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Research article

Development of microsatellite markers from an enriched genomic library for genetic analysis of melon (*Cucumis melo* L.)

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Abstract

Background: Despite the great advances in genomic technology observed in several crop species, the availability of molecular tools such as microsatellite markers has been limited in melon (*Cucumis melo* L.) and cucurbit species. The development of microsatellite markers will have a major impact on genetic analysis and breeding of melon, especially on the generation of marker saturated genetic maps and implementation of marker assisted breeding programs. Genomic microsatellite enriched libraries can be an efficient alternative for marker development in such species.

Results: Seven hundred clones containing microsatellite sequences from a *Tsp*-AG/TC microsatellite enriched library were identified and one-hundred and forty-four primer pairs designed and synthesized. When 67 microsatellite markers were tested on a panel of melon and other cucurbit accessions, 65 revealed DNA polymorphisms among the melon accessions. For some cucurbit species, such as *Cucumis sativus*, up to 50% of the melon microsatellite markers could be readily used for DNA polymophism assessment, representing a significant reduction of marker development costs. A random sample of 25 microsatellite markers was extracted from the new microsatellite marker set and characterized on 40 accessions of melon, generating an allelic frequency database for the species. The average expected heterozygosity was 0.52, varying from 0.45 to 0.70, indicating that a small set of selected markers should be sufficient to solve questions regarding genotype identity and variety protection. Genetic distances based on microsatellite polymorphism were congruent with data obtained from RAPD marker analysis. Mapping analysis was initiated with 55 newly developed markers and most primers showed segregation according to Mendelian expectations. Linkage analysis detected linkage between 56% of the markers, distributed in nine linkage groups.

Conclusions: Genomic library microsatellite enrichment is an efficient procedure for marker development in melon. One-hundred and forty-four new markers were developed from *Tsp*-AG/TC genomic library. This is the first reported attempt of successfully using enriched library for microsatellite marker development in the species. A sample of the microsatellite markers tested proved efficient for genetic analysis of melon, including genetic distance estimates and identity tests. Linkage analysis indicated that the markers developed are dispersed throughout the genome and should be very useful for genetic analysis of melon.

Background

The Cucurbitaceae family includes several species of cultivated plants of great economic importance, such as watermelon (*Citrullus lanatus* L.), squash (*Cucurbita maxima* L.), melon (*Cucumis melo* L.) and cucumber (*Cucumis sativus* L.). The morphological variation observed in germplasm accessions of melon and other cucurbit species is enormous. Gene flow between cultigenes is facilitated by the allogamous habit of the species [1]. Difficulties concerning intraspecific botanical classification in *C. melo*, based mainly on morphological characters, have been addressed in the last 40 years [2-4]. Cultivated melon morphotypes are usually classified in three major groups: *reticulatus*, *cantaloupensis* and *inodorus* [3,4]. More recently, the *reticulatus* group has been suggested to be part of the *cantaloupensis* group [1]. Wild type melons are classified in the *flexuosus*, *momordica*, *conomon*, *dudaim* and *chito* groups [4].

Melon varieties that belong to the *cantaloupensis* or *reticulatus* groups are the most cultivated in the United States, usually called "muskmelon" or "cantaloupe", producing aromatic fruits with a netted or ribbed coat and a sweet orange flesh. However, the yellow melons of "Valenciano" type are the most important in the Brazilian [5] and other markets. In European countries it is the second most commercialized type of melon [6]. Valenciano melons are classified in the *inodorus* group, showing non-smelling fruits with a smooth or wrinkled rind and white or green sweet flesh. Fresh melon production is important for internal market consumption in Brazil, but a significant percentage of the production is exported to other countries [7-9]. Melon is actually one of the most important agronomic products of Northeastern Brazil, where soil and climate conditions are suitable for commercial production. Fruit quality, especially flavor, is among the most valued commercial traits demanding further improvement [8]. The development of Valenciano melon varieties with improved fruit quality could be accelerated with the use of genomic technology. This would include, for example, molecular mapping of genomic regions associated with fruit quality control and, eventually, marker assisted selection.

Molecular markers have been widely used in genetic analysis and breeding of plant species, with a multitude of applications [10]. Among the various types of molecular markers available, microsatellites have received greater attention recently, especially for breeding purposes. Microsatellite markers, also known as simple sequence repeats or SSRs [11,12], are clusters of short (usually 2 to 6) tandemly repeated nucleotide bases distributed throughout the genome. Microsatellite markers distinguish themselves as co-dominant, multiallelic, highly polymorphic genetic markers, requiring small amounts of

DNA for straightforward PCR and gel electrophoresis analysis. Its main disadvantage is the high cost of the initial investment necessary for marker development [13]. The development of microsatellite markers can be based on DNA sequence information deposited in databases, or it could be based on the screening of genomic DNA libraries specifically constructed for the discovery of repeated sequences in the genome. The use of genomic libraries enriched for microsatellite sequences is a strategy devised to decrease the cost of marker development while increasing the opportunity for marker discovery [14].

The actual use of microsatellite markers in melon genetic analysis has been very limited. Only about 70 microsatellite markers have been described in the literature so far [15,16] and just a few used in linkage analysis. These markers were integrated in genetic maps built with morphological, isoenzimatic and other molecular markers such as RFLP (Restriction Fragment Length Polymorphisms), AFLP (Amplified Fragment Length Polymorphisms) and RAPD (Random Amplified Polymorphic DNA [16-28]. There is, therefore, great need to discover and develop more microsatellite markers for genetic and linkage analysis of melon.

The main objectives of this work were to develop new microsatellite markers for melon based on a genomic DNA library enriched for microsatellite sequences and test their usefulness: (a) in genetic linkage analysis; (b) to determine genetic distances between varieties of Valenciano melons and (c) as molecular markers in other cucurbit species.

Results and Discussion

Genomic library enriched for microsatellite sequences

A *Tsp*-AG/TC genomic library was constructed and a total of 700 positive colonies were identified as a result of the hybridization of 1600 clones with $(AG/TC)_{13}$ probes, most of them containing microsatellite repeat sequences. Anchored-PCR reactions confirmed microsatellite presence in 450 of the 700 clones and allowed the identification of the size, orientation and position of microsatellite sequences in these clones. This information was important for selecting the clones to be sequenced, reducing costs and time. Knowledge of the DNA sequences flanking the microsatellite region allowed for primer design and synthesis. A set of 237 primer pairs was initially developed based on information generated by the anchored-PCR approach and one hundred and forty-four primer pairs were synthesized (additional file 1). Fifty-nine of these markers had perfect AG or TC repeats (41%), 68 had complex repeats (47%) and 17 had compound repeats (12%) (additional file 1). A greater amount of perfect repeats was detected in this sample compared to what was observed for the 40 melon microsatellite markers

already published, which reported 34% of perfect repeats, 24% of compound repeats and 42% of complex repeats [16]. The number of repeat units observed in the new microsatellite markers varied from 3 to 40 (additional file 1), while in the markers already published the observed range was 7–15 (dinucleotides) and 5–10 (trinucleotides) [15,16].

Sixty-seven out of 144 primer pairs were immediately selected for genetic tests since their PCR conditions did not require further adjustments. The sixty-seven markers were tested on a panel of 11 varieties of six cucurbit species (*C. melo*, *Citrullus lanatus*, *Cucurbita maxima*, *C. moschata*, *C. pepo* and *Cucumis sativus*). Sixty-five markers detected DNA polymorphism between the five melon accessions analyzed (Figure 1).

Marker transferability to other cucurbit species
Thirty-three of the 67 new melon microsatellite markers tested on the cucurbit panel generated PCR products with sample DNA from cucumber (*Cucumis sativus*), 16 with watermelon DNA (*Citrullus lanatus*), 11 with *Cucurbita moschata* DNA and a smaller amount (8 primer pairs) with *C. maxima* and *Cucurbita pepo* DNA. Previously reported estimates of microsatellite marker transferability from melon to cucumber were similar to these results [16]. It seems, therefore, that the level of microsatellite marker transferability in the genus *Cucumis* resembles what is observed in other intragenus comparisons, such as *Glycine*, where up to 65% of the microsatellite markers developed for *G. max* could be transferred to other *Glycine* species [29]. This, of course, results in a rapid dissemination of marker use and facilitates studies on mapping and comparative genetics. Likewise, the use of melon microsatellite markers for DNA polymorphism assessment in cucurbit species represents a significant reduction of marker development costs and a shortcut to in-depth genetic analysis of these species.

Six markers (CMBR18, CMBR22, CMBR23, CMBR48, CMBR56, CMBR82) produced PCR products with sample DNA from all seven species. These sequences were compared with DNA sequences deposited at Genbank [30] and marker CMBR48 showed very significant homology with chloroplastic 16S ribosomal RNA gene sequences. This could explain the high level of transferability of this marker and, as expected, DNA polymorphism was not detected at this marker locus on melon accessions and other cucurbit species. On the other hand, three of the microsatellite markers detected in all six cucurbit species (CMBR18, CMBR56 and CMBR23) showed polymorphism between *Cucumis melo* accessions. The nature of these marker loci is not known at this point. The data indicates, however, that a larger and more diverse sample of each cucurbit species should be considered for microsatellite polymorphism analysis and marker transferability.

Genetic diversity analysis with microsatellite markers
A set of 25 microsatellite markers (Table 2) was selected at random to perform a diversity study of 40 accessions of melon. Without exception, the PCR products for the new markers had the expected fragment size when tested on DNA from accession AF 686. Allele number per locus varied from two (CMBR107, CMBR44, CMBR5, CMBR71) to six (CMBR20 and CMBR39). Expected heterozygosity varied from 0.45 (CMBR100) to 0.70 (CMBR25) (Table 2). The mean expected heterozygosity for these selected microsatellite markers was 0.52. Observations with six microsatellite markers [15] reported the number of alleles per locus varying from 3–5 and observed heterozygosity ranging from 0.49 to 0.75 (mean 0.61). Another study with 38 microsatellite markers detected 2–6 alleles per locus and observed heterozygosity ranging from 0.26–0.79 [16]. The number of alleles and observed heterozygosity estimated here are smaller than expected for an allogamous species such as melon. This is obviously related to the genetic relationships of the melon samples chosen for the study. Rather than selecting a very diverse germplasm for the analysis, a sample composed mostly of Valenciano accessions collected throughout Brazil, representing the commercial varieties planted in the country, predominated in the study. This sample represents, therefore, a small subset of the melon genetic pool, which could certainly explain the relatively lower than expected heterozygosity estimates. It should also be noted that endogamy depression is not significant in the cucurbit family [1,31-33]. Populations in cucurbits are generally derived from seeds obtained from one or few fruits in small populations, not only at farms but also in the wild, favoring endogamy [34].

Polymorphism Information Content (PIC) was estimated for the 25 markers (Table 2), with values ranging from 0.28 (CMBR100) to 0.65 (CMBR43). A group of these markers could certainly be selected for DNA fingerprinting purposes, including applications such as for identity tests, analysis of seed contamination in commercial seed production and variety protection. Based on the estimates of PIC and He (expected heterozygosity), ten of such markers were chosen and studied on 40 melon accessions (markers CMBR7, CMBR22, CMBR24, CMBR25, CMBR27, CMBR33, CMBR39, CMBR43, CMBR73, CMBR89). The probability of identical genotypes was estimated as 2.0×10^{-7}, based on the allelic frequency database constructed (Table 2). This suggests that DNA profiles based on a panel of selected microsatellite markers could be efficiently used to differentiate melon individuals and accessions.
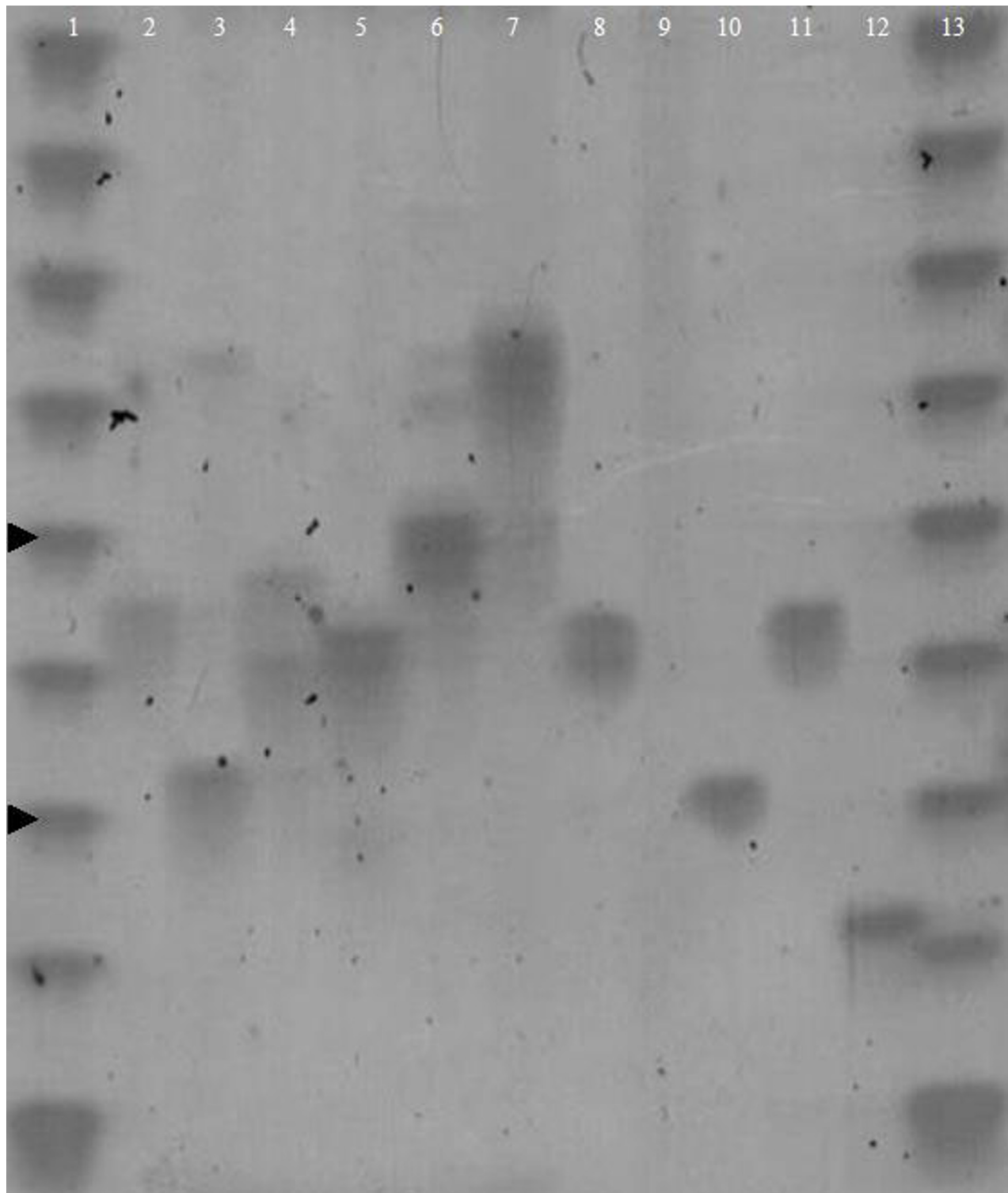
Figure 1
Microsatellite amplification products submitted to polyacrylamide gel (PAGE) electrophoresis and visualized by silver nitrate staining. From left to right: Lanes 1 and 13: ladder 10 bp; Lanes 2 to 7: melon accessions CNPH 82015, CNPH 84201, CNPH 03971, CNPH 84177 and CNPH 85205; Lanes 8 to 12: *Citrullus lanatus* (Crimson Sweet), *Cucurbita maxima* (Exposição) (no amplification), *C. moschata* (Menina Brasileira), *C. pepo* (Caserta) and *Cucumis sativus* (Caipira). Arrows indicate ladder fragments of 120 and 140 bp.

Table 2: Allele frequency database, expected heterozigosity (He), observed heterozygosity (Ho) and polymorphic information content (PIC) estimates for a random set of 25 microsatellite markers analyzed with a sample of 40 *C. melo* accessions.

Locus	Allele (bp)	Frequency	He	Ho	PIC	Locus	Allele (bp)	Frequency	He	Ho	PIC
CMBR1	140	0.03	0.53	0.27	0.47	CMBR33	180	0.15	0.65	0.22	0.57
	150	0.20					150	0.03			
	160	0.12					160	0.39			
	130	0.65				CMBR39	150	0.03	0.60	0.00	0.52
CMBR100	110	0.10	0.45	0.10	0.42		160	0.03			
	130	0.05					190	0.03			
	140	0.13					170	0.49			
	120	0.73					165	0.03			
CMBR107	170	0.53	0.51	0.18	0.37		180	0.41			
	180	0.47				CMBR42	110	0.11	0.44	0.00	0.40
CMBR108	160	0.03	0.30	0.06	0.28		150	0.14			
	200	0.03					160	0.73			
	170	0.11					100	0.03			
	180	0.83				CMBR43	230	0.08	0.68	0.22	0.65
CMBR14	130	0.39	0.53	0.39	0.42		280	0.03			
	140	0.04					240	0.25			
	150	0.57					290	0.15			
CMBR18	160	0.03	0.33	0.06	0.29		250	0.49			
	170	0.17				CMBR44	100	0.35	0.46	0.13	0.35
	150	0.81					110	0.65			
CMBR19	180	0.05	0.48	0.08	0.44	CMBR5	170	0.58	0.49	0.40	0.37
	150	0.01					180	0.43			
	160	0.05				CMBR56	180	0.01	0.36	0.08	0.31
	110	0.18					210	0.03			
	170	0.70					170	0.18			
CMBR20	150	0.08	0.50	0.05	0.46		150	0.78			
	180	0.01				CMBR64	130	0.05	0.53	0.06	0.48
	140	0.01					140	0.16			
	110	0.15					150	0.13			
	160	0.69					160	0.66			
	170	0.05				CMBR7	90	0.10	0.54	0.46	0.49
CMBR22	170	0.23	0.59	0.15	0.52		80	0.06			
	160	0.04					100	0.19			
	150	0.14					110	0.64			
	180	0.59				CMBR70	200	0.15	0.46	0.00	0.41
CMBR24	160	0.23	0.55	0.11	0.49		150	0.10			
	140	0.03					190	0.03			
	170	0.12				CMBR71	100	0.65	0.46	0.05	0.35
	180	0.62					110	0.35			
CMBR25	150	0.12	0.70	0.08	0.63	CMBR73	110	0.19	0.61	0.50	0.53
	160	0.13					100	0.28			
	170	0.39					120	0.54			
	180	0.36				CMBR89	120	0.06	0.63	0.00	0.56
CMBR27	240	0.01	0.55	0.40	0.45		130	0.51			
	260	0.05					150	0.11			
	250	0.38					140	0.31			
270	0.56										

Genetic distance estimates between the melon accessions
DNA polymorphism detected by 25 microsatellite markers allowed genetic distance estimates and clustering of the accessions into two major groups (Figure 2). The first group (Group 1) included mainly *inodorus* and *cantaloupensis/reticulatus* cultivated accessions, which clustered with melon accessions that seem to be derived from crosses between these two types. These accessions were classified as "intermediate" since they show morphological traits peculiar to both types. When morphological information was not available, the accession was classified as an "unknown" type. The second group (Group 2)

included a *conomon* accession, one *cantaloupensis* accession (W6) and also "intermediate" type accessions (Figure 2). A separation of cultivated melon types, such as *inodorus* and *cantaloupensis/reticulatus*, from non-cultivated types, such as *conomon*, was also observed in other melon studies based on DNA polymorphism [20,16]. Here, it was observed that in the group composed mostly of cultivated types (Group 1), the *cantaloupensis/reticulatus* accessions (CNPH 85205, CNPH 84201, CNPH 00884, CNPH 00885, CNPH 00853, CNPH 83062, CNPH 83102) can be easily separated from the *inodorus* accessions (Figure 2). Differentiation of some cultivated accessions into *canta-*
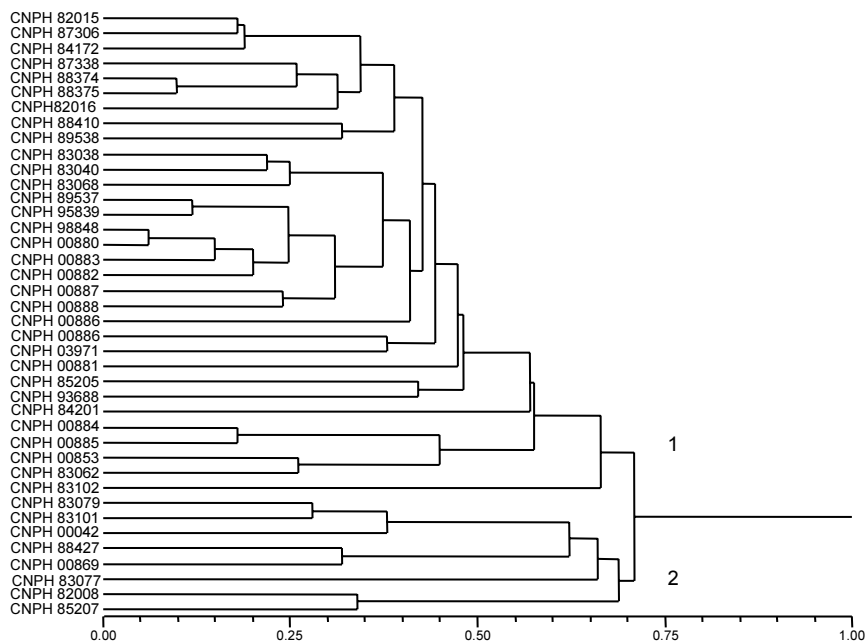
Figure 2
Dendrogram resulting from microsatellite based genetic distance analysis of 40 accessions of melon (*C. melo*), estimated by shared allele distance in pairwise comparisons. Cluster analysis used the neighbor joining method.

loupensis and *reticulatus* groups is not always detected by DNA polymorphism analysis [20,16]. On the other hand, separation of climacteric accessions (*cantaloupensis* and *reticulatus*) from non-climacteric (*inodorus*) is usually consistent in studies based on different types of molecular markers [35]. Together these data seems to support the classification of cultivated melon in only two types, *inodorus* and *cantaloupensis*, as already proposed [1]. Discrepancies between morphological classification and clustering based on DNA polymorphism could reflect recent genetic breeding, which probably combined germplasm of different origins [20,16]. A very obvious example is the classification of Galia varieties (*cantaloupensis*), clustering in different subgroups. Most pedigrees of Galia varieties report parents classified in different melon types, both in breeding programs of the public and private sectors.

The majority of the melon accessions included in this study was previously classified as *inodorus* (Table 1). Exceptions are CNPH 00884, CNPH 00885, CNPH 83102

and CNPH 84201 (*cantaloupensis*), seven other accessions that seem to be derived from germplasm combinations of two or more melon types and three accessions for which morphological data was not available (CNPH 88498, CNPH 89538 and CNPH 93688). Although there is no information about botanical types of CNPH 89538 and CNPH 93688, it seemed very clear from the microsatellite marker analysis that they could be classified as *inodorus*.

Thirty of the 40 melon accessions analyzed with SSR markers were also evaluated with RAPD markers (Figure 3). A -0.70 correlation coefficient was estimated between matrices generated by microsatellite (Figure 4) and RAPD (Figure 5) markers using the Mantel Test (t = - 5.0, P = 0.01). Correlations between matrices generated by RAPD and microsatellite markers on other species such as soybean, *Elymus* and wheat were very similar to the values obtained here [36-38]. RAPD analysis also splits the melon accessions into two major groups, one comprising *inodorus* and *cantaloupensis/reticulatus* melons and the other formed by accessions CNPH 83077, CNPH 83079

Table 1: Melon accessions used on microsatellite and RAPD based genetic distance analysis

| Germplasm Bank Number	Common Name	Country	Type	Marker Analysis
CNPH 82015	Amarelo	Brazil	*inodorus*	RAPD/microsatellite
CNPH 83038	Valência	USA	*inodorus*	RAPD/microsatellite
CNPH 83040	Napoletano	-	*inodorus*	RAPD/microsatellite
CNPH 83068	Progênie Valência	Brazil	*inodorus*	RAPD/microsatellite
CNPH 83077	USDA 164320	Índia	intermediate *	RAPD/microsatellite
CNPH 83079	USDA 255478	Korea	intermediate	RAPD/microsatellite
CNPH 83101	Golden Charm	USA	intermediate	RAPD/microsatellite
CNPH 83102	Farmies Yellow	USA	*cantaloupensis*	RAPD/microsatellite
CNPH 84172	Valenciano	Spain	*inodorus*	RAPD/microsatellite
CNPH 84201	B633.3	France	*cantaloupensis*	RAPD/microsatellite
CNPH 87306	Amarelo	Brazil	*inodorus*	RAPD/microsatellite
CNPH 87338	Eldorado 300	Brazil	*inodorus*	RAPD/microsatellite
CNPH 88374	Valenciano Verde Redondo	Brazil	*inodorus*	RAPD/microsatellite
CNPH 88375	Valenciano Verde Elíptico	Brazil	*inodorus*	RAPD/microsatellite
CNPH 88410	Progênie Valência	Brazil	inodorus	RAPD/microsatellite
CNPH 88427	Elizabeth 239	Japan	intermediate	RAPD/microsatellite
CNPH 89538	CNPH 89538	Brazil	unknown**	RAPD/microsatellite
CNPH 88498	Hoon	Portugal	unknown	RAPD/microsatellite
CNPH 89537	Panaú Amarelo	Brazil	*inodorus*	RAPD/microsatellite
CNPH 95839	Amarelo	Brazil	*inodorus*	RAPD/microsatellite
CNPH 98848	Amarelo Polpa Branca	USA	*inodorus*	RAPD/microsatellite
CNPH 00880	AF 682	Brazil	*inodorus*	RAPD/microsatellite
CNPH 00881	AF 646	Brazil	*inodorus*	RAPD/microsatellite
CNPH 00882	Rochedo	Brazil	*inodorus*	RAPD/microsatellite
CNPH 00883	Gold Mine	Brazil	*inodorus*	RAPD/microsatellite
CNPH 00884	Hi-Mark	Brazil	*cantaloupensis*	RAPD/microsatellite
CNPH 00885	Mission	Brazil	cantaloupensis	RAPD/microsatellite
CNPH 00886	Yellow King	Brazil	*inodorus*	RAPD/microsatellite
CNPH 00887	Yellow Queen	Brazil	*inodorus*	RAPD/microsatellite
CNPH 00883	Gold Pride	Brazil	*inodorus*	RAPD/microsatellite
CNPH 03971	Galileo	Brazil	*cantaloupensis*	microsatellite
CNPH 00042	Conomon	USA	*conomon*	microsatellite
CNPH 82016	Hales Best Jumbo	-	intermediate	microsatellite
CNPH 85205	Early Down Rockmelon	Australia	*cantaloupensis*	microsatellite
CNPH 93688	CNPH 93-688	-	unknown	microsatellite
CNPH 00853	Gália F$_1$	Brazil	*cantaloupensis*	microsatellite
CNPH 83062	WMR-29	USA	intermediate	microsatellite
CNPH 00869	Elizabeth PMR	Japan	intermediate	microsatellite
CNPH 82008	W6	USA	*cantaloupensis*	microsatellite
CNPH 85207	Rockmelon Gulf Coast H91/L1	Australia	intermediate	microsatellite

* accession presenting characteristics of both types, *inodorus* and *cantaloupensis* ** morphological information not available

and CNPH 83101, which clustered with the *conomon* accession (CNPH 00042) in the previous SSR analysis. The accessions CNPH 89538 and CNPH 93688, for which no morphological information was available, were also classified as *inodorus* based on the RAPD marker analysis.

Linkage analysis of microsatellite marker loci

An F2 progeny of 93 individuals derived from a cross between the lines CNPH 82015 and CNPH 84201 was used for linkage analysis of 55 microsatellite markers. Most primers showed segregation according to Mendelian expectations (Figure 6). Only 5% of the primers

(CMBR53, CMBR70 and CMBR147) presented segregation distortion (p < 0.001). Distortion can be caused by male and/or female gamete abortion, selective fertilization of specific gametic genotypes or abortion of zygotes and embryos during development [39]. In all three loci that showed distortion, the F2 genotypes were not distributed according to Hardy-Weinberg Equilibrium proportions, indicating that the nature of distortion is zygotic. The three loci are not linked, though, suggesting that if the distortion observed has a genetic basis, it is not caused by just one deleterious locus in the genome. It should be remarked that some level of distortion is usually expected
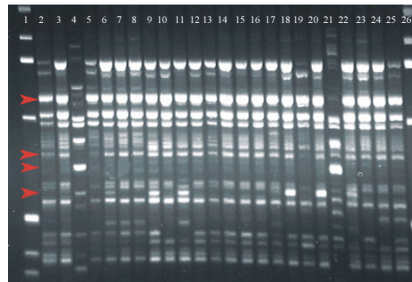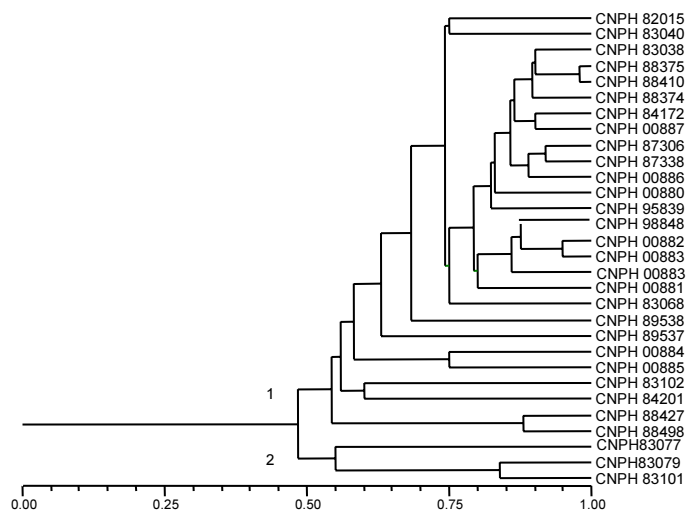
Figure 3
RAPD amplification products submitted to agarose gel electrophoresis and visualized by ethidium bromide staining under UV light. From left to right: Lanes 1 and 26: ladder 1 Kb; Lanes 2 to 27: melon accessions in the order of appearance on Table 1. Arrows indicate polymorphic markers used for analysis.
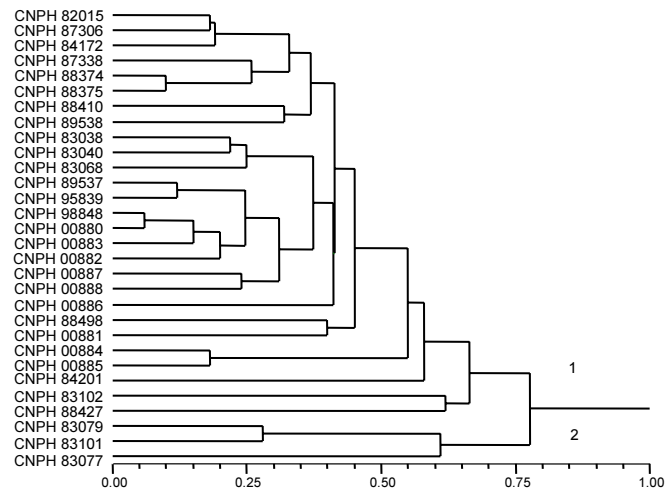
Figure 4
Dendrogram resulting from microsatellite based genetic distance analysis of 30 accessions of melon (*C. melo*), estimated by shared allele distance in pairwise comparisons. Cluster analysis used the neighbor joining method.

Figure 5
Dendrogram resulting from RAPD based genetic distance analysis of 30 accessions of melon (*C. melo*), estimated with the DICE genetic similarity coefficient. Cluster analysis was performed using the UPGMA algorithm.

in intraspecific crossings [40], and especially in interspecific crosses [39]. Possible causes of zygotic distortion include the deleterious effect of reproduction related genes that map in the genome region where the marker locus is located. Since in the three loci the genotype in excess was always heterozygote, hybrid vigor could potentially explain the observed zygotic distortion, such as observed in alfalfa [41] and potato [42].

Linkage analysis of microsatellite marker loci of *C. melo* detected linkage associations (Figure 7). Thirty-one microsatellite markers covering 583.6 cM were assembled into nine linkage groups. Although preliminary, genome coverage represents approximately 50% of the expected genome size (cM) of melon [23]. Twenty-four microsatellite markers did not show significant linkage disequilibrium with any of the markers tested. The data indicates that the microsatellite markers analyzed are distributed throughout the genome of *C. melo*. New markers from the *Tsp*-AG/TC genomic library are currently being tested in the segregating population aiming for the construction of a saturated genetic map based on microsatellite markers.

Conclusions

Genomic library microsatellite enrichment is an efficient procedure for marker development in melon. One hundred and forty-four new markers were developed from *Tsp*-AG/TC genomic library. This is the first reported attempt of successfully using enriched libraries for microsatellite marker development in the species. A sample of the microsatellite markers tested proved efficient for genetic analysis of melon, including genetic distance estimates and potential identity tests. Linkage analysis indicates that the markers developed are dispersed throughout the melon genome and are suitable for mapping purposes. The level of polymorphism observed at marker loci and genome coverage observed so far suggest that the microsatellite markers developed should be very useful for genetic analysis of melon.

Methods

Development of genomic library enriched for hypervariable sequences

Genomic DNA was extracted from fresh leaves of seedlings of the melon commercial hybrid AF 686 (Sakata Seed Company, Brazil) using the CTAB method [43] with
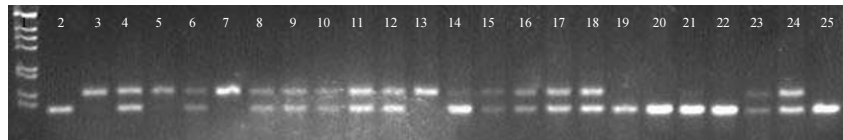
Figure 6
Segregation pattern of marker BRC83 based on polyacrylamide gel electrophoresis of PCR products using an F2 population derived from the cross between accessions CNPH 82015 and CNPH 84201. Lanes 1 and 26: 1 Kb marker; Lanes 2–4: CNPH 82015, CNPH 84201 and F1 hybrid; lanes 5–25: sample of the F2 population.

some modifications [10]. Enzymatic DNA digestions with Tsp 509 I, Sau III and Mse I were performed in order to select the enzyme yielding the greatest amount of fragments in the range of 300 to 800 bp. The Tsp 509 I and Mse I fragment profiles were selected and the digestion products separated in 3.5% agarose gel [44,45]. Fragments between 300 and 800 bp were recovered in DEAE-cellulose NA-45 membrane [13]. These selected fragments were precipitated in saline solution and linked to adaptors containing complementary sequences to the enzyme restriction site. The enrichment of the library with microsatellite sequences was performed through ligation of fragments to biotinilated oligonucleotides. The biotinilated fragments were then hybrized with $(AG)_{13}$ probes to develop the genomic library based on Tsp 509 I derived fragments selected with probes $(AG)_{13}$. Fragments potentially containing microsatellite sequences were recovered with magnetic beads [13]. Enrichment control was performed through PCR using primers complementary to the target site of the restriction enzyme. Southern Blot confirmed preferential recovery of fragments containing microsatellite regions. Fragments containing microsatellite sequences were cloned on pGEMT™ plasmids and used to transform XL1Blue *E. coli* cells (Promega, Madison, WI) through thermic shock [45], resulting in melon genomic library Tsp-AG/TC.

*Selection and identification of positive clones*
Clones of the genomic library containing microsatellite insertions were identified by Southern hybridization [46]. Positive clones were selected by orientation, size and position inside the plasmid insert using the anchored-PCR approach [13]. Plasmid DNA of selected clones was extracted and prepared for sequencing.

DNA sequencing and primer design
Sequencing reactions were performed with "Dye-Terminator" and "Big-Dye" kits (Applied Biosystems, Foster City, CA) using an Applied Biosystems 377 DNA sequencer. Specific primer pairs, unique and complementary to microsatellite flanking regions, were designed using the software PRIMER v.3.0 [46]. Primer designing considered restrictive parameters for primer auto-annealing, G+C content of ∼50% and annealing temperature for PCR reactions in the 55–60°C range.

Characterization and transferability of newly developed microsatellite markers
Primer pairs were synthesized and tested on three melon accessions (CNPH 82015, CNPH 84201 and CNPH 83102). The expected size of fragments resulting from amplification was observed on 3.5% agarose gel and, whenever necessary, adjustments on annealing temperature were performed. Sixty-seven out of 144 primer pairs were immediately selected for genetic tests since their PCR conditions did not require further adjustments. The sixty-seven markers were tested on a panel of 11 varieties of six cucurbit species (*C. melo*, *Citrullus lanatus*, *Cucurbita maxima*, *C. moschata*, *C. pepo* and *Cucumis sativus*), which included five accessions of melon. Sixty-five markers detected DNA polymorphism between the five melon accessions analyzed (Figure 1). The experiment was also repeated in polyacrylamide gel electrophoresis (PAGE) followed by silver nitrate staining, confirming the agarose results. PCR and electrophoresis conditions were the same as described below.
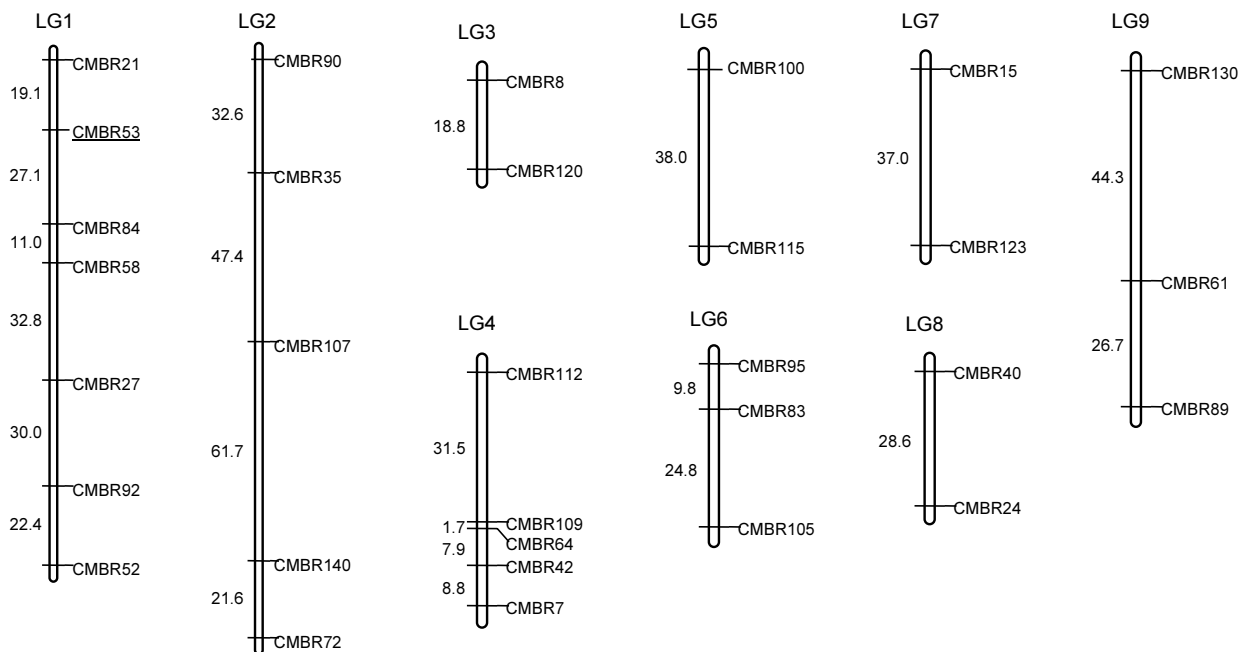
Figure 7
Partial microsatellite linkage map of *C. melo*. Fifty-five microsatellite markers were tested for linkage and thirty-one were assembled into nine linkage groups, which covered 583.6 cM of the melon genome. Map distances in cM are indicated on the left side of linkage groups and locus names are on the right. Underlined markers along linkage groups show segregation distortion

Microsatellite marker data analysis
Forty melon accessions (Table 1) were selected for genetic analysis using a random sample of 25 microsatellite markers extracted from the new microsatellite marker set (Table 2). One individual per accession was used in the analysis. The melon accessions used were mostly commercial Valenciano varieties planted in different areas of Brazil. This sample includes most of the commercial varieties planted in the country. For marker genotyping, young leaves of the 40 melon accessions were used for DNA extraction [10]. Microsatellite loci were genotyped using 13 μl PCR reactions containing DMSO 5%, dNTP 0.25 mM, primer pair 0.3 μM, Tris HCl 10 mM (pH 9.0), $MgCl_2$

1.5 mM, Taq-polymerase 1 U plus 7.5 ng of DNA template. Thermic variation cycles were 94°C for 4 min, 30 cycles of 94°C for 1 min, 50–58°C (depending on the primer) for 1 min, 72°C for 1 min and an extension final step of 72°C for 7 min. PCR was performed on a MJ Research thermocycler PTC 100. Amplification products were submitted to polyacrylamide gel containing polyacrylamide 4% and urea 7 M in TBE 1X on a GIBCO/BRL S42 model electrophoresis apparatus (Promega, Madison, WI). Gel dimensions were 20 cm × 34 cm × 0.4 mm. Electrophoresis was performed at 45-watt constant power and band visualization obtained by silver nitrate staining [48]. Alleles at marker loci were detected in base pairs. All ana-

lyzed microsatellite loci contained dinucleotide repetitive sequences. In addition to the ladder of known fragment sizes used to size alleles, the most frequent allele of a locus was used as a reference to adjust fragment size of the other alleles in the same locus.

Allele frequencies across loci were estimated using the software GDA-Genetic Data Analysis [49]. An allele frequency database for the 25 microsatellite loci genotyped on 40 varieties of melon was then constructed. Observed (Ho) and expected (He) heterozygosities as well as genetic parameters such as the size range and number of alleles per locus were estimated for each microsatellite locus (additional file 1) using the GDA software [49]. The percentage of observed heterozygosity was calculated as the ratio between heterozygote individual genotypes and the total number of analyzed genotypes for each locus. Expected heterozygosity was estimated based on the probability that two individuals taken at random from a given sample would have different alleles at a locus [50]. The arithmetic mean of the expected heterozygosity of multiple loci is defined as Hav = Hn / n where n is the total number of loci. The Polymorphism Information Content (PIC) was also estimated as the probability that an individual is informative with respect to the segregation of its inherited alleles [51].

A set of 10 new microsatellite loci (CMBR7, CMBR22, CMBR24, CMBR25, CMBR27, CMBR33, CMBR39, CMBR43, CMBR73, CMBR89) was selected to estimate the potential of these markers for line identification and variety protection in melon. The probability of identical genotypes [52], defined as $PI = \sum pi^4 + \sum (2pipj)^2$ was estimated for the selected loci individually, and later, for all 10 loci as a whole.

Genetic distance analysis with microsatellite markers
Genetic distance estimates between the 40 melon accessions were performed with microsatellite and RAPD markers. One individual per accession was used on genotyping experiments. Microsatellite genotyping followed the procedures described above. RAPD genotyping was based on data from an initial screening of eighty oligonucleotide primers (Operon Technologies, USA) tested on three melon accessions (CNPH 82015, CNPH 83038, CNPH 83040). Thirty-five oligonucleotide primers were selected. Primer sequences are available upon request. DNA amplifications were performed by PCR in 13 μl reaction volume containing 1.04 μg/μl purified BSA, 0.20 mM each dNTP, 0.5 μM primer, 10 mM Tris HCl (pH 9,0), 1.5 mM $MgCl_2$, 50 mM KCl, 1 U Taq-polymerase and 7.5 ng of extracted melon DNA [10]. Amplification conditions were 40 cycles of 94°C for 1 min, 35°C for 1 min, 35°C for 1 min, 72°C for 2 min and an extension final step of 72°C for 5 min. Amplification products were separated

on horizontal 1.5% agarose gel stained with ethidium bromide. Electrophoresis was performed at 100 volts constant power during 4–5 hours [10].

Genetic distances among melon accessions based on microsatellite marker polymorphism were estimated by shared allele distance in pairwise comparisons. The estimates are based on the sum of the proportion of common alleles between two melon accessions examined across loci (PS) divided by twice the number of tested loci [53,54]. Genetic distances were obtained by the parameter [-ln (PS)] using the Genetic Distance Calculator [55]. The diagonal matrix was then submitted to cluster analysis using the neighbor joining method and a genetic distance dendrogram built using the software NTSYS 2.02 g [56]. RAPD marker polymorphism was used to calculate pairwise distances among melon accessions with the DICE genetic similarity coefficient [57]. Cluster analysis was performed using the UPGMA method (NTSYS software) [56,58]. The correspondence between distance and similarity matrices based on microsatellite and RAPD markers was evaluated through the correlation coefficient estimated by the MXCOMP procedure of NTSYS software [56]. The program plots one matrix against the other, element by element, ignoring diagonal values and estimating the product moment correlation (r) between two matrices. Statistical significance was declared based on a t-test, comparing the observed Z value with its permutational distribution [56,59].

Linkage mapping analysis
An F_2 progeny of 93 individuals derived from a cross between the lines CNPH 82015 and CNPH 84201 was used for linkage analysis of 55 microsatellite markers reported here (additional file 1). Deviations from the expected Mendelian segregation ratios were calculated based on chi-square contingency tables. Marker loci eventually showing segregation distortion were further analyzed for the nature of distortion, if gametic or zygotic, based on two additional chi-square conditional [39]: (a) one testing the hypothesis p=q by the formula Chi-square $(GL = 1) = [(2np - n)^2 + (2nq - n)^2]/n$ [where p (A) = (AA + AB/2)/n and q (B) = (BB + AB/2)/n and n = total number of individuals] and (b) a second testing if genotypes are distributed as expected frequencies by Hardy-Weinberg equilibrium: Chi-square = + $[(AB - 2npq)^2]/ 2npq + [(BB - nq^2)^2]/ nq^2$. The relationships of these two tests allows for the classification of distortion as gametic or zygotic [39]. Linkage analysis was performed using the program Mapmaker v 2.0 [60]. Only markers with 81 or more scored F2 individuals were considered for analysis. A minimum LOD score of 4.0 and a maximum recombination frequency of 0.35 were used to group the microsatellite markers into potential linkage groups. Three-point and multipoint analyses were performed to find the most

probable locus order within eventual linkage groups observed. The final locus order of each linkage group was tested by removing one locus at a time and checking for inconsistencies. Double crossover events were examined, and the original scores re-checked before a final linkage group order was assigned. Recombination frequencies were corrected based on Haldane's map distance function as executed by Mapmaker.

Authors' contributions

PSR developed the microsatellite markers, the segregation population, carried out microsatellite analysis and drafted the manuscript. TCLL and RLT carried out the RAPD study and GSCB participated in RAPD data analysis and development of microsatellite markers. JAB helped on interpretation of RAPD and SSR diversity analysis data. MEF conceived the study, participated in its design and coordination, checked the data and reviewed the manuscript. All authors read and approved the final manuscript.

Additional material

Additional File 1

Repeat number and type, primer sequence, annealing temperature and expected fragment size of 144 new melon microsatellite markers. This table describes the repeat number and type, primer sequence, annealing temperature and expected fragment size of 144 new microsatellite markers developed from a Tsp *AG/TC enriched genomic library.*

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Acknowledgements

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