Floral tubes are often thought to be a consequence of adaptive specialization towards pollinator morphology. We explore floral tube length evolution within *Tritoniopsis revoluta* (Iridaceae), a species with considerable geographical tube length variation. We ask whether tube lengths of *T. revoluta* populations are associated with pollinator proboscis lengths, whether floral divergence occurs in the presence of different pollinators and whether floral convergence occurs between distantly related populations pollinated by the same pollinator. Finally, we ask whether tube length evolution is directional. Shifts between morphologically different pollinators were always associated with shifts in floral morphology, even when populations were very closely related. Distantly related populations had similar tube lengths when they were pollinated by the same pollinator. Shifts in tube length tended to be from short to long, although reversals were not infrequent. After correcting for the population-level phylogeny, there was a strong positive, linear relationship between floral tube length and pollinator proboscis length, suggesting that plants are functionally specialized on different pollinators at different sites. However, because tube length evolution in this system can be a bidirectional process, specialization to the local pollinator fauna is unlikely to result in evolutionary or ecological dead-ends such as canalization or range limitation.

1. Introduction

An outstanding feature of angiosperms is the diversity of their reproductive organs, flowers. Many floral and inflorescence traits appear to be primarily adapted for the attraction of pollen vectors to flowers, or to manipulate their foraging behaviour in ways that maximize pollen transfer efficiency ([1], but see [2]). Extensive variation in pollinator morphology and behaviour is thought to account for much of the richness in floral form. Pollinator species or functional groups of pollinators can be viewed as ecological niches, the exploitation of which often requires adaptive evolutionary shifts in floral design, resulting in floral diversification across heterogeneous pollinator landscapes [3,4]. This association between plants and their pollinators is thought to have been a key contributor towards the impressive radiation of angiosperms [5,6].

The primary conceptual model of pollinator-driven floral divergence has its roots in the writings of Grant [3] and Stebbins [7] and has been referred to as the Grant–Stebbins model of floral divergence [4] or the pollinator-shift model of floral divergence [8,9]. Here, geographically separated plant populations of the same species may experience different pollinator communities, and specialization on those different communities is thought to drive allopatric divergence [10–12]. This is expected to give rise to pollination ecotypes, geographically separated floral forms that differ because they are under selection from functionally different pollinators. Grant [3] and Stebbins [7] viewed ecotype formation as a primary and crucial step in the speciation process, a view that is shared by advocates of the ecological species concept [13]. Some of the strongest evidence for this comes from phylogenetic [14] studies on well-established species. For example, a review of macroevolutionary studies, mapping pollinators onto phylogenies, suggests that approximately 25% of diversification events are associated with
shifts in pollinator [14]. However, one draw-back of studying mechanisms of divergence in well-established species is that macroevolutionary patterns are not necessarily informative about factors underlying speciation events [15].

An alternative and more direct approach is to study selection and patterns of association between floral and pollinator phenotype in morphologically divergent populations of the same plant species [10]. Identifying factors associated with morphological change in populations that are not yet recognized as separate species presumably brings researchers closer to studying the initial stages of divergence. Despite the need to take a broad geographical, multi-population approach in the study of diversification (as emphasized by Thompson [16,17]), relatively few studies have done so for plant–pollinator relationships. Some of these studies have found strong associations between plant and pollinator traits across multiple populations [11,18–23], while others have demonstrated geographical mosaics in the patterns of selection imposed by pollinators on different plant populations [10,12]. Very few studies have integrated ecological data on pollination ecotypes with population-level phylogenetic/phylogeographic approaches (but see [24,25]). Such an integrated approach potentially enables researchers to control for the effects of relatedness on patterns of matching between plant and pollinator traits, and to ask questions about the directionality and frequency of trait evolution in the context of different kinds of pollinators.

Floral nectar tubes have been one of the most frequently studied traits in the context of pollinator-mediated selection and the match between nectar tube length and the length of pollinator mouthparts has been shown to be important in facilitating efficient pollen transfer between flowers and pollinators [9,26,27]. Floral nectar tube length is also sometimes considered a key innovation which enhances diversification rates [28] because long tubes reduce the number of pollinators able to use a flower and this consequent specialization is expected to intensify selection by pollinators [29]. In addition, diversification rates may be enhanced because specialization upon different pollinators may lead to reproductive isolation [30].

Radiations in the Iridaceae, and particularly in the genus Tritoniopsis (21 species, [31]) are associated with pollinator specialization and shifts between functionally different groups of pollinators that include nectar-foraging bees, oil-collecting bees, wasps, butterflies, birds, moths and flies [32,33]. Here, we examine tube length evolution and specialization towards morphologically different pollinators in populations of Tritoniopsis revoluta (Burm.f.). Tritoniopsis revoluta has a disjunct distribution across mountain ranges which generally run parallel with the coast of the Southern Cape, South Africa (figure 1). Tritoniopsis revoluta has pink

![Figure 1. A geographically orientated unrooted chloroplast haplotype network for Tritoniopsis revoluta. Populations of the same colour and patterning have identical haplotypes. All populations were fixed for a particular haplotype. The size of the circles represents the average tube length in each population. Numbers indicate base pair changes separating haplotypes. Filled black circles are populations that we were unable to find but which were recorded in the herbarium. (F...](http://rspb.royalsocietypublishing.org/Downloaded from http://rspb.royalsocietypublishing.org/ on April 18, 2017)
flowers with highly variable (40–70 mm) tube lengths [31], and their nectar is known to be consumed by long proboscid flies [32–34] and anthophorid bees [35]. Newman et al. [21] suggest that short- and long-tubed populations of *T. revoluta* are pollinator ecotypes, i.e. populations of the same plant species that are morphologically different because they are functionally specialized on different kinds of pollinator. Despite apparent morphological adaptations to long proboscid pollinators, bees with short proboscides are able to reach the nectar of long-tubed *T. revoluta* flowers when a scarcity of long proboscid pollinators allows the nectar to accumulate in the perianth tubes [35]. This stands in contrast to many other systems where short proboscid pollinators are not usually able to access the nectar from long-tubed flowers, while long proboscid pollinators can access nectar from both long- and short-tubed flowers [36]. Such an asymmetry in foraging ability may generate directionality in the evolution of tube lengths. For example, *Aquilegia* displays a unidirectional trend from short to long nectar spur lengths, with no reversals in spur length [8].

Through field observations and phylogenetic approaches, we examine functional specialization on pollinator morphology and its effects on tube length evolution. More specifically, we test the following hypotheses: if tube length variation is associated with pollination ecotypes, we expect that the floral tube lengths of *T. revoluta* populations should be closely matched with the proboscis lengths of their pollinators. This should hold, even after correcting for statistical non-independence of populations resulting from genetic relatedness (i.e. phylogenetic signal). We predict that shifts between pollinators with different proboscis length should be associated with differences in tube length morphology. Divergence in tube lengths should be found between closely related populations with different pollinators, and different lineages are expected to have converged in morphology when they are pollinated by the same pollinators. Distantly related lineages may also have similar morphology if ancestral character states are retained, in which case similarity would not be the result of convergence. Here, phylogeny can be used to distinguish between these two alternative hypotheses. Lastly, while directionality in tube length evolution (from short to long) may be expected, the trend may not be very strong due to the effects of nectar upwelling described above.

2. Material and methods

(a) Pollinator observations and trait measurements

We sampled seven of a total of 11 populations of *T. revoluta* represented in the collections of the Compton Herbarium (SA National Biodiversity Institute, Kirstenbosch). In addition, three new populations were discovered during field observations (figure 1). Pollinator observations were made in each of these populations. Floral visitors were considered as pollinators if they made contact with the reproductive parts of *T. revoluta* flowers in both the male and the female phases. Pollinator observations took place between 2005 and 2012 during the peak flowering time of each population (March and April). We found that pollinators were most active between 09.00 and 12.00, and so we confined our observations to this period. In each population, one or more observers would sit within a dense patch of flowers and record pollinating visitors. In the electronic supplementary material, table S1, we report the total number of days and observation hours made in each population. We captured fly and bee visitors from the populations and measured their proboscis lengths. Bee proboscis (glabrous antennae) lengths were measured after relaxing the pollinators in a humid jar for 2 days, which allows the tissue to soften and the proboscis to be pulled out using fine forceps. The distance from proboscis tip to head was measured with a steel ruler. Captured flies were killed using an ethanol injection and pinned while still fresh, stretching the centroparium of the proboscis to maximum extension. Proboscides were measured in this state of maximum extension. To avoid killing large numbers of individuals unnecessarily, proboscides of 19 flies from the Barry population were measured while the flies were still alive. Individuals were immobilized by holding each wing, and the centroparium of the proboscis was slowly extended as described above. The number of fly proboscides measured in each population was often restricted by the number of visitors observed and ranged between one and 27 individuals (see the electronic supplementary material, table S1 for details). Bee proboscis lengths did not vary much between and within populations, and we thus pooled measurements across all bee populations (see the electronic supplementary material, table S1). The tube lengths of 25 *T. revoluta* flowers were measured in each population. Measurements were from the point of the ovary to the top of the nectar tube, a point that corresponded to the point of fusion of the dorsal tepal.

(b) Evolutionary relatedness of *Tritoniopsis revoluta* populations

Fresh flower and leaf material was collected from randomly selected plants in nine *T. revoluta* populations during March 2007 and 2008, and from one additional population (Barry) in November 2013. DNA was extracted from 0.2 g of silica-dried plant material using the CTAB method [37] or DNeasy Plant Mini Kits (Qiagen). Extractions were purified using Illustra DNA and Gel Band Purification Kits (GE Healthcare UK Ltd) prior to amplification and sequencing where necessary. DNA was extracted from three individuals per population, except in the Gys population which had a bimodal tube length [38], where we sampled three short-tubed and three long-tubed individuals; and the Barry population, where two individuals were sampled.

For a phylogeographic analysis of *T. revoluta*, we amplified two chloroplast gene regions: (i) the trnV-ndhC intron, using the primers trnV and ndhC [39] and (ii) the trnG–trnS intergenic spacer, using the primers trnG and trnS [40]. PCR was performed using an Applied Biosystems thermal cycler (GeneAmp PCR System 2700) and reactions were prepared on ice in 30 μl volumes per reaction containing: 10–50 ng template DNA, 10× reaction buffer (Southern Cross Biotechnologies, Cape Town), 1 mM dNTP, 25 mM MgCl₂, 1 pmol μl⁻¹ of each primer and 1.5 units Taq (Southern Cross Biotechnologies, Cape Town). The program used for the DNA amplification consisted of an initial denaturation step of 5 min at 80 °C, followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and final elongation at 72 °C for 10 min. PCR products (30 μl) were run on a 1% agarose gel at 100 V for an hour. Successful PCR products were purified from the agarose gel, prior to sequencing, with the Illustra DNA and Gel Band Purification Kit (GE Healthcare UK Ltd). DNA sequencing was performed with the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer/Applied Biosystems), using the thermal cycle parameters at 96 °C for 10 s, at 55 °C for 5 s and at 60 °C for 4 min, for 25 cycles. These products were electrophoresed and detected on an ABI Prism 3100 automated sequencer (Central Analytical Facility, University of Stellenbosch), or purified PCR products were sent to Macrogen (Seoul, Korea) for sequencing. Sequences were edited, assembled and aligned in MEGA v. 4 [41]. *Tritoniopsis lata* was chosen as an outgroup taxon on the basis of morphological similarity [31] and a preliminary
Tritoniopsis genus tree provided by F. Forest (2009, unpublished data). While using a single outgroup taxon limits our ability to definitively confirm monophyly of *T. revoluta*, this should not impact our ability to detect multiple evolutionary shifts in response to pollinator morphology. Only four indels were present in the combined data matrix of 1411 bp and were excluded from the analyses. Appropriate substitution models for each gene region were determined using *MODELLTEST* v. 2.1.2 [42]. A GTR model with a proportion of invariant sites (I) and Gamma-distributed rate variation between sites (G) was specified for both gene regions. Phylogeny inference was carried out in BEAST [43] by specifying three different runs of 1 000 000 generations each, sampling every 1000th generation. The program Tracer was used to determine whether the effective sample sizes (ESSs) of model parameters from each run were sufficiently large to assume convergence on a likely tree topology. Once this was achieved, the three log files were combined using LogCombiner and annotated using TreeAnnotator. A maximum clade credibility tree was then constructed in FigTree [43] using a combined log file of all three runs, each with the first 25% of samples removed as burnin. To further explore relationships between chloroplast haplotypes, an unrooted haplotype network was constructed in TCS 1.21 using a parsimony approach [44].

To confirm that the resulting chloroplast gene tree was representative of population relationships, we generated an amplified fragment length polymorphism (AFLP) dataset. Details of the generation and analysis of this dataset are present in the electronic supplementary material, appendix S1. Briefly, AFLP markers were generated from four primer pairs using the standard Applied Biosystems AFLP Plant Mapping Protocol and scored across individuals using *GENEMAPPER* R (Applied Biosystems). The resulting binary dataset was converted to a Nt & Li [45] genetic distance matrix in Phylip [46] from which relationships were inferred using neighbour-joining. The degree of relatedness between populations had no effect on similarity of nectar tube lengths (phylogenetic signal, figure 3), although populations visited only by bees had short floral tubes (less than 40 mm). Overall, the tube length of *T. revoluta* is multimodal, reflecting the geographically variable landscape of pollinator morphology (figure 3). Data are available in the Dryad repository [56].

All *T. revoluta* populations were fixed for a single chloroplast haplotype (figure 1). Our phylogenetic analysis did not recover *T. revoluta* as monophyletic (figure 4), since the Gys south population is more closely related to outgroup *T. lata* than to other *T. revoluta* populations. Therefore, the Gys south population was excluded from PGLS and trait evolution analyses. The remaining populations of *T. revoluta* formed a well-supported clade comprising three geographically separated groups; one on the eastern Langeberg, one on the Swartberg and one on the western Langeberg and Potberg (figures 1 and 4). Neighbour-joining analysis of the AFLP dataset supported these groupings, although deeper nodes were not resolved (electronic supplementary material, appendix S1, figure S1).

**3. Results**

Field surveys identified four geographically separate species of long proboscid flies that visit *T. revoluta* populations (figures 2 and 3). These flies have different proboscis lengths and floral tubes always match the average proboscis length of the fly species present, irrespective of whether flies are the most or the least abundant visitors to *T. revoluta* (figures 2 and 3). In addition to flies, many populations are also visited by *Amegilla fallax* bees (Anthophoridae), which have very short (less than 10 mm) proboscides, and in three populations, only bees were observed as visitors. In all the cases, the proboscis lengths of the bees were much shorter than the tube lengths of the flowers in each population (figure 3), although populations visited only by bees always had short floral tubes (less than 40 mm). Lack of conflict between the AFLP and chloroplast datasets supports our use of the chloroplast gene tree as representative of evolutionary relationships between populations. Sequences are available at GenBank (http://www.ncbi.nlm.nih.gov/genbank/) under accession numbers KM362297–KM362420. The degree of relatedness between populations had no effect on similarity of nectar tube lengths (phylogenetic signal: mean $\lambda = 0.00098$, 95% CI from 1000 BEAST
trees $= -0.0009 - 0.003$; likelihood ratio tests—$\lambda$ significantly different from 1, but not 0) suggesting little phylogenetic constraint on nectar tube evolution in $T. revoluta$. Trait reconstruction confirmed the labile nature of tube length evolution in $T. revoluta$ (figure 4a; electronic supplementary material, figure S2) which was best approximated by a multiple optimum Hansen (OU) model of trait evolution (electronic supplementary material, table S2). This indicated two evolutionary optima (95% CI: ‘short’ tubes—24.3–25.1 mm, ‘long’ tubes—63.7–64.6 mm) and strong selection towards these optima ($\alpha = 197.01$, electronic supplementary material, table S2). Evolutionary transitions between optimal trait classes occurred three times on the tree, involving lengthening of nectar tubes on two occasions and shortening on one occasion. This finding was also robust to uncertainty in phylogeny, since no change in the lengthening/shortening ratio was observed over 1000 95% HPD BEAST trees. In addition, 56.4% (95% CI from 1000 BEAST trees $= 56.1\% - 56.7\%$) of recent tube length changes (i.e. between tips of the tree and the most recent shared ancestors) have involved nectar tube lengthening, which is significantly more lengthening events than expected by randomly reshuffled tube lengths on the tips of the tree (95% CI from 1000 randomized iterations $= 51.5\% - 52.2\%$), suggesting a slight tendency towards the evolution of longer nectar tubes in $T. revoluta$.

Shifts towards the pollinator with the longest proboscis ($P. longipennis$) occurred in two genetically distinct lineages, suggesting that tube lengths have converged (figure 4). Multiple instances appear to suggest that tube length divergence occurs when closely related lineages are pollinated by different species of fly pollinators with different proboscis lengths (figure 4). This is also apparent when considering that the ‘long’ and ‘short’ size classes equate to evolutionary optima imposed by proboscis lengths of ‘$P. longipennis$’ and ‘other’ pollinator classes. Finally, the proboscis lengths of insect species with the longest proboscides in each population were positively associated with perianth tube length in each population after accounting for genetic relatedness of the plant populations (PGLS, $R^2 = 0.80$, $p < 0.001$; OLS, $R^2 = 0.86$, $p < 0.001$). The exclusion of the bee-pollinated populations from trait matching analyses increased the strength of the plant tube length–pollinator proboscis length relationship (figure 4b; PGLS, $R^2 = 0.95$, $p < 0.0001$; OLS, $R^2 = 0.98$, $p < 0.0001$).

4. Discussion

(a) Is floral tube length variation related to pollinator proboscis length?

While $T. revoluta$ is pollinated by several insect species over its range, it is nevertheless highly specialized at the population level, where no more than two pollinator species (one fly and one bee) were ever recorded in any one population. Specialization was reflected in floral morphology: in populations where fly pollinators were captured, nectar tube lengths were closely matched to the proboscis lengths of the flies. This association was strong, irrespective of whether the data were adjusted for phylogeny or not. Although bees with much shorter proboscides were more abundant than flies in some populations, floral morphology always appeared to match fly morphology, rather than bee morphology (figure 3). We expect that this may be due to flies being more efficient pollinators than bees, whose efficiency as pollinators is often reduced through pollen grooming [57]. Alternatively, tube length adaptations to flies do not significantly reduce the effectiveness of bee pollinators.
(sensu [58]), whereas adaptations to bees do reduce the effectiveness of fly visitors. While similar matching of traits has also been found in populations of other plant–pollinator interactions [11,18–21], none of these studies have explicitly taken relatedness of populations into account. We find that closely related populations do not have more similar tube lengths than populations that are distantly related. Consequently, accounting for phylogeny does not alter interpretation of the pattern of association between plant and pollinator traits in *T. revoluta*. Other phylogenetically corrected studies of pollinator-specialized plant species also suggest that floral or inflorescence traits of plants and the corresponding traits of their pollinator species are adaptively matched [59,60]. These complementary patterns, at differing taxonomic levels, suggest that adaptation to pollinators may be important in driving floral morphological divergence of

![Figure 3. Frequency distributions of *T. revoluta* tube length for each population (n = 25 plants per population) and the mean ± minimum and maximum proboscis length of the pollinators found at each site. Circles represent long proboscid flies with different colours representing different species of fly. Light grey, *Prosoeca* sp. 1; dark grey, *Prosoeca* sp. 2; striped, *Prosoeca ganglbaueri*; black, *Prosoeca longipennis*. White squares represent *Amegilla fallax* bees. For bees, proboscis length measurements were pooled across multiple sites. Pie charts represent the relative abundances of different pollinators in each population. Population names correspond to the map in figure 1, and numbers alongside the pie charts are the total number of pollinators observed within the observation times recorded.](http://rspb.royalsocietypublishing.org/Downloaded from http://rspb.royalsocietypublishing.org/ on April 18, 2017)
populations and well-established species. Although our results suggest that floral tube lengths are adaptations to the mouth parts of their pollinators, one seemingly anomalous observation is that while populations visited only by bees always had short tubes, the floral tubes were always considerably longer than the bee proboscis lengths (figure 3). One explanation for this apparent maladaptation is that these represent populations where longer proboscid pollinators have become exceedingly rare or have gone extinct, and that further reductions in tube length may not always increase the efficiency of bee pollination. While we interpret these correlative results as floral adaptation to pollinators, correlations cannot distinguish cause from effect and there may be several alternative reasons for them [61].

(b) Is there directionality to tube length evolution? While our reconstruction of ancestral tube lengths suggests a general trend towards increasing lengths through time, we find several instances of tube length reduction, and in fact, only 56% of recent transitions involved tube lengthening. Consequently, the association of long tubes with more terminal nodes should not be regarded as an evolutionary dead end. One reason may be that longer tubes in this system do not result in increased ecological specialization because long tubes do not exclude nectar access by bees, which can still forage for nectar when it wells up the tubes in the absence or scarcity of long proboscid pollinators [35]. This welling up of nectar has also been noted in other iridaceae and is enhanced by narrowing of the perianth tube [33]. Nectar accumulation may also facilitate a general tube lengthening trend because long-tubed flowers can still be pollinated effectively by bees, and consequently selection on flowers to reduce their tube lengths in the presence of bees may be relatively weak. By contrast, short-tubed flowers visited by long proboscid insects may be expected to evolve longer tubes to maximize pollen transfer efficiency. This Darwinian explanation for the elongation of floral tubes has been demonstrated in numerous plant-pollinator relationships [20,26,27,62–65], and when measured it is usually associated with strong positive directional selection coefficients [20,27,64]. Thus, our finding of apparent dramatic reductions in tube length in some populations is contrary to expectations. This suggests that selection imposed by shorter proboscid pollinators, or alternately alleviation of the costs in producing long tubes, may favour tube length shortening, at least to a point. The differences in the relative strengths of selection when plants shift from short to long proboscid pollinators versus shifts from long to short proboscid pollinators probably determines the apparent directional biases in tube length evolution. Using phylogenetically controlled data, Whittall & Hodges [8] demonstrated directional shifts from short- to long-tubed flowers in the genus Aquilegia. Short spurred, blue/purple-coloured bee-pollinated species were ancestral to longer spurred, red/orange-coloured bird-pollinated species. Bird-pollinated species were ancestral to very long spurred, pale-coloured moth-pollinated species. In Aquilegia, the lack of tube length reversals could be explained by the fact that once certain pigment pathways are lost, they are difficult to re-evolve [66]. Consequently, directionality in Aquilegia tube length evolution is likely to be the result of its link to floral colour, rather than a directional mechanism relating to tube length alone. By contrast, directionality in the tube length evolution in T. revoluta is not confounded by associations with colour as all T. revoluta populations are pink.

5. Conclusion

These results support the idea that specialization is best studied and also defined at the population level, as
emphasized by Fox & Morrow [67]. It is at the population level, and not necessarily at the species level, where specialization will have either evolutionary or ecological consequences. Tube length in *T. revoluta* appears to be a highly labile trait and is associated with functional specialization to different pollinators. It shifts in either direction whenever populations are regularly visited by morphologically different pollinators, suggesting that the species is composed of multiple pollination ecotypes (as predicted by Newman et al. [21]). Closely related populations always have very different tube lengths when visited by pollinators with different proboscis lengths. This suggests that geographical mosaics in pollinator morphology can generate morphological divergence of plant populations. However, ancestral tube length states are retained in closely related populations if there is no shift in pollinators. Conversely, distantly related populations sometimes appear to have converged in their tube lengths when pollinated by the same pollinator. For example, extremely long-tubed flowers evolved independently on two occasions, probably in response to long proboscid flies. These results support those of other trait matching studies which, without a phylogenetic context, provide some evidence of divergence of tube length in populations exposed to pollinators with different traits, as well as convergence of tube length in populations pollinated by pollinators with similar traits [19–21]. Since tube length evolution can occur in both directions functional specialization on the proboscis lengths of pollinators in this system is not likely to constrain the ability of *T. revoluta* to adapt, shift pollinators or spread.

Data accessibility. The data and additional details of the approaches used in the supplementary material and at: DNA sequences: GenBank accessions KM362397–KM362420; trait matching and pollen data: Dryad doi:10.5061/dryad.g1vc4.

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