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Analysis of molecular diversity of *Capsicum* spp. to identify redundant accessions and duplicates within accessions from a germplasm bank

Paulo Henrique Marquiori Visacre[1], Claudete Aparecida Mangolin[2], Eliane Rodrigues Monteiro[3], Angela Celis de Almeida Lopes[3], Hugo Zeni Neto[4] and Maria de Fátima Pires da Silva Machado[2]*

*[1]Graduate in Genetics and Breeding; [2]Department of Biotechnology, Genetics and Cell Biology; [3]Department of Biology, Universidade Federal do Piauí, Teresina PI Brasil; [4]Department of Agronomy, Universidade Estadual de Maringá, 87020-900 Maringá PR Brazil. *E-mail: mfpsmachado@uem.br*

Abstract

Assessment of genetic diversity and identification of redundant accessions in seed banks are important to minimize the risk of gene loss in collections and to decrease costs in the management of germplasm banks. Current study used markers at DNA level to evaluate genetic diversity and identify whether there are duplicate accessions of *Capsicum* spp. in the collections of an active germplasm bank. Randomly amplified polymorphic DNA markers using touchdown-polymerase chain reaction method evaluated genetic diversity and identified duplicates in ten accessions of *Capsicum* spp. Higher genetic diversity has been detected in *C. annuum* rather than in the species *C. chinense* and *C. baccatum*. DNA amplified segments revealed high genetic divergence showing that accessions of *C. annuum*, *C. chinense* and *C. baccatum* were separately grouped after data analysis with three software programs. Accessions of *C. baccatum* var. *pendulum* and *C. annuum* var. *glabriusculum* were the least similar. Since rates were less than 60% between accessions of *C. chinense* and *C. annuum*, they revealed low identifity of amplified DNA segments among the three *Capsicum* species. Redundance in four accessions of *C. chinense* and in five accessions of *C. annuum* was not detected. On the other hand, duplicates within accessions *C. chinense* (50%), *C. annuum* var. *glabrisculum* (5%) and *C. baccatum* var. *pendulum* (75%) were reported in the *Capsicum* active germplasm bank.

Key words: Bell pepper, bishop's crown pepper, chili pepper, RAPD td-PCR method, seed bank

Introduction

Peppers of the *Capsicum* genus are important crops worldwide because of their pungency and high nutritional value. *Capsicum* species are marketed *in natura* and are important raw material for production of condiments, spices, seasonings and preserves in agroindustry (Rebouças *et al.*, 2013). Brazil is an important center of diversity for *Capsicum* spp. where four domesticated species *C. annuum* L. (bell pepper, sweet pepper), *C. chinense* Jaqc. (yellow lantern chili), *C. frutescens* L. (malagueta pepper) and *C. baccatum* L. (bishop's crown pepper) are widely grown and consumed.

Besides their nutritional value, species of *Capsicum* (*C. annuum*) are also appreciated for their high ornamental value (Pessoa *et al*., 2018). The fruits morphological variations are the main characteristics often employed to evaluate the genetic diversity of *Capsicum* species and organize seed banks (Quresh *et al*., 2015, Abhinaya *et al.*, 2016**;** Naegele *et al*., 2016; Araujo *et al*., 2018; Luitel *et al*., 2018; Medeiros *et al*., 2018; Moreira *et al*., 2018; Solomon *et al*., 2019).

Conservation of diversity in germplasm banks is greatly relevant due to over exploitation of plant species. A seed bank may be a source of genetic diversity and the basis of plant survival in nature and for crop improvement. Therefore, it is important to plan and monitor genetic diversity in seed banks. The importance of evaluating the genetic diversity of plants and the study of methods applied for such investment were themes highlighted in a review by Bhandari *et al*. (2017). The authors emphasized that genetic diversity is the basis for the survival of plants *in natura*, for conservation and breeding programs.

A seeds bank of peppers of the *Capsicum* genus [Banco Ativo de Germoplasma de *Capsicum* (BAGC)] has been established and it has received pepper samples that are often grown in community gardens in various regions in Brazil. Pepper samples deposited in the seed bank are identified by their popular and scientific name, place and date of collection. Morphological and agronomic traits of plant and fruit are often employed to evaluate the genetic diversity in *Capsicum* germplasm collections (Pessoa *et al*., 2015a; 2015b; 2018) and to organize BAGC (Monteiro *et al*., 2010). Biochemical markers have also been employed to evaluate the genetic diversity of *Capsicum* species in the BAGC. (Monteiro *et al*., 2013). However, the biochemical markers failed to identify duplicate samples of seeds in the *C. chinense*, *C. annuum* and *C. baccatum* existed in BAGC collections.

Markers at DNA level, such as randomly amplified polymorphic DNA (RAPD), have been largely employed to evalute genetic diversity and genetic divergence among accessions, to study genealogies and phylogenetic relationships, genetic mapping,

intercrossing rates, to identify cultivars and duplicates of accessions in *Capsicum* spp. germplasms (Renganathan *et al*., 2017; Taha *et al*., 2019). Our hypothesis in current study is that RAPD markers using touchdown-polymerase chain reaction (td-PCR) method may be useful to evaluate genetic diversity and identify duplicates in ten accessions of *C. chinense*, *C. annuum* and *C. baccatum* at BAGC. The annealing temperature in the td-PCR method is gradually decreased during the PCR. In the first PCR cycles, the annealing temperature is programmed at 5-10°C above the primer's melting temperature (Tm). The highest temperature favors pairing between the primer and specific bases of the template DNA. Only the specific product will be amplified. In the following cycles the annealing temperature is reduced to 2-5°C below Tm. Consequently, the specific products previously amplified, featuring a greater quantity, will be amplified preferentially. The td-PCR method reduces the amplification of unrepresentative RAPD markers. A combined use of RAPD analysis with td-PCR method was employed to safely analyze the genetic diversity in the ten accessions of *C. chinense*, *C. annuum* and *C. baccatum* collections at BAGC. Current analysis aims at evaluating genetic diversity using markers at DNA level to identify whether there are duplicate accessions of *C. chinense*, *C. annuum* and *C. baccatum* in the BAGC collections.

Materials and methods

***Capsicum* species:** Seeds of four accessions of *C. chinense* (BAGC 06, BAGC 07, BAGC 23 and BAGC 24), five *C. annuum* (BAGC 11, BAGC 36, BAGC 40, BAGC 59 and BAGC 67) and one *C. baccatum* (BAGC 26) from the seed bank BAGC were collected from home and community gardens and identified with their scientific and common names (Table 1). Fruits from each accession are shown in Fig. 1 (a-j). The seeds of 10 plants per accession were germinated in 128-cell trays using commercial substrates and maintained in an acclimatized chamber (Fig. 2a). After germination, seedlings (with 4-6 leaves) were transferred to pots (Fig. 2b) containing soil and substrate (1:1) and maintained in a house garden. Young leaves of each seedling were individually stored in labelled plastic screen bags to avoid the mixture of varieties, maintained at 4°C, and transferred to the laboratory. The samples were frozen in liquid nitrogen at the laboratory and stored at -80°C until DNA extraction.

DNA extraction: DNA was extracted from eight seedlings of each *Capsicum* species (10 accessions; Table 1) according to protocol originally described by Doyle and Doyle (1990).

Table 1. Seeds of accessions of *C. chinense* (BAGC 06, BAGC 07, BAGC 23, and BAGC 24), *C. annuum* (BAGC 11, BAGC 36, BAGC 40, BAGC 59, and BAGC 67), and *C. baccatum* (BAGC 26) identified with the scientific and common names from seed bank BAGC (Active *Capsicum* Germplasm Bank)

Accession	Common Name	Species
BAGC-06	Murici	*C. chinense*
BAGC-07	Girl's Chest	*C. chinense*
BAGC-11	Girl's Chest	*C. annuum* var. *glabriusculum*
BAGC-23	Fisheye	*C. chinense*
BAGC-24	Girl's Finger	*C. chinense*
BAGC-26	Bishop's Crown Pepper	*C. baccatum* var. *pendulum*
BAGC-36	Table Pepper	*C. annuum* var.*glabriusculum*
BAGC-40	Long Mexican Pepper	*C. annuum* var. *annuum*
BAGC-59	Unnamed	*C. annuum* var. *glabriusculum*
BAGC-67	Unnamed	*C. annuum* var. *glabriusculum*

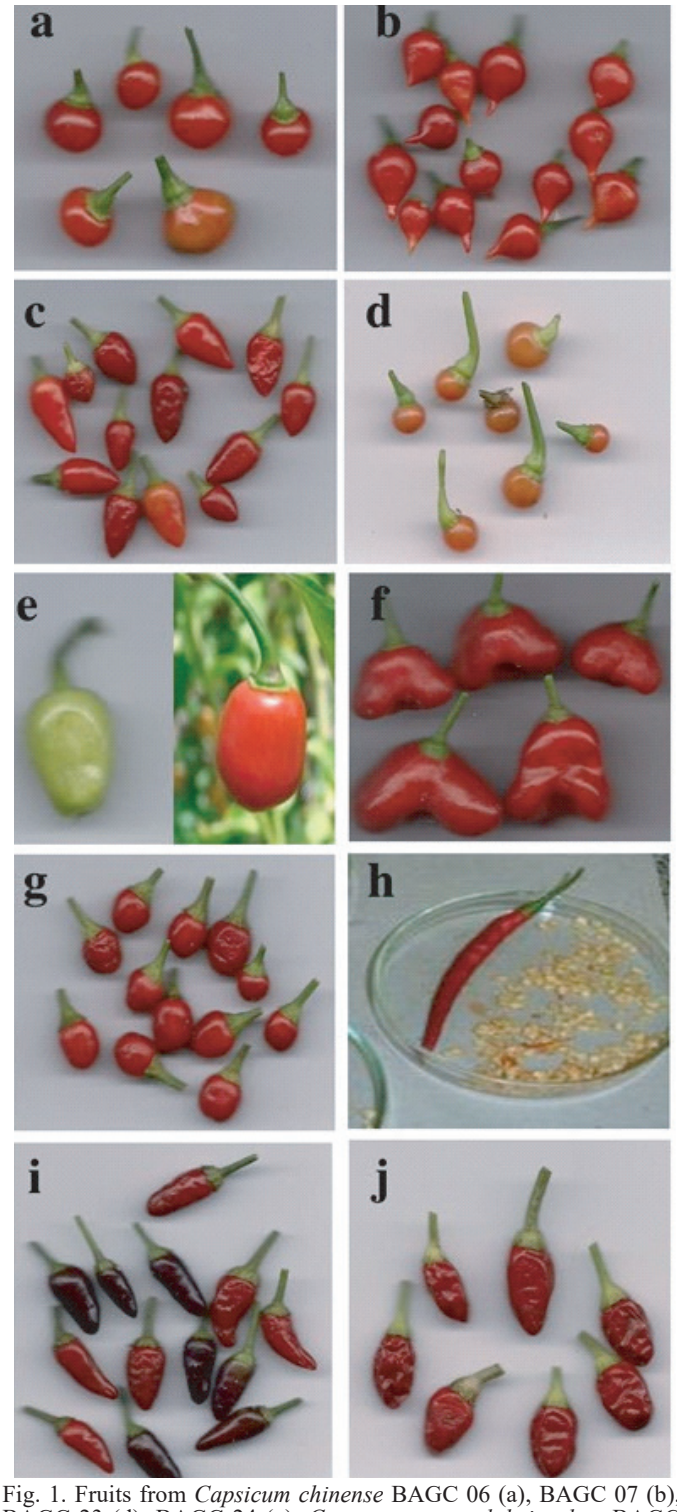

Fig. 1. Fruits from *Capsicum chinense* BAGC 06 (a), BAGC 07 (b), BAGC 23 (d), BAGC 24 (e), *C. annuum* var. *glabrisculum* BAGC 11 (c), *C. baccatum* var. *pendulum* BAGC 26 (f) and *C. annuum* var. *glabrisculum* BAGC 36 (g), BAGC 40 (h), BAGC 59 (i), BAGC 67 (j).

Reactions were carried out in microcentrifuge tubes (1.5 mL) containing 100 mg of leaves of each seedling pulverized with liquid nitrogen. The quality of the isolated DNA was determined by electrophoresis (80 V) in a 0.8% agar gel, using TAE (0.04 M Tris-acetate, 0.001 M EDTA) buffer. DNA quantification (10-100 ng·μL^{-1}) was given by visual comparison with known quantities (50, 100, 150 ng) of lambda phage DNA (Invitrogen). After electrophoresis, the gel was incubated with ethidium bromide (0.5 μg·mL^{-1}) and photographed with a L-Pix HE photo-documenter (Loccus) by L-Pix Image software.

Fig. 2. Seedlings of *Capsicum chinense* (BAGC 06, BAGC 07, BAGC 23, BAGC 24), *C. baccatum* var. *pendulum* (BAGC 26) and *C. annuum* var. *glabrisculum* (BAGC 11, BAGC 36, BAGC 40, BAGC 59, and BAGC 67) from seeds germinated in cell trays maintained in an acclimatized chamber (a) and transferred to pots containing soil and substrate (1:1) and maintained at a home garden (b).

Amplification reactions using td-PCR RAPD markers: Amplification reactions were performed in an aseptic chamber in a 20 µL volume containing 20 ng of genomic DNA, 2.0 µL reaction buffer (10 mM Tris–HCl, pH 8.8), 3.0 mM $MgCl_2$, 0.15 mM of dATP, dGTP, dCTP, and dTTP each, 0.3 µM primer, 1 unit of Taq Polymerase Platinum (Invitrogen) and Milli-Q water, to bring the reaction to the final volume. Twenty primers (OPA-01, OPA-07, OPA-09, OPA-10, OPA-12, OPA-13, OPA-17, OPA-18, OPA-19, OPA-20, OPB-01, OPB-04, OPB-05, OPB-08, OPB-10, OPB-17, OPC-08, OPC-11, OPC-20 and OPP-09; Operon Technologies Inc. Alameda CA, USA) were selected from 83 primers and used in the amplification reactions (Table 2).

Table 2. Primers (Operon Technologies Inc. Alameda CA, USA) employed in the amplification reactions, their sequences, number of total DNA amplified segments (NTS), number of polymorphic segments (NPS) and polymorphism (%) observed with use of each primers in accessions of *C. chinense*, *C. annuum*, and *C. baccatum* from seed bank BAGC (Active *Capsicum* Germplasm Bank)

Primers	Sequences	NTS	NPS	P(%)
OPA-01	5'-CAGGCCCTTC-3'	17	17	100
OPA-07	5'-GAAACGGGTG-3'	19	19	100
OPA-09	5'-GGGTAACGCC-3'	15	15	100
OPA-10	5'-GTGATCGCAG-3'	18	17	94.45
OPA-12	5'-TCGGCGATAG-3'	14	12	85.7
OPA-13	5'-CAGCACCCAC-3'	17	14	82.35
OPA-17	5'-GACCGCTTGT-3'	09	8	88.89
OPA-18	5'-AGGTGACCGT-3'	19	19	100
OPA-19	5'-CAAACGTCGG-3'	20	17	85
OPA-20	5'-GTTGCGATCC-3'	16	16	100
OPB-01	5'-GTTTCGCTCC-3'	15	13	86.67
OPB-04	5'-GGACTGGAGT-3'	12	11	91.67
OPB-05	5'-TGCGCCCTTC-3'	18	16	88.89
OPB-08	5'-GTCCACACGG-3'	16	15	93.75
OPB-10	5'-CTGCTGGGAC-3'	15	12	80
OPB-17	5'-AGGGAACGAG-3'	20	17	85
OPC-08	5'-TGGACCGGTG-3'	17	14	82.35
OPC-11	5'-AAAGCTGCGG-3'	19	18	94.74
OPC-20	5'-ACTTCGCCAC-3'	18	18	100
OPP-09	5'-GTGGTCCGCA-3'	19	18	94.74
Total		333	306	91.71

Selection of the 20 primers was performed to amplify peppers' DNA and produce clear-cut, accurate and separate bands on the gel. Amplifications were performed in duplicate with Techne TC-512 Thermocycler following method by Ince *et al*. (2010). Initially, PCR program conditions were denaturation for 3 min at 94°C; 10 cycles of denaturation at 94°C for 1 min; at 42°C for 45 seg and a final extension time at 72ºC for 2 min. The annealing temperature during the 10 cycles was reduced by 0.5ºC for each cycle, starting with an annealing temperature of 42ºC and the annealing temperature at 37ºC for the last cycle. After this initial step, 30 more cycles were performed with denaturation at 94°C for 1 min; at 37°C for 45 seg, and an extension for 1 min at 72°C. A final 10 min extension was carried out at 72°C.

The amplification products were separated by electrophoresis on a 1.7% agar gel and 0.5X TBE buffer (44.5 mmol·L^{-1} Tris, 44.5 mmol·L^{-1} boric acid, 1.0 mmol·L^{-1} EDTA) at 60V, for 5 hr. Gels were stained with ethidium bromide (0.5 mg·mL^{-1}) and the images were captured in a L-Pix HE photo-documenter (Loccus) by L-Pix Image software. A 1 Kb DNA Ladder (Invitrogen) was used as a size marker.

Data analysis: Polymorphisms from td-PCR RAPD markers were analyzed as dominant markers [(1) presence and (0) absence of amplified DNA segments]. Polymorphism was analysed using software R (R Core Team, 2019) with the packages: NbClust (Charrad *et al*., 2014), factoextra (Kassambara and Mundt, 2019), and dendextend (Galili, 2015). The distance similarity matrix was computed with Jaccard coefficient, followed by UPGMA clustering method, with resampling analysis using 5000 replications. A dendrogram was constructed based on a reference tree using software R. The NTSYS-pc package was also used to compare the 80 samples of *Capsicum* species.

Main component analysis (PCA) was performed to reduce the data set without losing important information from the original data of the 10 *Capsicum* acessions (BAGC-06, BAGC-07, BAGC-11, BAGC-23, BAGC-24, BAGC-26, BAGC-36, BAGC-40, BAGC-59, BAGC-67). The PCA groups specimens according to the variation of their characteristics. Analysis of molecular variance (AMOVA) was performed with GenAIEx 6.41 to explore the hierarchical partitioning of genetic variation within and between the 10 accessions of *Capsicum.*

Polymorphisms of randomly amplified DNA segments were also analyzed using STRUCTURE software 2.0, which evaluates the level of genetic admixture between the 80 samples of *Capsicum* species. The peppers were clustered, with number of clusters (K) ranging between 2 and 10 and were tested by the admixture model with a burn-in period of 3,000 repeats, followed by 30,000 Markov Chain Monte Carlo (MCMC) repeats, taking the presence and absence of alleles across the sample into account. The true number of populations (K) was identified by the maximal value of Δ (K) returned by the software. The graphical output display of the STRUCTURE results was taken as input data using the STRUCTURE HARVESTER, a website and software employed to visualize STRUCTURE output and to implement the Evanno method (Earl and vonHoldt, 2012) for graphic representation.

Results

Three hundred and thirty-three DNA segments were amplified by 20 RAPD primers (OPA-01, OPA-07, OPA-09, OPA-10, OPA-12, OPA-13, OPA-17, OPA-18, OPA-19, OPA-20, OPB-01, OPB-04, OPB-05, OPB-08, OPB-10, OPB-17, OPC-08, OPC-11, OPC-20 and OPP-09; Table 2). The number of bands produced for each primer ranged between 9 (OPA-17) and 20 (OPA-19), with an average of 16.65 amplicons per primer. The size of the amplified products ranged between 400 and 5000 bp. Primer OPA-19 generated the highest number of amplified segments (20), while the highest numbers of polymorphic segments (100%) in the 10 accessions of *Capsicum* were detected for OPA-01, OPA-07, OPA-09, OPA-18, OPA-20 and OPC-20 primers (Table 2).

Thus, primers OPA-01, OPA-07, OPA-09, OPA-18, OPA-19, OPA-20 and OPC-20 may be preferred to compare accessions of *C. chinense*, *C. annuum* and *C. baccatum* at BAGC.

The highest polymorphism rate was reported in BAGC-59 (8.7%; *C. annuum* var. *glabriusculum*), whilst the lowest occurred in BAGC-06 (0.6%; *C. chinense*) and BAGC-26 (0.6%; *C. baccatum* var. *pendulum*) accessions. Polymorphism rates were higher in *C. annuum* (1.2 to 8.7%) than in *C. chinense* (0.6 to 3.9%) accessions (Table 3). AMOVA showed higher genetic variation among (95%) than within (5%) the 10 *Capsicum* accessions.

Table 3. Number of total DNA amplified segments (NTS), number of polymorphic segments (NPS), and polymorphism (%) observed in each accessions of *C. chinense* (BAGC-06, BAGC-07, BAGC-23, BAGC24), *C. annuum* (BAGC-11, BAGC-36, BAGC-40, BAGC-59, BAGC-67), and *C. baccatum* (BAGC-26) from seed bank BAGC (Active *Capsicum* Germplasm Bank)

Accessions	NTS	NPS	P (%)
BAGC-06	333	02	0.60
BAGC-07	333	13	3.90
BAGC-11	333	04	1.20
BAGC-23	333	05	1.50
BAGC-24	333	05	1.50
BAGC-26	333	02	0.60
BAGC-36	333	11	3.30
BAGC-40	333	12	3.60
BAGC-59	333	29	8.70
BAGC-67	333	23	6.90

The estimated genetic divergence level for td-PCR RAPD markers of the 10 *Capsicum* accessions, represented by G_{ST}, was also remarkably high (G_{ST} = 0.9553). G_{ST} is defined as the proportion of genetic diversity that resides among populations and was estimated by Nei´s method with POPGENE 1.32. It is equivalent to Wright's F_{ST} when there are only two alleles at a locus and, in the case of multiple alleles, G_{ST} is equivalent to the weighted average of F_{ST} for all alleles. G_{ST} rates between 0.15 and 0.25 indicate high level of interpopulation divergence or high level of genetic differentiation between populations.

The genetic similarity rate estimated by Nei´s coefficient showed that accessions BAGC-23 (*C. chinense*) and BAGC-24 (*C. chinense*) were similar (I = 0.9183), whilst similarity between accessions BAGC-26 (*C. baccatum* var. *pendulum*) and BAGC-67 (*C. annuum* var. *glabriusculum*) was lowest (I = 0.4446) (Table 4).

Dendrogram generated by Nei´s coefficient from the analysis of individual seedlings (constructed from td-PCR RAPD data using NbClust package from R Development Core Team, 2019) identified two well-defined larger groups with smaller sub-groups within each group and one isolated group formed by accession *C. baccatum* (BAGC-26) (Fig. 3). Four sub-groups formed by accessions *C. chinense* (BAGC-06, BAGC-07, BAGC-23 and BAGC-24) were identified within group 1 and five sub-groups, formed by accessions *C. annuum* (BAGC-11, BAGC-36, BAGC-59, BAGC-67 and BAGC-40), were identified within group 2.

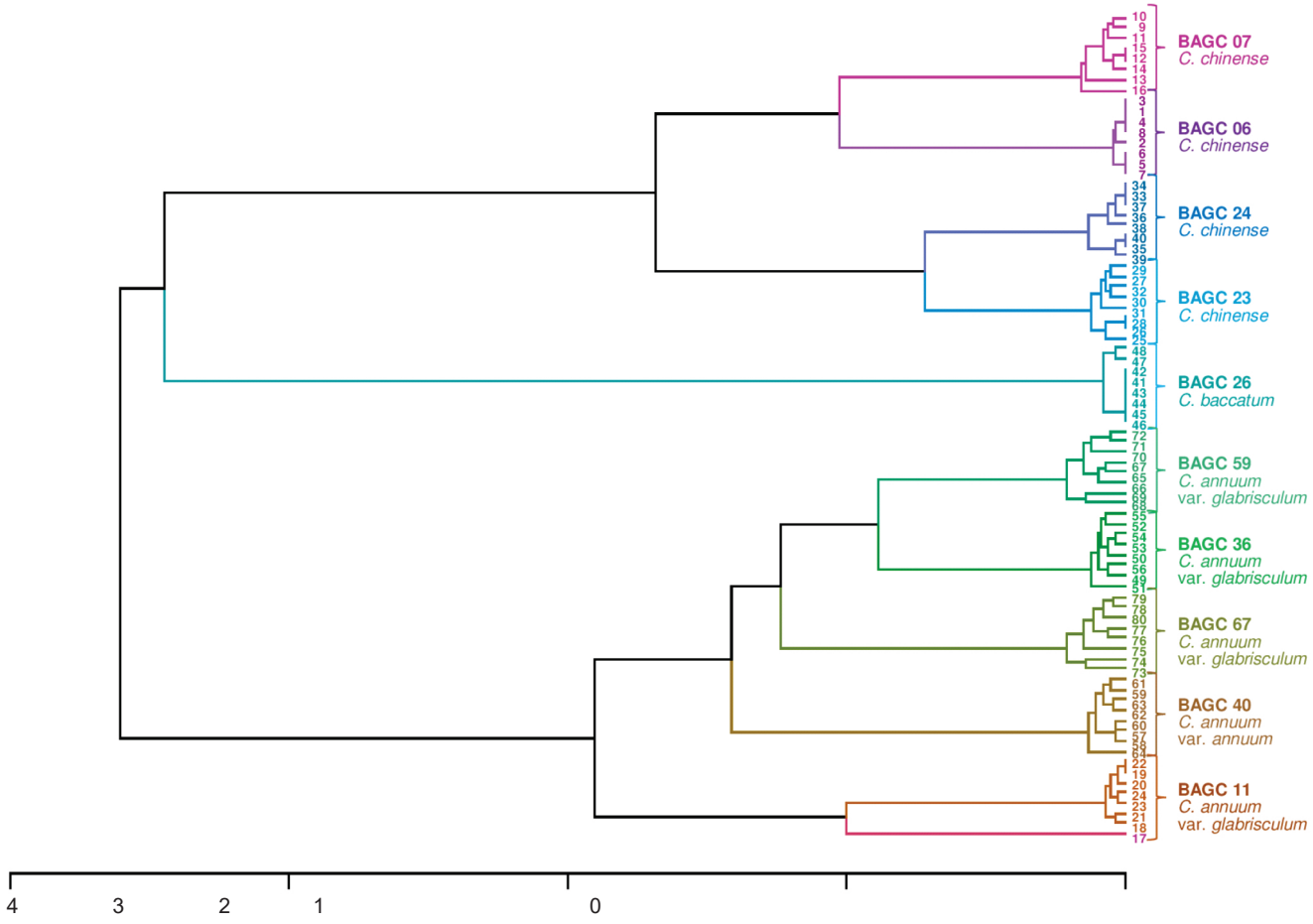

Fig. 3. Dendrogram generated by Nei´s coefficient from analysis of individual plants of *Capsicum chinense* (BAGC 06, BAGC 07, BAGC 23, BAGC 24), *C. baccatum* var. *pendulum* (BAGC 26) and *C. annuum* var. *glabrisculum* (BAGC 11, BAGC 36, BAGC 40, BAGC 59, and BAGC 67) constructed from data of twenty primers for RAPD touchdown-PCR markers using adegenet package from R Development Core Team (2019).

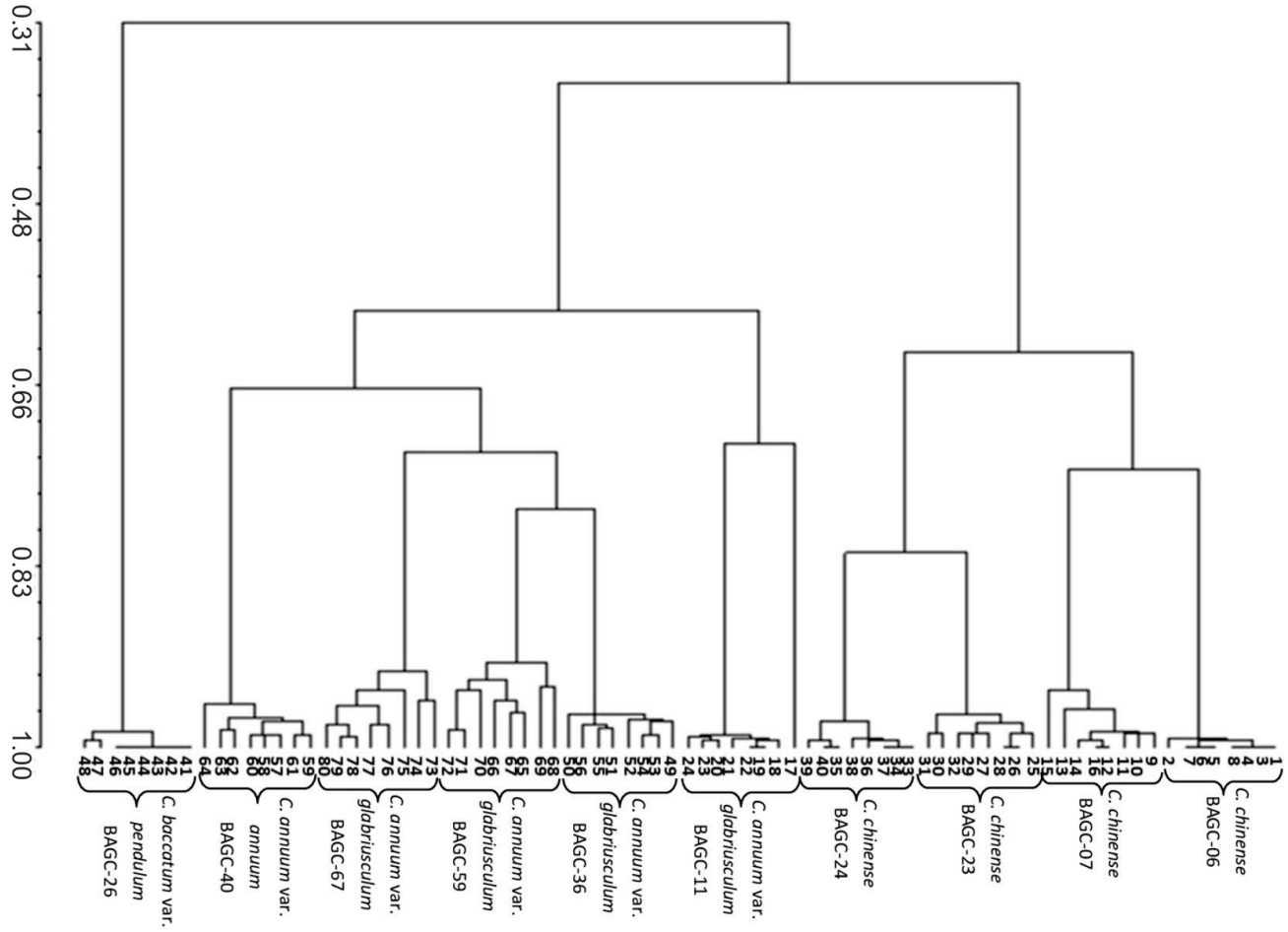

Fig. 4. Dendrogram generated by Jaccard coeficient, followed by UPGMA clustering method, from analysis of individual plants of *Capsicum chinense* (BAGC 06, BAGC 07, BAGC 23, BAGC 24), *C. baccatum* var. *pendulum* (BAGC 26) and *C. annuum* var. *glabrisculum* (BAGC 11, BAGC 36, BAGC 40, BAGC 59, and BAGC 67) constructed from data of twenty primers for RAPD touchdown-PCR markers using the NTSYS-pc package.

Dendrogram generated by Jaccard´s coefficient by analyzing each 80 pepper seedlings constructed by NTSYs pc package also showed two well-defined larger groups and smaller sub-groups within each larger group (Fig. 4). Four sub-groups formed by accessions *C. chinense* (BAGC-06, BAGC-07, BAGC-23 and BAGC-24) were identified within group 1, five sub-groups formed by accessions *C. annuum* (BAGC-11, BAGC-36, BAGC-59, BAGC-67 and BAGC-40), were identified within group 2, and accession *C. baccatum* (BAGC-26) formed one isolated group. Dendrograms showed homogeneous sub-groups formed by each accession and genetic identity (I = 1.0) within accession. The td-PCR RAPD pattern was *i.e.* tical in 50% of *C. chinense* seedlings, while the genetic identity in *C. annuum* reached only 5%. In the sub-group formed by BAGC 11 accession of *C. annuum* var. *glabriusculum,* the seedling 17 formed one isolated sub-group. Seedling 17 showed a pattern of td-PCR RAPD which is divergent from the pattern detected in the other seven plants of accession BAGC 11. The largest number of identical seedlings was observed in BAGC-06 accession of *C. chinense*. In the *C. baccatum* accession, the pattern of the td-PCR RAPD was identical in 75% of seedlings.

The principal component analysis (PCA) based on 333 molecular markers showed that total variance provided by first three components was 87.6% (PC1 = 60.6%; PC2 = 21.1%; PC3 = 5.9%). The first component (PC1) accounted for the highest rate 60.6% of total variation (Fig. 5).

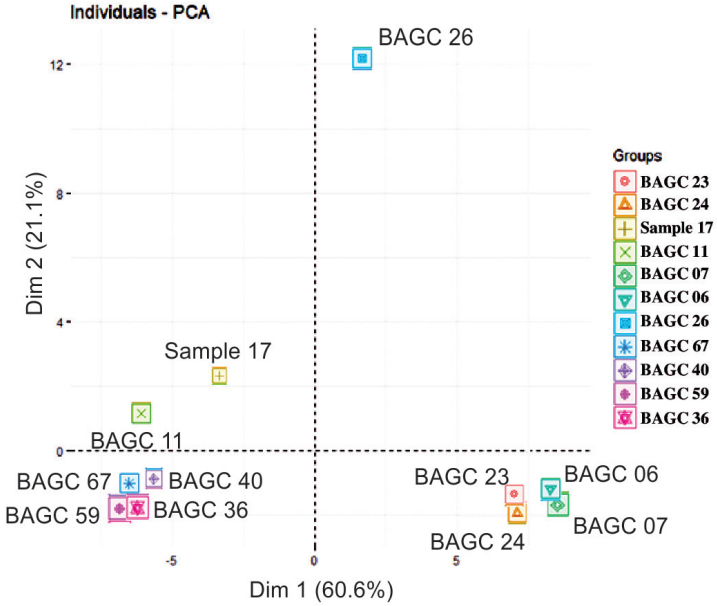

Fig. 5. The PCA groups individuals of *Capsicum chinense* (BAGC 06, BAGC 07, BAGC 23, BAGC 24), *C. baccatum* var. *pendulum* (BAGC 26) and *C. annuum* var. *glabrisculum* (BAGC 11, BAGC 36, BAGC 40, BAGC 59, and BAGC 67) constructed from data of twenty primers for RAPD touchdown-PCR markers according to the variation of their characteristics.

Fig. 6 shows the clustering of the 80 seedlings of 10 *Capsicum* accessions according to a model-based Bayesian algorithm. Each bar in the graph represents a plant, while colors represent different proportions of plants in each group. The optimal K rate determined

Table 4. Nei's similarity and genetic distance among 10 accessions of *C. chinense* (BAGC-06, BAGC-07, BAGC-23, BAGC24), *C. annuum* (BAGC-11, BAGC-36, BAGC-40, BAGC-59, BAGC-67), and *C. baccatum* (BAGC-26). Coefficients were calculated from analysis of 333 DNA segments amplified by td-PCR RAPD.

Acession	BAGC 06	BAGC 07	BAGC 11	BAGC 23	BAGC 24	BAGC 26	BAGC 36	BAGC 40	BAGC 59	BAGC 67
BAGC 06	*****	0.8632	0.5796	0.8043	0.7971	0.5149	0.5963	0.5800	0.5655	0.5656
BAGC 07	0.1471	*****	0.5665	0.8006	0.7885	0.4933	0.5798	0.5786	0.5514	0.5742
BAGC 11	0.5454	0.5682	*****	0.5328	0.5436	0.5284	0.7441	0.7149	0.7402	0.7950
BAGC 23	0.2178	0.2225	0.6467	*****	0.9183	0.4811	0.5679	0.5574	0.5522	0.5398
BAGC 24	0.2267	0.2376	0.6096	0.0852	*****	0.5014	0.5550	0.5697	0.5449	0.5447
BAGC 26	0.6638	0.7067	0.6380	0.7316	0.6904	*****	0.4878	0.4624	0.4569	0.4446
BAGC 36	0.5171	0.5452	0.2956	0.5658	0.5888	0.7178	*****	0.7928	0.8905	0.8092
BAGC 40	0.5447	0.5471	0.3356	0.5844	0.5626	0.7713	0.2321	*****	0.8049	0.8020
BAGC 59	0.5701	0.5952	0.3009	0.5939	0.6072	0.7833	0.1160	0.2170	*****	0.8786
BAGC 67	0.5699	0.5549	0.2295	0.6166	0.6075	0.8107	0.2117	0.2206	0.1295	*****

Nei's identity (I) above the diagonal and genetic distance (D) below the diagonal.

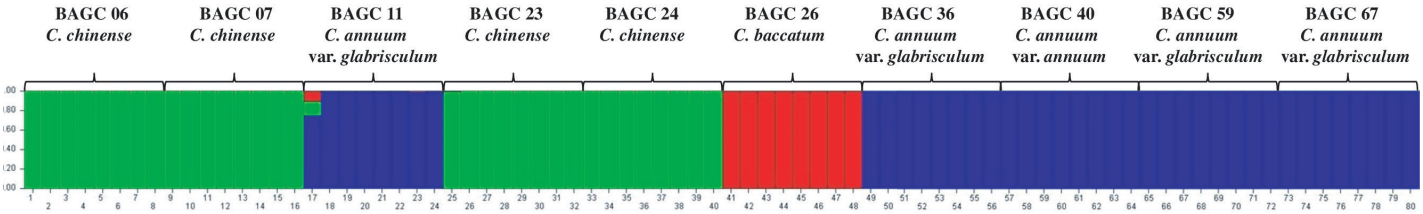

Fig. 6. Bar plot-like population structure based on RAPD *touchdown-PCR* markers for 80 plants of the *Capsicum chinense* (BAGC 06, BAGC 07, BAGC 23, BAGC 24), *C. baccatum* var. *pendulum* (BAGC 26) and *C. annuum* var. *glabrisculum* (BAGC 11, BAGC 36, BAGC 40, BAGC 59, and BAGC 67) within the K clusters. Each plant is represented by a single vertical bar broken in K colored segments (K = 3), with lengths proportional to each of the K inferred clusters. Each color represents the proportion of DNA segments for every individual represented by a vertical bar, in each group.

Table 5. Proportion of 80 pepper seedlings of *C. baccatum*, *C. annuum* and *C. chinenese* in each group (K = 3) analyzed by STRUCTURE software 2.0

Species	Number of plants	Group		
		Red	Green	Blue
C. *baccatum*	8	1.0000	0.0000	0.0000
C. annuum	40	0.0165	0.0175	0.9658
C. chinense	32	0.0010	0.9999	0.0000

by Bayesian analysis indicated that the 80 pepper seedlings were grouped into 3 subpopulations (ΔK2 = 0.00; ΔK3 = 2179.1710; ΔK4 = 6.3481; ΔK5 = 38.2466; ΔK6 = 0.6819; ΔK7 = 0.1970; ΔK8 = 0.6974; ΔK9 = 3.1237; ΔK10 = 0.00). The bar plot obtained for the K rate (K = 3; ΔK = 2179.1710) and results were consistent with the evidence that 100% of the *C. baccatum* seedlings belong to the red group; 96.58% of the *C. annuum* seedlings belong to the blue group; and 99.99% of the *C. chinense* seedlings belong to the green group (Table 5). The greatest mix of genome groups was detected in one seedling (sample 17) of *C. annuum* var. *glabriusculum*: 75, 14 and 10% of the blue, green and red groups, respectively.

Discussion

Polymorphism of td-PCR-RAPD markers detected in 10 accessions of *Capsicum* from seed bank BAGC showed higher genetic diversity in *C. annuum* than in *C. chinense* and *C. baccatum*. Genetic diversity in *C. annuum* which is higher than in *C. chinense* and *C. baccatum* has also been described in several studies comparing morphoagronomic traits (number of fruits per plant, size, shape, weight, length, surface and colour of fruits) and different molecular markers (Krishnamurthy *et al*., 2015; Zhang *et al*., 2016; López-Castilla *et al*., 2019). *C. annuum* species is the most widespread and economically important *Capsicum* species worldwide and the most used in commercial cultivar breeding programs (Bosland and Votava, 2012).

In spite of the continued selection to obtain *C. annuum* lines that may lead to dramatic reduction in the genetic basis (Tang *et al*., 2010; Sudré *et al*., 2010), high genetic diversity still has been related in several landraces and accessions. Aguilar-Meléndez *et al*. (2009) found an average reduction of only 10% in the diversity of domesticated relatives from the *C. annuum* species within Mexican populations.

High genetic diversity in *C. annuum* from different geographic regions has been reported for several morphoagronomic traits (Zhigila *et al*., 2014; Quresh *et al*., 2015; Abhinaya *et al*., 2016; Bracho-Gil *et al*., 2018), biochemicals (Abhinaya *et al*., 2016; Ridzuan *et al*., 2019) and molecular markers (Rana *et al*., 2014; Naegele *et al*., 2015; Tsaballa *et al*., 2015; Tsonev *et al*., 2017), as well as for associated morphological and molecular marker traits (Hayano-Kanashiro *et al*., 2015; Lee *et al*., 2016; Toledo-Aguilat *et al*., 2016; Rivera *et al*., 2016; Zhang *et al*., 2016; Du *et al*., 2019) and chemical and molecular traits (Taha *et al*., 2019). Toledo-Aguilat *et al*. (2016) revealed broad genetic diversity among *C. annuum* var. *annuum* populations from southern, central and northern Mexico.

In spite of lower genetic diversity in the four *C. chinense* accessions in current study, several studies have revealed high diversity in morpho-agronomic (Costa *et al*., 2015; Baba *et al*., 2016; Araújo *et al*., 2018) and molecular (Baba *et al*., 2016) traits. The great genetic variability in *C. chinense* has been reported in fruit colors and shapes (Luitel *et al*., 2018). In contrast to the low molecular diversity observed in *C. chinense* (BAGC-06, BAGC-07, BAGC-23 and BAGC-24), variability in the shape of fruits in accessions of *C. chinense* was also reported in our study. Four different shapes of fruits were identified in four accessions of *C. chinense* and four different shapes and colour of fruits were identified in four accessions of *C. annuum* var. *glabrisculum* (Fig. 1a-j). *C. annuum* var. *annuum* and *C. annuum* var. *glabrisculum* showed different shapes of fruits (Fig. 1h).

The well-defined separate groups of *C. chinense* (BAGC-06, BAGC-07, BAGC-23 and BAGC-24), *C. annuum* (BAGC-11, BAGC-36, BAGC-40, BAGC-59 and BAGC- 67) and isolated group of *C. baccatum* (BAGC-26) were generated from the analysis of individual seedlings and revealed high genetic divergence (G_{ST} = 0.9553) among the 10 analyzed accessions. High genetic divergence has been reported in landraces of *C. annuum* and *C. chinense* due to particular biological characteristics of the species, different center of domestication, or inbreeding (Castilla *et al*., 2019). Phenotypic similarity, biochemical and agromorphological properties analysed in other studies have shown accessions of *C. baccatum* as an isolated group of *C. annuum* accessions (Albrecht *et al*., 2012; Krishnamurthy *et al*., 2015). The esterase analysis showed high genetic diversity among the *C. chinense* and *C. annuum* samples and very high genetic differentiation among the *C. chinense, C. annuum* and *C. baccatum* accessions (Monteiro *et al.*, 2013). *C. baccatum* is considered underexploited for germplasm improvement (Albrecht *et al*., 2012) and more distant to the other *Capsicum* species (Mongkolporn *et al.*, 2015). Few studies are available on *C. baccatum* with regard to its breeding and genetics (Rodrigues *et al*., 2012; Oliveira *et al*., 2015). Molecular markers have shown that accessions of *C. annuum*, *C. chinense* and *C. baccatum* were separately grouped (Zhang *et al*., 2016). Sequencing analysis has also shown the *C. baccatum* cluster located between the *C. annuum* and *C. chinense* groups (Pereira-Dias *et al*., 2019).

Low identity rates (0.4446-0.5284) were observed between accessions of *C. baccatum* and *C. chinense,* and *C. baccatum* and *C. annuum*. Further, identity rates less than 60% have also been reported between accessions of *C. chinense* and *C. annuum* (0.5328-0.5963), demonstrating the low identity of amplified DNA segments among the three *Capsicum* species. Accessions BAGC-26 (*C. baccatum* var. *pendulum*) and BAGC-67 (*C. annuum* var. *glabriusculum*) were the least similar (I = 0.4446). The phenotypic and geographic diversity between *C. baccatum* and *C. chinense* accessions showed much phenotypic similarity, while *C. annuum* accessions distinguished themselves clearly (Zonneveld *et al*., 2015). Single-nucleotide polymorphisms (SNPs) analysis revealed a wide range of genetic diversity for different agro-morphological traits within *C. annuum* and *C. baccatum* (Solomon *et al*., 2019).

Rates of genetic identity within each *C. chinense* and *C. annuum* accessions may be useful to select parent genotypes in hybridization programmes. Genetic identity and distances within the four accessions of *C. chinense* and the five *C. annuum* estimated employing td-PCR-RAPD markers suggested that crosses between accessions *C. chinense* with the lowest genetic similarity (BAGC 07 × BAGC 24, BAGC 06 × BAGC 24; I < 0.80; Table 4) and between accessions *C. annuum* with the lowest genetic similarity (BAGC 11 × BAGC 40, BAGC 11 × BAGC 59, BAGC 11 × BAGC 36, BACG 11 × BAGC 67; I < 0.80; Table 4) may be useful in formulating a breeding program for the species. In fact, crosses between accessions with the lowest genetic similarity may provide a good possibility in obtaining unusual and favourable combinations of genes in a plant-breeding program.

In contrast to the symmetrical karyotypes and identical chromosome numbers reported for the acessions of *C. annuum*, *C. chinese* and *C. baccatum* (Sousa *et al*., 2015) including the accessions BAGC-07, BAGC- 49 (*C. chinense*), BAGC-36, BAGC-59 (*C. annuum*) and BAGC-26 (*C. baccatum*), a high molecular divergence was detected in randomly amplified DNA segments obtained by the td-PCR method. Redundance in four accessions of *C. chinense* (BAGC-06, BAGC-07, BAGC-23 and BAGC-24) and in five accessions of *C. annuum* (BAGC-11, BAGC-36, BAGC-40, BAGC-59 and BAGC- 67) was not detected. identifying redundant accesses is important to minimization the redundancy in a collection, to lessen the costs of gene bank management and the risk of gene loss in collections that are too large for proper maintenance. A good core collection is one that has no redundant accessions, is small enough to be easily managed, and represents high genetic diversity (Odong *et al*., 2013).

On the other hand, RAPD analysis with td-PCR method identified duplicates within accessions *C. chinense* (50%), *C. annuum* var. *glabrisculum* (5%) and *C. baccatum* var. *pendulum* (75%) from collection of the BAGC. Accessions of *C. baccatum* and *C. chinense* showed very lower molecular diversity than *C. annuum*. However, great caution is needed to minimize the number of seeds in *C. chinense* accessions. Although the characteristics of fruits were not analyzed in current study, it was preliminarily observed that *C. chinense* has an outstanding variation in fruit shapes. Since the morphological diversity of fruit is of high ornamental value, it is important to evaluate the diversity of fruits before minimizing the number of seeds of *C. chinense* due to their low molecular diversity.

RAPD analysis with td-PCR method also identified, from the Bayesian analysis, a mixture of genome groups in one pepper plant of *C. annuum* var. *glabrisculum* (sample 17) of the accession BAGC 11. Sample 17 is a promising element for germplasm bank BAGC. The other subgroup of *C. annuum* var. *glabrisculum* may be generated from pepper plant 17 after successive generations of self-fertilization occurring naturally. In fact, moderate to high level of endogamy is expected in *Capsicum* species. Self-compatibility has been described as the rule in the *Capsicum*. After successive generations of self-fertilization from pepper plant 17, the genome of the blue group, or of the red or green groups, may prevail on pepper plants. Alternatively, plant 17 of *C. annuum* var. *glabriusculum* may be used in crosses as a male parent (pollen donor) to explore genetic variability and as a potential for recombination with other genomes. The use of plant 17 as a pollen receptor may be also considered as a perspective to accentuate characteristic of morpho-agronomic importance (color, pungency, resistance to pests) when these are of maternal effect.

Combined use of RAPD analysis with td-PCR was useful to: i) emphasize the high genetic differentiation among *C. chinense*, *C. annuum* and *C. baccatum* accessions; ii) show no-redundance in four accessions of *C. chinense* (BAGC-06, BAGC-07, BAGC-23 and BAGC-24) and five accessions of *C. annuum* (BAGC-11, BAGC-36, BAGC-40, BAGC-59 and BAGC- 67); iii) identify duplicates within accessions *C. chinense* (50%), *C. annuum* var. *glabrisculum* (5%) and *C. baccatum* var. *pendulum* (75%); iv) identify plant with genome mixture; v) identify within each *C. chinense* and *C. annuum* accessions favourable combinations of genes for a plant breeding program; vi) guide future experiments of the keeper and curator of the BAGC *Capsicum* collection, and vii) monitor the level of homozygosity after successive

generations of self-fertilization or after cross-pollination in breeding programs. Analysis of molecular diversity of *Capsicum* spp. to identify redundant accessions and duplicates within accessions from BAGC revealed that the four accessions of *C. chinense* and five accessions of *C. annuum* should be mantained in the BAGC since redundance was not detected among the accessions of each species. On the other hand, duplicates were observed within each accession, so that the number of seeds may be reduced in each BAGC 07 and BAGC 23 (25%), BAGC 06 (87.5%), and BAGC 24 (62.5%) accession of *C. chinense*, in 75% of BAGC 26 accession of *C. baccatum* and 25% of BAGC 11 accession of *C. annuum.*

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