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Research Article

# Heterochromatin diversity and its co-localization with 5S and 45S rDNA sites in chromosomes of four *Maxillaria* species (Orchidaceae)

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## Abstract

We investigated four orchids of the genus *Maxillaria* (*M. discolor*, *M. acicularis*, *M. notylioglossa* and *M. desvauxiana*) in regard to the position of heterochromatin blocks as revealed using chromomycin $A_3$ (CMA) and 4'-6-diamidino-2-phenylindole (DAPI) fluorochrome staining and 5S and 45S rDNA sites using fluorescence *in situ* hybridization (FISH). The species showed differences in chromosome number and a diversified pattern of $CMA^+$ and $DAPI^+$ bands, including heteromorphism for $CMA^+$ bands. The 5S and 45S rDNA sites also varied in number and most of them were co-localized with $CMA^+$ bands. The relationship between 5S rDNA sites and $CMA^+$ bands was more evident in *M. notylioglossa*, in which the brighter $CMA^+$ bands were associated with large 5S rDNA sites. However, not all 5S and 45S rDNA sites were co-localized with $CMA^+$ bands, probably due to technical constraints. We compare these results to banding data from other species and suggest that not all blocks of tandemly repetitive sequences, such as 5S rDNA sites, can be observed as heterochromatin blocks.

*Key words:* heterochromatin, CMA and DAPI, 45S and 5S rDNA, *Maxillaria*.

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## Introduction

Secondary constrictions and heterochromatic regions are the most intensively studied and best known chromosome markers in both plants and animals. Secondary constrictions can be observed by conventional chromosome staining techniques, but are more easily identified by silver nitrate (Ag) staining or *in situ* hybridization with 45S rDNA probes (see *e.g.* Brasileiro-Vidal *et al.*, 2003). These constrictions are largely known as 45S rDNA location sites, although smaller or less active 45S rDNA sites may exist in chromosomes which apparently do not form secondary constrictions (Guerra *et al.*, 1996). On the other hand, heterochromatic regions are generally identified by either C-banding techniques or direct staining with base-specific fluorochromes, such as 4-6-diamidino-2-phenylindole (DAPI), Hoechst 33258, chromomycin A3 (CMA), mythramycin, quinacrin, and others (Schmid and Guttenbach, 1988; Sumner, 1990; Guerra, 2000). The reaction of these fluorochromes with the chromosomes depends mainly on the nitrogen base composition of the DNA molecule, in such a way that each region of the chromosome may show a positive (+), negative (-) or neutral (0) reaction with a given fluorochrome (Schweizer, 1981). In plants, the most used fluorochromes are CMA, which preferentially stains GC-rich DNA, and DAPI, which preferentially stains AT-rich regions. Using these two fluorochromes, heterochromatin blocks can be characterized as $CMA^+/DAPI^-$ (GC-rich), $CMA^-/DAPI^+$ (AT-rich) or neutral ($CMA^0$, $DAPI^0$) for either one or both fluorochromes (Guerra, 2000).

The origin of the differential staining of heterochromatin after C-banding is still unclear, although it is certainly related to one of the most universal characteristics of heterochromatin, *i.e.* the presence of one or more families of tandemly repetitive DNA sequences. Such repeat families can be recognized by different fluorochromes or by fluorescent *in situ* hybridization (FISH) (Cuadrado and Schwarzacher, 1998; Shibata *et al.*, 2005).

As the tandemly repetitive sequences are generally short and non-coding, heterochromatin is predominantly empty of genes. However, the heterochromatin adjacent to the secondary constrictions is an exception, being constituted of tandemly repetitive 45S rRNA genes, which forms large rDNA blocks identified as positive regions for C-banding and CMA staining (Fuchs *et al.*, 1998; Guerra and Felix, 2000). In a different manner, 5S rRNA genes are also tandemly organized, but they do not form constrictions in

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prophase or metaphase chromosomes and rarely are visible as heterochromatin blocks (Fuchs *et al*., 1998; Martins and Wasko, 2004).

During a cytotaxonomic analysis of about 30 *Maxillaria* Ruiz & Pavón species (unpublished data), four species belonging to different sections of the genus were selected to investigate the diversity and distribution of CMA or DAPI heterochromatin blocks and the position of 5S and 45S rDNA sites. *Maxillaria* is the fifth largest American orchid genus, harboring about 420 tropical species (Pabst and Dungs, 1975). Despite such high diversity in the neotropical flora, the cytology of the genus is almost unknown in that chromosome number data is available for only six species (Felix and Guerra, 2000). The four species analyzed in our study showed differences in chromosome number and revealed a diversified pattern of $CMA^+$ and $DAPI^+$ bands, having 5S and 45S rDNA sites co-localized with $CMA^+$ bands. The meaning of these results is discussed regarding the cytological characterization of heterochromatin.

## Materials and Methods

We collected four *Maxillaria* species in field excursions in different Brazilian states, the species analyzed being: *M. acicularis* Herb. (voucher 10830 EAN), collected in Ouro Preto, Minas Gerais state); *M. notylioglossa* Rchb. f. (voucher 10824 EAN), collected in Domingos Martins, Espírito Santo state; *M. desvauxiana* Rchb.f. (voucher 10914 EAN) and *M. discolor* (Lodd. ex Lindl.) Rchb. f. (voucher 10913 EAN), both collected in Jaqueira, Pernambuco state. The species were maintained in cultivation at the Experimental Garden of the Department of Botany of the Federal University of Pernambuco. Vouchers of all species are deposited in the EAN herbarium, Federal University of Paraíba.

For cytological analyses, root tips were pre-treated with 0.002 M 8-hydroxyquinoline at 8 °C for 20 h, fixed in ethanol/glacial acetic acid 3:1 (v/v) for two hours at room temperature and stored at -20 °C. Root tips were digested using a solution containing 2% cellulase and 20% pectinase (both w/v) for 90 min at 37 °C and the meristem dissected in 45% (v/v) aqueous acetic acid, squashed under a coverslip (subsequently removed by freezing in liquid nitrogen), air-dried and then aged for three days.

The aged preparations were stained at room temperature with 0.5 CMA mg $mL^{-1}$ for 60 min and then 2 μg $mL^{-1}$ DAPI for 30 min before being mounted in 1:1 (v/v) McIlvaine's pH 7.0 buffer/glycerol containing 2.5 mM $MgCl_2$ and aged for at least three days for fluorochrome stabilization. The best slides were analyzed using a Leica DMLB microscope and cell images captured with a Cohu digital camera using the QFISH software (Leica). Afterwards, the slides were de-stained at room temperature in 3:1 (v/v) ethanol:acetic acid for 30 min, dehydrated in absolute ethanol for two hours at room temperature, air-dried and stored at -20 °C until being subjected to fluorescence *in situ* hybridization (FISH) experiments.

For 45S rDNA FISH analyses we used the *Arabidopsis thaliana* R2 probe (Wanzenböck *et al.,* 1997) which was labeled by nick translation with digoxigenin, while for locating 5S rDNA we used the *Lotus japonicus* D2 probe (Pedrosa *et al.*, 2002) which was labeled by nick translation with biotin. The hybridization mixture contained 50% (v/v) formamide, 10 % (v/v) dextran sulfate, 2x SSC and 2 to 5 ng $\mu L^{-1}$ of each probe. The preparations were hybridized *in situ* overnight and the stringent wash was performed in 0.1x SSC at 42 °C. The final stringency was 76%. The 45S rDNA sites were detected with sheep anti-digoxigenin-FITC (avidin-fluorescein isothiocyanate) (Roche 1207741) plus anti-sheep-FITC (Dako F0135). The 5S rDNA sites were detected with mouse anti-biotin (Dako M0743) in combination with anti-mouse-TRITC (tetramethyl rhodamine isothiocyanate) (Dako R0270). The chromosomes were counterstained with 2 μg $mL^{-1}$ DAPI, mounted in Vectashield (Vector) and analyzed using the Leica microscope as described above.

## Results

All the species investigated exhibited stable chromosome numbers and had similar-sized chromosomes, about 4.5 μm long. After CMA/DAPI double-staining, some chromosome regions were $CMA^+/DAPI^-$ (*i.e.* brighter with CMA and duller with DAPI), whereas others were $CMA^-/DAPI^+$ or neutral with one of the fluorochromes ($CMA^0$ or $DAPI^0$). We observed chromosome pairs with both $CMA^+$ and $DAPI^+$ bands only in *M. acicularis* and *M. notylioglossa*. Heteromorphism for $CMA^+$ bands was detected in all species except in *M. discolor*. The largest blocks of 5S rDNA always coincided with $CMA^+/DAPI^-$ bands, while the sites of 45S rDNA always co-localized with $CMA^+$ blocks of different fluorescence intensity (Figure 1). On the other hand, $DAPI^+$ bands and rDNA sites were never found closely associated. The position of the fluorescent bands and rDNA sites and the number of chromosomes without band differentiation are shown in Figure 2, although chromosome size and centromere position are not indicated because they could not be consistently identified. A brief karyotype description of each species is presented below.

*Maxillaria discolor* (2n = 42) exhibited four terminal $CMA^+/DAPI^-$ bands (arrows in Figure 1a-c), two proximal $CMA^+/DAPI^0$ bands (arrowheads in Figure 1a-c) and at least 16 proximal $CMA^-/DAPI^+$ bands (half of them in metacentric chromosomes and half in submetacentric to acrocentric chromosomes - see Figure 2). Four 45S rDNA sites and two 5S rDNA sites were also observed (Figure 1d). After sequential staining the 45S site co-localized with

the terminal $CMA^+/DAPI^-$ bands and the 5S rDNA sites with the proximal $CMA^+/DAPI^0$ bands.

*Maxillaria acicularis* (2n = 38) exhibited 18-20 proximal $CMA^-/DAPI^+$ bands, all localized in submetacentric to acrocentric chromosomes, and at least four $CMA^+/DAPI^-$ bands (Figure 1e). In some cells up to three additional $CMA^+/DAPI^-$ bands were also observed (Figure 1h). After FISH, four 45S rDNA sites and four 5S rDNA sites were visible (Figure 1f, g), among these one locus of each different rDNA family was heteromorphic in size. The strongest signals were produced by three 45S rDNA sites and one 5S rDNA site which clearly coincided with the four

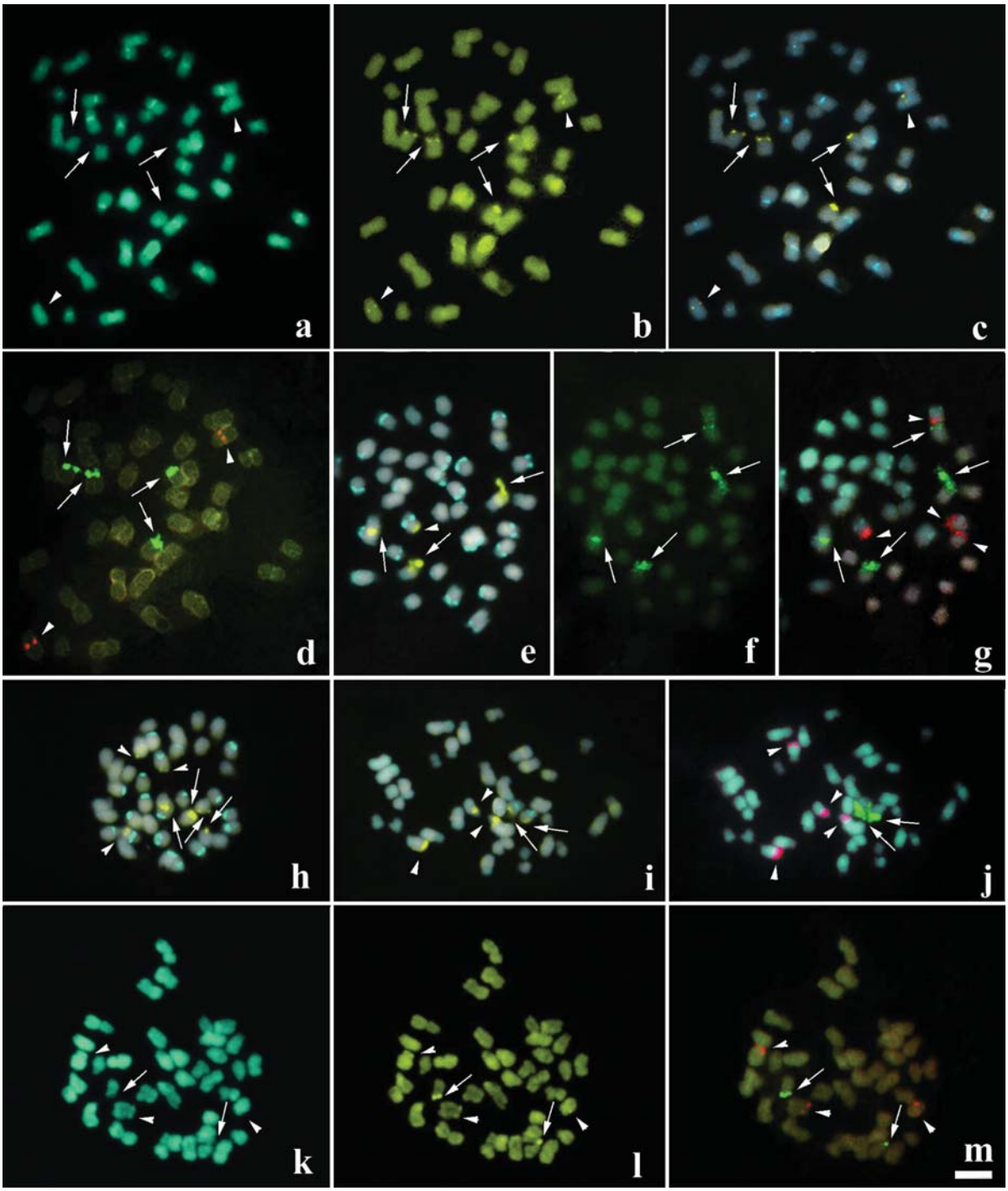


**Figure 1** - Metaphase cells of *Maxillaria discolor* (a-d), *M. acicularis* (e-h), *M. notylioglossa* (i, j), and *M. desvauxiana* (k-m) showing $CMA^+$ bands (yellow), $DAPI^+$ bands (blue), 5S and 45S rDNA sites (detected in red with TRITC, and in green with FITC, respectively). a, k, DAPI; b, l, CMA; c, h, i, CMA/DAPI overlay; f, FITC; d, m, DAPI/FITC/TRITC overlay. Metaphase in h displays three very small $CMA^+/DAPI$ bands (arrowheads). The gray color in some photographs (c, e, g-j) is due to the overlay of different colors. Arrowheads and arrows indicate the sites of 5S and 45S rDNA sites, respectively. Bar in m corresponds to 5 μm.

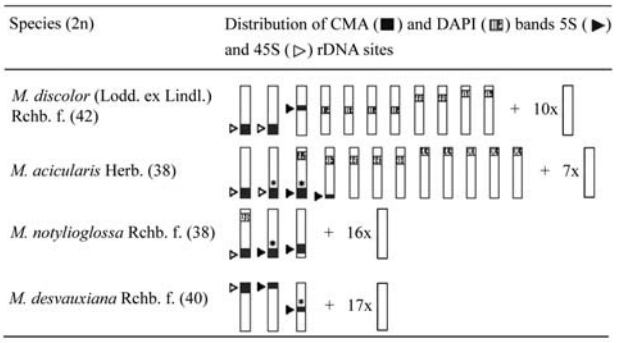


**Figure 2** - Chromosomal distribution of heterochromatic bands and 5S and 45S rDNA sites in the haploid chromosome complement of four *Maxillaria* species. Centromere position and chromosome size are not indicated, but the chromosomes are represented with the long arms at the bottom. Therefore, all chromosome bands in or close to the top are proximal bands of acrocentric chromosomes. Wherever band-size heteromorphism was observed (indicated by an asterisk) only the largest band variant was represented.

largest $CMA^+/DAPI^-$ bands (arrows and arrowhead in Figure 1e). Therefore, these four $CMA^+$ bands are distributed in three different chromosome pairs: one pair homozygous for a large 45S rDNA site, one pair heterozygous for a large 45S rDNA and a third pair heterozygous for a large 5S rDNA site. The three smallest 5S rDNA sites also seemed to co-localize with the smallest $CMA^+/DAPI^-$ bands, since both marks were located in a similar position on chromosomes with proximal DAPI bands. The smallest 45S rDNA site was probably too small to generate a $CMA^+$ signal.

*Maxillaria notylioglossa* (2n = 38) displayed three terminal and two subterminal $CMA^+/DAPI^-$ bands and two proximal $CMA^-/DAPI^+$ bands (Figure 1i and Figure 2), with up to 12 weaker $CMA^0/DAPI^-$ and $CMA^+/DAPI^0$ bands sometimes being visible (not shown). Three of the four 5S rDNA sites observed co-localized with the most evident $CMA^+/DAPI^-$ bands (arrowheads in Figure 1i, j), showing that they are localized on two different chromosome pairs (Figure 2). Only a single pair of 45S rDNA sites (Figure 1j) was observed, terminally located and coincided with a weak $CMA^+$ band (arrows in Figure 1i).

*Maxillaria desvauxiana* (2n = 40) had two terminal $CMA^+/DAPI^-$ bands (arrows in Figure 1k-l) and two terminal and one interstitial $CMA^+/DAPI^0$ bands (arrowheads in Figure 1k-l). Two terminal 45S rDNA sites and two terminal and one interstitial 5S rDNA sites were revealed by FISH (Figure 1m), all coinciding with $CMA^+$ bands.

## Discussion

Only *M. discolor* had been previously investigated regarding chromosome number (Felix and Guerra, 2000), our results confirm this to be 2n = 42. The chromosome numbers reported here for the other three species (2n = 38, 40) were similar to the few other counts for the genus, *i.e.* three species with 2n = 40 and three with 2n = 42 (Felix and Guerra, 2000). Similarly, most better-studied orchid genera, such as the horticulturally important genera *Laelia* and *Cattleya*, show little variation in their chromosome numbers (see Felix and Guerra, 2000; 2005). However, our analysis with base-specific fluorochromes showed more pronounced karyological differentiation among the four *Maxillaria* species, including variation in the number and location of heterochromatic bands with both CMA and DAPI staining. Kao *et al.* (2001) observed that in *Phalaenopsis* species heterochromatin is a major source of variation in DNA content, while Kondo *et al.* (1994) and Hoshi *et al.* (1995) have shown that in the orchid genus *Cypripedium* (which has large chromosomes) the amount and variability of heterochromatin was exceptionally high.

The bright $DAPI^+$ or $CMA^+$ bands observed in many plant genera (Guerra, 2000) reveal the presence of AT- or GC-rich repetitive DNA sequences, respectively (Schweizer, 1981). The unusual feature of the *Maxillaria* $CMA^+$ bands was that all of them coincided with 5S or 45S rDNA. It is known that 45S rDNA sites commonly stain positively after Giemsa C-banding or after direct staining with CMA, while 5S rDNA sites are usually visualized only by *in situ* hybridization (Guerra and Felix, 2000; Vanzela *et al.*, 2002; Carvalho and Guerra, 2002; Carvalho *et al.*, 2005). This relationship between 5S rDNA sites and $CMA^+$ bands was most evident in *M. notylioglossa*, in which the largest and brightest $CMA^+$ bands co-localized with 5S rDNA sites instead of 45S rDNA sites.

However, not all 5S and 45S rDNA sites in *Maxillaria* and in other genera generate $CMA^+$ bands, probably because the sites were too small to be detected with CMA (Zoldos *et al.*, 1999; Marcon *et al.*, 2003; 2005). Schmid and Guttenbach (1988) obtained positive mithramycin A staining of vertebrate nucleolus organizer region (NOR) in various species except humans, the authors attributing this exception to the low repeat number (40-46 repeats per locus) of human NORs. In *Maxillaria*, the rDNA site heteromorphism observed in three of the four species investigated may also be caused by differences in the number of rDNA repeats in some loci, as observed in *Arabidopsis* (Murata *et al.*, 1997) and *Trifolium* (Ansari *et al.*, 1999).

The co-localization of 5S rDNA sites with heterochromatic blocks has been rarely reported in plants, perhaps because only a few groups of plants have been simultaneously investigated by banding techniques and *in situ* hybridization with a 5S rDNA probe. In *Hypochaeris* (Cerbah *et al.*, 1998; Ruas *et al.*, 2005), *Quercus* (Zoldos *et al.*, 1999) and *Lilium* species (Siljak-Yakovlev *et al.*, 2003) both 5S and 45S rDNA sites were differentially stained by either CMA or C-banding followed by CMA/DAPI double-staining. In *Clivia*, 5S and 45S rDNA sites clearly coincided with Giemsa C-bands (Ran *et al.*, 2001).

Loci of 5S rDNA are constituted of repeats containing a transcribed region of about 120 bp and a non-transcribed spacer (NTS) of a very variable size, normally several hun-

dred base pairs (Gottlob-McHugh *et al.*, 1990). As the transcribed sequence is highly conserved, the difference in staining among 5S rDNA sites in different genera might be due to NTS sequence divergence. It is interesting to note that in all plant species in which 5S rDNA blocks have been observed the blocks within a chromosome complement seem to present the same reaction to the fluorochromes. This apparent intraspecific homogeneity may be due to the occurrence of interloci concerted evolution, as observed in cotton by Cronn (1996). However, differential intralocus homogenization has been demonstrated at the molecular level for several plant species, such as *Arabidopsis* (Cloix *et al.*, 2000) and *Nicotiana* (Matyásek *et al.*, 2002). Interestingly, the opposite situation is found in some vertebrates, in which different 5S rDNA loci of a single karyotype may show very distinct families of NTS repeats and may be differentially stained by FISH using NTS specific probes (Martins and Galetti, 2001; Martins and Wasko, 2004).

The correlation between sites of repetitive DNA sequences and heterochromatic bands may also depend on factors other than DNA sequence. For example, in the fish species *Astyanax scabripinnis* terminal C-bands that hybridized with an AT-rich satellite DNA sequence stained positively with mithramycin A in one population and negatively in another population (Mantovani *et al.*, 2004) and it has also been reported that in several *Citrus* species some 45S rDNA sites are $CMA^{+}$ while others are $CMA^{0}$ (Carvalho *et al.*, 2005). Furthermore, the rDNA may apparently exist in distinct chromatin conformations determined by specific epigenetic codes, such as cytosine methylation and post-translational changes in histones (Neves *et al.*, 2005), that could affect its reactivity with fluorochromes. These data suggest that, as with 5S rDNA sites, other tandemly repetitive sequences may exist in chromosomes without forming heterochromatic blocks.

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