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PHYLOGENETIC RELATIONSHIPS OF THREE SPECIES OF *CUCURBITA* SPP. FROM PORTUGAL EVALUATED BY SSR MARKERS

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Abstract

Cucurbita species were introduced in Europe after the discovery of America and are now used in many parts of the world. In this genera *Cucurbita pepo* L., *C. moschata* Duch. and *C. maxima* Duch. are the most economically important species. To evaluate the genetic diversity, 54 *C. pepo*, 21 *C. moschata* and 32 *C. maxima* populations, all from northern and inner center of Portugal, were studied using six microsatellite primers. The percentage of polymorphism found for the three species was 100%. The observed heterozygocity values for individual *loci* ranged from 0.065 to 0.540, with an average of 0.316, while expected heterozygocity ranged from 0.275 to 0.598 with a mean of 0.447. The polymorphism information content varied from 0.604 to 0.787 with an average of 0.688. AMOVA indicated that about 54% of variation in the data set was from genotypic variations among species and the remaining 46% of differences among and within populations, indicating a degree of population structure. A Neighbor-joining dendrogram based on shared allele distance showed a clear separation of the three species. Principal Coordinated Analysis showed that the three most informative principal coordinates explain 66.3% of the total variation and clearly separated the three species of *Cucurbita*. The genetic diversity found on these species of *Cucurbita* can provide relevant information for the diversity conservation and it will be useful to identify local selections for preservation and use in breeding programs.

Keywords: *C. maxima, C. moschata, C. pepo, microsatellites, genetic diversity*

Introduction

The three most economically important *Cucurbita* species are *Cucurbita pepo* L., *C. maxima* Duch. and *C. moschata* Duch. which have different climatic adaptations and are widely distributed in agricultural regions worldwide (Robinson and Decker-Walters, 1997). *Cucurbita* species were introduced in Europe after the discovery of America and are now used in many parts of the world. The genetic diversity within and among *Cucurbita* species has been evaluated using several molecular markers, such as SSR, RAPD, SRAP, ISSR and AFLP (Katzir et al., 2000; Paris et al., 2002; Hernandez and Eguiaete, 2002; Ferriol et al., 2003, 2004; Esteras et al., 2008; Gong et al., 2008; Tsivelikas et al., 2009; Inan et al., 2012). Comparisons of ribosomal DNA (Torres-Ruiz and Hemleben, 1991) and ISSR and SSR analysis (Katzir et al., 2000; Ferriol et al., 2003) were performed with the same purpose. However, the majority of these studies used improved commercial cultivars. Simple sequence repeat (SSR) markers are one of the major tools in the evaluation of genetic diversity and phylogenetic relationships of species based on sequence conservation because of its high efficiency, codominant nature, reproducibility, and high degree of polymorphism (Kalia et al., 2011). Genetic relationships among the species of *Cucurbita,* has been studied using SSR markers (Gong et al., 2012, 2013). The production of *Cucurbita* in Portugal is based in local cultivars, landraces, and is mainly for self-consumption (human food or animal feed) and is

sale on local markets. The landraces are a very important source of genetic diversity, and are an important genetic resource for plant breeders. This variability is maintained by deliberate selection for specific traits by farmers. At the research level, the diversity of genetic resources in germplasm collections may increase the efficiency of efforts to improve a species (Geleta et al., 2005). The aim of the present study was to evaluate the genetic diversity and relationships between three *Cucurbita* species from Northern and inner center of Portugal.

Materials and methods

A collection of 107 populations derived from three species of *Cucurbita* was evaluated in this study. It included 54 populations of *C. pepo*, 21 of *C. moschata* and 32 of *C. maxima* collected between 2011 and 2013. Total genomic DNA was extracted using the DNeasy kit (Qiagen) (Hilden, Germany), according to the manufacturer's instructions. A minimum of 20 seedlings per landrace were sampled in bulk, and six *Cucurbita* SSR markers were used in this study. The total volume of the PCR mixture was 20µl, and contained 12.5µl of 2x Qiagen multiplex PCR Master Mix (QIAGEN Multiplex PCR Kit), 2.5µl Q-Solution, 0.125µl each of forward and reverse primers and 10 ng of template DNA. The PCR program consisted in an initial denaturation of 15min at 94°C followed by 35 cycles of a 30s denaturation step at 94ºC, a 90s annealing step at the optimum annealing temperature, and a 30s extension step at 72ºC. There was a final 10min extension step at 72ºC. PCR products were separated on an ABI Prism 3100 Genetic Analyser (Applied Biosystems, FosterCity,CA). DNA fragment sizes were determined using GeneMapper software (Applied Biosystems). The population genetic analysis was performed using GenAlEx software package version 6.5 (Peakall and Smouse 2006) to calculate expected heterozygosity (H_e), observed heterozygosity (H_o) and the analysis of molecular variance (AMOVA) in order to partition of genetic variation among populations and within populations (Schneider et al. 2000). The significance of each variance component was tested with permutation tests (Excoffier et al. 1992). Wright's F_{ST} was used to estimate population differentiation. Genetic distances were estimated according to Nei (1978) and principal coordinate analyses (PCoA) (Gower 1966), was performed to identify genetic variation patterns among the *Cucurbita* genotypes. The polymorphism information content (PIC) value of a locus ranges from 0 (monomorphic) to 1 (highly informative), and it was calculated using The Excel Microsatellite Toolkit (Park 2001). Genetic similarity matrices based on the proportion of shared alleles and Neighbor-joining cluster analysis were used to construct genetic trees by Populations software. Dendrogram was visualized using TreeView (Win 16) 1.04 software (Page, R. 1997).

Results and discussion

Several powerful marker techniques are currently available for genetic analysis of plant species. The choice of the most appropriate technique for a specific study is not obvious and depends principally on the purpose of the research, the biology and the genetic structure of the species. In this study the 107 populations of three *Cucurbita* species evaluated by six SSR markers, showed a high genetic diversity. The observed heterozygocity (H_o) values for individual *loci* ranged from 0.065 to 0.540, with an average of 0.316, while expected heterozygocity (H_e) ranged from 0.275 to 0.598 with a mean of 0.447. These values can be considered high compared with other *Cucurbita* studies. Hernandez and Eguiaete (2002) and Inan et al. (2012) registered a $H_e = 0.40$ and $H_e = 0.30$ in 16 and 24 accession of *Cucurbita* species, respectively. In *C. pepo* Barzegar et al. (2013) found a H_e=0.40. The polymorphism information content varied from 0.604 to 0.787 with an average of 0.688 (Table 1).

Table1. Description of six SSR *loci* in three *Cucurbita* species

SSR *locus*	Ho	He	PIC
P61	0.468	0.562	0.787
M90	0.514	0.544	0.765
P5	0.215	0.331	0.631
P102	0.441	0.474	0.698
P98	0.194	0.319	0.604
M54	0.064	0.451	0.645
Mean	0.316	0.447	0.688

H_e, expected heterozygosity; H_o, observed heterozygosity;
PIC, polymorphic information content.

Barzegar et al. (2013) in *C. pepo* also reported a high mean value for PIC (0.779). Markers with high PIC values could be effectively used in genetic diversity studies of *Cucurbita* species. Boststein et al. (1980) suggested that PIC value >0.5 indicates a highly informative marker. The PIC values obtained suggested that SSR markers employed in the present study resulted adequate and efficient for the diversity analysis of the three species evaluated. AMOVA showed that about 54% of variation in the data set was from genotypic variations among species and the remaining 46% of differences within species, indicating a degree of population structure. Wright (1978) suggested that Fst values between 0.05 and 0.15 indicated moderate genetic differentiation while values above 0.25 indicated high differentiation. The Fst result obtained was 0.543, revealing a high differentiation. This result was in accordance with the values found by Barzegar et al. (2013) in *C. pepo* populations. A Neighbor-joining dendrogram based on shared allele distance showed a clear separation of *C. maxima, C. moschata* and *C. pepo* species (Figure 1). These results are in agreement with Gong et al. (2013), which found a clear separation of 88 accessions of *Cucurbita* in nine species. Principal Coordinated Analysis showed that the three most informative principal coordinates explained 66.3% of the total variation. The PCoA scatter plot showed a clear separation of the three species, spreading the 107 *Curcubita* populations into three groups (Figure 2). The first group comprised the *C. pepo* populations, the second group the *C. moschata* populations and the third group the *C. maxima populations*. The genetic diversity found on these species of *Cucurbita* can provide relevant information for the diversity conservation and it will be useful to identify local selections for preservation and use in breeding programs.

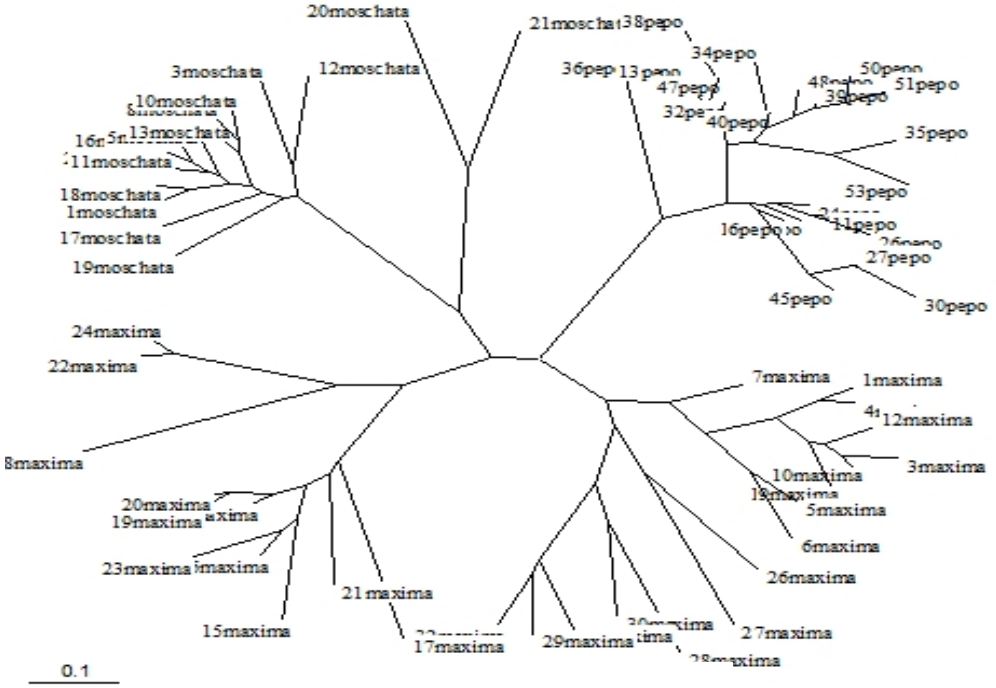

Figure1. NJ cluster analysis of 107 populations based on the proportion of shared allele distance for six dinucleotide containing SSRs.

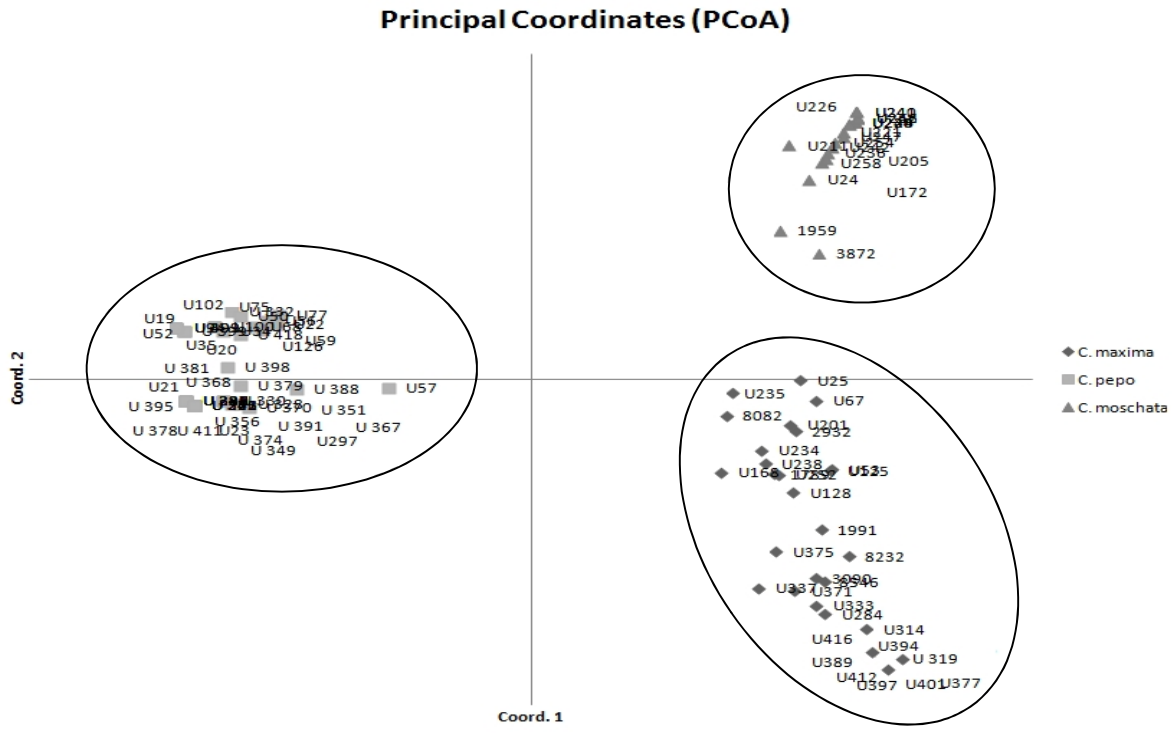

Figure2. Projection of the 107 *Cucurbita* populations in a two-dimensional graph defined by PC1 and PC2

Conclusions

Estimates of genetic similarity using genetic fingerprinting data are a useful tool in plant breeding. The knowledge of genetic variation and the genetic relationship between genotypes can be an important approach for efficient rationalization and utilization of *Cucurbita* sp. resources. The SSR markers used in this set of *Cucurbita* populations proved to be useful for analyzing the genetic diversity. They clear separated pumpkin populations belonging to the *C. maxima, C. pepo* and *C. moschata* species. The high genetic diversity found in this collection of Portuguese *Cucurbita* populations provide relevant information for future genetic and morphological-pathological studies.

The high genetic diversity found in this work can be important for the management and conservation of the material in a genebank.

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