

# Biodiversity and Evolution in the *Vanilla* Genus

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## 1. Introduction

Since the publication of the first vanilla book by Bouriquet (1954c) and the more recent review on vanilla biodiversity (Bory et al., 2008b), there has been a world regain of interest for this genus, as witnessed by the recently published vanilla books (Cameron, 2011a; Havkin-Frenkel & Belanger, 2011; Odoux & Grisoni, 2010). A large amount of new data regarding the genus biodiversity and its evolution has also been obtained. These will be reviewed in the present paper and new data will also be presented.

## 2. Biogeography, taxonomy and phylogeny

### 2.1 Distribution and phylogeography

*Vanilla* Plum. ex Miller is an ancient genus in the Orchidaceae family, Vanilloideae sub-family, Vanilleae tribe and Vanillinae sub-tribe (Cameron, 2004, 2005).

*Vanilla* species are distributed throughout the tropics between the 27th north and south parallels, but are absent in Australia. The genus is most diverse in tropical America (52 species), and can also be found in Africa (14 species) and the Indian ocean islands (10 species), South-East Asia and New Guinea (31 species) and Pacific islands (3 species) (Portères, 1954). From floral morphological observations, Portères (1954) suggested a primary diversification centre of the *Vanilla* genus in Indo-Malaysia, followed by dispersion on one hand from Asia to Pacific and then America, and on the other hand from Madagascar to Africa. This hypothesis was rejected following the first phylogenetic studies of the genus (Cameron, 1999, 2000) which suggested a different scenario with an American origin of the genus (160 to 120 Mya) and a transcontinental migration of the *Vanilla* genus before the break-up of Gondwana (Cameron, 2000, 2003, 2005; Cameron et al., 1999). The genetic differentiation between New World and Old World species observed would therefore be a consequence of the further separation of the continents. Our recent molecular phylogeny using chloroplastic *psaB*, *psbB*, *psbC*, and *rbcL* regions (Bouetard et al., 2010) supported the hypothesis of an American origin of the genus (figure 1). However, the recent discovery of a fossilized orchid pollinaria (20 Mya) (Ramirez et al., 2007) allowed the dating of Vanilloideae sub family at 72 Mya, well after the separation of Gondwana which questions the hypothesis of a vicariate evolution of the *Vanilla* genus (Bouetard et al., 2010).

Transoceanic dispersion appears more credible and would have been implied at least three times in the evolution of the *Vanilla* genus (figure 1). This was demonstrated by dating a *Vanilla* molecular phylogeny, testing these two extreme evolutionary scenarios (vicariate

versus transoceanic dispersion) (Bouetard et al., 2010) (figure 1). The Gondwanan dispersion scenario used 95 Mya as prior on the NW/OW node (the minimum age assumption for the break-up of Gondwana), whereas the NW/OW transoceanic dispersion scenario used 71 Mya as prior on the Vanilloideae node (a date estimated from fossil orchid pollinaria dating (Ramirez et al., 2007)) (figure 1). This provided evidence for at least three transoceanic dispersion events whatever the original scenario retained for the differentiation of NW versus OW species: from Africa to Asia, from Africa to the South West Indian Ocean Islands, and from Africa back to America (Caribbean region) (Bouetard et al., 2010) (figure 1).

## 2.2 Taxonomy and phylogeny

Taxonomic classification is based on morphological variations in vegetative and floral characters. Ephemeral flowers and their scarce availability in herbarium specimens associated with the fact that vegetative characters show important intra-specific variations are responsible for the difficulties in providing a clear taxonomic classification in *Vanilla* (Bory et al., 2010).

The first classification (Rolfe, 1896) distinguished two sections in the genus: section Foliosae, and section Aphyllae with leafy or leafless species, respectively. Portères (1954) then divided section Foliosae in three sub-sections: Papillosae, with thick leaves and a labellum with fleshy hairs, Lamellosae with thick leaves and a labellum with scaly lamellae, and Membranaceae with thin membranous leaves.

The *Vanilla* genus taxonomy has recently greatly benefited from molecular phylogenetics. The sequences used were chloroplastic *rbcL* (Cameron et al., 1999; Soto Arenas & Cameron, 2003), *psaB* (Cameron, 2004), *psbB* and *psbC* (Cameron & Molina, 2006), and the results obtained showed that Rolfe's sections and Portères' sub-sections classically used for taxonomy in *Vanilla* did not have a phylogenetic value. A recent study (Bouetard et al., 2010), based on these four markers combined, revealed three major clades in the genus, called groups  $\alpha$ ,  $\beta$ , et  $\gamma$  (figure 1). Group  $\alpha$  is represented by *V. mexicana* and is ancestral. Separation between group  $\beta$  (composed of New World/American Foliosae species) and group  $\gamma$  (composed of Old World/African and Asian Foliosae and American, Asian and African Aphyllae species) is more recent. This study confirmed an American origin of the genus, and also showed that the sections Foliosae and Aphyllae are not monophyletic (figure 1), a statement that questions the classical taxonomic treatment of the genus proposed by Rolfe (1896) and Portères (1954).

Recently, based on phylogenetic data of 106 species, (Soto Arenas & Cribb, 2010) proposed a new taxonomic classification, differentiating two sub-genera in the *Vanilla* genus. A group contains species previously classified as sub-section Membranaceae: *V. angustipetala*, *V. martinezii*, *V. inodora*, *V. mexicana*, *V. parviflora*, *V. edwalii* and the monospecific genus *Dictyophyllaria dietschiana* now *V. dietschiana* (Bouetard et al., 2010; Cameron, 2010; Pansarin, 2010a2010b; Soto Arenas & Cameron, 2003). It was named genus *Vanilla* sub-genus *Vanilla* as it contains the typus species for the genus (*V. mexicana*). It corresponds to the ancestral phylogenetic group  $\alpha$  (figure 1). The remaining *Vanilla* species are included in genus *Vanilla* sub-genus *Xanata*, which is further divided in two sections: section *Xanata* (corresponding to phylogenetic group  $\beta$ ) and section *Tethya* (group  $\gamma$ ) (figure 1). Within section *Xanata*, an early diverging group is noteworthy (figure 1) containing *V. palmarum*, *V. lindmaniana* and *V. bicolor* (Bouetard et al., 2010; Cameron, 2010; Soto Arenas & Cameron, 2003). This preliminary revised classification is a major step towards a needed complete revision of the genus based on molecular analyses.

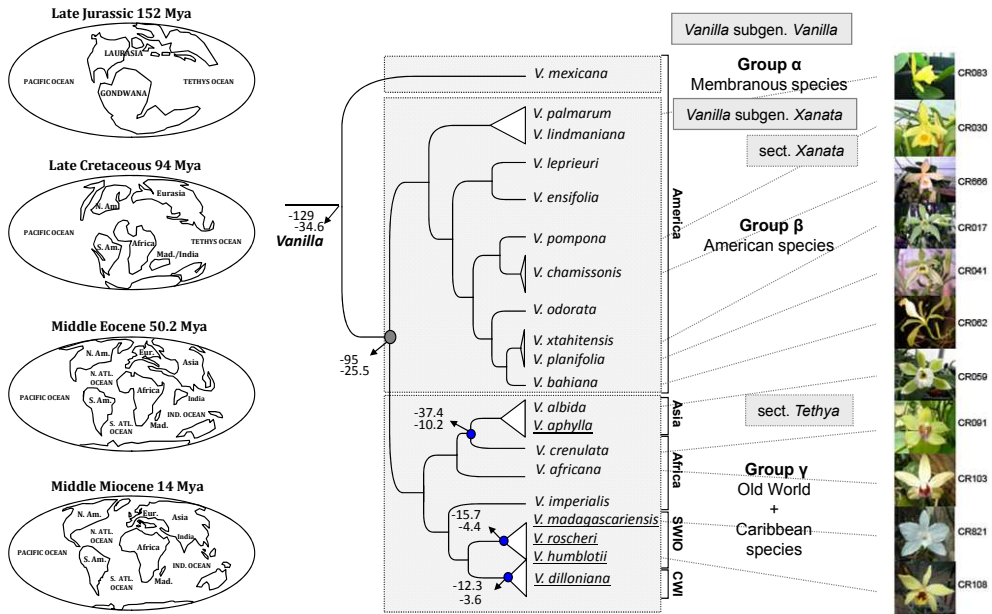


Fig. 1. Schematic representation of the molecular phylogeny of the *Vanilla* genus based on *rbcL*, *psaB*, *psbB* and *psbC* (Bouetard et al., 2010), distinguishing clades  $\alpha$ ,  $\beta$  and  $\gamma$ . The geographical origin of the species is indicated. Species underlined are from sect. *Aphyllae*, others are from sect. *Foliosae* (as per Rolfe’s classification). Taxonomic classification as per Soto Arenas & Dressler (2010) is indicated. Flowers of representative species and their voucher number (CR) in the BRC Vatel collection are presented (photographs: M Grisoni). Estimated divergence times (in Mya) derived from Bayesian relaxed clock analyses (uncorrelated exponential relaxed molecular clock model) (Bouetard et al., 2010) are indicated for key nodes: (i) origin of *Vanilla*, (ii) separation between New and Old World *Vanilla* species; (iii) separation between African and Asian species; origin of *Aphyllae* species (iv) in the South West Indian Ocean area and (v) in the Caribbean-West Indies area. Upper values correspond to the Gondwanan dispersion scenario and lower values correspond to the transoceanic dispersion scenario. Blue dots on clade nodes indicate transoceanic dispersion whatever the scenario tested. World maps at different geological times are provided.

In the first thorough taxonomic treatment of the genus published, Portères (Portères, 1954) described 110 species in the *Vanilla* genus. This number was reduced by different authors (Cameron et al., 1999; Soto Arenas, 1999, 2006; Soto Arenas & Dressler, 2010), but some species were not included (Hoehne, 1945) and new species have since been described (Z.J. Liu et al., 2007; Pignal, 1994; Soto Arenas, 2006, 2010; Soto Arenas & Cameron, 2003; Szlachetko & Veyret, 1995). There are to date more than 200 *Vanilla* species described (Bory et al., 2008b; Cameron, 2011b), but numerous synonymies remain and there is therefore an urgent need to thoroughly revise the taxonomic classification of the *Vanilla* species. We recently reviewed (Bory et al., 2010) the complexity of the processes involved in the evolution and diversification

of the *Vanilla* genus and concluded that *Vanilla* must be considered as a TCG, a “Taxonomic Complex Group” (Ennos et al., 2005). Indeed, it exhibits (i) an uniparental reproduction mode (vegetative growth) (Portères, 1954) (ii) interspecific hybridization in sympatric areas (Bory et al., 2010; Bory et al., 2008c; Nielsen, 2000; Nielsen & Siegismund, 1999) and (iii) polyploidy (Bory et al., 2010; Bory et al., 2008a; Lepers-Andrzejewski et al., 2011a; Lepers-Andrzejewski et al., 2011b). These mechanisms have profound effects on the organization of the biological diversity and have been described as responsible for the difficulty to define discrete, stable and coherent taxa in such TCGs (Ennos et al., 2005). *Vanilla* is a typical example of a genus for which the barcoding protocols (*matK* and *rbcL*) as proposed by the CBOL (M.L. Hollingsworth et al., 2009; P.M. Hollingsworth & CBOL Plant Working Group, 2009 ; Ratnasingham & Hebert, 2007), will therefore not be sufficient to revise the species taxonomy. The lack of genetic incompatibility between most *Vanilla* species (Bory et al., 2010) and the proven occurrence of inter-specific hybridizations in the genus (Bory et al., 2010; Bory et al., 2008c; Nielsen, 2000; Nielsen & Siegismund, 1999) will necessitate the obligate survey of nuclear regions in addition to cpDNA markers to resolve introgression patterns and correctly identify *Vanilla* species (Rubinoff, 2006). As an example, the species *V. ×tahitensis* was recently shown to be a *V. planifolia* x *V. odorata* hybrid using a combined ITS and chloroplastic phylogenetic analysis (Lubinsky et al., 2008b), when chloroplastic DNA alone repeatedly identified this species as identical to its maternal donor parent *V. planifolia* (figure 1). Moreover molecular genetic diagnostics can only be useful for barcoding biodiversity when species delimitations are either subtle or cryptic but nonetheless clear-cut. In a TCG, taxon limits are themselves diffuse, therefore genetic analysis alone might fail in the identification of discrete species (Ennos et al., 2005). A typical example of expected difficulties will be within the *V. pompona* species complex which was recently described as containing subspecies *pompona*, *pittieri*, and *grandiflora* based on ITS data, although the latter two are rather paraphyletic (Soto Arenas & Cribb, 2010) . In *Vanilla*, taxonomic revision of species will therefore have to use a combination of taxonomic, morphological, ecological, reproductive biology, cytogenetic (polyploidy estimates) and genetic (nuclear and chloroplastic) assessments.

### 3. *Vanilla* biodiversity in the wild

Most *Vanilla* species are hemiepiphytic vines climbing up to 30 meters high (*V. insignis*) (Soto Arenas & Dressler, 2010) and growing in tropical wet forests between 0-1000m (Portères, 1954). Only a few species are adapted to drier conditions (*V. calycullata*, (Soto Arenas & Dressler, 2010)), although extreme xeric adaptation is observed in the 18 leafless species of the genus (Portères, 1954). Vegetative reproduction (by natural stem cuttings) is the predominant reproduction mode adopted by most *Vanilla* species to develop settlements, such as *V. bahiana*, *V. chamissonis*, *V. madagascariensis*, *V. dilloniana*, *V. barbellata*, *V. claviculata* (reviewed in (Bory et al., 2010)). Some vines can grow up to 100 meters long (*V. insignis* (Soto Arenas & Dressler, 2010)) and in *V. planifolia* the same individual can cover up to 0.2ha (Soto Arenas, 1999). However a few species might be strictly sexually reproducing, such as *V. bicolor* and *V. palmarum* which are described as epiphytic on palm trees (Householder et al., 2010; Pignal, 1994), and *V. mexicana* (Bory et al., 2010; Cameron, 2010). Another notable exception is the species *V. dietschiana* which is non lianescent and 40 cm high, and has long been classified for these reasons as a different genus *Dictyophyllaria* (Pansarin, 2010a, 2010b; Portères, 1954).

In natural conditions, vanilla plant density can be extremely variable from being very high in certain areas (*V. trigonocarpa* (Soto Arenas & Dressler, 2010), *V. pompona* (Householder et al., 2010)) from very low as reported for wild *V. planifolia* in Mexico with less than one plant found per square kilometre (Soto Arenas, 1999). Some species are known to flower very frequently (*V. chamissonis*, (Macedo Reis, 2000)) to very un-frequently (*V. planifolia*, *V. hartii*, (Schlüter, 2002; Soto Arenas & Dressler, 2010)). A single flower per inflorescence generally opens in *Vanilla*, except 2-3 in some species (*V. odorata*, *V. martinezii*, *V. insignis*) and flowers are ephemeral (one day) except for some rare species such as *V. inodora* (2-3 days) (Soto Arenas & Dressler, 2010) or *V. imperialis* for which the flowers can be fertilized 4-5 days after opening (unpublished data). Seedlings can be found very frequently for species such as *V. bicolor* and *V. palmarum* (Householder et al., 2010) or be extremely rare as in *V. pompona* in Madre de Dios (Householder et al., 2010) or *V. planifolia* in Mexico (Schlüter, 2002). All these natural history traits will have deep effects on the levels of *Vanilla* species biodiversity that can be found in the wild. Particularly, the relative balance between vegetative and sexual reproduction and their relative efficiency will be of major importance in shaping populations genetic diversity. Exploring *Vanilla* species reproductive systems is therefore essential in this context.

### 3.1 *Vanilla* pollination

*Vanilla* species, like other orchids, are characterized by the presence of a rostellum membrane separating female and male reproductive systems, therefore limiting self-pollination. The diverse floral morphology observed in *Vanilla* species (figure 1) suggests that they have evolved to adapt to different pollinators (Soto Arenas & Cameron, 2003).

#### 3.1.1 Self-pollinating species

A few *Vanilla* species are described as spontaneously self-pollinating (Householder et al., 2010; Soto Arenas & Cameron, 2003; Soto Arenas & Dressler, 2010; Van Dam et al., 2010), as suggested by their abnormally high fruit set (table 1). This is consistent with general data in orchids showing that autogamous species display a much higher fruit set (77%) than cross pollinating species for which the majority show fruit set <20% (Tremblay et al., 2005). Based on high fruit set, these suggested autogamous species are *V. palmarum*, *V. savannarum*, *V. bicolor* (American species of the *V. palmarum* group), *V. guianensis*, *V. martinezii* (American species of the *V. mexicana* group) and *V. griffithii* (an Asian species). Possible self-pollination for *V. inodora* is also reported (Soto Arenas & Dressler, 2010), due to the large fruit set observed in some populations, although others have a fruit set as low as 2.5%.

Species	Natural fruit set (self-pollination)	Reference
<i>V. guianensis</i>	78%	(Householder et al., 2010)
<i>V. palmarum</i>	76%	(Householder et al., 2010)
<i>V. bicolor</i>	71%	(Householder et al., 2010)
<i>V. bicolor</i>	42.5% per raceme	(Van Dam et al., 2010)
<i>V. martinezii</i>	53% in a clone	(Soto Arenas & Dressler, 2010)

Table 1. Suggested self-pollinating *Vanilla* species and recorded natural fruit sets.

More precise observations are available for some of these species. *V. guianensis* is supposedly self-pollinated at early anthesis, as it was observed that the stigma and the

anther grew to contact one another; and no pollinators were observed despite the high fruit set recorded in Peru (Householder et al., 2010). The lack of observed local pollinators and the high fruit set also suggested that *V. bicolor* and *V. palmarum* were self-pollinating species in Peru (Householder et al., 2010).

Two mechanisms were proposed to account for self-pollination in *Vanilla* species (Van Dam et al., 2010): true self-pollination occurring by either stigmatic leak and/or the presence of a dehydrated or reduced rostellum, or agamospermy. In *V. bicolor*, pollen removal experiments showed that agamospermy was not the mechanism in play (Van Dam et al., 2010). Also all fertilized flowers showed fully developed rostellum. This suggested that a stigmatic leak, where stigma lobes release a fluid that contacts the pollen and induces germination of the pollen tubes (Van Der Pijl & Dodson, 1966) was the more likely explanation for self-pollination in this species (Van Dam et al., 2010). The observation of the occurrence of a thick rostellum in *V. palmarum* led to the suggestion of an identical mechanism (Householder et al., 2010). Our own observations on *V. palmarum* reveal self-pollination most likely due to a rostellum reduced in width, allowing pollinaria to get in contact with the stigmata on both sides of the rostellum (figure 2). A similar situation is found for the self-fertile species *V. lindmaniana* (data not shown).

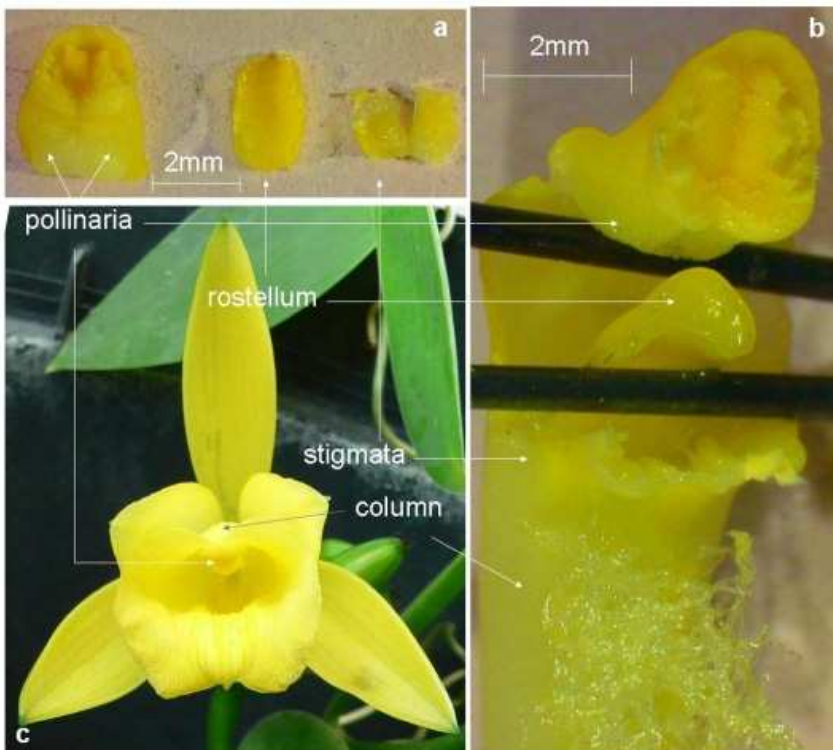


Fig. 2. Detailed structure of the pollinaria, rostellum and stigmata in the species *V. palmarum*: (a) and (b) accession CR0891, (c) accession CR0083, maintained in BRC Vatel (Reunion Island).

Spontaneous self-pollination is sometimes described even in classically outcrossing species. In Oaxaca plantations, cases of *V. planifolia* self-pollination are reported (Soto Arenas & Cameron, 2003) with rates reaching 6% of covered flowers giving fruit. Similar rates (6.06%) were reported for bagged *V. chamissonis* flowers in Sao Paulo (Macedo Reis, 2000). Nothing is known about the mechanisms involved in such exceptional cases.

### 3.1.2 Outcrossing species and pollinators

For the majority of *Vanilla* species, self-pollination does not occur due to an efficient rostellum and sexual reproduction therefore relies on the intervention of pollinators. Consequently, relatively low natural fruit sets are observed in natural conditions ((Bory et al., 2008b), table 2), consistent with the 17% median natural fruit set reported for tropical orchids (Tremblay et al., 2005). Reproductive success in orchids is pollination - rather than by resource - limited and could depend on pollinator effectiveness, abundance and diversity, and pollen quantity and quality (self *versus* allopollen) (Tremblay et al., 2005). This was demonstrated by crossing experiments in temperate and tropical orchids showing that cross hand-pollination shows significantly greater success (80%) than natural open pollination (26.6%) (Tremblay et al., 2005). Further studies are needed in *Vanilla* to determine the highest fruit sets achievable, but results on *V. barbellata*, *V. claviculata*, *V. dilloniana*, and *V. poitaei* have showed up to 100% fruit set under hand pollination experiments (Tremblay et al., 2005), and 75.76% in *V. chamissonis* (Macedo Reis, 2000), much higher values than what can be observed in natural conditions (table 2).

Species	Natural fruit set (open pollination)	Reference
<i>V. barbellata</i>	18.2 %	(Tremblay et al., 2005)
<i>V. chamissonis</i>	15%	(Macedo Reis, 2000)
<i>V. claviculata</i>	17.9 %	(Tremblay et al., 2005)
<i>V. crenulata</i>	0%	Johansson 1974, as cited in (Soto Arenas & Cameron, 2003)
<i>V. cristato-callosa</i>	6.6%	(Householder et al., 2010)
<i>V. dilloniana</i>	14.5 %	(Tremblay et al., 2005)
<i>V. planifolia</i>	1% to 1‰	(Soto Arenas, 1999)
<i>V. planifolia</i>	1%	(Childers & Cibes, 1948)
<i>V. planifolia</i>	1%	(Tremblay et al., 2005)
<i>V. planifolia</i>	1 à 3%	(Weiss, 2002)
<i>V. poitaei</i>	6.4 %	(Tremblay et al., 2005)
<i>V. pompona subsp. grandiflora</i>	0.9%	(Householder et al., 2010)
<i>V. riberoi</i>	1.1%	(Householder et al., 2010)

Table 2. *Vanilla* out-crossing species and natural fruit sets recorded.

If the pollinator of *V. planifolia* was long been considered as a social bee from the *Melipona* genus, as reported by Deltiel (as cited in (Rolfe, 1896)) and then mentioned in (Bouriquet, 1954a, 1954b; Stehlé, 1954), these records are now admitted as doubtful (Soto Arenas & Cameron, 2003; Van Der Cingel, 2001) as the bee is too small to perform the necessary

pollination steps (Lubinsky et al., 2006; Soto Arenas & Cameron, 2003). Lubinsky (2006), during observations of *V. planifolia* in Oaxaca (Mexico) and *V. pompona* subsp. *grandiflora* in Peru, indeed noticed *Melipona* visits, but no pollen movement was recorded. In tropical America (Guadeloupe (Stehlé, 1952) and Mexico (Stehlé, 1954)), authors have also reported the intervention of *Trigona* bees for *Vanilla* pollination, but this has never been confirmed. In Puerto Rico, leafless *Vanilla* species might be pollinated by *Centris* bees (Soto Arenas & Cameron, 2003). Hummingbirds are considered as vanilla pollinators in tropical America (Bouriquet, 1954a, 1954b; Stehlé, 1954). Lubinsky (2006) did indeed observe occasional *V. planifolia* visits by hummingbirds in Oaxaca, but with no pollen movement. Finally some authors (Dobat & Peikert-Holle, 1985; Geiselman et al., 2004) have suggested that the species *V. chamissonis* could be pollinated by two species of bats, although this fact was recently questioned (Fleming et al., 2009).

It is much more likely that in the American tropics, *Vanilla* is pollinated by large euglossine bees, as suggested by Dressler (1981) and demonstrated by such bees caught with *Vanilla* species pollinaria (Ackerman, 1983; Roubik & Ackerman, 1987). The principal reward offered by orchid flowers is nectar (Dressler, 1993), the most common reward for pollination (Van Der Pijl & Dodson, 1966). No *Vanilla* species has been described as producing floral nectar to our knowledge. However, the pollinators that visit orchid flowers can also obtain a variety of rewards (Singer, 2003; Tremblay et al., 2005) including oil, floral fragrances and, occasionally, pollen or stigmatic exudates (Bembe, 2004).

From years of observations in Mexico, Soto Arenas (Soto Arenas, 1999; Soto Arenas & Cameron, 2003) suggested the existence of three pollination systems for American *Vanilla* species (Bory et al., 2008b).

The first system relies on fragrance collection on flowers by male bees of the *Euglossa* genus, and has been suggested to concern the species of the *V. pompona* group as well as *V. hameri*, *V. cribbiana*, and *V. dressleri* (Soto Arenas, 1999; Soto Arenas & Cameron, 2003; Soto Arenas & Dressler, 2010). In this 'male euglossine syndrome' (Williams & Whiten, 1983) also referred to as 'perfume flower syndrome' (Bembe, 2004), now well known in many non nectar producing orchid species, male bees are attracted solely by the flower fragrance, and rub the surface of the flower with special tarsal brushes to collect fragrance materials, and subsequently store them in swollen glandular tibiae of the rear legs (Dodson et al., 1969). This fragrant orchid- male euglossine bee relationship is often highly specific (Dodson et al., 1969; Williams & Whiten, 1983). Bees then supposedly use these fragrance compounds as precursors for their own sex pheromones (Williams & Whiten, 1983) or in a "spraying" (of the fluid substances from their mid tibial tufts by vibrating action of their hind wings) behaviour as part of their courtship displays (Bembe, 2004). No study has so far been conducted to analyze *Vanilla* species flower fragrance compounds diversity and their relationship with pollinator specificity. This could give great insights on *Vanilla* evolution and diversity. On the other hand no direct evidence has been provided with regards to this male euglossine scent collection behaviour in any *Vanilla* flowers so far. Pollination of *V. trigonocarpa* by male *Euglossa asarophora* in Panama was reported (Soto Arenas & Dressler, 2010), with no information regarding scent collection behaviour. Male *Eulaema meriana* was identified as a possible pollinator for the species *V. pompona* subsp. *grandiflora* in Peru following observations of visits accompanied by pollen movement, but no scent collection behaviour was observed (Lubinsky et al., 2006). Similarly, some particularly fragrant flowers of this species were shown to attract two species of euglossine bees, *Eul. meriana* and *Eug. imperialis* (Householder et al., 2010). Only *Eul. meriana* was observed pollinating flowers on



two occasions, but no floral fragrance collection was recorded (Householder et al., 2010). This does not so far therefore confirm the suggested male euglossine syndrome within the *V. pompona* group. Most species seem to be pollinated under a deceptive system, as also suggested for *V. planifolia*, *V. odorata*, *V. insignis* and *V. hartii*, with flower visits by either male or female bees and an absence of reward (Soto Arenas, 1999; Soto Arenas & Cameron, 2003). This particular pollination system, using different strategies to lure pollinators, is mainly encountered in orchids with a third of the species in this family supposedly using this pollination system (Jersakova et al., 2006; Schiestl, 2005; Singer, 2003; Tremblay et al., 2005), particularly low density species (Ackerman, 1986), as it is the case for *V. planifolia* (Bory et al., 2008b; Soto Arenas, 1999). Soto Arenas considers the bee *Eugl. viridissima*, and maybe bees from the *Eulaema* genus, to be the real pollinators of *V. planifolia* (Bory et al., 2008b; Soto Arenas & Dressler, 2010). These species (as well as *Exeretes*) were recorded as occasional visitors of *V. planifolia* in Oaxaca (Mexico) without pollen movement (Lubinsky et al., 2006). *V. cribbiana* is reported to be pollinated by an unidentified *Eulaema* bee, *V. hartii* flowers are visited by female *Euglossa* bees and *V. insignis* flowers by male bees of *Eul. polychroma* (Soto Arenas & Dressler, 2010). The true pollinators of *V. planifolia* and most allied species therefore remain to be elucidated.

The last system might imply strong and large carpenter bees (*Xylocopa* species) and would concern the species *V. inodora*. This was suggested based on the peculiar floral structure of this species and allied Membranaceae (Soto Arenas & Cameron, 2003) characterized by a frontally closed labellum (the column apex lying on the lip) which is similar to that of other orchid species pollinated by carpenter bees (Soto Arenas & Cameron, 2003). These bees were observed visiting *V. inodora* but no proof of true pollination has been provided so far (Soto Arenas & Cameron, 2003; Soto Arenas & Dressler, 2010). The only data available on *Vanilla* potential pollinators, although partial, is therefore from America. There is a considerable lack of knowledge of potential *Vanilla* pollinators in other geographical areas. In Africa, euglossine bees do not occur, but other large bees may be pollinators there (Van Der Cingel, 2001). Despite three years of observation of the species *V. crenulata* in Africa, no pollinator visit was recorded (Johansson, 1974, as cited in (Soto Arenas & Cameron, 2003)). Observations in Madagascar of occasional natural fruit set in the introduced species *V. planifolia*, were attributed locally to sunbirds of the *Cynniris* genus (so called ‘Sohimanga’) (Bouriquet, 1954a). Similarly, in Reunion Island, rare natural pollination events of the introduced *V. planifolia* may be linked to noticed visits by the bird *Zosterops* (Zosteropidae) (Bory et al., 2008b), an Angraecoid orchid pollinator there (Micheneau et al., 2006). These hypotheses have not been confirmed, and remain unlikely as flower structure in *Vanilla* is indicative more of a bee pollination system (Dressler, 1981). Finally, a large bee of the *Aegilopa* genus was recorded pollinating *V. cf. kaniensis* in Papua New Guinea (Soto Arenas & Cameron, 2003). Although fruits of *V. albida* and *V. aphylla* from Java were described and illustrated in 1832, the introduced species *V. planifolia* did not naturally set fruit there, showing the need for different pollinators (Arditti et al., 2009). No other information is available regarding *Vanilla* pollinators in Asia (Van Der Cingel, 2001). It will be important to assess whether *Vanilla* species with higher fruit set (table 2) are characterized by reward pollination mechanisms as it was demonstrated that rewarding orchids show significantly higher fruit set than deceptive ones (twice as much) (Tremblay et al., 2005). Reproductive success might also be related to the fragrance attractiveness of flowers, even in a deceptive system. Further insights on this matter could be obtained by characterising *Vanilla* species floral fragrance and colour as well as identifying their respective pollinators and behaviour.

Partial information is available (Soto Arenas & Dressler, 2010) for *V. planifolia* stating the presence of 1-2-dimethyl-cyclopentane, ethyl acetate, 1-8-cineol and ocimene-trans, and for *V. insignis* possessing the same principal constituents although ocimene-trans is notoriously absent. 1-8-cineol is especially well known to be a strong attractant for euglossine bees (Soto Arenas & Dressler, 2010). Our own observations (unpublished data) show that the species *V. chamissonis* displays particularly strongly fragrant flowers (more than *V. planifolia*), this could explain why its fruit set is amongst the highest.

### 3.2 Myrmecology

An obvious interaction exists between *Vanilla* and ants, as also demonstrated for other orchid species (Peakall, 1994). Extrafloral nectar is produced in immature bud abscission layer in many *Vanilla* species such as *V. pompona*, *V. cristato-callosa* in Peru (Householder et al., 2010) and *V. planifolia* in Panama (Peakall, 1994) and ants were observed in these species feeding on sugary exudates. Ants were also reported visiting *V. planifolia* flowers in Oaxaca (Lubinsky et al., 2006), without pollination. *V. planifolia* also occasionally inhabits ant nests, and was also observed to support ant nests in its root mass (Peakall, 1994).

The benefit of the association is obvious for the ant (food and shelter), but the benefit (if any) for the *Vanilla* plant remains to be elucidated. In some orchid species, ants visiting extrafloral nectaries have been shown in some cases to protect them against herbivory or to be attractors to bird pollinators (Peakall, 1994). Close association between ant nests and orchids have also suggested a role of ants in seed dispersion particularly in orchids with oily seeds (Peakall, 1994). In fragrant *Vanilla* fruits, seeds are held in an oily matrix (Householder et al., 2010). Ants have been reported in vanilla crop to be important for humus disintegration (Stehlé, 1954). On the other hand, the presence of ants could simply be indicative of the presence of mealybugs, softscales or aphids rather than an indication of a mutualistic interaction (Chuo et al., 1994). In *V. planifolia*, associations between scale and the black ant *Technomyrmex albipes* in Seychelles, as well as between ants and the aphid *Cerataphis lataniae* have been reported (Risbec, 1954).

### 3.3 Fragrance and bees and fruit dispersion

Seed dispersal mechanism(s) of *Vanilla* remains enigmatic. Fruits reaching maturity in many *Vanilla* species show dehiscence (Bouriquet, 1954c). This character favours seed dispersal, although it is noticeably not interesting in fruit crop production. In aromatic fruits, *Vanilla* seeds are easily rubbed off and are extremely sticky due to a thin covering of oil, which may favour epizoochorous seed dispersal by any visitor, insect or vertebrate (Householder et al., 2010). Soto Arenas and Cameron (2003) mentioned that *Vanilla* species producing fragrant fruits are restricted to tropical America and proposed the designation of group  $\beta$  (figure 1) as the 'American fragrant species' group, but this should not include species from the *V. palmarum* group as these were described as non-fragrant ((Householder et al., 2010), see below). Fruit fragrance was described as a pleisiomorphic character in orchids as it is present in *Vanilla* and in three other primitive groups (*Cyrtosia*, *Neuwiedia*, *Selenipedium*) (Lubinsky et al., 2006).

It has been demonstrated that euglossine bees are attracted by fragrant *Vanilla* fruits and act as seed collectors and potential dispersers. Van Dam et al. (2010) have photographed male *Eul. cingulata* with a typical scent collection behaviour on *V. pompona* subsp. *grandiflora* fruits in Peru. Householder et al. (2010) also reported strong attractiveness of fruit of this species

to *Eul. meriana* and *Eug. imperiali* which may stay on the same fruit for 15 minutes displaying typical scent collection behaviour. They also observed a similar behaviour by a metallic green *Euglossa* sp. on old and dehiscent *V. cristato-callosa* fruits. This confirmed previous observations of euglossine bees brushing on *Vanilla* fruits (Madison, 1981) and demonstrated the particular attractiveness of these bees to fragrant *Vanilla* flowers as well as to fragrant fruits, an important evolutionary step in the orchid/orchid-bee relationship in *Vanilla*. As discussed by Lubinsky et al. (2006), this demonstrates that the orchid/orchid-bee relationship has evolved in *Vanilla* as a mode of flower pollination as well as fruit dispersion. *Trigona* bees were observed in Peru transporting sticky *V. pompona* seed packets on their hind tibia and often dropping them (Householder et al., 2010). These bees are not typical scent collectors and could just be interested in the nutritional value of the oils (Householder et al., 2010). One species of carpenter bee (*Xylocopa* sp) is also mentioned visiting *V. pompona* fruits (Householder et al., 2010).

Fruit dispersal by bats was suggested for *V. insignis* and observed for *V. pompona* (Soto Arenas & Dressler, 2010). Occasional total or partial herbivory of the fruit was also noticed for *V. pompona* in Peru, possibly attributed to bats or marsupials (Householder et al., 2010).

Bird dispersal is expected in some Asian species, as *V. abundiflora* and *V. griffithii*, as in the closely related Vanilloideae *Cyrtosia* genus (Soto Arenas & Dressler, 2010). However *Cyrtosia* has fleshy fruits like *Vanilla* but these are bright red presumably acting as an attractor to birds or mammals (Cameron, 2011b)

For some other *Vanilla* species however, fruits are non fragrant and seeds are not held in a particularly oily matrix. This is the case for *V. bicolor* and *V. palmarum* (Householder et al., 2010). Dehiscence of the fruits and canopy habitat suggested a different mechanism of seed dispersal in such species, by a combination of wind turbulence and gravity (Householder et al., 2010).

### 3.4 Conclusions

Many *Vanilla* species are threatened in the wild. This is particularly the case for *V. planifolia* in Mexico, its centre of origin. Proper conservation strategies need to be developed, but this will require gaining a better knowledge on the reproductive strategies and the derived levels of genetic diversity in these *Vanilla* species. This will include assessing the relative contribution of vegetative *vs* sexual reproduction, self-compatibility (auto *vs* allo fecundation success), pollination syndromes (pollinators, reward/deceit) and seed dispersion systems.

There is a considerable lack of genetic studies of *Vanilla* species biodiversity in the wild. The only published data concern the aphyllous species *V. barbellata*, *V. dilloniana* and *V. claviculata* on the island of Puerto Rico (Nielsen, 2000; Nielsen & Siegismund, 1999) using isozyme markers. Genotypic frequencies were in accordance with Hardy-Weinberg proportions for all species, which could suggest random crosspollination. High differentiation among populations was detected, supposedly attributed to limited seed dispersal by bees. Genetic drift was also demonstrated in some isolated populations (Nielsen & Siegismund, 1999). Soto Arenas also conducted *V. planifolia* population genetic studies in Mexico using isozymes (Soto Arenas, 1999), surprisingly demonstrating homozygous excess corresponding to preferential autogamous reproduction for this species. Development of suitable approaches to the analysis of genetic diversity in a spatial context, where factors such as pollination, seed dispersal, breeding system, habitat heterogeneity and human influence are appropriately integrated in combination with molecular

population genetic estimates, will be essential (Escudero et al., 2003) to provide new insights in the understanding of the mechanisms of maintenance and dynamics of *Vanilla* populations and to provide guidelines for their preservation.

#### 4. Vanilla biodiversity in cultivated conditions

*Vanilla* is the only orchid with a significant economic importance in food industry. It is cultivated for its aromatic fruit, a character restricted to some species from the American continent (Soto Arenas & Cameron, 2003). Only two species are grown to produce commercial vanilla: *V. planifolia* and *V. ×tahitensis*; with *V. planifolia* providing 95% of the world production, mainly originating from Madagascar, Indonesia, Comoros, Uganda and India (Roux-Cuvelier & Grisoni, 2010). Biodiversity in cultivated conditions depends on the level of diversity originally introduced and on cultivation practices used in different countries during domestication. Vanilla crops are established from stem cuttings of 8–12 nodes, collected from healthy and vigorous vines (Bory et al., 2008b; Bouriquet, 1954a; Purseglove et al., 1981; Soto Arenas & Cameron, 2003; Stehlé, 1952). As natural pollinators are absent in the areas of vanilla production, pollination is performed by hand following a simple method discovered by the slave Edmond Albius in Reunion Island in 1841 (Kahane et al., 2008). Given these cultivation practices, low levels of genetic diversity are expected in cultivation areas. However, for both species, different varieties, showing recognized but poorly defined morphological, agronomical and aromatic properties, are often cultivated by growers (Duval et al., 2006). Given the vegetative mode of propagation and the absence of pollinators, five hypotheses have been proposed to explain these variations (Bory et al., 2008b): (i) multiple introduction events, (ii) somatic mutations, (iii) sexual reproduction, (iv) polyploidy and (v) epigenetic modifications. In recent years, these hypotheses were explored, giving new insights on the processes involved during the dispersion and domestication of the two main cultivated *Vanilla* species. These results also give important clues to the understanding of *Vanilla* evolutionary processes in natural conditions.

##### 4.1 *V. planifolia* in Reunion Island

The species *V. planifolia* originated in Mesoamerica (Portères, 1954). Some of the history of vanilla follows the history of chocolate because vanilla was gathered from the wild for use in flavoring chocolate beverages in the pre-Columbian Maya and Aztec cultures of southeastern Mexico and Central America. However, the Totonac people of Papantla in north-central Veracruz (Mexico) were probably the first group to cultivate *V. planifolia* (Lubinsky et al., 2011). The species *V. planifolia* has an interesting history of dispersal to other tropical regions between 27° N and 27° S latitudes (Lubinsky et al., 2008a). After the discovery of the Americas by C. Columbus, the whole history of *V. planifolia* dissemination, following the discoveries of manual pollination by the slave Edmond Albius in 1841 and curing process by E. Loupy and D. De Floris is intimately linked to Reunion Island (Kahane et al., 2008). From then, *V. planifolia* was renowned as 'Bourbon Vanilla' since it was produced originally from Reunion Island (from 1848) and later from a cartel of Indian Ocean Island producers (Madagascar, Reunion, Comoros and Seychelles).

The true origin of cultivated vanilla outside of Mexico was unclear until AFLP and microsatellite markers were used to elucidate the patterns of introduction of *V. planifolia*. These studies showed that most of the accessions cultivated today in the islands of the Indian Ocean and worldwide (Reunion Island, Madagascar, French Polynesia, French West

Indies, Mexico) and of different morphotypes (from Reunion 'Classique', 'Mexique', 'Sterile', 'Grosse Vanille' (table 3) and from Mexico 'Mansa', 'Acamaya', 'Mestiza') (Bory et al., 2008c; Lubinsky et al., 2008a) derive from a single introduced genotype. It could correspond to the lectotype that was introduced, early in the nineteenth century, by the Marquis of Blandford into the collection of Charles Greville at Paddington (UK) (Portères, 1954). Cuttings were sent to the botanical gardens of Paris (France) and Antwerp (Belgium) from where these specimens were disseminated to Reunion Island (by the ordinance officer of Bourbon, Marchant) and then worldwide (Bory et al., 2008b; Kahane et al., 2008).

Consequently, cultivated accessions in Reunion Island exhibit extremely low levels of genetic diversity and have evolved by the accumulation of point mutations through vegetative multiplication (Bory et al., 2008c) (table 3). Maximum genetic distance ( $D_{max}$ ) was 0.106 and the majority of the polymorphic AFLP bands revealed had frequencies in the extreme (0-10% and 90-100%) ranges, therefore corresponding to rare AFLP alleles (presence or absence) a pattern typical of point mutations (Bory et al., 2008c). One peculiar and rare phenotype 'Aiguille' found in Reunion Island was shown to result from sexual reproduction (selfing) (Bory et al., 2008c) (table 3) as its AFLP pattern fell within a group of selfed progeny with  $D_{max}=0.140$  and showed a strong pattern of segregation bands. The hypothesis was that it resulted from manual self-pollination and subsequent seed germination from a forgotten pod (Bory et al., 2008c). Flow cytometry, microdensitometry, chromosome counts and stomatal lengths showed that polyploidization has been actively involved in the diversification of *V. planifolia* in Reunion Island (Bory et al., 2008a). Three ploidy levels (2x, 3x, 4x) were revealed that allowed to explain the features of the 'Sterile' type which is auto-triploid and of the 'Grosse Vanille' type, auto-tetraploid (Bory et al., 2008a). It was suggested that these resulted from the production of non-reduced gametes during the course of manual self-pollination performed by growers (Bory et al., 2010; Bory et al., 2008a).

As the particular phenotype 'Mexique' encountered in Reunion could not be explained by genetic or cytogenetic variations, we tested whether it could have resulted from epigenetic modifications as some studies showed that morphological variations in clonal populations could be explained by a combination of genetic and epigenetic factors (Imazio et al., 2002). Epigenetics corresponds to reversible but heritable modifications of gene expression without changes in the nucleotidic sequence (Mathieu et al., 2007; Wu & Morris, 2001), such as DNA methylation (Finnegan et al., 1998). Epigenetic modifications are heritable (Akimoto et al., 2007; Finnegan et al., 1996; Grant-Downton & Dickinson, 2006; Martienssen & Colot, 2001) and transmitted as well as by asexual propagation (Peraza-Echevarria et al., 2001).

Sometimes, a phenotypic reversion correlated with demethylation of the epi-mutated gene can occur and its expression is restored (Jaligot et al., 2004). These epigenetic mutations have important phenotypic as well as evolutionary consequences, this representing a current field of investigation (Finnegan, 2001; Kalisz & Purugganan, 2004; B. Liu & Wendel, 2003). DNA methylation proceeds by the addition in a newly replicated DNA of a methyl group by a DNA methyltransferase (Finnegan et al., 1998; Martienssen & Colot, 2001). Cytosine is the most frequently methylated base, resulting in 5-methylcytosine formation ( $5mC$ ) (Martienssen & Colot, 2001). Plant methylation is restricted to the nuclear genome and is concentrated in repeated sequence regions (Finnegan et al., 1998). Methylation is implied in many biological processes such as 'gene silencing', mobile DNA elements control, DNA replication duration, chromosome structure determination, and mutation frequency increase (Finnegan et al., 1998; Paszkowski & Whitham, 2001). Many spontaneous or induced epimutations are known in maize, Arabidopsis and other plant species and are responsible

Morphotypes	Characteristics	Diversity/genetics	Origin
'Classique'	The most cultivated type	Point mutations Dmax = 0.106	Mexico then Antwerp Botanical Gardens
'Aiguille'	Slender leaves and thin pods	As self progenies Dmax=0.140	Selfing of 'Classique'
'Sterile'	'Classique', but self-sterile	Same AFLP profile as 'Classique', auto-triploid (3x)	Selfing of 'Classique', unreduced gamete (2n x n)
'Grosse Vanille'	Bigger leaves, stems, flowers and fruits than 'Classique'	Same AFLP profile as 'Classique', auto-tetraploid (4x)	Selfing of 'Classique', unreduced gametes (2n x 2n)
'Mexique'	Darker bluish leaves with central gutter and curved sides, cylindrical pods	Same AFLP and MSAP profile as 'Classique'	Epigenetic or genetic single dominant mutation with pleiotropic effects

Table 3. *V. planifolia* morphotypes encountered in Reunion Island and their description.

Accession	Morphotype	Collection	Accession	Morphotype	Collection
CR0217	'Classique'	Provanille 3A11	CR0493	'Mexique'	Provanille 15A8
CR0218		Provanille 3A11	CR0494		Provanille 15A8
CR0219		Provanille 3A11	CR0495		Provanille 15A8
CR0343	'Classique'	Provanille 6A8	CR0334	'Mexique'	Provanille 6A5
CR0344		Provanille 6A8	CR0335		Provanille 6A5
CR0345		Provanille 6A8	CR0336		Provanille 6A5
CR0457	'Classique'	Provanille 15A6	CR0337	'Mexique'	Provanille 6A6
CR0458		Provanille 15A6	CR0338		Provanille 6A6
CR0459		Provanille 15A6	CR0339		Provanille 6A6
CR0563	'Classique'	Provanille 16B2	CR0001	'Mexique'	BRC Vatel
CR0564		Provanille 16B2	CR0002	'Mexique'	BRC Vatel
CR0565		Provanille 16B2	CR0627	'Mexique'	BRC Vatel StP
CR0340	'Classique'	Provanille 6A7	CR0649	'Mexique'	BRC Vatel StP
CR0341		Provanille 6A7	CR0632	'Mexique'	BRC Vatel StP
CR0342		Provanille 6A7	CR0711	'Classique'	BRC Vatel SteR
CR0647	'Classique'	BRC Vatel StP	CR0714	'Classique'	BRC Vatel SteR
CR0650	'Classique'	BRC Vatel StP			

Table 4. *V. planifolia* Reunion Island accessions surveyed in the MSAP analysis (StP: Saint Philippe; SteR: Ste Rose).

for the generation of mutant phenotypes (Finnegan et al., 1996; Martienssen & Colot, 2001). To assess whether 'Mexique' morphotypes might have resulted from epigenetic modifications, we selected the MSAP (Methylation-sensitive amplified polymorphism) method (Reyna-López et al., 1997), an AFLP-derived methodology which allows the visualization of a large number of markers revealing cytosine methylation state at each digestion site, without any *a priori* knowledge of genomic sequences. MSAP analyses were performed on a sample of 'Classique' and 'Mexique' accessions (table 4). Twenty-four accessions were collected in the collection of Provanille in Bras-Panon (Reunion Island), corresponding to 8 varieties with three cuttings. This was to verify if genetic or methylation polymorphism, if existing, is transmitted through vegetative multiplication. Others were

collected in vanilla plantations in Reunion Island (St-Philippe or Ste-Rose) and are maintained in the BRC Vatel collection.

We used the restriction enzyme *EcoRI* as well as *MspI* and *HpaII*, isochizomers that cut the same restriction site CCGG but show different sensitivity to methylation (table 5). The MSAP methodology used was as described in (Reyna-López et al., 1997). *HpaII* digests were repeated twice. The adaptators used are presented in table 6 and 8 *Eco/Hpa* primer combinations were used for selective amplification.

	<i>EcoRI/HpaII</i>	<i>EcoRI/MspI</i>	CCGG methylation
Case number 1	1	1	CCGG
Case number 2	1	0	<sup>5hm</sup> CCGG
Case number 3	0	1	C <sup>5m</sup> CCGG
Case number 4	0	0	<sup>5m</sup> C <sup>5m</sup> CCGG or <sup>5m</sup> CCGG

Table 5. Methylation sensitivity of *HpaII* and *MspI* (m : methylation; hm : hemimethylation).

The comparison of the profiles from the amplification after DNA digestion with *EcoRI/HpaII* and *EcoRI/MspI* gives informations on the methylation status of the internal cytosine in sequence CCGG (table 5). For example a band present in the *MspI* profile and absent in *HpaII* indicates a methylation of the internal cytosine, whereas the opposite situation indicates an hemimethylation of the external cytosine. A methylation event was considered as polymorphic when at least one accession differed from the others in its profile.

Name	Sequence (5'-3')
Double strand adaptators	
Ad <i>EcoRI</i> 1	CTC GTA GAC TGC GTA CC
Ad <i>EcoRI</i> 2	AAT TGG TAC GCA GTC
Ad <i>HpaII</i> 1	GAT CAT GAG TCC TGC T
Ad <i>HpaII</i> 2	CGA GCA GGA CTC ATG A
Pre-amplification primers	
Eco-A	GAC TGC GTA CCA ATT CA
Hpa-A	TCA TGA GTC CTG CTC GGA
Selective amplification primers	
Eco-AC	GAC TGC GTA CCA ATT CAC
Eco-AG	GAC TGC GTA CCA ATT CAG
Hpa-ATT	ATC ATG AGT CCT GT CGG ATT
Hpa-ATG	ATC ATG AGT CCT GT CGG ATG
Hpa-AAC	ATC ATG AGT CCT GT CGG AAC
Hpa-AAG	ATC ATG AGT CCT GT CGG AAG

Table 6. Adaptator and primer sequences used in MSAP analysis.

Between 48 and 70 fragments were revealed by primer combination. On the 483 CCGG sites observed, 188 were non methylated (38.9%), 36 were methylated (7.45%), with 5 sites only presenting methylation polymorphisms (1.03%) in 4 accessions. Accessions CR0340 and CR0341 were hypomethylated, they showed bands in both their *HpaII* and *MspI* profiles whereas the other accessions only presented these bands with *MspI*. CR0340 was

hypomethylated at locus *Eco-AG/Hpa-AAC/98bp* and CR0341 at locus *Eco-AC/Hpa-ATT/426bp*. Accessions CR0632 and CR0711 were hypermethylated, they presented some bands in their *MspI* profiles whereas the other accessions presented these bands in both their *HpaII* and *MspI* profiles. Accession CR0632 was hypermethylated at locus *Eco-AG/Hpa-ATG/205bp* and at locus *Eco-AG/Hpa-AAC/382bp*. Finally, accession CR0711 was hypermethylated at locus *Eco-AG/Hpa-AAG/393bp*.

These results showed that methylation is present in *V. planifolia* genome, with 7.45% of the fragments revealed being methylated. This value is in accordance with methylation rates reported in banana (7,5%, (Noyer et al., 2005)), but less than what is revealed in other conventionally propagated plant species such as rice (16.3%, (Xiong et al., 1999)), other bananas (18.4%, (Peraza-Echeverria et al., 2001)), apple (25%, (Xu et al., 2000)) and cotton (32%, (Keyte et al., 2006)).

A limited amount of methylation polymorphism (1%) was detected among 'Classique' and 'Mexique' accessions but the methylation patterns revealed were accession specific. Even for CR0340/0341/0342 which are three clones of the same accession, two different methylation polymorphisms were revealed in CR0340 and CR0341 and none in CR0342, showing that these methylation patterns are either not transmitted through asexual propagation, or have appeared after clonal propagation. In all cases, no methylation marker was identified which could allow to specifically distinguish the 'Classique' and 'Mexique' morphotypes. A similar conclusion was obtained in studies performed on vegetatively propagated plants such as banana (Baurens et al., 2003; Noyer et al., 2005). Methylation polymorphisms were revealed but could not be correlated to morphological variations.

We therefore conclude that the 'Mexique' morphotype showing no detectable AFLP or MSAP polymorphism is most probably the result of a limited genetic or epigenetic dominant mutation event with pleiotropic effects.

#### 4.2 *V. xtahitensis* in French Polynesia

The mysterious history of the origin of *V. xtahitensis*, the so called Tahitian vanilla, has partly been solved. As opposed to its allied species (*V. planifolia*) it cannot be found wild in tropical American forests (Moore, 1993; Portères, 1954; Soto Arenas & Cameron, 2003) but was described from cultivated material found in the Island of Raiatea (Lubinsky et al., 2008b), where it had been introduced via the botanical garden of Papeete from the Philippines in 1848 (Soto Arenas & Dressler, 2010). Molecular sequencing (ITS and cpDNA) have recently shown that *V. xtahitensis* would be a hybrid, intentional or not between *V. planifolia* and *V. odorata* dating from vanilla exploitation by Mayas in Mesoamerica between years 1359-1500 (Lubinsky et al., 2008b).

As much as 18 different morphotypes are described in *V. xtahitensis* in French Polynesia beside the most widely cultivated type 'Tahiti' (Lepers-Andrzejewski et al., 2011a). These include: 'Haapape' (the second most cultivated type because of its bigger fruits), 'Tahiti Court', 'Tiarei', 'Ofe Ofé', 'Oviri', 'Parahurahu' and 'Sterile'. A study of 16 different accessions using AFLP markers revealed a Dmax value of 0.150, a slightly higher value than what was revealed in *V. planifolia*. All accessions had patterns related to that of 'Tahiti' (either identical or showing missing bands) which led the authors to conclude of a single introduction event in French Polynesia of a 'Tahiti' vine, consistently with the fact that this accession is the oldest one recorded in Polynesia (Lepers-Andrzejewski et al., 2011a). Ten accessions showed more or less the AFLP profile of 'Tahiti'. These included 'Haapape' and 'Tiarei', which were shown to be autotetraploids based on flow cytometry and chromosome



counts (Lepers-Andrzejewski et al., 2011b). Similarly as in *V. planifolia* (Bory et al., 2008a), 'Sterile' morphotypes in *V. ×tahitensis* were also related to autotriploidy (Lepers-Andrzejewski et al., 2011b). It was hypothesized that they originated from a cross between the two most cultivated morphotypes 'Tahiti' (2x) and 'Haapape' (4x) (Lepers-Andrzejewski et al., 2011b). The remaining accessions showed a pattern related to 'Tahiti' but with 15 to 30 missing bands (Lepers-Andrzejewski et al., 2011a), a pattern consistent with segregation, as shown in *V. planifolia* for the 'Aiguille' morphotype or selfed progenies (Bory et al., 2010). For these accessions, graphical genotypes were constructed based an AFLP 'Tahiti' map and showed that morphotypes such as 'Parahurahu', 'Rearea', 'Oviri' and 'Tahiti court' displayed patterns consistent with an origin via self-pollination of 'Tahiti' (one single recombination event per bivalent) whereas others such as 'Popoti' and 'Parauti' most probably resulted from a second generation of self-pollination (two recombinations events in the same bivalent) (Lepers-Andrzejewski et al., 2011a).

### 4.3 Conclusions

These results therefore highlight two different domestication models. In both cases, the genetic base of the cultivated material is very narrow with obviously a single genotype introduced ('Classique' *V. planifolia* in Reunion Island and other cultivation areas; 'Tahiti' *V. ×tahitensis* in French Polynesia). Genetic variation revealed is however slightly higher in *V. ×tahitensis* than in *V. planifolia* because most of *V. ×tahitensis* morphotypes have resulted from selfing of the original 'Tahiti' (with sometimes more than one generation involved) (Lepers-Andrzejewski et al., 2011a). Only one rare case of self-pollination ('Aiguille') was detected in Reunion (Bory et al., 2010). This shows that deliberate or inadvertent seed germination has been strongly involved in the domestication of *V. ×tahitensis* in French Polynesia. In Reunion Island, the limited amount of variation revealed is more related to vegetative propagation and the consecutive accumulation of point mutations.

In both cases however, a noticeable diversification was achieved through polyploidy. Autotetraploidy generated varieties with bigger leaves and fruits, and autotriploidy generated self-sterile individuals. It is noteworthy that self-sterile *V. planifolia* varieties were also described in Mexico ('Oreja de Burro') (Castillo Martinez & Engleman, 1993; Soto Arenas & Dressler, 2010). It is most likely that these have resulted as well from autotriploidy. These results, as well as those that surveyed genome sizes in a wide range of *Vanilla* species (Bory et al., 2010) provide converging evidences for the importance of polyploidy and genome rearrangements during *Vanilla* evolution. Polyploidy can be of major importance in cultivation as well as in natural populations as triploidy and to a certain extent tetraploidy can be responsible for dramatic loss in fruit set. Further work is therefore needed to assess polyploidization consequences on *Vanilla* reproductive biology.

## 5. *Vanilla* genome dynamics

Concordant data obtained on *V. planifolia* as well as *V. ×tahitensis* demonstrated an abnormal mitotic behaviour in the *Vanilla* genus, with a combination of somatic aneuploidy and partial endoreplication (Bory et al., 2008a; Lepers-Andrzejewski et al., 2011b).

### 5.1 Somatic aneuploidy

Most data in the literature give a basic number  $n=16$  for *V. planifolia* with  $2n= 32$  (Chardard, 1963; Heim, 1954; Hoffmann, 1929, 1930; Martin, 1963). Hurel-Py (1938) was the first to show

the existence of a variable number of chromosomes in differentiated cells (13 to 32 chromosomes). Similarly, Nair & Ravindran (1994) described an important variation in chromosome numbers, from 20 to 32 with 28 being the most encountered. Recent analyses confirmed the existence of such somatic hypo-aneuploidy (i.e. chromosome number is always below an exact multiple of the usually haploid number) in root tip cells of *V. planifolia* (Bory et al., 2008a), *V. ×tahitensis* (Lepers-Andrzejewski et al., 2011b) as well as other *Vanilla* species (Bory, 2007). This aneuploidy could be explained by somatic associations of chromosomes (Nair & Ravindran, 1994) but as well by chromatin elimination (Lepers-Andrzejewski et al., 2011b). Interestingly, it was recently demonstrated that somatic aneuploidy is regulated between somatic and gametic cells in *V. ×tahitensis*, with the full genome complement present in germ cells (Lepers-Andrzejewski et al., 2011b). This suggests that a regulatory mechanism functions during meiosis to stabilize the genome and chromosome number

## 5.2 Progressively partial endoreplication

Flow cytometry genome size estimates and chromosome counts have been successfully used to demonstrate the occurrence of diploid, triploid and tetraploid accessions of *V. planifolia* in Reunion Island (Bory et al., 2008a) and *V. ×tahitensis* in French Polynesia (Lepers-Andrzejewski et al., 2011b). Genome size variations were also demonstrated in some other species of the *Vanilla* genus (Bory et al., 2010). Flow cytometry revealed endoreplication in somatic cells of *V. planifolia* and *V. ×tahitensis*. In *V. planifolia* the marginal replication ratio, which is the ratio between each peak position, was irregular with 1.43, 1.63, 1.76, 1.82 instead of 2.00 (Bory et al., 2008a). In *V. ×tahitensis* it was 1.38, 1.65, 1.77, 1.79 and 1.81 (Lepers-Andrzejewski et al., 2011b). The almost perfect linearity found between DNA content and the number of endoreplication cycles suggested that the same genome part (or chromosome batch) (P, figure 3) is amplified at each cycle. A matrix of only 43.73% and 38% of the holoploid nucleus is replicated at each cycle in *V. planifolia* and *V. ×tahitensis*, respectively (Bory et al., 2008a; Lepers-Andrzejewski et al., 2011b).

More importantly, this phenomenon is apparently present in all the *Vanilla* species surveyed so far. Flow cytometry genome size estimates for 38 accessions representing 17 different *Vanilla* species and 3 artificial inter-specific hybrids revealed, for each accession, fluorescence histograms with five endoreplicated peaks and the marginal replication ratio was still irregular (from 1.5 to 1.8 instead of 2) (Bory et al., 2010). Nothing is known concerning the mechanisms in play, whether it results from partial replication of the DNA or excision of DNA (possibly chromatin elimination) following whole genome replication, but they occur in many orchids (Bory et al., 2008a). It will be important in the near future to gain knowledge on this developmentally regulated “progressively partial endoreplication” phenomenon unique to orchids. Available data already show that it is vegetatively, as well as sexually transmitted as demonstrated by surveying interspecific hybrids, such as the natural hybrid *V. ×tahitensis* (Lepers-Andrzejewski et al., 2011b) and artificial hybrids (*V. planifolia* × *V. planifolia*, *V. planifolia* × *V. ×tahitensis*, *V. planifolia* × *V. phaeantha*) (Bory et al., 2010). This phenomenon is technically important as the first peak (2C) is often very small, and this was shown to be responsible for considerable errors in the genome size estimates that have been published in the literature for *Vanilla* species (Bory et al., 2008a; Lepers-Andrzejewski et al., 2011b). This phenomenon is also evolutionary important as it was shown to be a source of polyploidization in many plant species. However it cannot itself explain the origin of autotetraploid types in *V. planifolia* and *V. ×tahitensis* as these have

exactly double the amount of DNA than their diploids counterparts, unless endoreplication in meristematic cells is regulated (Lepers-Andrzejewski et al., 2011b).

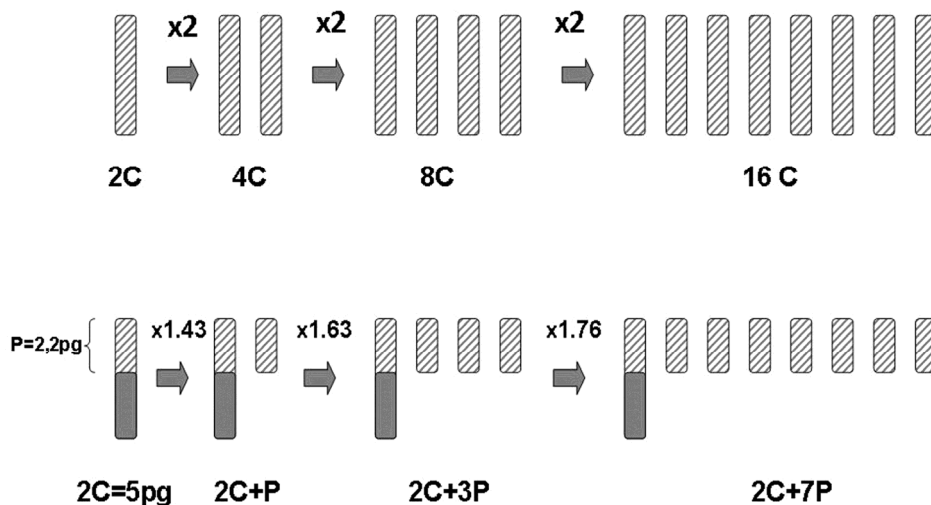


Fig. 3. Partial progressive endoreplication in *V. planifolia* (below) as compared to normal endoreplication (above). The replicated part (P) of the *V. planifolia* genome is indicated (hatched).

## 6. Conclusion

Although considerable progress has been made in recent years in the precision of the taxonomy and the discovery of evolutionary processes in the *Vanilla* genus (reproduction, genetic diversity, polyploidy, hybridization), many questions remain unanswered. These include elucidating the complex processes involved in genome dynamics and its possible implications on the genus diversification. Evolutionary pathways of important traits in the genus such as self-pollination ability and aromatic compounds accumulation in fruits, which are major targets for vanilla breeding, will need to be surveyed. Self-pollination appears as an ancestral character in the genus, shared by species from group  $\alpha$  and early diverging species from group  $\beta$ . Furthermore, although allied genera possess aromatic fruit, this character is found in *Vanilla* within American group  $\beta$ , but not in ancestral American nor in more recent species from Africa and Asia. The aromatic character of both flowers and fruit in *Vanilla* has evolved in a specialized relationship with euglossine bees involved in both flower pollination and fruit dispersion. This represents an exciting further area of investigation. Molecular and cytogenetic studies will have to be combined with morphological, history traits and ecological assessments to provide a thorough revision of the genus taxonomy. In particular, more data is needed to fully characterize the reproductive biology of *Vanilla* species and its implication on the levels of genetic diversity in natural populations. This will be essential to provide conservation guidelines for the many endangered species of the genus.

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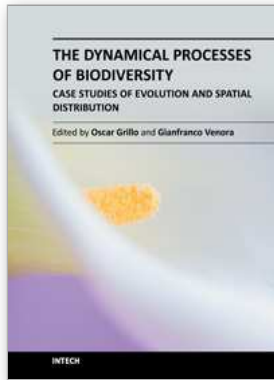
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## **The Dynamical Processes of Biodiversity - Case Studies of Evolution and Spatial Distribution**

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