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DNA barcode regions for differentiating *Cattleya walkeriana* and *C. loddigesii*

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ABSTRACT. Growers appreciate *Cattleya walkeriana* and *C. loddigesii* due to striking shape and rarity. Thus, this study aimed to evaluate the feasibility of DNA barcode regions, namely ITS1, ITS2 and *rpoC1*, to discriminate between *C. walkeriana* and *C. loddigesii* species. DNA barcode regions were successfully amplified using primers designed to amplify plants. We also included sequences from public databases in order to test if these regions were able to discriminate *C. walkeriana* and *C. loddigesii* from other *Cattleya* species. These regions, and their combinations, demonstrated that the ITS1+ITS2 had the highest average interspecific distance (11.1%), followed by *rpoC1* (1.06%). For species discrimination, ITS1+ITS2 provided the best results. The combined data set of ITS1+ITS2+*rpoC1* also discriminated both species, but did not result in higher rates of discrimination. These results indicate that ITS region is the best option for molecular identification of these two species and from some other species of this genus.

Keywords: orchid improvement, genetic variation, species separation.

Regiões de DNA barcode para diferenciar *Cattleya walkeriana* e *C. loddigesii*

RESUMO. As espécies *Cattleya walkeriana* e *C. loddigesii* são apreciadas pelos colecionadores devido às suas impressionantes forma e raridade. Este estudo teve como objetivo avaliar a viabilidade das regiões *DNA barcode*, ou seja, ITS1, ITS2 e *rpoC1*, para discriminar as espécies *C. walkeriana* e *C. loddigesii*. Regiões *DNA barcode* foram amplificadas com êxito utilizando os iniciadores desenhados para plantas. Nós também incluímos sequências de bases públicas de dados, a fim de testar se estas regiões foram capazes de discriminar *C. walkeriana* e *C. loddigesii* de outras espécies de *Cattleya*. Estas regiões e suas combinações demonstraram que o ITS1 + ITS2 teve a maior distância média interespecífica (11,1%), seguido por *rpoC1* (1,06%). Para a discriminação das espécies, ITS1 + ITS2 proporcionaram os melhores resultados. Os dados combinados dos ITS1 + ITS2 + *rpoC1* também discriminaram ambas as espécies, mas não resultaram em maiores taxas de discriminação. Estes resultados indicam que a região ITS é a melhor opção para a identificação molecular destas duas espécies e a partir de algumas outras espécies deste gênero.

Palavras-chave: melhoramento de orquídeas, variação genética, separação de espécies.

Introduction

Brazil has a great biodiversity of orchid species. Some of them, especially the epiphytes, are endangered. Thus, knowledge about genetic diversity is extremely valuable for the preservation of species at risk. Loss of genetic variability may reduce survival and evolution chances in the wilds. Conserving such hereditary legacy is crucial for long-term species survival (Muñoz, Warner, & Albertazzi, 2010). *Cattleya walkeriana* Gardner is now a threatened species due to forest fragmentation and predatory collection (Tambarussi et al., 2017). Growers appreciate *C. walkeriana* due to its diversity of forms, springing in beautiful and valuable flowers (Faria, Santiago, Saridakis, Albino, & Araújo, 2002). In recent years, collectors have been looking for plants with high genetic improvement (Menezes, 2011). Individuals with improved traits (rare color and good flower shape) are highly valued (Tambarussi et al., 2017). *Cattleya loddigesii* Lindl. occurs in the states of Minas Gerais, Paraná and São Paulo States in Brazil, and also in the Northeast of Argentina (Barbosa Rodrigues, 1996). These species are in the same background of modern *Cattleya* Alliance hybrids (American Orchid Society [AOS] (2016) and the growers have used these species to produce hybrids. Orchid growers accept this process

when the aims are produce pure plants (crosses among the same species).

Currently, the development of biotechnology, including several techniques manipulating DNA for differentiation purpose, are being applied to maintain genetic features, breeding programs, characterization of germplasm banks, and discrimination of hybrids (Cruz, Selbach-Schnadelbach, Lambert, Ribeiro, & Borba, 2011). Many molecular techniques help generating information and assessing polymorphism among individuals and populations (Qian, Wang, & Tian, 2013). Biotechnological techniques, such as the *in vitro* procedure (Faria et al., 2002), differentiation of natural populations, species delimitations in rare plants (Qian et al., 2013), and phylogeographical studies (Monteiro, Selbach-Schnadelbach, Oliveira, & van den Berg, 2010), have extensively contributed for understanding and saving orchid species. Molecular markers have been used for genetic analysis of the genus *Calanthe* (Qian et al., 2013), *Cattleya* (Almeida et al., 2013; Rodrigues et al., 2015), and many others.

Molecular identification of species has been extensively used in many organisms, such as animals, fungi, bacteria and even plants. Hebert, Cywinska, Ball, and deWaard (2003) proposed an identification of a biological system based on DNA sequences (DNA barcoding). In this context, they proposed that a small, but standardized region from the genome could be able to discriminate species. In animals, this region is Cytochrome Oxidase subunit 1(COX1; Hebert et al., 2003) and ITS (internal transcribed spacer; Schoch et al., 2012) region for fungi for example, but in plants several systems have been discussed, involving the sequencing of one or more standard genomic regions for species identification (Hollingsworth et al., 2009). For plants, the DNA barcodes (*rpoC1+rpoB+matK or rpoC1+matK+trnH-psbA*) (Kress & Erickson, 2009, Hollingsworth, Graham, & Little, 2011), and an internal transcribed spacer (ITS1+ITS2) (Chen et al., 2010; Selvaraj et al., 2012), have been suggested by different researches for plant species identification. In the Orchidaceae, the following loci [*rbcL, matK, atpF-atpH, psbK-psbI, trnH-psbA* and ITS] have been recommended as plant barcoding loci to discriminate among species within the genus *Holcoglossum* (Orchidaceae: Aeridinae) (Xiang, Hu, Wang, & Jin, 2011). However, DNA barcoding in Orchidaceae is very recent. Consequently, several studies have proposed a new system to discriminate between species based on infrageneric taxonomy, within few genus (Kim, Oh, Bhandari, Kim, & Park, 2014; van den Berg, 2014).

Thus, this research looked at the use of DNA barcode to differentiate the species *C. walkeriana* and *C. loddigesii*, and also to differentiate these two species from other *Cattleya* species.

Material and methods

Plant material and genetic analysis of DNA barcode candidates

Growers from the States of Minas Gerais (MG) and São Paulo (SP) supplied three *C. walkeriana* individuals, and one of *C. loddigesii*. Three other *C. loddigesii* individuals were sampled from the "Professor Paulo Sodero Martins" Orchid Collection of the Genetics Department (ESALQ/USP), *Universidade de São Paulo*, Piracicaba, São Paulo, Brazil (Table 1).

DNA was extracted from 100 mg samples per plant by the Doyle and Doyle (1990) method. Four *C. loddigesii* and three *C. walkeriana* individuals were genotyped. DNA barcodes from these species were tested and separated in order to use their sequences in further studies. Although the aim of this study was to test the DNA barcode discrimination between *C. loddigesii* x *C. walkeriana*, we also included GenBank sequences from other species of *Cattleya* and tested the discrimination of *C. loddigesii* and *C. walkeriana* against other species. Unfortunately, several species were represented by only one specimen, where it was not possible to evaluate the intraspecific species variation and also not able to concatenate the regions in order to test species discrimination. Sequences of the universal primers for evaluating DNA barcodes, including those for ITS1, ITS2 (ITS1+ITS2, herein named as ITS region) and *rpoC1*, and general PCR reaction conditions, were obtained from previous studies (Tokuoka & Tobe, 2006; Chen et al., 2010; Sharma, Folch, Cardoso-Taketa, Lorence, & Villarreal, 2012). All PCRs were performed in 25 µL reaction volumes with 12.5 µL of PCR Master Mix (Promega Corp., Madison, Wisconsin), 1.25 µL each of 10 µM primers (upstream and downstream), and 10 µL of diluted (10- to 100-fold) DNA template. PCR products were checked on a 1.0% agarose gel. The sequencing amplification protocol consisted of one cycle of 1 min at 96°C, followed by 30 cycles of 10 sec at 96°C, 5 sec at 55°C, and 4 min at 60°C, using the ABI Prism BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) following the manufacturer's instructions. Sequenced products were purified by EDTA-Sodium Acetate precipitation and run on a 3130XL sequencer (Applied Biosystems, USA).

Table 1. List of *Cattleya walkeriana* and *C. loddigesii* individuals with their respective varieties and source/origin and GenBank accession numbers.

Species/"clone"	Variety	Origin/provenance	Code	GenBank accession numbers	
				rpoC1	ITS region
C. walkeriana "Rayane"	tipo	Piumhi, MG⋆	Or-6	KY006864	KY006871
C. walkeriana "Marina"	alba	Abadia dos Dourados, MG⋆	Or-48	KY006863	KY006870
C. walkeriana "Matão"	albecens	Matão SP	Or-66	KY006865	KY006872
C. loddigesii	alba	Poços de Caldas, MG	Or-28	KY006860	KY006867
C. loddigesii	tipo	Cabralia Paulista, SP	Or-17	KY006859	KY006866
C. loddigesii	tipo	Piracicaba, SP	Or-30	KY006862	KY006869
C. loddigesii	tipo	Piracicaba, SP	Or-29	KY006861	KY006868

⋆Manzan (2014).

Statistical analysis of DNA barcodes

DNA barcodes candidates were edited with BioEdit program, version 7.0.9.0 (Hall, 1999). Informative polymorphic characters were identified by MEGA6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). Alignment of the sequences was executed by MUSCLE program (Edgar, 2004). Eventually manual adjustments were made through BioEdit software, version 7.0.9.0 (Hall, 1999). The different locus combinations were partitioned for independent model assessment at each marker. Diagnostic characters analysis was conducted for *rpoC1* gene according to BOLD Systems (Barcode of Life Data Systems). Pairwise genetic distances for each individual sequence data set, and all possible combinations of the three sequence data sets, were determined by Kimura 2-parameter (K2P) method (Kimura, 1980) using MEGA6 (Tamura et al., 2013). Neighbor-joining analysis (NJ), under MEGA6, was employed to assess whether the resulting sequence data sets in various combinations formed species-specific clusters. For NJ analyses, K2P distance matrices were used. A bootstrap (BS) analysis (Felsenstein, 1985) of 1000 replicates was conducted to evaluate support for clades using the same search parameters as in the previous NJ analysis. We tested two approaches for discrimination levels using the entire dataset for both regions and using only the pair *C. loddigesii* x *C. walkeriana*. In the first one, the degree of discrimination was successful when the minimum interspecific distance was larger than the maximum intraspecific distance, a similar approach proposed by Hollingsworth et al. (2009), but here we considered the K2P distance. Unfortunately, many species from GenBank were represented by a unique sequence, not representing the intraspecific variation. In the second one, the tree-based method (NJ) was only considered successful by the specific monophyletic groups for species for at least two specimens sequenced, and that showed bootstrap values ≥70% (as used by Zhang, Fan, Zhu, Zhao, & Fu, 2013 and Vivas et al., 2014). Cladograms were analyzed and edited with MEGA6 (Tamura et al., 2013).

Results and discussion

On the specimens used in this study, the assessed DNA barcode regions were successfully amplified using primers designed to evaluate plants (Tokuoka & Tobe, 2006; Chen et al., 2010; Sharma et al., 2012). Most samples were successfully amplified through direct sequencing of the PCR products using the same primer pairs, which generated high-quality bidirectional sequences. This indicates that the primers used for each DNA barcode region in this study are universally applicable to the genotypes of *C. walkeriana* and *C. loddigesii*. The lengths of aligned DNA fragments of *rpoC1* and ITS region were 518 and 678, respectively. ITS region provided a greater number of variable sites (29.8%) than *rpoC1* (2.3%) and also a higher interspecific mean distance (5.4%) (Table 2).

Table 2. Sequence characteristics of the regions tested.

	rpoC1	ITS region
Aligned sequence length (bp)	518	678
Number of variable sites	12	202
Mean intraspecific K2P distance % (range)	0.038 (0 - 0.15)	0.75 (0 - 1.64)
Mean interspecific K2P distance % (range)	0.22 (0 - 1.06)	5.4 (0 - 11.1)

Both regions were analyzed separately, for all samples, and combined only for the pair *C. loddigesii* x *C. walkeriana*, demonstrating a great discrimination of this pair of species (Table 3).

Table 3. Summary from analysis indicating resolution of regions tested for *Cattleya* genus.

	Resolution (%)		
	Intra x Interspecific distances		
	rpoC1	ITS region	*rpoC1*+ITS region
All dataset	52	82	-
C. walkeriana x *C. loddigesii*	100 ⋆	100	100
	Tree-based method ¶		
All dataset	-	54.5	-
C. walkeriana x *C. loddigesii*	100	100	100

⋆ See text for details, diagnostic character analysis incomplete sampling, only species with two or more sequences. Due to low variability of *rpoC1* gene, this item was not accessed for this region.

For all others, when the analysis was conducted on separated regions, *rpoC1* showed low variability (overall mean 0.3%). Despite its lower variability, it

is possible to distinguish *C. walkeriana* from *C. loddigesii* taking into account a SNP at positions 30 (G/C) and 66 (G/C) of the final alignment (Figure 1).

When compared with all public sequences, position 30 can be classified as partial diagnostic since it is possible to distinguish *C. walkeriana*, but not *C. loddigesii*, from all the other species except for *C. violacea* and *C. nobilior.* Also, position 66 is considered as diagnostic since it is possible to discriminate *C. walkeriana* from all the other species. On the other hand, ITS region was shown to be more efficient to discriminate species, with higher pairwise distances between them. These results were also discussed in the literature, with a lower variability being reported for *rpoC1* (Hollingsworth et al., 2009) and greater variability for ITS region (overall mean distance 7.05% for the ITS region and 0.35% for *rpoC1*; Chen et al., 2010). But in our study, although little variation was found in the *rpoC1* region, this region can indeed discriminate at least *C. walkeriana* from all other species. The NJ tree analysis for ITS region represented in Figure 2 clearly shows that *C. walkeriana* is distinguished from all other species, but *C. loddigesii* cannot be separated from 35.7% of the species tested (*C. bicolor, C. granulosa, C. leopoldii, C. forbesii, C. porphyroglossa, C. elongata, C. tenuis, C. velutina, C. harrisoniana, C. schilleriana, C. kerrii, C. amethystoglossa, C. guttata, C. dormaniana* and *C. intermedia*).

Furthermore, it is also possible to discriminate *C. elongata, C. lueddemanniana, C. maxima, C. porphyroglossa* and *C. trichopiliochila* from all other species. Similar results were found in *Gossypium*, as the ITS region is most suitable as a candidate DNA barcode for identification compared to the plastid regions, even between organisms of the same species (Ashfaq, Asif, Anjum, & Zafar, 2013). The plastid regions *rpoC1* discriminate more than 60% while ITS regions discriminate more than 90% of species in land plants (Kress & Erickson, 2007). There are several works trying to identify combinations of universal genes that allow separation of plant species. According to Kress et al. (2009), using multilocus combinations *rbcL* + *trnH-psbA* + *matK* regions is useful for the identification in plants. The combination of genes *rpoA, rpoB, rpoC1* and *rpoC2* is proposed as a phylogenetic marker in systematic and molecular phylogeny of flowering plants (Logacheva, Penin, Samigullin, Vallejo-Roman, & Antonov, 2007). The result from concatenated regions (*rpoC1*+ITS), tested only for *C. walkeriana* x *C. loddigesii* pair, discriminated both species with 100% resolution (Figure 3), with a mean distance of 4.4% in our dataset (Table 3).

Figure 1. Alignment detail of the *rPOC1* gene. This alignment includes 35 species including public sequences. Notice that for *C. walkeriana* it is possible to distinguish it from all other species based on SNP (C/T) at position 66 (classified as diagnostic character) from 518bp alignment. Also, the SNP (G/C) at position 30 can be classified as partial diagnostic to distinguish *C. walkeriana*, since *C. violacea* and *C. nobilior* share this same SNP.

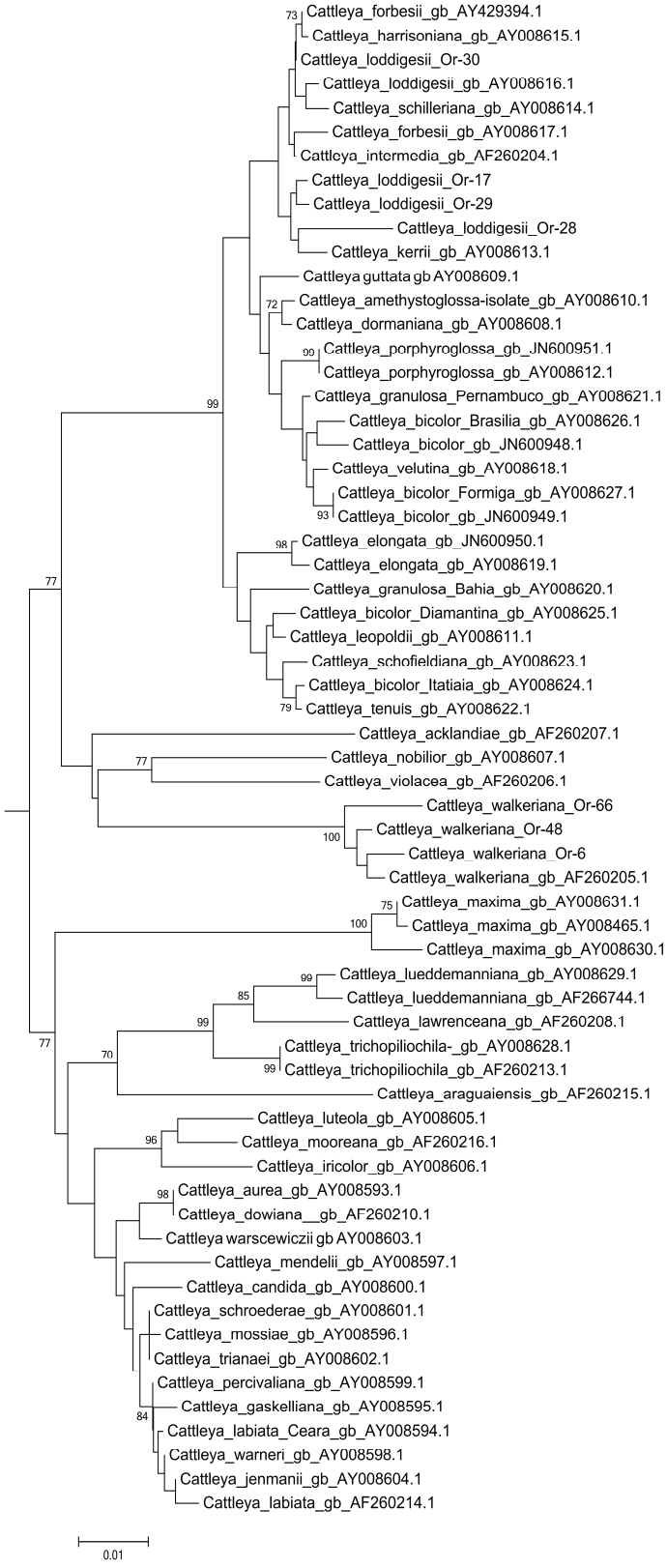

Figure 2. NJ tree including public records for ITS region. Numbers above the branches represent bootstrap values (≥70%). Genbank accession numbers are listed with samples.

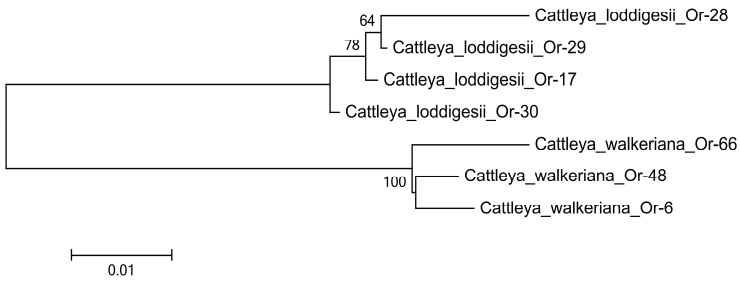

Figure 3. Neighbor-joining (NJ) tree based on the combined data sets of ITS region and *rPOC1* for only *Cattleya loddigesii* and *Cattleya walkeriana* species (public data not included). Numbers above the branches represent bootstrap values (≥50%).

Species resolution abilities of the DNA barcode regions and their combinations were proved through the methods tested, but the method used by Hollingsworth et al. (2009) returned the best results, reaching 82% based only on ITS region. High levels of species resolution for ITS and ITS2 have been reported in several previous plant barcode studies, but lower for *rpcC1* (Chen et al., 2010; Selvaraj et al., 2012; Little, 2014). In Orchidaceae, Xiang et al. (2011) reported the use of the regions *rbcL, matK, trnH-psbA*, and ITS, to discriminate among species of the genus *Holcoglossum* (Orchidaceae: Aeridinae). These were successfully implemented in barcoding species of the orchid genus *Dendrobium*. For other genus of Orchidaceae from Korea, Kim et al. (2014) used another combination of DNA barcodes, based on four regions combined, and reached a 98.8% species resolution

Conclusion

Although some methods have been proposed for the Orchidaceae, our work showed that it is possible to discriminate between *C. walkeriana* and *C. loddigesii* based only on ITS region, but the inclusion of other high variable markers could be valuable to discriminate all of the other species, as found by Kim et al. (2014).Therefore, taking into account the current economic importance and conservation status of both species, such region provides a rapid identification method to differentiate the species *C. walkeriana* and *C. loddigesii*, with a great power of discrimination and precise identification of these two orchid species.

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