Pollinator-driven floral variation in *Tritoniopsis revoluta*

by

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DECLARATION

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ABSTRACT

It is thought that a large proportion of the great variety of floral structures in flowering plants reflect adaptations to different biotic pollen vectors. Divergence in flower traits and pollinators is linked to speciation. Pollinator-driven speciation is thought to have played a large role in the spectacular floral diversity found in South African Iridaceae and the genus *Tritoniopsis* is a particularly good example of this. This study focuses on *Tritoniopsis revoluta*, a pink irid occurring in the Swartberg and Langeberg Mountains, as well as Potberg Mountain. I tested the hypothesis that variation in flower tube-lengths of *Tritoniopsis revoluta* are related to the geographic distribution of pollinators and the variation of their tongue lengths. It was determined that this species is highly variable in respect to corolla tube-length and is pollinated by different fly species across its range. Also, the tongue-lengths of the fly pollinators corresponded almost exactly with the tube-length of the flowers they were pollinating in each population. In some populations, where long-proboscid flies were absent, bees were observed visiting *T. revoluta* flowers. This presents evidence for pollinator-driven floral variation within a single plant species, and most of this vast diversification in floral morphology has probably been driven by morphological variation found within a single fly family. In one population I found variable tube-lengths which appeared to exhibit a bimodal distribution of corolla tube-lengths. I hypothesized that the two *Tritoniopsis revoluta* ecotypes at this population are pollinated by two different pollinators, leading to assortative mating, and ultimately strong inter-ecotype incompatibility. *Tritoniopsis revoluta* is self-incompatible and exists as two discrete entities (morphotypes) at the Gysmanshoek Pass site, and these entities differ in tube-length, color, nectar volume and sugar content. These morphotypes were not pollinated by long-proboscid flies, but seems to represent a recent shift to pollination by *Amegilla* bees. However, ecotypes are not reproductively isolated as short and long flowers can produce offspring, rather tube-length differences are possibly maintained through spatial separation. To compliment the correlatory data between flower tube-lengths and pollinator tongue-lengths, I used molecular tools (chloroplast markers and AFLPs) to elucidate the patterns of tube-length evolution in *Tritoniopsis revoluta*. I aimed to determine the directionality and frequency of transitions between tube-length categories. Tube-length transitions would be suggestive of flower morphology being labile, and together with the tube-tongue length correlation it suggests pollinator shifts may drive the changes in tube length. Character state reconstructions using tube-length as character determined that four evolutionary transitions to shorter tube-length categories and two transitions to longer categories occurred. I also tested whether morphological divergence between populations corresponds to patterns of divergence from

neutral genetic markers. Population genetic structure in this system showed that the different populations of *T. revoluta* are vicariant and tube-length differences between them could have evolved through selection.

OPSOMMING

Dit is ‘n algemene gedagte dat die groot verskeidenheid blom strukture in die angiosperme dui op aanpassings tot verskillende biotiese stuifmeel draers. Die diverse blom strukture in baie van die groot Kaapse genera kan verduidelik word deur aanpassings tot veranderinge in bestuiwings-sisteme. ‘n Aantal studies hieroor stel voor dat bestuiwers nie net die veranderinge in blom morfologie bewerkstellig nie, maar ook ‘n rol speel in die aanpassende uiteenlopendheid van blomplant kenmerke. Spesiasie bewerkstellig deur bestuiwers het moontlik ‘n groot rol gespeel in die blom-diversiteit wat gevind word in die Suid-Afrikaanse *Iridaceae* familie, en die genus *Tritoniopsis* is ‘n baie goeie voorbeeld hiervan. Hierdie studie fokus spesifiek op *Tritoniopsis revoluta*, ‘n pienk iris wat voorkom in die Swart- en Langeberge, asook by Potberg. Die hipotese dat die variasie in buis-lengtes van *T. revoluta* verwant is aan die geografiese verspreiding van bestuiwers en die variasie in hul tong-lengtes is hier getoets. Dit is bepaal dat hierdie spesie groot variasie toon in terme van buis-lengtes en bestuif word deur verskillende vlieg spesies regoor sy verspreiding. Die tong-lengtes van hierdie vlieë korrespondeer ook met die buis-lengtes van die blomme wat hul bestuif in elkeen van die *T. revoluta* populasies. In sommige van die populasies, waar lang-tong vlieë afwesig was, is bye wat die *T. revoluta* blomme besoek, opgemerk. Hierdie resultate lewer bewyse vir die hipotese dat bestuiwers blom morfologie kan beïnvloed; die interessante hiervan is dat die variasie in buis-lengtes in hierdie spesie heel moontlik te danke is aan die morfologies variasie wat gevind word in ‘n enkele lang-tong vlieg familie. In een van die populasies het ek ‘n bimodale verspreiding van buis-lengtes gevind. ‘n Logiese afleiding is dat hierdie twee verskillende buis-lengtes – ekotipes – deur twee verskillende bestuiwers besoek word, en dat dit lei tot sterk onversoenbaarheid tussen ekotipes. *Tritoniopsis revoluta* is nie instaat tot self-bestuiwing nie en die twee ekotipes verskil in terme van buis-lengtes, kleur, nektar volume en suiker inhoud. Kort- en lang-buis blomme word nie eksklusief bestuif deur lang-tong vlieë in die Gysmanshoek Pas nie, maar word in die algemeen ook bestuif deur bye van die genus *Amegilla*. Die twee ekotipes is in staat om te reproduseer met mekaar, so die buis-lengte verskille word moontlik in stand gehou deur hul geografiese skeiding. Om die korrelasie analise tussen blom buis-lengtes en vlieg tong-lengtes te komplimenteer, het ek molekulêre tegnieke (chloroplast merkers en AFLPs) gebruik om die patrone van buis-lengte evolusie in *Tritoniopsis revoluta* duidelik te maak. Ten eerste het ek bepaal of verkortings en verlengings van buis-lengtes een keer in die verlede gebeur het, of as meermalige gebeurtenisse. Meermalige veranderinge in buis-lengtes kan moontlik dui op verskuiwings tussen verskillende bestuiwers, asook taksonomiese verdelings wat korrespondeer met bestuiwer veranderinge. Ek het ook bepaal of

die buis-lengte verskille in die verskillende populasies toegeskryf kan word aan seleksie prosesse. Deur buis-lengte as karakter te gebruik, het ek karakter-status rekonstruksies gedoen en bepaal dat vier ewolutionêre transisies na korter buis-lengte kategorieë, en twee transisies na langer kategorieë plaasgevind het. Populasie genetiese struktuur in die sisteem dui daarop dat *T. revoluta* populasies geïsoleer is deur afstand. Die konklusie wat ek trek gebasseer op hierdie resultate is dat verskille in buis-lengtes in hierdie sisteem moontlik ontstaan het as gevolg van die verskillende bestuiwers wat aktief is in die verskillende *T. revoluta* populasies, en dat natuurlike prosesse nie die hoofrol spelers in hierdie sisteem is ten opsigte van buis-lengte evolusie nie.

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TABLE OF CONTENTS

GENERAL INTRODUCTION

The great variety of floral structures in flowering plants often reflect adaptations to different biotic pollen vectors (Stebbins 1970). Studies have shown that pollinator-mediated selection indeed plays an important role in shaping flower morphology (see Harder and Johnson 2009). Pollinators, especially those from different guilds, may differ drastically in their attributes, such as proboscis length, behavior, flower color preference and flight period. Apparent pollination syndromes develop as a result of pollinator preference for certain floral attractants and rewards. These attractants include perianth coloring, floral odor and flower shape, and rewards include nectar, pollen and in some cases, non-volatile oil (Goldblatt and Manning 2000; Goldblatt and Manning 2006).Floral diversity in many of the large Cape genera is explained by adaptation to changes in pollination systems (Johnson 1996).

Pollinators play a very important role in both the formation of new plant species, and maintaining reproductive barriers between different species (Waser 1998). The Biological Species Concept (BSC) states that a species consists of populations linked by gene flow that can therefore evolve as a unit (*sensu* Morjan and Rieseberg 2004). Speciation can then subsequently be described as the process that creates isolating barriers between these populations. Although pollinators facilitate pollen flow across landscapes and the possibility of gene flow between populations of the same species exists, it is prevented between species by nearly imperviable isolating barriers (*sensu* Johnson 2006). Pollinators may contribute towards gene-flow barriers in two ways: mechanical isolation of species means the floral structures and mechanisms of the species differ in such a way that no pollen from either plant can be deposited on the stigmatic surfaces of the opponent plant (Smith and Rausher 2007). Ethological isolation of species means that the pollinating animals do not roam from species to species, but have a preference for certain floral traits in a certain species (Grant 1949). Both mechanical and ethological isolation play an important role in the maintenance of different taxa living in overlapping geographical areas (Johnson 2006). A study done on *Pedicularis groenlandica* and *P. attollens* provides very convincing evidence of strong ethological isolation in plants, based on flower constancy by the particular pollinator (Grant 1994). Sister species of monkeyflowers, *M. lewisii and M. cardinalis* are pollinated by hummingbirds and bees, respectively, and pollinators play a considerable role in the isolation of these species in sympatry (Ramsey et al., 2003). Pollinator fauna also play a role in the maintenance of different floral phenotypes in a single species living in overlapping geographical areas (Ellis and Johnson 2009; Kennedy et al. 2006). There are other isolating mechanisms that may play a

role in isolating species, such as geographical isolation of populations. Allopatric speciation occurs when diverging populations are spatially isolated and do not exchange genes with each other (*sensu* Abbott et al. 2008). Populations may diverge so much allopatrically that when they come into contact again, they can not interbreed (Johnson 2006). Several studies have shown that divergence and allopatric speciation is promoted by pollinator mosaics, including those of long-proboscid fly species in southern Africa (Goldblatt and Manning 1996; Johnson 2006; Johnson and Steiner 1997).

The Cape Floristic Region of South Africa has extremely high floristic diversity (Goldblatt and Manning 2002). It comprises over 9000 species of which 70% are endemic (Goldblatt 1978; Linder 1991). The Iridaceae family is the third largest Cape plant family (Goldblatt and Manning 2002) as it comprises 65 genera and 1850 species (Goldblatt 1990; Rudall et al. 2003). Although Iridaceae is distributed worldwide, it is extensively prevalent in the southern and western areas of the Western Cape Province of South Africa. The wide variety of pollination strategies employed by Iridaceae may account for a large portion of the floral diversity of the family and the large number of species within the group (Goldblatt and Manning 2006). In contrast, a recent analysis suggests that the frequently cited reasons for high species richness in the Cape (including pollinator shifts) are not supported. Instead, reading between the lines it may be low extinction rates that result in high species diversity in the Cape. This of course does not state that pollinator shifts don't lead to speciation events – it just means that they are not unusually common in the Cape (Van der Niet and Johnson 2009).

This study particularly focuses on a flowering plant species in the Iridaceae, from the genus *Tritoniopsis*. This genus belongs to the subfamily Crocoideae (previously Ixioideae) (Goldblatt et al. 2008). It consists of 24 species and is endemic to South Africa (Manning and Goldblatt 2005). *Tritoniopsis* is sister to the remaining genera of the southern Africa and Cape Crocoideae. Crocoideae diverged from Nivenioideae approximately 37 million years ago, and *Tritoniopsis* diverged from the remaining genera 24 million years ago (Goldblatt et al. 2008). The *Tritoniopsis* lineage clearly differentiated from the remaining genera in Crocoideae at the time the climate of the world got cooler and drier (end of the Oligocene) (Zachos et al. 2001). The early radiation of *Tritoniopsis* in southern Africa took place as the landscape became more dry and seasonal and are at least 11 MY old (Goldblatt et al. 2008). A high degree of floral variation is exhibited by the genus, which includes species that are pollinated by a wide variety of pollinators (bees, flies, moths, birds) (Goldblatt et al. 2008; Manning and Goldblatt 2005). Manning and Goldblatt (2005) concluded that, on the insect-pollinated flower types, the length

of the floral tubes in *Tritoniopsis* match closely with the length of the mouthparts of the floral visitors.

Tritoniopsis is distributed throughout the winter rainfall zone of southwestern South Africa, and is characteristic of acidic oligotrophic soils. As most of the species are summer flowering, the leaves are usually dry and withered. All of the species in the genus have a deeply buried, short, vertical and swollen stem (called a corm) for storage. This enables the plant to survive extreme conditions (Manning and Goldblatt 2005). Very little data has been published on the pollination of *Tritoniopsis*, but in a study by Manning and Goldblatt (2005) it was determined that *Tritoniopsis* species are seasonal, the flowering stem produced is mostly unbranched, although a branched flowering stem is not uncommon, and flowering in a population is synchronized. Summer and autumn (December – April) is the main flowering seasons for *Tritoniopsis*. Early in the morning, flowers open sequentially and stay open. The perianth does not close at night, or anytime during the life of the flower. *Tritoniopsis* is a hermaphrodite, and also protandrous (Goldblatt and Manning 2006). The flowers stay fresh for up to 5 days, with the first 3 days being the male phase, followed by the female phase. After the perianth has withered, the stigma is still receptive for approximately a day.

One important mode of pollination amongst *Tritoniopsis* is long-tongued fly pollination. There are two families of long-proboscid flies, namely Nemestrinidae and Tabanidae, comprising of approximately 14 species. Flies from both these families can attain impressive tongue-lengths (15-80 mm long). Some flies from the family Nemestrinidae have proboscedes of up to 100 mm in length. These flies can consume rather large amounts of nectar, and they are active foragers. Adult flies depend largely or entirely on the nectar for their nutritional needs. Nemestrinid flies forage on a wide variety of flowers, but the most reliable ones that are thought to offer a definite reward are the long-tubed flowers, because the nectar is not accessible to other nectar-utilizing insects. The length of the perianth tube of the flower is (in most instances) somewhat longer than the proboscid of the pollinator (Goldblatt and Manning 2000; Goldblatt et al. 1995). No details of the life cycles of these flies are known, but all (studied) members of the family Nemestrinidae have parasitic larvae, often found on locusts (Goldblatt and Manning 2000). The *Prosoeca ganglbauri* pollination system is the most extensive of the long-tongued fly pollination systems. It manifests all through the highlands of eastern southern Africa, and reach a far as the southern coast and adjacent mountains of southern Africa. It pollinates a variety of summer- and autumn-flowering plant species (usually

comprising of white to pink flowers), mainly in the families Amaryllidaceae, Iridaceae and Orchidaceae. The *Prosoeca ganglbauri* pollination system consists of *Prosoeca ganglbauri, Prosoeca robusta* and *Prosoeca longipennis* (Goldblatt and Manning 2000; Goldblatt and Manning 2006).

This study focuses on *Tritoniopsis revoluta*, a pink irid occurring in the Swartberg and Langeberg Mountains, as well as near the coast at Potberg Mountain. The flower color of *T. revoluta* ranges from light to dark pink, with red streaks. *Tritoniopsis revoluta* emits no distinguishable fragrance, and it produces nectar as a reward to pollinators (Manning and Goldblatt 2005). The flower shape of *T. revoluta* is a long-tubed gullet (about 20-70mm long (Manning and Goldblatt 2005)), and the geographic variability of the tube lengths recorded by myself suggest that pollinators across the range of *T. revoluta* also have very variable mouthpart morphology. Two species of pollinator with very different mouthparts from the genus *Prosoeca* have been caught visiting *T. revoluta*: *P. ganglbaueri* (Manning and Goldblatt 2005) and *P. longipennis* (de Merxem et al. 2009).

To determine the patterns of tube-length evolution in *Tritoniopsis revoluta* and to resolve whether long-proboscid pollinators play a role in its evolution, this study was divided into three parts:

For the first part, I hypothesized that variation in flower tube-lengths of *Tritoniopsis revoluta* is related to the geographic distribution of pollinators and the variation of their tongue lengths. This hypothesis was tested by collecting tube- and tongue-length data from both flowers and pollinators from all known *T. revoluta* populations, and determining whether these were closely matched in each population.

For the second part of this study, I hypothesized that the two *Tritoniopsis revoluta* morphotypes at the Gysmanshoek Pass are pollinated by two different pollinators (with tongue-lengths corresponding to the tube-length of the ecotype they are pollinating), leading to assortative mating, and maintenance of phenotypic polymorphisms at this site. This hypothesis was tested by examining floral traits and pollinator species behavior and preferences at the Gysmanshoek Pass site. In addition, we tested the possibilities that divergent tube-lengths in the Gysmanshoek Pass population are maintained through selfing and genetic incompatibilities.

For the third part of the study, and in order to compliment the morphological data in Chapter 1, I used population genetics techniques to determine whether patterns of tube-length evolution are also suggestive of pollinator-driven variation. If not, alternative patterns could be suggestive of neutral processes in which case pollinator-driven variation can be rejected. Molecular tools were utilized to determine firstly whether the short and long tubed populations form distinct clades, or whether tube-length is evolutionary labile which would be suggestive of pollinator shifts; tube-length transitions that correspond to pollinator shifts would be suggestive of pollinator-driven variation. Secondly, it was determined whether genetic variance was structured morphologically (i.e. between long and short tubed populations) or geographically. If tube-lengths between populations are similar due to common descent, it may suggest that selection processes played a very small role (if any role at all) in the evolution of tube-lengths. However, in populations that are geographically isolated, differences in tube-lengths could have evolved due to selection processes.

CHAPTER 1

BROAD SCALE GEOGRAPHICAL PATTERNS OF MORPHOLOGICAL COVARIATION IN *T. REVOLUTA* AND ITS POLLINATORS

ABSTRACT

A large amount of the floral diversity in many of the large Cape genera is explained by adaptation to changes in pollination systems. Subsequent studies suggest that pollinator-driven selection drives changes in flower morphology (i.e. adaptive divergence of floral traits). These different forms of a plant, utilizing different pollinators, are called pollination ecotypes. Pollination ecotypes may be a result of plant distribution ranges being larger than the distribution range of a single pollinator species, or differences in the relative abundance and/or types of pollinators in different parts of their ranges. Pollinator-driven variation is thought to have played a large role in the spectacular floral diversity found in South African Iridaceae and the genus *Tritoniopsis* is a particularly good example of this. This study focuses on *Tritoniopsis revoluta*, a pink irid occurring in the Swartberg and Langeberg Mountains, as well as Potberg Mountain. I tested the hypothesis that variation in flower tube-lengths of *Tritoniopsis revoluta* is related to the geographic distribution of pollinators and the variation of their tongue lengths. This was done by collecting tube- and tongue-length data from both flowers and pollinators from all known *T. revoluta* populations, and determining whether these were closely matched in each population. It was determined that this species is highly variable in respect to corolla tube-length and is pollinated by different fly species across its range. Also, the tube-length of the flowers corresponded very closely to the tongue lengths of their fly pollinators. In some populations, where long-proboscid flies were absent, bees were observed visiting *T. revoluta* flowers. The results from this study present evidence for pollinator-driven floral variation within a single plant species, and much of this diversification in floral morphology has probably been driven by morphological variation found within a single fly family.

INTRODUCTION

It is thought that the great variety of floral structures in flowering plants reflect adaptations to different biotic pollen vectors (Stebbins 1970). A logical extension of this hypothesis would be that plant species sharing the same pollinator species should have similar shapes, structures, colours, smells and patterns, called pollination syndromes (Faegri and van der Pijl 1966). As a general rule, adaptations to the physical environment are reflected in variation of a plants' vegetative morphology, whereas variation in floral morphology reflects adaptations to the pollinator fauna (Stebbins 1970). These patterns of adaptation in either vegetative or floral characters can give us clues as to what environmental factors drive speciation in plants (Carson 1985). For example, Johnson (1996) compared the contributions of the physical and pollination environment in the diversification of Cape plants and concluded that the floral diversity in many of the large Cape genera is explained by adaptation to changes in pollination systems.

Johnson (1996) also identified a number of genera with very little vegetative, but considerable floral diversification. Subsequent studies supported the idea that the floral diversity of these genera is influenced by pollinators present across the geographical ranges of these flowering plants (Goldblatt and Manning 1996; Goldblatt et al. 2001; Johnson et al. 1998), and data from other systems (e.g. Grant 1949) also link floral diversification to specialized pollination systems. An example of this is flower tube-length evolution in plant populations that exhibit specialization for pollination by a long-proboscid fly species (Johnson and Steiner 1997). In *Aquilegia* pollination by long-proboscid insects has caused the significant lengthening of floral spurs (Whittall and Hodges 2007).

The effects of pollinator-driven selection on flower morphology may differ between populations, resulting in possible geographical variation in floral traits of the same species (Johnson 1997; Perez-Barrales et al. 2007; Rey et al. 2006). *Satyrium hallackii*, for example, is distributed from the north to the south of South Africa. Plants in the north, which are pollinated by hawkmoths, have long tubes corresponding to the long tongues of the moths. In contrast, the plants in the south have short tubes corresponding to the short tongue-lengths of their bee pollinators (Johnson 1997). Also, hummingbird-pollinated populations of tree tobacco (*Nicotiana glauca*) plants growing in Argentina and southern Bolivia differ in corolla length and width, and there is evidence that the flowers with longer tubes are matched to longer-billed pollinators (Nattero

and Cocucci 2007). These different forms of a plant species, utilizing different pollinators, are called pollination ecotypes.

In many instances pollination ecotypes are a result of plant distribution ranges being larger than the distribution range of a single pollinator species (Johnson 2006). Alternatively the relative abundance and/or types of pollinators may vary in different parts of their ranges. For example, populations of the amaryllid, *Narcissus papyraceus*, occur in the Strait of Gibraltar, where insect relative abundance is driving ecotype formation. In the areas close to the Strait, long-proboscid moths (feeding on nectar) are the dominant pollinators for *Narcissus papyraceus*, whereas short-proboscid flies (feeding on pollen) are the dominant pollinators in the populations on the outer boundaries of the Strait; long-proboscid moths, however, are rare in the outer boundaries of the strait (Perez-Barrales et al. 2007). Pollination ecotypes may diverge so much allopatrically that when they come into contact again, they can not interbreed, leading to allopatric speciation (Johnson 2006). Several studies have shown that divergence and allopatric speciation is promoted by pollinator mosaics (e.g. Haloin and Strauss 2008), including those of long-proboscid fly species in southern Africa (Goldblatt and Manning 1996; Johnson 2006; Johnson and Steiner 1997).

Pollinator-driven speciation is thought to have played a large role in the spectacular floral diversity found in South African Iridaceae (Goldblatt and Manning 2006). The genus *Tritoniopsis* consists of 24 species which display great floral variation (Manning and Goldblatt 2005) and many species are specialized to a single functional group of pollinators (bees, flies, moths or birds) (Manning and Goldblatt 2005). Here we investigated a single species, *Tritoniopsis revoluta*, which exhibits considerable variation in floral tube length across its range, which encompasses the Swartberg and Langeberg Mountains, as well as near the coast at Potberg Mountain (Figure 1). The flower color of *T. revoluta* ranges from light to dark pink, with red streaks. *Tritoniopsis revoluta* emits no distinguishable fragrance, and it produces nectar as pollinator reward (Manning and Goldblatt 2005). The flower shape of *T. revoluta* is a long-tubed gullet (about 20-70mm long), and the highly variable nature of the tube lengths suggest that different pollinators may be active in different parts of the species' range. Two species of pollinator from the genus *Prosoeca* have been captured on *T. revoluta*: *P. ganglbaueri* (Manning and Goldblatt 2005) and *P. longipennis* (de Merxem et al. 2009).

For this study, I hypothesized that variation in flower tube-lengths of *Tritoniopsis revoluta* is related to the geographic distribution of pollinators and the variation of their tongue lengths. This hypothesis was tested by collecting tube- and tongue-length data from both flowers and pollinators from all known *T. revoluta* populations, and determining whether these were closely matched in each population.

MATERIALS & METHODS

Floral variation

Locality data for *Tritoniopsis revoluta* were obtained from the Compton Herbarium, SANBI, Kirstenbosch. Seven populations of *Tritoniopsis revoluta* were identified for this study from collections housed at the Compton Herbarium and two were discovered during field observations. Flower tube-length data were collected for all nine *T. revoluta* populations during March 2007 and March 2008. The tubes of between 20 and 96 flowers per population were measured (Table 1) from the top of the ovary to the opening of the perianth tube, using a digital caliper. One population (the LW-2 population), which morphologically resembled *Tritoniopsis ramosa* based on its very short tube-length, was included in this study as genetic analyses (see Chapter 3) showed it to be nested within the *T. revoluta* populations sampled. To determine if there were differences in flower colour that could be perceived by insects, the spectral reflectance over the UV-visible range (300–700 nm) of *T. revoluta* flowers in SW-1, SW-2, LE-1 (both short and long flowers) and LW-2 populations was measured using an Ocean Optics (Dunedin, Florida, USA) S2000 spectrophotometer and Ocean Optics DT-mini deuterium tungsten halogen light source (200–1100 nm). Between 5 and 10 flowers were randomly collected from each site, tube-length was noted and the dorsal petal was used for colour analysis of all flowers. Readings were taken through a fibre-optic reflection probe (UV/VIS 400 micron) held at 45° and about 5 mm from the surface of the petal.

Pollinator variation

Pollinator data were collected from seven of the *T. revoluta* populations. Two hundred-and-five hours were spent on pollinator observations in these seven populations in the years 2007 and 2008 with an average of three observers per hour. Observations were done in sunny weather conditions, between 09h00 and 12h00 when insect pollinators are most active. Data collected included the number and type of pollinators, and the functional proboscis lengths measured with a digital caliper. Functional proboscis length is measured when the proboscis of the pollinator is fully extended (Anderson et al. 2005), and is an indication of tongue function as these pollinators have to probe flower tubes for nectar (Waddington and Herbst 1987).

To test whether populations differed significantly in tube- and tongue-length, I used a one-way ANOVA (STATISTICA 8 Statsoft Inc.) and a post hoc Tukey's (HSD) Test.

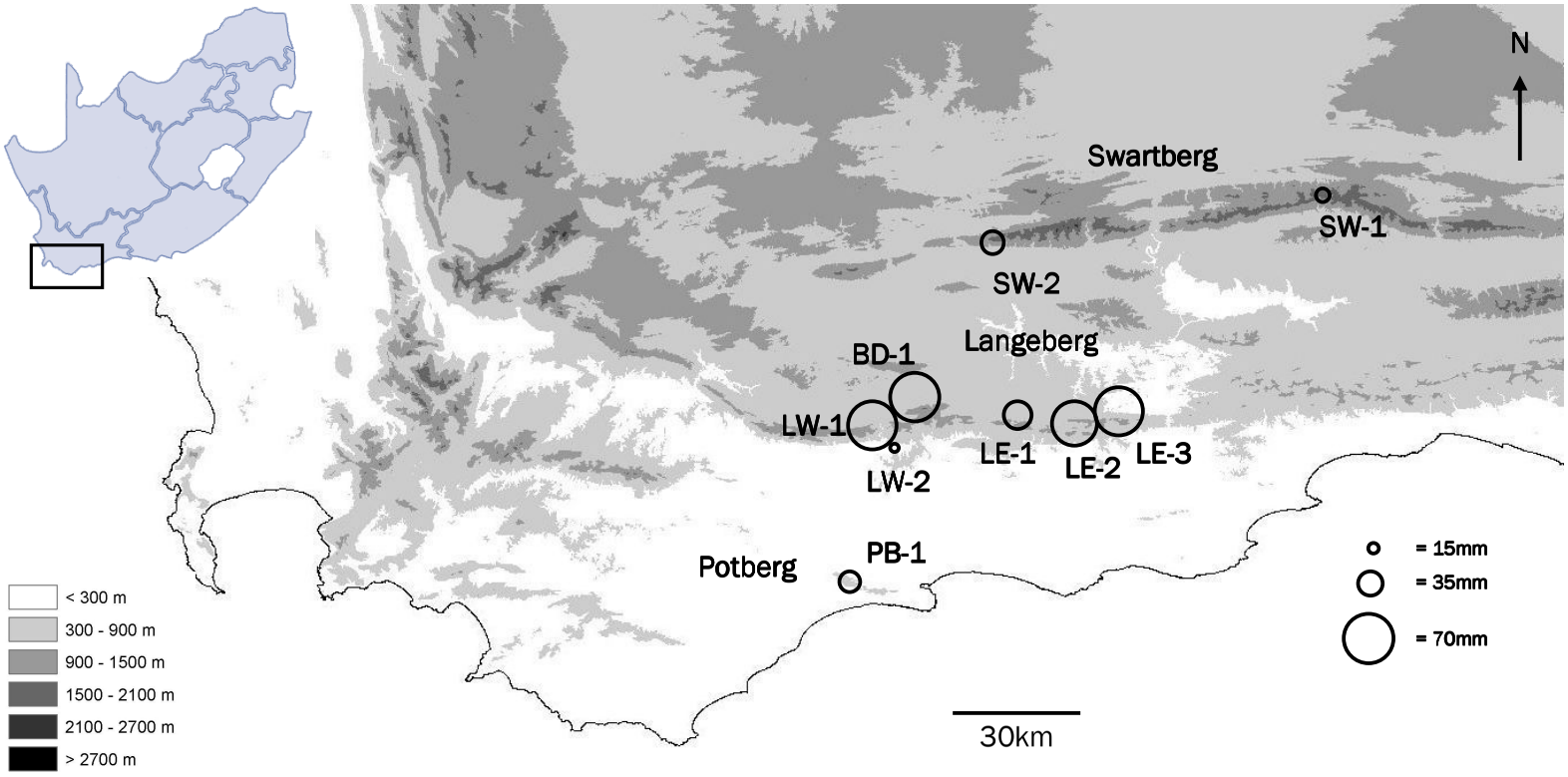
RESULTS

Floral variation

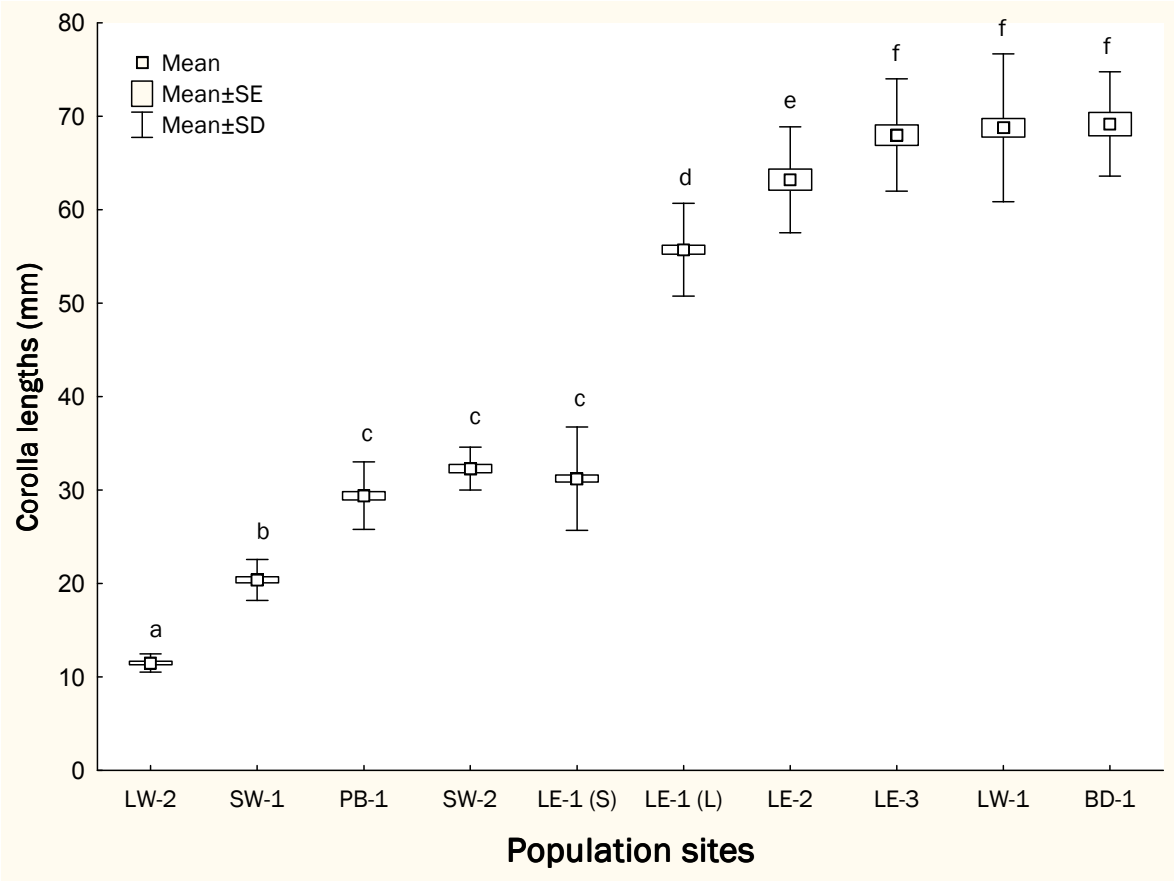
Different flower morphology (with respect to perianth tube-length) was found in the different populations (Figure 1). Longer-tubed flowers were found in four populations which are all located in the Langeberg Mountains. In contrast, flowers with shorter tubes were found in four populations; in three geographically separate areas, located on the southern slopes of the Swartberg Mountains, southern slopes of the Langeberg Mountains and on the flats south of Potberg Mountain (Table 1). The distribution ranges of longer- and shorter-tubed populations overlap at the LE-1 site which contained both short and long-tubed plants (see Chapter 2 for details). There are significant differences in tube-lengths between many *T. revoluta* populations ($F = 831.97$, $p = < 0.05$) (Figure 2). Spectrophotometer data for flower color show that the spectral profiles of most populations were very similar; populations only differed in reflectance intensity. There is one exception to this pattern – the LW-1 population had a peak at 425 nm and not 400 nm like the others (Figure 4).

Table 1: Locality data for *Tritoniopsis revoluta* including population sites, latitude, longitude, the number of individuals collected and the mean tube-lengths of flowers at each population

Population Sites	Pop abbr.	Latitude	Longitude	# individuals collected	Tube-length (mm) (mean±SE)
Gysmanshoek Pass I	LE-1	S 33º 55,943'	E 021º 04,336'	96	39.2±0.69
Garcia's Pass	LE-2	S 33º 57.278'	E 021º 13.544'	25	63.2±1.13
Langkloof	LE-3	S 33º 56.939'	E 021º 15.460'	30	68.0±1.09
Gysmanshoek Pass II	LS-1	S 33º 59.499'	E 020º 59.446'	4	21.0±2.00
Tradouws Pass I	LW-1	S 33º 56.813'	E 020º 41.987'	32	68.8±0.97
Buffelsrivierpoort	SW-2	S 33º 26.851'	E 021º 00.236'	27	32.3±0.44
Swartberg Pass	SW-1	S 33º 21.747'	E 022º 04.615'	47	20.4±0.32
Potberg	PB-1	S 34º 24,075'	E 020º 33,439'	24	29.4±0.44
Tradouws Pass II	LW-2	S 33º 59.229'	E 020º 42.726'	30	11.5±0.18
Barrydale	BD-1	S 33º 54.352'	E 020º 43.331'	20	69.2±1.25



▲ Figure 1: The 9 different *Tritoniopsis revoluta* localities, where circle size represents the mean tube-length at each population. Population names, abbreviations and numbers are in Table 1.



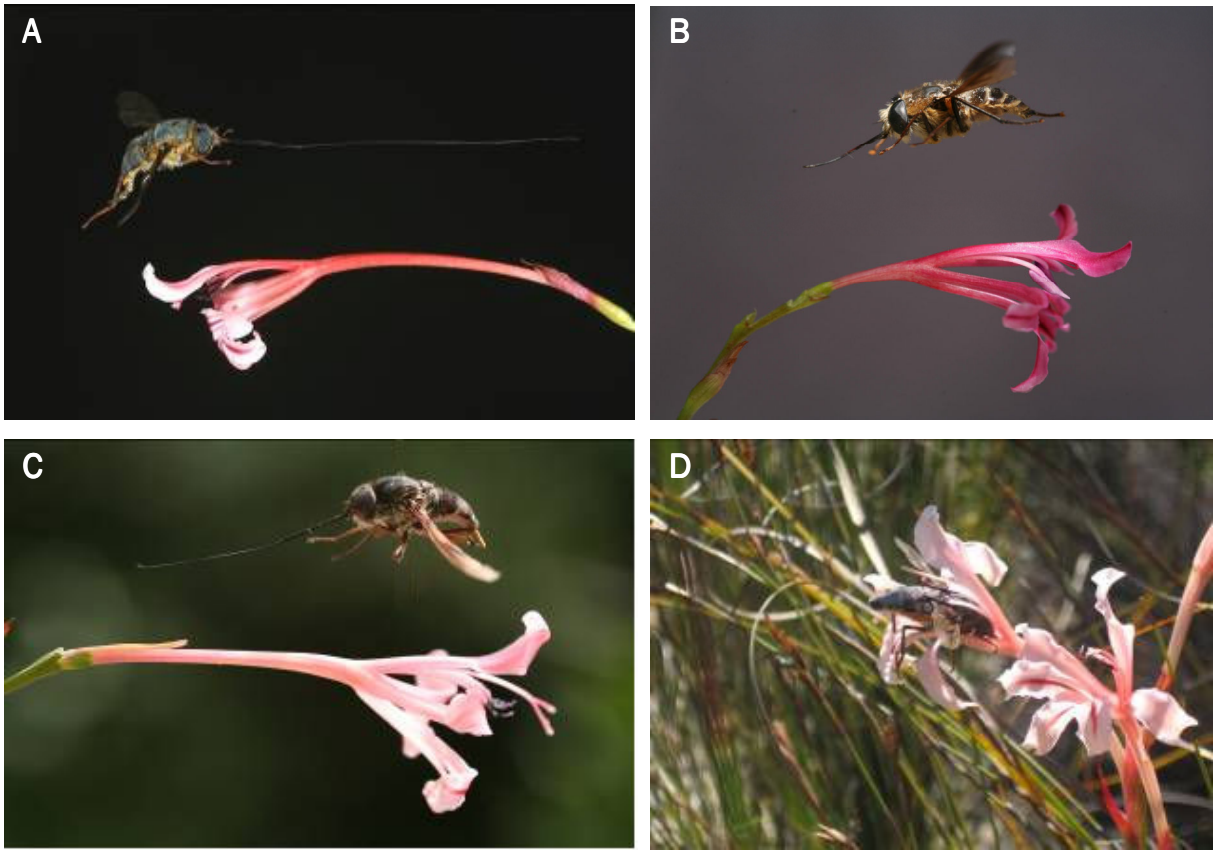
▲ Figure 2: Tube-length data of all nine *T. revoluta* populations. Letters denote significant differences between populations from ANOVA. The short (LE-1 (S)) and long (LE-1 (L)) tubed flowers at the Gysmanshoek Pass site were treated separately for this analysis (see Chapter 2 for bimodal distribution at this site).

Pollinator variation

A number of long-proboscid fly pollinators were captured at five of the nine *T. revoluta* populations (Figure 3, Table 2). *T. revoluta* pollen was present on all the captured flies from all five populations.

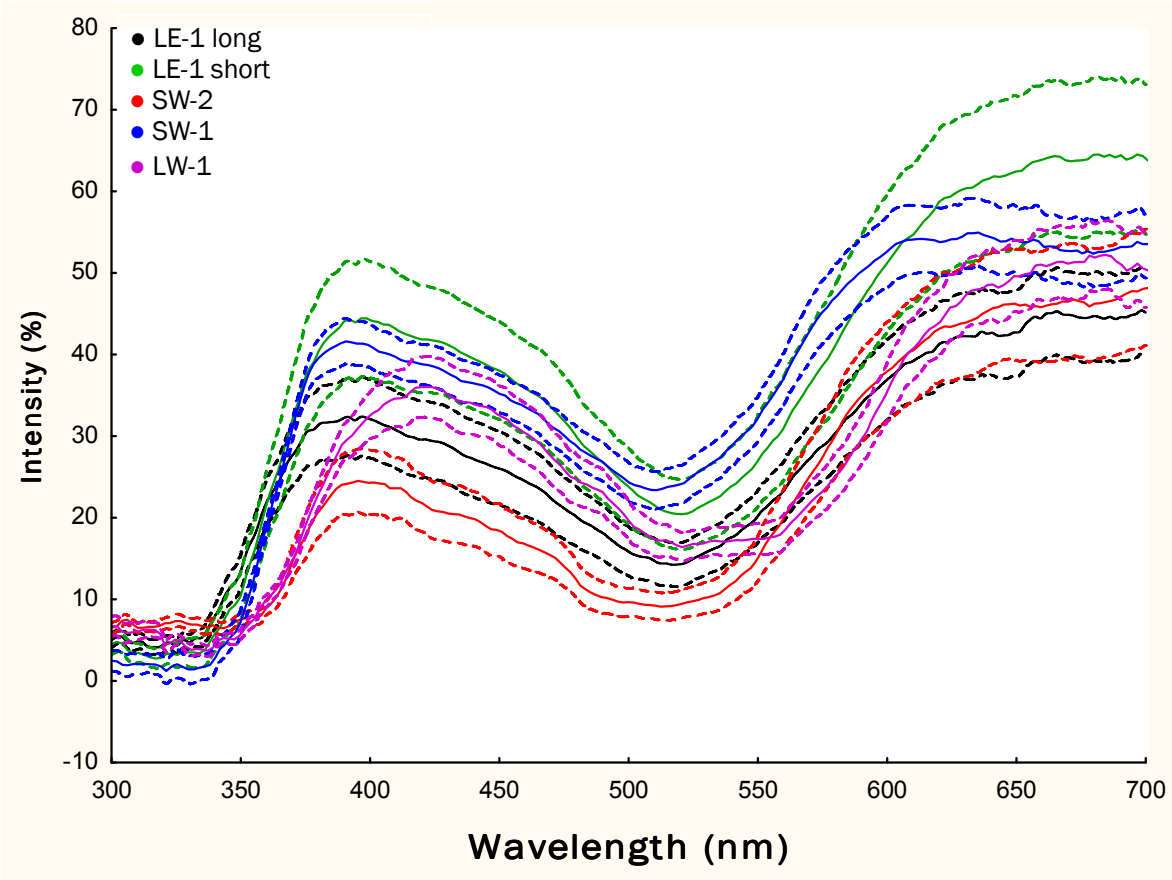
Table 2: Long-proboscid fly pollinator data for *Tritoniopsis revoluta*, including the population sites where long-proboscid flies were captured, the number, species names and tongue-lengths (T-L) of flies, observation hours per population and whether *Amegilla* bees were observed or not

Population sites	Fly pollinators collected	Pollinator species	T-L (mm) (mean±SE)	Observation hours	*Amegilla* bees observed
LE-1	0	none	-	50	Y
LE-2	0	none	-	5	-
LE-3	0	none	-	5	-
LS-1	0	none	-	20	Y
LW-1	1	*Prosoeca longipennis*	71	35	Y
SW-2	7	*Prosoeca ganglbaueri*	28.6±0.56	20	N
SW-1	4	*Prosoeca sp. 1*	18.2±0.94	20	N
PB-1	0	none	-	20	Y
LW-2	2	*Prosoeca sp. 2*	10.7±0.7	35	Y
BD-1	7	*Prosoeca longipennis*	56.8±0.83	15	Y



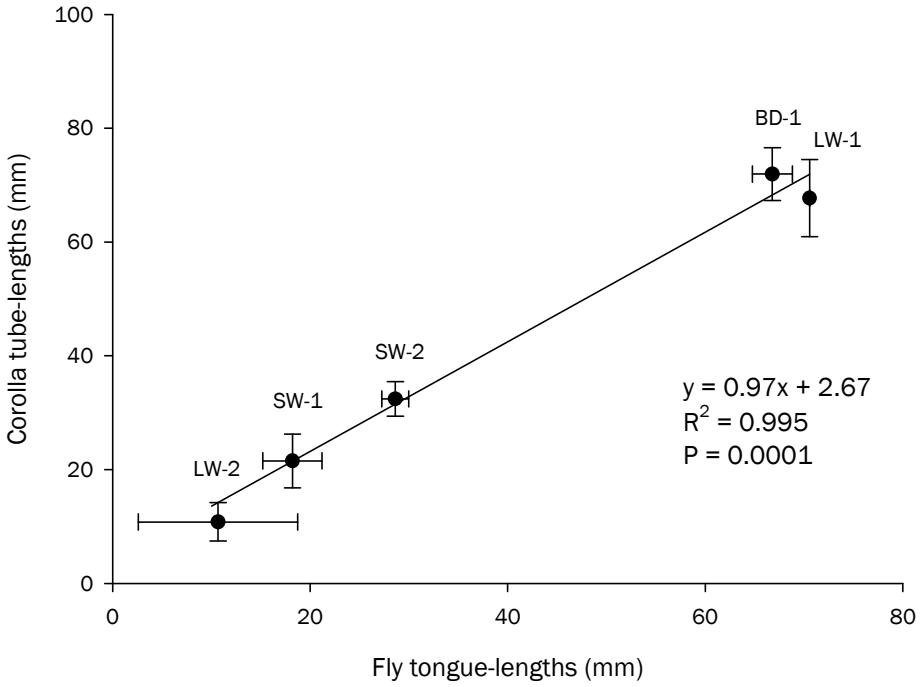
▲ Figure 3: *Tritoniopsis revoluta*; **A** Tube- and tongue-length of *Prosoeca longipennis* and *Tritoniopsis revoluta* from Tradouws Pass I, **B** Tube- and tongue-length of *Prosoeca sp novo* and short-tubed *Tritoniopsis revoluta* from Tradouws Pass II, **C** the match between *P. ganglbaueri* tongue-length and *T. revoluta* tube-length at Buffelsrivierpoort, **D** *Prosoeca longipennis* visits *Tritoniopsis revoluta* at the Barrydale site.

Pollinators captured at the SW-2 site were identified as *Prosoeca ganglbaueri*, whereas long-proboscid fly pollinators captured at the SW-1 and LW-2 sites are undescribed *Prosoeca* species. *Prosoeca longipennis* was observed and captured in the long-tubed LW-1 and BD-1 populations (see Table 2). Bee visitors have been observed and captured at the LE-1, PB-1, BD-1, LW-1 and LW-2 sites. However their tongue-lengths are much shorter than the tube-lengths of most *T. revoluta* populations so it could be a possibility that the role they may play in the evolution of the long-tubed plants is not major. All the pollinators captured were observed visiting *T. revoluta* flowers.



▲ Figure 4: Spectrophotometer data of 4 different populations (the LE-1 population is divided into short and long tubes, respectively). Solid lines represent the mean and dotted lines the SE.

The length of *T. revoluta* corolla tubes closely matches the tongue-length of their fly pollinators in each population ($R^2 = 0.97$, $p < 0.001$) (Figure 5), with the tube-lengths being longer than the proboscis length of the flies.



▲ Figure 5: Regression of *T. revoluta* tube-lengths and fly tongue-lengths at the LW-2, SW-1, SW-2, BD-1 and LW-1 sites. Mean tube- and tongue-lengths are represented by (●). Whiskers represent the SE.

DISCUSSION

Tritoniopsis revoluta exhibits remarkable variation in its tube-length across its range. Four broad non-overlapping tube-length morphotypes were discovered (10-20, 20-30, 30-40 and 60-75 mm). Floral morphology suggests that *T. revoluta* should be pollinated by insects with different tongue lengths at various sites. We caught fly pollinators with tongue-lengths which corresponded almost exactly to the four tube-length classes discovered. The captured pollinators, however, were not the same species throughout the whole range of *T. revoluta.* In the short-tubed SW-2 population in the Swartberg Mountains, *T. revoluta* is pollinated by *Prosoeca ganglbaueri*, a long-proboscid fly pollinator with a proboscis matching the tube-lengths of the flowers at that specific site. Two different undescribed long-proboscid flies (*Prosoeca sp*. 1 and 2) pollinate *T. revoluta* at the SW-1 and LW-2 sites, respectively. In the two long-tubed populations in the Langeberg Mountains, *T. revoluta* is pollinated by *Prosoeca longipennis*, a long-proboscid fly pollinator with a proboscis matching the long-tubed flowers in both the LW-1 and BD-1 sites.

Bees, but not flies, were observed visiting *T. revoluta* at some sites. However, since their tongues are much shorter than the tubes of the flowers in all but one population (the LW-2 site), they are unlikely to have played a role in the evolution of the very long corolla tubes found in *T. revoluta*. Nevertheless, they may still play an important role as pollinators, when flies are rare or absent (de Merxem et al. 2009), and may even represent a recent switch to bee pollination, to which not all flowers have responded. The flowers in the different *T. revoluta* populations are all pink and showed similarities in their light reflectance.

In the five *T. revoluta* populations where fly pollinators were caught, there is a positive correlation between tube-lengths of the flowers and the tongue-lengths of the fly pollinators. Although correlations between pollinators and their flowers may be caused by factors unrelated to adaptation of one species to another, evidence in other studies of similar systems suggest that trait matching is either a result of coevolution or unilateral evolution (e.g. Anderson and Johnson 2008; Johnson and Steiner 1997; Pauw et al. 2009). It is possible that the tube-length differences between the five populations arose through co-evolution of plants and pollinators (see Anderson and Johnson 2008; Pauw et al. 2009); since in many populations, flies were only observed on *T. revoluta* and there were few or no co-flowering long-tubed plant species present (also see Thompson 1999). In populations where flies were observed on more than one plant species (as in the LW-2 site), tube-length could have evolved unilaterally.

A number of studies have focused on the relationship between pollinators and plant species. Johnson and Steiner (1997) provided evidence for pollinator-driven selection on tube-length in their study on the *Disa draconis* complex. A shift from short-proboscid horseflies to long-proboscid nemestrinid flies in adjacent populations shows that selection favors the longer-tubed individuals when long-proboscid pollinators are more abundant and/or the only available pollinator. More evidence for pollinator-mediated selection is presented in a study on corolla tube-length in *Gladiolus longicollis* populations, which are pollinated by long-proboscid hawkmoth species in southern Africa (Alexandersson and Johnson 2002). Apparent pollination syndromes may develop as a result of pollinator preference for certain floral attractants and rewards, or because of a perfect morphological fit between plant and pollinator (Goldblatt and Manning 2000; Goldblatt and Manning 2006; Pauw 2006), so that different species of plants display similar floral traits.

For *T. revoluta*, multiple tube-length morphs correspond to visitation by different species of long-proboscid flies, with different proboscis lengths. The occurrence of pollination ecotypes in nature is quite frequent (Johnson 1997; Nattero and Cocucci 2007; Perez-Barrales et al. 2007; Robertson and Wyatt 1990) and variation in floral morphology may reflect adaptation to the local pollinator fauna (Stebbins 1970). The pattern of trait matching I present here is suggestive of pollinator-driven floral variation within a single plant species. Most surprising is the fact that much of this substantial variation in corolla length has probably been driven by morphological variation found within a single family of flies.

ACKNOWLEDGEMENTS

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CHAPTER 2

FACTORS MAINTAINING FLORAL TUBE-LENGTH MORPHOTYPES IN SYMPATRY IN A CAPE IRID

ABSTRACT

Species are often thought to be discrete units in nature where nearly impermeable isolating barriers prevent the flow of genes between species. However, in some cases, hybrid zones exist, usually consisting of two species that are interbreeding and subsequently producing hybrid organisms. The study of hybrid zones can contribute to the understanding of the relative importance of reproductive isolation and selection in the maintenance of species integrity. Pollinator-mediated assortative mating in populations of different species in sympatry plays an important role in reproductive isolation. I extend this reasoning to apply to populations of a single species, exhibiting variation in floral traits, living in overlapping geographical areas. Pollinator fauna may play a role in maintaining distinct morphological traits of a species in sympatry. Other factors, such as self-fertilization and genetic incompatibilities may also come into play. Here we investigated a single species that exhibits remarkable variation in its tube-length across its distribution range. *Tritoniopsis revoluta* (Iridaceae) occurs in the Swartberg and Langeberg Mountains, as well as near the coast at Potberg Mountain. Tube-length variation correlates closely with the proboscis length of the different pollinating species utilized by different populations of *T. revoluta*. In one population we found variable tube-lengths which appeared to exhibit a bimodal distribution of corolla tube-lengths. For this study, I hypothesized that the two *Tritoniopsis revoluta* morphotypes at the Gysmanshoek Pass are pollinated by two different pollinators, leading to assortative mating, and preservation of morphotypes in sympatry. This hypothesis was tested by examining floral traits, and pollinator species behavior and preferences at this site. In addition, we tested the possibilities that divergent tube-lengths in the Gysmanshoek Pass population are maintained through selfing and genetic incompatibilities, by conducting hand pollination experiments. We conclude from this study that *Tritoniopsis revoluta* is unable to self-fertilize without the aid of pollinators and exists as two overlapping entities (morphotypes) at the Gysmanshoek Pass site, and these entities differ in tube-length, colour, nectar volume and sugar content. These morphotypes do not seem to be pollinated by long-proboscid flies, but rather by *Amegilla* bees. Tube-length differences are possibly maintained through spatial separation.

INTRODUCTION

Species are often thought to be discrete units in nature (Coyne 1994; Coyne and Orr 1998) where nearly imperviable isolating barriers prevent the flow of genes between species, according to the Biological Species Concept (*sensu* Johnson 2006). Also determined by gene flow is whether, and to what extent, species-level traits are established in small founder populations, or across a vast geographical area. Traits at the species-level could also have developed during initial divergence, and occur in different populations mainly by the sharing of a common ancestor (the original founders) (Raven 1976). In an examination of genetic studies done on more than 250 plants species, it was determined that gene flow between populations in these species is inadequate to impede divergence through selection and genetic drift (Morjan and Rieseberg 2004) but connections between species may be sustained as highly advantageous alleles only require low levels of gene flow to spread. Johnson (2006) concluded that populations primarily diverge not because of interrupted gene flow, but because of a reaction to selection pressures. Carson (1985, p. 380) also argued that ''the integrity of either a plant or an animal species is maintained not by ad hoc mechanisms, but primarily by selection that serves to maintain and sharpen the adaptive norm that characterizes species.''

The study of hybrid zones can contribute to understanding the relative importance of reproductive isolation and selection in maintenance of species integrity (Mallet et al. 1990; Rieseberg and Carney 1998). A number of studies have been directed at examining contact / hybrid zones (Aldridge 2005; Aldridge and Campbell 2006; Aldridge and Campbell 2007; Campbell et al. 1997; Cruzan and Arnold 1994). Hybrid zones usually consist of two species that are interbreeding and subsequently producing hybrid organisms. Distributions of individual phenotypes at such contact sites where species co-exist in sympatry or parapatry, can be either unimodal, or bimodal (Aldridge 2005) and the frequency of intermediate individuals in a hybrid zone reflects the amount of reproductive isolation between species (Jiggins and Mallet 2000). Few intermediate individuals, with respect to parental numbers, indicate strong reproductive isolation between species (Harrison and Bogdanowicz 1997). These isolating mechanisms are thought to evolve in allopatry by processes such as genetic drift and selection (Ramsey et al. 2003), and include 'pre-zygotic' mechanisms (including gametic incompatibility, ecogeographic and temporal differences between species) and 'post-zygotic' mechanisms (including hybrid inviability, hybrid sterility and F_2 breakdown) (Coyne and Orr 1998; Rieseberg 1997). Speciation is complete when species are reproductively isolated and gene flow in sympatry ceases.

Studies of hybrid zones typically focus on two different species living in sympatry; however, sometimes such secondary contact sites can exist between allopatric populations of the same species that have made secondary contact. These populations may exhibit diverse floral phenotypes while still being one species (e.g. Ellis and Johnson 2009).

It is widely accepted that pollinator-mediated selection plays an important role in the diversity of floral characters, so that different populations of the same species may exhibit geographic variation in floral characters, in both flowering phenology (Olsson and Ågren 2002) and flower morphology (Johnson 1997; Perez-Barrales et al. 2007; Rey et al. 2006). This geographical variation in flower morphology is often a result of geographical differences in pollinator fauna (Boyd 2004; Johnson 1997). Assortative mating facilitated by pollinators may play a role in maintaining phenotypic polymorphisms in single populations of the same species (Anderson et al. 2009; Kennedy et al. 2006). Competition for the most efficient pollinator(s) can facilitate divergence of floral traits, aimed at attracting such a pollinator(s), for populations living in sympatry. Floral trait divergence occurring in allopatric populations due to pollinator-mediated selection may lead to assortative mating when these populations become sympatric (Martin et al. 2008).

A number of studies have been conducted that show the importance of pollinator behavior in reproductive isolation (Aldridge and Campbell 2007; Martin et al. 2008; Ramsey et al. 2003; Wendt et al. 2002). In some taxa, pollinator-mediated isolation is the main factor keeping species integrity intact (Kato et al. 2003; Kawakita and Kato 2009; Kawakita et al. 2004; Okuyama et al. 2008); in others it plays an important role in maintaining the integrity of different taxa living in overlapping geographical areas (Johnson 2006), as well as maintenance of distinct morphological traits in a species living in sympatry (Anderson In Press). Another factor possibly contributing to reproductive isolation and subsequently maintaining divergence is self-fertilization (Lloyd 1979). A number of possible reasons exist for the evolution of selfing, and include competition for pollinators, scarcity and unreliability of pollinators, and dispersal and fragmentation events (Levin 1971; Stebbins 1957). As there is no gene dispersal during self-fertilization, it may be an effective mechanism for isolating two inter-fertile, sympatric populations (Levin 1971). This reasoning may be extended to ecotypes of one species living in overlapping geographical areas; selfing may be a mechanism of preserving distinct morphological traits in these populations (in the absence of potential pollinators). Selfing is the most important mechanism of reproduction in three sympatric species of *Pitcairnia*, namely *P. albiflos, P. corcovadensis* and *P. flammea* (Wendt et al. 2002). This limits gene flow and subsequent hybridization between the sympatric populations. In self-compatible *Ipomoea*

hederacea, close clustering of anthers around the stigma (lack of herkogamy) prevents heterospecific pollen from reaching the stigma, leading to pre-zygotic isolation, as species have overlapping flowering phenologies and share bee pollinators (Smith and Rausher 2007). Also see similar studies on *Senecio* (Abbott and Lowe 2004).

Reproductive isolation between hybridizing species, or populations of the same species, can also be a result of genetic incompatibilities. The outcome of such crosses are a result of 'post-zygotic' isolating mechanisms, like hybrid inviability, hybrid sterility and F_2 breakdown (Coyne and Orr 1998; Rieseberg 1997). In recent years, it has been determined that genes, rather than chromosome rearrangements, play a role in post-zygotic reproductive isolation (Coyne and Orr 1998). Between-locus incompatibilities are the reason for the occurrence of sterile and inviable hybrids (Coyne and Orr 1998; Orr 1997). Genetic barriers can indeed play a role in reproductive isolation in plants species, as seen in two sister species of monkeyflowers, *Mimulus lewisii* and *M. cardinalis* (Schemske and Bradshaw 1999).

Here we investigate a single species that exhibits remarkable variation in its tube-length across its range. *Tritoniopsis revoluta* (Iridaceae) is a pink irid that occurs in the Swartberg and Langeberg Mountains, as well as near the coast at Potberg Mountain. Tube-length variation correlates closely with the length of the proboscides of different pollinating species utilized by different populations of *T. revoluta*. Whereas most *T. revoluta* populations exhibit limited variance in tube-length, one population in the Langeberg Mountains was found to exhibit substantial variation and appeared to comprise two sympatrically occurring tube-length morphotypes (see Chapter 1). Here we examine the patterns of variation within this population in detail and test the hypothesis that the two *Tritoniopsis revoluta* morphotypes at the Gysmanshoek Pass are pollinated by two different pollinators (with tongue-lengths corresponding to the tube-length of the morphotype they are pollinating). Such pollinator behavior can promote assortative mating, and possibly isolate the two morphotypes. This hypothesis was tested by examining floral traits and pollinator species behavior and preferences at the Gysmanshoek Pass site. In addition, we tested the possibilities that divergent tube-lengths in the Gysmanshoek Pass population are maintained through selfing and genetic incompatibilities.

METHODS

Patterns of variation in floral traits

The study site is at the top of the Gysmanshoek Pass, in an area of approximately 150 m long and 50 m wide. The highest point of the site is situated at 730 m above sea level, with the lowest one being at 686 m above sea level. To determine the spatial distribution of morphotypes at the Gysmanshoek Pass, we measured the corolla tube-lengths of randomly chosen *Tritoniopsis revoluta* flowers (N = 188) from the top of the ovary to the opening of the perianth tube, using a digital caliper. This distribution of corolla-tube lengths collected from this population was analyzed for bimodality, using equations outlined by Der and Everitt (2002). Skewness (M_3), kurtosis (M_4) and sample size (n) are used to calculate a coefficient of bimodality (b), where $b = (M_3^2 + 1)/(M_4 + 3(n -1)2/(n-2)(n-3))$. If b is greater than 0.55, frequency distributions are taken to be bimodal (Der and Everitt 2002). The spatial distribution of morphotypes at the Gysmanshoek Pass site was determined by walking a transect through the site from top to bottom (150 m) and measuring the tube length of all plants within 20m of the transect, as well as taking GPS readings (with an eTrex Vista® from Garmin) (n = 101).

Short morphotypes were identified as < 40 mm, and long as > 50 mm; the low number of individuals between these ranges were labelled as intermediates. To determine if there were differences in pollen grain size between the morphotypes, which is often indicative of ploidy differences (see Eenink 1980), microscope slides were created of pollen from short (N=6), intermediate (N=4) and long (N=6) flowers using glycerol gel infused with fuschin stain. Between 3-8 pollen grains were measured per flower using a microscope with a graduated eyepiece and pollen grain size was regressed against tube-length. To determine if there were differences in flower colour that could be perceived by insects, the spectral reflectance over the UV-visible range (300–700 nm) of both short- and long-tubed *T. revoluta* flowers in the Gysmanshoek Pass population was measured using an Ocean Optics (Dunedin, Florida, USA) S2000 spectrophotometer and Ocean Optics DT-mini deuterium tungsten halogen light source (200–1100 nm). Nine short and ten long flowers were randomly collected from the site, tube-length was noted and the top petal was used for colour analysis of all flowers. Readings were taken through a fibre-optic reflection probe (UV/VIS 400 micron) held at 45° and about 5 mm from the surface of the petal. To determine if there were any significant differences in nectar volumes and sucrose concentrations between short- and long-tubed flowers, nectar volumes were obtained by removing the ovaries and measuring the nectar with 10 µl capillaries.

Sucrose concentration for both morphotypes was measured with a handheld refractometer (from Bellingham & Stanley) and T tests were used for comparisons.

Pollinator observations

To determine the number, type and behavior of pollinators present at the Gysmanshoek Pass population, approximately 50 hours were spent on pollinator observations in 2007 and 2008. Observations were done in sunny weather conditions, between 09h00 and 12h00 when insect pollinators are most active. In addition, a flower-visitor preference test was conducted to determine whether pollinators preferentially visit short- and/or long-tubed flowers. The experimental set-up consisted of 5 short and 5 long inflorescences in vials filled with water, arranged in mixed pairs (one short, one long) in a half circle. Experiments were conducted at the top (short tubes) and bottom (long tubes) of the study site and observers noted all visits by all pollinators to arranged flowers and captured visitors after they made contact with the flowers. Ten hours were spent on observing choice pairs. Data collected included the number and type of pollinators, and T tests were used for comparisons of visitations rates to the two morphotypes. A number of the insects visiting *T. revoluta* flowers were caught and tested for pollen presence on their bodies.

Pollen limitation and autogamy

To determine whether *T. revoluta* is pollen limited, as well as self-compatible, bagging experiments were conducted for short and long morphotypes. Inflorescences with either two or three closed flower-buds on 40 short-, 20 intermediate- and 31 long-tubed plants were bagged. After 3-4 days, when the buds had opened, treatments were applied as follows: buds were cross-pollinated with pollen from a plant at least 1 m away, or left to self-pollinate without the aid of an external pollinator. Plants were clearly marked with bright yellow duct tape, and coloured wool was used to label each treatment. After treatments were applied, the mesh bags were put back over the plants and left for approximately a month. Seeds from treatments were harvested and counted, as well as seed that was naturally set. Possible pollen limitation was determined by comparing hand- and natural-pollination seed set between short and long morphotypes. Data were analysed using generalized linear models which assumed a Poisson distribution and a log link function and controlled for overdispersion of the data (STATISTICA 8 Statsoft Inc). Pollination types (hand- and natural) and tube-length categories (short and long) were used as independent categorical variables. Possible autogamy was determined by comparing natural-and autogamous pollination seed set between short and long morphotypes.

Generalized linear models (as described above) were used with pollination type (natural and autogamous) and tube-length (short and long) as independent categorical variables. Generalized linear models were used because data did not satisfy the assumptions of parametric statistics.

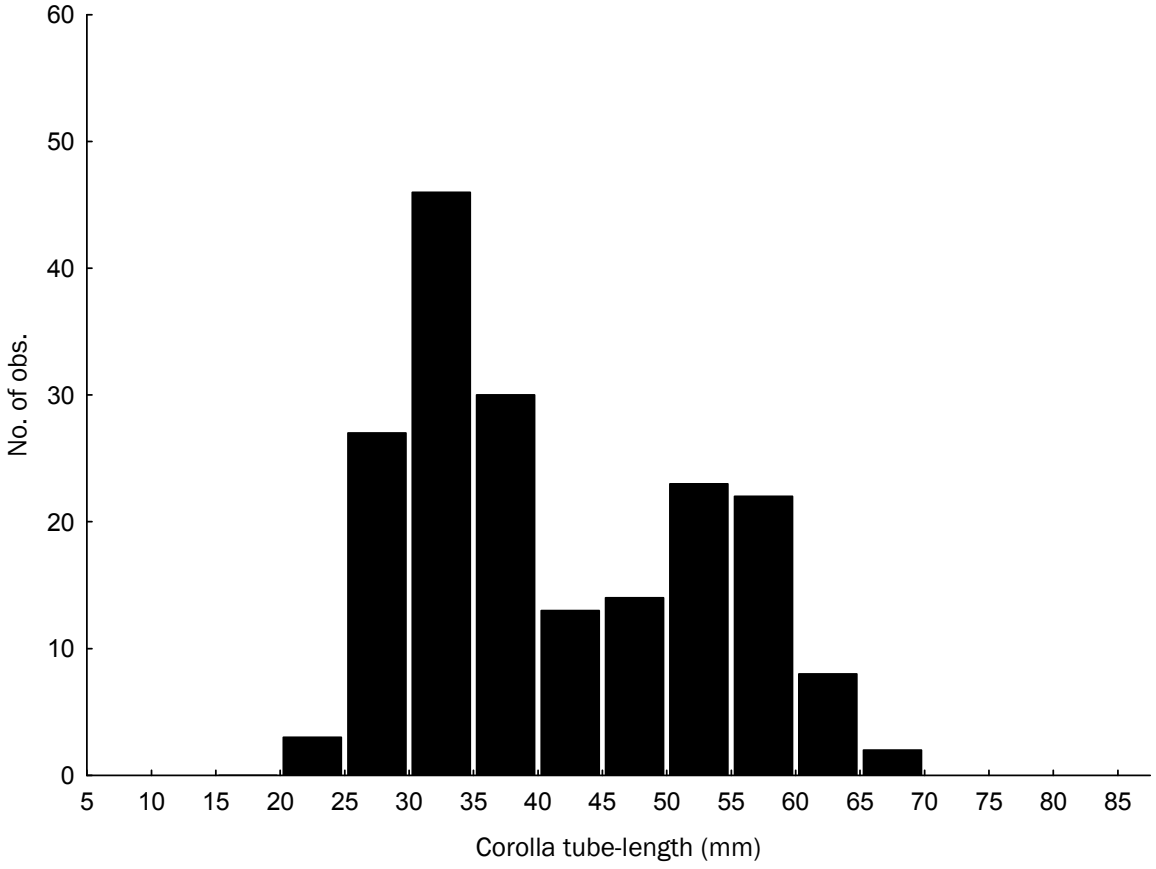
Genetic incompatibility

To determine whether the different *T. revoluta* morphotypes are reproductively compatible with one another, crossing experiments were conducted. I made nine types of crosses; short, intermediate and long individuals were pollinated with short, intermediate and long pollen, respectively. These treatments were applied to the same inflorescences as the breeding system experiments. Flowers were labelled and bagged as above, and after about a month, the seeds were harvested and counted. Mean seed set between short, intermediate and long individuals were compared. Data were analysed using generalized linear models which assumed a Poisson distribution and a log link function and controlled for overdispersion of the data (STATISTICA 8 Statsoft Inc). Ovule parents and pollen parents were used as independent categorical variables. Generalized linear models were used because data did not satisfy the assumptions of parametric stats. In addition, I also used Kruskal-Wallis rank sum tests to compare seed set in a subset of this data set where sample sizes were largest: between short ovule parents.

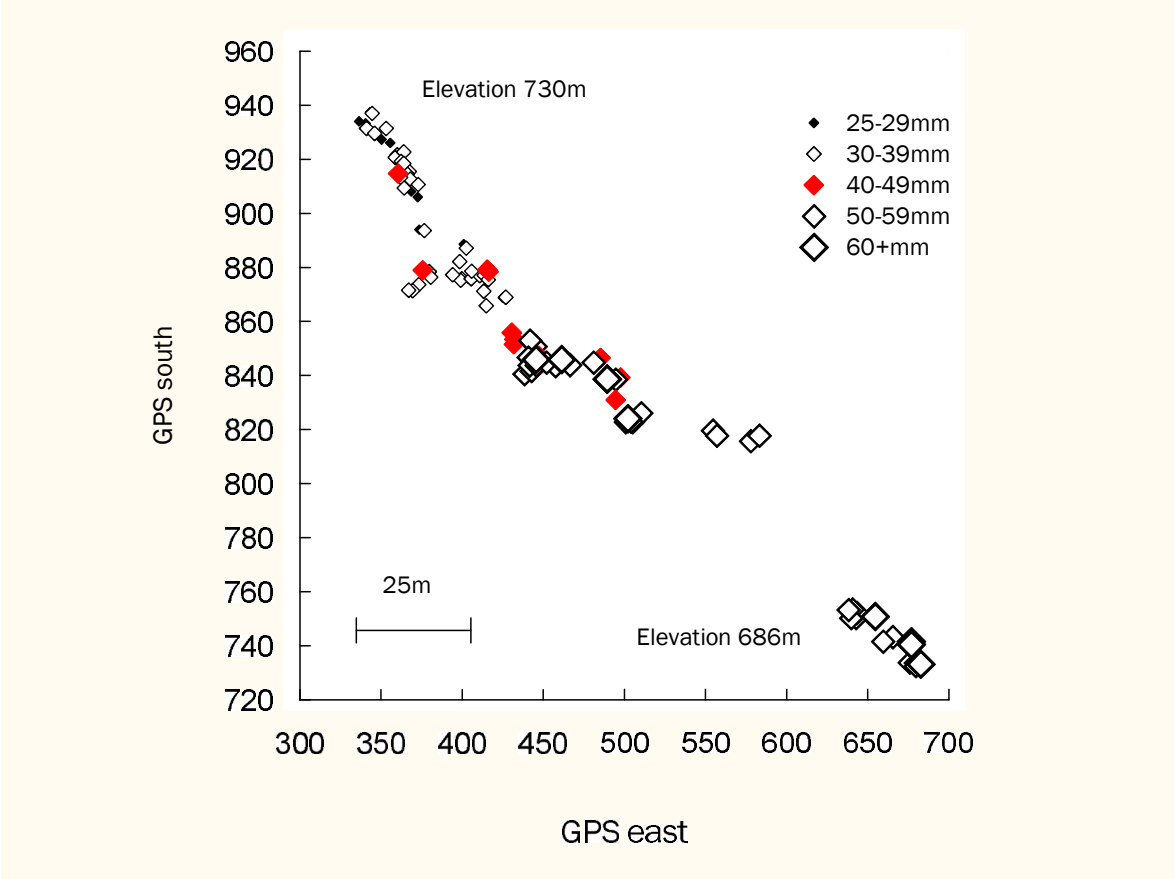
RESULTS

Patterns of variation in floral traits

The *Tritoniopsis revoluta* population at Gysmanshoek Pass has a significant bimodal distribution of perianth tube lengths (b = 0.62) (Figure 1). Tube lengths range from 20-70 mm, with a paucity of individuals in the 40-50 mm range. The distribution of morphotypes at this site shows a pattern of the shortest flowers (25-29 mm) growing at the highest elevation (730m above sea level) and, as the elevation decreases, the flower tube-lengths increases (Figure 2) with the longest flowers (60-70 mm) growing at 686 m above sea level. The distribution of the shortest flowers (< 30 mm) does not overlap with that of the longest flowers (> 60 mm).



▲ Figure 1: Histogram of the distribution of *T. revoluta* tube-lengths at the Gysmanshoek Pass site (N = 188). A clear bimodal distribution can be distinguished.



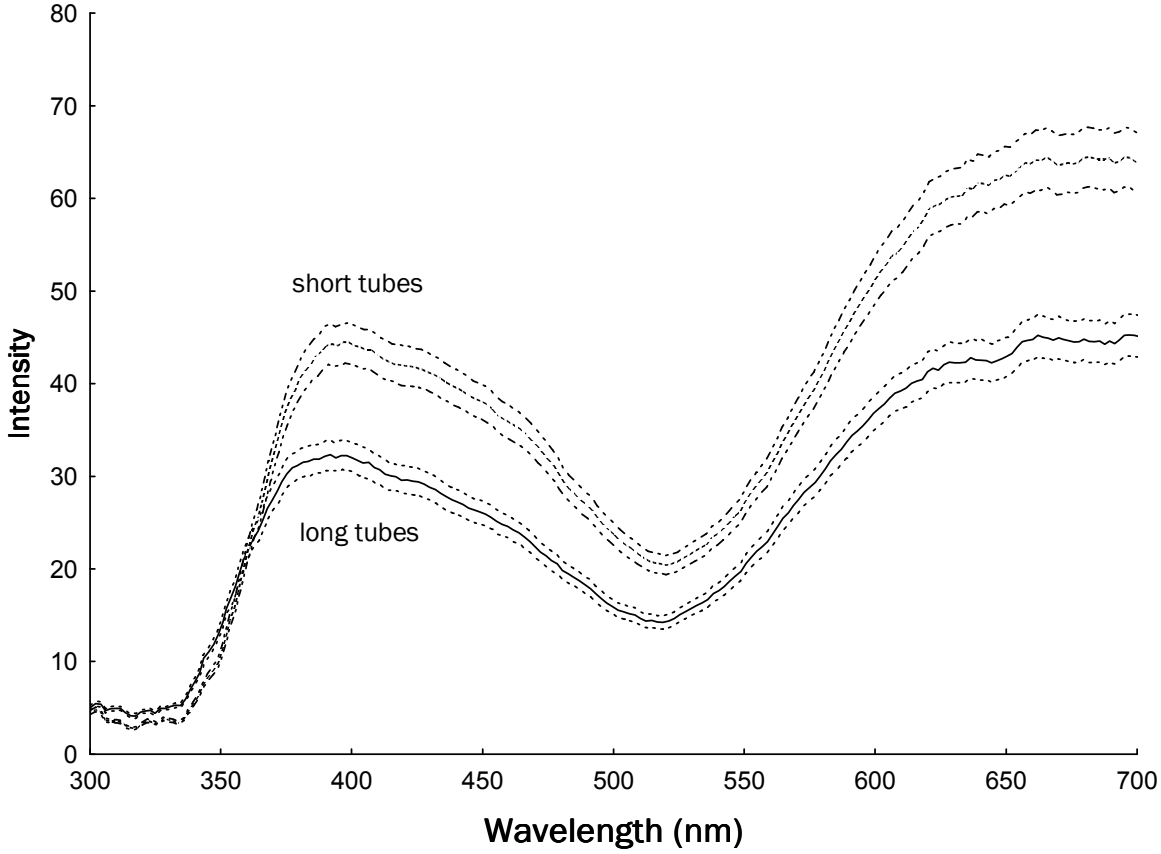
▲ Figure 2: Spatial distribution of *T. revoluta* tube-length categories at the Gysmanshoek Pass site.

There was no significant relationship between pollen grain size and tube-length (R^2 = 0.03, P = 0.15, data not shown). Spectrophotometer data showed that there is a slight difference in the reflectance pattern between short and long morphotypes (Figure 3), with short-tubed flowers being brighter than long-tubed flowers. It was also determined that long-tubed flowers have a significantly higher nectar volume (t = -7.46, df = 125, p < 0.05, Figure 4) as well as sugar content (% sucrose) than short-tubed flowers (t = -4.54, df = 123, p < 0.05, Figure 4).

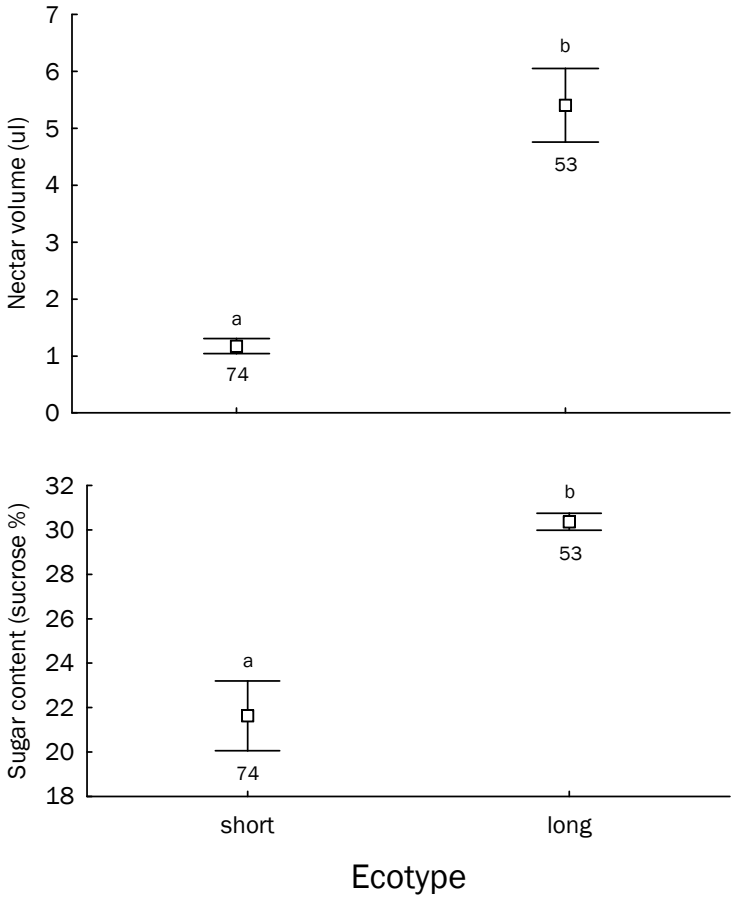
Pollinator observations

No long-proboscid flies were observed on *T. revoluta* at the Gysmanshoek Pass site. The only types of pollinators caught at the Gysmanshoek Pass site visiting *T. revoluta* flowers were insects from order Hymenoptera and Diptera (Table 1, Appendix A). Pollen was present on 81% of the captured *Amegilla fallax* bees, on 67% of the captured Halictidae (small black bees), on 11% of the captured short-proboscid black calliphorid flies (*Cosmina fuscipennis*) and absent from the one captured honeybee (genus *Apis*). Carpenter bees (genus *Xylocopa*) were very

occasionally observed visiting *T. revoluta* flowers but we were unable to capture them for pollen load analysis. It was determined that there are no significant differences in visitation rates of all bees to short and long morphotypes at the top of the gradient (t =-1.20, df = 100, p = 0.231), as well as at the bottom of the gradient (t = -0.33, df = 32, p = 0.741).



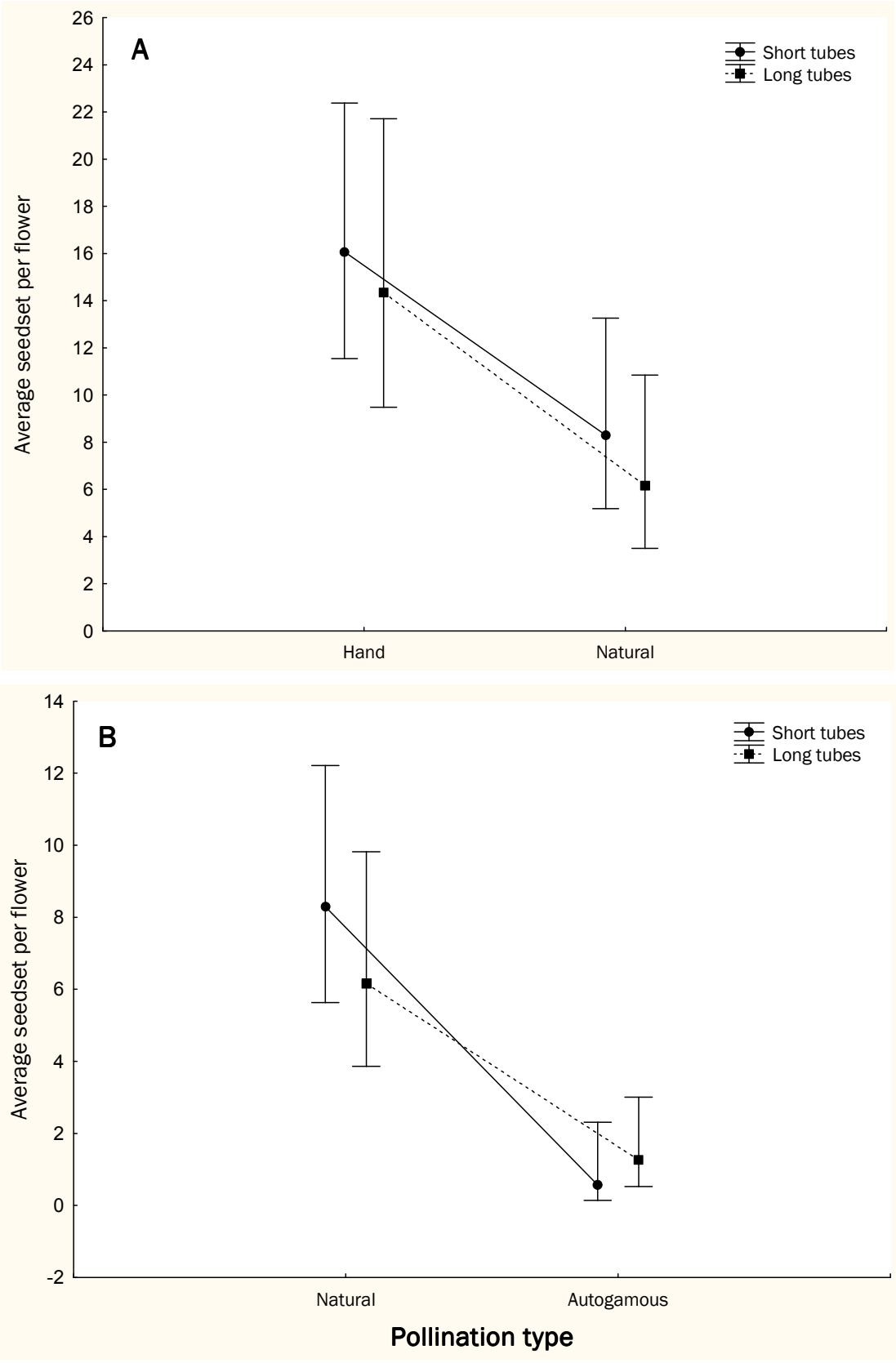
▲ Figure 3: Reflectance spectra of short and long tubed flowers at the Gysmanshoek Pass site. Solid lines represent the mean and dotted lines the SE.



▲ Figure 4: Nectar volumes and sugar content (% sucrose) of short and long-tubed flowers. Letters denote significant differences from T-tests. Whiskers are SE and numbers below each treatment indicate sample size.

Pollen limitation and autogamy

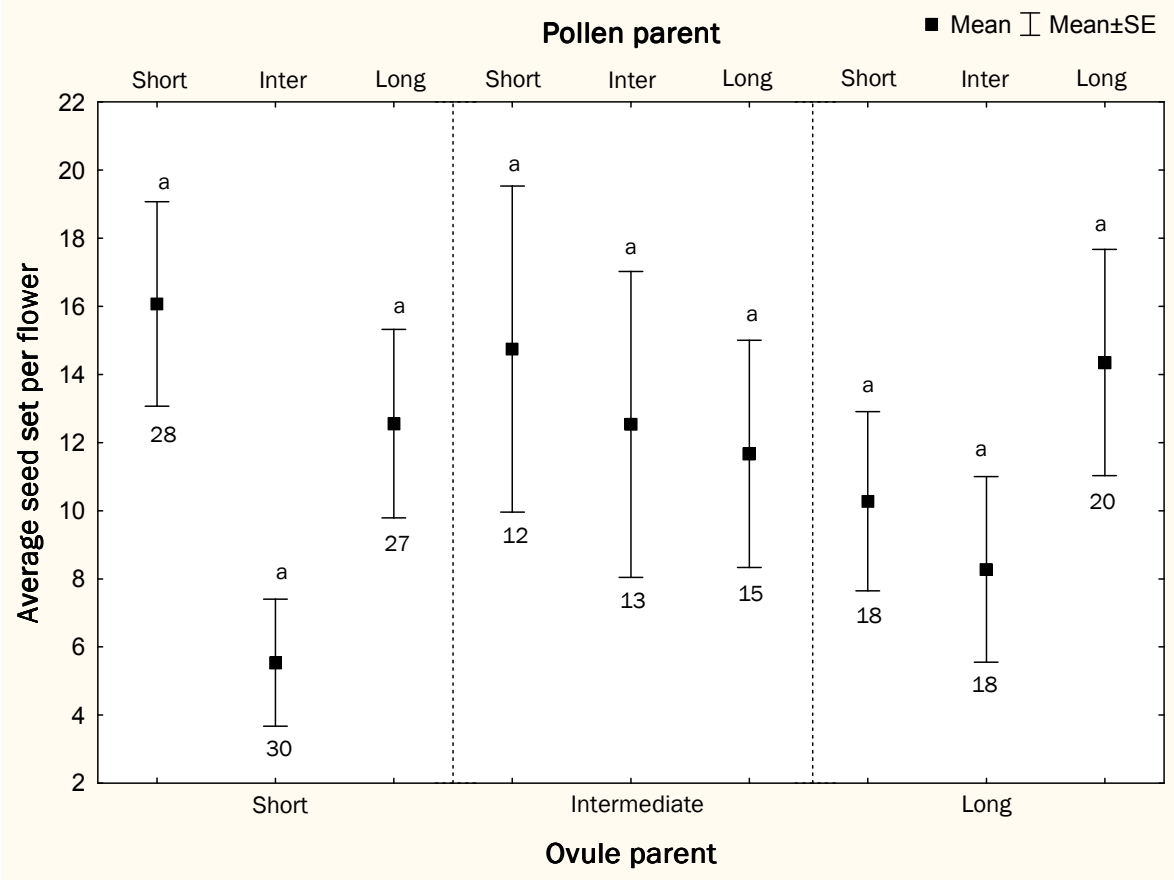
From the bagging experiments, it was concluded that *Tritoniopsis revoluta* is pollen limited. Analysis showed a treatment effect where hand pollination set more seed than natural (Wald $X^2(1) = 10.645$, $p = 0.001$, Figure 5a). However, there was no effect of tube length (Wald $X^2(1) = 0.792$, $p = 0.374$, Figure 5a) and there was also no interaction effect (Wald $X^2(1) = 0.159$, $p = 0.689$, Figure 5a). Also, *Tritoniopsis revoluta* is unable to self-fertilize efficiently without the aid of pollinators. Analysis showed a treatment effect where natural pollination set more seed than autogamous pollination (Wald $X^2(1) = 22.689$, $p < 0.001$, Figure 5b). However, there was no effect of tube length (Wald $X^2(1) = 0.309$, $p = 0.578$, Figure 5b) and there was also no significant interaction effect (Wald $X^2(1) = 1.488$, $p = 0.223$, Figure 5b).



▲ Figure 5: Bagging experiments for *T. revoluta* to test for pollen limitation and self-compatibility. Pollination type is represented on the x-axis and whiskers are SE.

Genetic incompatibility

Mean seed set per flower did not differ significantly between short, intermediate and long ovule parents when crossed with short, intermediate and long pollen. Analysis showed that there was no effect of ovule parents (Wald $X^2(1) = 0.996$, $p = 0.608$, Figure 6) or pollen parents (Wald $X^2(1) = 4.565$, $p = 0.102$, Figure 6). There was also no interaction effect (Wald $X^2(1) = 4.778$, $p = 0.311$, Figure 6). From figure 6 it seems that intermediate pollen sets fewer seeds when crossed with short individuals; pollen parent as independent variable crossed with only short individuals had a significant effect (Kruskal-Wallis $\chi^2 = 9.91$, df =2, $p = 0.007$, Fig. 6).



▲ Figure 6: Cross experiments for *T. revoluta*. Tube-length data is represented on the x-axis (top and bottom of graph) as ovule and pollen parents respectively, and seed set per flower on the y-axis. Letters denote significant differences using the Tukey test at $p<0.05$. Whiskers are standard error and numbers below each treatment indicate sample sizes.

DISCUSSION

T. revoluta exists as two overlapping floral morphotypes at the Gysmanshoek Pass site. These morphotypes differ in tube-length, colour, nectar volume and sugar content. The two *T. revoluta* morphotypes occurs at either side of the Gysmanshoek Pass; a short morphotype to the South of the pass, and a long one to the North. A very narrow contact zone exists where these two morphotypes meet and a few intermediate flowers are present here. More short- and long-tubed flowers were observed than intermediate-sized flowers. The presence of the few intermediate flowers suggests that some gene-flow occurs between the two morphotypes where the ranges of these morphotypes overlap.

Judging from previous studies on relationships between floral traits and pollinator proboscis lengths (Alexandersson and Johnson 2002; Anderson and Johnson 2008; Anderson and Johnson 2009; Johnson and Steiner 1997; Whittall and Hodges 2007) and Chapter 1 of this thesis, we hypothesized that long-proboscid flies are the primary pollinators of *T. revoluta* at the Gysmanshoek Pass site, with tongue-length variation being the force driving the length of the short and long flower tubes and assortative mating keeping the tube-length difference between morphotypes intact (Aldridge and Campbell 2007). This hypothesis may be supported by the fact that short- and long-tubed plants at this contact zone have different nectar volumes and concentrations which may be related to bee and fly preferences. In contrast the similar reflectance patterns for both short and long morphs lend no support to the "two pollinator" hypothesis, although the lack of differences in reflectance patterns do not necessarily means the two morphotypes utilize the same pollinator.

No long-proboscid fly pollinators were observed in this bimodal population, although the weather was favorable and flowers were numerous. Therefore it may seem that distinct morphotype morphology is not maintained in this population through assortative mating by two different long-proboscid fly pollinators. A number of plausible explanations may exist for the absence of long-proboscid fly pollinators. This long-tubed population could be at the edge of the long-proboscid fly pollinator distribution range and flies may be exceptionally uncommon here or even absent. Absence can be explained by dispersal out of the fly's range or local extinction (e.g. Steiner 1993). It is also possible that the two survey years were just poor for flies, as Manning and Goldblatt (2005) captured *P. longipennis* very close to the LE-2 population and so it is likely that the fly does occur in some of the eastern populations.

A number of bees and small flies were observed visiting the flowers at this site, as well as to the south of the hybrid zone where flowers have shorter tubes and bees can access the nectar (de Merxem et al. 2009). Short-tubed flowers and their different nectar qualities could be the result of allopatric adaptations to bees. de Merxem et al. (2009) found that bees could access the nectar in short flowers, but not in the long ones, and on the southern slopes where only short-tubed flowers were found, bee visitation was very high, as well as seed set. Bees were not common visitors to long morphotypes because the nectar is inaccessible, and probably avoided them because long morphs generally grow together. When short- and long-tubed flowers were presented together, the bees did not distinguish between them. It is therefore unlikely that bee choices are the cause for assortative mating in sympatry.

In the light of the pollinator community found at the Gysmanshoek Pass site, a logical question would be why some flowers have long tubes seemingly adapted to long-proboscid flies, and not short tubes adapted to bees. *Tritoniopsis revoluta* may be specialized for pollination by long-proboscid flies (*sensu* Stebbins' (1970) ''most effective pollinator principle''), while still being pollinated by bees, if there is no fitness cost to being adapted to both bees and flies (Aigner 2001; Aigner 2005). However, this does not seem to be the case at this site as bees appear to have learned to avoid areas with long-tubed individuals because they are unable to access the nectar within these flowers (de Merxem et al. 2009). Instead, this site may be an example of a *T. revoluta* population at the edge of its fly pollinators' range. Adaptations to bees may have driven tube-length shortening on one side of the Langeberg Mountain but this shortening may not have spread to the long tubed flowers on the northern slopes due to factors unrelated to pollinator-driven assortative mating, for example differences in flowering times, differences in spatial distribution, self-fertilization or genetic incompatibilities between the morphotypes. Pollen limitation in *T. revoluta* may also suggest that its specialist pollinators are absent from the landscape (see Larson and Barrett 2000). However, it is still possible that one (or two) long-proboscid fly species exist, but were not present during the two seasons encompassed by this study, based on the observation of *P. longipennis* by Manning and Goldblatt (2005) near Riversdale.

It was determined that the flowering times of the short and long morphotypes at the Gysmanshoek Pass site are identical; thus we can rule out assortative mating through differences in flowering time. The spatial distribution of morphotypes make it more likely for gene flow to occur between closely situated individuals, which tend to be of the same morphotype, and this could contribute towards assortative mating at the site (Cruzan and Arnold 1994; Ramsey et al. 2003). The spatial distribution of *T. revoluta* may be influenced by

limited seed dispersal of morphs, or possible effects of an environmental gradient where tube-length is phenotypically plastic. However, the gradient is very short and altitude does not differ much between short and long morphs; short and long morphs occasionally grow mere meters apart. *T. revoluta* seeds are also small and have sponge-type wings, and can possibly travel great distances through wind dispersal. Large seed dispersal distances would lend support to tube-lengths being a phenotypically plastic trait. The only way to determine whether variation in tube-length is a result of phenotypic plasticity is to do reciprocal transplant or common garden experiments, neither of which were done in this study. However, steep slopes at other sites investigated in Chapter 1 did not lead to variance in tube–length, suggesting phenotypic plasticity is an unlikely cause of tube-length variation at the Gysmanshoek Pass site.

Another possibility may be that all the seed set at this site is produced via autogamy, in which case there will be no mixing between the morphotypes, and short and long morphs will be retained. I found that autogamy contributes very little to seed set at this site. It is uncertain whether *Tritoniopsis* plants are self compatible as no studies have been published concerning this, but *T. revoluta* plants are unable to self-fertilize without the aid of pollinators as can be concluded from the low number of seeds being set after autonomous self-pollination. A pilot experiment conducted by myself (self-pollination of *T. revoluta* by hand) suggests that *T. revoluta* is indeed self-compatible; although a more thorough study of this is needed as my own sample sizes were very small. If *Tritoniopsis* plants are indeed self-compatible, then seeds set may be the result of geitonogamy. If all the seed set is through geitonogamy, morphotypes will also be maintained at this site. The only way to assess the impact of geitonogamy on seed set would be through emasculations, microsatellite- or allozyme analyses.

Preservation of morphs may also be through genetic incompatibility that acts as a gene flow barrier. From the crossing experiments it was determined that the short, intermediate and long morphotypes are fully inter-fertile. Also, the lack of relationship between tube-length and pollen grain size (suggesting similar ploidy levels between morphotypes (Eenink 1980)) did not support the genetic incompatibility hypothesis. Short, intermediate and long morphotypes produce offspring when crossed with short, intermediate and long pollen. Gene-flow between the short and long morphotypes appears to be taking place, and intermediate individuals aren't less fit than either the short or long morphotypes. Hybridization between the short and long morphotypes appears to be taking place, producing intermediate individuals. There are no significant differences in seedset between short, intermediate and long individuals, meaning the short and long *T. revoluta* entities are not reproductively isolated, and intermediates are

not hybrids. Subsequently, genetic incompatibility can not be accounted for in the maintenance of morphotypes in sympatry.

So how, then, did these tube-length differences between morphotypes arise? There are a few plausible explanations. The Gysmanshoek Pass site may be a secondary contact zone where *T. revoluta* morphotypes meet after having diverged in allopatry; long morphotypes may have historically experienced selection from long-proboscid flies which have been captured in the Langeberg mountain range (de Merxem et al. 2009). Morphotypes are hybridizing and producing intermediate morphotypes at LE-1. An alternative explanation is that *T. revoluta* is pollinated by two different long-proboscid fly species with differing tongue lengths in the Langeberg, transferring pollen assortatively, but visiting insects transfer pollen indiscriminately thus giving rise to intermediates occasionally. I found no evidence to support this, but have given a number of possible explanations for the lack of long proboscid fly pollinators at this site. Bees which seem to be common visitors to both morphotypes do not distinguish between the two when they are growing side by side. In conclusion *T. revoluta* morphotypes at the Gysmanshoek Pass site are not reproductively isolated; tube-length differences could possibly be maintained through slight differences in the spatial distribution of morphotypes and the fact that plants are most likely to mate with close neighbours which will normally have a similar tube length (Figure 2).

ACKNOWLEDGEMENTS

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APPENDIX A

Tables

Table 1: Pollinator observations at the Gysmanshoek Pass site.

Pollinator	Landed	Long Tube	Short Tube
Cosmina fuscipennis	57	19	38
Amegilla fallax	31	11	20
Halictidae	5	2	3
Xylocopa	2	1	1
Apis	7	3	4

CHAPTER 3

GENETIC PATTERNS OF TUBE-LENGTH EVOLUTION IN *TRITONIOPSIS REVOLUTA*

ABSTRACT

Pollinators play a role in the evolution of floral traits. *Tritoniopsis revoluta,* a pink irid occuring in the Western Cape Province of South Africa, exhibits highly variable tube-lengths. The mean tube lengths of populations correspond closely to the tongue-lengths of available pollinators, suggesting that pollinators may have driven divergence in floral traits in this system. This study aimed to elucidate the patterns of tube-length evolution in *Tritoniopsis revoluta* using phylogenetic methods (chloroplast markers and AFLPs) to determine the directionality and frequency of transitions between tube-length categories. Using character state reconstruction we determined whether tube-length transitions were associated with pollinator shifts, suggesting that tube length is labile and responds to variable selection from pollinators. From this it was determined that two evolutionary transitions to shorter tube-length categories and four transitions to longer categories occurred. Importantly, extremely long tubes evolved independently twice in this species, which may suggest a role for pollinator-mediated selection in tube length evolution. Population genetic structure in this system showed that populations of *T. revoluta* are isolated by distance; tube-length similarities in different populations are thus not due to common descent. I conclude that tube-lengths possibly evolved due to pollinator-mediated selection and that common ancestry or other environmental pressures are not the main determinants of tube-length evolution in this system.

INTRODUCTION

Floral trait variation between and within genera has been largely attributed to selective pressures imposed by pollinators (Campbell et al. 1997; Campbell 1996; Schemske and Bradshaw 1999). In contrast adaptations to the physical environment are often reflected in variation of plants' vegetative morphology (Stebbins 1970). Apparent ''pollination syndromes'' (Faegri and van der Pijl 1966; Fenster et al. 2004) may develop as a result of pollinator preference for certain floral attractants and rewards. It has been suggested that, in addition to influencing the evolution of flower structure, pollinators may also play a role in the evolution of other floral traits, like flower color (Campbell et al. 1997; Rausher 2008; Schemske and Bradshaw 1999). Floral traits may alternatively be influenced by other selective agents which include both biotic (e.g. nectar thieves, herbivores and seed predators) and abiotic (e.g. soil nutrients and moisture availability) factors (Galen 1999; Herrera 1996). In addition, genetic drift (Wright 1943), or selection on linked characters (Armbruster 2002) may also play a role in generating floral trait variation.

Perhaps one of the best ways of showing the effects of pollinators on floral morphology is through natural selection studies. It has been shown that pollinator proboscis length can exert strong selection pressures on flower spur length. For example, moth-pollinated *Platanthera* flowers with artificially shortened spurs had lower fruit set than flowers with longer spurs (Nilsson 1988). Also using this approach Johnson and Steiner (1997) determined that *Disa draconis* flowers with artificially shortened spurs had reduced fitness because their long proboscid fly pollinators did not make contact with their stigmas. They also showed a correlation between pollinator mouthparts and the tube lengths of the flowers in this system. Using translocation experiments, more evidence for the pollinator-mediated floral variation hypothesis was provided by studies on the long-proboscid fly pollinator *Prosoeca ganglbaueri* and two flowers pollinated by it (Anderson and Johnson 2008; Anderson and Johnson 2009). A study using natural variation was conducted on the South African iris, *Gladiolus longicollis*, which also exhibits significant variation in flower tube-length and is pollinated by the large hawkmoth *Agrius convolvuli* (Alexandersson and Johnson 2002). This study provides evidence for the hypothesis of pollinator-mediated selection on floral tube-length in a plant population that is specialized for pollination by a long-proboscid hawkmoth species. In a study on two species of *Ipomopsis*, pollination by hummingbirds and hawkmoths were studied (Campbell et al. 1997). Hummingbirds preferentially visited flowers with wide corollas and bright red coloration, producing directional selection, whereas hawkmoths rather visited flowers with

narrow corolla tubes; in areas where both pollinators were present, corolla width experienced disruptive selection. The problem with selection approaches is that often there is little trait variation within a population and so selection is difficult to detect. In addition the strength of selection on morphological traits has been found to change on an ongoing basis and so often studies do not find good evidence for selection on traits that seem to be obviously adaptive (Harder and Johnson 2009).

Pollinator-driven variation may also be inferred through correlation of adaptive traits of plants and their pollinators (Anderson and Johnson 2008; Anderson and Johnson 2009; Pauw et al. 2009; Steiner and Whitehead 1991). The effects of pollinator-driven selection on flower morphology may differ between populations, resulting in possible geographical variation in floral traits of the same species. An example of this is shown in a study on *Satyrium hallackii*. This orchid is distributed from the north to the south of South Africa, along the coast. *Satyrium hallackii* plants in the north, which are pollinated by hawkmoths, have long tubes corresponding to the long tongues of the moths. In contrast, the plants in the south have short tubes corresponding to the short tongue-lengths of their bee pollinators (Johnson 1997). In a correlatory study on *Zaluzianskya microsiphon* and its long-proboscid fly pollinator *Prosoeca ganglbaueri* (Anderson and Johnson 2008), it was shown that corolla tube-length and pollinator proboscis length were strongly correlated with each other. Correlations between pollinator and plant morphology were also found in the irid *Lapeirousia anceps* and its long-tongue fly pollinator (Pauw et al. 2009) as well as for the legs of oil collecting bees which are inserted into the matching spurs of *Diascia* (Steiner and Whitehead 1990; Steiner and Whitehead 1991). Correlations between plant and insect traits may not be the result of adaptations by plants to insects but rather by insects to plants. However this is usually thought not to be the case because of the asymmetry of specialization which has been shown to exist between plants and their pollinators. Here, specialized plants are usually visited by generalist insects (Johnson and Steiner 2000; Waser et al. 1996). In addition, other alternative mechanisms for trait correlations may also exist such as environmental differences which have similar effects on both plant and pollinator morphology (Galen 1999). For example, variation in flower size can't always be attributed to pollinator-mediated selection as there may be other ecological factors playing a role in this variation by focusing on the cost of exhibiting a floral display and the attraction of herbivores. Because of the shortcomings of the correlative approach, many such studies combine correlations with other approaches such as selection studies (Anderson and Johnson 2008; Anderson and Johnson 2009; Johnson and Steiner 1997; Pauw et al. 2009; Toju and Sota 2006a; Toju and Sota 2006b; Toju and Sota 2006c).

The Cape Floristic Region has unusually high species richness and a number of studies have focused on pollinators as drivers of variation within this region (Goldblatt et al. 1995; Johnson et al. 2007; Johnson and Steiner 1997; Pauw 2006). Few of these studies utilize phylogenetic methods to elucidate evolution of floral traits; however, one study in Iridaceae, on the genus *Moraea* (Goldblatt et al. 2002) used four plastid DNA regions to construct a chloroplast phylogeny and then map floral traits onto this phylogeny, where floral changes are thought to be associated with shifts in pollination systems. Also, in a study on *Disa*, a large orchid genus in southern Africa, morphological characters were used to construct a cladogram. Possible pathways of floral trait evolution were investigated by mapping the different pollination systems existing in this genus onto the phylogeny. In all, 19 different specialized pollination systems have been found in 27 species of *Disa* (Johnson et al. 1998). Floral evolution in this genus can thus be ascribed to shifts in pollination systems. In the columbine genus *Aquilegia* (Ranunculaceae), amplified fragment length polymorphisms were used to construct a phylogenetic framework to determine the history of the evolution of spur lengths (Whittall and Hodges 2007). Their phylogenetic evidence indicates that 73% of the total spur-length evolution occurs coincidently with changes in pollinator syndromes, and that tube-length evolution in this genus is directional and has occurred without reversals, resulting in the progressive lengthening of nectar spurs.

Phylogenetic methods have also been used extensively to study plant diversification in the Cape Floristic Region and add insight into the processes driving this diversification. In a recent study, parsimony optimization was used to identify succulent karoo- and fynbos-endemic lineages using dated phylogenies. All succulent karoo-endemic lineages are a product of recent radiation, whilst the fynbos-endemic lineages were mostly older (Verboom et.al 2009). Thus the species richness of the fynbos may be a result of a long history of speciation. A number of studies have also demonstrated a role for plant adaptation to edaphic environments. In a study on the miniature succulent, *Argyroderma pearsonii* in the Knersvlakte region of South Africa (Ellis et al. 2007), it was determined, using amplified fragment length polymorphisms (AFLP), that restricted gene flow between basins together with the spatial and ecological isolation has played an important role in the evolutionary radiation of this succulent. Another phylogenetic study, on the grass genus *Ehrharta*, used sequence data (nuclear ITS1 and plastid *trn*L-F) to determine if all members of this genus in the Cape are diversified from a single lineage, and if shifts in climate conditions played any role in the rapid radiation of this genus (Verboom et al. 2003). The constructed phylogeny of the African *Ehrharta* shows rapid speciation coinciding with a shift from wet, all-year rainfall habitats to more dry and arid conditions. Outside of the

CFR, it has also been shown how effectively molecular tools can be utilized to study the evolution of floral traits (e.g. Archibald et al. 2005 , Whittal and Hodges 2007).

In Chapter 1 of this thesis, I focused on the distribution and pollination of *Tritoniopsis revoluta*, a pink irid occuring in the Swartberg and Langeberg Mountains, as well as near the coast at Potberg Mountain. This species exhibits highly variable tube-lengths and this is suggestive of different pollinators operating in different parts of the flower's range. I showed that a clear relationship exists between the tube-lengths of *T. revoluta* and the tongue-lengths of its corresponding pollinators in the different populations (Chapter 1). In order to complement these correlative data, I aim to use population genetics techniques to determine whether patterns of tube-length evolution are also suggestive of pollinator-driven variation. Molecular tools were utilized to construct a phylogeny in order to understand the evolutionary history of *T. revoluta.* Pollinators were mapped on the phylogeny to determine, using a phylogenetic framework, whether shifts in tube-lengths are associated in pollinator shifts. I investigated whether the patterns of tube length divergence between populations are likely the result of selection by pollinators or other processes (neutral) by firstly determining patterns of transitions in tube length, and secondly the relationship between tube length and neutral genetic divergence. If tube-lengths between populations are similar due to common descent, it may suggest that selection processes played a very small role (if any role at all) in the evolution of tube-lengths. However, if tube-length differences arise in populations which do not form monophyletic groups, then this suggests independent evolutionary origins most probably connected to selective pressures operating in the isolated populations.

MATERIALS & METHODS

Population Sampling

Seven populations of *Tritoniopsis revoluta* were identified for this study from collections housed at the Compton Herbarium (SA National Biodiversity Institute, Kirstenbosch) and two were discovered during field observations. Population BD-1 is not included in this Chapter; however, population LS-1 is short-tubed and was discovered approximately one kilometre south of the LE-1 population and included in these analyses. Fresh flower material (one flower per plant) was randomly collected for all nine *T. revoluta* populations during March 2007 and March 2008; the tube-lengths of the collected flowers were recorded. The material was stored in envelopes and immediately dried with silica gel for DNA analysis. Whole DNA was extracted from nine *Tritoniopsis revoluta* populations (three individuals per population) occurring across the species range. DNA was extracted from 0.2g of dried flower material stored in silica gel using the CTAB method (Doyle and Doyle, 1987) as well as the DNeasy Plant Mini Kit (Qiagen). The dry flower material was ground to a fine powder in liquid nitrogen, and extractions of DNA were purified using Illustra™ DNA and Gel Band Purification Kits (GE Healthcare UK Ltd) prior to amplification and sequencing.

Chloroplast Sequencing

The two plastid gene regions amplified using PCR (polymerase chain reaction) were the 3'trnV-ndhC intron, using the primers *trnV* and *ndhC* designed by Shaw *et al.* (2007), and the *trnG-trnS* intergenic spacer, using the primers *trnG* and *trnS* designed by Hamilton (1999). PCR was performed using an Applied Biosystems thermal cycler (GeneAmp PCR System 2700) and reactions were prepared on ice in 30µl volumes per reaction with the following reaction components: 10-50 ng template DNA, 10X reaction buffer (Southern Cross Biotechnologies, Cape Town), 1 mM dNTP, 25 mM $MgCl_2$, 1 pmol/µl 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 minutes at 80°C, followed by 35 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 1 min. The final elongation step was at 72ºC for 10 min. Gel electrophoresis using a 1 % agarose gel was performed to check the success of the PCR amplification. The gel was loaded with the 30 µl PCR product and was allowed to run at 100V 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 BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer/Applied Biosystems), using the thermal cycle parameters at 96ºC for 10 s, at 55 ºC for 5 s and at 60ºC 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. Editing and assembling of the obtained sequences, as well as alignments, was performed in MEGA version 4 (Tamura et al. 2007). *Tritoniopsis antholyza* was used as outgroup taxon throughout the study, based on a *Tritoniopsis* genus tree constructed by Felix Forest (unpublished data).

Amplified Fragment Length Polymorphisms

Individuals were genotyped for AFLP markers according to the AFLP Plant Mapping Protocol from Applied Biosystems. One hundred ng genomic DNA was digested with restriction enzymes *Mse*I and *Eco*RI (New England Biolabs) and then ligated to doublestranded *Eco*RI and *Mse*I adapters. The restriction–ligation reactions were incubated for 2 hours at 37ºC. The ligation product was then pre-amplified using the PE Applied Biosystems (Foster City, CA, USA) Ligation and Preselective Amplification Module. Pre-selective PCR reactions were run using 10 µl reaction volumes. A total of 12 primer combinations were tested for eight samples. Of these combinations, four were selected for amplification of the full set of samples: E-ACT (FAM) & M-CAC, E-AAG (JOE) & M-CAA, E-ACT (FAM) & M-CAG and E-AGC (FAM) & M-CTA. Products from selective amplifications were separated on a LiCor 4200 DNA sequencer (Central Analytical Facility, University of Stellenbosch). Genescan-500-ROX (PE Applied Biosystems) was used as an internal lane size standard. Multiple runs of a number of samples were done to check consistency of results.

Genetic structure

1. Chloroplast Analysis

The two chloroplast intergenic spacer regions, *3'trnV-ndhC* and *trnG-trnS* were analyzed as a combined data matrix and used for the phylogeny reconstruction. Only four indels were present in the combined data matrix of 1411 bp and were excluded from the analysis. A phylogram of *Tritoniopsis revoluta* populations using chloroplast sequence data was constructed using Bayesian inference. MrBayes version 3.1 (Huelsenbeck and Ronquist 2001) was used for Bayesian analyses, which were performed on the combined chloroplast data matrix using a GTR+I model. This model was selected after sequence data had been tested in MrModelTest

(Posada and Crandall 1998). The Markov chain was run on 100,000 generations, sampling every 100th generation, thus providing 1000 trees from each run. The first 250 (25%) generations of each run were identified as the 'burn-in' period and thus discarded before the calculation of the posterior probabilities. The remaining 75,000 generations from each run, giving a total of 15,000 trees, were used to determine the posterior probabilities.

To depict relationships between chloroplast haplotypes, a haplotype network was constructed in TCS 1.21 (Clement et al. 2000). The program implements a parsimony approach to network construction including the estimation of the 95% confidence interval. In contrast to the tree building methods that estimate genetic divergence between individuals / species / taxa, this program collapses sequences into haplotypes and calculates the frequencies of the haplotypes in the sample.

2. AFLP Analysis

Potential AFLP loci were initially located automatically by searching for peaks higher than 75 fluorescence units within the size range 50– 500 base pairs, but scoring across all individuals for each fragment was done manually with Peakscanner Software v1.0 and Genemapper ® (Applied Biosystems). An overall present/absent binary matrix was constructed for the dataset from all four primer combinations. A cladogram of *Tritoniopsis revoluta* populations using AFLP data was constructed using PAUP* (Swofford 2001) using restriction-site distance methods (from Nei and Li 1979). A heuristic search was performed on the distance matrix data, with TBR and neighbor-joining sequence addition. Relative support for individual branches was estimated using bootstrap values (Felsenstein 1985). Bootstrap values were calculated using 1000 replicate full heuristic searches with MULTREES on and TBR branch swapping.

To determine the number of clusters present in the AFLP dataset (objectively) and to determine to what extent these genetic clusters correspond to geographic locations, populations or tube-length classes, we used the Bayesian clustering algorithms implemented in the program STRUCTURE (Falush et al. 2003; Pritchard et al. 2000). An admixture model was used which allows individuals to have mixed ancestry and assumed correlated allele frequencies. We used multiple runs of between 10,000 – 100,000 burn-in and between 10,000 – 100,000 data collection MCMC iterations to determine the posterior Bayesian probabilities of the existence of one through eleven clusters in the dataset, and the membership coefficients (Qs) to determine each individual's estimated membership fraction in each of the inferred clusters.

3. AMOVA

AMOVA (Analysis of Molecular Variance; Excoffier *et al.* 1992) was conducted for both chloroplast and nuclear datasets in Arlequin 3.1 (Excoffier et al. 2005) to investigate the structuring of genetic variation in the system. I compared two mechanisms potentially structuring genetic variation in the system: 1) vicariance (i.e. genetic divergence between spatially isolated lineages) and 2) genetic divergence between short and long-tubed lineages. To do this I examined the between group component of variance from AMOVAs; one partitioning genetic variation between mountain ranges (Swartberg, Langeberg, Potberg) and the other partitioning variance between four tube length classes (6-15 mm, 16-25 mm, 26-35 mm and > 50 mm). These tube-length categories reflect the natural discontinuity of tube-length distribution found in Chapter 1. Mantel's test (Manly 1991), as implemented in AIS (Miller 2005) was used to test for an isolation-by-distance pattern through regression of the genetic relatedness and geographical distance between pairs of individuals, for both chloroplast and AFLP datasets.

Patterns of tube-length evolution and pollinator switches

In order to elucidate patterns of tube-length evolution in this system, the chloroplast and AFLP datasets were combined. The congruence between chloroplast and AFLP datasets was analyzed according to the partition homogeneity test (Farris et al. 1994) implemented in PAUP* (Swofford 2001) to determine the combinability of the two datasets. The test was run with 1000 replicates, heuristic search, simple addition of sequences, and TBR. A phylogeny combining both chloroplast and AFLP datasets was then constructed using Bayesian inference, with populations as terminals. Each sampled population had a single chloroplast haplotype. Individuals varied a lot in terms of their AFLP profiles, so AFLP profiles for each population were bulked (see Beardsley et al. 2003) in order to derive population-level genotypic profiles. MrBayes version 3.1 (Huelsenbeck and Ronquist 2001) was used for Bayesian analyses, which were performed on the combined data matrix using a GTR+I model for the chloroplast dataset and a restriction-site model for the AFLP dataset. The analysis was run as described above, except the Markov chain was run on 10,000,000 generations, sampling every 1000th generation, thus providing 10,000 trees from each run.

The tubes of between 12 and 96 flowers per population were measured (table 1), from the top of the ovary to the opening of the perianth tube using a digital caliper. Pollinator data were collected from seven of the *T. revoluta* populations (see Chapter 1). To determine the

directionality of tube-length evolution and whether transition(s) between short and long flowers happened as a single event, or on multiple occasions, ancestral character state reconstructions for tube-length were performed with the 'reconstruct ancestral states' module implemented in Mesquite v 2.5 (Maddison and Maddison 2008) using 'Parsimony Ancestral States' as reconstruction method and a stepwise ordered state categorical model. A character matrix was created for all nine *T. revoluta* populations with mean tube-length as categorical character and the combined chloroplast-AFLP tree file from Mr. Bayes (10001 trees) was imported for reconstructions. The same four tube-length categories as above were used: 6-15mm, 16-25mm, 26-35mm and > 50mm. Pollinator species as well as their proboscis-lengths were also mapped onto the tree.

To further test whether patterns of tube length differentiation between populations correspond to the pattern of neutral evolution reflected by the genetic differentiation matrices, a Q_{ST} approach was used to build a tube-length difference matrix (differences in mean tube-lengths between populations, where tube-length is taken as a quantitative trait), and to correlate this matrix with a population-pairwise F_{ST} matrix (as an indication of genetic differentiation between populations) for both chloroplast and AFLP datatsets, respectively, using Mantel's test (Manly 1991), as implemented in Arlequin 3.1 (Excoffier et al. 2005).

RESULTS

Chloroplast Sequencing

Complete (forward and reverse) DNA sequences were obtained for three individuals from each of the nine *Tritoniopsis revoluta* populations for the two chloroplast introns *3'trnV-ndhC* and *trnG-trnS*. The G + C content in the *trnG-trnS* intergenic spacer of *Tritoniopsis revoluta* was ca. 27%. The final alignment of forward and reverse sequences resulted in a matrix of 797 characters, 25 of them informative. The final alignment of forward and reverse sequences resulted in a matrix of 641 characters, 12 of them informative. The combined alignment of these two introns consisted of 1438 characters initially, and 1411 after the four indels have been removed.

Amplified Fragment Length Polymorphisms

The four AFLP primer combinations used to analyze the nine *Tritoniopsis revoluta* populations produced a genetic matrix consisting of 387 AFLP loci scored across 33 individuals; 78 for primer pair E-ACT & M-CAC, 90 for primer pair E-AAG & M-CAA, 109 for primer pair E-ACT & M-CAG and 110 for primer pair E-AGC & M-CTA. All individuals had unique AFLP profiles with an average of 118.5 fragments per profile.

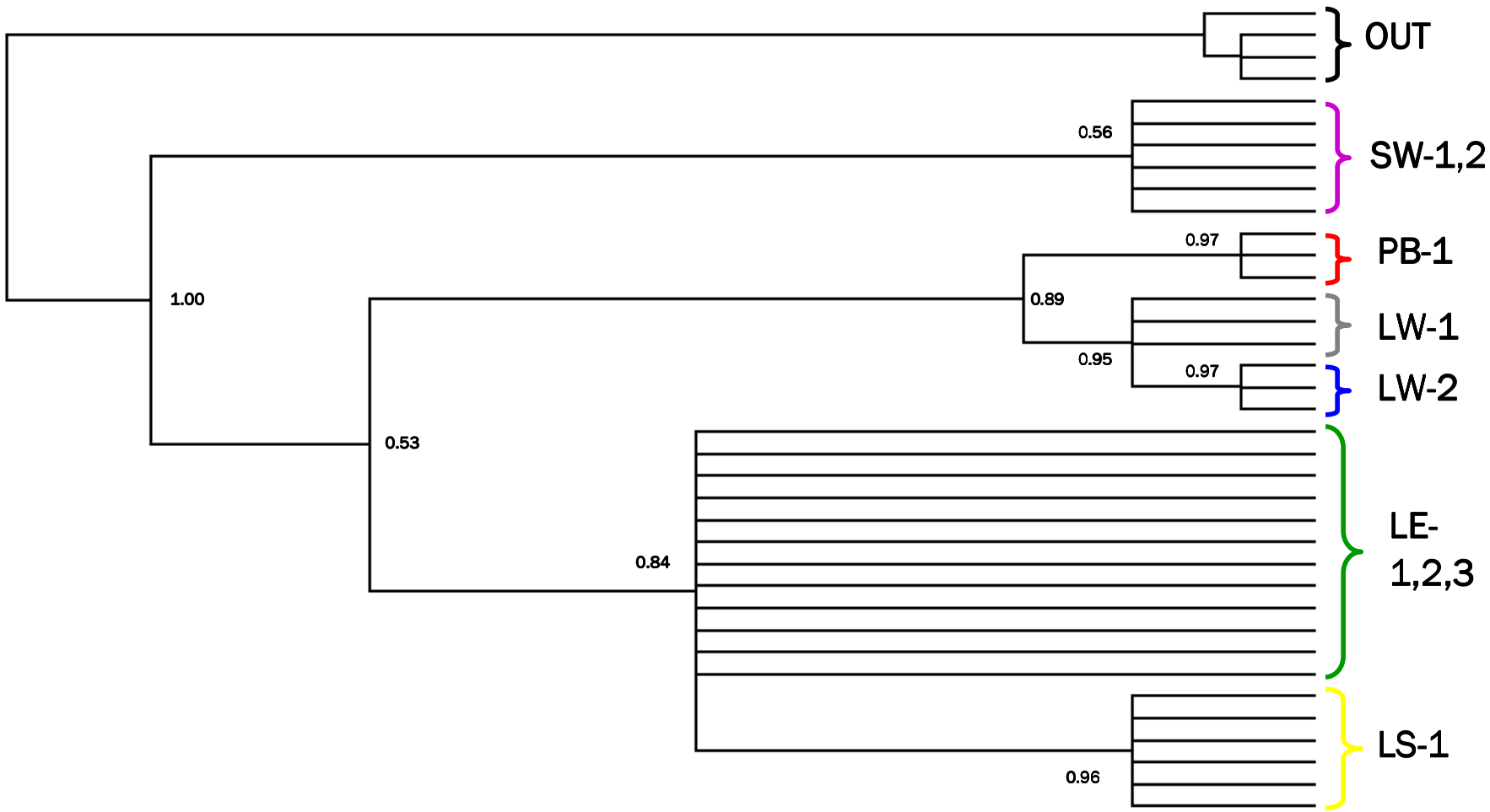
Genetic structure

1. Chloroplast Analysis

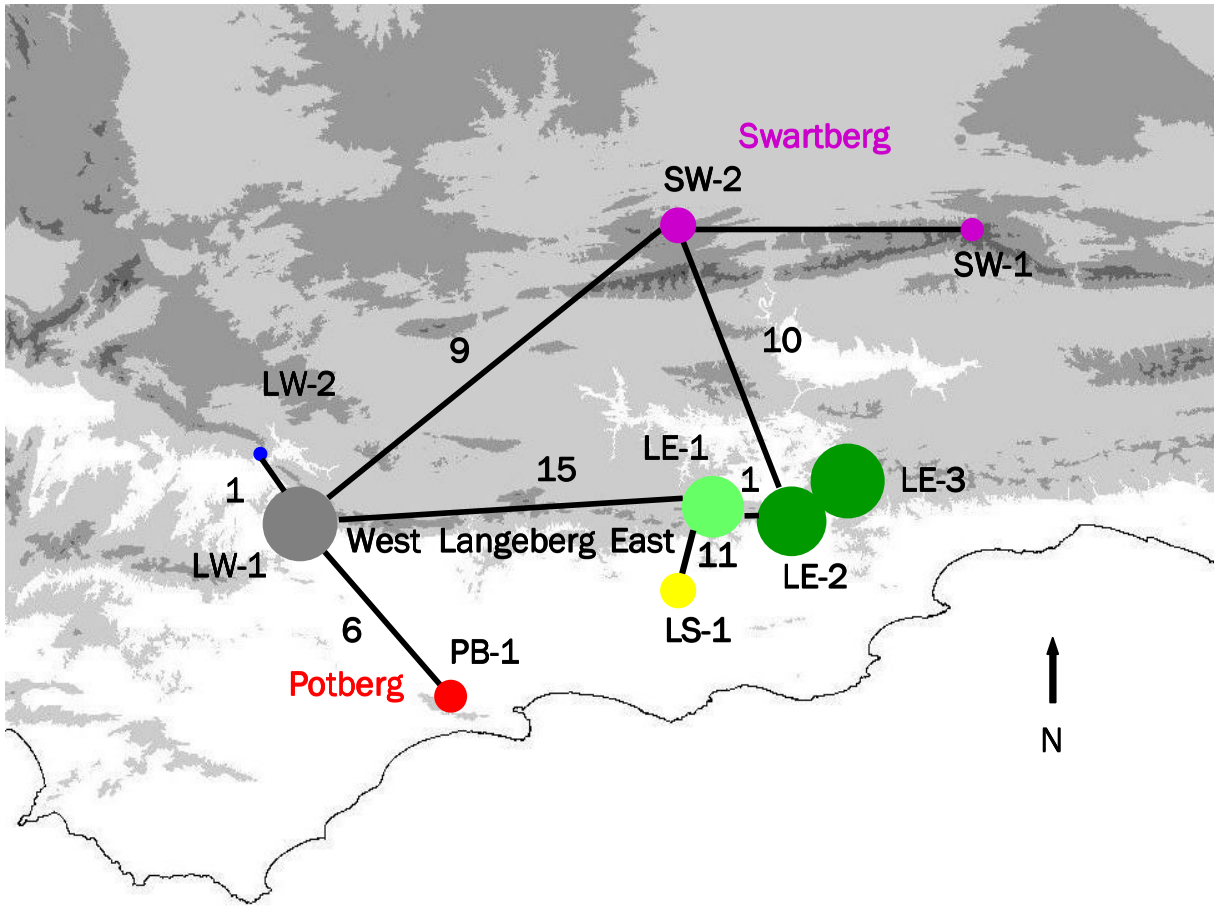
The strict-consensus tree (Figure 1) of the chloroplast dataset shows six major chloroplast lineages, and seven chloroplast haplotypes split between the nine sampled *T revoluta* populations (Figure 2). Populations never contained multiple haplotypes. Most sampled populations comprised a unique chloroplast haplotype, only the Swartberg populations (SW1, SW2) and two of the Langeberg East populations (LE2, LE3) shared haplotypes. Five divergent haplotypes were present in the Langeberg Mountains (LW-1, LW-2, LS-1, LE-1, LE-2, LE-3), whereas only one haplotype was present in both the Swartberg- (SW-1, SW-2) and Potberg- (PB-1) Mountains, respectively.

2. AFLP Analysis

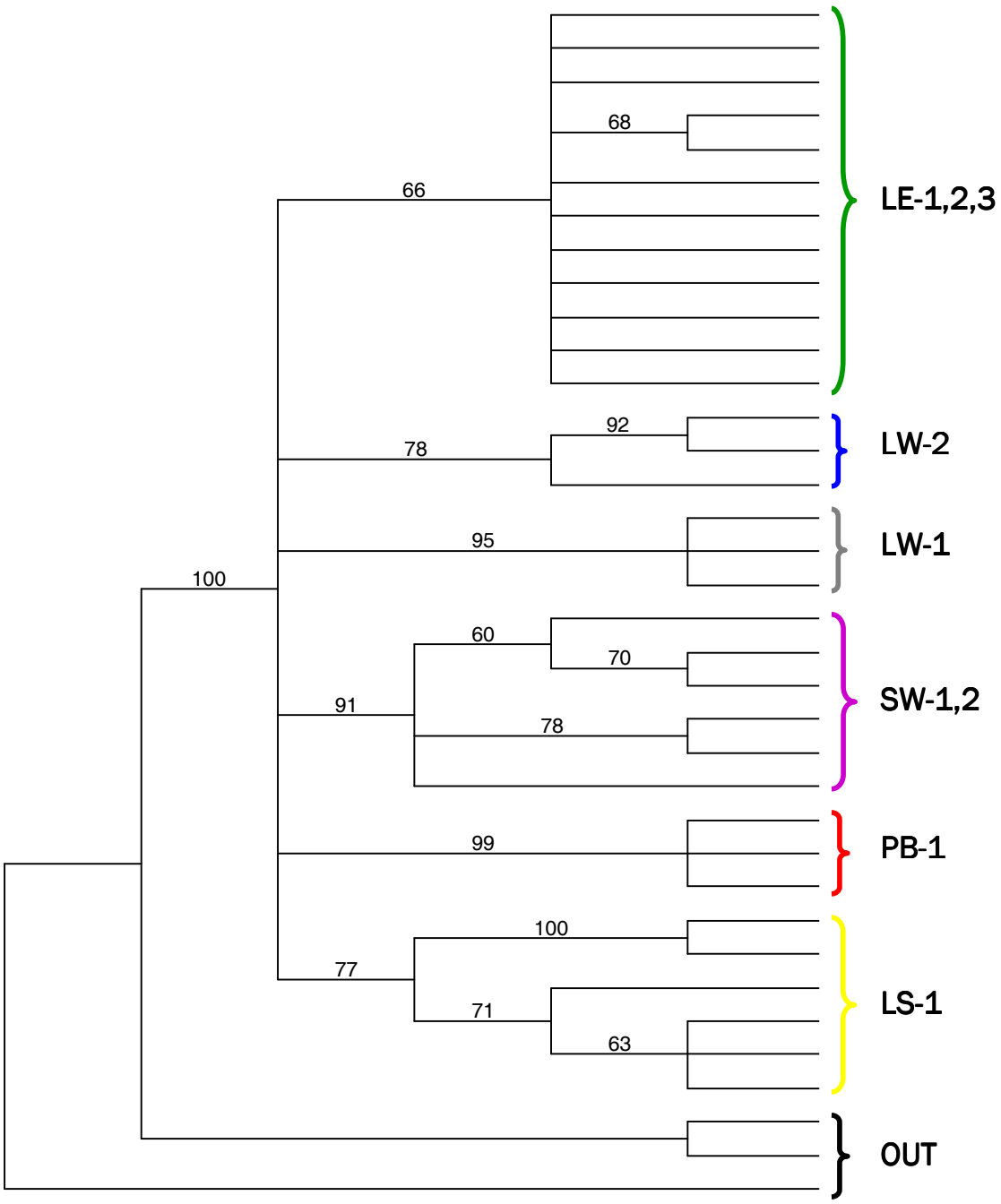
The Neighbor-Joining distance tree (Figure 3) of the AFLP dataset, shows six clusters in the nine different populations of *T revoluta*. One cluster is present in the Swartberg and Potberg Mountains (SW-1, 2 and PB-1 populations) respectively and two clusters are present in both the Eastern (LE-1, 2, 3 and LS-1) and Western Langeberg Mountains (LW-1 and LW-2). Bayesian clustering analysis yielded an unambiguous estimate of $K = 4$ clusters in the AFLP dataset. The Bayesian posterior probability of $K = 4$ given the data was φ1.0, whereas posterior probabilities for $K = 1, 2, 3, 5, 6, 7, 8, 9, 10$ and 11 ranged between 5.08E-281 and 1.03E-168. The four genetic clusters are not grouped by differences in tube-lengths but rather are structured geographically, with clusters comprising all individuals from the eastern Langeberg (LE-1, 2, 3), the southern Langeberg (LS-1), the Swartberg (SW-1, SW-2) and Potberg (PB-1) respectively (Figure 4). The Western Langeberg populations (LW-1, LW-2) on the other hand comprise admixed individuals with either eastern Langeberg and Potberg genotypes (LW1) or Langeberg (southern or eastern) and Swartberg genotypes (LW2).



► Figure 1: Bayesian consensus cladogram of chloroplast data from 9 *Tritoniopsis revoluta* localities using (four individuals of) *T. antholyza* as outgroup; three individuals from each population were used (with LS-1 as exception where 6 were used). See table 1 for abbreviations. The six major chloroplast lineages are shown in different colors and posterior probabilities are depicted on branches.



► Figure 2: Haplotype network. Each color (from figure 1) represents a different haplotype in an isolated geographical location. Populations are indicated by the solid circles and numbers are mutational steps between populations. Size of circles represents mean tube-length. See table 1 for population name abbreviations



► Figure 3: Neighbour-joining cladogram of AFLP data from 9 *Tritoniopsis revoluta* localities using (three individuals of) *T. antholyza* as outgroup. See table 1 for abbreviations. Numbers represent bootstrap values.

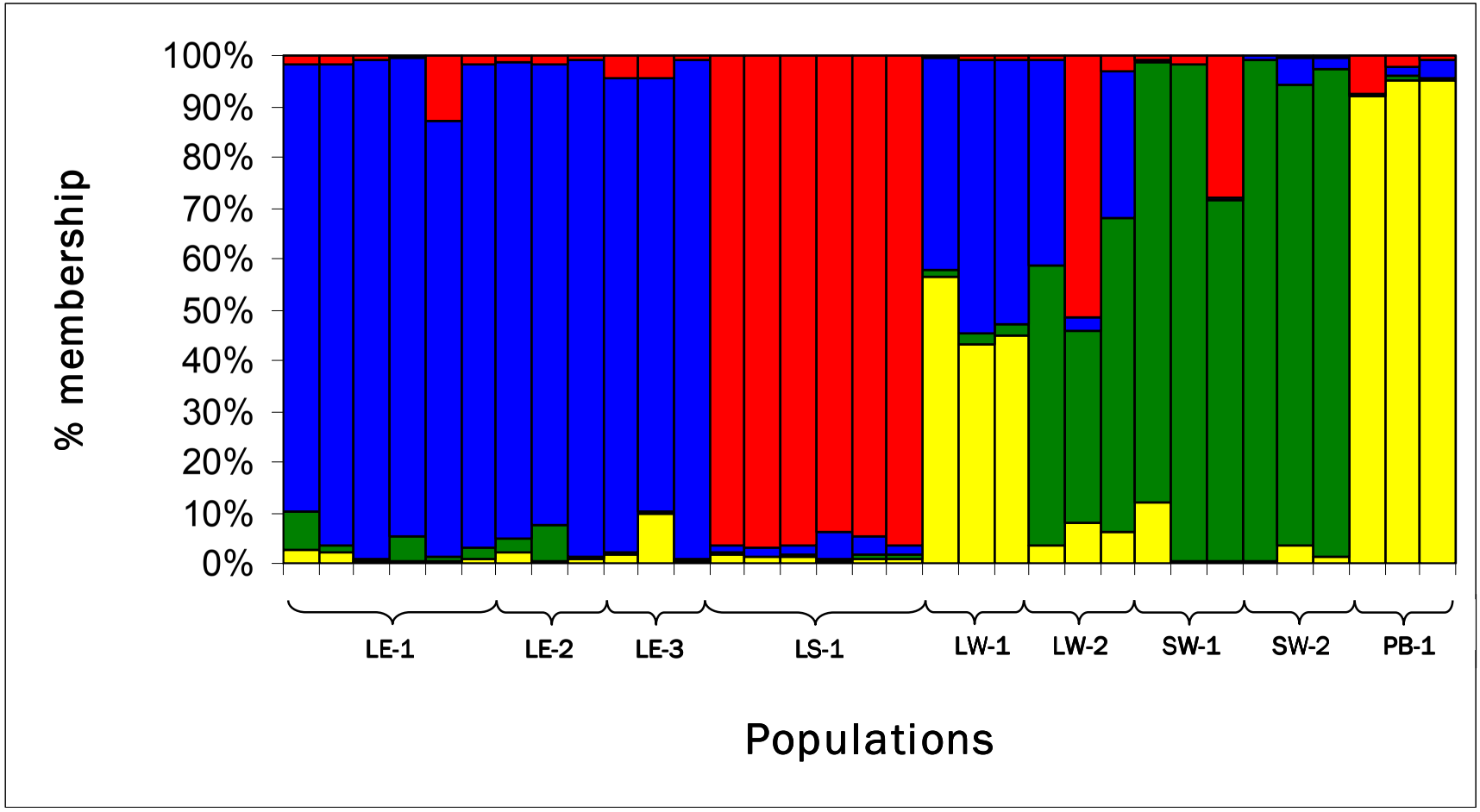


Figure 4: Plot illustrating the membership of sampled *Tritoniopsis revoluta* individuals in each of four genetic clusters (blue, yellow, red and green) identified from the AFLP dataset using Bayesian clustering algorithms. Vertical bars represent individuals and shaded segments represent their estimated membership percentage in each of the four inferred clusters. Individuals are ordered according to their population of origin.

3. AMOVA

AMOVA was performed to determine differentiation between populations according to their spatial distribution, as well as their morphological variation. The Gysmanshoek Pass I population (LE-1) was divided into short and long tube-length categories. AMOVA for the chloroplast dataset indicated 27.23% (F_{ST}=1.0, p<0.001) of the genetic variation as lying among the groups for the spatial analysis, compared to the 2.78% (F_{ST}=1.0, p<0.001) for the tube-length analysis. AMOVA for the AFLP dataset indicated 12.68% (F_{ST}=0.32, p<0.001) of the genetic variation is distributed among the groups for the spatial analysis, compared to the 2.16% (F_{ST}=0.27, p<0.001) for the tube-length analysis (Table 2). Most of the populations in the chloroplast dataset are completely differentiated from one another (F_{ST} =1.0, p<0.001), except for LE-2 & LE-3, and SW-1 & SW-2, where F_{ST} =0 (p<0.001). In the AFLP dataset, 72.94% of the variation (F_{ST}=0.27, p<0.001) is within populations. Genetic variation is clearly more strongly structured spatially than on the basis of tube-lengths for both the chloroplast and AFLP datasets. The isolation-by-distance pattern was confirmed by matrix correlation of genetic and geographic distances between individuals (r=0.322, p=0.001 for chloroplast and r=0.367, p=0.004 for the AFLP dataset).

Patterns of tube-length evolution and pollinator switches

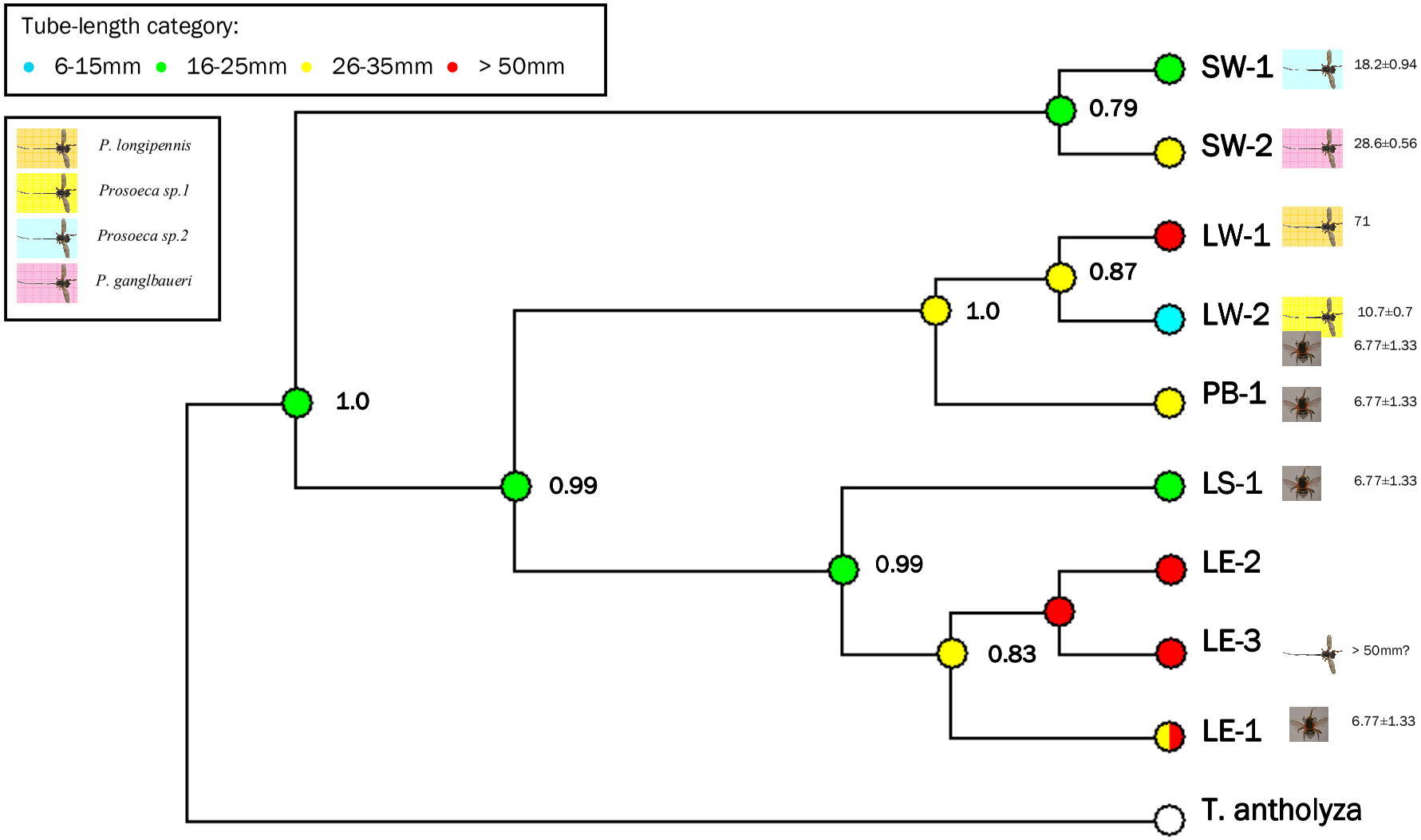
The partition homogeneity test indicated that the results of the analysis of the chloroplast and AFLP partitions did not differ significantly (p=0.976). It was thus possible to combine these datasets for further analyses. Neighbour-joining analysis of a Nei and Li distance matrix from the AFLP data yielded six clusters which correspond exactly to the major lineages in the chloroplast tree. The cladogram (Figure 5) of the combined chloroplast/AFLP dataset, showing posterior probabilities, shows the same six major clusters in the nine different populations of *T revoluta* as in both the separate chloroplast and AFLP analyses. The only major difference between the separate chloroplast and AFLP analyses versus the combined analysis is that in the former, the two populations in the Swartberg Mountains (SW-1 and SW-2) did not show differentiation. In the latter, these two populations show some distinction from one another.

Reconstruction of the ancestral character states for tube-lengths provide evidence that transitions between shorter and longer tube-lengths happened on more than one occasion, and not just as a single event (Figure 5). The character reconstructions suggest two evolutionary transition to shorter tube-length categories and four transitions to longer categories. Importantly, extremely long tubes evolved independently twice in this species. Main

nodes and the character state of each node are shown in Figure 5. Short-to-long tube transitions took place in the LW-1, and the three Langeberg East populations (LE-1, LE-2 and LE-3) populations (Figure 5). Transitions within the short category also took place, in the SW-2 and LW-2 populations. No tube-length transitions took place in the SW-2, LS-1 and PB-1 populations.

In a number of the populations (SW-2, LW-1, LW-2, LE-1, LE-2 and LE-3) tube-length transitions coincide with pollinator shifts. In all of these populations, with the exception of LW-2, tubes lengthened to fit the tongue-length of the respective pollinators. In LW-2, tubes shortened considerably.

Mean tube-length differences between populations were not correlated with genetic distance. This was evidenced by Mantel tests showing no apparent relationship between population-pairwise F_{ST}s and Q_{ST}s for both the chloroplast ($r=0.16$, $p= 0.17$) and the AFLP dataset ($r= 0.05$, $p=0.34$).



► Figure 5:. Combined chloroplast-AFLP cladogram. Colored circles represent ancestral states and posterior probabilities are depicted on branches. Pollinator and their proboscis-lengths are mapped on the right, next to population names. *Amegilla* bee tongue-lengths from de Merxem D.G. et al. (2009) and the possible long-proboscid fly at LE are based on the Manning and Goldblatt (2005) observation of *P. longipennis* near Riversdale, which is close to LE-2

DISCUSSION

Tritoniopsis revoluta exhibits highly variable tube-lengths across its range and I showed that a clear relationship exists between the tube-lengths of *T. revoluta* and the tongue-lengths of its corresponding pollinators in the different populations in Chapter 1. In this chapter, I showed that genetic diversity *in T. revoluta* is geographically structured. Analyses in this chapter suggest species originated in the Swartberg, dispersed (once) to the Western Langeberg, whence it subsequently dispersed to the Potberg and Eastern Langeberg Mountains. Multiple chloroplast lineages exist in the Langeberg Mountains, and these lineages are supported by the nuclear data as well. Also, tube-length is evolutionarily labile; exceptionally long tubes have evolved twice in this system.

Genetic structure and phylogeography

The six chloroplast lineages in the three geographically isolated mountain ranges are clearly differentiated from each other (posterior probability > 0.8). The populations in these chloroplast lineages have been reproductively isolated long enough that genetic differences can be resolved by chloroplast markers (see Groppo et al. 2008), and also nuclear analysis using AFLPs show distinct clusters in the dataset corresponding to the three main geographically isolated mountain ranges. The chloroplast and AFLP datasets exhibit similar patterns of genetic differentiation, suggesting comparable evolutionary histories for both the chloroplast and nuclear genomes. Utilizing a population perspective, AMOVA analyses of both datasets show a geographic component in the genetic structure of *T. revoluta* populations. The existence of six chloroplast lineages suggest that colonization of the different areas have occurred by the different lineages, and there appears to be limited gene flow between populations as well as a history of population isolation, particularly between the three mountain ranges, but also between regions within the Langeberg Mountains. Seed dispersal seems limited as evidenced by the confinement of haplotypes to individual populations or regions. As cpDNA is transmitted by seeds (Falchi et al. 2009), these results suggest that low or no seed flow was highly dependent on geographical constraints (also see Anderson et al. 2004).

Most of the *T. revoluta* lineages are significantly geographically isolated, but not all. One such exception is the LE and LS populations; divergence exists at a very local scale here as these haplotypes are included in a single clade by both the plastid and total data. This genetic

differentiation between nearby populations can potentially reflect founder effects (De Meester et al. 2002). Alternatively, these populations from different chloroplast lineages could historically have been isolated by distance, and the Langeberg Mountains is a secondary contact zone. The divergence in chloroplast DNA suggests they have been separated for a long time. It could also be a result of pollinator shifts leading to gene-flow barriers in either sympatry or parapatry; divergence in pollinators might be contributing to the prevention of hybridization between these populations. However, I found no evidence for this; bees were found to be the main pollinators in LS-1 and also important pollinators in both short and long tubed populations in the eastern Langeberg. Although long proboscid flies may be found in the eastern Langeberg as suggested by the *P. longipennis* specimens found very close by (near Riversdale which is at the start of Garcias Pass where LE-2 is located – Manning and Goldblatt 2005), pollinators seem unlikely to be effective disruptive agents in sympatry or parapatry because bees do visit both morphs.

The patterns of genetic admixture (AFLP) revealed by the Bayesian clustering analysis suggest pollen-mediated gene flow between chloroplast lineages in the Langeberg Mountains. Mixed ancestry between the two LW populations and the three LE populations (blue cluster) may suggest pollen mixing is taking, or has historically taken place in the Langeberg Mountains. However; mixed ancestry between population LW-1 in the Western Langeberg Mountains and the PB-1 population near Potberg Mountain (yellow cluster) may suggest the LW-1 population consists of intermediate genotypes, perhaps through pollen flow from the LE populations into (possible undiscovered) populations from the Potberg chloroplast lineage. An alternative interpretation is that they are just difficult to place with certainty, perhaps because they sit at a nexus in the population tree. In the same light, population LW-2 may be the product of hybridization between pollen flow from the LE populations and populations from the Swartberg chloroplast lineage. This corresponds in some degree to the study on the genus *Argyroderma* by Ellis et al. (2006) where genetic differentiation was geographically structured and populations had high (>80%) membership to a specific genetic cluster.

It is most probable that seed dispersal occurs through wind and pollen dispersal through long-proboscid fly or bee pollinators in this system (de Merxem et al. 2009 and Chapter 1). Divergence in chloroplasts suggests that populations of *Tritoniopsis revoluta* have been separated for a long time. However, other process may also be a reason for low diversity within populations, such as small population sizes, or they may experience frequent bottlenecks. The populations in the six distinct statistically supported chloroplast lineages are all genetically differentiated and each contain a single haplotype, suggesting no gene-flow between these

populations through seed dispersal. However, while the same six potential clusters are also present in the nuclear analysis, shared ancestry between populations may suggest gene flow via pollen dispersal between populations in at least the Langeberg Mountains. The genetic structure among the populations of *Tritoniopsis revoluta* suggests an interaction of limited seed dispersal and spatial isolation of the populations (Dane et al. 2007).

Tube-length evolution

To elucidate patterns of tube-length evolution in *Tritoniopsis revoluta*, ancestral character state reconstructions showed that for all populations present in this distribution range, the ancestral tube-length state was short. All the long-tubed *T. revoluta* populations are confined to the Langeberg Mountains (LE-1,2,3 and LW-1), which is the only region where flies with extremely long tongues were found. From the character state reconstructions, it seems that the longest-tubed phenotype has evolved independently on at least two occasions – in the LE populations and the LW-1 population. Reconstructions also indicate that transitions to shorter tube-lengths have taken place. These tube-length transitions coincide with transitions in pollinator species in a number of the populations and may suggest tube-length is very labile and may respond to variable selection from pollinators. Although the pattern observed in the long-tubed LW-1 and three LE populations suggest response to selection by very long-proboscid flies, these have only been observed in the LW-1 population, but are absent in all the LE populations. There are a number of possible explanations for the occurrence of long-tubed flowers in the LE populations despite the absence of long-proboscid pollinators. These long-tubed populations could be at the edge of the long-proboscid fly pollinator distribution range and flies may be exceptionally uncommon there or even absent. Absence can be explained by dispersal out of the fly's range or local extinction (e.g. Steiner 1993). I favor absence or rarity as the explanation in the LE-1 population since this study and the study of de Merxem et al. (2009) spent many hours of observation in this population, over several years, without ever observing a long-proboscid fly. The evolution of short-tubed flowers in this population also suggests pollination by long-proboscid flies is not important here. However, Manning and Goldblatt (2005) captured *P. longipennis* very close to the LE-2 population and so it is likely that the fly does occur in some of the eastern populations. The reason for not catching the flies in these populations is probably related to the low number of observation hours spent in LE-2 and LE-3 (see table 2, Chapter 1). The most favorable explanation, however, is that the LE populations were a founder event from LW seed. Presently, in the possible absence of long-proboscid fly pollinators, the long-tubed individuals are maintained in the landscape and have not shortened in response to the bee pollinators because the bees are visiting the long-tubed flowers less as

no nectar reward can be obtained from them. However, observations suggest that bees still visit them and thus they probably do exert a degree of selective pressure on the flowers, especially if they are the only pollinators (see Chapter 2 and de Merxem et al. 2009). They can, however, access the nectar in the short-tubed flowers. In other populations where bees are important (e.g. LS-1) flowers have much shortened corollas and nectar wells up in these tubes making it accessible to bees. These short tubes may be interpreted as an adaptation to the abundant bee pollinators at this site (de Merxem et al. 2009).

The intermediate genotypes of the LW-1 population suggests that the genetic code for long tube length may have entered this population via a pollen dispersal event from the long-tubed LE lineage (also see Gomez et al. 2009). Subsequently this adaptation may have spread through the entire population as a result of long proboscid pollinator presence. However, there seems to be little evidence to indicate that floral morphology genes moved between populations via pollen dispersal. Nuclear analysis suggests there may have been pollen dispersal from the Swartberg lineage to the LW-2 population, and the evolution and spread of very short tubes here may have been assisted by this gene flow. Flowers in this population match the length of the pollinator proboscides present in this area. *T. ramosa* (the LW-2 population) is included in this study as it actually is a highly derived *T. revoluta* population which has probably converged on the *T. ramosa* morphology because of the prevalence of *Amegilla* bees in this population (de Merxem et al. 2009) and short-proboscid *Prosoeca*, both of these have been recorded as pollinators on *T. ramosa* (Manning and Goldblatt 2005). Whichever the case, it can be concluded that tube length evolution is fairly flexible and shows considerable convergence and divergence.

Populations in the Swartberg (SW-1, 2) and Potberg (PB-1) Mountains are all short-tubed and the ancestral state is also short tubed. However, as in the Langeberg Mountains, these genetically distinguishable short-tubed populations differ significantly in their mean tube lengths (Chapter 1), suggesting that more subtle tube-length transitions have taken place between populations with different long-proboscid fly pollinators. Short tube-lengths of the SW-2 population seems to be derived from shorter tubes and probably lengthened to match the tongue-length of its fly pollinator; although phylogenetic uncertainty should be taken into account as support for some of the nodes in the species tree are weak. In the Swartberg Mountains, this presents possible evidence for pollinator-driven selection, as the SW-1 and SW-2 populations originate from the same chloroplast lineage and still exhibit considerable variation in tube-lengths. No tube-length transitions took place in the PB-1 population. Long-proboscid fly pollinators were also absent from this population and it may be that this

population is similar to the LS-1 one – nectar wells up in the tubes and is accessible to bees; subsequently they play a role in pollination of *T. revoluta* in this population.

It is thus very clear from this evidence that multiple tube-length transitions occurred in this system; both short to long and vice versa. As flower tube-lengths are closely matched with pollinator tongue-lengths in the majority of the populations, we conclude that tube-length shifts correspond closely to pollinator shifts. The possibility that tube-length variation may be influenced by phenotypic plasticity in reaction to environmental factors or selection on correlated traits cannot be excluded as it was not specifically tested for in this study. However, it is highly unlikely that tube-length variation seen in this system can be attributed to other factors than pollinator-mediated selection (*sensu* Johnson and Steiner 1997), as both short and long-tubed individuals occur in similar environmental regimes in the Langeberg Mountains, and tube-length varies independently from other floral characters (like inflorescence height and top petal length) (pers. obs). This study is similar to a study on *Aquilegia* (Whittall and Hodges 2007) which examines the evolutionary patterns of spur-lengths in this genus, where spur-length evolution occurs coincidently with changes in pollinator syndromes. Tube-length evolution in *Aquilegia* is directional and has occurred without reversals, resulting in the progressive lengthening of nectar spurs, whereas tube-length evolution in *T. revoluta* is reversible, and transitions happened in both directions. This reversibility in *T. revoluta* may be possible because tube-length differences in this system are not associated with colour changes which usually involve loss-of function mutations and are very difficult to regain (Rausher 2008). Similarly, in Loranthaceae certain floral traits (corolla color, flower symmetry, petal fusion) evolved due to interaction with its pollinators (primarily insects and birds) (Vidal-Russell and Nickrent 2008).

If tube-lengths in the different populations evolved neutrally and no selection pressures were at play, then one would expect to find a correlation between genetic distance and mean tube-lengths; i.e. short tube-lengths would be more related to short tube-lengths, and long to long. This is, however, not the case in *T. revoluta*. Mantel tests showed no correlation between genetic distance and mean tube-length differences for all populations in both the chloroplast and AFLP datasets. This suggests tube-lengths aren't short or long because of common descent or genetic similarities to other short or long populations. Analysis of both the chloroplast and AFLP datasets suggests a predominant influence of spatial structure on the partitioning of genetic differentiation between all populations present in this dataset. Genetic divergence occurs between all allopatric populations present in the three main geographically separate mountain ranges. This correlates closely to a study on the evolutionary radiation of

the genus *Argyroderma* by Ellis et al. (2006), where landscape structure also has a big influence on genetic divergence between and within specific drainage basins where this genus occurs. Also, a study on Joshua trees (*Yucca brevifolia)* and two sister species of pollinating yucca moths (*Tegeticula* spp.) provides molecular evidence that morphologically distinct populations are maintained as evolutionarily distinct groups by their specific pollinator species (Smith et al. 2008).

This study provides evidence that pollinators are important in generating divergent floral trait patterns in allopatry through pollinator shifts. Tube-length evolution in *Tritoniopsis revoluta* is thus not neutral, but tube-lengths possibly evolved due to pollinator-mediated selection. Also, the genetic variation in this system shows populations to be isolated by distance, and not related by tube-length. This is suggestive of selection playing an important role in the evolution of these tube-lengths, and not random processes.

ACKNOWLEDGEMENTS

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APPENDIX A

Table 1: Locality data for *Tritoniopsis revoluta*

Abbr.	Population sites	Latitude	Longitude	# individuals collected	Tube-length (mm) (mean±SE)
LE-1	Gysmanshoek Pass I	S 33° 55,943'	E 021° 04,336'	96	39.2±0.69
LE-2	Garcia's Pass	S 33° 57.278'	E 021° 13.544'	25	63.2±1.13
LE-3	Langkloof	S 33° 56.939'	E 021° 15.460'	30	68.0±1.09
LS-1	Gysmanshoek Pass II	S 33° 56.130'	E 021° 04.151'	12	22.2±0.64
LW-1	Tradouws Pass I	S 33° 56.813'	E 020° 41.987'	32	68.8±0.97
LW-2	Tradouws Pass II	S 33° 59.229'	E 020° 42.726'	30	11.5±0.18
SW-1	Swartberg Pass	S 33° 21.747'	E 022° 04.615'	47	20.4±0.32
SW-2	Buffelsrivierpoort	S 33° 26.851'	E 021° 00.236'	27	32.3±0.44
PB-1	Potberg	S 34° 24,075'	E 020° 33,439'	24	29.4±0.44

where L = Langeberg Mountains, E = East, W = West, S = South, SW = Swartberg Mountains and PB = Potberg Mountains

Table 2: Hierarchical AMOVAs indicating the distribution of genetic variation among predefined groups.

	Group partitioning	Source of variation	d.f.	Sum of squares	Variance components	% of variation	F_{ST}	p
CHL	Morphological	Among groups	3	69.091	0.17748	2.78	1	< 0.001
	Geographial	Among groups	2	64.157	2.102	27.23	1	< 0.001
AFLP	Morphological	Among groups	3	328.164	1.33244	2.16	0.27	< 0.001
	Geographial	Among groups	2	288.72	8.358	12.68	0.32	< 0.001

GENERAL CONCLUSION

Tritoniopsis revoluta exhibits remarkable variation in its tube-length across its range. Four non-overlapping tube-length ecotypes were discovered (10-20, 20-30, 30-40 and 60-75mm). I caught fly pollinators with tongue-lengths which corresponded almost exactly with the tube-length of the flowers they were pollinating. Only bee pollinators were observed and captured at several sites. However since their tongues are much shorter than the tubes of the flowers in all but one population, they are unlikely to have played a role in the evolution of the very long corolla tubes found in *T. revoluta*. Nevertheless, they may still play an important role as pollinators, when flies are rare or absent (de Merxem et al. 2009) and may select for shorter tubes in these situations. In the five *T. revoluta* populations where fly pollinators were caught, there is a positive relationship between tube-lengths of the flowers and the tongue-lengths of the fly pollinators. It is possible that the tube-length differences between the five populations arose through co-evolution of plant and pollinator (see Anderson and Johnson 2008), since in many populations, flies were highly dependent on *T. revoluta* as it was the only nectar source they were seen utilizing and *Prosoeca* were the only long proboscid pollinators observed in these populations. Reciprocal selection studies will have to be conducted to confirm whether this is the case. For *T. revoluta*, multiple tube-lengths probably correspond to visitation by different species of long-proboscid flies, with different proboscis lengths. The results from part one of this study present evidence for pollinator-driven floral variation within a single plant species.

Tritoniopsis revoluta exists as two overlapping morphological entities at the Gysmanshoek Pass site. These entities differ in tube-length, color, nectar volume and sugar content. These two *Tritoniopsis revoluta* entities exist at either side of the Gysmanshoek Pass; a short tubes to the South of the pass, and long ones to the North. A very narrow contact zone exists where these two tube lengths meet and a few intermediate flowers are present here (also see Ramsey et al. 2003). No long-proboscid fly pollinators were observed in this bimodal population. *Amegilla* bees were observed visiting the flowers at this site, as well as to the south of the hybrid zone.

The short and long *Tritoniopsis revoluta* morphotypes at the Gysmanshoek Pass site may be maintained by differences in spatial distribution rather than ethological barriers. Morphotypes are not differentially pollinated by long-proboscid flies or bees, but are more generalized to include pollination of both morphotypes by *Amegilla* bees. A number of plausible explanations exist for the co-occurrence of short and long ecotypes at this site, but genetic studies need be

conducted to clarify this phenomenon and continued observations have to be made to confirm the absence of the long-proboscid fly pollinator at the Gysmanshoek Pass.

Population genetic techniques were utilized to determine whether patterns of tube-length evolution are also suggestive of pollinator-driven variation. The chloroplast and AFLP datasets exhibit similar patterns of genetic differentiation, suggesting similar evolutionary histories. Analyses of both datasets show a definite presence of a geographic component in the genetic structure of *T. revoluta* populations. The genetic structure among the populations of *Tritoniopsis revoluta* suggests an interaction of limited seed dispersal and genetic isolation of the populations. From the character state reconstructions, it seems that two evolutionary transition to shorter tube-length categories and four transitions to longer categories occurred. The fact that tube-length transitions occurred on multiple occasions in different evolutionary lineages suggest convergent evolutionary responses to selection The possibility that tube-length variation may be influenced by phenotypic plasticity in reaction to environmental factors or selection on correlated traits cannot be excluded as it was not specifically tested for in this study. However, it is highly unlikely that tube-length variation seen in this system can be attributed to other factors than pollinator-mediated selection (Johnson and Steiner 1997), as both short and long-tubed individuals occur in similar environmental regimes in the Langeberg Mountains, and tube-length varies independently from other floral characters (like inflorescence height and top petal length) (Pers. Obs). If no selection pressures were at play in the evolution of tube-length in this system, one would expect to find a correlation between genetic distance and mean tube-lengths; this was, however, not the case in *T. revoluta*. No correlation was found between genetic distance and mean tube-length differences for all populations in both the chloroplast and AFLP datasets. This study provides evidence that tube-length evolution in *Tritoniopsis revoluta* is not neutral, but that tube-lengths possibly evolved due to pollinator-mediated selection. This is suggestive of selection playing an important role in the evolution of these tube-lengths, and not other processes being in charge of tube-length evolution.

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