Pollinator mediated selection in *Pelargonium reniforme* Curtis (Geraniaceae): patterns and processes.

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By

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Abstract

Pelargonium reniforme is currently divided into two subspecies, *P. reniforme* subsp. *reniforme* and *P. reniforme* subsp. *velutinum*. The species falls into section *Reniformia* along with the closely-related *P. sidoides*. Observations of the section showed some discrepancies in the current taxonomy; mainly floral variation that was not recorded in the descriptions of the subspecies of *P. reniforme*, particularly the differences in hypanthium lengths.

Patterns of variability were analysed using both morphometrics and inter simple sequence repeat (ISSR) data for *P. reniforme* (both subspecies), and *P. sidoides*. Results showed no support for the current subspecific division of *P. reniforme* but also no support for the current status of *P. sidoides* as a separate species. However, both morphometric and ISSR data show some evidence for the existence of two distinctly separate groups within *P. reniforme* subsp. *velutinum* as two distinct types have been recognized: those with bright pink flowers with long tubes (up to 70mm), and those with pale pink flowers with shorter tubes (as little as 8mm). These two forms have been found in a single population (Grahamstown), where they occur sympatrically, as well as in monomorphic populations. Analyses of the processes thought to be responsible for the observed patterns were conducted on the Grahamstown population. Observations of pollinators suggest that long-tongued insects (Butterflies, Nemestrinid flies) pollinate the long-tubed flowers and short-tongued insects (Bombyliid flies, long-tongued bees) pollinate short-tubed flowers. However, analysis of selection gradients in the population, indicate no directional selection is occurring for hypanthium lengths.

The results of this thesis show that selection is occurring within *Pelargonium reniforme*, but at this time, selection is not strongly directional and floral differences are maintained, even when floral forms occur sympatrically. It is suggested that a review of the taxonomy be undertaken as well as additional pollination and selection studies to confirm suspected taxonomic groupings and relationships between the floral forms respectively.

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Eddie Izzard on science: "I have this theory, now, I will prove it! With string, and iodine and a note from my mother!"

Chapter 1: Introduction

1.1. Study taxa

1.1.1. Species

Pelargonium reniforme belongs to the family Geraniaceae. There are over 200 species of *Pelargonium* worldwide, 80% of which are endemic to the Cape in South Africa and occur in the winter rainfall region (van der Walt & Vorster 1983). The pink flowered *Pelargonium reniforme* as well as the closely related black- to maroon-flowered *Pelargonium sidoides* (until recently classified as a single species) fall into section *Reniformia* (Dreyer & Marais 2000). This classification of *P. reniforme* is supported by a genetic review of the genus (Bakker *et al.* 2004). The centre of diversity for the section is in the Eastern Cape and *Pelargonium reniforme* occurs throughout the Eastern Cape (Figure 1.1) (Dreyer & Marais 2000). It is known as an East Cape near endemic (Dold & Cocks 2002) (see Figure 1.1).

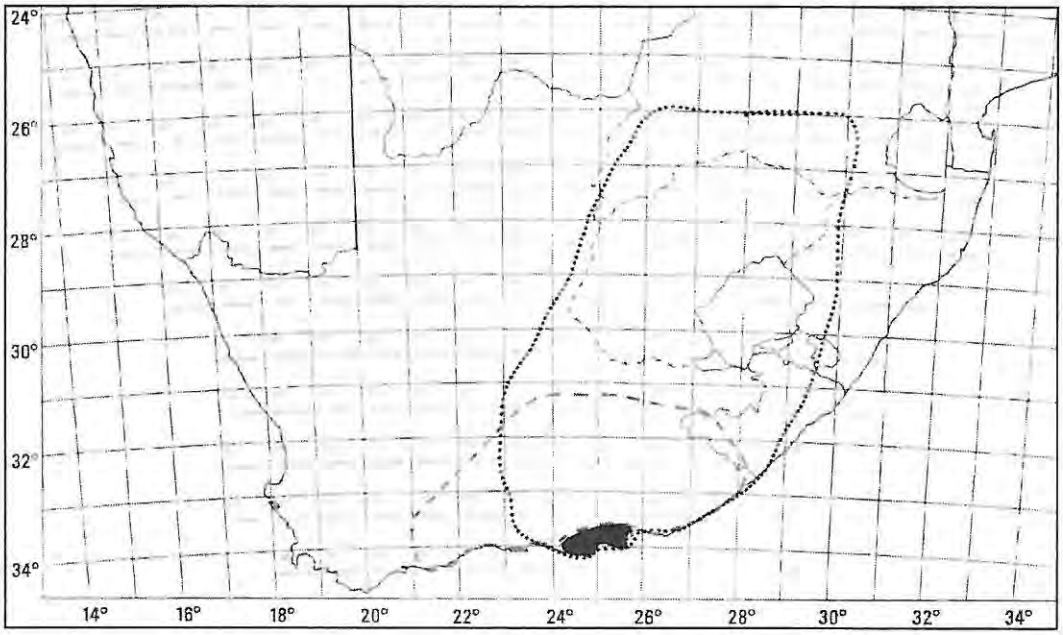

Figure 1.1: Map of distributions of *Pelargonium sidoides* (dotted line), *P. reniforme* subsp. *velutinum* (dashed line) and *P. reniforme* subsp. *reniforme* (shaded area).

Pelargonium reniforme is a pink flowering pelargonium (Figure 1.2D & E) with fairly long hypanthia although as is described below hypanthia length is exceptionally variable. As with most pelargoniums, the ovaries are situated at the top of the floral tube and the nectary near the base. The hypanthium is usually dark pink or green. Nectar is produced at the bottom of the hypanthium and is accessible only to those insects with a sufficiently long tongue to reach it. The green sepals are at the top of the hypanthium. Petals range from bright pink in colour to pale pink the lower three of the five petals bear nectar guides. Flowers are protandrous and the stigma usually becomes receptive only after the anthers have fallen off. Flowers are carried on a pseudoumble and buds open successively over a few weeks. Seeds are produced and held on the hypanthium until they have dried out and are carried off by the wind.

Pelargonium sidoides (Figure 1.2C) is very similar to *Pelargonium reniforme* in all vegetative characters. In fact, unless the plants are flowering, it is impossible to identify which species the specimen is. The floral characters are also very similar between the two

species. The striking difference is that *P. sidoides* has dark maroon to black flowers. The hypanthium in this species is always green rather than the same colour as the flowers as in *P. reniforme*. Flower shape and arrangement are the same as in *P. reniforme*.

1.1.2. Subspecies

Pelargonium reniforme is a morphologically variable species. This has been noted in descriptions of the species including the recent description of two recognized subspecies by Dreyer *et al.* (1995), who describe the occurrence of four morphologically defined groups throughout the range of the species. Although all four groups are described as having very different and easily differentiated morphological characters, only one of the groups is separated from the others geographically. It is this morphologically and geographically defined group occurring only on the coastal flats around Port Elizabeth that is known as *P. reniforme* subspecies *reniforme* (Figure 1.2A). The three additional groups are clumped together in a geographically undifferentiated second subspecies *Pelargonium reniforme* subsp. *velutinum* (Figure 1.2B, D & E).

Despite substantial vegetative variability the plants are claimed to be seldom variable when it comes to their flowers (Dreyer *et al.* 1995). This is clearly an invalid statement: floral variation has been one of the most striking characters about these plants. Unfortunately, the use of herbarium specimens for morphometric analysis removes one of the most important flower characters available: flower colour. During the course of this study I have found flower colour to be highly variable and it is well known that flower colour plays an important role in the differentiation of flowers for potential pollinating insects (Aragón & Ackerman 2004). Floral characters are not used by Dreyer *et al.* (1995) in their study and as these characters are described as invariable and their groups are defined on the basis of vegetative morphology. Dreyer *et al.* (1995) did not apply any objective morphometric analysis to their specimens in order to aid the formulation of groupings. They did however rely on some vegetative characters to discern their four groups as follows:

Group 1: "decumbent habit, elongated internodes, opposite leaves, reniform laminae and relatively short petioles"
Group 2: "rosulate habit, short internodes, ovate-cordate laminae and very long petioles"
Group 3: "rosulate habit, short internodes, small reniform laminae and long, thin, persistent petioles"
Group 4: "Dwarf form of group 2 but with laminae more reniform and petioles proportionally shorter".

Group 1 was separated to some degree geographically and described as a separate subspecies (subsp. *reniforme*) as discussed above (Dreyer *et al.* 1995). These authors say that "the sympatric distribution and great number of intermediate forms between the other three groups complicate further subspecific divisions" of *Pelargonium reniforme* subsp. *velutinum*. Certainly, the variation of vegetative morphology in the species is confounding. The authors also developed a key to be able to correctly assign a member of the species to one of the subspecies. Discriminating morphological traits for subspecies *reniforme* included elongated internodes, short petioles and reniform leaves; for subspecies *velutinum* these characters included short internodes, long petioles and leaves seldom reniform.

These defining characters are vague. Visits to Port Elizabeth to collect *P. reniforme* subspecies *reniforme* revealed that although the plants seem to have a different growth form to the other subspecies (*P. reniforme* subsp. *velutinum*) such as the plants occurring around Grahamstown, this seems to be largely caused by inherent phenotypic plasticity rather than a striking and consistent difference in vegetative morphology. We found plants of *P. reniforme* subsp. *reniforme* in the very same locality as the identified herbarium specimens (also *P. reniforme* subsp. *reniforme*) that seemed to be very different from *P. reniforme* subsp. *velutinum*, but others, on burnt areas took on the same growth form as *P. reniforme* subsp. *velutinum* observed in other localities in the Eastern Cape. This observation suggests that post-fire age complicates interpretation of morphology.

The subspecies seem to form geographically disjunctive groups. It seems that vegetative morphology is not different between the coastal *Pelargonium reniforme* subsp. *reniforme* and the inland *P. reniforme* subsp. *velutinum*. Flowers of *P. reniforme* subsp. *reniforme* resemble those of some *P. reniforme* subsp. *velutinum* plants. There is no variation in the floral forms of *P. reniforme* subsp. *reniforme*, whereas there is a great deal of floral variation in *P. reniforme* subsp. *velutinum*.

1.1.3. Forms of *Pelargonium reniforme* subsp. *velutinum*

Perhaps one of the most important observations of *Pelargonium reniforme* subsp. *velutinum* over the course of this study is that populations seem to be restricted to one of two flower types. The two different floral types are quite distinct. One floral form consists of relatively large flowers with very long hypanthia which are bright vivid psychedelic pink in colour. These long-tubed flowers have hypanthia that are also bright pink. The other form consists of smaller flowers with much shorter hypanthia and pale pink flowers (Figure 1.2F). Nectar guides are usually a darker pink than the petals but occasionally are white. The hypanthia colour in the short-tubed forms is usually green, rather than the same colour as the flowers.

One population in Grahamstown comprises both floral forms but no other population has been found that has both floral types occurring sympatrically. In this one sympatric population, intermediates exist between the long- and short-tubed forms. These have hypanthia lengths and flower colours intermediate between the bright and pale pink of the two flower forms. Other populations consist of either long-tubed, bright pink or short-tubed, pale pink flowers Intermediates are not found in populations that are exclusively long- or short-tubed. This floral variation of *Pelargonium reniforme* subsp. *velutinum* has not been noted in any previous taxonomic revisions of the species. This may be because flower colour is lost in herbarium specimens.

Observations also indicate that the two different floral forms are pollinated by different pollinators with tongue lengths corresponding to the flower tube lengths (Chapter 4). This

may indicate the importance of pollinators in the divergence of the species or maintaining gene flow between seemingly isolated populations, as pollinators are likely to play a pivotal role in selecting floral traits (Grant 1949).

Figure 1.2: Growth form and flowers of *Pelargonium reniforme* subsp *reniforme* (A), growth form of *P. reniforme* subsp *velutinum* (B) and flowers of *P. sidoides* (C). Short-tubed flowers and long-tubed flowers of *P. reniforme* subsp *velutinum* are shown in D and E respectively. A range of hypanthium lengths of *P. reniforme* subsp *velutinum* from a single site (old clay pigeon shooting range, Grahamstown) is shown in F.

1.2. Pollinator mediated selection

The huge variety of floral forms has long been attributed to the action of pollinators. The plants that are the best at attracting the most successful pollinators have become selected for (Stebbins 1970). Pollinator choice is one of the, if not the most important driving selective force of floral evolution countered by an opposing selective pressure. Schemske and Agren (1995) showed, (with the use of artificial flowers) that pollinators of *Begonia involucrate* consistently chose the larger flowers. This showed a distinct directional selection, countered by a trade off between the highest numbers of the largest flowers possible (Schemske & Agren 1995). Directional selection is clearly demonstrated in such a study and it is clear that the selection is affected by the pollinators. Pollinators were also shown to be important in the levels of pollen export in *Platanthera*, indicating that they play a pivotal role in speciation in plants (Maad & Nilsson 2004). Directional selection by pollinators was detected in *Platanthera bifolia* where larger inflorescences (more flowers) and longer tubes were selected for (Maad & Alexandersson 2004). Johnson and Steiner (1997) showed the importance of pollinators and spur length in divergence of the *Disa draconis* complex, and pollinators are also of utmost importance in the divergence of sexually deceptive orchids (Steiner *et al.* 1994).

1.3. Overall aims and objectives

This morphologically variable species with its taxonomic confusion and interesting pollination biology is an ideal organism for examining morphological and genetic diversity, and relating this to biological processes (selection). This thesis thus comprises two parts: Pattern-related studies and Process studies, which are used to explain the patterns.

1.3.1.1. Analysis/ Detection of pattern: morphometrics (Chapter 2)

Recent work has classified *Pelargonium reniforme* into two subspecies; however traditional taxonomy has ignored putatively important floral characters. Although *P. reniforme* subsp. *reniforme* is geographically isolated from the rest of the taxon, it shows few unique vegetative characters although it seems the flowers can be discerned from the darker pink *P. reniforme* subsp. *velutinum* flowers, but are very similar to the light pink *P. reniforme* subsp. *velutinum* flowers. Populations of *P. reniforme* subsp. *velutinum* tend to be restricted to one of two flower forms, indicating potential genetic isolation and pollinator-mediated selection.

There are clearly two different floral forms of *P. reniforme* subsp. *velutinum*. Populations of the subspecies tend to consist entirely of one of two floral forms. One floral form has long hypanthia and is bright pink in colour. The other flower form is light pink in colour with relatively short hypanthia. Only one population has been found where both of these floral forms occur sympatrically, usually either one form or the other comprises a population. In the mixed population, intermediates of the long- and short-tubed flowers are found in addition to the two distinct floral forms.

Chapter 2 explores the taxonomic delimitation of infraspecific entities within *Pelargonium reniforme* and its relationship to the closely related *P. sidoides*. In addition to this, the chapter also utilises characters such as petal colour that are unavailable on herbarium specimens to test the potential importance of these characters for taxonomy. The chapter makes use of numerical taxonomy rather than the 'common sense' taxonomy applied to the species and its relatives in the past (Dreyer *et al* 1995).

The chapter deals with three main analyses. The first of these utilises all possible herbarium specimens (139) in order to determine whether or not the four groups described by Dreyer *et al.* (1995) are circumscribed by objective morphometrics. The second analysis used 75 identified specimens in order to determine whether or not the same four groups described by Dreyer *et al.* (1995) were circumscribed using the same

specimens that they used. The third analysis utilised flower colour characters that are unavailable on herbarium specimens. This was done to determine whether or not both subspecies (*P. reniforme* subsp. *reniforme* and *P. reniforme* subsp. *velutinum*) would separate out with the addition of the colour characters or that the two floral forms of *P. reniforme* subsp. *velutinum* would separate out.

As Dreyer *et al.* (1995)'s groups were based on vegetative characters that are not clearly definable, it was expected that the four groups described by Dreyer *et al.* (1995) and comprising the two subspecies (*P. reniforme* subsp. *reniforme* and *P. reniforme* subsp. *velutinum*) would not be retrieved by the analyses. The similarity of *P. sidoides* to *P. reniforme* in terms of vegetative characters led to the expectation that the two species (*P. reniforme* and *P. sidoides*) would also not be adequately circumscribed by morphometric analyses. It was expected that the addition of the colour variables (one of the only very distinctly variable morphometrics measurements) to the analysis would separate the two floral forms of *P. reniforme* subsp. *velutinum* (short- and long-tubed flowers) into distinct clusters.

1.3.1.2. Analysis/ Detection of pattern: Inter simple sequence repeat (ISSR) markers (Chapter 3)

Genetics are widely accepted as the ultimate indication of relationships between individuals. Chapter 3 makes use of a DNA fingerprinting technique: inter simple sequence repeat (ISSR) markers. This chapter analyses a number of populations of *Pelargonium reniforme* subsp. *reniforme*, both floral forms of *P. reniforme* subsp. *velutinum* and one population of *P. sidoides*. The analysis was done in order to determine whether or not *P. sidoides* separated out from all other *P. reniforme* specimens. It was expected that, as *P. sidoides* is classified as a separate species, it would form a cluster of its own separated from the *P. reniforme* specimens. Similarly, the geographically separate subspecies of *P. reniforme* (*P. reniforme* subsp. *reniforme* and *P. reniforme* subsp. *velutinum*) were expected to form different groups. In addition to this, the two different

floral forms (long- and short-tubed) of *P. reniforme* subsp. *velutinum* were expected form distinct clusters even when in sympatry. Populations of each of these different taxa were expected to cluster together. The chapter also compares results from the ISSR study (the genetic groupings for the taxon) with those from the morphometrics study (chapter 2).

1.3.2.1. Processes: Pollination biology (Chapter 4)

Recognising patterns in populations of a species of plant is almost nonsensical without an attempt to explain the observed patterns. As most of the variation within *Pelargonium reniforme* may be attributed to floral characters, it is evident that pollinators must play an important role in the selection of the floral traits. The most important variation is that of the sympatric occurrence of the two floral forms (long- and short-tubed flowers) of *P. reniforme* subsp. *velutinum*. It was suspected that a difference in the pollinators was responsible for at least maintaining, and possibly driving divergence of two different floral forms of *P. reniforme* subsp. *velutinum*. Pollinator choice could result in pollinator-mediated selection for the different tube lengths and colours, effectively creating two largely genetically isolated groups of the same plant species.

Chapter 4 deals with the pollination biology of *Pelargonium reniforme* subsp. *velutinum*, specifically within one population where both the long- and short-tubed floral forms occur sympatrically. The action of pollinators is discussed and their potential role in gene flow or isolation between populations and floral forms. It was expected that insect visitors to the long- and short-tubed flowers would be different insects, and that pollen transfer between forms, although possible, occurred rarely.

Mechanical isolating mechanisms are reviewed. Colour and tube length and their differences between the forms are discussed. The role of nectar in attracting the most effective pollinator is also discussed. The breeding system of *Pelargonium reniforme* is examined in this chapter with attention given to the two different floral forms of *P. reniforme* subsp. *velutinum* and whether or not the two forms are compatible with one

another. In addition to this, the possibility of hybridization between *P. sidoides* and *P. reniforme* was tested in the breeding system experiment.

1.3.2.2. Processes: Directional selection (Chapter 5)

Chapter 5 uses statistical methods in order to determine which plant characters (both vegetative and floral) are potentially selected for. Fitness measures are regressed with various morphological traits to explain the selective effects of the pollinators on plants of both long- and short-tubed floral forms of *Pelargonium reniforme* subsp. *velutinum* in a population in which they occur sympatrically.

It was expected that directional selection driven by the pollinators was occurring in both the short- and long-tubed forms. It was suspected that pollinator-mediated selection has driven the two floral forms apart through selection of floral characters. The consistent choice of long-tongued pollinators for the darker pink long-tubed flowers would increase the fitness of the flowers with the longest hypanthia in comparison with those with shorter hypanthia thus resulting in directional selection for longer tubes. The consistent choice of short-tongued pollinators for the lighter pink short-tubed flowers would increase the fitness of the flowers with relatively short hypanthia. The two floral forms would then be consistently diverging in response to pollinator behaviour.

1.3.3. Conclusions

The final chapter, Chapter 6, summarises the conclusions from each of the other chapters. The chapter explains the processes described in chapters 4 and 5, and uses the results of these to explain patterns revealed in chapters 2 and 3. This last chapter comprises the overall conclusions of this thesis and discusses future investigations necessary to further elucidate patterns and processes of pollinator-mediated selection of *Pelargonium reniforme*.

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Pattern

Chapter 2: Morphometrics: examining the phenotypic variability of *Pelargonium reniforme*.

2.1. Introduction

2.1.1. *Pelargonium reniforme* – uncertain taxonomy and undescribed variation

Along with DNA data, morphometrics play an essential role in taxonomy. The approach is used to clarify relationships between organisms where simple observation is insufficient or may be considered too subjective. Morphometrics provides an invaluable and more objective tool for use in taxonomy. The wide range of phenotypic characters in *Pelargonium reniforme*, and the use of traditional 'common sense' taxonomy seem not to be able to delimit the sub-specific classification adequately. An objective elucidation of phenotypically defined groups would be invaluable for the clarification of the subspecific divisions of the species and provide an idea of which characters contribute most to the differences between phenotypic groups.

Current taxonomy of *Pelargonium reniforme* describes four main phenotypically distinct groups of the plant (Dreyer *et al.* 1995). Only one of these four groups is geographically isolated and thus described as a separate subspecies. The majority of the range of *P. reniforme* is occupied by *P. reniforme* subsp. *velutinum* while *P. reniforme* subsp. *reniforme* occupies the limited range of the coastal flats of Port Elizabeth, in the Eastern Cape, South Africa (Dreyer *et al.* 1995) (Chapter 1).

Observations of both herbarium specimens of *Pelargonium reniforme* as well as various populations of the plant throughout its range have indicated that the plant may be more variable than previously thought, especially in floral characters omitted in previous taxonomic assessment of the species. It was thought that an extensive and comparably objective review of the morphology of the species throughout its range may provide some

insight into the different groups of the plants and refine the groups already defined by Dreyer *et al.* (1995) (Chapter 1).

2.1.2. The role of morphology in species level taxonomy

Traditional morphometrics is defined by Jensen (2003 pg 664) as: 'the application of such multivariate tools as principal component analysis (PCA), cluster analysis, and canonical variates analysis to datasets based on continuous (e.g. linear measurements, angles) and meristic characters'. Numerical taxonomy such as traditional morphometrics and the more recently developed geometric morphometrics are especially useful for use with taxa at the species level or below (Jensen 2003). Many papers have utilised traditional morphometrics to circumscribe groups and clarify relationships (See examples listed in Jensen 2003).

There are two types of morphometric analysis: traditional morphometric techniques and geometric morphometrics. Traditional techniques involve the application of multivariate statistics on continuous variables such as linear measurements and angles (Jensen 2003). Geometric morphometrics, developed during the morphometric 'revolution' involves analysis of measurements between landmarks thought to describe the shape of organisms (Jensen 2003). Geometric morphometrics are heralded as the solution to the problem of size and shape inherent in traditional morphometrics (Rohlf and Marcus 1993). Geometric methods are not without their own controversy however. Gielis (2003) describes a means of transforming geometric morphometric data for better results and Rohlf (2003) describes an isotropic error model for estimates of mean shape in geometric morphometrics.

Many biologists have avoided the use of geometric morphometrics in favour of the traditional morphometric methods. Jensen (2003) cites superficial similarity as a reason why many biologists have steered clear of geometric morphometrics. In addition to this, landmarks used in geometric morphometrics are notoriously difficult to define and use in

studies of plant taxa. The definition of landmarks that are homologous throughout the taxa being studied often proves impossible (Jensen 2003). Methods have been developed to circumvent this problem, such as the use of elliptical Fourier functions. A study by Cannon and Manos (2001) uses these functions to compare morphometric shape descriptors in *Lithocarpus*. These involve complex measurements from the centre of the measured object. However, elliptical Fourier functions may not correspond with any real biological phenomena and thus are of little use as data to determine the effects of these phenomena (Jensen 2003). Truss measurements between landmarks may be used as a better indication of shape (Strauss & Bookstein 1982) (truss measurements involve as many measurements as possible across a shape defined by a set of landmarks; they are thought to better describe shape). The newer geometric morphometrics may thus not be applicable in many studies although they are potentially more informative than traditional methods and should be employed over traditional morphometrics when the researcher needs to test shape and size differences specifically. Although geometric morphometrics may provide insight in higher taxonomic levels, traditional methods may provide more resolution at the species level and below (Jensen 2003).

The use of morphometrics in systematics is invaluable and often overlooked by systematists in the face of an overwhelming wealth of information available since the development of genetic techniques (Jensen 2003). Systematics not only includes traditional taxonomy, but also speculative and realistic aspects of evolution and its mechanisms (Quicke 1993). If used in conjunction with other systematic methods, morphometrics form an integral part of any taxonomic study and may help to resolve issues of identification and classification that genetic data is unable to achieve (Jensen 2003).

Both traditional and geometric morphometrics are still important methodologies and form an important part of developing keys to distinguish closely related taxa and provide a much more objective classification technique than traditional taxonomy. In a recent study of the legume genus *Tylosemsa*, Castro *et al.* (2005) used traditional morphometrics in order to clarify the taxonomic boundaries of populations of the genus occurring in south

Angola. Distinct groups of three recognized taxa were found as well as a new species, which they describe. In addition these authors used key character identification in the morphometrics analysis to produce a key for these four taxa (Castro *et al.* 2005). Kores *et al.* (1993) describe a geometric morphometric study using thin plate spline and eigenvectors to analyse their data. They discover that two of the three species used in the study are distinctly different but that the third species is indistinguishable from one of the others (Kores *et al.* 1993).

In such a discipline as taxonomy, objectivity is valued by systematists as it provides a common base from which all taxonomies can be compared. Morphometrics, as a discipline within systematics imparts a large degree of objectivity onto classifications, which have been achieved traditionally by 'common sense' and the intrinsic pattern-discerning ability of researchers. Logically, an objective elucidation of taxonomic hierarchies within groups of organisms would be a much better representation of classifications. However, traditional taxonomic methods should not be overlooked in favour of mathematics and statistical analyses. Although it is often assumed that 'common sense' circumscriptions of taxa are biased, it seems that they might be more important than statisticians give them credit for (Jensen 2003). Gilmartin and Harvey (1976) found similar groupings in *Viola* using the characters used to denote species in 'common sense' taxonomies compared to those described by the use of morphometrics.

Objectivity in taxonomy has been further advanced by the recent introduction and use of genetic methods to determine classifications as DNA data is the ultimate objective standard in all biological systems. However it is virtually impossible to study the entire genome of all species and the avoidance of important taxonomic methods such as morphometrics to do so is largely nonsensical. Indeed, Mishler (2000) describes morphometrics as being as important as DNA data in systematics. He describes six properties that an ideal marker should posses in order to be utilised in systematics. These include complexity and comparability, discrete states, heritability, independence, low rate of change and many possible character states. Morphological characters posses complexity and comparability as well as many possible character states, DNA data has

discrete states and heritability. Both these types of data may not necessarily be independent or have low rates of change. Thus, separately, the two methods fulfil only two of the six properties but if used in conjunction have four out of six of the properties. Mishler (2000) suggests scientists combine the two markers to increase the reliability of the results. A study of morphometric data and allozyme markers shows that both molecular and morphometric methods can be used simultaneously (Leht & Jaaska 2002).

2.1.2.1. Problems with morphometrics

With a method as controversial as morphometrics, and the arguments that arise from different disciplines as to what is important in terms of calculations, come a slew of problems that have to be resolved before the method can be used with credibility. Morphometrics involves the application of complicated mathematics. The majority of biologists find such mathematics difficult if not impossible to apply but place a great deal of value on the objectivity it imparts on systematics and taxonomy, a notoriously subjective discipline. Jensen (2003, pg 664) poses the question: 'does the complexity of morphometric data represent an insurmountable obstacle?' It seems that although morphometrics is a bone of contention amongst many scientists, their use still provides a great deal of important information, especially in taxonomy.

2.1.2.1. a. Morphometrics and genetics

Morphometric data collected for any group of organisms is assumed to have an ultimately genetic basis, and there has been a great deal of discussion on the use of morphometrics and what it may mean (Sokal & Sneath 1963). With the development of numerical taxonomy came the discussion of many aspects of the method, some of which, especially the problems of size, shape and allometry, continued for years after the method became widely accepted and used. Discussions of the method cite correlations between characters, invariant characters, and the problem of weighting characters and measurement error (among other things) as problems that need to be resolved before

utilizing the method (Sokal & Sneath 1963). The nexus hypothesis, the hypothesis of nonspecificity and the hypothesis of the matches asymptote are discussed at length by Sokal and Sneath (1963).

The nexus hypothesis assumes that phenotypic characters used for taxonomy is likely to involve more than one gene and that one particular gene may affect a suite of characters. There is, as a result, a great confusion of cause and effect and morphometrics cannot assume to describe genetics but must rely on morphological characters (Sokal & Sneath 1963). The hypothesis of nonspecificity assumes there is no one particular group of genes controlling a suite of characters such as one specific region such as leaves. The objective of numerical taxonomy, as described by Sokal and Sneath (1963) is to be able to describe taxonomies objectively. The characters rest on the assumptions that they are to some degree a reflection of the genetics of the taxa being studied. However phenotypic plasticity may reduce a character's taxonomic utility as it obscures genetically based relationships (Davis 1983).

2.1.2.1. b. Characters

The raw data used for a numerical taxonomy study is a matrix consisting of various characters (Quicke 1993). Of all of the potential characters, including both discrete and continuous variables, binary characters are the most simple to work with (Quicke 1993). The choice of particular characters for morphometric analysis is also extremely important for the outcome of the analysis and grouping of taxa. Of course, there are various problems involved in the choice of characters and their effect on the resultant groupings. Endress *et al.* (2000) describe the problem of defining characters and characters states as crucial for all morphometric studies. If a numerical taxonomic analysis is conducted on a group of plants utilising only the characters used to define the taxa in a traditional taxonomic method, the result will give the same groupings originally described (Sokal & Sneath 1963). In order to avoid this potential bias, it is important not to limit the characters used for the analysis and to include as many as possible (Sokal & Sneath 1963). The inclusion of only specific characters known to specifically define certain

groups may result in spurious groupings. Thus it is clear that investigators should include as many characters as possible without introducing bias (Sokal & Sneath 1963).

For many of the methods employed in determining groupings of taxa according to the similarities of these characters, continuous characters have to be coded rather than left as raw measurements. This is a potential cause of problems as there is often no clear room for division of the continuous variables into discrete coded characters (Quicke 1993). Fortunately, analyses exist that can utilise both discrete and continuous variables within the same data set so long as the data set is transformed in some way to make the characters comparable.

Although the fact that bias must be avoided when it comes to utilising characters and the most accurate results will be given with the use of the maximum number of characters, the number of characters used in the study is also of utmost importance. This choice involves the hypothesis of nonspecificity as discussed above. The less well the hypothesis holds for a given sample, the more characters need to be used in the analysis (Sokal & Sneath 1963). Sokal and Sneath (1963) state that the number of characters used in a study must be carefully considered, bearing in mind the taxonomic units and affinities under exploration. The hypothesis of the matches asymptote (Sokal & Sneath 1963) is important for character utilisation. This hypothesis assumes that the value of the similarity coefficient becomes more constant with an increase in the number of characters used eventually a point will be reached where additional characters are not warranted as the maximum stability of the coefficient is reached (Sokal & Sneath 1963). As expected, there is no unequivocal solution to the question of the number of characters to be used in such an analysis, especially considering the subjective nature of choosing which characters to use in the analysis. In order to avoid bias sampling as much as possible, it has been suggested that the largest number of characters possible be used for the analysis (Sokal & Sneath 1963). Although it may be possible to test the hypothesis of the matches asymptote, the use of a large number of characters will ensure an objectivity that is impossible to gain with the use of traditional taxonomic methods or 'common sense'.

Numerical taxonomy is not concerned with characters that are used for the identification of taxa (Sokal & Sneath 1963). It is for this reason that characters used in morphometric studies are all given automatic equal weighting. Although weighting of characters has been identified as an issue, there seems to be no rational way of doing so and thus all characters are accorded equal weighting within the analysis.

Sokal and Sneath (1963) describe seven reasons for the equal weighting of characters in numerical taxonomy. These are:
1) The characters must be weighted on rational grounds, if this cannot be done then all must be considered equally.
2) Characters used to identify a taxon may be given higher weighting in the analysis, but this would mean a-priori classification into groups and thus undermine the very reason for conducting a numerical analysis.
3) Taxonomic importance is an arbitrary assignation.
4) There is no exact method for determining neither whether weighting should be used nor how to use it.
5) Weighting characters runs the risk of introducing differences in the method and is no longer a general arrangement with comparable results.
6) Each character forms a statement about the taxa being studied, whether any of these characters is more important than another is irrelevant.
7) The more characters that are used in the analysis, the less effect weighting of any of these characters has on the overall analysis and weighting thus becomes pointless.

2.1.2.1. c. Size, shape and allometry

One of the most commonly discussed problems in morphometrics is the problem of size and shape. Differences between operational taxonomic units (OTUs) are thought to have two components; size and shape (Jensen 2003). Often, the effects of size on the grouping of taxa should be removed to be able to observe properly the effect of shape on the differences between OTUs. Over the years, many methods have been developed to remove the effects of size and look only at shape as a differentiating character. Rohlf and

Bookstein (1987) describe size and shape as corresponding to overall factors and not specifically measured characters such as height. They also describe the importance of removing the effects of size from an analysis. Size is extremely variable where shape is not. This removal of the variability of shape would allow for the separate analysis of the less variable shape descriptors. There is potential for size to mask much more informative but subtle differences, thus it seems logical to try to remove size and concentrate on these potentially more important differences (Rohlf & Bookstein 1987). There are three main methods for removing size and analysing only shape as a result in morphometric analyses. These three methods are ratios, regressions and factor component analysis (Humphries *et al.* 1981).

Ratios are the most often debated of all of these techniques. The heated arguments regarding the use of ratios in morphometrics is described by Atchley and Anderson (1978 pg 71): 'Apparently, any criticism of the use of ratios by biologists elicits a Pavlovian response in some circles similar to that resulting from criticisms of motherhood, America and apple pie'. Humphries *et al.* (1981) give three reasons why ratios are insufficient as indicators of shape in an analysis: correlations of ratios with either the numerator or denominator could cause problems in the analysis, the ratio may not be independent of the denominator and finally, the use of ratios changes the covariance matrix. Although many researches feel that the problems associated with the use of ratios can be eliminated by log-transforming the data, Humphries *et al.* (1981) believe that they are not and advocate the elimination of the use of ratios from morphometrics. Albrecht (1978) discusses the use of ratios and determines that they may become confounding in an analysis: he argues that the numerator and denominator should not be correlated and that the use of ratios to correct for size results is both confusing and may invalidate analyses. Albrecht (1978) is adamant that ratios should not be used although their use can be justified and says that the effort needed to satisfy the assumptions the use of rations requires outweighs their usefulness. Atchley (1978) also supports the cautionary use of ratios in morphometrics and suggest that biologists utilise ratios without realising the affects of doing so. Atchley and Anderson (1978) also conclude that using ratios introduces unnecessary complications in principal component analysis. Bookstein (1989)

criticises the use of ratios as limiting and not informative. Hills (1978), is immovable in his opinion that ratios can and should be used in multivariate analyses and suggests that log-transforming ratios eliminates any problems there may be with correlations of numerator and denominator. James and McCulloch (1990) suggest the use of ratios as shape descriptors in multivariate analyses.

Ratios were not used in this study but are often not included as a size measure in morphometric analyses or make very little difference to the groups observed. Many recent studies ignore any problems previously discussed with elucidating shape and size in morphometric data. Some do not include any shape descriptors such as ratios and it is often assumed that the first axis of a principal components analysis describes size sufficiently. The scientists developing the methods cannot seem to come to a consensus for the elimination or elucidation of size variance in traditional morphometrics. Many modern studies skirt around the issue of ratios as a result and either use ratios or do not, without any reasoning for their doing so. Ratios are used in a number of studies without any explanation for their use (Holsinger 1985, Watson & Estes 1990, Ackerfield & Wen 2002, Selvi *et al.* 2002, Borazan & Bubac 2003). Others make no use of ratios and likewise do not mention the reason (Bruederle *et al.* 1989, Crompton *et al.* 1990, Ballard & Wujek 1994, Aldasoro *et al.* 1998, Abdel Khalik *et al.* 2002, Cupido 2003).

Regressions are also suggested as a means of removing size from important shape differences between OTUs in a morphometric analysis, regressions allow for the removal of the first PCA axis, which is regressed out and the PCA recalculated without this 'size' variable (Humphries *et al.* 1981). Humphries *et al.* (1981) describe this means of doing so as incompetent and say that regressions do not entirely remove the effects of size form the analysis. Atchley (1978) also slates the use of regressions to correct for size.

Often, as size forms the greatest degree of variation between OTUs in a morphometric analysis, it is thought to be exhibited mainly by the first axis in principal component analysis (Quicke 1993). This can be construed as a result of the allometric growth existing inevitably in the OTUs used in the study (Quicke 1993). If first component

loadings are similar in scale and have the same sign then the first axis may be considered as size. Since all axes of a PCA are orthogonal to one another, it follows then that the subsequent axes describe any differences but size. It is however, not that simple (Jensen 2003). Importantly, phenotypic plasticity in such a variable character as size is potentially confounding. Many reviews of the traditional morphometric method have suggested variation on methods to remove to effects of size from the analysis to be left with size-free shape as a more important descriptor.

Humphries *et al.* (1981) states that ratios are often used only because of their simplicity, rather than for reasonable statistical considerations. The authors determine that not only ratios are a poor estimation of size but also the use of discriminant functions and regression to determine size descriptors. The authors follow with a new method for separating size from shape using the assumption that a great deal of size is described in the first PCA axis. Humphries *et al.* (1981) suggest a method based on the assumption that the first PCA axis forms a good estimation of the degree of differentiation size plays in the analysis. The method they describe and say is the best developed so far for looking at size-free shape in morphometrics is Shearing. The method uses the first two components of PCA and identifies groups divided on the basis of shape and size. The size factor is removed from the analysis.

Shearing as a method for removing the effects of size is criticised by Rohlf and Bookstein (1987). Their study determines that the sheared components are not necessarily uncorrelated with size and thus the effects of size have not been removed from the analysis at all. They recommend Burnaby's (1966) method rather than the sheared components analysis of Humphries *et al.* (1981). Rohlf and Bookstein (1987) determine that the shearing method removes a projection of size, and not size itself, thus leaving the effect of size differences in the analysis. Rohlf and Bookstein (1987) also dispute the assumption that the first axis of principal component analysis can be attributed to size (an assumption of the sheared analysis) and say that although it describes the most amount of variation in the data this may not necessarily be size. Shearing is also used by Dickinson *et al.* (1987) in addition to their own method, the use of truss measurements between

landmarks in leaves to describe leaf shape. Their method adds numerous measurements to the analysis, thus according a more 'accurate' leaf shape measurement (Dickinson *et al.* 1987), however, this may not be a method so easily applicable to leaves and shearing is not necessarily a good shape discrimination technique.

Somers (1986) also describes a method for removing size so that shape can be analysed without the potential confounding effects of size. As in shearing, this method is also based on the first axis of a principal component analysis representing size. The method describes the use of a correlation matrix and the extraction of the first principal component axis and re-examining of the PCA without this purported size factor. It is also noted that the first PCA axis produced from a correlation matrix calculated from log-transformed data more accurately describes the size within the taxa, mostly because it automatically standardises the data. The method has limitations identified by the author as the assumption that a size vector actually does exist, that characters are correlated with size and that the relationships become linear when log-transformed. The author also cautions the use of PCA which assumes the data are linearity and normally distributed. As a consequence, PCA cannot describe non-linear patterns (Somers 1986). Somers (1989) cautions against the assumption that the first axis of a PCA describes only size as allometric size represents bivariate shape correlated with allometric size and thus the first PCA axis describes a combination of size and shape. Sundberg (1989) revises all of the methods used to eliminate size from a multivariate morphometric analysis and concludes that size should be retained in the analysis and that the first axis of PCA sufficiently explains the variation size has on the distribution of data along eigenvectors.

Generally, size is assumed to be described sufficiently by the first axis of a PCA (James & McCulloch 1990). If the components of this axis are all of the same sign and are correlated with the original data, then the axis describes size; this is however, only specifically true for a PCA constructed from a variance-covariance matrix, a correlation matrix standardises the data and removes differences in scale (size) from the data, this is somewhat alleviated if the data is log-transformed before the correlation matrix is constructed (James & McCulloch 1990). Bookstein (1989), after an exhaustive definition

of size and shape and what they mean in morphometric analyses, determines that the first axis of a PCA provides a sufficient estimation of size. He also explains that, as all axes subsequent to the first axis in PCA are orthogonal to the first axis and thus must to some extent describe the variability of shape in the data (Bookstein 1989).

2.1.3. Principal component analysis and cluster analysis

Morphometrics involves the use of multivariate statistical techniques that define particular groups from a matrix of measured variables. The raw data may be transformed, standardised and re-arranged to form a similarity matrix on which the final analysis is conducted. These methods include cluster analysis (CA) and principal component analysis (PCA) among other commonly used methods. Most recent taxonomic studies utilise both of these methods. (Ackerfield & Wen 2002, Cupido 2003, Castro *et al.* 2005). There is controversy over the methods but both cluster analysis and principal components analysis provide valuable information important for taxonomy. Controversies over these methods mainly lie with cluster analysis, specifically two points. The first is that cluster analysis cannot be used to infer phylogeny. The second is that even when only used to describe similarities without inferring relationships between OTUs, cluster analysis imposes a nested hierarchical structure on groups that may not necessarily actually exhibit these kinds of groups.

A critique of the utilisation of clustering techniques to resolve similarities between Operational Taxonomic Units (OTUs) through morphometrics was conducted by Quicke (1993). He concludes that it is impossible to infer evolutionary history from phenetic clustering. This stands to reason as it is impossible to determine the ancestral state of a suite of morphological characters (Quicke 1993). Quicke (1993) states emphatically that numerical taxonomy is not a good method for identifying relationships. Despite limitations of the method, numerical taxonomy proves an invaluable taxonomic tool for classification and identification without inferring evolution (Quicke 1993).

As discussed by Quicke (1993), a phenogram cannot be used to infer phylogeny. This sentiment is echoed by Jensen (2003), although he does say that numerical morphometrics have a valuable role to play in phylogenetics even if they cannot estimate evolutionary relationships. deQueiroz and Good (1997) describe in detail the problems of using phenetic clustering itself (even with genetic data) to infer phylogenetics. Zelditch *et al.* (1995, pg182) give three reasons why most morphological methods cannot be used to infer phylogenetic relationships. The three reasons given all relate to the test of similarity for homologies and are given as follows:

'1) Comparisons are based on linear combinations that optimise discrimination or variance of scores rather than on homology of parts.

2) Differences among organisms cannot be localised to parts of the organisms.

3) Manipulations of variables chosen in advance of analysis limits the possibility of assessing detailed similarity'.

Scotland *et al.* (2003) describes the main limiting factor of the use of morphology in phylogenetics as the limited number of unambiguous characters.

Zelditch *et al.* (1995) describe the reasons why phenetic analyses of morphometric data are inappropriate for phylogenetic analysis. Both traditional and geometric morphometric characters fail one of the tests for homology: the test of similarity, and thus cannot be used to infer phylogenetics. However, the thin-plate spline decomposed by its partial warps, a morphometric analysis applicable only to landmark data, manages to fulfil the test of similarity and may be used to infer phylogeny (Zelditch *et al.* 1995). Zelditch *et al.* (1995) states that thin plate spline analysis is able to identify differences in shape of particular regions, which are then comparable across specimens. No other morphometric analysis ensures homology so explicitly. The analysis must be performed on landmark data but, this often proves impossible with many studies, especially on plants where homologous landmarks are difficult to identify and use. It is thus a restricted methodology and only applicable in a restricted number of morphometric studies.

The restricted use of morphology in phylogeny reconstruction compared to DNA sequence data is discussed by Scotland *et al.* (2003). They suggest using fewer more

rigorously identified and measured characters rather than huge matrices of potentially problematic data. Certainly, some kind of use of the two methods in conjunction will better resolve classifications based on the morphological methods of traditional taxonomy and the new wealth of genetic phylogenies. Overall, phylogenetics should not be inferred from phenetic results of morphological studies.

deQueiroz and Good (1997) discuss at length the downfalls of cluster analysis as a phenetic method. Cluster analysis is often used to describe similarity without inferring phylogeny, but even in these cases it depends on the assumption of nested hierarchical structure, which does not allow for the accurate representation of similarity when relationships do not exhibit the nested hierarchical structure. The problems arise from the clustering method itself, rather than from the similarity matrix on which the clustering algorithm is performed, claim deQueiroz and Good (1997). PCA analyzes similarities without imposing a nested hierarchy (deQueiroz & Good 1997). Phenetic clustering should only be used to describe similarity relationships among OTUs within the assumption of nested hierarchical structure (deQueiroz & Good 1997). But even used simply as an analysis of similarity, the method is flawed. It is constrained by the production of results on the form of nested hierarchies. Despite the limitations of the technique as described by deQueiroz and Good (1997), it has none the less provided some insight into the results of morphometrics studies. Few recent studies utilise cluster analysis without comparing the results of that analysis with another, usually PCA. As with any statistical method, cluster analysis is a bone of contention among the scientists who use it. And, similar to the use of ratios, the use of cluster analysis is not discussed but simply applied without explanation.

In this study, it was thought best to utilise both principal component analysis and cluster analysis in order to compare the two methods and to determine whether or not the results agree with each other.

2.1.4. Aims and Objectives

This study had two main aims: The first was to determine whether or not traditional morphometrics could circumscribe the same groups described by Dreyer *et al.* (1995). Secondly, to determine if the addition of floral characters (so often necessarily omitted from taxonomic studies as they cannot be recorded in dried herbarium specimens) in the morphometrics data could circumscribe the differences in floral forms evident from fresh specimens.

Three main analyses were conducted. The first analysis uses all available herbarium specimens in order to determine whether or not the four groups described by Dreyer *et al.* (1995) are circumscribed by objective morphometrics. This first analysis is also used to determine whether or not the closely related *Pelargonium sidoides* groups with *P. reniforme* specimens.

The second analysis uses only those specimens already identified by Dreyer as part of her study or collected from the geographically isolated populations described by Dreyer *et al.* (1995). This was done in order to determine if the four groups defined by Dreyer *et al.* (1995) were circumscribed by the analysis using the same specimens on which the taxonomy was based. *P. sidoides* was also included in this analysis to determine if it formed its own species group.

The third analysis utilises additional colour characters unavailable on herbarium specimens. This was done to determine whether or not both subspecies (*P. reniforme* subsp. *reniforme* and *P. reniforme* subsp. *velutinum*) would separate out with the addition of the colour characters. Also, most importantly, the analysis was conducted in order to determine whether or not the different floral forms of *P. reniforme* subsp. *velutinum* (short- and long-tubed flowers) would separate out in the analysis with the addition of the putatively important colour variable.

As Dreyer *et al.* (1995)'s groups were based on vegetative characters that are not clearly definable, it was expected that the four groups described by Dreyer *et al.* (1995) and comprising the two subspecies (*P. reniforme* subsp. *reniforme* and *P. reniforme* subsp. *velutinum*) would not be detected by the analyses. The similarity of *P. sidoides* to *P. reniforme* in terms of vegetative characters led to the expectation that the two species (*P. reniforme* and *P. sidoides*) would also not be adequately circumscribed by the analyses. It was expected that the addition of the colour variables (one of the only very distinctly variable morphometrics measurements) to the analysis would separate the two floral forms of *P. reniforme* subsp. *velutinum* (short- and long-tubed flowers) into distinct clusters.

Secondary aims included a study of the methods of morphometrics themselves. This included a comparison of the groups defined by principal component analysis (PCA) and cluster analysis (CA), and a discussion on the affects of standardising data for use in a cluster analysis.

2.2. Materials and methods

2.2.1. Sampling

Analysis 1: All herbarium specimens. Analysis 1 was conducted on all (139) herbarium specimens and field collected specimens, comprising 19 variables for each of the specimens. Thirty-nine Specimens of *P. reniforme* were measured from the Selmar Schonland herbarium, none of which were previously identified to subspecies level. An additional eight specimens of *P. sidoides* were also included from the Selmar Schonland herbarium. *Pelargonium sidoides* specimens were included as the species was until recently considered a subspecies of *P. reniforme* and was lately assigned its own species mainly due to a difference in flower colour. Seventeen *P. reniforme* specimens were included from the Compton herbarium; these specimens were also not identified to subspecies level. Twenty-two specimens previously identified by Dreyer to subspecies level were measured from the Bolus herbarium and 16 also identified by Dreyer from the National herbarium. Specimens from each of the herbaria that were incomplete (those missing some important characters that would allow for all the morphometric characters to be measured) were not included in the morphometric study as most characters could not be measured. Each selected specimen comprised an operational taxonomic unit (OTU). A list of all of the specimens examined can be found in Appendix 1.

Analysis 2: Previously identified specimens. Analysis 2 was conducted on all specimens identified by Dreyer and on those field collected specimens from areas defined by Dreyer *et al.* (1995) as belonging to one of the two subspecies (i.e. *Pelargonium reniforme* subsp. *reniforme* and *P. reniforme* subsp. *velutinum*). It was predicted that, should Dreyer's groups be a true reflection of the morphometric groupings of the species, the specimens should cluster according to the four groups identified by Dreyer *et al.* in their review of the species.

Analysis 3: Living specimens. Analysis 3 was conducted only on field collected specimens and included an additional character (flower colour) which added two

variables and allowed for the classification of the specimens on the basis of potential floral variation observed in the field for the species. Measurements were done on 36 specimens collected throughout the range of *Pelargonium reniforme* (these were identified to subspecies level according to Dreyer *et al*'s geographic ranges). Vouchers are lodged in the Selmar Schonland herbarium, Grahamstown. Data was collected from four specimens from each of nine populations encompassing both of Dreyer's subspecies and the long- and short-tubed individuals of *Pelargonium reniforme* subsp. *velutinum*. These specimens are lodged in the Selmar Schonland herbarium. Again, each specimen constituted an OTU (see Appendix 1 for a list of the specimens).

2.2.2. Character selection

In Analysis 1, 19 morphological characters were coded for each specimen (Table 2.2). The characters included 17 quantitative characters and 2 ranked variables. Most of the measurements (Table 2.2) are standard, such as width and length of leaf blade. Order of branching is described as the number of branches before termination in an inflorescence. The first branching length is the length of the first branch that terminates in the inflorescence and the last branching length the length of the last branch, the branch bearing the inflorescence.

Two additional characters denoting colour (identified with an asterisk in Table 2.2) were added to Analysis 3. The two additional characters in Analysis 3 were measured from the specimens before they were pressed and dried. These two characters (denoting colour and usually used to place colours in a colour plane diagram) were measured with an Ocean Optics USB2000 spectrophotometer linked to a PC. An Ocean Optics Mini–D2T (Tungsten-Deuterium-Halogen) light source was used to illuminate the sample. The reflection probe (UV/VIS 400 micron) was orientated at 45° to the surface of the floral part being measured. Colours were summarised using the Endler segment classification method (Endler 1990). The method of Endler (1990) was used as it describes a general model for showing colour spectra. The commonly used alternative, the Chittka (1992)

method is based on the visual spectrum of hymenoptera. As this pollination system is not one restricted to hymenoptera pollinators only, the Endler method was the best means of summarising the spectra. The Endler (1990) segment classification takes the integral of light reflected from floral part and the light incident on the sample (the D65 norm-function in this case) for each of four equal segments between 300 and 700 nm. These values are divided by the integral for the entire spectrum of interest (300 – 700 nm) to separate colour from brightness and subtracted from one another to determine values for colour "opponents". The value for the medium-short wavelength segment is subtracted from the long wavelength to give a long-medium (LM) opponent and the short wavelength segment is subtracted from medium long segment to determine the medium short (MS) opponent. These two colour measurements were added to the morphometrics data set as additional characters.

2.2.3. Phenetic analysis: Principal Component Analysis (PCA) and Cluster Analysis (CA).

Phenetic analyses using multivariate and univariate methods were performed. The same statistical applications were used for both analyses. Multivariate analyses were carried out using NTSYS (Rohlf 2002). Two types of analyses were conducted on each of the data sets, principal component analysis (PCA) and cluster analysis (CA).

PCA:

In order to remove the effects of allometric growth, all the qualitative characters were $\log_{10}$-transformed. The raw data in the form of a taxa/character matrix is log-transformed to deal with homogeneity of variances and reduce the variance due to allometric growth that may be evident in the data (Humphries *et al.* 1981). Jolicoeur (1963) states that data such as this should be log-transformed as diverse distance measures relate log-linearly. A correlation matrix was then calculated for the variables. Because a correlation matrix automatically standardises the data (Somers 1986, James & McCulloch 1990), the data set was not standardised for the PCA analysis. The first three eigenvectors were extracted

and plotted in Microsoft Excel. Loadings on each of the PCA axes were examined. Loadings 'refer to the correlation of an original variable with one of the linear combinations constructed by the analysis' (James & McCulloch 1990 pg 135).

The first three eigenvectors were examined using ANOVA in order to determine whether or not these were significantly different between groups. In addition to this, a discriminant function analysis (DFA) was conducted to determine which variables contributed to the differences between groups.

CA:

For this analysis, data were not $\log_{10}$-transformed. There is no evidence to suggest that cluster analysis assumes normality and thus it becomes unnecessary to transform data (deQueiroz & Good 1997). Raw data were used for the cluster analysis for both standardised and unstandardised data in order to compare the phenograms constructed for both standardised and unstandardised data. There has been some argument about the necessity of standardising data for phenetics and it was considered important to compare methods to determine the advantages and effects of using standardisations in statistical applications for morphometric data. Standardisation effectively removes the effects of units on the raw data so that data are directly comparable. Results of an analysis conducted on unstandardised data are susceptible to outliers; this is not the case for standardised data (deQueiroz & Good 1997).

The most commonly used clustering algorithm is the unweighted pair group with arithmetic averaging (UPGMA). In this algorithm, OTUs are assigned to clusters on the basis of their average distance to other OTUs within the cluster (Quicke 1993). The most commonly used distance measure for similarities is the Euclidian distance (Quicke 1993). This is described by Quicke (1993) as the same as the physical distance separating the taxa if each axis is one normalised variable and each axis is perpendicular to all other axes. This distance data can be used to determine whether groups exist within the taxa being studied or to determine where an unclassified taxon may fall within already-defined groups (Quicke 1993).

A distance matrix based on average taxonomic distance was calculated for the cluster analysis. This matrix was then subjected to the unweighted pair group method with arithmetic averaging (UPGMA) clustering algorithm. Trees were labelled in Microsoft Powerpoint. A matrix of the co-phenetic values was then calculated and the correlation coefficient between the co-phenetic matrix and the distance matrix calculated. This was done to examine the goodness of fit of the cluster analysis to the distance matrix.

Discriminant function analysis (DFA) was conducted on the groups elucidated by the cluster analysis in Statistica. This statistical test was done in order to determine whether or not the phenogram groups were effective groupings and allowed for comparisons of the phenograms of standardised and unstandardised data for each of the three analyses. A forward stepwise discriminant function analysis gave the results. F to remove and F to add were used as the default values in Statistica. Missing values for certain measurements were replaced by mean substitution rather than the automatic case deletion method in Statistica. This was done as the program NTSYSpc used to construct the phenograms, does not delete cases with missing data. The most important value for the DFA is the first root from the canonical analysis. The canonical analysis takes the variables in the model (those that contribute most to the clusters) and assigns a value to them, much like the loading values of each of the characters in a PCA analysis. It is these roots then that are the most valuable in an analysis of the results of a DFA.

Table 2.1: Summary of the analyses conducted on three different groups of morphometric data.

Analysis	Variables	Numerical Analyses				Aims
		PCA	analysis of results	CA	analysis of results	
1	All herbarium specimens	- log_{10}-transformed - correlation matrix - eigenvectors extracted and plotted - loadings examined		- not log_{10}-tansformed - standardised vs unstandardised - UPGMA - Euclidian distances - Co-phenetic correlation for goodness-of-fit	- DFA (examination of phenogram groups)	Test the existence of groups identified by Dreyer *et al.* (1995) with as many specimens as possible.
2	All identified specimens	- log_{10}-transformed - correlation matrix - eigenvectors extracted and plotted - loadings examined	- ANOVAs on eigenvectors - DFA (differences between groups)	- not log_{10}-tansformed - standardised vs unstandardised - UPGMA - Euclidian distances - Co-phenetic correlation for goodness-of-fit	- DFA (examination of phenogram groups)	Test groups identified by Dreyer *et a.l* (1995) using specimens identified by the authors.
3	All freshly collected specimens	- log_{10}-transformed - correlation matrix - eigenvectors extracted and plotted - loadings examined	- ANOVAs on eigenvectors - DFA (differences between groups)	- not log_{10}-tansformed - standardised vs unstandardised - UPGMA - Euclidian distances - Co-phenetic correlation for goodness-of-fit	- DFA (examination of phenogram groups)	Test groupings with putative and previously overlooked important floral variation.

Table 2.2. Morphological characters used in the phenetic analysis of *Pelargonium* populations. The quantitative characters were measured in millimetres. Characters marked with an asterisk were used only in Analysis 3.

Character	Description
Leaf characters	
1.	Length of the leaf blade.
2.	Width of the leaf blade.
3.	Length from the base of the sinus to the junction with the petiole.
4.	Length of the petiole.
Characters of the whole plant	
5.	Habit: decumbent (1)/ rosulate (0).
6.	Internode length.
7.	Order of branching: inflorescence borne on the first branch (1), second (2), third (3).
Characters of flowers and inflorescence	
8.	First branching length of the inflorescence stalk.
9.	Last branching length of the inflorescence stalk.
10.	Inflorescence height/ length.
11.	Number of flowers per pseudoumble.
12.	Pedicel length.
13.	Sepal length.
14.	Flower width.
15.	Flower height.
16.	Length of middle lower petal.
17.	Width of middle lower petal.
18.	Hypanthium length (from the base of the sepals to the nectary).
19.	Functional hypanthium length (From the tip of the stigma to the nectary).
20.	*Flower colour variable 1
21.	*Flower colour variable 2

2.3. Results

2.3.1. Analysis 1: all herbarium specimens

2.3.1.1. Analysis 1: Principal Component Analysis

PCA was carried out on 139 specimens not all of which were identified to subspecies level (Appendix 2.1). The results of this analysis are given in Table 2.3. The first three axes account for 56.3% of the total variation. The first component explains 25.2% of the total variation and has a high positive loading for variables 1 (habit) and 5 (petal length) contrasted with the strongly negative loading for several of the remaining variables (variables 2, 11-14, 16, 18-19, Table 2.4). The second component explains 18.3% of the variation, and the third component explains 12.7% (the component loadings along the first three axes are presented in Table 2.4).

Table 2.3: Results of PCA on 19 variables of 139 specimens of *Pelargonium* (Analysis 1): eigenvalues, percentage of total variance explained by each axis and cumulative percentage along the first three axes.

Axis	Eigenvalues	Percentage of variance explained	Cumulative percentage of variance explained
1	4.79	25.25	25.25
2	3.47	18.27	43.52
3	2.42	12.75	56.27

Table 2.4: Results of PCA on 19 variables of 139 specimens of *Pelargonium* (Analysis 1): component loading along the three first axes.

Variable	Axis 1	Axis 2	Axis 3
1. habit	0.4332	0.0554	0.6463
2. sepal length	-0.3535	0.63	0.1332
3. flower width	-0.1447	0.6321	0.4065
4. flower height	-0.056	0.779	0.305
5. petal width	0.3057	0.449	0.1186
6. petal length	0.0694	0.3588	0.5053
7. hypanthium length	-0.1433	0.74	-0.4815
8. functional hypanthium length	-0.1538	0.7534	-0.4569
9. last branching length	-0.2353	0.131	0.4958
10. pedicel length	-0.2765	0.3683	-0.1914
11. leaf length	-0.907	-0.0209	-0.0074
12. leaf width	-0.9037	0.0035	0.0821
13. sinus	-0.6455	0.1785	-0.0248
14. number of flowers	-0.4408	-0.3224	0.2257
15. internode length	-0.1019	0.1454	0.7285
16. petiole length	-0.919	-0.0478	-0.0977
17. order of branching	-0.2199	-0.3487	0.3435
18. inflorescence height	-0.843	-0.1862	0.0176
19. first branching length	-0.5676	-0.3891	0.1375

The scatterplot of the first two components is illustrated in Figure 2.1, the first and third components in Figure 2.2 and the second and third components in Figure 2.3. It is clear that though there are no distinctly isolated groups, Figures 2.1 and 2.2 do describe vague groupings. *P. reniforme* subsp. *reniforme* and *P. sidoides* separate along PCA 1 with some overlap. *P. reniforme* subsp. *velutinum* occurs among both of these groups. Figure 2.3 shows no groupings at all and thus most of the variation can be attributed to PCA 1. Although the groups that should have been described by Analysis 1, (namely, *P. sidoides*, *P. reniforme* and the four groups described by Dreyer *et al.* (1995), including the two subspecies *P. reniforme* subsp. *reniforme* and *P. reniforme* subsp. *velutinum*) are not circumscribed by the analysis, there is some indication that *P. sidoides* forms a group.

Analysis of variance (ANOVA) and Discriminant function analysis (DFA) was not done on the results of the PCA for Analysis 1. Some of the specimens were not identified and did not form a natural group on which to base the analysis.

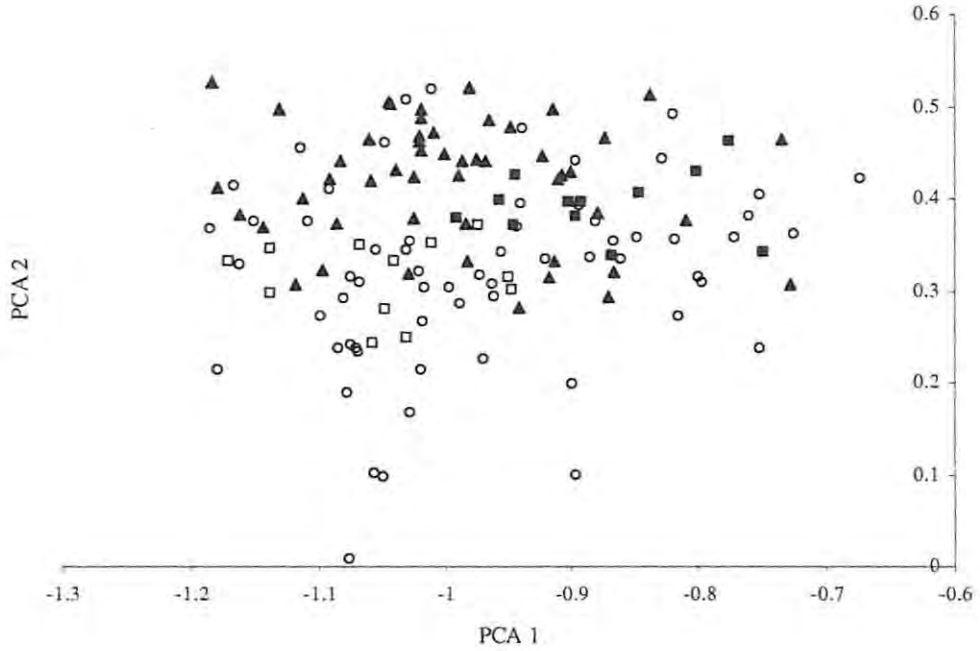

Figure 2.1: Scatterplot of first two components from a PCA performed with 19 variables and 139 specimens of *Pelargonium* (Analysis 1); *P. reniforme* subsp. *reniforme* (■), *P. reniforme* subsp. *velutinum* (▲), *P. sidoides* (□), unidentified specimens (○).

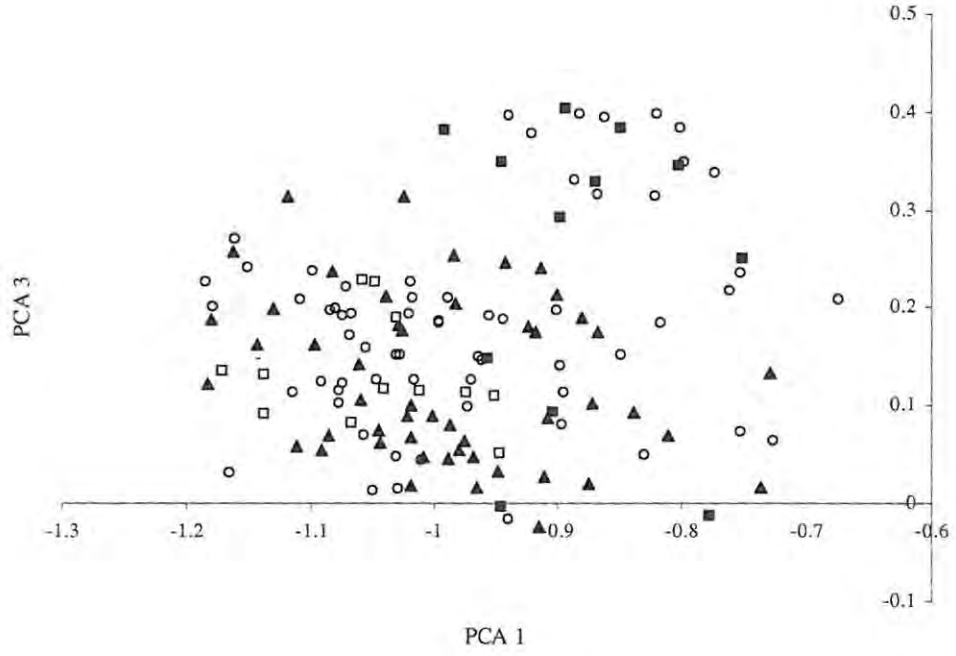

Figure 2.2: Scatterplot of components 1 and 3 from a PCA performed with 19 variables and 139 specimens of *Pelargonium* (Analysis 1); *P. reniforme* subsp. *reniforme* (■), *P. reniforme* subsp. *velutinum* (▲), *P. sidoides* (□), unidentified specimens (○).

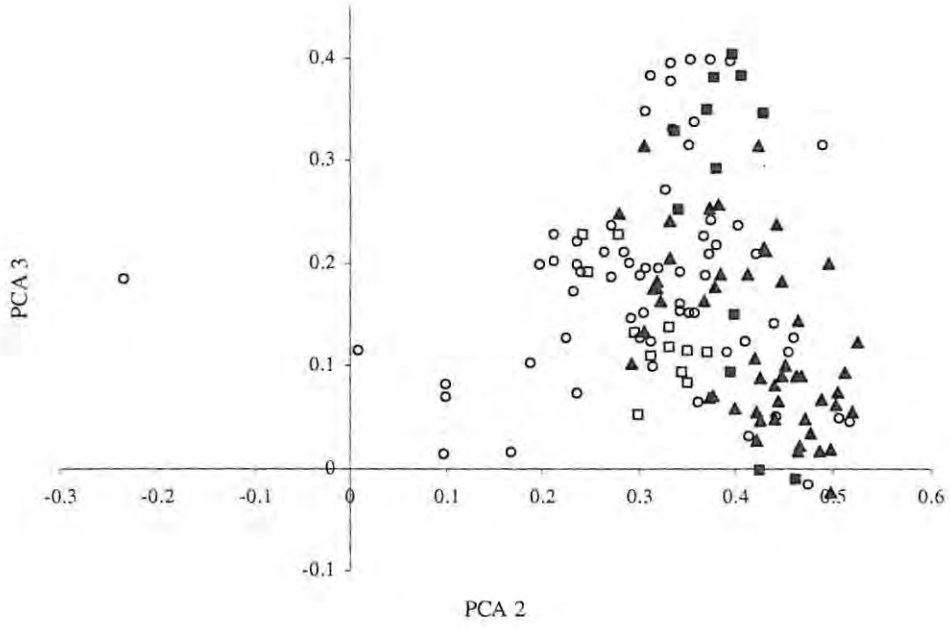

Figure 2.3: Scatterplot of components 2 and 3 from a PCA performed with 19 variables and 139 specimens of *Pelargonium* (Analysis 1); *P. reniforme* subsp. *reniforme* (■), *P. reniforme* subsp. *velutinum* (▲), *P. sidoides* (□), unidentified specimens (○).

2.3.1.2. Analysis 1: Cluster Analysis

Two separate cluster analyses were carried out using 19 variables and 139 specimens, as used in PCA. The first of these was conducted in the traditional method, by standardising the data prior to running the analysis. The phenogram can be seen in Figure 2.4. Co-phenetic correlation produced a value of $r = 0.75$ (t = 13.87, p = 1.0000) for Figure 2.4. Although the matrices are correlated, they are not significant, indicating that the phenogram is a good fit for the data, but not significant. The phenogram does not reveal expected groups; the different subspecies are not separated out.

The second of the cluster analyses was conducted on raw data (it was not standardised before-hand). The phenogram can be seen in Figure 2.5. Co-phenetic correlation produced a value of $r = 0.77$ (t = 18.29, p = 1.0000) for Figure 2.5. These matrices are correlated but also not significant, indicating that the phenogram is not a good fit for the data. The phenogram does not reveal expected groups.

Groups expected to be delimited by the cluster analysis included the four groups of *Pelargonium reniforme* defined by Dreyer *et al.* (1995) as well as *P. sidoides*. Although clear groups do exist in both phenograms (Figures 2.4 and 2.5), these do not correspond with what was expected.

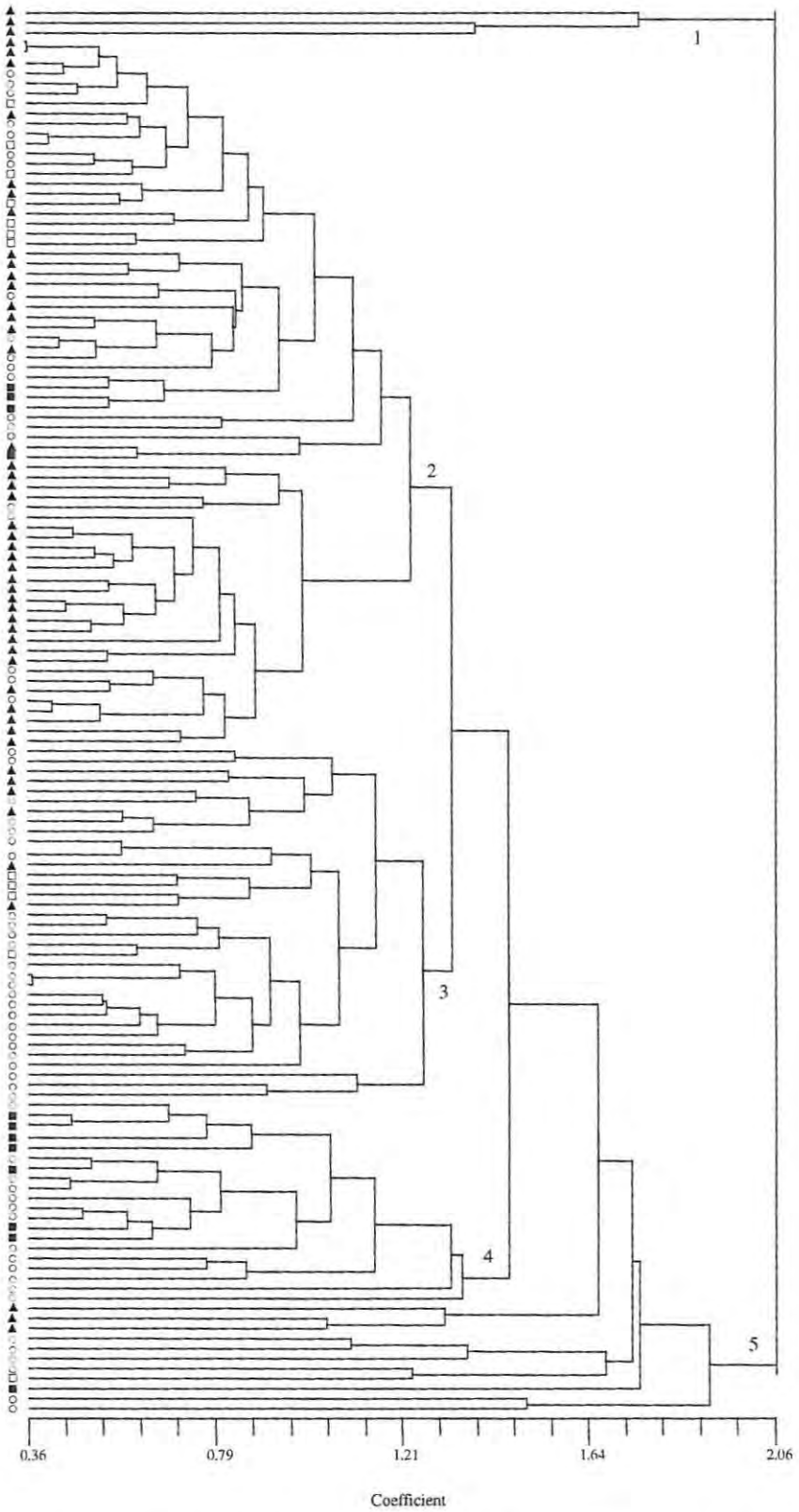

Figure 2.4: Phenogram obtained from CA with UPGMA clustering method with Euclidian distances performed on standardised data for 139 specimens of *Pelargonium* and on the basis of 19 variables (Analysis 1); *P. reniforme* subsp. *reniforme* (■), *P. reniforme* subsp. *velutinum* (▲), *P. sidoides* (□), unidentified specimens (○). Numbers 1-5 denote groups referred to in the text.

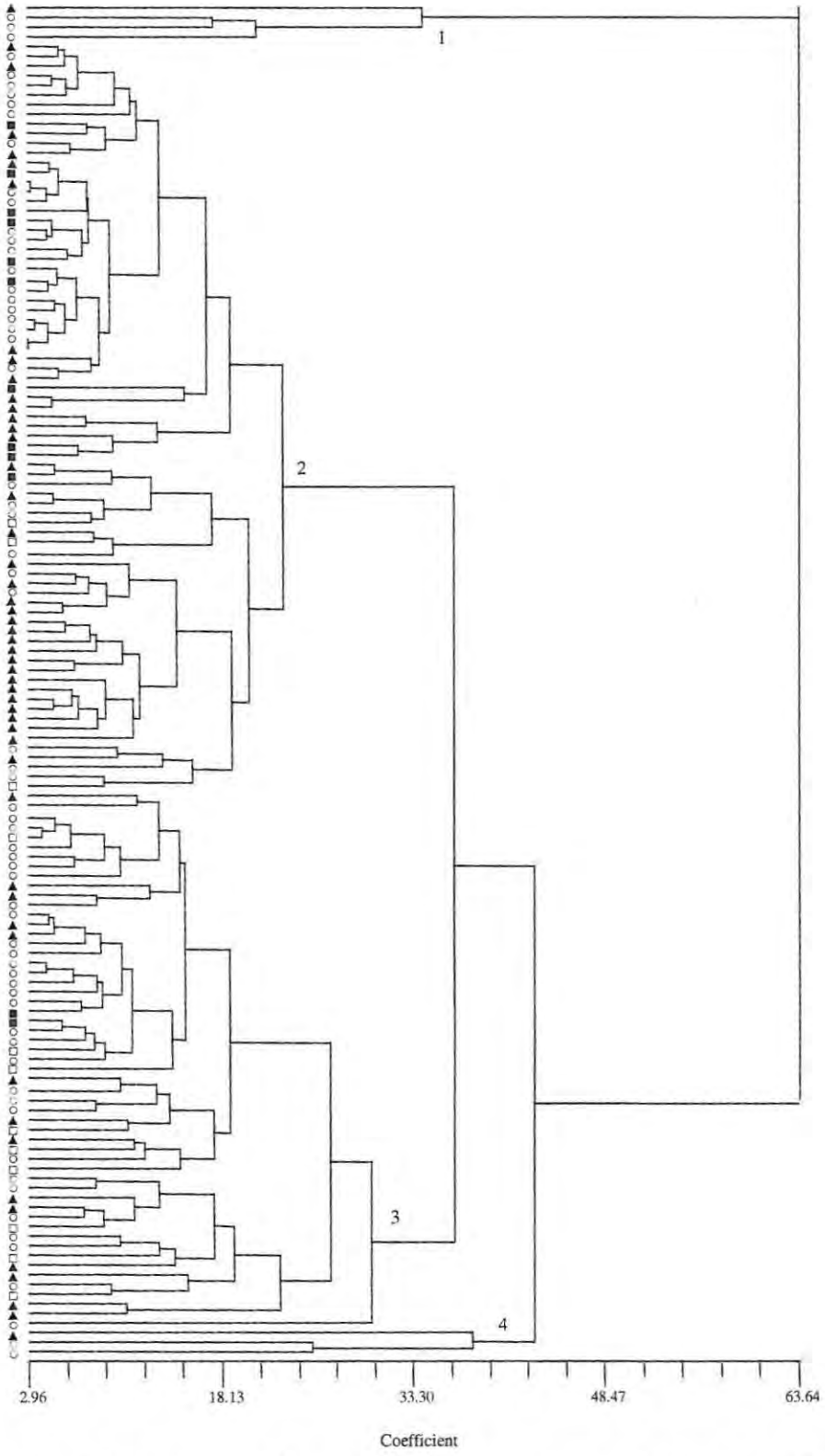

Figure 2.5: Phenogram obtained from CA with UPGMA clustering method with Euclidian distances performed on unstandardised data for 139 specimens of *Pelargonium* and on the basis of 19 variables (Analysis 1); *P. reniforme* subsp. *reniforme* (■), *P. reniforme* subsp. *velutinum* (▲), *P. sidoides* (□), unidentified specimens (○). Numbers 1-4 denote groups referred to in the text.

Although the groups delimited by the cluster analysis do not correspond with the existing taxonomy, a discriminant function analysis was conducted to determine the statistical significance of the groups outlined by the analysis.

The main groups were identified for each of the phenograms (see Figures 2.4 and 2.5). Figure 2.4 shows five main groups. The forward stepwise DFA resulted in 16 steps with 16 of the 19 variables in the model contributing to the grouping. Wilk's lambda = 0.003 approx. $F_{(64, 468)} = 24.91$, $p < 0.05$. The analysis determined that the specimens would be grouped into the correct groups 98.52%. (See the classification matrix, Table 2.5). Important characters in the model included habit, petal height and length, leaf length and indent length among others (the details of the variables in the model can be seen in Appendix 2.2). Canonical analysis was conducted as part of the DFA and was able to extract 4 roots, which show the loadings for each of the variables in the model. From the first (and most important statistically) root of the canonical analysis, it can be seen that the habit is the most important character, with a comparatively high value (Table 2.6).

Table 2.5: Classification matrix. Results from the Discriminant function analysis conducted on Figure 2.4 (all data standardised, Analysis 1). Rows: observed classifications. Columns: predicted classifications.

	Percent Correct	Group 1 p=.02158	Group 2 p=.49640	Group 3 p=.26619	Group 4 p=.13669	Group 5 p=.07914
Group 1	100.00	3	0	0	0	0
Group 2	98.55	0	68	0	0	1
Group 3	86.48	0	2	32	1	2
Group 4	100.00	0	0	0	19	0
Group 5	72.73	0	0	3	0	8
Total	93.53	3	70	35	20	11

Table 2.6: Raw coefficients (all data standardised, Analysis 1) for canonical variables.

	Root 1	Root 2	Root 3	Root 4
habit	10.35	-0.89	1.13	0.20
petal length	-0.22	-1.12	-0.76	0.12
petal width	0.07	0.61	0.25	-0.06
leaf length	0.04	0.03	0.08	0.06
sinus	-0.16	-0.07	0.09	-0.16
functional hypanthium length	-0.07	0.05	-0.19	0.01
no. of flowers	0.05	-0.11	0.23	0.09
pedicel length	0.02	-0.08	0.08	-0.26
petiole length	0.02	-0.00	0.01	-0.01
last branching length	0.00	0.01	-0.00	-0.02
sepal length	-0.10	-0.26	-0.39	-0.06
leaf width	-0.03	-0.05	-0.02	-0.02
flower height	0.00	-0.04	0.16	0.01
hypanthium length	0.07	-0.05	0.17	-0.03
internode length	0.06	0.03	-0.12	-0.12
flower width	-0.05	0.01	0.04	0.23
Constant	-1.53	2.01	-3.59	-0.41
Eigenval	13.00	3.48	2.44	0.53
Cum.Prop	0.67	0.85	0.97	1.00

Figure 2.5 shows four main groups. The forward stepwise DFA resulted in 14 steps with 14 of the 19 variables in the model (Wilks lambda = 0.09 approx. $F_{(42, 362)} = 10.82$, $p < 0.05$). The analysis determined that the specimens would be grouped into the correct groups 98.56% of the time (see the classification matrix, Table 2.7). Important characters in the model included inflorescence height and pedicel length among others (the details of the variables in the model can be seen in Appendix 2). Canonical analysis was conducted as part of the DFA and was able to extract three roots, which show the loadings for each of the variables in the model. From the first root of the canonical analysis (Table 2.8), it can be seen that order of branching and tube length are the most important characters, though their values are not much higher than those for the other variables.

Table 2.7: Classification matrix. Results form the Discriminant function analysis conducted on Figure 2.5 (all data unstandardised, Analysis 1). Rows: observed classifications. Columns: predicted classifications.

	Percent Correct	Group 1 p=.02878	Group 2 p=.55396	Group 3 p=.39568	Group 4 p=.02158
Group 1	100.00	4	0	0	0
Group 2	97.40	0	75	2	0
Group 3	100.00	0	0	55	0
Group 4	100.00	0	0	0	3
Total	98.56	4	75	57	3

Table 2.8: Raw coefficients (all data unstandardised, Analysis 1) for canonical variables.

	Root 1	Root 2	Root 3
inflorescence height	-0.02	0.01	-0.00
first branching length	-0.02	-0.01	-0.00
pedicel length	0.01	-0.04	0.01
functional hypanthium length	0.15	-0.03	0.10
no. of flowers	0.09	-0.11	0.04
flower height	0.05	-0.24	-0.11
petal height	-0.04	0.21	0.14
leaf length	0.05	-0.03	-0.15
leaf width	-0.06	0.01	0.08
last branching length	-0.07	0.07	0.17
order of branching	0.24	-0.51	-0.26
sinus	0.02	-0.07	0.10
petiole length	-0.01	0.01	0.01
hypanthium length	-0.13	0.06	-0.09
Constant	3.86	5.11	0.59
Eigenval	5.17	0.55	0.16
Cum.Prop	0.88	0.97	1.00

2.3.2. Analysis 2: Previously identified specimens

2.3.2.1. Analysis 2: Principal Component Analysis

For the second analysis, PCA was carried out on 75 herbarium and field collected specimens, and 19 variables (Appendix 2). The results of this analysis are given in Table 2.9. The first three axes account for 56.2% of the total variation. The first component explains 25.7% of the total variation and has a positive loading for variables 1 (habit) and

5 (petal width) contrasted with the negative loading of all other variables and the strong negative loadings of variables 11 (leaf length), 12 (leaf width) and 16 (petiole length) in particular. The second component explains 15.4% of the variation, and the third component explains 15% (the component loadings along the first three axes are presented in Table 2.10).

Table 2.9: Results of PCA on 19 variables of 75 identified specimens of *Pelargonium* (Analysis 2): eigenvalues, percentage of total variance explained by each axis and cumulative percentage along the first three axes.

Axis	Eigenvalues	Percentage of variance explained	Cumulative percentage of variance explained
1	4.88	25.71	25.71
2	2.93	15.43	41.14
3	2.856	15.03	56.17

Table 2.10: Results of PCA on 19 variables of 75 identified specimens of *Pelargonium* (Analysis 2): component loading along the three first axes.

Variable	Axis 1	Axis 2	Axis 3
1. habit	0.1495	0.1557	0.5674
2. sepal length	-0.5905	0.4983	0.1132
3. flower width	-0.295	0.6303	0.2916
4. flower height	-0.1854	0.7804	0.0386
5. petal width	0.3715	0.4295	-0.1804
6. petal length	-0.2	0.5331	0.381
7. hypanthium length	-0.1789	0.353	-0.8055
8. functional hypanthium length	-0.2066	0.3431	-0.7944
9. last branching length	-0.3338	0.3798	0.3933
10. pedicel length	-0.1605	0.4295	-0.3252
11. leaf length	-0.8913	-0.1362	-0.2118
12. leaf width	-0.9204	-0.068	-0.086
13. sinus	-0.5497	-0.2445	-0.0969
14. number of flowers	-0.5168	0.1445	0.3223
15. internode length	-0.4219	0.2405	0.4672
16. petiole length	-0.8538	-0.2013	-0.2418
17. order of branching	-0.3343	-0.3255	0.4835
18. inflorescence height	-0.7424	-0.3349	-0.0941
19. first branching length	-0.4705	-0.4397	0.1758

Figure 2.6 shows a scatterplot of component 1 vs. component 2. The figure shows that *Pelargonium sidoides* and *P. reniforme* subsp. *reniforme* tend to separate across both axes but are both in the midst of a group entirely consisting of *P. reniforme* subsp. *velutinum* specimens and do not resolve into well-defined groups. A similar pattern can be seen for PCA components 2 and 3 (Figure 2.7). No groups exist in Figure 2.8 which describes PCA components 1 and 3. PCA component 2 seems to be important in grouping these specimens.

A one-way analysis of variance (ANOVA) conducted on the first three components of the PCA revealed that there was a significant difference between groups of identified plants (*Pelargonium sidoides*, *P. reniforme* subsp. *velutinum* and *P. reniforme* subsp. *reniforme*) for all three axes (Wilks lambda=0.36, $F_{(6, 140)}$=15.34, $p<0.05$). Discriminant function analysis conducted on all variables for 44 identified plants (specimens with missing data

were excluded from the analysis) revealed that habit, flower width, indent length, order of branching and branching length all were significantly different between groups (*Pelargonium sidoides*, *P. reniforme* subsp. *velutinum* and *P. reniforme* subsp. *reniforme*) (Wilks' Lambda: 0.05 approx. $F_{(38, 46)}$=4.49 $p<0.05$).

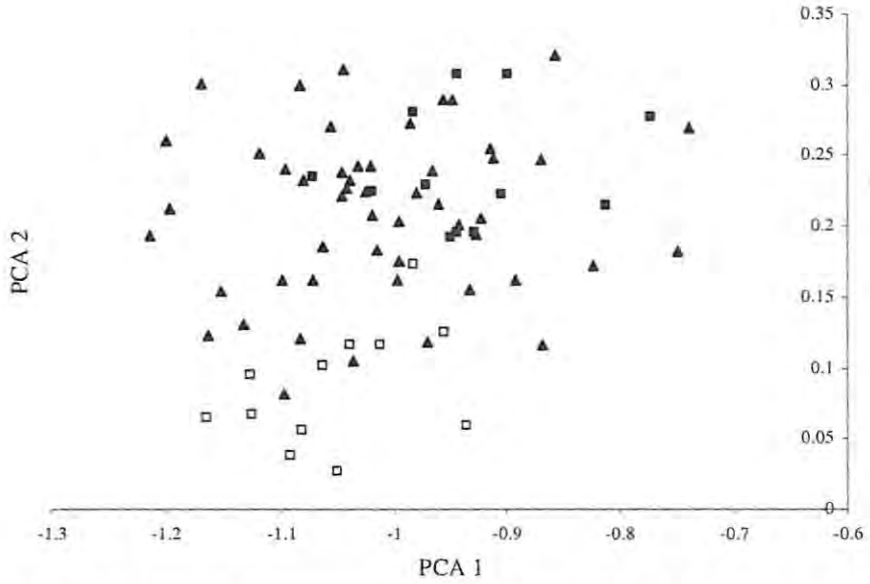

Figure 2.6: Scatterplot of first two components from a PCA performed with 19 variables and 75 identified specimens of *Pelargonium* (Analysis 2); *P. reniforme* subsp. *reniforme* (■), *P. reniforme* subsp. *velutinum* (▲), *P. sidoides* (□).

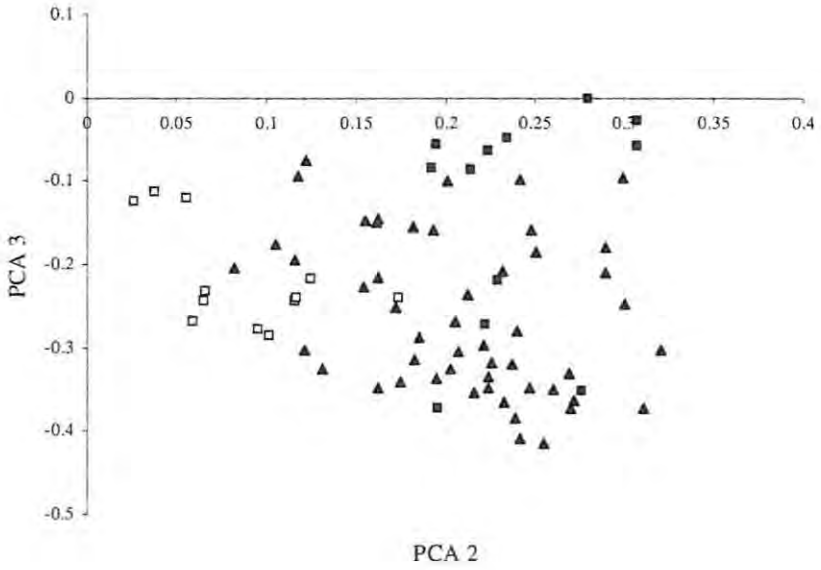

Figure 2.7: Scatterplot of components 1 and 3 from a PCA performed with 19 variables and 75 identified specimens of *Pelargonium* (Analysis 2); *P. reniforme* subsp. *reniforme* (■), *P. reniforme* subsp. *velutinum* (▲), *P. sidoides* (□).

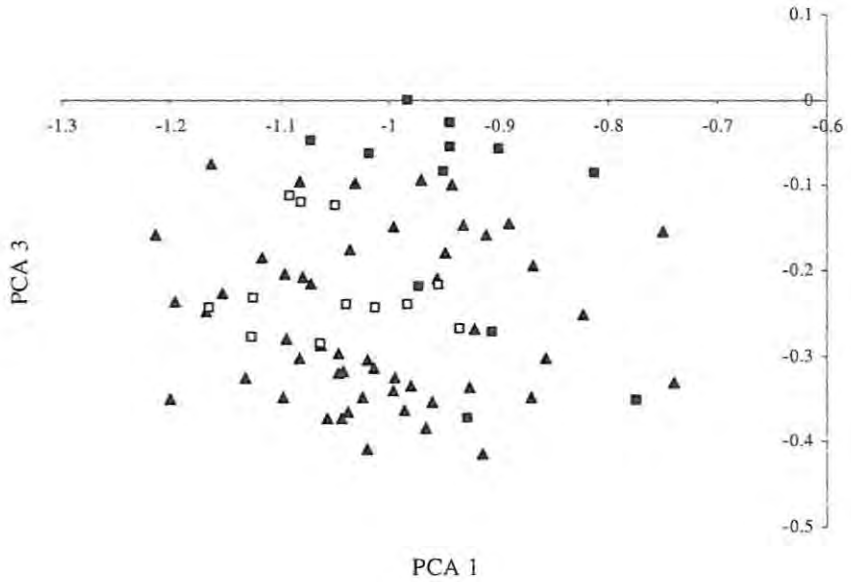

Figure 2.8: Scatterplot of components 2 and 3 from a PCA performed with 19 variables and 75 identified specimens of *Pelargonium* (Analysis 2); *P. reniforme* subsp. *reniforme* (■), *P. reniforme* subsp. *velutinum* (▲), *P. sidoides* (□).

2.3.2.2. Analysis 2: Cluster analysis

Two separate cluster analyses were carried out using 19 variables and 139 specimens, as used in PCA. The first of these was conducted on standardised data. The phenogram can be seen in Figure 2.9. Co-phenetic correlation analysis produced a value of $r = 0.74$ (t = 11.04, p = 1.0000) for Figure 2.9, the matrices are thus correlated but they are not significant, indicating that the phenogram is not a good fit for the data.

The second of the cluster analyses conducted on unstandardised (raw) data, produced the tree seen in Figure 2.10. Co-phenetic correlation produced a value of $r = 0.77$ (t = 13.09, p = 1.0000). Although the matrices are correlated, they are not significant, indicating that the phenogram is not a good fit for the data.

The cluster analysis was expected to delimit four distinct groups corresponding with the groups described by Dreyer *et al.* (1995). However, specimens belonging to these expected groups are scattered throughout each of the phenograms. Though distinct groups are clear in both phenograms (Figures 2.9 and 2.10), they are not formed by the expected individuals, as found in Analysis 1.

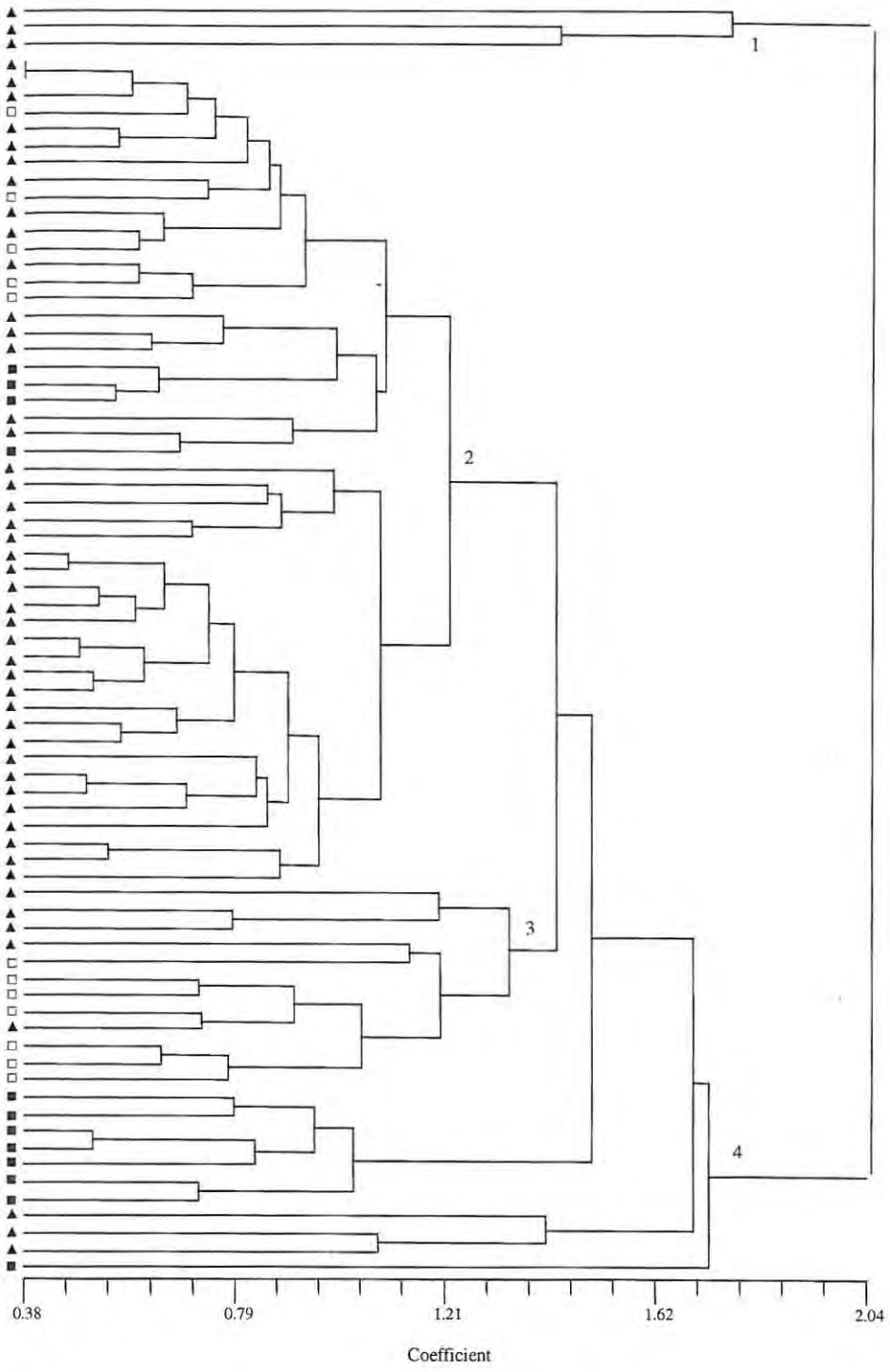

Figure 2.9: Phenogram obtained from CA with UPGMA clustering method with Euclidian distances performed on standardised data for 75 identified specimens of *Pelargonium* and on the basis of 19 variables (Analysis 2); *P. reniforme* subsp. *reniforme* (■), *P. reniforme* subsp. *velutinum* (▲), *P. sidoides* (□). Numbers 1-4 denote groups referred to in the text.

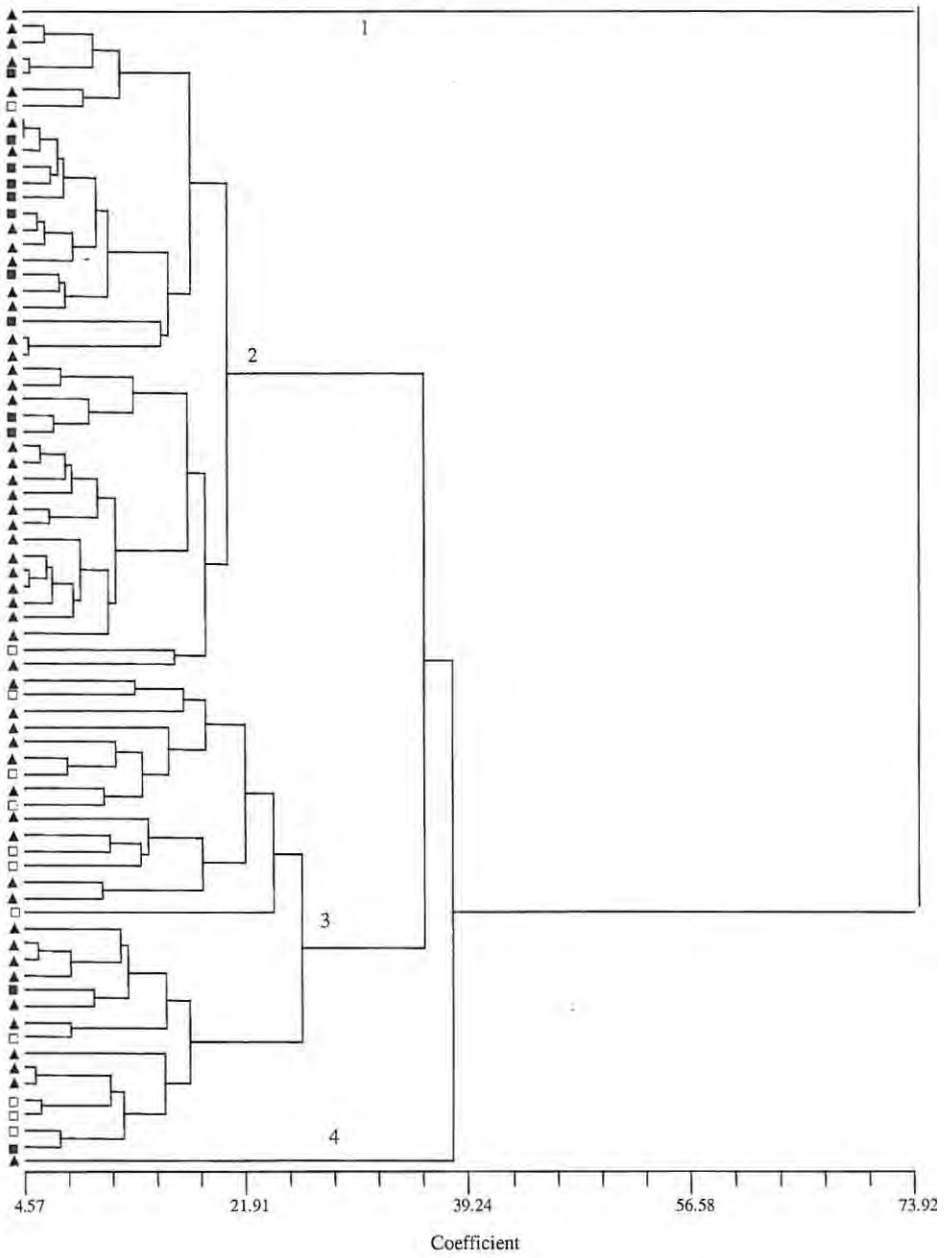

Figure 2.10: Phenogram obtained from CA with UPGMA clustering method with Euclidian distances performed on unstandardised data for 75 identified specimens of *Pelargonium* and on the basis of 19 variables (Analysis 2); *P. reniforme* subsp. *reniforme* (■), *P. reniforme* subsp. *velutinum* (▲), *P. sidoides* (□). Numbers 1-4 denote groups referred to in the text.

Although the groups delimited by the cluster analyses were not what were expected, a discriminant function analysis (DFA) was conducted to determine the statistical significance of the groups outlined by the analyses.

The main groups were identified for each of the phenograms (see Figures 2.9 and 2.10). Figure 2.9 shows four main groups. The forward stepwise DFA resulted in 14 steps with 14 of the 16 variables in the model. Wilks lambda = 0.004 approx. $F_{(42, 172)} = 22.81$, $p<0.05$. The analysis determined that the specimens would be grouped into the correct groups 98.56% of the time (see the classification matrix, Table 2.11). Important characters in the model include habit and petal width and length (Appendix 2.2). Canonical analysis was conducted as part of the DFA and was able to extract three roots. From the first root (Table 2.12), it can be seen that functional tube length is the most important character, with a comparatively high value.

Table 2.11: Classification matrix. Results form the Discriminant function analysis conducted on Figure 2.8 (Analysis 2). Rows: observed classifications. Columns: predicted classifications.

	Percent Correct	Group 1 p=.02878	Group 2 p=.55396	Group 3 p=.39568	Group 4 p=.02158
Group 1	100.00	4	0	0	0
Group 2	97.40	0	75	2	0
Group 3	100.00	0	0	55	0
Group 4	100.00	0	0	0	3
Total	98.56	4	75	57	3

Table 2.12: Raw coefficients (all data standardised, Analysis 2) for canonical variables.

	Root 1	Root 2	Root 3
inflorescence height	-0.73	0.26	-0.01
first branching length	-0.58	-0.22	-0.09
last branching length	0.09	-0.90	0.21
functional hypanthium length	2.29	-0.52	1.56
no. of flowers	0.27	-0.32	0.13
flower height	0.11	-0.59	-0.28
petal height	-0.05	0.24	0.16
leaf length	0.62	-0.37	-1.75
leaf width	-0.66	0.08	0.99
pedicel length	-0.17	0.17	0.42
order of branching	0.13	-0.27	-0.14
sinus	0.07	-0.25	0.36
petiole length	-0.18	0.27	0.38
hypanthium length	-1.94	0.91	-1.24
Eigenval	5.17	0.55	0.16
Cum.Prop	0.88	0.97	1.00

Figure 2.10 shows four groups. The forward stepwise DFA resulted in seven steps with seven of the 19 variables in the model. Wilks lambda = 0.20 approx. $F_{(7, 65)} = 36.19$, $p < 0.05$. The analysis determined that the specimens would be grouped into the defined groups 98.63% of the time (see the classification matrix, Table 2.13). Important characters in the model included inflorescence height (Appendix 2.2). Canonical analysis was conducted as part of the DFA and was able to extract only one root (table 2.14). From this root, it can be seen flower width is the most important of the characters although it does not have a much higher value than the other characters in the model.

Table 2.13: Classification matrix. Results form the Discriminant function analysis conducted on Figure 2.9 (Analysis 2). Rows: observed classifications. Columns: predicted classifications.

	Percent Correct	Group 2 p=.57534	Group 3 p=.42466
Group 2	100.00	42	0
Group 3	96.77	1	30
Total	98.63	43	30

Table 2.14: Raw coefficients (all data standardised, Analysis 2) for canonical variables.

	Root 1
inflorescence height	0.03
first branching length	0.02
flower width	0.30
leaf length	-0.02
flower height	-0.19
petal length	0.14
Internode length	0.06
Constant	-7.50
Eigenval	3.89
Cum.Prop	1.00

2.3.3. Analysis 3: Living specimens

2.3.3.1. Analysis 3: Principal Component Analysis

PCA was carried out with 31 specimens and 21 variables (Table 2.2). The results of this analysis are given in Table 2.15. The first three axes account for 58.8% of the total variation. The first component explains 26.4% of the total variation and has high negative loadings for variables 2 (sepal length) and 3 (flower width), contrasted with the strongly positive loading for several of the remaining variables (variables 1, 4, 9-10, 13-14, 16, 18 and 20, see Table 2.16). The second component explains 20.5% of the variation, and the third component explains 11.8% (the component loadings along the first three axes are presented in Table 2.16).

Table 2.15: Results of PCA on 21 variables of 31 specimens of *Pelargonium* (Analysis 3): eigenvalues, percentage of total variance explained by each axis and cumulative percentage along the first three axes.

Axis	Eigenvalues	Percentage of variance explained	Cumulative percentage of variance explained
1	5.55	26.42	26.42
2	4.31	20.52	46.94
3	2.48	11.83	58.77

Table 2.16: Results of PCA on 21 variables of 31 specimens of *Pelargonium* (Analysis 3): component loading along the three first axes.

Variable	Axis 1	Axis 2	Axis 3
1. habit	0.6895	0.2708	0.3364
2. sepal length	-0.4046	-0.581	-0.3233
3. flower width	-0.3717	-0.4307	0.039
4. flower height	0.6716	-0.1388	-0.3704
5. petal width	0.3356	0.3163	-0.7191
6. petal length	0.3372	0.5562	-0.5694
7. hypanthium length	0.1111	0.5049	-0.3708
8. functional hypanthium length	-0.0061	0.0721	-0.582
9. last branching length	0.6688	0.6452	0.1119
10. pedicel length	0.7257	0.5892	0.0829
11. leaf length	-0.0963	-0.2327	0.2097
12. leaf width	0.0119	0.629	0.3114
13. sinus	0.8754	-0.2762	0.1328
14. number of flowers	0.8038	-0.4053	0.148
15. internode length	0.1249	-0.1859	0.5792
16. petiole length	0.6313	-0.4585	0.0535
17. order of branching	0.285	-0.4509	0.1012
18. inflorescence height	0.8175	-0.1659	0.145
19. first branching length	0.0853	-0.6893	-0.2839
20. colour 1	0.6212	-0.488	-0.1211
21. colour 2	0.3577	-0.6178	-0.3812

The scatterplot of the first two components for all specimens is illustrated in Figure 2.11. Although there are no distinct clusters shown with these axes, there is definitely a separation across principal component axis one which separates the long- and short-tubed forms (with different colours) of *P. reniforme* subsp. *velutinum*. *P. reniforme* subsp. *reniforme* also separates across this axis from left to right. This shows a clear gradation of different observed floral forms of the species. Similar groupings can be seen in Figures 2.12 and 2.13 which are plots of PCA axes one and three, and two and three respectively. Figures 2.12 and 2.13 do not separate the groups as clearly as Figure 2.11.

A one-way analysis of variance (ANOVA) conducted on the first three components of the PCA revealed that there was a significant difference between groups of identified plants (*P. reniforme* subsp. *velutinum* (long-tubed), *P. reniforme* subsp. *velutinum* (short-tubed) and *P. reniforme* subsp. *reniforme*) for all three axes (Wilks lambda=0.09, $F_{(6, 52)}=21.01$, $p<0.05$). Discriminant function analysis conducted on all variables for 31specimens revealed that only leaf length was significantly different between groups (*P. reniforme* subsp. *velutinum* (long-tubed), *P. reniforme* subsp. *velutinum* (short-tubed) and *P. reniforme* subsp. *reniforme*) (Wilks' Lambda: 0.001 approx. $F_{(42,16)}=11.96$ $p<0.05$).

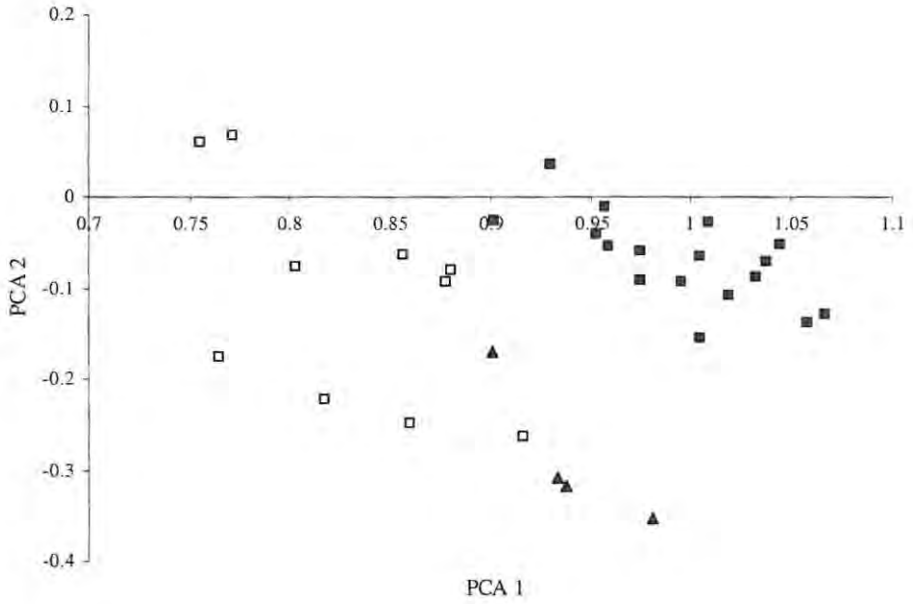

Figure 2.11: Scatterplot of components 1 and 2 from a PCA performed with 21 variables and 31 specimens of *Pelargonium* (Analysis 3); *P. reniforme* subsp. *reniforme* (□), *P. reniforme* subsp. *velutinum* long-tubed (■), *P. reniforme* subsp. *velutinum* short-tubed (▲).

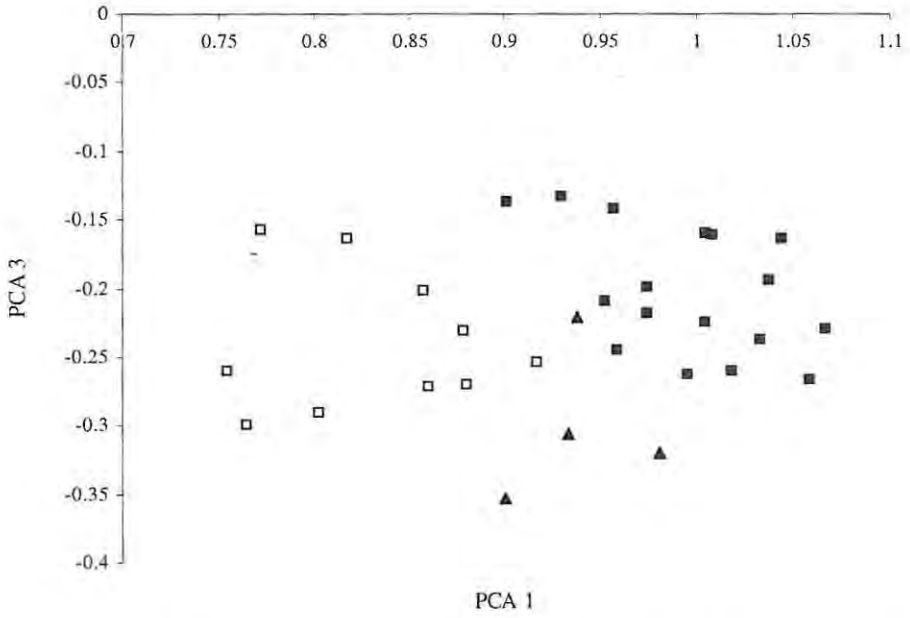

Figure 2.12: Scatterplot of components 1 and 3 from a PCA performed with 21 variables and 31 specimens of *Pelargonium* (Analysis 3); *P. reniforme* subsp. *reniforme* (□), *P. reniforme* subsp. *velutinum* long-tubed (■), *P. reniforme* subsp. *velutinum* short-tubed (▲).

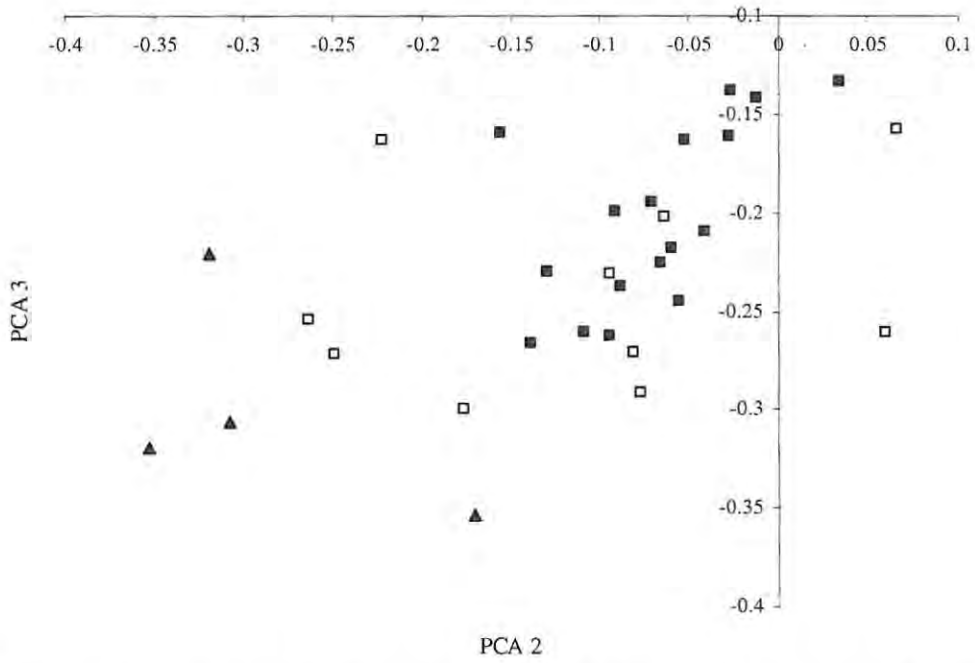

Figure 2.13: Scatterplot of components 2 and 3 from a PCA performed with 21 variables and 31 specimens of *Pelargonium* (Analysis 3); *P. reniforme* subsp. *reniforme* (□), *P. reniforme* subsp. *velutinum* long-tubed (■), *P. reniforme* subsp. *velutinum* short-tubed (▲).

2.3.1.2. Analysis 3: Cluster analysis

Two separate cluster analyses were carried out using 31 specimens and 21 characters and are illustrated in Figures 2.14 and 2.15. The first of these (illustrated in Figure 2.14), was conducted on standardised data. Co-phenetic correlation produced a value of $r = 0.77$ (t = 8.18, p = 1.0000). The goodness of fit test shows that thought the matrices are correlated; they are not significantly correlated, indicating that the phenogram is not a good fit to the data. The phenogram (Figure 2.14) shows a division into two groups, one of which consists of the long-tubed and dark pink *Pelargonium reniforme* subsp. *velutinum* (group 1 in Fig. 2.14) and the other a mixture of the short-tubed and lighter pink *Pelargonium reniforme* subsp. *velutinum* and the lighter pink *P. reniforme* subsp. *reniforme*. These groups show the same groups found in the principal components analysis. This is only partly what was expected.

The second analysis, conducted on unstandardised data, revealed a similar grouping but some of the expected groups are non-cohesive (Figure 2.15). Co-phenetic correlation produced a value of $r = 0.80$ (t = 8.57, p = 1.0000).

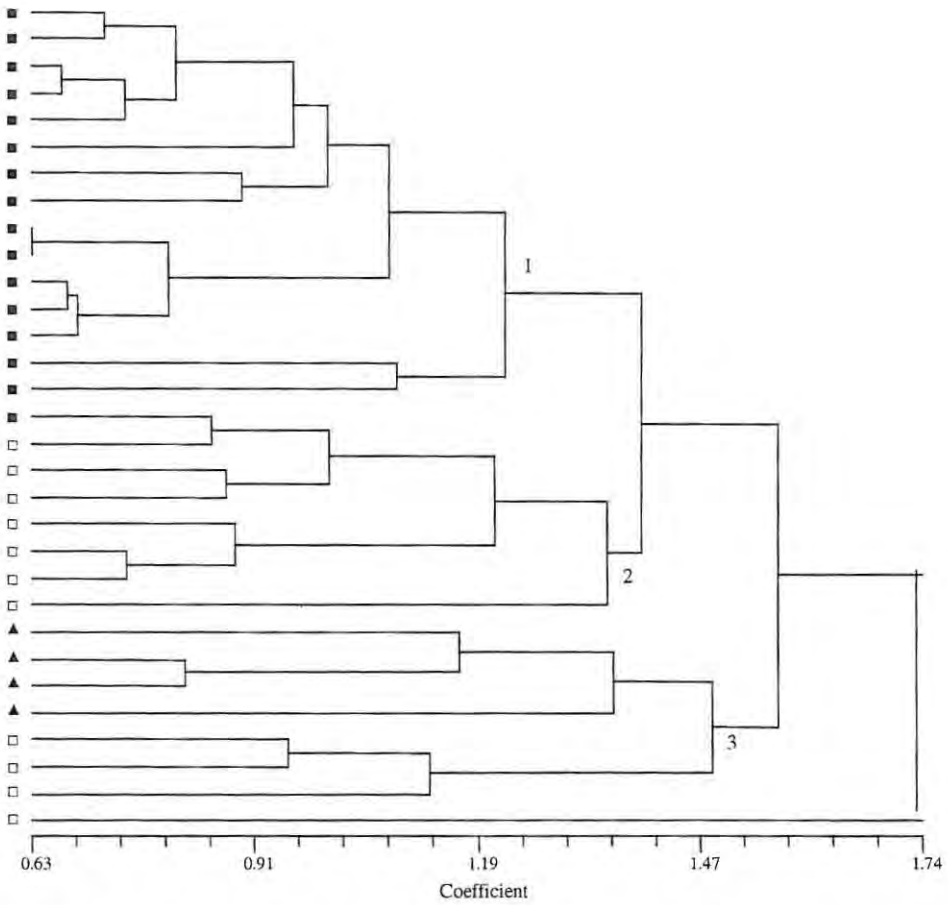

Figure 2.14: Phenogram obtained from CA with UPGMA clustering method with Euclidian distances performed on 31 specimens of *Pelargonium* and on the basis of 21 variables (Analysis 3); *P. reniforme* subsp. *reniforme* (□), *P. reniforme* subsp. *velutinum* long-tubed (■), *P. reniforme* subsp. *velutinum* short-tubed (▲). Numbers 1-3 denote groups referred to in the text.

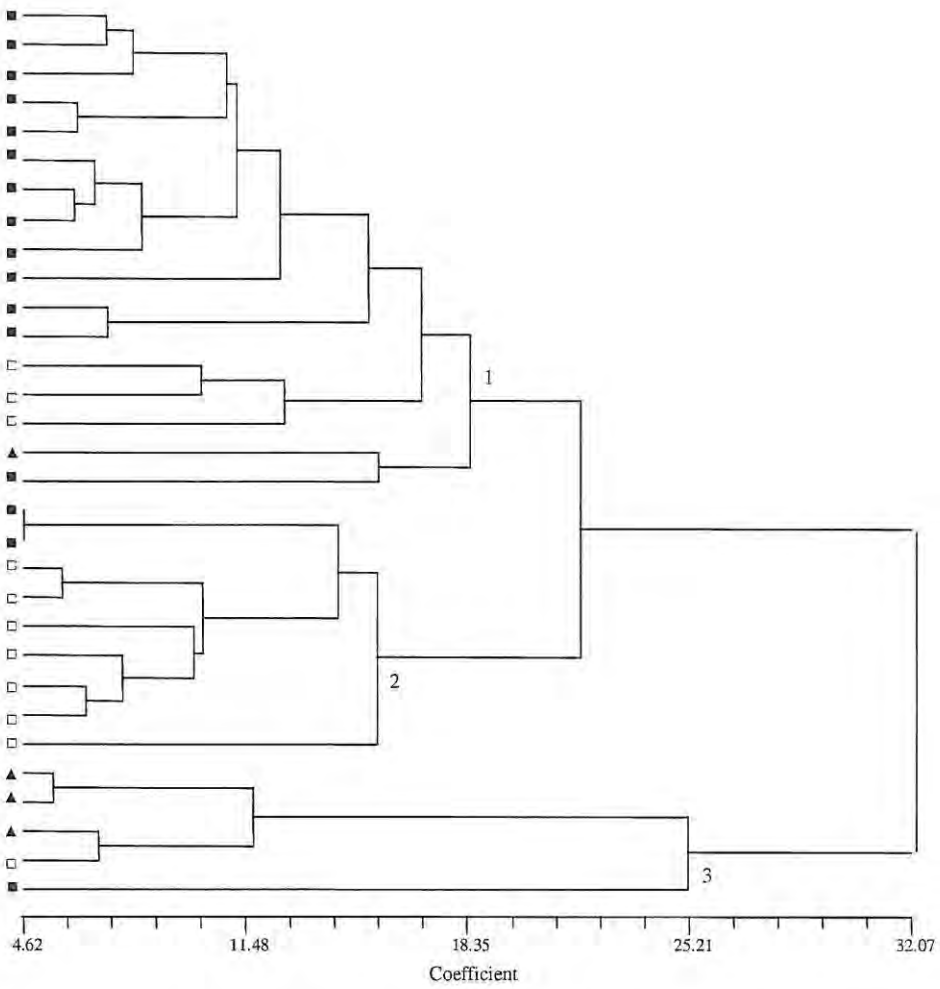

Figure 2.15: Phenogram obtained from CA with UPGMA clustering method with Euclidian distances performed on 31 specimens of *Pelargonium* and on the basis of 21 variables (Analysis 3); *P. reniforme* subsp. *reniforme* (□), *P. reniforme* subsp. *velutinum* long-tubed (■), *P. reniforme* subsp. *velutinum* short-tubed (▲). Numbers 1-3 denote groups referred to in the text.

The groups elucidated by the standardised data were exactly what were expected from the morphometrics measurements whereas the groups for the unstandardised data, although similar, were not exactly what were expected, as the two floral forms of *Pelargonium reniforme* subsp. *velutinum* did not form two distinct clusters. A discriminant function analysis (DFA) was conducted to determine details about the groups resulting from the cluster analysis.

The main groups were identified for each of the phenograms (Figures 2.14 and 2.15). Figure 2.14 showed three main groups. The forward stepwise DFA resulted in 11 steps with 11 of the 21 variables in the model (Wilks lambda = 0.01 approx. $F_{(22, 36)} = 15.21$ $p<0.05$). The analysis determined that the specimens would be grouped into the correct groups 100% of the time (see classification matrix, Table 2.17). Important characters in the model include colour and habit (Appendix 2). Canonical analysis was conducted as part of the DFA and was able to extract two roots. From the first root (Table 2.18), it can be seen that colour and habit are the most important characters.

Table 2.17: Classification matrix. Results form the Discriminant function analysis conducted on Figure 2.12 (Analysis 3). Rows: observed classifications. Columns: predicted classifications.

	Percent Correct	Group 1 p=.48387	Group 2 p=.25806	Group 3 p=.25806
Group 1	100.00	15	0	0
Group 2	100.00	0	8	0
Group 3	100.00	0	0	8
Total	100.00	15	8	8

Table 2.18: Raw coefficients (all data standardised, Analysis 3) for canonical variables.

	Root 1	Root 2
functional hypanthium length	-0.07	-0.03
first branching length	0.02	-0.02
colour 1	-7.47	-9.81
habit	3.23	-0.39
order of branching	1.07	-0.53
flower height	-0.55	0.32
leaf width	-0.03	-0.04
petal height	0.13	-1.64
flower width	0.16	0.97
last branching length	-0.02	0.03
sepal length	0.17	-0.84
Constant	8.31	1.98
Eigenval	13.33	6.39
Cum.Prop	0.68	1.00

Figure 2.15 shows three main groups. The forward stepwise DFA resulted in 11 steps with 11 of the 21 variables in the model. Wilks lambda = 0.02 approx. $F_{(22, 36)} = 11.11$, $p<0.05$. The analysis determined that the specimens would be grouped into the defined groups 100% of the time (see the classification matrix, Table 2.19). Important characters in the model included habit and sepal length among others (Appendix 2). Canonical analysis was conducted as part of the DFA and was able to extract two roots (Table 2.20). From the first root, it can be seen that colour is the most important of characters with its comparatively high value.

Table 2.19: Classification matrix. Results form the Discriminant function analysis conducted on Figure 2.12 (Analysis 3). Rows: observed classifications. Columns: predicted classifications.

	Percent Correct	Group 1 p=.54839	Group 2 p=.29032	Group 3 p=.16129
Group 1	100.00	17	0	0
Group 2	100.00	0	9	0
Group 3	100.00	0	0	5
Total	100.00	17	9	5

Table 2.20: Raw coefficients (all data standardised, Analysis 3) for canonical variables.

	Root 1	Root 2
inflorescence height	0.03	-0.02
functional hypanthium length	0.10	0.46
first branching length	0.04	-0.02
hypanthium length	-0.14	-0.47
flower width	-0.90	-1.51
habit (rosulate/recumbent)	-4.18	-3.78
sepal length	0.51	1.39
pedicel length	0.32	0.49
colour 1	7.76	8.39
sinus	-0.10	-0.16
petal height	0.23	1.13
Constant	-3.52	5.67
Eigenval	10.19	4.41
Cum.Prop	0.69	1.00

2.4. Discussion

2.4.1. Implications for current specific and subspecific groupings.

In both the PCA and CA conducted on all 139 specimens of *Pelargonium reniforme* and *P. sidoides* (Analysis 1), no clear groups were circumscribed by the analyses. Although it was expected that there would be a division along geographic lines or at least between *P. reniforme* and *P. sidoides*, there are no clearly defined groups. There are, however, gradations from *P. reniforme* to *P. sidoides* in the principal component analysis (Figures 2.1 to 2.3). The loadings for the PCA axes for all 139 specimens indicate that the most important characters contributing to the distribution of the specimens are leaf length, leaf width and petiole length. Although the four distinct groups defined by Dreyer *et al.* (1995) are not visible in the analysis, the most important characters are size characters corresponding to some of the characters cited as important in Dreyer *et al.* (1995). The same characters are identified as being the most important along the first axis of the PCA for 75 identified specimens (Analysis 2). In this, the second analysis, clearly defined groups are also not visible in the PCA (Figures 2.6, 2.7 and 2.8). What is clear is that *Pelargonium reniforme* subsp. *reniforme* separates completely from *P. sidoides* within a much larger and undifferentiated clump of *P. reniforme* subsp. *velutinum*. Leaf shape, internode length and petiole length are given as the most important characters by Dreyer *et al.* (1995). It is clear that Dreyer *et al.* (1995) did identify some of the important characters in discerning groups, especially petiole length. The first PCA axis, however, is used to describe the distribution of specimens according to size. And thus the leaf size and petiole lengths (also a size measurement) are the most important for the species. One of the four groups is described as a dwarf version of another. As size is adequately described by the first PCA axis, we can be certain that this group is not circumscribed by the first PCA axis in this study.

The second and third axes can account for a mixture of both size and shape. In the second axes for Analysis 1, are variables flower height, hypanthium length and functional hypanthium length. These variables too, are important for grouping the specimens.

Flower height is also the most important variable for Analysis 2. Axis three has the highest loading value for internode length for analysis 1 but hypanthium length is the highest loading value for Analysis 2. Again, although one of Dreyer *et al.* (1995)'s characters is determined as being important (i.e. internode length), previously overlooked floral traits become equally important in defining the distribution of the specimens in the orthogonal space of a principal component analysis. However, despite the defining characters of the species being a mixture of those described by Dreyer *et al.* (1995) and those floral characters suspected to be important in observations of the species, there are still no well-defined groups. The failure to obtain expected groups to be circumscribed by PCA is not unusual. 'Common sense' taxonomy often does not result in the same groups elucidated by the comparatively objective morphometrics.

Taxonomic groups are often not reflected in results of morphometric studies but it is the objectivity of morphometrics that is valued. A morphometric analysis conducted in order to determine whether or not objectively defined groups existed in *Crataegus* revealed four reasonably defined groups in the genus (Smith & Phipps 1988) who cited apomixis as the primary reason for the small number of groups identified by such an objective analysis

Often, in morphometric studies, current taxonomy is not reflected in morphometric results, and this study is certainly not an exception. Other studies also encounter no definitive groupings where authors have expected clusters of already-defined species. However, just as often as morphometrics fail to circumscribe the same groups as existing taxonomy, they also support some taxonomy. The method is even utilised to aid new taxonomies of groups. Traditional taxonomic methods (PCA and cluster analysis) defined 12 distinct groups with many intermediate specimens of the genus *Sorbus* (Aldasoro *et al.* 1998). This lack of definitive groups was attributed to the clonality, principally agamospermous nature of the genus and lack of reproductive isolation (Aldasoro *et al.* 1998). Continuous morphological variation is evident in the four major clusters elucidated by PCA and cluster analysis of *Marshellia* (Watson & Estes 1990). The four clusters in this traditional morphometric study correspond roughly to four described

species complexes within the genus. The authors determine that the pattern seen in the results of the analysis could be either due to recent isolation (and thus insufficient time for morphological variations to accumulate) or a more ancient divergence with little variation in morphology (Watson & Estes 1990). A study on the *Quercus* subgenus *Erythrobalanus* complex Jensen (1977), found the traditionally recognised series do not group together in the morphometric analysis. Groupings of 11 recognised core families of the Centrospermae were discovered in a morphometric analysis of the group, and the results of the study in conjunction with other supporting studies on genetics were used to circumscribe the group (Rodman *et al.* 1984). Jensen *et al.* (2002) utilise traditional morphometrics, geometric morphometrics and outline analysis to elucidate relationships between two species of *Acer* and their hybrid. The hybrid consistently appears between the two species for all analysis types (Jensen *et al.* 2002).

The use of an additional grouping technique namely, cluster analysis, also failed to circumscribe clear groups. Even when the analyses were conducted on both standardised and unstandardised data, groupings delimited were not those that were expected for either method. The discriminant function analysis (DFA) conducted on the main clusters of each of the phenograms did reveal which of the characters were responsible for the groupings visible in Figures 2.4, 2.5, 2.9 and 2.10. Habit is clearly the most important character for Figure 2.4 (standardised data, Analysis 1), whereas for Figure 2.9 (standardised data, Analysis 2) the most important character is clearly the functional hypanthium length. Theoretically, the defining character of both of these analyses should be the same. The fact that they are not indicates how delicate morphometrics studies are and cluster analysis as a method in particular. The lack of consistency also points to the lack of any clearly definable groupings and the characters chosen for this analysis.

The last analysis (Analysis 3), however describes very clear groups. The difference for this analysis is that fewer specimens were used (only 31 specimens), leaving the robustness of the results in doubt, and the addition of two characters denoting colour. The results of the PCA (Figures 2.11, 2.12 and 2.13), show clear groups defined by colour, namely the long-tubed and short tubed *Pelargonium reniforme* subsp. *velutinum* and *P.*

reniforme subsp. *reniforme*. The most important characters for the first axis are surprisingly a measure of leaf shape, the number of flowers and the inflorescence height. The second axis loadings for first and last branching lengths are the highest and for the third axis, the loading for petal width is the highest. Groups are clearly defined in this analysis. The addition of the two colour variables seems to have separated out groups that could not be defined by all other 19 measurements. Cluster analysis shows very similar groupings, with the short-tubed *P. reniforme* subsp. *velutinum* grouping with *P. reniforme* subsp. *reniforme*. This is clear in Figure 2.14 (standardised data). The DFA showed that the most important characters (root 1 of the canonical analysis) were colour and habit. Again, colour is exceptionally important for the grouping of these specimens.

Other morphometric studies have also discovered that colour is important. Hannan (1982) discovered a correlation of morphological characters with flower colour and geography in their morphometric study of *Platystemon californicus* although no distinct infraspecific taxa were identified in the analysis. Flower colour is certainly one of the most important traits for pollinators, and thus theoretically, one of the plant traits with the most selection pressure acting on it. It makes sense that such a trait would describe important differences in groups of plants within the same species that vegetative characters cannot describe.

Although there are no specific groups circumscribed by Analyses 1 and 2, the addition of a colour variable into the analysis was able to provide some groupings. The addition of colour allowed for groups, although not those described by Dreyer *et al.* (1995), to be elucidated by both the PCA and CA. The use of additional methods to morphometrics would prove invaluable to describing a species that is clonal and highly variable, making it difficult to categorise into groups. Morphometrics combined with other methods used to circumscribe taxa are useful in determining unequivocal taxonomic clarity. Wipff & Hatch (1994) used morphometrics in conjunction with reproductive and cytology studies to elucidate groupings within the grass genus *Digitaria*. Results of a PCA and cluster analysis of *Carex mitchelliana* correspond to allozyme data, chromosome counts and biogeography (Bruederle *et al.* 1989).

2.4.2. Statistics

Principal components analysis is one of the most widely accepted and utilised methods for use in traditional morphometric studies. PCA is straightforward both in its application and interpretation. Cluster analysis, on the other hand, is more controversial. Cluster analysis imposes hierarchical clusters onto the data, regardless of whether or not the data actually would exhibit this type of relationship. There is also a great deal of controversy when it comes to some of the more widely accepted statistical techniques and their application for cluster analysis. Two studies have done comparisons on different clustering algorithms and have found UPGMA to produce the highest cophenetic coefficients and thus to be the best algorithm to use for phenograms (Solvi *et al.* 2002, Cupido 2003). Not only are the specific algorithms important, so too is how the data is treated before it is used to construct the phenogram.

For the purposes of this study, the matrix calculated for the phenogram was a distance matrix. Euclidian distances were used. Traditionally, the skewing effects of differences in scale of measurements are eliminated by standardising the data before the phenogram is constructed. In the past, there has been much discussion on the necessity of standardising data for use in a phenetic analysis. It was for this reason that all of the phenograms for this study were computed for both standardised and unstandardised data.

The differences in phenograms for Analyses 1 and 2 cannot be clearly seen as the groupings for the specimens themselves are not clear. This is due, not to a problem with the method, but rather with the lack of clearly defined groups of morphologically different plants. The differences between the standardised and unstandardised data can be looked at for the first two analyses. Standardised data tended to result in more of the variables contributing to the grouping of the data (see Tables 2.6, 2.8, 12.2 and 2.14). Standardisation increases the effect most of the variables have on the analysis. Leaving the data unstandardised tends to reduce the number for characters contributing to the groupings as well as reducing the values of the first root for each of these characters.

It is most important though, to look at those phenograms that do describe groups so that the effect of standardised and unstandardised data can be compared sufficiently. The third analysis shows a definite difference in the phenograms for standardised and unstandardised data (Figure 2.14 and 2.15 respectively). Figure 2.14 (standardised data) shows three main clusters corresponding mainly to a-priori defined groups. Figure 2.15 (unstandardised data) shows the same a-priori groups distributed incongruently on branches of the phenogram. Although both discriminant function analyses show that the groups are 100% predicted by the developed model, there is a lack of congruence which can only be explained by standardisation or the lack of it. Table 2.18 (the roots for Figure 2.14), defines colour, habit, order of branching and flower height as the most important variables in the model. The root scores vary hugely between all of the variables in the model (Table 2.18), ranging from the highest: 3.23 for habit to the lowest: -7.47 for colour. The unstandardised results show much smaller variation: only two roots were extracted as opposed to the three for standardised data. Table 2.20 defines a much more limited range of coefficient scores than Table 2.18. Important characters include colour and habit. Similar characters are defined as important, but the question remains: why is there such a difference in the phenograms?

Firstly, the effect of standardisation on a data matrix should be analysed. Standardisation is most often employed to reduce the effect of scale on an analysis and reduces the effect of outliers. Along with most controversial statistical applications in morphometrics, reasons for the use of standardisation are more often than not, not given in the recent literature. Data is either standardised for use in cluster analysis (Abdel Khalik *et al.* 2002, Ackerfield & Wen 2002, Castro *et al.* 2005, Cupido 2003, Wipff & Hatch 1994, Solvi *et al* 2002) or not (Leht & Jaaska 2002). Data is however, more often than not, standardised for use in cluster analysis.

Standardisation effectively means that for each character, calculations are done so that there is a mean of 0 and a standard deviation of 1 (Sneath & Sokal 1973). Sneath and Sokal (1973) also define consequences of standardisation. These include the reduction of variability within characters so that they are all comparable, the correlation of the matrix

is reduced and the standard deviations of the correlation coefficients of standardised data tend to be larger and thus skewed. Cophenetic correlation coefficients are used to determine the goodness-of-fit of the cluster analysis. For all of the analyses (1 to 3) the r-value for the cophenetic correlation coefficients is consistently larger for unstandardised than for the standardised data, although not by much. Gilmartin (1974) suggests that by standardising data, one is removing valuable variation needed for the analysis to be accurate. The character composition of the matrix is also important for standardisation, binary or other simple coded variables do not need to be standardised as there is very little variation, with no differences in scale. For raw measurement data however, standardisation is statistically essential, especially when the matrix consists of both discrete and continuous data. Binary characters and measurements should be as comparable as possible for an analysis, and standardisation helps to achieve this.

Although better correlation coefficients are given for unstandardised data, the reason behind applying the standardisation must be examined. Perhaps for some studies making use of only coded characters standardisation is unnecessary, for continuous characters it becomes more important. When both discrete and continuous characters are used, it becomes necessary to standardise as the data are not directly comparable and the great deal of variation between characters causes problems with the analysis. In this study, it is clear that standardising the data produces a phenogram with more expected groupings than unstandardised data. The roots for the variables in the model for both standardised and unstandardised data are very similar although the same variables do not always occur in the model (Tables 2.18 and 2.20). With standardisation, the data are all comparable and produce the phenogram in Figure 2.14. Perhaps the only way to gain some clarity on which phenogram is the best indication of the natural groups is further study involving genetics. Certainly, there seems no clear reason for the differences in the phenograms when the DFA gives such similar results for the standardised and unstandardised data.

It would seem that the controversy surrounding the cluster analysis method is well-founded, especially as it creates such a difference in the results. Perhaps much can be said to consistency in the method. Most morphometrics studies standardise data as a matter of

protocol and it may give consistently better results that can be explained easily by the authors. Unstandardised data gives often confounding results and an explanation for such phenograms cannot be found. It seems that 'common sense' has an important role to play after all. As standardisation, by its very definition, makes data with different units comparable, I would recommend it for use on data consisting of continuous measured variables and data consisting of both coded characters and measured ones. If data consisting of only coded characters is used, then standardisation becomes unnecessary.

2.4.3. Conclusions

The existence of the four groups, or indeed the two subspecies defined by Dreyer *et al.* (1995) cannot be supported by the morphometric data used in this study. There is a concern about the importance of characters that cannot be measured from dry specimens, as was shown in analysis three, where the addition of floral traits circumscribed groups that were not resolved with the other analyses. Floral characters have the potential to be much more important than most of the vegetative characters often used in circumscribing taxa. In this case, suspected differences in colour allowed for the circumscription of groups. As floral characters are potentially much more important that vegetative characters in terms of directional selection of plants (usually by their pollinators), it may be that such characters should become more important in detecting and describing new taxa.

In the case of *Pelargonium reniforme*, there seems little basis to recognise the two existing subspecies in terms of morphometrics and the only remaining factor in favour of the current subspecific divide is the geographical isolation of *P. reniforme* subsp. *reniforme* from *P. reniforme* subsp. *velutinum*. If taxonomists were to consider a review of the species, it is suggested that the colour differences of populations of *P. reniforme* subsp. *velutinum* be taken into account. This has been done before, when *P. sidoides* was defined as an individual species and no longer as a subspecies of *P. reniforme*. It is

certainly clear that *P. sidoides* is indistinguishable from *P. reniforme* when only vegetative characters are taken into account.

It remains to be seen whether or not the differences in colour and thus potentially in pollinators in some *P. reniforme* plants is reflected in the genetics of the plant. The results from this morphometrics study certainly hint at the importance of floral characters in defining groups. These groups may even be so different that they have diverged genetically. This could be potentially attributed solely to pollinator mediated selection as many of the populations of the plant are sympatric and not isolated geographically or otherwise.

2.5. References

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Chapter 3: Gene flow and genetic similarity among populations of *Pelargonium reniforme* and *P. sidoides* as described by inter simple sequence repeat (ISSR) markers.

3.1. Introduction

3.1.1. Genetic description of *Pelargonium reniforme* and *P. sidoides*.

DNA data is increasingly being used in conjunction with other taxonomic methodologies to resolve relationships between organisms. The most commonly used genetic method used to resolve higher level taxonomy is DNA sequencing. Sequencing has proven to be very effective in aiding traditional taxonomy. However, sequencing may be insufficient for population-level studies within the same species. The regions usually sequenced in plants may show too little variation at such a low taxonomic level. Genetic fingerprinting however has been shown to be an effective means of determining species-level genetic relationships and inter simple sequence repeats (ISSRs) have become widely accepted as the best fingerprinting technique available (Bornet *et al.* 2002).

The lack of easily distinguishable morphological groups in *Pelargonium reniforme* populations (Chapter 2) highlights the need for a genetic analysis of the species. The use of traditional 'common sense' taxonomy has not been able to adequately resolve the species-limits. Even numerical taxonomic methods have failed to find clear boundaries between the putative subspecies *P. reniforme* subsp. *reniforme* and *P. reniforme* subsp. *velutinum* and between the species as a whole and the closely-related *P. sidoides*.

Current taxonomy of *Pelargonium reniforme* divides the species into two subspecies (Dreyer *et al.* 1995). *Pelargonium reniforme* subsp. *reniforme* is restricted to the coastal flats of Port Elizabeth and *P. reniforme* subsp. *velutinum* is distributed throughout the range of the plant (Dreyer *et al.* 1995). However, this conflicts with morphometric analyses (Chapter 2), which indicate that there may be two groups within *Pelargonium*

reniforme subsp. *velutinum* corresponding to floral characters. Certainly, it is clear that populations of *P. reniforme* subsp. *velutinum* consist of either one of two forms. Flowers are either dark pink and long-tubed or pale pink and short-tubed (Chapter1).

An objective elucidation of genetically defined groups would allow for a comparison of morphometric groups (Chapter 2) and the taxa defined by Dreyer *et al.* (1995). In addition to this, it would be able to determine whether or not populations of the species are genetically different and if the groups defined by the morphometric analysis (Chapter 2, Analysis 3) corresponding with flower type are defined by genetic data as well as morphology.

3.1.2. Inter simple sequence repeats (ISSRs)

SSRs (simple sequence repeat) markers are known to be powerful markers due to their co-dominance, high polymorphism and reproducibility but are difficult to utilise because of the time and cost to develop (Bornet *et al.* 2002). ISSR (inter simple sequence repeat) markers consist of PCR amplification of DNA sequences with inverted SSR units on each end. They are rapid and easy to develop and use. Primers consist of SSR units either with or without an anchoring end (Bornet *et al.* 2002).

Microsatellites or simple sequence repeats (SSRs) occur throughout the eukaryotic genome and are used extensively in plant breeding (Gupta and Varshney 2000). SSRs are a source of a great deal of genetic variation (Shlötterer 1998) and produce quantitative mutations that do not reduce fitness and may be a valuable source of molecular evolution information (Kashi *et al.* 1997). SSRs are used to assess genetic diversity in plants, Saghai Maroof *et al.* (1994) asses 207 barley accessions, Cipriani *et al.* (2002) use SSRs to identify olive (*Olea europaea*) cultivars. However, SSRs are difficult to use as specific sequences have to be known in order to choose the correct primers (e.g. Saghai Maroof *et al.* 1994). The utilisation of SSRs has been increased by making use of PCR based approaches. Among a great variety of these, which are a significant improvement on RAPD analysis, are ISSRs, which have a greater variability and reproducibility than

RAPDs. ISSRs are also known as anchored microsatellite-primed PCR (AMP-PCR), inter-microsatellite PCR (IM-PCR), inter SSR amplification (ISA) and anchored simple sequence repeats (ASSRs) (Gupta and Varshney 2000).

There are four main applications for ISSRs. These include agriculture, conservation, taxonomy and population biology. When first developed, ISSRs were used mainly to research genetic diversity within varieties of cultivated plants. The technique has been proven to be more effective than other fingerprinting techniques to determine within-species genetic diversity. For example, it was discovered that ISSR markers were the best markers for distinguishing between citrus cultivars (Fang and Roose 1997). ISSRs have also been used to identify cultivars of *Olea europaea* (Terzopoulos *et al.* 2005) and to identify potato cultivars (Prevost & Wilkinson 1999). Blair *et al.* (1999) discovered that ISSRs were the best fingerprinting technique to define 59 varieties of cultivated rice (*Oryza sativa* L.). Their study was extensive, and discovered that, through comparison of ISSR markers with RFLP (restriction fragment length polymorphism), isozyme markers and specifically AFLP (amplified fragment length polymorphism techniques) ISSR markers are the most polymorphic of all the marker types. They also concluded that ISSRs correlated with what is already known about the frequency of SSRs within the rice genome, validating the fingerprinting technique. Genetic variability within and between accessions of white lupin (*Lupinus albus*) was explored with the use of ISSR markers and a suggestion for screening for specific genotypes developed (Gilbert *et al.* 1999). ISSRs have been used to discern different variations of cotton (Liu & Wendel 2001). Joshi *et al.* (2000) used ISSRs to analyse genetic diversity in wild and cultivated *Oryza* and to infer phylogenetics. Kochieva *et al.* (2002) also used ISSR markers to analyse both cultivated and wild plants of the tomato genus *Lycopersicon* and the phylogeny generated by the ISSR markers was consistent with previous taxonomies of the genus.

Wolfe *et al.* (1998) were the first to use ISSRs to explore natural populations (population biology) in their study of *Penstemon* (Scrophulariaceae). The study was done in order to test for hybridisation and speciation within the group. Accessions were easily identified by 1 to 3 primers. The results show evidence of hybridisation as well as the importance of

pollinators for gene flow (Wolfe *et al.* 1998). Culley and Wolfe (2001) discovered a much higher genetic diversity within *Viola pubescens* than they expected and also the differentiation of the populations they studied. Similarly, a much greater amount of genetic variation than expected was discovered in the clonal *Calamagrostis porteri* ssp *insperata* using ISSR markers (Esselman *et al.* 1999). Camacho and Liston (2001) used ISSRs to investigate the genetic diversity of *Botrychium pumicola* (Ophioglossaceae) and the role of gemmae in a mostly clonal plant. They discovered that individuals tend to be clones and sexual reproduction is rare (Camacho & Liston 2001). Qian *et al.* (2001) used ISSRs to investigate populations of wild rice *Oryza granulate* and discovered a low genetic diversity in wild populations.

ISSRs are most often used to determine levels of genetic variation within groups of plants. An idea of the overall genetic diversity can give an indication of the need to conserve populations of a given species (Ge *et al.* 2005a). Low within population diversity in *Megacodon stylophorus* was discovered using ISSR markers and populations tagged for conservation due to this low diversity, probably affected by genetic drift of the small, isolated populations (Ge *et al.* 2005a). ISSRs were also utilized by Ge *et al.* (2005b) to determine the amount of genetic variation between two species of *Ammopiptanthus* with a view to conserving genetic diversity of the species. This study is important as this genus of desert plants are pinpointed as being essential for the prevention of further desertification in Northwest China. The study discovered that though there was a great deal of variation between the species, very little existed within species and within populations of those species (Ge *et al.* 2005b). Another study on a mangrove species *Aegiceras comiculatum* determined that there was very low genetic diversity both within the species in its entirety and between populations of the species, indicating that populations of the plant all descended from a founder population with very little polymorphism (Ge and Sun 1999). Genetic diversity in populations of *Oryza granulata* populations described by ISSR and RAPD markers reveal that there is little genetic diversity among populations and there is a need to conserve the existing populations (Wu *et al.* 2004). Both ISSR and AFLP markers were used to determine that conservation is necessary for the mainly agamic tree species *Zelkova abelicea* and *Z.*

sicula (Ulmaceae) (Fineschi *et al.* 2004). The genetic diversity within a clonal grass species *Psammochloa villosa* was found to be surprisingly large at the species level but relatively low at the population level, and ISSR markers provided more polymorphisms than other fingerprinting methods (Li & Ge 2001). There are many other studies that use ISSRs to determine whether or not conservation measures are necessary for certain species (e.g. Ge *et al.* 2003, Qiu *et al.* 2004, Chen *et al.* 2005).

ISSRs are often used to resolve taxonomy previously only described by morphological characters. Often these taxonomies are unresolved with the use of most genetic markers and even sequence data. A study on the genus *Vigna* was conducted using ISSR techniques (Ajibade *et al.* 2000), and discovered that ISSRs produced a great deal of polymorphism within the genus and the species defined by the method were the same as those defined by morphological data. The study utilised less powerful di-nucleotide primers and thus had few polymorphic bands, but sufficient to describe inter- and intra-species variation within the genus. The technique provided too much polymorphism to be used for subgeneric classification and it was suggested that ISSR markers be restricted to the delimitation of species and subspecies, rather than higher taxonomic levels such as genera. It was discovered that ISSR markers were more effective than RAPD markers in defining the species within the genus (Ajibade *et al.* 2000). Qian *et al.* (2001) stated that ISSR markers are superior to RAPD markers in terms of both reproducibility and polymorphisms.

ISSRs used in conjunction with morphological data have been used to prove the existence of hybrids in *Zaluzianskya* (Scrophulariaceae) despite fairly specialized pollination systems between day- and night-flowering species (Archibald *et al.* 2004). ISSRs have even been used to locate disease resistant gene clusters; Ratnaparkhe *et al.* (1998) used ISSR markers to locate disease resistant genes in chickpeas with some re-designing of the primers.

3.1.3. Methodological issues

Since their initial development ISSRs have been assumed to be powerful DNA markers and through their ease of use, been applied to various studies of plant relationships. In a study on different cauliflower lines, their efficacy was validated by sequencing of the ISSR fragments produced by the standard fingerprinting protocol (Bornet *et al.* 2002). The same study concluded that ISSRs occur in coding regions of the genome as several of the sequenced fragments corresponded to genes already identified in other plant species. Though ISSRs occur within SSR regions, they also contain SSR "hotspots" within their length. These repeated SSR units within the ISSR fragments were identified as an important source of variation within the ISSR sequences.

Although tiny differences in ISSR fragment length caused by only a few different base pairs may not be observable as a source of variation on the resolving media (both agarose and polyacrylamide gels), and may be a problem for coding the variation between individual plants, they form another source of variation within the ISSR fragments. Larger differences between ISSR fragments occur in the SSR units within the ISSR fragments, or simply through insertions, deletions or variations of non-repetitive elements within the DNA fragments. However, although small variation cannot be counted as a source of variation in gel-coding, most other variation between the ISSR fragment is sufficient to define them in the gel resolving medium (Bornet *et al.* 2002).

In a study conduced on several species of dicot plants, Bornet and Branchard (2001) studied the effects of changing parameters on the effectiveness and reproducibility of ISSR fingerprinting. They monitored the effects of changing DNA, primer, dNTP and DNA polymerase concentrations in addition to changing the number of PCR cycles, the primer annealing temperatures, the thermocyclers and the % agarose gel used for the electrophoresis. They showed that the banding pattern for all seven species of plants varied very little regardless of the parameters changed and the same polymorphic bands were observed for each of the plant species studied. In a study on cotton, Liu and Wendel

(2001) concluded that ISSR markers are an effective means of revealing variation and the banding patterns are highly variable. Liu and Wendel (2001) investigated a variation of ISSR techniques. Sequencing gels had better resolving power than agarose gels, especially when labelled with radioactive tags and double primers produced more bands (Liu & Wendel 2001). In comparing various resolving mediums and staining procedures for visualising the results of an ISSR-PCR, Wiesner and Wiesnerová (2003) concluded that polyacrylamide gels with SYBR Green I staining is the best for resolving PCR products for flax germplasm. Fang and Roose (1997) showed that ISSRs are highly reproducible in their study of citrus cultivars; they also proved that ISSR markers are by far the best to use to distinguish between cultivars often resulting from mutations rather than sexual reproduction.

One of the main factors affecting the banding pattern found when using ISSR primers is the primer annealing temperature; at very low temperatures non-specific amplification may increase, leading to the production of artefact bands (Bornet & Branchard, 2001). In addition to this, high concentrations of any of the PCR components result in smears which can be largely reduced by reducing the volume of PCR product loaded for gel electrophoresis (Bornet & Branchard, 2001).

Bornet *et al.* (2002) discovered that tri-nucleotide primers produce the most variable and polymorphic banding patterns in ISSRs and thus are the most powerful primers to use in ISSR studies on *Vigna*. In contrast, such primers were found to produce the lowest levels of polymorphism in a study on rice varieties (Blair *et al.* 1999); instead, dinucleotide primers were found to produce the most polymorphic bands. This pattern was correlated with what is already known about SSRs in the rice genome with dinucleotide repeats occurring most frequently of all possible repeats. Blair *et al.* (1999) also discovered incongruency related to 3'-and 5'-anchored primers that they attributed to loss of annealing sites at one end of ISSR fragments and slippage occurring during amplification for the 5'-anchored primers. The same study concluded, however, that ISSRs are a very powerful marker, and if the primer producing the most polymorphic bands is utilised, all of the 59 rice varieties can be distinguished using only one primer and one PCR.

Comparison of allozyme markers and ISSR markers used to determine genetic variation in the species *Viola pubescens*, revealed that both techniques produced a similar amount of variation within the species (Culley and Wolfe 2001). It was concluded that ISSRs would be best used when levels of allozyme variation are low. One such example is *Calamagrostis porteri* spp. *insperata*, which showed very little allozyme variation but much greater variation with both RAPD and ISSR markers in a study to determine the number of genets within populations of the clonal species; ISSRs produced the most variation (Esselman *et al.* 1999). Levels of allozyme variation is exceptionally low in the clonal grass species *Psammochloa villosa* and ISSRs produced much more genetic variation than the allozyme data (Li & Ge 2001). Li and Ge (2001) say 'the limited number of enzymes available and the small number of polymorphic loci detected make allozyme techniques inadequate for accurate identification of genotypes and population structures'. They determine that ISSRs are a much more efficient technique because of their basis in SSRs, which are extremely variable throughout the DNA and so, ISSRs form a powerful form of fingerprinting technique (Li and Ge 2001). Nagaoka and Ogihara (1997) compared ISSR markers to both AFLP and RAPD markers and discovered that ISSR markers produce more polymorphic bands than RAPD markers. The genetic relationships elucidated by the ISSR markers were similar to those produced by both AFLP and RAPD markers, indicating that ISSR markers are reliable to use for identification of wheat accessions (Nagaoka & Ogihara 1997).

In a study conducted by Bornet and Branchard (2001 pg 214) on seven dicot species concluded that "ISSR markers are universal, quick, easy to apply, highly reproducible, and polymorphous"; they go on to recommend ISSR markers for the "study of interspecific and intraspecific relationships and plant breeding". The method is widely accepted as being the easiest applicable and reproducible of all of the fingerprinting techniques. Although SSRs are more accurate and may provide better results, it is the ease of use of ISSR fingerprinting techniques that make the method useful. It also allows for the study of lower level taxonomic units such as species and subspecies and so is ideal for application in this case.

3.1.4. Aims and Objectives

This study had two main aims.

1. To determine if populations of *Pelargonium reniforme* are genetically distinct, or if there is any evidence of gene flow.

2. To assess the genetic validity and coherence of the two subspecies of *P. reniforme* and *P. sidoides* (i.e. do samples of each of these taxa form single lineages?). As it is a separate species, *P. sidoides* is expected to form its own cluster in this genetic analysis. In addition to this, the two subspecies described by Dreyer *et al.* (1995) are expected to form clusters consisting entirely of either *P. reniforme* subsp. *reniforme* or *P. reniforme* subsp. *velutinum*, giving genetic support to the subspecific division of the species. Alternatively, the two different floral forms of *P. reniforme* subsp. *velutinum* (long- and short-tubed flowers) may form their own clusters nested within the *P. reniforme* subsp. *velutinum* cluster, lending credence to the role of floral characters in reproductive isolation, even in sympatric multimorphic populations.

Populations sampled represented the subspecies defined by Dreyer *et al.* (1995), the two different flower types as described by the morphometric analysis (Chapter 2) and one population of the closely-related *P. sidoides* to determine whether or not this species was distinct from *P. reniforme* using ISSRs.

3.2. Materials and Methods

3.2.1. Sampling

Six populations of *Pelargonium reniforme* subsp. *velutinum* were sampled for this study (Table 1). One Grahamstown population (old clay pigeon shooting range) comprising both long and short-tubed forms was sampled (counted as two populations). In addition to this, two populations of each of the long-and short-tubed forms were sampled. Three populations of *P. reniforme* subsp. *reniforme* in and around Port Elizabeth were sampled. One population of *P. sidoides* was also sampled in order to determine the relationship of a different but closely-related species to *P. reniforme* (Figure 3.1).

15 to 20 samples were collected from each of these nine populations (Table 1). Plants spaced at least 5m apart were sampled (this was done because *Pelargonium reniforme* is clonal and sampling of genetically identical individuals was to be avoided). For each of the collected specimens for the long, short and additional subspecies populations, hypanthium length and flower colour was measured before the plants were pressed (Chapter 2). Leaves were then removed and stored in labelled envelopes in silica gel until extracted. Specimens were then pressed and lodged in the Selmar Schonland herbarium in Grahamstown.

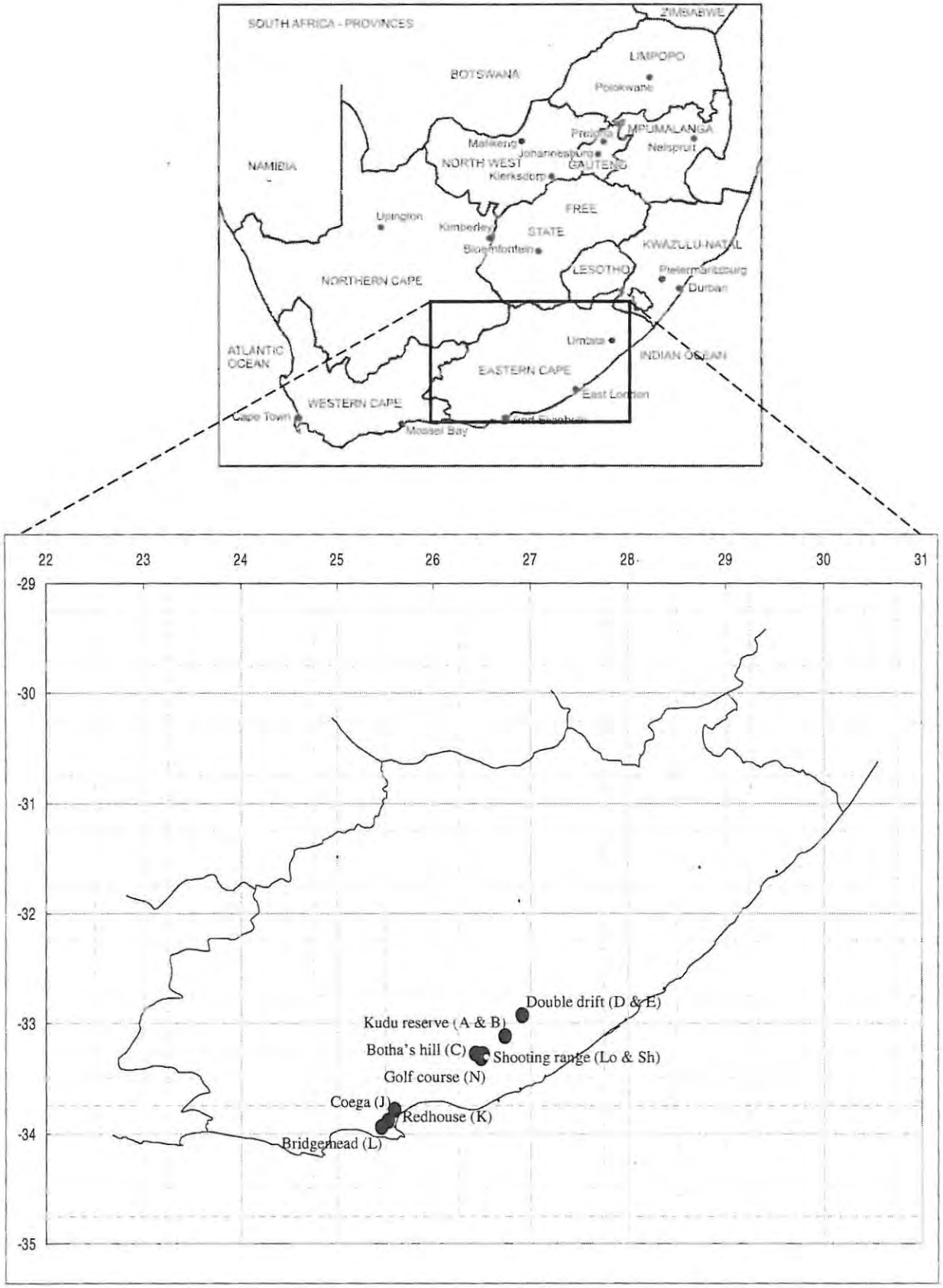

Figure 3.1: Map showing locations of all eight different populations used in the ISSR study. Population details are given in Table 3.1.

Table 3.1: List of populations of *Pelargonium* used in ISSR study

Population	Group (subspecies)	Code	Location	Samples used
Shooting range	*velutinum* long	Lo	33°16.527'S 26°29.610'E	118, 104, 103, 102, 101, 100, 95, 39, 31, 33
Shooting range	*velutinum* short	Sh	33°16.527'S 26°29.610'E	11, 10, 8, 7, 5, 76, 58, 78, 18, 59
Kudu reserve	*velutinum* long	A	33°07.554'S 26°43.124'E	11, 12, 13, 14, 15, 16, 17, 18, 19, 20
Kudu reserve	*P. sidoides*	B	33°07'554'S 26°43.124"E	6, 7, 8, 9, 10, 12, 13, 14, 15, 16
Botha's Hill	*velutinum* short	C	33°14'35"S 26°35'07"E	1, 2, 5, 11, 12, 13, 14, 17, 18, 20
Double Drift	*velutinum* long	D, E	32°59'56.7"S 26°51'29.9"E	D1, D2, D3, D4, D5, D6, D7, D8, E1, E2
Coega	*reniforme*	J	33°46'08.63"S 25°39'49.41"E	2, 3, 4, 5, 6, 7, 8, 9, 10, 11
Redhouse	*reniforme*	K	33°49'57.74"S 25°33'58.15"E	1, 2, 3, 4, 5, 6, 7, 8, 9, 10
Bridgemead	*reniforme*	L	33°55'25.22"S 25°28'20.68"E	1, 2, 3, 4, 5, 6, 7, 8, 9, 10
Golf course	*velutinum* short	N	33°17.548'S 26°30.201'E	1, 2, 3, 4, 5, 6, 7, 8, 9, 10

3.2.2. Total DNA extraction

Genomic DNA was extracted using the CTAB method (Doyle & Doyle 1987). DNA was extracted from a portion of each leaf by grinding up approximately 1g of each of the leaves with 1ml CTAB buffer, to which one drop of ß-Mercaptoethonol was added. All of the fluid was then decanted into a tube and incubated in a water bath at 60°C for 15 minutes. 500µl of CIA (chloroform: isoamyl alcohol, 24:1) was then added to each tube

and the tubes centrifuged at 13 000 rpm for one minute. 600µl of the clear aqueous phase was removed and transferred to a clean tube. To this, 400µl of ice cold isopropanol was added and the tubes left on ice overnight. The tubes were then centrifuged at 13 000rpm for 10 minutes. The liquid was then poured off, leaving the pellet of DNA behind. The pellet was then washed in 70% alcohol and left to air dry and then resuspended in 300µl of water. Once the DNA had dissolved, it was diluted 1:100 for use in the study.

ISSR-PCR amplifications were performed in a Corbett Research PC-960G Gradient Thermal Cycler. PCRs were run in 40µl volumes. The PCR included 35 cycles of 94°C for 45 seconds, optimum annealing temperature for 45 seconds, 72°C for three minutes followed by extension times of 72°C for 10 minutes and 25°C for one minute. Nine primers were initially screened to identify well amplified, polymorphic bands among populations. Out of the nine primers (all of which worked), five were identified as having the most polymorphic bands and were used for the study. Optimum conditions for each of the primers were different and these are listed in Table 3.2.

Table 3.2: Optimum conditions for each of the primers used in the ISSR analysis.

Primer name	Primer sequence (5'-3')	Annealing temperature (°C)	Magnesium concentration (mM) in 40µl PCR
Mao	5'-$(CTC)_4$-RC-3'	46	2.5
864	5'-$(ATG)_6$-3'	49	1.25
856	5'-$(AC)_8$-YA-3'	53	1.25
812	5'-$(GA)_8$-A-3'	48	1.25
841	5'-$(GA)_8$-YC-3'	54	1.25

Amplification products were electrophoresed on polyacrylamide gels at 100 V for 2 hours along with a DNA ladder (DNA Molecular Weight Marker III (0.12-21.2 kbp) Roche, Germany, 2006), stained with ethidium bromide and photographed with a UVtec UViprochemi camera on the ethidium bromide filter setting (UVTech 2006). For the polyacrylamide gels, 14ml mixture of polyacrylamide gel (sufficient for 2 gels) was used.

8ml per gel was loaded into the gel cassette (Biorad, 2006). In each mixture the following volume of reagents were used: 3.5ml acrylamide, 4.375ml 1.2M TRIS HCL, 6.125ml dH_2O, 9.8µl TEMED, and 140µl 10% ammonium persulphate.

3.2.3. Controls

In order to be sure that the data from each of the gels was accurate, several controls were executed. A PCR and gel were run without DNA to check for contamination. Several PCRs were repeated and the products run on gels to determine repeatability of the primers. In addition to this, individuals from one population were re-extracted and the PCR and gel re-run to check for possible mistakes in the extraction protocol.

3.2.4. Analysis of ISSR data

ISSRs are dominant markers. Amplified fragments were scored for presence (1) or absence (0) of homologous bands (Figure 3.2). This method of scoring gels is somewhat limited in its objectivity. Gels are scored manually. A method that was the most objective as possible had to be found. For this study, the gel images were stretched so that all of the ladders matched; the images were then printed out. The ladder was marked on a piece of clear plastic. Bands were then marked off on the same piece of plastic and presence or absence scored for each gel. This was repeated for each of the five primers for all of the populations.

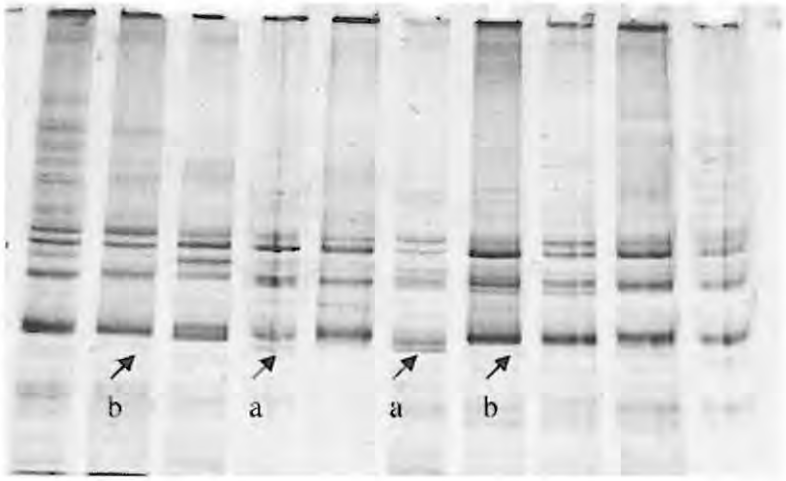

Figure 3.2: Example of a gel picture used for scoring and drawing up a presence/absence matrix (ladder not included in this picture). Gel images were inverted so bands appeared as black on a pale background. Arrows labelled 'a' indicate the presence of a band clearly absent in specimens labelled 'b' (see arrows).

The resultant presence/absence data matrix of the ISSR phenotypes was used to create a distance matrix using the Jaccard similarity coefficient in NTSYS-PC. The Jaccard similarity coefficient takes the shared presences and divides these by the total sample size with the number of shared absences subtracted (Rohlf 2002). The distance matrix was then used to construct an unweighted pair-group method with arithmetic means (UPGMA) phenogram using NTSYS-PC ver. 2 (Rholf 2002). Genetic diversity was measured by the percentage of polymorphic bands, which was calculated by dividing the number of polymorphic bands at population, species and subspecies level by the total number of bands surveyed. Although it is remarked by Esselman *et al.* (1999) and Wolfe *et al.* (1998) that only three primers are needed to sufficiently describe all variation, five primers were used in this study.

3.3. Results

3.3.1. ISSR polymorphism

Nine primers were evaluated for their ability to produce numerous, polymorphic bands. The five primers that produced the most variable bands were used for the study (Table 3.3). For the 100 samples, these five primers produced a total of 76 bands, all of which were polymorphic across all samples. The percentage of polymorphic bands (PPB) is 100% overall. Genetic diversity was not particularly variable across populations but was very high for each of the populations (Table 3.4). It ranged from the lowest in the Double Drift population (D) at 71.01% to the highest in the Kudu Reserve *Pelargonium sidoides* population (B) at 94.44%.

Controls showed repeatability of banding patterns for repeated PCRs (Figure 3.3), extractions (Figure 3.4) and no contamination in the blank control (Figure 3.5)

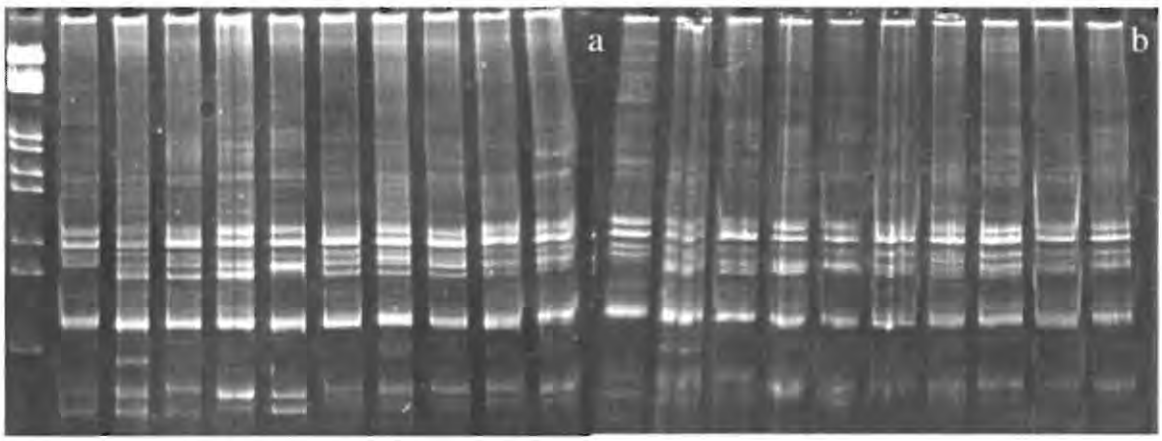

Figure 3.3: Repeat PCR control. Gel a and b were both run using different PCRs of the same DNA samples from population K run with the primer Mao.

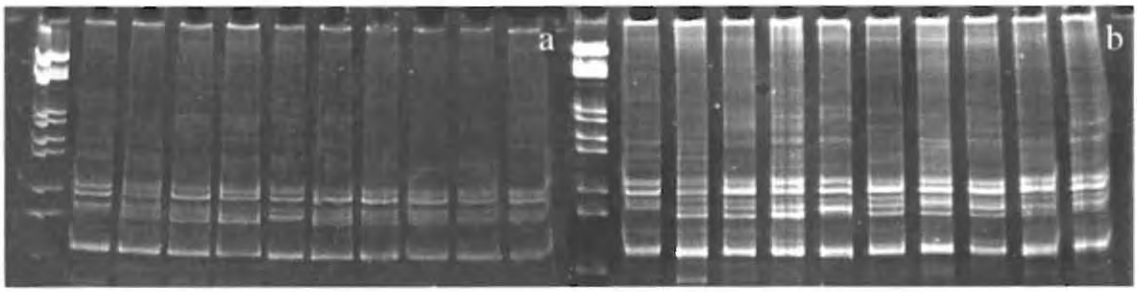

Figure 3.4: Repeat extraction control. Gel a and b are both run using different extractions of the same samples from population K run with the primer Mao.

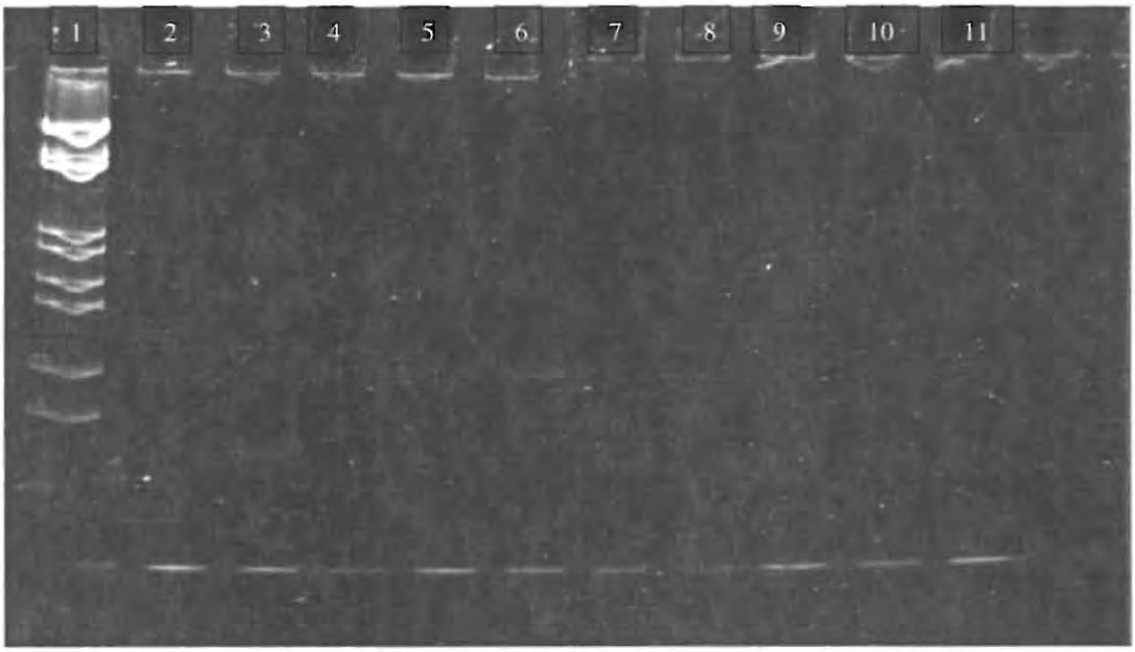

Figure 3.5: Blank control gel. Lane 1 is the ladder, lanes 2-11 contain PCR products run with no DNA

Table 3.3: 5 primers used for the ISSR analysis and the number of informative bands produced by each.

Primer name	No. of bands
Mao	23
864	9
856	17
812	14
841	13

Table 3.4: Percentage of polymorphic bands (PPB) for 9 *P. renforme* populations and 1 *P. sidoides* population.

Population	No. of bands	No. of polymorphic bands	PPB
Lo	63	50	79.37
Sh	70	59	84.29
A	67	60	89.55
B	72	68	94.44
C	71	57	80.28
D	69	49	71.01
J	67	58	86.57
K	69	54	78.26
L	70	58	82.86
N	60	50	83.33

3.3.2. Genetic structure of populations

A cluster analysis (Figure 3.6) with all of the 76 ISSR markers and all five primers indicated that there were no major groupings that correlated to expected divisions. Groups in the phenogram corresponded approximately with individual populations, each of which was separated geographically. Occasionally within each of the population groups is an individual from a different population can be found.

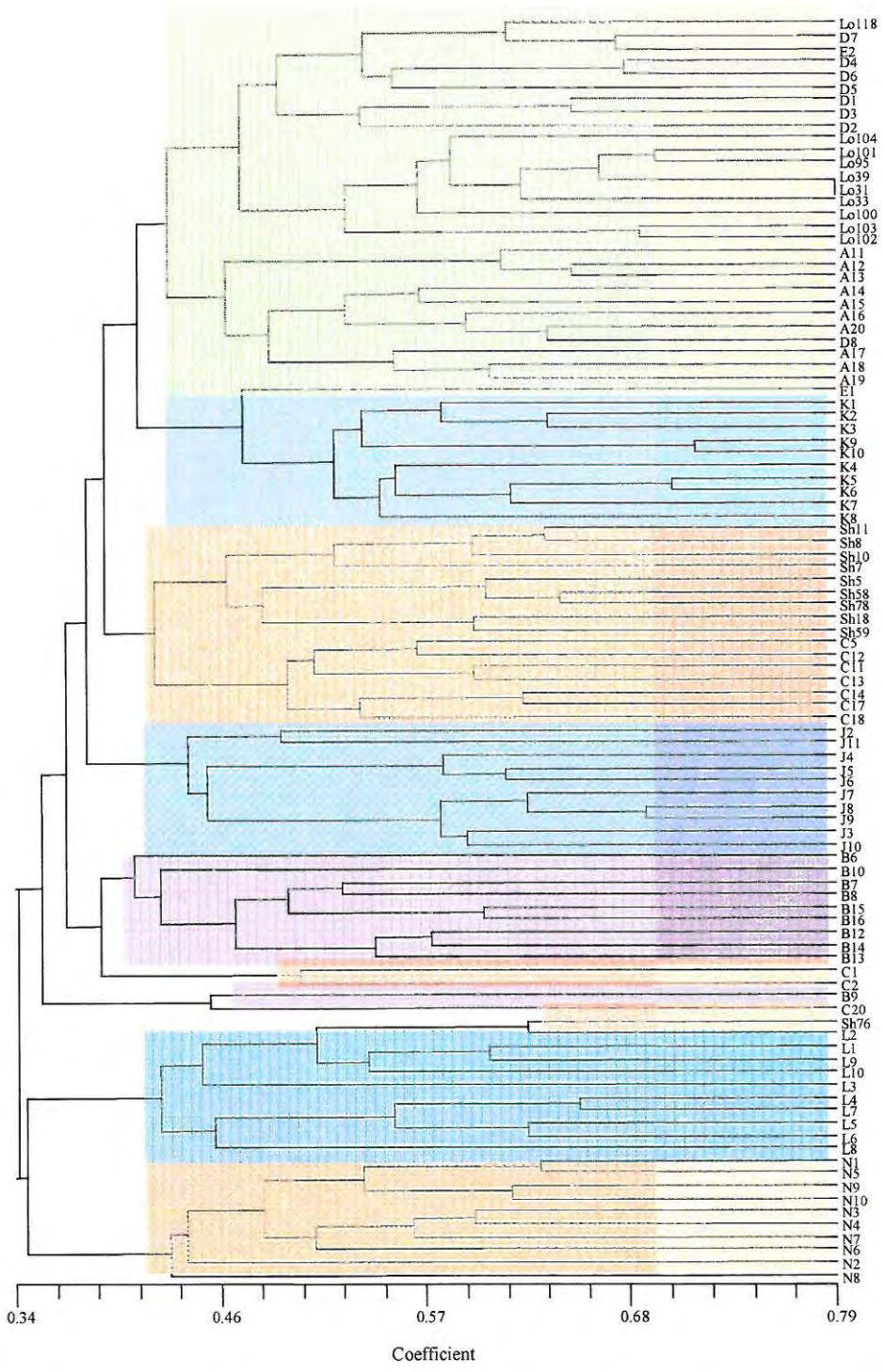

Figure 3.6: Phenogram illustrating genetic relationships among 100 individuals of nine populations of *Pelargonium reniforme* and one population of *P. sidoides*, generated by the UPGMA cluster analysis (NYSYS) with the Jaccard similarity coefficient calculated from 76 ISSR markers produced by five primers. Key to codes is given in Table 3.1. The key to colours is as follows: long-tubed *P. reniforme* subsp *velutinum* ☐, short-tubed *P. reniforme* subsp *velutinum* ☐, *P. reniforme* subsp *reniforme* ☐, *P. sidoides* ☐

3.4. Discussion

3.4.1. Genetic diversity and reproductive biology

The results of the ISSR analysis reveal that both within- and between-population diversity is surprisingly high for the species. The percentage of polymorphic bands is high for all populations and for the species as a whole (Table 3.4). As *Pelargonium reniforme* is a clonal plant, genetic diversity is expected to be low. Genetic diversity in clonal plants has shown to be much higher than anticipated in other species.

Esselman *et al.* (1999) found that sexual reproduction events in the past were likely to have contributed to the relatively high diversity shown by ISSR markers in the highly clonal grass *Calamagrostis porteri* ssp. *insperata*. A study of another clonal grass species *Psammochloa villosa*, revealed very little ISSR genetic diversity within populations but high genetic diversity between populations (Li & Ge 2001). Even for highly clonal species such as grasses, genetic diversity can be high, so it should be expected to be surprisingly high in moderately clonal species such as *Pelargonium reniforme*. High genetic diversity was also shown in the clonal fern *Pteridium aquilinum* (Parks & Werth 1993). Clonal plants can exhibit a range of clonal diversity from a very high level of clonal diversity (every individual has a different genotype) to a very low level of clonal diversity (every individual has the same genotype) (Ellstrand & Roose 1987). Ellstrand and Roose (1987) discovered that of 27 clonal plants surveyed, the vast majority were surprisingly genetically diverse. These authors indicated though, that increasing the number of characters in the study increased the amount of genetic diversity detected within clonal plant populations.

Genetic diversity is attributed to sexual reproduction events, whether they are rare or more frequent (Ellstrand & Roose 1987). It seems then, that clonality in a species of plant cannot be used to assume low genetic diversity. Certainly, *Pelargonium reniforme* shows an extremely high genetic diversity, more than would be expected for a clonal plant. Breeding system experiments in *Pelargonium reniforme* have indicated that reproduction

via seed may be quite low (Chapter 4) indicating that the slow-growing plants may rely heavily on clonal propagation (this may change with changing environmental conditions, but studies over more than one breeding system should be conducted). This kind of reproduction indicates that genetic diversity, especially within populations, may be quite low and between-population gene flow may be limited. Gene flow might occur regularly between populations however via sexual reproduction as the putative pollinators of the species (Nemestrinid flies and butterflies) are able to cover large distances.

As can be seen by Figure 3.4, populations tend to group together in the cluster analysis. A few of the groups include individuals from other populations but these are mostly specimens that would be expected to group together. For example, a long-tubed shooting range specimen grouped with a predominantly long-tubed double drift (D) population cluster at the very top of the phenogram. It was expected that *Pelargonium sidoides* would group separately to all other populations (all of which are *P. reniforme* populations). While the *P. sidoides* population (B) does occur as a cluster consisting almost entirely of *P. sidoides* individuals, it does not form a distinct cluster separate from all other populations. Instead, it is flanked by populations of *P. reniforme* subsp. *reniforme* and contains, within the *P. sidoides* cluster (B), some unexpected specimens. These out of place specimens included some from short-tubed *P. reniforme* subsp. *velutinum* populations Botha's Hill (C) and shooting range. Long-tubed *P. reniforme* subsp. *velutinum* populations form a clear cluster at the top of the phenogram the other expected groups are distributed unevenly throughout the phenogram. *P. reniforme* subsp. *reniforme* does not form its own cluster but instead is interspersed with short-tubed *P. reniforme* subsp. *velutinum* populations. Although it was expected that the subspecies would form their own clusters, as *P. reniforme* subsp. *reniforme* flowers more closely resemble those of short-tubed *P. reniforme* subsp. *velutinum* flowers, it makes sense that they would cluster together. The presence of *P. sidoides* in the middle of the same mixed cluster seems inexplicable. Despite the mixed positions of expected groups (apart from that of long-tubed *P. reniforme* subsp. *velutinum*) on the phenogram, the phenogram still shows that populations mainly form their own clusters. Interestingly, the short- and long-tubed *P. reniforme* subsp. *velutinum* from the same population (the shooting range, Sh

and Lo respectively), are completely separated in the phenogram. This indicates that there is very little or no gene transfer between the two floral types even though they occur sympatrically.

This arrangement of clusters indicates that though populations are more similar genetically than they are to the rest of the species, there probably is, or has been in the recent past, gene flow between populations. This is demonstrated by the similarity of populations, and the tendency of individuals to group with populations to which they do not belong. Certainly, the partitioning expected between species and subspecies is not evident, indicating that groups are far more similar to each other than current taxonomy indicates. Such similarity indicates gene flow between populations, reducing local adaptation and thus the development of separate taxonomic groups. Local adaptation may be limited if gene flow occurs between populations (Lenormand 2002). Adaptation is especially strongly counteracted in sparsely populated areas by gene flow from more dense populations. Gene flow is important for the between and among population genetic diversity (Lenormand 2002). Lack of gene flow between populations tends to result in rapid divergence of the isolated populations (García-Ramos & Kirkpatrick 1997). Even if gene flow was reduced between populations, allowing for potential local adaptation and thus genetic divergence, studies indicate that a decrease in genetic diversity and genetic drift in the population level may not occur (Young *et al.* 1996). Such genetic change within population would result in divergence and perhaps clearer taxonomic clusters. Even if the populations were isolated, however, it may be that genetic divergence has not occurred. In some instances, genetic diversity has increased in fragmented populations (Young *et al.* 1996). Gene flow from central populations may inhibit peripheral populations (such as the geographically isolated *P. reniforme* subsp. *reniforme*) from evolving (García-Ramos & Kirkpatrick 1997). It seems then, that if the populations described by the phenogram in this study are geographically isolated and that gene flow no longer occurs between populations, that they have only relatively recently been isolated. They have had insufficient time to diverge sufficiently for it to show in an ISSR analysis. However, the lack of clear divergence may be due to gene flow still occurring

between populations, but obviously to a lesser extent than that occurring within populations of the species.

Pollinators may be largely responsible for regulating gene flow between populations. Although fairly specialised pollination systems are suspected in this taxonomic group, it seems that either these are insufficient to isolate the populations, or that they have recently developed and the populations have had insufficient time to diverge. It does seem somewhat strange, however, that populations of *Pelargonium reniforme* subsp. *velutinum* consist of either long-, or short-tubed forms and rarely both. One population comprising both types of flowers (Grahamstown clay pigeon shooting range) was included in the analysis and the long- and short-tubed plants are genetically distinct from one another, forming groups comparable to those formed by geographically separated populations (Figure 3.4). Although short- and long-tubed populations surprisingly, do not group together as well-defined clades, it is clear that gene flow via pollinators between the two floral forms is likely to be rare. Gene flow via seed dispersal certainly seems not to occur as individuals within the populations are usually restricted to one flower form. A question arises then as to the origin of these two floral forms and the selection pressures that may have led them to develop.

Pollinators can be an important source of gene flow between populations; Wolfe *et al.* (1998) in their ISSR study, discovered that hummingbirds were the likely source of gene flow between populations of *Penstemon* (Scrophulariaceae). Hybridization and gene flow was investigated with the use of ISSRs between two species of *Zaluzianskya*, revealing that hybridization had occurred, indicating the importance of pollinators for gene flow (Archibald *et al.* 2004). Floral characters are essential for selection in plants. Pollinators play a huge role in gene flow and floral characters are responsible for the attraction of potential pollinators. It would follow then, that there is a high degree of heritability of floral characters in response to pollinator choice. This has been shown to be true in controlled experiments with *Saxifraga granulata* (Andersson 1996).

3.4.2. Taxonomic implications

Dreyer *et al.* (1995) defined two subspecies of *Pelargonium reniforme*: *P. reniforme* subsp. *velutinum* occurring throughout the range of the species and *P. reniforme* subsp. *reniforme*, restricted to the costal flats of Port Elizabeth. The division was made as *P. reniforme* subsp. *reniforme* is geographically isolated and thus most probably genetically isolated. *P. reniforme* subsp. *reniforme* does not however, group separately from the other subspecies, but is rather nested within (Figure 3.4). The same can be said for the closely-related species *P. sidoides*. It seems that all of these species are very closely related and are either recently diverged or gene flow still occurs between populations.

These results reflect those from the morphometrics analysis (Chapter 2) where there was no basis for the subspecies defined by Dreyer *et al.* (1995) and some basis for the classification of *P. reniforme* subsp. *velutinum* into long- and short-tubed flower forms. Here, specimens form population clusters. Long-tubed populations have all grouped together whereas short-tubed populations are distributed throughout the phenogram. Dreyer *et al.* (1995)'s subspecies *P. reniforme* subsp. *reniforme* are scattered between short-tubed populations of *P. reniforme* subsp. *velutinum* and *P. sidoides* population. Of course, in taxonomy, genetics are not the only tool to be used for the delimitation of taxa. Evidence from morphometrics, genetics and 'common sense' taxonomy can be used to define the group. Also, it is important to remember that selection is dynamic, thus making it difficult to classify rapidly radiating groups or those that have recently diverged.

There is thus morphological and genetic evidence to suggest that the subspecies defined by Dreyer *et al.* (1995) should not be retained. *Pelargonium sidoides* differs from *P. reniforme* only in flower colour. It was originally part of the same species and has only fairly recently been assigned to its own species. This study indicates that it may not be as different as was suspected.

3.4.3. Conclusions

It is clear that though the results of this analysis effectively separate the populations, it is relatively congruent with the results of the morphometric analysis (chapter 2). The morphometric analysis suggest no basis for the existence of the subspecies defined by Dreyer *et al.* (1995), the ISSR analysis also indicates that there is no reason to separate *Pelargonium reniforme* subsp. *reniforme* from the rest of the species. Similarly to the results of the morphometrics study, there seems to be a clear division of *P. reniforme* subsp. *velutinum* into long- and short-tubed forms. Certainly, long-tubed populations of the subspecies do group together at the top of the ISSR phenogram (Figure 3.6).

Pollinators are thought to play a role in both gene flow and isolation of the species. Certainly, the clear differences in flower form in populations of *Pelargonium reniforme* subsp. *velutinum* indicate a role of some sort of directional selection on floral characters. This can only be attributed to pollinators. The paradox lies in that there is no clear division of long- and short-tubed populations. Although long-tubed populations groups together, the short-tubed populations do not form their own cluster, nor do they exhibit geographical isolation. The question of the origin of these populations of clearly different flower forms with potentially different pollinators remains unanswered.

What is important, however, is the existence of two genetically distinct entities in one population. The Grahamstown population, consisting of both short- and long-tubed forms of *Pelargonium reniforme* subsp. *velutinum* was divided into these groups artificially. The two groups however, despite being from the same geographical location, and thus the most likely to be experiencing gene flow, behave exactly like they are isolated populations similar to the others. This clear genetic division between the two floral forms in the same geographical location leaves a number of questions that may be answered with closer investigation into the pollination biology of the population.

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Process

Chapter 4: Pollination biology: evidence of different pollinators for long- and short-hypanthia forms of *Pelargonium reniforme.*

4.1. Introduction

4.1.1. Pollination biology of *Pelargonium*

Pollination biology has likely played a crucial role in the diversification of *Pelargonium* and may account for the variety of flower forms in the genus (Struck & van der Walt 1996). All *Pelargonium* plants have zygomorphic flowers and the nectar tube is present in a modified receptacle. Section *Reniformia* (to which *Pelargonium reniforme* belongs) is noted as having long tubes. However, tube length seems to be highly variable in most species. Many *Pelargonium* species have long-tongued fly pollinators, forming part of a syndrome known as rhinomyophily (Struck & van der Walt 1996).

Few studies have been conducted on the pollination biology of *Pelargonium* species. Pollination of *Pelargonium* species by long-tongued flies has been noted in a series of studies conducted on the long-tongued fly pollination guild in the Cape, focusing on Iridaceae (Goldblatt *et al.* 1995, Manning & Goldblatt 1996, Goldblatt and Manning 1999). McDonald and van der Walt (1992) conducted a study on the pollination of *Pelargonium tricolour* but revealed little applicable to the genus as a whole. Another study suggests generalist honey bees (*Apis mellifera*) pollinate *Pelargonium dolomiticum* revealing very little about pollination of the genus either (Zietsman 1993).

No information on the pollination biology of *Pelargonium reniforme* exists. Floral form and initial observations suggest that it is pollinated by long-tongued flies and forms part of the rhinomyophily syndrome. The protandrous flowers of *Pelargonium reniforme* are pink, with a long floral tube called a hypanthium. Nectar is produced in a nectary located at the base of the hypanthium.

4.1.2. Long-tongued fly pollination guild

A number of studies have documented the long-tongued fly pollination guild (Goldblatt *et al.* 1995, Manning & Goldblatt 1996, Manning & Goldblatt 1997, Goldblatt & Manning 1999). In nearly every one of these studies the importance of *Pelargonium* species in the guild has been documented. The studies were broad as they encompassed a wide range of Iridaceae species and much of the extrapolation to Geraniaceae species was speculative. However, it seems clear that there is a guild of long-tongued fly pollinated plants in the Western Cape and all share very similar features. In the first study by Goldblatt *et al.* (1995), two guilds are identified. In these guilds, all of the flowers (both Iridaceae and Geraniaceae) are purple to red, with pale nectar guides and long perianth tubes. The authors outline the importance of pollen placement on pollinators' bodies within the guilds to prevent pollen wastage and stigma clogging. In these guilds, Iridaceae tended to deposit pollen on the top of the fly head and body whilst Geraniaceae pollen was deposited on the underside of the head and thorax of the flies, corresponding to the position of reproductive organs of the flowers in these groups. Nectar tubes of the flowers have evolved to fit the length of the tongues of the pollinators and vice versa, a rare case of a coevolutionary arms race. Floral traits such as tube length can be extremely variable and are easily manipulated by natural selection processes (Goldblatt *et al.* 1995).

Manning and Goldblatt (1996) reiterate many of these claims in another study discussing the 28 plant species pollinated by the Nemestrinid fly *Prosoeca peringueyi*. This species of fly has one of the longest recorded proboscis lengths for a fly. In this case, floral morphology is similar across families involved in the guild (again, Geraniaceae and Iridaceae are the most important). Pollen placement is, once again essential in preventing hybridisation between closely related species. The flowers examined in the study all exhibited specific characters constituting a specific pollination syndrome. They are characterised by a straight or slightly curved and extremely narrow floral tube, nectar is produced at the base of the tube, making it accessible only to long-tongued insects. Geraniaceae flowers have anthers that emerge from the top of the tube where they can be

brushed against visiting insects. Flowers tend to be unscented and are protandrous. Important in their discussion about the evolutionary development of the guild, they mention the importance of long-tongued flies visiting short-tubed flowers. Although they provide no experimental evidence of this, they suggest that it may be too wasteful for long-tongued flies to visit short-tubed flowers for the comparatively energy-poor nectar and thus they would not visit short-tubed flowers. This pollinator behaviour cements already developing reproductive isolation (Manning & Goldblatt 1996).

An additional study on the general long-tongued fly pollination guild within the Iridaceae was conducted by Goldblatt & Manning (1999). In this study, they comment again on the importance of the Geraniaceae within the guild. The Geraniaceae have the highest incidence of long-tongued fly pollinated flowers (25%). Long-tongued fly pollination has evolved repeatedly within the Geraniaceae from the ancestral bee-pollinated condition. The long-tongued fly pollination guild involves pollination by Bombyliidae, Nemestrinidae and Tabanidae, three families of long-tongued flies. Bees are noted visiting the flowers within this guild but do not in fact pollinate them (Goldblatt & Manning 1999).

Not only does long-tongued fly pollination occur in the Geraniaceae and Iridaceae, but also within the Orchidaceae (Johnson & Steiner 1997). Johnson and Steiner (1997) describe the importance of long-tongued fly pollinators in the evolutionary development of spur length in the *Disa draconi*s complex (Orchidacea). Here, allopatric reproductive isolation and hence, evolution has occurred. Spur length within the complex is described as being highly variable (Johnson & Steiner 1997), a trait shared by the hypanthium length of *Pelargonium reniforme*. Other studies also document pollination by long-tongued flies (e.g. Johnson 2000, Johnson *et al.* 2002, Johnson *et al.* 2003, Anderson *et al.* 2005, Johnson 2006 and Johnson & Morita 2006).

4.1.3. Pollinator-mediated selection of floral characters

Pollinator-mediated selection has been documented in numerous species which show adaptation to pollinators. Pollinator behaviour may even result in sympatric speciation (Maad & Nilsson 2004). Allopatric speciation in populations of the same species can occur as a result of pollinator choice. One such example is that of the development of different spur lengths in different populations of *Platanthera ciliaris* (Robertson & Wyatt 1990). Different populations developed spur lengths corresponding to those of the species of moth pollinators within each population. The populations thus become reproductively isolated except within the hybridisation zone, and speciation may occur within the geographically separated populations (Robertson & Wyatt 1990). A selection gradient is proposed (Blionis & Vokou 2002) in a study on *Campanula spatula* in Greece. Here, an ecological gradient is used to explain a different suite of pollinators with a rise in elevation and thus, the change in floral form in response to the actions and choice of the different pollinator suites.

A study on the sympatric speciation of two *Disa* species suggests that one instance of gene recombination may occur that shifts pollinators, especially in their example of sexual mimicry (Steiner *et al.* 1994). Most of the studies are explained using the 'most effective pollinator' principle (Stebbins 1970), where plants adapt to the traits favoured by the most effective pollinator. It is expected that flowers exhibit those morphological traits favoured by the pollinators that transfer the most pollen (Mayfield *et al.* 2001). The most effective pollinator principle, however, may not apply to all natural plant populations (Mayfield *et al.* 2001). Mayfield *et al.* (2001) found that comparatively rare flower visitors contributed the most to seed production in *Ipomopsis aggregata*. The authors cite two reasons for this contradiction of the most effective pollinator principle: either evolution for a very specific pollinator relationship is favoured but constraints prevent it from developing or, generalization is favoured by natural selection but plants can retain some feature of a specialist relationship (Mayfield *et al.* 2001). Maad and Nilsson (2004) described the most effective pollinator principle in explaining the movement from tongue to eye attachment in a moth pollinated *Platanthera* species. Most

effective pollinator is also used to explain the shift in *Penstemon* from bumblebee to bird pollinated flowers (Castellanos *et al.* 2003). In this example, the divergence of pollinator modes is explained by a trade-off between the pollinator characteristics of birds and bees and the change between them is explained by birds becoming more effective pollinators. Birds allow for the deposition of less pollen (as they do not clean it off, as do bees), and as a result, birds have a higher pollen transfer efficiency, increasing the number of effectively pollinated flowers (Castellanos *et al.* 2003).

Isolating mechanisms involving pollinators may be common. Often, pollinator constancy is one of the most important isolating mechanisms – insects are likely to stick to a floral from that they recognise and do not visit other species; such a type of isolation is termed ethological isolation (Grant 1949). Insects tend to visit flowers where they are assured of receiving nectar, but may switch flowers if the nectar source becomes unavailable or poor (Goulson 1999). Pollinator constancy has been shown to have little or no involvement in speciations or maintenance of separate species (Chittka *et al.* 1999). Various morphological contrivances are necessary to exclude unwanted visitors if flowers are mechanically isolated (as in long- and short-tubed forms of the same species; Grant 1949). More specialized pollination systems, including that of long-tongued flies, have much higher levels of floral morphology diversity than general syndromes (Grant 1949).

Directional selection (for example, for floral tube length) cannot occur infinitely; there are constraints that form the cut-off for evolutionary change. These are usually ecological constraints that involve resource limitation. For example, the number of flowers on an inflorescence may be increased if the individual flower size is decreased, and if the flower size increased, the number of flowers per inflorescence decreased (Schemske 1980). Schemske (1980) develops this idea in his paper about the floral evolution of *Brassavola nodosa* where a counter-selective force was responsible for the rarity of many-flowered inflorescences within populations of the orchid. There is selection pressure in plants for inflorescence size, or the number of flowers on a plant. In a study on *Brassavola nodosa*, Schemske (1980) describes how the larger the floral display, the higher the reproductive success individuals. However, despite the increased reproductive

success of larger floral displays, the plants consistently produce small floral displays indicating a selective trade-off that Schemske (1980) attributes to low survivorship of the plants.

4.1.3.1. Tube length

Floral traits important to pollinators, and thus likely to undergo high levels of natural selection include nectar, flower tube length and flower colour (Schemske & Bierzychudek 2001). Examples of divergence due to flower tube length include those described above (Robertson & Wyatt 1990, Johnson & Steiner 1997). Tube length is often noted as one of the most important characters upon which natural selection acts (Benitez-Vieyra *et al.* 2006, Johnson 2006).

Nilsson (1998) describes evolution of nectar tube length in most examples as one-sided. Flowers develop long tubes in response to pollinators but pollinators do not develop long tongues in response to flowers. This occurs as nectar is produced at the base of nectar tubes and the stigma or anther is situated at the top. Insects visit flowers for the nectar reward and so must have tongues long enough to reach the nectar at the base of the tube. In order to pollinate the flowers, however, the tongue must be short enough for the head of the insect to brush against the stigma or anthers whilst feeding. Flowers develop longer tubes in response to the long-tongues of the pollinators (Nilsson 1998). Jermy (1999, pg 136) states: 'deep flowers are for long tongues, otherwise such flowers could not exist. Long tongues, however, are not for deep flowers, as feeding with long tongues is possible also on shallow flowers.' Although this statement may be true, in cases where pollination systems are extremely specialised, evolution of tube length may not be entirely one-sided.

The evolution of long tubes is most often one-sided but, in exceptional cases, may in fact involve an evolutionary arms race. This occurs when the pollinator's tongue grows in length in response to the long floral tubes and the floral tubes become longer in response to the pollinator's tongue. Tube length can drastically affect particular plant species

resulting in such incredible cases of extremely long tongues and flower tubes such as that of the Malagasy orchid *Angraecum sesquipedale* and its hawkmoth pollinator *Xanthopan morganii praedica* with a tongue length of up to 23 centimetres (Nilsson 1998). Although long-tongued pollinators are able to reach nectar in short-tubed flowers, nectar in long-tubed flowers provides a resource that is not available to other insects that cannot reach it and so, it is more likely that evolutionary arms races occur for long-tubed flowers and their long-tongued pollinators (Nilsson 1988). Regardless of the reasons for the initial development of long-tongued insects, it is clear that coevolution occurs between tongues and flower tube lengths in natural plant populations (Nilsson 1988).

4.1.3.2. Colour

Advances in describing colour have enabled observers of pollination systems to avoid the reliance on human vision to distinguish colour (Endler 1990). It is possible to determine the visual spectra of pollinators (Endler 1990). Much research has been done into the colour vision of bees (Chittka 1996, Lunau *et al.* 1996, Kevan *et al.* 2001, Chittka & Tautz 2003, Dyer & Chittka 2004, Chittka & Raine 2006) but very little on the visual spectra of other insect groups. Although the ancestral insect visual spectrum is UV-blue-green-trichromacy, variation from this ancestral state can be extremely variable (Briscoe & Chittka 2001). However, the visual spectra of insects resulting from wavelength tuning of the photoreceptors, predates the evolution of flower colour (Chittka 1996). The visual spectra of the insects observed in this study are unknown. Thus any analyses of the colour data of the flowers can only be interpreted in general terms (general insect vision) rather than specific spectra (for example, of bees). Diagrams representing insect visual spectra have been developed (Endler 1990, Chittka 1992, Chittka *et al.* 1992), such as the hexagon diagram of Chittka (1992) based on the visual spectrum of the honey bee.

Colour is an important floral character for pollinators. Flower colour polymorphism can occur. *Linanthus parryae* has both white and blue flowers, thought to be a product of either genetic drift or natural selection (Schemske & Bierzychudek 2001). A long-term

study of the fitness of each of the colours showed that selection of flower colour varies over time and space in direction and magnitude. The authors conclude that flower colour polymorphism was not a result of isolation by distance, but could not determine the mechanism of selection (Schemske & Bierzychudek 2001). Melendez-Ackerman *et al.* (1997) showed that natural selection could possibly act separately on floral characters such as nectar, colour and shape. Their study showed that though hummingbirds usually chose to visit red flowers, this could be changed by increasing the reward in white flowers (Melendez-Ackerman *et al.* 1997). Colour may provide pollinators with a promise of a reward consistent with that colour and so is important for pollinator choice (Melendez-Ackerman *et al.* 1997). Bees visiting snapdragons (*Antirrhinum*) were shown to be able to influence the evolution of flower colour (a single-locus trait in this case) (Jones & Reithal 2001). Changes in rewards available in different colour flowers resulted in changes in pollinator behaviour; pollinators learn which colours denote the most rewarding flowers and consistently visit these flowers (Jones & Reithal 2001). Melendez-Ackerman and Campbell (1998) discovered that flower colour of *Ipomopsis* can affect fitness independently of any other floral traits and that traits such as nectar can increase fitness over and above the fitness advantage of certain colours.

4.1.3.3. Nectar

Nectar is a reward for flower visitors and is thus important in pollination biology. It is logical to assume that the greater the volumes and concentration of nectar available to flower visitors, the better the pollination. This is not necessarily true, Salguera-Faria and Ackerman (1999) discovered that there is no difference in pollination between flowers with large rewards and those with very little, indicating that volume of nectar may not be as important as it seems. Nectar concentration is also important, flowers of *Asclepias exaltata* with the most concentrated nectar produced the most seeds as the pollinating bees preferred concentrated nectar (Wyatt & Shannon 1986).

4.1.4. Aims and objectives

Two different floral forms of *Pelargonium reniforme* subsp. *velutinum* with floral characters (hypanthium length and colour) showing obvious variation may be maintained, if not driven, by pollinators. Results of an ISSR study on the *P. reniforme* group indicated that within one population (Grahamstown), consisting of both short- and long-tubed floral forms of *Pelargonium reniforme* subsp. *velutinum*, genetic isolation is occurring between groups. As variation occurs only in floral characters in the group, it is thought that pollinators play a major role in the apparent genetic isolation of the two floral forms.

The aims of this chapter were to further describe differences between the visually-defined long- and short-tubed groups of *Pelargonium reniforme* subsp. *velutinum*. This involved the analysis of three important floral traits: nectar, hypanthium length and colour. It was expected that each of these traits would show differences between the two floral forms. Colour and hypanthium lengths clearly are different between the two groups, but these had to be quantified. Nectar volume and sucrose concentration are important for pollinators and it was expected that these too, would be different for long- and short-tubed flowers if they are pollinated by different groups of insects that have different nectar requirements. In addition to determining the differences in these floral traits between long- and short-tubed flowers, aims also included the comparison of the colours of *P. sidoides* as this is the primary trait used in describing it as a different species to *P. reniforme* (Chapter 2). As the flowers of *P. reniforme* subsp. *reniforme* flowers resemble those of short-tubed *P. reniforme* subsp. *velutinum*, the colours of *P. reniforme* subsp. *reniforme* were also measured.

By examining the breeding system of the plants, I aimed to determine the degree to which the two different floral forms are compatible. This provided an indication of the level of genetic isolation between the long- and short-tubed flowers of *Pelargonium reniforme* subsp. *velutinum*. In addition, hybridisation experiments were done to determine the level of compatibility between *Pelargonium reniforme* and *Pelargonium sidoides* as the only defining character of the second species is flower colour.

4.2. Materials and Methods

4.2.1. The study area

The study area is the Grahamstown old clay pigeon shooting range (33°16.527'S, 26°29.610'E), where both long- and short-tubed floral forms of *Pelargonium reniforme* subsp. *velutinum* occur sympatrically. Clumps of the plant are restricted to one flower form, so observation of pollinators could only be done at either long- or short-tubed clumps rather than for both forms simultaneously. Despite an average of three hours a day three times a week spent in the field observing pollinators over one flowering season (2005-2006), few visiting insects were observed. Data are thus restricted to what little information was available from field observations.

4.2.2. Tube length and distribution

Hypanthium lengths were measured from 122 randomly selected flowering individuals in the Grahamstown study site. In order to provide a comparison, hypanthium lengths of 93 herbarium specimens were also measured in order to get an idea of the overall range and distribution of hypanthium lengths over the distribution of the species. Also for comparative purposes, 146 measurements were taken from an entirely long-tubed population (Kudu Reserve: 33°07.554'S 26°43.124'E).

To determine any geographical pattern to the distribution of populations consisting of long- and short-tubed individuals, hypanthium length of herbarium specimens was examined.

4.2.3. Colour

As long- and short-tubed flowers of *Pelargonium reniforme* subsp. *velutinum* are distinguishable by colour, an important character for pollinator choice, colours of the two different forms were measured. In addition to the floral forms of *P. reniforme* subsp. *velutinum*, individuals belonging to *P. reniforme* subsp. *reniforme* were also measured. This was done as the colour closely resembles that of the short-tubed *P. reniforme* subsp. *velutinum* flowers. As *P. sidoides* is defined as its own species mainly because of the huge difference in flower colour, individuals of *P. sidoides* were also measured.

Colour was measured with an Ocean Optics USB2000 spectrophotometer linked to a PC. An Ocean Optics Mini–D2T (Tungsten-Deuterium-Halogen) light source was used to illuminate the sample. The reflection probe (UV/VIS 400 micron) was orientated at 45° to the surface of the floral part being measured. Colours were summarised using the Endler segment classification method (Endler 1990). The method of Endler (1990) was used as it describes a general model for showing colour spectra. The commonly used alternative, the Chittka (1992) method is based on the visual spectrum of hymenoptera. As this pollination system is not one restricted to hymenoptera pollinators only, the Endler method was the best means of showing the spectra. The Endler (1990) segment classification takes the integral of light reflected from floral part and the light incident on the sample (the D65 norm-function in this case) for each of four equal segments between 300 and 700 nm. These values are divided by the integral for the entire spectrum of interest (300 – 700 nm) to separate colour from brightness and subtracted from one another to determine values for colour "opponents". The value for the medium-short wavelength segment is subtracted from the long wavelength to give a long-medium (LM) opponent and the short wavelength segment is subtracted from medium long segment to determine the medium short (MS) opponent.

4.2.4. Nectar

The nectar volume and concentrations for both long- and short-tubed flowers of *P. reniforme* subsp. *velutinum* were measured. *P. sidoides* flowers were also examined for nectar volume and concentration. This was done in order to determine if volumes and concentrations were significantly different and hence, the flowers pollinated by different pollinators with different nectar requirements.

Nectar was extracted from 10 long- and 10 short-tubed flowers with a 5µl capillary tube, and the volume measured. The % sucrose equivalents in the nectar were then measured with a hand-held refractometer. Statistical analysis consisted of two t-tests in order to determine whether or not sucrose concentration and nectar volume were significantly different between long- and short-tubed flowers.

4.2.5. Breeding system

Twenty of each long- and short-tubed plants of *Pelargonium reniforme* subsp. *velutinum* were monitored in a greenhouse. Large sections of tuber were collected and planted in five litre pots. Long-tubed plants were collected from the Kudu reserve population and short-tubed plants from the Botha's hill population. Plants were allowed about three months to acclimatise and flower. The greenhouse was used as breeding system experiments attempted in the field failed, possibly due to resource limitation. An additional 20 plants of *P. sidoides* were also monitored. The breeding system experiment was done on 50 different inflorescences each with 5 treatments. 15 groups of 5 treatments were done for each of the long- and short-tubed *P. reniforme* and *P. sidoides* plants, extra experiments were begun to account for possible abortion of buds common in the field. Inflorescences with 5 or more flowers were used. One of the flowers was self-pollinated. As the species is protandrous, pollen was collected when it was ripe and transferred to a clean eppendorf tube for each of the flower and labelled. These tubes were kept in the fridge until the stigma was receptive, and then pollinated. The second flower was simply

left unmanipulated in order to test for autogamy. The last three flowers were cross pollination experiments. Pollen was used from different plants. Flowers were cross-pollinated with long-tubed pollen, short-tubed pollen and *P. sidoides* pollen. Pollination was done by hand. The flowers were constantly monitored and when either the seeds were aborted or formed, the flower heads were removed. Those that had produced seeds were weighed in order to measure the biomass of the seeds produced.

The percentage of flowers that set seed for each of the treatments was calculated. This was done as previous attempts to conduct breeding system experiments in the field resulted in extremely low seed set (2% of the pollinated flowers). The breeding system was conducted on well-watered plants in the greenhouse to prevent the same low seed set observed in the field. In order to test for significant differences between pollination treatments for each of the flower types (long- and short-tubed *P. reniforme* subsp. *velutinum* and *P. sidoides*), ANOVAs were done on the seed mass data. The average time taken for the protandrous flowers to reach receptive female state from ripe male state was also calculated.

4.2.6. Insect species

Observations of insect foraging involved 3 hours a day about 3 times a week for the duration of one flowering season of the species. Observations were done extensively on the main population of *Pelargonium reniforme* subsp. *velutinum* at the old clay pigeon shooting range, Grahamstown and comprised both long- and short-tubed individuals of the subspecies. Insects observed visiting the flowers were captured and killed in a jar using ethyl acetate fumes. Location of pollen deposits was based first on visual observation of the captured insects and later on examination of pinned specimens. To prevent contamination of the body of an insect with pollen carried by another, the insects were all killed in separate clean jars.

Insects visiting long- and short-tubed flowers were recorded and their tongue lengths measured. Statistical analyses of tongue lengths consisted of t-tests to determine if the average tongue lengths of insects visiting short-tubed flowers were significantly different from those visiting long-tubed flowers.

4.3. Results

4.3.1. Tube length and distribution

Hypanthium length variation throughout the range of *Pelargonium reniforme* can be seen in the histogram of tube length measured from herbarium specimens (Figure 4.1A). Hypanthium lengths vary from very short (7.82mm) to very long (56.65mm) (Figure 4.1A). More short-tubed individuals exist throughout the range of the species (Figure 4.1A) even though a range of hypanthium lengths is evident throughout the distribution. The distribution of the short- and long-tubed individuals throughout the range of *Pelargonium reniforme* can be seen in Figure 4.2. Most quarter degree squares (14) comprise only short-tubed flowers there are four areas of long-tubed flowers. Two quarter degree squares comprise both short- and long-tubed flowers. One of these, with an even number of short- and long-tubed flowers is the Grahamstown (clay pigeon shooting range) population which is the subject of this study. One other quarter degree square consists mainly of short-tubed individuals with only a few long-tubed flowers (Figure 4.2). This second mixed population occurs near Port Elizabeth where *P. reniforme* subsp. *reniforme* is distributed, and may be a reflection of the relatively long-tubes of the subspecies when compared to the short-tubed *P. reniforme* subsp. *velutinum* flowers. This map may not be a true reflection of the distribution of tube length in the species as data was gathered from herbarium specimens and did not include huge numbers of individuals from the same populations.

A clear bimodal distribution of hypanthium lengths can be seen in the histogram of the Grahamstown population (Figure 4.1B). This population comprises both short- and long-tubed flowers. Both flower forms exist in the same population, the area consists of several clumps of the plants. Each clump consists of either long- or short-tubed individuals; they do not occur within the same clump but clumps do occur within metres of one another. This is the only population consisting of both floral forms that has been found. All other populations visited for the entire study comprise either short- or long-

tubed flowers but not both. For example, the Kudu reserve population consists entirely of long-tubed individuals (Figure 4.1C).

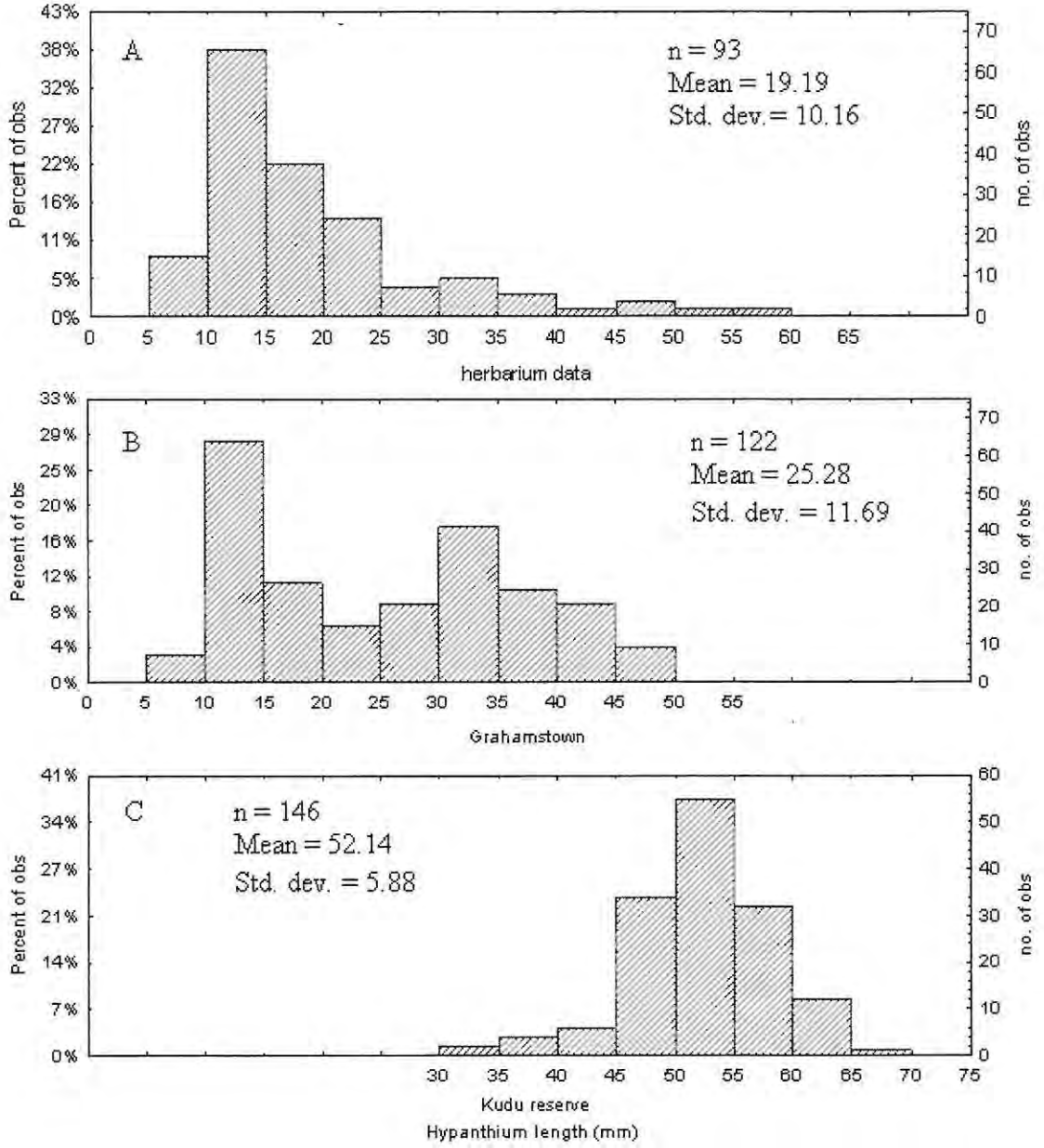

Figure 4.1: Histograms of hypanthium lengths of A) entire distribution of *Pelargonium reniforme* subsp *velutinum*, B) Grahamstown population and C) Kudu reserve population.

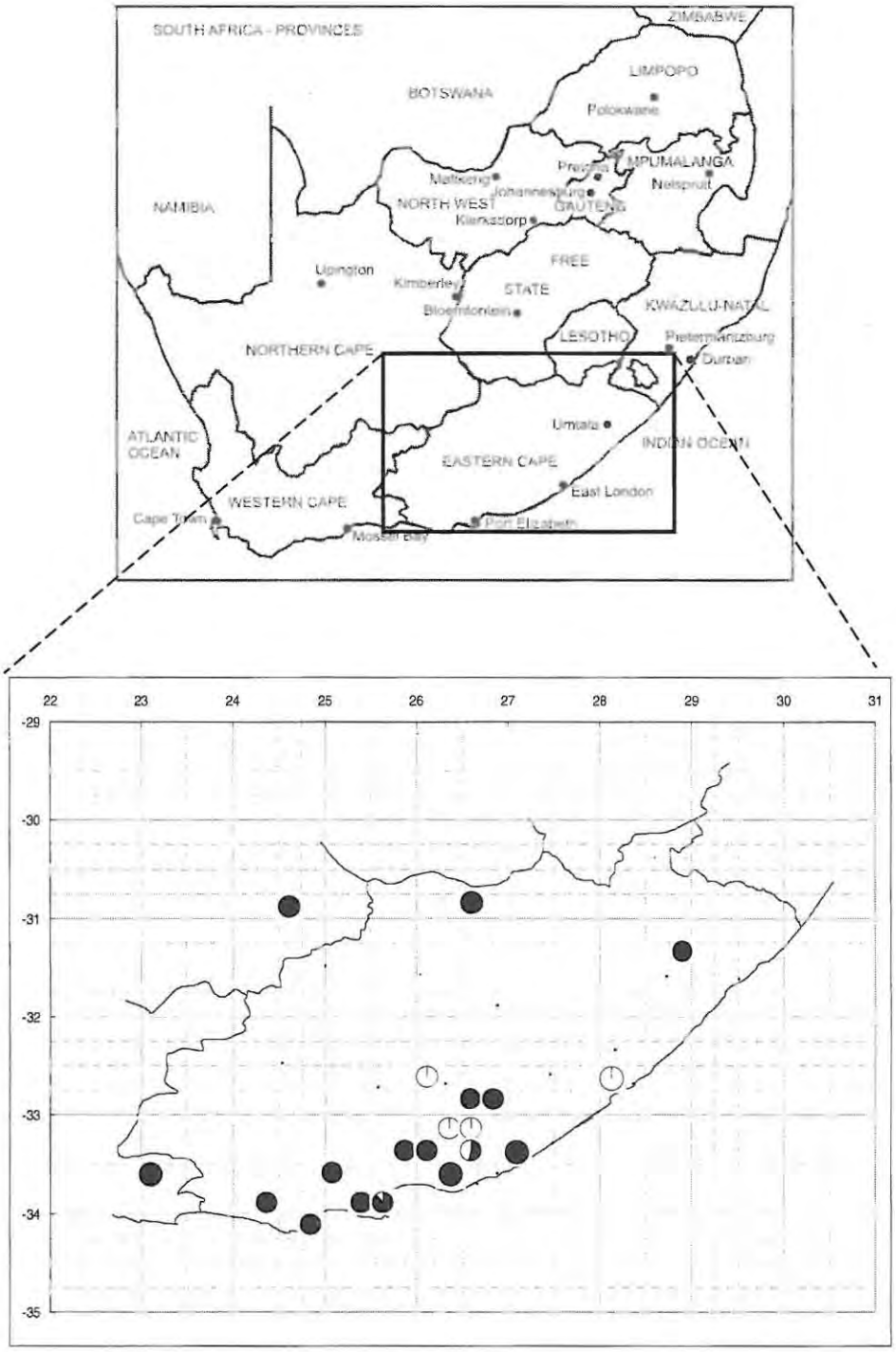

Figure 4.2: Distribution map of tube lengths of *Pelargonium reniforme* subsp. *velutinum* throughout the range of the subspecies. Open circles denote long-tubed flowers and closed circles short-tubed flowers. Pie charts are plotted in quarter degree squares. n=1 for all areas except the following: 3 (3325CD), 8 (3325DC), 2 (3326AB), 146 (3326BA), 140 (3326BC), 3 (3326CB), 2 (3424BB).

4.3.2. Colour

Colour was measured not only for the long- and short-tubed flowers of *Pelargonium reniforme* subsp. *velutinum* but also for the other subspecies described by Dreyer *et al.* (1995), *P. reniforme* subsp. *reniforme* as well as the closely-related black to dark-maroon *P. sidoides*. The results of the colour measurements can be seen in Figure 4.3. The colours of the flowers are very similar but some divisions can be seen. *P. sidoides* occurs at the top of the group and is clearly defined as a different colour. Short-tubed *P. reniforme* subsp. *velutinum* occur to the left of the distribution and long-tubed *P. reniforme* subsp. *velutinum* flowers to the right. *P. reniforme* subsp. *reniforme* shows a colour distribution very similar to that of the short-tubed *P. reniforme* subsp. *velutinum* flowers but the distribution lies towards the bottom of the cluster, indicating a slight difference in colour between the subspecies.

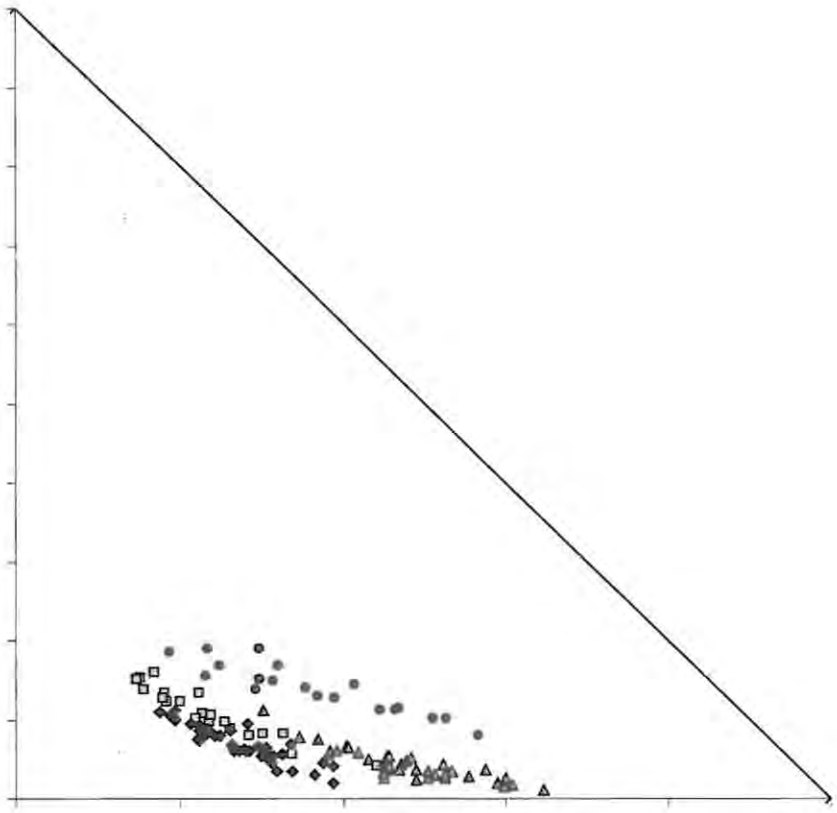

Figure 4.3: Endler colour diagram showing colours of *Pelargonium sidoides* (●), short-tubed *Pelargonium reniforme* subsp. *velutinum* (■), long-tubed *Pelargonium reniforme* subsp. *velutinum* (▲) and *Pelargonium reniforme* (♦).

4.3.3. Nectar

Concentration and volume of nectar was measured for both long- and shot-tubed *Pelargonium reniforme* subsp. *velutinum* flowers (Figure 4.4.). A t-test of nectar volume (n=10 for each form, df=18), showed a significant difference between long- and short-tubed flowers (t-value=9.65, $p<0.05$). A t-test of sucrose concentration (%, n=10 for each form, df=18) showed no significant difference between long- and short-tubed flowers (t-value=-1.44, p=0.1700). I attempted to measure nectar volume and concentration in *P. sidoides* but no nectar was present.

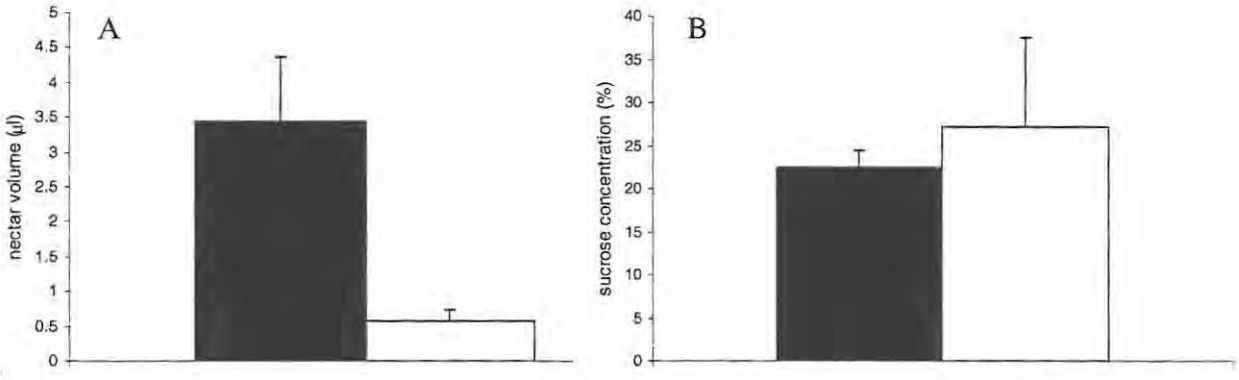

Figure 4.4: Graphs showing nectar volume (A) and sucrose concentration (B) in both long-tubed (shaded bars) and short-tubed (unshaded bars) flowers of *Pelargonium reniforme* subsp. *velutinum*. n=10 for each form.

4.3.4. Breeding system

Breeding system experiments on both long- and short-tubed *Pelargonium reniforme* subsp. *velutinum* as well as *P. sidoides* flowers showed possibilities for cross-pollination, autogamy, and the possibility of selfing (Figures 4.5 and 4.6). ANOVA results for differences between treatments showed significant differences between treatments for *P.*

sidoides ($p<0.05$, $F=3.78$, $n=15$, $d.f=70$). The significant difference is between the short-tubed crosses, which have much lower average seed mass than both the long-tubed *P. reniforme* subsp. *velutinum* and *P. sidoides* crosses. A post-hoc Scheffe's test showed no statistically significant differences between means for all treatments.

Long-tubed flowers have a lower level of autogamy than both short-tubed *P. reniforme* subsp. *velutinum* and *P. sidoides* flowers (Figures 4.5 and 4.6). Long-tubed *P. reniforme* subsp. *velutinum* flowers show the highest average seed mass for selfing (Figure 4.5), indicating that should autogamy occur more often, seed set as a result would be higher than that occurring naturally. Overall, crosses with the same flower type yielded the highest average seed mass (Figure 4.5). Flowers of all three plant groups are self-compatible (Figures 4.5 and 4.6), but produce lower percentage seed set for autogamy than all other treatments.

These results indicate (without taking into account possible mechanical or environmental isolating mechanisms) that long-tubed *Pelargonium reniforme* subsp. *velutinum* flowers are most successfully pollinated by other long-tubed flowers. Short-tubed *Pelargonium reniforme* subsp. *velutinum* flowers produce the most seed when pollinated by long-tubed *Pelargonium reniforme* subsp. *velutinum* flowers. *P. sidoides* flowers are most successfully pollinated by long-tubed *Pelargonium reniforme* subsp. *velutinum* flowers.

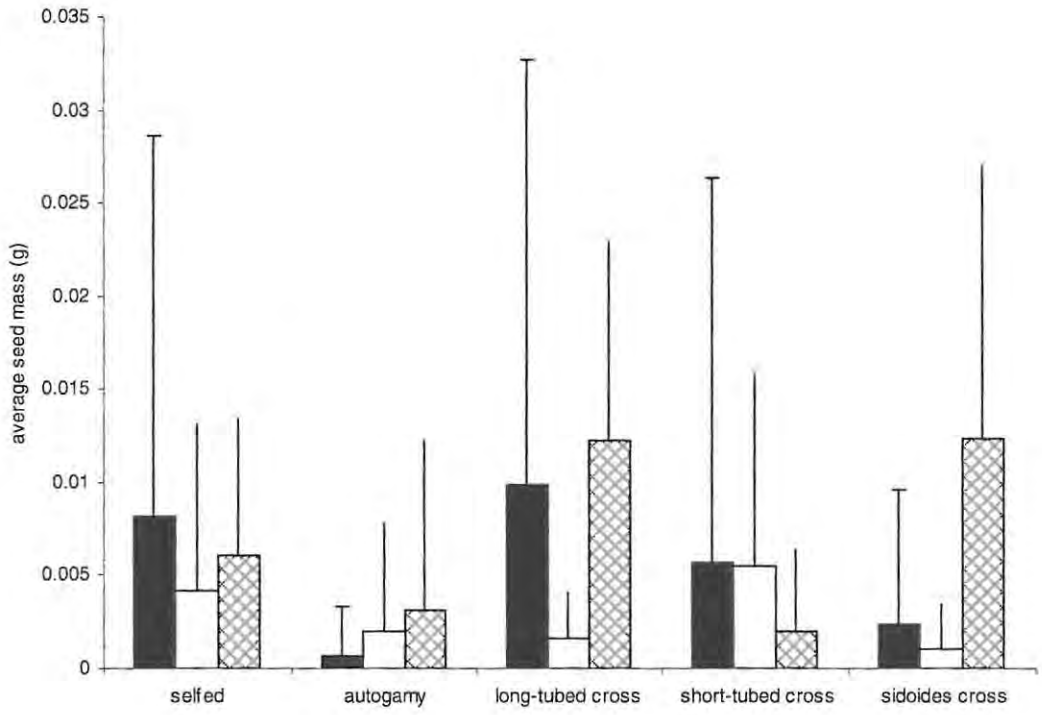

Figure 4.5: Graph of average seed mass of five different treatments for breeding system experiments. Results for long-tubed *P. reniforme* subsp. *velutinum* (shaded bars), short-tubed *P. reniforme* subsp. *velutinum* (unshaded bars) and *P. sidoides* (crosshatched bars).

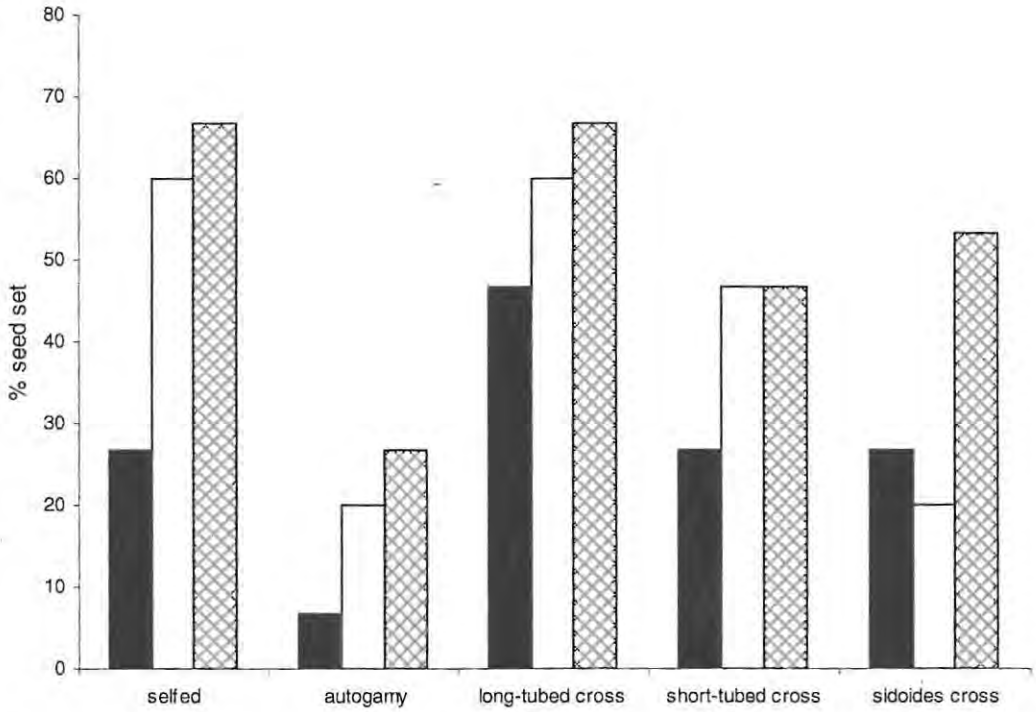

Figure 4.6: Percentage seed set for each of the breeding system treatments for long-tubed (shaded bar) and short-tubed *P. reniforme* subsp. *velutinum* (unshaded bar) and *P. sidoides* (crosshatched bar).

4.3.5. Visiting insect species

Observations of the insects visiting both long- and short-tubed *Pelargonium reniforme* subsp. *velutinum* flowers revealed very few visitors. During the time spent in the field, only five long-tongued Nemestrinid flies were caught visiting long-tubed flowers, two Bombyliids visiting short-tubed flowers and four butterflies (all Papillionidae) visiting long-tubed flowers (Figure 4.7 and 4.8). However more visitors were observed than caught (including bees visiting short-tubed flowers). Observations included numerous flies and butterflies visiting both long- and short-tubed flowers (Figure 4.9). Tongue lengths of insect visitors corresponded to tube lengths of the flowers they visited. A t-test conducted on the average tongue-length of insects visiting long- and short-tubed

Pelargonium reniforme subsp. *velutinum* flowers showed a significant difference ($t=4.14$, $d.f.=9$, $p<0.05$).

Position of pollen was assessed on pinned specimens. Little pollen was evident on butterfly species. Both Bombyliid and Nemestrinid flies caught visiting flowers were heavily loaded with pollen, which was deposited on the head and lower thorax of the body.

Observation of pollinators revealed the habit of short-tongued Nemestrinid flies (genus *Prosoeca*) flying from short-tubed to long-tubed flowers. The insects spent a long time probing short-tubed flowers but only probed long-tubed flowers for a few seconds before moving off. These observations infer that pollinators may not respond to colour differences in the flower forms, but rather respond to the accessible nectar.

Observations of insects visiting flowers in the Grahamstown population of *Pelargonium reniforme* subsp. *velutinum* showed visits to both long- and short-tubed flowers by nectar-robbing wasps. Most flowers observed in the field showed evidence of nectar being robbed from the base of the tube from holes chewed just above the nectary at the base of the hypanthium. This robbery was attributed by the wasps, which were the most common visitors to the flowers.

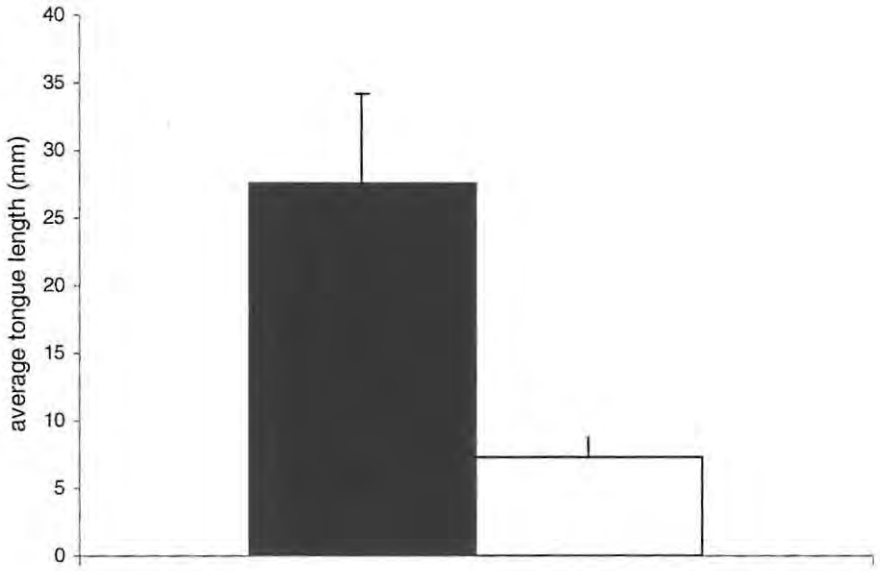

Figure 4.7: Average tongue lengths of insects caught visiting long-tubed (shaded bar, n=9) and short-tubed (unshaded bar, n=2) *P. reniforme* subsp. *velutinum* flowers.

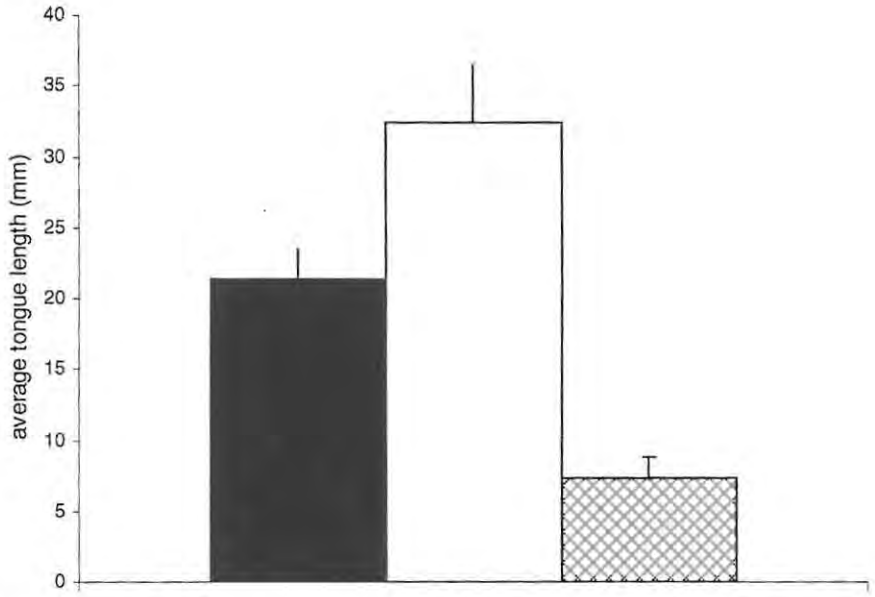

Figure 4.8: Average tongue lengths of insects caught visiting *P. reniforme* subsp. *velutinum* flowers, butterflies (shaded bar), nemestrinids (unshaded bar) and Bombyliids (crosshatched bar).

Figure 4.9: Pollinators of *Pelargonium reniforme* A) a Nemestrinid on a short-tubed flower, B) a Nemestrinid on a long-tubed flower, C) a Bombyliid on a short-tubed flower. Following its visit the Bombyliid repeatedly exposed the droplet of nectar at the end of its proboscis presumably to increase the concentration of the nectar through evaporation (D).

4.4. Discussion

4.4.1. Floral characters: hypanthium length, colour and nectar.

Floral characters may be the most important for pollinator-mediated selection. One of the most responsive characters is flower tube length. As can be seen by Figure 4.1, showing the distribution of hypanthium length in the Grahamstown population, there is a clear bimodal distribution. The population consists of both long- and short-tubed individuals of *Pelargonium reniforme* subsp. *velutinum* with a few individuals of intermediate hypanthium length (Figure 1.2 F). Figure 4.2 shows that only one other quarter degree square contains both long- and short-tubed flowers (not necessarily corresponding to a single population). All other quarter degree squares consist of either short- or long-tubed flowers and not both. The short-tubed form is common and the long-tubed form relatively rare and restricted to areas around Grahamstown. It is however, from this limited geographic pattern, not clear whether this population forms a hybrid zone, or the original area in which the two floral forms developed or an area of secondary contact between two usually separate forms. Despite this, it is clear that there are definitive differences in hypanthium length between the two floral forms within the same population.

Different floral tube lengths may be attributed to phenotypic plasticity. Such a hypothesis for the explanation of tube length difference was not explicitly tested in this study although repeat measures showed no variation in tube length over time. However, the co-occurrence of the different forms within the same population, and the unchanged floral characters of plant grown in a greenhouse for breeding system purposes indicated no evidence of phenotypic plasticity.

Flower colour of the two forms shows differences (Figure 4.3), where the short-tubed flowers occupied the paler pink side of the spectrum and long-tubed flowers occupied the darker pink side of the spectrum. This colour difference is obvious to human eyes in observed flowers. Figure 4.3 also shows a clear difference in colour for *Pelargonium sidoides* specimens. *P. reniforme* subsp. *reniforme* are, as expected, very similar to short-

tubed flowers of *P. reniforme* subsp. *velutinum*. Nectar also shows differences, but only in volume, not sucrose concentration. Nectar volume was much higher for long-tubed flowers (Table 4.2). This is expected as the much longer tubes are able to hold more nectar, but also indicate that the level of nectar within the tube may be such that insects with intermediate tongues may be able to reach it as well as long-tongued insects.

4.4.2. Breeding system

Seed set is extremely low for both natural and greenhouse populations of *P. reniforme*, but certainly much higher in hand-pollinated flowers than in those in the field, indicating either resource or pollinator limitation (seed set in the field was as low as 2%). Pollinator limitation may be one of the factors affecting the number of seeds produced; often hand-pollinated plants produce many more seeds than natural populations. This indicates that there may be a pollen limitation problem, which reveals that there is a lack of pollinator action (Campbell & Halama 1993). Campbell and Halama (1993) discovered this to be true for populations of *Ipomopsis aggregata*. It is likely to be the case for *P. reniforme* as well, very few insects were caught visiting the flowers in the study population and seed set was extremely low. However, adverse environmental conditions throughout the duration of the flowering season may also have contributed to the low level of seed set in the field. Reasons for the low seed set in well watered and fertilized greenhouse plants remain a mystery but show that seed set is likely to be low, even in optimum conditions.

Results of the breeding system experiments show that autogamy is likely to occur in both *P. sidoides* and short-tubed *P. reniforme* subsp. *velutinum* flowers, but is highly unlikely to occur in long-tubed *P. reniforme* subsp. *velutinum* flowers, despite the flowers being self-compatible (Table 4.3). It was observed that anthers always fell off before ripening of the stigma in long-tubed flowers, but often remained attached, bearing pollen in short-tubed and *P. sidoides* flowers. Both long- and short-tubed flowers are able to cross pollinate, levels of seed set are lower for long-tubed flowers crossed with short-tubed flowers than long-tubed flowers crossed with other long-tubed flowers. However, the

percentage seed set for short-tubed flowers crossed with other short-tubed flowers is lower than the percentage seed set for short-tubed flowers crossed with long-tubed flowers. As pollinators with long tongues are not able to pollinate short-tubed flowers with long-tubed pollen (it may be possible for them to pollinate long-tubed flowers with short-tubed pollen but this is unlikely, considering that pollen may not cling to the proboscis as well as it does to the hairy body of an insect), this stops cross pollination from long-tubed to short-tubed flowers. It is unnecessary for the short-tubed flowers to selectively differentiate long-tubed pollen as it is unlikely to encounter it. However, the possibility of a short-tongued pollinator pollinating a long-tubed flower is high, but percentage seed set for this cross is extremely low, indicating that there may be some sort of discrimination of short-tubed pollen by long-tubed flowers. In addition, both long- and short-tubed *Pelargonium reniforme* subsp. *velutinum* flowers are able to hybridise with *P. sidoides*.

4.4.3. Pollinators

Most insects tend to visit the same flower type consistently (Goulson 1999). This may be an important isolating mechanism between the two forms, where insects visit either long- or short-tubed flowers, but rarely both. Certainly, colour is sufficiently different between the two forms for pollinators to be able to choose either floral form consistently. However, no evidence was found to prove that insects distinguish between colours. Indeed, some insects such as short-tongued Nemestrinids tend to visit both floral forms to probe for nectar (unsuccessfully in the long-tubed form). Bombyliids, on the other hand, were only seen visiting short-tubed flowers. Colour is an important selective trait for pollinators. Wild radish (*Raphanus raphanistrum*), shows response to pollinator choice, with specific colour morphs clearly favoured by pollinators (Stanton *et al.* 1989). On the other hand, flower colour may have no influence on pollinators at all. (Aragón & Ackerman 2004) found no selection pressure favouring one flower colour variation over another in *Psychilis monensis* (Orchidaceae). More studies should be done on pollinator behaviour and choice in this population in order to determine insect response to colour.

It is unclear if these flowers are exclusively pollinated by long-tongued flies as not only flies visit *P. reniforme* flowers. Manning & Goldblatt (1996) indicate in their study of long-tongued fly pollination guild, that all flowers exhibiting what can only be described as the typical long-tongued fly pollination characteristics may in fact not be pollinated by long-tongued flies. They note that often nectar tubes are long and narrow but the base is so narrow that nectar is forced closer to the top of the tube and is thus accessible to other shorter-tongued insects. It was found that bees, butterflies and shorter-tongued Bombyliid flies could affect pollination in these species (Manning & Goldblatt 1996). Visitors to *P. reniforme* subsp. *velutinum* flowers are not restricted to long-tongued flies, and although more pollen is deposited on flies than other visitors, butterflies and long-tongued bees such as *Amegilla* do pollinate the flowers.

Long-tubed flowers contain large amounts of nectar that is inaccessible to short-tongued insects, making them an ideal food source for long-tongued flies. There is the potential for reproductive isolation as flower tubes become longer to accommodate effective long-tongued fly pollinators (Manning & Goldblatt 1996). Long-tubed *P. reniforme* subsp. *velutinum* flowers do contain large volume of nectar, and nectar is accessible only to insects with long tongues, most of which are long-tongued Nemestrinid flies.

Isolation of the long- and short-tubed floral forms may be due in part to pollinator constancy to one colour, and due to the location of nectar deep within the flower tube. But these floral characters may be insufficient to isolate the different floral forms. Long-tongued insects cannot pollinate short-tubed flowers, but short-tongued insects are able to pollinate long-tubed flowers. This seems to be prevented in part by the long-tubed flowers themselves by recognising pollen from short-tubed flowers as shown by the low levels of seed set for long-tubed flowers pollinated by short-tubed flowers in breeding system experiments.

Other cases of different tube lengths and corresponding pollinators have been documented. A study on the orchid *Disa draconis* showed that flowers formed short, long

and medium-length spurred populations. Pollinators tended to be more specific for the long-tubed populations, which were pollinated by a tangle-wing fly (Nemestrinidae). It was clear that selection favours longer spurs in the long-spurred population, with the selection being driven by the pollinator (Johnson & Steiner 1997). It is also mentioned that gene flow can occur between long- and short-tubed individuals as, though short-tubed flowers can only be pollinated by pollen from other short-tubed flowers, long-tubed flowers can be pollinated by both long- and short-tubed pollen (Johnson & Steiner 1997).

Another study on an orchid (*Platanthera ciliaris*), revealed that pollinator-mediated selection of spur length resulted in the existence of ecotypes (Robertson & Wyatt 1990). The floral types occupy separate geographical areas and are clearly pollinated by different butterfly species, with tongue lengths matching spur lengths of the flowers (Roberston & Wyatt 1990). Selection for longer flower tube lengths in *Gladiolus longicollis* (Iridaceae) in response to pollinator mediated selection by hawkmoths has been shown (Alexandersson & Johnson 2002). Although other hawkmoths with much shorter tongues do visit the plant, they were unable to reach the nectar and did not often carry pollen, shorter-tubed individuals also tended not to be pollinated as the head of the moth did not come into contact with anthers and stigma (Alexandersson & Johnson 2002).

4.4.4. Conclusions

The results of this study provide evidence of the processes behind the patterns described by Chapters 1 and 2, where evidence of differences between long- and short-tubed *Pelargonium reniforme* subsp. *velutinum* flowers is given. The genetic isolation of the two floral forms where they occur sympatrically in Grahamstown (chapter 3) is explained by floral morphology and pollinators. Tube lengths reduce the possibility of cross-pollination between floral forms, and where it is possible, it rarely occurs due to pollen discrimination. In addition to this, flower colour allows for the identification of the different forms by the pollinator. Although evidence of pollinator behaviour is limited

due to the lack of insects visiting the flowers, it seems that insects visit either long- or short-tubed flowers consistently. Cross pollination between the two floral forms would be rare. However, cross pollination may occur as there is evidence of intermediates between the two forms in the population (Figure 4.2 F). It may be that over time hybridisation may occur more often and a shift in pollinators may result.

Observations of pollinators and analysis of pollinator behaviour should be done before pollinators can be clearly painted as the isolating or hybridisation mechanism of the two floral forms. It is clear from analysis of floral characters, that assortative, pollinator mediated mating is the most likely mechanism for genetic isolation of the long- and short-tubed forms of *Pelargonium reniforme* subsp. *velutinum*.

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Chapter 5: Directional selection in a variable population of *Pelargonium reniforme*.

5.1. Introduction

5.1.1. Selection gradients and fitness surfaces

Natural selection acts on phenotypes, without regard to genetics (Arnold & Wade 1984). This kind of phenotypic selection can occur within one generation. Evolutionary (and thus genetic) effects of this selection can only be seen over a number of generations. Selection over one generation can thus be measured without inferring overall genetic change (Lande & Arnold 1983). Only recently have statistical methods been developed that allow for the description of directional selection (Brodie *et al.* 1995). These statistical methods allow for the description of directional selection over a number of generations, or even just one flowering season of a particular plant (Schemske & Horvitz 1989). Assumptions can then be made as to the driving force of this selection depending on what floral or vegetative traits are experiencing the directional selection (Brodie *et al.* 1995). These assumptions can then be tested experimentally to determine the driving forces of selection (Brodie *et al.* 1995).

The statistical applications that allow for the description of selection occurring within a population are regressions (Brodie *et al.* 1995). Directional selection is measured with a linear regression of various traits against relative fitness. The selection coefficient describes the nature of this selection and is a measure of the overall direction of the selection. Linear selection differentials (directional selection differentials) measure the overall change as a result of directional selection, the changes in the trait averages. The non-linear selection coefficient also describes the overall selection acting on a particular traits or pair of correlated traits. Non-linear selection differentials measure the changes in variance of particular traits or covariance between two traits (Brodie *et al.* 1995). Of course, this is not the only method that can be employed to estimate selection; even

principal component analysis has been used to determine the effect of pollinators on morphological traits (Galen 1989). However, the regression method developed by Lande and Arnold (1983) is the most effective and certainly the most commonly used method. And a variety of studies have used this in order to infer selection in plant populations.

Selective pressures within a population may act on both morphological, phenotypic and life-history traits, all of which can be measured (Kelly 1992). Most studies utilize only morphological traits as these are the easiest to measure (Conner 2001). Other characters can be used; O'Connell and Johnston (1998) used microhabitat as one of their characters and found it to be important in determining fitness for a deceptive orchid *Cypripedium acaule*. Flowering phenology was found to be evolutionarily unstable in the deceptive orchid *Tolumnia variegata*, this instability was attributed to the deceptive nature of the pollination system (Sabat and Ackerman 1996).

In the case of floral traits in plants with a clear specialist pollination system, the driving force of the selection is most often clearly the pollinators. For example Alexandersson and Johnson (2002) prove the presence of selection within a population of a *Gladiolus* species with a very variable flower tube length. The positive directional selection gradient formed by the statistical analysis of the fitness and floral characters was then related to the pollinators of the flowers (Alexandersson & Johnson 2002). Morgan and Schoen (1997) attribute selection of floral traits of *Asclepias syriaca* to pollinators.

Maad (2000) used the multiple regression method for determining the degree of selection on correlative characters as developed by Lande and Arnold (1983). The study involved the development of fitness surfaces of hawkmoth pollinated *Platanthera bifolia*, through measurements of the fitness of the flowers in relation to floral characters, and over two flowering seasons. Maad (2000) discovered that there is natural selection within the population, but this can vary over time. Another study along the same lines involved the study of selection within two species of *Lobelia* over one flowering season (a selection interval) (Johnston 1991). In this study, seed set is used as an indication of the fitness of the plant and may represent a fraction of the overall number of recruits produced as a

result of that particular flowering season. There was positive directional selection in both species but was different between them (Johnston 1991). Schemske and Horvitz (1989) discover in their study of *Calathea ovandensis* that no significant selection exists. Maad and Alexandersson (2004), show in their study of *Platanthera bifolia* that selection can differ if environmental conditions are altered. The study showed that selection gradients differed in a drought year for *Platanthera bifolia* (Maad & Alexandersson 2004).

It can be difficult to study selection on correlated traits and accurately predict the agents of that selection. Johnston (1991) described pollinators as the driving force of selection on floral traits of two *Lobelia* species with similar life histories and different pollinators. By evaluating only floral traits Johnston (1991) assumes that any selection can be directly attributed to pollinators, without regard for potential correlations with other vegetative traits. Campbell *et al.* (1997) studied pollinators and selection in a hybrid zone of two species of *Ipomopsis* (Polemoniaceae). The species were pollinated by hummingbirds and hawkmoths and there was clear evidence of hybrids in the overlapping distributions. Studies showed strong directional selection for wide corolla tubes and intense red flowers in the hummingbird-pollinated species, whereas in the hawkmoth-pollinated species selection was for narrower tubes. As was expected, the hybrid zone showed disruptive selection for corolla tube width (Campbell *et al.* 1997).

A statistical analysis of a variety of character traits and the corresponding fitness values may give an indication as to how natural selection is acting within the population. However, this type of study does not attempt to provide a mechanism for this selection, nor attempt to predict that it will occur over more than one breeding season.

5.1.2. Criticisms of the method

Schluter (1988) discusses the restrictions imposed by Lande and Arnold's (1983) methods as they do not take into account the possibility of non-normal distributions of character traits within a species. This study provides a non-parametric alternative that is

essential for non-normal distribution analysis as well as potentially more accurate than the parametric method of Lande and Arnold (1983). A non-parametric alternative is also given by Schluter and Nychka (1994). These non-parametric alternatives are multivariate cubic splines and eliminate the problem of the normality assumptions of the traditional regression analyses (Schluter 1988, Schluter & Nychka 1994). Indeed, in order to utilise Lande and Arnold's (1983) method, data must first be normalised, as done by Schemske and Horvitz (1989).

Mitchell-Olds and Shaw (1987), who warn against assumptions of overall evolution from evidence of directional selection, in addition to this, they discuss the importance of considering potentially correlated characters that have not been measured as well as potential environmental effect. Limitations of transformations of the data are discussed and necessity for using parametric statistical tests for non-normal data. The authors suggest a carefully considered selection of techniques to adequately describe selection in a population (Mitchell-Olds & Shaw 1987). They do advocate the use of regression analyses as it is the easiest of the statistical applications and can be applied to most populations with large sample sizes (Mitchell-Olds & Shaw 1987). Maad (2000) avoids these downfalls; the data in the study are normal and thus non-parametric tests were unnecessary. Brodie *et al.* (1995) review all the statistical methods of analysing natural selection to date and discuss the downfalls and merits of the methods. They also provide support for the accurate non-parametric method of Schluter (1988). Rausher (1992) cautions against the use of fitness measures as these are necessarily correlated with environmental variables. He suggests using genotypic and breeding values rather than phenotypic values. He suggests that regression analyses of fitness values be only preliminary and followed up with experimental confirmation using genotypic and breeding values (Rausher 1992). An alternative method, logistic regression, is advocated by Janzen and Stern (1998) although they do advocate the use of traditional linear regression techniques.

Overall, however, the linear and quadratic regression of Lande and Arnold (1983) is the most popular method and still utilized by the most recent studies. The problem of the

assumption of normality has been overcome in the use of this method by interpreting the results of the analyses only as estimations of fitness gradients and surfaces (Kelly 1992). The method makes estimations of selection accessible for selection episodes as short as one flowering season or as long as several generations, the latter allowing for the extension of the phenotypic selection described by the method to evolutionary change. The method gives an indication of the selection a certain population undergoes and allows for speculation as to mechanisms of the selection.

5.1.3. Potential for selection in a population of *Pelargonium reniforme*

One population of *Pelargonium reniforme* subsp. *velutinum* exists in Grahamstown where two different floral forms exist sympatrically. In this population, long-tubed bright vivid pink and short-tubed light pink flowers exist in sympatry (Chapter 1). Through pollinator observations (Chapter 4), it seems that mostly long-tongued insects visit long-tubed flowers and short-tongued insects visit short-tubed flowers. Short-tongued insects can pollinate both short- and long-tubed flowers but long-tongued insects can pollinate only long-tubed flowers however, it is suspected that short-tongued insects consistently choose short-tubed flowers and thus seldom pollinate long-tubed flowers. It is thus expected that tube length within the population should be undergoing disruptive selection. The longer-tubed and shorter-tubed flowers should have correspondingly larger fitness values than middle tube lengths due to pollinator action. The selection surface (quadratic function showing selection for the relationship between two traits) should look much like that of a hybrid zone, clearly disruptive at least for tube length. Certainly, genetic evidence (Chapter 3) indicates partitioning of the long- and short-tubed individuals within the population. Selection should be acting on each of the floral forms separately, showing disruptive selection. Traits other than tube length that are often positively selected for in plants are flower size, floral display and flower height. Directional selection for these traits is expected to be no different in *P. reniforme* subsp. *velutinum*.

5.1.4. Aims and objectives

This study was undertaken as a preliminary study utilising a new method for quantifying selection in a population of *Pelargonium reniforme* subsp. *velutinum*, where it was suspected that strong directional selection could be occurring. This preliminary investigation aimed to determine which traits could be measured for the study species, which measure of fitness is the easiest to determine and use, and the potential for extending the study over a number of flowering seasons.

The aims of this study are to detect any selection that may be occurring in a population of *Pelargonium reniforme* subsp. *velutinum* where both long- and sort-tubed floral forms occur sympatrically. It is expected that one of the major traits undergoing selection will be tube length as this is an important isolating floral character. Behaviour of pollinators indicates that there may directional selection for longer and shorter tubes, demonstrating disruptive selection within the population. Genetic data indicate separation of the two floral forms (Chapter 3). In addition to this, flower size and inflorescence size have been shown in some studies to be consistently positively selected for. It is expected that *P. reniforme* subsp. *velutinum* is no different and that larger flowers (i.e. larger area of petal display) and larger inflorescences will be selected for (i.e. they will have higher fitness compared to smaller flowers and smaller inflorescences).

5.2. Materials and methods

5.2.1. The study area

The study area is the Grahamstown old clay pigeon shooting range (33°16.527'S, 26°29.610'E), where both long- and short-tubed floral forms of *Pelargonium reniforme* subsp. *velutinum* occur sympatrically (Chapter 1). 200 Plants with buds were marked for measurements for the analyses. This was done as large sample sizes are statistically sounder than small sample sizes. The necessity for large sample sizes was emphasized by Johnston (1991), who discovered that the minimum sample sizes needed to reflect the true fitness gradients were all large (under 500) for two *Lobelia* species. Certainly the larger the sample size the more statistically robust the results of the study; the larger the sample size, the smaller the selection gradients (Conner 2001). Small sample sizes could give a false directional selection coefficient but Conner (2001) does warn against making the assumption that few studies with large sample sizes reflect selection occurring in nature. An extremely high level of bud abortion (usually of the entire inflorescence) resulted in only 55 plants being measured for the duration of the study. We suspected that environmental conditions for this particular flowering season resulted in the high level of floral abortion. Only 10 of the 55 plants set seed. Similar problems were found in Chapter 4, where there was limited seed set. The flowering season (2005-2006) was particularly poor.

5.2.2. Fitness and trait measures

As this was an exploration of a method, a number of characters were measured before a few were selected to be used in the analysis. Originally, the maximum number of traits possible was measured. It is possible that the results of the statistical analyses could point to the selection of a trait not measured. In the hope of avoiding this, as many characters as possible were included in the analysis. These original characters included: number of flowers per inflorescence, number of open flowers per inflorescence, inflorescence

height, inflorescence width, inflorescence height above ground, plant height above ground, lifespan of inflorescence (measured as the time from the first open flower to the last wilted flower), flower lifespan (measured as the time from first opening of the flower till wilting of the flower), time taken for seed set (measured as the time from wilting of the flower till seed set), average tube length (measured from all opened flowers in the inflorescence), flower height and flower width. Number of seeds per flower and seed mass were also identified as important measures with a view to using them as fitness components. However, due to the low level of seed set, however, none of the seed measures could be utilised. Traits used in other studies, and those often found to be undergoing directional selection were identified and used. These included flower height, size of individual flowers and inflorescences as well as flower tube length. These measures were isolated from the others to be used in the study. Four traits were finally chosen for analysis. These included flower size (a ratio of flower height and width was calculated to give this trait), inflorescence size (a ratio of inflorescence height and width), inflorescence height (the height of the highest point of the inflorescence above the ground) and average tube length (measured as an average of all the open flowers of each inflorescence).

Often, binary or alternative discrete measures of fitness are the only possible way to measure fitness as the measurement of lifetime fitness of numerous individuals in a population is impractical (Brodie & Janzen 1996). As few of the plants measured set seed, a fitness measure other than seed production had to be found. As abortion of buds was also common within the *Pelargonium reniforme* subsp. *velutinum* population, the number of flowers that opened per inflorescence divided by the number of buds for the same inflorescence was used as the fitness measure (a continuous measure of fitness, and so useable in parametric correlation analyses). In order to make this measure comparable between plants, relative fitness was calculated as the number of flowers opened per inflorescence divided by the average over all of the measured plants. Essential to the use of this fitness measure is the assumption that it is correlated with total fitness. Fitness measures that are continuous, rather than binary, can be used in parametric tests of fitness gradients (Brodie & Janzen 1996).

5.2.3. Statistical analyses

Terms used to describe selection gradients and differentials are misleading (Brodie *et al.* 1995). The terms directional, stabilising and disruptive selection are used in both the statistical sense (where they do not give an indication of the shape of fitness surfaces) and in the graphical sense (where they describe the shape of fitness surfaces). In the graphical sense, directional selection describes a straight line relationship between fitness and a defined trait. Similarly, stabilizing and disruptive selection describes features of selection surfaces such as peaks, valleys and saddles (Brodie *et al.* 1995). For this reason, the terms suggested by Brodie *et al.* (1995) are used for statistical results in this study. These include linear coefficients (directional selection) and non-linear coefficients (stabilizing/ disruptive selection). Traditional graphical terms are used to describe fitness surfaces these include directional, stabilizing and disruptive selection.

Descriptive statistics (means and standard deviations) of each of the traits were measured. As the method is based on calculating the effects of selection on correlated traits, correlations between the four traits were also tested for using Pearson's product moment coefficient. Correlated traits would by default experience similar selection directions due to this correlation. The distributions of each of the traits were also tested for normality using the Kolmogorov-Smirnov and Lilliefors tests for normality. Normality is largely unnecessary for regressions however, as they can still give an estimation of fitness gradients and surfaces without conforming to the assumption of normality (Kelly 1992).

Linear selection gradients (ß) were measured with a multiple regression in Statistica (Statsoft 2004). Relative fitness was regressed with the four traits and the gradients (ß) given by the slope of the regressions. Linear selection differentials (s) were given by the covariances of each of the traits and relative fitness. As linear regression only gives overall directional selection, quadratic regressions were also done in Statistica (Statsoft 2004). Non-linear selection gradients (γ) were given by the regression coefficient in quadratic regression. These gradients (γ) give the overall nature of non-linear selection,

that is, whether selection is disruptive or stabilizing. Non-linear selection differentials (C) were calculated as the covariances of each of the traits and their relationships and fitness. These differentials indicate the change in means of selection and the effect of correlated traits on selection.

In addition to calculating non-linear gradients and differentials, fitness surfaces were also plotted for the relationships between the traits and fitness. This allows for visual representation of selection, rather than its estimation by one number. Fitness surfaces are only the best estimate of the selection surface due to constraints of quadratic regression (Brodie *et al.* 1995). One of these constraints is the restriction to representation of only three axes at a time, and therefore cannot be used to visualise the overall selection surface (Brodie *et al.* 1995). Although other non-parametric techniques have been proposed (such as the multivariate cubic spline suggested by Schluter (1988)), the use of multiple regressions and quadratic regression is common and valuable for descriptions of selection (Kelly 1992). Selection surfaces allows for the interpretation of the phenotypic selection occurring over one selection episode (in this case, one flowering season) and estimations of stabilizing and disruptive selection can be made from the graphical representation.

5.3. Results

Of the plants initially marked (n=200), only 55 were used for the study. Although only 10 of these produced seeds, 55 produced flowers from the buds. Often buds were aborted before the flowers opened. This study was only done over one flowering season thus comparisons could not be made as to the normal seed production of the population. Various environmental aspects (such as poor rainfall or resource limitation) could have contributed to the low levels of seed production in this particular flowering season.

Basic statistics of each of the measured traits are given in Table 5.1. Correlations between each of the traits are given in Table 5.2, inflorescence size and inflorescence height is positively correlated, inflorescence size and average tube length is negatively correlated and inflorescence height and flower size are negatively correlated. Any selection, directional, stabilizing or disruptive that acts on any of these traits will also act on the traits correlated with them. Size of inflorescence as well as relative fitness failed the test for normality and permitted transformations (Lande & Arnold 1983) fail to normalise the data. All results for this trait then are estimations of the selection acting on it.

Table 5.1: Means and standard deviations (SD) of characters in the population of *Pelargonium reniforme* subsp. *velutinum* in one flowering season. n=55

Trait	Mean (mm)	SD (mm)
inflorescence display size	1.92	1.64
inflorescence height	182.36	44.74
average tube length	28.01	7.63
flower display size	1.36	0.28

Table 5.2: Conditional Pearson's correlation coefficients for all traits for *Pelargonium reniforme* subsp. *velutinum*. Correlations marked with a * indicate significance at the 0.05 level, ** at the 0.01 level and *** at the 0.001 level.

Trait	inflorescence size	inflorescence height	average tube length	flower size
inflorescence size	-			
inflorescence height	0.34*	-		
average tube length	-0.49***	-0.18	-	
flower size	-0.02	-0.36**	0.14	-

Selection gradients are given for one flowering season only, and thus no inference to the evolutionary effect of phenotypic selection. However, the forces involved in phenotypic selection over one flowering season can be reviewed. These are not necessarily consistent over more than one flowering season, but are an indication of the selection occurring in one season.

Linear selection gradients are given in Table 5.3. The only statistically significant gradient is that of inflorescence size, which is negatively correlated with the fitness measure. However, the differentials however, are quite low values, indicating little changes in mean value of the trait resulting from direct selection on that trait or on correlated characters. Other traits also show directional selection: this is positive for inflorescence height and negative for flower size. Average tube length shows the smallest directional selection gradient, tending towards 0, showing that there is very little directional selection acting on this trait.

Table 5.3: Directional selection gradients ß (Standard Error) and differentials (s) for the population of *Pelargonium reniforme* subsp. *velutinum* for one flowering season. Gradients marked with a * are significant at the $p<0.05$ level.

Trait	ß(SE)	s
inflorescence size	-0.45* (0.15)	0.012
inflorescence height	0.27 (0.14)	0.005
average tube length	0.01 (0.14)	0.000
flower size	-0.15 (0.13)	0.002

Non-linear selection gradients are given in Table 5.4. In addition to this, fitness surfaces are illustrated in Figures 5.1 to 5.6. Strong negative gradients are given for inflorescence size, indicating that stabilizing selection is occurring (Table 5.4). Negative gradients are also given for average tube length and flowering size, indicating direct stabilizing selection. Selection gradients for the relationship between inflorescence height and flower size were also strongly negative. Similarly to the direct selection gradients,

stabilizing/ disruptive differentials are very small, indicating little change in the mean of the gradients resulting from either direct or indirect selection on the traits (Table 5.4).

A high positive gradient is given for inflorescence height, for the relationship between inflorescence size and inflorescence height and the relationship between inflorescence size and flower size, indicating disruptive selection. Positive selection gradients are also given for the relationship between inflorescence size and average tube length and inflorescence height and average tube length. Low differentials indicate that selection is direct rather than indirect (through correlated traits).

Table 5.4: Stabilizing/disruptive selection gradients γ (SE) and differentials (C) for the population of *Pelargonium reniforme* subsp. *velutinum* for one flowering season.

Trait	γ (SE)	C
inflorescence size	-3.59 (3.06)	0.001877
inflorescence height	2.93 (2.42)	0.001989
average tube length	-0.78 (1.97)	0.000213
flower size	-1.55 (2.13)	0.000710
inflorescence size*inflorescence height	1.29 (1.14)	0.001760
inflorescence size*average tube length	0.36 (1.1.6)	0.000132
inflorescence height *average tube length	0.40 (1.07)	0.000190
inflorescence size*flower size	1.53 (1.5)	0.001414
inflorescence height*flower size	-1.11 (1.36)	0.000902
average tube length*flower size	-0.05 (1.46)	0.000001

Fitness surfaces help to visualise the stabilizing or disruptive selection occurring on each of the relationships described in Table 5.4. Figure 5.1 shows little effect on fitness for flower size, with increased fitness for either small or large flowers. Also, there is little effect on inflorescence height, with a slight increase in fitness for the tallest inflorescences. The relationship between the two traits indicates an increase in fitness for larger flowers and taller inflorescences. The overall selection gradient is negative (Table 5.4) indicating stabilising selection.

Figure 5.2 shows the fitness surface for flower size and inflorescence size. It shows highest fitness for larger flowers and smaller inflorescences. The selection gradient indicates that selection in this relationship is disruptive (Table 5.4).

Figure 5.3, describing the fitness surface of the relationship between inflorescence height and average tube length shows increased fitness for inflorescence height in the middle of the spectrum and very little change in fitness for average tube length. There is a very slight increase in fitness for very short and very long tubes. Overall, the relationship between the two traits is undergoing disruptive selection (Table 5.4).

Figure 5.4 shows the fitness surface of the relationship between inflorescence size and inflorescence height. Overall, the relationship is undergoing fairly strong disruptive selection. Fitness can be seen to increase slightly with an increase in inflorescence height and with smaller inflorescence size.

Figure 5.5 shows the fitness surface of the relationship between average tube length and inflorescence size. Overall, the relationship is undergoing slight disruptive selection (Table 5.4). The graph shows little effect of average tube length on fitness, the trend is towards increased fitness with an increase in tube length. In contrast, fitness clearly increases with increased inflorescence size.

The fitness surface of the relationship between average tube length and flower size is shown in Figure 6.6, overall, the relationship is undergoing stabilizing selection (Table 5.4). Individually, the surface shows little effect of average tube length on fitness, fitness remains much the same for all measures of the trait. Flower size is different, however, and the larger the flowers, the higher the fitness.

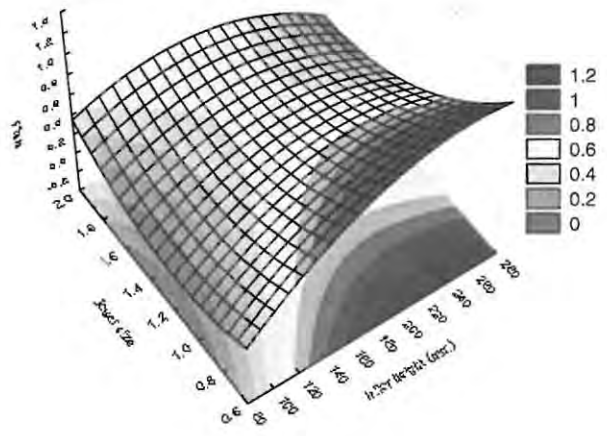

Figure 5.1: Fitness response surface of inflorescence height and flower size in *Pelargonium reniforme* subsp. *velutinum* during one flowering season. The legend describes the fitness surface, with the darkest grey indicating the highest levels of fitness.

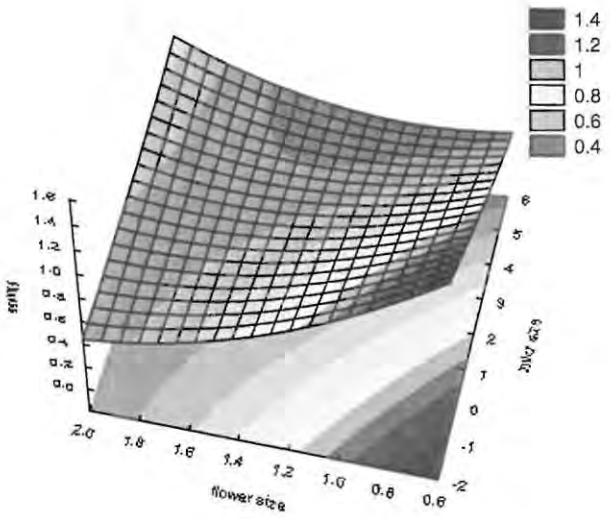

Figure 5.2: Fitness response surface of flower size and inflorescence size in *Pelargonium reniforme* subsp. *velutinum* during one flowering season. The legend describes the fitness surface, with the darkest grey indicating the highest levels of fitness.

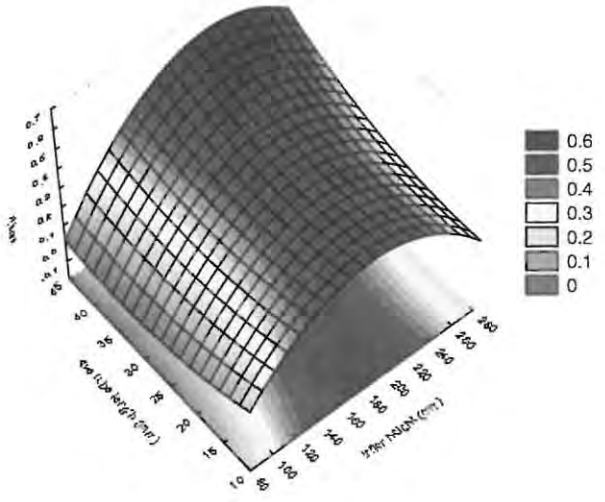

Figure 5.3: Fitness response surface of average tube length and inflorescence height of *Pelargonium reniforme* subsp. *velutinum* for one flowering season. The legend describes the fitness surface, with the darkest grey indicating the highest levels of fitness.

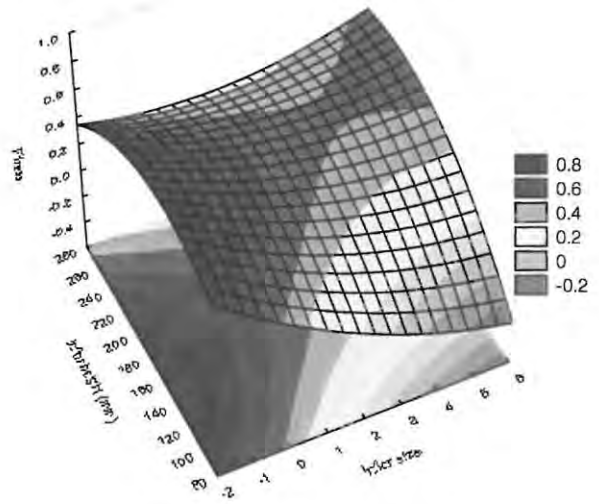

Figure 5.4: Fitness response surface of inflorescence size and inflorescence height of *Pelargonium reniforme* subsp. *velutinum* for one flowering season. The legend describes the fitness surface, with the darkest grey indicating the highest levels of fitness.

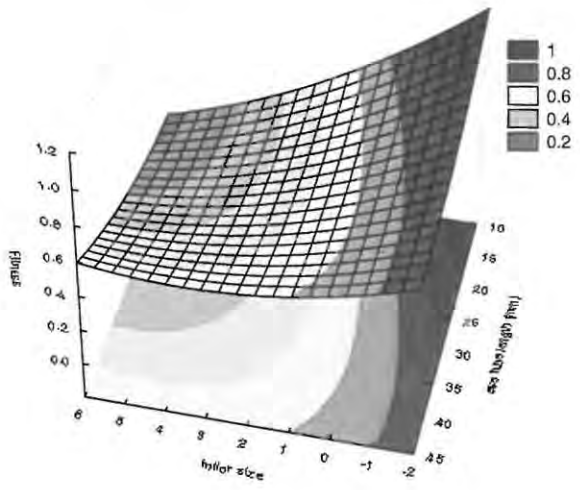

Figure 5.5: Fitness response surface of inflorescence size and average tube length of *Pelargonium reniforme* subsp. *velutinum* for one flowering season. The legend describes the fitness surface, with the darkest grey indicating the highest levels of fitness.

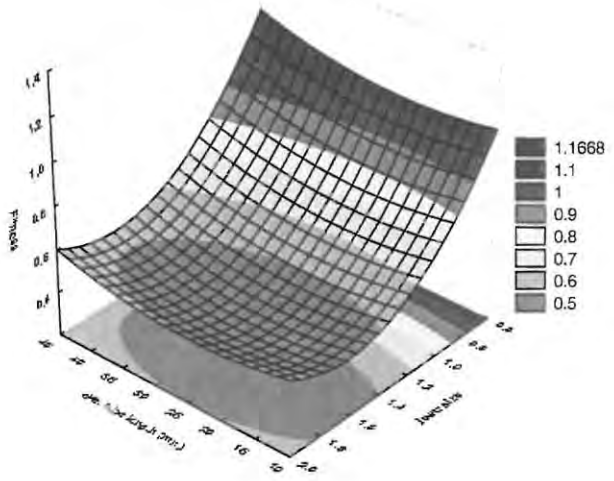

Figure 5.6: Fitness response surface of flower size and average tube length of *Pelargonium reniforme* subsp. *velutinum* for one flowering season. The legend describes the fitness surface, with the darkest grey indicating the highest levels of fitness.

5.4. Discussion

5.4.1. Selection in *Pelargonium reniforme* subsp. *velutinum*

Selection gradients (both linear and non-linear) are given for one flowering season only, and thus represent only the phenotypic selection occurring in this one flowering season. Should additional flowering seasons be included in a similar analysis, it may be possible to gauge the effects of continual directional selection on particular traits and this estimate the resultant evolutionary change within the population. It is important to be able to visualise the selection surfaces as well as review the selection gradients and differentials (Brodie *et al.* 1995). Although the gradients are able to describe the overall selection response, graphic representation allows for complete interpretation of the selection surfaces. This can be seen in Figures 5.1 to 5.6 which show the quadratic selection surfaces described by Table 5.4.

Assumptions of normality are violated for both relative fitness and one of the traits: size of inflorescence. As measures of fitness are usually discrete data, violations of the assumption of normality are common for fitness variables. In addition, results may still be interpreted as estimations of selection (Kelly 1992). The assumptions most selection studies quote, usually of normality, actually apply only to the assigning of significance to relationships, rather than the fitting of curves (Brodie & Janzen 1996). Kelly (1992) uses data that is not normally distributed in standard regression techniques and thus they are simply estimations of fitness gradients as the data do not fit the assumption of normality.

Expected linear (directional) selection occurred for inflorescence height, where higher inflorescences were positively selected for. Both inflorescence and flower size were negatively selected for. This is unusual, considering that often, size of floral display is heavily selected for. In this study, however, pollinators do not influence the fitness measure, and the resultant selection gradients can be attributed only to environmental factors. Flower number is positively selected for in *Lobelia* (Johnston 1991). Similarly, increased flower production was consistently selected for over three seasons for wild

radish (*Raphanus raphanistrum*), increased ovule number and flower size was also positively selected for through female fitness in two and one of the seasons respectively (Conner *et al.* 1996a). There is little evidence of selection for floral morphology through male fitness in the same species however, although flower number was also positively selected for (Conner *et al.* 1996b). Non-linear gradients show that stabilizing selection is occurring in inflorescence size and flower size and disruptive selection for inflorescence height. Interpretation of the fitness surfaces in figures 5.1 to 5.6, show a different selection trend for the traits. Large flower size is selected for, which is expected for most floral selection. Inflorescence height shows a little selection for taller inflorescences despite the large non-linear selection gradient for the trait.

Selection gradients however, do not always reflect expected directions. Often, selection gradients are completely different over more than one selection episode (usually one flowering season). These results could be an artefact of the environmental conditions of this particular flowering season, and could be reflect expected norms if measured in another selection episode. Kelly (1992) discovered both temporal and spatial variation in selection on morphological and life-history traits of *Chamaecrista fasciculata*, indicating that selection may not be consistent over a selection episode. Uneven phenotypic selection over successive selection episodes reflects only phenotypic selection. Overall directional selection over generations would result in evolutionary (genetic) change. This is only possible to show over a number of generations, rather than just one flowering season. The importance of continuing the study over a number of flowering seasons and more importantly, over a number of generations, is emphasised by Johnston (1991). Such an extended analysis would certainly indicate whether or not even weak selection occurs consistently over generations and thus could have a large evolutionary effect.

Little linear selection or non-linear selection occurs for floral tube length. As this was thought to be the primary isolating morphological trait between the two flower forms, disruptive selection classic to hybrid zones was expected to be exhibited for this population. Should the fitness measure have been influenced by pollinators, selection for tube length should have been large. The linear selection gradient is near to zero, the non-

linear selection gradient is also extremely low for this trait. Fitness surfaces also show very little selection for one particular tube size, where clear disruptive selection is expected for the trait. Results such as these may be attributed to only one flowering season, even if they had occurred for a fitness measure influenced by pollinators. In a study on selection of corolla length of the wasp-pollinated *Calathea ovandensis* Schemske and Horvitz (1988) discovered that there is little directional selection over three flowering seasons. A study of the behaviour of the pollinators revealed consistent more effective pollination by short-tongued insects and so it was hypothesised that the shorter the length of the pollinator's tongue, the more fruit set and thus the higher the fitness. This was shown to be true only for one of the flowering seasons, where one pollinating species was particularly abundant. The authors conclude that variation is not consistent over time because of changes in the pollinator assemblage. Although there was strong directional selection in one of the flowering seasons, the other two measured seasons showed no significant selection. The result is that although selection can occur over one flowering season, this does not necessarily translate to an evolutionary effect unless it occurs consistently over a number of generations (Schemske & Horvitz 1988). A positive selection for spur length was found to be consistent over three flowering seasons in the orchid *Platanthera bifolia* (Maad 2000). Positive selection for corolla tube width was discovered in response to pollinator action in *Ipomopsis aggregata* (Campbell *et al.* 1991). Strong selection in this *P. reniforme* population could occur for either long- or short-tubed flowers in other flowering seasons for a fitness measure involving the pollinators. Even the overall directional selection over generations (impossible for this study to estimate), may result in disruptive selection for either short- or long-tubed flowers rather than those in the middle.

Selection is often assumed to be weak for most traits. But, Kingsolver *et al*, (2001) discovered that selection may often be much stronger than authors describe. The average linear selection gradient is given as 0.16 in a study by Kingsolver *et al.* (2001) on 63 studies of selection. A selection gradient of this magnitude is often considered weak, but may be considered strong depending on the point of view, including heritability of the trait and the effects of correlated traits (Conner 2001). In this study, linear selection

gradients are particularly weak for average tube length, but particularly strong for both inflorescence size and height. The linear selection gradient for flower size is average. If these strong selection gradients were to occur over a number for generations, and the trait was highly heritable, the potential for evolutionary response to such selection is high.

Non-linear selection gradients average 0.1, indicating that quadratic selection is typically weak (Kingsolver *et al.* 2001). Kingsolver *et al.* (2001) discovered an overall equal frequency of stabilizing and disruptive selection (negative and positive non-linear selection gradients respectively). Conner (2001) attributes this partially to the tendency of researchers to choose traits that are expected to be undergoing directional selection as, according to evolutionary theory, stabilising selection should occur more often than disruptive selection. In this study, non-linear selection gradients are extremely high, with the highest value being 3.59 of inflorescence size. This indicates a strong selection of this trait for this particular flowering season. The lowest value is 0.05 of the relationship between average tube length and flower size, indicating very little selection occurring for this relationship. As with linear selection gradients, the effect of these values is better gauged over a number of generations of the same populations, which would indicate evolutionary response to selection.

5.4.2. Conclusions and future expansions

The results of this study, although a fair indication of selection occurring over one flowering season in one population of *Pelargonium reniforme* subsp. *velutinum*, may be biased as the fitness measure used was not a direct reflection of overall fitness for one flowering season. If in future flowering seasons, more flowers actually set seed, seed mass or number would be a far better reflection of overall fitness. As with all such studies, a larger sample size will ensure robust statistical analyses. Sample sizes should be as large as the population studied allows. More than one flowering season would indicate whether some of the linear and non-linear selection described in this study occurs consistently over time. Ideally, the study should be done over generations with

measures of lifetime fitness to be able to gauge evolutionary response. However, this is impossible for a long-lived clonal plant such as *P. reniforme*, as such a study will have to be conducted over an impractically long time period.

Although directional selection was shown for all investigated traits in the study, these may not necessarily occur consistently over a number of flowering seasons, which is important to the evolutionary response to this phenotypic selection. The study should ideally be continued over a number of flowering seasons utilising pollinator information to determine the driving force of any selection occurring over a number of selection episodes. This preliminary investigation showed some interesting results. Importantly, the investigation discovered which traits could be best measured and identified the use of seed set as a fitness measure as a problem.

5.5. References

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Chapter 6: Conclusions

Analyses and description of patterns reveal that there is no basis for the division of *Pelargonium reniforme* into two subspecies, namely *P. reniforme* subsp. *reniforme* and *P. reniforme* subsp. *velutinum*. This was shown both in analyses of morphometrics data (Chapter 2) as well as ISSR data (Chapter 3). In addition to this, both genetics and morphometrics data showed little reason apart from flower colour, to separate *P. sidoides* from *P. reniforme*.

If the section (*Reniformia*) to which both *P. reniforme* and *P. sidoides* belong, were to be reviewed, it is recommended that floral characters usually unavailable on herbarium specimens, should be considered. In addition to this, the basis for the current classification of *P. sidoides* is also weak. Future studies should be able to clarify these taxonomic affinities by assessing gene flow between taxa and assessing the criteria for classification of the taxa within section *Reniformia*.

Both morphometric (Chapter 2) and genetic (Chapter 3) data show a division of the short- and long-tubed floral forms of *P. reniforme* subsp. *velutinum*. As these groups were divided within the morphometric analysis primarily based on flower colour variables, flower colour is thus an important floral trait. ISSR data reflect this division of floral forms (Chapter 3). Long-tubed populations of *P. reniforme* subsp. *velutinum* form a separate cluster to the rest of *P. reniforme* and *P. sidoides*, which form dispersed population clusters. Short-tubed *P. reniforme* subsp. *velutinum* was grouped with populations of *P. reniforme* subsp. *reniforme*. This is interesting, especially considering the flower colours of these two forms are very similar (Chapter 4). There is thus more evidence from both morphometric and genetic data for the separation of the two floral forms of *P. reniforme* subsp. *velutinum* than for the division of the already-described subspecies themselves.

Genetic analyses of populations (Chapter 3), revealed a degree of population-level genetic isolation. Most interestingly, the Grahamstown population consisting of both

long-and short-tubed individuals of *P. reniforme* subsp. *velutinum*, formed two separate clusters, indicating that there is no gene flow between long- and short-tubed flowers despite them having a sympatric distribution (or coming into secondary contact). However, the use of additional primers may refute these results. There is a dramatic difference in the average hypanthium lengths, nectar volumes and colours of the long- and short-tubed flowers (Chapter 4). This kind of morphological differentiation can account for the lack of gene flow occurring between the two groups. Pollination is unlikely to occur between long and short-tubed flowers by long-tongued pollinators, but may be common by short-tubed pollinators. Long-tubed flowers, however, show comparatively low seed set for a cross with short-tubed flowers, indicating there may be some degree of incompatibility between the two forms. This would also account for the dramatic genetic division of two floral forms of the same species occurring sympatrically.

As there is clear genetic differentiation between long- and short-tubed flowers in the Grahamstown population, it was expected that each of the flower forms would be undergoing some directional selection. Certainly, pollinators are clearly the reason for the maintenance of floral differences, if not their initial divergence. An analysis of selection over one flowering season (Chapter 5) however, showed little directional selection acting on what was identified as the most important isolating floral trait: hypanthium length. The analysis was conducted over only one flowering season, selection for floral traits could change over a series of selection episodes (flowering seasons). Also, the sample size was small due to the low levels of flowering for the season, an increased sample size may provide more robust information on selection gradients.

Future studies on the Grahamstown population in which long- and short-tubed floral forms of *P. reniforme* subsp. *velutinum* occur sympatrically (Chapter 1), should include a continuation of the measurements of selection as well as more pollinator observations. This is important, as the flowering season (2005-2006) studied for this thesis was particularly poor, with relatively sparse flowering, very low levels of seed set and low pollinator activity relative to previous years (CI Peter pers. comm.). A continuation of the selection study (Chapter 5) over a number of flowering seasons, would give a much better indication of overall phenotypic selection within the population, and possible allow

the inference of evolutionary response of the species to phenotypic selection. The preliminary study allowed for the assessment of traits that can be easily measured for continued monitoring of the population. Further observations of pollinators and their behaviour would be valuable. Detailed information on pollinators of the two forms is lacking in this thesis (Chapter 4) due to the poor flowering season, as is information regarding pollinator constancy, choice and visitation to either long- or short-tubed flowers should be monitored.

The evidence suggests that at this time, selection is largely stabilising and floral differences are maintained, even when floral forms occur sympatrically. This genetic isolation is a result of the very different floral traits of the floral forms and the resulting response of pollinators. It is suggested that a review of section *Reniformia* into which both *P. reniforme* and *P. sidoides* fall, should be undertaken. Further studies of pollination biology and selection in populations of *P. reniforme* would help to clarify both patterns and processes.

Appendix 1: specimens used in morphometric analysis

Collector	Collector's number	coordinates	Locality	Identified
Selmar Shonland Herbarium, Grahamstown				
J Cruden	410		PE	
L Britten	2510		Bathurst	
LL Britten	1783		PE	
RA Dyer	1428		Botha's Hill	
RA Dyer	66		Grahamstown	
EEA Archibald	3930		Alexandria	
EEA Archibald	3970		Alexandria	
EEA Arcibald	4023		Alexandria	
R Schlechter	2682		Grahamstown	
MJ Wells	2934		Alexandria	
JE Reed	53		Grahamstown	
JE Reed	61		Grahamstown	
Troughton	417		PE	
E Brink	318		Grahamstown	
Booi	12		Grahamstown	
AR Palmer			Andries Vosloo Kudu Reserve	
TE Skotile	14	3326BC	Grahamstown	
W Howe		3326BC	Grahamstown	
GJ ne Bie	2		Settlers dam	
A Jacot Guillarmod	9275		Grahamstown	
RM Cowling		3424BB	Humansdorp	
RM Cowling	311	3424BB	Humansdorp	
B Schrire	1995	3325CA	Tafelberg	
JO Wirminghaus	55	3326BD	Round Hill Oribit reserve	
W Howe			Bloukranz reserve. Grahamstown	
MW Stork	54	3326BC	Grahamstown	
M Lipskey	13		PE	
t Dold	11		Grahamstown	
AR Palmer	2606	3326AC	Alicedale	
J Chan	171	3326BA	Ecca Reserve	
K Bowker		3326AB	Carlisle Bridge	
T Doubell	22		Grahamstown	
T Dold	1874	3327AC	Peddie	
T Dold	1667	3327DB	Peddie	
P brown	19980513/pb/1	3424BB	Humansdorp	
HH Burrows	5325	3325DC	PE	
CJ Kayombo	3733	3326BC	Grahamstown	
HJ Vanderplank			PE	
S Ramdhani and S vetter	361	3326AD	Salem	

Compton Herbarium				
W Tyson	2899	3227CD	Kingwilliamstown	
RH Compton	4194		Uniondale	
WF Burken	7828		Grahamstown	
WF Barker	6988		Albany	
B Maguine	537		Swellendam	
LE Taylor	5972	3326CA	Grahamstown	
MC Olivier	1745	3325BB	Somerset East	
L Hugo	792	3323DC	Louterwater	
L Hugo	1359	3325CC	PE	
DS Wagner			Humansdorp	
KA Dahlstrand	2712	3325DC	PE	
HG Fourcade	5937		Mond Plaate	
HG Fourcade	1144		Humansdorp	
E van Jaarsveld	7688	3324CA	Steytlerville	
A Batten	1/71		East London	
JM Wurts	2061		Humansdorp	
EJ van Jaarsveld	3716	3325DC	PE	
Bolus Herbarium				
H Bolus	8307	3325DC	PE	*P. reniforme* subsp. *reniforme* (det. Dreyer 1990).
CE Fries			PE	JJA van der Walt 1978
J Cook	1381/29	3325DC	PE	*P. reniforme* subsp. *reniforme* (det. Dreyer 1990).
FM Leighton	3074	3424BB	Humansdorp	*P. reniforme* subsp. *reniforme* (det. Dreyer 1990).
GE Gibbs Russell	3706	3126DD	Alice	
RH Compton	5221	3323DC	Lautewater	*P. reniforme* subsp. *velutinum* (det. Dreyer 1990),
E Esterhuysen	13637	3323DD	Joubertina.	*P. reniforme* subsp. *velutinum* (det. Dreyer 1990),
RH Compton	5295	3324CC	Uniondale div	*P. reniforme* subsp. *velutinum* (det. Dreyer 1990),
EE Galpin	184	3326CB	Alexandria	*P. reniforme* subsp. *velutinum* (det. Dreyer 1990),
Bertha Rogers		3326AB	Grahamstown	*P. reniforme* subsp. *velutinum* (det. Dreyer 1990),

West	223	3325DC	PE	*P. reniforme* subsp. *velutinum* (det. Dreyer 1990),
FM Leighton	3075	3326CA	Bushman's river	*P. reniforme* subsp. *velutinum* (det. Dreyer 1990),
C Goulinis	23866	3227CA	Kingwilliamstown	*P. reniforme* subsp. *velutinum* (det. Dreyer 1990),
HG Fourcade	1144	3324CD	Humansdorp	*P. reniforme* subsp. *velutinum* (det. Dreyer 1990),
E Esterhuysen	24, 220	3323DD	Jourbertina.	P. reniforme subsp. *velutinum* (det. Dreyer 1990),
E. Esterhuysen	7588	3327BA	Chalumna	*P. reniforme* subsp. *velutinum* (det. Dreyer 1990),
RH Compton	4149	3323DC	Lauterwater	*P. reniforme* subsp. *velutinum* (det. Dreyer 1990),
Hall	668/24	3226CA	Bedford	*P. reniforme* subsp. *velutinum* (det. Dreyer 1990),
FM Leighton	2784	3227AD	Cathcart	*P. reniforme* subsp. *velutinum* (det. Dreyer 1990),
HG Fourcade	1338	3323CB	Uniondale	*P. reniforme* subsp. *velutinum* (det. Dreyer 1990),
HG Fourcade	4316	3424BB	Jeffery's Bay	*P. reniforme* subsp. *velutinum* (det. Dreyer 1990),
WE Barker	1393	3227AC	Cathcart	*P. reniforme* subsp. *velutinum* (det. Dreyer 1990),
National Herbarium				
JL Sidey	695	3227AD	Cathcart	*P. reniforme* subsp. *velutinum* (det. Dreyer 1990),
EE Galpin	10663	3326CB	Alexandria	*P. reniforme* subsp. *velutinum* (det. Dreyer 1990),
Thode	A753	3324CD	Humansdorp	*P. reniforme* subsp. *velutinum* (det. Dreyer 1990),
M de Vries	132	3227DA	Amabele	*P. reniforme* subsp. *velutinum* (det. Dreyer 1990),
GE Gibbs Russell	3216	3226DD	Alice	*P. reniforme* subsp. *velutinum* (det. Dreyer 1990),
L. Hugo	792	3323DC	Louterwater	*P. reniforme* subsp. *velutinum* (det. Dreyer 1990),

van Breaa	1165	3323DD	Jourbertina	*P. reniforme* subsp. *velutinum* (det. Dreyer 1990),
GE Gibbs Russell	3706	3226DD	Alice	
E. Esterhuysen	24,220	3323DD	Uniondale	*P. reniforme* subsp. *velutinum* (det. Dreyer 1990),
L. Hugo	1359	3325CC	PE	
JE Victor and DB Hoare	478		Amatola mountains.	
JJA van der Walt	565		PE	*P. reniforme* subsp. *reniforme* (det. Dreyer 1990).
FA Ropens	22528		Plettenberg bay	*P. reniforme* subsp. *velutinum* (det. Dreyer 1990),
H Joffe	801	3324CD	Suuranysberge	
P Vorster	2290	3326BC	Grahamstown	
AJ Hall-Martin	6699	3325DA	Addo National Park.	
Field-collected specimens				
L. de Wet	G7	32°59'56.7"S 26°51'29.9"E	Double Drift game reserve	*P. reniforme* subsp. *velutinum* (det. de Wet 2006).
L. de Wet	G13	32°59'56.7"S 26°51'29.9"E	Double Drift game reserve	*P. reniforme* subsp. *velutinum* (det. de Wet 2006).
L. de Wet	G1	32°59'56.7"S 26°51'29.9"E	Double Drift game reserve	*P. reniforme* subsp. *velutinum* (det. de Wet 2006).
L. de Wet	G16	32°59'56.7"S 26°51'29.9"E	Double Drift game reserve	*P. reniforme* subsp. *velutinum* (det. de Wet 2006).
L. de Wet	E4	32°59'56.7"S 26°51'29.9"E	Double Drift game reserve	*P. reniforme* subsp. *velutinum* (det. de Wet 2006).
L. de Wet	K17	33°49'57.74"S 25°33'58.15"E	Redhouse, PE	*P. reniforme* subsp. *reniforme* (det. de Wet 2006).
L. de Wet	K5	33°49'57.74"S 25°33'58.15"E	Redhouse, PE	*P. reniforme* subsp. *reniforme* (det. de Wet 2006).
L. de Wet	K20	33°49'57.74"S 25°33'58.15"E	Redhouse, PE	*P. reniforme* subsp. *reniforme* (det. de Wet 2006).
L. de Wet	K18	33°49'57.74"S 25°33'58.15"E	Redhouse, PE	*P. reniforme* subsp. *reniforme* (det. de Wet 2006).
L. de Wet	I1	32°59'56.7"S 26°51'29.9"E	Double Drift game reserve	*P. reniforme* subsp. *velutinum* (det. de Wet 2006).
L. de Wet	I4	32°59'56.7"S 26°51'29.9"E	Double Drift game reserve	*P. reniforme* subsp. *velutinum* (det. de Wet 2006).

L. de Wet	I14	32°59'56.7"S 26°51'29.9"E	Double Drift game reserve	*P. reniforme* subsp. *velutinum* (det. de Wet 2006).
L. de Wet	I2	32°59'56.7"S 26°51'29.9"E	Double Drift game reserve	*P. reniforme* subsp. *velutinum* (det. de Wet 2006).
L. de Wet	C4	33°14'35"S 26°35'07"E	Botha's Hill	*P. reniforme* subsp. *velutinum* (det. de Wet 2006).
L. de Wet	C3	33°14'35"S 26°35'07"E	Botha's Hill	*P. reniforme* subsp. *velutinum* (det. de Wet 2006).
L. de Wet	C9	33°14'35"S 26°35'07"E	Botha's Hill	*P. reniforme* subsp. *velutinum* (det. de Wet 2006).
L. de Wet	C10	33°14'35"S 26°35'07"E	Botha's Hill	*P. reniforme* subsp. *velutinum* (det. de Wet 2006).
L. de Wet	L25	33°55'25.22"S 25°28'20.68"E	Bridgemead, PE	*P. reniforme* subsp. *reniforme* (det. de Wet 2006).
L. de Wet	L21	33°55'25.22"S 25°28'20.68"E	Bridgemead, PE	*P. reniforme* subsp. *reniforme* (det. de Wet 2006).
L. de Wet	L5	33°55'25.22"S 25°28'20.68"E	Bridgemead, PE	*P. reniforme* subsp. *reniforme* (det. de Wet 2006).
L. de Wet	L8	33°55'25.22"S 25°28'20.68"E	Bridgemead, PE	*P. reniforme* subsp. *reniforme* (det. de Wet 2006).
L. de Wet	J21	33°46'08.63"S 25°39'49.41"E	Coega, PE	*P. reniforme* subsp. *reniforme* (det. de Wet 2006).
L. de Wet	J6	33°46'08.63"S 25°39'49.41"E	Coega, PE	*P. reniforme* subsp. *reniforme* (det. de Wet 2006).
L. de Wet	J9	33°46'08.63"S 25°39'49.41"E	Coega, PE	*P. reniforme* subsp. *reniforme* (det. de Wet 2006).
L. de Wet	J25	33°46'08.63"S 25°39'49.41"E	Coega, PE	*P. reniforme* subsp. *reniforme* (det. de Wet 2006).
L. de Wet	D8	32°59'56.7"S 26°51'29.9"E	Double Drift game reserve	*P. reniforme* subsp. *velutinum* (det. de Wet 2006).
L. de Wet	D5	32°59'56.7"S 26°51'29.9"E	Double Drift game reserve	*P. reniforme* subsp. *velutinum* (det. de Wet 2006).
L. de Wet	D6	32°59'56.7"S 26°51'29.9"E	Double Drift game reserve	*P. reniforme* subsp. *velutinum* (det. de Wet 2006).
L. de Wet	D7	32°59'56.7"S 26°51'29.9"E	Double Drift game reserve	*P. reniforme* subsp. *velutinum* (det. de Wet 2006).

L. de Wet	E8	32°59'56.7"S 26°51'29.9"E	Double Drift game reserve	*P. reniforme* subsp. *velutinum* (det. de Wet 2006).
L. de Wet	E11	32°59'56.7"S 26°51'29.9"E	Double Drift game reserve	*P. reniforme* subsp. *velutinum* (det. de Wet 2006).
L. de Wet	E13	32°59'56.7"S 26°51'29.9"E	Double Drift game reserve	*P. reniforme* subsp. *velutinum* (det. de Wet 2006).
L. de Wet	E9	32°59'56.7"S 26°51'29.9"E	Double Drift game reserve	*P. reniforme* subsp. *velutinum* (det. de Wet 2006).
L. de Wet	A7	33°07.554'S 26°43.124'E	Kudu Reserve	*P. reniforme* subsp. *velutinum* (det. de Wet 2006).
L. de Wet	A6	33°07.554'S 26°43.124'E	Kudu Reserve	*P. reniforme* subsp. *velutinum* (det. de Wet 2006).
L. de Wet	A8	33°07.554'S 26°43.124'E	Kudu Reserve	*P. reniforme* subsp. *velutinum* (det. de Wet 2006).
L. de Wet	A1	33°07.554'S 26°43.124'E	Kudu Reserve	*P. reniforme* subsp. *velutinum* (det. de Wet 2006).

Appendix 2: Discriminant function analysis results

Table 1: Discriminant function analysis summary (Analysis 1: all data standardised) variables in the model. Step 16, 5 groups. Wilk's Lambda: 0.03 approx. $F_{(64, 468)} = 24.91$ $p<0.05$

	Wilks' Lambda	Partial Lambda	F-remove (4,119)	p-level	Toler.	1-Toler. (R-Sqr.)
habit	0.017091	0.176242	139.0524	0.000000	0.580845	0.419155
petal length	0.006317	0.476834	32.6407	0.000000	0.547494	0.452506
petal width	0.004674	0.644375	16.4187	0.000000	0.470218	0.529782
leaf length	0.003446	0.874123	4.2841	0.002834	0.155825	0.844175
sinus	0.004175	0.721546	11.4809	0.000000	0.627961	0.372039
functional hypanthium length	0.003205	0.939848	1.9041	0.114211	0.008958	0.991042
no. of flowers	0.004031	0.747210	10.0648	0.000000	0.686612	0.313388
pedicel length	0.003506	0.859153	4.8771	0.001121	0.778617	0.221384
petiole length	0.003401	0.885745	3.8376	0.005713	0.475648	0.524352
last branching length	0.003396	0.886885	3.7944	0.006114	0.719404	0.280596
sepal length	0.003367	0.894655	3.5030	0.009659	0.568623	0.431377
leaf width	0.003209	0.938739	1.9414	0.107996	0.158851	0.841149
flower height	0.003158	0.953711	1.4439	0.223774	0.359155	0.640845
hypanthium length	0.003148	0.956793	1.3434	0.257895	0.009183	0.990817
internode length	0.003170	0.950170	1.5602	0.189415	0.740636	0.259364
flower width	0.003114	0.967221	1.0082	0.406116	0.487595	0.512405

Table 2: Discriminant Function Analysis Summary (Analysis 1: all data unstandardised) variables in the model. Step 14, 4 groups. Wilk's Lambda: 0.09 approx. $F_{(42,362)} = 10.815$ $p<0.005$.

	Wilks' Lambda	Partial Lambda	F-remove (3,122)	p-level	Toler.	1-Toler. (R-Sqr.)
inflorescence height	0.117063	0.768097	12.27801	0.000000	0.487718	0.512282
first branching length	0.112492	0.799312	10.21041	0.000005	0.680372	0.319628
last branching length	0.114161	0.787627	10.96521	0.000002	0.700036	0.299964
functional hypanthium length	0.093487	0.961805	1.61494	0.189399	0.007879	0.992122
no. of flowers	0.096728	0.929581	3.08067	0.030033	0.691017	0.308983
flower height	0.099160	0.906777	4.18080	0.007428	0.649164	0.350836
petal length	0.091435	0.983390	0.68689	0.561726	0.646782	0.353218
leaf length	0.099588	0.902878	4.37450	0.005812	0.122416	0.877584
leaf width	0.095729	0.939279	2.62893	0.053266	0.120189	0.879811
pedicel length	0.093973	0.956824	1.83506	0.144339	0.741517	0.258483
order of branching	0.092872	0.968171	1.33695	0.265553	0.765746	0.234255
sinus	0.092815	0.968766	1.31114	0.273915	0.720016	0.279984
petiole length	0.092727	0.969688	1.27124	0.287321	0.414182	0.585818
hypanthium length	0.092563	0.971402	1.19724	0.313790	0.007828	0.992172

Table 3: Discriminant Function Analysis Summary (Analysis 2: identified standardised) variables in the model. Step 14, 4 groups. Wilk's Lambda: 0.004 approx. $F_{(42,172)} = 22.81$ $p<0.005$

	Wilks' Lambda	Partial Lambda	F-remove (3,58)	p-level	Toler.	1-Toler. (R-Sqr.)
habit	0.013285	0.286138	48.23326	0.000000	0.344604	0.655397
petal length	0.013901	0.273459	51.36588	0.000000	0.569823	0.430177
petal width	0.009242	0.411283	27.67410	0.000000	0.533680	0.466320
pedicel length	0.005285	0.719278	7.54546	0.000240	0.491276	0.508724
petiole length	0.004816	0.789315	5.16048	0.003135	0.362149	0.637851
first branching length	0.004482	0.848140	3.46166	0.021950	0.625945	0.374055
functional hypanthium length	0.004361	0.871577	2.84867	0.045201	0.009648	0.990352
leaf width	0.004689	0.810640	4.51613	0.006488	0.382589	0.617412
sepal length	0.004248	0.894745	2.27431	0.089456	0.584177	0.415823
internode length	0.004352	0.873420	2.80189	0.047778	0.650240	0.349760
last branching length	0.004469	0.850592	3.39593	0.023707	0.492082	0.507918
no. of flowers	0.004275	0.889169	2.40982	0.076128	0.613710	0.386290
order of branching	0.004186	0.908099	1.95656	0.130571	0.688956	0.311044
hypanthium length	0.004182	0.908921	1.93732	0.133591	0.009897	0.990103

Table 4: Discriminant Function Analysis Summary (Analysis 2: identified unstandardised) variables in the model. Step 7, 2 groups. Wilks' Lambda: 0.20 approx. $F_{(7,65)}$ =36.19 p<0.0

	Wilks' Lambda	Partial Lambda	F-remove (1,65)	p-level	Toler.	1-Toler. (R-Sqr.)
inflorescence height	0.351003	0.581649	46.75132	0.000000	0.613602	0.386398
first branching length	0.230777	0.884663	8.47428	0.004929	0.814080	0.185920
flower width	0.221797	0.920480	5.61530	0.020783	0.436361	0.563639
leaf length	0.211789	0.963978	2.42896	0.123968	0.711497	0.288503
flower height	0.213872	0.954591	3.09199	0.083386	0.379640	0.620361
petal width	0.209563	0.974219	1.72014	0.194288	0.648997	0.351003
internode length	0.209014	0.976778	1.54535	0.218289	0.849680	0.150320

Table 5: Discriminant Function Analysis Summary (Analysis 3: colour standardised) variables in the model. Step 11, 3 groups. Wilks' Lambda: 0.01 approx. $F_{(22,36)}$ =15.21 p<0.05

	Wilks' Lambda	Partial Lambda	F-remove (2,18)	p-level	Toler.	1-Toler. (R-Sqr.)
functional hypanthium length	0.012706	0.742301	3.124455	0.068426	0.726850	0.273150
first branching length	0.013335	0.707288	3.724667	0.044296	0.686670	0.313330
colour 1	0.015193	0.620786	5.497744	0.013692	0.484519	0.515481
habit	0.014658	0.643449	4.987113	0.018907	0.508954	0.491047
order of branching	0.011733	0.803889	2.195573	0.140206	0.583438	0.416562
flower height	0.013476	0.699877	3.859394	0.040290	0.369984	0.630016
leaf width	0.010866	0.868035	1.368242	0.279793	0.652096	0.347904
petal length	0.013747	0.686078	4.118048	0.033679	0.491567	0.508433
flower width	0.013691	0.688881	4.064668	0.034938	0.247035	0.752965
last branching length	0.012230	0.771215	2.669900	0.096511	0.585974	0.414026
sepal length	0.011552	0.816475	2.022992	0.161245	0.403046	0.596954

Table 6: Discriminant Function Analysis Summary (Analysis 3: colour unstandardised) variables in the mode. Step 11, 3 groups. Wilks' Lambda: 0.02 approx. $F_{(22,36)} = 11.11$ $p<0.05$

	Wilks' Lambda	Partial Lambda	F-remove (2,18)	p-level	Toler.	1-Toler. (R-Sqr.)
inflorescence height	0.025908	0.636614	5.13729	0.017175	0.737247	0.262753
functional hypanthium length	0.027969	0.589691	6.26224	0.008622	0.009629	0.990371
first branching length	0.023097	0.714079	3.60365	0.048274	0.424671	0.575329
hypanthium length	0.028335	0.582085	6.46165	0.007671	0.009395	0.990605
flower width	0.045306	0.364040	15.72255	0.000112	0.153834	0.846166
habit	0.038425	0.429225	11.96801	0.000495	0.207133	0.792867
sepal length	0.029405	0.560895	7.04577	0.005495	0.308380	0.691620
pedicel length	0.027116	0.608234	5.79694	0.011393	0.428674	0.571326
colour 1	0.024498	0.673248	4.36804	0.028417	0.213734	0.786266
Sinus	0.023337	0.706727	3.73476	0.043981	0.470170	0.529830
petal length	0.019743	0.835395	1.77335	0.198165	0.545488	0.454513