

Chromosome number in meiotic stage cells and pollen viability of *Vanhouttea hilariana* Chautems, *Vanhouttea brueggeri* Chautems and an interespecific hybrid (Gesneriaceae)

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Resumen

Número de cromosomas en las células meióticas y la viabilidad del polen de Vanhouttea hilariana Chautems, Vanhouttea brueggeri Chautems y un híbrido interespecifico (Gesneriaceae)

Vanhouttea (Gesneriaceae) es un género de las montañas rocosas de Brasil. Dos especies, *Vanhouttea hilariana* Chautems y *Vanhouttea brueggeri* Chautems, son muy similares, diferenciándose sólo por la morfología del cáliz. La hipótesis de la hibridación entre estas especies fue sugerido por muchos autores, principalmente por la observación de individuos con morfología intermedia dentro de las poblaciones. El objetivo de este trabajo fue determinar el número de cromosomas y evaluar el comportamiento meiótico y la viabilidad del polen de estas dos especies y los posibles híbridos. El análisis de los posibles padres y híbridos reveló que todos los individuos analizados tienen $2n=26$ y un comportamiento meiótico normal. Sin embargo, aunque los granos de polen de los posibles padres aparentemente eran viables, se observó alrededor de 92,87% de polen inviable en todos los posibles híbridos analizados, reforzando la hipótesis de la hibridación entre *V. hilariana* y *V. brueggeri*.

Palabras clave: Número de cromosoma, Gesneriaceae, Híbridos, Meiosis, Viabilidad del polen.

Abstract

Vanhouttea (Gesneriaceae) is a genus from Brazilian rock mountains. Two species, *Vanhouttea hilariana* Chautems and *Vanhouttea brueggeri* Chautems, are very similar, differentiated just by the calyx morphology. The hypothesis of hybridization between these species was suggested by many authors mainly by the observation of individuals in the populations with intermediate morphology. The aim of this work was to determine the chromosome number and to evaluate the meiosis behavior and pollen viability of these two species and the putative hybrid. The analyses of the putative parental and hybrid revealed that all individuals have $2n=26$ and a normal meiosis behavior. Nevertheless, although the pollen grains of possible parental has been viable, it was observed around 92.87% of unviable pollen from all putative hybrids analyzed which reinforce the hypothesis of hybridization between *V. hilariana* and *V. brueggeri*.

Key words: Chromosome number, Gesneriaceae, Hybrids, Meiosis, Pollen viability.

Introduction

Interspecific hybrids are normally a consequence of the mating of two species, normally from the same genus. The offspring normally displays traits and characteristics of both parents and they are usually sterile. Thus, hybrid sterility prevents the flow of between parental species contributing to their genetic isolation. In many cases sterility in hybrids is due to the different chromosome numbers displayed by the parents that lead to the generation of unbalanced gametes in the hybrids (Rieseberg and Carney, 1998). On the other hand, the viability of the hybrids can sometimes be reached by their genome duplication (Singh, 2002). In this case the difference between parental genomes and, consequently, the meiotic pairing can be used to study phylogenetic relationships among species and therefore to understand the evolution of a given genus (John, 1990; Singh, 2002; Soltis and Soltis, 2009).

Gesneriaceae is a pantropical plant family considered for many authors as a monophyletic group (Araujo et al., 2005; Chautems, 2002; Wanderley et al., 2003). The family possesses around 150 genera and 4000 species. Among them 23 genus and almost 200 species can be found in Brazil (Araujo et al., 2005; Souza and Lorenzi, 2005). Many species have been used as ornamental plants and few ones showed contraceptive and analgesic medicinal properties, being also used against injuries caused by snakebites (Heywood, 1993; Denham, 2004; Souza and Lorenzi, 2005).

Certain groups of vascular plants, including the Gesneriaceae family, have characteristics that facilitate the formation and maintenance of hybrids such as habitat fragmentation, ecological flexibility and vegetative reproduction, which can guarantee reproductive success even when sterile (Ellastrand et al., 1996; Sanmartin-Gajardo and Sazima, 2005; Wiehler, 1983).

The genus *Vanhouttea*, together with *Pal-iavana* and *Sinningia*, form the monophyletic Sinningeae tribe that belongs to subfamily Gesnerioideae (Araujo et al., 2005; Chautems, 2002; Perret et al., 2001; Perret et al., 2003). The genus comprises eight endemic species from Southeast Brazil (Chautems, 2002). Among them, *Vanhouttea hilariana* Chautems and *Vanhouttea brueggeri* Chautems were recently described in small localities of Mantiqueira Mountains Range and Ibitipoca State Park, at Minas Gerais State, Brazil

(Chautems 2002). Strong evidences favor the existence of natural hybridization between them, namely: populations are close together, they have similar flowers (the unique difference is the calyx), synchronic bloom (3-6 months), and pollination by the same three species of hummingbirds. Also, the putative hybrids exhibit an intermediate form of the calyx (size and morphology) between the possible parents (Chautems, 2002; Sanmartin-Gajardo and Sazima, 2005).

Cytogenetic analyses have been widely used as an important tool to study the hybridization process (Rieseberg and Carney, 1998; Soltis and Soltis, 2009). The determination of chromosome number in plants is a critical step to detect mechanisms that make feasible abrupt speciation such as hybridization, polyploidy, aneuploidy and dysploidy (Briggs and Walters, 1997; Guerra, 2008). Moreover, the knowledge of chromosome numbers and morphology and also their meiotic behavior may help to differentiate between allopolyploidy or autopolyploidy considering the differences or similarities between the genomes involved in the hybrid (Stebbins, 1971; Guerra, 2008). In addition, the meiotic chromosome behavior can affect pollen viability. If meiosis is irregular, with the chromosome pairs segregate abnormally, pollen sterility can be expected. When meiosis is normal, mechanisms such as inbreeding depression, pollen age and exposure to environmental stress (temperature and humidity) can also cause pollen sterility. All together these considerations can be a good indicator on the fertility of the hybrids and the corresponding parents (Krebs and Hancock, 1990; Husband and Schemske, 1996; Goodwillie, 2000; Boff and Schifino-Wittmann, 2002).

In this paper, the chromosome number, the meiotic behavior and also the pollen viability of populations of *V. hilariana*, *V. brueggeri* and the putative hybrid between them were described for the first time.

Material and methods

Biological material

Immature inflorescences of plants from two populations of *V. hilariana* (23 individuals) and *V. brueggeri* (14 individuals) and 10 individuals with intermediate morphology (putative hybrid) among these two species were collected in adjacent regions at the Ibitipoca State Park, Minas Gerais,

Brazil (21°42'S and 43°54'W). Inflorescences were fixed in fresh cold methanol:acetic acid solution 3:1 for at least for 24h. Vouchers were deposited at the Herbarium CESJ of Universidade Federal de Juiz de Fora, Juiz de Fora, Minas Gerais, Brazil according to usual procedures.

Pre-selection of inflorescences

To obtain cell suspensions in the adequate meiotic stages cells to determine the chromosome number and to analyze the meiotic behavior, the immature inflorescences were divided based on previously defined size that showed meiotic cells in different stages of development were chosen and their cells observed.

Cell suspensions and slides preparation

The cell suspensions used were prepared according to Viccini et al. (2005) with some modifications. About 40 anthers were excised from 12 flower buds and placed in a special microtube (0.5 ml) with a nylon screen attached (60 µm) at the bottom. The material was washed in distilled water to remove the fixative solution. The tube containing the anthers was immersed in an 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 obtain the pollen mother cells (PMCs). The cellular suspension obtained was centrifuged at 2000 rpm for 12 min. The slides were prepared by the air the drying technique (Caixeta and Carvalho, 2001) and stained with Giemsa solution during three min. Cell images were analyzed using Image Pro Plus software (Media Cybernetics, Silver Spring, MD, USA).

Pollen viability

Pollen grains were collected from 6 mature flowers buds of 10 individuals of each one the putative parentals populations and hybrids. The slides were prepared using a squash technique and their viability was estimated according to the differential staining they showed (Alexander, 1980). Digital images were obtained by means of a BX 51 microscope and analyzed by Image Pro Plus software (Media Cybernetics, Silver Spring, MD, USA). Ten slides were confectioned for each species, 16,785 grains for *V. hilariana*, 12,345 for *V. brueggeri* and 9,731 grains for the putative hy-

brids were scored.

Results

Chromosome number and meiotic behavior

The analysis of meiotic behavior in pollen mother cells (PMCs) at different stages of *V. hilariana* and *V. brueggeri* showed a regular chromosome pairing (Figs. 1a-b). Thirteen bivalents were observed during diplotene, diakinesis and metaphase I in all individuals studied indicating that the chromosome number of both species is $2n=26$. Regarding to the putative hybrid, we also observed $2n=26$, but about 12% of cells showed some univalents and trivalents at diakinesis (Fig. 1c) and normal cells in metaphase I and II and anaphase I and II.

Pollen viability

Alexander staining indicated high pollen viability for *V. hilariana* (91,80%±3,21) and *V. brueggeri* (97,20%±5,82). On the other hand, all supposed hybrids individuals showed about 92.87%±2,31 of nonviable pollen grains. In these individuals it was possible to see pollen grains, characterized by different sizes and morphologies (Figs. 1d-g).

Discussion

Gesneriaceae whole family possess chromosome numbers ranging from $n=8$ to 17, while in the sub-family Gesnerioideae the most common chromosome numbers are $n=9$ and $n=13$. Considering just the tribe Sinningieae, the most common chromosome number observed is $n=13$ (Skog, 1984; Weber, 2004). In concordance with previous reports for the tribe, the *Vanhouttea* species studied here showed $n=13$ chromosomes, reinforcing the relationships among the three genera of the tribe Sinningieae: *Vanhouttea*, *Paliavana* and *Sinningia*.

The species of the tribe Sinningieae have campanulate or tubular flowers often red and odorless, which suggest that the pollination is performed by hummingbirds (Chautems, 2002; Denham, 2004; SanMartin-Gajardo and Sazima, 2005). The nectar produced by the species of this tribe is rich in sucrose which reinforces the role of humming birds in pollination process (Perret et al., 2001). As the same bird species can pollinate different

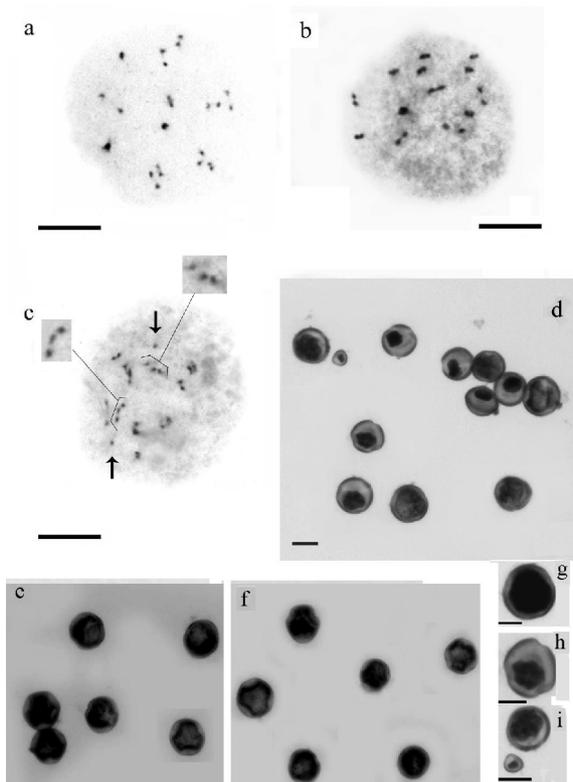


Figura 1. Diacinesis de *Vanhouttea brueggeri* (a), *Vanhouttea hilariana* (b) y posibles híbridos (c) (las flechas muestran los univalentes, en detalle se muestra la formación de los trivalentes); tinción diferencial de polen de los posibles híbridos mostrando gran porcentaje de granos inviábiles (d); tinción diferencial de polen de *V. brueggeri* (e) y *V. hilariana* (f); granos viables (g), granos inviábiles (h), granos inviábiles con diferentes diámetros (i). Bar=5 μ m

Figure 1. Diakinesis of *Vanhouttea brueggeri* (a), *Vanhouttea hilariana* (b) and putative hybrids (c) (arrows show the univalent, details show the trivalent formation); differential staining in putative hybrids showing great percentage of unviable grains (d); differential staining in *V. brueggeri* (e) and *V. hilariana* (f); viable grain (g), unviable grain (h), unviable grains with different diameter size (i). Bar=5 μ m

plant species it may result in a mechanism called 'Improper Pollen Transfer' (IPT) resulting in hybrid production (Dafni, 1992). It is also possible that same process has been occurred with *V. hilariana* and *V. brueggeri* from Ibitipoca State Park – MG because the occurrence of the same three different species of hummingbirds as pollinators (Chautems, 2002; Sanmartín-Gajardo and Sazima, 2005).

The species *V. hilariana* and *V. brueggeri* are defined as taxonomically distinct (Chautems, 2002). However, the meiotic behavior of the putative hybrids showed apparent regular microsporogenesis, indicating that they can produce viable

pollen grains from the cytogenetic point of view. Nevertheless, the palynological studies revealed the production of pollen grains with great morphometric variation, without nuclear content and some morphological changes (folds and cracks), indicating absence of viability and reinforcing the hypothesis of hybridization among the two *Vanhouttea* species (Conceição, 2006). These data suggest that *V. hilariana* and *V. brueggeri* can be under a speciation process, although the biological barriers to prevent intercrossing and consequently genetic isolation are not yet completely efficient.

The speciation, emergence of new species, occurs along with the gradual evolution of barriers that prevents the genetic exchange. In animals this process mostly occurs by the appearance of pre- and post-copulatory mechanisms of reproductive isolation. In plants, the main mechanisms for isolation are the inviability, and also the total or partial sterility of the hybrid (Stebbins, 1945; Futuyama, 1992; Singh, 2002; Soltis and Soltis, 2009). Substantial efforts have been done to understand the mechanisms of genic sterility or hybrid inviability. The most widely accepted model was first proposed by Dobzhansky (1937). In this model, a gene from one species interacts negatively with a gene from another species, causing some degree of unviability or sterility. A somewhat different model for the evolution of hybrid unviability or sterility is that a much larger number of diverging loci interact negatively in a hybrid genetic background, and that these weak interactions act cumulatively to cause unviability or sterility (Wu and Palopoli, 1994). Others models suggest that meiosis plays a key role in the evolution of postmating reproductive isolation. In this view, the sterility may be explained due to the differences of chromosome structure between species with different genomes with little or no affinity chromosomal interfering with meiotic pairing and forming genetically unbalanced gametes (Stebbins, 1945; Frank, 1991; Singh, 2002). In the present study, since the meiotic process seems normal with a low frequency of chromosome abnormalities observed in the putative hybrids, it is not possible to conclude that abnormalities in this process are the cause of the supposed hybrid sterility. Additional studies at molecular level should be carried out in order to really understand and explain the origin of the sterility mechanism involved.

Our data, including chromosome and palynological features, together with others previous studies on the composition of floral nectar and pollinators as well as the intermediate size of the calyx, reinforce the hypothesis of the hybridization between *V. brueggeri* and *V. hilariana*. The data also indicated a close relationship between these species and possibly that a speciation process may still be in progress. However, further studies, especially about phenology, reproduction mechanisms and other molecular aspects have to be done to confirm the origin of the hybrid. Cytologically, the hybridization studies using FISH (Fluorochrome in situ Hybridization) and mainly GISH (Genome in situ Hybridization) will be very useful to improve our knowledge and better understand the relationships among these two species and their putative hybrids.

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