Wild *Vanilla planifolia* and its relatives in the Mexican Yucatan Peninsula: Systematic analyses with ISSR and ITS

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Abstract

Background: *Vanilla planifolia*, a threatened species, is distributed naturally in semi-deciduous and evergreen rain forests of southeastern Mexico and parts of Central America. In the wild, it is difficult to diagnose from another sympatric *Vanilla* species, and individuals with reproductive structures are usually required.
Questions: Can ISSR discriminate wild individuals of *Vanilla planifolia* from other sympatric species of the genus? Can phylogenetic analyses of ITS recover the monophyly of *V. planifolia* and help identify *Vanilla* species?
Studied species: The vanilla (*V. planifolia*), the second-most important economically spice in the world.
Study site and years of study: Plant material was collected during 2014 in the Mexican Yucatan Peninsula.
Methods: We analyzed 88 wild individuals of several *Vanilla* species. Individuals of five species of *Vanilla* reported for the region were used as reference. 84 ISSR loci were analyzed using three clustering methods. A phylogenetic inference using ITS was performed.
Results: ISSR markers clearly discriminate wild *Vanilla planifolia*, finding definite genetic structure within the species. The three clustering methods identified genetic relationships with congruent patterns. Five groups were found and they corresponded with the species studied. Phylogenetic inference of ITS sequences supported the monophyly of the *Vanilla* and the resulting cladograms were coherent with the clustering pattern observed in the ISSR studies.
Conclusions: Both ISSR and ITS analyses are able to identify *V. planifolia*. Molecular data suggest the presence of *V. pompona* and a new species of *Vanilla* in the Yucatan Peninsula.
Keywords: Genetic identification, molecular markers, phylogenetic analysis, vanilla, wild populations.

Vanilla planifolia silvestre y sus parientes en la Península de Yucatán, México: análisis sistemáticos con ISSR e ITS

Resumen

Antecedentes: *Vanilla planifolia* es una especie amenazada que se distribuye naturalmente en las selvas tropicales perennifolias y subperennifolias del sureste de México y parte de Centroamérica. En estado silvestre, es difícil de diferenciar de otras especies simpátricas de *Vanilla*, y usualmente se requieren individuos con estructuras reproductivas.
Preguntas: ¿Pueden los marcadores ISSR discriminar individuos silvestres de *Vanilla planifolia* de otras especies simpátricas de vainilla?, ¿Puede el análisis de ITS recobrar la monofilia de *V. planifolia* y contribuir a identificar las especies de *Vanilla*?
Especie en estudio: Económicamente, la vainilla (*Vanilla planifolia*) es la segunda especia más importante en el mundo.
Sitio de estudio y fecha: La colecta se realizó en 2014 en la Península de Yucatán.
Métodos: Se analizaron 88 de individuos silvestres de especies de *Vanilla*. Se utilizaron como testigos individuos de cinco especies de vainilla reportadas para la región. 84 loci de ISSR fueron analizados usando tres métodos de agrupamiento. Se realizó una inferencia filogenética con ITS.
Resultados: Los ISSR discriminaron claramente a los individuos silvestres de *Vanilla planifolia*, detectando una estructura genética al interior de la especie. Los tres métodos de agrupamiento mostraron patrones congruentes, encontrándose cinco grupos que correspondieron con las especies estudiadas. El análisis filogenético de las secuencias de ITS mostró la monofilia del género *Vanilla*, y los cladogramas obtenidos fueron coherentes con los resultados obtenidos con los ISSR.
Conclusiones: Tanto ISSR como ITS identificaron a *Vanilla planifolia*. Los análisis moleculares sugieren la presencia de *V. pompona* y de una nueva especie de *Vanilla* en la Península de Yucatán, México.
Palabras clave: Análisis filogenético; identificación genética, marcadores moleculares, poblaciones silvestres, vainilla.

The genus *Vanilla* Mill. is composed of possibly more than 150 species. In 1954 Portères reported 110 distinct taxa but many more have been described since (*e.g*. Soto-Arenas & Dressler 2010), and several more still await formal taxonomic recognition. The genus features a Pantropical distribution and it probably originated in America (Cameron 2000, Ramírez *et al*. 2007, Bouetard *et al*. 2010). *Vanilla* taxonomy has been hampered by several factors, including the following:

- The difficulty of finding and collecting individuals with fertile structures in the field as a result of the ephemeral, gregarious flowering that usually happens high in the canopy of the forests (Schlüter 2002, Soto-Arenas & Dressler 2010).
- Flowers, which are fundamental for identification of species, are poorly preserved in herbaria. Usually, herbarium species are sterile or include a single flower, often poorly preserved and irretrievably flattened (or altogether glued to the herbarium sheet).
- The rarity or inaccessibility of many of the species.
- The fact that many species are vegetatively indistinguishable.
- The enormous vegetative variation and phenotypic plasticity associated with the hemiepiphyte growth habit including leaves of different morphologies and sizes in the same individual as a response to age, vegetative development, and light exposure (Putz & Holbrook 1986, Ray 1990).

Vanilla planifolia Andrews has great economic importance, being the source of approximately 95 % of the world's vanilla extract production (Lubinsky *et al*. 2008a). The vanilla is the second-most important spice in gastronomy and the cosmetic industry and is one of the most popular flavorings (Cid-Pérez & López-Malo 2011, Álvarez *et al*. 2013). Vanilla cultivation typically features vegetative propagation and hand-pollination, which ensures fruit production, albeit seeds are never harvested, and plants are never raised from seeds. Vegetative reproduction, plus the lack of new genotypes incorporated via seed, have in combination drastically reduced genetic variation in vanilla plantations (Smith *et al*. 1992, Soto Arenas 1999, Minoo *et al*. 2008). Because of this, FAO (1995) lists cultivated *V. planifolia* as species with a high degree of genetic erosion. Furthermore, wild populations of *V. planifolia* have been severely damaged by natural habitat destruction and illegal extraction for replenishment of commercial plantations, nearly driving the species to the brink of extinction, at least locally (Soto-Arenas 2006, Soto-Arenas & Solano-Gómez 2007, Menchaca 2010). Due to this, *V. planifolia* has a "special protection" status (SEMARNAT 2010) in Mexico. As a member of the Orchidaceae, it is included in Appendix 2 of CITES (CITES 2016).

Vanilla planifolia is reported as native from southeastern Mexico, Guatemala, Belize, and Costa Rica. However, a clear picture of its distribution is still uncertain (Soto-Arenas 1999) because relatively few wild individuals have apparently been collected (fewer than 30 individuals, according to Soto-Arenas 2006 and Schlüter *et al*. 2007). There are also several vegetatively similar species. To make matters worse, flowers in the herbaria are difficult to reconstruct; thus, a significant portion of specimens are probably misidentified as *V. planifolia* in many herbaria (Soto-Arenas 2009, Soto-Arenas & Dressler 2010). These errors of identification have also been reported in vanilla plantations in Mexico, as we have been able to corroborate in the Mexican states of Quintana Roo (Villanueva-Viramontes, pers. obs.), and Oaxaca (M. Hernández-Apolinar pers. Obs.). The literature also reports this from other countries, such as Ecuador and Guatemala (Soto-Arenas & Dressler 2010). Some of these plantations include, along with true *V. planifolia*, individuals of species such as *Vanilla insignis* Ames, *Vanilla cribbiana* Soto Arenas, *Vanilla odorata* C. Presl., *Vanilla pompona* Schiede, and *Vanilla sp. nov. aff. V. phaeantha* (Soto-Arenas & Dressler 2010).

In the Mexican Yucatan Peninsula (MYP), wild individuals of *Vanilla planifolia* have been collected, but only a few of them have been included in previous studies of this species (Cibrián 1999, Schlüter *et al*. 2007). Furthermore, at least four additional species of the genus have been reported as growing actually or potentially sympatrically with *V. planifolia* in this region: *Vanilla insignis*, *Vanilla odorata*, *Vanilla inodora* Schiede, and *Vanilla sp. nov. aff. V. phaeantha* (Carnevali *et al*. 2001, Soto-Arenas 2009). This sympatry complicates sampling of wild individuals of *V. planifolia* whenever fertile structures are wanting.

During the last few decades, DNA markers have proven to be a powerful tool for accurate

Author Contributions
Sara Villanueva-Viramontes: collected most of the botanical material, conceived, designed, and performed the experiments, analyzed the data, and drafted the first version of the paper as well as reviewed drafts of the manuscript.
Mariana Hernández-Apolinar: provided botanical materials and reviewed drafts of the manuscript.
Germán Carnevali Fernández-Concha: provided botanical materials, conceived and designed parts of the experiments and analyses. Translated and reviewed drafts of the paper. Funded the ITS analyses.
Alfredo Dorantes-Euán: Participated in the botanical collections, and reviewed drafts of the first version.
Gabriel Rolando Dzib: Participated in the botanical collections, and reviewed drafts of the first version.
Jaime Martínez-Castillo: conceived and designed parts of the experiments and reviewed drafts of the manuscript. Funded the ISSR analysis and the collecting trips.

taxonomic determination (Nagy *et al*. 2012). The ISSR (*Inter Simple Sequence Repeats*; Zietkiewicz *et al*. 1994) is one of these markers. They are especially robust because allow discriminating between individuals of genetically close species or between varieties of the same species (Pharmawati *et al*. 2005, Elmeer & Almaki, 2011, Reyes-Alemán *et al*. 2013). Also, the ISSR, compared with other molecular tools, generate a high number of molecular markers with little effort; and is a relatively simple, cost-effective technique (Godwin *et al*. 1997, Bornet *et al*. 2002). Verma *et al*. (2009) used 10 ISSR primers to assess the genetic relationships of seven species and two hybrids of *Vanilla*: *V. albida* Blume, *V. aphylla* Eggers, *V. andamanica* Rolfe, *V. planifolia*, *V. wightii* Lindl. *ex* Wight, *V. parishii* Rchb. f., *V. walkeriae* Wight, *V. x tahitensis* JW. Moore and the hybrid *V. planifolia*♀×*V. wightii*♂. They found low levels of variation among individuals of *V. planifolia*, *V. tahitensis*, and *V. aphylla*, suggesting close relationships whereas there was also high affinity among *V. planifolia* and *V. tahitensis*, and between *V. albida* y *V. aphylla*. *Vanilla andamanica* was only distantly related to the other species, possibly reflecting a more distant phylogenetic relationship. Ramos-Castella *et al*. (2016), using five of the same ISSR primers of Verma in three species of *Vanilla* from México, found a clear differentiation between *V. planifolia* and the other three cultivated species from the wild (*V. pompona*, *V. insignis*, and *V. aff. V. odorata*).

On the other hand, the sequences of the ITS (Internal Transcribed Spacer) 1 and 2 of the 18S - 26S region of ribosomal DNA, usually display a high variability that allows for the broader molecular identification of organisms (Coleman 2003). ITS have been used in several molecular phylogenies of Vanilloideae (Cameron 2009, Poeaim *et al*. 2011, Soto-Arenas & Dressler 2010), providing a rich source of variable characters at several taxonomic levels, including species.

Wild populations of *Vanilla planifolia* are of utmost importance for the species' conservation and its genetic improvement. As discussed earlier, the preservation of this species (and of related taxa) is currently hindered by the difficulties associated with identifying the species of *Vanilla* in the wild. Taking into account that ISSR molecular markers are relatively easy to use and powerful in genetic discrimination, they may provide accurate determinations of non-fertile, wild-collected individuals of *Vanilla* taxa, as well as of *V. planifolia* populations and varieties. Thus, the objectives of this study were the following:

1. To assess the power of ISSR markers to discriminate between wild individuals of *V. planifolia* and those belonging to other species growing naturally in the Mexican Yucatan Peninsula.
2. Contrast the results with those of a phylogenetic analysis of *Vanilla* ITS sequences.

Methods

Plant material. Leaves of 88 wild specimens of *Vanilla spp*. from the MYP were considered in this study (Table 1). These samples were deemed wild because they were collected in natural settings without evidence of human management. Hereafter, these samples will be referred to as MYP, followed by an identifying consecutive numbering plus a key to the region where they were collected (Table 1). Of all specimens studied, only two of them were found fertile and positively confirmed as *V. planifolia* ("MYP1-CY" and "MYP2-CY", both from the same population) (Figure 1-A). The other specimens were infertile and only leaves were collected from them. However, we could observe morphological differences in leaf and stem in some of these samples. The detailed information about the collecting site of the samples is not provided for protection reasons. Sterile vouchers were collected by location and deposited at herbarium CICY.

In the ISSR analysis, we included 11 cultivated individuals as controls (standard samples). These individuals belong to the four species of *Vanilla* reported for the MYP and distributed as follows:

1. Three individuals of *Vanilla planifolia*, originating from Puebla and Veracruz ("varieties" "Mansa" [M], "Variegata" [V], and "Oreja de Burro" [OB]).
2. Two individuals of *Vanilla insignis* (one from Puebla [P], another from CICY's Jardín Botánico Regional Roger Orellana [JBR-RO] – Figure 1-B).
3. A single individual of *Vanilla odorata* from Veracruz (Figure 1-C).
4. Four individuals of *Vanilla sp. nov. aff. V. phaeantha* grown at the JBR-RO (Figure 1-D).

Table 1. Geographical origin and description of the control samples and wild specimens of *Vanilla* collected in the MYP.

Species	Code	Number of samples	Origin	Taxonomic characteristics
V. planifolia				Soto-Arenas 2009
var. Mansa	M	1	Puebla; MH	
var. Variegata	V	1	Puebla; MH	
var. Oreja de Burro	OB	1	Veracruz; MH	
V. pompona		1	Puebla; MH	
V. odorata		1	Veracruz; C-AR	
V. insignis		2	Puebla; MH /JBR*; GC	
V. sp. nov. aff. V. phaeantha		4	CY- JBR; GC	No taxonomic determination
V. spp.	MYP	88	CY, CQ, SQ, SC y SET; SV	

Note: JBR: Jardín Botánico Regional Roger Orellana. MPY: Mexican Yucatan Peninsula. Regions: CY: Center of Yucatan; CQ: Center of Quintana Roo; SQ: Southern Quintana Roo; SC: South Campeche; and SET: Southeastern Tabasco. *: Unknown origin. MH: Mariana Hernández Collection. SV: Sara Villanueva Collection. GC: Germán Carnevali Collection. AR: Alma Ramos Collection.

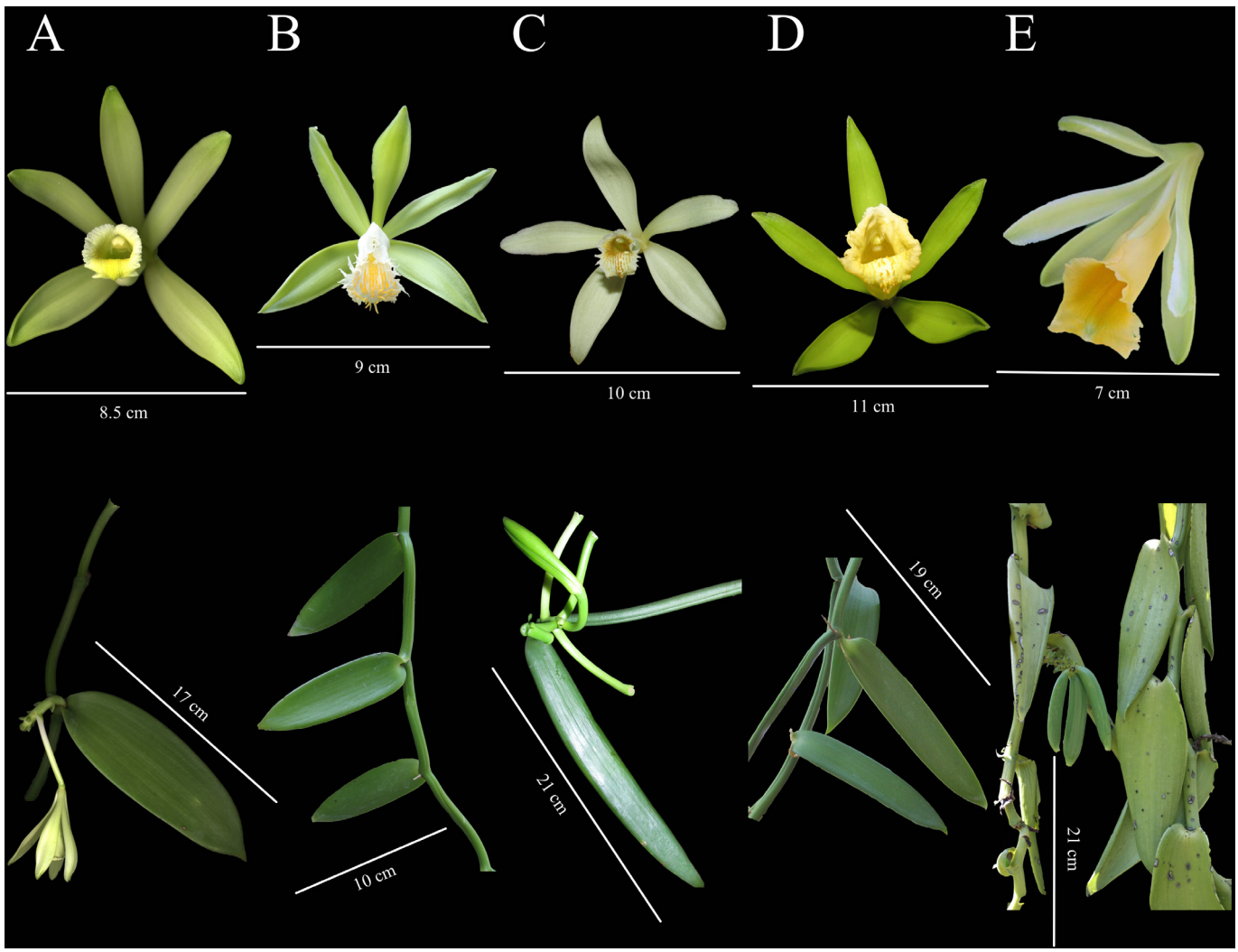

Figure 1. Flowers and vegetative structures of the *Vanilla* species reported for the Yucatan Peninsula and used in the analysis with ISSR molecular markers. **A)** *V. planifolia* wild (both photographs by Sara Villanueva [SV]). **B)** *V. insignis* (flower photography by Leon Ibarra González, and leaves photography by SV). **C)** *V. odorata* (flower photography by Manfred Speckmaier, and leaves photography by SV). **D)** *V. sp. nov. aff. V. phaeantha* (both photographs by SV). **E)** *V. pompona* (both photos by Verónica Borbolla).

These have locality data and have been documented in bloom. This taxon still awaits formal description.

Also, a single individual of *Vanilla pompona* originally from the state of Puebla (Figure 1-E), a species that could potentially occur in the MYP.

DNA extraction and ISSR technique. Total genomic DNA was extracted from fresh leaves following a modification of the Dellaporta *et al*. (1983) procedure, which involved a single protein washout using phenol:chloroform:isoamylic. DNA was quantified with an ND-1000 spectrophotometer (Nanodrop, USA) and its quality assessed by 0.8 % agarose gel electrophoresis (Sambrook *et al*. 1989) and by PCR amplification of the 18S constitutive gene.

Three of the 10 ISSR primers specifically designed for *Vanilla* by Verma *et al*. (2009) were employed in this study: C07, C09 y T06. Amplifications were performed in a total volume of 20 µl, including 20 ng/µl of ADN, 200 mM of dNTPs (Invitrogen), 1.5 mM $MgCl_2$, 60 pg. of primer, 2.5 µl buffer 10x of Taq ADN polymerase, and 1.5 U Taq DNA polymerase (Invitrogen). Amplification conditions were as follows: an initial step of 5 minutes at 94°C, followed by 35 cycles, each consisting of 30 seconds at 94°C for denaturing, 90 seconds at 50-54°C (depending upon primer used) for aligning and 90 seconds at 72°C for elongation, and 5 minutes at 72°C for final extension. Amplifications were performed at least twice and only reproducible products (bands) were considered for data analyses. Amplified PCR products were screened by agarose gel electrophoresis with 1.5% buffer TBE 0.5X (pH 8.3) and finally stained with ethidium bromide (Sambrook *et al*. 1989). Resulting gels were photographed and visually read with a 1 kb molecular marker as a reference for fragment length assessment.

Genetic relationships based on cluster analysis of the ISSR. Both monomorphic and polymorphic bands were considered for analyses and only well-defined bands (color intensity and reproducibility) included. The basic assumption was that bands of identical molecular weight regardless of whether belonging to individuals of the same or different population/species, represented the same allele. We compiled a database for each primer, recording the presence/absence of particular bands as 1or 0, respectively.

We performed a clustering analysis of both the standard samples as well as the MPY samples. Three different approaches followed for analyses:

1) An assignment test of individuals using a Bayesian approach implemented in Structure 2.3.1 (Pritchard *et al*. 2000). Because individuals included in the analyses were referable to several species, the program was run under the *no-admixture* model. This model assumes that all of the genetic material of a particular individual comes from a single genetic pool (*i.e*. that no individual is hybrid). Based on this, we used the sampling location as LOCPRIOR in all the analyses performed (Tucker *et al*. 2012). The program ran under the following specifications: independent allelic frequencies, burn-in a period of 100,000, and 200,000 iterations after this period to allow the Markov chain to reach stationarity. Because there are at least four different *Vanilla* species reported for the MYP (see above), the analysis tested several values of K (K = 4 through K = 10). The most likely K value (K_{optima}l) was estimated according to Evanno *et al*. (2005) implemented in the Structure-Harvester program (Earl & von Holdt 2012). Graphics output from Structure was edited in Microsoft PowerPoint 2013.
2) A Neighbor-Joining analysis (Saitou & Nei 1987) to generate similarity clusters with Jaccard's Similarity Index (Jaccard 1908). A frequency tree (Hampl *et al*., 2001) was constructed with the program FreeTree 0.9.1.50 (Pavlícek *et al*. 1999), with a bootstrap resampling of 1000 iterations. The output trees visualized in FigTree version 1.4.2 (Rambaut 2006-2014).
3) A Principal Coordinates Analysis (PCoA), which allows for the recognition of spatial clustering of genotypes, without altering the data and only inputting the genetic similarity matrix. This analysis was conducted in GenAlEx 6.2 (Genetic Analysis in Excel) (Peakall & Smouse 2006). The two-dimensional PCoA graphic was constructed with the Gnuplot 3.7 freeware <http://www.gnuplot.info/>.

Phylogenetic inference based on ITS. With the goal of obtaining an independent corroboration of the clustering patterns emerging from the ISSR analyses, we performed a phylogenetic analy-

Table 2. GenBank specimens used as outgroups in the analysis of ITS.

Accession	Species	Reference
EU498152.1	*C. paranaensis*	**Pansarin *et al.* 2008**
EU498153.1	*C. pusilla*	
EU498158.1	*C. uliginosa*	
FJ425836.1	*E. lucidum*	Cameron 2009
FJ425837.1	*E. subrepens*	
FJ425828.1	*E. parviflorum*	
FJ425838.1	*C. smilacifolium*	
FJ425835.1	*V. barbellata*	
FJ425830.1	*V. imperialis*	
FJ425834.1	*V. africana*	
FJ425840.1	*V. roscheri*	
GQ867246.1	*V, planifolia* - clone 19	**Belanger & Havkin-Frenkel 2011**
GQ867237.1	*V. pompona* – clone 8	
AF151006.1	*V. aphylla*	Cameron & Chase1999
JF825978.1	*V. siamensis*	**Li *et al.* 2011**
JF796930.1	*V. shenzhenica*	
AF391785.1	*V. hirsuta*	Clements *et al*. 2002
C-GC	*V. grandiflora*	**Panamá; Germán Carnevali Collection, 2016**
C-GC	*V. dressleri*	
C-GC	*V. cribbiana*	

Note: C-GC: Germán Carnevali Collection.

sis of the nuclear ITS (ITS 1 and 2) region. This study included all the individuals considered in the ISSR analysis and relevant outgroups (see below), representing both tribes (Pogonieae and Vanilleae) of Subfamily Vanilloideae (Chase *et al*. 2015). Taxon sampling, consisting of 121 terminals, putatively representing 25 species, was devised to test the following three hypotheses:

a) That *Vanilla* is monophyletic.
b) That the *Vanilla*'s Afro-Caribbean Clade (Cameron 1999) is monophyletic and sister to the rest of the genus.
c) That all individuals tentatively referred to *V. planifolia* (on morphological grounds or ISSR data) fall in a clade.

To polarize phylogenetically informative character states, a species of the distantly related genus *Cleistes* Rich. *ex* Lindl. (tribe Pogonieae), *Cleistes paranaensis* Schltr. was employed as a functional outgroup. This taxon was identified as basal in a recent phylogenetic analysis of the genus (Pansarin *et al*. 2008). The outgroup of the phylogenetic analysis consisted of the following taxa (Table 2):

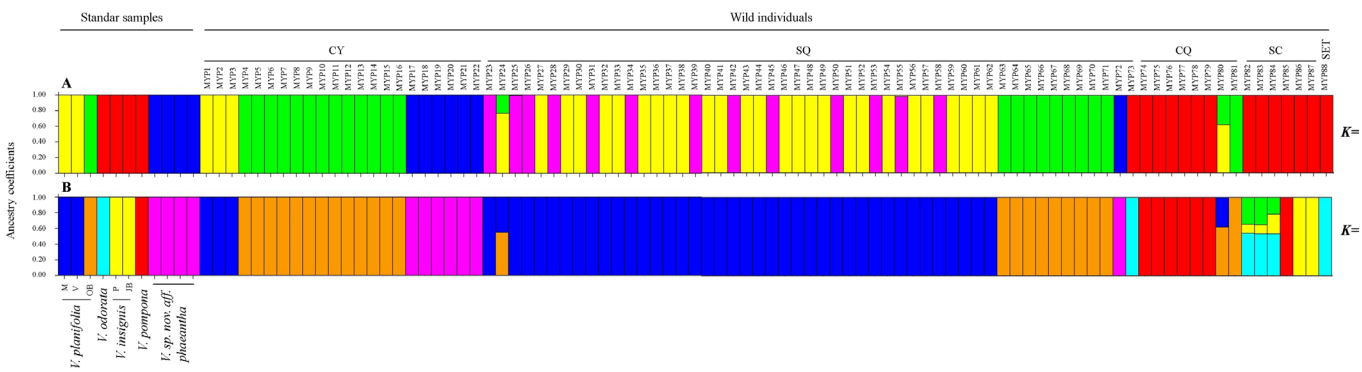

Figure 2. Assignment test of 88 wild individuals of *Vanilla spp*. collected in the Mexican Yucatan Peninsula and 11 standard samples of *Vanilla*, using ISSR markers and the Structure program. **A)** *K*=5 based in the Evanno's method. **B)** *K* = 7 (second K optimal). CY: Center of Yucatan; CQ: Center of Quintana Roo; SQ: Southern Quintana Roo; SC: South Campeche; and SET: Southeastern Tabasco.

1) Two species of *Cleistes* (Tribe Pogonieae): *C. uliginosa* Pabst and *C. pusilla* Pansarin.
2) Three species of *Epistephium* Kunth: *E. lucidum* Cogn., *E. subrepens* Hoehne, and *E. parviflorum* Lindl.
3) The single species of *Clematepistephium* N. Hallé: *C. smilacifolium* (Rchb. f.) N. Hallé.

The ingroup of the analysis consisted of several different *Vanilla* species, representing both morphological variation and geographical distribution of the genus, although Neotropical taxa thought to be related to *V. planifolia* constitute the bulk of the sample. Species included are:
1) Five members of the Afro-Caribbean clade of *Vanilla*: *V. roscheri* Rchb. f., *V. africana* Lindl., *V. siamensis* Rolfe ex Downie, *V. imperialis* Kraenzl. and *V. barbellata* Rchb. f.
2) Two taxa occurring in Asia: *V. shenzhenica* Z.J. Liu & X. Qi Chen and *V. aphylla*.
3) Nine Neotropical taxa occurring in the continental mainland: *V. sp*. (Venezuela), *V. grandiflora* Lindl. (Panama), *V. dressleri* Soto Arenas (Panama), *V. cribbiana* Soto Arenas (Panama), *V. planifolia* (Puebla-Veracruz; and Genbank's clone 19), *V. pompona* (Puebla; Genbank's clone 8), *V. odorata*, *V. insignis*, *V. sp. nov. aff. V. phaeantha*.
4) The hybrid *Vanilla* × *hirsuta* M.A Clem. & D.L. Jones (*V. odorata* × *V. planifolia*).
5) All the wild-collected *Vanilla* individuals also analyzed in the ISSR study.

The ITS region was amplified following Poeaim *et al*. (2011), and using primers ITS1 and ITS4 (White *et al*. 1990). Amplification reactions performed in 30 µl containing 50 ng/µl of ADN, 1.25 nm of dNTPs, 3 mM of $MgCl_2$, 20 pmol of each primer, 1.5 U Taq polymerase, and the buffer provided by the manufacturer (Qiagen). The PCR protocol was as follows: an initial denaturalizing at 95°C for 5 minutes, followed by a sequence of 30 cycles consisting each of 1.5 minutes of denaturalizing at 94 °C, 2 minutes of annealing at 55 °C, and 1 minute of extension at 72 °C, ending in a 10 minute final extension period at 72 °C. PCR products amplified were screened and evaluated in an agarose gel stained with ethidium bromide, using a molecular weight marker of 100 pb. PCR products were later sent for sequencing at Macrogen (http://www.macrogen.com).

DNA sequences were edited with Sequencher 5.3 <http://www.genecodes.com> (accessed:14, 2015). Alignments were performed with Muscle (Edgar 2004) with the default parameters as available at the CIPRES Science Gateway, Miller *et al*. 2010). Afterward, the resulting alignment was visually refined in BioEdit v. 5.0.6 (Hall 2001). Average sequence length was 746 bp. To select the model of nucleotidic substitution that best fit the data, jModelTest2 (Darriba *et al*. 2012, Guindon & Gascuel 2003) was employed. The model selected was GTR + G.

Nucleotide sequence data of the ITS region used for phylogenetic inferences were analyzed under three different paradigms: Bayesian Inference, Maximum Likelihood, and Maximum Parsimony. The first two were implemented through the CIPRES Science Gateway, Miller *et al*. 2010). Conditions for each analysis were as described below:
1. *Bayesian inference* (Yang & Rannala 1997): This analysis was conducted in MrBayes 3.2.6 (Ronquist *et al*. 2012). Two independent threads of four chains each were run with a MCMC chain length of 50,000,000 generations. Parameters and convergence of trees were assessed with Tracer v.1.6 (Rambaut *et al*. 2014).
2. *Maximum likelihood*: Implemented in RAxML 8 (Stamatakis 2014). The support for nodes was assessed with 1,000 bootstrap iterations.
3. *Maximum Parsimony*: The shortest trees were estimated with NONA (Goloboff, 1999) through the Winclada 1.00.08 shell (Nixon 1999-2000). 1,000 iterations of the Parsimony Ratchet (Nixon 1999, Vos 2003) algorithm were run. Characters were coded as unordered and of uniform weight (Fitch parsimony); gaps coded as missing characters. Support for clades was assessed with 1,000 iterations of a Jackknife analysis (Felsenstein 2004).

The primary objective of the three analyses was the qualitative assessment of the degree of congruence between the topologies yielded by them. Consensus trees were visualized in Figure 3 and edited with Paint of Microsoft Windows version 1,511 to include clade support values 80 % or higher of posterior probabilities, parametric bootstrap, and Jackknife.

Results

This section will present the results of the ISSR analyses first and then the phylogenetic ITS analysis, along with a description of their congruence or lack thereof.

Genetic relationships based on cluster analysis of the ISSR. 84 loci amplified with the three ISSR primers. Evanno's method estimated a $K = 5$, which correlate with the following color-coded aggregations (Figure 2A):

1. "YELLOW": This group aggregated some of the standard samples of *Vanilla planifolia* (the "Mansa" and "Variegata" cultivars) along with 31 wild-collected specimens from the MYP (including the MYP1-CY y MYP2-CY specimens identified as *V. planifolia* using floral structures). These individuals share characteristics in leaf morphology (elliptic-obtuse in shape) and smooth stem-like *V. planifolia*. Within this group, the individuals MYP24-SQ and MYP80-CQ presented some degree of "green" ancestry (25 and 38 % respectively).
2. "GREEN": This group aggregated the standard sample of *Vanilla planifolia* var. "Oreja de Burro" along with 23 additional wild specimens from the MYP. This group has the same leaf morphological characteristics of the "yellow" group.
3. "RED": This color signaled the standard samples of *Vanilla odorata*, *V. insignis*, and *V. pompona*, along with 14 wild-collected individuals. Within this group differences in leaf (falcate-lanceolate, linear, and elliptic) and stem (rough, thick, smooth, thin) morphology were found.
4. "BLUE": This color category characterized the JBR-RO cultivated specimens of *Vanilla sp. nov. aff. V. phaeantha* (originally from central Yucatan) and an additional seven wild individuals. All member of this group share characteristics in leaf shape (linear) and stem surface (rough-thick) morphology.
5. "FUCHSIA": This group consisted of 13 wild specimens. These specimens have a morphology similar to individuals assigned to *V. planifolia*.

According to Meirmans (2015), considering different values of *K* may reflect different genetic and demographic processes and thus ensure a better biological interpretation of the data. In this study, we found that the second-highest *K* value was 7 (Figure 2B):

1. "BLUE": In this group, the yellow and fuchsia previous color categories ($K = 5$; Figure 2A) were aggregated, including the standard samples of the "Mansa" and "Variegata" cultivars of V. planifolia.
2. "OCHRE": This group is practically equal that former green group ($K = 5$; Figure 2A) including standard sample of variety "Oreja de Burro" of *V. planifolia*. Within this group appear the wild individuals MYP24-SQ and MYP80-CQ, which have a higher degree "ochre" ancestry (45% and 30% respectively).
3. "AQUA": This color signaled the standard sample of *V. odorata* with five wild-collected individuals. Three of these (MYP82-SC, MYP83-SC, and MYP84-SC) individuals present different degree of "green" and "yellow" ancestry (*i.e*. admixed individuals). All these individuals were present in the former red group ($K = 5$; Figure 2A).
4. "YELLOW": This color category is composed by *V. insignis* specimens and two wild-collected individuals. These individuals also were grouped in the former red category ($K = 5$; Figure 2A).
5. "RED": This group consisted of the standard sample of *V. pompona* and seven wild specimens. In the same way, these individuals are grouped into the former red category ($K = 5$; Figure 2A).
6. "FUCHSIA": This group is maintained as in the $K = 5$ (the old blue group).
7. "GREEN": An unknown wild genotype that composes part of the ancestry of three individuals, namely MYP82-SC, MYP83-SC, and MYP84-SC (*i.e*. admixed individuals). These individuals were assigned to the "aqua" group according to their high ancestry coefficient derived from this group.

The Neighbor-Joining analysis aggregated the genotypes analyzed in two (A and B) highly supported (100 % bootstrap) major clusters (Figure 3), within which several subgroups were

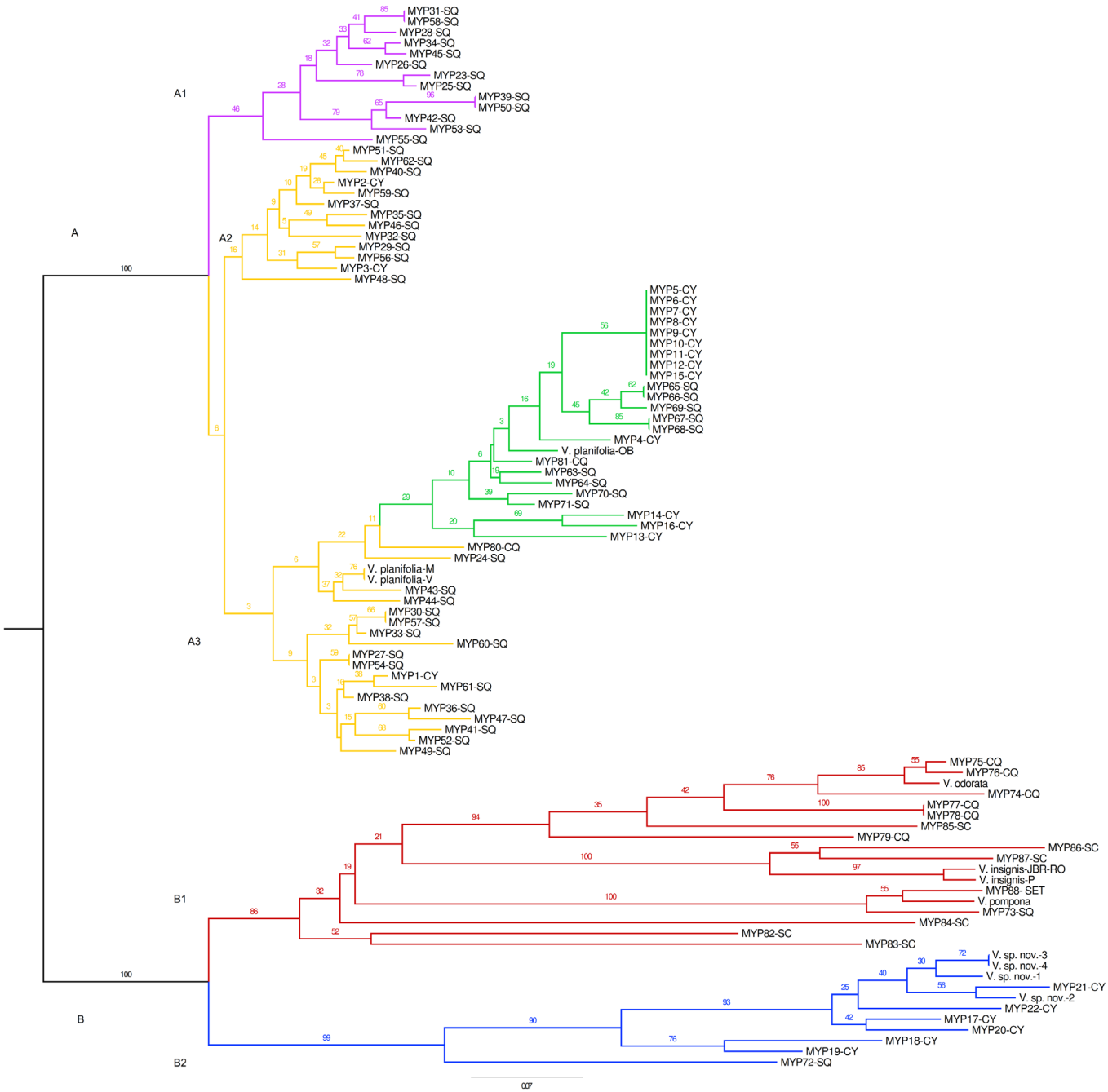

Figure 3. Neighbor-Joining dendrogram based on Jaccard similarity index (Jaccard 1908) of 88 wild individuals of the Mexican Yucatan Peninsula and 11 standard samples of *Vanilla*, using ISSR markers. The color in the branches is according to with the Figure 2A.

retrieved; these are color coded according to what was observed in the Structure analysis (Figure 2A).

1. The A cluster aggregated all the standard samples of *Vanilla planifolia* and 67 wild MYP specimens. Three subclusters were contained: A1 (13 individuals), A2 (13 individuals, including MYP2-CY, identified as *V. planifolia*), and A3 (41 wild individuals along with the standard samples of the three varieties of *V. planifolia*, and the MYP1-CY, also identified as *V. planifolia* using floral material).
2. The B cluster consisted of the remaining standard samples and further wild MYP specimens. Two highly supported subclusters were included: B1 (86 % support by the bootstrap) this

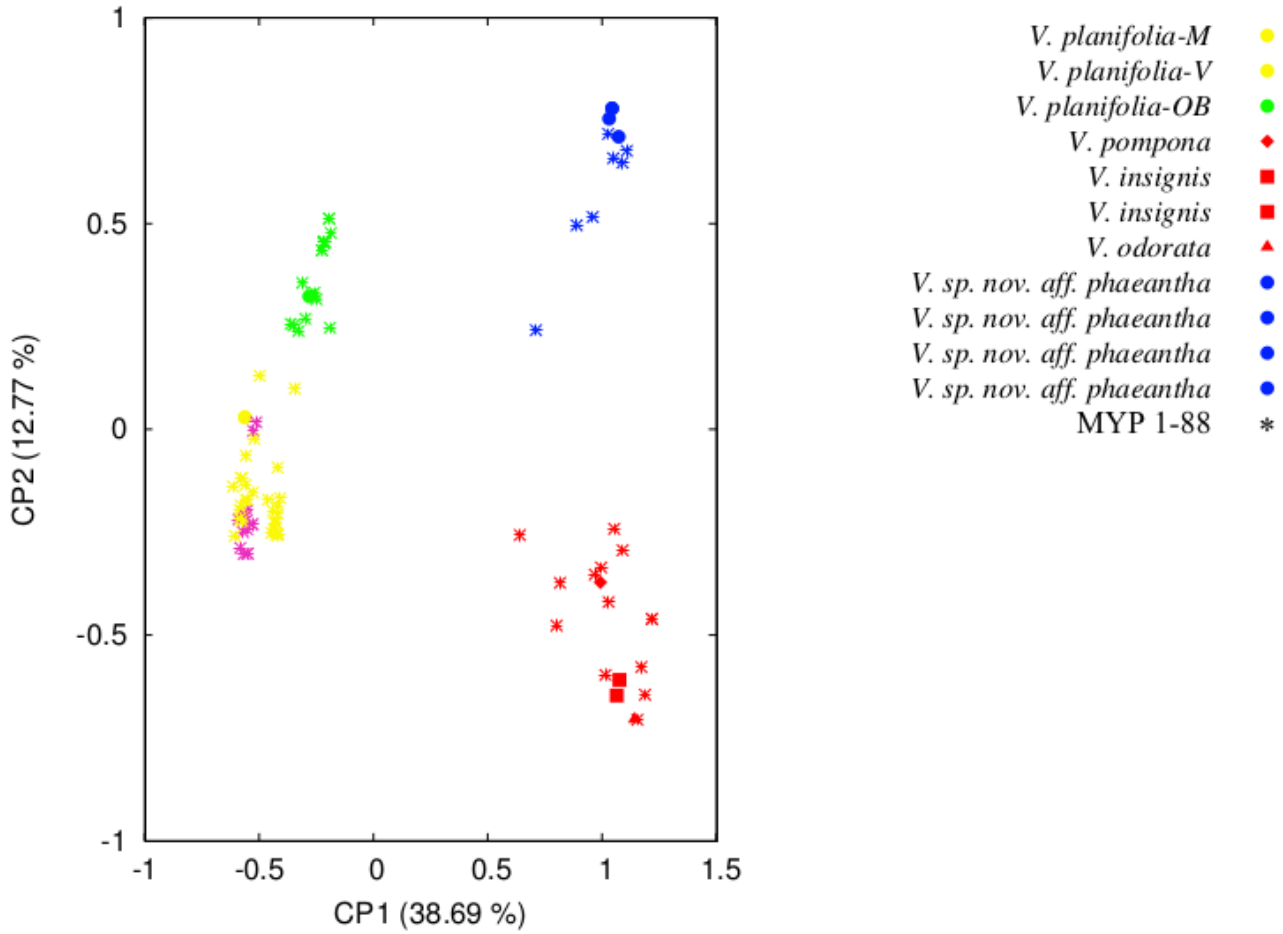

Figure 4. Principal Coordinates Analysis (PCoA) of 88 wild individuals of *Vanilla* of the Mexican Yucatan Peninsula and 11 standard samples of *Vanilla*, using ISSR markers. The color points are according to with the Figure 2A.

subcluster contained the standard samples of *V. odorata*, *V. pompona*, and *V. insignis*, along with 14 wild specimens. B2 (99 % bootstrap) included the JBR individuals of *Vanilla sp. nov. aff. V. phaeantha* and seven wild MYP samples.

The PCoA analysis identified four principal clusters (Figure 4). Also, color coded according to with the Figure 2A. These clusters were mostly "pure-colored"; except an assorted-color (yellow/fuchsia) cluster. The YELLOW/FUCHSIA group included the "Mansa" and "Variegata" cultivars of *Vanilla planifolia* and 44 wild-collected MYP specimens, the MYP1-CY and MYP2-CY among them. The GREEN group comprised the standard samples of *V. planifolia* var. "Oreja de Burro" and 23 additional wild MYP individuals. The BLUE group contained the standard samples of *Vanilla sp. nov. aff. V. phaeantha* and seven wild MYP individuals. Lastly, the RED group included *V. pompona*, *V. odorata*, and *V. insignis* with the remaining (14) wild individuals. These groups feature morphological coherence, except for the red group. The principal coordinates 1 and 2 explained 38.69 % and 12.77 % of total variation, respectively.

In summary, the three clustering methods identify genetic relationships with relatively congruent patterns where there are at least four genetic groups and *Vanilla planifolia* isolated from the other species that occur wild in the MYP. It also identifies a genetic group that appears to correlate with morphologically distinctive plants and that will eventually be proposed as a new taxon (*Vanilla sp. nov. aff. V. phaeantha*).

Phylogenetic inference of Vanilla based on ITS. Including gaps, the matrix analyzed consisted of 1,018 sites with an average of 698 pb per taxon. We show here the Bayesian inference ma-

jority (80 %) consensus tree (Figure 5), including the bootstrap values from the ML analysis and jackknife values exceeding 80 from the parsimony analysis (589 informative sites [57.7 %], L: 3775, CI: 35, RI: 54). Basal nodes were fully resolved and strongly supported. However, there was not resolution within the American *Vanilla* taxa, due to collapse/lack of support for several clades.

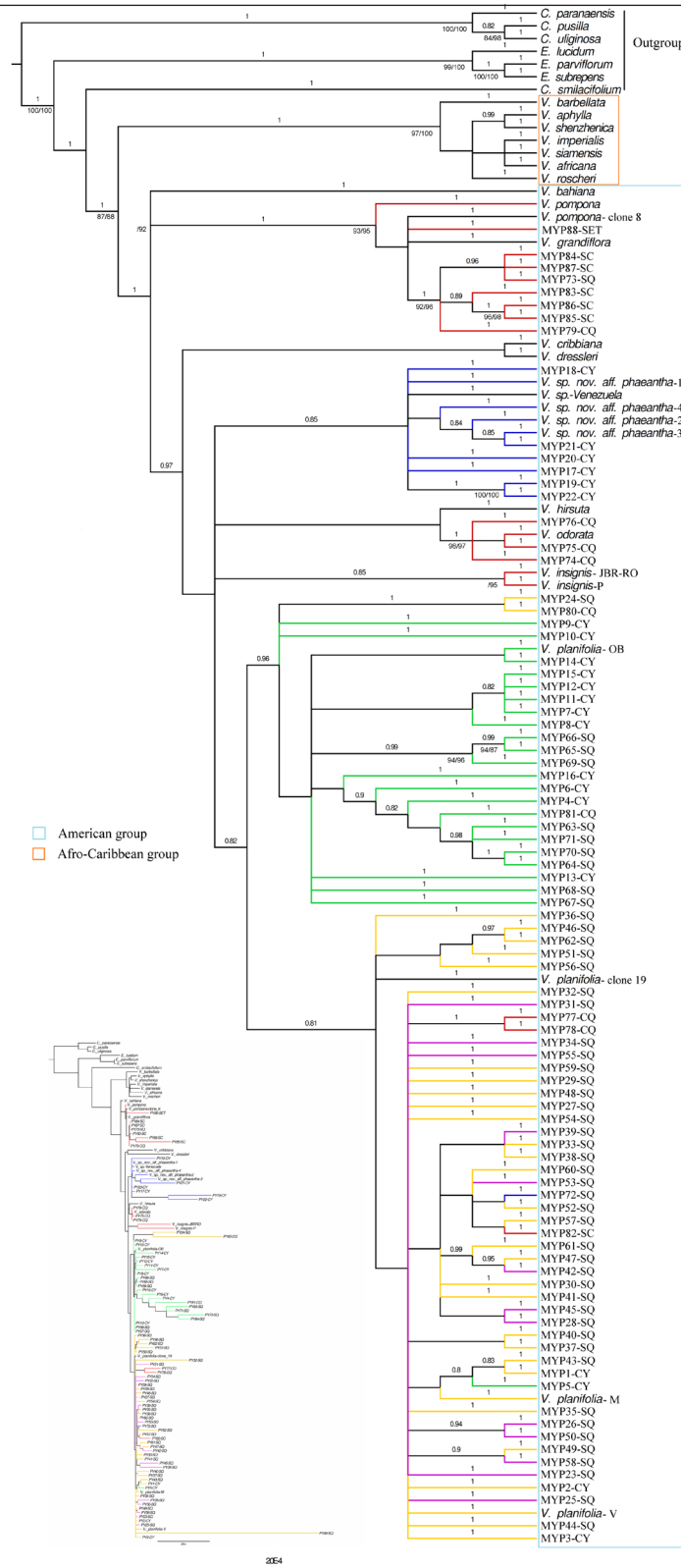

Figure 5. Vanilliodeae phylogeny. Tree with the highest posterior probability resulting from the Bayesian phylogenetic inference. ML bootstrap values to the left of the diagonal; Parsimony jackknife values to the right of the diagonal; and posterior probability values above the branches. The phylogram in the lower left corner shows the length of the branches. Trees were obtained from multiple sequence alignments of nucleotides of *Vanilla* species using ITS.

In our analyses, the most highly supported topology retrieves *Vanilla* as monophyletic (1.0:87/88) and sister to *Clematepistephium* (1.0), being both members of tribe Vanilleae, in congruence with previous analyses (*e.g*. Cameron 2009, Bouetard *et al*. 2010). The Afro-Caribbean clade, represented in this analysis by *Vanilla aphylla*, *V. shenzhenica*, *V. imperialis*, *V. siamensis*, *V. africana*, *V. roscheri* and *V. barbellata*, is strongly supported (1.0:97/100). This clade is sister to the remainder of the genus, composed in this study only of American species, hereafter referred to as the American Clade (AC). The Afro-Caribbean clade includes West Indian taxa as well as Paleotropical, mainly from Africa and tropical Asia, some of which are aphyllous.

The AC resolved as a trichotomy in our analyses, including *Vanilla bahiana* in an isolated position, along with a highly supported (p.p. 1.0) metaphyletic complex (*i.e*., monophyletic but lacking internal resolution) composed of *V. grandiflora*, *V. pompona* (Puebla and clone 8) and several MYP wild, sterile genotypes most likely attributable to *V. pompona* (1.0: 93/95). This clade is vegetatively characterized by succulent plants with large, massive leaves. It roughly corresponds with the "RED" group identified by the ISSR analysis. It is noteworthy that *V. pompona* has not been reported from the MYP, and if eventually confirmed by flowering material, it would constitute a first report for the area.

The third clade contains all the remaining species, represented by one or several samples, in the analysis. Within the third clade, two sister groups are readily identifiable. One of these clades is formed only by *V. dressleri* + *V. cribbiana* (DC-clade), composed of tropical rainforest, shade-loving species with thinly textured leaves and narrow stems. The DC-clade is sister to a poorly supported, metaphyletic group, consisting of four clades, one lacking support, whereas the other three have posterior probabilities equal or smaller than 0.85. Each of these clades roughly corresponds to additional four species of *Vanilla* known from the MYP. Similarly, these clades correspond with the color-coded groups recovered by the ISSR analyses. They are described below:

- The first, or BLUE-clade, includes all the samples positively identified as *Vanilla sp. nov. aff. V. phaeantha*, along with six wild MYP individuals, which most likely represent the same species. This clade includes a sample of a Venezuelan species (*Carnevali s.n*, CICY), also closely related to *V. phaeantha* but differing in floral details.
- RED-clade A: This highly supported (1.0:98/97) clade aggregates the standard sample of *V. odorata*, along with three wild MYP individuals (MYP76-CQ, MYP75-CQ, and MYP74-CQ). The analyses retrieve *V. hirsuta* as sister to the core of this clade. *Vanilla hirsuta* is thought to be a hybrid of *V. odorata* and *V. planifolia*. This relationship, however, is poorly supported.
- RED-clade B. This clade contains the two standard samples of *Vanilla insignis* and receives an intermediate to moderately high level of support (0.85: --/95).
- GREEN-YELLOW/FUCHSIA clade: This moderately supported (0.82) clade includes individuals positively identified or presumed to be *Vanilla planifolia*. Two subclades are retrieved, the first of which (green) includes the standard samples of *V. planifolia* var. "Oreja de Burro" along with 24 wild MYP individuals. Support is high at 0.96. The second subclade (yellow/fuchsia) contains the standard samples of the "Mansa" and "Variegata" cultivars of *V. planifolia*. It also includes 47 wild individuals, two of which are noteworthy (MYP1-CY, and MYP2-CY) because these have been seen in bloom and positively identified as *V. planifolia*.

Discussion

There is currently a void in our knowledge of the genus *Vanilla*. This vacuum ranges from basic biology including aspects of its systematics, phylogenetics, ecology, and physiology through more applied aspects such as the conservation status of its species (Gigant *et al*. 2011, Herrera-Cabrera *et al*. 2012, Azofeifa-Bolaños *et al*. 2014). At the most basic level, the identity, distribution, and circumscription of *Vanilla* taxa constitute the cornerstone to addressing problems related to the successful conservation and sustainable exploitation of the species of the genus.

Recently, it has become apparent that DNA molecular markers are powerful to help in the

characterization of *Vanilla* species. These markers provide a tool from the population to the species-level identification of individual plants (Nagy *et al*. 2012). This characteristic is particularly useful in this genus where plants are most frequently collected sterile, are intrinsically variable and phenotypically plastic according to growing conditions, are difficult to the get established and flowered in cultivation, and many species are vegetatively very similar or practically identical.

In *Vanilla*, only 47 of about 110 species recognized in the genus, have been included in any molecular analysis, and most of them only in phylogenetic studies (Soto-Arenas 2003, Minoo *et al*. 2008, Soto-Arenas & Cribb 2010; Soto-Arenas & Dressler 2010). A few studies have included purported *Vanilla* species (*e.g*. *V. tahitensis*; Besse *et al*. 2004) that were later more correctly identified as hybrid taxa (*e.g*. Lubinsky *et al*. 2008b). In other cases, these hybrids had proposed as new species only to be later more correctly recognized as nothotaxa (*e.g*. *V. hirsuta*; Soto-Arenas & Dressler 2010).

Molecular characterization for *Vanilla* species is particularly relevant for *V. planifolia*, the most commonly cultivated taxon of the genus and upon which most of the world's commercial production of vanilla based (Bory *et al*. 2008a; Lubinsky *et al*. 2008a). Furthermore, this characterization is very important because natural populations of this species have become severely threatened due to illegal extraction of wild individuals for introduction into plantations, both historically and recently (Soto-Arenas 2006). Moreover, destruction of natural habitats has recently become an even larger threat to the continued wild survival of *V. planifolia*. This study is the first to employ molecular markers to characterize a relatively large number of wild *V. planifolia* individuals, along with its sympatric congeners in an area of its natural distribution, the MYP.

Clustering pattern of Vanilla species in the MYP. The several approximations used in this study with ISSR markers retrieved largely congruent clustering patterns. *Vanilla planifolia* and *Vanilla sp. nov. aff. V. phaeantha* were clearly identified with the ISSR markers. The standard samples of *V. pompona*, *V. odorata*, and *V. insignis* were closely related to each other, probably because only a few individuals per species were tested. Three individuals of *V. odorata* identified by vegetative morphological features (mainly the narrow, falciform leaves), upon analyses, demonstrated to have a close genetic relationship with the group constituted by *V. pompona* - *V. insignis* - *V. odorata*. These individuals were included in the clade of *V. odorata* in the ITS phylogeny. The small number of wild-collected individuals in the MYP referable to these species suggest they are extremely rare in the area.

It is interesting to notice that the second highest delta *K* value obtained revealed a *K* = 7. These seven genetically different groups of individuals are ascribable to (and contain standard samples of) species previously known from the MYP (*V. planifolia*, *V. insignis*, *V. odorata*, and *Vanilla sp. nov. aff. V. phaeantha*). In the fifth group, a few wild MYP individuals clustered together with the *V. pompona* standard sample, and if morphologically confirmed, it would constitute the first record for this species that is mainly known from the western drainage of Megamexico. *Vanilla planifolia* var. "Oreja de Burro" was retrieved in the sixth group. And finally the seventh ancestral genotypic contribution (20–35 %, "green", as a yet unidentified wild genotype) is present in three individuals (MYP82, MYP83, and MYP84) from southern Campeche. These individuals include in their genetic makeup about 50 % *V. odorata* and a smaller genetic contribution from *V. insignis*. However, in the PCoA, these individuals are not genetically close to *V. odorata* even though featuring an ancestry coefficient exceeding 50 %. Similarly, this *K* = 7 brings together two wild individuals to *V. insignis* (MYP86-SC y MYP87-SC), which are placed by the PCoA closest to *V. pompona*. Interestingly, although there is no clear evidence that *V. pompona* occurs natively in the MYP (Carnevali *et al*., 2001, Soto-Arenas 2009), seven MYP wild individuals were identified as this species by the *K* = 7 (Figure 2B). We identified these sterile individuals as *V. planifolia* relying upon their vegetative structures. These two species are vegetatively similar, especially when juvenile, but eventually, *V. pompona* develops larger, heavier, much thicker leaves than those commonly found in *V. planifolia* (Figure 1-A and 1-E). Floral features of course, readily diagnose both species.

Regarding *Vanuilla planifolia*, the finding of three subgroups within this species is probably attributable to the inclusion of a larger number of samples with a similar vegetative morphol-

ogy, and therefore a greater probability of identifying an increased number of genotypes referable to the species and thus of detecting genetic or phylogenetic structure within the species. It is known that both self-compatible and self-incompatible individuals occur in *V. planifolia*. The last type, includes the entity commonly referred to as "Oreja de Burro" (Soto-Arenas 2009). Although the literature describes significant morphological features for the "Mansa" and the "Oreja de Burro" entities (Castillo & Engleman 1993), we were unable to identify wild MYP morphotypes unambiguously referable to them. However, the standard sample "Oreja de Burro" morphotype was retrieved widely apart from the standard samples of the "Mansa" and "Variegata" morphotypes in the Structure and the PCoA analyses, thus suggesting phylogenetic structure within *V. planifolia*.

A noteworthy finding from both the ISSR and the phylogenetic analyses was the precise identification of a group of individuals morphologically referred to *Vanilla sp. nov. aff. V. phaeantha*. This entity, albeit closely related to the West Indian *V. phaeantha* and initially identified as such (Soto-Arenas & Dressler 2010) it is easily differentiated by the presence of several prominent, deep yellow rows of teeth emerging from the labellum throat and reaching the labellum margin, which is also slightly fimbriated (Figure 1-D). This new taxon is in the process of being formally proposed.

Although the ISSR markers displayed high efficiency for the discrimination of individuals as belonging to clades or genetic groups associated with the standard samples of species involved in the study, some individuals were difficult to assign. A preliminary classification based on stem and leaf macro-morphology indicated that some wild individuals possibly belong to *V. insignis* but the genetic analysis indicates they are best referred (or genetically closer) to *Vanilla sp. nov. aff. V. phaeantha*, and secondly a cluster of eight individuals similar to *V. planifolia* but genetically related to the group comprised by *V. pompona-V. odorata-V. insignis*. These examples reveal how the comparison of individuals at different stages of development can be misleading in this genus, where plants are phenotypically plastic according to age and exposition, as previously pointed out by Putz &Holbrook (1986) and Ray (1990).

Phylogenetic relationships of the Vanilla species in the MYP. The most probable cladograms obtained in our phylogenetic inferences retrieved clades whose composition was entirely coherent with patterns of aggregation identified in the ISSR analysis. An example of this is the recovery of clades consisting of *Vanilla sp. nov. aff. V. phaeantha* and *V. planifolia* (Figs. 4 and 5). This finding reinforces the notion that the ISSR markers are robust as species-level discriminators of taxa, in this particular case in *Vanilla*.

Only a fraction of the species of the genus have been included in phylogenetic inferences, and there has not been an exhaustive phylogenetic analysis of *Vanilla* to date (Poeaim *et al*. 2011), the closest being the one by Soto-Arenas & Dressler (2010), which included only 15 species-level taxa. Furthermore, novelties within the genus are still being reported (*e.g. V. paludosa* Pansarin JM, Aguiar & A. C. Ferreira (Pansarin *et al*. 2012), *V. rivasii* Molineros, R. T. González, Flanagan & J. T. Otero (Molineros-Hurtado *et al*. 2014), *V. labellopapillata* A.K. Koch, Fraga, J.U. Santos & Ilk.-Borg. (Koch *et al*. 2013).

The resolution of the clades with the different methods evaluated agree with that reported by Cameron & Molina (2006), Pansarin *et al*. (2008), Cameron (2009), and Soto-Arenas & Dressler (2010). It is important to note that *Vanilla hirsuta* is synonymous with *V. tahitensis* J.W. Moore, which has a recent hybrid origin (*V. planifolia* x *V. odorata*; Soto-Arenas & Dressler, 2010). Therefore, its proximity to the individual genetic relationship of the standard sample of *V. odorata* and wild individuals identified as belonging to this species, it is not surprising. Moreover, *V. bahiana* described in Brazil, it appears to be very similar both morphologically and genetically with *V. phaeantha* (Soto-Arenas 2009). However, in each of the methods evaluated in this study, this species showed a closer relationship with *V. pompona*, which strengthens the hypothesis that *Vanilla sp. nov. aff. V. phaeantha* is a different species to *V. phaeantha*.

Except for *V. planifolia* "Oreja de Burro" (in the parsimony analysis), all the specimens referred by us to *V. planifolia* on morphological grounds constituted a monophyletic assemblage. Flowers of this entity have yet to be analyzed by us to confidently assess its relationships. Other individuals remained unidentified at this stage and placed at intermediate positions. The

phylogenetic analysis retrieved a clade consisting mainly of specimens of *Vanilla planifolia*. However, nested within it there were three individuals assigned to the *V. pompona-V. odorata-V. insignis* group (red group in $K = 5$) by the STRUCTURE analysis of the ISSR data. This same analysis (but with $K = 7$) grouped two of these individuals with *V. odorata* (red group). The remaining genotype is possibly a complex hybrid containing in its ancestry *V. odorata* (50% aqua of its genetic makeup), *V. sp*. (35 % green), and *V. insignis* (15 % yellow), where the unknown taxon is likely to be a wild genotype of *V. planifolia*. From this perspective, individuals that were retrieved in intermediate or conflicting positions could be interpreted either as belonging to additional *Vanilla* species. Yet to be formally identified (with floral material) in our area, or interspecific hybrids.

Natural hybridization has long been known to be frequent in several groups of the Orchidaceae (*e.g*. Adams & Anderson 1958) including *Vanilla* (Lubinsky *et al*. 2006). Examples include introgression of *V. claviculata* (W. Wright) Sw. with *V. barbellata* Rchb.f in Puerto Rico (Nielsen & Siegismund 1999, Nielsen 2000). Also, AFLP and SSR markers have suggested the possibility of interspecific hybrids between Neotropical species of *Vanilla* such as *V. bahiana*, *V. planifolia*, or *V. pompona* (Bory 2007, Bory *et al*. 2008b). So, interspecific hybridization may be common whenever closely related taxa occur in sympatry (Bory *et al*. 2008). Nevertheless, not all morphological variation (or polymorphisms) within *Vanilla* species is to be attributed to interspecific hybridization. As in other species, this variation could be due to genetic drift, disruptive selection, or even directional selection resulting from an evolutionary response to local conditions (Ackerman *et al*. 2011). Also, there is a component of this variation associated with phenotypic plasticity arising from differences in life stages and different growth conditions (van Kleunen & Fischer 2005).

Implications for the conservation and sustainable use of Vanilla. Wild relatives of crops constitute an important source of genes associated with the resistance to pests and diseases or with higher yields under a variety of conditions (Hajjar & Hodgkin 2007, Hunter & Heywood 2011, Fisher, 2012). They are also relevant as a source of nutrients, medicine and other traditional uses for communities and for the maintenance ecosystemic services (Hoyt & Brown 1992). However, before these wild relatives can be utilized in a sustainable manner, it is first necessary to determine which species belong.

In concordance with Verma *et al*. (2009), our study showed that the ISSR can be used to identify *Vanilla* species present in the MYP. We found higher levels of genetic variation in wild samples than commonly found in vanilla plantations and that might be a result of sexual reproduction and generation from seed, as indicated by the presence of pods in some of the wild populations studied. This point is an indicative of the relevance of these reservoirs of potentially important alleles for the improvement of *V. planifolia* as a crop, and as such, they should be protected.

Thus, the MYP, where the species is still plentiful (yet occurring as small, widely dispersed populations) should be considered of utmost importance for the conservation of *V. planifolia* as a genetically healthy wild taxon, and as a source of alleles for its improvement as a crop.

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