Pollination, mating and reproductive fitness in a plant population with bimodal floral-tube length

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Abstract

Mating patterns and natural selection play important roles in determining whether genetic polymorphisms are maintained or lost. Here, we document an atypical population of *Lapeirousia anceps* (Iridaceae) with a bimodal distribution of floral-tube length and investigate the reproductive mechanisms associated with this pattern of variation. Flowers were visited exclusively by the long-proboscid fly *Moegistorhynchus longirostris* (Nemestrinidae), which exhibited a unimodal distribution of proboscis length and displayed a preference for long-tubed phenotypes. Despite being visited by a single pollinator species, allozyme markers revealed significant genetic differentiation between open-pollinated progeny of long- and short-tubed phenotypes suggesting mating barriers between them. We obtained direct evidence for mating barriers between the floral-tube phenotypes through observations of pollinator foraging, controlled hand pollinations and measurements of pollen competition and seed set. Intermediate tube-length phenotypes produced fewer seeds in the field than either long- or short-tubed phenotypes. Although floral-tube length bimodality may not be a stable state over long timescales, reproductive barriers to mating and low 'hybrid' fitness have the potential to contribute to the maintenance of this state in the short term.

Introduction

The diversity of animal-pollinated flowers is often interpreted as an evolutionary outcome of heterogeneity in the pollination environment. When the pollinator composition differs across the geographical range of a plant species, mosaics of contrasting selection often result in morphologically different pollinator ecotypes (Grant & Grant, 1965; Anderson *et al.*, 2014; Boberg *et al.*, 2014; Newman *et al.*, 2014, 2015; Sun *et al.*, 2014). An extension of geographical pollinator mosaics is that if pollinators are rare or absent in some populations, plants may evolve traits that facilitate self-pollination (Barrett *et al.*, 1989; Moeller & Geber, 2005). Populations of the same species of pollinator can also differ geographically in morphology (Steiner & Whitehead, 1991; Anderson & Johnson, 2009; Newman *et al.*, 2014) or behaviour

(Newman *et al.*, 2012), which may be matched by striking patterns of geographical variation in floral traits. For example, coevolutionary races of reciprocal trait escalation have been implicated in driving the allopatric divergence of insect mouthparts and floral-tube length in several species (e.g. Anderson & Johnson, 2008; Pauw *et al.*, 2009; Paudel *et al.*, 2016). Variation in floral-tube length may often be an important target of natural selection, but little is known about the forces maintaining tube-length variation in instances where variation is large.

Recent theoretical work indicates that the interplay between coevolutionary races and assortative mating can generate divergence of plant or pollinator traits within populations, leading to the evolution of trait bimodality (Zhang *et al.*, 2012). Trait bimodality can also occur in sympatry if divergent allopatric populations make secondary contact (Anderson *et al.*, 2010). Of these two possibilities, sympatric origins are usually considered the least plausible because they are complicated by the difficulty in evolving strong gene flow barriers associated with disruptive selection in sympatry

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(reviewed in Coyne & Orr, 2004). Irrespective of the origins of bimodality, there are several potential outcomes of trait bimodality in sympatry: bimodality can escalate, can be maintained or can be lost. Assuming that the traits concerned are quantitatively inherited, most evolutionary theory predicts that the eventual outcome should reflect a balance between the opposing processes of gene flow and selection. In particular, assortative mating resulting from reproductive barriers between dissimilar phenotypes is expected to facilitate linkage disequilibrium between loci and the maintenance of trait variation by preventing the homogenizing effects of gene flow (Maynard Smith, 1966; Kirkpatrick & Ravigne, 2002; Gavrilets, 2004). Disruptive selection is important in driving further trait differentiation in sympatry by selecting for extreme phenotypes and against individuals with intermediate phenotypes (e.g. Mather, 1953; Maynard Smith, 1962; Rueffler *et al.*, 2006). Most models (e.g. Slatkin, 1981; Bolnick & Fitzpatrick, 2007) predict that trait bimodality can only be maintained in the face of gene flow under conditions of strong disruptive selection. Despite the plethora of theory on the balance between selection and gene flow in governing trait evolution, there have been few empirical studies (but see Linn *et al.*, 2003; Bolnick, 2004; Hendry *et al.*, 2009; Martin & Pfennig, 2009; Snowberg & Bolnick, 2008; Heystek *et al.*, 2014) on the mechanisms responsible for trait bimodality, particularly the forms of selection acting on the variation, and the occurrence of reproductive barriers that could potentially cause assortative mating.

Recent studies have revealed bimodality in the floral-tube length of flowers pollinated by insects with long proboscides, a pattern which appears to have arisen on multiple occasions in unrelated species (Anderson *et al.*, 2010; Ros *et al.*, 2011; Zhang *et al.*, 2012). In some cases, trait bimodality takes the form of very sharp clines suggesting recent contact zones between differentiated phenotypes (Anderson *et al.*, 2010), whereas in other cases this pattern of variation appears to have originated in sympatry by the spread of advantageous phenotypes (Ros *et al.*, 2011). Studies of floral-tube length bimodality in *Gladiolus longicollis* reported strong gene flow barriers resulting from flowering time differences between the floral tube-length phenotypes in some populations, but only weak barriers in others. Selection on floral-tube length in *G. longicollis* was seldom disruptive, and when it was, disruptive selection was relatively weak (Anderson *et al.*, 2010; Rymer *et al.*, 2010). Despite increased interest in the ecology and evolution of variation in floral-tube length, little is known about the reproductive consequences of trait bimodality in populations visited by a single species of pollinator, the main focus of our study.

We investigate pollination, mating and reproductive fitness in a population of *Lapeirousia anceps* (L.f.) (Iridaceae) with a bimodal distribution of floral-tube length. *Lapeirousia anceps* has pale, scentless flowers adapted for pollination by long-proboscid flies, the only recorded visitors to the species (Manning & Goldblatt, 1997). Three species (one Nemestrinidae and two Tabanidae) of allopatrically distributed long-proboscid flies pollinate *L. anceps,* and floral-tube length in populations closely matches the proboscis length of their local pollinators (Pauw *et al.*, 2009). Of these pollinators, *Moegistorhynchus longirostris* (Nemestrinidae) has the longest proboscis. Trait matching and measurements of reciprocal selection between *M. longirostris* proboscis length and *L. anceps* tube length suggest that these traits have reached extreme values as a result of a geographically structured coevolutionary race (Pauw *et al.*, 2009). This process has resulted in two-fold (43–86 mm), continuous variation in floral-tube length and fly proboscis length among populations. Furthermore, in populations pollinated by Tabanid flies, which have much shorter proboscides, floral-tube lengths of ~28 mm occur, suggesting that floral-tube length diversification also evolves through shifts between different pollinators, as generally emphasized for pollinator-driven floral diversification by Grant & Grant (1965) and Stebbins (1970). *Lapeirousia anceps* floral-tube length and *M. longirostris* proboscis length are quantitative traits, and in most populations, these traits are normally distributed (Pauw *et al.*, 2009). However, in October 2003 we discovered an unusually variable population of *L. anceps* near Mamre, Western Cape, South Africa, that exhibited a bimodal distribution of floral-tube length (and see Pauw *et al.*, 2009; Fig. 3; and Zhang *et al.*, 2012; Fig. 1). The occurrence of this population motivated the present study in an effort to understand the consequences of bimodality for the reproductive biology of the population and the mechanisms that may maintain it.

We used field experiments, glasshouse studies and marker-gene analysis to investigate the consequences of floral-tube length variation for pollination, mating and reproductive fitness. We first quantified the pattern of floral-tube length variation in the Mamre population to establish whether the trait was indeed bimodal and then addressed the following specific questions: (i) Does the distribution of floral-tube length match the distribution of pollinator proboscis length in the population? (ii) Is there genetic differentiation between the floral tube-length phenotypes, consistent with mating barriers? (iii) Could mating barriers be the result of pollinator preference for either of the two primary floral-tube phenotypes (long and short), and if so why does this occur? (iv) Is there evidence for post-pollination reproductive barriers between the floral tube-length phenotypes that might favour assortative mating? (v) How does reproductive fitness vary with floral-tube length, and specifically is there evidence for disruptive selection?
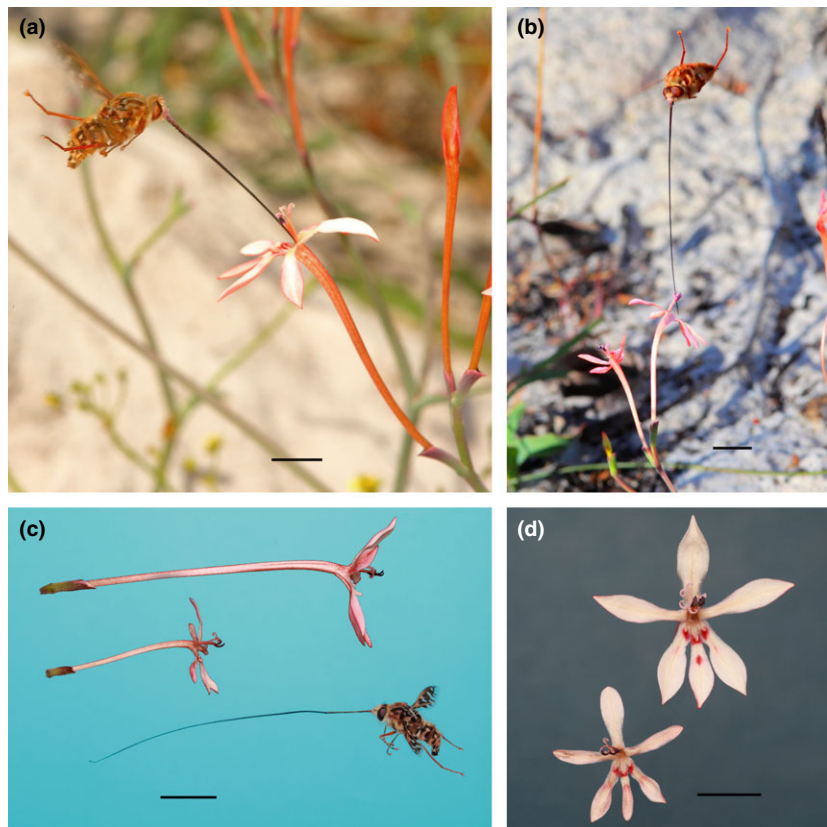
Fig. 1 Long- and short-tubed floral phenotypes of *Lapeirousia anceps* from the study population, with the sole pollinator *Moegistorhynchus longirostris* at the site. (a) long-tubed flower, (b) short-tubed flower. (c) Comparison of the floral tubes of long- and short-tubed phenotypes with the proboscis of *M. longirostris*. (d) Frontal view of long-tubed (right) and short-tubed (left) flowers. Scale bar = 1 cm.

Materials and methods

Study area

We investigated a *L. anceps* population located within sand plain fynbos vegetation near Mamre, Western Cape, South Africa (33°31′S, 18°28′E), measuring ~200 × 300 m in extent. Our studies were conducted during the flowering time of the species, from 2003 to 2004 during October–November. Zhang *et al.* (2012) found that the population appeared to be primarily composed of two floral tube-length phenotypes with modes corresponding to ~28 and 54 mm and a paucity of individuals with floral tubes ranging between 32 and 42 mm in length. Consequently we refer to floral tubes of 32–42 mm as intermediate in length, and floral tubes on either side of this range as short and long floral tubes. Bimodality of floral-tube length in *L. anceps* is not associated with polyploidy or genome size variation based on flow cytometric analysis (J. Suda, unpublished). Further information on the natural history and floral biology of *L. anceps* is available in Pauw *et al.* (2009).

Relation between floral-tube length in parents and offspring

We compared the floral-tube length of parents and their offspring to determine whether there is a heritable genetic component to variation in floral-tube length. We measured floral tubes using digital callipers on two flowers from 61 randomly selected plants in the population. Open-pollinated seed families from these plants were subsequently grown to flowering in 2005 under uniform glasshouse conditions at the University of Toronto. We measured two flowers per plant from 1 to 6 progeny per family and compared family means with parental values. A correlation between values would be indicative of a heritable component to variation in floral-tube length.

Floral-tube and pollinator proboscis length distribution

We measured floral-tube length on two flowers from 72 randomly chosen plants, each of which was separated by more than two metres. Floral-tube length was measured from the top of the ovary to the point at which the dorsal sepal splits from the adjacent sepals. During 270 h of fieldwork at the site, we captured a sample of 41 *M. longirostris* visiting flowers of *L. anceps*. We measured the length of the extended proboscis of each individual following methods in Pauw *et al.* (2009). We investigated whether the distribution of floral-tube length and pollinator proboscis length was unimodal or multimodal using the dip test of unimodality (Hartigan & Hartigan, 1985) under the null hypothesis that the distributions were unimodal. This analysis was

conducted using the statistical software R, version 2.9.1 (R Core Team, 2013).

Genetic differentiation of floral-tube phenotypes

To investigate whether the long- and short-tubed phenotypes in the population were genetically differentiated, we sampled open-pollinated progenies from the two phenotypes. Starch gel electrophoresis was used to identify four polymorphic allozyme loci. For complete methods, see Data S1.

We used HICKORY (version 1.1, Holsinger & Lewis, 2003) to estimate inbreeding (*f*) and measures of genetic variance between the floral-tube phenotypes. In this program, theta-I ($\theta^{(I)}$) is analogous to Wright's F_{st} and theta-II ($\theta^{(II)}$) to Weir and Cockerham's calculation, where values of one suggest complete barriers to gene flow. Comparisons between the full model, a model with inbreeding set to zero, and a model with genetic differentiation set to zero, allowed us to test for the significance of these parameters. For comparison, we also estimated Nei's genetic distance (GENDIST: PHYLIP Ver. 3.6, Felsenstein, 1993) based on polymorphism at the four loci.

Pollinator preferences and correlates of floral-tube variation

To investigate whether pollinators preferred long- versus short-tubed flowers, we used a modified version of the 'pollinator interview technique' (Thomson, 1988). We offered pollinators a choice between a long- or a short-tubed flower placed approximately 1 cm apart (*n* = 28 choices) on the end of a two-metre-long stick and recorded the pollinator's first choice and the total number of choices for each phenotype. Floral pairs were changed after every choice test, and the position (left vs. right) of long- and short-tubed flowers was swapped. We analysed deviation from the expected 1:1 visitation rate using a *G*-test (Sokal & Rohlf, 1995).

Next, we determined whether observed choice preferences in the previous experiment translated into non-random foraging in a natural population. We demarcated a 30 × 20 m plot containing a dense mixture of the two tube-length phenotypes (1001 long-tubed plants, 1633 short-tubed plants and no plants with intermediate tube length). Based on a sample of 24 individuals, long-tubed plants had a mean ± SD flower number of 3.04 ± 1.85, whereas short-tubed plants had a mean of 3.26 ± 2.28 flowers per plant (*n* = 27). From this, we calculated that there were approximately 5324 short-tubed flowers and 3043 long-tubed flowers in the plot. We waited for *M. longirostris* pollinators to visit the plot and recorded the floral-tube phenotype of each flower visited, as well as whether transitions were within or between phenotypes of flowers and plants. Observations were made over the course of a single day between 8 h00 and 13 h30 in October

2007. We tested whether pollinators made transitions between flowers and plants of the same phenotype (assortative foraging) more frequently than predicted by their relative abundance. Eighteen flies were observed in this manner (a total of 458 visits), and the proportion of transitions between and within each phenotype was recorded for each fly so that each was a replicate. We compared the expected transition rates between and within phenotype combinations to the observed transition rates of the flies using a one sample Wilcoxon test (data were not normally distributed) in SPSS Statistics (IBM Corp, 2012). The expected probability that a fly would move from a long-tubed flower to another long-tubed flower was calculated as the total number of long-tubed flowers in the plot minus one (i.e. the long-tubed flower that the fly was visiting), divided by the total numbers of flowers in the plot minus one. The probability that a fly moves from a long-tubed flower to a short-tubed flower would be one minus the number obtained above. Similarly, we were able to calculate expected rates of transitions from short-tubed flowers, as well as between plants.

To determine the possible reasons for any observed pollinator preferences, we investigated whether reward size was correlated with floral-tube length. Early in the morning before pollinators were active, we measured nectar volume and floral-tube length from 24 flowers using graduated 5-μL capillary tubes, and nectar concentration using a Bellingham and Stanley 0–50% refractometer. Total sugar was calculated following methods outlined by Bolton *et al.* (1979). We also determined whether flower size was correlated with floral-tube length by measuring the length of the dorsal sepal and floral-tube length of 74 flowers representing the range of floral-tube lengths found within the population.

Lastly, we investigated whether *L. anceps* was capable of setting seed in the absence of pollinators (autonomous self-pollination) and also whether differences in autonomous self-pollination were associated with differences in floral-tube length. At the Mamre site, we excluded pollinators from 47 plants with netting suspended over a wire frame. Plants were selected so that their floral-tube lengths spanned the entire range of variation for this trait. The tube lengths of three randomly selected flowers per plant (that matured under exclusion netting) were measured and their positions marked using sticky tape with each flower given a unique number. We harvested capsules just prior to dehiscence and seed set was recorded. We examined the relation between average seed set per capsule and floral-tube length among our sample of plants using correlation analysis in SPSS (IBM Corp, 2012).

Post-pollination reproductive barriers

We performed controlled hand pollinations under field and glasshouse conditions to investigate whether

reduced fertility occurs in crosses between the floral-tube phenotypes, relative to crosses within floral-tube phenotypes. In the field, we bagged whole plants of the long- and short-tubed phenotypes, as well as plants with intermediate floral-tube lengths. Nine buds per plant were emasculated to prevent autonomous self-pollination. When stigmas were fully expanded, we cross-pollinated each flower with pollen from either a long-tubed, intermediate or short-tubed phenotype (three flowers per treatment). Plants were then re-bagged, and we harvested capsules and counted seeds a month later. We analysed the normally distributed seed set data using a general linear model in which ovule and pollen parent were fixed factors and seed set per flower was the dependent variable. The model also included the interaction between ovule parent and pollen parent, with plant as a random factor. For this analysis, we used a single average seed set value per plant by averaging across the three capsules of each treatment sampled on each plant. The test was performed in SPSS Statistics (IBM Corp, 2012). To confirm the results of the field study, we repeated crosses under uniform glasshouse conditions, but using only the long- and short-tubed phenotypes. In the glasshouse study, only a single flower per plant was used for each pollination treatment. Sample sizes (numbers of plants used per treatment) for both crossing studies are presented in Fig. 5.

To determine whether pollen competition plays a role in reducing mating between long- and short-tubed phenotypes, we conducted a controlled pollination study under glasshouse conditions using allozyme markers and pollen mixtures from the two floral-tube phenotypes. Polymorphism at the *AAT-2* locus enabled us to detect the siring success in mixed pollen loads because the two floral-tube phenotypes were fixed for alternate alleles (long-tubed – FF, short-tubed – SS). We emasculated flowers one day before anthesis to prevent self-pollination. We used fine forceps to pollinate each of the three stigma lobes per flower using pollen mixed from three anthers per floral-tube phenotype. We were not able to ensure that there were equal numbers of pollen grains from each tube-length phenotype in the mixture. If unequal numbers of pollen grains have an important effect on siring success, we would expect one phenotype to consistently outperform the other, irrespective of which tube-length phenotype the pollen was applied to. We alternated which floral-tube phenotype was applied first to each lobe with the second donor applied immediately after the first. This order was also alternated between flowers. When capsules were mature, we collected and assayed seeds (total of 284 seeds from 26 capsules on 11 long-tubed plants and 170 seeds from 18 capsules on 8 short-tubed plants) to determine the floral-tube phenotype that sired each seed using the genotype identity at the *AAT-2* locus.

We analysed differences in siring success using a generalized linear model (SAS GLIMMIX Procedure: SAS 2011) and treated assortative (intraphenotype) vs. disassortative (interphenotype) mating as a binary response with data log odds transformed for the analysis. Seeds sampled from the same fruit were treated as a repeated measure and maternal floral-tube phenotype as a fixed effect. The back-transformed means and 95% confidence intervals are reported along with a test of significant difference between the levels of assortative mating of the two floral-tube phenotypes. If no post-pollination bias exists, we would expect equal siring success between the competing pollen donors (50%, 1:1 odds or the natural logarithm of the odds = 0.1391).

Estimating reproductive isolation

Using methods outlined by Ramsey *et al.* (2003), we calculated the separate and combined contributions to reproductive isolation by pollinator foraging patterns and the assortative siring ability of different floral tube-length phenotypes (See Data S2). These indices for reproductive isolation range from zero to one, where zero indicates no isolation and one indicates complete reproductive isolation.

Reproductive success and floral-tube length

We measured phenotypic selection on variation in floral-tube length in October 2003 by tagging 110 flowers (one flower per plant) on which we measured floral-tube length and seed set. Plants were selected so that their flowers spanned the range of floral-tube lengths as evenly as possible. Once fruits were mature, we collected the capsules and counted the number of seeds in each capsule. We used the seed set of a single flower per plant in our selection analysis as this fitness component provides a direct measure of the effectiveness of pollination. Although total seed production per plant would be a more accurate estimate of total fitness, we chose not to measure this fitness component because of the large number of flowers per plant and the difficultly in disentangling the relative importance of pollination effectiveness and resource availability in affecting variation in total seed production. Using the methods outlined in Lande & Arnold (1983) and Alexandersson & Johnson (2002), we used linear and quadratic regression to detect selection on floral-tube length (predictor variable) with seed set as the response variable. We calculated relative fitness by dividing an individual's seed set by the population average and standardized trait values by subtracting population mean values from individual values and dividing by the standard deviation. Selection coefficients for quadratic functions were doubled following Stinchcombe *et al.* (2008).

Results

Relation between floral-tube length in parents and offspring

There was a strong positive correlation ($b = 1.06$, $R^2 = 0.93$, $F = 28.42$, $P < 0.001$, $n = 61$ parent–progeny pairs) between the floral-tube length of maternal parents measured under field conditions and the floral-tube length of their glasshouse-grown progeny (Fig. S1).

Floral-tube and pollinator proboscis length distribution

The long-proboscid fly *M. longirostris* was frequently observed visiting flowers of both long- and short-tubed plants in the population (Fig. 1a,b). No other floral visitors were observed. The proboscis length of the fly had a unimodal distribution (dip test statistic = 0.046, $P > 0.05$, $n = 46$) and was normally distributed with a mean of $52.38 \pm$ SD 4.33 mm (Fig. 2). Floral-tube length was multimodal (dip test statistic = 0.094, $P < 0.001$) with two distinct peaks corresponding to long- and short-tubed phenotypes ($49.54 \pm$ SD 5.04 mm and $27.08 \pm$ SD 1.92, respectively; Figs 1c and 2). No plants with floral-tube lengths between 34 and 36 mm were evident in the sample (Fig. 2).

Genetic differentiation between floral-tube phenotypes

Analysis of allele frequencies in the population revealed that of the loci which could be reliably scored, four were polymorphic and 11 were fixed for a single allele.
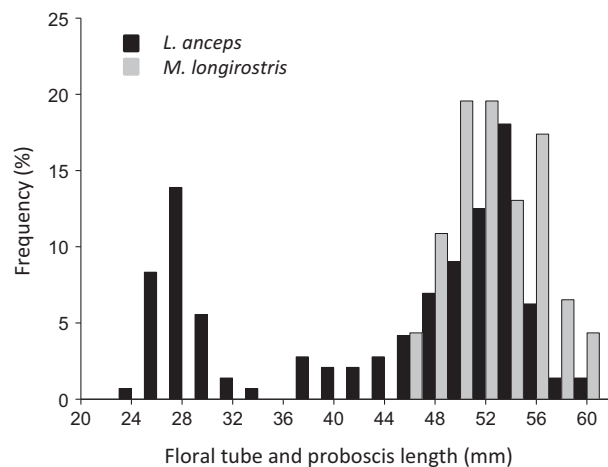

Fig. 2 The distributions of floral-tube length in *Lapeirousia anceps* and pollinator proboscis length of *Moegistorhynchus longirostris* in the Mamre population. Floral-tube length displays a bimodal distribution and proboscis length a unimodal distribution; $n = 72$ plants, 2 flowers per plant and $n = 46$ pollinators.

The four polymorphic loci exhibited either strong (*AAT-2*, *IDH:2*) or complete disequilibrium (*PGI-1*, *TPI-1*) between allele frequency and maternal floral-tube phenotype (Table 1). For example, at the *AAT-2* locus the genotype frequencies of progeny for the long- and short-tube phenotypes were $FF = 0.85$, $FS = 0.12$, $SS = 0.03$ and $FF = 0$, $FS = 0$, $SS = 1.0$, respectively, $\chi^2 = 407.3$, $P < 0.001$. The strong disequilibrium between allele frequencies and floral-tube phenotype resulted in a large genetic distance (D = 0.276) between the progeny of the long- and short-tube phenotypes. The proportion of genetic diversity due to differences among the phenotypes was significant ($\theta^{(I)} = 0.832$ SD = 0.065, 95% CI = 0.682–0.936; $\theta^{(II)} = 0.800$, SD = 0.069, 95% CI = 0.647–0.913), as was the estimate of inbreeding ($f = 0.653$, SD = 0.081, 95% CI = 0.482–0.797). Limited variation within the floral-tube phenotypes precluded distinguishing inbreeding and assortative mating.

Pollinator preferences and correlates of floral-tube variation

Moegistorhynchus longirostris exhibited a preference for long-tubed over short-tubed flowers when the two phenotypes were placed alongside each other. Flies first visited long-tubed flowers on 23 of 28 occasions ($G = 12.540$, $P < 0.001$), and long-tubed flowers received significantly more total visits than short-tubed flowers (27 vs. 14, respectively; $G = 4.194$, $P = 0.041$).

In the mixed population study of pollinator preference, 18 *M. longirostris* pollinators visited a total of 458 flowers on 361 plants. For both long-tubed and short-tubed flowers, over 70% of transitions occurred within phenotypes (71.5% between short-tubed phenotypes and 71.0% between long-tubed phenotypes). Analysed as transitions between plants, more than 60% of transitions occurred within phenotypes (66.1% between short-tubed phenotypes and 61.1% between long-tubed phenotypes. There were more transitions than expected from long-tubed flowers to other long-tubed flowers ($U = 163$, $n = 18$, $P = 0.001$, Fig. 3a), whereas transitions from long-tubed flowers to short-tubed flowers

Table 1 Allele frequencies at four polymorphic allozyme loci in a population of *Lapeirousia anceps* at Mamre containing a bimodal distribution of floral-tube phenotypes. Eleven loci were monomorphic (data not shown). For polymorphic loci, we assayed a total of 223 seeds from 23 long-tubed plants and 90 seeds from 9 short-tubed plants. Sample sizes for monomorphic loci were smaller and involved 80–120 seeds.

Phenotype	Allele	*AAT-2*	*IDH-2*	*PGI-1*	*TPI-1*
Long-tubed	F	0.846	0.069	1.000	1.000
	S	0.154	0.931	0.000	0.000
Short-tubed	F	0.000	1.000	0.000	0.000
	S	1.000	0.000	1.000	1.000

Flowers with longer floral tubes were associated with higher total sugar content than flowers with shorter floral-tube lengths ($R = 0.548$, $P = 0.006$), and the dorsal sepal length, a surrogate for flower size, was positively correlated to floral-tube length, with longer-tubed plants possessing larger flowers ($R = 0.872$, $P < 0.001$, Fig. 1d). Both long- and short-tubed flowers have the capacity for autonomous self-pollination, although long-tubed flowers tended to set fewer seeds than shorter-tubed flowers when not visited by a pollinator ($R = -0.323$, $P = 0.027$, Fig. 4).

Post-pollination reproductive barriers

Seed set
Controlled crosses conducted under field conditions among the floral-tube phenotypes indicated that the floral-tube identity of the maternal parent had a weakly significant effect on seed set ($F_{2,226} = 2.72$, $P = 0.068$). This probably results from the overall low seed set of intermediate floral-tube phenotypes. In contrast, the effect of paternal identity was not significant ($F_{2,226} = 0.44$, $P = 0.647$). Importantly, the particular crossing combination had a highly significant effect on seed set, where crosses between plants of the same floral-tube phenotype set significantly higher seed than crosses between phenotypes ($F_{4,226} = 10.059$, $P < 0.001$). Crosses between the two main floral tube-length phenotypes and plants with intermediate floral-tube length (see Fig. 5) resulted in significantly lower seed set than crosses within a particular floral-tube length class ($P < 0.05$, LSD test, Fig. 5). Parallel results were also obtained from crosses conducted under glasshouse conditions using only long- and short-tubed plants (Fig. 5).

Pollen competition
More seeds were sired by long-tubed than short-tubed pollen donors on long-tubed flowers than would be expected under an assumption of equal siring success (76.1%, SE = 4.30, 95% CI based on back-transformed logit values = 70.6–80.8%). Similarly, short-tubed pollen donors sired significantly more seeds than long-tubed pollen donors on short-tubed flowers (90.6%, SE = 5.56, 95% CI based on back-transformed logit values = 84.0–94.24%).

Estimating reproductive isolation

Reproductive isolation through assortative foraging on flowers was 0.77 (See Data S2). Taken on its own, reproductive isolation due to assortative siring success was calculated as 0.63 for long-tubed phenotypes and 0.89 for short-tubed phenotypes. As this barrier only acts on potential gene flow, which has not been prevented by earlier barriers (i.e. assortative pollinator foraging), its absolute contribution to reproductive isolation was calculated as 0.14 for the long-tubed
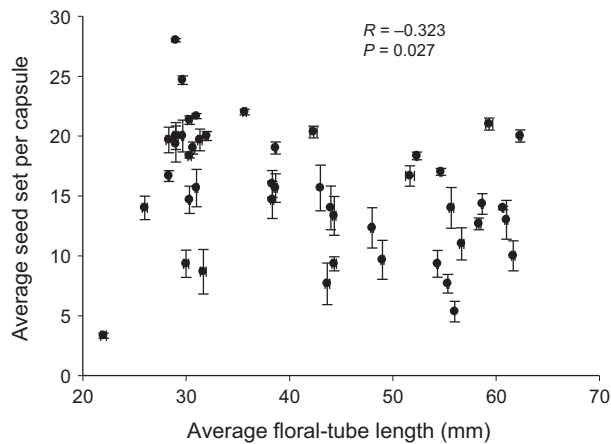
Fig. 3 The observed proportion of transitions among flowers of the long- and the short-tubed floral phenotypes made by 18 *Moegistorhynchus longirostris* pollinators (histogram), compared with the expected proportion of transitions (arrow). (a) Transitions from long-tubed flowers to other long-tubed flowers occurred more regularly than expected by chance. Transitions from long- to short-tubed flowers are not shown but were the inverse and therefore reciprocal of this figure. (b) Transitions from short-tubed flowers to other short-tubed flowers occurred as frequently as expected by chance, as were the proportion of transitions from short- to long-tubed flowers (inverse, reciprocal of this figure).

were less frequent than expected by chance ($U = 8$, $n = 18$, $P = 0.001$). In contrast, transitions among short-tubed flowers occurred as frequently as expected by chance ($U = 123$, $n = 18$, $P = 0.102$, Fig. 3b), as did transitions from short- to long-tubed flowers ($U = 50$, $n = 18$, $P = 0.102$). The patterns of movement between and within plant phenotypes were very similar to the patterns observed between and within floral phenotypes. Transitions among long-tubed plants occurred more frequently than expected ($U = 143$, $n = 18$, $P = 0.012$), and transitions from long- to short-tubed plants occurred less frequently than expected ($U = 28$, $n = 18$, $P = 0.012$). Transitions among short-tubed plants occurred as frequently as expected ($U = 112$, $n = 18$, $P = 0.248$), as did transitions from short- to long-tubed plants ($U = 59$, $n = 18$, $P = 0.248$).

Fig. 4 The association between floral-tube length and the ability of *Lapeirousia anceps* to set seed in the absence of pollinators (autonomous self-pollination). Floral-tube length and seed per capsule were averaged over three flowers per plant. Bars are standard errors.
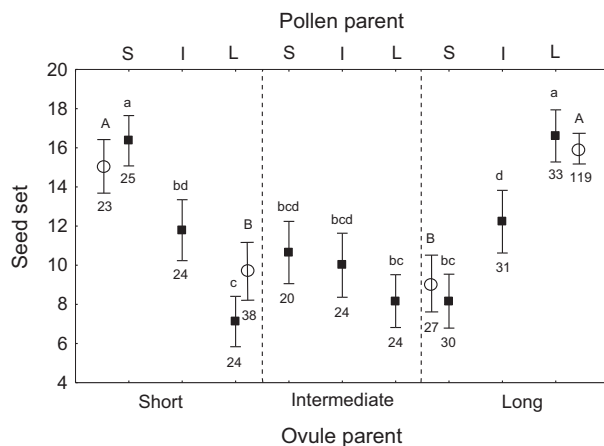

Fig. 5 Seed set per capsule following hand cross-pollinations among different floral-tube phenotypes of *Lapeirousia anceps*. Black squares are means (±SE) for crosses conducted in the field, and clear circles are for crosses conducted under glasshouse conditions. Numbers are the sample plants pollinated for each treatment; letters are significant differences (*P* < 0.05) following Fisher's LSD test.

phenotype and 0.20 for the short-tubed phenotype. The combined effect of these mechanisms of reproductive isolation was 0.91 for the long-tubed phenotype and 0.97 for the short-tubed phenotype.

Reproductive success and floral-tube length

Measurements of reproductive success in relation to floral-tube length indicated that long- and short-tubed flowers set more seed than flowers with intermediate floral-tube lengths ($\gamma \pm$ SE = 0.582 $\pm$ 0.096, *P* = 0.003, *n* = 110, Fig. 6). There was no evidence for directional
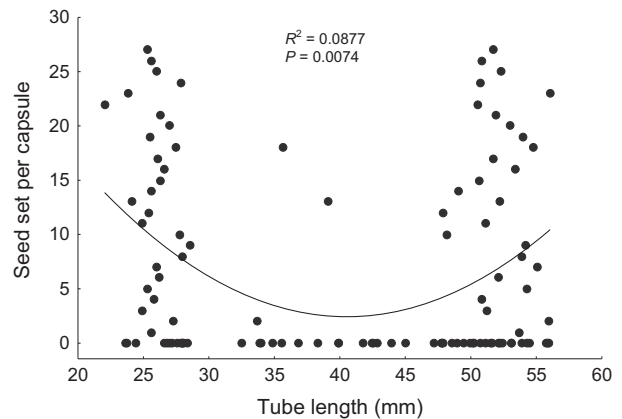

Fig. 6 The relation between floral-tube length and seed set per capsule in the Mamre population of *Lapeirousia anceps* in 2003. The fitted line was obtained using a quadratic function (*n* = 110 flowers).

selection on floral-tube length ($\beta \pm$ SE = −0.099 $\pm$ 0.096, *P* = 0.304, *n* = 110).

Discussion

The major findings of our investigation were as follows: (i) both floral tube-length phenotypes of *L. anceps* shared a single species of pollinator, the long-proboscid fly *M. longirostris;* (ii) despite a shared pollinator, mating within the population was nonrandom, with restricted gene flow between the two floral-tube phenotypes (Table 1); (iii) both pre- and post-pollination mechanisms contribute to partial reproductive barriers between the floral-tube phenotypes, including pollinator foraging patterns (Fig. 3), pollen competition favouring assortative mating and reduced fertility in crosses between the phenotypes (Fig. 5); (iv) floral-tube length was associated with differences in reproductive success in the field, where intermediate tube-length phenotypes produced fewer seed than phenotypes with either long or short floral tubes (Fig. 6). We now discuss the mechanisms that may account for our results and consider the ecological and evolutionary processes that may have given rise to floral-tube bimodality and the possible fate of this condition.

Mating patterns inferred from genetic markers

Strong linkage disequilibrium between floral-tube phenotypes and allele frequencies in open-pollinated progeny strongly support the occurrence of reproductive barriers between the floral-tube phenotypes. This pattern would be unlikely if mating were random in the population and is best explained as the consequence of mating restrictions between the floral-tube phenotypes, although viability selection against 'hybrid' offspring (e.g. Jiggins & Mallet, 2000) may also play some role.

The allelic differences associated with the long- and short-tubed phenotypes also clearly demonstrate that these phenotypes do not constitute a developmental polymorphism, but are genetically differentiated floral forms. The strong correlation between the floral-tube length of parents and offspring, despite growing in different environments, also supports the hypothesis that variation in this trait has a heritable genetic component (Fig. S1), although other mechanisms (e.g. maternal effects and/or epigenetic inheritance) could also contribute to this pattern. We now consider the potential causes of reproductive barriers between the floral-tube phenotypes.

Pollinator preferences and correlates of floral-tube variation

Partial assortative pollination appears to have contributed towards a preponderance of matings within rather than between the floral tube-length phenotypes. This is especially likely for the long-tubed phenotype for which flies have a preference. Because floral tubes are usually obscured from approaching flies by the sepals of a flower, it is unlikely that pollinators discriminate between flowers based on direct assessment of floral-tube length. However, as floral-tube length is correlated to flower size, it seems probable that pollinators use this trait to discriminate between flowers. Flower size has frequently been identified as a trait under selection by pollinators (reviewed in Harder & Johnson, 2009), and a manipulative study of variation in *Trollius* flowers demonstrated that flies in particular have a preference for larger flowers (Zhao & Huang, 2013).

Nonrandom patterns of pollinator foraging can also result from clumped distributions of flowers and plants in space. Because plants of *L. anceps* are self-compatible and produce multiflowered daily displays, within-plant foraging likely accounts for a component of nonrandom mating due to geitonogamy. However, at the plant level we also recorded nonrandom foraging on plants with long-tubed flowers. Although our data suggest assortative foraging, all foraging flies that we observed made some transitions between floral tube-length phenotypes. Therefore, foraging patterns are only partially assortative and pollen dispersal between the floral tube-length phenotypes certainly occurs.

Significantly, there is some indication that mechanical isolation (e.g. Grant, 1994; Kay, 2006) may also play a role in reducing cross-pollination between the floral tube-length phenotypes. Flowers of long-tubed plants deposited pollen on the forehead of flies during nectar feeding, whereas pollen was deposited midway along the proboscis during visits to short-tubed flowers (Figs S2 and S3). Further studies are required to confirm these observations and whether they represent an important mechanism promoting assortative pollen transfer.

Autogamous selfing can also contribute towards reproductive isolation and ecological differentiation in plant populations (Lowe & Abbott, 2004; Martin & Willis, 2007; Brys *et al.*, 2013). Although we were unable to estimate the contribution of self-pollination to seed set and mating patterns in *L. anceps*, our field bagging experiment demonstrated that both floral tube-length phenotypes are capable of autonomous self-pollination (Fig. 4), with long-tubed flowers less efficient at autonomous self-pollination than short-tubed flowers. Pauw *et al.* (2009) demonstrated that stigmas of short-tubed flowers received less pollen after being visited by *M. longirostris* than stigmas of long-tubed flowers. Consequently, we might predict that self-pollinated seed makes up a smaller component of the reproductive output in long-tubed than in short-tubed phenotypes, although levels of autogamy are likely to fluctuate in response to pollinator numbers.

Post-pollination reproductive barriers

Partial post-pollination reproductive barriers between the two phenotypes probably restrict the number of 'hybrid' progeny with intermediate floral-tube length. Partial interphenotype incompatibilities appear to lead to lower seed set in crosses between phenotypes than within phenotypes, and intermediate phenotypes set fewer seeds irrespective of the identity of the pollen donor. Furthermore, the application of mixed pollen loads accentuates the pattern of reduced fitness for interphenotype crosses, suggesting that pollen from the same phenotype is more competitive than pollen from the alternative phenotype.

Isolating barriers involving genetic incompatibilities are sometimes thought to evolve as nonadaptive byproducts of adaptive divergence in allopatry (Coyne & Orr, 2004). Consequently, gene flow barriers in sympatry may not require the same forces that originally drove divergence. For example, divergences driven by allopatric variation in pollinators are unlikely to require the same pollinators in zones of secondary contact for divergence to be maintained. In fact, studies of sympatric pollinator ecotypes often show very weak isolation by pollinators (Anderson *et al.*, 2010; Ros *et al.*, 2011), and other prepollination barriers (e.g. differences in flowering time) may be more pronounced (Anderson *et al.*, 2010). Interspecific studies frequently identify strong post-pollination isolating barriers including pollen-tube competition and incompatibilities expressed at the seed set stage (Rieseberg *et al.*, 1995; Carney *et al.*, 1996; Wolf *et al.*, 2001; Ramsey *et al.*, 2003; Moyle *et al.*, 2004; Kay, 2006). Importantly, Ramsey *et al.* (2003) have demonstrated that multiple, partial reproductive barriers may act in concert to generate complete or near complete reproductive isolation. Our study only investigated the effects of three reproductive barriers (assortative foraging, incompatibilities and interphenotypic pollen competition). Nevertheless, we found that in combination, they resulted in considerable reproductive isolation of the long- and short-tubed

phenotypes (0.91 and 0.97, respectively) and these levels of isolation were reflective of the strong gene flow barriers and levels of genetic differentiation that were revealed in our study using genetic markers ($\theta^{(I)} = 0.832$ and $\theta^{(II)} = 0.800$).

Reproductive success and floral-tube length

Under field conditions, we observed a significant decrease in seed set in plants with intermediate floral-tube lengths compared with long- and short-tubed phenotypes (Fig. 6). Although this type of pattern is often interpreted as disruptive selection, our data suggest another more likely mechanism. The controlled crossing data revealed that seed set of intermediate tube-length phenotypes was always lower compared with within-phenotype crosses, even when pollen was not limited (Fig. 5). Consequently, we suggest that the low seed set of intermediate floral-tube phenotypes in the field is likely to be the result of low hybrid fitness because of genetic incompatibilities. In contrast, the relatively high seed set of long- and short-tubed phenotypes may result from a reduction in the occurrence of interphenotypic incompatibilities through assortative mating. This mechanism differs from classical disruptive selection because the low fitness of intermediate phenotypes is not due to the expression of any trait associated with mating success, but rather stems from genetic incompatibilities between the floral tube phenotypes.

Disruptive selection is the least frequently reported of the various forms of selection, with most coefficient estimates clustered between 0 and 0.10 and a median of $\gamma = 0.10$ (reviewed in Kingsolver *et al.*, 2001; Harder & Johnson, 2009). The quadratic coefficient associated with floral-tube length variation and seed set in the Mamre population of *L. anceps* was considerably stronger ($\gamma = 0.5818 \pm 0.0962$) than for most disruptive selection coefficients. The combination of low hybrid fitness and assortative mating should promote the maintenance of distinct phenotypes in sympatry and play a similar ecological and evolutionary role to disruptive selection in our study population.

Origins and maintenance of bimodality

The average floral-tube length for the long-tubed phenotype in the Mamre population lies within the variation found for other populations pollinated by *M. longirostris*. In contrast, the short-tubed phenotype in the Mamre population has a floral tube similar in length to other short-tubed populations within 10 km of the Mamre site (A. Pauw, pers. obs.). No pollinators have been observed visiting flowers at these geographically close short-tubed populations (A. Pauw, pers. obs.), raising the possibility that short-tubed populations are largely selfing. In the Mamre population, short-tubed plants are associated with smaller flowers,

higher levels of autonomous selfing, reduced sugar content in nectar and lower attractiveness to pollinators in comparison with the long-tubed phenotype. The short-tubed phenotype in this population could have originally evolved in allopatry as an adaptation to a pollinator with a shorter proboscis, or alternatively as a response to insufficient pollinator service.

Zhang *et al.* (2012) demonstrated theoretically that coevolutionary races between *L. anceps* and *M. longirostris* can lead to the evolution of bimodality *in situ*. However, the geographical pattern of tube-length variation in the Mamre population suggests that the origins of bimodality may have been allopatric. Furthermore, if allozyme loci are largely selectively neutral (Lewontin, 1974), then the strong genetic differentiation that we report at these loci would not be expected to be maintained through selection. Consequently, divergence is more likely to have arisen through historical geographical isolation. Although allozyme loci are not always selectively neutral (reviewed by Eanes, 1999), the occurrence of several loci exhibiting similar patterns of allelic fixations in *L. anceps* suggests that these differences between tube-length phenotypes are the result of geographical separation, which would affect multiple loci. In this population, occasional crosses between the floral-tube phenotypes results in a low frequency of plants with intermediate phenotypes. These matings, if they were frequent or went on for a sufficient period of time, should lead to similar frequencies of alternate alleles at neutral loci, even if they were linked to loci under selection. The fact that this is not observed suggests that sympatry of the long- and short-tubed phenotypes of *L. anceps* may be relatively recent and represents a case of secondary contact between differentiated populations. In contrast, traits under selection may be able to persist under conditions of partial reproductive isolation if disruptive selection is strong enough (Kirkpatrick & Ravigne, 2002; Bolnick & Fitzpatrick, 2007). Accentuating the level of bimodality in a population would require gene flow barriers between the floral-tube phenotypes and also strong disruptive selection. Although our study demonstrates partial gene flow barriers, it is uncertain whether the fitness variation among *L. anceps* floral-tube phenotypes would accentuate the present levels of bimodality in the same manner as disruptive selection. Nevertheless, it is likely to prolong the existence of phenotypic differences and reduce the number of intermediate tube-length phenotypes.

Long-term persistence of phenotypic differences is also dependent on the use of different resources or reproductive niches if competitive exclusion is to be avoided (Coyne & Orr, 2004; Grant & Grant, 2008; Pauw, 2013). Long-term coexistence of the floral-tube phenotypes may be unlikely if long-tubed plants are more effective at being cross-pollinated by *M. longirostris,* by setting either more seed or more outcrossed seed. On the other hand, short-term coexistence may occur if pollinator numbers fluctuate annually allowing

occasional years in which short-tubed plants are favoured because they have a greater facility for autonomous self-pollination. Recent observations in 2015 of the Mamre population of *L. anceps* indicate persistence of the bimodal floral tube condition since it was first discovered in 2003, despite striking fluctuations in the size of the flowering population over the intervening years (B. Anderson, unpublished). Long-term studies would be required to determine whether assortative mating and tube-length associated fitness variation would be sufficient to ensure the long-term persistence of floral-tube length bimodality at the site.

Acknowledgments

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Data S1 Genetic differentiation of floral-tube phenotypes.

Data S2 Calculating reproductive isolation.

Fig. S1 Correlation between the mean maternal floral-tube length and the mean floral-tube length of open-pollinated progeny showing that there is a genetic component to tube length.

Fig. S2 The purple pollen of *Lapierousia anceps* is located predominantly on the forehead and mid proboscis of the fly (indicated by white arrows), corresponding to the floral-tube lengths of the two floral phenotypes.

Fig. S3 *Lapierousia anceps* pollen counts (average ± SE number of grains) on different parts of fly proboscides.