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BRIEF COMMUNICATION

Genetic diversity in *Passiflora* species determined by morphological and molecular characteristics

A.J.C. VIANA, M.M. SOUZA*, I.S. ARAÚJO, R.X. CORRÊA and D. AHNERT

Departamento de Ciências Biológicas, Universidade Estadual de Santa Cruz, Rod. Ilhéus-Itabuna, km 16, 45662-900, Ilhéus, Brasil

Abstract

Morphological and molecular characteristics were studied in six wild species of *Passiflora*. There were statistically significant differences among these six species for all characteristics studied. Intra-specific variability was observed for number of flowers, number of fruits, number of seeds, fruit length, fruit width and leaf area. Cluster analysis using morphological data showed three groups: *1*) *P. palmeri* var. *sublanceolata*, *P. morifolia* and *P. foetida* var. *foetida*, *2*) *P. coriacea* and *P. micropetala*, and *3*) *P. suberosa*. The dendrogram constructed using randomly amplified polymorphic DNA (RAPD) data showed six different groups for each species. The genetic distances among the 24 accessions ranged from 0.05 (between *P. morifolia* accessions P1 and P3) to 0.95 (*P. coriacea* accession 31 and *P. palmeri* var. *sublanceolata* accession 49). The species showed high morphological and molecular inter- and intra-specific variability.

Additional key words: passion fruit, polymerase chain reaction, RAPD markers.

The wild species in the genus *Passiflora* have important characters for breeding programs, especially for their ornamental value. There are more than 500 species in this genus and most originated in tropical America (Cervi 2005) and about 200 of these species are native to Brazil. *Passiflora* has wide morphological (Crochemore *et al.* 2003a) and genetic variability (Crochemore *et al.* 2003b, Viana *et al.* 2003). Molecular variability studies can help in the utilization and conservation of this wild germplasm, as they provide a background for the development of better strategies for germplasm maintenance. Most taxonomic studies on *Passiflora* are based on the morphological and agronomic characterization and the taxonomy of the *Passifloraceae* family has not been clarified yet.

Molecular markers used to assess inter- and intra-specific genetic diversity in *Passiflora,* have enabled the identification of hybrids and parents and can be used as a tool in selecting parents for breeding programs (Segura *et al.* 2002, Crochemore *et al.* 2003b, Viana *et al.* 2003). However, characterization of the genetic diversity using RAPD in *Passiflora* species has been limited to accessions of *P. edulis* f. *flavicarpa* and some wild species (Aukar *et al.* 2002, Crochemore *et al.* 2003b, Viana *et al.* 2003).

The aim of this study was to carry out morphological and molecular characteristics of wild *Passiflora* species in order to assess the genetic diversity that helps in selecting parents for inter-specific ornamental hybrids.

The plant material included plants of six *Passiflora* species maintained in the Germplasm Bank of the Universidade Estadual de Santa Cruz (UESC), Ilhéus, Bahia, Brazil (39° 10'W, 14° 39'S at 78 m above sea level). Four accessions were analyzed per species and ten replications per accession. Accession codes were P1ce, P2ce, P3ce and P5ce for *P. morifolia* Mast.; P1, P4, P5 and P16 for *P. foetida* var. *foetida* L.; P29, P30, P31 and P4ce for *P. micropetala* Mart.; P32, P33, P34 and P35 for *P. coriacea* Juss.; P44, P46, P48 and P49 for *P. palmeri* var. *sublanceolata* Rose and P56, P57, P58 and P59 for *P. suberosa* L.

Eleven variables were used in the morphological

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Abbreviations: RAPD - randomly amplified polymorphic DNA; UPGMA - unweighted pair-group method with arithmetic means.
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* Corresponding author: fax: (73) 3680-5226, e-mail: souzamagg@yahoo.com.br.

analysis (see Table 2). Leaf area was obtained using an *LI-3100* area meter (*Li-Cor*, Nebraska, USA). DNA was extracted from leaves of four randomly chosen individual plants of 24 accessions following the protocol of Doyle and Doyle (1990), with the modifications suggested by Viana *et al.* (2003). DNA quality and concentration was determined by 0.8 % gel electrophoresis in comparison to a DNA standard ladder (*Invitrogen*, Carlsbad, USA). After quantification, the DNA was diluted to 10 ng mm^{-3} and stored at -20 ºC until use. Fifteen 10-bp RAPD primers (Table 1) selected on the basis of showing a high level of polymorphism in *Passiflora* (Crochemore *et al.* 2003b, Aukar *et al.* 2002) were used. Amplifications were performed in PCR conditions following the protocol described by Williams *et al.* (1990) with modification suggested by Viana *et al.* (2003). The cycle program consisted of an initial denaturation step of 5 min at 94 ºC, followed by 35 cycles of 1 min at 94 ºC, 1 min at 32 ºC, and 2 min at 72 ºC, with a final extension step of 7 min at 72 ºC, using a thermocycler *Gene AMP PCR System 9700* (*Perkin Elmer*, Norwalk, CT, USA). Amplification products were analyzed by electrophoresis on 1.5 % agarose gels stained with ethydium bromide and photographed with the *Kodak Edas 120* system. Each locus was treated as a separate character and scored as present (1) or absent (0) and converted into a binary data matrix. The obtained data were submitted to analysis of variance and multivariate analysis. The variables were used to calculate Euclidean distances allowing the construction of a dendrogram by unweighted pair-group method with average means (UPGMA). A genetic similarity matrix was computed from the binary matrix using the Jaccard coefficient. Data analyses were conducted using the *Genes* software.

P. suberosa and *P. foetida* var. *foetida* showed the greatest number of flowers (15) opening in each day (NFl). Most of the species produced only one flower a day, except for *P. suberosa* that showed 3 flowers d^{-1}. *P. foetida* var. *foetida* produced also the greatest number of fruits (NF), with 32 fruits d^{-1}, while *P. suberosa*, the second species for NF, showed only half this value. The fruit from *P. morifolia* showed the greatest fruit diameter (FD) × fruit length (FL), 30.9 × 34.1 mm and were the heaviest (13 g). *P. micropetala* had the greatest number of seeds per fruit (NS = 198), differing from all the other species studied, and the second highest NS of 57 seeds $fruit^{-1}$ was reported for *P. coriacea.* The greatest leaf area (LA) was observed in *P. coriacea* (89.0 cm^2), which was statistically different from all other species assessed.

There were significant differences among the six species in the eleven morphological variables analyzed

Table 1. Primers, their nucleotide sequences, and numbers of interspecific polymorphic (NPB) and monomorphic (NMB) bands of wild *Passiflora* species. (a) - *P. coriacea*, (b) - *P. palmeri* var. *sublanceolata*, (c) - *P. suberosa*, (d) - *P. foetida* var. *foetida*, (e) - *P. micropetala*, (f) - *P. morifolia*.

Primer	Sequence 5´ - 3´	NPB	NMB
2	CCTGGGTTCC	11	3(a), 2(b), 1(c), 1(d), 1(e), 2(f)
3	CCTGGGTCCA	15	1(a), 3(b), 1(c), 1(d), 1(e), 4(f)
4	CCTGGGTGGA	18	1(b), 2(c), 5(d), 2(e), 3(f)
5	CCTGGGCCTC	11	1(a), 3(b), 1(c), 2(d), 2(e), 2(f)
6	GCCCGGTTTA	11	1(a), 2(d), 1(f)
7	TCCGGGTTTG	4	2(f)
11	CCGGCCTTAC	5	1(a), 2(b), 1(c), 1(d), 2(e), 1(f)
12	CCGGCCTTAG	7	2(a), 1(c), 1(d), 2(e), 2(f)
13	CCGGGGTTTT	3	1(c)
15	TTAACCGGGG	1	1(c)
16	TTCCCCGCGC	5	1(b), 1(d)
17	CTACCCGTGC	13	3(a), 2(b), 2(c), 3(d), 2(e), 6(f)
24	CCACAGCAGT	8	3(c), 1(d), 2(e), 2(f)
25	ACCCCCGCCG	15	3(a), 1(b), 2(c), 2(d), 3(e), 2(f)
26	GGACCCTTAC	7	1(d), 1(f)

Table 2. Mean values and mean square values for morphological characters assessed in six wild *Passiflora* species. NFl - number of flowers, NF- number of fruits, FM - fruit mass [g], FD - fruit diameter [mm], FL - fruit length [mm], NS - number of seeds [$fruit^{-1}$], SM - seed mass [mg], SL - seed length [mm], LW - leaf width [cm] , LL - leaf length [cm], LA - leaf area [cm^2]. Values in the same column followed by the same letter are not significantly different according to the Tukey test at 5 % probability level. SV - source of variation; DF - degree of freedom; CV - coefficient of variation (* - significant at 5 % probability level).

Species	NFl	NF	FM	FD	FL	NS	SM	SL	LW	LL	LA
P. suberosa	9.6^{a}	7.7^{b}	0.4^{c}	8.8^{e}	8.4^{f}	8.2^{e}	4.7^{e}	3.7^{d}	8.6^{c}	7.0^{b}	19.3^{d}
P. palmeri var. *sublanceolata*	2.0^{c}	2.1^{c}	5.1^{a}	20.8^{c}	31.3^{a}	31.7^{bc}	14.0^{b}	5.5^{a}	5.4^{e}	8.2^{a}	30.0^{c}
P. morifolia	1.6^{c}	1.9^{c}	6.0^{a}	24.1^{b}	29.2^{b}	24.5^{cd}	18.9^{a}	5.1^{b}	7.9^{cd}	6.6^{b}	31.1^{c}
P. foetida var. *foetida*	5.1^{b}	14.1^{a}	1.5^{b}	16.4^{d}	17.5^{e}	18.3^{de}	9.8^{c}	5.1^{b}	7.3^{d}	8.0^{a}	39.9^{b}
P. coriacea	1.9^{c}	1.4^{c}	2.4^{b}	17.8^{d}	19.2^{d}	37.8^{b}	9.7^{c}	5.1^{b}	15.8^{a}	4.6^{c}	50.4^{a}
P. micropetala	2.9^{c}	2.4^{c}	5.9^{a}	25.7^{a}	23.8^{c}	107.4^{a}	5.7^{d}	4.3^{c}	9.7^{b}	5.1^{c}	37.2^{b}

SV	DF	Mean square									
Genotype	5	380.2*	1020.2*	238.5*	1496.6*	2830.0*	50552.8*	1125.4*	17.5*	514.6*	88.9*	4404.9*
Residue	234	5.8	14.3	2.3	4.3	7.0	431.2	2.1	0.1	1.6	1.1	81.1
CV		62.4	77.1	43.4	4.3	12.3	54.7	13.9	5.6	13.8	16.3	25.9

($P < 0.05$; Table 2). Significant intraspecific differences were found in NFl, NF, NS, leaf length (LL), leaf mass (LM), and LA for *P. suberosa* and *P. micropetala*; *P. foetida* var. *foetida*; *P. suberosa* and *P. palmeri* var. *sublanceolata*; *P. morifolia*, *P. foetida* var. *foetida* and *P. micropetala*; *P. suberosa*, *P. morifolia*, *P. foetida* var. *foetida* and *P. micropetala*; *P. morifolia*, *P. foetida* var. *foetida* and *P. micropetala*, respectively. These characteristics also showed high coefficient of variation (CV). The results of the multivariate analysis indicate that the morphological variables that contributed the most to diversity among the species studied were NS with 80.6 % and LA with 7.0 % contribution to the diversity. The dendrogram shows three clusters (Fig. 1*A*), the first cluster comprised *P. coriacea* and *P. micropetala*, the second cluster consisted of *P. palmeri* var. *sublanceolata*, *P. morifolia* and *P. foetida* var. *foetida* and the third cluster included only *P. suberosa*. *P. suberosa* showed the highest value of genetic dissimilarity (0.99), compared to the other species. *P. palmeri* var. *sublanceolata* and *P. morifolia* showed greater genetic similarity (0.32), followed by *P. coriacea* and *P. micropetala* (0.68).

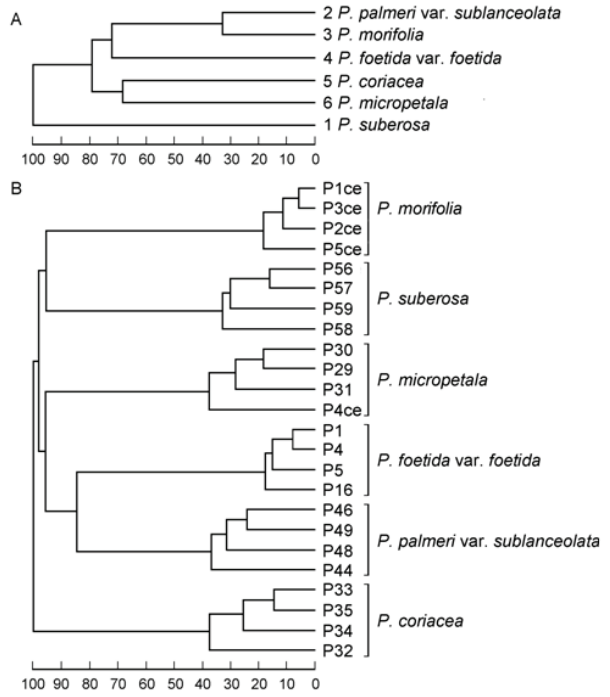

Fig. 1. UPGMA dissimilarity dendrogram based on Euclidean distances of 24 accessions showing clustering relationships of six *Passiflora* species: *A* - based on morphological characters, *B* - based on the bands generated using 15 RAPD primers.

The 15 RAPD primers generated a total of 134 bands that were polymorphic among the six species. The primers displaying the greatest potential as markers in the six species analyzed were primers 2, 3, 4, 5, 6, 11, 12, 16, 17, 24, 25, and 26 that yielded at least five or more polymorphic bands. Six clusters were derived from the clustering analysis (Fig. 1*B*), corresponding to the six species studied. Genetic distances among the 24 accessions ranged from 0.05 to 0.95. The smallest distance was observed between P1ce and P3ce accessions (0.05), both belonging to the same species, *P. morifolia*, and they were from the same geographic region. The greatest genetic distance (0.95) was observed between *P. coriacea* P32 and *P. palmeri* var. *sublanceolata* P49 accessions.

A high polymorphism observed among the six species contrasted with a low intraspecific polymorphism. Regarding the number of intraspecific bands in *P. morifolia* and *P. foetida* var. *foetida*, most bands were monomorphic (28 and 21, respectively) with few intraspecific polymorphic bands (Table 1). *P. coriacea*, *P. palmeri* var. *sublanceolata,* and *P. micropetala* exhibited greater polymorphism compared to remaining species studied. It is noteworthy that *P. coriacea*, *P. palmeri* var. *sublanceolata*, *P. suberosa,* and *P. micropetala* showed fewer intraspecific bands, both monomorphic and polymorphic, for the 15 primers used, when compared to *P. morifolia* and *P. foetida* var. *foetida*.

The variation observed in NF is very common, even at an intraspecific level (Martins *et al.* 2003). The high CV value observed for this variable may have been due to the environmental effect, or genetic difference, or both. According to Figueiredo *et al.* (1988), variations in the traits related to fruit result in differences in the maturation stage of the fruit, age of the plant, latitude, soil and climactic conditions and seed origin. In this study, variation in NF was probably due to the genotype, since the environmental conditions were the same and remained constant for all plants.

The high divergence between *P. suberosa* and *P. foetida* var. *foetida* observed by Crochemore *et al.* (2003a) was confirmed in this study as these species were on distant branches in the dendrogram. Assessments of morphological traits clustered the species according to their similarities. Primot *et al.* (2005) assessed the morphological diversity in *P. tripartita* var. *mollissima*, *P. tarminiana*, *P. mixta* and their hybrids and obtained a clear separation between these species and their hybrids. A similar results were reported by Cerqueira-Silva *et al.* (2009) who worked with *P. edulis* and *P. setacea* and Crochemore *et al.* (2003a) who worked with 11 species in this genus, including the two botanical forms of *P. edulis*, the yellow passion fruit and the purple passion fruit, and obtained a clear separation among the 11 species studied and between the two botanical forms.

In this study, the genetic diversity results from RAPDs markers differed from the diversity results from the morphological analysis. In the cluster analysis based on morphological characteristics, *P. suberosa* was the most dissimilar species, while in the molecular analysis *P. coriacea* was the most divergent species. The high similarity detected between *P. palmeri* var. *sublanceolata* and *P. morifolia* in the morphological analysis was not corroborated by the RAPD analysis that showed a large genetic distance between them. Fajardo *et al.* (1998)

studied 52 accessions using RAPD and also found some differences regarding the results of the classification based on morphological traits. Cassiano *et al.* (1998) analyzed the species *P. edulis*, *P. edulis* f. *flavicarpa, P. amethystina*, *P. caerulea*, *P. cincinnata*, *P. coccinea*, *P. serrato-digitata*, *P. foetida*, *P. maliformis*, *P. alata*, *P. giberti*, *P. laurifolia*, *P. macrocarpa*, *P. nitida*, *P. setacea*, *P. suberosa*, *P. ligularis*, *P. capsularis,* and *P. coriacea* using RAPD markers and characterized those species and reported their similarity.

Vieira *et al.* (1997) studied variability in passion fruit using RAPD markers and detected genus-specific and species-specific bands. In the present study, these markers enabled the delimitation of different accessions and species, and six groups were derived from the clustering analysis corresponding to the six species studied. The origin of the accessions influenced the clustering within the species, since accessions of the same origin were closer in the dendrogram. Previous studies on genetic diversity in *Passiflora* had reported that accession origin influenced intraspecific clustering (Viana *et al.* 2003, Bellon *et al.* 2007). In other groups of plants the accession origin did not influenced the intraspecific clustering (Jordán-Pla *et al*. 2009).

The high interspecific polymorphism observed was probably due to the diversity of species studied. Viana *et al.* (2003) used different accessions of the cultivated and some wild species of *Passiflora* and reported similar results. Different levels of intraspecific polymorphism found in the species studied in this research were also reported by Fajardo *et al.* (1998) who used RAPD markers in *Passiflora* species. These authors observed little intraspecific variation in *P. edulis* and *P. maliformis*, while *P. ligularis* and *P. adenopoda* exhibited great intraspecific variation. The high level of interspecific polymorphism observed allowed for a clear distinction between accessions (Fig. 1*A*). It was observed that the initial clustering of species with a high genetic distance that characterized the remarkable diversity among the species studied.

Based on these results, we conclude that the genus *Passiflora* shows high inter- and intra-specific morphological variation for traits of interest in ornamental plants. The data obtained with RAPD analyses indicated that there is polymorphism within and among the accessions of the species studied. The identification of the most divergent accessions was very important for the *Passiflora* breeding program, since the information will be used for selecting parents for interspecific crosses to produce ornamental hybrids.

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