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THE FRAGRANT ORCHID *VANILLA CHAMISSONIS*: AN APPRAISAL OF THE GENETIC STRUCTURE OF WILD POPULATIONS FROM OSUNUNÚ NATURAL RESERVE, MISIONES, ARGENTINA

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Abstract. The commercial crops of vanilla face a significant risk of genetic erosion due to various factors. *Vanilla chamissonis*, a species with promising bioeconomic compounds, is found in limited regions in Argentina, particularly in the Osununú Natural Reserve in San Ignacio, Misiones, where it grows wildly in sectors (nuclei). This locally adapted germplasm of *V. chamissonis* holds considerable value and needs preservation efforts. In this study, we genetically characterized unexplored *V. chamissonis* germplasm and identified specimens within a phylogenetic framework using nuclear and plastid markers. The genotyping revealed that at least four gene pools contribute to the genetic diversity of these plants, with approximately 81% of total genetic variation allocated within populations. Sexual reproduction seems to predominate, and population N1 is a candidate for conservation. There is a genetic discontinuity between northeastern and southwestern nuclei, due to their different genetic constitutions and the tenuous isolation by distance. Phylogenetic results confirm the surveyed vanilla germplasm as *V. chamissonis*. This research provides essential insights for guiding the ongoing conservation and management efforts at the Osununú Reserve.

Resumen. Debido a diversos factores los cultivos comerciales de vainilla son altamente vulnerables a la erosión genética. Se ha demostrado que la especie *Vanilla chamissonis* produce compuestos bioeconómicos interesantes que podrían diversificar el mercado. En Argentina, la distribución de esta orquídea fragante se restringe a regiones específicas del noreste, como, por ejemplo, la Reserva Natural Osununú (San Ignacio, Misiones) donde crece de manera silvestre en sectores (núcleos). Este germoplasma, inexplorado, representa un endemismo localmente adaptado y, por lo tanto, es un recurso valioso que amerita preservarse. En este trabajo se llevó a cabo la caracterización genética de germoplasma de *V. chamissonis.* Además, se realizó la identificación molecular de los especímenes en un contexto filogenético empleando tanto marcadores nucleares como plastidiales. El genotipado expuso que, al menos, cuatro acervos genéticos contribuyen a la diversidad genética de estas plantas, y que *ca.* del 81% de la variación genética total se asigna dentro de las poblaciones. Los resultados actuales sugieren un predominio de la reproducción sexual, que el núcleo N1 emerge como una unidad candidata a conservarse, y la ocurrencia de una discontinuidad genética entre las poblaciones del noreste y del suroeste, que muestran constituciones genéticas diferentes. También se develó la ocurrencia de un tenue aislamiento por distancia. Nuestros resultados filogenéticos sugieren que es apropiado considerar al germoplasma estudiado como *V. chamissonis*. El conocimiento básico generado en este trabajo ayudará a guiar la iniciativa de manejo y conservación *in situ,* en curso en la reserva de Osununú.

Keywords / Palabras clave: filogenética, genética poblacional, genotipado molecular, molecular genotyping, Misiones province, orquídeas silvestres, Osununú, phylogenetics, population genetics, provincia de Misiones, wild orchids

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Introduction. The genus *Vanilla* Plum. ex. Mill (Orchidaceae Juss.) consists of approximately 118 species worldwide, most remaining unexplored scientifically (Karremans *et al.* 2020). Perhaps because of this lack of information, only nine species have been included in the International Union of Conservation of Nature´s List of Threatened Species (IUCN 2022). *Vanilla planifolia* Andrews, *V. pompona* Schiede, and the hybrid *V.* × *tahitiensis* J.W.Moore, are extensively studied due to their commercial value as sources of natural vanillin (Cameron 2011). However, wild and cultivated populations of *V. planifolia* are highly vulnerable to genetic erosion due to the over-collection, habitat destruction, and the clonal management of the crop (Bramel & Frey 2021, Soto Arenas 2006). Consequently, there is a need for conservation strategies and alternative vanillin sources (da Silva Oliveira *et al.* 2022, Flanagan *et al.* 2018). *Vanilla chamissonis* Klotzsch is a fragrant, hemiepiphytic orchid native to South America with terrestrial and climbing growth (Soto Arenas 2003, Soto Arenas & Dressler 2010). Taxonomically, it has been placed in the subgenus *Xanata*, section *Xanata* by Soto Arenas & Cribb (2010) and Besse *et al.* (2021), while others recognize only the section shared with 38 species with fragrant fruits (Karremans *et al.* 2020). Indeed, it has been recently demonstrated that *V. chamissonis* and *V. bahiana* Hoehne synthetize aromatic compounds with promising bioeconomic potential (da Silva Oliveira *et al.* 2022). Furthermore, a recent study unveiled the pollination strategy of Neotropical vanillas, including *V. chamissonis*. It was found that these species employ a pollination strategy that entices euglossine bees with nectar rewards (Pansarin 2022). On a physiological level, it has been shown that sunlight directly and proportionally affects the leaf area, succulence, and chlorophyll content (Lopes *et al.* 2019). The geographical distribution of *V. chamissonis* ranges from northern South America, including French Guiana, to southeastern Brazil, Bolivia, and northeastern Argentina (Biganzoli & Múlgura de Romero 2004, Jørgensen *et al.* 2013, Soto Arenas & Cribb 2010, Szlachetko 2016). In Brazil, it is among the most frequent *Vanilla* species found in the Atlantic Forest, extending from Espírito Santo to Rio Grande do Sul (Reis *et al.* 2011). In Argentina, *V. chamissonis* is restricted to specific areas of the province of Misiones, with recorded occurrences in Isla Caraguatay Provincial Park (located in the Paraná River), the Teyú Cuaré Provincial Park and the adjacent private Osununú Natural Reserve (Reserva Natural Osununú; RNO) (Biganzoli & Múlgura de Romero 2004, Munno *et al.* 2011, M. A. Munno *et al.* unpub. data). Due to the ongoing fragmentation of natural habitats and the restricted distribution of *V. chamissonis*, this species has been classified as threatened in Argentina, listed on the Appendix II of Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES 2022). Therefore, a significant project is underway at the RNO to conserve and sustainably manage *V. chamissonis*. The objective of the present work was to genetically characterize the wild unexplored germplasm of *V. chamissonis* from the RNO. This step represents the initial phase in identifying priority areas for preserving locally adapted variants. To date, such genetic characterization has not been carried out. Additionally, the genetic data obtained could be valuable for future breeding programs. Molecular genotyping techniques allow revealing the genetic background of organisms through the scrutiny of highly polymorphic loci. These techniques have been applied in studying several plant species, including many orchids (George *et al.* 2009, Hou *et al.* 2012, Ma & Yin 2009, Qian *et al.* 2013, 2014, Verma *et al.* 2009). Insights on population structure and species identification are basic knowledge in defining the candidate units that merit *in situ* conservation. Notably, *Vanilla* species from Argentina have been documented under various names (Biganzoli & Múlgura de Romero 2004, Johnson 2001, Keller *et al.* 2019, Munno *et al.* 2011, M. A. Munno *et al.* unpub. data), many of which have been synonymized (e.g., Soto Arenas & Cribb 2010). Therefore, as part of the characterization of this genetic resource, we undertook its molecular identification within a phylogenetic context.

Materials and methods

Plant material.— The plant material was collected at the RNO in May 2015 and May 2019. Within the RNO, these climbing plants develop within sectors called nuclei. Four of the nuclei reached were outlined in the work of M. A. Munno *et al.* (unpub. data) (i.e., N1 to N4), and the additional nuclei were pinpointed by the RNO´s personnel, who identified these sectors during their vigilant monitoring activities. Given that

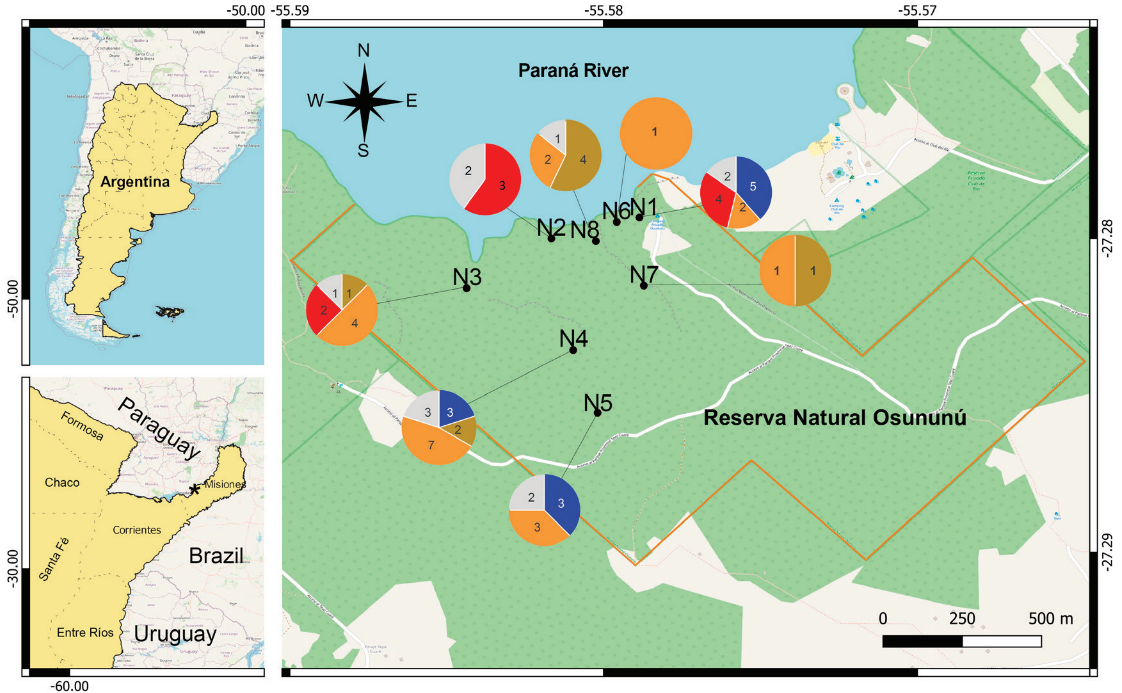


FIGURE 1. Nuclei location of sampled vanilla plants at the Reserva Natural Osununú, Province of Misiones, Argentina. Black dots indicate the nuclei. Each nucleus's relative gene pool composition is depicted in colored pie charts. The proportion of individuals assigned to each genetic cluster (DAPC clusters 1 to 4; see text) with a probability >0.90, is shown in blue, brown, orange, and red, respectively. The proportion of individuals with an ambiguous assignment (probability range: 0.483–0.815) is denoted in grey.

these plants may reproduce both clonally and sexually (Munno *et al.* 2011, M. A. Munno *et al.* unpub. data, Reis *et al.* 2011), each plant (stem) is considered –a priori– as an individual. The plants sampled from each nucleus are treated as a population (Appendix 1). Eight nuclei were reached, with a variable number of individuals collected from each nucleus. Leaf samples were collected from healthy and accessible plants at least 1.5–2.0 m apart, ensuring they were not interconnected, and were then preserved in silica gel. Out of the 72 individuals, those that dried without any apparent signs of oxidation were employed for total genomic DNA extraction (Appendix 1). This extraction process involved processing small leaf pieces with the DNeasy Plant Quick Extraction kit (QIAgen Inc., Duesseldorf, Germany) following the manufacturer's instructions.

Sampling site. — The private reserve RNO is located at the Municipio and Department of San Ignacio (Misiones, Argentina), and is adjacent to the Provincial Park Teyú Cuaré (Fig. 1); both the park and reserve are within the Paranaense Forest and show remnants of the Cerrado biome. The reserve involves 174 ha of undulating topography with heights up to 215 m; five distinct environments or landscape units were delineated within the RNO, encompassing wetlands, vegetation areas associated with exposed rocky outcrops or elevated sandstone formations, mixed forests, secondary successions, and forests situated in lowlands and slopes (Keller *et al.* 2019, Velazco *et al.* 2015). The mixed forest encompasses most of the area (79 ha). However, the *Vanilla* under study is associated with the arboreal vegetation of the Myrtaceae forests found in both lowlands and slopes. Additionally, it is associated with the Annonaceae trees located on the sandstone rocky outcrops slopes, where the soil is characterized by nutrient deficiencies (Keller *et al.* 2019, M. A. Munno *et al.* unpubl. data). The RNO exhibits a subtropical, humid temperate climate without a clearly defined dry season. The mean annual temperature is 21.86 ºC, while the

mean annual rainfall is 1644 mm. The rainiest period in the region comprises October to February.

Molecular genotyping with co-dominant markers.— Six heterologous simple sequence repeat (SSR) markers, originally designed for *V. planifolia* and reported showing amplification signals in *V. chamissonis* and *V. bahiana* by Bory *et al.* (2008a), were assayed on eight randomly chosen DNA samples. The SSR loci assayed were: 005, 015, 019, 025, 031 and 047. PCR amplifications were performed using 30–60 ng of genomic DNA, 60 ng of each primer, 1X PCR buffer (Invitrogen Thermo Fisher Scientific, California, USA), 2 mM $MgCl_2$, 0.2 mM of each dNTP and 0.5 unit of Taq DNA polymerase (Invitrogen Thermo Fisher Scientific), in a final volume of 25 µl. PCR cycles were as in Cascales *et al.* (2014), but the annealing steps were tested at 50°C, 55°C and 60°C. PCR products were checked by means of electrophoreses in 1.5% (w/v) agarose gels, stained with ethidium bromide, and photographed under UV light. Promising loci were amplified for all individuals and subjected to high-resolution electrophoreses. For this, SSR amplicons were mixed with an equal volume of 98% (v/v) formamide, heat-denatured, ice-cooled, and loaded into a 6% (w/v) polyacrylamide gel under denaturing conditions (8 M urea). Electrophoreses were carried out in 1X TBE buffer (0.09 M Tris, 0.09 M boric acid, 2 mM EDTA, pH 8.0) at 60 W for 4–5 hs. Bands were visualized by silver nitrate staining using the Silver Sequence Staining Reagents kit (Promega, USA) according to the manufacturer's instructions. Air-dried gels were then digitalized, and band sizes were estimated from gel images. A molecular weight standard (30–330 AFLP DNA Ladder; Invitrogen Life Technologies) was included in every run as a size reference and staining control.

Molecular genotyping with dominant markers.— A set of Inter-Simple Sequence Repeat (ISSR; Zietkiewicz *et al.* 1994) markers were screened on 13 randomly chosen genomic DNA samples; the primers assayed were: ISSR-P1: $(AG)_8Y$; ISSR-P3: $CT(GA)_8$; ISSR-P4: $(GA)_8C$; ISSR-P6: $(GACA)_4$; ISSR-P7: $(GA)_8T$; ISSR-P9: $(GATA)_4$; ISSR-P13: $(AG)_8YT$. PCR conditions were as in Paiva *et al.* (2020). Primers that produced more than five clear bands, between 300–1500 bp in size in agarose gels, were selected for further analyses. Aliquots of ISSR amplifications were prepared as above, separated through denaturing 5% (w/v) polyacrylamide gel electrophoreses, and visualized through silver staining procedures, as already stated. Fragment sizes were estimated from digitalized gel images by comparison against molecular weight standards (100 bp DNA Ladder –Productos Bio-lógicos, Argentina– and 30-330 AFLP DNA Ladder). For a given locus, each band was registered as present (1) or absent (0). These data were used to construct five binary matrices, one per ISSR primer plus a combined matrix comprising individuals with >50% of loci information. In addition, low-frequency bands (≤ 0.10) were removed to reduce errors generated during scoring. Genotyping error rates were determined for each ISSR marker by running duplicate, independent PCR amplifications for 4–5 individuals; errors were estimated as the ratio of the total number of loci with discordant scores (all individuals combined) to the product of the number of individuals by the total number of scored loci (Bonin *et al.* 2004).

Analyses of genotyping data.— The performance of the markers was evaluated through the polymorphism information content (*PIC*) calculated as in Roldán-Ruiz *et al.* (2000). Also, the power of the set of loci to discriminate between unique individuals (referred to as multilocus genotypes) was assayed through the genotype accumulation curve, with the function genotype_curve of the poppr package v. 2.9.4 (Kamvar *et al.* 2014, 2015) implemented in R4.3.1 (R Core Team 2023), setting the resampling to 1000. Partitioning of genetic variation within and among pre-defined populations was assessed by the analysis of the molecular variance (AMOVA; Excoffier *et al.* 1992) using GenAlEx 6.5 (Peakall & Smouse 2012); statistical significance of each variance component was assessed based upon 999 permutations in GenAlEx. To identify genetic clusters without any a priori group delimitation (for instance, populations), we conducted Discriminant Analysis of Principal Components (DAPC; Jombart *et al.* 2010) on the five matrices, using the adegenet 2.1.0 package (Jombart & Collins 2015) implemented in R 4.2.1 (R Core Team 2022). Genetic clusters were assessed using the find.clusters function with n.iter=100,000 and n.start=10, evaluating a range from 1 to 6, by the K-means algorithm (Legendre & Legen-

dre 1998). The optimum K value and number of discriminant functions were determined for each matrix.

Relationships among individual plants were investigated, as well, through a clustering distance-based analysis for the combined matrix. Distances were obtained applying the complementary of the Dice similarity index, in FAMD 1.31 (Schlüter & Harris 2006); a Neighbor-Joining unrooted phylogram was obtained and the bootstrapping was performed with 1000 pseudoreplicates with the same program. Resulting topology was visualized with FigTree v1.4.3 (Rambaut 2016).

A correlation analysis between pairwise genetic and geographical distance matrices was performed through a Mantel test in the Alleles In Space program (AIS; Miller 2005). The spatial genetic structure was approximated through the Genetic Landscape Shape interpolation procedure, as implemented in AIS, employing the geographical distances among the nuclei and the residual genetic distances among individuals to construct a connectivity network among the sampled points based on Delaunay triangulation. The X and Y coordinates are the midpoints of each edge in the triangulation, and the 'heights' in the landscape are reflections of the genetic distances (i.e., residual genetic distances) between the observations found at the vertices of the triangles. An inverse distance weighted interpolation (weighting parameter= 1.5) across a uniform grid size of 100 × 100, was set.

Phylogenetic analyses.— Nucleotide sequences of the entire nuclear Internal Transcribed Spacer region of the ribosomal DNA (namely, ITS1-5.8S-ITS2) and partial sequences of plastid genes coding for the Rubisco large subunit (*rcbL*) and the maturase K (*matK*) were obtained from three individuals representing different plant sectors or nuclei (namely, N1P374, N3P397, and N7P376). The ITSs were generated using primers ITS-4 and ITS-5 (White *et al.* 1990); for *rbcL* the Z1 and Z1351R primers (Soltis *et al.* 1992) were employed, and for *matK* the forward 5´-CGATCAACATCTTCTGGAGTGT-3´and reverse 5`-TGAACAGTTATGAATGAGCCACT-3´ primers, which were designed in this study with Primer3web (Untergasser *et al.* 2012). The sequencing service was provided by Macrogen Inc. (South Korea). Chromatograms were inspected in BioEdit (Hall 1999), and the consensuses between forward and reverse sequences were obtained with the same program.

The ITS sequences obtained here (OQ080023–OQ080025) were analyzed together with 77 ITS sequences retrieved from the Genbank belonging to 55 species of *Vanilla*, and 11 sequences from three genera that were used as outgroups (Appendix 2). Plastid genes were analyzed separately from the nuclear partition; the sequences generated here for *rcbL* and for *matK* (OR472978-OR472983) were analyzed with 38 *Vanilla* accessions derived from 28 species and two *Epidendrum* species used as outgroups (Appendix 2).

Sequences were aligned in MEGA 7.0.25 (Kumar *et al.* 2016) using the MUSCLE algorithm (Edgar 2004) with default parameters. A pairwise *p-distance* matrix was generated in MEGA to survey for duplicated sequences. Evolutionary model selection and maximum likelihood (ML) phylogenetic analyses were conducted on IQ-TREE v. 2.0.6 (Minh *et al.* 2020, Nguyen *et al.* 2015). Each segment of the ITS, namely ITS1, 5.8S, and ITS2, and each plastid gene had a distinct substitution model, selected through the ModelFinder algorithm (Kalyaanamoorthy *et al.* 2017) in IQ-TREE; when needed, the best partition scheme was selected with the same program. The command path line for the nuclear matrix was: *Iqtree2 -s inputfile.fas -keep-ident -p partition_file.txt -m TESTMERGE -pre outputfile -bnni -nt AUTO*. The command line was as above for the plastid genes, but the command *-s* was excluded and *-p* was used to call both matrices. Thus, the best model determined for ITS1 and ITS2, was Tamura-Nei with unequal base frequencies (F) and site rate heterogeneity accounted through a Gamma distribution with four categories (G4) that is, TN+F+G4 (*alpha* parameter= 1.91); the 5.8S partition received the model K2P+G4 (Kimura two parameters, *alpha* parameter= 0.26). The fittest model selected for *rbcL* was K3Pu+F+I (Kimura three parameters with unequal base frequencies and a proportion of invariant sites, I=0.8) and for *matK* was TVM+F+G4 (transversion model, *alpha* parameter= 0.56). Ten independent runs were carried out using the same multiple sequence alignment obtained for each marker, but with different orders of the taxa, to escape from local optima. In each run, support for internal nodes was estimated by ultrafast bootstrapping (Hoang *et al.* 2018) and by the Shimodaira–Hasegawa approximate likelihood ratio test

Table 1. ISSR characterization of *Vanilla chamissonis* plants from the Reserva Natural Osununú (Misiones, Argentina).

		Number of bands								
Matrix	Number of individuals	Raw matrix	Edited matrix	Missing data (%)	B/N [a]	Error [b]	PIC [c]	#GC [d]	Assign. Prob [e] (%)	Phi_{PT} [f] (%)
ISSR-P3	58	42	33	0	18.63	0.012	0.491	6	82.75	19.3
ISSR-P4	58	87	45	0	18.63	0	0.482	4	89.65	15.6
ISSR-P6	63	92	26	0	8.16	0	0.431	6	96.83	23.2
ISSR-P7	65	75	37	0	19.47	0.01	0.498	5	100	22.1
Combined	**59**	--	**136**	**3.24**	**62.22**	--	--	**4**	**79.66**	**18.7**

[a], average number of bands per individual; [b], refers to genotyping error; [c], polymorphism information content; [d], number of genetic clusters discriminated via DAPC; [e], percentage of individuals showing an assignment probability >90%; [f], the genetic differentiation among groups estimated through AMOVA (p-value ≤ 0.001), excluding N6 and N7 for having less than five individuals.

(SH-aLRT; Guindon *et al.* 2010) with 10,000 pseudoreplicates. The command path line used for each ITS´s run was: *Iqtree2 -s inputfile_runX.fas -keep-ident -p partition.best_scheme.nex -pre outputfile_runX -bnni -bb 10000 -wbt -alrt 10000 -nt AUTO*. The command *-p inputfile_runX.fas* was used for running the plastid markers, and the command *-p partition.best_scheme.nex* was excluded. Trees were visually inspected with Figtree. Afterwards, to detect differences among runs, the topology test of Shimodaira and Hasegawa (1999) and the Approximately Unbiased test (AU; Shimodaira 2002) were performed for ITS, with the command line: *Iqtree2 -s inputfile_runX.fas -p partition.best_scheme.nex -z 10_trees4test.trees -zb 10000 -zw -au -pre output_Tree_test -nt AUTO*. For the plastid genes, the command line was adjusted as outlined above. A full bootstrapping was performed after the best runs were selected (with command: *-b 10000*). These took 96 hr for ITS and 16 hr for the plastid genes, using 20 CPU cores. The final phylograms were rooted with *Epidendrum* species, following Pérez-Escobar *et al.* (2021), and visualized in Figtree.

Results. Ninety-three percent of the plants gathered yielded genomic DNA of good quality (N=67). Of the six heterologous SSR markers assayed, solely markers 019 and 031 yielded banding patterns compatible with co-dominant loci. The other four markers produced several bands (between 100–1000 bp in size) even under stringent amplification conditions (*i.e.,* 60°C), and thus were excluded. When markers 019 and 031 were assayed in high-resolution gels, no variation was evidenced among >60 individuals tested. Then, the plants were characterized through ISSR genotyping. Four of the seven examined primers exhibited reliable banding patterns (Appendix 3) and were successfully amplified across 58–65 individuals (Table 1). Out of 296 bands registered, 54% were excluded due to their appearance at a frequency below the preestablished threshold (i.e., <0.10). Each primer produced high PIC values (>0.43; Table 1), and solely for primers ISSR-P3 and ISSR-P7 it was detected a genotyping error (1.0–1.2%). The genotype accumulation curve displays that within the conditions of this study, complete discrimination (100%) of multilocus genotypes can be achieved by utilizing a minimum of 27 ISSR loci (see Appendix 4). The AMOVA results showed that most of the genetic variation appeared within populations ranging from 77% to 84% for single ISSR primer datasets and reaching 81.3% for the combined matrix (Table 1). The DAPC discriminated between four to six genetic clusters, according to the matrix considered, without any prior group indication (Table 1). The combined matrix showed that the individuals were assigned to four non-overlapping genetic clusters or gene pools (numbered 1–4; Fig. 2A), consisting of 11, 12, 23, and 13 plants, respectively. DAPC results also evidenced that 81% of the individuals showed >0.90 probability of membership to the cluster assigned, that is, most individuals have in their genetic makeup predominance from a single gene pool (Fig. 2B). Solely 11 individuals were ambiguously assigned, exhibiting membership probabilities ranging from 0.483 to 0.815, showing signals of genetic admixture (Fig 2B; Fig. 3, pie charts). Then, for the plants from nuclei N1, N3, and N4, at least three distinct gene pools have been estimated to contribute with high probability. In contrast, for the plants from N5, N7, and N8, the estimation indicates a genetic in-

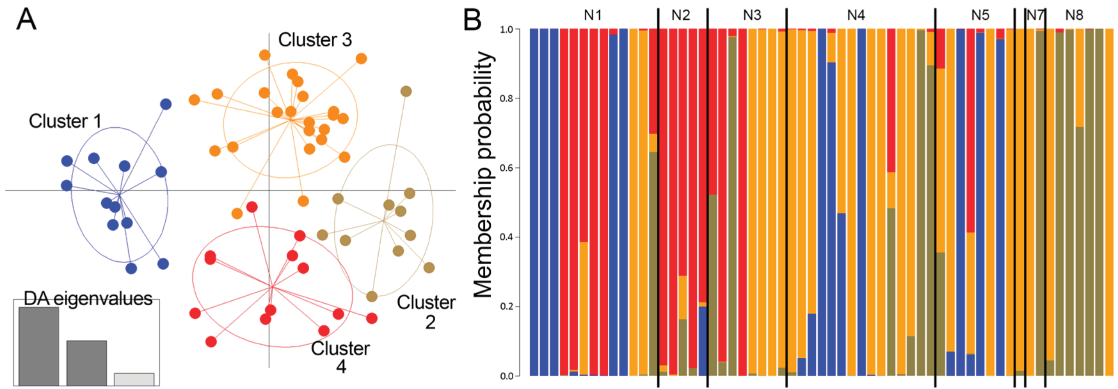


FIGURE 2. Genetic clustering and estimated genetic constitution of *Vanilla chamissonis* individuals from the Reserva Natural Osununú (Misiones, Argentina). **A**. Scatterplot of the Discriminant Analysis of Principal Components (DAPC) depicting relative intra-group and among-group genetic distances. Dots represent individual samples; clusters are indicated with colors (blue: cluster 1, brown: cluster 2, orange: cluster 3, and red: cluster 4). Inset indicates the amount of variance in the grouping variable explained by the predictors in the discriminant function (discriminant analysis eigenvalues 72.38, 41.82, and 11.35, respectively). **B**. Genetic background of each individual surveyed. Each individual plant is represented by a vertical bar, which could appear partitioned into colored segments that represent the estimated membership probability to each of the four genetic clusters obtained (K=4). The colors are the same as in the scatterplot.

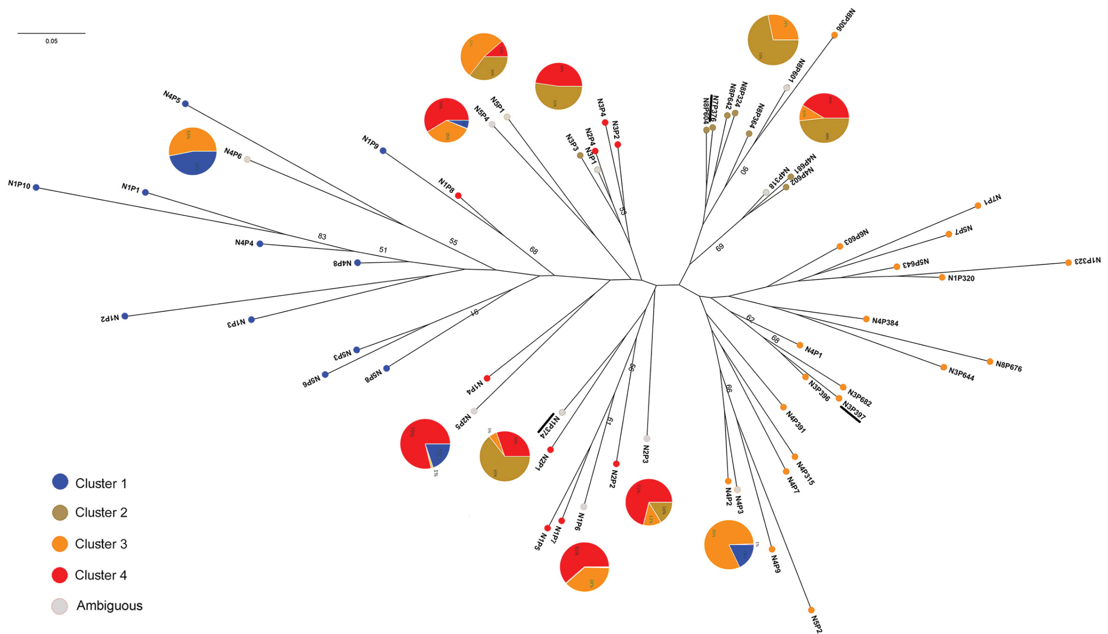


FIGURE 3. Neighbor Joining unrooted tree of *V. chamissonis* wild plants from the Reserva Natural Osununú (Misiones, Argentina), based on genetic distances derived from ISSR genotyping. Numbers on branches represent bootstrap values (values >50% are shown). The colored tip of each branch designates the DAPC cluster to which the individual was assigned with a probability > 90% (blue: cluster 1, brown: cluster 2, orange: cluster 3, red: cluster 4). Individuals with ambiguous assignments are denoted by grey dots, while their estimated genetic constitution is visually represented by colored pie charts located on the side. Underlined individuals were used for sequencing (see text).

put from two pools with high probability. For N2, there is a predominant assignment to a single gene pool (Fig. 1, pie charts; Fig. 2B).

The average genetic distance estimated among all individuals equals 0.3648. Plants from nucleus N1 yielded the highest average distance (*d*=0.4197), and those from N3 showed the smallest distance (*d*=0.2159). The population comparisons indicated that N1-N8 are the most dissimilar pair (*d*=0.4404), being linearly among the closest nuclei (154 m; Appendix 5). In contrast, N2-N3 appear as the most similar nuclei (*d*=0.2725), being 388 m apart. The unrooted neighbor-joining dendrogram showed groupings concordant with DAPC clusters 1, 2, and 3; while individuals assigned to cluster 4 appeared dispersed (Fig. 3). Still, very few nodes received bootstrap support values worth considering.

A slight but significant positive correlation between genetic and geographic distances was detected (r= 0.1724; P< 0.001). The genetic landscape shape interpolation analysis showed that the highest positive peaks, *i.e.,* the greatest genetic distances, are found among individuals from the northeastern region of the RNO (Appendix 6). Instead, valleys and negative peaks are apparent among the nuclei located towards the southwest. This landscape reveals a genetic discontinuity between both regions.

The alignment of 88 ITS sequences spanned 755 bp, of which 31.5% were constant sites. ITS sequences obtained from plants N1P374, N3P397, and N7P376 resulted identical. Likewise, the Genbank ITS sequences of *V. odorata* (accession numbers MN902028 and MN902032) on the one hand, and of *V. mexicana* Mill. (accession numbers MW829691 and MW829692) on the other, also resulted in being identical. As a result, only a single representative was subjected to further analysis for each of these species. Thirteen accessions belonging to different nominal taxa showed identical ITS sequences. Specifically, the pair *V. chamissonis* (MW829674) and *V. bahiana* (EU498163); the quartet *V. pompona* (GQ867234), *V. pompona* subsp. *pittieri* (Schltr.) Dressler (MN902062), *V. pompona* subsp. *grandiflora* (Lindl.) Soto Arenas (MN902058), and the hybrid *V.* × *tahitensis* (MN902063); and the triplet *V. planifolia* (U66819), *V. pompona* subsp. *pompona* (MN902053) and *V.* cf. *planifolia* (FJ425832). The pairs *V. norashikiniana* Go & Raffi (MH777755) and *V. griffithii* Rchb.f. (MH777726), as well as *V. shenzhenica* Z.J.Liu & S.C.Chen (JF796930) and *V. somae* Hayata (KY966687), also had identical ITS sequences but were kept for analyses. The ten independent runs yielded phylogenetic trees showing slight topological differences, although none in the clade containing *V. chamissonis;* statistical tests failed to discriminate among the topologies (SH p-value ≥ 0.381; AU p-value >> 0.05). The phylogram from run_7 was selected based on the highest logL, and lowest BIC and AIC values (Appendix 7); it shows that all *Vanilla* sequences formed three highly supported clades (Fig. 4), with their relationships poorly supported (30–64% bootstrap support value, BSV). One highly supported clade (94% BSV) comprises 44 sequences representing 18 taxa from North, Central, and South America, including *V.* × *tahitensis* and *V. hirsuta* M.A.Clem. & D.L.Jones from Oceania. The ITS sequences obtained in this study formed a monophyletic group (100% BSV) with *V. chamissonis* MW829674, *V. bahiana* EU498163, and the three sequences of *V. calyculata* Schltr. A second highly supported clade (94% BSV) involved 26 sequences from 20 Asian or African species plus three species from Central America (*V. barbellata* Rchb.f., *V. claviculata* Sw., and *V. dilloniana* Correll) whose position within this clade is uncertain. The third clade involves three American taxa (98% BSV). Noteworthy is the placement of *V. mexicana* (MN902027) and *V. planifolia* (MN902045); these accessions appeared nested among *Epidendrum* species, with full support (100% BSV).

The alignments of the plastid genes for 28 *Vanilla* species and two *Epidendrum* L. species (43 sequences, in total; Appendix 2) reached 1264 bp for *rbcL* and 840 bp for *matK*, and exhibited 93% and 79.3% of constant sites, respectively. The sequences obtained here for plants N1P374, N3P397, and N7P376 resulted identical for both genes. Among the other nucleotide sequences included, those attributed to *V. bahiana* from voucher specimens CR0099 and CR0668, exhibit identical sequences for both *rbcL* and *matK*; as well, the *rbcL* of *V. bahiana* is indistinguishable from those of *V. insignis* and *V. phaeantha*. Similarly, *V. madagascariensis* Rolfe and *V. humblotii* Rchb.f., on the one hand, and the five accessions of *V. planifolia* and *V. sotoarenasii* M.Pignal, Azof.-Bolaños & Grisoni (CR0068), on the other, show identical plastid sequences. No discernable

topological variations regarding sister-group relationships among the ten phylogenetic trees were identified. Furthermore, the statistical tests did not discriminate among the topologies (SH p-value ≥ 0.224; AU p-value > 0.05). The phylograms from run_0 and run_6 emerge equally likely based on their highest logL and lowest BIC and AIC values (Appendix 7). The plastid phylogram (Fig. 5) depicts a highly supported clade (98% BSV) comprising 12 taxa (25 sequences) from North, Central, and South America, and *V.* × *tahitensis*; within this clade, the sequences obtained in this study relate to the reference voucher *V. chamissonis* CR0666 (81% BSV). Another strongly supported clade (91% BSV) involves 15 Asian and African species that conform the sister group of the latter clade (92% BSV). Then, with full support, *V. mexicana* CR2144 appears as the sister taxon to all other vanillas. As expected, specimens with identical sequences formed clades with zero-length branches.

Discussion. The present work contributes basic and novel genetic knowledge for the understudied yet valuable germplasm of the fragrant orchid *Vanilla chamissonis*. The findings offer constructive insights for conserving and managing this species within the Reserva Natural Osununú (RNO), given that the natural habitat of *V. chamissonis* has been under anthropic reduction, mainly because of progressive deforestation. The utilitarian expression "nucleus", used to denote distinct groups of *V. chamissonis* plants, emerges as a conceptually relevant unit in planning management strategies. The RNO comprises a remnant of the Cerrado biome, unique within Argentina and intermingles with rupiculous vegetation; its biological diversity harbors a set of distinct local adaptations that serve as valuable genetic reservoirs. For instance, 23 to 38 plant species have been reported with a distribution constrained to the geographical boundaries of the Teyú Cuaré Provincial Park and the Reserva Natural Osununú (Keller *et al.* 2019, Velazco *et al.* 2015). To genetically characterize the *V. chamissonis* germplasm, firstly, we attempted the transferability of SSR markers that were documented as successful in this species (Bory *et al.* 2008a); however, our efforts failed to reproduce their results on *V. chamissonis*. Four of those SSR markers yielded banding patterns incompatible with microsatellite loci, even under highly stringent amplification conditions, and the other two markers produced monomorphic patterns in high-resolution polyacrylamide gels. Gigant *et al.* (2012) developed 19 microsatellite markers for *V. humblotii* and *V. roscheri* Rchb.f., successfully tested on 14 individuals of four American species. However, it is noteworthy that none was *V. chamissonis*. Then, Gigant *et al.* (2016), in trying to transfer SSR markers from the African vanillas to *V. mexicana*, had a mild success, ending with four reliable markers. Additionally, their attempt to transfer 14 SSRs of Bory *et al.* (2008a) from *V. planifolia* to *V. mexicana* proved unsuccessful. Since species-specific co-dominant markers are an ideal tool for conducting populational-based studies, developing novel homologous markers could significantly enhance such studies across the distribution range of *V. chamissonis*.

To gather genetic data that could unveil the genetic diversity, we genotyped the plants by applying anonymous dominant markers at hand, employing highly resolving and sensitive procedures, and following stringent inclusion criteria. The unsupervised DAPC genetic analysis allowed the detection of at least four gene pools that contribute to the genetic diversity of these plants. Also, each nucleus of vanilla plants varies in its genetic constitution, so that most of the total genetic variation (81%) was due to differences among individuals within each nucleus. Even though small populations of *V. chamissonis* have been reached in this study, present results suggest that future efforts should focus on preserving as many individuals as possible from each nucleus to maintain the existent diversity. *Vanilla* species form small populations in Colombia due to infrequent flowerings and rare pollination events; there is also a dependency on the forest dynamics´ (Flanagan *et al.* 2018). Studies on the floral biology of *V. chamissonis* from Brazil revealed a spontaneous fruit production rate of 21.2%; this rate was deemed high, considering the inability to ascertain the pollinating agents (Reis *et al.* 2011). At the RNO, the modestly sized populations of *V. chamissonis* exhibited reproductive strategies involving regeneration through seed germination and agamic reproduction (M. A. Munno *et al.* unpubl. data). Although that study needs formalization, at the time, those authors examined 30 plants from four nuclei, of which only nine showed ripened

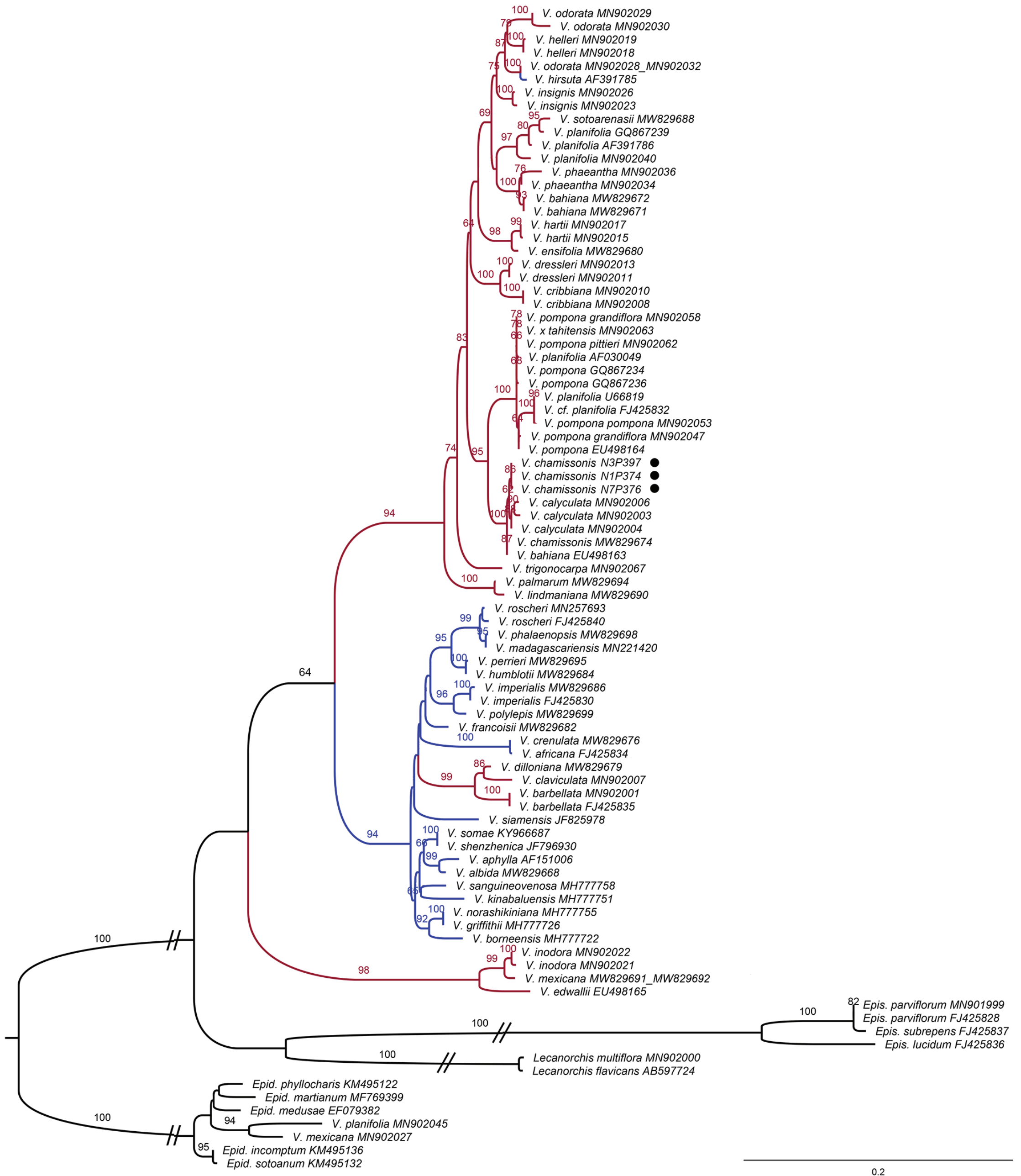


FIGURE 4. Maximum likelihood phylogram of ITS sequences from *Vanilla* species (LogL= -8010.7364). Southern, Central or Northern American species are denoted by purple branches, while blue branches indicate African or Asian species. Outgroup sequences are represented with black branches. Black dots mark the *V. chamissonis* individuals sequenced from the RNO, Argentina. Bootstrap support values > 60% are depicted above branches. The scale bar represents nucleotide substitutions per site.

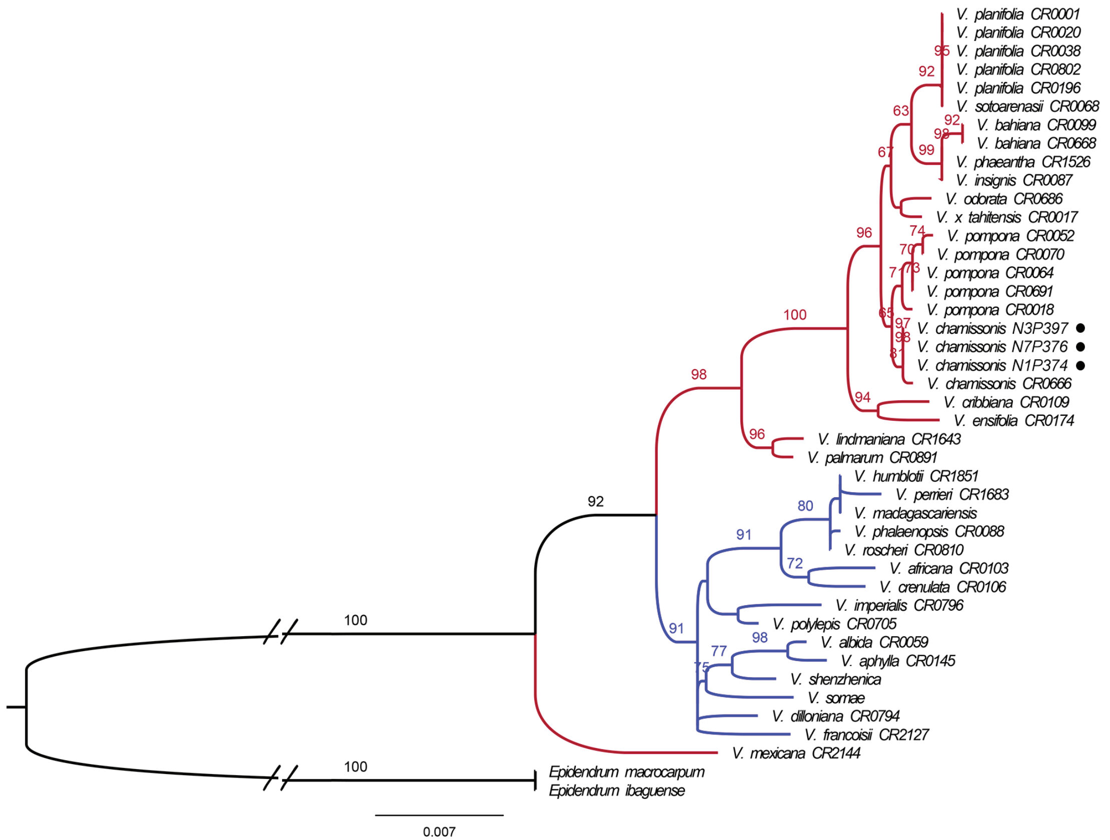


FIGURE 5. Maximum likelihood phylogram of plastid sequences *rbcL* and *matK* from *Vanilla* species (LogL= -4877.0334). Southern, Central or Northern American species are denoted by purple branches, whereas blue branches indicate African or Asian species. Outgroup sequences are represented with black branches. Black dots mark *V. chamissonis* individuals from RNO, Argentina, that were sequenced. Bootstrap support values > 60% are depicted above branches. The scale bar represents nucleotide substitutions per site.

pods. This suggests a higher fruit production rate (namely, 30%) than the rate documented by Reis *et al.* (2011). Thus, other factors may well be restricting the establishment of larger populations at RNO. For instance, the absence of suitable soil microbial communities wherever a seed is deposited (Mújica *et al.* 2009). The present study exposed the heterogeneity of the genotypes surveyed (yielding an average intraspecific genetic distance= 0.3648). This distance value is considerably higher than that reported by Verma *et al.* (2009), which estimated an overall interspecific mean distance of 0.45 among eight *Vanilla* species using ISSR markers. In addition, our investigation did not find evidence of vegetatively reproduced individuals (clones), as identical genotypes were not detected. Therefore, present results would suggest a predominance of sexually reproduced individuals, at least among the ones sampled so far.

Also, our survey allowed ascribing most individuals to one of the four distinct genetic clusters, or gene pools, with a high probability (>0.90). From a conservation standpoint, the nucleus N1 is an interesting unit because it receives input from three gene pools (DAPC clusters 1, 3, and 4); a fourth genetic input is also present but in a much lesser proportion (DAPC cluster 2; Fig. 1, 3). Furthermore, our research has revealed that a genetic discontinuity could exist between the nuclei in the northeastern sector of the RNO, where N1 is located, and the populations placed towards the southwest of the reserve (Ap-

pendix 6). Among the latter, the germplasm from N3 and N4 appear constituted by a different combination of three gene pools, and, therefore, its preservation might also be deemed valuable. As per the floristic survey of Keller *et al.* (2019), the northeastern RNO is dominated by a Myrtaceae forest, in which *Eugenia psidiiflora* O.Berg abounds. As well, the landscape features riparian "ararí" forests (*Calophyllum brasiliense* Cambess.), "pacurizales" (*Garcinia brasiliensis* Mart.), and rupicolous shrubby vegetation, although these are present in a much lesser extent. Moving southwards of RNO, the prominent plant community shifts to a xylopial forest (*Xylopia brasiliensis* Spreng.), as documented by Keller *et al.* (2019). Our findings suggest that sexual reproduction has shaped the observed genotypes among the examined plants. The distribution of this genetic variation may be attributed to pollen movement and ecto- and endozoochorous seed dispersal (Karremans *et al.* 2023, Pansarin 2021, 2022, Pansarin & Suetsugu 2022, Reis *et al.* 2011), which could be compatible with the tenuous isolation by distance and the low genetic differentiation (Phi_{PT} =0.187) evidenced among the populations. Thus, the present results highlight that the geographical proximity of *V. chamissonis* nuclei may not accurately reflect the plants' genetic closeness. Similar results were obtained for outcrossing *Zingiber* species (Huang *et al.* 2019), and for the primarily allogamous populations of seven wild *Vanilla* species from Madagascar (Andriamihaja *et al.* 2021), among other cases. According to Bory *et al.* (2008a), even rare sexual events have the potential to contribute to the genetic diversity within *Vanilla*, and diversity may play a role in succeeding by settling vegetatively. Further investigation is required to elucidate how the unique organismal communities (for instance, bats) and the distinctive topography jointly found at the RNO have shaped the historical and current distribution of *V. chamissonis*.

To attain the molecular identification of the plants under study, we employed the biparentally inherited nuclear ITS region and the maternally inherited plastid genes *rbcL* and *matK*. Pérez-Escobar *et al.* (2021) found high congruence between nuclear and organellar phylogenetic inferences in orchids. Nevertheless, due to interspecific hybridization and polyploidy in *Vanilla* (Hu *et al.* 2019), we carried out separate phylogenetic analyses to keep track of both evolutionary histories. Flanagan *et al.* (2018) highlighted the importance of the ITS region and the *matK* as barcoding loci, particularly for this genus. More recently, Besse *et al.* (2021) demonstrated that the ITS region resulted far more diverse than the plastid markers tested (namely, *matK, psaB, rbcL,* or *psbC*), and the most appropriate phylogenetic marker for the genus *Vanilla*. The ITS phylogenetic analysis presented here intertwined sequences derived from the seminal works of Soto Arenas and Dressler (2010) and Besse *et al.* (2021) (32 and 18 accessions, respectively), which, to the best of our knowledge, were not analyzed jointly before. The ML phylogram shows that the orchids from the RNO share a most recent common ancestor with *V. chamissonis* reference voucher CR0666 (Besse *et al.* 2021), *V. calyculata* (=*V. columbiana* Rolfe; Karremans *et al.* 2020) and a *V. bahiana* representative (Pansarin *et al.* 2008), with full support. Still, a 100% identity was evidenced between the Genbank sequences used here for *V. chamissonis* and *V. bahiana.* Likewise, other ITS accessions showed 100% sequence identity and formed well-supported clades. Another clade was formed by seven accessions of *V. pompona*, *V.* × *tahitensis*, and three *V. planifolia*. As to the latter three accessions, when a Blast search was performed, it showed that, in each case, the best 30 hits matched with *V. pompona* (96.53–99.41% identity; *e-value*=0.0). Similar results were obtained for the accession of *V.* × *tahitensis*. Noteworthy is the placement of *V. mexicana* MN902027 and *V. planifolia* MN902045 within the *Epidendrum* clade, as evidenced in our phylogenetic analyses. These "vanilla" ITS sequences were derived from Soto Arenas and Dressler (2010), where these suspicious sequences appeared as the sister group of the remaining *Vanilla* sequences since solely one *Epistephium* Kunth and one *Lecanorchis* Blume sequences were used as outgroups. The calling for paralogs as an explanation for that location (Soto Arenas & Dressler 2010) seems unlikely given that Blast searches with MN902027 and MN902045 as queries retrieved the first 27 best hits with *Epidendrum* accessions (99% identity; *e-value* range: $2e^{-170}$-$1e^{-152}$). An unnoticed mislabeling or even contamination with *Epidendrum* DNA could explain both results. The vouchers of these two "vanilla" accessions should be revisited to clarify their identification. Hence, it appears that several vanilla ITS accessions deposited in Genbank require careful curation.

Our findings regarding plastid markers are consistent with Besse *et al.* (2021) research, demonstrating that the variation exhibited by *matK* surpasses that of *rbcL*. In line with that work, the ITS sequences displayed the highest nucleotide variation among the three markers in the present study. As to the plants studied here, the plastid markers indicate that the organellar genome of *V. chamissonis* from Argentina may share a most recent common ancestor with the reference voucher´s sequences of *V. chamissonis*. The species compared comprise several lineages of maternally inherited plastid genes. Among these, the North, Central, and South American lineages appeared monophyletic, as do African and Asian species´ plastid genes; this clade conforms the sister group of the latter, while *V. mexicana* appears as the sister taxon to all other vanillas. This general pattern of relationships resembles that of Bouetard *et al.* (2010) based on four plastid genes and that of the ITS phylogram presented here, supporting the findings of Pérez-Escobar *et al.* (2021).

It is known that intraspecific phenotypic diversity, interspecific hybridization, and polyploidy prevail among species of *Vanilla*, possibly causing difficulties in species recognition (Bory *et al.* 2008b) and the instability of the taxonomic names associated with sequence data. This, of course, applies to *V. chamissonis* and allied species. Karremans *et al.* (2020) emphasized the necessity for in-depth investigations into living materials of *V. argentina* Hicken, *V. carinata* Rolfe, *V. chamissonis*, and *V. vellozii* Rolfe. These studies are crucial for clarifying their identity, relationships, and distribution. Similarly, further studies are needed for *V. edwallii* Hoehne and *V. rojasiana* Hoehne (=*V. angustipetala* Schltr.), previously reported in Northeastern Argentina (Johnson 2001, Soto Arenas & Cribb 2010). Unfortunately, sequence data is presently unavailable for the referred species, except for *V. chamissonis* (Genbank accessed August 2023).

Following a conservative approach, we deem it appropriate to continue considering the RNO-studied germplasm as *V. chamissonis* until more molecular data can be gathered from related species. This proposal is consistent with Biganzoli & Múlgura de Romero (2004), Munno *et al.* (2011), and M.A. Munno *et al.* (unpub. data), yet it diverges from Keller *et al.* (2019), who mentioned this orchid as *V. vellozi.* We strongly support Karremans *et al.* (2020), who highlighted the negative effects of inaccurate species identification on various biological aspects. This includes phylogenetic studies, species distribution and abundance estimation, and conservation and biodiversity assessment, all of which are affected.

Appendices

Appendix 1. Details of every nucleus of vanilla plants sampled at the Osununú Natural Reserve (Misiones, Argentina). It is indicated the nucleus identification, its corresponding Global Positioning System coordinates, the altitude (in meters above sea level), and the number of plants per nucleus, that were gathered, processed for DNA extraction, and effectively analyzed.

Nucleus	GPS coordinates	Altitude (m a.s.l.)	Number of plants		
			gathered	extracted	analyzed
N1	S 27° 16’ 46.2” W 55° 34’ 44”	188	16	14	13
N2	S 27° 16’ 48” W 55° 34’ 52”	90	6	6	5
N3	S 27° 16’ 54” W 55° 35’ 4”	85	8	8	8
N4	S 27° 17’ 0.8” W 55° 34’ 51.5”	189	20	20	15
N5	S 27° 17’ 08.0” W 55° 34’ 48.7”	177	9	9	8
N6	S 27° 16’ 46.1” W 55° 34 ‘46.5”	165	3	1	1
N7	S 27° 16’ 53.4” W 55° 34’ 43”	164	3	2	2
N8	S 27° 16’ 48.3” W 55° 34’ 48.9”	120	7	7	7
		Total	72	67	59

Appendix 2. Details of the Genbank accession numbers of ITS, *rbcL* and *matK* sequences of *Vanilla* and outgroup species used for the molecular characterization of vanilla´s germplasm from the Osununú Natural Reserve (Misiones, Argentina).

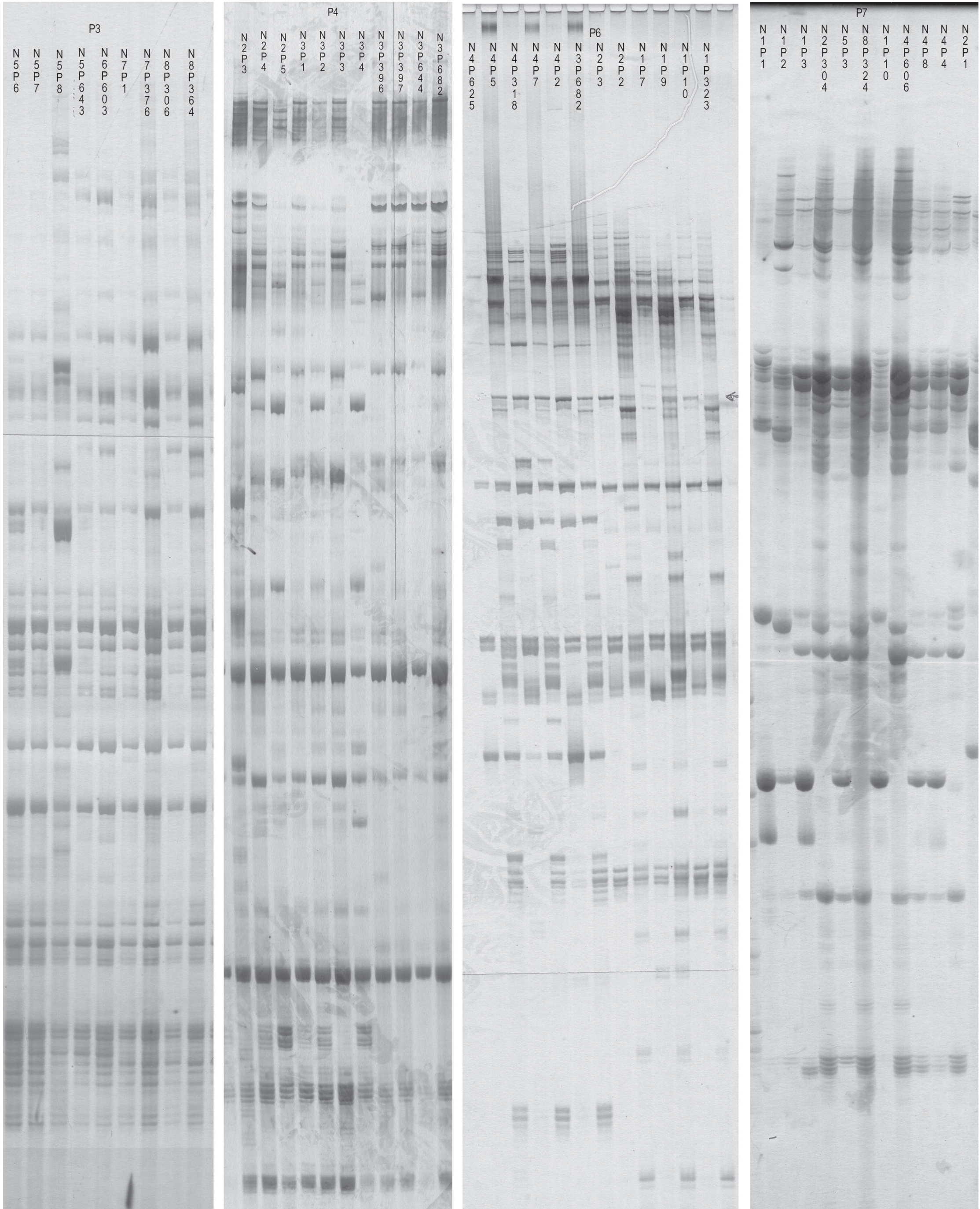
Species	**Genbank accession numbers**			**Code** [a]	**Origin** [b]
	ITS	***rbcL***	***matK***		
Vanilla africana Lindl.	FJ425834	FN545544	MW828231	CR0103	Asia/Africa
V. albida Blume	MW829668	MW828165	MW828232	CR0059	Asia/Africa
V. aphylla Blume	AF151006	MW828166	MW828233	CR0145	India
V. bahiana Hoehne	EU498163 MW829671 MW829672	MW828168 MW828169	MW828236 MW828237	CR0099 CR0668	Tropical South America
V. barbellata Rchb.f.	FJ425835 MN902001	--	--		Caribbean
V. borneensis Rolfe	MH777722	--	--		Southeastern Asia
V. calyculata Schltr.	MN902003 MN902004 MN902006	--	--		Southern North America and Central America
V. chamissonis Klotzsch	MW829674	FN545563	MW828238	CR0666	Tropical South America
V. claviculata Sw.	MN902007	--	--		Caribbean
V. crenulata Rolfe	MW829676	MW828170	MW828240	CR0106	Asia/Africa
V. cribbiana Soto Arenas	MN902008 MN902010	FN545546	MW828241	CR0109	Southern North America and Central America
V. dilloniana Correll	MW829679	FN545573	MW828243	CR0794	Caribbean
V. dressleri Soto Arenas	MN902011 MN902013	--	--		Central America and northern South America
V. edwallii Hoehne	EU498165	--	--		Tropical South America
V. ensifolia Rolfe	MW829680	FN545557	MW828244	CR0174	Northern South America
V. françoisii H.Perrier	MW829682	MW828172	MW828246	CR2127	Africa (Madagascar)
V. griffithii Rchb.f.	MH777745 MH777726	--	--		Southeastern Asia
V. hartii Rolfe	MN902015 MN902017	--	--		Southern North America and Central America
V. helleri A.D.Hawkes	MN902018 MN902019	--	--		Southern North America and Central America
V. hirsuta M.A.Clem. et D.L.Jones	AF391785	--	--		Oceania

V. humblotii Rchb.f.	MW829684	MW828173	MW828248	CR1851	Asia/Africa
V. imperialis Kraenzl.	FJ425830 MW829686	MW828174	MW828250	CR0796	Asia/Africa
V. inodora Schiede	MN902021 MN902022	--	--		Southern North America and Central America
V. insignis Ames	MN902023 MN902026	FN545537	MW828251	CR0087	Southern North America and Central America
V. kinabaluensis Carr	MH777751	--	--		Southeastern Asia
V. lindmaniana Kraenzl.	MW829690 MW829689	MW828175	MW828254	CR1643	America
V. madagascariensis Rolfe	MN221420	NC_046809*	NC_046809*	--	Asia/Africa
V. mexicana Mill.	MN902027 MW829691 MW829692	MW828177	MW828256	CR2144	Caribbean and northern South America
V. norashikiniana R.Go et A.Rafii	MH777755	--	--		Southeastern Asia
V. odorata C.Presl.	MN902028 MN902029 MN902030 MN902032	FN545567	MW828257	CR0686	Southern North America, Central America and South America
V. palmarum (Salzm. ex Lindl.) Lindl.	MW829694	MW828178	MW828258	CR0891	South America
V. perrieri Schltr.	MW829695	MW828181	MW828261	CR1683	Africa (Madagascar)
V. phaeantha Rchb.f.	MN902034 MN902036	MW828183	MW828263	CR1526	Southern North America and Central America
V. phalaenopsis Rchb.f.	MW829698	MW828184	MW828264	CR0088	Asia/Africa
V. planifolia Andrews	MN902045 AF030049 AF391786 GQ867239 MN902040 U66819	MW828163 MW828160 MW828161 MW828162 MW828164	MW828229 MW828226 MW828227 MW828228 MW828230	CR0196 CR0001 CR0020 CR0038 CR0802	Central America
V. cf. *planifolia*	FJ425832	--	--		
V. pompona Schiede	GQ867234 GQ867236 EU498164 MN902053	MW828187 MW828190	MW828268 MW828272	CR0064 CR0691	Southern North America
V. pompona subsp. *grandiflora* (Lindl.) Soto Arenas	MN902058 MN902047	MW828186 MW828188	MW828267 MW828269	CR0052 CR0070	Northeastern South America
V. pompona subsp. *pittieri* (Schltr.) Dressler	MN902062	MW828185	MW828266	CR0018	Central America
V. polylepis Summerh.	MW829699	FN545572	MW828265	CR0705	Asia/Africa
V. roscheri Rchb.f.	FJ425840 MN257693	FN545574	MW828274	CR0810	Asia/Africa

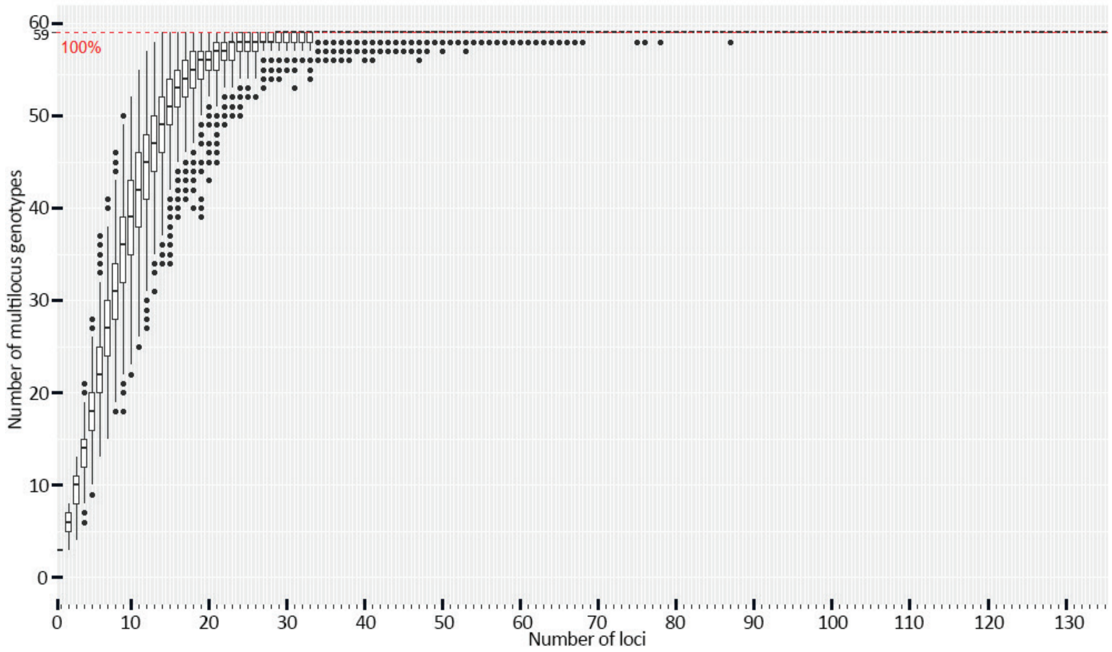
V. sanguineo-venosa Go et Rafii	MH777758	--	--		Southeast Asia
V. shenzhenica Z.J.Liu et S.C.Chen	JF796930	MK962478*	MK962478*		Asia
V. siamensis Rolfe ex Downie	JF825978	--	--		Asia
V. somae Hayata	KY966687	NC_079955*	NC_079955*		Asia
V. sotoarenasii M.Pignal	MW829688	FN545534	MW828252	CR0068	Central America
V. × *tahitensis* J.W. Moore	MN902063	MW828191	MW828275	CR0017	---
V. trigonocarpa Hoehne	MN902067	--	--		Northern South America
Lecanorchis flavicans Fukuy.	AB597724	--	--		---
L. multiflora J.J.Sm.	MN902000	--	--		---
Epistephium subrepens Hoehne	FJ425837	--	--		---
Epis. parviflorum Lindl.	MN901999 FJ425828	--	--		---
Epis. lucidum Cogn.	FJ425836	--	--		---
Epidendrum medusae (Rchb.f.) Pfitzer	EF079382	--	--		---
Epid. phyllocharis Rchb.f.	KM495122	--	--		---
Epid. martianum Lindl.	MF769399	--	--		---
Epid. sotoanum Karremans & Hágsater	KM495132	--	--		---
Epid. incomptum Rchb.f.	KM495136	--	--		---
Epid. macrocarpum Rich.	--	MH218792	MH218773	--	
Epid. ibaguense	--	MH218791	MH218772	--	

[a] Code number from the Biological Resources Center (BRC) Vatell *Vanilla* collection; the codes correspond to the voucher specimens from which the plastid sequences were obtained, as indicated in Genbank. [b] Geographic distribution data were compiled from the literature (Bouetard *et al.* 2010, Karremans *et al.* 2020, Soto Arenas & Cribb 2010, Soto Arenas & Dressler 2010). * the gene was extracted from the complete plastome.

Appendix 3. Examples of ISSR genotypes produced by primers ISSR-P3, ISSR-P4, ISSR-P6 and ISSR-P7, and obtained by means of PAGE and silver staining (see text for details). In each lane, the individual loaded is indicated by its identification number.



Appendix 4. Genotype accumulation curve. Number of multilocus genotypes (individuals) vs. number of loci sampled. Box plots were constructed from the observed genotypes considering a 1000 bootstrap pseudoreplicates.

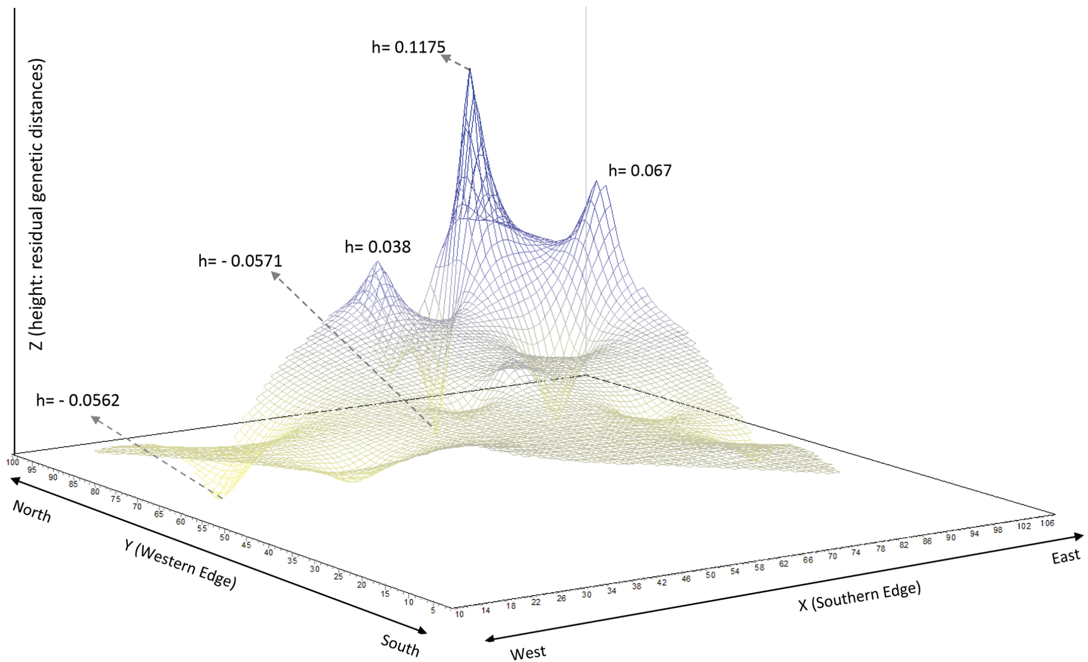


Appendix 5. Average genetic distances (complementary of Dice index) estimated within and among populations (N1-N8), and geographic distances (in meters) between populations.

	N1	N2	N3	N4	N5	N7	N8
N1	**0.4197**	225	604	503	696	235	154
N2	0.3729	**0.2498**	388	412	645	306	89
N3	0.3935	0.2725	**0.2159**	404	604	577	450
N4	0.4167	0.3358	0.3313	**0.3216**	236	326	394
N5	0.4304	0.3563	0.3609	0.3686	**0.3282**	478	607
N7	0.4354	0.3522	0.3127	0.364	0.3459	**0.3443**	227
N8	0.4404	0.3265	0.292	0.3574	0.4013	0.294	**0.2306**

In the diagonal, values (in bold) represent average genetic distances estimated within each nucleus of vanilla plants. Below the diagonal, values are between-nuclei average genetic distances. Above the diagonal, pairwise approximate geographic distances, estimated with Google Earth. The single plant from N6 was not considered here.

Appendix 6. Genetic Landscape Shape interpolation plot. The x- and y-axes represent the sampling area, and the z-axis depicts the residual genetic distances (h, height). Negative peaks and valleys depict areas of high genetic similarities, whereas positive peaks represent areas of genetic discontinuities.



Appendix 7. Details of the ten phylogenetic runs and of each resulting phylogram. Initial random seed (Rseed) of each run, log likelihood value (logL), and Bayesian and Akaike information criteria values (BIC and AIC, respectively) for the corresponding phylogram. In bold type, the best runs selected.

	Nuclear matrix (ITS)				**Plastid matrices *rbcL* and *matK***			
Run	**Rseed**	**logL**	**BIC**	**AIC**	**Rseed**	**logL**	**BIC**	**AIC**
0	32425	-8010.7972	17227.657	16385.5944	**252815**	**-4877.0334**	**10503.9232**	**9950.0669**
1	247024	-8011.2201	17228.5028	16386.4402	902305	-4877.0357	10503.9277	9950.0713
2	728175	-8011.2751	17228.6128	16386.5502	991013	-4877.0337	10503.9237	9950.0674
3	524613	-8011.2623	17228.5873	16386.5246	423930	-4877.0406	10503.9376	9950.0813
4	447898	-8011.2606	17228.5837	16386.5211	508043	-4877.0338	10503.9240	9950.0676
5	666861	-8011.4323	17228.9273	16386.8647	814976	-4877.0336	10503.9236	9950.0672
6	586907	-8011.4503	17228.9632	16386.9006	**762657**	**-4877.0334**	**10503.9232**	**9950.0669**
7	**522341**	**-8010.7364**	**17227.5353**	**16385.4727**	133019	-4877.0354	10503.9271	9950.0708
8	338432	-8011.2155	17228.4937	16386.4311	862700	-4877.0369	10503.9301	9950.0737
9	438538	-8011.2616	17228.5857	16386.5231	980979	-4877.0389	10503.9342	9950.0778

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LITERATURE CITED

Andriamihaja, C. F., Ramarosandratana, A. V., Grisoni, M., Jeannoda, V. H. & Besse, P. (2021). Drivers of population divergence and species differentiation in a recent group of indigenous orchids (*Vanilla* spp.) in Madagascar. *Ecology and Evolution*, 11(6), 2681–2700. doi: 10.1002/ece3.7224

Besse, P., Da Silva, D. & Grisoni, M. (2021). Plant DNA barcoding principles and limits: A case study in the genus *Vanilla*. *Methods in Molecular Biology*, 2222, 131–148. doi: 10.1007/978-1-0716-0997-2_8

Biganzoli, F. & Múlgura de Romero, M. E. (2004). Inventario Florístico del Parque Provincial Teyú Cuaré y alrededores (Misiones, Argentina). *Darwiniana*, 42(1–4), 1–24.

Bonin, A., Bellemain, E., Bronken Eidesen, P., Pompanon, F., Brochmann, C. & Taberlet, P. (2004). How to track and assess genotyping errors in population genetics studies. *Molecular Ecology*, 13(11), 3261–3273. doi: 10.1111/j.1365-294X.2004.02346.x

Bory, S., Da Silva, D., Risterucci, A. M., Grisoni, M., Besse, P. & Duval, M. F. (2008a). Development of microsatellite markers in cultivated vanilla: Polymorphism and transferability to other *Vanilla* species. *Scientia horticulturae*, 115(4), 420–425. doi: 10.1016/j.scienta.2007.10.020

Bory, S., Catrice, O., Brown, S., Leitch, I. J., Gigant, R., Chiroleu, F., Grisoni, M., Duval, M. F. & Besse, P. (2008b). Natural polyploidy in *Vanilla planifolia* (Orchidaceae). *Genome*, 51(10), 816–826. doi: 10.1139/G08-068

Bouetard, A., Lefeuvre, P., Gigant, R., Bory, S., Pignal, M., Besse, P. & Grisoni, M. (2010). Evidence of transoceanic dispersion of the genus *Vanilla* based on plastid DNA phylogenetic analysis. *Molecular Phylogenetics and Evolution*, 55(2), 621–630. doi: doi.org/10.1016/j.ympev.2010.01.021

Bramel, P. & Frey, F. (2021). *Global strategy for the conservation and use of Vanilla genetic resources*. Bonn: Global Crop Diversity Trust.

Cameron, K. (2011). *Vanilla orchids: natural history and cultivation*. Portland-London: Timber Press.

Cascales, J., Bracco, M., Poggio, L. & Gottlieb, A. M. (2014). Genetic diversity of wild germplasm of "yerba mate" (*Ilex paraguariensis* St. Hil.) from Uruguay. *Genetica*, 142(6), 563–573. doi: 10.1007/s10709-014-9804-3

CITES. (2022). Convention on International Trade in Endangered Species of Wild Fauna and Flora. Retrieved from https://checklist.cites.org/ Accessed December 2022.

da Silva Oliveira, J. P., Garrett, R., Bello Koblitz, M. G. & Furtado Macedo, A. (2022). *Vanilla* flavor: Species from the Atlantic Forest as natural alternatives. *Food Chemistry*, 375, 131891. doi: 10.1016/j.foodchem.2021.131891

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, 32(5), 1792–1797. doi: 10.1093/nar/gkh340

Excoffier, L., Smouse, P. E. & Quattro J. M. (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, 131(2), 479–491. doi: 10.1007/s00424-009-0730-7

Flanagan, N. S., Chavarriaga, P. & Mosquera-Espinosa, A. T. (2018). Conservation and sustainable use of *Vanilla* crop wild relatives in Colombia. In: D. Havkin-Frenkel & F. Belanger (Eds.), *Handbook of Vanilla science and technology* (pp. 85–110). Singapore: Wiley-Blackwell.

George, S., Sharma, J. & Yadon, V. L. (2009). Genetic diversity of the endangered and narrow endemic *Piperia yadonii* (Orchidaceae) assessed with ISSR polymorphisms. *American Journal of Botany*, 96(11), 2022–2030. doi: 10.3732/ajb.0800368

Gigant, R.L., Brugel, A., De Bruyn, A., Risterucci, A.-M., Guiot, V., Viscardi, G., Humeau, L., Grisoni, M. & Besse, P. (2012). Nineteen polymorphic microsatellite markers from two African *Vanilla* species: across-species transferability and diversity in a wild population of *V. humblotii* from Mayotte. Conservation Genetics Resources, 4(1),121–125. doi: 10.1007/s12686-011-9489-1

Gigant, R. L., Rakotomanga, N., Goulié, C., Da Silva, D., Barre, N., Citadelle, G., Silvestre, D., Grisoni, M. & Besse, P. (2016). Microsatellite markers confirm self-pollination and autogamy in wild populations of *Vanilla mexicana* Mill. (syn. *V. inodora*) (Orchidaceae) in the Island of Guadeloupe. In: I. Y. Abdurakhmonov (Ed.), *Microsatellite markers* (pp. 73–93). London: InTechOpen.

Guindon, S., Dufayard, J. F., Lefort, V., Anisimova, M., Hordijk, W. & Gascuel, O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. *Systematic Biology*, 59(3), 307–321. doi: 10.1093/sysbio/syq010

Hall, T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series,* 41, 95-98.

Hoang, D. T., Chernomor, O., Von Haeseler, A., Minh, B. Q. & Vinh, L. S. (2018). UFBoot2: Improving the ultrafast bootstrap approximation. *Molecular Biology and Evolution*, 35(2), 518–522. doi: 10.1093/molbev/msx281

Hou, B., Tian, M., Luo, J., Ji, Y., Xue, Q. & Ding, X. (2012). Genetic diversity assessment and ex situ conservation strategy of the endangered *Dendrobium officinale* (Orchidaceae) using new trinucleotide microsatellite markers. *Plant Systematics and Evolution*, 298(8), 1483–1491. doi: 10.1007/s00606-012-0651-3

Huang, R., Chu, Q. H., Lu, G. H. & Wang, Y. Q. (2019). Comparative studies on population genetic structure of two closely related selfing and outcrossing *Zingiber* species in Hainan Island. *Scientific Reports*, 9(1), 17997. doi: 10.1038/s41598-019-54526-y

Hu, Y., Resende, M. F. R., Bombarely, A., Brym, M., Bassil, E. & Chambers, A. H. (2019). Genomics-based diversity analysis of *Vanilla* species using a *Vanilla planifolia* draft genome and Genotyping-By-Sequencing. *Scientific Reports*, 9, 3416. https://doi.org/10.1038/s41598-019-40144-1

IUCN. (2022). The IUCN Red List of Threatened Species [online]. Available from https://www.iucnredlist.org/en [Accessed 14 Dec 2022].

Johnson, A. E. (2001). *Las orquídeas del Parque Nacional Iguazú*. Buenos Aires: L. O. L. A.

Jombart, T., Devillard, S. & Balloux, F. (2010). Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. *BMC Genetics*, 11(1), 94. doi: 10.1186/1471-2156-11-94

Jombart, T. & Collins, C. (2015). A tutorial for Discriminant Analysis of Principal Components (DAPC) using adegenet 2.0.0. [online]. Available from http://adegenet.r-forge.r-project.org/files/tutorial-dapc.pdf [Accessed 10 Aug 2022].

Jørgensen, P.M., Nee, M.H. & Beck., S.G. (eds.) (2013). Catálogo de las plantas vasculares de Bolivia. *Monographs in Systematic Botany from the Missouri Botanical Garden*, 127, 1-1741. Missouri Botanical Garden.

Kalyaanamoorthy, S., Minh, B. Q., Wong, T. K. F., Von Haeseler, A. & Jermiin, L. S. (2017). ModelFinder: Fast model selection for accurate phylogenetic estimates. *Nature Methods*, 14(6), 587–589. doi: 10.1038/nmeth.4285

Kamvar, Z.N., Brooks, J.C. & Grünwald, N.J. (2015). Novel R tools for analysis of genome-wide population genetic data with emphasis on clonality. *Frontiers in Genetics* 6: 208. doi:10.3389/fgene.2015.00208, https://doi.org/10.3389/fgene.2015.00208.

Kamvar, Z.N., Tabima, J.F.& Grünwald, N.J. (2014) Poppr: an R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. *PeerJ* 2:e281.<doi:10.7717/peerj.281>

Karremans, A. P., Chinchilla, I. F., Rojas-Alvarado, G., Cedeño-Fonseca, M., Damián, A. & Léotard, G. (2020). A reappraisal of neotropical *Vanilla*. With a note on taxonomic inflation and the importance of alpha taxonomy in biological studies. *Lankesteriana*, 20(3), 395–497. doi: 10.15517/lank.v20i3.45203

Karremans, A. P., Bogarín, D., Otárola, M. F., Sharma, J., Watteyn, C., Warner, J., Rodríguez Herrera, B., Chinchilla, I. F., Carman, E., Rojas Valerio, E., Pillco Huarcaya, R., & Whitworth, A. (2023). First evidence for multimodal animal seed dispersal in orchids. *Current Biology,* 33(2), 364-371.e3. doi:10.1016/j.cub.2022.11.041.

Keller, H. A., Keller, K. M. & Rojas, L. J. (2019). *Estudio florístico de la Reserva Natural Osununú (San Ignacio Misiones)*.

Ambientes, riqueza, especies con alto valor de conservación y plantas invasoras. Misiones: FCF-UnaM.

Kumar, S., Stecher, G. & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, 33(7), 1870–1874. doi: 10.1093/MOLBEV/MSW054

Legendre, P. & Legendre, L. (1998). *Numerical ecology*. Amsterdam: Elsevier.

Lopes, A. B., Silva, M. M. & Ferreira Melo Júnior, J. C. (2019). Estratégias funcionais de *Vanilla chamissonis* (Orchidaceae) em ambiente arbustivo e florestal de restinga. *Revista Brasileira de Geografia* Física, 12(2), 355–364. doi: 10.26848/rbgf.v12.2.p355-364

Ma, J.-M. & Yin, S.-H. (2009). Genetic diversity of *Dendrobium fimbriatum* (Orchidaceae), an endangered species, detected by inter-simple sequence repeat (ISSR). *Acta Botanica Yunnanica*, 31(1), 35–41. doi: 10.3724/sp.j.1143.2009.08076

Miller, M. P. (2005). Alleles In Space (AIS): Computer software for the joint analysis of interindividual spatial and genetic information. *The Journal of Heredity*, 96(6), 722–724. doi: doi.org/10.1093/jhered/esi119

Minh, B. Q., Schmidt, H. A., Chernomor, O., Schrempf, D., Woodhams, M. D., Von Haeseler, A. & Lanfear, R. (2020). IQ-TREE 2: New models and efficient methods for phylogenetic inference in the genomic era. *Molecular Biology and Evolution*, 37(5), 1530–1534. doi: 10.1093/molbev/msaa015

Mújica, E., Raventós, J. & González, E. (2009). Análisis de la selección de sustrato por parte de *Dendrophylax lindenii* (Orchidaceae) en Cabo San Antonio, Península de Guanahacabibes, Pinar del Río, Cuba. *Lankesteriana*, 9(3), 533–540. doi: 10.15517/lank.v0i0.12116

Munno, M. A., Espíndola, M. & Flores, R. (2011). Estudio de poblaciones de *Vanilla chamissonis* (Klotzsch) var. *chamissonis* en la Reserva Natural Osununú. In: M. E. Rodríguez (Ed.), *Boletín de la Sociedad Argentina de Botánica*. *Proceedings of the XXXIII Jornadas Argentinas de Botánica* (pp. 239–240). Córdoba: Sociedad Argentina de Botánica.

Nguyen, L. T., Schmidt, H. A., Von Haeseler, A. & Minh, B. Q. (2015). IQ-TREE: A fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Molecular Biology and Evolution*, 32(1), 268–274. doi: 10.1093/molbev/msu300

Paiva, D. I., Cascales, J., Rosetti, M. E. N., Scherer, R. A., Gauchat, M. E. & Gottlieb, A. M. (2020). Unraveling the genetic complexity of a cultivated breeding population of “yerba mate” (*Ilex paraguariensis* St. Hil.). *Anais da Academia Brasileira de Ciencias*, 92, e20190113. doi: 10.1590/0001-3765202020190113

Pansarin, E. R. (2021). Unravelling the Enigma of Seed Dispersal in *Vanilla*. *Plant Biology,* 23, 974–80. doi:10.1111/plb.13331

Pansarin, E. R. (2022). *Vanilla* flowers: Much more than food-deception. *Botanical Journal of the Linnean Society*, 198(1), 57–73. doi: 10.1093/botlinnean/boab046

Pansarin, E. R. & Suetsugu, K. (2022). Mammal-mediated seed dispersal in *Vanilla*: Its rewards and clues to the evolution of fleshy fruits in orchids. *Ecology*, 103. Doi: 10.1002/ecy.3701

Pansarin, E. R., Salatino, A. & Salatino M. L. F. (2008). Phylogeny of South American Pogonieae (Orchidaceae, Vanilloideae) based on sequences of nuclear ribosomal (ITS) and chloroplast (*psaB, rbcL, rps16*, and *trnL-F*) DNA, with emphasis on *Cleistes* and discussion of biogeographic implications. *Organisms, Diversity & Evolution,* 8, 171–181. doi:10.1016/j.ode.2007.09.003

Peakall, R. & Smouse, P. E. (2012). GenALEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics*, 28(19), 2537–2539. doi: 10.1093/bioinformatics/bts460

Pérez-Escobar, O. A., Dodsworth, S., Bogarín, D., Bellot, S., Balbuena, J. A., Schley, R. J., Kikuchi, I. A., Morris, S. K., Epitawalage, N., Cowan, R., Maurin, O., Zuntini, A., Arias, T., Serna-Sánchez, A., Gravendeel, B., Torres Jimenez, M. F., Nargar, K., Chomicki, G., Chase, M. W., Leitch, I. J., Forest, F. & Baker, W. J. (2021). Hundreds of nuclear and plastid loci yield novel insights into orchid relationships. *American Journal of Botany*, 108(7), 1166–1180. doi: 10.1002/ajb2.1702

Qian, X., Li, Q. J., Liu, F., Gong, M. J., Wang, C. X. & Tian, M. (2014). Conservation genetics of an endangered lady’s slipper orchid: *Cypripedium japonicum* in China. *International Journal of Molecular Sciences*, 15(7), 11578–11596. doi: 10.3390/ijms150711578

Qian, X., Wang, C.-X. & Tian, M. (2013). Genetic diversity and population differentiation of *Calanthe tsoongiana*, a rare and endemic orchid in China. *International Journal of Molecular Sciences*, 14(10), 20399–20413. doi: 10.3390/ijms141020399

R Core Team. (2022). R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. Retrieved from https://www.r-project.org/.

R Core Team. (2023). R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. Retrieved from https://www.r-project.org/.

Rambaut, A. (2016). FigTree v1.4.3 software. Institute of Evolutionary Biology, University of Edinburgh. Retrieved from http://tree.bio.ed.ac.uk/software/figtree/

Reis, C. A. M., Brondani, G. E. & De Almeida, M. (2011). Biologia floral, reprodutiva e propagação vegetativa de baunilha. *Scientia Agraria Paranaensis*, 10(1), 69–82. doi: 10.1818/sap.v10i1.5271

Roldán-Ruiz, I., Dendauw, J., Van Bockstaele, E., Depicker, A. & De Loose, M. (2000). AFLP markers reveal high polymorphic rates in ryegrass (*Lolium* spp.). *Molecular Breeding*, 6, 125–134. doi: 10.1023/A:1009680614564

Schlüter, P. M. & Harris, S. A. (2006). Analysis of multilocus fingerprinting data sets containing missing data. *Molecular Ecology Notes*, 6(2), 569–572. doi: 10.1111/j.1471-8286.2006.01225.x

Shimodaira, H. (2002). An approximately unbiased test of phylogenetic tree selection. *Systematic Biology*, 51(3), 492–508. doi: 10.1080/10635150290069913

Shimodaira, H. & Hasegawa, M. (1999). Multiple comparisons of log-likelihoods with applications to phylogenetic inference. *Molecular Biology and Evolution*, 16(8), 1114–1116. doi: 10.1093/oxfordjournals.molbev.a026201

Soltis, P. S., Soltis, D. E. & Smiley, C. J. (1992). An *rbcL* Sequence from a Miocene *Taxodium* (Bald Cypress). *Proceedings of the National Academy of Sciences of the United States of America*, 89(1), 449-451

Soto Arenas, M. A. (2003). *Vanilla*; tratamiento genérico. In: A. M. Pridgeon, P. M. W. Chase & F. N. Rasmussen (Eds.), *Genera Orchidacearum, 3. Orchidoideae (Part two), Vanilloideae* (pp. 321-334). Oxford: Oxford University Press.

Soto Arenas, M. A. (2006). La vainilla: retos y perspectivas de su cultivo. *Biodiversitas*, 66(2), 2–9.

Soto Arenas, M. A. & Cribb, P. (2010). A new infrageneric classification and synopsis of the genus *Vanilla* Plum. ex Mill. (Orchidaceae: Vanillinae). *Lankesteriana*, 9(3), 355–398. doi: doi.org/10.15517/lank.v0i0.12071

Soto Arenas, M. A. & Dressler, R. L. (2010). A revision of the Mexican and Central American species of *Vanilla plumier* ex Miller with a characterization of their ITS region of the nuclear ribosomal DNA. *Lankesteriana*, 9(3), 285–354. doi: 10.15517/lank.v0i0.12065

Szlachetko, D.L. (ed.) (2016). Orchids of the Guianas (Guyana, Suriname, French Guiana), 1, 1-461. *Koeltz Botanical Books*

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M. & Rozen, S. G. (2012). Primer3--new capabilities and interfaces. *Nucleic Acids Research* 40(15), e115

Velazco, S.J.E., Galvao, F., Keller, H.A., & Bedrij, N.A. (2015). Floristica e Fitossociologia de uma Floresta Estacional Semidecidual, Reserva Privada Osununú-Misiones, Argentina. *Floresta e Ambiente*, 22(1),1-12. Doi:10.1590/2179-8087.038513

Verma, P. C., Chakrabarty, D., Jena, S. N., Mishra, D. K., Singh, P. K., Sawant, S. V. & Tuli, R. (2009). The extent of genetic diversity among *Vanilla* species: Comparative results for RAPD and ISSR. *Industrial Crops and Products*, 29(2–3), 581–589. doi: 10.1016/j.indcrop.2008.11.006

White, T. J., Bruns, T., Lee, S. & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White (Eds.), *PCR protocols: a guide to methods and applications* (pp. 315–322). New York: Academic Press, Inc.

Zietkiewicz, E., Rafalski, A. & Labuda, D. (1994). Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics*, 20(2), 176–183. doi: 10.1006/geno.1994.1151