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H.S. Paris · N. Yonash · V. Portnoy
N. Mozes-Daube · G. Tzuri · N. Katzir

Assessment of genetic relationships in *Cucurbita pepo* (Cucurbitaceae) using DNA markers

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Abstract *Cucurbita pepo* (pumpkin, squash, gourd), an economically important species of the Cucurbitaceae, is extremely variable in fruit characteristics. The objective of the present study was to clarify genetic relationships across a broad spectrum of the *C. pepo* gene pool, with emphasis on domesticates, using AFLP, ISSR and SSR markers. Forty-five accessions were compared for presence or absence of 448 AFLP, 147 ISSR, and 20 SSR bands, their genetic distances (GDs) were estimated and UPGMA cluster analysis was conducted. The results obtained from these three marker systems were highly correlated ($P \ll 0.001$). Clustering was in accordance with the division of *C. pepo* into three subspecies, *fraterna*, *texana* and *pepo*, with the first two less distant to one another than to the last one. Within the clusters, sub-clustering occurred in accordance with fruit shape and size. The subsp. *texana* cluster consisted of six sub-clusters, one each for the representatives of its five cultivar-groups (Acorn, Crookneck, Scallop, Straightneck and Ovifera Gourd) and wild gourds. Within the subsp. *pepo* cluster, the representatives of two cultivar-groups (Zucchini and Orange Gourd) formed distinct sub-clusters and the representatives of two other groups (Cocozelle and Vegetable Marrow) tended to sub-cluster separately from one another but formed an assemblage with the representatives of the remaining group (Pumpkin). Within-group GDs were less than corresponding between-group GDs in nearly all comparisons. The smallest-fruited accession, 'Miniature Ball', appears to occupy a genetically central position within *C. pepo*.

Keywords Pumpkin · Squash · Gourd · Diversity · Taxonomy · Genetic distance · AFLP · ISSR · SSR

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H. S. Paris (✉) · V. Portnoy · N. Mozes-Daube · G. Tzuri
N. Katzir
Department of Vegetable Crops,
Agricultural Research Organization, Newe Ya'ar Research Center,
P. O. Box 1021, Ramat Yishay 30-095, Israel,
e-mail: hsparis@agri.gov.il
Fax: +972-4-983-6936

N. Yonash
The Israel Gene Bank for Agricultural Crops,
Agricultural Research Organization, Volcani Center, P. O. Box 6,
Bet Dagan 50-250, Israel

Introduction

Cucurbita pepo L. (pumpkin, squash, gourd), an economically important member of the Cucurbitaceae, is perhaps the most polymorphic species with respect to fruit characteristics (Duchesne 1786; Naudin 1856). The domesticates of *C. pepo* are extremely variable in fruit size, shape, and color, and nearly all of them have non-bitter fruit flesh that is thicker, more highly colored, and less fibrous than that of their wild relatives. Domesticates also have larger and fewer vegetative and reproductive parts (Whitaker and Bemis 1964). While most are grown for culinary purposes, some are grown for decoration. Generally, the edible round-fruited sorts are called "pumpkins" and the edible non-round-fruited sorts are called "squash"; the non-edible sorts are called "gourds."

Variations in allozymes are the basis of the current botanical classification of *C. pepo* into three subspecies: *C. pepo* subsp. *fraterna* (Bailey) Andres, *C. pepo* subsp. *texana* (Scheele) Filov and *C. pepo* subsp. *pepo* (Decker 1988). The first has been considered as the putative ancestor for the species as a whole and consists exclusively of wild gourds from northeastern Mexico (Andres 1987; Nee 1990). The second contains wild gourds from the United States (Decker 1988). *C. pepo* subsp. *pepo* has not been identified in the wild, but is presumed to have originated in the most southerly geographic area of the three subspecies. All of the domesticates (pumpkins, squash and ornamental gourds) are included in *C. pepo* subsp. *texana* and *C. pepo* subsp. *pepo*.

Variations in fruit shape, a highly polygenic characteristic that is easily observable, are the basis for the horticultural classification of *C. pepo* to eight cultivar-groups of edible-fruited domesticates (Paris 1986). To

these are added two cultivar-groups of ornamental gourds. The ten cultivar-groups are designated: 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), Straightneck (short, thick neck) and Ovifera Gourd (small, various shapes) (Paris 2000).

The botanical and horticultural classifications have been integrated. Accordingly, *C. pepo* subsp. *pepo* contains the Cocozelle, Pumpkin, Vegetable Marrow, Zucchini and Orange Gourd Groups, and *C. pepo* subsp. *texana* contains the Acorn, Crookneck, Scallop, Straightneck and Ovifera Gourd Groups (Paris 2000).

Recently, DNA polymorphisms have been applied toward assessing relationships in the genus *Cucurbita* L. These have included RFLP, RAPD and preliminary work with AFLP markers to study nuclear genetic variation within and among several species of *Cucurbita* (Torres Ruiz and Hemleben 1991; Stachel et al. 1998; Baranek et al. 2000; Brown and Myers 2000; Gwanama et al. 2000; Ferriol et al. 2001; Decker-Walters et al. 2002). Organellar genetic variation has also been reported (Wilson et al. 1992; Sanjur et al. 2002).

We have applied five ISSR primers to study polymorphism within a limited set of *C. pepo* and obtained results showing a dichotomy between accessions of *C. pepo* subsp. *pepo* and those of *C. pepo* subsp. *texana* and *C. pepo* subsp. *fraterna*, with cultivar-groups tending to form sub-clusters within their respective subspecies (Katzir et al. 2000). However, genetic distances among cultivar-groups have not been rigorously defined using any marker system, nor have results obtained from different marker systems been compared. The objective of the present study was to assess relationships within *C. pepo* through the use of AFLP, ISSR and SSR markers, with emphasis on the domesticates. The results were expected to clarify genetic relationships among subspecies, cultivar-groups and accessions across a broad spectrum of the cultivated *C. pepo* gene pool.

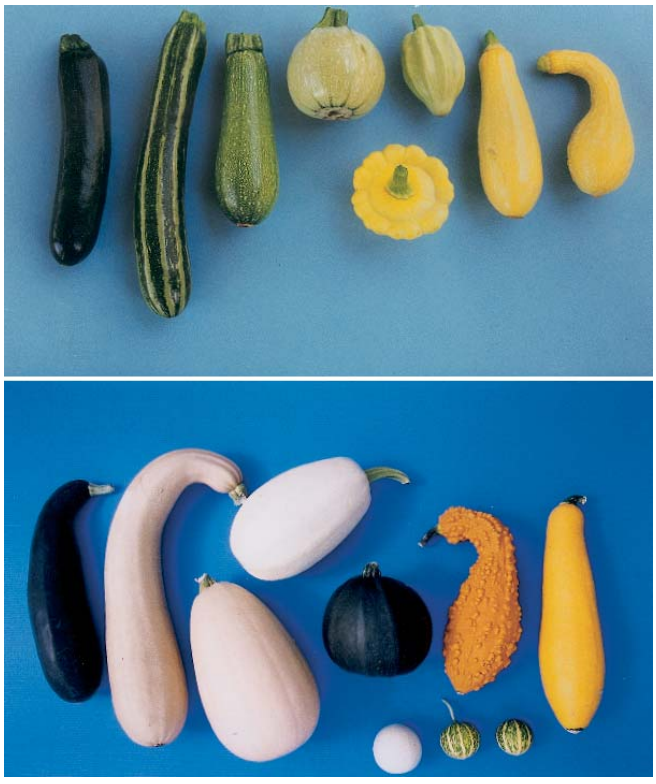

Fig. 1 Top: immature fruits (2–5 days past anthesis) of eight of the accessions studied. Left to right, the first four are *C. pepo* subsp. *pepo* and the latter four are *C. pepo* subsp. *texana*: 'Black Zucchini' (Zucchini Group), 'Striato d'Italia' (Cocozelle Group), 'Verte Petite d'Alger' (Vegetable Marrow Group), 'Tondo di Nizza' (Pumpkin Group), 'Yellow Bush Scallop' (Scallop Group), 'Royal Acorn' (Acorn Group), 'Early Prolific Straightneck' (Straightneck Group) and 'Yellow Summer Crookneck' (Crookneck Group). The cocozelle fruit is approximately 30-cm long. Bottom: mature fruits (>40 days past anthesis) of ten of the accessions studied. Left to right, the first five of the larger fruits are *C. pepo* subsp. *pepo* and the latter two are *C. pepo* subsp. *texana*: 'True French' (Zucchini Group), 'Lungo Bianco di Sicilia' (Cocozelle Group), 'Blanche non-coureuse' (Vegetable Marrow Group), 'Vegetable Spaghetti' (Vegetable Marrow Group), 'Tondo Verde Scuro di Piacenza' (Pumpkin Group), 'Early Crookneck' (Crookneck Group), 'Creamy' (Straightneck Group). The smaller fruits at the lower right are accessions Wild Texas (*C. pepo* subsp. *texana*), Wild Mexico 1 (*C. pepo* subsp. *fraterna*) and Wild Mexico 2 (*C. pepo* subsp. *fraterna*). The cocozelle fruit is approximately 50-cm long through its axis and that of Wild Mexico 2 is 52-mm long

Materials and methods

Plant material

Forty-five accessions representative of the phenotypic diversity of *C. pepo* were chosen for this study. The classification of these accessions to subspecies and group, as well as the sources of their seeds, are presented in Table 1. Of the 45 accessions, 30 were represented by plants grown from the original seeds obtained from seed companies or collectors. The remaining 15 accessions were represented by plants grown from seeds obtained from self- or sib-pollination of plants grown by us from the original seeds. Plants of all accessions had been grown to maturity in the field at least twice to confirm the identity and uniformity of fruit shape of the accessions. Immature and mature fruits of a few of the accessions are depicted in Fig. 1.

The four edible-fruited cultivar-groups of *C. pepo* subsp. *texana* as well as the cultivated and the wild gourds assigned to this subspecies had three representatives each (Table 1). To obtain fair representations, the three representatives included one that was less closely similar phenotypically to the other two. As our seed collection has only two open-pollinated (non-hybrid) cultivars of the *C. pepo* subsp. *texana* Straightneck Group, a widely grown hybrid ('Seneca Butterbar') was included as a representative of this group.

The four edible-fruited cultivar-groups of *C. pepo* subsp. *pepo* contain far more cultigens and have a wider distribution than any of those of *C. pepo* subsp. *texana* (Paris 2001). Thus, they were represented by a greater number of accessions in the present study (Table 1), in which the number of accessions representing each group was roughly in accordance with the amount of phenotypic diversity of the group. Four gourd cultivars classified in this subspecies were also included.

Seeds of the 45 accessions were planted in flats filled with a potting soil mix or in the field. Samples for analysis were taken from the first true leaf of four to eight plants approximately 13

Table 1 Classification and sources of seeds of 45 *C. pepo* accessions. Tripartite designation is for subspecies, group and abbreviated accession name, respectively. For subspecies: T = *texana*, F = *fraterna*, P = *pepo*. For Group: AC = Acorn, CN = Crookneck, SC = Scallop, SN = Straightneck, GO = Ovifera gourd, GT = *texana* wild gourd, GF = *fraterna* gourd, CO = Cocozelle, PU = Pumpkin, VM = Vegetable marrow, ZU = Zucchini, GP = Orange gourd

Accession	Designation	Country of origin
Royal Acorn	T-AC-RAC	U.S.A. (Twilley)
Sweet Dumpling	T-AC-SWD	U.K. (Thompson & Morgan)
Table Queen	T-AC-TQE	U.S.A. (Northrup King)
Golden Bush Scallop	T-SC-GBS	U.S.A. (Dessert)
White Patty Pan	T-SC-WBS	U.S.A. (Field)
Yellow Bush Scallop	T-SC-YBS	U.S.A. (Gurney)
Creamy	T-SN-CRE	U.S.A. (Park)
Early Prolific Straightneck	T-SN-EPS	U.S.A. (Ledden)
Seneca Butterbar	T-SN-SBU	U.S.A. (Gurney)
Early Crookneck	T-CN-EAC	U.S.A. (Field)
Early Summer Crookneck	T-CN-ESC	U.S.A. (Ledden)
Yellow Summer Crookneck	T-CN-YSC	U.S.A. (Ferry Morse)
Spoon	T-GO-SPN	Canada (Stokes)
Striped Pear	T-GO-STP	Canada (Stokes)
Shenot's Crown of Thorns	T-GO-SHC	Canada (Stokes)
Wild Arkansas	T-GT-WAR	U.S.A. (L.R. Oliver[1])
Wild Texas	T-GT-WTX	U.S.A. (H.D. Wilson[2])
PI 285213	T-GT-213	U.S.A. (North Central Plant Intro. Sta.)
Wild Mexico 1	F-GF-WMX1	Mexico (T.C. Andres[3])
Wild Mexico 2	F-GF-WMX2	Mexico (H.D. Wilson[4])
Long Cocozelle	P-CO-LCO	U.S.A. (Ledden)
Lungo Bianco di Sicilia	P-CO-LBS	Italy (S.A.I.S.)
Romanesco	P-CO-ROM	Italy (Semitalia)
Striato d'Italia	P-CO-STI	Italy (C.C.S.A. & S.A.I.S.)
Verte non-coureuse d'Italie	P-CO-VNI	France (Abondance)
Connecticut Field	P-PU-CTF	U.S.A. (Ledden)
Nonkadi	P-PU-NOK	Uzbekistan (Y. Asherov, I.V.S.Q.)
PI 442309	P-PU-309	Mexico (North Central Plant Intro. Sta.)
PI 442313	P-PU-313	Mexico (North Central Plant Intro. Sta.)
Small Sugar	P-PU-SSU	U.S.A. (Ledden)
Styria, Wies 371	P-PU-STY	Austria (P.S. Lichtenecker[5])
Tondo di Nizza	P-PU-TON	Italy (Ingegnoli)
Tondo Verde Scuro di Piacenza	P-PU-TOS	Italy (C.C.S.A.)
Blanche non-coureuse	P-VM-BNC	France (Gondian)
M2546	P-VM-546	Israel (A.R.O. Dept. Plant Intro.)
Vegetable Spaghetti	P-VM-VSP	Japan (Sakata)
Verte Petite d'Alger	P-VM-VPA	France (Abondance)
Black Zucchini	P-ZU-BZU	U.S.A. (Field)
Fordhook Zucchini	P-ZU-FZU	U.S.A. (Burpee)
Nero di Milano	P-ZU-NER	Italy (C.C.S.A.)
True French	P-ZU-TRF	U.K. (Thompson & Morgan)
Miniature Ball	P-GP-MNB	Canada (Stokes)
Orange Ball	P-GP-ORB	Canada (Stokes)
Orange Warted	P-GP-OWA	Canada (Stokes)
Rolet	P-GP-ROL	South Africa (unknown)

[1] Wild Arkansas was obtained from L.R. Oliver of the University of Arkansas, Fayetteville, in 1986

[2] Wild Texas was obtained from H.D. Wilson of Texas A&M University in 1986. From Bedias Creek, Madison County Texas, collection #5467, lab id TE 39, collected 22 March 1986

[3] Wild Mexico 1 was obtained from T.C. Andres of the New York State Agricultural Experiment Station in 1987. Collected in Tamaulipas, Mexico in 1985

[4] Wild Mexico 2 was obtained from H.D. Wilson of Texas A&M University in 1993, his designated no. FR1-1-2

[5] Styria was obtained from P.S. Lichtenecker and presumably is of an accession designated Wies 371 from H. Pelzmann of Wies, Austria

days after emergence. Leaf samples of each accession were bulked for analysis. Preparation of DNA from the 45 accessions was as described previously (Danin-Poleg et al. 1998; Katzir et al. 1998).

AFLP analysis

AFLP analysis was performed with the AFLP Analysis System I Kit (Gibco BRL) (Vos et al. 1995). Genomic DNA (40–50 ng) was restricted with *Eco*RI and *Mse*I (0.25 units each) in a restriction buffer (10 mM Tris-HCl pH 7.5, 10 mM Mg-acetate, 50 mM K-acetate) in a final volume of 5 µl. *Eco*RI and *Mse*I adapters were subsequently ligated to the digested DNA fragments in a final volume of 10 µl. The adapter-ligated DNA was diluted (1:10) and pre-amplified with AFLP primers, each having one selective nucleotide at its 3′ end. The pre-amplified DNA was diluted (1:50) and an aliquot was used for selective amplification with *Eco*RI and *Mse*I primers having three selective nucleotides at their 3′ ends. Fourteen combinations were used as listed in Table 2. Pre-amplification and selective amplification programs were as described by Vos et al. (1995). PCR products were separated on a 6% polyacrylamide gel containing 8 M urea and 1 × TBE, at 60 W constant power for 2.0 to 2.5 h. After drying, the gels were exposed to a Kodak BioMax MR film.

ISSR and SSR analyses

ISSR primers 807, 809, 810, 841, 842 and 855 from Kit #9, University of British Columbia, Canada, were applied (Zietkiewicz et al. 1994). ISSR reaction mixtures and protocols were as described by Katzir et al. (2000).

Of the seven melon and cucumber SSR primer pairs tested, five were CMAG59, CMTC51, CSTCC813, CSCTTT15a and CMGA15, as described by Katzir et al. (1996) and Danin-Poleg et al. (2001). Two previously undescribed primer pairs, CMAGN73 and CMTGN17, were employed. Respectively, their primer sequences (5′ to 3′) were: ATCCAACTCGACCAAGAAAC and CAGCTCTACAACAACATCTC, and CATGATGACCTTTTGGGCTG and GGGTAGGATTGAGTGAGAGT. SSR reaction mixtures and protocols were as described previously (Katzir et al. 1996; Danin-Poleg et al. 2001).

Band scoring and data analysis

For each type of marker, DNA fragments were scored as present (1) or absent (0), and a binary file was prepared. The SSR data were interpreted as dominantly inherited marker profiles because DNA pooling from four to eight plants per accession did not permit accurate determination of allele frequency. The analysis was conducted in two general stages: first, bootstrapping and calculation of genetic distances, and second, cluster analysis and drawing of phylogenetic trees. For each of the three types of markers, 1,000 bootstraps and genetic difference estimates, expressed as genetic distance (GD) values (Nei and Li 1979), were calculated using the PhylTools software (Buntjer 2000); genetic distance matrices are available from the authors upon request. Cluster analysis by the UPGMA method (Sneath and Sokal 1973) was conducted with NEIGHBOR and CONSENSE software (PHYLIP package) (Felsenstein 1993). Consensus trees were drawn using the TreeView software (Page 1996); those for the ISSR and SSR results are available from the authors. Estimates of similarity among genetic-distance matrices were calculated with Mantel matrix correspondence tests (Mantel 1967) using the Arlequin software (Schneider et al. 2000). Significance for the Mantel tests was determined by 1,000 permutations.

Table 2 *Eco*RI/*Mse*I primer pairs used for selective amplification (each primer having three selective nucleotides on its 3′ end) and the number of bands amplified in each combination for the 45 accessions

*Eco*RI (P33)	*Mse*I	No. bands	Percentage polymorphic
AAC	CAT	35	60.0
AAG	CAC	34	64.7
AAG	CAG	31	48.4
AAG	CTC	43	74.4
AAG	CTG	39	74.4
AAG	CTT	39	53.8
ACA	CAT	43	58.1
ACG	CAT	19	52.6
ACT	CAT	55	56.4
AGC	CAC	21	66.7
AGC	CAG	24	66.7
AGC	CTA	20	60.0
AGC	CTC	18	83.3
AGC	CTT	27	63.0
		Total	Mean
		448	62.5

Results

The 14 AFLP primer combinations yielded 18 to 55 amplification products each, of which 10 to 32 were polymorphic (Table 2). They produced a total of 448 well-spaced, easily scored bands of which 280 (63%) were polymorphic (Table 2). The number of reliably scoreable bands produced using ISSR primers ranged from 16 to 34, of which 15 to 23 were polymorphic. Of the 147 ISSR bands scored, 108 (74%) were polymorphic. The seven SSR primer pairs detected two to five size alleles among the 45 accessions. A total of 20 SSR amplification products were scored. Thus, the results are based on data from a large number of AFLP bands, a moderate number of ISSR bands and relatively few SSR bands.

The dendrograms and genetic distance matrices produced from the AFLP, ISSR and SSR data were similar. Correlation coefficients by Mantel tests were 0.95 between the AFLPs and ISSRs, 0.78 between the AFLPs and SSRs and 0.77 between the ISSRs and SSRs, all three with a significance of $P < 0.000001$. Given this high degree of similarity of the three genetic-distance matrices, and as the AFLP matrix was based on the highest number of loci, the results obtained from the AFLP data were selected for presentation (Fig 2), and a condensed AFLP genetic-distance matrix (Table 3) is presented to serve as an overview of the results.

Two clusters are evident in the dendrogram (Fig. 2), with the 20 accessions classified as subspecies *texana* and *fraterna* separated from 24 of the 25 accessions classified as subspecies *pepo*. GD values among groups within the *texana-fraterna* cluster ranged from 0.08 to 0.18. GD values among groups within the *pepo* cluster from 0.09 to 0.14. GD values among groups from different clusters were higher, ranging from 0.18 to 0.25 (Table 3).

Within the cluster of *C. pepo* subspp. *texana* and *fraterna*, the two accessions of subsp. *fraterna* paired tightly together (1,000 out of 1,000 bootstraps) (Fig. 2) with a GD value = 0.04 (Table 3). However, subsp. *fraterna* was unique from subsp. *texana*, as there was greater distance between the two *fraterna* accessions and all groups within subsp. *texana* (GD = 0.16 to 0.18) than there was among the *texana* groups (GD = 0.09 to 0.13). There was even greater distance between the accessions of subsp. *fraterna* and the groups of subsp. *pepo* (GD = 0.20 to 0.22).

A readily apparent feature of the *texana* cluster was the formation of six sub-clusters, one of each representing the five cultivar-groups (T-AC, T-CN, T-SC, T-SN T-GO) and the wild gourds (T-GT) (Fig. 2). The domesticated gourds (T-GO) occupied the basal position and had the highest GD values with respect to the other *texana* groups. This sub-clustering was often based on high bootstrap values. Furthermore, within-group GD values were less than those corresponding between-group GD values in all cases. The within-group GD value for the three wild *texana* gourds (T-GT) was 0.04, lower than that of any of the cultivar-groups of subspecies *texana* (Table 3).

Within the sub-clusters, the two accessions that were more similar phenotypically also had lower GD values between them than either did with the third accession. For example, of the Acorn Group (T-AC) accessions, T-AC-RAC and T-AC-TQE are more similar phenotypically than either of these two is to T-AC-SWD. Concordantly, the GD value between T-AC-RAC and T-AC-TQE was 0.05, which was less than the GD values between T-AC-RAC and T-AC-SWD (0.07), and between T-AC-TQE and T-AC-SWD (also 0.07).

The edible-fruited cultivar-groups formed distinct sub-clusters using the ISSR and SSR data, too (Table 3; data not presented). However, in the analysis with ISSRs, the wild gourds (T-GT) occupied the basal position within subsp. *texana* and had the highest GD values in pairwise comparisons among the *texana* groups. The AFLP, ISSR and SSR analyses showed that, on average,

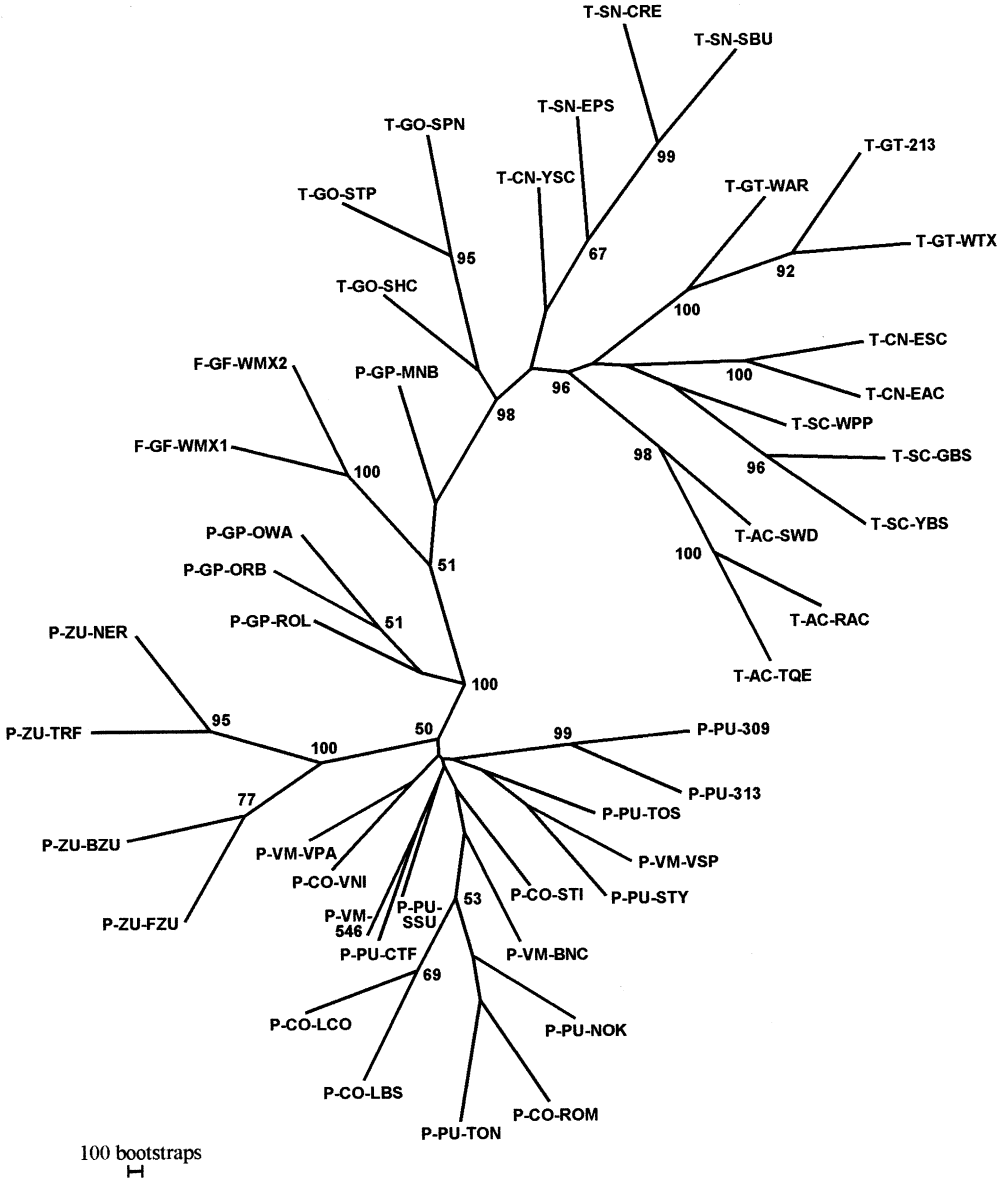

Fig. 2 Unrooted dendrogram derived from a UPGMA cluster analysis of 45 *C. pepo* accessions, using 448 AFLP bands. The tripartite designations are as in Table 1, with the *single letters* representing subspecies, the *double letters* representing groups, and the *triple letters* representing accessions. The *numbers* at the nodes are bootstrap percentages out of 1,000. Only values of ≥50% are indicated

Table 3 Genetic distance matrix among and within groups based on 448 AFLP bands. The dipartite designations are the first two parts of the tripartite designations listed in Table 1, with the single letters representing subspecies (T = *texana*, F = *fraterna*, P = *pepo*) and the double letters representing groups (AC = Acorn, SC = Scallop, CN = Crookneck, SN = Straightneck, GO = Ovifera gourd, GT = *texana* wild gourd, GF = *fraterna* gourd, GP = Orange gourd, PU = Pumpkin, VM = Vegetable marrow, CO = Cocozelle, ZU = Zucchini)

T-AC	0.06											
T-SC	0.09	0.07										
T-CN	0.09	0.09	0.07									
T-SN	0.11	0.11	0.09	0.07								
T-GO	0.11	0.11	0.11	0.13	0.10							
T-GT	0.09	0.09	0.08	0.12	0.11	0.04						
F-GF	0.18	0.17	0.17	0.17	0.17	0.16	0.04					
P-GP	0.19	0.19	0.19	0.19	0.18	0.22	0.20	0.12				
P-PU	0.22	0.22	0.22	0.21	0.20	0.25	0.22	0.12	0.08			
P-VM	0.21	0.21	0.22	0.20	0.19	0.24	0.22	0.12	0.09	0.09		
P-CO	0.22	0.22	0.22	0.21	0.20	0.25	0.20	0.12	0.08	0.09	0.07	
P-ZU	0.22	0.23	0.23	0.21	0.21	0.25	0.22	0.14	0.10	0.09	0.09	0.04
	T-AC	T-SC	T-CN	T-SN	T-GO	T-GT	F-GF	P-GP	P-PU	P-VM	P-CO	P-ZU

the GD values between the wild *texana* gourds and the Scallop Group (T-SC) were smaller than the GD values of pairwise comparisons between these gourds and all other cultivar-groups. The ISSR and SSR analyses also showed relatively small GD values between the Ovifera Gourd Group (T-GO), especially 'Spoon' (T-GO-SPN) and 'Striped Pear' (T-GO-STP), and the Scallop Group. However, moderate-to-high GD values were observed between the wild gourds and the Ovifera Gourd Group.

On average for the AFLP, ISSR and SSR analyses, the largest GD value among the subsp. *texana* pairwise comparisons was between the Straightneck Group (T-SN) and the wild gourds (Table 3; data not presented). In pairwise comparisons using the AFLP data, the Straightneck Group was most similar to the Crookneck Group (T-CN); however, using the ISSR and SSR data, the Straightneck Group was most similar to the Acorn Group (T-AC).

Within *C. pepo* subsp. *pepo*, three of the Orange Gourd Group (P-GP) accessions formed a sub-cluster separate from the edible-fruited groups (Fig. 2). The four representatives of the Zucchini Group (P-ZU) formed a distinct sub-cluster with relatively high bootstrap values. The GD value within this cultivar-group was 0.04 but between it and all others the values were more than double (0.09 to 0.14) (Table 3).

The remainder of the accessions of *C. pepo* subsp. *pepo* were representatives of the Cocozelle (P-CO), Pumpkin (P-PU) and Vegetable Marrow (P-VM) Groups. These formed a more mixed assemblage (Fig. 2), as evidenced by the GD values within cultivar-groups that, on average, were nearly as high as those among them (Table 3). Nonetheless, the two pumpkins from Mexico, P-PU-309 and P-PU-313, had relatively small GD values between them (0.05 for AFLP, 0.02 for ISSR, 0.20 for SSR). The two pumpkins from the U.S.A., P-PU-CTF and P-PU-SSU, had moderate values (GD = 0.08, 0.10 and 0.14, respectively). When the two accessions from Mexico were compared with the two from the U.S.A., the average GDs were larger (GD = 0.11, 0.11 and 0.33, for AFLP, ISSR and SSR, respectively). The remaining four pumpkins, from Europe and Asia, had moderate GD values in pairwise comparisons between them and those of both North American countries (average 0.09, 0.10 and 0.29, respectively). The cocozelles and vegetable marrows did not show consistent similarities with pumpkins of different geographic origins nor with each other. However, the cocozelle and vegetable-marrow representatives did not occur together with one another in any of the sub-clusters of the SSR dendrogram (data not presented).

Of all the accessions classified as subsp. *pepo*, 'Miniature Ball' (P-GP-MNB) had, on average, the greatest GD values in pairwise comparisons among the accessions of subsp. *pepo* (Fig. 2; data not presented). On the other hand, in pairwise comparisons, it had lower GD values with the accessions of subsp. *pepo* than did any accession of subsp. *texana* and lower GD values with the accessions of subsp. *texana* than did any accession of subsp. *pepo*. 'Miniature Ball' also had lower GD values with the cultigens of both subspecies than did the wild subsp. *fraterna*.

Discussion

The three types of DNA markers employed in this study, AFLP, ISSR and SSR, differ in the nature of the evolutionary mechanisms underlying their variation and their distribution in the genome (Powell et al. 1996; Staub et al. 1996). Nonetheless, the results obtained from these three marker systems were highly correlated. In a related species, *Cucumis melo* L. (melons, Cucurbitaceae), correlation coefficients of similar magnitude were found among AFLP, RAPD and RFLP markers (Garcia-Mas et al. 2000). AFLP marker analysis is advantageous for systematic studies because of its high efficiency in detecting polymorphism (Powell et al. 1996; Garcia-Mas et al. 2000), and AFLP markers have been observed to be evenly distributed throughout the genome of two cucurbits, melons (Périn et al. 2000) and cucumbers (*Cucumis sativus* L.) (Bradeen et al. 2001). In melons, a relatively low correlation coefficient was obtained between RAPD and SSR markers, even though the SSR markers were treated as codominant alleles (Staub et al. 2000). In the present study, the correlation coefficients obtained from the comparisons of the SSR results with the AFLP and ISSR results were high, in spite of the low number of SSR bands and their interpretation as dominant markers.

The results (Fig. 2, Table 3; ISSR and SSR are not presented) support the botanical (Decker 1988) and horticultural (Paris 1986) classifications of *C. pepo*. They are also consistent with observations based on variations in organellar DNA (Wilson et al. 1992; Sanjur et al. 2002) showing that *C. pepo* subsp. *texana* and *C. pepo* subsp. *fraterna* are more closely related to one another than either is to subsp. *pepo*. Likewise, the present results are consistent with the results based on variations in nuclear DNA (Torres Ruiz and Hemleben 1991; Katzir et al. 2000) and allozymes (Ignart and Weeden 1984; Decker 1988) that allowed for integration of the botanical and horticultural classifications (Paris 2000). The present results show that the cultivar-groups are genetically quite distinct from one another, and furthermore suggest possible genetic relationships among them.

Historically, the Scallop Group is old and presumed to have been developed by Native Americans directly from non-edible-fruited ancestors (Paris 1989). The results from the three marker systems (Table 3; data not shown) show that the scallops are the edible-fruited cultivar-group that is most closely related to the *texana* gourds, suggesting a link between this group and putative non-culinary ancestors.

Conversely, the Straightneck Group is the most recent of the subsp. *texana* cultivar-groups (Paris 2000) and the most dissimilar to the *texana* gourds (Table 3). This cultivar-group possesses the least phenotypic variation and consists of relatively few cultivars (Paris 2001), all of which may share a common ancestry. 'Early Prolific Straight-

neck' (T-SN-EPS) apparently was derived from 'Summer Crookneck' by outcrossing with an unknown parent (Paris 2000). Analysis of variation using AFLPs (Fig. 2) suggests that the Straightneck Group is most closely related to 'Yellow Summer Crookneck' (T-CN-YSC). However, ISSR and SSR analyses (data not presented) suggest that the Straightneck Group is most closely related to the Acorn Group. Hence, the most likely outcross donor parent of the Straightneck Group was an acorn squash cultivar.

The Zucchini Group is the most recent of the subsp. *pepo* cultivar-groups (Paris 2000), and likewise the most dissimilar to the gourds of its subspecies (Table 3). This cultivar-group was the most distinct of the edible-fruited cultivar-groups of subsp. *pepo*, having the lowest within-group and highest between-group GD values (Table 3), consistent with results obtained using allozymes (Ignart and Weeden 1984), RFLPs on nucleolar DNA (Torres Ruiz and Hemleben 1991) and ISSRs on nuclear DNA (Katzir et al. 2000). The Zucchini Group had less affinity to the Pumpkin Group, which is the oldest of the edible-fruited cultivar-groups (Paris 2000), than it did to the Cocozelle Group and the Vegetable Marrow Group.

The relationships within the Pumpkin Group and between it and the Cocozelle and Vegetable Marrow Groups were not as clearly defined by the results presented herein as were those of the other groups (Fig. 2; Table 3). As these three groups contain many old cultivars and greater phenotypic diversity than any of the others (Paris 2000, 2001), genotyping more cultivars of each might provide a better insight to their relationships.

Variation among wild populations of subsp. *texana* has led to the suggestion that gourds from Texas and other states in the U.S.A. should be treated as separate botanical varieties (Decker-Walters et al. 2002). In the present study, the average GD value for the wild *texana* accessions, two from Texas and one from Arkansas, was 0.04, equal to or less than the GD values obtained within all of the cultivar-groups (Table 3). However, a wider sampling of wild subsp. *texana* gourds has revealed greater genetic variation (Decker-Walters et al. 2002). The results of the present study define wide genetic variation among domesticates, consistent with their wide variation in fruit size and shape. Such variation is much more modest among wild populations.

'Miniature Ball' has been placed within the Orange Gourd Group of subsp. *pepo* on the basis of results obtained for 12 polymorphic allozyme loci (Decker and Wilson 1987). The present results obtained from three DNA marker systems showed 'Miniature Ball' to be closest to the Orange Gourd Group and the Pumpkin Group (data not shown). Our results also showed 'Miniature Ball' to have greater affinity to each of the two domesticated subspecies, *pepo* and *texana*, than does the wild subsp. *fraterna*. Furthermore, 'Miniature Ball' was more similar to the domesticated accessions of both subspecies than any accession of one was to the accessions of the other. Thus, 'Miniature Ball' appears to occupy a genetically central position in *C. pepo*.

The phenotypic characteristics of 'Miniature Ball' are much like those of wild accessions (Whitaker and Bemis 1964). However, it differs from them by possessing the dominant allele *D* for dark stems and peduncles (Paris and Nerson 1986), which is common among the edible-fruited domesticates. 'Miniature Ball' does not appear to be the product of introgression from domesticates into wild gourds. As fruit size is a highly polygenic characteristic, such introgression would be expected to result in larger fruits. On the contrary, the fruits of 'Miniature Ball' are even smaller than those of the wild subsp. *fraterna*. A wild ancestor for *C. pepo* subsp. *pepo*, and indeed of *C. pepo* as a whole, has not been identified (Sanjur et al. 2002). 'Miniature Ball' possesses wild-type characteristics and yet shows genetic affinity to a wide range of domesticated *C. pepo*, suggesting that it may represent the wild ancestor preserved in cultivation.

Fruit size separates the edible-fruited forms (pumpkins and squash), which have larger fruits, from the ornamental and wild forms (gourds), which have smaller fruits. This separation was reflected in the sub-clustering of the gourds separately from the pumpkins and squash (Fig. 2). Fruit shape separates the squash and pumpkins into eight cultivar-groups (Paris 1986). This separation was also reflected in the separate sub-clustering of the groups. Therefore, in *C. pepo*, DNA polymorphisms are quite consistent with phenotypic variation of two highly polygenic characteristics: fruit size and fruit shape.

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