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CELLULAR AND MOLECULAR BIOLOGY

The karyotype of *Adenia* and the origin of the base number x = 12 in Passifloroideae (Passifloraceae)

NATONIEL FRANKLIN DE MELO & MARCELO GUERRA

Abstract: *Adenia* is an Old World genus of Passifloroideae closely related to *Passiflora*. The two genera comprise the large majority of Passifloroideae species, although most studies are concentrated on *Passiflora*. Cytological analyses reveal that changes in chromosome numbers played an important role in the evolution of *Passiflora,* whereas in the remaining genera little is known, hindering the identification of the base number of the family. Here we analyzed the chromosome number and the 35S rDNA sites of three species of *Adenia* and reevaluated the base number (*x*) of the subfamily Passifloroideae and the family Passifloraceae, including chromosome data for Turneroideae and Malesherbioideae. The chromosome number of *Adenia* species seemed to be stable with 2*n* = 24 or 48 and one or two pairs of rDNA sites, very similar to *Passiflora* subgenus *Astrophea*, suggesting a common ancestral karyotype with *x* = 12. Differently, Turneroideae and Malesherbioideae present *x* = 7. A whole genomic duplication detected after the separation of Passifloroideae and Malesherbioideae suggests that the base number of Passifloraceae most probably was *x* = 7, which by dysploidy and polyploidy generated *x* = 12 for the subfamily Passifloroideae.

Key words: Cytotaxonomy, 35S rDNA sites, karyotype evolution, fluorescence *in situ* hybridization, Turneroideae, Malesherbioideae.

INTRODUCTION

Adenia Forssk. is the second largest genus of the subfamily Passifloroideae (Passifloraceae) with approximately 100 species distributed in the Old World tropics and subtropics, the large majority of them in Africa (Feuillet & MacDougal 2007). The genus presents an uncommon diversity in growth form and ability to explore very different habitats (Hearn 2006). It is closely related to *Passiflora*, the largest and best studied genus of the family with over 560 species (Krosnick et al. 2013). Phylogenetic analyses revealed that both genera are monophyletic (Hearn 2006). Morphological (Feuillet & MacDougal 2007) and molecular analyses (Maas et al. 2019) placed *Adenia* in an intermediate position between the two tribes of Passifloroideae: Passifloreae and Paropsieae. According to APG III (2009), the former families Passifloraceae, Turneraceae and Malesherbiaceae should be included into the family Passifloraceae, as subfamilies Passifloroideae, Turneroideae and Malesherbioideae.

Cytological analyses of *Passiflora* revealed that chromosome number changes played an essential role in the early diversification of the genus, resulting in subgenera with different chromosome base numbers (*x*). The species of *Passiflora* are currently subdivided into four subgenera, according to Feuillet & MacDougal (2003), with a fifth subgenus (*Tetrapathea*)

proposed latter by Krosnick et al. (2009). The two largest subgenera, *Decaloba* and *Passiflora,* have *x* = 6 and *x* = 9, respectively, whereas *Astrophea, Deidamioides, and Tetrapathea* possess *x* = 12 (Hansen et al. 2006), resulting in different interpretations of the base number of the genus (reviewed by Sader et al. 2019a). Differently, the chromosome number of *Adenia* species has been reported only for *A. lobata* (Jacq.) Engl. (2*n* = 24), *A. mannii* (Mast.) Engl. (2*n* = 24) and *A. rumicifolia* Engl. & Harms (2*n* = 48) (Mangenot & Mangenot 1962). In this sense, it would be important to have more chromosome counts of *Adenia* species to know if it experienced similar chromosome number radiation.

A key point to understand the chromosomal evolution of a taxon is the identification of its chromosome base number, which can be defined as the haploid number that most parsimoniously explains the cytological variability in a clade and shows a clear relationship with the base numbers of the closest related taxa (Guerra 2000). It may be inferred from a careful evaluation of the chromosome numbers reported for a clade, or it may be based on probabilistic models, some of them taking into consideration the possible ways of chromosomal evolution in that clade (Mayrose et al. 2010, Freyman & Höhna 2017). Since chromosome numbers are subjected to different rates of dysploidy and polyploidy and are under control of natural selection (Levin 2002), these probabilistic methods should be considered with caution. For *Passiflora,* the base number of each subgenus is clear since the haploid numbers do not vary, with a few exceptions, whereas the base number of the genus have been subject to a long debate. Strictly cytological analysis suggest *x* = 6 or *x* = 12 for the genus (reviewed by Melo et al. 2001), whereas probabilistic models suggest *x* = 6 (Hansen et al. 2006) or *x* = 12 (Mayrose et al. 2010, Sader et al. 2019a), depending on the algorithm used.

Beside the chromosome numbers, extensive genomic and cytomolecular studies have been done for *Passiflora* species (Melo & Guerra 2003, Munhoz et al. 2018, Pamponét et al. 2019, Dias et al. 2020, Xia et al. 2021), whereas nothing similar is known for *Adenia*. Most cytomolecular studies include the chromosome mapping of 5S and 35S rDNA sites by fluorescence *in situ* hybridization (FISH), bringing further details about the chromosome variability of the group (*e.g*., Melo & Guerra 2003, Silva et al. 2018, Sader et al. 2019b). The analysis of 20 species of *Passiflora* revealed that the number of 5S rDNA sites was generally proportional to the ploidy level of the species, while the number of 35S rDNA sites varied from 2 to 10 among diploid species (Melo & Guerra 2003).

In the present study, we analyzed the chromosome number and the distribution of the 35S rDNA sites in three *Adenia* species, aiming to evaluate the karyotype variability of the genus. Further, we reappraised the basic number of *Adenia*, *Passiflora*, Passifloroideae and Passifloraceae based on the most recent phylogenetic arrangements and genomic data.

MATERIALS AND METHODS

The three species analyzed, *Adenia fruticosa* Burtt Davy, *A. spinosa* Burtt Davy, and *A. glauca* Schinz, were grown in pots in the greenhouse of the Botanical Garden of the University of Vienna, Austria. Actively growing shoot meristems and young root tips were cut in small pieces and immediately pretreated with 8-hydroxyquinoline (0.002 M) for 5 h at 6 °C. After pretreatment they were washed in distilled water for 5 min, fixed in Carnoy solution [ethanol-acetic acid (3:1, v/v)] for 24 h at room temperature, and stored in the freezer at –20 °C.

For cytological preparations, the meristems were digested in 2% cellulase-20% pectinase at 37 °C for 90 min. The meristems were squashed in 45% acetic acid and the coverslips were removed in liquid nitrogen. The slides were air-dried and stained with 2 µg/ml DAPI–glycerol (1:1) to allow selection of the best preparations. The best slides were fixed again in Carnoy, for 30 min, dehydrated in 100% ethanol and stored at –20 °C until required for *in situ* hybridization.

For *in situ* hybridization, the same protocol described by Melo & Guerra (2003) for *Passiflora* species was used. Probes SK18S and SK25S containing, respectively, 18S and 25S rDNA of *Arabidopsis thaliana* L. (Unfried et al. 1989, Unfried & Gruendler 1990) were used to localize the 35S rDNA sites. They were labelled with biotin-11-dUTP and detected with TRITC (tetramethyl rhodamine isothiocyanate). Chromosomes were counterstained with DAPI and the slides mounted in Vectashield (Vector). Cells were photographed with a DMLB Leica epifluorescence photomicroscope using Kodak Ultra color film ASA 400. The images were later digitalized and edited in Adobe Photoshop CS3 version 10.0.

RESULTS AND DISCUSSION

The karyotype of *Adenia*

Adenia spinosa and *A. fruticosa* presented the same chromosome number, 2*n* = 24, with symmetrical karyotypes and small chromosomes, which were slightly smaller in the former, whereas *A. glauca* exhibited 2*n* = 48, with some chromosomes nearly twice as larger as the smaller ones (Figure 1a-d). These data reinforce the assumption that the basic chromosome

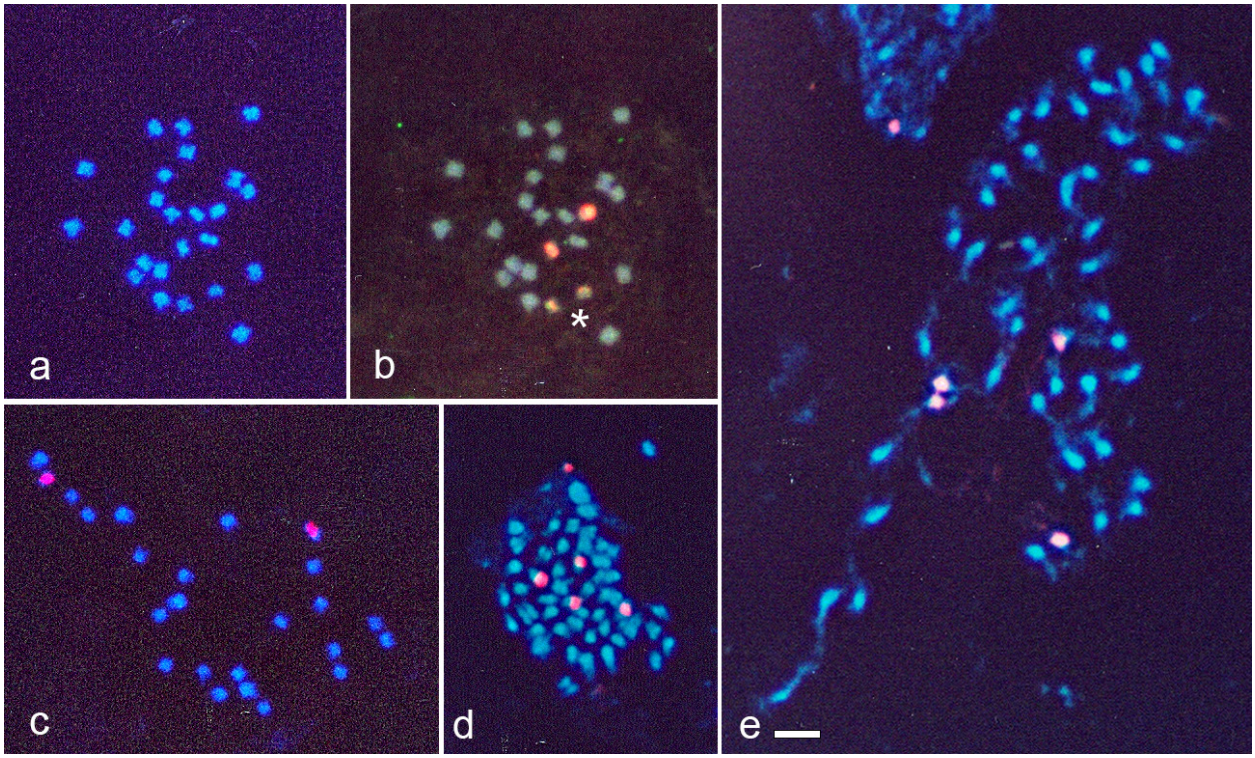

Figure 1. Chromosomes of *Adenia fruticosa (a-b*, 2n = 24), *A. spinosa (c*, 2n = 24), *and A. glauca (d-e*, 2n = 48). Note the similarity in chromosome size and morphology (a,b), the occurrence of four 35S rDNA sites (red) in b, d and e, and only two sites in c. Prophase chromosomes of *A. glauca* (e) show the chromosome condensation pattern. Bar in and corresponds to 5 µm.

number of the genus is *x* = 12. At prophase, most chromosomes exhibited less condensed terminal regions (Figure 1e), as observed in most *Passiflora* species (Melo et al. 2001). Noteworthy, the tetraploid *A. glauca* had a more asymmetrical karyotype than its sister species, the diploid *A. spinosa* (Hearn 2006), suggesting that *A. glauca* most probably is an allopolyploid derived from *A. spinosa* and another species with larger chromosomes. Likewise, *A. rumicifolia* (2*n* = 48) is the sister species of *A. lobata* (2*n* = 24) (Mangenot & Mangenot 1962, Hearn 2006), but in this case there is no information about their karyotype symmetry. A parental relationship between diploid and tetraploid sister species by allopolyploidy with increasing karyotype asymmetry has been demonstrated in several other genera (see, e.g., Moraes & Guerra 2010, Ibiapino et al. 2019).

The *in situ* hybridization experiment detected only two sites of 35S rDNA in *A. spinosa* (2x) and four sites in *A. fruticosa* (2x) and *A. glauca* (4x) (Figure 1), indicating instability in the number of rDNA sites between diploid species. Similarly, among diploid species of *Passiflora* the number of 35S rDNA sites varied from two to six with 2*n* = 12 or 18 (Melo & Guerra 2003, Viana & Souza 2012, Silva et al. 2018, Dias et al. 2020). However, in *Passiflora* subgenus *Astrophea,* the most basal lineage of *Passiflora*, the two species investigated had also 2*n* = 24 and four 35S rDNA sites (Melo & Guerra 2003), as *A. glauca*, emphasizing the similarity between the karyotype of these two taxa. Reduction of 35S rDNA sites to a single pair was observed in some species of *Passiflora* (Melo & Guerra 2003) as well as in most angiosperms (Roa & Guerra 2012).

The base number of Passifloraceae

The finding of three other species of *Adenia* with *n* = 12, 24, in the present work, reinforces the assumption that its ancestral base number is *x* = 12. However, the six species of *Adenia* cytologically investigated belong to Clade V, the largest and most diversified among the five clades of the genus, with approximately 25 species and all of them endemic to Madagascar, one of the two centers of diversity of the genus (Hearn 2006). Therefore, additional chromosome counts are necessary to confirm the apparent chromosome stability of *Adenia*.

The elevated base number *x* = 12 has probably been originated by the Whole Genomic Duplication (WGD) that occurred after the separation of *Passifloroideae* from the monospecific *Malesherbioideae* (One Thousand Plant Transcriptomes Initiative 2019). Figure 2 shows the phylogenetic relationships within Passifloraceae (modified from Maas et al. 2019), highlighting only the genera with known chromosome numbers. Violaceae, the sister group of Passifloraceae, has a huge variation in chromosome numbers and an uncertain basic number (Raven 1975). The two species of *Malesherbia* cytologically known displayed *n* = 7 and *n* =14 (Ricardi 1967). For the Turneroideae, the base number *x* = 7 occurs in *Piriqueta, Adenoa,* and in most series of *Turnera* (Shore et al. 2006, Gonzalez et al. 2012).

The base number *x* = 7 in Malesherbioideae and Turneroideae suggests that the WGD has occurred after the separation of Turneroideae and Passifloroideae with *x* = 12 (Figure 2). In this case, there are two alternative scenarios: the sister group of Turneroideae, with *n* = 7, experienced a descending dysploidy to *n* = 6 followed by a WGD generating *n* = 12, or, the sister group had a WGD, resulting in *n* = 14, which by descending dysploidy generated *n* = 12. Further chromosome counts for other genera of Turneroideae and Passifloroideae are necessary to elucidate this point.

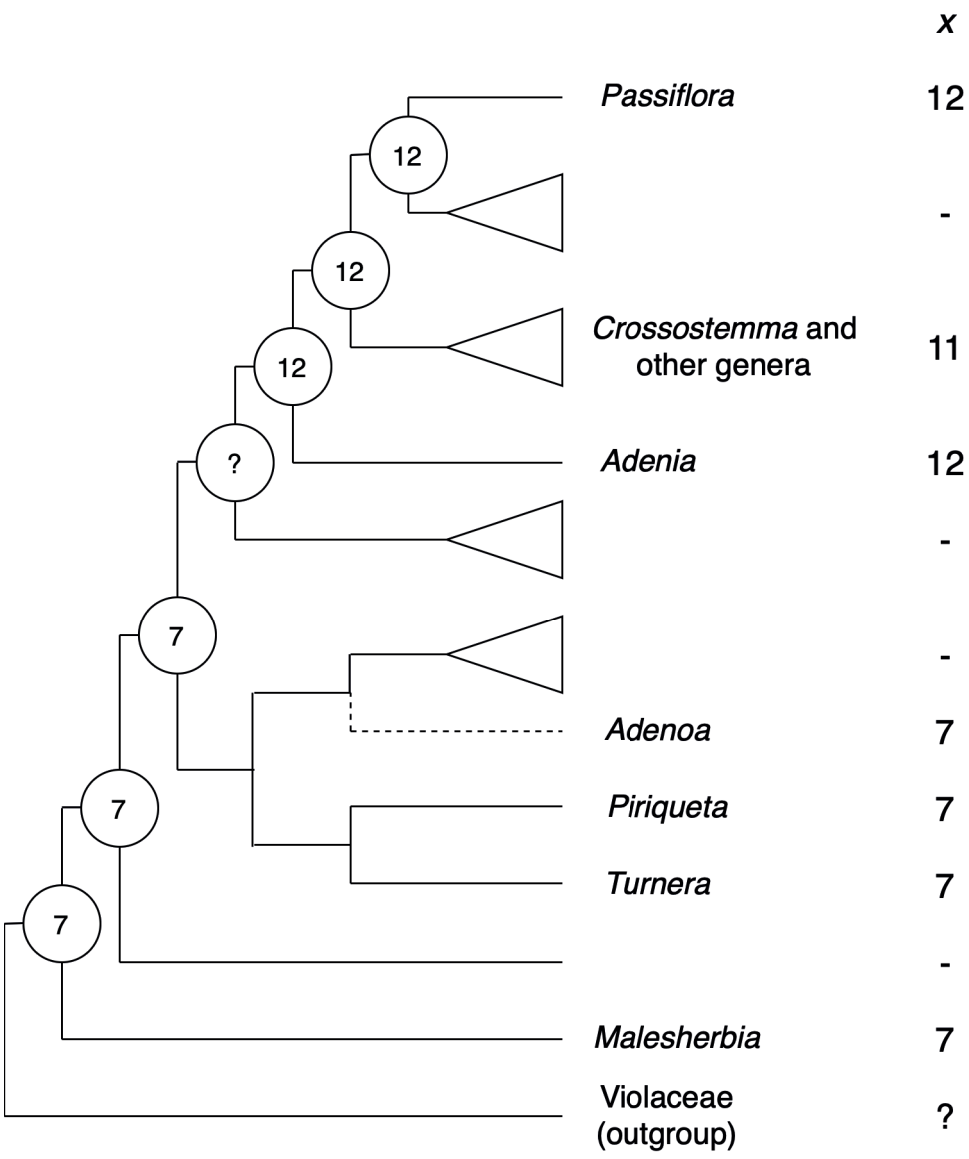

Figure 2. Cladogram of Passifloraceae (based on Maas et al. 2019) highlighting the genera with known base numbers. *Adenoa* was added to the cladogram as sister to *Turnera* and *Piriqueta*, according to Arbo et al. (2015). The base number of each clade is indicated within the circles. Triangles indicate three or more genera. The base number 11 refers to *Crassostemma* only.

Besides *Adenia* and *Passiflora*, the only other chromosome count for Passifloroideae is $n = 11$ for the monospecific genus *Crossostemma* (Gadella 1970), suggesting that $n = 12$, or near 12, was on the origin of several Passifloroideae genera (Figure 2). Within *Passiflora*, the number $n = 12$ seems to have been conserved in the subgenera *Astrophea*, *Deidamioides* and *Tetrapathea*, whereas the subgenera *Passiflora* and *Decaloba* evolved by descending dysploidies to $n = 9$ and $n = 6$, as indicated by recent genomic analyses of *P. edulis* Sims ($n = 9$) (Xia et al. 2021) and *P. organensis* Gardner ($n = 6$) (Costa et al. 2021). Intermediate numbers between the extremes of this dysploid series have been reported for a few species of *Passiflora* with $n = 11$, 10, and 7 (Melo et al. 2001), supporting the assumption that descending dysploidy played a central role on chromosome number variation and in the origin of the subgenera.

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Author contributions

MG and NFM designed the research, analyzed data and wrote the manuscript. The authors read and approved the manuscript.