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Comparative analysis of genetic diversity in Greek Genebank collection of summer squash (*Cucurbita pepo*) landraces using start codon targeted (SCoT) polymorphism and ISSR markers

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Abstract

The conservation and characterization of summer squash (*Cucurbita pepo*) genetic resources in germplasm banks has been the basis of their use in breeding projects, which resulted in the development of new cultivars. The genetic diversity of thirty six summer squash landraces from Greece was investigated here using start codon targeted (SCoT) polymorphism and inter-simple sequence repeat (ISSR) markers. In this study, we compared the informativeness and efficiency of the SCoT and ISSR molecular markers. ISSR markers were found to be more polymorphic with an average PIC value of 0.237, while SCoT markers showed the highest marker index (1.503). Cluster analysis on combined set of SCoT and ISSR genotyping data classified summer squash landraces in six distinct groups. The obtained PCoA (Principal Coordinate Analysis) scatter plots further supported the dendrogram results in a robust way. To our knowledge, this is the first detailed comparison of performance among the targeted DNA region molecular markers (SCoT) and the ISSR technique on a set of samples of *C. pepo.* The high genetic diversity existing in the Greek germplasm suggested that it would be beneficial to utilize this pool in summer squash breeding programs and germplasm management activities, in order to maximize genetic diversity in cultivated squash.

Keywords: *Cucurbita pepo*, genetic diversity, genotyping, molecular markers, informativeness.
Abbreviation: AFLP_amplified fragment length polymorphism, ISSR_inter simple sequence repeat, MI_Marker index, PCoA_ Principal coordinates analysis, PCR_polymerase chain reaction, PIC_polymorphic information content, RAPD_random amplified polymorphic DNA, RFLP_restriction fragment length polymorphism, R_p_Resolving power, SCoT_Start Codon Targeted, SRAP_ sequence related amplified polymorphism, UPGMA_Unweighted Pair-Group Method using arithmetic Average.

Introduction

Cucurbitaceae is a large family consisting of about 130 genera and 900 species. The Cucurbita genus harbours five domesticated species *C. argyrosperma* Huber, *C. ficifolia* Bouché, *C. maxima*, *C. moschata* and *C. pepo*, which are considered some of the earliest domesticated species and approximately ten wild species (Robinson and Decker-Walters, 1997; Smith, 2005). Greece hosts three domesticated species, *C. pepo*, *C. maxima* and *C. moschata*. Zucchini types of cultivars (*C. pepo*) are both the most popular types within the genus, and the highest-valued vegetables worldwide., In addition, they are one of the most variable amongst plant species (Paris, 1986) *C. pepo* fruits are consumed immature. *C. pepo* and *Cucurbita* species in general are consumed as food; their fruits they are rich in fat and vitamins and their seeds have high economic value especially the oil - seed industry. In Greece, summer squash is a widespread crop. The majority of production is based on landraces maintained over the years by local farmers, although hybrids are becoming popular. However, landraces of summer squash retain interesting horticultural characteristics, like resistance to diseases and pests. Moreover, landraces are an invaluable source of genes, important for breeding programs. The Greek Gene Bank is actively seeking to collect germplasm and, particularly in this case, *Cucurbita* spp. landraces, from all around Greece. Subsequently, a molecular characterization of this germplasm is needed, along with tests for biotic and abiotic stress tolerance, in order to render this collection useful to breeders and farmers (Tsivelikas et al., 2009). Lately, genetic diversity of crop plants is continuously being lost due to climate change, introduction of hybrids and improved cultivars. In this context, gene banks hold an extremely important role as reservoirs of biodiversity and source of alleles that can be relatively easily retrieved for genetic enhancement of crop plants. Increased efforts are being made to collect threatened landraces, cultivars that were obsolete, genetic stocks and wild relatives of cultivated

species (Ortiz and Engels, 2004). Different marker systems have already been used in the assessment of genetic diversity within *C. pepo*, like restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPDs), amplified fragment length polymorphism (AFLP) and inter simple sequence repeats (ISSRs), as reviewed in (Lebeda et al., 2006) and in (Esteras et al., 2011). Yet, these tools are not very well developed in the *Cucurbita* genus, which reduces the potential gene discovery and the process of breeding (Blanca et al., 2011). The recent development of genomics and DNA sequencing technologies resulted in exponentially increased DNA sequence data, allowing the development of functional markers located in or near candidate genes (Andersen and Lubberstedt, 2003). Start Codon Targeted (SCoT) polymorphism is a novel functional marker system targeting genes (Collard and Mackill, 2009). SCoT primers have been developed based on the short conserved region which flanks the ATG start codon in plant genes. SCoT markers are generally reproducible, and it is suggested that primer length and annealing temperature are not the sole factors determining reproducibility. To our knowledge, this is the first time SCoT analysis has been applied to summer squash genotypes. The present study attempts to make a critical assessment regarding the potential of SCoT and ISSR markers for genotyping *C. pepo* landraces. In addition, we present the classification of summer squash landraces in different groups. Finally, comparison of the characteristic properties for two types of marker assays, including polymorphic information content (PIC), resolving power (R_P) and marker index (MI), is reported.

Results

The oligonucleotide sequences of SCoT, ISSR primers, the resultant multiple band patterns and the data on computed Shannon's index, diversity index and marker index for SCoT and ISSR markers for the thirty six summer squash landraces are summarized in Table 1 respectively.

SCoT analysis

The PCR amplification using SCoT primer pairs resulted in generation of reproducible amplification products (Fig. 1a). A total of 78 bands were detected among 36 *C. pepo* landraces, using seven SCoT markers, of which 49 were polymorphic (Table 1). The number of polymorphic bands ranged from 14 (SCoT70) to 3 (SCoT61 and SCoT34) with an average of 7 per primer. The overall size of amplified products ranged from 250 to 3,800 bp. Percent polymorphism ranged from 30 to as high as 100 with average polymorphism of 62.82 % across all landraces. PIC values ranged from 0.103 (SCoT61) to 0.309 (SCoT33), with an average value of 0.207 per primer. The diversity of the Genebank collection was represented with Nei's gene diversity (GD), as well as Shannon's information index (I). Data for GD and I for all the thirty six landraces was analyzed using seven SCoT markers and their corresponding mean values were found as 0.251 and 0.393 respectively (Table 1). The resolving power R_P provided a modest indication of the ability of SCoT primers to distinguish among landraces. Thus, the R_P of each primer was estimated in order to determine the most informative ones for the discrimination between summer squash landraces. The R_P of the seven primers ranged from 0.612 for primer SCoT61 to 10.054 for primer SCoT13 (Table 1), with a mean value of 4.595. Three of the SCoT primers (SCoT13, SCoT70 and SCoT14) possessed high R_P values (10.054, 8.78 and 4.556, respectively) and are the most efficient for surveying genetic diversity in the thirty six landraces of the Genebank collection. The highest MI was observed with the SCoT primer 70 (3.094) and the lowest in the SCoT primer 61 (0.315).

ISSR analysis

The set of six ISSR primers used showed multiband patterns (Fig. 1b) for each landrace analysed (Table 1). This primer set amplified a total of forty two reliable and reproducible bands from the DNA of thirty six summer squash landraces tested. The total number of bands scored ranged from six to nine and the number of polymorphic fragments from three to seven per primer. Primers UBC811, UBC823, UBC827 and UBC834 resulted in the lowest number of fragments (three) and primer UBC860 and UBC881 generated the highest number of fragments (seven). The average number of fragments per primer was 7.1. Band size ranged from 600 bp (UBC811 and UBC834) to 4.0 kb (UBC860). Among the analysed landraces, 60.5% of the ISSR fragments were polymorphic. For the ISSR markers, GD and I were estimated to be 0.294 and 0.451 respectively (Table 1). The information obtained on the genetic profile of each landrace, using the six ISSR primers, was used to assess the marker performance through the evaluation of three parameters: PIC, MI and R_P. To determine PIC values for each ISSR primer, we analyzed the mean of PIC values for all loci regarding each ISSR primer. We obtained a high PIC value for the ISSR primer UBC834 (0.302) and a low PIC value for the ISSR primer UBC881 (0.189), (Table 1). The average value of PIC per primer was 0.237. Moreover, R_P and MI were used in order to determine the general usefulness of the system markers used, for each ISSR primer (Table 1). The average R_P was 3.350 per ISSR primer (Table 1). The highest R_P value was observed with the ISSR primer UBC881 (5.662) and the lowest with the ISSR primer UBC827 (1.504). The highest MI was observed with the ISSR primer UBC860 (1.330) and lowest with the ISSR primer UBC823 (0.672).

Cluster and PCoA analyses

Both molecular marker systems (SCoT and ISSR) were able to discriminate between 36 summer squash landraces. The level of polymorphism generated by SCoT markers (62.82%) was relatively similar to that of ISSR (60.5%) markers. Genetic relationships as determined by cluster analysis for SCoT, ISSR and pooled allelic data (SCoT + ISSR), are shown in Fig. 2 respectively. Dendrogrammes obtained by SCoT (Fig. 2a) and ISSR (Fig. 2b) markers were relatively similar and most of the landraces were placed in their respective groups. The values of the Mantel test showed positive correlation between the two marker types. The correlation coefficient was 0.25 between SCoT and ISSR, 0.78 between SCoT and pooled markers data (SCoT + ISSR) and 0.53 between ISSR and polled data. The dendrogramme from SCoT data was most consistent with the general dendrogramme from pooled (SCoT + ISSR) data. Cluster analysis using SCoT and ISSR data grouped the 36 squash genotypes into six and five distinct clusters, respectively (Fig. 2a and 2b). Genotypes belonging to cluster F in both SCoT and ISSR dendrogrammes were relatively similar. In the general dendrogramme (Fig. 2c), genotypes grouped in six distinct clusters (A-F). The detected polymorphic bands originating from the SCoT markers were used to calculate firstly: the Similarity Coefficient Matrix and then to construct a similarity dendrogramme using the UPGMA cluster algorithm. In this similarity dendrogram, the thirty six summer

Table 1. Details of SCoT and ISSR primers used, number of markers obtained, polymorphism and genetic diversity indices of SCoT markers from thirty six summer squash landraces.

Primer (SCoT)	Primer Sequence (5'-3')	Annealing Temperature (^oC)	Fragment size range (bp)	Fraction polymorphic fragments	Percentage Polymorphism (%)	Gene diversity (GD)	Shannon Index (I)	PIC	Resolving Power (R_P)	Marker Index (MI)
SCoT13	ACGACATGGCGACCATCG	50	500-2450	11/11	100	0.328	0.498	0.264	10.054	2.904
SCoT14	ACGACATGGCGACCACGC	50	510-1930	8/11	72.72	0.241	0.391	0.203	4.556	1.624
SCoT33	CCATGGCTACCACCGCAG	50	400-3000	3/10	30	0.393	0.575	0.309	3.166	0.927
SCoT34	ACCATGGCTACCACCGCA	50	450-2400	3/10	30	0.244	0.382	0.196	2.112	0.588
SCoT61	CAACAATGGCTACCACCG	50	310-3300	3/9	33.3	0.114	0.180	0.105	0.612	0.315
SCoT66	ACCATGGCTACCAGCGAG	50	250-3300	7/13	53.8	0.176	0.306	0.153	2.886	1.071
SCoT70	ACCATGGCTACCAGCGCG	50	270-3800	14/14	100	0.265	0.423	0.221	8.78	3.094
Mean	-	-	-	7/11.14	62.82	0.251	0.393	0.207	4.595	1.503
Primer (UBC)	Primer Sequence (5'-3')	Annealing Temperature (^oC)	Fragment size range (bp)	Fraction polymorphic fragments	Percentage Polymorphism (%)	Gene diversity (GD)	Shannon Index (I)	PIC	Resolving Power (R_P)	Marker Index (MI)
811	$(GA)_8C$	53	600-2400	3/9	33.3	0.320	0.490	0.259	2.334	0.777
823	$(TC)_8C$	53	800-3400	3/7	50	0.287	0.431	0.224	1.778	0.672
827	$A(CA)_7CG$	53	850-2100	3/6	50	0.321	0.490	0.259	1.504	0.777
834	$(AG)_8YT$	53	600-2700	3/7	42.8	0.374	0.560	0.302	4.712	0.906
860	$(TG)_8RA$	53	870-4000	7/7	100	0.237	0.370	0.190	4.112	1.330
881	$GGGT(GGGGT)_2G$	53	930-2650	7/7	100	0.229	0.368	0.189	5.662	1.329
Mean	-	-	-	4.3/7.1	60.5	0.294	0.451	0.237	3.350	0.965

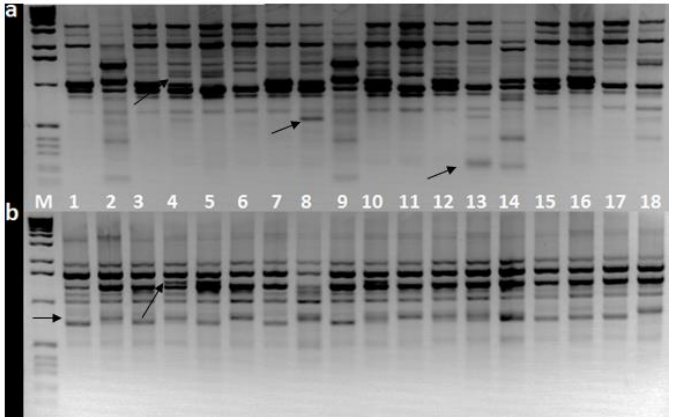


Fig 1. Amplification profile obtained with a) SCoT66 with the arrow we show certain distinctive bands allowing the discrimination of certain landraces. Landraces 14 (P54/06) shows a unique profile when SCoT66 was used which is also represented in the resulting UPGMA tree and b) UBC827 primers detected in 18 representative summer squash landraces the arrows show the band which allows the discrimination among the landraces.

Table 2. Summer squash (*C. pepo* ssp. *pepo*) landraces used for SCoT and ISSR genetic analysis.

No	Genebank Accession number	Origin	Common Name	Morphotype
1	GRC262/04	CHANIA		Zucchini
2	GRC597/04	AHAIA		Zucchini
3	GRC1025/04	AETOLOAKARNANIA		Zucchini
4	GRC1369/04	ARTA		Zucchini
5	ATS030/06	ANDROS ISL.		Zucchini
6	ATS63/06	TINOS		Zucchini
7	F104/06	KASTORIA		Zucchini
8	F135/06	KASTORIA	PITSIALA	Zucchini
9	K61/06	KOZANI		Zucchini
10	K161/06	GREBENA		Zucchini
11	K192/06	GREBENA		Zucchini
12	M47/06	LESVOS ISL.	MAGIATIKO	Zucchini
13	M166/06	LESVOS ISL.		Zucchini
14	P54/06	ARKADIA		Zucchini
15	P085/06	MESSINIA		Zucchini
16	P170/06	LAKONIA		Zucchini
17	P89/06	ARKADIA	KARDIA	Zucchini
18	SK067/06	SERRES		Zucchini
19	T24/06	KARDITSA		Zucchini
20	T121/06	KARDITSA		Zucchini
21	T223/06	KARDITSA		Zucchini
22	T373/06	TRIKALA		Zucchini
23	T528/06	TRIKALA	RIZITES	Zucchini
24	ANP39/07	AMORGOS ISL.		Zucchini
25	ANP163/07	NAXOS ISL.		Zucchini
26	AO59/07	AG. OROS		Zucchini
27	HL18/07	IRAKLION	FORMARES	Zucchini
28	HL195/07	LASITHI		Zucchini
29	KD14/07	KAVALA		Zucchini
30	LKK58/07	KALYMNOS ISL.		Zucchini
31	RK46/07	RODOS ISL	SARAVANES	Zucchini
32	ROX91/07	XANTHI		Zucchini
33	ROX139/07	RODOPI		Zucchini
34	SAS10/07	ALONNISOS ISL.		Zucchini
35	SAS85/07	SKOPELOS ISL.		Zucchini
36	XKA10/07	CHALKIDIKI		Zucchini

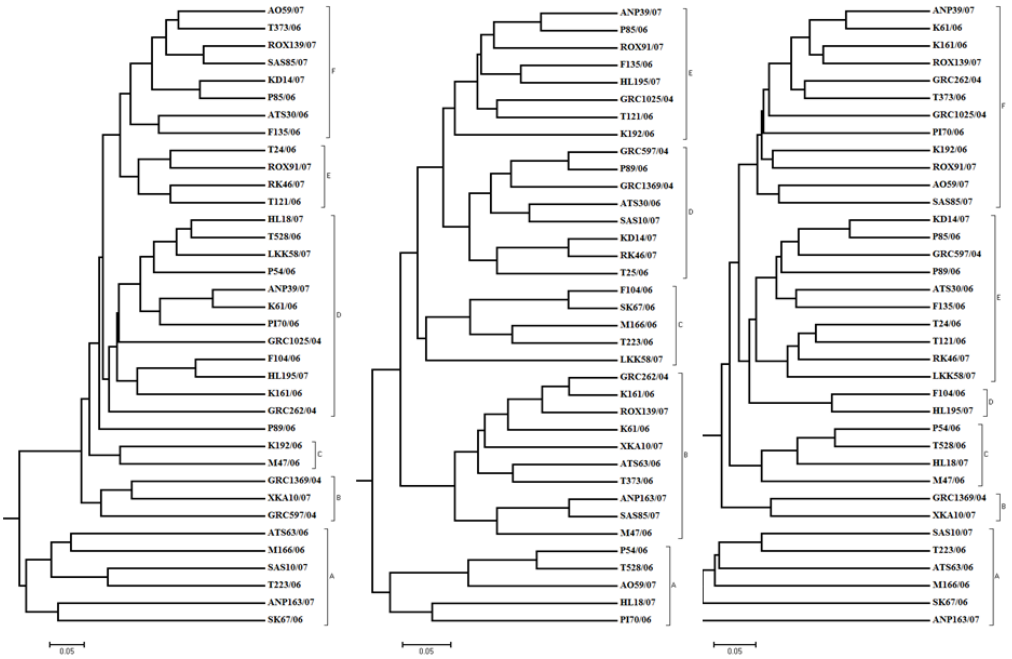


Fig 2. Dendrograme representation of the relationships between summer squash landraces using the genetic distance of UPGMA, based on SCoT (a), ISSR (b) and combined SCoT & ISSR (c) markers; letters show clusters. In general SCoT markers show higher discriminatory power since they divide the genotypes used in 6 groups the same as the combined analysis SCoT – ISSR. It is interesting to notice LKK58/07 from the island Kalymnos which shows a unique pattern probably due to the isolation.

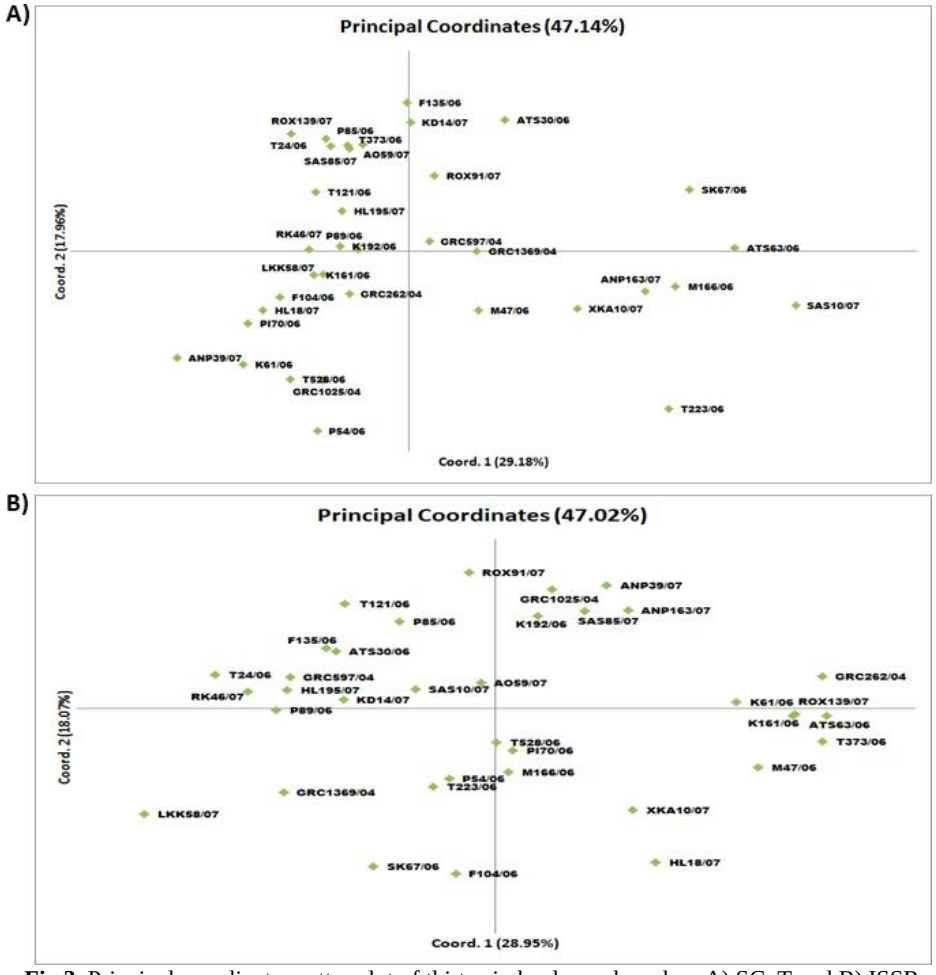
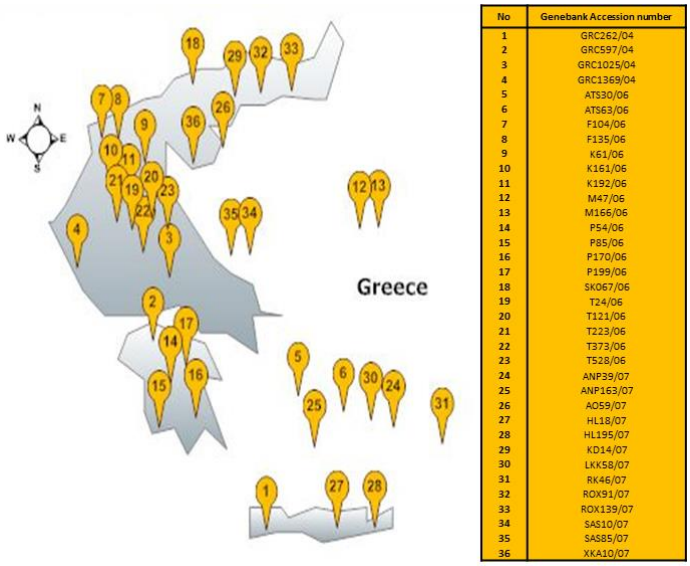


Fig 3. Principal coordinate scatter plot of thirty six landraces based on A) SCoT and B) ISSR markers.



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4	GRC1369/04
5	ATS30/06
6	ATS63/06
7	F104/06
8	F135/06
9	K61/06
10	K161/06
11	K192/06
12	M47/06
13	M166/06
14	P54/06
15	P85/06
16	P170/06
17	P199/06
18	SK067/06
19	T24/06
20	T121/06
21	T223/06
22	T373/06
23	T528/06
24	ANP39/07
25	ANP163/07
26	AO59/07
27	HL18/07
28	HL195/07
29	KD14/07
30	LKK58/07
31	RK46/07
32	ROX91/07
33	ROX139/07
34	SAS10/07
35	SAS85/07
36	XKA10/07

Fig 4. Collecting sites of summer squash landraces in Greece. Number indicated landraces name in the Table 1. The map was produced using SmartDraw2014.

summer squash landraces were classified into six clusters, with two major clusters of twelve and eight landraces each (Fig. 2a, clusters D and F, respectively), and three smaller clusters comprised of two to four landraces (Fig. 2a, clusters B, C and E); one landrace was unclassified (P89/06). On the other hand the thirty six summer squash landraces using the ISSR markers, were classified into five clusters, with three major clusters of ten, eight and eight landraces each (Fig. 2b, clusters B, D and E, respectively), and two smaller clusters comprised of five landraces (Fig. 2c, clusters A and C). Landraces KD14/07 and RK46/07 share high level of genetic relationship as revealed by their position on the dendrogramme. The two-dimensional PCoA plots (Fig. 3a and b) show the high percentage of the total genetic diversity (47.14% for SCoTs and 47.02% for ISSRs).

Discussion

The conservation, management and use of germplasm maintained in gene banks poses a number of challenges to the researchers dedicated to the investigation of plant genetic resources. Thorough knowledge of the genetic diversity of a crop is necessary for the parental selection in order to maximize genetic improvement. More accurate and complete descriptions of the genotypes and patterns of the genetic diversity could help determine future breeding strategies and facilitate the introgression of diverse germplasm into the current commercial summer squash genetic base. From all the results obtained, it is clearly evident that there is a considerable molecular variation among the summer squash landraces. *C. pepo* has been genotyped successfully before by SRAP, AFLP and ISSRs (Katzir et al., 2000; Paris et al., 2003), but no studies have been published yet using SCoT markers in order to characterize summer squash germplasm. In the present study GD and I which is independent of sample size (Cardoso et al., 1998), revealed high level of diversity among summer squash landraces (GD=0.251, I=0.393 for SCoTs and GD=0.254, I=0.451 for ISSRs). Our estimates are highly comparable with similar *Cucurbita* studies. For example, (Montes-Hernandez and Eguiarte, 2002) found GD = 0.40 in 16 populations of four *Cucurbita* species and (Inan et al., 2012) reported GD = 0.3, I = 0.46 in 24 accession of three *Cucurbita* species. Furthermore, (Yildiz et al., 2011) measured high genetic variation among Turkish melon genotypes (GD= 0.28 and I = 0.43) using 15 ISSR, 8 SRAP, and 17 RAPD primers. Calculated genetic diversity indices were also in agreement with other *Cucurbita* crops such as melon (Chen et al., 2010; López-Sesé et al., 2002; Staub et al., 2004). The genetic similarity matrix based on Nei/Li Dice coefficient was also subjected to PCoA analysis, for better visualization of the genetic structure and relationships among the landraces with seven SCoT and six ISSR markers, respectively (Fig. 3A and B). The PCoA results were in accordance with the dendrogramme cluster analysis but allowed a higher resolution. In a comparable study of apple cultivars, 40.43% could be accounted for by the first two coordinates (Song et al., 2006). Both dendrograms and PCoA analysis showed that there is no distinct classification of the 36 landraces between island and mainland for example, maybe because *C. pepo* has been introduced as a crop in Greece relatively recently. Mady et al. (2013) used RAPD markers to assess the genetic diversity of twenty summer squash landraces. In their study, a total of ten RAPD primers produced sixty five reproducible fragments, of which forty six (70.77%) were polymorphic. Here, a total of seven SCoT and six ISSR primers produced forty nine and twenty six polymorphic fragments, respectively (SCoT=62.82%; ISSR=60.5%). Genetic variation in traditional landraces is the crucial genetic pool for plant breeding. Farmers play a paramount and important role in conserving genetic variation by conserving the seeds and by phenotypic selection. Evaluation of the landraces in terms of phenotypic behavior and genetic variability needs to be performed before they are used in breeding programs to develop new cultivars that are more productive and of greater nutritional value. The identification and use of landraces of diverse genetic background is a critical issue for successful breeding programs through the selection of suitable parents. Current findings could support the development of breeding programs regarding *C. pepo* since we have found high genetic variability in Greek native *Cucurbita* landraces. Our results suggest that SCoT and ISSR molecular markers are an excellent choice for the evaluation of genetic diversity and the assessing differentiation among landraces belonging to different geographical regions.

Conclusion

The current report is the first study on the genetic diversity of a collection of Greek landraces belonging to the elongated forms of *C. pepo* ssp. *pepo*, using gene-based (SCoT) and neutral (ISSR) molecular markers. Moreover, improved preservation efficiency of germplasm as well as breeding efforts for summer squash will be enabled by the genetic grouping of landraces through the use of molecular markers. Molecular fingerprinting of the Genebank landraces will also allow the reduction of duplicates and ensure an adequate and balanced representation of the Greek summer squash gene pool. From our study, the delineation of the broader germplasm of summer squash landraces into gene pools could help guide introgression efforts to expand the genetic diversity within breeding materials and may lead ultimately to the development of more efficient strategies and greater genetic gain within future breeding programs.

Materials and Methods

Plant material and DNA isolation

Thirty six summer squash landraces collected from the Greek Gene Bank (GGB; http://envicom.wordpress.com/about-genebanks/the-greek-genebank/) were chosen for this study (Table 3; Fig. 4). Isolation of DNA from seeds was performed with the NucleoSpin Plant kit (Macherey-Nagel, Germany), according to the manufacturer's instructions. The DNA concentration was estimated by standard spectrophotometric methods at 260 nm and 280 nm UV lengths by an Eppendorf BioPhotometer and the integrity by gel electrophoresis in a 0.8% agarose gel. Samples were then diluted to 20 ng/μl work concentration.

SCoT and ISSR analysis

Seven SCoT primers were used for fingerprinting. Information on the primers used is listed in Table 11. PCR amplification was carried out in a total reaction volume of 15 μl using Eppendorf Mastercycler Gradient thermocycler (Brinkman Instruments, Westbury, NY), according to Collard and Mackill (2009). Standard PCR cycling parameters were used: in an initial denaturation step at 94°C for 4 min, followed by 35 cycles of 30 s at 94°C, 1 min at 50°C and 2 min at 72°C. The cycles were followed by 5 min at 72°C for final extensions.

For ISSR analysis, a set of 10 primers representing di-, tri-, tetra-, and pentamer repeats (UBC set #9, Table 1) was procured from the Biotechnology Laboratory, University of British Columbia, Canada. Following the optimization of polymerase chain reaction (PCR) conditions and prescreening of the first 10 primers on a sample set, 6 primers providing clear and informative amplicon profiles landraces were selected to survey ISSR variation in the landraces listed in Table 1. PCR reactions were composed as described previously by Ganopoulos et al. (2011). Amplification products of SCoT and ISSR molecular markers were separated by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. A 1 Kb DNA ladder (Invitrogen, USA) was used as size marker. Data points are the presence/absence of each distinguishable band across all samples for the same primer, in both replicate sets of amplifications. Gels and images were analyzed using UVIDoc software (UVItec, Cambridge, UK) to quantify signal intensity.

Data analysis

The number of polymorphic loci in the germplasm set of interest, analyzed per assay, called effective multiplex ratio (E) is estimated as: $E=n\beta$ where β is the fraction of polymorphic markers, estimated after considering the polymorphic loci (n_p) and non-polymorphic loci (n_{np}) as $\beta = n_p/(n_p+ n_{np})$. The utility of a given marker system is a balance between the level of polymorphism detected and the extent to which an assay can identify multiple polymorphisms. We calculated the polymorphism information content (PIC) according to (Botstein et al., 1980) for each primer using PICcalc (Nagy et al., 2012). Marker index (MI), as measured by PIC, and effective multiplex ratio may provide a convenient estimate of marker utility (Powell et al., 1996): MI = PIC x E. Nei's gene diversity (He) (Nei 1973) and Shannon's information index (I) (Lewontin 1972) were estimated via the POPGEN 1.32 software (Yeh and Boyle 1997). Resolving power (R_P) of a primer is: $Rp = \Sigma\ IB$ where *IB* (band informativeness) takes the value of: 1 – [2 x (0.5 – p)], p being the proportion of the 36 landraces analyzed containing the band (Prevost and Wilkinson 1999). The similarity between matrices based on different marker system (ISSR and SCoT) was calculated using the standardized Mantel coefficient (Mantel 1967). The SCoT and ISSR reproducible fragments were classified as present (1) or absent (0), and were typed into a computer file as a binary matrix, one for each molecular marker. The matrices were then analyzed by FreeTree v. 0.9.1.50 software (Hampl et al., 2001). Similarity of qualitative data was calculated using the Nei and Li/Dice similarity index (Nei and Li, 1979), and similarity estimates were analyzed using UPGMA (Unweighted Pair Group Method using Arithmetic Averages). The matrices of mutual coefficients of similarity calculated by FreeTree were converted to MEGA 4 v.4.1 software (Tamura et al., 2007) and resulting clusters were expressed as dendrogrammes. The robustness of the dendrogrammes was assessed by bootstrap analysis running 1000 iterations, also performed by FreeTree and MEGA 4 v.4.1. The binary data for individuals was subjected to the PCoA (Peakall and Smouse, 2006), and the first two principal coordinates were plotted to indicate the multilateral genetic relationships among the summer squash landraces.

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