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Inbreeding depression in crosses of *coerulea* clones of Walker's Cattleya (*Cattleya walkeriana* Gardner)

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Orchids are among the most beautiful flowers and endangered due to habitat destruction and over-collection. *Cattleya walkeriana* is one of the most beautiful flowers joining the small sized plant with medium large and heavily scented flowers. It is widely known and appreciated by its beautiful clones and it has much to offer to breeders because their plants have besides other attributes as small habit and big flowers, many colour variations, form and precocity, becoming flower only four years in *ex vitro* culture. However, in some of the original places it is becoming a red listed species. Notwithstanding, very little is known about the genetics of these flowers and the variability in the species that is widespread in the Brazilian territory. The aim of this work was to estimate the variability among cultivated materials using the F statistics and to verify if there was inbreeding in plant crosses with similar characteristics, employing as a tool the RAPD simple methodology. The results obtained showed that RAPD was good enough to estimate the variability in *C. walkeriana*. The selected primers were able to define colour group, especially the *coerulea*. Inbreeding will occur in crosses of clones with the same colour.

Key words: Orchidaceae, deoxyribonucleic acid (DNA), random amplified polymorphic DNA (RAPD), variability, domestication.

INTRODUCTION

Currently, molecular markers have been used in plant breeding for several objectives, which makes possible more detailed and consistent analysis of their genetics. The establishment of deoxyribonucleic acid (DNA) molecular patterns serves as a parameter for identification of clones and varieties and as a tool for a better taxonomical classification and variability determination in orchids (Chung et al., 2006; Minoo et al., 2006, 2008; Parab et al., 2008; Niknejad et al., 2009; Verma et al., 2009; Oliveira et al., 2010; Xue et a l., 2010; Machado Neto and Vieira, 2011; Sharma et al., 2011; Manners et al., 2013).

The variability estimated by these markers can be used, as any other markers, to estimate the population genetics (F statistics) (Wright, 1978). However, these tools (F statistics) are barely used to follow populations in plant breeding (Sallam et al., 2015). Marker Assisted Selection (MAS) has been a useful tool for plant breeders,

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but has had limited success in improving complex traits (Heffner et al., 2010).

According Minoo et al. (2008), random amplified polymorphic DNA (RAPD) technique has some advantages over other techniques as the easy, rapid achievement of polymorphic markers, low cost, absence of hybridization, detection of polymorphism in highly repetitive genome, and high level of polymorphism compared to other molecular markers. However, either RAPD or any other single primer amplification method (SPAR) (Gupta et al., 1994), present a few disadvantages, such as ambiguity in the interpretation of the bands co-migrating fragments of equal size or close and dominant character in most of the markers obtained, what cannot be a penalty for this (Simmons, 2007). In the orchids study, this method can be used to indicate genetic similarity between these plants, their hybrids and wild ancestors, to offspring prediction of a cross, based on the information from the genotype of early and efficiently, as well as helping in the species classification, or just to measure the raw variability (Costa et al., 2006; Minoo et al., 2008; Niknejad et al., 2009; Oliveira et al., 2010; Xue et al., 2010; Machado-Neto and Vieira, 2011; Manners et al., 2013).

The analysis at molecular level is an advantage for studies on perennial plants, enabling the evaluation of genetic similarity between genotypes (Ambiel et al., 2008, 2010; Machado-Neto and Vieira, 2011) and to collect information on the level of genetic diversity of wild orchids to enable a better conservation of the species (Manners et al., 2013).

In the world flora, orchids are the second largest family, with almost 736 genera and over 26,000 species (Chase et al., 2015), ranging from 7 to 10% of the flowering plant species (Cowan et al., 2006) distributed in five subfamilies: Apostasioideae, Cypripedioideae, Epidendroideae, Orchidoideae, and Vanilloideae (Cameron, 2006). *Cattleya* together with *Cymbidium*, *Oncidium*, *Phalaenopsis* and *Dendrobium* are important commercial ornamental species due to its large spectrum of colours and relatively high cross ability with other genera. Orchid commercialization, both pot plants and cut flowers, is highly significant worldwide, about US$504 million (De and Medhi, 2015) and is increasing year after year.

The number of described species in *Cattleya* is still a matter of debate, ranging from 49 to 114 species (excluding *Guarianthe* and *Cattleyella* and including *Sophronitis* (Van den Berg, 2014) and Brazil has 98 endemics (Forzza et al., 2013).

Cattleya walkeriana belongs to the unifoliate group of the genus and within which it can be considered a small plant. It has a stout rhizome, with three internode pseudobulbs, close to each other. It has long roots, thick and often branched (Menezes, 2011). It is widely known and appreciated by its beautiful clones. It is found in different regions of Brazil, growing over rocks or trees in the states of Goiás, São Paulo, Mato Grosso do Sul, and Minas Gerais, nearby lakes, rivers or swamps and it could be easily cultivated (Menezes, 2011). It has much to offer to breeders because their plants have small habit, large flowers, many colour variations (t*ype*: (pink), *alba, semi-alba* and *coerulea,* Figure 1), form and precocity (Menezes, 2011), becoming flower only four years in *ex vitro* culture. However, in some of the original places, it is becoming a red listed species (Brasil, 2008).

Very few is known about the genetics of *Cattleya* species; *Cattleya intermedia* (Machado-Neto and Vieira, 2011) using RAPD; *Catleya elongata* (Cruz et al., 2011) with isozymes and ISSR; *Cattleya coccinea* (Novello et al., 2013); with ISSR in *Cattleya bicolor*, *Cattleya labiata* and *Cattleya schofieldiana* (Fajardo et al., 2014) with SSR and one in *C. labiata* using RAPD and ISSR (Pinheiro et al., 2012). And lesser is known about the inheritance of some characters especially because these plants are perennials and the time between one generation and the following is almost 5 years.

Plant breeding and evolution are related for two reasons; the first is that plant breeding might be defined as evolution guided by man and the second is that both processes have their basis in, and a major effect on, biodiversity (Ceccarelli, 2009).

In populations submitted to constant selection, where just the superior individuals were promoted for reproduction, the alleles controlling characters of interest had their frequency increased, leading to diversity loss in crop plants. In orchids, flower shape and colours have been improved by breeding (Machado Neto and Vieira, 2011). While in wild specimens of *C. walkeriana,* another colour than the type are often not found; it is common to find *alba*, *coerulea* and different colours in bred *C. walkeriana* with exceptionally well-shaped flowers. This species has much to offer for breeders, but it counts just with 109 direct hybrid offspring and it is not much used as parent; for example two related species, *Cattleya loddigesii* and *C. intermedia*, counts with 230 and 217 primary hybrids respectively (RHS, 2016)..

This study aimed at the measurement of the variability and inbreeding in a population submitted to selection and directional crosses of *coerulea* clones of *C. walkeriana*, by means of F statistics (Wright, 1978; Sallam et al., 2015), and to evaluate the ability of RAPD markers in grouping phenotypes of these plants and measure the fixation of the *coerulea* characteristic in the species and among clones. To our knowledge, this work is the first of this kind with this species.

MATERIALS AND METHODS

Plant

In this work, young and adult plants of *C. walkeriana*, *coerulea* (blue) colour, type (pink), *semi-alba* and *alba* (white flowers), were used. Plants have diverse origin, except the offspring. They are listed in Table 1.

DNA extraction

DNA extraction and amplifications were done as in Machado-Neto

Figure 1. Colour forms of *Cattleya walkeriana* Gardner.

Table 1. List of *Cattleya walkeriana* plants, their colour and origin.

Plant	Colour	Origin
Patrícia (parental P_1)	*Coerulea*	Prata - Minas Gerais
Blue City (parental P_2) and ABC (parental for Backcross - BC_1)	*Coerulea*	Offsprings of selfed DICK (ESALQ) clone from Itajubá, MG.
F_1 (first generation, 7 plants)	*Type*	(Patrícia x Blue City)
F_2 (second generation, F_1xF_1, 11 plants)	Unknown	-
BC_1 (first backcross generation - 23 plants)	Unknown	(F_1 x ABC)
Rancho Sereno x Patrícia (4 plants) (RSP)	*Coerulea*	-
Alba (CA)	white	unknown
Puanani (Pu)	*Semi alba*	-
Unnamed clone (SA)	*Semi alba*	(*semi alba* "Goiaba" x *albescens* "Denise Cavasini" – from Guará-SP)
Twins (CWT)	Light pink	-

Prata and Itajubá are nearly 600 km apart.

and Vieira (2011). To identify markers, 120 primers from Operon (Alameda, USA) were initially tested in four plants, one representative of each generation (Parental, F1, F2, BC1) and 33 were primarily chosen (A1, A2, A5, A10, A11, A14, A18, A19, A20, C1, C2, C4, C5, C6, C7, C8, C11, C12, C14, C16, D1, D2, D13, G2, G3, G5, G6, G7, G8, G11, G12, G14, G16) as they were polymorphic. PCR was carried out in a reaction volume of 25 µl containing Tris buffer (20 mM Tris-HCl, pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.4 µM of primer, 0.2 µM of each dNTP, 1U Taq polymerase and two concentrations of template DNA 25 and 50 ng. RAPD amplifications were performed in a thermo cycler under the following conditions: 94°C for 3 min for initial denaturation and then 40 cycles of 1 min at 94°C, 1 min at 37°C for primer annealing and 90 s at 72°C for chain elongation in a PTC-100 Thermocycler. An extra step of 5 min at 72°C for final elongation was included.

Amplification products were separated by electrophoresis in 1.5% agarose gel. Gels were stained with ethidium bromide and visualized using Electrophoresis Analysis System (Biosystems). Each amplification reaction was repeated at least twice and only clearly distinct and reproducible bands were scored. Weak or low intensity bands were not considered to avoid ambiguous interpretations. The analysis of the bands was performed with the Quantum program - Capt (Vilber -Lourmat) to determine the electrophoretic pattern. The primers selected for the final analysis were

Table 2. Nucleotide sequences of the primers, number of bands, polymorphism and size of amplified fragments in *Cattleya walkeriana*.

Primers	Nucleotide sequence (5' 3')	Polymorphic bands	Fragment size (pb)
A2	TGC CGA GCT G	10	280 - 1380
A5	AGG GGT CTT G	9	300 - 1190
A10	GTC ATC GCA G	10	250 - 1370
G5	CTG AGA CCG A	10	280 - 1380
G11	TGC CCG TCG T	8	400 - 1460
G13	CTC TCC GCC A	7	350 - 1060
Total	-	54	-

A2, A5, A10, G5, G11 and G13, because they successfully amplified a total of 54 fragments. Polymerase chain reaction (PCR) was performed as described earlier.

Bands were used to construct a similarity matrix based on the Jaccard coefficient, coding 1 as presence and 0 as absence. The grouping analysis was done using the Unweighted Pair-Group Method Using an Arithmetic Average (UPGMA) algorithm. This analysis was performed with the software NTSYS 2.1 (Rohlf, 2004). Molecular variance analysis (AMOVA) was calculated by total decomposition of its components among and between accessions using the square distances with the Arlequin software (Excoffier et al., 2015).

The fixation index or F statistics of Wright (F_{ST}) was generated by the Arlequin software (v. 3.5). The inbreeding coefficient (F_{IS}) was calculated by the formula:

$$F_{IS} = 1 + \frac{(1 - F_{IT})}{(1 - F_{ST})}$$

and the general fixation index (F_{IT}) was calculated by the formula:

$$F_{IT} = 1 - (\frac{Ho}{He})$$

where *Ho* and *He* were the observed and waited heterozygosity respectively, obtained in Arlequin software.

RESULTS AND DISCUSSION

Table 2 shows the nucleotide sequences used. The fragments generated ranged from 150 to 2500 bp, lying within the boundaries according to Xue et al. (2010), in which the RAPD technique has good reproducibility.

After primers selection, the construction of a dendrogram was made with all RAPD markers selected. According to the band analysis, it was possible to estimate the ability of these markers to group plants by their colour, that efficiency is demonstrated by the dendrogram (Figure 2), which exhibit a group of *coerulea* plants, where P_1 and RSP_1 are in the same branch, but in a different location of P_2 and RSP (P). That both P1 and P2 plants could be regarded as *coerulea* but to different subclades, P1 for group IA and P2 for group IB.

It was expected that all plants from a cross between "Rancho Sereno" and "Patricia" were in the same branch, because both plants are *coerulea*. All *coerulea* plants showed up together in a larger branch. In another clade, F_1 plants, although the same phenotype, were separated by branches indicating genetic proximity, but no similarity (group II – Figure 2). The CWT plant showed next to *semi-alba* (group III – Figure 2), these last sharing the same clade, and the plant CA in other branch bellow, but close to the *semi-alba* (group IV – Figure 2). This figure shows the clustering of plants that have flourished (relatives, control and F_1) and plants that have not flowered (F_2 and BC_1 generations). Some F_2 plants were close to F_1 ones which indicates a probable phenotypic similarity. The proximity of these plants with those of already known phenotype may indicate that their flower will have the same colour.

As shown in Table 3, for the population studied, there was a high variability, indicated by the overall F_{ST} (0.017), considering Wright (1978) in which $F_{ST} > 0.25$ was considered low variability. However, even using *coerulea* of different origins an increase in the F_{ST} values was showed (0.337 for the parents, 0.539 for F_1 and 0.567 for F_2, 0.465 for all the *coerulea*, and 0.492 for BC_1) explained by the fact that plants with similar characteristics were crossed and there was a decreasing in the variability meaning a strong differentiation between those plants and the population (F_{ST} 0.017). The selection of plants with the same colour for the initial cross led to endogamy showed in this study by the cross between Patricia x Blue City (Table 1), but it could happen in nature, as a pollinator would choose flower with the same colour, by chance, creating an inbreed population.

In *Cattleya intermedia* (Machado-Neto and Vieira, 2011) and *Cattleya elongata* (Cruz et al., 2011) F_{ST} values were low indicating a high gene flux among plants (0.016 and 0.100 respectivelly), but for Fajardo et al. (2014) these values are much higher (from 0.177 in *Cattleya labiata* to 0.322 in *Cattleya granulosa*) indicating loss of variability in the last case.

The F_{IS} values shown (Table 3) are also very informative, as the values are closer to -1, in the overall sample, meaning that there is more heterozygosity in

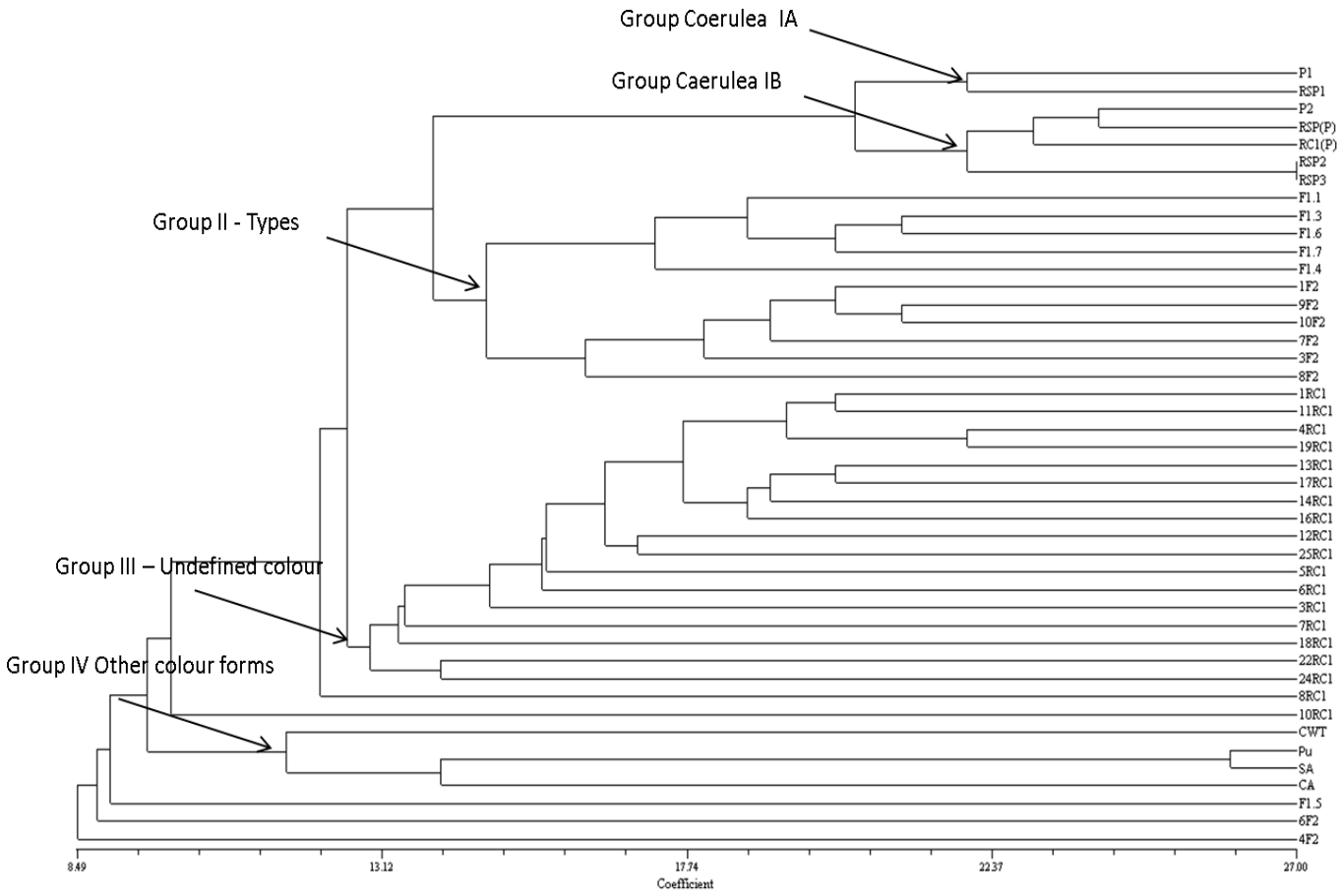

Figure 2. Grouping of plants of *Cattleya walkeriana* progeny F_1, F_2, BC_1 and other colour forms.

Table 3. Wright's measure of population differentiation (F_{ST}) and inbreeding (F_{IS}), observed (H_o) and estimated Heterosigosity (*He*) using RAPD markers for *Cattleya walkeriana* offsprings.

Source of variation	FST	FIS	HO (±SD)	HE (±SD)
Parents	0.377**	-0.403**	0.496 (0.273)	0.353 (0.140)
F_1	0.539**	-0.022	0.456 (0.197)	0.446 (0.126)
F_2	0.567**	-0.320**	0.454 (0.198)	0.344 (0.145)
Coerulea	0.465**	-0.243**	0.496 (0.213)	0.399 (0.135)
BC_1	0.492**	-0.447**	0.450 (0.211)	0.311 (0.149)
Overall	0.017	-0.734**	0.463 (0.211)	0.267 (0.153)

**P<0.01.

this. On the other hand, there was more homozygosis as the values approaches to zero, exemplified by -0.022 in the F_1 population. Intermediate values as the parents (-0.403), F_2 (-0.320), *coerulea* (-0.243) and BC_1 (0.447) were more heterozygous than F1. These kind of data are very useful for perennials (Guries and Ledig, 1981). The values found in this work for *Ho* and *He* were not statistically different for the populations. In both, F_{ST} and F_{IS}, there were indications that general population has a good gene flux and driven crosses led to gene diversity loss.

Li and Ge (2006) using RAPD markers found low genetic diversity within populations and high among the studied populations of *Changnienia amoena* (an orchid species). These results were due to small population size, the local extinction because of habitat destruction

and restricted gene flow.

In *Platanthera leucophaea*, another species of orchid, rare and endangered species, the values of F_{ST} for RAPD and isoenzymes (0.889 and 0.754, respectively) showed a large amount of inbreeding consistent with each other (Holsinger et al., 2002). Moreover, in a study by Pressoir and Berthaud (2004), the allelic fixation index in corn landraces showed little variation between populations (F_{ST} 0.003 to 0.011).

Ambiel et al. (2008, 2010) estimated in *Brachiaria brizantha*, an apomitic species, lower values of F_{ST} (0.216 and 0.276) indicate a high gene flow. According to Wright (1978) populations with low levels of selection showed lower values of F_{ST}. Sallam et al. (2015) used the F statistics to follow selection during a barley breeding process, so this could be a very useful tool to follow improvement during breeding program, especially in perennials as orchids.

Wild and cultivated populations differ statistically in various characters likely to be human selected, although some cultivated plants are morphologically indistinguishable from their relative wild plants (Pickersgill, 2007). So, the increase in the F_{ST} measured during the breeding generations followed in this work was there the indicator that there was an increase in the inbreeding and in the loss of variability. The targeted character (flower colour) was not being fixed in the generations after the initial cross, but the primers were good enough to group the plants according flower colours.

Conclusion

RAPD was good enough to estimate the variability in *C. walkeriana*. The selected primers were able to define colour group, especially the *coerulea*. F_{ST} was a good of inbreeding, occurring in crosses of clones with the same colour and and in the loss of variability driven by the selection.

Conflict of Interests

The authors have not declared any conflict of interest.

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