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Molecular polymorphism in seedlings of *Cattleya forbesii* resulting from *in vitro* germination

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Abstract: The aim of this study was to evaluate molecular polymorphism of *in vitro* samples of propagated *Cattleya forbesii* which are used to replace stocks and cultivation in nature. Molecular diversity in micro-propagated orchids has not been investigated. The genetic diversity of this species was studied using touchdown PCR (Polymerase Chain Reaction) for random amplified polymorphism DNA markers (td-PCR-RAPD). The 12 RAPD primers used in the present study generated 148 reproducible DNA segments, of which 132 were polymorphic. There were 89.19% polymorphism, and the number of bands for each primer varied from 7 to 17, with an average of 12.33 bands per primer. The large natural genetic variability previously described in studies examining populations distributed in their natural habitats was also observed in *C. forbesii* seedlings grown *in vitro*. The polymorphism of td-PCR-RAPD markers in *C. forbesii* seedlings from self-pollination and *in vitro* germination was high, indicating that the genetic diversity in this species does not suffer from the negative effects of self-pollination or *in vitro* propagation. *In vitro* culture of *C. forbesii* seeds was considered suitable to produce seedlings for replacing stocks, for cultivation in nature and to stimulate the production of hybrids.

Keywords: orchid micropropagation; td-PCR-RAPD markers; genetic polymorphism; genetic diversity.

Resumo: O objetivo no presente estudo foi avaliar o polimorfismo molecular em amostras de *Cattleya forbesii* propagadas *in vitro,* as quais são usadas para reposição de estoques e cultivo na natureza. A diversidade genética em orquídeas microporpagadas não tem sido investigada. A diversidade genética nesta espécie foi estudada usando *touchdown* PCR (*Polymerase Chain Reaction*) para analisar o polimorfismo em sequencias aleatórias de DNA amplificadas por PCR (*td*-PCR-RAPD). Os 12 *primers* de RAPD usados no presente estudo geraram 148 segmentos de DNA, dos quais 132 foram polimórficos. O polimorfismo de 89.19%, e o número de segmentos amplificados para cada *primer* variou de 7 a 17, com uma média de 12,33 segmentos por *primer*. A ampla variabilidade genética previamente descrita em estudos de populações de orquídeas distribuídas em seus habitats naturais foi observada também em plântulas de *C. forbesii* crescendo *in vitro*. O polimorfismo dos marcadores *td*-PCR-RAPD em plântulas de *C. forbesii* originadas de autopolinização e germinadas *in vitro* foi alto, indicando que a diversidade genética nesta espécie não sofre os efeitos negativos de autopolinização ou da propagação *in vitro*. A cultura *in vitro* de sementes de *C. forbesii* foi considerada

adequada para produzir plântulas para reposição de estoques, para o cultivo em condições naturais, e para estimular a produção de híbridos.

Palavras-chave: micropropagação de orquídeas; marcadores *td*-PCR-RAPD; polimorfismo genético; diversidade genética.

Introduction

Orchids of the genus *Cattleya* Lindl. comprise several species showing exuberant flowers that are economically important because are the species of orchids most highly commercialised (ZANENGA-GODOY & COSTA, 2003). *C. forbesii* is an epiphyte found on trees, rocks and bushes near streams and waterfronts, occurring in the states of Rio de Janeiro, São Paulo, Paraná, Minas Gerais, Espírito Santo, Rio Grande do Sul e Santa Catarina, in Brazil. *C. forbesii* plants bloom in late November, extending through the month of March; their pollination is made by bees (MEDEIROS *et al*., 2013). It has an average of 2-5 flowers per inflorescence, with 10-12.5 cm; the sepals and petals are green, and the lip is pink and brown with yellow in the centre (GROVES, 2009); the sepals are nearly equal in size, are wavy and obtuse, the lip is tri-lobed, yellow coloration, streaked in shades of reddish-brown (MEDEIROS *et al.*, 2013).

C. forbesii has an ornamental value; therefore, this plant is cultivated and appreciated for its blossoms. Self-compatibility and incompatibility are common in Orchidaceae, and the mating systems of *Cattleya* species reveal that self-compatibility and interspecific compatibility are the rule in this genus (STORT, 1986). Natural hybrids of the genus *Cattleya* have also been reported (MENEZES & CASTRO NETO, 2007). Although it few studies found in the specialized literature, species of the genus *Cattleya* and their hybrids are currently the most commercialised orchids in Brazil due to their large, colourful flowers. Orchids from *Cattleya* genus are currently threatened with extinction due to loss of habitat and intense action collectors (PINHEIRO *et al.*, 2012).

Mass propagation of these species has been necessary to replace these orchids in nature because large numbers of plants have been harvested by orchid collectors and producers. *In vitro* tissue culture is a powerful alternative tool for germination and initial growth of many orchid species. Knudson 'C' medium (KC medium), described by Knudson (1946), has been the primary medium utilised for *in vitro* germination of most orchid species. *In vitro* or micropropagation of orchids is particularly important as a tool of species conservation and recovery when working with highly valued ornamental plants and attempting to maintain population genetic diversity (REED *et al*., 2011).

In vitro propagation and development of orchids may be induced by using different supplements (vitamins, amino acids, sugar source and concentration, types and concentrations of growth regulators) in the culture medium and/or by using different light and temperature conditions during culture. The addition of different supplements in the culture medium has been particularly effective for the *in vitro* propagation of *Cattleya* species (KRAPIEC *et al*., 2001; 2003; PRIZÃO *et al*., 2012). Although the addition of different supplements is particularly effective for the *in vitro* propagation of

orchids (HAMADA *et al*., 2010; KATSUMI, 2007), the use of culture medium and supplements, as well as *in vitro* culture conditions, may select specific genotypes, leading to a reduction in the genetic diversity of species cultivated *in vitro*. Founder effects and genetic drift can be expected with *in vitro* culture, resulting in low genetic variation and the narrowing of the genetic basis within samples.

High and low genetic diversity have been revealed using molecular markers in different orchid species maintained in their natural habitats or in collections (2006; COZZOLINO & WIDMER, 2005; VERMA *et al*., 2009; FAY *et al*., 2009; DUFF *et al*., 2009; CAI *et al*., 2011). However, the genetic diversity in orchids from micropropagation has not been investigated. The genetic diversity of *C. forbesii* also remains unknown; thus, it is difficult to implement effective conservation strategies. Therefore, the objective of this study is to evaluate the genetic diversity in samples of *in vitro*-propagated *C. forbesii* used to replace stocks and cultivation in nature. The touchdown PCR (DON *et al.,* 1991) for RAPD markers (td-PCR-RAPD) was used in studying the genetic diversity of this species. In comparison with other molecular markers, the use of RAPD markers has some limitations (Random Amplification of Polymorphic DNA; WILLIAMS *et al*., 1990), such as occasional low reproducibility and amplification of artefact markers, which may discourage many investigators from using RAPD. The use of touchdown PCR (Polymerase Chain Reaction) may reduce the amplification of artefacts in RAPD markers (KARACA & INCE, 2008). The touchdown PCR program proposed by Don *et al*. (1991) employs an initial annealing temperature above the projected melting temperature for the primers used as well as a gradual decrease of the primer annealing temperature to ensure the effectiveness of the primer complementary to the DNA sample. The combination of RAPD with touchdown PCR ensures the fidelity of the amplified DNA segments. During the experimental procedures, all steps were carefully controlled, and the touchdown PCR-based RAPD (td-PCR-RAPD) markers were used to improve reproducibility and to eliminate artefact markers. Using td-PCR-RAPD under carefully controlled reaction conditions, reproducible and interpretable RAPD banding patterns can be obtained. We provide the first data about the genetic diversity of Cattle*ya forbesii*. We believe that this study will supply useful information for *C. forbesii* conservation programs.

Materials and Methods

The seeds used in our study were collected from *C. forbesii* maintained in its natural habitat. The mature capsules of *C. forbesii* from self-pollination, collected in a riparian forest (city of Maringá, Parana State, Brazil), were opened to remove the seeds. The seeds were germinated *in vitro* in vials containing KC medium (KNUDSON, 1946) modified by the addition of 0.6% agar (Himedia, Agar-Agar, Type 1), 3% sucrose and 10% banana pulp (*Musa* sp. variety "nanica") [maturation stage 7; scale 1-7 defined by Oliveira-Neto (2002), which corresponds to a yellow colour with light brown spots]. The addition of banana pulp has been shown to have positive effects on the germination and seedling growth of *Cattleya loddigesii*, *Dendrobium nobile*, *Encyclia randii* and *Hadrolaelia*

purpurata (SU *et al.,* 2012; GONÇALVES *et al*., 2012).

After inoculation, the seed-containing vials were maintained in a growth chamber at a temperature of 25 ± 3 °C with continuous illumination provided by fluorescent lamps (40 Watts). Figure 1 shows the *C. forbesii* seedlings growing in the culture medium six months after seed inoculation (Figure 1A). After 12 months, the seedlings were removed and freed of the agar by washing them in water and transferring them to polyethylene vials containing a mixture of sand and rock wool (over the sand) for the acclimation phase (Figure 1B).

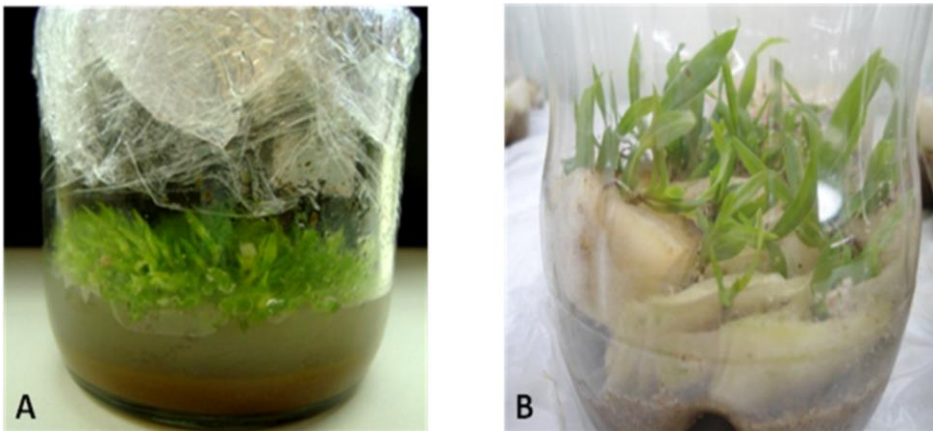

Figure 1. Seedlings of *Cattleya forbesii* growing in the culture medium six months after seed inoculation (A) and seedlings transferred after 12 months to polyethylene vials containing a mixture of sand and rock wool for the acclimation phase (B).

For DNA extraction, 43 *C. forbesii* seedlings originating from seeds germinated *in vitro* in the medium Knudson "C" were randomly selected. The genomic DNA extraction was performed using the CTBA method (cetyltrimethylammonium bromide) from Knapp and Chandlee (1996), adapted by Choi *et al*. (2006). After purification, DNA samples were quantified, and their quality was evaluated by electrophoresis on a 0.8% agarose gel.

Two individual plants were randomly selected to standardise the concentrations of DNA and $MgCl_2$ for use in the amplification of RAPD markers based on touchdown polymerase chain reactions (td-PCR-RAPD). The concentrations of DNA and $MgCl_2$ were based on the optimisation of RAPD-PCR using genomic DNA (10, 20, 30 ng) and $MgCl_2$ (1.5, 2.0, 2.5, 3.0 mM). The primers used for the test were RAPD primers from Random Primer Kits A (1 to 20), B (1 to 20), C (1 to 20), F (5 and 13), L (11), M (1 to 10) and P (2, 4, 7 to 11 and 17) (Operon Technologies, Alameda, California, USA).

The amplification reactions were performed in a total volume of 20 µL containing 20 ng of template DNA; 2.5 mM $MgCl_2$; 0.3 µmol/L primers (only one primer was used in each reaction); 2.0 µL of reaction buffer 10x (Invitrogen); 0.01 mmol each of dATP, dGTP, dCTP and dTTP; 1 unit *Taq* DNA polymerase (Invitrogen); and sterilised

water. PCR was carried out in a Techne TC-512 thermal cycler. In the td-RAPD, the PCR program was adjusted: the annealing temperature was modified in relation to conventional PCR. The reaction profile was as follows: 3 min at 94 °C, followed by a 10-cycle pre-PCR of 1 min at 94 °C for denaturation, 45 s at 42 °C for annealing, and 2 min at 72 °C for extension. A 42 °C temperature was used as the initial annealing temperature from which the temperature was decreased 0.5 °C after each cycle (10 cycles); then, PCR amplifications were continued for 30 cycles using 37 °C as the annealing temperature, with a final extension at 72 °C for 10 min.

After td-PCR, electrophoresis was performed in a 1.7% (w/v) agarose gel using 0.5 TBE buffer (44.5 mmol/L Tris, 44.5 mmol/L borate and 1 mmol/L EDTA) at 60 V for 3 h. Then, the gels were stained with ethidium bromide at 0.5 g/ml, and images were captured with a High-Performance Ultraviolet Trans-Illuminator – Edas 290 using the 1-D 3.5 Kodak program. The size of the fragments was determined using a 100 bp DNA ladder (Invitrogen).

From the 82 primers that amplified, 12 (OPB17, OPB18, OPC02, OPC05, OPC06, OPC07, OPC08, OPC19, OPM02, OPM03, OPM05 and OPP08) were chosen to amplify the 43 DNA samples of *C. forbesii* seedlings.

Amplified products were scored as present (1) or absent (0) to form a binary matrix. Smeared and weak bands were excluded. The binary data obtained were then analysed using the NTSYSpc version 2.1 program (ROHLF, 2000), which generates the genetic distance matrix (NEI & Li, 1979) and draws the UPGMA dendrogram.

Results and Discussion

The well-defined bands in the gel were observed using 20 ng of DNA and 2.5 mM $MgCl_2$ in the amplification reactions (Figure 2). The 12 primers (OPB17, OPB18, OPC02, OPC05, OPC06, OPC07, OPC08, OPC19, OPM02, OPM03, OPM05 and OPP08), which yielded repetitive patterns for all scored bands, were applied to all 43 seedlings of *C. forbesii*. Only one mix was prepared for each primer to simultaneously compare the individual DNA samples in the same amplification reaction.

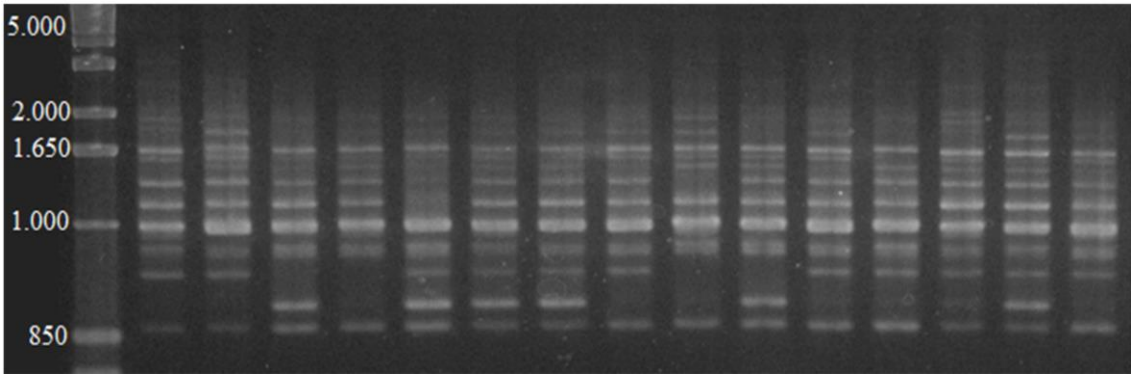

Figure 2. The DNA fingerprint based on td-PCR-RAPD in samples of *Cattleya forbesii* (2–16) shows well-defined amplified DNA segments using the OPC05 primer. Sample 1: *Ladder* 100 bp (Invitrogen).

The 12 primers generated 148 reproducible DNA segments (Table 1), of which 132 were polymorphic. Only those DNA segments that were strongly stained were scored. There was 89.19% polymorphism for the 43 seedlings of *C. forbesii.* The number of bands for each primer varied from 7 to 17, with an average of 12.33 bands per primer. The size of amplified products ranged from 200 to 5000 bp. The OPC02 primer generated the highest number of DNA segments (17 bands) among the tested primers, while primers OPC02, OPC07, OPC08, OPC19 and OPM03 showed the greatest capacity for discriminating polymorphic fragments (100% polymorphism).

Table 1. Number of primers and polymorphic segments of DNA amplified by td-PCR in seedlings of *Cattleya forbesii* obtained from *in vitro* seed germination.

Primer [5' → 3']	**Total of Amplified DNA Sequences**	**Number of Polymorphic Sequences**
OPB17 [5'-AGGGAACGAG-3']	16	14 (87,5%)
OPB18 [5'-CCACAGCAGT-3']	13	12 (92,3%)
OPC02 [5'-GTGAGGCGTC-3']	17	17 (100%)
OPC05 [5'-GATGACCGCC- 3']	13	11 (76,9%)
OPC06 [5'-GAACGGACTC-3']	9	07 (77,8%)
OPC07 [5'-GTCCCGACGA- 3']	11	9 (100%)
OPC08 [5'-TGGACCGGTG- 3']	16	16 (100%)
OPC19 [5' –GTTGCCAGCC- 3']	8	08 (100%)
OPM02 [5'-ACAACGCCTC- 3']	14	09 (64,3%)
OPM03 [5'-GGGGGATGAG- 3']	7	07 (100%)
OPM05 [5'-GGGAACGTGT- 3']	9	08 (88,9%)
OPP08 [5'-ACATCGCCCA- 3']	15	13 (86,7%)
Total	**146**	**132 (89,19%)**

The dendrogram generated from the Jaccard coefficient (Figure 3) showed several groupings of seedlings and demonstrated that the similarity among seedlings germinated *in vitro* ranged from 50% to 86%. The number of groups formed is indicative of many possibilities to perform crosses between

plants allocated in different groups when the goal is to generate intraspecific hybrids genetically divergent of *C. forbesii*. The similarity between 95% of seedlings descendent from self-pollination was lower than 0.85.

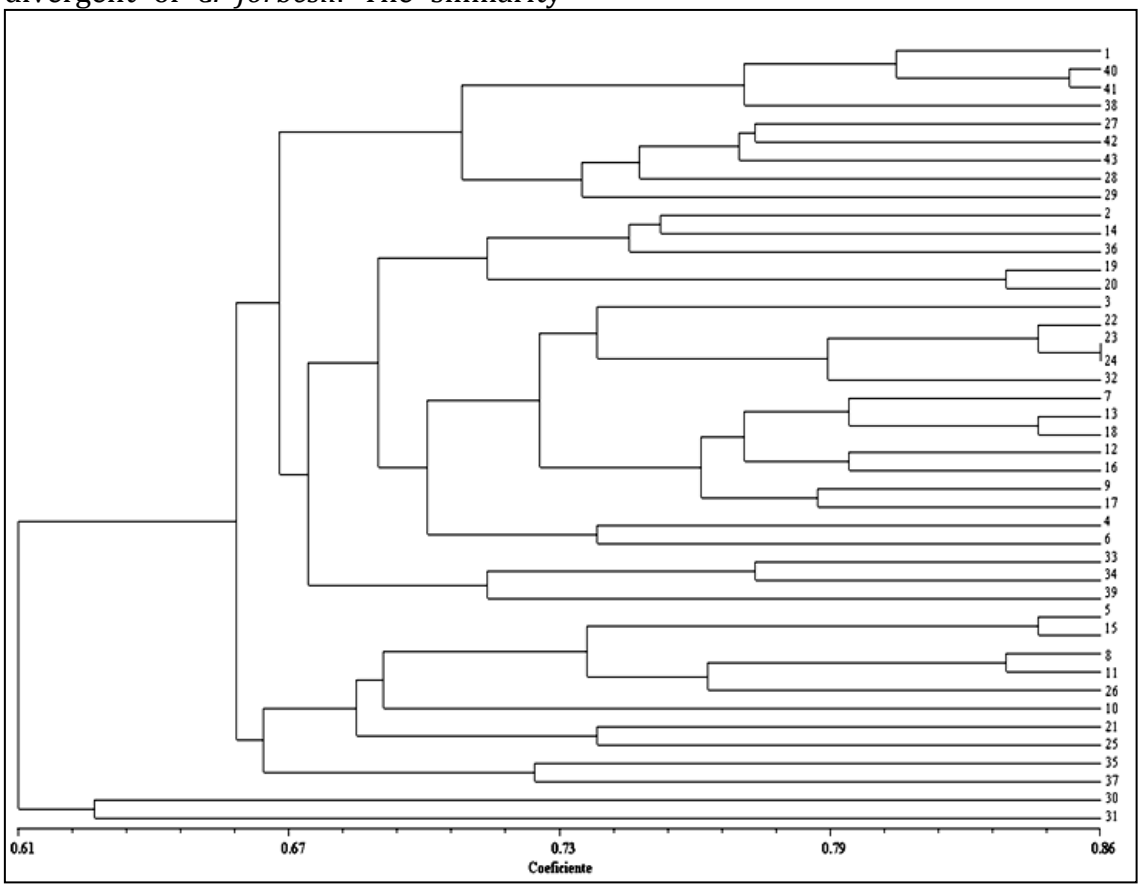

Figure 3. Dendrogram using the Jaccard coefficient showing the various groupings and the genetic basis (50% to 86% of genetic identity) observed among seedlings of *Cattleya forbesii* from *in vitro* germination.

Molecular polymorphism in *C. forbesii* has not been previously studied; however, the polymorphism of DNA segments using td-PCR in *C. forbesii* seedlings grown *in vitro* was as high as the polymorphism observed for other molecular markers in other species of orchids grown in their natural habitats or in collections (VERMA *et al*., 2009; DING *et al.,* 2008; CAI *et al*., 2011). In species of the *Vanila* genus, the polymorphism found was 83.24% and 86.11% from RAPD and ISSR markers, respectively (VERMA *et al*., 2009). Ding *et al*. (2008) found a high rate of diversity at the species level in *Dendrobium oficinalis* (88.07%), and Duffy *et al*. (2009) found an interpopulation variation of 72% in *Neotinea maculata* in Ireland and Italy.

A high level of genetic diversity in *Dendrobium londgesii* found using SRAP markers was suggested to be associated with different strategies of pollination and with a high dispersion of seeds. Neto & Vieira (2011) have observed a high level of polymorphism (90.3%) in *Cattleya intermedia*, using seven RAPD

primers. In *C. labiata* also was detecteda high level of genetic polymorphism (PINHEIROS *et al*., 2012). Although the *C. forbesii* seedlings analysed in our study resulted from self-pollination, studies examining floral biology and pollination in natural populations of *Cattleya* species point to pollination in the genus by bees of the subtribes Meliponina and Euglossina (WITHNER, 1988; JERSÁKOVÁ *et al*., 2006). Among the seven species of *Cattleya* whose floral biology was studied, three different bee groups were identified as pollinators: (*i*) Meliponina, which pollinates representatives of the section *Stellata,* the section with the smallest flowers in the genus (JERSÁKOVÁ *et al*., 2006); (*ii*) Euglossina, which pollinates species that have a velvety lip surface, such as section *Cattleya* (JERSÁKOVÁ *et al*., 2006); and (*iii*) Bombina, which pollinates species with a waxy lip surface (SMIDT *et al*., 2006).

The high diversity of the td-PCR-RAPD markers observed in the *C. forbesii* seedlings resulting from *in vitro* germination may be justified by cross pollination occurring in the natural habitats of these *Cattleya* species as well as by interspecific crossings. In the *C. tenuis* and *C. elongata* species, similarities have been shown between the flowers of both species, which have the same pollinator (*Bombus brevivillius*), presenting compatibility between their gametes (SMIDT *et al*., 2006). The td-PCR-RAPD method has analysed random amplified segments of DNA which are not directly (individually) affected by self-pollination, since they are not specific loci.

The highest level of genetic similarity between the *C. forbesii* seedlings from *in vitro* germination was 0.86. Most seedlings (95%) showed genetic similarity of less than 0.85. A level of genetic identity lower than 0.85 was considered by Thorpe & Solé-Cava (1994) to indicate geographically distant species in the process of speciation or co-generic species (different species of the same genus). According to that assumption, the genetic diversity in *C. forbesii* is as wide as it would be if they were plants of different species within a particular genus. A high diversity of RAPD markers (37–83%) has been found in different species of the *Catasetum* genus (OLIVEIRA *et al*., 2010).

The most relevant information resulting from this study is that the wide natural genetic variability that has been described in studies examining orchids populations distributed in their natural habitats was also observed in seedlings grown from seeds germinated in alkaline KC (KNUDSON, 1946). The *in vitro* germination of *C. forbesii* seeds (with the goal of producing seedlings to replace stocks for cultivation in nature or with the goal of preferentially performing crossings to stimulate the production of hybrids) does not provide sufficient selection pressure to result in a reduction in genetic diversity of the species. The genetic diversity of seedlings originating from *in vitro* germination using KC medium was wide (0.50 to 0.86), indicating that this germination process produces seedlings with wide genetic variability.

Another interesting aspect of the present study is that the primers OPC02, OPC07, OPC08, OPC19, OPM03, OPB18, OPM05, OPP08, and OPB17, which were more polymorphic and resulted in a larger number of amplified regions of DNA (higher number of bands), may be used as follows: (*i*) to study the genetic diversity in natural populations, (*ii*) to estimate the divergence between

populations, *(iii)* to check and monitor natural or artificial processes of hybridisation of *C. forbesii* with other species of the same genus or of *C. forbesii* with others orchids genera, or (*iv*) to check the effects of adding supplements, natural or synthetic, to increase or restrict the genetic variability of *C. forbesii* seedlings when cultured *in vitro*. Amplified DNA segments (well-defined and with high reproducibility) generated by the 12 primers [OPB (OPB17 and OPB18), OPC (OPC02, OPC05, OPC06, OPC07, OPC08, and OPC19), OPM (OPM02, OPM03 and OPM05), and OPP (OPP08) based on td-PCR-RAPD] may also be used as molecular markers in the identification of the *C. forbesii* genotype in natural hybrids.

The disclosure of these results is also very important since studies with *Cattleya* species which have high commercial value, has been rarely reported in the specialized literature. The large natural genetic variability previously described in studies examining orchid populations distributed in their natural habitats was also observed in seedlings of *C. forbesii* grown from seeds germinated in KC medium supplemented with 0.6% agar, 3% sucrose, and 10% banana pulp. The polymorphism of td-PCR-RAPD markers in the *C. forbesii* seedlings resulting from self-pollination and *in vitro* germination was sufficiently high, indicating that the genetic diversity in this species does not suffer negative effects resulting from self-pollination or *in vitro* propagation.

Conclusion

The genetic basis of seedlings obtained from seeds germinated *in vitro* using the KC medium was substantial (0.50 to 0.86), indicating that *in vitro* cultivation produces seedlings of *C. forbesii* with a high genetic variability. The *in vitro* germination and *in vitro* culture of *C. forbesii* seeds can be considered as suitable to produce replacement seedlings to be grown in nature, or with the prospect of performing preferential crosses to stimulate the production of hybrids in breeding programs.

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