PATTERNS OF INTRODUCTION AND DIVERSIFICATION OF V. PLANIFOLIA (ORTHIDACEAE) IN REUNION ISLAND (INDIAN OCEAN)1

SEVERINE BORy,2,6 PESACH LUBINSKY,3 ANGE-MARIE RISTERUCCI,4 JEAN-LOUIS NOYER,4 MICHEL GRISONI,2 MARIE-FRANCE DUVAL,5 AND PASCALE BESSE6,7

1 CIRAD, UMR PBVMBT Cirad/Université de la Réunion, Pôle de Protection des Plantes, 7 chemin de l’IRAT 97410 Saint-Pierre, La Réunion, France; 2Ellstrand Lab, Department of Botany and Plant Sciences, University of California, Riverside, California 92521-0124 USA; 3CIRAD, UMR DAP, Avenue Agropolis, TA A-96 / 03 34398 Montpellier Cedex 5, France; 4CIRAD, UPR Multiplication Végétative, Boulevard de la Lironde, TA A-7502 34398 Montpellier cedex 5, France; and 5Université de La Réunion, UMR PBVMBT Cirad/Université de la Réunion, 15 avenue René Cassin, BP 7151 97715 Saint Denis messag cedex 9, La Réunion, France

The cultivated species Vanilla planifolia is a typical example of a crop introduced from its area of origin (America) to new regions where natural pollinators are absent. Although the Vanilla cultivars are exclusively vegetatively propagated, a high degree of phenotypic variation is observed among the cultivars in their introduction areas such as Reunion Island. To test several hypotheses explaining this variation—different introduction events, somatic mutations and sexual reproduction (through manual pollination)—we used AFLP markers to elucidate the patterns of introduction of V. planifolia. Most of the accessions cultivated in the world were derived from a single accession, possibly the Mexican cultivar Mansa. The patterns of diversification of this cultivated species were also studied and compared with other cultivated (V. tahitensis) and wild (V. pompona and V. bahiana) species. Except for one particular phenotype (‘Aiguille’), which may come from sexual reproduction, cultivated accessions exhibit very low levels of genetic diversity. They have evolved by the accumulation of point mutations through vegetative multiplication. The genetic diversity revealed could not explain the phenotypic diversity, which may be related to epigenetics or polyploidy. This new understanding of the basis of genetic diversity of vanilla may assist to improve management of genetic resources.

Key words: AFLP; diversification; genetic diversity; Indian Ocean; Orchidaceae; Vanilla planifolia, vegetative reproduction.

Vanilla is the world’s most popular flavor and, by unit mass, is among the most valuable of the spice crops. The only true sources of natural vanilla are the cured fruits of two obligatorily hand-pollinated and clonally propagated orchids: ‘Bourbon/Mexican vanilla’ Vanilla planifolia G. Jackson, syn. V. fra-

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grans (Salisb.) Ames and ‘Tahitian vanilla’ (V. tahitensis J. W. Moore). Other aromatic species are grown locally or harvested in the wild, but have no economic importance, for example, V. pompona Schiede, called ‘Vanillon’ in the French West Indies and V. odorata C. Presl in America (Soto Arenas, 2003). Of the estimated 2300 T of vanilla beans sold on the international market in 2001 (Loeliet, 2003), roughly 95% derives from V. planifolia. The discovery in the mid-19th century of a practical artificial method to pollinate vanilla manually signaled, by the end of the 19th century, the rise and eventual dominance of the Indian Ocean (Madagascar, Reunion Island, Comoros) as the major vanilla exporting region in the world, a position it still maintains (Kouri, 2004).

In contrast to the enigmatic V. tahitensis, a species not known in the wild and found exclusively in cultivated or feral populations in French Polynesia and Papua New Guinea, V. planifolia is a rare (ca. 1 individual/2–10 km2) perennial herb endemic to humid evergreen forests in Mesoamerica (Portères, 1954; Soto Arenas, 1999; Hägsater et al., 2005). The aromatic fruit condition in the genus Vanilla Pluimier ex Miller (Orchidaceae, pantropical; ca. 110 species) is reported to be present in 18 (Portères, 1954) to 35 (Soto Arenas, 2003) species from the neotropics/Caribbean and could represent a synapomorphy for the genus (Soto Arenas, 1999; 2003; Lubinsky et al., 2006; Lubinsky, 2007).

As early as the mid-18th century, cuttings of V. planifolia began to be introduced into Europe, not from Mexico into Spain, where vanilla bean imports were centered (Kouri, 2004), but from the West Indies into England. When or how V. planifolia made its way to the West Indies is unknown and/or unrecorded, but most probably was an introduction postdating...
Spanish arrival in the New World. The lectotype of *V. planifolia* was based on a plant introduced by the Marquis de Blanford from the West Indies and cultivated in the greenhouse of the R. Hon. C. Greville in Paddington in the early 1800s. This same individual was propagated shortly thereafter throughout botanical institutions in continental Europe, and a widely subscribed viewpoint is that the entire stock of cultivated vanilla in the Indian Ocean and Indonesia derives from this single genetic individual (clone), the Blanford/Greville type, introduced into these regions essentially contemporaneously during the 1820s and 1830s by French and Dutch colonists, respectively (Correll, 1953; Bouriquet, 1954; Smith et al., 1992; Soto Arenas, 2003; Ecott, 2004; Bory et al., 2008).

In Reunion Island, five primary successive vanilla introductions are cited in the literature (Bory et al., 2008), but it is generally admitted that only one (by Marchant in 1822) was successful, which would correspond to the introduction of the Blanford/Greville type. Cultural management of vanilla includes a strict regime of asexual propagation via cuttings and the exclusive “selling” of flowers by artificial means (hand-pollination) to produce pods. In combination with its putative introduction history, diversity expectations are therefore very limited for Indian Ocean vanilla. Similar expectations have, however, not been found to hold when examining levels of genetic diversity in other clonally propagated crops of New World origin like potato (*Solanum*) (Brush, 2004; Spooner and Hetterscheid, 2006), cassava (*Manihot*) (Elias et al., 2000, 2004; Schaal et al., 2006), *Stenocereus* (Casas et al., 2006), *Opuntia* (Griffith, 2004), pepino (*Solanum*) (Blanca et al., 2007), and oca (*Oxalis*) (Emshwiller and Doyle, 2002; Emshwiller, 2006), which have been demonstrated to be not just multiclonal in constitution, but in many instances were shown also to maintain sufficiently high genetic variation so as to preclude the molecular detection of a domestication bottleneck. Often, different stable phenotypes of *V. planifolia* are described in cultivation in introduction areas. In Reunion Island, they differ by leaf color, shape, and thickness; pods shape; stem thickness; and self-fertility. Two types are widespread in cultivation: the Classique type has light green, flat leaves and pods that progressively narrow and the Mexique type has darker and more bluish leaves than Classique, with a central gutter and curved sides and pods that are cylindrical up to the stem. Four other minor types were also found in cultivation by prospecting with a focus on identifying peculiar phenotypes: the Aiguille type, distinguishable from the Classique type by its more slender leaves and a thin pod; the Grosse Vanille type, which resembles *V. pompona*, with bigger and thicker leaves and stem than Classique; the self-sterile Stéride type with the same morphological characteristics of Classique; and lastly, the Variegata type, which has leaves with white yellow stripes (Bory et al., 2008).

Molecular characterization (i.e., isozymes, RAPDs) of limited samplings of cultivated *V. planifolia* in the Indian Ocean and elsewhere (Besse et al., 2004; Divakaran et al., 2007), including Mexico (Cibrian Jaramillo, 1999; Soto Arenas, 1999; Schlüter, 2002), have already shown low levels of genetic diversity but have failed to discriminate distinct clusters of intraspecific genetic diversity. To refine genetic polymorphism resolution with a wider genome-scanning approach, we used amplified fragment length polymorphism (AFLP) markers. These markers are ideally adapted to appraise germplasm because the method can rapidly generate and detect numerous polymorphisms (Krauss, 2000) that are widely distributed throughout the genome and the results are highly reproducible (Vos et al., 1995; Jones et al., 1997). This AFLP diversity study was undertaken to elucidate the patterns of *V. planifolia* introduction and diversification in cultivation areas (Indian Ocean, Polynesia, West Indies), with a special focus on Reunion Island and the specific aim to explain the phenotypic variations observed in the vanilla crop. In a recent review (Bory et al., 2008), we suggested that these phenotypes could be the result (1) of different introduction events, (2) of an accumulation of mutations through successive rounds of vegetative propagation or (3) of sexual reproduction (through manual pollination or possible rare natural/accidental pollination events). To distinguish between clonal diversity and diversity representative of variations between nonclonal siblings, we genotyped selfed progenies of the Classique type. This genotyping allowed us to characterize variations arising through sexual reproduction as opposed to strict asexual reproduction. Furthermore, levels of genetic diversity between spontaneous accessions of two American *Vanilla* species: *V. bahiana* Hoehne and *V. pompona* were also assessed to allow a comparison between spontaneous and cultivated species. As a whole, these results also contributed to unravelling the mechanisms of evolution history of *V. planifolia* and provided guidelines for *Vanilla* genetic resources conservation and improvement.

**MATERIALS AND METHODS**

*Plant material and DNA extraction*—The study focused on *V. planifolia* with 289 accessions surveyed (Table 1). This sample includes the six phenotypes that are grown and identified in Reunion Island (Bory et al., 2008). These types were collected in Reunion Island fields and forests during extensive prospecting and are maintained ex situ in the CIRAD collection (Centre de coopération Internationale en Recherche Agronomique pour le Développement) (Grisoni et al., 2007) or in the private collection of Provanille, both located on Reunion Island. All the phenotypic variations are stable as they were observed in the field and were maintained when accessions were transferred to the ex situ collections. Some *V. planifolia* self-progeny obtained from hand-pollination in Reunion Island were also analyzed. Other *V. planifolia* cultivated samples came from the Indian Ocean area (Madagascar) or other introduction areas (French Polynesia, French West Indies/Guadeloupe) and were obtained from prospecting, exchanges with botanical gardens or private collections and added to the CIRAD collection. Mexican cultivated samples from the area of Veracruz (‘Acamaya’, ‘Colibrí’, ‘Mansa’, ‘Mestiza’, ‘Oreja de Burro’) and from Chiapas (‘Vainilla’) were provided by the University California at Riverside (USA) as DNA extracts. Finally, some spontaneous types collected in Costa Rica, Central America, Brazil, and Guatemala or obtained from exchanges with botanical gardens or private collections were also included in the CIRAD collection and studied.

Some specimens from the cultivated species *V. tahitensis*, mainly supplied by the private collection of Etablissement Vanille de Tahiti (EVT) in French Polynesia, were also studied to provide information on the levels of genetic diversity and diversification processes in another cultivated species.

Spontaneous accessions from the American species *V. pompona* and *V. bahiana* that are genetically (Soto Arenas, 2003) and morphologically (Portères, 1954) related to *V. planifolia* were also studied to allow a comparison of the levels of genetic diversity between cultivated and spontaneous species. Accessions of *V. bahiana* originated from recent prospecting and were obtained from the Museum national d’Histoire naturelle (MNHN; Paris). Accessions of *V. pompona* were obtained through prospecting (Guadeloupe, Reunion Island) or from exchanges with botanical gardens. Despite only a single accession of *V. odorata* was available, it was also added because of the potential that it is a parental species of *V. tahitensis* (Bory et al., 2008). Artificial interspecific hybrids from breeding programs in Madagascar (Delassus, 1960; Dequaire, 1976; POFIFA, 1990) were also studied (Table 1).

Finally, some unidentified accessions were obtained from botanical gardens and were also included in the analysis to unravel their taxonomic designation.

Accessions for which a species name was provided and/or identified based on morphological observations or additional information provided by growers or botanists, were coded as PL for *V. planifolia*, TA for *V. tahitensis*, PO for *V..
Table 1. Code, type, place of collection of the sample, and number of analyzed Vanilla samples listed by species. Accession numbers are from the CIRAD vanilla collection (CRXXXX numbers), but the CR code was replaced by a code corresponding to species names to improve clarity.

<table>
<thead>
<tr>
<th>Species</th>
<th>Code</th>
<th>Type</th>
<th>Origin</th>
<th>No. (+repeats)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V. planifolia</td>
<td>PL...</td>
<td>289 (+20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLac. ...</td>
<td>Acamaya</td>
<td>Mexico</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>PLco. ...</td>
<td>Colibri</td>
<td>Mexico</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>PLma. ...</td>
<td>Mansa</td>
<td>Mexico</td>
<td></td>
<td>17</td>
</tr>
<tr>
<td>PLme. ...</td>
<td>Mestiza</td>
<td>Mexico</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>PLob. ...</td>
<td>Oreja de Burro</td>
<td>Mexico</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>PLvn. ...</td>
<td>Vainilla</td>
<td>Mexico</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>PL ...</td>
<td>Aiguille</td>
<td>Reunion Island</td>
<td>153 (+11)</td>
<td></td>
</tr>
<tr>
<td>PL ...</td>
<td>Grosse Vanille</td>
<td>Reunion Island</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>PL ...</td>
<td>Mexique</td>
<td>Reunion Island</td>
<td>8 (+2)</td>
<td></td>
</tr>
<tr>
<td>PL ...</td>
<td>Magic</td>
<td>Reunion Island</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>PL ...</td>
<td>Seedlings from self-pollination</td>
<td>Reunion Island</td>
<td>10 (+1)</td>
<td></td>
</tr>
<tr>
<td>PLst. ...</td>
<td>Sterile</td>
<td>Reunion Island</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>PLv. ...</td>
<td>Vartegata</td>
<td>Reunion Island (1), unknown (2)</td>
<td>12 (+2)</td>
<td></td>
</tr>
<tr>
<td>PLw. ...</td>
<td>Wild?</td>
<td>Central America, Costa Rica, Guatemala</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>PL ...</td>
<td>Wild?</td>
<td>Madagascar</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>PL ...</td>
<td>Pacific Ocean</td>
<td>Reunion Island</td>
<td>10 (+1)</td>
<td></td>
</tr>
<tr>
<td>PL ...</td>
<td>Reunion Island</td>
<td>29 (+2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PL ...</td>
<td>West Indies</td>
<td>5 (+1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PL ...</td>
<td>Unknown</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V. tahitensis</td>
<td>TA ...</td>
<td>11 (+1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAh ...</td>
<td>Haapape</td>
<td>French Polynesia</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Tap ...</td>
<td>Parahuru</td>
<td>French Polynesia</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Tar ...</td>
<td>Rea Rea</td>
<td>French Polynesia</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Tat ...</td>
<td>Tahiti</td>
<td>French Polynesia</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>TA ...</td>
<td>Madagascar</td>
<td>1 (+1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TA ...</td>
<td>Papua New Guinea</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TA ...</td>
<td>Unknown</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Not det. = Not determined

pompona, BA for V. bahiana, OD for V. odorata, and HY for hybrids. Accesions for which species identification was doubtful or unknown, either because information regarding species name was not available or because morphology did not correspond to the species description (according to Portères [1954]) were coded as SP.

DNA was extracted according to Risterucci et al. (2000) and purified on spin columns 100 (Sigma, St. Louis, Missouri, USA). DNA concentrations of extracts were visually estimated by comparisons with dilutions of maize DNA samples of known concentration after electrophoresis on agarose gels.

AFLP assay—The AFLP assay developed by Vos et al. (1995) was performed with the following modifications: 250 ng total genomic DNA were endonuclease-digested with 2.5 U EcoRI and 2.5 U MseI with reaction buffer (AFLP Core Reagent Kit, Invitrogen, Carlsbad, California, USA) for 2 h at 37°C in a final volume of 25 µL. Enzymes were inactivated for 15 min at 65°C. Digestion products were ligated by adding EcoRI and MseI double-stranded adapters (AFLP Core Reagent Kit, Invitrogen), 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen), 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Core Reagent Kit, Invitrogen). 1 U T4 DNA ligase, and T4 DNA ligase reaction buffer (AFLP Co...
further analyses. An assignment test, using the program AFLPOP (Duchesne and Bernatchez, 2002), did not provide additional information to our results using SM distances because of the strong structure (high bootstrap values) of the NJ tree.

**Reproducibility**—The seven primer pairs selected for the AFLP analysis (Table 1) were included in the analysis to test the AFLP reproducibility. Dissimilarities between pairs of repeats ranged from 0% to 0.0194. This maximum value represented a discrepancy of 13 bands over 285 bands and was attributed to misalignments by the GelCompar software. Therefore, the error rate of the AFLP analysis was estimated to a maximum of 4.56%, which was considered acceptable for genetic diversity analysis.

**RESULTS**

**Interspecific AFLP diversity**—The number of amplified fragments on the whole sample studied (375 accessions plus 25 repeats) varied from 81 to 205 with an average of 135 fragments per primer pair (Table 3). The average percentage of polymorphism was 96.4%, ranging from 94.7% for E-AAC/M-CAT to 98.8% for E-AGG/M-CTA primer pair.

The structure of the tree that was based on dissimilarities reflected species delimitations with high bootstraps (100% for *V. pompona*, *V. bahiana*, and *V. tahitensis* and 80% for *V. planifolia*; Fig. 1). The species genetically closest to *V. planifolia* was *V. tahitensis* (*D* max = 0.241; Table 4), and *V. bahiana* and *V. pompona* were the most divergent species from *V. planifolia* (*D* max = 0.387 and 0.373 respectively; Table 4).

All the artificial hybrids had positions consistent with their known parental origin (Fig. 1). Hybrids ‘Manitra Ampotony’ *HY0130* and *HY0003* (PL × TA) were intermediate between the two parental species *V. planifolia* (*D* 0.079 and *D* 0.074, respectively) and *V. tahitensis* (*D* 0.104 and 0.107, respectively); the back-cross hybrid *HY0131* ‘Tsy Tairy’ ((PL × PO) × PL) was closer to *V. planifolia* (*D* 0.096) than to *V. pompona* (*D* 0.291) as expected, and the three-way hybrid HY0140 ((PL × TA) × PO) was in an intermediate position, closer to (PL × TA) hybrids (*HY0130* and *HY0003*) (*D* 0.185 and *D* 0.183, respectively) than to the *V. pompona* species (*D* 0.275).

Twenty-one unidentified accessions (SP) could be reclassified as *V. planifolia* (12 accessions), *V. pompona* (6 accessions), *V. bahiana* (2 accessions) and *V. tahitensis* (1 accession) according to their position in the AFLP tree. On the other hand, 10 unidentified accessions also occupied unresolved positions (Fig. 1). Some were close to artificial interspecific hybrids, such as SP0139 or SP0123 and SP0125, which were linked to HY0140 with a bootstrap of 94%. Others were linked to species such as SP0706, which was close to *V. bahiana*, or they were in intermediate positions between species: SP0124, SP0060, SP0068, SP0166, SP0173, SP0683 (Fig. 1).

**Intraspecific AFLP diversity**—Intraspecific analyses were carried out separately for each species studied (including the newly identified SP specimens). Studying each species separately refined the analysis as more band levels are revealed in the interspecific analysis, thus enhancing the risk of misalignment.

**Vanilla planifolia**—For the 303 accessions analyzed, the number of amplified fragments varied from 33 to 90 with a mean of 62 fragments per primer pair (Table 3). The mean percentage of polymorphism was 66.2%, ranging from 46.7% for E-AAC/M-CAT to 82.5% for E-AGG/M-CTA primer pair. The global intraspecific *D* max value was 0.220. The majority of the accessions (284) were placed in a group of 101 genotypes (one of these genotypes included 122 accessions) with little variability (*D* mean = 0.011 and *D* max = 0.106) (Fig. 2). It gathered accessions from different geographical origins (Reunion Island, Madagascar, French Polynesia, French West Indies, Mexico) and of different phenotypes, from Reunion (Classique, Mexique, Stéride, Grosse Vanille, Varigata) and from Mexico (Mansa, Acamaya, Mestiza, one accession of Colibri). Within these 101 *V. planifolia* genotypes, the relative frequencies of the 185 polymorphic bands (over 436 scored bands) were calculated (Fig. 3A), which showed that the majority of the polymorphic bands (172) had frequencies in the extreme 0%-10% and 90%-100% ranges.

In addition to this group, 19 unique genotypes were found (*D* mean = 0.105 and *D* max = 0.190) (Fig. 2). These were the Mexican types Oreja de Burro and Colibri (two accessions), the 10 self-progenies of Classique, two self-progenies of Tsy Tairy, and the two Aiguille types from Reunion Island. Self-progenies of Classique had a strong pattern of segregating bands and a higher AFLP diversity (*D* max = 0.140) than did the main group.

**Table 2**. Name and sequence of selective amplification primers used in AFLP study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eco-AAC</td>
<td>GAC TGC GTA CCA ATT GAA C</td>
</tr>
<tr>
<td>Eco-AGG</td>
<td>GTC TGC GTA CCA ATT GAC G</td>
</tr>
<tr>
<td>Mse-CAT</td>
<td>GAT GAG TCC GTA ACA A</td>
</tr>
<tr>
<td>Mse-CAG</td>
<td>GAT GAG TCC GTA ACA C</td>
</tr>
<tr>
<td>Mse-CTA</td>
<td>GAT GAG TCC GTA ACT A</td>
</tr>
<tr>
<td>Mse-CTT</td>
<td>GAT GAG TCC GTA ACT T</td>
</tr>
</tbody>
</table>

**Table 3**. Total number of AFLP fragments (TN), number of polymorphic fragments (NP), and percentage of polymorphism (P%) observed using seven AFLP primer pairs for the global analysis and for the intraspecific analyses of the four species of Vanilla.

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>All species</th>
<th>V. planifolia</th>
<th>V. tahitensis</th>
<th>V. pompona</th>
<th>V. bahiana</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TN</td>
<td>NP</td>
<td>P%</td>
<td>TN</td>
<td>NP</td>
</tr>
<tr>
<td>E-AAC/M-CAT</td>
<td>205</td>
<td>195</td>
<td>95.1</td>
<td>90</td>
<td>42</td>
</tr>
<tr>
<td>E-AAC/M-CTT</td>
<td>173</td>
<td>167</td>
<td>96.5</td>
<td>73</td>
<td>41</td>
</tr>
<tr>
<td>E-AAC/M-CTA</td>
<td>151</td>
<td>143</td>
<td>94.7</td>
<td>71</td>
<td>43</td>
</tr>
<tr>
<td>E-AAC/M-CAC</td>
<td>133</td>
<td>128</td>
<td>96.2</td>
<td>77</td>
<td>54</td>
</tr>
<tr>
<td>E-AAC/M-AGG</td>
<td>117</td>
<td>114</td>
<td>97.4</td>
<td>52</td>
<td>34</td>
</tr>
<tr>
<td>E-AGG/M-CTA</td>
<td>82</td>
<td>79</td>
<td>96.3</td>
<td>40</td>
<td>33</td>
</tr>
<tr>
<td>E-AGG/M-CAC</td>
<td>81</td>
<td>80</td>
<td>98.8</td>
<td>33</td>
<td>27</td>
</tr>
<tr>
<td>Mean</td>
<td>135</td>
<td>129</td>
<td>96.4</td>
<td>62</td>
<td>39</td>
</tr>
<tr>
<td>Total</td>
<td>942</td>
<td>906</td>
<td>96.2</td>
<td>436</td>
<td>274</td>
</tr>
</tbody>
</table>


(D max = 0.106). The relative frequencies of the 95 polymorphic bands (of 322 scored bands) were assessed in these self-progenies (Fig. 3B), showing that the majority of the polymorphic bands (80) have frequencies around 75% (60–90% classes). Because these polymorphic bands obviously represent segregating heterozygous loci, they can be used to estimate an observed heterozygosity (Ho) of 0.295 in V. planifolia.

Global D max values were 0.126 and 0.174 for V. planifolia accessions cultivated in Reunion Island (without self-progenies) and Mexico, respectively.

**V. tahitensis**—For the 12 accessions analyzed, the number of amplified fragments varied from 20 to 87 with an average of 50 fragments per primer pair (Table 3). The average percentage of polymorphism was 20.1%, ranging from 5.6% for E-AAC/M-CTA to 36.6% for E-AAC/M-CAG primer pair. The intraspecific D max value was 0.161. The NJ tree was structured by a main group of seven genetically identical accessions (D = 0) and one very close accession (D = 0.003) as well as four diverging accessions (0.08 < D < 0.161) (data not shown). There was no apparent relationship between the genetic profiles and the phenotypes (Haapape, Rea Rea, Parahurahu, and Tahiti). The relative frequencies of polymorphic bands in V. tahitensis could not be calculated due to the small sample size (6 genotypes).

**V. pompona**—For the 27 accessions analyzed, the number of amplified fragments varied from 32 to 99 with an average of 66 fragments per primer pair (Table 3). The average percentage of polymorphism was 68.8%, ranging from 5.6% for E-AAC/M-CTA to 36.6% for E-AAC/M-CAG primer pair. The intraspecific D max value was 0.161. The NJ tree was structured by a main group of seven genetically identical accessions (D = 0) and one very close accession (D = 0.003) as well as four diverging accessions (0.08 < D < 0.161) (data not shown). There was no apparent relationship between the genetic profiles and the phenotypes (Haapape, Rea Rea, Parahurahu, and Tahiti). The relative frequencies of polymorphic bands in V. tahitensis could not be calculated due to the small sample size (6 genotypes).
DISCUSSION

Patterns of introduction of *V. planifolia* on Reunion Island—The vast majority (284 of 303) of the *V. planifolia* accessions studied here were grouped with very low levels of genetic diversity. This group included different phenotypes: Mexican types (Mansa, Mestiza, Acamaya, Vainilla, one accession of Colibri), types from Reunion Island (Classique, Mexique, Grosse Vanille, Sterile, Variegata) and accessions from other introduction and cultivation areas (Madagascar, West Indies, French Polynesia). The Mexican cultivated clones from Veracruz studied here were only slightly more variable ($D_{\text{max}} = 0.174$) than those cultivated in Reunion Island ($D_{\text{max}} = 0.126$). Mexican vanilla plantations generally consist of plants obtained from a very small number of original clones (Schlüter, 2002), and the supposedly spontaneous accessions from Costa Rica, Guatemala, and Central America studied are obviously escaped from cultivation (Soto Arenas, 2003). The close genetic affinity of Mexican cultivars to Indian Ocean (as well as French Polynesia and West Indies) cultivars suggests that the same original type is propagated in commercial production in these regions.

Mansa is the most widespread cultivated type in Mexico. The genetic similarity between Mansa and the other *V. planifolia* accessions (284 of 303) were also clearly differentiated from the others with a bootstrap of 100%. Accessions introduced to Reunion Island and French Polynesia were clearly differentiated from one another and from the spontaneous accessions (Fig. 4). Within the 25 *V. pompona* genotypes, the relative frequencies of polymorphic bands (307 of 463) were calculated (Fig. 3C). Approximately one third of the polymorphic bands (101) were distributed as for *V. planifolia* within the extreme 10% presence/absence frequencies, but the remaining two thirds (206) were also found in the intermediate 10–90% frequency range.

**V. bahiana**—For the 18 accessions analyzed, the number of amplified fragments varied from 20 to 101 with an average of 57 fragments per primer pair (Table 3). The average percentage of polymorphism was 42.8%, ranging from 34.5% for E-AAC/M-CTA to 56.3% for E-AAC/M-CAG primer pair. The intraspecific $D_{\text{max}}$ value was 0.164. The radial representation of the NJ tree revealed no structure (data not shown). Within the 18 *V. bahiana* genotypes, the relative frequencies of polymorphic bands (169 of 402 bands scored) were distributed similarly to that of *V. pompona* (Fig. 3D), with approximately one third of the polymorphic bands (63) within the extreme 10% presence/absence frequencies.

Fig. 2. Diversity structure of 303 *Vanilla planifolia* accessions derived from the neighbor joining tree using the distance matrix of Sokal and Michener (1000 bootstraps, bootstraps probabilities <60% not shown). For the identity of all accessions in the 284 accessions group, see Appendix S1 (see Supplemental Data with the online version of this article).
cultivated accessions is in accordance with the hypothesis that Mansa was probably the clone introduced in the Indian Ocean, Asia, and Africa (Soto Arenas, 2003). This clone could correspond to the specimen that was introduced, early in the 19th century, by the Marquis of Blandford into the collection of C. Greville at Paddington, from where it would have been disseminated worldwide (Correll, 1953; Bouriquet, 1954) via the botanical gardens of Paris and Antwerp (Belgium). From the current study, it can be concluded that most cultivated *V. planifolia* in the Indian Ocean (Reunion Island, Madagascar) and in other studied introduction areas (West Indies, French Polynesia) have evolved from a narrow genetic base via a single accession introduced (possibly Mansa).

**V. pompona: Another case of a limited introduction event on Reunion Island**—Based on morphological observations, a few *V. pompona* specimens were suspected to be present in vanilla crops in Reunion Island (Table 1). AFLP analysis confirmed that these accessions were indeed *V. pompona* (Fig. 4). Furthermore, they had extremely similar genotypes, suggesting that a limited number of specimens were introduced to Reunion Island (Fig. 4). On Reunion Island, the *V. pompona* specimens encountered in crops, may be the remaining specimens of the second documented introduction of vanilla on the island (Bory et al., 2008) from Cayenne on 27 June 1819 by Commander Philibert and the botanist Perrotet. The specimen was described as a “big vanilla” and was supposed to have disappeared (Bouriquet, 1954). It is therefore apparently still present. Interestingly, *V. pompona* was also introduced in the EVT collection in French Polynesia, and the introduced specimens in both areas were obviously different.

**Patterns of diversification of *V. planifolia* on Reunion Island**—The low levels of genetic diversity revealed are consistent with the vegetative mode of propagation of *V. planifolia* crops. Despite the higher information level of the markers used, the lack of genetic diversity revealed by AFLP markers ($D_{\text{mean}} = 0.011$ and $D_{\text{max}} = 0.106$) is similar to the RAPD results of Besse et al. (2004), who found very limited levels of intraspecific variation ($D = 0.04$) in cultivated *V. planifolia* (Classique and Mexique types) in the introduction areas. The level is also similar to that revealed for other cultivated species such as fig or clementine (Khadari et al., 1995; Bretó et al., 2001; Cabrita et al., 2001) or introduced invasive species such as *Rubus alceifolius* (Amsellem et al., 2000) after vegetative reproduction.

The current study also strongly suggests that *V. planifolia* specimens in Reunion Island have evolved through the accumulation
of mutations during two centuries of vegetative propagation after the introduction of a single accession. AFLP pattern analysis for the group of 284 accessions indicates the occurrence of point mutations: the variations were due to the appearance or disappearance of fragments with frequencies below 10% (Fig. 3A). Although the high proportion of these low frequency polymorphic bands (172/185) could be due to an overestimate because of errors inherent in the method (amplification and misalignment errors), this proportion is much higher than the estimated error rate (4.56% based on replicated experiments). The occurrence of point mutations is therefore a characteristic of the *V. planifolia* group.

As a comparison, we have also studied self-progenies created by manual pollination in Reunion Island. These were characterized by a pattern of segregating bands compatible with a 75% presence/25% absence segregation ratio expected from heterozygous AFLP loci, as shown from polymorphic band distribution in Fig. 3B. This sexual reproduction enhances diversity levels ($D_{max} = 0.140$).

Furthermore, we have also studied spontaneous American accessions of the species *V. bahiana* and *V. pompona* collected in the wild to see how genetic diversity was structured compared to a vegetatively propagated cultivated species such as *V. planifolia*. *V. bahiana* accessions showed no genetic structure and exhibited a $D_{max}$ value of 0.164. This could be related with the limited distribution area of *V. bahiana* in northeastern Brazil (Pignal, 1994). On the contrary, *V. pompona* had the highest $D_{max}$ value (0.340) in the AFLP study, in accordance with its large area of distribution as described by Portères (1954) (Trinidad, southeastern Mexico, Nicaragua, Panama, Colombia, Venezuela, Ecuador, Bolivia, Brazil, French and Dutch Guyana, Paraguay, Martinique, and Guadeloupe). The genetic structure showed well-defined groups in accordance with the description of *V. pompona* as a species complex by Soto Arenas (2006). Furthermore, the frequency distribution of polymorphic bands was assessed in *V. pompona* and *V. bahiana* (Fig. 3C and 3D). This distribution was compared to the patterns revealed for vegetatively propagated (Fig. 3A) and sexually propagated by selfing (Fig. 3B) *V. planifolia* accessions.

This comparative analysis confirms that the pattern of diversification of *V. planifolia* in introduction areas such as Reunion Island is different from what exists for other species in areas of
natural dispersion: they are solely due to the accumulation of somatic mutations through vegetative propagation.

On the basis of the AFLP patterns revealed, none of the phenotypes on Reunion Island (Classique, Mexique, Sterile, Grosse Vanille, and Variegata) could be differentiated from one another (Fig. 2). The observed phenotypic variations are therefore not the result of an accumulation of somatic mutations. These phenotypes could be the result of dominant point mutations with pleiotropic effects (Krug and Carvalho, 1951; Manning et al., 2006). Because we were unable to recover AFLP bands unique to any of these Indian Ocean phenotypes, such a proposition, if accurate, will be very difficult to test with AFLPs. Therefore, the revealed mutations cannot explain the majority of the phenotypic diversity observed in Reunion Island. A similar pattern of diversification was observed for the other cultivated species V. tahitensis in French Polynesia, which was shown to be related to V. planifolia (Fig. 1); genetic similarities between V. tahitensis accessions were high ($D_{\text{max}} = 0.161$) despite an obvious phenotypic variations in pod size and shape, leaf length, intensity of flowering, maturity times, and disease susceptibility (Portères, 1951).

There was only one notable exception. This study provided evidence that the phenotype Aiguille from Reunion Island might result from a sexual event. Indeed, the type Aiguille was genetically different from the majority of the V. planifolia accessions (Fig. 2). When Aiguille is taken into account, the $D_{\text{max}}$ value for V. planifolia on Reunion Island goes from 0.106 to 0.126. Interestingly, the genetic variability of the V. planifolia self-progenies encompassed that of the Aiguille type (Fig. 2). These observations support the possibility of a rare sexual reproduction (selfing) in the diversification of types from Reunion Island. Possibilities for sexual reproduction of Vanilla in Reunion Island have previously been discussed (Bory et al., 2008), including germination of a forgotten pod in a cultivated field (obtained through hand self-pollination or a possible pollination by the bird Zosterops; Zosteropidae), followed by vegetative multiplication of a single successfully germinated seedling. The two Aiguille accessions are indeed almost genetically identical (Fig. 2), showing that they obviously derived from each other by vegetative multiplication and subsequent point mutations. This sexual reproduction could have given rise to an easily distinguishable Aiguille type, which resembles the Classique type although it has more slender leaves, thinner flowers with a curved central sepal, and thinner pods (Bory et al., 2008). Such a sexual reproduction event would however be exceptional because it is at the origin of only two accessions found in the same plot despite extensive prospecting on Reunion Island. Within the limits of the sampling, the Aiguille type from Reunion Island was not introduced from Mexico, but this possibility cannot be ruled out because Mexican samples included in this study represent only cultivated material from Veracruz, an area that is likewise nearly genetically uniform and poorly representative of total levels of diversity in Mexico (Lubinsky, 2007). Nevertheless, the owner of the plantation did not mention any recent foreign introduction of plant material. Moreover, if this type had been introduced a long time ago, it would be expected to have been multiplied and spread to other plantations on the island. This is not the case and therefore strongly supports the hypothesis that the Aiguille type arose from sexual reproduction on Reunion Island.

**Perspectives for vanilla breeding and conservation**—As in any organism, but especially one of economic importance, a level of genetic diversity approaching uniformity is a clear and common cause for concern, particularly with regards to disease outbreaks. Nevertheless, there is considerable opportunity to diversify the stock of cultivated vanilla, as attested by the prior breeding successes in forming interspecific hybrids (Delassus, 1960; Dequaire, 1976; FOFIFA, 1990), as well as the optimistic results of more recent attempts to cross V. planifolia with Fusarium-resistant species like V. aphylla in India (Divakaran et al., 2006). Because V. planifolia was demonstrated to be heterozygous, with seedlings from self-pollination having greater AFLP diversity ($D_{\text{max}} = 0.140$) than the other group of 284 V. planifolia accessions ($D_{\text{max}} = 0.106$), there exists as well a high potential to increase diversity (in terms of genetic combinations) through selfing alone. In addition, the AFLP tool should assist genetic resources curators in screening accessions and setting up core collections.

**Conclusions**—We have answered major questions regarding the patterns of dissemination and diversification of V. planifolia. We also raise new questions and issues that will have to be further investigated.

Although this study suggested the possible (but rare) role of sexual reproduction (selfing) in the diversification of the Aiguille type in Reunion Island, the source of the remaining phenotypic variations observed in crops remains unresolved at this stage. The limited genetic variation revealed cannot distinguish between the main phenotypes Classique (from Reunion Island) and Mansa (from Mexico) and the other phenotypes (Mexique, Sterile, Grosse Vanille, and Variegata in Reunion Island or Mestiza, Acamaya, and Vainilla in Mexico). Other possible explanations can be proposed to account for the phenotypic variations observed in a vegetatively propagated species. The yellow/white striped accessions Variegata and Acamaya may have resulted from a mutation giving deficient plastids in one or several layers of the foliar tissue, and this origin will have to be specifically assessed particularly at the chloroplast DNA level. Phenotypes could also be the result of epigenetic phenomena, which cause heritable but reversible modifications in gene expression without concomitant changes in DNA sequence (Wu and Morris, 2001). Mechanisms of epigenetic origin were recently discovered through the use of MSAP (methylation sensitive amplified polymorphism) in numerous plant species where genetic diversity shown by classical molecular markers was very low when compared to the observed phenotypic diversity (Xiong et al., 1999; Bretó et al., 2001; Imazio et al., 2002). Epigenetic modifications in vanilla in introduction areas might explain why the phenotypic variation is not congruent with the observed low molecular diversity. Recent work suggested preliminary evidence that the V. tahitensis cv. Haapape could be a tetraploid (Duval et al., 2006) and that similar variations of the ploidy level could exist in V. planifolia and be responsible for some of the phenotypic diversity. Both hypothesis (epigenetics and polyploidy) are currently being tested to further unravel patterns of diversification of V. planifolia in Reunion Island. Finally, despite the self-sterility trait shared between the Mexican Oreja de Burro type (Castillo Martínez and Engleman, 1993; Soto Arenas, 2003) and the Sterile type from Reunion Island, these accessions displayed very different genotypes. It will be necessary to find out if these two accessions are characterized by the same mechanism of self-sterility (self-incompatibility, male sterility, or other mechanism). If this is the same mechanism, this self-sterility has evolved independently in Mexico and Reunion Island from different genotypes.
Some accessions remained unidentified at this stage and occupied intermediate positions between V. planifolia, V. bahiana, V. pompona, and V. odorata. These could represent other American Vanilla species not sampled in this study or interspecific hybrids. Vanilla is indeed notable for providing one of the few cases in which natural hybridization in neotropical orchids has been reported (Lubinsky et al., 2006) as demonstrated between V. claviculata (W. Wright) Sw. and V. barbellata Rchb.f. in the Puerto Rico region (Nielsen and Siegismund, 1999; Nielsen, 2000) and suggested for other American sympatric species (Bory et al., 2008). All this strongly suggests that there is a crucial need for codominant markers for the Vanilla genus, such as microsatellite markers, which would be of great help to resolve possible interspecific hybridization events. Such events are essential to study because they could explain the apparent difficulties in providing a correct revision of the taxonomy of the Vanilla genus (Soto Arenas, 1999, 2003).

LITERATURE CITED


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