Phenotypic variation seems not to be associated with the genetic profile in Zygopetalum (Orchidaceae): a case study of a high-elevation rocky complex

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Abstract

Hybridization associated with polyploidy studies is rare in the tropics. The genus *Zygopetalum* (Orchidaceae) was here investigated as a case study of Neotropical plants. In the Ibitipoca State Park (ISP), Southeast, Brazil, occurring in outcrops of *campos rupestres,* it was observed a wide phenotypic variation involving *Z. maculatum* and *Z. triste*; individuals with intermediate colors and forms between the species are commonly identified. Chromosomal analysis and DNA amount showed a uniform population. Regardless of the aspects related to the color and shape of floral structures, all individuals presented 2*n* = 96 chromosomes and an average of 14.05pg of DNA. Irregularities in meiosis associated with chromosome number and C value suggest the occurrence of polyploidy. The genetic distance estimated using ISSR molecular markers revealed the existence of genetic variability, not related to morphological clusters. Morphometric measurements of the flower pieces revealed that *Z. maculatum* has greater variation than *Z. triste* but without a defined circumscription. The observed variation can be explained by the polyploid and phenotypic plasticity resulting from the interaction of the genotypes with the heterogeneous environments observed in the study area. The data together contributed to the understanding of evolutionary processes and the origin of diversity in tropical regions.

Key message

Variation in phenotypic and genetic profiles in *Zygopetalum* can be explained by the polyploid and phenotypic plasticity.

Introduction

During the last decade, the contribution of polyploidy (or whole genome duplication-WGD) to the evolutionary history of vascular plants has received increasing attention (Pavarese et al., 2013; Soltis et al., 2014; Wendel et al., 2016; Toda and Okamoto et al., 2019). Genome and transcriptome-based analyses suggest that a WGD (whole-genome duplication) event occurred shortly before the radiation of the angiosperms (Jiao et al., 2011; Amborella Genome Project 2013). Polyploidy and hybridization often provide genetic and phenotypic variability upon which evolutionary forces can act and are therefore considered fundamental evolutionary processes for diversification in vascular plants, resulting in plant adaptation to changing environmental conditions (Pavarese et al., 2013; Wendel et al., 2016; Kagawa and Takimoto 2018, Padilla-Garcia et al., 2023). The 'success' of polyploids is often attributed to an increase in genetic diversity within polyploids relative to their diploid progenitors. This genetic diversity may be manifested in novelty at the biochemical, physiological, morphological, and ecological levels, giving polyploids an advantage, at least in the short term, over their diploid parents. Polyploid species are common in all floras worldwide and are particularly abundant at high latitudes and high elevations (Soltis et al., 2015; Rice et al., 2019).

The high-elevation rocky complexes (HERCs) are among the most diverse areas in the Neotropical region (Safford, 1999; Alves et al., 2014). Brazil, the largest country in South America, has approximately 46850

species representing Algae, Angiosperms, Bryophytes, Fungi, Gymnosperms, Ferns, and Lycophytes (Flora do Brasil, 2020). As a megadiverse country, six phytogeographic domains host several endemic species (Forzza et al., 2012; Oliveira et al., 2017), offering uncountable opportunities to investigate patterns of floristic diversity and origin. In high elevations of mountain ranges dispersed across eastern and central Brazil, c*ampos rupestres* comprehend a spectacular environment, known for high species diversity and remarkable plant endemism (Silveira et al., 2016; Mucina et al., 2017; Morellato and Silveira 2018). In southern Brazil, HERCs are highly heterogeneous formations, where climate, geology (i.e. topography, soil depth, fertility, and drainage), and the surrounding vegetation define distinct floristic compositions (Safford, 1999; Benites et al., 2007; Alves and Kolbek 2010). Ibitipoca State Park (ISP) is located in southeastern Minas Gerais, in Bias Fortes, Santa Rita de Ibitipoca and Lima Duarte municipalities (21º40'-21º44'S; 43º52'-43º55'W). With different types of vegetation, HERC is the predominant scenario of ISP where 118 taxa of Orchidaceae were described (Menini Neto et al., 2007).

Orchidaceae is currently the largest family and one of the ecologically and morphologically most diverse families of flowering plants (Dressler, 1981; see Gustafsson et al., 2010) with several species occurring in HERCs (Borba et al., 2007). Polyploidy was also reported for different genera of the family (Felix and Guerra 2000; 2005; 2010). Ever since the first classification of Orchidaceae (Swartz 1800), systematics have sought to reveal the relationships within this large plant family (Freudenstein and Chase 2015) that show several examples of overlapped phenotypes (e.g. Leal et al., 2016). Morphological variation may arise not only from selection but also because of the emergence of polyploidy (Stebbins, 1974; Soltis et al., 2015). Species of *Zygopetalum maculatum* "complex" (Kunth) Garay (Epidendroideae, Zygopetalinae) represent one of the taxonomically more complex groups among Brazilian orchids. The species within this complex present terrestrial habit, occurring in sandy soils. Taxonomic circumscription is difficult, mainly due to overlapping in diagnostic characters related to leaf and flower morphology (Hoehne, 1953).

A natural population of *Zygopetalum maculatum is* "complex", including *Z. triste* Barb. Rodr. and *Z. maculatum* has been described in the ISP (Menini Neto et al., 2007). The individuals show morphological characters representing a gradient of variation; in only a few kilometers (~ 4420m) of trail on the west side of the park, it is possible to observe individuals with different flower shapes and colors.

Although the expression of morphological traits in hybrids is unpredictable, often resulting in a mosaic of parental, intermediate, and new characters (Rieseberg and Ellstrand, 1993), many natural hybrids in Orchidaceae have been described based only on the observation of intermediate phenotypes between putative parental species (e.g. Borba and Semir, 1998; Pupulin 2007; see Leal et al., 2016; Solano et al., 2019; Scopece et al., 2020).

Chromosome number and ploidal level associated with molecular markers and morphological characters constitute suitable tools for understanding the dynamic of natural processes such as hybridization, speciation, adaptation, and plasticity (e.g. Yan et al., 2016). They have been extensively

used to elucidate the genetic relationships within and between plant species including Orchidaceae (e.g. Paverese et al., 2013; Leal et al., 2016; Chao et al., 2018; Trávníček et al., 2019, Zhang et al., 2023).

In this study, we investigate the genetic and morphological variation of *Zygopetalum maculatum* "complex" over a gradient stream in a typical southern Brazilian HERC in Ibitipoca State Park (ISP). Genome size, karyotype analysis, floral morphometry, and ISSR markers were used to understand the phenotypic profile of *Zygopetalum* in the study area. More broadly, we intend to contribute to the knowledge about genotypic launch on the Z*ygopetalum* complex helping future research involving phenotypic variation in polyploid plants occurring in tropical altitude fields.

Material and methods

Study system

Zygopetalum maculatum and *Z. triste* are terricolous or epilithic orchids found in the Atlantic forest and Cerrado, predominantly occurring in outcrops of *campos rupestres* at 1000m.a.s.l. *Zygopetalum maculatum* (Fig. 1A, B) has a broader distribution occurring across the South, Southeast, and Northeast (only Bahia) of Brazil regions while *Z. triste* (Fig. 1C, D) was observed only in the Southeast Region. Both species share some vegetative and reproductive characteristics but are often distinguished by the general length of leaves and inflorescences (longer in *Z. maculatum*), size, morphology, and color of the perianth pieces. *Z. maculatum* often has bigger flowers; margins of sepals and petals are often plane and greenish with wide dark brown spots; lip is widely reniform, 2(-4)-lobed, white, and densely covered with dark purple veins (Fig. 1A, B). *Z. triste* has smaller flowers; margins of sepals and petals are often revolute, dark or pale brown to dark reddish-brown and green base; the lip is often narrower, widely obovate, sometimes 2(-4) lobed, entirely lilac to pink, sometimes with darker veins (Fig. 1C, D).

In addition to typical *Z. triste* and *Z. maculatum*, it is possible to observe individuals with intermediate characters. Some of them showed flowers with color patterns more similar to *Z. maculatum* and shapes more similar to *Z. triste*. These individuals were classified as *Z.* aff. *maculatum* (Fig. 1E, F). Others showed color patterns more similar to *Z. triste* and morphology more similar to *Z. maculatum* they were classified as *Z.* aff. *triste* (Fig. 1G, H).

Study area and sampling

The Ibitipoca State Park (ISP), located in the Southeast of Minas Gerais, is part of the Mantiqueira Complex (21º 40'- 21º 44' S; 43º 52' – 43º 55' W) (CETEC, 1983). ISP features high metamorphic and Quartzite Mountains immersed in a mosaic of vegetation that includes forests, highland fields, and mainly rock fields (Oliveira-Filho et al., 2013; Rocha 2013). Starting at 1000m, the highest point at ISP reaches 1784m. A cold and dry winter followed by a rainy summer characterize the climate of the study area (Rocha 2013). The *Zygopetalum* specimens were collected over a trail 4420m long and a gradient stream of 584m (Fig. 2).

Genome size estimation, karyotype analysis, floral morphometry, and genetic diversity were accessed for up to 89 individuals. We collected specimens at least 10m apart to avoid clonal plants due to sympodial fragmentation and apomixis (Arnaud-Haond 2007; Campacci et al., 2017). Voucher of the material was deposited, according to the usual techniques, in the Leopoldo Krieger Herbarium (CESJ-UFJF) under identification 66324, collector C. Nardy et al., (2017).

Flower morphometry

Three fresh flowers of 22 individuals representing the flower morphological variation (Menini Neto et al., 2007) were collected and fixed in 70% alcohol. The material was dissected, mounted on cardboard paper, and scanned on a flatbed scanner. The measurements of the floral pieces were taken from the scanned sheets using the program Image J (Rasband, 1997). The following floral variables were considered: dorsal sepal; left lateral sepal; right lateral sepal; left lateral petal; right lateral petal; lip and spine (S1). The multivariate analyses were performed using PAST software v.2.04 (Hammer 2008) according to Everitt (1978). Principal Component Analysis (PCA) aimed to show global morphological variation and relationships among individuals with distinct floral characteristics.

Genome size estimation

To estimate the genome size of 86 individuals, we macerated approximately 25 mg of leaf tissue with the same amount of the internal reference standard *Vicia faba* var equina cv Inovec (2C = 26.90 pg) (according to Doležel and Sgorbati, 1992). We macerated the leaves on a Petri dish containing 1 mL of cold LB01 buffer using a scalpel blade to release the nuclei into the suspension (Doležel et al., 1989). For nuclei staining, we added 25 µL of a 1 mg mL^{-1} solution of propidium iodide (PI, Sigma, USA) followed by 5 µL of RNase (100 µg mL^{-1}). The estimation of genome sizes was done using the BD FACS Canto™II (Becton, Dickinson and Company, USA) flow cytometer and the histograms were generated using Cell Quest software. The statistical analysis of histograms was performed using Flowing Software 2.5.1 (http://www.flowingsoftware.com). We considered three samples from each individual for genome size estimation and used analyses of variance (ANOVA) to describe variation in DNA content.

Chromosome counting and morphometry

Root meristems representative of each phenotype (Menini Neto et al., 2007), totaling 20 individuals were treated with 0.002M 8-hydroxyquinoline (Sigma®, USA) at 9 ^{o}C for 24h, fixed in Carnoy solution (ethanol: acetic acid, 3:1) and stored in a refrigerator, for at least 24h. The material was digested in enzymatic solution [2% (w/v) Onozuka R-10 (Serva®)/20% (v/v) cellulase pectinase (Sigma®)] at 37 ^{o}C for 7h. The slides were prepared according to Carvalho and Saraiva (1993) and stained with Giemsa 5% (Merck®). At least 20 cells of each individual were evaluated under an Olympus BX51 light microscope coupled to a scanning and image analysis system (Image Pro-Plus 4.5 Media Cybernetics™). Chromosome length, short and long arms, ratio between chromosome arms, and the index of karyotype asymmetry were determined according to Watanabe et al., (1999). The chromosomes were classified according to Levan et al., (1964).

Meiotic behavior

Flower buds of nine individuals were collected in the morning, fixed in Carnoy (ethanol/glacial acetic acid – 3:1, v/v), and stored at -20 ^{o}C for a minimum period of 24h. The slides were obtained according to Viccini et al., (2006). The analysis was performed under an optical microscope (Olympus BX-51) with a 100x objective. 100 cells/phase of each individual of all phases were evaluated, except for diakinesis for which 50 cells were evaluated. Representative pictures of the abnormalities were digitized through an Olympus DP72 camera coupled to the microscope and registered using Image Pro-Plus 4.5 software (Media Cybernetics).

DNA extraction, ISSR amplification, and data analysis

Total DNA was extracted from fresh leaves using the Genomic DNA extraction kit NucleoSpin® Plant II (Macherey-Nagel), following all manufacturer's specifications. After the extraction, the DNAs were solubilized and quantified using a Nanodrop Spectrophotometer (ThermoFisher Scientific, Inc., Wilmington, DE, USA), diluted to 25 ng µl, and kept at -20°C for subsequent use.

Eight ISSR primers were selected (Supplemental File 2). PCR was carried out in a DNA Thermal Cycler Mastercycler® (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) following the specifications given in the Supplementary Material (Supplemental File 2). The amplification products were identified on 2% agarose gels. The images of the gels were captured in a UVP GelDoc-It Imaging System transilluminator (Upland, CA, USA) and analyzed using the Vision Works LS program. The molecular weight of the fragments was estimated using 5µl of the 1kb Plus DNA Ladder marker (Invitrogen™).

The ISSR loci were visually encoded as the presence (1) or absence (0) of equal-sized bands, and a binary matrix was constructed. All bands of the same molecular size obtained with the same primer were considered homologous (Williams et al., 1993). The overall number of alleles, the heterozygosity, and the polymorphism information content (PIC) were estimated using Genes software (Cruz 2016). The heterozygosity and PIC were calculated according to Weir (1990) and Anderson et al., (1993), respectively. Based on the binary matrix, the Jaccard similarity coefficient between each pair of individuals was calculated. The dendrogram and principal coordinate analysis (PCA) were estimated using NTSYS software (Rohlf 2000). The bootstrap values were estimated by PAST software (Hammer et al., 2008).

The genetic structure within and among populations was investigated using Bayesian inference clustering as implemented in Structure 2.3.4 (http://pritchardlab.stanford.edu/structure.html; Pritchard et al., 2000); the final plots were produced using Structure Plots v2.0 (Ramasamy et al., 2014). We analyzed 86 accessions as dominant data, coded as presence/absence data, using the admixture model with uncorrelated allele frequencies (Stift et al., 2019). The Monte Carlo Markov Chain was run for 100,000 steps, following a burn-in of 10,000 steps. Simulations were performed for the number of groups (*K*) varying from 1 to 10. We used Structure Harvester (Earl and Von Holdt 2012) to calculate *ΔKm* (Evanno et al., 2005).

Results

Flower morphometry

An attempt of grouping individuals according to the visual classification of the flowers (color and shape) and respective measurements of perianth parts (sepal and petal), revealed that an association between flower morphology and taxa delimitation was not clear. The characters revealed a wider variation in *Z. maculatum* and *Z.* aff. *maculatum* making it difficult to establish a specific group. Those individuals were distributed over the other three quadrants. Taking all morphometric measurements together two principal components from PCA explained 87.6% of the total variance. However, it was possible to observe that, except for one individual, all *Z. triste* and *Z.* aff. *triste* were clustered together, showing that those individuals share similar phenotypic traits occupying mainly one quadrant of PCA (Fig. 3).

Genome size estimation

Overall, the DNA content ranged from 13.91 to 14.32 pg (Fig. 4A). It was not possible to observe variations in the C value comparing all samples investigated. Even considering all *Zygopetalum* "types" observed (Fig. 1), it was not possible to detect a significant variation among the samples (Supplemental File 3).

Cytogenetic analysis

A consistent chromosome number was observed ($2n = 96$) in all individuals analyzed (Fig. 4B), corroborating the genomic uniformity of the population indicated by flow cytometry. The same number of 5S and 45S marks were also observed using FISH (data not shown). An attempt at karyotype was made by taking $x_2 = 24$ as a basic number (Félix and Guerra 2010) (Fig. 4C).

Chromosome morphometry showed symmetric karyotypes (A = 0.19 and Ai = 4.05) with most of them being metacentric and submetacentric. The karyotype is composed of 18 metacentric chromosomes, 5 submetacentric, and 1 telocentric chromosome (Table 1).

Table 1
Average of chromosome morphometric data of *Zygopetalum* (2*n* = 96) from Ibitipoca State Park, Southeast, Brazil

Chrome	*sa*	*la*	Total length	r	*ci*	Classification
1	2.99	3.81	6.80	1.27	43.97	*M*
2	2.96	3.37	6.33	1.14	46.76	*M*
3	2.59	2.66	5.25	1.03	49.33	*M*
4	2.44	3.88	6.32	1.59	38.61	*M*
5	2.41	2.61	5.02	1.08	48.01	*M*
6	2.32	2.75	5.07	1.19	45.76	*M*
7	2.31	3.31	5.62	1.43	41.10	*M*
8	2.23	2.80	5.03	1.26	44.33	*M*
9	2.26	2.55	4.81	1.13	46.99	*M*
10	2.17	2.96	5.13	1.36	42.30	*M*
11	1.95	2.53	4.48	1.30	43.53	*M*
12	1.94	2.19	4.13	1.13	46.97	*M*
13	1.81	1.92	3.73	1.06	48.53	*M*
14	1.75	4.47	6.22	2.55	28.14	*Sm*
15	1.74	2.23	3.97	1.28	43.83	*M*
16	1.68	2.83	4.51	1.68	37.25	*M*
17	1.54	2.55	4.09	1.66	37.65	*M*
18	1.37	1.71	3.08	1.25	44.48	*M*
19	1.22	1.61	2.83	1.32	43.11	*M*
20	0.97	1.87	2.84	1.93	34.15	*Sm*
21	0.96	2.15	3.11	2.24	30.87	*Sm*
22	0.95	1.64	2.59	1.73	36.68	*Sm*
23	0.90	2.66	3.56	2.96	25.28	*Sm*
24	0.01	2.92	2.93	292.00	0.34	*T*

Chrom = Chromosome; *sa* = short arm; *la* = long arm; *ci* = centromeric index; *m* = metacentric; *sm* = submetacentric; *t* = telocentric

In total, around 750 meiocytes for each individual in different stages were analyzed representing all morphotypes previously mentioned (Menini Neto et al., 2007). All plants showed similar behavior with high indexes of abnormalities (Table 2). Abnormalities were observed in both meiosis I and meiosis II (Fig. 5).

Table 2
Average of abnormalities (%) in different phases of meiosis of *Zygopetalum* individuals from Ibitipoca State Park (ISP), Southeast, Brazil

Stage cell	Normal cells	Abnormal cells	Total	Abnormalities (%)
Prophase I (diakinesis)	0	50	50	100
Metaphase I	30	70	100	70
Anaphase I + Telophase I	122	78	200	39
Metaphase II	36	64	100	64
Anaphase II + Telophase II	126	74	200	37
Tetrad	47	53	100	53
Total	362	388	750	51.73

The most frequent alterations were those related to problems in mitotic spindle and adherent DNA. All individuals analyzed showed irregularities such as late segregation, spindle anomalies, adherent chromosomes, chromosomal bridges, asynchrony in the second division of meiosis, and micronuclei formation.

During cell division, we observed diakinesis with sticky chromosomes (Fig. 5A), delayed chromosomes (Fig. 5B-D), multipolarity (Fig. 5E), chromosomal bridge (Fig. 5F); metaphase II in T (Fig. 5G) and micronucleus (Fig. 5H). So many irregularities resulted in considerable indexes of abnormal tetrads, such as triads (Fig. 5I).

Molecular analysis

The eight selected primers produced a total of 58 bands, corresponding, on average, to 7.25 bands per primer, ranging in size from 200 to 950 bp. All 58 loci obtained were considered polymorphic.

The PIC mean value was 0.347 per primer and the heterozygosity mean value was 0.447 per primer (See Table 3 for a summary of ISSR primers). Most of the ISSR primers amplified multiple bands indicating relatively high heterozygosity and diversity.

Table 3
Genetic diversity of ISSR markers evaluated in *Zygopetalum* samples from Ibitipoca State Park, Southeast (ISP), Brazil

		Total		***Z. maculatum***		***Z. triste***	
Primer	Number of bands	H	PIC	H	PIC	H	PIC
UBC-808	5	0.439	0.342	0.412	0.326	0.419	0.331
UBC-818	5	0.439	0.342	0.440	0.342	0.459	0.353
UBC-823	6	0.471	0.360	0.468	0.358	0.447	0.347
UBC-835	11	0.461	0.354	0.478	0.364	0.473	0.361
UBC-842	14	0.444	0.345	0.440	0.342	0.438	0.341
UBC-847	6	0.457	0.353	0.393	0.314	0.426	0.334
UBC-851	4	0.425	0.334	0.443	0.344	0.352	0.290
UBC-857	7	0.441	0.343	0.421	0.331	0.382	0.307
Average	7.25	0.447	0.347	0.437	0.340	0.424	0.333
Total	58						
H = Observed Heterozigozity; *PIC* = Polymorphic Information Coefficient							

Primers UBC-842 and UBC-835 showed the highest polymorphism (14 and 11 respectively). The UBC-823 primer showed the highest heterozygosity and PIC values (0.471 and 0.360, respectively), while UBC-851 was the least informative primer, presenting only four bands and the lowest heterozygosity and PIC values (0.425 and 0.334, respectively).

The distribution of genotypes obtained by PCA showed a random distribution among the taxa (Fig. 6A). The dendrogram showing genetic distances based on the Jaccard coefficient of similarity showed some *Z. triste* individuals together (e.g. 132 and 144, 130 and 156, 200, and 201). However, most of the branches showed no association between morphological and molecular patterns. In general, low support was observed for the branches two of them higher than 50% and four higher than 75% (Fig. 6B). The Structure analysis using the *ΔKm* (Evanno et al., 2005) method indicated that the best number of groups is *K* = 2. This analysis revealed similar genetic structure among the accessions once the “morphotypes” were randomly distributed over the two groups (Fig. 6C).

Discussion

The occurrence of a wide morphological variation including two *Zygopetalum* species across an altitudinal gradient of 500m in the Ibitipoca State Park (ISP), Southeast, Brazil, represents a particular case to explore the relationship between phenotypic and genotypic variations in high-elevation rocky

complexes (HERCs). By accessing the genetic profile, the geographical distribution, and the morphological description we might be able to add new insights into the comprehension of tropical biodiversity, in particular, in South American orchids.

Here, for the first time, we describe the chromosome number for *Z. triste* ($2n = 96$), which agrees with the highest chromosome number reported for *Zygopetalum* so far (Felix and Guerra 2000; Gomes et al., 2018). Two different chromosome numbers ($2n = 48$ and $2n = 96$) were previously reported for *Z. maculatum* (Tanaka and Kamemoto, 1984; Brandham, 1999; Felix and Guerra, 2000) and no reports for *Z. triste* have been found so far. The divergence of previously reported chromosome numbers ($2n = 48$ and $2n = 96$) may be explained by the morphological similarity among some *Zygopetalum* species (Hoehne, 1953) that led to specimen misidentification.

The first DNA content here estimated for *Zygopetalum maculatum* "complex" from ISP revealed that all 86 individuals analyzed showed the same 2C value (2C = 14.05 pg) which agrees with another recent estimation for the genus (Gomes et al., 2018). The value is nearest to the smaller value (1.20 pg – *Oncidium leucochilum* Lindley) so far estimated for orchids than to the highest one (77.65 pg - *Cypripedium henryi* Rolfe) (Bennett and Leitch 2012). Many authors proposed $x = 5$, 6, and 9 as the inferior limit of the basic chromosome number in angiosperms (see Ehrendorfer et al., 1968; Stebbins, 1971; Raven, 1975; Grant, 1981). The basic chromosome number was estimated for orchids as $x = 7$. For the subtribe Zygopetalinae, where *Zygopetalum* is included, a putative basic number $x_2 = 24$ was proposed (Félix and Guerra 2000; 2005), suggesting that the individuals investigated from ISP are likely tetraploids.

The polyploidy in those individuals is reinforced by the meiotic behavior. A high number of abnormalities was identified in all individuals analyzed, suggesting an absence of disomic pairing, a typical scenario in autopolyploids that possess multiple sets of homologous chromosomes, resulting in a polysomic inheritance (Spoelhof et al., 2017). Autopolyploids may be affected by multivalent pairing, which contributes to the production of unviable aneuploid gametes and reduced fertility (Ramsey and Schemske 2002). It seems that this is the case for *Z. maculatum* "complex" taxa occurring at ISP. Around 82% of pollen grains were unviable (data not shown) suggesting that *Zygopetalum* might represent an autopolyploid complex. In addition, individuals with $2n = 48$ and 72 were recently described (Gomes et al., 2018) for the same genus, supporting the hypothesis of a polyploid series in *Zygopetalum*.

Polyploidization may cause dosage effects, novel allele combinations, and epigenetic patterns, rapidly changing the gene expression (Comai 2005; Buggs et al., 2014), which may result in different phenotypes or increased phenotypic variation (Ramsey and Schemske 2002; Adams and Wendel 2005; Chen 2007; Soltis et al., 2014; Soltis and Soltis 2016).

Over the trail at ISP, two intermediate phenotypes were observed (Menini Neto, personal communication) between two species, *Z. maculatum* and *Z. triste*. Two hypotheses could be addressed to explain the scenario: 1) the intermediate phenotypes are a consequence of hybridization between two *Zygopetalum* species, or 2) they are the expression of phenotypic plasticity.

Considering the two circumscribed taxa within the *Z. maculatum* "complex" (*Z. triste* and *Z. maculatum*) and the two intermediate forms (*Z.* aff *triste* and *Z.* aff *maculatum*), floral morphometry did not reveal any distinct grouping. However, *Z triste* individuals seem to be more similar to each other than the other individuals investigated from *Z. maculatum* and the two intermediate forms. In Orchidaceae, hybrids occasionally present intermediate morphological traits (e.g. Caputo et al., 1997; Nielsen 2000; Azevedo et al., 2006; Moccia et al., 2007). Intermediate morphological characters have been used for hybrid recognition, taking into account that these traits are supposedly inherent as polygenic characteristics with simple additive effects (Rieseberg et al., 2007; Kiær et al., 2007; Conceição et al., 2008). On the other hand, this classification can be also useless (Wallace 2006; Stahlberg and Hedren 2009; De Hert et al., 2011; López-Caamal and Tovar-Shachez 2014; Leal et al., 2016; Goulet et al., 2017) probably due to the unpredictability of gene expression in hybrids (Rieseberg and Ellstrand, 1993).

The combination of morphological and molecular data has been used in many cases to investigate new lineages, hybrid zones, and phenotypic variation (e.g. Marques et al., 2014; Pinheiro et al., 2015; Leal et al., 2016). Morphological and molecular data might converge, supporting the same result or not (Paverese et al., 2013; Leal et al., 2016; Pavani et 2018; Tasanakas et al., 2018; Wu et al., 2019). In our case, ISSR markers did not reveal a specific genetic profile associated with each of the taxa in the *Z. maculatum* "complex" or either intermediate forms, suggesting that all individuals investigated share the same genetic background. Even if only the two species are considered, it seems that we have only one population. The structure plot shows that accessions are admixed with no well-delimited group based on the genetic structure, in the same way that low levels of genetic diversity and similar genetic structure have been reported for other orchids, such as *Cattleya brevipedunculata, C. coccinea*, *C. labiatae*, and species of *Epidendrum* (Pinheiro et al., 2012; Pinheiro et al., 2014; Rodrigues et al., 2015; Leal et al., 2016). According to Li and Ge (2006), those results might be related to habitat fragmentation.

Taking all data into consideration (chromosome number, DNA content, genetic diversity, morphological analysis), there is no evidence to suggest intermediate individuals (here called *Z.* aff. *maculatum* and *Z.* aff. *triste*) constitute hybrids between *Z. maculatum* and *Z. triste*. It is more likely that phenotypic variation in the sympatry of *Z. maculatum* and *Z. triste* occurring at ISP is related to phenotypic plasticity within *Z. maculatum* “complex” gene pool.

At ISP, *Z. triste* is mainly observed at the top of the trail but no population structure was observed according to our data. Considering that population structure is strongly influenced by mating systems, bee pollination, and wind seed dispersal, long-lived plants with mating systems that favor outcrossing and long-distance dispersal tend to be less structured (Hamrick et al., 1991; Nybom 2004). In addition, we cannot discard the occurrence of gene flow among the individuals here investigated and also that apomixis might contribute to this genetic scenario (Campacci et al., 2017). The same chromosome number, DNA content, and genetic profile observed for both taxa in *Z. maculatum* "complex" support the hypothesis that morphological variation can be explained by phenotypic plasticity. Although the scenario among *Z. triste* and *Z. maculatum* specimens from ISP may be not completely resolved, we cannot

discard the situation where the ISP population is under a genetic homogenization process giving rise, in the future, to a distinct evolutionarily lineage.

At ISP, different processes should be responsible for the wide morphological variation, especially assuming a polyploid origin for the analyzed specimens. The wide morphological variation could be associated with phenotypic plasticity in response to environmental parameters, such as temperature, soil, humidity, and light. *Campos rupestres* present very low levels of essential nutrients such as phosphorus and nitrogen, they are well known to host heterogeneous environments and microhabitats that favor phenotypic plasticity leading the individuals to express a wide range of phenotypes, especially if they do not reduce fitness (Forsman 2014, Fernandes 2016, Mucina et al., 2018). Although we did not annotate climatic variables along the trail where samples were collected, natural light, moisture fluctuations, and temperature variations were evident. At the base of the trial, small trees and shrubs characterized forest vegetation, while at the top, rocky outcrops and grasslands were predominant.

On the other hand, intermediate phenotypes occurred across different microenvironments and were not related to any specific location. Differences in the environment strongly impact the evolution and differentiation of one population. Heterogeneous environments strongly impact the evolution and differentiation of a population, promoting local adaptation to microhabitats and changes in phenotype (Anderson et al., 2014; Baythavong 2011; Henneresse et al., 2018).

Orchidaceae floral characters are commonly used to identify different species, but variation in floral characters might be also due to environmental conditions (Geber and Eckhart 2005; Lambrecht and Dawson 2007) and therefore make problematic the delimitation and description of plant species (Paverese et al., 2013). Phenotypic plasticity occurs when variations in the environment induce only phenotypic variation, without significantly affecting the genome (Gratani 2014). In our samples, we did not identify clones but the genetic similarity among the individuals and the wide variation in floral morphology support the phenotypic plasticity hypothesis. Although more studies are needed, chromosome data including the number of 5S and 45S (data not shown) together with chromosome number and DNA content suggest an autopolyploid series for *Zygopetalum*. Autopolyploids fulfill the requirements of different species concepts (Soltis et al., 2007) and are frequently relegated as cytotypes due to their similar morphology. Cryptic polyploids are likely autopolyploids that will probably be challenging to recognize by morphological traits alone, but may fulfill the requirements of other species concepts (Barker et al., 2016).

The results of the genetic analyses demonstrated low genetic variation among individuals that probably constitute a single population resulting in either of hybridization or phenotypic plasticity. Little is known about the hybridization of wild species (Ramsey and Schemske, 1998) and few reports have been done about the phenotypic plasticity of orchids (e.g. Ackerman et al., 2011; Moreira et al., 2013; Leal et al., 2016) probably due to the lack of environmental/genetic control (Viccini et al., 2014) in natural populations.

This is the first report for *Zygopetalum* showing this variation. Clausen et al., (1945) emphasized that the morphology of living organisms is controlled by both genetic and ecologic factors; morphology was therefore a good indicator of the “more basic genetic-physiologic” characters of species. However, such a definition is sometimes not easy to identify mainly when polyploids are involved (Soltis et al., 2015). The *Zygopetalum* population of ISP represents a real example of the complex evolutionary process that drives species richness and biodiversity and more studies should be done to better understand the *Zygopetalum* “complex” diversification.

Declarations

Author contributions

S.S.L.G., L.M.N., and L.F.V. designed the research; S.S.L.G., J.M.L.L., E.M.M., and E.G.C. performed the research with the support of A.L.S.A., M.A.M., J.M.S.C., L.M.N., and L.F.V.. S.S.L.G., J.M.L.L., E.M.M., A.L.S.A., M.A.M., J.M.S.C., L.M.N., and L.F.V. analyzed the data; S.S.L.G., J.M.L.L., E.M.M., and L.F.V. wrote the article with input from all the other authors.

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Data availability

Datasets generated during this research are available through the corresponding author upon reasonable request.

Competing Interests

To the knowledge of the authors, no competing interest, financial or non-financial, is issued over this research.

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Figures

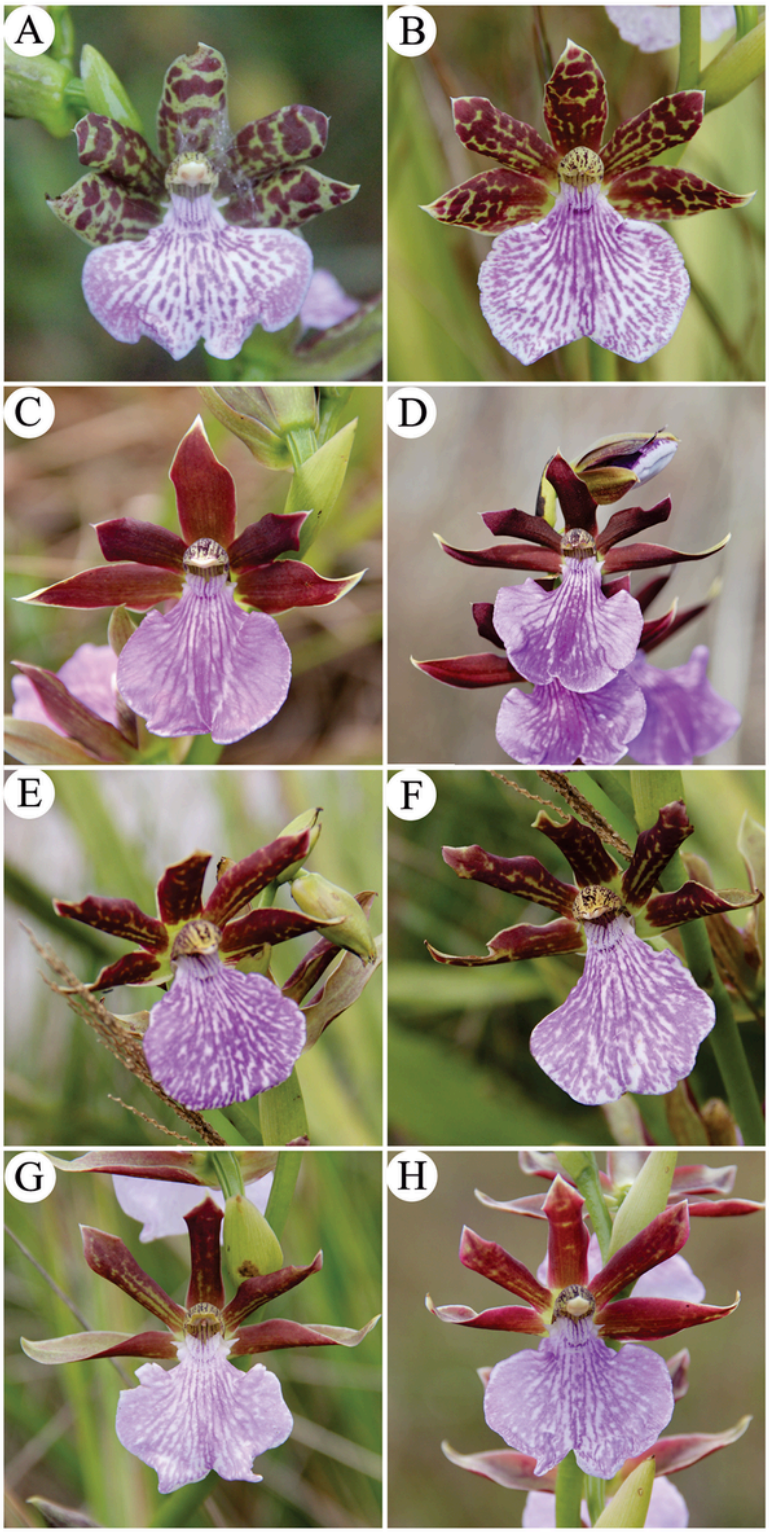

Figure 1

Morphological variation of flowers in individuals of *Zygopetalum* population from Ibitipoca State Park (ISP), Southeast, Brazil. Differences are observed in the coloring of floral pieces, distribution of macules, shape, and size of the flower. (**A**, **B**) *Zygopetalum maculatum*; (**C**, **D**) *Z. triste*; (**E**, **F**) *Z.* aff. *maculatum*; (**G**, **H**) *Z.* aff. *triste*

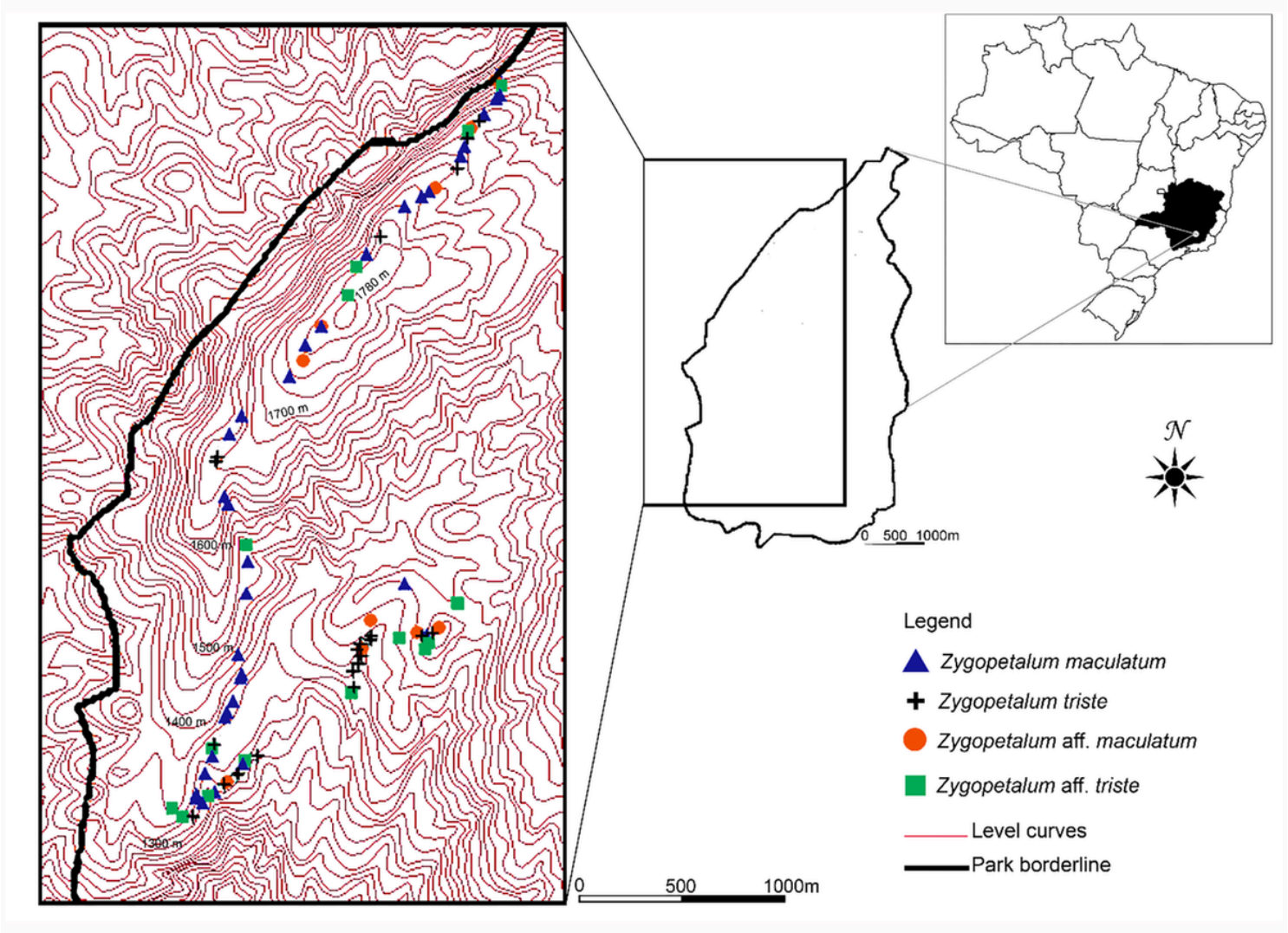

Figure 2

Collection area of *Zygopetalum*individuals from Ibitipoca State Park, Minas Gerais State, Southeast, Brazil

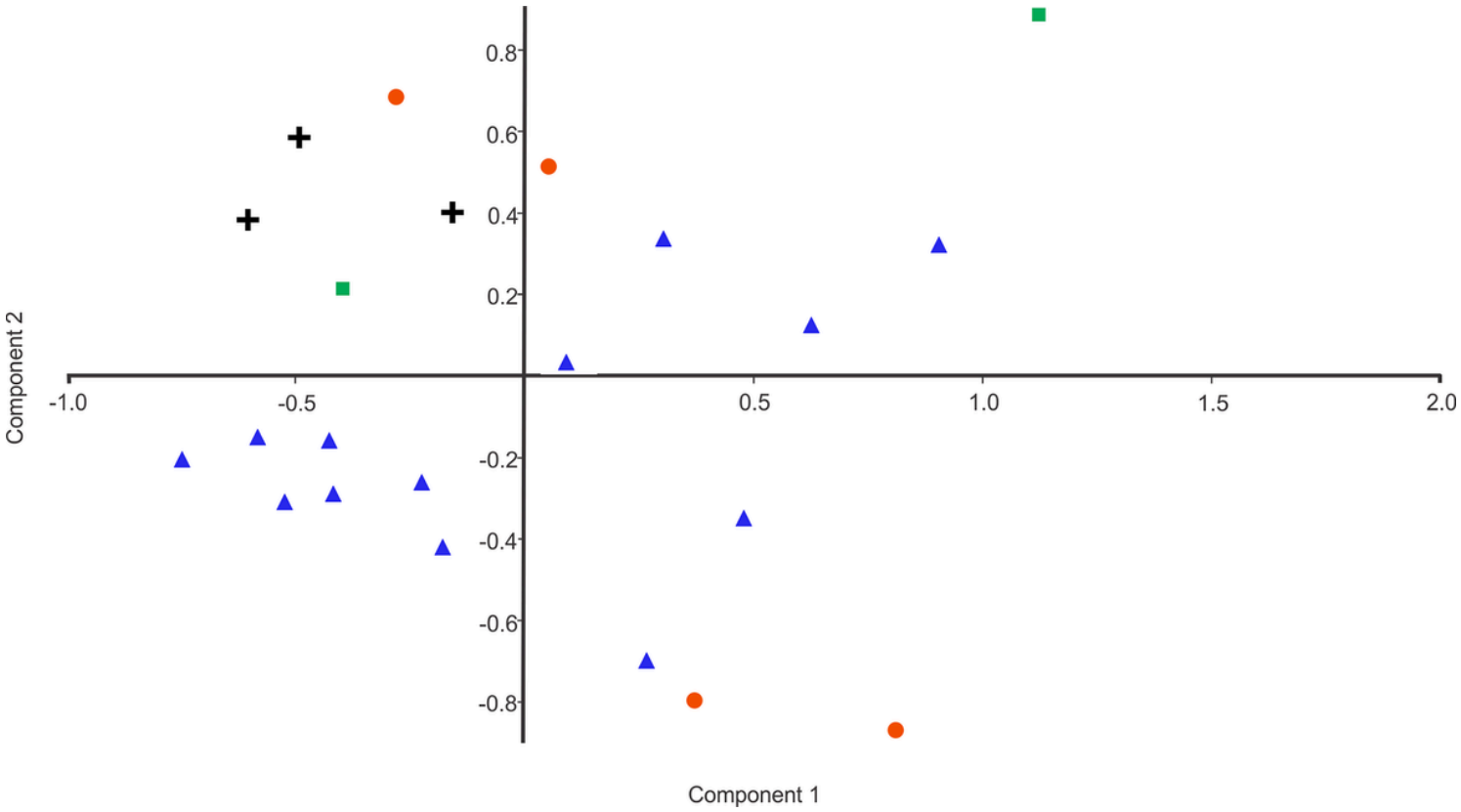

Figure 3

Principal component analysis of floral pieces of *Zygopetalum* from Ibitipoca State Park (ISP), Minas Gerais State, Southeast, Brazil

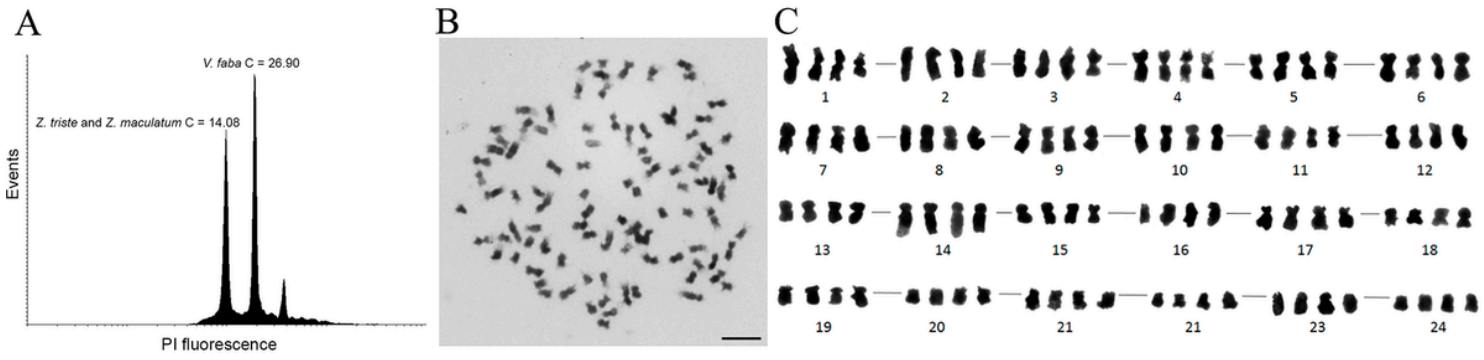

Figure 4

Representative histogram of DNA content and metaphase (2*n* = 96) of *Zygopetalum* (Orchidaceae) from Ibitipoca State Park (ISP), Southeast, Brazil. (**A**) - DNA amount of *Zygopetalum* and *V. faba* (internal reference standard); (**B**) mitotic metaphase; (**c**) attempt of karyotype considering the individuals as tetraploids. Bar = 10 µm

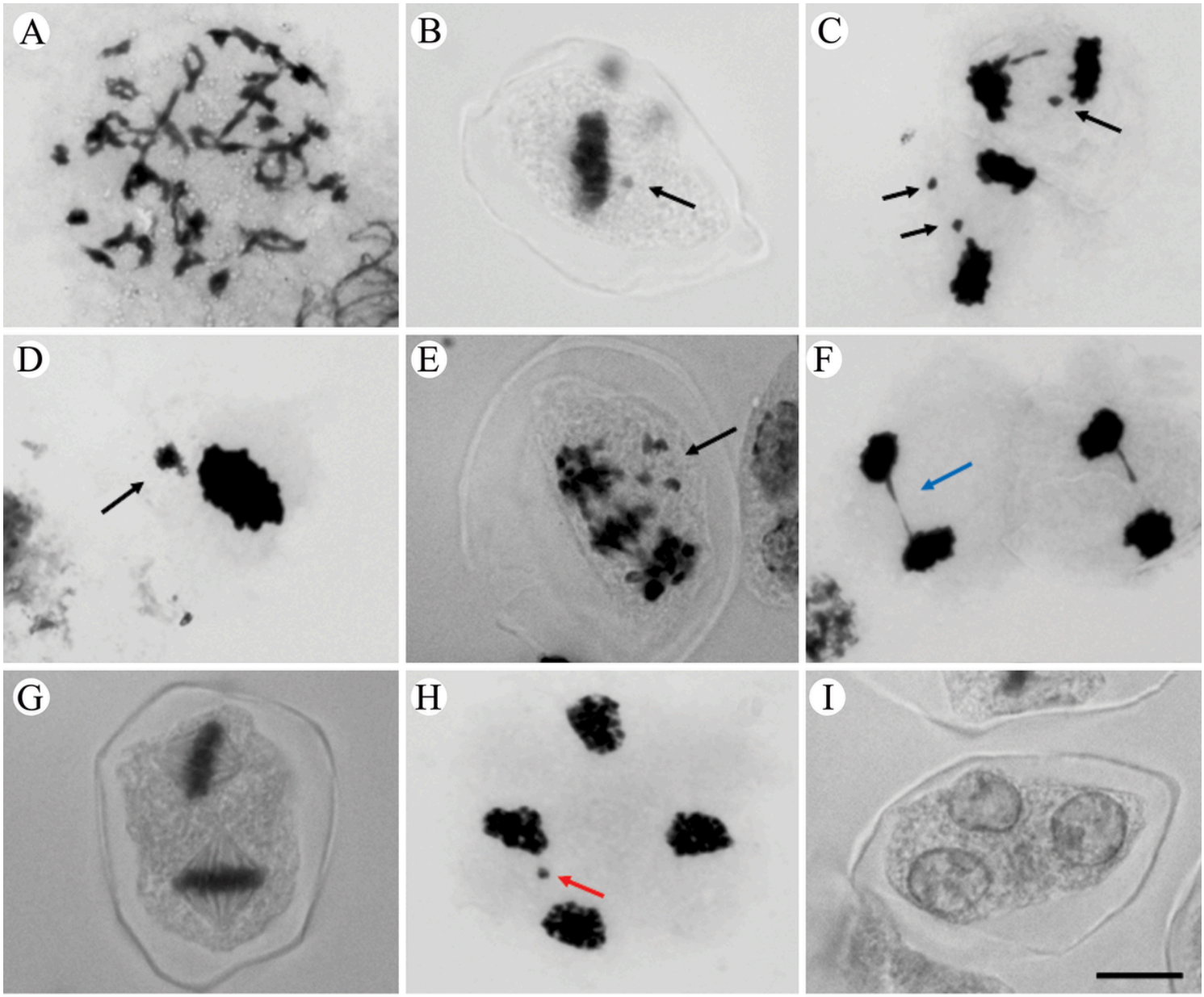

Figure 5

Abnormalities recorded in the gametogenesis of *Zygopetalum* from Ibitipoca State Park (ISP). (**A**) Diakinesis with adherent chromosomes; (**B**) metaphase I with delayed chromosome; (**C**) anaphase with delayed chromosomes; (**D**) metaphase I with delayed chromosomes; (**E**) multipolarity and delayed chromosomes; (**F**) telophase I with chromosomal bridge; (**G**) metaphase II in “T”; (**H**) telophase with micronucleus and (**i**) triad. Black arrows indicate delayed chromosomes; the blue arrow indicates chromosome bridge and the red arrow indicates micronucleus. Bar = 10 µm

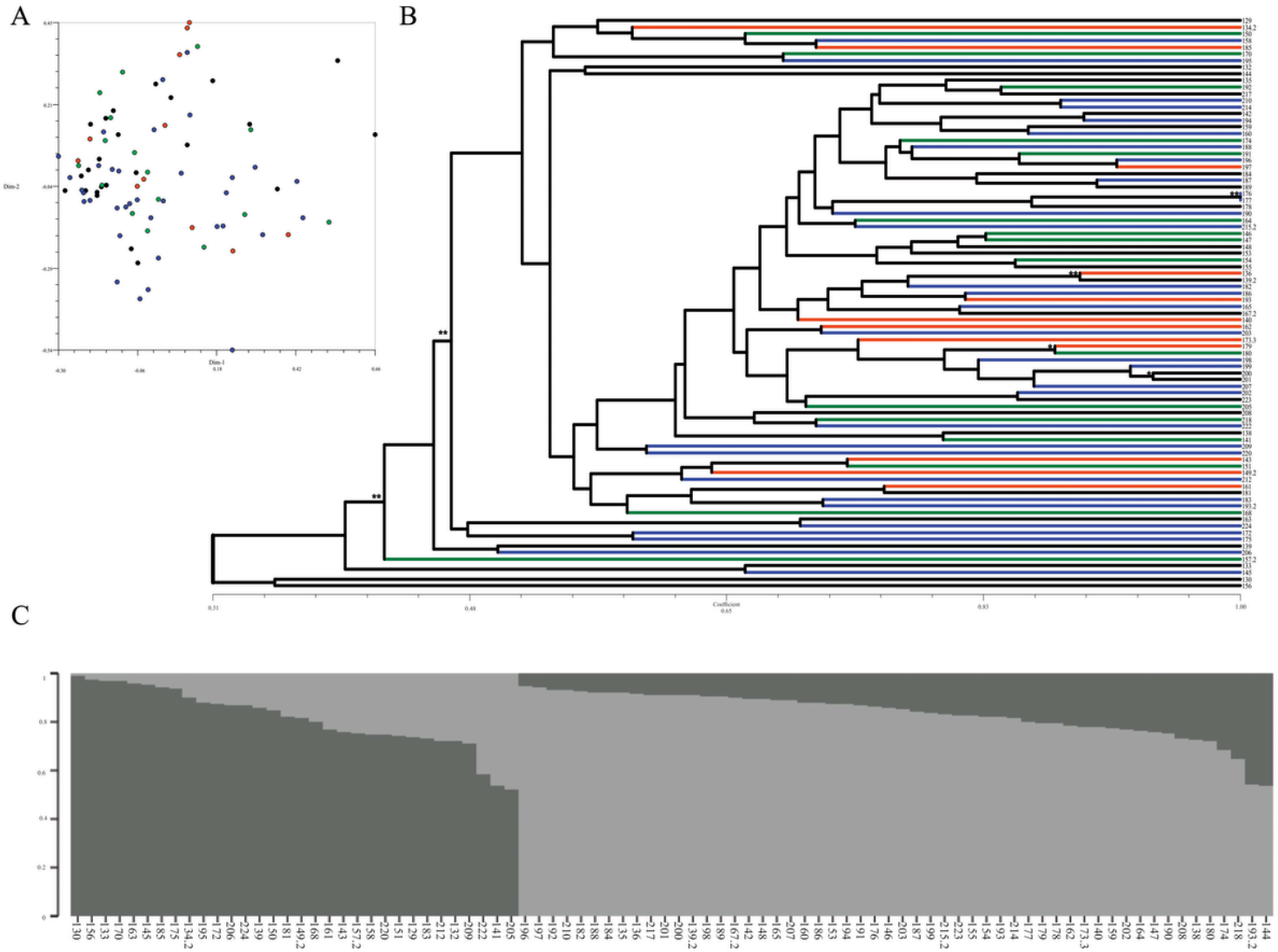

Figure 6

Molecular profile among 86 accessions of *Zygopetalum* from Ibitipoca State Park (ISP), Southeast, Brazil, based on eight ISSR markers. (**A**) 2D graph illustration of PCA (**B**) Dendrogram of genetic similarity using UPGMA and Jaccard coefficient. *represents bootstrap values above 50 and **represents bootstrap values above 75. Blue: *Z. triste*; green*: Z.* aff. *triste*; red: *Z. maculatum*; pink: *Z.* aff. *maculatum*. (**C**) Bayesian analysis of the genetic structure of 86 accessions of *Zygopetalum.* The gray shades represent the proportion of the genome shared by each individual

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.xlsx
- TableS2.docx