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Molecular Study on Endemic *Cattleya* Species from Brazilian Flora

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Abstract

The orchids are known for its beautiful flowers, the genetic recombination ability and the lack of its defined taxonomic classification of the Orchidaceae family. Phylogenetic studies using molecular technics allow additional taxonomy understanding. ISSR molecular markers can determine the phylogenetic relationships among species and individuals of the same species. In this study the patterns of polymorphism were compared in subjects of *Cattleya guttata*, *C. tigrina* and *C. leopoldii* in order to find variations in the germplasm which can contribute to elucidate phylogenetic issues. Results were obtained using 13 primers in ten subjects of each species. They increased 97 loci being 96.9% polymorphic for the three studied species. It is concluded that the protocol was effective for the identification of fragments in all studied species. The analysis of total banding pattern suggests that *C. guttata* and *C. leopoldii* are molecularly related to each other and more distant from *C. tigrina*.

Keywords: ISSR, taxonomy, genetic divergence, *Cattleya*

1. Introduction

Of great value and appreciation by the general population, the orchids are known for its beautiful flowers and the genetic recombination ability (Pasqual, 2011). However, the taxonomic classification of the Orchidaceae family is not well defined (Oliveira, 2010).

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Over the last 200 years the phylogenetic studies on orchids were based on morphological characters (Freudenstein and Chase, 2001), mainly using floral traits (Kores, 2001).

Biotechnology through the molecular technics allows fulfil the understanding of the plant phylogeny based on flower morphology and discrimination on doubtful identification and classification (Freudenstein and Chase, 2001). One of the many molecular technics employed to analyses DNA variation is the ISSR marker (inter-simple sequence repeat) (Hossain *et al.*, 2013).

The technic is simple to read in which common fragments among the genotypes illustrates similarity and different bands indicate different genotypes (Ferreira and Grattapaglia, 1998). Is fast, low cost, high reproducibility and there is no need for previous genomic sequence information (Martins, 2007; Reedy *et al.*, 2002).

The ISSR markers were useful in phylogenetic relations determination studies among species and subjects within the same species in orchids such as *Dendrobium* (Wang *et al.*, 2009), *Piperiayadonii* (George *et al.*, 2009), *Cymbidium*spp (Sharma *et al.*, 2013), *Cattleya*spp and *Brassavolatuberculata* (Fajardo *et al.*, 2014). In this study the ISSR markers were used for the purpose of comparing the polymorphic fragment pattern in commercialized plants named *Cattleya guttata, C. tigrina* e *C. leopoldii*, aiming to find variations in the germplasm.

2. Materials and Methods

2.1 Plant Material

Ten cultivated plants of each studied species (*C. guttata, C. tigrina* e *C. leopoldii*) were acquired in commercial grower and their name was maintained. The plants were identified according to the origin city and numbered sequentially (Table 1).

Table 1. Origin City, Code and Quantity of Acquired Plants of the Species *Cattleya guttata*, *C. Tigrina* and *C. leopoldii*

City (code)	*C. guttata*	*C. tigrina*	*C. leopoldii*
Santa Bárbara d'Oeste (A)	2	4	2
Bauru (B)	2	3	2
Assis (C)	3	-	3
Arealva (D)	3	3	3
Total plants	10	10	10

2.2 Genomic DNA Extraction

Genomic DNA was extracted from leave samples using a cetyltrimethyl ammonium bromide (CTAB) method described by Doyle & Doyle (1987) and modified by Novaes*et al.* (2009). Fresh leaves fragments from 200 to 300 mg of all the species were grounded in liquid nitrogen. To that, were add 1.0 mL of the CTAB 2X extraction buffer (1.4 M NaCl; 100 mMTris-Cl pH 8.0; 20 mM EDTA; 2% CTAB; 2% PVP-40; 2% β-mercaptoetanol) and 35 µL proteinase K (1 mg mL^{-1}). This mix was transferred to a 2.0 mL microtube and heated to 60 °C for 60 min. After cooling at room temperature, 600 µL of chloroform: isoamyl alcohol (24:1) were add to the samples mixing gently for 5 min, followed by centrifuging at 15,000 rpm for 15 min 4°C.

The supernatants were taken and transferred to new micro tubes which was add 140 µL of 10% CTAB (w/v) and 280 µL of 5M NaCl, then gently mixed to homogeneity. To that 600 µL of chloroform: isoamylalcohol (24:1) were add and again centrifuged. Supernatants were isolated to a new microtube and add equal volume of ice cold isopropanol; these were kept at -20°C for at least 12 h. For the pellet obtainment the samples were centrifuged at 15,000 rpm for 10 min. Pellets were three times washed with 70% ethanol, air dried and diluted in TE buffer (10 mMTris-HCl (pH 7.5), 1 mM EDTA). Samples stayed at -20°C to use.

Concentration and purity of total genomic DNA were determined using DNA electrophoresis with 0.8% agarose gel and spectrophotometry at 260/280 nm (range values 1.8 to 2.0).

For the ISSR analysis thirteen primers were evaluated (Table 2), as described by Wolfe (2000).

These markers presented positive results to the species *Cattleya coccinea* and *C. mantiqueirae* (Rodrigues, 2010). For primer validation, 10 subjects of each species were screened to check for polymorphism.

The PCR reaction mixture contained 10% buffer (v/v) (20 mMTris-HCl (pH 8.0), 500 mMKCl) 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 10 pmol of each primer, 1 unit of Taq DNA polymerase, 10 ng of genomic DNA. Amplification reaction was performed in the Applied Biosystems, Veriti 96-Well Thermal Cycler; cycle was performed as described by Wolfe (2000): 94°C/90 s; 35 cycles 94°C/40 s, primer specific annealing temperature/45 s, 72°C/90 s, 94°C/45 s, 44°C/45 s; 72°C/5 min.

The PCR products were separated on 1.5% agarose gels and analyzed using ethidium bromide staining (10 μg mL^{-1}). Results were observed under UV light and digitally recorded by a Loccus system, L PIX HE, with UVB transilluminator, Loccus, LTB-20x20 HE.

2.3 Data Analysis

Only unambiguous, intensively stained and clear fragments were used for statistical analysis. The presence of a fragment was scored as (1) and the absence as (0) resulting binary matrixes. Using Jaccard's index the similarity coefficients were calculated. Dendrograms were constructed by the UPGMA distance method. The applied program was the R Programming.

3. Results

Results were obtained with the thirteen tested primers in all ten samples for each one of the three species. Analysis reveals 97 fragments, with 94 polymorphic (96.9%) and only three monomorphic.

For *C. guttata* there were 70 fragments in which 92.9% shown polymorphic. In *C. leopoldii* 75 bands were polymorphic (92.0%) e five monomorphic (8.0%). The specie *C. tigrina* presented fewer bands, totalizing 55 with 92.7% of those being polymorphic.

Minimum amount of amplified fragments for the tested primers was three to Terry and maximum of 12 to 901, mean 6.53 loci per primer.

Fragments size vary from 100 to 1,000 bp. Biggest were observed in *C. guttata* using primer 7 (1,000 bp), followed by primer Mao in *C. leopoldii* (930 bp) and primer Omar in *C. tigrina* (810 bp). Smallest fragments in all three species appeared using primer Dat, approximate size 100 bp.

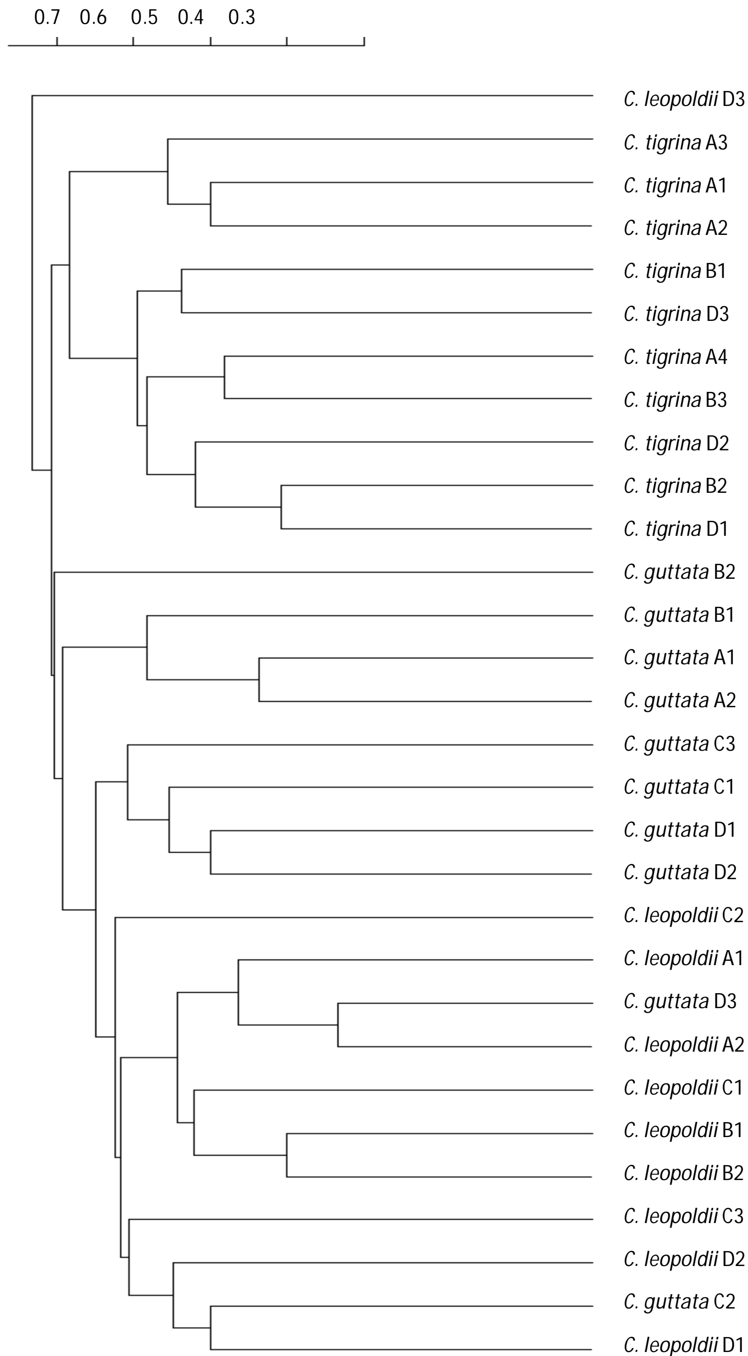
Dendrogram were plotted for all studied species in order to identify germplasm variation in the tested individuals. Based on the dendrogram genetic distances (GD) were evaluated. Among *C. guttata* specimens variation occurred from 0.439 to 0.778. *C. leopoldii* GD ranged from 0.400 to 0.792, while the observed values for *C. tigrina* were 0.407 to 0.806.

A dendrogram with the 30 tested plants, being 10 of each species was plotted, and the result was a GD ranging from 0.334 to 0.837. Two large groups could be observed,one contains all accesses of *C. tigrina* and the other contains 10 individuals of *C. guttata* and nine of *C. leopoldii*. The access *C. leopoldii* D3 differed from the others, positioning itself as the most distant of the main groups in the dendrogram.

Table 2: Primers List and Their Sequences, Optimized Annealing Temperature, Number of Fragments and Polymorphism Rates in *Cattleya guttata*, *C. leopoldii*and *C. tigrina*

Primer	Sequence	Tm optimized	Total fragments	Polymorphism rate
7	(CT)8RG	40°C	8	100%
901	(GT)6YR	52°C	12	100%
AW3	(GT)6RG	42°C	8	100%
DAT	(GA)7RG	47°C	10	100%
GOOFY	(GT)7YG	45°C	8	100%
MANNY	(CAC)4RC	45°C	5	80%
MAO	(CTC)4RC	46°C	11	100%
OMAR	(GAG)4RC	44°C	7	100%
TERRY	(GTG)4RC	52°C	3	66.7%
UBC814	(CT)8TG	42°C	9	100%
UBC843	(CT)8RA	50°C	5	100%
UBC844	(CT)8RC	41°C	5	80%
UBC899	(CA)6RG	36°C	6	100%

Figure 1: Dendrogram Plotted using UPGMA Method for all Subjects of *C. guttata*, *C. leopoldii* and *C. tigrina*.



4. Discussion

The ISSR markers use is effective to analyses genetic diversity in the *Cattleya* genus, as seen in other orchid genus as *Dendrobium*, especially due to the lack of total genome information (Wang *et al.*, 2009). The protocol used in this work was very efficient for extracting DNA from *Cattleya* leafs, even though it was described for stem bark of Leguminosae tree, presenting high polymorphism rates (96.9%). This polymorphism is justified by the use of the three species and the different accessions of each. High levels of polymorphism were found in other orchids genus such as *Dendrobium* (Wang, 2009), *Piperia* (George, 2009) and *Cymbidium* (Sharma, 2013).

Some fragments were unique to the species, in *C. guttata* with primer 7, UBC843, Manny and AW3 (one fragment in each), Goofy and Dat (two fragments each) and UBC844 (three fragments). *C. leopoldii* fragments were unique in UBC814, UBC899, Omar, and AW3 Dat, two in Manny and three in Mao. In the primers Dat and AW3 were observed just a unique fragment in each and two in UBC814, Mao and Goofy in the species *C. tigrina*. None was unique enough to identify a species-specific primer.

It was expected that accessions of *C. leopoldii* were closer to *C. tigrina* since *C. leopoldii* is heterotypic synonym of *C. tigrina*, however Figure 1 shows the similarity between *C. leopoldii* accesses with *C. guttata*. This fact may be related to the possibility of individuals being called *C. leopoldii* be a form or variety of *C. guttata* or even be a different although closer species to *C. guttata*.

The access *C. leopoldii* D3 was quite distant from other accessions of *C. leopoldii*. One explanation for this behavior would be that this access originated from a cross between a *C. leopoldii* pollen donor with a *C. tigrina* used as mother plant.

Davina (2009) points out that the complexity of studying the phylogeny of the orchid family is the wide morphological, cytological and ecological variety. States that the new molecular approaches help understanding that, however may present contradictions with classical analyzes.

The analysis of total banding pattern suggests that accessions called *C. guttata* and *C. leopoldii* are molecularly related to each other and more distant from *C. tigrina*.

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6. References

Daviña, J. R., Grabiele, M., Ceturtti, J. C., Hojsgaard, D. H., Almada, R. D., Insaurralde, I.S., Honfi, A. I. (2009). Chromosome studies in orchidacea from Argentina. Genet MolBiol, 32, 811-821.

Doyle, J. J., Doyle, J. L. (1987). A rapid DNA isolation procedure for small quantities of fresh life tissue.Phytochem Bull, 19,11-15.

Fajardo, C. G., Vieira, F. A., Molina, W. F. (2014). Interspecific genetic analysis of orchids in Brazil using molecular markers.Plant Systematic and Evolution, 300, 1825-1832.

Ferreira, M. E., Grattapaglia, D. (1998). Introdução ao uso de marcadores moleculares em análise genética (3rd ed.). Brasília, DF: Embrapa-Cenargen.

Freudenstein, J. V., Chase, M. W. (2001). Analysis of mitochondrial nad1b-c intron sequences in orchidaceae: Utility and coding of length-change characters. Systematic Botany, 26, 643-657.

George, S., Sharma, J., Yadon, V. L. (2009). Genetic diversity of the endangered and narrow endemic *Piperiayadonii* (Orchidaceae) assessed with ISSR polymorphisms. American Journal of Botany, 96, 2022-2030.

Hossain, M. M., Sharma, M., Pathak, P. (2013).*Invitro* propagation of *Dendrobiumaphyllum* (Orchidaceae) – Seed germination to flowering. Journal of Plant Biochemistry and Biotechnology, 22, 157-167.

Kores, P. J., Molvray, M., Weston, P. H., Hopper, S. D., Brown, A. P., Cameron, K. M., Chase, M. W. (2001). A phylogenetic analysis of *Diurideae* (Orchidaceae) bases on plastid DNA sequence data. Am J Bot, 88, 1903-1914.

Lu, J., Hu, X., Liu, J., Wang, H. (2011). Genetic diversity and population structure of 151 Cymbidium sinense cultivars. Journal of Horticulture and Forestry, 3, 104-114.

Martins, F. L. C. (2007). Análise molecular de espécies perenes e anuais do gênero *Dicerandra* (Lamiaceae) baseada em marcadores ISSR. Viçosa, Minas Gerais: UFV.

Novaes, R. M., Rodrigues, J. G., Lovato, M. B. (2009). An efficient protocol for tissue sampling and DNA isolation from the stem bark of Leguminosae trees. Genet Mol Res, 8, 86-96.

Oliveira, L. V. R. (2010). Genetic analysis of species in the genus *Catasetum* (ORCHIDACEAE) using RAPD markers.Braz Arch Biol Technol, 53, 375-387.

Pasqual, M., Soares, J. D. R., Rodrigues, F. A., Araujo, A. G., Santos, R. R. (2011). Influência da qualidade de luz e silício no crescimento *invitro* de orquídeas nativas e híbridas. Hortic Bras, 29, 324-329.

Reddy, M. P., Sarla, N., Siddiq, E. A. (2002). Inter-simple sequence repeat (ISSR) polymorphism and its applications plant breeding. Euphytica, 128, 9-17.

Rodrigues, J. F. (2010). Delimitação de espécies e diversidade genética no complexo *Cattleya coccinea* Lindl. e *C. mantiqueirae* (Fowlie) van den Berg (Orchidaceae) baseada em marcadores moleculares ISSR. Piracicaba, São Paulo: ESALQ, USP.

Sharma, S. K.,Kumaria, S., Tandon, P., Rao, S. R. (2013). Assessment of genetic variation and identification of species-specific ISSR markers in five species of *Cymbidium* (Orchidaceae). Journal of Plant Biochemistry and Biotechnology, 22, 250-255.

Wang, H. Z., Fenge, S. G., Lu, J. J., Shi, N. N., Liu, J. J. (2009). Phylogenetic study and molecular identification of 31 Dendrobium species using inter-simple sequence repeat (ISSR) markers. SciHortic, 122, 440-447.

Wolfe, Andrea D., (2000), ISSR protocols. [Online] Available: http://www.biosci.ohio-state.edu/~awolfe/ISSR/protocols.ISSR/html (August 10, 2012).