



Intraspecific variation and ecology of a highly restricted paleoendemic (*Witsenia maura*) in the South-Western Cape



Fig 1. *Witsenia maura* on Mt. Lebanon

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Systematics Thesis
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Abstract

Witsenia is a monospecific genus of the putatively basal group, the woody Iridaceae. This upright iris has extremely long black and yellow flowers (see fig. 1) that are thought to have been pollinated by an extinct Sunbird. The role of the unusual black floral colouration is investigated as this colour is seldom associated with bird pollination. This plant typically exists in discreet and restricted populations in wet habitats in the South Western Cape (South Africa). The restricted nature of the plant is peculiar as it occurs in either low or high altitudes, yet appears to be extremely sensitive to altitude. Popular belief suggests that *Witsenia maura* occurs in the Peninsula only, and results from this study show the Peninsula population to be genetically separate from other populations, reflecting a long term separation. Samples from nine populations are sequenced to investigate haplotypic variation within the species, and dispersal of ancestral populations. This thesis investigates the current knowledge of *Witsenia*, its ecology, history and distribution. An examination of flowers under UV light reveals the first evidence of UV nectar guides in an ornithophilous flower. Conservation issues are also addressed, and it is established that although small and apparently shrinking due to global warming, populations are nonetheless viable if managed properly. A molecular study of the species and examination of its variation revealed exceptional haplotype diversity. This diversity can best be explained by swamps acting as refugia during interglacial periods.

Keywords: paleoendemism, refugia, intraspecific variation, chloroplast DNA, vicariance distribution, biogeography, *Witsenia maura*, pollination.

Chapter 1: History and Morphology

Introduction

Witsenia maura is a unique member of the Subfamily Nivenioideae (the woody Iridaceae) of the Iridaceae. It is a monotypic genus characterised by its long and tubular yellow flowers, borne in pairs on long, woody stems. Small populations are scattered in isolated boggy wetlands, from the Peninsula through to Riversdale. Despite this seemingly wide distribution, only about 22 populations exist totalling a few thousand plants exist.

These populations are thought to be dwindling due to climate change and as a result of the pollinator having gone extinct. The proof for this extinct pollinator lies in the 6cm long flower being nearly twice as long as the beak of any contemporary potential avian pollinator. As this bird is not pollinating, the plant is said to be relying on resprouting from its woody base. Populations appear to comprise adults alone, typically up to two metres high. A systematic study enables us to look at a populations in which no gene flow is purported to be

taking place, and which have been subject to extreme isolation. This study looks at the historical establishment of the current populations through the use of advanced genetic techniques. Sequencing is used to identify variation in the chloroplast intron, PSBA-TrnH. Introns are non-coding regions that are useful as they evolve relatively rapidly as they are not subject to evolutionary pressures. This makes them useful for studies such as intraspecific phylogeography and resolving species level phylogenies.

My objectives for this thesis were thus to revise and expand upon the current knowledge of the species. The first chapter deals with the historical background, taxonomy and morphology of the species. The second chapter clarifies the myths and speculation surrounding the distribution, pollination and reproduction of the plant. I also examined the adaptive value of a bizarre black marking present on the tepal tubes of flowers. The third chapter deals with a molecular analysis of the plant, aimed to explain the distribution and paleohistory of the restricted populations of this species.

Historical background

The first finding and description of *Witsenia maura* goes to the Father of botanical exploration in the Cape, Linnaeus, in 1771. He called the plant *Antholyza maura* on account of its vague similarity to *Antholyza*, the synonym for some of the taller *Babiana* species. It was described near the end of his life with no type specimen on which his description was based (Goldblatt 1983). At this stage Linnaeus probably did not conceive that he had stumbled upon one of the most basal and enigmatic members of the Iridaceae.

Some eleven years later, in 1782, Thunberg collected the plant again, described it and placed in its own genus *Witsenia*, as he did not feel it correctly placed in *Antholyza*. The councillor to the Chief Magistrate of Amsterdam, Nicholas Witsen, was the patron of botany and the sciences in the 1770's. This dates the

age of travels and exploration of Thunberg, whom Witsen encouraged and supported. Indeed in 1782 Thunberg discovered and described the new genus to honour Witsens assistance, as the genus *Witsena* in *Novum Generum Plantarum* 33 (1782). Finally in 1784, studies of Linnaeus came posthumously to light, and *Witsena* was finally changed to *Witsenia* where it has remained since with one minor exception (Goldblatt, 1983). Subsequent to the name *Witsenia* (L.) given posthumously by Linnaeus, Lamarck considered *Witsenia* part of *Ixia*, and named it *Ixia disticha*. This treatment was ignored by contemporary botanists and thus the name *Witsenia* remained firmly entrenched in botanical literature (Goldblatt, 1983).

The name *maura* comes from the Greek for dark or obscure. This possibly alludes to the dark green perianth tube grading to black in some populations. (Goldblatt, 1983). Lemaire's interpretation from 1846, suggests that the yellow tepals resemble the turbans that Jews wore in North Africa in past times. Maurus was the adjective for Moorish in Latin, and these Jews were referred to as Moors. (Goldblatt, 1983) The common name bokmakierie's tail refers to the flower that strongly resembles the colouration of the birds tail with the same name. Unfortunately, this plant appears not to attract this insectivorous bird, nor is there any case of mimicry or interaction between the two species, either sexual or otherwise. The Afrikaans name "Waaiertjie" is unexplained in Goldblatt (1983) although it probably refers to the way the plant bows and nods before the blowing (waa-ing) wind.

In 1790, a collection by Francis Masson made the plant first known to British botanists. Subsequently around 10 years later, seed was sent by James Niven who grew the plant at Kennedy's Nursery in Hammersmith, London. It first flowered in 1814, (Edwards, 1815, in Goldblatt, 1983), where a striking illustration was made in the *Botanical Register*. Paxton's *Magazine of Botany* (1841) graphically illustrates one of the final years of a living specimen in Britain (Goldblatt, 1983).

In recent years only 2 major studies have been done on the species. Goldblatt (1983) published a revision of the woody Iridaceae in which much of the morphological aspects were addressed. This culminated in a phylogeny of the subfamily, based on morphological and karyological evidence. Finally, Wright *et al.* (1983) produced a thesis on the facultative pollination of *Witsenia maura*.

Morphology

The shrubby growth form that characterises the woody Iridaceae, is unique amongst the Iridaceae, and rare in the monocots. Adamson (1936, in Goldblatt 1983) found that the woodiness of the woody Iridaceae is basically the same mechanism for all woody monocots (Goldblatt, 1983). The woody Iridaceae produce aerial stems from a woody underground caudex. Multiple fast growing shoots arise from this caudex after to a fire. These stems grow to a height of around one metre after two to three years, whereupon they flower and shoot growth rapidly diminishes (Goldblatt, 1983). Branching primarily occurs in axils just beneath the inflorescence. With the exception of a few major shoots that continue growth, the majority of shoots remain short and stubby and are referred to as 'spur shoots'. Spur shoots produce flowers in the subsequent growing season (Goldblatt, 1983). These major axes become top heavy with age and tend to sprawl or incline with age.

The deep-green leaves are narrowly lanceolate to linear. They leaves are bifoliate, only opening around 1mm from the stem (Goldblatt 1983). Leaves that tightly clasp the stem may produce a strong architecture that typically remains at least until the growth of new leaves. The leaves are distichously arranged, overlapping up the stem in a single plane, yet slightly twisted. The corkscrew-like twisting of the leaves up the stem is a hitherto unexplored feature of growth that may be a consequence of plant architecture, but may also perform a function in the plant.

Flowering takes place from January to August (Hall, 1980), with the main flowering period being April to August (Goldblatt & Manning, 2000), with peak flowering in mid July (at Bettys Bay). Peak flowering time seems to vary from locality to locality, and this might be a reflection of genetic diversity, or a difference in altitude. Capsules ripen for several weeks, finally dispelling the seed approximately a month and a half after flowering. (pers. obs.)

The basic inflorescence unit in the Iridaceae is termed a rhipidium. This is an inflorescence consisting of a series of flowers enclosed within large bracts (spathes) and developing on opposite sides of the axis alternately (in many Iridaceae (Goldblatt, 1983). The basic rhipidium of Iridaceae consists of a pair of large, opposed, herbaceous bracts, traditionally called spathes. These spathes enclose a monochasial cyme of flowers (Goldblatt 1983). For the woody genera, Weimarck coined the term a 'binate rhipidium', referring to two flowers that are enclosed by two opposed spathes that are coriaceous (leathery textured). Thus there are four bract-like structures per rhipidium, with only one bract. This makes the four bracts of the 'binate rhipidium' of *Witsenia* intriguing, as a simple rhipidium with two flowers would have only 3 bracts: two spathes and a floral bract (Goldblatt 1983).

Witsenia has a pseudopanicule of binate rhipidial units. Outer spathe pairs are prominent, pale green and emarginate, and are much larger than the membranous floral bracts. The pseudopanicule of *Witsenia maura* and *Nivenia argentea* consist of short lateral branches. This is most similar to *Aristea* with its central axis and short lateral branches. Goldblatt (1983) thus regards this as the basal (synapomorphic) condition for the Nivenioidea and the woody genera.

An inflorescence consists of a two long-lasting flowers within a rhipidium. These sessile flowers have their bases firmly entrenched within the coriaceous (skin-like or leathery) spathes that are probably protection against opportunistic nectar

thieves. The floral tube is long (up to 80 mm) with an increasingly dark green band becoming almost a black band in some populations, ending about three millimetres from the pappilose outer tepals. This pappilosity is yellow and observations of Goldblatt (1983) record that even the inner tepals become pappilose where they are exposed to the light. These yellow tepals are considered to have an attractant function for the pollinators.

The anthers are positioned so that they are addressed to the inner tepals and are introrsely dehiscent. The tepals require substantial force to prize them apart, the only entrance being a small hole around where the style protrudes. Goldblatt (1983) states that the tepals are so firmly closed, that only birds could be strong enough to prize apart the tepals to get at the nectar. The tepals are also free for a small distance down the tube, facilitating the extraction of nectar if a bird were to probe the flower from above. Possible nectarivorous birds with ranges that coincide with *Witsenia maura* are the Lesser Double Collared Sunbird (*Nectarinia chalybea*), the Malachite Sunbird (*Nectarinia famosa*) and the cape Sugarbird (*Promerops cafer*).

The following questions are investigated:

- 1) Was *Witsenia maura* pollinated by an extinct Sunbird?
- 2) Is *Witsenia maura* being cross-pollinated and thus producing seedlings?
- 3) Is the black section of the flower UV-reflective and if so, what is producing the reflection?
- 4) Do the Peninsula populations form a separate clade to the other populations?
- 5) Can dispersal of the Ancestral *Witsenia maura* be inferred from chloroplast DNA?
- 6) Do the current distributions reflect dwindling refugia of a doomed species?
- 7) Can prior climatic conditions of the Cape be inferred from the current distribution and history of *Witsenia maura*?

Chapter 2 : Studies on ecology and distribution.

Introduction

The morphology of the Nivenioideae separates it from all the other Iridaceae in the world. In this chapter, we show that it is not just the woodiness that makes *Witsenia* unique, but a number of other floral characters too. It is these floral characters that give rise to a variety of fanciful theories regarding pollination of the extraordinarily long flowers. These flowers are so much longer than any other bird pollinated flower in the cape, that it was suggested that an extinct giant Sunbird is the only bird that could possibly access the flowers. This has led to further observations that the plant is no longer reproducing, and as a consequence is only surviving in two sites in the Cape Peninsula. I address these assumptions and conclusions, favouring other evidence that suggests that there may be more to *Witsenia*'s survival than an extinct Sunbird.

Ecology & Distribution

Habitat

Witsenia maura is found primarily in perennially waterlogged seepages in south-facing slopes. In montane areas these are often in the upper reaches of a catchment. The plants are often associated with seepage and riverine specialists, notably *Cliffortia graminea* and *Osmitopsis asteriscoides* that nearly always co-occur (pers. obs.), although these plants have a more expanded distribution. Other co-occurring species are further explored in the chapter on paleoclimate. *Witsenia maura* populations in these areas are seldom more than tens of metres long, with their roots in the permanent water. Populations often occupy a mere few metres of suitable habitat. Only one exception to this waterlogged favouritism was seen, where a small population occupied a position between two streams possessing *Witsenia* on Mt. Lebanon. Due to the generally tall, shrubby nature

of the competing plants in this moist habitat, woodiness may be an adaptation to coping with a lack of light due to dense overstorey shading. Resprouting after fire is another way of competing with dense fire recruitment in a moist, yet nutrient poor landscape.

Dispersal

Goldblatt (1983) refers to the seed as shield-shaped, due to the lateral compression of the seed that is presumably an adaptation for wind dispersal. They may however be water dispersed, as these cork textured seeds float on water. The most likely scenario is evident in post-fire environments where seedlings appear to emerge within several metres of the resprouting parent plant. What is uncertain however, is whether there are seeds dispersed further afield, but these are not visible as seedlings, as they are in an unfavourable habitat for germination or growth. Recruitment from seed appears to be non-existent away from perennially saturated bog areas. The widespread dispersal of *Witsenia* (Cape Peninsula to Swellendam) may be explained by its wind or water dispersed seeds. In the Bignoniaceae, wind and water dispersed species are the most wide ranging. This contrasts with the mammalian dispersed species which tend to have restricted ranges with a high degree of endemism (Gentry, 1974).

Wright *et al.* (1989) investigated the role of a small elaisome like appendage on the seed (reported by Bond & Slingsby, 1983) that may be an attractant in ant dispersal. Ant dispersal seems unlikely given the wetness of the habitat in which *Witsenia* exists. They found however that ants were of minor importance in the dispersal of seeds. Gawith (1983) soaked seeds in water for three months, reporting a subsequent germination of 37%. This supports the hypothesis that the seeds are water-dispersed, as seeds often become waterlogged and lose their viability after such an extended period of water saturation (A. Harrower, pers. comm.). As the plants are usually found in relatively flat areas, Gawith (1983) considers this mechanism of water dispersal to be conducive to the species survival, as seeds would remain in relatively favourable (moist) habitats.

Distribution

This section has been placed in order to give accurate information as to plant localities extant today. These descriptions are also given to give the reader some sense of how and where the populations grow. In order to sample as many populations as possible, localities and past population information was gained from such diverse sources as herbarium specimens, the PRECIS database, retired botanists (A. Hall), farmers (T. de Villiers, P. le Roux), personal communication with botanical society members (F. Woodvine, P. Drewe) and an interested medical doctor from Riviersonderend (Dr. Phillip). Several 'travels' (Hutchinson's, Burchell's and Thunberg's) were also trawled for information with no success. This map (Figure 2) reflects the best knowledge of the distribution of the species in any account. GPS co-ordinates for all the populations have also been taken such that all the populations can be re-located.

Witsenia maura occurs from the Cape Peninsula in the north-west, south to Hermanus and then East to Riversdale. It is both spatially and altitudinally segregated into small, localized populations. In the Cape Peninsula, *Witsenia maura* occurred in numerous sites in Cape Point Nature Reserve in the Southern tip of the Cape Peninsula, northwards through to Silvermine Nature Reserve at around 300m altitude. A site is said to exist above the dam at Silvermine, although extensive searching of likely places revealed no plants. Another site was given by E. Esterhuysen (4972) as midway in Silvermine Valley. This population has not been seen since, and much of the area has been destroyed through housing developments and a golf-course in the lower reaches. Midway between Silvermine and Cape Point lies a locality given as Klawersvlei. This vlei located above Simonstown has been the site of a military base for a number of years. Alien species have invaded the streams and swamps, and despite two expeditions to the area, and two other attempts by botanists familiar with the plant, no population could be located. It is therefore likely that the plants have

been destroyed, either by the military base, or by the *Acacia* spp. Invading the area.

The Peninsula is proposed to act as an island for populations of *Witsenia maura* due to the lowland belt of sand surrounding the Peninsula – the Cape Flats. This area is perennially flooded, but lacks a suitable sandstone habitat in which *Witsenia* typically grows. The only reconstruction of the Cape Flats is an old palynological paper (Schalke, 1973). A suitable moist climate, capable of supporting mixed *Podocarpus* forest in the central Cape Flats, was found to have occurred in the middle pleniglacial. These wet intervals, derived from radiocarbon dating, occurred approximately 45000 and 36 500 B.P. The Aeolian sands are said to have been laid down in the lower pleniglacial between 50 000 and 60 000 B.P. (Schalke, 1973). There is therefore a window of opportunity at this time in which *Witsenia* could have crossed the Cape Flats.

Immediately South of the Cape Flats lies the mountains of the Hottentots Holland range and the Kogelberg. This has been cited on a number of occasions as a hotspot for diversity and paleoendemics. *Witsenia maura* proves no exception, having not only the largest population seen (Dawidskraal), low, medium and high altitude sites, but all of the haplotypes are present (see discussion). Populations occur across the entire East-West range of the Kogelberg, from Hangklip in the west, through Bettys Bay, Kleinmond, up to Elgin and eventually to the highest population at Groenberg in the east. An even further East population is recorded from 5 Beacon Ridge. It also had the only known North-facing population recorded: a site near Sir Lowrys Pass where alien infestations have presumably terminated the population.

The Hangklip population was last reported (Hall, 1980) as searched for, but not relocated, and was therefore considered extinct. Not knowing this, I set about finding the population, and on the second attempt chanced upon a single plant growing in the wettest area at the head of the stream that flows into skilpadvlei,

no more plants were found lower down the stream. A follow up to collect more material turned up a further 7 plants in the area, widely scattered in the *Berzelia* thicket and appearing very straggly. A more recent search revealed only a single plant. This difficulty in finding *Witsenia* typifies the disappointment in not finding a population, and the elation in relocating a population last collected many years before. With the availability of GPS (global positioning system) units, all subpopulations should be accurately mapped to ensure future surveys can locate the populations.

30 Km south of the Kogelberg lies Babilonstoring at 1300 metres altitude. Here *Witsenia* occurs in moist south-facing valleys from about 1100 metres upwards. A large population exists on the west side of the summit. West of Babilonstoring lies the Onrustriver mountains. These relatively short mountains have a herbarium record from 300 feet (E. Esterhuysen 11564). Here, below the overhanging plateau, were 3 small populations of plants found in three adjacent streams. One population consisted of about seven plants, another of about 20 and the easterly most had about 40 plants in a stream. No more plants were seen below these populations, nor did searching the dense streams to the peak yield any more populations.

South-east of this mountain lies the Kleinrivier mountains, where no extant populations have been found. However, a herbarium record giving a general location of 'kleinriver mountains' exists. I have intensively searched 3 major streams and many swamps till Maanskijkop, the peak at 1300m to no avail. I assume therefore that the population is yet to be found, or that the herbarium record refers to a population at the foot of Voelklip. Of this population, only a single plant may remain as a transplanted specimen that was the orphan of an unfortunate placement of a supermarket development on the lowlands. A good locality was given to me by Frank Woodvine of the Botanical Society of Hermanus, but I could not find the plant.

Three years ago, while swimming in Quark pool, in Vogelgat (Hermanus) I was amazed to find a single *Witsenia* growing at the edge of the pool. I queried the warden about this unlikely find, and he informed me that this must be a plant remaining from the transplant operation of the Voelklip population. The following year a fire ravaged the mountainside, followed by a 1 in 50 year flood. Having decided upon my third year project, I returned to the site, swam in the same pool, and failed to find the plant. The fire had burnt the plant back to the stump, and the subsequent floods had eroded the banks of the river such that the plant was sent to a watery grave. The fate of this Voelklip population is therefore apparent. Transplanted plants in two localities have both succumbed to nature. Being too few specimens to reproduce, in a relatively hostile environment, no specimens remain of this population. This was a critical population to sample, as it represents a lowland population that is in the east. The value of this sample will be discussed in the discussion in section 3.

Eastwards and at high altitude exists several relatively recently discovered populations from the farm Boskloof in the Akkedisberg mountains. These sites are all located within around 100m of the highest peak in the range. Thys de Villiers, the owner of the farm, was kind enough to drive me up in his car on two separate occasions. The first occasion yielded low quality DNA, as I was still collecting leaves in silica gel. Two localities were found, one in the main stream about 100m below the highest peak. This locality consisted of two populations of about 5m diameter, perched in the sluggish water above waterfalls in the stream. One of these populations was growing mixed in with *Roridula gorgonias*. At the other site, plants were resprouting and reseeding from a site where they had been burnt in the previous dry season. A population in a nearby swamp 50m away that was confirmed by Mr. de Villiers as consisting of some 30 plants, had no survivors. However, further down the stream, some adult plants were located that Mr. de Villiers had not seen before.

Frank Woodvine was also fortunate enough to have come across a relatively large population of *Witsenia*, apparently in some Northern slopes approximately 10km North of Pearly Beach. Despite a good description of the locality on the farm DroeKloof, no *Witsenia* could be found. The dam around which the *Witsenia* was said to grow had been heavily grazed and disturbed. Despite two separate attempts to find this population, and further searching in swamps on the farm, no plants could be found. Although almost certainly extinct from this locality, a number of south facing slopes exist in the area that show immense promise for more populations. This was another immensely important locality, as it represents a low altitude, coastal site that was on a north facing slope. The closest extant lowland site is about 50km to the north in the Onrusriver mountains, or the extinct population slightly closer at Voelklip. Its closest known neighbour is the high altitude site at Akkedisberg.

East of Akkedisberg lies a vague locality for *Witsenia* possibly in the Caledon Swartberg. 30km east lies a record from the Riviersonderend mountains, and another 100km eastwards is a record from the Langeberg mountains above Swellendam. The lower slopes of the Riviersonderend mountains below Pilaarkop were scoured for *Witsenia maura*. In all, about 5 streams were followed to the source, and countless tributaries walked along. A good locality was given by the foreman below the mountain, who claims to have harvested the plant in the past. Despite a thorough search of the swamp and the surrounds just above the waterfall he indicated, no plants could be found. A positive sign was the discovery in the failing light, of a single specimen of *Roridula gorgonias*. This plant has occurred near *Witsenia* on three occasions, and with it on Mt. Lebanon and Akkedisberg. There is therefore a strong possibility of *Witsenia* being found near this locality.

What are the trends in distributions? Approximately half of the 40 odd populations for which specimens exist in the Bolus and Compton Herbaria have been visited. Of the visited populations, Nine are confirmed to be extant and

have been sampled. Seven further populations with good localities have been visited, and can in all likelihood be confirmed to be extinct. 7 localities have been recently reported to be surviving, but have either not been visited, or the populations were not found. Good Herbarium localities exist for another four populations, and about three vague but important localities are yet to be found. A total of between 15 and 20 populations probably exist that could be accounted for with our current knowledge. Of subpopulations visited (clusters of plants separated by a considerable barrier),

Predation

Extensive studies on the predation of seeds of *Witsenia maura* were done by Wright *et al.* (1989). They intimated that *Witsenia* is attacked at several levels of development. The flowers are eaten by the CMR beetle (*Mylabris ocularis*) that were said to be eating the flowers of *Witsenia maura*. This beetle has been observed by myself, by Gawith *et al.* (1993) as well as two somewhat squashed beetles of *Mylabris* with a herbarium specimen from Bettys Bay by Pillans (4742) where he described the beetles as "greatly damaging the flowers". This specimen dates back to 1920 which suggests that this beetle has been a long term predator of *Witsenia maura*. Gawith *et al.* (1983) also observed the damaging behaviour of ants to the flowers, as well as their role as nectar thieves. At the seed stage, Gawith *et al.* (1983) report low levels of pre-dihiscent seed predation. This is corroborated by observations in the field of a substantial portion of immature capsules with holes in the ovary walls. The leaves of some plants have been severely eaten. This is presumed to be of a minor influence in most populations, but in some with poor growth, for example at Kleinmond and Hangklip, this could lead to the eventual destruction of the plant. Gawith *et al.* (1993). also found low levels of rodent predation at Cape Point. This was in part explained by the low rodent levels present in the area.

Pollination of *Witsenia maura*

Introduction

With beaks that reach less than half way down a long tube, how then does a pollinating bird extract the nectar? Midgley & Bond (pers. comm) promote the theory that only an extinct giant nectarivorous bird could have a beak long enough for the flower. Due to the diversity of Sunbirds in the Cape, this is presumed to be a Sunbird. Although no fossil record exists for this extinct giant Sunbird, they confirm that *Witsenia maura* is no longer being actively pollinated. Bond, Midgley (pers. comm.) and Goldblatt (1983) claim that field observations have revealed no active pollination. Seeds are not being produced (Bond and Midgley, pers. comm.) and there are no seedlings of *Witsenia maura* (Bond & Midgley, pers. comm). *Witsenia maura* would therefore seem to be a relictual plant, reliant on an extinct pollinator, and only capable of short-term survival through resprouting.

A study by Wright *et al.* (1989) reveals that seed set exists, and is unaffected by the exclusion of birds. They conclude that facultative pollination by insects explains the majority of seed set of *Witsenia maura*. This is contrary to Goldblatt (1983) who claims that all but bird pollinators should be able to access the pollen. Yellow is an unusual colour for bird pollination, red and orange being more common in Africa (Goldblatt, 1983). The colours may then suggest insect pollination, but a long, broad, closed floral tube is extremely unusual in insect pollination. Also in question is the value to insects or birds of the green and black of the petal tube. It was suggested (A. Pauw. pers. comm.) that the coloration may contrast with the bright yellow, thereby enhancing the flower and particularly the tepals visibility.

Ultra violet guides are a familiar concept for insect pollination. Such species as *Gorteria diffusa* have been demonstrated to be physically altered to produce UV reflectance (Johnson & Midgley, 1997), these colours are typically purple to

black. However ordinarily bird pollinated flowers are orange or red and lack any recorded UV marking (Gentry, 1983). Flowers of *Spathodea* (Bignoniaceae) are bright red, often with a yellow margin, but this has not been demonstrated to reflect UV. They share a number of traits with other bird-pollinated flowers as well as *Witsenia*, in having sub-exserted anthers, frequently odourless flowers, and a large disc that produces copious nectar (Gentry, 1983).

Methods

An attempt at photographing pollinators in action led to a total of 12 hours observation time logged. This took place from sunrise through to midday. Observation took place from a single vantage point near a population so that photography could be made possible. Number of plants visited per visitation were logged, as well as the pollinating species.

A specimen of *Witsenia maura* was fixed in position against a UV-dark velvet cloth. The plant was angled, such that the maximum incidence of sunlight and hence UV rays were striking the plant. A graded scale of 100% UV reflective to 100% UV absorbance was placed next to the plant. A UV filter allowing light in the UV-A (320-400nm) region was obtained in order to examine this UV reflectance of the flower. Peering through the lens at the flower under harsh sunlight was enough to reveal a patch of UV reflection. This was assumed to be the bright yellow tepals at the tip, but to confirm, I obscured the patch with my finger. To my astonishment, it was not the bright yellow tepals I was covering, but the seemingly black band.

Inspired by this anecdotal evidence, I modified the filter to fit a Nikon Coolpix 4500 digital camera. Focussing was found to be extremely difficult with this camera as no image could be seen through the visually "dark" filter. The resulting image (Figure 2) was encouraging enough to warrant further photographic investigation. Subsequent attempts used the same filter placed over a pre-focussed Fuji Finepix 2100 digital camera. The aperture was placed at F8 to

obtain depth of field, but allow for significant light to enter the camera. The camera was also altered to ISO 400 to obtain greater CCD sensitivity. Exposure times of around four seconds were obtained using this setting.

In *Gorteria*, a xerophytic daisy, the nectar guide was found to be physically rough to reflect the UV (Johnson & Midgley, 1997). The flower of *Witsenia maura* was examined under Scanning Electron Microscope (SEM) to establish whether there was any physical mechanism to explain the reflectivity. A flower was cut longitudinally to form a section containing the tepals and the tube. The section was initially placed in FAA (70% ethanol: 40% Formalin: 30% glacial acetic acid = 85:10:1) before being dipped in increasing concentrations of ethanol to a concentration of 98% ethanol to dehydrate it. The tissue was then dried in a Balzers critical point drier using CO₂ as a carrier gas. A sputter coating of gold-palladium was then applied to the tissue which was fixed to the charge plate using an electrically conductive coating. The tepal was subsequently viewed using a Cambridge S200 microscope at 7KV and magnifications of 30-1000X.

Nectar volume was measured using calibrated micropipettes. The nectar was then diluted with equal volumes of water in order to make a reading possible with an Erma Hand Refractometer. Measurements of nectar volumes on flowers that had been picked three hours earlier were done using 5 µl micropipettes.



Figure 2 : A UV photo displaying fantastic reflection of UV light in the region of the black band.

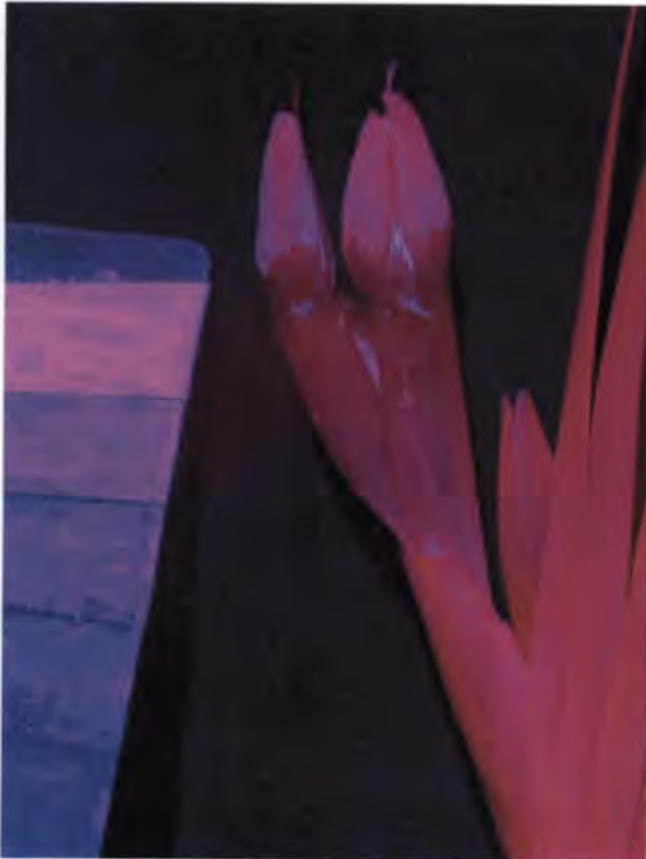


Figure 3: On the left is a flower of *Witsenia* under UV light. The scale on the left is graded at 10% intervals with the furry tepals reflecting approximately 90% of UV light. The black band can not be seen to reflect light much, although a bright band representing 100% UV reflectance is visible just below the furry tepals. The darkness of the band in the visible spectrum (on the right) is not

visible however, and this demonstrates that the band is not UV absorbing.



Fig 4 : Photo of *Witsenia* with SEM images superimposed. On the right are rough cells with a bump, and on the left, significantly fewer bumps are visible. Colours of "zoom" lines refer roughly to the colour of the part they are indicating.

Results

Observations revealed that Malachite Sunbirds (*Nectarinia famosa*) were observed to clasp the branch below the inflorescence before plunging their beak into the flowers situated above them. In this way two or three flowers per plant could be probed before the bird flew to the next plant. This is a commonly documented method of feeding as exemplified by the pollination by Hummingbirds of *Erythrina oliviae* (Toledo & Hernandez, 1979). Sugarbirds were also active in the area, mostly feeding on *Mimetes cucullatus*. It is suspected that they were also utilizing the flowers of *Witsenia* although no pollination of these birds were seen.

Both the yellow tepals and the black banding below the tepals were found to be significantly UV reflective at approximately 80% reflectivity. The rest of the plant also appeared to be UV reflective although less so.

The SEM results are inconclusive and more investigation is needed. The green section of the plant consists of elongate cells with little or no bump in the centre of the cell. The black section consists of roughly hexagonal cells with a conspicuous bump in the centre. The green section of tepal tube between the black and the yellow tepals was found to contain almost hexagonal cells with rare bumps in the centre. The anther demonstrates the largest bumps of all. Finally the papillose hairs were found to be worm like protrusions arising from cells.

The two flowers analysed provided nectar concentrations of 13 and 15%, and volumes of 46 and 52 μl .

Discussion

Comments on Herbarium records (Esterhuyzen 6790 (Bolus Herbarium), Salter 4672 (Compton Herbarium)) concur with our observations that Sunbirds and Sugarbirds were pollinating the flowers. As Sunbirds are extremely shy, and studies of facultative insect pollinators have already been done (Gawith, 1983), this aspect of pollination was not examined.

Toledo & Hernandez (1979) suggest that the large quantities of nectar (average volume = 230 μl , average sugar concentration = 11.7 %) produced by *Erythrina oliviae* may be a strategic response by the plant to provide water for the pollinators in a relatively hot and dry summer climate. Toledo & Hernandez (1979) also found that there were 20 amino acids present in the nectar, including those necessary for the growth and maintenance of birds.

Several methods were done to clarify the pollination syndrome of *Witsenia*. Large volumes of nectar were extracted from the flowers compared with typical insect-pollinated flowers (Johnson & Midgley, 1997). The concentration of the nectar was found to be within the range of both birds and insects (Johnson & Midgley, 1997). Flower length and shape, and the strength of the tepals, both suggest that the flower is bird pollinated. UV reflectance is usually thought of as being associated with insects, but both papers (Toledo & Hernandez, 1979; Sieberglied, 1979), and personal communication (P. Ryan) confirm that some birds can see UV.

Photography of the flowers of *Witsenia* under UV confirms that the flowers are UV reflective. The initial photograph (Figure 2) shows clearly reflectance in the UV range (purple) coming from the black section of the flower. Upon repeating the experiment using a superior camera, it was found that reflectance occurred equally over the entire corolla except for the pubescent tips. These outgrowths appear bright pink – with a brightness of about 90% UV reflectivity. Hill (1977) has found that specialized epidermal papillae combine physical and chemical properties to reflect UV. As the reflective tips are located at the entrance to the flower, it is suggested that these are the nectar guides of the flower.

The 'bumps' in figure 4 show that the tepals of the flower appear to be concentrated around the black band. These bumps may merely be enlarged nuclei and not responsible for reflectivity at all, as the anthers, organs unexposed to light, have the largest bumps of all. The compounds found to be chemically active in UV reflectance are flavonoids, particularly flavanol glucosides and anthochlors (Hill 1977). If the bumps are responsible for the reflection of light, they may be vacuoles containing these compounds, without the bumps responsible for physical reflection of UV as with *Gorteria* petals (Johnson & Midgley, 1997).

UV visibility for birds was confirmed by P. Ryan (pers. comm.). Thus not only the yellow tepals attract the birds, but also the dark petal tube. Yellow and violet flowers are the most common colours associated with UV reflectance, whereas green, blue or red flowers less frequently reflect UV (Guldberg & Atsatt, 1973). Silberglied (1979) has many references and techniques for viewing and quantifying UV reflectance. Toledo & Hernandez (1979) suggested that the contrasting orange of the standard against the bright red petals may act as a nectar guide in *Erythrina oliviae*. They commented that “this (nectar guides) is an unusual phenomenon in ornithophilous flowers”. This very same phenomenon, a dark tepal on a bright yellow background may also provide the contrast needed to attract birds from a distance. Evidence for UV sensitivity in vertebrates is scarce, although Sieberglied (1979) reports on two species of birds that have been confirmed to be UV sensitive: a Hummingbird and a homing pigeon. Since then though, many more UV sensitive birds may have been identified.

Silberglied(1979) provides an extreme example of a butterfly in which the visible spectrum is unrelated to the UV reflectivity. In *Hypolimnus misippus*, the butterfly has wings that are black and white, yet the UV reflectivity is equally strong on both a white and black background. This demonstrates that visible colouration can not reveal reflectivity with certainty. *Witsenia* thus seems to be demonstrating this same effect as the butterfly, with a dark area in visible light, being reflective under UV. Indeed, the equal overall brightness of the black band of the tepal, compared with the green of the tepal seems to confirm the reflectivity of this band.

Sieberglied (1979) waxes lyrical over the seductiveness that the flashing UV must pose to the receptive mate of the *Hypolimnus* butterfly as the wings open and close. Surely fanciful, it is enticing nonetheless to speculate upon a mechanism of attraction almost as irresistible to birds... Consider a UV receptive Sunbirds eye-view of a population of *Witsenia* sparkling in the wind, like

Noctiluca (sea-sparkle) flashing in the sea at night. This bright glittering of reflectance on the black band (figure 3), may catch a birds eye from a distance. The contrasting black on yellow then confirms the possibility of a reward at medium ranges. Finally the diffuse reflectance of the tepal tips acts as a nectar guide to direct the birds beak to the glucose-rich nectar.

The energetic costs of UV reflectance and UV absorbance are high (Guldburg & Atsatt, 1975) and thus these markings are only maintained through selective pressure. This selective pressure may, in the case of red bird pollinated flowers, be to prevent herbivorous insects from seeing the flower. *Witsenia maura* is thus taking a risk in making entirely UV reflective flowers, and the effect can be seen in the number of flowers that are destroyed by insects. However this risk may also be offset by the benefits gained by facultative pollination by insects (Wright *et al.*, 1983) or by enticing an avian pollinator.

All the characters seem to suggest that an Sunbirds and Sugarbirds are responsible for pollination of *Witsenia*, but their beaks are some 3cm short of the nectar. Somehow both birds must be adapted to extracting nectar that is beyond their reach. A. Pauw revealed that Sunbirds have a tubular tongue that allows them to suck up nectar that is beyond their beak, much like a straw. It is narrow, hollow tongue that enables the Sunbird to obtain nectar from the narrowest of bird pollinated flowers, *Microlooma spp.* Sugarbirds have a different mechanism for obtaining nectar from flowers. They have a tongue that is divided much like a mop. The tongue is dipped into the nectar, and the tongue squeezed between its beak to extract the nectar. This is done multiple times a second until the nectar is exhausted. This is the final piece in the puzzle of what pollinates *Witsenia maura*, and how it manages to do so with a beak far shorter than the length of the flower.

Chapter 3 : Molecular analysis

Introduction

Goldblatt (1983) suggests that the woody Iridaceae are amongst the basal members of the Iridaceae and represent an ancient evolutionary separation of the species. His conclusion was based on a combination of karyological and morphological work. This ancient distribution forms the basis for much of the variation anticipated within the species. Intraspecific variation has been regularly used to examine within and between population variation in animals, but direct sequencing has seldom been used to examine intraspecific variation in plants (Jordan *et al.* 1996). This intraspecific polymorphism forms an integral part of biodiversity (Fiedler & Jain, 1992), but is usually neglected due to the difficulty in quantifying this variation (Taberlet, 1998). This polymorphism is also of particular interest for biotechnologists wanting to use wild species' as genetic resources. The interaction between geography and phylogeny within a species has been termed intraspecific phylogeography by Avise *et al.* (1987).

Because cpDNA is maternally inherited, it can disperse in seeds but not in pollen. Contrasting the distribution of genotypes of biparentally inherited (nuclear DNA) variation in pollen with that of cpDNA can be used to infer the relative contributions of seed and pollen to total gene flow (McCauley, 1995). Only rarely has cpDNA been used by population biologists as it seldom shows the variation necessary for population level study (McCauley, 1995). The migration rate (M_c) of cpDNA in angiosperms is potentially much less than the migration rate of nuclear genes, provided that there is some dispersal of pollen. (McCauley, 1995). Recently however, several authors (Fuji *et al.*, 2002. Jordan *et al.* 1996) suggested that cpDNA could be used in population level analysis. The majority of papers examining variation in cpDNA have utilized RFLP's (eg. Lavin, Mathews and Hughes *et al.* 1991) and which produce degrees of similarities between populations and plants, but can not be used for phylogeography. The following

summaries of successful intraspecific phylogenies are given, not only as examples, but to draw comparisons with *Witsenia maura*.

Overview of successful intraspecific phylogenies using chloroplast genes.

Jordan *et al.* (1999) used the chloroplast *rpL16* gene to establish variation in *Lemna*. Despite having chosen the most variable *Lemna* using RFLP techniques, only 2 haplotypes were present from a species that spans the Eastern and Southern United States. As they are mostly vegetatively reproductive and thus largely confined to water bodies in which they exist, it was expected that these fern allies would show considerable haplotype variation across their range. Their sampling distances were always greater than 32km to avoid sampling from the same population and same river system. Their interpretation of the low molecular divergence of *Lemna* included the recent expansion from two refugial populations during the Pleistocene glacial maximum.

A study of the Japanese alpine plant *Pedicularis shamissonis* (Scrophulariaceae) done by Fujii *et al.* (1997) was found to be highly variable across its North-South range. From 24 populations, 17 distinct haplotypes could be discerned. They furthermore found that each haplotype was strongly geographically structured. This geographic structuring of variation may be used to infer historical biogeography of a species (Avice, 1994). Intra-population cpDNA variation was found for two sites, indicating a common link. Strongly monophyletic clades separated North and South clades, implying a common ancestor. The presence of a number of autapomorphies in eastern Hokkaido, suggest a unique evolutionary history. Less difference between haplotypes within the Northern clade compared with the Southern clade suggest that these haplotypes were more recently differentiated. Each branch of the Southern clade was supported by high bootstrap values with at least two synapomorphies. This potentially indicates haplotype diversification through geographical isolation.

A further study in this region (Fuji *et al.*, 1999) of a similarly distributed plant, *Primula cuneifolia*, recovered a very similar phylogeography. Plants could again be grouped into three similar clades: North, South and Hokkaido. The most likely hypothesis explaining the distributions of these haplotypes was a common ancestor that expanded and contracted through a number of glacial cycles during the Pleistocene. At least four glacial advances and retreats have been proposed by geologists, and these were confirmed with the potential mapping of the movement and subsequent separation of these ancestral populations through these epochs. From the haplotypic distributions, they inferred that the ancestors most likely colonized from the North, and moved South over time. These two studies by Fuji *et al.*, (1997 & 1999) demonstrate brilliantly the possibilities for a similar study on distributions of haplotypes of *Witsenia maura*. Explanations of movements of plants with subsequent contractions due to climatic cycles are also highly applicable to the refugia dwelling *Witsenia maura*.

Bernachez & Wilson (1998) discovered that in freshwater fishes, less polymorphism exists within a species at higher altitude than lower. A review of 8 species by Taberlet (1998) has confirmed this finding. This trend is particularly acute in areas that were glaciated during the Pleistocene. Most of the variation was found to be due to range contractions into refugia during this time. Taberlet (1998) questioned whether this same trend could be confirmed in other animals and in plants. A study by Soltis *et al.* (1997) on the flora of the northeastern coast of North America supports this trend as well as several other European studies (see Taberlet *et al.* 1998). An exception to this trend occurs if a suture-zone is present, where local intraspecific biodiversity can be elevated. This suture-zone is described by Remington (1968) as "a band of geographic overlap... between biotic assemblages including some pairs of species or subspecies that hybridize in the zone". Taberlet *et al.* (1998) extended this definition to the postglacial union of different populations due to postglacial expansions in their ranges.

Questions

These aim of the molecular work is to find out :

- 4) Do the Peninsula populations form a separate clade to the other populations.
- 5) Can dispersal of the Ancestral *Witsenia maura* be inferred from chloroplast DNA.
- 6) Do the current distributions reflect dwindling refugia of a doomed species.
- 7) Can prior climatic conditions be inferred from the current distribution and history of *Witsenia maura*.
- 8) Is there evidence to support the notion of *Witsenia maura* being a "paleoendemic".
- 9) Is there more polymorphism at low altitude than upper altitude as found by Bernachez & Wilson for freshwater fishes (1998).

Materials & Methods

Sample Collection and Identification

Witsenia maura was obtained in as many sites as could be found. Numerous localities were too vague to be found, and several sites have been destroyed through development and alien invasion. Leaves were collected in plastic *Ziplock*™ bags with a few drops of water to maintain freshness, as gel dried specimens were found to yield low quality DNA. Several plants were collected per population with substantial spacing per plants. When population structure existed, specimens were taken from all available subpopulations. Specimens were found to be unmistakable in the field and no further identification was necessary. Voucher specimens for new localities have been lodged in the Bolus and Compton Herbarium (DGE 1279).

Choice of DNA region

It was decided to use chloroplast DNA (cpDNA) in order to establish the separation of populations. TrnL-TrnF was initially chosen due to its proven variability and success in a number of intraspecific phylogenies. This region was found to be unco-operative due to problems with the tertiary structure. The next candidate that held promise for variation is the intron (non-coding region) of PSBA-TrnH. After sampling several populations and getting variation, we decided to continue with this region. Later other cpDNA regions may be used, to increase the resolution of variation, and nuclear DNA as recombinant DNA.

DNA extraction

Initial DNA extraction was done using a modified CTAB extraction method by Doyle and Dickson (1987). Leaf segments of around 0.75 cm² were ground in a plastic microfuge tube after freezing with liquid nitrogen. Around one spatula full of sterilized river sand was added to the microfuge tube before grinding to ease extraction. A hot CTAB extraction was later used as this was found to yield higher quantities of DNA. This method involves the pouring of 700µl of warm CTAB with 18µl of Mercaptoethanol per sample, into a pestle containing sand. PVP was added to make up a 1% solution with the CTAB. The ground solution was then placed in a warm incubation bath for at least 90 minutes with regular mixing by inversion every half hour. 24:1 isoamyl:alcohol was then added to the suspension and the samples hand-mixed by inversion for 5 minutes. The samples were subsequently centrifuged at 18 000 rpm for 5 minutes, and the supernatant placed in a new microfuge tube. Freezer chilled Isopropanol was added and the mixture hand-mixed by inversion for five minutes, before placing in a freezer at -5°C overnight to allow for precipitation of the DNA.

To recover the DNA pellet, the mixture was centrifuged at 18 000 rpm, the liquid then drained, and the tube allowed to air-dry inverted on tissue for 10 minutes. 95% ethanol was applied to the pellet twice as a wash, and the pellet dried for a period exceeding three hours in a sealed container with silica-gel as the

dessicant. 50µl of 0.1X TE (1mmol Tris-HCl pH 8.0; 0.1 mmol/L EDTA) was added to the dried pellet to create the stock DNA solution.

The DNA was run out on a mini-gel rig, as a check for DNA levels.

PCR amplification :

Amplification was done according to the following protocol (Table 1).

5ul	DNA TEMPLATE
29.7ul	Np H2O
5ul	NH4 BUFFER (10X – 1X)
5ul	MgCl2
2ul	DNTP'S
1.5ul	PRIMER X
1.5ul	PRIMER Y
0.3ul	TAQ
45.0ul	MMIX
5ul	DNA
50.0ul	REACTION

Table 1: Table of ingredients and volumes used in PCR reaction.

MgCL₂ was later increased to 7µl (H₂O = 25.5µl) as this was found to give better annealing. PCR was accomplished on a *Hybaid sprint*, 30 cycles being of the following thermal profile being applied to the samples: 94oC – 2 min to start; 94oC – 1 min; 52oC – 1 min; 72oC – 75 sec; 72oC – 7 min; hold at 4oC.

Raw DNA and PCR products were run on a *mini-XL* gel rig, using 0.5X TBE buffer and 1% EtBr.

PCR products were cleaned using Qaigen clean-up columns and protocol. The cleaned products were then run in the same PCR . The samples were then run on a ABI sequencer to obtain the final traces.

Data analysis

Sequences were assembled using a Apple Macintosh running "seqman" software, using the forward and reverse direction where possible. The resultant sequences were compared using "Megalign" software. Sequences were edited in PAUP. All dubious characters were trimmed from the alignment. A resulting 190 characters were used remaining, with 15 being informative. Of these 15 characters, four haplotypes could be discerned.

Results

Raw DNA of Iridaceae is difficult to use as a predictive tool for amplification purposes, as smears of DNA result. Working with Iridaceae provides many problems, as success at any stage is unpredictable.



Figure 5: A typical raw amplification gel showing gross smearing, and inconsistent brightness of bands. This is typical of the variable success when working with the Iridaceae.

A haplotype tree was constructed in order to summarize the genetic diversity at a locus. This reflects the evolutionary relationships among the alleles.

Haplotype Tree of *W. maura*

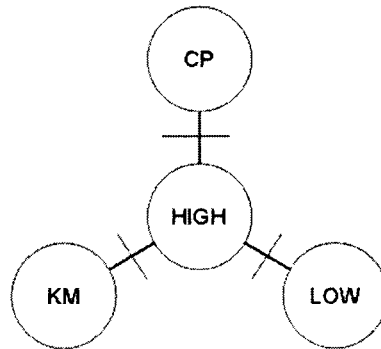


Fig 6: The following haplotypes are represented in the haplotype tree : CP = Cape Point, Silvermine and a single individual at Mt. Lebanon. High represents the high altitude sites = Mt. Lebanon, Babilonstoring and the Akkedisberg. Low contains low altitude sites = Hangklip, Bettys Bay, Onrus River mountains. KM is a haplotype represented by the single lowland population at Kleinmond. Lines represent a single mutational difference. All lines represent indels.

O'Donnell (1992) provided a simple formula to estimate the proportion of observed mutational events : $\text{proportion of mutational events} = (\text{TS} + \text{TV} + \text{ID}) / \text{L}$, where TS = number of observed transitions, TV = number of observed transversions, ID = number of observed indels, and L = the sequence length.

In *W. maura* for the region PSBA-TRNH, ID = 4, L = 360, the proportion of observed mutational events is therefore $4/360 = 0.011$

A total of four haplotypes was found from a 9 sites sampled (Table 2; Figure 7). Strong haplotype homogeneity existed within all populations, with only the Peninsula haplotype shared with a single individual from the north-west most population of Mt. Lebanon. The Peninsula populations (Silvermine & Cape Point) formed their own haplotype, with the high altitude haplotype (Mt. Lebanon, Babilonstoring & Akkedisberg) being the most similar. A coastal haplotype (Betty's Bay, Hangklip & Onrusriver mountains) formed another haplotype, with the Kleinmond population an endemic haplotype comprising a population of under ten plants.

Sub-
population
size

Population name	0	1-10	11-100	100-1000	1000-2000	Haplotype	Alt (m)
Cape Pt				1		C	50
Slvrmine			1			C	300
Hangklip		1				B	30
B' Bay				2		B	30
Klnmond		1				D	40
OnrusRiv		2	1			B	100
Mt Leb		2	2	1		A	750
B's Towr		1	1	2		A	1300
Ak'disBrg	1		3			A	700

Table 2: A summary of sites where plants were investigated. The numbers of sub-populations at each site is given according to the sub-population size. The haplotype is the genetic type to which the plant belongs (Chapter 3: this study), and the approximate altitude is included.

Discussion

The first objective the project aimed to do was establish whether any variation could be found using chloroplast markers, and if so, whether this variation is structured. In total, four haplotypes were found showing significant structuring. The only intra-population link is the Peninsula haplotype, found on Mt. Lebanon.

This shared haplotype indicates a common link between these two populations, and demonstrates that at some stage a founder individual traveled between the Peninsula and Mt. Lebanon or *visa versa*. This may have taken place in a single leap, in an incredibly strong wind. More likely is that a land bridge existed between the two sites, when the environment was wetter, and aeolian sand did not cover the Cape Flats in the mid-pleniglacial when the climate was moist. Ferns (Pterodophyta) and *Podocarpus* were at their maximum in this period, suggesting a suitable habitat for *Witsenia maura* to co-exist. This pattern closely

resembles the intermediately positioned populations of *Pedicularis chamissonis* of eastern Hokkaido (Fuji *et al.*, 1997).

The linking of lowland sites is to be expected as the fewest altitudinal barriers exist. A distance of around 50km of coastline links these populations. This corridor of movement is interrupted however by the unique and endemic haplotype at Kleinmond, midway between the lowland populations. This Kleinmond population may have been caused through a retreat and subsequent isolation of this population. In favour of this argument is the relictual and isolated pattern of each population. Alternatively, the Kleinmond population may represent an intrusion of another haplotype into a habitat vacuum produced by the extinction of the lowland haplotype in the area. The stream in which the Kleinmond population is growing, is linked indirectly to populations above in the Elgin Valley, above which the high altitude haplotype is found. The haplotype tree favours the latter hypothesis, with the Kleinmond differing from the high altitude plot by 1 indel, but by two indels from the lowland populations.

The high altitude haplotype is proposed to be the ancestral population due to it being the most widespread. The greatest barriers also separate the populations, and in order to invoke a long distance dispersal mechanism, seeds would have to travel across a great valley to Babilonstoring, and across another valley and mountain range to Akkedisberg. An ancestrally widespread distribution is the more parsimonious solution to the geographic structuring. As *W. maura* is a hydrophile, it is suggested that it would have been at its most abundant during a glacial maximum. The current population sizes appear to support this hypothesis that these plants are suffering in the interglacial periods. The Peninsula population perhaps geographically represents the strongest case for isolation. *Witsenia maura* has not been recorded on alkaline coastal sands – a formidable barrier of some 50km of this substrate separating the Peninsula from its nearest ancestor at Mount Lebanon.

As *Witsenia* is a hydrophile an alternative possibility of distribution exists. It currently grows in the seepages, but diaspores from these populations could move great distances downstream. These seeds would then require a seepage at the base of the mountain where the tallus begins. The problem then arises of how the plant can move upstream again. Ancient rivers of which today's rivers are mere remnants, may have to be taken into account when inferring phylogeography from current distributions of *Witsenia*.

If *Witsenia* has wind dispersed seeds, long distance dispersal due to a freak wind, may be possible. Capsules are held upright and don't dehisce completely. This may be an adaptation to wind dispersal to make sure seeds are only ejected at maximum wind velocity. This scenario may explain why all the high-altitude haplotypes are the same, as the specific high-altitude haplotype may not survive in the lower altitudes, and only the high altitude dispersed seed is thus revealed.

Our study appears to confirm the hypothesis of Bernachez & Wilson (1998) that polymorphism is higher at low altitude than high altitude. There are 3 high altitude sites distributed over 100km that have the same high altitude haplotype. This contrasts with the Bettys Bay region, where two haplotypes exist within a short distance (10km) of each other. The Peninsula is also counted as a low altitude site.

One of the primary aims of the work was to determine the dispersal and phylogeny of *Witsenia*. As only four haplotypes were uncovered, a rooted tree could not be created. This could be established with the inclusion of at least one outgroup of *Klattia* or *Nivenia*, the putative sister taxa. Rooting the tree should reveal which is the oldest haplotype and how the other haplotypes are derived. With the current information, two hypothesis can be suggested. A general East-West trend exists, with the high altitude locality in the East, and the low altitude localities to the west. The Peninsula population is a further haplotype formed by isolation. The Kleinmond haplotype is more closely related to the high altitude

(east) haplotype, although its origins may still either be as a result of separation from the high or the low altitude haplotype.

Other methods may also be used with success. Many comparative methods such as ISSR (Inter Sequence Simple Repeats), RFLP's (Restriction Fragment Length Polymorphism) and Microsatellite Sequencing (Hare, 2001) are more sensitive to variation and can be used for inferring relationships between individuals. However, due to problems in homology, only sequencing can be used for phylogeographic reconstruction.

Chapter 4: General discussion and conclusions

The boggy sites in which *Witsenia* grows are hotspots for endemics. Rare endemics such as *Mimetes hirtus*, *Cliffortia graminea*, *Lonchostoma sp.* and *Roridula gorgonias*, together with numerous *Berzelias* have demonstrated the importance of wetlands as refugia. Of particular interest is the remarkably similar distribution of *Roridula gorgonias*. This species was found in the same swamp in Mt. Lebanon and the Akkedisberg. Swamps nearby to where *Witsenia* has been found at Hermanus, Bettys Bay, Palmiet River (Kleinmond), and Riviersonderend. The localities are even co-incident in the extreme east of the range above Riversdale. It is suggested that these species are both paleoendemics that emerged from the same ancestral refugia, with almost identical ecological requirements. I speculate that these may be amongst the oldest surviving Angiosperms in the Cape Flora, and special attention should be given to them from a systematic (Biogeography, Phylogeography) and conservation point of view.

The altitudes at which populations of *Witsenia* grow are highly specific, yet appear to have little trend. Modelling the climatic envelope may prove useful in suggesting where populations may occur, whether haplotypes are constrained to a certain climatic envelope, and used for inferring past climates. However, as *Witsenia* occurs in extremely narrow ranges, no model is sensitive enough to capture this specificity. Furthermore, it occurs at many different altitudes in the same mountain (eg. At Kleinmond (30m), Houwhoek(700m) and then Mt. Lebanon(1200m)) which I believe would be difficult to explain using specific bioclimatic envelopes. Even searching swamps at the right altitude, with many of the indicator species present, proves fruitless most of the time. This is all part of *Witsenia's* charm and mystery, which make it such a challenge to understand.

It is expected that sequencing of nuclear DNA will paint a very different picture to that given by cpDNA. As seed appears to be transported only very short distances, there is a high degree of isolation. It is apparent that *Witsenia* is

pollinated by Sunbirds and Sugarbirds. These birds can move large distances, thereby visiting several populations in a day. It is expected therefore that there will be a lot more gene flow between populations, resulting in a more difficult phylogeny to reconstruct. The only populations that may be outside the range of frequent pollen flow, are the Peninsula populations from the mainland Kogelberg, and the Riversdale population from Riviersonderend. nuclear DNA may therefore re-inforce the finding of the isolation of the Peninsula.

Conservation

Of the sites, only two locations on the Peninsula are currently in conservation areas (Silvermine Nature Reserve & Cape Point Nature Reserve). The Hermanus (Voelklip) population made way in 1990 for a 7-11 supermarket chain. The population at Sir Lowry's Pass appears to have succumbed to alien invasion due to founders from a nearby pine and eucalyptus plantation. In Onrusriver mountains there are three populations with less than 10 individuals in two of the populations and less than 100 in the last population. Of these, all three are contending with *Pinus radiata*. Mount Lebanon has the highest number of small populations distributed over a substantial area. In this site there are few small populations (less than 20 plants) with several larger populations (< 100 plants). As this is bordering on a pine plantation, many populations have been destroyed already and the remaining populations have recently had aliens cleared from around them. If the alien removal is not followed up we can expect several of these populations to be outcompeted. Populations lower down in the valley (eg. Houwhoek) have not been located yet, although these have probably also made-way for orchards, plantations or escaped aliens. The Klawersvlei population may also have gone extinct due to the presence of aliens. The southern slopes of the Caledon Swartberg are also covered in a runaway growth of *Pinus*. If a population still exists here, it is likely that it is growing under these pines.

Populations at Bettys Bay are also severely threatened by development. Not only are streams being changed and canalised, but houses are being built on the wet

habitats of these plants and on existing populations (eg. Sunny Seas estate between Kleinmond and Bettys Bay). Kleinmond comprises a handful of plants. These are growing on a riverside verge several metres in from the roadside. These are subject to trampling, collecting of flowers and destruction of habitat through invasion and poor management. The collecting of flowers plays an increasingly risky role as populations come under threat. The flower has already proven itself as catching to the florists eye (a flowering specimen was rescued from a florist in Cape Town in 1924 by Compton). As the plants leaves are mostly high up on a branch, and is struggling to compete in some areas, it is suggested that harvesting may be fatal to some *Witsenia* individuals. Rampant mowing, both for roadside verges and for fire-breaks, also poses a threat to populations.

Being a resprouter, yet requiring a fire for successful germination of the seeds, an incorrect fire cycle may lead to mortality of the plants. It is suggested that fire be investigated as an important tool in leading to the medium term maintenance of populations. As it currently stands, the Bettys Bay population is broken by a fire-break which has potentially been placed there for its protection!

Several engineering projects are under investigation in the Cape Metropolitan Area examining the feasibility on groundwater extraction for supplying a burgeoning population. Research is currently underway examining the effects of groundwater extraction on plants in the area (Rob Taylor, 2003). *Witsenia maura* is clearly dependent on groundwater for its continued existence, and extraction will lead to a lowering of groundwater with subsequent destruction of entire populations.

Genetic and functional restraints may also play a role in the demise of populations. Inbreeding depression may result in inferior survival of a population. If a certain population size is required for successful pollination and seed set, populations may go extinct. This may be due to a lack of pollinators visiting due to too small a reward, or individuals flowering times not co-inciding. Franklin

(1980) developed the 50/500 rule, as a rule of thumb for maintaining and preserving genetic diversity. These numbers refer to the number of founding members as well as the total population of size necessary for survival. If this rule is to be applied, only a handful of populations exceed 500 individuals, would survive, as most populations contain less than 100 individuals. Fortunately, *Witsenia* may yet survive, as this rule has been debunked as too simplistic and general (Templeton, 1994). Population numbers may also be representative of past interglacial periods, from which *Witsenia* survived to expand and colonize areas, of which today's populations are mere fragments. This fragmentation is suggested by the genetic homogeneity present within all populations studied.

If the 50/500 rule were implemented, this could lead to many of these populations being deemed as too small to be viable. This could lead to only the largest populations being saved. This is unacceptable given the possible seedbank, the possibility of reviving populations through hand-pollination and manipulating fire regimes to suit the species. If evidence of extreme genetic variation is found, these populations could be useful for future biogeographic studies and are deserving of protection. Further pollination studies should also be initiated to enable wiser management of the species such as viable population sizes, possibility of cross pollination from nearby populations and examining the impact of housing and farming on pollinators.

Limitations of the study

Ideally all populations should be sampled. Due to the limitations of knowledge, time, and the difficulty in finding populations this cannot be so. Ideally more samples per population should be done to better ensure haplotype diversity per population. These problems were mitigated through the use of sampling as much spatial diversity within the population. Many populations also contain less than 10 plants. Because all population samples were homogenous, with the exception of a single individual, it is suggested that more extensive sampling per population

would not increase the rate of within population diversity. An increased number of chloroplast genes could also provide a better resolution and better confidence of haplotype diversity. There are potential problems with chloroplast genes such as tokogeny and Introgression (Mc Cauley, 1995). A nuclear gene would compliment the pattern given by the cpDNA by providing additional information on pollen flow.

The Future

In the future I intend to write a popular article in veld and flora on *Witsenia*, with the aim of promoting conservation through knowledge of this unusual species. This may also serve to locate populations which have not been made into herbarium specimens and thus have remained unknown to the botanical community. A formal study of population numbers and localities should take place in order to understand and conserve this and co-occurring species better. Future studies should take more populations and gene regions into account, and also compare other restricted paleoendemics such as *Roridula gorgonias*. Formal management on a local level should also be taken to ensure the survival of all of the populations.

As the pollen grains are unique, these plants grow in boggy areas, and the plants are highly specific in their moisture requirements, future palynologists working with sediments from wetlands should keep a key eye out for this species. I am convinced that an awareness of this species will not only serve to increase our knowledge of ancient distributions of this plant, but could lead to better understanding of our past climate.

Conclusion

To conclude, this species was found to be remarkably variable, and potentially phylogenetically informative. This suggests that intraspecific phylogenies using cpDNA are a possibility, given sufficiently rapidly evolving regions and a suitable subject. Although little dispersal of the ancestral *Witsenia* could be inferred, this

objective is obtainable with a few more samples and/or rooting of the tree using an outgroup. The Peninsula population has been found to be separate clade, with the exception of a single individual, re-enforcing the idea that the Peninsula is an island of biodiversity. The homogeneity of the populations strongly suggest bottlenecks in the past and present, and the differentiation of haplotypes over short distances suggests ancient bottlenecks and refugia. There is very little seed dispersal at present, but this may contrast with pollen dispersal, for which sequencing of nuclear DNA would have to be done. The high diversity of haplotypes seems to be similar to that of *Primula cuneifolia* (Fuji *et al.* 1999). I would suggest that the original dispersal of this *W. maura* took place at least as far back as the Pleistocene which is when *P. cuneifolia* is thought to have first diversified. *Witsenia maura* seems to follow the trend suggested by Taberlet (1998), that there is greater polymorphism at lower altitude than at higher altitude sites.

The pollinators of *Witsenia* are Sunbirds and Sugarbirds. These pollinators as well as facultative pollination by insects are causing a low seed set in *Witsenia maura*. This seed only successfully gives rise to adults under ideal conditions, and the main body of seed only germinate after a fire. The yellow tubular flower is unusual in bird pollinated flowers, and a UV reflective band as a nectar guide or attractant has been demonstrated. This reflectivity is most likely caused by chemical means.

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Distribution of *Witsenia maura*

