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Botanical aspects of *Heteropterys umbellata* (Malpighiaceae): a cytological and palynological approach

SAULO M. SOUSA[1], ARYANE C. REIS[1], SHAIANY S.L. GOMES[1], KARINA B. BERNARDO[2], FÁTIMA R.G. SALIMENA[3] and LYDERSON F. VICCINI[1]

[1]Instituto de Ciências Biológicas, Departamento de Biologia, Laboratório de Genética
Universidade Federal de Juiz de Fora (UFJF), Campus Universitário, Bairro Martelos
36036-900 Juiz de Fora, MG, Brasil
[2]Empresa Brasileira de Pesquisa Agropecuária, Centro Nacional de Pesquisa de Gado de Leite
Rua Eugênio do Nascimento, 610, Dom Bosco, 36038-330 Juiz de Fora, MG, Brasil
[3]Instituto de Ciências Biológicas, Departamento de Botânica, Universidade Federal de Juiz de Fora (UFJF)
Campus Universitário, Bairro Martelos, 36036-900 Juiz de Fora, MG, Brasil

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ABSTRACT

The genus *Heteropterys* is one of the major genera in Malpighiaceae. However, few cytological and palynological studies were reported. The present work described for the first time the chromosome number, heterochromatin pattern, meiotic behavior, pollen viability and palynological aspects of *Heteropterys umbellata*, a very spread species. One large Brazilian population was evaluated using conventional techniques for meiotic studies and acetolyse to access the pollen morphology. The species showed 2n = 20 chromosomes, normal meiotic development and viable pollens. Great blocks of heterochromatin were observed around the centromers. DAPI staining was positive for centromeric heterochromatin, while CMA_3 mark was observed just at terminal regions of one pair of homologues chromosomes. This result and the presence of one chromosome pair attached to the nucleoli during the pachytene and diakinesis suggested the presence of only one pair of NORs. Palynological analysis revealed that pollen grains are apolar, 6 porate and with colpoids associated to all pores. The pollen content was positive for the starch test, and the exine was rugulate with little psilate regions.

Key words: chromosome number, heterochromatin pattern, *Heteropterys umbellata*, Malpighiaceae, meiosis, pollen viability.

INTRODUCTION

The genus *Heteropterys* Kunth is one of the major genera in the Malpighiaceae family, followed in size *Byrsonima*, which is slightly smaller. With nearly 130 species found in different habitats, the species are distributed from the savannas and dry woodlands to rain forests in the New World tropics and subtropics, from northern Mexico and the West Indies to northern Argentina and southeastern Brazil, and can be also found at low wet places along the coast of West Africa from Senegal to Angola (Anderson 1993, Amorim 2002).

As well as other genera in Malpighiaceae, *Heteropterys* has each cincinnus reduced to one flower and dry schizocarpic fruits. The genus is defined by the presence of samaras, which are unique in the family. While in most Malpighiaceae genera the samaras have a dominant dorsal wing, thickened along the upper (adaxial) margin, and the parallel veins bend downward ending in the thinner abaxial margin, in *Heteropterys* the situation is reversed, with their adaxial margin being thinner than the abaxial one, and the wing bends more or less

Correspondence to: Saulo Marçal de Sousa
E-mail: saulo_marcal@yahoo.com.br

upward, with the veins following this curvature (Amorim 2002).

The taxonomy of *Heteropterys* is very complex, and innumerous approaches have been used to support inclusions in the genus and also in the Malpighiaceae family. In spite of the dorsal-winged samara, the overall morphology suggests that this genus is actually more closely related to genera with lateral wings, such as *Tetrapterys*, than to the large group with the usual type of dorsal-winged samara as *Stigmaphyllon* and *Banisteriopsis*, for example (Anderson and Davis 2007). Studies regarding chromosome number and karyotype have also helped the characterization of some clades in Malpighiaceae; nevertheless, few data about cytology of *Heteropterys* were observed. Only nine species have the chromosome number reported, and n = 10 was observed for most of them. Other haploid numbers were observed: n = 17 for *H. angustifolia* and n = 21, 28 and 29 for *H. leona* (Pal 1964, Semple 1970, Ormond et al. 1981, Anderson 1990, Lombello and Forni 2001).

The determination of chromosome number in plants is a critical step to detect processes that make feasible abrupt speciation such as polyploidy, aneuploidy and dysploidy (Briggs and Walters 1997, Guerra 2008). Moreover, the knowledge of chromosome number and morphology, plus the meiotic behavior, may help to differentiate between allopolyploidy or autopolyploidy considering the differences or similarities among the involved genomes (Stebbins 1971, Guerra 2008). Complementarily, the behavior of the chromosomes at meiosis can affect pollen viability. If meiosis is irregular, with the chromosomes pairs segregating abnormally, the sterility of the pollen grain can be expected to occur due to cytological reasons. Nevertheless, if meiosis is normal, other mechanisms as inbreeding depression, pollen age and its exposure to environmental stress such as temperature and humidity, can be the causes of pollen sterility in one species (Krebs and Hancock 1990, Willis 1993, Husband and Schemske 1996, Goodwillie 2000, Boff and Schifino-Wittmann 2002).

Besides the chromosome counts, other chromosome markers such as C-banding, Ag-NOR and fluorescent staining methods performed with counter-staining reagents are useful to obtain species-distinctive regions of chromosomes. They can also be applied to cytotaxonomy and chromosome evolution, including the comparison among individuals of the same species distributed into and among different populations (Kokubugata and Katsuhiko 1996, Sousa et al. 2009).

Among the *Heteropterys* species, *Heteropterys umbellata* Adr. Juss. is a widespread species that can be found at Brazilian savannas and can be also confused with *H. glabra* Hook & Arn., another similar but different species used in Brazilian folk medicine as a sedative and anxiolytic agent (Galietta et al. 2005). Many authors classified both species as synonyms due to their morphological similarity and probably also due to the lack of reports about other botanical basic aspects. So, additional data are necessary for an accurate conclusion about the correct status of this species and the genus.

The present work reported the chromosome number, the meiotic behavior and the heterochromatin patterns in *H. umbellata*. The palynomorphology and pollen viability were also showed.

MATERIALS AND METHODS

Biological Material

Immature inflorescences of 30 individuals were collected from one population of *H. umbellata* at Diamantina, Minas Gerais State, Brazil. Inflorescences were fixed in fresh cold methanol: acetic acid solution 3:1 for at least 24h. Vouchers were housed at the Herbarium CESJ of Universidade Federal de Juiz de Fora, Juiz de Fora, Minas Gerais, Brazil.

Pre-selection of Inflorescences

In order to obtain suspensions with meiotic stage cells appropriate to chromosome counts and analyses of meiotic behavior, the immature inflorescences were separated into arbitrary sizes, and the cells of each one were observed. Cell suspensions were made based on the previously defined size that showed meiotic cells in different stages of development.

Cell Suspensions and Slides Preparation

The cell suspensions were prepared according to Viccini et al. (2005) with some modifications. Nearly 40 anthers were excised from 12 flower buds and placed in a special microtube (0.5 ml) with a nylon screen attached (60μm).

The material was washed in distilled water to remove fix solution. The tube containing the anthers was immersed in enzymatic solution (Pectinex Novozymes, Bagsvaerd, Denmark®) and incubated at 34°C for 20 min. After enzymatic maceration, the anthers were washed in distilled water and mechanically fragmented to remove the pollen mother cells (PMCs). The obtained cellular suspension was centrifuged at 2000rpm for 12 min. For slides preparation, drops of the suspension were added to clean slides. Following this step the slides were air-dried and stained with Giemsa solution for three min. Cell images were analyzed using Image Pro Plus software (Media Cybernetics™, Silver Spring. MD, USA). At about 20 suspensions containing a mix of buds from all individuals were obtained. Around 12 slides were prepared from each suspension.

Chromosome Banding

For heterochromatin studies, the C-banding procedure was performed according to Schwarzacher et al. (1980) with minor modifications. Aged slides (3 days) were hydrolyzed in acetic acid 45% (60°C) for 12 min and, then, immersed in 5% Ba $(OH)_2$ at 28°C for 10 min. After that, the slides were incubated in 2× SSC at 60°C for 80 min, and stained with a 10% Giemsa solution for 30 min. The fluorochrome staining was performed according to Schweizer (1976). Aged slides (3 days) were doubled-stained with 0.5 mg/mL of chromomycin A3 (CMA) (90 min) and 2 μg/mL of diamidino-2-phenylindole (DAPI) (30 min), and immediately mounted in 1:1 (v/v) Mcllvaine's pH 7.0 bufer-glycerol. Slides were aged for 3 days before analysis, and the chromosomes were observed using an epifluorescence microscope (Olympus BX 51) with appropriate filter set.

Pollen Viability

Mature flower buds were collected and used to prepare the slides by squash technique. The viability was estimated according to differential staining (Alexander 1980). Digital images were taken with the BX 51 microscope and analyzed using Image Pro Plus software (Media Cybernetics™, Silver Spring. MD, USA). At about 20,000 grains were analyzed from 10 slides.

Palynology

Mature anthers were placed in glacial acetic acid for at least 24h and then acetolysed according to Erdtman (1960). The slides were mounted in glycerin jelly and examined using a BX 51 Olympus microscopy. The images were analyzed using Image Pro Plus software (Media Cybernetics™, Silver Spring. MD, USA). The material and the slides were stored at the Biology Department of Federal University of Juiz de Fora, Juiz de Fora, Minas Gerais State, Brazil. At least 20 slides were prepared, and measures of pollen diameters were obtained from at least 20 grains per slide, while for other characters about 10 grains were used per slide. The pollen classifications were made according to Punt et al. (2007).

Pollen Hystochemistry

Pollen grains were stained with Lugol (starch indicator) and with Sudan (lipids indicator) according to Baker and Baker (1979). Around 10 slides were prepared: one half was stained with Lugol and the other with Sudan. The slides stained with Lugol and Sudan were compared with slides not stained using a BX 51 Olympus microscopy. The results were registered and analyzed using Image Pro Plus software (Media Cybernetics™, Silver Spring. MD, USA).

RESULTS

Chromosome Number and Meiotic Behavior

The analysis of meiotic behavior of *H. umbellata* showed regular chromosome pairing and normal stage cells: interphase, zygotene, pachytene, initial diplotene, diplotene, diakineses, metaphase I, anaphase I, telophase I, metaphase II, anaphase II and telophase II (Fig. 1). Ten bivalents were observed during pachytene, diplotene and diakinesis in all the studied individuals, which indicates that the chromosome number of the species is 2n = 20.

Although we have been observed a normal chromosomal behavior during meiosis, little meiotic alterations as translocations, precocious migration, laggard chromosomes and chromosome bridges were also observed (Fig. 2 A-D). Considering meiosis I, laggard chromosomes were found in 11.20% of the cells, chromosome bridges in 13.21%, translocations in 7.36% and only

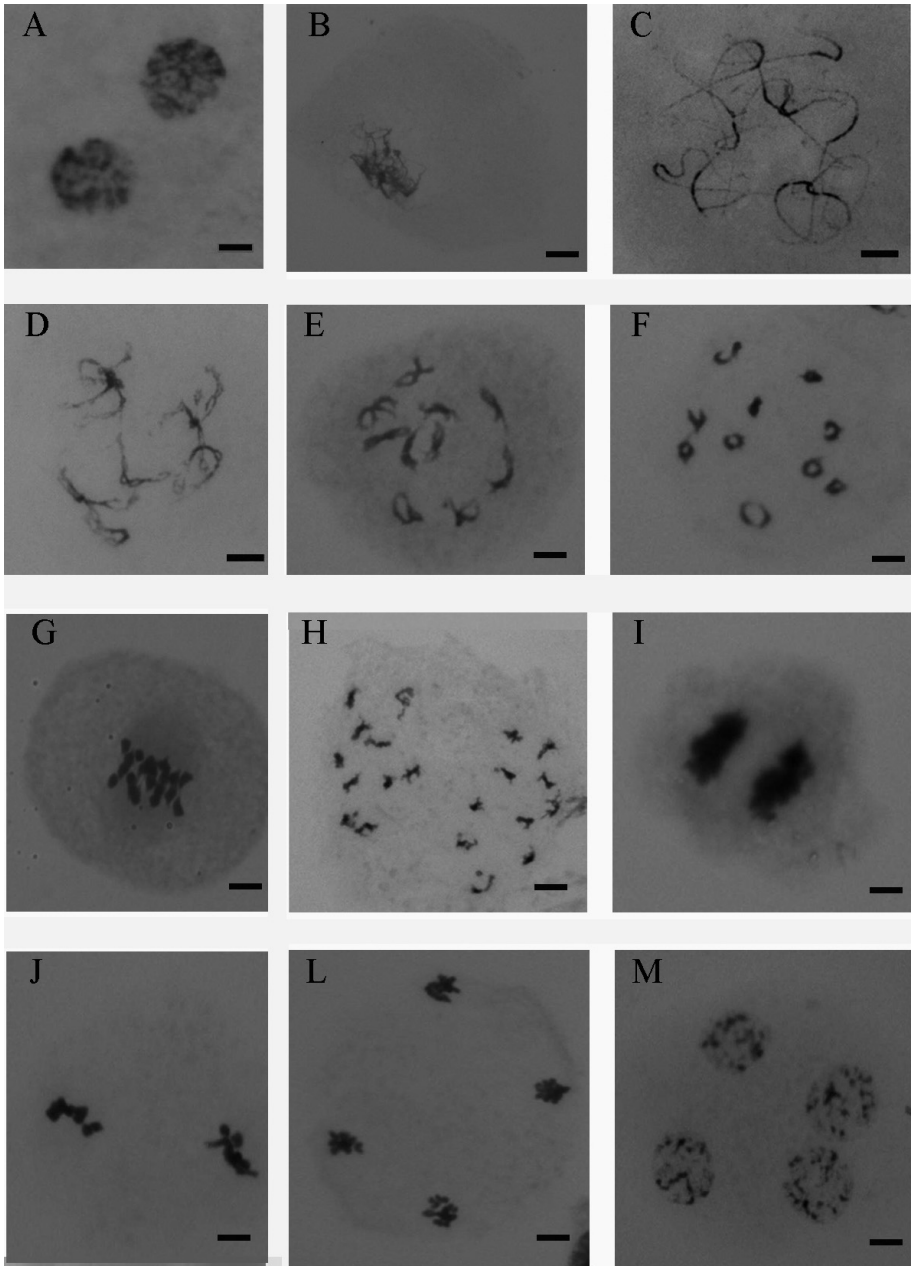

Fig. 1 – Meiosis of *Heteropterys umbellata* (A) Interphasic nucleus, (B) Zygotene, (C) Pachytene, (D) Initial diplotene, (E) Diplotene, (F) Diakinesis, (G) Metaphase I, (H) Anaphase I, (I) Telophase I, (J) Metaphase II, (L) Anaphase II, (M) Telophase II. Barr = 5 μm.

3.31% of cells with early segregation were observed. We didn't observe chromosome alterations in meiosis II.

Regarding the meiotic products, the Alexander solution indicated high pollen viability in *H. umbellata* (93.30%). It was also possible to observe that non-viable pollens were smaller than the viable ones (Fig. 2 E-F).

HETEROCHROMATIN QUALITY AND DISTRIBUTION

The meiotic chromosomes of *H. umbellata*, mainly during pachytene, showed naturally large, blocks of chromatin around the centromers strongly stained with Giemsa (Fig. 1C). These same regions, after a C-banding treatment, showed positive stained indicating that

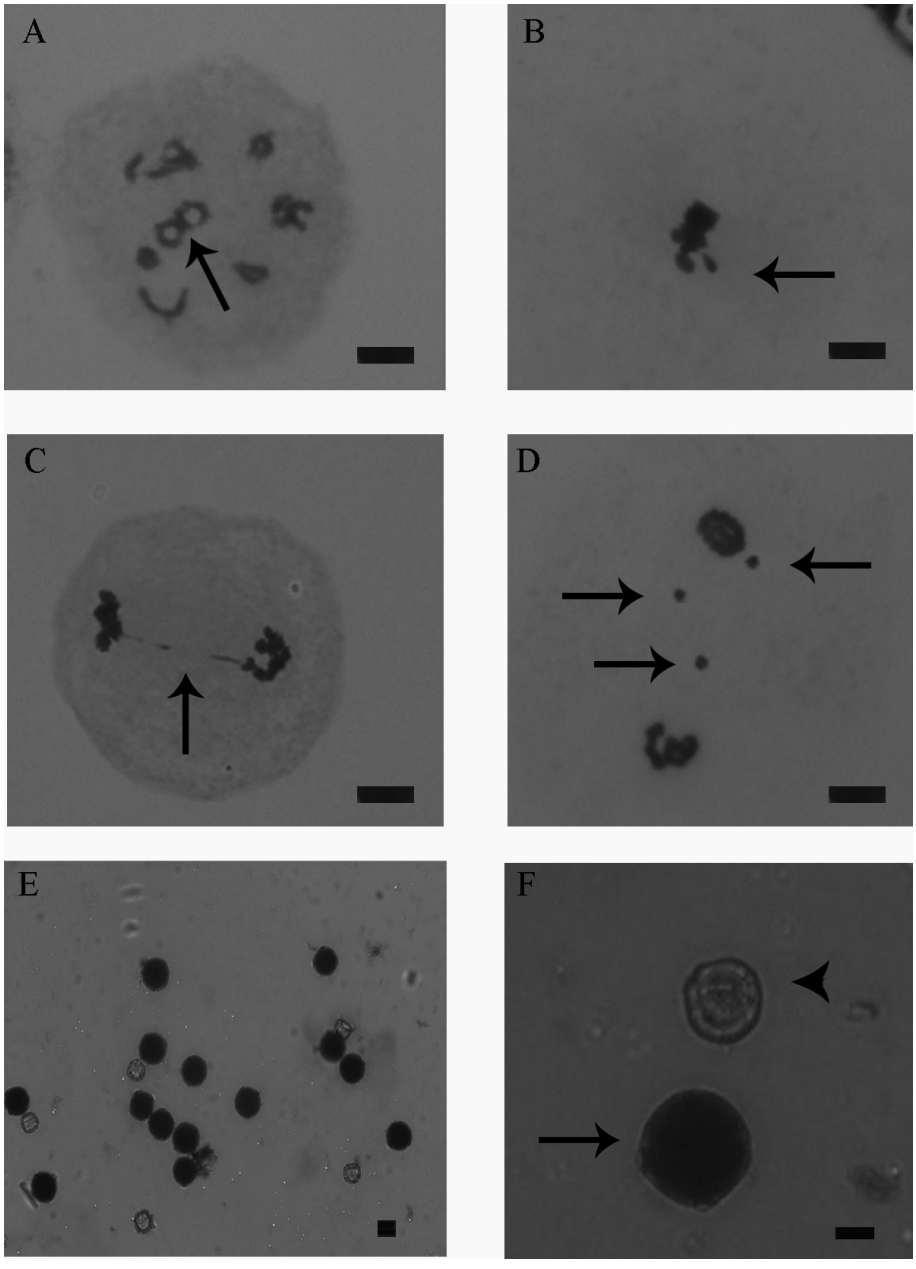

Fig. 2 – (A) Diakinesis with translocation (arrow), (B) Metaphase I with early segregaton (arrow), (C) Anaphase I with chromosome bridge (arrow), (D) Anaphase I with laggard chromosomes (arrows), (E) Pollen staining with Alexander solution, (F) Alexander staining: viable pollen (arrow) and non-viable pollen (arrow head). Barr = 5 μm.

these great blocks are rich in constitutive heterochromatin (Fig. 3A). In addition, when the slides were treated with DAPI and CMA_3 (specific stains to detect heterochromatin blocks rich in AT and CG, respectively), we observed that the great heterochromatic blocks around the centromers were $DAPI^+$, while for CMA_3 only one positive mark was observed at a subterminal region of one bivalent pachytene, diplotene and diakineses. During the pachytene and diplotene we observed a unique mark probably due to the great proximity of the chromosomes in these stages, while on diakineses we observed two marks, each one at one of the two homo-

logues of the same chromosome, and at the same position (Figs. 3 B-G and M). Regarding the interphase nucleus, the species showed great blocks of chromatin well distributed. When the nuclei were stained sequentially with DAPI and CMA_3, the blocks were positive for DAPI, while for CMA_3 we observed only two positive marks (Fig. 3 J-L).

Nucleolar Organizer Regions (NORs)

One chromosome pair attached to the nucleoli was observed with Giemsa stained in a great number of diakinesis cells, which indicates that, in *H. umbellata*, one pair of sites for NORs (rDNA 45S) are active and involved with the nucleolus formation. Moreover, we also observed that this site is subterminal in some pachytenes and diakinesis where one great attached nucleolus was observed (Fig. 3 H-I and N-O).

Palynology

The pollen of *H. umbellata* showed apolar morphology, with subspheroidals grains, 6 porates with colpoids and rugulate exine with few psilate areas (Fig. 4 A-D). The diameters of grains, D1 (25.19 ± 1.89 μm) and D2 (25.13 ± 2.08 μm) were very similar. The mean of sexine was 1.92 μm ($\pm$ 0.56) and nexine 1.33 μm ($\pm$ 0.39). The pollen stained was positive for Lugol and negative for Sudan, which indicates high starch content (Fig. 4 E-F). Regarding the apertures, the mean of pore diameter and the colpoidi size were 2.80 μm ($\pm$ 0.85) and 8.91 μm ($\pm$ 1.55), respectively. The margin of pores was thick and the colpoids were ever associated with the pores (Fig. 4D and G-H).

DISCUSSION

In all sexually reproducing organisms, meiosis is a complex process that helps to keep the chromosomal number constant from generation, to generation and ensures the operation of Mendel's laws of heredity (Singh 1993, Wendel 2000). The present study allowed us to determine, for the first time, the chromosome number of *H. umbellata* as 2n = 20, corroborating the hypothesis of Anderson (1990) that suggests the basic chromosome number for the genus x = 10.

The analysis of *H. umbellata* meiotic behavior showed that chromosome pairing was regular, and most of the cells showed 10II, although some irregularities such as chromosome bridges, translocations, early segregation and laggard chromosomes have been observed during the meiosis I. On the other hand, the high pollen viability that was observed can be a consequence of the checkpoint or nuclear restitution events during meiosis in *H. umbellata*, considering that the percentage of cells with laggard chromosomes observed in metaphase I and telophase II was, respectively, 11.20% and 0%. About these checkpoints, there are at least three known mechanisms that can act in meiosis: the first one is called "DNA damage checkpoints pathway" that causes arrest of cells to either G_1 or G_2 stage of the cell cycle (Weinert 1998); the second one states that recombination events can be completed before metaphase I start, while the third one states that all the chromosomes can be attached to the spindle fibers during metaphase I (Shaw and Moore 1998). However, we cannot explain the exact mechanism that works in *H. umbellata*. One or all of them can act in order to correct eventual disturbances and assure the normal development of pollen grain.

Regarding the pollen viability, we observed that Alexander solution was very useful, fast and practice to access it. Pollen viability has been carried out by different stains such as carmine (Pagliarine 2000), lugol (Johansen 1940), fluorescein diacetate (Heslop-Harrison et al. 1984, Shivanna and Rangaswamy 1992) and tetrazolium salts (Hauser and Morrison 1964). In the present study the Alexander solution was used because it provides a good contrast between the wall and cytoplasm, which allows a better visualization of the unviable grains. The non-viable pollen grains were smaller than the normal ones which suggests a problem during pollen formation after meiosis. Similar results were also observed by Souza et al. (2003) in *Passiflora edmundoi*, and by Palma-Silva et al. (2008) in *Vriesea gigantea*.

Due to the very low viability of the seeds that hinder the study of mitotic chromosomes, the present description of meiotic process provides an important information about the species. The banding pattern showed here was the first report for *Heteropterys* species. In *H. umbellata*, there are great blocks of heterochromatin revealed by C-banding technique, mainly around the centromere. This characteristic has been described for a great number of species but has not been

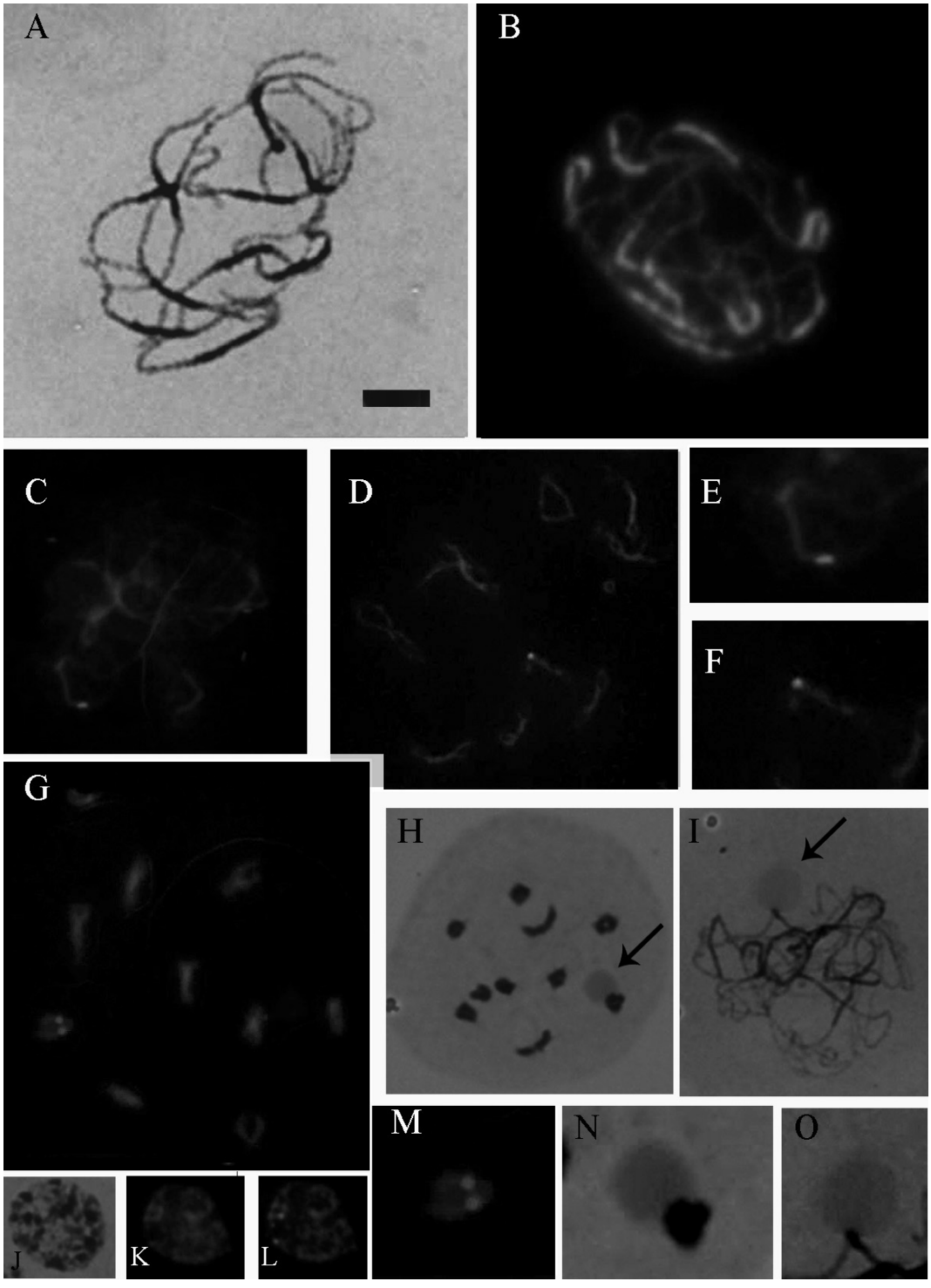

Fig. 3 – (A) Pachytene C-banded, (B) Pachytene DAPI stained showing positive marks around the centromers, (C) Pachytene stained with CMA_3, (D) Diplotene stained with CMA_3, (E) Detail of the positive CMA_3 mark onto pachytenic chromosome, (F) Detail of the CMA_3 positive mark onto diplotenic chromosome, (G) Diakinesis stained with CMA_3, (H) Diakinesis with one chromosome pair attached to the nucleolus (arrow), (I) Pachytene with one chromosome pair attached to the nucleolus (arrow), (J, K, L) Interphasic nucleus stained with Giemsa, DAPI and CMA_3 (see two positive marks), (M) Detail of CMA_3 positive marks onto the chromosomes at diakinesis, (N, O) Details of nucleolus attached to one chromosome pair during the diakinesis and pachytene, respectively. Barr = 5 μm.

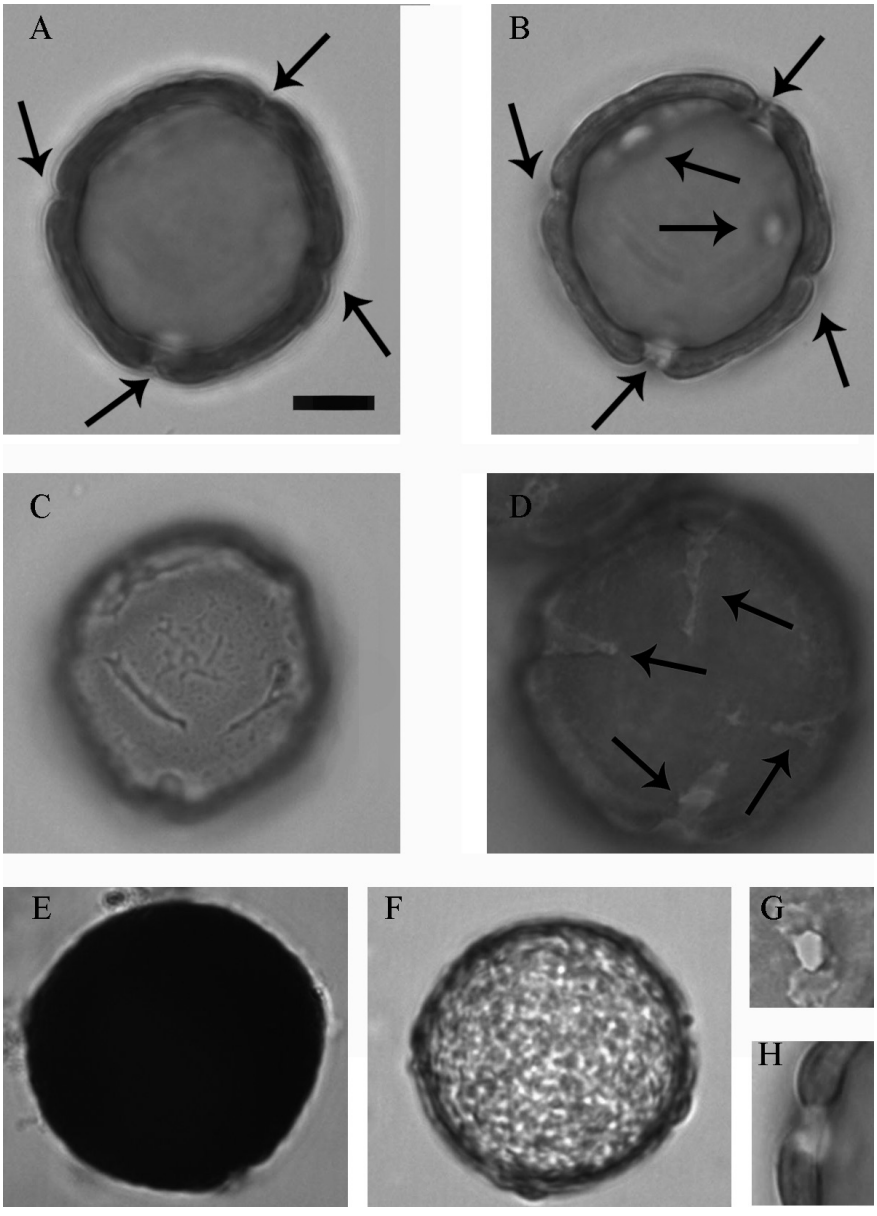

Fig. 4 – Acetolyzed pollen grains of *H. umbellata*, (A) Vision of 4 apertures (arrows), (B) Vision of 6 apertures (arrows), (C) Vision of exine, (D) Vision of colpoids (arrows), (E) Starch positive grain stained with Lugol, (F) Lipid negative grain stained with Sudan IV, (G-H) Details of pores. Barr = 5 μm.

reported earlier for Malpighiaceae (Brandão et al. 2007, Sousa et al. 2009). The heterochromatin is considered one of the most important factors in the evolution process (Edelman and Lin 1995) once it can interfere in many other mechanisms such as DNA replication, chromosomal structure, gene expression and the cell cycle (Redi et al. 2001, Stace 2000). Besides, variations in heterochromatin amounts have proved to be useful in karyosystematic and phylogenetic studies (Grif 2000, Sousa et al. 2009, Schwarzacher et al. 1980). Many studies indicate that the presence of few heterochromatin in one species is a plesiomorphic condition, while large blocks indicate an apomorphic condition (Greilhuber 1979, Redi et al. 2001).

The fluorochrome bandings were also very important to access the heterochromatin constitution of *H. umbellata*. The large positive blocks visualized by DAPI indicated that the species possesses large amounts of AT and few amounts of GC. This conclusion was also supported by the single positive signal observed after CMA_3 staining. Nevertheless, the association among CMA_3 marks (only one pair), their chromosome places (terminal regions) and the number of chromosomes attached to the nucleoli (only one pair) clearly indicated that the *H. umbellata* possesses only one chromosome pair with active sites for nucleolus organization. Schweizer (1976) described the relationship between GC specific stain and NORs (Nucleolar Organizer Regions) also using the CMA_3 positive bands. Sousa et al. (2009) also observed in *Lippia alba* (Verbenaceae) two pairs of chromosomes attached to one nucleolus, and CMA_3 marks at the same place of NORs. In addition, they observed by AgNOR banding that only one of these two pairs of chromosomes had active NORs sites, although the FISH technique had shown three pairs of NORs.

In addition to the cytological studies we also observed that pollen grains of *H. umbellata* are very similar to other species of Malpighiaceae, including species of the genus *Heteropterys*. Gonçalves-Esteves et al. (2007) described the pollen morphology of 5 species of *Heteropterys*, and reported that the diameter sizes (D1 and D2) ranged from D1 = 27.1μm and D2 = 27.8μm in *H. rufula* to D1 = 49.1μm and D2 = 45.3μm in *H. coleopteran*. According to the diameter of the grains, the authors classified the studied species as small and median pollen grains, respectively. Our results were very similar to those ones observed to *H. rufula*. Using the same system, the values of D1 = 25.19μm and D2 = 25.13μm allowed us to classify the pollen grains of *H. umbelllata* as small ones. On the other hand, regarding the pore diameter, we observed different results if they are compared with other *Heteropterys* species. While we observed a mean value of pore diameter equal to 2.80 μm, Gonçalves-Esteves et al. (2007) observed values that ranged from 5.80 to 7.30 μm. With respect to exine ornamentation and thickness, the present results were very similar to those ones observed by Gonçalves-Esteves et al. (2007) for other 5 species.

Concerning the chemical content of pollens from *H. umbellata*, it may be stated that this species has pollen grains that are rich in starch due to positive stained with Lugol and negative one with Sudan solution. Baker and Baker (1979) argued that starch pollen arose as an adaptation to deter pollen theft by non-pollinating insects, and lipid pollen arose as a plant adaptation to attract and reward pollen-feeding pollinators. There are no reports about the pollination in the genus *Heteropterys*. Our results about the pollen content suggest, according to Baker and Baker's hypothesis, that pollinators of *H. umbellata* are possibly non-pollen-feeding pollinators as wings, birds, moths and other animals that prefer non-starch pollen. Nevertheless, additional studies about the pollination systems in *Heteropterys* should be done to better understand how this system works.

Finally, our data constitute an important upgrade in the biology of the genus *Heteropterys*, and can be used to future taxonomic and phylogenetic studies. The use of the same approach with of other species will also be very interesting.

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RESUMO

O gênero *Heteropterys* é um dos maiores gêneros entre as Malpighiaceae. Entretanto, poucos estudos citológicos e palinológicos foram relatados. O presente trabalho descreveu pela primeira vez o número cromossômico, o padrão heterocromático, o comportamento meiótico, a viabilidade polínica e aspectos palinológicos de *Heteropterys umbellata*, uma espécie muito disseminada. Uma grande população brasileira foi estudada utilizando técnicas convencionais para o estudo de cromossomos meióticos e acetólise para acessar a morfologia polínica. A espécie apresentou 2n = 20 cromossomos, desenvolvimento meiótico normal e pólens viáveis. Grandes blocos de heterocromatina foram observados ao redor dos centrômeros. A coloração com DAPI foi positiva para a heterocromatina centromérica, enquanto marcas com CMA_3 foram observadas somente em um sítio terminal de um par de cro-

mossomos homólogos. Este resultado e a presença de um par de cromossomos associados a um nucléolo durante o paquíteno e diacinese sugerem a presença de somente um par de RONs. A análise palinológica revelou que os grãos de pólen são apolar, com 6 poros e colpóides associados a todos eles. O conteúdo polínico foi positivo para o teste de amido e a exina mostrou-se rugulada com poucas regiões psiladas.

Palavras-chave: número cromossômico, padrão heterocromático, *Heteropterys umbellata*, Malpighiaceae, meiose, viabilidade polínica.

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