



Original Paper

Campomanesia adamantium and *Campomanesia pubescens* are distinct species? Use of palynology and molecular markers in taxonomy

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Abstract

The species *Campomanesia adamantium* and *C. pubescens* present a great morphological variability, with the existence of morphotypes and the indication of a possible hybridization process occurring between them. Thus, the aim of this study was to characterize the *Campomanesia adamantium* species and morphotypes of species *Campomanesia pubescens* through palynological reviews and with the use of molecular markers to assist the taxonomic description of the species. The plant material was collected in the experimental field of Federal University of Jataí, in bloom of 2014, and analyzes were performed in the Morphology Laboratory of Plant Taxonomy and in the Laboratory of Genetics and Plant Breeding belonging to the same institution. Analysis of scanning electron microscopy was performed at the Federal University of Lavras. Important information was found that reinforce the possibility of cross between *Campomanesia adamantium* and *Campomanesia pubescens*, generating morphotypes intermediaries, indicating a possible hybridisation process. It can be concluded that *Campomanesia adamantium* is farthest from the morphotype 2 from *Campomanesia pubescens*, and morphotypes 1 and 3 are intermediate between them, possibly being hybrids.

Key words: gabirola, hybridization, pollen grains, taxonomy.

Resumo

As espécies *Campomanesia adamantium* e *C. pubescens* apresentam uma grande variabilidade morfológica, com a existência de morfotipos e, a indicação de um possível processo de hibridação ocorrendo entre elas. Assim o objetivo deste estudo foi caracterizar a espécie *Campomanesia adamantium* e os morfotipos da espécie *Campomanesia pubescens* através de análises palinológicas e com uso de marcadores moleculares para auxiliar na descrição taxonômica das espécies. O material vegetal foi coletado no campo experimental da Universidade Federal de Jataí, na floração de 2014 e as análises realizadas no Laboratório Morfologia e Taxonomia Vegetal e no Laboratório de Genética e Melhoramento de Plantas da mesma instituição. As análises de microscopia eletrônica de varredura foram realizadas na Universidade Federal de Lavras. Foram encontradas informações importantes que reforçam a possibilidade de cruzamento entre *C. adamantium* e *C. pubescens*, gerando morfotipos intermediários, indicando um possível processo de hibridação. Pode-se concluir que *C. adamantium* está mais distante do morfotipo 2 de *C. pubescens* e os morfotipos 1 e 3 são intermediários entre eles, sendo possivelmente híbridos.

Palavras-chave: gabirola, hibridização, grãos de pólen, taxonomia.

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Introduction

The Myrtaceae family is one of the main botanical families, according to Flora of Brazil (2020b, under construction) presents 23 genera and 1,031 species, being 793 endemic to Brazil. We highlight the genus *Campomanesia*, being represented by 42 species in all the phytogeographic domains of Brazil, being 32 endemic (Flora of Brazil 2020a, under construction).

Known as gabirobeiras, these native Brazilian species are widely distributed throughout the national territory (Flora of Brazil 2020a, under construction). Its species cannot be easily identified often being confused, as in the case of *Campomanesia adamantium* (Cambess.) O.Berg and *Campomanesia pubescens* (Mart. ex DC.) O.Berg, which are morphologically similar, and the basic difference is that the latter has trichomes on sepals and leaves (Arantes & Monteiro 2002; Landrum 1986).

Based on original descriptions and with the aid of field work and herbarium materials, Amaral *et al.* (2016) identified three distinct morphotypes for the species *C. pubescens*, and morphotype 2 fits the original descriptions of Lima *et al.* (2011) and Landrum & Kawasaki (1997), and morphotypes 1 and 3 present of intermediate characters with *C. adamantium*, suggesting possible hybridization process occurring between which has already been recognized by Landrum (1986), where he stated that part of the variability among *C. pubescens* may be due to the occurrence of hybridization. This process occurs by interspecific cross-pollination, which may be one of the factors that lead to the appearance of variability in the morphological characters within the species. In a study conducted by Borém (2009) indicates that the sexual system of *C. pubescens* is allogene, which allows inferring about the existence of gene flow. The absence of effective isolating barriers may lead to hybridization procedure (Wendt *et al.* 2008).

Another factor that may favor the hybridization mechanism is the number of chromosomes, which varies little in the *Campomanesia* genus, as observed by Costa & Forni-Martins (2006) in diploid ($2n = 22$) populations of *C. adamantium* and *C. pubescens*.

With this, it is important to know characteristics that provide subsidies to verify the occurrence of possible crosses between species, explaining the formation of intermediate morphological characters. One of the works that can help is the study of the morphology of pollen

grains, since it is based on the typical differences presented by each plant species, mainly with respect to size and shape (Bauermann & Neves 2005). In this sense, current palynology is an important tool for taxonomic and environmental studies (Cancelli *et al.* 2007), as was done by Tuler *et al.* (2016) for species of the *Psidium* genus, and by Correa *et al.* (2018) where the pollen morphology showed to be a significant source of information for taxonomic purposes.

In addition, with the use of molecular tools, it is possible to quantify genetic diversity through molecular markers (Brandão 2008), having access to information directly from the DNA, without environmental influences and the stage of development of the plant (Tuler *et al.* 2015) a better characterization of the species of this genus, even those presenting near morphological characteristics, since they may have be affected by the influence of the environment, being useful tools for the taxonomy. The literature found some work using molecular tools to evaluate the genetic variability contained in some species of the genus *Campomanesia* including *C. adamantium* and *C. pubescens* (Assis *et al.* 2013; Miranda *et al.* 2016), but none with focus molecular characteristics to aid in the taxonomy of the genus.

This work aimed to contribute to the knowledge of the diversity of the genus *Campomanesia* in the state of Goiás, through the study of pollen and molecular analyzes, seeking information on reproductive compatibility between *C. adamantium* and *C. pubescens*, according to studies by Amaral *et al.* (2016) and Assis *et al.* (2013), in addition to records in the Jataiense Herbarium (HJ), these are the two species of almost prevailing occurrence in the state. In addition, Amaral *et al.* (2016) describes the existence of three morphotypes for the species *C. pubescens*, which are accepted and studied in this work.

Materials and Methods

The plant material was collected from the experimental field of the Federal University of Jataí (UFJ), where there are accessions of *Campomanesia* collected in 17 municipalities in the state of Goiás, from the Southwest region to the surroundings of the Federal District. In addition to collections made in other forests in the municipality of Jataí. All the collected material was deposited at the Herbarium Jataiense (HJ) of UFJ. It was used a classification proposed by Amaral *et al.* (2016) with the help of specialists, specific bibliography and

comparison with botanical material, accepting the existence of three morphotypes for *C. pubescens*.

Campomanesia adamantium (Cambess.) O. Berg.

Material examined: BRAZIL. GOIAS: Jataí, Woods of Queixada, 18.VIII.2010, fl., *E.V.E.J. Amaral 160* (HJ); 18.VIII.2010, fl. e fr., *E.V.E.J. Amaral 161* (HJ); 18.VIII.2010, fl., *E.V.E.J. Amaral 164* (HJ); 18.VIII.2010, fl., *E.V.E.J. Amaral 166* (HJ); 18.VIII.2010, fl., *E.V.E.J. Amaral 168* (HJ); 9.X.2010, fr., *E.V.E.J. Amaral 234* (HJ); 9.X.2010, fr., *E.V.E.J. Amaral 235* (HJ); 9.X.2010, fr., *E.V.E.J. Amaral 237* (HJ); 9.X.2010, fr., *E.V.E.J. Amaral 238* (HJ); 9.X.2010, fr., *E.V.E.J. Amaral 239* (HJ); 6.XI.2010, fr., *E.V.E.J. Amaral 259* (HJ); 6.XI.2010, fr., *E.V.E.J. Amaral 261* (HJ); 9.XI.2010, fr., *E.V.E.J. Amaral 263* (HJ); 9.X.2010, fr., *E.V.E.J. Amaral 267* (HJ). 41° BIMTZ, 20.VIII.2010, fl., *E.V.E.J. Amaral 171* (HJ); 27.IX.2010, fl., *E.V.E.J. Amaral 192* (HJ); 20.IX.2010, fl. e fr., *E.V.E.J. Amaral 197* (HJ). UFJ, 23.XI.2001, fl., *L.F. Souza 554* (HJ); 25.XI.1999, fl., *L.F. Souza 187* (HJ).

Campomanesia pubescens (DC.) O. Berg.

Material examined: BRAZIL. GOIAS: Jataí, Woods of Queixada, 18.VIII.2010, fr., *E.V.E.J. Amaral 163* (HJ); 18.VIII.2010, fl., *E.V.E.J. Amaral 165* (HJ); 18.VIII.2010, fr., *E.V.E.J. Amaral 167* (HJ); 13.X.2010, fr., *E.V.E.J. Amaral 280* (HJ); 6.XI.2010, fl., *E.V.E.J. Amaral 258* (HJ); 6.XI.2010, fr., *E.V.E.J. Amaral 262* (HJ); 9.XI.2010, fr., *E.V.E.J. Amaral 266* (HJ). 41° BIMTZ, 20.VIII.2010, fl., *E.V.E.J. Amaral 172* (HJ); 20.VIII.2010, fl., *E.V.E.J. Amaral 174* (HJ); 9.XI.2010, fr., *E.V.E.J. Amaral 190* (HJ); 20.IX.2010, fl. e fr., *E.V.E.J. Amaral 191* (HJ); 20.IX.2010, fl. e fr., *E.V.E.J. Amaral 195* (HJ); 20.IX.2010, fl. e fr., *E.V.E.J. Amaral 198* (HJ); 20.IX.2010, fl. e fr., *E.V.E.J. Amaral 199* (HJ); 8.X.2010, fr., *E.V.E.J. Amaral 230* (HJ); 8.X.2010, fl., *E.V.E.J. Amaral 232* (HJ); 9.XI.2010, fr., *E.V.E.J. Amaral 268* (HJ); 9.XI.2010, fl., *E.V.E.J. Amaral 270* (HJ); 9.XI.2010, fl., *E.V.E.J. Amaral 272* (HJ); 18.VIII.2010, fl., *E.V.E.J. Amaral 162* (HJ); 20.VIII.2010, fl., *E.V.E.J. Amaral 170* (HJ); 20.VIII.2010, fl., *E.V.E.J. Amaral 173* (HJ); 20.IX.2010, fl., *E.V.E.J. Amaral 193* (HJ); 20.IX.2010, fl. e fr., *E.V.E.J. Amaral 194* (HJ); 20.IX.2010, fl. e fr., *E.V.E.J. Amaral 196* (HJ); 8.X.2010, fr., *E.V.E.J. Amaral 233* (HJ); 9.XI.2010, fr., *E.V.E.J. Amaral 264* (HJ); 9.XI.2010, fl., *E.V.E.J. Amaral 265* (HJ); 9.XI.2010, fl., *E.V.E.J. Amaral 269* (HJ); 9.XI.2010, fl., *E.V.E.J. Amaral 271* (HJ). Farm São Domingos, 19.X.2010, fl., *E.V.E.J. Amaral 253* (HJ); 15.XI.2010, fr., *E.V.E.J. Amaral 277* (HJ). UFJ, 15.IX.2010, fl., *E.V.E.J. Amaral 6584* (HJ); 28.XI.2008, fl., *L.F. Souza, 2620* (HJ).

Pollen viability and stigmatic receptivity

The analyses of pollen viability were carried out using approximately 10 flowers in anthesis of *C. adamantium* and of each of the morphotypes

of *C. pubescens* studied. The pollen grains were spread out on slides, covered with an aqueous sugar solution at 20% on the Brix scale as the substrate, left at ± 25 °C for 24 h (adapted from Dafni & Husband 2005), and were subsequently analyzed. The percentage of germinated pollen grains was assessed under a binocular optical microscope, *i.e.*, the percentage of germinated pollen grains was assessed from 100 pollen grains counted at random. A pollen grain was considered to be germinated when the pollen tube exceeded the diameter of the pollen grain (Chia *et al.* 2009).

The receptivity of the stigma was verified in the field and observed with a hand-held magnifying glass and hydrogen peroxide (adapted from Dafni & Husband 2005). A drop of hydrogen peroxide was placed on the stigma of flowers in anthesis and the magnifying glass was used to observe whether or not bubbles were present. Bubbles would not be observed when the stigma was no longer receptive. We evaluated an average of 10 flowers of each species for 10 days, always around 8 am.

Morphological characterization of pollen grains

At least five fresh flowers in a state of pre-anthesis were used for the morphological analysis of the pollen grains of each species and the respective morphotype was evaluated, except in the case of morphotype 3, for which dried flowers were rehydrated and used as no fresh flowers were available. The anthers were macerated to obtain the pollen grains, which were then subjected to acetolysis (Erdtman 1960, adapted from Dafni *et al.* 2005). The pollinic material was fixed in 70% alcohol and transported to the Federal University of Lavras (UFLA).

The analyses were performed using a scanning electron microscope at the Electron Microscope and Ultra-structural Analysis Laboratory of the Phytopathology Department of UFLA, where the images were captured using a digital camera coupled to the microscope with Leica Application Suite software (LAS EZ).

The pollen grains were described using terminology and classification criteria in the well-known studies of Barth (1965) and Barth & Barbosa (1972).

For the pollinic morphometric analysis by optical microscope (OM), fresh flowers were mounted on slides in glycerin, and pollen grains were photomicrographed and measured with software specific to the equipment (Leica

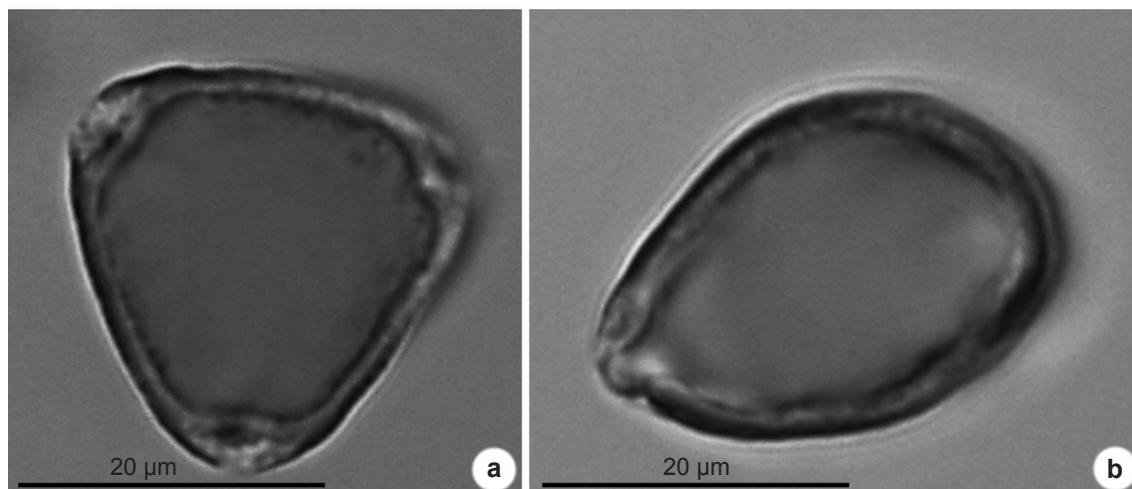


Figure 1 – a-b. Pollen grains of species of *Campomanesia* photomicrographed using an optical microscope, objective 100x – a. polar view; b. equatorial view.

Table 1 – Description of ISSR primers, with their respective numbers, names, melting temperatures, polymorphic amplifications (PA), monomorphic amplifications (MA), total amplifications (TA), and the percentage of polymorphic amplifications (%PA) used for the molecular analysis of the species *Campomanesia adamantium* (*C.ad.*), *C. pubescens* morphotype 1 (*C.p.1*), *C. pubescens* morphotype 2 (*C.p.2*) and *C. pubescens* morphotype 3 (*C.p.3*).

N°	Primer*	Tm	Experimento total				AT*			
			AP	AM	AT	% AP	<i>C.ada.</i>	<i>C.p.1</i>	<i>C.p.2</i>	<i>C.p.3</i>
1	(AC) ₉ T	46 °C	12	1	13	92,31	9	6	9	9
2	(AG) ₈ YC	46 °C	6	5	11	54,55	9	9	7	9
3	(AG) ₈ YT	42 °C	11	1	12	91,67	10	7	7	4
4	(CA) ₇ YC	42 °C	11	3	14	78,57	11	8	7	11
5	(CA) ₇ YG	42 °C	5	2	7	71,43	6	4	4	6
6	(CT) ₈ GC	46 °C	7	4	11	63,64	8	7	8	6
7	(CA) ₈ T	46 °C	12	1	13	92,31	9	6	3	7
8	(CA) ₈ G	46 °C	12	1	13	92,31	10	4	8	5
9	(CTCT) ₄ RC	42 °C	11	1	12	91,67	8	6	10	5
10	(GA) ₈ YC	42 °C	6	4	10	60,00	7	7	9	6
11	(TC) ₈ G	42 °C	8	3	11	72,73	7	3	9	10
12	GAC(CAA) ₅	42 °C	9	2	11	81,82	5	5	8	9
13	TA(CAG) ₄	42 °C	5	6	11	45,45	9	8	10	8
Total			115	34	149	77,18	99	74	90	86

*Degenerate primers: R = A, G; Y = C, T.

Application Suite - LAS EZ). All pollen grain measurements were expressed in micrometers (μm) and sizes according to the classification proposed by Barth (1965).

On average, ten pollen grains of five different flowers were analyzed for a total of 50 random pollen grains. For morphotype 3, due to the unavailability of the material, pollen grains subjected to acetolysis were used and mounted in glycerinated gelatin to create permanent slides.

The length and width of the pollen grains were measured in the polar (P) view (Fig. 1a) and equatorial (E) view (Fig. 1b), and the arithmetic mean (\bar{X}), the standard deviation ($s\bar{X}$), with the aid of the Systat software and the averages compared by the Tukey test at 5% probability, in addition to the amplitude of the size of the pollen grain, described in this order, with the range values in parentheses. The form of the pollen grain is given by the ratio of the major polar axis divided by the major equatorial axis (P/E).

Based on the morphological data of the pollen grain, multivariate analysis was performed, by principal components. Multivariate analysis was performed in the Genes program (Cruz 2013).

Molecular characterization

Molecular analyses were performed at the Genetic and Plant Improvement Laboratory of the Federal University of Jataí. Leaves in a state of intermediate maturation were collected from 10 individuals of the species *C. adamantium* and three of *C. pubescens* morphotypes to form the bulk DNA sample used in the polymerase chain reaction (PCR).

DNA was extracted using the CTAB method as previously described Carvalho *et al.* (2012). Thirteen ISSR *primers* were used for the molecular analyses (Tab. 1). ISSR amplification reactions were carried out in a final volume of 13 μL , containing 5 μL Master Mix (*Taq* DNA polymerase, PCR buffer, and dNTPs), which corresponds to 1X of the solution, 3 Mm MgCl_2 , 1 μL BSA (0.25 mg/ μL), 0.4 μM *primer*, and 20 ng DNA.

The amplifications were carried out in Veriti thermocyclers (Applied Biosystems) under the following conditions: 95 °C for 4 min (1 cycle), 94 °C for 60 s, 42–46 °C for 45 s (depending on the *primer*), 72 °C for 60 s (40 cycles), and a final extension at 72 °C for 07 min (1 cycle). Electrophoresis was carried out in a 2.5% agarose gel for three h at 70 volts.

The generated ISSR markers were converted to a binary matrix from which the genetic distances between the species studied were estimated using the Jaccard similarity coefficient complement. The matrix of genetic dissimilarity was used to estimate the main coordinates for the species *C. adamantium* and the three *C. pubescens* morphotypes, and the scores were projected in three-dimensional space. The analyses of the main coordinates were carried out using Genes (Cruz 2013).

Results and Discussion

Pollen viability and stigmatic receptivity

All flowers in anthesis around 8 a.m. had receptive stigmas and pollen grains with an average viable of 23% to *C. adamantium* and 27% were viable for the *C. pubescens* morphotypes, the which led to conclusion that there is no temporal difference in the maturation of the reproductive organs of the two species analyzed.

Franzon & Raseira (2006) also found similar values for the germination *in vitro* of *C. xanthocarpa* pollen collected from flower buds and values near 50% from flowers in anthesis cultivated through different means. In contrast, Borém (2009) found the viability of *C. pubescens* reached pollen grains to be as high as 90% in a test done using acetic carmine to identify the viable pollen grains. However, Baez *et al.* (2002) concluded that the use of dyes in testing pollen grain viability results in an overestimate compared to germination *in vitro*, as the dye is absorbed by non-aborted pollen grains which may not all be viable.

Therefore, the two species observed have receptive stigmas and fertile pollen grains concurrently. It was observed that the two species open few flowers daily for many days, which was also observed by Proença & Gibbs (1994) in *C. pubescens* increasing cross-pollination rate, both intra-species and inter-species, as it increases the reproductive period of the plant as well as the movement of pollinators for resources, favoring a possible inter-species movement of pollen since there is similarity between flowers. In addition, it reinforces that there is no temporal barrier to cross-fertilization between the two species.

Cross-pollination is a natural mechanism for increasing genetic variation, reducing the chances of inbreeding depression, and allowing the greater adaptation of the population to environmental changes (Morran *et al.* 2009). It also

promotes genetic recombination and contributes to morphological variation, given that individuals that cross-fertilize generally have different characteristics.

The formation of fruit in *C. pubescens* is statistically higher by cross pollination, as observed by Proenca & Gibbs (1994) as well as *C. phaea*, fruits have not occurred in the training manual self treatments Cordeiro (2015). Thus, we can say that these plants reproduce by cross-pollination without controlling the origin of the pollen and with the possibility of cross-fertilization between the species *C. adamantium* and *C. pubescens*, as there are no isolating barriers to cross-fertilization. This could generate individuals with characteristics intermediate to the two species, thus forming morphologically different individuals as found in the field for *C. pubescens* individuals.

Morphological characterization of pollen grains

Campomanesia adamantium

Presents small pollen grains, isolated in monads¹, with radial symmetry², isopolar³, triangular, suboblate⁴, P/E = 0.79 ± 0.13 (0.57 – 1.22), aperturate⁵, tricolpate⁶, convex apocolpate⁷ (Fig. 2a). P = 15.37 ± 2.09 (11.59–21.60), E = 11.93 ± 1.49 (10.00–17.10).

Campomanesia pubescens

morphotype 1

Presents small pollen grains, isolated in monads, with radial symmetry, isopolar, triangular, suboblate, P/E = 0.80 ± 0.14 (0.59–1.27), aperturate, tricolpate, convex apocolpate (Fig. 2b). P = 14.97 ± 2.37 (11.56–22.12), E = 11.89 ± 2.30 (09.10–19.01).

Campomanesia pubescens

morphotype 2

Presents small pollen grains, isolated in monads, with radial symmetry, isopolar, triangular,

suboblate, P/E = 0.82 ± 0.14 (0.53–1.17), aperturate, tricolpate, convex apocolpate (Fig. 2c). P = 17.96 ± 1.88 (12.88–24.42), E = 14.73 ± 2.30 (11.05–23.10).

Campomanesia pubescens

morphotype 3

Presents small pollen grains, isolated in monads, with radial symmetry, isopolar, triangular, suboblate, P/E = 0.76 ± 0.08 (0.67–0.93), aperturate, tricolpate, convex apocolpate (Fig. 2d). P = 18.61 ± 1.33 (16.29–20.86), E = 13.98 ± 1.75 (11.54–17.10).

Note that the pollen grains of the species of *Campomanesia* analyzed did not present morphological variation, and even with the means of the polar and equatorial axes varied between groups, all fitted into the classification of Barth (1965) as small in size, pollen grains with their longer axis varying between 10 and 25 μm .

These results confirm the description of Stanski (2014) and de Silva *et al.* (2010) for *Campomanesia* species, with the analyzed pollen grains showing the same morphological patterns and small dimensions, indicating little variation in pollinic morphology within this genus. But Stanski (2014), the P/E values varied from 1.01 to 1.13 μm , classified as prolate-spheroidal; these values differed from those in the present study, although they are still classified as pollen grains of small size.

Regarding the shape of the pollen grain Thornhill *et al.* (2012) found round pollen grains for *C. guazumifolia* (Cambess.) O.Berg, differing from the triangular grains found in the species studied. According to the Flora of Brazil (2020a, under construction) *C. guazumifolia* is occurring in the Northeast, Southeast and South regions, not having records of it for the state of Goiás, suggesting that there is no overlap with the species studied.

In the analysis of morphometric pollen grains, there was difference between length (Fig. 3a) and width (Fig. 3b) of the pollen grain samples analyzed in the polar view and length (Fig. 3c) and width (Fig. 3d) in the equatorial view of *C. adamantium* and the three *C. pubescens* morphotypes.

The pollen grain analyses show the formation of two groups: one comprising *C. adamantium* and morphotype 1 of *C. pubescens* and the other comprising morphotypes 2 and 3 of *C. pubescens*, with similarities existing between the two groups.

The species *C. adamantium* is found to be closer to morphotype 1 of *C. pubescens* with no difference in width observed in the polar view. Morphologically, these plants are also more similar (Tab. 2) having rounded sepals, the presence of

¹ Monads: Pollen grain that disperses as individual unit.

² Radial symmetry: pollen grain that has several planes of symmetry.

³ Isopolar: pollen grain with similar distal and proximal poles.

⁴ Suboblate: P/E ratio varying between 0,75–0,87 μm .

⁵ Aperturate: pollen grain provided with openings.

⁶ Tricolpate: pollen grain that has three openings in the form of a colporo (compound opening, formed by a colpo provided with one or more endo-openings).

⁷ Convex apocolpate: area at the poles, delimited by imaginary lines that connect the apexes of the colporos in pollen grains zonoaperturates (pores located in the equatorial region), which is convex in shape.

two bracteoles at the base of the buds that are smaller than the bud, and scamiform bracts at the base of the stems. They differ only in the presence of trichomes, absent in *C. adamantium* and small in morphotype 1 of *C. pubescens* (Amaral *et al.* 2016). Similarly, plants of morphotypes 2 and 3 of *C. pubescens* are very similar with triangular sepals, bracteoles at the base of the buds that are larger than the bud, and foliiform bracts at the base of the stems. They differ in the presence of a gland at the apex of the anther, which is found only in morphotype 3 (Amaral *et al.* 2016), suggesting the possibility of cross-fertilization between these

morphotypes and *C. adamantium* giving rise to intermediate characteristics through the formation of hybrids, as observed in sympatric palm species of the genus *Euterpe* (Wendt *et al.* 2011).

Barth & Barbosa (1972) concluded that the plant of family Myrtaceae represent a group, where the differences in pollinic morphology are limited to secondary characteristics, such as the dimensions of the grains varying between small and medium in size. In this case, where all the pollen grains analyzed are characterized as small, cross-pollination may be facilitated, whether intra- or inter-species.

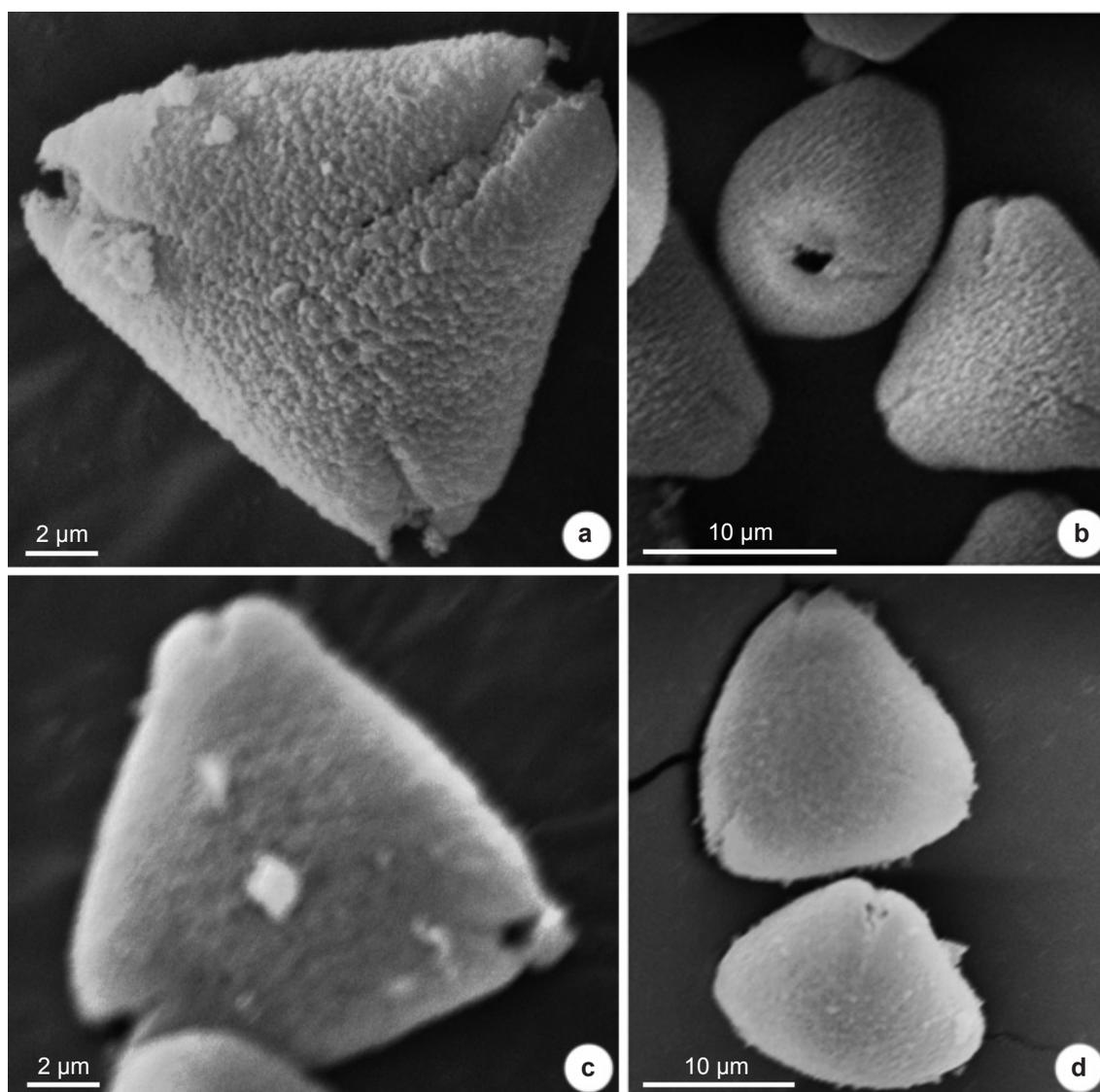


Figure 2 – a-d. Morphology of pollen grains shown by scanning electron microscope – a. *Campomanesia adamantium*; b. *C. pubescens* morphotype 1; c. *C. pubescens* morphotype 2; d. *C. pubescens* morphotype 3.

Table 2 – Morphological differences between *Campomanesia adamantium*, and morphotypes *C. pubescens* 1, *C. pubescens* 2 and *C. pubescens* 3.

Character	<i>Campomanesia adamantium</i>	<i>Campomanesia pubescens</i> 1	<i>Campomanesia pubescens</i> 2	<i>Campomanesia pubescens</i> 3
Sepals	rounded	rounded	triangular	triangular
Bracteoles	smaller than the bud	smaller than the bud	larger than the bud	larger than the bud
Bracts	scamiform	scamiform	foliform	foliform
Trichomes	absent	small	long	long
Gland at the apex of the anther	absent	absent	absent	present

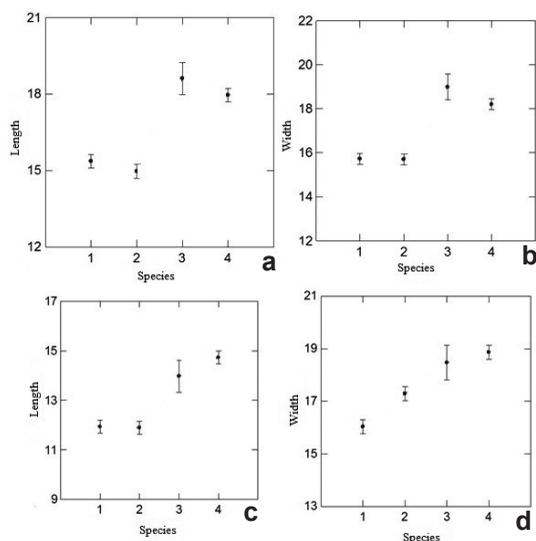


Figure 3 – a-d. Comparison of average morphometric measures of pollen grains of species of *Campomanesia* – a. length in polar view; b. width in polar view; c. length in equatorial view; d. width in equatorial view. (1 = *C. adamantium*; 2 = *C. pubescens* morphotype 1; 3 = *C. pubescens* morphotype 2; 4 = *C. pubescens* morphotype 3).

There proximity of *C. adamantium* and morphotype 1 of *C. pubescens* and of morphotypes 2 and 3 of *C. pubescens* (Fig. 4).

Based on the morphology of pollen grains, *C. adamantium* and *C. pubescens* morphotype 3 appear to be the most distantly related, and *C. pubescens* morphotypes 1 and 2 may be hybrids between them. These data are consistent with the results found by Amaral *et al.* (2016) for morphological characteristics of the plants, which

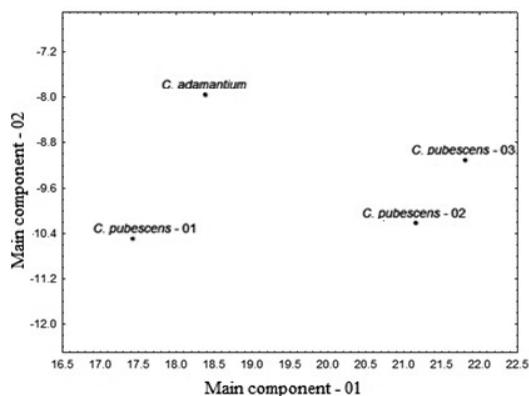


Figure 4 – Scatter plot in two dimensions of the species *Campomanesia adamantium* with the morphotypes of the species *C. pubescens*, obtained using the morphological characteristics of the pollen grain.

revealed that the species *C. adamantium* does not have the hairiness on the leaf and branch surfaces and *C. pubescens* morphotype 3 has a gland at the apex of the anther, making them more distantly related than the other morphotypes that possess intermediate morphological characteristics.

The hybridization process occurs naturally in some species, such as two species of palms, *Euterpe espirosantensis* and *Euterpe edulis*, (Wendt *et al.* 2011) have been observed hybridization events between the two species, which important morphological and reproductive characteristics, similar to observed *Campomanesia*. The authors also recognized the difficulties of identifying palm species based on only morphological characteristics, indicating the need for complementary studies to aid in the correct differentiation of the species.

Currently, the majority of species are defined based on morphological characteristics owing to a lack of information on phylogenetics and reproductive behavior. Thus, morphological discontinuities reveal the limits of a species (Wendt *et al.* 2011) and complementary studies are needed to determine where phenotypic plasticity within a species ends and where a new species begins.

Molecular characterization

The PCR reaction showed excellent quality amplification patterns, which can be observed in Figure 5. Among the *primers* (ISSR markers) used it can be observed that the *primers* 01, 08 and 09 were those that showed amplifications (tags) that can be used in the identification of the species and in the differentiation of the morphotypes. The *primer* that deserves attention is P-08 that presented an easy-to-view amplification pattern for the characterization of the three morphotypes.

Where the occurrence of monomorphic marks found by the amplification of *primer* 08 (P-08), can be used to differentiate the morphotypes of *C. pubescens*, since these marks occur individually in only one of the morphotypes. It can be observed in the P-08 the occurrence of three monomorphic marks that present approximately 1,000pb (base pairs), 700bp and 600bp, occurring respectively in the morphotypes *C. pubescens* 1, *C. pubescens* 2

and *C. pubescens* 3, and these three *C. adamantium*, showing that this species shows morphological characters occurring in the three morphotypes of *C. pubescens*, reinforcing the possibility of hybridization between them.

The thirteen ISSR *primers* used produced a total of 149 amplifications among the species studied, having a mean of 12 bands per ISSR (Tab. 1). A polymorphism level corresponding to 77.18% was observed, similar to that found in the work using another molecular technique (Assis *et al.* 2013), which obtained a 60% polymorphism rate in *Campomanesia* sp.

It was observed that the dissimilarity between species *C. adamantium* and *C. pubescens* morphotypes varied from 0.51 to 0.60, with an average of 0.55. This variation, although small, reveals differences between the molecular species.

It is worth noting that the greatest similarity was between the species *C. adamantium* and *C. pubescens* morphotype 1 and the greatest dissimilarity was between morphotypes 2 and 3 of *C. pubescens* based on the principal coordinates (Fig. 6), confirming the results found in the morphometry of pollen grains.

This genetic divergence confirms the results of (Amaral *et al.* 2016), confirming the greater proximity of *C. adamantium* with morphotype 1 *C. pubescens* and its greatest distance towards morphotypes 2 and 3 *C. pubescens*.

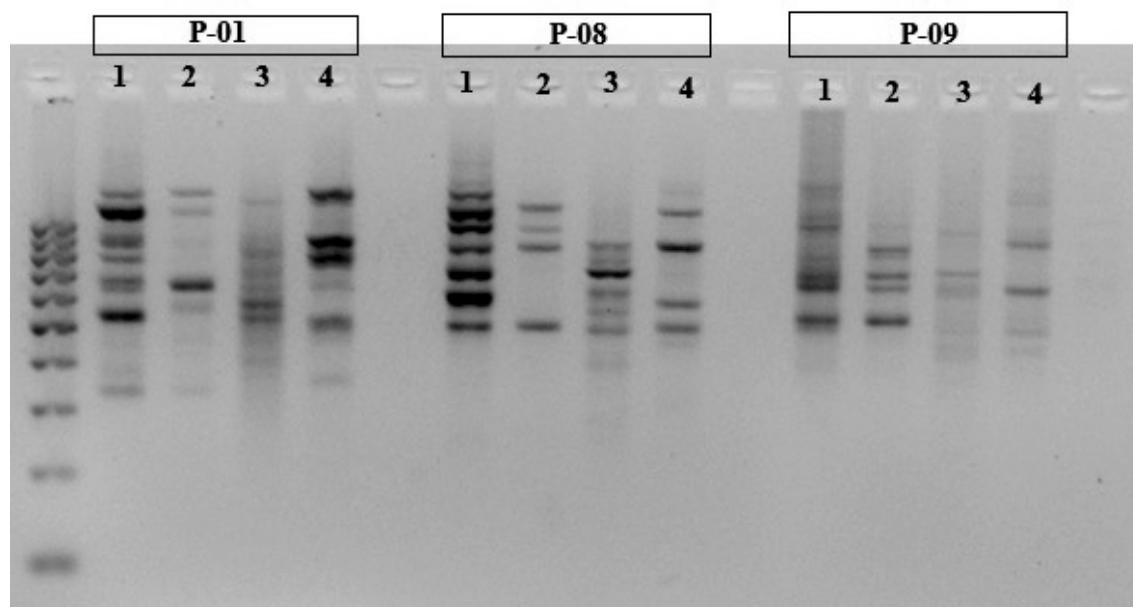


Figure 5 – Amplification pattern of three ISSR *primers* (P-01, P-08 and P-09). (1 = *C. adamantium*; 2 = *C. pubescens* morphotype 1; 3 = *C. pubescens* morphotype 2; 4 = *C. pubescens* morphotype 3).

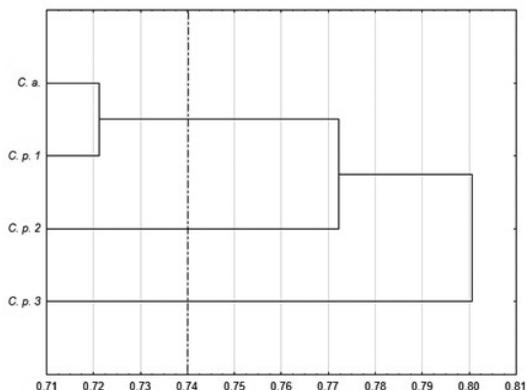


Figure 6 – Dendrogram based on the dissimilarity matrix generated from amplifications of 115 to polymorphic species *Campomanesia adamantium* and the three morphotypes *C. pubescens*. Vertical dotted line = cutting point in the dendrogram.

The existing descriptions for the species *C. pubescens* (Landrum 1986; Landrum & Kawasaki 1997) are more consistent with morphotype 2; however, the existence of a gland on the apex of the anther in morphotype 3 suggests that it is more distant from the others. Thus, it is reasonable to propose that the species *C. adamantium* and *C. pubescens* morphotype 2 are species and the other morphotypes are hybrids of them.

The absence of effective isolating barriers may lead to the formation of hybrids in nature, as observed in different species of bromeliads (Wendt *et al.* 2008) where the barriers found were too weak to prevent hybridization. For the *Campomanesia* species analyzed, no isolating reproductive barrier to prevent hybridization was found, since they are sympatric, flower simultaneously, and their flowers are reproductively viable at the same time, barrier to cross between them, suggesting cross between the species and the formation of hybrids, leading to a possible process of speciation, which deserves to be better studied.

Conclusions

Analysis of the morphology of the pollen grains in scanning electron microscopy, show no difference between *C. adamantium* species and morphotypes *C. pubescens* studied. The analyses morphometry of pollen grains indicated that all have the same standard size, with grouping of *C. adamantium* and morfotipo1 *C. pubescense* between morphotypes 2 and 3 *C. pubescens*.

The use of 13 ISSR *primers* reinforced the existing grouping among them being the *C. adamantium* species and morfotipo 3 *C. pubescens* the farthest, with morphotypes 1 and 2 presenting between them, with intermediate characters, possibly being hybrids.

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