Genetic variability of a Brazilian *Capsicum frutescens* germplasm collection using morphological characteristics and SSR markers

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ABSTRACT. Characterization studies provide essential information for the conservation and use of germplasm in plant breeding programs. In this study, 103 *Capsicum frutescens* L. accessions from the Active Germplasm Bank of Embrapa Hortaliças, representative of all five Brazilian geographic regions, were characterized based on morphological characteristics and microsatellite (or simple sequence repeat - SSR) molecular markers. Morphological characterization was carried out using 57 descriptors, and molecular characterization was based on 239 alleles from 24 microsatellite loci. From the estimates of genetic distances among accessions, based on molecular characterization, a cluster analysis was carried out, and a dendrogram

was established. Correlations between morphological and molecular variables were also estimated. Twelve morphological descriptors were monomorphic for the set of *C. frutescens* accessions, and those with the highest degree of polymorphism were stem length (14.0 to 62.0 cm), stem diameter (1.0 to 4.2 cm), days to flowering (90 to 129), days to fruiting (100 to 140), fruit weight (0.1 to 1.4 g), fruit length (0.6 to 4.6 cm), and fruit wall thickness (0.25 to 1.5 mm). The polymorphism information content for the SSR loci varied from 0.36 (EPMS 417) to 0.75 (CA49), with an overall mean of 0.57. The correlation value between morphological and molecular characterization data was 0.6604, which was statistically significant. Fourteen accessions were described as belonging to the morphological type tabasco, 85 were described as malagueta, and four were malaguetainha, a morphological type confirmed in this study. The typical morphological pattern of malagueta was described. Six similarity groups were established for *C. frutescens* based on the dendrogram and are discussed individually. The genetic variability analyzed in the study highlights the importance of characterizing genetic resources available for the development of new *C. frutescens* cultivars with the potential for various niche markets.

Key words: Peppers; Polymorphism; Microsatellites; Descriptors; Germplasm bank; Multivariate analysis

INTRODUCTION

Peppers belong to the family Solanaceae and the genus *Capsicum*, which contains approximately 35 taxa (species and varieties), among which 30 are wild and five are domesticated: *Capsicum chinense* Jacq, *C. frutescens* L., *C. annuum* L. var. *annuum*, *C. baccatum* L. var. *pendulum* (Willd.) Eshbaugh, and *C. pubescens* Ruiz et Pav (Eshbaugh, 1980). Three of the domesticated species, *C. annuum*, *C. chinense*, and *C. frutescens*, form a closely related group that evolved in the lowlands of the tropics of Latin America and the Caribbean, with *C. annuum* predominating in Mexico, *C. frutescens* in the Caribbean, and *C. chinense* in the Amazon basin (Pickersgill et al., 1979).

The most common morphological types of *C. frutescens*, one of the most cultivated *Capsicum* species in Brazil, are malagueta pepper (in Brazil) and tabasco pepper (in Mexico and the USA). The main difference between these types is the size and color of the fruit, which varies according to the ripening stage. For malagueta, color changes directly from green (unripe fruit) to red (ripe fruit) and, in some cases it steps by an intermediate stage that occurs before the fully ripe fruit, corresponding to a light red color. For tabasco, the color transition has more steps, beginning with light green, followed by yellow, orange, and light red that deepens to red (ripe fruit). Furthermore, the two types differ in fruit size: the fruits range from 1 to 3 cm long and 0.4 to 0.5 cm wide for malagueta, whereas, for tabasco, they range from 2.5 to 5 cm long and are 0.5 cm wide (Carvalho et al., 2014). Producers have mentioned a third morphological type, named malaguetainha (small malagueta), but the literature on this type is very rare. Characteristics of malaguetainha are small-sized, highly pungent fruits presenting upright position, elongated or short cylindrical shape, red color when ripe (Ribeiro et al., 2008; Rêgo et al., 2012).

In Brazil, the morphological type malagueta is mainly grown in small family-run farms (Ribeiro et al., 2008), especially in the States of Minas Gerais, Bahia, Goiás, Sergipe, and Roraima, the latter located on the northern edge of the Brazilian Amazon basin. The *C. frutescens* (malagueta pepper) and *C. chinense* (murupi pepper and olho-de-peixe pepper) peppers are the morphological types that are most traditionally consumed and shared by the indigenous communities of the Brazilian Amazon basin due to the high pungency of their fruits (Barbosa et al., 2002, 2010).

Plants of *C. frutescens* are typically erect, and fruits are erect, soft-fleshed, and deciduous. They are usually small and conical, have very thin walls, are red when ripe, have high capsaicin content and are sold fresh or dried in markets or processed into liquid sauces, preserves, jams, and pastes (Ribeiro et al., 2008).

The genetic variability of *C. frutescens* has been little explored in plant breeding programs. Consequently, few cultivars of this species are commercialized worldwide: ‘tabasco’, ‘green-leaf tabasco’, ‘malagueta’, and ‘siling labuyo’ (DeWitt and Bosland, 2009). Besides, there are many misidentifications and many cultivars listed in seed catalogs as *C. frutescens* are in fact *C. annuum*.

Many Brazilian farmers who cultivate malagueta select fruits that they have produced for seed extraction aiming the next planting. These seeds often have poor phytosanitary and physiological quality and a slow and less uniform germination, culminating in low crop productivity. Furthermore, malagueta pepper cultivars found in the Brazilian market have a poor uniformity of plant, fruit, and productivity, reinforcing shortage of malagueta pepper cultivars with superior plant and fruit characteristics (Ribeiro et al., 2008).

The development of new malagueta pepper cultivars and hybrids with desirable agronomic and industrial characteristics depends on the genetic variability available. Embrapa Hortaliças *Capsicum* Active Germplasm Bank (AGB) located in Brasília, Distrito Federal, Brazil, was started 36 years ago and has consolidated *ex situ* conservation strategies including the characterization of the variability found in the genus *Capsicum*. Currently, the AGB has over 4000 accessions, comprising the five *Capsicum* domesticated species and dozens semi-domesticated and wild species from various regions of Brazil and other countries. Among all conserved accessions, the AGB has 103 accessions identified as *C. frutescens*.

The aim of this study was to evaluate the genetic variability of 103 *C. frutescens* accessions from the *Capsicum* AGB, based on morphological characteristics and microsatellite molecular markers, thus enhancing knowledge regarding the *C. frutescens* genetic variability available for breeding programs.

MATERIAL AND METHODS

Genetic material

A total of 103 *C. frutescens* accessions of Embrapa’s AGB from the five different Brazilian regions were morphologically evaluated: North, Northeast, Center-West, Southeast, and South. For the molecular analysis, accessions from other species were included: 15 *C. chinense*, one *C. baccatum* var. *pendulum*, one *C. praetermissum*, two *C. annuum* var. *annuum*, and one *C. annuum* var. *glabriusculum*, resulting in a total of 123 accessions (Table 1).

Table 1. *Capsicum* accessions (123) of Embrapa Hortaliças Active Germplasm Bank used for a study on the *Capsicum frutescens* variability.

	CNPH	Origin (region)	Morphological type	Species
1	63	Southeast	Malagueta	<i>C. frutescens</i>
2	287	Center-West	Malagueta	<i>C. frutescens</i>
3	595	Southeast	Malagueta	<i>C. frutescens</i>
4	597	Center-West	Malagueta	<i>C. frutescens</i>
5	1386	Northeast	Malagueta	<i>C. frutescens</i>
6	2631	Southeast	Malagueta	<i>C. frutescens</i>
7	2744	North	Malagueta	<i>C. frutescens</i>
8	2841	North	Malagueta	<i>C. frutescens</i>
9	2866 A	North	Malagueta	<i>C. frutescens</i>
10	2866 B	North	Similar to Habanero	<i>C. chinense</i>
11	2869	North	Malagueta	<i>C. frutescens</i>
12	2870	North	Malagueta	<i>C. frutescens</i>
13	2871	North	Similar to Malagueta	<i>C. chinense</i>
14	3241	Center-West	Malagueta	<i>C. frutescens</i>
15	3257	Northeast	Malagueta	<i>C. frutescens</i>
16	3286	North	Malagueta	<i>C. frutescens</i>
17	3349	Southeast	Malagueta	<i>C. frutescens</i>
18	3374	Center-West	Malagueta	<i>C. frutescens</i>
19	3399	North	Malagueta	<i>C. frutescens</i>
20	3410	Center-West	Malagueta	<i>C. frutescens</i>
21	3414	Southeast	Malagueta	<i>C. frutescens</i>
22	3440	Southeast	Malagueta	<i>C. frutescens</i>
23	3446	Southeast	Malagueta	<i>C. frutescens</i>
24	3448	North	Malagueta	<i>C. frutescens</i>
25	3453	North	Similar to Malagueta	<i>C. chinense</i>
26	3462	North	Malagueta	<i>C. frutescens</i>
27	3470	North	Malagueta	<i>C. frutescens</i>
28	3484	North	Malagueta	<i>C. frutescens</i>
29	3499	North	Malagueta	<i>C. frutescens</i>
30	3535 A	North	Malagueta	<i>C. frutescens</i>
31	3535 B	North	Similar to Murupi	<i>C. chinense</i>
32	3539	North	Malagueta	<i>C. frutescens</i>
33	3546	North	Malagueta	<i>C. frutescens</i>
34	3550	North	Malagueta	<i>C. frutescens</i>
35	3606 A	North	Malaguetainha	<i>C. frutescens</i>
36	3606 B	North	Similar to Malaguetainha	<i>C. chinense</i>
37	3612	North	Malagueta	<i>C. frutescens</i>
38	3621	Southeast	Malagueta	<i>C. frutescens</i>
39	3630	USA	Malagueta	<i>C. frutescens</i>
40	3645	Northeast	Malagueta	<i>C. frutescens</i>
41	3646	Northeast	Malagueta	<i>C. frutescens</i>
42	3647	Northeast	Malagueta	<i>C. frutescens</i>
43	3648	Northeast	Malagueta	<i>C. frutescens</i>
44	3649	Northeast	Malagueta	<i>C. frutescens</i>
45	3667	Southeast	Malagueta	<i>C. frutescens</i>
46	3696	Center-West	Malagueta	<i>C. frutescens</i>
47	3697	Southeast	Malagueta	<i>C. frutescens</i>
48	3698	Center-West	Malagueta	<i>C. frutescens</i>
49	3715	North	Malagueta	<i>C. frutescens</i>
50	3716	North	Malagueta	<i>C. frutescens</i>
51	3746	-	Malagueta	<i>C. frutescens</i>
52	3804	Center-West	Malagueta	<i>C. frutescens</i>
53	3805	Center-West	Malagueta	<i>C. frutescens</i>
54	3806	Center-West	Malagueta	<i>C. frutescens</i>
55	3813	Center-West	Malagueta	<i>C. frutescens</i>
56	3815	Center-West	Malagueta	<i>C. frutescens</i>
57	3816	Center-West	Malagueta	<i>C. frutescens</i>
58	3818	Center-West	Malagueta	<i>C. frutescens</i>
59	3819	Southeast	Malagueta	<i>C. frutescens</i>
60	3820	Northeast	Malagueta	<i>C. frutescens</i>
61	3821	Northeast	Malagueta	<i>C. frutescens</i>
62	3835	Southeast	Malagueta	<i>C. frutescens</i>
63	3847	Southeast	Malagueta	<i>C. frutescens</i>
64	3861	Northeast	Tabasco	<i>C. frutescens</i>
65	3880	North	Malagueta	<i>C. frutescens</i>

Continued on next page

Table 1. Continued.

	CNPH	Origin (region)	Morphological type	Species
66	3885	North	Malagueta	<i>C. frutescens</i>
67	3891	North	Malagueta	<i>C. frutescens</i>
68	3894	North	Malagueta	<i>C. frutescens</i>
69	3906	Center-West	Malagueta	<i>C. frutescens</i>
70	3932	North	Malagueta	<i>C. frutescens</i>
71	3944	Northeast	Tabasco	<i>C. frutescens</i>
72	3984	South	Malagueta	<i>C. frutescens</i>
73	4005	North	Malagueta	<i>C. frutescens</i>
74	4011	Center-West	Malagueta	<i>C. frutescens</i>
75	4020	Southeast	Malagueta	<i>C. frutescens</i>
76	4037	North	Malagueta	<i>C. frutescens</i>
77	4052	Northeast	Malagueta	<i>C. frutescens</i>
78	4069	Center-West	Malagueta	<i>C. frutescens</i>
79	4082	Center-West	Dedo-de-moça	<i>C. baccatum</i> var. <i>pendulum</i>
80	4083	Center-West	Malagueta	<i>C. frutescens</i>
81	4084 A	Center-West	Malagueta	<i>C. frutescens</i>
82	4084 B	Center-West	Malagueta	<i>C. frutescens</i>
83	4085	Center-West	Malagueta	<i>C. frutescens</i>
84	4095	South	Malagueta	<i>C. frutescens</i>
85	4105	Center-West	Malagueta	<i>C. frutescens</i>
86	4138	North	Malagueta	<i>C. frutescens</i>
87	4154 A	Southeast	Malagueta	<i>C. frutescens</i>
88	4154 B	Southeast	Similar to Malagueta	<i>C. chinense</i>
89	4161	Southeast	Tabasco	<i>C. frutescens</i>
90	4184	North	Malagueta	<i>C. frutescens</i>
91	4191	North	Malagueta	<i>C. frutescens</i>
92	4195	North	Malagueta	<i>C. frutescens</i>
93	4212	Center-West	Ornamental	<i>C. annum</i> var. <i>glabriusculum</i>
94	4224	Center-West	Malagueta	<i>C. frutescens</i>
95	4231	Center-West	Malagueta	<i>C. frutescens</i>
96	4237	Center-West	Malagueta	<i>C. frutescens</i>
97	4263	Northeast	Tabasco	<i>C. frutescens</i>
98	4264	Northeast	Tabasco	<i>C. frutescens</i>
99	4265	Northeast	Tabasco	<i>C. frutescens</i>
100	4266	Northeast	Tabasco	<i>C. frutescens</i>
101	4267	Northeast	Tabasco	<i>C. frutescens</i>
102	4268	Northeast	Tabasco	<i>C. frutescens</i>
103	4269	Northeast	Tabasco	<i>C. frutescens</i>
104	4270	Northeast	Tabasco	<i>C. frutescens</i>
105	4271	Northeast	Tabasco	<i>C. frutescens</i>
106	4272	Northeast	Tabasco	<i>C. frutescens</i>
107	4273	Northeast	Tabasco	<i>C. frutescens</i>
108	4274	Center-West	Bode	<i>C. chinense</i>
109	4283	North	Malagueta	<i>C. frutescens</i>
110	4304	North	Malagueta	<i>C. frutescens</i>
111	4353	North	Malagueta	<i>C. frutescens</i>
112	4364	North	Malagueta	<i>C. frutescens</i>
113	30062	Center-West	Jalapeño	<i>C. annum</i> var. <i>annuum</i>
114	40013	North	Bell pepper	<i>C. annum</i> var. <i>annuum</i>
115	3825	Southeast	Cumari	<i>C. praetermissum</i>
116	4315	North	Olho de peixe	<i>C. chinense</i>
117	4316	North	Similar to Malagueta	<i>C. chinense</i>
118	4325	North	Similar to Malagueta	<i>C. chinense</i>
119	4327	North	Similar to Habanero	<i>C. chinense</i>
120	4328	North	Similar to Bode	<i>C. chinense</i>
121	4332 A	North	Similar to Cayenne	<i>C. chinense</i>
122	4360	North	Murupi	<i>C. chinense</i>
123	4361	North	Similar to Tabasco	<i>C. chinense</i>

Approximately 45 days after sowing, five seedlings per accession were transplanted to the soil in a greenhouse in the experimental area of Embrapa Vegetables, Brasília, DF, Brazil, located at 15°56'S, 48°08'W, and 998 m in altitude; plants were kept from September 2009 to March 2010, spaced 1.5 m between rows and 0.60 m within a row. Drip irrigation was used

in the trial, and plant cultivation followed technical recommendations for the cultivation of *Capsicum* (Ribeiro et al., 2008).

Morphological characterization

Morphological characterization was carried out using 53 descriptors usually recommended for *Capsicum* (International Plant Genetic Resources Institute - IPGRI, 1995) and four descriptors were added for this study: fruit position, pungency, aroma, and segregation. The set of descriptors included 17 passport/vegetative part descriptors, 16 inflorescence/seed descriptors, and 24 fruit descriptors (Table 2).

Table 2. Passport data and morphological descriptors used for the characterization of *Capsicum frutescens* accessions.

Passport/vegetative part	Inflorescence/seed	Fruit
Origin	Male sterility	Fruit persistence
Species	Calyx margin	Number of locules
Plant height	Number of flowers/axil	Fruit wall thickness
Plant width	Calyx pigmentation	Fruit pedicel length
Leaf color	Flower position	Fruit weight
Leaf shape	Stigma exertion	Fruit width
Leaf density	Calyx annular constriction	Pungency
Stem shape	Corolla spot color	Fruit shape
Stem color	Anther color	Days to fruiting
Stem length	Filament color	Fruit color at immature stage
Stem diameter	Corolla color	Fruit color at mature stage
Branching habit	Days to flowering	Placenta length
Nodal anthocyanin	Corolla shape	Aroma
Growth habit	Seed color	Fruit length
Tillering	Number of seeds/fruit	Fruit blossom end appendage
Leaf pubescence	Seed surface	Varietal mixture condition
Stem pubescence		Segregation
		Fruit shape at pedicel attachment
		Fruit position
		Anthocyanin spot
		Neck at base of fruit
		Fruit shape at blossom end
		Cross-sectional corrugation
		Fruit surface

Molecular characterization

Samples of leaflets from two plants of each accession were collected individually for DNA extraction using the cetyltrimethylammonium bromide (CTAB) 2% protocol, with modifications. The concentration of DNA in each tube was estimated by electrophoresis on a 1.0% (w/v) agarose gel by comparing the fluorescence intensity of each sample stained with ethidium bromide with different concentrations of lambda DNA standards. Each sample was diluted to 3.0 ng/ μ L.

The amplification reactions applied to the DNA samples from two plants from each of the 123 accessions were carried out using 24 pairs of SSR primers, 19 from Carvalho et al. (2015) and Buso et al. (2016) (CA19, CA20, CA26, CA27, CA29, CA41, CA49, CA52, CA56, CA62, CA79, CA88, CA96, CA131, CA159, CA167, CA172, CA174, and CA178) and five from Nagy et al. (2007) (EPMS 331, EPMS 376, EPMS 386, EPMS 417, and GPMS 112). The reaction was carried out in a total volume of 10 μ L containing 10% (v/v) reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂), 1 U Taq DNA polymerase,

200 μ M of each dNTP, 0.2 mg/mL bovine serum albumin, 0.15 μ M of each primer labeled with 6-FAM (blue), HEX (green), or NED (yellow) fluorescence and 3 ng DNA template. The reactions were carried out in a PT-100 thermal controller (MJ Research, Waltham, MA, USA) using the following conditions: 15 min at 95°C (one cycle); 0.5 min at 95°C, 1.30 min at 56°C, 1 min at 72°C (30 cycles); and 50 min at 60°C (one cycle).

The 123 accessions were genotyped using an ABI 3730 automated sequencer (Applied Biosystems, Foster City, CA, USA). Samples were prepared by the polymerase chain reaction (PCR) by mixing 1 μ L reaction with 10 μ L denaturing agent formamide (HiDi) and 1 μ L of the molecular weight standard (ROX). The mixture was then denatured for 5 min at 95°C.

After passage through the sequencer, the fluorescence peaks and the alleles detected to perform the genotyping were demarcated manually with the help of the GeneMapper software version 4.1 (Applied Biosystems). The allele sizes were rounded using the AlleloBin software. A spreadsheet was then created that identified each plant analyzed and the respective alleles present in each of the analyzed 24 SSR loci.

The genetic distances obtained from the microsatellite markers were calculated with the help of the Genes software based on the following formula:

$GD_{ij} = 1 - (NCL/TNL)$, where GD_{ij} = genetic distance between i and j accessions; NCL = number of coincident loci; TNL = total number of loci.

The NCL is the sum of the allelic coincidences of each analyzed locus, and each coincidence can assume a value of 1 (two coincident alleles), 0.5 (one coincident allele), or 0 (no coincident allele).

The matrix of genetic distances was used to carry out the cluster analysis with the dendrogram using the unweighted pair group mean averaging (UPGMA) method as the clustering criterion and the SAS and Statistica programs.

The correlation and its significance (*t*-test) were estimated between the calculated genetic distances based on the SSR molecular markers and the calculated distances based on the set of morphological descriptors through a simple correlation analysis using Pearson's correlation coefficient, with the help of the statistical program Genes.

RESULTS

Of the 57 descriptors used in the morphological characterization, 12 descriptors were monomorphic for the 103 *C. frutescens* accessions characterized: stem pubescence (sparse), leaf shape (ovate), leaf pubescence (sparse), flower position (erect), corolla color (white-green), corolla spot color (spot absent), calyx pigmentation (absent), fruit blossom end appendage (absent), neck at base of fruit (absent), seed color (deep yellow), seed surface (smooth), and male sterility (absent). Among the *C. frutescens* accessions evaluated, 14 were described as belonging to the morphological type tabasco, 85 as malagueta, and four as malaguetinha, a morphological type confirmed in this study. To describe the typical morphological type malagueta, the most frequent characteristics of the 85 accessions considering 55 of the 57 morphological descriptors (excluding origin and species), are presented in Table 3.

The morphological descriptors with the highest degree of polymorphism (i.e., presenting the largest number of classes or categories), for the 103 *C. frutescens* accessions, were the following: stem length (14 to 62 cm), stem diameter (1 to 4.2 cm), days to flowering (90 to 129 days), days to fruiting (100 to 140 days), fruit weight (0.1 to 1.4 g), fruit length (0.6 to 4.6 cm), and fruit wall thickness (0.25 to 1.5 mm).

Table 3. Morphological descriptors for *Capsicum* and the most frequent forms presented by 85 accessions of malagueta peppers (*Capsicum frutescens*).

Descriptor	Most frequent form	Occurrence of the most frequent form (%)
Stem color	Green	98
Nodal anthocyanin	Green	69
Stem shape	Cylindrical	73
Stem pubescence	Sparse	100
Plant height (cm)	100-125	52
Growth habit	Intermediate	75
Plant width (cm)	100-125	42
Stem length (cm)	15-30	40
Stem diameter (cm)	1-2	76
Branching habit	Dense	70
Tillering	Intermediate	50
Leaf density	Dense	70
Leaf color	Dark green	68
Leaf shape	Ovate	100
Leaf pubescence	Sparse	100
Days to flowering	Up to 100	42
Number of flowers/axil	2	59
Flower position	Erect	100
Corolla color	White-green	100
Corolla spot color	Absent	100
Corolla shape	Campanulated	53
Anther color	Pale blue	86
Filament color	Light purple	75
Stigma exertion	Exserted	95
Calyx pigmentation	Absent	100
Calyx margin	Entire	95
Calyx annular constriction	Absent	91
Days to fruiting	>122	38
Fruit color at immature stage	Green	99
Fruit position	Erect	98
Fruit color at mature stage	Red	47
Fruit shape	Elongated	94
Fruit length (cm)	2-3	63
Fruit width (cm)	0.5-0.7	65
Fruit weight (g)	0.5-0.7	43
Fruit pedicel length (cm)	2-3	76
Fruit wall thickness (mm)	0.5-0.8	56
Fruit shape at pedicel attachment	Obtuse	74
Neck at base of fruit	Absent	100
Fruit shape at blossom end	Blunt	43
Fruit blossom end appendage	Absent	100
Cross-sectional corrugation	Slight corrugated	93
Number of locules	2	95
Fruit surface	Semi-wrinkled	78
Fruit persistence	Persistent	61
Placenta length	>½ Fruit length	99
Pungency	Highly spicy	89
Aroma	Low	89
Seed color	Deep yellow	100
Seed surface	Smooth	100
Number of seeds/fruit	<20	69
Segregation	Absent	100
Varietal mixture condition	Absent	93
Male sterility	Absent	100
Anthocyanin spot	Absent	99

The 24 SSR loci used for molecular characterization generated 239 alleles that allowed the discrimination of *C. frutescens* among the 123 accessions with a dissimilarity value of 0.75 (Figure 1). For the SSR loci analyzed in this study, the PIC ranged from 0.36 (EPMS 417; Nagy et al., 2007) to 0.75 (CA49; Carvalho et al., 2015), with an overall mean of 0.57. Of the 24 SSR loci, 18 (75%) presented a PIC greater than 0.5, highlighting the presence of genetic variability among the analyzed accessions. Null distances were only obtained between

replicates of plants from the same accession, eliminating the possibility of duplicates. The correlation between the morphological and molecular characterization data had a value of 0.6604, which was significant (t -test 1%).

The dendrogram based on molecular data (Figure 1) shows the discrimination among species. *C. praetermissum* (CNPB 3825) appeared to be the most distinct species (dissimilarity greater than 0.90 compared with the other accessions), followed by *C. chinense* (dissimilarity around 0.83), *C. baccatum* var. *pendulum* (CNPB 4082, 0.80 dissimilarity), *C. annuum* (CNPB 30062, CNPB 4212, and CNPB 40013, dissimilarity slightly below 0.80), and finally, the formation of a large group of *C. frutescens* (0.75 dissimilarity).

Figure 1. Genetic variability of *Capsicum* spp assessed using 24 microsatellite loci (Ca - *Capsicum annuum*, Cb - *C. baccatum* var. *pendulum*, Cc - *C. chinense*, Cf - *C. frutescens*, and Cp - *C. praetermissum*).

By establishing a cut-off point in the genetic dissimilarity value equivalent to 0.5 (Figure 1, vertical dotted line), the 103 *C. frutescens* accessions were divided into six groups that varied in number from one accession (Group 5, corresponding to 0.97% of the *C. frutescens* accessions) to 60 accessions (Group 2, 58.2% of the *C. frutescens* accessions). Group 6 was the most dissimilar (dissimilarity around 0.75), followed by Group 1 (dissimilarity around 0.60). Groups 2 and 3 were separated from Groups 4 and 5 (by approximately 0.55 dissimilarity), and the distance from Group 2 to Group 3, as well as from Group 4 to Group 5, was slightly above (0.50 dissimilarity). Two accessions (CNPH 3804 and CNPH 4005) had samples clustered into two different groups (Groups 2 and 3, for both cases). These accessions were considered as belonging to both groups.

Group 6, consisting of nine accessions (CNPH 1386, CNPH 2841, CNPH 2869, CNPH 2870, CNPH 3484, CNPH 3546, CNPH 3612, CNPH 3649, and CNPH 3715) from the North and Northeast regions, was the only group that reflected a relationship with the geographical origin, containing the highest proportion of accessions from the North region among all the established groups. The proportion of accessions from the North region accounted for 77.8% of the accessions in Group 6, while this proportion was 32% in the set of 103 *C. frutescens* accessions studied. Nineteen morphological characteristics were shared among the genotypes in Group 6, demonstrating the genetic similarity among them. Group 6 exhibited the highest concentration of accessions with erect growth habit (88.9% accessions in the group), a characteristic present only in 19.4% of the *C. frutescens* accessions studied, and fruits with low persistence (88.9% of the accessions in Group 6), a characteristic shared with the accessions in Group 4 (77.8% of the Group 4 accessions).

Group 1 consisted of 12 accessions, 11 of which (CNPH 63, CNPH 595, CNPH 597, CNPH 2631, CNPH 3448, CNPH 3241, CNPH 3257, CNPH 3499, CNPH 3746, CNPH 3819, and CNPH 3820) exhibited a morphological pattern similar to “malagueta”, but one accession (CNPH 3630) exhibited tabasco characteristics. Group 1 accessions shared nine morphological characteristics, all with an occurrence of 100%. The main discriminatory characteristics of this group in relation to the others were a high concentration of accessions with early flowering (75% of the group accessions) and early fruiting (66% of the group accessions) beginning at 30 and 60 days after transplantation, respectively; fruits with intermediate persistence (50% of the group accessions), i.e., medium ease of detachment of the ripe fruit, and especially, a largest variation in values for the aroma of the fruits, which ranged from low to high, while this value was consistently low in the other groups.

Accessions in Groups 2 and 3 shared 21 morphological characteristics and presented the most dissimilar patterns in comparison to the typical “malagueta” (Table 3). Both groups contained at least one accession with the following unique characteristics, unusual for *C. frutescens*: presence of calyx annular constriction (CNPH 3804, CNPH 3805, CNPH 3806, CNPH 3813, CNPH 3815, and CNPH 3818, corresponding to 7.8% of the accessions within the two groups), yellow-ripe fruits (CNPH 3804, CNPH 3813, and CNPH 3818, 2.9% of the accessions), and rectangular fruits (CNPH 3804). Group 2 was the largest group (Figure 1), formed by 60 accessions (CNPH 0287 to CNPH 4364) that shared nine morphological characteristics. In a large portion of their accessions, Groups 2 and 3 had a similar dense branching habit occurrence (93.3 and 80%, respectively), as well as dense leaf density (93.3 and 80%, respectively).

Group 2 differed from all others for presenting the highest concentration of accessions with dark green leaves (86.6% compared with 61% among the 103 *C. frutescens* accessions),

and the only orange (CNPH 3805 and CNPH 3806) and triangular (CNPH 3805, CNPH 3815, and CNPH 3818) fruits.

Group 3 consisted of five accessions (CNPH 3716, CNPH 3804, CNPH 4005, CNPH 4084B and CNPH 4304). Unexpectedly, one of the replicates of CNPH 3804 and CNPH 4005 was clustered in Group 2 and therefore separated by the dissimilarity value corresponding to the separation between the two groups (approximately 0.53). This result, although unexpected, emphasizes the genetic closeness between Groups 2 and 3. Eight characteristics were shared by Group 3 accessions. Group 3 differed from the other groups because it predominantly exhibited delayed flowering (40% compared with 18.4% of the *C. frutescens* accessions) and delayed fruiting (40% compared with 50% of the *C. frutescens* accessions), beginning at 90 and 120 days after transplantation, respectively. This group included the only accessions with plants exhibiting dark purple nodal anthocyanin (CNPH 3716 and CNPH 4304), fruits with a pendant (CNPH 4084B) and an intermediate (CNPH 3716) position, fruits with 4.1 cm in length (CNPH 3716), consistently longer than the mean determined for the *C. frutescens* accessions (2.6 cm) and the accessions with the greatest fruit wall thickness (CNPH 3716 and CNPH 4084B), ranging from 1 to 1.3 mm, thicker than the mean determined for the *C. frutescens* accessions, 0.57 mm.

The accessions in Groups 4 and 5 shared 13 characteristics and presented accessions with the most similar morphological pattern to the typical malagueta (50 and 63% of the accessions belonging to each group, respectively; Figure 2).

Figure 2. *Capsicum frutescens* fruits typical of the morphological type malagueta (accession CNPH 3894).

Group 4 consisted of 18 accessions that shared 15 characteristics. Group 4 differed from the other groups because it had the highest concentration of accessions with thick stems (3 cm in diameter compared with a mean of 1.9 cm for the *C. frutescens* accessions), plants with a single flower per reproductive node, purple filament, intermediate calyx margin, green-yellow fruit at the immature stage, and the highest concentration of accessions with the lowest fruit weights (16.6% of accessions weighing less than 0.3 g compared with 13.6% of the *C. frutescens* accessions), as well as accessions with the greatest fruit weights (77.8% of accessions with fruits weighing between 0.7 and 1.2 g compared with 26.2% of the set of *C. frutescens* accessions). The mean fruit weight among the 103 *C. frutescens* accessions

was 0.58 g. Groups 4 and 1 had the highest concentration of accessions with a plant height exceeding 150 cm, among which 66.6% corresponded to Group 4 and 33.3% to Group 1, in contrast to the mean value of 135 cm determined for the set of *C. frutescens* accessions.

Two morphological types (two subgroups) composed Group 4, separated by a dissimilarity value close to 0.4. The first subgroup (4a) comprised genotypes CNPH 3606 A, CNPH 3894, CNPH 4037, and CNPH 4353, and one of the replicates of CNPH 3861. The first four listed accessions characterize the morphological type malaguétinha (Figure 3).

Figure 3. *Capsicum frutescens* fruits typical of the morphological type malagueta (accession CNPH 3820, left) and malaguétinha (accession CNPH 3894, right), with a comparatively smaller size.

One of the samples of accession CNPH 3861, which was characterized morphologically as tabasco, clustered within this subgroup. The other sample of CNPH 3861 was clustered with the immediately adjacent subgroup (4b). This second subgroup (4b) was formed by 14 accessions corresponding to the morphological type tabasco (CNPH 3861, CNPH 3944, CNPH 4161, and CNPH 4263 to CNPH 4273; Figure 4), with green-yellow immature fruit (Figure 5) and accessions with fruit weighing between 0.7 and 1.3 g (100% of the accessions in the subgroup compared with 26.2% in the total set of *C. frutescens* accessions evaluated), characteristics that differentiated them from all other studied accessions.

Group 5 consisted of only one accession (CNPH 3821). Although clustered alone, CNPH 3821 displayed 63% of the characteristics associated with the malagueta typical morphologic pattern, but a unique shorter placenta length, $\frac{1}{4}$ to $\frac{1}{2}$ of the fruit length; in all other accessions, this trait was more than $\frac{1}{2}$ of the fruit length. Accession CNPH 3821 also exhibited other characteristics that were less common in the other accessions, such as intermediate fruit cross-section corrugation and fruit with a sunken blossom end shape (Figure 6), while most of the accessions (94.2%) had blunt or pointed blossom ends. These factors together explain the observed genetic dissimilarity, favoring the discrimination of this single accession concerning the others.

Figure 4. *Capsicum frutescens* fruits typical of the morphological type tabasco (accession CNPH 3861).

Figure 5. *Capsicum frutescens* fruits at different stages of maturity; on the left is the morphological type tabasco (accession CNPH 3861); on the right is the morphological type malagueta.

Figure 6. *Capsicum frutescens* fruits typical of the morphological type “malagueta”, displaying a sunken blossom end shape (accession CNPH 3821).

DISCUSSION

This study is the first in-depth characterization of such a large sample of the Brazilian *C. frutescens* germplasm, representing all Brazilian five geographic regions. Molecular and morphological characterizations carried out with this germplasm provide insight into the genetic variability of the *C. frutescens* in Brazil.

Twelve morphological descriptors were monomorphic (stem pubescence, leaf shape and pubescence, flower position, corolla and corolla spot color, calyx pigmentation, fruit blossom end appendage, neck at base of fruit, seed color, seed surface, and male sterility), i.e., invariable or unable to discriminate accessions within *C. frutescens* species, and thus could be eliminated in a future intraspecific characterization. Flower position is one of the minimum descriptors for *C. frutescens* selected by Silva et al. (2013), suggesting that these descriptors have discriminative capacity when applied to distinguish *Capsicum* species, but not accessions within *C. frutescens*. According to Baral and Bosland (2004), to differentiate among species of *Capsicum*, the inflorescence-related descriptors are essential, such as the flower position and the presence of calyx annular constriction, which are used to distinguish between *C. frutescens* and *C. chinense*. Qualitative descriptors related to reproductive parts are useful for the identification of *Capsicum* species, whereas intraspecific variability is best assessed by quantitative multivariate analysis based on the fruit length/width ratio, days to flowering and leaf, anther, filament and pedicel length (Ortiz et al., 2010).

In this study, genetic variability was mainly observed for quantitative traits, and the morphological descriptors with the highest degree of polymorphism were stem length and stem diameter, days to flowering, days to fruiting, fruit weight, fruit length, and fruit wall thickness. Jarret et al. (2007) evaluated fruit traits of 40 accessions of *C. frutescens*, including two accessions from Brazil, and observed a large variation of fruit length from 1 to 8.5 cm, fruit width from 0.5 to 1.5 g, and fruit weight from 0.18 to 4.04 g. Barbosa et al. (2010) studied 182 pepper accessions of two *C. annuum* subspecies (*C. annuum* var. *annuum* and *C. annuum* var. *glabriusculum*) and two species, *C. baccatum* var. *pendulum* and *C. frutescens*, from Roraima, Brazil. Size (length and width) and weight of fruits of the 18 *C. frutescens* accessions studied had the lowest values compared to the other accessions, with an average of 1.7 cm in length, 0.48 cm in width and 0.30 g fresh mass.

Morphological characterization evidenced a similar morphological pattern among *C. frutescens* accessions, despite the variations observed especially among the fruits of malagueta and tabasco (Figure 5), and allowed the identification of 85 accessions of the morphological type malagueta (Figure 2), presenting erect, elongated, and red fruit, with 1.2 to 4.2 cm in length and 0.4 to 1.1 cm wide, fruit weight varying from 0.2 to 1 g and fruit wall thickness with a range of 0.3 to 1.3 mm. The 14 accessions classified as tabasco differed from the morphological type malagueta by presenting fruit color transition beginning with light green, followed by yellow, orange, and light red to red (ripe fruit), fruit length ranged from 1.3 to 4.6 cm and fruit width from 0.5 to 1.4 cm, fruit weight varied from 0.7 to 1.3 g, and fruit wall thickness ranged from 0.2 to 1.2 mm.

Morphological and molecular analyses were significantly correlated, although they presented a medium magnitude (0.6604), demonstrating the differences between the two types of characterization. Baba et al. (2016) found no significant correlation between the fruit morphological descriptors and amplified fragment length polymorphism molecular markers in *C. chinense* germplasm from different geographical regions of Brazil. The low magnitude or

even the lack of correlation between the assessments should not be considered a limitation of these tools to quantify genetic variability. In contrast, the lack of correlation suggests that both types of characterization are important and play a complementary role in providing a better understanding and differentiation of the germplasm (Sudré et al., 2010; Oh et al., 2012).

Although molecular characterization showed genetic variability among and within the six established groups of *C. frutescens*, the formation of a large group (Group 2), with dissimilarity lower than 0.55 shows morphological and genetic similarity for most studied accessions. This finding leads to another issue, as previously observed by Smith and Heiser Junior (1951) among others (Pickersgill, 1971, 1984; Jarret et al., 2007) concerning the highly conserved morphological pattern of *C. frutescens* fruits. However, no logical explanation has been found for the origin of the *C. frutescens* conserved morphological pattern, in contrast to the wide variability expressed by other *Capsicum* species.

The dendrogram resulting from molecular data allowed a clear grouping of the *C. frutescens* types found in Brazil, specifically malagueta and tabasco peppers. There were questions regarding whether malagueta (Figure 3) constituted a varietal type of *C. frutescens* or whether the small fruits (results obtained from the four accessions studied ranging from 0.6 to 1.9 cm long, 0.3 to 0.7 cm wide, 0.14 to 0.4 g, and fruit wall thickness from 0.3 to 0.6 mm) were the result of a viral infection or rejection during the production process. However, a subgroup specific for the four malagueta accessions identified was established within Group 4 of the dendrogram (Figure 1), thus confirming its genetic identity, i.e., its identification as a morphological type within *C. frutescens*. Tabasco accessions were also clustered in Group 4, indicating the genetic proximity of this morphological type to malagueta.

Due to the visible difference between these two types of peppers, the inclusion of tabasco accessions in the same group as malagueta was not expected. Nine morphological characteristics displayed similar results between the fruits of the malagueta and tabasco subgroups: erect and elongated fruit presenting a slightly corrugated cross-section, low fruit persistence, two locules, placenta length greater than half of the fruit size, semi-wrinkled surface, strong pungency, and weak aroma.

Group 6 in the dendrogram (Figure 1) had accessions with the largest number of descriptors (19 morphological characteristics) with similar results, which contributed to making the group discrepant concerning the others. Additionally, this was the only group that showed some relationship to the geographical origin, with the highest proportion of accessions from the North region. Different from this result, the study by Finger et al. (2010) did not show a relationship between the geographic distance and the estimated diversity of 49 accessions of *C. chinensis* from Brazil. They reported that the diversity might be a reflection of genetic drift and plant selection in different environments rather than the geographic location. Another explanation is the occurrence of cross-pollination in a rate varying in *Capsicum* from 0.5 to 70%, contributing to the genetic contamination of the seeds. Genotypes presenting characteristics that are unusual for *C. frutescens* are described and its putative position within species genetic variability (Figure 1) is reported. Namely, a subgroup in Group 2 of the dendrogram presenting orange and triangular fruits, and, in Group 3, accessions with fruits presenting pendant and intermediate positions, greater length, and increased fruit wall thickness. Moreover, Groups 2 and 3 had accessions that shared rare characteristics, such as calyx annular constriction, yellow and rectangular fruits.

Some of these characteristics are directly related to domestication practices, such as the human selection of fruits for increased wall thickness, greater length, and different

colors (yellow and orange), corresponding to the original or wild plants, and the possibility of interspecific hybridization between *C. frutescens* and *C. chinense*, whether natural or artificial. This hypothesis was raised due to the presence of calyx annular constriction in *C. frutescens* genotypes (CNPH 3804, CNPH 3805, CNPH 3806, CNPH 3813, CNPH 3815, and CNPH 3818) since this characteristic is distinctive of the *C. chinense* species (Baral and Bosland, 2004). The presence of the calyx annular constriction mistakenly led the Embrapa Vegetable team to identify such accessions as *C. chinense* initially. However, they were all grouped together with *C. frutescens* specimens in the molecular analysis. Moreover, their passport data indicated proximity between *C. chinense* and *C. frutescens* cultivation fields, reinforcing the possibility of cross-fertilization.

The North, Northeast, and Center-West regions of Brazil may have played an important role in the genetic differentiation of *C. frutescens*. Accessions collected in the Amazon basin probably underwent incipient domestication by Amazonian indigenous populations, potentially with selection for plant populations that were adapted to different ecological conditions, but with no obvious alterations of the morphological pattern. Thus, accessions with different adaptive abilities are of extreme interest to breeding programs aimed at obtaining cultivars that are adjusted to different regions and farming systems in the country.

During the development of new pepper cultivars, a plant breeder should consider, in addition to the distance between accessions, adaptation to different farming systems, specific morphological characteristics of each varietal group, and the requirements and preferences of the market. The malagueta pepper market in Brazil includes the commercialization of fresh and dried fruits and fruits processed into preserves or liquid sauces (Ribeiro et al., 2008). The germplasm of *C. frutescens* evaluated in this study has considerable genetic variability that can be exploited for the development of new cultivars intended for different niche markets.

For the commercialization of fresh fruits, fruits with a larger size and weight and easy detachment of the calyx are preferred, for which the following accessions stood out in this study: CNPH 3649 (fruit length = 3.5 cm; width = 1 cm; fresh weight = 1.3 g), CNPH 3944 (fruit length = 3.7 cm; width = 1.1 cm; fresh weight = 1.4 g), and CNPH 4161 (fruit length = 3.2 cm; width = 0.8 cm; fresh weight = 1.1 g).

When processed into preserves, the fruits of malagueta pepper are used whole, so they should be suitable for packaging and have the desired commercial characteristics, i.e., small, resistant to cracks, free of spots, a deep red color, and good organoleptic properties (taste, aroma, and pungency). Furthermore, the fruit for the industry of preserves and sauces should be easily detached from the calyx (low persistence) to avoid injuries and darkening of the fruits after packaging and to avoid the additional step of peduncle removal. The presence of these characteristics for the production of preserves was found in accessions CNPH 2869, CNPH 3484, CNPH 3546, CNPH 3612, CNPH 3715, and CNPH 3894. Flesh firmness is also critical, but this characteristic has not been evaluated in this study.

One of the major routes of *Capsicum* pepper consumption in Brazil and worldwide is the liquid sauce. Peppers presenting larger fruits with a fleshy pulp, red color, and intense pungency are used. Malagueta and tabasco peppers, despite their small fruits, are used alone or in blends with other peppers in the preparation of highly spicy sauces (Ribeiro et al., 2008). For this niche market, the accessions CNPH 2631, CNPH 3499, CNPH 3630, CNPH 3645, CNPH 3646, CNPH 3649, CNPH 3819, CNPH 3820, and CNPH 3944 were prominent.

Association of morphological and molecular characterization provided knowledge on the variability of *C. frutescens* accessions representing the Brazilian diversity of *C.*

frutescens. This study showed genetic variability that can be used for developing cultivars for different market niches. Three morphological types were confirmed: malagueta, tabasco, and malagueta. This study confirmed that *C. frutescens* has highly preserved morphological characteristics and this needs to be further studied and possibly different hypothesis formulated, invoking domestication, artificial selection, and other anthropogenic elements.

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