Quest Journals
Journal of Research in Agriculture and Animal Science
Volume 11 ~ Issue 6 (2024) pp: 14-18
ISSN(Online) : 2321-9459
www.questjournals.org

Research Paper

Morphological and Molecular Characterization of Five Morphotypes of *Vanilla planifolia*

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ABSTRACT: *Vanilla, Vanilla planifolia Jacks ex Andrews, is a plant native to Mesoamerica. Its processed fruit has been used since pre-Hispanic times as a flavoring and in medicinal uses by the Aztecs, Mayas, and Totonacs. Today, it is highly valued in the international market. At the Ixtacuaco Experimental Field (CEIXTA) of National Institute of Forestry, Agricultural, and Livestock Research (INIFAP), located in the municipality of Tlapacoyan, Veracruz, there is a vanilla germplasm bank containing rescued genetic material. The objective of this study was to characterize phenotypically and molecularly five morphotypes of Vanilla planifolia ("Mansa", "Rayada", "Oreja de Burro", "Polen Duro", and "Chinanteca"). The "Mansa" morphotype is the one commercially cultivated in Mexico; however, other plant types with undesirable characteristics for producers can be found within plantations. For example, the "Rayada" is very susceptible to diseases, and the "Oreja de Burro" aborts its fruits between 42 and 60 days after manual pollination. Sometimes, due to lack of knowledge, producers have bought and planted propagation material of "Oreja de Burro" only to realize the fruit abortion problem three years later when production begins. The morphotypes "Polen Duro" and "Chinanteca" were recently discovered in the Totonacapan region in Papantla, Veracruz, and in Chinantla region in San Felipe Usila, Oaxaca, respectively. Molecular characterization results through DNA amplification by PCR and sequencing of two gene regions [chloroplast gene matK (maturase K) and the ITS region of rRNA] indicated genetic differences among the five morphotypes. However, other molecular markers such as RAPD or ISSR need to be employed to explain this existing variation.*

KEYWORDS: *matK, ITS, morphotypes, diversity*

R*eceived 03 June, 2024;* ***R****evised 12 June, 2024;* ***A****ccepted 14 June, 2024 © The author(s) 2024.*
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I. INTRODUCTION

Biodiversity in cultivated areas depends on the level of originally introduced variation and on the cultivation practices used during domestication. Due to the way vanilla has been managed, low levels of genetic diversity are expected [1]. However, Mexican producers, in addition to the cultivated vanilla known as "Mansa," recognize other types in *V. planifolia* such as "Oreja de Burro", "Rayada" or "Variegata", "Acamaya", "Polen Duro", and "Chinanteca". These may represent genetically different individuals referred to as clones, morphotypes, cultivars, or landraces, which have not been manipulated or genetically improved in Mexico. "Oreja de Burro" can occasionally be found in the plantations of Papantla, Veracruz, and is difficult to distinguish from "Mansa" vanilla except by experienced producers. This cultivar aborts 80 to 100% of its fruits three months after manual self-pollination due to genetic self-incompatibility [2, 3]. "Mansa" shares the same morphological characteristics with "Oreja de Burro," but with significant differences in leaf width, stem diameter, and fruit length [4]. Lubinsky (2003) indicates that there is no scientific evidence to determine if these vanilla varieties are clones within the same species or hybrids resulting from crosses between close relatives [5]. There appear to be no genetic differences between "Mansa" and "Rayada" analyzed with molecular markers, suggesting that "Rayada" may be a somatic mutation [6, 7]. However, a more recent study suggests that "Rayada" is not the result of a mutation, but rather sexual reproduction between "Mansa" and a wild species, or an epigenetic phenomenon. A thorough study is needed to determine the origin of the "Rayada" type [8].

Bory et al. (2008) mention four hypotheses to explain the origin of morphotypes in cultivated areas: 1) the existence of different introduction events, 2) the accumulation of somatic mutations through vegetative

propagation, 3) the possible effect of sexual recombination through pollination, and 4) the existence of epigenetic phenomena [9]. Additionally, polyploidization could explain the morphological variation in cultivated species [10] (Duval et al., 2006). It is important to inform producers about the characteristics of morphotypes because, in our country, there is no institution or company that offers propagation material guaranteeing the purity of the recommended variety for planting ("Mansa"). In commercial plantations where cuttings are obtained to establish new crops, they are mixed with other undesirable types such as "Oreja de Burro", leading to failures for some producers who unknowingly acquired and used this genetic material [3, 11, 12]. Therefore, the objective of this study was to phenotypically and molecularly characterize five morphotypes of *Vanilla planifolia* ("Mansa", "Rayada", "Oreja de Burro", "Polen Duro", and "Chinanteca") preserved in the vanilla germplasm bank at CEIXTA.

II. MATERIALS AND METHODS

Collection of Vanilla Morphotypes

The vanilla morphotypes "Mansa", "Rayada", "Oreja de Burro", "Polen Duro", and "Chinanteca" were collected between 2017 and 2019 from the plots of producers in the municipalities of Papantla, Tihuatlán, and Nautla in the state of Veracruz, and in San Felipe Usila, Oaxaca. These collections are preserved in the vanilla germplasm bank located at CEIXTA (20°18'37" N and 96°59'29" W, 105 masl), km 4.5, Martínez de la Torre-Tlapacoyan road, Veracruz. The general characteristics of the different morphotypes were described using the UPOV document [13] as a guide and complemented with reports from other researchers [2, 6, 11]. Observations were also made on the rescued clones regarding tolerance to pests, diseases, and drought, flowering period, fruit quality, among other attributes.

Extraction of Genomic DNA from Vanilla

Young leaves from the apical region of two different plants of each of the five morphotypes were collected in plastic bags and stored at 4°C in the CEIXTA Laboratory until processing. Before DNA extraction, the material was washed with a 1% sodium hypochlorite solution followed by 70% ethanol for one minute each. A 9 mm disc was taken using a hole punch and placed in a mortar where it was ground with liquid nitrogen, and the ground tissue was transferred to a 2 ml microtube. DNA extraction was performed using the CTAB protocol with some modifications [14, 15].

PCR Amplification and Sequencing of *matK* and 18S rRNA

Partial sequences of the chloroplast gene *matK* (maturase K) and the ITS region of rRNA (ITS1 + 5.8S + ITS2) were amplified by PCR using the primers matK743F and matK1520R for *matK*, and AB101F and AB102R for ITS [16]. PCR reactions were performed in a thermal cycler (Thermo Scientific, MiniAmp Plus, USA) with a final volume of 50 µl. The PCR conditions for *matK* were 94 °C for 2 min, 40 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 45 s, finishing with a step at 72 °C for 10 min. The PCR conditions for ITS were 94 °C for 2 min, 35 cycles at 94 °C for 1 min, 59 °C for 1 min, and 72 °C for 1 min, finishing with a step at 72 °C for 7 min. PCR products were analyzed by electrophoresis on 1.5% agarose gels. The DNA bands of interest were cut and purified using a commercial kit (Wizard SV Gel and PCR Clean-Up System, Promega, USA). Purified DNA products were sequenced by IPICYT (San Luis Potosí). The raw DNA sequences were manually edited with PreGap and Gap (http://staden.sourceforge.net) to obtain consensus sequences, which were then analyzed with the NCBI Blast program (http://www.ncbi.nlm.nih.gov) to determine similarity percentages with other sequences.

Phylogenetic Analysis

The gene sequences were individually aligned with ClustalW [17], then manually edited in MEGA X [18], and subsequently concatenated into SequenceMatrix v.1.8 [19] (Vaidya et al. 2011). The concatenated sequences were analyzed under the Maximum Parsimony (MP) criterion to infer phylogenetic relationships. MP analyses were performed in MEGA X using a heuristic search with TBR branch swapping, excluding sites with gaps and non-informative characters. Node support was determined by bootstrap values with 1000 replicates. The tree was visualized in Mesquite v2.75 [20]. The "Mansa" morphotype was selected as the outgroup.

III. RESULTS AND DISCUSSION

Morphological and Agronomic Characteristics of *Vanilla planifolia* Morphotypes

V. planifolia "Mansa" is the species and cultivar typically planted by producers. It is characterized by fleshy, elliptical, lanceolate green leaves; yellow-green flowers; and fruits with three concave sides, cylindrical in shape and green in color. The collected morphotypes exhibit similar characteristics with some morphological and agronomic variations, which are described as follows:

Rayada (R). This plant is easily identifiable by its leaves, which have longitudinal yellow-green bands. Occasionally, parts of the same plant produce leaves and stems without the striped coloration. It is more susceptible to root and stem rot and less productive, although its fruits are aromatic. It appears to be a natural mutation [2, 3].

Oreja de Burro (OB). This morphotype aborts up to 100% of the developing fruit between 42 and 60 days after manual pollination due to genetic self-incompatibility [21, 22]. Although OB plants occasionally have large, elongated leaves resembling donkey ears (hence the name), they are difficult to distinguish in the vegetative stage. The most reliable way to identify OB is by the cream color at the base of the flower's perianth. OB plants are susceptible to diseases but grow at the same rate as "Mansa." However, literature suggests that they grow better and remain healthy because the plants do not weaken by aborting their fruits.

Polen Duro (PD). This accession was collected from plots in Papantla, Veracruz. It is characterized by flowers that do not fully open or open very late; its pollinia are hard, making manual pollination difficult and resulting in poor fruit set. Occasionally, up to five flowers per inflorescence open in a day, whereas typically only one flower opens. The petals and sepals are dark green, and the ovary is initially slightly curved. The top third of the fruit (from the peduncle to the apex) is thin and lighter. Discovered six years ago, this genotype appears to require optimal soil moisture, relative humidity, and temperature conditions for normal flower opening. Due to these undesirable traits, up to 50% of the flowers are not pollinated, reducing fruit production.

Chinanteca (CH). This landrace variety was located in its natural habitat in the Chinantla region of Oaxaca. The fruits of this collection have two stripes or grooves along their length, leading locals to refer to it as striped vanilla. The naturally pollinated fruits are harvested for local use or sold to collectors. The desirable or undesirable attributes of this cultivar are still unknown as it was recently discovered.

Molecular Characterization of Five *V. planifolia* Morphotypes

The five morphotypes of *V. planifolia* were molecularly characterized by PCR amplification and sequencing of two widely used gene regions in the *Vanilla* genus: the chloroplast gene *matK* (maturase K) and the ITS region [16]. A BLAST analysis was performed with the obtained sequences to confirm the identity of the five morphotypes. Coverage percentages for the ITS region ranged from 95% to 99%, and identity percentages from 88% to 100% compared to *V. planifolia* "Mansa." For the *matK* gene, coverage ranged from 97% to 100%, with identity percentages from 92% to 100%, also compared to *V. planifolia* "Mansa" (Table 1).

Table 1. BLAST Analysis Showing Coverage and Identity Percentages of *V. planifolia* Morphotypes Compared to NCBI GenBank Sequences.

Morphotype	*matK*[a] (%)		ITS[b] (%)	
	Coverage	Identity	Coverage	Identity
"Oreja de Burro" 13 OB	100	100	97	99.86
"Oreja de Burro" 40 OB	100	100	96	100
"Rayada" 25 R	99	99.21	95	93.73
"Mansa" 23 M	98	96.72	99	88
"Polen Duro" 106 PD	97	92.25	95	95.94
"Chinanteca" 79 CH	-	99.1	96	100
"Chinanteca" 125 CH	99	94.1	95	99.87

[a]*matK* = maturase K, [b]ITS = Internal transcribed spacer

Subsequently, a Maximum Parsimony analysis was performed using the concatenated sequences of *matK* and ITS to determine if the morphotypes previously associated phenotypically could be grouped using these gene regions. The results show that the morphotypes "Oreja de Burro" and "Chinanteca" form well-supported clades with Bootstrap values of 99 and 93, respectively. Meanwhile, the "Polen Duro" morphotype remains the basal clade of the "Chinanteca", "Rayada", and "Oreja de Burro" morphotypes, with the "Rayada" morphotype as the basal clade of the "Oreja de Burro" (Fig. 1).

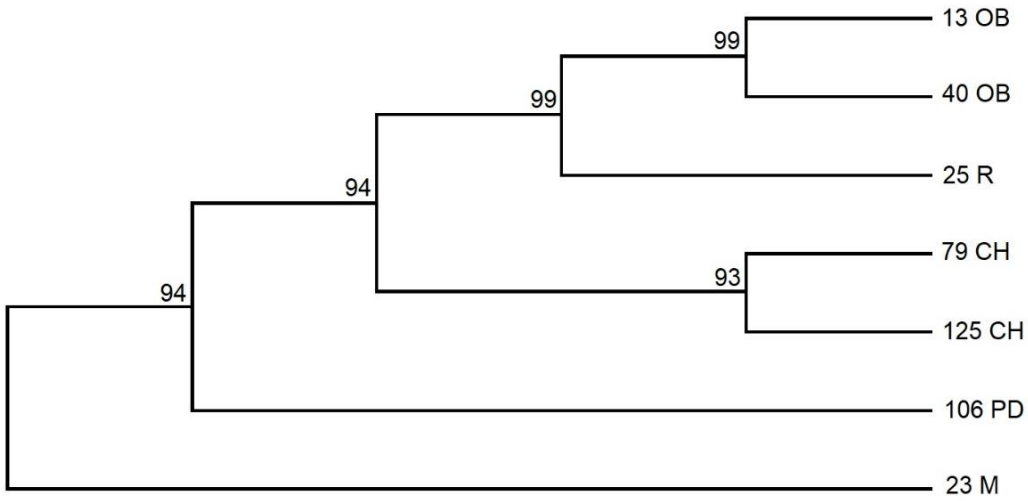

Figure 1. Phylogenetic tree based on the Maximum Parsimony analysis, illustrating the relationships between the different morphotypes of *V. planifolia*. One of the three most parsimonious trees using concatenated sequences of the *matK* and ITS gene regions. The consistency index is (0.889401), the retention index is (0.913357), and the composite index is 0.842187 (0.812341) for all parsimoniously informative sites. Bootstrap values >80% (1000 replicates) are shown above the internodes. All positions with gaps or missing data were eliminated, leaving a total of 879 positions in the final dataset. The "Mansa" morphotype was selected as the tree's outgroup. CH = "Chinanteca", M = "Mansa", OB = "Oreja de burro", PD = "Polen duro", R = "Rayada".

IV. CONCLUSION

In general, the results indicate that there are genetic differences among the five morphotypes studied. However, the data presented here are preliminary since the gene regions used are primarily employed to determine the evolutionary relationships among species of the *Vanilla* genus and not specifically to analyze the genetic structure of *V. planifolia* populations. Therefore, studies on genetic diversity using molecular markers such as RAPD (Random Amplified Polymorphic DNA) or ISSR (Inter-Simple Sequence Repeats) are necessary to clarify the variation present among the morphotypes in the Vanilla Germplasm Bank.

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