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Characterization of *Capsicum* species using anatomical and molecular data

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ABSTRACT. *Capsicum* species are frequently described in terms of genetic divergence, considering morphological, agronomic, and molecular databases. However, descriptions of genetic differences based on anatomical characters are rare. We examined the anatomy and the micromorphology of vegetative and reproductive organs of several *Capsicum* species. Four *Capsicum* accessions representing the species *C. annuum* var. *annuum*, *C. baccatum* var. *pendulum*, *C. chinense*, and *C. frutescens* were cultivated in a greenhouse; leaves, fruits and seeds were sampled and their organ structure analyzed by light and scanning electronic microscopy. Molecular accession characterization was made using ISSR markers. Polymorphism was observed among tector trichomes and also in fruit color and shape. High variability among accessions was detected by ISSR markers. Although the species studied

present a wide morphological and molecular variability, this variability was not reflected in anatomical features.

Key words: Genetic diversity; Sweet and chili pepper; ISSR markers; Micromorphology; Anatomy

INTRODUCTION

The *Capsicum* species are members of the Solanaceae family (tribe Solaneae, subtribe Capsicinae), which includes tomato, potato, tobacco, and petunia. This genus contains about 31 species (Moscone et al., 2007) of which five are domesticated, namely *C. annuum* L., *C. frutescens* L., *C. chinense* Jacq., *C. baccatum* L., and *C. pubescens* R. (IBPGR, 1983). The production of sweet and chili pepper crops is an important agribusiness worldwide, where this market stimulates family farming and increased employment and income generation from agriculture (Reifschneider and Ribeiro, 2008).

The main producing region in the world is Asia, especially represented by China which produced approximately 254 thousand tons of hot and sweet peppers in 2008, followed by India producing 1.23 million tons (FAOSTAT, 2010). Reifschneider and Ribeiro (2008) argue that in Brazil this is a market that moves around 100 M USD per year, including domestic consumption and exports. Also according to these authors, red peppers account for third place in production and consumption of seasoning vegetables in Brazil. Therefore, this market stimulates agriculture in Brazil (Vilela et al., 2008).

Capsicum is native to Central and South America (Perry et al., 2007), where this genus is believed to have been selected in two areas of origin, one called the primary center and then introduced to other regions called secondary centers (Mongkolporn and Taylor, 2011). Brazil is considered a secondary center of diversity of this genus.

Due to the selection process, varieties with new morphological characters arose in these new areas (Clement et al., 2010), and their genetic variability is poorly understood. Many varieties have overlapping morphological character states, potentially leading to unresolved or erroneous species identification. The great importance of correct species identification can be exemplified by the knowledge of the anatomical and morphological characteristics that are necessary for studies on the interactions between plants and herbivores and other natural enemies (Price, 1997). Additionally, a correct botanical species classification is essential for the proper management of germplasm collections. An erroneous identification of species maintained in gene banks can lead to losses ranging from the propagation and inadequate conservation of accessions to the delivery of misidentified genetic material to other institutions, resulting in a waste of time and financial resources (Sudré et al., 2010).

The genus *Capsicum* has a very complex taxonomy, and its circumscription into one species or another can vary considerably based on the characteristics of the leaves, flowers and fruits, and these variations are often factors related to the geographic and weather conditions where the plants grow (Petters, 2002). In general, the identification of this genus and species is carried out by morphological features observed mainly in flowers (Sudré et al., 2010). However, flower characteristics are not enough and, in general, a combination of diagnostic characters associated with genetic characteristics is usually required to identify and differentiate *Capsicum* species.

The characterization and evaluation of domesticated *Capsicum* species are particularly interesting for gene bank curators, since a wide variability, not yet fully known and exploited, is available for these species (Ince et al., 2009). Despite the accuracy in estimating genetic divergence among accessions by molecular markers, knowledge of the phenotype given by morphological and molecular descriptors is still important. Besides the aspect of correct species identification, the characterization and evaluation of conserved genotypes are of fundamental importance for improving our knowledge and making it possible to detect better genotypes for use in breeding programs and duplicates (Laurentin, 2009).

Thus, this study aimed to evaluate the anatomy and micromorphology of vegetative and reproductive organs of 4 species of *Capsicum*, and to detect their special chemical constituents, providing data to assist in the understanding of these species, for ecological and medicinal studies. In addition, we determined the genetic divergence between the four accessions of *Capsicum* spp based on morphological and molecular data and estimated the relation between genetic distances obtained based on morphological characteristics by inter simple sequence repeat (ISSR) markers.

MATERIAL AND METHODS

Plant materials

Four accessions from the *Capsicum* gene bank of Centro de Ciências e Tecnologias Agropecuárias of the Universidade Estadual do Norte Fluminense were studied. Seeds of each accession were sown in a polystyrene seed tray with 128 cells filled with organic substrate and grown until seedling stage. Seedlings were individually transferred to 5-L plastic pots filled with a mixture of organic substrate:sand (3:1, w/w). Plants were kept in a greenhouse, fertilized only once and exposed to daylight illumination during the experimental period. Completely expanded leaves, ripe fruits and seeds of the species *C. annuum* (accession No. UENF1381), *C. baccatum* (accession No. UENF1732), *C. chinense* (accession No. UENF1755), and *C. frutescens* (accession No. UENF1775) were sampled and used for anatomical characterization. These materials were fixed immediately after collection and processed as described in the following sections. These accessions showed remarkable phenotypic differences in growth form, corolla shape and color, fruit shape and color and seed color (Table 1) (Moscone et al., 2007). For the genetic divergence study, seeds were sown and seedlings were individually transferred to 0.5-L plastic pots and kept as described before for a period of 3 weeks. Young leaves of 5 plants of each species were collected, frozen in liquid nitrogen and stored at -70°C until used.

Table 1. Inventory of the recognized *Capsicum* taxa in relation to their growth form, corolla shape and color, fruit shape and color, and seed color.

Capsicum sp	Growth form	Corolla shape and color	Fruit shape and color	Seed color
C. chinense	Herb or sushrub (0.5-2 m)	Stellate white or cream	Sherical or conical; red, orange, yellow, white or brown	Yellowish
C. annuum	Herb or sushrub (1-2 m)	Stellate white or cream (expectionally violet)	Highly variable shape, red	Yellowish
C. baccatum	Shurb (0.6-2 m)	Stellate white with greenish spots in the throat	Ovoid or fusiform red	Yellowish
C. frutescens	Herb or sushrub (1-2 m)	Stellate white or cream	Elongate red	Yellowish

Adapted from Moscone et al., 2007.

Light microscopy

Samples from each plant organ were immediately fixed for 2 h in a solution of 2.5% glutaraldehyde and 4.0% formaldehyde in a 0.05 M sodium cacodylate buffer at pH 7.2 (Klein et al., 2004). Afterwards, the samples were washed three times with buffer for 30 min and then fixed for 2 h at room temperature with 1.0% osmium tetroxide in the same buffer. The fixed samples were dehydrated using an acetone series and then embedded in epoxy resin (Epon®). Thin sections (4.0 µM) were then made and stained with 0.05% toluidine blue (O'Brien et al., 1964). The glass slides were sealed with Entellan® (Merck), and the material was examined with an Axioplan microscope (ZEISS). Images were obtained with a Canon PowerShot A640 camera and the Axiovision software (ZEISS).

Scanning electron microscopy

Samples were fixed, post-fixed and dehydrated, as done for the light microscopy study. The samples were then critical point dried with CO_2, sputter coated with 20 nm gold, and observed using a ZEISS DSEM 962 scanning electron microscope.

Molecular analysis

The ISSR marker was used for molecular characterization. Besides the four *Capsicum* accessions, a tomato (*Solanum lycopersicon*) cultivar (Rio Grande) was used as an outgroup control. Three hundred milligrams of macerated leaf of each sample were transferred to 1.5-mL tubes and immersed in liquid nitrogen for extraction of DNA according to the protocol by Doyle and Doyle (1990) with modifications as described in the following. The sample was mixed with 1 mL preheated extraction buffer containing 2% CTAB, 1.4 mM NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0, 1% PVP, and 0.2% β-mercaptoethanol, along with 5 µL 10 mg/mL proteinase K. These samples were incubated at 37°C for 30 min with gentle stirring every 10 min and subsequently incubated at 65°C for 30 min. The samples were then centrifuged at 8000 *g* for 10 min, and the supernatant (about 800 µL) was transferred to a new tube, to which was added an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). The phases were mixed by gentle inversion for approximately 10 min until becoming cloudy. The supernatant (aqueous phase) was separated by centrifugation at 8000 *g* for 10 min and transferred to a new tube, to which 200 mL 2.0 M NaCl containing 4% PEG (final concentration) were added for complete removal of proteins and DNA recovery. The samples were then incubated for 15 min at 4°C and centrifuged again at 8000 *g* for 10 min. Nucleic acids were precipitated by adding two-thirds volume (400 µL) cold isopropanol and incubating for 20 min at -70°C. The precipitates were pelleted by centrifugation at 8000 *g* for 10 min. The supernatants were discarded and the precipitates washed twice with 200 µL 75% ethanol with ammonium acetate, to remove salt (between each wash, the tubes were centrifuged at 8000 *g* for 5 min). After discarding the last supernatants, the samples were dried at room temperature, until ethanol was removed. The samples were resuspended in 100 µL TE solution (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) with RNase A at a final concentration of 10 mg/mL and incubated in a water bath at 37°C for 30 min and then the samples was stored at -20°C until use. DNA concentration was determined NanoDrop spectrophotometer (Thermo Scientific) and adjusted to 1 µg/µL.

PCR was performed according to the protocol by Williams et al. (1990) with some

modifications. The final reaction volume was 13 µL containing the following: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.4 mM $MgCl_2$, 100 µM of each deoxyribonucleotide (dATP, dCTP, dGTP, and dTTP), 0.4 µM oligonucleotide primers, 10 ng genomic DNA, and 0.75 U Taq DNA polymerase. First, 2 µL DNA were placed in the tube, followed by 11 µL previously described mix. PCR (StepOne Plus Thermal Cycler - Applied Biosystems) was as follows: 3 min at 94°C for the initial denaturation; 40 cycles consisting of 94ºC for 1 min, 40-50°C for 1 min (depending on the primer), and 72°C for 3 min; and a final extension step of 72°C for 7 min. The amplified fragments were separated on a 1.5% agarose gel, stained with GelRed, and visualized under UV light (photodocumented with Minibis Pro, Bio-imaging System).

The amplification conditions were optimized for each primer to determine the best temperature for amplification. We used 41 primers (Kumar et al., 2011; Refaat et al., 2007; Patel et al., 2011) as described in Table 2 (Primers, Columbia, Canada).

Table 2. ISSR primers used, optimal annealing temperatures, and number of polymorphic and monomorphic bands generated in the study of 4 accessions of *Capsicum* and 1 accession of *Solanum lycopersum*.

No.	Sequence (5'-3')	Ta (°C)	Polymorphic bands	Monomorphic bands	Total of bands
1	$(AC)_8CT$	47	9	2	11
2	$(GA)_8YT$	47	9	1	10
3	$(GT)_8YC$	47	11	1	12
4	$(AC)_8YA$	47	13	0	13
5	$(GT)_8YG$	47	11	0	11
6	$(AC)_8YT$	47	10	2	12
7	$GAC(CAA)_5$	47	6	0	6
8	$CTC(GT)_8$	47	14	0	14
9	(GAA) EAA	49.5	14	0	14
10	$(AG)_8TG$	47	10	0	10
11	$(CCA)_7$	47	9	1	10
12	$(GCC)_5$	47	4	0	4
13	CGA_7	50	14	0	14
14	$T(TTA)_4TT$	46	14	1	15
15	$(GTG)_4RC$	49.5	14	0	14
16	$CG(A)_7$	50	24	1	25
17	$(GC)_2CGCCGCCGCC$	50	17	3	20
18	$(GT)_8CTC$	50	0	6	6
19	$TACA(GCA)_3G$	50	19	0	19
20	CT_8AC	50	20	0	20
21	CT_8GC	50	27	0	27
22	$(CA)_6AG$	50	13	0	13
23	$(GT)_6GG$	50	13	20	0
24	$CGAA(TTA)_3TT$	50	10	0	10
25	$(AA)_2(TAA)_3T$	44	5	1	6
26	$(AA)_2AT(AAT)_3$	48	13	2	15
27	TG_8	50	*	*	*
28	TG_7T	50	*	*	*
29	TG_9T	50	*	*	*
30	$CT(ATT)_4$	41	*	*	*
31	$TCA(TTA)_3TT$	50	*	*	*
32	$(CT)_8AGG$	50	*	*	*
33	GA_8T	50	*	*	*
34	$(GGGTG)_3$	50	*	*	*
35	CT_8TG	50	*	*	*
36	$(CA)_6AC$	50	*	*	*
37	$(GT)_6CC$	50	*	*	*
38	$CA(CCA)_2CGC$	50	*	*	*
39	$GA(GGA)_2GGC$	50	*	*	*
40	$GA(ATT)_4$	50	*	*	*
41	$(AG)_8CG$	50	*	*	*
	Total of bands/column		323	41	331

Ta = annealing temperature.

The statistical analysis of the data was performed considering a binary matrix that was constructed using the value '1' to indicate band present and '0' to indicate band absent. The monomorphic bands were eliminated. The binary data were submitted to analysis using the GENES program (Cruz, 2006), and a dendrogram was constructed using the R program (R Development Core Team, 2009). Clustering was performed using unweighted paired group method with arithmetic averages, and the results were validated by calculating the cophenetic correlation coefficient.

RESULTS AND DISCUSSION

Leaf anatomy

The leaf epidermis of the four species studied displayed a uniseriate epidermis covered by a slightly thicker cuticle on the adaxial surface. The epidermal cells had a rectangular shape, and the adaxial surface cells were larger than the abaxial cells (Figure 1A). The external periclinal cell walls of the adaxial epidermal cells of *C. baccatum* and *C. frutescens* were slightly convex. The shape of the external periclinal cell walls of the adaxial epidermal cells of both these species appeared to be related to the radiation intensity and the ability of plants to capture sunlight, because these plants are found in the forest. Epidermal cells with convex periclinal walls may be advantageous in shaded environments, because they capture light more efficiently (Smith et al., 1997). Convex periclinal walls of the epidermal cells have been observed in other species of *Psychotria* that grow in the understorey (Moraes et al., 2011).

On the adaxial surface of the species, the epidermal cells showed sinuous anticlinal walls (Figure 1B). The sinuous outline of the anticlinal walls has been previously described in other genera of Solanaceae, such as *Physalis* and *Nicandra,* and in other sections of the subgenus *Solanum* and *Geminata*, such as *S. dulcamara* L., *S. nigrum* L., *S. seaforthianum* Andrews, *S. swartzianum* Roem. & Schult., *S. tuberosum* L., and *S. pseudocapsicum* L. Cosa et al. (2002) reported that the outline of the anticlinal epidermal walls shows varying degrees of sinuosity according to the species and the surface observed. For example, in *S. palinacanthum*, *S. elaeagnifolium* and *S. juvenal*, this outline is slightly sinuous, whilst in *S. sisymbriifolium* it is strongly sinuous, for both leaf surfaces.

The presence of anomocytic stomata on both surfaces of the four species studied classified the leaves as amphistomatic (Figure 1B and C). According to Metcalfe and Chalk (1950), amphistomatic leaves are common in Solanaceae, but Cosa et al. (2002) described hypostomatic leaves in some species of this family.

Polymorphism was observed for trichomes. The species *C. chinense* and *C. baccatum* showed tector trichomes, and in *C. baccatum* the trichomes were hooked. Glandular trichomes were observed in *C. annuum*, *C. frutescens* and *C. baccatum* (Figure 1D and E). According to Metcalfe and Chalk (1983), in some cases, trichomes may serve to characterize some families. The development of trichomes from the epidermis usually results from differential enlargement and subsequent divisions of the epidermal cells (Carlquist, 1958). The presence of glandular trichomes is a characteristic of the species of the genus *Solanum* and many other Solanaceae as well, with the known exception of *Nicotiana glauca* and *Solandra nitida* (Maiti et al., 2002). Glandular trichomes are characterized by having specialized cells that contain or exude, on contact with pests, sticky and/or toxic exudates that may entrap, irritate or potentially kill some pests (Simmons et al., 2003).

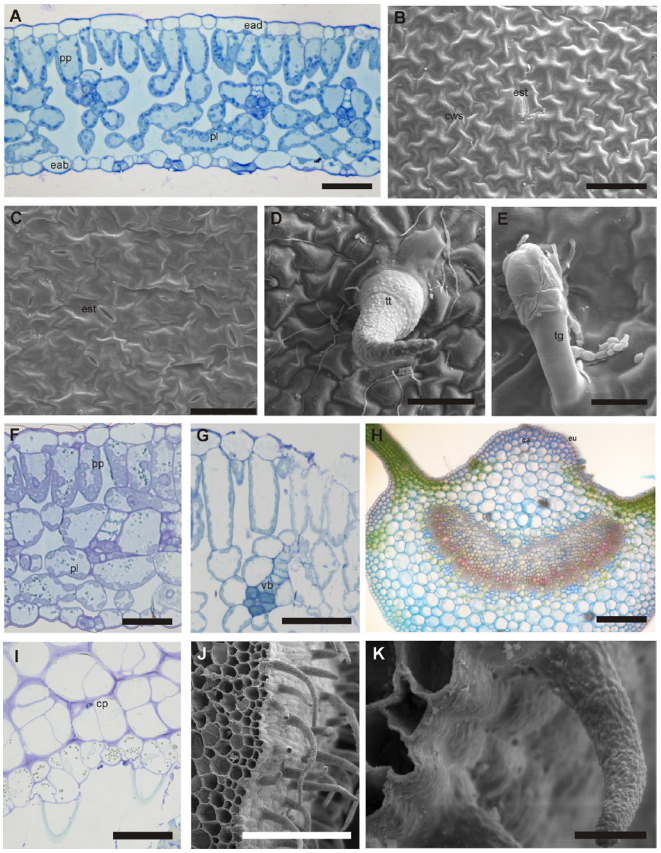

Figure 1. A. Cross-section of leaf from *Capsicum chinense*. **B.** Abaxial surface of the *C. baccatum* with ornamentation and fine cuticular wax layer epiticular. **C.** Cuticular ornamentation on the abaxial surface of *C. annum*. Anomocytic stomata on both leaf surfaces. **D.** Tector trichome-ornamented surfaces of the leaf the *C. chinense*. **E.** Cuticular ornamentation on the adaxial surface of the *C. frutescens*. Glandular trichome-ornamented surfaces of the leaf. **F.** Detail of the leaf blade. **G.** Detail of the vascular system the *C. baccatum*. **H.** Anatomical cut petiole the *C. chinense*. **I.** Tector trichome-ornamented surfaces of the petiole. **J.** Cross-section of petiole, the scanning electron microscope showing tector trichomes. **K.** Detail the tector trichomes ornamented. Bars: A and H = 50 µM; B and C = 25 µM; D to G, I, and K = 20 µM; J = 200 µM. pp = palisade parenchyma; ead = adaxial epidermis; pl = spongy parenchyma; eab = abaxial epidermis; est = stomata; cws = sinuous cell wall; tt = tector trichome; tg = glandular trichome.

In several plant species where the composition of the secretion has been characterized, this secretion is composed of different substances belonging to diverse chemical classes. Moreover, the secretion has been related to plant defense against pathogens and pests as indicated by biological activity across different organisms (Amme et al., 2005).

No polymorphism was detected for leaf blade. The four *Capsicum* species studied showed a dorsiventral mesophyll leaf blade (Figure 1F), which has been described by several authors for Solanaceae family (Cosa et al., 2002; Granada-Chacón et al., 2004). Dorsiventral mesophyll is the most frequent in the Solanaceae (Cosa et al., 2002; Granada-Chacón et al., 2004). The palisade parenchyma consisted of one layer of elongated cells and the spongy parenchyma showed 4-5 layers of cells with varying shapes and noticeable intercellular spaces (Figure 1A).

The bicollateral vascular bundles were immersed in the mesophyll of the four species studied, with intraxylary phloem (Figure 1G), which is a characteristic of the Solanaceae (Metcalfe and Chalk, 1983). Vesque (1875) discovered this type of cambium for the first time in the Solanaceae, Asclepiadaceae and Apocynaceae. Although intraxylary phloem, which is unidirectional and differentiated only into phloem elements in the centripetal direction, is reported in several other families, there are very few species that develop internal cambium at the pith margin.

The petiole of the 4 species studied, when viewed in cross-section and at the level of leaf insertion on the branches, exhibited a concave-convex contour. The epidermis was uniseriate and its cells were covered with a thin cuticle. The cortex was composed of 4-5 subepidermal layers of the angular collenchyma represented by 2-3 cells layers (Figure 1H). The parenchyma cells displayed a isodiametric shape, which varied in size and exhibited intercellular spaces of the meatus type (Figure 1I). The vascular system was arranged in an arc, with the phloem surrounding the xylem on both sides, featuring a bicollateral vascular system in the four species studied. Trichomes with verrucose ornamentation on the surfaces of the petiole were observed in the species *C. annuum*, *C. chinense* and *C. frutescens* (Figure 1J and K).

Seed anatomy

The seeds of the species studied were similar in structure and shape, but varied in relation to size (Table 3). These seeds were campylotropous, (Figure 2A), ellipsoid, long, and broad oval in longitudinal section (Figure 2B), elliptical in cross-section, with plicata on the longitudinal plane and constituted of embryo, endosperm and a mantle consisting of a silver film.

Table 3. Mean length, width and thickness of seeds from *Capsicum* species.

Species	Length (mm)	Width (mm)	Thickness (mm)	Weight of 1000 seeds (g)
C. chinense	4.37	3.92	0.82	6.57
C. annum	2.94	3.23	0.71	5.18
C. baccatum	2.71	2.84	0.77	5.91
C. frutescens	2.69	3.18	0.61	4.29

N = 100 individuals.

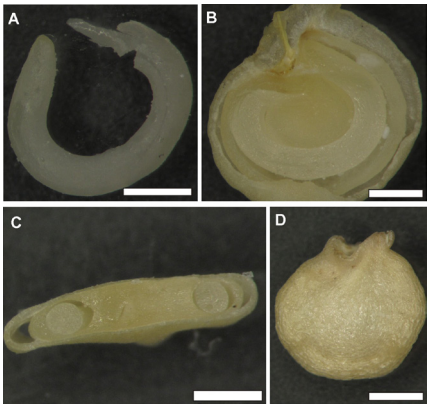

Figure 2. *Capsicum* sp. **A.** Longitudinal section of the embryo axis of circinate type. **B.** Longitudinal section of entire seed showing its ellipsoids, long, broad oval shape. **C.** Cross-section of entire seed showing its elliptic shape. **D.** Seeds of the *C. chinense*. Bars: A, C and D = 1.6X (1 mm); B = 1.2X (1 mm).

The seeds of the four species of *Capsicum* were albuminous (endosperm persists during the development of the embryo), with abundant endosperm, semi-transparent and whitish coloration (Figure 3A). In the literature, there are no reports of seeds with colors darker than the embryos. All four species had a circinate embryo (Figure 3A and B), and in general, the forms described fall within the characteristics described by Barroso et al. (1999) for the family Solanaceae. The tegument was reduced to an inner and outer layer with collapsed mesophyll (Figure 3C). The outer epidermis was uniseriate and juxtaposed, and their cells showed smooth external periclinal walls slightly undulated. The conspicuous thickening of the inner periclinal wall is highlighted in Figure 3, which was lignified as evidenced by the greenish-blue color with toluidine (Figure 3D). The epidermis consisted of an inner layer of cells with long, flat and rectangular shapes (Figure 3E). These characteristics coincide with those observed with scanning electron microscopy (Figure 3C). The tegument epidermis was differentiated in the four species, when observed by scanning electron microscopy. These differences are features that can aid the taxonomic identification of these species, as also stated by Castellani et al. (2008).

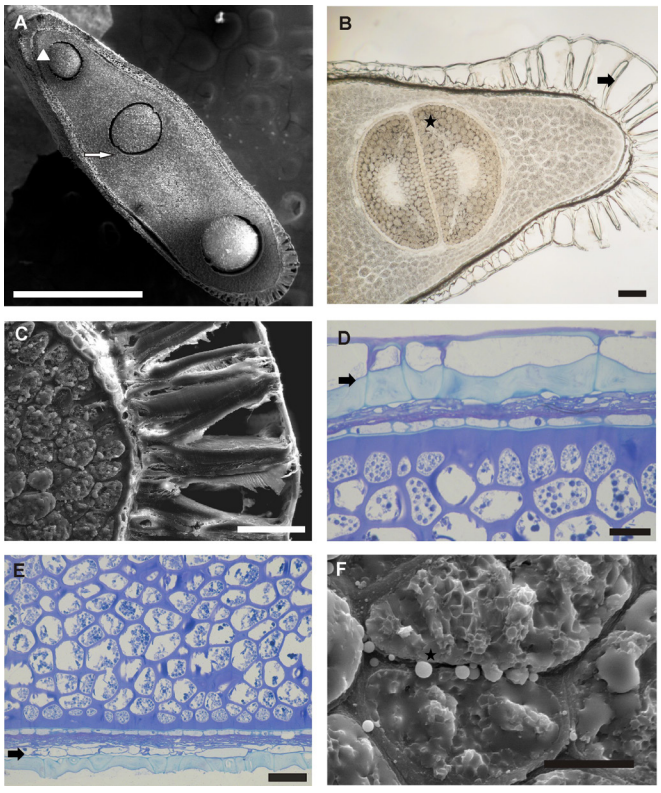

Figure 3. A. Cross-section of seed visualizing the embryonic axis (triangle) and a double cotyledon (arrow). **B.** Cross-section of seed visualizing the cotyledon (star) and testa (arrow). **C.** Surface of the outer epidermis of the integument with strongly undulating anticlinal wall. **D.** Epidermis external layer formed from a compact cell more or less thickened wall cells and undulated (arrow). **E.** Epidermis, which the internal layer is generally rectangular and with elongated cells (arrow). **F.** Detail of starch granules in the endosperm (star). Bars: A = 1 mm; B = 200 µM; C = 50 µM; D and E = 20 µM; F = 10 µM.

Fruit anatomy

According to Knapp (2002), pepper fruits in the conventional sense are classified as berries, that is, simple indehiscent fruit with pericarp that contains many seeds embedded in a solid mass and fleshy, with epicarp less than 2 mm in thickness and with air space between the seeds and pericarp.

Generally, the fruits of the four species investigated are small to medium, ranging from 1 to 8 cm in diameter (Costa et al., 2009). The fruits have many variations in color and shape (Moscone et al., 2007). They may be red, for example, *C. annuum*, *C. baccatum* and *C. frutescens*, or brown like *C. chinense*. The pericarp is composed of three clearly distinct areas: the exocarp, mesocarp and endocarp.

The exocarp of the fruits of the four studied species had uniseriate and smooth epidermis, with no overlapping tabular cells and with dense-textured and cellulosic walls typically found in berries. The cuticle was highly variable and usually thick as shown in the fruit of *C. chinense* (Figure 4A). In all species, the cuticle layer was present between the epidermal cells. Immediately below the epidermis, a differentiated hypodermis comprising several layers of a collenchyma was observed in *C. frutescens* (Figure 4B).

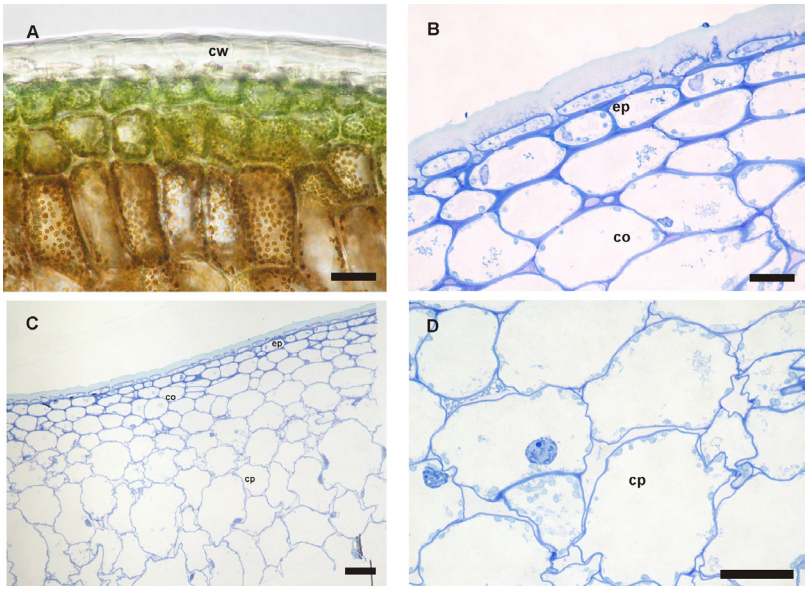

Figure 4. Cross-sections of the fruit of *Capsicum* sp. **A.** Detail of the cuticle of the fruit. **B.** Exocarp. **C.** Mesocarp. **D.** Detail of the endocarp. cw = cell wall; ep = epidermis; co = collenchyma; cp = cortical parenchyma. Bars: A and B = 20 µM; C = 10 µM; D = 40 µM.

The epidermis and hypodermis constitute a unit, the exocarp, which generally have layers with gradually decreasing degrees of lignification from the outside to inside of the fruit. Normally, in immature fruits, the cell layers located below the epidermis show chloroplasts and chromoplasts. In ripe fruits, chloroplasts disappear and the cells become compressed. The collenchyma is always present, with the number of layers and degree of lignification varying according to the species (Chiarini and Barboza, 2008).

The mesocarp comprised two histologically distinct zones: an external (immediately below the hypodermis) and an internal one. The external zone may have two forms, according to the kind of cell arrangement (Chiarinini and Barboza, 2008). The greater the number of mesocarp layers, the greater the thickness of the pericarp was. Fruits with a thick pericarp usually had more than 10 layers. The four species of *Capsicum* studied showed mesocarp composed of five collenchyma layers, followed by the parenchyma and vascular bundles accompanied by external fibers (Figure 4C).

Finally, no specific particularities were observed in the endocarp. This layer, which is very difficult to observe due to its delicate structure, was uniseriate and lacked stomata in all species studied. It appeared to be composed of parenchyma cells with different sizes and shapes and thin walls (Figure 4D). It is a well-known fact that the collenchyma is followed by a thick-walled parenchyma and that it is also difficult to draw a line between the two tissue types (Chiarini and Barboza, 2008).

Molecular analysis

A total of 41 primers were tested, and of these, 26 were selected and evaluated in regard to the number of bands generated and also the polymorphism observed with these bands. We obtained a total of 331 bands, of which 323 (91.2%) were polymorphic and 41 monomorphic (8.5%) (Table 2).

The average number of polymorphic bands produced by the primers was 12.03. The most polymorphic primer was $(CT)_8GC$ (indicated by number 21, Table 2), generating 27 polymorphic bands, followed by primer CGA_7 (indicated by number 16, Table 2), generating 24 polymorphic bands, $(CT)_8AC$ (indicated by number 20, Table 2), generating 20 polymorphic bands, $(GT)_6GG$, $TACA(GCA)_3G$ (indicated by number 19, Table 2), generating 12 polymorphic bands, and (GAA)EAA, CGA_7, $T(TTA)_4TT$ and $(GTG)_4RC$ (indicated by numbers 13, 14 and 15, Table 2), generating 14 polymorphic bands.

The dendrogram generated based on the molecular data indicated the formation of two main groups (Figure 5), the first one containing accessions of *C. annuum*, *C. baccatum* and *C. frutescens* and the second group containing only the accessions of *C. chinense*. In the first group, although it had been separated by species, the accessions formed 2 subgroups, one gathering accessions of *C. frutescens* and the other gathering the species *C. baccatum* and *C. annuum*. The dendrogram generated by the molecular characters also showed a cluster pattern different from the proposed division of the *Capsicum* genetic complex (Pickersgill, 1991). In this proposal, the genetic complex of *C. annuum* encompasses the domesticated species of *C. annuum* var. *annuum*, *C. chinense* and *C. frutescens*, which demonstrates the existence of a great proximity and higher possibility of gene exchange between these species, which also happens in the genetic complex of *C. baccatum*, which includes the *C. baccatum* varieties *pendulum*, *baccatum* and *praetermissum* and also *C. tovarii* (Tong and Bosland, 1999).

In our study, molecular analyses clustered *C. baccatum* with *C. annuum* and *C. frutescens*, separating the latter two species from *C. chinense*. The same result was observed by Costa et al. (2009) working with RAPD markers and morphoagronomic descriptors to estimate the genetic diversity between *Capsicum* accessions. In analyzing only morphoagronomic descriptors, these authors found accessions from *C. baccatum*, *C. annuum* and *C. frutescens* in the same cluster, while *C. chinense* accessions were placed in a different cluster. Also, the

authors hypothesized that this clustering could indicate some closeness and the possibility of gene exchange between *C. baccatum*, *C. annuum* and *C. frutescens*. The results obtained by Monteiro et al. (2011) support this hypothesis, since fertile hybrids were obtained between the species *C. annuum* var. *annuum* (sweet or hot pepper) and *C. baccatum* var. *pendulum* with pollen viability exceeding 90%. Moreover, Potnis et al. (2012) transferred one gene that controls resistance to bacterial spot from *C. baccatum* to *C. annuum,* showing that gene exchange between different *Capsicum* species is quite feasible.

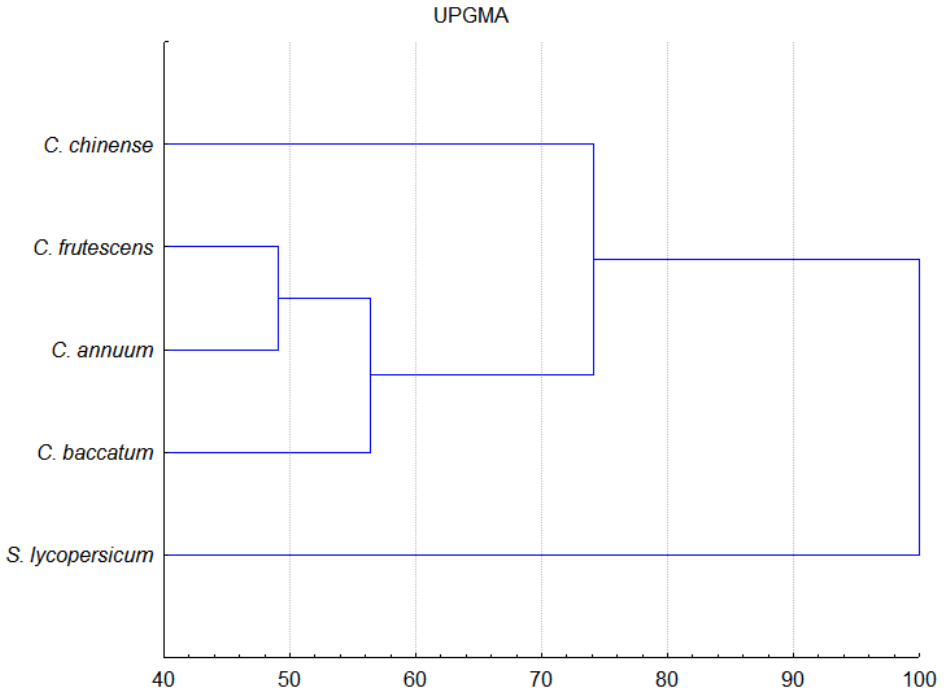

Figure 5. Dendrogram obtained by the UPGMA method based on molecular markers among the 4 accession of *Capsicum* spp belonging to the germplasm bank collection of Universidade Estadual do Norte Fluminense.

Despite the extensive polymorphism observed for ISSR markers, along with polymorphism for fruit and other agronomic traits, these differences were not reflected in the large variability for anatomical descriptors. Some studies concluded that the association between morphological, agronomic and molecular data is the most suitable approach to estimate *Capsicum* genetic divergence (Costa et al., 2009) or that joint analysis of quantitative and qualitative data resulted in greater efficiency in the determination of genetic divergence among the *Capsicum* accessions (Moura et al., 2010). Multivariate strategies such as the Ward-MLM methodology in data analysis for morphoagronomic characterization of accessions have allowed, with some level of efficiency, the separation of *Capsicum* species with the simultaneous use of morphological and agronomic variables (Sudré et al., 2010). However, Sudré et al. (2010) observed that only morphological descriptors can efficiently discriminate between *Capsicum* species and their botanical varieties.

CONCLUSION

Taking into consideration the complexity of the taxonomic classification of the genus *Capsicum* (Barbosa et al., 2006), the comparative study of the anatomy of four species of *Capsicum* demonstrated the existence of some anatomical variations between *C. baccatum*, *C. annuum*, *C. chinense*, and *C. frutescens*. However, many characters were present in the four species and may be typical of the genus. Polymorphism in the morphology and type of trichomes of *C. baccatum*, *C. annuum*, *C. chinense*, and *C. frutescens* can result in differential characters of these species. Some of these characters are valid in distinguishing the species of *Capsicum* and can also contribute to the taxonomic studies of Solanaceae plants. Other descriptors should be taken into consideration, such as flower morphology, to help in describing and discriminating *Capsicum* accessions. As expected, ISSR markers were able to detect a high level of polymorphism, but it was not reflected in anatomical ultrastructural characters.

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