



Karyotype analysis in Bignoniaceae (Bignoniaceae): chromosome numbers and heterochromatin

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ABSTRACT

Chromosome numbers and heterochromatin banding pattern variability have been shown to be useful for taxonomic and evolutionary studies of different plant taxa. Bignoniaceae is the largest tribe of Bignoniaceae, composed mostly by woody climber species whose taxonomies are quite complicated. We reviewed and added new data concerning chromosome numbers in Bignoniaceae and performed the first analyses of heterochromatin banding patterns in that tribe based on the fluorochromes chromomycin A3 (CMA) and 4'-6-diamidino-2-phenylindole (DAPI). We confirmed the predominant diploid number $2n = 40$, as well as variations reported in the literature (dyploidy in *Mansoa* [$2n = 38$] and polyploidy in *Dolichandra unguis-cati* [$2n = 80$] and *Pyrostegia venusta* [$2n = 80$]). We also found a new cytotype for the genus *Anemopaegma* (*Anemopaegma citrinum*, $2n = 60$) and provide the first chromosome counts for five species (*Adenocalymma divaricatum*, *Amphilophium scabriusculum*, *Fridericia limae*, *F. subverticillata*, and *Xylophragma myrianthum*). Heterochromatin analyses revealed only GC-rich regions, with six different arrangements of those bands. The A-type (one large and distal telomeric band) were the most common, although the presence and combinations of the other types appear to be the most promising for taxonomic studies.

Key words: Cytotaxonomy, fluorochromes, neotropical lianas, ploidy variation.

INTRODUCTION

Bignoniaceae is the largest tribe in Bignoniaceae, comprising more than 393 species in 21 genera (Lohmann and Ulloa 2017). While Bignoniaceae is Pantropical, Bignoniaceae is exclusively Neotropical, occurring from southern United States through

northeastern Chile and central-northern Argentina; several of its species are restricted to Brazil (Gentry 1980, Lohmann and Taylor 2014). The tribe is characterized by lianas (sometimes shrubs or small trees), wood with cambial variants forming four to 32 phloem arcs or wedges, compound and opposite leaves with the terminal leaflet often replaced by a tendril, and septicial capsules (Lohmann and Taylor 2014).

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Phylogenetic studies in Bignoniaceae have shown that Bignoniaceae is a strongly supported clade, sister to a not well-supported clade with two lineages mostly composed of arboreal species: one lineage with Catalpeae and Oroxyleae, and the other with the informal Crescentiina clade (Olmstead et al. 2009). Although the tribe Bignoniaceae is very well established, the classification of its genera has always been challenging (Gentry 1980, Lohmann and Taylor 2014). Molecular phylogenetic studies have shown that previous generic system did not reflect evolutionary relationships between lineages within the tribe. Forty-seven genera were previously recognized, but only 21 lineages were retrieved (Lohmann 2006). Among those lineages, six reflected genera that kept their previous circumscriptions (*Anemopaegma*, *Lundia*, *Martinella*, *Pyrostegia*, *Styzyphyllum*, and *Tynanthus*). The remaining species were combined into 15 genera with broader circumscriptions that are now recognizable by previously unused synapomorphies (Lohmann and Taylor 2014). Despite this new and robust system, some of the genera whose circumscriptions were altered are morphologically very close, and it remains difficult to distinguish them (Lohmann and Taylor 2014).

Most Bignoniaceae species have very stable chromosome numbers. Approximately 85% of the species studied showed $2n = 40$ (Goldblatt and Gentry 1979, Piazzano 1998, Firetti-Leggieri et al. 2011, 2013, Piazzano et al. 2015, Cordeiro et al. 2016a), although some species show variant ploidies, such as *Pyrostegia venusta* (Ker Gawl) Miers ($2n = 40, 60, \text{ and } 80$; Joshi and Hardas 1956, Piazzano 1998, Cordeiro et al. 2016a), *Dolichandra unguis-cati* (L.) L. G. Lohmann ($2n = 40 \text{ and } 80$; Goldblatt and Gentry 1979, Piazzano 1998, Cordeiro et al. 2016a), in addition to some *Anemopaegma* species with $2n = 40 \text{ and } 80$ (Firetti-Leggieri et al. 2011, 2013). Dysploidy was also observed in *Mansoa*, with $2n = 38$ being observed in *M. hymenaea* (DC.) A. H. Gentry (Simmonds

1954) and *M. difficilis* (Cham.) Bureau & K. Schum. (Cordeiro et al. 2016a). It is also speculated that the chromosome numbers in some species of *Anemopaegma* (Firetti-Leggieri et al. 2011, 2013) may reflect allopolyploidy, as some individuals of distinct species have intermediate morphologies.

In addition to morphology and chromosome numbers, important tools for karyotypic characterization in plants are variations in the quantities, distributions, and compositions of their constitutive heterochromatin. The fluorochromes Chromomycin A3 (CMA) and 4'-6-diamidino-2-phenylindole (DAPI) are widely used for those studies. Both fluorochromes are used with the same specimens, resulting in differential coloring patterns: CMA stains GC-rich regions, whereas DAPI stains only AT-rich regions (Guerra 2000). This type of analysis has been used to distinguish the cytotypes of species belonging to different hierarchical levels, such as families (Cordeiro et al. 2017), species of the same genus (Almeida et al. 2007, 2016, Cordeiro et al. 2016b), and even cultivars and populations of the same species (Dematteis et al. 2006, Romero-da Cruz et al. 2015, Begum and Alam 2016).

We considered here the heterochromatin patterns of 24 species of Bignoniaceae based on the fluorochromes chromomycin A3 (CMA) and 4'-6-diamidino-2-phenylindole (DAPI), as well as variations in the chromosome numbers of 62 species of the tribe (including variations due to polyploidy and dispolyploidy). We discuss the variations found among heterochromatin patterns as well as those of chromosome numbers in the tribe, based on the new findings provided here and records gathered from literature.

MATERIALS AND METHODS

TAXON SAMPLING

The chromosome numbers of 62 species in 17 genera of Bignoniaceae were determined, eight of

them were new counts; 16 species whose counts were previously published in the literature were counted again, and 38 species were considered only from published literature. Among them, 18 species had more than one sample included in the analysis. Taxon names including authorities, chromosome numbers, and references are listed on Table SI - Supplementary Material.

The heterochromatin banding patterns of 24 species belonging to 12 genera of lianas and shrubs (mostly from northeastern, southeastern, mid-western Brazil) were examined. Taxon names, vouchers, collection sites, vegetation types, and karyological details are listed in Table SII. On the average, three specimens of each species were germinated in plastic pots in the experimental garden of the Centro de Ciências Agrárias of the Universidade Federal da Paraíba. When their roots reached 2 cm in length, fifteen roots tips per specimen were excised and analyzed.

CYTOGENETIC ANALYSES

Mitosis was examined in root tips that had been pre-treated with 0.002 M 8-hydroxyquinoline (8-HQ) for 24 hours, fixed in Carnoy's solution (absolute ethanol: glacial acetic acid; 3:1, v/v) for 30 minutes and subsequently stored at -20 °C. The root tips were then digested in a solution of 2% cellulase and 20% pectinase at 37 °C for 40 minutes. The samples were crushed between slides and coverslips in 45% acetic acid and subsequently submerged in liquid nitrogen to remove the coverslips. The samples were then stained with DAPI (2 µg/ml): glycerin (1:1, v/v), and the best samples were selected. The samples selected were fixed again in Carnoy's solution (3:1, v/v) for 30 minutes at room temperature, and then kept in absolute ethanol for two hours. The slides were then dried for three days at room temperature, and each sample was subsequently stained with 10 µL of CMA (0.1 mg/mL) for one hour and then with 10 µL of DAPI (1 µL/mL) for 30 minutes. The slides

were mounted with glycerin/ McIlvain buffer at pH 7,0 (1:1, v/v) and kept in dark for three days (Guerra and Souza 2002).

Images of three slides with at least ten cells in metaphase per slide were captured using an Axio Cam MRC5 digital camera with AxioVision 4.8 software (Carl Zeiss Microscopy GmbH, Jena, Germany). Final documentation was prepared using Photoshop CS3 Extended 10.0 software (Adobe Systems Incorporated, San Jose, USA). Chromosome measurements were made using Image Tool 3.0 software (Donald et al. 2008). Chromosome morphologies were determined using the centromeric index, following Guerra (1986). Classification of the heterochromatin banding patterns followed Guerra (1993) and Cornélio et al. (2003).

RESULTS

CHROMOSOME NUMBERS

The chromosome numbers of 62 species of Bignoniaceae (Bignoniaceae) were analyzed; 55 species (88.7%) showed $2n = 40$; six (9.67%) showed $2n = 80$, two (3.22%) showed $2n = 60$; and two (3.22%) showed $2n = 38$. Two species have reported intraspecific variations: *Dolichandra unguis-cati* ($2n = 40$ and 80) and *Pyrostegia venusta* ($2n = 40$, 60 and 80). Polyploidy was also reported in *Anemopaegma* ($2n = 60$ and 80), while disploidy has only been reported in *Mansoa* ($2n = 38$) (Table SI).

CYTOGENETIC ANALYSES AND HETEROCHROMATIN PATTERNS

Most species sampled here were diploids, with $2n = 40$ (Table SII; Figures 1 and 2). Two species were tetraploids (*D. unguis-cati* and *P. venusta*, $2n = 80$, Figures 1i and 2i respectively); one was triploid (*Anemopaegma citrinum*, $2n = 60$, Figure 1H); and one dysploid (*Mansoa difficilis*, $2n = 38$, Figure 2h). The karyotypes showed small chromosomes (following Guerra 2000). The mean sizes ranged between 1.36 µm (± 0.22) in *Adenocalymma*

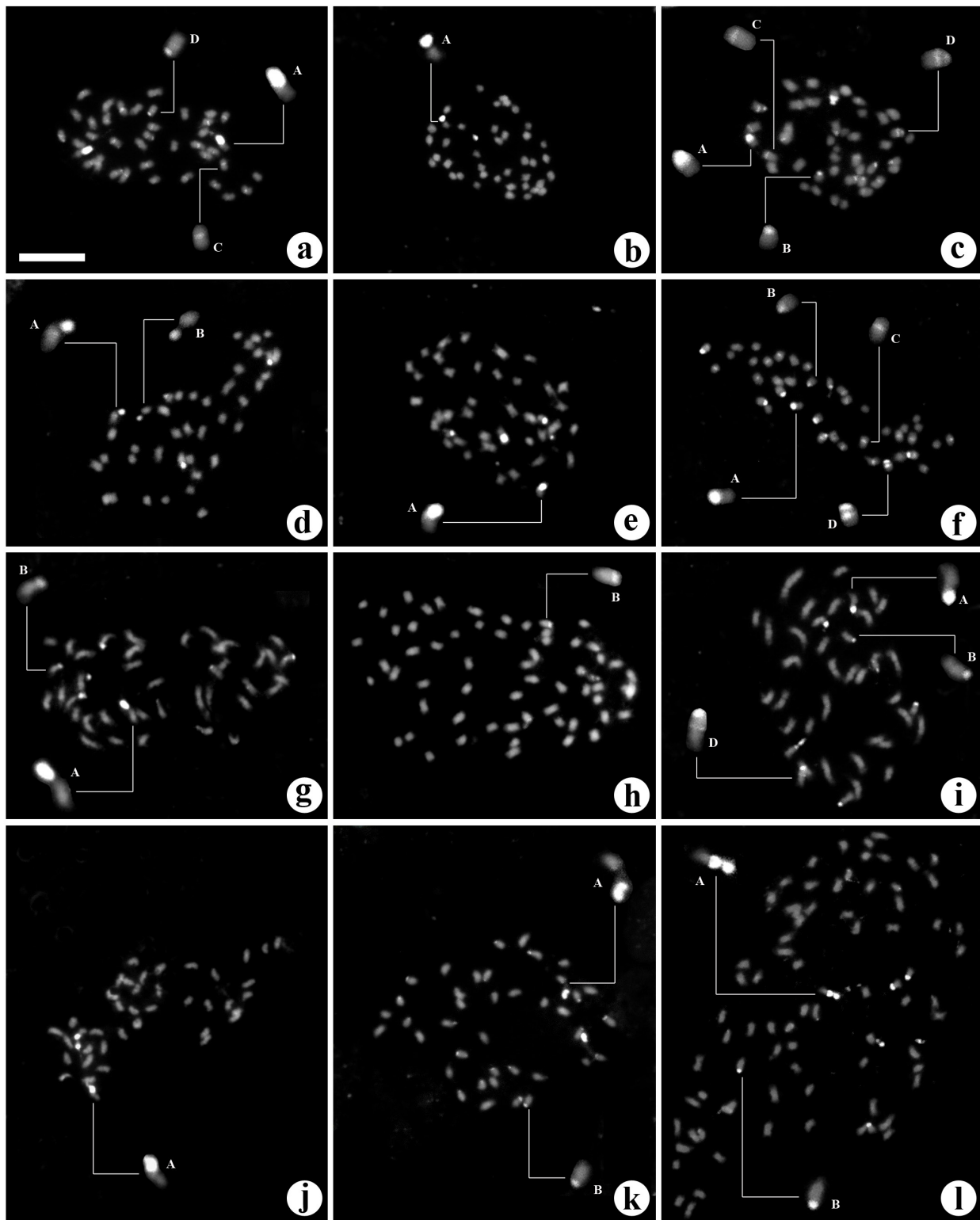


Figure 1 - Distribution of CMA⁺ bands in Bignoniaceae (Bignoniaceae) species: **a.** *Adenocalymma divaricatum*, **b.** *A. imperatoris-maximilianii*, **c.** *Amphilophium bauhinioides*, **d.** *A. crucigerum*, **e.** *A. elongatum*, **f.** *A. scabriusculum*, **g.** *Anemopaegma album*, **h.** *A. citrinum*, **i.** *A. leave*, **j.** *Cuspidaria laterifolia*, **k.** *Dolichandra quadrivalvis*, **l.** *D. unguis-cati*. Scale bar in **a** corresponds to 10 μ m.

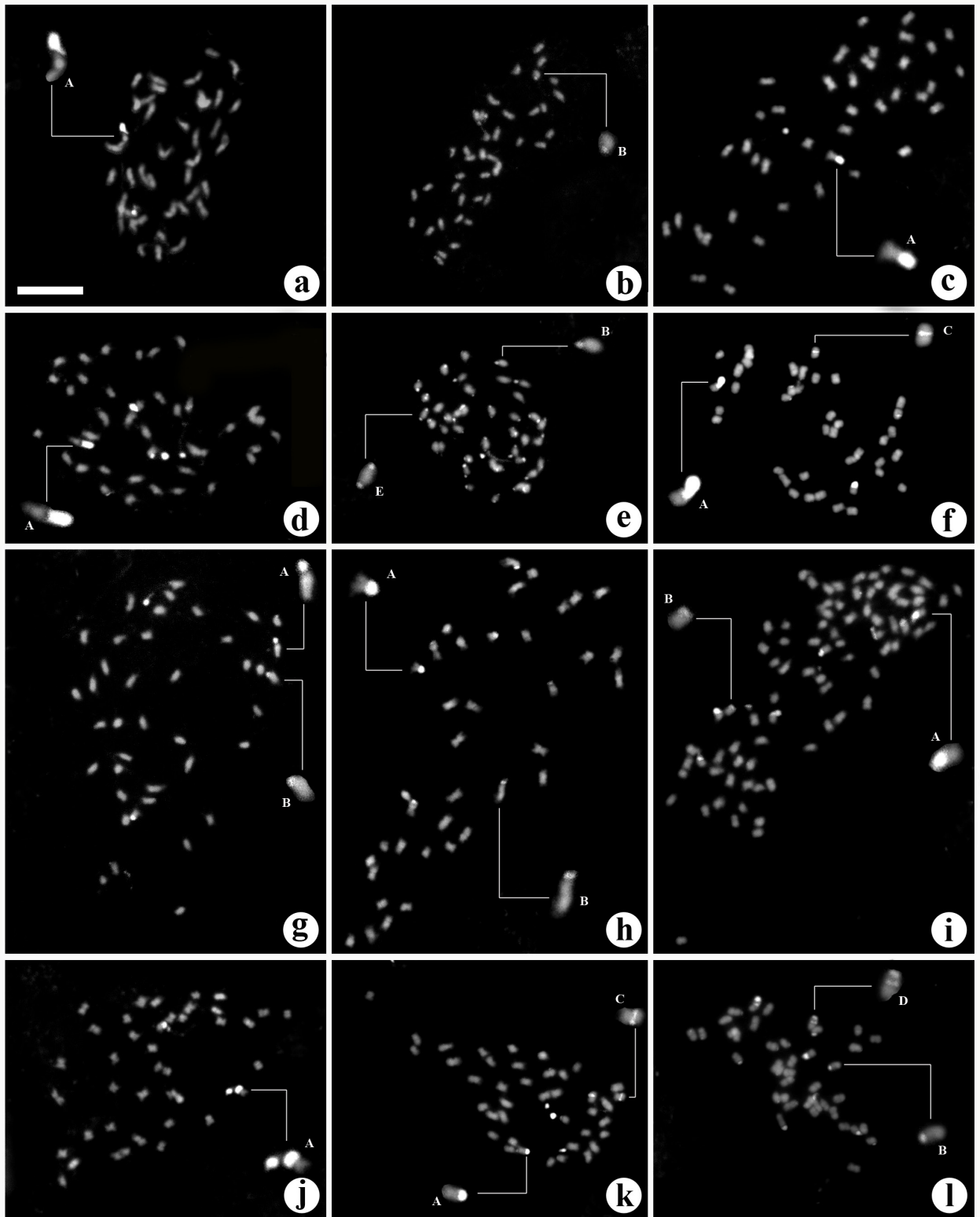


Figure 2 - Distribution of CMA⁺ bands in Bignoniaceae (Bignoniaceae) species: **a.** *Fridericia dichotoma*, **b.** *F. erubescens*, **c.** *F. limae*, **d.** *F. platyphylla*, **e.** *F. pubescens*, **f.** *F. subverticillata*, **g.** *Lundia longa*, **h.** *Mansoa difficilis*, **i.** *Pyrostegia venusta*, **j.** *Stizophyllum riparium*, **k.** *Tanaecium selloi*, **l.** *Xylophragma myrianthum*. Scale bar in **a** corresponds to 10 μ m.

imperatoris-maximilianii and $2.62 \mu\text{m}$ (± 0.42) in *Anemopaegma laeve* (Table SII). The chromosome morphologies were mainly symmetrical for all species, being metacentric or sub-metacentric.

The heterochromatin of all of the species showed exclusively GC-rich bands ($\text{CMA}^+/\text{DAPI}^-$), with no AT-rich bands ($\text{CMA}^-/\text{DAPI}^+$) being found. The GC-rich bands were observed in the proximal or terminal portions of the chromosome arms (Figures 1 and 2, Table SII). Six patterns were identified among the karyotypes of Bignoniaceae species based on the sizes and distributions of their chromosomal bands (Figure 3, Table SII): A) one large and distal telomeric band (observed in 19 species); B) one small or very small distal band (in 14 species); C) one small proximal band (in six species); D) two small bands, one being proximal and the other one distal in the same arm of the

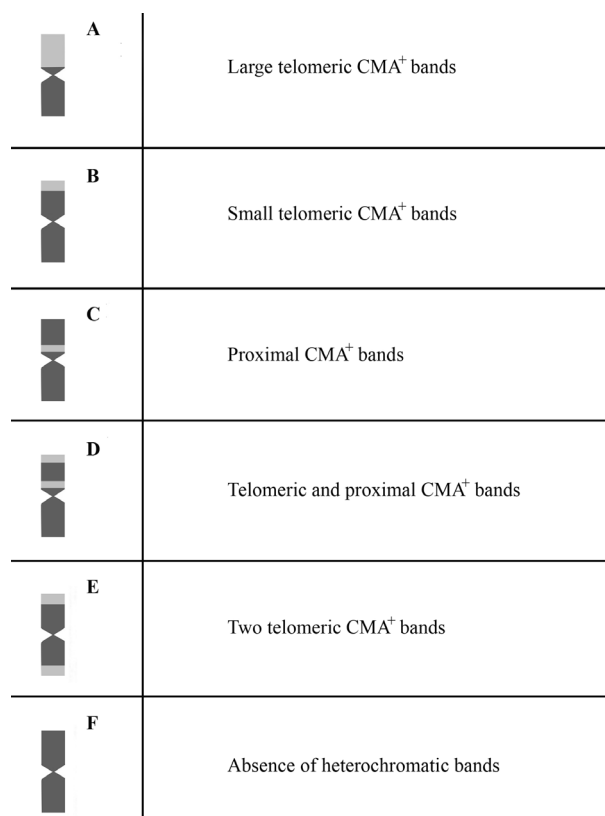


Figure 3 - Main chromosome types in Bignoniaceae (Bignoniaceae) according to the sizes and positions of the CMA^+ bands.

chromosome (in five species); E) two small distal bands (only in *Fridericia pubescens*, Figure 2e); and f) a lack of any heterochromatic bands.

DISCUSSION

The chromosome number $2n = 40$ was found in $\sim 90\%$ of the species of Bignoniaceae previously analyzed (Table SI). Few species are $2n \neq 40$, although polyploidy is quite important in *Anemopaegma* (Firetti-Leggieri et al. 2011, 2013), and dispolyploidy seems to be important in *Mansoa*. The prevalence of $2n = 40$ has also been observed in other supra-generic groups of the family Bignoniaceae, such as in Catalpeae and the *Tabebuia* alliance clade (Goldblatt and Gentry 1979, Piazzano 1998). Diploid numbers are variable in other clades of Bignoniaceae, however, such as in the tribes Oroxyleae ($2n = 14$ and 15 ; Goldblatt and Gentry 1979), in Tecomeae *sensu* Olmstead et al. (2009) ($2n = 22$, 36 and 38 ; Goldblatt and Gentry 1979, Piazzano 1998, Chen et al. 2004, Piazzano et al. 2015), in Jacarandae ($2n = 36$; Cordeiro et al. 2016b), and in the genera *Argylia* D. Don ($2n = 30$; Goldblatt and Gentry 1979) and *Delostoma* D. Don ($2n = 42$; Goldblatt and Gentry 1979).

Our data corroborated previous records of chromosome numbers in Bignoniaceae. Five of seven new records had $2n = 40$ (*Adenocalymma divaricatum*, *Amphilophium scabriusculum*, *Fridericia limae*, *F. subverticillata*, and *Xylophragma myrianthum*). Additionally, a new cytotype is described here for the genus *Anemopaegma*, more specifically for *A. citrinum* ($2n = 60$), since previous counts for that genus were $2n = 40$ and 80 (Firetti-Leggieri et al. 2011, 2013). Gentry (1973) noted that *Anemopaegma* is one of the most complicated genera of Bignoniaceae because of the wide phenotypic plasticity of some species – which has led several authors to consider different phenotypes as different species. *Anemopaegma citrinum* is restricted to

dry regions of Brazil, whereas a morphologically close species, *A. chamberlaynii* (Sims) Bureau & K. Schum., is widely distributed in South America (see Lohmann and Taylor 2014). Both species show wide variations in leaf and calyx shapes and sizes; it is possible to distinguish between them because of variations in the morphologies of their inflorescences as well as the larger prophylls in *A. chamberlaynii* (smaller or missing in *A. citrinum*). There different chromosome numbers can also aid in distinguishing between them, with $2n = 40$ in *A. chamberlaynii* (Goldblatt and Gentry 1979, Firetti-Leggieri et al. 2011) and $2n = 60$ in *A. citrinum*. Further studies, including samples from different populations, will still be needed to determine if triploidy is a common feature in *A. citrinum*.

Disploidy is only found in *Mansoa* ($2n = 38$) in the Bignoniaceae. That genus was recently re-circumscribed based on molecular and morphological evidence and now includes species previously placed in *Pachyptera*, such as *Mansoa hymenaea*, whereas *M. difficilis* had long been placed in *Mansoa* (Lohmann and Taylor 2014). The similar chromosome numbers of those two taxa seem to represent additional evidence corroborating the synonymization of most species of *Pachyptera* into *Mansoa*.

Some species of *Anemopaegma*, *Dolichandra*, and *Pyrostegia* have putative polyploidy. All known polyploid species of *Anemopaegma* are from the Brazilian Cerrado and belong to a morphologically similar complex of species named the “*Anemopaegma arvense* complex” (Firetti-Leggieri et al. 2011, 2013). Some samples of *A. arvense* show us a continuum of otherwise distinct morphological features, which may indicate hybridization. Therefore, the $2n = 80$ record probably represents allopolyploidy (Firetti-Leggieri et al. 2011, 2013). The new record found here of $2n = 60$ in a species outside the “*Anemopaegma arvense* complex” (*A. citrinum*), on the other hand, may represent the fusion of a regular reduced gamete (n)

with an unreduced gamete ($2n$) of the same species, generating the triploid sample analyzed here. In addition to *A. citrinum*, $2n = 60$ was also observed in *Pyrostegia venusta* (Joshi and Hardas 1956). Even though triploids are sterile, triploid gametes can be fertilized by regular reduced gametes (n) and generate fertile tetraploids (Levin 2002). The generation of tetraploids involving triploid bridges is a well known mechanism (see de Wet 1971 for a review study) and can play an important role in generating polyploidy in plants (Soltis et al. 2007, Mason and Pires 2015). *Pyrostegia venusta*, for example, also has records of $2n = 40$ (Goldblatt and Gentry 1979, Piazzano 1998) and $2n = 80$ (Cordeiro et al. 2016a). This reinforces the hypothesis that polyploidy involving triploid bridges is an evolutionary mechanism acting in Bignoniaceae.

Another example of polyploidy was recorded for *Dolichandra unguis-cati*. This species is widely distributed in wet and dry forests in the neotropical region, from the southern United States through Argentina (Lohmann and Taylor 2014). Polyploidy is very common feature of invasive species (see Beest et al. 2012 for a review), and *Dolichandra unguis-cati* is an important invasive species in Australia, the United Arab Emirates, and South Africa (Fonseca and Lohmann 2015). The records of $2n = 40$ and 80 for this species may have resulted from autopolyploidy, although its sympatric distribution with the closely related species *D. quadrivalvis*, associated with its wide distribution and potential as an invasive plant, suggests allopolyploidy. Further studies involving reproductive biology and population genetics of these closely related species will be essential to understanding how that species has fixed a tetraploid karyotype.

Fluorochromes have been shown to be a powerful cytotaxonomic tool in karyotype analysis. The distinct staining patterns of heterochromatin revealed when using this technique are useful for distinguishing between plant groups with very stable chromosome

numbers (Guerra 2000). At least six different GC-rich band patterns are observed in Bignoniaceae, and they are usually telomeric. Chromosome type A is the most common pattern, occurring in 79.16% of the species studied. This large band may be related to nucleolar organizer regions (NORs) because their placement at, or close to, telomeric portions of the chromosomes. Chromosomes types B, C, D and F were variable in terms of presence/absence and numbers among the different species, and therefore appear to be the most suitable for cytotaxonomic analysis within the Bignoniaceae. Chromosome type E was the rarest, being observed only in *Fridericia pubescens*. The variations of the numbers and placements of heterochromatic bands may reflect satellite DNA amplification, retrotransposons, and co-amplification of tandem repeats, and/or other transposable elements (Eickbush and Eickbush 2007, Hobza et al. 2015, Evtushenko et al. 2016). Despite the multitude of mechanisms capable of producing different patterns, variations in heterochromatin patterns have been used to confirm the taxonomic placements of numerous taxa. *Citrus* is a good example, as the seven chromosome types described by Guerra (1993) and Cornélio et al. (2003) have been widely used to distinguish between different specimens, cultivars, and hybrids – even species of related genera (Cornélio et al. 2003, Carvalho et al. 2005, Barros e Silva et al. 2010).

According to Guerra (2000), heterochromatin is not homogeneous and may vary qualitatively and quantitatively between taxa, making it potentially useful for taxonomic purposes. Although the species of some genera have constant patterns of heterochromatic bands, such as *Crinum* (Ahmed et al. 2004), *Lycium* (Stiefkens et al. 2010), and *Pereskia* (Castro et al. 2016), no genus of Bignoniaceae that has had more than one species sampled has been observed to demonstrate any particular banding pattern; identical karyotypes, on the other hand, have been observed in species of distinct genera. The karyotype 2 A + 38 F, for

example, is shared by *Adenocalymma imperatoris-maximilianii*, *Cuspidaria lateriflora*, *Fridericia dichotoma*, *F. limae*, and *Stizophyllum riparium*; the karyotype 4 A + 36 F is shared by *F. platyphylla* and *Amphilophium elongatum*; and 2 A + 2 C + 36 F is shared by *F. subverticillata* and *Tanaecium selloi*. The most common karyotypes are based on chromosomes types A, B, and F, with variable numbers of types B and F (more specifically 2 A + 2–10 B + 28–36 F). This karyotype is shared by *Amphilophium crucigerum*, *Anemopaegma album*, *Dolichandra quadrivalvis*, *Lundia longa*, and *Mansoa difficilis*. In addition to those taxa, two of the three polyploid species (*Dolichandra unguis-cati* and *Pyrostegia venusta*) show the same patterns when the formula is multiplied by the ploidy number. Eleven species have exclusive karyotypes, including *Fridericia pubescens* (2 B + 10 E + 8 F), with the exclusive chromosome type E. Our results suggest that there are significant variations in heterochromatin banding patterns in the family Bignoniaceae, although their future utility will depend on further studies, including more samples per species. Wider studies would allow us to better understand variations in the chromosome types of species having the 2 A + 2–10 B + 28–36 F karyotype (i.e., if this karyotype is stable within the same species), and help ensure that there are no variations in the karyotypes of species having exclusive karyotypes. This will certainly be an interesting investigative line to follow.

CONCLUSIONS

The chromosome number $2n = 40$ is predominant in the cytogenetics of Bignoniaceae. Some variations were found among the species of *Mansoa* (possibly due to dispolyploidy [$2n = 38$]), in some species of *Anemopaegma* and *Pyrostegia venusta* ($2n = 40, 60$ and 80), and in *Dolichandra unguis-cati* ($2n = 40$ and 80) – possibly due to allopolyploidy or autopolyploidy.

Staining with the fluorochromes CMA/DAPI demonstrated only GC-rich bands (CMA⁺/DAPI⁻) in all species. Six chromosomes types were identified based on the sizes and distributions of their heterochromatin bands. Type A chromosomes, have large CMA⁺ and telomeric bands (occurring in ~80% of the species) that probably represent nucleolar organizer regions (NORs). Chromosome types B, C, D and F were variable in terms of their presence/absence and numbers of CMA⁺ bands among the different species, and therefore may be suitable for cytotaxonomic analyses of Bignoniaceae species.

No genus of Bignoniaceae with more than one sampled species showed a unique banding pattern, and similar karyotypes were occasionally observed in species from distinct genera. Therefore, there are variations in heterochromatin bands in the Bignoniaceae, but the utility of those analyses will still depend on further studies to ensure that the karyotypes are stable within the same species and that there are no variations in the karyotypes of species that show exclusive karyotypes.

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SUPPLEMENTARY MATERIAL

TABLE SI - Chromosome numbers recorded for the tribe Bignoniaceae and their respective bibliographic references.

TABLE SII - Species of the tribe Bignoniaceae (Bignoniaceae) analyzed and their main karyological parameters. Legend: A - large telomeric CMA⁺ bands, B - small telomeric CMA⁺ bands, C - proximal CMA⁺ bands, D - telomeric and proximal CMA⁺ bands, E - two telomeric CMA⁺ bands, F - absence of heterochromatic bands.