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Evaluation of molecular variability in germplasm of vanilla (*Vanilla planifolia* G. Jackson in Andrews) in Southeast Mexico: implications for genetic improvement and conservation

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Abstract

Molecular variability of vanilla (*Vanilla planifolia*) and three wild species was assessed to explore the possible sources of variation that can be used for crop improvement. A total of 154 ISSR loci were analysed by the UPGMA, assignment tests of individuals (STRUCTURE) and indices of genetic diversity. The assignment tests were done at two levels: first considering the four species and then only the accessions of *V. planifolia*. The molecular analysis indicated 99.3% polymorphism among all species and 70.45% within *V. planifolia*. The UPGMA showed the separation of these four species into three groups and grouped *V. planifolia* accessions into three subgroups. The more genetically differentiated accessions were of the Rayada morphotype and a wild accession was from Oaxaca, followed by a wild accession from Quintana Roo; all the commercial accessions of *V. planifolia* (Mansa morphotype) were grouped together. The STRUCTURE analysis differentiated between *V. planifolia* and the three wild species, and among the accessions of the Mansa and Rayada morphotypes and the wild accessions. The STRUCTURE analysis also indicated the presence of mixed individuals. These results are of great importance since the accessions of *V. planifolia* that are genetically more differentiated are the most threatened due to the scarcity of these individuals, the destruction of habitat

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and replacement by the commercial morphotype. These individuals should be salvaged and used to expand the genetic background of vanilla.

Keywords: commercial accessions; genetic diversity; ISSR; variegate; wild accessions

Introduction

The genus *Vanilla* Plum. ex Mill comprises 106 species (Soto Arenas and Cribb, 2010) distributed in the tropical regions of America, Asia, Africa and islands of the Indian and Pacific Oceans (Portéres, 1954). However, only three species have economic value: *V. planifolia*, *V. pompona* and *V. tahitensis*. Of these, commercial vanilla (*V. planifolia*) occupies 95% of the market (Bory *et al*., 2008a) and is used in food, perfumes and pharmaceuticals (Krushnamurthy *et al*., 2013).

V. planifolia is native to southern Mexico and Central America, and southern Mexico has been identified as a possible domestication centre (Soto Arenas, 1999; Lubinsky *et al*., 2008a). In Mexico, this wild material is a species of low abundance, with only one individual per 2–10 km^2 in well-conserved regions (Soto Arenas, 1999). This material is extremely important as a source of genetic variability to broaden the genetic base within the commercial cultivars of *V. planifolia*. As revealed by RAPD (random amplified polymorphic DNA) and AFLP (amplified fragment length polymorphism) studies, the majority of the cultivated plantations in the world are derived from one to three genotypes of the Mexican morphotype Mansa (Schluter *et al*., 2007; Minoo *et al*., 2008; Bory *et al*., 2008b).

This lack of variability is also thought to be a primary contributor to the low tolerance to biotic and abiotic stresses, which have caused fruit drop and significant yield losses worldwide. The main problems are high sensitivity to drought and temperature changes and susceptibility to diseases caused by *Fusarium*, *Phytophthora* and *Glomerella* and by cymbidium mosaic virus (CymMV) (Duval *et al*., 2006; Minoo *et al*., 2008; Bory *et al*., 2008a; Hernández, 2011). To improve the tolerance of commercial cultivars and reduce losses, the wild species and their nearest relatives must be genetically characterized in depth to make full use of the germplasm.

Although the genetic variability of *V. planifolia* has been evaluated with different markers, inter-simple sequence repeats (ISSRs) have not been sufficiently explored. ISSR markers combine most of the advantages of microsatellites (SSRs) with those of AFLPs and the universality of RAPDs. The dominant ISSR markers are highly polymorphic, can track sequences found abundantly in the eukaryotic genome, and evolve quickly, yielding robust results (Zietkiewicz *et al*., 1994; Fang and Roose, 1997; Reddy *et al*., 2002).

In a comparison between ISSR and RAPD markers to detect the genetic diversity between nine species of *Vanilla* (*V. planifolia*, *V. tahitensis* and seven wild Asian species), it has been found that the ISSR detected more polymorphisms (Verma *et al*., 2009). They have also been used to characterize the germplasm of various species such as *Zingiber officinale* (ginger) (Kizhakkayil and Sasikumar, 2010) and *Nelumbo* spp. (lotus) (Zuo *et al*., 2010).

The Instituto de Biotecnología y Ecología Aplicada (INBIOTECA-Universidad Veracruzana) has collected 20 accessions of wild and cultivated germplasm of *V. planifolia* in southeastern Mexico. The objective of this study was to characterize this germplasm molecularly and morphologically to identify the sources of intra- and interspecific variation that can be used in breeding programmes and in the conservation of this valuable resource.

Materials and methods

Plant material

The collection sites were selected according to the previous information that indicated the presence of genetic variation in *V. planifolia* (Soto Arenas, 1999). Also, we considered the information provided by producers about the presence of wild individual or different morphotypes of *V. planifolia*. Wild species of *Vanilla* were collected from the commercial cultivars of *V. planifolia* or at sites nearby.

In each site, we collected healthy cuttings of length 80 cm. The number of individuals collected in each site depended on the abundance and quality of plants. The cuttings were taken from different plants. Each plant was considered as an individual. Each individual or group of individuals of one species or morphotype from one site was labelled as an accession. Their passport data were registered and the cuttings were established under the greenhouse condition to integrate the genebank of INBIOTECA.

V. planifolia had both wild and cultivated accessions. The wild accessions were from Agua Blanca, Quintana Roo (QS) and an accession was from Armadillo Chico Oaxaca known as 'Rajada' because its fruits had a dehiscence more pronounced than other morphotypes of *V. planifolia*. The majority of the accessions of *V. planifolia* had the Mansa morphotype (Fig. 1). This is a commercial

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Fig. 1. Collection sites. ●, Cultivated accessions; +, wild accessions. The letters represent the names of the locations of the colleted sites: A, Armadillo Chico; B, La Bandera; E, Emiliano Zapata; F, Francisco Sarabia; J, Jalcomulco; JP, Joloapan; L, La Gran Lucha; M, Mesa de Guadalupe; O, Ojital Chico; P, Potingo; QC, Calderitas; QS, Agua Blanca; R, Rivera de Puntilla de Aldama; U, La Unión.

morphotype from which commercial plantations have been derived worldwide. Also, we collected the vanilla known as 'Colibri' from Oaxaca. Finally, we collected an uncommon commercial morphotype known as 'Rayada' or 'Acamaya', which had stems and leaves variegated with green and yellow stripes. It is similar in appearance to the genotype known as variegated in Reunion Island (Fig. 2).

We analysed 20 accessions that comprised 108 individuals of *V. planifolia*, one individual of *V. pompona*, three of *V. insignis* and one of *V.* affin. *odorata* from the INBIOTECA Genebank. These accessions were collected from 14 sites in the states of Veracruz, Oaxaca and Quintana Roo in Mexico (Table 1).

DNA extraction and generation of gene pools

DNA was extracted from young leaves following the method of Doyle and Doyle (1987). The DNA obtained was treated with 0.5 µl RNase (Invitrogen, Carlsbad, CA, USA) per 50 ml DNA suspension. The concentration and integrity of the DNA were verified by electrophoresis on 1.5% (w/v) agarose gels. As *V. planifolia* is a species that is established by cuttings, we can observe the presence of the same genotype in a plantation and even in several plantations. Therefore, we performed genetic pools to detect the genetic variation between the collection sites. For the molecular analysis of gene pools, the total number of individuals of a collection site was divided into two to three groups, each containing equal amounts of DNA from three to five individuals. The method of Gilbert *et al.* (1999) was used to generate 33 gene pools (Table 1).

DNA amplification

The reaction mixture for PCR was prepared in a final volume of 20 µl, containing 30 ng genomic DNA, 15.2 µl ultrapure water, 2 µl of 1× *Taq* buffer, 0.8 µl $MgCl_2$ (50 mM), 0.4 µl dNTPs (10 mM), 0.4 µl primer (10 mM) and 0.2 µl of *Taq* polymerase (5 U/ml) (Promega, Madison, WI, USA). A negative control, without any genomic DNA, was run to rule out any self-amplification of primer or genomic DNA contamination. Six ISSR primers were used: five were designed by Verma *et al.* (2009) and the primer (017) was designed by Martínez-Castillo (2005) (Table 2). Amplifications were performed in a

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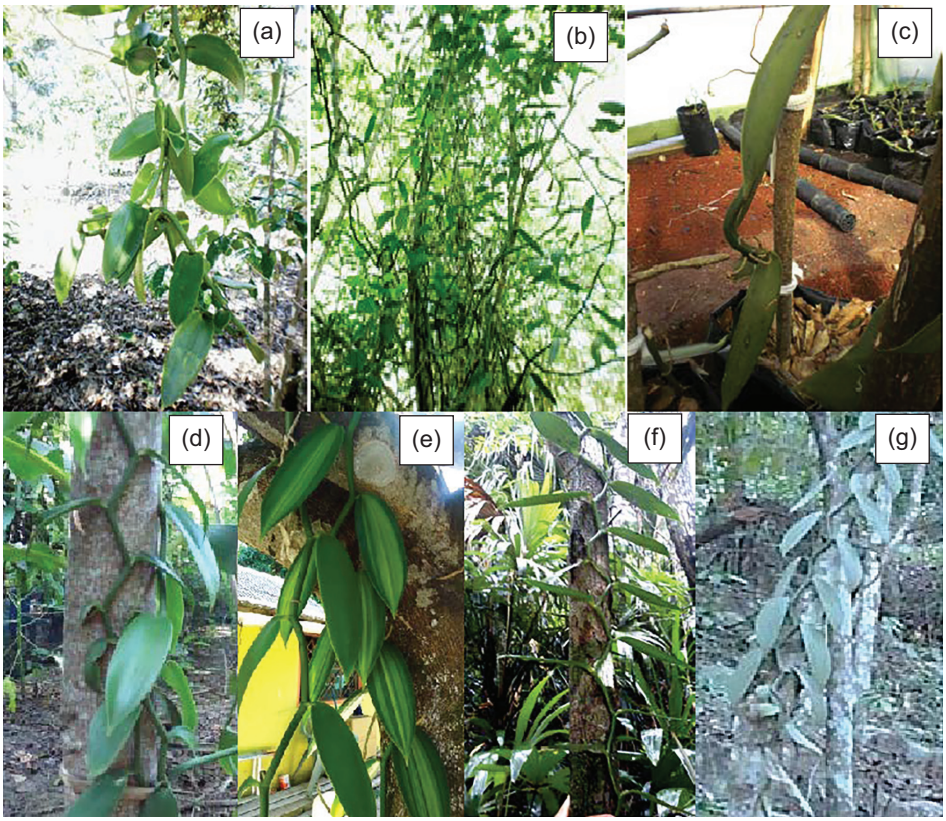

Fig. 2. Plant material. (a) *V. pompona*, (b) *V. insignis*, (c) *V.* affin. *odorata*, (d) *V. planifolia* (Mansa morphotype), (e) *V. planifolia* (Rayada morphotype), (f) *V. planifolia* (wild accessions from Quintana Roo) and (g) *V. planifolia* (wild accessions commonly known as Rajada).

GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) using the following programme: an initial denaturation step of 7 min at 94°C, 34 cycles of 30 s of denaturation at 94°C, 45 s alignment with temperatures between 47 and 54°C depending on the primer used, and 63 s extension at 72°C and a final extension of 10 min at 72°C.

Separation and visualization of products

Each amplification product (5 µl) was mixed with 4 ml bromophenol blue and separated electrophoretically on 6% polyacrylamide gels for 13 h at 350 V in a vertical electrophoresis chamber (SQ3 sequencer; Hoefer Scientific Instruments, San Francisco, CA, USA). Bands were visualized using the silver staining technique (Martinez-Castillo *et al.*, 2008).

Data analysis

For the four species, the clustering patterns and genetic relationships among accessions were represented by dendrograms constructed using the Jaccard similarity coefficient and the UPGMA (unweighted pair group method with arithmetic mean) algorithm. Their reliability was assessed by bootstrap analysis with 1000 replicates using the program Past v. 2.17 (Hammer *et al.*, 2001). Thereafter, an individual assignment test was performed using the program STRUCTURE v. 2.3.3 (Pritchard *et al.*, 2000). STRUCTURE uses a clustering model based on a Bayesian approach to infer the number of *K* groups (i.e. populations or accessions) by means of an adjustment to the Hardy–Weinberg and linkage disequilibrium. The model used was that of ancestry admixture with correlated allele frequencies. Values of *K* from 1 to 10, with five independent simulations for each value of *K*, were evaluated. To obtain reliable data, the analyses were made with replications for a burn-in period of 10,000 and a run length of 100,000. With the results generated, the optimal value of *K* (number of genetically distinct groups or subgroups) was determined according to the method of Evanno *et al.* (2005) using the program Structure Harvester v. 0.6.93 (Earl and VonHoldt, 2012). Finally, the graphs of ancestry for the optimum value of *K* were generated using STRUCTURE.

For *V. planifolia*, the following indices of genetic diversity were estimated using the Popgene program

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Table 1. Location and accession information and number of gene pools by site for individuals used in the molecular analysis

Species	Status of accession (morphotype)	Abbreviation	Location, municipality, state	No. of gene pools per accession (no. of individuals per gene pool)
V. pompona	W	VP	Mesa de Guadalupe, Alto Lucero, Ver.	1 (1)
V. insignis	W	VIJ	El Naranjo, Jalcomulco, Ver.	1 (1)
V. insignis	W	VIE	El Palmar, Emiliano Zapata, Ver.	1 (1)
V. insignis	W	VIA	Armadillo Chico, Valle Nacional, Oax.	1 (1)
V. affin. *odorata*	W	VOC	Calderitas, Othon P. Blanco, QR.	1 (1)
V. planifolia	W	RJ	Armadillo Chico, Valle Nacional, Oax.	1 (1)
V. planifolia	W	QS	Agua Blanca, Othon P. Blanco, QR	1 (5), 1 (5)
V. planifolia	C (RY)	RYP	Potingo, Nautla, Ver.	1 (1)
V. planifolia	C (RY)	RYU	La Unión, Tihuatlan, Ver.	1 (1)
V. planifolia	C (Co)	A	Armadillo Chico, Valle Nacional, Oax.	1 (5), 1 (5), 1 (5), 1 (1)
V. planifolia	C (Co)	L	La Gran Lucha, Valle Nacional Oax.	1 (5), 1 (5)
V. planifolia	C (M)	B	La Bandera, Actopan, Ver.	1 (5), 1 (5)
V. planifolia	C (M)	QC	Calderitas, Othon P. Blanco, QR	1 (3)
V. planifolia	C (M)	E	El Palmar, Emiliano Zapata, Ver.	1 (5), 1 (5)
V. planifolia	C (M)	F	Francisco Sarabia, Papantla, Ver.	1 (5), 1 (5)
V. planifolia	C (M)	J	El Naranjo, Jalcomulco, Ver.	1 (4)
V. planifolia	C (M)	JP	Joloapan, Papantla, Ver.	1 (3), 1 (4)
V. planifolia	C (M)	M	Mesa de Guadalupe, Alto Lucero, Ver.	1 (1)
V. planifolia	C (M)	O	Ojital Chico, Papantla, Ver.	1 (3), 1 (3)
V. planifolia	C (M)	P	Potingo, Nautla, Ver.	1 (3), 1 (3)
V. planifolia	C (M)	R	Rivera de Puntilla de Aldama. Nautla, Ver.	1 (5), 1 (5)

W, wild; VP, *V. pompona*; Ver., Veracruz; VIJ, *V. insignis* Jalcomulco; VIE, *V. insignis* Emiliano Zapata; VIA, *V. insignis* Armadillo Chico; Oax., Oaxaca; VOC, *V.* affin. *odorata*, Calderitas; QR, Quintana Roo; RJ, Rajada; QS, Agua Blanca; C, crop; RY, Rayada morphotype; RYP, Rayada Potingo; RYU, Rayada La Unión; Co, Colibrí; A, Armadillo Chico; L, La Gran Lucha; (M), Mansa morphotype; B, La Bandera; QC, Calderitas; E, Emiliano Zapata; F, Francisco Sarabia; J, Jalcomulco; JP, Joloapan; M, Mesa de Guadalupe; O, Ojital Chico; P, Potingo; R, Rivera de Puntilla de Aldama.

Table 2. Results of molecular characterization using ISSR molecular markers, separated on polyacrylamide gels and silver-stained, at two levels [among four *Vanilla* species (*V. planifolia, V. insignis, V.* affin. *odorata* and *V. pompona*) and among *V. planifolia* accessions]

Primer (sequence)	Total bands		Percentage of polymorphic bands		Range of band sizes (bp)	
	All four species	*V. planifolia*	All four species	*V. planifolia*	All four species	*V. planifolia*
T06 (5′-AGAGAGAGAGAGAGT-3′)	26	13	100	53.9	201–2057	420–2057
C03 (5′-TGTCACACACACACAC-3′)	32	19	100	84.2	407–2583	510–2583
T05 (5′-CGTTGTGTGTGTGT-3′)	14	9	100	11.1	434–1634	434–1634
C07 (5′-GAGAGAGAGAGAGAC-3′)	29	11	100	63.6	327–2829	432–1476
C09 (5′-CAGATGGGAGTCAAGTCAAC-3′)	30	24	100	95.8	434–3196	434–3196
017 (5′-GACACGACAC-3′)	23	11	95.7	66.7	446–2952	446–2706
Total	154	87	99.3	71.26	201–3196	420–3196

v. 1.31 (Yeh and Boyle, 1999): number of polymorphic loci; percentage of polymorphic loci; dominant nature of the ISSR; Shannon–Weaver diversity index. The analysis was conducted at three levels, within an accession, between groups and among all accessions, considering each accession as an independent population. Results of UPGMA and STRUCTURE were considered for determining the groups.

Results

The six primers generated 154 loci, of which 99.3% were polymorphic. Loci ranged from 201 to 3196 bp. An average of 25.6 bands was obtained per primer. Primers C03 and C09 detected more loci (Table 2). Within the group of *V. planifolia*, 88 loci were obtained, with 70.45% of these being polymorphic. The size of the sampled loci ranged from 420 to 2706 bp (Table 2).

The UPGMA (Fig. 3) showed three distinct groups for the four species of *Vanilla* evaluated. Group I represents *V. pompona* as a distant group separated from the other accessions. Group II comprised four accessions of the species *V. insignis* and *V.* affin. *odorata*. Group III comprised all accessions of *V. planifolia*, with three subgroups designated as IIIA, IIIB and IIIC. Subgroup IIIA comprised individuals of the Rayada morphotype (variegata) (RYP and RYU) and a wild individual of Oaxaca (RJ). Subgroup IIIB comprised wild individuals of Agua Blanca, Quintana Roo, and subgroup IIIC comprised all cultivated accessions of the Mansa morphotype from Veracruz and Quintana Roo, and accessions of Oaxaca (vanilla colibrí).

The method of Evanno *et al.* (2005) yielded an optimal value of $K = 2$, indicating the existence of two genetically distinct groups. Figure 4 shows the grouping pattern generated by STRUCTURE based on $K = 2$. Individuals of wild species (*V. insignis*, *V.* affin. *odorata* and *V. pompona*) belonged to one group and all accessions of *V. planifolia* belonged to the other group. Figure 4 also shows the existence of some accessions with some degree of ancestry from both groups. These are the wild accession from Oaxaca (RJ) (approximately 50% ancestry from the group of wild species and the group of *V. planifolia*) and individuals of the Rayada morphotype of *V. planifolia* (RYP and RYU).

Genetic diversity in V. planifolia

The accession of *V. planifolia* with the greatest genetic diversity was Armadillo Chico (A) of Oaxaca, with a Shannon–Weaver diversity index of 0.0957 and 14.94% polymorphism, followed by Ojital Chico from Papantla Veracruz with a Shannon index of 0.0487 and 8.05%

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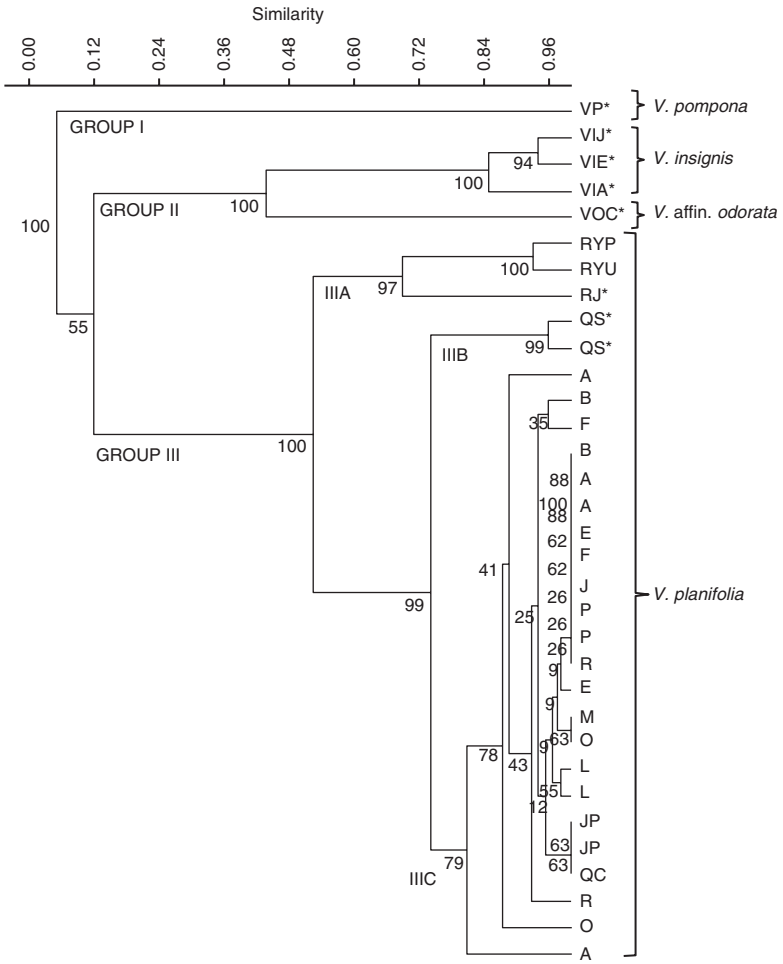

Fig. 3. Dendrogram generated from the Jaccard similarity coefficient. The numbers indicate the values generated with 1000 bootstrap repetitions. The asterisk (*) denotes the accessions of wild origin. Group I consisted of VP (*V. pompona*). Group II consisted of VIJ (*V. insignis* Jalcomulco), VIE (*V. insignis* Emiliano Zapata), VIA (*V. insignis* Armadillo Chico) and VOC (*V.* affin. *odorata*, Calderitas). Group III consisted of all the accessions of *V. planifolia*. Subgroup IIIA consisted of RYP (Rayada Potingo), RYU (Rayada La Unión) and RJ (Rajada Armadillo Chico). Subgroup IIIB consisted of QS (Agua Blanca, Quintana Roo). Subgroup IIIC consisted of A (Armadillo Chico), B (La Bandera), E (El Palmar), F (Francisco Sarabia), J (Jalcomulco), JP (Joloapan), L (La Gran Lucha), M (Mesa de Guadalupe), O (Ojital Chico), P (Potingo), R (Rivera de Puntilla de Aldama) and QC (Calderitas).

polymorphism at the group level. The greatest genetic diversity according to the Shannon index was found in group IIIA ($R = 0.1536$). Although group IIIC had a higher percentage of polymorphic loci (31.03%), group IIIB had less genetic diversity with a Shannon index of 0.0139. Considering all accessions, a Shannon index of 0.2143, with 71.26% of polymorphic loci, was obtained (Table 3).

Discussion

Molecular characterization of all species

The use of the ISSR in conjunction with polyacrylamide gel electrophoresis is a technique that provided a significant increase in the mean number of bands. Verma *et al.* (2009) used agarose gels, obtaining a mean of 13.4 bands with primers C03, C07, C09, T05 and T06. In our study using polyacrylamide gels and those five primers, the mean was 25.6. The products with primer T05 were the least polymorphic and increased by only three bands in the polyacylamide gels.

The four interspecific relationships, the four species of *Vanilla* could be differentiated despite their close relationship. The genetic relationships among the species closely related to *V. planifolia* have been studied with different molecular markers (AFLP, RAPD, ITS (internal transcribed spacer) and plastid *trnH-psbA*). Although results varied in previous reports, *V. odorata* and *V. tahitensis* appear to be the closest species to

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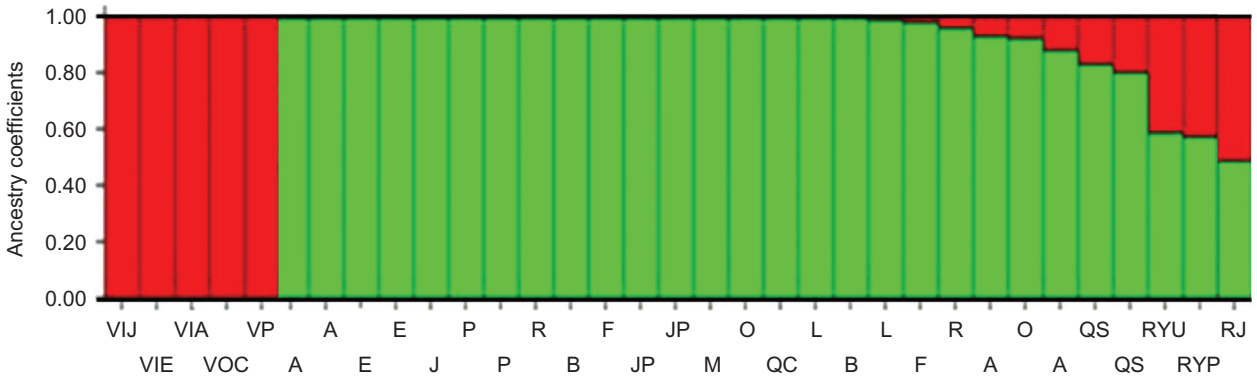

Fig. 4. Analysis of assignment of individuals. The red group comprised the accessions of wild origin: VIJ, *V. insignis* Jalcomulco; VIE, *V. insignis* Emiliano Zapata; VIA, *V. insignis* Armadillo Chico; VOC, *V.* affin. *odorata*, Calderitas; VP, *V. pompona* Mesa de Guadalupe. The green group comprised the cultivated accessions of *V. planifolia*: A, Armadillo Chico; B, La Bandera; E, El Palmar; F, Francisco Sarabia; J, Jalcomulco; JP, Joloapan; L, La Gran Lucha; M, Mesa de Guadalupe; O, Ojital Chico; P, Potingo; QC, Calderitas; R, Rivera de Puntilla de Aldama. Mixed individuals are as follows: RJ, Rajada Armadillo Chico; RYP, Rayada Potingo, Rayada morphotype (variegata); RYU, Rayada La Unión, Rayada morphotype (variegata); QS, Agua Blanca; A, Armadillo Chico; O, Ojital Chico; R, Rivera de Puntilla de Aldama.

V. planifolia. *V. tahitensis* is an interspecific hybrid between *V. planifolia* and *V. odorata* (Soto Arenas, 1999; Schluter *et al*., 2007; Lubinsky *et al*., 2008a, b; Bory *et al*., 2008b).

Molecular characterization of V. planifolia

With respect to the genetic diversity of *V. planifolia*, our results indicate that the wild individual from Armadillo Chico, Oaxaca (RJ) was the most genetically differentiated accession. This accession is locally known as 'Rajada' because its fruits have a dehiscence more pronounced than other morphotypes of *V. planifolia*. Also, the fruit dehiscence starts when the fruit is not yet fully mature, so the producers consider that these fruits have no commercial value. It is important to mention that this individual was the only one present in the field crop where it was collected; the producers were exterminating it because such dehiscence was undesirable for commercialization. From the data generated in this study, we cannot determine whether this individual represents a new species of *Vanilla* or is a wild individual of *V. planifolia*.

Furthermore, the accessions of the morphotype Rayada (acamaya or variegata) are extremely important for breeding programmes and conserving diversity. In this study, we found the first clear molecular distinction between the Rayada and Mansa morphotypes. The Rayada accessions (RYP and RYU) had numerous unique loci, which were shared only with the wild accession of Oaxaca (Rajada RJ). Therefore, we can rule out the suggestion that the Rayada morphotype has arisen by somatic mutations from a Mansa morphotype as reported by other authors (Soto Arenas, 1999; Bory *et al*., 2008b). In addition, the genetic relationship of the Oaxaca wild individual may be an indication that the Rayada morphotype is the product of sexual reproduction between the Mansa morphotype and some wild material. However, a thorough investigation is required to classify the origin of the Rayada morphotype in Mexico. However, it is noteworthy that this pattern has not been observed in other studies, which could be due to the type of marker (isozyme, AFLP and RAPD) used in other studies or to different Rayada genotypes studied (Soto Arenas, 1999; Schluter *et al*., 2007; Bory *et al*., 2008b). Although these results are very interesting and novel, we should not rule out the possibility that epigenetic variation in Rayada vanilla (variegata) could be responsible for the change in leaf colour in response to light. We observed that a variegated plant can have green shoots when it is exposed to a higher intensity of light. Thus, a plant can present variegated shoots and completely green.

Another important source of genetic variation was observed in the wild material from Agua Blanca, Quintana Roo. This material has several unique loci but is more close to cultivated accessions that are of the Rayada morphotype and the wild individual from Oaxaca. We must take into account the hypothesis of Lubinsky *et al*. (2008a) that vanilla cultivation may have originated in the Maya region and subsequently was successfully adapted in the Veracruz region under the care of the Totonacos. However, we must also consider that our results may indicate that the material from Veracruz is more closely related to the material from Oaxaca. Other studies of genetic diversity in vanilla have suggested that individuals in the states of

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Table 3. Indices of diversity among 16 accessions of *V. planifolia* collected from three states in Mexico

	I	No. of polymorphic loci	Percentage of polymorphic loci
Accessions			
A	0.0957	13	14.94
B	0.0139	2	2.3
E	0.007	1	1.15
F	0.0278	4	4.6
J	0	0	0
JP	0	0	0
M	0	0	0
L	0.007	1	1.15
O	0.0487	7	8.05
P	0	0	0
QC	0	0	0
QS	0.0139	2	2.3
R	0.0209	3	3.45
RYP	0	0	0
RYU	0	0	0
RJ	0	0	0
UPGMA groups			
IIIA	0.1536	21	24.14
IIIB	0.0139	2	2.3
IIIC	0.0779	27	31.03
All accessions	0.2143	62	71.26

I, Shannon index; A, Armadillo Chico; B, La Bandera; E, Emiliano Zapata; F, Francisco Sarabia; J, Jalcomulco; JP, Joloapan; M, Mesa de Guadalupe; L, La Gran Lucha; O, Ojital Chico; P, Potingo; QC, Calderitas; QS, Agua Blanca; R, Rivera de Puntilla de Aldama; RYP, Rayada Potingo; RYU, Rayada La Unión; RJ, Rajada.

Oaxaca, Chiapas and Quintana Roo are genetically very similar (Soto Arenas, 1999; Schluter *et al.*, 2007). However, we found marked differences between the genotypes of Quintana Roo and Oaxaca, so a more detailed analysis of the diversity of vanilla in the Maya region and northern Oaxaca is needed.

The northern region of Oaxaca has been cited as harbouring great diversity in the genus *Vanilla* (Schluter *et al.*, 2007; Lubinsky *et al.*, 2008a; Soto Arenas and Dressler, 2010). Oaxaca is the second largest producer of vanilla in Mexico. and therefore it has a greater influence on the forms of production in the Veracruz region. In this region, cuttings of *V. planifolia* from wild individuals (known as vanilla colibrí) are grown with commercial cuttings from Veracruz. In our results, we found that some accessions from Armadillo Chico are genetically distinct individuals, while others are genetically identical to the Veracruz accessions. Individuals from La Gran Lucha, Oaxaca, are genetically separated from other accessions, but are very close to the Veracruz material. However, this region of Oaxaca remains strategic as a source of diversity for the genus *Vanilla*, and studies on the population genetics of the material from this area are needed to help determine strategies for the use and conservation of wild genotypes.

For commercial individuals grown in the state of Veracruz, we note that the region of Nautla has been popular as a commercial source of cuttings, which is reflected by accessions from different backgrounds having the same genetic basis. We know that vanilla production at El Palmar (E) was established over 50 years ago in central Veracruz from five cuttings from San Rafael (near Nautla). However, we observed some differences between the gene pools of this accession that can be attributed to somatic mutations. Such mutations also explain the differences between accessions from Rivera de Puntilla de Aldama (R), La Bandera (B) and Francisco Sarabia (F). Variability in *V. planifolia* generated by somatic mutations has been reported by several authors for accessions from the Asian-Pacific region (Duval *et al.*, 2006; Minoo *et al.*, 2008; Bory *et al.*, 2008b). The material grown in Calderitas Quintana Roo (QC) is identical genetically to the material grown in Joloapan Veracruz (JP). This site is very close to the town of Primero de Mayo from Papantla, Veracruz, which is also a popular source of cuttings.

Conclusions

The ISSR analysis used in conjunction with other markers allowed us to differentiate the species of *Vanilla* and provided a better detail to detect the variation among the accessions of *V. planifolia*. Thus, we consider this technique to be suitable for characterizing germplasm and to study the population genetics of *Vanilla*.

Some authors have shown the potential importance of using other species of *Vanilla* as sources to genetically improve *V. planifolia*, e.g. for breeding for tolerance against various pathogens. For example, *V. andamanica* (a distant species from *V. planifolia*) is tolerant to *Fusarium oxysporum* and is being used in hybridization work with *V. planifolia* (Minoo *et al.*, 2008). Duval *et al.* (2006) mentioned *V. pompona* and *V. tahitensis* as species tolerant to CymMV. Other close wild relatives of *V. planifolia* (e.g. *V. insignis*) should also be explored for any evidence of tolerance to biotic and abiotic stresses. Promising materials should then be characterized and evaluated for use as parental lines for hybridization work.

Among the accessions of *V. planifolia* are at least four that have greater genetic variability that can be used to improve the genetic diversity in the crop. As discussed already, the most diverse materials proved to be a wild individual from Oaxaca (Rajada), two

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accessions of the Rayada morphotype (variegata) and a wild accession from Quintana Roo.

The individual with dehiscent fruits from Oaxaca and the accession from Quintana Roo should be analysed primarily for their tolerance to factors that negatively affect cultivated *V. planifolia* and to broaden the genetic base of the cultivated species.

Vanilla Rayada accessions (variegata) are of great interest for genetic improvement because of their variability and commercial value (similar to the Mansa morphotype). The Rayada morphotype is unfortunately rare in Mexico. Among the Asociación Veracruzana de Vainilleros are only four producers that have any individuals of this morphotype, and Rayada accessions are also present in the genebanks of INBIOTECA and Benemérita Universidad Autónoma de Puebla. In other vanilla-producing areas, such as the Asian-Pacific region, there are more records of variegated accessions. Accessions of the Rayada morphotype and those of wild origin should be given priority in conservation programmes because they are the most locally endangered ones due to the scarcity of individuals, destruction of habitat and replacement by the commercial morphotype. Together with breeding programmes in INBIOTECA, we work on micropropagation (Ramos-Castellá *et al.*, 2014) and *in vitro* conservation (Bello-Bello *et al.*, 2015). This is intended to promote a comprehensive programme of conservation and breeding of this important resource.

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