

# Variability of the 5S and 45S rDNA Sites in *Passiflora* L. Species with Distinct Base Chromosome Numbers

NATONIEL FRANKLIN DE MELO<sup>1,\*</sup> and MARCELO GUERRA<sup>2</sup>

<sup>1</sup>Embrapa Semi-Árido, C.P.23, 56302-970, Petrolina, Pernambuco, Brazil and <sup>2</sup>Department of Botany, CCB, Federal University of Pernambuco, Rua Nelson Chaves s/n, 50670-420, Recife, Pernambuco, Brazil

Received: 18 December 2002 Returned for revision: 12 March 2003 Accepted: 4 May 2003

Cytologically, the species of *Passiflora* with known chromosome number can be divided into four groups: (1) 2n = 12, 24, 36; (2) 2n = 24; (3) 2n = 18, 72; and (4) 2n = 20. The base chromosome number proposed for the genus is x = 6, with x = 9, x = 10 and x = 12 being considered secondary base numbers. In the present study, variability of 5S and 45S rDNA sites was investigated in 20 species of these four groups to check the reliability of this hypothesis. In the group with x = 6, five diploid species (2n = 12) exhibit two 5S rDNA sites and two (P. capsularis, P. morifolia and P. rubra) or four (P. misera 2x and P. tricuspis) 45S rDNA sites. The hexaploid cytotype of P. misera had 12 45S rDNA sites and six 5S rDNA. A tetraploid species, P. suberosa, had ten 45S rDNA sites and four 5S rDNA sites, both in the same chromosomes as the 45S rDNA sites. In the group with x = 9, P. actinia, P. amethystina, P. edmundoi, P. elegans, P. galbana, P. glandulosa and P. mucronata displayed six 45S rDNA sites, whereas P. alata, P. cincinnata, P. edulis f. flavicarpa, P. edulis var. roxo and *P. laurifolia* had four sites. In this group, all species were diploid (2n = 18) and had only two 5S rDNA sites. *Passifiora foetida*, the only species with 2n = 20, had six 45S rDNA sites and four 5S rDNA sites. The species with x = 12 (2n = 24), *P. haematostigma* and *P. pentagona*, showed four 45S rDNA sites and two 5S rDNA. In general, the number and location of 5S and 45S rDNA sites were consistent with the hypothesis of x = 6 as the probable ancestral genome for the genus, while the groups of species with x = 9, x = 10 and x = 12 were considered to be of tetraploid origin with descending dysploidy and gene silencing of some redundant gene sites, mainly those of 5S rDNA. © 2003 Annals of Botany Company

Key words: Passiflora, chromosome base number, FISH, rDNA, karyotype.

#### INTRODUCTION

*Passiflora* L. is the largest genus of the family Passifloraceae, with approx. 465 species distributed mainly in tropical regions (Vanderplank, 1996; Cervi, 1997). Most of these species are herbaceous, although some representatives are shrubs or trees. Many species of *Passiflora* are cultivated as ornamentals or for their edible fruits and medicinal properties.

Cytologically, the species of Passiflora for which chromosome numbers are known can be divided into four karyological groups, represented by x = 6, x = 9, x = 10 and x = 12. Most species of *Passiflora* are diploid, with 2n = 12, 2n = 18 or 2n = 20, although some tetraploids (2n = 24), hexaploids (2n = 36) and octoploids (2n = 72) have been recorded (Snow and MacDougal, 1993; Melo et al., 2001). Different chromosome base numbers (x = 3, 6, 9) have been proposed for the genus, without a clear understanding of this variation and of the phylogenetic relationships among the species (Storey, 1950; Raven, 1975; Morawetz, 1986; Snow and MacDougal, 1993). Recently, Melo et al. (2001), revising the cytotaxonomy of the group, considered x = 6 as the most probable base number for the genus, whereas x = 9, x = 10 and x = 12 were considered secondary base numbers. However, the second-most probable base number, x = 12,

\* For correspondence. Fax + 55 87 3862 1744, e-mail natoniel@cpatsa. embrapa.br appears to have played an important role in the evolution of the group because it is better represented in other genera of the family. Therefore, with regards to the cytotaxonomy of this group, it is important to know whether x = 6 is derived from x = 12, or vice versa. The mechanisms of chromosome alteration most probably related to these changes are descending dysploidy (x = $12 \rightarrow 6$ ) or polyploidy ( $x = 6 \rightarrow 12$ ) (Guerra, 2000). In this case, any additional indication of polyploidy or dysploidy may be important for understanding the evolution of this group.

In the genus Aloe, Adams et al. (2000) found a correspondence between the number of 5S rDNA sites revealed by FISH (fluorescent in situ hybridization) and the ploidy level of several species. Diploid species displayed two 5S rDNA sites, tetraploids four sites and hexaploids six sites. A similar correspondence was found in Saccharum (D'Hont et al., 1998). However, more often, this relationship has been not observed, mainly because the number of rDNA sites can vary among diploid species. In diploid species of Paeonia, for instance, the number of chromosome pairs carrying 45S rDNA sites varied from three to five (Zhang and Sang, 1999). Exceptionally, the number of 5S and 45S rDNA sites can vary widely within a single species, as in Crocus vernus (Frello and Heslop-Harrison, 2000). Nevertheless, the number of 5S or 45S rDNA sites is generally larger in tetraploids than in related diploids. In

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| Taxon  | 2 <i>n</i> | Number<br>and position<br>of 5S<br>rDNA sites | Number<br>and position<br>of 45S<br>rDNA sites | Voucher<br>number | Provenance*                                     |
|--|------------|---|--|-------------------|---|
| Subgenus Astrophea (DC.) Mast.   |            |   |  |                   | *   |
| Section Pseudoastrophea (Harms) Killip   |            |   |  |                   |   |
| P. haematostigma Mart. ex Mast.  | 24         | 2 (st)  | 4 (st)   | PAS-1641          | Caeté, MG                                       |
| P. pentagona Mast.   | 24         | 2 (st)  | 4 (st)   | TSAH-1736         | Barra da Estiva, BA                             |
| Subgenus <i>Plectostemma</i> Mast.<br>Section <i>Cieca</i> (Medic.) Mast.                        |            |   |  |                   |   |
| P. morifolia Mast.   | 12         | 2 (st)  | 2 (st)   | UFP-31195         | Águas de Lindóia, SP                            |
| P. suberosa L.   | 24         | 4 (st)  | 10 (st; p)                                     | TSAH-1742         | Porto Alegre, RS                                |
| Section Decaloba (DC.) Mast.   |            |   |  |                   |   |
| P. misera Kunth  | 12         | 2 (st)  | 4 (p)  | TSAH-1737         | Foz do Iguaçu, PR                               |
|  | 12         | 2 (st)  | 4 (p)  | TSAH-1746         | Cachoeira do Sul, RS                            |
|  | 36         | 6 (st)  | 12 (st; p)                                     | TSAH-1741         | Recife, PE                                      |
|  | 36         | 6 (st)  | 12 (st; p)                                     | UFP-31197         | Caruaru, PE                                     |
| P. tricuspis Mast.   | 12         | 2 (st)  | 4 (p)  | UFP-31201         | São José do Rio Preto, SP                       |
| Section Xerogona (Raf.) Killip   |            |   |  |                   |   |
| P. capsularis L.   | 12         | 2 (st)  | 2 (st)   | TSAH-1745         | Aguas de Lindóia, SP                            |
| P. rubra L.  | 12         | 2 (st)  | 2 (st)   | TSAH-1744         | Mata do Saltinho, Rio Formoso, PE               |
| Subgenus Distephana (Juss.) Killip   |            | -   | 10-10-10-10-10-10-10-10-10-10-10-10-10-1       |                   |   |
| P. glandulosa Cav.   | 18         | 2 (st)  | 6 (st)   | UFP-31196/1       | Cultivated: Embrapa CNPMF, Cruz das Almas, BA   |
| C. 1. D. 10  | 18         | 1 (st)  | 6 (st)   | UFP-31196/2       | Cultivated: Embrapa CNPMF, Cruz das Almas, BA   |
| Subgenus Passiflora  |            |   |  |                   |   |
| Series Quadrangulares (Harms) Killip   | 10         | 2 ( )   | 1 ( )  |                   |   |
| P. alata Curtis  | 18         | 2 (st)  | 4 (st)   | —                 | Cultivated: Embrapa CNPMF, Cruz das Almas, BA   |
| Delawifelia L  | 10         | 2 (-+)  | 4 (-4)   |                   |   |
| F. laurijolia L.<br>Sarias Passiflora  | 10         | 2 (St)  | 4 (St)   | _                 | Cultivated: Embrapa CNPMF, Cruz das Almas, BA   |
| P cincinnata Most  | 19         | 2 (st)  | 4 (ct)   | LIED 17402        | Duíque DE                                       |
| P edulis Sims f flavicarna Deg   | 18         | $\frac{2}{2}$ (st)                            | 4 (st)<br>4 (st)                               | UFF-1/402         | Cultivated: Embrana CNDME Cruz das Almas PA     |
| P edulis Sims var roxo   | 18         | 2(st)   | 4 (st)   | _                 | Cultivated: Embrana CNPME Cruz das Almas, BA    |
| Series Kermesinge Killin ex Cervi  | 10         | 2 (31)  | - (St)   |                   | Cuntvaled. Embrapa Civi Wi , Ciuz das Annas, DA |
| P edmundoi Sacco   | 18         | 2 (st)  | 6 (st)   | LIFP_31199        | Palmeiras BA                                    |
| Series Simplicifoliae (Harms) Killin   | 10         | 2 (50)  | 0 (50)   | 011 511))         | Tumonus, Dry                                    |
| <i>P. actinia</i> Hook.  | 18         | 2 (st)  | 6 (st)   | PAS-1592          | Porto Alegre, RS                                |
| P. galbana Mast.   | 18         | 2 (st)  | 6 (st)   | UFP-31200         | Mamanguape, PB                                  |
| P. mucronata Lam.  | 18         | 2 (st)  | 6 (st)   | TSAH-1740         | Igarassu, PE                                    |
| Series Lobatae (Harms) Killip  |            | - ()  | - ()   |                   | -6  |
| P. amethystina Mikan var. amethystina  | 18         | 2 (st)  | 6 (st)   | UFP-31202         | Águas de Lindóia, SP                            |
| an an ann an Chairte ann an Chairte an Chairte an Chairtean an Anna Anna Anna Anna Anna Anna Ann | 18         | 2 (st)  | 6 (st)   |                   | Domingos Martins, ES                            |
| P. elegans Mast.   | 18         | 2 (st)  | 6 (st)   | PAS-1591          | Porto Alegre, RS                                |
| Subgenus Dysosmia (DC.) Killip   |            |   |  |                   | - 1 121   |
| P. foetida L.  | 20         | 4 (st)  | 6 (p)  | TSAH-1735         | Petrolina, PE                                   |

 TABLE 1. Passiflora species analysed with respective chromosome numbers, number and position of 45S and 5S rDNA sites, herbarium vouchers and provenances

Species are organized according to the infrageneric division proposed by Killip (1938).

St, Subterminal; p, proximal.

\* BA, Bahia; ES, Espírito Santo; MG, Minas Gerais; PB, Paraíba; PE, Pernambuco; PR, Paraná; SP, São Paulo, RS, Rio Grande do Sul.

*Rhynchospora*, the number of 45S rDNA site pairs varied from two to four among diploids, and from four to 15 among polyploids (Vanzela *et al.*, 1998). A similar variation in number of 5S and 45S rDNA sites has been described in diploid and tetraploid species of *Hordeum* (Taketa *et al.*, 1999). Therefore, in general, polyploids have a higher average number of rDNA sites (mainly 45S rDNA) than diploids.

In the present study, the variability of 5S and 45S rDNA sites was investigated in 20 species of *Passiflora*, representing different base numbers, to help identify the base number of the genus and relationships among the haploid numbers.

## MATERIALS AND METHODS

Material was collected in the field or was obtained from the germplasm bank of Embrapa Mandioca e Fruticultura Tropical—CNPMF (Table 1). Voucher specimens of all materials are deposited at the UFP Herbarium (Federal University of Pernambuco, Brazil), or at TSAH of Embrapa Semi-Árido (Petrolina-PE, Brazil).

For cytological analysis, young root tips were pre-treated with 0.002 M 8-hydroxyquinoline at 8 °C for 24 h, and fixed overnight at room temperature in Carnoy 3 : 1 (ethanol : acetic acid). Floral buds of *P. pentagona* Mast. were collected in the field and fixed directly in Carnoy. Root tips were then stored at -20 °C. Root tips were digested in 2 % cellulase (Sigma, St Louis, Mo, USA) and 20 % pectinase (Sigma) solution for 2 h at 37 °C, and squashed in 45 % acetic acid. Coverslips were removed by freezing in liquid nitrogen and the slides were air-dried. Slides were stained briefly with DAPI (4',6-diamidino-2-phenylindol)/glycerol and the best ones were re-fixed in Carnoy 3 : 1 for 30 min, dehydrated in 100 % ethanol and stored at -20 °C until required for *in situ* hybridization.

To locate the 45S rDNA sites, probes SK18S and SK25S were used, containing 18S and 25S rDNA of Arabidopsis thaliana L. (Unfried et al., 1989; Unfried and Gruendler, 1990), kindly supplied by Professor Dieter Schweizer (University of Vienna, Austria). The 5S rDNA was obtained from total genomic DNA of P. edulis Sims by PCR using the primers 5'-GTGCGATCATACCAGC(AG)(CT)TAATG-CACCGG-3' 5'-GAGGTGCAACACGAGGAand CTTCCCAGGAGG-3'. The in situ hybridization procedure followed that of Moscone et al. (1996). Probes SK18S and SK25S were labelled with biotin-11-dUTP and detected with TRITC (tetramethyl rhodamine isothiocyanate), while the 5S rDNA probe was labelled with digoxigenin-11-dUTP and detected with FITC (fluorescein isothiocyanate). Chromosomes were counterstained with DAPI and the slides mounted in Vectashield H-1000.

Cells were photographed with a DMLB Leica epifluorescence microscope, using Kodak Ultra colour film ASA 400, or their images captured with a Cohu-CCD video camera using Leica QFish software.

### RESULTS

Karyologically, the species were distributed into four groups, in agreement with the base number: six species with x = 6 (2n = 12, 24, 36), 11 with x = 9 (2n = 18), one with x = 10 (2n = 20) and two with x = 12 (2n = 24). Chromosome morphology varied from metacentric to submetacentric. Size chromosome asymmetry was more evident in the group of species with x = 6, especially in *P. suberosa*, *P. capsularis* and *P. morifolia*. Most of the populations had not been analysed previously by Melo *et al.* (2001), but they exhibited the same number of chromosomes and chromosome morphology as previous samples. Only *P. haematostigma*, with 2n = 24 (Fig. 1M), was investigated for the first time.

The positions of 5S and 45S rDNA sites are summarized in Table 1. Figures 1 and 2 illustrate the main results. Note that filter Leica I3 used to excite FITC (green) also excites TRITC (red), changing the colour of the signals captured with filter N2·1 for TRITC. Consequently, the 45S rDNA sites are shown twice and in different colours.

The number of 5S rDNA sites was almost constant, usually a pair for each species, except in *P. foetida* (2n = 20) and in the tetraploid *P. suberosa* L., with four sites, and *P. misera* Kunth (6x), with six sites (Table 1). The position of these sites often seemed to be subterminal and, at least in the species with x = 6, it was always observed on chromosome VI (the smallest pair) or V. In several species, visualization of the 5S rDNA site was limited by its small size. For instance, in *P. alata* Curtis, the 5S rDNA sites were

observed as minuscule points inside the chromosome mass (Fig. 2O). In a single individual of *P. glandulosa* Cav., the 5S rDNA site of one homologue was not detected, possibly owing to its very small size (Fig. 2K). The interphase nuclei of this sample also displayed only one fluorescent spot. In another individual of this same species, the two 5S rDNA sites showed size heteromorphism (Fig. 2L).

The small chromosome size and the tendency of fluorescence to expand beyond the region marked by the probe did not allow us to distinguish clearly between terminal and subterminal rDNA sites. Since, in some cases, the 45S rDNA sites were located within a subterminal secondary constriction, all sites are referred to as subterminal in Table 1, although some of these may be terminal. In general, 45S rDNA signals were larger, more numerous and more variable than 5S rDNA signals.

In the group of diploid species with x = 6, the 45S rDNA sites varied from one pair in *P. capsularis*, *P. morifolia* and *P. rubra*, to two pairs in *P. misera* 2x and *P. tricuspis* (Fig. 1A–I; Table 1). In polyploids, this number was higher. In *P. misera* 6x, the number and position of rDNA sites were the expected multiple of the diploid form, with 12 45S rDNA sites and six 5S sites. However, while all 45S rDNA sites in *P. misera* 2x were the same size (Fig. 1H), *P. misera* 6x showed six large and six small blocks of 45S rDNA (Fig. 1J), and six 5S rDNA sites distributed in three very small chromosome pairs. DAPI bands observed in the diploid cytotype were also observed in the hexaploid one (Fig. 1G).

The tetraploid P. suberosa displayed an asymmetrical karyotype, with eight to 12 larger chromosomes and 12 to 16 smaller ones. It had ten 45S and four 5S rDNA sites. Six 45S rDNA sites were located on the smallest chromosomes: two in the proximal region and four in the subterminal region. These latter four chromosomes also showed the 5S rDNA sites (Fig. 1K and L). The largest 45S rDNA site was located on the second largest chromosome pair. Passiflora tricuspis, although showing a karyotype very similar to that of P. misera 2x, displayed 45S rDNA in chromosome pairs II and III, and 5S rDNA in pair V (Fig. 1F), whereas in P. misera these sites were located in pairs II, IV or V (45S) and VI (5S). Passiflora capsularis, P. rubra and P. morifolia Mast. had only two 45S rDNA sites, located on the short arm of chromosome pair I in the first two species (Fig. 1B and D), and on the metacentric pair V in P. morifolia (Fig. 1E).

In the two species of the group with x = 12, *P. haematostigma* and *P. pentagona*, both with 2n = 24, four 45S rDNA sites and two 5S rDNA sites were observed, all terminally located (Fig. 1M and N). They had larger chromosomes than the species with x = 6.

Chromosomes of *Passiflora foetida* (x = 10; 2n = 20) were larger than those of species with x = 6 and smaller than those of species with x = 9. Six proximal 45S rDNA sites and four terminal or subterminal 5S rDNA sites were observed (Fig. 1P and Q).

In the group of x = 9, the number of 45S rDNA sites varied from two to three pairs (Table 1), all apparently subterminals (Fig. 2). Of the species with three pairs of sites, *P. edmundoi*, *P. elegans* and *P. mucronata* were similar in



FIG. 1. Fluorescent *in situ* hybridization with 5S (green) and 45S (red or yellow) rDNA probes, in *Passiflora* species with x = 6, x = 10 or x = 12. A–C, *P. capsularis*. Note chromosomes stained with DAPI in A, 45S rDNA sites in B and a pair of 5S rDNA sites in C (arrows). D, *P. rubra*. Arrows indicate 5S rDNA. E, *P. morifolia*. Arrows indicate 5S rDNA, F, *P. tricuspis*. Note a longer pair of chromosomes with terminal regions characteristically less condensed, two pairs with proximal blocks of 45S rDNA, with one of the chromosomes being distended (arrowhead), and a pair with sub-terminal 5S rDNA (arrows). G–I, *P. misera* 2x. Note the terminal DAPI<sup>+</sup> bands in almost all chromosomes in G, 45S rDNA sites apparently sub-terminal in one pair of chromosomes and proximal in the other one in H, and 5S rDNA sites in I (arrows). J, *P. misera* 6x. Broken arrows and arrowhead indicate small 45S rDNA sites. K and L, *P. suberosa*. Note that three of the five pairs of 45S rDNA sites are located in the smallest chromosome, two of which also have 5S rDNA (arrows). M, *P. haematostigma*. Arrows indicate terminal 5S rDNA sites. N, *P. pentagona*. Arrows indicate 5S rDNA sites. O–Q, *P. foetida*. Note the secondary proximal constrictions in three chromosome pairs in O, three pairs of 45S rDNA sites in P, and two pairs of 5S rDNA sub-terminal sites in Q (arrows). All chromosomes were counterstained with DAPI (blue). Bar in Q represents 5  $\mu$ m.

having 45S rDNA sites in chromosome pairs I and III, whereas the third pair was located on chromosome VII, IX or VIII, respectively (Fig. 2A, C and E). The 5S rDNA was

located on the short arm of pair II in *P. edmundoi* (Fig. 2B), on the long arm of pair VI in *P. elegans* (Fig. 2D) and on the long arm of pair VII in *P. mucronata* (Fig. 2F).



FIG. 2. Fluorescent *in situ* hybridization with 5S (green) and 45S (red or yellow) rDNA probes, in *Passiflora* species with x = 9. *P. edmundoi* (A and B); *P. elegans* (C and D); *P. mucronata* (E and F); *P. galbana* (G and H); *P. actinia* (I and J); *P. glandulosa* (K and L); *P. amethystina* (M); *P. alata* (N, O); *P. edulis* (P); *P. laurifolia* (Q); and *P. cincinnata* (R). Arrows indicate 5S rDNA sites while broken arrows indicate weak signals of 45S rDNA. All chromosomes were counterstained with DAPI (blue). Bar in R represents 5  $\mu$ m.

In *P. galbana* two larger blocks of 45S rDNA were observed, located on pairs II and IV, and a smaller one on the pair V (Fig. 2G and H). *Passiflora actinia* had a symmetrical karyotype with 5S rDNA signals observed as very weak dots (Fig. 2I and J). In *Passiflora glandulosa*, 45S rDNA sites (Fig. 2K and L) were located on chromosomes V, VII and VIII, and 5S rDNA sites on one or two chromosomes of pair VI. In *P. amethystina* (Fig. 2M), the 45S rDNA sites were located on pairs V, VI and IX, whereas the 5S rDNA sites were observed in the sub-terminal region of pair VII.

*Passiflora alata* had the largest chromosomes of the group with x = 9, with 45S rDNA located on the terminal region of pairs V and VIII, and 5S rDNA subterminally located on the short arm of pairs III or IV (Fig. 2N and O). In *Passiflora edulis* f. *flavicarpa* there was a large and a small 45S rDNA site, located on the long arm of pairs VII and IX, respectively, and 5S rDNA was located on the long arm of pair V (Fig. 2P). In *P. edulis* var. roxo, the number and position of the rDNA sites were the same as those in *P. edulis* f. *flavicarpa* although it was impossible to define the chromosome morphology. *Passiflora cincinnata* and

a tendency to reduce the number of duplicated sites owing to diploidization mechanisms and gene silencing (Leitch and Bennett, 1997). The selection pressure to reduce the rDNA site number to a single pair seems to be smaller in 45S rDNA owing to the existence of more efficient inter-loci homogenization mechanisms for this sequence than for 5S rDNA (Cronn *et al.*, 1996). Therefore, the probability of finding the duplicated 5S rDNA sites would be larger in infraspecific polyploids or in neopolyploids, than in paleopolyploids. Vogel *et al.* (1999) observed that silencing isoenzyme markers also is more evident in the paleopolyploids than in the neopolyploids.

The only two samples analysed here that showed site duplication proportional to the ploidy level (*P. misera* and *P. suberosa*) were also the only examples of infraspecific polyploidy. Multiplication of the 5S rDNA site number has also been found in other intraspecific polyploids, such as *Aloe* (Adams *et al.*, 2000), or in neopolyploids, as in common wheat (Mukai *et al.*, 1990). On the other hand, the occurrence of a single 5S rDNA site in *P. haematostigma* and *P. pentagona* seems to reflect an oldest origin of polyploidy in these species, as observed in typical paleopolyploids, such as *Hevea* (Leitch *et al.*, 1998) and *Manihot* (Carvalho and Guerra, 2002).

Although these data corroborate the hypothesis of  $x_1 = 6$ as being the primary base number of Passiflora and Passifloraceae, the hypothesis that the base number is 12 still has in its favour the fact that this is the best represented haploid number among other genera and it is also the base number of Astrophea, a subgenus of shrubby and arboreal species, considered one of the most primitive of Passiflora. However, since polyploidy is a highly recurrent phenomenon in angiosperms, this widespread representation may be due to independent paleopolyploid lines. Guerra (2000) observed that in many taxa, the most primitive representatives are paleopolyploids that conserve ancient characteristics of the group. The polyploid origin of these representatives is suggested by the frequent occurrence of the chromosome number corresponding to the haploid (n = 6,in the case of Passiflora) in close taxa, which would hardly be justified by descending dysploidy (see, for example, Félix and Guerra, 2000).

#### ACKNOWLEDGEMENTS

The authors are grateful to Dr Armando Carlos Cervi (Federal University of Paraná) for species identification, to Fernando Campos (Zoo-botanical Garden of Belo Horizonte and Petrobras) for several samples, and to the National Council of Scientific and Technological Development (CNPq) for financial support.

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