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Assessing the genetic diversity of Spanish *Allium cepa* landraces for onion breeding using microsatellite markers

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ABSTRACT

Onion (*Allium cepa* L.) is one of the most valuable vegetables in the world. However, despite its global culinary and economic significance, the knowledge about onion genetic diversity and resources is limited. The Vegetable Germplasm Bank of Zaragoza (BGHZ) (Spain) holds an important *A. cepa* L. collection, where most of the Spanish onion variability is represented. Since the genetic diversity of Spanish onion germplasm is an unexploited resource for onion breeding, a total of 85 Spanish onion landraces (*A. cepa* L.) and 6 related *Allium* outgroups from the BGHZ collection were studied by means of SSR markers. The results showed that 12 out of the 18 SSR markers amplified were useful and polymorphic to distinguish all the studied onion accessions, allowing the detection of 47 alleles, with an average of 3.9 alleles per SSR, ranging from 2 to 7. Within related *Allium* species, the total number of detected alleles was 45, with an average of 3.7 alleles per SSR, ranging from 1 to 10. Specific alleles were obtained both in the Spanish onion landraces and in related *Allium* species, with cross transferability rates ranging from 25.0% to 91.7% in the six *Allium* species assayed. The resulting UPGMA dendrogram grouped the 91 *Allium* accessions according to their taxonomical classification, producing 6 main clusters, with all the Spanish onion landraces included in one cluster at a genetic distance of 0.69. These results revealed an interesting reservoir of genetic variability, useful for onion breeding, and confirmed the need to preserve these irreplaceable genetic resources.

1. Introduction

Onion (*Allium cepa* L.) is one of the most valuable vegetables in the world, only surpassed by tomato and watermelon (FAOSTAT, 2013). However, despite its global culinary and economic significance, the genetic research of onion has greatly lagged behind that of other major vegetable crops (McCallum et al., 2008a). The knowledge of onion genetic diversity and resources is limited, mainly due to a paucity of public markers and germplasm resources and their out-breeding, biennial habit (McCallum and Havey, 2006).

Studies of genetic diversity in onion have been hampered by a lack of portable codominant molecular markers (McCallum, 2007). Although a variety of molecular markers has been successfully used to solve issues of genetic diversity and relatedness at the species level in *Allium* (Klaas and Friesen, 2002), identifying robust and informative markers within *A. cepa* has proved much more challenging.

Microsatellites (SSR) have been widely used in the last decade, acquiring an increasing significance because of their broad range of applications, based on a high degree of intraspecific polymorphism, codominant genetics and their higher reliability and reproducibility as compared to other molecular DNA markers (Jones et al., 1997; Powell et al., 1996). Fischer and Bachmann (2000) were the first to develop SSR markers for onion and used 30 primer pairs flanking microsatellite motifs to assess interspecific taxonomic analyses in *Allium*. As a result of complex amplification requirements, these markers have not proved sufficiently portable to enable their wider use in mapping and diversity studies, although Masuzaki et al. (2006) reported the optimization and chromosomal allocation of a subset of these. SSR markers have been developed from genomic onion (Baldwin et al., 2012) and 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., 2006; McCallum and Havey, 2006) and cultivar discrimination (Jakše et al., 2005; McCallum et al., 2008b; Kisha and Cramer, 2011; Khar et al., 2011).

According to Vavilov (1926) the Southwest Asian gene centre is proposed as the primary centre of domestication and variability of onion. Since onions have been cultivated for so long, and their bulb and inflorescence development must be closely adapted to the

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temperatures and photoperiods prevailing in their regions of growth, a huge range of cultivars and landraces have been developed over the centuries, to fit the diverse climates and food preferences all over the world (Brewster, 1994). Onions show particular diversity in the Mediterranean countries and these regions are, therefore, important sources of genetic diversity (Astley et al., 1982).

The availability of genetic variability within crops, both in ex situ and in situ collections, is of pivotal importance for a sustainable agriculture since the improvement of any crop is proportional to the magnitude of its genotypes' genetic variability. Germplasm characterization and evaluation is an unavoidable requirement for its utilization, specially after taking into account that old varieties are being replaced by the modern varieties sold by international seed companies, in particular F1 hybrids which have a narrow genetic base. Therefore, many potentially valuable adaptive genes contained in the old varieties are in danger of being lost. This fact stresses the importance of characterization, collection, preservation and regeneration of these old varieties and landraces' seeds and vegetable clones (Astley, 1990).

In Spain, onions are grown from the arid South to the lush green and mountainous Northwest, through the high inland plains, as well in the Mediterranea Balearic Islands and the Atlantic Canary Islands. The Vegetable Germplasm Bank of Zaragoza (BGHZ) (Spain) holds an important *A. cepa* L. collection where most of the Spanish onion variability is represented (Carravedo and Mallor, 2007). Some of these traditional onion landraces are still grown in some regions of Spain due to their high quality and acceptance by local markets, but others have already disappeared (Mallor and Sales, 2012). A representative sample of the BGHZ onion collection has already been characterized using morphological and physico-chemical descriptors (Mallor et al., 2011). Thus, the goal of this study was to explore for the first time the genetic diversity of Spanish onion landraces using SSR markers, including 85 accessions supplied by the BGHZ. In addition, *A. cepa* SSRs were cross-amplified in related *Allium* species to assess their reliability in *Allium* classification.

2. Materials and methods

2.1. Plant material

A total of 85 Spanish onion landraces (*A. cepa* L.) (Table 1) and 6 related *Allium* species (*A. cepa* L. *aggregatum* group [shallot], *A. ampeloprasum* L. var *porrum* [leek], *A. cristophii* Trautv., *A. sphaerocephalon* L., *A. ramosum* L., and *A. senescens* L.) were used for this study. Spanish onion accessions are local landraces collected as seeds from farmers in the main growing regions of Spain between 1981 and 2006. All accessions were supplied by the BGHZ (Spain) and all of them have been previously characterized by using morphological and physico-chemical descriptors (Mallor et al., 2011). The onion seeds were grown in the same season in a randomized block design with three replications at the CITA de Aragon (Spain) (latitude 41° 39′ N), where soils have a loamy texture, moderately basic pH and relatively high salt contents.

2.2. Genomic DNA isolation and SSR analysis

The total DNA was extracted from young leaves following a CTAB method (Doyle and Doyle, 1987) with minor modifications (Arnedo-Andrés et al., 2002; Garcés-Claver et al., 2007). The quality and concentration of DNAs were evaluated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The final DNA concentrations were adjusted to 10 ng μL^{-1} with a MTE Buffer (10 mM Tris–HCl, pH 8.0; 1 mM EDTA) for PCR amplifications and stored at −20 °C until used. For SSR analysis, a set of 18 SSR markers, 16 EST-SSRs (ACM004, ACM006, ACM045, ACM046, ACM091, ACM101, ACM119, ACM124, ACM134, ACM138, ACM146, ACM187, ACM227, ACM235, ACM300, and ACM303; McCallum et al., 2008b) and 2 genomic SSRs (AMS12 and AMS21; Fischer and Bachmann, 2000) were used. SSR markers were selected for their higher polymorphism, according to the studies quoted above. PCR amplification was carried out in a final 12.5 μL solution containing 12.5 ng of genomic DNA, 1× PCR buffer (20 mM of Tris–HCl pH 8.4 + 50 mM of KCl), 2 mM of $MgCl_2$, 65 μM of each dNTP (Invitrogen), 0.25 μL of DMSO (≥99.9% Sigma), 0.2 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), 0.0625 μM of the forward primer and 0.25 μM of each reverse and M13-forward primer (5′-CACGACGTTGTAAAACGAC-3′) labelled at the 5′ end with one of the 4 fluorescent dyes (6-FAM, VIC, PET or NED; Applied Biosystems, Foster City, CA, USA). Forward primers were designed with a 19-bp long M13F-tail sequence added to their 5′ end (5′-CACGACGTTGTAAAACGAC-3′) (Schuelke, 2000). PCR reactions were performed in a thermocycler (model 9700; Perkin-Elmer Corp., Norwalk, CT, USA) under the following cyclic conditions: 5 min denaturation at 94 °C followed by 4 cycles of 30 s at 94 °C, 45 s at 62 °C (with each cycle, the annealing temperature decreasing 1 °C) and 45 s at 72 °C. This was followed by 36 cycles at 94 °C for 30 s, 58 °C for 45 s and 72 °C for 45 s, with a final extension at 72 °C for 10 min. Amplified fragments were separated by capillary electrophoresis on an ABI 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA). The raw data produced was analyzed using PEAK SCANNER software (Applied Biosystems).

2.3. Data analysis

All polymorphisms were scored as presence (1) vs. absence (0) of a specific allele and an accession was assigned a null allele where an amplification product could not be detected for a particular genotype-marker combination. The informativeness and discriminatory power of the microsatellite markers were found out by calculating the polymorphic information content (PIC) for the SSR locus. PIC was calculated according to the formula (Weir, 1996): $PIC = 1 - (\sum P_i^2)$, where P_i is the frequency of the ith allele in the set of the Spanish onion landraces and the related *Allium* species genotyped.

A cluster analysis was performed using both distance and similarity matrices on the basis of the UPGMA algorithm. For the PCoA, Eigen-vectors were calculated from the matrix used for the cluster analysis. The PCoA results are shown in two dimensional plots with the first two principal coordinates. The cluster and principal coordinate analyses (PCoA) were performed using the NTSYSpc v2.1 (Rohlf, 2000).

3. Results and discussion

3.1. Microsatellite analysis

The results obtained with the 18 SSR markers (16 EST-SSRs and 2 gSSRs) showed that 12 EST-SSRs were able to amplify in onion landraces (Table 2) and in related *Allium* species (Table 3). The EST-SSR markers showed a percentage of amplification (75%) higher than those previously reported (Santos et al., 2010; Khar et al., 2011) using also the EST-SSRs developed by Jakše et al. (2005). Only one EST-SSR primer (ACM300) gave monomorphic bands in the Spanish onions but it revealed polymorphism between onions and other *Allium* species. This pattern of amplification agrees with that found by Khar et al. (2011) using the same marker (ACM300) to study the genetic diversity of tropical Indian onions and related *Allium* spp. Regarding the two gSSRs used (AMS12 and AMS21), neither of them amplified in our plant material, despite the fact that