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Genetic diversity, population structure, and conservation of *Cattleya trianae* (Orchidaceae) through molecular marker analysis

María Eloísa Aldana-Jauregui[1]*; https://orcid.org/0009-0001-7601-9061
Héiber Cárdenas-Henao[2]; https://orcid.org/0000-0003-2823-8443
Nelson Toro-Perea[2]; https://orcid.org/0000-0002-2835-7285

1. Departamento de Biología, Facultad de Ciencias, Universidad del Tolima, Calle 42 # 1-02, Ibagué, Colombia; ealdana@ut.edu.co (*Correspondence)
2. Departamento de Biología, Facultad de Ciencias Naturales y Exactas, Universidad del Valle, Calle 13 # 100-00, Cali, Colombia; heiber.cardenas@correounivalle.edu.co, nelson.toro@correounivalle.edu.co

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ABSTRACT

Introduction: *Cattleya trianae* Linden & Rchb., celebrated for its botanical splendour, holds a revered status as the national floral emblem of Colombia, commonly known as the "Flor de Mayo" (Flower of May). However, its survival is threatened by a variety of environmental and anthropogenic pressures, needing urgent and tailored conservation and management strategies to conserve the remarkable genetic diversity of *C. trianae* in the central Andean range of Colombia.

Objective: To elucidate the genetic architecture of *C. trianae* populations using random amplified polymorphic DNA (RAPD) and chloroplast microsatellite repeat (cpSSR) markers, providing critical insights for conservation planning, sustainable management, and the use of this species.

Results: RAPD analysis reveals unexpectedly high levels of heterozygosity, suggesting considerable genetic variability. Genetic distances and dendrogram topologies indicate significant relatedness between populations, while analysis of molecular variance (AMOVA) reveals considerable population structuring, primarily due to intrapopulation differentiation. F_{ST} and Nm values challenge the assumption of isolation by distance, reflecting a complex genetic landscape. Notably, despite the limited population sizes, there is no substantial evidence of genetic erosion.

Conclusions: The results reveal alarmingly low intraspecific genetic diversity within these orchid populations, highlighting their vulnerability to environmental change and stochastic events. Furthermore, the marked genetic divergence between populations suggests the influence of multiple evolutionary forces shaping the genetic structure and distribution of *C. trianae* in Colombia. These findings underscore the urgency for tailored conservation strategies to mitigate potential extinction risks.

Key words: biodiversity; endangered species; genetic analysis; nuclear markers; cpSSR; Orchidaceae.

RESUMEN

Diversidad genética, estructura poblacional y conservación de *Cattleya trianae* (Orchidaceae) mediante el análisis de marcadores moleculares

Introducción: *Cattleya trianae* Linden & Rchb., célebre por su esplendor botánico, ostenta un venerado estatus como emblema floral nacional de Colombia, comúnmente conocida como la "Flor de Mayo". Sin embargo, su supervivencia está amenazada por una variedad de presiones ambientales y antropogénicas, lo que requiere

estrategias de conservación y manejo urgentes y adaptadas para conservar la notable diversidad genética de *C. trianae* en el área de distribución andina central de Colombia.
Objetivo: Dilucidar la arquitectura genética de las poblaciones de *C. trianae* utilizando marcadores de ADN polimórfico amplificado al azar (RAPD) y microsatélites de cloroplasto repetidos (cpSSR), proporcionando información crítica para la planificación de la conservación, la gestión sostenible y la utilización de esta especie.
Resultados: El análisis RAPD revela niveles inesperadamente altos de heterocigosidad, lo que sugiere una variabilidad genética considerable. Las distancias genéticas y las topologías de los dendrogramas indican un parentesco significativo entre poblaciones, mientras que el análisis de la varianza molecular (AMOVA) revela una considerable estructuración de las poblaciones, debida principalmente a la diferenciación intrapoblacional. Los valores de F_{ST} y Nm desafían la hipótesis del aislamiento por distancia, reflejando un paisaje genético complejo. Cabe destacar que, a pesar del limitado tamaño de las poblaciones, no existen pruebas sustanciales de erosión genética.
Conclusiones: Los resultados revelan una diversidad genética intraespecífica alarmantemente baja dentro de estas poblaciones de orquídeas, destacando su vulnerabilidad al cambio ambiental y a los eventos estocásticos. Además, la marcada divergencia genética entre poblaciones sugiere la influencia de múltiples fuerzas evolutivas que moldean la estructura genética y la distribución de *C. trianae* en Colombia. Estos hallazgos subrayan la urgencia de estrategias de conservación a medida para mitigar los riesgos potenciales de extinción.

Palabras clave: biodiversidad; especies amenazadas; análisis genético; marcadores nucleares; cpSSR; Orchidaceae.

INTRODUCTION

Cattleya trianae Linden & Rchb., an exemplar of botanical beauty, occupies a prominent place as the national floral emblem of Colombia, affectionately known as the 'Flor de Mayo' (Flower of May). This horticultural tribute pays homage to the eminent Colombian botanist José Jerónimo Triana (Ossenbach & Jenny, 2021).

Nestled in the heart of the Central Cordillera, *C. trianae* thrives on the Eastern slopes of the Magdalena River basin in Colombia, within an elevation range of 700 to 1 400 m.a.s.l. This region, within the Colombian Andes region, is a testament to the dynamic forces of anthropogenic change. The relentless growth of the human population, the encroachment of once virgin forests, extensive land use practices and the expansion of illicit crops have combined to create a landscape deeply affected by human activities. These disruptions have cast a shadow over the once undisturbed habitats of *C. trianae* and forced us to take a critical look at the genetic diversity within the populations of this unique endemic species (Reina-Rodríguez et al., 2017).

In a world increasingly concerned with biodiversity conservation, the utility of molecular markers has become paramount. These markers provide a means of objectively quantifying genetic diversity, a prerequisite for efficient prioritisation, cost-effective resource allocation and judicious optimisation of management strategies within conservation programmes (Salgotra & Chauhan, 2023). The advent of Polymerase Chain Reaction (PCR) technology, as pioneered by Powell et al. (1995), marked a watershed in genetic analysis. This monumental scientific achievement paved the way for the development of techniques such as Random Amplified Polymorphic DNA (RAPD), which have the invaluable advantage of not requiring prior knowledge of the target organism's genome (Nadeem et al., 2018).

C. trianae, classified in the global category of the International Union for Conservation of Nature (IUCN), faces the ominous prospect of possibly joining the ranks of endangered species, as highlighted in the "Red Book" of Colombian plants (Calderón-Sáenz, 2007) and in the Plan for the study and conservation of Orchids in Colombia (Ministerio de Ambiente y Desarrollo Sostenible & Universidad Nacional de Colombia, 2015). This categorisation casts a gloomy light on the vulnerability of this species, with the main threat coming from the ongoing degradation of its habitat quality. In this

context, the economic importance of *C. trianae* in the fields of floriculture and orchidology is significant. Paradoxically, however, many species within the genus *Cattleya* remain shrouded in commercial obscurity, lacking the benefit of efficient propagation protocols and, regrettably, often overshadowed by conservation efforts. This unfortunate situation has paved the way for a worrying trend: the illegal collection of plants from wild populations. This clandestine practice, which has been going on for more than a century and a half, has inflicted severe damage on the species. It has not only taken its toll in terms of population decline, but also in the gradual erosion of the natural habitats that *C. trianae* calls home (Salazar-Mercado & Vega-Contreras, 2017).

In this context, the primary aim of our study was to elucidate the current status of *C. trianae* populations. We sought to provide essential information not only to safeguard the existing genetic diversity within this endangered orchid species, but also to provide the basis for the development of sustainable management strategies. In this respect, our research plays a pivotal role in the conservation and rejuvenation of the ecological environment of these orchids.

MATERIAL AND METHODS

Study areas and collection of *C. trianae*: Sampling was carried out in the upper Magdalena Basin, located in the central Colombian Cordillera, and covering the geographical coordinates 4-4°48'0" N & 75-75°18'0" W. This study covered an elevation range of 800 to 1 700 m.a.s.l. focusing on montane forest vegetation. A total of ten to 30 samples were meticulously collected, ensuring plant viability by taking a single leaf from each plant. These samples were then carefully preserved in silica gel with a cobalt indicator, following the protocol outlined by Chase & Hills (1991). The harvested tissues were then dehydrated, macerated in liquid nitrogen and securely stored at -70 °C.

Geospatial processing and analysis: The georeferencing coordinates were meticulously documented and this data was systematically organised using ArcView version 3.2 ® software. Subsequent analysis of the datasets was carried out using ArcView 3.2 ®, supplemented by the Spatial Analyst and 3D Analyst add-ons. Distance analysis was carried out by considering population distribution and road coverage with the ArcView Find Distance function. To ensure consistency and compatibility, all information was reprojected into the Universal Transverse Mercator (UTM) coordinate system, specifically within Zone 18 North using the WGS-84 datum. Distance calculations between locations were skilfully performed using the Distance Matrix extension within the ArcView platform.

Taxonomic identification: The information about orchid species documented in the upper Magdalena Basin, located in the central Colombian Cordillera, their distribution in the study area, their geographical and altitudinal range, and the habitat requirements were obtained during the fieldwork, and the revision of herbarium material. Herbarium specimens were examined according to the standard procedures. Every studied sheet was photographed, and the data were taken from the labels. Both vegetative and reproductive characters of each plant were studied. All information was complemented by data obtained from the literature, mostly protologues and Neotropical orchid floras (Bateman et al., 2003; Cuatrecasas, 1958; Dodson & Luer, 2010; Espinal & Montenegro, 1977).

DNA extraction: For DNA extraction, 70 mg of finely powdered leaf tissue was processed according to the method originally described by Kobayashi et al. (1998), adapted for recalcitrant plant tissues. Notable modifications to the protocol were the inclusion of Proteinase K at a concentration of 60 µg/ml in buffer 2 and the addition of RNAse at 20 µg/ml in the final step. The extracted DNA was then stored at -20 °C. DNA concentration was quantified by spectrophotometric analysis using a Pharmacia

Biotech Gene Quant kit. Simultaneously, spectrophotometric measurements were used to assess protein absorbance, which provided an indication of the purity of the DNA samples. DNA integrity was check through an electrophoresis in 0.8 % agarose gel stained with ethidium bromure.

Random Amplified Polymorphic DNA (RAPD): The RAPD-PCR reaction protocol for *Cattleya* was standardised according to the method described by Weeden et al. (1992). Amplification was performed in a GeneAmp PCR System 9700 thermal cycler from Applied Biosynthesis. PCR conditions included an initial denaturation at 94 °C for 4 minutes, followed by 40 cycles of 1 minute at 94 °C, 1 minute at 35 °C and 2 minutes at 72 °C, culminating in a final extension at 72 °C for 7 minutes. PCR amplification was performed using a mixture containing 1X buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.8 µM oligonucleotide, 0.6 µg/µl BSA, 0.8 U Taq polymerase and 2.5 ng DNA, adjusted with ultrapure water to a final volume of 12 µl. The reproducibility of the markers was ensured by repeating all PCRs.

Amplification of cpSSR: The PCR conditions were as follows: 4 minutes of denaturation at 94 °C, followed by 30 cycles of denaturation at 94 °C for 1 minute, annealing at 54 °C for 1 minute, and extension at 72 °C for 2 minutes. This was followed by a final extension at 72 °C for 7 minutes. PCR amplification was performed using 1X PCR buffer, 3 mM $MgCl_2$, 0.25 mM dNTPs, 0.2 µM forward and reverse oligonucleotides, 0.6 µg/µl BSA, 0.75 U Taq polymerase, and 0.4 ng/µl DNA. The total volume was 20 µl, adjusted with ultrapure water. Reproducibility was confirmed for all amplifications. The PCR primers for cpSSR were developed by another study of the author that is currently being published.

Evaluation of gene flow patterns via pollen and seed dispersal: We performed a comparative analysis of gene distances derived from RAPD and cpSSR data to identify potential correlations. Our aim was to determine the interaction between pollen and seed gene flow. To achieve this, we implemented the formula introduced by Ennos (1994), denoted in equation 1.

$$r = \frac{mp}{ms}$$

Where: *mp* and *ms*, represent the migration rates of pollen and seeds, respectively. These values were calculated by extrapolation from the F_{ST} estimates obtained in AMOVA for both the RAPD and cpSSR datasets.

Association between genetic distances and geographical distances: We carried out a comprehensive analysis of Jaccard distances among populations of each species studied. These distances were calculated from RAPD and cpSSR data. The aim was to investigate potential relationships between genetic distances and geographical distances separating localities, thus evaluating the isolation-by-distance model. To facilitate this investigation, we used the Mantel test as explained by Sokal & Rohlf (1995).

Correlation between anthropogenic influence and the genetic diversity of the *Cattleya* populations: We conducted an analysis to detect correlations between genetic diversity, as measured by expected heterozygosity values derived from RAPD and cpSSR data, and the proximity of sampling sites to the nearest road and human population centres. This study served as an indirect assessment of the potential anthropogenic impact on the plant populations studied.

Data analysis: We calculated F_{ST} and φST for all locations and location pairs, assuming RAPDs followed Hardy-Weinberg equilibrium and random mating (F_{IS} = 0). For cpSSR data, which considers maternally inherited alleles, we estimated gene flow and migration between populations using Wright´s (1931) island model. Notably, we adjusted the Nm calculation for chloroplast data, using a multiplication

factor of 1 / 2 instead of 1 / 4 due to the haploid nature of the data. Molecular analysis of variance (AMOVA) was then used with parameters from Excoffier et al. (1992) using GENALEX 6.0® software, following the instructions of Peakall & Smouse (2006). Population structure was assessed at two levels: between sites and within sites. We evaluated variance components and fixation indices through 999 permutations and calculated ΦST for a measure analogous to F_{ST}, using the same software and interpretation of population differentiation according to Wright (1978). Finally, a DAPC was made with the RAPD matrix using the package Adegenet (Jombart, 2008) from R software.

RESULTS

Study areas and population collection: Populations inhabited patches of secondary forest ranging from five to ten hectares in size, with canopies over 25 metres high. These areas were interspersed with other soil uses such as mixed crops, coffee plantations and mining areas. The region's altitudes ranged from 854 to 1 550 m.a.s.l. and temperatures ranged from 17 to 25 °C, with an average annual temperature of 25.3 °C. Annual rainfall averaged between 1 700 and 2 400 mm. According to Holdridge's ecological formations (Holdridge, 1982), these zones straddle the tropical dry forest (bs-T) and the very humid premontane forest (bmh-PM). The Magdalena Basin comprises agroecosystems of mechanised rice (*Oryza sativa* L.), coffee (*Coffea arabica* L.), diversified peasant systems and undifferentiated rural areas.

Plant habitat and lyophytic occurrence: Individuals of *C. trianae* were found on various tree species including *Anacardium excelsum* Skeels., *Guazuma ulmifolia* Lam., *Samanea saman* (Jacq.) Merr., *Cordia alliodora* (Ruiz & Pav.) Oken, *Jacaranda caucana* Pittier., *Ficus* spp., *Pseudolmedia rigida* (Klotzsch & H.Karst.) Cuatrec., *Trichilia pallida* Sw., *Guarea gigantea* Triana & Planch. and *Guarea* spp. at heights between 15 and 35 metres. We also confirmed the presence of lyophytic habitats, as plants were also identified on rocks.

Chloroplast microsatellites (cpSSR): Of the 14 chloroplast genome sequences analysed, four showed repetitive variable regions in the different *Cattleya* species analysed, from which specific oligonucleotide pairs were designed for the amplification of chloroplast microsatellites for this plant genus.

Estimation of cpSSR frequencies in *C. trianae* populations: The rps16 and matK 5 loci were found to be monomorphic. Three alleles were found for matK 3, with allele 100 exhibiting the highest frequency (0.97948). The rbcL-atpB system was the most polymorphic with three alleles (124, 125, 126), with allele (126) being the highest frequency (0.9793). Although the absence of heteroplasmy is generally reported in cpSSRs (Provan et al., 2001), in *C. trianae* heteroplasmy of MatK 3 was identified in the La Chapa and Boquerón populations and another in an individual from La Chapa for rbcL-atpB.

Analysis of RAPD and cpSSR molecular data for *C. trianae* populations: The RAPD results (Table 1) showed that these populations, despite occupying disturbed and relatively small areas, had remarkable genetic diversity values (He = 0.31).

The genetic diversity from RAPD in Table 1, shows the expected heterozygosity for each of the populations of *C. trianae*, showing similar values and an average value for the total population of 0.31, highlighting those obtained for the populations: El Pital, Congoja, Gaviota and Boquerón. Furthermore, Genetic diversity from cpSSR, shows the heterozygosity values obtained, which were very low (He = 0.013), while the percentage of polymorphic loci observed per population was less than 40 % and for five populations of *C. trianae* (Table 1).

The RAPD-derived *FST* values (Table 2) correspond to moderate to very high genetic differentiation, indicating different levels of gene flow between the pairs of *C. trianae*

Table 1
Genetic diversity values in *C. trianae* based on RAPD and cpSSR.

Locations	Individuals	RAPD		cpSSR	
		Average expected heterozygosity unbiased Nei (1978)	% of polymorphic loci	Average estimated heterozygosity unbiased Nei (1978)	% of polymorphic loci
Porvenir	14	0.27	83.7	0.028	20
La Argentina	16	0.24	76.0	0.000	0
Congoja	20	0.28	90.4	0.020	20
San Jacinto	13	0.25	82.7	0.000	0
Guayabal	15	0.25	80.8	0.028	20
El Pital	20	0.30	93.3	0.000	0
El Agrado	13	0.29	76.9	0.000	0
Gaviota	22	0.29	89.4	0.036	40
Chapa	20	0.25	75.0	0.020	20
Boquerón	20	0.29	89.4	0.010	10
Totumo	19	0.25	78.8	0.000	0
Total/Average	192	0.31	83.3	0.013	60

Table 2
F_{ST} and Nm values between pair of populations of *C. trianae* using RAPD markers. Below the diagonal, F_{ST} values, above the diagonal, Nm values.

Populations	Porv.	Argen.	Congoja	San Jacinto	Guay.	El Pital	El Agrado	Gaviota	Chapa	Boquer.	Totumo
Porvenir	-	1.4	2.6	2.2	1.9	2.0	0.9	0.7	0.9	1.1	2.1
Argentina	0.1511	-	3.8	2.1	1.8	1.4	0.8	0.7	0.7	0.8	1.0
Congoja	0.0879	0.0611	-	6.1	3.2	2.0	1.0	0.7	0.8	0.9	1.4
San Jacinto	0.1036	0.1068	0.0393	-	3.6	2.4	1.0	0.6	0.8	0.8	1.2
Guayabal	0.1177	0.1204	0.0728	0.0642	-	3.3	0.8	0.6	0.7	0.8	1.1
El Pital	0.1106	0.1515	0.1094	0.0959	0.0698	-	2.8	1.4	1.2	1.5	1.2
El Agrado	0.2193	0.2435	0.1931	0.1993	0.2279	0.0826	-	0.9	1.1	1.0	0.7
Gaviota	0.2649	0.2665	0.2610	0.2801	0.2811	0.1528	0.2227	-	1.1	1.1	0.6
Chapa	0.2246	0.2608	0.2323	0.2371	0.2580	0.1678	0.1907	0.1838	-	1.4	0.7
Boquerón	0.1821	0.2451	0.2131	0.2374	0.2284	0.1431	0.1949	0.1803	0.1493	-	1.0
Totumo	0.1064	0.2065	0.1488	0.1700	0.1910	0.1766	0.2505	0.2915	0.2649	0.1962	-

populations studied. The RAPD dendrogram of Jaccard distances (Fig. 1) shows the differentiation of the populations.

Expected heterozygosity values calculated with cpSSR and range from 0 to 0.036, indicating that cpSSR detects little polymorphism and very little genetic variation at the cytoplasmic level (Table 1). The results derived from cpSSR in *C. trianae* (Table 1, Fig. 2) indicate that there is no significant differentiation between populations. The dendrograms obtained showed high similarity of the chloroplast genome. The F_{ST} (Table 3) indicated low to moderate differentiation between populations, which would be due to frequent chloroplast alleles in the populations, possibly derived from a common maternal ancestor.

Similarity, genetic distance and DAPC: Fig. 1 shows the Jaccard distance-based dendrogram for RAPD markers in *C. trianae*. Three groups can be identified: one for El Agrado, El Boquerón, La Chapa, and La Gaviota; one for El Pital, Guayabal, La Argentina,

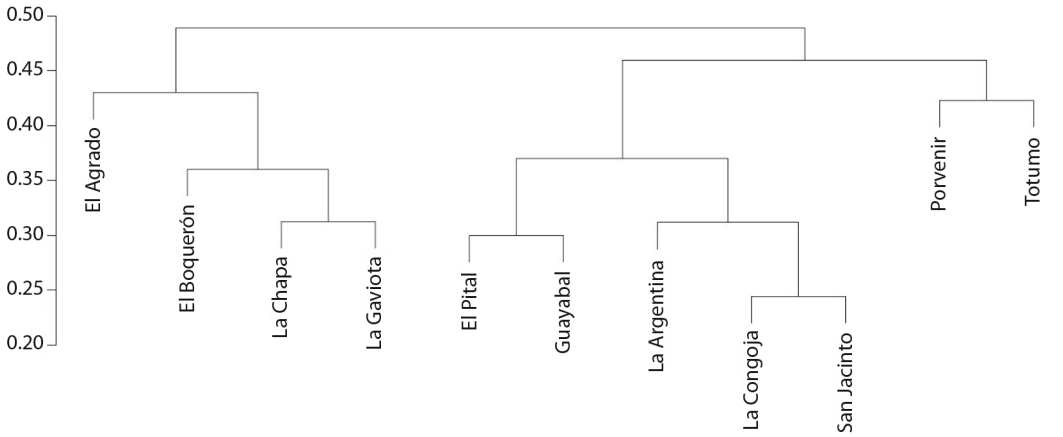

Fig. 1. Dendrogram according to Jaccard distances, using RAPD markers in *C. trianae*.

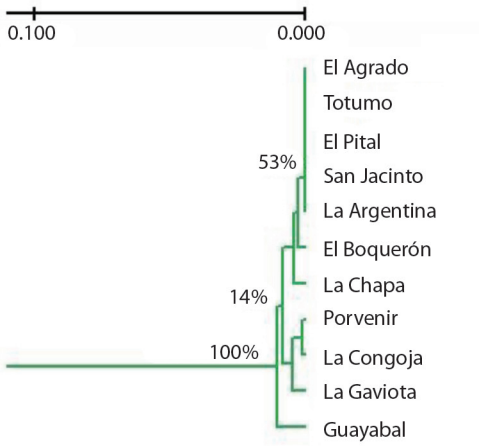

Fig. 2. UPGMA dendrogram according to Nei (1978), genetic distance among populations from cpSSR in *C. trianae*. Values above the nodes indicate the percentage of consistency of each cluster, based on bootstrap analysis with 1 000 replicates.

La Congoja, and San Jacinto; and one for Porvenir and Totumo. DAPC analysis suggests a structure of three populations (k = 3), and the membership probability plot (Fig. 3) reflects the same population configurations for the three groups as the Jaccard distances. The populations of Porvenir and El Pital showed the highest intrapopulation variance.

Fig. 2 shows the UPGMA dendrogram of Nei (1978), specifically in terms of the genetic distance between populations from the cpSSR in *C. trianae*. The values above the nodes indicate the percentage of agreement for each grouping, according to bootstrap analysis with 1 000 replicates. From cpSSR, estimated F_{ST} values between pairs of *C. trianae* populations ranged from 0 to 0.027. Except for nine out of 55 population pairs where there is little differentiation, the values hover around 0, indicating no genetic differentiation. This is supported by the fact that for the populations evaluated, all four cpSSR systems, matK 5 and rps16 were monomorphic, matK 3 and rbcL-atp each had 3 different alleles, but one of the three had the highest frequency (0.98). As all F_{ST} values are less than 0.33, the estimated values are greater than one migrant per generation, again indicating very little differentiation of the chloroplast genome in *C. trianae* (Table 3, Fig. 2).

The population structure, analysed by means of an AMOVA analysis of variance with two hierarchical levels, showed that there were significant differences between the populations of *C. trianae* (Table 4), but that the variance within populations was greater than the variance between populations. At this level, the La Congoja, El Pital, Gaviota and Boquerón populations are the ones that contribute the most to the within-population variance (Table 4). It should be noted that the populations with a

Table 3
F_{ST} and Nm values between pair of populations of *C. trianae* using cpSSR markers.

Populations	Porv.	Argen.	Congoja	San Jacinto	Guay.	El Pital	El Agrado	Gaviota	Chapa	Boquer.	Totumo
Porvenir	–	50	–	–	–	18.2	–	–	124.5	42.6	18,2
Argentina	0.010	–	–	–	110.6	–	–	–	–	–	–
Congoja	-0.059	-0.012	–	–	199.5	–	–	–	–	–	–
San Jacinto	-0.006	0.000	-0.023	–	–	–	–	–	–	–	–
Guayabal	0.000	0.005	0.003	-0.010	–	24.5	–	–	199.5	59.7	24,5
El Pital	0.027	0.000	0.000	0.000	0.020	–	–	–	–	–	–
El Agrado	-0.006	0.000	-0.023	0.000	-0.010	0.000	–	–	–	–	–
Gaviota	-0.043	-0.015	-0.034	-0.026	-0.041	-0.004	-0.026	–	–	–	–
Chapa	0.004	-0.012	0.00	-0.023	0.003	0.000	-0.023	-0.001	–	–	–
Boquerón	0.012	-0.012	0.000	-0.023	0.008	0.000	-0.023	-0.003	-0.034	–	–
Totumo	0.027	0.000	0.000	0.000	0.020	0.000	0.000	-0.004	0.000	0.000	–

Below the diagonal, F_{ST} values, above the diagonal, Nm values.

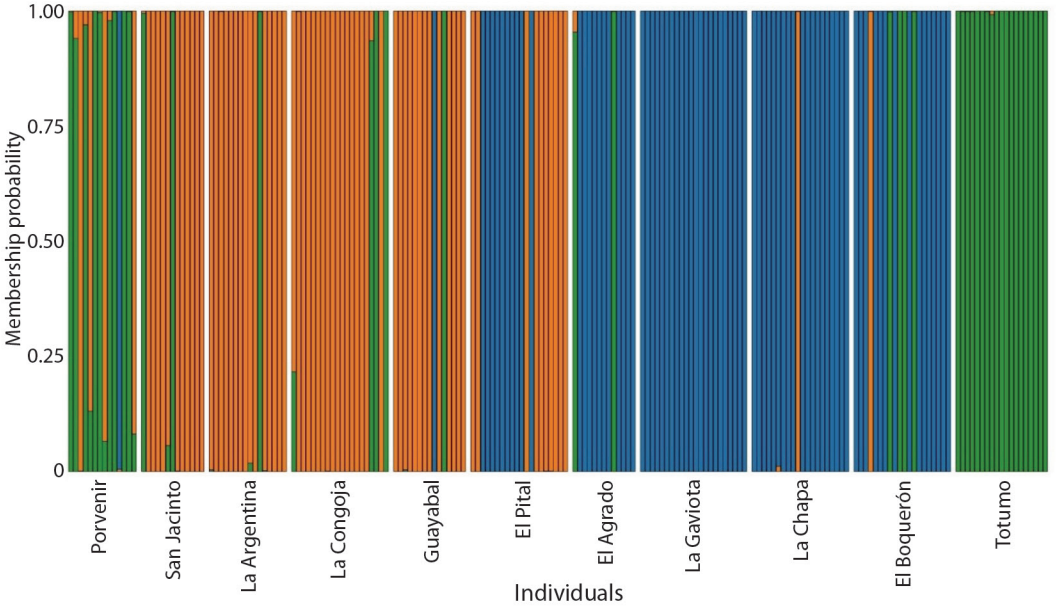

Fig. 3. Membership probability plot from the DAPC (k=3) for populations of *C. trianae*.

lower contribution to the variance have a lower number of individuals. The ΦST estimator of population structure indicates high genetic differentiation between populations (Fig. 1, Fig. 2).

The AMOVA of the cpSSR data with two levels of hierarchy showed that, according to the ΦST value, there is no significant variation between populations, while the P value within populations is significant (between individuals), indicating that the variation between individuals is high, suggesting variation at the local level. La Gaviota population contributes the most to the variation, followed by El Porvenir, Guayabal and La Chapa. The contribution to variation is zero for La Argentina, San Jacinto, El Pital, El Agrado and Totumo (Fig. 1, Fig. 2).

Using RAPD data, the F_{ST} estimator showed that genetic differentiation among pairs

Table 4
AMOVA for *C. trianae* using RAPDs and SSRs markers.

Source of variation	g.l	Sum of Square	Mean square	Variance component	% total	P-value
			RAPDs			
Between populations	10	761.4	76.3	3.5	18.6	< 0.001
Between individuals	181	2 764.2	15.3	15.3	81.4	< 0.001
			cpSSR			
Between populations	10	0.55	0.06	-0.0007	-1.10	0.87
Between individuals	182	12.29	0.07	0.0675	101.10	< 0.001

of *C. trianae* populations ranges from moderate to very high, with only the Congoja-San Jacinto pair showing low differentiation. The pairs with very high genetic differentiation are Gaviota with Porvenir, Argentina, Congoja, San Jacinto and Guayabal and La Chapa with Argentina and Totumo.

Gene flow Nm is less than three individuals per generation for most population pairs. Population pairs exchanging ≤ 10 individuals per ten generations generally included El Agrado, Gaviota, La Chapa, Boquerón and Totumo (Fig. 2). In the remaining pairs, the number of migrants per generation varied up to a maximum of 6 individuals per generation, as can be observed between La Congoja and San Jacinto.

When correlating genetic diversity values derived from RAPD or cpSSR with distance to the nearest road or distance to the nearest town or urban centre, in both cases the correlation coefficient is not significant, i.e. there is no significant association between the pairs of variables considered. The p-values indicate that there is no correlation between the genetic diversity data obtained from RAPD and cpSSR and the distance of each plant population studied to the nearest road or highway and to the nearest urban centre, so it can be postulated that the distance to roads does not influence the genetic composition of the populations and that the proximity to human settlements does not influence the genetic composition of the populations of *C. trianae*.

Genetic distances derived from RAPD data and cpSSR data showed no correlation ($R^2 = 9.3 \times 10^{-4}$ and p = 0.8253). This may indicate that RAPD genetic distances derived from nuclear genomes of both maternal and paternal origin, and those derived from chloroplasts of maternal origin, follow different patterns, which may be explained by the mechanisms of transmission of this genetic information from one generation to the next.

The Mantel test to correlate RAPD and geographical distances showed no significant correlation values (R = 0.0497). Similarly, when contrasting cpSSR genetic distances and geographical distances, there were no significant correlation values (R = -0.1916, p = 0.0850).

DISCUSSION

Pollination of *Cattleya* species: Studies of *Cattleya* species have recognised Euglossina bees as their pollinators (Braga, 1977; van der Pijl & Dodson, 1966). In the subgenus *Cattleya*, male *Euglossina* bees facilitate cross-pollination, thereby promoting variation within populations, a pattern that may also apply to *C. trianae*. The fact that *C. trianae* populations show less genetic differentiation than other endangered orchid species may be due to the particular mechanisms of gene flow through anemophilous seed dispersal and pollen flow mediated by euglossine bees (Wu et al., 2023; Zhang et al., 2019).

On the other hand, Euglossines, which pollinate Neotropical orchids, not only travel long distances, but also adapt to scattered microhabitats in the landscape and colonise forested areas during regeneration processes, so they would be able to move through the matrices within which forest patches are found (Cândido et al., 2021; Ulyshen et al., 2023).

The Ennos test (Ennos, 1994) concluded that seed dispersal predominates, a result similar to that found by Hedrén & Lorenz (2019) for the orchid *Epipactis helleborine*, which seems to be a peculiarity of the orchid family, which produces a large number of very light seeds that favour their anemophilous dispersal over long distances (Brzosko et al., 2017). Fruiting within and between populations can be influenced by pollinator diversity and activity, can vary between 13 and 70 % positively related to visitation rate, and in *C. trianae*, fruit size increases as the number of pollinia applied increases (Zhang & Gao, 2021).

This could be explained by the fact that for many of the pollinating bees, movement along a route is preferred because more plant resources are available to collect substances from flowers, and regardless of the distance between populations, there would be higher or lower levels of flow, resulting in different degrees of differentiation found in the populations.

Analysis of RAPD and cpSSR molecular data for *C. trianae* populations: The genetic diversity obtained by RAPD exceeded those reported for other *Cattleya* species in disturbed habitats, such as *Cattleya lobata* Lindl. (He = 0.262) (Lemos-Gomes et al., 2018) and *Cattleya elongata* Barb. Rodr. (He = 0.175) (Ueno et al., 2015) but similar to those found by Pinheiro et al. (2012) for *Cattleya labiate* (He = 0.30) in Brazil They were significantly higher than the values reported for other orchid species in disturbed habitats, including *Paphiopedilum micranthum* Tang & F. T. Wang (Li et al., 2020), *Platanthera leucophaea* Lindl. (Bell et al., 2021) and *Changnienia amoena* S. S. Chien (Qian et al., 2014).

The levels of genetic diversity found in this research for *Cattleya*, compared to the work described above, could be due to the fact that in this genus the combination of sexual and asexual reproduction and the longevity of the plants can ensure that genotypes are maintained over generations, reducing the impact of dispersal, even in affected populations (Lemos-Gomes et al., 2018) and that cross-pollination is favoured, associated with long distances travelled by pollinators and successive visits to flowers of the same species. In fact, Wong & Sun (1999) and Sun & Wong (2001) reported for the orchids *Goodyera procera* Hook., *Zeuxine gracilis* (Breda) Blume, *Eulophia sinensis* Miq. and *Zeuxine strateumatica* (L.) Schltr., with different mating mechanisms, that genetic diversity differs greatly both at the species level (He = 0.144 ~ 0.293) and at the population level (He = 0.011 ~ 0.181) (Table 1).

The genetic diversity values obtained from cpSSR (average He = 0.013), indicated a little genetic variation at the chloroplast DNA level (Table 1). To contrast these data with others for orchids, only the data reported by Squirrell et al. (2001), where core diversity values from allozymes and (cpDNA RFLPs) from the orchid *E. helleborine* (L.) Crantz. showed equivalent levels of genetic diversity.

The chloroplast genome is haploid, does not recombine and has a low mutation rate compared to the nucleus, as noted by Vu et al. (2020), chloroplast markers have different resolutions for different orchid genus. van den Berg et al. (2009) showed that the combination of different chloroplast microsatellites is very useful for phylogenetic studies of Laleliinae. Sequencing of the chloroplast genome of *Cattleya crispata* showed that it is similar to that of the genus *Cymbidium* and that the differences are in the order of some genes, which is useful for population studies (da Rocha-Perini et al., 2016). However, the presence of rare and private alleles (Jin et al., 1996) in two of the four cpSSR loci in *C. trianae* and heteroplasmy (Fig. 1, Fig. 2) may indicate an origin by recent mutations in the tested regions of the chloroplast genome and may be related to the fact that mutation rates of microsatellite length variation have been found to be higher (10–2–10–6) than point mutation rates (Amos, 2016; Wheeler et al., 2014).

The levels of genetic structure generated by RAPD (ΦST = 0.186) correspond to high genetic differentiation and are highly significant; the greatest contribution to genetic differentiation is within populations, suggesting that

there is under-structuring within populations, requiring microgeographic evaluation of localities. These values are lower than those reported for threatened orchids such as *P. leucophaea* (Φ ST = 0.21) and *Platanthera integrilabia* (Φ ST = 0.27) (Wooten et al., 2020) and the orchid *C. amoena* (Φ ST = 0.43) (Tikendra et al., 2021).

The Mantel test showed that for the two species there was no correlation between the genetic distances obtained with the two markers and the geographical distances, so there is no isolation by distance, a result consistent with that reported for endangered orchids such as *C. amoena* and *P. leucophaea*, where a lack of gene flow between populations or genetic drift within populations has been suggested (Qian et al., 2014).

Our research, with the primary aim of elucidating the current status of *C. trianae* populations, has provided essential information that not only safeguards the existing genetic diversity within this endangered orchid species, but also serves as a cornerstone for the development of sustainable management strategies. This study plays a pivotal role in the conservation and rejuvenation of the ecological environment in which these orchids thrive.

The genetic diversity and structure analyses using RAPD and cpSSR molecular markers revealing that the genetic diversity in the populations of *C. trianae* is not as low as is expected for species affected by habitat fragmentation. Additionally, the pronounced divergence among these populations is staggering, implying that various factors have significantly shaped the genetic composition and distribution of Colombian *C. trianae*.

This study is only the first step in understanding this native orchid species. To further our understanding, it is imperative to expand our investigation to include a larger and more geographically diverse sample representing the entire country. Using co-dominant markers and delving into sequencing data from cpDNA or nrDNA (ITS) is emerging as a promising avenue, with the potential to unravel the intricate mechanisms governing seed and pollen gene flow. This approach not only advances our understanding of population biology but also provides critical insights into the context of invasive orchids, with far-reaching implications for conservation and ecological management.

Implications for conservation: The comprehensive analyses presented in this study clearly demonstrate the profound influence of dominant habitats on the striking genetic differentiation within *C. trianae* populations. This research serves as a clarion call, highlighting the transformative effects of disturbance on the genetic fabric of these populations, and underscoring the urgent need for conservation action.

The implications for the conservation of this species are clear. Urgent and concerted efforts are imperative to mitigate the genetic differentiation between *C. trianae* populations, with a primary focus on the rapid restoration of ecological integrity. In situ conservation is of paramount importance, not only to prevent further habitat fragmentation, but also to protect the complex ecological relationships involving fungi and pollinators.

Considering these findings, a strong and effective ban on the unsustainable collection of *C. trianae* resources, particularly during the flowering season, is imperative. This measure is a linchpin in facilitating increased gene flow and ensuring the continued survival of these invaluable orchids.

Taken together, these findings highlight the urgent need for proactive and strategic conservation approaches. This study lays the groundwork for the formulation of effective management and conservation protocols, reflecting our unwavering commitment to the preservation of these threatened orchid populations. In its guiding role, this research not only secures the future of *C. trianae* but also revitalises the ecological sanctuaries they inhabit.

Ethical statement: The authors declare that they all agree with this publication and made significant contributions; that there is no conflict of interest of any kind; and that we followed all pertinent ethical and legal procedures

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