



Local adaptation: Mechanical fit between floral ecotypes of *Nerine humilis* (Amaryllidaceae) and pollinator communities

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Geographic variation in floral morphology is often assumed to reflect geographic variation in pollinator communities and associated divergence in selective pressures. We studied populations of *Nerine humilis* (Amaryllidaceae) to assess whether geographic variation in floral form is the result of local adaptation to different pollinator communities. We first tested for associations between floral traits and visitor communities, and found that populations with similar floral morphologies were visited by similar insect communities. Mean style length in each population was also closely associated with the mean body length of the local visitor community. A reciprocal translocation experiment demonstrated that native phenotypes set more seed than translocated phenotypes. Single visitation experiments showed that native flowers received more pollen, and set more seed per visit, than introduced phenotypes in both populations. This suggests that the effectiveness of pollinator visits is determined by the degree of mechanical fit between flowers and visitors. We provide strong evidence that the observed among-population variation in floral traits is an adaptive response to geographic variation in the pollinator community.

KEY WORDS: Advergent evolution, ecological speciation, long-proboscid fly, mechanical fit, pollination ecotypes, reciprocal translocation.

Pollinators select on floral traits through their behavioral preferences or via the mechanical fit between pollinator and floral morphology (Anderson et al. 2010a; Nattero et al. 2010; Van der Niet et al. 2014a). Geographic mosaics in the composition of pollinator communities can generate geographically divergent selective pressures among plant populations (Stebbins 1970). Divergent selection on floral morphology is expected to lead to morphologically distinct pollination ecotypes, and may potentially drive speciation events (Van der Niet et al. 2014a). This is sometimes referred to as the Grant–Stebbins model of pollinator-driven divergence (Johnson 2006, 2010). Phylogenetic studies showing associations between pollinator shifts and speciation events suggest

that pollinators may have driven up to 25% of speciation events in animal-pollinated angiosperms (Kay and Sargent 2009; van der Niet and Johnson 2012). However, studies of associations between divergence events and pollinators are seldom informative about the actual processes that drove the initial stages of divergence (Nosil 2012). To bridge this gap between macroevolutionary patterns and the processes that drove them, it is important to study selection and adaptation in plant populations that are in the process of diverging (Sobel and Streisfeld 2014). Our approach in this article is to determine first whether divergent floral morphology among plant populations is associated with variation in the local pollinator fauna, a pattern that is suggestive of local adaptation to

geographically variable pollinators. We then attempt to determine the mechanisms behind the identified patterns through reciprocal translocations between populations.

We investigate local adaptation in populations of the South African geophyte *Nerine humilis* (Jacq.) Herb. (Amaryllidaceae) across a large part of its range. *Nerine humilis* is part of a pollination guild that includes 17 other plant species that are visited by the long-proboscid fly *Prosoeca longipennis*. *Prosoeca longipennis* has a patchy distribution range in Southern Africa, and nine of the 17 plant species (including *N. humilis*) appear to display shifts to other pollinator species in populations outside the range of the fly (Newman et al. 2014). *Nerine humilis* exhibits significant geographic variation in the length of stamens and styles. In particular, Newman et al. (2014) documented populations with either short styles (grand mean \pm SE: 27.44 \pm 0.74 mm, $n = 4$ populations) or much longer styles (grand mean \pm SE: 43.14 \pm 0.53 mm, $n = 3$ populations). They hypothesized that these floral differences represented adaptive responses to differences in pollinator assembly (i.e., pollination ecotypes), and that geographic differences in pollinator preference or morphology drove the divergence of floral traits. This hypothesis forms the core of the article. We test the hypothesis by investigating patterns of association between floral phenotype and visitor fauna, and present data from experimental approaches aimed at determining the mechanisms behind those patterns.

PATTERNS OF ASSOCIATION BETWEEN FLORAL PHENOTYPE AND POLLINATORS

If pollinator mosaics drive the divergence of floral morphology, then we can expect that populations with similar floral morphology should share similar pollinators. Furthermore, the pollinator traits that drive floral divergence (e.g., proboscis length or color preference) should coincide with the floral traits that are presumed to be under selection in each population (e.g., tube length or floral color). Several studies have described geographic patterns of association between plant and pollinator morphology, indicative of floral adaptations to local differences among pollinator communities (Boberg et al. 2014; Johnson et al. 2014; Newman et al. 2014; Sobel and Streisfeld 2014; Van der Niet et al. 2014b). This pattern-based approach has often revealed very close trait-matching between plants and pollinators (Anderson et al. 2010b), supporting the idea that floral morphology converges when populations are pollinated by similar pollinators, and that it diverges when populations are pollinated by different pollinators (Anderson et al. 2014). Correlative patterns of association are a useful starting point in the study of floral trait divergence as they are able to demonstrate that trait matching is replicated across multiple populations. Although falsification of the trait-matching expectation will reject the hypothesis that pollinator mosaics drive floral diversification,

correlative studies are not definitive evidence for the hypothesis because they are limited by the inability to distinguish cause and effect, making it necessary to study the mechanisms behind those patterns (Nuismer et al. 2010).

MECHANISMS BEHIND PATTERNS OF ASSOCIATION

Reciprocal translocations are useful for identifying divergent patterns of selection and for demonstrating local adaptation of putative ecotypes (reviewed in Kawecki and Ebert 2004), and some pollination studies have used them to demonstrate that local floral forms have greater female fitness than introduced floral forms (Robertson and Wyatt 1990; Streisfeld and Kohn 2007; Sun et al. 2014). However, a limitation of many reciprocal translocation studies is that while they may suggest differing selection gradients on floral traits among populations, it is often difficult to distinguish the agents of selection (Van der Niet et al. 2014a). To overcome this problem, reciprocal translocation studies can also be used to examine the effects of floral phenotypes on specific subcomponents of fitness (e.g., visitation rates and pollen deposition), which are unlikely to be affected by anything other than pollinators. As well as identifying pollinators as the agents of selection, this directly links patterns of local adaptation to traits that enhance attraction (e.g., color or scent, see Newman et al. 2012) or traits that affect the degree of morphological or behavioral fit between flowers and their pollinator selection agents (e.g., Aigner 2001; Nattero et al. 2010). Whenever plants are introduced into novel populations, the use of seed set as a measure of female fitness may be confounded if incompatibilities exist between native and introduced phenotypes, making it essential to undertake preliminary studies on compatibility between the phenotypes used in reciprocal translocations. Furthermore, both pollen deposition and seed set may be influenced by the position of pollen placement in the host population. In such instances, introduced phenotypes may be pollen limited because plants in the host population deposit pollen on a different part of the pollinator and not because their morphology does not fit with the pollinators' morphology. Consequently, assessing whether pollinators make contact or not with the reproductive parts of flowers can be useful in determining whether reduced seed set or pollen deposition of introduced phenotypes are the results of maladaptation to the local pollinator fauna. Although well-designed reciprocal translocation experiments can be very informative about local adaptation and even about the agents of selection, they are labor-intensive and are thus normally limited to just a few populations. Consequently, it is useful to combine pattern-based approaches that infer local adaptation in multiple populations, with reciprocal translocations that are able to demonstrate it in selected study populations.

We divide this article into two sections that are repeated throughout, and that reflect these two different approaches

commonly used to study local adaptation: In the first section, we describe patterns of association between pollinators and floral morphology. We predict that if pollinator mosaics have generated floral divergence, then (1) populations with similar visitor assemblages will share similar floral morphology, and (2) that style length in each population should match the functional body length of floral visitors in each population. In the second section, we identify the mechanisms behind the described patterns. An association between pollinator and plant traits may arise if local pollinator preferences or morphology differ among populations and also select for divergent traits. If so, then (3) local phenotypes should perform better than morphologically different introduced phenotypes. Furthermore, (4) if local adaptation is due to differences in the preferences of pollinators, then we expect local pollinators to visit local phenotypes more frequently than introduced phenotypes. Finally, (5) if local adaptation is due to differences in the fit between plant and pollinator, then we predict that pollen transfer between local plants and pollinators will be more effective than that between introduced plants and pollinators. In particular, the stigmas of local (but not introduced) flowers are expected to make frequent contact with floral visitors. The mechanical fit between pollinator and flower should affect pollen receipt and seed set so that local forms receive more pollen and produce more seeds than introduced forms.

Methods

STUDY SPECIES

Nerine humilis is a self-incompatible geophyte (Newman, E., unpubl. data) from Southern Africa that flowers in the wild from late March to early June. The umbellate inflorescences carry from one to eight pale to deep pink, zygomorphic flowers lacking any fragrance discernible to the human nose. The flowers lack a nectar tube and nectar is secreted from septal nectaries in the inferior ovary at the base of the polypetalous perianth. Nectar is easily accessible to most floral visitors because a long proboscis is not required to reach it (Fig. 1). Six anthers and a single stigma are borne at the ends of slender filaments and style, respectively, both of which display significant variation in length across the geographic range of the species (Goldblatt and Manning 2000). Flowers of *N. humilis* are protandrous, with distinct male and female phases, and the flowers last between three and seven days each. The male phase is initiated by dehiscence of the anthers, and terminated by recurving of the filaments. Following the male phase, the style elongates and the stigmatic surface becomes receptive, occupying the former position of the anthers. *Nerine humilis* is relatively widespread through the Cape Floristic Region with a range stretching across ± 700 km (Goldblatt and Manning 2000).

PATTERNS OF ASSOCIATION BETWEEN FLORAL PHENOTYPE AND POLLINATORS

Visitor observations

We recorded the abundance and identity of all visitors to *N. humilis* flowers in 11 populations across the Western and Eastern Cape of South Africa (Table S1), from March toward the end of May. Visitor observations in each population were conducted for 10 h across two days, between 09:00 h and 14:00 h on days when ambient temperatures were at least 20°C. For each population, 5 h of observation were completed in 2012 and another 5 h in 2015. In each population, observations were conducted on approximately 300 flowers located within a radius of 2 m, so that a single observer was able to survey the entire demarcated patch without moving.

Measurements of floral morphological traits

Four floral traits (functional style length, tepal length, nectar volume and nectar concentration) were measured for 12–45 flowers at each study locality using one mature flower per arbitrarily selected plant. Table 1 details the exact replication for the measurement of each trait in each population. Trait measurements were standardized by using only mature flowers in the female phase, recognized by their strongly trilobed stigmas. Tepal length, a surrogate for floral size, was determined as the straight line distance between the base and apex of the dorsal tepal, using digital calipers for 19–27 flowers per population (Table 1). Nectar concentration and volume were measured in 12–37 flowers from each locality (Table 1). Measurements were performed early in the morning before visitors started foraging, and before evaporation could potentially alter nectar readings. Measurements were taken using a 1–5 μ L graduated micropipette (Drummond Scientific Company, Broomall, PA, USA) and a 0–50 % Bellingham–Stanley refractometer (Bellingham and Stanley, Tunbridge Wells, U.K.). Functional style length was recorded from 19 to 45 flowers in each of the 11 *N. humilis* populations (Table 1). Functional style length was determined as the distance from the nectary to the tip of the stigma (see Appendix S1). The abdomens of floral visitors routinely make contact with the stigmas (Fig. 1A), making functional style length a likely trait to be selected on by visitor morphology. Floral color does not generally differ among most populations in this study, but flowers from the Skurwekop (SK) population used in reciprocal translocations were unusually pale. Consequently, in the two reciprocal translocation populations (SK and Nuwekloof Pass [NKP]), we used a spectrometer (Dunedin, Florida) to measure the dominant tepal color (principal attractive surface) across the range 300–700 nm. These measurements were averaged over five individual flowers from SK and seven individual flowers from NKP.

Table 1. Short and long-style *Nerine humilis* study localities, with population means, SEs, and sample sizes for floral traits. Statistical differences between trait magnitudes of short and long-style phenotypes from the linear mixed model (LMM) are given in bold. Populations 4 and 9 represent reciprocal translocation localities SK and NKP.

Statistics	Population	Phenotype	Style length (mm)	Tepal length (mm)	Nectar volume(μL)	Nectar concentration (%)	Functional visitor length (mm)	Latitude (mm)	Longitude
	1	Short	21.56 ± 0.91 (20)	20.80 ± 0.94 (20)	0.67 ± 0.17 (20)	23.86 ± 2.48 (18)	15.16	33.54	20.60
	2	Short	23.42 ± 0.89 (21)	18.87 ± 0.89 (21)	0.56 ± 0.16 (21)	15.19 ± 2.29 (21)	16.83 ± 0.67	34.07	20.39
	3	Short	24.09 ± 0.78 (27)	20.65 ± 0.78 (27)	0.75 ± 0.10 (24)	17.85 ± 1.73 (37)	18.11 ± 1.61	33.70	19.07
	4	Short	24.44 ± 0.61 (45)	21.61 ± 0.61 (20)	0.42 ± 0.74 (31)	10.80 ± 2.35 (20)	15.86 ± 1.06	34.46	19.91
	5	Short	25.20 ± 0.85 (23)	21.46 ± 0.85 (23)	0.76 ± 0.18 (18)	15.00 ± 2.48 (18)	14.11 ± 0.32	34.48	19.92
	6	Short	27.44 ± 0.70 (34)	29.42 ± 0.70 (19)	0.83 ± 0.17 (20)	17.89 ± 2.48 (18)	15.33 ± 0.71	34.19	20.29
	7	Short	28.46 ± 0.83 (24)	25.60 ± 0.83 (24)	0.70 ± 0.07 (21)	17.58 ± 3.04 (12)	18.56 ± 1.97	34.41	19.77
	8	Short	29.24 ± 0.93 (19)	24.10 ± 0.93 (19)	0.87 ± 0.16 (23)	25.52 ± 2.30 (21)	15.78 ± 5.23	34.44	19.74
	9	Long	41.23 ± 0.87 (22)	29.92 ± 0.74 (22)	3.86 ± 0.14 (27)	16.96 ± 2.10 (25)	31.49 ± 6.36	33.51	23.64
	10	Long	42.15 ± 0.74 (30)	29.92 ± 0.74 (26)	2.36 ± 0.18 (18)	33.38 ± 2.63 (16)	37.56 ± 0.08	33.39	22.56
	11	Long	42.22 ± 0.81 (25)	29.10 ± 0.81 (25)	1.85 ± 0.15 (24)	27.06 ± 1.83 (33)	54.78 ± 1.80	34.02	20.60
Grand means (short)			25.48 ± 2.66	22.81 ± 3.39	0.70 ± 0.05	17.96 ± 4.78	16.22 ± 0.54	–	–
Grand means (long)			41.87 ± 0.55	29.65 ± 0.47	2.69 ± 1.04	25.80 ± 8.28	41.28 ± 6.98	–	–
P (LMM)			<0.001	0.009	<0.001	0.080	–	–	–
Total sample size			290	246	247	239	133	–	–

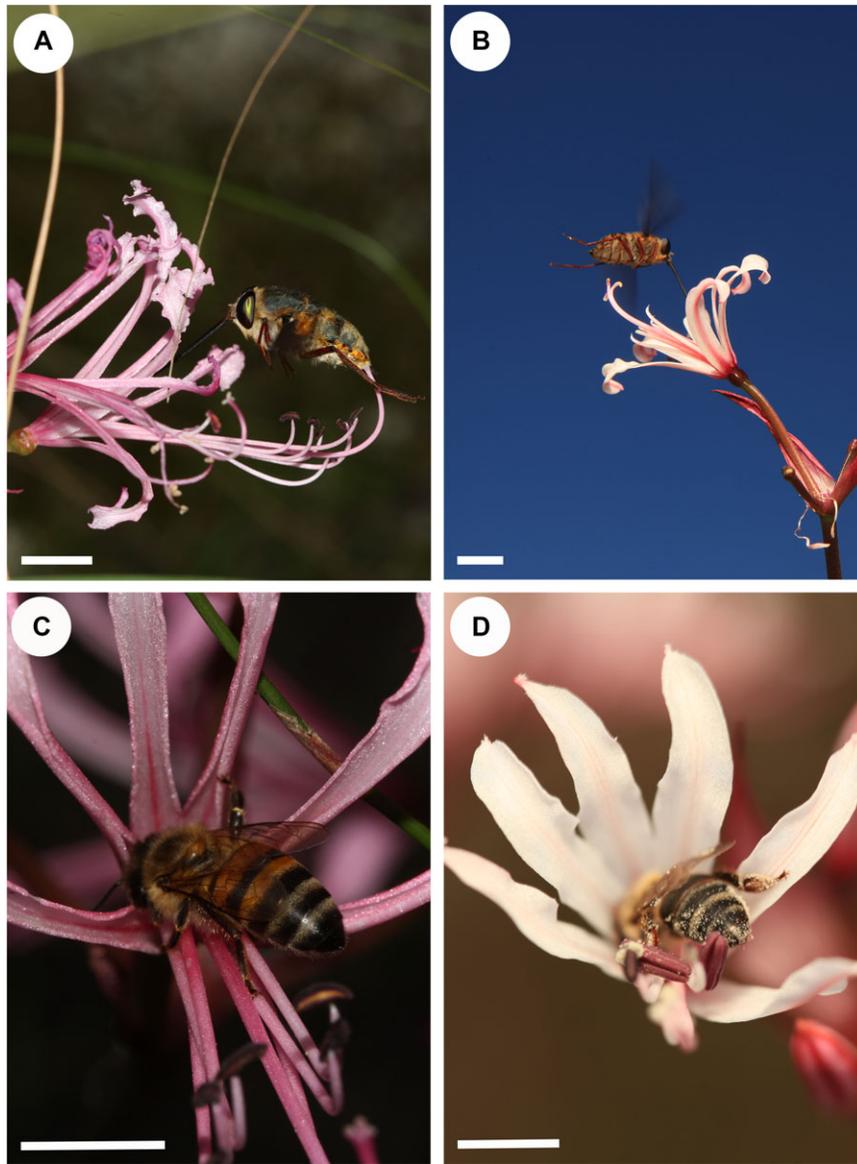


Figure 1. Flowers from the two translocation populations (NKP and SK), being visited by long proboscid flies and honey bees. (A) The long-proboscid fly *Prosoeca longipennis* visiting the native long-style phenotype (population NKP) of *Nerine humilis*, where the stigma makes contact with the abdomen of the fly. (B) *Prosoeca longipennis* visiting an introduced short-style phenotype (from population SK) during a single visitation experiment. Here, the fly is thieving nectar because it makes no contact with the mature stigma. Note that the anthers have been removed. (C) Honey bees, *Apis mellifera*, were occasionally observed visiting the native long-style phenotype (population NKP), where they seldom make contact with the reproductive parts of the flowers and pollen was not placed on their abdomens. (D) A honey bee visiting the native short-style phenotype (population SK) of *N. humilis*. Notice pollen deposition on the abdomen of the bee. Scale = 10 mm.

Measurements of visitor morphological traits

In all 11 populations, functional visitor length was calculated as the combined length of body plus proboscis. We considered this length to be the pollinator trait to which functional style length may be adapted. Functional visitor lengths were measured with digital calipers from a total of 133 insects (Table S2) that had been killed using potassium cyanide. Functional visitor length was

determined by the angle at which insects held their proboscides while foraging (see Appendix S1).

Is there an association between floral phenotype and visitor assemblage?

We generated a preliminary Bray–Curtis similarity matrix, which has no *a priori* assumptions about grouping, to identify *N. humilis*

populations that were associated by their similarities in visitor fauna. The Bray–Curtis similarity matrix was based on the similarities of visitor communities among all 11 *N. humilis* population pairs (Table S1) and the Bray–Curtis metric accommodates the relative abundance of different visitors at each site and not just their presence or absence. The matrix was used to plot an unweighted pair-group mathematic averaging (UPGMA) cluster analysis, which grouped populations in the form of a neighbor-joining tree. The neighbor-joining tree identified two distinct clusters of populations based on their visitor assemblages; one group comprising eight populations (Group 1) and another of three populations (Group 2; Fig. S1). We tested for a significant difference between the visitor faunas of the two groups using a one-way Analysis of similarity (ANOSIM). Significance was estimated by comparing the calculated global *R* value with randomly generated *R* values (10,000 permutations) (Clarke and Warwick 2001). A Similarity Percentages (SIMPER) analysis determined that the presence or absence of long proboscis flies was the most important factor in generating these two groups. Results were visualized using a non-metric multidimensional scaling plot (NMDS).

To test whether similarities in visitor fauna were associated with similarities in floral morphology, we determined whether floral traits (style length, tepal length, nectar volume and nectar concentration—see Table 1) differed significantly between the two groups identified by the preliminary analysis (i.e., populations with and without long proboscis flies). Following a Bray–Curtis similarity matrix on these floral traits, significant differences in the floral traits of these two groups were determined using a one-way ANOSIM as above. We also performed a SIMPER analysis to determine which floral traits were the most important associates with the two insect visitor groups. PRIMER 5 version 5.2.9 (Primer-E, Ltd., Plymouth, U.K.; Clarke and Warwick 2001) was used for all analyses above.

We also tested for a correlation between the Bray–Curtis differences in floral morphology and visitor assemblage between each pair of populations. Associations between these two matrices were tested using a Mantel test in Excelstat (2014) version 2014.6 package of Microsoft Excel (Redmond, Washington: Microsoft). Our expectation was that if populations are locally adapted to different floral visitor communities, then the matrices should be correlated (i.e., populations with similar floral traits should share similar visitor communities).

To determine specifically which traits differed and how these traits differed between populations with and without long proboscis flies, we used a linear mixed model (LMM) with phenotype as a fixed factor and locality as a random factor, with phenotype nested within locality. The traits examined were style length, tepal length, nectar volume and nectar concentration.

Do floral and visitor morphology correlate across populations?

Here we focused on a single floral trait (functional style length) and a single pollinator trait (functional visitor length) to determine the existence of morphological correlations across the 11 populations. Mean functional length of each visitor species was weighted by multiplying its abundance in each population (Table S1) with its mean functional length for that population (Table S2) to obtain a weighted mean for each visitor species in each population. The weighted means of visitors were summed for each population and divided by the total number of visitations observed for all insects within each population to provide a weighted grand mean of functional visitor length in each population (Table S2). SEs of the weighted means were calculated using the methods of Gatz and Smith (1995). The relationship between mean functional style length and mean functional visitor length was analyzed using a univariate regression with functional style length as the dependent variable, and the grand mean functional visitor length as the predictor variable. For this analysis of pattern, all floral visitors were included, even those that did not regularly make contact with the reproductive parts of the flowers. We did this because we were interested in whether floral morphology was reflective of the potential selective agents in each population, thereby avoiding the circularity of using only visitors that made frequent contact with the reproductive parts of flowers.

MECHANISMS BEHIND PATTERNS OF ASSOCIATION

Are floral phenotypes locally adapted?

To experimentally investigate whether plant populations with short and long styles are adapted to their local environments, we conducted reciprocal translocation experiments between one long-style and one short-style population. The short-style population was located at SK near Napier in Western Cape and the long-style population was at NKP in the Baviaanskloof Mountains in Eastern Cape, approximately 380 km distant (see Fig. 2). Eighteen cut inflorescences with flowers in bud were translocated from SK to NKP, and 15 similar inflorescences were translocated from NKP to SK. Translocating inflorescences did not affect their ability to set seed (see Appendix S2). In each reciprocal translocation population, cut inflorescences were arranged in pairs comprising one native and one introduced inflorescence and placed in water-filled test tubes mounted on skewer sticks, providing a total of 33 inflorescence pairs across the two reciprocal translocation localities.

At each reciprocal translocation locality, inflorescence pairs were arranged at the same height and had the same number of flowers (one to four). Within replicated pairs, native and introduced inflorescences were arranged approximately 30 cm apart.

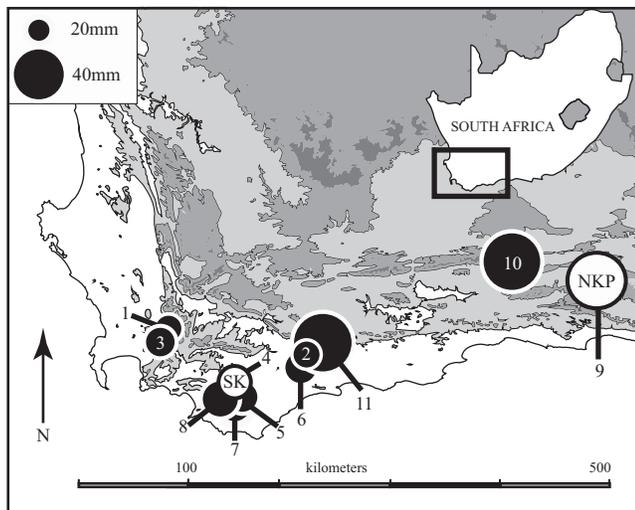


Figure 2. The localities of all study sites used, representing the range of *Nerine humilis* in the Western Cape, South Africa. Circle diameters represent mean style lengths per population. Localities highlighted in white, labeled SK (Skurwekop) and NKP (Nuwekloof Pass) are where translocation studies were performed.

For the purpose of recording observations for choice experiments (see below), pairs were arranged approximately 2 m apart in a half circle in front of the observer. Buds were allowed to open in the field and water was changed every three days. Anthers of all experimental plants in the study were removed while flowers were in bud to prevent contamination of the local gene pool. This process of anther removal had little effect on visitor positioning because the visitors of these flowers were not observed to actively harvest pollen. Furthermore, the anthers usually dehisce and abscise by the time the mature stigma occupies the position formerly held by the anthers. As *N. humilis* is self-incompatible (Newman, E., unpubl. data), all seeds produced by experimental plants were the result of cross-pollination. Inflorescence pairs were left in the field until they showed signs of withering. At this stage they were taken back to the laboratory for fruits to develop, where the proportion of fertilized ovules out of the total number of ovules was determined. Ovule development continued normally in water (Appendix S2) and fertilized ovules were clearly distinguished from aborted ovules by their green and swollen appearance (>3 mm diameter). In contrast, unfertilized ovules fail to develop beyond small, transparent spheres ± 1 mm diameter. These differences were readily determined with the naked eye (Fig. S2). Furthermore, 18 flowers from SK and 15 flowers from NKP had similar numbers of ovules ($t = 0.86$, $P = 0.66$).

We used generalized estimating equations (Liang and Zeger 1986) to determine whether native inflorescences produced a higher proportion of fertilized ovules than introduced inflorescences. For each flower, the proportion of fertilized ovules (de-

pendent variable) was calculated using the total number of events (fertilized ovules) occurring in a set of trials (total number of ovules). Because each inflorescence had several flowers, an exchangeable correlation matrix was used to account for repeated measures of seed set within an inflorescence, which additionally accounts for possible correlations of increased visitations to inflorescence pairs with larger display sizes (Galen and Newport 1987). We used a binomial distribution with a logit-link function with factors, source locality, and phenotype treated as interaction terms in the model (source locality \times phenotype). Furthermore, we also performed pairwise contrasts on the interaction term (source locality \times phenotype) using Sequential Sidak statistics on the estimated marginal means. Hand crosses between plants from SK and NKP showed that the two phenotypes were fully cross-compatible and consequently incompatibilities do not confound reciprocal translocation results (Appendix S3).

Are floral phenotypes locally adapted to visitor preferences (Sensory fit)?

The previous experiment was capable of demonstrating local adaptation, but it could not identify which traits are being selected upon, or what the agents of selection are. To determine whether differences in seed set are due to traits that vary in their attractiveness to visitors (e.g., color or size), we recorded visitation rates to local and introduced inflorescences within the same experimental pairs as above, with a total of 65 visits at NKP, and 70 visits at SK. We excluded no floral visitors from the analyses. The total number of visits for long and short style phenotypes within inflorescence pairs (replicates) was recorded for three days at each reciprocal translocation locality between 09:00 and 14:00 h. At the end of each day, the positions of the introduced and local individuals within each pair were swapped around. Total visits to pair members were analyzed using GEEs with a Poisson distribution and a log-link function, with factors source locality \times phenotype treated as interaction terms. The model used an exchangeable correlation matrix to account for non independence of foraging pollinators. Significance was assessed using generalized score statistics.

Are floral phenotypes locally adapted to visitor morphology (mechanical fit)?

To investigate whether plants are locally adapted to fit mechanically with local visitor morphology, we performed single visitation experiments for a single day in each reciprocal translocation population. This experiment used different flowers from the previous experiments. Flowers were bagged in each reciprocal translocation population, and as soon as stigmas were receptive, inflorescences with a single receptive flower were cut and offered to visitors. Developing anthers were removed from all flowers

in bud, thus preventing gene pool contamination. Virgin flowers from each phenotype were offered to honey bees where short-style plants were native, or to long proboscis flies where long-style plants were native. In the short-style population (SK), we offered 33 short-style flowers and 12 long-style flowers to bees. In the long-style population (NKP), we offered 31 short-style flowers and 14 long-style flowers to long-proboscid flies (*P. longipennis*). We solicited single visitations by placing flowers in the natural *N. humilis* populations in water-filled test tubes, mounted on skewer sticks. The flowers were monitored continuously and each flower was permitted only a single visitation, at which time we recorded whether floral visitors made contact with the mature stigmas of the flowers or not. After a single visit, the inflorescence was placed in the shade and bagged to prevent further visitation. Visited flowers were labeled and taken back to the laboratory where pollen grains were counted on each stigma under a dissecting microscope. Stigmas were not cut or stained as we did not want to compromise seed set. After pollen counting, the inflorescences were placed in regularly changed water until fruits were formed.

We analyzed “contact” versus “no contact” by visitors to native and introduced flowers using a generalized linear model (GLM) with a binomial distribution and a logit-link function, with source locality × phenotype as interaction terms in the model. Pollen counts from single visitation experiments were analyzed using a GLM with a Poisson distribution and a log-link function, with source locality × phenotype as the interaction term in the model. The proportion of fertilized ovules from single visitations was analyzed using a GLM with a binomial distribution and a logit-link function. Source locality × phenotype were treated as interaction terms. Pairwise contrasts on the interaction terms of all single visitation analyses described above were investigated using Sequential Sidak and LSD (least significant difference) statistics. For graphic representation of all models depicted above, we used back-transformed values of the adjusted marginal means, which resulted in asymmetric SEs. See Table S3 for details.

Results

PATTERNS OF ASSOCIATION BETWEEN FLORAL PHENOTYPE AND POLLINATORS

A total of 537 observations of 18 insect species were recorded across 11 populations of *N. humilis* (Table S1). Long-proboscid flies, *P. longipennis* and *P. ganglebauri* (Nemestrinidae) visited all long-style populations (9, 10, and 11) and were the most abundant visitors. Long-style populations were also visited at lower frequencies by short-proboscid insects (Table S1). Long-proboscid flies were never observed at the short-style populations. Fourteen species of short-proboscid insects

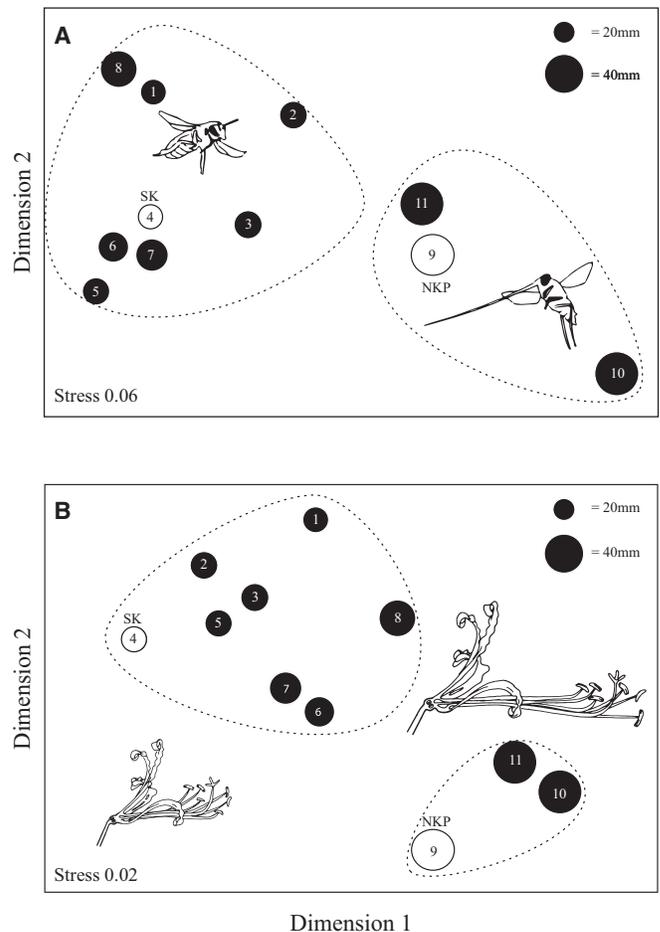


Figure 3. Non-metric multidimensional scaling plot of associations between visitor community composition and floral morphology. (A) Visitor composition of short- and long-style *Nerine humilis* populations clustered as two significantly different groups in non-metric multidimensional space. Long-style populations cluster primarily as a result of long-proboscid fly visitors and short-style populations cluster primarily due to an abundance of honey bee visitors. (B) Populations with and without long-proboscid flies cluster as two distinct groups on the basis of floral traits. Style length was the primary trait generating the clustering of populations visited by long-proboscid pollinators and populations without long-proboscid pollinators. Circles represent mean style lengths of *N. humilis* populations, while numbers refer to the geographic positions of populations identified in Figure 2.

visited the short-style populations (1–8), with honey bees (*Apis mellifera*) the only species seen at all short-style populations.

Is there an association between floral phenotype and visitor assemblage?

Spatial clustering suggested that the two groups of plant populations identified in the UPGMA analysis differed significantly in their visitor assemblages (ANOSIM: Fig. 3A; $R = 0.88$, $P = 0.006$). Honey bees were responsible for $59.33 \pm 1.04\%$

(SD) of the similarity among the eight short-style populations, whereas the long-proboscid fly *P. longipennis* was responsible for $74.18 \pm 0.89\%$ (SD) of the similarity among the three long-style populations.

The type of visitor community (i.e., with or without long-proboscid pollinators) was associated with specific sets of floral traits (ANOSIM: Fig. 3B; $R = 0.95$, $P = 0.006$). The most important of these traits was style length, which accounted for $39 \pm 12.51\%$ (SD) of the similarity among populations without long-proboscid flies, and $44.6 \pm 34.14\%$ (SD) of the similarity among populations with long-proboscid pollinators (SIMPER analysis). Populations without long-proboscid pollinators also clustered through similarities in tepal length ($34.4 \pm 15.31\%$ of similarity) and nectar volume ($24.93 \pm 5.92\%$ of similarity). Similarly, populations visited by long-proboscid flies also clustered by larger tepals ($31.55 \pm 23.57\%$ of similarity) and higher nectar volumes ($21.69 \pm 4.02\%$ of similarity). Furthermore, increasing similarity in visitor assemblages between population pairs was correlated with greater similarity in floral morphology (style length, tepal length, nectar volume, nectar concentration) between populations (Fig. S3; Mantel, $r = 0.66$, $P < 0.001$).

Populations with long-proboscid pollinators had significantly longer styles ($F_{1,8.64} = 109.51$, $P < 0.001$), longer tepals ($F_{1,8.76} = 11.09$, $P = 0.009$), and larger nectar volumes ($F_{1,9.2} = 33.08$, $P < 0.001$) than populations without long-proboscid pollinators. There was no significant difference in nectar concentrations among populations with and without long-proboscid fly pollinators (Table 1; $F_{1,8.71} = 3.92$, $P = 0.080$). Although tepal color did not appear to differ among populations with and without long-proboscid flies, clear color differences between the two populations used in the choice experiment could be seen with the human eye and with the aid of a spectrophotometer (Fig. S4).

Do floral and visitor morphology correlate across populations?

We observed a strong match between functional style length and functional visitor length across populations (Fig. 4; $R^2 = 0.75$, $P = 0.001$). Populations with short style lengths were associated with visitors that had short functional body lengths, and populations with long style lengths were associated with visitors that had longer functional body lengths.

MECHANISMS BEHIND PATTERNS OF ASSOCIATION
Are floral phenotypes locally adapted?

A significant source locality \times phenotype interaction (Table 2, Fig. 5; $\chi^2 = 12.09$, $P < 0.007$) in the reciprocal translocation experiments provides strong evidence for local adaptation (Kawecki and Ebert 2004). All pairwise contrasts for this experiment were

Table 2. Pairwise contrasts on the interaction terms of local adaptation experiments, and similar experiments investigating mechanisms underlying local adaptation, significant values are indicated in bold.

Test	Experiment	Experimental localities	Phenotype	Locality \times phenotype (χ^2)	Locality \times phenotype (P)	Contrasts within source locality (P)	Contrasts between source localities (P)
Local adaptation	Reciprocal translocation	NKP SK	Long Short	12.09	0.007	< 0.001 0.019	< 0.001 0.002
Sensory fit	Preference experiment	NKP SK	Long Short	5.47	0.141	- -	- -
Mechanical fit	Single visitations (contact)	NKP SK	Long Short	19.70	< 0.001	0.004 0.006	0.001 0.024
Mechanical fit	Single visitations (pollen deposition)	NKP SK	Long Short	1697.00	< 0.001	< 0.001 < 0.001	< 0.001 < 0.001
Mechanical fit	Single visitations (seed set)	NKP SK	Long Short	120.90	0.001	< 0.001 < 0.001	< 0.001 < 0.001

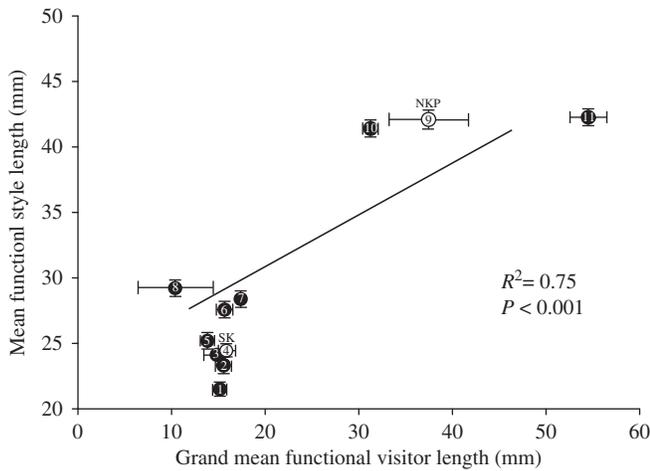


Figure 4. Mean functional style length with SEs, plotted against grand mean functional visitor length (adjusted to incorporate the relative abundance of different visiting species) with weighted SEs. Numbers represent study populations and reciprocal translocation localities SK and NKP are indicated by white circles.

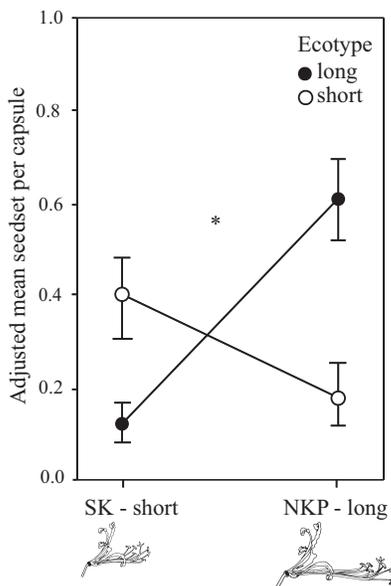


Figure 5. Reciprocal translocations measuring the effects of floral phenotype and locality on female fitness (proportion of fertilized ovules). Here, access to flowers by pollinators was not controlled. A significant interaction between source locality and floral phenotype (short- and long-style flowers) indicates that native flowers set a higher proportion of fertilized ovules than introduced flowers. Significant contrasts (Table 2) suggest local adaptation in both populations. * indicates significance at $P = 0.007$

significant (Table 2; $P < 0.02$), suggesting that both long- and short-style phenotypes are locally adapted.

Are floral phenotypes locally adapted to visitor preferences (sensory fit)?

A total of 65 choices were observed at NKP (59 from *P. longipennis*, three from *Prosoeca willowmorensis*, and three from *A. mellifera*) and 70 choices at SK (61 from *A. mellifera*; six from *Lasioglossum sp.*; and single choices from *Eristalinus tenax*, *Anthene definita*, and *Amegilla spilostoma*). Although there appeared to be a pattern of local adaptation in which local phenotypes received more visits than introduced phenotypes, this pattern was not significant (Table 2, Fig. 6A; interaction term $\chi^2 = 5.47$, $P = 0.141$).

Are floral phenotypes locally adapted to visitor morphology (mechanical fit)?

Single visitation experiments suggest that a greater proportion of floral visitors make contact with the stigmas of native phenotypes than with introduced phenotypes (interaction term: Fig. 6B; $\chi^2 = 19.70$, $P < 0.001$). All contrasts were significant (Table 2; $P < 0.025$). This translated into local phenotypes receiving more pollen grains per visit than introduced phenotypes (interaction term: Fig. 6C; $\chi^2 = 1697.00$, $P < 0.001$). All contrasts were significant (Table 2; $P < 0.001$). Differences in pollen deposition also translated into differences in seed set (interaction term: Fig. 6D; $\chi^2 = 120.90$; $P = 0.001$), with all contrasts significant (Table 2; $P < 0.001$).

Discussion

Several lines of correlative evidence suggest that floral traits in populations of *N. humilis* visited by long proboscis flies differ from those in populations not visited by long proboscis flies. Furthermore, experimental evidence suggests that geographic differences in pollinator morphology drive local adaptation of floral traits through the mechanical fit between floral and pollinator morphology. Theoretically, divergence in floral traits can arise through genetic drift, phenotypic plasticity, or natural selection (Herrera et al. 2006). Taken together, our evidence suggests that the floral traits of *N. humilis* populations have diverged as a result of natural selection imposed by different pollinator communities. Below, we discuss the hypothesized links between floral morphology, visitor composition, and visitor morphology as correlative support for pollinator-driven trait divergence. We then discuss experimental evidence for local adaptation of *N. humilis* populations to different kinds of pollinators, addressing the mechanisms behind the observed patterns.

PATTERNS OF ASSOCIATION BETWEEN FLORAL PHENOTYPE AND POLLINATORS

Nerine humilis populations across the range of the species are visited by two distinct visitor communities, and the type of visitor

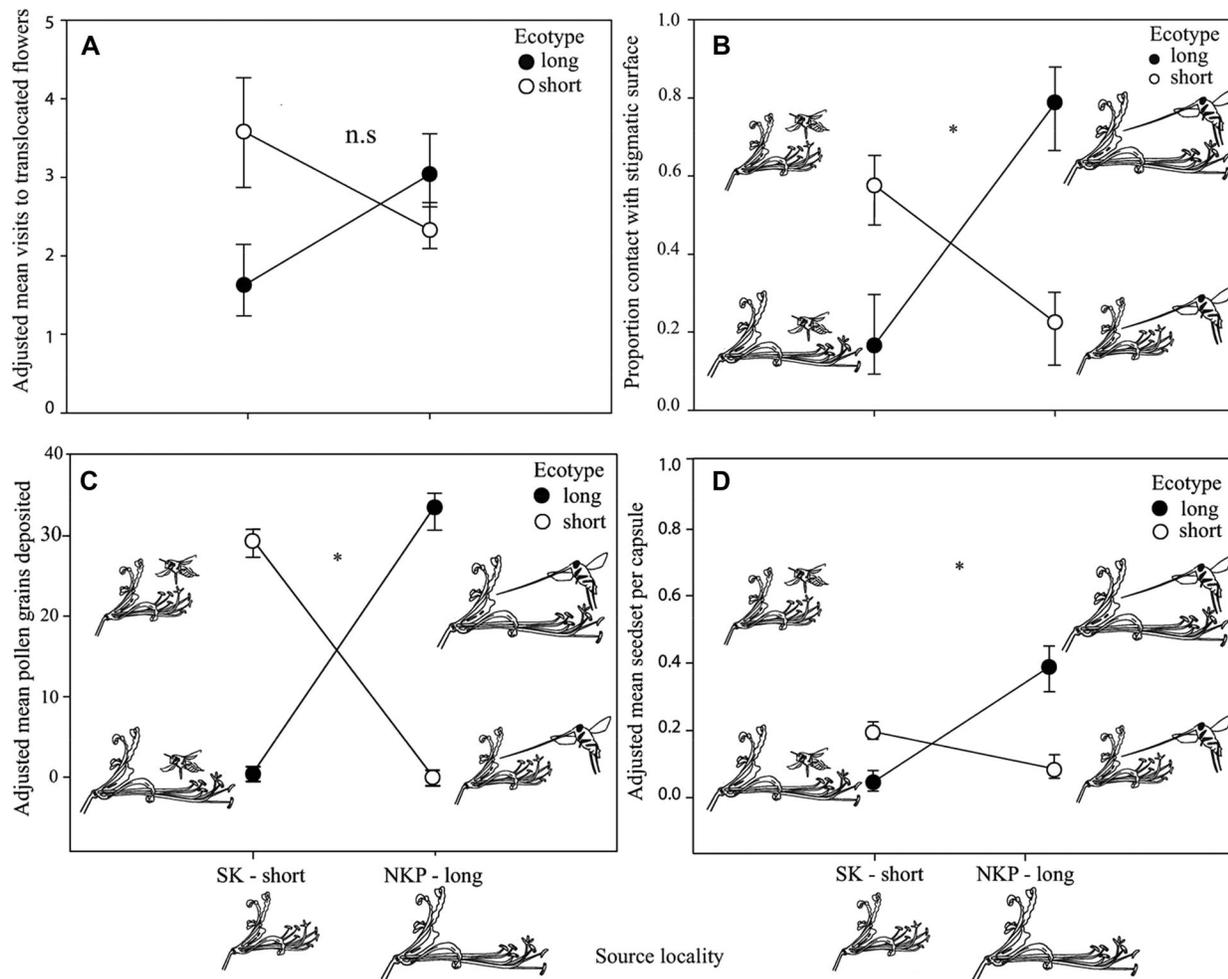


Figure 6. Reciprocal translocations on subcomponents of female fitness, designed to distinguish the kinds of traits being selected on, and the agents of selection. (A) A nonsignificant interaction effect suggests that floral traits involved with pollinator attraction play little or no role in explaining local adaptation. (B) Pollinators had a significantly higher rate of making contact with stigmas of their native phenotypes than introduced phenotypes, suggesting that the mechanical fit between plant and pollinator may affect pollen transfer in both populations. (C) After single pollinator visits (by bees at SK and by long proboscis flies at NKP), pollen was consistently deposited more effectively on native phenotypes than introduced phenotypes. This suggests that the identity of the pollinator affects the efficiency of pollen transfer. (D) Differences in the efficiency of pollen transfer translated into differences in the proportion of seeds produced after single visitations by either bees or long-proboscis flies. Illustrations on the x-axis represent the style lengths of the experimental source localities. *indicates significant interactions at $P < 0.001$. Non significance is indicated by n.s. See Table 2 for contrasts and Table S3 for adjusted means and asymmetrical SEs.

community is predicted by the floral morphology of each population. Populations with long styles, higher volumes of nectar, and larger flowers are visited predominantly by long proboscis flies, while populations with short styles, less nectar, and smaller flowers are visited only by functionally smaller pollinators. Moreover, we show that the greater the morphological similarity between any pair of populations, the greater the similarity in the visitor fauna. Of the floral traits under study, we focused on the trait most closely associated with the functional size of the visiting insect, namely style length. We found a strong positive association between average style length of *N. humilis* flowers

and the weighted grand mean of visitor functional body length in each population. This type of geographic trait matching among pollinator–plant populations has been found in other studies (e.g., Steiner and Whitehead 1990; Anderson and Johnson 2008; Pauw et al. 2009; Newman et al. 2014) and appears to be a general trend among specialist plant species and their pollinators (Anderson et al. 2010b; Armbruster et al. 2014). Geographic associations between plant morphology and pollinator species, as well as trait matching at the population level, are both expected outcomes of local adaptation in plant–pollination interactions (Van der Niet et al. 2014a). They provide supportive evidence that plants are

locally adapted to phenotypic differences in their visitors at each site, or that visitors are locally adapted to plants at each site (Boberg et al. 2014; Sun et al. 2014). Alternatively, both plant and visitor traits may be phenotypically plastic and similarly affected by an external factor, such as temperature or altitude (Strauss and Whittall 2006). Although we have not demonstrated a genetic basis to the trait differences that we identified, several published studies show heritability in floral tube length (Campbell 1996; Worley and Barret 2000), a trait which is frequently correlated with style length. In addition, we observed that considerable interpopulation variation in style length in *N. humilis* is maintained in the bulb collections of growers, suggesting a heritable component to this trait.

Despite multiple possible sources of variation in floral morphology, floral form has frequently been used to make predictions about pollinator identity, and associations between the two have been used to infer pollinator-driven evolution (Valente et al. 2012). A prominent example is pollination syndromes, defined as suites of floral traits associated with particular pollinators (Johnson 2010). Thus, sunbird pollinated plants often have unscented, red, tubular flowers, plenty of nectar, and a structure for birds to perch on while foraging (Anderson et al. 2005a), whereas moth pollinated flowers are often recognized by pale flowers with long tubes and nocturnal fragrance (Johnson 2010). Although the concept of pollination syndromes is pervasive in the pollination literature, their predictive power remains controversial among some authors (Rosas-Guerrero et al. 2014). Here, we show that syndromes may extend to the level of populations in *N. humilis*, and that floral morphology still has persuasive predictive power about the identity of important floral visitors even at the population level. Nevertheless, patterns of association on their own do not actually show that there is, or has been, divergent selection by pollinators acting on floral traits. Nor are correlative patterns able to determine whether plants are adapting to local pollinator traits or vice versa (Nuismer et al. 2010). For this, we used experimental approaches to elucidate the mechanisms behind the patterns of association.

MECHANISMS BEHIND PATTERNS OF ASSOCIATION

We demonstrated at both sites that local phenotypes set more seed than introduced phenotypes, suggesting that populations of *N. humilis* are locally adapted to each site. Through mechanical fit experiments, we showed that plants are in fact locally adapted to floral visitors, thereby enabling us to identify them as the agents of selection. In preference experiments, the site-by-phenotype interaction was not significant, despite the appearance of a local adaptation pattern (Fig. 6A). This suggests either that differences in floral phenotypes are not the result of differences in pollinator choice or that pollinator choice plays a weak role in driving floral divergence of these two populations. The weakness of the

interaction effect is unlikely to be an artifact of low replication because the replication in this experiment was more than three times greater than any of the other experiments presented here. Instead, it suggests that performance differences in reciprocal translocations may rather be explained by traits relating to other mechanisms such as the mechanical fit between pollinators and flowers (cf. Figs. 5 and 6D).

We found long-proboscid flies to be much more effective visitors of the long-style phenotype, making frequent good contact with the stigmas of the flowers (Figs. 1A and 6B), resulting in good pollen deposition (Fig. 6C) and high seed set (Fig. 6D). In contrast, long-proboscid flies seldom made contact with the stigmas of short-style flowers (Figs. 1B and 6B), seldom deposited pollen on these stigmas (Fig. 6C), and their visits resulted in a lower proportion of fertilized ovules (Fig. 6D). The opposite was true of honey bees that frequently made contact with short-style flowers (Fig. 1D, Fig. 6B) but not long-style flowers (Figs. 1C and 6B), resulting in good pollen deposition (Fig. 6C) and a higher proportion of fertilized ovules in short-style flowers than in long-style flowers (Fig. 6D). This suggests that the proportion of fertilized ovules produced may be strongly influenced by the morphological match between flower and pollinator. Because anthers are positioned similarly to stigmas in *N. humilis*, the “lock-and-key-fit” is also likely to affect components of male fitness, such as pollen export. Consequently, aspects of male fitness may also select on floral morphology such as the length of the anther filaments and their reciprocal correspondence with style length. Contact data demonstrated that performance differences in pollen receipt and seed set are not just the result of introduced phenotypes being in a population in which pollen is placed on some other part of the pollinator body. Instead, performance differences are the result of introduced phenotypes having a poor morphological fit with the local pollinators.

An alternative pollinator-driven explanation for variation in floral morphology is that floral traits evolve to exclude less efficient pollinators (e.g., Johnson et al. 2006) rather than being adaptations to a particular pollinators. Tube length is a case in point: it may have evolved either to exclude inefficient pollinators (e.g., Borrel 2005) or as an adaptation to the morphology of a specific pollinator (sensu Darwin 1862). In reality, it is often difficult to disentangle these differences. Style length variation in *N. humilis* is an unusually informative trait to study because its origins are less ambiguous. Style length does not inhibit access of pollinators to nectar, and so variation in this trait is most likely to influence only the efficacy of pollen transfer and not the likelihood that different pollinators will visit. Because long styles (unlike long nectar tubes) do not restrict access to nectar, style length is unlikely to select for longer proboscis lengths, whereas tube length may (e.g., Pauw et al. 2009). Nevertheless, our data suggest that the functional length of insects may be an important

selective force on style length in *N. humilis*. Thus, style variation among populations appears to represent adaptation of plants to pollinators and not vice versa. In a similar way, the spurs of nectarless orchids appear to track the coevolutionary races between rewarding plants and their pollinators, without selecting on the pollinators themselves (Anderson et al. 2005b; Anderson and Johnson 2009; Anderson et al. 2010b; Thompson 2013). This so-called adergent evolution (Anderson et al. 2005b) is in contrast to coevolution where both pollinators and plants adapt to one another, as has been proposed for many long-tubed plant systems (Darwin 1862; Muchhala and Thomson 2009; Pauw et al. 2009).

Our findings lend support to the pattern-based approaches of macroevolutionary studies, which so frequently associate shifts in pollinators with plant speciation (van der Niet and Johnson 2012). Most importantly, we illustrate how floral morphology affects the efficiency of pollen transfer, and that the presence of morphologically different visitors selects for differences in floral morphology. This positively identifies pollinators as agents of floral selection in *N. humilis*, and demonstrates some of the mechanisms behind morphological trait matching in pollinators and plants. We show that adaptations to pollinators can lead to geographic divergence in floral traits, and that different forms of *N. humilis* can be considered as pollination ecotypes (Van der Niet et al. 2014a), namely local forms that have diverged as a result of pollinator differences.

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DATA ARCHIVING

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Cluster analysis based on the relative abundances of different visitors to each population.

Figure S2. Fruits used to determine the proportion fertilized ovules in field and laboratory experiments.

Figure S3. Pairwise correlation of population similarity of floral traits (style length, tepal length, nectar volume, and nectar concentration) and pairwise population similarity of visitor composition used in the Mantel test.

Figure S4. Reflectance spectra indicating color differences of flowers native to reciprocal translocation localities (A) NKP, Nuwe Kloof Pass and (B) SK, Skurwekop.

Table S1. Observation data, documenting all visitors recorded from short-style (S) and long-style (L) localities one to 11.

Table S2. Means \pm SEs, and (number) of insect visitors measured to obtain the functional visitor length in (mm) at short (S) and long-style (L) localities used in the linear regression analysis.

Table S3. Adjusted means and SEs from back-transformed values of the linear predictor variable, used in analysis of local adaptation and the underlying mechanisms of local adaptation.

Appendix S1. Measurements of functional floral and visitor traits.

Appendix S2. Effects of cut stems and travel on seed set.

Appendix S3. Testing for interphenotype incompatibilities.