ASSESSMENT OF THE CURRENT POPULATION STATUS, GENETIC DIVERSITY AND PHYLOGENETIC AFFINITIES OF THE RARE SOUTH AFRICAN MOSS, *ZYGODON LEPTOBOLAX*

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ABSTRACT

The rare moss *Zygodon leptobolax* is endemic to Table Mountain, where it is found growing on alien *Quercus* hosts. The present study aims to investigate this species, place it within a phylogenetic framework and answer the key questions 1) what is the current status of *Z. leptobolax*? 2) what level of genetic diversity exists in the remaining populations? and 3) should these populations be afforded conservation status? This study used repeated censuses over a period of two years to investigate the population status and inter-annual dynamics of the species. In the first census, 43 populations were found, a number which was reduced to 37 after the second census. All populations centred around a single ravine, First Waterfall Ravine, on Table Mountain. Populations varied in size, but the majority were small (fewer than 20 shoots) to medium (between 20 and 50 shoots) in size. In addition, populations were found to be producing sporophytes freely. The plastid region *trn*L-*trn*F was used to generate a phylogenetic framework for *Z. leptobolax*. The species was found to be most closely related to *Z. inermis*, a species from South America, and not closely related to the morphologically similar *Z. intermedius*. The South African species of *Zygodon* did not form a clade, and were spread out over the phylogeny. ISSR (inter simple sequence repeat) data was used to investigate the level of genetic diversity in the current populations of *Z. leptobolax*. Levels of genetic diversity were found to be moderate, with no association between genetic diversity and geographic distance. In addition to this, the moss was used as a case study for the application of some currently available tools for the evaluation of conservation targets. Phylogenetic diversity indices were calculated for *Z. leptobolax*, and it was determined that this species represents a number of features unique within the South African *Zygodon* species. Hence, this species should be afforded conservation status if biodiversity is to be maintained within the genus *Zygodon*, ad well as among the South African bryophytes.

Key words: conservation genetics, bryophytes, Zygodon leptobolax

GENERAL INTRODUCTION

Extinction of species is a natural occurrence which has been happening ever since life evolved. It is part of the natural cycle of nature that some species survive and thrive, while others dwindle in number until they disappear altogether. The fossil record indicates that the Earth has experienced several mass extinctions, the most widely recognised of these is that of the dinosaurs during the late Cretaceous Period (± 144 – 65 mya)(Ehrlich & Ehrlich, 1981). These mass extinctions are usually followed by periods with above-average speciation rates. Over time, new species evolved to fill the gaps left by those that died off (Raup, 1979, 1986).

However, the current rate of extinction is not natural. The earth's biodiversity is being diminished at an ever-increasing rate as the result of human activity, and extinction rates are estimated to be 1 000 to 10 000 times higher than they would be without the influence of humans (Wilson, 1989; Soulé, 1983). The fossil record suggests an average "existence span" of 1 – 10 million years per species. However, rates of documented bird and mammal extinctions over the past century suggest existence spans of only 10 000 years per species (Lawton & May, 1995). Most species evolve gradually, taking hundreds of thousands to millions of years to fully separate. Even the most rapid speciation could only occur over the course of 500 000 years (Futuyma, 1998). Thus, even with the highest rates of speciation, the number of new species evolving cannot match the number being destroyed concurrently (Smith, 1993). As a consequence, any hope of maintaining reasonable levels of biodiversity requires intervention of some sort.

Biodiversity can be considered on different levels, including species, genetic and ecosystem levels (Meffe & Carroll, 1994). It is important to conserve biodiversity for several reasons, and the destruction of another species that has just as much right to be here as we do cannot be justified. There are many different approaches to biodiversity conservation. These differ in several fundamental ways. Traditional conservation biology involves the creation of management plans to preserve threatened species based on

ecology and population biology, while other approaches base their management plans on factors such as genetic diversity or ecosystem approaches.

The present thesis examines the status of the very rare and range-restricted moss *Zygodon leptobolax* Mull. Hall. Currently restricted to the eastern slopes of Table Mountain, it may rely on the presence of alien *Quercus* species for its survival. The current state of the populations is assessed, as are indicators of genetic health. This rare moss is then used as a case study for the application of some of the currently-available tools for making conservation choices.

1.1 Conservation biology - a brief overview

Conservation, in one form or another, has been in existence for some time. In the past, conservation meant ensuring certain species could provide a constant harvest (e.g. tress for timber, trout), or the establishment of private manor land and royal preserves (Meffe & Carroll, 1994). Since then, the field of conservation has changed. During the 1970s, it was realised that humans were having a very large and negative effect on the world's biodiversity, and that something needed to be done to slow down or stop the process (Takacs, 1996). The First International Conference on Conservation Biology took place in 1978, which provided a platform for scientists to discuss this common concern (Gibbons, 1992). From these early beginnings, the field of conservation biology developed into the discipline it is today (Wilson, 1992; Western & Pearl, 1989; Soulé & Wilcox, 1980).

Conservation biology deals with limiting the damaging effect of humans on nature, and protecting those species that have already been affected (Moritz, 2002). Soulé described conservation biology as a "crisis discipline". That is, if species are threatened and in danger of extinction, action must be taken quickly before it is too late for any meaningful preservation (Soulé, 1985; 1991). Conservation biology will remain a crisis discipline unless the rate of extinctions caused by humans is reduced drastically (Meffe & Carroll, 1994). Since the rate of increase of the human population is showing no sign of slowing,

our negative effect on the environment and biodiversity will be continue to be felt (Smil, 1999). Therefore, plans must be made to conserve biodiversity and protect threatened species. The basis for any form of conservation plan aimed at maintaining biodiversity is the knowledge of the status of the organisms in question (Sabovljević et. al., 2001). However, conservation decisions often need to be made quickly, and complete species information is often lacking, especially when the species in question are members of relatively poorly-known groups like the bryophytes. Individuals and populations of species that have already been affected are studied briefly, and classified according to IUCN (World Conservation Union) guidelines (IUCN, 1996). This classification system provides a method of quickly identifying which species are in need of protection from further damage, based on their distributions and population size and number (Table 1). Measures may then be taken to ensure their survival. Hallingbäck et. al. (1998) outlined a modified set of IUCN guidelines for use in classifying bryophyte species, which takes into account the differing life cycles of bryophytes, among other things. This may prove useful in future bryophyte conservation efforts.

Ecology and population genetics both play a large role in conservation biology (Soltis & Gitzendanner, 1999). Ecological factors are an important part of planning and executing conservation management plans. A complete picture of the processes surrounding a species can be determined by examining the environment it occurs in, its specific habitat requirements, its biotic interactions, and its demography (Primack, 1998). A good management plan requires knowledge of these factors in order to maintain the critical ecological processes that will allow the persistence of a threatened species. Ecology often forms the basis for population level conservation action (Gilpin & Soulé, 1986). However, it is not enough to know the ecology of a threatened species. It is also necessary to know whether a species can survive in a given environment. Ecosystem approaches to conservation may conserve a wider range of organisms, but they are perhaps a little too broad in scope. Conservation resources are limited and it is important to know whether money will be well spent i.e. does the species have a reasonable chance of long term survival or not? If the answer is not, then any resources spent on developing and implementing conservation plans will be wasted. This can be determined through

population viability analysis (Shaffer, 1981; Boyce, 1992; Burgman, Ferson & Akçakaya, 1993).

In recent years, it has been realised that molecular systematics can offer information that is equally useful as that provided by ecology and population genetics (Avise & Hamrick, 1996). For example, if a population is conserved in a healthy environment, it may still go extinct if the genetics aren't equally healthy (Saccheri, 1998). Genetic data can add extra information and insight to conservation, and can be used to make informed management decisions. Combining conservation biology, which deals with endangered individuals and populations, and genetics, which deals with genes and inheritability within populations, resulted in the field of conservation genetics.

1.2 Conservation genetics

The field of conservation genetics is a mixture of many disciplines, including population genetics, systematics, ecology, statistics and biological modelling (Frankham, Ballou & Briscoe, 2004). The main concerns of this field have revolved around the maintenance of fitness, and the capacity for evolutionary change (Frankel & Soulé, 1981; Franklin, 1980). Maintenance of genetic diversity has long been considered essential for the survival of species (Lesica & Allendorf, 1992). Hence, when resources for conservation are limited, it is important to be able to identify the species, or populations, that are most genetically diverse (Lande & Barrowclough, 1987). Presumably, these would be the ones that are most likely to survive, and represent the best "investment" of resources. Alternatively, or in addition, one might also seek to ensure that the maximum spread of diversity is maintained i.e. that populations or species with unique diversity (even if the overall diversity within those is lower) should be targeted for conservation. It is for this reason that it is important to identify those species that are most diverse within a given environment. The relative value of these species should also be evaluated in a global context. It is no good to conserve a species that is diverse and locally rare, when it is abundant globally.

Table 1: Classification criteria for species according to the IUCN (after Frankham, Ballou & Briscoe, 2004). Species falling into the first two categories require immediate action if they are to avoid extinction, species in the third category are likely to require action in the near future

Criteria	**Critically endangered**	**Endangered**	**Vulnerable**
Actual or projected reduction in population size	80% decline over 10 years or 3 generations	50%	20%
Area of occurrence	<100km2 or <10km2 and any two: i) severely fragmented or known from a single location ii) continuing declines iii) extreme fluctuations	<5000km2 or <500km2 and i) known from only 5 locations	<20 000km2 or <2000km2 i) known from only 10 locations
Population numbering	<250 mature individuals and continuing decline	<2500	<10000
Population estimated to number	<50 mature individuals	<250	<1000
Probability of extinction in the wild	50% within 10 years or 3 generations	20% in 20 years or 5 generations	10% in 100 years

Human influence can reduce the number of populations representing a species and the size of these populations. When the number of individuals in a population decreases, genetic diversity within the species is also often reduced (Ridley, 1996). Without sufficient genetic diversity, surviving individuals and populations are more susceptible to factors such as inbreeding depression and are at a greater risk of extinction (Lacy, 1987). In addition, there is no further evolution and species lose the ability to adapt to change. This makes them susceptible to catastrophic events that could wipe out the entire species in one go (Young, 1994). These effects are avoidable if sufficient genetic diversity is maintained within populations as well as within species. This is where genetic information can help conservationists to make informed choices.

This is not the only way in which genetics can be useful to conservation, and where conservation genetics provides insight into populations where other approaches to conservation biology can't. In order to conserve a species, the species and units of conservation, usually populations, must be defined and identifiable (Frankham, Ballou & Briscoe, 2004). If a species is not clearly delimitated, the incorrect species may be conserved, and more seriously, money may be spent on a species that doesn't need conservation. Genetics can be of use in resolving any taxonomic questions surrounding the target species (Soltis & Gitzendanner, 1999). For example, a species may be incorrectly identified as a rare new species which warrants immediate conservation, when in fact it could be a hybrid species or just misidentified (Small et. al, 1998). Genetics may help clarify these questions. In much the same way, genetics can identify the units for conservation. Resources may not be available to conserve the entire species once it has been targeted for conservation. Genetics can be used to identify the populations within the species which are most in danger, and which ones would maximise the genetic diversity within the species. None of these things would be possible within the usual realm of conservation biology.

Direct observation of a population can only give limited insight into the biology of the species. Genetics can be used to gain further insight into biology and population structure (Hendrick, 2000). This information can be of great use when formulating conservation

management plans, especially in deciding how species are to be conserved. If breeding programs are initiated, genetics plays a further role, as genetic diversity is essential for the success of captive breeding programs.

1.3 Choice of conservation targets

Before anything else can be done, the actual targets for conservation action must be chosen. There are a few different methods of choosing targets, each with a different reasoning behind it. The first of these, as mentioned previously, is basing choice on threat and distribution data, and using this data to classify species. The species classified as critically endangered, endangered or threatened are afforded conservation priority (Table 1). This method may be scientific, but it appears a bit too simplistic and doesn't include any genetic data. Other approaches include conservation of diversity "hot spots" and ecosystem approaches. While these methods both have their advantages, neither takes into account that a "hot spot" of species diversity may contain many species, but these species are not necessarily the most genetically diverse. Another area with fewer species may harbour a species containing a lot of genetic diversity. This needs to be taken into account. The remaining conventional ways for choosing conservation targets are all based on the perceived value of the species in question (Bisang & Hedenäs, 2000). Many species are of economic value e.g. those used in pharmaceuticals (Balick, 1996). It is of benefit to many that these species are conserved and continue being a harvestable resource. However, we have no way of telling which species may become of economic value in the future and we can only protect those that already have economic value (Plotkin, 1988). In basing our choice solely on this value, we may let species go extinct because we have not yet discovered their potential value.

In addition to economic value is ecological value. Many species have an ecological value i.e. they play a crucial role in maintaining ecosystems. Conservation targets may be chosen because of these roles they play, but this alone cannot be justification for conserving a species. If it were, we would have to conserve every insect that pollinates our crops and every plant that cycles nutrients into the soil (Barbier, 1994). Another,

more subjective value is the aesthetic or cultural value assigned to species deemed to be unusual, beautiful or culturally significant. Conservation targets are chosen for their appearance, popular appeal, or as "flagships". These species are often used to represent wide reaching conservation campaigns and to garner public support for conservation of certain areas (Bisang & Hedenäs, 2000). While this may be a good way of boosting support for conservation initiatives, it is a highly subjective method of decision and only aims to conserve species as they currently exist.

The great appeal of genetics is that it promises help with the choice of conservation targets, by providing an objective and scientific priority to each potential target. When combined with other data and assigned values, genetic data can make the final decision much easier. Genetic methods of determining conservation priority are particularly useful when species have no associated economic or other value, as in the case of many bryophytes. However, many of the more recently developed tools have yet to be applied to bryophytes.

1.3.1 Bryophyte conservation

Bryophyte conservation has gained more interest in recent years. The IUCN has even formed a special branch devoted to bryophyte conservation - the IUCN Species Survival Commission Bryophyte Specialist Group (www.artdata.slu.se). Their function is to unite bryologists working on conservation programs around the world, and provide a world red list for endangered bryophytes. At present, there are only 92 species on the list (www.iucnredlist.org). There are also several specialist bryophyte conservation programs run in different locations around the world, especially in Europe and America (see the IUCN website for more details: www.iucn.org). However, in South Africa there are currently no bryophyte conservation programs, but this does not mean that there are no bryophytes in need of conservation within our borders. One of the aims of this study is to bring attention to bryophyte conservation in South Africa, and alert people to the fact that many bryophytes are on the brink of extinction, yet nothing is being done to conserve them. The focus of conservation in the Western Cape is very much on fynbos. Yet

bryophytes are the ones responsible for maintaining the micro environments in which many fynbos species grow. Perhaps it is time to start looking a little closer.

1.4 The study plant

1.4.1 The genus *Zygodon*

The genus *Zygodon* belongs to the bryophyte family *Orthotrichaceae*. The exact number of species contained in *Zygodon* is debatable, especially in light of recent taxonomic reviews. In the first genus wide review, done by Malta in 1926, 77 species were recognised (Malta, 1926). However, Index Muscorum (1969) recognised 90 species and Vitt (1982) recognised 52 (Wijk et. al, 1969; Vitt, 1982). More recent taxonomic reviews have focused on small subsections of the genus, advocating either the reclassification of species currently recognised as belonging to *Zygodon*, or describing new *Zygodon* species (Wilbrahm & Long, 2005; Goffinet, 1998; Ignatov, 1999, Matcham & O'Shea, 2005). A comprehensive review has not been performed since that of Malta. A modern taxonomic review is needed and would shed light on the species diversity encompassed by this genus, and their relationships.

Malta divided his 77 species into 4 sections, these being *Euzygodon*, *Stenomitrium*, *Bryoides* and *Obtusifolii*. *Euzygodon* contained the majority of the species. Goffinet and Vitt (1988) segregated the species of section *Obtusifolii* into the genus *Bryomaltaea* (Goffinet & Vitt, 1998). In fact there was only one species in this section, *Z. obtusifolius* Hook, which was renamed *Bryomaltaea obtusifolia*. Since then, this species has been transferred into another new genus, based on morphological and genetic data, and is now called *Leratia obtusifolium* (Goffinet, 2004).

When Malta performed his review of the genus, he created the section *Bryoides*. In this section he included the type specimen for the existing genus *Codonoblepharum*, *C. menziesii* (Schwägr.) Arnott. In their review of *Orthotrichaceae*, Goffinet and Vitt (1998)

recognised sufficient differences between species previously designated as *Codonoblepharum* that were placed in *Zygodon* section *Bryoides*, and the rest of the species in *Zygodon*, that they resurrected the genus. Almost all of the species placed in section *Bryoides* by Malta were reassigned to the genus *Codonoblepharum*. The exception to this was *Z. forsteri* Dicks. This species was deemed to have additional morphological features that suggest it belongs to neither genus, while the molecular data suggest that it too should be reassigned to *Codonoblepharum*. At the present time, it remains part of *Zygodon*. An additional review was done by Matcham & O'Shea (Matcham & O'Shea, 2005). New combinations were created for several members of *Zygodon*, separating smooth-celled species out of *Zygodon* and into *Codonoblepharum*. Certainly more work should be done to further evaluate this classification.

Zygodon species are found mostly in temperate and tropical regions of the southern hemisphere. The type species is European, but the centre of diversity is considered to be South America, where almost half of the known species can be found (Lewinsky, 1989). Even though most of the known species are South American in origin, *Zygodon* species can be found throughout Europe, Asia, Australasia and Africa.

1.4.2 *Z. leptobolax* – a narrow Cape Endemic

Several species are evident in South Africa. In his review of the South African bryophyte flora, Sim (1926) noted 7 species, these being *Zygodon africanus* Sim, *Z. dixonii* Sim, *Z. erosus* Mitt., *Z. leptobolax* Müll. Hall., *Z. runcinatus* Müll. Hall., *Z. transvaaliensis* Sim and *Z. trichomitrius* Hook & Wilson. However, more recent reviews have placed *Z. transvaaliensis* in the synonymy of *Z. intermedius* B.S.G., and *Z. africanus* in *Z. erosus*. In addition, *Z. dixonii* is recognised as a synonym of the newly erected *Codonoblepharum microtheca* (Dixon ex Malta) Matcham & O'Shea. *Z. dixonii* was described by Sim shortly after *Z. microtheca* was described by Dixon, hence the new combination name (Matcham & O'Shea, 2005).

The most recent review recognises five species of *Zygodon* in South Africa: *Z. erosus*, *Z. intermedius, Z. leptobolax*, *Z. runcinatus* and *Z. trichomitrius* (Magill & van Rooy 1998). Of these *Z. trichomitrius* and *Z. leptobolax* are endemic to South Africa, but where *Z. trichomitrius* can be found throughout the country, *Z. leptobolax* is restricted to the Western Cape.

This project focuses on the genus *Zygodon* in South Africa and, more specifically, on *Zygodon leptobolax*. The species, first described in 1899 (Müller), is based on a type collected in the Rondebosch area of Cape Town, in the Western Cape region of South Africa. It is very similar to the widespread *Z. intermedius*. (occurring in the in the Eastern and Western Cape regions of South Africa (Magill & Van Rooy, 1998), as well as in Asia, tropical America and Australasia (Allen, 2000) and the two differ only in the grouping of the sexual organs of the gametophyte. *Z. intermedius* is dioicous, having archegonia and antheridia on separate plants, while *Z. leptobolax* is synoicous, having both archegonia and antheridia in the same inflorescence on each plant. All subsequent collections of *Z leptobolax* have been from a very narrow area on the eastern slopes of

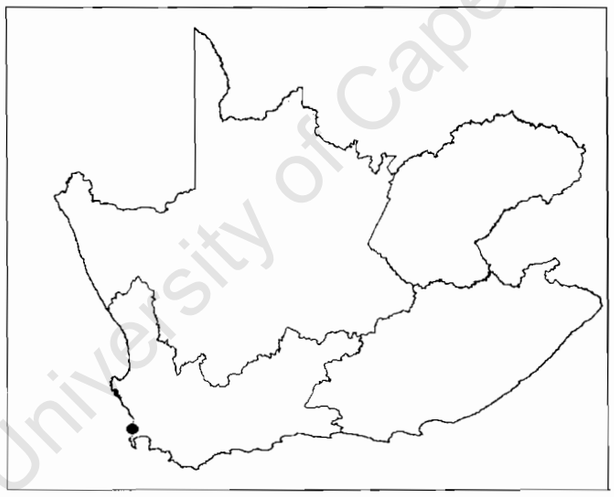

Figure 1: Distribution of *Z. leptobolax* in South Africa.

Table Mountain and the adjacent Devils Peak (Figure 1). Records from outside this range have all been based on misidentifications of *Z. intermedius*.

Z. leptobolax can occur as single shoots or in small to medium sized loose cushion-like aggregations that are epiphytic (Sim, 1926). The formal description, after Magill & van Rooy (1998) describes *Z. leptobolax* as having stems, averaging 0.5cm (but can be up to 1cm) in length, bear small oblong-lanceolate to lanceolate shaped leaves, ending in an acute tip. Leaves are costate, with the costa ending before the apex. Leaf margins are entire and plane, but sometimes decurrent below, and densely papillose. Leaves are erecto-patent to spreading when wet and appressed and twisted when dry. Laminal leaf cells are typically rounded and densely papillose, becoming smaller near the apex, while basal cells are more oblong in shape and also densely papillose. *Z. leptobolax* is synoicous and bears terminal inflorescences. A narrowly pyriform capsule, with eight ribs, is borne upon a seta which averages 1cm in length. A single peristome is present. Papillose spores averaging 17 – 28 μm are dispersed through the operculum.

The host species of the original collection is unknown and recent collections have all been found on the bark of large specimens of *Quercus* species. *Quercus* species are not indigenous to South Africa and were introduced to the region in the 19th century. It appears that *Z. leptobolax* grows on large individuals of *Quercus* species, due to similarities between them and the original indigenous host species, and because of the suitable microhabitat created by the vertical grooves in the trunk bark (pers. obser). In the southern Cape region of South Africa, several other species of *Zygodon* grow on large *Podocarpus* individuals, such as *P. latifolius* (Thunb.)R.Br. ex Mirb., including the very similar *Z. intermedius* which also grows on *Quercus*. Extensive logging took place on the slopes of Table Mountain in the 19th century, and few large trees of indigenous species that might have been hosts occur now in the areas where *Z. leptobolax* has been known to grow. If the indigenous host of *Z. leptobolax* was in fact a species of *Podocarpus*, there would be very few hosts left on Table Mountain. Hence the likelihood of finding a colonised indigenous host is very remote. The idea that *Podocarpus* is the original host species is further supported by the fact that isotypes at the Pretoria Herbarium and British

Museum are presented on peeled bark strips. These strips are not likely to belong to a *Quercus* species, but are highly consistent with the bark of *Podocarpus* species (Professor T. Hedderson, pers.comm.).

1.5 Conservation of *Z. leptobolax*

South Africa is one of the world leaders in the control of alien invasive plants. After several studies showed that invasive alien trees growing in mountain water catchment areas reduce stream flow, aggressive removal campaigns were initiated (Le Maitre et. al. 2002). The removal efforts focused on a few specific invasive species e.g. *Acacia* and *Pinus* spp. *Quercus* species, not usually invasive in the Western Cape, were exempt from such plans. However, recent plans have been initiated to remove all alien plants from Table Mountain, not just the invasive species. This may pose a serious conservation threat to *Z. leptobolax*, as most of its current known populations grow on *Quercus* species. As host trees are removed, so too are populations of *Z. leptobolax*. In the absence of new colonisation sites, and larger core populations, the species is likely to become extinct in the next few years. If a species of *Podocarpus* is indeed the original host, it will take some time before existing specimens reach a sufficient size, as *Podocarpus* species have slow growth rates, taking many years to reach maturity (Palmer & Pitman, 1972).

1.6 Aims and objectives

Given the similarity of *Zygodon leptobolax* to *Z. intermedius*, its value as conservation target might be questioned. Furthermore the small population size and the possibility of extensive self-fertilisation (because of the autoicous sexual condition) raise the possibility that the species might be genetically depauperate. In this thesis I use the *Z. leptobolax* as a case study in the application of prioritisation criteria and genetic measures of diversity. I attempt to answer the following key questions:

1) What is the current status of *Z. leptobolax*?

2) What level of genetic diversity exists in the remaining populations?
3) Should these populations be afforded conservation status?
4) How can this be decided?

This moss is then used as a case study for testing some of the currently-available tools for making conservation choices.

STATUS OF CURRENT POPULATIONS AND AN ASSESSMENT OF INTER-ANNUAL DYNAMICS

2.1 Introduction

Currently, populations of *Z. leptobolax* are known only from the eastern slopes of Table Mountain, where they grow on the bark of forest trees. Populations have been recorded from localities within Newlands Forest, as well as from the many gullies and kloofs on the eastern slopes of the mountain (T. Hedderson, pers. comm.). The distribution and extent of the species has never been determined, nor has the number of constituent populations been recorded. It is currently accepted that *Z. leptobolax* is endemic to Table Mountain, but the actual distribution of populations on the mountain remains to be determined. Little is known about the spatial dynamics of *Z. leptobolax*, and the range of the species is also currently unknown. A population census would provide this information and further our knowledge of the spatial patterns of the species.

A population census can be very informative to conservationists. For conservation action to take place, conservationists need to know what the species is, where it is, how much there is of it and how it is threatened (Meffe & Carroll, 1994). Defining a species is the role of taxonomists, but the other factors can be determined by a population census. A population census of *Z. leptobolax* would provide information on the number of populations, how they are distributed and what their current status is. The environmental envelope of *Z. leptobolax* could also be determined through a population census. All previously observed populations of *Z. leptobolax* were found to be growing on non-indigenous host trees (T. Hedderson, pers. comm.). A close examination of the individual populations could reveal the indigenous host species, or at the very least offer a plausible explanation as to why populations have previously only been found on non-indigenous hosts.

A population census can also be useful in identifying any threats that a species is currently experiencing. This information is vital for the construction of conservation

management plans. In the case of *Z. leptobolax*, current practises adopted in the Table Mountain National Park (one of the locations where *Z. leptobolax* has been observed) may threaten the non-indigenous host species of this bryophyte. It is currently unknown whether *Z. leptobolax* grows exclusively on non-indigenous species, or if other populations are present on indigenous host trees. These practises may be a large threat to the survival of *Z. leptobolax* if the former is true. This information can be provided by a population census. Additional threats may also be revealed during the course of a census. For example, it will become apparent if the species distribution occurs mainly in a highly fragmented or degraded habitat type.

Population censuses can be of use in other ways. In addition to providing the answers to the questions posed by conservationists, they can be used to form the basis for further investigation into the population dynamics and demographics of a species. If population censuses are repeated at regular intervals, conservationists can get a good understanding of how populations fluctuate over time, which is necessary for conservation of those populations (Meffe & Carroll, 1994). Repeated censuses can give insight into the changing distributions of target species as well as into the status of the individual populations within the distribution. Any size reduction or loss of populations can be placed in context. In this way, it is easier to identify whether the observed loss is due to natural fluctuation or to a negative outside influence.

2.1.1 Aims and objectives

This section of the study aims to determine the extent of *Z. leptobolax* in its native habitat, by means of two population censuses. The number of populations, their size and location will also be determined by the two censuses. This will attempt to answer the key questions: What is the current distribution of *Z. leptobolax*? How many populations are there and how dynamic are they? Which species of host is most prevalent and can populations be found on both indigenous and non indigenous hosts?

2.2 Methods

2.2.1 The study area

This study was conducted on the eastern slopes of Table Mountain, where populations of *Z. leptobolax* have previously been observed, as well as in the surrounding areas. An area extending from Groote Schuur Estate (33° 46.78S, 018° 57.26E) to the area above Kirstenbosch Botanical Gardens (33° 59.17S, 18° 25.99E), including Newlands Forest, was included in the study. This area was selected so as to include all known remaining populations of *Z. leptobolax*. The study area encompassed a variety of vegetation types and plant species. Starting at Rhodes Memorial, on Groote Schuur Estate, the vegetation tends to be mostly alien pines, oaks, grasses etc. in a park like setting. This gives way to more indigenous fynbos above the monument, once the contour path is reached. From there onwards, heading towards Newlands Forest, the vegetation remains mixed, with poplars, gums, oaks and other indigenous trees growing together. Newlands forest itself was once a pine plantation and the remains of this history are evident at the lower levels of the forest, where the dominant species is still *Pinus*. However, large parts of the forest remain indigenous, especially those parts situated in the many gullies and ravines formed by the mountain.

This area experiences a mild Mediterranean climate, with wet winters and warm to hot summers. Being situated so close to Table Mountain, this area probably receives slightly more precipitation than other areas in the Cape, and there are several seasonal river beds situated in the ravines.

2.2.2 Population census

This section of the study consisted of two parts i.e. two censuses. The first population census was conducted during the summer in September to October of 2004/2005, and the second during the summer in October to January of 2005/2006. This provided replication and allowed the examination of growth and distribution trends among populations.

The study area was divided into sections of roughly similar size, and trees in each section (both alien and indigenous) were examined for the presence of *Z. leptobolax*. Once a population was located, the host tree was identified and the coordinates of the location were recorded. Additional details of the host bark texture, location of the population on the trunk and size of the population were recorded. It was also recorded if sporophytes were present and what condition the host tree seemed to be in. Population size was categorically recorded, with size being divided into four classes. Populations consisting of a few single shoots on a trunk were placed in class one, populations consisting of fewer than 20 shoots were placed in class two, and populations of between 20 and 50 shoots were placed in class three. Extensive populations that covered large areas of the tree trunk and consisted of more than 50 shoots were placed in class four.

The second population census was conducted during the summer of 2005/2006, in the same study area previously covered by the first population census. The procedure was the same as that followed for the first census. However, during the second census, previously observed populations were revisited to see if any changes had occurred in either the host trees or the populations themselves e.g. population expansion, host damage etc.

2.2.3 Data analysis

The extent of *Z. leptobolax* was determined by mapping the GPS coordinates of located populations. GPS location data were first converted into standard decimal coordinates before a distribution map was created using Geographical Information Systems (GIS) software (ArcView v3.2, Environmental Systems Research Institute, Inc. Populations were also subdivided into three categories – those that were found in both censuses, those that were only found in the first census and those that were only found in the second census. This information was also plotted onto the map.

A graph was constructed to show the number of populations per size class per year. A second graph was constructed to show the number of mortalities per size class. A third graph was constructed to compare the number of populations with sporophytes per year,

to give an indication of how this can vary. In addition, the number of sexually reproducing populations was broken down by population size class for each year, in order to determine whether any one population size class produced more sporophytes than the others.

2.3 Results

During the first population census, conducted in the summer of 2004/2005, the populations that were expected to be alive and healthy in Newlands Forest were found to be dead. The host trees had been ring barked, presumably as part of the alien removal campaign initiate. This was a large setback. However, further examination of the areas adjacent to Newlands Forest i.e. sections of Groote Schuur Estate yielded more populations. *Z. leptobolax* was found to have a very restricted range, with populations being found between First Waterfall Ravine and Duiwekloof Ravine in census one (Figure 2). This range shrank to encompass only First and Second Waterfall Ravines after the second census was completed. Populations appeared to be clustered in these ravines on host trees that grow close to seasonal rivers.

During the first population census a total of 43 trees was found to be hosting populations of *Zygodon leptobolax*. All host trees recorded were non-indigenous *Quercus* species, and the individuals are between 100 and 150 years old (Dr. E. February, pers. comm.). No additional populations were found on indigenous host species. The majority of populations (74.4%) examined were small in size, consisting of fewer than 20 shoots per population (Figure 3). This category contained almost three quarters of all populations recorded during this census. Most of the remaining populations fell into the medium category (11.6%), with only 2 populations, (4.7%) classified as large.

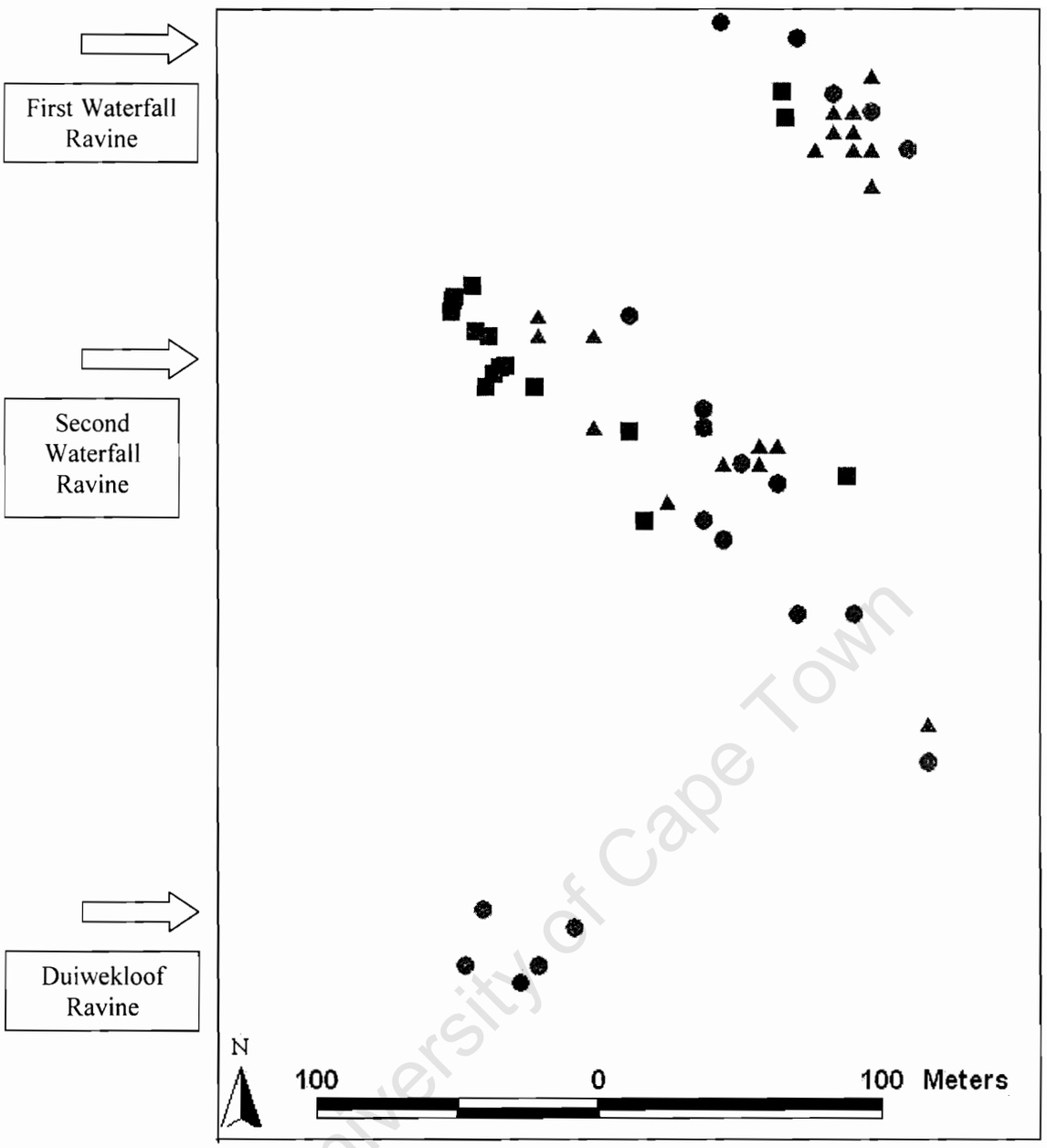

Figure 2: Map showing the geographical distribution of trees on the eastern slopes of Table Mountain hosting populations of *Zygodon leptobolax*. Circles represent populations found only during census 1, squares represent new populations in census 2 and triangles are the populations recorded in both censuses. All three clusters were on similar soil, near to seasonal riverbeds.

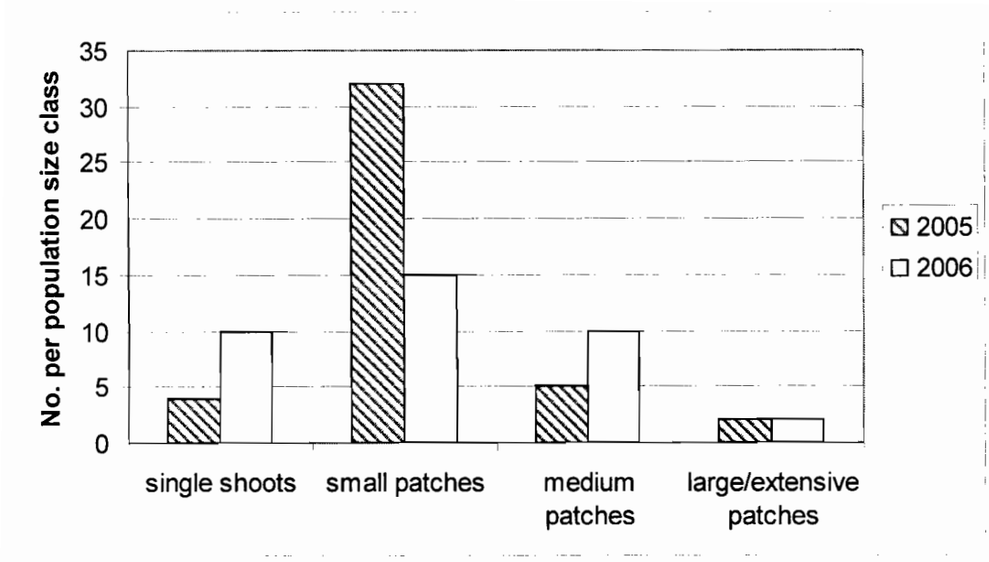

Figure 3: Number of populations falling into each size category for the first census versus the second census. Small patches consisted of fewer than 20 shoots, medium patches of between 20 and 50 shoots and large patches consisted of more than 50 shoots.

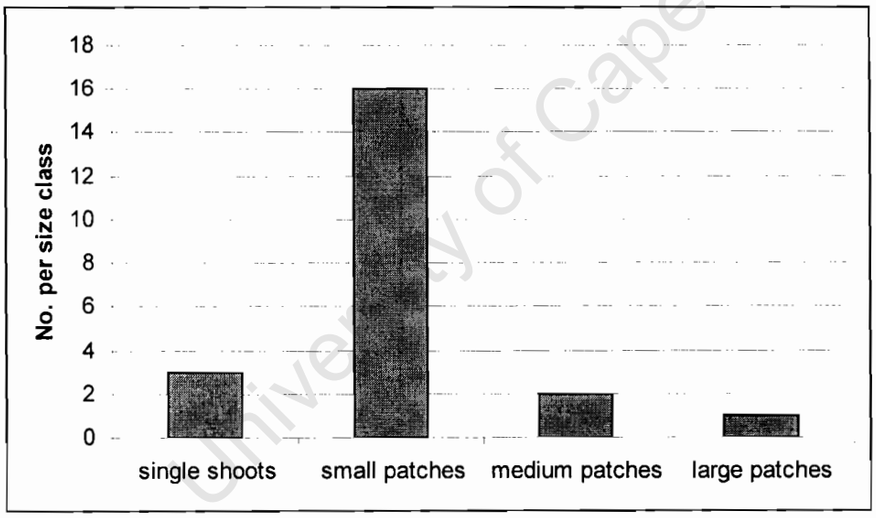

Figure 4: Mortality per size class for the populations not located during the second census.

During the second population census, a total of 37 trees was found to be hosting populations of *Zygodon leptobolax*. This is six populations fewer than were recorded during the first census where 43 populations were recorded (Figure 2). All host trees recorded were one again found to be non-indigenous *Quercus* species, and the individuals aged between 100 and 150 years old (Dr. E. February, pers. comm.). Populations examined during census two were more evenly distributed among size classes than those examined during census one. As with the first census, the majority of populations fell into the small category. But while three quarters of all populations in census one fell into this category, only 40.5% of all populations from census two fell into this category. Both the single shoots and medium patch (containing between 20 and 50 shoots) categories contained 27.02% of the populations observed during the second census. Once again, only two populations were classified as large (Figure 3).

Of the original 43 populations found during census one, only 21 (48.8%) survived to be recorded during census two. The remaining 22 original populations could not be found during the second census and were presumed to have died. Three (13.6%) of the populations presumed dead fell into size class one (single shoots), 16 (72.7%) fell into class two, 2 (9.2%) fell into class three and only 1 (4.5%) fell into class four (large/extensive patches) (Figure 4).

Of the 37 populations found during census two, 16 (43.3%) were new populations not previously recorded. As all trees in the areas where populations were found during census 1 were examined and found to be free of the moss, this indicates that some colonisation events must be occurring. These could be the result of spore dispersal and germination or of vegetative means of reproduction e.g. gemmae. The majority of these newly colonised populations fell into size categories one and two i.e. single shoots and small patches. Only two fell into the third category (medium patches). Of the 21 populations recorded in both surveys, a third grew in size i.e. more individual shoots were successfully produced

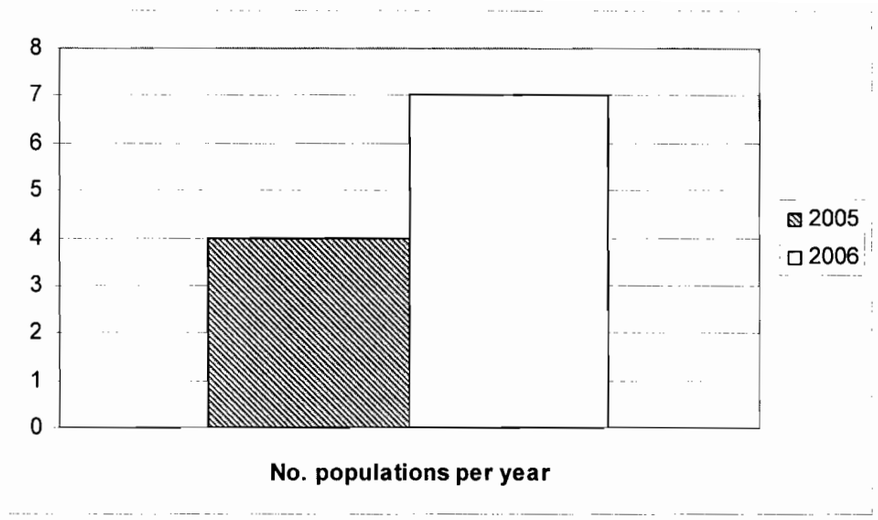

Figure 5: The number of populations with sporophytes per year

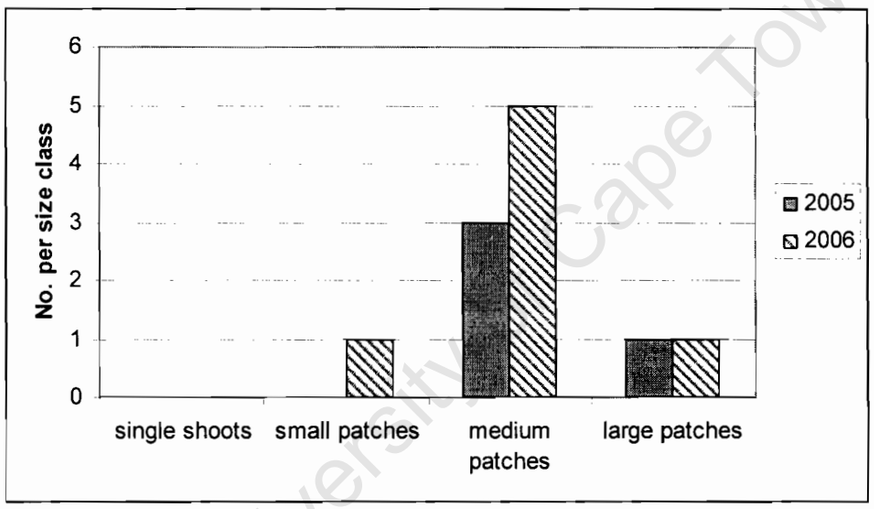

Figure 6: The number of populations with sporophytes per size category for the first census versus the second.

within those populations, a third decreased in size i.e. there was some shoot mortality within those populations and a third remained the same size.

During the first census, four (9.3%) of the populations were found to have sporophytes. However, during the second census, seven (18.9%) populations were found to have sporophytes (Figure 5). This represents a doubling in sexual reproductivity. The majority of populations with sporophytes in both censuses were medium sized patches. During 2005, the only populations to reproduce sexually were medium and large in size. A similar pattern is true for those populations reproducing sexually in 2006. However, during 2006, one small patch was found to have sporophytes. In the populations where sporophytes were produced they were numerous, with roughly one sporophyte per 10 – 15 shoots (Figure 6).

Even though the species seems capable of sexual reproduction, and it would appear that the populations are healthy, the host trees are not fairing as well. During census one, only 6.9% of host trees were recorded as damaged i.e. by dry rot or physical damage, and all populations of *Z. leptobolax* had a healthy appearance. However, during census two, the percentage of trees damaged had increased to 18.9% (Figure 7). Also, some of the populations in the vicinity of Duiwekloof Ravine, on Devil's Peak were intermingled with black algae that were growing on the host bark and covering populations in places.

2.4 Discussion

Z. leptobolax was found to have a very narrow distribution range. Populations were found only in three ravines on Table Mountain during the first census, conducted in 2005. This range was reduced to only two ravines after the 2006 census. However, only five populations were found in the third ravine during the first census, and the actual distribution may vary to include the third ravine or not, depending on dispersal and germination of spores. The range remains very limited, whether or not the third ravine is included, and the species' distribution appears to be centred in First and Second Waterfall Ravines on Table Mountain. *Z. leptobolax* was only found on non-indigenous host

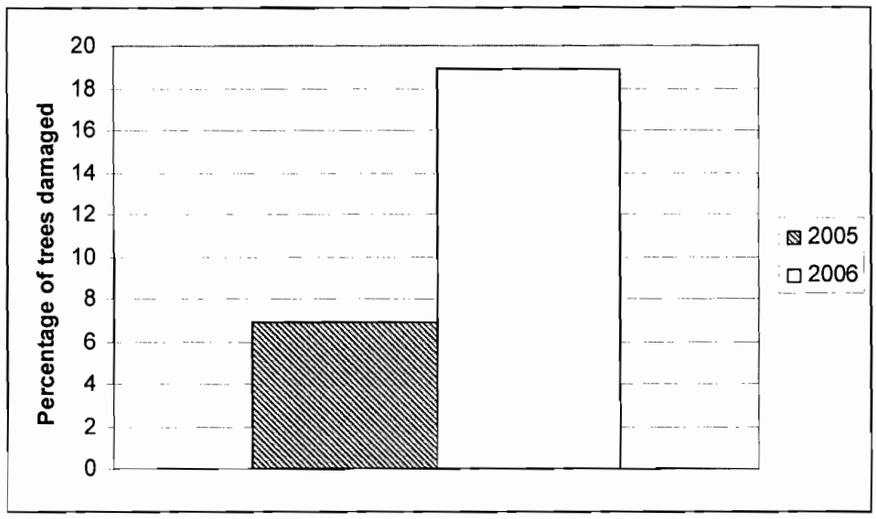

Figure 7: The percentage of host trees that were found to be damaged during each census.

species of *Quercus*, and then only on older specimens. It seems that *Z. leptobolax* only grows in the corrugation-like grooves formed in the bark of mature oaks. This is a very specific habitat, made more specific by the fact that oak trees do not naturally occur in the area. The total number of populations varied slightly between censuses, but this could be attributed to natural variation rather than as a sign of population decline.

While the total number of populations remained relatively constant, the composite populations varied greatly between censuses. *Z. leptobolax* is a very dynamic species, with much recruitment and population mortality occurring. The results from both censuses showed that the majority of populations were small, but a greater proportion of the total number of populations located during the first census fell into this size class. Populations found during the second census were more evenly distributed among the size classes, indicating a slight shift towards a more normal distribution, rather than a distribution skewed toward newly recruited populations. The number of large populations remained constant, while there was an increase in the number of single shoots, as well as in the number of medium sized patches. This indicates that some recruitment occurred, and some small patches increased in size. However, mortality was also greatest in the

small patches category, and least in the medium and large categories. This suggests that while a few small patches increased in size, to become medium sized, others died off. This could indicate that most single shoot patches survive past recruitment and become small patches. Then other factors, such as demographic or abiotic factors, become involved to threaten the survival of these populations and prevent them from becoming medium sized. The implication being that not many populations reach medium size, which is the observed pattern. More recruitment is required to survive and grow to fill the gap left by the deceased small sized populations. If this doesn't happen, the species could be reduced to a few large populations, and the survival of the species could be threatened (Söderström, 1995; Söderström & During, 2005).

Evidence of reproduction is good. Almost 20% of all populations found during the second census had produced sporophytes. This represents a doubling in the number of populations with sporophytes relative to the first census. Most of these populations were medium or large sized, with only one small patch producing sporophytes throughout the entire study period. This suggests that plants need to reach a certain age threshold before they can produce sporophytes (Benson-Evans, 1964; Hassel, Pederson & Söderström, 2005). In the meantime, they may reproduce via asexual means e.g. via gemmae, in order to increase population size (Kimmerer, 1994). This would allow an increase in population size from single shoots to small populations, and then a further increase to medium sized populations of a sexually reproductive age. However, this would mean that most small patches would consist of clones of the first plant recruited at that particular site, making them more vulnerable to demographic factors, such as pathogens (Meffe & Carroll, 1994). It is important to note that sporophytes are being formed, and spores are being dispersed to new recruitment sites. This is important for the replacement of the large number of small populations that die off.

Despite the relatively stable number of populations, the active reproduction and recruitment, the survival of *Z. leptobolax* may still be threatened. The loss of suitable hosts plays a very important role in the survival of the species (Vanderpoorten, Engels & Sotiaux, 2004). The results of this section of the study show that the number of host trees

that have been damaged has increased almost threefold. *Z. leptobolax* is a species with very specific habitat requirements. This section of the study aimed to determine if populations could be found on an indigenous host, however, none was found. This implies that *Z. leptobolax* also has very specific habitat requirements. If potential hosts are damaged or removed, the species could face a possible crisis in the coming years.

A familiar model of classifying species as rare is that of Rabinowitz (Rabinowitz, 1981). Seven types of rarity were recognised, based on combinations of three criteria: narrow geographic range; high habitat specificity and frequently small populations. Being restricted to a single host species and being found at a single location is enough to classify *Z. leptobolax* as rare. However, the data also show that the majority of populations are also small, which puts the species into the rarest category. Rare species, by their nature, are more likely to be threatened than more common species (Longton & Hedderson, 2000). It seems that *Z. leptobolax* has a delicate balance between recruitment and mortality, and any changes to the host availability or population structure could result in a large population decline. Sporophytes are only produced by plants of a certain age, in larger populations. These spores have to germinate as recruitments are needed to grow into small populations in order to replace the large proportion of small populations that die off. If there are no suitable hosts, recruitment won't take place, plants won't grow to a size where they are capable of sexual reproduction and the species won't be able to persist. On the other hand, if the large populations producing sporophytes are removed, by death of the host, for example, no further recruitment will take place until new recruitments reach an age where they can start producing sporophytes. However, the number of recruits that actually reach this age is low, and the species may go through a bottleneck. With so few populations, a bottleneck could result in the elimination of the species.

Overall, the current status of the species can be said to be rare, and threatened. If action is not taken to preserve the hosts or the species itself, *Z. leptobolax* may not survive for many more years.

PHYLOGENETICS, DIVERSITY INDICES AND THE CONSERVATION VALUE OF ZYGODON LEPTOBOLAX

3.1 Introduction

Traditional methods of choosing conservation targets are often biased or too subjective to be deemed scientific. As mentioned previously in this study, there are many ways to choose targets, but for conservation to be successful on a large scale there needs to be a uniform method for choosing these targets. Ultimately, all of the traditional methods of choice have the same goal – preserving a portion of nature from further man-made damage and maintaining it as it is today (Moritz, 2002). This would be acceptable if we wanted to preserve the environment as it is presently, without any changes. But nature is not static and neither are species. Species must have the ability to adapt to environmental and circumstantial change, and to evolve (Frankel & Soulé, 1981; Franklin, 1980). To allow change and evolution to continue happening, it would be more advantageous to preserve the underlying processes that resulted in the species and environments we see, rather than trying to conserve static species in a dynamic environment (Cowling & Pressey, 2001; Frankham, Ballou & Briscoe, 2004).

By preserving the processes that shape species, the future potential, or evolutionary potential, of those species can be preserved. The raw material driving evolution is genetic diversity (Ridley, 1996). In order to preserve the evolutionary processes, and hence evolutionary potential, shaping species we must conserve the maximum genetic diversity within those species. This concept can be extrapolated to a larger scale, where the evolutionary potential of a monophyletic group can be conserved by the preservation of the maximum genetic diversity within that group (Crozier, 1997; Faith, 1992). This can be done by conserving the most genetically diverse, or distinct, species within the group. This idea is analogous with the method of choosing reserve areas that maximise biodiversity, as described by Margules (Margules, Nicholls & Pressey, 1988).

When aiming to maximise biodiversity of a group of species, the phylogenetic relationships among them can be used as an indicator of underlying genetic diversity (Faith, 1992; Faith, 1994a; Faith 1994b). The actual pattern of the phylogeny can be used to indicate biodiversity. This is the basis of the phylogenetic criteria of target choice.

Phylogenetic criteria for conservation choice have been the subject of debate for several years (Faith, 1992, 1994a, b; Faith, Reid & Hunter, 2004; Vane-Wright, Humphries & Williams, 1991; Crozier, 1992, 1997; Nee et. al, 1994; Soltis & Gitzendanner, 1997; Owens & Bennet, 2000; Posedas, Esquivel & Crisci, 2001; Moritz, 1995). In order to select a species for conservation using the phylogenetic criteria, a value must be placed on all the species in the selected monophyletic group. This ranking method should favour the retention of the maximum amount of genetic diversity represented in that group; in order to conserve the evolutionary processes (Crozier, 1992). This is done by quantifying the amount of biodiversity each species harbours, as more diversity equals more evolutionary potential. In the past, biological diversity has been referred to as the "option value" of a species – the "safety net of biological diversity for responding to unpredictable events" (McNeely et. al., 1990; Faith, 1992). In this study the term "evolutionary potential" refers to the same quality.

3.1.1 Overview of phylogenetic analyses

Evolution is descent with modification. Evolution can result in speciation, and each species has a relationship to other species, as well as an evolutionary path that resulted in these relationships (Futuyma, 1998). Evolution is such that if you follow the ancestry and evolutionary history of two species, they will eventually have a common ancestor. Phylogenetic systematics is a method of classification that deals with these evolutionary relationships, and represents them in phylogenetic trees. Phylogenetic trees are simply visual representations of the relationships among species, essentially showing the shared history of species, and their common ancestry (Hillis, Moritz & Mable, 1996). Competing phylogenetic hypotheses (trees) are evaluated under certain criteria. The most commonly used of these is the criterion of parsimony, under which the tree requiring the fewest

changes in character states to give the observed data is preferred (Swofford, Olsen & Waddell, 1996). Another means of evaluating phylogenetic trees is under the likelihood criterion (Lewis, 1998). Parsimony assumes a relatively simple model when evaluating phylogenies. We know a great deal about molecular data and this knowledge can be incorporated into a more complex model to determine phylogenies – the likelihood model. Rather than choosing the tree that represents the fewest changes, as under parsimony, likelihood chooses the tree that has the highest probability of resulting in the DNA sequences for the species being examined (Lewis, 1998).

Phylogenies can be constructed from morphological or molecular characters, or from a combined data matrix. Over the years there has been much debate as to which type of data set is the best to use (Patterson, Williams & Humphries, 1993). All of them have their pros and cons; however, basing a phylogeny on morphological characters that may be homoplasious could result in a false topology (Wendel & Doyle, 1998). This is because morphological characters can be interpreted differently by different taxonomists e.g. what one person interprets as a lanceolate leaf shape, another may describe as more oblong. A systematist recreating the evolutionary history may place such a species more basally in a tree, as it would appear to be similar to the basal species, rather than in its correct place further up the tree. Molecular data can overcome such problems, as a large number of informative characters can be relatively easily achieved. In addition, character states can be clearly defined within a group of group of study organisms (Kitching et. al., 1998). There has also been debate around the issue of combining different data sources and whether they should be analysed separately or simultaneously. Combined data matrices, incorporating morphological and molecular data, often result in more resolved phylogenies with fewer changes than either data set alone would give (Nei & Kumar, 2000).

3.1.2 Phylogeny –based diversity measures

The quantification of biodiversity is done by examining "features" of the species in the phylogeny. These features may be visible morphological characters or genetic characters.

Genetic features are changes among the sequenced DNA regions of the species that are represented in the phylogeny. The more unique genetic features a species has, the more different it will be, and the higher its conservation priority (Faith 1992, 1994). However, quantifying biodiversity is not as simple as counting the changes between species on a phylogeny. There are many other factors, such as the probability of change along a branch and the actual branch lengths themselves, involved. Hence, a diversity index is calculated for each species in the phylogeny. These indices may be calculated in several different ways. Once these indices have been calculated, the species with the highest ranking, or value, will be the one on which to focus conservation efforts.

The first papers touching on this topic were published in the early 1990's, and involved very simplistic models. Each node in a phylogeny was assigned a value according to how many branches arose from it. The values of terminal taxa were determined by adding all values for the nodes on the braches leading up to them (May, 1990). This was very easy to do, but required a fully resolved, rooted tree, which was not always available. This idea was later revised and modified by Vane-Wright and others, resulting in an approach where all that was required was to count the nodes between the taxon in question and the root of the tree. The values of terminal taxa were then said to be proportional to the inverse of their node count (Vane-Wright, Humphries & Williams, 1992). This model also had its limitations i.e. basal species were naturally assigned low priorities, and the scores changed with topology. Hence, a fully resolved, rooted tree was also required.

These methods were both purely cladistic in nature. Crozier argued that the ranking procedure should favour the retention of genetic diversity (Crozier, 1992). Previous methods had problems – apart from needing to know the root of the tree, branch lengths were not included in the calculations. If branch lengths are not included in the ranking procedure, the species with the highest genetic diversity may not be prioritised. This is because species on long branches have more time to accumulate changes, but don't have a high node count (Crozier, 1992). Species on shorter branches may have more nodes leading to them, and thereby are afforded a higher priority according to the previous models, even though they may not have as much diversity as the species on the longer

branches. The new model used genetic data to estimate branch length, and a formula to determine the "uniqueness" value of each species i.e. how many different features each species had. The species with the highest uniqueness values were then the ones prioritised for conservation.

This model was further refined so that topology and branch length were combined to assign priority for conservation (Faith 1992, 1994). Faith's model for assigning conservation priority is based on the path in a tree with the fewest branches connecting all species. He called this the minimum spanning path. Phylogenetic diversity (PD) is then determined by adding all branch lengths in a given set. Conservation priority can then be determined by adding potential conservation target species and recalculating the gain in PD they offer. The species that adds the largest gain in PD is assigned the highest conservation status. This information can then be added to existing traditional threat/vulnerability data and a final decision can be made. He also reworked the uniqueness formula of Crozier, to include this newly developed measure of diversity (Faith, 1994).

Faith's measure of phylogenetic diversity has been applied to several bryophyte examples. Bisang and Hedenas presented an excellent paper on this topic, where they presented examples of the use of PD both at and above the species level (Bisang & Hedenas, 2000). They demonstrated the use of the principles of PD in assigning conservation priority to species within the genus *Didymodon.* In addition, they demonstrated the versatility of this method by using PD to assign priority at a higher taxonomic level i.e. genera within families.

Overall, a method that includes both topology and branch length appears to be the most robust. The problems of rooting the tree and incorrect ranking are overcome. This method is also universal - it can be applied to bryophytes as easily as it can be applied to animals and other plants, as well as at different taxonomic levels. All that is required is the molecular sequences and a phylogeny to describe the relationships among the species in question.

3.1.3 Phylogenetic relationships in *Zygodon*

The genus *Zygodon* has not been reviewed in its entirety since 1926 (Malta, 1926). Since then, new species have been discovered, others renamed and some moved out of the genus. At present, it is unclear how large the genus is, or what the relationships among species are. However, the world list of mosses (Crosby et. al., 2004) lists 91 valid names although many of these will eventually fall into synonymy.

While there have been several taxonomic studies on species of *Zygodon*, the majority of these only examined morphological characters (Lewinsky, 1989; Wilbrahm & Long, 2005; Ignatov, 1999, Karttunen, 1984). To date, there have been very few molecular studies conducted on species of *Zygodon*, and the majority of these have focused more on the placing of *Zygodon* within a broader class or family classification, rather than defining the relationships within the genus (Goffinet, Bayer & Vitt, 1998; Goffinet & Vitt, 1998; Goffinet et. al. 2004). Relationships among the South African representatives of *Zygodon* are also unclear. A full genus wide molecular review could certainly help to sort out the relationships among the South African species of *Zygodon*, as well as their relationships to the rest of the genus.

3.1.4 Aims and objectives

This section of the study aims to place the South African *Zygodon* species into a global framework phylogeny for the genus. The resulting phylogenetic framework will then be used in the application of some of the diversity measures discussed above, to evaluate the conservation priority of *Z. leptobolax*. The difference between nucleotide and morphologically based diversity measures will also be compared. This is in an attempt to address the key questions 1) Will the loss of *Z. leptobolax* results in a loss of feature diversity? and 2) Does *Z. leptobolax* afford conservation priority?

3.2 Methods

3.2.1 Phylogeny reconstruction

3.2.1.1 Taxon and DNA sampling

Sampling was done across the morphological diversity of the genus, as well as across the geographical range. Samples of all of the South African species were included in the study, as were representative species from South America, Europe, Asia and Australia. Additional samples and sequences were kindly supplied by Bernard Goffinet. The primers *trn*F and *trn*C were used to amplify a plastid region of the genome, *trn*L-*trn*F (Gielly & Taberlet, 1994). This region has been used extensively in phylogenetic studies, as it displays enough change to be of use at the species level (Clegg, et. al., 1994; Bohle, et. al., 1994; Ham et. al., 1994; Taberlet et. al., 1991; Golenberg et. al., 1993; Sang, et. al., 1997).

3.2.1.2 DNA extraction, amplification and sequencing

Molecular analysis was performed on 6 herbarium samples, as well as on 15 fresh samples of *Z. leptobolax* that were collected from the eastern slopes of Table Mountain and air dried. Approximately 0.5ml of each dried specimen was placed in a 1.5ml micro-centrifuge tube for extraction. DNA was extracted following a modified version of the protocol outlined by Gawel and Jarret (1991). Each sample was ground in a mortar and pestle with 700µl of hexadectyltrimethylammonium (CTAB) and 1µl of β-mercaptoethanol. Once ground, the samples were returned to the 1.5ml micro-centrifuge tubes and heated in a water bath at 65°C for approximately 30 minutes. 600µl of chloroform-isoamyl alcohol (24:1 v/v) was added to each sample and mixed by inversion. Samples were spun in a micro-centrifuge at 12 000 rpm for 5 minutes, after which the supernatant was transferred to clean 1.5ml micro-centrifuge tubes. An equal volume of ice-cold isopropanol was added and mixed briefly by inversion. Samples were placed in the fridge for 1 hour to precipitate the DNA.

Chilled samples were spun at 12 000 rpm for 5 minutes to recover DNA. The resulting DNA pellets were washed with 250µl of 75% ethanol, which was discarded, and then left to air dry. DNA was re-suspended in 100µl of autoclaved double-distilled water (PCR water) and stored in the fridge. Dilutions of the raw DNA solution were made by adding 45µl of DNA solution to 5µl of PCR water. Dilutions and raw DNA extract were stored at 4°C.

The target region was amplified using the Polymerase Chain Reaction (PCR). 3µl of each DNA template was placed in a micro-centrifuge tube with 27µl of master mix, containing 14.65µl PCR water, 3µl 10x NH_4 buffer, 6µl 25mM $MgCl_2$, 1.2µl dNTP's, 1µl of each relative primer and 0.15µ SUPERTHERM^{TM} (Bioline) DNA polymerase. Thermo-cycling consisted of the following conditions: initial denaturation at 94°C for 2 minutes, followed by 30 cycles of 94°C for 1 minute, 52°C for 1 minute and 72°C for 2 minutes. After 30 cycles, there was a final polymerisation step at 72°C for 7 minutes.

Amplified DNA was cleaned using a GFX^{TM} PCR DNA and Gel Band Purification Kit (Amersham Biosciences). Each GFX column consisted of a glass fibre matrix and a collection tube. 500µl of capture buffer, consisting of a buffered solution containing acetate and chaotrope, was placed in each column to which the amplified DNA solution was added. The columns were spun at 14 000 rpm for 30 seconds, and the resulting flow-through was discarded. The glass fibre matrix was then washed with 500µl of wash buffer, consisting 10mM Tris-HCl and 1mM EDTA diluted to 80% with 100% ethanol. 30µl of PCR water was applied directly to the top of the glass fibre matrix, and the columns were left to stand at room temperature for 5 minutes. They were then spun at 14000 rpm for 1 minute to recover the purified DNA.

Cycle sequencing was performed on cleaned templates in 10µl volumes. Each 10µl sample contained 1-4µl of DNA template, 2µl BigDye® Terminator v3.1 Cycle Sequencing TRR (Applied Biosystems), 1µl 10x NH_4 buffer, 0.16µl primer and the

remaining volume of PCR water. Cycle sequencing products were resolved on an ABI PRISM 3100 Genetic Analyser.

Sequences were assembled using SeqMan (LaserGene System Software, DNAStar, Inc.). These were then entered into MegAlign (LaserGene System Software, DNAStar, Inc.), where they were aligned automatically. The alignment of sequences was optimised manually. The additional sequences kindly supplied by Bernard Goffinet, were trimmed using EditSeq (LaserGene System Software, DNAStar, Inc.) and added to the alignment. All sequences were then realigned automatically, and checked manually.

3.2.1.3 Morphological character scoring

A morphological character matrix was generated to supplement the molecular data. Thirty characters were scored from the existing taxonomic literature for species of *Zygodon* (Sim, 1926; Lewinsky, 1989; Sainsbury, 1955; Watson, 1955; Braithwaite, 1895; Dixon, 1924; Magill & Van Rooy, 1998; Smith & Sowerby, 1941; Smith, 1978; Ignatov et. al, 1999; Scott & Stone, 1976; Allen, 2002; Wilbraham & Long, 2005; Eddy, 1996). This, rather than direct scoring from specimens, was necessary as, due to time constraints of this study, and logistically difficulties, no material could be sourced for some of the species. A matrix of character information (Appendix A) for 17 taxa was entered into MacClade (Madison & Madison, 1992).

3.2.2 Phylogenetic analysis

Molecular and morphological data were further analysed using PAUP – phylogenetic analysis using parsimony (v4.06 for Macintosh; Swofford, 2002). Molecular and morphological data were analysed both separately and together in a combined data matrix. The purpose of the combined analysis was to provide the phylogenetic framework for *Zygodon*, while the individual analyses were to provide contrasting approaches to phylogenetic diversity indices.

The combined molecular and morphological data set was analysed under the parsimony criterion. A heuristic search using TBR (tree bisection and reconnection) was performed using simple addition. Since multiple parsimonious trees were recovered (see results), successively approximated weights were used in an attempt to downweight characters by their homoplasy. The rescaled consistency index was used as a weighting factor, and optimal trees were sought with this weighting scheme. This process was repeated until there was no change in tree length over two successive iterations. Nodal support for the topology was determined by means of bootstrap values, using 1000 replicates.

The molecular and morphological data partitions were optimised onto the single most parsimonious tree retained under the SAW (successive approximate weighting) approach (Farris, 1969). Branch lengths for each of the separate partitions, as well as the combined data, were used to calculate diversity indices. All characters were given a weight of one for this optimisation

3.2.3 Quantification of biodiversity

Phylogenetic diversity was calculated according to the method outlined by Faith, 1992 (Faith, 1992). In this method, phylogenetic diversity (PD) of a given set of taxa is equal to the sum of all branch lengths in the tree. All branch lengths were added and PD values were obtained for each of the combined, molecular and morphological data sets. PD was then recalculated for each data set without *Z. leptobolax*. In this way, the amount of PD, and therefore biodiversity, represented by *Z. leptobolax* in South Africa could be quantified. In addition to this, the percentage of diversity represented by each of the four other South African species of *Zygodon*, and hence the amount of diversity that would be lost if any of them were to go extinct, was calculated for each of the data partitions. This was done for both the entire set of species as well as for just the set of South African species.

3.3 Results

Under equal weights, ten equally most parsimonious trees of length 337 were retained, the strict consensus of which is shown in Figure 8. Under successive weights, a single most parsimonious tree was recovered (Figure 9). In both sets of analyses, *Z. leptobolax* was placed with strong support as sister to the South American species, *Z. inermis*. *Z. intermedius,* thought on morphological grounds to be the most similar to *Z. leptobolax*, was placed in a clade, containing *Z. baumgartneri*, *Z. viridissimus*, and *Z. trichometrius,* sister to the one containing *Z. leptobolax* and *Z. inermis*. The remaining species of *Zygodon* found in South Africa were not placed in a clade together, but were spread out over the tree. *Codonoblepharum menziesii* and *C. pungens* formed a clade together with *Z. forsteri* and *Z. bartramiodes*, and this was placed sister the rest of the *Zygodon* species included in this study.

The same tree was also used to show how well the morphological and molecular data fit the topology, as well as to examine the proportion of total diversity that could be attributed to molecular and morphological characters (Figure 10 and Figure 11). Branch lengths are shown, and these were used to calculate the phylogenetic diversity indices for the trees, including and excluding *Z. leptobolax*. The percentage of total, molecular and morphological diversity was calculated from the change in total PD calculated with and without *Z. leptobolax* (Table 2). If *Z. leptobolax* was to go extinct, 2.27% of the total diversity in the study group of *Zygodon* species would be lost. Similarly, 2.26% of the total molecular diversity in the group would be lost, but only 1.5% of total morphological diversity would be lost from the whole group. The same procedure was followed to calculate just the percentage of diversity that would be lost from the South African *Zygodon* species (Table 3). *Z. leptobolax* was found to represent 5.53% of the total diversity, both morphological and molecular characters, in *Zygodon* in South Africa. If it was to go extinct, 6.09% of molecular diversity would be lost while only 3.05% of morphological diversity would be lost.

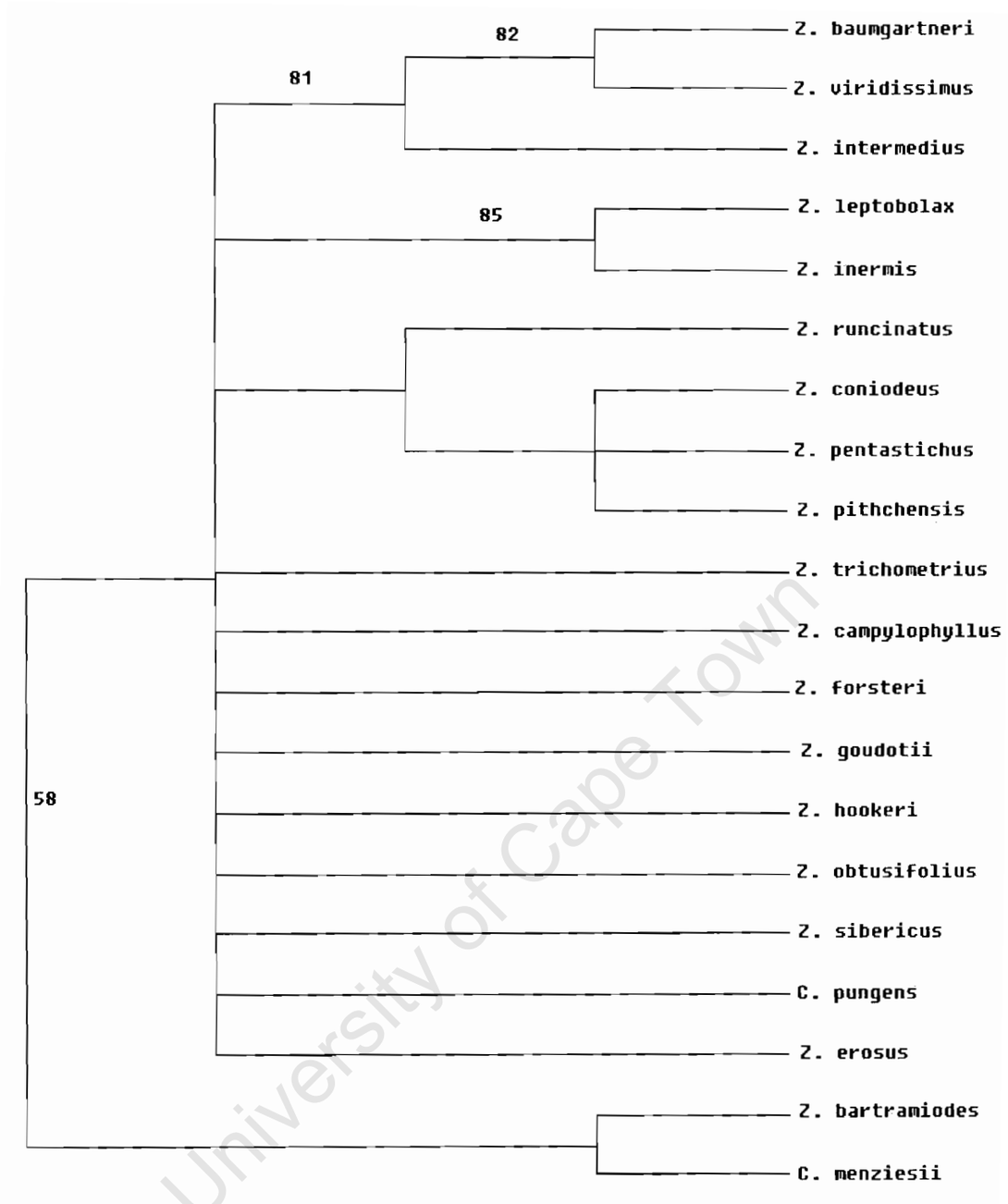

Figure 8: Strict consensus of the 10 most parsimonious trees retained under equal weighting, showing bootstrap nodal support.

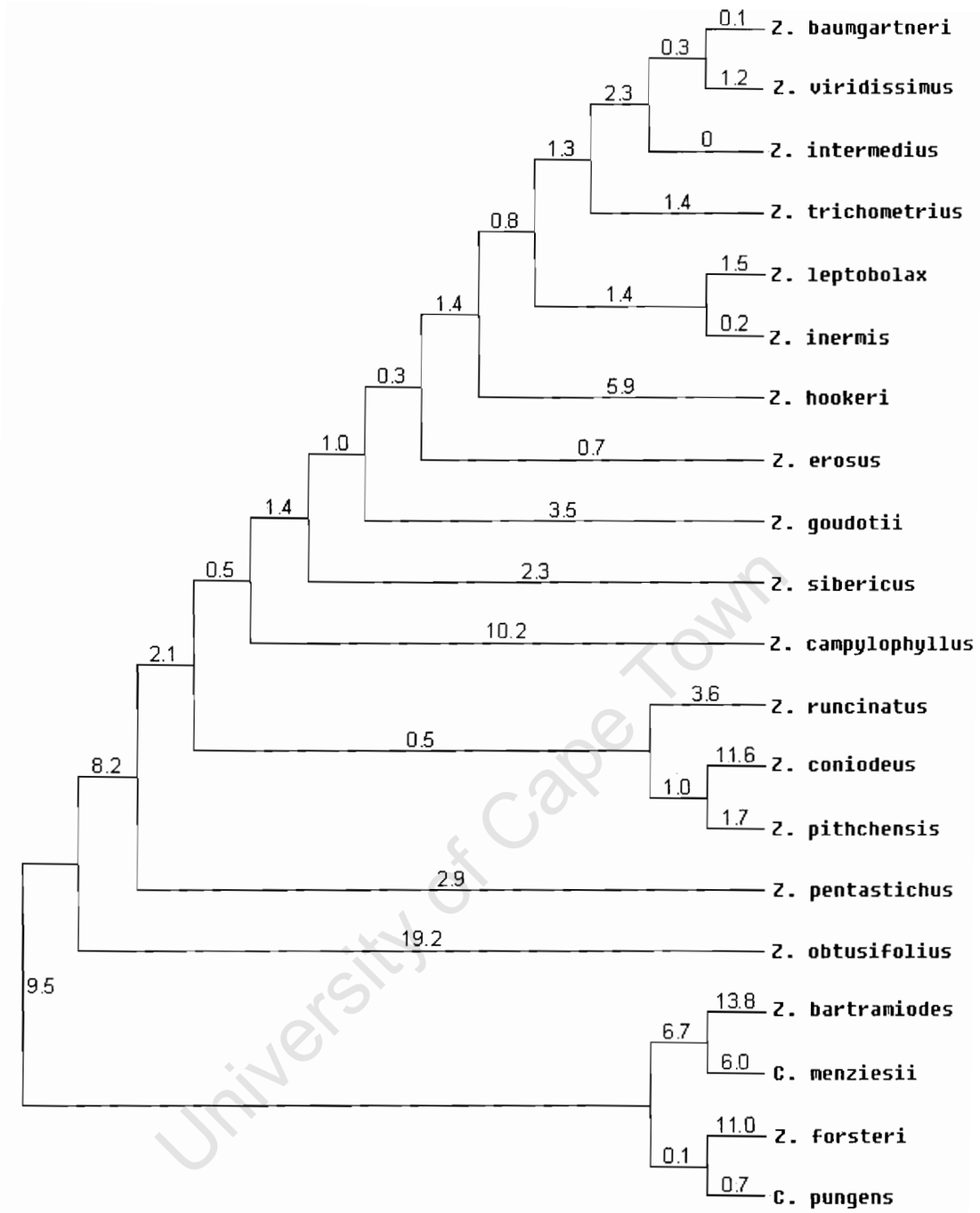

Figure 9: Single most parsimonious tree based on the combined molecular and morphological character set. Branch lengths are calculated from the combined data set.

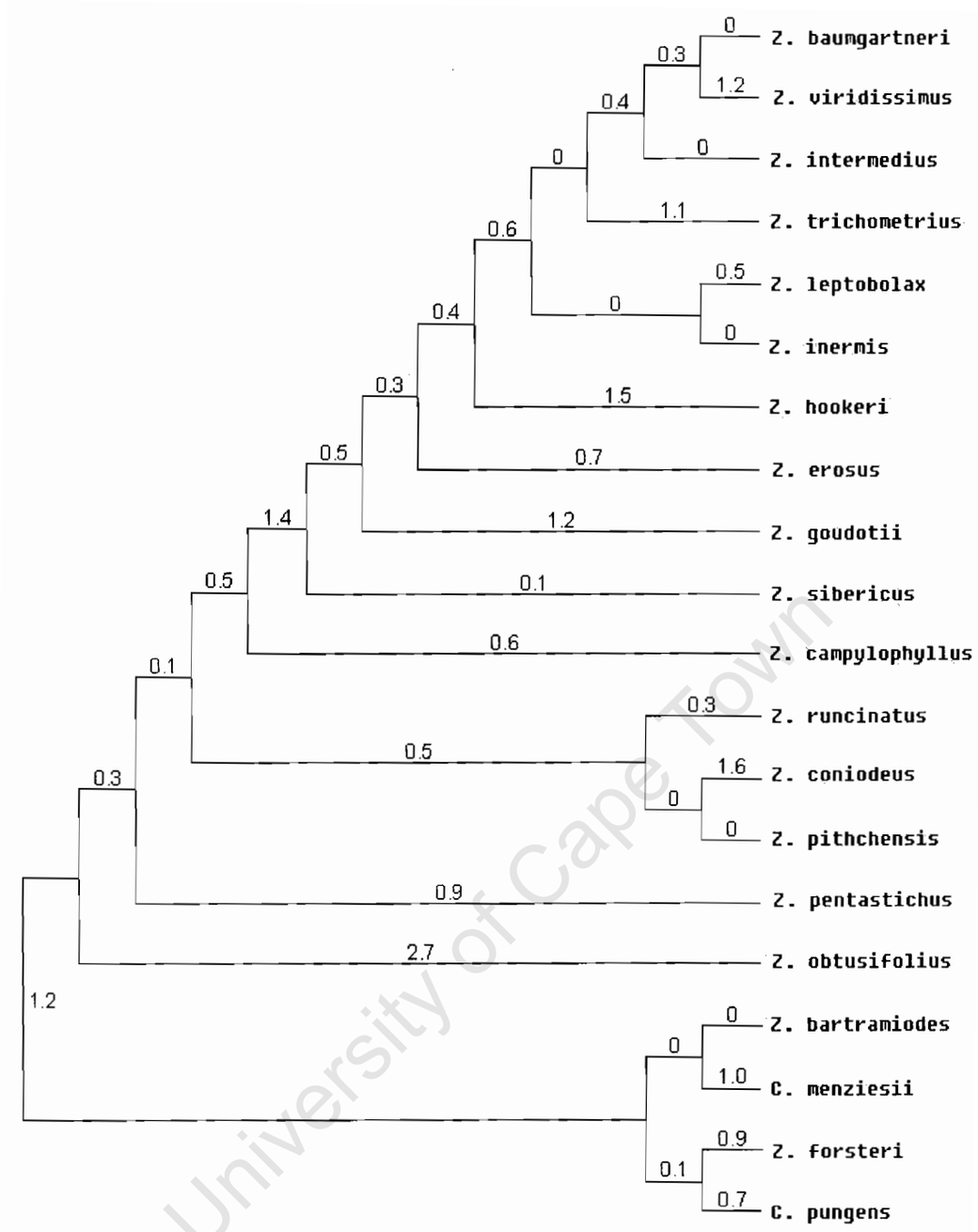

Figure 10: Single most parsimonious tree based on the combined molecular and morphological character set. On this tree, only morphological characters are maximised and branch lengths are accordingly calculated from only the morphological data set.

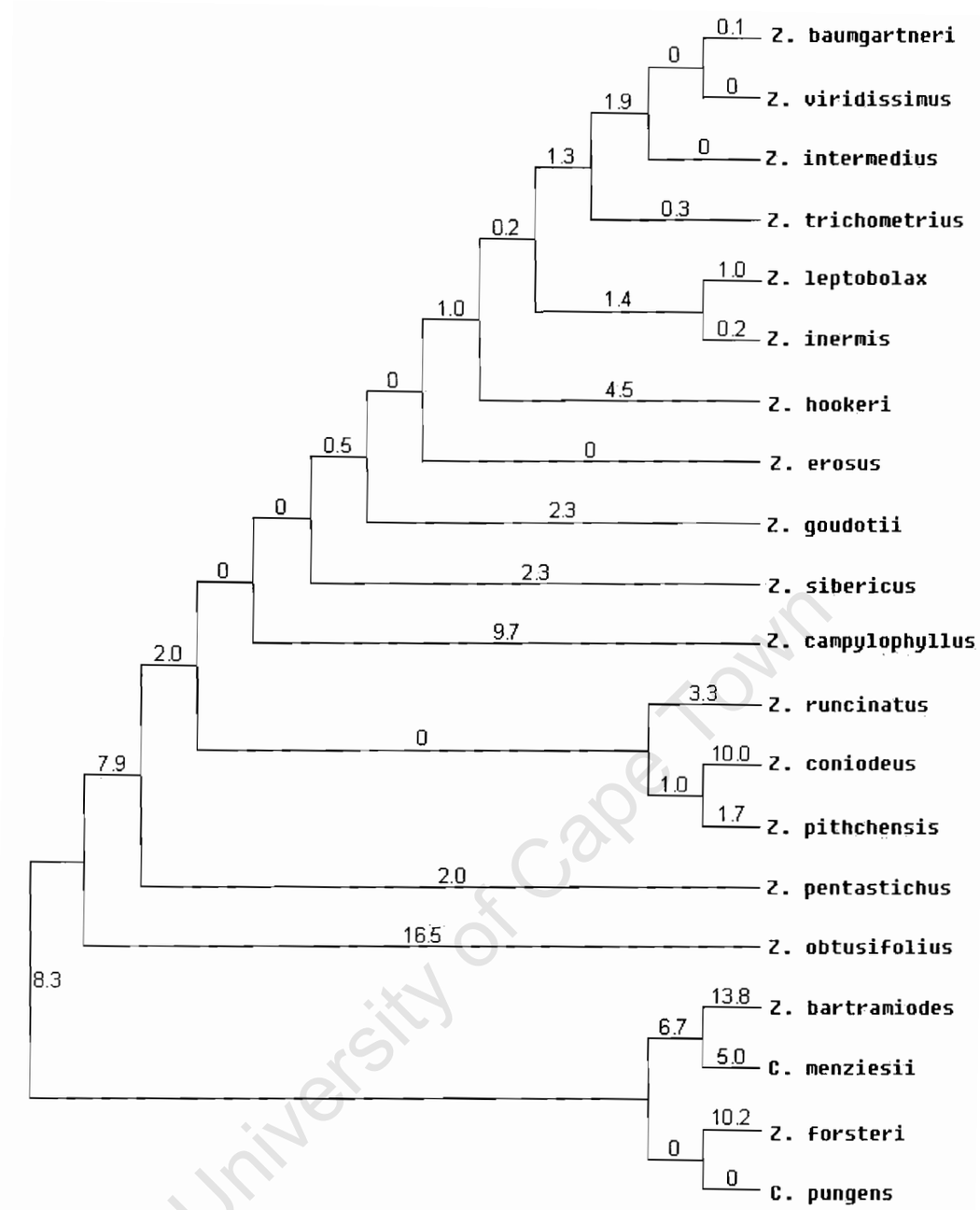

Figure 11: Single most parsimonious tree based on the combined molecular and morphological character set. On this tree, only molecular characters are maximised and branch lengths are accordingly calculated from only the molecular data set.

The average percentage of total, molecular and morphological diversity was similarly calculated from the change in total PD calculated with and without each of the South African species of *Zygodon* (Table 4). The average percentage of the total diversity lost if any South African *Zygodon* species went extinct is 1.28%, while 1.56% of total morphological diversity would be lost, and only 1.06% of the molecular diversity. The average amount of total diversity lost from the sub-set of South African *Zygodon* species if any one was to go extinct is 5.13%, while 3.16% of total morphological diversity would be lost, and only 2.89% of the molecular diversity represented by the South African *Zygodon* species (Table 5).

3.4 Discussion

This section of the study aimed to place the South African *Zygodon* species into a framework phylogeny for the genus. In addition, this section aimed to use the resulting phylogeny in the calculation of phylogenetically based diversity measures, which could be used to evaluate the conservation priority of *Z. leptobolax*. The

3.4.1 Phylogenetic relationships

Despite the evidence suggesting that the region of the genome used, *trn*L-*trn*F, displays enough change to be of use at the species level, under equal weighting resolution was poor. This may be because the region is not variable enough to resolve relationships among bryophytes. The evidence suggesting the region would be variable enough is all based on analysis of angiosperm data, and perhaps the region is not as variable in bryophytes as it appears to be in angiosperms. However, under successive weighting, the region was useful enough to produce an informative phylogeny. In the phylogeny, the South African species of *Zygodon* were not found to form a distinct clade. Rather, they were placed throughout the phylogeny, suggesting that clades in the phylogeny do not correspond to geographical distribution. If this was the case, all of the South African species would have formed a single clade. In fact, there appears to be no association

between geographic distribution and phylogenetic relationship for any of the species examined. For example, a species only found in Russia (*Z. sibericus* Ignatov) was found to be most closely related to a predominantly South American species (*Z. goudotii* Hampe), which is sister to a clade including the South African species *Z. erosus* in its basalmost part. The centre of diversity, in regard to numbers of species, for this genus may be South America, but the phylogeny suggests that this region was not the site of origin for all diversity. It appears that species in different areas, and in many cases on different continents, are related in ways that precede the separation of the areas of the current genus' mainly Gondwanan distribution. The phylogeny shows that rather than the genus originating in South America, being dispersed to other continents and subsequently speciating in the new areas, species diversified first, and then the areas separated. However, a fully resolved and dated phylogeny would be required to support this idea.

Z. leptobolax is morphologically very similar to another South African species – *Z. intermedius*. Yet *Z. leptobolax* is placed with strong nodal support as sister to *Z. inermis* Malta, a South American species. Clearly *Z. leptobolax* is not as similar as *Z. intermedius* as their morphology would suggest. The phylogeny shows that *Z. leptobolax* is not a slightly altered version of *Z. intermedius*, perhaps adapted to specific conditions on Table Mountain. Instead, it appears to be more closely related to *Z. inermis*. It is possible that *Z. inermis* became established in South Africa, possibly recently or possibly in the distant past, and adapted slightly to the different conditions here, which resulted in a new species - *Z. leptobolax*. Experiments in spore dispersal have shown that spores can travel large distances in the wind to colonise new areas, which may have been how *Z. inermis* was introduced to the Western Cape region of South Africa (van Zanten, 1978). The similarities between *Z. leptobolax* and *Z. intermedius* could be the result of convergent evolution and adaptation to similar habitats and life strategies.

Apart from setting the South African species of *Zygodon* into a global framework, the phylogeny also provides a few insights into taxonomic debates. There has been recent debate about the placing of several species of *Zygodon* into the genus *Codonoblepharum*, as discussed previously. This phylogeny supports the renaming of *Z. menziesii* as *C.*

Table 2: PD values with and without *Z. leptobolax*, for the three data partitions considered.

	Combined data set	**Morphological data set only**	**Molecular data set only**
PD including *Z. leptobolax*	136.3	33.3	114.8
PD excluding *Z. leptobolax*	133.2	32.8	112.2
% diversity represented by Z. leptobolax	2.27%	1.5%	2.26%

Table 3: PD values with and without *Z. leptobolax* for set of South African *Zygodon* species. The bottom row indicates the percentage of diversity in *Zygodon* species in South Africa that will be lost if *Z. leptobolax* goes extinct.

	Combined data set	**Morphological data set only**	**Molecular data set only**
PD including *Z. leptobolax*	56.1	16.4	42.7
PD excluding *Z. leptobolax*	53.0	15.9	40.1
% diversity represented by Z. leptobolax	5.53%	3.05%	6.09%

Table 4: The percentage of diversity lost from the study group if any of the South African *Zygodon* species went extinct, for each data partition.

	Combined data set	**Morphological data set only**	**Molecular data set only**
Z. erosus	0.5%	3.3%	<0.01%
Z. runcinatus	2.64%	0.9%	2.8%
Z. trichometrius	1.02%	2.1%	0.26%
Z. intermedius	<0.01%	<0.01%	<0.01%
Z. leptobolax	2.27%	1.5%	2.26%
Average	**1.28%**	**1.56%**	**1.06%**

Table 5: The percentage of diversity lost from the study group if any of the South African *Zygodon* species went extinct, for the set of South African species.

	Combined data set	**Morphological data set only**	**Molecular data set only**
Z. erosus	1.25%	4.26%	<0.01%
Z. runcinatus	6.42%	1.8%	7.7%
Z. trichometrius	2.49%	6.71%	0.7%
Z. intermedius	<0.01%	<0.01%	<0.01%
Z. leptobolax	5.53%	3.05%	6.09%
Average	**5.73%**	**3.16%**	**2.89%**

Apart from setting the South African species of *Zygodon* into a global framework, the phylogeny also provides a few insights into taxonomic debates. There has been recent debate about the placing of several species of *Zygodon* into the genus *Codonoblepharum*, as discussed previously. This phylogeny supports the renaming of *Z. menziesii* as *C. menziesii* and *Z. pungens* as *C. pungens*. These species form a distinct clade that is sister to the main clade containing the rest of the species of *Zygodon* included in this study. However, included in this clade are *Z. bartramiodes* Malta and *Z. forsteri* (Dicks ex. With) Mitt. The later of which was excluded from *Codonoblepharum* on the basis of rhizoid colour, laminal cell width and its reaction to 2% KOH (Matcham & O'Shea, 2005). This phylogeny suggest that this species, as well as *Z. bartramiodes* warrant further examination, and possible renaming, and supports the work of Goffinet and others (Goffinet et. al., 2004). This phylogeny also adds clarity to the taxonomic debate as to whether *Z. intermedius* and *Z. hookerii* Hampe are the same species and should be made synonymous (Lewinsky, 1989). Clearly they are separate species.

3.4.2 Diversity indices

How much biodiversity will be lost if *Z. leptobolax* goes extinct? *Z. leptobolax* represents a fairly small amount of the total diversity in the genus probably because of its molecular similarity to *Z. inermis* and its morphological similarity to both *Z. inermis* and *Z. intermedius*. However, *Z. leptobolax* represents a greater proportion of the total diversity among South African species. A small, but significant part of the diversity in *Zygodon* in this country will be lost if *Z. leptobolax* goes extinct. Even though it is morphologically similar to *Z. intermedius*, it harbours a distinct number of molecular features that are unique among South African species of *Zygodon*. This is reflected in the slightly larger proportion of molecular diversity, as opposed to morphological diversity, that would be lost both globally and locally. Because of the unique molecular features, *Z. leptobolax* has unique evolutionary potential among the South African *Zygodon* species, which could be lost in the near future. By removing this diversity from South Africa, we are effectively lessening the option value of the entire genus in South Africa. In addition to this, the percentage of diversity lost from both the study group and the sub-set of South

African species is greater if *Z. leptobolax* is removed than when any other South African *Zygodon* species is removed, that is to say, PD and total diversity decrease more when *Z. leptobolax* is removed than when any other South African species is removed from the phylogeny. Based on this and PD values, *Z. leptobolax* is worth conservation priority.

GENETIC VARIATION AND STRUCTURE AS INFERRED FROM ISSR DATA

4.1 Introduction

Population genetics is the field of study that examines population structure and the evolutionary history of populations (Meffe & Carroll, 1994). This is usually done by using molecular data to estimate parameters such as gene diversity and gene flow Population structure and history can be determined from these parameters, which in turn can also be used to answer other questions about the populations being studied (Excoffier et. al., 1992). For example, population structure can indicate the extent of sexual reproduction in a species i.e. are new individuals the result of sexual or asexual reproduction? These parameters can also inform us about the amount of genetic diversity within a species or between populations (Bornet & Branchard, 2004). This is important for several reasons, one of which is conservation (Holsinger & Gottlieb, 1991).

If conservation resources are limited, only a few populations may be conserved (Vane-Wright, Humphries, & Williams, 1991). It would be advantageous to conserve those that have the most genetic diversity, rather than those that essentially consist of clones with very little, or no genetic diversity. In addition to this is the question of viability. Some populations are more viable than others, and in conservation, the most viable populations are the ones to conserve (Lande & Barrowclough, 1987). If a species or population consists only of clones, it becomes more susceptible to demographic effects. For example, a population may go through a bottleneck, and all but one individual survives. This individual could repopulate the area by vegetatively reproducing clones of itself. However, if the original survivor harboured a gene that made it susceptible to a certain pathogen, the possibility exists that the entire species could be wiped out by a single infection (Frankham, Ballou, & Briscoe, 2004). Only rarely is there the opportunity to preserve all populations, so the choice must be made (Moritz, 1999). We must select those that allow survival of the species in the short term and diversification in the long term (Mortiz, 1999). In the same way, other demographic effects can be magnified in

small populations and those with very few haplotypes, inbreeding depression for example.

Population structure is also important in species that exhibit phenotypic plasticity. By estimating the parameters such as genetic diversity, it can be deduced whether the observed morphological differences are reflected in levels of actual genetic diversity or not (Zhu, Degnan & Moritz, 1998). The evolutionary history of populations is also useful in much the same way. Evolutionary history can identify the processes that shaped current populations, and give an indication of any geographical association with certain genes. This is useful in conservation, once again, to identify the populations for conservation, as well as in breeding programs, where genetic diversity is important (Tan et. al., 2005). In general, population genetics can be a very useful tool.

4.1.1 Bryophyte population genetics and conservation

Population genetics and parameters are important factors in determining bryophyte survival, distribution and rarity (Söderström & During, 2005). It is more likely that a rare species will go extinct than a more common one. Hence, knowledge of species rarity and the distribution and amount of genetic diversity are vital for conservation planning (Wyatt, 1992). Rarity in bryophytes has been linked to the lack of sporophyte production (Longton & Hedderson, 2000) as well as to the lack of available suitable habitats (Birks et. al. 1998; Herben, Rydin & Söderström, 1991). Either way, it is important to know the extent of genetic diversity in order to evaluate population viability and conservation worth (Shaffer, 1981). Bryophytes, on average, have levels of genetic diversity similar to those of higher, diploid-dominant plants (Stoneburner, Wyatt & Odrzykoski, 1991; Montagnes, Bayer & Vitt, 1993). It has been said that bryophytes, like higher plants, show a relationship between biological features, such as breeding systems, and the amount of genetic diversity within species (Loveless & Hamrick, 1984). Certainly, species that reproduce solely asexually will have very low levels of genetic diversity. The only diversity will be the result of accumulated mutations (Akiyama, 1999). Monoicous bryophytes can be expected to exhibit less genetic variation than dioicous bryophytes,

due to at least part-time self-fertilisation (Wyatt, Odrzykoski, Stoneburner, 1989). However, this is not enough information to base conservation decisions on. Only rarely is there the opportunity to preserve all populations, so the choice must be made as to which ones are the best for conservation (Moritz, 1999).

4.1.2 Marker Choice - why ISSRs?

ISSR – inter simple sequence repeats – are quickly evolving regions of DNA (Bornet & Branchard, 2001). They can be used to identify polymorphism in all genomes, but in particular they are capable of identifying polymorphism throughout the whole nuclear genome (Wolfe & Liston, 1998). ISSRs are small, microsatellite-like regions of DNA, consisting of di- or tri-nucleotide repeats e.g. AG or AGA. These regions are usually in groups of four to ten repeats each, but can be much longer. They are highly variable and differ within populations and between species. Primers, consisting of a few repeat units combined with a few arbitrary bases, have been designed. These primers are used in PCR reactions, where they able to identify the repeated sections in the DNA. When two of these repeat groups are sufficiently close together on the genome, and the corresponding antisense strand is reversed relative to the sense strand, the region between them is amplified. In this way, many different sections are amplified depending on how many times the specific repeat region is present in the genome. This results in bands of amplified DNA, each of a different molecular weight. These can be separated out and visualised on an agarose gel. Each band that is generated is treated as a "locus", and these are treated as having two alleles, i.e. "present" or "absent" (Zietkiewicz, 1994; Wolfe & Liston, 1998).

In organisms, where the dominant life phase is diploid, the loci are regarded as dominant and genotypic allele information cannot be determined i.e. when a band is present, it is impossible to determine whether the individual was homozygous or heterozygous. Therefore, estimation of allele frequencies (which form the basis for many population genetic measures) requires the assumption that populations are in Hardy-Weinberg equilibrium. In organisms where the haploid phase is dominant, such as in bryophytes,

this is not a problem as there is usually only one copy at each locus. Hence, the presence of a band can be interpreted as being "dominant", while the absence implies that the region definitely was absent in the genome. Therefore allele (i.e. present versus absent) frequencies can be estimated directly, without making assumptions about population structure.

The high level of polymorphism found in ISSRs makes this method ideal for studying genetic diversity within populations as well as determining the evolutionary history of those populations (Zhang, Li & Qiu, 2005). ISSR data have been used for separating actual genetic diversity from phenotypic plasticity (Zhu, 1998), as well as for quantifying this genetic diversity (Bornet & Branchard, 2004; Kolodinska Brantestam et. al., 2004; Ge & Sun, 1999) and identifying hybrid species (Wolfe, Xiand & Kephart, 1998). In addition, ISSRs have proven useful in the genotyping of agricultural cultivars (Fang, 1997; Levi, 1997), as well as in identifying clonal versus asexual reproduction (Robinson, 1997) and in conservation (Maunder, 1999; Wu, 2004).

4.1.3 *Z. leptobolax* – A genetically depauperate species?

Z. leptobolax has an extremely small range. In addition to this, it is synoicous and capable of self-fertilising. These factors suggest that we could expect low levels of genetic variation both among and between populations (Söderström & During, 2005). There is also the possibility that the species went through a bottleneck when host trees were removed from the area to supply timber to the expanding city of Cape Town during the 19th and 20th centuries. If this is the case, then the level of genetic diversity will be extremely low, and certain alleles may have become fixed for the remaining populations.

4.1.4 Aims and objectives

This section of the study aims to determine the population structure of *Z. leptobolax*, and to examine patterns of genetic similarity among the extant populations on Table Mountain. In addition, the amount of genetic diversity will be estimated, and it will be determined whether the populations are the result of sexual reproduction or if they are clones of one another. This will attempt to answer the key questions 1) What is the extent of genetic variation and structuring in *Z. leptobolax*? 2) Are the patterns of genetic variation consistent with predominantly asexually reproducing or self-fertilizing populations, or do they suggest that the populations are predominantly outcrossed?

4.2 Methods

4.2.1 ISSR band amplification

ISSR analysis was performed on DNA extracted from 15 samples collected on Table Mountain. During the second populations census performed on the species, 15 small samples were collected, placed in paper envelopes and left to air-dry upon return to the laboratory. These 15 samples were used to generate molecular data for the species.

For six of the populations, ISSR analysis was performed on four individuals. The remaining nine populations were represented by one individual each. A total of 15 primers were screened, of which three were selected for analysis (Table 6). These primers were chosen from set nine obtained from the University of British Columbia Biotechnology Laboratory. PCR amplifications were done in 25µl volumes. 4µl of each DNA template was placed in a micro-centrifuge tube with 21µl of master mix.

Table 6: List of ISSR primer sequences used in this study. The nucleotide Y represents either a C (cytosine) or a T (thiamine) base.

Primer	Sequence
835	AGA GAG AGA GAG AGA GYC
841	GAG AGA GAG AGA GAG AYC
856	ACA CAC ACA CAC ACA CYA

The volume of the individual master mix components varied according to the concentration of the primers used. For a primer with a concentration of 10mM, 21µl of master mix, containing 13.35µl PCR water, 2.5µl 10x NH_4 buffer, 3.0µl 25mM $MgCl_2$, 1.0µl dNTP, 1.0µl 10mM primer and 0.15µl SUPERTHERM^{TM} (Bioline) DNA polymerase, was placed in the tube with 4µl of DNA. For a primer with a concentration of 15mM, 21µl of master mix, containing 13.95µl PCR water, 2.5µl 10x NH_4 buffer, 3.0µl 25mM $MgCl_2$, 1.0µl dNTP, 0.4µl 10mM primer and 0.15µl SUPERTHERM^{TM} (Bioline) DNA polymerase, was placed in the tube with 4µl of DNA. Optimised thermo-cycling conditions consisted of the following: initial denaturation at 94°C for 1.5 minutes, followed by 35 cycles of 49°C for 1 minute, 72°C for 1 minute and 94°C for 30 seconds. After 35 cycles there was a polymerisation step at 47°C for 2 minutes followed by two sets of final polymerisation steps at 72°C for 3 minutes each. PCR products were separated vertically on a 1.5% agarose gel running at 60V for 80 minutes. Amplified product bands were visualised under UV light and digital images of the gels were recorded. Amplified ISSR fragments were manually scored into a data matrix as either present (1) or absent (0) for each of the samples in all 15 populations (Zietkiewicz, 1994; Bornet & Branchard, 2004).

4.2.2 Data analysis

Genetic diversity was quantified as i) the percentage of polymorphic bands and ii) Nei's gene diversity (Nei, 1973) using the program POPGENE version 1.32 (Yeh et. al. 1997).

POPGENE was also used to examine the structure between populations. Pair-wise similarities were calculated using the Simple Matching coefficient (Sokal & Michener, 1958). The resulting similarity matrix was clustered using the Unweighted Pair Group Mean Analysis (UPGMA) algorithm, also implemented in POPGENE (Sneath & Sokal, 1973).

The presence of genetic structure was evaluated by analysis of molecular variance (AMOVA) performed in ARLEQUIN version 2.0 (Schneider, Roessli & Excoffier, 2000). AMOVA variance components were used as estimates of the genetic diversity within and between populations. ARLEQUIN was also used to generate a matrix of pair-wise differences between populations, based on Nei's unbiased measures of genetic distance (Nei, 1978). These distances were then contrasted against geographical distance between populations, to see if there was any correlation between genetic and geographical distances.

4.3 Results

Among 15 populations, three ISSR primers generated 69 bands, of which 68 (98.5%) were found to be polymorphic. A total of 69 bands were scored manually into a data matrix, which corresponds to an average of 23 bands per primer. Within populations, the percentage of polymorphic bands (PPB) showed an average of 57.7% (Table 8).

Nei's genetic diversity statistics (Nei, 1973), indicated a moderate level of gene diversity within individual populations, with an average genetic diversity index (GD) of 0.2361 ± 0.2026 (Table 8). Among the six populations containing multiple individuals, population 3 showed the lowest level of variability (PPB = 39.1%, GD = 0.1558 ± 0.1981) and population 13 showed the highest level of variability (PPB = 72.5%, GD = 0.2989 ± 0.1920).

In order to show the relationships among all sampled populations, cluster analysis (UPGMA) was used to generate a dendrogram (Figure 13), based on Nei's unbiased

genetic measures between populations (Table 9). The populations with the largest genetic distance between them are populations 1 and 2, while the populations with the least genetic distance between them are populations 10 and 13 (Table 9). This is also evident from the dendrogram, where the populations were divided into four clusters. However, further examination revealed that the clusters were not related to geographic distance. The populations that were furthest apart geographically were populations 2 and 10 (139.94m), and those closest together were populations 5 and 12 (5.57m) (Table 9). These geographic distances are not represented in the dendrogram. A graphical plot of geographic versus genetic distance supports these findings (Figure 12). The graph showed that there was no significant relationship between the two variables ($r^2 = 0.0402$, $p > 0.05$).

In addition to the previous tests, an AMOVA analysis was performed on the data. This analysis provided evidence to support the genetic structure suggested by Nei's genetic diversity statistics (Table 7). There were significant ($p < 0.05$) genetic differences among populations as well as within populations. Of the total genetic diversity observed, 22.47% was the result of among population diversity, while 77.53% was the result of within population diversity.

Table 7: Analysis of molecular variance (AMOVA) within/among *Z. leptobolax* populations.

Source of Variation	d.f	SSD	Variance component	Total variance	*p*
Among populations	5	38.625	0.675	22.47 %	< 0.05
Within populations	42	97.750	2.327	77.53 %	< 0.05

Table 8: Analysis of genetic variation among the six populations with multiple individuals.

Sample	% Polymorphic Bands	Genetic diversity (Nei)
Population 3	39.1%	0.1558 ± 0.1981
Population 8	63.8%	0.2627 ± 0.2047
Population 9	65.2%	0.2627 ± 0.1978
Population 10	55.1%	0.2301 ± 0.2140
Population 13	72.5%	0.2989 ± 0.1920
Population 14	50.7%	0.2065 ± 0.2088
Average	57.7%	0.2361 ± 0.2026

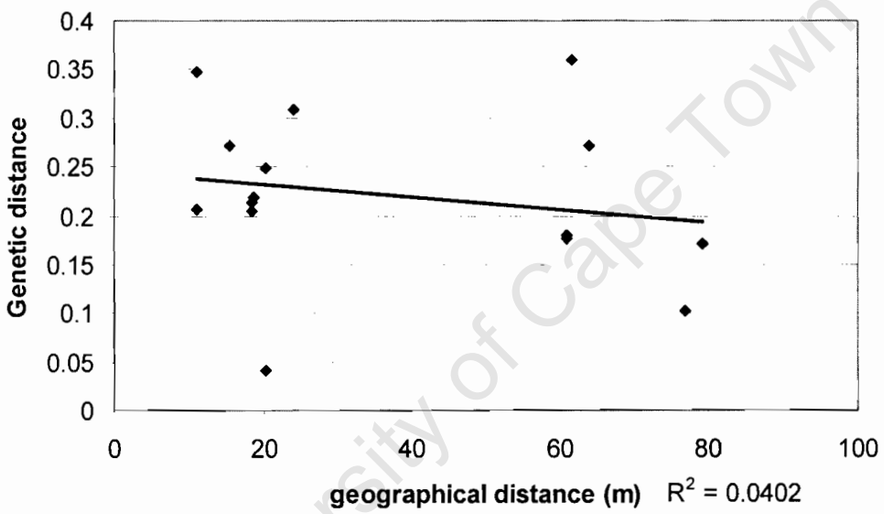

Figure 12: The relationship between genetic difference and geographical distance.

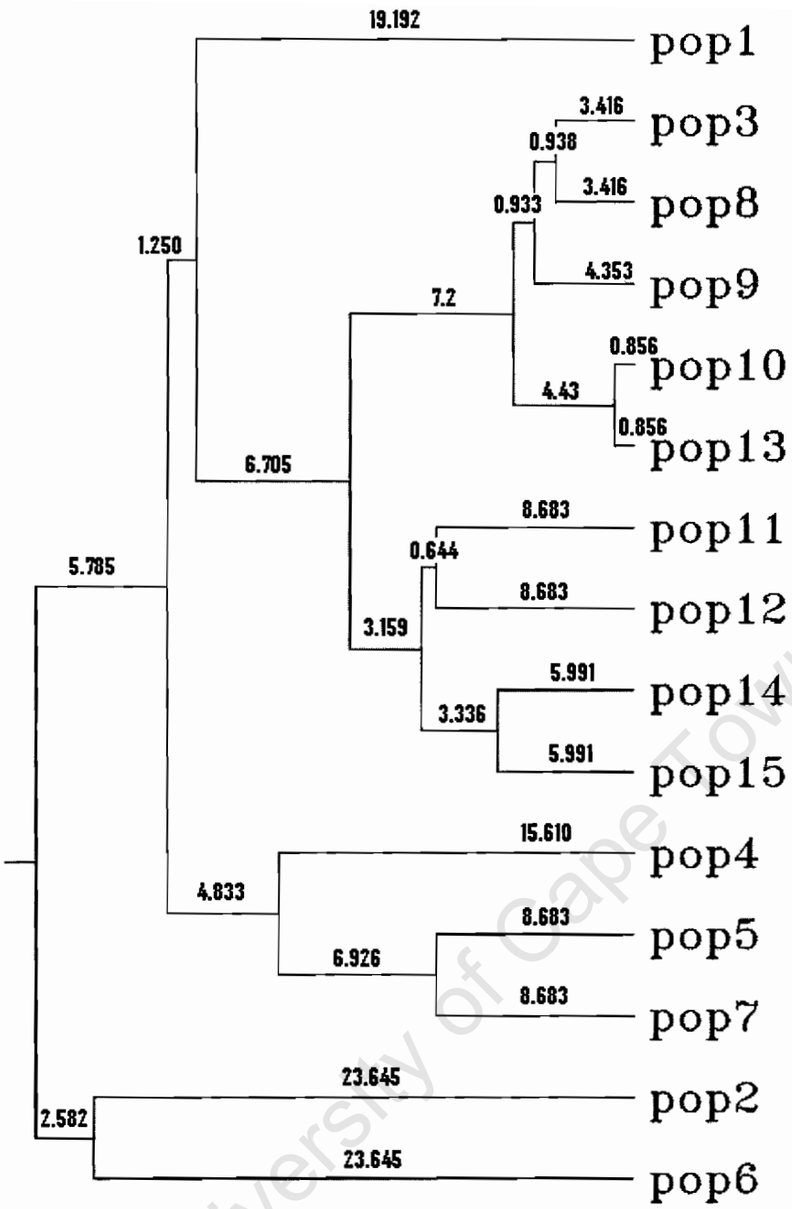

Figure 13: Dendrogram of 15 populations of *Z. leptobolax* using UPGMA cluster analysis, based on Nei's unbiased genetic distances.

Table 9: Nei's unbiased measures of genetic distance among 15 populations of Z. leptobolax, as generated by ISSR data (below the diagonal) and actual geographic distance between populations, in meters (above diagonal).

Pop-ulation	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	*****	78.68	51.63	15.39	22.26	35.93	37.14	40.17	64.29	71.88	24.10	27.82	51.63	59.25	97.73
2	0.80	*****	121.87	63.72	77.09	46.15	79.56	64.79	138.46	139.94	61.52	77.69	121.87	124.76	131.83
3	0.32	0.62	*****	65.40	44.78	75.77	46.00	61.04	18.49	20.27	60.48	44.66	0.10	11.24	68.21
4	0.62	0.62	0.45	*****	27.06	24.10	40.20	37.10	79.16	85.43	18.55	31.80	65.40	71.90	103.62
5	0.52	0.57	0.29	0.30	*****	31.00	14.88	21.39	61.67	63.32	15.83	5.57	44.78	48.52	76.93
6	0.62	0.47	0.39	0.43	0.52	*****	35.95	24.10	92.33	94.18	15.38	32.14	75.77	79.18	96.42
7	0.59	0.59	0.34	0.32	0.17	0.49	*****	15.39	64.31	61.56	24.14	9.31	46.00	46.20	63.57
8	0.22	0.53	0.11	0.35	0.33	0.35	0.35	*****	79.16	76.95	18.56	17.97	61.04	61.59	72.31
9	0.35	0.49	0.12	0.34	0.30	0.37	0.39	0.15	*****	18.56	76.95	62.25	18.49	24.09	80.35
10	0.31	0.49	0.13	0.39	0.32	0.42	0.36	0.09	0.16	*****	79.16	62.26	20.27	15.36	63.61
11	0.57	0.47	0.37	0.47	0.38	0.74	0.45	0.39	0.42	0.23	*****	17.97	60.48	64.32	87.40
12	0.55	0.59	0.33	0.49	0.45	0.49	0.52	0.31	0.39	0.18	0.17	*****	44.66	47.12	71.86
13	0.36	0.45	0.18	0.41	0.29	0.41	0.34	0.16	0.19	0.07	0.24	0.21	*****	11.24	68.21
14	0.44	0.47	0.18	0.62	0.35	0.59	0.41	0.27	0.22	0.17	0.23	0.23	0.16	*****	57.76
15	0.47	0.62	0.33	0.62	0.39	0.74	0.49	0.37	0.36	0.22	0.19	0.14	0.20	0.14	*****
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**

4.4 Discussion

This section of the study aimed to determine the population structure of *Z. leptobolax*, and to examine the relationships among the extant populations on Table Mountain. *Z. leptobolax* is capable of self-fertilization as a means of reproduction. In species where this is possible, it is usually the main method of reproduction, as is an easy and guaranteed method of self perpetuation (Mischler, 1988). Reproduction by this method often results in low genetic diversity, both among and between populations, as new plants are essentially clones of the parent plant. Low genetic diversity was expected to be seen in the sampled populations, showing that they rarely reproduce sexually, by means other than self-fertilisation. However, this section of the study suggests the opposite is true.

Gene diversity was found to be moderately high within populations. This implies that the populations of *Z. leptobolax* on Table Mountain are actively reproducing by sexual means, including self-fertilisation.

The results of the AMOVA also support this. A larger percentage of the total genetic diversity could be attributed to within population diversity, rather than to among population diversity. This implies that between different populations, there is a high degree of similarity, while within individual populations there is a low degree of similarity among individual plants. If *Z. leptobolax* was only reproducing by self-fertilisation, then a high level of similarity could be expected both between and within populations. Essentially, the majority of plants would be clones, and exhibit very low genetic diversity. They would also have a very low percentage of polymorphic bands. This is not the case. As mentioned previously, genetic diversity was moderately high. Of the ISSR bands generated in this study, 98.5% were polymorphic between populations, while within populations the percentage of polymorphic bands ranged from 39.1% to 72.5%. Even if some of the ISSR fragments of similar length may be non-homologous, meaning that the presence of some of the similar bands is not comparable, there is enough diversity to still support these conclusions. Non-homologous banding is unlikely though, and these plants are clearly not clones.

If *Z. leptobolax* was only reproducing sexually, then a moderate to low level of genetic similarity could be expected both between and within populations (Van der Velde et.al., 2001). This is because all plants would have to be the result of gene recombination. A moderate level of similarity would result in populations where spores were not dispersed, and all germinated in the same population as the parent. Even so, they would not be very similar as one plant may be fertilised by several others, resulting in sporophytes containing spores of differing genetic makeup. As we know that *Z. leptobolax* is synoicous, it would be a far stretch of the imagination to believe that self-fertilisation never occurs and all sporophytes are the product of sexual reproduction. Hence, this explanation should be rejected.

The population structure that would explain the observed results the best is a combination of sexual and clonal reproduction. For there to be high genetic diversity within populations, there must be some sexual reproduction other than self-fertilisation, occurring (Hassel et. al., 2005). And for genetic diversity to be low among populations there must be some self-fertilisation or asexual (vegetative) reproduction. From the view point of genetics, even though self-fertilisation is sexual reproduction, the net effect of repeated self-fertilisation is similar to that of vegetative reproduction (Pfeiffer et. al., 2006). Sexual reproduction results in gene flow and genetic recombination. This alone, as mentioned above, would result in high diversity among and within populations. However, if there was some self-fertilisation, and these "clonal" spores were dispersed and developed into plants in other populations, we would see a greater level of similarity among populations. This is because the "clone" spores would introduce foreign DNA into neighbouring populations, thereby raising the number of gene variants those populations share. This would also increase the diversity within the population receiving the foreign DNA. This combination of events would result in the observed pattern of low among and high within population genetic diversity.

This model suggests that there should be a geographic component to genetic diversity, which there isn't. This can be explained by the fact that in any given season, only a few populations produce sporophytes. These spores may be dispersed to neighbouring populations, or may form new populations. If spores are only dispersed in a local capacity, and only neighbouring populations receive the lowered genetic diversity, over several seasons, the patterns may not be clear anymore. There appears to be no obvious reason why certain populations produce sporophytes and others don't, but this randomness over several seasons may make it difficult to see any association between genetic and geographical distances. If the spores that are dispersed are capable of travelling some distance, there would be no association with geographic distance as spores may germinate anywhere near or far from the parent (Thingsgaard, 2001; Zartman, McDaniel & Shaw, 2006). In addition to this, *Z. leptobolax* reproduces asexually, via gemmae. These gemmae are also clones of the parent plant and may act in the same way as self-fertilised spores in reducing among population diversity.

Cluster analysis also suggests that there is a complex population structure occurring. Populations were grouped into four clusters, yet none of these related to geographic distance. It appears that there is no relation between physical distance and genetic distance. Populations may be grouped together on the basis of genetic similarity, but these similarities are the result of something other than geographic distance. What is causing the grouping is unclear, as there are no obvious physical factors, host features or distinguishing characteristics that could result in the observed pattern.

In general, the few remaining populations of *Z. leptobolax* appear to harbour a large amount of genetic diversity over a small area and among relatively few populations. This evidence refutes the idea that biological factors such as breeding system are correlated to the amount of genetic diversity in a species (Hamrick & Godt, 1996). *Z. leptobolax* is monoicous, yet has a moderate degree of genetic diversity. This is significant information for determining whether *Z. leptobolax* warrants conservation status. As populations retain a moderate amount of genetic diversity, they also retain a degree of evolutionary flexibility, which is vital for both long and short term viability. This diversity also indicates that the species is still actively reproducing with spores. Spores are more able to colonise new hosts than gemmae are, due to their ability to travel longer distance and survive in the diaspore bank for longer periods of time, which suggests that given more hosts the species would expand its range (Jonsson, 1993).

GENERAL DISCUSSION AND CONCLUSIONS

The aim of this study was to investigate the current status of the moss *Zygodon leptobolax*, determine what level of genetic diversity exists in remaining populations, place the species into phylogenetic context, and evaluate whether this species should be afforded conservation status.

The second chapter in the present study addressed the status of current populations, by means of paired censuses performed annually. From these censuses it was determined that *Z. leptobolax* is extremely range and habitat restricted – only growing in the vicinity of First and Second Waterfall Ravines on Table Mountain, and only on mature *Quercus* specimens. An average 40 populations exist on the mountain. While they are mostly relatively small, they are dynamic, with a delicate balance between recruitment and mortality rates. The third chapter dealt with the species on a higher level and placed *Z. leptobolax*, along with the other four South African *Zygodon* species, into a phylogeny for the genus. From the phylogeny it was determined that *Z. leptobolax* is not as closely related to the rest of the South African *Zygodon* species as previously thought. In fact *Z. leptobolax* harbours several, mostly molecular, features that are unique among the South African flora. However, the species does not represent a large number of unique features within the genus. Despite this, calculations of the phylogenetic diversity index, according to the method outlined by Faith (Faith, 1994a, 1994b), suggests that diversity in *Z. leptobolax* may be important for sustaining the option value of the South African species. The fourth chapter focused back on the individual populations of *Z. leptobolax* and determined the level of genetic diversity both within and among populations, by analysis of ISSRs. From these analyses it was determined that the populations have a moderately high level of genetic diversity, and that this is the result of sexual recombination. *Z. leptobolax* does not rely only of self-fertilisation as a means to perpetuate, but rather combines sexual and asexual reproduction.

Is *Z. leptobolax* on the verge of extinction? Evolutionary processes have different effects on small populations than on larger populations (Berger, 1990). Low genetic diversity becomes more of a problem, and the effects of genetic drift are magnified – alleles may become fixed more rapidly, which decreases genetic diversity. In small populations, there are fewer possible breeding partners. Hence, inbreeding, and inbreeding depression, is more common. Inbreeding depression decreases the number of offspring and increases the mortality rate of the offspring, which, in turn, results in even smaller populations. These smaller populations are more susceptible to environmental and demographic stochasticity, and evolutionary effects have an even larger effect. This cycle is repeated, until, eventually, the species falls out of existence. This is known as the extinction vortex (Frankham, Ballou, & Briscoe, 2004).

Zygodon leptobolax may be on the verge of extinction. The only known host is a species of *Quercus*, an alien species on Table Mountain. Large, spore producing populations and their hosts may be removed from the Table Mountain National Park in the near future, as part of a conservation initiative for the reserve. In the absence of suitable colonisation sites that meet the specific habitat requirements, and reduced numbers of spore producing individuals, the existence of this species will be severely threatened. This is just the first step toward extinction. As mentioned previously, there are several steps toward extinction. With each progressive step, it becomes more difficult to turn back, and conservation and restoration become increasingly difficult to implement. If host trees are removed from Table Mountain National Park, the likelihood of extinction for *Z. leptobolax* will increase substantially. This presents a dilemma to conservation efforts – remove the aliens and potentially lose an endemic species.

Are the remaining populations viable? *Z. leptobolax* consists of roughly 40 populations, ranging in size from single shoots to large populations of many more than 50 individuals. In order to avoid the effects of inbreeding depression, animal populations require more than 50 reproductive adults (Franklin, 1980; Soulé, 1980). In the case of *Z. leptobolax*, the majority of populations are small (consisting of fewer than 20 individuals) to medium (consisting of between 20 and 50 individuals) sized. Few are large, consisting

of many more than 50 individuals. This suggests that some inbreeding depression may be unavoidable. However, *Z. leptobolax* is capable of self-fertilization as well as vegetative reproduction by means of gemmae, which indicates that there must be a level of tolerance or adaptation to inbreeding. Besides which, the minimum viable populations numbers have not been assessed for bryophytes, and may be very different from those for animals and higher plants. Genetic diversity is moderately high within populations of *Z. leptobolax*, suggesting that even though there are few individuals, they have retained enough genetic diversity to maintain a degree of evolutionary potential. Usually, when a species is reduced to so few populations, the genetic diversity of the remaining individuals is extremely low. It may be so low that the entire species consist of clones. If this is the case, no further evolution is possible for that species, as all alleles become fixed. The only evolution would be through the accumulation of mutations, which are quickly removed from the population, through drift, almost as soon as they arise. This is not the case for *Z. leptobolax*. There is considerable diversity within the species. If sufficient suitable hosts are available, *Z. leptobolax* should be able to persist, evolve and adapt to future conditions. It also indicates that inbreeding is not such a large problem as it would be in similarly sized animal populations. This evolutionary potential is important for the long term survival of the species.

At present the number of species in the genus is undetermined, as are the phylogenetic relationships among them. A full revision may help to resolve some of the taxonomic uncertainties surrounding the genus. This is definitely required before a conservation plan can be formed. But by the time that is completed, it may be too late for *Z. leptobolax*. As Soulé stated, conservation biology (and genetics) is a crisis discipline. Decisions must be made on the basis of available information, if we are to preserve anything. Based on the available information, *Z. leptobolax* presents as a phylogenetically distinct South African species, with evolutionary potential. Populations are healthy and producing sufficient numbers of sporophytes for expansion of current populations, as well as for future colonisation. In light of this, it seems likely that *Z. leptobolax* will survive, given adequate hosts and protection from further host and key population removals. However, if these key populations i.e. those that contain individuals of a sexually reproductive age,

are removed, the species may not be able to replace populations lost due to environmental and demography stochasticity. In addition, if hosts are removed, no new colonisations can occur, and population numbers will be reduced. *Z. leptobolax* may be able to survive this for a few seasons, but eventually, the lack of hosts will result in the extinction of the species. How important is *Z. leptobolax* really? In a local context, it appears to be a good example to raise awareness of bryophyte conservation. Certainly, it is an important species for the Western Cape in terms of its interesting genes and questionable origins. In addition to this it raises an interesting question about the interactions between bryophytes and host species – how exactly can organisms switch hosts? If this is possible, it may provide the starting point for a new method of ex situ conservation for epiphytic mosses, and possibly other epiphytic species. More information is needed to place *Z. leptobolax* into a broader context though. As mentioned previously, at present there are no bryophyte conservation initiatives in place, and there has been no evaluation of the relative conservation value of South African bryophyte species as a whole. Perhaps this needs to be done before the true conservation value of *Z. leptobolax* can be determined.

In conclusion, the rare and endangered moss *Z. leptobolax* has the potential to thrive, given suitable habitat. Unfortunately, the only species found to host the moss is an alien *Quercus* species. Perhaps it is time to reconsider the implications of aggressive alien removal campaigns. Surely when a rare endemic is threatened by removal of alien species, the removal should be reconsidered? For this interesting moss to avoid extinction, a conservation plan must be devised. Either an ex situ strategy must be implemented, for example, spore banks or the removal and relocation of the surviving populations. However, relocation is never easy, and has not been attempted with bryophyte species. Or, the removal of the alien *Quercus* species must be reconsidered, and a management solution incorporating both the indigenous vegetation and the alien hosts must be devised. To lose *Z. leptobolax* would be a potential blow to South African bryophyte diversity.

APPENDIX A

MORPHOLOGICAL CHARACTERS USED IN PHYLOGENETIC ANALYSIS

1) **Form of populations.** 0 = cushion, 1 = dense tuft, 2 = open tuft, 3 = sparsely clustered shoots
2) **Stem length.** 0 = <1cm, 1 = 1-2cm, 2 = >2cm
3) **Leaf length.** 0 = <1mm, 1 = 1-2mm, 2= >2mm
4) **Shape of leaf apex.** 0 = acute, 1 = acuminate, 2 = obtuse, 3 = mucronate
5) **Shape of leaf.** 0 = linear lanceolate, 1 = lanceolate acuminate, 2 = oblong lanceolate, 3 = ovate lanceolate, 4 = ligulate
6) **Curve of leaf margins.** 0 = plane, 1 = incurved to involute, 2 = recurved to revolute.
7) **Leaf margin denticulation.** 0 = entire, 1 = crenulated, 2 = serrate, 3 = denticulate
8) **Leaf margin undulation.** 0 = not undulate, 1 = undulate
9) **Leaf margin papillose.** 0 = not papillose, 1 = papillose
10) **Length of costa.** 0 = ecostate, 1 = costa percurrent, 2 = costa decurrent, 3 = costa ends in the middle of the leaf.
11) **Presence of papillae on leaf cells.** 0 = smooth, 1 = papillose
12) **Shape of basal leaf cells.** 0 = rounded, 1 = oblong 2 = rectangular, 3 = hexagonal
13) **Hyaline basal cells present.** 0 = hyaline cells present, 1 = hyaline cells absent
14) **Sexual orientation.** 0 = monoicous, 1 = dioicous
15) **Length of seta.** 0 = < 1mm, 1 = 1 – 10mm, 2 = > 10mm
16) **Colour of seta.** 0 = pale yellow, 1 = yellow, 2 = orange red, 3 = brown,
17) **Shape of capsules.** 0 = cylindrical, 1 = oblong, 2 = pyriform
18) **Presence of a peristome.** 0 = peristome absent, 1 = single peristome present, 2 = double peristome present
19) **Size of spores.** 0 = < 10µm, 1 = 10 - 20µm, 2 = >20µm
20) **Substrate.** 0 = bark only, 1 = rocks only, 2 = both bark and rocks
21) **Leaf orientation when wet.** 0 = erect, 1 = sqaurrose, 2 = spreading

22) **Leaf orientation when dry.** 0 = appressed, 1 = loosely twisted around stem, 2 = spreading
23) **Methods of vegetative reproduction used.** 0 = no vegetative reproduction, 1 = via brood bodies or gemmae
24) **Costa papillose.** 0 = smooth, 1 = papillose
25) **Basal leaf cells papillose.** 0 = smooth, 1 = papillose
26) **Colour of stem leaves.** 0 = yellow-green, 1 = olive green, 2 = brown-green, 3 = black-green
27) **Spores papillose.** 0 = smooth, 1 = papillose
28) **Branching of stems.** 0 = unbranched, 1 = multiple branches, 2 = dichotomous branching
29) **Shape of leaf cells.** 0 = isodiametric, 1 = rhomboidal, 2 = round
30) **Presence of leaf rhizoids.** 0 = absent, 1 = few, sparse rhizoids, 2 = plentiful rhizoids forming a tomentum

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