Phenotypic and Genetic Diversity in Pumpkin Accessions with Mutated Seed Coats

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Abstract. The increasing importance of pumpkin (Cucurbita pepo L.) cultivars for seed production has led to considerable breeding efforts for novel high-yielding and disease-resistant cultivars lacking seedcoats. Because it is very important to use genetically diverse genotypes for the development of cultivars with a broad genetic and phenotypic base, this study focused on phenotypic and genetic diversity within and among available pumpkin accessions with mutated seedcoat phenotypes. Fifty-one accessions were collected from various sources and countries, which showed a wide variety of seedcoat types. Genetic analysis with 18 simple sequence repeat (SSR) markers revealed that 37.59% of the total genetic diversity was attributable to interpopulation differentiation and 62.41% to individual differentiation within populations. The average genetic differentiation between accessions (Fst) was from 0.030 to 0.760, whereas expected heterozygosity (He) was between 0.048 and 0.491 and observed heterozygosity (Ho) between 0.056 and 0.522. Based on unweighted pair group method with arithmetic mean (UPGMA) analysis, the genetic relationship among accessions reflects the primary geographical origin of accessions. Marker amplification yielded a total of 109 alleles with an average number of alleles per locus of 6.06. Gene diversity per locus varied between 0.027 and 0.879, whereas the polymorphism information content (PIC) varied between 0.027 and 0.867. This is the first report about intra-accession phenotypic and genotypic variability of pumpkins with mutated seedcoats cultivated for their seeds, which are today used in the baking industry, seed oil production, and in traditional and modern medicine.

The genus Cucurbita (Cucurbitaceae) includes a vast variety of fruits, including gourds, squashes, and pumpkins. One of the economically most important species of the genus is Cucurbita pepo L., which produces plants with a wide range of growth habits, fruit sizes, shapes, and colors. The edible round-fruited types of C. pepo are called “pumpkins,” the edible non-round types are called “squash,” whereas the non-edible sorts are called “gourds” (Paris et al., 2003). Based on fruit shape, the species is divided into eight edible-fruit cultivated groups and two cultivar groups of ornamental gourds: Pumpkin (round), Cocozelle (long, bulbous cylindrical), Vegetable Marrow (short, tapered cylindrical), Zucchini (uniformly cylindrical), Orange Gourd (small, round), Acorn (turbinate, furrowed), Scallop (flat, scalloped), Crookneck (long, narrow neck), Straghineck (short, thick neck), and Ovifera Gourd (small, various shapes) (Paris, 1986, 2000).

At the end of the 19th century, a mutation in the gene (genes) responsible for pumpkin seedcoat development occurred in Styria, and phenotypes with collapsed testa layers segregated from normal field pumpkins (Teppner, 2000). The absence of four lignified seedcoat layers revealed the dark green color of the innermost layer—the chlorenchyma—composed of cells with high levels of protochlorophyll pigment (Kreft et al., 2009). Since then, these genotypes have been cultivated and bred for the production of pumpkin seeds that are used as snacks and in the baking industry or further processed to extract the oil. The oil from roasted pumpkin seeds is particularly valued in central European cuisine as a result of its strong nut-like taste and dark green color. The seeds are also used in traditional medicine and are present in several medicinal products because of their demonstrated therapeutic properties against benign prostatic hyperplasia and other diseases (Gossell-Williams et al., 2006; Shirvan et al., 2014; Yadav et al., 2010). Despite the increasing economic significance of pumpkin cultivars for seed production, there is no information available about population structure within and among accessions with mutated seedcoats and the range of inbreeding present in these accessions. Previous genetic studies have been mainly focused on interspecific diversity within the genus Cucurbita or genetic diversity among C. pepo subspecies with an emphasis on other cultivar groups (Physset al., 2003; Formisano et al., 2012; Gong et al., 2008a, 2012, 2013; Mady et al., 2013; Paris et al., 2003).

SSR (microsatellites) are very popular molecular markers because they are highly informative, multiallelic, polymerase chain reaction (PCR)-based, and codominant. In this study, SSRs were used to evaluate genetic variability within and among 51 C. pepo accessions with wild-type or mutated seedcoats, the latter commonly referred to as hull-less seeds, thin-coated seeds, or naked seeds. These accessions are valuable resources for breeding purposes and the obtained genetic information will complement phenotypic characterization during selection.

Material and Methods

Fifty-one accessions originating from five genebanks (U.S. Department of Agriculture, Agricultural Research Service, North Central Regional Plant Introduction Station, Iowa State University, Regional Plant Introduction Station, Ames, IA; Institute of Special Crops, Agricultural Research Center Styria, Austria; Arche Noah Association, Austria; Crop Research Institute, Czech Republic; CRA Consiglio per la ricerca e la sperimentazione in agricoltura, Italy), three seed companies (Semenara Ljubljana, Slovenia; Saatzucht Gleisdorf, Austria; H.S.C. New Zealand), a Slovenian plant breeder (Prof. Dr. Anton Ivančič) and from a commercial package of snack seeds imported from China were included in the study (Table 1).

The type of seedcoat was determined for each accession based on the classification of Murovec et al. (2012). Five seeds per accession were germinated and total genomic DNA was extracted from the leaf tissue of individual plants by a modified CTAB method (Kump and Javornik, 1996). PCR was performed as described by Gong et al. (2008a) and the amplified products were separated by capillary electrophoresis using an ABI PRISM 3100 Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA). Electropherograms were analyzed with GeneMapper software Version 3.5 (Applied Biosystems, Foster City, CA) and GeneScanTM 600 LIZ® Size Standard was used as an internal standard. Results were recorded as allele lengths and converted for further analyses to appropriate matrices with the software CONVERT Version 1.31 (Glaubitz, 2004).

Genetic diversity among and within the 51 accessions of C. pepo was determined through allele analysis of 18 SSR markers (Table 2). The total number of amplified alleles, the effective number of alleles, the Nei’s and He, and PIC for each SSR marker were determined with PowerMarker Version 3.25 software (Liu and Muse, 2005) and POPGENE Version 1.31 (Yeh et al., 1999). For each accession, the number of polymorphic...
Table 1. Results obtained for 51 C. pepo accessions collected and analyzed for seedcoat characteristics and genetic variability.

| Accession | Country of origin | Accession type | Seed providery | Seed type | No of polymorphic loci | No of alleles per accession | No of alleles per locus | Effective no of alleles per locus | Observed heterozygosity | Expected heterozygosity | Fixation index |
|-----------|------------------|----------------|----------------|-----------|------------------------|----------------------------|-------------------------|--------------------------------|----------------------------|-------------------------|--------------|
| 01-Gleisdorfer Ölkurbis Cultivar AUT SG J | 13 | 46 | 2.556 | 2.119 | 0.367 | 0.361 | –0.022 |
| 02-WIES-SK5-Gleisdorfer Ölkurbis Cultivar AUT ISC J | 13 | 43 | 2.389 | 1.850 | 0.322 | 0.307 | –0.055 |
| 03-WIES-SK9-Gleisdorfer Ölkurbis Cultivar AUT ISC J | 12 | 38 | 2.111 | 1.635 | 0.322 | 0.268 | –0.169 |
| 04-WIES-SK25-Gleisdorfer Ölkurbis Steirer-SZ Gleisdorf Cultivar AUT ISC I | 14 | 37 | 2.056 | 1.707 | 0.433 | 0.341 | –0.268 |
| 05-ARCHE-KU025-Feldkuerbis ohne schale Cultivar AUT ANA I, J | 8 | 29 | 1.611 | 1.437 | 0.156 | 0.196 | 0.281 |
| 06-PI 615133-Gleisdorfer Ölkurbis Cultivar AUT USDA J | 13 | 40 | 2.222 | 1.741 | 0.389 | 0.327 | –0.189 |
| 07-Gleisdorfer Opal Hybrid AUT SG J | 11 | 35 | 1.944 | 1.622 | 0.422 | 0.289 | –0.429 |
| 08-Gleisdorfer Diamant Hybrid AUT SG J | 10 | 31 | 1.722 | 1.597 | 0.422 | 0.282 | –0.496 |
| 09-WIES-SK10-Gleisdorfer Ölkurbis Wieser Busch Landrace AUT ISC I | 14 | 37 | 2.056 | 1.689 | 0.378 | 0.321 | –0.168 |
| 10-WIES-SK22-Gleisdorfer Ölkurbis Dirnberger Landrace AUT ISC J | 11 | 36 | 2.000 | 1.683 | 0.367 | 0.299 | –0.230 |
| 11-ARCHE-KU046-Olkurbis Riegelnegg Unknown AUT ANA I, J | 5 | 23 | 1.278 | 1.229 | 0.122 | 0.123 | 0.068 |
| 12-ARCHE-KU070-Tschermak Ölkurbis Unknown AUT ANA I | 3 | 21 | 1.167 | 1.080 | 0.367 | 0.123 | 0.068 |
| 13-WIES-SK6-Olkurbis Wies 371 Cultivar AUT ISC J | 12 | 40 | 2.222 | 1.906 | 0.400 | 0.340 | –0.182 |
| 14-Snack seeds Unknown CHI / D | 10 | 31 | 1.722 | 1.449 | 0.222 | 0.236 | 0.056 |
| 15–09H4200822-Opavska Cultivar CZE CRI H, I | 10 | 32 | 1.778 | 1.543 | 0.278 | 0.267 | –0.084 |
| 16-Ames29179-GSMO 1–4 Landrace Geo, SO USDA A, F, L | 6 | 26 | 1.444 | 1.340 | 0.156 | 0.156 | 0.001 |
| 17-PI 209783-Gießenner Ölkurbis Cultivar GER USDA C, I, J | 11 | 31 | 1.722 | 1.551 | 0.344 | 0.280 | –0.222 |
| 18-PI 266925-Gießenner Buscholkurbis Cultivar GER USDA A, I | 14 | 39 | 2.000 | 1.862 | 0.400 | 0.379 | –0.182 |
| 19-PI 531323-Szentesi Oliva Cultivar HUN USDA J, L | 11 | 33 | 1.833 | 1.567 | 0.278 | 0.267 | –0.075 |
| 20-WIES-SK8-Olkurbis Kakai Cultivar HUN ISC J | 14 | 36 | 2.000 | 1.613 | 0.422 | 0.307 | –0.075 |
| 21-WIES-SK7-Ungarischer Ölkurbis Landrace HUN ISC I, J | 12 | 40 | 2.056 | 1.613 | 0.378 | 0.321 | –0.168 |
| 22-PI 364241-Hejatlan Magn Unknown HUN USDA C, J, L | 15 | 37 | 2.056 | 1.736 | 0.378 | 0.321 | –0.168 |
| 23-Zucca stiriana 49 Unknown ITA CRA J | 8 | 26 | 1.444 | 1.312 | 0.203 | 0.176 | –0.140 |
| 24-PI 379308-Furazna Landrace MAC USDA A | 11 | 34 | 1.889 | 1.613 | 0.222 | 0.196 | –0.182 |
| 25-PI 379309-Volovska Landrace MAC USDA A, F | 14 | 38 | 2.111 | 1.906 | 0.400 | 0.340 | –0.182 |
| 26-PI 379310-Zeljanka Landrace MAC USDA A | 10 | 32 | 2.000 | 1.613 | 0.378 | 0.321 | –0.168 |
| 27-PI 379319-Golosemenka Landrace MAC USDA G, H, I | 10 | 29 | 1.613 | 1.406 | 0.222 | 0.196 | –0.182 |
| 28-PI 311741-Pulawska Unknown POL USDA I | 10 | 32 | 1.778 | 1.543 | 0.278 | 0.267 | –0.075 |
| 29-PI 285611 Unknown POL USDA B, I, J | 10 | 32 | 1.613 | 1.406 | 0.222 | 0.196 | –0.182 |
| 30-PI 364240-Gribovskaja 14 Cultivar RUS USDA A, F | 3 | 21 | 1.167 | 1.080 | 0.367 | 0.299 | –0.230 |
| 31-Slovenska golica Cultivar SLO SLI C | 7 | 25 | 1.389 | 1.350 | 0.211 | 0.183 | –0.165 |
| 32-PI 164997-Tergomlek Landrace TUR USDA A | 15 | 46 | 2.556 | 1.926 | 0.439 | 0.384 | –0.137 |
| 33-PI 420328-Turkey #1 Landrace TUR USDA A | 10 | 36 | 2.000 | 1.638 | 0.367 | 0.270 | –0.364 |
| 34-PI 420329-Turkey #2 Landrace TUR USDA A, F | 8 | 30 | 1.667 | 1.518 | 0.267 | 0.229 | –0.154 |
| 35-PI 420330-Turkey #3 Landrace TUR USDA A, F | 8 | 30 | 1.667 | 1.426 | 0.289 | 0.196 | –0.391 |
| 36-PI 406679 Landrace TUR USDA A, F | 12 | 37 | 2.056 | 1.698 | 0.400 | 0.341 | –0.137 |
| 37-PI 490278-Butterball Cultivar USA USDA A, I, K, L | 16 | 44 | 2.444 | 1.961 | 0.511 | 0.422 | –0.194 |
| 38-PI 615102-Naked Seed Cultivar USA USDA E | 7 | 27 | 1.500 | 1.325 | 0.211 | 0.159 | –0.279 |
| 39-PI 615104-Prostate Cultivar USA USDA I | 5 | 23 | 1.278 | 1.114 | 0.056 | 0.074 | 0.167 |
| 40-PI 267664-Yellow Long Unknown USA USDA D, G, K | 4 | 22 | 1.222 | 1.164 | 0.056 | 0.089 | 0.397 |

(Continued on next page)
Results

Seed types. Seeds were collected from several sources to have a comprehensive collection of seeds with mutated seedcoats. The collected seeds originated from 14 different countries with the highest number of accessions from Austria followed by the United States and Turkey (Table 1). Visual evaluation based on seedcoat characteristics revealed that a wide variety of seed types was present in our collection. They were categorized into 12 different seed types (Fig. 1), as described in Murovec et al. (2012), and labeled from A to L, where the letters indicate: A = wild type, B = central sclerenchyma, C = marginal sclerenchyma 1, D = marginal sclerenchyma 2, E = distorted sclerenchyma, F = aerenchyma, G = undulated, H = collapsed 1, I = collapsed 2, J = thin-coated, K = partial hull-less, and L = white hull-less. The results of the seed types observed in each accession are presented in Table 1. All the different seed types were further analyzed with scanning electron microscopy and the results presented in Murovec et al. (2012).

Genetic diversity based on microsatellite data. Amplification of 18 microsatellite loci from the 51 accessions listed in Table 1 yielded a total of 109 alleles, which ranged in size from 108 bp (markers CMTm115 and CMTm131) to 264 bp (marker CMTp177). The number of alleles per locus varied between two (marker CMTm115) and 16 (marker CMTp79) with an average of 6.06. Hs of markers ranged from 0.004 (marker CMTm239) to 0.888 (marker CMTp235), whereas He ranged from 0.028 (marker CMTm239) to 0.879 (marker CMTp79). PIC of markers varied between 0.027 (marker CMTm239) and 0.867 (marker CMTp79) (Table 2). The intra-accession genetic variability was estimated by the number of polymorphic loci, number of alleles per accession and locus, effective number of alleles, mean observed and expected heterozygosity, the fixation index (F), and the FST between accessions were calculated by GenALEx 6.5 (Peakall and Smouse, 2006, 2012). The analysis of molecular variance (AMOVA) was performed using Arlequin 3.5 (Excoffier et al., 2005). The pairwise genetic differences among accessions were estimated according to the Nei (1973) coefficient as implemented in PowerMarker Version 3.25. The resulting distance matrix was used for hierarchical clustering using the UPGMA analysis algorithm as implemented in PowerMarker Version 3.25 and visualized by MEGA5 software (Tamura et al., 2011).

Discussion

Seed characterization revealed that a wide variety of different seed types was present in our collection. Although most literature data describe only the most common three types of C. pepo seeds, the presented results show that at least 12 different seed types are present (Fig. 1). Ten of them show mutant seedcoat phenotypes with the outer four testa layers collapsed in different ways revealing the innermost green chlorenchyma layer, as...
Table 2. Amplification results for 18 simple sequence repeat (SSR) markers on 51 accessions of *C. pepo*.

| SSR marker | Number of alleles reported in Gong et al. (2008a) | Observed number of alleles | Effective number of alleles | Allele size range (bp) | Observed heterozygosity | Expected heterozygosity | PIC |
|------------|-----------------------------------------------|---------------------------|-----------------------------|-----------------------|------------------------|------------------------|-----|
| CMTp55     | 5                                             | 3                         | 1.932                       | 134 144               | 0.315                  | 0.482                  | 0.382 |
| CMTp79     | 6                                             | 16                        | 8.239                       | 184 222               | 0.538                  | 0.879                  | 0.867 |
| CMTp80     | 5                                             | 3                         | 2.724                       | 167 171               | 0.314                  | 0.633                  | 0.555 |
| CMTp88     | 5                                             | 5                         | 1.899                       | 173 194               | 0.327                  | 0.473                  | 0.439 |
| CMTp109    | 5                                             | 5                         | 1.390                       | 118 144               | 0.127                  | 0.281                  | 0.264 |
| CMTm115    | 3                                             | 2                         | 1.503                       | 108 109               | 0.139                  | 0.335                  | 0.279 |
| CMTm125    | 5                                             | 3                         | 2.356                       | 120 133               | 0.310                  | 0.576                  | 0.485 |
| CMTm131    | 5                                             | 3                         | 1.392                       | 108 134               | 0.211                  | 0.282                  | 0.252 |
| CMTp131    | 3                                             | 7                         | 1.268                       | 122 150               | 0.068                  | 0.211                  | 0.202 |
| CMTp142    | 7                                             | 8                         | 3.554                       | 164 212               | 0.441                  | 0.719                  | 0.677 |
| CMTp175    | 5                                             | 3                         | 1.852                       | 159 175               | 0.298                  | 0.460                  | 0.363 |
| CMTp176    | 6                                             | 12                        | 1.176                       | 202 264               | 0.131                  | 0.316                  | 0.200 |
| CMTp193    | 5                                             | 14                        | 3.258                       | 160 221               | 0.359                  | 0.693                  | 0.649 |
| CMTp224    | 5                                             | 7                         | 2.090                       | 155 173               | 0.061                  | 0.522                  | 0.470 |
| CMTp235    | 6                                             | 12                        | 6.428                       | 145 178               | 0.888                  | 0.844                  | 0.827 |
| CMTm239    | 1                                             | 3                         | 1.028                       | 181 184               | 0.004                  | 0.028                  | 0.027 |
| CMTp245    | 5                                             | 5                         | 2.345                       | 132 151               | 0.325                  | 0.574                  | 0.485 |
| CMTp252    | 5                                             | 4                         | 1.221                       | 175 208               | 0.052                  | 0.181                  | 0.173 |
| Average    | 4.778                                         | 6.056                     | 2.541                       | ——                   | 0.303                  | 0.466                  | 0.422 |

PIC = polymorphism information content.
HSC151," grouped with European accessions. Some genetic relatedness was expected still to exist because the mutation in gene (genes) for the lignification of the seedcoat occurred in central Europe (Styria). Accessions from Turkey and the Republic of Macedonia clustered into a well-defined cluster (from “41-PI 420331-Turkey #4” to “37-PI 164997-Tergomlek”) of 11 accessions with most seeds of Types A (wild=type) and F (aerenchymal). At the bottom of the tree, below a small cluster composed of a Russian and a Georgian–South Ossetia accession, are all the eight North American accessions with several seedcoat types.

An evaluation of a comprehensive collection of \emph{C. pepo} accessions with mutated seedcoats is presented in this study. The results of morphological characterization of seeds showed that a wide variety of different seed types exists among the studied accessions (Fig. 1; Table 1). These data, in combination with the results of genetic analysis, will be useful for future breeding programs. As previously reported (Gong et al., 2012; Kosmrlj et al., 2013), there is high genetic similarity within the gene pool of hull-less pumpkins cultivated today and the discovery of genetically more distant accessions could overcome this drawback.

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Fig. 2. Dendrogram of genetic relationships among 51 C. pepo accessions based on Nei's coefficient (Nei, 1973) and unweighted pair group method with arithmetic mean (UPGMA) cluster analysis. Origins of accessions: (AUT) Austria, (CHI) People’s Republic of China, (CZE) Czech Republic, (Geo, SO) Georgia, South Ossetia, (GER) Germany, (HUN) Hungary, (ITA) Italy, (MAC) Republic of Macedonia, (NZ) New Zealand, (POL) Poland, (RUS) Russia, (SLO) Slovenia, (TUR) Turkey, (UN) Unknown, (USA) United States of America. Seed types: (A) wild type, (B) central sclerenchymal, (C) marginal sclerenchymal 1, (D) marginal sclerenchymal 2, (E) distorted sclerenchymal, (F) aerenchymal, (G) undulated, (H) collapsed 1, (I) collapsed 2, (J) thin-coated, (K) partial hull-less, and (L) white hull-less.
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