Genetic Diversity Analysis and Single-nucleotide Polymorphism 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 (Peefley 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
(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).

**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* | Genbank accession no. | Forward primer sequence | Reverse primer sequence | Expected size (bp) |
|------------|-------|-----------------------|-------------------------|-------------------------|-------------------|
| ACM004     | EST-SSR | BI095629 | TCGTTCTTTAGAAGGACGTAGG | GTCGGCGGATATAGTGACA      | 214               |
| ACM018     | EST-SSR | CF438995 | GGGGAATGGGTAGGAATAGA   | AACAGAGAAGAGGAGGCG       | 300               |
| ACM046     | EST-SSR | CF439398 | TCCTGTCACCAACCACAG     | CGTGGAGGATACGGGAG         | 280               |
| ACM050     | EST-SSR | CF438491 | GTTCTCTGTTGGGACA       | CCGGTCCGCTACCTTTGTAT      | 277               |
| ACM065     | EST-SSR | CF449328 | GCTCTGATGGGAGATGGTGC   | CTTGCCATCTTTGTCG          | 197               |
| ACM068     | EST-SSR | CF450463 | GAAGGGTAAAGGTGTCAGG    | GATGATGTTGATGGCATTGA      | 261               |
| ACM183     | EST-SSR | CF443106 | GATGATGTTGATGGCATTGA   | CAAATGGCTCAATAAGGCA       | 213               |
| ACM187     | EST-SSR | CF446468 | GATGATGTTGATGGCATTGA   | CAAATGGCTCAATAAGGCA       | 246               |
| ACM227     | EST-SSR | CF439865 | GAGGTCGGAGAAGGAGGAGT    | TGATAGCCAGTTGAGTCG        | 194               |
| ACM238     | EST-SSR | CF443464 | GTACGTGCTCAATACCAAGGAGGAGG | 261               |
| ACM240     | EST-SSR | CF445554 | GTCCAACCTCAAGAGAAAGAGGAGG | 146               |
| ACM243     | EST-SSR | CF445129 | ATCAAGGAGGGTCAGGAGGAAA  | TCAATGCTGATACCTTGGG       | 141               |
| ACM295     | EST-SSR | CF437320 | AGATCGCTTCCATGAAGACT   | GATCAGCTCTGTTGAAAAATCTCG  | 146               |
| ACM300     | EST-SSR | CF448987 | AGGTGCATGTTTGTGAGG     | TTAGCTGTCAGTGTAAGTGTG     | 154               |
| ACM301     | EST-SSR | CF434545 | TGAGTCAAAAGGTTGAGGAA   | TTCTGTAGTCCCTGGTTTGG      | 109               |
| ACM303     | EST-SSR | CF437320 | AGATCGCTTCCATGAAGACT   | GATCAGCTCTGTTGAAAAATCTCG  | 146               |
| ACM315     | EST-SSR | ES449741 | TTCTGTCACCTTACGAGCAG   | AGAAATGTGTTGAGGATG        | 269               |
| ACM316     | EST-SSR | ES449430 | TGGATCATAAAAAGGGGCGCAT  | GAAAGGCTTTTTCGGGACT        | 204               |
| ACM318     | EST-SSR | ES449367 | TCTCTTCCAACCAACATC     | GATCAGAAAGAACGAGCGTC       | 243               |
| ACM326     | EST-SSR | CF437364 | AAACCCAGCAACAACCAAATG   | AAAATTGGAGAGCAGGCAA       | 200               |

*EST-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.  

| Primer set | Repeat motif | $P_p$ | *A. fistulosum* allele sizes (bp) | *A. roylei* 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     | (CT)$_6$     | 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)$_6$    | 0.87  | 272                              | 272                         | 272                     | 226, 269, 271, 273, 275     |
| ACM045     | (TC)$_6$     | 0.84  | NULL                             | 226                         | 226                     | 293, 296, 299, 302          |
| ACM046     | (TCC)$_9$    | 0.51  | 287                              | 287, 299                    | 293, 296, 299, 302      | 215, 218                    |
| ACM061     | (AGC)$_6$    | 0.63  | 210                              | 207                         | 207, 215                | 215, 218                    |
| ACM063     | (TA)$_6$     | 0.50  | 251                              | 251                         | 251                     | 240, 251, 253               |
| ACM066     | (TA)$_5$N$_{59}$ (CT)$_7$ | 0.17 | NULL                             | NULL                        | 209                     | 209, 211                    |
| ACM068     | (TA)$_6$     | 0.48  | 284                              | 289                         | 281, 285                | 281, 285                    |
| ACM071     | (AG)$_{10}$  | 0.14  | NULL                             | 167                         | 170                     | 166, 170, 172, 174          |
| ACM078     | (TCG)$_7$    | 0.14  | NULL                             | 167                         | 170                     | 286, 292                    |
| ACM091     | (TG)$_6$     | 0.68  | 206                              | 209                         | 209, 206                | 197, 200, 203, 206, 209, 212 |
| ACM093     | (CCA)$_7$    | 0.59  | NULL                             | 209                         | NULL                    | 156                         |
| ACM094     | (TGG)$_5$    | 0.27  | NULL                             | 209                         | NULL                    | 156                         |
| ACM099     | (ATC)$_6$    | 0.91  | NULL                             | 209                         | NULL                    | 136                         |
| 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                         | 209                     | 203, 205                    |
| ACM115     | (CAC)$_6$    | 0.84  | 241                              | 241                         | 241                     | 243, 246                    |
| ACM119     | (AAT)$_8$    | 0.57  | 245                              | 244                         | 244, 249                | 241, 250, 259               |
| ACM121     | (TAT)$_6$    | 0.81  | 165                              | 168                         | 165, 168                | 168                         |
| ACM124     | (AAAG)$_5$   | 0.80  | 221                              | 224                         | 221, 226                | 224, 226, 228, 233, 250     |
| ACM125     | (GTAT)$_3$   | 0.93  | NULL                             | 220                         | 220                     | 216, 220, 224               |
| ACM132     | (ACAT)$_{14}$AC(CATG)$_4$ | 0.91 | NULL                             | 228                         | 220                     | 220, 224, 226, 228, 230, 232, 236 |
| ACM133     | (CA)$_3$(CG)$_6$ | 0.74 | 186 | 206, 210 | 198, 200, 206, 210, 212 |
| ACM134     | (GA)$_8$     | 0.74  | 198                              | 206                         | NULL                    | 198                         |
| ACM138     | (CTGC)$_{11}$ | 0.70 | 252 | 252 | 242, 247, 250, 252, 255, 259, 262, 270, 274, 278, 286 |
| ACM146     | (ACA)$_{15}$ | 0.91  | 233                              | 233                         | 233, 239, 242           | 239, 242                    |
| ACM147     | (CTC)$_5$    | 0.79  | NULL                             | 270                         | 270                     | 267, 270, 273               |
| ACM151     | (ACA)$_{15}$ | 0.48  | NULL                             | 267                         | 267                     | 267, 269                    |
| ACM154     | (AGA)$_{10}$ | 0.84  | 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                             | 263                         | 263                     | 263, 265, 269, 271          |
| ACM170     | (TTC)$_6$    | 0.46  | NULL                             | 241                         | NULL                    | 231, 241, 244               |
| Primer set | Repeat motif | $P_p$ | A. fistulosum allele sizes (bp) | A. roylei 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$_2$ (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     | (TATG)$_{4}$ | 0.18  | 316                           | 292                       | 296, 300                 | 292, 296, 300, 304             |
| ACM243$^*$ | (TGA)$_{6}$  | 0.00  | 162                           | 165                       | 162                      | 159                          |
| ACM251     | (ATTCC)$_{4}$ | 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)$_{5}$  | 0.21  | 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$_{41}$ (TCC)$_{4}$ | 0.00 | 263                           | 263                       | 263, 266                 | 266                          |
| ACM326$^*$ | (CTT)$_{4}$  | 0.00  | NULL                          | 224                       | 224                      | 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)$_{5}$ | 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 tagged forward plus reverse primers at 0.5 μM and fluorescently labeled universal primer at 0.2 μM. A “PIG-tail” sequence (GTFTTCTT) 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 \( P_\pi \) (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

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Fig. 1. Distribution of proportion of polymorphic populations \( P_\pi \) 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 \( P_\pi \) for 89 bulb onion accessions (seven doubled haploid, 12 inbred, 25 landrace, and 45 open-pollinated or hybrid) 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 $P_l$ 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 $P_l$ ranging from 0.12 to 0.84 (Fig. 2). Median $P_l$ in landrace and OP or hybrid populations was 0.7 compared with 0.43 in the sample of inbred lines. The range of $P_l$ observed in the noninbred populations is at the high end of the range observed in isozyme studies of outcrossing species (Berg and Hamrick, 1997;
Hamrick and Godt, 1989). The wide range of $P_l$ 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 $P_l$ 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 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 $P_l$, 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 3. DNA sequence polymorphisms detected in regions flanking simple sequence repeats (SSRs) amplified by onion expressed sequence tag (EST)-SSR markers.

| Primer set | Reference sequence | 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 |

*No polymorphisms were identified in regions flanking SSRs in the markers ACM033, ACM066, ACM094, ACM115, ACM154, ACM229, and ACM245.*

### 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) | Taq1 CAPS                           |
| ACM018           | —                 | CF438995           | T124C                 | See Table 1           | Taq1 CAPS                           |
| ACP014           | ACM183            | CF443106           | G105A                 | GACGACAAACATGATAATAGATAGTCG AATGGTTGCGAGGTCCATT | Xho1 dCAPS |
| ACP015           | ACM093            | CF445996           | C58T                  | TATATACCCGCCACCACGCA TATCTCCTCGGCGTTTCGGA | MboI dCAPS |
| ACP017           | ACM227            | CF439865           | G99A                  | ACCCTCCCCAACCACACGC GAGGTCCGGAGAAGGAGGAG | HhaI dCAPS |
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. | 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               | India             |                  | Open-pollinated       | Havey (2000)                            |
| AC319 (Red pinoy)    | AVRDC               | Philippines       |                  | Open-pollinated       | Havey (2000)                            |
| AC452 (AgriFound Light Red) | AVRDC | India            | TA000200         | Open-pollinated       | Havey (2000)                            |
| AC63 (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        | T000265          | 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             | T000415          | Open-pollinated       | Havey (2000)                            |
| AC595 (Kalpatiya)    | AVRDC               | Sri Lanka         | T000541          | Open-pollinated       | Havey (2000)                            |
| Bombay Red           | AVRDC               | India             | PI 531171        | Open-pollinated       | Havey (2000)                            |
| Hygro                | Bejo Zaden*         | Holland           |                  | Hybrid                | Havey (2000)                            |
| NYS3E21B             | Cornell Univ.       | USA               |                  | Inbred                | http://www.cals.cornell.edu/            |
| YIX A                | Cornell Univ.       | USA               |                  | Open-pollinated       | 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)                      |
| DH2112               | 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       | Leite et al. (1999)                     |
| Shallot 66-1002      | Hebrew University,  | Thailand          |                  | Outgroup              | http://departments.agri.huji.ac.il/fieldcrops/ staff-eng/rabinowitch.html |
| *A. fistulosum* D1s-1s | NIVTS*              | Japan             |                  | Outgroup              | Ohara et al. (2005)                     |
| LC2062               | INTA                | Argentina         |                  | Open-pollinated       | http://www.inta.gov.ar                  |
| Jumbo                | Plant Research      | Netherlands       |                  | Open-pollinated       | van Heusden et al. (2000)              |
| *A. roylei*          | Plant Research      | India             |                  | Outgroup              | van der Meer and de Vries (1990)       |
| Senshu Ki            | Shippo Seed Co.     | Japan             | G 28985          | Open-pollinated       | Havey (2000)                            |
| Pukekohe Longkeeper  | Terranova Seeds     | New Zealand       |                  | Open-pollinated       | http://www.tnseeds.com                  |
| Kiwi Gold            | Terranova Seeds     | New Zealand       |                  | Open-pollinated       | http://www.tnseeds.com                  |
| May & Ryan Regular   | Terranova Seeds     | New Zealand       |                  | Open-pollinated       | http://www.tnseeds.com                  |
| Solara               | Tropicasem          | Senegal           |                  | Hybrid                | http://tropicasemgenel.com/produits/prdts.html |
| Red Creole           | Tropicasem          | Senegal           |                  | Open-pollinated       | http://tropicasemgenel.com/produits/prdts.html |
| Violet de Galmi      | Tropicasem          | Senegal           |                  | Open-pollinated       | http://tropicasemgenel.com/produits/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-grin.gov/                |
| PI 164970            | USDA-ARS            | Turkey            | PI 164970        | Landrace              | http://www.ars-grin.gov/                |
| PI 168960            | USDA-ARS            | Turkey            | PI 168960        | Landrace              | http://www.ars-grin.gov/                |

*continued next page*
| Description                  | Source                  | Country of origin | Accession ID no. \(^{a}\) | 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                | Argentina         | 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 Portuguese             | 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               | G 18545                    | Open-pollinated | Bark and Havey (1995) |
| Southport Red Globe          | USDA-ARS                | USA               | PI 546137                  | Open-pollinated | Bark and Havey (1995) |
| Stuttgarter                  | 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) |
| B1794B                       | USDA-ARS                | USA               |                           | Inbred          | Braden and Havey (1995a) |
| B1826B                       | USDA-ARS                | USA               |                           | Inbred          | Braden and Havey (1995a) |
| MSU2399B                     | USDA-ARS                | USA               |                           | Inbred          | Braden and Havey (1995a) |
| MSU6111-B                    | USDA-ARS                | USA               |                           | Inbred          | Braden and Havey (1995a) |
| MSU8151B                     | USDA-ARS                | USA               |                           | Inbred          | Braden and Havey (1995a) |
| MSU826B                      | USDA-ARS                | USA               |                           | Inbred          | Braden and Havey (1995a) |
| Beltsville Bunching          | USDA-ARS                | USA               |                           | Outgroup        | Jones and Clarke (1942) |
| AC43                         | USDA-ARS                | USA               |                           | Inbred          | King et al. (1998) |
| BYGI5-23                     | USDA-ARS                | USA               |                           | Inbred          | King et al. (1998) |
| Striginowski                 | USDA-ARS                | USSR              |                           | Open-pollinated | Leite et al. (1999) |

\(^{a}\)Accession ID numbers for USDA-ARS (http://www.ars-grin.gov/) or AVRDC (http://www.avrdc.org/germplasm.html) accessions.

\(^{b}\)Asian Vegetable Research and Development Center (The World Vegetable Center) http://www.avrdc.org/.

\(^{c}\)http://www.bejo.com.

\(^{d}\)National Institute of Vegetable and Tea Science (NIVTS), National Agriculture and Bio-oriented Research Organization (NARO), 360 Kusawa, Ano, Mie, 514–2392, Japan.

\(^{e}\)Instituto Nacional de Tecnología Agropecuaria (INTA), La Consulta CC8, San Carlos, Mendoza (5567), Argentina.