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THE INFLUENCE OF POLLINATOR PHYLOGEOGRAPHY AND MATE PREFERENCE ON FLORAL DIVERGENCE IN A SEXUALLY DECEPTIVE DAISY

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Divergent mate preferences and subsequent genetic differentiation between populations has been demonstrated, but its effects on interspecific interactions are unknown. Associated species exploiting these mate preferences, for example, may diverge to match local preferences. We explore this idea in the sexually deceptive, fly-mimicking daisy, *Gorteria diffusa*, by testing for association between genetic structure in the fly pollinator (a proxy for mate preference divergence) and geographic divergence in floral form. If genetic structure in flies influences interactions with *G. diffusa*, we expect phylogeographically distinct flies to be associated with different floral forms. Flies associated with forms exploiting only feeding behavior often belonged to several phylogeographic clades, whereas flies associated with forms exploiting male-mating behavior always belonged to distinct clades, indicating the possibility of pollinator-mediated floral divergence through phylogeographic variation in mating preferences of male flies. We tested this hypothesis with reciprocal presentations using male flies from distinct clades associated with separate floral forms. Results show that males from all clades exhibit similar preferences, making pollinator driven divergence through geographic variation in mate preference unlikely. Males, however, showed evidence of learned resistance to deceptive traits, suggesting antagonistic interactions between plants and pollinators may drive deceptive floral trait evolution in *G. diffusa*.

KEY WORDS: **Coevolution, floral diversification, learning, mate preferences, pollinator-mediated selection, sensory trap..**

Adaptation to local environments often results in genetic divergence between populations, at least at selected loci (Galen et al. 1991; Carroll et al. 1997; Quinn et al. 2000). Such local adaptation can facilitate reproductive isolation when, for example, it influences the evolution of mating signals and the perception of signal receivers (e.g., sensory drive; Boughman 2002). Under this hypothesis, the effectiveness of a signal will likely depend on how well it matches the receiver's perceptive abilities and these elements of communication systems will codiverge between populations experiencing different environments (Boughman 2001; Seehausen et al. 2008). Genetic divergence in neutral markers typically follows (Seehausen et al. 2008) and may therefore potentially be indicative of divergent mate preferences. Some studies investigating this link within invertebrates have found that preference for local mates over foreign ones is stronger with increased genetic divergence between them (Sutherland et al. 2010). Local mate preference has also been reported in vertebrate taxa comprising genetically distinct groups (Knight and Turner 2004; Wong et al. 2004).

If phylogeographic variation does reflect divergent mate preferences, it could also have interspecific effects by driving phenotypic divergence in closely interacting species that exploit

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these mating preferences. Sexually deceptive plants in particular present a promising system for investigation of this idea. The flowers of these plants actively mimic female-specific olfactory (Schiestl et al. 2003) and visual (De Jager and Ellis 2012) signals, which male insects respond to and achieve pollination through successful elicitation of mating behavior from mate-searching males. These flowers are therefore acting as a sensory trap, exploiting biases within males that evolved outside of a foraging context. Because pollination success is directly correlated with the intensity and frequency of mating behavior that plants elicit from male pollinators (Ellis and Johnson 2010; Gaskett 2011), sexually deceptive flowers will be subject to sexual selection exerted by males through mate preference. Variation in mating preferences between geographically separated populations of their male pollinators may subsequently lead to divergence in the flowers that are under selection to deceive these males (Mant et al. 2005), explaining why floral phenotypes of some sexually deceptive plants vary across their ranges.

Gorteria diffusa Thund., a South African daisy comprising 14 geographically distinct floral forms (Ellis and Johnson 2009), elicits mating behavior from its male pollinators with fly-mimicking spots on its ray florets (Ellis and Johnson 2010). The bee fly *Megapalpus capensis* Wiedemann pollinates all of the *G. diffusa* floral forms and is its main and often only visitor throughout the flowering season (Ellis and Johnson 2009). Although feeding behavior in both male and female flies is elicited by all floral forms, only three forms elicit mating behavior from *M. capensis* males (Ellis and Johnson 2010), which actively search for female flies and often interact with potential mates within inflorescences (De Jager and Ellis 2012). These sexually deceptive forms are separated geographically and differ significantly in floral phenotype, including the petal spot ornaments with which *M. capensis* males attempt to mate (Ellis and Johnson 2009).

One explanation for this pattern could be that phylogeographically distinct *M. capensis* males from different areas vary in mate preference and thus exert differential selective pressures on their local *G. diffusa* populations, thereby contributing to diversification of the sexually deceptive forms. To test this intriguing hypothesis systematically, we first investigated genetic structure within *M. capensis* and determined whether genetically similar flies are associated with the same floral forms of *G. diffusa*, and whether this association is stronger for sexually deceptive forms than for the less specialized feeding forms. We then conducted reciprocal presentation experiments with genetically distinct males, which may represent divergent mating preferences, to determine if males exhibit more mating behavior on the fly-mimicking spots of their local sexually deceptive floral forms. Such a pattern would offer support for local adaptation to male-mating preferences driving floral diversification in sexually deceptive *G. diffusa*.

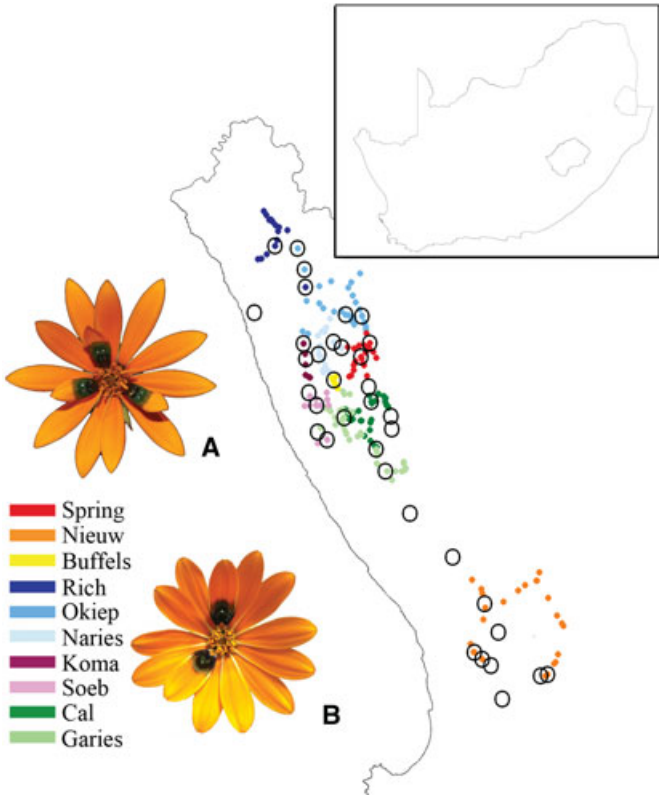

Figure 1. The range of *G. diffusa* within Namaqualand in South Africa, color coded by the floral forms described in Ellis and Johnson (2009). Black rings indicate locations where we sampled and sequenced their pollinator, *M. capensis*. The two sexually deceptive forms investigated in our reciprocal pollinator behavior experiments are shown in (a) Spring and (b) Nieuw.

Materials and Methods

GENETIC SAMPLING AND LABORATORY PROTOCOL

M. capensis individuals of both sexes were collected from 36 sites across Namaqualand in South Africa where its range coincides with *G. diffusa*'s (Fig. 1). In addition, outgroup samples (*Corsomyza* sp. in the Bombyliid subfamily Mariobezziinae with *Megapalpus*) were collected at two locations. Fly vouchers are held in the AGE collection at Stellenbosch University (Matieland, South Africa). For each fly sample we recorded the local floral form of *G. diffusa* that grows in the area where it was caught. All samples were preserved in 95% ethanol and we extracted genomic DNA from each fly following the Promega Wizard Genomic DNA purification kit (Madison, WI) protocol. Fragments of both mitochondrial (*cox1* and *cox2*) and nuclear genes (*EF-1α*) were amplified. A total of 92 samples were amplified for *cox1* with a universal primer pair (LCO1490 5′-GGTCAACAAATCATAAAGATATTGG-3′ and HCO2198 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) yielding a 623bp fragment of the gene. We selected a subset of

31 representatives from this dataset (representing all the major *cox1* clades) for the amplification of additional genes, including *cox2* (C457B 5′-AACTAGTATCCTTTCATGAYCAYGC-3′ and C457C 5′-GTGATTAGCACCGCARATYTC-3′), which yielded a 478bp fragment and the intronless nuclear *EF-1α* gene (EF-F05 5′-CCTGGACATCGTGATTTCAT-3′ and EF-F06 5′-TTACCTTCAGCGTTACCTTC-3′), which yielded a 303bp fragment of DNA. Sequences are available at GenBank (http://www.ncbi.nlm.nih.gov/genbank/) under accession numbers KC561981-KC562140.

Every 50 μl PCR reaction contained 1.5 mM $MgCl_2$, 1 μM of the forward and reverse primers each, 0.2 mM of each dNTP, 1 unit Taq polymerase (Super-Therm JMR-801; Southern Cross Biotechnologies, Cape Town, South Africa), 5 μl 10× Buffer (Southern Cross Biotechnologies, Cape Town, South Africa), 25 μl dH_2O and about 100 ng (1 μl) of DNA template. For the PCR amplifications we used the following thermal regimes (*cox1/cox2/EF-1α*): a denaturation step of 94°C for 5 min/4 min/3 min followed by 30 cycles of 94°C for 1 min/30 sec/30 sec, 50°C for 30 sec/50°C for 1 min/45.9°C for 30 sec, and 72°C for 1 min/2 min/90 sec. All were followed by a final elongation step at 72°C for 5 min/10 min/10 min. Amplifications were performed on a Labnet Multigene gradient PCR thermal cycler (Sigma-Aldrich, St. Louis, MO). PCR products were stained with ethidium bromide and run on a 1% agarose gel for confirmation under ultraviolet light. Products were purified using QIAquick® spin columns (Qiagen, Valencia, CA) and sequenced in the forward direction only with a BigDye terminator kit version 3.0 (Applied Biosystems, Foster City, CA) and analyzed on an ABI 3130XL Genetic Analyzer (Applied Biosystems) as preliminary sequencing in both directions yielded clean and unambiguous sequences. Any ambiguous sites in the nuclear dataset were coded using the IUPAC codes. Trace files were imported into MEGA v.4 (Tamura et al. 2007) and edited by hand before alignment with ClustalW (Thompson et al. 1994).

PHYLOGEOGRAPHIC ANALYSIS

We conducted two analyses, one using only the *cox1* dataset (*Cox1*) and the other using only the representatives that had been sequenced for both mitochondrial genes, as well as the nuclear gene (*Combined*). Using the Akaike information criterion (AIC; Akaike 1974) as implemented in jModelTest v0.1.1 (Posada 2008), the HKY + I + G model of sequence evolution was selected for the *Cox1* analysis. The dataset was partitioned into three codon positions with substitution rates, rate heterogeneity, and base frequencies unlinked across codon positions and run with a strict molecular clock and an uncorrelated lognormal relaxed molecular clock using a mean substitution rate of 1.7% sequence divergence per million years for both clock runs (*Drosophila* mtDNA; Brower 1994). Bayes factors were used to determine which model performed best (with $2\ln BF_{10} \geq 2$ indicating positive support for model 1 over model 0; Nylander et al. 2004). We also ran the best model without partitioning by codon positions and compared it with the partitioned model using Bayes factors.

For the *Combined* analysis we partitioned the various genes and selected the HKY + I + G, HKY + G, and HKY + G models for the mitochondrial (*cox1*, *cox2*) and nuclear (*EF-1α*) genes, respectively, as determined by AIC. We partitioned each gene by the 1st + 2nd and 3rd codon positions and ran it with an uncorrelated lognormal relaxed molecular clock using a mean substitution rate of 1.7% for the mitochondrial genes and 1.1% for the nuclear gene (*Drosophila* nuclear DNA; Tamura et al. 2004). We also ran this model without partitioning by codon positions and used Bayes factors to determine which model performed the best given our data. Markov chain Monte Carlo (MCMC) were run for 20 million (*Cox1*) and 60 million (*Combined*) generations in BEAST v1.5.3 (Drummond and Rambaut 2007), sampling parameters every 2000 or 6000 states, respectively. Starting trees were randomly generated and a constant size coalescent prior was selected for all tree models. Results were checked in Tracer v1.4 (Rambaut and Drummond 2007) for reliable effective sample sizes and convergence of MCMC likelihoods. We ran the preferred models identified with Bayes factors twice and combined results with Log Combiner v1.5.3, discarding the first 10% of samples as the burnin phase in each case. Resulting trees were summarized with TreeAnnotater as part of the BEAST package and viewed and edited in FigTree v1.3.1 (Rambaut 2006).

ASSOCIATION BETWEEN *M. CAPENSIS* PHYLOGEOGRAPHIC CLADES AND *G. DIFFUSA* FLORAL FORMS

To determine whether flies associated with the same *G. diffusa* forms grouped together genetically, we employed randomization tests where we shuffled the floral forms associated with our fly samples across the tips of our *Cox1* tree (see Fig. 2). Only flies associated with a *G. diffusa* floral form were used, and only floral forms associated with more than one fly sample were shuffled. We randomized this dataset for 1000 iterations in Excel. During each we calculated, for every floral form, the percentage of associated flies that fell within each of the clades (determined in our phylogeographic analysis as all samples sharing a common ancestor within each of the main Namaqualand groups), controlling for sample size differences in the number of flies caught within the range of every floral form. For every floral form we then collected during each iteration the maximum percent clade membership (MPCM—the percentage of flies that belonged to the fly clade most frequently associated with that floral form) to create a null distribution of the expected probability of each MPCM category in 10% increments. Significant deviation from random

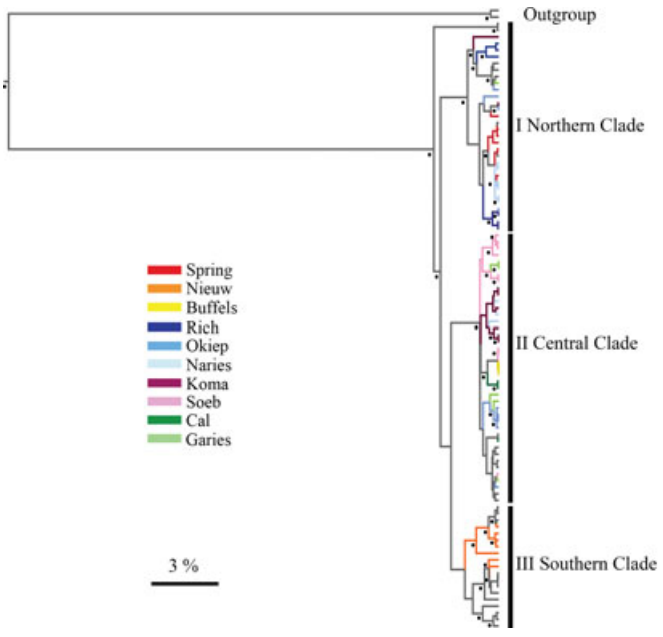

Figure 2. Maximum credibility tree based only on the *Cox1* dataset with posterior probabilities above 0.9 indicated by dots alongside nodes. Sampled *M. capensis* individuals resolve into three Namaqualand clades, as well as a basal coastal group comprising a single population. The floral form of *G. diffusa* associated with each fly sample is indicated using the colors from Figure 1. Tips in gray represent flies caught in areas where no *G. diffusa* grows. Scale bar represents percent divergence.

association was determined using one-tailed tests (i.e., flies within the range of a given floral form exhibit significantly higher MPCM than expected). We also calculated the mean of MPCM categories across all floral forms from randomizations and compared this to the observed average MPCM of flies associated with sexually deceptive floral forms that mimic female-mating signals and feeding floral forms that do not.

In addition to our randomization tests, we ran an analysis of molecular variance (AMOVA; Excoffier et al. 1992) in Arlequin 3.10 (Excoffier et al. 2005) on our only dataset with sufficient sample size (*Cox1*), using sampling sites as populations to determine whether genetic variation of the flies was structured by the local floral form present in each area (i.e., most genetic variation is found between flies associated with different floral forms). Fly samples that were not associated with *G. diffusa* were excluded and all samples were grouped by the floral form they were associated with.

LOCAL ADAPTATION EXPERIMENTS

To test if variation in pollinator mate preferences can drive the divergence of sexually deceptive floral phenotypes, we investigated whether phylogeographically distinct male flies associated with different sexually deceptive floral forms exhibit more mating behavior on the fly-mimicking spots of their local floral form. We employed reciprocal presentation experiments where we exposed male flies from two different clades to two floral arrays in random order; one containing a local floral form they are familiar with and the other a foreign form they had not encountered before. We used the Spring and Nieuw floral forms (Fig. 1) which are widely separated geographically (over 150 km) and differ significantly in floral phenotype, including their fly-mimicking spots (Ellis and Johnson 2009). *M. capensis* males from both these areas regularly exhibit mating behavior in response to these spots (Ellis and Johnson 2010).

Male flies were caught close to the towns of Springbok (29°39′14.5″S, 17°53′20.9″E, $N = 16$) and Nieuwoudtville (31°22′46.8″S, 19°5′38.1″E, $N = 15$). We released each male into a 1 m^3 pollinator cage containing a floral array composed of 12 fresh inflorescences (replaced as necessary) of a given floral form for 10 min, before resting it for at least 10 min and exposing it to the floral array of the second floral form for 10 min. We recorded how many visits each male made to the inflorescences within each array, as well as their behavior during these visits. Behavior was categorized as sitting (inactive or grooming), feeding (actively consuming pollen or nectar with an extended proboscis), or mating. Mating behavior was only exhibited on the fly-mimicking spots and is composed of various motor responses including *inspecting* (quick landings on spots < 1 second), *changing* (flitting between different spots within an inflorescence), *hopping* (repeatedly hopping and arching abdomen downwards on the spot), and *turning* (rotating on the spot). We also recorded the amount of time males spent exhibiting the various behaviors during each visit, as well as whether they landed on the fly-mimicking spots when visiting the inflorescences of a given array. We compared all results of individual male fly behavior between the two floral arrays using paired *t*-tests or Wilcoxon matched pairs test, depending on the normality of the data.

In addition to these reciprocal presentation experiments with experienced males from within the ranges of the Spring and Nieuw floral forms, we also used the same experimental design to investigate males from an area between their respective ranges where no *G. diffusa* occurs (30°12′33.3″S; 18°2′58.4″E, $N = 10$). To these males, both the Spring and Nieuw forms are foreign and this experiment thus investigates the innate preference of naïve males for the female fly cues which *G. diffusa* mimics. For this experiment, we also employed paired *t*-tests/Wilcoxon matched pairs tests. Using naïve males in an identical experimental design also offers us the opportunity to investigate any putative differences between experienced and naïve males regarding their response to sexually deceptive inflorescences. To do this we compared the pollination behavior and time spent per visit exhibiting these behaviors between naïve and experienced males from different areas on both the Spring and Nieuw forms using *t*-tests for independent

samples or Mann–Whitney *U*-tests where applicable. We used the SPSS 19 statistical package for all analyses (SPSS Inc., Chicago, IL) and conducted all experiments at the Succulent Karoo Knowledge Centre in Kamieskroon (30°12′20.6″S, 17°56′12.1″E) and in Nieuwoudtville (31°22′46.8″S, 19°5′38.1″E) during August and September 2009 and 2010 on warm sunny days.

Results

PHYLOGEOGRAPHIC ANALYSIS

Our *Cox1* dataset contained DNA sequences from 92 *M. capensis* flies from 36 locations (median of three flies per location) and 15.41% of the characters were Parsimony-Informative. Bayes factors indicated that the uncorrelated lognormal relaxed molecular clock analysis performed better than both the strict clock analysis ($2\log_e BF_{1-0} = 5.29$) and the uncorrelated lognormal relaxed molecular clock without partitioning by codon position analysis ($2\log_e BF_{1-2} = 11.02$). Based on our Bayesian analysis of the *Cox1* dataset *M. capensis* is monophyletic and separates into three main Namaqualand clades (northern, central, and southern), as well as a fourth basal group sister to these clades, which comprises samples from a single population along the Namaqualand coast (Fig. 2). For the *Combined* dataset (containing 31 *M. capensis* sequences from 25 locations) 9.13% of the *cox1* characters, 9.83% of the *cox2* characters and 5.94% of the *EF-1*α characters were Parsimony-Informative. The uncorrelated lognormal relaxed molecular clock analysis with partitioning by two codon position also performed better than the same analysis without partitioning ($2\log_e BF_{1-0} = 9.24$). From the *Combined* analysis we retrieved the same clades as with the *Cox1* analysis with better nodal support (Fig. 3).

The northern clade of *M. capensis* flies in our *Cox1* analysis was associated with the Rich, Spring, Garies, Naries, Okiep, and Koma forms of *G. diffusa*, some of which exist in northern Namaqualand (see Fig. 1). The Central clade was associated with the Soeb, Garies, Naries, Koma, Buffels, Cal, and Okiep forms, which mostly exist in central Namaqualand, including the coastal plain to the west. The Southern clade was associated only with the Nieuw form, which exists in a wide distribution within southern Namaqualand. The association between the phylogeographic pollinator clades from our *Cox1* analysis and the floral forms of *G. diffusa*, however, was clearly not absolute, as flies associated with four of the 10 floral forms belonged to more than one clade of *M. capensis* (see Table 1 for the number of flies analyzed for each floral form). Our randomization tests revealed that flies associated with two sexually deceptive floral forms and two feeding forms showed significant genetic structuring by exhibiting a higher observed MPCM than expected under random association (Table 1). It is, however, important to note that for all sexually deceptive floral forms the flies caught within their respective ranges belonged to single clade (i.e., MPCM = 100%), although this only occurred for less than half of the feeding forms. The average observed MPCM of flies associated with sexually deceptive *G. diffusa* also exhibited overall significant genetic structuring (Table 1). All flies associated with the sexually deceptive Spring form belonged to the Northern Namaqualand clade, whereas all flies associated with the sexually deceptive Buffels and Nieuw forms belonged to the Central and Southern Namaqualand clades, respectively (Fig. 3). Male flies from these three clades were chosen to be used in our reciprocal presentation experiments and

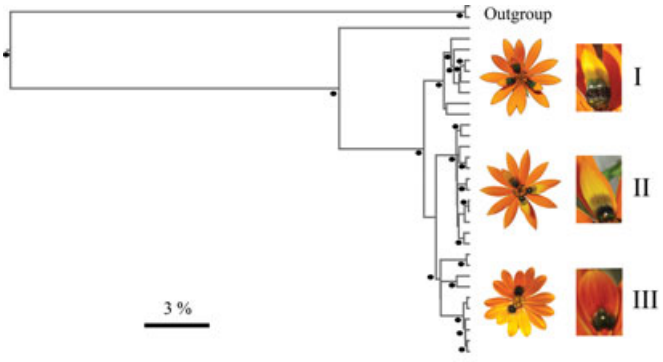

Figure 3. **Maximum clade credibility tree based on the *Combined* dataset showing the same topology as the *Cox1* dataset. Nodes with higher than 0.9 posterior probability are indicated with dots. Namaqualand clades designated in the *Cox1* tree are indicated by Roman numerals. The sexually deceptive floral forms associated with each of these clades are depicted to the right; with a close-up of the petal spot of each form that *M. capensis* males attempt to copulate with. Scale bar represents percent divergence.**

Table 1. **The observed maximum percentage clade membership (MPCM) of flies caught (*N*) within the range of every *G. diffusa* floral form and the expected probability of that percentage occurring under random association between flies and floral forms; **P* < 0.05.**

Flies from (*N*)	Functional type	Observed MPCM	Expected probability
Spring (6)	Sexual	100	**0.0140***
Nieuw (7)	Sexual	100	**0.0080***
Buffels (3)	Sexual	100	0.1980
Rich (6)	Feeding	100	**0.0120***
Okiep (9)	Feeding	56	0.3990
Naries (9)	Feeding	67	0.2460
Koma (8)	Feeding	75	0.1300
Soeb (9)	Feeding	100	**0.0040***
Cal (3)	Feeding	100	0.2010
Garies (6)	Feeding	83	0.1360
Average for	Sexual forms	100 ± 0	**0.0455***
Average for	Feeding forms	83 ± 18	0.0502

from here on we refer to them as "Spring," "Buffels," and "Nieuw" clade males, respectively.

The AMOVA analysis revealed that genetic variation was significantly structured by the floral form with which flies were associated, with 29.07% of the variation found among groups ($F_{CT} = 0.291$, $P < 0.001$). Other major sources of genetic variation were found among populations within groups at 28.39% ($F_{SC} = 0.400$, $P < 0.001$) and within populations at 42.53% ($F_{ST} = 0.575$, $P < 0.001$).

LOCAL ADAPTATION EXPERIMENTS

Results from our experiments investigating the preferences of genetically distinct *M. capensis* males for the fly-mimicking spots of *G. diffusa* revealed that there is no difference between phylogeographic clades. Experienced males from both the "Spring" (Wilcoxon Matched Pairs Test $Z = 2.98$, df $= 15$, $P = 0.003$) and "Nieuw" ($Z = 2.47$, df $= 14$, $P = 0.013$) clades exhibited significantly higher proportions of total visits including mating behavior on the Spring floral array (Fig. 4A, B). In addition, naïve males from the "Buffels" clade also exhibited significantly higher proportions of mating visits on the Spring array ($Z = 2.55$, df $= 9$, $P = 0.011$; Fig. 4C), indicating that the Spring floral form is more effective at exploiting innate mating preferences of *M. capensis* males and that these preferences are essentially the same throughout its range in Namaqualand. Experienced "Spring" and "Nieuw" clade males also spent significantly more time per visit exhibiting mating behavior on the Spring array ($Z = 2.90$, df $= 15$, $P = 0.004$; $Z = 2.12$, df $= 14$, $P = 0.034$, respectively), despite making the same number of visits to each array (Table 2). In line with the Spring form being a more successful fly-mimic, "Nieuw" clade males landed on the fly-mimicking spots of Spring more often ($Z = 2.50$, df $= 14$, $P = 0.012$).

"Nieuw" clade males, however, spent significantly more time per visit being active on and feeding from the Nieuw form ($Z = 2.56$, df $= 14$, $P = 0.011$; $Z = 2.61$, df $= 14$, $P = 0.009$, respectively). The naïve males from the "Buffels" clade mirrored this pattern and spent more time per visit being active on and feeding from the Nieuw form ($Z = 1.99$, df $= 9$, $P = 0.047$; $Z = 2.09$, df $= 9$, $P = 0.037$, respectively), suggesting that Nieuw is relatively better at eliciting a feeding response from male flies and may be a better food source. In contrast to the similar number of visits made by experienced males from the "Spring" and "Nieuw" clades to the two floral forms, naïve "Buffels" clade males made almost fourfold as many visits to the Spring array (Paired *t*-tests $t = -2.54$, df $= 9$, $P = 0.032$). During visits they also spent more than five times as much time exhibiting mating behavior on the Spring array ($Z = 2.67$, df $= 9$, $P = 0.008$). This result confirms that the Spring form is clearly more effective at eliciting mating responses from *M. capensis* males in all clades.

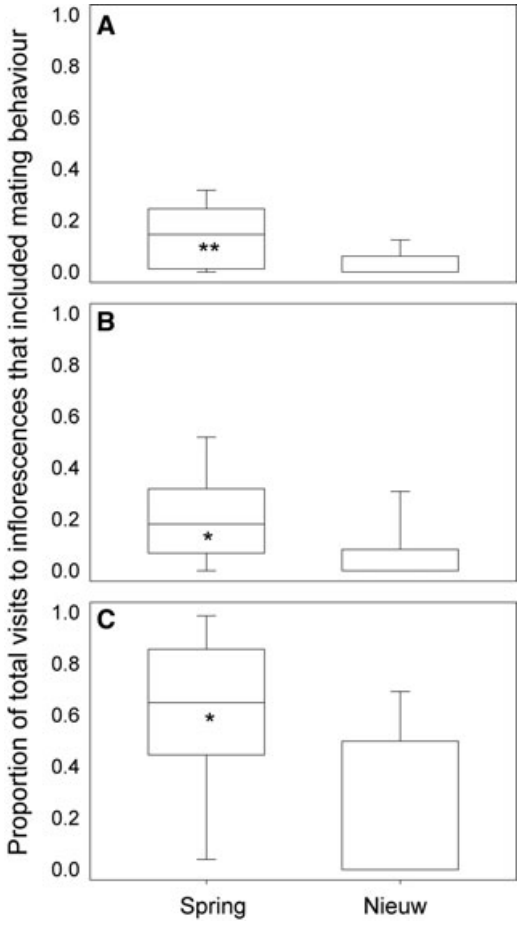

Figure 4. The proportion of total visits to inflorescences of each sexually deceptive *G. diffusa* floral form that included mating behavior by (A) experienced "Spring" clade males, (B) experienced "Nieuw" clade males, and (C) naïve "Buffels" clade males. Medians with 5th and 95th percentiles are shown and significance is indicated by *$P < 0.05$ and **$P < 0.005$.

Our results also revealed that there are significant differences between the behavior of naïve males and males experienced with the fly-mimicking spots of *G. diffusa*. Experienced males landed on the deceptive spots of the Spring form significantly less often than naïve males (Mann–Whitney *U*-test $U = 37$, df $= 25$, $P = 0.023$; Fig. 5). They also exhibited mating behavior in much lower proportions of all visits ($U = 14$, df $= 25$, $P < 0.001$), and spent less time exhibiting mating behavior per visit than naïve males ($U = 29.0$, df $= 25$, $P = 0.007$). This is despite the fact that they did not exhibit any differences in the total number of visits they made, or the amount of time they were active per visit on the arrays (*t*-test $t = 1.45$, df $= 25$, $P = 0.159$; Mann–Whitney *U*-test $U = 46$, df $= 25$, $P = 0.073$, respectively). In contrast, there was no difference between naïve and experienced males on the Nieuw form for any of these measures, indicating that this form does not induce putative learned avoidance of mating behavior within *M. capensis* males.

Table 2. The number of visits made to inflorescences in each *G. diffusa* array by males from different clades, the proportion of visits where they landed on the fly-mimicking spots and the amount of time (sec) they were active per inflorescence visit, as well as the amount of time spent exhibiting feeding and mating behavior. Medians and quartile ranges (75th–25th percentile) are displayed; $*P < 0.05$, $**P < 0.01$.

Male clade	*G. diffusa* array	Total visits made	Prop visits landed on spot	Time active per visit	Time feeding per visit	Time mating per visit
Exp "Nieuw"	Nieuw	11 (12)	**0.4 (0.4)***	**37.8 (29.7)***	**37.5 (29.6)****	**0 (0.1)***
($N = 15$)	Spring	11 (18)	**0.8 (0.3)**	**15.3 (19.5)**	**15.2 (21.2)**	**0.3 (0.4)**
Exp "Spring"	Nieuw	10.5 (10.5)	0.51 (0.2)	23.2 (27.6)	23.2 (27.8)	**0 (0.1)****
($N = 16$)	Spring	14 (6.5)	0.61 (0.2)	20.5 (21.3)	20.3 (22.4)	**0.2 (0.8)**
Naïve "Buffels"	Nieuw	**5.5 (6)***	0.6 (0.6)	**19.1 (37.7)***	**19.1 (37.8)***	**0 (0.6)****
($N = 10$)	Spring	**20 (18)**	0.9 (0.4)	**8.3 (12.5)**	**2.3 (13.8)**	**2.3 (4)**

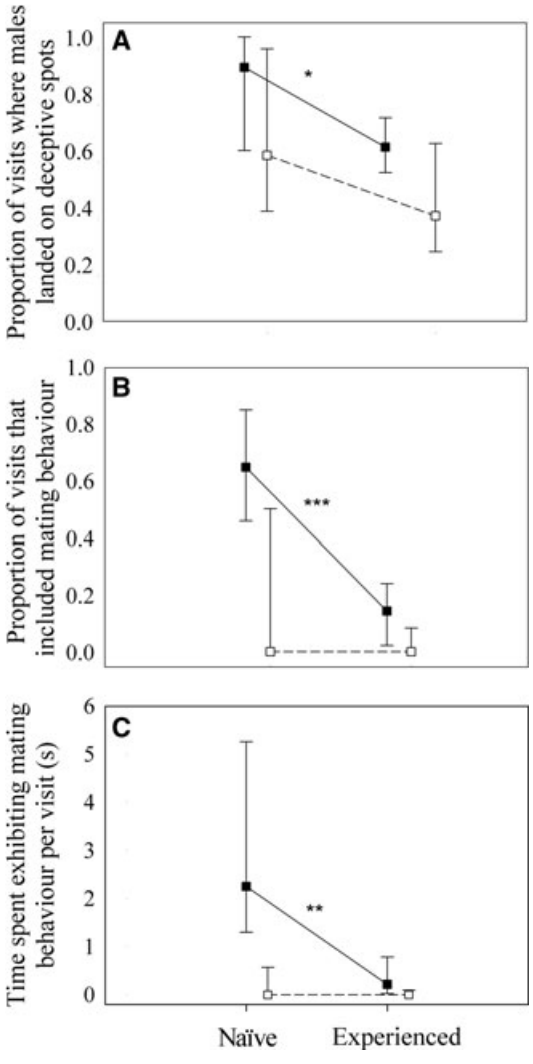

Figure 5. The difference between naïve and experienced male flies in (A) the proportion of visits where they landed on the deceptive spots, (B) the proportion of visits where they exhibited mating behavior, and (C) the amount of time they spent exhibiting mating behavior per visit on the sexually deceptive Spring (black squares) and Nieuw (open squares) forms. All plots show medians with their upper and lower quartiles; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Discussion

Our results show that *M. capensis* flies within Namaqualand fall into three well-supported phylogeographic clades. The association between genetically similar flies and *G. diffusa*'s floral forms was not absolute, as flies associated with four of the 10 floral forms we investigated belonged to more than one clade. Such patterns of incomplete association between plants and their pollinators are often reported within the well-studied yucca–yucca moth system (Leebens-Mack and Pellmyr 2004; Smith et al. 2009). Flies associated with sexually deceptive floral forms that elicit mating behavior from male flies, however, exhibited overall significant genetic structuring. This pattern may suggest a role for phylogeographic variation in male-mating preferences as a driver of floral divergence between sexually deceptive floral forms.

Our behavioral experiments clearly rejected this hypothesis as male flies from three distinct clades all exhibited significantly more mating behavior on the Spring form of *G. diffusa*. These results indicate either that there is no geographic variation in male-mating preferences in *M. capensis*, or that *G. diffusa*'s mimicry of *M. capensis* females has not responded to this level of variation. This latter explanation seems somewhat unlikely, as sexually deceptive *G. diffusa* are under selection to elicit copulation attempts from male flies that consistently contribute to significantly higher levels of pollen export compared to nonmating male or female visits (Ellis and Johnson 2010). Attempted copulation with fly-mimicking spots will only occur once males have selected the "female" (deceptive spot) on which to focus their efforts. Because the deceptive spots of the Spring form of *G. diffusa* elicits strong mating responses from males from all three of the *M. capensis* phylogeographic clades, any variation not mimicked by the Spring form is likely to contribute little to overall mate preference within *M. capensis* males.

Our results thus suggest that neutral genetic divergence within species does not necessarily indicate the potential for divergent mate preferences, and that mate preference within *M. capensis* males are uniform, although experiments with real females are required for confirmation. Some studies have reported

that preference for local mates over foreign ones only occurs after considerable genetic divergence between them (Sutherland et al. 2010). Within *Drosophila*, however, females have also failed to exhibited preference for essential sexual traits in local males over those of foreign males that are phylogeographically highly divergent (Klappert et al. 2007). Such patterns might be the result of selection to promote outbreeding through reduced sibling mating, as has been suggested for solitary bees that exhibit preference for exotic mates over local, presumably genetically similar, mates (Vereecken et al. 2007). These bees pollinate sexually deceptive orchids in the genus *Ophrys* and their preference for exotic sexual signals may considerably affect floral divergence in these plants (Vereecken et al. 2007). This mechanism is unlikely to drive plant–pollinator interactions within our study system as all *M. capensis* males exhibit clear and uniform preference for the female-mimicking spots of a single floral form. This preference is independent of geographic origin and genetic association of the males, indicating that phylogeographic structure does not play a large role in mating preferences within *M. capensis*. Rather, males throughout the landscape have similar preferences that appear to have driven the evolution of complex female-mimicking forms in allopatric populations of *G. diffusa*.

This is supported by the observation that only sexually deceptive forms of *G. diffusa* possess raised, multicellular papillate trichomes within their spots (Ellis and Johnson 2009), which have been shown to be crucial for the attraction of male, but not female flies (De Jager and Ellis 2012). This may of course also be the result of sharing a recent common ancestor and the evolutionary history of these forms is currently under investigation. Although the fly-mimicking spots of sexually deceptive forms share such similarities, there are nonetheless significant differences in their overall spot phenotype (Ellis and Johnson 2009). This implies that the same preference in a pollinator may result in similar, but not identical, phenotypes in different populations, determined by which mutations happen to occur in each. This phenomenon is also important in the sexually deceptive orchid genus *Ophrys*, where geographically separated species sometimes evolve similar phenotypes to exploit the mating preferences of a single pollinating species (Mant et al. 2005; Paulus 2006).

Although such one-sided evolution is a likely scenario, floral diversity may also be the result of more interactive relationships between plants and pollinators. If male pollinators suffer potential costs when deceived (Gaskett et al. 2008), they may learn to reduce the amount of mating behavior they exhibit on sexually deceptive flowers with time. Because the reproductive success of these plants is determined by the intensity and frequency of mating behavior they elicit from their male pollinators (Ellis & Johnson 2010; Gaskett 2011), this might exert selective pressure on flowers to increase deceptiveness (Wong and Schiestl 2002). This scenario may be less likely within *Ophrys* where deceived males quickly decrease their mating behavior on deceptive flowers with repeated exposure, but will renew their mating efforts if a new flower is introduced (Ayasse et al. 2000; Paulus 2006). This strongly suggests that they learn the identity of individual flowers, but not the actual signals used to mimic females and will therefore remain effective pollinators for other flowers in the population. In our study, however, experienced males were tested in a new location on inflorescences of the sexually deceptive Spring form they had not encountered before and still they exhibited significant reductions in mating behavior relative to naïve males, which implies they have learned to discriminate the deceptive spots of this form as female mimics.

This will certainly pose a reproductive cost for sexually deceptive *G. diffusa*, which achieve increased levels of pollen export only when they elicit mating behavior from male flies (Ellis and Johnson 2010). The costs suffered by male pollinators due to sexual deception have rarely received investigation and little is known about their strength and frequency (Gaskett 2011), which will likely determine the scope for antagonistic coevolution to operate within these systems. This process is important in pollination systems involving long-tubed flowers that place their pollen on the bodies of long-proboscid flies searching for nectar within their tubes. The fitness of both plant and pollinator is determined by the difference between their floral tube lengths and proboscid lengths and each benefits by outdistancing the other, resulting in a coevolutionary arms race (Pauw et al. 2009). Such arms races generate floral diversity between isolated populations of many long-tubed angiosperms, each linked to the proboscid length of their local pollinators (Anderson and Johnson 2009).

Similar processes may also promote floral divergence between isolated populations of sexually deceptive flowers, dependent on male pollinators possessing the necessary learning abilities to avoid deceptive flowers. Pollinator learning appears to be common within sexually deceptive pollination systems (Gaskett 2011) and learning in insects is known to comprise genetically based, and therefore heritable, variation (reviewed in Dukas 2008). This, together with the fact that *M. capensis* males show substantial variation in their mating responses toward a given sexually deceptive *G. diffusa* form, and that both *G. diffusa* and *M. capensis* have short annual life cycles, may make antagonistic coevolution likely between these two species. Owing to the allopatric nature (Ellis and Johnson 2009) and very low dispersal ability (M. de Jager, pers. obs.) of *G. diffusa* forms, putative arms races in different populations will run along different trajectories, which could promote floral diversification. Although coevolution is an established mechanism generating diversity within interacting species, its role in deceptive systems has not received much attention and its importance, as well as the potential cause and effect of pollinator learning requires much needed experimental investigation.

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