Exploring floral evolution in *Pelargonium* (Geraniaceae)

linking shapes and macro-evolution

Sara J. van de Kerke

Thesis committee

Promotor
Prof. Dr M.E. Schranz
Professor of Biosystematics
Wageningen University & Research

Co-promotor
Dr F.T. Bakker
Assistant professor, Biosystematics Group
Wageningen University & Research

Other members
Prof. Dr B.J. Zwaan, Wageningen University & Research
Prof. Dr S. Nadot, Université Paris-Sud, France
Prof. Dr R. Etienne, University of Groningen, Groningen
Dr F. Forest, Royal Botanic Gardens, Kew, UK

This research was conducted under the auspices of the Graduate School of Experimental Plant Sciences.

Exploring floral evolution in *Pelargonium* (Geraniaceae)

linking shapes and macro-evolution

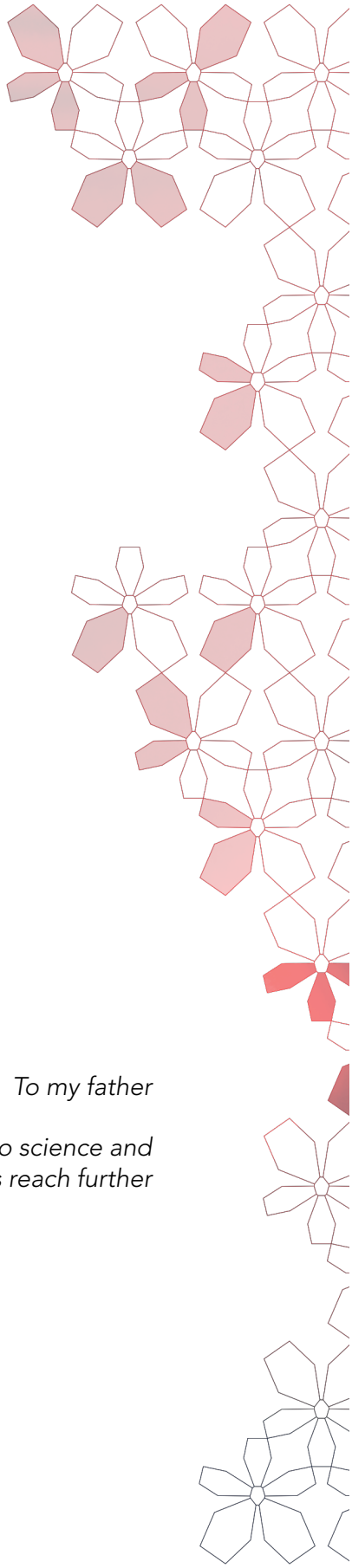
Sara Jacoba van de Kerke

Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Wednesday 20 November 2019
at 11 a.m. in the Aula.

Sara J. van de Kerke
Evolution of floral shape in *Pelargonium* (Geraniaceae); linking shapes and macro evolution
178 pages.

PhD thesis, Wageningen University, Wageningen, the Netherlands (2019)
With references, with summary in English

ISBN 978-94-6395-059-6
DOI 10.18174/497382



To my father

who nudged me into science and
encouraged me to always reach further

CONTENTS

Chapter 1

General Introduction

1

ONCE UPON A TIME...

It is in the nature of humans to wonder and try to make sense off everything they see and all things they encounter (Bod, 2019). Already since the start of humankind, humans have been observing, describing, and recording patterns in the natural world around them. 500.000 years ago, *Homo erectus* scratched a geometric pattern on a shell (Bod, 2019), of which the meaning is unclear. 40.000 years ago, humans recorded the lunar cycle by carving stripes of different lengths on a mammoth tusk. The grouping of stones in a circle, attributed to Neolithic communities tracking the summer and winter equinox, is another fascinating example. For instance Stonehenge, and many more of these stone groupings, scattered around the British landscape.

EVER SINCE DARWIN

i.a. Aristotle, Goethe, de Candolle, Cuvier, L'Héritier have all contributed to our understanding of species, morphology, and nature in a large way (Wheeler, 2012), and Linnaeus already started to systematically order all the natural diversity known (Linnaeus, 1758). But the person in whose work pattern recognition and interpretation all came together was Darwin who saw the underlying patterns and formulated his theory of the process of evolution and natural selection (Darwin, 1859). His theory is well illustrated by the well-known example of the Galápagos finches, whereby every island is occupied by a different species of passerine bird with a differently shaped beak that is adapted to the food available on the island (Darwin, 1836; Lack, 1947, 1940; Petren et al., 1999; Ponting et al., 2013). Darwin's ideas caused a paradigm shift in the way we look at the world (Berra, 2008). Although 'standing on the shoulders of giants', ever since Darwin's *On the Origin of Species* (Darwin, 1859) researchers have been seized by the tremendous variety in shape among living organisms (i.a Briggs and Walters, 2016; Endler, 1977; Tremblay et al., 2005; Winemiller, 1990).

SHAPE

But what is shape? Mathematically, shape is defined as what is left when we subtract the size component from form (Goodall, 1991; Klingenberg, 2016; Zelditch, 2012). In other words, shape is the true variation in morphological parts, irrespective of the size of the individuals studied (Klingenberg, 2016). There is tremendous variation in shape within and among all living organisms that have ever existed. Birds and flowers are excellent examples of clades that are able to utilize and showcase their morphological potential.

The variation in shape within a clade is captured in what is called the morphospace. The morphospace encompasses all known shapes of species within a clade (or, for that matter, in any group of individuals) and represents the realized morphological diversity that has evolved (Lowery and Fraass, 2019). When reconstructing the morphospace of a clade, a coherent picture of shape variation is formed. This allows the exploration of possible avenues of change and how different components of shape may be correlated (Whibley et al., 2006). This can also help give insight into how various shapes have evolved, as in the case of Darwin's finches where beak shape is a direct adaptation for food availability. Or the way bivalves are shaped

in order to cope with different beach types (McLahlan et al., 1995). However, as has been pointed out in the past, one should be cautious when attributing such direct cause – effect relations to potential drivers of shape (Gould and Lewontin, 1979; Prum, 2012). Not everything has to be adaptive, as has never been Darwin's intention to convey (Gould and Lewontin, 1979; Prum, 2012).

Sometimes, a part of the potential morphospace has not been realized by the clade due to several reasons. First, the non-sampled morphospace is biologically not possible (a 'forbidden area'). An example is given in foraminiferal shells whereby in a forbidden area the shells chambers did not touch (Tyszka, 2006). Second, it might be the case that species have gone extinct that once occupied the now empty part of the morphospace. For instance the hunt for the missing shape in human evolution has long been thought to solve the evolutionary gap between great apes and Hominoids (Johanson et al., 1990). A major influence on these 'gaps' are mass extinction events whereby abruptly an enormous amount of shape variation is wiped out and only a limited range is left over (Gould, 1990; Lowery and Fraass, 2019; Raup, 1994, 1986). Following from these extinctions, it might be the case that the clade has not yet 'recolonised' the empty part of the morphospace. The shape variation of the species in this clade is possible, but there has not yet been adequate pressure on the clade to mine this potential shape variation, or the genetic variation in order to 'get there' has been lost. Under influence of outside pressure, shape might change and this could lead to the formation of new species.

SPECIATION IN PLANTS

For flowering plants, there are multiple potential influences and drivers of speciation (Figure 1). For example, phylogenetic 'canalisation' means that certain clades will never be able to evolve certain morphologies because of genetic constraints, i.e. a species is part of a genomic make-up and cannot escape even over long evolutionary time periods. On the other hand, shifts in pollinator pressure can lead to rapid speciation. Pollinator-induced changes are often highly dynamic and often associated with climatic changes.

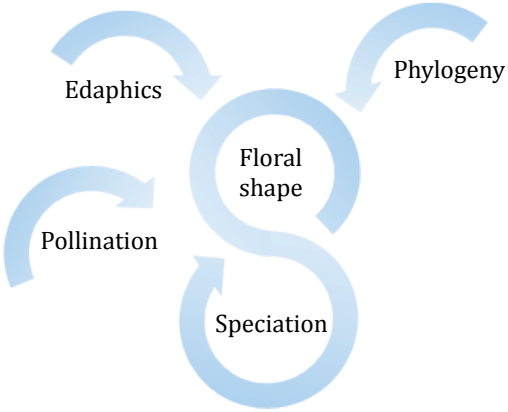


Figure 1. A conceptual visualisation of how floral evolution is driven, and how it ties in with speciation.

Phylogeny

Flowering species have to 'deal' with relicts from the past and are thus constrained by their phylogenetic placement. Evolutionary changes that at one time were preferable to increase fitness, might in the present circumstances turn out to no longer be beneficial. A curious example of this is the section *Otidia* in the genus *Pelargonium*. Species in this section all have nectar spurs that are typical for *Pelargonium* and must have developed in deeper evolutionary time. On the other hand, characteristic for this section are the 'ears' on the petals which are thought

to close off the nectar spur. Why it is beneficial for these species to deny their pollinators access to the spur is not known, but for some reason a more recent evolutionary event has triggered the development of these structures which seems to give the species in this clade some advantage.

Pollinator speciation

The relation between a flower and its pollinator is a special one. Darwin already proposed that the floral characteristics of a plant are shaped by their interaction with pollinators (Darwin, 1877a, 1877b). Since then, pollination biologists have categorised sets of floral traits shaped by pollinator-driven selection into pollinator syndromes (Faegri and van der Pijl, 2013). The idea being that a certain pollinator group has a specialised preference for a certain set of floral traits collectively referred to as a pollinator syndrome. Therefore, plants sharing a pollinator syndrome will be pollinated by the same set of pollinators and are referred to as 'guilds' (Faegri and van der Pijl, 2013; Fenster et al., 2004; Schiestl and Johnson, 2013). An example of this is the bombyliid fly *Megapalpus capensis* (characterised by a fairly short proboscid) which is known to pollinate *i.a. Gorteria* (Asteraceae), *Ursinia* (Asteraceae), *Dimorphotheca* (Asteraceae), and a number of *Pelargonium* species. All these species are characterised by dark 'eye spots' on their petals that resemble female *Megapalpus* flies (Jager and Ellis, 2017; Johnson, 2010; Johnson and Midgley, 1997; Röschenbleck et al., 2014; Struck, 1997), making this guild one of the few examples of sexual deception outside of the Orchids (Ellis and Johnson, 2010). Other examples of specialised preferences of pollinators groups are those attracted by specific scents (Johnson and Hobbhahn, 2010; Shuttleworth and Johnson, 2010; van der Niet et al., 2010), flower colours (Newman et al., 2014), and nectar guide patterns (Medel et al., 2003; Schiestl and Johnson, 2013).

Spur pollination

In contrast with generalist pollination syndromes, spur pollination is specialised in that it spatially separates pollination reward (the nectar) from the corolla. This is considered to result in a Darwinian 'arms-race' between plants and pollinators, increasing effective pollen transfer, and, ultimately, fitness for both (Whittall and Hodges, 2007). It has been shown for *Aquilegia* that there is an evolutionary trend towards longer nectar spurs (Whittall and Hodges, 2007). This trend could be explained by pollinator fidelity (Kay and Sargent, 2009). Plants with longer nectar spurs will not be pollinated by insects with shorter proboscids because they are not able to reach the nectar reward at the base of the nectar spur. Thus, the flowers will solely be pollinated by insects that are able to reach the reward. Therefore, by elongating their spur, flowers apply a 'pollinator-filter' keeping often more generalist pollinators out (Struck, 1997) and effectively forming a relationship with a more specialised pollinator that gives a higher pollination efficiency (Kay and Sargent, 2009). Hodges (1997) compared the twelve known 'spurred' Angiosperm clades with their unspurred sister-clades and concluded that floral nectar spurs can be considered as evolutionary key innovation, as spurred clades mostly are more species rich than their unspurred sister-clades.

A unique type of spur-pollination is found in *Pelargonium* that has a sepal-spur growing adnate to the pedicel, not found in its smaller sister-clade (i.e. the remainder

of Geraniaceae). Bakker et al. (2005), inferred spur length evolution in *Pelargonium* and concluded bees to have been ancestral and that three switches towards longer-tongued pollinators had occurred during *Pelargonium* clade proliferation. Whereas an overall trend towards longer spurs was found, speciation rate and spur lengths are negatively correlated, i.e. highly specialised flowers are found in smaller clades (Ringelberg, 2012). This is the opposite trend as was observed in most other angiosperm clades (Hodges, 1997).

PELARGONIUM, A BUDDING MODELSYSTEM

I have touched upon the genus *Pelargonium* above, giving a few concrete examples of how evolution has proceeded. In this section, I give an overview of the general research on *Pelargonium*, providing background information on the genus. We are aware botanically speaking *Pelargonium* does not possess a 'nectar spur' (Tsai et al., 2018) but rather, ontogenetically the nectar tube is an outgrowth of the receptacle. However, given the functional similarity between the structures as well as the frequent use of the word 'spur' and 'spur pollination' in literature regarding *Pelargonium*, we here use these terms as substitutes. In addition, when using the term 'spur evolution' we mean 'spur-like evolution'.

Taxonomy

In the seventeenth century, *Pelargonium* species where discovered around the Cape of Good Hope during voyages of the Dutch East India Company (VOC, Miller 2002). Linnaeus, after having been sent material of *Pelargonium triste* (the only species that managed to survive the journey to Europe due to its tuberous roots), assigned it to the genus *Geranium* within the Geraniaceae in 1753. The Dutch botanists Johannes and Nicolaas Burman determined this classification was incorrect, and the species occurring around the South African Cape should form a genus separate of *Geranium*. This view was shared by the Frenchman Charles Louis L'Héritier, in whose manuscript the names *Pelargonium, Geranium and Erodium* were established between 1787-1788 (Miller, 2002). Unfortunately, L'Héritier was killed in the French revolution before he was able to publish his manuscript but it was incorporated in the publication of Aiton (1789).

Pelargonium has subsequently become an extremely popular clade for plant breeders for over a century (James, 2002) and it is a ubiquitous component of flower boxes around the world. Thus, there has been and continues to be a need for correct descriptions and classification of the species. Over the years, a general consensus established by the *Pelargonium* research community concerning the taxonomic units. After L'Héritier described *Pelargonium*, many new species where discovered and described by various collectors exploring the Cape flora. The first serious taxonomic classification was done by Sweet in 1820, who created ten new genera related to *Pelargonium*. Not long after, De Candolle demoted these to section level within the genus (Miller, 2002). Growth form was the foundation of his infrageneric classification (Miller, 2002; Röschenbleck et al., 2014). Other classifications followed based on ecological parameters, morphology, and karyology (Miller, 2002; Röschenbleck et al., 2014). The most recent classification was by Röschenbleck, who recognised sixteen sections based on molecular phylogenetic

analyses (Röschenbleck et al., 2014). Currently ~280 taxa (at varying taxonomic levels) are accepted for *Pelargonium* (Bakker et al., 2005; Röschenbleck et al., 2014; Weng et al., 2012) with continual changes and additions (Manning et al., 2015; Manning and le Roux, 2016; Marais, 2017, 2016).

Overall morphology

The genus *Pelargonium* is sister to all other genera within the Geraniaceae: *Erodium* L'Héritier in Aiton (Aiton, 1789), *Geranium* Linnaeus (Linnaeus, 1799), *Monsonia* Linnaeus (Linnaeus, 1799), and *California* (Aldasoro et al., 2001; Fiz et al., 2008; Price and Palmer, 1993). Morphologically, *Pelargonium* is distinguished from the rest of the family by having a nectar spur that is adnate to the pedicel, zygomorphic flowers, and a hypanthium (Albers and van der Walt, 2007; Röschenbleck et al., 2014).

The ~280 species in the genus are morphologically grouped in sixteen sections according to Röschenbleck (Röschenbleck et al., 2014). Among these sections, there is extensive morphological variation not only in flowers, but also in growth form (Albers and van der Walt, 2007; Bakker et al, 1999b). For example, the sections *Peristera* and *Campylia* form large herbaceous structures, while the sections *Myrrhidium*, *Jenkinsona*, and *Chorisma* form woody shrubs (Jones et al., 2009). *Pelargonium* also displays a wide-range of leaf morphology (Figure 2), which is not

Figure 2. Sample of variety in leaf shape in *Pelargonium*. After Nicotra et al. (2011).

constrained by environmental conditions or relationships (Jones et al., 2009; Nicotra et al., 2011). In addition, there is a wide variety of scents reported for numerous species, ranging from spicy-clover like (i.a *P. triste* and *P. lobatum;* Röschenbleck et al., 2014) to heave rose scented (as in *P. graveolens;* Boukhris et al., 2013).

Floral morphology and pollination

While most flowers of Geraniaceae are actinomorphic, almost all *Pelargonium* species have generally zygomorphic flowers which are arranged in inflorescences (Figure 3; Albers and van der Walt, 2007). Each flower usually consists of five petals and five sepals which are arranged as two posterior and three anterior petals. The two posterior petals are most often clearly distinguishable from the other three; be it in size, colour, or by petal markings. The variation in petal copy number that occurs in *Pelargonium* is present in the number of anterior petals. In some species (*P. tetragonum.*), the middle anterior petal is largely reduced, giving the illusion the petal is absent. In other species the petal actually is not present. This variation in petal copy number can occur within a species and even within one plant (i.a. *P. caucalifolium* and *P. myrrhifolium*). The orientation of the petals also varies and ranges from highly zygomorphic (*P. fulgidum*) to almost actinomorphic (*P. cotyledonis*). Also, the shape of the petals is highly variable: from slender and elongated (*P. paniculatum*) to almost round (*P. inquinans*).

Figure 3. Sample of variety in floral shape in *Pelargonium*. **Top row**: (Left) *P. lobatum* (15204363); (middle) *P. candicans* (38062357); (right) *P. fulgidum (*24075355). **Bottom row**: (Left) *P. myrrhifolium* (36584104); (middle) *P. australe* (4269162); (right) *P. asarifolium* (14175021). Source:www.inaturalist.org.

Pelargonium is well-known for its brightly coloured flowers and it is no surprise flower colour is an important target for plant breeding (Forkmann, 1991). In *Pelargonium*, especially red or pink coloured flowers are common, while yellow and blue flowers are rare (Sukhumpinij et al., 2012). The colour is due to the accumulation of the anthocyanin pelargonidin (named after *Pelargonium*; Forkmann, 1991; Rausher, 2008). Colour is also affected by the accumulation of betalains and carotenoids in in different ratios and assemblies. The colour of the pigments is effected by cellular pH of the petals, since some of the compounds display slightly different colours under a pH gradient (Mitchell et al., 1998). An example of a rare lightly yellow coloured *Pelargonium* species is *P. quinquelobatum* (Sukhumpinij et al., 2012).

Pelargonium essential oils and compounds are widely used for commercial purposes (Blerot et al., 2015). In the cosmetics industry, so called 'Rose scented' *Pelargonium* are used in soaps and perfumes (Bendahmane et al., 2013). In pharmaceutical research, *Pelargonium* has been in use as a model system. Based on its use as a traditional medicine, *Pelargonium* has been found to contain many compounds that are antimicrobial (Boukhris et al., 2013; Lis-Balchin et al., 1998a; Lis-Balchin and Deans, 1996; Mativandlela et al., 2006) and antibacterial in particular (Ghannadi et al., 2012; Kayser and Kolodziej, 1997; Kolodziej et al., 2003; Lewu et al., 2007; Lis-Balchin et al., 1998b). In addition, *P. sidoides* has been proposed as a candidate for anti-HIV therapy (Helfer et al., 2014).

The nectar spur is a distinctive feature in *Pelargonium.* Spurs are not common in angiosperms but are present in for example *Aquilegia* (Ranunculaceae), Tropaeolaceae and Orchidaceae (Weberling, 1992; Whittall and Hodges, 2007). The nectar spur is an outgrowth of floral parts (usually petals), which is formed into a hollow tube (Endress, 2001; Hodges and Arnold, 1995). In *Pelargonium*, the spur is formed by one of the posterior sepals (Miller, 2002). Spurs are a way to increase the distance between the flower show and reward apparatus. This is critical for controlling the type of pollinator that is able to reach the reward. They therefore are an important component of reproductive isolation and thus speciation (Hodges and Arnold, 1995). In most cases, for example in *Aquilegia*, the nectar spurs are quite prominent. In *Pelargonium* however, the spur is much less obvious by being adnate with the pedicel of the flower (Hodges, 1997; Hodges and Arnold, 1995; but see Tsai et al., 2018). Often, spurs contain nectar which doesn't have to be produced within the spur itself (Endress, 2001; Struck and Van der Walt, 1996), but in *Pelargonium* nectar is produced in the hypanthium which is fused with the pedicel at the base of the spur (Miller, 2002). The length of the spur relative to the pedicel is often used as a way to distinguish between species (Miller, 2002).

The combination of reward and show in *Pelargonium* is important for pollination. The role of the show apparatus (the corolla) is to lure possible pollinators. Not only overall flower colour is important for this, but also the nectar guides that often decorate the petals are thought to play an important role (Hansen et al., 2012; Röschenbleck et al., 2014). These, and other spatial cues, are thought to align the pollinator, helping it to forage on the flower (Hansen et al., 2012; Kaczorowski et al., 2012).

Since *Pelargonium* is a morphological diverse genus, it is not surprising that there are a number of different pollination syndromes described for it. Also, many multiple transitions between syndromes have occurred (Struck, 1997). The first one to research these syndromes in *Pelargonium* was Delpino in the 1870s, where he described sphingophilous (hawkmoth pollination) and melittophilous (bee pollination) syndromes for a number of *Pelargonium* species (Struck, 1997). Later, Vogel (1954) added the psychophilous (butterfly pollination), ornithophilous (bird pollination), and myiophilous (fly pollinated) syndromes (Struck, 1997). Manning, Goldblatt and Bernhardt described long-tongued hovering fly pollination guilds for Southern Africa, and included a number of *Pelargonium* species in their analysis (Goldblatt et al., 1995; Goldblatt and Manning, 2006; Manning and Goldblatt, 1996).

Phylogenetics

Since 1993, much molecular work has been done to infer a correct phylogenetic tree for the genus *Pelargonium*. Price and Palmer (1993) published the first phylogenetic inference of the Geraniaceae studying the relationships between the five genera within the family using *rbc*L. They found that *Pelargonium* is the first splitting genus within the Geraniaceae. Later, a subgeneric split was inferred based on chromosome size variation into large and small chromosome clades (Bakker et al., 1999). In 1996, Jones and Price performed the first sequence-based analysis based on the rDNA ITS region for thirteen species. This study was expanded by Bakker et al. who added the cpDNA trnL-F markers, first for seventeen species from the section *Peristera*, and subsequently using seventy-three species from the small chromosome clade (Bakker et al., 1999, 1998). In 2000, Bakker et al. conducted the first study that included the mitochondrial marker *nad1* in combination with the chloroplast *trn*L-F region for twenty large chromosome species in addition to five species from the small chromosome clade using the extraordinary high substitution levels found in *Pelargonium* mitochondrial genomes (see below). The first genus-wide phylogenetic study came in 2004, and included markers from all genomic compartments: *nad1* (mitochondrial), *trn*L-F (chloroplast) and *ITS* (nuclear) for 149 *Pelargonium* species and four outgroups (Bakker et al., 2004). In this study, Bakker et al. proposed the division of the genus in an A, B, and C clade: (((A1,A2),B),(C1,C2)). In 2009, Jones et al. used the data of Bakker et al. (2004) for a new phylogenetic tree but with a different inference method (MrBayes instead of PAUP*). With this analysis, they confirmed the previously found division of clades (A1, A2, B, C1 and C2). However, *P. nanum* was omitted from this analysis because it was found to obstruct convergence of the MCMC's used. In 2012, Weng et al. (2012) expanded the gene-set, but achieved only a limited taxon sampling with only fifty-eight species. Röschenbleck based his phylogenetic inference on the *atp*B-*rbcL* spacer and *trnL-F* data of 104 species. Röschenbleck also found the A, B, and C clades as did previous studies, and recognized these as subgenera. In addition, he divided the C clade is a C1 and C2 clade, thus forming four subgenera. These subgenera were further divided in sixteen clades. These divisions are supported by floral morphological data (Röschenbleck et al., 2014).

Genomic instability

In addition to the phylogenetic studies describes above, much research has been performed on the cytogenetics of *Pelargonium.* For example, studies of chromosome numbers, ploidy level, and chromosome size have been performed (Gibby et al., 1990; Van der Walt et al., 1990; Van der Walt and Vorster, 1983, 1981; Weng et al., 2012). The most basal split in the genus, between clade AB and C, is based on chromosome size and divides the genus in a large (1.5-3 µm) and small (<1.5 µm) clade (Bakker et al., 2005, 2004; Röschenbleck et al., 2014; Weng et al., 2012). As described earlier, this split is confirmed by phylogenetic studies, but is not taxonomically formalised since there is no morphological basis found (Röschenbleck et al., 2014). In addition to nuclear differences, there are major differences in their cytoplasmic genomes.

In contrast to most angiosperms, *Pelargonium* (and Geraniaceae in general) possess atypical organellar genomes (Ruhlman and Jansen, 2018). *Pelargonium* x *hortorum* contains one of the largest plastomes on the planet (~218kb, compared with an average of ~151kb for angiosperms) and expansion of the Inverted Repeat (IR) has been a major contributor (Ruhlman and Jansen, 2018). The *Pelargonium* genome has been as a result found to be exceptionally rearranged, containing many changes in gene order, and gene loss and duplication (Chumley et al., 2006; Guisinger et al., 2011; Palmer et al., 1987). The mitochondrial silent substitution rate is exceptionally elevated (Bakker et al., 2006; Parkinson et al., 2005) and the mitochondrial genome also suffered extensive gene loss (Parkinson et al., 2005). Nucleotide substitution in mitochondrial and plastid genomes are accelerated (Weng et al., 2012). Genomes in *Pelargonium* have thus been found to be relatively unstable (Guisinger et al., 2008).

A beautiful example of genomic instability in *Pelargonium* is the incompatibility between the different genomes to correctly communicate, which is a critical issue for breeding (Baur, 1908; Weihe et al., 2009). Due to cytonuclear incompatibility, the leaves of F1 progeny of *Pelargonium* crosses can display variegation: yellow or white zones in their green leaves, petioles, and even meristems (Breman, pers. comm; Greiner and Bock, 2013; Ruhlman and Jansen, 2018). This phenomenon is related with biparental inheritance of the plastome that occurs in *Pelargonium* (Greiner and Bock, 2013; Weihe et al., 2009).

Historical biogeography and the Greater Cape Floristic Region

The bulk of the genus *Pelargonium* occurs in the South African Cape Floristic Region. This region is known to be a biodiversity hotspot, with high levels of endemism not only on species, but also on generic and family level (Cowling et al., 2009; Goldblatt and Manning, 2002; Linder, 2005; Linder and Hardy, 2004; van der Niet and Johnson, 2009; Verboom et al., 2009b, 2009a). *Pelargonium* is one of the prime examples of a nested radiation in this region (Bakker et al., 2005). Thirty of these radiations make up half of the species richness of the Cape flora (Linder, 2005).

Since the coming of phylogenetic analyses, the composition of the Cape flora is found the be much more complex than initially thought. At first, it was thought

that the flora was influenced by three components. The 'Antarctic' (a relic of the Cretaceous Gondwanan flora), the African (shared with tropical Africa), and the Eurasian (migrated south along the African mountains; Linder, 2005). But now, it is found that a number of lineages have a close relationship with sister lineages on all other continents, but that the relation with Australia is the most common. An example of this is *Pelargonium*, with the Australia based clade *Peristera* (Linder, 2005). This phenomenon is not unique, the flora of New Zealand is another example (Linder, 2005).

Bakker et al. (2005) are the first who attempted a (narrative) historical biogeographical analysis for *Pelargonium* (Figure 4). According to their analysis, the deepest split in *Pelargonium* (between the AB and C clades) occurred 30 Mya in the Oligocene mesic subtropical climate. This split, which divides the genus into the large and small chromosome clades, could be the result of climatic changes during that time. Although only species from clade B and C occur outside southern Africa, this split is not a direct geographical indication of the region of origin of *Pelargonium* since these all have sister species occurring in the CFR. The split between clade A and B seems to be the result of contrasting life strategies: while clade A species are predominantly perennial woody shrubs, clade B species are mostly annual herbs.
Although the vast majority of species occurs in the CFR, there is a number of remarkable 'escapees' from this region. One of the clearest is the dispersal to

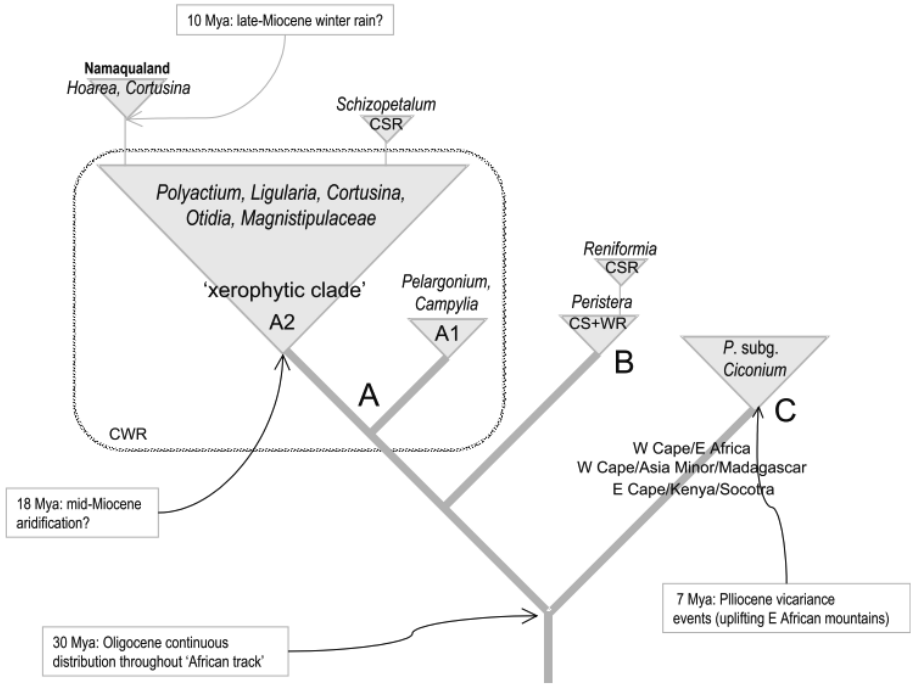


Figure 4. Narrative historical biogeography of *Pelargonium*. After Bakker et al. (2005).

Australia of a small group of species from within the B clade section *Peristera*. Also found in the B clade are two island endemics: *P. cotyledonis* on St. Helena and *P. grossularioides* on Tristan da Cunha. Also, a number of species occur in Kenya, Tanzania and Ethiopia and even as far north as Yemen and Asia minor.

AIM OF THE THESIS

This thesis aims to bring together multiple layers of potential influences on floral shape in *Pelargonium* in order to paint a comprehensive picture of the evolution of this clade. I accomplish this by studying the historical biogeography and ancestral conditions of the genus, within- and between species differences in floral shape, and their relation with the adaptation of *Pelargonium* species to local conditions. By building upon the extensive knowledge on speciation processes in the Greater Cape Floristic Region, we are now able to mine this research and infer speciation processes for the *Pelargonium* clade.

THESIS OUTLINE

The chapters in this thesis all have their own focus, but build upon each other and together paint the intricate picture of speciation in *Pelargonium*.

Chapter 2 presents a new phylogenetic tree for *Pelargonium* based on 74 plastome exons and nuclear rDNA ITS regions for 120 species. We used material obtained from the wild as well as botanical gardens, which gave us the opportunity to represent 43% of the genus. Phylogenetic analyses of nucleotide, amino acid, and ITS alignments resolved relationships within the genus and a dating analysis examined the timing of the major radiations.

In chapter 3 we use this newly formed, time-calibrated phylogenetic tree to infer the ancestral area as well as ancestral climatic conditions of *Pelargonium*. We manually craft an additional 136 species to the plastome-based backbone based on known phylogenetic and taxonomic relationships, composing a 256 terminal phylogenetic tree. We use available distribution data for these species to infer the historical biogeographical events that occurred within the genus and use BIOCLIM data for the georeferenced coordinates to infer paleo-climatic patterns.

In Chapter 4 we quantify floral shape by using geometric morphometrics on a 3D model based on 2D photographical data and demonstrate its performance in capturing shape variation. We quantify *Pelargonium* floral shapes using 117 landmarks and show similarities in reconstructed morphospaces for spur, corolla (2D datasets), and a combined 3D dataset. Through our approach, we find that adding the third dimension to the data is crucial to accurately interpret the manner of, as well as levels of, shape variation in flowers.

In Chapter 5 we dive into this variation in shape and explore the *Pelargonium* floral morphospace and examine to what extent the floral parts within the *Pelargonium* flower are integrated, i.e. whether they evolve in concert, and whether different, show-, reward-, and transfer- apparatus specific, selective pressures may exist.

Finally, in Chapter 6 we bring together all existing knowledge on these different aspects of plant speciation specifically for the Cape lineage *Pelargonium* and explore the relation between historical-biogeography, edaphic influences, pollinators, flowering time, and floral shape in a multi-variate analysis. We want to know to what extend there is a relation between floral shape and the spatial distribution of the species and whether local environmental conditions are an influence on floral shape. We find speciation in *Pelargonium* to be a complex patchwork of interaction between environmental conditions, pollinator distributions, flowering time, and historical biogeographical influences.

Chapter 2

Plastome based phylogenetics and younger crown node age in *Pelargonium*

Sara J. van de Kerke[1], Bikash Shrestha[2], Tracey A. Ruhlman[2], Mao-Lun Weng[2,4], Robert K. Jansen[2,5], Cynthia S. Jones[3], Carl D. Schlichting[3], Samin Hosseini[1,6], Setareh Mohammadin[1], M. Eric Schranz[1], & Freek T. Bakker[1]

Published in *Molecular Phylogenetics and Evolution*, 2019, 137; 33-43.
DOI: 10.1016/j.ympev.2019.03.021

[1]Biosystematics, Plant Science Group, Wageningen University, Droevendaalsesteeg 1, 6708 PB, Wageningen, The Netherlands
[2]Department of Integrative Biology, University of Texas at Austin, 205 W. 24th St. Stop C0930, TX 78712, USA
[3]Department of Ecology & Evolutionary Biology, University of Connecticut, 75 N. Eagleville Road, Storrs, CT 06269 USA
[4]Department of Biology and Microbiology, South Dakota State University, Brookings, SD 57006, USA
[5]Department of Biological Sciences, Faculty of Science, King Abdulaziz University, P.O. Box 80141, Jeddah 21589, Saudi Arabia
[6]Department of Plant Protection, College of Agriculture, Vali- e-Asr University of Rafsanjan, Rafsanjan, Iran

ABSTRACT
The predominantly South-African plant genus *Pelargonium* L'Hér. (Geraniaceae) displays remarkable morphological diversity, several basic chromosome numbers as well as high levels of organelle genomic rearrangements, and represents the 7th largest Cape Floristic Region clade. In this study, we reconstructed a phylogenetic tree based on 74 plastome exons and nuclear rDNA ITS regions for 120 species, which represents 43 % taxon coverage for *Pelargonium*. We also performed a dating analysis to examine the timing of the major radiations in the genus.
Phylogenetic analyses of nucleotide, amino acid, and ITS alignments confirmed the previously-documented subgeneric split into five main clades ((C1,C2),(B(A1,A2))) although clade only A1 received low bootstrap support.
Using calibration evidence from a range of sources the *Pelargonium* crown age was estimated to be 9.7 My old, much younger than previous estimates for the genus but similar to recent studies of other Cape Floristic lineages that are part of both Fynbos and Succulent Karoo biomes.

Keywords: *Pelargonium*; Geraniaceae; phylogeny; plastome; time-calibrated
Abbreviations: *Polyactium-Otidia-Cortusina* clade (POC clade), Greater Cape Floristic Region (GCFR)

INTRODUCTION

The predominantly South African genus *Pelargonium* L'Hér. (Geraniaceae) is morphologically diverse both in life forms, ranging from herbaceous annuals, woody (sub)shrubs, geophytes, rosette herbs to stem succulents and by remarkable variation in floral and leaf morphology (Bakker et al., 2005, 1999, Jones et al., 2009, 2003; Nicotra et al., 2008; Röschenbleck et al., 2014). *Pelargonium* is also characterised by extensive genomic variability, with six different basic chromosome numbers (Bakker et al., 2005), substantial variation in nuclear genome size (Weng et al., 2012), the independent occurrence of several polyploid series and unprecedented levels of variation in organelle genomes (Bakker et al., 2006; Chumley et al., 2006; Guisinger et al., 2008, 2011; Mower et al., 2007; Parkinson et al., 2005; Weng et al., 2014; Weng et al., 2012). In addition, many species exhibit biparental inheritance and cytonuclear incompatibility (Ruhlman and Jansen, 2018). *Pelargonium* is one of a handful of speciose Cape lineages that span multiple biomes in Greater Cape Floristic Region, making this clade a promising model system for testing ecological and evolutionary hypotheses (e.g. Moore et al., 2018; Verboom et al., 2009).

Of the ~280 species of *Pelargonium*, approximately 200 species occur in the Greater Cape Floristic Region (GCFR) in South Africa (Linder, 2003; Manning and Goldblatt, 2012; Snijman, 2013) and have been well-documented taxonomically (Van der Walt and Vorster, 1977, 1981, 1988). Morphological, palynological, phytochemical and karyological data have been used in an extensive range of taxonomic studies (e.g. Van der Walt et al., 1990; Van der Walt and Vorster, 1983, 1981; see Röschenbleck et al., 2014 for an overview). New species are still being described, especially in the geophytic sect. *Hoarea* (Manning et al., 2015; Marais, 2016), which was considered a non-adaptive radiation nested within an adaptive radiation by Bakker et al. (2005). Their appeal as garden plants (dating back to Victorian times, Sweet (1826)) has led to interspecific crosses of *Pelargonium* that have resulted in a wide variety of highly valued commercial cultivars (Albers & van der Walt, 2007), especially in the *P.* x *hortorum* hybrid complex, *P. peltatum*, *P. cucullatum,* and *P. tricolor*, and some species such as *P. citronellum* and *P. graveolens* are important for essential oil production (Blerot et al., 2015).

Phylogenetics of *Pelargonium* has been investigated in a series of studies spanning the last three decades. Price and Palmer (1993) published the first DNA-based generic-level phylogenetic tree of Geraniaceae and found *Pelargonium* to be sister to the rest of the family (except *Hypseocharis*) and inferred a subgeneric split that correlated with chromosome size. Subsequent phylogenetic studies using increased taxon sampling confirmed this pattern for both the internal transcribed spacer (ITS) of nuclear ribosomal DNA (rDNA) and the plastid *trnL-F* group I intron and *trnL-F* spacer regions for small and large chromosome species (Bakker et al., 1999, 1998; Jones and Price, 1996). Making use of the remarkably elevated substitution rates in Geraniaceae mitochondrial DNA (mtDNA), Bakker et al. (2000) found species-level phylogenetic resolution using exons 4 and 5 of mitochondrial-encoded *nad*1, which is otherwise fairly conserved across angiosperms. Using markers from all three genome compartments (*nad*1, *trnL-F*, and rDNA ITS) for 149 *Pelargonium* species (i.e. 53% taxonomic sampling), Bakker et al. (2005, 2004b) inferred a phylogeny

and proposed a (C(B,A)) division of the genus with A and C clades each divided into two main clades. This analysis used weighted parsimony and heavily relied on non-coding sequences and recoded insertions and deletions (indels; Bakker et al., 2004), which were found to provide ~20% of the total plastid DNA signal (Bakker & al. 1999). The ((C1,C2),(B(A1,A2))) *Pelargonium* phylogenetic pattern was later confirmed using Bayesian Inference on the same data (Jones et al., 2009).

Weng et al. (2012) noted the lack of overlap in taxonomic sampling of the different gene sequences in previous studies and recommended expanded gene and taxon sampling. The authors partially fulfilled this need by adding additional gene sequence data for the markers nad5, *ndhF*, *rbcL*, *mat*K, *rpo*C1, in addition to *trnL-F*. However, *Pelargonium* taxon sampling remained limited (only 21%) in that study. By adding the atp*B-rbcL* spacer to an increased taxon sampling for the *trnL-F* data set of Bakker et al. (2004), Röschenbleck et al. (2014) achieved a taxonomic coverage of 38% and confirmed a ((C1,C2),(B(A1,A2))) (but not A1 and A2) topology. However, nuclear and mitochondrial data were not included. Röschenbleck et al. (2014) proposed raising these four clades to the subgenus level. These subgenera were then further divided into sixteen sections, most of which confirmed the previously-proposed sectional classification (Bakker et al., 2004), and are supported by floral morphological data (Röschenbleck et al., 2014), although a general morphology based key has not been developed for *Pelargonium*.

Phylogenetic studies focusing on single *Pelargonium* clades have been performed for Clade C (James et al., 2004, based on plastome RFLPs), sect. *Hoarea* (Touloumenidou et al., 2003, based on rDNA ITS sequences), clade B (Bakker et al., 1998) and the Australian clade (Nicotra et al., 2016, using a population genomic approach). For sect. *Otidia,* an AFLP approach was used to study relationships in the *P. carnosum - P. paniculatum* and *P. alternans* complexes (Becker and Albers, 2010, 2009).
Four molecular dating studies have been performed in *Pelargonium,* but the results are contradictory. In 2005, using r8s and the non-parametric rate smoothing approach on *trnL-F* and nuclear rDNA ITS sequences, Bakker et al. estimated the *Pelargonium* crown node to have originated around 30 Mya. Based on the same data, but using BEAST analyses, Verboom et al. (2009) came to a similar estimate of 34.54 My, making it the oldest Fynbos biome clade giving rise to Succulent Karoo clades (Verboom et al., 2009). The deepest split into clades A,B versus C (with different chromosome size), would coincide with climatic changes in the Oligocene/Miocene (Goldblatt et al., 2002). Additionaly, the estimated age of the winter-rainfall clade A2 of 22 My coincides with the Early Miocene and was interpreted to be linked with the emergence of summer drought by Bakker et al. (2005). During the Miocene-Pliocene climate change around 10 Mya, upwelling of the cold Benguela current was established and, in combination with a strengthened South Atlantic pressure cell, could well have caused dry summers along the west coast of southern Africa. This is considered to be associated with the formation of the Succulent Karoo biome and the radiation of new, xerophytic, *Pelargonium* species (Bakker et al., 2005; Linder, 2003; Verboom et al., 2009). In contrast, both Fiz et al. (2008) and Palazzesi et al. (2012) estimated the date for

the *Pelargonium* crown node to be 10-15 Mya, corresponding with a transition to a drier and colder climate in the mid-Miocene (Linder, 2003). These studies both used fossilized pollen as evidence for their analyses (see Supplementary Table S1 for an overview of these studies). Given the discrepancies among *Pelargonium* dating studies, an update using increased numbers of characters and taxa is needed.

The aims of this study are to resolve phylogenetic relationships within *Pelargonium* using extended character sampling (74 plastome protein-coding genes as well as nuclear rDNA ITS) and to provide robust age estimate. Previous *Pelargonium* phylogenetic studies have relied on plastome intergenic and Group I intron sequences (such as *trnL-F* and the *atpB-rbcL* spacer), in addition to indels in the same spacers. Our approach using exons provides sufficient data to resolve remaining phylogenetic issues in *Pelargonium*, and results in an improved phylogenetic framework for future genomic, morphological and evolutionary studies.

MATERIAL AND METHODS

Taxon sampling

Leaf material was obtained from various sources (see Supplementary Table S2), including plants obtained in the field and from botanical gardens. One collection series was sampled from various wild populations collected across South Africa and was silica gel dried by Schlichting, Jones and collaborators during 2012-2015. Vouchers are deposited at CONN. Another series comes from the Jansen lab with material obtained from Geraniaceae.com. Plants were maintained in the University of Texas at Austin greenhouse and vouchers were made for each species and deposited in TEX-LL. Based on previous phylogenetic studies, we selected samples to represent all five major clades within *Pelargonium*. This resulted in a combined set of 148 accessions representing 120 species (approximately 43% of all *Pelargonium* species). We chose two accessions of *Hypseocharis biloba* (NC_023260.1; (Bakker et al., 2016) as outgroups.

DNA isolation, Illumina sequencing and plastome assembly

DNA from the wild collected samples was isolated from silica gel dried leaf material in the Bakker lab using a modified CTAB protocol (Bakker et al., 1998; Doyle, 1991) after grinding in liquid nitrogen. Following Isopropanol precipitation, the Wizard® DNA Clean-Up System was used to further purify the samples. DNA quantity was determined using a Qbit spectrophotometer. Samples yielding > 20 ng were shipped to BGI Hong Kong for library preparation and Illumina paired end (PE) sequencing. A few accessions with low total yield (between 20 and 50 ng) underwent a whole genome amplification step.

For the plastome dataset, Illumina PE reads were assembled using IOGA, an automated bioinformatics pipeline (Bakker et al., 2016), which uses both *de novo* and reference-based assembly, by mapping reads against a panel of reference genomes that need not be closely related to the target. As a reference library, the same reference plastomes as in Bakker et al. (2016) were used, including complete plastomes of *P. alternans* (NC_023261.1) and *P.* x *hortorum* (DQ897681.1). IOGA uses SOAPdeNovo (Xie et al., 2014) in order to assemble mapped reads into

contigs. A range of k-mer sizes was used (33, 55, 75, 95) to optimise the assembly, assuming an insert size of 250 bp. Plastome-derived reads remaining in the initial total read pool that overlapped with the assembled contigs were mapped to the contigs and assembled *de novo*. New iterations of mapping and assembly were then performed until no new reads could be added to the contigs. Final assembly, usually producing a range of contigs, was performed using SPADES (Bankevich et al., 2012) as implemented in IOGA, followed by selection of candidate assemblies using either Assembly Likelihood Estimation (ALE) score (Clark et al., 2013), overall coverage or N50. When plastome read coverage was exceptionally high for a particular sample (i.e. > 1000), a subsample of one to five million reads was taken before re-assembly.

The methods for DNA isolation, Illumina sequencing, assembly and annotation for the 61 species contributed from the Jansen collection are described in Blazier et al. (2016a) and Weng et al. (2014). For 21 of the 61 *Pelargonium* species, complete plastomes were completed and 74 protein coding genes were extracted. For the remaining species, the genes were extracted from contigs of draft genome assemblies.

For the nuclear rDNA dataset, both the Bakker and Jansen collection underwent the IOGA assembly procedure as described above. A collection containing all previously published rDNA ITS accessions for *Pelargonium* available in GenBank was used as reference.

Annotation and gene selection

All plastid assemblies from the Bakker lab were annotated in Geneious 8.1.6 (Kearse et al., 2012) using *P. alternans* (Weng et al., 2014) as reference and setting the sequence similarity threshold at 75%. In total, a set of 74 protein coding genes was extracted from the assembly data and gene alignments were compiled. Each gene alignment was split into separate intron/exon alignments with the use of the TAIR webtool (https://www.arabidopsis.org/index.jsp; Supplementary Table S3 and S4). The complete plastome sequence of *Hypseocharis biloba* (NC_023260.1) chosen as outgroup underwent the same procedure of gene extraction and alignment.

All nuclear rDNA assemblies were annotated using *Brassica rapa* (KM538956.1) as reference for the ribosomal (18S, 5.8S, and 26S) as well as ITS1 and ITS2. In cases when not all components of the rDNA region could be retrieved in one piece, we used the universal primers as designed by White et al. (1990) to find the ITS1 and ITS2 boundaries.

Alignment and data matrix construction

For both plastome and nuclear data, MAFFT v. 7 was used for optimising each alignment under 'auto' settings (Katoh and Standley, 2013). All alignments were visually inspected in Mesquite v. 3.04 (Maddison and Maddison, 2015) and manually adjusted where needed. Reading frames were set for all coding region alignments using the 'Minimize stop codons' function. Alignments were trimmed accordingly to reading frame in order to eliminate incomplete codons. A 'Plastome Introns

and Exons' (PIE) matrix included all above described alignments, concatenated using SequenceMatrix (Vaidya et al., 2011) into a single alignment. In addition, a 'Plastome Exons AminoAcid' (PE-A) matrix contained an amino-acid version of the exon-only data. The number of parsimony informative sites was calculated using PAUP* (Swofford, 2002).

Phylogenetic analyses

Maximum likelihood-based phylogenetic analysis of plastome matrices was performed using RAxML v. 8.2.8 on the XSEDE supercomputer at the CIPRES Science Gateway platform (Miller et al., 2010; Stamatakis, 2014). Two partition schemes for the PIE matrix were compared: 1. unpartitioned and 2. partition assigned by PartitionFinder v. 1.1.1 (Lanfear et al., 2012), which selects from alternative gene- or codon-position level partitioning on the basis of the Bayesian Information Criterion (BIC). The PE-A matrix was analysed under an unpartitioned model (using the PROTGAMMADAYHOFF amino acid substitution model) as optimising AA models in multiple partitions is computationally prohibitive. RAxML analyses of DNA sequence data was performed using the GTR+GAMMA model. All analyses included inference of the 'best tree' as well as generation of 1000 bootstrap trees, to obtain node support measures. In addition, we used MrBayes v. 3.2.6 (Huelsenbeck and Ronquist, 2001; Ronquist et al., 2012) for a Bayesian inference of our plastome alignments (500 million generations, nruns=2, four chains, sampled every 30.000th generation, nst=mixed, temp=0.05/0.2).

Phylogenetic analysis of the rDNA ITS matrix was performed under ML using IQ-TREE with standard settings on the IQ-TREE web server (iqtree.cibiv.univie.ac.at) generating 1000 bootstrap trees (Hoang et al., 2018; Kalyaanamoorthy et al., 2017; Nguyen et al., 2015; Trifinopoulos et al., 2016). The analysis includes Ultrafast model selection (ModelFinder) and Ultrafast bootstrap (UFBoot).
All resulting phylogenetic trees were visualised using TreeGraph2 (Stöver and Müller, 2010).

Divergence date estimates

We used BEAST v1.8.4 (Drummond et al., 2012) to infer a time-calibrated phylogenetic tree of *Pelargonium* using the PIE matrix, adding fourteen Geraniales genomes in order to accommodate all available fossil calibrations (Supplementary Table S5). We used three calibration methods: (1) Fossil calibration, using estimated ages of available fossils of *Geranium*, *Erodium*, Vivianaceae and *Pelargonium* set with a log-normal distribution for each calibration prior which has an unbound tail reflecting the uncertainty of the maximum age of the node (Ho and Phillips, 2009; Palazzesi et al., 2012). (2) Secondary calibration, in which the crown node age corresponding to Geraniales as estimated by Wang et al. (2009) was used to calibrate our phylogenetic tree using a normal prior. (3) Ecological calibration, in which we assumed that clade A2 (the 'Winter Rainfall Region' clade in Bakker et al. 2005) could have emerged in response to the establishment of the Mediterranean type climate in the South Western Cape, which has been estimated as late-Miocene: the tertiary fossil record of southern Africa suggests that the earliest summer-drought conditions became established approximately 10 Mya (Linder 2003). We therefore

Table1. Prior setting for calibration evidence for different calibration combinations.

Node	Fossils		2nd		Ecological	
	Age[1]	Prior	Age	Prior	Age	Prior
GAL			99-109	Normal 104 (2)[3]		
G	7.25 (0.005)	Lognormal 7.25 (4, *7.24*)[2]				
E	7.25 (0.005)	Lognormal 7.25 (4, *7.24*)[2]				
MFV	10 (0.3)	Lognormal 10 (2.5, *9.7*)[2]				
GexH	28.4 (0.1)	Lognormal 28.4 (3,*28.3*)[2]				
A2					8-10	Normal 9 (0.4)[3]
	Combination I		Combination II		Combination III	
	Combination IV					
			Combination V			
	Combination VI					

[1]Age range in Mya;

[2] mean, sd, hard minimum bound (= *offset*) (in real space),

[3]mean, sd; G, E, MFV, GexH, A2 and GAL indicate calibrating node.

calibrated the A2 node with this age using a normal prior distribution. In order to assess possible calibration incongruence, we explored the following calibration combinations: Fossils, 2nd, and Ecological calibration separate, the combination of Fossils and 2nd calibration, the combination of 2nd and Ecological calibration, and all three methods combined (Table 1).

We used the uncorrelated lognormal relaxed molecular clock (UCLD) models to account for rate variability among lineages and chose the Yule speciation model, which is considered the most appropriate model for species-level datasets (Bouckaert et al., 2014). We set the prior distribution for mean rate of the clock model as recommended by Ferreira and Suchard (2008) and used the GTR substitution model and assumed site rates to be 4Γ distributed (as suggested by IQ-tree, not shown). We performed one MCMC analysis per dating scenario of 400 million generations each, sampling every 10.000 steps. For the scenario combining all calibration methods, we performed four additional MCMC analyses.

We combined log and tree files using LogCombiner v.1.8.4. (Drummond et al., 2012) and checked for convergence using VMCMC (Visual Markov chain Monte Carlo, Ali et al., 2017) to diagnose global convergence of the whole MCMC chain to the target distribution by calculating Gelmen-Rubin and Gewek parameters (Ali et al., 2017). Split frequency plots that measure topological differences among chains were generated in RWTY (Warren et al., 2017). In case of appropriate convergence, frequencies in the cumulative plot should level off, indicating that clade/split is present in both posterior distributions. We used TreeAnnotator v1.8.4 (implemented in BEAST tools package) with a burn-in of 10% to summarize the tree results.

RESULTS

Assembly and alignments

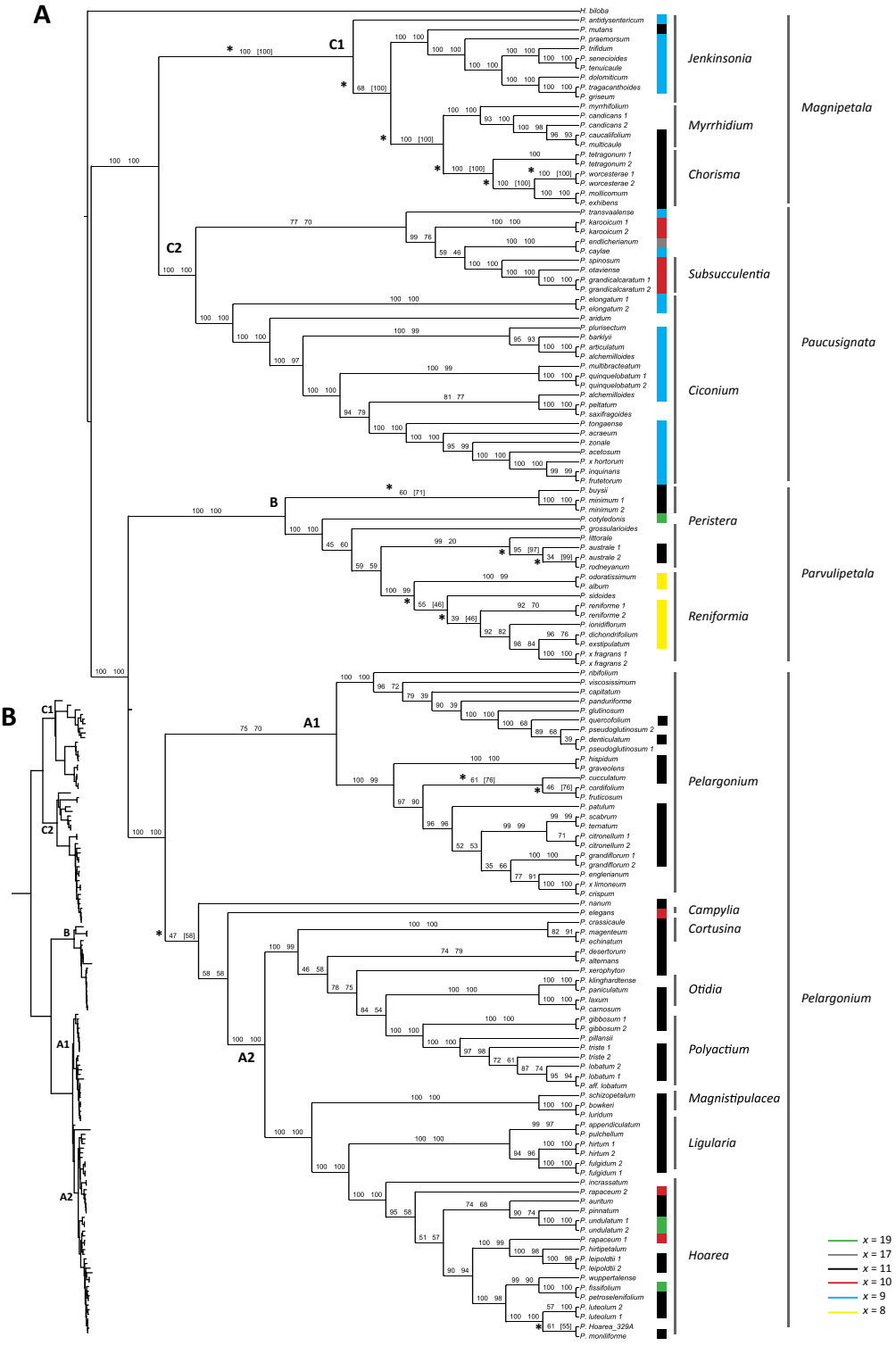
In the Bakker lab, 80 new *Pelargonium* specimens were sampled for DNA extraction and Illumina sequencing in this study. After library preparation and sequencing, the total number of reads ranged from 5,286,525 (*P. minimum*) to 29,102,984 (*P. saxifragoides*). For the specimens from the Jansen lab sequencing depth was much higher, around 60M reads each (see Supplementary Table S6). Average assembly size of the plastomes was 154,624 bp with an average read coverage of 690 after sub-sampling for the Bakker lab samples and on average over 1500X for those from the Jansen lab. The total concatenated PIE alignment (Plastome Introns and Exons) was 64,388 bp in length with 6,305 (9.8%) potentially parsimony informative sites, and covered 43% of all known *Pelargonium* species, whereas the PE-A matrix contained 18,800 amino acid residues of which 3,187 (17%) were parsimony informative. All sequences have been deposited in GenBank (Supplementary Table S7) and the final PIE and ITS alignments and resulting phylogenetic trees can be found in Supplementary File S8 and S9 and under TreeBase Submission ID 24185.

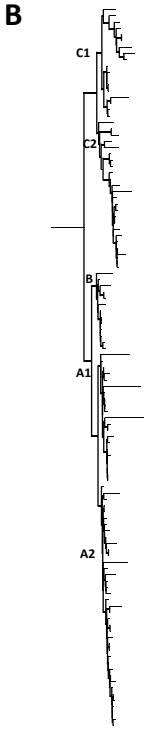
Phylogenetic patterns

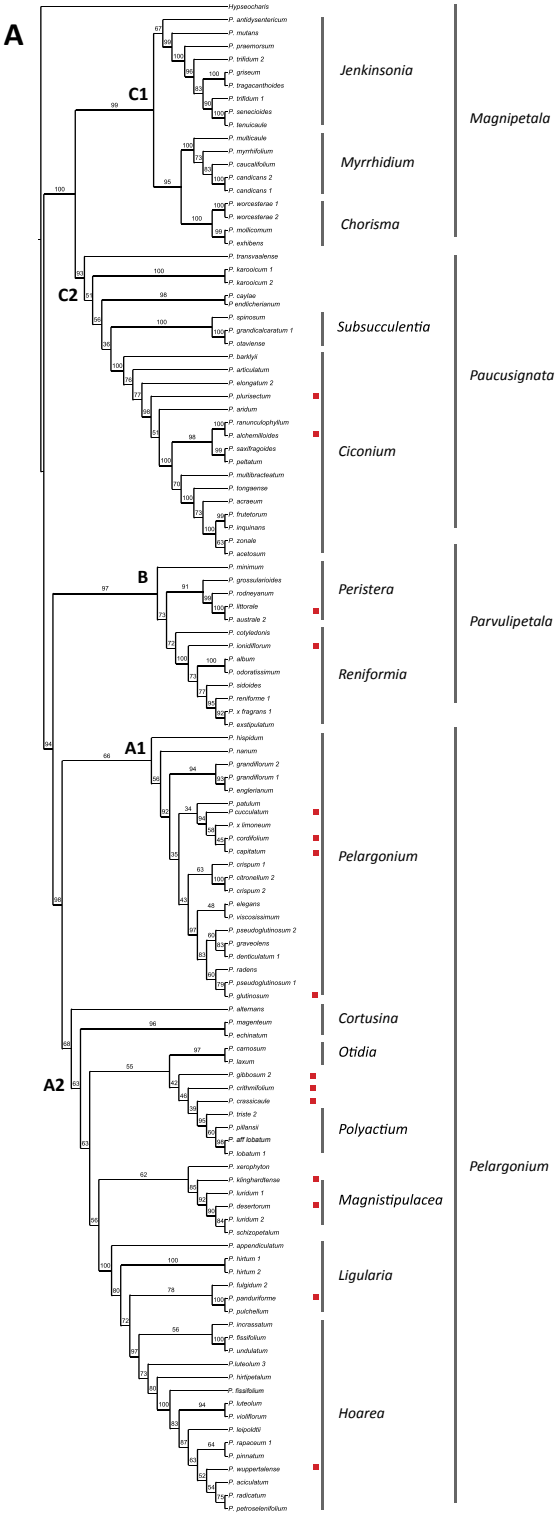
PartitionFinder analysis suggested the data be partitioned over 22 different partitions (Supplementary Table S10 and S11) that corresponded to codon position rather than gene functional group as in Guisinger et al. (2008).

Comparisons of ML tree topologies and support values for the unpartitioned PIE matrix, the partitioned PIE matrix and the unpartitioned PE-A matrix detected few topological discrepancies (indicated by * in Figure 1): five within clade C1, five within clade B, two within the sect. *Pelargonium* and one within the *Hoarea* clade. When bootstrap support values differed, higher values were generally obtained for the unpartitioned PIE data set. Tree topologies for MrBayes analyses were congruent with those for RAxML. The phylogenetic tree inferred from the nuclear rDNA ITS matrix produced the same topology as the plastome matrices, for major clades of *Pelargonium* although the topologies of species within clades were different (Figure 2). For example, based on plastome sequences *P. plurisectum* is, together with *P. barklyi*, *P. articulatum*, and *P. alchemilloides*, confidently placed as sister to the remainder of the clade corresponding to the section *Ciconium* while the nuclear rDNA patterns suggests *P. plurisectum* to be placed more central in the clade. The same small species-level shift within clades occurred for *P. cucculatum*, *P. cordifolium*, *P. capitatum*, *P. glutinosum*, *P. ionidiflorum*, *P. alchemilloides*, and *P. wuppertalense*. Larger incongruences occur for *P. klinghardtense* which shifts from the clade corresponding to its taxonomic section in the plastome based phylogeny to sister species of the section *Magnistipulacea* based on nuclear rDNA. The reverse

Figure 1. ***(next page)*** (A) RAxML tree based on matrix PIE (unpartitioned) and PE-A matrices (GTR+GAMMA), in cladogram style. Bootstrap values indicate support at node for PIE/PE-A analysis respectively. Brackets indicate conflict between analyses. Clade labels sensu section- and subgenus level classification of Röschenbleck et al. (2014). Capital letters correspond to main clades. (B) Same tree as A showing branch lengths in nucleotide substitutions per site (outgroup pruned from tree).


A
B
C1
C2
A1
A2
B
H. bilobs
P. antidysentericum
P. mutans
P. praemorsum
P. trifidum
P. senecioides
P. tenuicaule
P. dolomiticum
P. tragacanthoides
P. grisseum
P. myrrhifolium
P. candicans 1
P. candicans 2
P. caucalifolium
P. multicaule
P. tetragonum 1
P. tetragonum 2
P. worcesterae 1
P. worcesterae 2
P. mollicomum
P. exhibens
P. transvaalense
P. karooicum 1
P. karooicum 2
P. endlicherianum
P. caylae
P. spinosum
P. otaviense
P. grandicalcaratum 1
P. grandicalcaratum 2
P. elongatum 1
P. elongatum 2
P. aridum
P. plurisectum
P. barklyii
P. articulatum
P. alchemilloides
P. multibracteatum
P. quinquelobatum 1
P. quinquelobatum 2
P. alchemilloides
P. peltatum
P. saxifragoides
P. tongaense
P. acraeum
P. zonale
P. acetosum
P. x hortorum
P. inquinans
P. frutetorum
P. buysii
P. minimum 1
P. minimum 2
P. cotyledonis
P. grossularioides
P. littorale
P. australe 1
P. australe 2
P. rodneyanum
P. odoratissimum
P. album
P. sidoides
P. reniforme 1
P. reniforme 2
P. ionidiflorum
P. dichondrifolium
P. exstipulatum
P. x fragrans 1
P. x fragrans 2
P. ribifolium
P. viscosissimum
P. capitatum
P. panduriforme
P. glutinosum
P. quercofolium
P. pseudoglutinosum 2
P. denticulatum
P. pseudoglutinosum 1
P. hispidum
P. graveolens
P. cucculatum
P. cordifolium
P. fruticosum
P. patulum
P. scabrum
P. ternatum
P. citronellum 1
P. citronellum 2
P. grandiflorum 1
P. grandiflorum 2
P. englerianum
P. x limoneum
P. crispum
P. nanum
P. elegans
P. crassicaule
P. magenteum
P. echinatum
P. desertorum
P. alternans
P. xerophyton
P. klinghardtense
P. paniculatum
P. laxum
P. carnosum
P. gibbosum 1
P. gibbosum 2
P. pillansii
P. triste 1
P. triste 2
P. lobatum 2
P. lobatum 1
P. aff. lobatum
P. schizopetalum
P. bowkeri
P. luridum
P. appendiculatum
P. pulchellum
P. hirtum 1
P. hirtum 2
P. fulgidum 2
P. fulgidum 1
P. incrassatum
P. rapaceum 2
P. auritum
P. pinnatum
P. undulatum 1
P. undulatum 2
P. rapaceum 1
P. hirtipetalum
P. leipoldtii 1
P. leipoldtii 2
P. wuppertalense
P. fissifolium
P. petroselenifolium
P. luteolum 2
P. luteolum 1
P. Hoarea_329A
P. moniliforme
Jenkinsonia
Myrrhidium
Chorisma
Magnipetala
Subsucculentia
Ciconium
Paucusignata
Peristera
Reniformia
Parvulipetala
Pelargonium
Campylia
Cortusina
Otidia
Polyactium
Magnistipulacea
Ligularia
Hoarea
x = 19
x = 17
x = 11
x = 10
x = 9
x = 8


B
C1
C2
B
A1
A2


A
C1
C2
B
A1
A2
Jenkinsonia
Myrrhidium
Chorisma
Magnipetala
Subsucculentia
Ciconium
Paucusignata
Peristera
Reniformia
Parvulipetala
Pelargonium
Cortusina
Otidia
Polyactium
Magnistipulacea
Ligularia
Hoarea
Pelargonium

is the case for *P. desertorum* whose position is likewise flexible but also has the status of 'unassigned species' within the subgenus *Pelargonium* (Röschenbleck et al., 2014). Also *P. panduriforme* ends up in the 'wrong' clade based on nuclear rDNA sequences. The trio *P. gibbosum, P. crithmifolium*, and *P. crassicaule* group together between the clades of their respective taxonomic sections, albeit based on rather low bootstrap support.

Divergence date estimates

In order to compare the calibration results for the single MCMC runs based on different combinations of calibration methods, we focus on the *Pelargonium* main crown nodes (Figure 3), i.e. 'Winter-rainfall' clade A, clade A1, 'xerophytic' clade A2, clade B and clade C . The estimated age of *Pelargonium* nodes for the Fossil and Ecological calibration separately are overall comparable, while the 2nd calibration methods shows quite a different pattern with much older age estimates. In addition, the range of HPD's is much larger. When combining the Fossil and 2nd calibration methods, age estimates appear to be predominantly influenced by the 2nd calibration evidence. The combination of 2nd and Ecological calibration does not appear to be subject to this influence as results are comparable with the separate Ecological calibration results. The result of the combination of all three calibration methods fits in this pattern with mean age estimates again congruent with the Ecological calibration.

We consider the scenario including fossil, secondary, as well as ecological calibration as the final result because it is based on maximum evidence (Figure 4). Based on the four extra MCMC runs for this scenario, Geraniaceae crown node appears to have proliferated in the Middle Eocene (~35.8, 95% HPD = 29.5-45.1 Mya) with the *Pelargonium* crown node proliferating in the Late Miocene (9.7 Mya, 95% HPD = 9.0-10.5 Mya). Based on our results, the crown of the oldest clade of *Pelargonium*, clade C, diverged around 8.6 Mya (95% HPD = 7.5-9.7 Mya) while the diversification of B, A1 and 'xerophytic' A2 occurred in the Early Pliocene and the Late Miocene (4.5 Mya, 95% HPD = 2.7-6.3 Mya, 4.5 Mya, 95% HPD = 2.8-6.2 Mya, and 5.3 Mya, 95% HPD = 3.9-6.7 Mya, respectively).

DISCUSSION

Pelargonium has been the focus of an expanding series of phylogenetic studies (Bakker et al., 2004, 1999, 1998; Jones et al., 2009; Price and Palmer, 1993; Röschenbleck et al., 2014; Weng et al., 2012). In those studies, an increasing number of phylogenetic markers has been utilized from all three genomic compartments and taxonomic coverage has been substantially expanded up to 53%. However, a common set of markers needed to link these studies has so far been missing,

Figure 2. ***(previous page)*** (A) RAxML tree based on ITS matrix (IQ-TREE), with bootstrap values indicated. Clade labels sensu section- and subgenus level classification of Röschenbleck et al. (2014). Red squares indicate species-level plasto-ribo incongruence. Capital letters correspond to main clades. (B) Phylogram showing branch lengths in substitutions per site (outgroup pruned from tree) resulting from the RAxML analyses on ITS matrix (IQ-TREE).

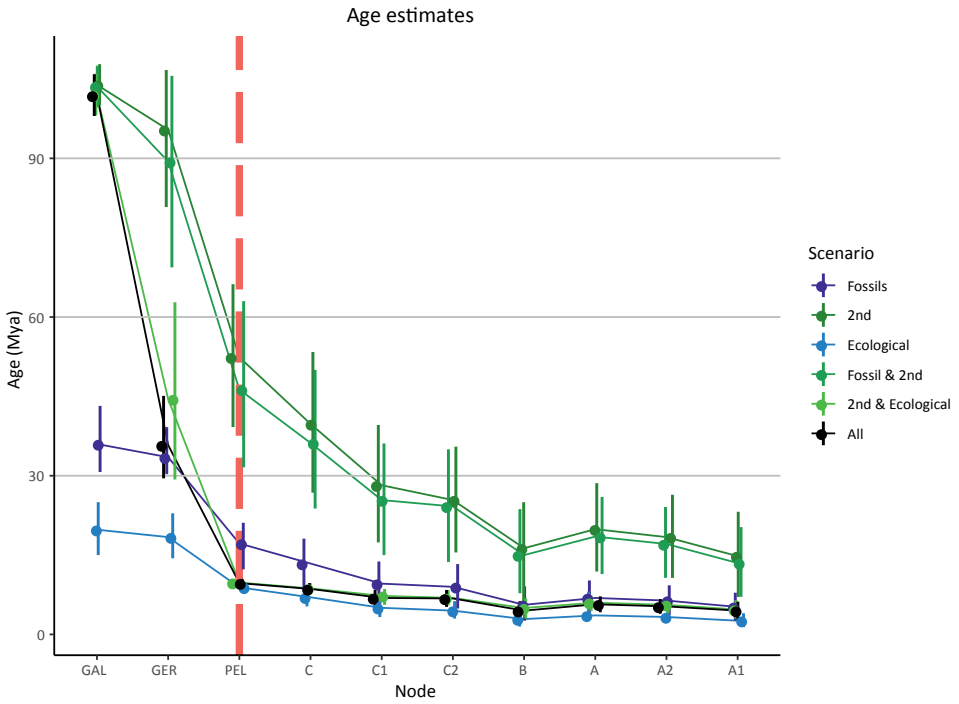


Figure 3. Boxplot of divergence date estimations for main nodes, based on the six combinations of calibration evidence, coloured as indicated: Fossils, 2nd, and Ecological calibration sepa- rate, the combination of Fossils and 2nd cali- bration, the combination of 2nd and Ecological calibration, and all three methods combined. Error bars represent HPDs.

leaving unclear to what extent missing data in the phylogenetic matrices has been influential and whether inter-genomic topological incongruence may have occurred. We compiled a matrix of 74 plastid genes as well as the nuclear ITS region for 120 *Pelargonium* species, achieving 43% taxonomic coverage of the genus. Although our taxon coverage is far from complete, our extensive gene sampling includes species representing all previously reported main clades.

Bakker et al. (2004) found that incongruence between phylogenetic trees generated from nuclear rDNA and plastome sequences was limited to the species-level and occurred predominantly within clades that corresponded to previously described taxonomic sections. Our findings reveal the same pattern detected by Bakker et al. (2004): incongruence between phylogenetic trees generated from nuclear rDNA and plastome sequences is limited to the species-level and occurs predominantly within clades (Figures 1 and 2). Overall, we feel there are no major incongruences between the plastome and nuclear rDNA perspective and, considering the relatively low bootstrap support values for the latter, decided focus on the plastome markers, leaving the nuclear and mitochondrial perspectives for future studies. Arguably, combining all genomic compartments in an overarching phylogenetic analysis would require a species tree estimation approach using multi-species coalescent methods (Liu et al., 2009).

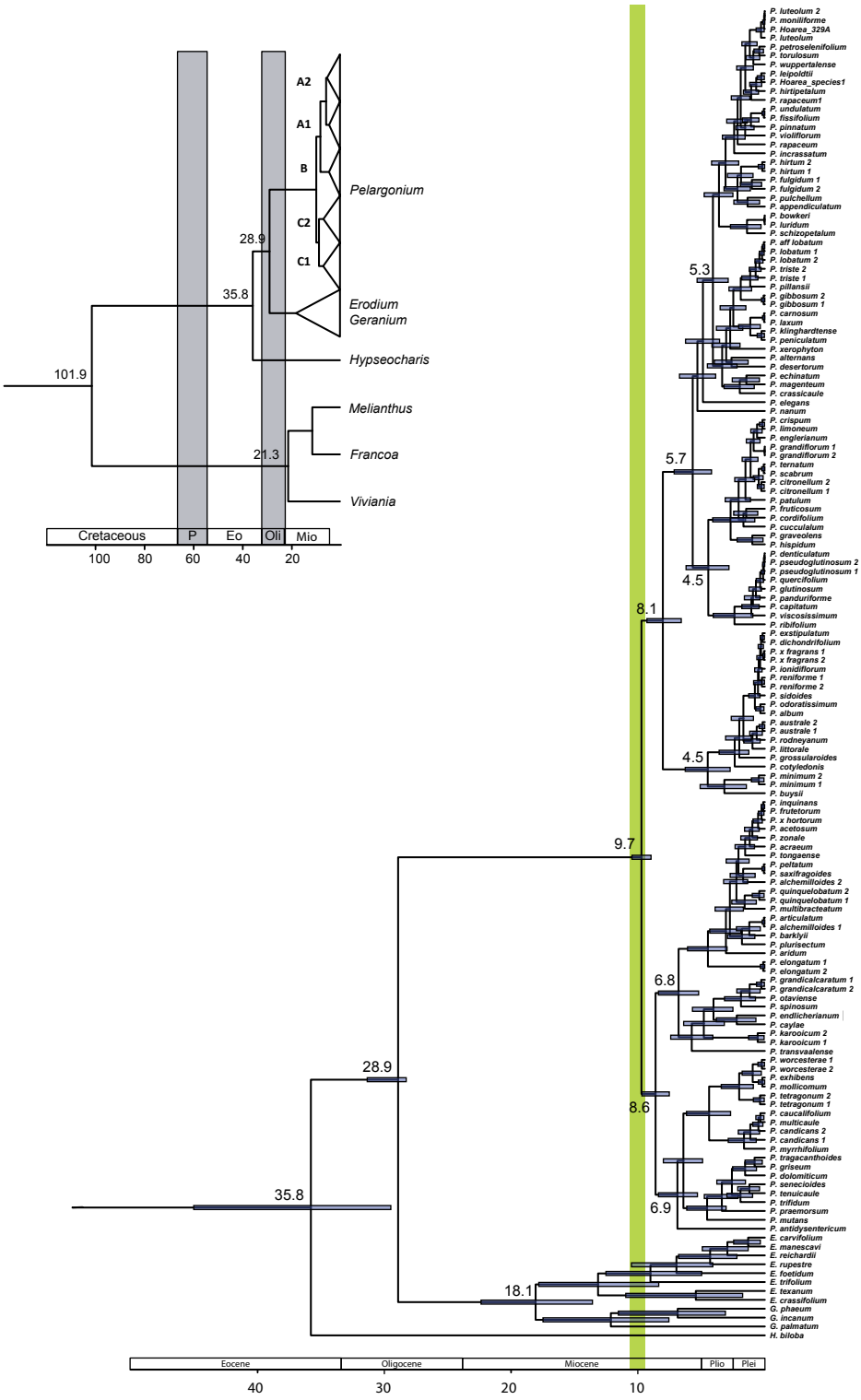

A2
A1
B
C2
C1
Pelargonium
Erodium
Geranium
Hypseocharis
Melianthus
Francoa
Viviania
28.9
35.8
101.9
21.3
Cretaceous
P
Eo
Oli
Mio
100
80
60
40
20
P. luteolum 2
P. moniliforme
P. Hoarea_329A
P. luteolum
P. petroselenifolium
P. torulosum
P. wuppertalense
P. leipoldtii
P. Hoarea_species1
P. hirtipetalum
P. rapaceum1
P. undulatum
P. fissifolium
P. pinnatum
P. violiflorum
P. rapaceum
P. incrassatum
P. hirtum 2
P. hirtum 1
P. fulgidum 1
P. fulgidum 2
P. pulchellum
P. appendiculatum
P. bowkeri
P. luridum
P. schizopetalum
P. aff lobatum
P. lobatum 1
P. lobatum 2
P. triste 2
P. triste 1
P. pillansii
P. gibbosum 2
P. gibbosum 1
P. carnosum
P. laxum
P. klinghardtense
P. peniculatum
P. xerophyton
P. alternans
P. desertorum
P. echinatum
P. magenteum
P. crassicaule
P. elegans
P. nanum
P. crispum
P. limoneum
P. englerianum
P. grandiflorum 1
P. grandiflorum 2
P. ternatum
P. scabrum
P. citronellum 2
P. citronellum 1
P. patulum
P. fruticosum
P. cordifolium
P. cucculatum
P. graveolens
P. hispidum
P. denticulatum
P. pseudoglutinosum 2
P. pseudoglutinosum 1
P. quercifolium
P. glutinosum
P. panduriforme
P. capitatum
P. viscosissimum
P. ribifolium
P. exstipulatum
P. dichondrifolium
P. x fragrans 1
P. x fragrans 2
P. ionidiflorum
P. reniforme 1
P. reniforme 2
P. sidoides
P. odoratissimum
P. album
P. australe 2
P. australe 1
P. rodneyanum
P. littorale
P. grossularoides
P. cotyledonis
P. minimum 2
P. minimum 1
P. buysii
P. inquinans
P. frutetorum
P. x hortorum
P. acetosum
P. zonale
P. acraeum
P. tongaense
P. peltatum
P. saxifragoides
P. alchemilloides 2
P. quinquelobatum 2
P. quinquelobatum 1
P. multibracteatum
P. articulatum
P. alchemilloides 1
P. barklyii
P. plurisectum
P. aridum
P. elongatum 1
P. elongatum 2
P. grandicalcaratum 1
P. grandicalcaratum 2
P. otaviense
P. spinosum
P. endlicherianum
P. caylae
P. karooicum 2
P. karooicum 1
P. transvaalense
P. worcesterae 1
P. worcesterae 2
P. exhibens
P. mollicomum
P. tetragonum 2
P. tetragonum 1
P. caucalifolium
P. multicaule
P. candicans 2
P. candicans 1
P. myrrhifolium
P. tragacanthoides
P. griseum
P. dolomiticum
P. senecioides
P. tenuicaule
P. trifidum
P. praemorsum
P. mutans
P. antidysentericum
E. carvifolium
E. manescavi
E. reichardii
E. rupestre
E. foetidum
E. trifolium
E. texanum
E. crassifolium
G. phaeum
G. incanum
G. palmatum
H. biloba
5.3
5.7
4.5
8.1
4.5
9.7
6.8
8.6
6.9
28.9
35.8
18.1
Eocene
Oligocene
Miocene
Plio
Plei
40
30
20
10

Figure 4. ***(previous page)*** **(**A) Time-calibrated phylogenetic tree of Pelargonium. Horizontal bars represent 95% highest posterior density (HPD) around mean node ages. Green line indicates Miocene-Pliocene climate change, used for ecological calibration (see text). (B) Cartoon style phylogenetic tree of Pelargonium showing outgroups used (dates are based on this study). Capital letters correspond to main clades: V=Viviania,F=Francoa,M=Melianthus,H=Hypseocharis biloba,G=Geranium, E=Erodium,P=Pelargonium.

We restricted our plastome-based analyses to predominantly protein-coding exon sequence data, ignoring fast-evolving spacer regions (i.e. the Plastome Exon and Intron (PIE) data partition). Spacer regions have been useful at the species level in *Pelargonium* and, for instance, the length variation present in the *trnL-F* regions (using indel coding) represented 20% of the phylogenetic signal (Bakker et al., 1999). However, the high frequency of rearrangements and indels observed in Geraniaceae plastomes (Guisinger et al., 2011) can confound homology assessment among sites in most spacer regions. Instead we relied here on the 6,305 informative characters residing in the 74 plastome exons and 10 corresponding introns, and expect that adding additional spacer regions or indel characters would not significantly alter the results. The resulting tree topology for Bayesian and maximum likelihood analyses were congruent and overall comparable to previous studies (Bakker et al., 2004; Röschenbleck et al., 2014; Weng et al., 2012).

Pelargonium species – level patterns

Although in some phylogenetic studies deeper nodes appear to be well-supported, studies using larger taxon sampling (e.g. Bakker & al. 2004) show poor support for these nodes, suggesting the high-support for deep nodes in these low taxon sampling studies is artefactual. The subgeneric split into a small and large chromosome clade was confirmed here (Bakker et al., 2004; Price and Palmer, 1993; Van der Walt et al., 1990; Weng et al., 2012). We found 100% bootstrap support for four of the five major clades. Support was low for clade A1 (75/70%) similar to all previous studies, challenging its validity . Clade A1 includes species from sect. *Pelargonium* that are characterised by shrub and sub-shrub life-forms making them a well-defined clade morphologically. This clade is the type section for *Pelargonium* (De Candolle, 1824; Röschenbleck et al., 2014; Sweet, 1822; van der Walt, 1985) but apparently its distinctness is not supported by DNA data in all cases.

The position of *P. nanum* has been a longstanding issue in *Pelargonium* phylogenetics (Bakker et al., 1999). *Pelargonium nanum* has floral and vegetative morphology more typical of species in clade B, with small, bicolored flowers and an annual habit (Röschenbleck et al., 2014). However, over the years, it has been proposed as part of clade A1 (Bakker et al., 2004), sister to clade A2 (Weng et al., 2012) or sister to the entire clade A (Röschenbleck et al., 2014). The inclusion of *P. nanum* prevented Markov Chain convergence in Jones et al. (2009), suggesting possible conflicting signals in the sequence. Our analyses agree with the findings of Weng et al. (2012) and place *P. nanum* sister to the rest of clade A2, albeit with poor support. This finding is in conflict with other studies (Bakker et al., 2004; Röschenbleck et al., 2014) that used plastome indel coding as well as rDNA ITS sequence data. Therefore, confirmation from additional nuclear genomic data is needed.

The placement of *P. karooicum* within section *Subsucculentia* based on *rbcL* data and chromosome number (*x* = 10) by van der Walt et al. (1995) has been problematic. Section *Subsucculentia* species were previously considered monophyletic based on a shared base chromosome number of *x* = 10 (van der Walt et al., 1995). This was in conflict with findings from previous cpDNA based phylogenetic studies that indicated *P. karooicum* (*x* = 10) is part of a clade including *P. quercetorum* (*x* = 17), *P. endlicherianum* (*x* = 17) and *P. caylae* (*x* = 9) (Bakker et al. 2004, 2000a; Röschenbleck et al., 2014) Our analyses place *P. karooicum* as sister to a small clade formed by these *x* = 9, 10 17 species, still making the *x* = 10 species paraphyletic. This unresolved placement, the shared base chromosome number with sect. *Subsucculentia* but similar morphology to sect. *Jenkinsonia* species and multiple ribotypes (Bakker, unpubl. data), suggests that *P. karooicum* might be the result of an ancient hybridisation event (Röschenbleck et al., 2014; van der Walt et al., 1995). In contrast to Röschenbleck et al. (2014), sections *Ligularia* and *Hoarea* are each monophyletic and we recovered increased resolution for the section *Ligularia*. This finding is important as the evolution of the formation of tunicate tubers coupled with a geophytic growth form in sect. *Hoarea* can now be studied in a proper sister-group context.

Although the existence of a *Polyactium-Otidia-Cortusina* clade has been disputed by Röschenbleck et al. (2014) and Weng et al. (2012), we find a highly supported (100%) *POC* clade that also includes *P. desertorum, P. alternans* and *P. xerophyton*. The latter three were designated 'unplaced' taxonomically within subgenus *Pelargonium* by Röschenbleck et al. (2014). *Pelargonium desertorum* as well as *P. xerophyton* have previously been assigned to section *Cortusina sensu stricto* (based on vegetative characters, Dreyer et al., 1992; Röschenbleck et al., 2014) and *P. alternans* for a long time has been part of the sect. *Otidia* based on its succulent stems (Röschenbleck et al., 2014). Rather than leaving them 'unplaced', we suggest restoring these species to their respective sections taxonomically and affirm the *POC* clade including *P. desertorum*, *P. xerophyton* and *P. alternans*. Upon further character and taxon sampling, it is possible that these species will be resolved in their respective *Otidia* and *Cortusina* clades.

Section *Campylia*, here represented by only *P. elegans*, appears to be sister to clade A2, which is in line with findings by Röschenbleck et al. (2014). However, as with *P. nanum* and its placement sister to clade A1, support for this finding is surprisingly weak. Again, data from different genomic compartments may help to clarify the phylogeny of this section. In addition, the inclusion of remaining species from the section is desirable since this will help to resolve the phylogenetic placement of the species in this section.

As in Röschenbleck et al. (2014), we retrieved the species *P. transvaalense*, *P. caylae*, *P. endlicherianum* and *P. karooicum* taxonomically unplaced as sister species to a clade formed by section *Subsucculentia*. We realize that phylogenetic patterns alone may be insufficient evidence to change existing taxonomic opinion and that corroboration from morphology and other evidence is necessary. With all the resources now at our disposal, it would be desirable to develop and classify all

known species in the genus *Pelargonium*, i.e. to avoid having unplaced species. This would mean having a broader concept for groups such as sect. *Subsucculentia*.

***Pelargonium* dating**

In our dating analysis, the influence of the 2nd calibration method on estimation of dates is apparent (Figure 3). In the analysis for the calibration methods separately, the age estimates resulting from this method are much older compared with the Fossil and Ecological calibration methods. In combination with Fossil evidence, there still is a heavy influence of the 2nd calibration method visible in the resulting dates. We consider these results with some hesitancy because of the known problems with dating analyses based on solely 2nd calibration (Schenk, 2016), such as "false impression of precision" and "age estimates shifting away from those based on primary calibration".

Compared with the influence of 2nd calibration, the influence of Fossil evidence on date estimates is much less evident. We expected larger uncertainty in the Fossil based age estimates since all available fossils correspond to clades that are rather distantly related to *Pelargonium*. For example, since the Vivianaceae fossil dated at ~10 Mya is on a relatively long branch from *Pelargonium* it could be expected to introduce considerable dating uncertainty.

The inclusion of ecological calibration (based on climatic data) seems to have a much larger influence on the age estimates. In the separate analysis, the results are in the same range as the Fossil based results. The combination with 2nd calibration caused the (otherwise much older date estimates) to be dramatically lowered.

Based on the total evidence scenario (which we prefer since it is most inclusive, Figure 4), our findings are similar to Fiz et al. (2008) and Palazessi et al. (2014) who estimated an age of 10 – 15 My old for the *Pelargonium* crown node age based on pollen fossils, but have lower estimated node ages than in Bakker et al. (2005) and Verboom et al. (2009). The latter estimated *Pelargonium* crown to be approximately 30-35 Mya, and *Pelargonium* was considered to be older than most CFR lineages included in that study. Our findings, however, indicate the *Pelargonium* crown node originated around 9.7 Mya, which would be consistent with the average age of Fynbos lineages of 8.5±1.85 Mya, and that of Succulent Karoo lineages of 5.17±0.64 Mya as inferred across CFR clades by Verboom et al. (2009). In our study the Xerophytic clade A2 crown node, harbouring many Succulent Karoo species, was dated 5.3 Mya, consistent with the radiations found for other typical Cape Floristic Region clades (Bouchenak-Khelladi and Linder, 2017; Hughes et al., 2015; Linder, 2008, 2003; Linder and Verboom, 2015). As suggested previously, the pattern of nested radiations in *Pelargonium* Winter-rainfall region clade A2 could be the result of a radiation in response to aridification in the mid-Miocene , in addition to the ensuing fragmentation of niches, and could be an explanation for the high number of growth forms found in *Pelargonium* (Bakker et al., 2005; Verboom et al. 2009).

These finding shed new light on the remarkable biogeographic disjunctions in Cape – non Cape sister species distributions found in *Pelargonium*. Several *Pelargonium* species, especially from clade C, occur in high-altitude East African regions, extending to Ethiopia and Asia Minor, and stemming from Eastern Cape affinities

(Bakker et al. 2005). These splits with such divergent distributions have all become much more recent compared with findings of Bakker et al. (2005). For example, the disjunction of *P. karooicum* (Cape) – *P. caylae* (Madagascar) – *P. endlicherianum* (Asia Minor) has now become as recent as ~5 Mya (early Pliocene). This and other occurrences of *Pelargonium* species outside the Greater Cape Floristic Region could be consistent with a 'Cape to Cairo' scenario as hypothesised for *Erica*, grasses and other clades in which the East African mountain range (starting from the Drakensbergen) provides a corridor across the equator (Galley et al., 2007). Whether the ancestral area for *Pelargonium* would have been inside or outside of the CFR remains unsolved.

CONCLUSIONS

Pelargonium phylogenetic relationships were estimated using a plastome-based data set including 74 plastid genes as well as the nuclear ITS region for 120 *Pelargonium* species, covering 43% of known species and 100% of known main clades. All species were retrieved within their expected major clade, i.e. consistent with previous phylogenetic studies. Resolution within clades has been increased compared to the last and most-inclusive study by Röschenbleck et al. (2014). We used different calibration approaches that have so far not been combined in one dating analysis yielding a crown node age for *Pelargonium* of 9.7 My, a much younger than previously expected. We present an improved, time-calibrated, phylogenetic framework for *Pelargonium* that can serve a diverse array of future studies. In particular we find the *Pelargonium* crown clade to be significantly younger than previously estimated, which makes it 'fit in' hypotheses of Fynbos and Succulent Karoo evolution much better. In order to arrive at a monophyletic section-level classification more sequence data from additional genomic compartments is needed. Ideally, a combination of population sampling and multispecies coalescent analysis (Kubatko and Degnan, 2007) yielding formal species trees would form the basis for such a classification.

ACKNOWLEDGEMENTS

The Bakker lab thanks Di Lei for her contribution to the DNA labwork and Verena Berchtold from the Botanical Garden in Zürich. C. D. Schlichting and C. S. Jones thank Cape Nature, the Northern Cape Province and the Eastern Cape of South Africa for permission to collect leaf samples. We also thank the Department of Botany, University of Cape Town, and especially Edwina Marinus at the Compton Herbarium, Kirstenbosch for logistical support. We thank Kerri Mocko, Hugo Martinez-Cabrera, Timothy Moore and Caroline Chong for their time spent collecting and preparing samples and voucher specimens. Support for this project was provided by a National Science Foundation Dimensions of Biodiversity grant (DEB-1046328) to C. D. S, C. S. J. and colleagues Kent Holsinger and John Silander. The Jansen lab thanks TACC at the University of Texas at Austin for access to supercomputers, Robin Parer from Geranicaeae.com for providing live plants of selected species and Plant Resources Center at the University of Texas at Austin for storage of voucher specimens. Support for this project was provided by a National Science Foundation grant (IOS-1027259) to R.K.J. and T.A.R. and the S. F. Blake Centennial Professorship to R.K.J.

Supplemental Files
All Supplemental Files are available under DOI: 10.1016/j.ympev.2019.03.021.

Chapter 3

Historical biogeography and ancestral conditions in *Pelargonium*

Sara J. van de Kerke[1,2], Merlijn Schram[1], M. Eric Schranz[1], & Freek T. Bakker[1]

Submitted

[1]Biosystematics Group, Wageningen University and Research, Droevendaalsesteeg 1, 6708 PB, Wageningen, The Netherlands
[2]Hortus Botanicus Amsterdam, Plantage Middenlaan 2A, 1018 DD, Amsterdam, The Netherlands

ABSTRACT

The Greater Cape Floristic Region (GCFR) is known for its hyperdiverse flora with extraordinary high levels of endemism and species richness. Although being a hyperdiverse region, the GCFR is dominated by a relatively small number of clades, one of which is *Pelargonium*. Biogeographic distribution patterns in *Pelargonium* are striking and complex and the ancestral geographic range of the genus is circumspect. Within *Pelargonium*, a number of long-range dispersal events towards Australia, St. Helena, Eastern Africa and Asia minor appear to have happened, predominantly within main clades B and C. The aim of this study is to infer the ancestral range of *Pelargonium* and assess whether dispersal and/or vicariance has been the cause of the current species distribution patterns. In addition, we infer ancestral environmental conditions. We find the ancestral geographic range of *Pelargonium* to be a range including the Winter and Summer rainfall region, the Karoo region, and the Natal region in South Africa. Long-range dispersal events appear to have occurred mainly in clade B, C1, and C2.

INTRODUCTION

The Greater Cape Floristic Region (GCFR) is known for its hyperdiverse flora with extraordinary high levels of endemism and species richness and has been named one of the six Floristic Kingdoms of the world (Born et al., 2007; Goldblatt, 1978; Goldblatt et al., 2000; Morrone, 2015; Takhtajan et al., 1986). The GCFR comprises two biomes: the Fynbos (containing heathy, small-leaved sclerophyllous evergreen shrubs) and the Succulent Karoo (containing leaf-succulent shrubs tolerant to extreme drought; Linder and Verboom, 2015). The unprecedentedly high numbers of species in this relatively small area (~9000 in 90.000 km^2) has been the subject of numerous studies (e.g. Barthlott et al., 2005; Verboom et al., 2009) and is attributed to high rates of speciation thought to be driven by adaptation to highly variable local environmental conditions resulting in distinct niches and combined with low extinction rates (Ellis et al., 2014; Goldblatt and Manning, 2002; Linder, 2003; van der Niet and Johnson, 2009). Although being hyperdiverse at the species-level, the GCFR is dominated by a relatively small number of plant clades (Linder, 2003). These so called 'Cape lineages' include *Disa* (Orchidaceae), *Erica* (Ericaceae), *Oxalis* (Oxalidaeceae), and *Moraea* (Iridaceae), *Aspalathus* (Fabaceae), *Cliffortia* (Rosaceae), *Restio* (Restionaceae), *Agathosma* (Rutaceae), *Senecio* (Asteraceae), and *Pelargonium* (Geraniaceae). *Pelargonium* is well known for its stunning floral and vegetative diversity across its ~280 species (Bakker et al., 2005, 1999; Jones et al., 2009, 2003; Linder, 2003; Linder and Verboom, 2015; Manning and Goldblatt, 2012; Nicotra et al., 2008; Röschenbleck et al., 2014; Struck, 1997). Reconstruction of phylogenetic relationships within the genus have recently been expanded and refined (Chapter 2; van de Kerke et al., 2019). Roughly 70% of the genus occurs in the GCFR (Linder, 2003; Manning and Goldblatt, 2012; Snijman, 2013), with other species occurring in Mozambique, Madagascar, Tanzania, Kenya, Ethiopia, Somalia, Yemen, Oman, Turkey, Namibia, and Australia (Dreyer et al., 1992; Van der Walt et al., 1990; Van der Walt and Vorster, 1983).

Distribution patterns in *Pelargonium* are striking and complex due to multiple disjunct sister-species distributions (Bakker et al., 2005, 1998). In addition, the ancestral geographic range is circumspect (Bakker et al., 2005, 1998). The majority of *Pelargonium* species occur predominantly in the GCFR, which has been hypothesised to be the centre of divergence for the genus (Bakker et al., 2005; Fiz et al., 2008). In addition, the ancestral lineage for Geraniaceae has been inferred to be in southern Africa (Fiz et al., 2008). However, the outgroup *Hypseocharis* is currently distributed in the South American Andes that started to form during the Oligocene (around 33 Mya; Fiz et al., 2008; Taylor, 1991). It has been hypothesised previously that the ancestral lineage of *Hypseocharis* and Geraniaceae had a wide distributional range over southern America, Antarctica, and southern Africa (F.T. Bakker, pers. comm.). The current pattern of distributions would then be the result of vicariance due to the breakup of Gondwanaland. Whereas the continental breakup started 145 Mya, the divergence between the two lineages is dated at only ~36 Mya (Chapter 2; van de Kerke et al., 2019). Therefore, the break-up of a widespread ancestor is increasingly unlikely. Another scenario for the arrival of *Pelargonium* in South Africa might be a (single) long-range dispersal event from South America, which could explain the disjunct distribution with *Hypseocharis* in the Andes. Such a scenario is not implausible considering the more recent history of *Pelargonium*.

Within *Pelargonium*, a number of long-range dispersal events appear to have happened, predominantly within main clades B and C. In clade B, the section *Peristera* escaped the GCFR east towards Australia/New Zealand as well as well as west onto the Atlantic ocean islands of St. Helena and Tristan da Cunha (Bakker et al., 2005). In main clade C, the distribution of *P. aridum* (eastern Cape region), sister species to *P. quinquelobatum* (east Africa) and *P. insularis* (Socotra) was hypothesised to be the result of the uplift of the East African Rift during the Pleistocene that acted as migratory corridor (Axelrod and Raven, 1978; Bakker et al., 2005). This disjunct distribution hypothesised for *Pelargonium* has been attributed to the so called 'East African highway' (Bakker et al., 2005). The uplift of the East African plateau during the mid-Miocene (~15 Mya; Linder, 2017) is thought to have provided a refuge for many plant lineages from the (sub)tropical climate of the African lowlands and deserts (Axelrod and Raven, 1978; Koch et al., 2006). In addition, these mountains acted as a corridor across Africa connecting the hyperdiverse GCFR with the north (Clark et al., 2011; Galley et al., 2007; Schwery et al., 2015).

The dated plastome-based phylogenetic tree (Chapter 2; van de Kerke et al., 2019) places these observations in a new light. First and foremost because the age of *Pelargonium* has become much younger (9.7 My instead of 30 My as estimated by Bakker et al. (2005)). Thus, the *Pelargonium* evolutionary history is now more congruent with the general pattern for GCFR lineages and the average age of other Fynbos lineages (8.5 ± 1.85 Mya; Verboom et al., 2009). In addition, the radiation of the Xerophytic A2 main clade (5.3 Mya, harbouring many Succulent Karoo species), is congruent with the inferred age of other Succulent Karoo lineages (5.17 ± 0.64 Mya; Verboom et al., 2009).
Second, van de Kerke et al. (Chapter 2; 2019) and Röschenbleck et al. (2014) find a number of subtle topological differences in the phylogenetic tree compared with earlier studies (Bakker et al., 2005) that are relevant for the historical biogeographical patterns of the genus. For example, the disjunct distribution of *P. caucalifolium – P. longicaule* (Cape) vs *P. whytei* (East Africa) and *P. tetragonum* (Cape) vs (*P. boranense*) as described by Bakker et al. (2005) is no longer applicable as they are no longer sister species (Chapter 2; van de Kerke et al., 2019). The same applies to the disjunction in the above-mentioned *P. aridum* (East Cape), *P. quinquelobatum* (Kenya), and *P. insularis* (Socotra) that no longer have a direct sister-relationship.

In order to interpret these historical biogeographical patterns and give meaning to the dispersal events that likely occurred, phyloclimatic studies are increasingly needed (Yesson and Culham, 2006a, 2006b). In phyloclimatics, the phylogenetic tree of a clade is combined with present climatic data for the terminals in order to infer ancestral states for these climatic conditions. This helps to shed light on past distributions and shifts therein. In combination with bioclimatic models, ancestral climate envelopes and distributions can be inferred which will aid our understanding of the clades resilience in current climatic changes (Keppel et al., 2012; Pearman et al., 2008). The aim of this study is to infer the ancestral range of *Pelargonium* and find out whether dispersal and/or vicariance has been the cause of the current species distribution patterns given the dated plastome study of van de Kerke et al. (Chapter 2; 2019). In addition, we infer ancestral climate to explore under what conditions shifts occurred.

MATERIAL AND METHODS

Ancestral geographic range reconstruction

We reconstructed ancestral geographic ranges using the program BioGeoBEARS (Matzke, 2014), which is event-based and offers the opportunity to include a 'jump dispersal' (J) parameter that treats dispersal as a cladogenetic process and that has been shown to have a significant effect on the likelihood of tested models (Matzke, 2014). Models included the following parameters: the dispersal, extinction, vicariance, and cladogenesis. We tested the DEC (Dispersal-Extinction-Cladogenesis; Ree and Smith, 2008), DIVA (Dispersal-Vicariance Analysis; Yu et al., 2010) and BayArea (Landis et al., 2013) models, with and without the J parameter. Likelihoods for different models are compared using AIC values. In addition to the ancestral state estimation we ran a biogeographical stochastic mapping analysis for the best fitting model (BayArea + J; Dupin et al., 2017) with 50 replicates. The purpose of this is to estimate the number and type of biogeographical events that led to the current species distribution of *Pelargonium*.

The division in floristic patterns in South Africa is complicated and thus we decided to follow Linder (2014, 2003). We identified 12 geographic areas (Figure 1) relevant for the distribution of *Pelargonium* and local climatic and geographical boundaries: (A) South African Winter Rainfall region, (B) South African Summer Rainfall region, (C) South Africa Karoo Region, (D) Natal, (E) Mozambique and Southern Malawi, (F) Tanzania, Kenya and Northern Malawi, (G) Ethiopia, (H) Madagascar, (I) Socotra, (J) Asia minor, (K) St. Helena, (L) Australia + New Zealand. We included South America in an exploratory analysis as geographic area for the outgroup *Hypseocharis*, but we decided to exclude this taxon and corresponding area from the analysis once we found it did not affect ancestral geographic range estimations (not shown). Areas are based on differences in seasonality in rainfall (A and B) and niche type (D). Area C is largely enveloped by the areas A, B, and D and corresponds to the Karoo region. No definite northern boundary was defined (gradient in Figure 1). Areas E, F, and G cover the eastern branch of the East African Rift system (EAR, Koptev et al., 2018; Linder, 2017, 2014), whereby E and F relate to the East African tropical lowland, and G to the Ethiopian highland. For areas E and F no western boundary was defined, corresponding with the colour gradient (Figure 1). Species distribution were obtained from the Global Biodiversity Information Facility (GBIF, www.gbif.org) and from primary taxonomic sources (see Supplementary Table S1) and were verified by hand to ensure accurate species distributions.

Phylogenetic relationships

We used the *Pelargonium* and *Hypseocharis* lineages from our time-calibrated phylogenetic tree (Chapter 2; van de Kerke et al., 2019) as the basis for our historical biogeographical study. We grafted species not present in our phylogenetic analysis, but with distribution data, halfway between their terminal sister-species based on previous phylogenetic and taxonomic findings (Bakker et al., 2005; Becker and Albers, 2009; Jones et al., 2009; Nicotra et al., 2016; Röschenbleck et al., 2014; van der Walt, 1985). When sister-species relations were not exactly known (i.e. were part of a polytomy), the species was included in the clade corresponding to that known polytomy in a sister group position with 0.01*branch-length distance. Otherwise,

the species was grafted to the base of the clade representing their taxonomic section *sensu* Röschenbleck et al. (2014; Supplementary File S2). We chose to include species in this way because subsequent historical biogeographic analyses require fully bifurcating phylogenetic trees. This resulted in two phylogenetic trees: (1) the original plastome phylogenetic tree (106 terminals) and (2) an extended phylogenetic tree including all species with georeferenced distribution datas (258 species). Analyses were performed using the packages 'phytools' and 'APE' in R (Paradis et al., 2004; Revell, 2012). Supplementary Figure S3 shows the two phylogenetic trees as used in this study.

Ancestral environment

We chose three environmental variables that are thought to correlate with many other environmental factors (T.M. Moore, pers. comm): Mean annual precipitation, mean annual temperature and elevation. Mean annual precipitation and mean annual temperature were selected because they give a good indication of the type of climatic conditions at the locality of the plant, and elevation because it highly correlates with general conditions on site and with potential pollinators in a generic way. We extracted these climatic variables for all georeferenced coordinates found for the current species distributions. We performed an ancestral state reconstruction using square change parsimony optimisation on our continuously-distributed characters in Mesquite v. 3.6 (Maddison and Maddison, 2018) to infer ancestral states for each of these variables.

RESULTS

Historical biogeographical model testing

Including jump dispersal parameter along with the dispersal, extinction, and cladogenesis parameters in the tested models, gave a significantly better model. The BayAreaJ model (containing dispersal and allowing for jumps, LnL = -724, P = 1.20E-10) was the best model for both phylogenetic trees. The inferred ancestral geographic range estimation for almost all nodes are identical for BayArea and BayAreaJ. Figure 1 shows the 258-terminal phylogenetic tree showing ancestral geographic range estimations under the BayAreaJ model at selected nodes. Ancestral geographic range for the core and extended phylogenetic trees gave corresponding results. We based all subsequent ancestral geographic range discussions on the extend phylogenetic tree constructed on the BayAreaJ model (Figure 1 and 2).

The 50 biogeographical stochastic mappings (BSM) in BioGeoBEARS provided probability distributions across the branches of the extended phylogenetic tree for each of the different cladogenetic events. Given the parameters of this model, 91.98% of cladogenetic events involve sympatry, 0% involve vicariance, and 8.02% involve jump-dispersals (Figure 3).

Biogeographical patterns

When we include the geographic area of outgroup *Hypseocharis* in the biogeographical analysis (South America), the ancestor of the two lineages is

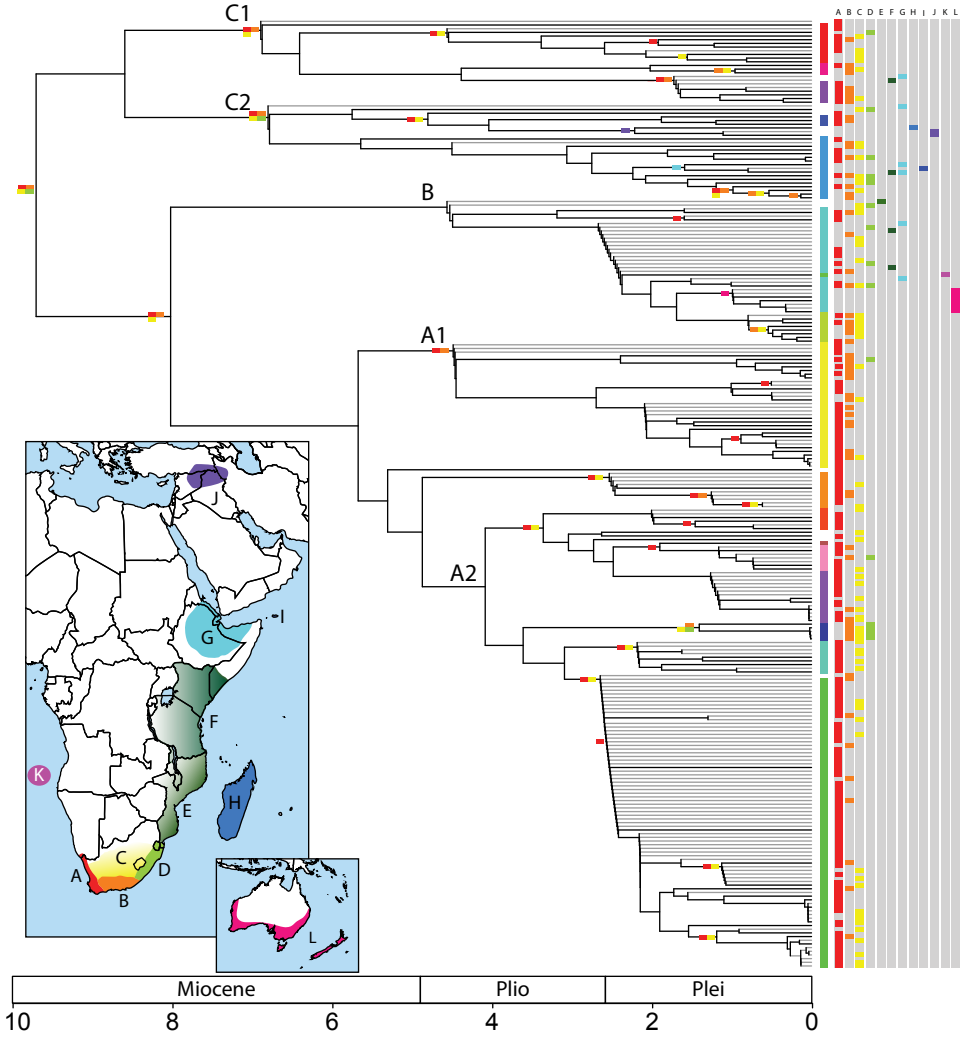


Figure 1. Historical biogeographical history of *Pelargonium*. Grafted phylogenetic tree of *Pelargonium* based on plastome core by van de Kerke et al. (2019; with core terminals in black and grafted terminals in grey) showing estimated ancestral history of the genus. Coloured blocks refer to areas as defined for this study and are shown on map.

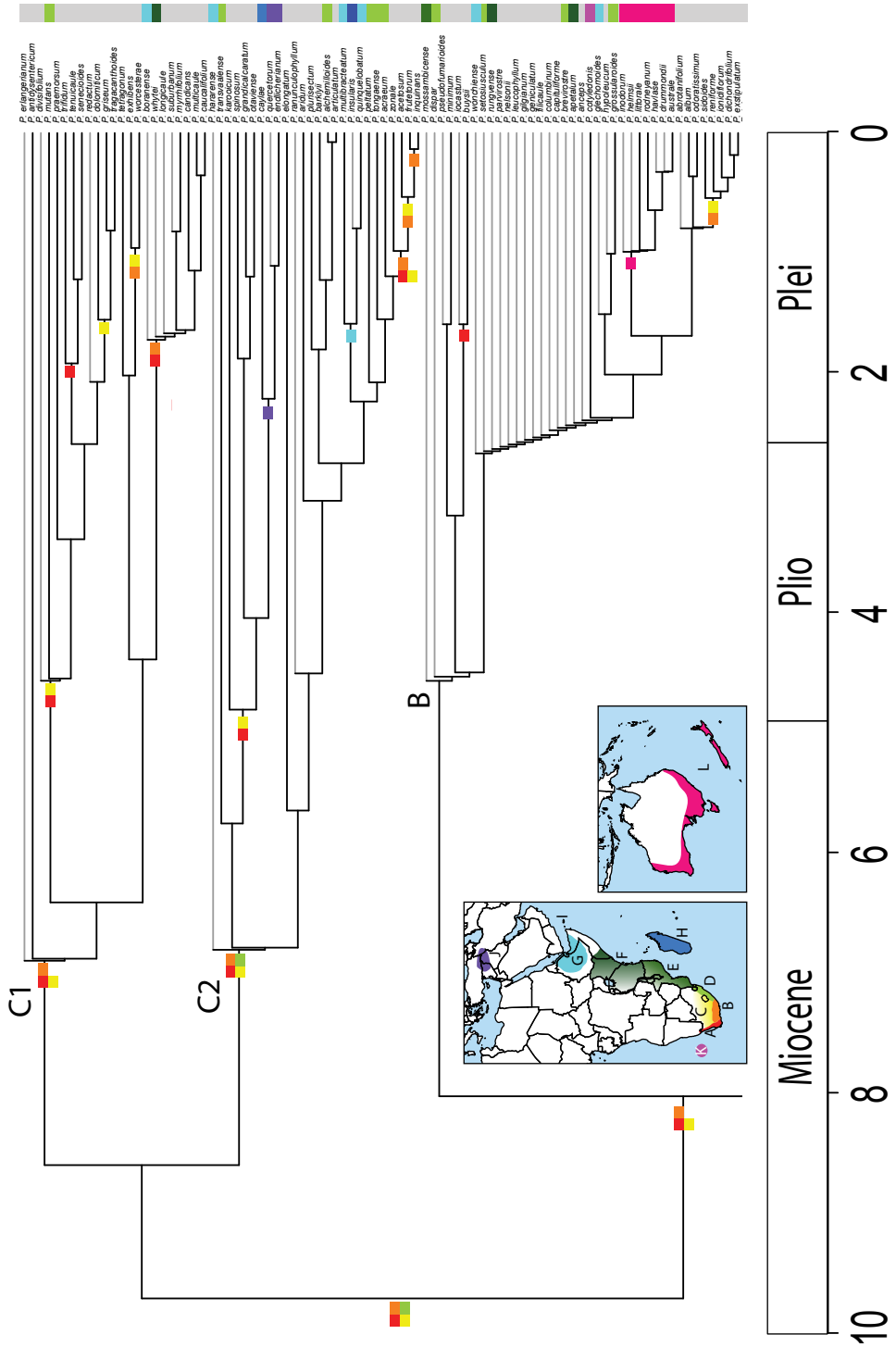

C1
C2
B
Miocene
Plio
Plei
10
8
6
4
2
0
A
B
C
D
E
F
G
H
I
L

Figure 2. ***(previous page)*** Historical biogeographical history of *Pelargonium* for main clades C1, C2, and B. Grafted phylogenetic tree of *Pelargonium* based on plastome core by van de Kerke et al. (2019; with core terminals in black and grafted terminals in grey) showing estimated ancestral history of the genus. Coloured blocks refer to areas as defined for this study and are shown on map.

estimated to have occurred in an area encompassing all of South America, the South African Winter Rainfall region, the South African Summer Rainfall region, the South Africa Karoo Region, as well as the South Africa Eastern Cape (not shown). Overall, the majority of species reside in a combination of the South African Winter and Summer Rainfall region, and the South Africa Karoo Region (Figure 1).

Distribution patterns in Clade A are quite conservative. An overwhelming majority of species occurs in the South African Winter Rainfall region, sometimes in combination with the South African Summer Rainfall or Karoo region. Only in a few occurrences a species is only present in one of the latter areas. The taxonomic section *Magnistipulacea,* in particular appears to have 'escaped' the South African Winter rainfall region and moved into the South African Eastern Cape region (Figure 1).

In the diversification of the main clade with the oldest crown node within *Pelargonium*, clade C, multiple shifts from this ancestral range into the East African Highway and Asia Minor have occurred: *Pelargonium mutans* into the South African Eastern Cape area, *P. whytei* into the Tanzania, Kenya and Northern Malawi area, *P. boranense*, *P. multibracteatum* and *P. hararense* to the Ethiopia area, *P. caylae* to Madagascar, *P. quercetorum* and *P. endlicherianum* into Asia minor, and *P. quinquelobatum* into both the Tanzania, Kenya and Northern Malawi area as well as the Ethiopia area (Figure 2).

Species from clade B also predominantly occur in the South African CFR regions (Figure 2). This clade is dominated by two jump dispersal events towards the Australia + New Zealand area by species from sect. *Peristera* (1 Mya in the Pliocene) and an independent event towards St. Helena by *P. cotyledonis* (3 Mya in the Pleiocene).

Figure 3 shows the flow and direction between areas as calculated by BioGeoBEARS (founder events per area, Supplementary Table S4). A relatively high number of events occurred based on areas A, B, and to a lesser extent C. We considered this to be driven by the comparatively high number of species in this area. We find there is a lot of flux of species from area A into all other areas, but there is also movement of species back into area A from area B and C. Dispersal into areas E, F, and H also appear to have occurred from A, B, and C. The event of *Pelargonium* arriving in Socotra (I) we find has probably happened out of Ethiopia (G). Dispersal into Madagascar (H) is more complex because we find influx from multiple sources as plausible (A, C, and/or J). The pattern of the ancestral geographic range reconstruction with an event out of Asia Minor by *P. caylae* is corroborated, but there also is a chance of movement in the other direction.

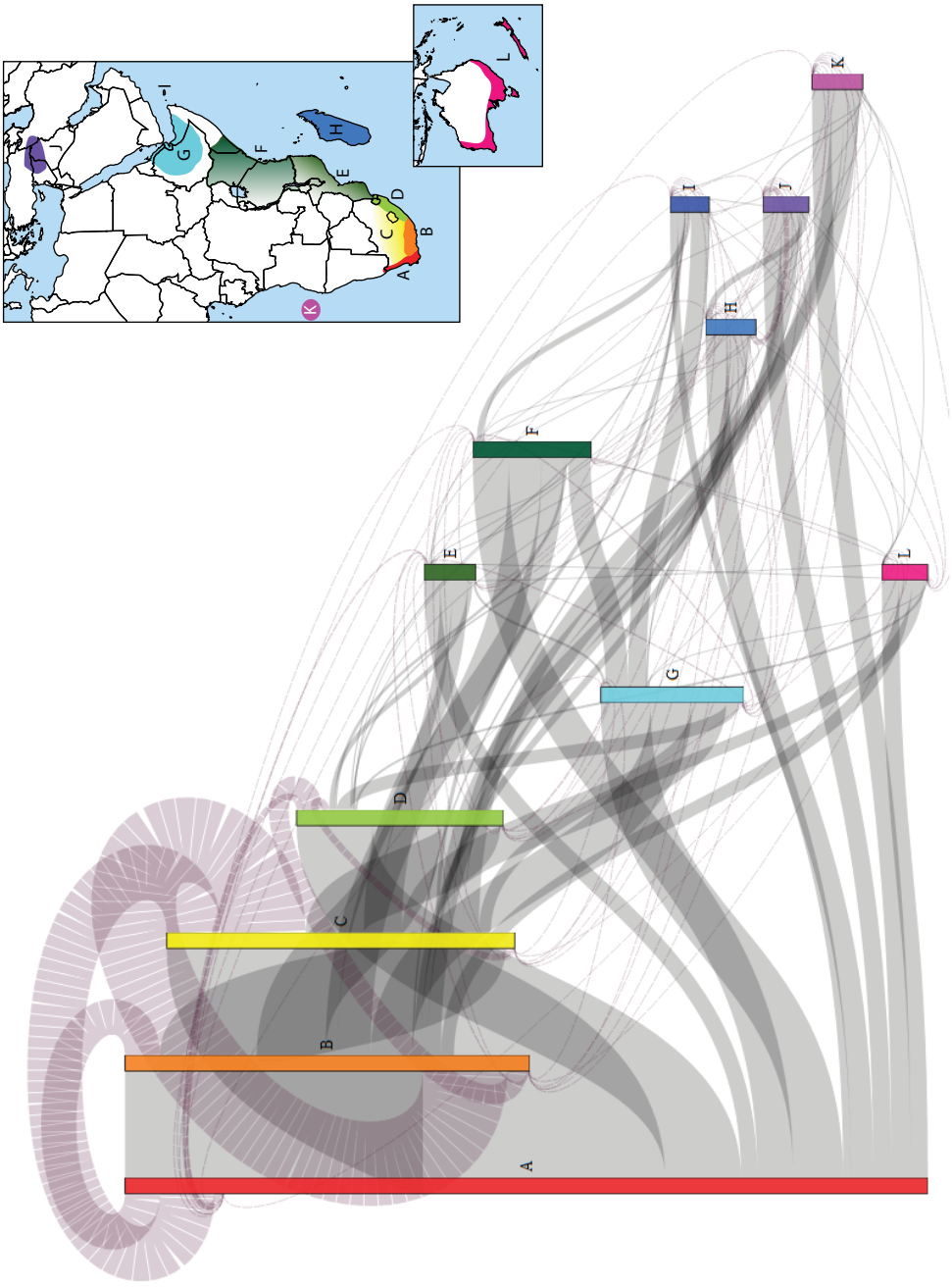

A
B
C
D
E
F
G
H
I
J
K
L

Figure 3. ***(previous page)*** River plot summarising dispersal events estimated with stochastic mapping. Lines correspond with size and direction of flow between areas as defined for this study and shown on map (see Supplementary Table S4).

Ancestral environment

We reconstructed ancestral conditions for mean annual temperature, mean annual precipitation, and elevation for all *Pelargonium* species present in the extended phylogenetic tree using georeferenced occurrence data obtained from GBIF. We infer the ancestral lineage leading to *Pelargonium* to have occurred at between ~600 – 900 m elevation, around ~18°C, and around 550 mm annual precipitation (Figure 4). Most clades quickly shifted to lower temperatures (~13°C, Figure 4B), and lower precipitation (~400 mm, Figure 4C) while estimated elevation remains similar with a few jumps of major clades to lower altitudes (~300 – 600 m, Figure 4A). The overall pattern for elevation seems to be that for the deep nodes conditions are stable between 300 – 900 m. Within clades, there are jumps of species shifting to either lower (19 – 300) or higher altitudes (900 – 3250 m). Switches are spread evenly over the phylogenetic tree.

With respect to temperature, the ancestral lineage for clade C1 and C2 is inferred to have remained the same as for the ancestral *Pelargonium* lineage (~18°C, Figure 4B). The other clades have shifted to a cooler temperature of ~13°C (Figure 4B), which has remained steady for most lineages in *Pelargonium*. Again, we find multiple transitions towards either cooler (7 - 13°C) or warmer (18 – 26.5°C) climates.

Precipitation levels have shifted much more in our ancestral state reconstruction in comparison with elevation and temperature. Conditions for the ancestral lineage of clade C1 and C2 are inferred to have remained the same as the ancestral *Pelargonium* lineage (~550 mm annual precipitation) while conditions for the ancestral lineage of all other clades have shifted to ~400 mm. Especially in clade A1 and A2, a large number of switches towards even lower precipitation levels (39 – 300 mm annually) happened while in clade C1, C2, and B relatively more species switched to wetter climates.

DISCUSSION

We find the ancestral geographic range of *Pelargonium* to include the Winter and Summer rainfall region, the Karoo region , and the Natal region of South Africa and which started to diverge around 9.7 Mya (Chapter 2; van de Kerke et al., 2019). This diversification coincides with the average age for other Fynbos lineages (Verboom et al., 2009) and hence provides additional support of these general patterns. The climatic conditions underlying the current Mediterranean type climate were already established at that time (Axelrod and Raven, 1978; Bakker et al., 2005).

Including the outgroup lineage *Hypseocharis* in the analysis simply stretches the ancestral range estimation to include the Andes region and seems to suggest a common ancestor on Antarctica, as has been inferred for Palms (Baker and Couvreur, 2013). However, an ancestral *Pelargonium* lineage on Antarctica seems to be highly unlikely given the crown clade estimate age of 9.7 Mya (Chapter 2; van de Kerke et al., 2019). Although the African, South American, and Antarctican continents where separated ~100 Mya (Jokat et al., 2003), Antarctica only became fully covered in ice

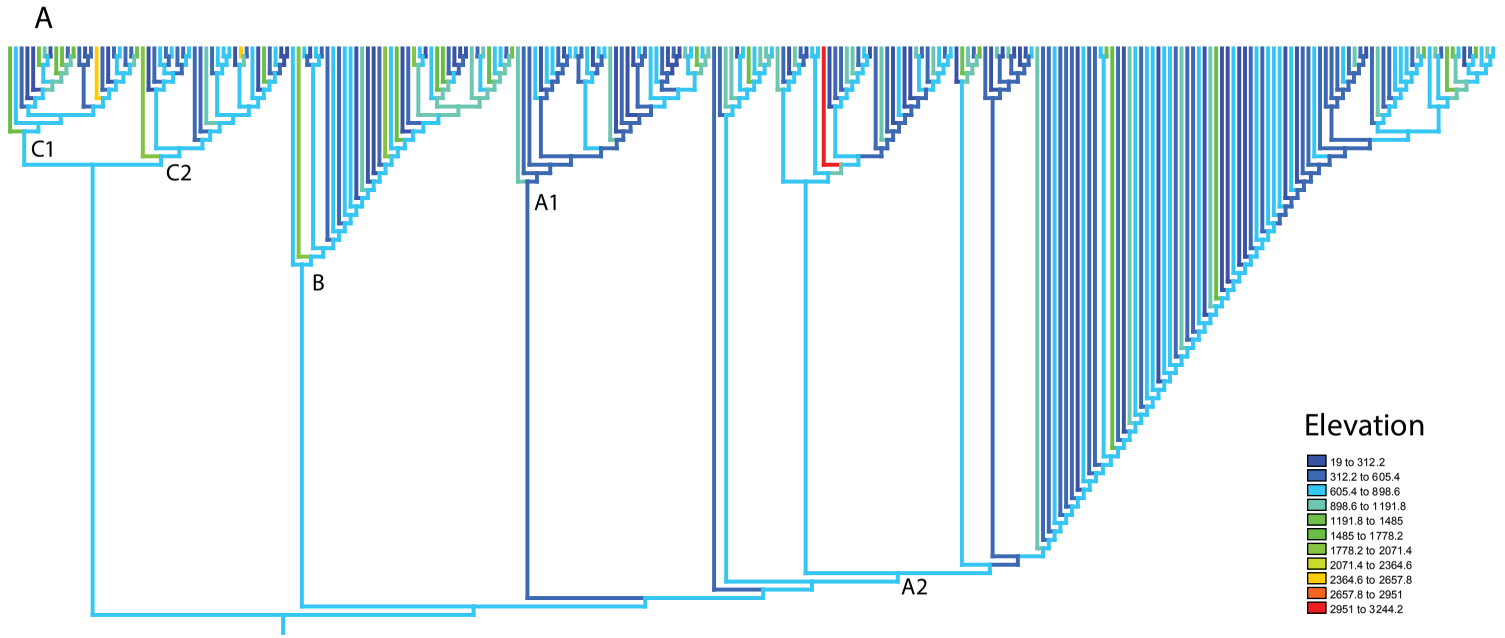
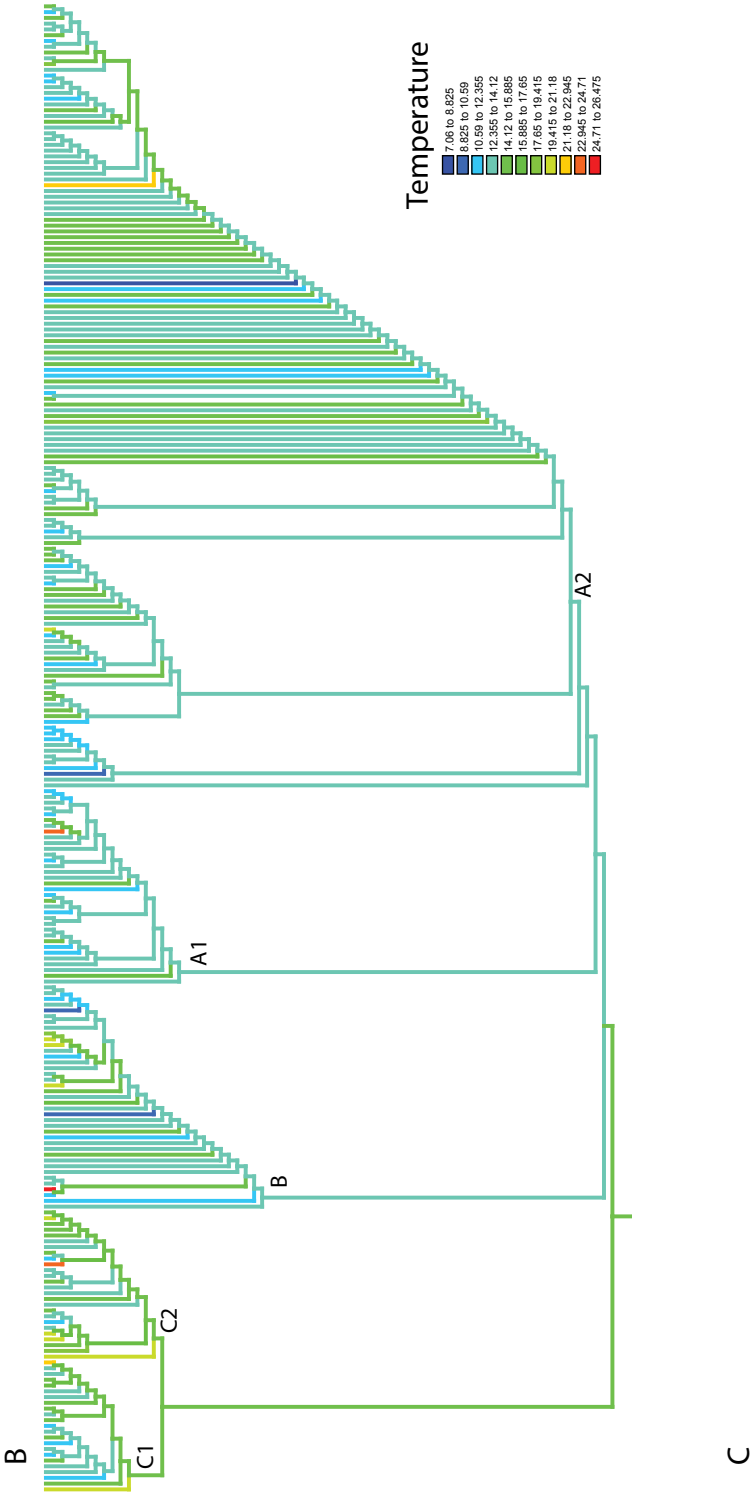
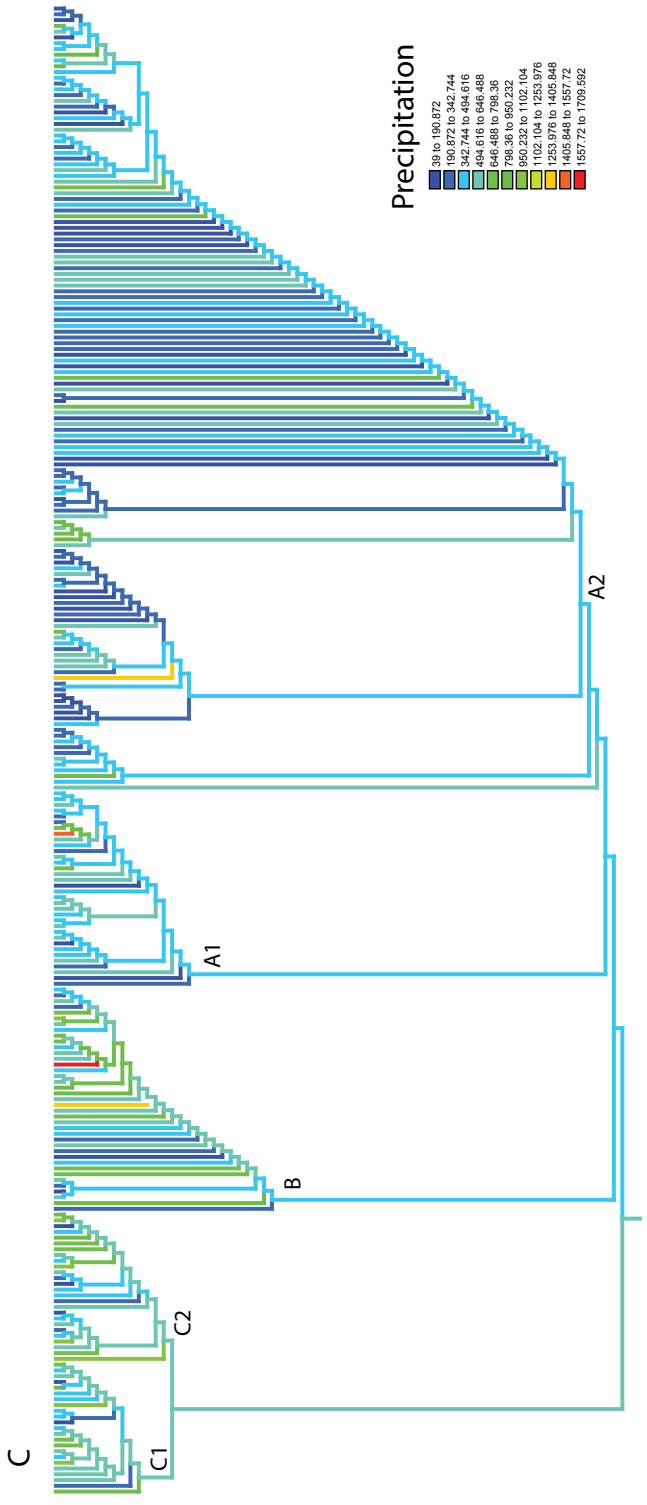


Figure 4. ***(continues on next pages)*** Parsimony reconstruction of mean elevation (A), mean annual temperature (B), and mean annual precipitation (C) over the *Pelargonium* phylogenetic tree performed in Mesquite. Labels refer to main clades as defined in *Pelargonium*.


Temperature
7.06 to 8.825
8.825 to 10.59
10.59 to 12.355
12.355 to 14.12
14.12 to 15.885
15.885 to 17.65
17.65 to 19.415
19.415 to 21.18
21.18 to 22.945
22.945 to 24.71
24.71 to 26.475
A2
A1
B
C2
C1
B
C


Precipitation
39 to 190.872
190.872 to 342.744
342.744 to 494.616
494.616 to 646.488
646.488 to 798.36
798.36 to 950.232
950.232 to 1102.104
1102.104 to 1253.976
1253.976 to 1405.848
1405.848 to 1557.72
1557.72 to 1709.592
A2
A1
B
C2
C1
C

around 35 Mya (Carter et al., 2017). Since this is congruent with the divergence date of *Hypseocharis* and the lineage leading to the Geraniaceae, a long-range event during the Oligocene of an ancestral *Pelargonium* or Geraniaceae lineage from Antarctica into the Cape region is still possible and would coincide with the arrival of Iridaceae lineages during a mediterranean type climate period (Linder, 2003).

Pelargonium as a clade started to diversify around 9.7 Mya. Climatic conditions in the Late Miocene (i.e. 11.63 to 5.3 Mya) were comparable with modern conditions after having gone through major changes during the Early Miocene (Linder, 2003). At that time, climatic conditions globally became tropical and a concomitant vegetation with palms established in the CFR (Linder, 2003). During the Middle and Late Miocene, these tropical conditions were replaced by the Mediterranean type climate still seen today. The upwelling of the Benguela current around 10 Mya, accentuated by the glaciation of Antarctica during that time, coincided with offshore blowing trade winds (removing moisture from land) and the southern Atlantic pressure cell that limits moist airflow, resulted in aridification. This formed the current east-west rainfall gradient. Around the same time (10 Mya) the Great Escarpment lifted in the central southern African plateau. The eastern margins (Drakensbergen) raised more than the margings in the west (Cederbergen), and the east-west rainfall gradient became more pronounced. 'Consequently the eastern CFR (windward slope of the escarpment mountains) would have become wetter, while summer aridity in Namaqualand and the succulent Karoo (leeward side) would have been enhanced' (Linder, 2003). Our imaginary ancestral *Pelargonium* could then have come out of a mountain refuge, where the moderate Cape flora established in the Early Miocene had retreated from the tropical conditions (van der Niet and Johnson, 2009). This coincides with the inferred elevation of the ancestral *Pelargonium* lineage, which is found to be between ~600 – 800 meters (Figure 4A). The inferred mean temperature for the ancestral lineage is relatively low (~18°C), which is congruent with a cooler, mountainous climate (Figure 4B). This scenario is congruent with those suggested for other Cape lineages, as for example Restionaceae (Linder and Hardy, 2004).

The ancestral geographic range estimation (Winter and Summer rainfall region, the Karoo, and the Natal) is congruent with the current distribution of *Pelargonium* species, not surprisingly especially for clade A1 and A2 of which an overwhelming majority of species occur in the Winter and Summer rainfall regions and the Karoo. These clades were referred to previously as the "Winter Rainfall region clade" and are characterised by a wide array in life forms including woody shrubs, stem succulents, geophytes, and herbaceous annuals (Bakker et al., 2005; Figure 1). Only the small clade corresponding with the section *Magnistipulaceae* (a.o. *P. luridum* and *P. caffrum*) appear to have escaped the Winter rainfall region and migrated to the Natal region on the South African East coast. This coincides with higher levels of precipitation we find for the clade. The *Magnistipulaceae*, as well as the section *Polyactium* occurring in the Winter rainfall region, was inferred to have shifted to hawkmoth pollination (Bakker et al., 2005), with its concomitant night-scentedness and dull petal colours. The competition in pollinator accessibility between these two clades might well have resulted in this geographic split.

The clade B species are well based in the ancestral range of Winter - Summer rainfall and Karoo regions, although we find more disjunct distributions than in clade A. Whereas clade A species have a solid base in the Winter rainfall region and from there seem to expand into other regions, clade B species more often occur in sub-areas of the ancestral range. A number of species have escaped the ancestral range altogether, moving into Mozambique (E; *P. mossambicense*), Tanzania, Kenya and Northern Malawi (F; *P. rungvense* and *P. apetalum*), Ethiopia (G; *P. wonchiense* and *P. glechomoides*), and most notably St. Helena (K; *P. cotyledonis*) and Australia + New Zealand (L, i.a. *P. australe*, *P. littorale*, and *P. rodneyanum*). For most of these species in this main clade, we can only note that they have a disjunct distribution but cannot go any further because they reflect species that are grafted to the phylogenetic tree and actual sister-species relations are unknown (grey lineages, Figure 2). Only the distribution patterns of *P. cotyledonis* and the 'Australia' clade are based on phylogenetic analyses, but how these relatively close species dispersed in such opposite directions remains elusive. Long-range dispersal by birds seems unlikely since *Pelargonium* does not bear any appetising fruits. A scenario of *Pelargonium* seeds sticking to the fur of prey eaten by birds and deposited either on St. Helena or in Australia is similarly farfetched. More plausible could be a scenario whereby a combination of thermodynamics and wind currents drifted *Pelargonium* seeds across the respective oceans (Bakker et al., 1998; van der Does et al., 2018).
The oceanic island endemic *P. cotyledonis*, occurring on St. Helena (K, ~2.5 Mya) has long been hypothesised to have 'reverted back' to a symplesiomorphic *Geranium* flower shape, having lost its characteristic *Pelargonium* zygomorphy and nectar spur. However, we found that other *Pelargonium* species have highly similar floral shapes to *P. cotyledonis*, undermining its presumed uniqueness (Chapter 2; van de Kerke et al., 2019). Apparently, the ancestral lineage of *P. cotyledonis* arriving on St. Helena has not been in the position to diversify on the island (although two forms have been recognised within this species) and until now remains the only species in the *Isopetalum* section (Röschenbleck et al., 2014).
Similar to the dispersal of *P. cotyledonis*, but around ~1.5 Mya, an ancestral lineage dispersed east toward Australia. Contrary to *P. cotyledonis*, this lineage did diversify in the new area (Bakker et al., 1998; Nicotra et al., 2016). Arguably, the climatic conditions this clade found itself in are rather similar to those of the South African Cape region, which is generally corroborated in the ancestral climate reconstruction. Radiation into this new biome does not appear to have happened on a large scale. The notable exception is *P. helmsii*, which occurs in an area with high precipitation levels. This would suggest that the ancestral lineage dispersing to Australia, which indeed escaped from the ancestral geographic range of Winter - Summer rainfall and Karoo regions, was not able to adapt to other environmental circumstances.

Similarly disjunct as the species distribution of clade B is that of clade C, which is the earliest diverging lineage and has the oldest crown-node age (~9 My) of the *Pelargonium* main clades. Also, the most shifts relative to the estimated ancestral range of Winter - Summer rainfall, Karoo and Natal regions have occurred within this clade. The first of these is the loss of the Natal region in the ancestral geographic range reconstruction for the entire C1 main clade, with only one escapee (*P. mutans*) into the Natal area. Most of the remainder of the shifts in this clade are within sub-

areas of the estimated ancestral range of Winter - Summer rainfall, Karoo and Natal regions, with distributions often becoming increasingly narrower.

The disjunct distribution patterns of *P. karooicum* (Winter rainfall area), *P. caylae* (Madagascar), and *P. endlicherianum – P. quercetorum* (Asia minor) has previously been attributed to the uplift of the East African plateau (Bakker et al., 2005). The distribution of this group of species was thought to suggest a step-like spread of species from the Cape region, over de Drakensbergen in the Natal region, over the East African plateau into Ethiopia and onwards to Asia minor via Yemen. The timescale this was hypothesised to have occurred at was much larger and allowed for extinct species 'filling the gaps' between the previously mentioned three steps (this disjunction was then dated at 18 Mya, Bakker et al., 2005). In light of the more recent dating analysis by van de Kerke et al. (Chapter 2; 2019) who found the diversification of *P. karooicum* to be ~5 Mya and the *P. caylae* (Madagascar) vs *P. endlicherianum - P. quercetorum* split around ~ 2.5 My, this scenario is now unlikely. The uplift of the East African plateau took placing during the middle Miocene and would have been well established around the divergence times of these *Pelargonium* species (Linder, 2017). Contrary to previous findings, of a step like dispersal from Cape to Madagascar to Asia Minor, our results suggest *P. caylae* dispersed back from Asia Minor to Madagascar around ~ 2.5 Mya (Figure 2) since we find the ancestral geographic range of *P. caylae* (Madagascar) vs *P. endlicherianum - P. quercetorum* to be Asia Minor.

The disjunct dispersal hypothesised for *P. aridum* (East Cape), *P. quinquelobatum* (Kenya), and *P. insularis* (Socotra) as suggested by Bakker et al. (2005) is not supported by the plastome data (Chapter 2; van de Kerke et al., 2019). Instead, we now encounter a clade including ((*P. quinquelobatum*, *P. multibracteatum* (Ethiopia)), and *P. insularis* (Socotra)) for which the ancestral geographic range is found to be Ethiopia (*P. aridum* is now in a sister-species position at the base of the clade). A single long-range dispersal event would then underlie the jump from the Cape region into Ethiopia around 2 Mya, from where a lineage leading to *P. insularis* would have dispersed onto Socotra around 1 Mya. The inclusion of other *Pelargonium* species occurring in this region and their correct placement in the core phylogenetic tree (as well as that of *P. hararense*) will undoubtedly shed more light on these dispersals. Unfortunately, pollinators for these species are unknown and therefore we cannot find an explanation for the dispersal events in that area. However, the seeds in sect. *Ciconium* are reported to be rather sticky and the species make for notoriously good cuttings which opens the possibility of hitchhiking with an unknowing carrier (F.C. Breman, pers. comm.).

The other species disjunctions previously hypothesised for *Pelargonium* (*P. caucalifolium – P. longicaule* (Cape) vs *P. whytei* (East Africa) and *P. tetragonium* (Cape) vs (*P. boranense*) (Bakker et al., 2005) similarly are not supported by our analysis. This is due to topological shifts in the plastome phylogenetic tree, but also potentially because a number of them were not included in the plastome phylogenetic analyses and were grafted to the phylogeny (Figure 2, grey terminals). Although we have made an effort to phylogenetically place these species as accurately as possible in the grafted phylogenetic tree, relationships inferred in other studies where not always fully resolved (Bakker et al., 2005; Röschenbleck et al., 2014). However, contrary to the study by Bakker et al. (2005) we retrieve *P.*

boranense and *P. whytei* in the same clade, dated ~1.5 Mya. We hypothesise their distribution to be the result of a single dispersal event, but in this case (given the long, empty branch leading to this clade) a step-like distribution with now extinct species spreading from the Cape over the Drakensbergen into the East African plateau would be equally plausible.

Disjunct distribution and migration patterns have been inferred for numerous African plant clades. Sanmartín et al. (2010) found that while exchange between northern and southern Africa appears very low, the migration that took place seems to have gone through the east. Ali et al. (2013) found several dispersal events in the Hyacinthaceae subfamily Urgineoideae from South Africa to eastern Africa. These patterns match well with our inferences of *Pelargonium*. It is suggested that the Pleistocene uplift in Africa might have formed a corridor through and over the tropical forests that acted as a barrier (Axelrod and Raven, 1978). Bellstedt et al. (2012) identified five migrations from south-west to north-east Africa in the plant family Zygophylloideae that can be explained by a migration corridor in eastern Africa. Chala et al. (2017) showed that drought-tolerant plant species inhabiting alpine environments could have used grassland and forest present in the Pleistocene to cross otherwise inhabitable mountain ridges. More evidence of an Arid belt across eastern Africa was collected by Jürgens (1997), who demonstrated that floral habitats show patterns of fragmentation, suggesting that what was once an arid migration corridor has now broken up into several habitable ranges. It is possible that the trail of *Pelargonium* species across Eastern and Northern Africa have been similarly divided by habitat fragmentation in an Arid belt.
Jump dispersal is thought to be the cause behind the North-Eastern Euryops species (Devos et al., 2010). These species, however, form a monophyletic group, and this is not the case with the Ethiopian *Pelargonium* species. Several dispersal events in the same direction suggest a pattern, which means that these events were possibly facilitated in some way by external factors. One possible influence on *Pelargonium* dispersal could therefor well be the East African Highway (Bellstedt et al., 2012).

We find that long range dispersal seems to be the prime cause of the disjunct distribution patterns in *Pelargonium*, both to Australia, Madagascar, along the East African Highway, and into Asia minor. Long distance dispersal of plants to Australia is not rare (Bergh and Linder, 2009). Crisp and Cook (2013) studied 85 Australian plants clades and found that 48% arrived in Australia through long-distance dispersal. Concordant dispersal patterns, dispersal facilitated by various abiotic factors, are also present in plants (Sanmartín and Ronquist, 2004). The expansion into New-Zealand may be explained by Trans-Tasman winds (Pole, 2001).
There are multiple ways for a plant to disperse over long distances (wind, water, fur or feathers, and droppings) and the general dispersal ability of a plant can often be linked to seed morphology (Heleno and Vargas, 2015). However, seed morphology has been found not to be a reliable predictor of long range dispersals. Rather, long range dispersals in plants are often attributed to different methods than the 'standard' for the clade (Heleno and Vargas, 2015; Higgins et al., 2003; Myers et al., 2004; Nathan, 2006). In addition, unpredictable and rare dispersal mechanisms can be a cause of these chance, long range, dispersals. Extreme weather can carry

seeds, even those that are not normally dispersed by wind, much further than normal (Heleno and Vargas, 2015; Nathan et al., 2008; Waters and Roy, 2004). Extreme weather may even offer the benefit of disturbing the environment it deposits the seeds in, giving the invading species an advantage (Wu et al., 2018). In addition, natural rafts can be carried by ocean currents while preserving the seeds they carry. It has been shown for Urticaceae that non-germinated seeds can survive in sea water long enough to travel long distances to isolated islands (Wu et al., 2018). These could all have been options for *Pelargonium*, although general durability of *Pelargonium* seeds in these rough (wet) conditions has not been studied.

One disadvantage of dispersal through extremely rare events is that it often takes multiple seeds for a species to settle, especially if plants are not self-compatible, or dioecious (De Waal et al., 2014; Wu et al., 2018). While the focus in historical biogeography studies tends to lie on the journey to different locations, the establishment of a species in a new location can be just as challenging. In *Pelargonium* sect. *Peristera* the species that have diverged in Australia are small weeds that can colonise new regions relatively easily and have been found to have increased levels of self-compatibility, a strategy often used in newly-diverged species to ensure establishment (Bakker et al., 1998; Nicotra et al., 2016).

CONCLUSION

We find the ancestral geographic range of *Pelargonium* to be a range including the Winter (A) and Summer (B) rainfall region, the Karoo region (C), and the Natal (D) region in South Africa. The inferred environmental conditions for *Pelargonium* concur with existing knowledge on local climatic conditions (Linder, 2003). A long-range event during the Oligocene of an ancestral *Pelargonium* and/or Geraniaceae lineage from Antarctica into the Cape region (Linder, 2003) is still possible based on our data and reconstructions. The ancestral geographic range estimation is congruent with the current distribution of *Pelargonium* species, especially for the species rich clades A1 and A2. Dispersal events appear to have occurred mainly in clade B, C1, and C2. Understanding the *Pelargonium* biogeographic history enables researchers to understand trait evolution within the genus.

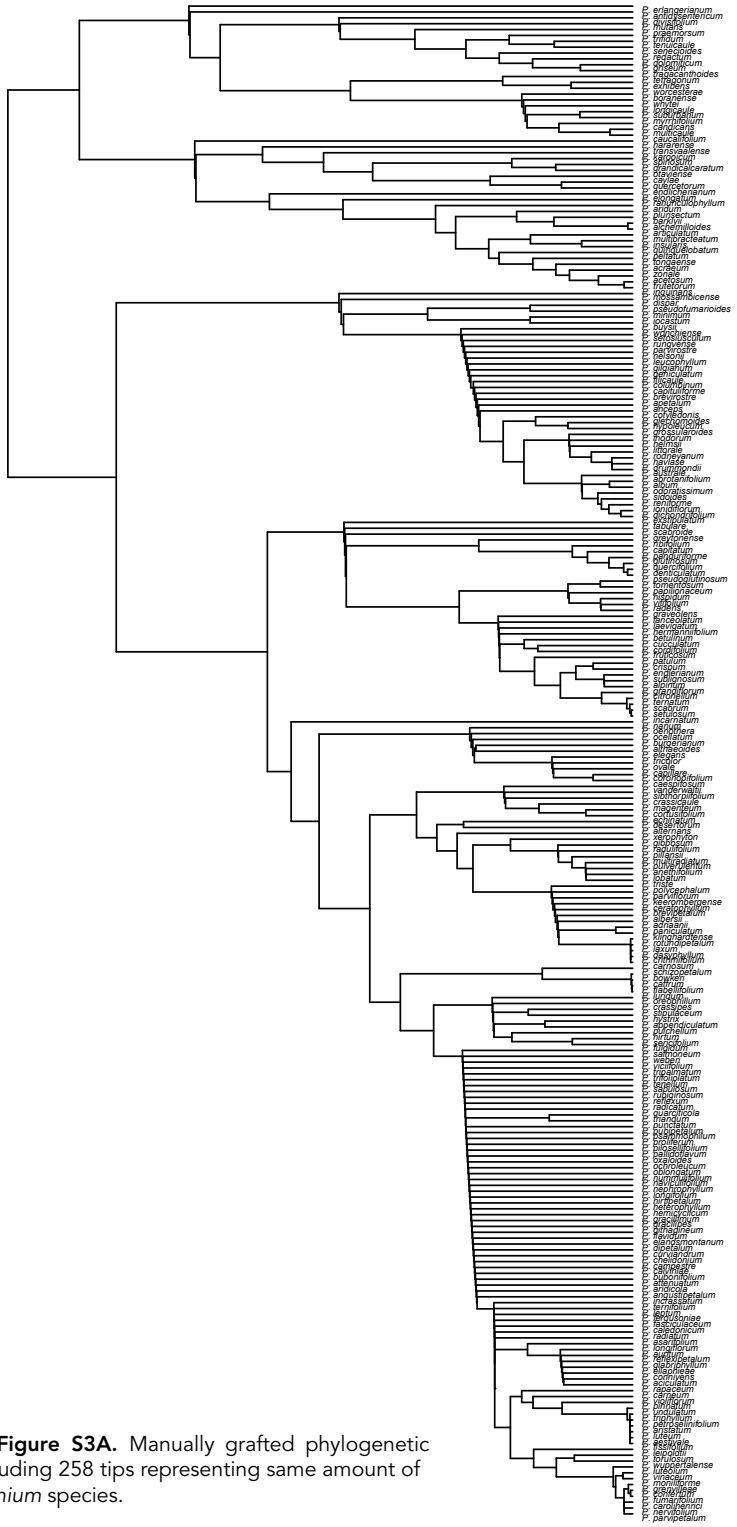
ACKNOWLEDGEMENTS

Many thanks to Cynthia Jones and especially Timothy M. Moore of University of Connecticut, Storrs for their help and discussion of ideas. Thanks to Sander Rodenburg (WUR) for the computational help and to Prof. Dr Chatrou for reading an earlier version of this manuscript.

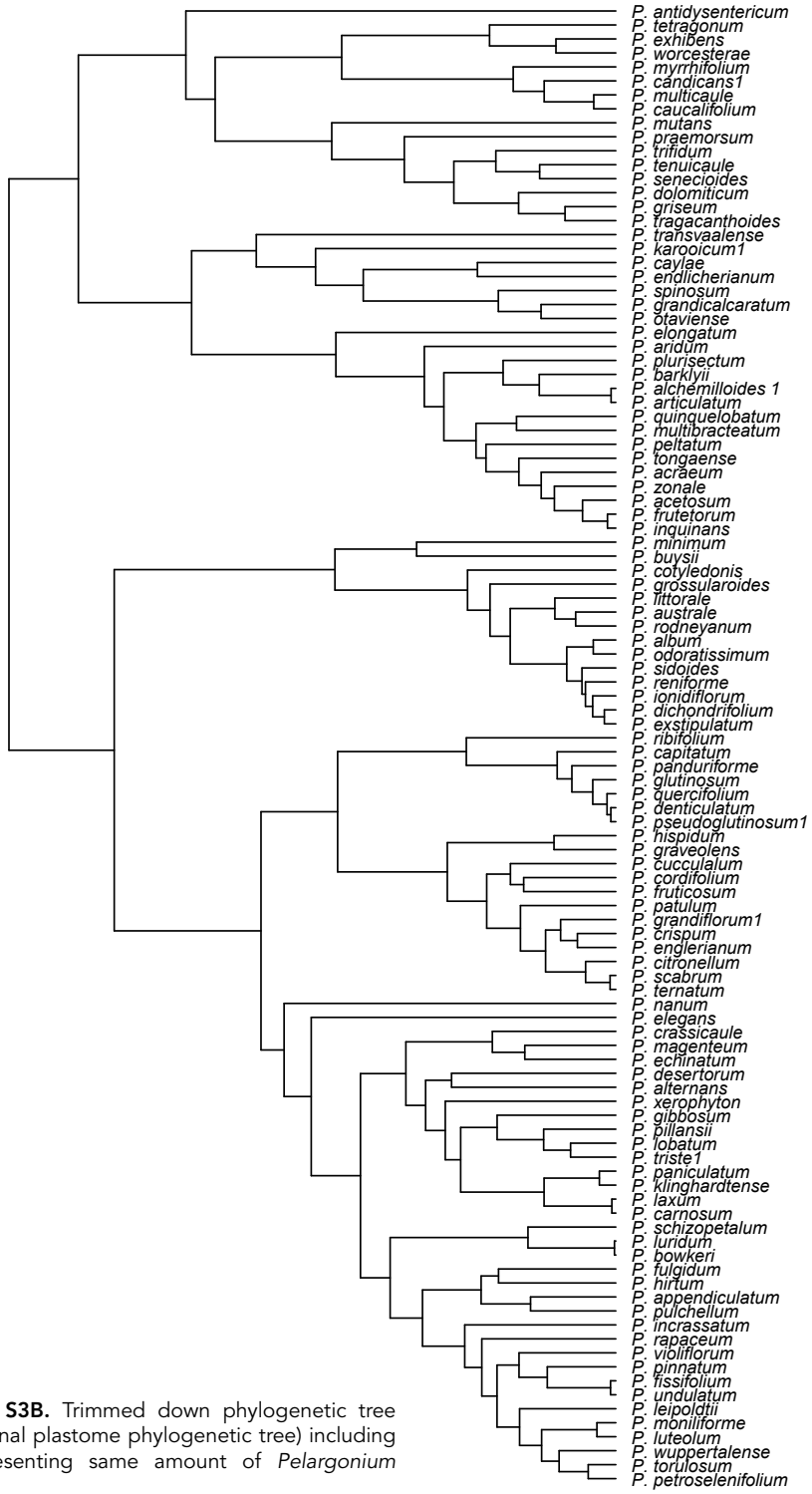
Supplemental Files

Suppl. Table S1
Overview of taxonomic primary sources. Available upon request.

Suppl. Table S2
Overview of taxonomic placement for species grafted to phylogenetic tree. Available upon request.



Suppl. Figure S3A. Manually grafted phylogenetic tree including 258 tips representing same amount of *Pelargonium* species.



Suppl. Figure S3B. Trimmed down phylogenetic tree (based on original plastome phylogenetic tree) including 106 tips representing same amount of *Pelargonium* species.

Supplementary Table S4. Founder events per area as calculated by BioGeoBEARS. Letters correspond with areas in Figure 1.

	A	B	C	D	E	F	G	H	I	J	K	L
A		12.4	10.1	3.18	0.68	1.5	2.08	0.94	0.32	0.76	0.8	0.72
B	1.46		4.04	2.88	0.5	1.38	1.74	0.28	0.08	0.28	0.56	0.54
C	2.28	3.52		2.44	0.64	0.94	1.42	0.54	0.16	0.5	0.6	0.5
D	0.26	0.82	0.28		0.2	0.12	0.56	0.08	0.06	0.02	0.04	0.1
E	0.06	0.06	0.04	0.02		0.04	0.06	0	0.02	0.02	0.02	0
F	0.02	0.02	0.04	0	0.02		0.06	0.02	0.24	0.02	0.02	0
G	0.02	0	0	0.08	0.1	0.94		0.02	0.76	0.04	0.02	0.04
H	0	0	0	0.02	0	0	0		0	0.26	0	0
I	0	0.02	0.02	0.02	0	0	0.02	0		0	0	0
J	0	0	0	0	0	0.02	0.02	0.22	0		0.06	0
K	0	0.02	0	0	0.02	0	0.02	0	0	0		0.02
L	0.04	0	0	0	0	0	0	0	0	0.02	0	

Chapter 4

Capturing variation in floral shape; a virtual3D based morphospace for *Pelargonium*

Sara J. van de Kerke[1,2], Tiemen van Engelenhoven[1], Anne van Es[1], Laura Schat[1], Lisa van Son[1], Sverre Vink[1], Lia Hemerik[3], Robin van Velzen[1], M. Eric Schranz[1], & Freek T. Bakker[1]

In revision

[1]Biosystematics Group, Wageningen University and Research, Droevendaalsesteeg 1, 6708 PB, Wageningen, The Netherlands
[2]Hortus Botanicus Amsterdam, Plantage Middenlaan 2A, 1018 DD, Amsterdam, The Netherlands
[3]Biometris, Wageningen University and Research, Department of Mathematical and Statistical Methods, P.O. Box 16, 6700AA Wageningen, The Netherlands

ABSTRACT

Background. Variation in floral shapes has long fascinated biologists and its modelling enables testing of evolutionary hypotheses. Recent comparative studies that explore floral shape have largely ignored 3D floral shape. We propose quantifying floral shape by using geometric morphometrics on a 3D model based on 2D photographical data and demonstrate its performance in capturing shape variation.

Methods. This approach offers unique benefits to complement established imaging techniques i) by enabling adequate coverage of the potential morphospace of large and diverse flowering-plant clades; (ii) by circumventing asynchronicity in anthesis of different floral parts; and (iii) by incorporating variation in copy number of floral organs within structures. We demonstrate our approach by analysing 90 florally-diverse species of the Southern African genus *Pelargonium* (Geraniaceae). We quantify *Pelargonium* floral shapes using 117 landmarks and show similarities in reconstructed morphospaces for spur, corolla (2D datasets), and a combined 3D dataset.

Results. Our results indicate that *Pelargonium* species differ in floral shape, which can also vary extensively within a species. PCA results of the reconstructed 3D floral models are highly congruent with the separate 2D morphospaces, indicating it is an accurate, virtual, representation of floral shape. Through our approach, we find that adding the third dimension to the data is crucial to accurately interpret the manner of, as well as levels of, shape variation in flowers.

INTRODUCTION

Variation in floral form continues to be an inspiration for a wide variety of research fields, ranging from taxonomy (Linnaeus, 1758), developmental biology (Carr and Fenster, 1994; Coen and Meyerowitz, 1991; Cubas et al., 1999; Fenster et al., 1995; Luo et al., 1995; Mummenhoff et al., 2009; Parenicova et al., 2003), evolution (Darwin, 1877a; Reyes et al., 2016; Sauquet et al., 2017), adaptation, to pollination biology and speciation (Darwin, 1877a, 1877b; Fernández-Mazuecos et al., 2013; Gómez et al., 2016; Grant, 1949; van der Niet and Johnson, 2012). The term 'form' refers to a combination of size and shape (Goodall, 1991; Zelditch, 2012). Whereas allometry is the study of the effect of size on the variation in morphological traits (Klingenberg, 2016) , shape is defined as "those geometrical attributes that remain unchanged when the figure is translated, rotated and scaled" (Goodall, 1991).
The total variation in shape of a clade after scaling and aligning forms its 'morphospace' (Chartier et al., 2014), which can change depending on the taxa included in the study. Traditional versus geometric morphological methods (GMM) have been the subject of debate (see Adams et al., 2004; Rohlf and Marcus, 1993). In GMM, landmarks placed on homologous structures capture the geometry of the studied object. Shape is maintained throughout the analyses, preserving the geometric relationships between structures (Adams et al., 2004; Rohlf and Marcus, 1993).

Challenges for any morphometric study are measurement accuracy and precision. For accuracy, including as many taxa as possible seems important in GMM studies, because the aim is to cover variation in shape. Taxonomic coverage is often used here as a proxy to determine inclusiveness or accuracy. However, more important might be to include a broad representation of the expected morphological diversity in the sampling, irrespective of phylogenetic diversity. In general, larger clades are considered to be more informative because more taxa means more data, likely increasing the accuracy in measuring the studied shape variation. But when the taxon- and total potential morphospaces cannot be adequately covered, studying large clades is less meaningful.

When a floral GMM analysis is performed on a plant clade , maintaining the precision of gathering the data poses an additional challenge. Since plant morphology can be considered "a process" (Sattler, 1996, 1990), i.e. development, it is important to make sure that there is no noise from developmental signals in the data and its resulting morphospace, and hence that comparisons are made for the same ontogenetic stage across individual flowers. Ontogenetic noise can be prevented by deciding on a particular developmental stage for all individuals when measured. Full anthesis of the corolla is an example thereof (Gómez et al., 2016). However, studies have shown that different floral parts are not synchronised in their development (Ronse De Craene, 2018) and that species differ in the synchronisation of their floral parts (van de Kerke, unpublished data). Therefore, the floral parts of all individuals included in the study should be captured during the same ontogenetic stages, which poses a practical problem in data gathering.

Another, practical, challenge in floral GMM is the variation in copy number of included structures. For example, a species can display a range in number of stamens or petals within its flowers. This can be problematic because GMM studies are based on capturing homologous structures and therefore retaining accurate homology assessment is essential. Simply omitting copy number-variable structures from the analysis is not desirable since they represent evidence on shape. Assuming serial homology and 'filling in' missing copies could be one solution but the ensemble shape may be affected. Thus, how to handle such morphs and their varying copy numbers is not straight-forward.

We aim to address the GMM challenges outlined above, using the predominantly South African genus *Pelargonium* (Geraniaceae) as a model. The genus is known for its stunning floral and vegetative diversity across its ~280 species (Bakker et al., 2005, 1999, Jones et al., 2009, 2003; Nicotra et al., 2008; Röschenbleck et al., 2014; Struck, 1997, Figure 1) and has been the subject of wide-spread breeding and horticulture (Becher et al., 2000; James, 2002; Miller, 2002). Roughly 70% of the genus occurs in the South African Greater Cape Floristic Region (GCFR; Linder, 2003; Manning and Goldblatt, 2012; Snijman, 2013), other species occur in eastern Africa, Namibia, Asia Minor, the Arabian peninsula, Madagascar and Australia (Bakker et al., 2005). Phylogenetic relationships within the genus are well known (Bakker et al., 2005; Röschenbleck et al., 2014; Chapter 2; van de Kerke et al., 2019) and show a pattern of deep splits as well as more recent species radiations (i.e. the geophytic sect. *Hoarea*; Bakker et al., 2005).

Pelargonium flowers are specialised when compared with the remainder of the Geraniaceae clade (i.e. *Geranium*, *Erodium*, *Monsonia* and *California*), as they exhibit strongly zygomorphic corollas and possess nectar spurs that are formed adnate to the pedicels (Albers and van der Walt, 2007; Bakker et al., 2005; Goldblatt et al., 2000; Hodges, 1997; Hodges and Arnold, 1995; Manning and Goldblatt, 2012; but see Tsai et al., 2018; Van der Walt and Vorster, 1988), which is unique in angiosperms (Hodges, 1997; but see Tsai et al., 2018). Throughout *Pelargonium*, variation in floral shape occurs in a number of ways. Most strikingly, the orientation of the petals ranges from highly zygomorphic (*P. fulgidum*) to almost actinomorphic (*P. cotyledonis*). Secondly, the variation in petal copy number occurs between and within a species (i.e. *P. caucalifolium*) and alters between five (the 'standard' in Geraniaceae), four (*P. tetragonum*), two (*P. dipetalum*), and can even be missing *(P. apetalum*). Third, the shape of the petals varies tremendously: from slender and elongated (*P. paniculatum*) to almost round (*P. inquinans*). *Pelargonium* exhibits a range of pollination syndromes, including species of long-tongued hovering flies (*Tabanidae*, *Bombyliidae*, and *Nemestrinidae*), bees (*Apidae*, *Anthophoridae*, *Megachilidae*), wasps (*Vespidae*), and beetles (*Scarabaeidae*; Struck, 1997). Some syndromes are highly-specialised, as in the oceanic island endemic *P. cotyledonis* (occurring on St. Helena) where the nectar spur is reduced to a few millimetres. Another extreme example is the geophytic *P. appendiculatum* (with a limited distribution range along the South African west coast (see Marais, 1999)) which has a nectar spur of 10 cm long, while no pollinator with a suitable proboscid is known. Spur length in *Pelargonium* appears to be a driver of speciation rate, whereby speciation rate seems to decrease with an increase in spur length and is associated

Figure 1. Variation in *Pelargonium* floral shape. (A) *P. caucalifolium,* (B) *P. sidoides,* (C) *P. caffrum,* (D) *P. cotyledonis*, (E) *P. columbinum*, (F) *P. tricolor.* Pictures by F.T. Bakker and S.J. van de Kerke.

with small clade size (Ringelberg, 2012). The wide variety of known pollinators for *Pelargonium* is reflected in spur length, whereby the spur matches the proboscis of the pollinator species. The extent to which the pedicel is covered by the spur differs greatly among species (Bakker et al., 2005; Manning and Goldblatt, 2012; Tsai et al., 2018). This could indicate pedicel length is independent from spur length, and thus is a potential constraint on spur length change.

In this study, we infer the floral morphospace for the corolla and the nectar spur across *Pelargonium*. We use two-dimensional (2D) photographs to form three-dimensional (3D) representations of virtual flowers in order to quantify floral shape in 90 *Pelargonium* species. We explore the diversity of floral forms within the genus and using this dataset as a case study we apply GMM methods to determine and compare natural variation in floral shape.

MATERIALS & METHODS

Flower data sampling

Floral shape was compared for 90 *Pelargonium* species growing in living collections in The Netherlands, Germany and in South Africa (see Supplementary Table 1 for an overview of species, numbers of individuals, and location). The sampling covers approximately 32% of known species in the genus and includes 378 individual flowers. We covered the potential morphospace as adequately as possible (based on known extreme floral forms from taxonomic studies; Albers et al., 1995; van der Walt, 1985; van der Walt and Boucher, 1986; van der Walt and van Zyl (nee Hugo), 1988) but not-necessarily representing phylogenetic diversity.

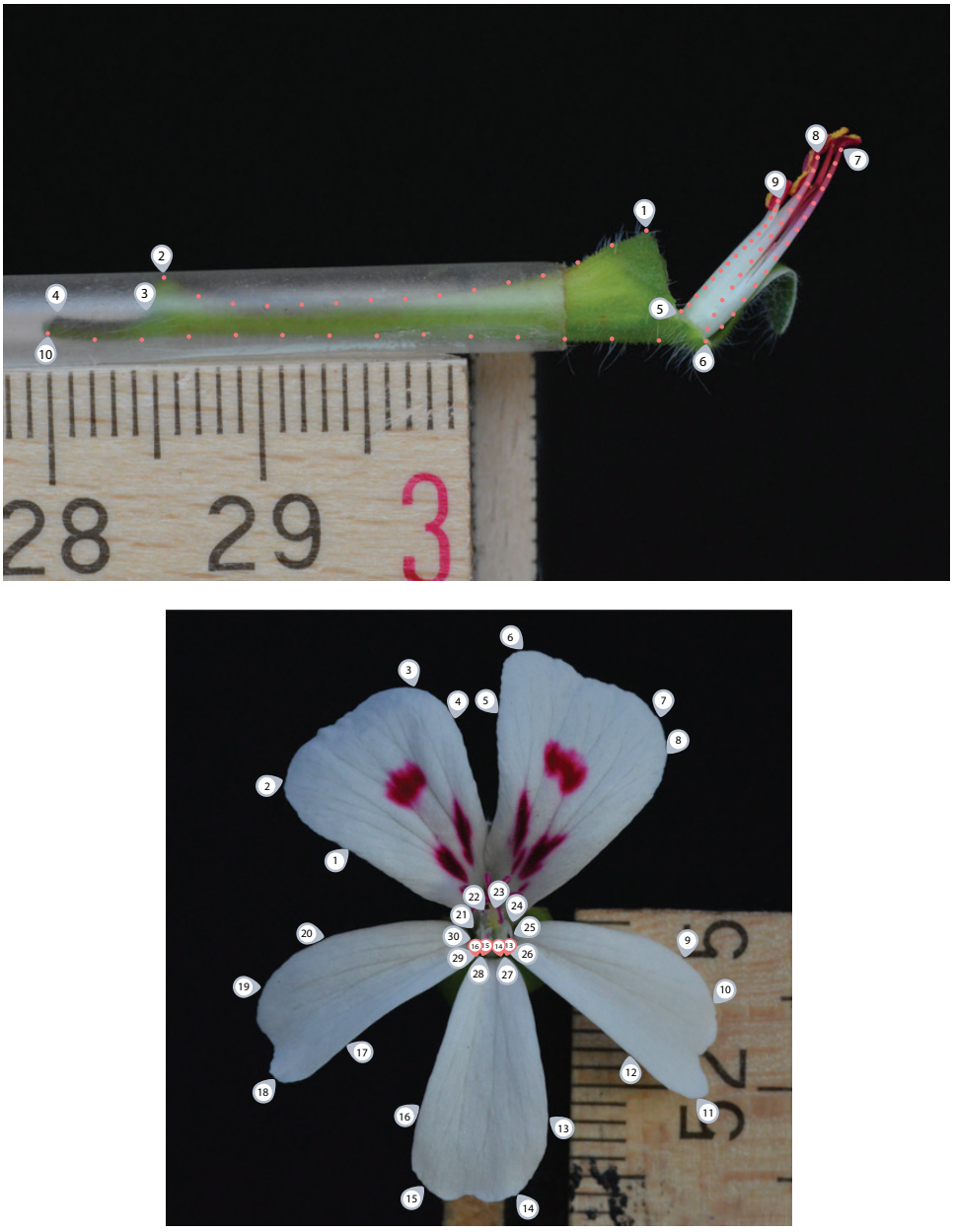


Figure 2. Landmark placement for the SPUR (A) and PETAL (B) datasets. For the SPUR dataset 10 landmarks and 75 sliding landmarks covering the spur outline and tracking its curvature, as well as that of the shortest, longest and an average stamen (grey labels) are defined. For the PETAL dataset 32 landmarks were placed along the outline of the corolla and the opening of the nectar spur using midrib, primary and secondary veins and petal attachment as a guide (grey labels). For specimens with four petals, we assumed that for the middle anterior petal the meristem is present but does not develop and landmarks allocated for this petal were placed with zero length from the petal base (red labels).

Geometric morphometric data collection

We selected flowers with corollas and flowers with stamens (used a proxy for full anthesis of the spurs, which was confirmed by eye) in full anthesis separately to limit possible ontogenetic effects on measured shape. We digitally photographed each flower using a standardised procedure in front and side view to avoid positional effects on measured shape. For each photograph, we defined a set of landmarks to provide comprehensive coverage of the specimen. We used both primary landmarks on homologous positions as well as sliding landmarks along a curve between two fixed primary landmarks. A datafile was created using tpsUtil (Rohlf, 2004) and landmarks were placed using tpsDig v. 232 (Rohlf, 2010).

For the side view photograph, covering the spur aspect, we defined a set of 10 landmarks and 75 sliding landmarks covering the spur outline and tracking its curvature, as well as that of the shortest, longest and an average stamen (Figure 2A; grey labels). We labelled this data set SPUR (containing 134 individuals, Supplementary Table S1).

For the front view photograph, we followed the corolla shape landmarks as defined by Gómez et al. (2006) and placed 32 landmarks along the outline of the corolla and the opening of the nectar spur using midrib, primary and secondary veins and petal attachment as a guide (Figure 2B; grey labels). We labelled this data set PETAL (containing 287 individuals, Supplementary Table S1). For specimens with four petals, we assumed that for the middle anterior petal the meristem is present but does not develop (Ronse De Craene, 2018). Therefore, landmarks allocated for this petal were placed but with zero length from the missing petal base (Figure 2B (pink labels)). A 5 mm scale bar was included in each picture to be able to represent all landmark coordinates on the same interval scale.

Creating 3D virtual representation from two 2D photographs

To be able to understand how shape variation happens at the level of the complete flower we linked individuals from both datasets at the species level. One-on-one pairing of individuals in the separate SPUR and PETAL databases was not possible because the flowers we used are not the same for both datasets (as a result of the separate sampling in order to avoid of asynchronisation), nor were individuals sampled from the same plant. Therefore, we designed a random sampling bootstrapping method based on the SPUR and PETAL datasets (see below, Figure 3).

First, we reoriented all individuals in both SPUR and PETAL dataset in the same position before we connected them to assure a virtual 3D flower that is as congruent with actual morphology as possible. To that extent, we performed an initial Generalised Procrustes Analysis (GPA) on the SPUR and PETAL datasets separately in order to align specimens and remove size components. Subsequently, we reintegrated the size component in order to retain actual size of the individual when coupling them from SPUR and PETAL datasets (via an anchor point, see below and Figure 3D). This was accomplished by multiplying each individual with its calculated centroid size. In this way, we orientated all specimens in the same position based on their landmarks, without removing size information (Figure 3B).

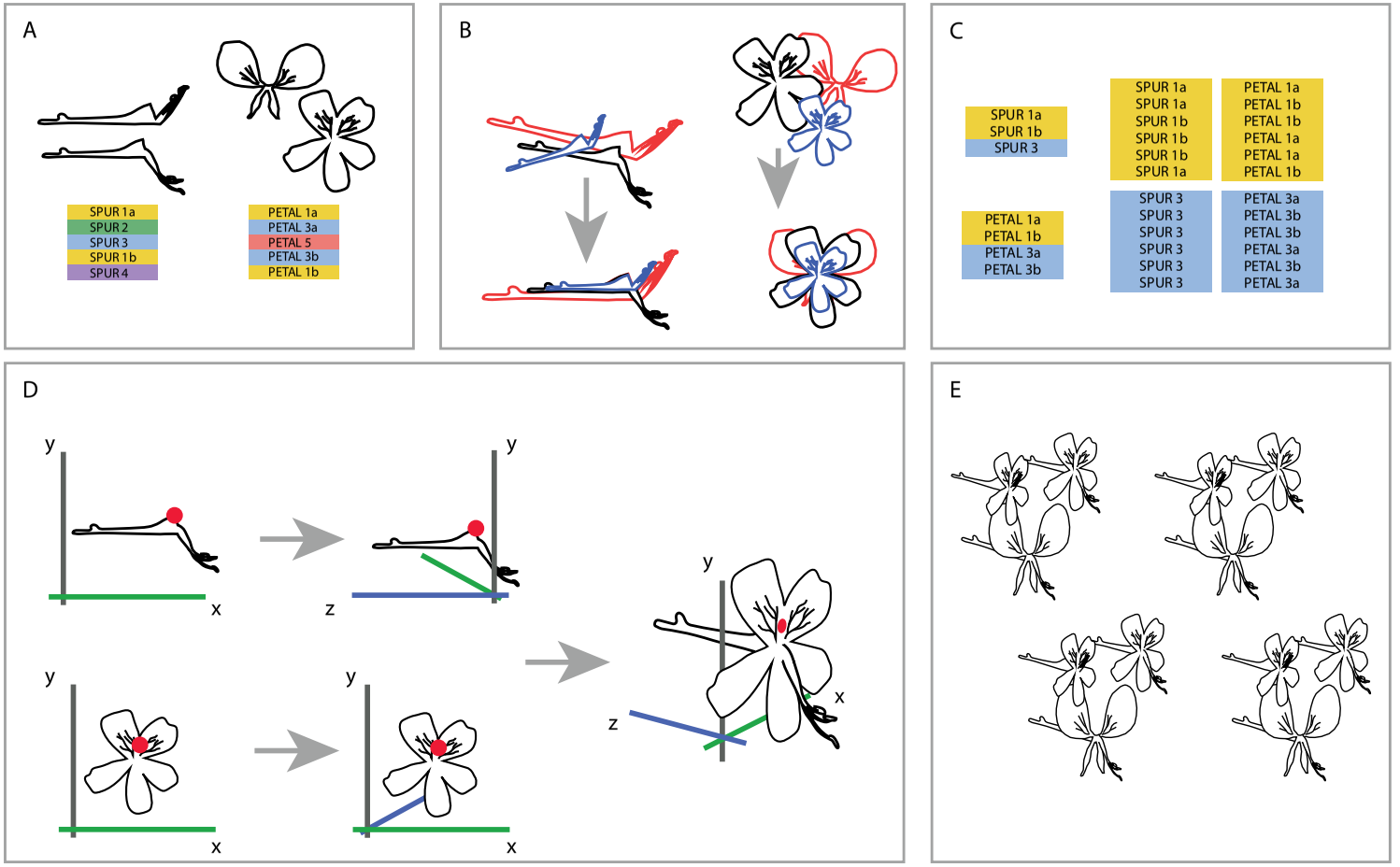

A
SPUR 1a
SPUR 2
SPUR 3
SPUR 1b
SPUR 4
PETAL 1a
PETAL 3a
PETAL 5
PETAL 3b
PETAL 1b
B
C
SPUR 1a
SPUR 1b
SPUR 3
PETAL 1a
PETAL 1b
PETAL 3a
PETAL 3b
SPUR 1a SPUR 1a SPUR 1b SPUR 1b SPUR 1b SPUR 1a
PETAL 1a PETAL 1b PETAL 1b PETAL 1a PETAL 1a PETAL 1b
SPUR 3 SPUR 3 SPUR 3 SPUR 3 SPUR 3 SPUR 3
PETAL 3a PETAL 3b PETAL 3b PETAL 3a PETAL 3b PETAL 3a
D
y
x
z
E

Figure 3. ***(previous page)*** Creating one 3D virtual flower from two 2d photographs. (A) The two separate datasets (SPUR and PETAL), with limited overlap between and within species. (B) Generalised Procrustes Analysis is performed on the SPUR and PETAL datasets separately in order to filter out all non-shape variation. Size component is then reintegrated by multiplying each individual with it calculated centroid size. In this way, all specimens are aligned based on their landmarks, without removing size information. (C) Species present in both SPUR and PETAL datasets are selected. In order to link species in both data sets, a random individual from dataset SPUR is then drawn for the first species and combined with a random individual of the same species from dataset SPUR. This was done six times per species, with replacement. (D) To integrate the two 2D datasets into a single 3D dataset, a common anchor point is defined in both the SPUR and the PETAL datasets, corresponding here with the top of the opening of the spur. A third coordinate is then added to the coordinate data, effectively making it 3D. see text for further details (E) This process is repeated for all individuals in the set selected in the linking step.

Next, we selected the species present in both SPUR and PETAL datasets. For each species, the number of individuals in each dataset was counted and we recorded at which row in the dataset a new species starts. In a linking step, a random individual from a certain species in dataset SPUR was then drawn and combined with a random individual of the same species from dataset PETAL. This was done six times per species, with replacement (Figure 3C).

To integrate the two 2D datasets into a single 3D dataset, a common anchor point was defined in both the SPUR and the PETAL datasets, corresponding to the top of the opening of the spur. In the SPUR dataset, the first landmark was chosen as anchor and for PETAL we defined the anchor to be the average of landmarks 22 and 23, as these anchors are homologous (Figure 3D).

A third coordinate was then added to the two 2D coordinate datasets (PETAL and SPUR), effectively making a virtual 3D image (dataset VIRTUAL3D). For the PETAL data set we kept the original x and y values and add a z = 0 coordinate to all landmarks. In this way, we 'forced' the corolla of the flower to be flat because we do not have data on the curvature of the petals. For SPUR the coordinate system was altered from x,y to z,y, which effectively becomes the depth of the flower. This alteration is relative to the coordinate combination of the anchor point defined previously, landmark SPUR 1 and landmarks PETAL 22-23, i.e. they are placed perpendicular to each other, around the anchor. Therefore the coordinates became negative for the spur and positive for the stamens. The value x = 0 was added for all SPUR landmarks, again resulting in a flat object. The new x and y SPUR coordinates were then transposed relative to the landmarks 22-23 anchor point of PETAL (Figure 3D), with which they were subsequently combined. The new 3D coordinates were written to a file using a format that is suitable for later analysis with Geomorph. This process was repeated for all combinations of individuals in the set selected in the linking step described above (Figure 3E).

This process is repeated 20 times to assess the structure in the virtual 3D flower data, and hence its stability, resulting in 20 bootstrap pseudoreplicate datasets containing 6 x 68 species = 408 virtual flowers, which we label $VIRTUAL3D_i$ (with i =1, …,20). We combined all resulting 8160 virtual flowers in VIRTUAL3D, a dataset which we use for further analyses.

Morphometric analysis

Landmark coordinates in the SPUR, PETAL, VIRTUAL3D, and all VIRTUAL3D$_i$ datasets were each aligned using a final Generalised Procrustes Analysis, extracting the shape information (Rohlf and Slice, 1990). Results were projected into tangent space to summarise and explore actual (SPUR, PETAL) and virtual (VIRTUAL3D) floral shape variation across *Pelargonium* species. Shape changes associated with principal components where illustrated using thin-plate spline deformation plots.

We conducted a Principal Component Analysis (PCA) on the GPA-aligned coordinates for each of the VIRTUAL3D$_i$ datasets. PCA results for the 20 VIRTUAL3D$_i$ datasets are highly congruent (results not shown, data will be made available). This indicates that there is high consistency in our data and that bootstrap subsampling seems justified for connecting the differently samples SPUR and PETAL datasets. We therefore decided to continue our analyses with the VIRTUAL3D dataset including all 8160 virtual flowers, as this dataset assures an even coverage of all included species and is the most inclusive.

Spurs occur adnate to the pedicel in *Pelargonium* species and pedicels can be 'occupied' by spurs to varying degrees. As this may in fact present limits to spur length it could constrain spur evolution and be relevant to floral shape exploration. We therefore decided to extract 'spur-filling' levels from our data in the following way: for each individual in the SPUR dataset, we extracted relative spur and pedicel length from the SPUR dataset using the function 'interlmkdist'. We calculated the ratio between the spur and pedicel length as a measure for the 'filling' of the pedicel by the spur. This ratio is visualised in the SPUR PCA plot as the transparency of the individual.

All analyses were performed in R v.3.2.2 (R Core Team, 2015) using the Geomorph library v.3.0 (Adams and Otárola-Castillo, 2013). All R scripts can be found in Supplementary R scripts S2, S3, and S4.

RESULTS

Our analysis on 68 *Pelargonium* species identified a wide variety of floral shapes across and within the species examined (see also Fig. 1). Supplementary Figure S5 shows the mean consensus configuration and Procrustes residuals (i.e. differences between observed and estimated value) calculated for the SPUR and PETAL datasets using the generalised Procrustes analysis (GPA). The figure illustrates the variability in landmarks around the calculated mean shape (in blue). What is striking is that halfway through the spur we see a constrained area where variation is limited compared with the base of the pedicel (Sup. Fig S5A). In addition, in Figure S5B it is conspicuous that the anterior petals are more restricted in shape variation than the posterior petals.

We conducted a Principal Component Analysis (PCA) on the GPA aligned coordinates for each of the SPUR, PETAL, and VIRTUAL3D datasets in order to assess variation in shape. For the SPUR dataset, the first PC accounts for 47% of the total variation present across the species and the first four axes explaining more than 90% of the data (Figure 4A, Supplementary Figure S6). The first two PCs and corresponding shape outlines of the extremes are plotted in Figure 4A and 4B, respectively. The

variation in shape explained by the first PC corresponds with the coverage of the spur relative to the pedicel. On the negative extreme of the axis, spurs are elongated and are the same length as the pedicel. On the positive extreme, spurs are much shorter than the length of the pedicel and, in addition, the opening of the spur is wide. PC2 corresponds with the curve of the stamens. Individuals on the negative extreme of the PC have stamens that are so curved they are doubled up on themselves, while those on the positive side have elongated stamens (Fig 4B, PC2). In Fig 4A some species, represented by multiple samples, are spread in varying degrees around the morphospace, such as *P. mutans* (in green) and *P. crithmifolium* (in red) along both PC1 and PC2. Other species appear to be much more clustered, such as *P. triste* (in brown) and *P. pseudoglutinosum* (in orange). Overall, a clear pattern emerges of individuals distributed along a trajectory corresponding with the ratio between the length of the spur and pedicel (indicated by transparency of the markers in Fig 4A). with individuals with a low spur-pedicel ratio occupying the lower region of the PC plot while individuals towards the top have an increasingly higher spur-pedicel ratio, i.e. each having nearly the same length, towards a boundary reflecting a physical barrier. This boundary is also reflected in the 'avoided area' in the *Pelargonium* SPUR morphospace just above it. In this area, the spur of a hypothetical flower would be longer than the pedicel of that individual, and this is not possible for *Pelargonium* flowers as spurs and pedicels are adnate.
Compared with the results of the SPUR dataset, the PCA results of the PETAL dataset are more centralised. In Figure 4C and 4D, the first two PCs and shape outlines are plotted. The first PC (explaining 40% variation, Supplementary Figure S6) corresponds with the position and number of petals in the flower. On the negative extreme of the axis, flowers consist of five petals with the two posterior ones close together and the three anterior petals spread out. On the positive extreme, the two posterior petals are enlarged and only two anterior petals appear to be present. PC2 (13%) corresponds with the distribution of the petals over the corolla. On the positive extreme of the PC, the posterior petals are narrow and overlap, while on the negative side the posterior petals are rounded. Overall, individuals cluster around the mean shape (as *P. multibracteatum* (yellow)) while other species show within-species variation with individuals that spread toward the positive extreme of PC1 (*P. myrrhifolium* (darkgreen)). A few species (as *P. mutans* (green)), with high within-species variation, found across the entire PCA spectrum.

For the VIRTUAL3D dataset, containing 8160 virtual flowers, the first PC accounts for 41% of the total variation present across species, with the first 5 axes collectively explaining > 80% of the data (Figure 5A, Figure 5B, and Supplementary Figure S6). Shape outlines illustrating the extreme forms are shown in Figure 5C. The variation in shape explained by the first PC corresponds with a zygomorphic flower, with corolla size varying with regards to pedicel length. On the positive extreme, individuals have a short pedicel and spur and a large corolla while flowers on the negative extreme show a more elongated spur and a relatively small corolla. Individuals from all species are spread along this axis, showing a high variability in spur and pedicel elongation and no clustering. PC2 (19%) corresponds with the length and curvature in stamens, with virtual flowers on the negative extreme showing straight stamens and those on the positive extreme showing highly curved ones. More importantly,

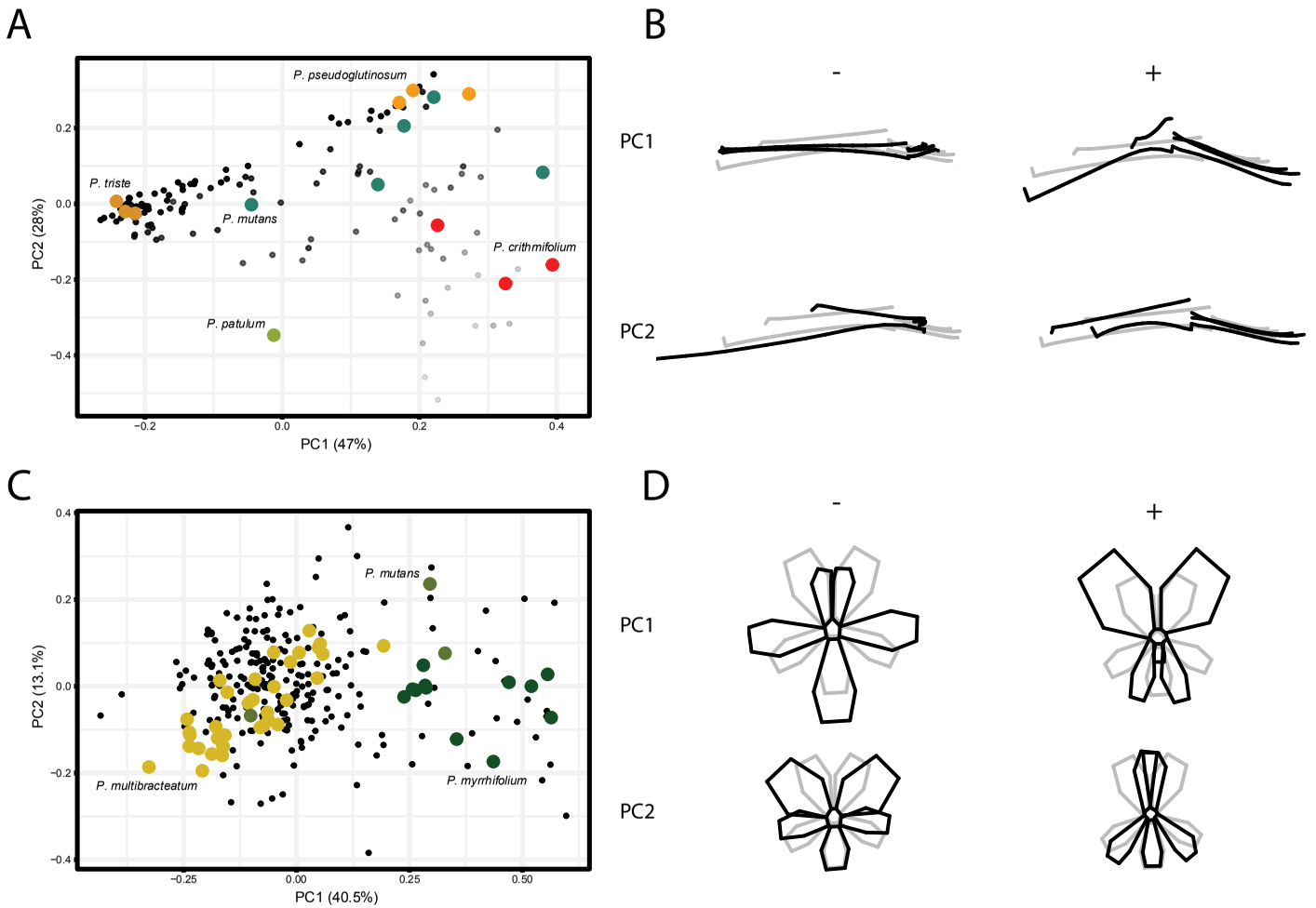

A
P. pseudoglutinosum
P. triste
P. mutans
P. crithmifolium
P. patulum
PC2 (28%)
PC1 (47%)
B
-
+
PC1
PC2
C
P. mutans
P. multibracteatum
P. myrrhifolium
PC2 (13.1%)
PC1 (40.5%)
D
-
+
PC1
PC2

Figure 4. ***(previous page)*** PCA analysis on SPUR and PETAL datasets. (A) PC1 and PC2 of PCA on SPUR dataset. Colours correspond with selected species: *P. triste* (brown), *P. mutans* (blue), *P. patulum* (green), *P. crithmifolium* (red), and *P. pseudoglutinosum* (orange). (B) Shape outlines corresponding to extremes on axes for PC1 and PC2 of SPUR dataset showing calculated mean shape (grey) and warped extreme shape (black). (C) PC1 and PC2 of PCA on PETAL dataset. Colours correspond with selected species: *P. multibracteatum* (yellow), *P. myrrhifolium* (darkgreen), and *P. mutans* (green). (D) Shape outlines corresponding to extremes on axes for PC1 and PC2 of PETAL dataset showing calculated mean shape (grey) and warped extreme shape (black).

this PC appears to correspond with the 'filling' of the pedicel by the spur, whereby we either see a long pedicel and relatively short spur (positive side) or a spur that 'spills over' the pedicel (negative side). Individuals from all species are spread along the axis but with an emphasis toward the negative extreme, suggesting a trend towards individuals with a high filling ratio. PC3 (14%) again (as PC1) appears to correspond with the filling of the pedicel by the spur as well as the length and orientation of the stamens. In individuals toward the positive end of this axis, the spur completely fills the pedicel and stamens are stretched out. On the negative side, only a small part of the pedicel is taken up by the spur and stamens are small. No clustering is observed and individuals are spread along the axis but with a strong emphasis on the negative end of the spectrum. Individuals within species are spread in varying degrees around the morphospace, such as *P. mutans* (in green) along PC1, PC2, and PC3. Other species vary along a number of PC axes, as *P. crithmifolium* (in dark blue) is variable along PC1 and PC3, but not along PC2. Lastly, some species are overall much more clustered, such as *P. pseudoglutinosum* (in orange).

DISCUSSION

In this study, we explore the potential of combining two 2D photograph-based datasets of floral morphology into a single 3D virtual flower giving us the opportunity to bring together multiple layers of shape variation. Using this method, we are able to investigate the tremendous floral diversity of *Pelargonium* species using 3D geometric morphometrics based on the spur plus corolla perspective. Our virtual 3D dataset gives a more nuanced view on shape variation in *Pelargonium* than the separate SPUR and PETAL perspectives, as we find the corolla perspective to be of less importance (see below). Our approach can serve as a low-cost alternative to emerging high-tech robotic and photogrammetry- based approaches to 3D geometric morphometrics.

Geometric morphometrics

Pelargonium flowers exhibit high variability in their floral shape with species ranging between zygomorphic to near-actinomorphic corolla shape (*P. cotyledonis*), varying in petal copy number (between five (most common in Geraniaceae), four (i.e. *P. caucalifolium*), two (in *P. dipetalum*; not included) and zero (in *P. apetalum*; not included)), and with lengths of nectar spurs varying between zero to ten cm (*P. appendiculatum*; not included). The variation in floral shape present in the VIRTUAL3D dataset as depicted in Figure 5 corresponds with this known variation in *Pelargonium* flowers, as well as with the separate PETAL and SPUR datasets (Figure

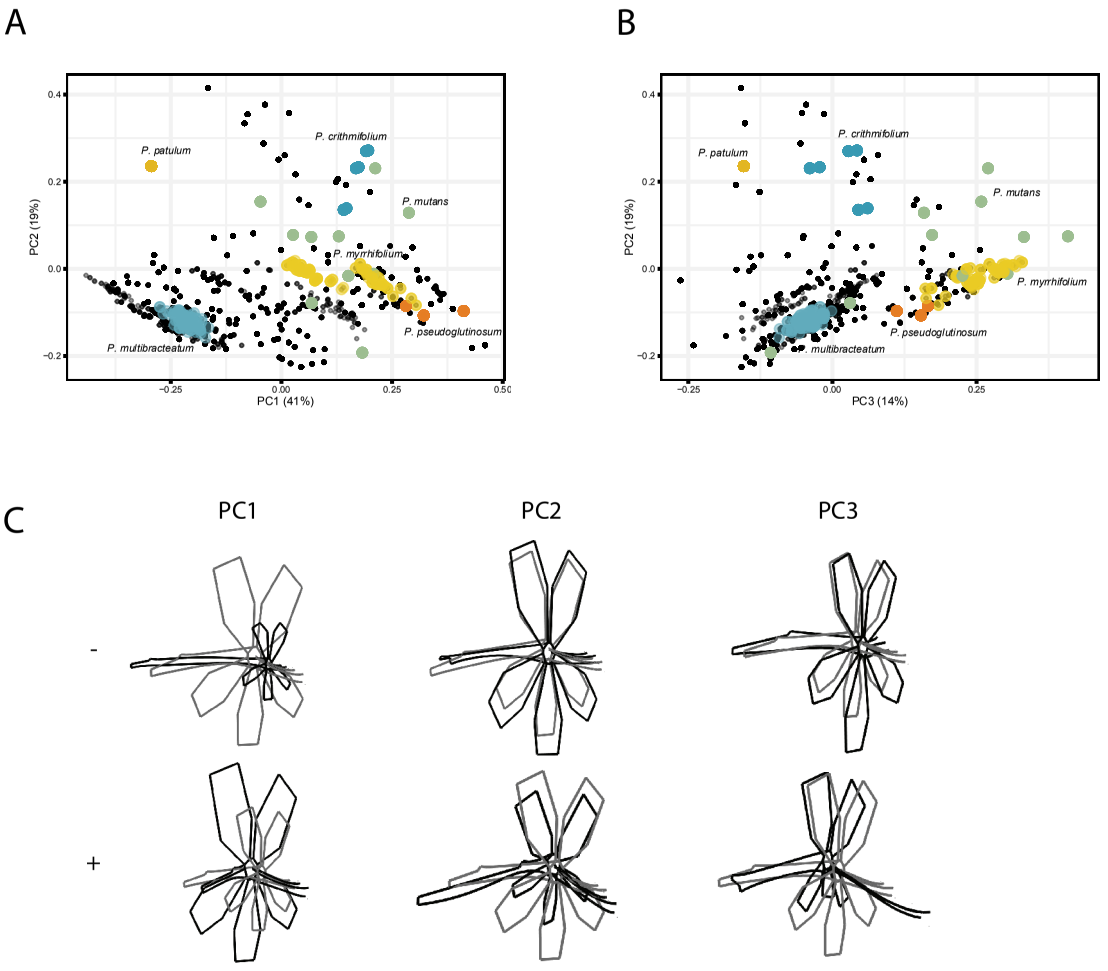

A
B
C
P. patulum
P. crithmifolium
P. mutans
P. myrhifolium
P. pseudoglutinosum
P. multibracteatum
PC1 (41%)
PC2 (19%)
PC3 (14%)
PC1
PC2
PC3
-
+

Figure 5. ***(previous page)*** PCA analysis on VIRTUAL3D datasets. (A) PC1 and PC2 and (B) PC3 and PC2. Colours correspond with selected species: *P. multibracteatum* (light blue), *P. triste* (brown), *P. mutans* (grey), *P. myrrhifolium* (yellow), *P. patulum* (brown), *P. crithmifolium* (dark blue), and *P. pseudoglutinosum* (orange). Intensity of colours indicated number of individuals stacked. (C) Shape outlines corresponding to extremes on axes for PC1, PC2, and PC3 of VIRTUAL3D dataset showing calculated mean shape (grey) and warped extreme shape (black).

3 and 4). Findings of the separate PETAL and SPUR datasets have now been put into perspective, giving us a better understanding of which changes in *Pelargonium* floral shape are relevant.

Resembling the results of the SPUR dataset, the elongation of the spur and size of the corolla are the most variable traits among the species included in the VIRTUAL3D morphospace (PC1, 41%). This trait corresponds with the unique spur pollinator syndrome featured in *Pelargonium* and correlates with their highly variable pollinator types (Struck, 1997, 1994). We know in some species the spur is almost completely missing (Figure 5C, as for example in the oceanic island endemic *P. cotyledonis*, probably pollinated by bees) or in *P. hirtum* with 3 mm short spurs. The latter is closely related to *P. appendiculatum* (probably pollinated by long-tongued hovering flies) where the spur is elongated to almost ten cm length (Struck, 1997).

Corresponding to PC2 (19%), and linked to inferred shifts in pollinators, is the curvature of the stamens. Along the PC, we find a shift of stamen shape ranging from short and straight to long and curved. For some hovering pollinator species, the stamens are thought to 'move out of the way' of the spur entrance by means of a large curve in the filament, both increasing accessibility to the flower (Goldblatt and Manning, 1999; Manning and Goldblatt, 1996) and enhancing contact of anthers and insect abdomen and head (Goldblatt and Manning, 1999). This would correspond to the long and curved stamens of *Pelargonium* species pollinated by long-tongued, hovering insects such as species from the *Tabanidae*, *Bombyliidae*, and *Nemestrinidae* (Struck, 1997). The short and straight stamens on the other end of the spectrum would then correspond with the association with short-proboscid, landing pollinator species, such as *Anthophoridae*, *Megachilidae*, and *Vespidae* to increase potential pollen transfer.

The 'filling' of the pedicel by the spur, which corresponds to both the second as well as the third PC (11%) in the VIRTUAL3D as well as the SPUR dataset is a relatively unexplored trait in *Pelargonium* literature. Recent studies found spur length to be dependent on both rate of cell division and duration of spur growth (Tsai et al., 2018). As the authors indicate, these mechanisms do not fully account for differences in spur length, suggesting other evolutionary influences. Ringelberg (2012) found spur length to be significantly correlated with speciation rate, whereby speciation rate appeared to decrease with increased spur length.

The distribution of virtual flowers over the first three PCs of the VIRTUAL3D morphospace varies and appears to be the results of interaction between the SPUR and PETAL morphospaces. In the SPUR morphospace, we see a clear boundary

limiting the distribution of individuals based on the ratio of spur and pedicel length (Figure 4A). In the PETAL morphospace on the other hand, the majority of species cluster together around the mean shape, indicating that there is variation to a limited extend. Some species in the VIRTUAL3D morphospace are highly variable and occur throughout large areas of the morphospace (for instance *P. mutans* (grey)) while others occupy a much smaller area (e.g. *P. multibracteatum* (light blue)). The former pattern does not directly correspond with a high individual count in PETAL and SPUR datasets. Certainly, in cases as *P. crispum* the low variability is the consequence of there being only one individual in the PETAL and SPUR datasets. As a result, over all the bootstrap iterations, only a single virtual-flower is included in the final analysis. But in other cases, as for instance with *P. multibracteatum*, multiple individuals are included in the separate datasets and still we find a narrow distribution in the morphospace.

Surprisingly, the results of the VIRTUAL3D dataset as discussed above are highly congruent with the results of the SPUR dataset while the PETAL dataset does not appear to have much influence since we do not find the variation in shape along PC1 in the PETAL dataset (variability in length of the fifth petal) until the third PC (14%). Rather the size of the corolla relative to the length of the spur is found to be of more influence in the VIRTUAL3D dataset. The variability in spur and stamens, combined with this relative size difference of the corolla, thus seem to be more relevant for distinguishing different shapes and presumably for attraction.

3D connection of 2D data sets

The combining of separate 2D datasets into a single 3D dataset by creating virtual flowers as we demonstrate here complements existing 3D approaches (van der Niet et al., 2010). We find the main PCs of the VIRTUAL3D dataset summarise the variability in shapes as presented in the separate SPUR and PETAL datasets and accurately portray the natural variation found in *Pelargonium* flowers (based on visual inspection). Having rendered the flower in 3D, we can now investigate the interaction between floral parts in more detail.

Our method enables us to circumvent a main issue in morphometric studies on flowers and thus to increase the precision of the data: asynchronicity in anthesis of floral parts. The moment of anthesis of floral parts differs both between and within species. This makes it impossible to pinpoint an ontogenetic stage for the entire flower that is the same for all species. We argue that anthesis is the most relevant ontogenetic stage for reproduction as well as pollinator attraction and thus is the most meaningful stage to include in our study. Following other plant studies (Gómez et al., 2006, 2014; Savriama et al., 2012; Savriama and Klingenberg, 2011), we decided to include all floral parts at their own, separate, anthesis. This results in the separate datasets of the SPUR (containing the spur and stamens) and the PETAL (containing the corolla). We consider the combination of spur and stamen floral parts in the SPUR dataset plausible since we suspect the flower's reward system to develop approximately in concert with the contact apparatus, in order to 'fit' the visiting pollinator. A drawback of combining the different floral parts each at their own anthesis is that we construct 'virtual-flowers' from our data. As a result, the morphospace is arguably

not biologically and temporally accurate. However, we argue that gathering the data in the same ontogenetic stage gives us the advantage of not polluting our data with unwanted developmental signal and enables the testing of evolutionary hypotheses regarding dynamic (un)coupling of compartments (van de Kerke et al., unpublished data).

Another problematic issue in plant geometric morphometrics is the variability in copy number within floral parts. A striking example of this phenomenon in *Pelargonium* is the variability in petal number, varying to four from the symplesiomorphic five. This variability makes it seemingly impossible to include all intended landmarks since they have to be placed on homologous structures. Not including these landmarks in the study is not desirable as they represent an important difference in shape between species. Likewise, it is not an option to treat these landmarks as 'missing' or 'NA' since the flower did not drop the petal by accident, but it is simply not present. Ideally, we would like to confirm the presence of petal primordia in an electron microscopy study. Based on literature describing the occasional loss of petals (Ronse De Craene, 2018, 2015), we now chose to simulate this 'missing' petal as if it is present, but with a length of zero (Figure 2). The influence of this simulation on morphospace results is limited since the variation between four and five petals is only visible on the fifth PC (4%) of the VIRTUAL3D dataset. We admit this approach is conceptually problematic because we assume the petal to be present, but operationally warranted because we find the corresponding difference in shape to be rather unimportant. Therefore we conclude this is a justifiable decision that can be put into practise for other similar cases that will occur in plant morphometric studies.

Unfortunately, we were not able to achieve complete matching in taxonomic coverage between the separate SPUR and PETAL datasets because sometimes there were no flowers in anthesis available for both datasets. The separate morphospaces therefore have a higher taxonomic sampling than the VIRTUAL3D dataset (68 for the VIRTUAL3D compared with 82 in SPUR and 90 in PETAL). This is an insurmountable drawback in combining the datasets, since in morphometric studies all landmarks need to be present in all included specimens. Estimating missing landmarks, as is available in the geomorph package, is not desirable when a large part of the studied shape of an entire species is missing because then the average *Pelargonium* shape is superimposed on a set of individuals and their unique shape is lost.

More important than high taxonomic coverage in the VIRTUAL3D dataset is to ensure accuracy of the data by good coverage of morphological extremes in the morphospace, which is not driven by the number of species included but by the shapes. In the case of *Pelargonium*, we have several 'missing' shapes that we were not able to include in the sampling (we did not encounter them while flowering) that will probably change the morphospace were they to be included. For example, we did not have the opportunity to include species such as *P. endlicherianum* and *P. dipetalum*, that only have two posterior petals. Likewise, we could not include species showing highly reflexed petals (for example *P. luridum*) as well as the peculiar, keel-flowered shaped *P. rapaceum* and the allopolyploid *P. quercetorum*.

Notwithstanding these gaps in the prospective morphospace, we are confident we reconstructed a fair representation of overall variability in floral shape found in *Pelargonium* and therefore provide a solid base for exploring floral shape in this clade.

CONCLUSIONS

This study provides a new approach for geometric morphometrics to analyse floral shape in 3D. Our method uses a semi-automated approach to combine 2D shape data of various data sets to include multiple morphological modules. It offers unique benefits to complement established imaging techniques by i) providing a bootstrapping method to help acquire adequate coverage of the potential morphospace of diverse flowering-plant clades when sampling of individual parts is unequal; (ii) by circumventing asynchronicity in anthesis of different floral parts; and (iii) by incorporating variation in copy number of parts within structures. This approach, for which the code is available as supplementary material, can be used for any flower as well as numerous plant structures and can be used to form an appropriate basis for future geometric morphometric and related studies starting from 2D pictures.

ACKNOWLEDGEMENTS

Many thanks to Walter den Hollander, Reinout Havinga and Carlien Blok from the Hortus Botanicus Amsterdam for taking in and maintaining the *Pelargonium* collection. In addition, we thank Melanie Wiethölter and Kai Müller from Münster Botanical Garden, Martin Smit and Peter Jansen from Stellenbosch Botanical Garden, Anthony Hitchcock and Roger Oliver from Kirstenbosch Botanical Garden (Cape Town), and Werner Voigt and Ricardo Riddles from Karoo Desert National Botanical Garden (Worcester) in South Africa for granting me access to their collections. Luuts and Riet Feenstra, thank you so much for welcoming us in your home and opening your collection so many times. Also the Family Mijnbergen, Arjen de Graaf, and Esther van der Velde, thank you for having us. In addition, we thank all other members of the Nederlandse Pelargonium Vereniging for their kind interest. Liesbeth Sluiter, Ronald Bakker, Ronald Flipphi, Rolf Roos, Paul Raadschelders, thank you for the amazing trip. We thank Nynke Groendijk-Wilders for her help with the digitalisation process and many thanks to Floris Breman for granting us access to his collection of *Pelargonium* flowers at Syngenta.

Supplemental Files

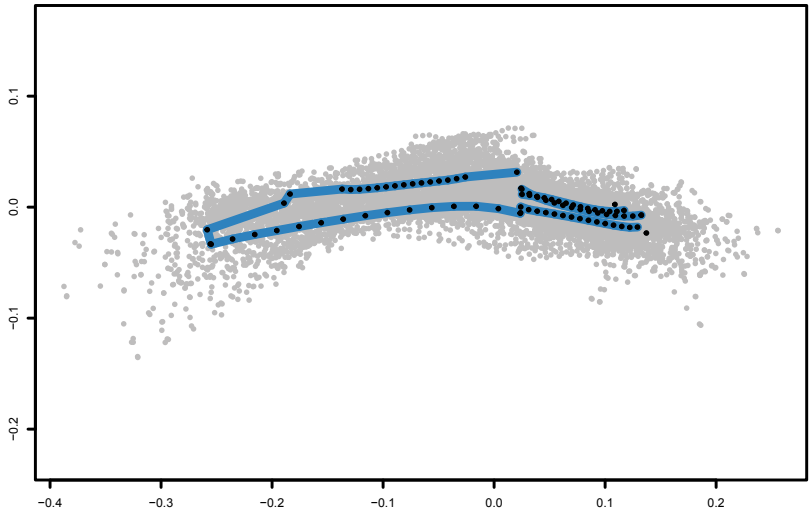
Suppl. Table S1
Taxonomic sampling of species included in the study. Including individuals per SPUR/PETAL dataset, with locality data and individual number/STEU number when available. Available upon request.

Suppl. R script S2
Supplementary R script describing steps for the analysis of floral morphology on SPUR and PETAL datasets separately using Geometric Morphometric Modelling. Available upon request.

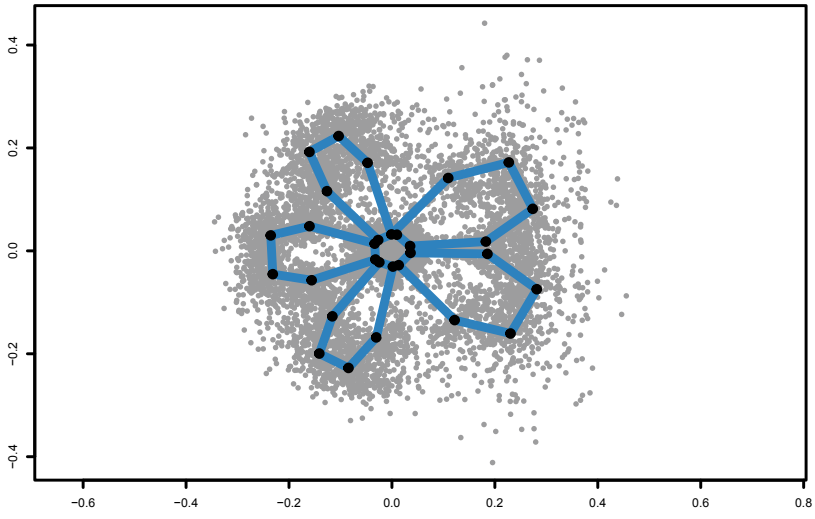
Suppl. R script S3
Supplementary R script describing the steps developed to combine the separate SPUR and PETAL datasets into virtual3D individuals and perform a bootstrapping sampling to evaluate consistancy in the virtually created flowers. Available upon request.

Suppl. R script S4
Supplementary R script describing steps for the analysis of floral morphology on virtual3D using Geometric Morphometric Modelling. Available upon request.

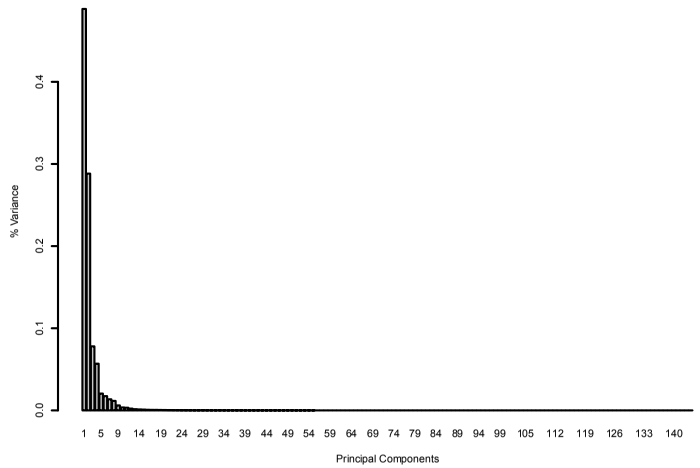
A

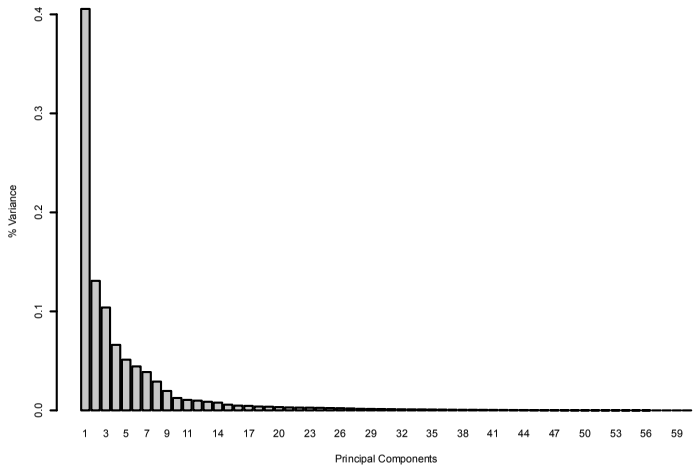


B

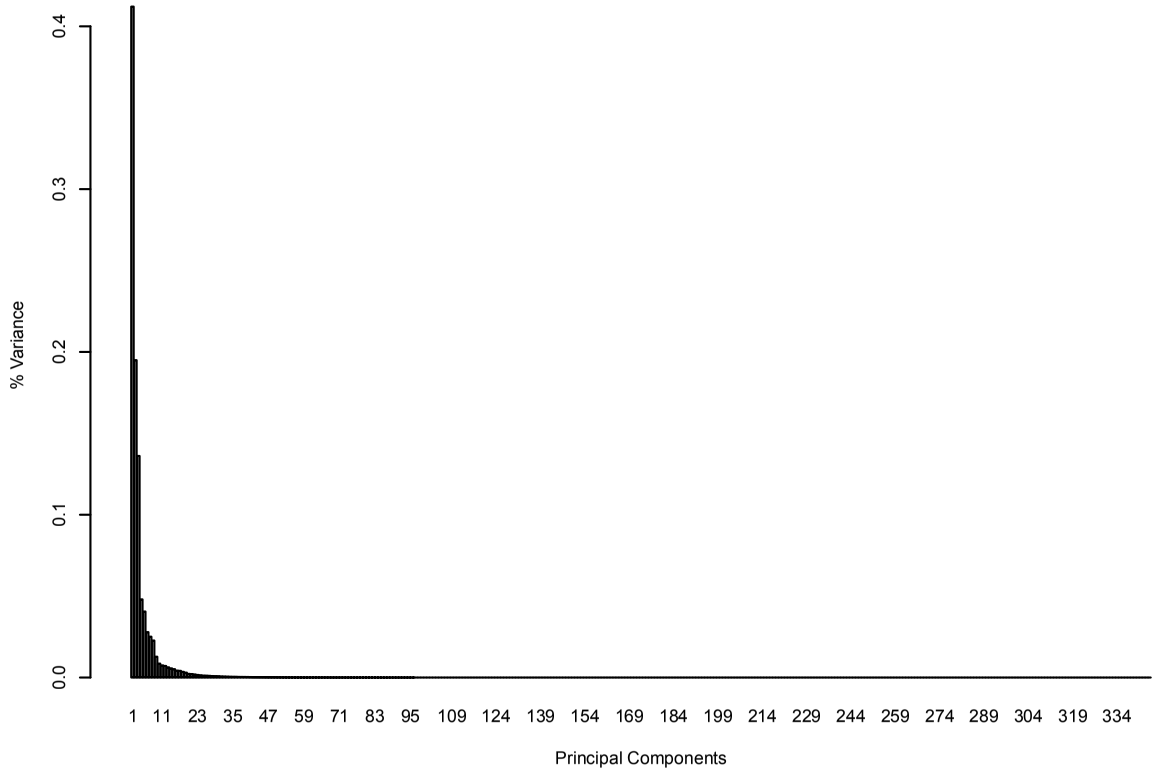


Suppl. Figure S5. Supplementary figure showing mean shape and spread of Procrustes residuals per landmark for (A) SPUR and (B) PETAL datasets





Suppl. Figure S6a. PC scores for SPUR and PETAL datasets.



Suppl. Figure S6b. PC scores for virtual3D dataset.

Chapter 5

Morphological integration of floral modules in *Pelargonium*

Sara J. van de Kerke[1,2], M. Eric Schranz[1], & Freek T. Bakker[1]

[1]Biosystematics Group, Wageningen University and Research, Droevendaalsesteeg 1, 6708 PB, Wageningen, The Netherlands
[2]Hortus Botanicus Amsterdam, Plantage Middenlaan 2A, 1018 DD, Amsterdam, The Netherlands

ABSTRACT
Flowers are functional modules composed of parts that co-operate to make pollination as effective as possible. We explore the *Pelargonium* floral morphospace to address three goals: (1) quantify the amount of morphological variation in *Pelargonium* flowers; (2) determine to what extent the different modules (i.e. show-, reward-, and transfer- apparatus) are integrated; and (3) examine to what extent flower shape is constrained by phylogenetic signal and what the relation is between floral shape and phylogeny. We do this by quantifying floral shape and by calculating integration and modularity for 68 *Pelargonium* species. The results indicate that each of the modules within *Pelargonium* is highly modular but also show high integration among modules within the flower. We show that floral shape is not related to phylogenetic placement, and examples of convergent and divergent evolution are nicely illustrated. We find that relation between floral shape and phylogeny is highly dependent on phylogenetic clade and differs per module.

INTRODUCTION

Flowers are complex structures that are essential for plant reproduction as they increase the fitness of the species by optimising the match with a prospective pollinator (Sattler, 1978). Effective pollination is characterised by a close morphological 'fit' between flower and pollinator, as well as a temporal match (Armbruster et al., 2004; Armbruster and Muchhala, 2009). Pollinators, considered to be drivers of change in floral morphological traits (Berg, 1960; Fenster et al., 2004; Gómez et al., 2014; Ordano et al., 2008; Schiestl and Johnson, 2013), can cause directional selection increasing the morphological integration of floral traits. Morphological integration between traits occurs when changes in one trait induce changes in others within a morphological structure. There is modularity when morphological integration is compartmentalised within specific parts of the structure, called modules (Klingenberg, 2014). The parts that compose these modules can thus be morphologically integrated to varying degrees, often under the direct influence of the type of pollinator (Figure 1; Gómez et al., 2014; Rosas-Guerrero et al., 2014). For example, in specialised pollination systems (by animals) morphological integration is thought to be higher than in more generalist wind- and self-pollinated species (Gómez et al., 2014).

A number of modules are present in all flowers. The 'show' apparatus, the corolla plus in many cases scent, generates maximum effect in attracting as many potential pollinators as possible from some distance as primary visual (and olfactorial) attractor. It is in this apparatus that the most splendid variation in form can be found. The 'transfer' apparatus, consisting of the stamens and stigma, is where transfer of pollen to and from the pollinator is maximised. In most species a third apparatus, containing the pollinator's reward, is present either localised, as nectar glands, or distributed across florets, or contained in a spur. Nectar guides, pointing to the locality of nectar and usually present on petals, are part of the reward unit too. It is the functional as well as ontogenetic synchronisation of these show-, reward-, and transfer-apparatuses that makes them integrated and thus important for effective pollination.

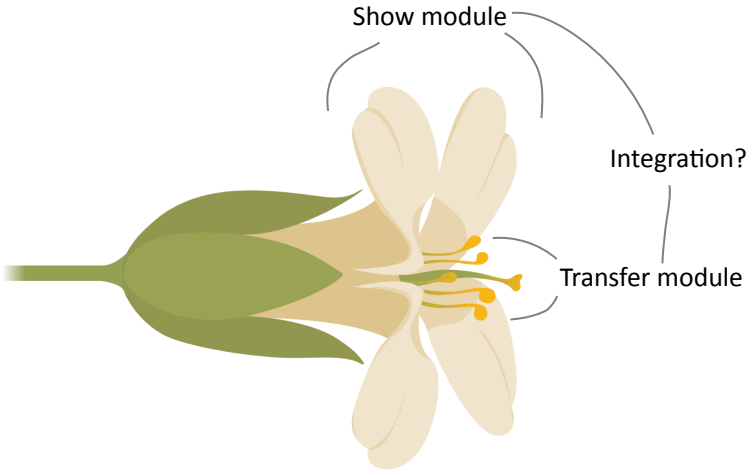


Figure 1. A conceptual visualisation of floral modules (consisting of parts) that may be integrated.

The integrated sets of floral traits that are associated with the attraction of pollinators are united in what has been referred to as pollination syndromes (Faegri and van der Pijl, 2013; Fenster et al., 2004; Dodson, 1966). Syndromes can be selected for in pollination guilds, where convergence of floral shapes and pollinator types can occur (Goldblatt and Manning, 1999; Johnson and Steiner, 2000). In contrast with more generalist pollination syndromes (usually characterised by actinomorphic flowers that are easily accessed by a large range of pollinators; Armbruster and Muchhala, 2009; Harder and Johnson, 2009; Rosas-Guerrero et al., 2014), spur pollination spatially separates reward (the nectar) from the show apparatus or corolla. Well-known examples of spur pollination are *Aquilegia*, where each petal has its own petal-spur, species of Orchidaceae, and *Tropaeolum* (Weberling, 1992; Whittall and Hodges, 2007). Hodges (1997) compared the 15 known 'spurred' Angiosperm clades with their unspurred sisters and found five of them to be larger and to have proliferated more; thus concluding that floral nectar spurs can be considered as evolutionary key innovations. Geraniaceae was the exception as the sister group of *Pelargonium* (i.e. the remainder of Geraniaceae) harbours several times more unspurred species. Whittall and Hodges (2007) hypothesized that speciation in spur pollination can happen in two ways, by gradual change or by jump-like evolution. In the first mode, for a flower – pollinator pair, a gradual change in flower (and pollinator) morphology can occur as the results of selection in order to maximise pollination success, i.e. 'pollinator-filtering', whereby low-efficient pollinators are kept out. A Darwinian 'arms race' between plant and pollinator would follow, increasing effective pollen transfer, and, ultimately, fitness for both. In the second mode, a pollinator shift can happen where the flower 'jumps' to another shape as the result of the presence of a new pollinator or the extinction of an existing pollinator (Whittall and Hodges, 2007).

In addition to pollinator pressure, floral shape change is constrained by phylogeny (Gómez et al., 2016, 2015; Sauquet et al., 2017). A flower in the Eurosid II will not suddenly evolve a daisy-like flower structure and as sister species evolve, it is highly likely they have similar floral shape because they share a close phylogenetic history (Schnitzler et al., 2011). For deep-level patterns this seems straight-forward as the amount of change both in copy number and of synteny of key flower-development gene families like MADS-box or Cycloidia may have changed considerably (Zhao and Schranz, 2019). At the species-level however, gene-copy and synteny have probably not significantly diverged and other mechanisms, such as promoter elements or DNA methylation, control (switches in) floral shape in closely-related species (Corley et al., 2005; Cubas et al., 1999). In addition, because they share a phylogenetic history, it might be more favourable for sister species to ensure a much different shape in order to maximise attractability (van der Niet et al., 2006, 2014). van der Niet et al. (2006) showed that, based on sister-species comparisons in Geraniaceae, Iridaceae, and Orchideae in the Cape Floristic Region, shifts in pollinator system would have occurred after shifts in edaphic conditions. This finding shows the high dynamics of floral shape.

We address these questions using the predominantly South African genus *Pelargonium* (Geraniaceae), known for its stunning floral and vegetative diversity

across its ~280 species (Bakker et al., 2005, 1999; Jones et al., 2009, 2003; Nicotra et al., 2008; Röschenbleck et al., 2014; Struck, 1997). Roughly 70% of the genus occurs in the South African Greater Cape Floristic Region (GCFR; Linder, 2003; Manning and Goldblatt, 2012; Snijman, 2013), considered a hot-bed of plant evolution (Verboom et al., 2009) having led to high levels of endemicity and plant species hyperdiversity. The latter (~9000 species in 90.000 km^2) has been attributed to high rates of speciation driven by adaptation to local environmental conditions and lack of extinction (Goldblatt and Manning, 2002; Linder, 2003). Other *Pelargonium* species occur in eastern Africa, Namibia, the Arabian peninsula, Australia, Asia minor, and St. Helena (Dreyer et al., 1992; Van der Walt et al., 1990; Van der Walt and Vorster, 1983). Phylogenetic relationships within the genus are well known (Chapter 2, van de Kerke et al., 2019).

Pelargonium flowers exhibit specialised pollination syndromes when compared with the remainder of the Geraniaceae clade (i.e. *Geranium*, *Erodium*, *Monsonia* and *California*), as they exhibit a strong zygomorphic corolla shape and possess nectar spurs that are formed adnate to the pedicels. Throughout *Pelargonium*, variation in floral shape appears to occur in a number of aspects. First, and perhaps most striking, the variation in petal copy number altering between five (>95% of species) and four, occurring between and even within a species (i.e. *P. caucalifolium*), two in *P. dipetalum* and zero in *P. apetalum*. Second is the orientation of the petals which ranges from highly zygomorphic (*P. fulgidum, P. acetosum, P. oblongatum*) to almost actinomorphic (*P. cotyledonis*). Also, the shape of the petals varies tremendously: from slender and elongated (*P. paniculatum*) to almost round (*P. inquinans*).

As indicated above, *Pelargonium* has a unique spur pollination syndrome with the spur growing adnate to the pedicel (Albers and van der Walt, 2007; Goldblatt et al., 2000; Hodges, 1997; Hodges and Arnold, 1995; Manning and Goldblatt, 2012; but see Tsai et al., 2018). Whereas this is a synapomorphy for the entire clade, spur lengths vary greatly among species, matching pollinator type. In the ocean-island St. Helena endemic *P. cotyledonis* the nectar spur seems to have almost disappeared, probably due to a switch to a new pollinator species present on St. Helena. While in *P. appendiculatum* the nectar spur is 10 centimetre in length, but no pollinator with such length proboscid is known. In addition, the extent to which the pedicel is 'filled' by the spur differs greatly among species (Chapter 4) and there appears to be a trend towards as long as possible nectar spurs, but being constrained by pedicel length.

Among the spurred clades, *Pelargonium* is unique as it is actually smaller than its sister clade (i.e. the remainder of Geraniaceae), and its spur is a sepal-spur, growing adnate to the pedicel. Bakker & al. (2005), based on parsimony, inferred spur length evolution in *Pelargonium* and concluded bees to have been ancestral and that three switches towards longer-tongued pollinators had occurred during clade proliferation. Based on QuaSSE modelling, an overall trend towards longer spurs could be inferred, but speciation rate and spur lengths were found to be negatively correlated, i.e. highly specialised flowers are found in smaller clades (Ringelberg, 2012).

In order to further explore the phenomenon of modularity within a flower, we wanted to know to what extent the modules within the *Pelargonium* flower are integrated, i.e.

whether they evolve in concert, and whether different, apparatus-specific selective pressures may exist. The goals of this study are therefore to: (1) quantify the amount of morphological variation in *Pelargonium* flowers; (2) determine to what extent the different modules (i.e. show-, reward-, and transfer- apparatus) are integrated; and (3) examine to what extent flower shape is constrained by phylogenetic signal and what the relation is between floral shape and phylogeny. We would expect three integrated apparatus to be present in the *Pelargonium* flower, corresponding with the show-, transfer-, and reward- apparatuses as described above. We expect the transfer- and reward apparatuses to show less individual modularity and be more dependent on each other than either is with the show apparatus, since they are both critical to pollination success and need to work in concert. We expect that overall floral shape is constrained by phylogenetic signal and expect to find specific floral shapes corresponding with clades.

MATERIAL AND METHODS

Flower data sampling and geometric morphometric data collection

Floral shape was compared for 68 *Pelargonium* species obtained from living collections in various locations in Western Europe and South Africa (see Chapter 4 and Supplementary Table S1 for an overview of species, numbers of individuals, and location). Sampling and analysis was as in Chapter 4. The sampling covers approximately 25% of the species in the genus and includes 388 individual flowers. Sets of landmarks include 32 co-planar landmarks covering the corolla shape as well as 10 landmarks and 75 sliding landmarks covering the spur outline and tracking its curvature, as well as that of the shortest, longest and an average stamen (Figure 2). We included as many different corolla shapes as possible, maximally covering the potential morphospace (based on known extreme forms), and not-necessarily representing phylogenetic relationships. For details, see Chapter 4.

Floral shape variation, disparity and integration

All analyses were performed in R v.3.2.2 (R Core Team, 2015) using the Geomorph package v.3.0 (Adams and Otárola-Castillo, 2013). From the 3D coordinates of the landmarks, we extracted shape information using the generalized Procrustes analysis (GPA) superposition method and we calculated the mean shape for each species. We performed a principal component analysis (PCA, Figure 3) to explore floral shape variation across all *Pelargonium* individuals as well as for mean species shape. For each individual in the dataset, we extracted relative spur and pedicel length using the function 'interlmkdist'. We calculated the ratio between the spur and pedicel length as a measure for the 'filling' of the pedicel by the spur. This ratio is visualised in the PCA plot as the size of the dot corresponding to the individual (Figure 3A&B), and ratio frequencies are plotted in Figure 3D.

To test whether there are statistical differences in floral shape between species present in the data, we used the Procrustes ANOVA function with the F-test to test for significant differences in shape between species based on Procrustes distances (Goodall, 1991). The advanced Procrustes ANOVA test was used for pairwise comparisons between species. Statistical significance of the ANOVA was assessed using 500 random permutations using RRPP (Residual Randomization, Collyer et al.,

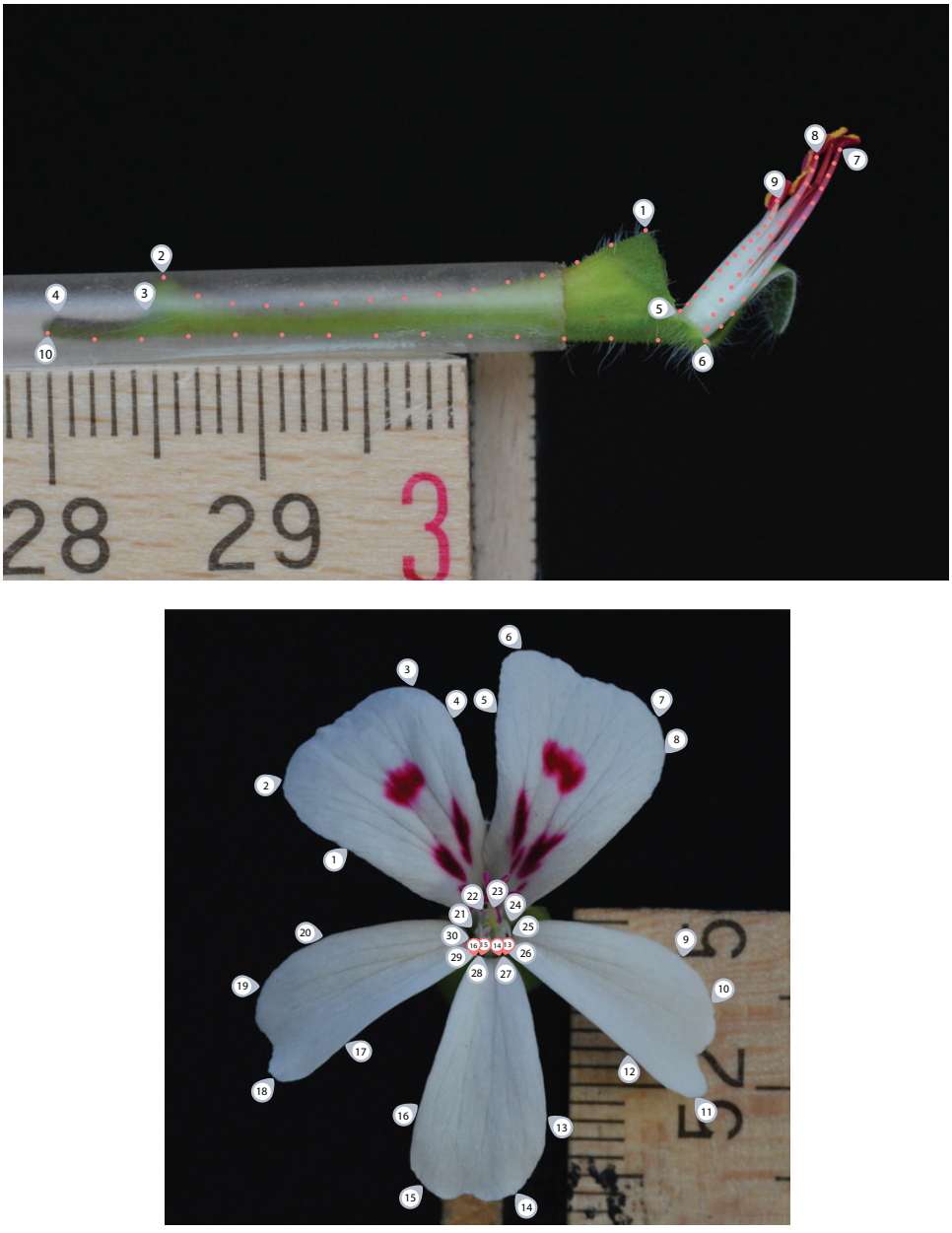


Figure 2. Landmark placement for the SPUR (A) and PETAL (B) datasets. For the SPUR dataset 10 landmarks and 75 sliding landmarks covering the spur outline and tracking its curvature, as well as that of the shortest, longest and an average stamen (grey labels) are defined. For the PETAL dataset 32 landmarks were placed along the outline of the corolla and the opening of the nectar spur using midrib, primary and secondary veins and petal attachment as a guide (grey labels). For specimens with four petals, we assumed that for the middle anterior petal the meristem is present but does not develop and landmarks allocated for this petal were placed with zero length from the petal base (red labels).

2015). We tested whether there is modularity between show-, transfer-, and reward-apparatus using 'modularity.test'. This function quantifies modularity in partitions of Procrustes aligned coordinates using the average pairwise covariance ratio (CR) coefficient (the ratio of the covariation within and between modules, Adams, 2016) and test this distribution against randomly chosen subsets of coordinates. We then tested whether there is morphological integration between show-, transfer-, and reward-apparatuses by using the function 'integration.test', and all combinations of modules using the 'two.b.pls' function. These functions quantify the extent to which partitions of Procrustes aligned coordinates are morphologically integrated, based on a partial least squares analysis of trait covariation (Adams and Collyer, 2016).

Phylogenetic relationships

In order to explore correlation with DNA-based phylogenetic relations in our floral shape dataset, we used the time calibrated phylogenetic tree as constructed by van de Kerke et al. (Chapter 2; 2019) as basis for our comparative study. Some species in our morphological dataset were not present in the van de Kerke et al. (Chapter 2; 2019) study. When possible, we grafted these species to their sister species based on earlier phylogenetic and taxonomic findings (Bakker et al., 2005; Becker and Albers, 2009; Jones et al., 2009; Nicotra et al., 2016; Röschenbleck et al., 2014; van der Walt, 1985). When sister-species relations were not exactly known (i.e. were part of a polytomy), the species was included in the clade corresponding to that known polytomy, in a sister group position. Otherwise, the species was grafted to the base of the clade representing their taxonomic section sensu Röschenbleck et al. (2014) since van de Kerke et al. (Chapter 2; 2019) found these to correspond (Supplementary File S2). We chose to include the species in this way because subsequent analyses require fully bifurcating phylogenetic trees. We trimmed the resulting phylogenetic tree to only include species present in the morphological dataset. These analyses were performed using the packages 'phytools' and 'APE' in R (Paradis et al., 2004; Revell, 2012). Supplementary Figure S3 shows the phylogenetic tree as used in this study.

Phylogenetic signal

To further explore the relation between floral shape and phylogeny, and to assess to what extent the show-, transfer-, and transfer-apparatuses are influenced by it, we calculated the phylogenetic signal we find in the overall data and for the modules separately. We calculated the K_{mult} statistic (Adams, 2014) using the function 'physignal' in the Geomorph R package to find to what degree phylogenetic signal constrains floral shape in general (Adams and Otárola-Castillo, 2013). The *K* statistic is based on Blomberg 2003's *K* statistic test and is related to Pagel's (1999) covariance-based lambda statistic. Basically, the K_{mult} statistic measures phylogenetic signal in highly multivariate data such as comparative analysis of shapes (Adams, 2014). A value of $K = 0$ indicates there is no influence of phylogenetic signal on the shape data, $K < 1$ indicates less influence of phylogenetic signal than can be expected under Brownian motion, and $K > 1$ indicates more influence of phylogenetic signal than can be expected under Brownian motion. The statistical significance of *K* was calculated for the total shape dataset and show-, reward-, and transfer functional modules separately based on 10000 iterations.

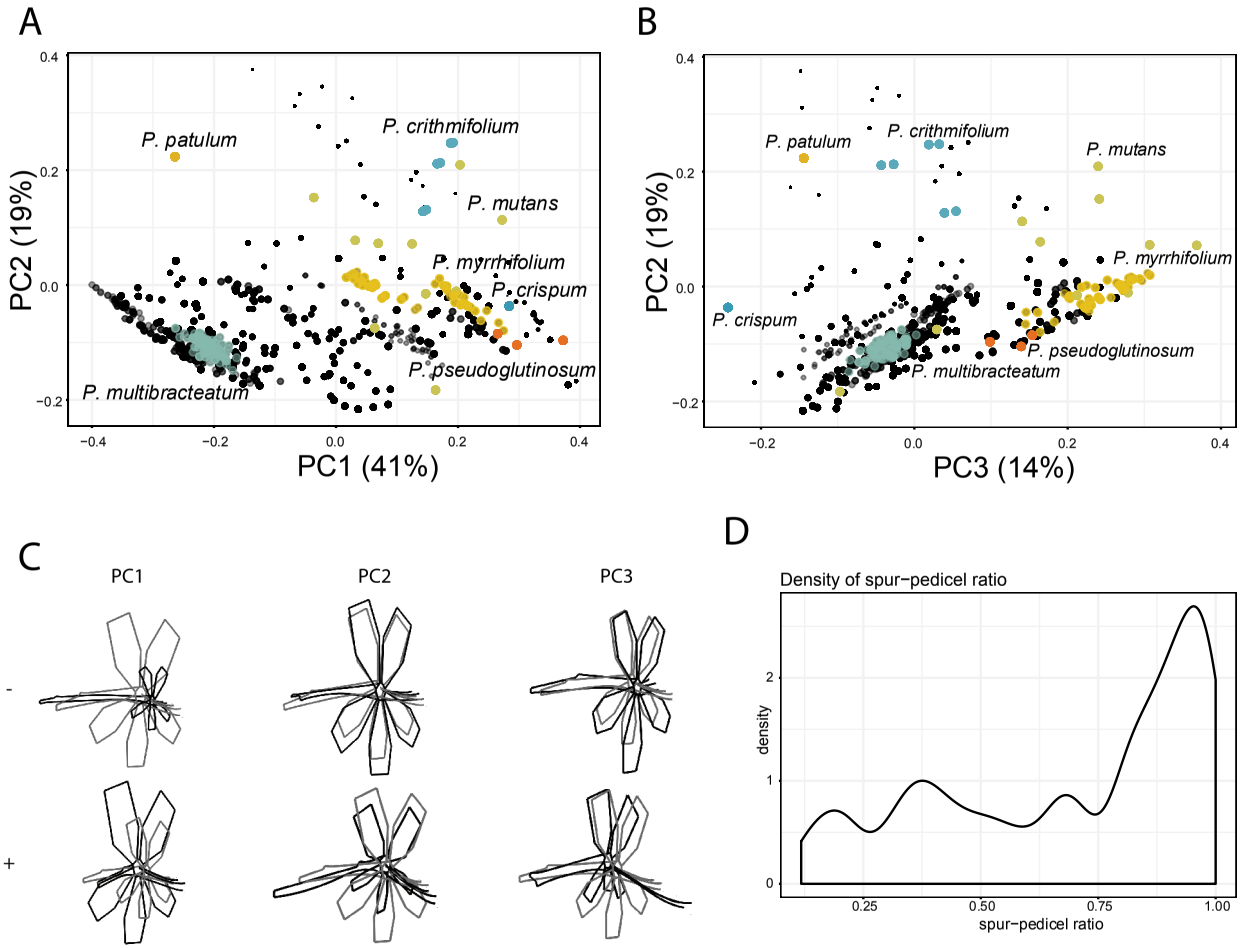
RESULTS

Variation in floral shape in a phylogenetic perspective

Figure 3 presents results of the Principal Component Analysis (PCA) on the GPA aligned coordinates of virtual individuals as in Chapter 4. The variation in shape explained by the first PC axis corresponds with a strongly zygomorphic corolla, with individuals on the negative side having a smaller corolla relative to the length of the pedicel and on the positive end a relatively large corolla (Figure 3C). PC2 corresponds with the length and curvature in stamens, with individuals on the negative end showing straight stamens and on the positive end highly curved ones. PC3 (14%) again (as in PC1) appears to correspond with the filling of the pedicel by the spur as well as the length and orientation of the stamens. Overall, a pattern seems to emerge of individuals distributed around the morphospace but avoiding certain areas. From the PCA in Figure 3A especially *P. patulum* appears to be rather isolated, although this is put in perspective in the PCA plot (Figure 3B) where it groups with individuals from other species. The reverse seems to be the case for *P. crispum*, which is surrounded by individuals from other species in Figure 3A but is quite isolated in the alternative perspective of Figure 3B. A clearly 'avoided area' in Figure 3A is the lower left corner, which corresponds with the lower right corner in Figure 3B. Here we see what appears to be a shape boundary individuals cannot cross, with filling ratios of the pedicel by the spur, whereby individuals at this boundary have a ratio approaching 1. As Figure 3D presents (showing relative frequencies of filling ratios), we find that higher ratios are more frequent than lower ones. For details, see Chapter 4.

When we examine the phylogenetic distribution of shapes (Supplementary Figure S4, S5, and S6), we find that all species are to some degree spread along these first 2 PC axes. Clade C2 in particular appears to be localised on the lower end of both PC1 and 2, while clade C1 is oriented more towards the positive end of PC1 and also slightly more along PC2. Clade A1 in contrast seems to have a more conservative distribution along the first PC axis (with a few outliers) and be more variable along PC2. Clade B and A2 are more evenly distributed along both axes. When we compare clade pairs C1-C2 and A1-A2, we find that certain areas in the morphospace are clade specific, while there is an overlap region of shapes occupied by both clades as well as outliers on both sides.

Figure 3. ***(next page)*** PCA analysis. (A) PC1 and PC2 and (B) PC3 and PC2. Colours correspond with selected species: *P. multibracteatum* (light blue) *P. triste* (brown), *P. mutans* (grey), *P. myrrhifolium* (yellow), *P. patulum* (brown), *P. crithmifolium* (dark blue), and *P. pseudoglutinosum* (orange). Intensity of colours indicated number of individuals stacked. Size of the dots corresponds with the 'filling' of the pedicel by the spur, as calculated by the ratio between the length of spur and pedicel. (C) Shape outlines corresponding to extremes on axes for PC1, PC2, and PC3 showing calculated mean shape (grey) and warped extreme shape (black). (D) Frequency plot of ratio between the length of spur and pedicel.


A
P. patulum
P. crithmifolium
P. mutans
P. myrrhifolium
P. crispum
P. pseudoglutinosum
P. multibracteatum
PC2 (19%)
PC1 (41%)
B
P. patulum
P. crithmifolium
P. mutans
P. myrrhifolium
P. crispum
P. pseudoglutinosum
P. multibracteatum
PC2 (19%)
PC3 (14%)
C
PC1
PC2
PC3
-
+
D
Density of spur−pedicel ratio
density
spur−pedicel ratio

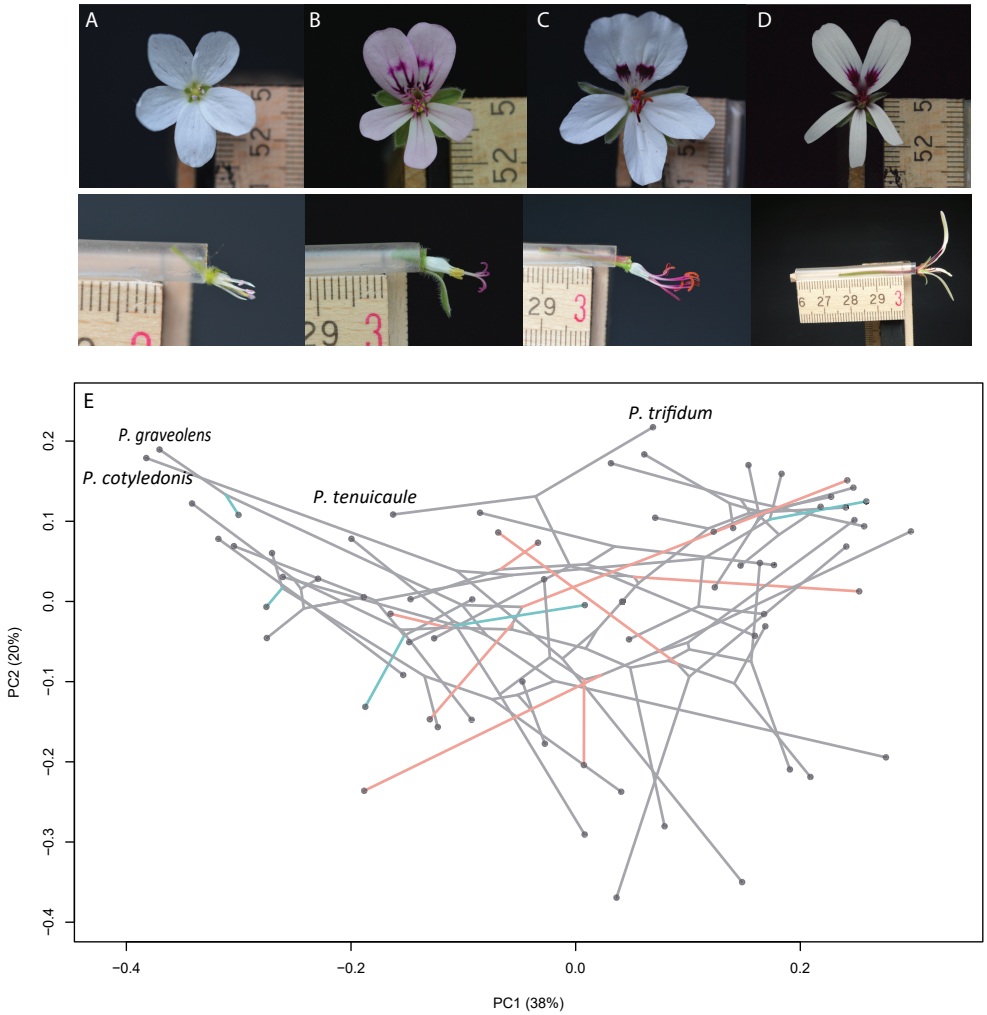


Figure 4. Phylomorphospace showing the relationship between phylogenetic relatedness and floral shape of *Pelargonium* species included in this study. (A) *Pelargonium cotyledonis* (taxonomic section *Isopetalum*, clade B). (B) *Pelargonium graveolens* (taxonomic section *Pelargonium*, clade A1). (C) *Pelargonium tenuicaule* (taxonomic section *Jenkinsonia*). (D) *Pelargonium trifidum* (taxonomic section *Jenkinsonia*). (E) PC 1 and 2 of morphospace per species (calculated average across individuals). Colours indicate confidence in grafted terminals with green: based on sister-species relation and red: based on section.

Figure 4E shows the morphospace per species (calculated average across individuals) including the reconstructed phylogenetic tree, illustrating variation in floral shape found in *Pelargonium* in a phylogenetic framework. We find a number of sister-species pairs residing in rather different parts of the morphospace, while we also encounter species occupying the same area in the morphospace that are not closely related phylogenetically. For example, the island endemic *P. cotyledonis* (clade B, Figure 4A) and *P. graveolens* (clade A1, Figure 4B) are not closely related but occupy the same region in the morphospace. Both species have a short pedicel and a rather short spur and rounded, quite evenly distributed, petals. On the other hand, *P. tenuicaule* (Figure 4C) and *P. trifidum* (Figure 4D) are sister species in our reconstructed phylogenetic tree (and in other studies occupy the same taxonomic section, *Jenkinsonia* (Röschenbleck et al., 2014; Chapter 2; van de Kerke et al., 2019)), but occupy different, non-overlapping, parts of the morphospace. *P. trifidum* has an elongated pedicel and spur and narrower petals than *P. tenuicaule.*

In Figure 5 we plotted phylogenetic distance (measured as sum of branch lengths between tips) against morphological distance (as calculated by the pairwise advanced ANOVA) per species. We found that morphological distance varies for all phylogenetic distances. The taxa with the largest phylogenetic distance are represented in 'band' of individuals at the utmost right. Here, distance between tips includes the deepest node in *Pelargonium*. All other band represent other, shallower nodes and corresponding distance. To the left, we find distances between sister species and other close relations. For some species pairs we could assess divergent evolution patterns as for example for *Pelargonium parviflorum - P. carnosum* and *P. carnosum - P. laxum*. For other species pairs, we find they are highly similar in their morphology while being phylogenetically not closely related, suggesting convergent evolution. Examples of this are *P. tongaense - P. abrotanifolium*, *P. abrotanifolium - P. desertorum*, and *P. desertorum - P. aridum*.

Morphological modularity and integration

We find that the CR coefficient calculated to test for modularity within our dataset for our partitioning over show-, transfer-, and reward-apparatuses was significantly lower than 1 (CR = 0.6591, P = 0.001). This indicates that there is independence between the show-, transfer-, and reward-apparatuses and that there is no other, randomly assigned, alternative partitioning for *Pelargonium* plausible.

For overall floral shape over the partitions of show-, transfer-, and reward-apparatuses we find that morphological integration was large and highly significant (Table 1). In addition, we find that for all pairwise combination of show-, transfer-, and reward-apparatuses, morphological integration was large and highly significant (Table 1).

Table 1. Integration between overall floral shape and combinations of show-, transfer-, and reward-apparatuses.

	Overall	Spur	Stamens
Overall	0.817 (p=0.001)		
Corolla		0.936 (p=0.001)	0.761 (p=0.001)
Spur			0.813 (p=0.001)

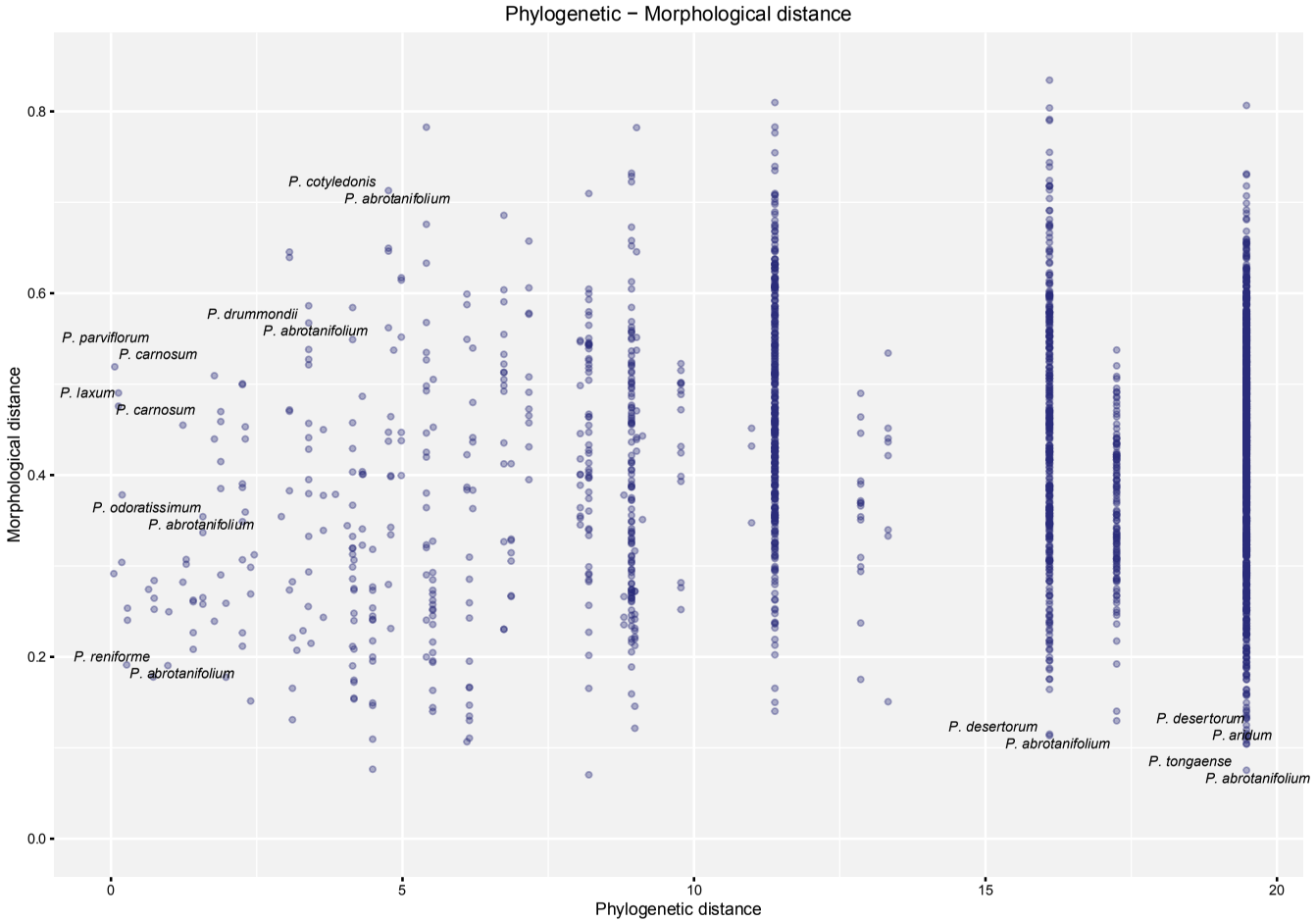


Figure 5. Dotplot showing phylogenetic distance against morphological distance (as calculated by the pairwise advanced ANOVA) per *Pelargonium* species included in this study.

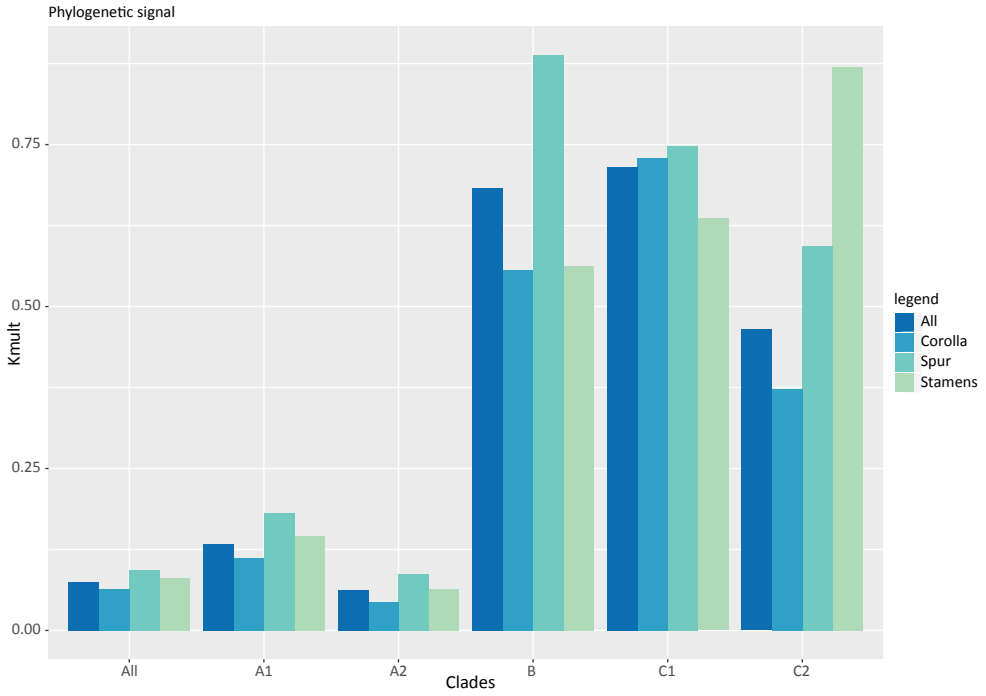


Figure 6. Barplot showing Kmult values for *Pelargonium* species included in this study for the modules corolla (light blue), spur (turquoise), stamens (light green), and all combined (dark blue) per phylogenetic main clade.

Phylogenetic signal in modules

We wanted to further explore the relation between floral shape and phylogeny, and assess to what extent the show-, transfer-, and reward-apparatuses are constrained by it. We therefore have calculated the phylogenetic signal we find for the overall floral shape data and the three modules separately for all species and per main clade (Figure 6). We find that for all species combined there is no phylogenetic pressure on floral shape, but that there are huge discrepancies between main clades. Clades B, C1, and C1 display a K_{mult} value of around 0.5 and higher for all modules indicating there is limited but significant influence of phylogenetic signal on floral shape. For the clades A1 and A2, harbouring extensive variation both morphologically and phylogenetically, K_{mult} values are close to zero, indicating an absence of phylogenetic constraint in the shape data for these clades.

DISCUSSION

In this study, we (1) quantify the amount of morphological variation in *Pelargonium* flowers; (2) determine to what extent the different modules (i.e. show-, reward-, and transfer- apparatus) are integrated; and (3) examine to what extent flower shape is constrained by phylogenetic signal and what the relation is between floral shape and phylogeny. Combined, this work provides new insight in the coordinated evolution of modules and their phylogenetic constrains.

Morphological variation

The range of floral diversity found in *Pelargonium* is immense, including varying numbers of petals from five (most species in Geraniaceae), to four (i.e. *P. caucalifolium*), two (i.e. *P. bipetalum*), and even zero (*P. apetalum*), corolla shapes varying from highly zygomorphic to almost actinomorph (i.e. *P. cotyledonis*), and pedicel length ranging from zero to 100 mm (i.e. *P. appendiculatum*). In addition, there is extensive petal nectar guide and colour variation (which was not taken into account in this study).

Indeed, the variation in floral shape as depicted in Figure 3 seems to have captured the variation in *Pelargonium* flowers outlined above. The elongation of the spur and size of the corolla are the most variable traits among the species included in the morphospace (PC1, 41%). This trait corresponds with the unique spur pollinator syndrome featured in *Pelargonium* and correlates with their highly variable pollinator types (Struck, 1997, 1994). Spur length evolution appears highly dynamic, with in some species the spur almost completely missing, as for example in *P. hirtum* (which has 3 mm short spurs) while in its closely related *P. appendiculatum* (probably pollinated by long-tongued hovering flies) the spur is elongated to almost ten cm length (Struck, 1997). Along PC2 (19%) we found a shift of stamen shape ranging from short and straight to long and curved. This corresponds with the variety of stamen shape in *Pelargonium*: the species with long and curved stamens are pollinated by long-tongued, hovering insects (such as species from the *Tabanidae*, *Bombyliidae*, and *Nemestrinidae;* Struck, 1997) while the short and straight stamens correspond with the association with short-proboscid, landing pollinator species (such as *Anthophoridae*, *Megachilidae*, and *Vespidae*). Figure 3 further illustrates a clearly 'avoided area' that individuals do not seem able to enter, which corresponds with filling ratios of the pedicel by the spur. At this boundary, individuals have a ratio approaching 1 which would suggest there is no pedicel left for the spur to further increase in size. In this sense, the pedicel length forms a clear physical boundary constraining spur length. In addition, there seems to be a trend towards higher filling ratios (Figure 3D), implying an overall trend towards as long as possible spur lengths. See Chapter 4 for more detail.

We explored the relation between floral shape and phylogenetic placement in detail by plotting the reconstructed phylogenetic tree in the morphospace resulting from a PCA analysis on the mean shapes per species (Figure 4). Here we found there is no patterning of species that are phylogenetically closely related. Examples of convergent and divergent evolution are nicely illustrated. For example, the distantly related *P. cotyledonis* (Figure 4A) and *P. graveolens* (Figure 4B), have

similar converging shapes. This is somewhat surprising, as *P. cotyledonis* has long been considered to be a morphological outlier in the *Pelargonium* floral spectrum. Being isolated on the remote island of St. Helena, *P. cotyledonis* is hypothesised to have 'reverted back' to a base *Geranium*-like shape in the absence of pressure by a clear pollinator. This is reflected in the almost actinomorphic distribution of petals in the corolla, the white petal colouration as well as the virtual loss of the, for *Pelargonium* so characteristic, nectar spur (Bakker et al., 1998; Van der Walt and Vorster, 1983). The closeness of *P. graveolens* in the floral morphospace we obtain, appears predominantly to be the result of the length of the pedicel and nectar spur related to the size of the corolla. These features approximate *P. cotyledonis.* Also, the shape of the petals as we have defined them here is strikingly similar.

On the opposite side of the spectrum, we found *P. tenuicaule* (Figure 4C) and *P. trifidum* (Figure 4D) with highly diverged floral shapes. A clear difference between these species is the length of the pedicel, which is much elongated in the case of *P. trifidum*. This difference in pedicel and spur length could reflect differences in primary pollinators. Struck (1997) determined *P. tenuicaule* to be part of a floral guild of *Pelargonium* species that are primarily or exclusively pollinated by the bombyliid fly *Megapalpus capensis*, which is characterised by a fairly short proboscid (reflected in the 'limited' pedicel and spur length of the flower). The exact pollinator species of *P. trifidum* is yet unknown, but could be one of the long-proboscid hovering flies that are known to pollinate other species of this section (Struck, 1997).

We did not see a clear patterning of species from a main clade occupying a particular part of the morphospace (Supplementary Figure S3,4, and 5). However, when looking in more detail we did find that the two sister-pairs of main clades (A1-A2 and C1-C2) in some degree are divided in the morphospace. The individuals belonging to clade C2 dominate the lower left quartile of the PC plot, while the individuals of clade C1 tend to be retrieved toward the positive ends of both PC axes, albeit with a large transition zone. We found the same phenomenon over PC1 for clades A2-A2, with a massive outlier in the avoided area for clade A1 by *P. lanceolatum*.

The finding that there is no direct correlation between phylogeny and floral shape is corroborated when we plot phylogenetic versus morphological distance (Figure 5). We can clearly see there that an increased phylogenetic distance between species does not mean they are morphologically more diverse. Rather, morphological distance seems to be highly dynamic. This is illustrated by the species *P. abrotanifolium*, which shows a wide variety in morphological distance even with relatively closely related species as *P. cotyledonis* and *P. drummondii* while over a larger phylogenetic distance (i.e. toward *P. desertorum* and *P. tongaense*) morphological distance is remarkably low. This would suggest phylogenetic placement is not a strong driver of floral shape and that changes in floral shape can happen independently from phylogenetic signal, a finding already hinted at by previous studies comparing sister species in *Pelargonium* (van der Niet et al., 2006).

We have to add the critical note that petal reflexion, which is known to be an important aspect of *Pelargonium* shape, was not accounted for in our GMM analyses. In addition, these results are often based on a single individual flower for both datasets. Within species variation is therefore not yet accounted for. When we are able to do so, we might find a shift in the morphospace.

Modularity in concert?

Shifts in floral shape can be caused by pollinator pressure. In the case of the specialised spur-pollinator syndrome, we are uncertain to what extent the different modules (show-, transfer-, and reward- apparatuses) are influenced by this pressure. To some extent, these apparatuses have to be integrated to maximise fertilisation success. These structures could be highly modular and all take their own evolutionary path, but this would probably not increase the fitness of the overall plant. When there is conflict between the optima of the different functional modules, or when they work in such a way that they start to oppose one another, this modularity is no longer in the species best interest and will thus be constrained.

We indeed found modularity within our floral data when partitioned over show-, transfer-, and reward- apparatuses, which suggests there are three evolutionary layers 'happening' alongside each other. Each of these lines will undergo evolutionary pressure by a range of causes and each of these lines will thus undergo changes in shape. However, since we also found strong integration between these modules, independent evolution only goes to a certain extent and evolution therefore appears to be a sort of balancing act between the show-, transfer-, and reward-apparatuses. Because what may be beneficial for one of the apparatuses, may be sub-optimal for the others. This interdependency of modules can be explained by the 'tailored fit' around the pollinator that is needed to ensure optimal pollination. All spatial aspects of the pollinator need to be met by the flower in order to maximise fitness. To some extent we saw this occurring within the reward apparatus (Figure 3). There appears to be a tendency toward longer spur lengths because this would be beneficial in securing pollinators. Long spurs are associated with a specialised pollination syndrome, acting as 'pollinator-filters'. However, in *Pelargonium* spur length will always be constrained by pedicel length. The length of the pedicel ultimately determines the maximum length of the spur, since a spur longer that the pedicel is not a possibility within *Pelargonium* (as it is in other flowering clades as *Aquilegia*). Therefore, the spur evolutionary trajectory will always be in some way limited by the pedicel evolutionary trajectory.

This raises the question whether there is an optimum for pedicel length? The pedicel is a hugely important floral structure of which the length is known to be influenced by temperature and differences in the *LEAFY* gene expression (Catley et al., 2002; Yamaguchi et al., 2012). Whether pedicels in *Pelargonium* are involved in the Darwinian arm's race as suggested by Whittall and Hodges (2007) for spurs, will be interesting to test.

In addition to defining spur length, the pedicel also plays a critical role in the positioning of the flower. During development of the inflorescence, the individual flowers develop one by one and successively reach full anthesis. However, this is

of no meaning without the pedicel to lift the flower up and orient them in the position that is most beneficial for pollinator access. We saw as a result there are large differences in pedicel orientation, ranging from short and straight to long and curved (in effect turning the flower upside-down).

Alternatively, and in a less adaptationist interpretation, an explanation for shifts in floral shape can possibly be found in population genetics. Floral shape shifts could be the result of genetic drift within small, isolated populations (effectively forming islands), indeed a feature of many *Pelargonium* species in the winter rainfall region. Due to reproductive isolation, changes in morphology can then more easily become established in the population. This could ultimately lead to speciation. We know that in *Pelargonium* post-zygotic barriers such as cytonuclear incompatibility and male sterility are a major influence on reproductive isolation (Chase, 2007; Weihe et al., 2009). The variety in floral shape we found in our data might therefore not only be driven by pollinator pressure, but simply be the result of chance.

Phylogenetic signal in modules

From a Geometric morphometric analysis perspective we found modules within the overall flower structure seem to be highly integrated, and we assessed the influence of phylogenetic signal on this data. We found that the relation between floral shape and phylogeny is highly dependent on clade and differs per module.

For example, stamen shape, when measured over all clades, has an extremely low K_{mult} value, indicating variation in shape is completely random and not under selective pressure. However, when we zoom in at the main clade level we found similar results for the A1 and A2 clades, but an increase of the K_{mult} value for the B, C1, and C2 clades. We found the same pattern for the corolla and spur. Surprisingly enough, it appears to vary across modules in which main clade selective pressure is highest. For stamens it is clade C2, for spur B, and for corolla C1 (although not significant).

So the B, C1, and C2 clades appear to be under phylogenetic constraint, which, as we have seen in the PCA analysis, does not result in different shapes. Rather there appears to be a path towards the 'typical' *Pelargonium* flower. Both clade A1 and A2 on the other hand do not appear to be under any type of phylogenetic pressure at all. Rather their form and potential shifts therein appear to happen at random. This finding is reflected in the morphospace (Supplementary Figure S4, S5, and S6) where we saw an enormous spread in distribution of A1 and A2 species.

A possible explanation for this finding can be found in the relative young age of the *Pelargonium* 'winter-rainfall' clade A (Chapter 2; van de Kerke et al., 2019). *Pelargonium* is found to have originated around 9.7 Mya, which would set the origin of clade A at 5.7 Mya (with 4.5 Mya for A1 and 4.8 for clade A2). Within this clade, itself being a radiation of species and main life forms, a second, non-adaptive, radiation consisting of geophytic species from *P.* sect. *Hoarea*, is thought have occurred (Bakker et al., 2005). These 'nested radiations' are associated with the high number of growth forms found in *Pelargonium* (Bakker et al., 2005; Chapter 2; van de Kerke et al., 2019; Verboom et al., 2009), and could also be correlated

with the diversity of floral shapes found in this clade. Clades B, C1, and C2, being older and less speciose, could have had time to 'settle down' or canalise within their phylogenetic constraints on floral shape, converging around a 'typical' *Pelargonium* flower. While under the influence of this double burst of increased speciation rate in clades A1 and predominantly A2, floral shape could hitherto be highly dynamic. One explanation for this finding is that floral shape happens 'just because', i.e. not as adaptation. As if the clade is exploring all possibilities available in the morphospace at random, and time will filter out all freak accidents. Time will 'weed out' all experiments that have gone bad or will not stand a chance (bad horses). Another explanation could be that this highly dynamic floral shape is the result of high mutation rates in *Pelargonium,* especially in the mitome (Bakker et al., 2006; Mower et al., 2007) that could lead to rapid reproductive isolation (post-zygotic).

This finding also raises the question to what extent *Pelargonium* flowers are adapted to their pollinators. Given the lack of correlation between phylogenetic signal and floral shape in clades A1 and A2, to what extent can this variation be explained by adaptation to potential pollinators? One might hypothesise about the relation between floral shape and pollinator in the light of mate choice, i.e sexual selection. Because the pollinator is the primary modus for the flower to ensure fertilisation, transmission of the pollen by the pollinator in a sense is the way two flowers mate. The sexual attraction in this system is not flower-flower, but rather flower-pollinator, which would mean the flower has to 'seduce' the insect and that sexual selective forces may be in place.
We found the relation between flower and pollinator in *Pelargonium* to be highly specialised in the spur pollination syndrome. We showed this is not the only variation present in *Pelargonium*. There is variation in petal size and shape, curvature of the pedicel, petal nectar guides, size of the opening of the spur. This raises the question, what is all that variation for? We suggest this variation is a result of the mate-choice of the pollinators. *Pelargonium* occurs in the highly competitive environment of the Cape Floristic Region where species have to fight for a select range of available pollinator species. In some way, the species have to ensure their flowers are more attractive than those of the competing flowering plants nearby. Much like birds have developed their extensive range in sexually attractive displays. For example, the dense inflorescences some species in the *Pelargonium* sect. *Hoarea* form might in fact be a way to form a type of 'superflower' that is more attractive for potential pollinators. When the pollinator has been seduced to approach this superflower, the more close-contact signals kick-in to direct the pollinator to the flower.

CONCLUSION

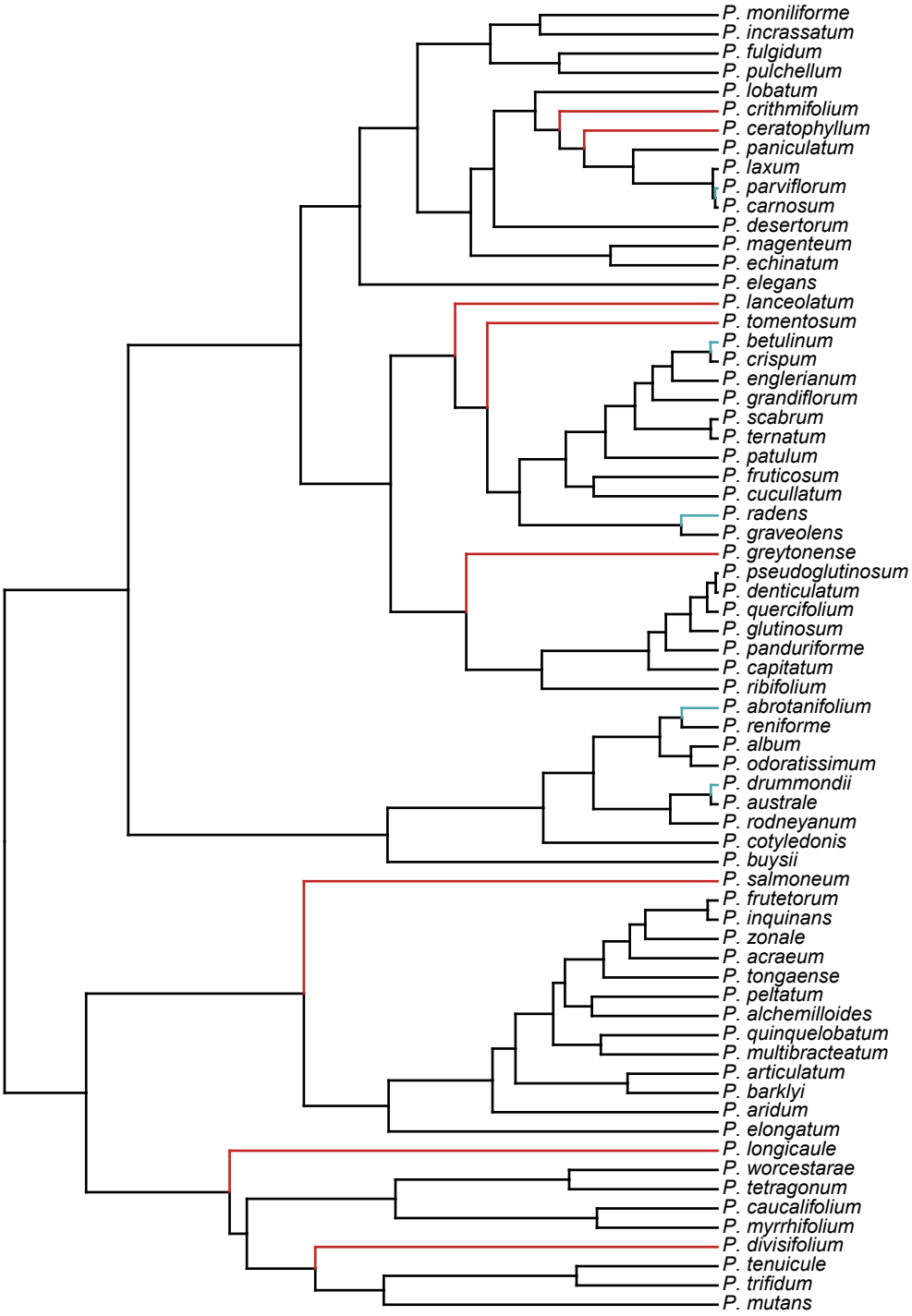
We explored the relation between floral shape and phylogenetic placement in detail by plotting the reconstructed phylogenetic tree in the morphospace resulting from a PCA analysis on the mean shapes per species (Figure 3). Here we found there is no patterning of species that are phylogenetically closely related. The finding that there is no direct correlation between phylogeny and floral shape is corroborated when we plot phylogenetic versus morphological distance. This finding suggests that the morphological structures within these modules are more integrated with each other than between modules. We found that relation between floral shape and phylogeny is highly dependent on phylogenetic clade and differs per module.

ACKNOWLEDGEMENTS
Many thanks to Walter den Hollander, Reinout Havinga and Carlien Blok from the Hortus Botanicus Amsterdam for taking in and maintaining the *Pelargonium* collection. Many thanks to Robin van Velzen for his help with R and Niké Groot for proofreading a version of this manuscript.

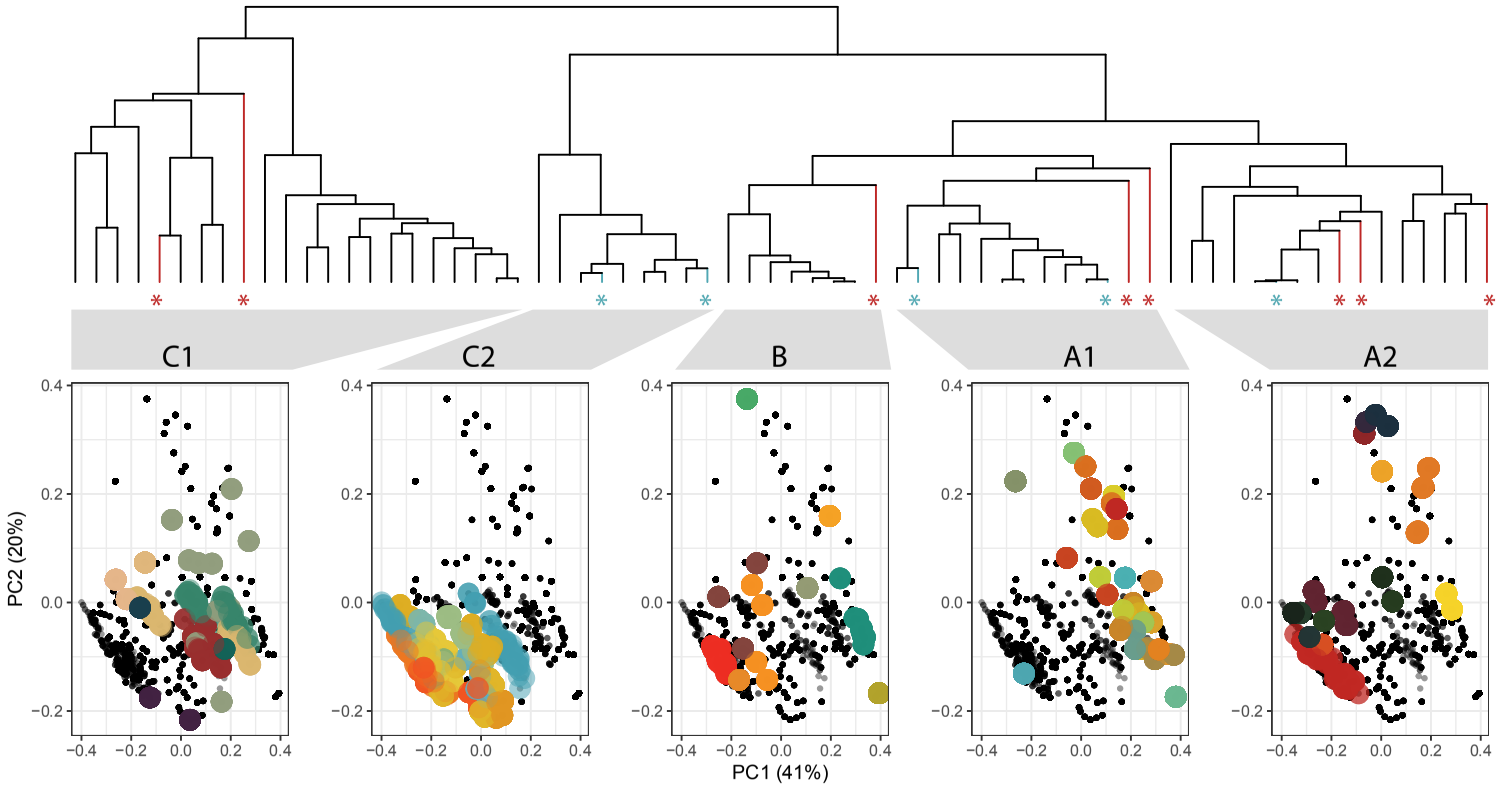
Supplemental Files

Suppl. Table S1
Taxonomic sampling of species included in the study. Including locality data and individual number/STEU number when available. Available upon request.

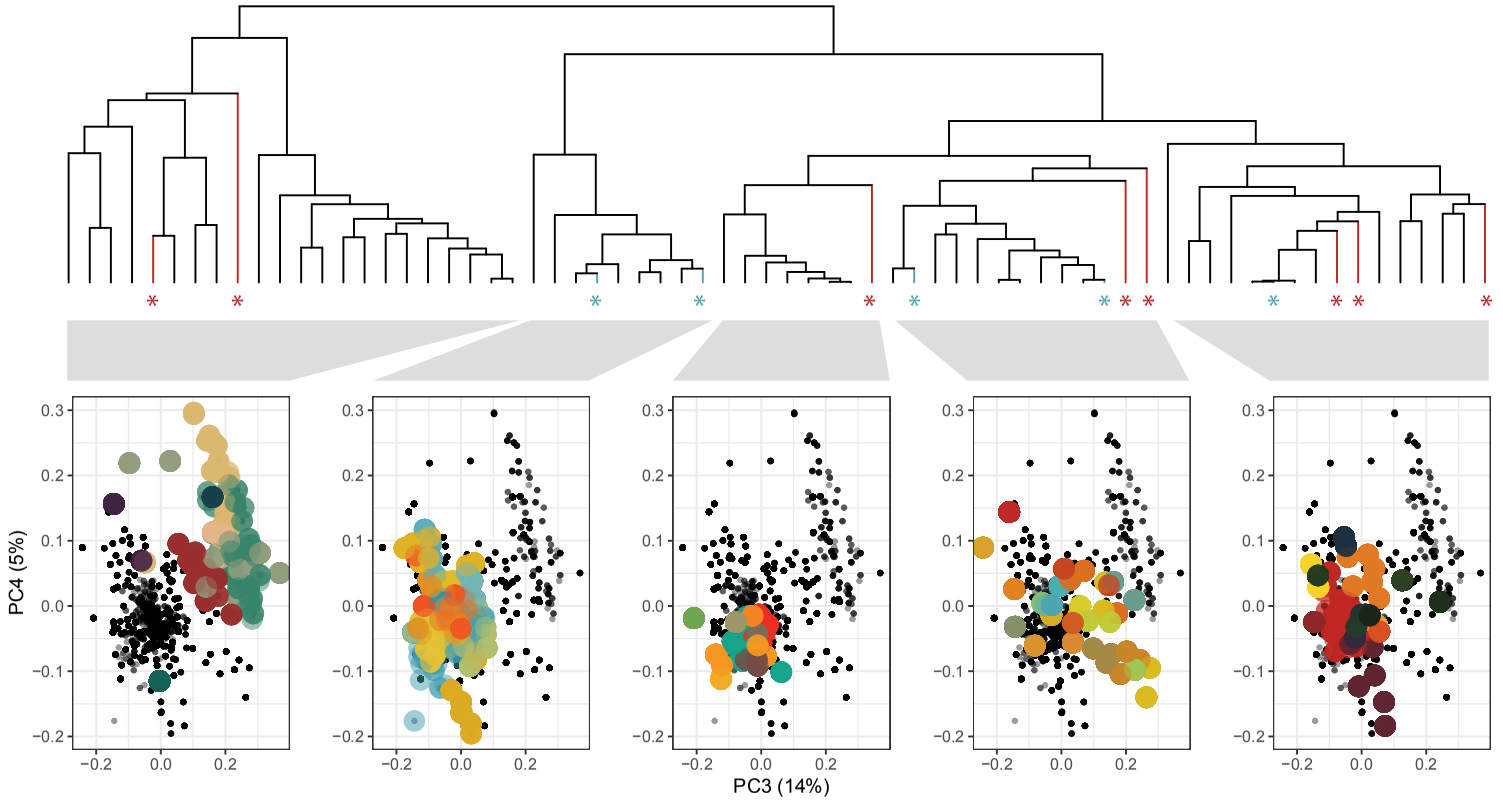
Suppl. Table S2
Overview of positioning of species grafted to the phylogenetic tree. Available upon request.



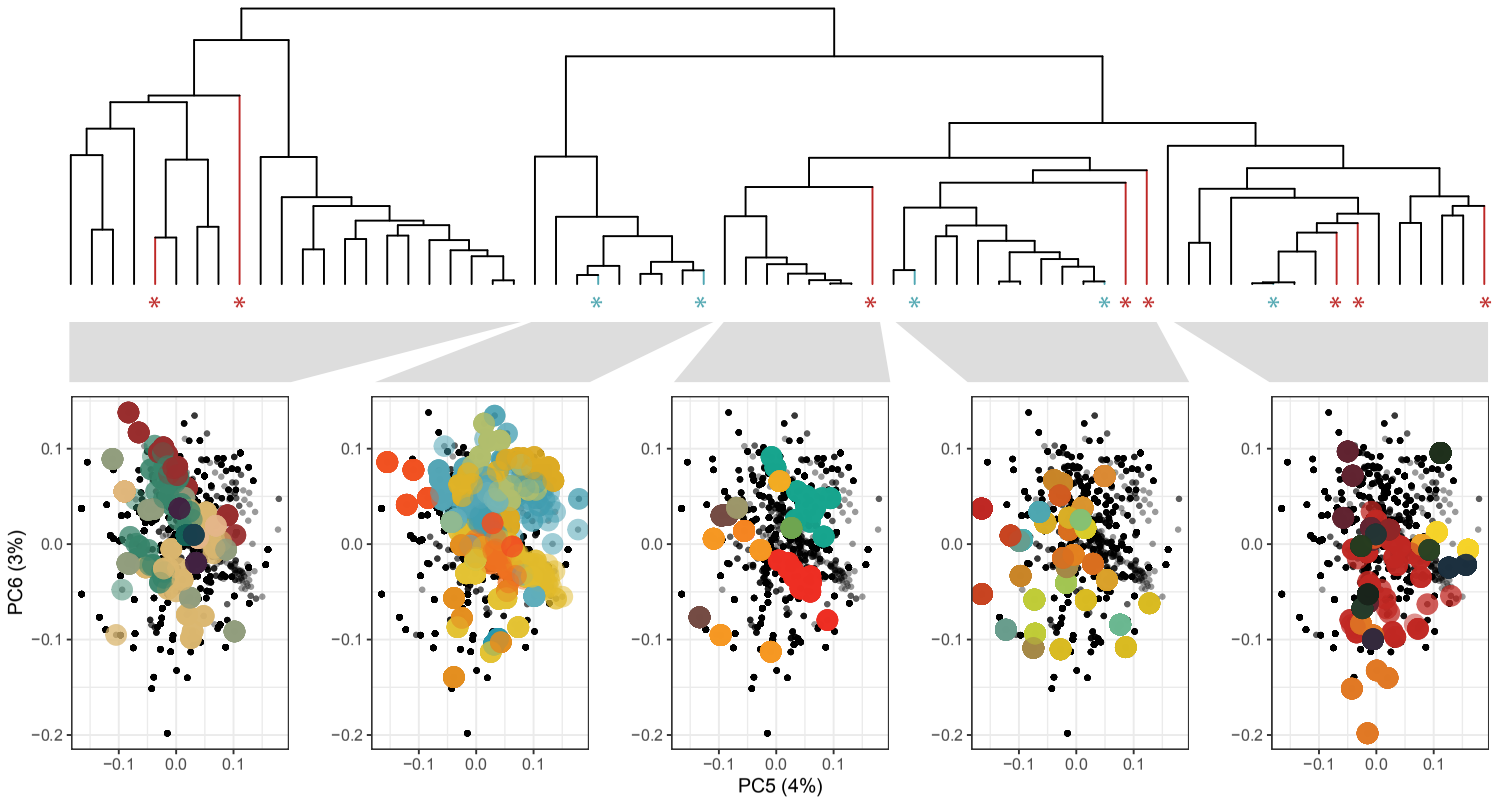
Suppl. Figure S3. Phylogenetic tree as used for this study, including grafted species based on known sister-species relations (blue) and other taxonomic information (red).



Suppl. Figure S4. PC analysis. Showing PC1 and PC2, divided per main clade and coloured per species.



Suppl. Figure S5. PC analysis. Showing PC3 and PC4, divided per main clade and coloured per species.



Suppl. Figure S6. PC analysis. Showing PC5 and PC6, divided per main clade and coloured per species.

Chapter 6

Pelargonium floral shape in space and time

Sara J. van de Kerke[1,2], M. Eric Schranz[1], & Freek T. Bakker[1]

[1]Biosystematics Group, Wageningen University and Research, Droevendaalsesteeg 1, 6708 PB, Wageningen, The Netherlands
[2]Hortus Botanicus Amsterdam, Plantage Middenlaan 2A, 1018 DD, Amsterdam, The Netherlands

ABSTRACT
Speciation in flowering plants is a complex and intricate process. It can be triggered by an enormously diverse set of factors such as shifts in environmental conditions, geological events, and pollinator interactions. In this chapter, we assess existing knowledge on these different aspects of plant speciation for the Cape lineage and radiation of *Pelargonium* in order to (1) examine whether there is a relation between floral shape, species distributions and environmental conditions and (2) determine to what extent aspects of floral shape are drivers of speciation rate in *Pelargonium*. We find speciation in *Pelargonium* to be a complex web of interactions between environmental conditions, pollinator distributions, flowering time, and historical biogeographical influences. Especially in the Cape region, *Pelargonium* appears to make the most of the circumstances. Species can occur in wet, dry, cold, warm, high elevation, and low elevation conditions and in all possible combinations of them. Floral shape seems to vary independently from these conditions. We find only the subset of corolla shape to have an influence on the speciation rate in our *Pelargonium* species. Floral shape appears to be an 'extra layer' of diversity in *Pelargonium* and seems not to be a driving force for speciation.

INTRODUCTION

Speciation in flowering plants is a complex and intricate process (Greiner et al., 2011; Rieseberg and Wendel, 2004; Rieseberg and Willis, 2007). It involves many facets and can be triggered by a diverse set of factors such as shifts in environmental conditions, geological events, pollinator interactions, and others (Dieckmann and Doebeli, 1999; Nosil, 2012; Nosil et al., 2017; Rundle and Nosil, 2005; Schulter, 2001; Tank et al., 2015; Turelli et al., 2001; Whittall and Hodges, 2007).

The unevenness in plant species distribution around the world and its relation with environmental conditions has long intrigued scientists (Abbott et al., 2013; Kier et al., 2005; Martínez-Cabrera and Peres-Neto, 2013; Mutke and Barthlott, 2005). Shifts to new habitats, climatic (in)stability and fragmented landscapes are important for species diversification, i.e. ecological speciation (van der Niet and Johnson, 2009; Warren et al., 2011). A classic and often cited example of repeated ecological speciation is of the numerous independent lineages with hyper-diversity found in the Greater Cape Floristic Region of South Africa. The unprecedentedly high numbers of species in this relatively small area (~9000 in 90.000 km^2) has been attributed to high rates of speciation driven by adaptation to local environmental conditions (Goldblatt and Manning, 2002; Linder, 2003). The idea being that there are so many distinct micro-niches formed by combinations of soil types, rainfall, altitude, frequently-occurring fires, different flowering times, and seasonality that closely related species can adapt to localised environmental conditions (Ellis et al., 2014; van der Niet and Johnson, 2009; Warren et al., 2011).

In addition to current environmental conditions, past conditions have also played an important role in triggering the emergence of what we now recognise as clades. For instance, geological and paleo-climatic events during a radiation can have a major influence on speciation patterns and the evolutionary history of a clade (Barba-Montoya et al., 2018; Li et al., 2019). The break-up of Gondwanaland as well as the occurrence of mass extinction events (such as the K/P boundary ~60-50Mya) have had a tremendous influence on the diversity of life on earth (Barreda et al., 2015; Berger et al., 2016; Chartier et al., 2017; Gould, 1990; Meredith et al., 2011). And, on a shorter evolutionary scale, the rise of numerous mountain ranges (i.a. Linder, 2017). Due to these geological events, new niches become available and populations become reproductively isolated, facilitating the formation of new species (Simões et al., 2016).

The interaction between a flower and a pollinator is another critical factor considered to be a possible driver of plant speciation (Faegri and van der Pijl, 2013; Dodson, 1966; Whittall and Hodges, 2007). Darwin proposed that the floral characteristics of plants are shaped by their interaction with pollinators (Darwin, 1877). Since then, pollination biologists have categorised sets of floral traits shaped by pollinator-driven selection in pollinator syndromes or guilds (Faegri and van der Pijl, 2013). The idea being that a certain pollinator group has a specialised preference for a certain set of floral traits. Therefore, plants belonging to the same pollinator guild (having the same syndrome) will be pollinated by the same set of pollinators (Fenster et al., 2004; Schiestl and Johnson, 2013). To allow for the functional attraction between

flower and pollinator there needs to be an anatomical match in morphology of both to guarantee effective pollen transfer. The pollinator must have sufficient access to the reward (i.e. nectar), otherwise pollinators will avoid these flowers in the future (Alexandersson and Johnson, 2002; Forest et al., 2014; Kay and Sargent, 2009; Pauw et al., 2009; van der Niet et al., 2014; Whittall and Hodges, 2007). Switches between pollinator syndromes have been found to be drivers of speciation and to correlate with clade radiations (Givnish and Sytsma, 2000; Johnson, 2010; Kay and Sargent, 2009; Weller and Sakai, 1999). A prime example is the South African genus *Disa* (Orchidaceae) where a pollinator switch took place in almost every speciation event (Johnson et al., 1998). Goldblatt and Manning (2006) found a pollinator switch for every five to six species for the genera *Gladiolus* and *Babiana* (Iridaceae).
The modularity of a flower and the potential independent evolution of these modules, whether or not under the influence of pollinator pressure, adds to the complexity of speciation in flowering plants (Edwards and Weinig, 2011; Wagner, 1996; Wagner et al., 2007). When integration among floral traits within a module is high, independent evolution of traits within a module is found to be limited to prevent maladapted trait combinations (Edwards and Weinig, 2011; Klingenberg, 2008; Wagner, 1996). Hence, the general adaptability of a flower is limited to fit the demands of the pollinator which may limit the flexibility to switch pollinator type. In contrast, the separate modules *an sich* follow their own evolutionary path (Chapter 5; Wagner, 1996). As we have hown in Chapter 5, in *Pelargonium* (Geraniaceae), the flower itself consists of a number of modules that evolve independently.

Another important factor for speciation is divergence of flowering time. Flowering time is a critical component of a plants' life cycle and determines reproductive success (Dreyer et al., 2006). Flowering time has been found to be subject to numerous influences including pollinator availability, soil moisture, and phylogenetic constrain (Brody, 1997; Dreyer et al., 2006; Johnson, 1992). Sympatric speciation can occur if two lineages have non-overlapping phenologies, since there will be no opportunity for pollen exchange. This can also reduce competition for the same set of pollinators. Shifts in flowering time are an important mechanism for plants to occupy new niches and ensure reproductive success in the area they occur in (Brody, 1997; Du et al., 2015; Kessler et al., 2010; Ollerton and Lack, 2014).

Separate factors of environmental conditions, geological events, and pollinator interactions also influence each other. For example, elevation is thought to have an influence on speciation patterns. Harsh conditions at higher elevations often select for species to form single, relatively large, flowers (Fabbro and Körner, 2004; Herrera, 2005), possibly influencing pollinator attraction and thus reproduction. But altitude is also known to be highly correlated with pollinator occurrence (Arnold et al., 2009; Koski and Ashman, 2015).

We study the interplay between environmental conditions, geological events, and pollinator interactions using the predominantly South African genus *Pelargonium* (Geraniaceae). *Pelargonium* is well-known for its stunning floral and vegetative diversity across its ~280 species (Bakker et al., 2005, 1999; Jones et al., 2009, 2003; Nicotra et al., 2008; Röschenbleck et al., 2014; Struck, 1997). Phylogenetic

relationships within the genus are resolved (Chapter 2; van de Kerke et al., 2019). Roughly 70% of the genus occurs in the South African Greater Cape Floristic Region (GCFR; Linder, 2003; Manning and Goldblatt, 2012; Snijman, 2013). Other species occur in eastern Africa, Namibia, Australia, the Arabian peninsula, Asia minor, and St. Helena (Dreyer et al., 1992; Van der Walt et al., 1990; Van der Walt and Vorster, 1983). Throughout *Pelargonium*, variation in diverse aspects of floral shape can be observed. First, and perhaps most striking, the orientation of the petals ranges from highly zygomorphic (*P. fulgidum*) to almost actinomorphic (*P. cotyledonis*). Second, the variation in petal copy number occurs between and within a species (i.e. *P. caucalifolium*) and alters between five (the 'standard' in Geraniaceae), four (*P. tetragonum*), two (*P. dipetalum*), and can even be missing *(P. apetalum*). Third, the shape of the petals varies tremendously: from slender and elongated (*P. paniculatum*) to almost round (*P. inquinans*).

Pelargonium has a unique spur pollination syndrome with the spur growing adnate to the pedicel (Albers and van der Walt, 2007; Goldblatt et al., 2000; Hodges, 1997; Hodges and Arnold, 1995; Manning and Goldblatt, 2012; but see Tsai et al., 2018) which is a synapomorphy for the entire clade. A range of pollinators matching varying spur length has been recorded for *Pelargonium*, including species of long-tongued hovering flies (*Tabanidae*, *Bombyliidae*, and *Nemestrinidae*), bees (*Apidae*, *Anthophoridae*, *Megachilidae*), wasps (*Vespidae*), and beetles (*Scarabaeidae*; Struck, 1997). In some cases, the spur pollination syndrome is highly specialised. For example, in the Atlantic Ocean island-endemic *P. cotyledonis* the nectar spur seems to have almost disappeared probably due to the loss of the ancestral pollinator from mainland Africa and a subsequent switch to a new pollinator species present on St. Helena. While in *P. appendiculatum* the nectar spur is 10 centimetres in length, but no pollinator with such a long proboscid is known. In addition to varying spur length, the extent to which the pedicel is covered by the spur (pedicel occupation or filling) differs greatly among species (Chapter 4). When a trend towards longer spurs within a clade is found, speciation rates and spur lengths are negatively correlated (i.e. highly specialised flowers are found in smaller clades; Ringelberg, 2012). This would indicate that spur length is an important driver of speciation, but does not necessarily result in the radiation of large clades (Ringelberg, 2012).

In this chapter we explore the relation between environmental conditions, geological events, pollinator interactions, flowering time, and floral shape in a multi-variate analysis for the GCFR lineage *Pelargonium*. The goals of this study are therefore to: (1) examine whether there is a relation between floral shape, species distributions and environmental conditions; and (2) determine to what extent aspects of floral shape are drivers of speciation rates in *Pelargonium*.
We would expect there to be a relation between floral shape and species range distributions because of a relation with local pollinator populations (Forest et al., 2014; Goldblatt and Manning, 1999; Manning and Goldblatt, 2005; van der Niet and Johnson, 2012). We expect that aspects of floral shape are correlated with climatic variables. In addition, we expect overall floral shape to be a driver of speciation, since all three functional units play an important role in ensuring pollination success. However, we expect the reward unit (i.e. the spur) will be the most important driver because of its highly specialised pollination function (Ringelberg, 2012).

MATERIAL AND METHODS

We included results from the historical biogeographical and ancestral environmental conditions study (Chapter 3) as well as the analysis on floral shape and modularity (Chapter 4 and 5). For details on the methods and results, we refer to these studies.

Environmental data

We chose three environmental variables that are thought to correlate with many other environmental factors and are linked to pollinator type. Mean annual precipitation (MAP) and mean annual temperature (MAT) were selected because they are good indicators of the type of climatic conditions at the locality of the plant. We selected elevation because it correlates with potential pollinators in a generic way. We extracted these three climatic variables for the 68 species for which floral morphological data was analysed (Chapter 4 and 5) and for all biogeographic coordinates previously used (Chapter 3). We performed an ANOVA to test whether there were significant differences in mean floral shape per species using these variables and areas (scored by geographical area as in Chapter 3).

Pollinator perspective

We compiled a list of all known pollinators of *Pelargonium* and being as specific as possible. For these pollinator species, we extracted distribution data from GBIF and scored in which of the twelve geographic areas (as defined for the Ancestral area reconstruction in Chapter 3) they occur in. We graphed this data using the package 'ggplot' in R (Wickham, 2009).

Flowering time

Flowering time was scored according to the month a species is known to flower for all species as reported by Van der Walt and Vorster (1988). We visualised this data using the package 'ggplot' in R (Wickham, 2009).

Floral shape and speciation rate estimates

We used the QuaSSE model (Quantitative State Speciation and Extinction; Fitzjohn, 2010) to test trait-dependent diversification related to floral shape. QuaSSE calculates the probability of a phylogenetic tree, a morphological trait, and a model of cladogenesis using likelihood (Fitzjohn, 2010). Both speciation and extinction rates in the birth-death model may vary, and are based on the evolution of the morphological trait which is assumed to evolve under a diffusion model and can have a constant, linear, sigmoid, or parabolic effect on speciation and extinction rates. We chose the best fitting model based on AIC score, and performed a chi-square test to test for significantly different results compared to the null model.

Since we did not measure single traits but overall floral shape based on landmarks, we instead decided to extract PC values as calculated in the PC analysis in Chapter 4 along the first PC axes and rescale these values on a scale ranging from 0 to 1 (Dr. Kaliontzopoulou, pers. comm.). We kept the extinction rate constant, because we do not have sufficient fossil data to properly use them for the estimation. We

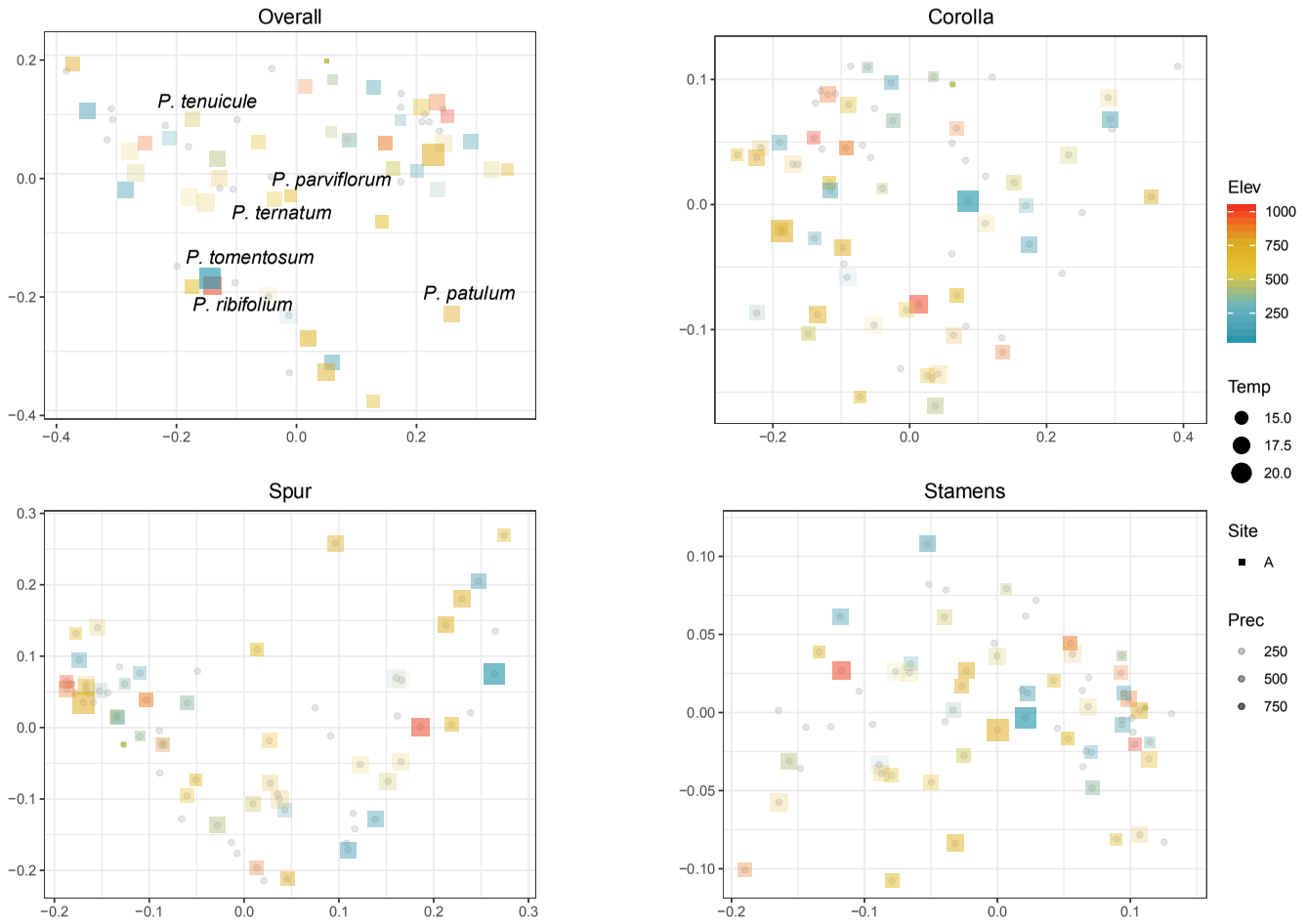


Figure 1. Figure showing PC1 and PC2 of PCA on overall, corolla, spur, and stamen shape. Individuals occurring in the South African winter rainfall region are highlighted and coloured according to mean elevation, temperature and precipitation.

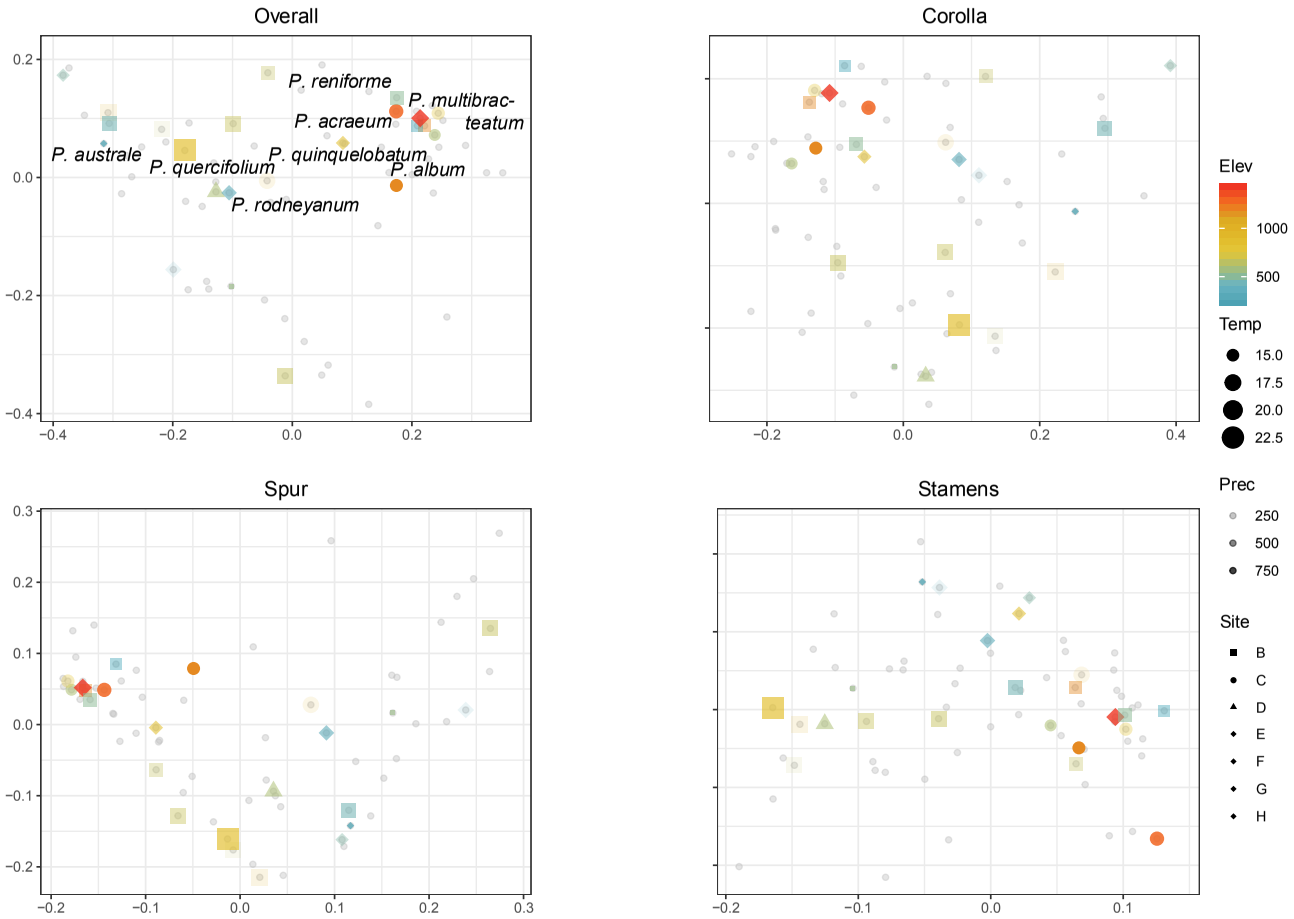


Figure 2. Figure showing PC1 and PC2 of PCA on overall, corolla, spur, and stamen shape. Individuals occurring outside of the South African winter rainfall region are highlighted and coloured according to mean elevation, temperature and precipitation.

performed both 'normal' and 'split' QuaSSE analyses, where we used previous knowledge on diversification rate shifts within the *Pelargonium* genus at the base of clade A to define the split (Ringelberg, 2012). No meaningful diversification rate analysis could be performed given our limited taxon sampling for our morphological data (data not shown). We performed both types of analyses for overall floral shape, as well as for the show-, transfer-, and reward-units separately.

RESULTS

Environmental data

We tested for a relationship between overall floral shape, and the separate floral modules, with specific environmental conditions using ANOVA (Table 1 and Supplementary File S1). The significant relation between stamen shape and the interaction between temperature and site is illustrated in Figure 1 and 2. We see that lower temperatures correspond with a higher PC2 value (shape aspect). We find this to be the case for the non-Cape areas. The relation between spur shape and the interaction between temperature and precipitation shows diverse patterns. Within the Cape region (Figure 1), species that occur in places with higher temperature correlate with a positive value on PC1. The species occurring at sites with lower precipitation are found around the centre of PC2 and precipitation values go up towards the extremes of the axis. Non-Cape species show a much more mixed pattern (Figure 2). The relation between corolla shape, elevation, and precipitation is most clearly shown for non-Cape species; an increase in PC2 correlates with more extreme elevations (both low and high). This pattern is less clear for the Cape species, indicating the importance of Site. Overall floral shape correlates with the interaction between temperature, precipitation, elevation, and site and is a combination of all above named separate floral parts and interactions.

Pollinator perspective

We found pollinator data records for 57 *Pelargonium* species (Supplementary Table S2). Overall, data on pollinator species is scarce for *Pelargonium* and resources are limited to a select number of publications (e.g. Combs and Pauw, 2009; Goldblatt et al., 2000; Struck, 1997). The majority of *Pelargonium* species are pollinated by long-proboscid flies (40 out of the scored 57), sometimes in combination with other pollinator types (beetles, bees, flies, and wasps). Bees also pollinate a number of *Pelargonium* species (19 recorded). *Pelagronium flabellifolium* and *P. fulgidum* are the only species reported to be pollinated by sunbirds (*Nectarinia*), and only *P. minimum* is reported to be pollinated by ants (unknown species, Figure 3).

Table 1. Results of ANOVA test for a relationship between overall floral shape, and the separate floral modules, with specific environmental conditions.

	All	Corolla	Spur	Stamens
Temperature	0.1989	0.4397	0.0627	0.037
Precipitation	0.9834	0.523	0.5522	0.114
Elevation	0.4256	0.1967	0.1101	0.3653
Site	0.8749	0.1136	0.6289	0.3948
Temp*Prec	0.2866	0.3635	0.0448	0.0922
Temp*Elev	0.0861	0.5008	0.5743	0.7507
Prec*Elev	0.0977	0.0824	0.5328	0.7857
Temp*Site	0.2693	0.2435	0.2558	0.0335
Prec*Site	0.2338	0.8969	0.4915	0.6434
Elev*Site	0.7621	0.4013	0.8894	0.7535
Temp*Prec*Elev	0.1909	0.3286	0.3838	0.5584
Temp*Prec*Site	0.0666	0.7272	0.8274	0.8413
Temp*Elev*Site	0.3133	0.2169	0.7744	0.841
Prec*Elev*Site	0.0428	0.0405	0.1312	0.132
Temp*Prec*Elev*Site	0.0026	0.0558	0.309	0.5035

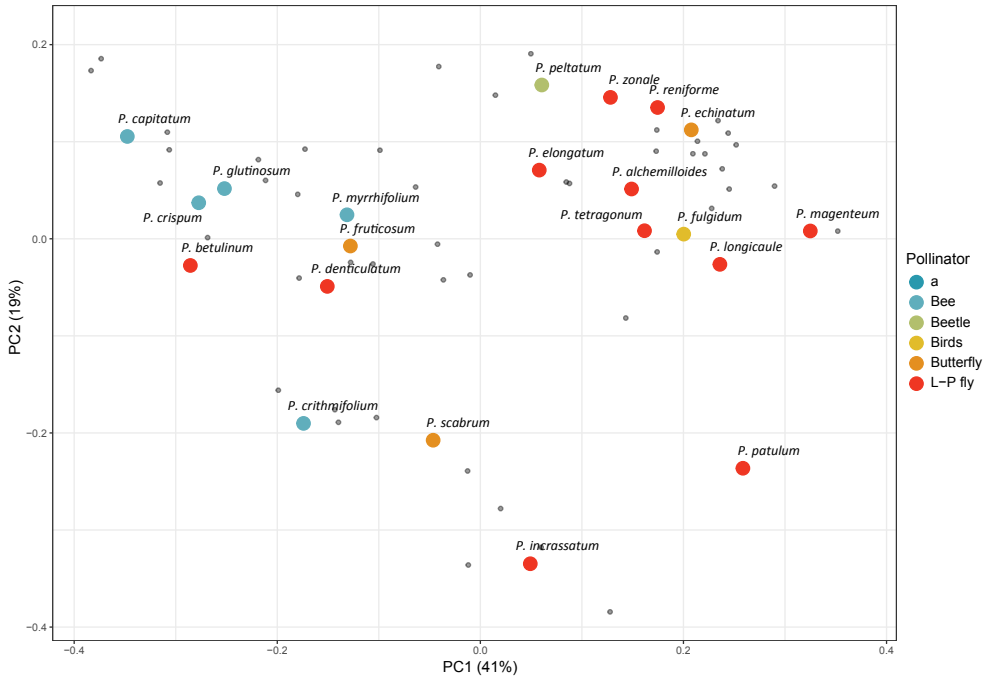


Figure 3. Figure showing PC1 and PC2 of PCA on overall shape with individuals coloured based on pollinator type.

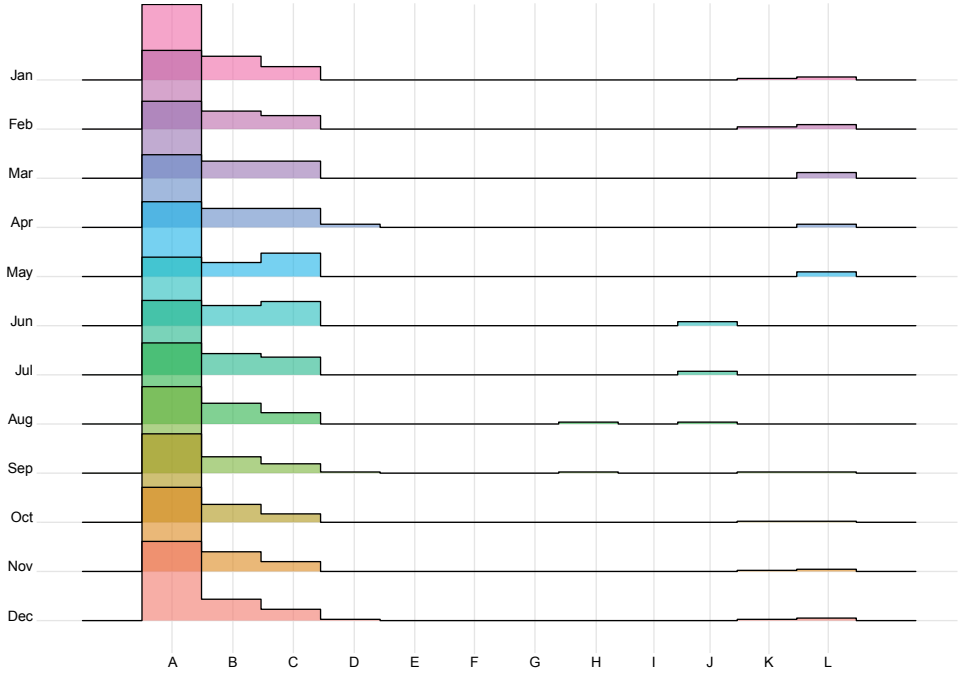


Figure 4. Figure showing amount of species flowering per month in areas as defined in Chapter 3.

Flowering time

We scored flowering time for 60 species (Supplementary Table S3). We find that for areas A, B, and C (as defined in Chapter 3) species flower throughout the year (Figure 4). For area D, H, J, K, and L we cannot infer a reliable pattern because we do not have sufficient data. For all other areas, no flowering time data was available.

Speciation rate estimates

We tested the influence of floral shape on speciation rate using QuaSSE (Fitzjohn, 2010). For the overall floral dataset, as well as the spur and stamen functional units, we found no significant relationships (Supplementary Table S4). We did find the corolla morphological subset to have an influence on linear speciation rate in our *Pelargonium* species (linear = 0.029, drift.linear = 0.025). The negative value of the drift parameter (linear = -0.021) indicates that there is a trend towards lower values of the variable tested. This suggests there is a trend towards smaller corollas (Figure 5, Chapter 4).

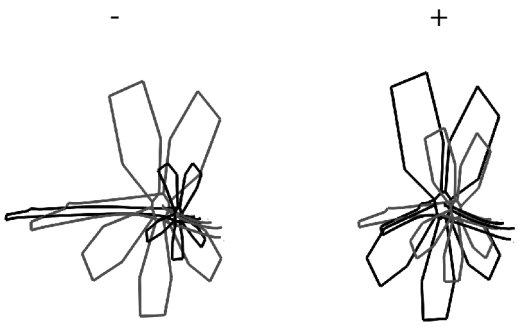


Figure 5. Visualisation of floral shapes corresponding to extremities of PC values.

DISCUSSION

We have examined the relation between environmental conditions, geological events, pollinator interactions, flowering time, and floral shape for the GCFR lineage of *Pelargonium*. We wanted to examine the relation between floral shape and the spatial distribution of the species and whether local environmental conditions are an influence on floral shape. In other words, do *Pelargonium* species have locally adapted floral shapes, suggesting climate to be a driver of floral shape? Or can we find an influence of pollinator species distributions and/or flowering time on floral shape patterning? In addition, we want to know if the shape of the functional units within the *Pelargonium* flower are drivers of speciation, i.e. whether clade proliferation is caused by overall flower shape, or whether single or multiple integrated units are pushing for shifts in speciation rate.

Environmental link

We found that environmental variables had an influence on different aspects of floral shape. Stamen shape is for instance correlated with temperature and with the interaction between temperature and site. Spur shape showed a significant relation with the interaction between temperature and precipitation. We also found that the interaction between site, precipitation, and elevation is correlated significantly with corolla shape. For overall floral shape, there is a significant relation with the interaction between site, precipitation, temperature, and elevation as determined for these species in Chapter 3.

All combinations of climatic variables, interactions between them, and the correlation with geography, in the relation with the different aspects of floral shape, paint quite a complicated picture. When we plot the variables over the respective morphospaces, an obvious pattern does not appear (Figure 3). CFR *Pelargonium* species (Figure 3) are present throughout the morphospace. We do not find a clear patterning of species occurring in the CFR clustering together. In the CFR, apparently, all floral shapes are possible. This would be consistent with the Cape region being the 'cradle' of *Pelargonium*, the home base where such an overwhelming majority of species occurs and we estimate to be the ancestral region of *Pelargonium* (see Chapter 3). This is especially true for species from the main A1 and A2 clades, for

which we in Chapter 5 found floral shape not to be under phylogenetic constraint. Floral shape appears to happen at random for these species and is attributed to the nested radiation of clades A and A2 (Chapter 5). These findings are congruent with findings of Moore et al. (2018), who found leaf traits to be differentiated among main clades.

The variation in climatic variables that go with this broad spectrum of floral shape on the other hand are quite homogeneous. Temperature for all species (size of the dot) is overall more or less the same and only a few species occur in areas with a higher precipitation level (transparency; Figure 1). Only a few of these species coincide with more extreme elevation levels, either low (blue, *P. tomentosum*) or mountainous (red, *P. ribifolium*). Overall, the levels of these variables over the morphospace is rather homogeneous. In comparison, the levels of the variables for the species occurring outside of the Cape region are much more extreme. Species are able to deal with extremely low levels in temperature, coinciding with a low precipitation or elevation (*P. australe*) or can be found on much higher elevations in combination with high precipitation (*P. multibracteatum*).
Even though these 'out of Cape' species are also spread across the morphospace, there is no clear clustering of species from particular areas. This suggests floral shape in itself is not area or clade specific. Rather the circumstances where the species occur have become more extreme, as a way for the species to distinguish itself from their Cape region counterparts. This patterns of 'escapees' is reflected in the ancestral condition reconstructions (Chapter 3) where we found single species to shift their distribution areas and thus general conditions, rather than differentiation of clades on a larger scale.

Pollinator perspective
The diversity in flowering plants in the GCFR is enormous, with an estimated ~9000 species in 90.000 km^2 (Linder, 2003). The diversity in pollinator species is much lower in comparison (Ellis et al., 2014). We attempted to analyse the relation between *Pelargonium* species distributions and the distribution patterns of their known or hypothesised pollinator species. The first hurdle was the poorly studied relation between *Pelargonium* and its pollinator species. Only a few field studies have been able to observe visitations of a number of insect species on *Pelargonium* species (Combs and Pauw, 2009; Goldblatt et al., 2000; Struck, 1997). However, often a deduction based on other known pollinators and floral characteristics, such as a red colour, have been used to infer a pollinator (Struck, 1997). In these cases, often only a pollinator type (long-proboscid fly, bee, or bird) was inferred (Struck and Van der Walt, 1996).
The majority of *Pelargonium* species are pollinated by a range of long-proboscid flies (*Bombyliidae*, *Nemestrinidae*, and *Tabanidae*; Figure 3 and Supplementary File S2). These flies are well-known pollinators of flowering plant species in the Cape region and pollinator guilds are known to have formed across a diverse group of plants displaying similar floral characteristics (Goldblatt and Manning, 2006; Jager and Ellis, 2017; Johnson and Midgley, 1997; Newman et al., 2014). A striking example of this is the wide-spread Bombyliid fly *Megapalpus capensis*, one of the few examples of sexual deception in plants outside of the Orchids (Ellis et al., 2014;

Ellis and Johnson, 2010). *Megapalpus capensis* pollinates species in the Succulent Karoo as well as the Fynbos region, including *Gorteria diffusa* (Asteraceae) and *Pelargonium longifolium, P. tricolor*, and *P. tenuicaule* (de Jager and Ellis, 2017; Struck, 1997). This guild is characterised by the warty thickening on the base of some or all of the petals, resembling a *M. capensis* female (Ellis and Johnson, 2010). Other, well studied examples of pollinator guilds *Pelargonium* species are part of the long-proboscid flies *Philoliche gulosa* (Combs and Pauw, 2009) and *Prosoeca peringueyi* (Manning and Goldblatt, 1996).
The *Pelargonium* species that occur outside of the estimated ancestral area (Winter - Summer rainfall and Karoo regions) appear to be pollinated by wide-spread pollinator species. An example of this is the pollinator *Nectarinia famosa*, a wide-spread sunbird that occurs throughout South Africa, Mozambique, Kenya, and Ethiopia. However, within *Pelargonium* it only pollinates *P. flabellifolium* which only occurs in the Natal region while it is known to pollinate many other species (Geerts and Pauw, 2009).

In contrast, for the Winter rainfall region (arguably the most diverse *Pelargonium* region) we find pollinator species to be more exclusive to the area. For example, the wasp species *Celonites bergenwahliae* and *Celonites wahlenbergiae* as well as the long-proboscid fly *Parisus eurhynatus* only occur in the Cape region while some of the *Pelargonium* species they pollinate (*P. praemorsum* and *P. capitatum*) are much more wide-spread and occur even in Australia. In these cases, where the distribution of the pollinator species is limited, we find the pollinator to be part of a larger set of pollinators that all pollinate the same *Pelargonium* species.

The dispersal of *Pelargonium* towards Australia must have been challenging, especially with respect to the availability of pollinators in the region. The dispersal event itself being a chance event, how likely would it have been to find a suitable pollinator ready to go on site? From *Pelargonium* species in botanical gardens throughout western Europe, as well as the occasional escapee (*P. candicans*) and the average 'garden geranium', we know *Pelargonium* in fact are quite easily pollinated by insect species that are present. Indeed, highly specialised *Pelargonium* species as *P. appendiculatum* (with a nectar spur of 10 cm) will not easily find a new pollinator and can be seen in living collections to have 'reverted back' to selfing. But *Pelargonium* species with readily available nectar seem to thrive in regions they would not naturally occur. A scenario similar to that of *P. appendiculatum* seems to have unfolded for the lineage that diversified over Australia for whose species an increased level of self-compatibility and autogamy have been found (Nicotra et al., 2016).

Flowering time
Diversity in flowering time is another mechanism for plants to occupy new niches and ensure reproductive success in the area they occur (Brody, 1997; Dreyer et al., 2006; Johnson, 1992). This also appears to be the case for *Pelargonium* species. We find a number of species to flower throughout the year, with a peak in spring (*P. denticulatum*) and in the case of *P. acraeum* in autumn as well (Supplementary Table S3). Other species have a more restricted flowering time and flower for only

two- or three-months during the spring (*P. alchemilloides* and *P. incrassatum*). We find that for most areas *Pelargonium* species flower throughout the year (Figure 4). Surprisingly, the influences of local seasonality and conditions do not mean they only flower at particular seasons, but rather flower year-round. For example, the rainfall seasonality that is so characteristic for the Winter (A) and Summer (B) rainfall regions is not reflected in the flowering times of their respective species. In the both the Winter (A) and Summer (B) rainfall regions, there are flowering species year-round. This would suggest that in these regions *Pelargonium* species maximally exploit the local conditions and are adapted to changes in seasonality in order to occupy a new niche in the area as was previously described (Warren et al., 2011).

Speciation in *Pelargonium*

Pelargonium speciation is a complex patchwork of interaction between environmental conditions, pollinator distributions, flowering time, and historical biogeographical influences. One can only image the spectrum the ancestral *Pelargonium* lineage had before the radiation of the clade upon arrival in the South African Cape, around 9.7 Mya in the Late Miocene. *Pelargonium* as a clade started to diversify around 9.7 Mya (Chapter 3). Climatic conditions in the Late Miocene where comparable with modern conditions, after having gone through major changes during the Early Miocene (Linder, 2003). As a result, local conditions became both topologically and climatically complex, and in that regard the ancestral *Pelargonium* had ample diversification potential.

This diversification potential is reflected in the diversity of climatic and topological conditions *Pelargonium* species occur in. Especially in the Cape region, *Pelargonium* appears to make the most of the circumstances. Species can occur in wet, dry, cold, warm, high, and low conditions as well as in all possible combinations of them. We do not find a clear correlation between these conditions and floral shape (Figure 1 and 2). Rather, floral shape seems to vary independently from these conditions. We find species with highly similar shapes occurring in different environmental conditions (both low and high altitudes (*P. tomentosum* and *P. ribifolium*)) while species with highly diverged shapes occur in the same environmental conditions (*P. patulum* and *P. graveolens*). We also encounter species with similar shapes occurring in the same environmental conditions (*P. parivflorum* and *P. ternatum*).
Outside of the Cape region, *Pelargonium* species appear to occur in more extreme conditions; lower and higher elevations, temperature and precipitation all play a role. Again, there is no clear link with floral shape as all combinations of these environmental conditions occur throughout the morphospace. However, we only have a limited morphological sampling for these species.
Unfortunately, we are only able to link the additional information layers of pollinator diversity and flowering time to a limited extent because of insufficient overlap between datasets (Figure 3). However, the way pollinator type can influence the availability of a new niche is nicely illustrated in this figure. The species group *P. denticulatum*, *P. fruticosum*, and *P. myrrhifolium* have comparable floral shapes and all three occur in the Cape region, but they are pollinated by different types of pollinators: long-proboscid fly, butterflies, and bees respectively. This is a clear example of species in sympatry tapping into a new niche by being able to switch

to different pollinators. As all three species have similar leaf shape, a differentiation on that level seems irrelevant. What makes for the exact differences between these species that causes this separation remains to be discovered, as there is great overlap in flowering time between these species.

The general pattern forms when we join all evidence layers: we find that all types of influences that have been described to play a role in the Cape region to influence *Pelargonium*. Species adapt their life habit by dispersing to new regions, switching pollinators, and changing flowering time. In that sense, all the processes that are proposed to play a role in Cape flora speciation are influences on drivers of differences in *Pelargonium* species. Contrary to what we had expected, there does not seem to be a clear link with floral shape. Rather, floral shape appears to be an 'extra layer' of diversity *Pelargonium* lineages can tap into in order to set them apart.
Then, how does the diversity in (aspects of) floral shape influence speciation rates in *Pelargonium*? Surprisingly, we find only the corolla morphological subset to have an influence on the speciation rate in our *Pelargonium* species (Table 2). The negative value of the drift parameter (linear = -0.021) indicates that there is a trend towards smaller corollas (Figure 5, Chapter 4). We had expected spur length to be highly important for speciation rate since it is directly correlated with pollinator type. Previous findings have corroborated this hypothesis (Ringelberg, 2012). An explanation why we do not find any indication for such a relation, could be our species sampling. In the gathering of our data, we have been limited by readily and 'easy-to-grow' species predominantly in western Europe. The harder-to-grow species, especially in the taxonomic section *Hoarea*, is where more of the variation in spur length can be found. This variation could be a potential source for driving speciation rate in *Pelargonium*.

CONCLUSION

We find speciation in *Pelargonium* to be a complex patchwork of interaction between environmental conditions, pollinator distributions, flowering time, and historical biogeographical influences. Especially in the Cape region, *Pelargonium* appears to make the most of the circumstances. Species can occur in wet, dry, cold, warm, high, and low conditions as well as in all possible combinations of them. Floral shape seems to vary independently from these conditions. We find only the corolla morphological subset to have an influence on the speciation rate in our *Pelargonium* species. Rather, floral shape appears to be an 'extra layer' of diversity *Pelargonium* and seems not to be a driving force for speciation.

ACKNOWLEDGEMENTS

Many thanks to Walter den Hollander, Reinout Havinga and Carlien Blok from the Hortus Botanicus Amsterdam for taking in and maintaining the *Pelargonium* collection. Many thanks Dr. Antigoni Kaliontzopoulou for her advice, Dr. Robin van Velzen for his help with R, Merlijn Schram for assembling part of the data, Loes de Heus for proofreading a version of this manuscript, and especially Jens Ringelberg for all his help and advice.

Supplemental Files

Suppl. Table S1
Values for environmental variables mean annual temperature, mean annual precipitation, and mean elevation. Available upon request.

Suppl. Table S2
Overview of pollinator data scored for 57 *Pelargonium* species. Available upon request.

Suppl. Table S2
Overview of flowering time scored for 60 *Pelargonium* species. Available upon request.

Chapter 7

General Discussion

In this thesis, I examine multiple layers of influence on floral shape change in *Pelargonium* in order to paint a comprehensive picture of the evolution of floral diversity of this clade. I accomplished this by studying the historical biogeography and potential ancestral climate conditions for the clade, within- and between species differences in floral shape, and their relation with the possible adaptation of *Pelargonium* species to local conditions. By building upon the extensive knowledge on speciation processes in the Greater Cape Floristic Region, I have been able to research and infer speciation processes for the *Pelargonium* clade. Working with living material certainly has it's analytical and practical challenges, and while I have overcome several of these, there is still room for improvement and new questions to explore. In this General Discussion I reflect on my findings on the relation between variation of floral shape and speciation in *Pelargonium*, and place them in the broader perspective of plant evolution in the Greater Cape Floristic Region.
Here, I again want to stress we are aware that, botanically speaking, *Pelargonium* does not have a spur but rather a nectar tube developed from the receptacle (Tsai et al., 2018) and that we use these terms as substitutes, as we do with 'spur-evolution' and 'spurlike-evolution'. We do not aim to make any morphological or developmental claims with our terminology, but rather use 'spur' in a functional sense, irrespective of its ontogeny.

MAIN FINDINGS AND CONCLUSIONS

This thesis starts with the construction of a new, time-calibrated, phylogenetic tree based on 74 plastome exons and nuclear rDNA ITS regions for 120 *Pelargonium* species that resolved relationships within the genus and helped clear up remaining species-level incongruences (**Chapter 2**). I find that the *Pelargonium* crown node originated around 9.7 Mya, in line with earlier estimates (Fiz et al., 2008; Palazzesi et al., 2012). This places the age of *Pelargonium* in agreement with general findings for other Fynbos (8.5 ± 1.85 Mya) and Succulent Karoo lineages (5.17 ± 0.64; Verboom et al., 2009).

My new age calibration makes *Pelargonium* around 25 My younger than previously thought and opened up new opportunities for a historical biogeographical analysis. **Chapter 3** describes the first formal biogeographical analysis for the clade (aside from the narrative by Bakker et al. (2005)). I found the ancestral geographic range of *Pelargonium* to include the Winter and Summer rainfall region, the Karoo region, and the Natal region in South Africa. I found that long range dispersal seems to be the prime cause of the disjunct distribution patterns in *Pelargonium* main clades, both to Australia, Madagascar, along the East African Highway, and into Asia minor.

Pelargonium flowers exhibit great variability in their floral forms. In **Chapter 4** I combined two 2D photograph-based datasets of floral morphology into a single 3D virtual flower and subsequently used this to examine multiple layers of shape variation. This approach offered unique benefits to complement established imaging techniques in a number of ways. Through my approach, I found that adding the third dimension to the data was crucial to accurately interpret the manner of, as well as levels of, shape variation in flowers.

The morphospace, based on virtual3D flowers as reconstructed in Chapter 4, formed the basis for the exploration of floral shape and floral modularity in **Chapter 5**. I wanted to know to what extent the modules (show-, reward-, and transfer-apparatus) within the *Pelargonium* flower are integrated (i.e. whether they evolve in concert, and whether different, apparatus-specific selective pressures may exist). I indeed found there is modularity within the floral data when partitioned and also that they are highly integrated. In addition, I found that the relation between floral shape and phylogeny was highly dependent on the main clade and that it was different per module.

In the final research chapter (**Chapter 6**) I examined whether there is a relation between floral shape, species distributions and environmental conditions and then determined to what extent aspects of floral shape (build in the previous chapter but also adding new evidence) are drivers of speciation rates in *Pelargonium*. I found that speciation in *Pelargonium* is a complex patchwork of interactions between environmental conditions, pollinator distributions, flowering time, and historical biogeographical influences and that corolla shape seemed to have an influence on the speciation rate of *Pelargonium* species. I concluded that floral shape appears to be an 'extra layer' of diversity of *Pelargonium* lineages, which set them apart, rather than the major driving force for their diversification.

PLANT SPECIATION IN GENERAL

For flowering plants, there are multiple potential influences and drivers of speciation. For example, due to phylogenetic 'canalisation' a species has a particular genomic make-up that it cannot escape, and hence cannot evolve certain morphologies due to genetic constraints, even over long evolutionary time periods. On the other hand, rapid speciation can occur due to shifts in pollinators which are often highly dynamic. A highly specialised form of pollination is spur pollination whereby pollination reward (the nectar) and the corolla are spatially separated. This is considered to result in a Darwinian 'arms-race' between plants and pollinators, increasing effective pollen transfer, and, ultimately, fitness for both (Whittall and Hodges, 2007).

There are several other factors that also have to be taken into account. For example, there could very well be a difference in 'types' of speciation when regarded in an evolutionary timeframe. For example, a radiation-type speciation event (adaptive or not) triggers a sudden burst of new species (Hughes and Eastwood, 2006). While on an often slower pace, there will be speciation on a lower level for example due to local adaptations such as shifts in phenology and/or pollinators (Forest et al., 2014; Warren et al., 2011). In addition, species can react to changes in environment by either adapting or moving away (Warren et al., 2011)

POST-ZYGOTIC BARRIERS IN *PELARGONIUM*

So far, I have only considered possible pre-zygotic barriers (such as pollination and flowering time) in the speciation process of *Pelargonium*, while post-zygotic barriers also play an important role in plant speciation in general and *Pelargonium* in particular (Greiner et al., 2011; Lowry et al., 2008; Rieseberg and Willis, 2007; Weihe et al., 2009; Widmer et al., 2009). One example of post-zygotic isolation,

by which reproductive isolation (RI) and hence speciation in *Pelargonium* could occur, is through genomic incompatibility, i.e. between organelle and the nuclear genome (Barnard-Kubow et al., 2016; Greiner et al., 2011; Greiner and Bock, 2013). After successful pollination and the establishment of a zygote, when the individual genomic compartments are not able to (correctly) communicate, an individual with low fitness can result. Incompatibility between the nucleus and organelle genomes has been referred to as cytonuclear incompatibility (CNI) and is common in *Pelargonium* (Weihe et al., 2009). Incompatibility between the nucleus and the chloroplast can be detected by leaf variegation (the occurrence of white or yellow zones on green leaves or other plant structures (Greiner and Bock, 2013; Weihe et al., 2009)). In case of yellow zoning (or not that much white zoning), offspring can grow into a viable plant. However, when the result of fertilisation is a truly white individual, most likely it will not be able to survive. An example of CNI at the mitome level is male sterility of offspring (Chase, 2007). This is a phenomenon that also occurs in *Pelargonium*, although it might be subject to temperature fluctuations (Breman, pers. comm.). In these ways, CNI forms a barrier for fertilisation and hybridisation (Breman, pers. comm.; Greiner and Bock, 2013).

Cytonuclear incompatibility is of particular relevance in *Pelargonium* because it is known that plastid inheritance is biparental (Tilney-Bassett et al., 1992; Tilney-Bassett and Almouslem, 1989; Tilney-Bassett and Birky, 1981; Weihe et al., 2009). This means that the plastid composition of offspring is often a mix of both parental lines, which could increase the chances of (a level of) CNI (Ruhlman and Jansen, 2018). On the other hand, precisely because there are plastome copies from both parents present in the offspring, it is likely that at least part of them are able to communicate correctly and in this way act as an escape from CNI (Barnard-Kubow et al., 2017).

Pelargonium is also well-known for having increased levels of substitution rates of the mitome (Bakker et al., 2006; Guisinger et al., 2008; Parkinson et al., 2005; Weng et al., 2012). The combination of high rates of nucleotide substitutions, biparental inheritance, and CNI may be causes for the relatively small population sizes found for most *Pelargonium* species (Van der Walt and Vorster, 1988). Thus, there is ample opportunity for genetic drift (Crespi and Nosil, 2013). These diverse genomic conflicts and post-zygotic factors may in fact drive speciation. In small populations, the increased substitution rates in *Pelargonium* may cause rapid changes in mitome genomes on such a scale that they quickly become so different from other (small) populations that they are subject to CNI. Any morphological changes that occur may simply be a result of genetic drift since they could easily become established in the small populations and result in the rapid formation of a new species. Biparental inheritance then could act as a form of escape, enabling these diverged populations/species to still form (a kind of) viable offspring. This could give rise to a hybrid zone between the populations, itself again a potential source for speciation.

ECOLOGICAL SPECIATION IN *PELARGONIUM*

Another, important, mode for speciation in plants is through ecological speciation (Nosil, 2012; Rundle and Nosil, 2005; Schulter, 2009, 2001). Although arguably

pre-zygotic reproductive isolation induced by pollinator pressure is a form of ecological speciation (van der Niet et al., 2010, 2014a, 2014b), here I want to make the distinction between them and look at speciation as a result of environmental circumstances separately from pollinator driven speciation. The former being a direct reaction on (changes in) local condition, while the latter is a somewhat more indirect effect.
Ecological speciation in *Pelargonium* has been found to occur on different levels (Bakker et al., 2005; Jones et al., 2009; Moore et al., 2018). Bakker et al. (2005) tied the radiation of main clade A2 to the adaptation to a xerophytic life habit triggered by the formation of stem- and leaf succulence, woody subshrubs and the formation of tubers. This was suggested to be a reaction to the aridification of the Cape region during the mid-Miocene, which Bakker et al. found to coincide. Within clade A2, including a number of xerophytic strategies as listed above, an specialised adaptation of section *Hoarea* forming tunicate tubers could be pointed out (Bakker et al., 2005).

Research into the leaf functional traits and niches of *Pelargonium* have corroborated these findings. For the xerophytic A2 clade as well as for sect. *Hoarea* it has been found that there are few direct relations between traits and environment, suggesting that the adaptation of xerophytic strategies allowed species to be somewhat independent of the environment (Moore et al., 2018). This is in contrast with the findings for clade A2, where a strong relation between especially leaf area and environment is found (Martínez-Cabrera et al., 2012). This suggests a direct influence of the environment on the formation of these species (Moore et al., 2018). The general pattern for *Pelargonium* is that the environmental niches species occur in are quite independent of phylogenetic placement (Jones et al., 2013; Martínez-Cabrera et al., 2012). Closely related species thus do not necessarily occur in similar climatic niches, although there appears to be a greater tendency toward niche conservatism than divergence (Martínez-Cabrera et al., 2012).

HOW DO THEY DO IT?

I have built upon the extensive body of literature on the Greater Cape Floristic Region (GCFR) to try and give a holistic perspective on speciation in *Pelargonium*. When I set out this project, I believed that changes in floral shape were triggered by pollinator pressure and that this was the driver of speciation processes for this clade. However, I found that floral shape may not be as important for *Pelargonium* speciation patterns as I had assumed. Rather, *Pelargonium* species occur in a wide range of wet, dry, cold, warm, high, and low altitude conditions as well as in all possible combinations of them (Jones et al., 2013, 2009, 2003; Mitchell et al., 2015; Mocko et al., 2017; Moore et al., 2018; Nicotra et al., 2008). Floral shape seems to vary independently from these conditions. In addition, floral shape does not seem to be phylogenetically canalised (i.e. there are no clear shape differences over the main clades) but some clades appear to be phylogenetically constrained to some degree. Whether this is the result of the relatively young age of the genus (having exploded in all possible floral shapes that can occur in the current potential morphospace) or is a non-adaptive response still remains to be tested.

How then do the patterns of speciation in *Pelargonium*, i.e. whereby floral shape does not seem that important, compare with other GCFR lineages? Much research has been done on the hyperdiverse flora of the GCFR, one of the six Floristic Kingdoms and Mediterranean-climate biodiversity hotspots of the world (Barthlott et al., 2007, 2005; Linder, 2005, 2003; Morrone, 2015; Takhtajan et al., 1986). It has been found that the GCFR comprises a limited number of founding lineages, but that all of these have contributed the extraordinary high levels of endemism and species richness of the region (Born et al., 2007; Goldblatt, 1978; Goldblatt et al., 2000; Linder, 2003; Sauquet et al., 2009). In comparison, Southwest Australia (another Mediterranean type climate vegatation, called the Kwongan) is much more phylogenetically diverse in numbers of founding lineages, but many of them show lower diversification rates (Sauquet et al., 2009). The Mediterranean Basin 'Maquis' vegetation may show lower levels of diversity because it has been a less stable environment for a shorter period of time, leading to fewer opportunities for diversity to build-up (Valente and Vargas, 2013). In general, differences between these mediterranean type vegatation Floristic Kingdoms in terms of diversity, and the potential causes thereof, are suggested to be found in terms of rates in speciation and extinction, geological stability of the region, and differences in local versus long-distance colonisation (Hughes, 2017).

Within the GCFR, ecological speciation in combination with reduced levels of extinction was inferred (largely from comparative species-level analyses) to be the primary driver of plant diversity (the 'hot-bed' vs evolutionary 'museum' models (Pirie et al., 2016; Verboom et al., 2009; and see Ellis et al., 2014 for an overview). It is hypothesized that the mosaic of different habitats, geologies and soil types (Goldblatt and Manning, 2002) in combination with regular fire-cycles, would have driven this mode of speciation. In what way ecological speciation, as opposed to post-zygotic barriers and/or genetic drift, has resulted in such high levels of species numbers is still under debate as the interaction between ecological axes makes it difficult 'to tease apart the influences of diversification drivers' (Ellis et al., 2014). An additional factor that should be noted is what appears to be a general tendency in species distributions in the GCFR, namely that the diversity of clades declines along a west-east gradient (named Levyns' Law (Cowling et al., 2017; Warren et al., 2011)). In addition, as Bouchenak-Khelladi et al. (2015) point out, both extrinsic conditions as well as intrinsic traits are required for a radiation to occur. Meaning, the opportunity has to be there for the potential to proliferate. I agree with the suggestion made by Ellis et al. that an answer for hyperdiversity in general may not necessarily be found in looking in all the ecological axes separately. Rather, understanding the complete GCFR ecological mosaic, as highlighted above, as well as non-adaptive responses, will give us more insight in the speciation processes in the GCFR.

That ecological speciation is the primary driver of diversity in the GCFR does not necessarily imply that all lineages do 'it' exactly in the same way. Determining direct drivers of speciation for a clade is difficult and often many theories and possibilities are suggested, researched, and discarded one-by-one (van der Niet and Johnson, 2009). For example, the massive radiation of *Erica* (the largest of the

Cape lineages; Linder, 2003) has been attributed to mycorrhizal interactions which could have resulted in increased soil fertility. However, using orchids (for which the same relation was suggested) and global ericoid mycorrhizal interactions, this hypothesis has been overturned (Ellis et al., 2014). Now, it is proposed that *Erica* (and Ericaceae in general) have been able to radiate into a nutrient poor sandstone soil type, triggered by the ability to form though leathery leaves with high leaf longevity, a characteristic feature of plants in oligotrophic habitats, and of Fynbos plants in general (Schwery et al., 2015; van der Niet and Johnson, 2009).

Whereas *Erica* thus appears to be constrained to a single soil type, the Restionaceae in comparison appear to utilise a different strategy: they seem to sample a wide array of different habitat types (Bouchenak-Khelladi and Linder, 2017). For Poaceae and Cyperaceae (Poales), the radiation is attributed to the development of CO_2-concentrating mechanisms (C_4 and CAM) in an environment that became increasingly aridified (Bouchenak-Khelladi et al., 2014). And although the diversity in pollination systems in Sub-Saharan Iridaceae could help explain part of the species diversity, it is suggested to be playing a secondary role (Goldblatt and Manning, 2006). But what then is the primary facilitator of this radiation remains unclear. Also the *Protea* radiation has been labelled to be an adaptive response of vegetative traits on climate (Mitchell et al., 2018; Onstein et al., 2016). When taking all this into consideration, it becomes apparent that radiations in the GCFR do not show a clear-cut pattern. There does not seem to be a 'one size fits all' type of solution explaining why and how the region has become so hyperdiverse. But maybe it is also not that complicated. It was previously suggested in the 'Levyns-Goldblatt' hypothesis: the species richness of the (G)CFR flora is the result of radiation into aridifying habitats in the western CFR (Linder and Hardy, 2004). Geophytes, and the ability to make tubers, seems to be a typical 'Cape response' in this respect (Jones et al., 2009; Moore et al., 2018; Oberlander et al., 2009; Procheş et al., 2006, 2005). And from the summary above we see indeed just that. All of these Cape lineages have found a strategy to successfully radiate in the arid conditions of the habitat they occur in, and they all found their own way.

One of the factors to take into consideration are the regular (seasonal) fires that occur in the Cape area and the influence these have on the vegetation. It has been suggested that the relative absence of forests in the Cape region is enhanced by the regular occurring fire cycle (Bond et al., 2003). However, recently it has been shown this might not necessarily be the case. Rather, the difference between open vegetation (i.e. Fynbos) and forest-type vegetation seems to be induced by a difference in soil nutrient levels (Cramer et al., 2019). Many of the Cape lineages have alternative fire survival strategies ('seeders versus resprouters) which have been suggested to be the result of divergent selection under pressure of these fires (Cowling et al., 2018; Ellis et al., 2014). Seeders do not survive the fires, but need them to regenerate their populations and sometimes need smoke to break seed dormancy (Brown and Botha, 2004; Cowling et al., 2018). Resprouters on the other hand are resistant to fire. Regular fires with short intervals between them has favoured the resprouter strategy (fire frequency hypothesis, Ellis et al., 2014). What this means for the vegetation composition in light of urbanisation and climate

change (will there be no opportunity for reseeders as a result of scarce fires, that in addition do not keep the resprouters in check?) and how it could influence plant speciation should definitely be researched more (Jones et al., 2013).

Another important component of speciation is pollination, which has been extensively researched for the Cape region (i.a. Bouman et al., 2017; Combs and Pauw, 2009; Forest et al., 2014; Goldblatt and Manning, 1999; Johnson et al., 2011; Johnson and Midgley, 1997; Manning and Goldblatt, 2011, 1997; Schiestl and Johnson, 2013; van der Niet et al., 2014b, 2014a). Sister-species switches between pollinator types are numerous, whereby it is suggested that the adaptation to specific pollinators can lead to reproductive isolation enhancing speciation (Forest et al., 2014; Linder, 2003; van der Niet et al., 2006; Welsford et al., 2016). Although for some clades, adopting a single pollination strategy appears to work (as for example for the wind-pollinated Restionaceae and *Cliffortia* (Johnson, 2010; Linder, 2003)). But generally, multiple pollination strategies are adopted within a clade, and often numerous and repeated switches occur between them (Forest et al., 2014). All in all, pollination seems to be an import component of speciation within the GCFR which can be expressed on both a deeper (radiation) as well a shallower (sister-species) level.
Of importance here is to note that, although the Cape region is hyperdiverse in plant species, this is not the case for insects (Ellis et al., 2014). As a result, an additional level of competition for the attention of the pollinators seems to be added (Ellis et al., 2014; Forest et al., 2014; Jager and Ellis, 2017). This is reflected in the convergence of flowering plant species, rising above family level, in the same pollinator guild (de Jager and Ellis, 2017; Goldblatt, 1978; Manning and Goldblatt, 1997, 1996).

I started this project with the understanding that *Pelargonium* was a prime example of a clade that had diverged driven by pre-zygotic speciation. i.e. For which speciation was driven by shifts in floral shape triggered by pollinator pressure and that especially spur length was a defining feature because of its direct relation with pollinator type. However, now that we have sampled the *Pelargonium* morphospace and have been able to put it in a broader context (in an evolutionary and geological sense as well as ecologically) I no longer think this necessarily is the case. Yes, *Pelargonium* displays a tremendous amount of floral diversity. However, as we have shown, this does not go hand in hand with segregation along any of the evolutionary axes I have compared it with (Chapter 6). Rather, floral shape appears to happen 'just because' and appears not to be induced by pollinator or environmental pressure, nor the result of phylogenetic canalisation (Chapter 5). The continued sampling of species for the floral morphospace, irrespective of their phylogenetic placement, could be a result of a nested radiation of *Hoarea* species nested in a deeper *Pelargonium* Xerophytic clade. These radiations have previously been attributed to the adaptation to a xerophytic life habit triggered by the formation of stem- and leaf succulence, woody subshrubs and the formation of tubers. The geophytic *Hoarea* radiation was suggested to be vegetative non-adaptive and florally adaptive driven by pollinator pressure (Bakker et al., 2005). Based on our analyses, where we find that floral shape appears not to be under phylogenetic constraint (Chapter 5), I think each of these radiations (whether or not an adaptive response in itself)

might have 'reset' the potential for floral diversity in the ensuing clade, allowing for repeated sampling of the floral morphospace. Genomic changes driven by TE mobile elements could also have played a role here (Lindqvist and Rajora, 2019). This view is further supported by the finding that different species with the same floral shape can occur in the same area while not being pollinated by the same pollinator type (Chapter 6). Aspects of leaf physiology and/or performance might be more important aspects for speciation in *Pelargonium* (Jones et al., 2013, 2009; Martínez-Cabrera et al., 2012; Mitchell et al., 2015; Mocko et al., 2017; Moore et al., 2018; Nicotra et al., 2011).

I think speciation on the level of radiations should not be mistaken for speciation on a sister-species level. The finding that a clade has radiated on a deeper phylogenetic level does not mean the speciation process stopped there. The combination of opportunity (extrinsic conditions) and event (intrinsic traits) that come together (in whatever order) can give rise to an adaptive radiation (Bouchenak-Khelladi et al., 2015). Thus, the adaptation of the clade to circumstances (or the presence of the adaptation when circumstances changed) give the potential of the clade to 'reset' and reuse it's full morphological potential. The findings for *Pelargonium* as presented in this thesis together with the potential for speciation driven by post-zygotic barriers as outlined above, combined with the plethora of research on speciation in other Cape lineages, will help us understand the process of speciation in the context of the GCFR.

REFLECTIONS

The expression 'hindsight is 20/20' is often applicable to a research project and looking back there are always new insights or 'could-have, would-have, should-haves'. Without wanting to undermine the content and findings of this thesis, I do want to subject them to some reflection.

The basis of my study is the floral morphospace that I have constructed in Chapter 4. Over the course of 3 years of data gathering, I have been able to include floral shapes for 68 species in the virtual3D dataset (with 134 for the SPUR and 287 for the PETAL datasets). While this corresponds with ~24% of known species, as I argue chapter 4, a good taxonomic coverage does not reflect a good morphological coverage. So, how good is my morphological sampling? Based on a general sense of floral diversity in *Pelargonium*, I feel we have been able to include a good portion of the variation in floral morphology in the genus. I was definitely able to sample the quintessential *Pelargonium* flower shape, including a wide range of different corolla shapes. One of the corolla types that I unfortunately was not able to include are the fibrillate species (i.a. *P. caffrum* and *P. bowkeri*). Although plants of these species were available, I did not include them for practical purposes. It would be impossible to correctly landmark the fibrillate petals because a clear homology is lost. However, I do not think including these species would have a major impact on the morphospace as the general morphology is largely congruent with a 'standard' *Pelargonium* floral shape. A number of species from the geophytic *P.* sect. *Hoarea* (comprising 77 species) were included, and for some of the missing ones it could be imagined they would have had affected the resulting floral morphospace. For instance, the keel-

flowered *P. rapaceum*, *P. oblongatum* or the conspicuous *P. asarifolium* may well be expected to occupy 'new' parts of the resulting morphospace.
One major aspect of floral shape that I was not able to include are the 'ears' on the petals of many sect. *Otidia* species. This outgrowth of the pedicel is thought to close the nectar spur. Why it is beneficial for these species to deny their pollinators access to the spur is not known, but for some reason a more recent evolutionary event has triggered the development of these structures which seems to give the species in this clade some advantage. I was not able to include these structures in my current analysis because of the way landmarks are placed (i.e. based on venation patterns). However, it would be possible to include the curve of this particular part of the petal in future studies.

For spurs, I do think including a wider range and more extreme spur lengths would have had a major influence. I have now been fairly limited in the variation in spur and pedicel length, with *P. stipulaceum* as notable outlier. However, I found spur length to be such an important feature in *Pelargonium* floral shape that extending the included range would undoubtedly result in a shift in the generated morphospace. In addition, I expected spur length to be a major influence on speciation rates in *Pelargonium* (based on the study by (Ringelberg, 2012). That I do not find a similar pattern in my QuaSSE models is, I think, a direct result of the limited range in spur lengths sampled in the analysis. While the Ringelberg study included over 1500 specimens with spur length ranging from 1 to ~85 mm, our study has been fairly limited in that regard (including 'only' 134 specimens in the SPUR dataset).
I could partially overcome this problem in the same way as I solved the addition of missing species for both the SPUR and PETAL datasets (i.e. missing from one of them). I have now made the decision to include only those species we have full coverage of in our virtual3D reconstruction. However, there could be made a case for generating an average shape per dataset (SPUR and PETAL) and use that as place holder to complement the sampled counterpart. The reason I have decided in this study not to do this is because I felt the number of individuals I have sampled was not sufficient to warrant relying so heavily on an average shape. However, when I would be able to sample more individuals per species, I would have a much more solid foundation allowing for such a solution.

A feature of *Pelargonium* floral shape I was not been able to include is the reflexedness of the petals, although we do know there is quite some variation between species in this regard. The sect. *Polyactium* species for example all display highly-reflexed petals when in anthesis. As a result, I was not able to include these correctly in this study because landmarking petals with such divergent orientation was not feasible. How I could overcome this issue is for future experiments.

I have tried to reflect the pollinator perspective by including their distribution patterns in the overarching speciation chapter (Chapter 6). However, a direct and clear link between pollinators and floral shape cannot be made. Since one-on-one relations between *Pelargonium* floral shape and pollinator species cannot be made until more visitations have been recorded, I should now turn to pollinator-derived features of floral shape such as the nectar guides (also known as petal markings) which

are extensive and conspicuous in *Pelargonium*, floral colour, nectar composition, and scent. Floral scent has not been recorded for many *Pelargonium* species. *Pelargonium transvaalense* is recorded as the only day-scented species, while sect. *Magnistipulaceae* and *Polyactium* species are night-scented (Röschenbleck et al., 2014). Scent, as well as floral shape, generally can be used to infer a pollinator type. More specifically, petal markings can be used to identify a particular pollinator guild (as the *Megapalpus capensis* guild that is characterised by the warty petal markings).

IN AN IDEAL WORLD...

In addition to upgrading our current research on influences on the evolution of *Pelargonium* and the role floral shape plays therein, there are a number of new paths we could (or should?) begin exploring. A low-hanging fruit would definitely be performing a proper phyloclimatic analysis (Yesson and Culham, 2006a, 2006b). The ancestral state reconstruction for selected environmental variables as I have performed now gives good insight in the course of conditions for *Pelargonium*. The advantage of a phyloclimatic analysis in not only that a broader range of variables can be included, but an entire 'climatic niche' for ancestral nodes can be inferred. Combining this with a species distribution and niche modelling program such as Maxent, would give the opportunity to infer ancestral ranges for selected nodes in the *Pelargonium* phylogenetic tree based on climatic conditions (Phillips et al., 2006).

Another low-hanging fruit would be to explore how *Pelargonium* floral shape shifted through time. I have now focussed on current morphological variation and how that relates with phylogenetic relations. However, I did not yet look at what the ancestral *Pelargonium* flower looked like, and how the morphospace was filled in an evolutionary timeframe. This could be done using my current morphological data and phylogenetic framework to extrapolate the shape of an ancestral node.

Combining phyloclimatic analysis and ancestral shapes would really give great insight in the development of *Pelargonium* as a clade through time. In addition, it would help to further understand specific dispersal events. Did the long-range jump to Australia really correspond with a morphological bottleneck? Can we say the same for the Asia minor species after we have added them to our dataset? These types of analyses should be complemented with a deeper understanding of pollinator diversity for *Pelargonium*. As I have found, only for a select number of *Pelargonium* species do we know by what type of pollinator they are visited, and even then often only on a non-species level (Chapter 6). Thus, attempting to expand our knowledge (for example by observing pollinator visitations) would be an obvious first step. However, this type of research has been notoriously difficult (T. van der Niet, pers. comm.).

In addition to my floral morphology analyses and their potential relation with speciation in *Pelargonium*, quite some research has been done on the leaf trait aspects in this clade (Jones et al., 2013, 2009; Martínez-Cabrera et al., 2012; Mitchell et al., 2015; Mocko et al., 2017; Moore et al., 2018; Nicotra et al., 2011).

Although there is scarce overlap between the underlying datasets, I find comparable patterns. Now that I have done an initial analysis into the ancestral conditions of *Pelargonium*, I can broaden both the floristic as well as the leaf perspective by combining these separate lines of evidence in an overarching analysis. This would be a great opportunity to study pre-, post-, and ecological speciation processes within one clade. After all, the plant is a process.
As many factors with their associated uncertainties are involved, a Bayesian approach using RevBayes seems an appropriate avenue to explore speciation in *Pelargonium* in a truly holistic approach (Hohna et al., 2016). Using RevBayes, I would be able to include all evidence lines in one, overarching, interacting analysis instead of using a phylogenetic hypothesis as basis and constraint for all subsequent analyses. The advantage of this approach thus seems to be that the evidence lines can complement each other (Hohna et al., 2016).

The NSF-funded Dimensions of Biodiversity project on parallel evolutionary radiations of the University of Connecticut was an unique example of a project comparing patterns and processes over multiple GCFR clades (*Protea* and *Pelargonium*). It would be time for other such overarching studies to be undertaken. As described above, there is a plethora of research on evolution and speciation within GCFR clades, but what is the bigger picture?

Another avenue of opportunity would be morphogenesis modelling (Coen et al., 2017; Coen and Rebocho, 2016; Rebocho et al., 2017; Tavares et al., 2018). In morphogenesis modelling, the development of an organism ontogenetically can be simulated and growth of certain organs can be tracked over time (Green et al., 2010; Kennaway et al., 2011; Prusinkiewicz and Runions, 2012). When comparing the approaches of Morphometrics modelling with Morphogenesis modelling for one and the same group of species, the latter method offers an important advantage as it takes the developmental process into account and is, in principle, capable of inferring stable versus unstable (morphological) equilibria. Morphogenesis modelling tools, such as GFTbox, have been developed by prof. Enrico Coen's group at the John Innes Centre in Norwich, UK. The approach is considered to be highly effective in finding the genetic variation (genes, alleles) behind specific switches in shape change.
For *Pelargonium*, this will be an interesting approach because, apart from learning more about the development of nectar spurs and petal nectar guides, morphogenesis modelling enables (in theory) inference of stable versus unstable equilibria in *Pelargonium* floral switches. The latter would be of great value in order to understand floral response to pollinator availability. A first step in a project like this will be to produce a Morphogenesis model for the *Pelargonium* spur, as mentioned before unique in the angiosperms, and infer whether the switches in spur length and shape, observed across the *Pelargonium* clade, are stable or not, and what parameters of the model are most sensitive. It has been found that the development of the *Pelargonium* spur follows a different pattern (i.e. cell division and elongation) opposed to the development of *Aquilegia* and *Linaria* that only display one or the other (E. Cullen, pers. comm.). Exploring the actual development of the *Pelargonium* spur could be an exciting addition to our understanding of it.

Comparative transcriptomics would be an obvious approach to study floral developmental stages and find out which genes play a key role in floral shape formation. Based on the geometric morphometric study, we found a number of species pairs of which the two sisters represent contrasting floral shapes (Chapter 5). For instance, the formation of petal nectar guides could be an aspect of floral shape that could be studied in this way.

Extensive studies have been conducted into the genetics and biosynthetic pathways underlying floral colouration and pigmentation (Agati et al., 2012; Elomaa and Holton, 1994; Forkmann, 1991; Giusti et al., 1999; Grotewold, 2006; Holton and Cornish, 1995; Mol et al., 1998; Petroni and Tonelli, 2011; Quattrocchio et al., 2001; Rausher, 2008; Tanaka, 2006; Wang et al., 2004; Winkel, 1991; Zhao and Dixon, 2010). Therefore, there is quite a good understanding of the genes and pathways involved, for plants in general but also some for *Pelargonium* more specifically (Johnson and Özhatay, 1988; Mitchell et al., 1998; Palumbo et al., 2007; Sukhumpinij et al., 2012). These form a good basis for finding out more about the genetic triggers that result in differences between petal marking syndromes. In one entire *Pelargonium* clade (the subgenus 'Paucisignata' sensu Röschenbleck et al. 2014, including the horticulturally important sect. *Ciconium* clade), petal markings are generally lacking or inconspicuous, which enables pathway comparisons with 'guided' petal species.

In addition, we could study the volatiles composition and metabolomics involved in the differences in petal nectar guide colours and colour gradients. This could allow us to better understand how and what genetic switches may be underlying the colour differences observed in the species.

IN THE END

The aim of my thesis was to bring together multiple layers of potential influences on floral shape in *Pelargonium* in order to paint a comprehensive picture of the evolution of this clade. Now that I have done so, I find that speciation in this clade may not be as clear-cut as we had expected. My findings give us the opportunity to place *Pelargonium* in a broader, GCFR-wide, evolutionary perspective. I have definitely been able to take a step in that direction. The suggestions for future research, as outlined above for *Pelargonium* but also on a broader scale enveloping multiple GCFR clades and incorporating pre-, post-, and ecological speciation, could be undertaken to extend our current knowledge. The drastic changes in climate that are already showing in the Cape region (for example in terms of extended periods of drought) will only become more prominent. What effect these changes will have on the extraordinary diversity in plants and animals in the GCFR, should be (and remain) closely observed.

References

Abbott, R., Albach, D., Ansell, S., Arntzen, J. W., Baird, S. J. E., Bierne, N., … Zinner, D. (2013). Hybridization and speciation. *Journal of Evolutionary Biology*, *26*(2), 229–246. https://doi.org/10.1111/j.1420-9101.2012.02599.x

Adams, D. C. (2014). A generalized K statistic for estimating phylogenetic signal from shape and other high-dimensional multivariate data. *Systematic Biology*, *63*(5), 685–697. https://doi.org/10.1093/sysbio/syu030

Adams, D. C. (2016). Evaluating modularity in morphometric data: Challenges with the RV coefficient and a new test measure. *Methods in Ecology and Evolution*, *7*(5), 565–572. https://doi.org/10.1111/2041-210X.12511

Adams, D. C., & Collyer, M. L. (2016). On the comparison of the strength of morphological integration across morphometric datasets. *Evolution*, *70*(11), 2623–2631. https://doi.org/10.1111/evo.13045

Adams, D. C., & Otárola-Castillo, E. (2013). Geomorph: An R package for the collection and analysis of geometric morphometric shape data. *Methods in Ecology and Evolution*, *4*(4), 393–399. https://doi.org/10.1111/2041-210X.12035

Adams, D. C., Rohlf, F. J., & Slice, D. E. (2004). Geometric morphometrics: Ten years of progress following the 'revolution.' *Italian Journal of Zoology*, *71*(1), 5–16. https://doi.org/10.1080/11250000409356545

Agati, G., Azzarello, E., Pollastri, S., & Tattini, M. (2012). Flavonoids as antioxidants in plants: location and functional significance. *Plant Science*, *196*, 67–76. https://doi.org/10.1016/j.plantsci.2012.07.014

Aiton, W. T. (1789). *Hortus Kewensis.* London: George Nicol.

Albers, F., & van der Walt, J. J. A. (2007). Geraniaceae. In *The Families and Genera of Vascular Plants* (pp. 157–167). Springer Berlin Heidelberg.

Albers, F., van der Walt, J. J. A., Gibby, M., Marschewski, D. E., Price, R. A., & du Preez, G. (1995). A biosystematic study of *Pelargonium* section *Ligularia*: 2 Reappraisal of section *Chorisma*. *South African Journal of Botany*, *61*(6), 339–346.

Aldasoro, J. J., Navarro, C., Vargas, P., J, L. S., & Aedo, C. (2001). *California*, a new genus of Geraniaceae endemic to the Southwest of North America. *Anales Del Jardín Botánico de Madrid*, *59*(2), 209–216.

Alexandersson, R., & Johnson, S. D. (2002). Pollinator-mediated selection on flower-tube length in a hawkmoth-pollinated *Gladiolus* (Iridaceae). *Proceedings of the Royal Society B: Biological Sciences*, *269*(1491), 631–636. https://doi.org/10.1098/rspb.2001.1928

Ali, R. H., Bark, M., Miró, J., Muhammad, S. A., Sjöstrand, J., Zubair, S. M., … Arvestad, L. (2017). VMCMC: A graphical and statistical analysis tool for Markov chain Monte Carlo traces. *BMC Bioinformatics*, *18*(1), 1–8. https://doi.org/10.1186/s12859-017-1505-3

Ali, S. S., Pfosser, M., Wetschnig, W., Martínez-Azorín, M., Crespo, M. B., & Yu, Y. (2013). Out of Africa: Miocene dispersal, vicariance, and extinction within Hyacinthaceae subfamily *Urgineoideae*. *Journal of Integrative Plant Biology*, *55*(10), 950–964. https://doi.org/10.1111/jipb.12065

Armbruster, S., Pelabon, C., Hansen, T., & Mulder, C. (2004). Floral integration, modularity, and accuracy: distinguishing complex adaptations from genetic constraints. In M. Pigliucci & K. Preston (Eds.), *Phenotypic integration: studying ecology and evolution of complex phenotypes* (pp. 23–49). New York, NY: Oxford University Press.

Armbruster, W. S., & Muchhala, N. (2009). Associations between floral specialization and species diversity: Cause, effect, or correlation? *Evolutionary Ecology*, *23*(1), 159–179. https://doi.org/10.1007/s10682-008-9259-z

Arnold, S. E. J., Savolainen, V., & Chittka, L. (2009). Flower colours along an alpine altitude gradient, seen through the eyes of fly and bee pollinators. *Arthropod-Plant Interactions*, *3*(1), 27–43. https://doi.org/10.1007/s11829-009-9056-9

Axelrod, D. I., & Raven, P. H. (1978). Late Cretaceous and Tertiary vegetation history of Africa. In M. J. A. Werger (Ed.), *Biogeography and ecology of southern Africa* (pp. 77–130).

Baker, W. J., & Couvreur, T. L. P. (2013). Global biogeography and diversification of palms sheds light on the evolution of tropical lineages. II. Diversification history and origin of regional assemblages. *Journal of Biogeography*, *40*(2), 286–298. https://doi.org/10.1111/j.1365-2699.2012.02794.x

Bakker, F. T., Breman, F., & Merckx, V. (2006). DNA sequence evolution in fast evolving mitochondrial DNA nad1 exons in Geraniaceae and Plantaginaceae. *Taxon*, *55*(4), 887–896.

Bakker, F. T., Culham, A., Daugherty, L. C., & Gibby, M. (1999). A trnL-F based phylogeny for species of *Pelargonium* (Geraniaceae) with small chromosomes. *Plant Species Biology*, *216*, 309–324.

Bakker, F. T., Culham, A., Gomez-Martinez, R., Carvalho, J., Compton, J., Dawtrey, R., & Gibby, M. (2000). Patterns of nucleotide substitution in angiosperm cpDNA trnL (UAA)-trnF (GAA) regions. *Molecular Biology and Evolution*, *17*(8), 1146–1155.

Bakker, F. T., Culham, A., Hettiarachi, P., & Touloumenidou, T. (2004). Phylogeny of *Pelargonium* (Geraniaceae) based on DNA sequences from three genomes. *Taxon*, *53*(1), 17–28.

Bakker, F. T., Culham, A., Hettiarachi, P., Touloumenidou, T., & Gibby, M. (2004). Phylogeny of *Pelargonium* (Geraniaceae) based on DNA sequences from three genomes. *Taxon*, *53*(1), 17–28.

Bakker, F. T., Culham, A., Marais, E. M., & Gibby, M. (2005). Nested radiation in Cape Pelargonium. In F. T. Bakker, L. W. Chatrou, B. Gravendeel, & P. B. Pelser (Eds.), *Plant species-level systematic: new perspectives on pattern & process* (pp. 75–100).

Bakker, F. T., Culham, A., Pankhurst, C. E., & Gibby, M. (2000). Mitochondrial and chlorplast DNA-based phylogeney of *Pelargonium* (Geraniaceae). *American Journal of Botany*, *87*(4), 727–734.

Bakker, F. T., Gibby, M., & Culham, A. (1999). Phylogenetics and diversification in *Pelargonium*. In *Molecular systematics and plant evolution* (pp. 353–374). Chapman & Hall. https://doi.org/10.1201/9781439833278.ch16

Bakker, F. T., Hellbrügge, D., Culham, A., & Gibby, M. (1998). Phylogenetic relationships within *Pelargonium* sect. *Peristera* (Geraniaceae) inferred from nrDNA and cpDNA sequence comparisons. *Plant Systematics and Evolution*, *211*, 273–287.

Bakker, F. T., Lei, D., Yu, J., Mohammadin, S., Wei, Z., van de Kerke, S., … Holmer, R. (2016). Herbarium genomics: plastome sequence assembly from a range of herbarium specimens using an Iterative Organanelle Genome Assembly pipeline. *Biological Journal of the Linnean Society*, *117*, 33–43.

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., … Pevzner, P. A. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *Journal of Computational Biology*, *19*(5), 455–477.

Barba-Montoya, J., dos Reis, M., Schneider, H., Donoghue, P. C. J., & Yang, Z. (2018). Constraining uncertainty in the timescale of angiosperm evolution and the veracity of a Cretaceous Terrestrial Revolution. *New Phytologist*, *218*(2), 819–834. https://doi.org/10.1111/nph.15011

Barnard-Kubow, K. B., McCoy, M. A., & Galloway, L. F. (2017). Biparental chloroplast inheritance leads to rescue from cytonuclear incompatibility. *New Phytologist*, *213*(3), 1466–1476. https://doi.org/10.1111/nph.14222

Barnard-Kubow, K. B., So, N., & Galloway, L. F. (2016). Cytonuclear incompatibility contributes to the early stages of speciation. *Evolution*, *70*(12), 2752–2766. https://doi.org/10.1111/evo.13075

Barreda, V. D., Palazzesi, L., Tellería, M. C., Olivero, E. B., Raine, J. I., & Forest, F. (2015). Early evolution of the angiosperm clade Asteraceae in the Cretaceous of Antarctica. *Proceedings of the National Academy of Sciences*, *112*(35), 201423653. https://doi.org/10.1073/pnas.1423653112

Barthlott, W., Hostert, A., Kier, G., Küper, W., Kreft, H., Mutke, J., … Sommer, J. H. (2007). Geographic patterns of vascular plant idversity at continental to global scales. *Erdkunde*, *61*(4), 305–315.

Barthlott, W., Mutke, J., Rafiqpoor, D., Kier, G., & Kreft, H. (2005). Global centers of vascular plant diversity. *Nova Acta Leopoldina NF*, *92*(342), 61–83. https://doi.org/10.1177/1354066101007003001

Baur, E. (1908). Das Wesen und die Erblichkeitsverhältnisse der „Varietates albomarginatae hort." von *Pelargonium zonale*. *Zeitschrift Für Induktive Abstammungs- Und Vererbungslehre*, *1*(1), 330–351. https://doi.org/10.1007/bf01990603

Becher, S. A., Steinmetz, K., Weising, K., Boury, S., Peltier, D., Renou, J. P., … Wolff, K. (2000). Microsatellites for cultivar identification in *Pelargonium*. *Theoretical and Applied Genetics*, *101*(4), 643–651. https://doi.org/10.1007/s001220051526

Becker, M., & Albers, F. (2009). Taxonomy and phylogeny of two subgroups of *Pelargonium* section *Otidia* (Geraniaceae). 1. The *Pelargonium carnosum* complex. *Bothalia*, *39*(1), 73–85.

Becker, M., & Albers, F. (2010). Pollinator shift and speciation in *Pelargonium alternans* (Geraniaceae). *Schumannia*, *6*, 207–218.

Bellstedt, D. U., Galley, C., Pirie, M. D., & Linder, H. P. (2012). The migration of the Palaeotropical arid flora: Zygophylloideae as an example. *Systematic Botany*, *37*(4), 951–959. https://doi.org/10.1600/036364412x656608

Bendahmane, M., Dubois, A., Raymond, O., & Bris, M. Le. (2013). Genetics and genomics of flower initiation and development in roses. *Journal of Experimental Botany*, *64*(4), 847–857. https://doi.org/10.1093/jxb/ers387

Berg, R. L. (1960). The ecological significance correlation pleiades. *Evolution*, *14*, 171–180.

Berger, B. A., Kriebel, R., Spalink, D., & Sytsma, K. J. (2016). Divergence times, historical biogeography, and shifts in speciation rates of Myrtales. *Molecular Phylogenetics and Evolution*, *95*, 116–136. https://doi.org/10.1016/j.ympev.2015.10.001

Bergh, N. G., & Linder, H. P. (2009). Cape diversification and repeated out-of-southern-Africa dispersal in paper daisies (Asteraceae-Gnaphalieae). *Molecular Phylogenetics and Evolution*, *51*(1), 5–18. https://doi.org/10.1016/j.ympev.2008.09.001

Berra, T. M. (2008). Charles Darwin's paradigm shift. *Records of Museums and Art Galleries of the Northern Terrirory*, *24*(1960), 1–5.

Blazier, C. C., Guisinger, M. M., & Jansen, R. K. (2011). Recent loss of plastid-encoded *ndh* genes within *Erodium* (Geraniaceae). *Plant Molecular Biology*, *76*, 263–272.

Blazier, J. C., Jansen, R. K., Mower, J. P., Govindu, M., Zhang, J., Weng, M.-L., & Ruhlman, T. A. (2016). Variable presence of the inverted repeat and plastome stability in *Erodium*. *Annals of Botany*. https://doi.org/10.1093/aob/mcw065

Blazier, J. C., Ruhlman, T. A., Weng, M.-L., Rehman, S. K., Sabir, J. S. M., & Jansen, R. K. (2016). Divergence of RNA polymerase α subunits in angiosperm plastid genomes is mediated by genomic rearrangement. *Scientific Reports*, *6*, 24595.

Blerot, B., Baudino, S., Prunier, C., Demarne, F., Toulemonde, B., & Caissard, J.-C. (2015). Botany, agronomy and biotechnology of *Pelargonium* used for essential oil production. *Phytochemistry Reviews*, *15*(5), 935–960.

Bod, R. (2019). *Een wereld vol patronen; De geschiedenis van kennis*. Prometheus Amsterdam.

Bond, W. J., Midgley, G. F., & Woodward, F. I. (2003). What controls South African vegetation - climate or fire? *South African Journal of Botany*, *69*(1), 79–91. https://doi.org/10.1016/S0254-6299(15)30362-8

Born, J., Linder, H. P., & Desmet, P. (2007). The Greater Cape Floristic Region. *Journal of Biogeography*, *34*(1), 147–162. https://doi.org/10.1111/j.1365-2699.2006.01595.x

Bouchenak-Khelladi, Y., & Linder, H. P. (2017). Frequent and parallel habitat transitions as driver of unbounded radiations in the Cape flora. *Evolution*, *71*(11), 2548–2561. https://doi.org/10.1111/evo.13364

Bouchenak-Khelladi, Y., Muasya, A. M., & Linder, H. P. (2014). A revised evolutionary history of Poales: origins and diversification. *Botanical Journal of the Linnean Society*, *175*(1), 4–16. https://doi.org/10.1111/boj.12160

Bouchenak-Khelladi, Y., Onstein, R. E., Xing, Y., Schwery, O., & Linder, H. P. (2015). On the complexity of triggering evolutionary radiations. *New Phytologist*, *207*(2), 313–326. https://doi.org/10.1111/nph.13331

Bouckaert, R., Heled, J., Kühnert, D., Vaughan, T., Wu, C. H., Xie, D., … Drummond, A. J. (2014). BEAST 2: A Software Platform for Bayesian Evolutionary Analysis. *PLoS Computational Biology*, *10*(4), 1–6. https://doi.org/10.1371/journal.pcbi.1003537

Boukhris, M., Simmonds, M. S. J., Sayadi, S., & Bouaziz, M. (2013). Chemical composition and biological activities of polar extracts and essential oil of rose-scented Geranium, *Pelargonium graveolens*. *Phytotherapy Research*, *27*(8), 1206–1213. https://doi.org/10.1002/ptr.4853

Bouman, R. W., Steenhuisen, S. L., & van der Niet, T. (2017). The role of the pollination niche in community assembly of Erica species in a biodiversity hotspot. *Journal of Plant Ecology*, *10*(4), 634–648. https://doi.org/10.1093/jpe/rtw068

Briggs, D., & Walters, S. M. (2016). *Plant variation and evolution.* Cambridge University Press.

Brody, A. K. (1997). Effects of pollinators, herbivores, and seed predators on flowering phenology. *Ecology*, *78*(6), 1624–1631.

Brown, M., & Botha, P. A. (2004). Smoke seed germination studies and a guide to seed propagation of plants from the major families of the Cape Floristic Region, South Africa. *South African Journal of Botany*, *70*(4), 559–581. https://doi.org/10.1016/S0254-6299(15)30194-0

Carr, D. E., & Fenster, C. B. (1994). Genetical Society of Great Britain Levels of genetic variation and covariation for Mimulus (Scrophulariaceae) floral traits. *Heredity*, *72*(6), 606–618.

Carter, A., Riley, T. R., Hillenbrand, C. D., & Rittner, M. (2017). Widespread Antarctic glaciation during the Late Eocene. *Earth and Planetary Science Letters*, *458*, 49–57. https://doi.org/10.1016/j.epsl.2016.10.045

Catley, J. L., Brooking, I. R., Davies, L. J., & Halligan, E. A. (2002). Temperature and irradiance effects on *Sandersonia aurantiaca* flower shape and pedicel length. *Scientia Horticulturae*, *93*(2), 157–166. https://doi.org/10.1016/S0304-4238(01)00324-7

Chala, D., Zimmermann, N. E., Brochmann, C., & Bakkestuen, V. (2017). Migration corridors for alpine plants among the 'sky islands' of eastern Africa: do they, or did they exist? *Alpine Botany*, *127*(2), 133–144. https://doi.org/10.1007/s00035-017-0184-z

Chartier, M., Jabbour, F., Gerber, S., Mitteroecker, P., Sauquet, H., von Balthazar, M., … Schönenberger, J. (2014). The floral morphospace - a modern comparative approach to study angiosperm evolution. *New Phytologist*, *204*(4), 841–853. https://doi.org/10.1111/nph.12969

Chartier, Marion, Löfstrand, S., von Balthazar, M., Gerber, S., Jabbour, F., Sauquet, H., & Schönenberger, J. (2017). How (much) do flowers vary? Unbalanced disparity among flower functional modules and a mosaic pattern of morphospace occupation in the order Ericales. *Proceedings of the Royal Society B: Biological Sciences*, *284*(1852), 20170066. https://doi.org/10.1098/rspb.2017.0066

Chase, C. D. (2007). Cytoplasmic male sterility: a window to the world of plant mitochondrial-nuclear interactions. *Trends in Genetics*, *23*(2), 81–90. https://doi.org/10.1016/j.tig.2006.12.004

Chumley, T. W., Palmer, J. D., Mower, J. P., Fourcade, M. H., Calie, P. J., Boore, J. L., & Jansen, R. K. (2006). The complete chloroplast genome sequence of *Pelargonium x hortorum*: organization and evolution of the largest and most highly rearranged chloroplast genome of land plants. *Molecular Biology and Evolution*, *23*(11), 2175–2190.

Clark, S. C., Egan, R., Frazier, P. I., & Wang, Z. (2013). ALE: a generic assembly likelihood evaluation framework for assessing the accuracy of genome and metagenome assemblies. *Bioinformatics*, *29*(4), 435–443. https://doi.org/10.1093/bioinformatics/bts723

Clark, V. R., Barker, N., & Mucina, L. (2011). Taking the scenic route – the southern Great Escarpment (South Africa) as part of the Cape to Cairo floristic highway. *Plant Ecology & Diversity*, *4*(4), 313–328. https://doi.org/10.1080/17550874.2011.619584

Coen, E., Kennaway, R., & Whitewoods, C. (2017). On genes and form. *Development*, *144*(23), 4203–4213. https://doi.org/10.1242/dev.151910

Coen, E., & Rebocho, A. B. (2016). Resolving Conflicts: Modeling Genetic Control of Plant Morphogenesis. *Developmental Cell*, *38*(6), 579–583. https://doi.org/10.1016/j.devcel.2016.09.006

Coen, E. S., & Meyerowitz, E. M. (1991). The war of the whorls: genetic interations controlling flower development. *Nature*, *353*, 31–37.

Collyer, M. L., Sekora, D. J., & Adams, D. C. (2015). A method for analysis of phenotypic change for phenotypes described by high-dimensional data. *Heredity*, *115*(4), 357–365. https://doi.org/10.1038/hdy.2014.75

Combs, J. K., & Pauw, A. (2009). Preliminary evidence that the long-proboscid fly, *Philoliche gulosa*, pollinates *Disa karooica* and its proposed Batesian model *Pelargonium stipulaceum*. *South African Journal of Botany*, *75*(4), 757–761. https://doi.org/10.1016/j.sajb.2009.06.015

Corley, S. B., Carpenter, R., Copsey, L., & Coen, E. (2005). Floral asymmetry involves an interplay between TCP and MYB transcription factors in *Antirrhinum*. *Proceedings of the National Academy of Sciences*, *102*(14), 5068–5073. https://doi.org/10.1073/pnas.0501340102

Cowling, R. M., Bradshaw, P. L., Colville, J. F., & Forest, F. (2017). Levyns' Law: explaining the evolution of a remarkable longitudinal gradient in Cape plant diversity. *Transactions of the Royal Society of South Africa*, *72*(2), 184–201. https://doi.org/10.1080/0035919X.2016.1274277

Cowling, R. M., Gallien, L., Richardson, D. M., & Ojeda, F. (2018). What predicts the richness of seeder and resprouter species in fire-prone Cape fynbos: rainfall reliability or vegetation density? *Austral Ecology*, *43*(6), 614–622. https://doi.org/10.1111/aec.12606

Cowling, R. M., Procheş, Ş., & Partridge, T. C. (2009). Explaining the uniqueness of the Cape flora: Incorporating geomorphic evolution as a factor for explaining its diversification. *Molecular Phylogenetics and Evolution*, *51*(1), 64–74. https://doi.org/10.1016/j.ympev.2008.05.034

Cramer, M. D., Power, S. C., Belev, A., Gillson, L., Bond, W. J., Hoffman, M. T., & Hedin, L. O. (2019). Are forest-shrubland mosaics of the Cape Floristic Region an example of alternate stable states? *Ecography*, *42*(4), 717–729. https://doi.org/10.1111/ecog.03860

Crespi, B., & Nosil, P. (2013). Conflictual speciation: Species formation via genomic conflict. *Trends in Ecology and Evolution*, *28*(1), 48–57. https://doi.org/10.1016/j.tree.2012.08.015

Crisp, M. D., & Cook, L. G. (2013). How was the Australian flora assembled over the last 65 million years? A molecular phylogenetic perspective. *Annual Review of Ecology, Evolution, and Systematics*, *44*(1), 303–324. https://doi.org/10.1146/annurev-ecolsys-110512-135910

Cubas, P., Vincent, C., & Coen, E. (1999). An epigenetic mutation responsible for natural variation in floral symmetry. *Nature*, *401*(6749), 157–161. https://doi.org/10.1038/43657

Darwin, C. (1836). *Narrative of the surveying voyages of His Majesty's ships adventure and Beagle, between the years 1826 - 1836, describing their examination of the southern shores of South America, and the Beagle's circumnavigation of the globe. ournal and remards*. London: Colburn.

Darwin, C. (1859). *On the origin of species by means of natural selection, or the preservation of favoured races in the struggle for life*. London: John Murray.

Darwin, C. (1877a). *The different forms of flowers on plants of the same species.* John Murray.

Darwin, C. (1877b). *The various contrivances by which orchids are fertilised by insects.* John Murray.

De Candolle, A. P. (1824). Geraniaceae. Prodromus systematis naturalis regni vegetabilis, vol. 1, *Treutel* &, 637–682.

de Jager, M. L., & Ellis, A. G. (2017). Evolutionary history of a keystone pollinator parallels the biome occupancy of angiosperms in the Greater Cape Floristic Region. *Molecular Phylogenetics and Evolution*, *107*, 530–537. https://doi.org/10.1016/j.ympev.2016.12.004

De Waal, C., Rodger, J. G., Anderson, B., & Ellis, A. G. (2014). Selfing ability and dispersal are positively related, but not affected by range position: a multispecies study on southern African Asteraceae. *Journal of Evolutionary Biology*, *27*(5), 950–959. https://doi.org/10.1111/jeb.12368

Devos, N., Barker, N. P., Nordenstam, B., & Mucina, L. (2010). A multi-locus phylogeny of *Euryops* (Asteraceae, Senecioneae) augments support for the "Cape to Cairo" hypothesis of floral migrations in Africa. *Taxon*, *59*(1), 57–67. https://doi.org/10.1002/tax.591007

Dieckmann, U., & Doebeli, M. (1999). On the origin of species by sympatric speciation. *Nature*, *400*(6742), 354.

Dodson, C. H. (1966). *Orchid flowers: their pollination and evolution*. Fairchild Tropical Garden and the University of Miami Press.

Doyle, J. (1991). DNA protocols for plants. *Molecular Techniques in Taxonomy*, 283–293.

Dreyer, L. L., Albers, F., van der Walt, J. J. A., & Marschewski, D. E. (1992). Subdivision of *Pelargonium* sect. *Cortusina* (Geraniaceae). *Plant Systematics and Evolution*, *183*(1–2), 83–97.

Dreyer, L. L., Esler, K. J., & Zietsman, J. (2006). Flowering phenology of South African *Oxalis*-possible indicator of climate change? *South African Journal of Botany*, *72*(1), 150–156. https://doi.org/10.1016/j.sajb.2005.06.009

Drummond, A., Suchard, M., Xie, D., & Rambaut, A. (2012). Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology And Evolution*, *29*, 1969–1973.

Du, Y., Mao, L., Queenborough, S. A., Freckleton, R. P., Chen, B., & Ma, K. (2015). Phylogenetic constraints and trait correlates of flowering phenology in the angiosperm flora of China. *Global Ecology and Biogeography*, *24*(8), 928–938. https://doi.org/10.1111/geb.12303

Dupin, J., Matzke, N. J., Särkinen, T., Knapp, S., Olmstead, R. G., Bohs, L., & Smith, S. D. (2017). Bayesian estimation of the global biogeographical history of the Solanaceae. *Journal of Biogeography*, *44*(4), 887–899. https://doi.org/10.1111/jbi.12898

Edwards, C. E., & Weinig, C. (2011). The quantitative-genetic and QTL architecture of trait integration and modularity in *Brassica rapa* across simulated seasonal settings. *Heredity*, *106*(4), 661–677. https://doi.org/10.1038/hdy.2010.103

Ellis, A. G., Brockington, S. F., de Jager, M. L., Mellers, G., Walker, R. H., & Glover, B. J. (2014). Floral trait variation and integration as a function of sexual deception in *Gorteria diffusa*. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *369*(1649), 20130563–20130563. https://doi.org/10.1098/rstb.2013.0563

Ellis, A. G., & Johnson, S. D. (2010). Floral mimicry enhances pollen export: the evolution of pollination by sexual deceit outside of the Orchidaceae. *The American Naturalist*, *176*(5), E143–E151. https://doi.org/10.1086/656487

Ellis, A. G., Verboom, G. A., van der Niet, T., Johnson, S. D., & Linder. (2014). Speciation and extinction in the Greater Cape Floristic Region. In *Fynbos: Ecology, Evolution, and Conservation of a Megadiverse Region* (pp. 119–141). https://doi.org/10.1093/acprof

Elomaa, P., & Holton, T. (1994). Modification of flower colour using genetic engineering. *Biotechnology and Genetic Engineering Reviews*, *12*(1), 63–88. https://doi.org/10.1080/02648725.1994.10647909

Endler, J. A. (1977). *Geographic variation, speciation, and clines.* Princeton University Press.

Endress, P. K. (2001). Origins of flower morphology. *The Journal of Experimental Zoology*, *291*(2), 105–115. https://doi.org/10.1002/jez.1063

Fabbro, T., & Körner, C. (2004). Altitudinal differences in flower traits and reproductive allocation. *Flora*, *199*(1), 70–81.

Faegri, K., & van der Pijl, L. (2013). *Principles of pollination ecology.* Elsevier.

Fenster, C. B., Armbruster, W. S., Wilson, P., Dudash, M. R., Thomson, J. D., Fenster, B., … Thomson, D. (2004). Pollinatation Syndromes and Floral Specialization. *Annual Review of Ecology, Evolution, and Systematics*, *35*, 375–403. https://doi.org/10.2307/annurev.ecolsys.34.011802.30000015

Fenster, C. B., Diggle, P. K., Barrett, S. C. H., & Ritland, K. (1995). The genetics of floral development differentiationg two species of Mimulus. *Heredity*, *74*(3), 258–266.

Fernández-Mazuecos, M., Blanco-Pastor, J. L., Gómez, J. M., & Vargas, P. (2013). Corolla morphology influences diversification rates in bifid toadflaxes (Linaria sect. Versicolores). *Annals of Botany*, *112*(9), 1705–1722. https://doi.org/10.1093/aob/mct214

Ferreira, M. A. R., & Suchard, M. A. (2008). Bayesian analysis of elapsed times in continuous-time Markov chains. *Canadian Journal of Statistics*, *36*(3), 355–368. https://doi.org/10.1002/cjs.5550360302

Fitzjohn, R. G. (2010). Quantitative traits and diversification. *Systematic Biology*, *59*(6), 619–633. https://doi.org/10.1093/sysbio/syq053

Fiz, O., Vargas, P., Alarcón, M., Aedo, C., García, J. L., & Aldasoro, J. J. (2008). Phylogeny and historical biogeography of Geraniaceae in relation to climate changes and pollination ecology. *Systematic Botany*, *33*(2), 326–342.

Forest, F., Goldblatt, P., Manning, J. C., Baker, D., Colville, J. F., Devey, D. S., … Buerki, S. (2014). Pollinator shifts as triggers of speciation in painted petal irises (*Lapeirousia*: Iridaceae). *Annals of Botany*, *113*(2), 357–371. https://doi.org/10.1093/aob/mct248

Forkmann, G. (1991). Flavonoids as flower pigments: the formation of the natural spectrum and its extension by genetic engineering. *Plant Breeding*, *106*(1), 1–26.

Galley, C., Bytebier, B., Bellstedt, D. U., & Linder, H. P. (2007). The Cape element in the Afrotemperate flora: from Cape to Cairo? *Proceedings of the Royal Society B: Biological Sciences*, *274*(1609), 535–543. https://doi.org/10.1098/rspb.2006.0046

GBIF. (2013). Global Biodiversity Information Facility. Retrieved July 8, 2014, from http://www.gbif.org/species/3112

Geerts, S., & Pauw, A. (2009). Hyper-specialization for long-billed bird pollination in a guild of South African plants: the Malachite Sunbird pollination syndrome. *South African Journal of Botany*, *75*(4), 699–706. https://doi.org/10.1016/j.sajb.2009.08.001

Ghannadi, A., Bagherinejad, M. R., Abedi, D., Jalali, M., Absalan, B., & Sadeghi, N. (2012). Antibacterial activity and composition of essential oils from *Pelargonium graveolens* L'Her and *Vitex agnus-castus* L. *Iranian Journal of Microbiology*, *4*(4), 171–176.

Gibby, M., Albers, F., & Prinsloo, B. (1990). Karyological studies in *Pelargonium* sectt. *Ciconium*, *Dibrachya*, and *Jenkinsonia* (Geraniaceae). *Plant Systematics and Evolution*, *170*(3), 151–159.

Giusti, M. M., Rodriquez-Saona, L. E., & Wrolstad, R. E. (1999). Molar absorptivity and color characteristics of acylated and non-acylated Pelargonidin-based anthocyanins. *Journal of Agricultural and Food Chemistry*, *47*(11), 4631–4637.

Givnish, T. J., & Sytsma, K. J. (2000). *Molecular evolution and adaptive radiation*. Cambridge University Press.

Goldblatt, P. (1978). An analysis of the flora of southern Africa: its characteristics, relationships, and origins. *Annals of the Missouri Botanical Garden*, *65*(2), 369–436.

Goldblatt, P., & Manning, J. C. (1999). The long-proboscid fly pollination system in *Gladiolus* (Iridaceae). *Annals of the Missouri Botanical Garden*, *86*(3), 758–774.

Goldblatt, P., & Manning, J. C. (2002). Plant diversity of the Cape Region of southern Africa. *Annals of the Missouri Botanical Garden*, *89*(2), 281–302.

Goldblatt, P., Manning, J. C., & Bernhardt, P. (1995). Pollination biology of *Lapeirousia* subgenus *Lapeirousia* (Iridaceae) in southern Africa; floral divergence and adaptation for long-tongued fly pollination. *Annals of the Missouri Botanical Garden*, *82*(4), 517–534.

Goldblatt, P., Manning, J. C., & Bond, P. (2000). *Cape Plants. A Conspectus of the Cape Flora of South Africa*. (P. Goldblatt & J. Manning, Eds.).

Goldblatt, P., Savolainen, V., Porteous, O., Sostaric, I., Powell, M., Reeves, G., … Chase, M. W. (2002). Radiation in the Cape flora and the phylogeny of peacock irises *Moraea* (Iridaceae) based on four plastid DNA regions. *Molecular Phylogenetics and Evolution*, *25*(2), 341–360. https://doi.org/10.1016/S1055-7903(02)00235-X

Goldblatt, Peter, & Manning, J. C. (2006). Radiation of pollination systems in the Iridaceae of sub-Saharan Africa. *Annals of Botany*, *97*(3), 317–344. https://doi.org/10.1093/aob/mcj040

Gómez, J. M., Perfectti, F., & Camacho, J. P. M. (2006). Natural selection on Erysimum mediohispanicum flower shape: insights into the evolution of zygomorphy. *The American Naturalist*, *168*(4), 531–545. https://doi.org/10.1086/507048

Gomez, J. M., Perfectti, F., & Klingenberg, C. P. (2014). The role of pollinator diversity in the evolution of corolla-shape integration in a pollination-generalist plant clade. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *369*(1649), 20130257–20130257. https://doi.org/10.1098/rstb.2013.0257

Gómez, J. M., Perfectti, F., & Klingenberg, C. P. (2014). The role of pollinator diversity in the evolution of corolla-shape integration in a pollination-generalist plant clade. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *369*(1649), 20130257. https://doi.org/10.1098/rstb.2013.0257

Gómez, J. M., Perfectti, F., & Lorite, J. (2015). The role of pollinators in floral diversification in a clade of generalist flowers. *Evolution*, *69*(4), 863–878. https://doi.org/10.1111/evo.12632

Gómez, J. M., Torices, R., Lorite, J., Klingenberg, C. P. ., & Perfectti, F. (2016a). The role of pollinators in the evolution of corolla shape variation, disparity and integration in a highly diversified plant family with a conserved floral bauplan. *Annals of Botany*, *117*(5), 889–904. https://doi.org/10.1093/aob/mcv194

Gómez, J. M., Torices, R., Lorite, J., Klingenberg, C. P., & Perfectti, F. (2016b). The role of pollinators in the evolution of corolla shape variation, disparity and integration in a highly diversified plant family with a conserved floral bauplan. *Annals of Botany*, *117*(5), 889–904. https://doi.org/10.1093/aob/mcv194

Goodall, C. (1991). Procrustes methods in the statistical analysis of shape. *Journal of the Royal Statistical Society. Series B (Methodological)*, *53*(2), 285–339.

Gould, S. J. (1990). *Wonderful life: the Burgess Shale and the nature of history*. WW Norton & Company.

Gould, S. J., & Lewontin, R. C. (1979). The spandrels of San Marco and the Panglossian paradigm: a critique of the adaptationist programme. *Proceedings of the Royal Society London B*, *205*(1161), 581–598.

Grant, V. (1949). Pollination systems as isolating mechanisms in angiosperms. *Evolution*, *3*(1), 82–97.

Green, A. A., Kennaway, J. R., Hanna, A. I., Bangham, J. A., & Coen, E. (2010). Genetic ontrol of organ shape and tissue polarity. *PLoS Biology*, *8*(11), e1000537. https://doi.org/10.1371/journal.pbio.1000537

Greiner, S., & Bock, R. (2013). Tuning a ménage a trois: co-evolution and co-adaptation of nuclear and organellar genomes in plants. *BioEssays*, *35*(4), 354–365. https://doi.org/10.1002/bies.201200137

Greiner, S., Rauwolf, U., Meurer, J., & Herrmann, R. G. (2011). The role of plastids in plant speciation. *Molecular Ecology*, *20*(4), 671–691. https://doi.org/10.1111/j.1365-294X.2010.04984.x

Grotewold, E. (2006). *The Science of Flavonoids*.

Guisinger, M. M., Kuehl, J. V., Boore, J. L., & Jansen, R. K. (2008). Genome-wide analyses of Geraniaceae plastid DNA reveal unprecedented patterns of increased nucleotide substitutions. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(47), 18424–18429.

Guisinger, M. M., Kuehl, J. V., Boore, J. L., & Jansen, R. K. (2011). Extreme reconfiguration of plastid genomes in the angiosperm family Geraniaceae: rearrangements, repeats, and codon usage. *Molecular Biology and Evolution*, *28*(1), 583–600.

Hansen, D. M., van der Niet, T., & Johnson, S. D. (2012). Floral signposts: testing the significance of visual "nectar guides" for pollinator behaviour and plant fitness. *Proceedings of the Royal Society of London*, *279*(1729), 634–639. https://doi.org/10.1098/rspb.2011.1349

Harder, L. D., & Johnson, S. D. (2009). Darwin's beautiful contrivances: evolutionary and functional evidence for floral adaptation. *New Phytologist*, *183*(3), 530–545. https://doi.org/10.1111/j.1469-8137.2009.02914.x

Heleno, R. H., & Vargas, P. (2015). How do islands become green? *Global Ecology and Biogeography*, *24*(5), 518–526. https://doi.org/10.1111/geb.12273

Helfer, M., Koppensteiner, H., Schneider, M., Rebensburg, S., Forcisi, S., Müller, C., … Brack-Werner, R. (2014). The root extract of the medicinal plant *Pelargonium sidoides* is a potent HIV-1 attachment inhibitor. *PLoS ONE*, *9*(1). https://doi.org/10.1371/journal.pone.0087487

Herrera, J. (2005). Flower size variation in *Rosmarinus officinalis*: individuals, populations and habitats. *Annals of Botany*, *95*, 431–437. https://doi.org/10.1093/aob/mci041

Higgins, S. I., Nathan, R., & Cain, M. L. (2003). Are long-distance dispersal events in plants usually caused by nonstandard means of dispersal? *Ecology*, *84*(8), 1945–1956. https://doi.org/10.1890/01-0616

Ho, S. Y. W., & Phillips, M. J. (2009). Accounting for calibration uncertainty in phylogenetic estimation of evolutionary divergence times. *Systematic Biology*, *58*(3), 367–380. https://doi.org/10.1093/sysbio/syp035

Hoang, D. T., Chernomor, O., von Haeseler, A., Minh, B. Q., & Vinh, L. S. (2018). UFBoot2: Improving the ultrafast bootstrap approximation. *Molecular Biology and Evolution*, *35*(2), 518–522. https://doi.org/10.1093/molbev/msx281

Hodges, S. A. (1997). Floral nectar spurs and diversification. *International Journal of Plant Sciences*, *158*(6), 81–88.

Hodges, S. A., & Arnold, M. L. (1995). Spurring plant diversification: are floral nectar spurs a key innovation? *Proceedings of the Royal Society of London B: Biological Sciences*, *262*(1365), 343–348.

Hohna, S., Landis, M. J., Heath, T. A., Boussau, B., Lartillot, N., Moore, B. R., … Ronquist, F. (2016). RevBayes: Bayesian phylogenetic inference using graphical models and an interactive model-specification language. *Systematic Biology*, *65*(4), 726–736. https://doi.org/10.1093/sysbio/syw021

Holton, T. A., & Cornish, E. C. (1995). Genetics and biochemistry of anthocyanin biosynthesis. *The Plant Cell*, *7*(7), 1071–1083.

Huelsenbeck, J. P., & Ronquist, F. (2001). MrBayes: Bayesian inference of phylogenetic trees. *Bioinformatics*, *17*(8), 754–755. https://doi.org/10.1093/bioinformatics/17.8.754

Hughes, C. E. (2017). Are there many different routes to becoming a global biodiversity hotspot? *Proceedings of the National Academy of Sciences*, *114*(17), 4275–4277. https://doi.org/10.1073/pnas.1703798114

Hughes, C. E., & Eastwood, R. (2006). Island radiation on a continental scale: exceptional rates of plant diversification after uplift of the Andes. *Proceedings of the National Academy of Sciences*, *103*(27), 10334–10339. https://doi.org/10.1073/pnas.0601928103

Hughes, C. E., Nyffeler, R., & Linder, H. P. (2015). Evolutionary plant radiations: Where, when, why and how? *New Phytologist*, *207*(2), 249–253. https://doi.org/10.1111/nph.13523

James, C. M., Gibby, M., & Barrett, J. A. (2004). Molecular studies in Pelargonium (Geraniaceae). A taxonomic appraisal of section Ciconium and the origin of the "Zonal" and "Ivy-leaved" cultivars. *Plant Systematics and Evolution*, *243*(3–4), 131–146.

James, J. (2002). Cultivation and sales of *Pelargonium* plants for ornamental use in the UK and worldwide. In M. Lis-Balchin (Ed.), *Geranium and Pelargonium. The genera Geranium and Pelargonium* (pp. 80–91). Taylor & Francis.

Johanson, D., Edey, M., & Edey, M. A. (1990). *Lucy: The beginnings of humankind.* Simon and Schuster.

Johnson, M. A. T., & Özhatay, N. (1988). The distribution and cytology of Turkish Pelargonium (Geraniaceae). *Kew Bulletin*, *43*(1), 139–148.

Johnson, S. D. (1992). Climatic and phylogenetic determinants of flowering seasonality in the Cape flora. *Journal of Ecology*, *81*, 567–572.

Johnson, S. D. (2010). The pollination niche and its role in the diversification and maintenance of the southern African flora. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, *365*(1539), 499–516.

Johnson, S. D., & Hobbhahn, N. (2010). Generalized pollination, floral scent chemistry, and a possible case of hybridization in the African orchid *Disa fragrans*. *South African Journal of Botany*, *76*(4), 739–748. https://doi.org/10.1016/j.sajb.2010.07.008

Johnson, S. D., Linder, H. P., & Steiner, K. E. (1998). Phylogeny and radiation of pollination systems in Disa (Orchidaceae). *American Journal of Botany*, *85*(3), 402–411. https://doi.org/10.2307/2446333

Johnson, S. D., & Midgley, J. J. (1997). Fly pollination of *Gorteria diffusa* (Asteraceae), and a possible mimetic function for dark spots on the capitulum. *American Journal of Botany*, *84*(4), 429–436. https://doi.org/10.2307/2446018

Johnson, S. D., Peter, C. I., Ellis, A. G., Boberg, E., Botes, C., & van der Niet, T. (2011). Diverse pollination systems of the twin-spurred orchid genus Satyrium in African grasslands. *Plant Systematics and Evolution*, *292*(1–2), 95–103. https://doi.org/10.1007/s00606-010-0411-1

Johnson, S. D., & Steiner, K. E. (2000). Generalization versus specialization in plant pollination systems. *Trends in Ecology and Evolution*, *15*(4), 140–143. https://doi.org/10.1016/S0169-5347(99)01811-X

Jokat, W., Boebel, T., König, M., & Meyer, U. (2003). Timing and geometry of early Gondwana breakup. *Journal of Geophysical Research: Solid Earth*, *108*(B9). https://doi.org/10.1029/2002jb001802

Jones, C., & Price, R. (1996). Diversity and evolution of seedling *baupläne* in *Pelargonium* (Geraniaceae). *Aliso*, *14*(4), 281–295. https://doi.org/10.5642/aliso.19951404.07

Jones, C. S., Bakker, F. T., Schlichting, C. D., & Nicotra, A. B. (2009). Leaf shape evolution in the South African genus Pelargonium L' Hér. (Geraniaceae). *Evolution*, *63*(2), 479–497.

Jones, C. S., Cardon, Z. G., & Czaja, A. D. (2003). A phylogenetic view of low-level CAM in *Pelargonium* (Geraniaceae). *American Journal of Botany*, *90*(1), 135–142.

Jones, C. S., Martínez-Cabrera, H. I., Nicotra, A. B., Mocko, K., Marais, E. M., & Schlichting, C. D. (2013). Phylogenetic influences on leaf trait integration in Pelargonium (Geraniaceae): convergence, divergence, and historical adaptation to a rapidly changing climate. *American Journal of Botany*, *100*(7), 1306–1321. https://doi.org/10.3732/ajb.1200526

Jürgens, N. (1997). Floristic biodiversity and history of African arid regions. *Biodiversity and Conservation*, *6*, 495–514. https://doi.org/10.1023/A:1018325026863

Kaczorowski, R. L., Seliger, A. R., Gaskett, A. C., Wigsten, S. K., & Raguso, R. A. (2012). Corolla shape vs. size in flower choice by a nocturnal hawkmoth pollinator. *Functional Ecology*, *26*(3), 577–587. https://doi.org/10.1111/j.1365-2435.2012.01982.x

Kalyaanamoorthy, S., Minh, B. Q., Wong, T. K. F., von Haeseler, A., & Jermiin, L. S. (2017). ModelFinder: Fast model selection for accurate phylogenetic estimates. *Nature Methods*, *14*(6), 587–589. https://doi.org/10.1038/nmeth.4285

Katoh, K., & Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Molecular Biology and Evolution*, *30*(4), 772–780.

Kay, K. M., & Sargent, R. D. (2009). The role of animal pollination in plant speciation: integrating ecology, geography, and genetics. *Annual Review of Ecology, Evolution, and Systematics*, *40*(1), 637–656. https://doi.org/10.1146/annurev.ecolsys.110308.120310

Kayser, O., & Kolodziej, H. (1997). Antibacterial activity of extracts and constituents of *Pelargonium sidoides* and *Pelargonium reniforme*. *Planta Medica*, *63*(6), 508–510. https://doi.org/10.1055/s-2006-957752

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., … Drummond, A. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, *28*(12), 1647–1649.

Kennaway, R., Coen, E., Green, A., & Bangham, A. (2011). Generation of diverse biological forms through combinatorial interactions between tissue polarity and growth. *PLoS Computational Biology*, *7*(6), e1002071. https://doi.org/10.1371/journal.pcbi.1002071

Keppel, G., Van Niel, K. P., Wardell-Johnson, G. W., Yates, C. J., Byrne, M., Mucina, L., … Franklin, S. E. (2012). Refugia: identifying and understanding safe havens for biodiversity under climate change. *Global Ecology and Biogeography*, *21*(4), 393–404. https://doi.org/10.1111/j.1466-8238.2011.00686.x

Kessler, D., Diezel, C., & Baldwin, I. T. (2010). Changing pollinators as a means of escaping herbivores. *Current Biology*, *20*(3), 237–242. https://doi.org/10.1016/j.cub.2009.11.071

Kier, G., Mutke, J., Dinerstein, E., Ricketts, T. H., Küper, W., Kreft, H., & Barthlott, W. (2005). Global patterns of plant diversity and floristic knowledge. *Journal of Biogeography*, *32*(7), 1107–1116. https://doi.org/10.1111/j.1365-2699.2005.01272.x

Klingenberg, C. P. (2008). Morphological integration and developmental modularity. *Annual Review of Ecology, Evolution, and Systematics*, *39*(1), 115–132. https://doi.org/10.1146/annurev.ecolsys.37.091305.110054

Klingenberg, C. P. (2014). Studying morphological integration and modularity at multiple levels: concepts and analysis. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *369*(1649), 33–35. https://doi.org/10.1098/rstb.2013.0249

Klingenberg, C. P. (2016). Size, shape, and form: concepts of allometry in geometric morphometrics. *Development Genes and Evolution*, *226*(3), 113–137. https://doi.org/10.1007/s00427-016-0539-2

Koch, M. A., Kiefer, C., Ehrich, D., Vogel, J., Brochmann, C., & Mummenhoff, K. (2006). Three times out of Asia Minor: The phylogeography of *Arabis alpina* L. (Brassicaceae). *Molecular Ecology*, *15*(3), 825–839. https://doi.org/10.1111/j.1365-294X.2005.02848.x

Kolodziej, H., Kayser, O., Radtke, O. A., Kiderlen, A. F., & Koch, E. (2003). Pharmacological profile of extracts of Pelargonium sidoides and their constituents. *Phytomedicine : International Journal of Phytotherapy and Phytopharmacology*, *10*, 18–24.

Koptev, A., Cloetingh, S., Gerya, T., Calais, E., & Leroy, S. (2018). Non-uniform splitting of a single mantle plume by double cratonic roots: insight into the origin of the central and southern East African Rift System. *Terra Nova*, *30*(2), 125–134. https://doi.org/10.1111/ter.12317

Koski, M. H., & Ashman, T. L. (2015). An altitudinal cline in UV floral pattern corresponds with a behavioral change of a generalist pollinator assemblage. *Ecology*, *96*(12), 3343–3353. https://doi.org/10.1890/15-0242.1

Kubatko, L. S., & Degnan, J. H. (2007). Inconsistency of phylogenetic estimates from concatenated data under coalescence. *Systematic Biology*, *56*(1), 17–24.

Lack, D. (1940). Evolution of the Galapagos finches. *Nature*, *146*(3697), 324–327. https://doi.org/10.1038/146324a0

Lack, D. (1947). *Darwin's finches*. Cambridge University Press.

Landis, M. J., Matzke, N. J., Moore, B. R., & Huelsenbeck, J. P. (2013). Bayesian analysis of biogeography when the number of areas is large. *Systematic Biology*, *62*(6), 789–804. https://doi.org/10.1093/sysbio/syt040

Lanfear, R., Calcott, B., Ho, S. Y. W., & Guindon, S. (2012). PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular Biology and Evolution*, *29*(6), 1695–1701.

Lewu, F. B., Grierson, D. S., & Afolayan, A. J. (2007). Morphological diversity among accessions of *Pelargonium sidoides* DC. in the Eastern Cape, South Africa. *Genetic Resources and Crop Evolution*, *54*(1), 1–6. https://doi.org/10.1007/s10722-006-9000-0

Li, H.-T., Yi, T.-S., Gao, L.-M., Ma, P.-F., Zhang, T., Yang, J.-B., … Li, D.-Z. (2019). Origin of angiosperms and the puzzle of the Jurassic gap. *Nature Plants*, *5*(5). https://doi.org/10.1038/s41477-019-0421-0

Linder, H. P. (2003). The radiation of the Cape flora, southern Africa. *Biological Reviews*, *78*(4), 597–638.

Linder, H. P. (2005). Evolution of diversity: the Cape flora. *Trends in Plant Science*, *10*(11), 536–541. https://doi.org/10.1016/j.tplants.2005.09.006

Linder, H. P. (2008). Plant species radiations: where, when, why? *Philosophical Transactions of the Royal Society B: Biological Sciences*, *363*(1506), 3097–3105. https://doi.org/10.1098/rstb.2008.0075

Linder, H. P. (2014). The evolution of African plant diversity. *Frontiers in Ecology and Evolution*, *2*(38), 1–14. https://doi.org/10.3389/fevo.2014.00038

Linder, H. P. (2017). East African Cenozoic vegetation history. *Evolutionary Anthropology*, *26*(6), 300–312. https://doi.org/10.1002/evan.21570

Linder, H. P., & Hardy, C. R. (2004). Evolution of the species-rich Cape flora. *Philosophical Transactions of the Royal Society B, Biological Sciences*, *359*(1450), 1623–1632. https://doi.org/10.1098/rstb.2004.1534

Linder, H. P., & Verboom, G. A. (2015). The evolution of regional species richness: the history of the southern African flora. *Annual Review of Ecology, Evolution, and Systematics*, *46*(1), 393–412. https://doi.org/10.1146/annurev-ecolsys-112414-054322

Lindqvist, C., & Rajora, O. P. (Eds.). (2019). *Paleogenomics: Genome-Scale Analysis of Ancient DNA*. Springer.

Linnaeus, C. (1758). *Systema naturae, vol. 1.*

Linnaeus, C. (1799). *Species plantarum*. Impensis GC Nauk.

Lis-Balchin, M., Buchbauer, G., Hirtenlehner, T., & Resch, M. (1998). Antimicrobial activity of *Pelargonium* essential oils added to a quiche filling as a model food system. *Letters in Applied Microbiology*, *27*(4), 207–210.

Lis-Balchin, M., Buchbauer, G., Ribisch, K., & Wenger, M. T. (1998). Comparative antibacterial effects of novel *Pelargonium* essential oils and solvent extracts. *Letters in Applied Microbiology*, *27*(3), 135–141.

Lis-Balchin, M., & Deans, S. G. (1996). Antimicrobial effects of hydrophilic extracts of *Pelargonium* species (Geraniaceae). *Letters in Applied Microbiology*, *23*(4), 205–207.

Liu, L., Yu, L., Kubatko, L., Pearl, D. K., & Edwards, S. V. (2009). Coalescent methods for estimating phylogenetic trees. *Molecular Phylogenetics and Evolution*, *53*(1), 320–328. https://doi.org/10.1016/j.ympev.2009.05.033

Lowery, C. M., & Fraass, A. J. (2019). Morphospace expansion paces taxonomic diversification after end Cretaceous mass extinction. *Nature Ecology and Evolution*, *3*(6), 900. https://doi.org/10.1038/s41559-019-0835-0

Lowry, D. B., Modliszewski, J. L., Wright, K. M., Wu, C. A., & Willis, J. H. (2008). The strength and genetic basis of reproductive isolating barriers in flowering plants. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *363*(1506), 3009–3021. https://doi.org/10.1098/rstb.2008.0064

Luo, D., Carpenter, R., Vincent, C., Copsey, L., & Coen, E. (1995). Origin of floral asymmetry in Anthirrhinum. *Nature*, *383*(6603), 794–799.

Maddison, W. P., & Maddison, D. R. (2008). Mesquite: a modular system for evolutionary analysis. Version 3.6. *Evolution*, *62*, 1103–1118.

Manning, J. C., Euston-Brown, D. I. W., & Magee, A. R. (2015). *Pelargonium uliginosum* (Geraniaceae: Section *Hoarea*), a new species from Western Cape, South Africa, and an updated key to the species of the *P. dipetalum* group. *South African Journal of Botany*, *97*, 204–207. https://doi.org/10.1016/j.sajb.2015.01.016

Manning, J. C., & Goldblatt, P. (2011). *Pelargonium elandsmontanum* (Geraniaceae: Section *Hoarea*), a new species from the Western Cape Province, South Africa. *South African Journal of Botany*, *77*(1), 241–243. https://doi.org/10.1016/j.sajb.2010.07.021

Manning, J.C., & Goldblatt, P. (1996). The *Prosoeca peringueyi* (Diptera: Nemestrinidae) pollination guild in southern Africa: long-tongued flies and their tubular flowers. *Annals of the Missouri Botanical Garden*, *83*(1), 67–86.

Manning, J.C., & Goldblatt, P. (1997). The Moegistorhynchus longirostris (*Diptera: Nemestrinidae*) pollination guild: long-tubed flowers and a specialized long-proboscid fly pollination system in southern Africa. *Plant Systematics and Evolution*, *206*, 51–69.

Manning, J.C., & Goldblatt, P. (2012). Plants of The Greater Cape Floristic Region 1: The Core Cape Flora. *Strelitzia*, *29*, 1–870. https://doi.org/10.1017/CBO9781107415324.004

Manning, J.C., & le Roux, A. (2016). Pelargonium conradiae (Geraniaceae), a new species in section Ligularia from Worcester, Western Cape, South Africa. *South African Journal of Botany*, *105*, 313–316. https://doi.org/10.1016/j.sajb.2016.03.014

Manning, John C., & Goldblatt, P. (2005). Radiation of pollination systems in the Cape genus *Tritoniopsis* (Iridaceae: Crocoideae) and the development of bimodal pollination strategies. *International Journal of Plant Sciences*, *166*(3), 459–474. https://doi.org/10.1086/428703

Marais, E. M. (1999). The taxonomic position of Pelargonium appendiculatum (Geraniaceae). *South African Journal of Botany*, *65*(5–6), 404–406. https://doi.org/10.1016/S0254-6299(15)31030-9

Marais, E. M. (2016). Five new species of *Pelargonium*, section *Hoarea* (Geraniaceae), from the Western and Northern Cape Provinces of South Africa. *South African Journal of Botany*, *103*, 145–155.

Marais, E. M. (2017). Three new geophytic species of *Pelargonium* (Geraniaceae) from the Western Cape Province, South Africa and their relationships within section *Hoarea*. *South African Journal of Botany*, *113*, 261–269. https://doi.org/10.1016/j.sajb.2017.09.005

Martínez-Cabrera, H. I., & Peres-Neto, P. R. (2013). Shifts in climate foster exceptional opportunities for species radiation: The case of South African Geraniums. *PLoS ONE*, *8*(12), e83087. https://doi.org/10.1371/journal.pone.0083087

Martínez-Cabrera, H. I., Schlichting, C. D., Silander, J. A., & Jones, C. S. (2012). Low levels of climate niche conservatism may explain clade diversity patterns in the South African genus *Pelargonium* (Geraniaceae). *American Journal of Botany*, *99*(5), 954–960. https://doi.org/10.3732/ajb.1100600

Mativandlela, S. P. N., Lall, N., & Meyer, J. J. M. (2006). Antibacterial, antifungal and antitubercular activity of (the roots of) Pelargonium reniforme (CURT) and Pelargonium sidoides (DC) (Geraniaceae) root extracts. *South African Journal of Botany*, *72*(2), 232–237. https://doi.org/10.1016/j.sajb.2005.08.002

Matzke, N. J. (2014). Model selection in historical biogeography reveals that founder-event speciation is a crucial process in island clades. *Systematic Biology*, *63*(6), 951–970. https://doi.org/10.1093/sysbio/syu056

McLachlan, A., Jaramillo, E., Defeo, O., Dugan, J., de Ruyck, A., & Coetzee, P. (1995). Adaptations of bivalves to different beach types. *Journal of Experimental Marine Biology and Ecology*, *187*(2), 147–160. https://doi.org/10.1016/0022-0981(94)00176-E

Medel, R., Botto-Haman, C., & Kalin-Arroyo, M. (2003). Pollinator-mediated selection on the nectar guide phenotype in the Anean monkey flower, *Mimulus luteus*. *Ecology*, *84*(7), 1721–1732.

Meredith, R. W., Janecka, J. E., Gatesy, J., Ryder, O. A., Fisher, C. A., Teeling, E. C., … Murphy, W. J. (2011). Impacts of the Cretaceous terrestrial revolution and KPg extinction on mammal diversification. *Science*, *334*(6055), 521–524. https://doi.org/10.1126/science.1211028

Miller, D. M. (2002). The taxonomy of Pelargonium species and cultivars, their origins and growth in the wild. In *Geranium and Pelargonium.* (pp. 61–91). CRC Press.

Miller, M. A., Pfeiffer, W., & Schwartz, T. (2010). Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In *2010 gateway computing environments workshop (GCE)* (pp. 1–8). Ieee.

Mitchell, K. A., Markham, K. R., & Boase, M. R. (1998). Pigment chemistry and colour of *Pelargonium* flowers. *Phytochemistry*, *47*(3), 355–361.

Mitchell, N., Carlson, J. E., & Holsinger, K. E. (2018). Correlated evolution between climate and suites of traits along a fast–slow continuum in the radiation of Protea. *Ecology and Evolution*, *8*(3), 1853–1866. https://doi.org/10.1002/ece3.3773

Mitchell, N., Moore, T. E., Mollmann, H. K., Carlson, J. E., Mocko, K., Martinez-Cabrera, H., … Holsinger, K. E. (2015). Functional traits in parallel evolutionary radiations and trait-environment associations in the Cape Floristic Region of South Africa. *The American Naturalist*, *185*(4), 525–537. https://doi.org/10.1086/680051

Mocko, K., Nicotra, A. B., & Jones, C. S. (2017). Extent of solar tracking differs between two co-occurring congeneric geophytes that differ in leaf shape. *International Journal of Plant Sciences*, *179*(2), 162–173. https://doi.org/10.1086/695698

Mol, J., Grotewold, E., & Koes, R. (1998). How genes paint flowers and seeds. *Trends in Plant Science*, *3*(6), 212–217. https://doi.org/10.1016/S1360-1385(98)01242-4

Moore, T. E., Schlichting, C. D., Aiello-Lammens, M. E., Mocko, K., & Jones, C. S. (2018). Divergent trait and environment relationships among parallel radiations in Pelargonium (Geraniaceae): a role for evolutionary legacy? *New Phytologist*, *219*(2), 794–807. https://doi.org/10.1111/nph.15196

Morrone, J. J. (2015). Biogeographical regionalisation of the world: a reappraisal. *Australian Systematic Botany*, *28*(3), 81. https://doi.org/10.1071/sb14042

Mower, J. P., Touzet, P., Gummow, J. S., Delph, L. F., & Palmer, J. D. (2007). Extensive variation in synonymous substitution rates in mitochondrial genes of seed plants. *BMC Evolutionary Biology*, *7*(135).

Mummenhoff, K., Polster, A., Mühlhausen, A., & Theißen, G. (2009). *Lepidium* as a model system for studying the evolution of fruit development in Brassicaceae. *Journal of Experimental Botany*, *60*(5), 1503–1513. https://doi.org/10.1093/jxb/ern304

Mutke, J., & Barthlott, W. (2005). Patterns of vascular plant diversity at continental to global scales. *Biologiske Skrifter*, *55*(4), 521–531. https://doi.org/10.3112/erdkunde.2007.04.01

Myers, J. A., Vellend, M., & Gardescu, S. (2004). Seed dispersal by white-tailed deer: implications for long-distance dispersal, invasion, and migration of plants in eastern North America. *Oecologia*, *139*(1), 35–44. https://doi.org/10.1007/s00442-003-1474-2

Nathan, R. (2006). Long-distance dispersal of plants. *Science*, *313*(5788), 786–788.

Nathan, Ran, Schurr, F. M., Spiegel, O., Steinitz, O., Trakhtenbrot, A., & Tsoar, A. (2008). Mechanisms of long-distance seed dispersal. *Trends in Ecology and Evolution*, *23*(11), 638–647. https://doi.org/10.1016/j.tree.2008.08.003

Newman, E., Manning, J. C., & Anderson, B. (2014). Matching floral and pollinator traits through guild convergence and pollinator ecotype formation. *Annals of Botany*, *113*(2), 373–384. https://doi.org/10.1093/aob/mct203

Nguyen, L. T., Schmidt, H. A., von Haeseler, A., & Minh, B. Q. (2015). IQ-TREE: A fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Molecular Biology and Evolution*, *32*(1), 268–274. https://doi.org/10.1093/molbev/msu300

Nicotra, A. B., Chong, C., Bragg, J. G., Ong, C. R., Aitken, N. C., Chuah, A., … Borevitz, J. O. (2016). Population and phylogenomic decomposition via genotyping-by-sequencing in Australian Pelargonium. *Molecular Ecology*, *25*(9), 2000–2014. https://doi.org/10.1111/mec.13584

Nicotra, A. B., Cosgrove, M. J., Cowling, A., Schlichting, C. D., & Jones, C. S. (2008). Leaf shape linked to photosynthetic rates and temperature optima in South African *Pelargonium* species. *Oecologia*, *154*(4), 625–635.

Nicotra, A. B., Leigh, A., Boyce, C. K., Jones, C. S., Niklas, K. J., Royer, D. L., & Tsukaya, H. (2011). The evolution and functional significance of leaf shape in the angiosperms. *Functional Plant Biology*, *38*(7), 535–552.

Nosil, P. (2012). *Ecological speciation.* Oxford University Press.

Nosil, Patrik, Feder, J. L., Flaxman, S. M., & Gompert, Z. (2017). Tipping points in the dynamics of speciation. *Nature Ecology & Evolution*, *1*(2), 1–8. https://doi.org/10.1038/s41559-016-0001

Oberlander, K. C., Emshwiller, E., Bellstedt, D. U., & Dreyer, L. L. (2009). A model of bulb evolution in the eudicot genus *Oxalis* (Oxalidaceae). *Molecular Phylogenetics and Evolution*, *51*(1), 54–63. https://doi.org/10.1016/j.ympev.2008.11.022

Ollerton, J., & Lack, A. (2014). Relationships between flowering phenology, plant size and reproductive success in *Lotus corniculatus* (Fabaceae). *Plant Ecology*, *139*(1), 35–47. https://doi.org/10.2307/20050693

Onstein, R. E., Jordan, G. J., Sauquet, H., Weston, P. H., Bouchenak-Khelladi, Y., Carpenter, R. J., & Linder, H. P. (2016). Evolutionary radiations of Proteaceae are triggered by the interaction between traits and climates in open habitats. *Global Ecology and Biogeography*, *25*(10), 1239–1251. https://doi.org/10.1111/geb.12481

Ordano, M., Fornoni, J., Boege, K., & Domínguez, C. A. (2008). The adaptive value of phenotypic floral integration. *New Phytologist*, *179*(4), 1183–1192. https://doi.org/10.1111/j.1469-8137.2008.02523.x

Palazzesi, L., Gottschling, M., Barreda, V., & Weigend, M. (2012). First Miocene fossils of Vivianiaceae shed new light on phylogeny, divergence times, and historical biogeography of Geraniales. *Biological Journal of the Linnean Society*, *107*, 67–85.

Palmer, J. D., Nugent, J. M., & Herbon, L. A. (1987). Unusual structure of geranium chloroplast DNA: A triple-sized inverted repeat, extensive gene duplications, multiple inversions, and two repeat families. *Proceedings of the National Academy of Sciences*, *84*(3), 769–773. https://doi.org/10.1073/pnas.84.3.769

Palumbo, R., Hong, W.-F., Wang, G.-L., Krause, C., & Tay, D. (2007). Target Region Amplification Polymorphism (TRAP) as a tool for detecting genetic variation in the genus *Pelargonium*. *HortScience*, *42*(5), 1118–1123.

Paradis, E., Claude, J., & Strimmer, K. (2004). APE: analyses of phylogenetics and evolution in R language. *Bioinformatics*, *20*, 289–290.

Parenicova, L., de Folter, S., Kieffer, M., Horner, D. S., Favalli, C., Busscher, J., … Colombo, L. (2003). Molecular and phylogenetic analyses of the complete MADS-box transcription factor family in Arabidopsis: new openings to the MADS world. *The Plant Cell*, *15*(7), 1538–1551. https://doi.org/10.1105/tpc.011544.these

Park, S., Grewe, F., Zhu, A., Ruhlman, T. A., Sabir, J., Mower, J. P., & Jansen, R. K. (2015). Dynamic evolution of *Geranium* mitochondrial genomes through multiple horizontal and intracellular gene transfers. *New Phytologist*, *208*(2), 570–583.

Parkinson, C. L., Mower, J. P., Qiu, Y.-L., Shirk, A. J., Song, K., Young, N. D., … Palmer, J. D. (2005). Multiple major increases and decreases in mitochondrial substitution rates in the plant family Geraniaceae. *BMC Evolutionary Biology*, *5*(73).

Pauw, A., Stofberg, J., & Waterman, R. J. (2009). Flies and flowers in Darwin's race. *Evolution*, *63*(1), 268–279. https://doi.org/10.1111/j.1558-5646.2008.00547.x

Pearman, P. B., Guisan, A., Broennimann, O., & Randin, C. F. (2008). Niche dynamics in space and time. *Trends in Ecology and Evolution*, *23*(3), 149–158. https://doi.org/10.1016/j.tree.2007.11.005

Petren, K., Grant, B. R., & Grant, P. R. (1999). A phylogeny of Darwin's finches based on microsatellite DNA length variation. *Proceedings of the Royal Society B: Biological Sciences*, *266*(1417), 321–329. https://doi.org/10.1098/rspb.1999.0641

Petroni, K., & Tonelli, C. (2011). Recent advances on the regulation of anthocyanin synthesis in reproductive organs. *Plant Science*, *181*(3), 219–229. https://doi.org/10.1016/j.plantsci.2011.05.009

Phillips, S. J., Anderson, R. P., & Schapire, R. E. (2006). Maximum entropy modeling of species geographic distributions. *Ecological Modelling*, *190*(3–4), 231–252. https://doi.org/10.1016/j.ecolmodel.2005.03.026

Pirie, M. D., Oliver, E. G. H., Mugrabi De Kuppler, A., Gehrke, B., Le Maitre, N. C., Kandziora, M., & Bellstedt, D. U. (2016). The biodiversity hotspot as evolutionary hot-bed: spectacular radiation of Erica in the Cape Floristic Region. *BMC Evolutionary Biology*, *16*(1), 190. https://doi.org/10.1186/s12862-016-0764-3

Pole, M. S. (2001). Can long-distance dispersal be inferred from the New Zealand plant fossil record? *Australian Journal of Botany*, *49*(3), 357–366. https://doi.org/10.1071/BT00022

Ponting, C. P., Teiling, C., Affourtit, J., Clabaut, C., Abzhanov, A., Rands, C. M., … Webster, M. T. (2013). Insights into the evolution of Darwin's finches from comparative analysis of the *Geospiza magnirostris* genome sequence. *BMC Genomics*, *14*(1), 95. https://doi.org/10.1186/1471-2164-14-95

Price, R. A., & Palmer, J. D. (1993). Phylogenetic Relationships of the Geraniaceae and Geraniales from *rbcL* Sequence Comparisons. *Annals of the Missouri Botanical Garden*, *80*(3), 661–671.

Procheş, Ş., Cowling, R. M., & Du Preez, D. R. (2005). Patterns of geophyte diversity and storage organ size in the winter-rainfall region of southern Africa. *Diversity and Distributions*, *11*(1), 101–109. https://doi.org/10.1111/j.1366-9516.2005.00132.x

Procheş, Ş., Cowling, R. M., Goldblatt, P., Manning, J. C., & Snijman, D. A. (2006). An overview of the Cape geophytes. *Biological Journal of the Linnean Society*, *87*(1), 27–43. https://doi.org/10.1111/j.1095-8312.2006.00557.x

Prum, R. O. (2012). Aesthetic evolution by mate choice: Darwin's really dangerous idea. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *367*(1600), 2253–2265. https://doi.org/10.1098/rstb.2011.0285

Prusinkiewicz, P., & Runions, A. (2012). Computational models of plant development and form. *New Phytologist*, *193*(3), 549–569. https://doi.org/10.1111/j.1469-8137.2011.04009.x

Quattrocchio, F., Baudry, A., Lepiniec, L., & Grotewold, E. (2006). The regulation of flavonoid biosynthesis. In *The science of flavonoids* (pp. 97–122). Springer, New York, NY.

Raup, D. M. (1986). Biological Extinction in Earth History. *Science*, *231*(4745), 1528–1533.

Raup, D. M. (1994). The role of extinction in evolution. *Proceedings of the National Academy of Sciences*, *91*(15), 6758–6763. https://doi.org/10.1073/pnas.91.15.6758

Rausher, M. D. (2008). Evolutionary transitions in floral color. *International Journal of Plant Sciences*, *169*(1), 7–21. https://doi.org/10.1086/523358

Rebocho, A. B., Kennaway, J. R., Bangham, J. A., & Coen, E. (2017). Formation and shaping of the *Antirrhinum* flower through modulation of the CUP boundary gene. *Current Biology*, *27*(17), 2610-2622.e3. https://doi.org/10.1016/j.cub.2017.07.064

Ree, R. H., & Smith, S. A. (2008). Maximum likelihood inference of geographic range evolution by dispersal, local extinction, and cladogenesis. *Systematic Biology*, *57*(1), 4–14. https://doi.org/10.1080/10635150701883881

Revell, L. J. (2012). phytools: an R package for phylogenetic comparative biology (and other things). *Methods in Ecology and Evolution*, *3*(2), 217–223. https://doi.org/10.1111/j.2041-210X.2011.00169.x

Reyes, E., Sauquet, H., & Nadot, S. (2016). Perianth symmetry changed at least 199 times in angiosperm evolution. *Taxon*, *65*(5), 945–964. https://doi.org/10.12705/655.1

Rieseberg, L. H., & Wendel, J. (2004). Plant speciation - rise of the poor cousins. *New Phytologist*, *161*(1), 3–8. https://doi.org/10.1111/j.1469-8137.2004.00957.x

Rieseberg, L. H., & Willis, J. H. (2007). Plant Speciation. *Science*, *317*(5840), 910–914.

Ringelberg, J. (2012). *Floral evolution in Cape* Pelargonium *(Geraniaceae) - Inferring shifts in nectar spur length and pollination*. Wageningen University & Research.

Rohlf, F. J. (2004). tpsUtil, file utility program. version 1.26. Department of Ecology and Evolution, State University of New York at Stony Brook.

Rohlf, F. J. (2010). TPSDig2 Version 2.16. Department of Ecology and Evolution, Stony Brook University, NY, USA.

Rohlf, F. J., & Marcus, L. F. (1993). A revolution in morphometrics. *Trends in Ecology & Evolution*, *8*(4), 129–132.

Rohlf, F., & Slice, D. (1990). Extensions of the Procrustes method for the optimal superimposition of landmarks. *Systematic Biology*, *39*(1), 40–59. https://doi.org/10.2307/2992207

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., … Huelsenbeck, J. P. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, *61*(3), 539–542. https://doi.org/10.1093/sysbio/sys029

Ronse De Craene, L. (2015). Meristic changes in flowering plants: how flowers play with numbers. *Flora: Morphology, Distribution, Functional Ecology of Plants*, *221*, 22–37. https://doi.org/10.1016/j.flora.2015.08.005

Ronse De Craene, L. (2018). Understanding the role of floral development in the evolution of angiosperm flowers: clarifications from a historical and physico-dynamic perspective. *Journal of Plant Research*, *131*(3), 367–393. https://doi.org/10.1007/s10265-018-1021-1

Rosas-Guerrero, V., Aguilar, R., Martén-Rodríguez, S., Ashworth, L., Lopezaraiza-Mikel, M., Bastida, J. M., & Quesada, M. (2014). A quantitative review of pollination syndromes: Do floral traits predict effective pollinators? *Ecology Letters*, *17*(3), 388–400. https://doi.org/10.1111/ele.12224

Röschenbleck, J., Albers, F., Müller, K., Weinl, S., & Kudla, J. (2014). Phylogenetics, character evolution and a subgeneric revision of the genus *Pelargonium* (Geraniaceae). *Phytotaxa*, *159*(2), 31–76.

Ruhlman, T. A., & Jansen, R. K. (2018). Aberration or Analogy? The Atypical Plastomes of Geraniaceae. In *Advances in Botanical Research* (1st ed., Vol. 85, pp. 223–262). Elsevier Ltd. https://doi.org/10.1016/bs.abr.2017.11.017

Rundle, H. D., & Nosil, P. (2005). Ecological speciation. *Ecology Letters*, *8*(3), 561–580. https://doi.org/10.18960/seitai.66.3_561

Sanmartín, I., Anderson, C. L., Alarcon, M., Ronquist, F., & Aldasoro, J. J. (2010). Bayesian island biogeography in a continental setting: the Rand Flora case. *Biology Letters*, *6*(5), 703–707. https://doi.org/10.1098/rsbl.2010.0095

Sanmartín, I., & Ronquist, F. (2004). Southern hemisphere biogeography inferred by event-based models: plant versus animal patterns. *Systematic Biology*, *53*(2), 216–243. https://doi.org/10.1080/10635150490423430

Sattler, R. (1978). "Fusion" and "continuity" in floral morphology. *Notes of the Royal Botanic Garden Edinburgh*, *36*, 397–405.

Sattler, R. (1990). Towards a more dynamic plant morphology. *Acta Biotheoretica*, *38*(3–4), 303–315. https://doi.org/10.1007/BF00047245

Sattler, R. (1996). Classical morphology and continuum morphology: Opposition and continuum. *Annals of Botany*, *78*(5), 577–581. https://doi.org/10.1006/anbo.1996.0163

Sauquet, H., Weston, P. H., Anderson, C. L., Barker, N. P., Cantrill, D. J., Mast, A. R., & Savolainen, V. (2009). Contrasted patterns of hyperdiversification in Mediterranean hotspots. *Proceedings of the National Academy of Sciences*, *106*(1), 221–225. https://doi.org/10.1073/pnas.0805607106

Sauquet, Hervé, Von Balthazar, M., Magallón, S., Doyle, J. A., Endress, P. K., Bailes, E. J., … Schönenberger, J. (2017). The ancestral flower of angiosperms and its early diversification. *Nature Communications*, *8*, 16047. https://doi.org/10.1038/ncomms16047

Savriama, Y., Gomez, J. M., Perfectti, F., & Klingenberg, C. P. (2012). Geometric morphometrics of corolla shape: dissecting components of symmetric and asymmetric variation in Erysimum mediohispanicum (Brassicaceae). *New Phytologist*, *196*(3), 945–954.

Savriama, Y., & Klingenberg, C. P. (2011). Beyond bilateral symmetry: geometric morphometric methods for any type of symmetry. *BMC Evolutionary Biology*, *11*(1), 280.

Schenk, J. J. (2016). Consequences of secondary calibrations on divergence time estimates. *PLoS ONE*, *11*(1), e0148228. https://doi.org/10.1371/journal.pone.0148228

Schiestl, F. P., & Johnson, S. D. (2013). Pollinator-mediated evolution of floral signals. *Trends in Ecology and Evolution*, *28*(5), 307–315. https://doi.org/10.1016/j.tree.2013.01.019

Schnitzler, J., Barraclough, T. G., Boatwright, J. S., Goldblatt, P., Manning, J. C., Powell, M. P., … Savolainen, V. (2011). Causes of plant diversification in the Cape biodiversity hotspot of South Africa. *Systematic Biology*, *60*(3), 343–357. https://doi.org/10.1093/sysbio/syr006

Schulter, D. (2001). Ecology and the origin of species. *Trends in Ecology and Evolution*, *16*(7), 372–380.

Schulter, D. (2009). Evidence for ecological speciation and its alternative. *Science*, *323*(5915), 737–742.

Schwery, O., Onstein, R. E., Bouchenak-Khelladi, Y., Xing, Y., Carter, R. J., & Linder, H. P. (2015). As old as the mountains: the radiations of the Ericaceae. *New Phytologist*, *207*(2), 355–367. https://doi.org/10.1111/nph.13234

Shuttleworth, A., & Johnson, S. D. (2010). Floral scents of chafer-pollinated asclepiads and a potential hybrid. *South African Journal of Botany*, *76*(4), 770–778. https://doi.org/10.1016/j.sajb.2010.07.023

Simões, M., Breitkreuz, L., Alvarado, M., Baca, S., Cooper, J. C., Heins, L., … Lieberman, B. S. (2016). The evolving theory of evolutionary radiations. *Trends in Ecology and Evolution*, *31*(1), 27–34. https://doi.org/10.1016/j.tree.2015.10.007

Snijman, D. A. (ed. . (2013). *Plants of the Greater Cape Floristic Region 2: The Extra Cape Flora*. South African National Biodiversity Institute.

Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics*, *30*(9), 1312–1313.

Stöver, B. C., & Müller, K. F. (2010). TreeGraph 2: combining and visualizing evidence from different phylogenetic analyses. *BMC Bioinformatics*, *11*(1), 7.

Struck, M. (1994). Flowers and their insect visitors in the arid winter rainfall region of southern Africa: observations on permanent plots. Insect visitation behaviour. *Journal of Arid Environments*, *28*(1), 51–74.

Struck, M. (1997). Floral divergence and convergence in the genus *Pelargonium* (Geraniaceae) in southern Africa: ecological and evolutionary considerations. *Plant Systematics and Evolution*, *208*, 71–97.

Struck, M., & van der Walt, J. J. A. (1996). Floral structure and pollination in *Pelargonium*. In L. J. G. van der Maesen (Ed.), *The Biodiversity of African Plants* (pp. 631–638). https://doi.org/10.1007/978-94-009-0285-5_77

Sukhumpinij, P., Kakihara, F., Hondo, K., & Kato, M. (2012). Analysis of pigments in yellow-flowered Pelargonium section Hoarea. *Acta Horticulturae*, *937*, 711–716.

Sweet, R. (1822). Geraniaceae 1. *Ridgway, J.*, 1–100.

Swofford, D. L. (2002). Phylogenetic Analysis Using Parsimony (* and other methods). *Options*. Sunderland, Massachusetts: Sinauer Associates. https://doi.org/10.1007/BF02198856

Takhtajan, A., Crovello, T. J., & Cronquist, A. (1986). *Floristic regions of the world*. Berkeley: University of California Press.

Tanaka, Y. (2006). Flower colour and cytochromes P450. *Phytochemistry Reviews*, *5*(2–3), 283–291. https://doi.org/10.1007/s11101-006-9003-7

Tank, D. C., Eastman, J. M., Pennell, M. W., Soltis, P. S., Soltis, D. E., Hinchliff, C. E., … Harmon, L. J. (2015). Nested radiations and the pulse of angiosperm diversification: Increased diversification rates often follow whole genome duplications. *New Phytologist*, *207*(2), 454–467. https://doi.org/10.1111/nph.13491

Tavares, H., Whibley, A., Field, D. L., Bradley, D., Couchman, M., Copsey, L., … Coen, E. (2018). Selection and gene flow shape genomic islands that control floral guides. *Proceedings of the National Academy of Sciences*, *115*(43), 11006–11011. https://doi.org/10.1073/pnas.1801832115

Taylor, D. W. (1991). Paleobiogeographic relationships of Andean angiosperms of Cretaceous to Pliocene age. *Palaeogeography, Palaeoclimatology, Palaeoecology*, *88*(1–2), 69–84. https://doi.org/10.1016/0031-0182(91)90015-J

Tilney-Bassett, R. A. E., & Almouslem, A. B. (1989). Variation in plastid inheritance between pelargonium cultivars and their hybrids. *Heredity*, *63*(2), 145–153. https://doi.org/10.1038/hdy.1989.86

Tilney-Bassett, R. A. E., Almouslem, A. B., & Amoatey, H. M. (1992). Complementary genes control biparental plastid inheritance in *Pelargonium*. *Theoretical and Applied Genetics*, *85*(2–3), 317–324. https://doi.org/10.1007/BF00222876

Tilney-Bassett, R. A. E., & Birky, C. W. (1981). The mechanism of the mixed inheritance of chloroplast genes in *Pelargonium* - evidence from gene frequency distributions among the progeny of crosses. *Theoretical and Applied Genetics*, *60*(1), 43–53. https://doi.org/10.1007/BF00275177

Touloumenidou, T., Bakker, F. T., Marais, E. M., & Albers, F. (2003). Chromosomal evolution interpreted from the rDNA ITS phylogeny for Pelargonium sect. Hoarea (Geraniaceae). *Schumannia*, *4*, 1–14.

Tremblay, R. L., Ackerman, J. D., Zimmerman, J. K., & Calvo, R. N. (2005). Variation in sexual reproduction in orchids and its evolutionary consequences: a spasmodic journey to diversification. *Biological Journal of the Linnean Society*, *84*, 1–54.

Trifinopoulos, J., Nguyen, L. T., von Haeseler, A., & Minh, B. Q. (2016). W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. *Nucleic Acids Research*, *44*(W1), W232–W235. https://doi.org/10.1093/nar/gkw256

Tsai, T., Diggle, P. K., Frye, H. A., & Jones, C. S. (2018). Contrasting lengths of *Pelargonium* floral nectar tubes result from late differences in rate and duration of growth. *Annals of Botany*, *121*(3), 549–560. https://doi.org/10.1093/aob/mcx171

Turelli, M., Barton, N. H., & Coyne, J. A. (2001). Theory of speciation. *Trends in Ecology & Evolution*, *16*(7), 330–343. https://doi.org/10.1016/S0169-5347(01)02177-2

Tyszka, J. (2006). Morphospace of foraminiferal shells: Results from the moving reference model. *Lethaia*, *39*(1), 1–12. https://doi.org/10.1080/00241160600575808

Vaidya, G., Lohman, D. J., & Meier, R. (2011). SequenceMatrix: concatenation software for the fast assembly of multi-gene datasets with character set and codon information. *Cladistics*, *27*(2), 171–180.

Valente, L. M., & Vargas, P. (2013). Contrasting evolutionary hypotheses between two mediterranean-climate floristic hotspots: the Cape of southern Africa and the Mediterranean Basin. *Journal of Biogeography*, *40*(11), 2032–2046. https://doi.org/10.1111/jbi.12156

van de Kerke, S. J., Shrestha, B., Ruhlman, T. A., Weng, M.-L., Jansen, R. K., Jones, C. S., … Bakker, F. T. (2019). Plastome based phylogenetics and younger crown node age in Pelargonium. *Molecular Phylogenetics and Evolution*, *137*, 33–43. https://doi.org/10.1016/j.ympev.2019.03.021

van der Does, M., Knippertz, P., Zschenderlein, P., Harrison, R. G., & Stuut, J.-B. W. (2018). The mysterious long-range transport of giant mineral dust particles. *Science Advances*, *4*(12), eaau2768. https://doi.org/10.1126/sciadv.aau2768

van der Niet, T., & Johnson, S. D. (2009). Patterns of plant speciation in the Cape floristic region. *Molecular Phylogenetics and Evolution*, *51*(1), 85–93. https://doi.org/10.1016/j.ympev.2008.11.027

van der Niet, T., & Johnson, S. D. (2012). Phylogenetic evidence for pollinator-driven diversification of angiosperms. *Trends in Ecology and Evolution*, *27*(6), 353–361. https://doi.org/10.1016/j.tree.2012.02.002

van der Niet, T., Johnson, S. D., & Linder, H. P. (2006). Macroevolutionary data suggest a role for reinforcement in pollination system shifts. *Evolution*, *60*(8), 1596. https://doi.org/10.1554/05-705.1

van der Niet, T., Jürgens, A., & Johnson, S. D. (2010). Pollinators, floral morphology and scent chemistry in the southern African orchid genus Schizochilus. *South African Journal of Botany*, *76*(4), 726–738. https://doi.org/10.1016/j.sajb.2010.07.004

van der Niet, T., Peakall, R., & Johnson, S. D. (2014). Pollinator-driven ecological speciation in plants: New evidence and future perspectives. *Annals of Botany*, *113*(2), 199–211. https://doi.org/10.1093/aob/mct290

van der Niet, T., Pirie, M. D., Shuttleworth, A., Johnson, S. D., & Midgley, J. J. (2014). Do pollinator distributions underlie the evolution of pollination ecotypes in the Cape shrub Erica plukenetii? *Annals of Botany*, *113*(2), 301–316. https://doi.org/10.1093/aob/mct193

van der Niet, T., Zollikofer, C. P. E., Ponce De Leon, M. S., Johnson, S. D., & Linder, H. P. (2010). Three-dimensional geometric morphometrics for studying floral shape variation. *Trends in Plant Science*, *15*(8), 423–426. https://doi.org/10.1016/j.tplants.2010.05.005

van der Walt, J. J. A. (1985). A taxonomic revision of the type section of *Pelargonium* L ' Hérit . *Bothalia*, *15*(3/4), 345–385.

van der Walt, J. J. A., Albers, F., & Gibby, M. (1990). Delimitation of *Pelargonium* sect. *Glaucophyllum* (Geraniaceae). *Plant Systematics and Evolution*, *171*(1–4), 15–26.

van der Walt, J. J. A., Albers, F., Gibby, M., Marschewski, D. E., & Price, R. A. (1995). A biosystematic study of *Pelargonium* section *Ligularia*: 1. A new section *Subsucculentia*. *South African Journal of Botany*, *61*(6), 339–346.

van der Walt, J. J. A., & Boucher, D. A. (1986). A taxonomic revision of the section *Myrrhidium* of *Pelargonium* (Geraniaceae) in southern Africa. *South African Journal of Botany*, *52*(5), 438–462. https://doi.org/10.1016/s0254-6299(16)31508-3

van der Walt, J. J. A., & van Zyl (nee Hugo), L. (1988). A taxonomic revision of *Pelargonium* section *Campylia* (Geraniaceae). *South African Journal of Botany*, *54*(2), 145–171. https://doi.org/10.1016/s0254-6299(16)31345-x

van der Walt, J. J. A., & Vorster, P. J. (1981). Typification of the genus *Pelargonium* L'Herit. (fam. Geraniaceae). *Taxon*, *30*(1), 307.

van der Walt, J. J. A., & Vorster, P. J. (1983). Phytogeography of *Pelargonium*. *Bothalia*, *14*(3/4), 517–523.

van der Walt, J. J. A., & Vorster, P. J. (1988). Pelargoniums *of southern Africa. Vol I-III*. Annals of Kirtenbosch Botanic Gardens.

Verboom, G. A., Archibald, J. K., Bakker, F. T., Bellstedt, D. U., Conrad, F., Dreyer, L. L., … Nowell, T. L. (2009). Origin and diversification of the Greater Cape flora: ancient species repository, hot-bed of recent radiation, or both? *Molecular Phylogenetics and Evolution*, *51*(1), 44–53. https://doi.org/10.1016/j.ympev.2008.01.037

Verboom, G. A., Dreyer, L. L., & Savolainen, V. (2009). Understanding the origins and evolution of the world's biodiversity hotspots: the biota of the African "Cape Floristic Region" as a case study. *Molecular Phylogenetics and Evolution*, *51*(1), 1–4. https://doi.org/10.1016/j.ympev.2009.03.001

Wagner, Günter P. (1996). Homologues, natural kinds and the evolution of modularity. *American Zoologist*, *36*(1), 36–43.

Wagner, G. P., Pavlicev, M., & Cheverud, J. M. (2007). The road to modularity. *Nature Reviews Genetics*, *8*(12), 921–931. https://doi.org/10.1038/nrg2267

Wang, H., Moore, M. J., Soltis, P. S., Bell, C. D., Brockington, S. F., Alexandre, R., … Soltis, D. E. (2009). Rosid radiation and the rapid rise of angiosperm-dominated forests. *Proceedings of the National Academy of Sciences*, *106*(10), 3853–3858.

Wang, L., Burhenne, K., Kristensen, B. K., & Rasmussen, S. K. (2004). Purification and cloning of a Chinese red radish peroxidase that metabolise pelargonidin and forms a gene family in Brassicaceae. *Gene*, *343*(2), 323–335. https://doi.org/10.1016/j.gene.2004.09.018

Warren, B. H., Bakker, F. T., Bellstedt, D. U., Bytebier, B., Claßen-Bockhoff, R., Dreyer, L. L., … Hawkins, J. A. (2011). Consistant phenological shifts in the making of a biodiversty hotspot: the Cape flora. *BMC Evolutionary Biology*, *11*(1), 39.

Waters, J. M., & Roy, M. S. (2004). Out of Africa: The slow train to Australasia. *Systematic Biology*, *53*(1), 18–24. https://doi.org/10.1080/10635150490264671

Weberling, F. (1992). *Morphology of flowers and inflorescences*. CUP Archive.

Weihe, A., Apitz, J., Pohlheim, F., Salinas-Hartwig, A., & Börner, T. (2009). Biparental inheritance of plastidial and mitochondrial DNA and hybrid variegation in Pelargonium. *Molecular Genetics and Genomics*, *282*(6), 587–593. https://doi.org/10.1007/s00438-009-0488-9

Weller, S. G., & Sakai, A. K. (1999). Using phylogenetic approaches for the analysis of plant breeding system evolution. *Annual Review of Ecology and Systematics*, *30*(1), 169–189. https://doi.org/10.1146/annurev.phyto.40.030402.090559

Welsford, M. R., Hobbhahn, N., Midgley, J. J., & Johnson, S. D. (2016). Floral trait evolution associated with shifts between insect and wind pollination in the dioecious genus *Leucadendron* (Proteaceae). *Evolution*, *70*(1), 126–139. https://doi.org/10.1111/evo.12821

Weng, M.-L., Blazier, J. C., Govindu, M., & Jansen, R. K. (2013). Reconstruction of the ancestral plastid genome in Geraniaceae reveals a correlation between genome rearrangements, repeats, and nucleotide substitution rates. *Molecular Biology and Evolution*, *31*(3), 645–659.

Weng, M.-L., Ruhlman, T. A., Gibby, M., & Jansen, R. K. (2012). Phylogeny, rate variation, and genome size evolution of *Pelargonium* (Geraniaceae). *Molecular Phylogenetics and Evolution*, *64*(3), 654–670. https://doi.org/10.1016/j.ympev.2012.05.026

Wheeler, W. C. (2012). *Systematics: a course of lectures*. Wiley-Blackwell.

Whibley, A. C., Langlade, N. B., Andalo, C., Hanna, A. I., Bangham, A., Thébaud, C., & Coen, E. (2006). Evolutionary paths underlying flower color variation in *Antirrhinum*. *Science*, *313*(5789), 963–966. https://doi.org/10.1126/science.1129161

White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols*, *18*(1), 315–322. https://doi.org/10.1016/B978-0-12-372180-8.50042-1

Whittall, J. B., & Hodges, S. A. (2007). Pollinator shifts drive increasingly long nectar spurs in columbine flowers. *Nature*, *447*(7145), 706–709. https://doi.org/10.1038/nature05857

Wickham, H. (2009). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York. Retrieved from http://ggplot2.org

Widmer, A., Lexer, C., & Cozzolino, S. (2009). Evolution of reproductive isolation in plants. *Heredity*, *102*(1), 31–38. https://doi.org/10.1038/hdy.2008.69

Winemiller, K. O. (1990). Spatial and temporal variation in tropical fish trophic networks. *Ecological Monographs*, *60*(3), 331–367.

Winkel, B. S. J. (1991). The biosynthesis of flavonoids. In E. Grotewold (Ed.), *The Science of Flavonoids* (pp. 71–95). Springer.

Wu, Z. Y., Liu, J., Provan, J., Wang, H., Chen, C. J., Cadotte, M. W., … Milne, R. I. (2018). Testing Darwin's transoceanic dispersal hypothesis for the inland nettle family (Urticaceae). *Ecology Letters*, *21*(10), 1515–1529. https://doi.org/10.1111/ele.13132

Xie, Y., Wu, G., Tang, J., Luo, R., Patterson, J., Liu, S., … Wang, J. (2014). SOAPdenovo-Trans: *de novo* transcriptome assembly with short RNA-Seq reads. *Bioinformatics*, *30*(12), 1660–1666.

Yamaguchi, N., Yamaguchi, A., Abe, M., Wagner, D., & Komeda, Y. (2012). LEAFY controls *Arabidopsis* pedicel length and orientation by affecting adaxial-abaxial cell fate. *Plant Journal*, *69*(5), 844–856. https://doi.org/10.1111/j.1365-313X.2011.04836.x

Yesson, C., & Culham, A. (2006a). A phyloclimatic study of *Cyclamen*. *BMC Evolutionary Biology*, *6*(1), 72. https://doi.org/10.1186/1471-2148-6-72

Yesson, C., & Culham, A. (2006b). Phyloclimatic modeling: combining phylogenetics and bioclimatic modeling. *Systematic Biology*, *55*(5), 785–802. https://doi.org/10.1080/10635150600815 70

Yu, Y., Harris, A. J., & He, X. J. (2010). S-DIVA (Statistical Dispersal-Vicariance Analysis): a tool for inferring biogeographic histories. *Molecular Phylogenetics and Evolution*, *56*(2), 848–850. https://doi.org/10.1016/j.ympev.2010.04.011

Zelditch, M. L., Swiderski, D. L., & Sheets, H. D. (2012). *Geometric Morphometrics for Biologists*. Academic Press.

Zhao, J., & Dixon, R. A. (2010). The "ins" and "outs" of flavonoid transport. *Trends in Plant Science*, *15*(2), 72–80. https://doi.org/10.1016/j.tplants.2009.11.006

Zhao, T., & Schranz, M. E. (2019). Network-based microsynteny analysis identifies major differences and genomic outliers in mammalian and angiosperm genomes. *Proceedings of the National Academy of Sciences*, *116*(6), 2165–2174. https://doi.org/10.1073/pnas.1801757116

Summary

It is in the nature of humans to wonder about and try to make sense off everything they see and all things they encounter. Humans have always been observing, describing, and recording patterns in the world around them. Particularly since Darwin's *On the Origin of Species*, researchers have been trying to interpret both the function and origins of the tremendous variety in shape among living organisms. But what is shape? Mathematically, shape is defined as what is left when we subtract the size component from form. In other words, shape is the true variation in morphological parts, irrespective of the size of the individuals studied. Under the influence of external pressure, shape might change and this could lead to the formation of new species. However, as has been pointed out in the past, one should be cautious when attributing direct cause – effect relations to potential drivers of shape.

For flowering plants, there are multiple drivers of shape change that effect the process of speciation. In the background, species are constrained by their phylogenetic history, i.e. evolutionary changes that were advantageous to their ancestors, might in the present circumstances turn out to no longer be beneficial. At the same time, speciation brought about by pollinator pressure is highly dynamic and can happen quickly because changes in floral shape are triggered by current events. Spur pollination is a highly specialised pollination form in that it spatially separates pollination reward (the nectar) from the corolla. This is considered to result in a Darwinian 'arms race' between plant and pollinator, increasing effective pollen transfer, and, ultimately, fitness for both.

In this thesis, I aim to bring together multiple layers of influence on floral shape in *Pelargonium* in order to paint a comprehensive picture of the evolution of floral diversity in this clade. I accomplish this by studying the historical biogeography and ancestral conditions of the genus, within- and between species differences in floral shape, and their relation with the adaptation of *Pelargonium* species to local conditions. By building upon the extensive knowledge on speciation processes in the Greater Cape Floristic Region, I am now able to research and infer speciation processes for the *Pelargonium* clade.

This thesis starts with the construction of a new phylogenetic tree based on 74 plastome exons and nuclear rDNA ITS regions for 120 *Pelargonium* species (**Chapter 2**). This phylogenetic reconstruction including nucleotide, amino acid, and ITS alignments, resolved relationships within the genus and helped resolve several incongruences. I confirm the subgeneric split into a small and large chromosome clade and retrieved 100% bootstrap support for four of the five major clades The long standing issue of the position of *P. nanum,* now sister to the remainder of clade A2, is still uncertain. *Pelargonium karooicum* ($x = 10$) I find is sister of a small clade consisting of $x = 9$, 10, and 17 species. I find both sect. *Ligularia* and *Hoarea* to be monophyletic and confirm the existence of a *Polyactium-Otidia-Cortusina* clade. I find sect. *Campylia* to be sister to clade A2 but with surprisingly weak support. I find *Pelargonium* crown node to have originated around 9.7 Mya, which places the age

of *Pelargonium* in line with general findings for other Fynbos (8.5 ± 1.85 Mya) and Succulent Karoo lineages (5.17 ± 0.64).

These new calibrations formed a great opportunity for a historical biogeographical analysis (**Chapter 3**). I find the ancestral geographic range of *Pelargonium* to include the Winter and Summer rainfall region, the Karoo region, and the Natal region in South Africa. This is congruent with the current distribution of *Pelargonium*, especially for clade A1 and A2 species (characterised by a wide array in life forms including woody shrubs, stem succulents, geophytes, and herbaceous annuals). Clade B species more often occur in subsets of the ancestral range. A number of species has escaped the ancestral range altogether, moving into Mozambique, Tanzania, Kenya and Northern Malawi, Ethiopia, and most notably St. Helena and Australia + New Zealand. Within clade C (containing a number of previously hypothesised disjunct distribution patterns) the most shifts relative to the estimated ancestral range for this clade of Winter - Summer rainfall, Karoo and Natal regions have occurred. I find that a single long-range dispersal event underlies the jump from the Cape region into Ethiopia around 2 Mya, from where a lineage would have dispersed onto Socotra around 1 Mya. I find that long range dispersal seems to be the prime cause of the disjunct distribution patterns in *Pelargonium*, both to Australia, Madagascar, along the East African Highway, and into Asia minor.

Pelargonium flowers are highly variable in their floral shape, with species ranging from zygomorphic to near-actinomorphic corolla shape, varying in petal copy number, and with lengths of nectar spurs varying between zero to ten cm. In **Chapter 4** I explore the potential of combining two 2D photograph-based datasets of floral morphology into a single 3D virtual flower. This provides a method for bringing together multiple layers of shape variation which offers unique benefits to complement established imaging techniques. I analyse separate datasets for the side and front view of the flower, and a combined dataset based on virtual3D flowers. PCA results of the reconstructed 3D floral models are highly congruent with the separate 2D morphospaces, indicating it is an accurate, virtual, representation of floral shape. Through my approach, I find that adding the third dimension to the data is crucial to accurately interpret the manner of, as well as levels of, shape variation in flowers.

The morphospace based on virtual3D flowers as reconstructed in Chapter 4 formed the basis for the exploration of floral shape and floral modularity in **Chapter 5**. I wanted to know to what extent the modules (show-, transfer-, and reward- apparatus) within the *Pelargonium* flower are integrated, i.e. whether they evolve in concert, and whether different, apparatus-specific selective pressures may exist. I indeed find there is modularity within our floral data when partitioned over show-, transfer-, and reward- apparatuses which suggests there are three evolutionary layers happening alongside each other. Each of these lines will undergo evolutionary pressure by a range of causes and each of these lines will thus undergo changes in shape. However, since I also find there is strong integration between these modules, independent evolution only goes to a certain extent and therefore appears to be a sort of balancing act. Based on this, I find that the relation between floral shape and

phylogeny is highly dependent on clade and different per module. The B, C1, and C2 clades appear to be under phylogenetic constraint, while both clade A1 and A2 do not appear to be under any type of phylogenetic pressure. A possible explanation for this finding can be found in the relatively young age of the *Pelargonium* 'winter-rainfall' clade A.

In my final research chapter (**Chapter 6**), I examined whether there is a relation between floral shape, species distributions and environmental conditions and determine to what extent aspects of floral shape are drivers of speciation rates in *Pelargonium*. I bring together the separate layers developed in the previous chapters, and add pollinator diversity and flowering time. I find that speciation in *Pelargonium* is a complex patchwork of interactions between environmental conditions, pollinator distributions, flowering time, and historical biogeographical influences. Species can occur in wet, dry, cold, warm, high, and low conditions as well as in all possible combinations of them and floral shape seems to vary independently from these conditions. I find only corolla shape to have an influence on speciation rates of *Pelargonium* species. Rather, floral shape appears to be an 'extra layer' of diversity *Pelargonium* lineages can tap into in order to set them apart. Now that I have brought together multiple layers of potential influences on floral shape in *Pelargonium*, I find that speciation in this clade may not be as clear-cut as expected. My findings give the opportunity to place *Pelargonium* in a broader, GCFR-wide, evolutionary perspective.

Acknowledgements

A PhD project is a mountain to climb and in the past five years I have enjoined (almost) every step of the way. Especially 'the last stretch', when the story written in this book finally started to unfold, has been an absolute pleasure. Luckily, us candidates are supported along the way by many people.

First, I want to thank my promotor Eric Schranz for giving me this amazing opportunity. When we first met, you immediately nicknamed me 'Sara Vanilla' based on the topic of my MSc thesis. I love that you are the laidback boss that you are, allowing your people to move out of the strict office culture. Let's explore the space!

Maybe most important, I want to thank my daily co-promotor Freek Bakker, the conceptual father of this project. I cannot thank you enough for offering this project to me, guiding me along the way. I love that in our meetings, first we would talk an hour about our music before we remembered we should be discussing science :) Your endless knowledge on *Pelargonium* and everything else is an inspiration to all of us. And I'm so happy you found happiness with Onkamon and your 'new-daughter' Parita. They bring out the best in you.

Next I want to thank the members of my PhD committee: Sophie Nadot, Bas Zwaan, Rampal Etienne, and Félix Forest. I am honoured you have taken the time and effort to read my thesis in such detail. Thank you for all your helpful comments and suggestions.

Also many thanks to my co-authors Cindi Jones, Carl Schlichting, Bob Jansen, Bikash Shrestha, Tracey Ruhlman, Mao-Lun Weng, Robert Jansen, Samin Hosseini, and Lia Hemerik. Together, we soldier through! And although at the time I maybe not always appreciated your comments, I now appreciate how much you have improved my work. Many, many thanks for your thoughts and time!

To all the people at Biosystematics, still there and in the past five years: thank you for all the 'gezelligheid'. Floris, my *Pelargonium* buddy: do not let them get to you! You are amazing and kind the way you are. It has been a pleasure to work, take trips, and complain with you!
Wilma... the group does not function when you are away. Do not underestimate how much people depend on you, even though they do not show it. You are amazing, and kind, and balanced, and thank you for all our talks about what is wrong and unfair in the world.
Dêêdi, you have undergone a true transformation: from the timid girl I picked up at the bus stop to a woman filled with confidence. Your unwavering focus is amazing to witness. You can do anything you set your mind to!
Ronald, Roel, and Nynke, thank you for letting me be part of all your teaching. It has been an inspiration!
Robin; many, many thanks for your endless patience in teaching me R! I would have been nowhere without your help.
Thijmen! Please send me pictures of your shoes!?!?

Dear Lars (or should I say Professor Chatrou?), thank you for all your support! You were always there for a critical note, a great laugh, and a big hug! I was so sad to see you go, but am so happy you are so happy with your new 'bureau' in Gent. I love that in the past few months I (and my family!) have had the pleasure of getting to know you (and your family). Griete and Korneel: thank you for your support when I really needed it and Griete: you are the most down-to-earth person I know, thank you for our talks!

Now for my paranymphs: Setareh and Sanne. I am honoured to have been paranymph for the both of you, and so happy you are mine. I love we will be three female Sses on stage during my defence, and in a bit all three a doctor! We will show them!
Setareh: thank you for taking me under your wing when I was your student. And thank you for all the amazing times we have had together. With you I can laugh, and cry, and watch terrible movies (picked by me...), and geek out over knitting, and complain, and eat, and send pictures of the cakes we have baked that week. And even though we do not work together any more, thank you I can always come to you with my problems and for giving me advice.
Sanne, sweet Sanne! I have said it many times before, you are the most together person I know. I am amazed by how you are so kind, and thoughtful, and organised, and always seem to know exactly what is going on behind all the screens. All the times we have been making music together, whether it was classical in the WSKOV and the HanzeOrkest, or pop in the SteenGroeve Orkest, or jiggs in the Music show, it has been awesome! I'm so happy you and Wouter have had your long time wish fulfilled this year and have been joined by your boys Daniël and Tobias. Let's play together again soon, I miss you!

I am eternally grateful to all my students: Laura, Anne, Lisa, Tiemen, Sverre, and Merlijn. It really is true: this thesis would never have been possible without your efforts, thank you! I loved our times in the greenhouse and all the trips we took to take pictures of flowers. It has been a pleasure to have seen all of you grow during your projects and a privilege to have been your supervisor.

Another group of people I'm indebted to are the members of the Dutch Pelargonium Vereniging species group. Thank you for having me during your meetings! Especially Luuts: thank you for welcoming us in your home and greenhouse so many times. I'm still in awe of your collection (and secretely jealous) and tell everyone who wants to hear (and also when they don't) about you and your plants. Also many thanks to the family Mijnsbergen and Esther van der Velde for allowing us in your plant collections.

To my friends in the Bazen group: Corné & Niké, Sander & Daniëlle, Tobie & Janita, and Loes. You have been amazing friends the past 11 (!) years and I love we still have dinner almost every week. Loes and Niké: thank you for reading versions of the chapters in this book! And especially Sander and also Corné: thank you for all the times you came to babysit so I could work extra. I'm really touched by the amount of support from all of you these past months and hope we still go on our trips when we are old and grey.

Attila; many, many thanks for creating the beautiful cover of this book! You took my weird idea of 'lots of flowers' and made a stunning tribute to *Pelargonium*. Thank you!

Thank you Rolf, Ronald & Ronald, Liesbeth, Paul, Reinout, Pieter, Martin, and Stuart for an amazing trip to South Africa! It has been an inspiration to be part of your enormous enthusiasm for everything that grows.
Martin: thank you for your hospitality during our stay and driving Reinout and me in your Fiat Panda and introducing us to so many people you know! It has been one of the greatest experiences of my life and actually 'feeling' the Fynbos has been a great help in completing this project.
Reinout and Walter, thank you for saving the *Pelargonium* collection! I'm honoured you were willing to take on this enormous project and I hope I can honour you, the Hortus Amsterdam, and your effort with this book.

Mom, thank you for soldiering through. I know it hasn't been easy these past years but I am really proud of you and the joy you seem to have found in Ella. I love you. Titus, my big little brother, I'm so proud you are not bothered at all by other people but find your own way through life. You and Sophie will create the perfect world for yourselves! Han, Wout, Vera, and Opa: thank you for taking me into your family! From the beginning, it has been an enormously warm bath. To many more vacations, drinks, and Italian sunshine!

And last: Rick. You are my life, my best friend. Thank you for letting me be myself, peculiar as that can be. You have given me the strength to believe in myself, to find my inner power, and be ok with who I am. We will find our way, no matter where it gets us! And thank you for our Ella. She is so much a perfect hybrid of the both of us, it is scary!
Ella, my sweet, strong-headed, happy, and beautiful little ball of energy... Never change, you are perfect.

Cheers!
Sara

List of publications

S.J. van de Kerke, M.E. Schranz, F.T. Bakker. *Morphological integration of floral modules in* Pelargonium. *In preparation.*

S.J. van de Kerke, M.E. Schranz, F.T. Bakker. Pelargonium *floral shape in space and time. In preparation.*

F. Breman, J. Hofs, **S.J. van de Kerke**, M.E. Schranz, F.T. Bakker. *Evolutionary relationships within a complex of African* Accipiter. *In preparation.*

S.J. van de Kerke, M. Schram, M.E. Schranz, F.T. Bakker. *Historical Biogeography and ancestral conditions of* Pelargonium. *Submitted.*

S.J. van de Kerke, T. van Engelenhoven, A. van Es, L. Schat, L. van Son, S. Vink, L. Hemerik, M.E. Schranz, F.T. Bakker (2019). *Capturing variation in floral shape; a virtual3D based morphospace for* Pelargonium. Peer*J, in revision*.

S.J. van de Kerke, B. Shrestha, T.A. Ruhlman, M.-L. Weng, R.K. Jansen, C.S. Jones, C.D. Schlichting, S. Hosseini, S. Mohammadin, M.E. Schranz, F.T. Bakker (2019). *Plastome based phylogenetics and younger crown node age in* Pelargonium. Molecular phylogenetics and evolution 137: 33-43.

S. Mohammadin, K. Peterse, **S.J. van de Kerke**, L.W. Chatrou, A.A. Dönmez, K. Mummenhoff, J. C. Pires, P. P. Edger, I.A. Al-Shehbaz, M.E. Schranz (2017). *Anatolian origins and diversification of Aethionema, the sister lineage of the core* Brassicaceae. American Journal of Botany, 104.7: 1042-1054.

F.T. Bakker, D. Lei, J. Yu, S. Mohammadin, Z. Wei, **S.J. van de Kerke**, B. Gravendeel, M. Nieuwenhuis, M. Staats, D.E. Alqueza-Planas, R. Holmer (2015). *Herbarium genomics: plastome sequence assembly from a range of herbarium specimens using an Iterative Organelle Genome Assembly pipeline*. Biological Journal of the Linnean Society. 2010;1-11

About the author

Sara was born on the 11th of December 1988 in Amsterdam, The Netherlands. She lived there with her family for 18 years. She attended the Barlaeus Gymnasium and received her high school diploma in 2007.

After taking a gapyear, Sara started her BSc Biology at Wageningen University and Research in 2008. During her BSc, Sara completed her thesis project at the Laboratory of Entomology. She continued her studies in Wageningen in her MSc Biology, majoring in Evolution and Biodiversity. She completed two thesis projects at the Biosystematics group. The first project was in cooperation with the Wageningen Herbarium Vadense and was entitled *"Systematics of African* Vanilla *Orchids"*, supervised by Dr. J.J. Wieringa, Dr. F.T. Bakker, and T. Damen. For this project, she was awarded a grant by the Alberta Mennega Foundation to visit the herbarium in Paris, as well as the herbarium at Kew Gardens London and the Natural History Museum. Her second project, entitled *"Historical Biogeography of Brassicaceae"*, was supervised by Dr. F.T. Bakker and Dr. S. Mohammadin.

During her studies, Sara was an active member of the Wageningen Student Choir and Orchestra Association (WSKOV) where she played the viola and was Secretary of the board for a year. In addition, she participated in the Dutch Student Orchestra (NSO) and was guestplayer in a variety of ensembles.

After completing her MSc studies, Sara got the opportunity to start her PhD project at the Biosystematics Group of Wageningen University & Research with Prof. M.E. Schranz and Dr. F.T. Bakker. During her PhD, Sara was member and chair of the Wageningen Evolution and Ecology Seminars (WEES) committee and participated in numerous musical projects.

Education Statement of the Graduate School
Experimental Plant Sciences

Issued to: Sara van de Kerke
Date: 20 November 2019
Group: Biosystematics
University: Wageningen University & Research

1) Start-Up Phase	date	cp
► **First presentation of your project**		
Modelling floral morphology in *Pelargonium*	22 Apr 2015	1.5
► **Writing or rewriting a project proposal**		
Pelargonium Project Proposal	Sept 2014 - Apr 2015	6.0
► **Writing a review or book chapter**		
► **MSc courses**		
Subtotal Start-Up Phase		7.5

2) Scientific Exposure	date	cp
► **EPS PhD student days**		
EPS PhD GetTogether, Soest	29 & 30 Jan 2015	0.6
EPS PhD GetTogether, Soest	28 & 29 Jan 2016	0.6
► **EPS theme symposia**		
EPS theme 4 symposium, University of Amsterdam	15 Dec 2015	0.3
EPS theme 4 symposium, University of Amsterdam	25 Sep 2018	0.3
► **Lunteren Days and other national platforms**		
Annual Experimental Plant Sciences meeting, Lunteren	13 & 14 Apr 2015	0.6
Annual meeting Nationale Pelargonium Vereniging, Wageningen	25 Apr 2015	0.3
Annual Experimental Plant Sciences meeting, Lunteren	11 & 12 Apr 2016	0.6
Annual Experimental Plant Sciences meeting, Lunteren	10 Apr 2017	0.3
Annual meeting Nationale Pelargonium Vereniging, Wageningen	3 Nov 2018	0.3
► **Seminars (series), workshops and symposia**		
Seminar: Lee Dugatkin, WEES	20 Nov 2014	0.1
Seminar: Koos Biesmeijer, WEES	18 Dec 2014	0.1
Seminar: George Coupland, EPS Flying seminar	19 Jan 2015	0.1
Seminar: Kevin Foster, WEES	22 Jan 2015	0.1
Seminar: Yves van de Peer, EPS Flying seminar	3 Feb 2015	0.1
Seminar: Doug Landis, WEES	19 Feb 2015	0.1
Seminar: Michael Pirie, EPS Flying seminar	18 Mar 2015	0.1
Seminar: Hanna Kokko, WEES	19 Mar 2015	0.1
Workshop: IOGA workshop	9 Apr 2015	0.2
Seminar: Yolanda Chen, WEES	21 May 2015	0.1
Seminar: Micheal Begon, WEES	25 Jun 2015	0.1
Seminar: Siobhan Brady, EPS Flying seminar	9 Sep 2015	0.1
Seminar: Christian C. Voigt, WEES	17 Sep 2015	0.1
Seminar: Tinde van Andel, WEES	17 Dec 2015	0.1
Seminar: Arnold van Vliet, WEES	21 Jan 2016	0.1
Seminar: Olivier Hamant, EPS Flying seminar	14 Mar 2016	0.1
Seminar: Etienne Danchin, WEES	17 Mar 2016	0.1
Seminar: Sophie Nadot, EPS Flying seminar	20 May 2016	0.1
Seminar: Dan Tawfik, WEES	16 Jun 2016	0.1
Seminar: Peter Linder, pre-promotion lecture	6 Oct 2017	0.1
Seminar: Johan Bucher, PBR Monday Seminar	24 Oct 2016	0.1
Seminar: Johan Bucher, PBR Monday Seminar	21 Nov 2018	0.1
► **Seminar plus**		
Workshop: WEES workshop Koos Biesmeijer	18 Dec 2014	0.1
Workshop: WEES workshop Tinde van Andel	17 Dec 2015	0.1
► **International symposia and congresses**		
Plant Diversity in the GCFR: From Genomes to Biomes, CapeTown, South Africa	20-22 Jul 2015	0.9
EMBO|EMBL Symposium: New Model Systems for Linking Evolution and Ecology, Heidelberg, Germany	8-11 May 2016	1.2
II Iberian Symposium of Geometric Morphometrics, Madrid, Spain	9-10 Jun 2016	0.6
3rd UK Plant Evolution Meeting, Kew, United Kingdom	3-5 Apr 2019	0.9
Congress of the European Society for Evolutionary Biology (ESEB), Turku, Finland	19-24 Aug 2019	1.6

►	**Presentations**		
	Poster: Modelling floral morphospace in *Pelargonium*, a new system for testing evolutionary ecological hypotheses, Annual Experimental Plant Sciences meeting	13 Apr 2015	1.0
	Presentation: Aligning the IOGA output at gene level, IOGA workshop	9 Apr 2015	1.0
	Presentation: Modelling floral morphology in *Pelargonium*, annual meeting Nationale Pelargonium Vereniging	25 Apr 2015	1.0
	Poster: Plastome sequencing in *Pelargonium* Phylogenetics, NSF Dimensions of Biodiversity Project 'Parallel Evolutionary radiations in Protea and Pelargonium in the Greater Cape Floristic Region' Project meeting	21 Jul 2015	1.0
	Poster: Plastome-based Phylogenetics in *Pelargonium*, annual Experimental Plant Sciences meeting & EMBO|EMBL Symposium: New Model Systems for Linking Evolution and Ecology	10 May 2016	1.0
	Poster: Modelling Floral Evolution in *Pelargonium*, II Iberian Symposium of Geometric Morphometrics	9 Jun 2016	1.0
	Presentation: Geometric morphometrics in *Pelargonium*, annual meeting Nationale Pelargonium Vereniging	3 Nov 2018	1.0
	Presentation: Evolution of floral shape in *Pelargonium*, 3rd UK Plant Evolution Meeting	4 Apr 2019	1.0
	Poster: Evolution of floral shape in *Pelargonium*, Congress of the European Society for Evolutionary Biology (ESEB)	19-24 Aug 2019	1.0
►	**IAB interview**		
►	**Excursions**		
	Visit Dümmen Orange and Flower Trials	15 Jun 2018	0.3
	Subtotal Scientific Exposure		20.9

3) In-Depth Studies		*date*	*cp*
►	**Advanced scientific courses & workshops**		
	Introduction to Geometric Morphometrics, Transmitting Science, Barcelona, Spain	27-31 Jul 2015	1.8
	Introduction to R for Statistical Analysis, Wageningen	20-21 Feb 2019	0.6
	Carpentries Workshop: Genomics Data, Wageningen	5 Feb 2019	0.3
►	**Journal club**		
	Biosystematics group Journal Club	2014-2019	3.0
►	**Individual research training**		
	Subtotal In-Depth Studies		5.7

4) Personal Development		*date*	*cp*
►	**General skill training courses**		
	Adobe InDesign Essential Training, Wageningen	7 & 8 Nov 2016	0.6
	Brainfriendly working and writing, Wageningen	31 Oct 2018	0.3
	Scientific Writing, Wageningen	Oct 2018 - Jan 2019	1.8
►	**Organisation of meetings, PhD courses or outreach activities**		
	Chair of Wageningen Evolution and Ecology Seminars (WEES)	Jan 2015 - Sep 2016	1.5
►	**Membership of EPS PhD Council**		
	Subtotal Personal Development		4.2

TOTAL NUMBER OF CREDIT POINTS*	**38.3**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS with a minimum total of 30 ECTS credits.

** A credit represents a normative study load of 28 hours of study.*

This work was supported by and carried out at the Biosystematics Group, Wageningen University and Research, The Netherlands.

This project has been financially supported by the Alberta Mennega Foundation.

Cover design: A.M. Houtkooper

Printed by: DIGIFORCE