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Spatial patterns of AFLP diversity in *Bulbophyllum occultum* (Orchidaceae) indicate long-term refugial isolation in Madagascar and long-distance colonization effects in La Réunion

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Abstract

Bulbophyllum occultum, an epiphytic orchid mainly distributed in the rainforests of (north)eastern Madagascar and La Réunion, represents an interesting model case for testing the effects of anthropogenic vs historical (e.g., climate-induced) habitat isolation and long-distance colonization on the genetic structure of plant species with disjunct distributions in the Madagascan region. To this aim, we surveyed amplified fragment length polymorphisms (AFLPs) across 13 populations in Madagascar and nine in La Réunion (206 individuals in total). We found overall high levels of population subdivision ($\Phi_{PT} = 0.387$) and low within-population diversity (H_E , range: 0.026–0.124), indicating non-equilibrium conditions in a mainly selfing species. There was no impact of recent deforestation (Madagascar) or habitat disturbance (La Réunion) detectable on AFLP diversity. *K*-means clustering and BARRIER analyses identified multiple gene pools and several genetic breaks, both within and among islands. Inter-island levels of population genetic diversity and subdivision were similar, whereby inter-individual divergence in flower colour explained a significant part of gene pool divergence in La Réunion. Our results suggest that (i) *B. occultum* persisted across multiple isolated ('refugial') regions along the eastern rainforest corridor of Madagascar over recent climatic cycles; and (ii) populations in La Réunion arose from either single or few independent introductions from Madagascar. High selfing rates and sufficient time for genetic drift likely promoted unexpectedly high population genetic and phenotypic (flower colour) differentiation in La Réunion. Overall, this study highlights a strong imprint of history on the genetic structure of a low-gene-dispersing epiphytic orchid from the Madagascan region.

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Data archiving

Data available from the Dryad Digital Repository: (<http://dx.doi.org/10.5061/dryad.1q34p>)

Conflict of interest

The authors declare no conflict of interest

Keywords

AFLP; *Bulbophyllum occultum*; genetic structure; habitat fragmentation; La Réunion; long-distance colonization; Madagascar

Introduction

The tropical rainforests of Madagascar represent one of the world's greatest repositories of plant diversity (*ca* 12,000 species) and endemism (*ca* 70–80%; Myers *et al.*, 2000; Goodman and Benstead, 2005) but also rank among the most seriously impacted ecosystems, where human-induced landscape changes occur at an unprecedented rate (Cable, 2011). However, population genetic studies of Madagascan plant species are extremely rare and mostly limited to trees and shrubs (Dawson and Powell, 1999; Amsellem *et al.*, 2000; Voigt *et al.*, 2009; Rivers *et al.*, 2011; Krishnan *et al.*, 2013), with only few having dealt with taxa restricted to the island's eastern rainforest ecosystems (Andrianoelina *et al.*, 2006, 2009; Shapcott *et al.*, 2007, 2012). With their main focus on conservation, these studies have provided some first and important insights into present-day patterns of intra-specific genetic variation, yet suggesting only limited impact of recent loss of vegetation on molecular genetic parameters (Shapcott *et al.*, 2007; Andrianoelina *et al.*, 2009). Moreover, there is a significant gap in our knowledge regarding the historical factors influencing past population dynamics (e.g., habitat fragmentation, colonization) and, in turn, the present-day genetic structure of Madagascan plant species, with implications for evolutionary biology. Somewhat surprisingly, the orchid family (Orchidaceae) has hitherto attracted little attention in this context, despite showing highest species richness (*ca* 1,000 spp.) and degree of endemism (*ca* 85%) among all vascular plant families in Madagascar (Hermans *et al.*, 2007; Cable, 2011; Madagascar Catalogue, 2013).

Because mainly thriving as epiphytes, tropical orchids are generally thought to be less resistant to both anthropogenic and natural changes in the environment when compared to their terrestrial, mostly temperate counterparts (Wiegand *et al.*, 2013). For instance, host tree availability (and possibly specificity) plays a fundamental role in determining the distribution and abundance of epiphytic orchids, often resulting in their patchy distribution across geographic areas and along microenvironments, even within a single tree (Nieder *et al.*, 2000; Winkler *et al.*, 2009; Trapnell *et al.*, 2013; but see Wagner *et al.*, 2015). Furthermore, epiphytic orchids exclusively obtain their moisture and nutrients from air, rain and debris, and thus must be able to withstand periods of extreme water stress between rainfall events (e.g., by development of water-storing pseudobulbs). These intrinsic physiological constraints may further enhance their susceptibility to the genetic consequences of habitat fragmentation, including reduced intra-population diversity and increased population divergence, mainly because of genetic drift and inbreeding (Honnay and Jacquemyn, 2007; Tremblay *et al.*, 2008; Leimu *et al.*, 2010). However, determining these processes remains challenging because levels of genetic diversity of post-fragmentation populations are also likely influenced by other factors, such as physical features of the landscape and species-specific traits, in particular longevity, mating system, and dispersal ability (Honnay and Jacquemyn, 2007; Aguilar *et al.*, 2008; Yuan *et al.*, 2012).

This study focuses on the population genetic structure of an epiphytic orchid, *Bulbophyllum occultum* Thouars (Epidendroideae), which is mainly distributed in the rainforests of northern and eastern Madagascar and the small Mascarene island of La Réunion (2,512 km²), ca 680 km to the East (Figure 1). There are several reasons why this orchid represents an interesting model case to gain insights into the effects of recent (anthropogenic) vs historical (e.g., climate-induced) habitat isolation and long-distance colonization on the genetic structure of plant species with a disjunct distribution in the Madagascan region. First, the extant rainforest habitats of *B. occultum* have been impacted severely by agricultural practices (e.g., ‘slash and burn’, livestock production, logging), with only about 10% of the primary vegetation remaining in Madagascar (Burney *et al.*, 2004; Harper *et al.*, 2007; Cable, 2011) and about 25% in La Réunion (Macdonald *et al.*, 1991; Thébaud *et al.*, 2009). Although much of this deforestation occurred only recently (i.e., over the last 100 yr), its genetic impact may already have become apparent in species with relatively short generation times (Aguilar *et al.*, 2008; Montoya *et al.*, 2008), such as *B. occultum*, which may require only about five years to reach the reproductive stage as observed for Asian relatives (e.g., *B. lobbia*) under cultivation (GA Fischer, U Jaros, personal observations). On the other hand, the species' potential for self-pollination (Jacquemyn *et al.*, 2005; Gamisch *et al.*, 2014, 2015; see below) and supposedly high dispersal ability (via dust-like, wind-dispersed seeds) may have provided intrinsic buffers to the loss of genetic diversity (Honnay and Jacquemyn, 2007).

Second, in the more distant past, natural processes of rainforest fragmentation may also have significantly influenced the genetic structure of *B. occultum*. For example, Quaternary periods of drier and cooler climate (broadly equivalent to glacials at high latitudes; Bennett and Provan, 2008) are believed to have had a major impact on the geographic distribution and within-species genetic structure of Madagascan rainforest biota (Andrianoelina *et al.*, 2006, 2009; Wilmé *et al.*, 2006; Vences *et al.*, 2009; Rakotoarisoa *et al.*, 2012, 2013; Rakotoarinivo *et al.*, 2013). Notably, in Madagascar, the extant range of *B. occultum* extends across five (out of 12) ‘Centres of Endemism’ (COEs) as defined by Wilmé *et al.* (2006) on the basis of modern land vertebrate records (see Figure 1). These COEs generally circumscribe river catchments in the coastal and montane portions of the island, and have been hypothesized to coincide with glacial refuge locations that fostered allopatric population divergence and speciation of locally endemic taxa (Wilmé *et al.*, 2006; Rakotoarinivo *et al.*, 2013). While recent population genetic studies in lemurs support this hypothesis (Markolf and Kappeler, 2013; Perry *et al.*, 2013), it remains to be established whether COE boundaries significantly contribute to the spatial genetic structure of a plant species as well, and for which *B. occultum* is a suitable test case along the island’s eastern rainforest corridor.

Finally, founder effects may have exerted a strong influence on the distribution of genetic variance among populations of *B. occultum* in La Réunion. Molecular phylogenetic studies have shown that this species (as represented, however, only by material from Madagascar) firmly nests within a clade of Madagascan congeners (with *B. bicoloratum* as likely sister; Fischer *et al.*, 2007; Gamisch *et al.*, 2015). Moreover, preliminary chloroplast DNA surveys indicate that La Réunion populations of *B. occultum* are almost fixed for the most common and widespread haplotype in Madagascan conspecifics (U Jaros, unpublished data).

Together, these data suggest that *B. occultum* colonized La Réunion from Madagascar. In case this occurred relatively recently, we would expect La Réunion populations to be little differentiated from their putative source population(s) in Madagascar, and harbour less diversity relative to the latter (Barrett *et al.*, 1996; Frankham, 1997). On the other hand, recurrent population founding has been shown to minimize bottlenecks created during colonization or even to promote population genetic differentiation (Whitlock and McCauley, 1990; Le Corre and Kremer, 1998; Vandepitte *et al.*, 2012). Notably in this context, La Réunion *B. occultum* exhibits an unusual flower-colour dimorphism, with both red- and yellow-flowered individuals often occurring in sympatry (Cadet, 1989; T Pailler, U Jaros, personal observation). The evolutionary significance of such dimorphisms has been discussed at length (reviewed by Wessinger and Rausher, 2012), but in most instances the mechanism(s) responsible for their maintenance (e.g., various forms of selection or genetic drift) remain unclear. However, for *B. occultum*, the null hypothesis would be that the genetic consequences of colonization (e.g., founder effects, drift, inbreeding) also affected the distribution of flower colour variation among populations in La Réunion.

In this study, we analyzed patterns of genetic structure in populations of *B. occultum* from Madagascar and La Réunion, using amplified fragment length polymorphisms (AFLPs), a dominant marker system with a wide coverage of the nuclear genome. Our primary aim was to elucidate the extent to which patterns of AFLP variability are shaped by the effects of intrinsic properties (mating system, dispersal ability), drift/migration dynamics (e.g., isolation-by-distance), recent human impact, historical habitat fragmentation, and long-distance colonization. More specifically, we addressed the following questions: (1) Are there discernable effects of deforestation or habitat disturbance on levels of within-population genetic diversity in each island? (2) Is there a spatial component to the genetic structure in Madagascar explicable in terms of COE regions and/or glacial refugial isolation? (3) What is the extent of genetic differentiation between Madagascar and La Réunion populations? (4) Which past demographic processes have shaped population genetic diversity and structure in La Réunion (e.g., single or recurrent population founding, founder effects, and subsequent genetic drift)? And (5) what is the extent of genetic differentiation between the two flower-colour morphs in the latter island? Overall, this study may serve as a first genetic baseline for gaining insights into the population divergence history of an orchid species from the eastern rainforests of Madagascar and with a disjunct distribution in the Mascarenes.

Materials and Methods

Study species

Bulbophyllum occultum is a herbaceous perennial orchid, with a diploid chromosome number ($2n = 36$; Pridgeon *et al.*, 2014; M Kiehn, Vienna University, unpublished data) and an epiphytic, or rarely lithophytic way of life (Cadet, 1989; GA Fischer, personal observation). Based on the 22 sample locations of this study (Table 1) plus 78 additional records based on all available geo-referenced herbarium specimens (mostly collected between 1932 and 1989; BR, G, K, MO, P, SZU, WU, and www.tropicos.org) and our own field data (all coordinates available upon request), the species is mainly distributed in the (sub)humid coastal and montane forests of northern and eastern Madagascar (0–1500 m; 76

known sites) and (south)eastern La Réunion (0–620 m; 15 sites), with rare occurrences in Mauritius (5) and the Comoro Islands (4) (Figure 1). The conservation status of *B. occultum* has not been assessed yet by the IUCN (International Union for Conservation of Nature), but there is little doubt that ongoing deforestation activities especially in Madagascar have led to populations becoming progressively more vulnerable and threatened with extinction (GA Fischer, personal observation).

Bulbophyllum occultum produces one or rarely few inflorescences, which arise from pseudobulbs and bear many (*ca* 20–40) small flowers (*ca* 5 mm). The flower colour appears to be uniformly red in Madagascar (Cribb and Hermans, 2010; GA Fischer, personal observation), but individuals with either red or yellow flowers occur in La Réunion, yet without any other discernable morphological difference (Cadet, 1989; T Pailler, U Jaros, personal observation). Most species of *Bulbophyllum* are self-compatible and fly (or rarely bee) pollinated (van der Cingel, 2001; Tan *et al.*, 2006; Liu *et al.*, 2010; Humeau *et al.*, 2011). However, direct observations of insect visitors in *B. occultum*, or related species from the study area, are lacking. In fact, field observations, pollination/bagging experiments and floral-morphological studies in *B. occultum* indicate that most, if not all, individuals from La Réunion are capable of autonomous within-flower self-pollination ('auto-pollination' *sensu* Catling, 1990) due to the lack of the 'rostellum' (i.e., a modified stigmatic lobe that usually serves as a physical barrier to prevent auto-pollination), whereas both outcrossing and selfing morphs (with and without a rostellum, respectively) occur in Madagascar, albeit at unknown frequencies (Jacquemyn *et al.*, 2005; Gamisch *et al.*, 2014, 2015; U Jaros, personal observation). We therefore anticipated that the genetic data of this study would provide us with some additional clues about the species' mating system, while realizing that dominant AFLPs are less suited for this purpose compared to co-dominant markers.

Plant material and sampling design

In 2008, silica-dried leaf material of *B. occultum* was collected from 13 populations in Madagascar (M1–13; $n = 133$) and, in 2010, from nine populations in La Réunion (R1–9; $n = 92$), that is, a total of 22 populations and 225 individual plants (see Figure 1; Table 2). All necessary collecting permits were obtained by the Département des Eaux et Forêts (Madagascar) and the Parc National de La Réunion (sampling permit code: DIR/I/2010/028). *Bulbophyllum occultum* has short internodes and thus probably little potential of vegetative propagation through fragmentation of creeping rhizomes (Batygina *et al.*, 2003). Different individuals are easily discriminated by their characteristic growth habit comprising the whole spectrum from small to larger bulbs (GA Fischer, U Jaros, personal observation). Nonetheless, care was taken to collect leaf material from putative genets from different host trees or widely separated branches within a tree. Mean (\pm SD) geographical distances between sampling sites were 331.8 (\pm 213.4) km in Madagascar, and 12.4 (\pm 8.2) km in La Réunion. In Madagascar, our sampling of northern (M1–7), central (M8–11), and southern (M12/13) populations covers three (out of five) COE regions in the eastern portion of the island as defined by Wilmé *et al.* (2006), i.e., South Bernarivo/North Mangoro (COE code no. 2), South Mangoro/North Manampatrana (no. 3), and South Mananara (no. 5), respectively (see Figure 1; Table 1). Within each La Réunion site, we recorded the flower colour of each sampled individual in flower (red or yellow; $n = 61$ in total). Spirit-preserved

vouchers of populations analysed in this study are held at the herbarium of Salzburg University (SZU).

DNA extraction and AFLP genotyping

For each individual, total genomic DNA was extracted from the dried leaf tissue using the CTAB method (Doyle, 1991). The AFLP procedure followed the protocol of Vos *et al.* (1995) with minor modifications that included the use of fluorescent-dye-labelled primers (MWG Biotech, Ebersberg, Germany). An initial primer trial was conducted on eight individuals from two geographically distant populations (M13, R8) using nine selective primer pair combinations with three selective nucleotides (data not shown). Based on this, the following primer combinations that produced the best results with respect to polymorphism and clarity of AFLP profiles were chosen for the complete survey: *EcoRI*-AGA/*MseI*-CTG (6-FAM), *EcoRI*-AGG/*MseI*-CAG (VIC), and *EcoRI*-AGC/*MseI*-CTC (NED). Each PCR plate (48 slots) contained one positive control and one blind sample. The selective PCR products were purified using Sephadex G-50 Superfine (GE Healthcare BioSciences, Uppsala, Sweden) following the manufacturer's instructions. AFLP fragments were separated on a MegaBACE™ 1000 (GE Healthcare Biosciences, Pittsburgh, PA, USA), using the ET550-R-Rox-MegaBACE™ sizing standard, and manually scored with the aid of DAX version 8.0 (Van Mierlo Software Consultancy, Eindhoven, The Netherlands). Each marker was coded as 1 or 0 for presence or absence in an individual, resulting in a binary data matrix. We also performed separate test runs with 13 individuals (from 11 different populations) used as replicates. This resulted in a low overall error rate (2%), calculated as the percentage of differently scored loci over all replicates and the three primer pair combinations (Bonin *et al.*, 2004). In total, 206 individuals were finally available for data analysis because 19 individuals failed to amplify in at least one of the three primer combinations. Accordingly, the mean (\pm SD) sample size was 9.4 (\pm 2.4) individuals per population (see Table 2).

Genetic diversity analyses of the sampled populations

In order to assess levels of genetic diversity, we calculated for each population the observed number of alleles (N_A), the percentage of polymorphic fragments (*PPF*), and the number of AFLP phenotypes, using GENALEX version 6.5 (Peakall and Smouse, 2012). A fragment was considered polymorphic if at least one individual showed a variant pattern. In addition, Nei's (1987) unbiased gene diversity (H_E) for small sample sizes and frequency down-weighted marker (*DW*) values (or 'rarity-indices') were estimated using the AFLPDAT R package (Ehrich, 2006). Large values of *DW* imply a high amount of rare AFLP markers as expected for long-term isolated populations, whereas populations affected by genetic drift and/or founder effects exhibit small *DW* values (Schönswetter and Tribsch, 2005; Skrede *et al.*, 2009). For *DW*, 95% confidence intervals (CIs) were obtained by 9999 bootstrap replicates over loci. Significantly small or large values of *DW* (lying outside the 95% CIs) were assessed by a permutation procedure in AFLPDAT using 1000 replicates. Significant differences in mean genetic diversities between Madagascar and La Réunion were tested by an exact Wilcoxon rank-sum test in R version 3.0.3 (R Development Core Team, 2012).

Testing effects of deforestation and habitat disturbance on genetic diversity

For the Madagascan populations, we used linear regression models in R to test for a negative relationship between measures of diversity (N_A , PPF , H_E , DW), as response variables, and the (log-transformed) percentage of deforestation (LPD) in sampled areas for the period 1950 to 2005, as possible predictor variable (see Table 1). Normality of the distribution of the response variables was confirmed using the Shapiro-Wilk test in R (all P values > 0.1). For estimating LPD , we used forest cover and change data for Madagascar (Harper *et al.* (2007; https://learning.conservation.org/spatial_monitoring/Pages/data_access.html), including digitalized aerial maps of 1950 (Humbert and Cours Darne, 1965) and Landsat-type images from NASA's GeoCover Project of 2005 (Tucker *et al.*, 2004), to calculate the relative change (i.e., loss) in forest cover within a 10-km radius around each site over this period (1950–2005). As similar data are not available for La Réunion, we classified each population there according to three categories of 'habitat transformation' (HT) proposed for this island by Strasberg *et al.* (2005) (see also Lagabrielle *et al.*, 2011): (1) relatively untransformed (native canopy still pristine and not invaded); (2) moderately degraded (native biota dominated by alien plants); and (3) transformed (urban or cultivated areas, and alien secondary vegetation) (see Table 1). Significant differences in genetic diversity parameters among these categories were assessed using a Kruskal-Wallis rank-sum test in R.

Population genetic structure, boundaries, and relationships

To identify the most likely number of genetic groups (K) in the entire AFLP dataset, we applied the K -means clustering technique of Hartigan and Wong (1979) using the R script of Arrigo *et al.* (2010). Based on initial trials, the number of K was restricted to vary from 1 to 10 (i.e., less than the number of populations sampled), first, to avoid unstable groups of small numbers of individuals with increasing numbers of K (set up to 22; data not shown), and, second, to capture the major underlying population structure of the data but without overestimating it (Pritchard *et al.*, 2000). For each value of K , we performed 100000 independent runs, starting at different random points. To identify the most likely number of groups, we calculated values of K (Evanno *et al.*, 2005) following the method of Arrigo *et al.* (2010). As a proxy of clustering accuracy, we used the inter-group inertia criterion, i.e. the average of the distances between the centroid of each cluster and each sample contained in it (Kergoat and Alvarez, 2008; Espíndola and Alvarez, 2011). We opted for this non model-based K -means algorithm because, in contrast to alternative model-based algorithms such as implemented in STRUCTURE (Pritchard *et al.*, 2000), it does not assume populations to be in Hardy-Weinberg equilibrium, an assumption probably violated in *B. occultum* due to its potential capacity of auto-pollination (Gamisch *et al.*, 2014, 2015; see above).

We used the program BARRIER version 2.2 (Manni *et al.*, 2004) to identify geographical locations where major genetic boundaries among populations might occur, based on their pairwise genetic (F_{ST}) distances (Supplementary Table 1). The number of barriers was set to vary from 1 to 10, reflecting their descending order of relative importance ('priority') for genetic dispersion (Manni *et al.*, 2004). F_{ST} was calculated using a fragment-frequency approach in AFLP-SURV version 1.0 (Vekemans *et al.*, 2002). This non-allelic method assumes fixed homozygosity at each locus (e.g., resulting from self-pollination), which may overestimate F_{ST} for highly outcrossing species, but is more likely to meet expectations for

B. occultum (see above). Statistical support for each barrier was tested by means of 1000 bootstrapped genetic distance matrices generated in AFLP-SURV. Barriers with high (> 80%) bootstrap support (BS) were retained for further analysis.

To visualize the genetic (F_{ST}) distances between populations, we performed a principal coordinates analysis (PCoA) in GENALEX. In addition, we constructed a mid-point rooted neighbor-joining (NJ) phenogram in PHYLIP version 3.69 (Felsenstein, 2005) based on pairwise F_{ST} values, with bootstrap support obtained by resampling with replacement over loci (1000 replicates).

Population genetic differentiation and tests of isolation-by-distance

Non-hierarchical analyses of molecular variance (AMOVA) were performed in GENALEX to estimate genetic differentiation among populations (Φ_{PT}), overall and within each island. For each dataset, Φ_{PT} values were also calculated in a pairwise fashion. Hierarchical AMOVAs were further used to quantify the partitioning of genetic variance between pre-defined groups of populations (Φ_{CT}), and between populations within such groups (Φ_{SC}), with populations grouped according to island, areas delimited by genetic (BARRIER) boundaries, or COE region (only Madagascar). Significance levels of both Φ -statistics and pairwise Φ_{PT} values were based on 9999 permutations. Effects of isolation-by-distance (IBD), as usually caused by restricted gene flow (Dainou *et al.*, 2010), were tested for each island separately by regressions of $\Phi_{PT}/(1-\Phi_{PT})$ against log-transformed geographic distance for all pairs of populations (Rousset, 1997). Significance of regression slopes was evaluated by Mantel tests with 9999 random permutations in GENALEX.

Comparison of AFLP variation and colour morph differentiation in La Réunion

To examine the genetic divergence between red and yellow flowered morphs from La Réunion, we computed a PCoA in GENALEX on the AFLP-derived Euclidian distance matrix between individuals and labelled with the K -means groups. Statistical association was further assessed through a contingency table by comparing the occurrence of colour morphs and K -means groups using chi-squared (χ^2) tests.

Results

Population genetic diversity

Scoring the sampled material of *B. occultum* (22 populations, 206 individuals) for three AFLP primer combinations resulted in 102 unambiguous fragments between 75 to 538 bp, of which 88 were polymorphic ($PPF = 86.3\%$; Table 2). There was some variation in the number of polymorphic fragments per primer pair ('6-FAM': 36; 'VIC': 30; 'NED': 36), with an average of 34.0 (± 3.5 SD) fragments per combination.

Notably, 139 individuals surveyed had unique AFLP phenotypes (i.e., fragment combinations), while the remainder (67) had redundant ones. However, the great majority of these redundant phenotypes (18 in total) were either scattered across multiple populations (even between islands) or mostly occurred in only two copies if confined to a single population (data not shown). When combined with the species' low capacity for rhizome

fragmentation (see Materials and Methods), these patterns strongly suggest that the redundant AFLP phenotypes observed do not result from vegetative propagation but rather reflect low resolution of the AFLP markers in combination with high levels of inbreeding (Dasmahapatra *et al.*, 2008; O'Brien *et al.*, 2014; see also Discussion).

Levels of variation within populations (in terms of N_A , PPF , and H_E) varied among populations but were generally low (Table 2). For example, within-population gene diversity (H_E) ranged from 0.026 to 0.124, with both values recorded in La Réunion (pops. R3 vs R8), and averaged 0.072 (± 0.028 SD) across all populations. The total (species-wide) H_E was estimated to be 0.116. Populations in La Réunion displayed on average slightly lower H_E values (0.063) than those in Madagascar (0.079), but the differences were not significant ($P = 0.164$), and the same was true for N_A and PPF ($P = 0.105$ and 0.098 , respectively). The mean proportion of rare AFLP markers was of comparable magnitude in Madagascar ($DW = 0.394 \pm 0.069$ SD) and La Réunion ($DW = 0.482 \pm 0.208$ SD). However, DW values varied considerably in the latter island, where two populations featured significantly small (R3, R6) and three large (R1, R8, R9) values. In Madagascar, only two populations (M3, M9) had significantly small DW values (Table 2).

Effects of deforestation and habitat disturbance on genetic diversity

In Madagascar, the mean log-transformed percentage of deforestation (LPD) for the period 1950–2005, as estimated across the 13 sample sites, was 63% ($\pm 31\%$ SD) (see Table 1). However, linear regression models revealed no significant relationship between measures of within-population genetic diversity (N_A , PPF , H_E) or allelic rarity (DW) and LPD ($P = 0.131$ – 0.335). In La Réunion, we classified four of the nine sample sites as being ‘untransformed’ (R5–7, R9) and the remaining ones as ‘moderately degraded’ or ‘transformed’ (only R2) (see Table 1). Yet, again, none of the above genetic diversity parameters were significantly correlated with these qualitative measures of habitat transformation (Kruskal-Wallis tests: $P = 0.134$ – 0.867), whereby results remained unchanged after excluding R2 ($P = 0.080$ – 0.885).

Population genetic structure, boundaries and relationships

Based on the K -means analysis of the entire dataset, $K = 2$ was suggested as the most appropriate number of groups (highest K value), but this grouping identified only certain populations from the South of Madagascar (i.e., M8–12, but excepting M13) as genetically distinct or admixed; by contrast, values of the inter-group inertia (viz. clustering accuracy) progressed to a plateau for levels of $K \approx 6$ (see Supplementary Figure 1). We decided on $K = 6$ as a reasonable solution as the geographical structure captured by this finer grouping also appeared to be associated with several genetic boundaries detected with BARRIER (Figure 2; see below). In detail, all six gene pools (A – F) were present in Madagascar, where most individuals were assigned to groups A (45%) or B (28%), and to a lesser extent C (11%) and D (9%), whereas the remainder (E , F) accounted for 5% of the regional total (Figure 2). Although gene pool A occurred in most populations (except M10/11), it was clearly predominant in the North-Central Madagascar, whereas group B was represented in the South (M8–M12). Gene pools C (11%) and D (9%) occurred at relatively high frequency in populations from the North (M2) and Northeast (M3), respectively, and the same applied to

group *E* in the Far South (M13). Four out of the six gene pools (excepting *B* and *D*) were also present in La Réunion, but here the majority of individuals there were assigned to gene pools *F* (67%) and *E* (25%). Individuals of gene pool *E* group were mainly confined to the South (R6–9), where groups *A* and *C* also occurred at low frequencies (8%).

The BARRIER analysis identified seven barriers (1–7) of high support (BS 81–100%) that documented an abrupt rate of change in the genetic profile of populations across the species' range (see Figure 2). In detail, two barriers of upper (1) and high (3) priority were found in the South of La Réunion, and isolated, respectively, a population (R8) fixed for gene pool *E*, and two nearby 'admixed' populations (R6, R7) comprised of several gene pools. In Madagascar, the strongest genetic boundary (2) separated North-Central (M1–9) and South (M10–13) populations, whereas barriers of lesser priority marked-down the peripheral populations in the Northeast (4: M3) and Far South (5: M13). Only the sixth boundary separated Madagascan and La Réunion populations, whereas the seventh further isolated a single population (M12) in the South of Madagascar (Figure 2).

In the two-dimensional PCoA plot (Figure 3A), the three genetically deviant populations from the South of La Réunion (R6–8) were grouped, along the first axis, either among populations from Madagascar (R7, R8) or between the latter and the rest of La Réunion populations (R6). However, taking the third axis into account (Figure 3B), populations R7 and R8 were clearly distinguishable from their Madagascan conspecifics. In the NJ phenogram (Figure 4), all populations from La Réunion (R1–9) formed a group nested within those from Madagascar, but without receiving strong support (BS 30%). Nonetheless, this analysis confirmed (i) the distinct genetic identity of populations M10–12 from South Madagascar in general (89%), and M10/11 in particular (100%); and (ii) the existence of a local cluster of highly differentiated or 'admixed' populations in the South of La Réunion (R6–8; 46%), different from the one in adjacent areas or further north (R1–5, R9; 100%; see also Figure 2).

Population genetic differentiation and isolation-by-distance

Non-hierarchical AMOVAs (Table 3) confirmed the high genetic differentiation over all populations of *B. occultum* ($\Phi_{PT} = 0.387$), but further revealed very similar levels of population subdivision in Madagascar and La Réunion ($\Phi_{PT} = 0.341$ vs 0.359). At the range-wide scale, 203 of 231 pairwise Φ_{PT} comparisons (88%) had permutation test *P*-values < 0.05, and the number of significant pairwise values was also high within Madagascar (62/78 = 79%) but lower in La Réunion (24/36 = 67%) (Table S1). When accounting for the species' significant inter-island substructure ($\Phi_{CT} = 0.153$, $P < 0.001$), overall levels of population divergence still remained high ($\Phi_{SC} = 0.332$) (Table 3). However, the strongest signature of range-wide spatial differentiation was obtained ($\Phi_{CT} = 0.330$) when populations were assigned to the eight areas delimited by genetic (BARRIER) boundaries, i.e., five in Madagascar and three in La Réunion (Figure 2B). When Madagascan populations were analysed separately, and grouped according to these genetic boundaries, the extent of regional subdivision was much stronger ($\Phi_{CT} = 0.306$) compared with the grouping based on COEs ($\Phi_{CT} = 0.073$). In each island, there were no detectable effects of isolation-by-distance (IBD) (Madagascar/La Réunion: $P = 0.144/0.464$).

Colour morph differentiation in La Réunion and its association with AFLP variation

Across the nine La Réunion populations, we censused 33 individuals that had red flowers, and 28 that bore yellow flowers (Figure 5A), based on a sample size range of 1–10 individuals per population (Table 1). Notably, all flowers within a given individual displayed the same colour (U Jaros, personal observation). Among the eight populations surveyed with $n = 4$, four were mixed (red/yellow) and two each were uniformly coloured red or yellow, whereby yellow individuals appeared to be more frequent in the South (Figure 5B). A PCoA conducted on the AFLP profiles available for 33 red and 28 yellow coloured morphs (Figure 5C) revealed they were largely distinguishable along the first axis, with only few exceptions. We also tested whether the PCoA results changed as we entered the AFLP data of the remaining 18 individuals from La Réunion with unknown flower colour, but the clustering patterns were essentially the same (Supplementary Figure 2). As apparent from Figure 5C, red morphs were almost exclusively associated with gene pool *F* (32 individuals, plus one with *C*), whereas yellow morphs were mostly assigned to *E* (15) but also *F* (8) and *A* (5). These associations were highly significant when analysed both globally ($\chi^2 = 35.23$, $df = 3$, $P < 0.001$) and for only the predominant gene pools, *E* and *F* ($\chi^2 = 25.50$, $df = 1$, $P < 0.001$).

Discussion

In Madagascar, the population divergence history of rainforest-dwelling pant species is still poorly known because of the scarcity of population genetic studies of respective keystone species across the eastern escarpment of the island (see Andrianoelina *et al.*, 2006, 2009; Shapcott *et al.*, 2007, 2012). This study is the first to provide insights into the patterns of population genetic structure of an epiphytic orchid, *Bulbophyllum occultum*, which is mainly distributed across the eastern rainforest corridor of Madagascar but also occurs at relatively high frequency in the small Mascarene Island of La Réunion. However, there are potential limitations of this study, including the limited number of polymorphic AFLP fragments scored, and the small sample sizes of some populations (especially in Madagascar). With these caveats in mind, our results nonetheless suggest that the overall genetic structure and diversity of this orchid conforms to expectations for a mainly selfing species with a non-equilibrium (i.e., historically drift-dominated) population system. Rather than revealing effects of recent human impact, this genetic structure contains signatures of long-term habitat isolation in Madagascar and long-distance colonization effects in La Réunion. Somewhat unexpectedly, our data revealed no reduced genetic diversity in the latter island but rather strong population subdivision, which tends to be associated with a divergence in phenotype (flower colour). In the following, we discuss the evidence for these inferences and their wider implication for a general understanding of the species' population history.

Population differentiation and within-population diversity

Our survey of AFLP variation in *B. occultum* revealed exceptionally high levels of population subdivision (Φ_{PT}), both at the species' range-wide scale (0.387) and within each island (Madagascar/La Réunion: 0.341/0.359; Table 3). These values are at variance with the rather low genetic differentiation among populations reported for orchids in general (e.g., Tremblay *et al.*, 2008) and epiphytic species in particular (e.g., mean $F_{ST} = 0.146$ vs 0.109 at

allozyme loci; Phillips *et al.*, 2012), which is usually ascribed to extensive long-distance gene flow facilitated by the dust-like and wind-dispersed seeds of these plants (Arditti, 1992; Trapnell and Hamrick, 2004; Vanden Broeck *et al.*, 2014; but see Kartzinel *et al.*, 2013; Pinheiro *et al.*, 2013). In addition, we observed remarkably low levels of genetic diversity within all populations of *B. occultum* (e.g., H_E , range: 0.026–0.124, mean: 0.072; Table 2). These values are much lower than those observed from allozyme analyses in pollinator-dependent *Bulbophyllum* species from South America (H_E , range: 0.391–0.612; Azevedo *et al.*, 2007; see also Ribeiro *et al.*, 2007). Rather, they are strikingly similar to the average value obtained for mainly selfing species screened with dominant marker systems ($H_E \approx H_{POP} = 0.12$; Nybom and Bartish, 2000; Nybom, 2004). This is in further agreement with field, experimental and morphological observations that most individuals of *B. occultum* in La Réunion, and a yet undetermined proportion in Madagascar, are capable of auto-pollination (Jacquemyn *et al.*, 2005; Gamisch *et al.*, 2014, 2015; U Jaros, personal observation).

Taken at face value, the above results suggest that restricted gene flow via pollen *and* seed, combined with high rates of selfing, are major determinants of the relatively increased population divergence and reduced within-population diversity observed in *B. occultum*, when compared with most other orchid species (e.g., Tremblay *et al.*, 2008; Phillips *et al.*, 2012; and above). We caution, however, that dominant AFLP markers provide no measure of inbreeding, and it remains to establish whether our inferences on mating system are robust to surveys using co-dominant markers. In addition, our data cannot fully disentangle the relative contributions of contemporary vs historical factors (e.g., gene flow/drift vs range fragmentation/colonization dynamics) on the species' current genetic structure. Remarkably, however, in each island, we did not detect a pattern of isolation-by-distance (IBD). When combined with the high overall values of population subdivision (see above), and the findings based on pairwise Φ_{PT} comparisons (Table S1), these data suggest that the great majority of *B. occultum* populations in Madagascar, and still a considerable number of those in La Réunion, are effectively isolated from each other, and that genetic drift, in general, had a larger historical role in shaping population structure compared to limited gene flow alone (Hutchison and Templeton, 1999). Hence, as further discussed below, certain historical events accompanied by drift, must have resulted in non-equilibrium situations that are still detectable in the current genetic structure of this tropical orchid.

Effects of recent habitat fragmentation and disturbance

Barring undetected levels of inbreeding (see above), the consistently low within-population genetic diversity observed in *B. occultum* could result from recent human-mediated deforestation and increased risks of habitat disturbance. The underlying rationale is that decreasing population size in fragmented or otherwise impacted populations may result in genetic erosion through the loss of alleles by drift, especially rare ones (Young *et al.*, 1996; Erikson and Ehrlén, 2001; Vandepitte *et al.*, 2012). However, we did not find a significantly negative effect of recent (1950–2005) deforestation (*LPD*) on allelic rarity (*DW*) in Madagascar, nor was such effect registered by other measures of genetic diversity despite nearly all sites were affected by deforestation to considerable extent (Table 1). Likewise, we did not find an effect of habitat transformation (measured as categorical variable) on any

genetic diversity parameter in La Réunion. Comparable to this study, no significant differences in genetic diversity values were recently found between codified ‘fragmented’ and ‘non-fragmented’ populations of *Dalbergia monticola* (Fabaceae), an endangered insect-pollinated rosewood tree of the eastern rainforest of Madagascar, using nuclear and chloroplast microsatellites (Andrianoelina *et al.*, 2009). It was suggested that habitat fragmentation has occurred too recently (< 100 years) in this tree species relative to the number of generations passed (about two) so that the effects of genetic drift have not become apparent yet (see also Young *et al.*, 1993; Victory *et al.*, 2006; Kramer *et al.*, 2008). A similar explanation, however, appears less likely for *B. occultum*, given its probably short generation time (see Introduction). Rather, we suspect that the species’ capacity for auto-pollination has buffered genetic responses to such recent fragmentation, as reported for other mainly selfing species (Honnay and Jacquemyn, 2007). Hence, based on the present AFLP data, it appears that recent (anthropogenic) habitat fragmentation and degradation had no effects on the genetic diversity characteristics of *B. occultum*, even though this requires further testing using both an extended population sample and additional genetic markers.

Inferences of population history in Madagascar

Most relevant to inferring the historical population dynamics of *B. occultum* are the spatial patterns of AFLP diversity revealed by the *K*-means clustering and BARRIER analyses, which identified remarkably high numbers of gene pools (*A–F*) and genetic boundaries (*1–7*) across the species’ range (Figure 2). Considering Madagascar first, BARRIER detected five zones of abrupt genetic change across the eastern rainforest corridor, but none of those breaks seems to coincide with apparent landscape features that may act as physical barriers to gene flow (i.e., certain mountain ranges, river systems, etc.). In addition, there was little spatial component to the genetic structure of Madagascan populations when they were grouped according to ‘Centres of Endemism’ (COEs; $\Phi_{CT} = 0.073$) (Table 3), i.e., hypothesized Quaternary glacial refuge locations of endemic vertebrate taxa (Wilmé *et al.*, 2006). This weak concordance may simply reflect that plant and animal species varied in their patterns of distribution over the last glacial cycles, but could also mean that the COEs are too broadly defined in space relative to the more fine-scaled genetic structure identified here. Nonetheless, our data seem to support the hypothesis that Madagascan *B. occultum* persisted across multiple isolated regions along the eastern rainforest throughout recent climatic cycles, leading to genetic divergence among populations from the Northeast, North-Centre, South, and Far South (Figure 2). This scenario of long-term population isolation (or ‘insularization’) is also in agreement with the lack of IBD at high Φ_{PT} (see above) and the broadly equivalent levels of diversity observed among these populations (Table 2). For example, in case of post-glacial (re-)colonization of the eastern corridor from a single refuge area, such as the one proposed recently on the basis of palaeo-climatic data for palm trees in the Northeast of Madagascar (Rakotoarinivo *et al.*, 2013), we would have expected a latitudinal cline in diversity in the direction of spread (e.g., Hewitt, 2004), but this is not observed (e.g., regression of H_E on latitude: $R^2 = 0.0104$, $P = 0.741$). Even though the primary vicariant factors initiating these multiple genetic boundaries in Madagascan *B. occultum* remain unclear, it is feasible that they relate to rainforest habitat contraction and isolation events caused by aridification of coastal/lowland sites during glacial maxima (Wilmé *et al.*, 2006; Vences *et al.*, 2009; Rakotoarinivo *et al.*, 2013). This scenario is perhaps

best exemplified by the genetic distinctiveness of a coastal population (M3) from the Northeast (Figure 2). However, in contrast to the ‘watershed’ hypothesis (Wilmé *et al.*, 2006), the present data predict that during glacial maxima, when the climate conditions were probably cooler and drier in Madagascar (Burney *et al.*, 2004), rainforest species tolerant to water stress, such as epiphytic bulbous orchids (Hermans *et al.*, 2007; Silvera *et al.*, 2009), were able to persist not only in coastal/lowland areas associated with riverine habitats of locally more mesic conditions but also at higher elevation/montane sites along the island’s eastern escarpment.

Colonization history and population differentiation in La Réunion

Hierarchical AMOVA (Table 3) suggested moderately strong differentiation between *B. occultum* populations from Madagascar and La Réunion ($\Phi_{CT} = 0.153$), which were also separated by BARRIER (Figure 2). Although the *K*-means analysis indicated that four out of the six Madagascar gene pools (*A–F*) were also present in La Réunion (excepting *B* and *D*), most individuals there were assigned to gene pools *E* and *F*, which otherwise were extremely rare in Madagascar (Figure 2). Finally, the PCoA (Figure 3) highlighted a gradual pattern of inter-island population divergence, while the NJ analysis (Figure 4) identified all La Réunion populations as a weakly supported group, nested among Madagascar conspecifics. Together, these partly conflicting results are probably best explained by a scenario where La Réunion populations were founded by a single or few independent introductions from Madagascar. However, in case of multiple independent colonizations from genetically different source populations, and/or hybridization with other *Bulbophyllum* species in La Réunion (*ca* 10; Sieder *et al.*, 2007), we would have expected to see increased levels of genetic diversity in this latter island (e.g., Dlugosch and Parker, 2008; Gaudeul *et al.*, 2011), but for which we found no evidence (i.e., inter-island comparisons in all diversity measures were non-significant). On the other hand, this latter evidence makes it unlikely that populations in La Réunion arose from a relatively recent, single population introduction, given that founder events almost always generate genetic bottlenecks, at least in the short term (e.g., Frankham, 1997; Dlugosch and Parker, 2008). This leaves us with the alternatives of (i) a relatively ancient single introduction; or, (ii) a few independent introductions from genetically similar source populations, whereby, in either instance, mutation and gene flow restored diversity to similar levels as that found in Madagascar. The present data do not allow further distinguishing among these hypotheses. Moreover, we are presently unable to assign any potential source population(s) in Madagascar, neither can we date such putative colonization event(s). These issues will likely be solved only through increased sampling and molecular dating approaches based on DNA sequence data.

Also difficult to interpret is the remarkably high genetic differentiation observed among populations from La Réunion ($\Phi_{PT} = 0.359$; Table 3). According to the *K*-means, BARRIER, and NJ analyses (Figures 2 and 4), this subdivision is largely due to the segregation of two predominant gene pools (*E*, *F*), resulting in strong genetic differentiation between certain southern populations (R6–8) and the remainder. In fact, the relevant barriers (*1* and *3*) are suggestive of a very localized, essentially sympatric transition (‘hybrid’) zone transecting one of the least disturbed areas of La Réunion, the Mare Longue Forest-Nature Reserve (Florens *et al.*, 2012). In lieu of evidence for strong founder effects (i.e., reduced genetic

diversity), these patterns may be explained by two complementary processes: (i) high selfing rates that increased population genetic structure viz. the variance among gene pools (i.e., through reductions of inter-population gene flow via pollen; Wright *et al.*, 2013) when the species colonized the island (Whitlock and McCauley, 1990; Giles and Goudet, 1997; García-Verdugo *et al.*, 2010; Horn *et al.*, 2014); and (ii) sufficient time for genetic drift to generate such differentiation since population establishment (see also Gaudeul *et al.*, 2011). Basically the same processes might be responsible for the species' phenotypic variation in flower colour (see below).

Genetic differentiation among flower colour morphs in La Réunion

In general, a flower colour transition as observed in *B. occultum* from La Réunion, i.e., from red to yellow (or white), is common to many animal-pollinated angiosperm taxa, including orchids, and most often involves the loss of floral anthocyanins (Wessinger and Rausher, 2012). In fact, shifts to pale flower colours seem to be common in oceanic islands (Carlquist, 1974; Barrett *et al.*, 1996; Juillet *et al.*, 2010), and this phenomenon has so far been observed also in other species of *Bulbophyllum* from La Réunion (i.e., *B. clavatum*, *B. densum*, *B. incurvum*, *B. variegatum*; Cadet, 1989; T Pailler, personal observation) and Madagascar (e.g., *B. erectum*; A Gamisch, personal communication), but has never been studied in detail. In *B. occultum*, the relatively high frequency of yellow-flowered variants (*ca* 47%) observed in La Réunion (Table 1; Figure 5B) makes it unlikely that they have been produced by repeated mutations alone (Dormont *et al.*, 2010). Other mechanisms to explain this dimorphism include shifting selection regimes (e.g., in response to new pollinator environments), negative frequency dependent selection (NFDS), or genetic drift (Gogord *et al.*, 2001; Ackerman and Carronero, 2005; Jersáková *et al.*, 2006; Delle-Vedove *et al.*, 2011). Although there is a growing body of investigations directly demonstrating the operation of natural selection on flower colour variation (Wessinger and Rausher, 2012), we may render any form of pollinator-mediated selection (including NFDS) less likely in what appears to be a mainly selfing, pollinator-independent orchid species. Also, it has been suggested that NFDS may not be commonly operational in tropical orchids, given their usually small and scattered distributions (Ackerman and Carronero, 2005). Rather, based on the previous section, we favour the hypothesis that flower colour divergence in *B. occultum* is maintained by genetic drift or linkage to pleiotropic traits experiencing selection (Wessinger and Rausher, 2012). Intriguingly, our AFLP data provided some first evidence for some genetic differentiation among these red vs yellow morphs (Figure 5C) and their significant association with certain gene pools, especially *F* and *E*, respectively. Overall, these results raise the possibility that reproductive isolation factors have accumulated among these sympatric colour morphs, perhaps (in)directly promoted by selfing (Wright *et al.*, 2013) and/or pleiotropic fitness traits associated with morph colour (e.g., seed viability; Jersáková *et al.*, 2006). But evidently, much further field, experimental, and genetic work is required to test these hypotheses.

Conclusions

Our AFLP analyses of *B. occultum* showed that, contrary to what has frequently been observed in other tropical orchids (Phillips *et al.*, 2012), this species has limited gene flow

abilities in both Madagascar and La Réunion. Further studies using nuclear and chloroplast markers are required (and currently underway) to infer the relative contributions of gene flow by seed vs pollen in the population genetic structure of this species, along with estimates of inbreeding based on nuclear microsatellite markers. Despite these and other limitations (see above), our results lend support to the hypothesis that two major historical, drift-mediated processes had the greatest effect on spatial patterns of genetic diversity in this study system. First, in Madagascar, we found evidence to support the notion that *B. occultum* not only exists as largely isolated populations under current climatic conditions but also persisted across multiple isolated ('refugial') regions along the eastern rainforest corridor over recent climatic cycles. And second, our data suggest that populations of *B. occultum* in La Réunion arose from either single or few independent introductions from Madagascar. Rather than recent founder effects, high selfing rates and sufficient time for genetic drift to operate are likely synergistic factors explaining the unexpectedly high population genetic and phenotypic (flower colour) differentiation observed in La Réunion. These populations should represent promising candidates to gain further insights into the mechanisms of (incipient) sympatric speciation in small oceanic islands (e.g., Papadopulos *et al.*, 2011; García-Verdugo and Fay, 2014; Mallet *et al.*, 2014). Overall, our study shows that the genetic structure of a low-gene-dispersing epiphytic orchid from the Madagascan region bears a strong imprint of history, tremendous human impact on its rainforest habitat notwithstanding.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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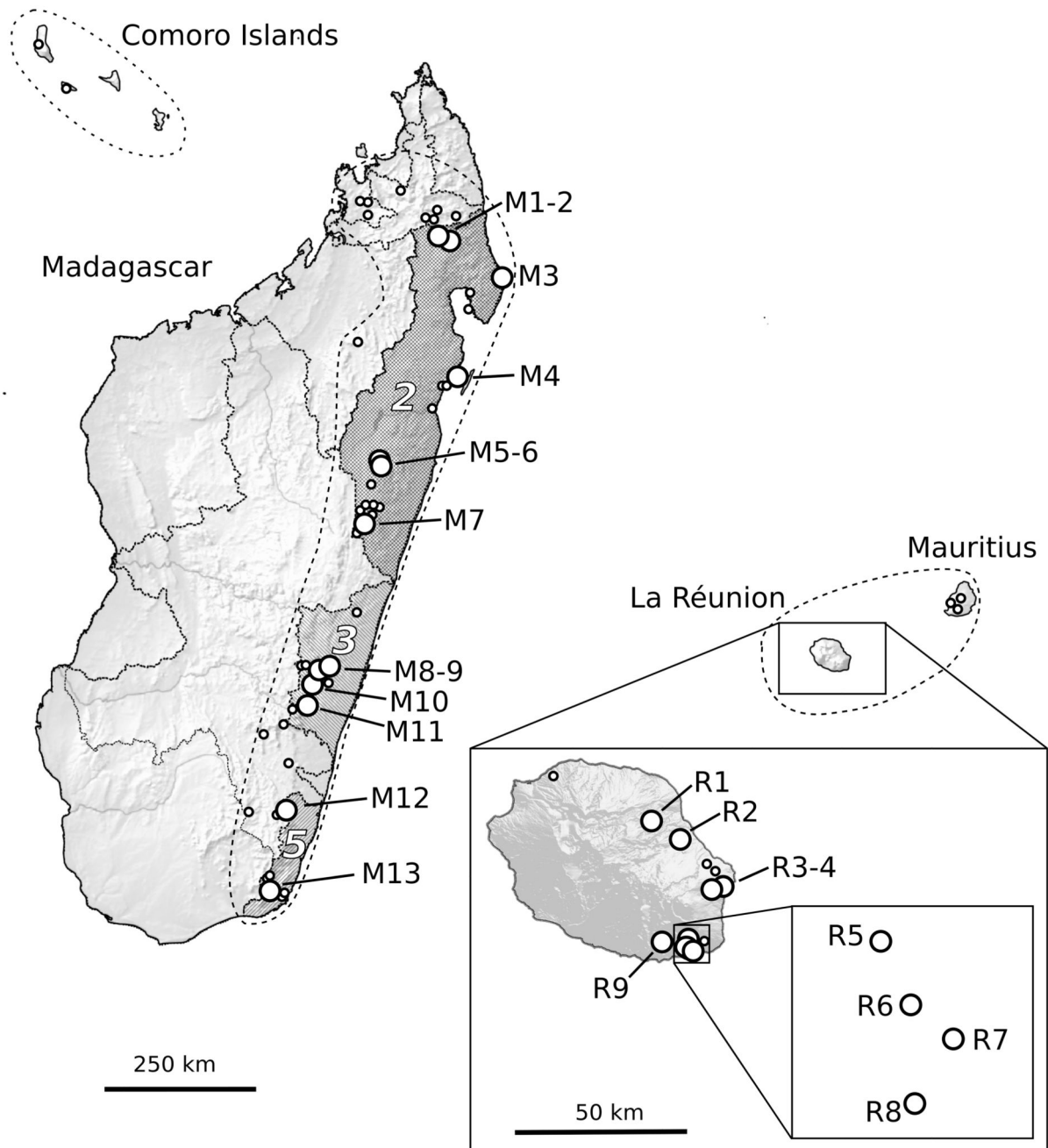


Figure 1.

Geographical distribution of *B. occultum* in Madagascar, the Mascarenes (La Réunion, Mauritius), and the Comoro Islands (stippled lines) based on the 22 sample locations of this study (large circles) plus 78 geo-referenced herbarium records and own field data (small circles). The dotted lines in Madagascar indicate 12 'Centres of Endemism' (COEs *sensu* Wilmé *et al.*, 2006), out of which three (dark shaded areas coded 2, 3 and 5) include sample localities for *B. occultum* (see Table 1 for identification of population codes).

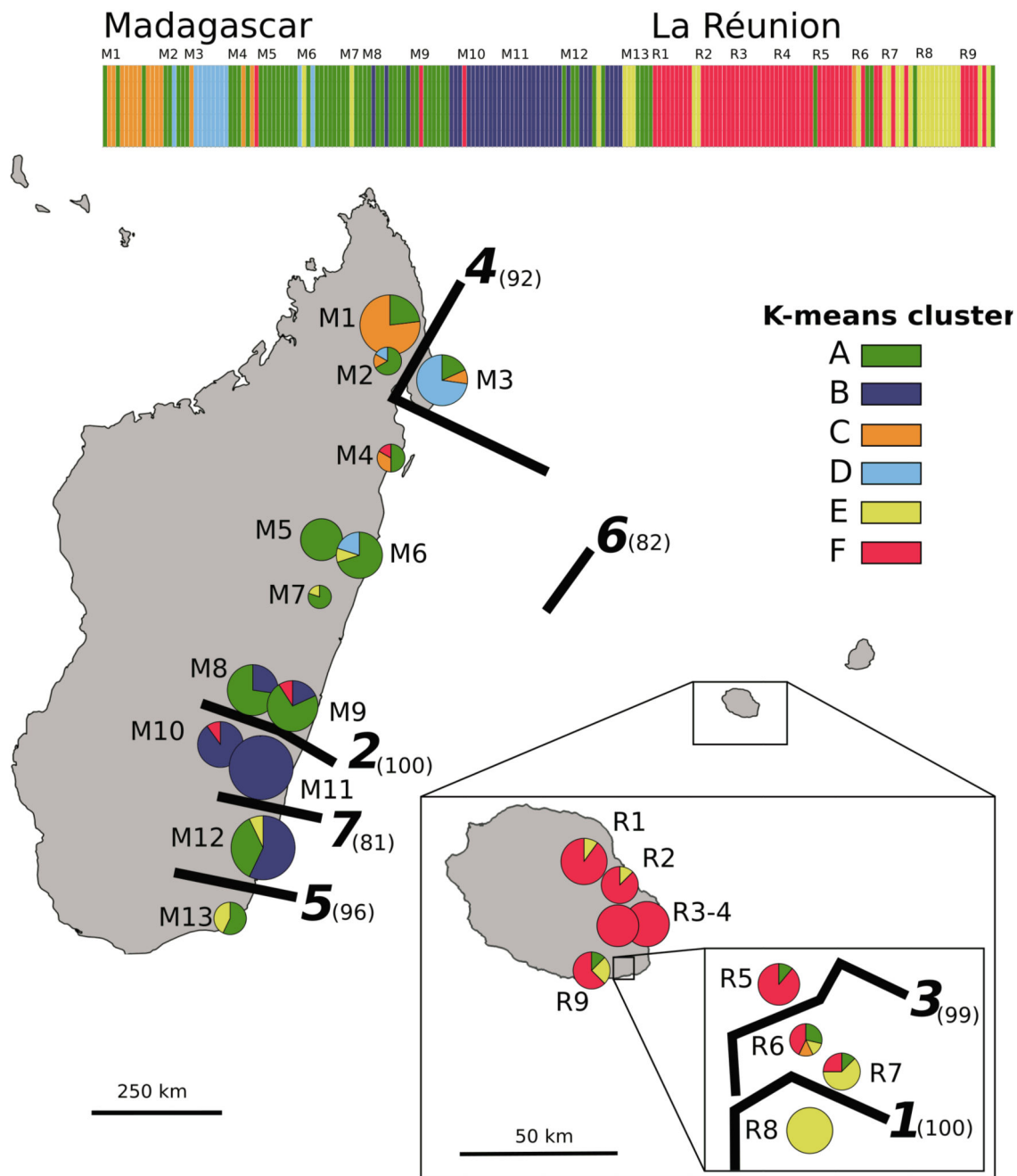


Figure 2. Distribution of six AFLP gene pool clusters (A–F) within and among 22 populations (206 individuals) of *B. occultum* from Madagascar (M1–13) and La Réunion (R1–9) as identified by *K*-means clustering, and with pie charts representing the percentage of individuals. The sizes of the pie charts are proportional to sample size (*n*), with the smallest circle representing *n* = 5 and the largest representing *n* = 14 (see Table 2). The barplot at the top displays the assignment of individuals to the six *K*-means clusters, with the smallest vertical bar representing one individual. Significant regions of genetic discontinuity (black lines)

identified by BARRIER are numbered (1–7) in descending order of relative importance ('priority'), with bootstrap values (> 80%) shown in parentheses (1000 replicates).

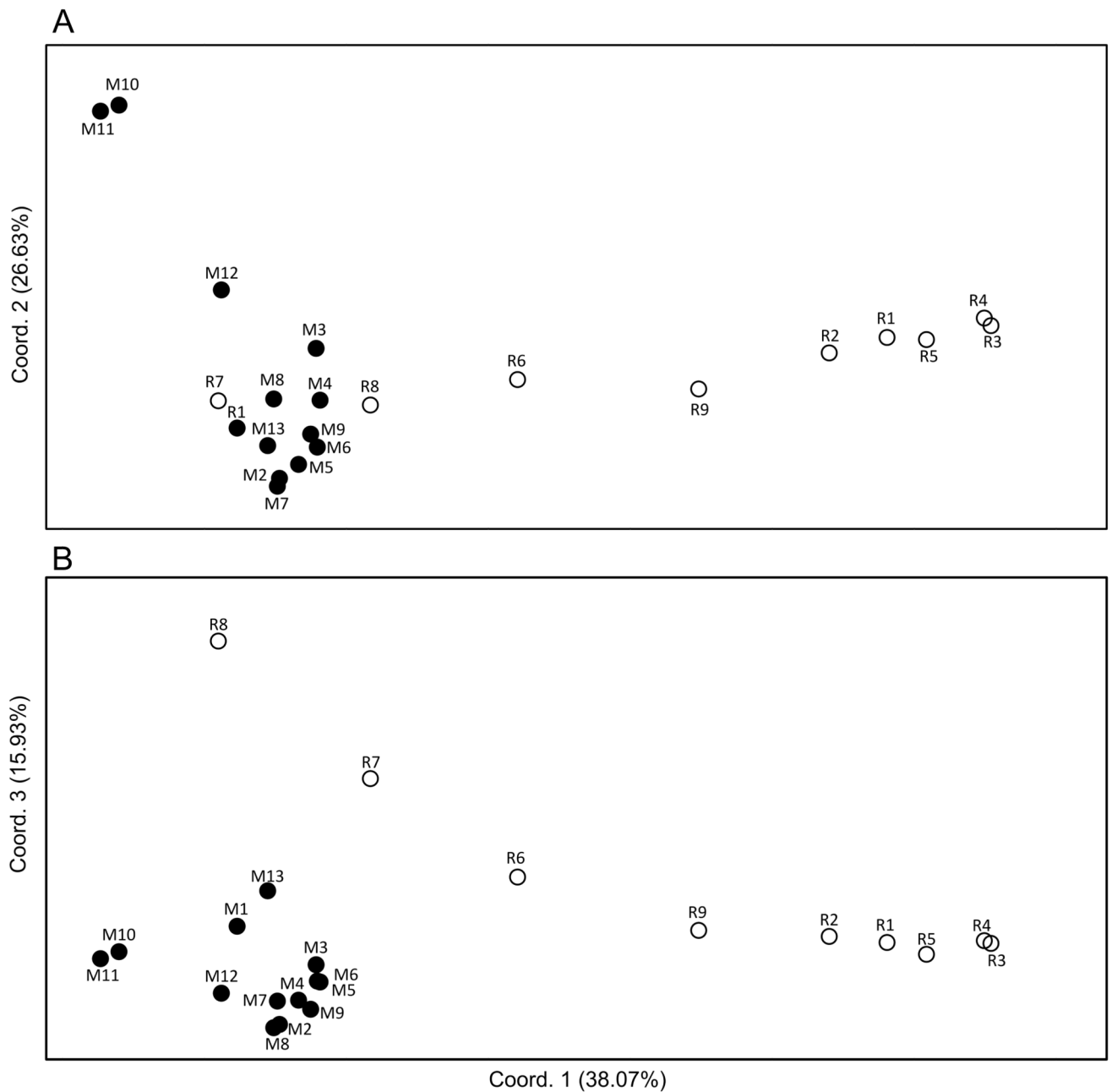


Figure 3.

Plots of (A) the first and second, and (B) the first and third principal coordinates of 22 populations of *B. occultum* from Madagascar (M1–13; filled circles) and La Réunion (R1–9; open circles) based on pairwise F_{ST} values derived from fragment frequencies of 102 AFLP markers using AFLP-SURV. See Table 1 for explanation of population codes.

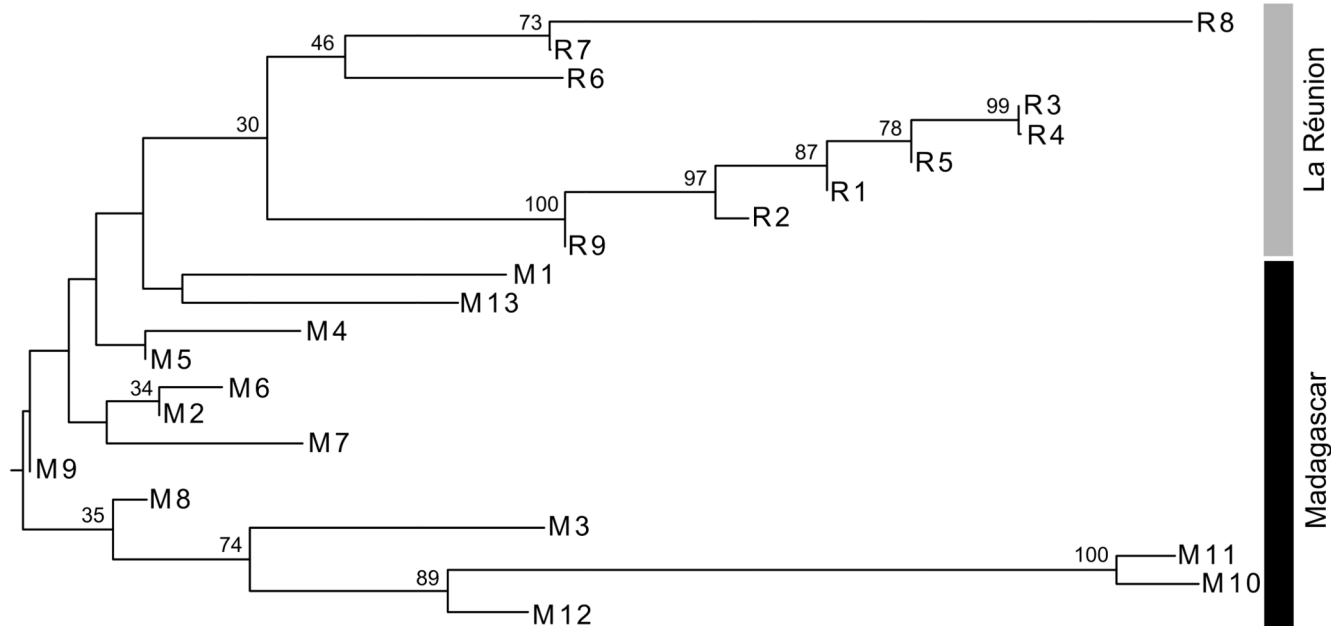


Figure 4. Mid-point rooted neighbor-joining (NJ) phenogram depicting genetic (F_{ST}) distances between 22 *B. occultum* populations from Madagascar (M1–13) and La Réunion (R1–9). Numbers above branches indicate bootstrap values (30%) based on 1000 replicates. See Table 1 for explanation of population codes.

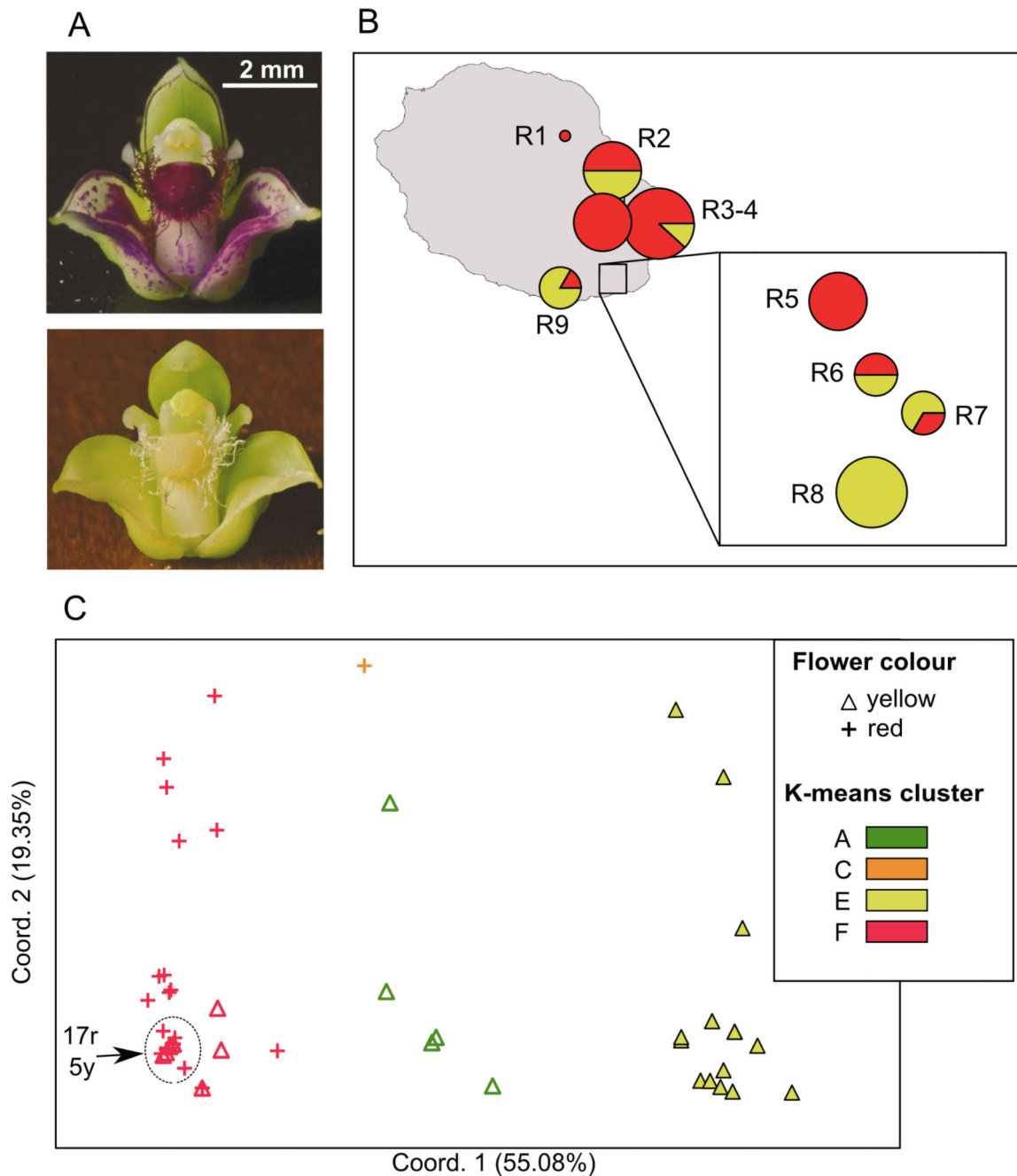


Figure 5.

(A) Red and yellow flower colour morphs of *B. occultum* from La Réunion. Photo credit: Joseph Vaillant (Parc National de La Réunion). (B) Their distribution (denoted by red vs yellow colour) censused across the nine populations (R1–9) surveyed for AFLP variation in La Réunion. The sizes of the pie charts are proportional to sample size (n), with the smallest circle representing $n = 1$ and the largest $n = 10$ (total $n = 61$; see Table 1). (C) Plot of the first and second principal coordinates of 33 red morphs (crosses) and 28 yellow morphs (triangles) from populations R1–9 based on Euclidian distances derived from AFLP data.

Individuals are colour-labelled according to the four AFLP groups detected by K -means clustering (see Figure 2). Numbers refer to densely-clustered individuals with red (r) vs yellow (y) flower colour morphs within the dotted circle.

Table 1
Information on sample locations for 22 populations of *B. occultum* from Madagascar (M1–13) and La Réunion (R1–9) surveyed for AFLP variation

Population code	Locality (COE)	Longitude	Latitude	Altitude (m)	LPD/HT	$N_{R/Y}$
<i>Madagascar</i>						
M1	Andapa 1 (2)	49.6556	-14.710	560	58.8	–
M2	Andapa 2 (2)	49.6556	-14.711	534	50.2	–
M3	Ambodirafiana (2)	50.4582	-15.269	20	77.4	–
M4	Pointe à Larée (2)	49.7447	-16.796	10	70.5	–
M5	Didy 1 (2)	48.5583	-18.133	1160	15.4	–
M6	Didy 2 (2)	48.5569	-18.069	1160	14.9	–
M7	Moramanga (2)	48.3664	-19.075	1004	51.9	–
M8	Ifanadiana 1 (3)	47.7086	-21.338	479	96.1	–
M9	Ifanadiana 2 (3)	47.6192	-21.326	468	97.7	–
M10	Tolongoina (3)	47.5478	-21.599	336	89.9	–
M11	Ikongo (3)	47.4575	-21.852	252	87.7	–
M12	Midongy (5)	47.1289	-23.499	140	83.8	–
M13	Andohahela (5)	46.8597	-24.747	547	18.8	–
<i>La Réunion</i>						
R1	Jardin l'Eden	55.6271	-21.026	616	2	1/0
R2	Ravine Françoise	55.6970	-21.085	290	3	0/4
R3	Bois Blanc 1	55.8116	-21.193	217	2	9/1
R4	Bois Blanc 2	55.8085	-21.193	280	2	8/0
R5	Mare Longue 1	55.7379	-21.351	288	1	8/1
R6	Mare Longue 2	55.7398	-21.356	192	1	4/3
R7	Mare Longue 4	55.7447	-21.359	127	1	2/4
R8	Mare Longue 3	55.7431	-21.364	36	2	0/10
R9	Basse Vallée	55.7071	-21.361	268	1	1/5

Abbreviations: COEs, 'Centres of Endemism' in Madagascar (Wilmé *et al.*, 2006), with code numbers given in parentheses (see Figure 1 and text for details); *LPD*, log-transformed percentages of deforestation for the period 1950–2005 per site in Madagascar; *HT*, levels of habitat transformation per site in La Réunion, with categories (1–3) according to Strasberg *et al.* (2005): 1, relatively untransformed (native canopy still pristine and not invaded); 2, moderately degraded (native biota dominated by alien plants); 3, transformed (urban or cultivated areas, and alien secondary vegetation); $N_{R/Y}$, number of red vs yellow flower-coloured individuals censused per site in La Réunion.

Table 2
Summary of AFLP variation for 22 populations (206 individuals) of *B. occultum* from Madagascar (M1–13) and La Réunion (R1–9)

<i>Population code</i> ^a	<i>N</i>	<i>N_A</i>	<i>PPF</i>	<i>H_E</i>	<i>DW</i> ^b
<i>Madagascar</i>					
M1	13	1.078	36	0.095	0.422
M2	6	0.863	17	0.069	0.434
M3	11	0.951	28	0.090	0.273 ^S
M4	6	0.922	21	0.091	0.466
M5	9	0.912	22	0.076	0.319
M6	10	0.892	18	0.075	0.438
M7	5	0.794	13	0.061	0.539
M8	11	1.000	29	0.100	0.357
M9	11	1.020	30	0.090	0.305 ^S
M10	10	0.863	18	0.045	0.363
M11	14	0.931	26	0.050	0.385
M12	14	1.029	30	0.103	0.396
M13	7	0.863	19	0.079	0.492
Regional total	127	1.56	71	0.115	–
Regional mean	9.8 (3.0)	0.932 (0.081)	24 (6.7)	0.079 (0.031)	0.394 (0.069)
<i>La Réunion</i>					
R1	10	0.941	22	0.055	0.838 ^L
R2	8	0.902	21	0.059	0.425
R3	10	0.765	11	0.026	0.316 ^S
R4	9	0.755	13	0.032	0.315
R5	9	0.824	16	0.039	0.379
R6	7	0.922	26	0.117	0.247 ^S
R7	8	1.010	30	0.124	0.441
R8	10	0.814	13	0.044	0.750 ^L
R9	8	0.863	16	0.070	0.626 ^L
Regional total	79	1.49	61	0.089	–
Regional mean	8.8 (1.1)	0.866 (0.085)	19 (6.5)	0.063 (0.026)	0.482 (0.208)
Species total	206	1.84	86	0.116	–
Species mean	9.4 (2.4)	0.91 (0.087)	22 (6.9)	0.072 (0.028)	0.432 (0.147)

Abbreviations: *N*, number of individuals assayed; *N_A*, observed number of alleles; *PPF*, percentage of polymorphic fragments; *H_E*, Nei's (1987) unbiased gene diversity; *DW*, frequency down-weighted marker values.

^aPopulation codes are identified in Table 1.

^bSignificantly small or large *DW* values are indicated by superscripts 'S' and 'L', respectively.

Note: Standard deviations are given in parentheses.

Table 3
Non-hierarchical and hierarchical AMOVAs for AFLP variation based on several groupings of *B. occultum* populations from Madagascar and La Réunion

Grouping/source of variation	df	Sum of squares	Variance components	Percentage of total variance	Φ -statistic ^c
<i>Total</i>					
Among populations	21	533.183	2.33	38.7	$\Phi_{PT} = 0.387$
Within populations	184	677.71	3.68	61.3	NC
<i>Madagascar</i>					
Among populations	12	290.335	2.05	31.9	$\Phi_{PT} = 0.341$
Within populations	114	447.161	3.96	68.1	NC
<i>La Réunion</i>					
Among populations	8	143.034	1.7	35.9	$\Phi_{PT} = 0.359$
Within populations	70	211.953	3.03	64.1	NC
<i>Madagascar vs La Réunion</i>					
Among regions	1	118.41	1	15.3	$\Phi_{CT} = 0.153$
Among populations	20	414.773	1.83	28.1	$\Phi_{SC} = 0.332$
Within populations	184	677.71	3.68	56.6	$\Phi_{PT} = 0.430$
<i>Eight barriers (total) ^a</i>					
Among regions	7	406.405	2.1	33	$\Phi_{CT} = 0.330$
Among populations	14	126.778	0.6	9.3	$\Phi_{SC} = 0.142$
Within populations	184	677.71	3.68	57.7	$\Phi_{PT} = 0.420$
<i>Five barriers (Madagascar) ^a</i>					
Among regions	4	201.057	2.03	30.6	$\Phi_{CT} = 0.306$
Among populations	8	70.682	0.52	7.8	$\Phi_{SC} = 0.110$
Within populations	114	465.757	4.09	61.6	$\Phi_{PT} = 0.380$
<i>Three COEs (Madagascar) ^b</i>					
Among regions	2	79.277	0.45	7.3	$\Phi_{CT} = 0.073$
Among populations	10	192.462	1.61	26.2	$\Phi_{SC} = 0.281$
Within populations	114	465.757	4.09	66.5	$\Phi_{PT} = 0.340$

Abbreviations: AMOVAs, analyses of molecular variance; df = degrees of freedom; NC = not computed.

^aPopulation grouping based on areas defined by the BARRIER analysis (see Figure 2).

^bPopulation grouping based on three 'Centres of Endemism' (COEs; Wilmé *et al.*, 2006) (see Table 1).

^cAll Φ values were significant ($P < 0.001$) based on 9999 permutations.