The genetic diversity and conservation biology of the rare terrestrial snail genus *Prestonella*.

By

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Abstract

Prestonella bowkeri and *Prestonella nuptialis* are montane specialists endemic to the southern Great Escarpment of South Africa. Phylogeographic analyses of these species based on mitochondrial markers CO1 and 16S reveal extremely high levels of divergence between populations indicating a lack of gene flow between populations. This is not surprising, because *P. nuptialis* and *P. bowkeri* have limited dispersal capacity, low vagility, a highly fragmented distribution and are habitat specialists that are restricted to isolated mesic refugia associated with waterfalls and montane seepages. A relaxed Bayesian clock estimate suggests that populations diverged from one another during the mid-late Miocene (12.5-7 MYA) which coincides with the modern trends of seasonal aridity which began during the Miocene. This result should be viewed with caution because the rates used are at best imprecise estimates of mutation rates in snails. There is no clear dichotomy between the two species and *P. bowkeri* is paraphyletic with respect to *P. nuptialis*, as a consequence the taxonomy is unclear. Due to the high levels of sequence divergence between populations they may be considered as evolutionary significant units (ESU's). An assessment of haplotype diversity (*h*) and nucleotide diversity (π) reveals that populations in the western part of the Great Escarpment are more genetically depauperate than populations in the east. Correlations between genetic diversity and climatic variables show that genetically depauperate populations are found in areas that have lower annual rainfall, less reliable rainfall and higher potential evaporation, all factors associated with a drier, less mesic environment that increases the chances of a population bottleneck. This indicates that a shift towards a more arid environment may be a driver of genetic erosion. Historical climate change may thus have affected the amount and distribution of genetic diversity across the Great Escarpment since the Miocene. This has serious future implications for the survival of *Prestonella.* With predicted increase in global temperatures, climate change in South Africa is likely to result in range contraction and an eastward range shift for many species in the drier central and western areas (Erasmus *et al.* 2002) and regions along the Great Escarpment are likely to become more arid. *Prestonella* populations found living on inselbergs along the Great Escarpment are already restricted to site specific watercourses and seepages. An increase in the periods between stream flow, and increasing rainfall variability and mean annual potential evaporation are likely to have an adverse affect on species living in these habitats, resulting in further bottlenecks and possibly local extinction. An IUCN assessment of *P. nuptialis* and *P. bowkeri* suggests that these two species are probably endangered. The issue surrounding the conservation of *Prestonella* species is that they are threatened by global climate change, which cannot be simply restricted or prevented, which makes dealing with the threat of climate change difficult. Assisted migration (MA) may be considered as a method to prevent possible future extinctions of *Prestonella* populations, but will only be considered as a last resort. The thermal tolerance (Arrhenius breaking temperature and flat-line temperature) of individual snails from three *Prestonella* populations (one forest population and two thicket populations) were assessed using infrared sensors that detected changes in heart rate with increasing temperature. The forest population had a significantly lower Arrhenius breaking temperature (ABT) and flat-line temperature (FLT) than the two thicket population ($p<0.05$). Our results do not show a correlation between upper thermal limits and maximum habitat temperatures or other climatic variables in *Prestonella* populations. Although no correlation is found between ABT and maximum habitat temperature, it is likely that the differences seen between these populations are due to local micro-climate adaptation. The climatic variables used in this experiment are coarse estimates from GIS data and do not reflect actual microhabitat conditions. Forest environments are less heat stressed than thicket environments due to the forest canopy which may explain the lower ABT and FLT of the forest population.

Declaration

I declare that this work is my own and has not been submitted in any form to another University. It has been accordingly acknowledged in the text where I have used the work of others.

Signed ……………………………………………………

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Chapter 1: Introduction

Introduction

This thesis presents the results of a phylogeographic study of the snail genus *Prestonella*, which comprises three species, two of which are known to be extant. The results from the phylogeographic study informed a subsequent exploration of physiological responses to temperature / heat stress. Finally, these results were used to re-assess the IUCN conservation category of these species. As explained below, this snail represents an unusual, possibly unique, model organism to explore many of the issues affecting speciation (and conservation) of the southern African biota.

This thesis also explores aspects that challenge the maintenance of both genetic and species integrity; issues such as specialized life history traits, habitat fragmentation and isolation, low vagility and hence limited dispersal ability, physiological limitations, and predicted responses to climate change.

As is typical of many molluscs, this snail has very low vagility, Studies on the phylogeography of animal species with limited vagility (such as snails) are rare, even though studies of these species may reveal different historical patterns of migratory routes and refugia more readily than those of more mobile species (Pfenninger and Posada 2002, Fiorentino *et al.* 2008). The range of most terrestrial snails is often quite limited due specific habitat requirements and poor dispersal ability and only a few snail species have managed to become widespread due to anthropogenic displacement (Pfenninger *et al.* 1996, Pfenninger and Posada 2002). Despite their suitability for phylogeographic study, only a few phylogeographic studies have been carried out on terrestrial snails (Pfenninger *et al.* 2003). Most of these show strong genetic structuring and high levels of genetic divergence (e.g. Ross 1999, Davidson and Chiba 2008, Johnson *et al.* 2010), which are often associated with accelerated rates of mtDNA evolution (e.g. Thomaz *et al.* 1996, Chiba 1999, Pinceel *et al.* 2005).

Prestonella is a distinctive component of the southern African terrestrial malacofauna, and is restricted to relictual habitats associated with the southern edge of the Great Escarpment in South Africa (Herbert 2007). The genus is thought to be a Gondwanan relic of considerable age and a recent phylogenetic study shows that it represents a fascinating and ancient lineage. The distribution of the species suggest that the genus was once widespread across the mountains of the southern Great Escarpment, and underwent extreme fragmentation such that it now ekes out an existence in small isolated populations. Due to its highly specialized habitat and apparent isolation of populations, *Prestonella* may show strong phylogeographic signal.

The aims of this thesis are thus to:

1. Assess the genetic diversity and phylogeographic relationships within and between populations of *P. bowkeri and P. nuptialis* (Chapter 2).
2. Examine the differences in thermal tolerance between *Prestonella* populations from across its distribution range (Chapter 3).
3. Assign *P. bowkeri* and *P. nuptialis* to a category on the IUCN red data list (Chapter 4).

Introducing *Prestonella*

Phylogenetic analysis of the Stylommatophora using nuclear DNA sequence data places an Australian clade comprising of *Bothriembryon* and *Placostylus* as the sister group to *Prestonella* (Herbert and Mitchell 2009). This genus thus represents the only surviving African element of a Gondwanan lineage *Bulimulidae* that has survived in isolation for more than 95 million years in post-Gondwanan Africa (Herbert 2007). Molecular and morphological evidence suggests that *Prestonella* belongs to the family *Bulimulidae s.l.* (superfamily Orthalicoidea) as *Prestonella* exhibits many morphological character states thought to be plesiomorphic, suggesting a relationship with this group.

According to the currently accepted taxonomy, *Prestonella* comprises three species: *Prestonella bowkeri* (Sowerby 1890), *P. nuptialis* (Melvill and Ponsonby 1894) and *P. quadingensis* (Connolly 1929) (Herbert 2007).

Prestonella bowkeri (Figure 1.1 A)

Herbert (2007) describes this species as follows: *P. bowkeri* is the largest of the three species and is up to 23.25 mm in length, with a width up to 13.5 mm. The shell is succineiform to elongate-conical in shape. Apical whorls corneous orange-brown, becoming paler with growth; surface layer of body whorl whitish, underlying layers apricot-coloured. Fresh specimens have a straw-brown periostracum. The body of the snail is buff to yellow in colour (Herbert, 2007). Body whorl length is approximately 81.4–90.4% of shell length; with an aperture length of approximately 48.2–62.9% of shell length The protoconch is approximately 1.6-2.2 mm, with two whorls, the first is set at a pronounced angle, while the start of the second whorl is initially flat-sided becoming more rounded towards the end of the whorl, with widely spaced collobral axial riblets.

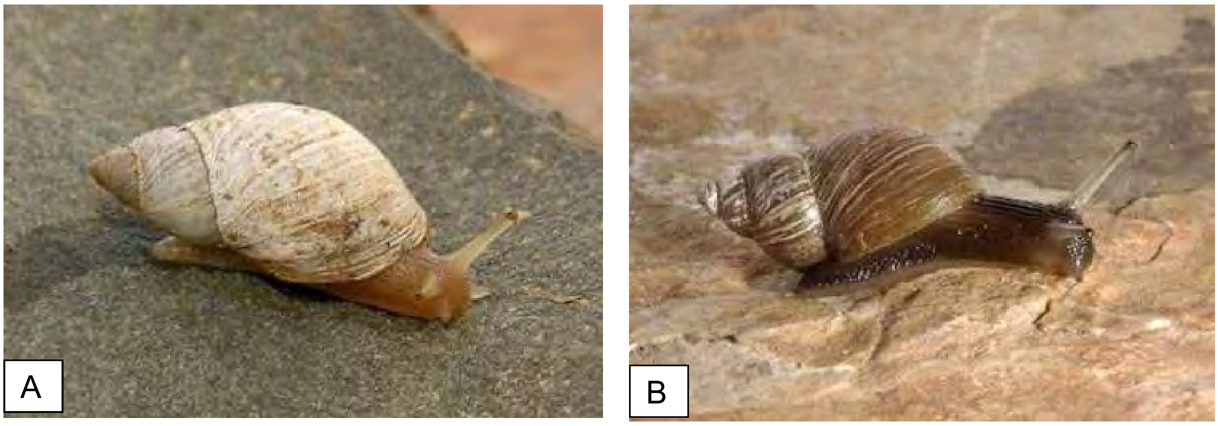

Figure 1.1, A : *Prestonella bowkeri* . *B: Prestonella nuptialis* .

Prestonella nuptialis (Figure 1.1 B)

Herbert (2007) describes this species as follows: *P. nuptialis* is up to 15.5 mm in length, with a width up to 10.0 mm. The shell is succineiform to bulimiform in shape. The shell is translucent when fresh, overlain by yellow-ochre to ochre-brown periostracum. The periostracum and underlying shell is often heavily eroded, particularly near apex; frequently with adherent particles of debris. The body of the snail is dark brown in colour. Body whorl length is approximately 78.5–87.3% of shell length; with an aperture length approximately 45.3–58.3% of shell length. The protoconch is approximately 1.5-2.0 mm, with 1.75-2.0 whorls. The basic shape is similar to *P. bowkeri*, but lacking axial riblets.

Prestonella quadingensis

The third species (*P. quadingensis*) is known only from type material and has not been collected since its original discovery in Quthing Lesotho in 1929 despite repeated efforts (including during this study) to locate it. Herbert (2007) describes this species as follows: *P. quadingensis* is extremely similar to *P. nuptialis* due to overlapping ratios of shell proportions, from which it differs mainly by the presence of fine, spiral threads on the teleoconch whorls, and axial riblets on the protoconch. The whorls may be slightly more obese. Colour of living animal unknown. *P. quadingensis* is the smallest of the three species and is up 12.2 mm in length, with a width up to 8.0 mm. Body whorl length is approximately 84.9–87.1% of shell length; with an aperture length of approximately 55.8–64% of shell length.

Habitat Preferences of *Prestonella*

Prestonella bowkeri and *P. nuptialis* are habitat specialists being found almost exclusively in isolated mesic environments in the southern and south-eastern regions of the Great Escarpment, where rivers, in traversing sharp topographical gradients at the edge of escarpments, form waterfalls and have cut steepsided ravines. These ravines are vegetated by indigenous forest or mesic riparian ticket/incipient forest (*P. bowkeri* Figures 1.2 A-B, *P. nuptialis* Figures 1.2 C-D) (Herbert 2007). The snails are found almost exclusively on near-vertical rock faces above water courses and on the shaded (south-facing) rock faces at altitudes between 1000 and 1600 m a.s.l. (Herbert 2007). They are often found in small aggregations hiding under overhangs and in cracks and crevices (Figure 1.2 E). The rock faces are mostly bare of macroscopic vegetation and superficial encrustations, but snails can often be found hiding under vegetation growing off the rock faces (Figure 1.2 F) (Herbert 2007).

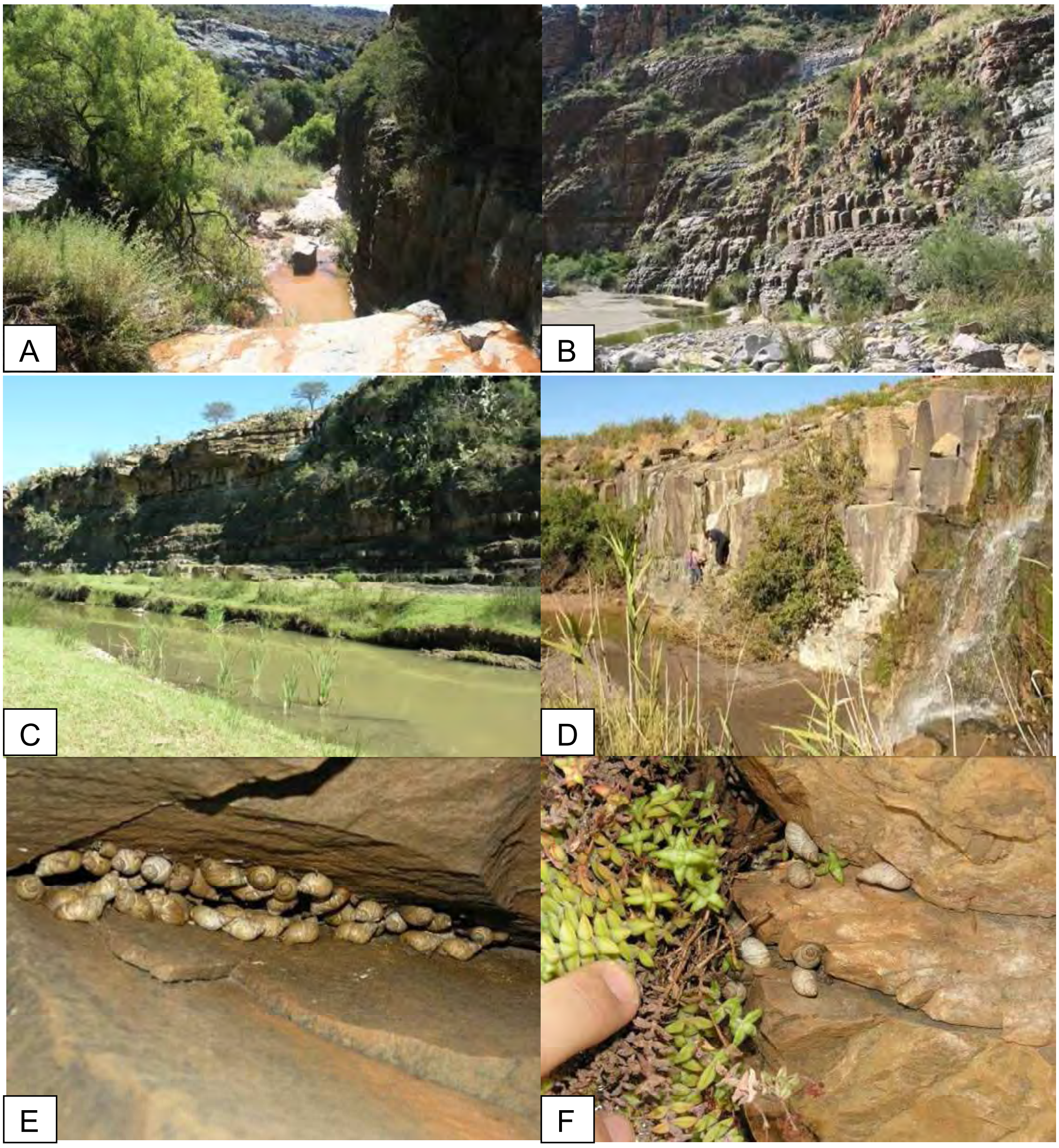

Figure 1.2 A-F, A: Habitat of *P. bowkeri* Rooivalle, Karoo National Park (Beaufort West area), B: habitat of *P. bowkeri*, at Wilge River Canyon, Nieu-Bethesda, C: habitat of *P. nuptialis*, at Kamala game reserve, Somerset East, D: habitat of *P. nuptialis*, at Molteno Pass, Beaufort West area, E: *P. nuptialis* in situ at Kamala Game Reserve , F: *P. bowkeri* in situ hiding under vegetation.

Distribution of *Prestonella*

Prestonella bowkeri and *P. nuptialis* have been found in several locations along the southern Great Escarpment extending from the Beaufort West area in the west to the Cradock and Fort Beaufort area in the east (Figure 1.3). The two species are sympatric in distribution at a broad spatial scale and although they have the same habitat requirements and seem to occupy very similar niches they have never been found occupy the same micro habitat and do not seem to be sympatric at the local scale.

Due to the habitat specificity of *Prestonella*, populations of these two species are found in small microhabitats. Such habitat fragmentation can create large distances between suitable habitats, resulting in genetically isolated populations as has been found in other terrestrial snails (Holland and Hadfield 2002, Hugall *et al.* 2002, Pfenninger and Posada 2002). The genetic diversity and structure of *Prestonella* is investigated in the next chapter.

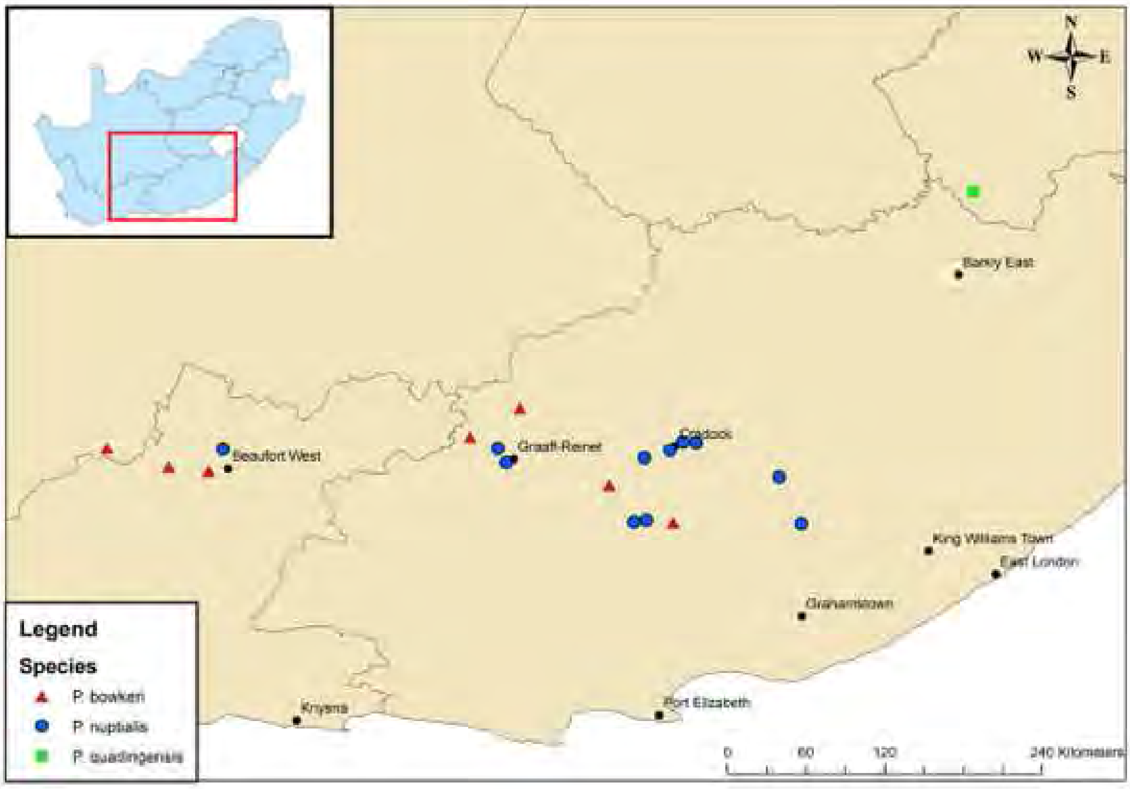

Figure 1.3: The distribution of *Prestonella* species in Southern Africa. Blue circles- *P. nuptialis*, red triangles- *P. bowkeri*, green square- *P. quadingensis*.

Chapter 2: The phylogeography of *Prestonella*

Introduction

There are several ways to examine the evolution of a species, including anatomy, fossil records, molecular sequences and genomic characters (allozymes and chromosomes) (Medina and Collins 2003). In the past, anatomy alone has been used for the phylogenetic inference and classification of species. However, in terrestrial snails the variation in size and shape of a shell may be environmentally and/or genetically determined which suggests that study of morphology alone may not be reliable (Goodfriend 1986). Molecular sequences such as mitochondrial DNA (mtDNA) can be used to examine the phylogenetic relationships of taxa but what is more intriguing is that molecular sequences can be used to construct haplotype phylogenies, which can be used examine the geographic distribution of genetic variation, a method known as phylogeography (Avise *et al.* 1987).

Over the past two decades phylogeography has grown as a discipline (Hare 2001, Beheregaray 2008, Avise 2009) and mammals have played a major role in the development of the theory and practice of phylogeography (Beheregaray 2008). The phylogeographic method deals with the spatial distributions of gene lineages and provides a means of investigating the historical genetic exchange between populations, with the potential to distinguish biogeographic patterns of genetic variation (Avise 2001), geographical distribution of major gene lineages and evolutionary relationships of populations (Arbogast and Kenagy 2008).

Phylogeography provides a framework for testing alternative hypotheses about genetic isolation and the timing and pattern of divergence without the limitations of taxonomic preconceptions that are based solely on morphological divergence (Richards *et al.* 2007, Schaal *et al.* 1998). Animal phylogeography has relied predominantly on mtDNA (Beheregaray 2008) and the success of these mtDNA based studies has led to the improved description and relative roles of gene flow, bottlenecks, population expansion and vicariant events in shaping geographical patterns of genetic variation, geographic distributions and genetic distances among evolutionary lineages, which in turn, has led to a better understanding of regional biogeography and endemism (Bermingham and Moritz 1998, Arbogast and Kenagy 2008).

Phylogeographic analysis relies on interpreting patterns of congruence (or lack of congruence) between the geographical distribution of haplotypes and their genealogical relationships (Schaal *et al.* 1998). A pattern of congruence is observed if groups of closely related haplotypes are geographically restricted and occur in proximity to each other (Schaal *et al.* 1998), which reveals a long standing pattern of highly restricted gene flow (novel mutations that remain localised in relation to their origins; Schaal *et al.* 1998).

Bermingham and Moritz (1998) acknowledged that phylogeography has been highly criticized for being overly reliant on using a single gene system as a marker of evolutionary descent. Any single gene tree represents only a tiny part of the full genetic history which may give a misleading representation of the population's history (Hare 2001). The possible problems associated with this limited method can be overcome by testing for phylogenetic congruence across several markers (Bermingham and Moritz 1998). The use of multiple markers (mtDNA and nrDNA markers) has its own advantages: more phylogenetically informative characters and the ability to detect different histories of the datasets which can be extremely powerful and highly sensitive (McDade *et al.* 2000). The use of multiple markers can overcome the issues associated with stochastic biological sampling and can reveal nonconcordant characters that are usually often biologically fascinating (Sunnucks 2000), thus it is important for the use of one or more mitochondrial markers and one or more nuclear markers in any phylogenetic/phylogeographic study.

Suitable molecular markers for phylogeographic studies of Molluscs

An ideal molecular system for phylogeographic study needs to meet certain criteria. Avise *et al.* (1987) suggest that a molecule should be: (1) distinctive, and widely distributed; (2) easy to isolate and assay; (3) have a fast evolutionary rate such that new character states may occur within the lifespan of the organism; (4) display a simple mode of genetic transmission, without recombination or other genetic rearrangements; (5) have an uncomplicated genetic structure deficient of problematic characteristics such as repetitive DNA, pseudogenes, introns and transposable elements; (6) provide sets of qualitative character states that are meaningful for microevolutionary analysis. Mitochondrial DNA of higher animals to someextent meets all of the above criteria, but there has been some evidence of recombination events (Eyre-Walker *et al.* 1999, Hagelberg *et al.* 1999).

Mitochondrial markers (mtDNA)

In higher animals, mitochondrial DNA (mtDNA) is a small, covalently closed circular molecule, about 16-20 kilobases long (Avise *et al.* 1987). Mitochondrial DNA has been preferentially used for reconstructing historical patterns of population demography, admixture, biogeography and speciation (Hurst and Jiggins 2005), and has proven to be a powerful tool for detecting population genetic structure at the intraspecific level (Avise *et al.* 1987; Moritz *et al.* 1987), and has been used extensively in taxonomic studies to describe species (Hurst and Jiggins 2005). In fact, the majority (>80%) of phylogeographic studies have relied upon the analysis of mitochondrial (mtDNA) variation, mainly because of processes of evolution that are conducive to providing a strong geographic signal (Avise 2001). Mitochondrial DNA has a high evolutionary rate, thought to be 5 to 10 times faster than nuclear DNA (Brown *et al.* 1979) and a lower effective population size than nuclear markers which allows us to investigate recent historical events (Hurst and Jiggins 2005). Different regions of the mtDNA genome evolve at different rates so suitable regions can be used for specific studies (Saccone *et al.* 1991). Commonly used mtDNA markers for animal invertebrate phylogenetic studies include mitochondrial markers cytochrome oxidase 1 (CO1), 16S rDNA, 12S rDNA and cytochrome b gene.

mtDNA is maternally inherited so unlike nuclear DNA, mutations arising in different individuals are not recombined during sexual reproduction (Avise *et al.* 1987). Importantly, no molecular system is perfect for phylogeographic study and mtDNA does have several limitations that need to be recognized, such as heteroplasmy, homoplasy, selection versus neutrality and lineage sampling bias (Avise *et al.* 1987). It has been argued that the pervasive nature of direct and indirect selection on this molecule makes any conclusion derived from it uncertain (Hurst and Jiggins 2005). Furthermore, studies on arthropods have shown that maternally inherited symbionts which are micro-organisms that exist inside the cells of their hosts and pass from female to her progeny through the egg can confound the inference of an organism's evolutionary history due to symbiont-driven increases and decreases in diversity, symbiont-driven changes in mtDNA variation over space and symbiont-associated paraphyly of mtDNA, thus mtDNA is not a suitable marker on its own for phylogeographic and phylogenetic studies in arthropods (Hurst and Jiggins 2005).

Nuclear markers (nrDNA)

The use of nuclear markers increased in popularity during the 1990's, whereas the proportion of studies based exclusively on uniparental inherited markers decreased from around 90% to 62% during the period between 1998 to 2008 (Beheregaray 2008). In many taxa, a lower substitution rate in nuclear DNA (nDNA) minimizes the back and parallel mutations that can lower phylogenetic resolution in mitochondrial DNA data sets. Therefore, in data with low homoplasy, even a single fixed difference can produce a statistically strong result at the intraspecific level, despite bootstrap support (Harris and Hey 1999). Commonly used nuclear markers include nuclear regions 28S rDNA, 18S rDNA and 26S rDNA and internal transcribed spacer (ITS) regions such as ITS1 and ITS2 and microsatellites. Noncoding nDNA typically has more variation than does the adjacent coding regions (Hare 2001).

According to Hare (2001) phylogeographic structure is expected to be less pronounced at diploid nuclear loci compared with cytoplasmic loci because of their different effective population sizes. There are a few concerns surrounding the use of nrDNA for phylogeographic studies, such as greater coalescent time of nuclear sequences as compared with mitochondrial genes, the difficulty in isolating DNA haplotypes and poor resolution could result from low mutation rates, leading to insufficient informative polymorphisms (Bermingham and Moritz 1998, Hare 2001) and recombination which could impede gene tree reconstruction (Ruths and Nakhelh 2005, Sota and Sasabe 2006). Recent methodological developments allow for the detection of recombination in different ways (Sota and Sasabe 2006). The prior assessment of recombination in sequence data may improve data analysis or the interpretation of results, thus several studies have considered the incidence of recombination in phylogenetic analyses of closely related species (e.g Broughton and Harrison 2003, Machado and Hey 2003).

Phylogeography has been applied in conservation biology with emphasis on testing for monophyletic groups, because evidence for long term isolation argues strongly for the evolutionary distinctiveness of a population (Avise 2001). However, if a population is isolated for long enough genetic drift will inevitably lead to neutral gene monophyly. Depending on the age of the group nuclear DNA may evolve too slowly to provide deep intraspecific partitions like those found in animal mtDNA (Hare 2001). Nuclear DNA with relatively deep coalescent times may thus be more useful to answer questions about ancient populations (Harding *et al.* 1997, Fu and Li 1999).

Although nuclear DNA has several draw backs as mentioned above the majority of ongoing research projects using nuclear DNA markers involve microsatellite DNA (Zhang and Hewitt 2003). According to Zhang and Hewitt (2003) among the 1758 primary papers and primer notes published between 1994 and 2003 in the journal *Molecular Ecology* of which 42.5% of these were indexed with microsatellite DNA markers while only 29.8% were indexed with mitochondrial DNA. Microsatellites have mutation rates significantly higher than nuclear gene sequences and even mitochondrial sequences and are the most revealing DNA markers available so far for inferring population-genetic structure and dynamics (Zhang and Hewitt 2003).

Although both nrDNA and mtDNA each have their own set of problems, comparisons of nrDNA and organellar DNA can identify historical hybridization, introgression events, asymmetrical mating preferences or provide evidence of ancestral polymorphisms when compared with discordant nrDNA (Palmer 1987, Sunnucks 2000). There is thus a definite necessity to use more than just one set of genetic sequence data, especially when inferring taxonomic status (Tautz 2003) and phylogeographic history.

Phylogeography of terrestrial snails

Phylogeography has proven to be a useful tool in detecting patterns of gene flow, historical range fragmentation, hybridization, range expansion, and speciation among many taxa (Avise 2001). Land snails provide a perfect model for the study of phylogeographic patterns and evolutionary processes as these are generally animals with low vagility and dispersal potential (Pfenninger and Posada 2002, Fiorentino *et al.* 2008). Most phylogeographic studies on terrestrial snails show extreme genetic structuring and high levels of genetic divergence (e.g. Ross 1999, Goodacre 2001, Davidson and Chiba 2008, Johnson *et al.* 2010), often in association with accelerated rates of mtDNA evolution (e.g. Thomaz *et al.* 1996, Chiba 1999, Pinceel *et al.* 2005), habitat fragmentation (e.g. Holland and Hadfield 2002) and climate change (e.g. Hugall *et al.* 2002, Pfenninger and Posada 2002, Pinceel *et al.* 2005).

Island species in particular show high levels genetic diversity mostly due to long periods of isolation and associated genetic drift and thus contribute to our understanding of speciation

(Davidson and Chiba 2008). Examples include *Amplirhagada* land snails from several different islands and mainland areas of the Bonaparte Archipelago, in the Kimberley region of northern Western Australia (Johnson *et al.* 2010), partulid land snails on Pacific Islands (Goodacre 2001), *Leptaxis* (*azorica* and *caldeirarum* types) from the Azores (Van Riel *et al.* 2005) and three endemic Cretan land snail species of the genus *Mastus* from the eastern part of Crete and the island group of Koufonisi (Parmakelis *et al.* 2003). Evolutionary rates for island species are often accelerated; the endemic land snail genus *Mandarina* of the oceanic Bonin Islands shows deep divergence and has an extremely fast mtDNA evolutionary rate that is more than 20 times faster than the average rate for animals (Chiba 1999). Likewise the extreme divergence seen between pulmonate land snails in Europe is most likely due to exceptionally rapid rates of mtDNA evolution (Thomaz *et al.* 1996). Chiba (1999) suggests that numerous founder effects can accelerate the rate of mtDNA evolution and frequent expansion and contraction of populations in the small islands may also accelerate mtDNA evolution in *Mandarina*. Having such high mutation rates means that phylogeographic studies on snails can be used to investigate more recent historical events than is possible with other taxa, and thus be able to inform on events that other taxa cannot.

Characteristics such as low vagility and poor dispersal ability inhibit terrestrial snails from escaping ecological change and it is commonplace to assume that historic climate change has led to many mollusc extinctions (Pfenninger and Posada 2002), population fragmentation and speciation. Phylogeographical patterns based on mtDNA sequences have shown that Pleistocene glaciations have played an important role in influencing the current distribution of many terrestrial snails in the northern hemisphere (e.g. Pfenninger and Posada 2002, Pinceel *et al.* 2005, Fiorentino *et al.* 2008). High levels of divergence are often found in northern hemisphere terrestrial snails as a result of population fragmentation during glacial periods and subsequent post-glacial re-colonization from several glacial refugia, as seen for *Candidula unifasciata* across its European range (Pfenninger and Posada 2002), Tyrrhenian land snails in the Italian Peninsula (Fiorentino *et al.* 2008), and the terrestrial slug *Arion subfuscus* across its native distribution range in Western Europe (Pinceel *et al.* 2005). Habitat specialists may be especially sensitive to climate change and it has been reported that bulimulid species in the Galapagos Islands have a limited distribution due to specific microclimate requirements. Specialized habitat restriction may thus greatly influence species distribution (Coppois 2003). Habitat specialists such as the Iowa Pleistocene snail (Ross 1999) and European spring snail genus *Bythinella* (Benke *et al.* 2009) show high levels of genetic divergence due to long periods of isolation and show similar patterns to that of island species.

The majority of phylogeographic research has focused on plant and animal species from temperate continental regions in the northern hemisphere (Hewitt 2004, Beheregaray 2008). However, the response of species in the southern hemisphere (especially tropical and island species) to past climate change is far less well known, with a single study on the land snail species *Gnarosophia bellendenkerensis* found in the rainforests of North Queensland, Australia. This phylogeographic study revealed six major lineages that are each restricted to a single biogeographic subregion (Hugall *et al.* 2002), and it was postulated that fluctuations in rainforest area due to climate change has led to range contraction and extinction followed by range expansion and dispersal from multiple refugia (Hugall *et al.* 2002).

The terrestrial snails of southern Africa

Southern Africa has a remarkably diverse terrestrial malacofauna with over 650 species occurring south of the Cunene and Zambezi rivers (van Bruggen 1978, Herbert 1998), of which 90% exhibit a high degree of regional endemicity (van Bruggen 1978, Herbert 1998). This presents a wealth of species for phylogenetic and phylogeographic inquiry. To date, however, there are few phylogenetic studies on southern African land snails such as the orthalicoid family Bulimulidae *s.l.* (Herbert and Mitchell 2009) and the carnivorous snail genus *Natalina* (Moussalli *et al.* 2009). Other African examples include the freshwater gastropod family Viviparidae from the African rift valley lakes (Sengupta *et al.* 2009), the *Bulinus* species complex in the Albertine rift freshwater bodies (Nalugwa *et al.* 2010), freshwater snail genera *Ceratophallus* and *Afrogyrus* (Brown 2001), *Bulinus sp.* populations in Cameroon crater lakes (Ndassa *et al.* 2007*)* and the genus *Bellamya* in Lake Malawi (Schultheiss *et al.* 2011). To our knowledge, no phylogeographic studies have been published on southern African terrestrial snails and in fact there are only a handful of phylogeographic and phylogenetic studies on South African invertebrates (Table 2.1). Phylogeographic studies on terrestrial snails in other parts of the world are fortunately more numerous. These largely treat taxa from Europe, Australasia and tropical islands and archipelagos. South Africa thus possesses a uniquely diverse malacofauna that has the potential to elucidate the effects of past climates, climate change and habitat fragmentation in the region.

Aims

The aims of this chapter are to assess the genetic diversity and phylogeographic relationships within and between populations of *P. bowkeri* and *P. nuptialis*.

Table 2.1: Molecular studies focusing on terrestrial invertebrates in southern Africa..

Taxon	**Group**	**Study**	**Reference**
Potamonautes spp.	Decapoda	Phylogenetic	Daniels *et al.,* 2001
Potamonautes perlatus	Decapoda	Phylogenetic	Daniels*,* 2003
Potamonautes spp.	Decapoda	Phylogenetic	Daniels *et al.,* 2002
Potamonautes spp.	Decapoda	Phylogenetic	Daniels *et al.,* 2006
Potamonautes spp.	Decapoda	Phylogenetic	Daniels *et al.,* 2003
Elporia barnardi	Insecta	Phylogeographic	Wishart and Hughes, 2001
Mantophasmatodea	Insecta	Phylogenetic	Klass *et al.*, 2003
Platypluera spp.	Insecta	Phylogenetic	Villet *et al.*, 2004
Platypleura stridula	Insecta	Phylogeographic	Price *et al.*, 2007
Platypleura plumosa complex	Insecta	Phylogeographic	Price *et al.* 2010
Scarabaeus spp.	Insecta	Phylogeographic	Sole *et al.*, 2005
Scarabaeini	Insecta	Phylogenetic	Forgie *et al.,* 2006
Mantophasmatodea	Insecta	Phylogenetic	Damgaard *et al.,* 2008
Mesamphisopus spp.	Isopoda	Phylogenetic	Gouws *et al.*, 2005
Mesamphisopus spp.	Isopoda	Phylogenetic	Gouws *et al.*, 2004
Mesamphisopus spp.	Isopoda	Phylogenetic	Gouws *et al.*, 2010
Prestonella spp.	Gastropoda	Phylogenetic	Herbert and Mitchell, 2009
Natalina spp.	Gastropoda	Phylogenetic	Moussalli *et al.*, 2009

Methods

Sample Collection

A total of 165 specimens of *P. nuptialis* and *P. bowkeri* were collected from 11 localities spanning the known distribution range of the genus. These localities, some of which were previously known (6), others of which were discovered during the course of this study (5), extend across the southern part of the Great Escarpment in South Africa (Figure 2.1). Table 2.2 provides details of the localities. When populations were large (more than a 100 individuals) 10 or more individuals were taken, when populations were small (less than 100 individuals) less than 10 snails were taken. Whole specimens were preserved in 80%-100% ethanol. All the voucher specimens are kept at the Natal Museum in Pietermaritzburg, South Africa.

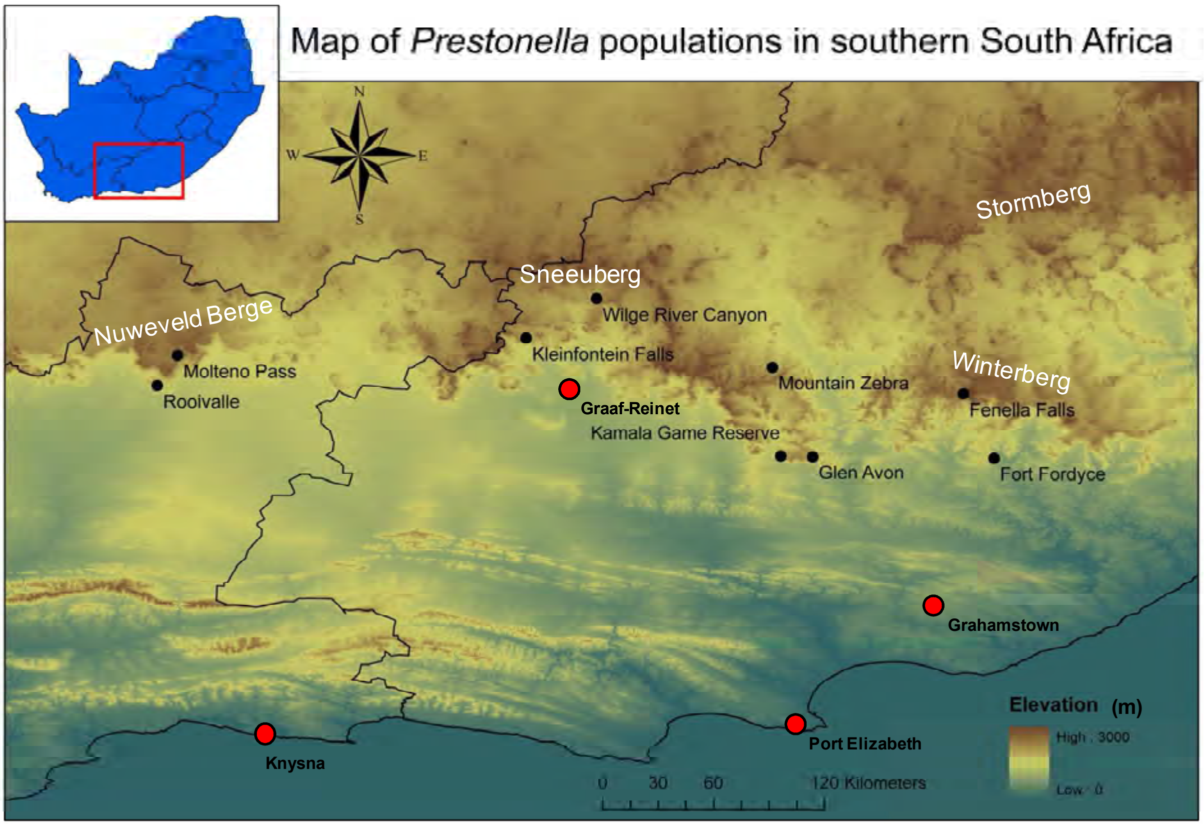

Figure 2.1: Map of South Africa showing the sampling localities (indicated by black dots) for *P. nuptialis* and *P. bowkeri* on mountain ranges along the southern edge of the Great Escarpment. The Kamala Game Reserve and Mountain Zebra populations are split into three populations.

Table 2.2: Locality and habitat data for *Prestonella* species.

Species	Locality & Voucher No.	Latitude	Longitude	Altitude (m)	Habitat	Geology
bowkeri	Somerset East, Glen Avon Falls, base of falls (V9816).	32° 40' 1.2"	25° 38' 9.5994"	1100	On large rocks in indigenous forest at base of falls	Dolerite
bowkeri	Nieu Bethesda area, Wilge River canyon, Ganora Farm (W6096)	31° 53' 29.83"	24° 35' 3.3"	1350	On sheer mudstone walls, in crevices up to 20m above river	Red, purple, grey and blue-green mudstone, subordinate sandstone
bowkeri	Beaufort West area, Karoo Nat. Park, Rooivalle (W1479).	32° 19' 31.9002"	22° 26' 56.4"	1130	Rocky kloof	Mudstone (red in places), sandstone
nuptialis	Fort Beaufort area, Fort Fordyce (W6649).	32° 41' 12.98"	26° 30' 51.99"	950	Indigenous forest, on vertical rock faces	Dolerite
nuptialis	Mountain Zebra Nat. Park, Boesmanskloof, site 1(W6115).	32° 14' 2.868"	25° 26' 21.048"	1400	Rock walls beside water course, in vertical crevices	Mudstone, greenish grey red in places; shale, sandstone
nuptialis	Mountain Zebra Nat. Park, waterfall top, site 2	32° 10'14.6"	24°25'59.87”	1258	Rock walls beside water course, in vertical and horizontal crevices	Mudstone, greenish grey red in places; shale, sandstone
nuptialis	Mountain Zebra Nat. Park, waterfall bottom, site 2	32°10'10.7"	24°26'03.62”	1249	Rock walls beside water course, in vertical and horizontal crevices	Mudstone, greenish grey red in places; shale, sandstone
nuptialis	Fenella Falls, Adelaide area (W5507).	32° 22' 1.19"	26° 22' 1.2"	1300	In crevices in rocks behind high waterfall	Sandstone, red and greenish grey mudstone
bowkeri	Kleinfontein Falls, between Graaff-Reinet and Murraysburg (W7319).	32°05'21"	24°14'29"	1281	On boulders at base of falls. Mesic riparian ticket/incipient forest at base of falls.	Dolerite
bowkeri	Molteno Pass, Beaufort West Area	32° 10' 26.8854"	22° 32' 51.18"	1545	In crevices in rock wall beside water course	Mudstone (red in places), sandstone
nuptialis	Somerset East, Kamala game reserve, site 1(W6734).	32° 40' 28.992"	25° 22' 13.728"	1260	Mid-level, S-facing rocky ridge	Mudstone, greenish grey red in places; shale, sandstone
nuptialis	Somerset East, Kamala game reserve, site 2 (W6742).	32° 39' 42.5514"	25° 27' 20.772"	830	Rock outcrop on bank of Little Fish River	Mudstone, greenish grey and red; sandstone
nuptialis	Somerset East, Kamala game reserve, site 1b	32° 41' 31.992”	25° 25 20.432"	832	S-facing rocky ridge	Mudstone Greenish grey red

DNA Extraction

To overcome problems associated with the inhibition of polymerase chain reaction (PCR) by mucopolysaccharides, DNA was extracted using a modified CTAB (hexadecyltrimethyl-ammonium bromide) extraction protocol described by Goodacre and Wade (2001). For each sample a small piece of foot tissue was finely chopped up and placed in 1000 μl CTAB extraction buffer (100mM Tris/HCL, 1.4M $NaCl_2$, 20mM ethylenediaminetetraacetic acid, 2% CTAB, 0.2% b-mercaptoethanol with 0.01mg Proteinase K) and incubated at 60°C for 1-2 hours, vortexing or shaking the tubes regularly. Proteins were removed by adding 500μl of CIA (chloroform:isoamyl alcohol, 24:1) and centrifuged at 13,000 rpm for 1 minute. The clear aqueous layer was removed and 400 μl of ice cold isopropanol was added to it and mixed. The tubes were left in a -20°C deep freeze for 30 minutes. The tubes were then centrifuged at 13,000 rpm for 10 minutes. The grey-white DNA pellets were then dried and resuspended in 300 μl water.

DNA Amplification and Isolation

This study used DNA sequence data from two mitochondrial markers (partial cytochrome oxidase 1 gene; CO1 and partial 16S rDNA) and one nuclear marker (28S rDNA).

A standard 50 μl PCR reaction included; 5 μl 10x NH_4 standard reaction buffer (Bioline), 0.2 μM of each primer, 0.2 mM dNTP (Bioline), 2 μl Bovine serum albumin (BSA), 2.5 mM $MgCl_2$, 2 Units of Taq DNA polymerase, 5 μl DNA and 26 μl-28 μl water.

Mitochondrial Cytochrome Oxidase 1 (CO1) region:

The primer pair LCO 1490 (Folmer *et al.* 1994) and H7005 (Donald *et al.* 2005) was used to screen samples of *P. nuptialis* and *P. bowkeri*, but based on limited PCR success we designed improved primers (Table 2.3).

The PCR protocol for *P. nuptialis* consisted of initial denaturation at 95°C (10 mins) followed by 40 cycles of; denaturation at 94°C (45 secs), annealing at 50°C (45 secs), elongation at 72°C (3 mins) and a final extension phase at 72°C (10 mins). The PCR protocol for *P. bowkeri* was identical to that of *P. nuptialis* but an annealing temperature of 48°C was used.

Table 2.3: Primers use to amplify the CO1, 16S and 28S regions for *P. nuptialis* and *P. bowkeri*.

<table>
<tr><td colspan="4">Mitochondrial Cytochrome oxidase 1</td></tr>
<tr><td>Primer</td><td>Direction</td><td>Use</td><td>Sequence</td></tr>
<tr><td>LCO 1490</td><td>Forward</td><td>PCR/Sequencing</td><td>5’- GGT CAA CAA ATC ATA AGA TA-3’</td></tr>
<tr><td>H7005</td><td>Reverse</td><td>PCR/Sequencing</td><td>5’-CCGGATCCACANCRTARTANGTRTCRT-3’</td></tr>
<tr><td>P. nupt CO1 F2</td><td>Forward</td><td>PCR/Sequencing</td><td>5’-AGATAAAACAATTCCAGTTAAT-3’</td></tr>
<tr><td>P. nupt CO1 IR</td><td>Reverse</td><td>PCR/Sequencing</td><td>5’- GCGGAGCAGCAGTAGATTTAG-3’</td></tr>
<tr><td>P. bowk IR</td><td>Reverse</td><td>PCR</td><td>5’-GGATCTCCTCCTCCTGC-3’</td></tr>
<tr><td>P. bowk IF</td><td>Forward</td><td>PCR</td><td>5’-GTTTATTTGTTTGATCAATTTTGG-3’</td></tr>
<tr><td colspan="4">Mitochondrial 16S rDNA</td></tr>
<tr><td>Primer</td><td>Direction</td><td>Use</td><td>Sequence</td></tr>
<tr><td>16Sar</td><td>Forward</td><td>PCR/Sequencing</td><td>5’- CGCCTGTTTATCAAAAACAT-3’</td></tr>
<tr><td>16Sbr</td><td>Reverse</td><td>PCR/Sequencing</td><td>5’-CCGGTCTGAACTCAGATCACGT-3’</td></tr>
<tr><td>16Smh1</td><td>Forward</td><td>PCR/Sequencing</td><td>5’- TTTGTACCTTTTGCATAATGG-3’</td></tr>
<tr><td>16Smh2</td><td>Reverse</td><td>PCR/Sequencing</td><td>5’-TAAGGTCCTTTCGTACTA-3’</td></tr>
<tr><td colspan="4">Nuclear 28S rDNA</td></tr>
<tr><td>Primer</td><td>Direction</td><td>Use</td><td>Sequence</td></tr>
<tr><td>LSU5</td><td>Forward</td><td>PCR/Sequencing</td><td>5’-TAGGTCGACCCGCTGAAYTTAAGC A-3’</td></tr>
<tr><td>LSU1600</td><td>Reverse</td><td>PCR/Sequencing</td><td>5’-AGCGCCATCCATTTTCAGG-3’</td></tr>
</table>

Mitochondrial 16S region:

The mitochondrial 16S rDNA region of both *P. bowkeri* and *P. nuptialis* was amplified with two primer pair combinations (Table 2.3). Universal primer pair 16Smh1 and 16Shm2 (Moussalli *et al.* 2009) was used initially to amplify this region, but for those specimens that didn’t amplify the primer pair 16Sar and 16Sbr (Palumbi 1996) was used.

The PCR protocol for 16S consisted of initial denaturation at 95°C (10 mins) followed by 38 cycles of; denaturation at 94°C (45 secs), annealing at 46°C (45 secs), elongation at 72°C (3 mins) and a final extension phase at 72°C (10 mins).

Nuclear 28S rDNA:

The 28S rDNA region was amplified for both *P. nuptialis* and *P. bowkeri* using primer pair LSU5 (Littlewood *et al.* 2000) and LSU1600 (Williams *et al.* 2003) (Table 2.3).

The PCR protocol for 28S consisted of initial denaturation at 96°C (5mins) followed by 25 cycles of; denaturation at 96°C (15 secs), annealing at 50°C (10 secs), elongation at 60°C (4mins) and a final extension phase at 72°C (10 mins).

Five μl of loading dye (499 μl bromophenol blue and xylene cyanol blue dye, 500μl DMSO and 1 μl SYBR green – a DNA detection dye) was added to 5 μl of PCR product and electrophoresed on 1% agarose gel which consisted of 0.5g agarose powder and 50 ml TBE buffer (10.8g Tris(hydroxymethyl) aminomethane, 5.5g Boric Acid and 0.93g EDTA made to 1L with water). DNA was visualized under a UV transilluminator. Clear, bright bands were recorded as a positive result and kept for sequencing, while smears and samples that didn't amplify were regarded as useless and PCR conditions were altered by changing magnesium (Mg) concentrations, the number of cycles or the annealing temperature. In some cases faint bands were pooled together to increase the yield and kept for sequencing.

PCR products were purified using Invitek MSB® Spin PCRapace purification kit according to manufactures instructions. Purified products were run on a 1% agarose gel and verified under a UV transilluminator.

Purified PCR product was sequenced as follows:

A typical 20 μl sequencing reaction mixture consisted of 2 μl sequencing mix, 3 μl sequencing buffer, 0.5 μl primer, 1-4 μl of DNA made up to volume with distilled water. For purified DNA products of low DNA yield, 4 μl of purified DNA was used, where as 1 μl of purified DNA was used when purified DNA products of high yield were sequenced. The sequencing reaction consisted of initial denaturation of 95°C for 1 minute followed by 30 cycles of 96°C (30 secs), 50°C (15 secs) and 60°C (4 mins).

Sequencing products were precipitated as follows; 50 µl of 99% ethanol, 2 µl EDTA and 2 µl NaAC was added to each reaction, mixed, and left to incubate for 15 minutes at room temperature. The samples were then centrifuged at 13 000 rpm for 15 minutes. The supernatant was carefully removed so as not to disturb the DNA pellet and 150 µl of 70% ethanol was added and centrifuged at 13 000 rpm for 10 minutes. The ethanol is carefully removed and the DNA pellet was left to air dry. The DNA samples were sequenced using an ABI Prism 3100 Genetic Analyzer.

Sequence editing and alignment

Assembled sequences (contiguous sequences) were constructed from sequence chromatograms and edited using Sequencher version 4.0. The sequences were checked for base-calling errors and corrected. The assembled sequences were imported and aligned using McClade version 3.0 (Maddison and Maddison 1992). Alignments are presented in Appendix 1. Owing to the range of primers used, as well as (in some cases) poor sequence data at the start or end of the chromatogram, several sequences in the alignment were shorter than other sequences. The alignment was thus shortened (between 10 and 100 base pairs had to be removed). No missing data was thus present in any of the alignments used in subsequent analyses.

Phylogenetic Analysis

Out group Selection

Herbert and Mitchell (2009) have shown that, based on a phylogeny of the gastropod order Stylommatophora, species of *Placostylus* (viz. *P. ambagiosus* and *P. eddystonensis)* fall within the same clade as *Prestonella*, suggesting that *Placostylus* and *Prestonella* are closely related. *Placostylus bivaricosus* (Lord Howe flax snail) from Australia was selected as the outgroup, because GenBank had sequence data for both CO1 (GenBank Accession: AY165841.1) and 16S (GenBank Accession: AY165850.1) for this species.

Phylogeographic Reconstruction

Four methods of phylogeographic reconstruction were used; Neighbor-Joining method, Maximum Parsimony, Bayesian Inference and Maximum Likelihood.

Neighbor-Joining method (NJ)

Neighbor-Joining is a distance based method of analysis and a tree is constructed from a set of distance measures (Hall 2001). The aligned sequences are converted into a distance matrix of pair wise differences. Unlike UPGMA the Neighbor-Joining method assumes that all taxa are equidistant from the root. Distance matrix methods can handle large volumes of data and use less time to compute than parsimony methods. The Jukes-Cantor model is the classic null model for DNA sequence evolution and assumes that all base positions in a sequence have the same expected rates of substitution (Huelsenbeck and Kirkpatrick 1996). Using software PAUP* a Jukes-Cantor NJ tree with additional bootstrap support was generated for all datasets. Bootstrap support was calculated from 1000 replicates.

Maximum Parsimony (MP)

Parsimony is a character based method where the most parsimonious tree is the one that requires the least number of changes or steps. This implies that the simplest hypothesis is the preferred one (Hall 2001). Using software PAUP* a HEURISTIC search was performed on each dataset using TBR branch swopping. Constant and uninformative characters were excluded. Strict consensus trees were produced from the set of equally most parsimonious trees obtained. 1000 Bootstrap replicates were carried out with MAXTREES set to1000.

Bayesian Inference (BI)

In Bayesian analysis, inference from a phylogeny is based upon posterior probabilities of phylogenetic trees (Huelsenbeck and Ronquist 2001). Bayesian Inference is thought to be advantageous over other methods of phylogenetic inference because the program has computational advantages and the ability to include priors (Huelsenbeck and Ronquist 2001). Bayesian inference also allows a user to specify an evolutionary model.

Phylogenetic analysis was conducted using MrBayes version 3.1.2 (Huelsenbeck and Ronquist 2001), with model selection determined by the Akaike Information Criterion (AIC) in MrModel Test v2.3 (Nylander 2004). This was done for each gene separately and for a combined mtDNA dataset which combined both genes. The AIC was selected over the hLRT because it typically selects more complex models than alternative approaches and does not have drawbacks associated with the hLRT (Ripplinger and Sullivan 2007). An ILD (Incongruence Length Difference) test was performed to confirm combinability of the two datasets. Multi-gene datasets were partitioned and analysed with selected models of sequence evolution. The analysis was run for 20 000 000 generations (one tree saved every 2000 generations). Convergence of the Bayesian runs was checked with Tracer v1.4.1 (Rambaut and Drummond 2007). Trees generated before stationarity had been reached were discarded as burn-in (first 10% of the sampled trees). Only trees sampled after this burn-in period were used to determine posterior probabilities of model parameters (bpp), branch lengths and clades, by generating a consensus tree with MrBayes version 3.1.2. The consensus BI trees were viewed in FigTree v .3.1 (Rambaut 2006).

Maximum Likelihood (ML)

Maximum likelihood analysis is a statistical procedure that estimates the likelihood of a hypothesis. The advantages of this analysis are that it allows a user to specify an evolutionary model and always produces a single tree. A disadvantage of this method is that it is considerably slower than Neighbor-Joining and parsimony methods and may be limited by the computing capacity of the computer running the analysis (Hall 2001).

MrModeltest v2.3 (Nylander, 2004) was used to identify the best model of DNA substitution for each dataset. The Maximum likelihood analyses were submitted and run on the Oslo University Bioportal. A Bootstrap tree (1000 replicates) was obtained and viewed in FigTree v1.3.1(Rambaut 2006).

Isolation by Distance

Isolation by distance (IBD) is a term used to describe a pattern in population genetic variation which is a consequence of limited gene flow between populations, as genetic similarity decreases between populations the geographic distance between populations increases (Holland and Cowie 2007). An isolation by distance test was conducted for the 11 populations of *Prestonella* and for each separate species using software IBD (Bohonak 2002) to determine if there is a correlation between genetic distance and geographic distance. Linear geographic distance was measure by determining the shortest straight line distance (kilometres) between each population using software in Google Earth. Genetic distance (nucleotide divergence) for the mitochondrial dataset between each population was calculated using software DNAsp v5.0 (Librado and Rozas 2009). A nonparametric Mantel test was performed to test for non-random associations between a matrix of genetic distances between all population pairs and a matrix of pairwise geographic distances, using the program IBD with 30 000 randomizations.

Molecular estimates of Divergence Times

MrModeltest (Nylander, 2004) was used to find the best nucleotide substitution model for the combined mitochondrial dataset (CO1 and 16S). The sequences divergence rates and molecular clock (relaxed uncorrelated lognormal clock) were set using BEAUTI and the divergence times were computed using Software BEAST (run on the Cornell University BioHPC server) (Drummond *et al.* 2002). The analysis was run for 20 000 000 generations and a tree was saved every 2000 generations. Sequence divergence rates based on several different published rates for gastropods were employed. Evolutionary rates of gastropod mitochondrial DNA vary considerably and are often estimated at 2% pairwise sequence divergence per million years for invertebrates (Desalle *et al.* 1987). Rates have been clocked anywhere between 0.5-2% sequence divergence per million years (e.g. Ozawa and Okamoto 1993) to accelerated rates of 10–12.9 % per million years in some land snails (Chiba 1999, Thomaz *et al.* 1996, Hayashi and Chiba 2000). Based on the evolutionary rates of terrestrial snails from other studies we have used average rates to calculate the divergence times. Sequence divergence rates for mtDNA of 10% per million years or more are rare and generally only found for terrestrial snails on volcanic archipelagos (e.g. *Mandarina*: Chiba 1999, *Leptaxis* (*azorica* and *caldeirarum* type): Van Riel *et al.* 2005). It is also unlikely that the rates of these snails are slow due to the large genetic divergence seen between even locally close populations, therefore a rate of 2%-5% (e.g. Van riel *et al.* 2005) sequence divergence was used for the CO1 partition and 2% for the 16S partition (e.g. Rumbak *et al.* 1994, Pinceel *et al.* 2005). Bayesian runs were checked with Tracer v1.4.1 (Rambaut and Drummond 2007). Trees generated before stationarity were discarded as burn-in (first 20% of the sampled trees). Trees were constructed using software TreeAnnotator v 1.5.4 (Rambaut and Drummond 2002) and viewed using FigTree v 1.3.1(Rambaut 2006).

Genetic Diversity

A coalescent estimation approach was employed to compare fixation indices F_{ST} (Hudson *et al.* 1992) among population, using DNAsp (Librado and Rozas 2009). Probability for F_{ST} values was obtained by the permutation test with 3000 replicates. DNAsp was also used to estimate population-level parameters and their variances, including the coefficient of gene differentiation G_{ST} (Nei 1982), pairwise nucleotide diversity (π) and haplotype diversity (h). Due to high level of divergence seen in phylogenetic analyses the population at Kamala Game reserve site 1 was divided into two populations found on either side of the ravine. Mountain Zebra National Park site 2 was also divided into two populations, one population upstream the other downstream, thus the total number of populations for this analysis is 13.

Determining climatic variables and correlating these to genetic diversity

ArcMap v9.0 GIS maps for annual precipitation, mean annual potential evaporation (how much water can potentially evaporate in a year), mean maximum and minimum temperatures, relative humidity and the coefficient of variation (CV%) of annual precipitation (how variable/reliable rainfall is in an area) for the southern Great Escarpment in South Africa were obtained from the South African Atlas of Agrohydrology and Climatology (Schultz 1997). Climatic values for each *Prestonella* population were extracted from these maps and plotted against gene diversity and nucleotide diversity for these populations and a linear regression was calculated using Microsoft Excel. We chose these climatic variables because we considered them to be variables that would affect sedentary molluscs, and as such would be good indicators of the climatic stress that populations of *Prestonella* face, which may potentially drive natural selection.

Results

The Maximum Parsimony, Maximum Likelihood and Neighbor-Joining trees presented in this section are reduced or summary trees, and only the full Bayesian Inference results are presented. Complete trees of the other analyses are given in Appendix 2.

The DNA dataset statistics are shown in Table 2.4. Maximum Parsimony analysis for the CO1 dataset included 647 base pairs of which 480 were uninformative and excluded, leaving 167 informative characters. The Maximum Parsimony tree length is 382 steps with a retention index (RI) of 0.977 and a consistency index (CI) 0.620. The model HKY + G was selected by the Akaike Information Criterion (AIC) in MrModeltest as the best model for the Bayesian and Maximum Likelihood analyses (Nylander 2004).

The Maximum Parsimony analysis for 16S included 559 base pairs of which 444 were uninformative and excluded, leaving 115 informative characters. The Maximum Parsimony tree has a length of 222 steps with a retention index (RI) of 0.983 and consistency index (CI) of 0.721. MrModeltest identified the best model as GTR+G for the Bayesian and Maximum Likelihood analyses.

Maximum Parsimony analysis for the combined mitochondrial dataset included 1209 base pairs of which 924 were uninformative and excluded, leaving 282 informative characters. The Maximum Parsimony tree has a length of 612 steps with a retention index (RI) of 0.978 and a consistency index (CI) of 0.649. MrModeltest identified the best model for the combined mitochondrial dataset as GTR+G for the Bayesian and Maximum Likelihood analyses.

Table 2.4: Statistics for CO1, 16S and MtDNA datasets.

Dataset	**Number of Base Pairs**	**Uninformative Characters**	**Informative Characters**	**Tree Length**	**Retention Index (RI)**	**Consistency Index (CI)**
CO1	647	480	167	382	0.977	0.620
16S	559	444	115	222	0.983	0.721
Mitochondrial	1206	924	282	612	0.978	0.649

Cytochrome oxidase 1 Dataset

The Maximum Likelihood (Fig 2.3A), Maximum parsimony (Fig 2.3B), Neighbor-Joining (Fig 2.3C) and Bayesian Inference (Fig 2.3D) trees all show strong genetic structuring and deep genetic divergence between the populations as indicated by long branch lengths. Each population is well supported with high bootstrap (BS) and posterior probabilities (PP) (BS=94%-100%, PP=0.97-1.0). There are manyincongruencies between the trees produced by the different analyses for the CO1 dataset, but there is poor support for the basal nodes that form the backbone of the tree. The Maximum Likelihood analysis has no support for the backbone of the tree while the other analyses have very weak support, providing little integrity for the structure of the trees.

16S Dataset

The Maximum Likelihood (Fig 2.4A), Maximum parsimony (Fig 2.4B), Neighbor-Joining (Fig 2.4C) and Bayesian Inference (Fig 2.4D) trees, like these from the CO1 dataset, all show strong genetic structuring and deep genetic divergence between the individual populations as indicated by long branch lengths. Each population is genetically distinct and is well supported by high bootstrap values and posterior probabilities (BS= 87%-100%, PP=0.96-1.0). The trees produced by the different analyses for the 16S dataset show many incongruencies and are less resolved than the CO1 dataset. The bootstrap and posterior probability support for the basal nodes that form the backbone of the trees are mostly absent from the analyses or extremely poor, giving no integrity to the structure of the tree.

Combined Mitochondrial dataset (CO1 and 16S)

The analyses for the combined mitochondrial dataset (Fig 2.5 A, B, C and D) like the analyses for the single markers show strong genetic divergence where each population is genetically distinct and well supported by high bootstrap values and posterior probabilities (BS=90%-100%, PP=1.0). The long branch lengths indicate that these populations have been genetically isolated for a long period of time. The combined trees are more resolved than the single marker trees and show stronger support for basal nodes. The Maximum Parsimony and Maximum Likelihood trees still show poor support for the backbone of the tree, however the Neighbor-Joining and

Bayesian Inference trees show stronger support. The node that groups the population of Kleinfontein Falls and all the populations of *P. nuptialis* has a good bootstrap support of 94% on the Neighbor-Joining tree and an excellent posterior probability support of 1.0 on the Bayesian tree. Similarly the node that groups the populations of Kleinfontein Falls, Wilge River Canyon and all the populations of *P. nuptialis* has good bootstrap and posterior probability support (BS=98%, PP=0.99). The GTR +G model was selected for the combined analyses. A summary consensus tree from the results of the four different analyses is shown in Figure 2.6.

28S Dataset

The 28S dataset proved to be highly conserved with no base pair differences across all populations sampled. Due to its uninformative nature it is not discussed further here. An alternative, more variable nuclear marker is needed in order to provide an independent test of the mtDNA results. Internal Transcribed Spacer (ITS) regions 1 and 2 have been used in other studies on gastropods to elucidate phylogeographic relationships (e.g. Mavarez *et al.* 2002, Pfenninger *et al.* 2003, Van Riel *et al.* 2005)

Isolation by Distance

The isolation by distance test produced a correlation coefficient (r) value of 0.565 (Z= 103.7361, $p < 0.1660$) for populations of *P. bowkeri* and a value of 0.271 (Z = 200.2714, $p< 0.5180$) for populations of *P. nuptialis*. These values reveal that geographic distance is not correlated with genetic distance. The two populations from Mountain Zebra National Park are both *P. nuptialis* and are geographically very close (only a few kilometres apart), but genetically they are very different (as seen on Figure 2.7), although not as distant as the other two examples.

Divergence Times

The relaxed clock based on the combined dataset with a mutation rate of 5%for the CO1 partition and 2% for the 16S partition yielded molecular divergence dates (given in millions of years) for each significant node as shown in Figure 2.8. This tree indicates that most of the divergence between populations took place between 11 and 6 MYA during the Miocene epoch. Support for the backbone of this tree is fairly weak with the exception of the first node (PP=1).

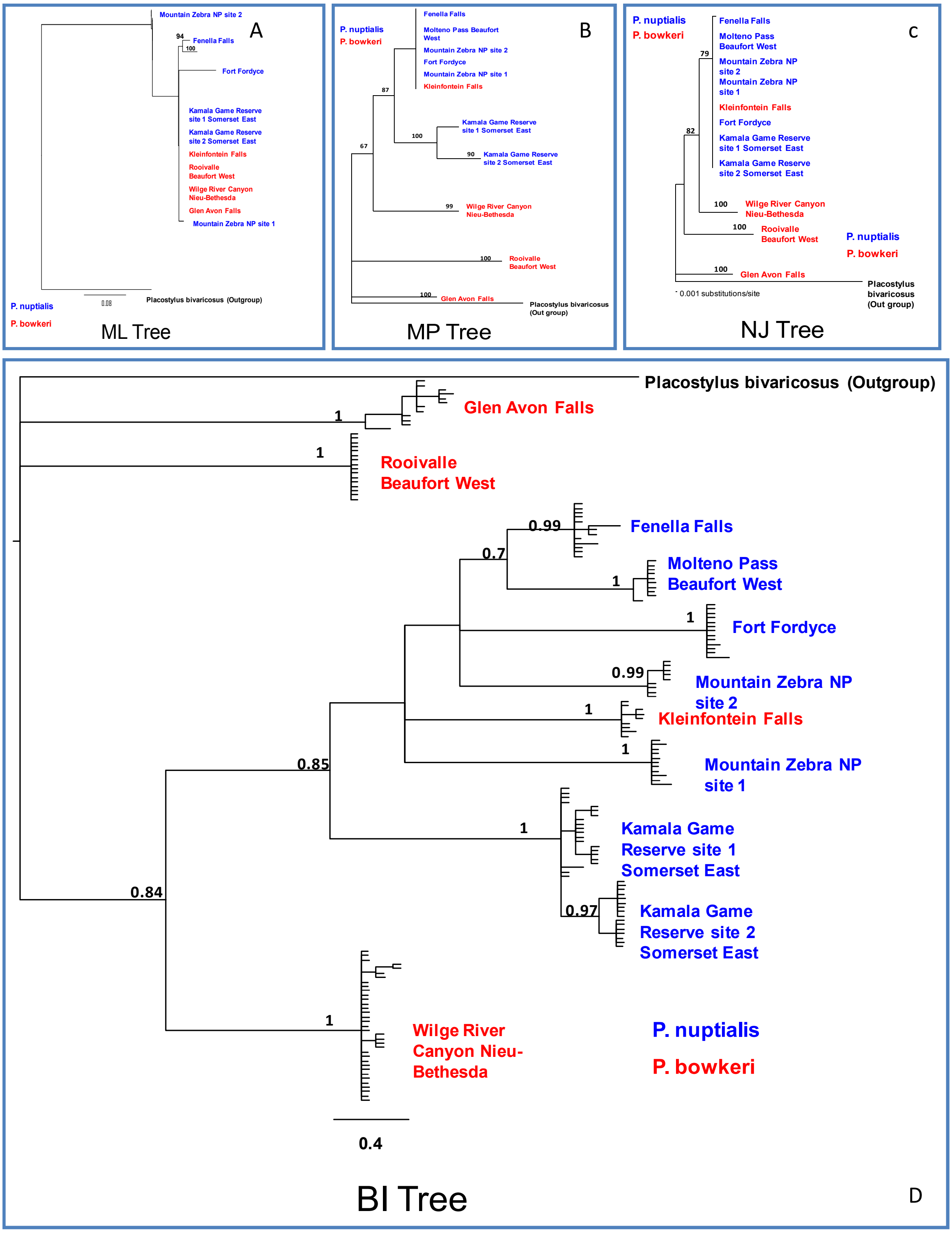

Figure 2.3: Maximum Likelihood (A), Maximum Parsimony (B), Neighbour-Joining (C) and Bayesian Inference (D) trees for the CO1 dataset for *P. bowkeri* (red) and *P. nuptialis* (blue) . Maximum Likelihood, Maximum Parsimony and Neighbour-Joining trees are reduced. Bootstrap and posterior probabilities are seen above the branches.

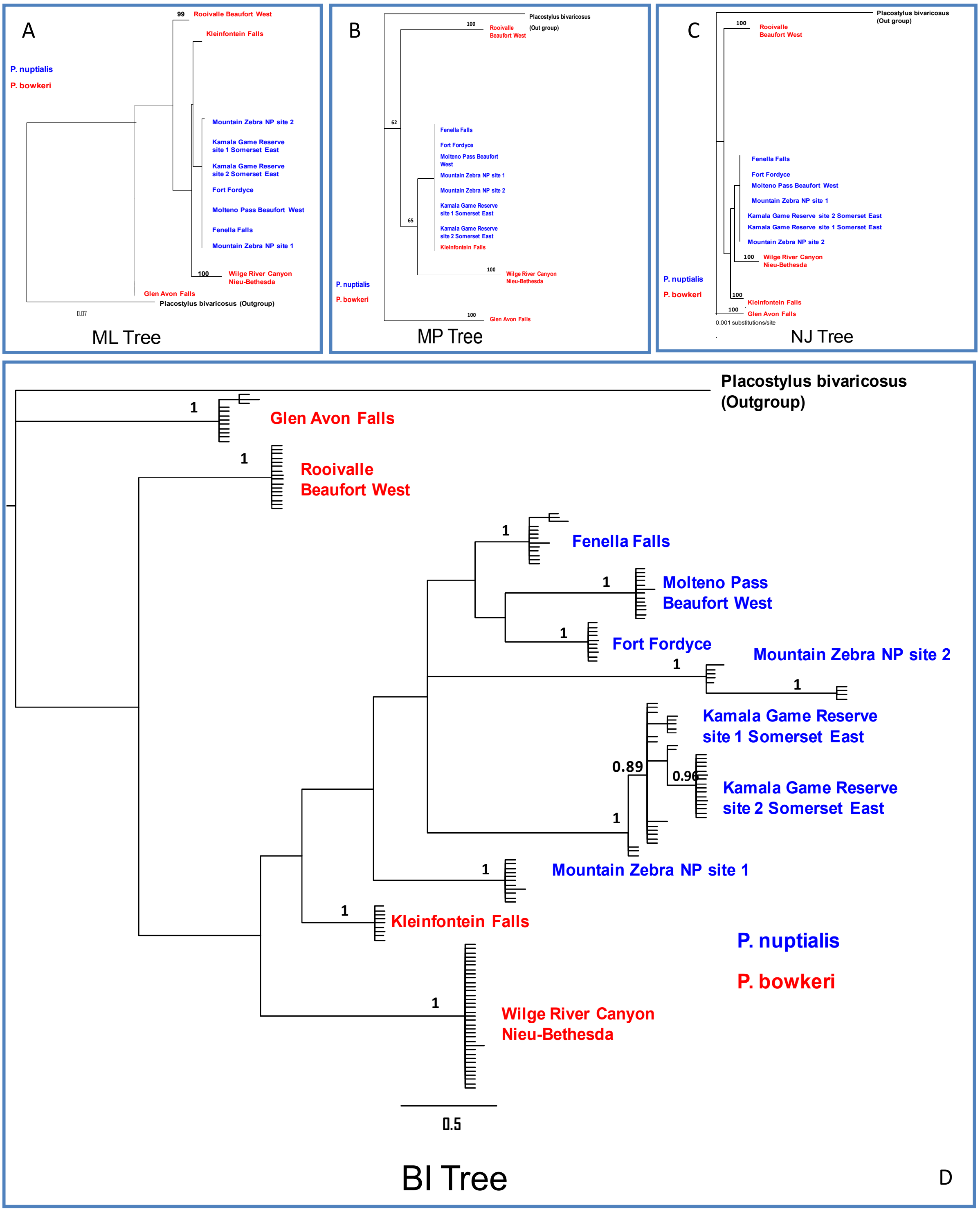

Figure 2.4: Maximum Likelihood (A), Maximum Parsimony (B), Neighbour-Joining (C) and Bayesian Inference (D) trees for the 16S dataset for *P. bowkeri* (red) and *P. nuptialis* (blue) . Maximum Likelihood, Maximum Parsimony and Neighbour-Joining trees are reduced. Bootstrap and posterior probabilities are seen above the branches.

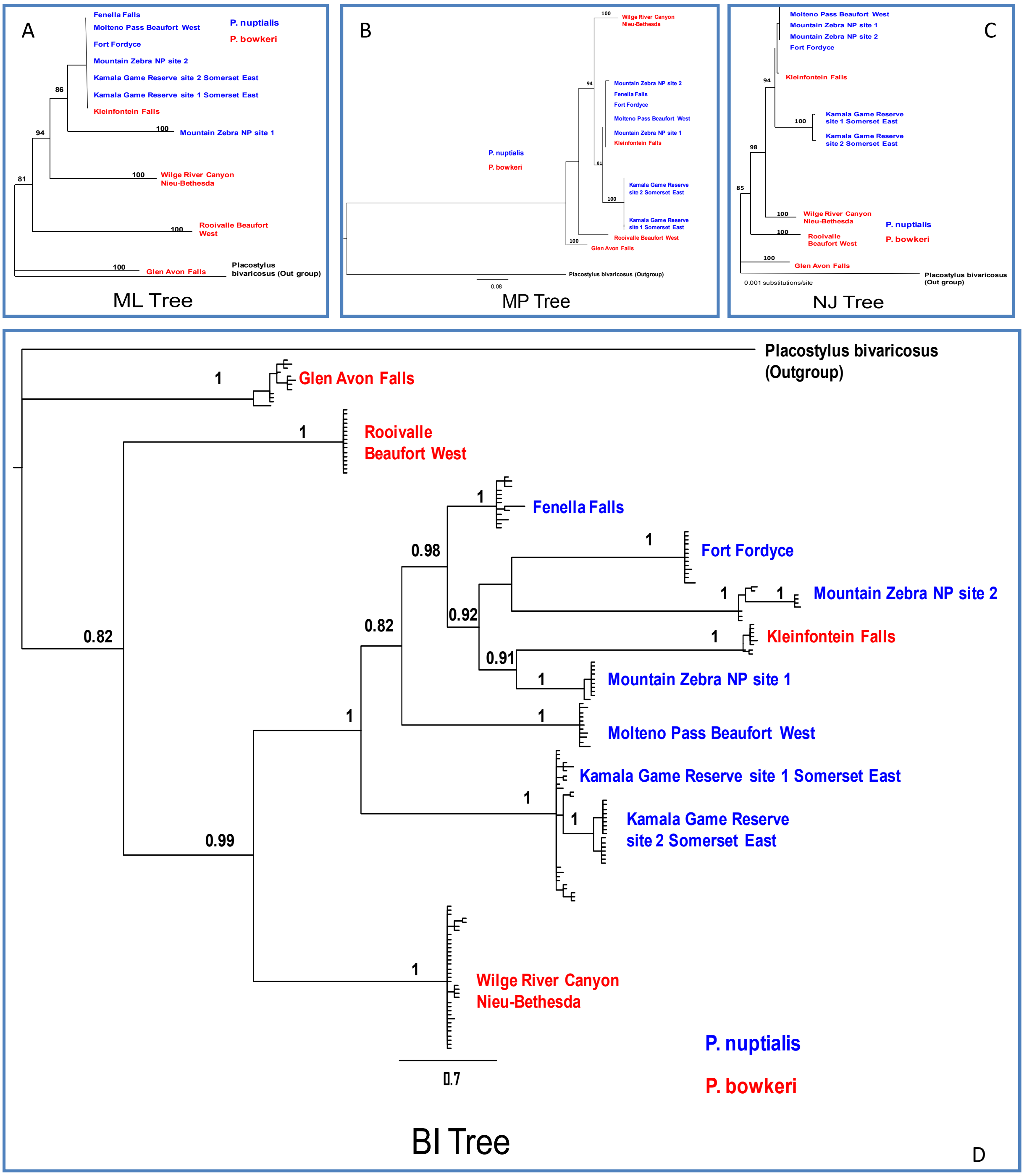

Figure 2.5: Maximum Likelihood (A), Maximum Parsimony (B), Neighbour-Joining (C) and Bayesian Inference (D) trees for the combined mitochondrial dataset for *P. bowkeri* (red) and *P. nuptialis* (blue) . Maximum Likelihood, Maximum Parsimony and Neighbour-Joining trees are reduced. Bootstrap and posterior probabilities are seen above the branches.

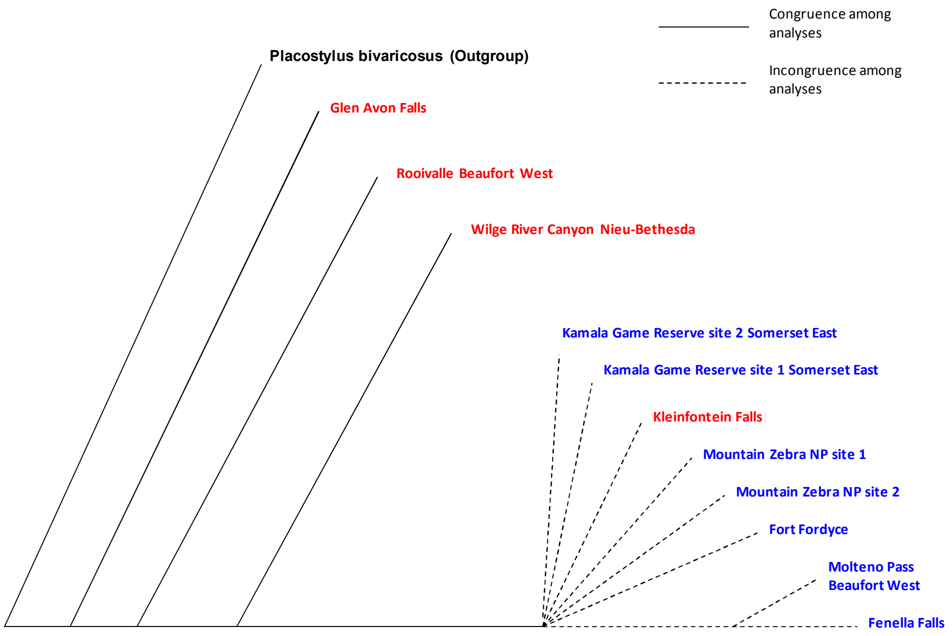

Figure 2.6: A consensus tree summarizing the four different analyses for the combined mitochondrial dataset.

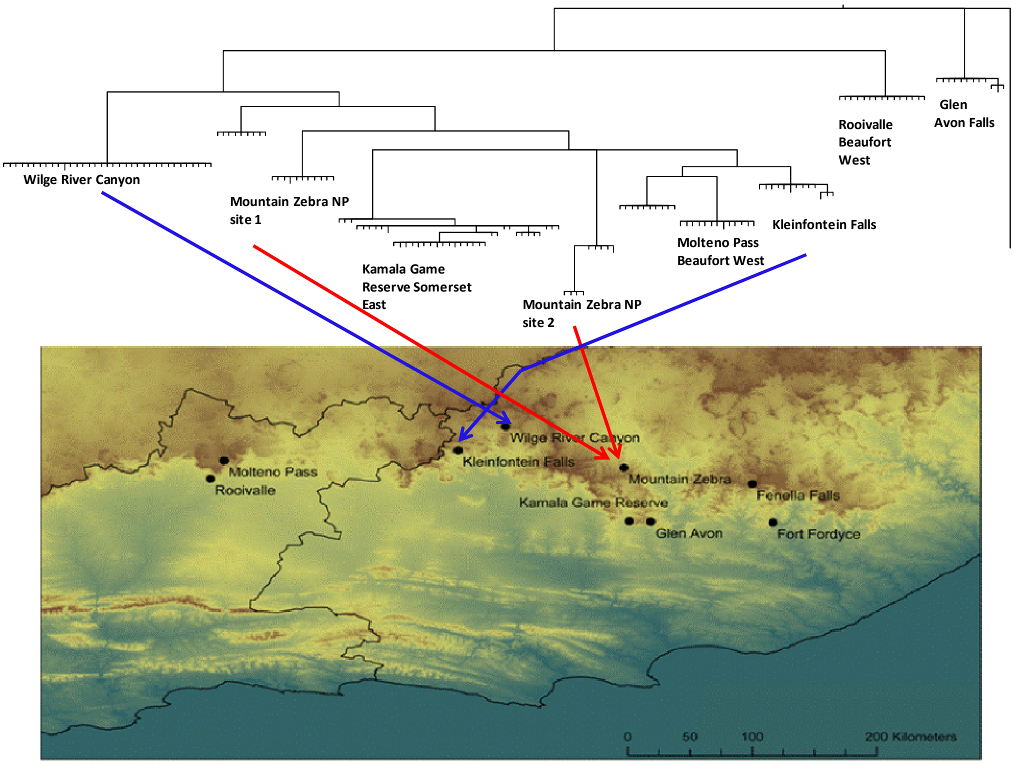

Figure 2.7: Isolation by distance, the relationship between geographic and genetic distance for populations of *Prestonella*. The coloured arrows indicate the location of a population on the Bayesian Inference tree of the combined mitochondrial dataset in relation to its geographic location in South Africa. This figure shows that there is no correlation between genetic and geographic distance. For example, the blue arrows show that the populations of Wilge River Canyon and Kleinfontein Falls (*P. bowkeri*) are genetically distant but geographically close.

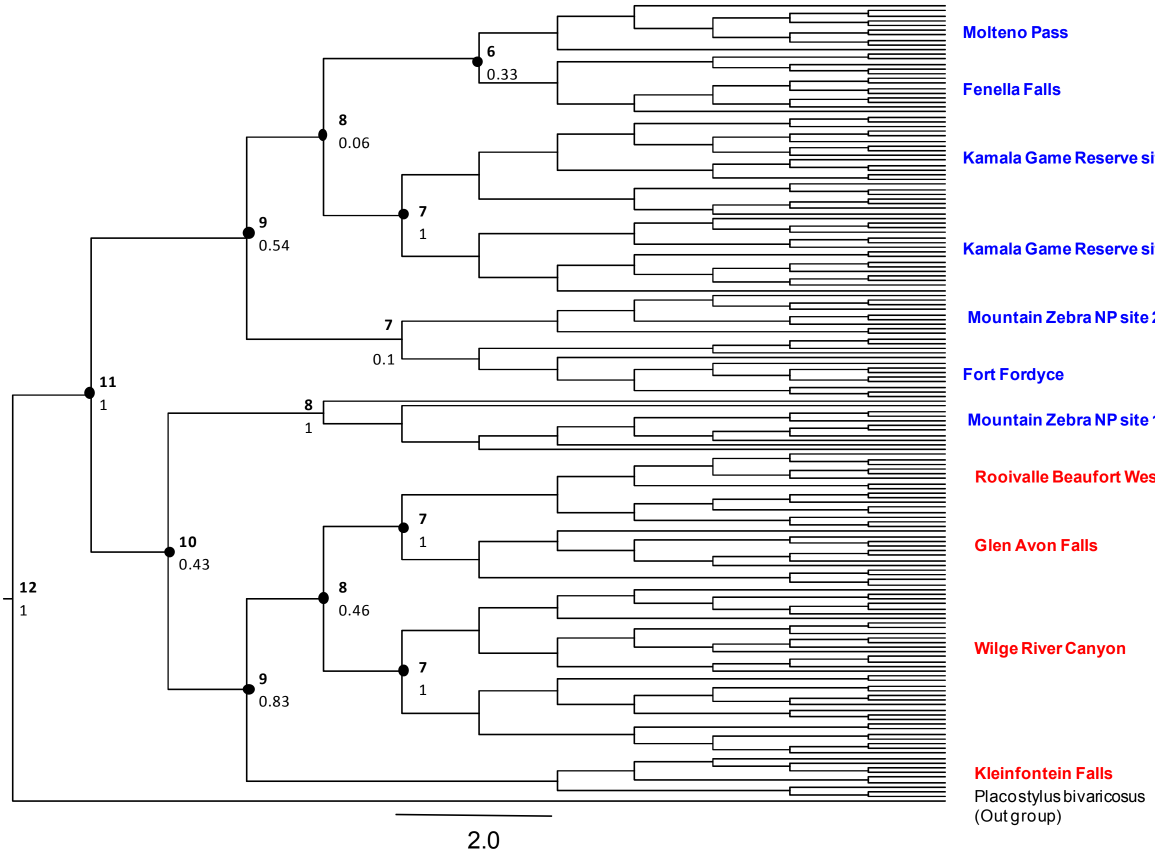

Figure 2.8: BEAST tree based on the combined mitochondrial dataset, sequence divergence rate of 5% per million years for the CO1 partition and 2% per million years for the 16S partition. Coloured dots are significant nodes and indicated when populations diverged from one another. Divergences times are indicated in bold above nodes (Node label scale = millions of years). . Posterior probabilities are indicated below nodes.

Genetic diversity

Table 2.5 shows the number of haplotypes, haplotype diversity (*h*) and pairwise nucleotide diversity (π) (collectively known as genetic diversity) for each population of *Prestonella* for the CO1, 16S and combined datasets. The average genetic diversity of all three datasets show that the most western populations including Rooivalle (Beaufort West) and Molteno Pass (Beaufort West) have the lowest number of haplotypes (mean: 1 and 2 respectively), lowest haplotype diversity (mean: 0 and 0.1) and the lowest nucleotide diversity (mean: 0 and 0.00016) followed by Wilge River Canyon and Mountain Zebra site 2b. Whereas, the more eastern populations of Fenella Falls, Fort Fordyce and Glen Avon Falls have the highest number of haplotypes (mean: 4, 5 and 5 respectively), the highest haplotypes diversity (0.661, 0.565 and 0.623) and some of the highest nucleotide diversities (0.00166, 0.00108 and 0.00174). These values indicate that the western populations are more genetically depauperate when compared to the eastern populations. F_{ST} (Fixation Index) or G_{ST} (coefficient of gene differentiation) estimates of population differentiation should decrease from a maximum of 1 (complete fixation of different alleles in each population) to 0 (no population subdivision). Pairwise F_{ST} comparisons (Table 2.6a and b) indicate that there was significant ($p<0.001$) genetic differentiation between allpopulations of *P. bowkeri* (CO1: 0.966-0.997 mean 0.986, 16S: 0.990-1.000 mean 0.995) and *P. nuptialis* (CO1: 0.972 -0.999 mean 0.984, 16S: 0.382-1.000 mean 0.995) and among all populations of *Prestonella* (CO1: 0.548 -0.999 mean 0.974, 16S: 0.382-1.000 mean 0.981).

Table 2.5: The number of haplotypes, haplotype diversity (h) and pairwise nucleotide diversity (π) for each population of *Prestonella,* for the CO1, 16S and combined datasets.

Population	No individuals	No haplotypes CO1	No haplotypes 16S	No. haplotypes MtDNA	Haplotype diversity (*h*) CO1	Haplotype diversity (*h*) 16S	Haplotype diversity (*h*) mtDNA	Nucleotide diversity (π) CO1	Nucleotide diversity (π) 16S	Nucleotide diversity (π) mtDNA
Rooivalle Beaufort West	16	1	1	1	0.000	0.000	0.000	0.00000	0.00000	0.00000
Fenella Falls	12	4	4	8	0.651	0.526	0.808	0.00193	0.00129	0.00176
Fort Fordyce	13	5	2	5	0.756	0.154	0.786	0.00191	0.00029	0.00104
Glen Avon Falls	12	5	3	3	0.718	0.439	0.712	0.00127	0.00108	0.00117
Kleinfontein Falls	9	3	1	3	0.639	0.000	0.670	0.00112	0.00000	0.00058
Molteno Pass	10	2	1	2	0.200	0.000	0.200	0.00032	0.00000	0.00017
Mountain Zebra 1	11	4	2	5	0.491	0.182	0.618	0.00113	0.00034	0.00077
Mountain Zebra 2a	5	2	2	3	0.200	0.400	0.700	0.00093	0.00075	0.00085
Mountain Zebra 2b	4	2	1	2	0.500	0.000	0.500	0.00078	0.00000	0.00042
Kamala Game Reserve 2	15	4	1	6	0.733	0.000	0.819	0.00117	0.00000	0.00106
Kamala Game Reserve 1a	6	2	4	3	0.333	0.659	0.730	0.00052	0.00171	0.00079
Kamala Game Reserve 1b	16	2	2	2	0.525	0.600	0.525	0.00163	0.00081	0.00089
Wilge River Canyon	35	4	2	5	0.392	0.057	0.440	0.00082	0.00045	0.00050

Values of Nei's (1982) coefficient of gene differentiation G_{ST} for among populations of *P. bowkeri* were 0.152-0.578 (mean 0.374) for CO1 (Table 2.6a) and 0.603 -1.000 (mean 0.784) for 16S (Table 2.6b). G_{ST} values among populations of *P. nuptialis* were 0.100-0.580 (mean 0.300) for CO1 (Table 2.6a) and 0.260 -1.000 (mean 0.578) for 16S (Table 2.6b). Among all populations GST values were 0.119-0.708 (mean 0.328) for CO1 and 0.260-1.000 (mean 0.646) for 16S.

Correlations between Genetic Diversity and Climatic Variables

Maps of the different climatic variables for the southern Great Escarpment are shown in Figures 2.10A, B and C. Out of the six different climatic variables selected for this study, three variables that best explain the differences in genetic diversity are presented her. These are the total annual precipitation (Figure 2.10A), coefficient of variation of annual precipitation (Figure 2.10B) and mean annual potential evaporation (Figure 2.10C). Figure 2.11 shows a positive, but weak correlation between total annual precipitation and haplotype diversity, the low R^2 (coefficient of determination) value suggests that the goodness of fit is weak (R^2= 0.435). Figure 2.12 shows a positive, but weak correlation between total annual precipitation and nucleotide diversity, the R^2 value suggests that the goodness of fit is weak (R^2= 0.523), but slightly better than the relationship between haplotype diversity. Figure 2.13 shows a negative correlation between the coefficient of variation of annual precipitation and haplotype diversity, the low R^2 value suggests that the goodness of fit is weak (R^2= 0.533). Figure 2.14 shows a negative correlation between the coefficient of variation of annual precipitation and nucleotide diversity. This correlation has a R^2 value of 0.718 which suggests that this climatic variable is a much better predictor of nucleotide diversity and that 71.8% of the variation in nucleotide diversity is explained by variation in rainfall reliability. This indicates that populations in areas that have less reliable rainfall are more genetically depauperate than those populations in areas with more reliable rainfall. Figure 2.15 shows a negative correlation (R^2= 0.577) between annual mean annual potential evaporation and haplotype diversity, the low the R^2 value suggests that the goodness of fit is weak Figure 2.16 shows a good negative correlation between annual mean potential evaporation and nucleotide diversity (R^2= 0.846), thus 84.6% of the variation in nucleotide diversity is explained by variation in mean annual potential evaporation. This suggests that mean annual potential evaporation is a good predictor of genetic diversity and implies that populations in areas that experience higher potential evaporation are more genetically depauperate than those populations that experience less potential evaporation. The above correlations used the combined mtDNA dataset which for the most part showed the best goodness of fit values. The CO1 dataset showed R^2 values between 0.655 and 0.720, while the 16S dataset showed lower R^2 values below 0.600. No correlation was seen between genetic diversity and relative humidity or temperature (max and min).

Table 2.6a: F_{ST} and G_{ST} values for CO1 among populations of *Prestonella*. F_{ST} values above the blank diagonal gap, G_{ST} values below the blank diagonal gap.

Populations	1	2	3	4	5	6	7	8	9	10	11	12	13
(1) Rooivalle Beaufort West		0.986	0.993	0.985	0.992	0.991	0.900	0.994	0.989	0.979	0.985	0.985	0.966
(2) Fenella Falls	0.608		0.999	0.813	0.986	0.985	0.985	0.988	0.984	0.978	0.980	0.978	0.994
(3) Fort Fordyce	0.476	0.219		0.548	0.992	0.991	0.991	0.995	0.991	0.982	0.986	0.983	0.998
(4) Glen Avon Falls	0.453	0.197	0.119		0.984	0.983	0.984	0.987	0.983	0.977	0.978	0.973	0.993
(5) Kleinfontein Falls	0.578	0.262	0.172	0.152		0.900	0.989	0.992	0.988	0.982	0.982	0.875	0.997
(6) Molteno Pass	0.847	0.449	0.333	0.312	0.415		0.988	0.990	0.986	0.982	0.981	0.973	0.996
(7) Mountain Zebra NP 1	0.646	0.324	0.288	0.207	0.279	0.481		0.990	0.987	0.981	0.984	0.977	0.995
(8) Mountain Zebra NP 2a	0.642	0.251	0.163	0.147	0.222	0.447	0.277		0.991	0.982	0.984	0.972	0.999
(9) Mountain Zebra NP 2b	0.708	0.255	0.177	0.162	0.243	0.486	0.295	0.100		0.979	0.981	0.981	0.996
(10) Kamala Game Reserve 1a	0.471	0.265	0.146	0.126	0.176	0.331	0.231	0.163	0.175		0.977	0.975	0.984
(11) Kamala Game Reserve 1b	0.785	0.350	0.245	0.277	0.324	0.580	0.384	0.388	0.413	0.220		0.973	0.993
(12) Kamala Game Reserve 2	0.584	0.309	0.221	0.199	0.256	0.431	0.318	0.237	0.247	0.228	0.329		0.992
(13) Wilge River Canyon	0.560	0.333	0.255	0.232	0.267	0.411	0.326	0.226	0.227	0.272	0.303	0.348	

Table 2.6b: F_{ST} and G_{ST} values for 16S among populations of *Prestonella*. F_{ST} values above the blank diagonal gap, G_{ST} values below the blank diagonal gap.

Populations	1	2	3	4	5	6	7	8	9	10	11	12	13
(1) Rooivalle Beaufort West		0.988	0.997	0.990	1.000	1.000	0.997	0.995	1.000	0.992	0.992	1.000	0.999
(2) Fenella Falls	0.607		0.977	0.940	0.983	0.965	0.979	0.985	0.991	0.973	0.972	0.986	0.986
(3) Fort Fordyce	0.868	0.492		0.991	0.996	0.995	0.993	0.992	0.998	0.987	0.987	0.997	0.996
(4) Glen Avon Falls	0.670	0.347	0.546		0.991	0.992	0.990	0.995	0.925	0.986	0.985	0.993	0.992
(5) Kleinfontein Falls	1.000	0.534	0.830	0.603		1.000	0.995	0.995	1.000	0.990	0.990	1.000	0.998
(6) Molteno Pass	1.000	0.549	0.838	0.617	1.000		0.996	0.994	1.000	0.989	0.989	1.000	0.999
(7) Mountain Zebra NP 1	0.855	0.465	0.712	0.512	0.818	0.826		0.992	0.997	0.986	0.986	0.997	0.996
(8) Mountain Zebra NP 2a	0.754	0.311	0.568	0.365	0.720	1.000	0.551		0.982	0.994	0.989	0.989	0.994
(9) Mountain Zebra NP 2b	1.000	0.405	0.740	0.476	1.000	0.727	0.728	0.640		0.993	0.993	1.000	0.990
(10) Kamala Game Reserve 1a	0.569	0.294	0.461	0.327	0.490	0.506	0.443	0.280	0.361		0.328	0.873	0.989
(11) Kamala Game Reserve 1b	0.630	0.260	0.479	0.305	0.483	0.593	0.459	0.327	0.482	0.087		0.987	0.983
(12) Kamala Game Reserve 2	1.000	0.607	0.869	0.670	1.000	1.000	0.855	0.754	1.000	0.569	0.556		0.999
(13) Wilge River Canyon	0.910	0.604	0.809	0.649	0.874	0.883	0.786	0.632	0.779	0.582	0.588	0.914	

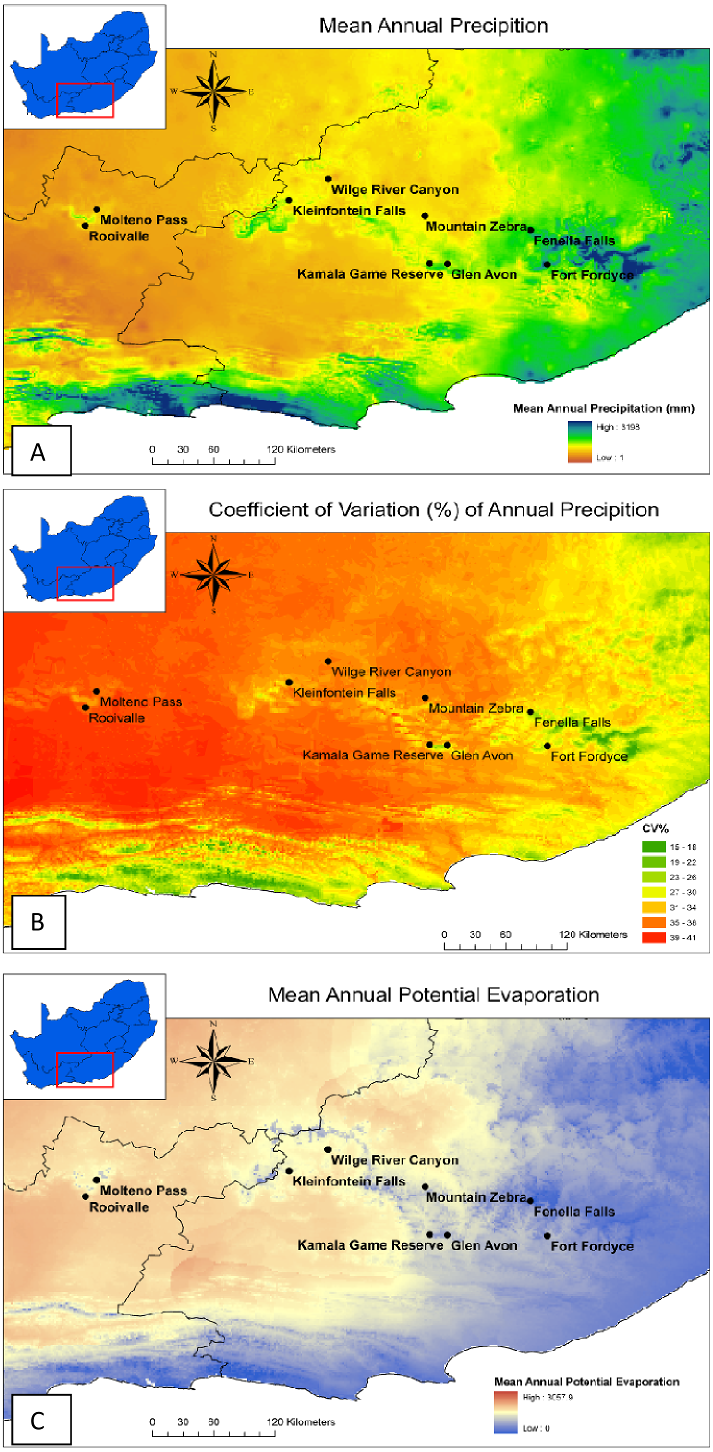

Figure 2.9: GIS maps of the three climatic variables used in this study for the southern Great Escarpment. Data was taken from the South African Atlas of Agrohydrology and Climatology (Schultz 1997). A: mean annual precipitation, B: coefficient of variation of annual precipitation, C: mean annual potential evaporation

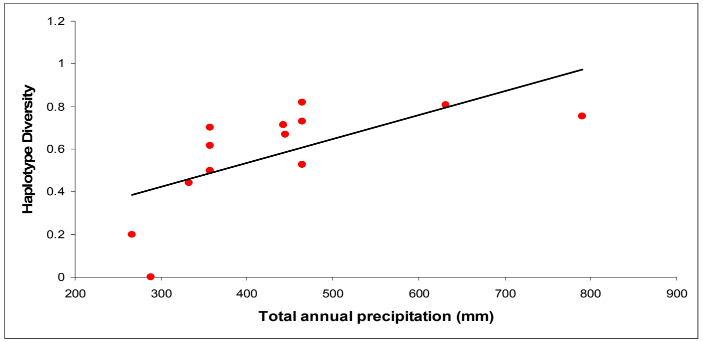

Figure 2.10: Correlation between total annual precipitation and haplotype diversity (*h*) for the combined mtDNA dataset (R^2= 0.435).

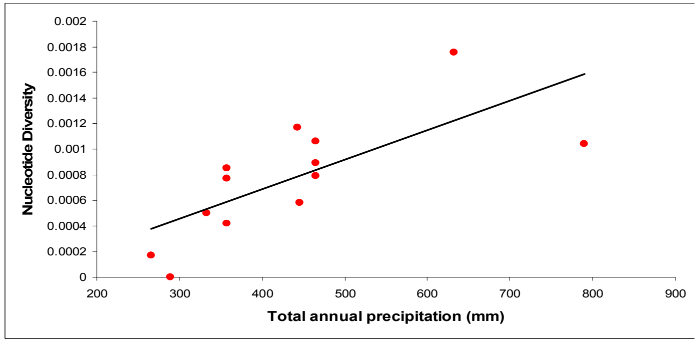

Figure 2.11: Correlation between total annual precipitation and nucleotide diversity (π) for the combined mtDNA dataset (R^2= 0.523).

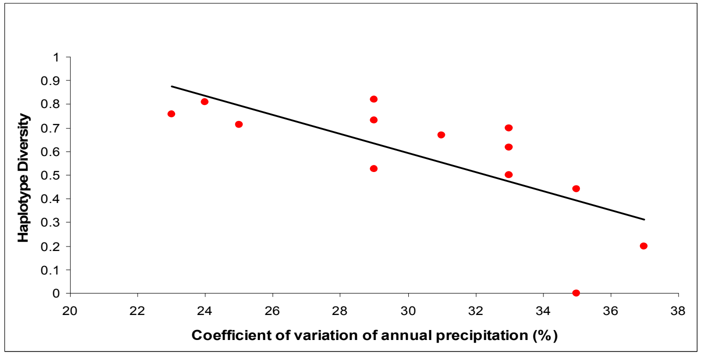

Figure 2.12: Correlation between coefficient of variation of annual precipitation and haplotype diversity for the combined mtDNA dataset (R^2= 0.533).

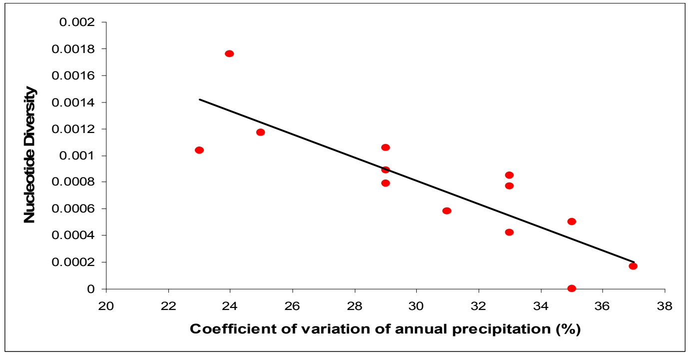

Figure 2.13: Correlation between coefficient of variation of annual precipitation and nucleotide diversity for the combined mtDNA dataset (R^2= 0.718).

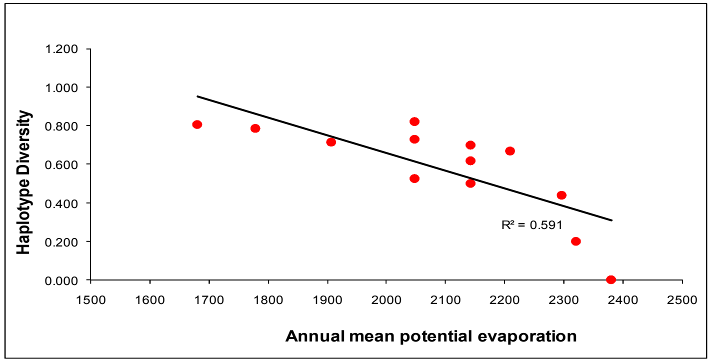

Figure 2.14: Correlation between mean annual potential evaporation and haplotype diversity for the combined mtDNA dataset (R^2= 0.577).

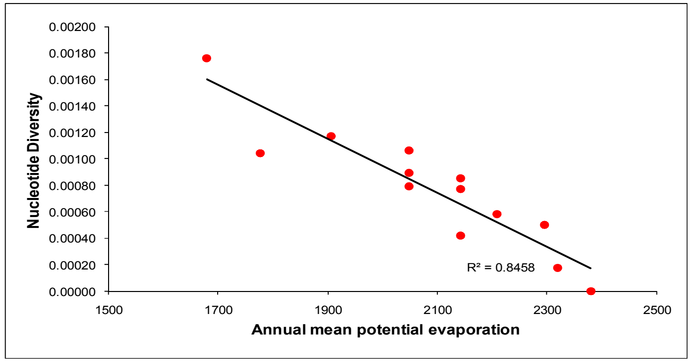

Figure 2.15: Correlation between mean annual potential evaporation and nucleotide diversity for the combined mtDNA dataset (R^2= 0.846).

Discussion

Taxonomic implications

The 16S dataset suggests that there may be two separate lineages represented by the two species, however, this is weakly supported and renders *P. bowkeri* paraphyletic. The CO1 dataset and the combined mitochondrial datasets show that all the *P. bowkeri* populations except for Kleinfontein Falls are basal to a well supported *P. nuptialis* clade. The phylogenetic analyses thus indicate that there is no clear dichotomy between the two species, which suggests that the current taxonomy is uncertain, or that the characters on which identifications are based on such as the morphology of the shell, protoconch, radula and reproductive tract may well be plesiomorphic in the case of *P. bowkeri*. There is no doubt that the Kleinfontein Falls population is morphologically *P. bowkeri,* thus there may be something else at play such as retention of ancestral polymorphisms and the lack of reciprocal monophyly. Sauer and Hausdorf (2010) suggest that ancestral polymorphisms are a frequent cause for nonmonophyly of species with a strongly subdivided population structure in gene trees. Reciprocal monophyly was defined in the context of a model of mitochondrial evolution that explains how incongruence between species trees and gene tress may arise (Avise 2001). Phylogenetic analyses of mtDNA nucleotide sequences from individuals representing early periods in the history of sister species may result in trees that suggest paraphyly for one or both species and only after sufficient complementary haplotype extinction will species become monophyletic with respect to each other through a process known as lineage sorting (Kizirian and Donnelley 2004). It is possible that retention of ancestral polymorphisms and the lack of reciprocal monophyly may explain why the Kleinfontein Falls populations groups with the *P. nuptialis* populations, and why *P. bowkeri* is paraphyletic. Although there is some support for a two species concept it should be treated with caution.

The Glen Avon population could possibly represent a third species (though it will always remain the true *P. bowkeri* as this is the type locality), because it is always the most basal population on the phylogeny and is frequently part of an unresolved polytomy. If the Glen Avon population is considered as a separate species, then the Rooivalle and Wilge River Canyon populations may need to be considered separate species as well.

Mean values of Nei's coefficient of gene differentiation G_{ST} (Nei 1982) between populations of *P. bowkeri* were (CO1: 0.374 , 16S: 0.784) and between populations of *P. nuptialis* were (CO1: 0.300, 16S: 0.578), higher than those seen within other terrestrial snail species (e.g. *Succinea caduca* Holland and Cowie 2007) and fresh water species (e.g. *Bythinella hansboetersi* Falniowski *et al.* 2009), thus genetic markers point to divergences between populations (within so-called species) that are possibly more reminiscent of the divergences normally observed between species. In this case, we suggest that confinement to isolated mesic environments on mountain slopes has promoted extraordinary intraspecific genetic divergence, which is contradictory to a two-species model. This raises the question whether there are in fact two species in this genus, or whether these lineages are divergent enough to be considered as separate species. A Bayesian analysis using the combined mtDNA data set without an out group and with mid-point rooting shows two clades, one clade includes all the *P. nuptialis* species together with one of the *P. bowkeri* populations (Kleinfontein Falls) and the other clade includes all the other *P. bowkeri* populations. This suggests that there are two distinct clades and support for a two species model. Although the Kleinfontein population has been classified as *P. bowkeri* it may need revision.There is much uncertainty concerning the two species model and it may be better to consider each population as an evolutionarily significant unit (ESU). An ESU is a distinct population within a species that is genetically unique and warrants its own management and conservation agenda. Much of the apparent uncertainly here could result from the fact that we only have mtDNA and the use of nuclear markers may resolve this issue.

***Prestonella* as a series of genetically distinct populations**

All of the phylogeographic analyses indicate strong genetic structuring and high levels of genetic divergence. Likewise, mean F_{ST} values among all populations of *P. nuptialis* and among all populations of *P. bowkeri* are extremely high (CO1: 0.974, 16S: 0.981) and significant ($p<0.001$) suggesting substantial structure, fixed differences among populations and strong genetic partitioning. Similar F_{ST} values have been found for endemic island snail species such as *Succinea caduca* (Holland and Cowie 2007) and *Mandarina* species (Davidson and Chiba 2008).

Thomaz *et al.* (1996) suggested three reasons why terrestrial snails may exhibit high levels of genetic diversity, including: (1) a rapid mtDNA mutation rate; (2) very old isolation events; and

(3) a population structure conducive to high variation. They concluded that the third hypothesis is most likely due to the fact that terrestrial snails usually have several populations with low dispersal and migration rates. In the case of *P. bowkeri* and *P. nuptialis*, numerous populations with low dispersal and migration rates are evident suggesting that hypothesis three is appropriate. However, it is likely that hypothesis two may also be at play. If populations experienced very old isolation events which prevented gene flow over a very long period of time, each population will evolve independently, allowing unique mutations to accumulate in individual populations, hence the large genetic differences seen between populations. Climatic transitions and habitat fragmentation act as barriers to gene flow and are potential agents for vicariant speciation (as in Tolley *et al.* 2010a). It is plausible to link the genetic differentiation among the populations to fragmentation due to historical climate changes, as suggested for other southern African fauna (e.g. chameleons: Tolley *et al.* 2006, cicadas: Price *et al.* 2007, southern rock agama: Swart *et al.* 2009, dwarf mountain toads: Tolley *et al.* 2010b).

A study on Hawaiian tree snails (*Achatinella mustelina*) has shown that long-term isolation of subpopulations within a species may be reflected in high levels of sequence divergence (Thacker and Hadfield 2000). Dispersal may have been common in populations that were historically widespread where suitable habitat would have been more extensive and continuous; subsequently populations have become isolated due to habitat fragmentation (Holland and Hadfield 2002). One may speculate that *Prestonella* is historically similar to *A. mustelina*, in that it too may have once been widespread throughout the Great Escarpment and that fragmentation of suitable habitat in association with habitat specialisation of the snails has led to genetic isolation of populations and consequentially high levels of genetic divergence. Large lowland gaps that separate inselbergs (or mountain blocks) provide significant barriers to gene flow (as seen in *Capensibufo:* Tolley *et al.* 2010b). In fact, even small distances that separate waterfalls and montane seepages are sufficient enough to prevent gene flow, as seen for the two populations of *P. nuptialis* at Mountain Zebra (sites 1 and 2) that are only 1.69 kilometres apart. Even within one site (Mountain Zebra site 2), snails from downstream (site 2b) are a few hundred meters apart yet are genetically different from snails upstream (site 2a). This clearly demonstrates the fine scale of population structure in these snails.

Species in mountain regions are typically restricted to relatively narrow and well-delineated altitudinal bands and as a result most montane populations are highly fragmented (Jump *et al.*

2009). Limited dispersal within fragmented mountain habitats can strongly influence phylogeographical patterns, as seen in the amphibian genus *Capensibufo* (Tolley *et al.* 2010b). The same processes may be reflected in the distribution of other southern African mountain-dwelling taxa such as the elephant shrew, *Elephantulus edwardii* (Smit *et al.* 2007) and the red rock rabbit, *Pronolagus rupestris* (Matthee and Robinson 1996). The habitat specific lifestyle of strict montane endemics makes these species prone to poor dispersal, resulting in strong genetic structuring. Terrestrial snails in general have low vagility and are often restricted by their particular habitat requirements (particularly so in the case of *Prestonella*) which make them poor dispersers (e.g. Schilthuizen and Lombaerts 1994, Pfenninger *et al.* 1996, Holland and Hadfield 2004, Ketmaier *et al.* 2006). The strong genetic divergence seen among populations of *Prestonella* is attributed to the fact that *Prestonella* is both a montane endemic, a terrestrial snail and a habitat specialist, which makes this genus particularly restricted by low vagility and limited dispersal.

Isolation-by-distance

The model of isolation-by-distance is recognized for many groups of land snails (Madec *et al.* 2003) and is a common pattern found for example in *Discus macclintocki* (Ross 1999), *Trochoidea geyeri* (Pfenninger *et al.* 1996) and *Marmorana* spp. (Fiorentino *et al.* 2008). This pattern however, was not found for *Prestonella* species. The isolation-by-distance analysis showed that the geographic pattern of genetic population structure does not conform to a stepwise or island-hopping model of haplotype distributions (Holland and Cowie 2007) The lack of congruence between geographical and genetic patterns is also seen for other land snail species such as *Candidula unifasciata* (Pfenninger and Magnin 2001), *Arianta arbustorum* (Haase *et al.* 2003) and *Succinea caduca* (Holland and Cowie 2007). Incongruence between geographical and genetic patterns is often attributed to species with high dispersal abilities (Hastings and Harrison 1994), but this is highly unlikely in this case due to the low vagility and dispersal powers of land snails.

The results can be interpreted in three ways. Firstly, cladogenesis could have occurred either simultaneously or in rapid succession (Hoelzer and Melnick 1994). Price *et al.* (2007) suggest that simultaneous and near-simultaneous isolation may be responsible for the patterns seen for montane clades of the cicada *Platypleura stridula* in South Africa. Simultaneous isolation results in a polychotomy where practically no opportunity exists for character-state changes to occur, or in which there is no clade structure to resolve (Page and Phelps 1994). A polychotomy may be seen for the clade that incorporates the *P. nuptialis* populations and the Kleinfontein Falls population (mtDNA dataset) and for the other *P. bowkeri* populations, however, the topology in general shows a fair amount of structure (although not well supported in the single gene trees) which suggests that simultaneous isolation may not be entirely responsible for the lack of congruence between genetic and geographic patterns. It has been argued that explanations involving simultaneous differentiation should be considered only as a last resort (DeSalle *et al.* 1994).

Secondly, the lack of congruence between geographic and genetic patterns may be due to gene saturation where the rapid evolution of mtDNA has reduced the resolution of deeper branches (Avise 2001). This is unlikely however, because there is no evidence of saturation in either gene (Appendix 3).

Thirdly, the results could be explained by haplotype fixation and we suggest that this is the most likely explanation. Early fixation of mtDNA haplotypes during the fragmentation of a haplotypically diverse ancestral populations might have allowed the persistence of several haplotypes in *Prestonella* populations (Prentice *et al.* 2003). Isolated populations may also have experienced one or more bottlenecks due to historic habitat fragmentation and climate change, which ultimately reduces long-term effective population size and genetic variation due to drift. Although genetic drift reduces genetic variability and ultimately leads to fixation in small populations, fixation of different haplotypes in different isolated populations may potentially serve to maintain haplotypes within a species or metapopulation, provided the populations are persistent.

Molecular dating and biogeographic scenarios

It is not possible to undertake a phylogeographic study of a South African taxon without an understanding the palaeoclimatic and ecological history of the region. The relaxed Bayesian clock estimates suggest that populations diverged from one another during the mid-late Miocene (11-6MYA)

The critical period for *Prestonella* diversification began during the Miocene, and is coincident with a period of increasing aridity in southern Africa that has impacted on much of its biota (examples of this are discussed below). We thus argue that divergence during the Miocene is probable, and invoke an explanation rooted in vicariance/ fragmentation driven by climate change.

Climatic changes during the Cenozoic in the Southern Hemisphere were relatively complex, with large scale fluctuations between warm mesic periods during the Palaeocene and Early Miocene, and warm dry periods during the Oligocene and Middle Miocene to the present day (Deconto and Pollard 2003). The Early Oligocene had climatic conditions similar to present day conditions, with low sea-surface temperatures and vast Antarctic glaciations (Udeze and Oboh-Ikuenobe 2005). The instability of the climate during the Miocene led to major habitat changes in southern Africa (deMenocal 2004). The early Miocene had subtropical and mesic conditions (Udeze and Oboh-Ikuenobe 2005) and consequentially a mixture of forest vegetation dominated southern Africa. During this period subtropical forest species would have flourished (e.g. dwarf chameleons; Tolley *et al.* 2006). It is possible that the climate during the late Oligocene and early Miocene would have provided more mesic environments along the southern Great Escarpment that would have been conducive to a widespread ancestor of *Prestonella* in this region or to two widespread ancestors if the two species concept is correct.

The modern trend of seasonal aridity was triggered during the Miocene by an increase in the strength of the south Atlantic high-pressure cell and glaciations in the Antarctic 14 MYA (Zachos *et al.* 2001). These changes have been linked to the expansion of the savanna biome, and consequent vertebrate radiations. For example, the major clades of the Mimosoideae (Leguminosae), a lineage that includes all of the African *Acacia* species originated in the late Oligocene-early Miocene (about 25 MYA; Bouchenak-Khelladi *et al.* 2010). The transitions from closed to open habitats occurred during the Miocene for at least four mimosoid lineages and are thought to be a response to increased seasonality leading to fire climates and drying trends, which gave rise to open habitats, such as savannas (Bouchenak-Khelladi *et al.* 2010). Co-incidentally bovid subfamilies experienced rapid radiation 23 MYA near the Oligocene/Miocene boundary, where several speciation events occurred following immigration into Africa due to adaptations to grassland savannah (Matthee and Davis 2001).

Further increases in aridity in Africa near 2.8 MYA, 1.7 MYA, and 1.0 MYA, are concurrent with the intensification of high-latitude glacial cycles (deMenocal 2004). The Early to Late Pliocene was warm-temperate with several dry intervals (Udeze and Oboh-Ikuenobe 2005), the wind-driven Benguela Current up-welling system gradually intensified and the sea-surface temperatures decreased markedly (deMenocal 2004). Aridification in southern Africa during the Late Pliocene dramatically increased due to the closure of the Panama seaway (Marlow *et al.* 2000, deMenocal 2004). However the timing and consequences of the emergence of the Isthmus of Panama, which closed the seaway, remain controversial (Keigwin 1982, Keller *et al.* 1989, Duque-Caro 1990, Maier-Reiner *et al.* 1990, Collins *et al.*1996). Aridification shifts during the Mid-Pliocene (3.2- 2.6 MYA) are thought to have favoured the evolution of arid adapted fauna and possibly influenced the evolution of early hominids (deMenocal 1995, Vrba 1995, Bobe *et al.* 2002). Changes in African vegetation during the Pleistocene were primarily regulated by changes in tropical and south Atlantic sea-surface temperatures (deMenocal 2004). It is likely that savannah and forest species in more temperate regions of Africa have been repeatedly subjected to habitat fragmentation due to cyclical habitat contraction of moist habitats during drier periods and subsequent habitat expansion during wetter periods (Kingdon 1990), consequently many species would have been confined to refugia during these periods.

These climatic fluctuations are predominantly responsible for the speciation and radiation of many animal taxa in southern Africa. Although radiations started as early as the Miocene in southern Africa (e.g. dwarf chameleons: Tolley *et al.* 2006, sand lizards: Makokha *et al.* 2007), most modern species evolved during the Pliocene-Pleistocene, and environmental theories of African faunal evolution suggest that significant evolutionary changes occurred during the Pliocene and Pleistocene, as a consequence of climate change or variation in climate (deMenocal 2004). However, the extent of climate change during the Pliocene and Pleistocene in southern Africa has been highly debated. Tyson (1999), Richardson *et al.* (2001) and Cowling *et al.* (2005) suggest rapid and dramatic climate shifts; whereas Weaver *et al.* (1998), Meadows and Baxter (1999), Dynesius and Jansson (2000) and Barraclough (2006) advocate that the Cape region was relatively stable. The diversification of several animal clades (sand lizards, southern rock agama, dwarf chameleons) in South Africa corresponds well to global cooling and habitat shifts during the Pliocene and Pleistocene (Makokha *et al.* 2007, Swart *et al.* 2009, Tolley *et al.* 2006). Climatic changes during the Pleistocene resulted in fluctuations in the abundance of animal species (e.g. rodents: Thackeray 1987) and several faunal species emerged during the Pleistocene including the southern African scrub hare (*Lepus saxatilis*), several clades of dwarf chameleons (*Bradypodion*), a few lineages of cicadas (*Platypleura*), at least four clades of the rock elephant shrew (*Elephantulus edwardii*), several major lineages of

Eastern Cape redfin (*Pseudobarbus afer*) and fiery redfin (*Pseudobarbus phlegethon*) and a few clades of southern rock agama (*Agama atra*) (Kryger *et al.* 2004, Tolley *et al.* 2006, Price *et al.* 2007, Smit *et al.* 2007, Swartz *et al.* 2007, Swart *et al.* 2009), almost every southern African animal taxon for which a phylogeographic study has been undertaken.

This increasing aridity reduced the extent of mesic environments along the Great Escarpment which most likely resulted in the reduction of suitable habitat for *Prestonella* species, leading to fragmentation and isolation of populations. If aridification is an important driver of ancestral fragmentation and diversification in *Prestonella*, then this aridification can still be expected to be playing a role in present day and future population processes that affect genetic diversity.
However, it must be stressed that although this hypothesis best fits our data, these results should be viewed with caution because the rates used are at best imprecise estimates of mutation rates in snails, and these rates are known to be extremely variable across lineages.

Genetic diversity in populations of *Prestonella*

Genetic diversity is high within several populations of *Prestonella* with 40 haplotypes for CO1 and 26 haplotypes for 16S spread across 13 populations (164 individuals). Ross (1999) has shown extremely high levels of genetic diversity within populations of *Discus macclintocki* (also a habitat specialist) with 40 haplotypes for 16S spread across 10 populations (173 individuals). If we look at *Prestonella* species, *P. nuptialis* has high genetic diversity with 35 haplotypes for CO1 and 23 haplotypes for 16S spread across 9 populations (93 individuals), whereas *P. bowkeri* has lower genetic diversity with 13 haplotypes for CO1 and 7 haplotypes for 16S spread across 4 populations (72 individuals). Although total genetic diversity within *Prestonella* species are not as high as that found for *Discus macclintocki*, several populations of *P. bowkeri* and *P. nuptialis* including Fenella Falls (*h*= 0.52), Glen Avon Falls (*h*= 0.43), Mountain Zebra site 2a (*h*= 0.40) and Kamala Game Reserve sites 1a and 1b (*h*= mean: 0.63) show similar haplotype diversity for 16S to that of the average haplotype diversity of *Discus macclintocki* populations (*h*= mean: 0.46

Genetic diversity among populations of *P. nuptialis* and *P. bowkeri* vary greatly from high levels of genetic diversity to extremely low levels of genetic diversity, where several populations are only represented by one haplotype (one for CO1 and five for 16S). In small populations, many factors influence the amount of genetic variation and distribution within a species, including mutation rates, dispersal and isolation (Ross 1999). Population size and habitat area may also influence within population genetic diversity. In larger populations alleles are less likely to drift to fixation and larger habitats have the potential to hold larger populations (Ross 1999). However it is unknown whether population size and habitat area has influenced the genetic diversity within populations of *P. nuptialis* and *P. bowkeri.* Another factor that may affect genetic diversity within populations of snail is self-fertilization which is absent in many other animal species. Selfing is expected to reduce genetic diversity within populations (Charlesworth *et al.* 1993). However, there is a complete lack of information on the reproductive biology of *P. nuptialis* and *P. bowkeri*.

Our results show that genetically depauperate populations are found in areas that have lower annual rainfall, less reliable rainfall and higher potential evaporation, all factors associated with a drier, less mesic environment that increases the chances of a population bottleneck. Nevo (2009) discovered that genetic diversity (both allozymes and DNA) was generally higher on the more heterogeneous and stressful region of the Evolution Canyons in Israel in 11 out of 16 test organisms (14 species) including beetle, snail, fly and earthworm species. Nevo's (2009) results conform to the niche width variation hypothesis (Van Valen 1965) or more generally the environmental theory of genetic diversity predicting positive correlation between stress and genetic diversity (Nevo 1988a, 1988b, 2001, 2006). In contrast, our results show the opposite, and suggest that a shift towards a more stressful arid environment has resulted in a decrease in haplotype and genetic diversity. Small populations are especially influenced by increases in climate stress due to climate change because of the associated effects of inbreeding depression and genetic drift. The effect of genetic drift and inbreeding depression on small populations is manifold; the loss of genetic variability can limit the ability of an organism to adapt to a changing environment (limit natural selection) which may increase extinction risk or genetic drift can lead to rapid evolution possibly enhanced by a selective pressure (Van Riel *et al.* 2001). The latter is important for conservation biology because small and isolated populations often possess unique genetic diversity that is an important constituent of biodiversity. Genetic diversity levels are thought to be directly linked to population fitness (Reed and Frankham 2003), which means decreases in haplotype and nucleotide diversity could result in reduced fitness in small populations. Populations with reduced fitness are susceptible to environmental changes (such as climate change) and have increased extinction risks (Van Riel *et al.* 2001). Fixation of haplotypes and decreased diversity within populations of *Prestonella* may be due to small-scale, possibly seasonal bottlenecks in association with poor short distance dispersal (as seen for Hawaiian Amber snail: Holland and Cowie 2007).

Conclusion

Using Prestonella as a representative and model organism, it is hypothesised that historical climate change has affected the amount and distribution of genetic diversity of numerous taxa across the Great Escarpment since the Miocene. This has serious future implications for the survival of not just *Prestonella*, but many other taxa too. Montane and alpine ecosystems are especially affected by climate change, because most montane species are already restricted to near-summit areas and cannot migrate higher to escape increasing temperatures. Evidence suggests that many montane species have already responded to global warming by dispersing to higher altitudes (Parmesan 2006, Jump *et al.* 2009). This trend has been recognized mostly for woody plants at their upper altitudinal range boundaries, where Increasing temperatures are driving rapid upward range shifts of montane species (Jump *et al.* 2009). However upward dispersal may be hampered by declines in the quantity and quality of suitable habitat, which may be particularly acute for species with narrow vertical distributions (Colwell *et al.* 2008). With predicted increase in global temperatures, climate change in South Africa is likely to result in range contraction and an eastward range shift for many species in the drier central and western areas (Erasmus *et al.* 2002) and regions along the Great Escarpment are likely to become more arid. However According to South Africa's second national communication under the United Nations framework convention on climate change (2011) surface air temperatures over land have changed with statistical significance since 1950 across South Africa and the coast is likely to warm by around 1-2ºC, and the interior by around 2-3ºC by 2050. Established but incomplete GCM projections for the winter rainfall region consistently suggest future rainfall decreases, while summer rainfall region projections deviate less from present climate. There will be an increases in the duration of dry spells. *Prestonella* species found living on inselbergs along the Great Escarpment are already restricted to site specific watercourses and seepages. An increase in the periods between stream flow, and increasing rainfall variability and Mean Annual Potential Evaporation are likely to have an adverse affect on species living in these habitats (Herbert 2007), resulting in further bottlenecks and possibly local extinction. Decreases in genetic diversity due climate change have been found for other invertebrate taxa. For example, Rodriguez-Trelles and Rodriguez (1998) found that chromosomal diversity in *Drosophila* has decreased in response to climate warming over the past 16 years. With limited vagility and specific habitat requirements it is unlikely that *Prestonella* will be able to disperse into favourable habitats to escape climate change, thus it is likely that future climate change will further reduce the genetic diversity and fitness of *Prestonella*. Populations with the lowest genetic diversity are likely to be more prone to extinction and might be viewed as a conservation priority. However, from a pragmatic perspective, it may be too late for some of these populations and conservation efforts should perhaps thus focus on those populations that are still genetically diverse and from regions where the climatic variables are not close to extinction thresholds. Refer to Chapter 4 for details on the conservation of *Prestonella.*

Chapter 3: The thermal tolerance of *Prestonella* in the context of evolutionary responses to climate change

Introduction

Habitat temperature is crucial for ectothermic animals, such as invertebrates, because of the effect that temperature has on all biological processes (Hochachka and Somero 2002). Changes in habitat temperature can occur at varying temporal scales, from hourly fluctuations between night and day (Stillman and Somero 1996) to those that might occur over a long period of time such as global climate change. Understanding the roles played by physiological adaptations to heat tolerance has regained new interest due to the potential effects of global warming (Portner 2002, Somero 2005). Recent meta-analyses have shown that climate change is responsible for widespread changes in ecosystems, such as changes in species distribution and the timing of annual migrations and reproduction (Hughes 2000, Parmesan and Yohe 2003, Root *et al.* 2003).

Species show a wide range of evolutionary responses to climatic variables and hence sensitivity to climate changes. These responses range from molecular to physiological to behavioral (Hochachka and Somero 2002, Kingsolver and Huey 1998, cited in Stillman 2004). Taxa primarily respond to rapid climate change in three ways: altering their distribution and abundance without evolving, going extinct, or evolving (Holt 1990). There is evidence to suggest that animals evolve in response to climate change and studies from a wide range of taxa have shown that polymorphisms at several loci are correlated with climatic variables (Parsons 1988, cited in Holt 1990).

Using controlled-environment chambers that mimic field conditions Bradshaw and Holzapfel (2001) have studied the variation in photoperiodic response of multiple populations of the pitcher-plant mosquito, *Wyeomyia smithii* over a wide geographic range in eastern North America. Over the last 30 years, the genetically controlled photoperiodic response of the *W. smithii*, has shifted toward shorter, more southern daylengths as growing seasons have become longer. This shift was detectable over a time period as short as 5 years (Bradshaw and Holzapfel 2001). Faster evolutionary response has occurred in northern populations where selection is stronger and genetic variation is greater than in southern populations (Bradshaw and Holzapfel 2001).

Populations of the frog *Rana sylvatica* in North America have undergone localized evolution in thermal tolerance (Skelly and Freidenburg 2000, Skelly *et al.* 2007), temperature-specific development rate and thermal preference (Freidenburg and Skelly 2004, Skelly *et al.* 2007) in response to altered temperature in their wetlands in less than 40 years.

Results from laboratory experiments on *Drosophila melanogaster* kept at 25°C, 27°C and 30°C show that after 15 generations the flies kept at 27°C and 30°C are smaller than those kept at 25°C (Good 1993). As treatment temperatures increase, flies have more intense search behaviour, prefer higher temperatures and are smaller (Good 1993). These behaviours were shown to have genetic components, indicating that differences were created in the laboratory through evolutionary adaptations to temperature (Good 1993).

The effect of temperature on the distribution and physiology of invertebrates

Several studies have shown that the limits of a species distribution are correlated with thermal tolerance limits (e.g. Barnacles: Wethy 1983, Porcelain crabs: Jensen and Armstrong 1991; Stillman and Somero 2000). The effects of climate change on the distribution of species will depend on the species' capacity to adjust their thermal limits through acclimation (Stillman 2003). Studies on invertebrates such as crabs (Stillman 2003), crayfish (Layne *et al.* 1985), planarians (Tsukuda and Ogoshi 1978), copepods (Bradley 1978), flies (Levins 1969) and slugs (Rising and Armitage 1969) have investigated the effects of thermal acclimation to heat tolerance. However, only a few studies have investigated the effect of acclimation temperature of multiple species adapted to different thermal conditions (e.g. porcelain crabs (*Petrolisthes*): Stillman and Somero 2000, Stillman 2004, marine snails (*Tegula*): Stensburg *et al. 2005*). These studies suggest that some species may be near their current habitat temperature maxima, and may be at highest risk in the event of global climate change.

Physiological acclimatization to a wide range of temperatures appears to correlate with increasing genetic differentiation between populations of the same species along latitudinal or altitudinal clines (e.g. *Arenicola manna* (lugworm): Hummel *et al.* 1997, *Drosophila* *melanogaster*: McCabe and Partridge 1997). Acclimatization refers to adjustments of an organism to an environmental complex, presumably a multistress environment, which includes seasonal and climatic changes (Hart 1957, Prosser 1958, Eagan 1963). Assessment of phosphoglucose isomerase (PGI) genotypes among montane populations of the willow beetle (*Chrysomela aeneicollis)* reveal functional and physiological differences among PGI genotypes, which suggest populations of this beetle are locally adapted to temperature (Dahlhoff and Rank 2000)

Heart function in response to increasing temperature

Thermal tolerance limits are established by several physiological, biochemical and morphological factors. Heart function is one such factor that can be directly measured and allows for the immediate detection of physiological change during changing environmental conditions (Marshall and McQuaid 1992, Stensburg *et al. 2005*). The molluscan heart is myogenic and the rhythm of the heart beat is controlled by the action of the neurotransmitters (Kobayashi 1987). Cardiac responses to changing environmental conditions have been recorded for a variety of mollusc species and there is a general tendency for the heart rate to increase in response to situations that would increase the load on the respiratory and excretory systems (Koester *et al.* 1979). In all species of molluscs that have been examined heart rate increases in response to increased temperature (Koester *et al.* 1979). Stensburg *et al.* (2005) found a strong positive correlation between the upper cardiac thermal tolerance limits of *Tegula* congeners and their vertical position in the intertidal. In *T. funebralis* (the higher-dwelling congener) heart failure occurred at significantly higher temperatures than the two subtidal species. Although this is a marine example, correlations between species distribution and thermal limits are also likely to be evident in terrestrial species.

In the context of *Prestonella* there are probably two main climatic variables that affect its survival: temperature and water / moisture availability. The importance of the latter in the context of genetic diversity was demonstrated in the previous chapter, where population genetic diversity was correlated to annual precipitation, coefficient of variation of annual precipitation and mean annual potential evaporation. While we did not find a correlation between temperature and genetic diversity, it is most likely that temperature in conjunction with other climatic variables such as water availability play a crucial role in local adaptation of organisms (in particular invertebrates) to climate.

Aims

The aim of this chapter is to compare the thermal tolerances of three different populations of *Prestonella* from different localities across their distribution range by measuring Arrhenius Breaking Temperatures (ABT) and Flat-Line Temperatures (FLT).

Methods and Materials

Measuring Heart Rates

Snails from three different populations were used for this experiment. Unfortunately, the number of snails used varied due to the availability of snails. The populations selected were: Fort Fordyce (9 snails), Mountain Zebra National Park site 2 (5 snails) and Glen Avon Falls (8 snails). Mountain Zebra National Park and Glen Avon Falls populations are found in habitats dominated by thicket vegetation, whereas the Fort Fordyce population is found in a habitat dominated by afromontane forest (Table 3.1). The maximum and average habitat temperatures were taken from the GIS layers obtained from the South African Atlas of Agrohydrology and Climatology (Schultz 1997) (as used in the previous chapter) are shown in Table 3.1.

Table 3.1: Habitat type, maximum and average temperatures (Degrees Celsius) for ESU's from Fort Fordyce, Mountain Zebra NP and Glen Avon Falls.

Population	Species	Habitat	Maximum Temperature ^{o}C	Average Temperature ^{o}C
Fort Fordyce	*P. nuptialis*	forest	42.6	17.2
Mountain Zebra NP	*P. nuptialis*	thicket	37.1	14.6
Glen Avon Falls	*P. bowkeri*	thicket	41.2	15.6

Prior to the experiment snails were kept in a temperature regulated oven at 30°C to induce aestivation. Once aestivating, the snails were secured by prestik (sticky putty) on an infrared sensor (Vishay Semiconductor, CNY70) which was used to detect the movement of the snail's heart. The resulting signals were amplified, filtered and digitally logged using an ADInstruments PowerLab™ 4/SP Data Acquisition System. A smoothed cardiogram trace (triangular Bartlett smoothing) was derived on a separate channel. A schematic diagram of the equipment used is shown in Figure 3.1.

Heart rate was measured over a temperature range as follows: Snails were placed in a plastic bag lined with absorbent paper to prevent them from getting wet. This setup accommodated a maximum of 2 animals per run. The plastic bag was then immersed in a temperature contolled waterbath. The temperature was initially held constant at 20°C for 20 minutes, then increased by 1°C every 12 minutes, a rate similar to that of natural daily temperature increases. Heart rate measurements were made every minute for the duration of the run. The run was stopped once the animals reached flat-line temperature (FLT); when the heart stops and death occurs.

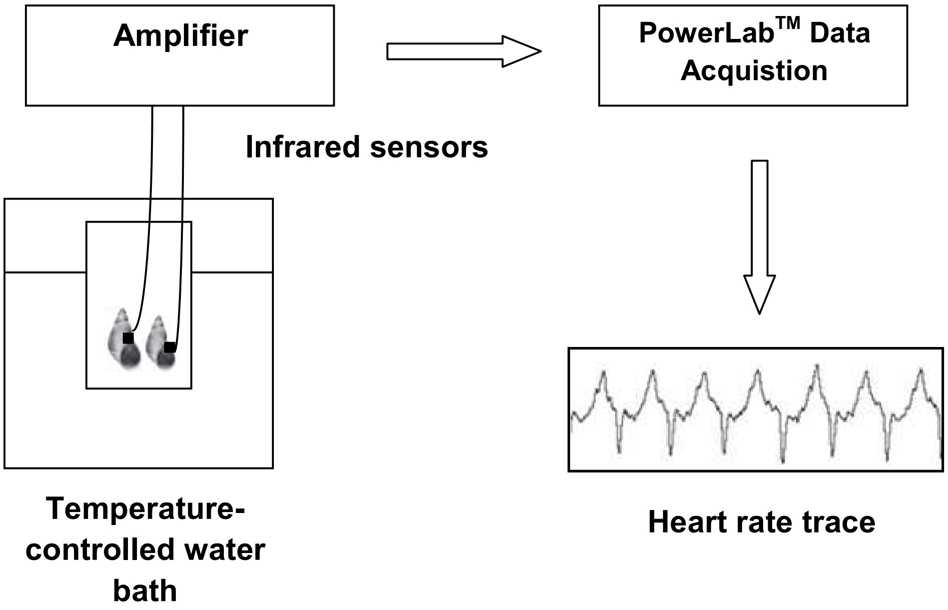

Figure 3.1: Schematic diagram demonstrating design of equipment for temperature stress experiments.

Data analysis

Heart rate traces were viewed in LabChart7. The raw heart rate data was edited and graphs were created using Microsoft Excel 2007. Arrhenius plots were generated for each animal by converting heart rate values to the natural logarithm of beats per minute and plotting these values against a temperature. The temperature measurements were transformed by dividing 1000 by the temperature in Kelvin (1000/K). The Arrhenius plot is used to study the effect temperature has on heart reaction rates. Arrhenius break temperatures (ABTs) were determined using regression analyses to generate the best fitting line on both sides of an inflection point (determined by observation) (Figure 3.2). The intersection of the two regression lines was taken as the ABT. Flat-line temperatures (FLTs) were also determined as the temperature at which the heart stopped beating. Critical temperatures (Arrhenius break temperatures and flat-line temperatures) were determined for each individual and the averages calculated for each population. To determine statistical significance between populations one-way ANOVA's were performed using STATISTICA 8.

To establish if there is a correlation between upper thermal limits and climatic variables, ABT and FLT values for each individual snail were plotted against annual precipitation, coefficient of variation of annual precipitation, mean annual potential evaporation, relative humidity and maximum and average temperature for each locality.

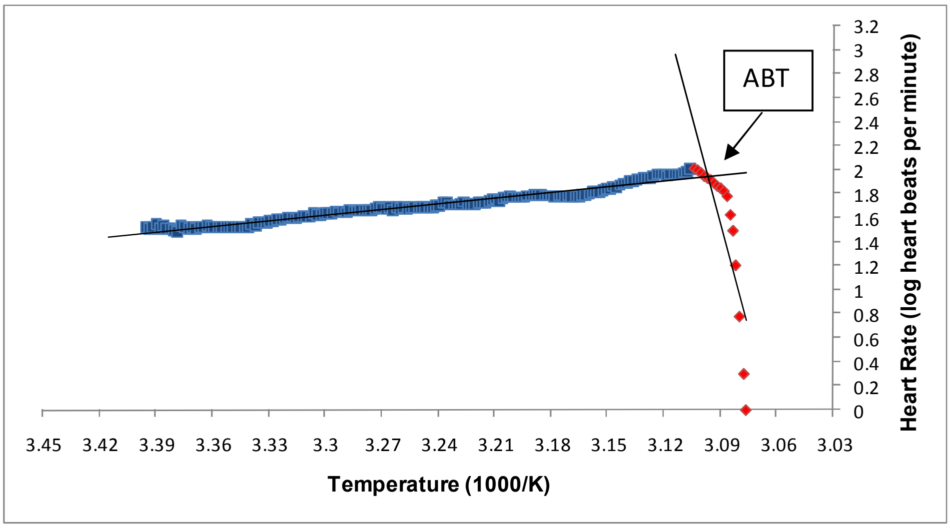

Figure 3.2: Arrhenius plot of a typical response of heart rate to increasing temperature. Inflection point was determined by visual inspection, and linear regression was performed on both sides of this point. ABT was calculated as the intersection of the two trend lines.

Results

Heart rates of snails from all three populations increased in response to heat stress, up to the cardiac limit (Arrhenius Breaking Temperature), after which the heart rate declined to zero (Figure 3.3 A, B and C). Heart rate increased slowly between 20°C and 40°C by about one beat per degree, followed by a rapid increase in heart rate between 40°C and ABT (approximately 50°C) by five beats per degree. All populations showed a similar pattern.

Arrhenius breaking temperatures and flat-line temperatures

ABTs were easily determined by generating Arrhenius plots for each specimen (Figure 3.2). The average ABTs for Fort Fordyce, Glen Avon Falls and Mountain Zebra NP were 47.2°C, 49.08°C and 48.8°C, respectively (Figure 3.4). The ABT of Fort Fordyce is significantly different to Mountain Zebra NP (Tukey HSD test: $p< 0.05$) and Glen Avon Falls (Tukey HSD test: $p< 0.05$). The average flat-line temperatures (FLT) for Fort Fordyce, Glen Avon Falls and Mountain Zebra NP were 49.6°C, 51.8°C, and 50.5°C, respectively (Figure 3.5). Flat-line temperatures showed more variation between populations than Arrhenius break temperatures. Fort Fordyce is significantly different from Glen Avon Falls (Tukey HSD test: $p< 0.001$), but not significantly different to Mountain Zebra NP. Glen Avon Falls is also significantly different to Mountain Zebra NP (Tukey HSD test: $p< 0.05$). The summary of the Post-hoc tests are shown in Tables 3.2a and b. These results do not show a correlation between upper thermal limits and maximum habitat temperatures or other climatic variables in *Prestonella* populations (graphs can be viewed in Appendix 4). However, there is a slight correlation between ABT and both annual precipitation ($R^2= 0.628$) and relative humidity ($R^2=0.628$), but these relationships are weakly supported.

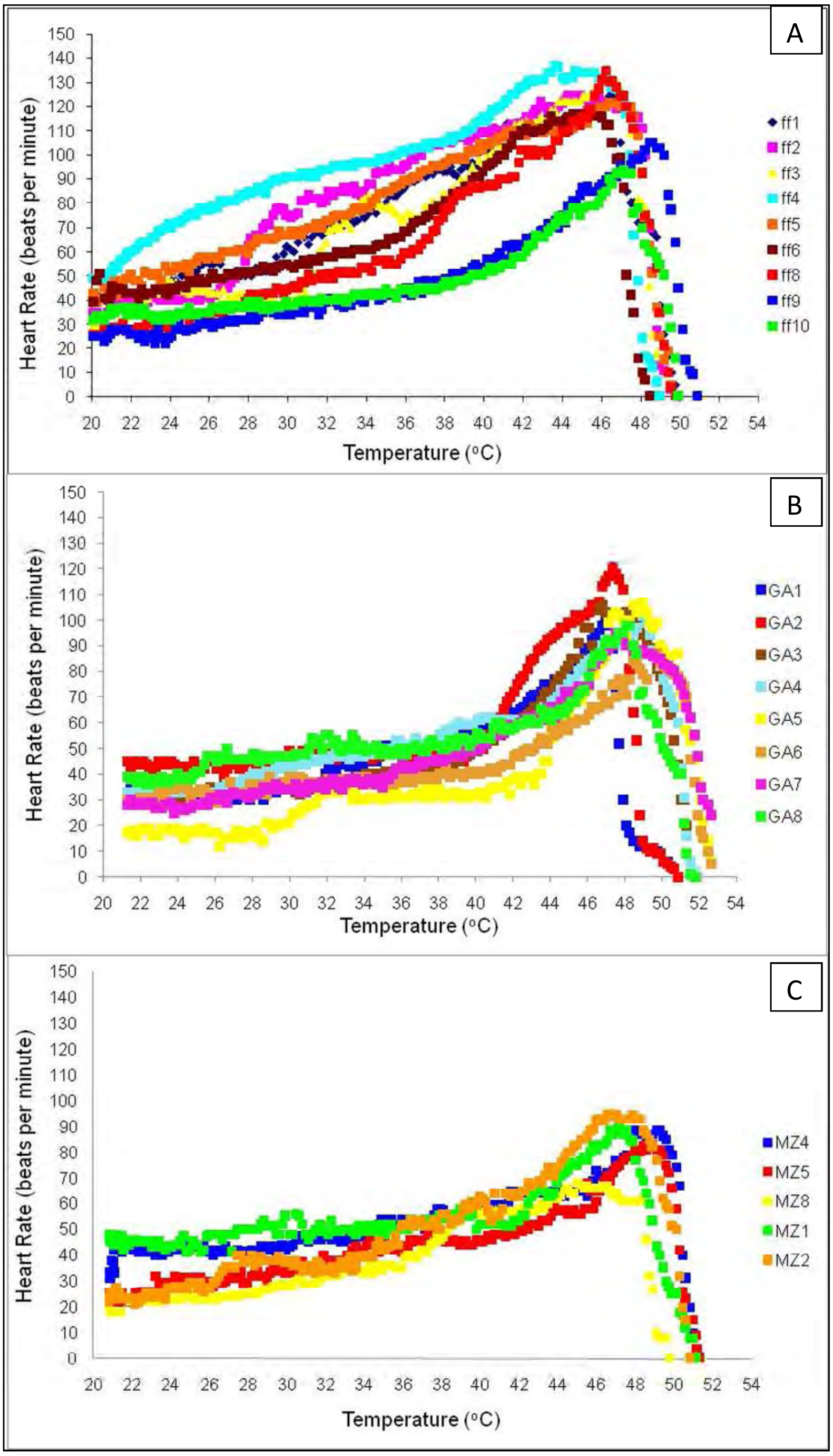

Figure 3.3 A,B and C: Effect of increasing temperature on the heart rates of snails from three different populations.: Fort Fordyce (A), Glen Avon Falls (B), Mountian Zebra National Park (C).

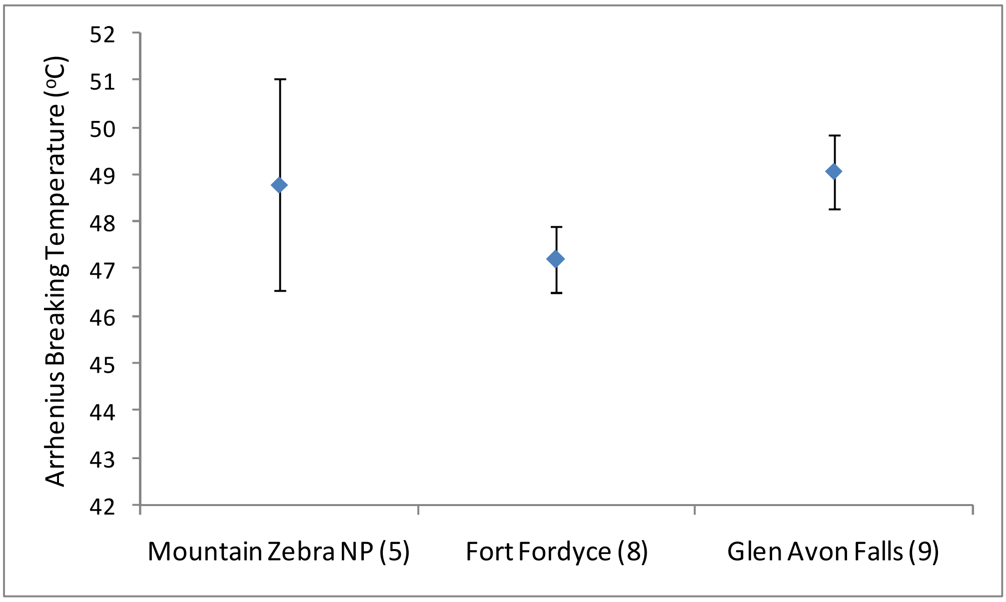

Figure 3.4: Average ABTs (Arrhenius breaking temperatures) for three *Prestonella* populations; Mountain Zebra NP (thicket), Fort Fordyce (forest) and Glen Avon Falls (thicket). Standard deviation indicated by error bars. Population sample size is indicated in brackets behind population name.

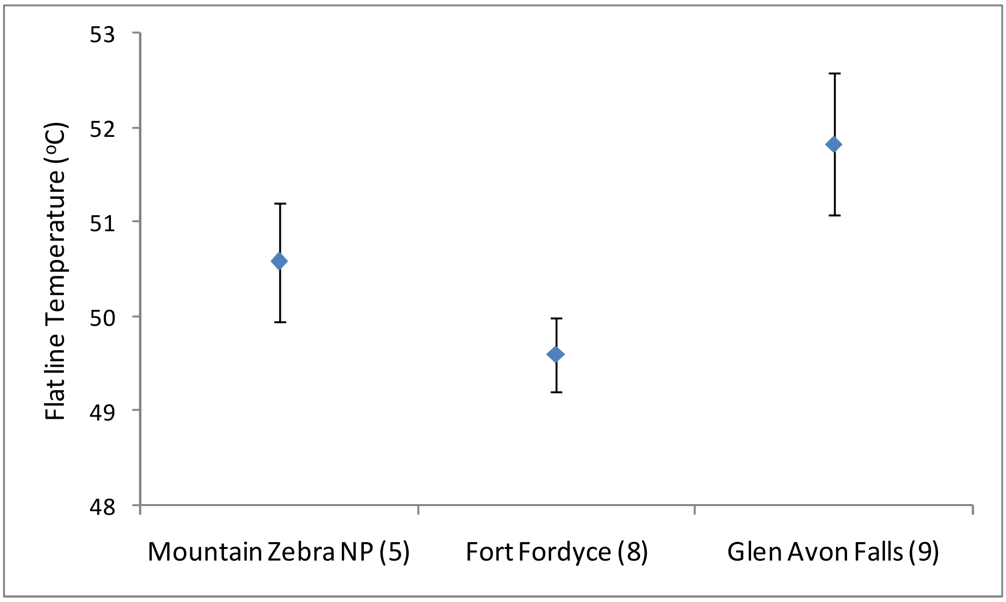

Figure 3.5: Average FLTs (Flatline temperatures) for three *Prestonella*: ESU's; Mountain Zebra NP (thicket), Fort Fordyce (forest) and Glen Avon Falls (thicket). Standard deviation indicated by error bars. population sample size is indicated in brackets behind population name.

Table 3.2a: Post-hoc Tukey HSD test: Arrhenius Breaking Temperature (Degrees Celsius) for Fort Fordyce, Mountain Zebra NP and Glen Avon Falls. Approximate Probabilities for Post Hoc Tests Error: Between MS = 1.0620, df = 22.000. Red numbers indicate significant differences.

Population	Fort Fordyce	Mountain Zebra NP
Fort Fordyce		
Mountain Zebra NP	0.0270	
Glen Avon Falls	0.0059	0.9497

Table 3.2b: Post-hoc Tukey HSD test: Flatline Temperature (Degrees Celsius) for Fort Fordyce, Mountain Zebra NP and Glen Avon Falls. Approximate Probabilities for Post Hoc Tests Error: Between MS = 50754, df = 22.000. Red numbers indicate significant differences.

Population	Fort Fordyce	Mountain Zebra NP
Fort Fordyce		
Mountain Zebra NP	0.0592	
Glen Avon Falls	0.0001	0.0125

Discussion

The upper thermal limits (point at which the heart stops functioning properly) of the three *Prestonella* populations are much higher than the maximum habitat temperatures associated with those sites (based on extrapolated data from the GIS layers). Upper thermal tolerance limits and plasticity of thermal limits are high because exposure to a temperature above the upper themal tolerance limit will be lethal and could reduce population fitness to zero especially if the exposure is just before reproduction (Stillman 2002). There is thus a huge selective advantage for individuals in a population to have thermal tolerance limits greater than the maximal habitat temperatures (Stillman 2002). *Prestonella* populations do not experience maximal habitat temperatures near to their thermal tolerance, however, individuals will experience a decrease in fitness below the upper thermal limit where the slope of the curve is the steepest and for most of the individual snails this was between 42°C and 48°C. Since maximum habitat temperatures for Fort Fordyce and Glen Avon Falls are close to 42°C, snails will experience a decrease in fitness at these temperatures which indicates that these snails live close to their physiological limits.

Adaptation to Micro-habitats

Temperature is arguably the most important abiotic stress that ectothermic organisms experience (Hochachka and Somero 1984). As a consequence species distribution is often correlated with thermal tolerance limits as shown by several studies on marine invertebrates (e.g. Wethy 1983, Jensen and Armstrong 1991, Stillman and Somero 2000). The results for *Prestonella,* however, do not show this pattern and upper thermal limits do not correlate with maximum habitat temperatures (see Appendix 4). Arrhenius break temperatures (upper thermal limit) showed variation between the populations and the Fort Fordyce population was significantly different to both the Mountain Zebra NP and Glen Avon Falls populations ($p<0.05$). The temperature measurements used in this study were extracted from GIS maps extrapolated from data obtained from weather stations across the country and may be too coarse to reveal actual micro-habitat climates that the snails live in. Also, the temperatures are measurements of air temperature and not surface temperatures. Rock faces that snails live on are likely to get much hotter than the air temperature on hot days. These factors may explain why upper thermal limits do not correlate with maximum habitat temperatures.

Although no correlation is found between ABT and maximum habitat temperature, it is likely that the differences seen between these populations are due to local micro-climate adaptation. Biological adaptation is a property of phenotypic features of organisms relative to selection demands of the environment.

Relatively few studies have been devoted to the microgeographic evolutionary adaptation of organisms to temperature at a local scale (e.g. Nevo 1997). Upper thermal tolerance limits often closely match the microhabitat conditions a species or population is likely to encounter (e.g. weevils: van der Merwe *et al.* 1997, *Petrolisthes*: Jensen and Armstrong 1991, Stillman and Somero 1996, 2000). Local thermal gradients can be formed by fine-scale variation in, for example, solar radiation, rainfall and altitude (Nevo 1997). *Prestonella* snails at Fort Fordyce have the lowest thermal tolerance (47.2^{o}C) and live in afromontane forest on south facing rock faces, often near to seeps and drip lines. Forest understories have lower light intensities than at the canopy top; less than 2% of the solar radiation above the canopy reaches the forest floor (Valadares *et al*. 2002). As a consequence of reduced solar radiation, forest understories do not heat up as rapidly, have a higher humidity and dry out more slowly than exposed areas which make forest understorey habitats less heat stressed. It is likely that the lower thermal tolerance observed in the Fort Fordyce population is a micro-habitat adaptation to reduced thermal stress in such a forest understorey habitat. The populations of Glen Avon Falls and Mountain Zebra NP on the other hand are found in exposed thicket habitats where solar radiation and heat exposure is much greater. It is reasonable to hypothesise that they have adapted to an environment that is more heat stressed and explains why these populations have higher thermal limits. Analyses of upper thermal limits in populations of *Prestonella* are consistent with the hypothesis that genetically based differences account for interspecific variation in heat tolerance. This hypothesis has been proposed for other invertebrates such as congeners of *Petrolisthes* (Porcelain crabs: Stillman and Somero 2000). A study on the thermal tolerance of snails has shown that the upper thermal limits for *T. pisana* snails from Spain are higher than those of snails from South Wales and it is suggested that these differences may have a genetic basis (Cowie 1985).

Evolutionary response to future climate change

Future climate change will undoubtedly affect micro-habitat conditions and influence local adaptation. An increase in maximal thermal temperatures due to global warming will likely increase thermal tolerances in populations of *Prestonella* over time. Changes in thermal tolerance in response to recent climate change have been shown for several species. The frog *Rana sylvatica* was shown to have undergone localized evolution in response to climate change and thermal tolerance in less than 40 years due to altered temperature in their wetland habitat (Skelly and Friendenburg 2000, Skelly *et al.* 2007). Studies on *Drosophila melanogaster* have shown changes in thermal tolerance after as few as 10 generations (Good 1993).

How *Prestonella* responds to future climate change will depend on how fast it can evolve in the face of rapid climate change. There are a few potential constraints on evolutionary responses, such as lag time between the change and response and the lack of, or erosion of, genetic variability (Skelly *et al.* 2007). Some taxa, such as species with small effective population sizes and long generation times (e.g. forest trees, large mammals) may not be able to respond to climate change fast enough and extinction will occur quickly (Davis *et al.* 2005). Evidence from past climatic change suggests that many species take hundreds to thousands of years to adapt to a new climate regime (Skelly *et al.* 2007). In small populations, evolutionary rates can be restricted by the amount of genetic variation maintained in the face of genetic drift or by the amount of novel genetic mutations (Holt 1987, Lande and Barrowclough 1988). If a novel selective pressure decreases the effective population size, genetic drift will diminish additive genetic variance (Holt 1990). Reduced genetic variation limits a species' ability to adapt to climatic change. If a population has nonadditive genetic variance in fitness, a bottle neck that lasts a few generations may convert this into additive genetic variance (Goodnight 1988). Adaptive evolution involving major mutations seems to be most common in highly disturbed populations (Holt 1990). In many organisms including *Drosophila*, rates of mutation and recombination increase in stressed populations (Parsons 1987, 1988).

Recommendation for future study

It is necessary to collect ABT and FLT data from other populations along the Great Escarpment, to see if similar trends exist. It is essential to collect micro-habitat specific climatic variables for each population which are more accurate than data from GIS maps. iButtons (computer chip data loggers) could be secured to rock faces to accurately measure the climate of each *Prestonella* population. Using accurate data other trends and correlations may emerge that may better explain our current results.

Conclusion

Differences in thermal tolerance observed between populations of *Prestonella,* although not correlated with maximum temperatures for the area, are most likely attributable to adaptations to micro-habitats. Lower thermal tolerances are associated with forest habitats, a consequence of adaptations to a less temperature-stressed environment. Populations that live in thicket habitats are more exposed than forest habitats and have greater temperature stress; hence they have higher thermal tolerances. This adaptation ensures that maximal habitat temperatures never reach thermal tolerance temperatures. If *Prestonella* evolves fast enough ABT's will increase in response to global warming. On the other hand if populations of *Prestonella* cannot evolve fast enough thermal tolerance limits will be approached and as a consequence populations *of Prestonella* are likely to go extinct. The rate of change may be the key factor in deciding *Prestonella's* fate. How fast *Prestonella* evolves in response to climate change will depend on genetic variation and effective population size of each population.

Chapter 4: Conservation status of *Prestonella nuptialis* and *Prestonella bowkeri*

Introduction

Molluscs are an extremely diverse group of animals, with estimates of the total number of valid described and un- described mollusc species ranging between 50,000 to 200, 000 (van Bruggen 1995). Global estimates of non-marine molluscan species richness vary considerably, but the estimated number of extant terrestrial species with valid descriptions is approximately 24,000 and that of fresh water species is approximately 7000 species (Lydeard *et al.* 2004). Lydeard *et al.* (2004) suggest that the number of extant mollusc species is probably underestimated due to the continual discovery of small, narrow range endemics throughout the world particularly in the tropics. There are thus a predicted 11, 000 to 40, 000 undescribed terrestrial species and 3000 to 10,000 undescribed freshwater species.

Despite the large number of known mollusc species only a small proportion of these (<3%) have had their conservation status assessed (Ballie *et al.* 2004). A total of 708 freshwater and 1222 terrestrial mollusc species were included in the 2002 IUCN Red List of Threatened Species (Lydeard *et al.* 2004). The total number of threatened non-marine molluscs is nearly half the number of all known amphibian species and twice the number of shark and ray species (Lydeard *et al.* 2004). In contrast only 41 marine molluscs make the IUCN Red List, despite their greater overall diversity (>120,000 species). The large number of nonmarine molluscs listed as threatened on the IUCN Red List is not merely a correlate of species richness, indicating that terrestrial mollusc taxa are considerably more threatened than would otherwise be expected.

To answer questions surrounding which groups of molluscs are most likely to go extinct, Kay (1995) compiled a database that included molluscs considered to be endangered, threatened or rare (within the last 200 years). The database included aspects such as geography and biological characteristics of each species. Out of the 1130 species (or subspecies) included in the data base just about all them have restricted distributions (Kay 1995). Island species in particular have restricted distributions. The Bulimulidae of the Galapagos, the Achatinellidae and Amastridae of Hawaii and the Partulidae of Tahiti and Guam are, predominantly, single island endemics, often found only on single mountain ridges or in a single valley (Kay 1995).

The database also revealed that threatened or endangered mollusc species are usually specialists in habitat and food habits. For example species of the Hydrobiidae are found in single drainage basins, springs, caves and other subterranean habitats (Ponder and Clark 1990), the Pleuroceridae and Unionidae from the United States of America are specialists in the shoal waters of the rivers (Stansberry 1971) and the Rhytididae from New Zealand are found deep in acidic leaf mould where they feed on earthworms and millipedes (Meads *et al.* 1984).

The limited distribution and habitat restriction of many mollusc species makes them particularly vulnerable and it is thus not surprising that molluscs have the dubious honor of having the highest number of documented extinctions of any major taxonomic group (Lydeard *et al.* 2004). Since the beginning of the 16th century, a recorded 693 animals species have gone extinct, 42% of which are molluscs (260 gastropods and 31 bivalves; Lydeard *et al.* 2004). More than 70% of known mollusc extinctions have taken place on oceanic islands such as Hawaii, French Polynesia, and the Mascarene Islands (Regnier *et al.* 2009). Marine habitats seem to have experienced little extinction in comparison (Regnier *et al.* 2009), which further emphasizes how much terrestrial and freshwater molluscs are threatened.

IUCN assessments of South African snails

As of January 2011 the IUCN redlist of threatened species lists 70 South African non-marine gastropods species that have had their conservation status assessed, of which 16 are terrestrial species. Five of these species are endangered (*Gulella caustralis, Gulella aprosdoketa, Laevicaulis haroldi, Chlamydephorus purcelli*, *Trachycystis haygarthi*) and another six are critically endangered (*Gulella puzeyi, Gulella salpinx, Natalina beyrichi, Tomichia tristis, Trachycystis clifdeni, Trachycystis placenta). Prestonella nuptialis* and *P. bowkeri* are currently not listed on the IUCN redlist of threatened species. In 2007 Herbert considered the status of *P. nuptialis* and *P. bowkeri* as Data Deficient, information permitting an informed categorization at a more specific threat level was not available at that time. No past conservation efforts have ever been made concerning *P. nuptialis* and *P. bowkeri*, and only recently has the level of threat to *P. nuptialis* and *P. bowkeri* been realized. In, fact very little is known about the biology and ecology of *Prestonella*. Until Herbert's publication on the revision of the genus in 2007 the only information known about the genus *Prestonella* was contained in the original description provided by Connolly in 1929.

Aims

The aim of this chapter is to assess the conservation status of *P. bowkeri* and *P. nuptialis* by designating them to an IUCN Red List category.

Defining *Prestonella* species

According to the molecular work, *P. bowkeri* is paraphyletic with respect to *P. nuptialis*, hence *P. bowkeri* appears to be non-monophyletic. However, this finding is based on mtDNA alone, therefore the evidence for this is not particularly strong, hence *P. nuptialis* and *P. bowkeri* will be considered separate species for the purpose of this chapter.

Habitat of *Prestonella*

P. nuptialis and *P. bowkeri* are microhabitat specialists found in isolated mesic habitats in an otherwise relatively dry region. They are almost always found on shaded south-facing slopes in ravines and gorges near waterfalls or montane seepages. Refer to chapter 1 for more details on the habitat of *P. nuptialis* and *P. bowkeri.*

Distribution of *Prestonella*

As mentioned in chapters 1 and 2, populations of *P. nuptialis* and *P. bowkeri* have been found across the southern Great Escarpment in South Africa, where the distribution of both species is fragmented. To date, only seven populations of *P. bowkeri* and 11 populations of *P. nuptialis* have been recorded (Figure 4.1). It is difficult to locate populations of these snails as they tend to have very small microhabitats which are easy to miss.

To calculate the Extent of Occurrence (EOO) of *P. nuptialis* and *P. bowkeri* a polygon boundary around the known localities was created using ArcMap (version 9.0) of each species (Figure 4.2). To calculate the area within the imaginary boundary 11 km x 11 km grid squares were used. The EOO was calculated by adding the number of grids that were full, half full and quarter full. Using this method, *P. bowkeri* has an EOO of 19 844 km^2 and *P. nuptialis* has an EOO of 19 118 km^2.

The Area of Occupancy (AOO) of *Prestonella* is very restricted due to their specific habitat requirements. Snails are often found occupying a few crevices in an area that is only a few square meters in extent. Other populations are a little more extensive and may occupy a larger area along a ravine or river (approximately 0.2-0.5 kilometers squared). Due to the inaccessibility of rocky crevices and vertical cliff faces it is difficult to assess the vertical distribution of *Prestonella*, and because of this we have assigned an Area of Occupancy (AOO) of 0.5 km^2 to each subpopulation. However this is probably grossly over estimated for most subpopulations. Hence the AOO of *P. nuptialis* is 5.5 km^2 and the AOO of *P. bowkeri* is 3.5 km^2.

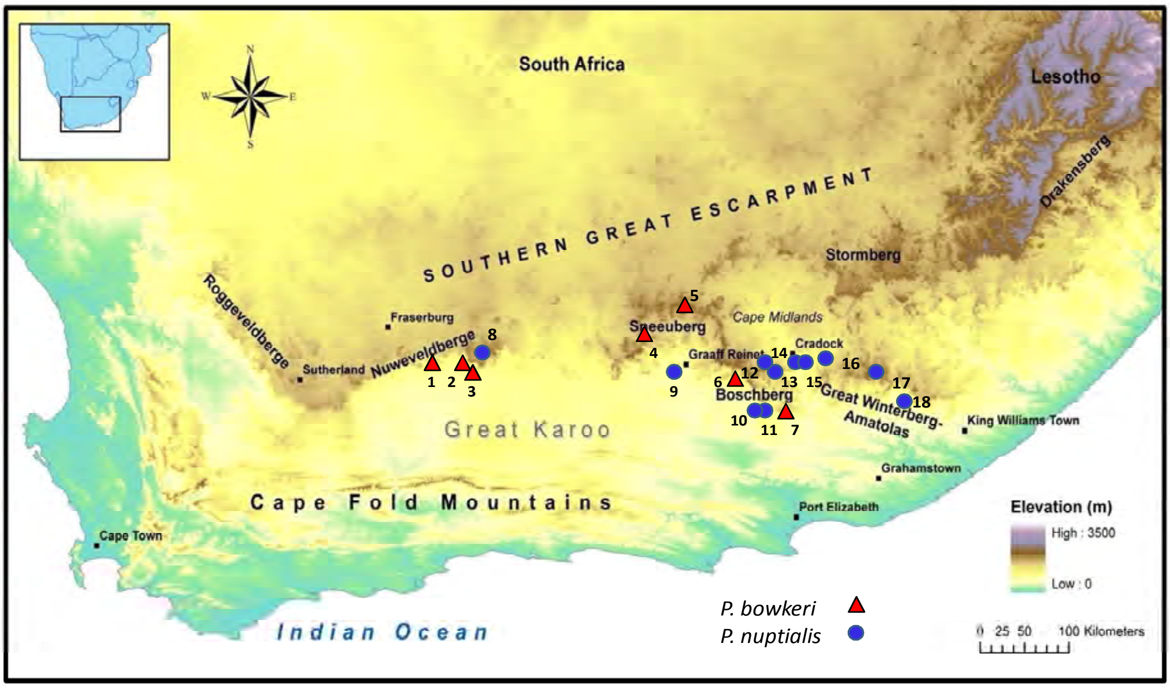

Figure 4.1: The known distribution of *P. nuptialis* (blue circles) and *P. bowkeri* (red triangles) in southern Africa. 1-Ou Kloof, Nuweveldberge, 2- Fonteintjies Kloof, Karoo Nat. Park, 3- Rooivalle, Karoo Nat. Pak, 4- Kleinfontein Falls, 5- Wilge River Canyon, 6- Boesmanshoek south of Coetzeesberg, 7- Glen Avon Falls, 8- Molteno Pass, 9- Valley of Desolation, Camdeboo Nat. Park, 10- Kamala Game Reserve site 1, 11-Kamala Game Reserve site 2, 12- Mountain Zebra Nat. Park site 1, 13- Mountain Zebra Nat. Park site 2, 14- Cradock area, inselberg south of Tarkastad, 15- Cradock, 16- Elandsberg, Cradock area, 17- Fenella Falls, Adelaide area, 18- Fort Fordyce.

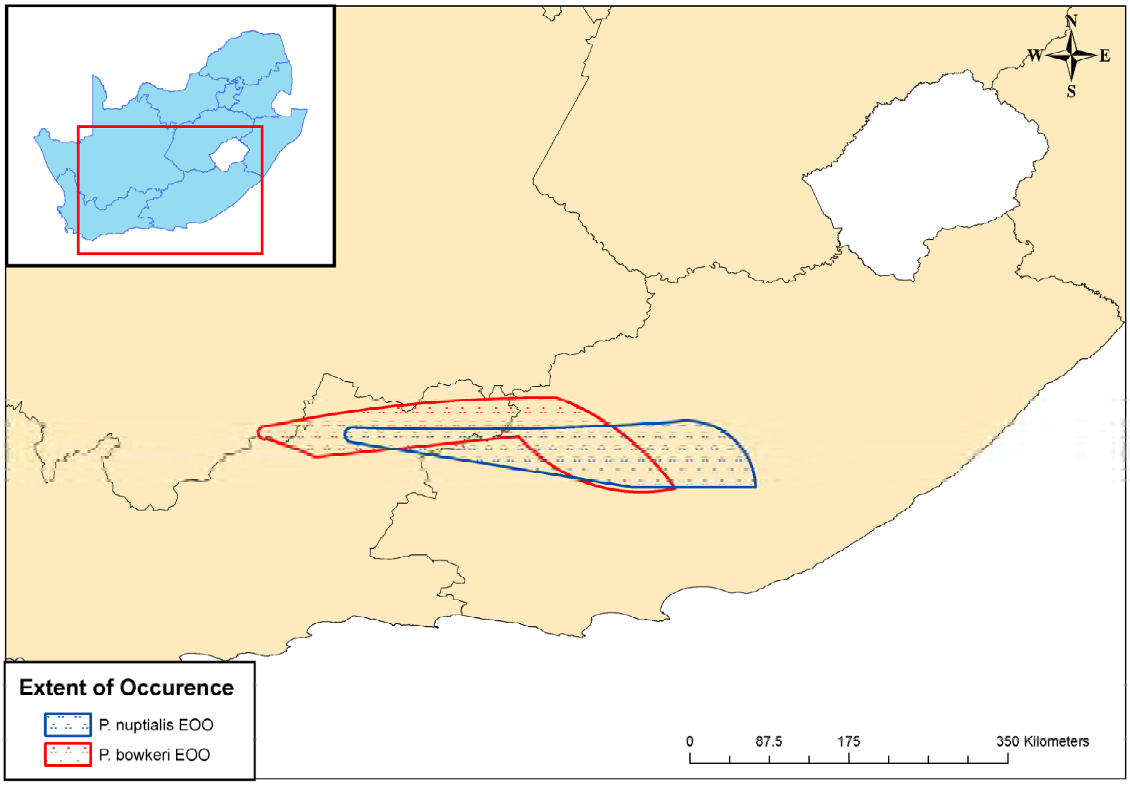

Figure 4.2: The Extent of Occurrence (EOO) of *P. nuptialis* (blue) and *P. bowkeri* (red) in the southern Great Escarpment of South Africa. To calculate the area within the imaginary boundary of the EOO a 11 x 11 km^2 grid square was used.

Population size of *Prestonella*

The term ‘population’ is used in a specific sense in the Red List Criteria that is different to its common biological usage. The usage of the term population in this chapter is defined as the total number of individuals of the taxon (IUCN 2001). A subpopulation is defined as geographically or otherwise distinct group in the population between which there is little demographic or genetic exchange (IUCN 2001).

The global population size is currently unknown because a complete population survey has never been carried out. However, subpopulation surveys have been conducted for four populations of *P. bowkeri* and six populations of *P. nuptialis.* It is extremely difficult to find these snails because they tend to live high up vertical rock faces which are inaccessible or tend to live deep within rocky crevices especially during hot dry weather. These surveys involved estimates of population size by roughly counting the number of individuals we could find in the field during site visits. Only one visit was made per site, so no follow-up counts were made. Population sizes ranged from less than 50 individuals to over 100 individuals in some subpopulations, although no population seemed to have more than 200 individuals (Table 4.1). Using these figures the global population size (juveniles and adults) of *P. bowkeri* probably ranges between 500-800 individuals and that of *P. nuptialis* ranges between 1000-1400 individuals. No data exists on changes in population size over time.

Table 4.1: Estimated population sizes for four populations of *P. bowkeri* and six populations of *P. nuptialis.* Population sizes were estimated by roughly counting the number of snails found. The population size includes the total number of adults and juveniles.

Population	**Species**	**Population Size**
Ou Kloof, Nuweveldberge between Fraserburg and Beaufort West	*P. bowkeri*	Unknown
Fonteintjieskloof, Karoo Nat. Park, Beaufort West area	*P. bowkeri*	Unknown
Sneeuberg, immediately S of Coetzeesberge, Boesmanshoek, Farm 24	*P. bowkeri*	Unknown
Glen Avon Falls "The Gorge", Somerset East,	*P. bowkeri*	<100
Wilge River Canyon, Nieu Bethesda area, Ganora Farm	*P. bowkeri*	100-200
Rooivalle, Karoo Nat. Park, Beaufort West area	*P. bowkeri*	<50
Kleinfontein Falls, between Graaff-Reinet and Murraysburg	*P. bowkeri*	<100
Cradock area, Elandsberg mountain	*P. nuptialis*	Unknown
Cradock area, inselberg south of Tarkastad road	*P. nuptialis*	Unknown
Graaff-Reinet, Camdeboo Nat. Park, Valley of Desolation	*P. nuptialis*	Unknown
Cradock	*P. nuptialis*	Unknown
Fort Fordyce, Fort Beaufort area	*P. nuptialis*	<100
Mountain Zebra Nat. Park, site 1	*P. nuptialis*	<100
Mountain Zebra Nat. Park, site 2	*P. nuptialis*	100-200
Fenella Falls, Adelaide area	*P. nuptialis*	Unknown
Molteno Pass, Beaufort West Area	*P. nuptialis*	<50
Kamala Game Reserve, Somerset East, site 1	*P. nuptialis*	<100
Kamala Game Reserve, Somerset East, site 2	*P. nuptialis*	100-200

Possible Threats to *Prestonella*

1) Genetic load

In general populations of conservation concern have small effective population sizes (Skelly *et al.* 2007) that are susceptible the effects of genetic drift and inbreeding depression (Van Riel *et al.* 2001). In small populations of randomly mating individuals, all individuals may suffer from inbreeding depression because of the cumulative effects of genetic drift that reduces the fitness of all individuals in a population (Hedrick and Kalinowski 2000). Inbreeding depression reduces genetic variation leading to genetic problems that can limit evolutionary response (Futuyma 2006). In some cases genetic drift can result in haplotype fixation which can limit natural selection (Burger and Lynch 1995). The maintenance of at least constant levels of genetic diversity are essential for the long term survival of a species and populations with reduced genetic diversity are suspected to lack adequate capacity to adapt to their environment (e.g. O'Brien et al. 1985, Ellstrand and Elam 1993). It is thought that inbreeding depression is greater in more stressful environments (such as those experiencing rapid ecological changes) (Frankham *et al.* 2002). However, over a wide range of taxa, studies that find statistically increased inbreeding depression under environmental stress are slightly less than those that do not, thus this subject remains highly controversial (Armbruster and Reed 2005). Limited gene flow is thought to be a trigger for genetic erosion in isolated populations. Habitat fragmentation is one factor responsible for the decline in gene flow between populations (e.g. Clarke and O'Dwyer 2000, Schmitt and Seitz 2002). Montane species often have highly fragmented and isolated populations due to the large distance that separate mountain blocks or peaks and are thus susceptible to the associated effects of inbreeding depression and genetic drift. In the case of *Prestonella*, in addition to being a fragmented montane species, is susceptible to climate driven population bottlenecks which reduce population size leading to inbreeding depression. A historic climate shift from one of a more mesic climate to that of seasonal aridity has driven genetic erosion in the most western populations of *Prestonella,* it is evident from Chapter 2 that populations in regions that are drier, have less reliable rainfall and higher potential evaporation are more genetically depauperate.

2) Land degradation

Land degradation also poses a threat to the survival of *Prestonella*. A large portion of the Great Escarpment falls within the Eastern Cape region which is one of the poorest provinces in South Africa. Much of the Eastern Cape is subjected to intensified degradation due to informal grazing and crop farming (Meadows and Hoffman 2003). Land degradation in the form of rill and gully erosion is extensive in the Sneeuberg uplands of the Great Karoo due to extensive stock farming (Boardman *et al.* 2003). Erosion in this area is accompanied by the replacement of grasslands by shrub vegetation through the process of bush encroachment (Boardman *et al.* 2003). This change in vegetation may affect stream flow dynamics and stream flow seasonality, thus affecting the populations of *Prestonella*.

3) Climate change

It is poorly known how the South African fauna will respond to climate change. Using a multivariate climate envelope approach for a suite of 179 South African animal species Erasmus *et al.* (2002) have predicted that the impact of climate change varies from little to no impact (3%) to local extinctions (2%). Some 78% of animal species show range contraction and the majority of range shifts are in an easterly direction with the highest species losses in the west (Erasmus *et al.* 2002). Small populations are especially susceptible to climate change due to the effects of genetic drift and inbreeding depression which may ultimately result in mountain top extinction (Colwell *et al*. 2008).

Although there is a degree of uncertainty as to how individual species will respond to climate change, these snails are habitat specialists found living on escarpment inselbergs are already restricted to specific sites associated with water courses with poor dispersal abilities and it is unlikely that they will be able to migrate to new habitats, and they may face extinction.

Status Designation

Based on the IUCN criteria, both *P. nuptialis* and *P. bowkeri* qualify for the Vulnerable (VU) category (*P. nuptialis*: (VU B1ab(iii,iv); D2), *P. bowkeri*: (VU B1ab(iii,iv); D1+2)) as shown in Tables 4.2 and 4.3. These assessments will be submitted to the IUCN for official status designation. It must be noted that both *P. nuptialis* and *P. bowkeri* qualify as Critically Endangered (CR) under criterion B2. For invertebrates, where there is seldom any reliable information on population size or possible decline, it is often the score under Criterion B that is given most emphasis when determining the level of threat.

Table 4.2: IUCN Assessment of *Prestonella bowkeri.*

IUCN Assessment of *Prestonella bowkeri*	
Criterion A: Declining population in the past or future?	NO
Population trends are unknown. Although it is likely that severe droughts may cause dramatic population fluctuations. The DNA data shows that the more the climate fluctuates, the more population bottlenecks are likely to occur	
Criterion B: Small distribution, population fragmented, or in a few locations, and continuing decline or fluctuation?	YES
Extent of Occurrence meets the threshold requirements for Vulnerable (VU) (<20,000 km^2) (VU B1). The Area of Occupancy is less than 10km^2 and meets the threshold requirements for Critically Endangered (CR B2). Populations of *Prestonella bowkeri* are extremely fragmented and genetically isolated (B1a+B2a). Global warming is likely to increase aridity along the Great Escarpment which will reduce habitat quality by changing stream flow dynamics and stream flow seasonality. Owing to their limited dispersal capacity and low vagility it is unlikely that subpopulations will be able to migrate to new habitats, thus it is likely that the number of subpopulations will decrease (B1b(iii,iv) + B2b(iii,iv)).	
Criterion C: Small population size and decline?	YES
The total number of mature adults is unknown, however, the total population size of *P. bowkeri* is estimated between 500-800 individuals, of which 50% or more are mature adults. This meets the criterion C threshold for Endangered (EN). There is insufficient information to estimate what rate of population decline can be expected, so Criterion C1 cannot be used. However, a continuing population decline is projected due to the expected impacts of global warming (EN C2). Mature adults are spread across several populations and 95% of adults do not occur in one subpopulation, therefore Criterion EN C2a(i) does not apply. There is no evidence of extreme population fluctuation, so Criterion C2b cannot be used. But the species already qualifies for Endangered (EN) under Criterion C (EN 2a(i)).	
Criterion D: Very small or restricted populations?	YES
Total population sizes ranges between 500-800 individuals which exceeds the D1 threshold for Endangered, thus *P. bowkeri* qualifies for Vulnerable (D1 population size <1000 individuals). As far as we know *P. bowkeri* is restricted to seven populations that have an area of occupancy less than 20 km^2 and are threatened by stochastic events associated with climate change (D2). This species qualifies for Vulnerable under Criterion D (VU D1+D2).	
Criterion E: Quantitative analysis?	NO
A quantitative analysis has not been carried out.	

Table 4.3: IUCN Assessment of *Prestonella nuptialis.*

IUCN Assessment of *Prestonella nuptialis*	
Criterion A: Declining population in the past or future?	NO
Population trends are unknown. Although it is likely that severe droughts may cause dramatic population fluctuations.	
Criterion B: Small distribution, population fragmented, or in a few locations, and continuing decline or fluctuation?	YES
Extent of Occurrence meets the threshold requirements for Vulnerable (VU) (<20,000 km^2) (VU B1). The Area of Occupancy is less than 10km^2 and meets the threshold requirements for Critically Endangered (CR B2). Populations of *Prestonella nuptialis* are extremely fragmented and genetically isolated (B1a+B2a). Global warming is likely to increase aridity along the Great Escarpment which will reduce habitat quality by changing stream flow dynamics and stream flow seasonality. Owing to their limited dispersal capacity and low vagility it is unlikely that subpopulations will be able to migrate to new habitats, thus it is likely that the number of subpopulations will decrease (B1b(iii,iv) + B2b(iii,iv)).	
Criterion C: Small population size and decline?	YES
The total number of mature adults is unknown, however, the total population size of *P. nuptialis* is estimated between 1000-1300 individuals, of which 50% or more are mature adults. This meets the criterion C threshold for Endangered (EN). There is insufficient information to say what rate of population decline can be expected, so Criterion C1 cannot be used. But, a continuing population decline is projected due to the expected impacts of global warming (EN C2). Mature adults are spread across several populations and 95% of adults do not occur in one subpopulation, therefore Criterion EN C2a(i) does not apply. There is no evidence of extreme population fluctuation, so Criterion C2b cannot be used. But the species already qualifies for Endangered (EN) under Criterion C (EN 2a(i)).	
Criterion D: Very small or restricted populations?	YES
Total population sizes ranges between 1000-1300 individuals which exceeds the D1 threshold for Vulnerable (D1 population size <1000 individuals). As far as we know the current population of *P. nuptialis* is restricted to 11 subpopulations that have an area of occupancy less than 20 km^2 and are threatened by stochastic events associated with climate change, thus *P. nuptialis* qualifies for Vulnerable under criterion D2.	
Criterion E: Quantitative analysis?	NO
A quantitative analysis has not been carried out.	

Conservation and Management of *Prestonella*

An IUCN red list assessment of *Prestonella bowkeri* and *Prestonella nuptialis* indicates that these species probably face a considerable threat to their continued survival as a result of global warming. Future climate change in South Africa is likely to result in range contraction and an eastward shift for many species, reflecting the west-east aridity gradient across the country, with the highest species losses in the west (Erasmus *et al.* 2002). According to South Africa's second national communication under the United Nations framework convention on climate change (2011) it has been observed that surface air temperatures over land have changed with statistical significance since 1950 across South Africa. These changes are consistent with, and have sometimes exceeded, the rate of mean global temperature rise. Future warming due to increased international greenhouse gas emissions is projected to be greatest in the interior of South Africa and least along the coast. Using Global Climate Models (GCMs) and assuming a moderate to high growth in greenhouse gas by 2050 the coast is likely to warm by around 1-2ºC, and the interior by around 2-3ºC. Established but incomplete GCM projections for the winter rainfall region consistently suggest future rainfall decreases, while summer rainfall region projections deviate less from present climate. There will be an increases in the duration of dry spells. Open water evaporation over South Africa is likely to increase by 5-10% by 2050, and by 15-25% by 2100 due to higher temperatures, this suggests that evaporation over the land will also likely increase.

An increase in aridity, evaporation and periods between rainfall events along the Great Escarpment is likely to affect the seasonality of rainfall and stream flow dynamics of waterfalls and seepages associated with populations of *Prestonella.* In conjunction with limited dispersal capacity and low vagility it is unlikely that snails such as *Prestonella* will be able to migrate to new habitats and may face extinction. Based on the climate correlation in Chapter 2 there is clear evidence of the effects of past climate-induced bottlenecks and extinctions, hence it is likely that some of the most threatened populations may go extinct in the near future. However According to South Africa's second national communication under the United Nations framework convention on climate change (2011) the geographic pattern of temperature change varies spatially and seasonally across South Africa. There is reliable but incomplete evidence showing lower rates of warming in the higher altitude eastern interior, relative to the coast and to the lower-lying western interior and does not fully conform with future projections of temperature change where most warming is projected to take place in the interior. This suggests that climate change along the Great Escarpment may not be as serious as previously predicted, however this is not certain.

Land snails are very sensitive to environmental conditions and many snail species have gone extinct as a result of climate change (Moreno-Rueda *et al.* 2009). The only known population of Aldabra banded snail *Rhachistia aldabrae* from the Seychelles declined through the late twentieth century until its extinction in the late 1990's (Gerlach 2007). The extinction of this snail is attributed to decreasing rainfall on the Aldabra atoll, associated with changes in regional rainfall patterns (Gerlach 2007). A field survey revealed that the land snail *Arianta arbustorum* had become extinct at 16 (55.2%) out of 29 localities in the surroundings of Basel (Switzerland) between 1908 and 1991 (Baur and Baur 1993). Extinction of eight of the populations is attributed to habitat destruction by urban development. However, *A. arbustorum* also became extinct at eight localities, all of which were covered by suitable habitat. Built-up areas affect adjacent vegetation by emitting thermal radiation, thus Baur and Baur (1993) suggest that these populations went extinct as a result of local warming due to extensive urban development.

Future monitoring and management

Most of the known populations of *P. bowkeri* and *P. nuptialis* were only discovered within the last five years. Given the amount of likely habitat still unexplored, there are almost certainly more populations of both species that have not yet been found. Suitable habitats may be located by aerial photographs and detailed topographic maps. Known populations need to be monitored for population decline which can be achieved through periodic surveys. Data also needs to be collected on the ecology and biology of populations, certainly with emphasis on demography, feeding behavior and reproduction. This can also be achieved through periodic surveys. To acquire more accurate measures of population size it may be necessary to conduct mark-recapture experiments. The Petersen method and Schnabel (1938) method could be used. Both models are useful when estimating population size in closed populations (populations of *Prestonella* are presumably closed due to the high levels of genetic divergence between them). The Petersen method is a simple mark-and-recapture method based on a single episode of marking animals and a second single episode of recapturing individuals. The Schnabel (1938) method is an extension of the Petersen method and uses multiple sampling to derive an estimate of population size. A more sophisticated method such as the Jolly-Seber method could be used which allows for both death and immigration (although immigration is highly unlikely for *Prestonella*) (Jolly 1982). Although this method requires a large number of parameters to be estimated it is easy to apply (Jolly 1982). These methods have been used to estimate population size of different snail species (e.g. *Euchemotrema hubrichti:* Anderson 2007, *Plicopupura pansa:* Michel-Morfin *et al.* 2009), however other studies, certainly more recent ones tend to use more sophisticated and robust models (e.g. *Vespericola cf depressa*: Ramsey and Severens 2010, *Discus macclintocki*: Clark *et al.* 2008).

The fact that, several populations of *P. bowkeri* and *P. nuptialis* are found in National Parks or private game reserves does not guarantee any form of long term protection or conservation. The issue surrounding the conservation of *Prestonella* species is that they are threatened by global climate change, which cannot be simply restricted or prevented as can habitat destruction and over harvesting which are more direct human associated threats. One cannot simply put up a fence to protect species against changing climate. Species threatened by climate change could be moved to new ecosystems through a method known as assisted migration (AM), which aims to save species from the effects of climate change by removing individual plants and animals from an area which has, or will become, unsuitable due to climate change, and moving them to a new site where they have not previously occurred, but where they are expected to survive as temperatures increase (Sax *et al.* 2009). Assisted migration is considered a radical type of human intervention and has become a hot topic in the ecological sciences as well as the biological management community (Schwartz *et al.* 2009). Ricciardi and Simberloff (2009) argue that species translocations are not a viable conservation strategy to deal with threats such as climate change, because conservation biologists lack sufficient understanding of the associated risks, such as biological invasions and ecosystem degradation. Vitt *et al.* (2009), Sax *et al.* (2009) and Schlaepfer *et al.* (2009) disagree with Ricciardi and Simberloff (2009), although they agree that there are risks associated with assisted migration, they suggest that it is still a viable option to save endangered species. The probability of translocated organisms causing significant damage to native species might be overstated by Ricciardi and Simberloff (2009) (Schlaepfer *et al.* 2009) and we know more about the impacts of species invasions than Ricciardi and Simberloff (2009) suggest (Sax *et al.* 2009). Ricciardi and Simberloff (2009) focus on extreme examples of species translocations, including the ‘re-wilding’ of North America with African mammals (Vitt *et al.* 2009). Translocations within a species former range were not included in Ricciardi and Simberloff’s (2009) analyses and are likely to have even lower probabilities of adversely affecting the ecosystem (Schlaepfer *et al.* 2009). Vitt *et al.* (2009) suggests that assisted migration should mimic the natural dispersal of some species across landscapes, tracking the leading edge of the shifting bioclimatic envelopes.

Evidence shows (Chapter 2) that *Prestonella* populations in the driest regions of the Great Escarpment are more genetically depauperate (lower genetic diversity) than populations in less arid environments. Historic climate change has driven genetic erosion and it is likely that future climate change due to global warming will do the same. Low genetic diversity limits evolutionary response and populations *in situ* may not be able to adapt in time in response to global warming. Vitt *et al*. (2009) suggest that assisted migration should only be undertaken if a species is not capable of migration, plastic response or adaptation *in situ*. Since populations of *Prestonella* are not capable of migration and in some cases are most likely incapable of *in situ* adaptation they could be considered as candidates for assisted migration. Populations of *Prestonella* in the western region of the Great Escarpment that are most at risk could be relocated to suitable habitats higher up in the eastern part of the Great Escarpment. Due their low vagility, dispersal capacity and habitat it is highly unlikely that relocated populations will become invasive. There is no strategy that accommodates all species, thus each case requires independent consideration (Vitt *et al.* 2009). Assisted migration could be considered as a method to save *Prestonella* species. However it will need a detailed risk assessment and possibly only considered as a last resort.

Thesis summary

Phylogeographic analyses of *P. bowkeri* and *P. nuptialis* using mitochondrial markers CO1 and 16S indicate that these populations have a high degree of sequence divergence. Likewise, mean F_{ST} values among all populations of *P. nuptialis* and among all populations of *P. bowkeri* are extremely high and significant ($p<0.001$) suggesting substantial structure, fixed differences among populations and strong genetic partitioning. These results indicate a lack of gene flow between populations, a consequence of habitat fragmentation in a montane ecosystem, in association with limited vagility, limited dispersal capacity and specialised habitat requirements (which are not uncommon in land snails).

The phylogenetic analyses reveals that there is no clear dichotomy between the two species and *P. bowkeri* is paraphyletic with respect to *P. nuptialis*, which suggests that the current taxonomy is uncertain, or that the characters on which identifications are based on may well be plesiomorphic in the case of *P. bowkeri*. There may be some evolutionary process at play such as retention of ancestral polymorphisms and the lack of reciprocal monophyly which are confounding the phylogeny. Mean values of Nei's coefficient of gene differentiation G_{ST} between populations of *P. bowkeri* and between populations of *P. nuptialis* are higher than those seen within several other terrestrial snail species, thus genetic markers point to divergences between populations (within so-called species) that are possibly more reminiscent of the divergences normally observed between species. It is possible that confinement to isolated mesic environments on mountain slopes has promoted extraordinary 'within-species' genetic divergence. Which raises the question whether or not these populations divergent enough to be considered as separate species? However, there is some support for a two species model which may be confounded by certain evolutionary processes, hence much of the apparent uncertainly here could result from the fact that we have only used mtDNA and the use of nuclear markers may resolve this issue.

The isolation-by-distance analysis showed that the geographic pattern of genetic population structure does not conform to a stepwise or island-hopping model of haplotype distributions for *P. nuptialis* and *P. bowkeri*. This may be due to simultaneous isolation, saturation in either gene or fixation of ancestral haplotypes; we suggest that the latter is the most likely explanation.

A relaxed Bayesian clock estimates suggest that populations diverged from one another during the mid-late Miocene (12.5-7 MYA). The climate of southern Africa shifted from a more mesic climate to one of seasonal aridity during this period. The divergence of *Prestonella* populations during this period is rooted in vicariance/ fragmentation of suitable habitat as a consequence of climate change.

Assessment of the genetic diversity within populations of *P. nuptialis* and P*. bowkeri* reveal that populations in the western region of the Great Escarpment have less genetic diversity than populations in the east. Correlation are found between genetic diversity (haplotype and nucleotide diversity) and annual precipitation, coefficient of variation of annual precipitation and mean annual potential evaporation, which shows that genetically depauperate populations are found in regions that are drier, have less reliable rainfall and higher potential evaporation, all factors that increase the chance of a population bottleneck. Historical climate change in southern Africa resulted in a shift towards seasonal aridity that has increased the chance of population bottlenecks leading to reduced genetic diversity in the most western populations. If increases in aridity are drivers a genetic erosion, then future climate change will likely adversely affect populations of *Prestonella.* Since these snails have low vagility, limited dispersal capacity and specialised habitat requirements it is unlikely that they will be able to migrate to new habitats. *In situ* adaptation will be necessary for the survival of these populations in the face of global warming. However, several of these populations are already genetically depauperate and the loss of genetic variability can limit the ability of an organism to adapt to a changing environment, thus these populations may face extinction if they cannot evolve fast enough.

Significant differences ($p<0.05$) were seen between the Arrhenius breaking temperatures (ABT's) and flat-line temperatures (FLT's) of three populations of *Prestonella.* The forest population had a lower ABT and FLT than the two thicket populations. Forest understories have reduced solar radiation which makes forest understorey habitats less heat stressed. It is likely that the lower thermal tolerance observed in the forest population is a micro-habitat adaptation to reduced thermal stress in such a forest understorey habitat. The two thicket populations on the other hand are found in exposed thicket habitats where solar radiation and heat exposure is much greater. It is reasonable to hypothesis that they have adapted to an environment that is more heat stressed and explains why these populations have higher thermal limits. Although no correlation was found between ABT and maximum habitat temperatures, it is likely that the differences seen between these populations are due to local micro-climate adaptation, because the temperature measurements used in this study were extracted from GIS maps extrapolated from data obtained from weather stations across the country and are too coarse to reveal actual micro-habitat climates that the snails live in. It is essential to collect micro-habitat specific climatic variables for each population. By using accurate data other trends and correlations may emerge that may better explain our current results.

An IUCN assessment of *P. nuptialis* and *P. bowkeri* suggests that these two species are probably Vulnerable (VU) due to their small population size, small Extent of Occurrence and fragmented/ isolated distributed. However, *P. nuptialis* and *P. bowkeri* are considered Critically Endangered under criterion B2. The issue surrounding the conservation of *Prestonella* species is that they are threatened by global climate change, which cannot be simply restricted or prevented, which makes dealing with the threat of climate change difficult. Assisted migration (MA) may be considered as a method to prevent possible future extinctions of *Prestonella* populations, but will only be considered as a last resort.

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Appendix 1: CO1 and 16S McClade alignments

Co1 sequences

PlacostylusbivaricosusOutgroup GAGGAGCTTCT-
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ATTAGCAGGTGCTATTACTATACTTTTAACAGATCGAAATTTTAATACTTCATTTTTTGATCCAG--
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BeaufortWestRooivallerv1 GCGGAGCAGCC-
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MountainZebraNat1108034
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MountainZebraNat1208034
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MountainZebraNat1mz14
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MountainZebraNat1mz16
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MountainZebraNat2mz21t
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MountainZebraNat2mz23t
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SomersetEast11003
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SomersetEast18003
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SomersetEast19003
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SomersetEast115003
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SomersetEast12003A
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16S Sequences

PlacostylusbivaricosusOutgroup ---
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BeaufortWestRooivallerv1
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FenellaFallsff2
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FenellaFallsff3
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FenellaFallsff7
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FenellaFallsff12
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FenellaFallsW5507
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FortFordycefda
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FortFordycefdl
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GlenAvonFallsga2
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GlenAvonFallsga7
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SomersetEast11003
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SomersetEast16003
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SomersetEast117003
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WilgeRiverCanyon3wr33
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Appendix 2: Phylogenetic trees of the MP, ML, NJ and BI analyses of the CO1, 16S and combined datasets

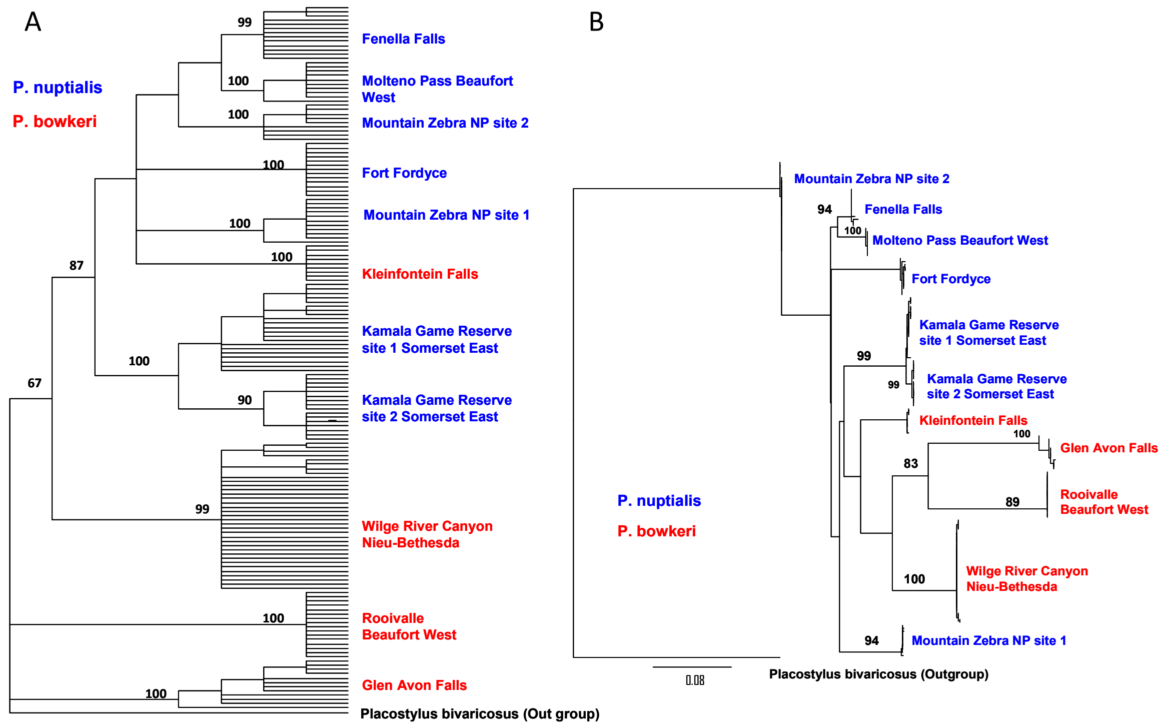

Figure 5.1. A: CO1 dataset: Strict consensus Maximum Parsimony tree. B: CO1 dataset: Maximum Likelihood tree. (Bootstrap support above branches).

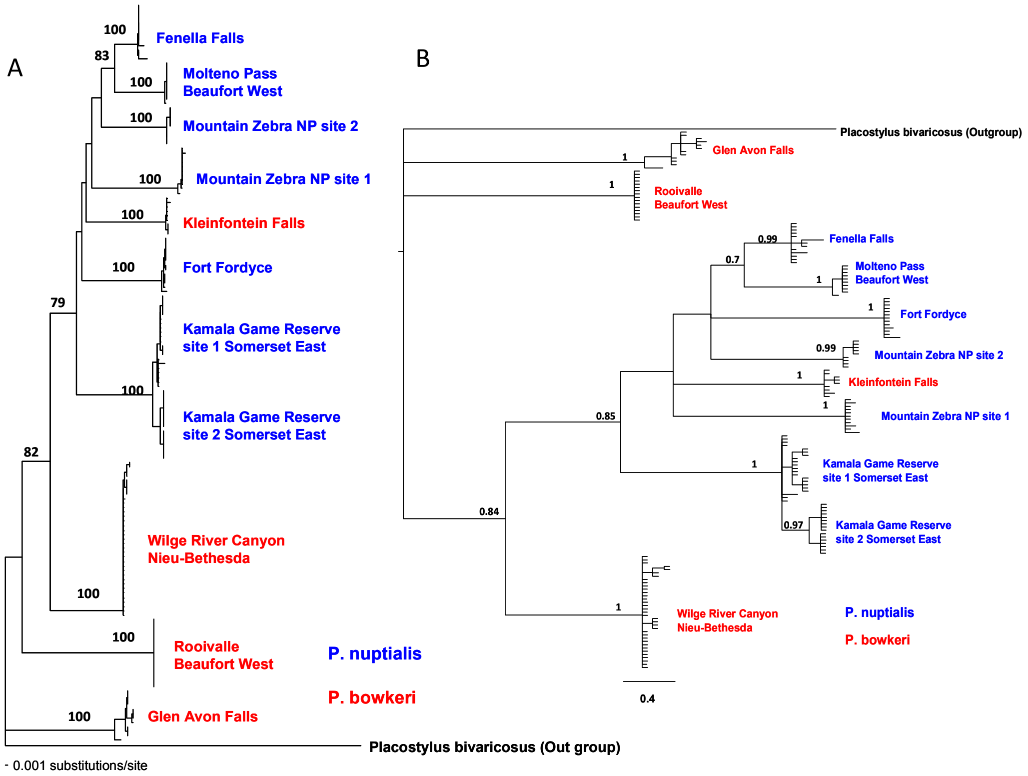

Figure 5.2. A:CO1 dataset: Neighbor-Joining tree. B: CO1 dataset: Bayesian Inference tree. (Bootstrap and Posterior support above branches).

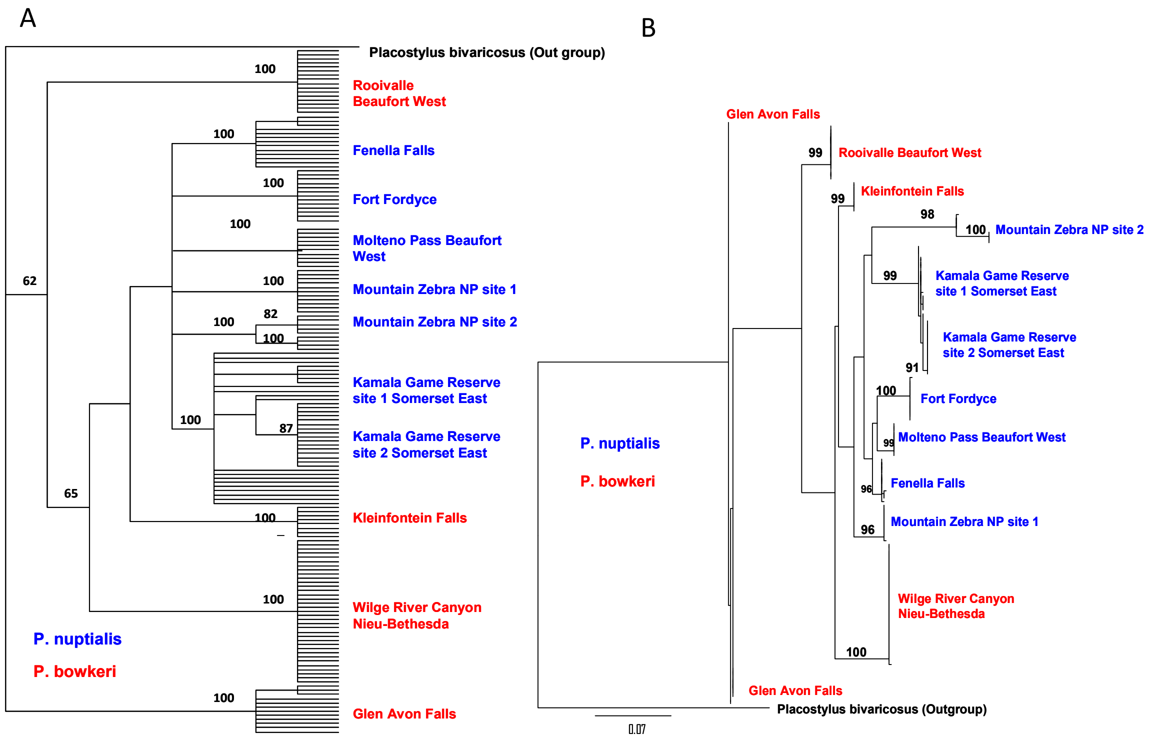

Figure 5.3. A: 16S dataset: Strict consensus Maximum Parsimony tree. B: 16S dataset: Maximum Likelihood tree. (Bootstrap support above branches).

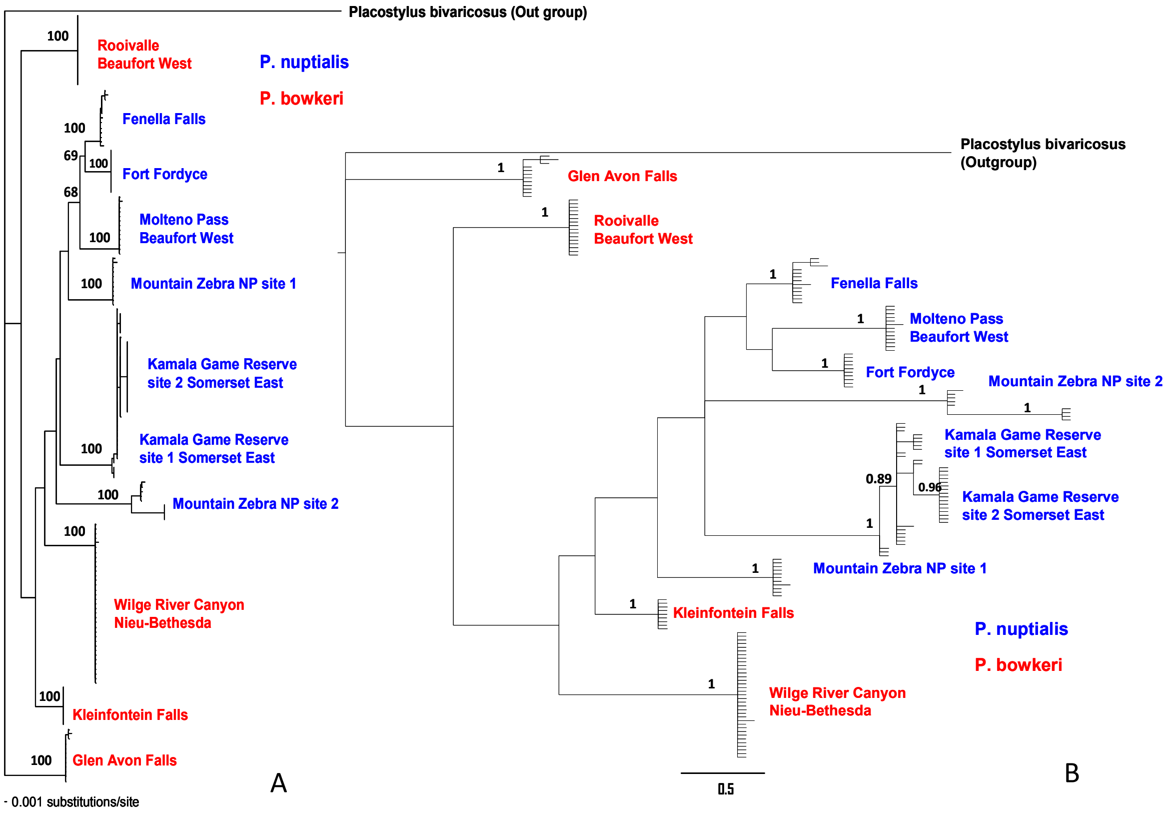

Figure 5.4. A: 16S dataset: Neighbor-Joining tree. B:16S dataset: Bayesian Inference tree. (Bootstrap support above branches).

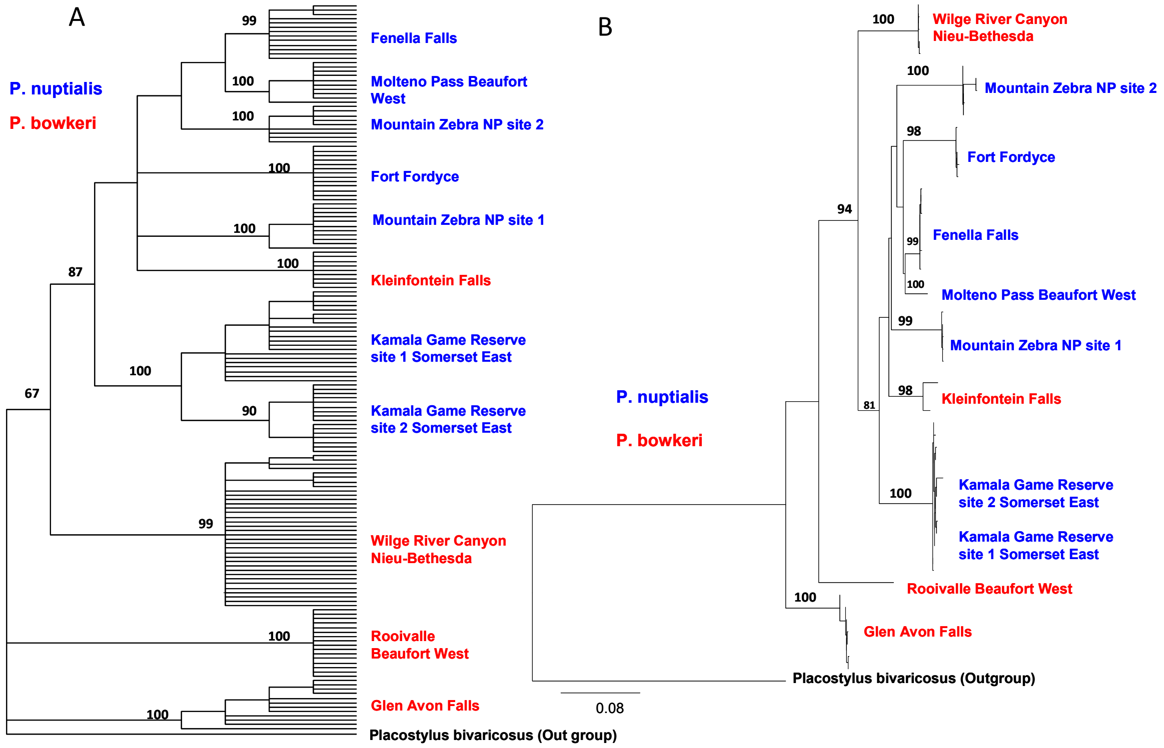

Figure 5.5, A: combined mitochondrial dataset: Strict consensus Maximum Parsimony tree. B: combined mitochondrial dataset: Maximum Likelihood tree. (Bootstrap support above branches).

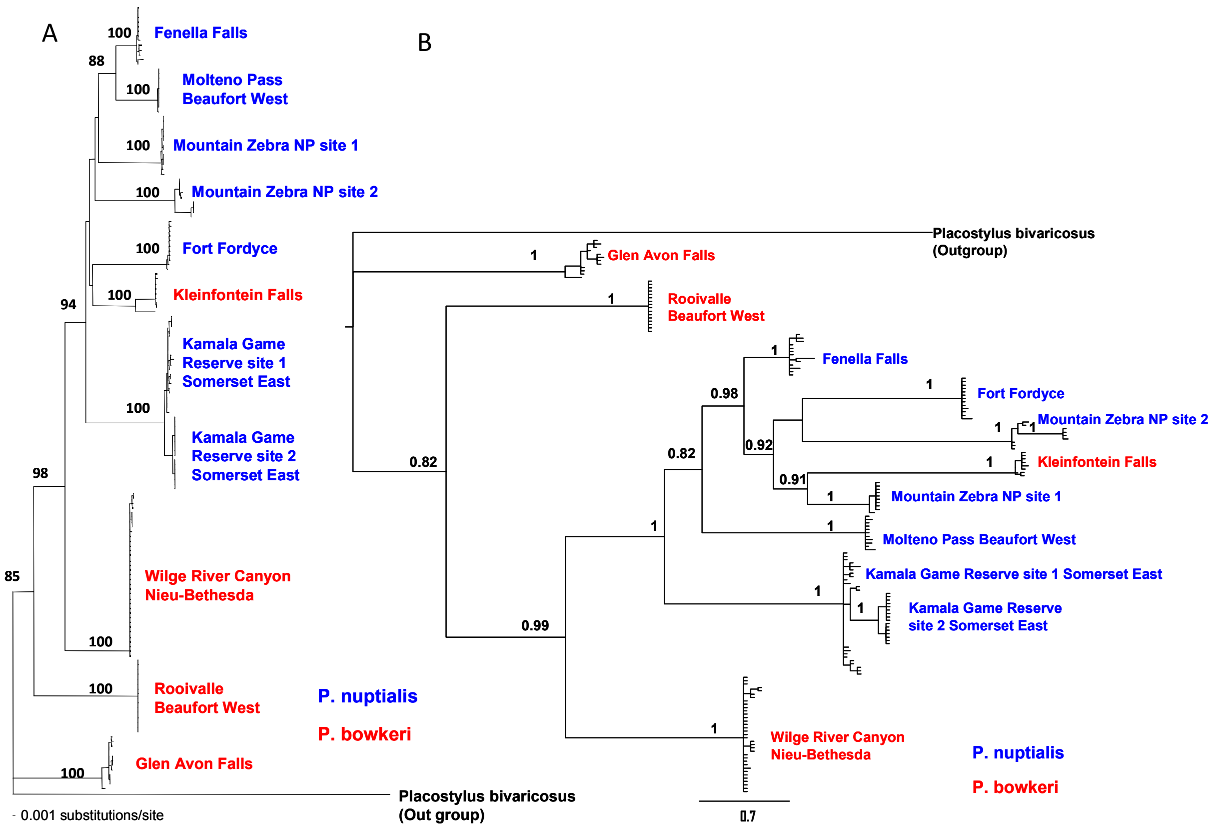

Figure 5.6. A: combined mitochondrial dataset: Neighbor-Joining tree. B: combined mitochondrial dataset: Bayesian Inference tree. (Bootstrap support above branches).

Appendix 3: Saturation Plots for CO1 and 16S

A saturation plot contrasts the phyletic and percentage pairwise genetic distances between pairs of isolates (Vuillaumier *et al.* 1997). The phyletic distance used was the Maximum Likelihood (ML) estimate of genetic distance between two isolates produced in the ML analyses, in nucleotide substitutions per site. The percentage pairwise genetic distance between two sequences was calculated as the percentage of non-gapped nucleotide positions at which the two sequences differed. The slop on a regression line drawn through a saturation plot will have a slope of 1 when phyletic distance and pairwise genetic distance are identical (Bush and Everett 2001). Phyletic distances increase more rapidly than pairwise distances when multiple nucleotide changes per position occur over time (Bush and Everett 2001). As the frequency of multiple mutations per position increases, the correlation between the phyletic and pairwise distance declines (Bush and Everett 2001). Mutational saturation is said to have occurred when there is no correlation between phyletic distance and percentage pairwise genetic distance. An assessment of 20 pairs of isolates for CO1 (Figure 6.1) and 16S (Figure 6.2) show that the regression lines drawn through the saturation plots have slopes almost equal to 1 which suggests that no saturation has occurred.

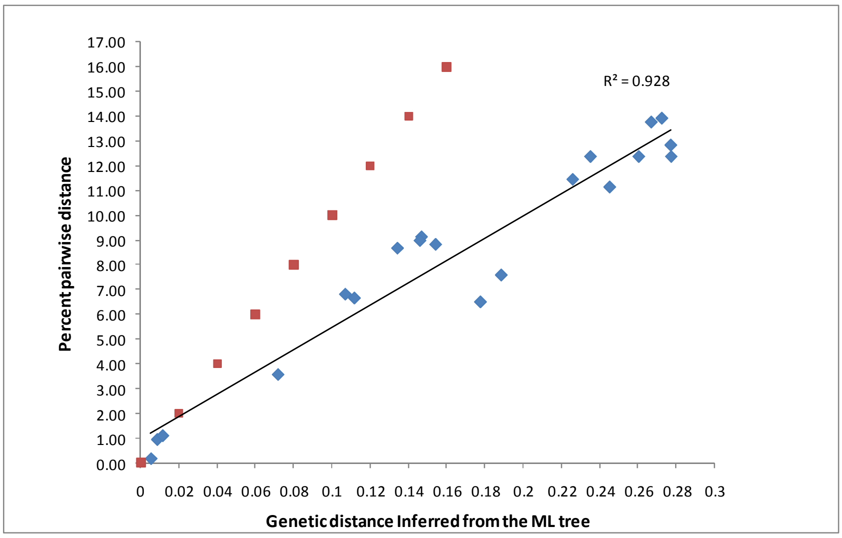

Figure 6.1: Mutational saturation plots contrasting phyletic distance and percentage pairwise genetic distances between 20 pairs of isolates from the CO1 dataset. Phyletic distance used was the Maximum Likelihood (ML) estimate of genetic distance between two isolates produced in the ML analyses, in nucleotide substitutions per site. The percentage pairwise genetic distance between two sequences was calculated as the percentage of non-gapped nucleotide positions at which the two sequences differed. The dashed line represents the ideal case where phyletic distance and percentage pairwise genetic distances are equal.

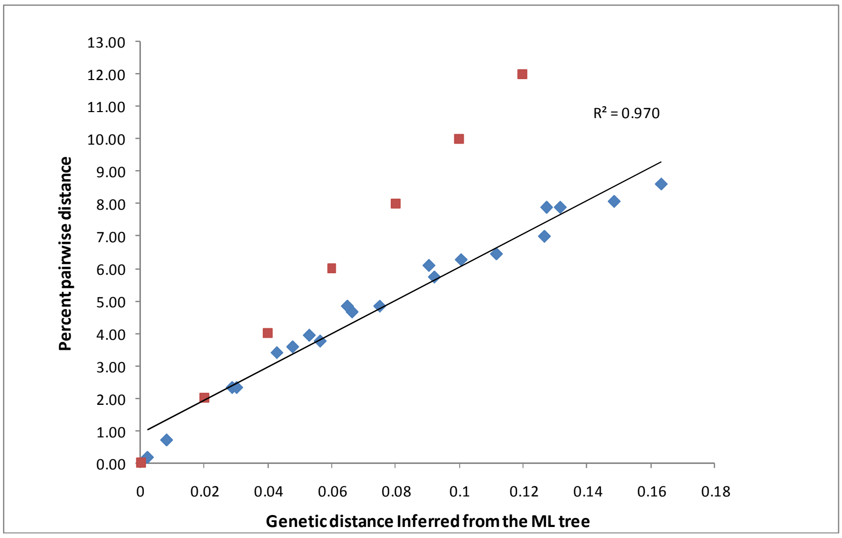

Figure 6.2 Mutational saturation plots contrasting phyletic distance and percentage pairwise genetic distances between 20 pairs of isolates from the 16S dataset. Phyletic distance used was the Maximum Likelihood (ML) estimate of genetic distance between two isolates produced in the ML analyses, in nucleotide substitutions per site. The percentage pairwise genetic distance between two sequences was calculated as the percentage of non-gapped nucleotide positions at which the two sequences differed. The dashed line represents the ideal case where phyletic distance and percentage pairwise genetic distances are equal.

Appendix 4: Graphs showing correlations between climatic variables and thermal tolerance (ABT and FLT) of *Prestonella* populations.

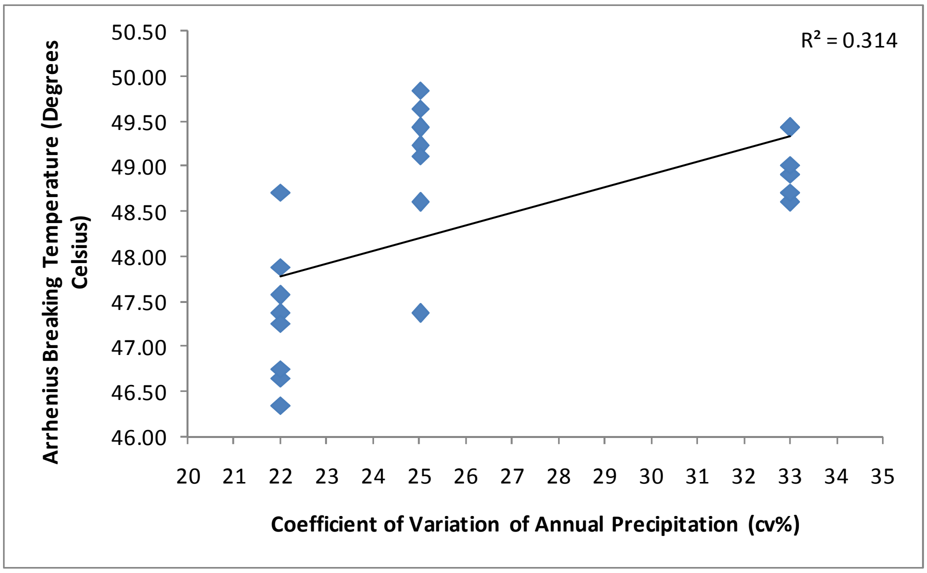

Figure 7.1: Correlation between Arrhenius Breaking Temperature and Coefficient of Variation of Annual Precipitation for individuals from *Prestonella* populations.

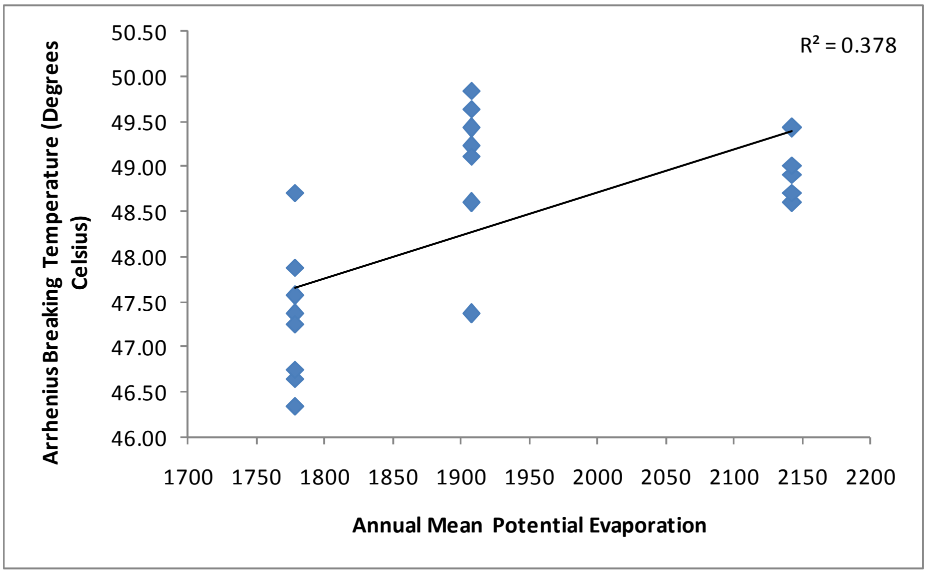

Figure 7.2: Correlation between Arrhenius Breaking Temperature and Annual Mean Potential Evaporation for individuals from *Prestonella* populations.

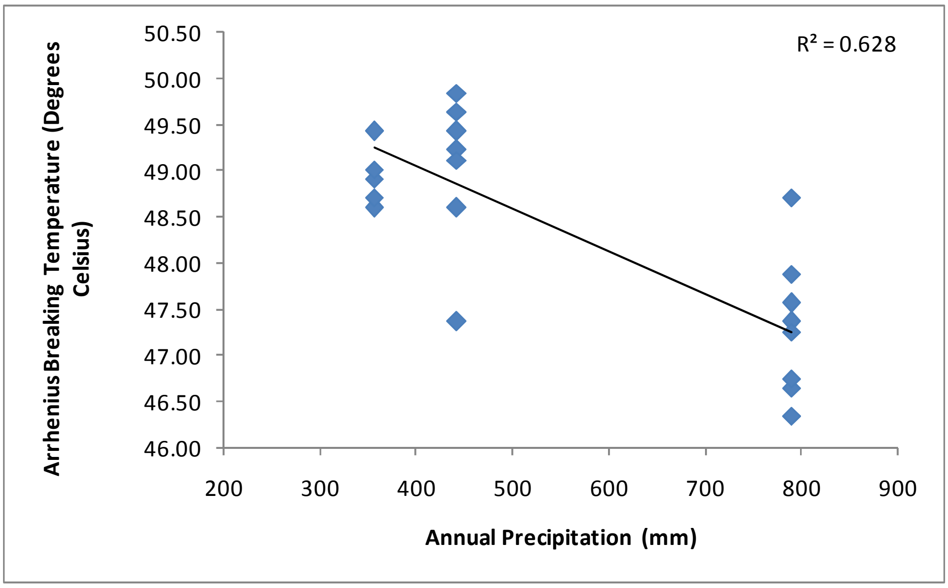

Figure 7.3: Correlation between Arrhenius Breaking Temperature and Annual Precipitation for individuals from *Prestonella* populations.

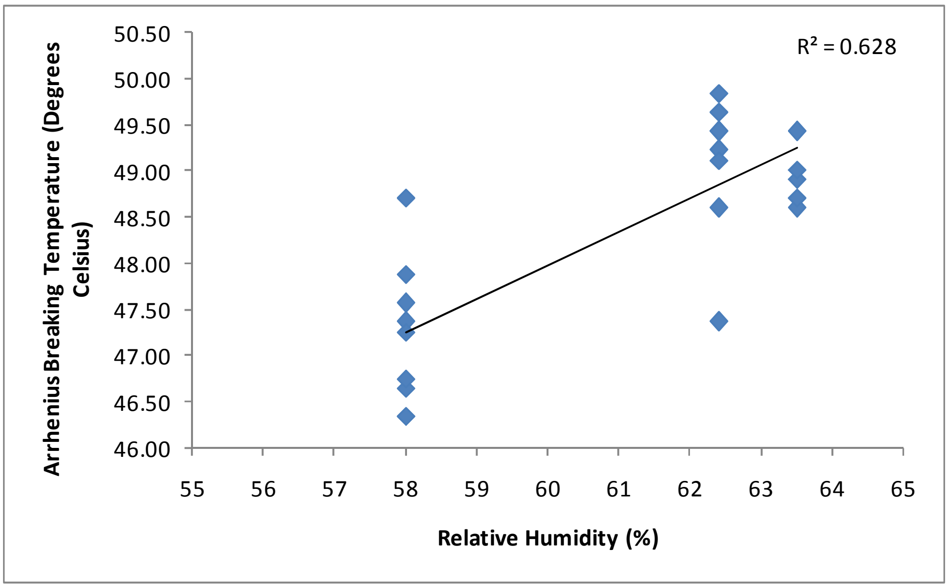

Figure 7.4: Correlation between Arrhenius Breaking Temperature and Relative Humidity for individuals from *Prestonella* populations.

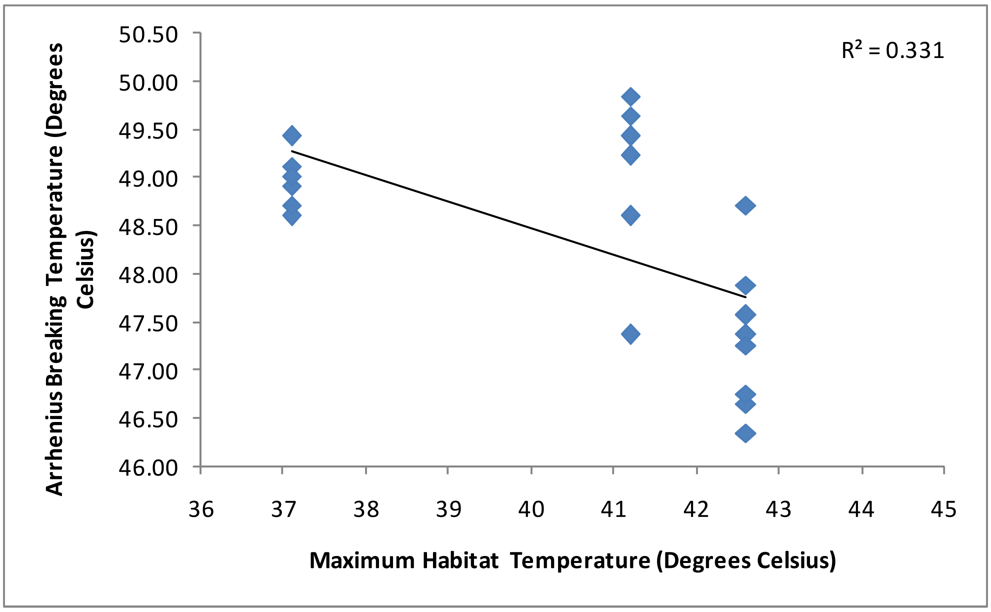

Figure 7.5: Correlation between Arrhenius Breaking Temperature and Maximum Habitat Temperature for individuals from *Prestonella* populations.

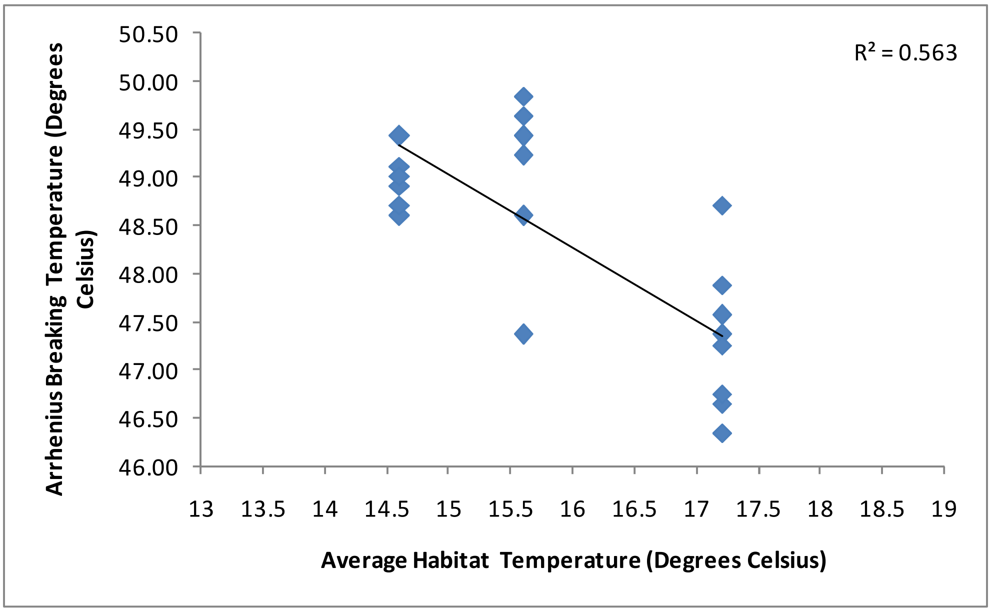

Figure 7.6: Correlation between Arrhenius Breaking Temperature and Average Habitat Temperature for individuals from *Prestonella* populations.

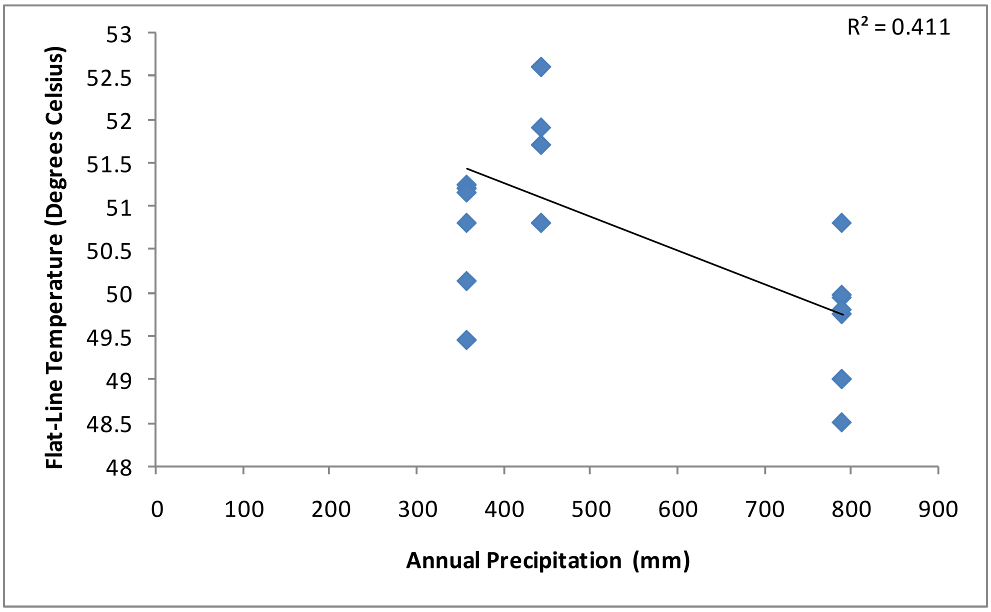

Figure 7.7: Correlation between Flat-Line Temperature and Annual Precipitation for individuals from *Prestonella* populations.

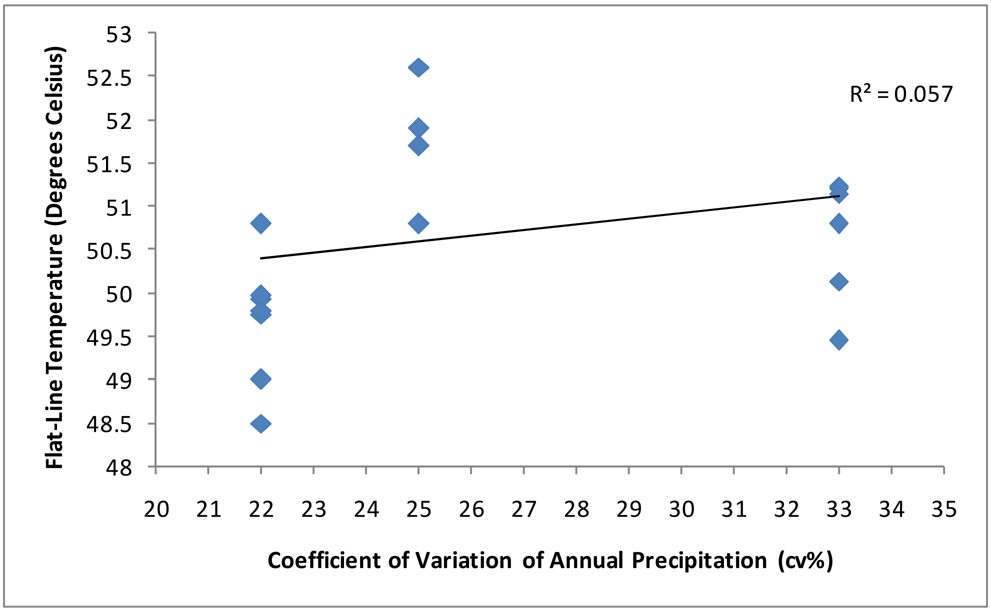

Figure 7.8: Correlation between Flat-Line Temperature and Coefficient of Variation of Annual Precipitation for individuals from *Prestonella* populations.

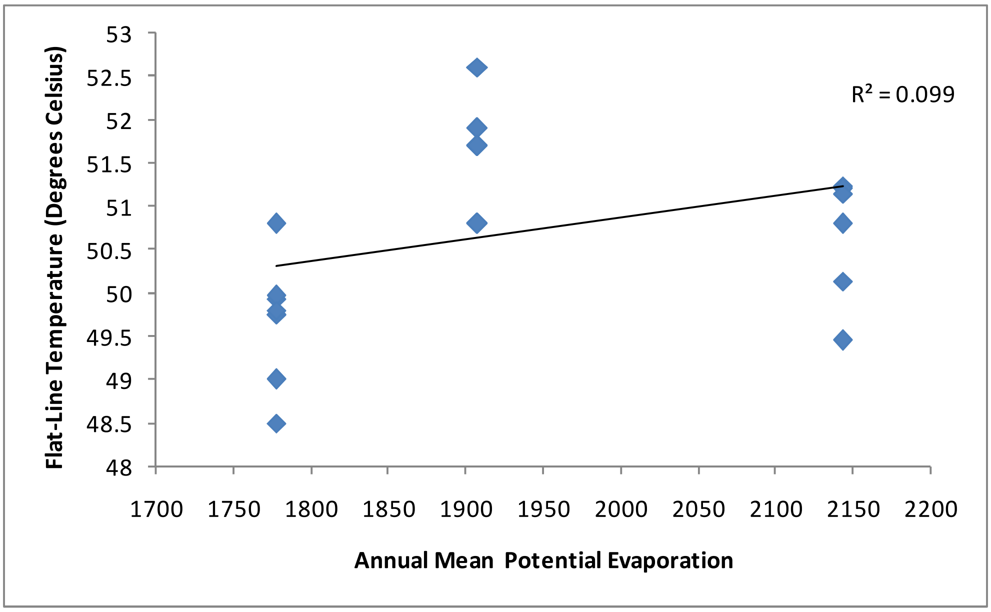

Figure 7.9: Correlation between Flat-Line Temperature and Annual Mean Potential Evaporation for individuals from *Prestonella* populations.

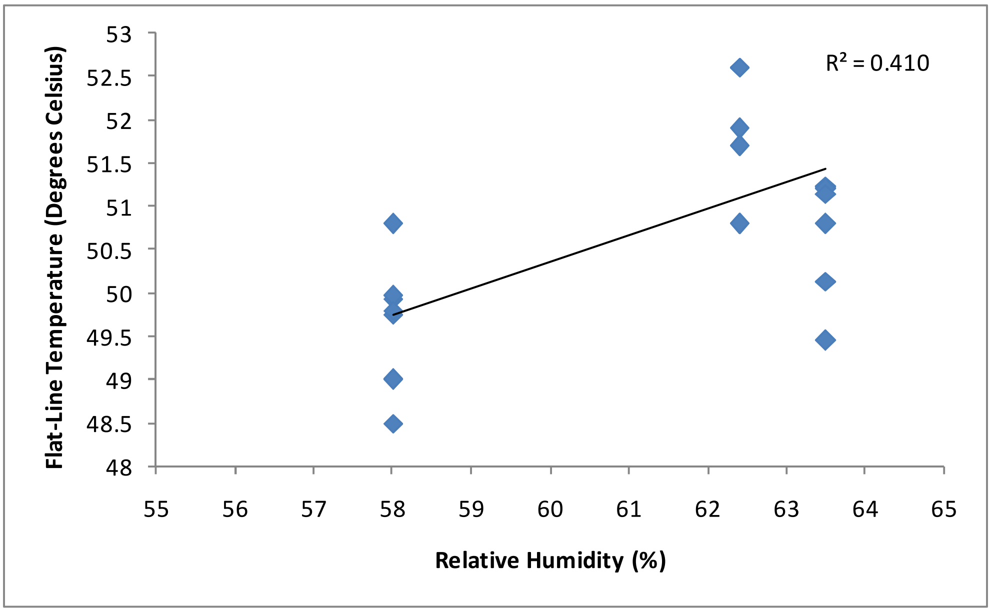

Figure 7.10: Correlation between Flat-Line Temperature and Relative Humidity for individuals from *Prestonella* populations.

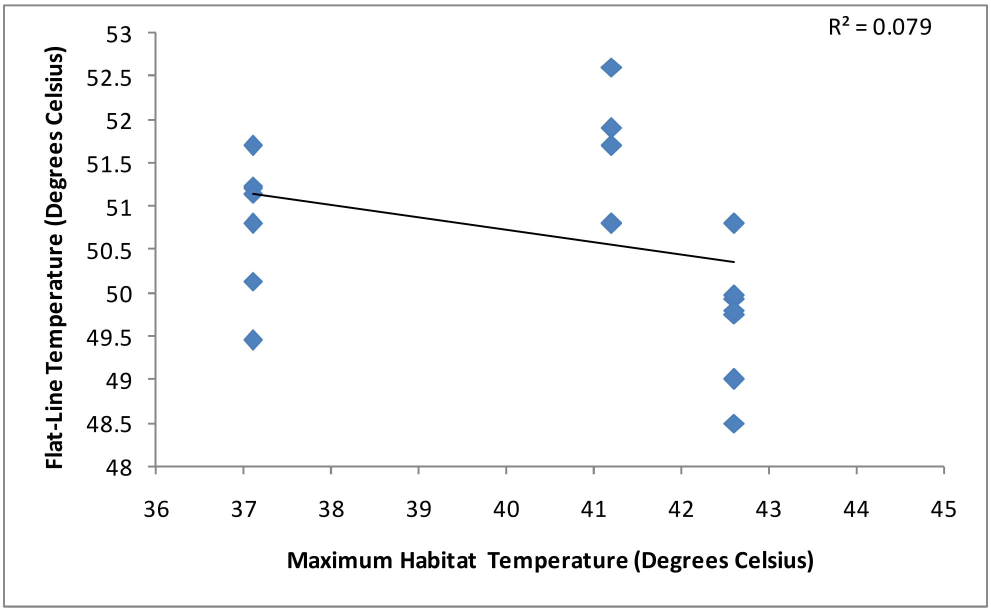

Figure 7.11: Correlation between Flat-Line Temperature and Maximum Habitat Temperature for individuals from *Prestonella* populations.

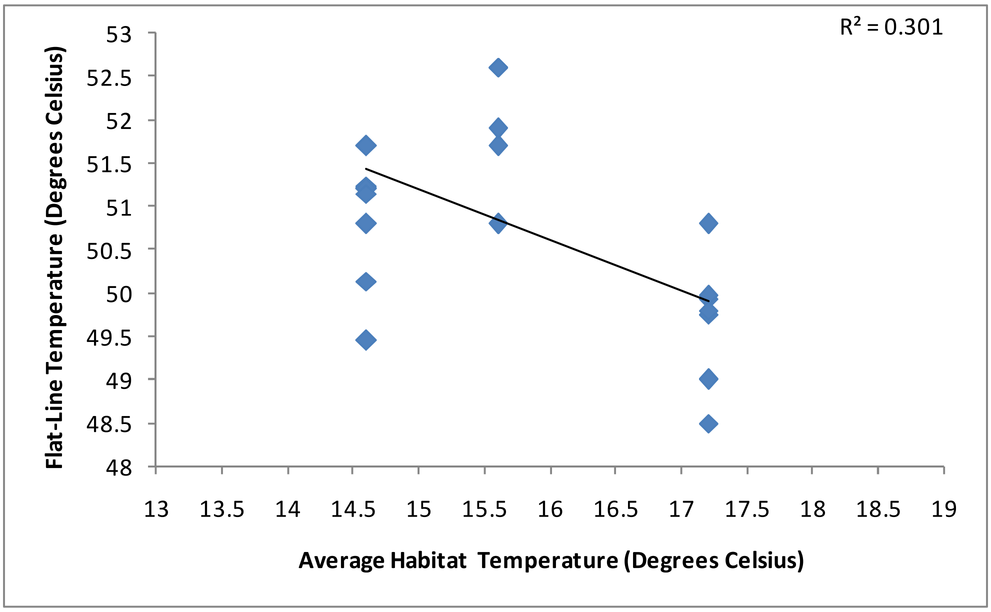

Figure 7.12: Correlation between Flat-Line Temperature and Average Habitat Temperature for individuals from *Prestonella* populations.