asgrowanddekalb.com/
Pusa White	AVRDC ^y	India		Open-pollinated	Havey (2000)
AC319 (Red pinoy)	AVRDC	Philippines		Open-pollinated	Havey (2000)
AC452 (AgriFound Light Red)	AVRDC	India	TA00202	Open-pollinated	Havey (2000)
AC463 (Pusa Madhvi)	AVRDC	India	TA00419	Open-pollinated	Havey (2000)
AC464 (Arka Niketan)	AVRDC	India	TA00420	Open-pollinated	Havey (2000)
AC477 (Nasik Red)	AVRDC	India	A00052	Open-pollinated	Havey (2000)
AC479 (Taherpuri)	AVRDC	Bangladesh	TA00265	Open-pollinated	Havey (2000)
AC503 (Pusa Red)	AVRDC	India	TA00418	Open-pollinated	Havey (2000)
AC591 (Poona Red)	AVRDC	India	A00031	Open-pollinated	Havey (2000)
AC593 (N-53)	AVRDC	India	TA00415	Open-pollinated	Havey (2000)
AC595 (Kalpatiya)	AVRDC	Sri Lanka	TA00541	Open-pollinated	Havey (2000)
Bombay Red	AVRDC	India	PI 531171	Open-pollinated	Havey (2000)
Hygro	Bejo Zaden ^s	Holland		Hybrid	Havey (2000)
NY53E21B	Cornell Univ.	USA		Inbred	http://www.cals.cornell.edu/
YIX A	Cornell Univ.	USA		Inbred	http://www.cals.cornell.edu/
Cayuga	Cornell Univ.	USA		Open-pollinated	http://www.cals.cornell.edu/
Iroquois	Cornell Univ.	USA		Open-pollinated	http://www.cals.cornell.edu/
DH2104	Cornell Univ.	USA		Doubled haploid	Alan et al. (2004)
DH2107	Cornell Univ.	USA		Doubled haploid	Alan et al. (2004)
DH2110	Cornell Univ.	USA		Doubled haploid	Alan et al. (2004)
DH2122	Cornell Univ.	USA		Doubled haploid	Alan et al. (2004)
DH2150	Cornell Univ.	USA		Doubled haploid	Alan et al. (2004)
DH2178	Cornell Univ.	USA		Doubled haploid	Alan et al. (2004)
DH from YIX A	Cornell Univ.	USA		Doubled haploid	Alan et al. (2004)
Crop Early Globe	Crop & Food Research	New Zealand		Open-pollinated	Leite et al. (1999)
Alfa Tropical	EMBRAPA	Brazil		Open-pollinated	Leite et al. (1999)
Beta Cristal	EMBRAPA	Brazil		Open-pollinated	http://departments.agri.huji.ac.il/fieldcrops/staff-eng/rabinowitch.html
Shallot 66-1002	Hebrew University, Rehovot	Thailand		Outgroup	
<i>A. fistulosum</i> D1s-15s					
LC2062	NIVTS ^w	Japan		Outgroup	Ohara et al. (2005)
Jumbo	INTA ^v	Argentina		Open-pollinated	http://www.inta.gov.ar
<i>A. roylei</i>	Plant Research International	Netherlands		Open-pollinated	van Heusden et al. (2000)
Senshu Ki	Plant Research International	India		Outgroup	van der Meer and de Vries (1990)
Pukekohe Longkeeper (PLK)	Shippo Seed Co.	Japan		Open-pollinated	Havey (2000)
Kiwi Gold	Terranova Seeds	New Zealand		Open-pollinated	http://www.tnseeds.com
May & Ryan Regular PLK	Terranova Seeds	New Zealand	G 28985	Open-pollinated	http://www.tnseeds.com/
Solara	Terranova Seeds	New Zealand		Open-pollinated	http://tropicasenegal.com/products/prdts.html
Red Creole	Tropicasem	Senegal		Hybrid	http://tropicasenegal.com/products/prdts.html
Violet de Galmi	Tropicasem	Senegal		Open-pollinated	http://tropicasenegal.com/products/prdts.html
W202A	Univ. of Wisconsin	USA		Inbred	Goldman (1996)
Primorska	Univ. of Ljubljana	Slovenia		Open-pollinated	Leite et al. (1999)
Chinese Purple	USDA-ARS	China	PI 432715	Landrace	http://www.ars-grn.gov/
PI 164970	USDA-ARS	Turkey	PI 164970	Landrace	http://www.ars-grn.gov/
PI 168960	USDA-ARS	Turkey	PI 168960	Landrace	http://www.ars-grn.gov/

continued next page

Supplemental Table 1. Continued.

Description	Source	Country of origin	Accession ID no. ^z	Type	Reference or URL
PI 174019	USDA-ARS	Turkey	PI 174019	Landrace	http://www.ars-grin.gov/
PI 175571	USDA-ARS	Turkey	PI 175571	Landrace	http://www.ars-grin.gov/
PI 177247	USDA-ARS	Syria	PI 177247	Landrace	http://www.ars-grin.gov/
PI 179167	USDA-ARS	Iraq	PI 179167	Landrace	http://www.ars-grin.gov/
PI 181929	USDA-ARS	Syria	PI 181929	Landrace	http://www.ars-grin.gov/
PI 207456	USDA-ARS	Afghanistan	PI 207456	Landrace	http://www.ars-grin.gov/
PI 210994	USDA-ARS	Afghanistan	PI 210994	Landrace	http://www.ars-grin.gov/
PI 222228	USDA-ARS	Iran	PI 222228	Landrace	http://www.ars-grin.gov/
PI 222698	USDA-ARS	Iran	PI 222698	Landrace	http://www.ars-grin.gov/
PI 222764	USDA-ARS	Iran	PI 222764	Landrace	http://www.ars-grin.gov/
PI 235353	USDA-ARS	Syria	PI 235353	Landrace	http://www.ars-grin.gov/
PI 239633	USDA-ARS	Iran	PI 239633	Landrace	http://www.ars-grin.gov/
PI 243008	USDA-ARS	India	PI 243008	Landrace	http://www.ars-grin.gov/
PI 247067	USDA-ARS	Italy	PI 247067	Landrace	http://www.ars-grin.gov/
PI 249901	USDA-ARS	Portugal	PI 249901	Landrace	http://www.ars-grin.gov/
PI 251509	USDA-ARS	Iran	PI 251509	Landrace	http://www.ars-grin.gov/
PI 269415	USDA-ARS	Pakistan	PI 269415	Landrace	http://www.ars-grin.gov/
PI 271309	USDA-ARS	India	PI 271309	Landrace	http://www.ars-grin.gov/
PI 271312	USDA-ARS	India	PI 271312	Landrace	http://www.ars-grin.gov/
PI 274782	USDA-ARS	India	PI 274782	Landrace	http://www.ars-grin.gov/
PI 280554	USDA-ARS	Soviet Union	PI 280554	Landrace	http://www.ars-grin.gov/
PI 288274	USDA-ARS	India	PI 288274	Landrace	http://www.ars-grin.gov/
Chinese Yellow	USDA-ARS	China	PI 432713	Open-pollinated	http://www.ars-grin.gov/
Downing Yellow Globe	USDA-ARS	USA	G 29261	Open-pollinated	http://www.ars-grin.gov/
Southport White Globe	USDA-ARS	USA	PI 546207	Open-pollinated	http://www.ars-grin.gov/
Walla Walla Sweet	USDA-ARS	USA	G 29209	Open-pollinated	http://www.ars-grin.gov/
White Portugal	USDA-ARS	USA	PI 433342	Open-pollinated	http://www.ars-grin.gov/
Yellow Sweet Spanish Winegar	USDA-ARS	USA	PI 546188	Open-pollinated	http://www.ars-grin.gov/
Brigham Yellow Globe	USDA-ARS	USA	PI 546132	Open-pollinated	Bark and Havey (1995)
Italian Red	USDA-ARS	USA		Open-pollinated	Bark and Havey (1995)
Southport Red Globe	USDA-ARS	USA	G 18545	Open-pollinated	Bark and Havey (1995)
Stuttgart	USDA-ARS	Germany	PI 321386	Open-pollinated	Bark and Havey (1995)
White Ebenezer	USDA-ARS	USA	PI 546137	Open-pollinated	Bark and Havey (1995)
Yellow Globe Danvers	USDA-ARS	USA	PI 546151	Open-pollinated	Bark and Havey (1995)
Zittauer Gelbe	USDA-ARS	Germany	PI 264632	Open-pollinated	Bark and Havey (1995)
BI794B	USDA-ARS	USA		Inbred	Bradeen and Havey (1995a)
BI828B	USDA-ARS	USA		Inbred	Bradeen and Havey (1995a)
MSU2399B	USDA-ARS	USA		Inbred	Bradeen and Havey (1995a)
MSU611-1B	USDA-ARS	USA		Inbred	Bradeen and Havey (1995a)
MSU8155B	USDA-ARS	USA		Inbred	Bradeen and Havey (1995a)
MSU826B	USDA-ARS	USA		Inbred	Bradeen and Havey (1995a)
Beltsville Bunching	USDA-ARS	USA		Outgroup	Jones and Clarke (1942)
AC43	USDA-ARS	USA		Inbred	King et al. (1998)
BYG15-23	USDA-ARS	USA		Inbred	King et al. (1998)
Striginowski	USDA-ARS	USSR		Open-pollinated	Leite et al. (1999)

^zAccession ID numbers for USDA-ARS (<http://www.ars-grin.gov/>) or AVRDC (<http://www.avrdc.org/germplasm.html>) accessions.^yAsian Vegetable Research and Development Center (The World Vegetable Center) <http://www.avrdc.org/>.^x<http://www.bejo.com>.^wNational Institute of Vegetable and Tea Science (NIVTS), National Agriculture and Bio-oriented Research Organization (NARO), 360 Kusawa, Ano, Mie, 514-2392, Japan.^vInstituto Nacional de Tecnología Agropecuaria (INTA), La Consulta CC8, San Carlos, Mendoza (5567), Argentina.