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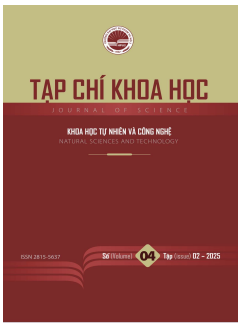

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Evaluation of hybridization potential and identification of molecular markers for early recognition of f1 hybrids between *Cymbidium aloifolium (L.)* Sw. 1799 and *Cymbidium finlaysonianum* Lindl. 1833

Phuong-Thu Pham*, Kim-Anh Duong Thi, Huyen-My Tran

Hanoi Pedagogical University 2, Phu Tho, Vietnam

Abstract

This study focuses on evaluating the hybridization potential between two native Vietnamese orchid species, ♀ *Cymbidium aloifolium* (L.) Sw. 1799 and ♂ *Cymbidium finlaysonianum* Lindl. 1833, and identifying specific SSR molecular markers for early recognition of F1 hybrids. The hybridization process was carried out using emasculation and cross-pollination methods, followed by the development of F1 populations under in vitro conditions. After eight months, the hybrid fruits were harvested, and their seeds germinated successfully on MS medium supplemented with 3% sucrose and 5 g/L agar. A substantial number of F1 individuals were obtained, of which 250 were selected for SSR molecular analysis. Eighteen SSR markers were tested; eight primers exhibited clear amplification, and two primers (CS16 and CS7) showed distinct polymorphism between parental samples. Based on the analysis, eight representative F1 individuals were selected and further evaluated using the CS7 marker, confirming allele inheritance from both parents and thereby validating the success of hybridization. These results contribute valuable molecular tools for early-stage identification of true hybrids and provide a scientific foundation for breeding programs and conservation efforts targeting native Cymbidium germplasm in Vietnam.

Keywords: Orchid hybridization, SSR molecular markers, *Cymbidium aloifolium*, *Cymbidium finlaysonianum*, F1 hybrids

1. Introduction

In recent years, the orchid market has shown substantial consumer demand with a steadily increasing volume of global transactions. Orchids are widely traded in two primary forms: cut flowers and potted

* Corresponding author, E-mail: phamphuongthu@hpu2.edu.vn

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plants [1]. Beyond their aesthetic appeal, orchids are extensively utilized in industries such as medicine, food, and beverages, due to their bioactive compounds, including polysaccharides and alkaloids [2]. In addition to their economic and ornamental value, orchids possess significant cultural importance, being revered in classical Chinese literature as one of the "Four Noble Plants," alongside plum, chrysanthemum, and bamboo [3], [4].

The expansion of the global economy has further accelerated the demand for orchids, both in terms of quantity and variety, thereby necessitating scientific advancements in breeding new cultivars with unique floral characteristics, improved disease resistance, and enhanced flowering performance [5].

In Vietnam, Cymbidium species are highly valued, ranking second in production volume after chrysanthemums and roses. These orchids hold considerable economic potential due to high market demand, complementing their ornamental and cultural significance. They have been cultivated for centuries, leading to the establishment of major production regions including Lao Cai, Quang Ninh, Gia Lam (Ha Noi), Phu Yen, and Da Lat. Each year, numerous new Cymbidium hybrids are introduced from countries such as China and Thailand. These imported varieties are widely favored in the domestic market, particularly during festive seasons and the Lunar New Year [5], [6].

Vietnam is also home to a rich diversity of native wild orchids, including 24 indigenous Cymbidium species distributed across the country [5]. These native orchids are distinguished by their unique beauty and captivating fragrance. However, their populations in natural habitats are being rapidly depleted due to overexploitation and habitat destruction [7]. Despite the abundant germplasm resources, there has been a lack of significant research efforts on the hybridization of indigenous Cymbidium species in Vietnam, highlighting an urgent need for systematic breeding programs.

Recent international studies have demonstrated that the integration of conventional hybridization techniques with molecular marker-assisted selection significantly enhances the efficiency and precision of orchid breeding [8]. Among various molecular markers, Simple Sequence Repeat (SSR) markers have emerged as highly effective tools for early hybrid verification, owing to their high polymorphism, co-dominant inheritance, and reproducibility [9]. These markers enable early identification of true hybrids at the seedling stage, thereby overcoming the limitations of traditional morphological evaluation, which often requires several years until flowering.

Given this context, the objectives of this study were: (1) to evaluate the hybridization potential between ♀ Cymbidium aloifolium (L.) Sw. 1799 and ♂ Cymbidium finlaysonianum Lindl. 1833; and (2) to identify specific SSR molecular markers for the early-stage recognition of F1 hybrids, contributing to the conservation and commercial development of Vietnam's native Cymbidium germplasm.

2. Materials and Methods

2.1. Materials

Seed parent (♀): *Cymbidium aloifolium* (L.) Sw. 1799 is a robust and disease-free species. This native orchid species of Vietnam was collected from mountainous regions in northern Vietnam. The chromosome number of the seed parent is 2n = 40 [10], [11].

Figure 1. Floral morphology of *Cymbidium aloifolium* (L.) Sw. 1799.

Pollen parent (♂): *Cymbidium finlaysonianum* Lindl. 1833 is a robust and disease-free species. This native orchid species of Vietnam was collected from mountainous regions in northern Vietnam. The chromosome number of the pollen parent is 2n = 40 [10], [11].

Figure 2. Floral morphology of Cymbidium finlaysonianum Lindl. 1833.

Eighteen pairs of SSR primers (Microsatellite Markers) [9] were published and utilized to analyze the polymorphism among *Cymbidium* samples, thereby supporting the molecular identification and selection of F1 plants. The primer names, sequences (5'-3' orientation), annealing temperatures, and expected product sizes are presented in Table 1.

Table 1. SSR molecular markers used for the analysis and identification of F1 hybrids.

No.	Primer Name	Primer Sequence (F-R)	Tm (°C)	Product Size (bp)
1	CS1	5'-AAAACCACAGCTACAGGGC-3' / 5'-ATGGCCCCAAGTTCAGAC-3'	55	251-379
2	CS2	5'-AGCCAAAGGTCTGCCTTC-3' / 5'-TCCAACATAGACGTCGCC-3'	55	265
3	CS3	5'-CTTATGGCAACGAGCAGG-3' / 5'-GTAACCTCCGTCGCTCCT-3'	55	245
4	CS4	5'-GCCTCTTGGAGGCTTGTT-3' / 5'-TGGCGAAGAGAATGAGGA-3'	53	270
5	CS5	5'-CTCATCGCCTTGCTTGAG-3' / 5'-TCGATTCTCAATGGCACC-3'	53	276
6	CS6	5'-TTTTAGAGGAGGGCGGAG-3' / 5'-CAAGCGACCTCAACTTGC-3'	54	223
7	CS7	5'-CCGTCACGACTAGCGAAG-3' / 5'-GGGAATCCTCGCCGT-3'	55	264
8	CS8	5'-TCTCGACATCCAACACCTG-3' / 5'-TCCCGAGGTGTGAAGAAA-3'	53	294
9	CS9	5'-CGTTTCTGGTGAGGGACA-3' / 5'-CCAAGGCTTCACATCCAA-3'	53	253
10	CS10	5'-TAGCAGAAGCTATGCCCG-3' / 5'-TCGGAGGAGAGACCACTG-3'	55	209
11	CS11	5'-AACACAGCTCAGGCTCCA-3' / 5'-TGTTTCCATTTCGCTGCT-3'	53	255

No.	Primer Name	Primer Sequence (F-R)	Tm (°C)	Product Size (bp)
12	CS12	5'-CTTCTCCACCACCACTGC-3' / 5'-GACGACCACAAGGCAGAA-3'	58	279
13	CS13	5'-AATCTGGGAATGATCGCA-3' / 5'-TCACAGCTCATAACAGAAGCA-3'	48	277
14	CS14	5'-GAAGGAGAAGAAGGCGGA-3' / 5'-CGGCTTCTTGTTGTAGCG-3'	54	286
15	CS15	5'-AGCAAACGGCAAGTCATGG-3' / 5'-ATTCGACTACCAGCCGGAC-3'	53	200-300
16	CS16	5'-CGTTGCTCTCTGTATGACCG-3' / 5'-TCGACCAAATTGCCTGTC-3'	54	281
17	CS17	5'-TGCGGTGAATTTGAGCTT-3' / 5'-GCAGTTTGCTGGTCGGTA-3'	54	292
18	CS18	5'-GATCAGCGGGCGAGA-3' / 5'-AAGGCCACCCTTTGTTGT-3'	55	248

The SSR primer sequences used in this study were developed by Moe et al. [9].

2.2. Research Methodology

2.2.1. Selection of parental plants for hybridization

The selection of parental plants was conducted based on the method proposed by De [12]. Healthy and vigorous plants free from diseases were selected as hybridization materials, while female plants flowering for the first time were excluded. A healthy female plant is capable of producing 2–3 seed pods without negatively impacting its overall growth and development [12].

Parental materials were selected according to key agronomic characteristics, including plant vigor, flowering ability, flower color, floral morphology, flower longevity, and disease resistance. Following the approaches described by De et al. [12] and Griesbach [13], selecting parental plants with compatible genetic backgrounds is crucial for ensuring superior offspring quality in terms of growth performance and commercial traits. These principles were applied to identify suitable hybrid combinations, thereby optimizing the orchid breeding process.

2.2.2. Hybridization procedure

The hybridization process for cymbidiums followed the method described by Dongarwar and Thakur [14]. Prior to blooming, flowers were enclosed in paper bags to prevent self-pollination.

Steps of the Hybridization Process:

Emasculation: Use sterilized forceps or tweezers to remove the pollen from the female flower, ensuring that pollen does not accidentally fall onto the stigma. The procedure must be performed carefully to avoid damaging other floral structures [14].

Pollen Collection: Use new or sterilized tweezers to carefully open the anther cap of the male flower. The inner surface of the anther cap will reveal two round yellow structures containing pollen. If removal of the pollen mass is difficult, it can be facilitated by lightly touching the pollen with a moistened toothpick. Gently place the collected pollen onto a clean Petri dish or paper [14].

Pollination: Using a moistened toothpick, lightly press the pollen and carefully place it onto the stigma of the female flower. Gently push the pollen as close to the ovary as possible. Pollination is best performed in the morning or late afternoon when the flower is completely dry [14].

Flower and Bud Trimming: Remove any unpollinated flowers or buds on the inflorescence to ensure that the plant can concentrate nutrients for fertilization and seed pod development. After completing the process, bag the pollinated flowers in a paper bag with small perforations to prevent contamination from pollen of other flowers [14].

Labeling: Attach a tag to the pollinated flowers, recording the date, the cross between the two parents, and the hybrid pair code for tracking purposes.

2.2.3. Development of F1 hybrid population

The F1 hybrid population was established through traditional hybridization methods. Hybrid pods were sterilized, and seeds were extracted for in vitro germination on MS medium supplemented with 3% sucrose and 5 g/L agar. The resulting F1 hybrid lines were subsequently propagated in vitro.

The in vitro propagation process was carried out following the micropropagation protocol proposed by Arditti [15], in combination with the findings reported by Hemanta et al. [16]. Tissue culture experiments were performed under controlled environmental conditions, including a room temperature of 25°C, relative humidity of 65%, and a light intensity of 2000 lux with a 16-hour photoperiod. The culture medium was adjusted to pH 5.8 and sterilized at 121°C under 1.1 atm pressure for 30 minutes prior to use.

2.2.4. Selection of F1 plants using SSR molecular markers

a) Sample collection and DNA extraction:

Leaves from the second node of F1 plants were sampled for DNA extraction, which was performed using a modified CTAB protocol as described by Doyle [17].

b) PCR procedure:

The PCR reaction was conducted in a total volume of 20 µL, containing: 20 ng DNA, 0.1 mM dNTPs, 0.1 µM forward and reverse primers, 2 µL 10X Dream Taq Buffer, 0.2 µL Dream Taq DNA polymerase (5 u/µL), and Milli Q sterilized water.

The thermal cycling protocol consisted of: Initial denaturation at 95°C for 5 minutes, 30 cycles of 95°C for 30 seconds, annealing temperature (Ta) for 30 seconds, and 72°C for 30 seconds, Final extension at 72°C for 5 minutes.

PCR products were analyzed via electrophoresis on 2.5% agarose gel, visualized under UV light, and processed using Fire Reader software. A DNA size marker (Low gene ruler, 100 bp) was used for reference.

2.3. Research timeline and location

The hybridization of parental cymbidium species was conducted in spring (February) 2023 at the Faculty of Biology, Hanoi Pedagogical University 2. The F1 lines were transplanted and evaluated in the spring of 2024. Molecular biology experiments for F1 plant selection were conducted at the Molecular Biology Laboratory, Agricultural Genetics Institute (AGI), Hanoi, Vietnam.

3. Results and Discussion

3.1. Fruit-Setting ability of the hybrid combination between Cymbidium aloifolium (L.) Sw. 1799 and Cymbidium finlaysonianum Lindl. 1833

The fruit-setting ability of the hybrid combination between ♀ Cymbidium aloifolium (L.) Sw. 1799 and ♂ Cymbidium finlaysonianum Lindl. 1833 was evaluated. The results demonstrated successful cross-pollination, as evidenced by the development of fruit pods after the hybridization process. A fruit-setting rate of approximately 65% was recorded among the pollinated flowers, indicating a high degree of cross-compatibility between the two native species.

Further analysis of the F1 hybrids confirmed that the fruit-setting resulted from successful fertilization, rather than self-pollination. This suggests that the genetic divergence between the two species was sufficient to enable interspecific hybridization without reproductive barriers. Such high fruit-setting ability provides an important foundation for generating viable F1 populations for breeding and conservation programs.

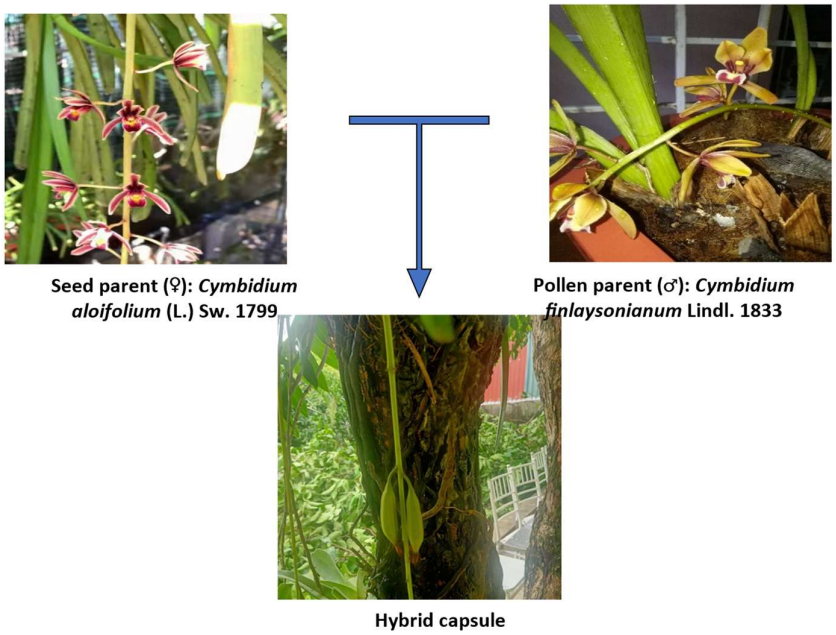

Figure 3. Hybridization results of ♀ *Cymbidium aloifolium* (L.) Sw. 1799 x ♂ *Cymbidium finlaysonianum* Lindl. 1833.

After eight months of pollination, the hybrid capsules were harvested for subsequent studies. As reported by Alghamdi et al. [18], Li et al. [19], Rasmussen [20], and Arditti and Ernst [21], orchid seeds contain minimal endosperm, which makes natural germination highly challenging. Successful germination typically requires a symbiotic association with mycorrhizal fungi and specific environmental conditions, as noted by Smith and Read [8].

To enhance germination efficiency, *in vitro* culture was employed using MS medium supplemented with 3% sucrose and 5 g/L agar, following the micropropagation protocol proposed by Arditti and Ernst [21]. This approach not only improved germination rates but also accelerated seedling development under controlled environmental conditions, thereby minimizing the effects of external biotic and abiotic stresses.

According to Zhang et al. [22], *Cymbidium* is a taxonomically complex genus, particularly during the pre-flowering stage. Identifying F1 hybrids based on conventional morphological characteristics is highly difficult and requires an extended period, as F1 plants typically take about four years to reach the flowering stage. Therefore, to ensure that the seedlings obtained were the result of cross-pollination rather than self-pollination, molecular analysis using SSR markers was employed to rapidly and accurately verify hybrid status at the seedling stage, thus overcoming the limitations associated with traditional morphology-based identification.

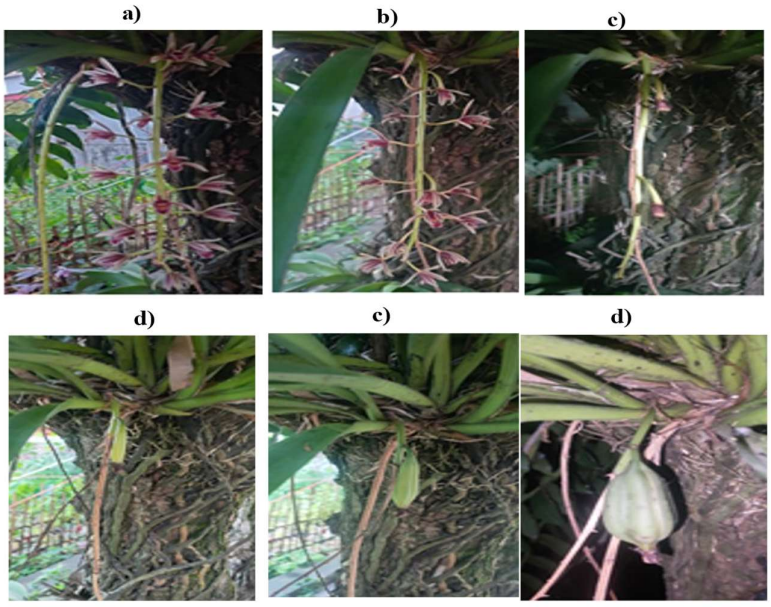

Figure 4. Illustrative images of the hybridization process from the female parent to fruit collection for the cross ♀ *Cymbidium aloifolium* (L.) Sw. 1799 x ♂ *Cymbidium finlaysonianum* Lindl. 1833.
Note: The process of hybridization is illustrated as follows: **a)** shows the inflorescence of the ♀ Cymbidium aloifolium (L.) Sw. 1799 immediately after pollination. **b)** captures the inflorescence 3 days after pollination. **c)** displays two flowers of the ♀ Cymbidium aloifolium successfully fertilized, with their ovaries enlarging, while unfertilized flowers wilt and drop after 10 days. **d, e, and f** depict the development of hybrid fruit pods at 1 month, 3 months, and 6 months after pollination, respectively.

3.2. Analysis and identification of the F1 population using SSR molecular markers

The F1 hybrid pods were sterilized, and the seeds were extracted and germinated in vitro on MS medium supplemented with 3% sucrose and 5 g/L agar to stimulate seed germination. Once germinated, the F1 hybrids were propagated in vitro to establish a robust F1 population for further research. A total of approximately 250 F1 seedlings were initially obtained through this protocol, from which representative individuals were selected for molecular analysis.

3.2.1. Analysis of polymorphism between the parental lines to identify SSR markers for distinguishing polymorphisms

A total of eighteen SSR molecular markers were employed to assess polymorphism between the parental lines. The analysis of polymorphism between ♀ *Cymbidium aloifolium* (L.) Sw. 1799 and ♂ *Cymbidium finlaysonianum* Lindl. 1833 revealed that 8 out of 18 primers successfully amplified DNA fragments in both parental samples. The PCR products exhibited distinct and clear bands, with sizes

approximately matching the expected product sizes, thus indicating successful amplification without non-specific products.

The electrophoresis results of the PCR products from these 8 markers are shown in Figure 5 (illustrated with clear labeling of parental samples and marker bands). Among them, 2 primers, CS16 and CS7, demonstrated distinct polymorphism between the seed and pollen parents. For Marker CS16, the PCR product from ♀ *Cymbidium aloifolium* was approximately 295 bp, while the product from ♂ *Cymbidium finlaysonianum* was around 280 bp. Similarly, for Marker CS7, the PCR product from ♀ *Cymbidium aloifolium* was approximately 240 bp, whereas the product from ♂ *Cymbidium finlaysonianum* was about 255 bp.

It should be emphasized that these product sizes were estimated based on visual comparison with a DNA ladder ranging from 200 bp to 300 bp, without software-based calculation. Therefore, the indicated sizes are approximate values.

The observed polymorphisms between the parental lines detected with CS7 and CS16 confirm that these markers are effective tools for distinguishing genetically similar individuals. These findings are crucial because distinguishing hybrids from self-pollinated progeny at early developmental stages is particularly challenging in *Cymbidium*, where morphological differentiation is often unobservable until several years post-germination. Thus, CS7 and CS16 were selected for subsequent hybrid verification in the F1 population.

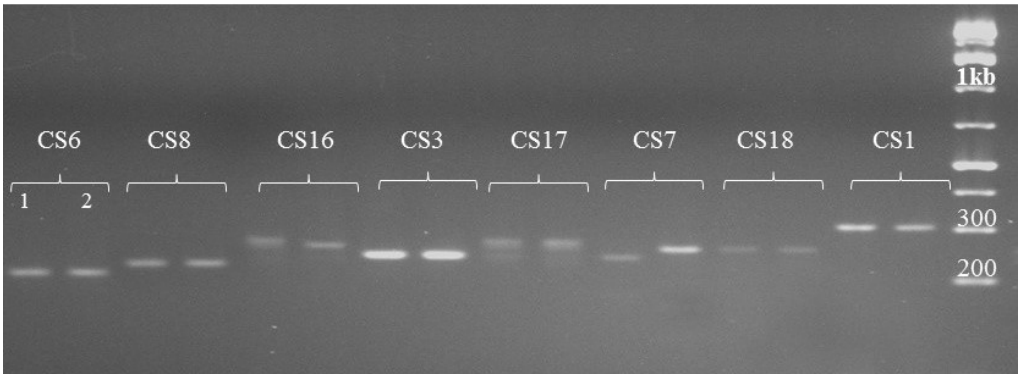

Figure 5. Results of polymorphism analysis between the parents of the hybrid pair ♀ *Cymbidium aloifolium* (L.) Sw. 1799 x ♂ *Cymbidium finlaysonianum* Lindl. 1833 using SSR molecular markers.
Note: *1- DNA of ♀ Cymbidium aloifolium. 2- DNA of ♂ Cymbidium finlaysonianum*
Ladder: 1 kb.

3.2.2. Verification of F1 individuals using SSR molecular markers

The SSR markers that exhibited polymorphism in the hybrid pair were utilized to analyze the genetic relationship between F1 individuals and their parents. Specifically, the molecular marker CS7 was employed to analyze the F1 hybrid lines.

The electrophoresis results of PCR products from 8 F1 individuals of the ♀ *Cymbidium aloifolium* (L.) Sw. 1799 × ♂ *Cymbidium finlaysonianum* Lindl. 1833 cross are presented in Figure 6. The results show that all 8 DNA samples from the F1 individuals contained both parental alleles, with approximate sizes estimated at about 255 bp (♂) and 240 bp (♀), confirming successful hybridization (Figure 6). It should be noted that these sizes were estimated based on visual comparison with the 200 bp and 300 bp bands of the DNA marker, without software-assisted calculation.

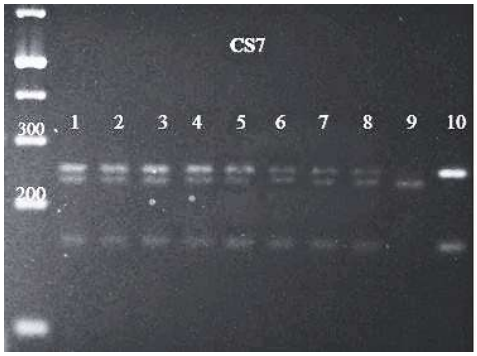

Figure 6. Electrophoresis results of PCR products from F1 individuals of the hybrid pair ♀ *Cymbidium aloifolium* (L.) Sw. 1799 x ♂ *Cymbidium finlaysonianum* Lindl. 1833 using the molecular marker CS7.
Note: 1–8: DNA of F1 hybrids; 9: DNA of ♀ *Cymbidium aloifolium*; 10: DNA of ♂ *Cymbidium finlaysonianum*. *Ladder: 1 kb.*

4. Conclusion

The F1 hybrid population was successfully created from the hybridization between the two native *Cymbidium* species from the northern mountainous regions of Vietnam ♀ *Cymbidium aloifolium* (L.) Sw. 1799 and ♂ *Cymbidium finlaysonianum* Lindl. 1833. An analysis of 18 SSR molecular markers for polymorphism in the parental lines revealed that 8 out of 18 markers successfully matched the DNA of both parents. The PCR products exhibited clear bands with sizes consistent with predictions, among which 2 markers (CS16 and CS7) demonstrated distinct polymorphism between the parents.

Analysis of DNA samples from the F1 population using marker CS7 confirmed that the F1 hybrids inherited loci from both parents. These results validate the success of the hybridization process between ♀ *Cymbidium aloifolium* (L.) Sw. 1799 and ♂ *Cymbidium finlaysonianum* Lindl. 1833, resulting in a robust F1 hybrid population. These findings provide essential foundational material for the selection and development of new *Cymbidium* varieties in future research.

Acknowledgements

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