Combining pollination ecology and fine-scale spatial genetic structure analysis to unravel the reproductive strategy of an insular threatened orchid

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A B S T R A C T

In *Vanilla* species (Orchidaceae), the influence of sexual and asexual mating systems on the spatial structuring of population genetic diversity is understudied. These elements are crucial in restoration program to limit inbreed-ing depression and to maintain the genetic diversity of natural populations. In the remnant fragments of tropical dry forest in Mayotte (Comoros Archipelago, Indian Ocean), the clarification of the reproductive strategies of the orphan leafless *Vanilla humblotii* Rchb. f. will provide a better understanding of its fine-scale spatial genetic struc-ture. Approaches combining reproductive biology and fine-scale spatial genetic structure analyses using ten mi-crosatellite markers in 49 individuals sampled in the only remaining large population of *V. humblotii* were employed to unravel the reproductive strategies of this species. The results showed that *V. humblotii* displays un-scented flowers and is allogamous and pollinator-dependent although also self-compatible. A total absence of pollen movements and a low level of natural fruit set (~1%) are reported, although a wild bee (*Allodape obscuripennis* Strand, Xylocopinae) and a bird (*Nectarinia coquerelli*, Nectarinidae) visited the flowers. A high ge-notypic diversity (G/N = 0.88) and a phalanx clonal growth are detected, and seed dispersal is higher than pollen dispersal. The phalanx distribution of the repeated genotypes (ramets arisen from the same genet) is responsible for significant autocorrelations detected at small distances. Limited inbreeding was detected although geitonogamy could have been enhanced by vegetative reproduction. This study highlights the need to perform interdisciplinary studies to unravel the reproductive strategy of clonal plant species with a deceptive pollination system.

1. Introduction

Vanilla Plum. ex Miller genus is a monophyletic primitive lineage of the Orchidaceae family (Cameron, 2004, 2005) that contains over 100 species widely distributed throughout the tropics in America, Asia and Africa (Portères, 1954). Most of *Vanilla* species are hemi-epiphytic vines with a mixed type of reproduction predominated by vegetative propagation, due to natural stem cuttings, in addition to sexual reproduction (Bory et al., 2010; Gigant et al., 2011a). Clonal patches of *Vanilla planifolia* G. Jackson covering up to 0.2 ha have been described (Gigant et al., 2011a; Soto Arenas, 1999a) therefore vegetative propagation plays a major role in the installation and survival of *Vanilla* individuals in the wild. The flowers of most species exhibit an efficient rostellum resulting in a pollinator-dependent system for sexual reproduction (Ackerman, 1983; Bory et al., 2008c; Bourriquet, 1954; Dobat and Peikert-Holle, 1985; Gigant et al., 2011a; Soto Arenas, 1999b; Soto Arenas and Cameron, 2003; Soto Arenas and Dressler, 2010; Stéhlé, 1954). Only few species are spontaneous self-fertilisers due to a reduced rostellum or stigmatic leak and display high (up to 78%) natural fruit set (Gigant et al., 2011a).

In other species, sexual reproduction involves an insect-dependent system for pollination, although other pollinators such as hummingbirds are also suspected (Gigant et al., 2011a). So far, pollinators identified for *Vanilla* species concern some American species and involve mainly euglossine bees (Bory et al., 2008b; Gigant et al., 2011a; Householder et al., 2010; Lubinsky et al., 2006; Soto Arenas, 1999b; Soto Arenas and Cameron, 2003; Soto Arenas and Dressler, 2010). In

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such nectarless American *Vanilla* species, those bees would collect the flower fragrance to make pheromons. This 'male euglossine syndrome' (Bembé, 2004; Williams and Whitten, 1983) was hypothesized for species with fragrant flowers (Dodson et al., 1969), such as *Vanilla trigonogarpa* Hoehne (Soto Arenas and Dressler, 2010) and *Vanilla pompona* Schiede subsp. *grandiflora* (Lubinsky et al., 2006). However, as a third of known orchids species (Jersáková et al., 2006; Schiestl, 2005; Singer, 2003; Tremblay et al., 2005), most *Vanilla* species are more likely pollinated by a deceptive system (Gigant et al., 2011a; Soto Arenas, 1999b; Soto Arenas and Cameron, 2003). For Asian *Vanilla* species, the only reported pollinator is a large Aegilopa bee for the species *V. cf. kaniensis* Schltr. in Papua New Guinea (Cameron and Soto Arenas, 2003). In Africa, until recently, nothing was known about the reproductive system of any *Vanilla* species (Van Der Cingel, 2001). Our recent study conducted on *Vanilla roscheri* in South Africa (Gigant et al., 2014) suggested two pollination mechanisms with (1) a generalized food deceptive system attracting a large pollinator spectrum, of which the anthophorine bees are the main pollinators and (2) a rewarding system, where the pollen is the reward for pollen-collecting bees, such as female allodapine bees (*Allodapula variegata* and *Allodape rufogastra*, Xylocopinae).

Vanilla humblotii Rchb. f. is an African leafless *Vanilla* species closely related to *V. roscheri* (Bouetard et al., 2010), found in the South West Indian Ocean (SWIO) area in the Comoros Archipelago (Mayotte, Moheli, Anjouan, Grande Comore) and also possibly in Madagascar (Cribb et al., 2009; Lecoufle and Bosser, 2011; Portères, 1954). A recent survey of the microsatellite genetic diversity of *V. humblotii* populations in the four islands of the archipelago reveals the small size of wild populations, with 18 populations with less than 20 individuals on the 21 remnant populations known (unpubl. res.). In Mayotte, the species is scarce and protected since 2006 (Arrêté Préfectoral APn° 42/DAF/2006 3rd may 2006) and the application of the IUCN Red List criteria at regional level classifies this species as endangered (B1ab (i,ii,iii,iv,v) + 2ab (i,ii,iii,iv,v), unpubl. res.). However, the finding of a relatively large *V. humblotii* population growing in the lowland mesophyllous forest of Sohoa in Mayotte provides an opportunity of choice to study the reproductive biology and the fine-scale genetic structure of the species. On the other islands of the archipelago, *V. humblotii* conservation status is uncertain but the species might be on the edge of extinction (L Gigord, CBNM, Réunion, France, unpubl. res.).

Defining a conservation plan for this species is therefore considered as a priority, and requires prior knowledge on its genetic diversity and reproductive biology. What is the extent of clonality of this species in the wild? Does it perform sexual reproduction and does it require a pollinator to do so? Is inbreeding occurring in such an isolated population within an island where forests are highly fragmented? Is geitonogamy happening (which can be enhanced depending on the flowering characteristics of the species *e.g.* simultaneously opened flowers from the same genotype and the extent of clonality *e.g.* size of clonal patches)? To address all these questions, we therefore conducted reproductive biology field experiments combined with a fine-scale microsatellite genetic analysis. Because at a fine scale, the spatial structure of genotypes allows learning more about both the nature and the relative importance of different reproductive strategies, *i.e.* the influence of the mating system, the gene dispersal (by pollen or seed), as well as the structure caused by spread of vegetative clones (Alberto et al., 2005; Debout et al., 2011; Epperson and Allard, 1989; Hamrick and Trapnell, 2011; Vallejo-Marín et al., 2010; Vekemans and Hardy, 2004).

2. Methods

2.1. V. humblotii in Mayotte Island

2.1.1. Study site

The Comoros Archipelago is composed of four main islands (Mayotte, Moheli, Anjouan, Grande Comore) emerged from the drift of the Somali plate on top of a hot spot plume (Emerick and Duncan, 1982; Späth et al., 1996) and located in the northern part of the Mozambique Channel between Madagascar and Mozambique. The French Overseas Territory Mayotte (379 km^2) is the southernmost (15° 33′ S, 54° 31′ E) and oldest island of the archipelago (around −10 Myrs) culminating at 660 m at the Benara Peak. The strong volcanic geomorphology is still visible by old craters and an undulating topography of elongated hills formed by lava flows (Audru et al., 2010). Primary natural forests have become rare in Mayotte and persist mainly on hilltops. The conservation of these forest patches is a priority given that the majority of the floral richness of the archipelago, in native and endemic plants, is concentrated in these remnant forests and distributed in only 15 km^2 of the whole land area of Mayotte (Vos, 2004).

The Sohoa forest reserve covers 208 ha with an average elevation of 190 m (Fig. 1). Sohoa is mainly composed uphill by a rainforest and by a fragment of mesophilous forest extending down to the sea, in which we found a large population of *V. humblotii*. The mesophilous forest is the least preserved of all natural formations in Mayotte, covering nowadays only 85 ha in Mayotte and reduced to two relict fragments in Sohoa and Dapani (Laybourne, 2010; Pascal et al., 2001). This plant formation used to be much more extended before the intense lowland clearings in the nineteenth century for agriculture (Guéneau, 2006; Pascal et al., 2001). In addition to the study of the Sohoa population of *V. humblotii*, the small remnant populations (Boungoudranavi, M'Bouzi, Chiconi, Choungui, Moya, M'Tsamoudou and Saziley) and the accession CR0108 (collected in Grande Comore, Union des Comores) from the Biological Resource Centre Vatel of Reunion Island (Roux-Cuvelier and Grisoni, 2010) were also included to perform the reproductive biology experiments (Fig. 1 and Table 1). The low number of individuals of those seven supplemental populations did not permit to include them into the following autocorrelation analyses.

2.1.2. Study species

Belonging to the genus *Vanilla* (Vanilloideae sub-family, Vanilleae tribe and Vanillinae sub-tribe), *V. humblotii* is a leafless hemi-epiphytic vine found from 0 to 600 m altitude both in extremely dry conditions

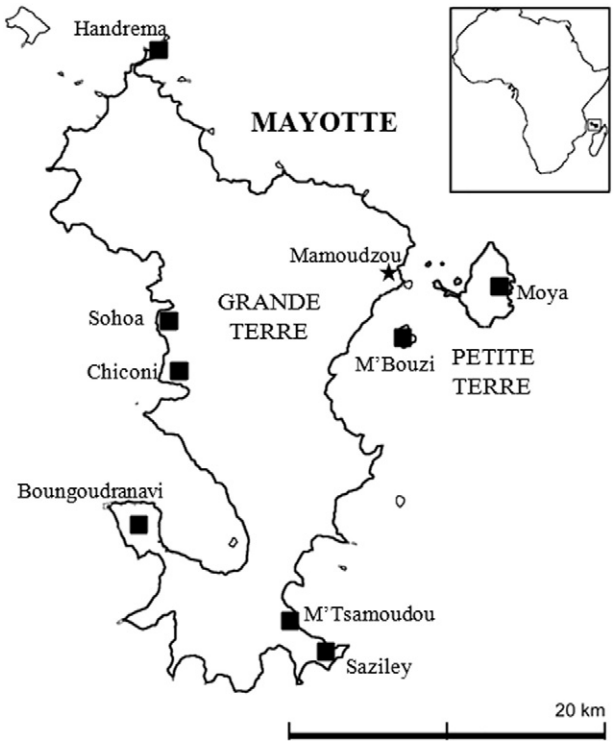

Fig. 1. Map of Mayotte showing the study sites of *V. humblotii*.

Table 1
Summary of the experiments conducted to define the reproductive biology of *V. humblotii*. The values represent the number of individuals (1), the number of populations (2) or the number of flowers employed for these experiments, which are spread over 2010 and 2012 for fruit set evaluation and pollinator observations.

	Floral measures[1]	Inflorescences measures[1]	SPME[1]	Pollinator observations[2]		Natural fruit set[1]		Breeding experiments[3]
				2010	2012	2010	2012	
Boungoudranavi	5	5	1	x		6	4	14
Chiconi							1	
Choungui							1	
CR0108			2					
M'Bouzi	1	1				1		
Moya	1	3		x		3	1	5
M'Tsamoudou	9	3	2	x	x	9	12	43
Saziley						1		
Sohoa	2	7		x	x	2	11	12

CR0108 is a BRC Vatel accession of *V. humblotii* from Grande Comore (Union des Comores).

on rocky environments (Portères, 1954) and in mesophilous forest. The stem is large and glaucescent (Portères, 1954) with occasionally wart-like spots. Flowering occurs preferentially on canopy or opened area, beginning with the onset of the rains in austral summer (November) and can last up to April (pers. observ). The inflorescences carry 40–50 flowers and the large canary-yellow flowers are characterized by a red-velvet lip (Portères, 1954). The anther is composed by four yellow and limp pollinia separated by a rostellum from the stigma which is situated below and ventrally. The cylindrical fruits are straight or a little arched and measure in mean 18–20 cm but up to 25 cm (Portères, 1954).

2.2. Reproductive biology

2.2.1. Floral measurements

In November–December 2010, 15 flowers were randomly collected from 13 inflorescences and stored in 70% ethanol prior to measurements. Floral characters were measured to the nearest 0.01 mm using a digital caliper (Fig. 2). A total of 41 inflorescences from 18 individuals were used to measure the mean length of an inflorescence using a tape measure (in centimetre) and the mean number of flowers per inflorescence (Table 1). The number of inflorescences per individual and the number of simultaneous opened flowers per individual were assessed on 19 individuals (Table 1). Floral volatiles were analysed using the solid phase microextraction (SPME) technique (Zhang and Pawliszyn, 1993) with 3 exposures realised on *in situ* individuals and 2 realised on flowers of the *ex situ* accession CR0108 (Table 1). Fibres were exposed to the flower headspace in a glass bell-jar for 3 to 8 h30 between 10 am and 7 pm.

2.2.2. Pollinator observations

Pollinator observations were realized using two hard-disk camcorders (Sony DCR-SR90E and Sony DCR-SR72E) mounted on tripods with long-lasting batteries (NP-FP90, NP-NH100 InfoLithium® P and

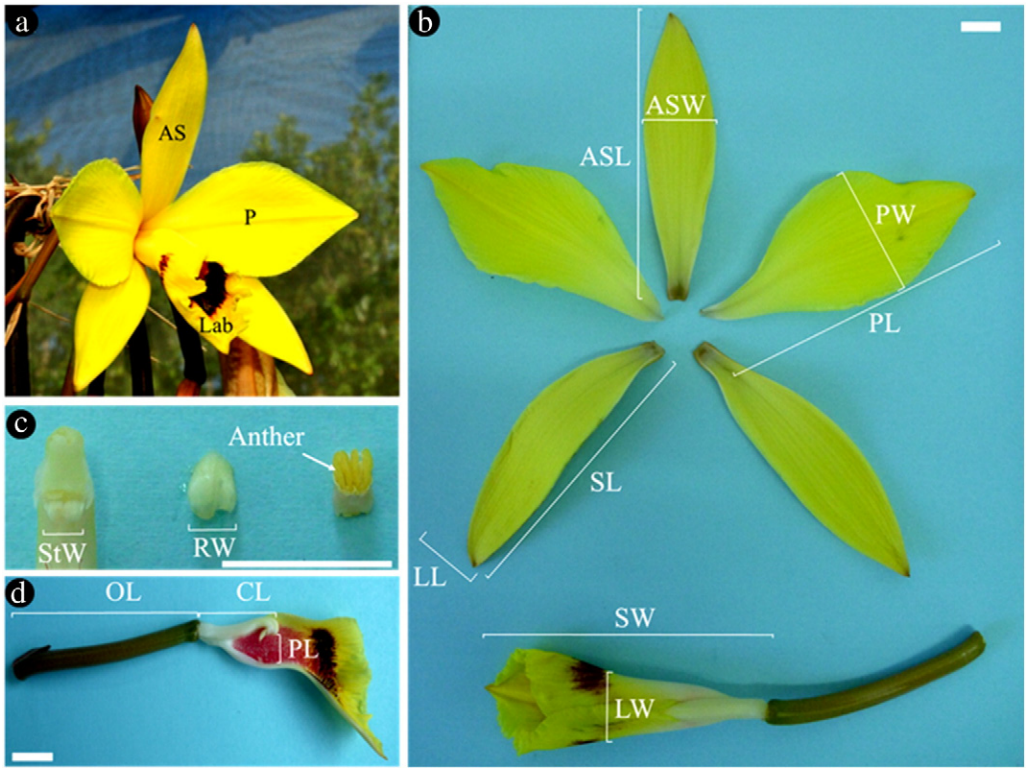

Fig. 2. Floral characters measured on *V. humblotii* (A); (B) adaxial sepal length (ASL) and width (ASW); petal length (PL) and width (PW); sepal length (SL) and width (SW); Labellum length (LL) and width (LW); (C) column width (CW) and ovary width (OW); (D) stigma width (StW) and rostellum width (RW); (E) ovary length (OL), column length (CL) and pollinator linked trait (PL), bars = 1 cm. Photos R. Gigant.

H series rechargeable battery), and protected with a waterproof casing (Sony SPK-HCB marine sport pack). Videotape sessions were conducted in November–December 2010 on three study sites in Grande Terre (Boungoudranavi, M'Tsamoudou and Sohoa) and one in Petite Terre (Moya) and in December 2012–January 2013 on two study sites in Grande Terre (M'Tsamoudou and Sohoa). The target flower was examined for pollen removal and/or deposition before and after each videotape session.

2.2.3. Natural fruit set

The number of fruits produced per flower per inflorescence (evaluated by the floral scars) was measured to assess the mean natural fruit set. A total of 72 inflorescences from 22 individuals in 2010 and 111 inflorescences from 30 individuals in 2012 were assessed (Table 1). The mean natural fruit sets of the two years were compared using the Fisher exact test of the software R 2.12.2 to see any statistical differences of proportions (R Development Core Team, 2010).

2.2.4. Breeding systems and compatibility of V. humblotii

The breeding system of *V. humblotii* was assessed by *in situ* and *ex situ* experiments (Table 1). Young opened-flowers were manually pollinated (or not) and the inflorescences were isolated from insect visits using insect-proof bags. Spontaneous self-fertility was tested on 30 flowers from eight inflorescences (one inflorescence per individual) by the exclusion of insect visits using the insect-proof bags. Self-compatibility was tested by hand self-pollinations using 22 virgin flowers from seven inflorescences (1–2 flowers per inflorescence with one inflorescence per individual). Eighteen cross-pollination treatments were performed as described in Nielsen (2000). Both male and female parents were carefully recorded and further microsatellite analyses (see below) permitted to differentiate 15 true cross-pollinations from 3 geitonogamous pollinations (involving different 'ramets' of the same 'genet' *i.e.* clones), considered *a posteriori* as 3 tests of self-compatibility. To avoid limitation of fructification due to resource allocation, only inflorescences without fruit were used and no more than three flowers per inflorescence were employed to receive pollinia. Inflorescences were protected by an insect-proof bag. Early abortions were recorded after 12–18 days after pollination experiments. Unsuccessful pollinations can be identified a few days after pollinations by the fall of the faded flower and the absence of swelling of the ovary (Lecomte and Chalot, 1901; Shadakshari et al., 2003). The breeding experiments revealed no significant different results between the two years (data not shown), which allowed us to concatenate the observations of the two years and perform the comparisons between treatments on a total of 70 flowers (Table 1). Then, the comparisons between self- and cross-pollinations were performed using Fisher exact tests using the software R 2.12.2 (R Development Core Team, 2010).

2.3. Genetic analyses

2.3.1. Sampling of V. humblotii in Sohoa population

The patch-like distribution of the individuals is probably related to the vegetative propagation as described in many *Vanilla* species (Bory et al., 2010; Gigant et al., 2011a). Without *a priori* knowledge on the extent of clonality in *V. humblotii*, our sampling was based on a transect through the forest from end to end in order to reveal potentially the maximum genetic diversity of this population. From this transect, multiple orthogonal transects were done where all separated (physically) and accessible ramets were sampled. This irregular sampling schema permitted to include a maximum of neighbour distances, which is considered as more efficient for pattern detection (Fortin et al., 1989). Given the difficulty to distinguish between vines which have arisen vegetatively from those arisen from seedlings, an inter-sampling of 5 m was defined for individuals in continuity, and 1 m when they were on different supports. This sampling permitted to avoid oversampling clones by considering only the individuals isolated (from their mother plant) and circumscribe physically in a given space. Following this compromise to detect both genetic diversity and clonality, the genetic homogeneity of large patchy individual (>5 m) was verified, and the true clones (arisen from vegetative reproduction and physically separated), were also detected. In total, 49 ramets were sampled in the population of Sohoa and their spatial coordinates were carefully recorded. Stem fragments were collected and stored in silica gel for further DNA analyses.

2.3.2. DNA isolation and genotyping

DNA extractions were made from the CTAB protocol (Risterucci et al., 2000) adapted for 1 g of lyophilized stem for the leafless *Vanilla* species. A total of 10 microsatellite markers from *V. humblotii* and *V. roscheri* Rchb. f. (Gigant et al., 2011b) and mVplCIR031 from *V. planifolia* G. Jackson (Bory et al., 2008a) were used. The recommendations of Gigant et al. (2011b) and Bory et al. (2008a) concerning the end-labelling of those markers with a fluorescent dye and the PCR conditions were followed for multiplexing these loci in a minimum number of assay. However, simplex amplifications were needed to confirm all the different alleles for each locus.

2.3.3. Genetic diversity

To measure the extent of clonality in the population of Sohoa and its influence on the genetic structure, two datasets were created: a dataset 'ramets' composed by the entire set of sampled individuals and a dataset 'genets' composed by the different genotypes after clone exclusion (one individual was randomly selected for each clone). The clones were identified using the multilocus analysis of clonality available in GenAlex 6.41 (Peakall and Smouse, 2006). Individuals with genotypes lacking at least one marker were excluded from this analysis. Using GenAlex, the probability (*Pse*) was calculated for each repeated multilocus to exclude the possibility that a specific repeated multilocus could be generated by sexual reproduction under random mating. The genotypic diversity, as assessed for clonal plants by G/N (Ellstrand and Roose, 1987), was calculated as the number of 'genets' (G) divided by the number of 'ramets' (N). Using GenClone 2.0 (Arnaud-Haond and Belkhir, 2007), the genotypic richness R and the adapted Simpson index for genotypic diversity D^* were also calculated. The average maximal clonal patch size was estimated from the pairwise geographical distance option from GenAlex, between each ramet with the same genotype. Genetic variability was estimated for each microsatellite locus using GenAlex on the dataset 'genets' by the number of different alleles (N_a) and the number of effective alleles (N_e). Heterozygosity observed (H_O), heterozygosity expected under random mating (H_E) and fixation indices (F_{IS}) were estimated using the software Genepop 4.1 (Rousset, 2008). Deviations from Hardy–Weinberg equilibrium (HWE) were tested at marker and population levels by the exact P-values estimations of the Markov chain method proposed by Guo and Thompson (1992) implemented in Genepop (1000 dememorizations, 100 batches and 1000 iterations per batch). Evidence of null allele was estimated with Microchecker 2.2.3 (Van Oosterhout et al., 2004) and linkage disequilibrium over loci was tested using Fisher's exact tests of Genepop 4.1 and Bonferroni correction for multiple comparisons.

2.3.4. Fine-scale spatial genetic structure

The criteria for the application of the method satisfied the recommendation of autocorrelation analyses at individual level by Spagedi 1.3 (Hardy and Vekemans, 2002), with a slightly lower number of pairwise comparisons nonetheless in accordance with the threshold of 30 pairwise comparisons in each distance class (Wartenberg, cited in Waser and Mitchell (1990)), commonly accepted for numerous analyses of genetic and spatial autocorrelations (Brennan et al., 2003; Fortin et al., 1989; Gonzales et al., 2010; Haas et al., 2010; Torres et al., 2003). Therefore, fine-scale spatial genetic structure (FSGS) analysis was performed for the Sohoa population. A single marker (mVroCIR05) presented a significant deviation from HWE. Removing it from the global analysis, the overall significant departure from HWE detected at the

population level became not significant (data not shown), so this marker was excluded from the analysis. Autocorrelation analyses based on the multilocus pairwise kinship coefficients of Loiselle (Loiselle et al., 1995) were calculated using Spagedi. The coefficient values were regressed on the linear and natural logarithm of the geographical distances between the individuals. The respective slopes of those two regressions were b_d and b_{Ld} and their corresponding coefficients of determination R^2_d and R^2_{Ld}. Five individuals at the limit of the area of the distribution of the population were excluded from the analysis (>500 m from most individuals) to avoid stretching the distance classes. Eight distance classes were defined automatically by the software Spagedi, to equilibrate the number of pairwise comparisons between the distance classes. The average multilocus kinship coefficients were calculated for each distance class defined separately in each dataset. The distance classes calculated for the two datasets 'ramets' and 'genets' were similar with 0–10 m, 10–22 m, 22–32 m, 32–42 m, 42–53 m, 53–63 m, 63–75 m and 75–105 m for the dataset 'ramets' and 0–10 m, 10–23 m, 23–33 m, 33–43 m, 43–54 m, 54–63 m, 63–75 m, 75–105 m for the 'genets'. Standard errors for the multilocus estimates of the kinship coefficients per distance class were assessed by jackknifing data over the loci. The significance of the kinship coefficients and slope estimates (b) were tested by comparing the observed values with those obtained after 10,000 random permutations of the individuals among positions.

The Sp statistic (Vekemans and Hardy, 2004) was calculated to quantify the strength of the spatial genetic structure of the two datasets. It is defined by $Sp = b_{Ld} / (F_1 - 1)$, where F_1 is the mean kinship coefficient between pairs of neighbours in the first distance class (<10 m).

2.3.5. Two-dimensional local spatial autocorrelation analyses

Given the patchy distribution of the individuals, we further examined the spatial genetic structure of the individuals used in the FSGS analysis, by the heuristic two-dimensional local spatial autocorrelation analyses (2D LSA) (Double et al., 2005) as implemented in GenAlex. This method based on pairwise comparisons estimated the local autocorrelation between a pivotal point and its nearest neighbours. Permutation testing and a 1-tailed test at $P = 0.05$ was used. The 2D LSA was computed for a range of nearest neighbours from 1 to 25, but only the result with the maximum number of significant autocorrelations is shown.

2.3.6. Estimation of gene dispersal

An iterative approach implemented in Spagedi was used to estimate jointly the neighbourhood size (Nb) and the gene dispersal (σ_g) in the restricted distance range $\sigma_g > d_{ij} > 20\ \sigma_g$, knowing the density of individuals (D) in the population of Sohoa and with d_{ij} the geographical distance for i–j pairs of individuals. D was estimated for *V. humblotii* in Sohoa as 65.5 ind. ha^{-1} as given by the number of genets sampled per hectare. Obviously, this value does not represent the density of *V. humblotii* in Mayotte (due to large agricultural landscapes) but it reflects the density of genets in the same fragment of primary forest. Neighbourhood size (Nb) was approximated from the global natural regression slope of kinship coefficients by $(F_1 - 1) / b_{Ld}$. As an approximation of D_e, the effective density determined from D (N_e/N), where N_e/N is the effective population size per the population size, $D/2$, $D/4$ and $D/10$ were used because demographic studies have shown previously that N_e/N varied from 0.1 to 0.5 in adult plant populations. Gene dispersal (σ_g) was estimated from Nb and D_e using $\sigma_g = (Nb / 4\pi D_e)^{1/2}$. The procedure of estimation of the parameters by iterations failed to converge when the regression of autocorrelations became null at one step, or when σ_g became larger than the distance between i–j pairs of individuals in the range $\sigma_g > d_{ij} > 20\ \sigma_g$.

The relative contributions of pollen (σ_p) and seed (σ_s) dispersals to the total gene flow were estimated on the dataset 'genets' following the method of Heuertz (2003). A cubic regression was fitted on the average kinship coefficients per distance class and the logarithm of the distance. The shape of the regression can be described by the k-value, the second derivative of the term of the second and the third power of the cubic regression (Jacquemyn et al., 2006; Vekemans and Hardy, 2004). A concave shape (k-value > 0) signifies a leptokurtic gene flow implying a restricted seed dispersal compared to pollen dispersal ($\sigma_s \ll \sigma_p$) in the case of a convex shape (k-value < 0) $\sigma_s \gg \sigma_p$.

3. Results

3.1. Reproductive biology

3.1.1. Floral characteristics of V. humblotii

The mean length of an inflorescence was estimated at 37.5 (±10.4) cm with 33.9 (±12.4) flowers per inflorescence. An individual carried 5.3 (±3.7) inflorescences on average. A mean number of 1.84 (±2.1) simultaneously opened flowers on the same individual was estimated. Floral features of *V. humblotii* are reported in Table 2. Observations and measurements of the flowers fitted with the description of *V. humblotii* by Portères (1954). The rostellum (2.7 ± 0.4 mm) is much larger than the stigma (1.8 ± 0.4 mm) and covers its entire surface. The height between the lip and the pollinarium was measured at 5.5 (±0.5) mm and the inside width at 7.1 (±01.5) mm. No volatile components were detected after SPME analysis in any of the flowers tested.

3.1.2. Breeding systems and natural fruit set

Pollinator exclusion tests indicated the need for pollinators for fruit production (Table 3). Self-compatibility of the species was verified and no significant differences were detected with the cross-pollination treatments ($P = 1$). In 2010 and 2012, the natural fruit sets were estimated at 0.62% and 1.2% on 2179 and 2991 flowers respectively, with no significant differences between the two years.

3.1.3. Pollination ecology

The lifespan of unpollinated flowers of *V. humblotii* was estimated to be 1 day. These observations supported our choice to realize the floral scent captures and video recordings during the day. Recordings (approximately 60 h) spanned over 16 days of observation. Two visitors were identified: a small female allodapine bee identified as *Allodape obscuripennis* Strand (Apidae, Xylocopinae, Allodapini; Fig. 3 a and b) and a female sunbird (*Nectarinia coquerelli*, Passeriformes, Nectariniidae; Fig. 3 c). No pollen movement was observed neither in the recorded flowers nor in any flower examined during field observations. A total of 23 visits of *A. obscuripennis* were recorded in two sites (Boungoudranavi and Sohoa) with a mean visit duration of 29 s. Although no pollen movement was observed, one recording permitted to film the arrival of an *A. obscuripennis* on a *V. humblotii* flower with pollinia on the mesonotum (upper surface of the mesothorax) (Fig. 3 b and see [SUPPORTING INFORMATION — Movie 1]). The single visit by the sunbird was in Moya study site and lasted for 38 s. The recording showed that perched on an inflorescence of *V. humblotii*, it was looking for food all around the flowers, in the insertion of the petals where sometimes small insects can be found [see SUPPORTING INFORMATION — Movie 2]. Otherwise, flowers of *V. humblotii* were commonly visited

Table 2
Floral morphology of *V. humblotii*. The values are the means (±SE) of floral measurements in millimetres (N = 15) in two dimensions of length and width for each floral organ.

Floral segments	Length	Width
Adaxial sepal	66.0 (±1.2)	15.5 (±4.7)
Lateral sepal	65.2 (±2.1)	17.6 (±4.5)
Petal	69.3 (±3.7)	29.0 (±1.5)
Lip	60.1 (±0.8)	9.8 (±1.5)
Column	24.8 (±0.2)	3.5 (±0.5)
Ovary	56.7 (±6.5)	4.7 (±0.4)
Rostellum	–	2.7 (±0.4)
Stigmata	–	1.8 (±0.4)

Table 3
Summary of the breeding experiments. The success of fruit production is expressed by the proportion of fruits produced after pollination relative to the total number of flowers used for the given experiment.

	No of flowers	Fruit set (%)
Pollinator excluded	30	6.7
Self-pollination (combined)	25	88.0
True self-pollination	22	90.9
Geitonogamy	3	66.7
Cross-pollination	15	86.7

by small arthropods, ants, midges and predatory spiders, but there were no interaction with the reproductive structures despite the high frequency and duration of their visits.

3.2. Genetic analyses of the Sohoa population

3.2.1. Genetic diversity

Among the 49 *V. humblotii* individuals collected in Sohoa population, one was excluded because genotypes lacked for at least one marker to identify the clones. A total of 42 genets were revealed, with a genotypic diversity (G/N) of 0.88, a genotypic richness R of 0.87 and a Simpson index D* of 0.99, representing 12.5% of clonality ($Pse < 0.001$). Therefore all the repeated genotypes found in Sohoa were considered as derived from vegetative reproduction. Clones were represented by four repeated genotypes (Fig. 4) and presented a phalanx distribution where clonal ramets are spatially aggregated (Lovett-Doust, 1981). The average maximal clonal patch size was measured at 4.6 ($\pm$2.7) m. On the other hand, spatial distribution showed that neighbour individuals were not necessarily clones, which *a posteriori* justified our sampling strategy.

Genetic variability estimates for the 'genets' of the Sohoa population (42 individuals) are reported in Table 4. Neither significant genotypic linkage disequilibrium between markers after Bonferroni correction nor significant levels of null alleles were detected in the study.

3.2.2. Fine-scale spatial genetic structure of Sohoa population

The spatial structure in the population of Sohoa showed a significant linear relationship for the two datasets (ramets and genets) whatever the linear regression or the natural logarithm regression, with a decrease of kinship coefficients correlated with an increase of geographical distance (Fig. 5 and Table 5). Furthermore, where the correlogram intercepts the *X*-axis or switches sign permitted to define the average patch size of autocorrelation which dimensions are determined by mating systems and dispersal (Epperson and Clegg, 1986), and influenced by density (Antonovics and Levin, 1980). The average autocorrelation patch size was estimated graphically at around 50 m for the two datasets (Fig. 5).

The autocorrelation between individuals separated by less than 10 m (distance class one, F_1) for the 'ramets' is significant and more than 4-fold greater than for the 'genets' subset (Table 5; Fig. 5). Consistently, the *Sp* statistic calculated for the 'ramets' was 2-fold higher than for the 'genets', which indicated a stronger FSGS for the 'ramets' than for the 'genets' (Table 5). This suggests a strong influence of the repeated multilocus genotypes (clones) in defining a strong spatial genetic structuring in the first distance classes.

3.2.3. Two-dimensional local spatial autocorrelation analyses

The results of 2D LSA showed local positive genetic autocorrelations for 19 nearest neighbours between 'genets' (genetically more similar than average) in the population of Sohoa, therefore exclusively due to sexual reproduction. The graphical representation highlighted three patches of autocorrelations composed not only by nearest neighbours but also by more distant individuals (Fig. 6).

3.2.4. Gene dispersal

Dispersal parameters converged for the two ratios *D*/2 and *D*/4 (as approximations of D_e) but the procedure failed to estimate the parameters based on *D*/10 (Table 5). Neighbourhood size estimates were higher for the 'ramets' than for the 'genets' (Table 5). Gene dispersal estimates were in the same range of variation regardless of the datasets but slightly greater at small distance for the 'ramets' than for the 'genets'

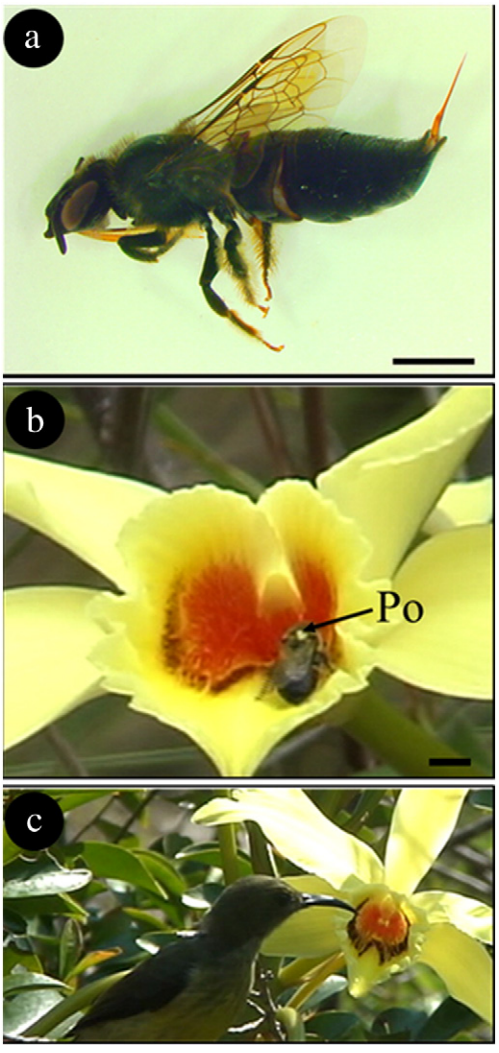

Fig. 3. Visitors of *V. humblotii* flowers: (A) *Allodape obscuripennis* sampled visiting a flower of *V. humblotii*, bar = 2 mm; (B) *A. obscuripennis* landing on *V. humblotii* flowers with pollinia (Po) on the mesonotum, bar = 3 mm; (C) female *Nectarinia coquerelli* probably foraging insects on *V. humblotii* flowers. Photos (A) A. Franck; (B) and (C) R. Gigant.

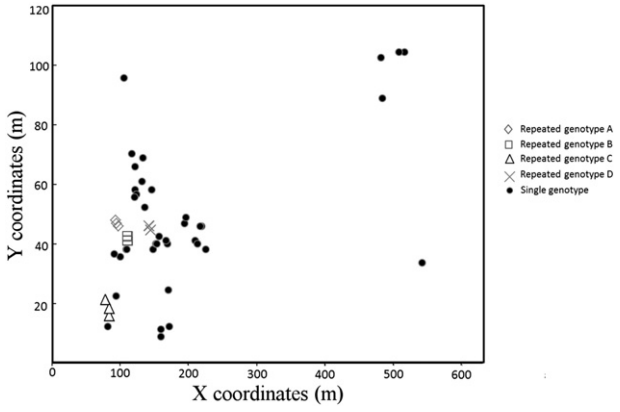

Fig. 4. Spatial distribution of the ramets sampled in the Sohoa population with the positions of each clone identified after multilocus analyses.

Table 4
Genetic variability per marker and across markers calculated for 11 polymorphic microsatellite loci in the *V. humblotii* Sohoa population. Name and Genbank code in parenthesis of each locus, number of different alleles (N_a), number of effective alleles (N_e), heterozygosity observed (H_O), heterozygosity expected under random mating (H_E) and fixation indices (F_{IS}) are precised. Global F_{IS} value for Sohoa population was calculated from the mean H_O and H_E values.

Locus	N_a	N_e	H_O	H_E	F_{IS}
mVplCIR031 (*EF486655*)	2	1.237	0.214	0.193	−0.108
mVhuCIR03 (*JN222562*)	4	1.913	0.381	0.484	0.213
mVhuCIR04 (*JN222563*)	2	1.690	0.429	0.413	−0.038
mVhuCIR06 (*JN222564*)	2	2.000	0.667	0.504	−0.323
mVhuCIR07 (*JN222565*)	2	1.825	0.405	0.458	0.117
mVhuCIR08 (*JN222566*)	2	1.024	0.024	0.024	0.000
mVhuCIR09 (*JN222567*)	5	2.633	0.548	0.629	0.129
mVroCIR09 (*JN222578*)	2	1.825	0.357	0.459	0.222
mVroCIR05 (*JN222575*)	3	2.201	0.452	0.553	0.183*
mVroCIR03 (*JN222573*)	6	2.621	0.500	0.628	0.203
mVroCIR04 (*JN222574*)	4	2.479	0.548	0.605	0.094
Mean Sohoa population (±SD)	3.09 (±1.45)	1.950 (±0.522)	0.411 (±0.175)	0.450 (±0.187)	0.086[a]

[a] Indicates significant deviation from Hardy Weinberg equilibrium ($P < 0.05$).

(Table 5). The shape of the regression of the kinship coefficients and the logarithm of the distance for the dataset 'genets' was convex (*k*-values > 0) indicating a pollen dispersal more restricted than seed dispersal ($\sigma_p \ll \sigma_s$).

4. Discussion

4.1. Vegetative reproduction and its impact on the spatial genetic structure

For plants with both sexual and asexual reproduction, the vegetative reproduction impacts on the spatial genetic structure of populations (Chung and Epperson, 1999; Hossaert-Mckey et al., 1996; Shapcott, 1995). At fine scale, the spatial genetic structure is expected to be higher for the 'ramets' than the 'genets' in case of significant structuring effect of clonal growth (Heywood, 1991). In Sohoa population, 12.5% of the individuals were clones deriving from vegetative reproduction (Fig. 4). This value is an agreement with the proportion of clones (6–25%) detected in two other leafless *Vanilla* species from Puerto Rico, *Vanilla claviculata* Sw. and *Vanilla barbellata* Rchb. f. (Nielsen, 2000). Following the FSGS analysis, the main difference observed between 'genets' and 'ramets' occurs in the first distance class for which, after exclusion of the repeated multilocus genotypes, the autocorrelation became not significant (Table 5). In the dataset 'ramets', individuals separated by short distances (<10 m) are more genetically related than those that are further apart, as evidenced by *Sp* statistics (Table 5). The phalanx distribution of the clonal architecture (Lovett-Doust, 1981) (Fig. 4) is mainly responsible for this prominent structuring in the first distance class (Vallejo-Marín et al., 2010).

4.2. Allogamous sexual reproduction in V. humblotii

As for most *Vanilla* species (Bory et al., 2008b; Gigant et al., 2011a), *V. humblotii* exhibited two reproduction modes: asexual and sexual. Hand-crossing experiments revealed that *V. humblotii* is self-compatible (Table 3). Spontaneous self-pollination is however prevented by a large rostellum covering the entire stigmatic surface (Table 2), therefore the species depends on pollinators for fruit production. However, 6.7% of bagged flowers gave fruits which revealed unexplained spontaneous self-pollination, as already observed in *V. planifolia* and *Vanilla chamissonis* and allogamous *Bulbophyllum* species (Gamisch et al., 2014; Macedo Reis, 2000; Soto Arenas and Cameron, 2003), all pollinator-dependant species. Nothing is known about the mechanisms involved in such exceptional cases. The *Sp* estimates (*Sp* (genets) = 0.020, Table 5) are concordant with the range of variation found in species with animal-dispersed pollen (mean 0.017 as reviewed in Vekemans and Hardy (2004)). The average F_{IS} value measured at 0.086 and the relatively high gene diversity ($H_E = 0.450$, Table 4) witnessed a sexual reproduction with a predominant allogamy. However, this high gene diversity is associated with a low level of allelic diversity (mean $N_e = 1.95$ and Mean $N_a = 3.09$; Table 4) which could be due to the isolation of the population and genetic drift threatening the long-term survival of the population (Bradshaw and Holzapfel, 2001; Frankham, 2005; Gienapp et al., 2008; Umina et al., 2005).

4.3. Pollinators of V. humblotii and natural fruit set

The female allodapine bee (*A. obscuripennis*) and the sunbird (*N. coquerelli*) were identified as visitors but may be considered as putative pollinators of *V. humblotii* in Mayotte. Indeed in South Africa, the closely related leafless *V. roscheri* is also possibly pollinated by allodapine bees (Gigant et al., 2014). In addition, (i) *A. obscuripennis* body size measures compatible with pollen movements in *V. humblotii* flower, (ii) direct captures of pollinia on the mesonotum (Fig. 3B), and (iii) foraging behaviour for nest feeding (Hargreaves et al., 2010), are all elements that strengthen the hypothesis that the allodapine may be a *V. humblotii* pollinator. Furthermore, our indirect estimation of the neighbourhood size ($18.6 < Nb < 21.6$; Table 5), is consistent but lower than the neighbourhood size of 50 for bee-pollinated plants having gravity-dispersed seeds (Chung and Epperson, 2000).

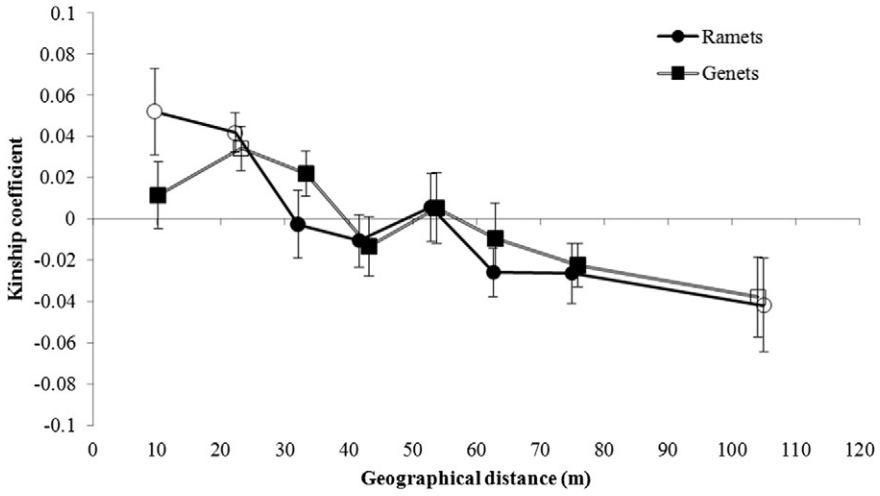

Fig. 5. Correlograms showing the spatial structure for the two datasets 'ramets' and 'genets' of the population of Sohoa, with average Loiselle kinship coefficients over all loci (±SD) plotted as a function of the geographical distance in metres. Significant values of kinship coefficients are precised by empty symbols (P (2-sided test) < 0.05).

Table 5
Estimates of Fine-scale spatial genetic structure and gene dispersal parameters for the two datasets 'ramets' and 'genets' of *V. humblotii* Sohoa population: Loiselle's average kinship coefficient between individuals separated by less than 10 m (F_1) and its standard deviation (SD), slope of linear regression (b_d) and slope of log-normal regression (b_{Ld}) with their respective determination coefficient R^2_d and R^2_{Ld}. The intensity of the spatial genetic structure (Sp), neighbourhood size (N_b), gene dispersal distance (σ_g) and standard error in parenthesis for three effective densities (D_e) estimated from the density of individuals (D) are also given. Lack of value (−) indicates a failure of the estimation procedure. N_b is a value obtained from the mean estimate under the three assumed D_e with standard deviation (SD) in parenthesis.

Pop	F_1 (SD)	b_d (R^2_d)	b_{Ld} (R^2_{Ld})	Sp	N_b (SD)	σ_g (m) ($D_e = D/2$)	σ_g (m) ($D_e = D/4$)	σ_g (m) ($D_e = D/10$)
Ramets	0.052^{**} (0.015)	$-1.0 \times 10^{-3***}$ (3.1×10^{-2})	$-4.3 \times 10^{-2***}$ (3.8×10^{-2})	0.045	21.6 (4.1)	25.2 (7.8)	33.0 (3.4)	44.1 (−)
Genets	0.012 (0.016)	$-7.0 \times 10^{-4**}$ (1.6×10^{-2})	$-1.9 \times 10^{-2*}$ (8.8×10^{-3})	0.020	18.6 (1.6)	21.2 (3.29)	31.6 (6.2)	45.0 (−)

* *P-value* < 0.05.
** *P-value* < 0.01.
*** *P-value* < 0.001.

Bird pollination of *Vanilla* species has long been suspected in Tropical America (Bouriquet, 1954; Stéhlé, 1954) and interactions were observed between *V. planifolia* and hummingbirds in Mexico (Lubinsky and Seung-Chul, 2006). In Mayotte, the sunbird *N. coquerelli* is mainly known to be nectarivorous (Pailler et al., 2002), but its diet is also composed of invertebrates (Louette, 1988). Large tubular scentless red/yellow flowers, as *V. humblotii* flowers, are known to attract birds for pollination (Hingston and Quillan, 2000), foraging for nectar. However, without nectar reward in *V. humblotii*, the sunbird may be attracted by the small invertebrates (midges, spiders and ants) constantly present in the flowers. This is supported by its foraging behaviour recorded in the Movie 2.

Whatever the pollinators of *V. humblotii*, they show low abundance or effectiveness given the absence of pollen movement and the low natural fruit set (0.8%). However, this value is in agreement with the 70% of orchid species whose the fruit set is below 10%, of which deceptive rewarding species have lower fruit set than rewarding species (Tremblay et al., 2005). In *V. humblotii*, like in many rewardless orchids, fructification is rather pollinator than resource limited (Tremblay et al., 2005), as demonstrated by the success of our hand-pollination experiments. Such low fruit sets (≤1%) were reported for some leafy American *Vanilla* species, as reviewed in Gigant et al. (2011a).

4.4. Impact of the sexual reproduction on the genetic structure of Sohoa population

Without vegetative reproduction, the spatial genetic structure of the population depends mainly on pollen transfer and subsequent seed dispersal and recruitment. The 2D LSA analysis illustrated the pattern of autocorrelations between nearest neighbours related to sexual reproduction (the dataset 'genets') in Sohoa population (Fig. 6). Three main patches of autocorrelations can be identified comprising not only nearest neighbours but also distant ones and some nearest neighbours in each patch are not systematically autocorrelated. These results highlight the non-significant autocorrelations obtained at the shortest distance (<10 m) for the 'genets' based on Loiselle coefficients (Fig. 5). Given that we detected a more important seed flow than pollen flow in Sohoa, we hypothesize that pollen flow is probably restricted between near neighbours in the population (due to bee-pollination), but that a mechanism of seed dispersal is responsible for the absence of significant structuring in the shortest distance class (*i.e.* no or few seedlings beneath the mother plant).

For epiphytic orchids, given the height of canopy and the dust-like seeds character of most orchids (Dressler, 1981), dispersal by wind and gravity or water movements on hillsides will involve a subsequent recruitments of individuals not necessarily close to the mother plant. In *Vanilla* species, blooming and fruiting occur preferentially on canopy, up to 8 m high for *V. humblotii* in Mayotte depending on the vegetation. Most *Vanilla* seeds are associated with a moist pulp (Madison, 1981), but in *V. humblotii*, this character is noticeable in the first days after dehiscence then the fluid ultimately dries up releasing the seeds (pers. obs.). In *V. bicolor* Lindl., Householder et al. (2010) described the non-oily character of the fruits and suggested a mixture of wind and gravity for seed dispersal. This could also be the case for *V. humblotii.*

However, our model is density-dependent so we may not generalize the local genetic structure values of Sohoa population to other populations in the overall distribution of the species (Jin et al., 2012). Moreover, for some species with wind/gravity seed dispersal, the absence of autocorrelations in the first distance class can be related to the escape hypothesis (Connell, 1971; Janzen, 1970; Nathan et al., 2000), where light conditions (Acherar et al., 1984; Schiller, 1979), spatiotemporal variations of competition and availability of safe sites (Hamrick and

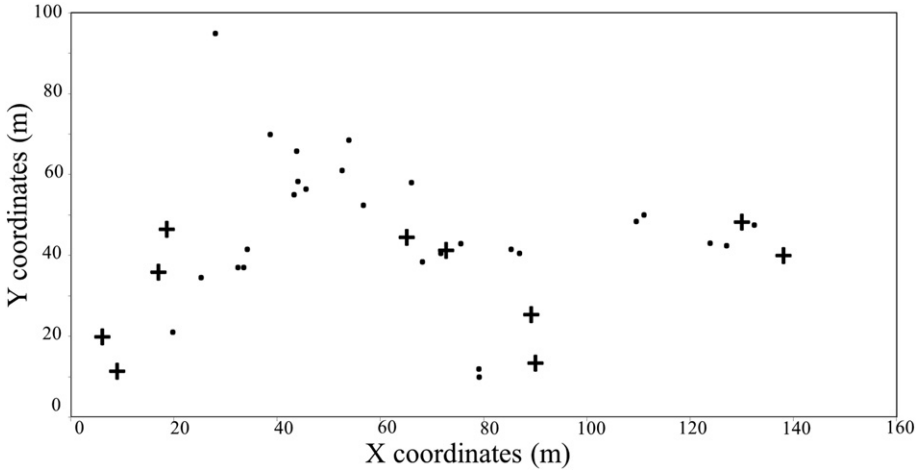

Fig. 6. Spatial distributions of the significant autocorrelations (+) detected in the dataset 'genets' using 2D LSA analysis ($P < 0.05$).

Trapnell, 2011; Nathan et al., 2000) induce a high mortality in the vicinity of adult plants. It is also important to remember that long-distance dispersals are overlooked deliberately in fine-scale studies, whereas they influence the recruitment pattern in plant species. Complemental empirical fine-scale spatial genetic structure analyses and direct seed dispersal measures are thus needed to better understand the underlying mechanisms responsible for the spatial structure of *V. humblotii* populations.

4.5. Geitonogamy avoidance of V. humblotii

Despite a rostellum promoting outcrossing by a pollinator-dependent system, geitonogamous inbreeding was expected in *V. humblotii* population because the flowers are self-compatible, as for most orchids (Tremblay et al., 2005). In a bee-pollinated species with limited pollen transfer, geitonogamous matings are highly probable when multiple inflorescences and flowers are available simultaneously (Eckert, 2000). This is enhanced by the phalanx architecture of clonality since the mating opportunities between clones increase when clones are clumped together (Charpentier, 2002; Reusch, 2001). In Sohoa, the significant but weak signs of inbreeding ($F_{IS} = 0.086$), with one marker showing significant departure from HWE, and most of them displaying moderate heterozygote deficits, may be the result of a Wahlund effect, a genetic consequence of the restricted dispersal (Wahlund, 1928). However, the genotypic diversity parameters (G/N = 0.88 and R = 0.87) revealed a low proportion of clonal genotypes in Sohoa, and the probability that two randomly selected genotypes in this population are different is close to 1 ($D^* = 0.99$). Indeed, the genotypic diversity is high in Sohoa (G/N = 0.88) compared to other clonal species (mean genotypic diversity = 0.42 (Vallejo-Marín et al., 2010)) but similar to leafless *Vanilla* species of Puerto Rico that did not deviate from Hardy Weinberg proportions (Nielsen and Siegismund, 1999). The high clonal diversity could contribute to reduce the probability of geitonogamy since the clonal diversity was described as inversely correlated with selfing rate (Albert et al., 2008; Eckert, 2000). Natural selection promotes characters increasing the clonal diversity and the establishment of a high number of genotypes within a patch (Albert et al., 2008): in accordance with the geitonogamy-avoidance hypothesis (Dressler, 1981; Jersáková and Johnson, 2006; Jersáková et al., 2006; Johnson and Nilsson, 1999; Johnson et al., 2004; Smithson and Gigord, 2001). Scentless and rewardless flowers, as in *V. humblotii*, are such characters driven by pollinator learning behaviour, that limit pollinator visits between close inflorescences (Internicola et al., 2006). By these mechanisms, the plant species experience a deceptive pollination system limiting the genetic consequences of inbreeding at the scale of a population (Johnson et al., 2004). Interestingly, wild populations of *V. planifolia* from Mexico (Soto Arenas, 1999b) revealed high inbreeding ($F_{IS} \approx 1$), which was attributed to the patchy distribution of the individuals (a single clone covering 0.2 ha), the low population density (<1 ind. km^{-2}) (Soto Arenas, 1999b) and the fragrant flowers containing 1-8-cineol, a strong attractant for euglossine bees (Soto Arenas and Dressler, 2010). This demonstrates empirically that some *Vanilla* species may experience strong inbreeding by geitonogamy. On the contrary, in *V. humblotii*, the combination of a high clonal diversity and scentless and rewardless flowers may limit geitonogamous matings which should have been favoured by clonality and self-compatibility.

5. Conclusions

For the first time, knowledge was provided on the reproductive biology and its influence on the genetic diversity and on the spatial structuring of an African endangered *Vanilla* species. As most *Vanilla* species, *V. humblotii* exhibited a mixed mode of reproduction: an allogamous sexual reproduction with a deceptive-system (with ~1% natural fruit set) and a vegetative reproduction with a phalanx distribution due to natural stem cuttings (accounting for 12.5% of the individuals) (Bory et al., 2008b; Bory et al., 2008c; Gigant et al., 2011a; Tremblay et al., 2005). Whether the allodapine bee *A. obscuripennis* and the sunbird *N. coquerelli* are pollinators of *V. humblotii* will need to be confirmed by further observations and evidences of pollen movements. This could be a difficult task given that pollen movements seem uncommon in Mayotte, and that the average natural fruit set measured is low (~1%). Here, we supported the pollinator identification by a comprehensive study of the genetic structure at population scale. Evaluating the gene dispersal and the overall pattern of isolation-by-distance, the genetic analyses brought complementary highlights in line with the direct observation of the pollination system. In Sohoa population of *V. humblotii*, we suggest that the reproductive strategy of *V. humblotii* consists of a phalanx clonal growth structuring the diversity at small distances (<10 m), counteracted by a high genotypic diversity and a deceptive pollination system allowing geitonogamy avoidance.

More field observations have to be made to confirm our hypotheses regarding pollen and seed dispersal in *V. humblotii* but our results represent a crucial step towards a conservation plan for this species in Mayotte and provide key elements to the general study of the reproductive strategy of clonal plant species with a deceptive pollination system.

Supporting information consists of 620 two video recordings showing the plant pollinator interactions. Supplementary data associated with this article can be found in the online version, at doi:http://dx.doi.org/10.1016/j.sajb.2016.02.205.

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