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Genetic diversity between native and improved *Cattleya walkeriana* Gardner famous clones

Evandro Vagner Tambarussi[1*], Elizabeth Ann Veasey[2], Lou Menezes[3], Bruna Ibañes[2], Kátia Cylene Lombardi[1] and Roland Vencovsky[2]

*[1]Universidade Estadual do Centro-Oeste, PR-153, Km 7, 84500-000, Irati, Paraná, Brazil. [2]Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo, Piracicaba, São Paulo, Brazil. [3]Orquidário Nacional do Ibama, Brasília, Distrito Federal, Brazil. *Author for correspondence. E-mail: tambarussi@gmail.com*

ABSTRACT. The aim of this study was to evaluate the genetic diversity among native plants and some individuals obtained from crosses with unknown genealogy of *C. walkeriana* as well as *C. loddigesii* and *C. nobilior* and to advance towards solving the question of the genetic purity of the "Orchidglade" clone. Eight microsatellite loci were used to evaluate the genetic diversity between individuals of *C. walkeriana*. Microsatellites were not efficient in determining the genetic diversity between *C. walkeriana* groups (native and improved). The difficulty in determining the genetic distance between the different genotypes can be attributed to the complex mating system of the species and to a weak genetic barrier that facilitates the development of hybrids. Our analysis revealed smaller genetic distances between the "Orchidglade", "Equilab", "Kenny" and "Pedentive" clones and the species *C. loddigesii* and *C. nobilior*. Native *C. walkeriana* plants were genetically more distant from the *C. loddigesii* and *C. nobilior* species.

Keywords: orchid improvement, genetic variation, genetic differentiation, *Cattleya loddigesii*, *Cattleya nobilior*.

Diversidade genética entre clones famosos nativos e melhorados de *Cattleya walkeriana* Gardner

RESUMO. O objetivo desta pesquisa foi o de avaliar a diversidade genética entre plantas nativas e indivíduos de genealogia desconhecida de *C. walkeriana*, bem como *C. loddigesii* e *C. nobilior*, e também avançar na solução do dilema da origem do clone "Orchidglade" de *C. walkeriana*. Oito locos microssatélites foram utilizados para avaliar a diversidade genética entre indivíduos de *C. walkeriana*. Os marcadores microssatélites não foram eficientes na determinação da diversidade genética entre os grupos *C. walkeriana* (nativas e melhoradas). A dificuldade em determinar a distância genética entre os genótipos diferentes pode ser devida a um sistema complexo de reprodução das espécies e devido a uma fraca barreira reprodutiva facilitando o desenvolvimento de híbridos. Nossa análise revelou menores distâncias genéticas entre os clones Orchidglade, "Equilab", "Kenny" e "Pedentive" e as espécies *C. loddigesii* e *C. nobilior*. As *C. walkeriana* nativas se mostraram geneticamente mais distantes das espécies de *C. loddigesii* e *C. nobilior*.

Palavras-chave: melhoramento de orquídeas, variação genética, diferenciação genética, *Cattleya loddigesii*, *Cattleya nobilior*.

Introduction

Cattleya walkeriana Gardner is appreciated by growers because of its diversity of forms and its beautiful and valuable flowers (Da Silva & Milaneze-Gutierre, 2004). In recent years, collectors have been looking for plants with high levels of genetic improvement (Menezes, 2011), and individuals with improved traits (rare colour and good shape of the flower) are highly valued.

Biotechnology has helped in many different ways to improve the understanding and preservation of orchid species, through *in vitro* techniques, the differentiation of natural populations, species delimitations in rare plants (Rodrigues, Borges, Neto, Boaretto, & Oliveira, 2015), phylogeography (Pinheiro et al., 2012) and hybridization (Azevedo, Borba, & Berg, 2006). Molecular markers have been used for the genetic analysis of many orchids, such as *Cypripedium* and *Calanthe* (Qian, Wang, & Tian, 2013), *Cattleya* (Almeida et al., 2013; Novello et al., 2013; Tambarussi et al., 2017), and *Liparis* (Broeck et al., 2014), among others.

The hybridization process occurs in all living organisms, including plants (Anamthawat-Jónsson, 2001). The use of molecular tools has shown that interspecific hybridization is even more prevalent than indicated by morphological and cytogenetic evidence (Kaplan & Fehrer, 2007). This type of hybridization

has helped to increase genetic diversity and plant speciation (Arnold, Cornman, & Martin, 2008). Natural hybrids have also been described in several species of orchids, for example, *Cattleya* (Neto, Motte, & Dubuisson 2012) and *Paphiopedilum* (Parveen, Singh, Raghuvanshi, Pradhan, & Babbar, 2012).

Hundreds of different hybrid combinations were produced by artificial crosses in the genus *Cattleya*. Artificial crosses are a standard practice in the multi-million-dollar orchid agribusiness and even among enthusiasts. Orchid growers accept this process when the aim is to produce hybrid individuals; however, it is not very well accepted in intraspecies breeding such as *C. walkeriana* when the aim is to produce "pure plants". When there is introgression, the second species in these hybridization programs and the newly formed species become indistinguishable based on a "normal" flower shape. This process is not welcome among orchid collectors, and many of them believe that *Cattleya walkeriana* alba "Orchidglade" is not natural, as historically assumed for years. These collectors believe that it is due to a hybridization event, which has never been confirmed.

There has been much discussion among orchid growers about possible contaminants in *C. walkeriana* species such as Orchidglade and many other clones. Microsatellites have high power for identifying hybrid plants (Rodrigues, Neto, & Schuster, 2008). Thus, the objective of this research was to estimate the genetic distance/similarity between famous clones of native and improved plants of *C. walkeriana* and a few accessions of *C. loddigesii a*nd *C. nobilior* using microsatellite markers.

Material and methods

Plant materials and microsatellite analysis

A total of 25 individuals of *C. walkeriana*, four of *C. loddigesii* and three of *C. nobilior* were genotyped for eight microsatellite loci. All *C. walkeriana* individuals and two *C. loddigesii* individuals were kindly provided by growers from the States of Minas Gerais, São Paulo and Goiás and are at different levels of improvement. These individuals are stored in private collections due to the high value of the plants. One *C. loddigesii* (34033) and two *C. nobilior* (30665 and 5652) were randomly collected from the "Professor Paulo Sodero Martins" Orchids Collection of the Genetics Department (ESALQ/USP), University of São Paulo, Piracicaba, São Paulo State, Brazil (Table 1).

DNA extraction, amplifications and microsatellite loci scoring steps were performed following Tambarussi et al. (2017). Eight microsatellite loci (Cw01, Cw02, Cw03, Cw04, Cw05, Cw07, Cw08, and Cw09) specific for *C. walkeriana* were used (Tambarussi et al., 2017). Allele scoring was performed using a 10 bp DNA Ladder (Invitrogen) as the size standard.

Table 1. List of *Cattleya walkeriana* genotypes and other *Cattleya* species studied, with the respective variety and source/origin.

Name of the clone	Variety	Origin/provenance
Native plants		
"Bandida"	Albecens	Nova Serrana/MG
Native concolor	Concolor	**
"Gravatinha"	rosada-delicata	Rio Verde/GO
"Marina"	alba	Abadia dos Dourados/MG
"Matão"	albecens	Matão/SP
"Meire"	albecens	Arcos/MG
"Raquel Nazar"	albecens	Batatais/SP
"Rosangela" (RO)	alba	Piumhi/MG
"Rainha da Canastra" (RC)	alba	Delfinópolis/SP
"São Francisco"	semi-alba	Três Marias/MG
"Nomura"	albecens	**
Crosses from native plants		
"Hebe Manarini"	semi-alba	Poços de Caldas/MG
"JK" x "Marina"	?	Uberaba/SP
"RC" x "Marina"	?	Uberaba/SP
"RC" x "Rosangela"	?	Uberaba/SP
Plants with unknown genealogy		
"Equilab"*	alba	Poços de Caldas/MG
"Kenny"	semi-alba	Poços de Caldas/MG
"Laina"	alba	Poços de Caldas/MG
"Orchidglade"*	alba	Catanduva/SP
"Orchidglade"*	alba	Uberaba/SP
"Orchidglade"*	alba	Poços de Caldas/MG
"Pedentive"	alba	Belo Horizonte/MG
"Tokio"*	semi-alba	Uberaba/MG
"Tokio"*	semi-alba	Munhuaçu/MG
"Puanani"*	semi-alba	Munhuaçu/MG
Other Cattleya spp. *(external group)*		
C. loddigesii	alba	Poços de Caldas/MG
C. loddigesii	tipo	Cabrália Paulista/SP
C. loddigesii	tipo	Piracicaba/SP-ESALQ 34033
C. loddigesii	tipo	Poços de Caldas/MG
C. nobilior "gracinha"	alba	Poços de Caldas/MG
C. nobilior	tipo	Piracicaba/SP-ESALQ_30995 33303099530995
C. nobilior	tipo	Piracicaba/SP-ESALQ 5652

?= there was no bloom;* = contradictory origin.

Statistical analysis

To evaluate the genetic distances between the genotypes of *C. walkeriana*, *C. loddigesii* and *C. nobilior,* we estimated the number of alleles and their frequencies for eight microsatellite loci. From these frequencies, we estimated all possible pairs of genotypes by Rogers (1972) genetic distances modified by Wright (1978) as follows:

$$d_{ij} = \sqrt{\frac{1}{2n}\sum_{k}(x_{ki} - x_{kj})^2},$$

where: n = number of loci, χ_{ki} and χ_{kj} = frequency of *k-th* allele of individuals *i* and *j*, which were used to cluster genotypes through UPGMA (Unweighted Pair-Group Method with Arithmetical Means). These analyses were performed using Tools for

Population Genetics Analyses (TFPGA) software version 1.3 (Miller, 1997).

To assess the genetic diversity of *C. walkeriana* accessions, we separated the individuals into two groups: Group I consisting of native plants and Group II of individuals with some level of improvement and individuals with unknown genealogy. The genetic differentiation between groups was estimated by FSTAT (Goudet, 1995). Genetic differentiation was estimated by Nei's (1978) statistics, where H_T is the total genetic diversity, H_s is the genetic diversity within groups, D_{ST} is the genetic diversity among groups, and G_{ST} is the proportion of genetic differentiation among groups. The G_{ST} genetic diversity estimation was standardized according to Hedrick (2005) and calculated as follows: $G'_{ST} = G_{ST}(1+H_S)/(1-H_S)$. To measure the genetic differentiation of the two groups, the method developed by Hedrick (2005) was chosen.

Results and discussion

The genetic distances between individuals ranged from 0.0833 to 0.8527 (data not shown). The highest values were observed across species. In the cluster analysis performed on the basis of genetic distances, no well-defined groups were observed (Figure 1). Unlike Jin, Naito, and Matsui (2004), we could not separate the *Cattleya loddigesii* and *C. nobilior* species from *C. walkeriana* individuals. By separating the accessions into two groups, Group I consisting of 17 native individuals of *Cattleya walkeriana* (68%) and Group II of the other plants of *C. walkeriana* (32%) (including individuals with some level of improvement and some from unknown genealogy), we found that the majority (98.1%) of the genetic diversity ($\hat{H}_T = 0.570$) is distributed within groups ($\hat{H}_S = 0.559$), while only 1.93% is distributed among groups ($\hat{D}_{ST} = 0.011$) (Table 2). Similar results were obtained by Pinheiro et al. (2012) for 130 genotypes of *Cattleya labiata*.

Low genetic variation was detected among loci, and no genetic diversity was found for loci Cw01, Cw03, Cw07, and Cw08 for $\hat{G}_{ST}$ (Table 2). The estimation of $G`_{ST}$, according to Hedrick (2005), is considered a more accurate parameter. The advantage of G'_{ST} is that it is suitable as an analogue of F_{ST} for multiple alleles (microsatellite alleles). This estimation of allelic frequencies also considers the different alleles present in the population. However, some aspects of Nei's (1978) estimates are also shown at a comparison level, when using codominant markers. Considering G'_{ST}, the difference between groups was 0.038. Therefore, the reproductive system has a significant impact on the distribution of genetic variability and consequently on population genetic diversity (Nybom & Bartish, 2000). Brzosko, Wróblewska, Jermakowicz, and Hermaniuk (2013), while studying 11 natural populations of *Goodyera repens*, detected greater genetic diversity within populations and low but significant genetic differentiation between them. The genetic variation in neutral loci depends on gene flow patterns; therefore, the dispersion and the founding of new populations are limited. This limitation will contribute to a positive correlation between the geographic and genetic distances between species (Kimura & Weiss, 1964; Slatkin, 1985, Alexandersson & Ågren, 2000).

Table 2. Genetic diversity parameters: number of alleles in Group 1 (k_{G1}) and Group 2 (k_{G2}), total ($\hat{H}_T$), within ($\hat{H}_S$) and among group ($\hat{D}_{ST}$) diversity, as well as Nei's (1978) ($\hat{G}_{ST}$) and Hedrick's (2005) ($\hat{G}'_{ST}$) statistics, respectively, between the two groups in *Cattleya walkeriana* based on eight microsatellite loci.

Locus	k_{G1}	k_{G2}	$\hat{H}_S$	$\hat{H}_T$	$\hat{D}_{ST}$	$\hat{G}_{ST}$	$\hat{G}'_{ST}$
Cw01	3	4	0.572	0.571	0.000	0.000	0.000
Cw02	8	5	0.835	0.839	0.004	0.005	0.009
Cw03	3	3	0.407	0.406	0.000	0.000	0.000
Cw04	7	7	0.814	0.851	0.037	0.044	0.084
Cw05	2	2	0.339	0.396	0.056	0.142	0.249
Cw07	5	5	0.750	0.735	0.000	0.000	0.000
Cw08	4	4	0.564	0.556	0.000	0.000	0.000
Cw09	1	2	0.190	0.206	0.016	0.078	0.144
Overall	33	32	0.559	0.570	0.011	0.019	0.038

Our results showed that the breeding process appears to be little influenced by the loss of alleles, as both groups presented almost the same number of alleles (Table 2). However, for locus Cw2, this allelic loss appears to be more pronounced (Tables 2 and 3). Indeed, 80% of the alleles for this locus are unique in Group 1. This difference in the presence of these alleles in the native plants may reflect higher allelic variability in plants not yet subjected to domestication (Upadhyaya et al., 2008). Genetic diversity variation can be influenced by various factors, with the breeding system having a particularly significant effect (Nybom & Bartish, 2000).

The small genetic distances observed between Groups I and II (Figure 1) may reflect the low genetic barrier in the species. The Orchidaceae family is known for the large number of hybrids between species and related genera, formed through artificially induced pollination.

Table 3. Presence (+) or absence (-) of alleles in two groups of *C. walkeriana*. Group I consists of native plants, and Group II consists of individuals with some level of improvement and individuals with unknown genealogy. Only the contrasting alleles are presented from each locus.

Molecular size of the allele	Presence (+) or absence (-) of specific alleles	
	Group 1	Group 2
Locus Cw01		
146	+	-
154	-	+
160	-	+
Locus Cw02		
200	+	-
232	+	-
234	-	+
310	+	-
330	+	-
Locus Cw04		
180	+	-
194	-	+
Locus Cw09		
222	-	+

Cattleya is a key genus used in the production of artificial hybrids. However, in nature, at least 89 natural hybrids have been found with established parents and 36 hybrids of uncertain origin as well as five natural hybrids with other genera (ex. *Brassavola*, *Laelia* and *Encyclia*) (Monteiro, Selbach-Schnadelbach, Oliveira, & van den Berg, 2010). For plants with unknown genealogy, one from each Orchidglade and Equilab clones were allocated differently from the other two "Orchidglade" clones. Indeed, all of these plants were separated from *C. loddigesii alba*. This fact may indicate a lower genetic distance between *C. loddigesii alba* and these clones.

Among growers, it is widely speculated that the plants called "Orchidglade" were first described in a Rio de Janeiro Botanical Garden, but this fact has never been proven. What we found in this molecular analysis is that these plants are genetically different. This difference may occur because growers change their names (intentional or unintentionally) in their greenhouses. An important fact about *C. walkeriana* is that the native clone called "São Francisco" (*C. walkeriana* var. *princeps* L.C. Menezes) was grouped with a clone that escapes the typical standard of the *C. walkeriana* flower, called "Laina". Many growers believe that Laina is a hybrid between *C. walkeriana* and *Cattleya* x *dolosa* (*C. walkeriana* x *C. loddigesii*).

As expected, there is lower genetic distance between individuals and their parents. The clones called "Rosangela", "Marina" and "Rainha da Canastra" (RC) were genetically closer to the seedlings of the crosses "JK" x Marina, RC x "Marina" and RC x "Rosangela", which may be a proof of the effectiveness of our markers.

With a detailed analysis, we noticed that allele 160 of locus Cw01 found in the clone "Pedentive" from Group 2 was detected only in *Cattleya loddigesii*. We also detected, in Group 2, that allele 232 of locus Cw02 was found in three individuals ("Gravatinha", "Puanani" and "Rachel Nazar") and in the *C. nobilior* group (Table 3). This result can be explained by the genetic proximity of these two *Cattleyas* (Braem, 1984). This similarity may influence the grouping of two "Orchidglade" clones with *C. nobilior* and *C. loddigesii* (Figure 1). However, "Pedentive" presents morphological traits that differ substantially from the *C. walkeriana* species. "Pedentive" was once considered a hybrid by other authors (Jin et al., 2004), which agrees with our analysis (Figure 1).

Traits such as time of flowering, shape, size and lip colour, scent of flowers and shape of the pseudobulbs from individuals stemming from "F1" crosses for the "Pedentive" clone showed strong traces of hybridization (Furusu, 2000). Allele number 222 (loci Cw09), found in Group 2, is exclusive to only one of each of the three clones called "Orchidglade" and "Kenny" and it is not present in any other genotyped individual. "Kenny" and "Pedentive" both have morphological traits that distinguish them from typical *C. walkeriana*. In 2009, the American Orchid Society considered that *C. walkeriana* Kenny appeared to be *Cattleya* Snowblind *(C. angelwalker*[1] x *C. walkeriana* Pendentive) and recommended changing its name. Another allele present only in Group 2 is allele 194 of locus Cw04. This allele appears at high frequency (0.389, data not shown) in Group 2 but is present only in "Equilab", Orchidglade and "Pedentive". In our analysis, "Orchidglade" and "Kenny" are closer than the native plants, which typically may be a signal of the introgression of genes from other species. Gene flow between species by natural introgression is a common event, especially in the genomes of species that are permeable to other closely related species (Russell et al., 2010). The three sources of "Orchidglade" that were analysed appear to be genetically closer to the *C. loddigesii* specimens and to individuals with hybrid traits ("Pedentive" and "Kenny") than to native *C. walkeriana* plants. For our genotypes, microsatellites were not efficient for determining the genetic similarity between *C. walkeriana* groups (native *vs* improved). The difficulty in determining the genetic distance between these different genotypes can be attributed to the complex mating system in the orchid species, presenting a weak or non-existent genetic barrier, and facilitating the development of artificial and natural hybrids.

[1]http://www.orchid.or.jp/orchid/people/hashizume/kakeizu/C_Angelwalker.htm

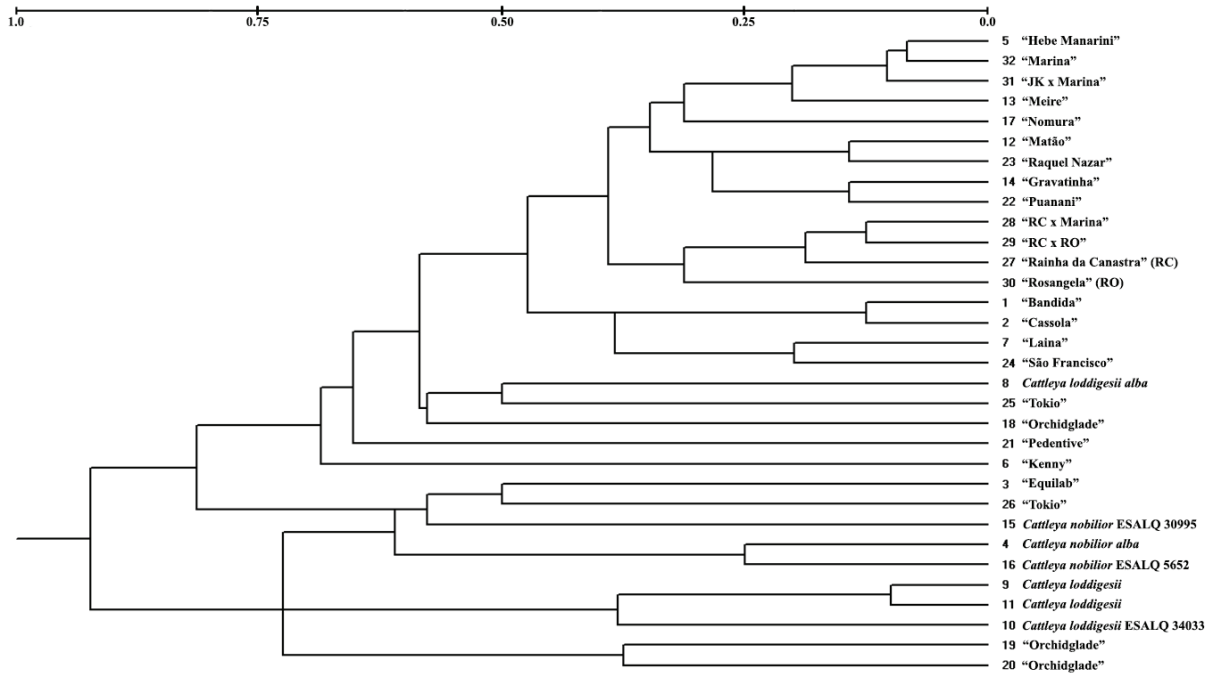

Figure 1. Dendrogram of 32 *Cattleya* genotypes, based on microsatellite data obtained by the UPGMA clustering method, based on the matrix obtained using the Rogers modified distance.

Conclusion

Our analysis revealed smaller genetic distances between the "Orchidglade", "Equilab", "Kenny" and "Pedentive" clones and the species *C. loddigesii* and *C. nobilior*. Our results also showed that native plants of *C. walkeriana* are genetically more distant from *C. loddigesii* and *C. nobilior*.

We still cannot verify that the clone "Orchidglade" underwent hybridization. However, new microsatellite markers and DNA barcodes are being developed to continue these studies.

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