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RESEARCH ARTICLE

Spanish melons (*Cucumis melo* L.) of the Madrid provenance: a unique germplasm reservoir

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Abstract Melon (*Cucumis melo* L.) landraces of the Madrid provenance, Spain, have received national distinction for their high fruit quality and sensorial attributes. More specifically, a unique array of Group Inodorus landraces have been continuously cultivated and conserved by farmers in the municipality of Villaconejos since the 19th century. Their genetic relationships to other Group Inodorus and Flexuous melon market classes is not known, and, thus, a study was designed to determine their genetic relationships using 52 simple sequence repeat (SSR) markers, and then make genetic comparisons between these accessions and a previously published "Standard Reference Germplasm Array" (RA) containing Group Inodorus (14 Spanish and one USA), Flexuosus (1 Spanish), and Cantalupensis (2 USA) melon accessions. This subset consisted of 15 Spanish Group Inodorus landraces that circumscribed the genetic variation of major Spanish melon market classes (Groups Inodorus and Flexuosus), and USA commercial varieties (Groups Cantalupensis and Inodorus). Based on genetic distances, Villaconejos (Madrid) genotypes differed substantially from RA subset accessions, thus defining their genetic uniqueness. Principal component analysis (PCA) partitioned the accessions examined into four distinct groups revealing that Villaconejos black epidermis melons (landraces 'Largo', 'Largo Negro Escrito' and 'Puchero') were distinctly different from all other accessions examined, as cluster analysis separated Rochet market type Villaconejos' accessions (landraces 'Mochuelo', 'Mochuelo Tradicional' and 'Melón de Villaconejos') from RA of the same market type. Genetic assessment of principal Spanish market classes revealed comparatively low intra-market heterogeneity in Piel de Sapo type accessions and high heterogeneity in Black and Yellow market type accessions. While a relatively high level of genetic introgression was detected between Yellow and Green market types, black epidermis market types

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were genetically unique. Given the uniqueness and high genetic diversity resident in Villaconejos landraces, this germplasm pool should be considered as a genetic source for broadening the comparatively narrow genetic base of Group Cantalupensis and Inodorus melon market types, especially standard commercial Spanish Group Inodorus market types (e.g., Piel de Sapo, Rochet, and Canari).

Keywords *Cucumis melo* · Exotic germplasm · Genetic distance · Market class · Molecular markers · SSRs

Introduction

Melon (*Cucumis melo* L., 2n = 2x = 24) is a horticultural crop species in the Cucurbitaceae family that has worldwide economic importance (FAO 2008). Several morphologically distinct wild (subsp. *agrestis*) and cultivated (subsp. *melo*) types exist having diverse geographical origins. Cultivated melon market types have been variously classified (taxonomically and horticulturally) based primarily on fruit morphology. In one popular classification, the original melon groupings of Naudin (1859) were subdivided by Munger and Robinson (1991) into seven horticultural groups including: (1) *C. melo* var. *agrestis* Naud. (wild melon); (2) *C. melo* var. *flexuosus* Naud. (snake melon); (3) *C. melo* var. *conomon* Mak. (pickling melon, Chinese white cucumber); (4) *C. melo* var. *cantalupensis* Naud. (cantaloupe or muskmelon); (5) *C. melo* var. *inodorus* Naud. (winter melons, honeydew, Casaba); (6) *C. melo* var. *chito* (mango melon) and var. *dudaim* Naud. (Queen's pocket melon), and; (7) *C. melo* var. *momordica* (Phoot or snap melon). More recently, Pitrat et al. (2000) proposed a synthesis of the infraspecific classification of melons based on the identification of the different synonymous epithets used in the literature. They identified 16 groups, where five were assigned to subsp. *agrestis* Jeffrey and 11 to the subsp. *melo* Jeffrey. Those groups were denominated *varietas* or variety following the International Code of Botanical Nomenclature (ICBN) rules for the lower-ranking taxa (Greuter et al. 2000; Pitrat et al. 2000). Subspecies *agrestis* include, among others, the varieties *conomon* Thunberg and *momordica* Roxburgh, and subspecies *melo* Naudin include the varieties *cantalupensis* Naud., *inodorus* Jacquin, *flexuosus* L., *dudaim* L., and *chito* Morren (Jeffrey 1980; Naudin 1859; Pitrat et al. 2000). In a monograph of *Cucumis*, Kirkbride (1993) recognized *C. melo* subspecies (supporting Jeffrey 1980), but indicated that "horticultural types" be treated under the rules of cultivated plant nomenclature. Although these associated informal *C. melo* taxonomic groups were more formalized by Jeffrey (2001), a recent review of genetic diversity of horticultural groupings was given by Burger et al. (2010) indicates the myriad of horticultural types recognized by Kirkbride (1993) and Pitrat et al. (2000). A single taxonomic classification of melon horticultural types has, however, not been universally accepted. Regardless of the infraspecific classification of melons (Pitrat et al. 2000), this can be made in accordance with cultivated plant nomenclature (Brickell et al. 2009). Herein we follow the subspecies classification of *C. melo* ssp. *melo* and *agrestis* as summarized in Burger et al. (2010) which supports that of Jeffrey (1980) and Pitrat et al. (2000), where horticultural group names Cantalupensis, Inodorus, and Conomon are typified by such representatives as Charentais, Tendral Verde Tardio, and Freeman's cucumber, respectively. These horticultural groupings are in accordance with and defined by previous genetic diversity analyses in melon (Mliki et al. 2001; Staub et al. 2000, López-Sesé et al. 2002, 2003).

Group Cantalupensis and Inodorus market types are of special interest in the USA, as well as in many European, Mediterranean, and Asiatic countries because of their unique culinary attributes (e.g., sweet taste). The fruit morphology within these groups is diverse, and depends on geographic origin (i.e., adaptation to environmental factors), cultural traditions, culinary attributes, and market characteristics and requirements (Staub et al. 2000). Commercial breeders and traditional farmers consider these factors during germplasm enhancement, which has led to a diverse array of market classes within a horticultural group [e.g., Charentais, Galia, Shipping, Ananas, and Ogen are Group Cantalupensis market types, and Honeydew and Casaba (White, Yellow, Tendral, Green, Piel de Sapo, and Rochet) are Group Inodorus market types (Nuez et al. 1994)].

Europe is characterized by extraordinary geographic, agricultural, and cultural diversity. Spain is a secondary centre for melon diversity (Esquinas-Alcázar 1977; McCreight et al. 1993), where commercial Group Cantalupensis and Inodorus melon

production is ~1.142 mil tons/year, which constitutes about 4% of the world's production (FAO 2008). Group Flexuosus market types are also cultivated for sale in local markets in eastern Spain. Group Inodorus market types differ in melon shape, skin color, appearance, and flesh color (Gómez-Guillamón et al. 1985; Nuez et al. 1986). The genetic base of these market classes has been continuously enriched by the introgression of traits from traditional landraces, which has resulted from long-term selection by farmers for specific culinary traditions, sensorial richness, and ease of preservation (Esquinas-Alcázar 1977).

Initially, morphological assessment initially led to the taxonomic and horticultural classification of Spanish melon germplasm (Gómez-Guillamón et al. 1985, 1998; Molina et al. 1986; Nuez et al. 1986, 1988, 1994). Isozyme analyses (Esquinas-Alcázar 1977; Staub et al. 1997; Akashi et al. 2002) have also been used to estimate genetic diversity in various melon market types, and García et al. (1998) used random amplified polymorphic DNA markers (RAPDS) to differentiate Spanish elite melon germplasm. More recently, López-Sesé et al. (2003) used RAPDs to assess the regional diversity of Spanish cultivars and developed a Standard Reference Germplasm Array (RA) that circumscribed the genetic diversity of Spanish melons.

About 1% of the Spanish melon production is harvested in the Madrid provenance, where it is the most economically important crop species (MARM 2007). This provenance has had a rich agricultural history due to its historically strategic geographic and political location, where commodities have been traded nationally for hundreds of years (Labajos and Laca 2007).

Since the 19th century Villaconejos, a village near Madrid (~50 km south), has produced a diverse array of landraces melons possessing unique culinary attributes (Ruiz and Escalona 2002; Escribano and Lázaro 2009). Since the genetic relationships among of these landraces and between other Group Inodorus and Flexuous Spanish melon market classes are not known, a study was designed to assess their genetic constitutions using simple sequence repeat (SSR) markers, and then compare this variation to that observed in the RA (Group Inodorus, Flexuosus, and Cantalupensis) previously published by López-Sesé et al. (2003). This evaluation will determine the potential usefulness of Villaconejos germplasm for melon breeding.

Materials and methods

Germplasm

Thirteen morphologically different Villaconejos landraces and 18 RA accessions [USA (3), Spain (15)] (Table 1; Staub et al. 2000; López-Sesé et al. 2003) were genotyped using 483 SSR markers. This phenotypically diverse RA was defined based on its representation of major Spanish market classes (López-Sesé et al. 2003; Table 2). The USA Group Inodorus and Cantalupensis accessions were chosen based on their previously described relationships with other major Group Inodorus, Flexuosus, and Cantalupensis germplasm (Staub et al. 2000, 2004; Mliki et al. 2001) and designated USA RA accessions. Seeds of USA accessions were obtained from the U.S. Department of Agriculture, Agriculture Research Service (USDA, ARS) melon breeding program, in Madison, Wisc., and Spanish melon accessions were received from the Madrilean Research Institute for Rural Development, Agriculture and Food (IMIDRA; Villaconejos landraces) and the Experimental Station 'La Mayora' of the Spanish Council for Scientific Research (CSIC), in Málaga, Spain (RA accessions). Spanish accessions were originally selected and maintained by local farmers for many generations.

DNA extraction

Twenty seeds of each accession were germinated in a greenhouse at the University of Wisconsin, Madison, and DNA was extracted according to Staub et al. (1996). Individual DNA was quantified and diluted to 25 ng/μl. Subsequently, DNA from 15 plants was bulked such that each bulk contained five individuals whose final DNA concentration was adjusted to 5 ng/μl.

SSR amplification and selection

Initially, an array of 15 SSRs previously employed for genetic assessment of Spanish melon landraces [designated Standard Marker Array (SMR) by Staub et al. 2000; López-Sesé et al. 2002] was used to genotype 26 samples (two individuals of each of 13 Villaconejos accessions) in order to determine their potential discriminatory power. This initial marker screening indicated that a broader screening would be required to identify polymorphic markers needed for

Table 1 Thirty-one melon (*Cucumis melo* L.) varieties, including a subset of previously defined reference array accessions (RA) and Villaconejos's landraces

Origin	Region[a]	ID[b]	Registration number[c]	Seed source[d]	Accession name[e]	Taxon[f]	Market class[g]
USA	Western	TM	46	USDA	Top Mark	C	U.S. western market (RA)[h]
	Eastern	WI-998		USDA	WI-998	C	Muskmelon (RA)[i]
	–	HD	45	HM	Green Flesh Honeydew	I	Honeydew (RA)
Spain	1	C2	2	CSIC	Negro	I	B (RA)
	1	C8	8	CSIC	Negro	I	B (RA)
	1	C31	31	CSIC	ANC-46. Piel de Sapo	I	PS (RA)
	1	C98	98	CSIC	CA-101084-1-C. Amarillo	I	Y (RA)
	1	C100	100	CSIC	CA-111084-3-C	I	G (RA)
	1	C116	116	CSIC	Severiano de Jata	I	R (RA)
	2	C190	190	CSIC	Mochuelo	I	G (RA)
	1	C225	225	CSIC	CA-1311-1-C	I	G (RA)
	1	C250	250	CSIC	ANC-29	I	Y (RA)
	3	C268	268	CSIC	CM-C-1	I	W (RA)
	4	C358	358	CSIC	CC-22	I	R (RA)
	5	C403	403	CSIC	EC-19	I	PS (RA)
	5	C413	413	CSIC	EC-16	I	Y (RA)
	6	C444	444	CSIC	VC-51	F	W (RA)
	7	C647	647	CSIC	Piel de Sapo	I	PS (RA)
	8	LNE	1	IMIDRA	Largo Negro Escrito	I	B
	8	M	2	IMIDRA	Mochuelo	I	R
	8	T	3	IMIDRA	Tempranillo	I	Y
	8	P	4	IMIDRA	Puchero	I	B
	8	AV	5	IMIDRA	Amarillo de Villaconejos	I	Y
	8	PN	6	IMIDRA	Pata Negra	I	PS
	8	F	7	IMIDRA	Felipe	I	R
	8	A	8	IMIDRA	Alfonso	I	PS
	8	R	9	IMIDRA	Reyes	I	PS
	8	L	69	IMIDRA	Largo	I	B
	8	MV	70	IMIDRA	Melón de Villaconejos	I	R
	8	MT	125	IMIDRA	Mochuelo Tradicional	I	R
	8	PST	126	IMIDRA	Piel de Sapo Tradicional	I	PS

[a] Geographic origin in Spain (region), where 1 = Andalucía; 2 = Madrid; 3 = Castilla La Mancha; 4 = Cataluña; 5 = Extremadura; 6 = Valencia; 7 = Aragón; 8 = Villaconejos

[b] Identification used in this study by accession number or accession name abbreviation

[c] Number given by the Melon Germplasm Bank of origin

[d] *USDA* United States Department of Agriculture, Agricultural Research Service, Salinas, Calif., USA; *HM* Harris Moran Seed, Modesto, Calif., USA; *CSIC* Agricultural Science Section of the Spanish Council for Scientific Research, Experimental Station la Mayora, Spain; *IMIDRA* Madrilean Research Institute for Rural Development, Agriculture and Food, Comunidad de Madrid, Spain

[e] Accession name given by the company, breeder or local farmer

[f] Taxonomic Group where: *C* Cantalupensis; *I* Inodorus; *F* Flexuosus

[g] *B* Black (Negro); *PS* Piel de Sapo; *Y* Yellow (Amarillo); *G* Green (Verde); *W* White (Blanco); *R* Rochet; *RA* Reference accession. All the Spanish accessions were Inodorus type, except C-444

[h] RA as defined in Staub et al. 2000 and López-Sesé et al. 2003

[i] Owens et al. 1980; Peterson et al. 1983

Table 2 Fruit characteristics of melon (*Cucumis melo* L.) groups separated into taxonomic and horticultural groupings

Taxonomic group[a]	Market class	Fruit shape	Fruit weight (Kg)	Fruit skin color	Fruit flesh color	Fruit warty skin[b]	Fruit spotted skin[b]	Fruit netted skin[b]
Cantalupensis	U.S. western market	Globular	1.0–2.2	Tan	Orange	A	A	P
Cantalupensis	Muskmelon	Globular	1.2–2.0	Dark yellow	Orange	P	A	P
Inodorus	Honeydew	Globular	1.1–2.2	Ivory	Green	A	A	A
Inodorus	Casaba (Black)	Elongate	1.0–3.5	Very dark green (Black)	White	P	A	P
Inodorus	Casaba (Piel de Sapo)	Elliptical	1.0–4.5	Green	Yellow	P	P	D
Inodorus	Casaba (Rochet)	Globular	1.0–5.0	Light green	White	A	P	P
Inodorus	Casaba (Green)	Oblong	1.5–3.5	Light green	White	P	A	P
Inodorus	Casaba (Yellow/Canari)	Ovate	1.0–3.0	Yellow	White	D	A	A
Inodorus	Casaba (White)	Oblong	1.0–3.0	White	Green	A	A	A
Flexuosus	White	Very elongate	–	White	Green	P	P	A

[a] According to Staub et al. (1997), Escribano and Lázaro (2009) and López-Sesé (unpublished data, 2009)

[b] *A* Absent; *P* Present, and; *D* Depending on the accession

genotype analysis, and, thus, 462 previously published cucurbit [squash (*Cucurbita* ssp.), melon and cucumber (*C. sativus* L.) single sequence repeat markers (SSRs) (19, Danin-Poleg et al. 2000, 2001; 3, Fazio et al. 2002; 31, Chiba et al. 2003; 143, Ritchel et al. 2004; 84, Gonzalo et al. 2005; 182, Fukino et al. 2007) and 21 EST-SSR markers (Kong et al. 2006, 2007)] were examined. Amplification reactions were performed according to Cuevas et al. (2008) using a range of primer annealing temperatures (50°C–65°C). The polymerase chain reaction (PCR) products were electrophoresed using 4% (w/v) Metaphor agarose (Lonza, NJ), stained with ethidium bromide, and then photographed using GelExpert Software (Nucleo-Tech Corporation, 1996, San Mateo, Calif.).

Primers that amplified bright, unique, and polymorphic PCR products were selected for a second series of evaluations using two sets of repeated DNA amplifications of Villaconejos samples (three bulks), Spanish RA (two bulks), and USA RA (single bulk) accessions. These amplifications identified consistent and non-redundant banding patterns, where band sizes were defined in reference to a standard 100 bp DNA ladder. Those SSR primers that were highly polymorphic were then reexamined (employing at least six plants/accession) using capillary electrophoresis to identify allelic variation (band size) using a Liz 500 size standard latter (Applied Biosystems, UK) and GeneScan Analysis Software Version 3.1 (Perkin-Elmer Hispania, S.A., Madrid, Spain).

Analytical procedures

Three kinds of genetic comparisons were made: (1) Contrasts among all accessions examined; (2) Evaluation of Villaconejos and Spanish RA accession differences, and then; (3) Assessment of fruit type variation between commonly cultivated Spanish melons and Villaconejos forms as evaluated by subgroup comparisons based on geographical origin and fruit type.

Bands were scored as present (1) or absent (0), and then data were transformed into a binary matrix for analysis. A data matrix of putative allele sizes was constructed according to their migration distances after capillary electrophoresis. The binary matrix was then used in principal component analysis (PCA) by employing NTSYS-PC software (Version 2.0; Rohlf 1997). This analysis identified the most informative SSR primers and provided a three-dimensional graphic for visual inspection of genetic relationships.

Genetic distances (GD) among Spanish accessions and between these and the RA accessions were calculated using Jaccard's similarity coefficient (Jaccard 1908). This genetic similarity estimator was used based on its utility in previous melon diversity analyses (Staub et al. 2000) and its concordance with other distance estimators (García et al. 1998; Mliki et al. 2001). Genetic similarity estimates were calculated as the complement of each coefficient ($1-J_{ij}$) as described by Spooner et al. (1996).

Additionally, Nei's (1973) genetic distance coefficients were estimated for comparative analyses with distance estimates obtained by López-Sesé et al. (2002).

The unweighted pair-group method using an arithmetic average cluster analysis (UPGMA) contained in NTSYS-PC (Version 2.0; Rohlf 1997) was employed to provide a graphic visualization of inter- and intra-accession relationships. Data were also subjected to 1,000 re-sampling bootstrap analysis to define cluster pattern reliability.

The population structure of Villaconejos germplasm was investigated using POPGENE Version 1.31 software, which estimates the observed number of alleles per locus, unique alleles, degree of heterogeneity using Shannon's Information Index (SI) (Shannon and Weaver 1949), and percentage of polymorphic loci (Lewontin 1974; Yeh et al. 1997). Although the assessment of diploid data and associated co-dominant markers typically allows for the identification of two alleles per locus, bulk sample analysis occasionally resulted in the detection of more than two alleles at some loci. In these cases, the two most distinct and frequent banding morphotypes were selected for inclusion in the data matrix.

Results

Microsatellite variability

Ninety-two of the 483 (21 + 462) primers (19 %) examined were polymorphic in the initial survey of 26 samples (13 accessions, 2 individuals per accession). A subsequent inspection of a larger array of melon germplasm (i.e., the 31 accessions examined) identified 52 primers (11 %) that were reproducible and provided additional discriminating power (Table 3). In fact, 185 putative polymorphic alleles (86–515 bp) were detected by this refined group of primers (i.e., 2–8 alleles/locus, where 82.7% > 3 alleles). There was an average of 3.4 alleles per locus, where an average of 2.6 alleles per locus were uniquely specific to Villaconejos landraces.

Nine SSRs (CMN21_41, CMN61_44, CMN62_08, CMBR002, CMBR026, CMBR33, CMBR132, CMTCN44, and TJ4), which were primarily di-, tri-, and tetra-nucleotide repeats, were particularly discriminating (i.e., unique or high number of alleles per locus), and, thus, were evaluated in subsequent analyses by capillary electrophoresis (Table 3).

PCA analysis

PCA ordination explained 34.6% of the variability among accessions, which was determined principally by 24 primers (i.e., 46.1 % of 52 primers used) (Fig. 1, Table 4). The accessions examined were partitioned into four distinct groups: PCA Group 1 [Group Flexuosus, accession 'C444'], PCA Group 2 [Group Cantalupensis accessions 'Top Mark' and 'WI-998', and Inodorus accession 'Green Flesh Honeydew'], PCA Group 3 [Villaconejos black melon epidermis fruit types (i.e., landrace cultivars 'Largo', 'Largo Negro Escrito', and 'Puchero'; hereafter referred to as Black melon)] and PCA Group 4 [24 accessions including Piel de Sapo, Yellow, Green, White and Rochet market types (i.e., consisting of Spanish RA accessions and several Villaconejos landraces)].

Accessions in Group 4 possessed the greatest heterogeneity (SI = 0.57, where 100% of the loci examined were polymorphic) when compared to the other groups examined as defined by PCA (Table 4). Moreover, the type (i.e., three unique putative alleles) and level of polymorphism (heterozygosity) detected in Group 3 accessions, which includes three Villaconejos melons, was also remarkably high (SI = 0.34). Likewise, Group Flexuosus and Cantalupensis accessions in PCA Groups 1 and 2 possessed considerable heterogeneity (i.e., 9 and 16 unique putative alleles, respectively), which resulted in a lack of genetic affinity among the Group Inodorus accessions examined.

Genetic distances among accessions

Genetic distances among the accessions examined ranged from 0.14 (most related = landraces 'Reyes' and 'Piel de Sapo Tradicional') to 0.83 (most unrelated = 'Top Mark' and landrace 'C116') (Table 5). The average GD ± standard deviation between USA and Spanish varieties was 0.74 ± 0.05, ranging from 0.62 to 0.83. The average GD between Spanish RA accessions and Villaconejos accessions taken collectively was 0.57 ± 0.14, ranging from 0.26 to 0.81.

The average GD among Villaconejos landraces was 0.55 ± 0.17, ranging from 0.14 to 0.81 (i.e.,

Table 3 Simple sequence repeat markers selected to assess the genetic variability among melon (*Cucumis melo* L.) genotypes

SSR Marker[a]	Allele number	Allele sizes (bp)	Linkage group[b]
CM17	2	128, 130	I
CM33	2	118, 120	I
CM49	2	238, 240	I
TJ27	4	170, 172, 174, 176	I
CMMS4_3	4	180, 190, 192, 194	I
CMN01_15	4	196, 198, 200, 220	II
CMBR041	4	163, 165, 168, 170	II
CMBR083	2	140, 144	II
CMBR095	3	110, 118, 120	II
CMBR026	6	104, 110, 112, 114, 116, 122	III
CMBR044	3	102, 106, 108	III
CMBR105	4	140, 146, 150, 152	III
CMN08_50	3	250, 254, 258	IV
CMN21_06	4	190, 202, 210, 214	IV
CMN23_48	3	188, 190, 194	IV
CMNB_10	3	236, 240, 242	IV
CMTCN44	4	156, 170, 172, 174	IV
CM15	3	232, 236, 240	IV
CMBR116	3	196, 200, 202	IV
CM16	3	190, 194, 196	V
CMTAA166	4	174, 176, 186, 188	V
CMMS2_3	6	190, 198, 200, 210, 212, 216	V
CMBR107	3	172, 176, 178	V
CMBR002	2	113, 118	VI
CMN21_41	4	274, 278, 282, 284	VII
CM05	3	192, 194, 198	VII
CM26	4	150, 152, 154, 158	VII
TJ4	4	118, 121, 124, 136	VII
CMBR022	2	166, 170	VIII
CMBR025	4	160, 164, 170, 172	VIII
CMBR079	4	86, 94, 100, 102	VIII
CMBR109	4	142, 144, 160, 162	VIII
CMN04_09	3	292, 296, 300	X
CMN22_05	3	290, 292, 296	X
CM38	4	136, 140, 142, 144	X
CMGAN12	3	162, 170, 176	XI
CMATN89	3	136, 164, 168	XI
CMBR132	5	166, 168, 169, 175, 178	XI
CMN61_44	7	184, 194, 229, 232, 241, 244, 268	XII
CMN62_08	8	444, 445, 453, 488, 491, 498, 501, 515	XII
CMBR040	2	160, 164	XII
CMBR051	3	168, 170, 175	XII
CMBR014	3	140, 160, 162	XII
CMBR077	4	170, 172, 176, 178	XII
CMBR097	3	176, 178, 180	XII
CMN05_82	4	190, 210, 212, 218	–
CMN23_42	2	136, 138	–
CM46	3	148, 152, 154	–
CM53	2	160, 162	–
CMTC51	3	160, 162, 164	–
CMBR033	6	144, 147, 150, 166, 168, 172	–
CMBR054	4	124, 126, 130, 132	–

[a] SSR markers from Danin-Poleg et al. (2000, 2001), Fazio et al. (2002), Chiba et al. (2003), Ritchel et al. (2004), Gonzalo et al. (2005), Kong et al. (2006, 2007) and Fukino et al. (2007)

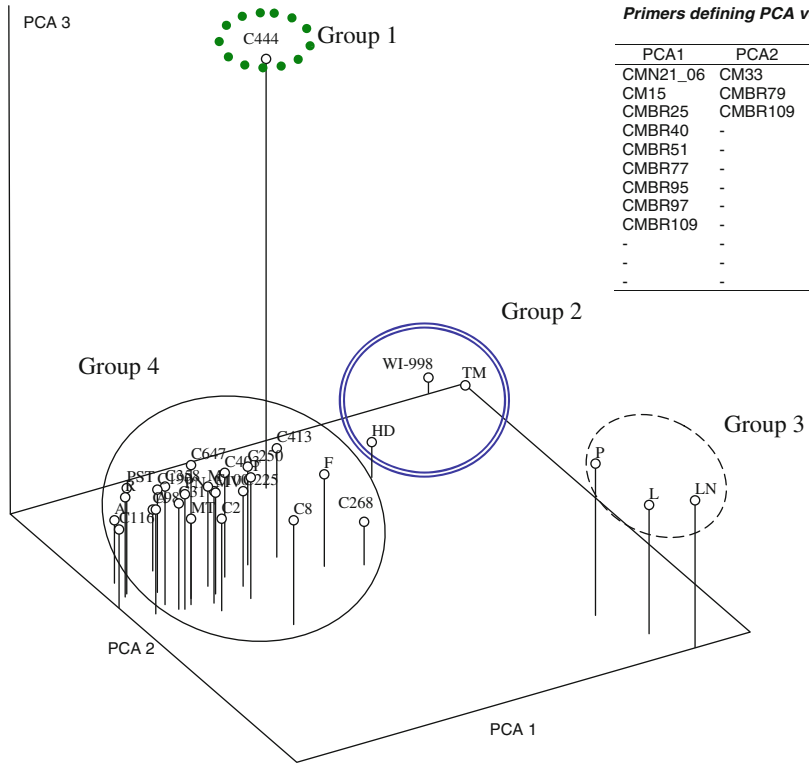
[b] Linkage group number according to Perin et al. (2002), Eduardo et al. (2007), Cuevas et al. (2008) and Fukino et al. (2008)

landrace 'Largo Negro Escrito' and 'Amarillo de Villaconejos' accessions) (Table 5). Three of these landraces ('Largo', 'Largo Negro Escrito', and 'Puchero') differed dramatically from the other Villaconejos landraces examined (i.e., average GD = 0.72 ± 0.05; range = from 0.63 to 0.81).

The mean GD among the Spanish RA accessions was 0.56 ± 0.09, where differences ranged from 0.30 (between 'C31' and 'C116') to 0.77 (between 'C268' and 'C444') (Table 5). Accessions 'C31' (Piel de Sapo market type) and 'C116' (Rochet market type) were the most similar accessions (GD = 0.30) within the RA. Accession 'C444' possessed the least genetic affinity with any PCA grouping [average Jaccard's GD = 0.68 ± 0.06 (Nei's GD = 0.51 ± 0.15); range among groups = Jaccard's GD 0.61 to 0.77 (Nei's GD 0.20 to 0.89)] (Fig. 1, Table 5).

Among the Group Inodorus market classes, Piel de Sapo and Rochet types possessed the greatest genetic affinity (average GD = 0.46 ± 0.10) (Table 6). In contrast, Black and Yellow fruit type melons were most distant (average GD = 0.68 ± 0.10).

The GD among accessions within a market class differed dramatically (e.g., average GD for Piel de Sapo = 0.36 ± 0.12, for Yellow = 0.55 ± 0.06, for



Primers defining PCA vectors

PCA1	PCA2	PCA3
CMN21_06	CM33	CMN01_15
CM15	CMBR79	CMN21_41
CMBR25	CMBR109	CM26
CMBR40	-	CM38
CMBR51	-	CM46
CMBR77	-	TJ4
CMBR95	-	CMTAA166
CMBR97	-	CMBR33
CMBR109	-	CMBR95
-	-	CMBR105
-	-	CMBR107
-	-	CMBR116

Fig. 1 Ordination of the 31 genotypes of melon (*Cucumis melo* L.) after principal component analysis (PCA; identification according Table 1)

Rochet $= 0.47 \pm 0.12$, for Green $= 0.53 \pm 0.07$, and for Black $= 0.59 \pm 0.18$) (Table 6). The comparatively high level of homogeneity (SI $= 0.291$) detected among Piel de Sapo accessions was due mainly to the presence of Piel de Sapo type Villaconejos accessions in that market class group (i.e., average GD among Piel de Sapo Villaconejos accessions $= 0.24 \pm 0.06$ vs. GD $= 0.49 \pm 0.11$ among Spanish RA Piel de Sapo accessions). In contrast, Villaconejos black epidermis accessions formed the most heterogeneous market class (SI $= 0.553$) of the accessions examined. This heterogeneity could be due to the genetic differences that exited between Black melons of the RA Spanish and Villaconejos accessions (average GD $= 0.7 \pm 0.3$) in the germplasm array examined.

Genetic clustering among accessions

Cluster analysis resulted in the partitioning of the accessions examined into two principal clades (Fig. 2). In the first clade, USA varieties ('Top Mark', 'WI-998', and 'Green Flesh Honeydew') were differentiated from all the other accessions in Node 1 (GD ≤ 0.76). Likewise, the Group Inodorus 'Green Flesh Honeydew' accession examined was differentiated from Group Cantalupensis 'Top Mark' and 'WI-998' in Node 2 (GD ≤ 0.53).

The second clade (Node 3; GD ≤ 0.72) was also partitioned into two nodes (Fig. 2). One clade contained the Black market type landraces 'Largo', 'Largo Negro Escrito', and 'Puchero', and Node 4 separated the accesion Black accession 'Puchero' from all other Madrilean Black melons (GD ≤ 0.55).

The second primary clade contained all the other Group Inodorus accessions and the single Flexuosus accession examined (Fig. 2). The Group Flexuous accession was genetically different from the Inodorus accessions (Node 5; GD ≤ 0.67). The Spanish Group Inodorus accessions (24) in this clade constituted of a large cluster grouping (Nodes 6–13), which were differentiated by similarity coefficients greater than

Table 4 Observed genetic population parameters of melon (*Cucumis melo* L.) groupings (G1, G2, G3, and G4) after PCA analysis

PCA grouping[a]	Average number of alleles observed per locus	Percentage of polymorphic loci	Unique alleles[b]	Shannon′s information index[c]
G1	1.05	5.77	9	0.0375
G2	1.57	44.23	16	0.3404
G3	1.61	50.0	3	0.3435
G4	2.82	100.0	35	0.5734

[a] According to Fig. 1, where G1 contains C444; G2 TM, WI-998 and HD; G3, L, LNE and P; G4, C2, C8, C31, C98, C100, C116, C190, C225, C250, C268, C358, C403, C413, C647, M, T, AV, PN, F, A, R, MV, MT and PST

[b] Number of alleles that were observed only in a sub-clustering group, where accession identification is according Table 1

[c] Shannon and Weaver (1949)

0.53. The only Group Inodorus white epidermis accession was distinguished at Node 6 (GD $\leq$ 0.72). Likewise, the only Rochet Villaconejos market type melon 'Felipe' was differentiated from all other Rochet types at Node 7 (GD $\leq$ 0.59). Node 8 (GD $\leq$ 0.56) separated the branch with two accessions, where RA Piel de Sapo 'C403' and yellow epidermis 'C413' accessions were uniquely defined (Node 8; GD $\leq$ 0.56), and which were themselves genetically different (Node 9; GD $\leq$ 0.52). The yellow epidermis types Villaconejos accession 'Tempranillo' and the RA 'C250' possessed considerable genetic affinities (GD $\leq$ 0.45). Likewise, the green epidermis RA type 'C225' was genetically defined at Node 11 (GD $\leq$ 0.53). While RA black epidermis melon accessions 'C2' and 'C8' possessed some genetic affinities (Node 12; GD $\leq$ 0.51), Piel de Sapo melon types were dissimilar from Villaconejos Rochet forms and two yellow and green RA epidermal types (GD $\leq$ 0.46), which were themselves similar (GD $\leq$ 0.47; Node 13).

The most homogeneous landraces were RA accessions 'C8' (black epidermis), 'C403' (Piel de Sapo), and 'C413' (yellow epidermis) (GD = 0.03 to 0.02; SI = 0.03), while Villaconejos 'Pata Negra' (Piel de Sapo) was the most heterogeneous (SI = 0.30). Although the level of intra-accession polymorphism among the accessions examined was relatively low (average polymorphism $\sim$12%), some accessions demonstrated comparatively high genetic variability (e.g., 4–21% in Villaconejos landraces, data not shown). In fact, given their relative lack of genetic affinities, the genetic variations among the Spanish landraces examined must be considered to be broad (i.e., GD among accession bulks = 0.03 to 0.17).

Genetic diversity among populations

Assessment of genetic diversity between Villaconejos accessions and those cultivated in other Spanish growing regions (RA) was accomplished by geographical origin and fruit type subgroup comparisons. Villaconejos landraces were initially compared to RA and then the five market class-based subgroups were compared among themselves (i.e., Piel de Sapo, Rochet, Black, Green, and Yellow types).

Villaconejos accessions versus Spanish reference accessions

The average GD between Villaconejos and Spanish Group Inodorus RA subgroups was 0.57 $\pm$ 0.14. Given their common SI of 0.59 and average of 2.6 alleles per locus, these subgroups must be considered to possess similar levels of heterogeneity. In fact, the polymorphism level and number of unique alleles (i.e., not present in the other subgroup) associated with Villaconejos and Spanish Inodorus RA was 99% and 10, and 96% and 18, respectively.

Market class diversity

A genetic assessment of market class types revealed that Black melon accessions possessed the greatest heterogeneity (average of 2.1 alleles per locus, 76.9 % polymorphic loci, SI = 0.55) (Table 7). In contrast, the Piel de Sapo accessions examined possessed the lowest heterogeneity (average of 1.7 alelles per locus, 55.7% polymorphic loci; SI = 0.29). Rochet, and green and yellow epidermis melon accessions possessed a common average of 2.0 alleles per locus.

Table 5 Pairwise genetic distance values (Jaccard′s coefficient) between melon (*Cucumis melo* L.) genotypes using SSR markers (accession identification according to Table 1)

	TM	WI-998	HD	PN	F	A	R	L	MV	MT	PST	LNE	M	T	P	AV	C2	C8	C31	C98	C100	C116	C190	C225	C250	C268	C358	C403	C413	C444	C647
TM	0																														
WI-998	0.36	0																													
HD	0.58	0.52	0																												
PN	0.78	0.71	0.66	0																											
F	0.72	0.70	0.64	0.57	0																										
A	0.82	0.76	0.66	0.26	0.54	0																									
R	0.81	0.74	0.64	0.33	0.57	0.22	0																								
L	0.76	0.74	0.73	0.63	0.66	0.69	0.74	0																							
MV	0.77	0.73	0.76	0.39	0.57	0.46	0.52	0.66	0																						
MT	0.75	0.75	0.72	0.32	0.61	0.39	0.49	0.69	0.30	0																					
PST	0.79	0.71	0.65	0.24	0.54	0.27	0.14	0.73	0.51	0.44	0																				
LNE	0.79	0.78	0.73	0.66	0.70	0.73	0.76	0.18	0.70	0.71	0.75	0																			
M	0.74	0.73	0.68	0.40	0.55	0.45	0.51	0.67	0.32	0.26	0.47	0.72	0																		
T	0.81	0.77	0.68	0.49	0.60	0.46	0.49	0.67	0.53	0.52	0.48	0.68	0.51	0																	
P	0.81	0.81	0.74	0.70	0.67	0.70	0.76	0.59	0.79	0.77	0.74	0.49	0.76	0.68	0																
AV	0.79	0.78	0.65	0.45	0.61	0.37	0.36	0.79	0.55	0.54	0.39	0.81	0.53	0.52	0.81	0															
C2	0.80	0.77	0.72	0.49	0.58	0.36	0.43	0.69	0.53	0.50	0.46	0.73	0.53	0.54	0.73	0.50	0														
C8	0.82	0.80	0.76	0.51	0.68	0.43	0.55	0.65	0.56	0.51	0.55	0.69	0.60	0.59	0.70	0.59	0.40	0													
C31	0.80	0.76	0.66	0.34	0.60	0.31	0.30	0.67	0.53	0.50	0.29	0.70	0.47	0.50	0.74	0.34	0.48	0.53	0												
C98	0.77	0.71	0.68	0.54	0.62	0.47	0.51	0.79	0.52	0.47	0.51	0.80	0.49	0.53	0.81	0.49	0.53	0.62	0.51	0											
C100	0.79	0.74	0.71	0.44	0.59	0.50	0.53	0.68	0.49	0.46	0.50	0.70	0.49	0.56	0.71	0.52	0.48	0.55	0.46	0.39	0										
C116	0.83	0.78	0.65	0.35	0.65	0.27	0.26	0.77	0.50	0.43	0.28	0.78	0.49	0.57	0.77	0.31	0.40	0.51	0.30	0.47	0.49	0									
C190	0.75	0.71	0.66	0.36	0.51	0.41	0.38	0.69	0.49	0.45	0.35	0.72	0.42	0.56	0.78	0.49	0.49	0.60	0.39	0.46	0.45	0.42	0								
C225	0.78	0.75	0.65	0.47	0.56	0.50	0.51	0.70	0.53	0.50	0.52	0.73	0.57	0.54	0.74	0.54	0.59	0.51	0.58	0.57	0.57	0.55	0.56	0							
C250	0.75	0.75	0.69	0.53	0.59	0.51	0.57	0.76	0.54	0.51	0.57	0.78	0.46	0.45	0.70	0.55	0.63	0.66	0.61	0.55	0.65	0.62	0.59	0.57	0						
C268	0.72	0.74	0.70	0.63	0.61	0.61	0.69	0.69	0.68	0.64	0.67	0.74	0.64	0.65	0.73	0.66	0.61	0.61	0.66	0.68	0.67	0.68	0.64	0.59	0.68	0					
C358	0.80	0.74	0.65	0.30	0.58	0.35	0.36	0.67	0.47	0.48	0.30	0.70	0.47	0.52	0.75	0.46	0.52	0.59	0.32	0.55	0.44	0.36	0.33	0.54	0.60	0.65	0				
C403	0.79	0.75	0.73	0.50	0.59	0.49	0.50	0.77	0.55	0.57	0.51	0.79	0.56	0.59	0.78	0.60	0.61	0.56	0.55	0.58	0.63	0.60	0.49	0.57	0.56	0.60	0.60	0			
C413	0.71	0.66	0.62	0.51	0.65	0.54	0.55	0.67	0.60	0.56	0.54	0.73	0.61	0.52	0.80	0.61	0.58	0.49	0.56	0.64	0.54	0.59	0.52	0.56	0.65	0.61	0.54	0.53	0		
C444	0.77	0.73	0.74	0.61	0.69	0.64	0.65	0.79	0.67	0.70	0.62	0.81	0.65	0.65	0.79	0.71	0.70	0.73	0.65	0.72	0.66	0.71	0.63	0.68	0.68	0.77	0.63	0.68	0.64	0	
C647	0.83	0.81	0.68	0.44	0.55	0.39	0.41	0.71	0.59	0.53	0.40	0.73	0.52	0.51	0.75	0.49	0.49	0.56	0.37	0.54	0.49	0.48	0.41	0.57	0.64	0.65	0.42	0.56	0.57	0.61	0

Table 6 Genetic distance (Jaccard′s Coefficient) among principal Spanish melon (*Cucumis melo* L.) market classes

Melon type[a]	Black	Piel de Sapo	Rochet	Green	Yellow
Black	*				
Piel de Sapo	0.63 ± 0.12	*			
Rochet	0.65 ± 0.10	0.46 ± 0.10	*		
Green	0.65 ± 0.09	0.48 ± 0.07	0.49 ± 0.06	*	
Yellow	0.68 ± 0.10	0.51 ± 0.07	0.53 ± 0.07	0.54 ± 0.05	*

[a] Melons market classes contain the following accessions: Black (C2, C8, LNE, P and L); Piel de Sapo (C31, C403, C647, PN, A, R and PST); Rochet (C116, C358, M, F, MV, and MT); Green type (C100, C190, and C225); Yellow (C98, C250, C413, T, and AV) according to Table 1

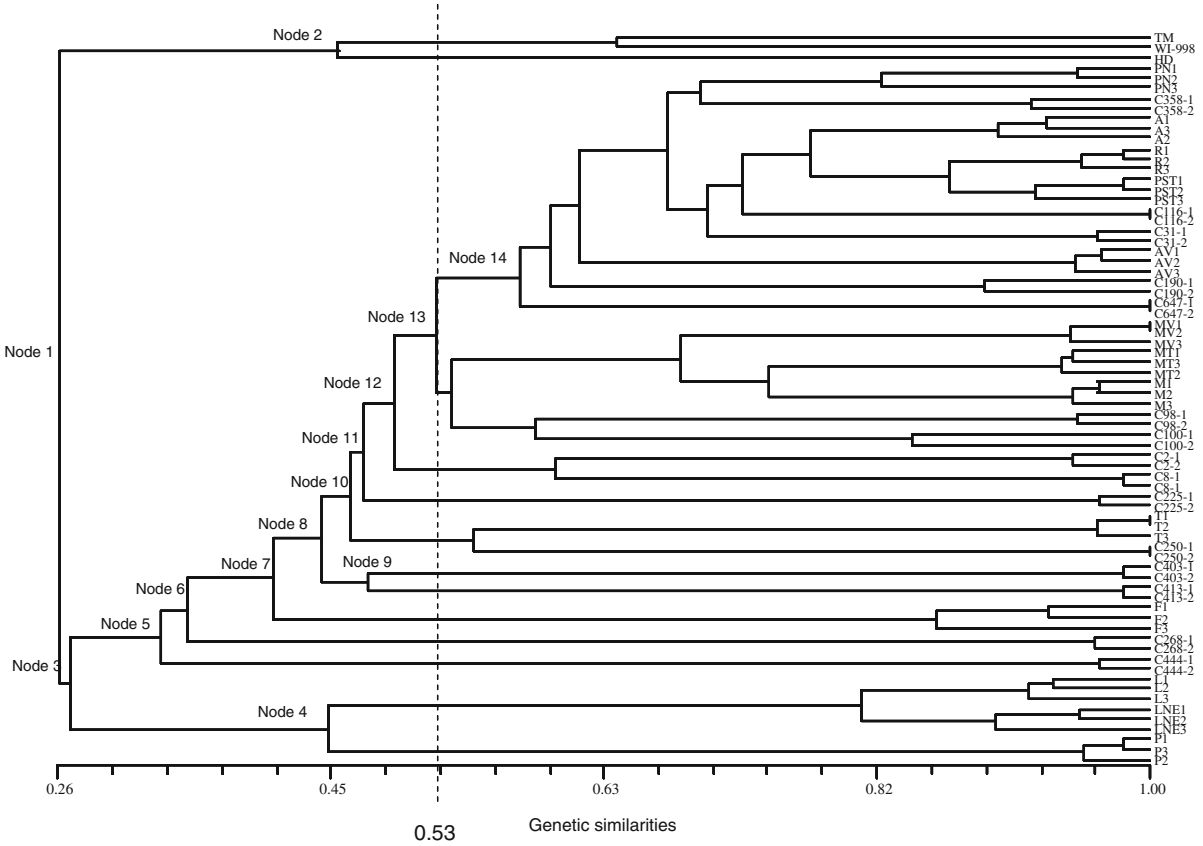


Fig. 2 Cluster analysis of 31 melon (*Cucumis melo* L.; Table 1) accessions as bulked samples by UPGMA using SSR-derived genetic similarities (Jaccard′s coefficient) as framing criteria

Discussion

The domestication of plants has "resulted from long periods of intimate coevolution between plants and man" (Harlan 1995). However, the continued displacement of locally adapted landraces by elite cultivars constitutes genetic erosion of primary gene pools and supports an *ex situ* conservation of genetic resources (Harlan 1971). The identification and assessment of unique landrace gene pools to define their structure in relation to locally used elite germplasm allows for the development of breeding strategies for genetic conservation (Laghetti et al. 2008; Cowling et al. 2009). In the case of Spanish

Table 7 Population parameters defining principal Spanish melon (*Cucumis melo* L.) market classes

Melon type[a]	Percentage of polymorphic loci	No. of unique alleles[b]	Shannon's information index[c]
Black	76.9	8	0.553
Piel de Sapo	55.8	1	0.291
Rochet	73.1	1	0.452
Green	69.2	4	0.455
Yellow	75.0	4	0.494

[a] Melons market classes contain the following accessions: Black (C2, C8, LNE, P and L); Piel de Sapo (C31, C403, C647, PN, A, R and PST); Rochet (C116, C358, M, F, MV, and MT); Green type (C100, C190, and C225); Yellow (C98, C250, C413, T, and AV) according to Table 1

[b] Number of alleles that were observed only in that market class

[c] Shannon and Weaver (1949)

melon, previously published broad marker-based assessments of the genetic structure of important landraces and elite germplasm provided the basis for comparative analysis conducted herein (Staub et al. 2000; López-Sesé et al. 2002). These analyses led to the characterization of unique melon landrace germplasm found in Villaconejos, Spain.

Single sequence repeat markers have been shown to be effective in detecting melon polymorphisms in melon (Staub et al. 2000; López-Sesé et al. 2002; Chiba et al. 2003; Monforte et al. 2003; Ritchel et al. 2004; Gonzalo et al. 2005; Fukino et al. 2007; Kong et al. 2006, 2007), where an informative unique germplasm array (Katzir et al. 1996; Danin-Poleg et al. 2001) has been constructed and used to discriminate a broad array of genotypes (Staub et al. 2000; López-Sesé et al. 2002; Nakata et al. 2005). In fact, the genetic structure of Villaconejos landraces and their relationship to elite germplasm examined herein was defined in terms of previously published broad-based intra-specific melon relationships because common markers (e.g., SSRs) were employed (García et al. 1998; Stepansky et al. 1999; Staub et al. 2000; Mliki et al. 2001; Monforte et al. 2003). Banding morphotypes were used to define the genetic diversity among 13 Group Inodorus landraces, where ~19% of the primers employed detected polymorphisms among the Villaconejos accessions examined. This set of primers was adequate for the discrimination of four Villaconejos Casaba market class melons grown in a relatively small geographic area (35.7 km^2). However, only one of these primers (CMTAA166) belonged to the original microsatellite SMR used by Staub et al. (2000), López-Sesé et al. (2002), and Nakata et al. (2005).

López-Sesé et al. (2002) determined that GD estimates of melon germplasm can differ depending upon the type and number of markers employed. The evaluation conducted herein used 11 of the 15 melon accessions employed by these authors. They defined the average GD (Nei's distance coefficient) among those accessions using 26 RAPDs as 0.42 ± 0.09, where the range among accessions was 0.14–0.72. Subsequently, an assessment of 125 Spanish landraces [including the 14 accessions examined by López-Sesé et al. (2002)] by López-Sesé et al. (2003) used 19 RAPDs to define the average GD between accessions examined as 0.31 ± 0.08, where GD between any two pairs of accessions ranged from 0.01 to 0.58. In contrast, estimates of the average and range of GD (Nei's coefficient) values among these accessions as assessed by the 52 SSR employed herein was 0.51 ± 0.15 and 0.20 to 0.89, respectively. Thus, these results support the assertion by Staub (1999), Staub et al. (2000) that GD estimation for use in plant variety protection in melon must be judiciously applied.

Variations of the bulking method defined by López-Sesé et al. (2002) have been successfully used for germplasm diversity appraisal in melon (Staub et al. 1997, 2000; Danin-Poleg et al. 2001; Mliki et al. 2001; López-Sesé et al. 2002; Monforte et al. 2003; this study). The use of bulk sampling increases the probability of identifying unique banding morphotypes (i.e., multiple alleles at a locus) and reduces laboratory costs. The bulking procedure employed by López-Sesé et al. (2002) was improved (i.e., greater reliability and accuracy) herein by extraction and dilution of each DNA sample followed by mixing to

obtain bulks consisting of mixed equal proportions of DNA from each plant sample.

The 52 SSR primers used herein supported the morphologically-based appraisal of Escribano and Lázaro (2009) of Villaconejos landraces as phenotypically and genotypically distinct from other Spanish Inodorus melons (García et al. 1998; Staub et al. 2000, 2004; López-Sesé et al. 2003; Monforte et al. 2003). Furthermore, they partitioned the melon accessions examined into Flexuosus, Inodorus, and Cantalupensis Groups and concomitant horticultural type classifications (i.e., market types; Fig. 1, Table 4). This analysis confirmed López-Sesé et al. (2003) assessment of Spanish melon germplasm as a unique gene pool and defined the distinctive genetic characteristics of Black Villaconejos landraces (i.e., 'Largo', 'Largo Negro Escrito' and 'Puchero'). Thus, these SSR markers will likely be useful for subsequent genetic analysis of Group Inodorus melons, especially those of Spanish origin.

Villaconejos landrace accessions were generally partitioned into independent groups differing from RA accessions after cluster analysis (Fig. 2). Genetic differences between the Villaconejos landraces and other accessions examined herein were defined by the presence of 10 alleles not founded in any Spanish RA melon accessions. For instance, Group Inodorous Piel de Sapo (*C. melo* L. var. *inodorus* Naud.; Pitrat et al. 2000) accessions grouped as one cluster, indicating their market class homogeneity, which themselves were located in sub-clusters distinct from Villaconejos accessions and RA. Although a majority of Villaconejos Rochet (*C. melo* L. var. *inodorus* Naud.) melons showed considerable genetic intra-market class homogeneity, they were clearly separated from the Spanish Rochet RA (GD = 0.47 ± 0.02). Thus, they should be considered a genetic pool unique within this market class, which will be decidedly appreciated by melon consumers (Escribano et al. 2010). The Group Inodorus Green (*C. melo* L. var. *inodorus* Naud.) and Yellow (*C. melo* L. var. *inodorus* Naud.) melon market types examined, however, were not genetically unique, suggesting the possibility of previous historic introgression with other Spanish market types.

García et al. (1998) and Staub et al. (2000) assessed the genetic diversity of a diverse array of elite Spanish melon germplasm (i.e., varied horticultural market classes) to define average GD as 0.33 ± 0.09 and 0.21 ± 0.04, respectively. In contrast, the average GD among the Casaba types examined herein was appreciably larger (GD = 0.56 ± 0.13; Table 5) than those elite germplasms. In fact, the SSR-based genetic diversity housed in Black (*C. melo* L. var. *inodorus* Naud.) Villaconejos melons (average GD from all other accessions = 0.73 ± 0.05) was considerably greater than Casaba melons of Villaconejos origin (GD = 0.55 ± 0.17) and Spanish RA accessions (GD was 0.54 ± 0.08). Likewise, Villaconejos Black melons possessed little genetic affinity other RA Black melon accessions ('C2' and 'C8'; GD = 0.7 ± 0.03). Such genotypic and phenotypic (Escribano and Lázaro 2009) differences expressed by Black and Rochet Villaconejos/Madrid melons are indicative of the historic horticultural/culinary specialization and seed preservation of the local melon farmers. Thus, the Villaconejos melons examined herein should be considered an important germplasm pool for use in plant improvement. This contention is supported by the unique alleles and relative general genetic richness detected within Villaconejos landraces in general (RA SI = 0.5991 vs. Villaconejos SI = 0.5974), and the fact that this germplasm pool has not been widely utilized by modern urban consumers (Escribano and Lázaro 2009).

The identification and characterization of the ancestral and potentially valuable Villaconejos melon germplasm pool suggests that collection and analysis of other under utilized Spanish landraces should be considered. One strategy for the design of future collection expeditions would be the study of ethnobotanical and agricultural records to optimize the recovery and preservation of genetically unique landraces. The retention of such germplasm would certainly diversify Spanish national germplasm collections and form the basis of core collections and test arrays for future genetic analysis.

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