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ORIGINAL ARTICLE

Cytogenetics of Amaryllidaceae species: heterochromatin evolution in different ploidy levels

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Abstract Species belonging to the Amaryllidaceae (*Zephyranthes* and *Habranthus*) were analyzed by banding with chromomycin A3 (CMA)/4,6-diamidino-2-phenylindole (DAPI) fluorochromes. The patterns of bands were studied in seven species of *Zephyranthes* Herb. and one of *Habranthus* Herb. Subterminal and interstitial DAPI+ bands were observed in *Z. robusta* 2n = 12 and *Z. brachyandra* 2n = 24. Other species showed no AT-rich heterochromatin. In species with 2n = 12, CMA+ bands were observed on one chromosome pair of *Z. robusta* and *Zephyranthes* sp., while in *Z. sylvatica* an additional small terminal band in the fifth chromosome pair was observed. *Z. rosea* and *Z. grandiflora* presented with 2n = 24 and had four CMA+ bands, while in *Z. brachyandra*, with 2n = 24 + 1B, there were eight interstitial dot bands and a larger terminal band in the short arm of the B chromosome. *Z. candida* with 2n = 38 presented CMA+ heterochromatin blocks on the long arms of five metacentric pairs and in the short arm of one of the submetacentric pairs; in addition a terminal band was observed on the long arm of one of the homologues of a larger submetacentric pair. *H. itaobinus* showed a heterozygous pair revealing a strong CMA+ band in only one of the homologues, likely a nucleolus organizing region. Taxonomic implications and karyotype evolution of this group are discussed and correlated with previous data from the literature.

Keywords CMA/DAPI · Polyploidy · Chromosome · Heteromorphism · B chromosome

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Introduction

The genus *Zephyranthes* Herb. comprises about 65 species of mostly Neotropical distribution (Hutchinson 1959; Judd et al. 1999) from the southern United States to southern Chile and Argentina, with 36 species reported from Brazil, some of which have great potential for ornamental use (Dutilh 2005). The genus is included in tribe Hippeastreae where three clades form subtribe Zephyranthinae subtribe. It is included in the Amaryllidaceae family and related to genus *Habranthus* Herb. (Meerow et al. 2007), the latter often considered a *Zephyranthes* synonym. For some authors *Zephyranthes* has flowers and erect stamens, while in *Habranthus* flowers and stamens are declined, although often these characteristics overlap (Arroyo 1981). In the single phylogenetic analysis that included some species of the genus, three clades were formed: one with species distributed in the southern United States and Mexico, another, along with *Habranthus*, formed

by species from South America, and a third one involving species from the southern United States and South America (Meerow et al. 2000a).

Most species recognized in *Habranthus* present 2n = 12 or similar numbers, whereas species recognized in *Zephyranthes* present with 2n = 24 or multiples of this

The members of Hippeastreae differ from the other Amaryllidaceae from South America by diploid karyotype and deletions in a set of bases in both ITS on the rDNA 45S (Meerow et al. 2000b). *Zephyranthes* taxonomically is a very complex genus, with poorly defined morphological boundaries between species. A chromosome record is available for 50 species out of about 65 known (Greizerstein and Naranjo 1987). Most species recognized in *Habranthus* present 2n = 12 or similar numbers, whereas species recognized in *Zephyranthes* present with 2n = 24 or multiples of this (Oliveira 2006). However, it is a very variable genus in terms of chromosome numbers, with counts from 2n = 10 in *Z. seubertii* (Daviña 2001) to 2n = 200 in an interspecific horticultural hybrid (Flory and Smith 1980).

The variability in numbers is due to events of polyploidy, aneuploidy and disploidy, in addition to the presence of B chromosomes, which are frequent in the genus (Greizerstein and Naranjo 1987; Felix et al. 2008). Chromosome records on intraspecific numerical variations and B chromosomes in species such as *Z. aff. mesochloa*, *Z. brasiliensis*, *Z. grandiflora*, *Z. chlorozolen*, *Z. candida*, and *Z. sylvatica* (Raina and Khoshoo 1971; Bhattacharyya 1972; Greizerstein and Naranjo 1987; Felix et al. 2007, 2008) are well documented and indeed confirm the great numerical chromosome variability of the genus. However, the only record involving banding techniques with base-specific fluorochromes (Daviña 2001) is restricted to a single species and does not provide an overview of the structural cytological variability of the genus.

In this work seven species of *Zephyranthes* and one of *Habranthus* were analyzed by cytological banding with the chromomycin A3 (CMA) and 4,6-diamidino-2-phenylindole (DAPI) fluorochromes to further characterize the karyotypes and to increase our understanding of karyotype evolution in these species.

Materials and methods

A total of eight Amaryllidaceae species, seven of *Zephyranthes* and one of *Habranthus*, were studied. Table 1 summarizes the information about species examined, synonym, provenance (origin), karyotype formula, and chromosome number. All material was grown in pots in the experimental garden of the Plant Cytogenetics Laboratory, Centre for Agrarian Sciences, Federal University of Paraíba. The samples were deposited in the Jayme Coelho

Table 1 Species of the *Zephyranthes* and *Habranthus* genus (Amaryllidaceae) and obtained data

Species	Synonym	Provenance city	Karyotypic formula	2n chromosome numbers
Z. robusta (Herb. ex Sweet) Baker	*H. robusta* Herb.	Posadas de Missiones, Argentina	4M + 2SM	12
Zephyranthes sp.		Wanderlândia,TO	4M + 1SM + 1A	12
Z. sylvatica (Mart. ex Schult. & Schult.f.) Baker	*Z. franciscana* (Baker) Baker *H. sylvatica* Ravena *H. cearensis* Herb.	Petrolina, PE	1M + 5SM; "3M + 15SM"	12, 18
Z. sylvatica Baker		Pariconhas, AL		12
Z. brachyandra (Baker) Backer	*H. brachyandra* (Baker) Sealy	Posadas de Missiones, Argentina	4M + 3SM + 5A + (1B)	24 + 1B
H. itaobinus Ravena		Campina Grande, PB	5 M, 12SM, 5A + (1B)	44 + 1B
Z. rosea Lindl.[a]	*Z. bifolia* (Aublet) Roem.	Areia, PB	4M + 7SM + 1A	24
Z. rosea Lindl.[a]		Belém, PA	4M + 7SM + 1A	24
Z. aff. rosea Lindl.[a]		Areia, PB	4M + 7SM + 1A + (1M)	25
Z. grandiflora Lindl.[a]	*Amaryllis carinata* Spring. *Z. carinata* Herb.	São Paulo, SP	2M + 5SM + 5A	24
Z. candida Herb.[a]	*Amaryllis candida* Herb.ex Lindl.	Campina Grande, PB	9M + 5SM + 5A	38

[a] Cultivated species

de Moraes Herbarium at the Center for Agrarian Sciences, Federal University of Paraíba, and also at the Agronomic Institute of Campinas, São Paulo.

For mitotic analysis, root tips were obtained and pretreated with 8-hydroxyquinoline (2 mM) overnight at 10°C, fixed in Carnoy fixative 3:1 (ethanol:acetic acid v/v) for 4 h at room temperature, and stored in a freezer at −20°C. The material was washed in distilled water and digested in an enzyme solution [2% cellulase Onozuka (Serva)-20% pectinase (Sigma) (w/v)] for 1 h at 37°C. Next, slides were prepared via crushing in a drop of 45% acetic acid, and the cover slides were removed in liquid nitrogen. The slides were air-dried and aged for 3 days at room temperature.

The Schweizer (1976) protocol was followed with minor modifications. After the plant material was aged for 3 days, it was stained with 0.5 mg/ml CMA for 1 h and 2 μg/ml DAPI for 30 min, then mounted with 10 μl of McIlvaine-glycerol (1:1, v/v). A Leica DMRB photomicroscope equipped with ultraviolet light and Leica DC 300F video camera were used to capture the images, which were processed using Image Manager 50 software.

Results

Seven *Zephyranthes* species were analyzed, four of them from a native field and three cultivated as ornamentals, together with one *Habranthus* species (*H. itaobinus*). *Z. robusta* (Fig. 1a, 2a) presented 2n = 12 and karyotype formula 4M + 2SM, and it exhibited strong interstitial DAPI+ bands in the short arms of one submetacentric pair,

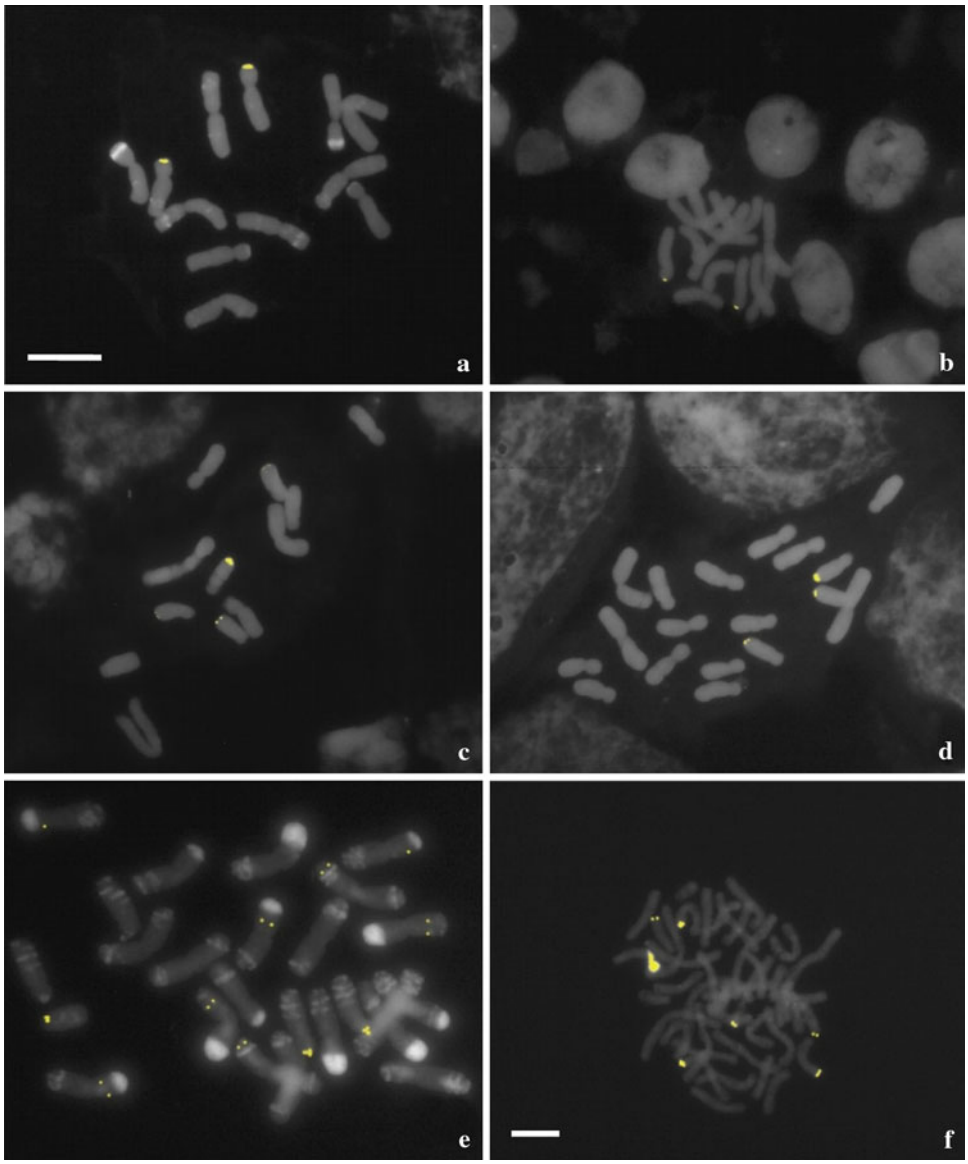

Fig. 1 Mitotic metaphases of wild species. **a** *Zephyranthes robusta*, 2n = 12, DAPI+ (*white*)/CMA−, DAPI−/CMA+ (*yellow*); **b** *Zephyranthes* sp., 2n = 12, CMA+/DAPI−; **c** *Z. sylvatica* 2n = 12, CMA+/DAPI−; **d** *Z. sylvatica* 2n = 18, CMA+/DAPI−; **e** *Z. brachyandra* 2n = 24 + 1B, DAPI+/CMA−, CMA+/DAPI−, **f** *H. itaobinus*, 2n = 44 + 1B, CMA+/DAPI−. *Bars* 10 μm

and other interstitial DAPI+ bands of varying intensity in all chromosomes except the number five chromosome pair. A strong CMA+ band was observed at the sixth chromosome pair localized in a terminal position. *Zephyranthes* sp., with 2n = 12 and karyotype formula 4M + 1SM + 1A, showed a subterminal CMA+ heterochromatin band in the short arm of acrocentric pair (Fig. 1b). *Z. sylvatica*, with 2n = 12 and karyotype formula 1M + 5SM, had a stronger heteromorphic subterminal CMA+ band in the long arm of the sixth pair, plus a small CMA+ band terminal in the long arm of pair five (Fig. 1c). This same species showed a triploid cytotype with identical karyotype though formed by cracks, with a heteromorphic CMA band in the sixth crack (Figs. 1d and 2c). *Z. brachyandra*, with 2n = 24 + 1B and formula 4M + 3SM + 5A + (1B), stood out by presenting a complex pattern of DAPI+ heterochromatin (Figs. 1e and 2d, f). Large subterminal bands were observed in the short arms of five acrocentric pairs and in the long arms of a large submetacentric pair. In addition to these bands, there were minor interstitial bands on both arms of pairs 1, 3, 9, 11 and 12 and in the long arms of other chromosomes. Strong CMA+ heterochromatin bands were observed on pair eight, apparently a satellite pair, and on the short arm of the B chromosome. Small dot bands were visualized in the interstitial region of the short arms of pairs one, four, and six and in the long arm of pair five.

H. itaobinus presented 2n = 44 + 1B and karyotype formula 5M + 12SM + 5A + (1B) and showed four pairs with subterminal CMA+ bands. In another pair a strong CMA+ band was observed in one of the homologues of the submetacentric pair, presumably a nucleolus organizer region (NOR) extended and amplified in only one of the homologous chromosomes, or heterozygous pair (Fig. 1f).

Among the cultivated species, both cytotypes were observed in *Z. rosea*, one with 2n = 24 and another with 2n = 25 and formulas 4M + 7SM + 1A and 4M + 7SM + 1A + (1 M). In both cytotypes from different populations, a CMA band was observed at the short arm terminals of pairs five and six (Fig. 3a, b). *Z. grandiflora*, with 2n = 24 and karyotype formula 2M + 5SM + 5A, showed CMA bands at the long arm terminals of pairs three and four (Fig. 3e, f). In *Z. candida* with 2n = 38 and karyotype formula 9M + 5SM + 5A, CMA+ heterochromatin blocks were observed on the long arms of five metacentric pairs and in the short arm of a submetacentric pair, in addition to a terminal band on the long arm of one of the homologues of a larger submetacentric pair (Fig. 3g, h).

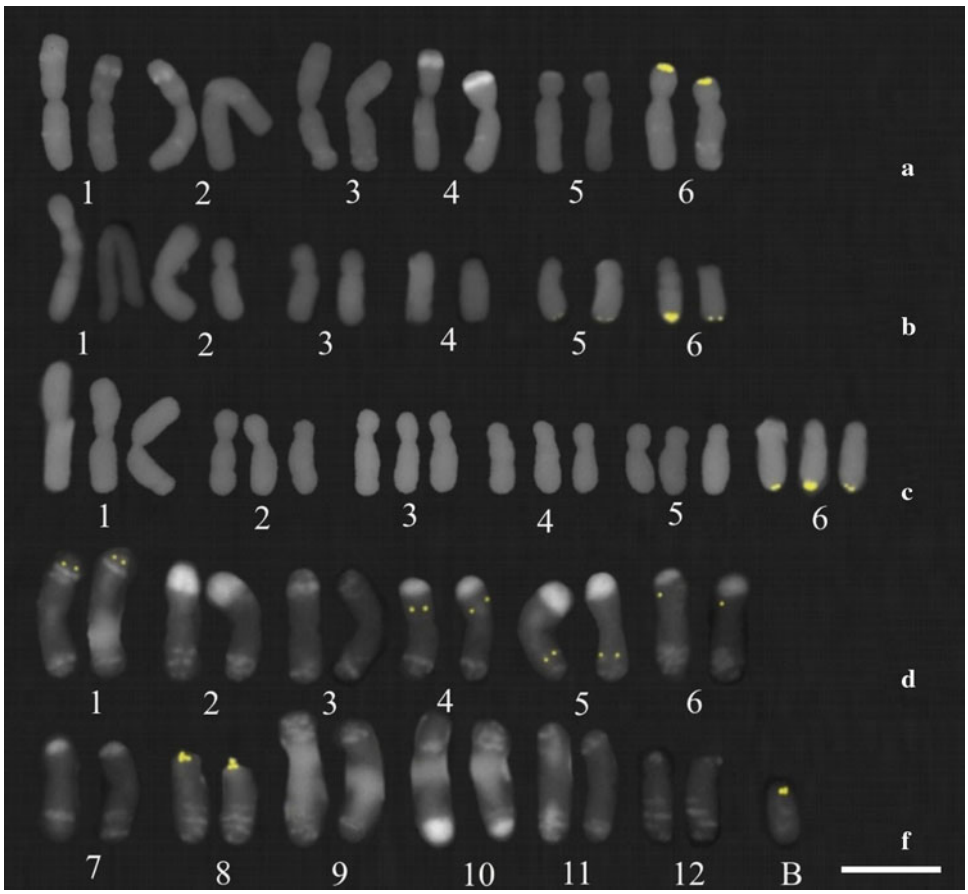

Fig. 2 Karyograms of *Zephyranthes robusta*. *a* 2n = 12, DAPI+/CMA−, DAPI−/CMA+; *Z. sylvatica,* 2n = 12 *b* CMA+/DAPI−; *Z. sylvatica,* 2n = 18 *c* CMA+/DAPI− and *Z. brachyandra* with 2n = 24 + 1B *d, f* DAPI+/CMA−, CMA+/DAPI−. *Bar* 10 µm

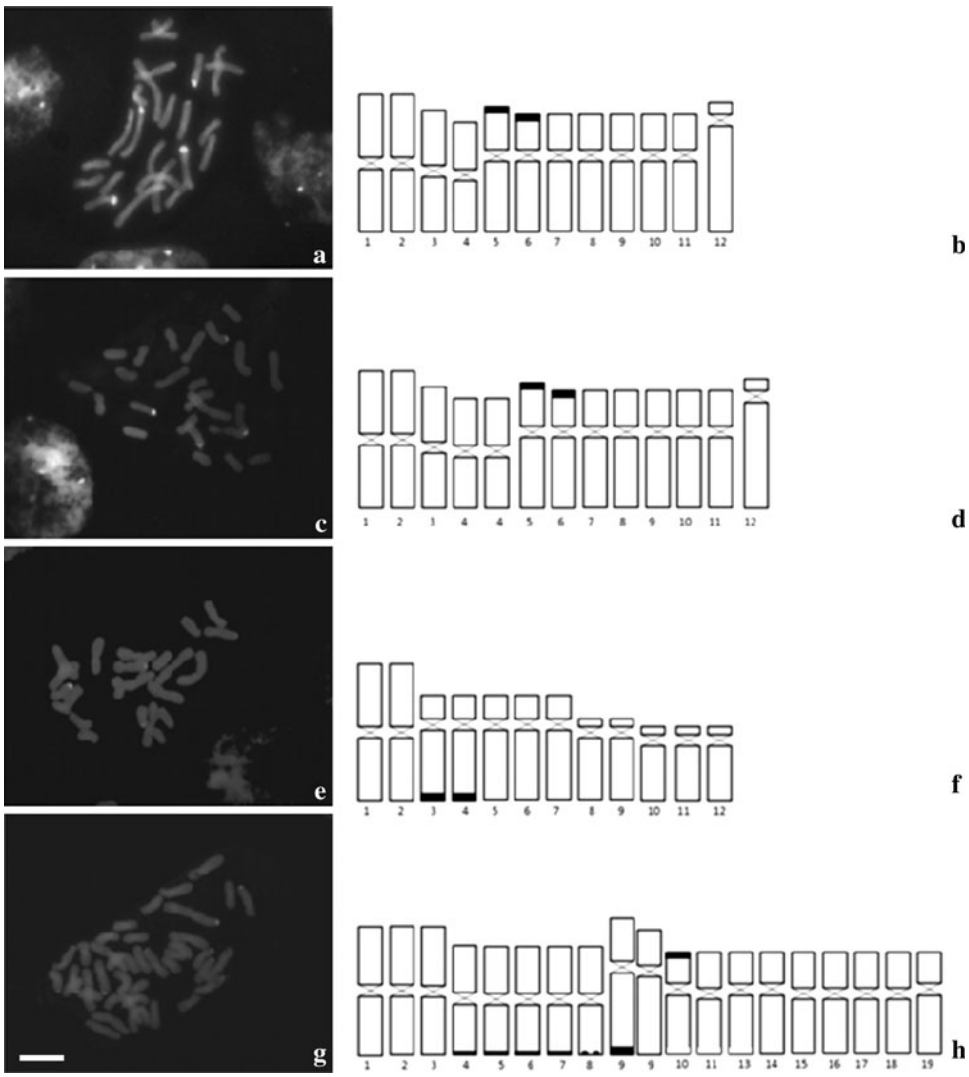

Fig. 3 Mitotic metaphases and ideograms of cultivated species. **a, b** *Zephyranthes rosea* 2n = 24, from Areia/PB population; **c, d** *Z. rosea* from Belém/PA population with 2n = 24; **e, f** *Z. grandiflora*, 2n = 24, and **g, h** *Z. candida*, 2n = 38. *Black markers* on the ideograms indicate CMA+ bands. *Bar* 10 μm

Discussion

The genus *Zephyranthes* is considered quite closely related to the genus *Habranthus*, and they have often been considered congeneric (Dutilh, personal communication). In phylogenetic analysis for the Amaryllidaceae from the Americas (Meerow et al. 2000a), it was demonstrated that *Zephyranthes* is polyphyletic and that some species from the southern United States and Mexico are sisters of *Habranthus*, reinforcing the idea of proximity of some species of both genera. In this analysis, *Z. brachyandra* and *Z. robusta*, which are sometimes included in *Habranthus*, differed by having AT-rich bands. However, *H. itaobinus* (Ravena 1999) exhibited only CMA bands similar to other species of *Zephyrathes*, suggesting that this characteristic is variable in the genus. Both genera are distinguished morphologically by presenting flowers and stamens declined (Oliveira 2006). Bentham (1883) considered all *Habranthus* species to be *Zephyranthes* synonyms, which is also reinforced based on overlapping anatomical and morphological characters (Arroyo 1981). However, *H. itaobinus* cannot be included in *Zephyranthes*, since it has not been recombined yet.

The genera *Zephyranthes* and *Habranthus* are known to have a wide variation in the number of chromosomes, often observed in a single population. Species such as *Z. grandiflora* (Greizerstein and Naranjo 1987) and *Z. sylvatica* in northeast Brazil (Felix et al. 2008) sometimes exhibit intrapopulation numerical variation. This variability also seems to occur in the chromosome structure. In *Z. sylvatica*, the only triploid individual examined showed a different CMA band pattern than that expected for a supposed self-triploid. In this case, it seems there has been loss of a CMA band in the establishment of this individual in the field. Similarly, *Z. rosea* also showed numerical variation among different populations with 2n = 24 and 2n = 25.

Among species with 2n = 12, *Z. robusta* differed from other species by presenting a set of DAPI+

heterochromatin blocks widely distributed across all chromosomes. Although it is the only species known from Argentina with 2n = 12, this pattern of heterochromatic bands clearly differed from other species from northern and northeastern Brazil that have this chromosome number, as they were devoid of AT-rich heterochromatin. Among Brazilian species with 2n = 12, *Z. sylvatica* showed a CMA band pattern distinct from *Zephyranthes* sp., species that are quite related morphologically, with two pairs with subterminal bands on the long arms in the former and only one pair on the short arm of the acrocentric pair in the latter. For the species group with 2n = 12, the CMA+ heterochromatin distribution seems to be a good indicator of the ploidy level. This is remarkable in *Zephyranthes* sp. and *Z. robusta* with only two CMA+ blocks. On the other hand, *Z. sylvatica* had four CMA blocks of which one pair was strongly heteromorphic, displaying a large block and another small one, suggesting there have been unequal translocations involving these heterochromatin regions.

The other *Zephyranthes* species can be divided into two groups based on chromosome number: one group with 2n = 24 (*Z. brachyandra*, *Z. rosea*, and *Z. grandiflora*) and another one with 2n = 38 (*Z. candida*). In the first group, *Z. brachyandra*, an Argentinean species, differed from other species by having DAPI+ heterochromatin spread among all chromosomes of the complement forming subterminal and interstitial bands. Among the species examined in this sample, only *Z. robusta* with 2n = 12 showed DAPI bands in the interstitial regions in most chromosomes of the complement. Both species occur in Argentina and the existence of DAPI bands in both suggests that they may be phylogenetically related. In this case, *Z. brachyandra* would be a tetraploid that would have extended additional DAPI+ blocks through structural changes. However, *Z. brachyandra* was distinguished from all species analyzed by presenting small punctate interstitial CMA blocks in several chromosomes of the complement, in addition to terminal blocks of the heterochromatic NOR, which was also observed in the supernumerary chromosome. The other two species with 2n = 24 showed no DAPI bands and both had only two pairs with CMA bands, which were probably retained from a diploid ancestral stock with $x = 6$. These blocks were located on the short arm of *Z. rosea* and in the long arm of two submetacentric pairs of *Z. grandiflora*. On the other hand, *Z. candida*, which probably constitutes a hexaploid based on $x_1 = 6$, presented CMA blocks that were translocated to other chromosomes, but preferentially retained the location on the chromosome long arm in at least five metacentric and one submetacentric pair. Blocks observed in the short arms of a metacentric pair and in a single chromosome of the larger heteromorphic submetacentric pair may be the result of translocations and inversions that occurred during this species' evolution. *Z. candida* is a remarkable species in terms of its variation in chromosome numbers (Daviña 2001), and the heteromorphism of heterochromatic blocks and chromosomal size is probably related to rearrangements that can also result in numerical changes.

H. itaobinus, with 2n = 44 + 1B, had the largest number of chromosomes in this sample and probably represents a hexaploid based on $x_1 = 7$ with diploid gain of one chromosome pair and a small metacentric B. The species had seven CMA+ blocks that were heteromorphic for an extensive expansion of the NOR (CMA+) on one of the homologous chromosomes of the satellite pair and also the loss of a CMA block in a homologue of another chromosome pair. This pattern of CMA bands did not correspond to the number of bands expected for a hexaploid species, indicating that it is going through a diploidization process. In this process a reduction in chromosome number can occur, especially in species currently diploided that had a large genomic size reduction, as demonstrated in *Arabidopsis thaliana* and other Brassicaceae (Lysaker et al. 2006). Other species have undergone a massive silencing and loss of redundant gene loci but suffered no significant reduction in genome size and chromosome number (Adams and Wendel 2005). Possibly, a similar process may be underway in *H. itaobinus*, where the loss of several duplicated CMA+ heterochromatin loci can clearly be observed. These blocks often correspond to 45S rDNA loci and are located in the terminals of the short chromosome arms (Guerra 2000), as observed in the genus *Manihot* (Carvalho and Guerra 2002).

The species studied in this work presented some trends in heterochromatin evolution, more easily observable in the species with 2n = 24, where CMA blocks always corresponded to the tetraploid level. A similar trend was observed in diploid species with 2n = 12, which always presented with a CMA block in a chromosome pair, except *Z. sylvatica* where there was a possible translocation of a small CMA block to another chromosome pair that was not observed in the triploid cytotype. This pattern was useful in separating *Z. sylvatica* from the related *Zephyranthes* sp., which showed a different number and distribution of CMA+ heterochromatin blocks. Two other species (*Z. robusta* and *Z. brachyandra*) stood out in terms of the occurrence of subterminal and interstitial DAPI bands, suggesting an ancestor with x = 12, similar to *Z. robusta*. *H. itaobinus* and *Z. candida* were distinguished by having marked heteromorphism in their CMA+ heterochromatin distribution.

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