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A genome-wide assessment of the genetic diversity, evolution and relationships with allied species of the clonally propagated crop *Vanilla planifolia* Jacks. ex Andrews

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Abstract The *Vanilla* genus is a complex taxonomic group characterized by a vegetative reproduction mode combined with intra- and inter-specific hybridizations, and polyploidy events. These factors strongly impact the diversification of the genus and complicate the delimitation of taxa. Among the hundred *Vanilla* species, *Vanilla planifolia* Jacks. ex Andrews and *Vanilla x tahitensis* J. W. Moore are the main cultivated aromatic species. We applied Genotyping-by-Sequencing to explore the genetic diversity

of these two cultivated vanilla species, seven closely related species and nineteen interspecific hybrids. The inter- and intra-specific relationships of 133 vanilla accessions were examined based on 2004 filtered SNPs. Our results showed a strong genetic structuring between the nine species studied, with wild species showing much lower heterozygosity levels than cultivated ones. Moreover, using Bayesian clustering analyses, the kinship of several hybrids could be verified. We evidenced in particular that *Vanilla sotoarenasii* and *Vanilla odorata* C.Presl may be the parental species of *V. x tahitensis*. The analysis of 1129 SNPs for 84 *V. planifolia* accessions showed a clear genetic

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demarcation between the vegetatively propagated traditional vanilla cultivars compared to the accessions derived from sexual reproduction, and a higher genetic diversity and lower heterozygosity of the latter ($H_o=0.206$) compared to the former ($H_o=0.362$). Our data are consistent with a single-step domestication for *V. planifolia* in accordance with the recent history of its cultivation. It also opens avenues to breed new *V. planifolia* varieties adapted to biotic and abiotic constraints and to reduce mutational load induced by clonal propagation.

Keywords Breeding · Domestication · *Vanilla × tahitensis* · *Vanilla planifolia*

Introduction

The genus *Vanilla* Plumier ex Miller belongs to the Orchidaceae family and is composed of about 120 species, among which 18 (Portères 1954) to 35 (Soto Arenas 2003) are considered to bear aromatic fruits. The main cultivated species is *Vanilla planifolia* Jacks. ex Andrews that contributes to more than 95% of the world's vanilla production. Native to Mesoamerican tropical forests, *V. planifolia* has been vegetatively propagated and cultivated in the Eastern coast of Mexico since the mid eighteenth century in response to the growing demand for vanilla pods in Europe. Vanilla cuttings were subsequently transferred into European botanical gardens, then reached the Indian Ocean region where no natural pollinator was present (Bory et al. 2008b; Lubinsky et al. 2010). The discovery in 1841 of an easy manual pollination technique by Edmond Albius in Reunion Island has led to the fast diffusion of *V. planifolia* into the south west Indian ocean region (Madagascar, Reunion Island, Comoros). *Vanilla × tahitensis* J.W.Moore, is mostly cultivated in French Polynesia and Papua New Guinea. It is supposed to have been introduced to Tahiti Island from the Philippines in 1848 (Constantin and Bois 1915). However, *V. × tahitensis* is no longer found in the wild and its origin has long been debated. Based on morphological characteristics, a hybrid origin between *V. planifolia* and *Vanilla pompona* Schiede (Portères 1954) or *Vanilla odorata* C.Presl (Portères 1954; Soto Arenas 1999) was suggested. A genetic analysis using the nuclear Internal Transcribed Spacer (ITS) and plastid DNA sequences

rather suggested a hybrid origin between *V. planifolia* as the maternal parent and *V. odorata* as the paternal one (Lubinsky et al. 2008). Other aromatic species are cultivated or harvested in the wild at small scales in some localities such as *V. pompona* in the French Caribbean islands and *V. odorata* in Central and South America (Soto Arenas 1999).

Domestication, according to Martínez-Ainsworth and Tenaillon (2016), can be described as a set of consecutive stages that begins with the onset of domestication followed by an increase in the frequency of a set of desirable traits. McKey et al. (2010) highlighted the lack of knowledge on the evolutionary ecology of domesticated plants that are clonally-propagated. Vanilla is no exception and the impact of domestication on the genetics of cultivated *Vanilla* has received little attention. From 1793 to 1875, five introduction events of *V. planifolia* cuttings into Reunion Island were reported, but only one introduction in 1822 by Marchant from Europe is supposed to have been successful and to be at the origin of vanilla cultivation in Reunion Island (Bory et al. 2008b). From a historical perspective and given the very limited number of introductions, “single-step domestication” (i.e. identification of interesting genotype and direct clonal propagation) might be the rule in *V. planifolia*, which would generate a crop that remains close to wild progenitors (Zohary 2004). Low levels of genetic diversity are therefore expected in *V. planifolia* in cultivation areas such as Reunion Island, in accordance with the vegetative mode of multiplication of vanilla vines, and their recent introduction in the Indian Ocean region. Random amplified polymorphic DNA (RAPD) (Besse et al. 2004), amplified fragment length polymorphism (AFLP) (Bory et al. 2008c) and microsatellite (SSR) (Bory et al. 2008a) markers succeeded to discriminate the species and confirmed the genetic uniformity of most *V. planifolia* cultivars in the Indian Ocean and other cultivation areas. AFLP patterns of variation suggested that *V. planifolia* has evolved in introduction areas by the accumulation of point mutations through vegetative multiplication. However, these markers, and even those based on methylation patterns (MSAP) (Gigant et al. 2011), have failed to identify clusters of intraspecific genetic diversity congruent with the phenotypic variations described in cultivation (Bory et al. 2008b, c; Gigant et al. 2011). On the contrary, based on AFLP studies and linkage mapping, varieties described in

V. × tahitensis were shown to result mainly from self-pollination or full sib crosses of plants belonging to the most ancient ‘Tahiti’ morphotype, with subsequent heterozygous selection (Lepers-Andrzejewski et al. 2012).

Genotyping-by-sequencing (GBS) is able to generate thousands of Single Nucleotide Polymorphisms (SNPs) markers by applying massively parallel sequencing and multiplexing methods (Elshire et al. 2011). GBS-generated SNP markers are useful to explore the genetic diversity and structure of population in order to better define phylogeny, adaptation of plants to their environment or domestication (Favre et al. 2021). Previous studies validated the efficiency of GBS to characterize *Vanilla* genetic diversity and to identify hybrids (Hu et al. 2019; Alomia et al. 2021). Herein, we developed SNP markers derived from GBS data to study intraspecific diversity and enlighten the evolutionary history of cultivated vanilla in its introduction areas. Our study focused on genetic diversity of *Vanilla* resources conserved in the Biological Resources Centers (BRCs) Vatel and Etablissement Vanille de Tahiti (EVT) (Roux-Cuvelier et al. 2021): cultivated *V. planifolia* and *V. × tahitensis* species, seven wild relatives originating from tropical America (Soto Arenas 2003) and selfed-progenies of *V. planifolia* and interspecific hybrids. This well characterized germplasm constitutes a material of choice to assess the genome-wide genetic diversity, the impact of domestication processes and breeding on genetic diversity levels in cultivated *Vanilla* compared to wild genotypes.

Materials and methods

Plant material and DNA extraction

A panel of 137 *Vanilla* spp. accessions was used for GBS sequencing, including (i) the two cultivated species *V. planifolia* (88 accessions) and *V. × tahitensis* (7 accessions), (ii) 7 closely related species *V. odorata* (8 accessions), *Vanilla cribbiana* Soto Arenas (4 accessions), *V. sotoarenasii* (4 accessions), *Vanilla insignis* Ames (2 accessions), *V. pompona* (2 accessions), *Vanilla bahiana* Hoehne (2 accessions), *Vanilla helleri* A.D. Hawkes (1 accession) and (iii) 19 interspecific hybrids (Supporting Information Table S1). All accessions were grown in

shade house or in vitro in Reunion Island and French Polynesia and were selected from a large collection of over 700 accessions conserved in the French BRCs Vatel (CIRAD, Reunion Island) and EVT (Raiatea) (Roux-Cuvelier et al. 2021). Accessions were selected in order to maximize variability for origin, variety and ploidy level and better evaluate the species diversity. Traditional cultivars of *V. planifolia* and *V. × tahitensis* collected in fields were classified as vegetatively (asexually) propagated clones or ‘cuttings’. Accessions obtained by sexual reproduction (selfed-progenies or intra-specific hybrids) were classified as ‘seedlings’. All accessions were clonally propagated by cuttings or micro cuttings in vitro for their conservation in BRCs Vatel and EVT (Supporting informations S1 and S2). High molecular weight DNA of each accession was extracted from 25 mg of lyophilized young leaves using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Genomic DNA was quantified using a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and normalized at 50 ng/μL. DNA homogeneity and quality was assessed by enzymatic digestion with *HindIII* (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and run in a 2% agarose gel.

Library preparation and sequencing

Library preparation was performed by the Regional genotyping technology platform (UMR AGAP, CIRAD, Montpellier, France) as described by Elshire et al. (2011). Two kinds of adapters are used for constructing GBS libraries, a common adapter and a sample-specific barcode adapter. Both adapters are designed to fit with Illumina sequencing. Adapters were mixed together in a 1:1 ratio and plated into two 96-well plates, so that each well contained one specific barcode. Extracted DNA was added into the 96-well plates and digested with *PstI* methylation-sensitive restriction enzyme (New England Biolabs, Ipswich, Massachusetts, USA). Adapters were ligated to the ends of the DNA fragments using a T4 ligase (New England Biolabs, Ipswich, Massachusetts, USA). Samples were then pooled together, amplified by Polymerase Chain Reaction (PCR) and purified to remove unreacted adapters. The GBS library was sequenced on Illumina HiSeq3000 sequencer (Illumina Inc., San

Diego, California, USA) with DNA-seq single-read protocol at the GeT-PlaGe platform (INRAE, Toulouse, France).

Sequence analysis and SNP calling

Sequence quality was checked with FastQC (Andrews 2010). Low-quality reads, reads with uncalled bases and reads with Illumina adapter sequences were removed using the Cutadapt software (Martin 2011). The remaining reads were assigned to each sample using the GBS barcode splitter tool (<https://sourceforge.net/projects/gbsbarcode/>). Demultiplexed sequences were trimmed to 140 bp to normalize the length between individuals. SNP calling was performed using STACKS de novo pipeline (Catchen et al. 2013) and identified SNPs were converted into Variant Call Format file (VCF; Danecek et al. 2011). Low-quality SNPs were filtered out with vcfr package 1.11.0 (Knaus and Grünwald 2017) from Rstudio (version 3.6.3) (R Development Core Team 2010) and using successive filters: minimum minor allele frequency < 10%, missing data per site > 30% and up to 3 SNPs per locus. SNPs with an allele frequency below 10% were discarded because these very rare variants probably resulted from genotyping errors, while retaining rare alleles that are associated to under-represented samples in the dataset. The *V. planifolia* accessions were used to study structure within such a clonally propagated vanilla, and only polymorphic SNP markers were kept. Loci with unfiltered SNP markers and filtered SNPs were mapped over the *Daphna V. planifolia* chromosomes (Hasing et al. 2020) to check SNP distribution and density. The genotyping data sets were converted into biallelic tables by vcfr package (Knaus and Grünwald 2017), which were used for both phylogenetic relationships and population structure analysis.

Genetic diversity and phylogenetic analysis based on SNP markers

The number of effective alleles (N_e), Shannon's information index (I), observed heterozygosity (H_o) and percentage of polymorphic loci (P) were calculated using GenAIEx (version 6.502) (Peakall and Smouse 2012). The values were compared across the nine species and interspecific hybrids using the complete genotyping data set, and across different types of *V.*

planifolia cultivars. From the complete genotyping dataset, a dissimilarity coefficient was calculated with *DarWIN* software (Perrier and Jacquemoud-Collet 2006) using the simple matching index (Sokal and Michener 1958): $d_{ij} = 1 - \frac{1}{L} \sum_{l=1}^L \frac{m_l}{\pi}$ where d_{ij} is the dissimilarity between units i and j ; L the number of loci; m_l the number of matching alleles for locus l ; and π the ploidy. Distance trees were constructed from 1000 bootstrap replicates using the Unweighted Neighbor-Joining method (Saitou and Nei 1987). Trees were then converted into *Phylip* file and plotted with *FigTree* software (Rambaut 2006).

Population structure analyses

Principal coordinates analyses (PCoA) were performed using the complete genotyping data set and the *V. planifolia* data set with GenAIEx (version 6.502) (Peakall and Smouse 2012). The population structure was analyzed to identify clusters of genetically related individuals using the Bayesian clustering method implemented in *STRUCTURE* (version 2.3.4) (Pritchard et al. 2000). The *STRUCTURE* analysis was first performed between *V. odorata*, *V. planifolia*, *V. pompona*, *V. × tahitensis* and hybrids, and then between all the *V. planifolia* accessions. The admixture model of *STRUCTURE* was chosen on the assumption that each individual had ancestry from one or more of K genetically distinct sources. Ten independent runs were performed for each K from $K=1$ to $K=10$, with a burn-in period of 10,000 and 100,000 Markov-chain Monte Carlo (MCMC) iterations after burn-in. The best number of K was chosen with the ΔK method (Evanno et al. 2005) by running the *STRUCTURE HARVESTER* (Earl and vonHoldt 2012). For *STRUCTURE* analysis within *V. planifolia*, accessions were assigned to one cluster if their probability of belonging to this cluster is higher than 60%.

Results

Sequencing and SNP calling

The sequencing of GBS libraries resulted in 1,143,846,935 single-reads of 150 bp for 137 *Vanilla* accessions (Supporting information Tables S1, S2,

S3 and S4). After the cleaning step, 554,565,581 reads of 140 bp were demultiplexed for each individual (51.5% of the reads were discarded). CR0026, CR0151 and CR2564 samples, with a low sequencing depth (0; 6377 and 2657 reads, respectively), were removed from the study. The de novo pipeline reconstructed 828,215 loci including 81,987 identified as biallelic (10.3%) with 225,857 raw SNPs. Among the 80,203 loci with a sequencing depth ≥ 5 reads, 32% mapped on the 14 chromosomes of the published *V. planifolia* Daphna cv genome (Hasing et al. 2020) (Supporting information Fig. S1). The 194,625 SNPs distributed on these loci were filtered out based on: minimum minor allele frequency (MAF) $< 10\%$, missing data per site $> 30\%$, and a maximum of 3 SNPs per locus. The data set obtained consisted of 2040 high-quality filtered SNPs. The accession *V. planifolia* CR0844 was removed due to a missing data rate $> 45\%$. The final data set consisted of 133 genotyped vanilla samples using 2004 filtered SNPs, with a mean heterozygosity of $19.4\% (\pm 16.7\%)$ and a mean missing data rate of $19.5\% (\pm 6.3\%)$. A specific subset of 84 *V. planifolia* individuals using 1129

SNPs was produced from the complete genotyping matrix using the same filters, with a mean heterozygosity of $43.6\% (\pm 14.9\%)$ and a mean missing data rate of $15.2\% (\pm 7.5\%)$. Among the 2004 and 1129 SNPs, 1916 (95.6%) and 1081 (95.8%) mapped onto the 14 chromosomes of the *V. planifolia* cv Daphna genome. For the 2004 SNP matrix, the density of SNPs per chromosome ranged from one SNP every 527 kb (chromosome 2) to one SNP every 230 kb (chromosome 1), with an average of one SNP every 359 kb (Fig. 1a). For the 1129 SNP matrix, the lowest density of SNPs was detected on chromosome 3 (one SNP every 1024 kb) and the highest on chromosome 1 (one SNP every 429 kb), with an average of one SNP every 641 kb (Fig. 1b).

Genetic relationships across species within the genus *Vanilla*

The unweighted Neighbor-Joining (NJ) tree built with the complete genotyping matrix revealed two major groups statistically supported with bootstrap values equal to 1. On one side of the tree, a group

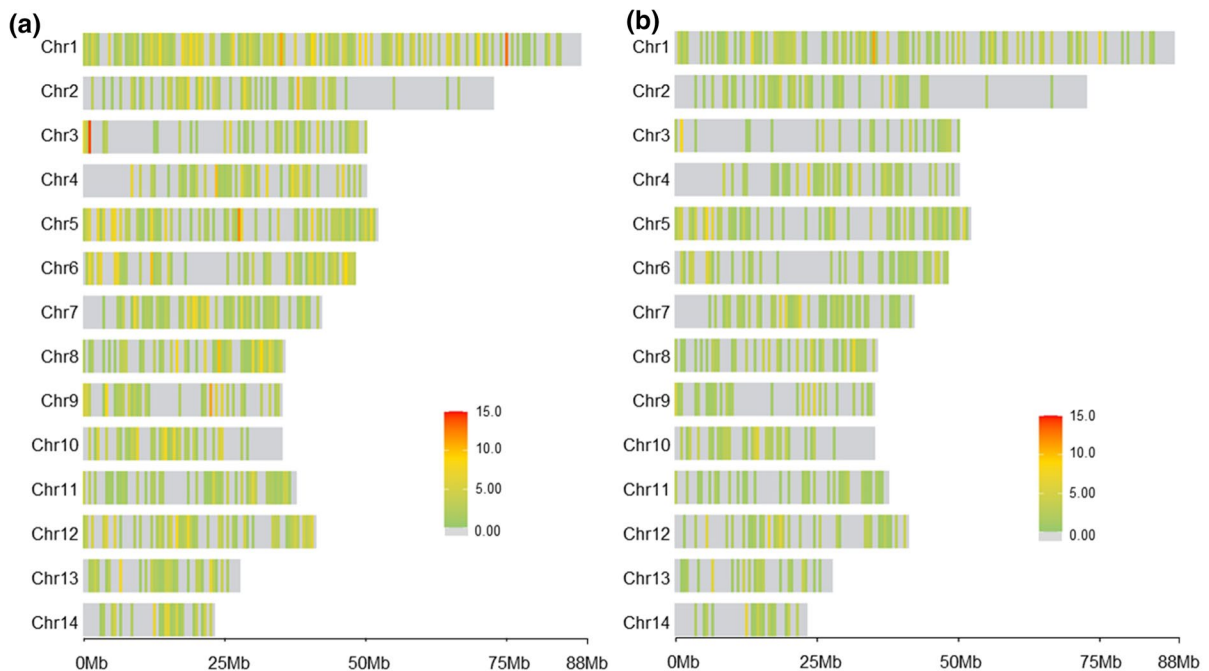


Fig. 1 Genotyping-By-Sequencing SNP distribution and density on *V. planifolia* cv Daphna chromosomes. **a** From 2004 filtered SNPs dataset. **b** From 1129 filtered SNPs dataset. Horizontal axis displays the chromosome length. The density scale

indicates the number of SNPs within 500 Kb window size. The plot shows the distribution of GBS SNPs across the 14 chromosomes

comprised the *V. planifolia* accessions plus six hybrids, and on the other side, a group comprised the accessions from the wild relative species (*V. bahiana*, *V. cribbiana*, *V. helleri*, *V. insignis*, *V. odorata* and *V. pompona*) (Fig. 2). *V. × tahitensis* accessions and most of the interspecific hybrids branched in the tree in intermediate positions between the two major groups, with bootstrap values ranging from 0.6 to 1. *V. sotoarenasii* accessions were closer to the *V. planifolia* group than to wild relatives but were clearly individualized (bootstrap value=1). The wild relative species group was divided into four subgroups statistically supported by bootstrap values >0.9. The first subgroup comprised *V. pompona*, the second

subgroup comprised *V. cribbiana*, the third subgroup comprised *V. odorata* accessions, *V. helleri* CR3614 and *V. insignis* cf. CR2688, and the last one comprised *V. bahiana* accessions, *V. insignis* cf. CR0087 and two hybrids having at least one *V. bahiana* parent. PCoA analyses showed a similar clustering of the accessions (Supporting information Fig. S2). The first coordinate of the PCoA explained 44.49% of the genetic variability and separated *V. planifolia* from the wild species, with hybrids in intermediate position. The second coordinate, explaining 7.03% of the genetic variability, separated *V. × tahitensis* from the other accessions. Genome-wide heterozygosity (Ho) per species was calculated using the complete

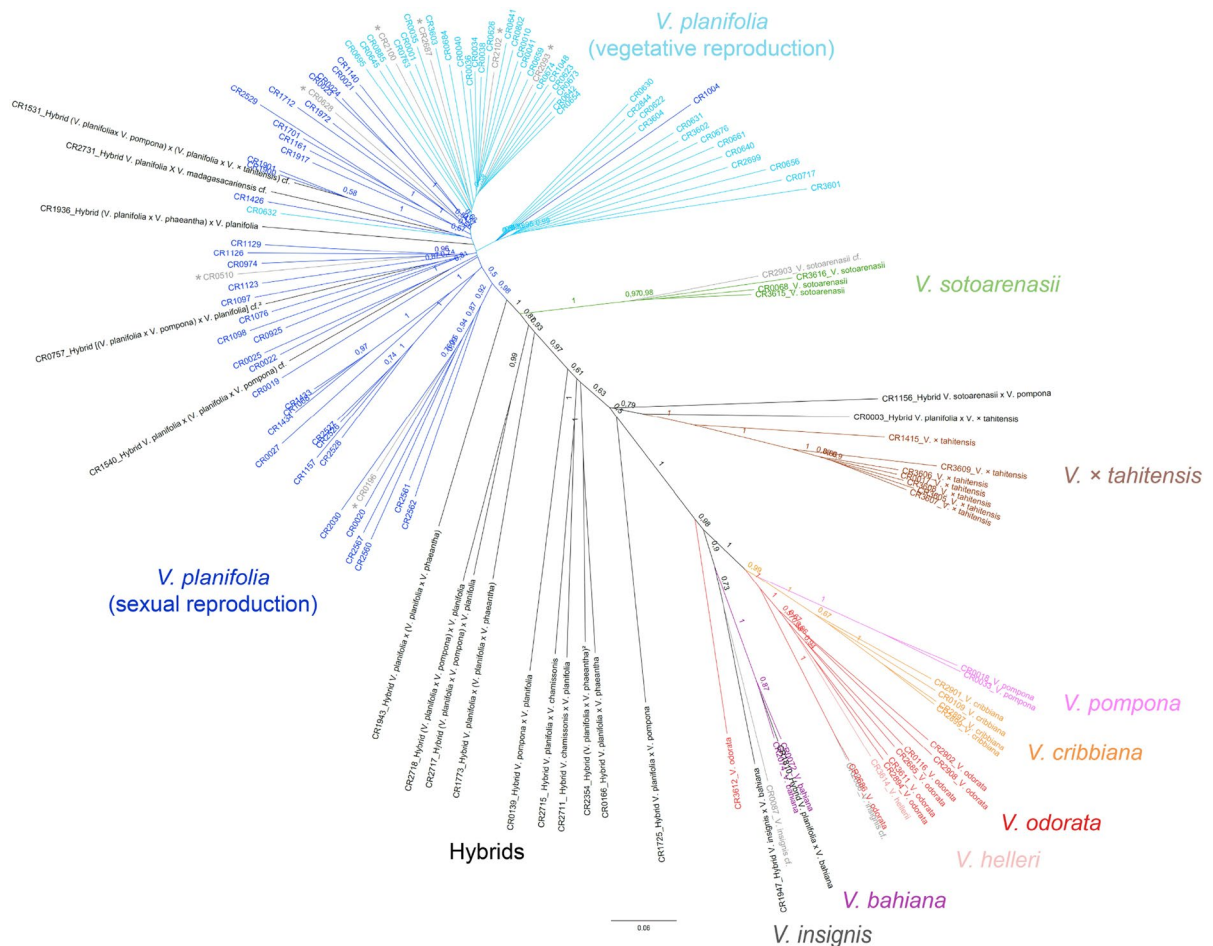


Fig. 2 Phylogenetic structuration between cultivated vanillas and wild relative species. Unweighted Neighbor-Joining tree constructed from 1000 bootstrap replicates using 2004 SNPs and 133 accessions. Bootstraps values higher than 0.5 are

shown between *V. planifolia*, *V. × tahitensis*, wild species and hybrids. Scale bar shows genetic distance. **V. planifolia* accessions with unknown reproduction mode

Table 1 Diversity indexes in Vanilla species using 2004 SNPs identified across all species

Species	N	Ne	(SE)	I	(SE)	Ho	(SE)	P (%)
<i>V. planifolia</i>	84	1.522	(0.011)	0.374	(0.008)	0.287	(0.001)	56.34
Vegetative	38	1.515	(0.011)	0.369	(0.008)	0.362	(0.008)	55.24
Sexual	39	1.498	(0.010)	0.368	(0.007)	0.206	(0.005)	56.24
Unknown	7	1.483	(0.010)	0.358	(0.007)	0.350	(0.008)	54.69
<i>V. x tahitensis</i>	7	1.192	(0.013)	0.205	(0.007)	0.239	(0.009)	30.89
<i>V. bahiana</i>	2	0.782	(0.009)	0.003	(0.001)	0.002	(0.001)	0.45
<i>V. cribbiana</i>	4	0.825	(0.009)	0.012	(0.002)	0.001	(0.000)	2.10
<i>V. helleri</i>	1	0.746	(0.010)	0.001	(0.001)	0.002	(0.001)	0.20
<i>V. insignis</i>	2	0.923	(0.008)	0.031	(0.003)	0.018	(0.002)	5.04
<i>V. odorata</i>	8	1.068	(0.007)	0.110	(0.005)	0.002	(0.001)	22.36
<i>V. pompona</i>	2	0.679	(0.011)	0.004	(0.001)	0.002	(0.001)	0.60
<i>V. sotoarenasii</i>	4	1.056	(0.009)	0.094	(0.005)	0.031	(0.002)	15.57
Interspecific crossing	19	1.664	(0.007)	0.551	(0.004)	0.246	(0.004)	96.56

N number of accessions, *Ne* number of effective alleles, *I* Shannon's information index, *Ho* observed heterozygosity, *SE* standard error, *P* percentage of polymorphic SNPs (%). Vegetative, sexual and unknown indicate the reproduction mode of the *V. planifolia* accessions

genotyping matrix (Table 1). *Ho* values were of similar range in the cultivated species, *V. planifolia* (0.287 ± 0.001) and *V. x tahitensis* (0.239 ± 0.009), and on average a hundred times higher than the *Ho* values observed in wild relative species (ranging from 0.001 ± 0.000 in *V. cribbiana* to 0.031 ± 0.002 in *V. sotoarenasii*). The mean *Ho* of vegetatively propagated *V. planifolia* (0.362 ± 0.008) was significantly higher than the mean *Ho* in selfed-progenies (0.206 ± 0.005). Hybrids revealed *Ho* levels (0.246 ± 0.004) close to those observed in cultivated vanilla. Shannon's information index (*I*) was highest for interspecific hybrids (0.551 ± 0.551), followed by *V. planifolia* and *V. x tahitensis* (0.374 ± 0.008 and 0.205 ± 0.007 respectively). Among the wild species, *I* values ranged from 0.001 ± 0.001 in *V. helleri* to 0.110 ± 0.005 in *V. odorata*. The percentage of polymorphic SNPs was higher in the cultivated vanillas and in interspecific hybrids (> 30%), compared to wild relatives (< 23%).

Genetic relationships between *V. planifolia* and several of its hybrids

In the light of our GBS-generated SNPs we explored the relationships between several species and derived interspecific hybrids. A Bayesian clustering analysis based on 2004 SNPs (Fig. 3a) was performed on a reduced dataset including six hybrids, two *V. pompona* (Fig. 3b), seven *V. odorata* (Fig. 3c), seven *V. x tahitensis* (Fig. 3d), four *V. sotoarenasii* (Fig. 3e), and a representative subset of 12 *V.*

planifolia accessions (Fig. 3f) selected among subgroups identified in Fig. 2. The estimated likelihood was greatest for *K*=4, suggesting the presence of 4 clusters corresponding to the 4 species *V. odorata*, *V. planifolia*, *V. pompona* and *V. sotoarenasii*. For supposed first-generation hybrids, we were expecting a probability of 0.50 for each parental genome assignment. The seven *V. x tahitensis* accessions had in average 56.5% of their SNPs attributed to *V. sotoarenasii* and 43.5% to *V. odorata* (X-squared=1.69, *df*=1, *p*-value=0.1936), confirming the interspecific hybrid status of this species. The accession CR1415 (CR0017 x CR0017) had similar proportions in its genome (53.6/46.4%) than its parent *V. x tahitensis* CR0017 (55.9/44.1%). For the *V. pompona* x *V. sotoarenasii* hybrid CR1156, 61.4% of the SNPs were assigned to *V. sotoarenasii* and 38.6% to *V. pompona*. These proportions did not fit the hypothesis of first-generation hybrid (X-squared=5.20, *df*=1, *p*-value=0.0226). The SNPs of hybrid CR1725 were assigned to *V. pompona* (67.6%) and *V. planifolia* (32.4%) which was also inconsistent with hypothesis of 0.50/0.50 distribution of parental genomes (X-squared=12.39, *df*=1, *p*-value=0.0004). The SNPs of hybrid CR0139 were assigned to *V. planifolia* (54.2%), *V. pompona* (38.0%) and *V. sotoarenasii* (7.80%). Therefore, these three first-generation hybrids showed parental inheritances of SNPs significantly deviating from the hypothetical ratio of 0.5/0.5.

For back-cross hybrids, we were expecting 0.75/0.25 distribution of the parental genomes. The hybrid CR2717 (*V. planifolia* x *V. pompona*)

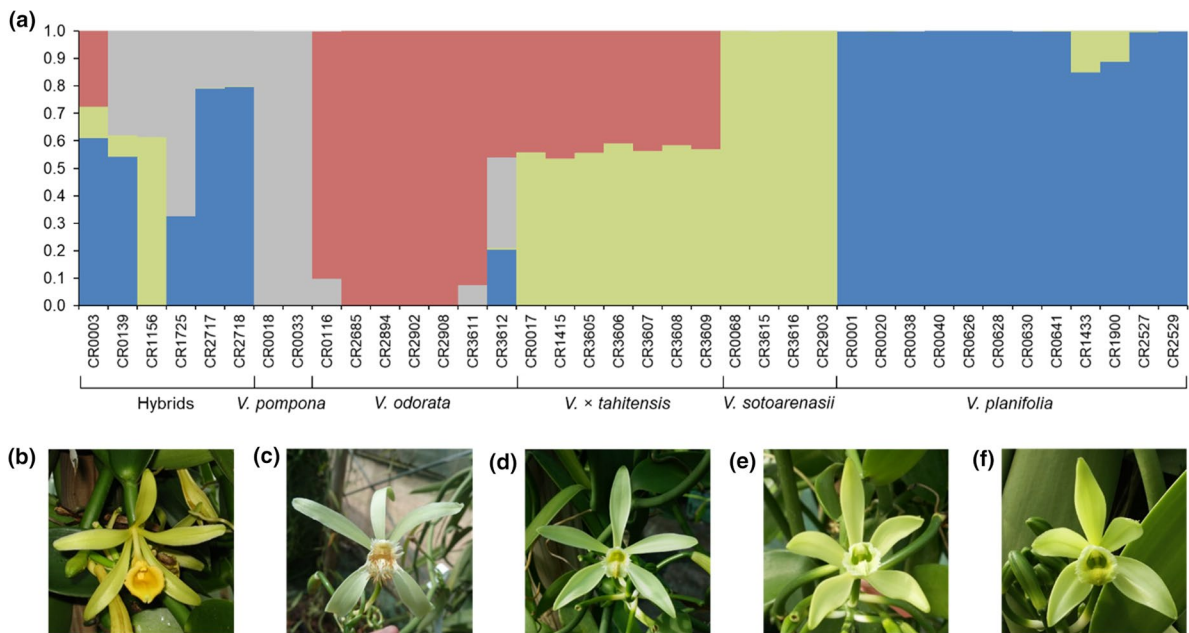


Fig. 3 Origin of *V. x tahitensis* shown by bayesian clustering and comparison of morphological traits. **a** Population structure of 38 vanilla accessions using 2004 significant SNPs. Colours represent different assigned clusters. The X-axis provides accession and species names and the y-axis provides the proba-

bility of each accession belonging to the assigned cluster. Front view of entire flowers of **b** *V. pompona* CR0018, **c** *V. odorata* CR0116, **d** *V. x tahitensis* CR0017, **e** *V. sotoarenasii* CR0068 and **f** *V. planifolia* CR0040

$\times V. planifolia$) had 79.1% of its genome assigned to *V. planifolia* and 20.8% to *V. pompona*, which was consistent with back-cross ratio hypothesis ($X^2=0.93$, $df=1$, $p=0.3347$). The hybrid CR2718, which is a cutting of CR2717 showed similar results (79.7% assigned to *V. planifolia* and 20.2% to *V. pompona*, and $X^2=1.22$, $df=1$, $p=0.2699$). The hybrid CR0003, that comes from a *V. planifolia* \times *V. x tahitensis* cross, revealed ancestry from *V. planifolia* (60.9%), *V. odorata* (27.6%) and *V. sotoarenasii* (11.5%) as expected, but the proportions (60.9/27.6/11.5%) were inconsistent with the 50/25/25% proportions expected for such a cross ($X^2=9.94$, $df=2$, $p=0.0070$).

Intraspecific genetic structuration within *V. planifolia*

The *V. planifolia* accessions derived from sexual reproduction showed some structuration in the NJ tree (Fig. 2) with subgroups supported by bootstrap values equal to 1, whereas no genetic structuration was supported by bootstraps values in vegetatively propagated accessions. Among the accessions with

unknown reproduction mode, CR2093, ‘Colibri’ CR2687, CR2100 and CR2102 branched within cuttings, while CR0510, CR0196 and CR0628 (‘Aiguille’) branched within seedlings. The tetraploids ‘Grosse Vanille’ CR0802 and CR0641 and the triploids ‘Sterile’ CR0645 and CR0630, were grouped together with cuttings without significant structuration between these accessions. The cultivar ‘Petite Mexique’ CR0632, supposed to derive from clonal propagation, was branched with accessions derived from sexual reproduction.

The first and second coordinates of the PCoA of *V. planifolia* accessions explained 13.92% and 8.29% of the genetic variability, respectively (Supporting information Fig. S3a). These values were lower than those observed from the complete dataset PCoA (Supporting information Fig. S2), indicating a low structuration within the *V. planifolia* group. Individuals obtained by vegetative propagation were clustered along the first axis, while individuals derived from sexual reproduction were much more dispersed throughout the plan. No structuration related to geographic origin or the ploidy level of cultivars could

be evidenced by PCoA (Supporting information Fig. S3b and c).

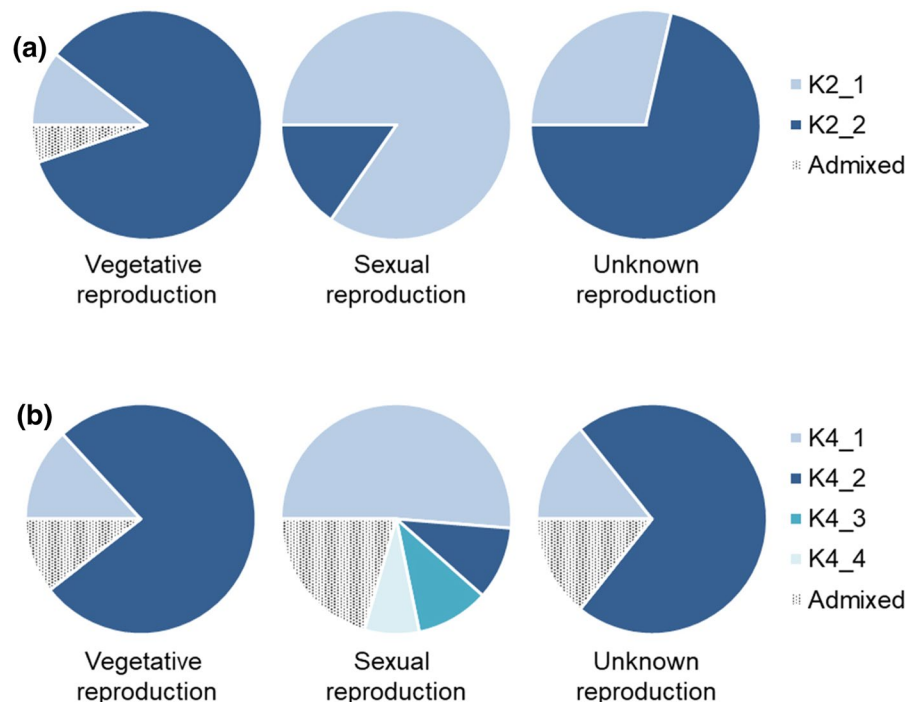
Genetic structure was explored by a Bayesian clustering analysis with 84 *V. planifolia* accessions using the 1129 SNP matrix (Supporting information Fig. S4a). The estimated likelihood was the greatest for $K=2$, $K=3$ and $K=4$, suggesting the presence of 2 to 4 genetic clusters (Supporting information Fig. S4b and c). Accessions were assigned to one specific cluster if their probability of belonging to this cluster was higher than 60%, in order to facilitate interpretation based on a majority rule basis. If not, they were considered admixed. For $K=2$, the majority (84.21%) of the accessions derived from vegetative propagation were assigned to cluster K2_2, whereas 84.62% of the accessions derived by sexual reproduction were assigned to cluster K2_1 (Fig. 4a). For $K=4$, 76.31% of the accessions derived from vegetative propagation were assigned to the cluster K4_2, whereas admixture and a greater diversity of clusters were observed in accessions derived from sexual reproduction (Fig. 4b). Among unknown accessions, five were assigned to the ‘vegetative’ clusters (K2_2 and K4_2), one to the ‘sexual’ clusters (K2_1 and K4_1), and one appeared admixed for $K=4$ (Supporting information Fig. S4a).

Discussion

Our study confirmed that GBS is a powerful genomic tool for the identification of highly informative SNPs for the study of the inter- and intra-specific genetic diversity in the *Vanilla* genus. Here, we applied GBS to explore the genetic relationships and genetic structure of the two cultivated *Vanilla* species, seven closely related species, and 19 interspecific hybrids from the well documented vanilla collections maintained ex situ at BRCs Vatel and Vanille de Tahiti. GBS genotyping yielded 2004 high quality filtered SNPs for the *Vanilla* genus, among those a subset of 1129 SNPs was used for *V. planifolia*. The majority of the SNPs (95%) successfully mapped on the published *V. planifolia* cv Daphna genome (Hasing et al. 2020), except for the extremity of the chromosome 2 not covered with GBS-generated SNPs. This might result from erroneous chromosome 2 assembly in the Daphna genome or a richness in repetitive elements and low-complexity regions in this chromosome part.

Our GBS data supported the current taxonomy for most of the species studied (Bouetard et al. 2010). As expected, *V. planifolia* and wild relative species were strongly separated. Hybrids from crosses between *V. planifolia* and a wild species were in intermediate

Fig. 4 Genetic structure of 84 *V. planifolia* using 1129 informative SNPs. **a** Proportion of accessions assigned to one of the two clusters determined by STRUCTURE for $K=2$, according to mode of reproduction. **b** Proportion of accessions assigned to one of the four clusters determined by STRUCTURE for $K=4$, according to mode of reproduction. The dotted ground indicates accessions classified as admixed



position on the NJ tree or close to the *V. planifolia* parent. The *V. pompona* group was the most distant from the *V. planifolia* group in agreement with plastid DNA analysis (Bouetard et al. 2010). Accessions attributed to the species *V. odorata* formed a large and structured group, except CR3612 which probably has a hybrid origin. Indeed, morphology of flowers and fruits obtained recently for CR3612 suggested a kinship with a species close to *V. pompona*, while the long and narrow leaves were related to *V. odorata* (Supporting information Fig. S5a). *V. odorata* cf. CR2686 was probably misidentified and should rather be classified within *V. insignis*, since it is genetically close to *V. insignis* CR2688 (Fig. 2) and showed broad leaves and rough stem typical of this species. This would make *V. insignis* a very close but nevertheless distinct group (bootstrap = 1) from *V. odorata*. Accession CR0087 identified as *V. insignis* cf. showed proximity in the NJ tree to the *V. bahiana* group and is very close to its hybrid with *V. bahiana*. The flowers of CR0087 differ from those of *V. bahiana* by their slightly larger size but above all by the presence of very developed orange papillae on the lip of the labellum (Supporting information Fig. S5b). The *V. helleri* accession CR3614, nested within the *V. odorata* group, could be conspecific to this species. The *V. ×tahitensis* accessions formed a large and structured group close to, but clearly distinct from its hybrids and from the *V. planifolia* group, contrary to what was observed with plastid DNA analysis (Bouetard et al. 2010).

The *V. sotoarenasii* group was closely related but distinct from *V. planifolia* (bootstrap value = 1), as previously established (Bory et al. 2008c; Bouetard et al. 2010; Azofeifa-Bolaños et al. 2017). *V. sotoarenasii* did not appear like a hybrid in the STRUCTURE analyses, but like a distinct genetic group with specific SNPs. In a commendable effort to limit specific inflation in the genus *Vanilla*, it was recently proposed that *V. sotoarenasii* is conspecific to *V. planifolia* (Karremans et al. 2020). The authors based their proposal on the absence of genetic differences between the two species and argue that the morphological variations of vanilla vines in the Cahuita area (Costa Rica), where large populations of *V. sotoarenasii* have been described (Azofeifa-Bolaños et al. 2017), do not allow to distinguish one species from the other. These two arguments do not hold. First, as indicated previously

(Azofeifa-Bolaños et al. 2017), a small (2 nucleotides) but steady difference separates the ITS sequences of *V. sotoarenasii* from those of *V. planifolia*. The GBS data presented here, based on 2004 SNPs, provided clear evidence of genetic differentiation between *V. planifolia* and *V. sotoarenasii*. Second, the argument that the morphological variability of *V. planifolia* contains that of *V. sotoarenasii* is not supported by any quantitative data. On the contrary, the photograph of numerous fruits produced by the authors (Karremans et al. 2020) to show the wide variability in *V. sotoarenasii* demonstrated that the fruits of *V. sotoarenasii* were always between 9 and 15 cm in length, and indehiscent, which is much less than the 21 cm average length of *V. planifolia* fruits, that are moreover very predominantly dehiscent (Díaz-Bautista et al. 2018). In addition, the variations in shape and color of the fruits are much more certainly due to different stages of maturity, and incomplete natural pollinations, than to phenotypic plasticity that remains to be demonstrated. Similarly, variations in leaf shape and color are common in vanilla plants in relation to biotic and abiotic factors during their growth and have little taxonomic value (Soto Arenas and Cribb 2013). The other objection of these authors that the flower size measurements of *V. sotoarenasii* (Azofeifa-Bolaños et al. 2017) would be biased by the fact that they were made on an accession grown under controlled conditions is not supported by any measurement of variability in wild populations. Unlike vegetative organs, the morphology of reproductive organs is little impacted by environmental conditions and therefore has a better taxonomic value. On the other hand, as mentioned previously (Azofeifa-Bolaños et al. 2017) and documented recently in *V. pompona* (Watteyn et al. 2022) flower size is, among others, an important trait affecting reproductive isolation. In the case of difficult groups such as the genus *Vanilla*, alpha-taxonomy is often not very discriminating or even risky, and an integrative taxonomy approach (Andriamihaja et al. 2022) allows more effectively to dissect the relationships between closely related taxa. In this perspective, the bundle of genetic, morphologic and ecologic arguments clearly plead in favor of the recognition of *V. sotoarenasii* as a valid species which might have recently evolved from *V. planifolia* by geographic and/or reproductive isolation. However,

the hypothesis of a hybrid origin of *V. sotoarenasii* involving *V. planifolia* and a species not included in our study, cannot be completely ruled out.

Bayesian clustering analyses based on GBS-generated SNPs proved to be a powerful tool to assess parental genetic contributions of hybrids, in case of lack of information on a genetic resource, mislabelling or uncontrolled pollination. In this study, we confirmed, or infirmed the taxonomic position and kinship for several species and hybrids. However, some first-generation hybrids did not show the expected 50/50 parental assignment probabilities. Most of the hybrids assessed have *V. pompona* as parental species, which is known to have a much larger genome than *V. planifolia* ($2C=8.18$ to 10.72 pg) (Bory 2007). The observed deviations could therefore be explained by parental genome complexity. For instance, molecular cytogenetics studies on polyploid sugarcane cultivars, deriving from a few interspecific hybridization events performed a century ago by breeders, highlighted an uneven contribution of each parental genome, with 75–85% of their chromosome originating from one parental species (*S. officinarum*) and 15–25% from the other parent (*S. spontaneum*), with some chromosomes derived from interspecific recombination (Piperidis and D'Hont 2020). A recent GBS study (Alomia et al. 2021) also included some *V. planifolia* x *V. pompona* hybrids, but the parental contributions were unfortunately not quantified, preventing any comparison. Nevertheless, GBS based Bayesian clustering enabled us to undoubtedly identify the parental origin of all the hybrids studied. Our results open important questions about possible genome rearrangement in interspecific crosses between relatively distant species in the genus *Vanilla*, that will need to be addressed further.

Previous assessment of *V. x tahitensis* origin, based on nuclear (ITS, GBS) and plastid (*rbcL*) loci, suggested a hybrid origin between *V. planifolia* and *V. odorata* (Lubinsky et al. 2008; Hasing et al. 2020; Alomia et al. 2021). Our GBS data confirmed at the genomic level (whole genome scale) the hybrid origin of Tahitian *Vanilla* evidenced in Lubinsky et al. (2008) study, and the proportions of genetic parental contributions (close to 0.50 for all accessions) were compatible with the hypothesis of a first-generation hybrid. However, *V. sotoarenasii* material was missing in Lubinsky et al.'s work. We contributed to clarify the question of the origin of *V. x tahitensis*

by including for the first time well characterized *V. sotoarenasii* accessions and genomic data. According to our analysis, *V. odorata* is indeed one parent, but the second parent is closer to *V. sotoarenasii* than to *V. planifolia* as previously stated. The flower morphology of *V. x tahitensis* (Fig. 3c), with traits that are close to *V. odorata* (Fig. 3b) and to *V. sotoarenasii* (Karremans et al. 2020), supports this observation (Fig. 3d). Alomia et al. (2021) identified, by GBS, *V. planifolia* wild accessions from Belize (referred to as Type 2) as the possible true parent of *V. x tahitensis*. These accessions appeared very close genetically to *V. sotoarenasii* (Alomia et al. 2021). This observation reinforces our hypothesis and we suggest that the Type 2 *V. planifolia* from Belize in Alomia et al. (2021) could possibly be *V. sotoarenasii*. Recent data from Chambers et al. (2021) indeed showed that Type 2 *V. planifolia* and *V. sotoarenasii* cannot be separated genetically in PCA and Structure analyses. A morphological characterization of these Belize accessions could also allow to further verify this hypothesis. Karremans et al. (2020) have also argued that *V. sotoarenasii* is not a species at all, but most likely an introgressed hybrid involving *V. odorata*, *V. x tahitensis*, and/or *V. planifolia*. Our results clearly rather show that *V. sotoarenasii* is a species close to *V. planifolia*, and that *V. x tahitensis* is a hybrid between *V. sotoarenasii* and *V. odorata*. Nevertheless, even if *V. sotoarenasii* was dismissed as a species as argued by Karremans et al. (2020), although we do not agree with this, the maternal origin of *V. x tahitensis* was more precisely addressed by our study and should be searched for in *V. sotoarenasii*-like populations. According to low divergence of ITS sequence data, *V. x tahitensis* appeared to be evolutionarily recent and it was suggested that it resulted from natural or man-mediated pollination in Mesoamerica (Lubinsky et al. 2008). *V. sotoarenasii* is present in southwestern Costa Rica (Azofeifa-Bolaños et al. 2017), and may also be also present in the north of Costa Rica. It was observed in northeastern Colombia (Choco, MG personal observation) and if it is confirmed that it is also present in Belize, this would suggest that the geographic range of *V. sotoarenasii* is much wider in America than the Caribbean area of Costa Rica where it has been reported so far. The other parent of *V. x tahitensis*, *V. odorata*, has a large distribution area covering Central and tropical South America. Thus, the hybrid origin of *V. x tahitensis*

is compatible with the sympatric range of both parents. Historically, the species is said to have been introduced from America to French Polynesia via the Philippines by Amiral Hamelin in 1848 (Correll 1953; Portères 195). The history of migrations and exchanges between the Viceroyalty of New Spain, the Philippines and Pacific islands between the sixteenth and eighteenth centuries (Merrill 1954) is compatible with this hypothesis. Indeed, it can be suggested that the shipment of pods or cuttings could have occurred 300 years ago on board of the Manila Galleons, the first Spanish ships that crossed the Pacific Ocean and introduced many plants from America to Asia and Oceania (Merrill 1954; Lubinsky et al. 2008).

Genome-wide H_o levels in wild species were very low ($H_o=0.001$ to 0.031) compared to cultivated species ($H_o=0.287$ and $H_o=0.239$), suggesting frequent inbreeding in the wild. Although vanilla flowers possess a rostellum preventing self-pollination, most species studied are self-compatible and selfing can occur via geitonogamy between different flowers from the same individual (Gigant et al. 2016). Further population genetic studies are needed to precisely assess possible genetic threats such as inbreeding on these species in the wild. The very low H_o in wild species can also be shown when observing GBS data in Alomia et al. (2021) and Hu et al. (2019), although the authors did not discuss this result. *V. planifolia* in cultivation was shown to have very low levels of diversity (AFLP, RAPD, SSR) (Besse et al. 2004; Bory et al. 2008a, c), and a single introduction in the south western Indian ocean area was suggested (Bory et al. 2008c). This pattern, compatible with a single-step domestication, was confirmed for *V. planifolia* using GBS. As clonal propagation is known to increase heterozygosity by the accumulation of point mutations (Balloux et al. 2003), single-step domestication process is always associated with high levels of heterozygosity, as shown for cassava (Elias et al. 2004) and hops (Jakše et al. 2001). Long established clones were indeed highly heterozygous as shown in cassava landraces ($H_o=0.50$ to 0.71) (Pujol et al. 2005). The high genome-wide H_o levels detected (0.362) in cultivated clonal *V. planifolia* varieties worldwide are in accordance with this hypothesis. Most vanilla cultivars indeed result from almost 200 years of intense clonal propagation of the cuttings initially introduced to La Reunion in 1822. The present values are indeed high compared to those reported in *V. planifolia*

wild populations using 15 allozyme loci ($H_o=0$ to 0.078) (Soto Arenas 1999). They are higher than those revealed by 14 SSR markers ($H_o=0,154$) (Bory et al. 2008a), but close to those estimated from AFLP ($H_o=0,295$) (Bory et al. 2008c) in a similar set of *V. planifolia* varieties. SSR markers' length evolves more quickly than point mutation, and SSR were shown to reveal higher H_o than SNPs in population genetics studies (Fischer et al. 2017). However, in our particular case, if the levels of heterozygosity are due to the accumulation of mutations during clonal propagation (rather than from demographic and reproductive history as in natural populations), only SNPs can detect such mutations, since SSR only assess length variations in the number of microsatellite repeats. The H_o value obtained from dominant AFLP markers was deduced from the proportions of segregating bands in self-progenies (Bory et al. 2008c) and appears slightly underestimated. Our H_o levels are much higher than those revealed by Hu et al (2019) ($H_o=0.0322$ to 0.0457) but this might be due to their SNP filtration of $H_o>0,2$ (Hu et al. 2019) because the same accessions studied in a recent GBS analysis (Alomia et al. 2021) showed much higher H_o levels, compatible with our results. Interestingly, we therefore demonstrate here, thanks to a well characterized set of accessions from BRC Vatel, that H_o levels can be used to differentiate wild from cultivated vanillas. This could be very useful for less characterized collections. Indeed, this might indicate that the "hidden diversity" detected in *V. planifolia* by Alomia et al. (2021) using GBS could rather simply correspond to cultivated ($H_o>0.20$, Type 1) compared to wild ($H_o<0,03$, Type 2) accessions. Type 3 accessions with intermediate H_o values (0.08 to 0.15) could be cultivars naturalized in the wild, or wild accessions with high natural cloning rate.

Based on AFLP analysis, *V. × tahitensis* was suggested to have a different domestication history than *V. planifolia* (Gigant et al. 2011; Lepers-Andrzejewski et al. 2011, 2012). Although a single introduction origin was also suggested, it has been followed by one or two generations of self-pollination, as shown by the detection of recombination events using AFLP graphical genotypes (Lepers-Andrzejewski et al. 2012). *V. × tahitensis* therefore rather fits the pattern of a single-step domestication (one introduction) followed by subsequent recombination-and-selection cycles (McKey et al. 2010). This hypothesis is supported

by genome-wide H_o that is lower in *V. × tahitensis* ($H_o=0.239$) than in *V. planifolia* ($H_o=0.362$).

Analysis of 1129 SNPs for 84 *V. planifolia* accessions showed a clear demarcation between the vegetatively propagated traditional vanilla cultivars compared to the accessions derived from sexual reproduction. GBS-generated SNPs could be used efficiently to better define the origin of unknown accessions: CR0196, CR2093, CR2100, CR2102 and ‘Colibri’ CR2687 were assigned to vegetatively propagated accessions, CR510 was assigned to accessions derived from sexual reproduction and CR0628 remains unknown. The cultivar ‘Petite Mexique’ CR0632 supposed to be a traditional cultivar with clonal propagation was branched and clustered with accessions derived from sexual reproduction.

Selfed-progenies and intra-specific crosses in *V. planifolia* showed an increased level of diversity (Fig. 4, Supporting information Fig. S3a, S4) as previously suggested by AFLP study (Bory et al. 2008c). They also showed a reduced genome-wide heterozygosity ($H_o=0.206$) as compared to the original parental cuttings group ($H_o=0.362$), as expected following selfing. Although heterozygosity is a favorable trait (ie hybrid vigor), the heterozygosity born from strict clonal propagation leads to the accumulation of deleterious mutations (McKey et al. 2010). Therefore *V. planifolia* plantations sustainability could be threatened by this mutational load (McKey et al. 2010). To preserve the adaptive potential of *V. planifolia*, the maintenance of mixed clonal/sexual systems is considered the best strategy (McKey et al. 2010). Efforts that have been engaged at BRCs Vatel and EVT to create new varieties from selfing will contribute to increase diversity levels, and also to reduce heterozygosity levels and therefore release mutational load. This has been a very successful strategy to create *Fusarium* resistant plant (Handa) (Grisoni and Dijoux 2017) from the selfing of a *V. planifolia* parent. Our study clarified the origin of *Vanilla* spp. from a large collection and their genetic diversity and structure, and provides new informations for breeding programs that contribute to the enhancement and protection of these materials. Tahitian vanilla, known to be more fragrant and fruity, illustrates the potential of hybridization to select new varieties.

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Author contributions CC, MG and CJ conceived and designed the experiment. JBD produced hybrids and grew the plant material at CRB Vatel. SL provided *V. × tahitensis* from the Etablissement Vanille de Tahiti (EVT) and provided her expertise on the genetics of *V. × tahitensis*. JH extracted DNA, RR produced GBS libraries, CJ performed the SNPs calling and QP the genomic mapping of SNPs. FF and CC analyzed the data and wrote the manuscript. PB, MG and CJ contributed to and edited the manuscript. All authors have reviewed and approved the final manuscript.

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Data availability The raw sequence data have been deposited in the Short Read Archive under NCBI BioProject ID PRJNA756864. Biological samples used in this study have been deposited in the BioSamples Database under the accession identifier SAMN_20,927,883 to 20,928,015, and SAMN20981991 to SAMN20981994.

Declarations

Conflict of interest The authors declare that they have no conflict of interests.

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