CLIMBIÊ FERREIRA HALL

SISTEMÁTICA FILOGENÉTICA, CITOGENÉTICA E TAXONOMIA DE ZYGOPETALINAE (ORCHIDACEAE), COM ÊNFASE EM *Koellensteinia*

SÃO PAULO
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SISTEMÁTICA FILOGENÉTICA, CITOGENÉTICA E TAXONOMIA DE ZYGOPETALINAE (ORCHIDACEAE), COM ÊNFASE EM *Koellensteinia*

Tese apresentada ao Instituto de Botânica da Secretaria do Meio Ambiente, como parte dos requisitos exigidos para a obtenção do título de DOUTOR em BIODIVERSIDADE VEGETAL E MEIO AMBIENTE, na Área de Concentração de Plantas Vasculares em Análises Ambientais.

Orientador: Dr. Fábio de Barros

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It’s a dangerous business, Frodo, going out your door. You step onto the road,

and if you don’t keep your feet, there’s no knowing where you might be swept off to

J.R.R. Tolkien, The Lord of the Rings

RESUMO

A subtribo Zygopetalinae (Orchidaceae) tem 36 gêneros e cerca de 450 espécies, apresentando distribuição Neotropical. Quando Zygopetalinae foi criada sua circunscrição era muito diferente da atual. Vários gêneros, agora tratados dentro da subtribo, eram inseridos em Huntleyinae, e outros estavam espalhados por diferentes subtribos. Filogenias com base em dados moleculares confirmaram que a subtribo Zygopetalinae é monofilética e composta por dois grandes grupos, os clados de *Huntleya* e *Zygopetalum*. Embora os relacionamentos dentro do último não fossem claros, vários gêneros eram distintamente não monofiléticos. Assim, o presente estudo, teve como objetivo esclarecer as relações filogenéticas especialmente dentro do clado *Zygopetalum* e analisar taxonomicamente o gênero *Koellensteinia* e gêneros próximos, resolvendo, quando possível, questões taxonômicas pendentes. Desse modo, os capítulos que compõem a presente tese têm os seguintes objetivos: inferir relações filogenéticas entre os gêneros de Zygopetalinae com foco no clado de *Zygopetalum* usando conjuntos de dados moleculares combinados de genomas plastidiais (Capítulo 1); determinar o número cromossômico de espécies da subtribo Zygopetalinae ocorrentes no Brasil (Capítulo 2); apresentar uma sinopse do gênero *Koellensteinia* como é concebido em sua atual circunscrição (Capítulo 3); propor o restabelecimento de um nome no gênero *Zygopetalum* (Capítulo 4); descrever *Koellensteinia dasilvae* e *Koellensteinia lilacina*, duas novas espécies da flora brasileira (Capítulos 5 e 6); e elaborar um guia de campo de espécies brasileiras de Zygopetalinae (Capítulo 7). A hipótese filogenética aqui apresentada confirma que a subtribo Zygopetalinae, assim como o clado de *Huntleya* e o clado de *Zygopetalum* são monofiléticos. Vários grupos com alto suporte surgiram dentro da subtribo. *Aganisia* e *Acacallis* não são agrupados e não devem ser tratados como um único gênero. *Koellensteinia*, *Zygopetalum* e *Zygosepalum* são polifiléticos e necessitam de reavaliação taxonômica de sua circunscrição. Duas espécies de *Koellensteinia* foram combinadas em *Paradisanthus*. O presente estudo representa mais um passo no conhecimento taxonômico e sistemático de Zygopetalinae e, assim, de Orchidaceae neotropicais. O volume de coletas realizadas e a grande quantidade de dados moleculares gerados permitirão a continuidade dos estudos com o grupo e os trabalhos já realizados darão subsídios para futuros estudos de biogeografia, evolução e taxonomia do grupo.

Palavras-chave: Orchidaceae; Zygopetalinae; Filogenia; NGS; Citogenética; Taxonomia.

ABSTRACT

The subtribe Zygopetalinae (Orchidaceae) has 36 genera and about 450 species, presenting Neotropical distribution.When Zygopetalinae was created its circumscription was very different from the current. Several genera, now treated within the subtribe, were previously inserted into Huntleyinae, and some other genera were spread into other subtribes. Phylogenies based on molecular data confirmed that the subtribe Zygopetalinae is monophyletic and comprises two large groups, the clades of *Huntleya* and *Zygopetalum*. Although relationships within the last were unclear, several genera are regarded as not monophyletic. Therefore, in this study we have the aims of clarify the phylogenetic relationships especially within the clade *Zygopetalum* and analyze taxonomically *Koellensteinia* and related genera, solving, where possible, pending taxonomic issues. Thus, the chapters of this thesis have the following approaches: examine the relationships within Zygopetalinae focused on the *Zygopetalum* clade using combined molecular data sets of plastidial genomes (Chapter 1); determine the chromosomic number of species of the subtribe Zygopetalinae occurring in Brazil (Chapter 2); present a taxonomic synopsis of the genus *Koellensteinia* as presently conceived (Chapter 3); propose the reinstatement of a name in the genus *Zygopetalum* (Chapter 4); describe *Koellensteinia dasilvae* and *Koellensteinia lilacina*, two new Brazilian species (Chapters 5 and 6); elaborate a field guide of Brazilian species of Zygopetalinae (Chapter 7). The phylogenetic hypotesis here presented confirms that the subtribe Zygopetalinae, *Huntleya* clade and *Zygopetalum* clade are monophyletic groups. Several highly supported groups were formed within the subtribe. *Aganisia* and *Acacallis* are not grouped together and should not be treated as a single genus. *Koellensteinia*, *Zygopetalum* and *Zygosetalum* are polyphyletic and need revision. Two species of *Koellensteinia* were transfered to *Paradisanthus*. This study represents another step in the taxonomic and systematic knowledge of Zygopetalinae and thus of

Neotropical Orchidaceae. The amount of samples and the large quantity of generated molecular data will allow the continuation of studies with the group and the studies already carried out will provide insights for future studies of biogeography, evolution and taxonomy of the group.

Keywords: Orchidaceae; Zygopetalinae; Phylogeny; NGS; Cytogenetics; Taxonomy.

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INTRODUÇÃO GERAL

A família Orchidaceae

Orchidaceae é uma das maiores famílias de plantas do mundo, possuindo cerca de 25.000 espécies distribuídas em 850 gêneros (Chase *et al.* 2003, Dressler 2005, Pupulin 2009). No Brasil, ocorrem cerca de 235 gêneros e 2.500 espécies de orquídeas, das quais cerca de 1.600 são endêmicas (Barros *et al.* 2015) e novas espécies são descritas frequentemente. A família possui ampla distribuição, mas concentra-se especialmente nas regiões tropicais. A maioria das orquídeas é epífita, porém, existem espécies rupícolas, terrícolas e saprófitas (Dressler 1993).

As primeiras análises filogenéticas realizadas com Orchidaceae foram baseadas em caracteres morfológicos (*e.g.* Burns-Balogh & Funk 1986, Dressler 1993, Szlachetko 1995). Porém, filogenias ajudaram a separar os efeitos da evolução paralela e convergente em grupos de plantas altamente diversificadas(Simon *et al.* 2011, Clark *et al.* 2012, Specht *et al.* 2012), incluindo orquídeas (Waterman *et al.* 2009, Papadopulos *et al.* 2013, Xiang *et al.* 2013).

Filogenias moleculares

Quando comparadas aos caracteres morfológicos, as seqüências de nucleotídeos apresentam a vantagem de serem pouco homoplásicas (Palmer *et al.* 1988), dando, portanto, origem a filogenias mais confiáveis. Outra vantagem das filogenias moleculares é a grande quantidade de caracteres que pode ser obtida com o seqüenciamento do DNA. Com o desenvolvimento das técnicas de filogenia molecular, o número de estudos com esse enfoque aumentou rapidamente (Cameron 2004, Freudenstein *et al.* 2004). Dessa forma, relações evolutivas dentro de grupos de Orchidaceae de diferentes níveis hierárquicos têm sido estabelecidas, incluindo táxons da região Neotropical, como por exemplo: tribo Pogonieae (Pansarin *et al.* 2008); subtribos Laeliinae (Berg *et al.* 2009) e Zygopetalinae (Whitten *et al.* 2005); gêneros *Galeandra* (Monteiro *et al.* 2010) e *Dichaea* (Neubig *et al.* 2009)

A partir da descoberta do primeiro fóssil de orquídea (polínia de uma espécie de Goodyerinae) foi possível realizar a primeira datação molecular confiável da família (Ramirez *et al.* 2007). Estudo mais recente, utilizando os três fósseis conhecidos de Orchidaceae estimou o surgimento da família há cerca de 77 milhões de anos atrás durante o Cretáceo Superior (Gustafsson *et al.* 2010).

O genoma de cloroplasto é uma importante fonte de informação para estudos de evolução vegetal em diferentes níveis taxonômicos (*e.g.* Byrne *et al.* 2002, Bausher *et al.* 2006, Jansen *et al.* 2006, McPherson *et al.* 2013, Ma *et al.* 2014, Panero *et al.* 2014). Sistematas têm usado DNA plastidial para inferir filogenias por diversas razões: o genoma é uniparental, resultando em um menor tamanho populacional efetivo comparado com o genoma nuclear e assim possui tempo de coalescência mais curto (Birky *et al.* 1983), não recombina (Schaal *et al.* 1998), possui estrutura única, e estrutura de genoma conservado (Palmer *et al.* 1988). No entanto, o genoma plastidial possui taxa de evolução mais lenta e, assim, pequeno nível de variação quando comparado com o DNA nuclear (Schaal *et al.* 1998). Consequentemente, técnicas Sanger necessitam de investigações preliminares para identificar regiões variáveis e um grande número de marcadores são necessários para obtenção de informação suficiente para gerar árvores bem suportadas (McPherson *et al.* 2013). Desse modo, todo o processo pode ser muito caro e demorado (Shendure & Ji 2008).

Técnicas de Next generation sequencing (NGS) resolvem o principal problema do uso de DNA plastidial, porque possuem baixo custo por par de base sequenciado e possibilidade de investigar todo o genoma de cloroplasto ao mesmo tempo (Nock *et al.* 2011, Straub *et al.* 2012). Mesmo que o custo por base seja menor com NGS do que em técnicas Sanger, o investimento total ainda é consideravelmente alto (Egan *et al.* 2012) e técnicas de sequencia de captura atualmente parecem ser a alternativa mais viável economicamente.

Citogenética em Orchidaceae

Menos de 1% das espécies de orquídeas possuem número cromossômico conhecido, sendo que a maioria dos estudos foi realizada apenas em gêneros economicamente importantes (*e.g.* Yamagishi-Costa & Forni-Martins 2009). Para *Koellensteinia*, por exemplo, apenas duas espécies possuem número cromossômico conhecido (Tanaka & Kamemoto 1984, Félix & Guerra 2000). O processo de análise cromossômica que apresenta os melhores resultados e é mais utilizado para a maioria das famílias vegetais, é a germinação de sementes seguida da coleta da radícula recém germinada. Porém, estudos com Orchidaceae possuem a vantagem de que a coleta de meristemas de raízes pode ser facilmente realizada em indivíduos adultos. Após tratamento adequado das células meristemáticas, é possível a contagem do número cromossômico de espécies, a construção de ideogramas, e a aplicação de técnicas como bandamento cromossômico e hibridação *in situ*. Essas técnicas e informações têm sido utilizadas dentro da citotaxonomia para auxiliar a classificação e o melhor posicionamento taxonômico das espécies (Stace 1989, *e.g.* Koehler *et al.* 2008, Pinheiro 2009).

Zygopetalinae

A subtribo Zygopetalinae (Orchidaceae, Epidendroideae, Cymbidieae) possui cerca de 450 espécies (WCSP 2015) distribuídas em 36 gêneros (Pupulin 2009). O grupo apresenta distribuição Neotropical, do sul do México ao norte da Argentina, com centro de diversidade nos Andes. As espécies de Zygopetalinae são muito variáveis morfologicamente, porém possuem algumas características comuns como flores maculadas em variações de violeta, coluna com quilha ventral, dois pares de polínias sobrepostas, dorsiventralmente achatadas, e estigma transversalmente achatado (Pupulin 2009).

Quando a subtribo Zygopetalinae foi criada por Schlechter (1915), sua circunscrição era muito diferente do atual. Vários gêneros, tratados atualmente dentro da subtribo, pertenciam anteriormente a Huntleyinae, enquantro outros gêneros que eram distribuídos por outras subtribos. Ao longo dos anos, foram propostos vários sistemas de classificação para as espécies de Zygopetalinae, alguns agrupando as espécies em grupos informais dentro da subtribo (*e.g.* Garay 1973, Dressler 1993), outros quebrando o grupo em pequenas subtribos (*e.g.* Szlachetko 1995).

Filogenias moleculares confirmaram que a subtribo é monofilética sendo constituída por dois grandes grupos: o clado de *Zygopetalum* com pseudobulbos proeminentes e folhas plicadas e o clado de *Huntleya* com pseudobulbos ausentes ou extremamente reduzidas e folhas conduplicadas (Whitten *et al.* 2005). O estudo de Whitten *et al.* (2005) usou apenas três marcadores genéticos e focou no clado de *Huntleya*, assim, as relações entre os gêneros dentro do clado de *Zygopetalum* continuam incertas, mesmo que haja a indicação de que vários gêneros não são monofiléticos

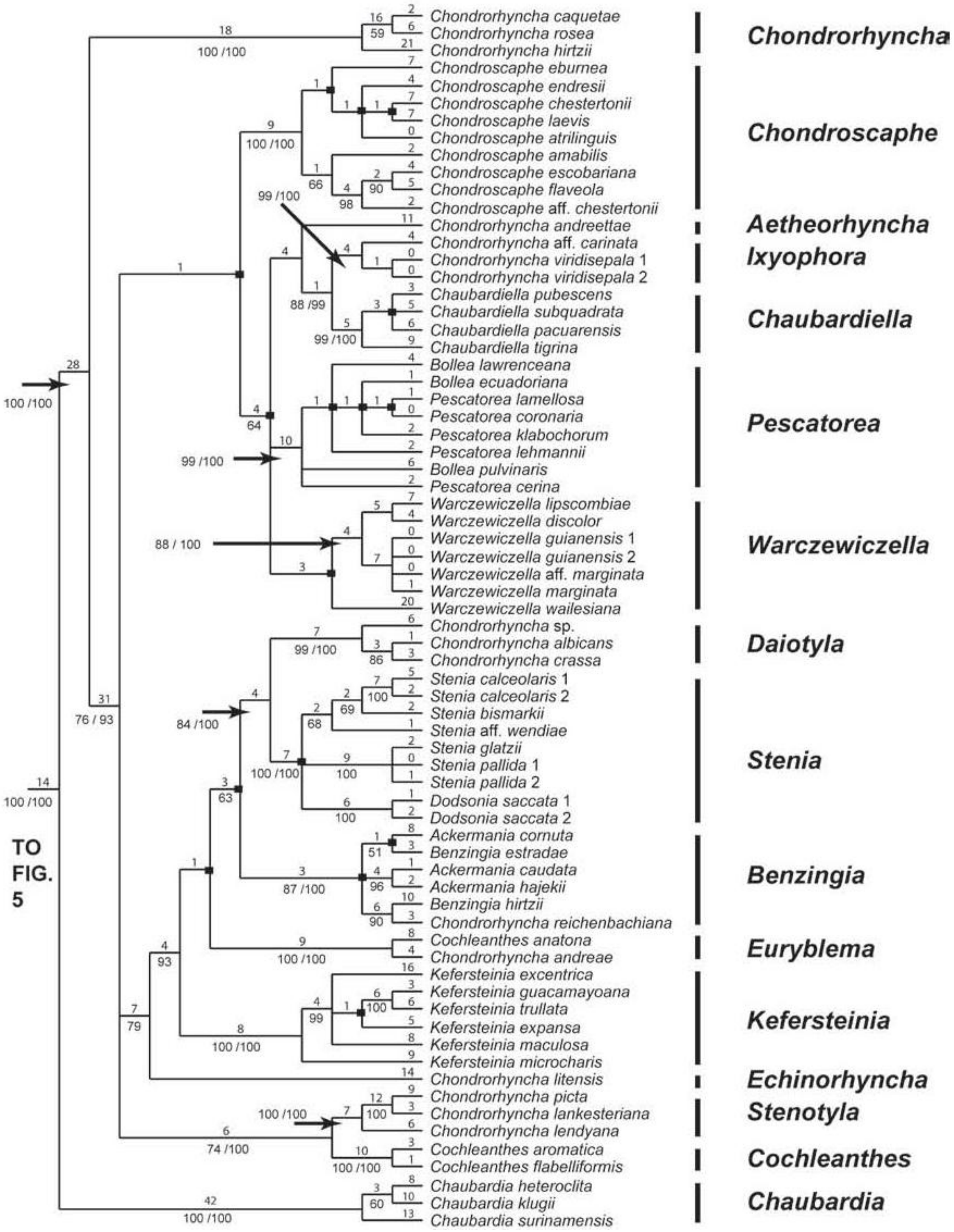

Figura 1 - Árvore presente no estudo de Whitten et al. (2005) contendo espécies do clado de *Huntleya*. Análise baseada em dados combinados de *matK*/*trnL-F*/ITS.

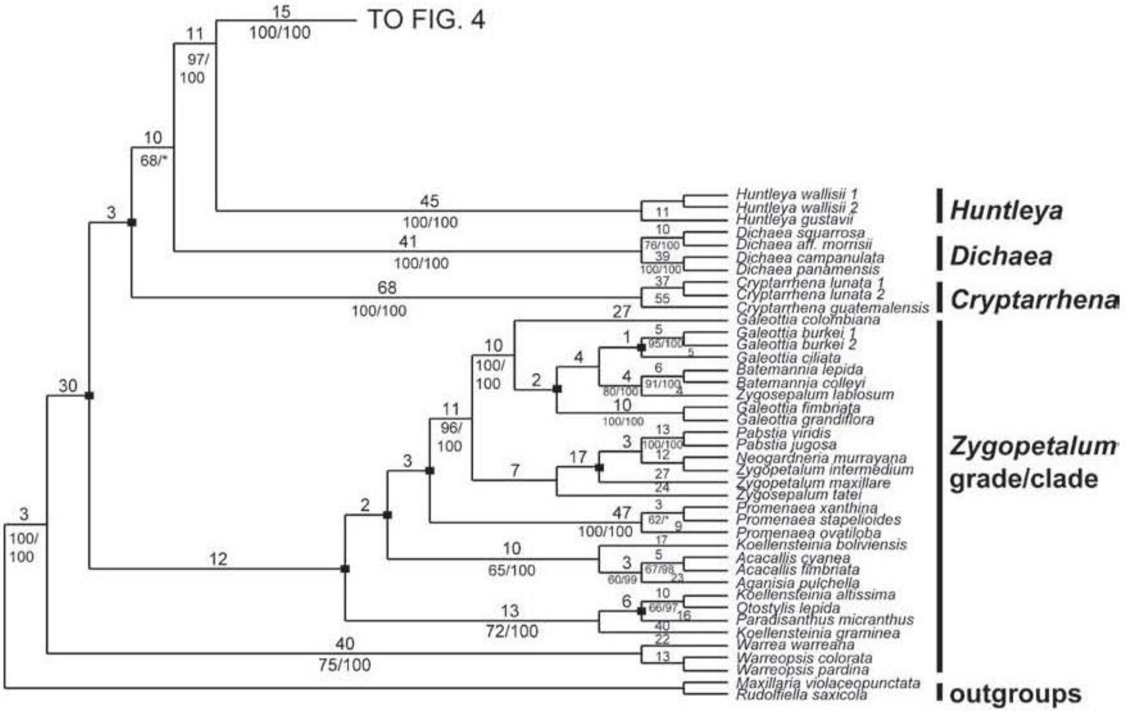

Figura 2 - Árvore presente no estudo de Whitten et al. (2005) contendo espécies do clado de *Zygopetalum*. Análise baseada em dados combinados de *matK*/*trnL-F*/ITS.

O gênero *Koellensteinia* Rchb. f.

Um dos gêneros que Whitten *et al.* (2005) mostraram não ser monofilético é *Koellensteinia*, aspecto re-afirmado no presente trabalho, pois as três espécies incluídas no estudo surgiram em dois clados distintos. *Koellensteinia boliviensis* (Rolfe ex Rusby) Schltr. surgiu como espécie irmã de *Aganisia*, enquanto as outras duas espécies surgiram em um clado juntamente com *Paradisanthus* e *Otostylis.* Ambos os clados possuem baixo suporte e a relação entre eles ainda é incerta, assim como em relação aos outros grupos de Zygopetalinae que compõe o chamado Clado de *Zygopetalum*.

O gênero *Koellensteinia*, em sua circunscrição atual possui cerca de 15 espécies distribuídas pela América do Sul (Pupulin 2009, WCSP 2015). Porém, o status de algumas espécies não é claro. Dentre as espécies do gênero, cerca de dez estão presentes no Brasil, país com maior número de espécies endêmicas (Pabst & Dungs 1977, Barros *et al.* 2015, WCSP 2015). No entanto, também há espécies endêmicas da Bolívia, Colômbia e Venezuela (WCSP 2015). A maior parte das espécies do gênero ocorre em florestas e savanas do norte da América do Sul, mas há espécies presentes na Mata Atlântica e Cerrado (Carnevali *et al.* 2003, Barros *et al.* 2015, WCSP 2015).

O gênero é caracterizado por espécies de hábito terrícola, saxícola ou epífita; folhas plicadas, estreitadas na base em um pecíolo; inflorescência em racemo lateral; flores ressupinadas, alvas, cremes, verdes ou amarelas, normalmente manchadas em rosa, púrpura ou vinho; labelo trilobado, articulado com o pé da coluna; disco com calo bi-lobado (Pupulin 2009).

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CAPÍTULO 1

Phylogenetic Analyses of Zygopetalinae (Orchidaceae, Epidendroideae), based on Chloroplast DNA, using Next-Generation Sequencing

(Formatado de acordo com as normas da Systematic Biology)

Phylogenetic Analyses of Zygopetalinae (Orchidaceae, Epidendroideae), based on Chloroplast DNA, using Next-Generation Sequencing

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Abstract

Chloroplast markers provide an important source of information for studies on plant evolution. However, plastidial genome has low level of variation compared to nuclear and consequently, a great number of markers are needed to obtain enough information. The great amount of sequences generated through next generation sequencing (NGS) solves this main problem of the use of plastidial DNA. Phylogenies based on molecular data confirmed that the subtribe Zygopetalinae (Orchidaceae) is monophyletic and comprises two large groups, *Huntleya* and *Zygopetalum* clades. Although, relationships within the last were unclear, several genera are clearly not monophyletic. Therefore, in this study we examine the relationships within Zygopetalinae focused on the *Zygopetalum* clade using combined molecular data sets of plastidial genomes. Sampling of 52 ingroup accessions were obtained from silica-gel dried leaves from wild-collected, cultivated or herbarium material. The chloroplast genome of *Phalaenopsis aphrodite* subsp. *formosana*, available at GenBank, was used as reference for the probe design for gene enrichment. Sequencing was performed on an Illumina platform. A phylogenetic analysis was performed using Bayesian inference implemented on MrBayes. The total number of polymorphic sites was 2863 from 103126 sites. Consensus tree showed Zygopetalinae, *Huntleya* clade and *Zygopetalum* clade as monophyletic groups. Several high supported groups were formed. *Otostylis* is sister of a group formed by *Warrea* and *Warreopsis*. *Aganisia* and *Acacalis* are not grouped together. *Koellensteinia*, *Zygopetalum* and *Zygosetalum* are polyphyletic and need revision.

Key-words: NGS; Chloroplast genome; Phylogeny; Orchidaceae; Zygopetalinae.

Introduction

The genome of chloroplast provides an important source of information for studies of plant evolution in different taxonomic levels (e.g. Byrne et al. 2002; Bausher et al. 2006; Jansen et al. 2006; McPherson et al. 2013; Ma et al. 2014; Panero et al. 2014). Systematists uses chloroplast DNA sequences to infer phylogenies for several reasons: this kind of genome is uniparental, resulting in a smaller effective population size compared with nuclear genomes and thus shorter coalescent time (Birky et al. 1983), does not recombine (Schaal et al. 1998), has single structure, and gene content and genome structure highly conserved (Palmer et al. 1988). However, plastidial genome has slow rate of evolution, and therefore, low level of variation compared to nuclear (Schaal et al. 1998). Consequently, Sanger techniques needs preliminary investigations to identify variable regions and a great number of markers are needed to obtain enough information to generate well supported trees (McPherson et al. 2013), thus, all the process can be time and resource intensive (Shendure and Ji 2008).

Next generation sequencing (NGS) solves the main problem of the use of plastidial DNA, because of the low cost per base pair, high yield and the possibility of investigate the whole genome of chloroplast at the same time (Nock et al. 2011; Straub et al. 2012). Even thought the cost per base of sequence is lower in NGS than in Sanger sequencing, the overall investment is still considerably high (Egan et al. 2012) and sequence capture techniques currently appears to be the most cost effective alternative. Using sequence capture, the number of targeted sequences can be very high, the DNA libraries are indexed, and multiplexing of individuals is possible, reducing the amount of work and time required to obtain a large amount of data, while also reduces sequencing costs when combined with NGS platforms such as Illumina (Lemmon et al.

2012). As sequence capture uses fragmented DNA, a beneficial side effect of the method is a better yield of herbarium material DNA (Staats et al. 2013)

The subtribe Zygopetalinae (Orchidaceae, Epidendroideae, Cymbidieae) has 36 genera (Pupulin 2009) and about 450 species (WCSP 2015), presenting Neotropical distribution, from Southern Mexico to Northern Argentina with the center of diversity in the Andes. The species of Zygopetalinae show wide morphological variations in both floral and vegetative structures. The main characteristics of the subtribe are the flowers spotted or flushed in violet; column with ventral keel; four superposed, dorsiventrally flattened pollinia; and the transverse, narrow, slit-like stigma. When Zygopetalinae was created by Schlechter (1915), its circumscription was very different from the current. Several genera, now treated within the subtribe, were inserted into Huntleyinae, and some other genera were spread in other subtribes. Several classifications were proposed for the species of the subtribe either grouping species in informal clades (e.g. Garay 1973; Dressler 1993) or as separate smaller subtribes (e.g. Szlachetko 1995)

Molecular phylogenies confirmed that the subtribe Zygopetalinae is monophyletic (Whitten et al. 2000, 2005) and has two large groups: the *Zygopetalum* clade with prominent pseudobulbs and plicate leaves and the *Huntleya* clade with pseudobulbs absent or extremely reduced and conduplicated leaves. Moreover, there was indication that several genera are not monophyletic within *Zygopetalum* clade. Therefore, in this study we examine the relationships within Zygopetalinae focused on the *Zygopetalum* clade using Bayesian analyses of combined molecular data sets of whole plastidial genomes. The main objectives of this study were: 1) To infer a phylogenetic hypothesis of *Zygopetalum* clade using NGS sequencing of whole chloroplast genomes; 2) To test the monophyly of major genera within *Zygopetalum* clade; 3) To position the genera *Cheiradenia* and *Hoehneella*; 4) To discuss the taxonomy and circumscription of genera in the light of phylogenetic results.

Material and Methods

Taxon sampling

Samples were obtained from wild-collected, cultivated or herbarium material. Sampling included 52 accessions of Zygopetalinae. Voucher information and GenBank accession numbers are given in Table 1. Vouchered taxa were deposited at the SP and GB herbaria. Selected species included 40 samples of the *Zygopetalum* clade, comprising representatives of all currently recognized genera and twelve species of the *Huntleya* clade from ten different genera. *Hoehneella* and *Cheiradenia* were sampled for the first time for phylogenetic study. Three outgroup taxa, two from other subtribes of Cymbidieae were included. One of this outgroups sequences, *Erycina pusilla* (L.) N.H.Williams&M.W.Chase, was obtained at GenBank (NC_018114.1). Leaf pieces from all collected samples were dried in silica-gel.

Probe design

The complete chloroplast genome of *Phalaenopsis aphrodite* subsp. *formosana*, available at GenBank (NC_007499.1) was used as reference for the design of MYcroarray RNA hybridization probes. This sequence was divided in blocks of 360 bp. Every other block was deleted and not used as reference. Sections of the genome with more than six repeated bases were avoided as reference. In total, 63720 bp of *P.*

aphrodite chloroplast were used. To ensure that the whole genes were covered the probes were designed in a tiling manner.

Sampling and library construction

DNA was extracted from dried leaf tissue using the Dneasy Plant Mini Kit (Qiagen). DNA of herbarium material was extracted using the same kit, but with the addition of protease. Extractions were measured in a Nanodrop 2000c instrument for selection of best samples. Genomic DNA was sheared with a Covaris S220 instrument. A DNA library was constructed with the NEXTflexTM DNA Sequencing Kit and NEXTflexTM Barcodes (BIOO Scientific). Agencourt AMPure XP magnetic beads (Beckman Coulter) were used for DNA purification and fragment size selection, excluding fragments under 300 bp and over 400 bp. To obtain a higher concentration of DNA for gene enrichment, a PCR run of 14 cycles was performed on indexed samples, with the following program: 98°C, 2'; 14 × (98°C, 30"; 65°C, 30"; 72°C, 60"); 72°C, 4'. Concentrations of the amplified library were measured in a NanoDrop 2000c instrument. Prior to gene enrichment, DNA was concentrated using a SpeedVac instrument.

Gene enrichment and sequencing

Gene enrichment was carried through the MYBaits target enrichment system (MYcroarray). Equimolar amounts of each amplified library were pooled in reactions of eight indexed samples each. Individuals were pooled according to phylogenetic proximity suggested by previously published phylogenetic trees (Whitten et al. 2005).

Hybridisation reactions were performed at 65°C for 36 hours. Probes were recovered with Dynabeads® MyOne™ Streptavidin C1. For better yield of the sequencing, a PCR run of 14 cycles was performed for each hybridisation reaction using Herculase II Fusion DNA Polymerase and the following program: 98°C, 30"; 14× (98°C, 20"; 60°C, 30"; 72°C, 60"); 72°C, 5'. High-throughput 150 bp paired-end reads were produced on a MiSeq Illumina platform at the Genomics Core Facility of the University of Gothenburg, Sweden.

Matrix assembly

A trimming was performed in the CLC Genomics software. Reads were stripped of adapter sequences and filtered for quality with a threshold of 20 for phred-scores, and then mapped towards the reference sequences also using CLC Genomics. Consensus sequences out of the mapped reads were obtained using SAMTools software. Alignment of the species sequences was performed on MAFFT - Multiple Sequence Alignment Software Version 7 (Katoh and Standley 2013) and checked on the program Geneious 8. To obtain an annotated alignment, the 54 sequences of this study were aligned with the chloroplast genome of *Erycina pusilla* (L.) N.H.Williams&M.W.Chase (available at GenBank).

The aligned sequences were partitioned into 42 gene files and two concatenated intron files, one of introns with secondary structure, and one with intergenic sequences and pseudogenes. Both intron files were ran on trimAl v1.2 (Capella-Gutiérrez et al. 2009) allowing different settings of levels of gaps (no gaps, and 10%, 20%, 30%, 40%, and 50% of gaps allowed). The original intron files and the ones generated on trimAl were evaluated on jModelTest-2.1.4 (Guindon and Gascuel 2003; Darriba et al. 2012) under the Akaike information criterion (Akaike 1974). All files had GTR + I+ G as model with best fit. For both type of intron files (with secondary structure and the one with intergenic sequences and pseudogenes) the one with maximum value of the alpha parameter of the gamma distribution were selected (files with no gaps allowed in both cases). The model of nucleotide evolution was also evaluated for each gene using jModelTest 2.1.4. The MEGA program (Tamura et al. 2013) was used to obtain the number of polymorphic sites and the average frequency of each base of the analyzed sequences.

Phylogenetic analysis

A phylogenetic analysis of the composite matrix based on the selected models was performed using Bayesian inference implemented on MrBayes version 3.2.3 (Ronquist and Huelsenbeck 2003). Data was analyzed using the 44 partitions: 42 partitions of single genes; introns with secondary structure + tRNA + rRNA; and intergenic regions + pseudogenes. Two simultaneous analyzes, each consisting of four Markov chains were performed to 10 million generations, with trees sampled every 100 generations. The first 10 thousand generations of each run were discarded as burn-in and the remaining trees of both analyzes were combined to construct a majority consensus tree. Posterior probabilities (PP) of 0.95-1.00 were considered as strong support, 0.85-0.94 as moderate support and <0.85 as weak support.

Results

The total number of polymorphic sites of exons was 1965 from 65247 sites (3,01%) and for introns 898 from 37879 sites (2,37%). The alignment of concatenated data demonstrated an average nucleotide composition of A = 30.1%, C = 20.7%, G = 29.1% and T = 20.1%. The tree resulting from the Bayesian majority-rule consensus that represents our phylogenetic hypothesis is shown in figure 1. Two herbarium samples, *Cryptahrrena* and *Koellensteinia kelneriana* appears together and outside the subtribe Zygopetalinae, this probably happened due to the bad quality of extractions, with over-fragmented DNA that resulted in incomplete sequences that led to long branch attraction.

Zygopetalinae formed a monophyletic group with strong support (1.00 of posterior probability (PP); Fig. 1, clade A) that is divided into two groups: the highly supported *Huntleya* clade (1.00 PP; Fig. 1, clade B) and the less supported *Zygopetalum* clade (0.89 PP; Fig. 1, clade C). All internal monophyletic subgroups of the *Huntleya* clade were highly supported (>0.95 PP). *Hoehneella* and *Chaubardia* formed a monophyletic group (1.00 PP). In the two genera that had more than one species sampled (*Dichaea* and *Kefersteinia*), the species were grouped together. *Zygopetalum* clade is divided into two main groups: the highly supported *Zygopetalum* alliance (sensu Garay 1973) (1.00 PP; Fig. 1, clade E) and the less supported *Warrea* alliance (0.88 PP; Fig. 1, clade D). *Warrea* and *Warreopsis* are clustered together with high support (1.00 PP), and *Otostylis* seems to also be part of the *Warrea* alliance (0.88 PP). The genus *Promenaea* is sister group of all other species in the *Zygopetalum* alliance. A strongly supported group (1.00 PP; Fig. 1, clade F) was formed by those species of the genus *Aganisia* that were previously assign to *Acacallis,* and a sister group formed by most of the species of *Koellensteinia*. All internal nodes of *Acacallis* were highly supported (1.00 PP). Populations of *Koellensteinia eburnea*, *Koellensteinia lilacina* and *Koellensteinia boliviensis* formed a monophyletic complex (1.00 PP) of species of unclear relationships. *Koellensteinia kelneriana* is sister of this complex (1.00 PP). This clade formed by most *Koellensteinia* and *Acacallis* (clade F) is sister of a different highly supported group (1.00 PP; Fig. 1, clade G) that have two subgroups, one formed by *Cheiradenia*, *Aganisia*, *Paradisanthus* and three other species of *Koellensteinia* (1.00 PP; Fig. 1, clade H) and other formed by *Batemannia*, *Galeottia*, *Neogardneria*, *Pabstia*, *Zygopetalum* and *Zygosepalum* (1.00 PP; Fig. 1, clade I). None of the species of *Koellensteinia* in clade H are grouped in monophyletic subgroups. Current positioning of *Koellensteinia graminea* within clade H, as sister group of remaining species (except for *Cheiradenia*), is not well supported. Clade I has two subgroups, one composed by *Batemannia*, *Galeottia* and *Zygosepalum* (1.00 PP; Fig. 1, clade J) and other formed by *Neogardneria*, *Pabstia* and *Zygopetalum* (1.00 PP; Fig. 1, clade K). Both *Zygosepalum* and *Zygopetalum* appears as non-monophyletic groups, mixed with nearby genera.

Discussion

Establishing the phylogenetic hypothesis of large and morphologically diversified groups is crucial for our understanding of the mechanisms associated with the evolution of such groups. Phylogenies have helped to disentangle the effects of parallel and convergent evolution in highly diversified plant groups (Simon et al. 2011; Clark et al. 2012; Specht et al. 2012), including orchids (Waterman et al. 2009; Papadopulos et al. 2013; Xiang et al. 2013). Independent derived characters present opportunities to explore the origin and the role of morphological traits in plant evolution. In this context, robust phylogenetic hypothesis and genomic techniques are promising tools to investigate the evolution of plant groups showing complex morphological patterns originated by convergence or reversions. The increased sampling effort and the genomic approach adopted in this study clearly disentangled the phylogenetic position of different clades within subtribe Zygopetalinae, with special focus in the *Zygopetalum* clade. Zygopetalinae was recovered as a well supported monophyletic group, which is followed by the subdivision of *Huntleya* and *Zygopetalum* clades (Fig. 1). The monophyly of different genera such as *Zygopetalum*, *Zygosepalum* and *Koellensteinia* was not confirmed, and most floral and vegetative characters in such groups appear to be homoplasious. Strong biogeographic patterns were detected in some groups, such as in clade K, with species endemic to the Brazilian Atlantic Forest and rock fields of Eastern Brazil, and clade J composed mostly by Amazonian species. Our results not only recover and strongly support clades recognized in previous studies (Whitten *et al.* 2000, 2005), but also revealed several major groups within *Huntleya* and *Zygopetalum* clades.

Limits of Zygopetalinae

Zygopetalinae appearing as a strongly supported monophyletic group (1.00 PP; Fig. 1, clade A) corroborates the study of Whitten *et al.* (2005). Nevertheless, several groups that were recognized as separate subtribes like Huntleyinae, Warreinae, Dichaeinae (Szlachetko 1995) or as its correspondent informal groups (Garay 1973; Dressler 1993), have support as separated groups (Figure 1, clades B-E). However, we believe that a broader circumscription as a single subtribe better serves the purposes of taxonomic classification.

Huntleya clade (clade B)

Huntleya clade forms a strongly supported monophyletic group (1.00 PP) (Fig. 1, clade B). Our sampling did not focus in this group of Zygopetalinae as it was very well sampled and resolved in the phylogeny of Whitten *et al.* (2005). Our data corroborates the circumscription adopted in that study, but includes a genus that was not sampled in it, the monospecific *Hoehneella*, endemic from the Brazilian Atlantic Forest in Brazil. Our phylogeny corroborates the morphological data, with *Hoehneella* appearing as sister group of *Chaubardia*, two genera vegetatively similar with inconspicuous pseudobulbs, but differentiated by the not gibbous lateral sepals, sessile labellum, column not winged and crested anther (Garay 1973).

We were unable to obtain fresh extractable material of the genus *Cryptarrhena*. The chloroplast sequence assembled from extractions of herbarium material of *Cryptarrhena* did not have good quality and appear as sister group of another herbarium material (*Koellensteinia kelneriana*) outside the subtribe, probably due to long branch attraction. The morphologically anomalous genus *Cryptarrhena*, emerged in the study of Whitten *et al.* (2005) within the subtribe, isolated on a very long branch. The genus has unresolved placement as sister group of all other genera in the *Huntleya* clade and morphological characters similar to the genera in that position: anchor-shaped labellum similar to that of *Dichaea* and column with a conspicuous clinandrium (hood) similar to those of *Huntleya*.

Zygopetalum clade (clade C)

In the study of Whitten *et al.* (2005) the *Zygopetalum* clade emerged as sister group of the *Huntleya* clade, but without good support. Collapsing the branches that did not have good support, the base of the phylogeny of the subtribe presents a polytomy with several clades of the *Zygopetalum* clade. In the present study, *Zygopetalum* clade forms a middling supported monophyletic group (0.89 PP) (Fig. 1, clade C), that is sister group of the *Huntleya* clade.

Within the *Zygopetalum* clade, the study of Whitten *et al.* (2005) presented two well supported clades, one formed by *Neogardneria*, *Pabstia* and *Zygopetalum*, and other composed by *Batemannia*, *Galeottia* and *Zygosepalum*. Both clades remained as monophyletic groups in the current phylogeny (Fig. 1, clades J and K). Other less supported groups like the one formed by *Koellensteinia*, *Otostylis* and *Paradisanthus* and the one of *Acacallis* and *Aganisia* were not sustained in the current study.

The *Zygopetalum* clade is composed by the genera that are traditionally considered as part of Zygopetalinae (e.g. Szlachetko 1995) that have as main characteristics developed pseudobulbs and plicate leaves. Brazil is the country that is home of most species of the *Zygopetalum* clade, and the group has two main centers of diversity the first in the Brazilian Atlantic Forest of Eastern Brazil and the second in the Amazon Rainforest and Guiana Shield in the Northern South America (Pupulin 2009; Barros et al. 2015; WCSP 2015).

Warrea alliance (clade D)

Garay (1973) circumscribed the *Warrea* alliance as a separate group from the so-called *Zygopetalum* aliance, including species of *Cheiradenia, Otostylis*, *Warrea*, *Warreella* and *Warreopsis*, genera with homoblasticous pseudobulbs. Szlachetko (1995) considered the clade as a separated subtribe, Warreinae, but did not include *Cheiradenia* as part of it. Whitten *et al.* (2005) only sampled species of *Otostylis*, *Warrea* and *Warreopsis*, but *Otostylis* was positioned as close related to species of *Koellensteinia* and *Paradisanthus* in their phylogeny. *Warrea* and *Warreopsis* formed a well supported clade that seems to be sister group of all other Zygopetalinae. Our phylogeny grouped *Otostylis*, *Warrea* and *Warreopsis* in a reasonably supported monophyletic group (0.88 PP) (Fig. 1, clade D), with genera that have homoblasticous pseudobulbs and multiple plicate leaves. We were unable to obtain extractable material of *Warreella*, but based on its morphology, this genus probably forms a clade, with *Warrea* and *Warreopsis*, that has *Otostylis* as sister group. *Cheiradenia* was positioned within a different group and consequently the homoblasticous pseudobulbs is a homoplasy. Most of the species of the *Warrea* alliance grows in Central America and Northern South America. Two taxa that have wider distribution are *Otostylis* (reaching Northern and Western Brazil) and *Warrea warreana* (ranging from Colombia to Argentina) (Pupulin 2009; WCSP 2015).

Promenaea

The *Zygopetalum* alliance, sensu Garay (1973), forms a highly supported clade (1.00 PP) (Fig. 1, clade E). The first lineage to arise in this clade is the one formed by the genus *Promenaea*. The phylogeny of Whitten *et al.* (2005) included two species of the genus that formed a strongly supported group, that had unclear relationship with other species of the *Zygopetalum* clade. Our study only included one species of

Promenaea, but the positioning of the genus seems clear. *Promenaea* is a genus endemic from the Brazilian Atlantic Forest characterized by epiphytic plants, with 1-2-flowered inflorescence produced from mature pseudobulbs, large cymbiform bracts, lateral sepals connate with the column foot, and complex callus (Pupulin 2009).

Koellensteinia and related genera (clades F and H)

The study of Whitten *et al.* (2005) included a scattered sampling of the *Zygopetalum* clade, but one of the main results arising from it was that *Koellensteinia* is polyphyletic. Our results confirm this polyphyly, presenting the species of *Koellenteinia*, as currently circunscribed, emerging in two different highly supported clades (Fig. 1, clade F and H), and forming four monophyletic lineages.

The first strongly supported clade (1.00 PP) (Fig. 1, clade F) is the one formed by those species of *Aganisia* previously assigned to the genus *Acacallis*, as sister group of a clade with most of the species of *Koellensteinia*. Species of *Koellensteinia* of this clade, form a well supported group (1.00 PP) that includes the type species *Koellensteinia kelneriana* Rchb.f., and most of the species of the genus. The three species of *Koellensteinia* that emerged in a different clade are the ones that have the most distinctive morphology. *Koellensteinia graminea* (Lindl.) Rchb.f. is the smallest species of the genus, have much reduced pseudobulbs, multiple conduplicate leaves, few flowered inflorescence, and is epiphytic. *Koellensteinia altissima* Pabst and *Koellensteinia spirallis* Gomes Ferreira & L.C.Menezes are by far the species with tallest inflorescence in the genus, but with a very congested short rachis.

The genera *Acacallis* and *Aganisia* have been always considered as closely related, and recently both genera have been lumped together (Pupulin 2009), specially after molecular data corroborated this circumscription (Whitten et al. 2005). However, our data strongly supports the separation of *Acacallis* from *Aganisia* in two separate genera. Morphologically, *Acacallis* can be differentiate from *Aganisia* by its pending inflorescence, epichile of labellum cochleate (vs. concave), column with a long foot (vs. short foot) and pollinia over short stipe (vs. long stipe).

The second highly supported clade (1.00 PP) (Fig. 1, clade H) that contains species assigned to *Koellensteinia* is the one formed by *Cheiradenia*, *Aganisia*, *Paradisanthus*, *K. altissima*, *K. graminea* and *K. spirallis*. However, none of the *Koellensteinia* species is grouped in monophyletic sub-groups. The three first lineages to emerge in this group compose a grade of small epiphytic, few-flowered, mostly Amazonian plants (*Aganisia*, *Cheiradenia* and *K. graminea*) and the three more recent lineages forms a clade of terrestrial herbs, with multi-flowered inflorescence, endemic from the Brazilian Atlantic Forest (*K. altissima*, *K. spirallis* and *Paradisanthus*). *Cheiradenia* is a monospecific genus from forests of the Guiana Shield and Northern Brazil, characterized by its reduced homoblastic pseudobulbs, 1-3 conduplicate leaves, small size of the flowers and 5-toothed callus. *Koellensteinia graminea* is closely related to *Koellensteinia graminoides* D.E. Benn. & Christenson a species endemic from Peru not sampled in this study. Both species can be differentiated from the remaining in the aforementioned clade by its much reduced pseudobulbs, 1-3 conduplicate linear leaves, and small flowers with 2-lobed callus. *Aganisia* is another monospecific genus of the clade. It can be differentiated from nearby genera by its developed pseudobulbs with one plicate leaf and cristate callus. Interestingly *K. graminea* and *Aganisia pulchella* present a similar disjunct distribution, occurring in the Amazon and in the Brazilian Atlantic Forest of the state of Bahia, Brazil. The clade formed by *K. altissima*, *K. spirallis* and *Paradisanthus* presents terrestrial herbs with multi-flowered inflorescence and labellum with a macula (restrict to the base in *K. altissima* and *Paradisanthus*). Both *K. altissima* and *K. spirallis* present very long inflorescences (reaching several meters in *K. spirallis*) with congested flowers over a short rachis of just a few centimeters and flowers with 2-lobed callus. Species of *Paradisanthus* present much shorter inflorescences with lax flowers over a medium sized rachis, flowers with maculated sepals and petals, and crenulate callus.

Zygosepalum + Galeottia + Batemania (clade J)

A mostly Amazonian clade formed by *Zygosepalum*, *Galeottia* and *Batemania* is highly supported (1.00 PP). Although our sampling within this clade was scarce, there is a clear inconsistency with the current generic concept of *Zygosepalum*. Our phylogenetic hypothesis placed *Zygosepalum labiosum* (Rich.) C.Schweinf. as sister group of all other species in the clade and *Zygosepalum lindeniae* (Rolfe) Garay & Dunst. as sister group of *Batemannia*. These inconsistencies corroborate the study of Whitten *et al.* (2005) that found indications of polyphyly in both *Galeottia* and *Zygosepalum*. A *Zygosepalum* species (*Zygosepalum tatei* (Ames & C.Schweinf.) Garay & Dunst.) was placed as sister of *Zygopetalum* species by Whitten *et al.* (2005), relationship not found in our study. Romero-González and Fernández-Concha (2010) created *Weidmannia*, genus of the Guayana Highlands, transferring *Z. tatei* and *Z. angustilabium* (C. Schweinf.) Garay to this new genus, trying to have a monophyletic *Zygosepalum*. Such attempt would not solve all the problems of the genus as *Z. lindeniae* would still need a new combination.

Zygopetalum + Pabstia + Neogardneria (clade K)

Zygopetalum, *Pabstia* and *Neogardneria* forms a highly supported clade (1.00 PP). Whitten *et al.* (2005) also found a similar pattern, but without support. Most species of the clade are endemic from Brazilian Atlantic Forest and rock fields of Eastern Brazil, with exception of *Zygopetalum maculatum* (Kunth) Garay that also occurs in Bolivia and Peru, and *Zygopetalum maxillare* G.Lodd. that reaches Argentina and Paraguay. *Zygopetalum* is therefore polyphyletic. The small *Zygopetalum sellowi* Rchb.f. is the first lineage to arise in the clade, followed than by the lineage of *Zygopetalum maxillare*. *Pabstia* forms a monophyletic clade, but appears with *Neogardneria* inside the current circumscription of *Zygopetalum*. Both *Pabstia* and *Neogardneria* were included in *Zygopetalum* (name that have priority) in previous studies, and Schlechter (1915) affirmed that there were no features that justify the separation of *Pabstia* from *Zygopetalum*. Therefore, a broader circumscription of *Zygopetalum* could be the new direction of the group.

Conclusions and perspectives

Our results indicate that the *Zygopetalum* clade forms a monophyletic lineage that is sister group of the *Huntleya* clade. The precise relationship between some genera, especially from the clade formed by *Batemannia*, *Galeottia* and *Zygosepalum* are still unclear, because of the limited sampling of the group. However, *Zygosepalum* is clearly non-monophyletic. Additionally, the inclusion of NGS data of nuclear genome can lighten some of the relationships of clades that currently do not have a high support. Moreover, the extensive genomic data produced here are promising resources for population level and phylogeographic studies, which may clarify the evolutionary patterns within species complexes and close related genera.

Our study greatly increases the amount of molecular data available in GenBank for Zygopetalinae, both in number of species and sequences. Almost all sequences previously available came from the study of Whitten *et al.* (2005), that did not focus in the *Zygopetalum* clade, our main focus. The topology of the tree indicates an origin in Northern South America for the group, but further studies, including molecular dating, are needed to better understand the biogeography of the group. Finally, in our phylogenetic hypothesis of the *Zygopetalum* clade, we could identify several groups that need revision and have in this study a good basis for guidance. Especially because genus delimitation in Zygopetalinae is traditionally very messy, due to several homoplasies and hard to find synapomorphies, what leads to circumscriptions with several small or monospecific genera. Our analyses show that the taxonomy of the infrageneric taxa of Zygopetalinae is more complicated than previous results have indicated, and their taxonomic status needs to be reassessed and redefined.

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Table 1 - Voucher information of the species used in the study.

Species	**Voucher**
***Zygopetalum* clade**	
Acacallis cyanea Lindl.	Hall 961 (IBt)
Acacallis fimbriata (Rchb.f.) Schltr.	Hall 780 (IBt)
Acacallis hoehnei Schltr.	Hall 909 (IBt)
Acacallis rosariana V.P.Castro & J.B.F.Silva	Hall 794 (IBt)
Aganisia pulchella Lindl.	Hall 777 (IBt)
Batemannia colleyi Lindl.	Hall 979 (IBt)
Cheiradenia cuspidata Lindl.	Hall 986 (SP)
Galeottia ciliata (Morel) Dressler & Christenson	Hall 814 (SP)
Koellensteinia altissima Pabst	Hall 746 (SP)
Koellensteinia boliviensis (Rolfe ex Rusby) Schltr.	Without number
Koellensteinia dasilvae C.F. Hall & F. Barros	Hall 1111 (SP)
Koellensteinia eburnea (Barb.Rodr.) Schltr. [GO]	Hall 772 (IBt)
Koellensteinia eburnea (Barb.Rodr.) Schltr. [MG]	Hall 1030 (SP)
Koellensteinia eburnea (Barb.Rodr.) Schltr. [MS]	Hall 852 (IBt)
Koellensteinia eburnea (Barb.Rodr.) Schltr. [MT]	Hall 881 (SP)
Koellensteinia graminea (Lindl.) Rchb.f.	Hall 743 (SP)
Koellensteinia kellneriana Rchb.f.	Pessoa 1152 (SP)
Koellensteinia lilacina C.F. Hall & F. Barros	Hall 1080 (SP)
Koellensteinia spiralis Gomes Ferreira & L.C.Menezes	Hall 955 (SP)
Neogardneria murrayana (Gardner ex Hook.) Schltr. ex Garay	IBt 18232
Otostylis brachystalix (Rchb.f.) Schltr. [MT]	Hall 904 (SP)
Otostylis brachystalix (Rchb.f.) Schltr. [RR]	Pessoa 750 (UFP)
Pabstia jugosa (Lindl.) Garay	IBt 1577
Pabstia modestior (Rchb.f.) Garay	Without number
Pabstia schunkiana V.P. Castro	Hall 727 (SP)
Paradisanthus bahiensis Rchb. f.	Hall 956 (SP)
Paradisanthus micranthus (Barb. Rodr.)Schltr.	Hall 734 (SP)
Paradisanthus mosenii Rchb. f.	Hall 818 (IBt)
Promenaea rollissonii (Lindl.) Lindl.	IBt 12878
Warrea warreana (Lodd. ex Lindl.) C. Schweinf.	IBt 5502
Warreopsis pardina (Rchb.f.) Garay	Molau et al. (GB3017)
Zygopetalum crinitum Lodd.	IBt 10222
Zygopetalum maxillare Lodd.	Without number
Zygopetalum maculatum (Kunth) Garay	Hall 975 (SP)
Zygopetalum pedicellatum (Sw.) Garay	IBt 17752
Zygopetalum sellowii Rchb. f.	IBt 1338
Zygopetalum triste Barb. Rodr.	IBt 10543
Zygosepalum labiosum (Rich.) C.Schweinf.	Hall 781 (SP)
Zygosepalum lindeniae (Rolfe) Garay & Dunst.	Without number

***Huntleya* clade**	
Chaubardia surinamensis Rchb. f.	Hall 813 (SP)
Chondrorhyncha lendyana Rchb. f.	Hall 1088 (GB)
Cochleanthes flabelliformis (Sw.) R.E. Schult. & Garay	IBt 17928
Cryptarrhena guatemalensis Schltr.	Harling & Andersson (GB16513)
Dichaea cogniauxiana Schltr.	Hall 848 (IBt)
Dichaea pendula (Aubl.) Cogn.	Hall 849 (SP)
Hoehneella gehrtiana Ruschi	Without number
Huntleya meleagris Lindl.	Hall 733 (SP)
Kefersteinia pulchella Schltr.	Without number
Kefersteinia sanguinolenta Rchb.f.	Without number
Pescatoria wallisii Linden & Rchb. f.	Hall 1087 (GB)
Warczewiczella discolor (Lindl.) Rchb.f.	IBt 1982
Outgroups	
Erycina pusilla (L.) N.H.Williams & M.W.Chase	GenBank NC_018114.1
Hoffmannseggella milleri (Blumensch. ex Pabst) V.P.Castro & Chiron	Hall 1085 (GB)
Stanhopea tigrina Bateman ex Lindl.	Hall 1086 (GB)

IBt - Live collection of the Instituto de Botânica.

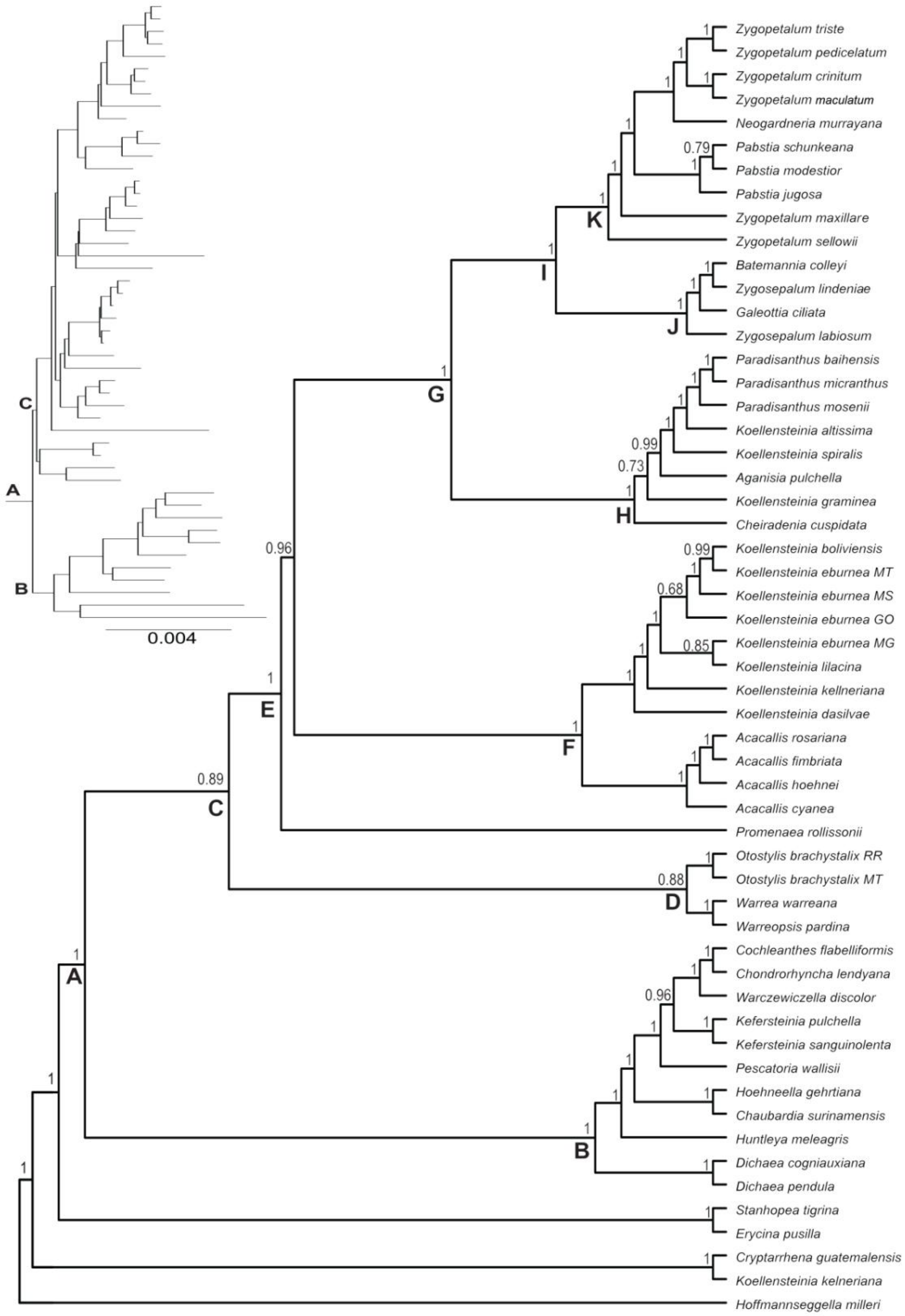

Figure 1 - Bayesian majority-rule consensus tree from complete chloroplast sequence dataset. Posterior probabilities are shown next to nodes. The same consensus tree, modified to just show ingroups of Zygopetalinae, with proportional branch lengths is shown in the upper left-hand corner. Capital letters for selected monophyletic groups are shown in both trees.

CAPÍTULO 2

Chromosomal studies in Zygopetalinae (Orchidaceae)

(Formatado de acordo com as normas da Brazilian Journal of Botany)

Chromosomal studies in Zygopetalinae (Orchidaceae)

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Running title: Chromosomal studies in Zygopetalinae

Abstract

This study aimed at the cytotaxonomic analysis of species of the subtribe Zygopetalinae occurring in Brazil, based on the determination of their diploid chromosomic numbers and presentation of ideograms. Root tips were pre-treated with 0,002 M 8-hydroxyquinoleine, fixed in 3:1 ethanol-acetic and transferred to 70% ethanol. For the slides preparation, the roots were hydrolysed in 5 N HCl and the squash preparations were made in 45% acetic acid. Cells were stained following the Giemsa method and mounted with Entellan. Chromosome counts were made in 20 species belonging to nine genera: *Cochleanthes* (1 species), *Dichaea* (3), *Huntleya* (1), *Pabstia* (1), *Paradisanthus* (1), *Promenaea* (4), *Warczewiczella* (2), *Warrea* (1) e *Zygopetalum* (6). Ideograms are presented for 14 species. Among the chromosomic numbers presented, 13 are previously unpublished counts, one for a genus. Seven counts are of species whose numbers had already been indicated in the literature, but only three of these confirmed the previously published numbers. Except for the polyploidy $2n = 92$, all other numbers found ($2n = 46, 48, 50, 52$) had been previously reported for the subtribe.

Keywords: cytotaxonomy, ideogram, neotropics, Orchidaceae, Zygopetalinae.

Introduction

The subtribe Zygopetalinae (Orchidaceae, Epidendroideae, Cymbidieae) has 36 genera (Pupulin 2009) and about 450 species (WCSP 2015), presenting Neotropical distribution, from southern Mexico to northern Argentina and center of diversity in the Andes. The species of Zygopetalinae show wide morphological variations, but the main characteristics of the subtribe are the flowers spoted or flushed in violet; column with ventral keel; four superposed, dorsiventrally flattened pollinia; and the transverse, narrow, slit-like stigma (Pupulin 2009).

When Zygopetalinae was created by Schlechter (1915), its circumscription was very different from the current. Several genera, now treated within the subtribe, were inserted into Huntleyinae, as well as in other subtribes. Over the years, several classification systems have been proposed for the species of Zygopetalinae (e.g. Garay 1973; Szlachetko 1995). Molecular phylogenies confirmed that the subtribe is monophyletic (Whitten et al. 2000, 2005) and comprises two large groups: the *Zygopetalum* clade with prominent pseudobulbs and plicated leaves and the *Huntleya* clade with pseudobulbs absent or extremely reduced and conduplicated leaves (Whitten et al. 2005).

The determination of chromosome numbers provide important information for a better understanding of the relationships and evolution of clades in a phylogeny, helping the classification and taxonomic positioning of the species (e.g. Koehler et al. 2008; Penha et al. 2011; Pinheiro et al. 2009). Despite this, only about 1% of orchid species have chromosome number known and most of the studies focus in economically important genera. In the case of the subtribe Zygopetalinae, chromosome numbers are known only for about 28 of the 450 species, and even with a good representativeness of genera, some are still missing (Pupulin 2009). The genera of Zygopetalinae with known chromossomic numbers are: *Dichaea*, *2n* = 52 (Félix & Guerra 2000; Tanaka & Kamemoto 1984); *Huntleya*, *2n* = 46 (Dematteis & Daviña 1999); *Koellensteinia*, *2n* = 96 (Tanaka & Kamemoto 1984; Félix & Guerra 2000); *Promenaea*, *2n* = 46, 48 (Tanaka & Kamemoto 1984; Aoyama et al. 1994); *Warrea*, *2n* = 48, 52 (Aoyama 1989; Daviña et al. 2009); *Warczewiczella*, *2n* = 48, 50 (Tanaka & Kamemoto 1984; Aoyama et al. 1994); *Zygopetalum*, *2n* = 48, 96 (e.g. Blumenschein 1960; Daviña et al. 2009); *Aganisia*, *2n* = 70; *Batemannia*, *2n* = 38; *Cochleanthes* *2n* = 50; *Galeottia*, *2n* = 48; *Kefersteinia*, *2n* = 53, 60; *Neogardneria*, *2n* = 48; *Pabstia*, *2n* = 48; *Pescatorea*, *2n* = 50; and *Stenia*, *2n* = 50 (Aoyama et al. 1994).

The aim of this study was to investigate the variation in chromosome number and describe ideograms of species Zygopetalinae, in order to assist in the taxonomic positioning of species.

Material and methods

The 20 species of Zygopetalinae used (Table 1) were mainly collected from the orchid collection at the "Núcleo de Pesquisa Orquidário do Estado", Instituto de Botânica of São Paulo, Brazil. Voucher specimens were deposited in the Herbarium SP, and the living specimens were cultivated in the greenhouse of the Institute.

Root tips were pre-treated with 0,002 M 8-hydroxyquinoleine for 24h at 4 ºC and fixed in 3:1 ethanol-acetic acid for 24h. Roots were transferred to 70% ethanol and freezer stored at -16 ºC. For the slides preparation, the roots were hydrolysed in 5 N HCl for 25 min at room temperature and the squash preparations were made in 45% acetic acid. Coverslips were removed with liquid nitrogen and cells were stained following the Giemsa method described by Guerra (1983), and mounted with Entellan. Chromosome counts were made on at least ten cells in metaphases of each species. The mitotic cells were examined and documented with an optic microscope and digital camera Evolution MP 5.0 Mp.

The chromosome size and centromeric index (CI) were defined using the Adobe Photoshop Program. For each cell, chromosome pairs were defined according to their chromosome length and centromeric position. The mean ideograms for each species were calculated. Total chromatin length (TCL), defined as the sum of the length of all of chromosomes of a cell, was calculated according to Huziwara (1962). The morphological classification of the chromosomes followed the nomenclature proposed by Guerra (1986).

Results

The chromosome numbers of 20 species belonging to nine genera of the subtribe Zygopetalinae are shown in table 2 and figure 1. Ideograms of 14 of these species are presented in figure 2. Metacentric and sub-metacentric chromosomes predominated in all species in which morphological characterization was possible. Only *Zygopetalum pedicellatum* and *Zygopetalum sellowii* presented acrocentric chromosomes. No telocentric chromosomes were observed.

Counts were performed in five genera of the *Zygopetalum* clade (*Pabstia*, *Paradisanthus*, *Promenaea*, *Warrea* and *Zygopetalum*). The chromosome number ranged from $2n$=46 to $2n$=52, besides the polyploid $2n$=92. The chromosomes of the species of the clade varied in length from 1.83 µm in *Pabstia schunkiana* to 0.39 µm in *Warrea warreana*. The largest TCLs were observed in *Zygopetalum pedicellatum*

(36.02 µm), because of its high chromosome number (*2n* = 66) and in *Pabstia schunkiana* (33.96 µm) because of its bigger chromosomes. The smallest TCL was observed in *Promenaea ovatiloba* (18.68 µm).

For the *Huntleya* clade, counts were performed in four genera (*Huntleya*, *Dichaea*, *Cochleanthes* and *Warczewiczella*). The chromosome numbers of the species in this group were practically constant, *2n* = 50, varying only in *Huntleya meleagris*, with *2n* = 46. The chromosomes of the species of the clade varied in length from 1.48 µm in *Warczewiczella wailesiana* to 0.40 µm in *Dichaea cogniauxiana*. Both species also represent the extremes of variation in TCL. The largest TCL was observed in *W. wailesiana* (25.44 µm) and the smallest TCL was observed in *Dichaea cogniauxiana* (16.21 µm).

Discussion

Molecular phylogenies of Whitten et al. (2005) and Hall et al. (Unpublished data) showed that Zygopetalinae comprises two large groups: the *Zygopetalum* clade and the *Huntleya* clade. Several groups can be identified within the *Zygopetalum* clade. The first lineage to emerge in the clade is the *Warrea* alliance sensu Garay (1973), group that is sister of all other species of the clade in a group that Garay (1973) named *Zygopetalum* aliance. *Promenaea* is sister group of all other species in the *Zygopetalum* aliance. A clade formed by *Batemannia*, *Galeottia*, *Neogardneria*, *Pabstia*, *Zygopetalum* and *Zygosepalum* forms the core of the *Zygopetalum* aliance. *Koellensteinia* is polyphyletic and some its members form a clade with *Acacallis* and others form a clade with *Aganisia*, *Cheiradenia* and *Paradisanthus*

The *Warrea* alliance includes the genera *Otostylis*, *Warrea*, *Warreella* and *Warreopsis*. Only *Warrea* has known chromosome numbers. The count of *2n* = 52 for *W. warreana* is different from that found in the literature for the species, *2n* = 48 (Daviña et al. 2009), but similar to that found by Aoyama (1989) in *Warrea costaricensis* Schltr.

The four species of *Promenaea* studied here, *P. ovatiloba*, *P. rollissonii*, *P. silvana* and *P. xanthina*, have *2n* = 46. *Promenaea ovatiloba* and *P. silvana* did not had published numbers, but the numbers here presented are the same of that published for *P. rollissonii* (Tanaka & Kamemoto 1984; as *Promenaea citrina* W. Bull ex Regel). The number found here for *P. xanthina* is different from the literature, *2n* = 48 (Aoyama et al. 1994).

Paradisanthus micranthus, *2n* = 46, is the first species of de genus with known chromosome number. Comparing with related genera, the number is quite different: *Koellensteinia graminea* presents *2n* = ca. 96 (Tanaka & Kamemoto 1984), *Koellensteinia tricolor* also *2n* = ca. 96 (Félix & Guerra 2000) and *Aganisia cyanea* (Lindl.) Rchb.f. presents *2n* = 70 (Aoyama et al. 1994).

The count of *2n* = 48 for *Pabstia schunkiana* had already been reported for *Pabstia jugosa* (Lindl.) Garay, as well as the numbers found in *Galeottia grandiflora* A. Rich. and *Neogardneria murrayana* (Gardner ex Hook.) Schltr. ex Garay (Aoyama et al. 1994). On the other hand, for the genus *Zygopetalum* we found two different chromosome numbers: *2n* = 46 for *Z. crinitum*, *Z. maxillare* and *Z. sellowii*, and *2n* = 92 for *Z. maculatum*, *Z. pedicellatum* and *Z. triste*. Both numbers are new to the genus. For *Z. crinitum*, two different numbers were published in the literature, both different from those reported here, *2n* = 48 (Aoyama et al. 1994) and *2n* = 96 (Tanaka & Kamemoto 1984). The count presented here for *Z. maxillare* differs from all previous counts, always with $2n = 48$ (Blumenschein 1960; Daviña et al. 2009; Aoyama et al. 1994, as *Zygopetalum gautieri* Lem.). For *Z. maculatum* the number presented in the literature is also different from here, n = ca. 24 (Tanaka & Kamemoto 1984; as *Zygopetalum mackayi* Hook). *Batemannia colleyi* Lindl. have a rather different chromosome number from *Pabstia* and *Zygopetalum*, $2n = 38$ (Aoyama et al. 1994). *Zygosepalum* lacks known chromosome numbers and needs further studies.

Pabstia schunkiana, *Z. crinitum* and *Z. maxillare* showed the biggest chromosomes, which could be an indication of the closeness of the genera *Pabstia* and *Zygopetalum*. Among the four species of *Zygopetalum* in which morphological characterization was possible, *Z. sellowii* and *Z. pedicellatum* have morphological characteristics very different from the other species of the genus, which also occurs with the chromosomal characteristics. *Zygopetalum sellowii* showed the smallest chromosome size, and also differs morphologically from the other *Zygopetalum* species by its smaller size. *Zygopetalum pedicellatum*, a polyploid species, has monopodial growth, a unique feature for the species of the *Zygopetalum* clade.

For the *Huntleya* clade, counts were performed in four genera, *Cochleanthes*, *Dichaea*, *Huntleya* and *Warczewiczella*. *Dichaea* and *Huntleya* are the two first lineages to emerge in the clade, with *Dichaea* being sister group of all other species of the group. *Cochleanthes* and *Warczewiczella* are genera from the core of the clade, along with genera such as *Chondrorhyncha*, *Kefersteinia*, *Pescatoria* and *Stenia* (Whitten et al. 2005; Hall et al. unpublished data). With the exception of *H. meleagris*, $2n = 46$, the chromosome number of all species of the *Huntleya* clade in this study was $2n = 50$, corroborating what was shown for several species of the clade (Aoyama 1989). However, *Huntleya meleagris* showed the same chromosome number described in the literature by Dematteis and Daviña (1999).

Dichaea are here represented by three species, all with previously unpublished chromosome numbers, *2n* = 50. This number is different from that presented in the literature for *Dichaea muricata* (Sw.) Lindl. (Tanaka & Kamemoto 1984) and *Dichaea panamensis* Lindl., both *2n* = 52 (Félix & Guerra 2000).

With the recognition of *Warczewiczella* again as a separate genus (Whitten et al. 2005), *Cochleanthes* now has only two species (Pupulin 2009). The previously unpublished chromosome number of *C. flabelliformis*, *2n* = 50, confirms what was found in *Cochleanthes aromatica* (Rchb.f.) RE Schult. & Garay (Aoyama et al. 1994; as *Chondrorhyncha aromatica* (Rchb.f.) PH Allen). *Warczewiczella* is a genus of ten species, two of them here studied, both *2n* = 50, *W. candida* and *W. wailesiana*. The chromosome number of *W. candida* was previously unknown. The number *2n* = 50 corroborate what is presented in the literature for *W. wailesiana* (Aoyama et al. 1994; as *Chondrorhyncha wailesiana* (Lindl.) Hawkes AD), but are different from the number of *Warczewiczella discolor* (Lindl.) Rchb.f., *2n* = ca.48 (Tanaka & Kamemoto 1984).

The chromosome number, *2n* = 50, of *Cochleanthes*, *Dichaea* and *Warczewiczella* corroborate the data of literature for the species of the *Huntleya* clade for: *Pescatoria cerina* (Lindl. & Paxton) Rchb. f., *P. dayana* Rchb.f., *P. lawrenceana* (Rchb. f.) Dressler, *P. violacea* (Lindl.) Dressler e *Stenia guttata* Rchb. f. (Aoyama et al. 1994). Thus, *2n* = 50 is the most common chromosome number of the *Huntleya* clade, x = 25 is most likely the basic number of the clade, and the only species of the clade that present different numbers are: *Dichaea muricata*, *D. panamensis*, *2n* = 52 (Félix & Guerra 2000; Tanaka & Kamemoto 1984), *Huntleya meleagris 2n* = 46 (Dematteis & Daviña 1999), *Kefersteinia graminea 2n* = 53 or 60 (Aoyama et al. 1994) and *Warczewiczella discolor 2n* = ca.48 (Tanaka & Kamemoto 1984). All species of the

Huntleya clade exhibit very similar ideograms to each other, so that taxonomic groupings based on the ideograms are complicated.

With the exception of the polyploid, $2n = 92$, all other numbers found ($2n = 46$, 48, 50 = 52) had been previously reported for the subtribe. However, this study expands the cytogenetic knowledge of the subtribe. For 13 species and one genus the chromosome number have not been previously published, three species presented numbers different of those of the literature and the ideograms here published are the first for the subtribe Zygopetalinae.

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Table 1 – Sample information of the species of Zygopetalinae used in the study.

Species	Live collection number
Cochleanthes flabelliformis (Sw.) R.E. Schult. & Garay	17928
Dichaea cogniauxiana Schltr.	15168
Dichaea pendula (Aubl.) Cogn.	14816
Dichaea trulla Rchb.f.	17539
Huntleya meleagris Lindl.	17312/17951/18147
Pabstia schunkiana V.P. Castro	203*
Paradisanthus micranthus (Barb. Rodr.)Schltr.	902D / 18093
Promenaea ovatiloba (Klinge) Cogn.	202*
Promenaea rollissonii (Lindl.) Lindl.	12878
Promenaea silvana F. Barros & Cath.	201*
Promenaea xanthina (Lindl.) Lindl.	13424
Warczewiczella candida (Lindl.) Rchb. f.	12536
Warczewiczella wailesiana (Lindl.) E. Morren	13620
Warrea warreana (Lodd. ex Lindl.) C. Schweinf.	117*
Zygopetalum crinitum Lodd.	10222
Zygopetalum maculatum (Kunth) Garay	17760
Zygopetalum pedicellatum (Sw.) Garay	17762
Zygopetalum sellowii Rchb. f.	17503
Zygopetalum maxillare Lodd.	Without number
Zygopetalum triste Barb. Rodr.	10543

*Species under cultivation in the private collection of V. T. Rodrigues. All other samples from the Orquidário “Frederico Carlos Hoehne”, Instituto de Botânica, SP.

Table 2 – Chromosome number and karyotype characterization of species of subtribe Zygopetalinae.

Species	*2n*	Chromosome length (µm)	TCL (µm)	karyotype formula
Cochleanthes flabelliformis	50*	1,06 – 0,53	19,07	9M+16SM
Dichaea cogniauxiana	50*	0,93 – 0,40	16,21	13M+12SM
Dichaea pendula	50*	1,04 – 0,41	18,87	16M+9SM
Dichaea trulla	50*	-	-	-
Huntleya meleagris	46	-	-	-
Pabstia schunkiana	48*	1,83 – 1,03	33,96	11M+13SM
Paradisanthus micranthus	46**	1,34 – 0,67	21,59	18M+5SM
Promenaea ovatiloba	46*	1,03 – 0,55	18,68	11M+12SM
Promenaea rollissonii	46	1,38 – 0,90	25,78	17M+6SM
Promenaea silvana	46*	-	-	-
Promenaea xanthina	46#	1,48 – 0,92	27,00	18M+5SM
Warczewiczella candida	50*	-	-	-
Warczewiczella wailesiana	50	1,48 – 0,62	25,44	23M+2SM
Warrea warreana	52#	1,13 – 0,39	20,45	7M+19SM
Zygopetalum crinitum	46#	1,60 – 0,76	26,99	16M+7SM
Zygopetalum maculatum	92*	-	-	-
Zygopetalum maxillare	46#	1,63 – 1,07	29,82	22M+1SM
Zygopetalum pedicellatum	92*	1,14 – 0,52	36,02	25M+20SM+1A
Zygopetalum sellowii	46*	1,15 – 0,73	18,79	13M+9SM+1A
Zygopetalum triste	92*	-	-	-

Unpublished counts for the species * and for the genus * *; # numbers different of the presented in the literature; TCL = total chromatin length; M = Metacentric, SM = Submetacentric, A = Acrocentric.

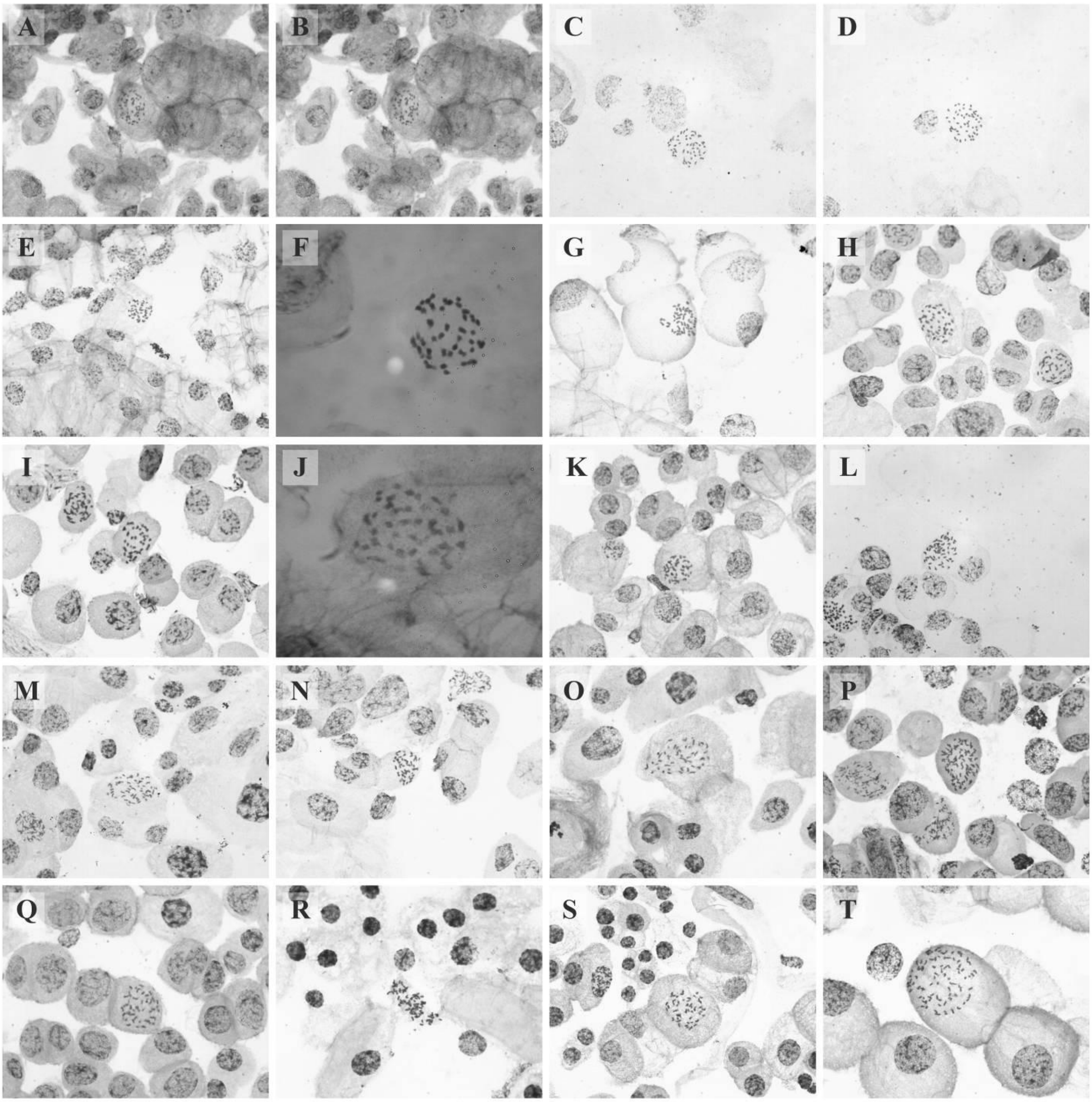

Figure 1 - Chromosomes of species of Zygopetalinae studied. A) *Cochleanthes flabelliformis* (*2n* = 50); B) *Dichaea cogniauxiana* (*2n* = 50); C) *Dichaea pendula* (*2n* = 50); D) *Dichaea trulla* (*2n* = 50); E) *Huntleya meleagris* (*2n* = 46); F) *Pabstia schunkiana* (*2n* = 48); G) *Paradisanthus micranthus* (*2n* = 46); H) *Promenaea ovatiloba* (*2n* = 46); I) *Promenaea rollissonii* (*2n* = 46); J) *Promenaea silvana* (*2n* = 46); K) *Promenaea xanthina* (*2n* = 46); L) *Warczewiczella candida* (*2n* = 50); M) *Warczewiczella wailesiana* (*2n* = 50); N) *Warrea warreana* (*2n* = 52); O) *Zygopetalum crinitum* (*2n* = 46); P) *Zygopetalum maculatum* (*2n* = 92); Q) *Zygopetalum maxillare* (*2n* = 46); R) *Zygopetalum pedicellatum* (*2n* = 92); S) *Zygopetalum sellowii* (*2n* = 46); T) *Zygopetalum triste* (*2n* = 92).

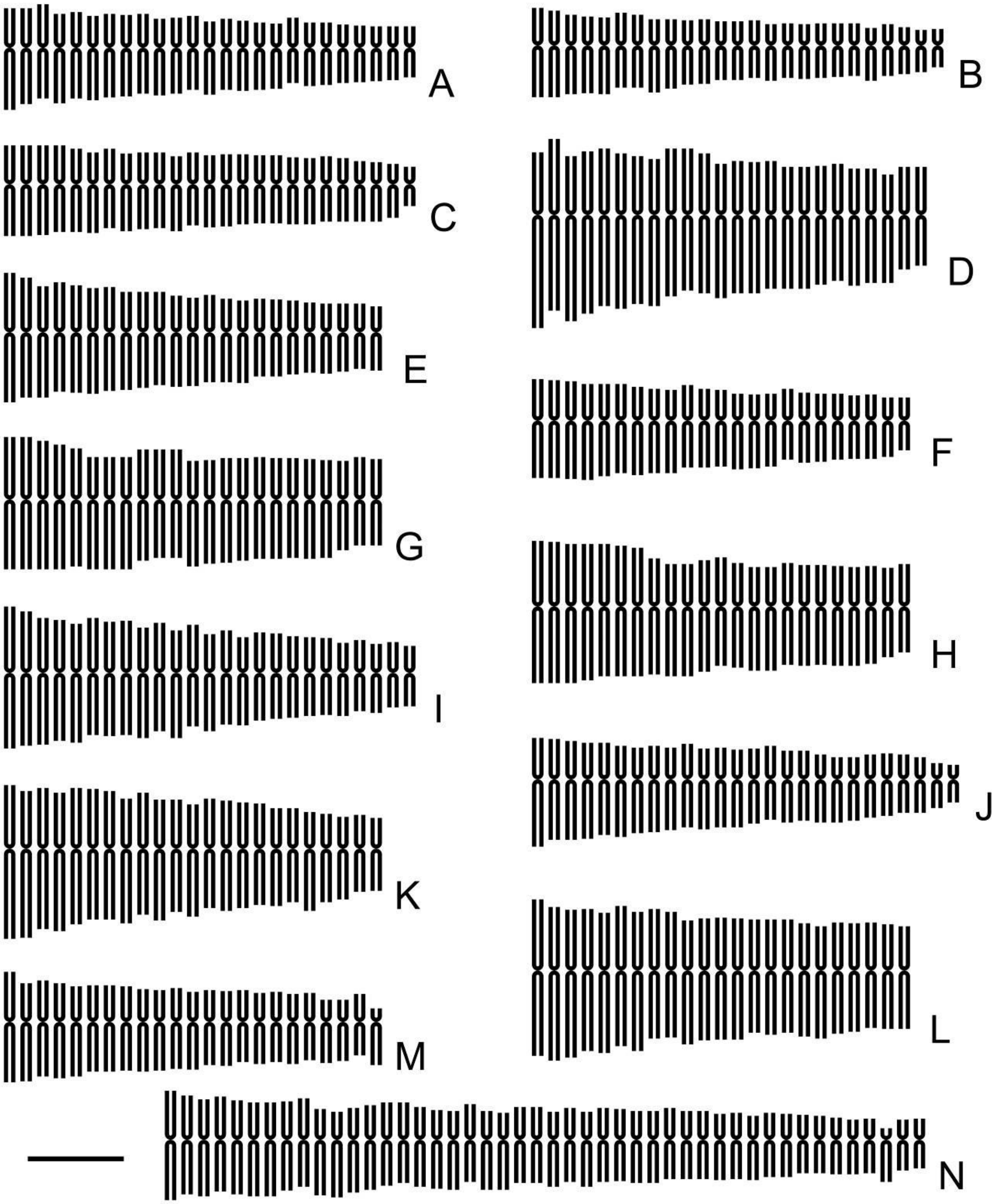

Figure 2 - Ideograms of species of Zygopetalinae studied. A) *Cochleanthes flabelliformis* (*2n* = 50); B) *Dichaea cogniauxiana* (*2n* = 50); C) *Dichaea pendula* (*2n* = 50); D) *Pabstia schunkiana* (*2n* = 48); E) *Paradisanthus micranthus* (*2n* = 46); F) *Promenaea ovatiloba* (*2n* = 46); G) *Promenaea rollissonii* (*2n* = 46); H) *Promenaea xanthina* (*2n* = 46); I) *Warczewiczella wailesiana* (*2n* = 50); J) *Warrea warreana* (*2n* = 52); K) *Zygopetalum crinitum* (*2n* = 46); L) *Zygopetalum maxillare* (*2n* = 46); M) *Zygopetalum sellowii* (*2n* = 46); N) *Zygopetalum pedicellatum* (*2n* = 92). Bar = 1 µm.

CAPÍTULO 3

A taxonomic synopsis of *Koellensteinia* (Zygopetalinae, Orchidaceae)

(Formatado de acordo com as normas da Hoehnea)

A taxonomic synopsis of *Koellensteinia* (Zygopetalinae, Orchidaceae)

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Título resumido: Synopsis of *Koellensteinia*.

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Abstract

(A taxonomic synopsis of *Koellensteinia* (Zygopetalinae, Orchidaceae)) Molecular phylogenies showed that *Koellensteinia* is not monophyletic and the circumscription of the genus needed revision. Therefore, in this paper we present a synopsis of the genus *Koellensteinia* as presently conceived. *Koellensteinia* as here accepted comprises ten species. One species only known by its lost type and one only known by its original illustration were considered as doubtful. Three species were transferred to *Paradisanthus*. Three other species should be transferred from *Koellensteinia*, but more studies are needed to clarify their taxonomic position.

Key words: lectotypifications, new combinations, new synonyms, nomenclature.

Resumo

(Sinopse taxonômica de *Koellensteinia* (Zygopetalinae, Orchidaceae)) Filogenias moleculares mostraram que *Koellensteinia* não é monofilético e a circunscrição do gênero necessitavam ser revisadas. Portanto, neste artigo apresentamos uma sinopse taxonômica do gênero *Koellensteinia* como atualmente concebido. *Koellensteinia* em sua nova circunscrição compreende dez espécies. Uma espécie conhecida apenas pelo seu tipo perdido e uma conhecida apenas pelo sua ilustração original foram consideradas como duvidosas. Três espécies foram transferidos para *Paradisanthus*. *Koellensteinia graninea* e espécies relacionadas devem ser transferidas de gênero, mas mais estudos são necessários para esclarecer a sua posição taxonômica.

Palavras-chave: lectotipificações, nomenclatura, novas combinações, novos sinônimos

Introduction

Koellensteinia Rchb.f. belongs to the subtribe Zygopetalinae (Orchidaceae), being positioned with most of the Brazilian species of the subtribe within the *Zygopetalum* clade, group characterized by rhizomatous plants with heteroblastic pseudobulbs and axillary inflorescences produced in developing pseudobulbs (Garay 1973, Whitten *et al.* 2005).

The Neotropical genus *Koellensteinia* has currently about 15 recognized species distributed in South America and Caribbean (Pupulin 2009, WCSP 2015). Brazil is home to most reported species, about ten, being considered the main center of endemism (Pabst & Dungs 1977, Barros *et al.* 2015, WCSP 2015). Most species occurs in forest or savannah areas from Northern South America, but there are some species in the Brazilian Atlantic Forest and Cerrado (Carnevali *et al.* 2003, Barros *et al.* 2014, WCSP 2014).

The genus is characterized mainly by its plicate leaves, narrowed at base into a petiole; lateral inflorescence, in raceme; flowers resupinate, white, cream, green or yellow, normally flushed and/or spoted or striped with pink, purple or violet; trilobed labellum articulated with the column foot; disc with a bilobed callus; and two subequal pairs of pollinia (Pupulin 2009).

Molecular phylogenies of Whitten *et al.* (2005) and Hall *et al.* (unpublished data) showed that *Koellensteinia* is not monophyletic and the circumscription of the genus needs revision. Therefore, in this paper we present a synopsis of the genus *Koellensteinia* as presently conceived, with comments on the taxonomic position of most species, and some needed new combinations.

Material & methods

Type data were taken from the protologues and from the labels in the type material. The circumscription of the genus was based on the results of the molecular phylogeny of Hall *et al*. (unpublished data). Synonymy and distribution data were based on distribution databases (Barros *et al.* 2015, WCSP 2015) and specialized literature (Pupulin 2009). Acronyms of herbaria names followed Thiers (2012). Abbreviations of authorities for plant names followed Brummitt & Powell (1992), updated in the IPNI (2014).

The taxonomic study of *Koellensteinia* is complicated by the loss of all the holotypes of Schlechter's species due to the bombing of the Berlin Herbarium during the World War II (Merrill 1943, Butzin 1978, Hiepko 1987) and loss of voucher specimens of Barbosa Rodrigues stored in the basement of the house of his son, that was destroyed by a flash flood (Cribb & Toscano de Brito 1996). Luckily, some isotypes and paratypes, and some published or unpublished illustrations are preserved and can be used as taxonomic references.

Taxonomy

Recent molecular phylogenies of Whitten *et al*. (2005) and Hall *et al.* (unpublished data, chapter 1 of this thesis) showed that *Koellensteinia* is not monophyletic and the circumscription of the genus needs revision. Species of *Koellensteinia* used in the study of Hall *et al.* (unpublished data, chapter 1 of this thesis) appears in two different clades. The first clade is the one that has the type species *Koellensteinia kelneriana* Rchb.f. and therefore, will retain the name *Koellensteinia*. Other species present in the study that appear in this clade are *Koellensteinia boliviensis*

(Rolfe ex Rusby) Schltr., *Koellensteinia dasilvae* C.F. Hall & F. Barros (sp. nov., see chapter 5 of this thesis), *Koellensteinia eburnea* (Barb.Rodr.) Schltr. and *Koellensteinia lilacina* C.F. Hall & F. Barros (sp. nov., see chapter 6 of this thesis). Three species of *Koellensteinia* that did not appear together with the other species and should be transferred from the genus are: *Koellensteinia graminea* (Lindl.) Rchb.f., *Koellensteinia altissima* Pabst, and *Koellensteinia spirallis* Gomes Ferreira & L.C.Menezes.

Koellensteinia in its new circumscription comprises ten species. One species only known by its lost type and one only known by its original illustration were considered as doubtful. Three species and five names were transferred to *Paradisanthus*. Three species should be transferred from *Koellensteinia,* but more studies are needed to clarify their taxonomic position, as a separate genus or within a previously known different genus.

1. *Koellensteinia* Rchb.f., Bonplandia (Hannover) 2: 17 (1854).

1.1. *Koellensteinia boliviensis* (Rolfe ex Rusby) Schltr., Orchis 12: 32 (1918) ≡ *Aganisia boliviense* Rolfe, Bulletin of the New York Botanical Garden 4: 448. 1907.

Type:— BOLIVIA. 1883 - 1889, *Bang 2909* (MO).

Distribution:— Bolivia.

1.2. *Koellensteinia carraoensis* Garay & Dunst., Venez. Orchids Ill. 6: 166 (1976).

Type:— VENEZUELA. Río Carrao, n/d, *Dunsterville 306* (AMES).

Distribution:— French Guiana, Suryname, Venezuela.

1.3. *Koellensteinia dasilvae* C.F. Hall & F. Barros, sp. nov.

Type:— BRAZIL. Amapá: Serra do Navio, 0°56'10.93"N, 51°54'51.15"W, 170 m, 15 October 2014, *C.F. Hall & A.K. Koch 1110* (SP).

Distribution:— Brazil (AP).

Note:— *Koellensteinia dasilvae* is morphologically similar to *K. hyacinthoides*, specially by the yellow flowers. However, differs mainly by: smaller size of the plant and leaves, larger inflorescence, >1/2 as long as the leaves with peduncle larger than raquis, and margin of labellum minutely erose. Additionally, *K. dasilvae* specimens were found growing as saxicolous or epiphyte in upland forest, while *K. hyacinthoides* are mainly terrestrial of white sand savannahs (e.g. Carnevali *et al*. 2003).

1.4. *Koellensteinia eburnea* (Barb.Rodr.) Schltr., Orchis 12: 28 (1918) ≡ *Cyrtopodium eburneum* Barb.Rodr., Gen. Spec. Orchid. 2: 182 (1882).

= *Cyrtopodium album* Barb.Rodr., Vellosia, ed. 2, 1: 127 (1891).

= *Koellensteinia peruviana* Schltr., Orchis 12: 28 (1918).

Type:— BRAZIL. Minas Gerais, São João Del Rey, s.d., *Barbosa Rodrigues s.n.* (lectotype original illustration in Barbosa Rodrigues 1882: tab. 208, here designed).

Distribution:— Brazil, Guyana, Suriname, Venezuela, Peru.

Note:— *Koellensteinia eburnea* herbarium material from central Brazil are commonly misidentified as *Koellensteinia tricolor*. However, the type of *Koellensteinia tricolor* from Guyana and is described as having green flowers with white labellum. Populations from central Brazil have white to cream flowers and should be treated as *Koellensteinia eburnea*.

1.5. *Koellensteinia hyacinthoides* Schltr., Beih. Bot. Centralbl. 42(2): 126 (1925).

Type:— BRAZIL. Amazonas, Taracua, Rio Uaupes, March 1924, *Hübner 183* (B).

Distribution:— Brazil, Colômbia, French Guyana, Suriname and Venezuela.

Note:— The type of *Koellensteinia hyacinthoides* was destroyed in Berlin. *Koellensteinia hyacinthoides* is morphologically similar to *K. dasilvae*, especially by the yellow flowers. However, differs mainly by: larger size of the plant and leaves, smaller inflorescence, <1/2 as long as the leaves, with peduncle larger than rachis, and labellum with midlobe margin entire, and lateral lobes margin dentate. Additionally, *K. hyacinthoides* are mainly terrestrial of white sand savannahs (e.g. Carnevali *et al*. 2003), while *K. dasilvae* specimens were found growing as saxicolous or epiphyte in upland forest.

1.6. *Koellensteinia ionoptera* Linden & Rchb.f., Gard. Chron. 1871: 1451 (1871) ≡ *Aganisia ionoptera* (Linden & Rchb.f.) G.Nicholson, Ill. Dict. Gard. 1: 35 (1885) ≡ *Paradisanthus ionopterus* (Linden & Rchb.f.) Schltr., Orchideen Beschreib. Kult. Zücht.: 419 (1914).

Type:— PERU. s.d., *Wallis s.n.* (W).

Distribution:— Ecuador and Peru.

1.7. *Koellensteinia kellneriana* Rchb.f., Bonplandia (Hannover) 2: 17 (1854).

Type:— VENEZUELA. Trujillo, December 1850, *Wagener 19* (W).

Distribution:— Brazil, Colômbia, French Guyana, Guyana, Suriname and Venezuela.

Note:— *Koellensteinia kellneriana* is closely related to *Koellensteinia tricolor* a species with type described from Guyana. Both species have green flowers with white labellum and the exact relationship between them still need to be clarified.

1.8. *Koellensteinia lilacina* C.F. Hall & F. Barros, sp. nov.

Type:— BRAZIL. Mato Grosso, Chapada dos Guimarães, Ribeirão do Rocandor, 15°6'30.96"S, 55°6'33.61"W, 08 February 2014, *C.F. Hall & A. Francener 1080* (SP).

Distribution:— Brazil

Note:— *Koellensteinia lilacina* is closely related to *K. eburnea* and *K. lineata* by its flower size and shape of sepals and petals. However, *Koellensteinia lilacina* can be differentiated mainly by its flowers that are tighter, lilac, and with a yellow callus in the labellum, the arcuate inflorescence, and the pseudobulbs that are normally semi-spherical.

1.9. *Koellensteinia lilijae* Foldats, Bol. Soc. Venez. Ci. Nat. 22: 263 (1961).

Type:— VENEZUELA. Amazonas, Laja Cabezón, on the banks of the River Cabezón, near its confluence with River Atabapo, 11 September 1960, *Foldats 3899* (VEN).

Distribution:— Venezuela.

Note:— *Koellensteinia lilijae* is a rare species endemic from Venezuela that is easily recognized by its 3-lobed callus.

1.10. *Koellensteinia tricolor* (Lindl.) Rchb.f. in W.G.Walpers, Ann. Bot. Syst. 6: 552 (1863) ≡ *Zygopetalum tricolor* Lindl., Edwards's Bot. Reg. 32: t. 64 (1846).

Type:— GUYANA. s/d, *Loddiges 931* (K).

Distribution:— Brazil, Guyana, Peru and Venezuela.

Note:— *Koellensteinia tricolor* is a name commonly used to identify specimens of *Koellensteinia* from central Brazil. However, this is a misidentification of *Koellensteinia eburnea*. The type of *Koellensteinia tricolor* is from Guyana and is described as having green flowers with white labellum. In this sense, *Koellensteinia tricolor* seems to be closely related to *K. kellneriana*, however, the exact relationship between these two species still needs to be clarified.

2. Doubtful taxa

2.1. *Koellensteinia lineata* (Barb.Rodr.) Garay, Orquideologia 8: 23 (1973) ≡ *Cyrtopodium lineatum* Barb.Rodr., Contr. Jard. Bot. Rio de Janeiro 1: 52 (1901).

Type:— BRAZIL. Minas Gerais: São João Del Rey. s.d., *Barbosa Rodrigues s.n.* (lectotype original illustration in Barbosa Rodrigues 1901: tab. VI, here designated).

Distribution:— Brazil.

Note:— This taxon was considered as doubtful because it is only known from the original illustration of Barbosa Rodrigues (1901). This species is probably a synonym of *Koellensteinia eburnea*, but the original illustration shows a spotted pattern in the petals, which is not found in *K. eburnea*.

2.2. *Koellensteinia roraimae* Schltr., Orchis 12: 29 (1918).

Type:— GUYANA. Roraima mountain, December 1909, *Ule 8582* (B). Neotype original illustration in Schlechter 1918: 27, here designated).

Distribution:— Guyana.

Note:— This taxon was considered as doubtful because it is only known from the type specimen, which was destroyed in Berlin.

3. Excluded taxa

Two species of *Koellensteinia* appears as close related to *Paradisanthus* in the molecular phylogeny of Hall *et al.* (unpublished data, chapter 1 of this thesis), *Koellensteinia altissima* Pabst and *Koellensteinia spiralis* Gomes Ferreira & L.C.Menezes. Both species and related ones should be transferred to *Paradisanthus*.

3.1.1. *Paradisanthus altissimus* (Pabst) C.F. Hall & F. Barros, **comb. nov.** ≡ *Koellensteinia altissima* Pabst, Anais Acad. Brasil. Ci. 34 (Res. Comm.): xlv (1962).

= *Koellensteinia abaetana* L.P.Queiroz, **syn. nov.**, Revista Brasil. Bot. 10: 21 (1987).

= *Koellensteinia colnagoi* Ruschi, Bol. Mus. Biol. Prof. Mello-Leitão. Sér. Bot. 80: 1 (1974).

Type:— BRASIL. Bahia, Porto Seguro, September 1961, *Duarte s.n.* (HB)

3.1.2. *Paradisanthus floridus* (Rchb.f.) C.F. Hall & F. Barros, **comb. nov.** ≡ *Promenaea florida* Rchb.f., Hamburger Garten- Blumenzeitung 19: 12. 1863 ≡ *Koellensteinia florida* (Rchb.f.) Garay, Orquideologia 8 (1): 23. (1973)

Type:— BRASIL. S. loc., s.d., *Schiller s.n.* (W).

3.1.3. *Paradisanthus spiralis* (Gomes Ferreira & L.C.Menezes) C.F. Hall & F. Barros, comb. nov. ≡ *Koellensteinia spiralis* Gomes Ferreira & L.C.Menezes, Bol. CAOB 29: 87 (1997).

Type:— BRAZIL. Bahia: Feira de Santana, March 1997, *Manoel Silva s/n* (UFP).

In the molecular phylogeny of Hall *et al.* (unpublished data, chapter 1 of this thesis) *Koellensteinia graminea* (Lindl.) Rchb.f., appears apart from all other species of *Koellensteinia*. However, its positionig as a new genus or lumped with other genera is still uncertain. Two other species close related to *K. graminea* should also be excluded from *Koellensteinia sensu stricto*: *K. elegantula* and *K. graminoides*.

3.2.1. *Koellensteinia elegantula* Schltr., Repert. Spec. Nov. Regni Veg. Beih. 7: 161 (1920).

Type:— COLOMBIA. Cauca, 300m, s.d., *Madero s.n.* (B).

Distribution:— Colombia

3.2.2. *Koellensteinia graminea* (Lindl.) Rchb.f., Bonplandia (Hannover) 4: 323 (1856) ≡ *Aganisia graminea* (Lindl.) Benth., J. Linn. Soc., Bot. 18: 321. (1881) ≡ *Maxillaria graminea* Lindl., Edwards's Bot. Reg. 21: sub t. 1802. (1836) ≡ *Promenaea graminea* (Lindl.) Lindl., Edwards's Bot. Reg. 29 (misc.): 13. 1843.

Type:— GUYANA. Demerara, January 1835, *Loddiges s.n.* (K).

Distribution:— Bolivia, Brazil, Colombia, Ecuador, French Guyana, Guyana, Peru, Puerto Rico, Suriname and Venezuela,

3.2.3. ***Koellensteinia graminoides*** **D.E.Benn. & Christenson**, Brittonia 46: 37 (1994).

Type:— PERU. Loreto, Iquitos, 250m, May 1965, *Dodson ex Bennett 1483* (USM).

Distribution:— Peru.

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CAPÍTULO 4

Reinstatement of the name *Zygopetalum mosenianum* Barb.Rodr. (Orchidaceae), an overlooked name formerly synonymized to *Zygopetalum pedicellatum* (Thunb.) Garay

(Formatado de acordo com as normas da Phytotaxa)

Reinstatement of the name *Zygopetalum mosenianum* Barb.Rodr. (Orchidaceae), an overlooked name formerly synonymized to *Zygopetalum pedicellatum* (Thunb.) Garay

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Abstract

In this paper we propose the restatement of the name *Zygopetalum mosenianum* Barb.Rodr. (Orchidaceae) an overlooked name formerly synonymized to *Zygopetalum pedicellatum* (Thunb.) Garay.

Key words: taxonomy, nomenclature, *Zygopetalum*

Introduction

Shortly after its creation, the genus *Zygopetalum* Hooker (1827: t. 2748) included several members of related Zygopetalinae genera, such as *Warczewiczella* Reichenbach (1852: 635) and *Koellensteinia* Reichenbach (1854: 17). However, Garay (1973a) reviewed the genus and placed it close to its current circumscription. Today, *Zygopetalum* comprises about 14 South American species, occurring in Argentina, Bolivia, Brazil, Paraguay and Peru (Pupulin 2009), eleven of which are endemic from Brazil (Barros *et al.* 2013).

When Garay (1973b) proposed to transfer *Cymbidium pedicellatum* Thunb. (1818: 19) to *Zygopetalum*, apparently he did not consider that this name is a later homonym of the previous published *Cymbidium pedicellatum* Swartz (1799: 224). Thus, the name *Cymbidium pedicellatum* Thunb. is illegitimate according to the article 53 of the International Code of Nomenclature for algae, fungi, and plants (McNeill *et al.* 2012), and therefore the name *Zygopetalum pedicellatum* (Thunb.) Garay is also illegitimate.

It is worth noting that the new combination of Garay (1973b) was based on the name of Thunberg, because there are indexes that indicate the new combination as *Zygopetalum pedicellatu*m (Sw.) Garay, assuming therefore that Garay (1973b) proposed the new combination based on the name established by Swartz (1799). The combination *Zygopetalum pedicellatum* (Sw.) Garay, is therefore also illegitimate, being a later homonym of *Zygopetalum pedicellatum* (Thunb.) Garay, even though the later name is itself illegitimate.

In this situation, the reinstatement of the name *Zygopetalum mosenianum* Barbosa Rodrigues (1877: 108) is necessary. This name, currently treated as a synonym, is the first available valid name for the species here concerned. *Zygopetalum mosenianum* was established by Barbosa Rodrigues (1877) and illustrated in the plate

234, of the fifth volume of his "Iconographie des Orchidées du Brésil", reproduced in Sprunger *et al.* (1996).

***Zygopetalum mosenianum* Barb.Rodr**., Genera et Species Orchidearum Novarum 1: 108. 1877. Type:—BRAZIL. São Paulo: Santos, *H. Mosén ex Regnell 3227* (holotype S!).

Synonyms: = *Zygopetalum caulescens* Rolfe, Gard. Chron., III, 7: 544. 1890.
= *Cymbidium pedicellatum* Thunb., Plantarum brasiliensium: decas prima 2: 19. 1818 [non *Cymbidium pedicellatum* Sw., Journal für die Botanik 2: 224. 1799, nec *Cymbidium pedicellatum* Finet, Bulletin de la Société Botanique de France 47: 268. 1900] ≡ *Zygopetalum pedicellatum* (Thunb.) Garay, Bradea 1: 305. 1973.

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CAPÍTULO 5

Koellensteinia dasilvae (Zygopetalinae-Orchidaceae), a new species from the Brazilian Amazon

(Formatado de acordo com as normas da Nordic Journal of Botany)

Koellensteinia dasilvae (Zygopetalinae-Orchidaceae), a new species from the Brazilian Amazon

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A new species of *Koellensteinia* from the Brazilian Amazon is described and illustrated. *Koellensteinia dasilvae* C.F. Hall & F. Barros (Orchidaceae) grows as epiphyte or saxicolous, close to streams in upland forests (terra firme) of the state of Amapá, Northern Brazil. The new species resembles *Koellensteinia hyacinthoides*, sharing morphological affinities with this species, but differs by growing inside forests; the smaller size of plant, leaves and petiole; the larger inflorescence with large peduncle and small rachis; labellum, when spread, broader between the lateral lobes than at the midlobe; margin of labellum minutely erose; and short wings of the column.

Keywords: Neotropics, orchid, taxonomy.

Introduction

Koellensteinia Rchb.f. belongs to the subtribe Zygopetalinae (Orchidaceae), being positioned with most of the Brazilian species of the subtribe within the "*Zygopetalum* complex" in the "*Zygopetalum* grade". This grade is characterized mainly by rhizomatous plants with heteroblastic pseudobulbs, axillary inflorescences produced in developing pseudobulbs and plicate leaves (Garay 1973, Whitten et al. 2005, Pupulin 2009). The study of Whitten et al. (2005) included three species of *Koellensteinia* in the molecular phylogeny of Zygopetalinae, and showed that the genus in its current circumscription is polyphyletic, with the sampled species falling into two distinct clades, presenting unclear relationships with the genera *Aganisia*, *Otostylis* and *Paradisanthus*.

This small-sized Neotropical genus has about 18 recognized species distributed from Central America and the Caribbean to South America (Pupulin 2009, WCSP 2015). Brazil is home to most reported species, about ten, being considered the main center of endemism (Pabst and Dungs 1977, Pupulin 2009, Barros et al. 2015, WCSP 2015). However, there are also endemic *Koellensteinia* species from Bolivia, Colombia and Venezuela (WCSP 2015), most of the species occurs in forest or savannah areas from Northern South America, but there are some species in the Atlantic Rainforest and Cerrado (Carnevali et al. 2003, Barros et al. 2014, WCSP 2014).

The genus is characterized mainly by its terrestrial, saxicolous or epiphytic habit; pseudobulbs often rudimentary or cryptic, heteroblastic, concealed by sheaths when young; plicate leaves, narrowed at base into a petiole; lateral inflorescence, in raceme; flowers resupinate, white, cream, green or yellow, normally flushed and/or spoted or striped with pink, purple or violet; trilobed labellum articulated with the column foot; disc with a bilobed callus; and two subequal pairs of pollinia (Pupulin 2009).

During the preparation of a molecular and taxonomic study of *Koellensteinia*, which included the examination of herbarium specimens and new collections, we found plants that were distinct from the remaining species of the genus. Morphologically, the specimens are similar to *Koellensteinia hyacinthoides* Schltr. (1925: 126), nevertheless proved to belong to a new species. Therefore, here we describe a new *Koellensteinia* species from the Brazilian Amazon.

Material & methods

This study was developed by the examination of specimens collected in situ and grown in greenhouses of the Instituto de Botânica, where flourished. Based on morphological studies of dry and living material, we described and elaborated nankin illustrations of this new species of *Koellensteinia*. Voucher specimens were pressed according to Fidalgo and Bononi (1984) and deposited at the herbarium SP. General morphological terms were based on Radford et al. (1974), Stearn (1983) and Harris and Harris (1994). Terms for specific features of Orchidaceae were based on Dressler (1993).

Taxonomy

Koellensteinia dasilvae C.F. Hall & F. Barros, sp. nov. (Figs. 1 a–f, 2 a-b)

Similis Koellensteinia hyacinthoides sed plantis minoribus, foliis minoribus, inflorescentia majori, pedunculo inflorescentiae quam rachis longiori, distantia inter *apices loborum lateralium labelli distentii majori quam latitude lobi medii, margine labelli minute eroso, alis gymnostemii brevioribus differt.*

Koellensteinia dasilvae is closely related to *K. hyancinthoides* differing by the smaller size of the plant; smaller leaves; larger inflorescence with the peduncle larger than the rachis; labellum, when spread, broader between the lateral lobes than at the midlobe, margin of labellum minutely erose; and short wings of the column.

Type: Brazil. Amapá: Serra do Navio, 0°56'10.93"N, 51°54'51.15"W, 170 m, flowering in cultivation, 15 Oct 2014, *C.F. Hall & A.K. Koch 1110* (holotype SP!).

Herbs, epiphyte or saxicolous, pseudobulbous, caespitose. Roots grayish-white, produced on the rhizome at the base of the pseudobulbs, 0.3–0.4 cm diam., cylindrical, filiform, glabrous. Rhizome short. Pseudobulbs ca. 2.0–2.5 × 0.4 cm, cylindrical, sometimes a little swollen at base, heteroblastic, 1-leaved, enveloped by leaf sheaths. Sheaths papyraceous, multiveined, wrapping up the petiole, those of older pseudobulbs shredded, the largest 6.0–9.5 × 0.6–0.7 cm, oblong, apex acute. Leaves glaucous-green, flexuous, narrowed to the base in a petiole; petiole 4.0–6.0 cm long, conduplicate, apically articulated with the leaf blade and basally with the pseudobulb; leaf edblade herbaceous, plicate, veins abaxially ribbed, 17.3–25.0 × 3.7–6.5 cm, elliptic, margin entire, apex acute. Inflorescence in a lateral raceme, 17.0-21.5 cm long, cylindrical-erect, lax, 5-10-flowered; peduncle vinaceous, turning green towards the apex, 12.5–15.0 cm long; rachis green, 4.5–9.5 cm long; bracts of the peduncle scarious, 0.5–1.5 × 0.3–0.6 cm, broadly triangular to triangular, base amplexicaul to truncate, apex acute to acuminate; floral bracts green, 0.3–0.7 × 0.2–0.3 cm, triangular, base truncate, apex acute. Flowers yellow, resupinate, pedicellate; ovary with pedicel 9.0–11.0 mm long, cylindrical, slightly thickened towards the apex; dorsal sepal yellow, 1.1–1.4 × 0.4–0.5

cm, elliptic-lanceolate, margin entire to minutely erose near the apex, apex acute; lateral sepals yellow, 1.0–1.2 × 0.5 cm, oblong-lanceolate to obovate-lanceolate, margin entire to minutely erose near the apex, apex acute; lateral petals yellow, 1.0–1.1 × 0.4–0.5 cm, oblong, slightly falcate, margin entire to minutely erose near the apex, apex acute; labellum yellow with brown-red blotches in the base, 3-lobed, 0.6–0.8 × 0.9–1.2 cm, conduplicate near the apex, margin minutely erose, articulated with the column foot; midlobe ca. 0.4 × 1.0 cm, reniform, apex acute; lateral lobes ca. 3.0–4.0 × 3.0 mm, widely oblong, apex rounded; callus prominent, bilobed, with dorsal brown-red blotches; column greenish-yellow, 4.0–5.0 × 3.0 mm, clavate, slightly curved, winged; column foot r brown-red, 2.0 mm long; wings bilobate; anther terminal, operculate; anther cap 2-locular, ca. 2.0 × 2.5 mm, apex gibbous; clynandrium shallow, margin prominent at apex; pollinarium 2.0 × 2.0 mm with two pairs of sub-equal, dorsi-ventraly flattened, clavate pollinia, the largest ca. 1.5 × 0.5 mm, on a rhomboid structure formed by a stipe and viscidium; rostelum 3-toothed; stigma transverse, narrow. Fruit green, 1.2–2.2 × 0.3–0.7 cm, fusiform.

Distribution and habitat

Koellensteinia dasilvae is known only from the municipality of Serra do Navio, state of Amapá, Brazil (Fig. 3), growing as epiphyte or saxicolous, close to streams. The vegetation of the collection site is an upland forest (terra firme) in the Araguari River Basin. The specimens were collected in the field in August, when there were still individuals with fruits. Individuals, that were gathered in situ, flourished in greenhouses from October to December.

Etymology

The new species honors João Batista F. da Silva, a Brazilian orchidologist, the first to recognize this species as a possible novelty and who gave information on its habitat.

Conservation assessment

Koellenteinia dasilvae is known to only one locality in the Amazon. The population, represented by a few aggregated individuals, is found in an area relatively close to the Montanhas do Tumucumaque National Park. Even though this population was found near to a conservation unit, there is no proof that it occurs within its borders. Historically many botanists visited the Amazon looking for orchids, but other populations of the species were never found. Additionally, only a few individuals were observed in the wild, and only one occurrence area is known, therefore this species should be considered Critically Endangered (CR) according to the IUCN (2014) criteria.

Affinities and notes on critical characters

Koellensteinia dasilvae is morphologically similar to *K. hyacinthoides*, specially by the yellow flowers. However, differs mainly by: smaller size of the plant, 23–33 cm tall (vs. 45–60 cm tall in *K. hyacinthoides*); smaller and proportionally broader leaves, 21–31 × 3.7–6.5 cm (vs. 39–55 × 8 cm); inflorescence 6–12-flowered (vs. 10–20-flowered), larger (17–21.5 cm vs. 10–16 cm long), >1/2 as long as the leaves; peduncle vinaceous, turning green towards the apex, larger than raquis; labellum, when spread, broader between the lateral lobes than at the midlobe, margin of labellum minutely erose (at stereo microscope); short wings of the column. *Koellensteinia hyacinthoides* is a larger plant with larger leaves; inflorescence pale green, <1/2 as long as the leaves, with peduncle shorter than raquis; labellum, when spread, narrower between the lateral lobes than at the midlobe, margin of midlobe entire and margin of lateral lobes dentate; larger wings of the column (Schlechter 1925, Dunsterville and Garay 1976, Carnevali et al. 2003). Additionally, *K. dasilvae* specimens were found growing as saxicolous or epiphyte in upland forest, while *K. hyacinthoides* are mainly terrestrial of white sand savannahs (e.g. Carnevali et al. 2003).

Additional specimens examined (paratypes)

Brazil: Amapá: Serra do Navio, ca. 0°56'10.93"N, 51°54'51.15"W, 170 m, fr., 7 Aug 2013, *C.F. Hall & A.K. Koch 1000* (SP); flowering in cultivation, 17 Oct 2014, *C.F. Hall & A.K. Koch 1111* (SP); 13 Nov 2014, *C.F. Hall & A.K. Koch 1112* (SP); 29 Nov 2014, *C.F. Hall & A.K. Koch 1113* (MG); 5 Dec 2014, *C.F. Hall & A.K. Koch 1114* (MG).

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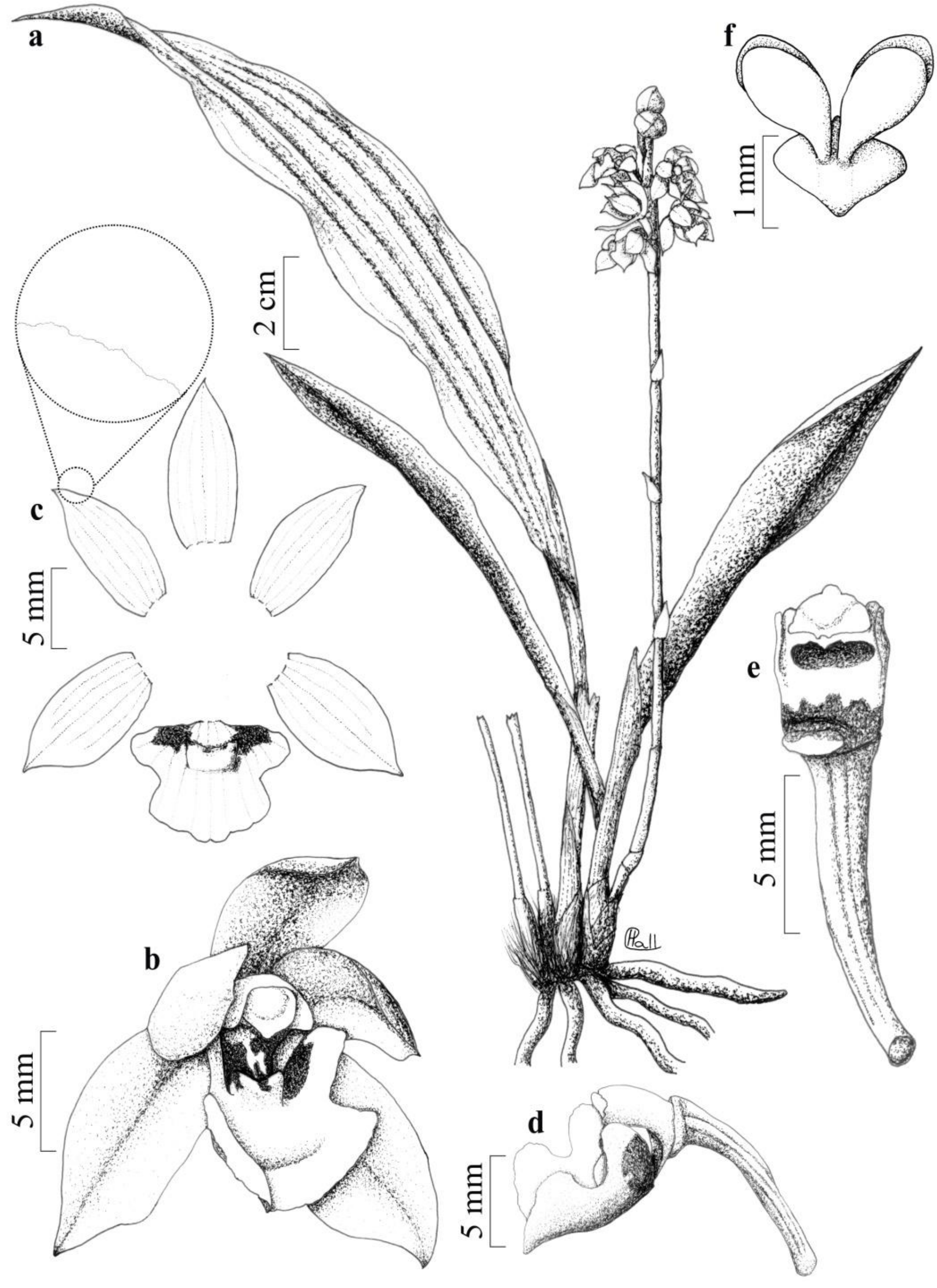

Figure 1. *Koellensteinia dasilvae* sp. nov. (a) Habit, (b) Detail of flower, (c) Dissected flower, (d) Column+labellum (lateral view), (e) Column front view, (f) Pollinarium. Illustrations by Climbiê Ferreira Hall.

Figure 2. *Koellensteinia dasilvae sp. nov.* (a) Habit, (b) Detail of flower. Photos by Climbiê Ferreira Hall.

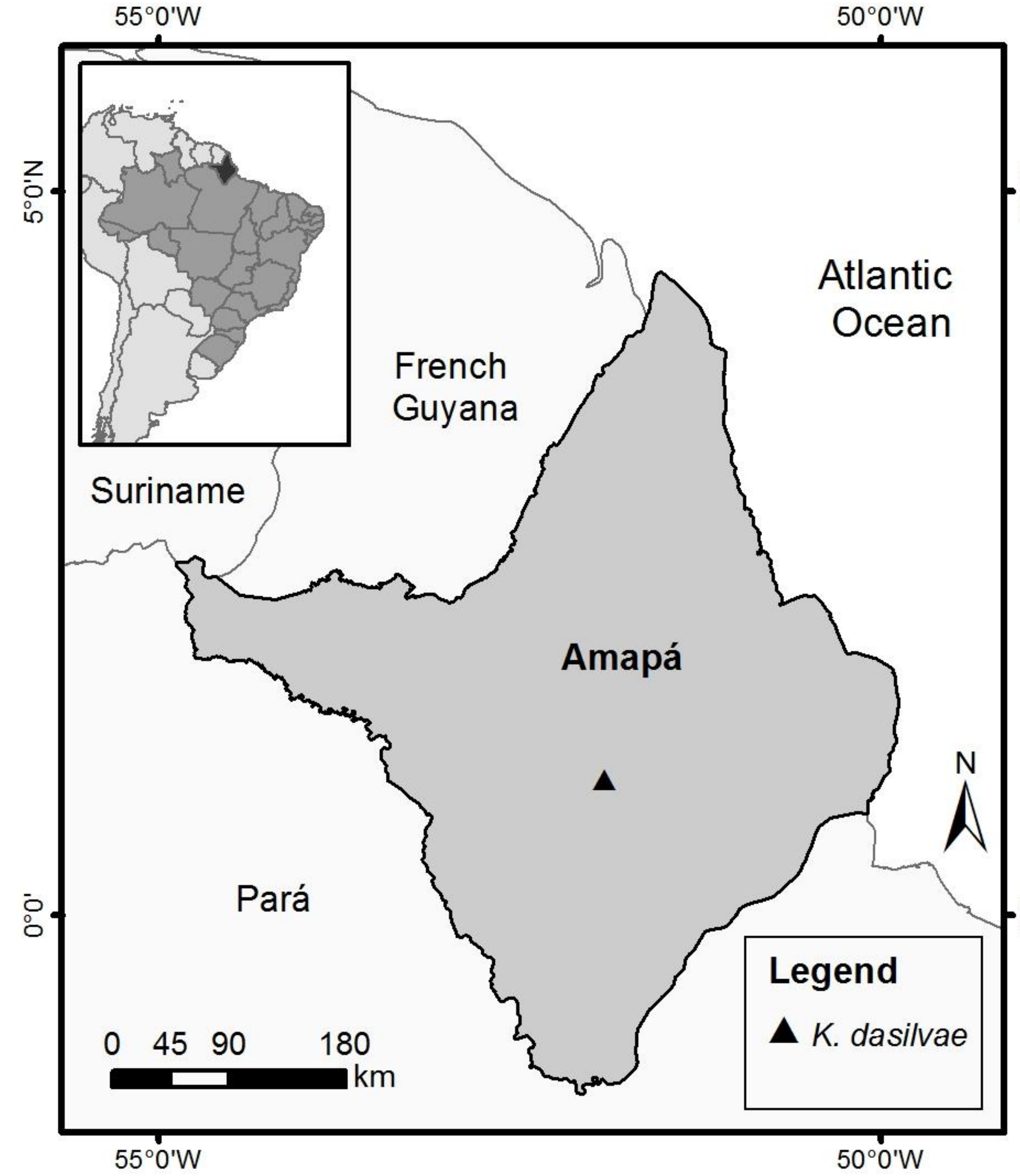

Figure 3. Geographic distribution of *Koellensteinia dasilvae* sp. nov.

CAPÍTULO 6

Koellensteinia lilacina (Zygopetalinae-Orchidaceae), a new species from the Brazilian Cerrado

(Formatado de acordo com as normas da Phytotaxa)

Koellensteinia lilacina (Zygopetalinae-Orchidaceae), a new species from the Brazilian Cerrado

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Abstract

Koellensteinia lilacina C.F. Hall & F. Barros (Orchidaceae), a new species from Mato Grosso, Brazilian Midwest, is described and illustrated. This species grows as terrestrial, near streams of water in riparian forests of the Cerrado biome. The new species is related to *Koellensteinia eburnea* and *Koellensteinia lineata,* sharing morphological affinities with them, but differs mainly by the tighter lilac flowers with yellow callus.

Keywords*:* Cerrado biome, Neotropics, Brazilian Midwest, Taxonomy.

Introduction

Koellensteinia Reichenbach f. (1854: 17) is a Neotropical genus with about 15 species distributed in South America and the Caribbean (WCSP 2015). Brazil is the center of endemism of the genus, with about 10 species recorded, growing in the Amazon, Brazilian Atlantic Forest and Cerrado (Barros *et al.* 2014; Pabst & Dungs 1977; WCSP 2014).

The most common features of the genus are the plicate leaves, lateral inflorescence in raceme, flowers normally flushed and/or spotted or striped with pink, purple or violet, 3-lobed labellum and disc with a 2-lobed callus (Pupulin 2009). *Koellensteinia* belongs to the subtribe Zygopetalinae and is positioned with most Brazilian species of the group within the *Zygopetalum* clade (Garay 1973; Whitten *et al.* 2005).

During examination of herbarium specimens and new collections of *Koellensteinia* we found some specimens that are distinct from the remaining species of the genus, although morphologically similar to *Koellensteinia eburnea* Schltr. (1918: 28) and *Koellensteinia lineata* (Barb. Rodr.) Garay (1973: 23). These specimens, collected in the Cerrado Biome, state of Mato Grosso, Central Brazil, were considered as a new species, which is described and illustrated here.

Material & methods

We performed this study by examining individuals that were previously collected *in situ* and flourished in greenhouses of the Instituto de Botânica, São Paulo, Brazil. Based on morphological studies of dry and living material, we described and illustrated this new species. Voucher specimens were pressed according to Bridson & Forman (1992) and deposited at the Herbarium SP. Morphological terms were based on Radford et al. (1974), Stearn (1983), Harris & Harris (1994) and Dressler (1993).

Taxonomy

Koellensteinia lilacina **C.F. Hall & F. Barros,** ***sp. nov***. (Figs. 1 and 2 a-b).

Koellensteinia lilacina is closely related to *K. eburnea* and *K. lineata*, differing mainly by its flowers that are tighter, lilac, and with a yellow callus in the labellum, the arcuate inflorescence, and the pseudobulbs that are normally semi-spherical.

Type:— BRAZIL. Mato Grosso: Chapada dos Guimarães, Ribeirão do Rocandor, 15°6'30.96"S, 55°6'33.61"W, flowering in greenhouse in February 08, 2014, *C.F. Hall & A. Francener 1080* (holotype SP!).

Herbs, terrestrial, caespitose. Roots grayish-white, cylindrical, filiform, glabrous, 0.2–0.3 cm diam., produced on the base of pseudobulbs. Rhizome short. Pseudobulbs sub-spherical to pyriform, heteroblastic, 1-leaved,1.0–2.0 × 0.9 cm, enveloped by leaf sheaths. Sheaths linear, papyraceous, multiveined, 5.0–8.7 × 0.6–1 cm, wrapping up the petiole, apex slightly acuminate. Leaf lanceolate to oblanceolate, green, arched, apex acute; base attenuate in a petiole, 4.2–8.5 cm long, conduplicate; leaf blade, 16–32 × 2–3.9 cm, sub-cartaceous, plicate, veins abaxially ribbed. Inflorescence in raceme, lateral, ca. 40 × 0.3 cm, cylindrical, arcuate, lax, ca. 12-flowered; peduncle wine colored, turning green towards the apex, ca. 29 cm long; rachis green, ca. 11 cm long; bracts of the peduncle wine colored-green, 1.1–1.2 × 0.5–0.7 cm, amplexicaul, broad triangular, apex cuspidate; floral bracts green, 0.3–0.6 × 0.1 cm, narrowly triangular, apex acute.

Flowers lilac, resupinate, pedicelate; ovary+pedicel cylindrical, slightly clavate, green, 10–16 × 1.5–3 mm; dorsal sepal lilac with whitish base, obovate, 5-veined, 1.4 × 0.5 cm, apex acute to slightly apiculate; lateral sepals lilac with whitish base, obovate, 5–7-veined, 1.1–1.2 × 0.55–0.6 cm, apex acute to slightly apiculate; petals lilac, purple at base, obovate, 5-veined, 1.0–1.1 × 0.4–0.45 cm, apex slightly apiculate; labellum white with lilac to purple stripes, 3-lobed, ca. 5.0 × 8.0 mm, attached to the column foot; midlobedepressed-obovate, 4.0 × 8.0 mm, apex mucronate; lateral lobes asymmetrically depressed-obovate, 3.0 × 3.5–4.0 mm; callus yellow with light lilac stripes, prominent, bilobed, 4× 4mm; column shortly clavate, white turning wine colored near the base, ca. 6.0 × 6.0 mm, ventrally concave, slightly curved, winged; column foot ca. 5.0 × 3.0 mm, vinaceous; anther incubent, operculate, anther cap 2-locular, ca. 1 × 1.8mm; clinandrium shallow, margin erose, prominentat apex; pollinarium with two pairs of sub-equal, dorsi-ventrally flattened, obovate pollinia, 1–1.2 × 0.6–0.8mm, stipe and viscidium not seen; rostelum prominent, cartilaginous, with an aciculate central projection; stigma transversely elliptic. Fruit not seen.

Etymology:—The chosen epithet is a reference to the lilac color of the flowers.

Distribution and habitat:—*Koellensteinia lilacina* was collected in the municipality of Chapada dos Guimarães in Mato Grosso state, Brazil (Fig. 3). It grows as terrestrial in the shadow of trees near streams of water. The vegetation of the collection site is a thin riparian forest, close to a savanna at about 650 m of altitude. Individuals that were gathered *in situ* flourished in greenhouses in February.

Conservation assessment:—*Koellensteinia lilacina* is known, up to now, from only one locality in the Cerrado of Mato Grosso. The area is surrounded by several cotton and soybean farms, suffering great agricultural pressure, as most of the Brazilian Cerrado vegetation (Silva *et al.* 2013), although the Cerrado Biome is considered a hotspot for conservation (Mittermeier *et al.* 2005). The population of *K. lilacina*, represented by a few aggregated individuals, grows in an area nearby the National Park of Chapada dos Guimarães, however there is no proof that it occurs within its borders. Therefore, *Koellensteinia lilacina* should be considered as Critically Endangered (CR) according to the IUCN (2014) criteria.

Discussion

Koellensteinia lilacina is similar to *K. eburnea* (Fig. 2 c-d).and *K. lineata* by its flower size and shape of sepals and petals. All three species occur in the state of Mato Grosso, but *K. eburnea* has broader distribution, through states of central Brazil. However, *Koellensteinia lilacina* can be differentiated mainly by its flowers that are tighter, lilac, and with a yellow callus in the labellum, the arcuate inflorescence, and the pseudobulbs that are normally semi-spherical. On the other hand, *K.eburnea* has flowers whitish to cream with petals painted in purple or violet, with a white callus in the labellum, the inflorescence is erect, and the pseudobulbs are piriform. *Koellensteinia lineata* has white flowers, petals with purple spots and labellum with narrow midlobe and a white callus, the inflorescence is erect, and the pseudobulbs are piriform. The three species can be separated by the characters in the following couplet:

Key to differentiate *Koellensteinia lilacina* from related species

1. Pseudobulbs semi-spherical; inflorescence arcuate; flowers tighter and lilac; callus of labellum yellow………..……......................……………………………..........*K. lilacina*

- Pseudobulbs piriform; inflorescence erect; flowers open and white or whitish to cream; callus of labellum white..2

2. Petals with purple spots; labellum with narrow transversely obovate midlobe……………………………...………………………….................…......*K. lineata*

- Petals painted in purple; labellum with depressed obovate midlobe..............*K. eburnea*

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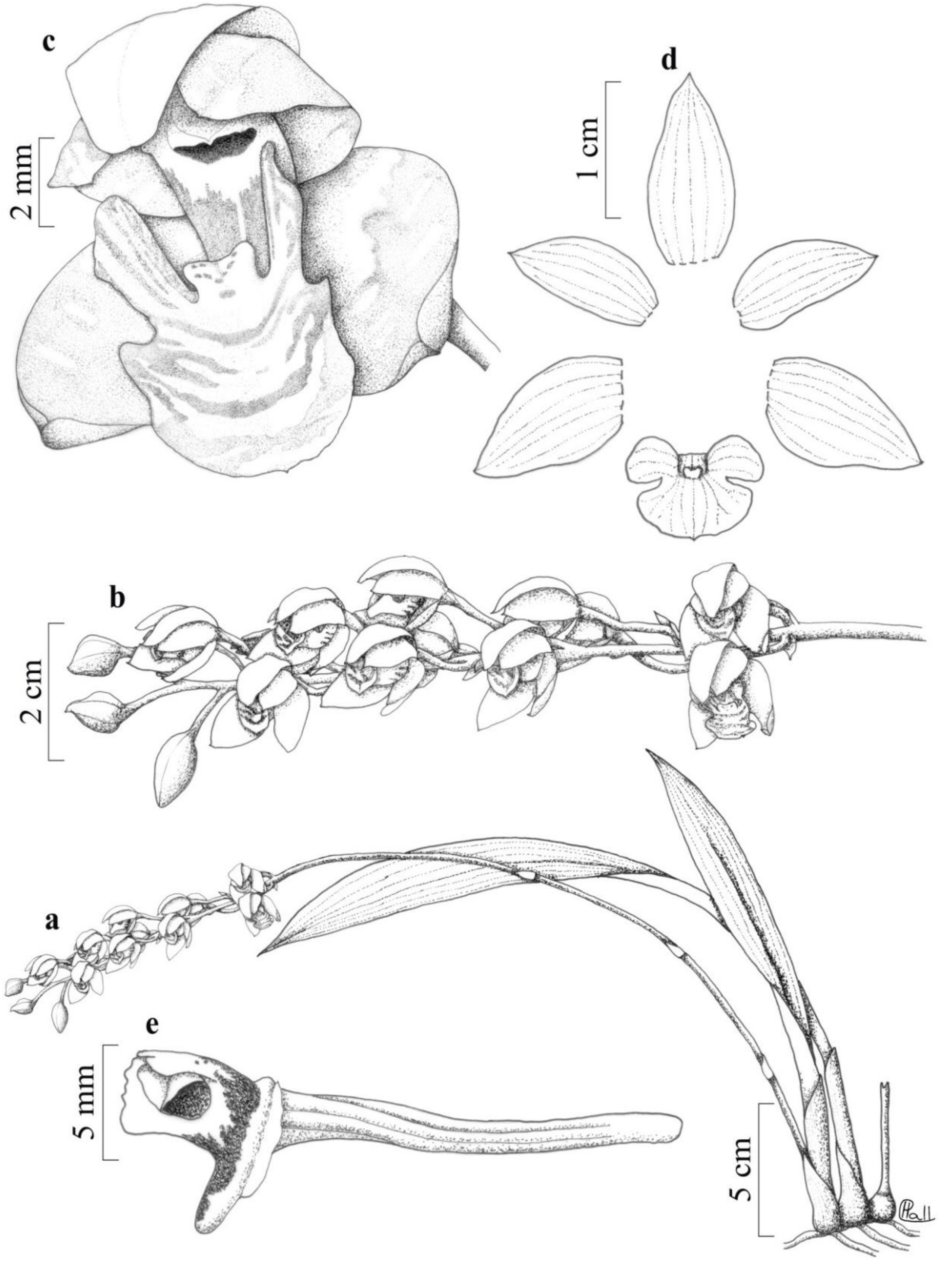

FIGURE 1. *Koellensteinia lilacina*: **a)** Habit; **b)** Rachis of the inflorescence; **c)** Flower; **d)** Dissected flower; **e)** Column (lateral view). Illustrated by Climbiê Ferreira Hall from the holotype**.**

FIGURE 2. a-b) *Koellensteinia lilacina*: **a)** General aspect; **b)** Flower; c-d) *Koellensteinia eburnea*: **c)** General aspect; **d)** Flower. Photo by Climbiê Ferreira Hall from the holotype**.**

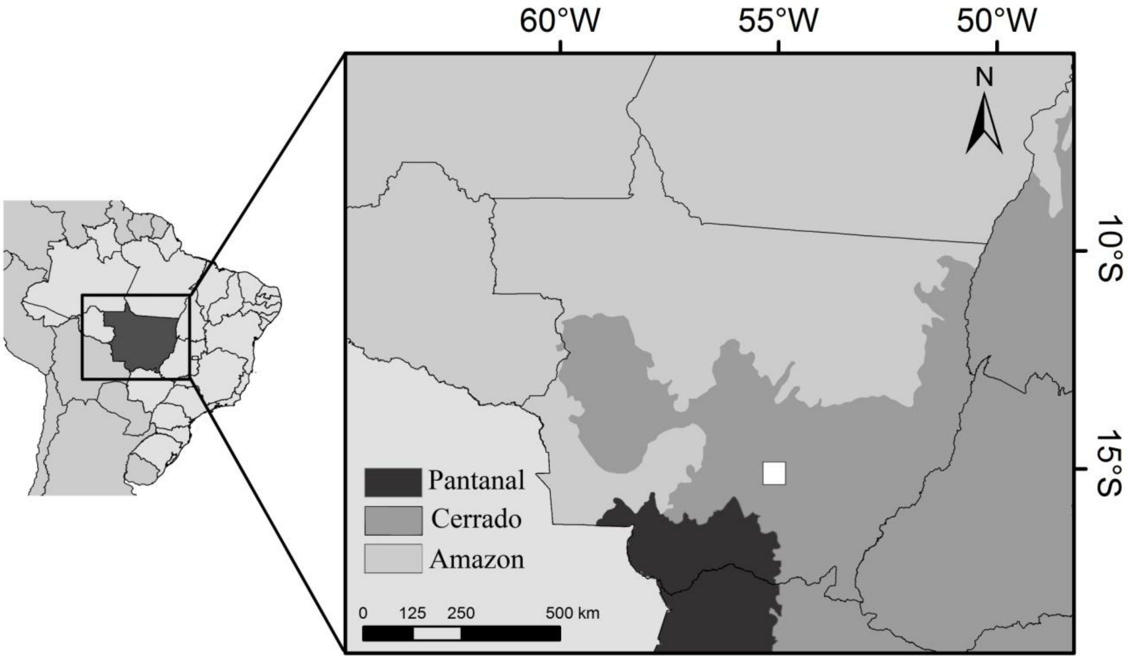

FIGURE 3. Geographic distribution of *Koellensteinia lilacina*.

CAPÍTULO 7

Field Guide: Brazilian species of the subtribe Zygopetalinae (Orchidaceae)

(Formatado de acordo com as normas do Field Museum)

Brazilian species of the subtribe Zygopetalinae (Orchidaceae)

Zygopetalinae of Brazil

1

Climbiê Ferreira Hall[1], Felipe Fajardo Villela Antolin Barberena[2], Sérgio Queiroz[3], Vinícius Trettel Rodrigues[1], Ana Kelly Koch[1] & Fábio de Barros[1]
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[fieldguides.fieldmuseum.org] **Color Guide #** version **1** 05/2015


1 ***Aganisia*** *cyanea*


2 ***Aganisia*** *cyanea*


3 ***Aganisia*** *cyanea*


4 ***Aganisia*** *fimbriata*


5 ***Aganisia*** *fimbriata*


6 ***Aganisia*** *fimbriata*


7 ***Aganisia*** *pulchella*


8 ***Aganisia*** *pulchella*


9 ***Aganisia*** *rosariana*


10 ***Batemannia*** *colleyi*


11 ***Batemannia*** *colleyi*


12 ***Batemannia*** *lepida*


13 ***Batemannia*** *lepida*


14 ***Batemannia*** *lepida*


15 ***Chaubardia*** *klugii*


16 ***Chaubardia*** *klugii*


17 ***Chaubardia*** *klugii*


18 ***Chaubardia*** *surinamensis*


19 ***Chaubardia*** *surinamensis*


20 ***Chaubardia*** *surinamensis*

Brazilian species of the subtribe Zygopetalinae (Orchidaceae)

Zygopetalinae of Brazil

Climbiê Ferreira Hall[1], Felipe Fajardo Villela Antolin Barberena[2], Sérgio Queiroz[3], Vinícius Trettel Rodrigues[1], Ana Kelly Koch[1] & Fábio de Barros[1]
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21 ***Cheiradenia*** *cuspidata*


22 ***Cheiradenia*** *cuspidata*


23 ***Cheiradenia*** *cuspidata*


24 ***Cochleanthes*** *amazonica*
Photo: M.A. Campacci


25 ***Cochleanthes*** *flabelliformis*


26 ***Cochleanthes*** *flabelliformis*


27 ***Cochleanthes*** *flabelliformis*


28 ***Dichaea*** *cogniauxiana*


29 ***Dichaea*** *cogniauxiana*


30 ***Dichaea*** *panamensis*


31 ***Dichaea*** *panamensis*


32 ***Dichaea*** *panamensis*

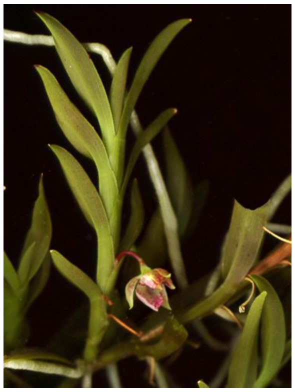

33 ***Dichaea*** *panamensis*


34 ***Dichaea*** *panamensis*


35 ***Dichaea*** *pendula*


36 ***Dichaea*** *pendula*


37 ***Dichaea*** *pendula*


38 ***Dichaea*** *pumila*
Photo: M.A. Campacci


39 ***Dichaea*** *pumila*
Photo: M.A. Campacci


40 ***Dichaea*** *rendlei*

Brazilian species of the subtribe Zygopetalinae (Orchidaceae)

Zygopetalinae of Brazil

Climbiê Ferreira Hall[1], Felipe Fajardo Villela Antolin Barberena[2], Sérgio Queiroz[3], Vinícius Trettel Rodrigues[1], Ana Kelly Koch[1] & Fábio de Barros[1]
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41 ***Dichaea*** *rendlei*


42 ***Dichaea*** *rendlei*


43 ***Dichaea*** *rendlei*


44 ***Dichaea*** *trulla*


45 ***Dichaea*** *trulla*


46 ***Dichaea*** *trulla*


47 ***Dichaea*** *trulla*


48 ***Galeottia*** *ciliata*


49 ***Galeottia*** *ciliata*


50 ***Galeottia*** *ciliata*


51 ***Galeottia*** *ciliata*


52 ***Huntleya*** *meleagris*


53 ***Huntleya*** *meleagris*


54 ***Koellensteinia*** *altissima*


55 ***Koellensteinia*** *altissima*


56 ***Koellensteinia*** *altissima*


57 ***Koellensteinia*** *altissima*


58 ***Koellensteinia*** *eburnea*


59 ***Koellensteinia*** *eburnea*


60 ***Koellensteinia*** *eburnea*

Brazilian species of the subtribe Zygopetalinae (Orchidaceae)

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Zygopetalinae of Brazil

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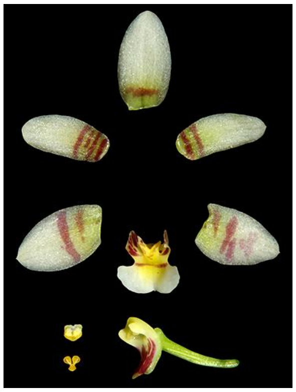
61 ***Koellensteinia*** *eburnea*


62 ***Koellensteinia*** *eburnea*


63 ***Koellensteinia*** *graminea*


64 ***Koellensteinia*** *graminea*

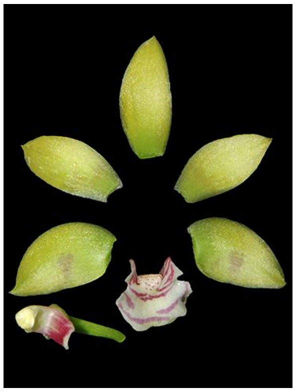

65 ***Koellensteinia*** *graminea*


66 ***Koellensteinia*** *graminea*


67 ***Koellensteinia*** *graminea*


68 ***Koellensteinia*** *spiralis*


69 ***Koellensteinia*** *spiralis*


70 ***Koellensteinia*** *spiralis*


71 ***Neogardneria*** *murrayana*
Photo: M.A. Campacci


72 ***Neogardneria*** *murrayana*
Photo: T.L. Vieira


73 ***Neogardneria*** *murrayana*
Photo: T.L. Vieira


74 ***Neogardneria*** *murrayana*
Photo: T.L. Vieira


75 ***Otostylis*** *brachystalix*
Photo: T.L. Vieira


76 ***Otostylis*** *brachystalix*
Photo: T.L. Vieira


77 ***Otostylis*** *brachystalix*
Photo: T.L. Vieira


78 ***Otostylis*** *brachystalix*
Photo: T.L. Vieira


79 ***Pabstia*** *modestior*
Photo: M.A. Campacci


80 ***Pabstia*** *modestior*
Photo: M.A. Campacci

Brazilian species of the subtribe Zygopetalinae (Orchidaceae)

Zygopetalinae of Brazil

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81 ***Pabstia*** *schunkeana*


82 ***Pabstia*** *schunkeana*


83 ***Pabstia*** *schunkeana*

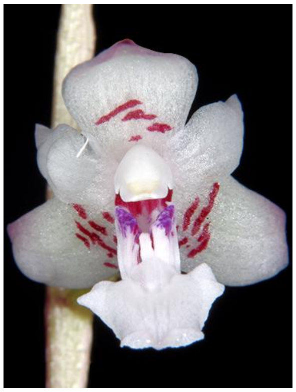

84 ***Pabstia*** *viridis*


85 ***Pabstia*** *viridis*
Photo: M.A. Campacci


86 ***Pabstia*** *jugosa*


87 ***Pabstia*** *jugosa*


88 ***Paradisanthus*** *baihensis*


89 ***Paradisanthus*** *baihensis*


90 ***Paradisanthus*** *baihensis*


91 ***Paradisanthus*** *micranthus*


92 ***Paradisanthus*** *micranthus*

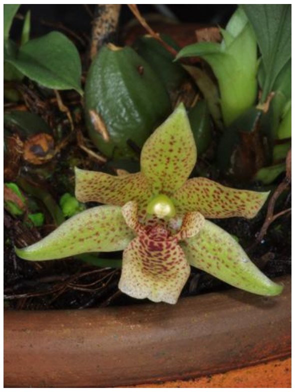

93 ***Paradisanthus*** *micranthus*


94 ***Pescatorea*** *violacea*


95 ***Promenaea*** *guttata*


96 ***Promenaea*** *guttata*


97 ***Promenaea*** *guttata*


98 ***Promenaea*** *rollissonii*


99 ***Promenaea*** *rollissonii*


100 ***Promenaea*** *rollissonii*

Brazilian species of the subtribe Zygopetalinae (Orchidaceae)

Zygopetalinae of Brazil

6

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101 ***Promenaea*** *silvana*
Photo: P. Leitman


102 ***Promenaea*** *silvana*
Photo: P. Leitman


103 ***Promenaea*** *stapelioides*


104 ***Promenaea*** *stapelioides*


105 ***Promenaea*** *stapelioides*


106 ***Promenaea*** *viridiflora*


107 ***Promenaea*** *xanthina*


108 ***Promenaea*** *xanthina*


109 ***Promenaea*** *xanthina*


110 ***Promenaea*** *xanthina*


111 ***Promenaea*** *xanthina*


112 ***Warczewiczella*** *candida*
Photo: F. Vinhos


113 ***Warczewiczella*** *wailesiana*
Photo: M.A. Campacci


114 ***Warczewiczella*** *wailesiana*


115 ***Warczewiczella*** *wailesiana*


116 ***Warrea*** *warreana*
Photo: L. Zandoná


117 ***Warrea*** *warreana*
Photo: L. Zandoná


118 ***Warrea*** *warreana*
Photo: L. Zandoná


119 ***Warrea*** *warreana*
Photo: M.A. Campacci


120 ***Zygopetalum*** *crinitum*

Brazilian species of the subtribe Zygopetalinae (Orchidaceae)

Zygopetalinae of Brazil

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121 ***Zygopetalum*** *crinitum*


122 ***Zygopetalum*** *maculatum*


123 ***Zygopetalum*** *maculatum*


124 ***Zygopetalum*** *maxillare*


125 ***Zygopetalum*** *maxillare*


126 ***Zygopetalum*** *maxillare*


127 ***Zygopetalum*** *pedicellatum*
Photo: M.A. Campacci


128 ***Zygopetalum*** *pedicellatum*
Photo: F. Vinhos


129 ***Zygopetalum*** *sellowii*


130 ***Zygopetalum*** *sellowii*
Photo: L.S. Guimarães


131 ***Zygopetalum*** *sellowii*


132 ***Zygopetalum*** *sellowii*


133 ***Zygopetalum*** *triste*


134 ***Zygopetalum*** *triste*


135 ***Zygopetalum*** *triste*


136 ***Zygosepalum*** *labiosum*


137 ***Zygosepalum*** *labiosum*


138 ***Zygosepalum*** *labiosum*


139 ***Zygosepalum*** *labiosum*


140 ***Zygosepalum*** *lindeniae*

CONSIDERAÇÕES FINAIS

Para a realização dessa tese de doutorado foi necessário um grande esforço de coleta. Foram visitados mais de 120 municípios pertencentes a 17 estados brasileiros, além de localidades na Colômbia e Bolívia. Tal esforço de coleta foi recompensado, pois o sequenciamento realizado a partir de material coletado em campo e seco em sílica-gel teve resultados muito melhores que os obtidos a partir de material de herbário, mesmo com o uso de técnicas de NGS, cujo rendimento, quando utilizadas em material de herbário,é considerado superior ao de técnicas Sanger.

A opção de se utilizar técnicas de NGS ao invés de técnicas Sanger revelou-se acertada, pois a árvore gerada na análise Bayesiana apresentou, em sua grande maioria, clados sustentados por altos valores de probabilidade a posteriori. A árvore de consenso obtida mostrou que Zygopetalinae, clado de *Huntleya* e clado de *Zygopetalum* são grupos monofiléticos. Vários clados com alto suporte se formaram dentro da subtribo, porém, *Aganisia* e *Acacallis* não são agrupados e não devem ser tratados como um único gênero. *Koellensteinia*, *Zygopetalum* e *Zygosepalum* são polifiléticos e necessitam de revisão.

Koellensteinia em sua nova circunscrição fica apenas com dez espécies, incluindo as duas descritas nessa tese. Duas espécies de *Koellensteinia* foram transferidas para *Paradisanthus*. Um dos poucos posicionamentos mais complicados foi a relação entre *Koellensteinia graminea* e os gêneros *Cheiradenia* e *Aganisia*, relação que poderá ser resolvida com a utilização de dados nucleares de NGS.

Apesar das grandes vantagens de NGS, o uso desse tipo de técnica apresenta algumas dificuldades de aplicação. Por ser uma técnica ainda nova e que gera uma grande quantidade de dados, exige utilização massiva da bioinformática; isso torna a análise dos dados bastante complexa, aumentando sensivelmente o tempo necessário para obtenção dos resultados. Durante o meu estágio na Universidade de Gotemburgo, Suécia, sequenciei DNA plastidial e nuclear. Porém, optou-se por focar, em um primeiro momento, na análise apenas do DNA de cloroplasto por dois motivos: 1) trata-se de um genoma haplóide; 2) possibilita o uso de um genoma de referência completo com introns e exons. Mesmo com essa opção, a análise dos dados, desde o recebimento das sequências até a obtenção de uma árvore obtida de uma análise bayesiana, demorou cerca de quatro meses e meio.

O grande esforço realizado nas coletas, se por um lado tomou bastante tempo nas primeiras fases de desenvolvimento da tese, por outro lado permitiu a obtenção de espécimes raramente coletados e nunca anteriormente incluídos em análises filogenéticas. Essas plantas, que agora estão em cultivo nas estufas do núcleo do Orquidário do Instituto de Botânica, estão sendo utilizadas também para um aumento do conhecimento citogenético de Zygopetalinae. O capítulo de citogenética da tese compreende a contagem cromossômica para 20 espécies, das quais o presente estado de enraizamento permitiu a coleta de pontas de raízes para a análise citogenética. Dentre essas 20 espécies, 13 foram contagens inéditas, incluindo a primeira contagem para o gênero *Paradisanthus*. Para as espécies de *Koellensteinia*, houve um problema com a baixa condensação dos cromossomos, que não permitiu uma contagem confiável. Esperamos, em futuro próximo, testar diferentes protocolos para coleta de raízes do grupo para uma contagem eficiente.

O presente estudo representa mais um passo no conhecimento taxonômico e sistemático de Zygopetalinae e, assim, de Orchidaceae neotropicais. O volume de coletas realizadas e a grande quantidade de dados moleculares gerados permitirão a continuidade dos estudos com o grupo e os trabalhos já realizados darão subsídios para futuros estudos de biogeografia, evolução e taxonomia do grupo.