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Evaluation of *Cucurbita pepo* germplasm for staminate flower production and adaptation to the frozen food industry

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ABSTRACT

Cucurbita pepo is the most economically important species of the genus *Cucurbita* (Cucurbitaceae). Its edible-fruited cultivated germplasm has been grouped into eight morphotypes (cultivar-groups) divided between two subspecies: *pepo* and *ovifera*. In this work, 93 accessions representative of all morphotypes were grown to investigate their genetic variability and to evaluate their potential for production and suitability of male flowers to be used as an Italian frozen food specialty. Results provid the first indications of a plant ideotype for such use. Principal coordinates analysis (PCoA) with nine SSR markers clearly separated the two subspecies, and yielded information on genetically similar *vs* distant genotypes to be used for parent choice in breeding programs. Analysis of morphological data allowed identification of seven accessions that met all the criteria for the specialty use of male flowers for the frozen food industry: production of many flowers, corolla length of 6–8 cm; and reduced spininess of the foliage. When accessions representing different morphotypes were compared for average male corolla length and average number of male flowers per plant, some accessions of the Pumpkin cultivar-group were observed to be the most suitable for the purpose. Overall, the information collected in the present work is a starting point for exploitation of *C. pepo* biodiversity in future breeding programs aimed at the production of male flowers for use by the frozen-food industry.

1. Introduction

Cucurbita pepo L. (2n = 40), an economically important crop species, is one of the most variable species with respect to fruit characteristics within the Cucurbitaceae (Paris, 2001). On the basis of fruit shape, cultivated edible-fruited *C. pepo* germplasm can be grouped in eight morphotypes or cultivar-groups divided between the two subspecies: subsp. *pepo* L. with morphotypes Pumpkin (round or nearly round), Vegetable Marrow (short, tapered cylindrical), Cocozelle (long, bulbous cylindrical) and Zucchini (uniformly cylindrical); and subsp. *ovifera* L. with morphotypes Scallop (flat, lobed), Acorn (turbinate, furrowed), Crookneck (long, narrow necked), and Straightneck (long, with proximal constriction) (Paris, 1986). *C. pepo* plants are monoecious, producing staminate and pistillate flowers. The bright yellow-orange flowersdifferentiate, in the leaf axils, at the junctions of the stem with the bases of leaf petioles. Male flowers differentiate at the first leaf axils on a long stalk, while female flowers differentiate at subsequent leaf axils but develop faster, on a short stalk (Paris and Hanan, 2010).

The production of female or male flowers and floral sex ratio are determined by a complex interplay of environmental, hormonal and genetic factors, often dependent on crop management. Low temperatures was reported to inhibit the development of male flowers and increase the number of female flowers per plant (Wien et al., 2004), whereas high temperature induced both a change of female flowers into male ones, and a partial transformation of female flowers into hermaphroditic ones (Peñaranda et al., 2007). Manzano et al. (2011) showed that ethylene has a much greater effect on sex expression and flower development in *Cucurbita pepo* than brassinosteroids. As far as genetic factors are concerned, the cocozelle 'Striato d'Italia' was reported to produce more than twice as many male flowers as the zucchini 'Zucchini Black', whereas production of female flowers was the same (El-Keblawy and Lovett-

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Doust, 1996). Moreover, staminate and pistillate flower counts were reported to vary depending both on cultivar and time of planting, indicating that maintaining production over a range of planting dates depends on careful cultivar selection (NeSmith et al., 1994). The harvest of immature fruits increased the production of both male and female flowers, and the development of additional stems and leaves (El-Keblawy and Lovett-Doust, 1996).

The main economic value of this species is based on its use as immature fruit vegetables, often referred to as "summer squash"; however, members of the Pumpkin and Acorn cultivar-groups have also a widespread use as mature fruit vegetables, known as "winter squash". Mature ripe *Cucurbita pepo* fruits are also popular as ornamentals, and seeds of *C. pepo* are widely used as snacks, as source of edible oil and protein for human and animal consumption, and in the pharmaceutical industry (Blanca et al., 2011). The Mediterranean traditional culinary use of flowers, staminate as well as pistillate, dates to the 16th century in Italy (Paris and Janick, 2004) and has since increased in popularity (Lust and Paris, 2016). There is a variety of recipes for squash flowers: deep-fried, stuffed, sautéed, baked or used in soups and pasta sauces.

For deep fried commercial preparations, the flowers are coated with breading or batter and then frozen. Staminate flowers, especially those with relatively small corollas of about 6–8 cm, are much more preferred by the food industry. Notwithstanding such a promising market for male flowers, still double purpose (flower and fruit) genotypes of *C. pepo* are grown nowadays, and male flowers for the production of specialty foods are harvested as a side-product. As far as the authors know, no specific plant breeding program exists, aiming at increasing male flower production of *C. pepo*. Therefore, the main purpose of the present study was to investigate both molecular and horticulturally relevant morphological variability of a *C. pepo* germplasm collection, constituted *ad hoc* in order to start exploitation of *C. pepo* biodiversity for the production of male flowers for specialty foods. SSR (Simple Sequence Repeat) markers were used to identify the level of genetic diversity among the accessions, and a number of traits (number of male *vs* female flowers per plant, corolla length, presence of spines on petioles, plant growth habit and vigor) were recorded in a field trial in Italy in order to identify the characteristics of a plant ideotype most suitable for this particular use.

2. Materials and methods

2.1. Plant material and field growth conditions

A collection of 93 accessions of *C. pepo* comprising old and modern cultivars, hybrid cultivars, landraces, breeding and cultivated materials, and accessions of unknown status, representative of the eight morphotypes of the species, were used in a field trial in Northern Italy and characterized for their morphological and genetic variability. Table 1 summarizes i the observed morphotypes, country of origin and source of the genetic materials. Seeds were kindly provided by the Germplasm Resources Information Network (GRIN, http://www.ars-grin.gov/, last accessed 30 June 2016) of the United States Department of Agriculture (USDA), by the Institute of Plant Genetics and Crop Plant Research (IPK, http://gbis.ipk-gatersleben.de/) (Gatersleben, Germany), and by European seed companies (La Semiorto Sementi, HM-Clause, Isi Sementi, Seminis, Semencoop, Enza Zaden, Euroselect and Sativa). A complete list of materials, reporting name of each accession, its abbreviation code, morphotype, country of origin and seed source, is shown in Table 2.

A field experiment was carried out in 2013 at Sant'Agata Bolognese, Northern Italy (44° 38′ N, 11° 8′ E; 22 m amsl). The site of cultivation has a sub-continental climate with total annual rainfall of 600–700 mm, and an average day temperature of 14.5 °C. The soil is classified as Calcic Hyposalic and Haplic Vertisol (IUSS Working Group WRB, 2006).

All accessions were sown in polystyrene trays (41.5 cm x 31 cm x 6.5 cm) with 40 cells each. Seedlings were grown for 3 weeks in a greenhouse with a 12-h photoperiod, day/night temperatures of 28/20 °C and a relative humidity of 60%. Before transplant, leaf samples were collected from three plants and bulked for each genotype, then conserved at −80 °C for gDNA extraction. Seedlings were transplanted into the open field from greenhouse on May 15, 2013, at the stage of 3rd leaf. Within a row, the transplants were spaced 0.85 m apart with 2.15 m between each row (density 0.53 plants m^{-2}). The experiment was performed as a randomized complete block design with seven plants per plot and three replications.

The squash plots were irrigated immediately after transplanting, and the following drip-irrigations were practiced on an 'as-needed' basis throughout the dry season. Eighty, 160 and 115 kg ha^{-1} of N, P and K respectively were added to the soil before transplanting. Moreover, during the growing season, nitrogen fertilizer (additional 100 kg ha^{-1} of N) was applied periodically as ammonium nitrate through fertigation. Weeds were managed using plastic mulch 100 cm wide, and, when necessary, herbicides. Pests (mainly aphids) and diseases were controlled by chemical treatments according to production rules for squash crop of Emilia Romagna Region, Italy.

2.2. Genotypic characterization and genetic diversity analysis

DNA was extracted from young leaves of three plants per accession using a modified CTAB method (Stein et al., 2001). A set of nine SSRs selected from those developed by Gong et al. (2008) were used for the analysis (listed in Table 3). The PCR reactions were performed in 25 μl volume containing 20 ng of template DNA, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of dNTPs, 0.04 μM of forward primer with an M13 tail added to its 5′ end (5′-CACGACGTTGTAAAACGAC-3′), 0.2 μM of reverse primer, and 0.32 μM of fluorescent labelled M13 tail (FAM, NED, VIC synthesized by MWG, Ebensburg, Germany), 1 unit of DreamTaq™ DNA Polymerase (Thermo Scientific, USA). The PCR profile was set as follows: denaturation at 95 °C for 3 min, followed by ten cycles of 30 s at 95 °C, 30 s at 65 °C (with each cycle the annealing temp decreases 1 °C), and of 30 s at 72 °C. Products were subsequently amplified for 30 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 7 min. The PCR was performed using Applied Biosystems thermal cycler GeneAmp PCR system 2720. The fluorescently labelled amplification products were analyzed by capillary electrophoresis on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

For each microsatellite marker, the number of alleles observed (n_a) and the number of rare alleles were recorded. An allele was considered to be rare if its frequency was lower than 0.05 (Roussel et al., 2004). Moreover the number of effective alleles per locus ($n_e = 1/\Sigma pi^2$, where pi is the frequency of the i^{th} allele), the mean observed homozygosity (H_o) and the Shannon's diversity index ($H' = -\Sigma pi \ln pi$) were calculated for each locus. Finally the Nei expected heterozygosity ($H_e = 1 - \Sigma pi^2$) and the allelic polymorphism information content (PIC; Botstein et al., 1980) were calculated using the free online software PICcalc (Nagy et al., 2012).

For the investigation of genetic relatedness of accessions, every SSR allele was scored as present (1) or absent (0) through all accessions to create a binary data matrix. The binary data were then subjected to principal coordinate analysis (PCoA) to partition the variance using PAST software version 2.14 (Hammer et al., 2001), after using Jaccard's similarity index to calculate genetic similarity. Only coordinates with eigenvalues >1 were considered.

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