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Genetic diversity of a germplasm collection of *Cucurbita pepo* using SRAP and AFLP markers

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Abstract *Cucurbita pepo* is a highly polymorphic species. The cultivars can be grouped into eight morphotypes in two subspecies, ssp. *pepo* and ssp. *ovifera*. A collection of 69 accessions representative of the morphotypes and some unclassified types was used for analysing the morphological and molecular diversity of this species. This collection includes commercial cultivars and Spanish landraces, which represent the great diversification of types that have arisen in Europe after this species arrived from America. For the molecular variability studies, two PCR-based systems were employed, AFLP and SRAP, which preferentially amplify ORFs. Principal coordinates analysis and cluster analysis using the UPGMA method clearly separate the accessions into the two subspecies through the use of both markers. However, the gene diversity and the genetic identity values among morphotypes and subspecies varied between the two marker systems. The information given by SRAP markers was more concordant to the morphological variability and to the evolutionary history of the morphotypes than that of AFLP markers. In ssp. *ovifera*, the accessions of the different morphotypes were basically grouped according to the fruit colour. This may indicate different times of development and also the extent of breeding in the accessions used. This study has allowed identification of new types that can be employed for the development of new cultivars. The landraces of the spp. *ovifera*, used as ornamental in Europe, have proved to be of great interest for preserving the diversity of *C. pepo*.

Keywords *Cucurbita pepo* · Germplasm collection · AFLP markers · SRAP markers

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Introduction

Cucurbita pepo L. is one of the most variable species in the plant kingdom (Naudin 1856). Taxonomically, cultivated *C. pepo* is considered to comprise two subspecies, ssp. *pepo* and ssp. *ovifera* (Decker 1988). Three wild forms related with ssp. *ovifera* have been described to-date, based on alloenzymatic studies (Decker and Wilson 1987), comparisons of chloroplastic DNA (Wilson et al. 1992), mitochondrial DNA (Sanjur et al. 2002) and ribosomal DNA (Jobst et al. 1998), ISSR (Katzir et al. 2000) and RAPD markers (Decker-Walters et al. 2002). Nevertheless, wild forms related to ssp. *pepo* are not known. These studies indicate, probably, that at least two independent domestications of *C. pepo* arose in different parts of America. Archaeological records are consistent with the evidence of the cultivation of this species in southern Mexico for at least 10,000 years (Smith 1997), and in northeastern Mexico and eastern USA for over 4,000 years (Decker 1988).

Morphologically, *C. pepo* displays a great diversity of types. The edible forms of this species can thus be grouped into eight morphotypes: Pumpkin, with round or nearly round fruits; Vegetable Marrow, with short, tapered and cylindrical-shaped fruits; Cocozelle, with long bulbous fruits; Zucchini, with uniformly cylindrical-shaped fruits; Acorn, with furrowed, turbinate fruits; Scallop, with flat, scalloped fruits; Crookneck, with narrow, usually curved and warted, necked fruits and Straightneck, with short-necked or constricted fruits, usually warted (Paris 1986). Pumpkin, Vegetable Marrow, Cocozelle and Zucchini, as well as spherical and warted ornamental gourds, like "Orange", "Warty Hardhead" and "Miniature Ball", correspond to ssp. *pepo*. Scallop, Acorn, Crookneck and Straightneck, as well as oviform and pyriform ornamental gourds, correspond to ssp. *ovifera* (Paris 2001). The great variability of types represented in the Botanical works of the 16th and 17th centuries, and the observations of the first European explorers in America, seem to indicate that Pumpkins, Scallops and Acorns were developed under the guidance

of Native Americans in pre-Columbian times (Paris 2001). The subsequent arrival in Europe of *C. pepo* fruits and seeds generated an extraordinary variability of new phenotypes through hybridisation and recombination. New cultivars were developed, particularly the elongated forms of ssp. *pepo*: Vegetable Marrow, Cocozelle and Zucchini. The origin of the Crookneck squash seems to be North American, since it was cultivated in the interior of this continent at the beginning of the 19th century. Some isolate depictions in European botanical works suggest even older origins. The commercial cultivars of Straightneck were later derived from Crookneck by outcrossing, selection and breeding.

The Genebank of the Center for Conservation and Breeding of Agricultural Diversity (COMAV), held at the Polytechnic University of Valencia (Spain), owns one of the most important collections in Europe of squashes and pumpkins (Nuez et al. 2000). This collection includes about 400 accessions of *C. pepo* from different Spanish provinces, collected since the eighties. Most of them are landraces, cultivated in small orchards, basically grown for self-consumption or sale in local markets. Good characterization of this germplasm is needed in order to be useful for breeders and farmers throughout the world. In addition to morphological characterization, molecular characterization is essential for elucidating the genetic relationships among the different groups of this species. Ignart and Weeden (1984), Decker (1985) and Decker-Walters et al. (1990) used alloenzymatic systems for analysing the relationships among different horticultural groups of *C. pepo*, resulting in a clear subspecific classification. More recently, comparisons of ribosomal DNA (Torres-Ruiz and Hemleben 1991) and ISSR and SSR analysis (Katzir et al. 2000, 2002) were performed with the same purpose. However, the majority of these studies used improved commercial cultivars. We have no knowledge of any study of detailed morphological and molecular characterization of germplasm collections for *C. pepo* made up of landraces, which in most cases cannot be assigned to a given known morphotype and can therefore increase the genetic basis of squash.

The aim of the present work is to analyze the variability of a collection of landraces, mainly from Spain, and some commercial cultivars belonging to both *C. pepo* subspecies. In addition to the morphological analysis, two molecular marker systems are employed: AFLP markers (amplified fragment length polymorphism; Vos et al. 1995) and SRAP markers (sequence-related amplified polymorphism; Li and Quiros 2001), which preferentially amplify open reading frames (ORFs). SRAP markers were previously employed for analyzing the genetic variability among accessions of *Cucurbita maxima* (Ferriol et al. 2003). The comparison between the two marker systems is discussed.

Materials and methods

Plant material

Sixty nine accessions of *C. pepo*, with representatives of each morphotype, ornamental gourds and cultivars which cannot be included in any of the described types, were employed in this study (Table 1). Forty four of the accessions belonged to the *C. pepo* collection at COMAV (10% of this collection). This sample was selected in order to represent the variability of the whole collection. Moreover, some forms with little representation at COMAV (fundamentally Acorn, Crookneck and Straightneck) were obtained from other Genebanks: the Institute for Plant Genetics and Crop Plant Research of Germany (IPK), the North Central Regional Plant Introduction Station of USA, the National Center for Genetic Resources Preservation of USA and the Research Institute of Vegetable Crops of Rumania.

Morphological characterization

The morphological characterization of ten plants per accession was accomplished in an open field during the spring and summer seasons, using the IPGRI descriptor for Cucurbitaceae (Esquinas-Alcázar and Gulick 1983). Seven qualitative characters (growth habit; shape, ribbing and fruit colour; texture and hardness of the skin and flesh colour), and ten quantitative characters (weight, length and width of the fruit; length/width ratio; skin and flesh thickness and length, width, thickness and weight of the seeds) were employed in this analysis. This characterization allowed the accessions to be grouped according to morphotype (Table 1).

DNA extraction

Genomic DNA was isolated from leaves using the modified CTAB method of Doyle and Doyle (1990). For each accession, 0.5 g of ground leaf tissue from a bulk of ten plants was suspended in 2.5 ml of extraction buffer [20 mM of EDTA, 0.1 M of Tris-HCl (pH 8), 1.4 M of NaCl, 2% CTAB and 5 μl of beta-mercaptoethanol]. The suspension was mixed well, incubated at 60 °C for 30 min, followed by chloroform-isoamyl alcohol (24:1) extraction and precipitation with 2/3 of the volume of isopropanol at –20 °C. The pellet formed after centrifugation at 2,000 rpm for 5 min was washed with 1 ml of 76% ethanol and 10 mM of ammonium acetate. The DNA was then suspended in TE buffer. The resulting DNA concentration was measured in a 1% agarose-gel stained with ethidium bromide using 1-D Manager (2.0), in comparison with the known concentration of *Arabidopsis thaliana* DNA (AFLP Core Reagent Kit of Invitrogen). The DNA was stored at –20 °C.

SRAP analysis

The SRAP technique consists of preferential amplification of ORFs using PCR. For this purpose, combinations of two types of primers were employed. The first type of primer (forward) is 17 bp long, and contains a fixed sequence of 14 nucleotides rich in C and G, and three selective bases at the 3' end. This primer preferentially amplifies exonic regions, which tend to be rich in these nucleotides. The second type of primer (reverse), with 18 bp, contains a sequence of 15 nucleotides, rich in A and T, and three selective bases at the 3' end. This primer preferentially amplifies intronic regions and regions with promoters, rich in these nucleotides. The observed polymorphism fundamentally originates in the variation of the length of these introns, promoters and spacers, both among individuals and among species (Li and Quiros 2001).

In this assay, 11 different combinations were employed using five forward primers and five reverse primers (Table 2). Each 25-μl PCR reaction mixture consisted of 20 ng of genomic DNA, 200 μM of dNTPs, 1.5 mM of $MgCl_2$, 0.3 μM of primer, 10 × *Taq* buffer and 1 unit of *Taq* polymerase (Boehringer Mannheim). Samples

Table 1 Passport data of the accessions grouped in morphotypes

Morphotype	Accession	Origin		Cultivar name or local name
		Province or state	Country	
Pumpkin (ssp. *pepo*)	E-25[1]*	Extremadura	Spain	–
	S-4[1]	Santander	Spain	Morcillera
	CM-39[1]	Castilla-la-Mancha	Spain	Calabaza
	AS-3[1]*	Asturias	Spain	de Pravia amarilla
	AN-21[1]*	Andalucía	Spain	Roteña
	S-1[1]	Santander	Spain	Asar
	ECU-282[1]	Loja	Ecuador	Calabaza
	PI-171628[2]*	Ordu	Turkey	–
Vegetable Marrow (ssp. *pepo*)	AN-113[1]*	Andalucía	Spain	Calabacín freír
	AN-120[1]	Andalucía	Spain	Calabaza
	CM-37[1]*	Castilla-la-Mancha	Spain	Calabacín
	V-21[1]	Valencia	Spain	Calabacín
	AFR-12[1]*	Khmelat	Morocco	Calabacín
	CA-84[1]*	Canarias	Spain	Calabaza
Cocozelle (ssp. *pepo*)	V-185[1]*	Valencia	Spain	Calabacín
	V-98[1]*	Valencia	Spain	Calabacín
	V-112[1]*	Valencia	Spain	Calabacín
	GRECIA-6[1]*	Agias Paraskeris	Grecia	–
	PI-261610[2]*	Castilla-León	Spain	Calabacín
	PI-357962[2]*	Novo Selo	Macedonia	Novo seslka
	PI-379307[2]*	Prizren	Yugoslavy	Tikvica
Zucchini (ssp. *pepo*)	MU-16[1]*	Murcia	Spain	Calabacín
	E-27[1]*	Extremadura	Spain	Calabacín
Scallop (ssp. *ovifera*)	V-196 A[1]*	Valencia	Spain	Calabaza ornamental
	V-196 B[1]	Valencia	Spain	Calabaza ornamental
	V-203[1]*	Valencia	Spain	–
	V-204[1]	Valencia	Spain	–
	NSL-5209[3]*	Colorado	USA	Benning Green Tint Scallop
	PEP-16[4]	Adelaide	Australia	Patty Pan Scallop
	USA-3[1]*	California	USA	Golden Scallopini Bush Squash
Acorn (ssp. *ovifera*)	V-142[1]*	Valencia	Spain	Calabacetas adorno
	PI-615111[2]	Iowa	USA	White Acorn
	PI-518687[2]*	New York	USA	Acorn squash
	NSL-32665[3]	Maryland	USA	Royal large (Acorn)
	NSL-5233[3]*	California	USA	Ebony Acorn Munger Strain
Crookneck (ssp. *ovifera*)	NSL-106681[3]*	California	USA	Ranger Crookneck
	NSL-42793[3]	Michigan	USA	Summer Crookneck 15
	NSL-5227[3]*	California	USA	Early Summer Crookneck Improvd
	NSL-5206[3]*	Colorado	USA	Early Summer Yellow Crookneck
	PEP-11[4]*	Missouri	USA	Early Summer Crookneck
	USA-2[1]*	California	USA	Yellow Crookneck Heirloom Squash
Straightneck (ssp. *ovifera*)	V-202[1]	Valencia	Spain	–
	PEP-15[4]*	Adelaide	USA	Yellow Crookneck
	PEP-17[4]*	California	USA	Early Prolific Straightneck
Ornamental	V-205[1]	Valencia	Spain	Spoon
	V-82[1]	Valencia	Spain	Adorno
	CL-4[1]*	Castilla-León	Spain	Adorno
Unclassified	V-4[1]*	Valencia	Spain	Adorno
	B-4[1]*	Baleares	Spain	Adorno
	CM-21[1]*	Castilla-la-Mancha	Spain	de 21 días
	V-200[1]	Valencia	Spain	–
	V-201[1]	Valencia	Spain	–
	V-116[1]*	Valencia	Spain	Parda
	CM-47[1]	Castilla-la-Mancha	Spain	Calabacín
	V-206[1]*	Valencia	Spain	–
	V-207[1]	Valencia	Spain	–
	A-19[1]*	Aragón	Spain	Forrajera
	V-31[1]*	Valencia	Spain	Porquines
	CA-35[1]*	Canarias	Spain	Calabacín
	C-72B[1]*	Cataluña	Spain	Marranera
	CA-154[1]*	Canarias	Spain	Bubango
	V-171[1]*	Valencia	Spain	Calabaza
	C-1[1]*	Cataluña	Spain	Calabacín negro

Table 1 (continued)

Morphotype	Accession	Origin		Cultivar name or local name
		Province or state	Country	
Unclassified	PI-169462[2]	Canakkale	Turkey	Sakiz Kabak
	PI-204698[2]	Malatya	Turkey	–
	PI-490279[2]*	New Hampshire	USA	Sweetnut (Acorn)
	PEP-307[4]	Bayreuth	Germany	Black Zucchini
	PEP-625[4]*	Ipswich	UK	Vegetable Spaghetti
	GO-44[5]	Thesprotia	Rumania	Zucchini

[1] COMAV
[2] North Central Regional Plant Introduction Station
[3] National Center for Genetic Resources Preservation
[4] Institute for Plant Genetics and Crop Plant Research
[5] Research Institute of Vegetable Crops
* Accessions used for AFLP analysis

Table 2 Primer sequences used for SRAP analysis

Primer	Type	Sequence (5'–3')
ME-1	Forward	TGA GTC CAA ACC GGA TA
ME-2	Forward	TGA GTC CAA ACC GGA GC
ME-6	Forward	TGA GTC CTT TCC GGT AA
ME-7	Forward	TGA GTC CTT TCC GGT CC
ME-8	Forward	TGA GTC CTT TCC GGT GC
EM-1	Reverse	GAC TGC GTA CGA ATT CAA
EM-2	Reverse	GAC TGC GTA CGA ATT CTG
EM-3	Reverse	GAC TGC GTA CGA ATT CGA
EM-5	Reverse	GAC TGC GTA CGA ATT CAA
EM-6	Reverse	GAC TGC GTA CGA ATT CCA

were subjected to the following thermal profile for amplification in an oven thermocycler (Eppendorf Mastercycler Gradient): 5 min of denaturing at 94 °C, five cycles of three steps: 1 min of denaturing at 94 °C, 1 min of annealing at 35 °C and 2 min of elongation at 72 °C. In the following 30 cycles the annealing temperature was increased to 50 °C, with a final elongation step of 5 min at 72 °C. Separation of amplification fragments was accomplished on 12% polyacrylamide gels [acrylamide-bisacrylamide (29:1), 1 × TBE] at 500 V for 11 h. The gels were dried overnight.

Sequencing of SRAP fragments

Some of the amplified fragments using SRAP markers were recovered from the dried acrylamide gel, re-amplified and sequenced as described by Stumm et al. (1997). The sequences were compared with the nucleotide and amino-acid sequences included in different databases maintained in the NCBI (National Center for Biological Information) and in the plant EST database maintained in TIGR (The Institute for Genomic Research). Searches were conducted with the FASTA (Pearson 1994) and BLAST (Altschul et al. 1990) algorithms.

AFLP analysis

In relation to the molecular analysis using AFLP, because of the greater complexity of this technique, 47 accessions, representing all the morphological groups, were included. These were selected according to the results obtained with SRAP markers and attempting to maximize the diversity of the collection (Table 1).

The digestion and ligation of DNA was accomplished using the AFLP Core Reagent Kit of Invitrogen. 250 ng of genomic DNA was double-digested using the restriction enzymes *Eco*RI and *Mse*I. The DNA fragments were ligated to *Eco*RI and *Mse*I adaptors. The adaptor-ligated DNA was diluted 10-times with TE buffer and subjected to pre-selective amplification.

Table 3 Primer sequences used for AFLP analysis

Primer	Enzyme	Sequence (5'–3')
*Eco*RI-ACA	*Eco*RI	GAC TGC GTA CCA ATT CAC A
*Eco*RI-AAC	*Eco*RI	GAC TGC GTA CCA ATT CAA C
*Eco*RI-AGG	*Eco*RI	GAC TGC GTA CCA ATT CAG G
Mse-CT	*Mse*I	GAT GAG TCC TGA GTA ACT
Mse-CA	*Mse*I	GAT GAG TCC TGA GTA ACA
Mse-CG	*Mse*I	GAT GAG TCC TGA GTA ACG
Mse-CC	*Mse*I	GAT GAG TCC TGA GTA ACC

Pre-selective reactions were performed in a 25-μl volume containing 5 μl of diluted DNA, 400 μM of dNTPs, 2.5 mM of $MgCl_2$, 0.2 μM of each *Mse*I adaptor+C and *Eco*RI adaptor+A primers, 10 × *Taq* buffer and 1 unit of *Taq* polymerase (Boehringer Mannheim). The PCR amplifications were subjected to 20 cycles of 94 °C for 20 s, 56 °C for 30 s and 72 °C for 25 s, with a final elongation step of 30 min (Eppendorf Mastercycler Gradient). The DNA was diluted 10-times in water.

The selective amplification mixture (25 μl) was prepared with 2 μl of diluted DNA, 400 μM of dNTPs, 2.5 mM of $MgCl_2$, 0.5 μM of *Mse*I+2 and 0.05 μM of *Eco*RI+3 primers labelled with three different 5' fluorescent dyes (Perkin Elmer Applied Biosystems) (Table 3), 10 × *Taq* buffer and 1 unit of *Taq* polymerase (Boehringer Mannheim). This amplification was carried out by programming a touch-down cycle profile as follows: 94 °C for 20 s, 66 °C (–1 °C/cycle) for 30 s and 72 °C for 25 s over ten cycles, until reaching the optimal annealing temperature of 56 °C. At this temperature, 25 more cycles were performed to complete the second amplification, with a final elongation step of 30 min.

Two microliters of the selective amplification product were mixed with 12 μl of de-ionized formamide and 0.25 μl of the HD400ROX (Perkin Elmer Applied Biosystems) internal size standard. Gel electrophoresis was conducted using an ABI PRISM 310 Genetic Analyzer (Perkin Elmer Applied Biosystems). Raw data were analyzed with GeneScan 3.1.2 analysis software (Perkin Elmer Applied Biosystems) and the resulting GeneScan trace files were introduced into Genographer 1.6.0. The AFLP fragments between 60 and 380 bp were scored in Genographer as present (A) or absent (B). Scores were recorded (by changing an A to 1 and a B to 0) and used for analysis.

Data analysis

Principal components analysis and principal coordinates analysis (PCAs) were performed using standardized morphological quantitative data and molecular data respectively, to obtain a graphical representation of the relationship structure of the 69 accessions employed. Computations were done using the procedures in the NTSYS pc 2.0 statistical package.

In the SRAP and AFLP molecular analysis, genetic distances (1 – S_{ij}) among genotypes were calculated according to the Nei and Li (1979) similarity coefficient S_{ij} = 2a/(2a+b+c), where S_{ij} is the similarity between two individuals i and j; a is the number of shared bands; b is the number of bands exclusive of i, and c is the number of bands exclusive of j. The distance matrix was first subjected to cluster analysis by the unweighted pair-group method (UPGMA, Sneath and Sokal 1973), and a tree was constructed using NTSYS pc 2.0. The goodness of fit of the clustering to the data matrix was calculated by the COPH and MXCOMP programs. The reliability and robustness of the dendrograms were tested by bootstrap analysis with 1,000 replications to assess branch support using PHYLIP 3.6 software. One accession of *Cucurbita maxima* was used as an outgroup.

Gene diversity (Nei 1973) and genetic identities (Nei 1972) were estimated using POPGENE 32.

Results and discussion

Morphological characterization

Two of the 69 accessions did not produce fruit (PEP-625 "Vegetable Spaghetti" and GO-44 "Zucchini") and one did not agree with the morphological data supplied by the donor banks (PEP-307 "Black Zucchini"). These three accessions were therefore considered as unclassified (Table 1). Moreover, PEP-15, catalogued in the passport data as "Yellow Crookneck", displayed the typical traits of Straightneck. Thus, it was included in this group.

A principal component analysis was carried out using the quantitative characters from the morphological characterization (Fig. 1a, b). The first component, which accounted for 56.1% of the total variation, grouped the accessions fundamentally according to the fruit weight and size. The two subspecies were clearly separated, with ssp. *pepo* fruits being bigger and heavier than those of ssp. *ovifera*. The second component, which accounted for 18.7% of the total variation, grouped the accessions according to the fruit shape (length/width ratio). The morphotypes with long fruits (Crookneck, Straightneck, Vegetable Marrow, Cocozelle and Zucchini) were separated from those with round or flattened ones (Pumpkin, Acorn and Scallop).

The Pumpkin accessions used in this analysis were highly variable, so they could be morphologically subclassified according to Paris (2001). ECU-282, from Ecuador, with a thick and lignified rind, could belong to the Mexican or Guatemalan subgroup. These Pumpkins are used for culinary purposes when immature or for seed consumption. The European and Asiatic Pumpkins subgroup, derived from the North American ones, could be represented by some different forms: E-25 and S-1, with a non-lignified rind and with a green and yellow band pattern, and CM-39, AS-3, AN-21 and S-4 with a lignified rind. The accession PI-171628, from Turkey, is of great interest because of the intense orange colour of its flesh, very unusual in *C. pepo* and indicative of a high carotenoid content. A similar pumpkin was described by A.N. Duchesne, who represented the European variability of the fruits of *C. pepo* in 1771 (Paris 2000). Although the accessions of Vegetable Marrow displayed variable colours and ribbing, they all had a lignified rind and a bushy growth habit, indicative of their recent development (Paris 1998). Contrarily, the accessions of Cocozelle were more variable according to the colour, ribbing and growth habit. Currently, this morphotype may harbor more genetic variation than any other group of summer squash (Paris 1998). In contrast, the accessions of Zucchini were very uniform, in agreement with their recent origins and degree of breeding.

In ssp. *ovifera*, the accessions of Scallop displayed variable fruit colouring, although they were uniform according to the plant characters, all of them being bushy. In the Acorn morphotype, PI-615111 ("White Acorn") and V-142 showed a pale-yellow colour at maturity, unlike the remaining accessions and the majority of commercial cultivars, usually dark (Paris 2001). Similarly, among the accessions of Crookneck, PEP-11 displayed a dark-green colour, unlike the majority of commercial cultivars belonging to this morphotype, orange at maturity (Paris 2001). Among the accessions of Straightneck, PEP-17 and PEP-15 were yellow at maturity, while V-202 was green.

In the collection, a high number of accessions might be considered as intermediate forms between different groups, according to the characterization data (C-72, GO-44, PI-169462, PI-204698, CA-35, PEP-307 and PEP-625 between Pumpkin and Vegetable Marrow; B-4 and V-200 between Straightneck and round or flattened forms; V-116 and V-171 between Zucchini and Cocozelle; PI-490279 between Acorn and Pumpkin, etc). Some accessions could not be included in any known morphotype. For example, A-19, V-31 and CA-154 displayed very large fruits, a non-lignified rind and white or yellow flesh. These squashes are employed for cattle consumption. V-4, CM-21 and V-201 had fruits similar to some elongated and warted ones drawn by A. N. Duchesne in 1770, currently thought to have disappeared (Paris 2000). This shows that most of the great variability displayed by the European cultivars as a consequence of the diversification of *C. pepo* after their appearance in Europe is maintained nowadays in the Spanish landraces.

Among the ornamental accessions, a "Spoon" gourd (V205) was included, belonging to ssp. *ovifera*, as well as two round, small and orange gourds, similar to "Orange Ball" and "Orange Warted" (V-82 and CL-4 respectively), considered to belong to ssp. *pepo* (Decker 1988).

Molecular characterization

SRAP analysis

Analysis of the 69 *C. pepo* accessions with 11 SRAP primer combinations identified a total of 88 reproducible fragments (Table 4). Among them, 64 were polymorphic (72.7%), ranging in size from 154 bp to 653 bp. The number of fragments detected by an individual primer combination ranged from 4 (for combinations ME-8/EM-1 and ME-2/EM-1) to 15 (ME-7/EM-5), with an average

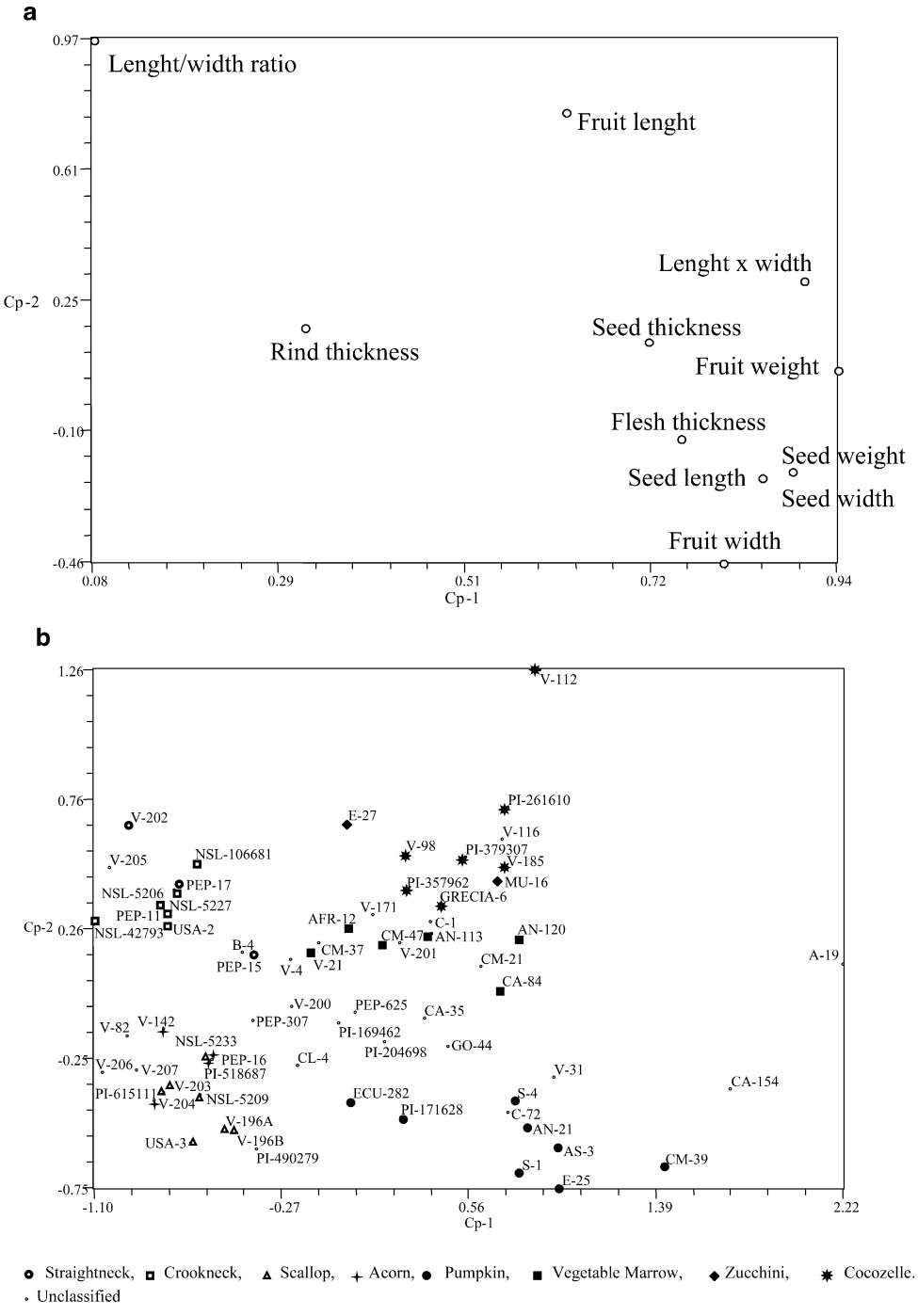

Fig. 1 **a** Diagram showing the relationships among the quantitative characters used for the characterization of 69 accessions of *C. pepo* based on principal component analysis. **b** Diagram showing the relationships among the 69 accessions of *C. pepo* based on principal component analysis using morphological characterization

of 8. The number of polymorphic fragments for each primer combination varied from 1 (ME-8/EM-1) to 14 (ME-7/EM-5), with an average of 5.8. Based on the percentage of polymorphic fragments, different levels of polymorphism ranging from 25% (ME-8/EM-1) to 100% (ME-2/EM-1) were detected. Gene diversity ranged from 0.09 (ME-8/EM-1) to 0.48 (ME-2/EM-1), with an average of 0.25 per primer combination (Table 4).

The percentage of polymorphic fragments, as well as gene diversity, was greater in ssp. *pepo* than in ssp. *ovifera* (0.19 and 0.16 respectively). This result is expected, as most of the ssp. *pepo* accessions used are landraces, whereas those of the ssp. *ovifera* are mainly improved commercial cultivars. In addition, these results agree with the fact that the ssp. *pepo* morphotypes possess a much larger number of cultivars and more phenotypic variation than the ssp. *ovifera* ones (Paris 1998). This greater phenotypic variation is also observed in our study.

A bootstrap analysis was performed using the Nei and Li distance (1979) and the UPGMA method. The dendrogram grouped the different accessions in two major clusters (bootstrap = 51) (Fig. 2). The cophenetic

Table 4 Number of total and polymorphic fragments and gene diversity estimates in all accessions, ssp. *pepo* and ssp. *ovifera*, using SRAP

Combination	Total fragments	Total			ssp. *pepo*			ssp. *ovifera*		
		Pol[a]	% pol[b]	GD[c]	Pol[a]	% pol[b]	GD[c]	Pol[a]	% pol[b]	GD[c]
ME-8/EM-1	4	1	25	0.09	0	0	0.00	1	25	0.12
ME-7/EM-5	15	14	93.3	0.29	10	66.7	0.22	13	86.67	0.26
ME-2/EM-1	4	4	100	0.48	4	100	0.49	4	100	0.38
ME-7/EM-6	7	5	71.4	0.21	5	71.4	0.24	4	57.14	0.11
ME-2/EM-2	9	7	77.8	0.19	3	33.3	0.10	5	55.56	0.14
ME-2/EM-6	11	8	72.7	0.30	6	54.5	0.21	5	45.45	0.13
ME-6/EM-5	8	7	87.5	0.26	6	75	0.19	4	50	0.20
ME-8/EM-3	11	5	45.5	0.20	4	36.4	0.13	2	18.18	0.05
ME-1/EM-2	8	4	50	0.25	3	37.5	0.11	3	37.5	0.09
ME-2/EM-3	5	4	80	0.20	4	80	0.25	1	20	0.05
ME-6/EM-6	6	5	83.3	0.30	4	66.7	0.24	3	50	0.22
Total	88	64	–	–	49	–	–	45	–	–
Average	8	5.8	72.7	0.25	4.5	56.5	0.19	4.09	49.6	0.16
SD	3.4	3.3	22.6	0.20	2.5	27.8	0.20	3.27	25.77	0.19

[a] Number of polymorphic fragments, [b] Percentage of polymorphic fragments, [c] Gene diversity

coefficient was 0.76, indicating a moderate fit. Cluster I included the accessions of ssp. *pepo*, while cluster II included those of ssp. *ovifera*. In cluster I, the accessions of the different morphotypes did not cluster together, except those pertaining to Vegetable Marrow, with uniform morphological characters. Katzir et al. (2000), using ISSR among morphotypes, observed that different commercial cultivars of Zucchini clustered together, as well as those of Cocozelle, unlike the classification obtained with SRAP. However, in both studies, the accessions of Pumpkin, which is the oldest and most variable morphotype, were dispersed among the remaining morphotypes. Cluster I also includes all the unclassified accessions, except PI-490279, morphologically similar to Acorn cultivars. The landraces used for cattle consumption (A-19, CA-154 and V-31) clustered together in the dendrogram. This was not the case for the accessions displaying long, warted fruits (V-201, V-4 and CM-21).

In ssp. *ovifera*, a sub-clustering according to morphotype was more clearly observed than in ssp. *pepo*, as with the results obtained using ISSR (Katzir et al. 2000). The Acorn accessions were grouped in two different subclusters, fundamentally according to the mature fruit colour: dark green (NSL-32665, NSL-5233 and PI-518687, bootstrap = 70.1) and pale yellow (PI-6151111 and V-142, bootstrap = 76.2). The same seems to occur with the remaining morphotypes. The four pale Scallop accessions clustered together (NSL-5209, V-196A, PEP-16 and V-196B, bootstrap = 56.1), separately from V-203, V-204 (bootstrap = 67) and USA-3, with intense colours. Likewise, the two Straightneck yellow accessions (PEP-15 and PEP-17, bootstrap = 68) clustered separately from V-202, of green colour. Finally, the orange accessions of Crookneck (NSL-106681, NSL-5227, NSL-5206 and USA-2, bootstrap = 50) clustered together, separately from the green PEP-11. It is unlikely that this grouping is due to the fruit colour, as this character is controlled by a few genes (Paris and Nerson 1986; Clayberg 1992). On

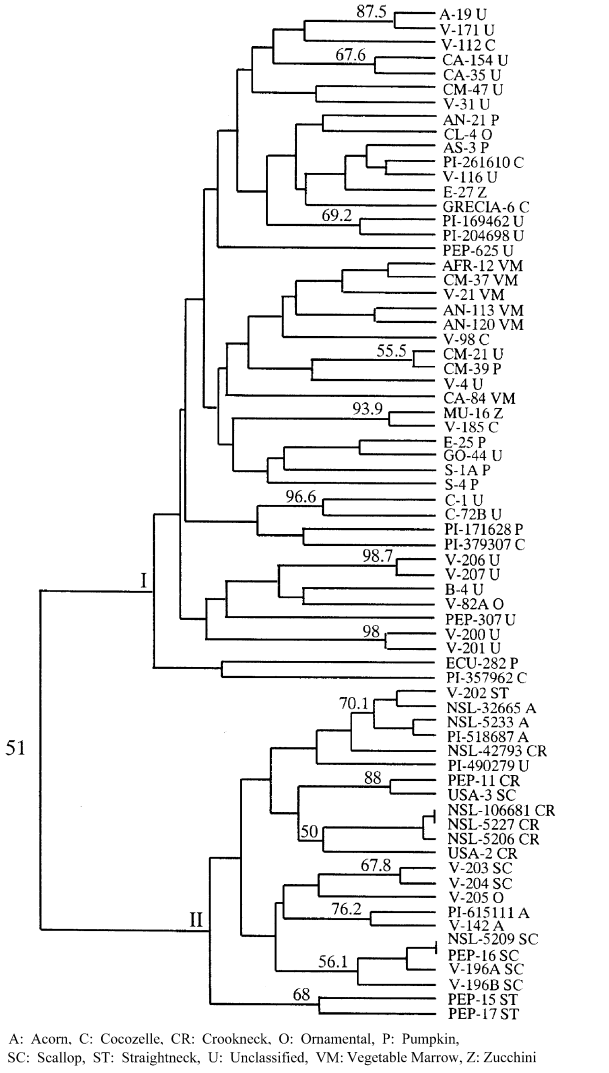

Fig. 2 Dendrogram showing relationships among 69 accessions of *C. pepo* using SRAP markers based on DICE Distance and UPGMA. Bootstrap values over 50 are indicated and are based on 1,000 re-samplings of the data set

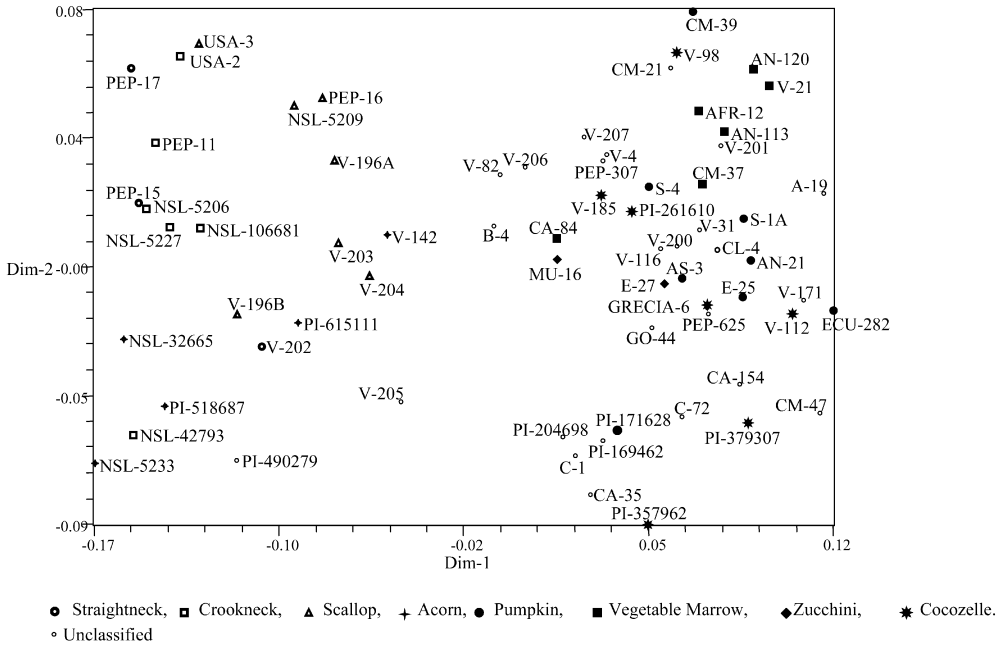

Fig. 3 Diagram showing the relationships among 69 accessions of *C. pepo* based on principal coordinates analysis using SRAP

the contrary, this grouping may indicate the degree of breeding in the accessions used, as in America, the most important market for ssp. *ovifera*; one of the current breeding aims is the development of cultivars with intensely and uniformly coloured fruits (Paris 2001). Thus, the majority of the Crookneck and Straightneck commercial cultivars are actually orange or yellow. Among the Scallop cultivars, pale colours have been preferred for many years, but now intense orange and yellow are the most popular and are becoming increasingly more so. In contrast, the majority of the actual Acorn cultivars are dark green.

Regarding the ornamental gourds, the pyriform "Spoon" V-205 clustered among the ssp. *ovifera* accessions, while CL-4 and V-82 clustered among those of ssp. *pepo*. This agrees with the morphological characterization and previous classifications (Decker 1988). The ornamental cultivars appeared dispersed among the edible forms, like the grouping obtained using RAPD (Decker-Walters et al. 2002).

Figure 3 represents the distribution of the different accessions according to the two principal axes of variation using principal coordinates analysis (PCA). On the basis of the first coordinate, which accounted for 52.8% of the total variation, the accessions were clearly grouped according to subspecies. Generally, within each subspecies, a sub-grouping according to morphotype was also observed in the first coordinate. In ssp. *ovifera*, Crookneck and Straightneck were grouped separately from Scallop, while the Acorn accessions were more dispersed. In ssp. *pepo* the majority of the Pumpkin, Vegetable Marrow and Zucchini accessions were closely grouped, while the Cocozelle accessions were more dispersed.

This grouping corresponded with the genetic identities (Nei 1972) obtained among morphotypes. The lowest values (0.71 to 0.8) were obtained, as expected, between the morphotypes belonging to different subspecies. Within ssp. *ovifera*, the genetic identities ranged from 0.85 to 0.91. Crookneck and Straightneck were the closest morphotypes. This result agrees with the fact that the first Straightneck commercial cultivars were derived from the Crookneck ones at the end of the 19th century, by outcrossing and subsequent inbreeding (Paris 2001). In contrast, the lowest values were obtained between Crookneck, Scallop and Acorn, which are the oldest morphotypes of ssp. o*vifera* and were probably grown by the Native Americans in pre-Columbian times (Paris 2001). Within ssp. *pepo*, the genetic identities ranged from 0.88 to 0.95. Pumpkin and Cocozelle were the closest morphotypes, while the lowest values were obtained between Zucchini and the remaining morphotypes. This agrees with the recent origin of Zucchini (Paris 1989). However, the low values obtained between Acorn and Scallop (0.85) and Acorn and Pumpkin (0.72) did not agree with the suggestion that Acorn could have been derived from a non-intentional cross between a Pumpkin cultivar and a Scallop one (Paris 1989).

Sequencing of amplified fragments

In order to determine the nature of the amplified fragments using SRAP markers, some of them, obtained with different primer combinations, were sequenced. The sequenced fragments were both monomorphic, appearing in all accessions, and polymorphic, appearing more frequently in one of the two subspecies. A monomorphic fragment of 528 nucleotides (ME8/EM1) showed a high homology (identity 74%, E = 1.7e-34) with two EST fragments of *Zea mays* (TC58173, TIGR), similar to the

Table 5 Number of total and polymorphic fragments and gene diversity estimates in all accessions, ssp. *pepo* and ssp. *ovifera* using AFLP

Combination	Total fragments	Total			ssp. *pepo*			ssp. *ovifera*		
		Pol[a]	% pol[b]	GD[c]	Pol[a]	% pol[b]	GD[c]	Pol[a]	% pol[b]	GD[c]
Eco-ACA × *Mse*-CG	60	43	71.67	0.24	32	53.33	0.15	34	56.67	0.17
Eco-ACA × *Mse*-CT	120	66	55.00	0.18	49	40.83	0.11	44	36.67	0.12
Eco-AAC × *Mse*-CG	92	46	50	0.18	31	33.70	0.09	34	36.96	0.16
Eco-AAC × *Mse*-CC	54	14	25.93	0.08	7	12.96	0.02	11	20.37	0.06
Eco-AGG × *Mse*-CA	67	37	55.22	0.19	28	41.79	0.12	19	28.36	0.09
Eco-AGG × *Mse*-CT	83	47	56.63	0.20	35	42.17	0.13	36	43.37	0.14
Total	476	253	–	–	182	–	–	178	–	–
Average	79.33	42.17	52.41	0.18	30.33	37.46	0.10	29.67	37.07	0.12
SD	24.48	16.89	14.90	0.05	13.59	13.55	0.04	12.21	12.48	0.04

[a] Number of polymorphic fragments, [b] Percentage of polymorphic fragments, [c] Gene diversity

protein SSRP1 (structure-specific recognition protein) of maize. Using the Fastax algorithm, the homology with the SSRP protein was studied (identity 63.3%, E = 8.5e-18). A monomorphic fragment of 384 nucleotides (ME2/EM2) also showed significant homology (identity 71%, E = 8e-24) with an EST (TC91641) corresponding to the MAP3K epsilon protein kinase located in chromosome 3 of *Arabidopsis thaliana*. Using the Blastx algorithm, the homology with this protein was studied, and an identity of 69% was obtained (E = 4e-30). Finally, a monomorphic fragment of 332 nucleotides (ME2/EM2) showed an identity of 87% (E = 1e-68) with a cDNA of the CsPK2.1 protein (AB001588) of *Cucumis sativus*. This is a kinase protein involved in the auxin and giberellin response in cucumber hypocotyls (Chono et al. 1998).

Some polymorphic fragments, appearing more frequently in a given subspecies, were sequenced. Thus, a fragment of 282 nucleotides (ME2/EM6), very frequent in ssp. *pepo* and almost absent in ssp. *ovifera*, showed high homology (identity 72%, E = 2.3e-21) with an EST (TC117656) of *A. thaliana* similar to the malate synthetase protein, and with the malate synthetase protein (identity 69%, E = 3e-24) isolated from *Cucurbita* ssp. (Gi 126767). Likewise, a fragment of 207 nucleotides (ME6/EM6), very frequent in ssp. *pepo* and absent in ssp. *ovifera*, showed homology (identity 60%, E = 0.099) with an EST of *Glycine max* of unknown function (BG510038).

The fact that all of the sequenced fragments showed high homology with known sequences agrees with the results reported in previous studies with other species (Li and Quiros 2001; Ferriol et al. 2003), and ratifies the usefulness of the SRAP markers as measuring the genetic variability at the ORFs level.

AFLP analysis

For analysis using AFLP markers, 47 accessions were selected according to the SRAP grouping. They represent the different morphotypes and the unclassified forms. In this analysis, six primer combinations were used. A total of 476 reproducible fragments, ranging in size from 60 bp to 360 bp, were identified, of which 253 (53.15%) were polymorphic (Table 5). The number of fragments detected by an individual primer pair ranged from 54 (for Eco-AAC/Mse-CC) to 120 (Eco-ACA/Mse-CT), with an average of 79, thus confirming the high multiplex ratio produced by AFLP markers. The number of polymorphic fragments for each primer pair varied from 14 (Eco-AAC/Mse-CC) to 66 (Eco-ACA/Mse-CT), with an average of 42. Different levels of polymorphism ranging from 71.7% (Eco-ACA/Mse-CG) to 25.9% (Eco-AAC/Mse-CC) were detected. Gene diversity ranged from 0.08 (Eco-AAC/Mse-CC) to 0.24 (Eco-ACA/Mse-CG), with an average of 0.18 per primer combination (Table 5).

The percentage of polymorphic fragments and the gene diversity estimates were similar in both subspecies, unlike the results obtained with SRAP markers (0.12 for ssp. *ovifera* and 0.10 for ssp. *pepo* using AFLP, in comparison with 0.17 and 0.26 respectively using SRAP, considering only the accessions common to both SRAP and AFLP analyses). This could be due to the fact that SRAP markers preferentially amplify ORFs, and can thus better reflect the morphological diversity of the accessions used, greater in ssp. *pepo* than in ssp. *ovifera*. In this sense, recent studies carried out in tomato have shown that AFLP markers obtained using *Eco*RI/*Mse*I enzymes are not uniformly distributed over the genetic map, being mainly clustered in the centromeres (Bonnema et al. 2002). This is not so evident in melon, belonging to the same family as *Cucurbita* (Wang et al. 1997), but in this case the genetic map is less saturated.

A bootstrap analysis was performed using a modified Nei and Li distance (1979) (Restdist Phylip 3.6) and the UPGMA method (Fig. 4). The cophenetic coefficient was 0.93, indicating a very good fit. As with the results obtained from SRAP markers, the dendrogram formed two major clusters with a high bootstrap support (= 100). Cluster I included the accessions of ssp. *ovifera*, while cluster II included those of ssp. *pepo*. These results support the hypothesis of independent domestication for the different subspecies of *C. pepo*, suggested by Decker (1988) and Kirkpatrick and Wilson (1988), and supported by previous molecular studies using allozymes (Decker 1985), a comparison of chloroplastic DNA (Wilson et al. 1992) and mitochondrial DNA (Sanjur et al. 2002), and RAPD markers (Decker-Walters et al. 2002).

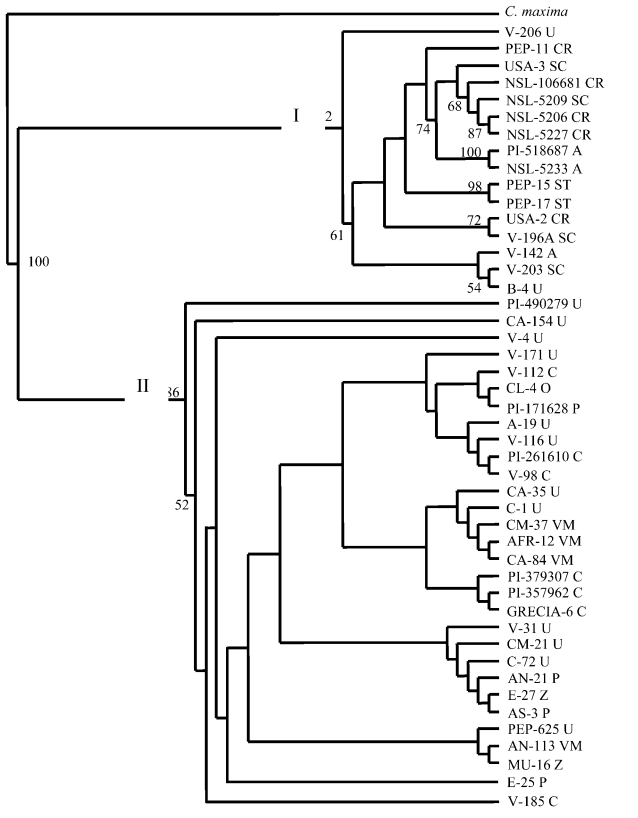

Fig. 4 Dendrogram showing relationships among 47 accessions of *C. pepo* using AFLP markers based on DICE Distance and UPGMA. Bootstrap values over 50 are indicated and are based on 1,000 re-samplings of the data set

As with the results obtained from SRAP, in ssp. *ovifera* a sub-clustering according to morphotype was more clearly observed than in ssp. *pepo*, where only two nodes, which separated two accessions from the rest, showed a significant bootstrap support. However, this grouping according to morphotype was less clear if compared with SRAP. Within ssp. *ovifera*, most of the nodes that showed high bootstrap values involved pairs of accessions. Thus, the Acorn acessions PI-518687 and NSL5233, dark green in colour, clustered together (bootstrap = 100) separately from the yellow cultivar, V-142, as with SRAP. Two Straightneck cultivars, PEP-15 and PEP-17, also clustered together (bootstrap = 97.9). The same occurs with two Crookneck orange cultivars, NSL-5206 and NSL-5227 (bootstrap = 87.4), being separate from the green cultivar PEP-11. Unlike the grouping obtained with SRAP, two unclassified accessions were included in ssp. *ovifera*. V-206, morphologically intermediate between Straightneck and Pumpkin, clustered separately from the remaining ssp. *ovifera* accessions (bootstrap = 82), while B-4, morphologically similar to Straightneck, was included among them.

Within ssp. *pepo*, PI-490279, morphologically similar to Acorn, clustered separately from the remainder of the accessions belonging to the same subspecies (bootstrap = 86.3). Probably, this accession, which was included within ssp. *ovifera* in the grouping obtained using SRAP markers, arose from the crossing of the two subspecies. This agrees with the intermediate morphological traits displayed by this accession. CA-154, used for cattle consumption, also clustered separately from the remainder accessions belonging to ssp. *pepo* (bootstrap = 52.1). The majority of the unclassified accessions clustered within this subspecies, including the squashes used for cattle consumption, the long, warted ones and almost all of the possible crosses between morphotypes. The ornamental pumpkin CL-4 clustered in ssp. *pepo*, as with SRAP markers.

On the basis of the first coordinate of the PCA, which accounted for 63% of the total variation, the accessions were more clearly grouped according to subspecies than with SRAP markers (Fig. 5). Therefore, the accessions clearly represented between the two subspecies could have been derived from crosses between them. However, within each of the subspecies, a grouping according to morphotypes was not observed, unlike the results obtained with SRAP. This difference could be due to the fact that, unlike AFLP markers, SRAP markers preferentially amplify ORFs, which in turn may be involved in the morphological traits that characterise the different morphotypes.

The genetic identity values among morphotypes (Nei 1972) were greater using AFLP markers than using SRAP markers. The lowest values (0.79 to 0.82) were obtained between the morphotypes of the different subspecies, as with the results obtained using SRAP. Within ssp. *ovifera*, the values ranged from 0.9 to 0.96. Crookneck and Scallop were the closest morphotypes, while Acorn and Straightneck were the most distant ones. Within ssp. *pepo*, the genetic identities ranged from 0.94 to 0.97. The greatest values were obtained between Cocozelle and Pumpkin and Vegetable Marrow, while the lowest values were obtained between Zucchini and Pumpkin and Vegetable Marrow. These results are less in agreement with the history and evolution of the *C. pepo* morphotypes proposed by Paris (1989) than those obtained using SRAP.

Possibility of a subspecific classification of the unclassified accessions using SRAP and AFLP

Using both SRAP and AFLP markers, some fragments were uniquely amplified from single accessions belonging to different morphotypes. In addition, a SRAP fragment of 338 nucleotides (ME2/EM6) appeared only in the accessions of ssp. *ovifera*. Its sequence showed limited homology (identity 31%, E = 1.5) with an *Oryza sativa* (GI 9558509) hypothetic protein. Using AFLP, as many as six different fragments appeared uniquely in one of the two subspecies, three in ssp. *ovifera* (91 bp in Eco-ACA ×

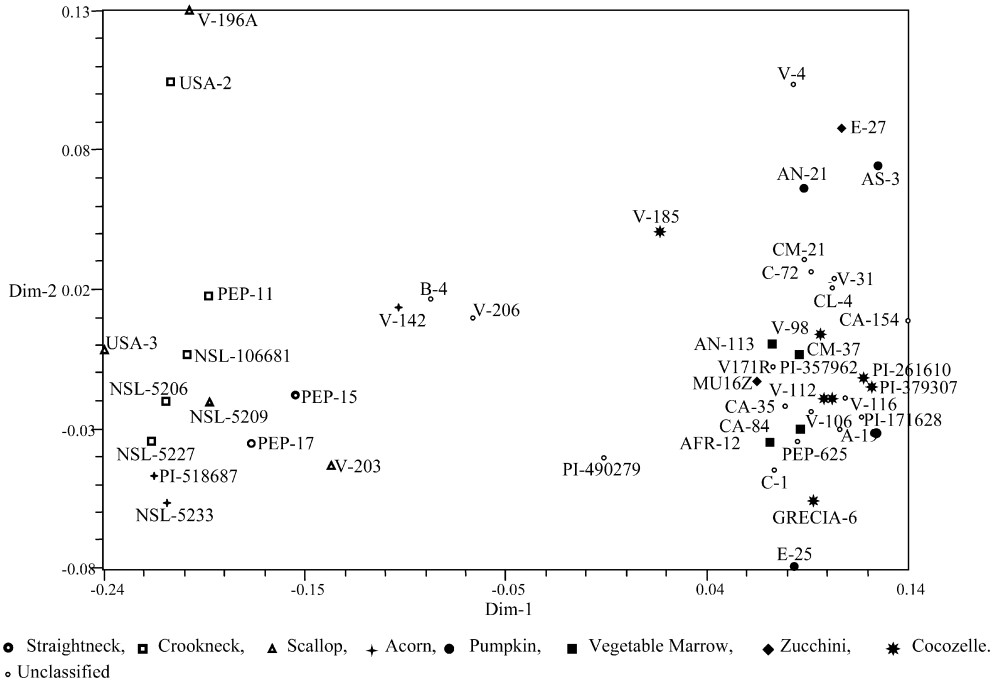

Fig. 5 Diagram showing the relationships among 47 accessions of *C. pepo* based on principal coordinates analysis using AFLP

Mse-CT, 276 bp in Eco-ACA × Mse-CT and 172 bp in Eco-AAC × Mse-CG) and three in ssp. *pepo* (230 bp in Eco-AAC × Mse-CC, 169 bp in Eco-AGG × Mse-CA and 167 bp in Eco-AGG × Mse-CT). However, fragments that appeared only in a given morphotype were not observed in either the SRAP or AFLP analyses.

Using both markers, those fragments specific to a given subspecies, as well as those very frequent in one subspecies and absent in the other, allowed tentative grouping of the unclassified accessions on the subspecific level. The majority of these accessions showed fragments specific to ssp. *pepo*, including the accessions that did not produce fruits (GO-44 and PEP-625), those used for cattle consumption (A-19, CA-154 and V-31), the two long, warted accessions (V-4, CM-21) and the accessions showing intermediate morphological traits between different morphotypes of ssp. *pepo* (C-72, PI-204698, V-116, CM-47, V-171, CA-35 and PEP-307). B-4 only showed fragments exclusive to ssp. *ovifera*. However, with both SRAP and AFLP, as well as with the morphological characterization, it displayed intermediate traits between the two subspecies. Finally, some accessions displaying intermediate morphological and molecular traits were considered to have originated from crosses between both subspecies, such as PI-169462 (similar to Pumpkin), PI-490279 (similar to Acorn), V-200 and V-206 (similar to Straightneck), V-207 (with some typical Scallop and Pumpkin traits) and V-201 (elongated and warted).

In the present study, the genetic diversity of a germplasm collection of *C. pepo*, mainly composed of Spanish landraces, has been analyzed. This collection could be representative of the great diversification that has taken place in this species after the first *C. pepo* fruits and seeds arrived in Europe from America. Given the historical importance of Spain in the Discovery of America and its geographical situation, it acted as a bridge between Europe and America, thereby spreading new types selected in the two continents both ways. The majority of the accessions used in this study are open-pollinated cultivars, as most of the Spanish production is for self-consumption or for sale in local markets. This generates a great diversity of phenotypes maintained as landraces, most of them being intermediate forms between morphotypes belonging to the same or different subspecies. However, since we began the collecting expeditions of germplasm in the eighties, we have detected an important genetic erosion process. Thus, this diversity is endangered, being replaced by more competitive hybrid cultivars. The lesser representation of ssp. *ovifera* in the collection held at COMAV is due to the use of the majority of the cultivars as ornamentals, in contrast with the use for the human consumption characteristic of North America. However, these minor cultivars collected in Spain could be essential for preserving the variability of this subspecies, as the corresponding American commercial cultivars tend to be more uniform.

The molecular tools developed in this study can be applied for characterizing other germplasm collections of *Cucurbita*.

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