Chapter 4

Microsatellite Markers Confirm Self-Pollination and Autogamy in Wild Populations of *Vanilla mexicana* Mill. (syn. *V. inodora*) (Orchidaceae) in the Island of Guadeloupe

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Additional information is available at the end of the chapter

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Abstract

The study aimed at evaluating the mating system of *Vanilla mexicana* (Orchidaceae) in natural populations in the island of Guadeloupe. A total of 132 *V. mexicana* samples were collected from 12 sites in Guadeloupe (Basse-Terre). Five other samples coming from Martinique and Mexico completed our analyses. Reproductive biology experiments excluding pollinators with bagged flowers revealed 53.9% fruit set, a value identical to the natural fruit set measured in the populations. These results suggested that *V. mexicana,* unlike most *Vanilla* species, was reproducing by self-pollination and autogamy. Due to lack of specific DNA markers for *V. mexicana*, microsatellite markers, previously developed in other *Vanilla* species, were used for the genetic analyses. Only 6 out of the 33 markers tested were transferable and polymorphic in *V. mexicana*. A panel of 51 *V. mexicana* samples genotyped with 3 polymorphic loci was finally retained for Guadeloupe population genetic analyses. A heterozygote deficiency was detected, and the selfing rate was estimated to 74%. These results confirmed the reproductive biology results as self-pollination and autogamy were the most likely explanation for this deficit. Results were compared to those from allogamous wild *Vanilla* species and discussed in the light of suggested existence of a pollinator for *V. mexicana* in other areas (Mexico).

Keywords: autogamy, genetic diversity, Guadeloupe, microsatellites, *Vanilla mexicana*

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1. Introduction

Knowledge and management of agricultural genetic resources (AGR) and of their wild relative species [referred to as Crop Wild Relatives (CWR)] are of major importance to ensure the preservation of natural resources, development of sustainable agriculture and food security in a global climate change context. The extremely low genetic diversity in the cultivated vanilla species *V. planifolia* G. Jacks. worldwide has been demonstrated [1–5], and this genetic erosion is a major limit for genetic improvement, particularly with regard to pathogen outbreaks. Vanilla wild relatives can be used for breeding interspecific hybrid varieties. For example, resistance to the virus CymMV was reported for *V. pompona* Schiede [6], and resistance to the fungus *Fusarium* was reported for *V. pompona, V. phaeantha* Rchb. f., *V. barbellata* Rchb. f., *V. aphylla* Blume, *V. andamanica* Rolfe, *V. crenulata* Rolfe, and *V. bahiana* Hoehne [7–10]. As *V. planifolia* wild populations, which are in danger of extinction in Mexico [11], some of the populations of vanilla wild relatives are threatened by deforestation, over-collection, and climate change [12]. This is the case for example for *V. humblotii* Rchb. f., endangered (EN) in Mayotte [13]. Vanilla wild relatives therefore deserve special attention. To date, there is still an important lack of knowledge of genetics and ecology, including breeding systems of vanilla CWR, despite their importance for the improvement of vanilla.

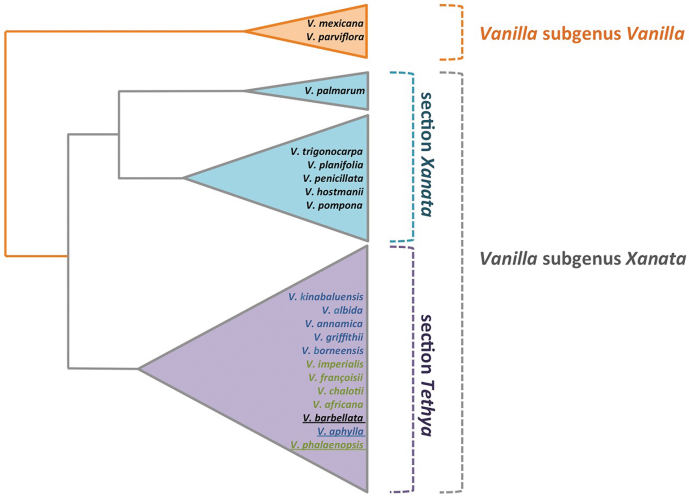

Figure 1. Synthetic representation of the phylogenetic groups in the genus *Vanilla* in relation to the new taxonomic classification proposed by Soto Arenas and Cribb [16]. The 20 species groups defined [16] are also indicated within each clade (without phylogenetic meaning in their order of appearance). American species are in black, African species in green, and Asian species in blue, and aphyllous species are underlined.

Vanilla mexicana Mill. is a distant wild relative of the cultivated vanilla species *Vanilla planifolia*. The *Vanilla* Plum. ex Mill. genus is a primitive lineage in the Orchidaceae family, Vanilloideae subfamily, Vanilleae tribe, and Vanillinae subtribe [14, 15]. In 2010, Soto Arenas and

http://dx.doi.org/10.5772/64674

Cribb [16] proposed a revision of the early taxonomic classification by Portères [17] of the genus *Vanilla*, based on eco-morphological and phylogenetic data, which has been confirmed by other independent studies [18]. This major work proposed taxonomic keys to resolve the 100+ species recognized in the genus into 20 very handy morphological informal species groups, which can in turn be classified phylogenetically into two subgenera, one being the subgenus *Vanilla* including *V. mexicana* (**Figure 1**). The subgenus *Vanilla* comprises two species morphological groups: the *V. parviflora* and *V. mexicana* groups. The *V. mexicana* group includes the species *V. mexicana*, but also *V. costaricensis* Soto Arenas ined, *V. guianensis* Splitg., *V. inodora* Schiede, *V. martinezii* Soto Arenas ined, *V. methonica* Rchb. f. & Warsz., *V. oroana* Dodson, and *V. ovata* Rolfe. These species are distributed in the neotropics from South America, Central America to southern Mexico [16]. Although distinct in this revision [16], but as suggested [17] and confirmed [19], *V. mexicana* and *V. inodora* should be considered as synonymous species.

Geographically, *V. mexicana* is distributed in the northern part of South America (Venezuela, Trinidad, and Tobago), Central America, the Caribbean islands (Cuba, Puerto Rico, Haïti and Guadeloupe), towards Florida in North America [16, 17] (**Figure 2**). Within our current efforts to determine the reproductive biology and genetic diversity in vanilla CWR, which led us so far to study *V. roscheri* Rchb. f. in South Africa [20] and *V. humblotii* in Mayotte [13, 21], we focused on wild populations of *V. mexicana* occurring in the island of Guadeloupe (French west indies) to unravel its mating system.



Figure 2. Geographical distribution of *V. mexicana* (from [16, 17]).

The vast majority of *Vanilla* species displays a mixed reproductive mode [1, 4] with both asexual and sexual reproduction. *Vanilla* species are hemi-epiphytic vines, and asexual reproduction is performed by means of natural stem cuttings [1]. It is a very efficient way for the plant to develop settlements and implies that vanilla plants are long-lived as they can indefinitely propagate. In *V. humblotii* in the island of Mayotte, it was shown that 12.5% of the individuals in the Sohoa forest were vegetative clones deriving from vegetative reproduction [13], a similar

value to what was observed in Puerto Rico for *V. claviculata* Sw. and *V. barbellata* with 6–25% vegetative clones [22]. Spatial genetic analysis also revealed that vegetative clones showed a phalanx (aggregated) distribution and the average maximal clonal patch size was measured at 4.6 ± 2.7 m in *V. humblotii* [13]. However, these patches can be much bigger as observed in Mexico for *V. planifolia* G. Jackson with the same vegetative clone covering up to 0.2 ha [4, 23].

In *Vanilla* species, sexual mating system is either allogamous or autogamous (**Table 1**), the most common system being allogamous and pollinator-dependent. Allogamous species are, however, self-compatible as demonstrated by manual self-pollination experiments giving up to 100% fruit set in *V. barbellata*, *V. claviculata*, *V. dilloniana* Correll, and *V. poitaei* Rchb. f. [24], *V. chamissonis* Klotzsch [25], *V. roscheri* [20], *V. humblotii* [13] and many other species of the genus (our unpublished self-pollination experiments in the shade-houses of BRC Vatel [26]). Manual self-pollination is also the method used to produce fruits in *V. planifolia* cultivation areas in the absence of natural pollinators. Allogamy is only guaranteed because of the floral structure presenting a rostellum, acting as a physical barrier between male and female reproductive organs [4]. Pollinators are needed to ensure pollination of allogamous species. As reviewed in [4], *Vanilla* subgenus *Xanata* section *Xanata* American species are most likely mainly pollinated by Euglossine bees.

***Vanilla* subgenus**	**Section**	**Taxonomic group**	**Species**	**Natural fruit set (%)**	**Mating system**
Xanata	*Tethya*	*V. africana*	*V. crenulata*	0.0[a,b]	Allo
		V. barbellata	*V. barbellata*	18.2[c]	Allo
		V. barbellata	*V. claviculata*	17.9[c]	Allo
		V. barbellata	*V. dilloniana*	14.5[c]	Allo
		V. barbellata	*V. poitaei*	6.4[c]	Allo
		V. phalaenopsis	*V. humblotii*	0.8[d]	Allo
		V. phalaenopsis	*V. roscheri*	26.3[e]	Allo
Xanata	*Xanata*	*V. pompona*	*V. chamissonis*	15.0[f]	Allo
		V. pompona	*V. pompona* ssp. *grandiflora*	0.9[g]	Allo
		V. planifolia	*V. cristato-callosa*	6.6[g]	Allo
		V. planifolia	*V. planifolia*	0.1–1.0[c,h,i,j]	Allo
		V. planifolia	*V. ribeiroi*	1.1[g]	Allo
		V. palmarum	*V. bicolor*	42.5[k]–71.0[g]	Auto
		V. palmarum	*V. palmarum*	76.0[h]	Auto
Vanilla		*V. mexicana*	*V. guianensis*	78.0[g]	Auto
		V. mexicana	*V. martinezii*	53.0[m]	Auto
		V. parviflora	*V. edwallii*	15.0[l]	Allo

References cited are: [a]Johansson 1974 as cited in [b][12]; [c][24]; [d][13]; [e][20]; [f][25]; [g][28]; [h][23]; [i][29]; [j][30]; [k][31]; [l][32]; and [m][11].

Table 1. Natural fruit set of some allogamous and autogamous *Vanilla* species (completed from [4]).

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In Africa (subgenus *Xanata* section *Tethya* species), it was recently demonstrated that pollinators might be Allodapine bees [13, 20]. On the other hand, some species of the genus, such as *V. palmarum*, *V. bicolor*, *V. guianensis* Splitg., *V. martinezii* Soto Arenas were determined to be autogamous (reviewed in [4] and **Table 1**). *Vanilla* autogamous species are characterized by much higher fruit sets *(*53.0% for *V. martinezii* to 78.0% for *V. guianensis)* than allogamous species (0.0% for *V. crenulata* to 26.3% for *V. roscheri)* (**Table 1**). These fruit sets are in accordance with known data on tropical orchids showing around 77.0% fruit set for autogamous species and less than 20.0% for allogamous species [24]. *V. savannarum* Britton, *V. griffithii* Rchb. f., and *V. mexicana* were also suggested as autogamous due to the high fruit sets reported [11, 12, 27]. Soto Arenas and Dressler [11], however, also mentioned that in Mexico, besides *V. mexicana* populations with high fruit sets, others have fruit sets as low as 2.5%. *V. mexicana* seems therefore to present also allogamy with potential pollinators supposedly being carpenter bees *Xylocopa* sp. [11, 12]. Measures of natural fruit set in wild populations, in addition to reproductive biology experiments, should therefore give us insights on the mating system of *V. mexicana*.

The use of codominant neutral genetic markers such as microsatellites to perform genetic analyses on natural populations [33, 34] is also a method of choice to estimate mating system parameters such as inbreeding rate [35–38]. As no specific markers were available for *V. mexicana*, we used microsatellite markers previously developed in other *Vanilla* species: the cultivated species *V. planifolia (*an American species from the genus *Vanilla* subgenus *Xanata* section *Xanata*) [2], *V. humblotii* and *V. roscheri* (African species from the genus *Vanilla* subgenus *Xanata* section *Tethya*) [21]. We performed genetic analyses and conducted reproductive biology experiments on *V. mexicana* wild populations from the island of Guadeloupe (French West Indies) to unravel its mating system.

2. *V. mexicana* mating system in Guadeloupe

2.1. Material and methods

2.1.1. Study species

V. mexicana is a vigorous hemi-epiphytic vine with a long stem reaching 10 m. Leaves are longer than internodes (7.5 cm long). Inflorescences are 3–12 cm long racemes bearing 3–5 flowers. Petals and sepals are greenish and very undulate, and labellum is white with a yellow crest. Fruits are nonaromatic, 10–25 cm long and thin [11, 17, 19] (**Figure 3**).

To precisely record morphological descriptors of the studied species, characters were measured to the nearest 0.01 mm using a digital caliper. Floral characters were measured from 11 flowers collected on three sites [Habituée (5), Mazeau (3), and Moreau (3)]: petal and sepal length and width as well as labellum, column and ovary length, width and thickness. The length and diameter of five eight-month-old fruits were also measured from one individual plant (Mazeau). Vegetative characters were assessed (four measures per plant on rank 4–7 leaves and internodes) on 16 plants from four sites [Mazeau- Solitude (6), Moreau (4),

Desbordes (3), and Habituée (3)]: internode length, stem diameter, leaf length, leaf width at 43 mm of the apex, and leaf maximum width (LMW).



Figure 3. *V. mexicana* inflorescences (A), flower (B), and 1-month-old fruits (C). Photographs by Nicolas Barre.

2.1.2. Study site

Sampling was performed in 2013 by the Association Guadeloupéenne d'Orchidophilie (AGO) mandated by the National Park of Guadeloupe (PNG). According to the inventory of *V. mexicana* in Guadeloupe, based on 22 traces representing 135 km around the Basse-Terre mountain in Guadeloupe [39], *V. mexicana* is mainly found in windward (west) mid-altitude (150–750 m) areas with a preferred altitudinal zone of 300–350 m (**Figure 4**). *V. mexicana* was most frequently found in secondary forests climbing on the following tree species : *Miconia mirabilis, Swietenia macrophylla* (Mahogany), and *Cyathea muricata* (Tree fern). *V. mexicana*

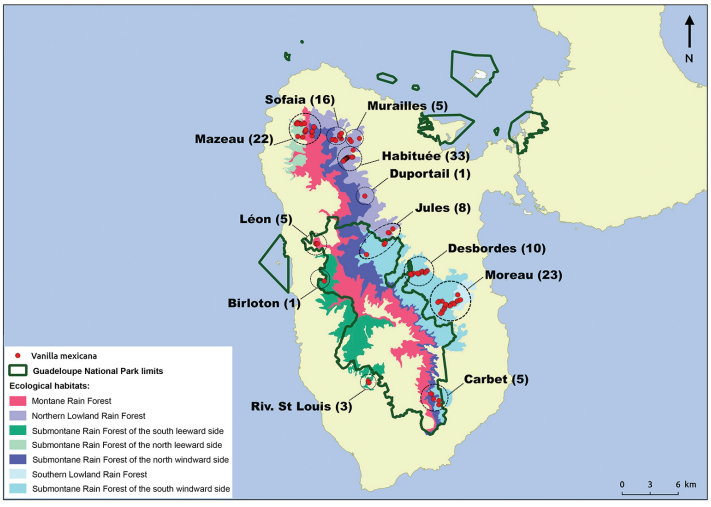

Figure 4. Red dots show the localization of the 132 *V. mexicana* accessions collected from 12 sites in Basse-Terre (Guadeloupe) with numbers of individuals in parenthesis. Ecological habitats [40] and the borders of the National Park of Guadeloupe are indicated.

http://dx.doi.org/10.5772/64674

preferably grows under medium shading (25–50%), and as a consequence, it is found mainly in opened habitats such as along forest tracks [39].

2.1.3. Plant sampling

Leaves were sampled from 132 accessions of *V. mexicana* collected from 12 different sites (populations) in Basse-Terre (**Figure 4**). Samples were dehydrated using silica gel for storage. Individual samples were deposited in the Biological Resource Centre (BRC) Vatel vanilla germplasm collection in Réunion Island [26] under accessions number CR2203 to CR2334.

GPS coordinates of each accession were recorded. Populations were named according to the locality (site) where they were collected (**Figure 4**). For the genetic analyses, two other *V. mexicana* accessions from Martinique (CR2352 and CR2353) and three from Mexico (CR2651, CR2658, and CR2665), maintained in the BRC Vatel, were also used.

2.1.4. Reproductive biology experiments and fruit set measurements

Flowering rates and season were estimated from June 2014 to June 2015 by surveying on average 96 plants each month in four sites [Habituée (40 plants in mean surveyed per month), Mazeau (22), Moreau (21), and Desbordes (13)]. Plants were checked for the presence of flowers. The lifespan per flower was estimated on 11 flowers from one plant (Desbordes) by measuring the time-laps between flower opening and its wilting.

From June to July 2014, fruit sets were precisely measured from 16 inflorescences (86 flowers in total) on two accessible Mazeau population plants, which were located at about 2 km distance from each other. Eight inflorescences were covered before flower opening by an insect-proof bag to exclude insect visits, while the other eight inflorescences (control) were not bagged. Inflorescences being always at the canopy (10–20 m high), access to flowers had to be performed using a 2 × 8-m-high ladder.

Fruit set was estimated as the ratio of the number of fruits developed at 30 days by the number of flowers at day 0. The natural fruit set (unbagged lowers) was then compared to the spontaneous fruit set observed in bagged flowers using a Student's test with the software *R v. 3.1.1* [41].

Natural fruit set was also assessed globally from June 2015 to June 2016 on 103 inflorescences from 32 plants in four different sites (9 from Habituée, 4 from Desbordes, 8 from Mazeau, and 11 from Moreau), by counting maturing fruits visible using Leica 10 × 40 binoculars. The fruit set was measured as the ratio of the mean number of fruits per inflorescence by the mean number of flowers produced by inflorescence (as determined from the previous Mazeau experiment).

2.1.5. DNA extraction

DNA was extracted from each accession from 0.020 to 0.025 g of dehydrated leaf material. Tissues were grinded using a *TissueLyser II* apparatus (Qiagen, Hilden/Germany) and DNA extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden/Germany). DNA was resuspended

in 70 µl of elution buffer and its quantity and quality evaluated both on a 2% agarose gel and by Nanodrop V8000 (Thermo Fisher Scientific, Waltham/USA). If the ratio of the OD 260/280 was not in the adequate 1.7–2 range, further purification was performed using the GeneClean® TurboKit (MP Biomedicals, Santa Ana/USA).

2.1.6. Microsatellite analyses

Fourteen microsatellite markers isolated from *V. planifolia* [2] and 19 microsatellite markers isolated from *V. humblotii* and *V. roscheri* [21] were tested in *V. mexicana*. Only six markers (from *V. humblotii* and *V. roscheri*) were transferable to *V. mexicana,* giving readable and repeatable amplifications and were used for subsequent PCR amplifications. These were HU03, HU04, HU06, HU07, HU09, and RO05 using appropriate fluorochrome dyes (see [21] for primer sequences and dyes). PCR volume was 15 µl including 7.5 µl of 2X Qiagen multiplex PCR Master Mix buffer (Qiagen, Hilden/Germany), 0.2 µl of each primer at 20 µM, 5.1 µl HPLC water, and 2 µl DNA (10 ng μl^{-1}). Amplifications were run on a Applied Biosystem GeneAmp® *PCR System 9700* (Thermo Fisher Scientific, Waltham/USA) thermocycler, using the following program: 2 min of predenaturation at 95°C, 45 cycles of 30 s at 95°C, 45 s at 57°C and 1 min at 72°C and a final elongation step for 7 min at 72°C. Amplification success was controlled by migration on a 2% agarose gel (1 h 30 min., at 110 V). PCR products were then diluted (1/10, 1/20, 1/30, or 1/40) depending on the intensity of the bands on the agarose gel. Then, 1 µl of the diluted amplification products were mixed with 10.3 µl formamide and 0.7 µl Gene Scan 500 Liz Size Standard (Applied Biosystems, Foster City/USA) and migrated on a *ABI 3130Xl* (Applied Biosystems, Foster City/USA) sequencer. Microsatellite alleles were visualized using the *GeneMapper v.4* software (Applied Biosystems) and manually scored.

2.1.7. Genetic analyses

An extended dataset comprising all studied accessions from Guadeloupe, Martinique, and Mexico (137 individuals) for the 6 microsatellite loci was used to calculate the total number of alleles for each locus (N_a), the number of private alleles per population (N_p) using the *GenAlex v.6.4* software [42, 43] and to study the levels of polymorphism at the regional scale.

Then, accessions from Martinique and Mexico were excluded from the dataset to calculate for each locus the observed heterozygosity (H_O), expected heterozygosity under Hardy-Weinberg (HW) equilibrium (H_E) and fixation index (F_{IS}) as in [44], using the online version of *Genepop v.4.2* [45]. These parameters and a global fixation index (F_{ST}) as in [44] were also calculated using *Genepop v.4.2* at the population level using a complete dataset (no missing data) with 3 markers (HU03, HU07, and HU09) and 51 individuals (11 populations). The fixation index F_{IS} or inbreeding coefficient is determined by a ratio of H_E and H_O, which indicates a heterozygote deficit or excess in the studied populations. It gives information on the reproduction regime in the populations, and the selfing rates were estimated by hand from F_{IS} using the equation $s = 2 \times F_{IS}/(1 + F_{IS})$ [46]. *Genepop v.4.2* was used to test for deviation from the HW equilibrium using multi-locus exact P-values estimations of the Markov chain method proposed by [47] (with default values).

http://dx.doi.org/10.5772/64674

Linkage disequilibrium between loci was tested using a probability test in *Genepop v.4.2* and Bonferroni correction for multiple comparisons. All loci were also tested for large-allele dropout using *Micro-Checker v. 2.2* [48]. The possible presence of null alleles was assessed with *Micro-Checker v. 2.2* using the Brookfield null estimator 1 [49] with each single locus complete dataset. The occurrence of null alleles was also verified by the program *INEst v.2.0* (Inbreeding/Null allele Estimation) [50], adapted for inbred populations, using the individual inbreeding model (IIM) with 200,000 MCMC iterations, 1000 thinning, and 20,000 burnin. *INEst* uses data from different loci simultaneously, which allows to estimate null allele frequencies at each locus together with the average level of inbreeding. We tested combinations of datasets with no missing data involving 2 to 3 loci of the 4 polymorphic in Guadeloupe and maximizing the number of individuals (35–107 depending on the dataset, datasets with $N < 15$ were not used).

2.2. Results

2.2.1. Reproductive biology

Morphological character measurements from reproductive and vegetative organs (**Table 2**) fitted the botanical description of *V. mexicana* [11, 17, 19]. The lifespan of a flower (from just-opened to wilted) was estimated to be 6.7 ± 1 days, the flower remaining fully opened for one to three days. Variations in flowering rates assessed on a mean of 96 plants on four sites each month for 1 year revealed that the species flowered almost all year-round, with a peak season in May–July with a maximum flowering rate at the beginning of June where 15.5% of plants were in flowering stage (**Figure 5**). In Guadeloupe, the May–July season is characterized by an increase in temperatures and rainfall.

Organ	Length	Width	Thickness	Diameter
Sepal	44.5 (±6.3)	12.5 (±1.8)		
Petal	44.4 (±5.4)	10.9 (±1.9)		
Labellum	25.8 (±2.0)	11.2 (±0.9)	11.2 (±0.5)	
Column	23.5 (±1.5)	2.4 (±0.3)	2.2 (±0.4)	
Ovary	40.6 (±10.1)	2.6 (±0.4)	2.5 (±0.4)	
Fruit	160 (±18.7)			10.2 (±0.3)
Stem	96.2 (±25.2)^{IL}			4.9 (±1,2)
Leaf	183.4 (±30.4)	48.5 (±8,9)^{LW}		
		82.1 (±21,1)^{LMW}		

The values are the means (±SE) of floral (N = 11), fruit (N = 5), and organ (N = 64) measurements in millimetres. ^{IL}internode length, ^{LW}leaf width at 43 mm from the apex, ^{LMW}leaf maximum width.

Table 2. Flower, fruit, and vegetative organ morphology of *V. mexicana*.

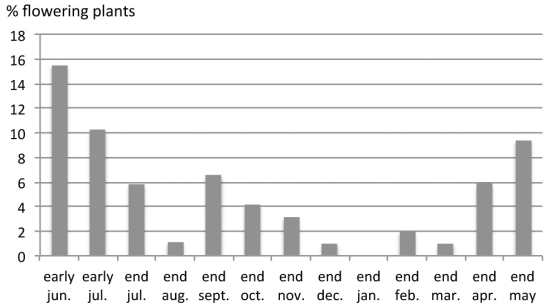

Figure 5. Annual variation in flowering rates in *V. mexicana* in Guadeloupe (June 2014–June 2015).

Results from the reproductive experiments (bagged and unbagged inflorescences) performed on 86 flowers from the Mazeau site are shown in **Table 3**. The mean number of flower per inflorescence in *V. mexicana* was 5.38 ± 0.93. There was no significant difference between the natural fruit set (53.7 ± 21.1%) and the spontaneous selfing rate obtained from bagged flowers (pollinators excluded), which was 53.9 ± 25.3% (**Table 3**). Both values showed important standard errors (SE), witnessing the fact that fruit set ranged from one to maximum six flowers becoming fruits, depending on the inflorescence. The natural fruit set observed in Mazeau was confirmed by visual observations of other 103 inflorescences from four different sites (Habituée, Desbordes, Mazeau, and Moreau), revealing that the mean number of fruits per inflorescence was 2.62 ± 1.72 (again with a high SE). If taking 5.38 as the mean number of flower per inflorescence (as determined in Mazeau), this gave a global natural fruit set estimation of 48.7%.

	Day 0		**Fruit set at day 30 (%)**	
	Control	**Bagged**	**Control**	**Bagged**
Individual	Nb_fl	Nb_fl		
Mazeau 16	6	6	50.0	50.0
	6	5	83.3	80.0
	6	5	66.7	60.0
	6	6	50.0	50.0
Mazeau 4	6	3	33.3	100.0
	5	6	80.0	50.0
	6	4	16.7	25.0
	4	6	50.0	16.7
Total	45	41		
Mean ± SE	5.63 ± 0.7	5.13 ± 1.05	53.7 ± 21.1	53.9 ± 25.3
t test			0.30 (NS)	

Control—inflorescences without protection. Bagged—inflorescence with insect-proof bag, Nb_fl—number of flowers, Mean ± SE—mean number of flower per inflorescence and standard error, mean fruit set value, and standard error, *t* test —*p* value of the Student's test, NS —nonsignificant

Table 3. Mating system of two individuals from *V. mexicana* in Guadeloupe (Mazeau population).

http://dx.doi.org/10.5772/64674

2.2.2. Genetic analyses

A total of 23 alleles were revealed for the 6 loci in the analyses on the complete dataset (**Table 4**), with a mean of 3.67 allele per locus, of which nine were private: four alleles to Mexico (with frequencies >0.1), and one in each of the Guadeloupe populations (with $N \geq 5$) of Desbordes, Habituée, Léon, Moreau, and Sofaia (with frequencies >0.01). The six loci were polymorphic at the regional scale (Guadeloupe, Martinique, Mexico), and only four were polymorphic in Guadeloupe. Eighteen alleles were revealed in Guadeloupe (**Table 4**), with a mean of 3 alleles per locus.

Locus	**HU03**	**HU04**	**HU06**	**HU07**	**HU09**	**RO05**
N_a (Guad)	4(4)	3(1)	4(4)	3(3)	6(5)	3(1)
Size (bp)	119–127	150–161	252–260	165–171	109–203	178–180
Pol_Reg	Yes	Yes	–	Yes	Yes	Yes
Pol_Guad	Yes	No	Yes	Yes	Yes	No
N (Guad)	113(111)	42(40)	43(43)	57(55)	126(125)	48(47)
Guadeloupe						
$Null^{MC}$	0.00	–	0.19	0.34	0.14	–
$Null^{IIM}$	0.01	–	0.12	0.02	0.02	–
H_E	0.333	0.000	0.515	0.525	0.503	0.000
H_O	0.342	0.000	0.227	0.000	0.296	0.000
F_{IS}	-0.026	–	0.559	1.000	0.412	–
HW	NS	–	***	***	***	–

N_a (Guad)—total number of alleles at the regional scale (with total number of alleles in Guadeloupe in parenthesis) per locus. Size (bp)—size range of alleles. Pol_reg—regional polymorphism. Pol_Guad—polymorphism in Guadeloupe. N (Guad)—total number of individuals at the regional scale (total number of individuals in Guadeloupe in parenthesis). Guadeloupe indices: $Null^{MC}$—null allele frequency estimated by *Micro-Checker*. $Null^{IIM}$—mean null allele frequency estimated by *INEst* from various complete multi-locus datasets with $N > 30$, H_E—expected heterozygosity, H_O—observed heterozygosity, F_{IS}—fixation index, HW—Hardy-Weinberg equilibrium deviation, with significant p value *<0.05, **<0.01, ***<0.001 and NS (nonsignificant) for p value > 0.05.

Table 4. Genetic diversity indices per locus defined by *GenAlex* and *Genepop* on the extended dataset.

Except for HU03, all other 3 polymorphic loci (HU06, HU07, and HU09) deviated significantly from HW expectations due to strong heterozygote deficits in Guadeloupe. The remaining two monomorphic loci (HU04, RO05) were also homozygous in Guadeloupe (**Table 4**), but not in Mexico (data not shown).

The test for genotypic disequilibrium for each pair of locus revealed no significant linkage between loci ($p > 0.05$). No large-allele dropout was detected.

Possible null alleles were detected with *Micro-Checker* for 3 loci (HU06, HU07, and HU09) (**Table 4**), with high frequency (0.14–0.34). However, using *INEst*, which accounts for possible

inbreeding, the null allele frequencies calculated became close to zero for HU07 and HU09. For HU06, the frequency was lower than with *Micro-Checker,* but there still remained possibilities of null allele. This marker was therefore excluded from further population genetic analyses.

The analyses per population on the selected complete dataset of 51 individuals for 3 loci (HU03, HU07, and HU09) revealed that the three studied populations with N > 5 individuals (Mazeau, Moreau, and Sofaia) deviated significantly from HW expectations due to a heterozygote deficit (**Table 5**). Deviation from HW expectations was also significant at the scale of Guadeloupe (**Table 5**). Selfing rate was estimated as 79% in Mazeau and 74% in Guadeloupe as a whole (**Table 5**). Global diversity H_E was 0.44 (**Table 5**). F_{ST} value across all populations was calculated as 0.157 using *Genepop*.

Population	N	N_a	A_p	H_E	H_O	F_{IS}	S	HW
Mazeau	14	6	0	0.342	0.119	0.652	0.79	**
Moreau	13	7	0	0.350	0.205	0.415	0.59	**
Sofaia	7	6	0	0.389	0.143	0.633	0.78	**
Guadeloupe	51	9	2	0.438	0.183	0.582	0.74	**

N—number of individuals, N_a—total number of alleles per population for the 3 loci studied, A_p—number of private alleles, H_E—expected heterozygosity, H_O—observed heterozygosity, F_{IS}—fixation index, S—selfing rate, HW—Hardy-Weinberg equilibrium deviation, with significant *p* value *<0.05, **<0.01, ***<0.001 and [S1] NS (nonsignificant) for *p* value > 0.05.

Table 5. Genetic diversity indices per population defined by *Genepop* on the complete dataset for locus HU03, HU07, and HU09 for populations with N > 5 and at the scale of Guadeloupe (51 individuals).

2.3. Discussion

V. mexicana flowers remained opened for 1–3 days, as previously suggested [12]. The flowering season was determined from our measurements to occur between May and July. It allowed to precise previous observations on flowering season, which was described as yearly, but more particularly between May to December [51]. Also in Mexico the species was only described as flowering without a defined period [11]. Reproductive biology experiments were performed during the flowering peak season identified.

Autogamy and self-pollination (53.9% fruit set in bagged inflorescences) explained the totality of the observed natural fructifications (53.7%) for the species *V. mexicana* in the Mazeau site in Guadeloupe. We, therefore, demonstrated that *V. mexicana* is reproducing mainly by autogamy in Mazeau, without the need for a pollinator. The natural fruit set estimated at a larger scale on four sites (but less precisely) was in the same range (48.7%). Both values were in the same order of magnitude of what was observed for autogamous *Vanilla* species (42.5–78%) and tropical orchids [24], therefore, confirming the autogamous mating system proposed for *V. mexicana* in Guadeloupe (**Table 1**). We noticed important standard errors in the mean fruit set estimates, which could be due in part to *Acromyrmex octospinosus* (cassava ant), a neotropical

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species introduced in Guadeloupe. This insect was observed on many occasions predating some flowers, which can be destroyed in a few hours (N. Barre, personal observation). Natural fruit set may also be underestimated for this reason.

It is noteworthy that it was suspected that *V. mexicana* could not perform asexual reproduction by stem cuttings and was strictly reproducing sexually [1, 11, 27]. This was confirmed by the impossibility to multiply this species by stem cuttings in laboratory conditions (Feldmann and Reyes-Lopez, personal communication, and unpublished observations).

Autogamy is therefore found either in subgenus *Vanilla* (in the *V. mexicana* species group) or in the *V. palmarum* species group of subgenus *Xanata* sect. *Xanata* (**Table 1**, **Figure 1**), two early diverging groups in the phylogeny of the genus. Spontaneous self-pollination is, therefore, an ancestral character in *Vanilla* shared by most, but not all, primitive species. Indeed, *V. edwallii*, from subgenus *Vanilla*, *V. parviflora* group, is not capable of self-pollination and requires a pollinator, supposedly the bee *Epicharis* (*Hoplepicharis) affinis* [32]. Autogamy in *V. bicolor* was explained by stigmatic fluids [28, 31], and agamospermy was ruled out [31]. For *V. palmarum,* both a narrow rostellum [4] and stigmatic leak [28] were noted. Our observations under dissecting microscope of *V. mexicana* flowers (data not shown) showed a glandulous and sticky rostellum (which could be due to stigmatic leak) on which the pollinaria are stuck, allowing their contact with the stigmata which they cover entirely (N. Barre, personal communication). Some rare cases of spontaneous self-pollination (6%) in some bagged flower experiments have also been reported for some allogamous species such as *V. planifolia, V. chamissonis,* and *V. humblotii* [12, 13, 25], but the mechanisms involved are unknown.

Population genetic parameters indicated a significant deviation from HW equilibrium and/or a homozygote excess for five loci out of six tested (not for HU03) in Guadeloupe vanilla population. Deviation from HW equilibrium was also detected in all the populations with more than five individuals studied, including Mazeau in which reproductive biology experiments were conducted. On the contrary, populations from allogamous species *V. barbellata* and *V. dilloniana* from Puerto Rico did not deviate from HW equilibrium [52] as expected for random mating. Deviation from HW for *V. mexicana* was due to heterozygote deficiency and F_{IS} value at the scale of Guadeloupe (0.582) allowed estimating selfing rate at 74.0%. This result is, as expected, very different from the one detected in the allogamous *V. humblotii* in Mayotte with a F_{IS} of 0.086 [13], which would correspond to a selfing rate of 15.8%. This Mayotte population slightly deviated from HW equilibrium due to limited selfing through geitonogamy between flowers on the same plant or from the same clonal patch [13]. Our genetic results, therefore, confirmed autogamy as the major mating system in *V. mexicana* in Guadeloupe as previously suggested [11, 27].

Deviation from HW equilibrium and homozygote excess could be due not only to homozygosity but also to null alleles, commonly encountered with microsatellite markers. This possibility was therefore also tested. *Micro-Checker* detected possible null alleles with high frequency for loci HU06, HU07, and HU09, but these were the 3 loci that also deviated from HW equilibrium (**Table 3**). This null allele test (like most) is not adapted for populations that do not comply with HW equilibrium, particularly due to inbreeding [53, 54], which is the case in *V. mexicana* populations as demonstrated by the reproductive biology experiments. This

often implies overestimation of null allele frequencies in such inbred populations [53, 54]. Van Oosterhout et al. [54] proposed a way to avoid this drawback in *Micro-Checker,* but it requires to have estimated the fixation index values by other markers, which was not possible for the present study. We, therefore, tested the IIM model proposed in the *INEst* software [50] which takes both inbreeding and null alleles into account in a Bayesian multilocus approach and this showed that frequency of null alleles dropped close to zero for the two loci, HU07 and HU09. Homozygote excess in populations of our selected dataset (HU03, HU07, and HU09) was therefore explained by inbreeding, not null alleles.

In autogamous species, only plant seeds ensure efficient gene dispersion whereas pollen also contributes in allogamous species [55, 56]. This has important consequences on the genetic diversity organisation, with autogamous species populations being more strongly differentiated, but less variable than populations from allogamous species [55, 56]. A metadata analysis [55] confirmed that annual or autogamous plants, or with gravity-dispersed fruits, allocate genetic variability among populations rather than within, with therefore high F_{ST} (0.34–0.42) and low H_E (0.41–0.47). On the contrary, long-lived or allogamous taxa, or with wind or ingested dispersed seeds, are more variable within populations than between and show low F_{ST} (0.13–0.22) and high H_E (0.61–0.68). The calculated F_{ST} value in *V. mexicana* (0.157) was, however, similar to the ones revealed in allogamous *Vanilla* species such as *V. humblotii* (F_{ST} = 0.120, [13]), *V. barbellata* (F_{ST} = 0.158) and *V. claviculata* (F_{ST} = 0.123) [52]. These F_{ST} values are moderate and in the range of what would be expected from allogamous species. Between populations differentiation is, therefore, lower than expected in *V. mexicana;* it may be because of a more efficient wind or animal-mediated seed dispersal system, which is still to be elucidated.

Intra-population diversity (H_E) value in *V. mexicana (*H_E = 0.438) was in the range of expected values for self-pollinating species [55], but similar to that of allogamous *V. humblotii* (H_E = 0.450, [13]). H_E values should have been higher for allogamous *V. humblotii*. Most allogamous *Vanilla* species are nevertheless self-compatible, and some degree of selfing can occur by geitonogamy. They are long-lived, thanks to their vegetative propagation capacity. Both factors could diminish intra-population diversity [55], associated in the case of *V. humblotii* with the loss of allelic diversity and the small size of fragmented populations [13]. Counterintuitive situations are not uncommon in *Vanilla* species. *V. roscheri* in South Africa was clearly allogamous with Allodapine pollinators and a relatively high fruit set (20%), but the isolated population near Lake Sibaya showed no diversity and was totally homozygous for the set of microsatellite markers employed, because of its range-edge distribution [20]. *V planifolia*, in the wild in Mexico, although allogamous and requiring pollinators, showed a F_{IS} of 1, witnessing high inbreeding probably through geitonogamy due to large size clonal patches and the scarcity of individual genotypes in the area [23].

It was suggested that *V. mexicana* could, in some populations in Mexico, also be allogamous because of a low fruit set observed [11] and carpenter bees were suggested as pollinators [11, 12]. It is possible that mating systems differ according to the geographical distribution. Evolution towards autogamy of allogamous but self-compatible species is often observed after colonization of isolated islands, a process associated with strong reproductive constraints often

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due to the absence or scarcity of adapted pollinators or partners [24, 57–60]. This could be the case for *V. mexicana* after colonization of the island of Guadeloupe. This was observed in *Eichhornia paniculata* (Spreng.) Solms (Pontederiaceae), this species was allogamous in Brazil but autogamous in Caribbean islands [61]. Autogamy is predominant also in orchids on islands [24], and this was the case for example for Angraecoideae (Vandeae, Orchidaceae) from Réunion island [59, 62, 63] that colonized the island from Madagascar.

From the set of 14 microsatellites developed from the *Vanilla* subgenus *Xanata* section *Xanata* American species *V. planifolia*, only two (mVplCIR025 and mVplCIR031) were transferable to African species from the subgenus *Xanata* section *Tethya* [2]. Here we demonstrated that none of them were transferable to *Vanilla* subgenus *Vanilla*. On the other hand, the 19 microsatellite markers developed from the *Vanilla* subgenus *Xanata* section *Tethya* African species *V. humblotii* and *V. roscheri* were highly transferable to other species from the same section (18 markers in mean were transferable) as well as to various American species from section *Xanata* (with however a slightly lower mean of 15.7 transferable loci) [21]. We showed that only six of them were transferable to *Vanilla* subgenus *Vanilla*. This reflects the important phylogenetic distance separating the primitive subgenus *Vanilla* from the subgenus *Xanata* species (**Figure 1**) [16, 18]. This preliminary study using these 6 transferable markers allowed the confirmation of the mating system revealed with reproductive biology experiments in *V. mexicana*. However, it is clear that further population genetic studies in *V. mexicana* to resolve more complex questions regarding gene flow, population differentiation, or spatial structuring of the populations will require more numerous loci to be analyzed and will therefore necessitate isolating *V. mexicana*-specific microsatellites through an enriched library construction or NGS (next-generation sequencing). Further studies should also be enlarged to other populations from regions other than Guadeloupe to cover the species distribution range (**Figure 2**) and should include as well reproductive biology experiments and measurements to further unravel *V. mexicana* possibly different mating system in other areas.

3. Conclusion

Our preliminary results obtained with the set of 6 heterologous microsatellite primers allowed the confirmation of the reproductive biology results and showed that *V. mexicana* is mainly reproducing by autogamy via spontaneous self-pollination in Guadeloupe. This trait can be of interest to *V. planifolia* breeding. Indeed, the major constraint to vanilla production is the time-consuming hand pollination. *V. planifolia* flowers are ephemeral and must be self-pollinated by hand every morning during the 2–3 months flowering season. Breeding of self-pollinating vanilla cultivars would first necessitate validating the heritability of the autogamous trait of *V. mexicana.* It could then be envisaged using backcross breeding between *V. mexicana* and *V. planifolia* as recurrent parent (to regain characters associated with fruit quality and aroma lacking in the donor parent). This would be a long, but worthwhile, process (5–7 years between each generation from seed germination to flowering). These results demonstrate the strong interest in pursuing the effort of characterization of wild vanilla populations.

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