Phylogeography of *Scarabaeus (Pachysoma)* Macleay (Scarabaeidae: Scarabaeinae).

By

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To David, Gillian, Michael and Ian with love……

"Do molecules and morphology give the same picture of the history of life, or two or more distorted views of the same picture, or two quite different pictures?"
Patterson (1988)

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Abstract

Scarabaeus (Pachysoma) consists of 13 flightless dung beetle species endemic to the arid west coast of southern Africa. *Scarabaeus (Pachysoma)* are unique in their feeding and foraging habits, in that they randomly search for dry dung/detritus which, when found, is dragged forwards, and buried in a pre-constructed holding chamber, as opposed to the convention of rolling it backwards. This action is repeated to provision the chamber after which the nest is expanded to below the moisture line to allow the stored food to re-hydrate. Poor vagility, taxonomic contention - seen in *Scarabaeus* taxonomy - and conservation concern, made *Scarabaeus (Pachysoma)* an ideal group of beetles to study both the phylogenetics and potential influences that anthropogenic and environmental changes have had on structuring the species and populations thereof.

Both molecular and morphological data were used as individual datasets and combined in a total evidence approach. Biogeographic inferences were made based on recent detailed Namib biogeography and the ages of the species were estimated using the molecular clock method. A phylogeographic study was done on three of the species of *Scarabaeus (Pachysoma)* – *S. (P.) hippocrates*, *S. (P.) gariepinus* and *S. (P.) denticollis* - that had previously shown south-north morphological clinal variation. Lastly, an attempt was made to isolate microsatellite loci for *Scarabaeus*, in the hope of characterising genetic diversity within and between populations of the same species.

Scarabaeus (*Pachysoma)* was found to be monophyletic within *Scarabaeus* and was therefore classified as a derived subgenus thereof. Morphologically *Scarabaeus (Pachysoma)* was shown to have 13 species while at a molecular level strong resolution for 11 of the 13

was obtained. *S. (P.) hippocrates* and *S. (P.) glentoni* formed a species complex the hippocrates/glentoni complex. The combined phylogenetic tree showed good overall support for all 13 species. Both the morphological and molecular data partition phylogenies show congruence with the combined phylogeny, lending support for combining datasets.

Scarabaeus (Pachysoma) appears to have arisen 2.9 million years ago. The formation of advective fog is a consistent water source for Desert dwelling organisms and appears to be associated with *Scarabaeus (Pachysoma)* radiation into inhospitable areas. Analysis of gene flow revealed large amounts of south-north movement, lending support for movement of psammophilous taxa with their substratum, the barchan dune.

Population demographics of the three species, *S. (P.) hippocrates, S. (P.) gariepinus* and *S. (P.) denticollis*, chosen for this study differed greatly except in areas of geographic similarity. Major rivers appear to have acted as gene barriers, allowing for distinct genetic entities to be identified within the three species. Phylogeographic partitioning was supported by an AMOVA analysis. All three species were shown to have undergone historical population expansion dating back to the Pleistocene era. Nested Clade Analysis indicated that allopatric speciation; isolation by distance and continuous range expansion could be the factors having affected overall population structure. Recent events show that human induced factors, environmental barriers and reduced vagility have influenced the species population structure.

Four potentially polymorphic loci were isolated for *Scarabaeus* using the FIASCO protocol. Identification of at least one additional locus is needed in order to obtain statistical significance for future studies directed at uncovering recent population dynamics.

Keywords: *Scarabaeus*, Cytochrome oxidase I, Morphology, Phylogeny, Combined, Phylogeography, Namib Desert, Total Evidence, Microsatellites, Coleoptera

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Disclaimer

The present study is a continuation of a study done by James du Gueslin Harrison (1999), all the morphological data was provided by him. Each of the chapters within this study, except for Chapter 5, have been written up in paper format for different journals, hence the format for each chapter may differ slightly.

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Chapter I

General Introduction:
Rationale for investigating the phylogeny and phylogeography of *Scarabaeus (Pachysoma)* MacLeay (Scarabaeidae: Scarabaeinae).

Conservation Genetics, Pattern and Process

"The overarching aim of conservation biology is to protect biological diversity and the processes that sustain it in the face of perturbations caused by human activity," (Moritz, 2002). Challenges we face are therefore threefold, 1) our knowledge of pattern and process is incomplete, 2) natural and anthropogenic change are bound to occur within a system and 3) conflict between human societies and biological needs is inevitable and reconciliation will only be achieved through trade-offs and priority setting (Moritz, 2002).

Conservation biology is therefore aptly described as a "crisis discipline." The magnitude of this crisis is evident by the large number of species being endangered or facing extinction. Presently 713 species are categorised as extinct/extinct in the wild, 5483 species are classified as critically endangered, endangered or vulnerable and 12,716 species as lower risk/conservation dependent, near threatened, data deficient and least concern (according to IUCN redlist of Threatened Status Category (2005): Summary for all Classes and Orders: www.redlist.org). In an attempt to prevent crisis management we need to understand the patterns and processes that conservation biology aims to describe by including detailed and comprehensive studies of organisms to date (DeSalle & Amato, 2004). The idea, therefore, is that conservation genetics aims at creating an accurate picture of pattern and process in the endangered species.

Conservation biology thus far is expanding to incorporate many disciplines, which allow for conservation biologists to more effectively address critical problems regarding the management of endangered species and critical areas. Genetic information not only allows for many conservation decisions to be placed in context but also adds unprecedented precision and understanding to decision making (DeSalle & Amato, 2004).

The integration of demographic factors (biology of population growth and life history) and genetic approaches often allow for strong inferences to be made regarding conservation biology. Conservation genetics allows for the quantification of processes, such as inbreeding depression, effective population size, minimum viable population size, levels of genetic variation and gene flow, that may all affect endangered populations. Conservation

decisions often rely on the designation of species boundaries, which in itself is a contentious issue in both systematic and evolutionary biology. The delineation of conservation units - environmentally significant units (ESU's) and management units (MU's) (Moritz, 1994a & b) may help designate conservation priorities and are thought to be of paramount importance while other units such as semi-species, incipient species and subspecies are thought to be of lesser concern due to high levels of confusion surrounding their definitions. The challenge in conservation genetics is to firstly integrate the genetic data with both biological and non-biological data and secondly to use the results obtained from these studies in the implementation of a successful conservation decision in the context of social, cultural and political issues.

Phylogeography, Molecules and Morphology

Phylogeography is the study of genes and geography. By overlaying molecules and geographic data over time and space, historical inferences about evolutionary processes at the population level can be inferred (Avise, 2000). Inferences include the restriction of gene flow by geographical and historical barriers, colonisation success of some lineages and the effects of population bottlenecks (Diniz-Filho *et al*., 1999).

Phylogeography, by revealing divergent evolutionary lineages often overlooked by traditional taxonomy and by identifying biotic processes, can help direct conservation biology (DeSalle & Amato, 2004). A crisis discipline often sees periods of expansion for tools used to solve problems that the crises pose. Proliferation of the technologies for genomics, systematics and population biology over the past decade has been a key factor for the integration of genetics into conservation biology (DeSalle & Amato, 2004).

DNA sequence data from the mitochondrial genome are being increasingly used to estimate phylogenetic relationships between taxa. The use of DNA sequence data provides an empirical means of understanding the processes governing the evolution and inheritance of DNA. Mitochondrial genes are chosen for study as they are easy to manipulate, clonally inherited, single copy, non recombining and abundant (Simon et al., 1994). Accurate estimates of species limits are imperative for biodiversity assessments especially in areas of endemism. Species are the basic units of biodiversity on which evolutionary biology focuses (Puorto *et al*., 2001). Given the fact that morphology and molecules evolve at different rates, these characters within the same taxa will have been exposed to similar vicariant biogeography as well as climatic changes and will therefore exhibit similar histories.

The overall availability of the number and diversity of characters is increasing at a remarkable rate in phylogenetic studies. How, therefore, to successfully integrate molecular and morphological data is one of the challenges of phylogenetics today. Different data sets often exhibit similar topologies with differences restricted to the positions of a few taxa, so may tell us different stories (Baum, 1992; Marshall, 1992). Three approaches have been suggested when combining datasets: (1) separate analysis, where trees are estimated separately from each partition, and the different estimates compared using taxonomic congruence (Miyamoto & Fitch, 1995); (2) the total evidence approach, whereby all available data are combined in a simultaneous analysis (Kluge, 1989); and (3) conditional data combination, whereby only homogenous data partitions (estimated by a statistical test of homogeneity) are combined in a simultaneous analysis (Bull *et al*., 1993; de Queiroz *et al*., 1995). It is desirable to know, when combining data sets, how each data partition contributes to the final tree topology. This can be achieved by comparing the overall tree topology with the individual trees of each data partition (Creer *et al*., 2003).

Inferences in evolutionary history are often based on the determination of genetic relatedness among individuals and the extent of the differences between them. The patterns of relatedness are often a result of processes occurring over two time scales: evolutionary time that encompasses broad-scale changes in prevailing environmental conditions, and ecological time over which population processes (e.g. migration, local extinction and colonisation) occur (Martin & Simon, 1990). Evolutionary biology, therefore, aims to unravel these interactions and assess the importance of short- and long- term processes. Understanding of evolutionary processes can be brought about by the study of closely related taxa representing a spectrum of divergence levels (Martin & Simon, 1990).

Genetic structure of a population is generally a result of both biogeographical factors and ongoing ecological and demographic processes (Carisio *et al*., 2004). Our understanding of species formation from an evolutionary paradigm is based on the foundation of population level comparisons. By examining the variation among populations, their historical associations and the processes of genetic restructuring, what may have lead to speciation can often be revealed (Wright, 1931).

Scarabaeus (Pachysoma) MacLeay (1821)

Dung beetles are probably the first insects to be considered divine. In ancient Egypt the beetles were worshiped in the form of the solar deity, Khepera who controlled the sun's daily path across the sky, where the beetle represents the sun god 'Ra' and the ball the sun moving

across the sky (Forgie, 2003). The rolling of the dung ball is one of the methods used by dung beetles to move and process dung. Dung represents a patchy, ephemeral and limited food source. These characteristics would have been the most probable factors allowing for the diversity in morphology, behaviour and ecology presently seen. Although many species form balls and roll them backwards with their hind limbs exceptions to this exist in that some species drag preformed dung pellets/detritus forward (Scholtz, 1989; Philips *et al*., 2002) while others may carry dung pellets with their front legs and sometimes heads (Halffter & Matthews, 1966; Zunino *et al*., 1989; Philips *et al*., 2002).

Scarabaeus (Pachysoma) is a subgenus of the Scarabaeini (Scarabaeidae: Scarabaeinae), a tribe whose members are found in moist savanna through to drier regions including very hot dry deserts (Scholtz, 1989) of the Afrotropics and southern latitudes of the Palaearctic. Scarabaeines predominantly feed on dung, but have also been known to feed on humus, carrion and fungi (Scholtz & Chown, 1995). Scarabaeini are one of 12 tribes in the Scarabaeinae that are differentiated in part by behavioural trichotomy between those that breed inside the dung pad (endocoprids), those that bury the dung in preformed burrows at the food source (paracoprids), and those that remove the dung and bury it some distance from the food source (telecoprids) (Balthasar, 1963; Halffter & Edmonds, 1982; Scholtz & Holm, 1985; Hanski & Cambefort, 1991).

Members of *Scarabaeus* (*Pachysoma)* are flightless and have feeding and foraging adaptations that are unique within the Scarabaeinae (Scholtz, 1989). *Scarabaeus* (*Pachysoma)* is an exception to the conventional backward dung ball rolling of the Scarabaeini. The beetles randomly move in search of dry dung pellets or plant matter (detritus) which, when found, is gathered up and held in the long comb-like setae on the hind limbs and dragged forwards to be buried in a preconstructed holding chamber (Scholtz, 1989; Harrison, 1999). This is repeated to provision the holding chamber. The nest is then expanded to below the moisture line (Scholtz, 1989). Moisture from the surrounding soil re-hydrates the stored food supply making it suitable for consumption.

The Namaqualand and the Namib Desert

Scarabaeus (Pachysoma) distribution extends from just north of Cape Town, in South Africa, to Walvis Bay, in Namibia and encompasses three distinct biomes. The southern tip comprises the western extreme of the fynbos biome, the area up to the Orange River is geographically considered to be Namaqualand and the section north of the Orange River to Mossamedes in Angola is considered Namib Desert (van Zinderen Bakker, 1975; Rutherford

& Westfall, 1994; Pickford & Senut, 1999). Widespread aridity on the west coast of Africa is related to the up welling of cold surface water, the Benguela Current, and the continental rain shadow – rain originates from moist air blown in from the Indian Ocean, east coast. Aridity becomes more intense as one moves northward, culminating in the Namib Desert (Tankard & Rogers, 1978). Rainfall is minimal but constant moisture is available to the fauna and flora through the formation of coastal fog banks, which are wind blown up to 50km inland (Logon, 1960; Seely & Louw, 1980). Presently the Namib Desert is one of the driest parts of the African continent and from a taxonomic point of view one of the richest deserts in the world (van Zinderen Bakker, 1975). The evolutionary processes resulting in the great number of endemic taxa points to a great age of the Namib with an undisturbed climatic history (van Zinderen Bakker, 1975). However some physical, chemical and biological attributes suggest that the aridity is youthful, developing progressively since the Miocene (Tankard & Rogers, 1978), indicating that relatively rapid radiation has occurred in most taxa found in this area.

Adaptations to the desert and flightlessness

The evolution of flight is thought to have contributed to the diversity and evolutionary success of insects. Flight allows for certain benefits including dispersal, the successful searching for mates, food and habitats (Roff, 1990, Scholtz, 2000). Contrary to these benefits certain species have secondarily become flightless (Scholtz, 2000). Some of the factors said to influence flightlessness are habitat persistence or environmental heterogeneity, geographic variables, alternative modes of migration and taxonomic variation (Roff, 1990).

Deserts are thought to pose considerable constraints on organisms occurring there. Many morphological, behavioural and physiological adaptations exist within desert animals permitting them to survive under harsh conditions. For all desert arthropods living in arid environments life is complicated by being small and having a relatively large surface area, which in turn leads to rapid exchange of heat and water with the surrounding area (Nicolson, 1990).

A possible physiological advantage of wing loss is that it allows an insect to divert energy associated with the wing and wing muscle development to some other use such as increased fecundity. Wing muscles are relatively massive structures within insects comprising 10 – 20% of the body mass of most insects (Roff, 1990). It has been shown that many insects histolyse their wing muscles during egg production, leading us to believe that this is a means to increase egg/sperm/offspring production, thereby increasing their overall fecundity (Roff, 1990).

Morphologically, flightlessness is associated with a secure joining of the elytra along the midline. The fusion of the elytra creates a hermetically sealed chamber called the subelytral cavity (Byrne & Duncan, 2003). This fusion of the elytra is thought to be a modification to prevent water loss through evaporation (Chown *et al*., 1998; Scholtz, 2000). Many desert beetles have a subelytral cavity with representatives being found in tenebrionids, carabids and scarabs (Byrne & Duncan, 2003). Flightless beetles have been shown to exhibit unidirectional tidal airflow (forward airflow, i.e. airflow from the posterior to the anterior body) as opposed to the previously thought convention of respiratory airflow moving from the anterior to posterior of the body (Duncan, 2003). The combination of tidal airflow and a subelytral cavity has allowed for arid-dwelling beetles to reduce water loss by releasing respiratory CO_2 via a single mesothoracic spiracle into the atmosphere (Byrne & Duncan, 2003). In this way water loss is, therefore, confined to a small area of the total respiratory system, with beetles losing up to 4% total water as opposed to 74% if all the spiracles were exposed to the atmosphere (Duncan, 2002; Duncan, 2003).

The species of *Scarabaeus* (*Pachysoma)* feed on dry rodent or herbivore pellets and/or detritus. Due to the dryness in the desert, rates of decay are slowed down considerably so insects feeding on detritus, carcasses or the persistent parts of desert plants have their food sources persist for long periods of time (Roff, 1990; Scholtz, 2000). *Scarabaeus* (*Pachysoma)* beetles drag the dry dung or detritus to below the moisture line allowing for re-hydration (Scholtz, 1989). Most beetles do not take advantage of the hygroscopic water absorption by detritus as they feed only during the day, in which the detritus has only 2% water content. If the beetles were to feed on the detritus when the fog was present they could be consuming detritus containing 60% water (Nicolson, 1990). This could be one of two reasons for *Scarabaeus* (*Pachysoma)* beetles dragging the dry dung or detritus to below the moisture line prior to feeding on it. Another reason for feeding below the moisture line could be that they are dependent on micro-organisms such as fungi and bacteria in the dry dung or detritus for food but these need moisture for development (Scholtz pers. comm.).

Systematic concerns

The diversity we see today and the uniqueness of its components is one of the more remarkable aspects of life. No two individuals in a sexually reproducing population are the same, nor are any two populations, species or higher taxa. According to Mayr & Ashlock (1991), '*Taxonomy is the theory and practise of classifying organisms'* and much, if not all, biological research is based on a sound phylogeny. Taxonomy *s.l.* serves not only to identify

and classify organisms but also allows for the comparative study of organisms as well as the role of lower and higher taxa in nature and evolutionary history (Mayr & Ashlock, 1991). Delimiting a species is important for understanding many evolutionary mechanisms and processes. Species are also used as the fundamental units of analysis in biogeography, ecology, macroevolution and conservation biology (Sites & Marshall, 2003). Two goals for systematic studies are to: 1) discover monophyletic groups at higher levels and 2) discover lineages (i.e. species) at lower levels (Sites & Marshall, 2003). A good phylogeny is therefore of paramount importance if good phylogeographic and population studies are to follow.

The genus *Pachysoma* was first described by MacLeay (1821). *Pachysoma* was defined by aptery, absence of humeral calli, semi-contiguous mesocoxae and short mesosterna (Ferreira, 1953). An evaluation by Holm & Scholtz (1979) concluded that these characteristics were either due to convergence or were too variable and inconsistent to use as the justification for a genus. In spite of this its generic status was maintained. The genus was later synonomised with *Scarabaeus* Linnaeus, 1758 by Mostert & Holm (1982). Endrödy-Younga (1989) and Scholtz (1989) questioned the synonymy of *Pachysoma* with *Scarabaeus* as the former have a unique set of morphological and behavioural apomorphies including unique feeding and foraging biology, a rounded body shape due to flightlessness and are restricted to the south-west coast of Africa. In a recent phylogenetic analysis of *Scarabaeus* (*Pachysoma)* by Harrison & Philips (2003) *Pachysoma s.l* forms a distinct clade within *Scarabaeus* and is therefore considered a subgenus thereof.

Relevance of this study

Habitat destruction and or deterioration are arguably the greatest threats to insect diversity (Samways, 1994). *Scarabaeus (Pachysoma)* occurs in the Succulent Karoo, Fynbos and Desert biomes (Rutherford & Westfall, 1994). Within this large range the species exhibit discontinuous distribution owing to their low vagility. Their distribution therefore consists of pockets of isolated populations some of which are threatened by the removal of the natural vegetation for large scale wheat farming in the south-western Cape, commercial development on the West Coast for holiday and recreational purposes e.g. Lambert's Bay and Strandfontein, mining for diamonds and other minerals and by exotic plant invaders e.g. Port Jackson (*Acacia saligna*) and Rooikrans (*Acacia cyclops*), modifying dune systems. Furthermore, some of the species are potentially threatened through their collection and sale to collectors (Harrison, 1999). Therefore, knowledge of their habitat requirements, taxonomy,

behaviour and distribution is of vital importance for the initiation of conservation strategies to ensure their survival.

Key Questions of this thesis:

Given the background above the objectives and key questions of the present study were:

***Chapter 2* - Phylogeography of the Namib Desert dung beetles *Scarabaeus (Pachysoma)* MacLeay (Coleoptera: Scarabaeidae).**

Key Questions

Q1. To resolve the relationships of the 13 species of *Scarabaeus (Pachysoma)* based on mitochondrial cytochrome oxidase I.

Q2. To estimate the divergence times and ages of the species within *Scarabaeus (Pachysoma)* and to relate these to past geological and climatic events

Chapter 3 - **Testing for congruence between morphological and molecular characters of *Scarabaeus (Pachysoma)* MacLeay (Coleoptera: Scarabaeidae).**

Key questions:

Q1. To resolve the phylogenetic relationships between the 13 species of *S. (Pachysoma)* using Parsimony and other methods based on both morphological and molecular data partitions.

Q2. To test for monophyly of *Scarabaeus (Pachysoma)* within *Scarabaeus*

Q3. To test whether there is congruence between the morphological and molecular datasets using the total evidence approach.

Chapter 4 - **Phylogeographic patterns of *Scarabaeus (Pachysoma)* (Coleoptera: Scarabaeidae) inferred from gene genealogies and coalescent theory.**

Key Questions

Q1. To what degree has geographic isolation led to the genetic restructuring between populations of the same species.

Q2. What is the extent of gene flow between populations of the same species and does it correlate with patterns of geographic proximity?

Q3. Where geographically did *Scarabaeus (Pachysoma)* originate and how are the populations of each species related to one another?

Q4 What are the effective/actual population sizes of the species in question?

***Chapter 5* – Isolation of microsatellite markers from *Scarabaeus (Pachysoma)* MacLeay (Scarabaeidae: Scarabaeinae).**

Key Questions

Q1. To successfully optimise the FIASCO enrichment protocol for the genus *Scarabaeus*.

Q2. To design at least five polymorphic microsatellite loci for the genus *Scarabaeus*.

***Chapter 6* – Concluding comments**

Based on the key questions above the essence of this project was three-fold. It was: firstly, to resolve the phylogenetic relationships between the 13 species of *Scarabaeus (Pachysoma)*; secondly, to elucidate phylogeographic patterns of the species through inferences from historical population dynamics; and lastly to identify and delineate genetically meaningful conservation units, environmentally significant units (ESU's) and management units (MU's) (Moritz, 1994a & b) within the different species. This information would be useful for developing sound conservation management recommendations, as they would be based on a good phylogeny with both strong molecular and morphological inferences as well as ecological data.

Thesis outline

Each of the chapters of this thesis has been compiled as a separate paper for publication purposes. Chapter 2 has been published in the Journal of Biogeography and is formatted for the journal. Chapter 3 has been submitted to Molecular Ecology. Chapter 3 and all the other chapters were formatted for Molecular Ecology. Chapter 4 comprises three sub-chapters based on the three species identified for population analysis. At the start of chapter 4 there is a general introduction and methods used for each species, each sub-chapter has a short introduction, results and discussion. Each chapter contains its own set of references and all appendices can be found at the end of the thesis. Both the general introduction and conclusion are tailored from the respective chapters, which give an overview of what to expect within the thesis and what conclusions were drawn.

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Chapter II

Phylogeography of the Namib Desert dung beetles *Scarabaeus (Pachysoma)* MacLeay (Coleoptera: Scarabaeidae)

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Abstract

Aim Namib Biogeography in many instances remains reliant on advanced and detailed systematic studies. This study attempts to combine molecular phylogenetic data, geology and palaeo-climatic data to, firstly, resolve the relationships of the 13 morphological species of *Scarabaeus (Pachysoma)* and, secondly, to relate their evolution to past climatic and geological events.

Location South Africa and Namibia

Methods Sequencing of an 1197 bp segment of the mitochondrial cytochrome oxidase I (COI) gene of the 13 species within *Scarabaeus (Pachysoma)* was undertaken. Analyses performed included Parsimony and Maximum Likelihood as well as imposing a molecular clock.

Results The molecular phylogeny showed strong support for 11 of the 13 morphological species. The remaining two species, *S. (P.) glentoni* and *S. (P.) hippocrates,* formed a complex and could not be assigned specific status on the basis of the COI gene phylogeny. Strong support for the three species formerly classified within the genus *Neopachysoma* was consistently obtained. The subgenus appears to have arisen approximately 2.9 million years ago. Species within the subgenus arose at different times, with the common ancestor to *Neopachysoma* and the hippocrates complex having evolved 2.65 and 2.4 million years ago respectively. *S. (P.) denticollis*, *S. (P.) rotundigenus*, *S. (P.) rodriguesi* and *S. (P.) schinzi* are some of the youngest species having diverged between 2 million and 600 000 years ago.

Main conclusions *Scarabaeus (Pachysoma)* is a derived monophyletic clade within the Scarabaeini. The subgenus appears to be young in comparison with the age of the Namib Desert, which dates back to the Miocene (*ca* 15 Ma). The psammophilous taxa are shown to disperse with their substratum and habitat, barchan dunes. Clear south/north evolutionary gradients can be seen within the species of this subgenus, which are consistent with the unidirectional wind regime. Species with a suite of mostly plesiomorphic characters have a southerly distribution while their derived psammophilous relatives have central to northern Namib distributions. Major rivers such as the Orange, Buffels and Holgat appear to be gene barriers to certain species as well as areas of origin of speciation events.

Keywords Coleoptera, Scarabaeidae, *Scarabaeus (Pachysoma),* Aptery, Endemic, Namib Desert, Biogeography, Phylogeny, Mitochondrial DNA, Cytochrome Oxidase I (COI).

Introduction

Scarabaeus (Pachysoma) MacLeay (1821) represents a group of 13 atypical flightless dung beetle species belonging to the ball-rolling Scarabaeini (Scarabaeidae: Scarabaeinae) that are distributed along the west coast of southern Africa from Cape Town in South Africa (S33°56'-E18°28') to the Kuiseb River (S22°58'-E14°30') in Namibia (Harrison *et al*., 2003). Individual species, however, usually have very restricted distributions. Flightlessness has resulted in atypical morphology in these species such as the absence of humeral calli, semi-contiguous mesocoxae and short mesosterna (Harrison *et al*., 2003). Their biology is also highly unusual as they feed on dry herbivore dung pellets and detritus that they drag forwards (Scholtz, 1989) whereas their *Scarabaeus* relatives form balls from wet herbivore dung, which they roll backwards. Their dung-burial activity also differs from other ball-rolling dung beetles. *Scarabaeus (Pachysoma)* first locate food, dig a burrow, then forage repeatedly using polarized light for orientation (Dacke *et al.,* 2002), until they have collected sufficient dung fragments or bits of detritus. Related rollers locate dung, form a ball at the source and roll it away to be buried in a suitable place. *Pachysoma* species are restricted to sandy coastal habitats whereas *Scarabaeus* species have a much wider habitat tolerance (Harrison & Philips, 2003). These morphological and biological differences have led to contention about *Pachysoma/Scarabaeus* taxonomy over the years. *Pachysoma* has been treated as a separate genus (Ferreira, 1953), as a synonym of *Scarabaeus* (Mostert & Holm, 1982) and more recently, as a result of a morphology based phylogenetic analysis of the tribe Scarabaeini, it has been accorded subgeneric status (Harrison & Philips, 2003). It is hypothesized to be a monophyletic group and sister to the main *Scarabaeus sensu stricto* lineage that radiated in the Namib Desert after the onset of hyper-aridity in the region.

The narrow, low-lying, coastal strip between the Atlantic Ocean and the Great Escarpment of southern Africa (Fig. 1) stretching from Cape Town in the south to the Carunjamba River in Angola (S15°10'00" – E12°15'00") extends over roughly 2000 km of arid, sandy regions and encompasses three distinct biomes (Rutherford & Westfall, 1994). The southern tip of this area comprises the western extreme of the Fynbos Biome and the enormously species-rich Cape Floristic Region. The area up to the Orange River (S28°40' – E16°30'), which divides South Africa and Namibia, comprises elements of the Succulent Karoo Biome, and is geographically considered to be Namaqualand. The area north of the Orange River and stretching

into Angola is treated as Desert Biome and comprises the Namib Desert. Geologically, however, the region from the Olifants River (S31°42' – E18°11') to the Carunjamba River is considered to be the Namib Desert (Pickford & Senut, 1999). All three regions are characterized by a sandy substrate and aridity, which has been maintained by the cold Benguela Current flowing up the west coast of the continent since the Miocene, 15 million years ago (Mya) (Pickford & Senut, 1999). Aridity increases from south to north. The southern half falls in a winter rainfall regime whilst the northern half receives rain in summer. Rainfall, however, is very low throughout the region but moisture is available to plants and animals in the form of regular dense fogs (Seely & Louw, 1980). The whole area is biologically characterized by exceptionally high plant and animal endemicity. Many of the adaptations seen in animals and plants can be attributed to the harsh conditions to which they are exposed.

Namib Desert beetles are amongst the animal groups with high endemicity and with a suite of morphological, behavioural and physiological characters that adapt them to these conditions (Endrödy-Younga, 1982; Crawford *et al*., 1990; Hanrahan & Seely, 1990; Nicolson 1990). Amongst these are several groups of Scarabaeoidea, including *Scarabaeus (Pachysoma)* (Holm & Scholtz, 1979; Scholtz, 1989; Dacke *et al*., 2002; Harrison *et al*., 2003).

The Namib Desert has been an evolutionary hotspot since the Miocene because of dramatic geological and climatic changes that have selected for taxa capable of withstanding hyper-aridity and barren, mostly sandy, landscapes. The area is currently characterized by barren, sand and gravel plains, extensive dune seas and rocky outcrops interspersed by wide beds of ancient rivers. These westward-directed rivers cut deep courses across the Namib, apparently in response to epeirogenic uplift in the Late Tertiary, possibly during the Pliocene 3-5 Mya (Ward & Corbett, 1990). This resulted in the availability of considerable sediment for transporting back onshore under the influence of the southerly palaeo-wind regime and arid climate. Since at least Late Miocene times, southerly winds have dominated the climate of the near shore parts of the southern Namib. Currently these winds are still some of the most persistent on earth (Pickford & Senut, 1999). They have contributed significantly to depositing the massive sea of mobile sands of the Central Namib, the 40 000 km^2 Sossus Sand Formation or, as it is colloquially known, the Namib Sand Sea.

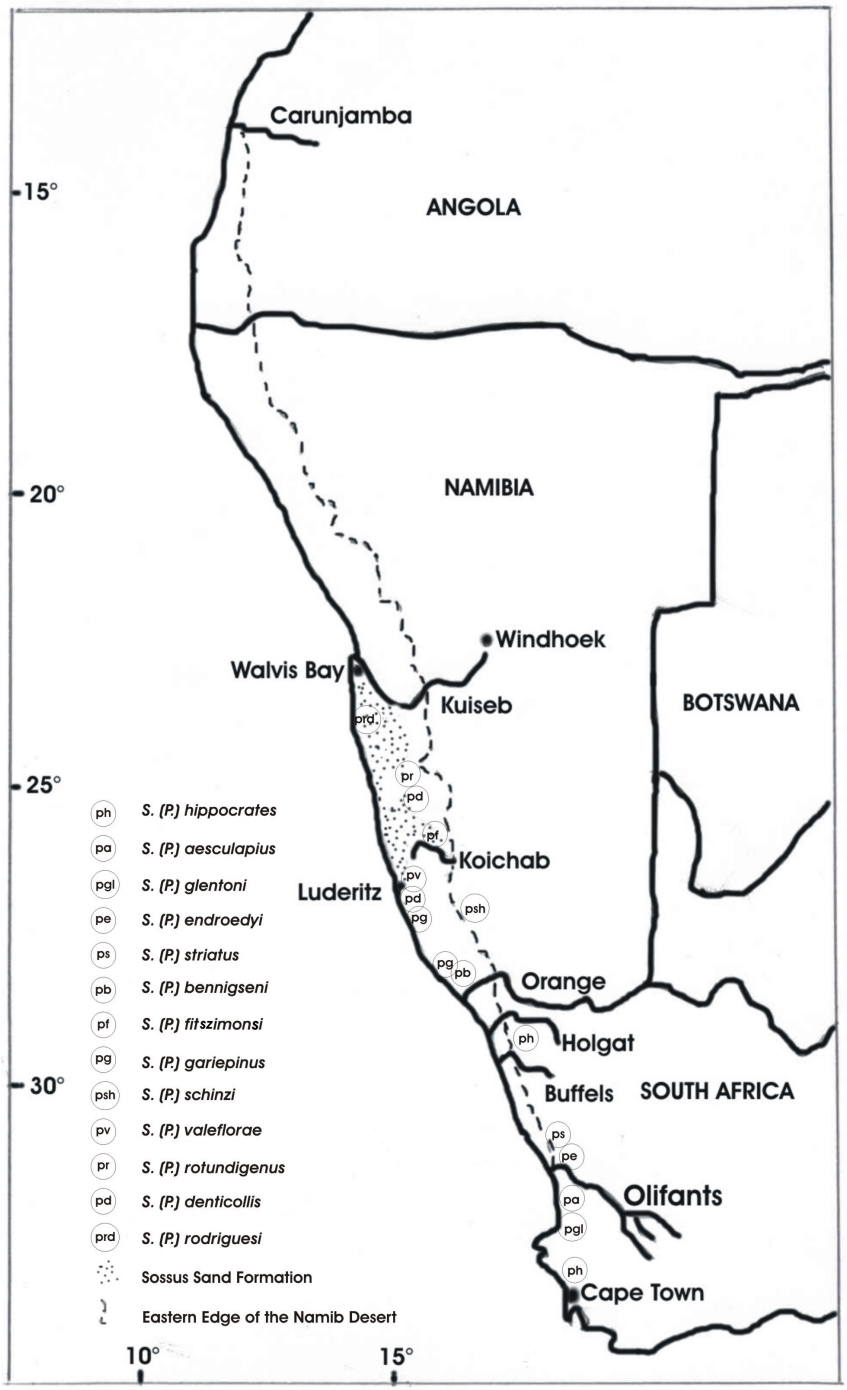

Figure 1. The Namib Desert, extending from the Olifants River, in South Africa, to the Carunjamba River, in Angola, indicating specimen collection sites for this study.

Although a recent morphological phylogeny of *Scarabaeus (Pachysoma)* exists (Harrison & Philips, 2003) it is unable to answer questions regarding the age of lineages or speciation events. However, radiation of the species and their biogeographical history may now be inferred because a comprehensive history of the geology and palaeo-climate of the Namib Desert is available (Pickford & Senut, 1999). In addition, molecular analyses allow estimates of lineage ages by applying a molecular clock (Zuckerkandl & Pauling, 1965; Tajima, 1993). Consequently, this study was aimed at resolving relationships between the 13 morphological species of *Scarabaeus* (*Pachysoma*) at a molecular level and at estimating the divergence times and ages of the species within the subgenus in relation to past geological and climatic events.

Methods

Representative taxa

In-group taxa **-** All 13 species of the subgenus *Pachysoma* were used to infer the phylogeny. These are *S. (P.) aesculapius* (Olivier), *S. (P.) bennigseni* (Felsche), *S. (P.) denticollis* (Péringuey), *S. (P.) endroedyi* Harrison, Scholtz & Chown, *S. (P.) fitzsimonsi* (Ferreira), *S. (P.) gariepinus* (Ferreira), *S. (P.) glentoni* Harrison, Scholtz & Chown, *S. (P.) hippocrates* (MacLeay), *S. (P.) rodriguesi* (Ferreira), *S. (P.) rotundigenus* (Felsche), *S. (P.) schinzi* (Fairmaire), *S. (P.) striatus* (Castelnau) and *S. (P.) valeflorae* (Ferreira).

Out-group taxa – Two flighted *Scarabaeus* species, *S. proboscideus* and *S. rugosus*, characterized in a separate study (Forgie, 2003), that occur sympatrically with *Pachysoma*, were used. The phylogenetic relatedness of these taxa falls within the selection criteria discussed by Nixon and Carpenter (1993) and by Wheeler (1990) to effectively polarize the in-group character sets.

Sampling and nucleic acid extraction

Twelve of the 13 species of *Scarabaeus (Pachysoma)* were collected along the west coast of southern Africa from the West Coast National Park in the Cape Province to the Kuiseb River just south of Walvis Bay (Fig. 1), in Namibia (Summarized in Table 1). For each species, individual's representative of diverse localities, were collected, and preserved in absolute ethanol. Two museum specimens of *S. (P.) valeflorae* were obtained from the National

Collection of Insects (NCI) at the Agricultural Research Council (ARC) in Pretoria, South Africa. Identification of three morphologically similar species, *S. (P.) hippocrates, S. (P.) endroedyi* and *S. (P.) glentoni*, was confirmed by James du G Harrison of the Transvaal Museum using male genitalia.

Where possible, at least three individuals per locality and per species were selected for genetic characterization of the mitochondrial Cytochrome Oxidase subunit I (COI) gene (Avise *et al*., 1987; Simon *et al*., 1994). For the specimens preserved in ethanol muscle tissue from the thorax was used for DNA extraction whilst DNA from dried specimens was extracted from the tarsus of one leg. DNA was ultimately extracted from 46 individuals representing the 13 species (Table 1) using the Dneasy Tissue Kit (Qaigen).

Genomic amplification and nucleic sequence determination

Primers used for amplification of contemporary DNA were TL2–N-3014 and C1–J–1718 (Simon *et al*., 1994), which target a 1345-bp fragment. For the dried museum material, *Scarabaeus* (*Pachysoma)* specific primers were designed to amplify regions of between 300 and 600-bp. Two forward primers - C-301-F and C-526-F - and two complimentary reverse primers - C-409-R and C-602-R - were designed on the basis of aligned *Scarabaeus* (*Pachysoma)* sequences generated in this study (all primers are summarized in Table 2).

PCR was performed using a Perkin Elmer Gene Amp 2400 in a final volume of 50µl containing 20pmol of each primer, 10mM dNTP's and 1 X buffer in the presence of 1 unit of *Taq* DNA polymerase (Takara). BSA was added to improve the sensitivity of the reaction when the dried material was amplified (Higuchi, 1991). Thermal cycling parameters comprised an initial denaturation for 90 seconds at 94°C followed by 35 cycles of 94°C for 22 seconds, 48°C for 30 seconds and 72°C for 90 seconds with a final elongation step at 72°C for 1 min. The amplified COI gene products were purified from the tube using the High Pure PCR Product Purification kit (Roche) according to manufacturer specifications.

Sequencing reactions were performed at an annealing temperature of 48°C with versions 2.0 and 3.0 of the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer). Each amplicon was sequenced with the external PCR primers plus two internal primers, C1-J-2183 and a modified version of C1-N-2329 (Simon *et al*., 1994; Table 2).

Table 1. Summary of the 46 *Scarabaeus* (*Pachysoma)* individuals characterized in this study.

Species	Specimen ID	Locality	Co-ordinates	GenBank Accession No.
S. (P.) aesculapius	LA01	10km W Leipoldtville	S32°13'06.3" - E18°26'06.8"	AY258214
S. (P.) aesculapius	LA02	10km W Leipoldtville	S32°13'06.3" - E18°26'06.8"	AY258213
S. (P.) glentoni	LEIP02	10km W Leipoldtville	S32°13'06.3" - E18°26'06.8"	AY258226
S. (P.) glentoni	LEIP03	10km W Leipoldtville	S32°13'06.3" - E18°26'06.8"	AY258227
S. (P.) glentoni	LEIP04	10km W Leipoldtville	S32°13'06.3" - E18°26'06.8"	AY258228
S. (P.) hippocrates	WC02	West Coast National Park	S33°48' - E18°27'	AY258215
S. (P.) hippocrates	WC10	West Coast National Park	S33°48' - E18°27'	AY258216
S. (P.) hippocrates	WC11	West Coast National Park	S33°48' - E18°27'	AY258217
S. (P.) hippocrates	PN01	Port Nolloth	S29°14'12.9" - E16°52'01.1"	AY258221
S. (P.) hippocrates	PN03	Port Nolloth	S29°14'12.9" - E16°52'01.1"	AY258222
S. (P.) hippocrates	SK01	Kleinsee - Sandkop	S29°40'03" - E17°12'13.2"	AY258218
S. (P.) hippocrates	SK02	Kleinsee - Sandkop	S29°40'03" - E17°12'13.2"	AY258219
S. (P.) hippocrates	SK03	Kleinsee - Sandkop	S29°40'03" - E17°12'13.2"	AY258220
S. (P.) endroedyi	KOEK01	Koekenaap	S31°30'32.7" - E18°12'29.2"	AY258223
S. (P.) endroedyi	KOEK04	Koekenaap	S31°30'32.7" - E18°12'29.2"	AY258224
S. (P.) endroedyi	KOEK10	Koekenaap	S31°30'32.7" - E18°12'29.2"	AY258225
S. (P.) striatus	KOEKN02	Koekenaap	S31°30'32.7" - E18°12'29.2"	AY258250
S. (P.) striatus	KOEKN03	Koekenaap	S31°30'32.7" - E18°12'29.2"	AY258251
S. (P.) striatus	KOEKN04	Koekenaap	S31°30'32.7" - E18°12'29.2"	AY258252
S. (P.) gariepinus	OBI02	Obib Dune Fields	S28°01'03.5" - E16°39'03.8"	AY258235
S. (P.) gariepinus	OBI03	Obib Dune Fields	S28°01'03.5" - E16°39'03.8"	AY258236
S. (P.) gariepinus	OBI07	Obib Dune Fields	S28°01'03.5" - E16°39'03.8"	AY258237
S. (P.) gariepinus	KHM06	Klingharts Mountains	S27°24'18" - E15°37'25.6"	AY258232
S. (P.) gariepinus	KHM08	Klingharts Mountains	S27°24'18" - E15°37'25.6"	AY258233
S. (P.) gariepinus	KHM14	Klingharts Mountains	S27°24'18" - E15°37'25.6"	AY258234
S. (P.) gariepinus	DBD09	Daberas Dune Fields	S28°11'20.6" - E16°46'59.9"	AY258231
S. (P.) schinzi	10KSAUS01	10km S Aus	S26°47'14.2" - E16°17'46.6"	AY258247
S. (P.) schinzi	10KSAUS02	10km S Aus	S26°47'14.2" - E16°17'46.6"	AY258248
S. (P.) schinzi	10KSAUS10	10km S Aus	S26°47'14.2" - E16°17'46.6"	AY258249

S. (P.) fitzsimonzi	GPS01	Namib Rand Road	S25°32'19.4" - E16°16'29.9"	AY258229
S. (P.) fitzsimonzi	GPS02	Namib Rand Road	S25°32'19.4" - E16°16'29.9"	AY258230
S. (P.) denticollis	NR05	Namib Rand	S25°12'52.5" - E16°01'10"	AY258255
S. (P.) denticollis	NR06	Namib Rand	S25°12'52.5" - E16°01'10"	AY258256
S. (P.) denticollis	LT12	Luderitz - Agate Beach	S26°41'17.1" - E15°15'50.1"	AY258253
S. (P.) denticollis	LA11	Luderitz - Agate Beach	S26°41'17.1" - E15°15'50.1"	AY258254
S. (P.) rotundigenus	NR03	Namib Rand	S25°12'52.5" - E16°01'10"	AY258241
S. (P.) rotundigenus	NR05	Namib Rand	S25°12'52.5" - E16°01'10"	AY258242
S. (P.) rotundigenus	NR11	Namib Rand	S25°12'52.5" - E16°01'10"	AY258243
S. (P.) bennigseni	DBD01	Daberas Dune Fields	S28°11'13.4" - E16°47'03.2"	AY258238
S. (P.) bennigseni	DBD02	Daberas Dune Fields	S28°11'13.4" - E16°47'03.2"	AY258239
S. (P.) bennigseni	DBD04	Daberas Dune Fields	S28°11'13.4" - E16°47'03.2"	AY258240
S. (P.) rodriguesi	GOB01	Gobabeb	S23°39'53.1" - E15°12'48.1"	AY258244
S. (P.) rodriguesi	GOB02	Gobabeb	S23°39'53.1" - E15°12'48.1"	AY258245
S. (P.) rodriguesi	GOB03	Gobabeb	S23°39'53.1" - E15°12'48.1"	AY258246
S. (P.) valeflorae	RT01	Rotkop	S26°43' - E15°23'	AY258257
S. (P.) valeflorae	RT02	Rotkop	S26°43' - E15°23'	AY258258

Table 2. Summary of oligonucleotide primers used in this study.

Primer	Primer sequence	Length	Position (§)	Reference
C1-J-1718	5' GGAGGATTTGGAAATTGATTAGTTCC 3'	26mer	1651-1676	Simon et al., 1994
C1-J-2183	5' CAACATTTATTTTGATTTTTTGG 3'	23mer	2219-2241	Simon et al., 1994
C1-N-2329	5' ACTGTA AATAT**G**TGATGAGCTCA 3'	23mer	2287-2309	Simon et al., 1994 modified by Forgie and Bloomer (unpubl.)
TL2-N-3014	5' TCCAATGCACTAATCTGCCATATTA 3'	25mer	3323-3302	Simon et al., 1994
C-301-F£	5' CAACAGGAATAACTTTTGATCGTA 3'	25mer	2014-2039	Sole and Bastos, unpubl.
C-409-R£	5' GATGTATTTAAR(A/G)TTTCGATCTGT 3'	25mer	2122-2147	Sole and Bastos, unpubl.
C-526-F£	5' GGATTTGGR(A/G)ATAATTTCTCATAT 3'	23mer	2239-2262	Sole and Bastos, unpubl.
C-602-R£	5' CCAATAGTTATTATAGCATAAAT 3'	23mer	2315-2338	Sole and Bastos, unpubl.

£ Denotes the *Pachysoma* specific primers. § Refers to the corresponding position in *Locusta migratoria* (Genbank accession no. NC_001712).

For the dried museum material up to six primers were used for amplification and sequencing purposes. Both the external amplification primers and the three additional internal forward and reverse primers, C1-J-2183 (Simon *et al*., 1994), C-301-F and C-409-R and, where necessary, C-526-F, were used.

Phylogenetic analysis

Sequence chromatograms were visualized and edited in Chromas (Version 1.43) and were subsequently aligned using Clustal X (Thompson *et al*., 1997). A homologous region of 1197 base pairs (bp) corresponding to nucleotide positions 1713-2910 of *Locusta migratoria* Linneaus (Flook *et al*., 1995) was used for phylogenetic analysis. Both Maximum Parsimony (MP) and Maximum Likelihood (ML) were used to infer the phylogenetic relationships between the species of *Scarabaeus* (*Pachysoma*) (PAUP*4.08b; Swofford, 1998). An initial un-weighted parsimony analysis of the sequences from all individuals was performed, employing branch and bound searches and heuristic searches with 10 random addition sequences for each of 1000 bootstrap replicates (Farrell, 2001).

A posteriori and *a priori* weighting schemes such as the successive approximations weighting method (Farris, 1969; Park & Backlund, 2002) and positional weighting (Huelsenbeck *et al*., 1994; Krajewski & King, 1996) were investigated. In the former approach weights were applied according to the rescaled consistency index (RC), consistency index (CI) and the retention index (RI), whilst with the latter, first, second and third base positions were assigned weights of 4, 1 and 15.7, respectively.

In order to determine the model of sequence evolution, which best fits the COI data at hand, hierarchical likelihood ratio tests were performed using Model Test 3.0 (Posada & Crandall, 1998). Parameters from Model Test were used in a ML heuristic search in PAUP* and nodal support was estimated following 500 bootstrap pseudoreplications.

To use genetic data to infer evolutionary rates the data needs to meet two criteria: firstly, rates of genetic evolution among organismal lineages need to be consistent with a molecular clock model and secondly, the availability of a reliable fossil record (Yoder *et al*., 2000). Equality of evolutionary rates between lineages was assessed with Phyltest 2.0 (Kumar, 1996). In addition rate heterogeneity was investigated by comparing branch lengths and log-likelihood ratios estimated in PAUP* on the most parsimonious tree using the HKY85 model of sequence evolution, with and without the constraint of a molecular clock (Hasegawa & Kishino, 1994). Divergence times were estimated from uncorrected pairwise -

distances in MEGA version 2.1 (Kumar *et al*., 2001) and calibrated on arthropod mtDNA where a 2.3% pair-wise divergence per million years is postulated (Brower, 1994).

Results

Of the 434 variable sites identified across the 46 taxa used in this study, 408 sites were informative and 26 were singletons. The proportion of nucleotide mutations at first, second and third base positions was 19 %, 5 % and 76 % respectively and base composition over the 1197 base pairs was 39.2 %, 16.1 %, 30.5 % and 14.2 % for T, C, A and G respectively

Maximum Likelihood and Maximum Parsimony Analyses

The un-weighted parsimony analysis resulted in three trees with a length of 1711, consistency index (CI) of 0.381, a retention index (RI) of 0.742 and rescaled consistency index (RC) of 0.283. Weighted parsimony searches using CI, RI and RC resulted in the recovery of a single most parsimonious tree, whereas, positional re-weighting did not improve resolution despite accounting for saturation at the third base position. A single ML tree was obtained assuming the GTR model (Rodriguez *et al*., 1990) with 52.4% invariant sites, a transition-transversion ratio of 1.2 and a gamma distribution shape parameter of 0.77. Weighted parsimony analysis using the rescaled consistency index gave a single tree of length 490.52, CI of 0.54, RI of 0.82 and RC of 0.45 (Fig. 2). This MP tree had a similar topology to those trees obtained following Neighbour Joining (NJ), Minimum Evolution (ME) and ML analyses (results not shown).

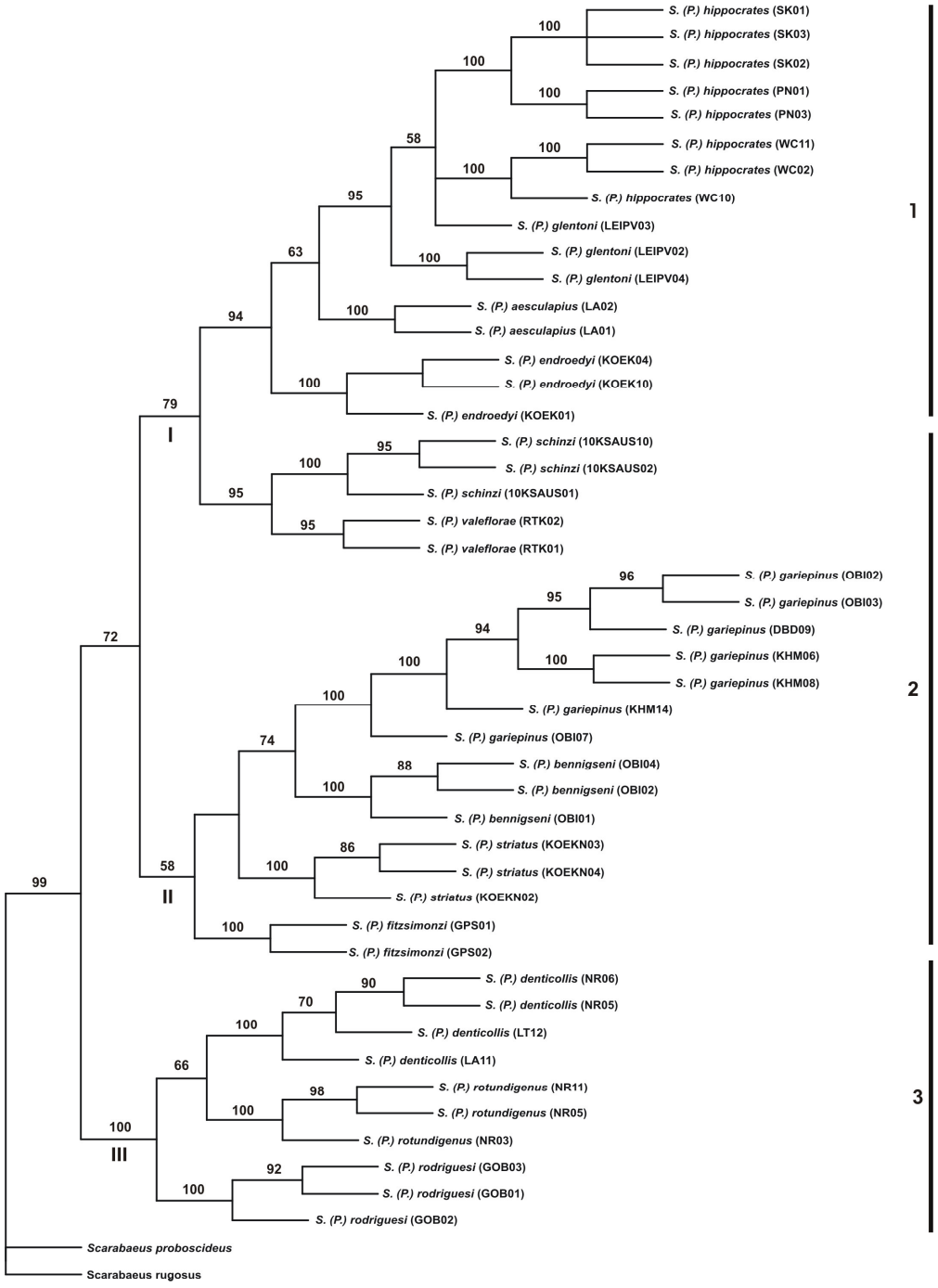

Figure 2. Phylogram of COI gene phylogeny of *Scarabaeus (Pachysoma).* Parsimony tree obtained following successive weighting using RC (tree length = 490.52, CI = 0.54 and RI = 0.82).

The COI gene phylogeny (Fig. 2) reveals the presence of three distinct clades (labelled I, II and III, respectively). Clade I, which has 79% bootstrap support, comprises 21 individuals, representative of six morphological species, namely *S. (P.) hippocrates, S. (P.) glentoni, S. (P.) aesculapius*, *S. (P.) endroedyi, S. (P.) valeflorae and S. (P.) schinzi*. Although there is high bootstrap support (between 94 % - and 100 %) for four of the six morphological species in this clade, a single individual *S. (P.) glentoniLEIPV03* does not group with the other two representatives of this morphological species. Instead a species complex comprising 11 individuals of *S. (P.) glentoni* and *S. (P.) hippocrates,* henceforth referred to as the hippocrates/glentoni complex was recovered (95% bootstrap support). Clade II supports four species (58% bootstrap support), *S. (P.) fitzsimonzi, S. (P.) gariepinus, S. (P.) bennigseni and S. (P.) striatus,* each with 100% bootstrap support. Clade III supports three species each with 100% bootstrap support, namely *S. (P.) denticollis, S. (P.) rotundigenus* and *S. (P.) rodriguesi*, which were formerly placed in the genus *Neopachysoma.*

Numbers 1 through 3 (right hand side of Fig.2) correspond to the species occurring in three areas differing in aridity as follows: Number 1; *S. (P.) hippocrates*, *S. (P.) glentoni, S. (P.) endroedyi* and *S. (P.) aesculapius* occur within the Fynbos and Namaqualand south and have the most southerly distribution of *Scarabaeus (Pachysoma)*. Number 2; *S. (P.) fitzsimonzi, S. (P.) gariepinus, S. (P.) bennigseni, S. (P.) striatus, S. (P.) valeflorae* and *S. (P.) schinzi* corresponds to those species that occur across two biomes and occupy the central part of the *Scarabaeus (Pachysoma)* distributional range, *S. (P.) striatus* occurs only in the Namaqualand while *S. (P.) fitzsimonzi, S. (P.) gariepinus, S. (P.) bennigseni, S. (P.) valeflorae* and *S. (P.) schinzi* can be found in the southern part of the Desert biome. Number 3; *S. (P.) denticollis, S. (P.) rotundigenus* and *S. (P.) rodriguesi* have the most northerly Desert Biome distribution and are the three ultrapsammophilous species, and also those species formerly classified as *Neopachysoma* (Clade III, Fig. 2).

Imposing a Molecular Clock

The likelihood of the tree with and without enforcing a molecular clock was -log 7205.1461 and –log 7167.44184 respectively. The difference was not significant according to the likelihood ratio test ($p<0.05$). In addition, rate constancy could also not be rejected using PHYLTEST ($p < 0.05$). As both results indicate that the molecular clock hypothesis cannot be rejected, a rate of 2.3% sequence divergence per million years was used to infer a molecular clock (Brower, 1994).

The subgenus is estimated to have arisen about 2.9 million years ago. The hippocrates complex (consisting of *S. (P.) hippocrates, S. (P.) glentoni* and *S. (P.) endroedyi* (Harrison *et al*., 2003)), and *S. (P.) aesculapius* appear to have diverged approximately 2.66 Mya and species of the former genus *Neopachysoma* appear to have diverged approximately 2.4 Mya. The youngest species of *Scarabaeus (Pachysoma)* include *S. (P.) schinzi, S. (P.) rodriguesi, S. (P.) rotundigenus, S. (P.) denticollis*, *S. (P.) bennigseni, S. (P.) aesculapius* and *S. (P.) fitzsimonzi*, and are estimated to have arisen between 200 000 and 600 000 Ya.

Discussion

Palaeontological History

The ball-rolling dung beetles of the tribe Scarabaeini comprise 146 species belonging to five genera and three subgenera. Their distribution extends throughout the Afrotropical region (including Madagascar) and southern latitudes of the Palaearctic (Forgie, 2003). Diversification of the Scarabaeini was thought to coincide with the radiation of both Angiosperms (Eocene: 50 Mya) and mammalian herbivores (lower Oligocene: 35 Mya), with a shift from saprophagy to mycetophagy to coprophagy by adults and larvae (Cambefort, 1991b; Scholtz & Chown, 1995). The Scarabaeini appear to have evolved during the Cenozoic from ancient scarabaeoid lineages dating back to the lower Jurassic ca. 180 – 200 Mya (Crowson, 1981; Cambefort, 1991a; Scholtz & Chown, 1995). The flightless Scarabaeini are monophyletic and contain the most derived members within the tribe with *Scarabaeus (Pachysoma)* representing the most highly evolved of the lineages (Forgie, 2003).

Ideas about rates of evolution of the rich, endemic Namib fauna and flora fall broadly into two schools of thought. Some authors argue that the desert must be very ancient (Cretaceous) in order for the specialized fauna and flora to have had time to evolve. For these scientists, the rates of evolution envisaged are extremely slow. For the second group who consider that the desert is appreciably younger (Miocene), rates of evolution are postulated to have been much more rapid (Pickford & Senut, 1999). However, the various authors have essentially been arguing about different taxa and different hierarchical levels. Some ancient lineages of Late Cretaceous proto-Namib desert ancestry are identifiable amongst insects, for example Lepismatidae (Thysanura: Insecta) (Irish, 1990), but the fauna associated with the post-Miocene Namib Desert Phase (Ward & Corbett, 1990) is logically much younger. Now that we know the hyperaridity of the Namib is no older than the Middle

Miocene (*ca* 15 Mya) (Pickford & Senut, 1999) it is evident that rates of evolution have been orders of magnitude more rapid. This could therefore imply more severe selection pressures and perhaps enhanced generation of genetic variability in desert environments, or a combination of both (Pickford & Senut, 1999).

Biogeographical Inferences

Endrödy-Younga (1978) coined the term "pocket speciation" to describe processes resulting from the numerous small dunes and dune fields of Namib or Kalahari sand origins which have been isolated from the main sand systems and occur throughout southern Namibia and the northern Cape (Koch, 1962). Most of these are alluvial sands that originate at the mouths of the large Tertiary rivers. Any separation of sand dunes from a major system could constitute a vicariance event (Prendini, 2001). These isolated sand dunes are often encountered in unlikely places on the flats and as deposits against mountain slopes. This sand is clearly wind-blown from major dune fields, so the possibility exists that psammophilous taxa may extend their distribution, following pockets of sand to their eventual destinations and thus becoming completely isolated from main populations in time. Endrödy-Younga (1982) provided evidence for this process by demonstrating that, over 11 years, barchan dunes in the southern Namib moved considerable distances across gravel plains together with their associated Tenebrionidae fauna. Dispersal of these species could be attributed not to the movement of individuals but to the movement of their substratum and habitat, the dune. Clear south to north evolutionary gradients in the majority of ultrapsammophilous taxa can be adequately explained in terms of sand movement of barchan dunes, which have been shown to move 10-100 $m.yr^{-1}$ within historical time (Penrith, 1979; Prendini, 2001).

Due to the low, unpredictable rainfall in the Namib since the advent of hyperaridity in the Miocene the fauna is and probably always has been, dependent on the regular, dense fogs that represent virtually the only free water available to it (Seely & Louw, 1980). The fogs have become frequent along the Namib coast since the Early Pleistocene (1.8.Mya) when cold upwellings from the Benguela Current caused cold air that condenses to form fog in contact with the warm air off the land (Pickford & Senut, 1999). This may have been the main environmental parameter that permitted dispersal into, and subsequent radiation, in areas that may have been inhospitable until then.

Speciation Events

It is around the riverbeds and in the deep loose sand of the Sossus Sand Formation, that speciation in *Scarabaeus (Pachysoma)* seems principally to have occurred. The rivers probably presented barriers to the spread of some of the species during the Plio-Pleistocene, and may have vicariously split populations of some others that lead to speciation events. The areas around these riverbeds have high species numbers, and some still appear to be barriers to further range expansion. Isolated populations that occur on sandy plains and in dune fields interspersed by dry riverbeds, gravel plains and rocky outcrops represent the current distribution of most species. Exceptions to this are the ultra-psammophilous species that occur throughout much of the Namib Sand Sea (Harrison *et al*., 2003). As the dune fields shifted and became more continuous through the southern and central Namib, so this allowed for the movement of these isolated populations in a northerly direction. Psammophilous taxa evolved subsequent to establishment of these systems, speciating after initial dispersal events into an environment that had previously constituted a barrier. The older species seem to have inhabited the Karoo (interior Cape Province of South Africa), the southern parts of Namibia and/or the Kaokoveld (north-western Namibia). These are areas of rocky, not excessively sandy substrates indicating that these conditions probably prevailed in much of the Gondwana Desert (Irish, 1990).

The Olifants, Buffels, Holgat, Orange and Kuiseb Rivers (see Fig. 1), which still flow, all affect *Scarabaeus (Pachysoma)* in some way. The Orange River appears to have been of lesser or sporadic importance as a gene barrier, since many psammophilous southern Namib species, for example *S. (P.) gariepinus* and *S. (P.) bennigseni*, occur on both sides of the river. The boundary between related Namib and Namaqualand species lies further south at the Holgat and Buffels Rivers (Irish, 1990). The Buffels River appears to be the southern limit for *S. (P.) gariepinus.* The Holgat River appears to be the barrier to *S. (P.) striatus* from extending its distribution northwards and *S. (P.) bennigseni* from moving southwards. *S. (P.) striatus, S. (P.) gariepinus* and *S. (P.) bennigseni* probably speciated around the Olifants, Buffels and Holgat Rivers, respectively and then moved northwards with the sand. The evolution of *S. (P.) endroedyi* could have resulted from a vicariance event caused by the Olifants River splitting the *S. (P.) aesculapius* population into two and thereby allowing for the speciation of *S. (P.) endroedyi* (For detailed distribution maps of *Scarabaeus (Pachysoma)* see Harrison *et al*., 2003).

Regarding the hippocrates/glentoni complex, *S. (P.) glentoni* is distributed along the Olifants River, from Lambert's Bay, inland to Clanwilliam as opposed to the wider

distribution of *S. (P.) hippocrates.* In some localities they occur sympatrically. *S. (P.) glentoni* prefer the firm vegetated sand of riverbanks and coastal hummocks while *S. (P.) hippocrates* prefer soft to firm sand of coastal hummocks and hillocks on the periphery of dune systems, and river beds and banks. *S. (P.) hippocrates* shows south/north morphological clinal variation implying that the species might be undergoing speciation (Harrison *et al*., 2003). Distances between populations of these two species can range from a few metres to about 40km. The overall small distance between populations and the young age of *S. (P.) glentoni* may underlie the lack of resolution of these two species with the molecular data. Increasing the number of individuals from different localities of the two species and use of an alternative gene marker may help resolve the species complex.

Inferences from the Molecular Clock

Phylogenetic analysis indicates that the psammophilous and ultrapsammophilous species of *Scarabaeus (Pachysoma)*, formerly placed in the genus *Neopachysoma*, are the most derived and have the most northerly distribution in the Sossus Sand Formation which is consistent with the findings of Irish (1990). One may therefore safely assume that psammophilous taxa evolved from an older non-psammophilous ancestor (Irish, 1990). Three of the species of *Scarabaeus (Pachysoma)* show distinct morphological south/north clinal variation, *S. (P.) hippocrates, S. (P.) gariepinus* and *S. (P.) denticollis* (Harrison *et al*., 2003). The clear south/north morphological clinal variation shows strong support for the movement of taxa with the wind blown sand from the barchan dunes. The distribution of *Scarabaeus (Pachysoma)* is halted at the Kuiseb River.

Rapid radiation of most of the species and/or their ancestors, between 2.35 Mya and 2.66 Mya, can clearly be seen within the subgenus and may be linked to the reliability of regular fog in the Pleistocene. Formation of regular fogs would constitute a consistent and reliable form of water. All of the species of *Scarabaeus (Pachysoma)* occur within the fog belt except for *S. (P.) schinzi,* which is confined to the areas around Aus on the Huib-Hoch Plateau, indicating it must be dependent on rainfall. This area is approximately 100km inland from the coast. Rainfall increases while the fog decreases as one moves inland. As seen here and in other insect groups (for examples see Irish, 1990), the distinction between coastal and inland fauna is not absolute as coastal species penetrate inland due to the shared similarities between the slips face/dune-crest habitats of the inland and coastal dunes. The reverse is not true (inland species are absent from the coast). Historical separation appears to be the primary cause of this east/west distributional gradient. One can clearly see the importance of coastal

dunes as a species reservoir and a dispersal vessel, since wherever this sand and its associated fauna have been blown inland, new taxa have evolved.

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Chapter III

Testing for congruence between morphological and molecular data partitions of *Scarabaeus (Pachysoma)* (Scarabaeidae: Scarabaeinae).

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Running Title: Congruence between data partitions of *Scarabaeus (Pachysoma)* (Scarabaeidae: Scarabaeinae).

Abstract

Scarabaeus (Pachysoma) comprises 13 species endemic to the west coast of southern Africa. A species level phylogenetic analysis was conducted using 64 morphological characters and 1197 bp of the Cytochrome Oxidase I (COI) gene. All 13 in-group and eight out-group species were included in the analyses. Morphological and molecular data sets were analysed both separately and combined, using the total evidence approach. Strong support is shown for all 13 species within *Scarabaeus (Pachysoma)* and its monophyly within *Scarabaeus* is confirmed. The COI sequence data had high inter- and intra-specific sequence divergence as well as a high A/T bias. All trees generated using Parsimony, Maximum Likelihood, Neighbor-Joining and Bayesian methods exhibited similar topologies. The morphological and molecular data partition phylogenies showed congruence with the combined phylogeny, lending strong support for combining datasets using total evidence. Phylogenetic trees based on combined data partitions were relatively more resolved than those based on the individual data analyses. The relative contribution of each data partition to individual nodes was assessed using Bremer and Partitioned Bremer Support. The morphological dataset, though small, was not overshadowed by the large molecular dataset in the combined analysis. A strong association between the phylogenies and geographic distribution over the total *Scarabaeus (Pachysoma)* distribution was demonstrated. This study was contrasted with other phylogenetic studies done on *Scarabaeus (Pachysoma)* as well as other insect orders. Lastly *Scarabaeus (Pachysoma)* mtDNA variation was compared within and between the orders Coleoptera, Lepidoptera, Hymenoptera and Diptera.

Keywords -Scarabaeidae, *Scarabaeus*, total evidence, cytochrome oxidase I, morphology, congruence

Introduction

Scarabaeus (Pachysoma) MacLeay 1821 is a group of the tribe Scarabaeini (Scarabaeidae: Scarabaeinae). Members of the Scarabaeini are found in moist savanna through drier regions to very hot dry deserts (Scholtz, 1989) of the Afrotropics and southern latitudes of the Palaearctic. The Scarabaeini comprise some 146 species of ball-rolling dung beetles belonging to two genera (Forgie *et al*., 2005). Diversification of scarabaeines was thought to coincide with the diversification of angiosperms and mammalian herbivores resulting in a shift of their feeding habits from saprophagy and mycetophagy to coprophagy (Cambefort, 1991; Scholtz & Chown, 1995). Scarabaeines predominantly feed on dung, but have also been known to feed on humus, carrion and fungi (Scholtz & Chown, 1995). Most scarabaeine species are adapted to open habitats and feed on resources that are usually patchy and ephemeral. Although true food specialisation is uncommon, it does exist. *Scarabaeus* (*Sceliages)* (Forgie *et al*., 2005), are specialist necrophages where both adults and larvae feed only on dead millipedes (Forgie *et al*., 2002) while the flightless *Scarabaeus* (*Pachysoma)* utilise dry dung pellets or detritus (Holm & Scholtz, 1979; Scholtz, 1989). In contrast to feeding specialisation, generalist - *Scarabaeus (Scarabaeolus)* contains species that will utilise dung or carrion - and opportunistic - *Scarabaeus rubripennis* has been observed rolling pieces of millipede along as it would a dung ball (Mostert & Scholtz, 1986) - feeders also exist within this tribe (Forgie *et al*., 2005).

MacLeay (1821) described the genera *Pachysoma* and *Mnematium* for all flightless species of the Scarabaeini that occur in south-west and north Africa, respectively. The genus *Neopachysoma* was created by Ferreira (1953) for the species of *Pachysoma* inhabiting the central Namib Desert. *Pachysoma* was defined by aptery, absence of humeral calli, semi-contiguous mesocoxae and short mesosterna (Ferreira, 1953). An evaluation by Holm & Scholtz (1979) concluded that these characteristics were either due to convergence or were too variable and inconsistent to use as the justification for a genus. They also found no justification for the separation of *Neopachysoma* and *Mnematium* and consequently synonomised both with *Pachysoma*. *Pachysoma* was tentatively maintained as a genus due to its unique biology. However, the genus was later synonomised with the genus *Scarabaeus* Linnaeus (1758) by Mostert & Holm (1982) an act that was questioned by Endrödy-Younga (1989) and Scholtz (1989) because of *Pachysoma's* unique set of morphological and behavioural apomorphies.

Scarabaeus (Pachysoma) is represented by 13 species, endemic to the west coast of southern Africa. Their southerly distribution begins near Cape Town, in South Africa (S33°56'-E18°28'), with their northerly distribution being halted at the Kuiseb River (S22°58'-E14°30'), in Namibia, which marks the end of the central Namib dune sea. Southern and eastern expansion by *Scarabaeus* (*Pachysoma)* is confined by the Cape Fold Mountains and escarpments, which act as topographical and climatic barriers (Harrison *et al*., 2003). *Scarabaeus (Pachysoma)* species are, therefore, restricted to the arid or semi-arid sandy regions of south-western Africa and psammophily is readily apparent as seen by the long setal hairs on the middle and hind limbs. Little is known about their biology (Scholtz *et al*., 2004), but they are unique in their food relocation strategy. They utilize dry dung pellets or detritus, which they randomly search for, and bury in a pre-constructed burrow. Dry dung pellets or detritus are gathered up in the setal fringes of their hind limbs and, dragged forward to be buried below the moisture line, in the pre-constructed holding chamber (Holm & Scholtz, 1979; Scholtz, 1989; Harrison *et al*., 2003).

In a recent revision of *Scarabaeus* (*Pachysoma*) by Harrison & Philips (2003) the phylogenetic validity of *Pachysoma* was evaluated using cladistic methods. Harrison & Philips (2003) maintained the synonymy of *Neopachysoma* Ferreira with *Pachysoma* while *Mnematium* MacLeay was regarded as a synonym of *Scarabaeus.* *Pachysoma* was confirmed as being a distinct monophyletic clade within *Scarabaeus* and was therefore classified as a derived subgenus thereof (Harrison *et al*., 2003; Forgie *et al*., 2005). Based on Harrison & Philips's (2003) phylogeny, Sole *et al*. (2005) re-examined *Scarabaeus (Pachysoma)* at a molecular level using Cytochrome Oxidase I (COI) mitochondrial sequence data. Eleven of the 13 species were supported at a molecular level with *S. (P.) hippocrates* and *S. (P.) glentoni* forming a species complex. *Scarabaeus (Pachysoma)* was confirmed as being monophyletic within *Scarabaeus*. The synonymy of *Neopachysoma* with *Pachysoma* was supported even though it is clearly a distinct lineage within *Scarabaeus (Pachysoma)* (Sole *et al*., 2005; Forgie *et al*., 2005).

In this study we firstly, re-construct the phylogeny of *Scarabaeus (Pachysoma)* using both morphological (Harrison *et al*., 2003) and molecular (Sole *et al*., 2005) data partitions and secondly, by using the total evidence approach we test for congruence between the two data partitions. In this way the relative overall contribution of these character sets to the combined phylogeny could be assessed.

Material and Methods

Taxa

In-group taxa - All 13 species of the subgenus *Pachysoma* were used to infer the phylogeny. These are: *S. (P.) aesculapius* (Olivier), *S. (P.) bennigseni* (Felsche), *S. (P.) denticollis* (Péringuey), *S. (P.) endroedyi* Harrison, Scholtz & Chown, *S. (P.) fitzsimonsi* (Ferreira), *S. (P.) gariepinus* (Ferreira), *S. (P.) glentoni* Harrison, Scholtz & Chown, *S. (P.) hippocrates* (MacLeay), *S. (P.) rodriguesi* (Ferreira), *S. (P.) rotundigenus* (Felsche), *S. (P.) schinzi* (Fairmaire), *S. (P.) striatus* (Castelnau) and *S. (P.) valeflorae* (Ferreira).

Out-group taxa - The following species were included as they are atypical (see Table 1) and their taxonomy was controversial in the past (Forgie *et al*., 2005): *Scarabaeus* [*Drepanopodus*] *proximus* Janssens, *Scarabaeus rugosus* (Hausman), *Scarabaeus* [*Neateuchus*] *proboscideus* (Guérin), *Scarabaeus galenus* (Westwood), *Scarabaeus* (*Scarabaeolus*) *rubripennis* (Boheman), *Scarabaeus* (*Sceliages*) *brittoni* zur Strassen, *Scarabaeus rusticus* (Boheman) and *Scarabaeus westwoodi* Harold all from the tribe Scarabaeini. The out-group representatives were chosen based on relationships indicated by recent phylogenetic studies (Harrison *et al*., 2003; Forgie *et al*., 2005) and taking into account selection criteria of Nixon & Carpenter (1993).

All species mentioned above were included in the molecular, morphological and combined data analyses. Synonyms of *Scarabaeus* used in this study are indicated in square brackets and include *Neateuchus* Gillet (synonomised by Mostert & Scholtz, 1986), *Neopachysoma* Ferreira (synonomised by Holm & Scholtz, 1979) and *Drepanopodus* Jannsens (synonomised by Forgie *et al*., 2005). Table 2 includes all the species used in this study, the data partitions used, the source of the data and accession numbers.

Phylogenetic Analysis

Statistics

The molecular data were subjected to preliminary sequence analyses prior to phylogenetic analysis. The best model of sequence evolution, the proportion of invariable sites and the α parameter of the distribution of rate variation among sites (Yang *et al*., 1994) were estimated in Modeltest 3.0 (Posada & Crandall, 1998). The average nucleotide and amino acid p-distances

were calculated in MEGA version 2.1 (Kumar *et al*., 2001), within and between both out - and in - group taxa. MEGA was also used to calculate sequence divergence values.

Molecular data

The total aligned molecular matrix consists of 1197 base pairs (bp), corresponds to bases 1713 to 2910 of the Cytochrome Oxidase I gene of *Locusta migratoria* Linneaus (Genbank Accession No. NC_001712). A total of 54 individuals were used for this study, of which 46 (accession numbers on GenBank AY258214 – AY258258) where in-group taxa and 8 identified as out-group taxa. The laboratory procedures for amplifying and sequencing followed standard protocols described previously (Sole *et al*., 2005). Sequences were aligned in Clustal X (Thompson *et al*., 1997) and subsequent analyses were performed in PAUP*4.0b1 (Swofford, 1998)**.**

Both Maximum Likelihood (ML) and Maximum Parsimony (MP) methods were used to infer phylogenetic relationships between species. The robustness of the results was assessed by means of bootstrap analysis (Felsenstein, 1985), using 1 000 pseudoreplicates and branch-and-bound searching (nucleotides treated as unordered characters). A single representative from each species was included in the ML analysis, except for *S. (P.) glentoni* for which two specimens were included one of which no resolution had been previously obtained (see results below and Chapter 2 (Sole *et al*., 2005) for details). The parameters estimated by Modeltest were used in a Maximum Likelihood heuristic analysis with 1000 pseudoreplicates.

Table 1. Summary of the wing status, feeding specialisation and modes of dung removal for the species used in this study.

Taxa	Distribution	Wing Status	Feeding Specialisation	Modes
S. [*Neateuchus*] *proboscideus* (Guérin)	Afrotropical (W South Africa, Kalahari)	Macropterous	wet dung	Rolling
S. [*Neopachysoma*] *denticollis* (Péringuey)	Afrotropical (Namib desert)	Apterous	dry dung pellets/detritus	Dragging
S. [*Neopachysoma*] *rodriguesi* (Ferreira)	Afrotropical (Namib desert)	Apterous	dry dung pellets/detritus	Dragging
S. [*Neopachysoma*] *rotundigenus* (Felsche)	Afrotropical (Namib desert)	Apterous	dry dung pellets/detritus	Dragging
S. (*Pachysoma*) *aesculapius* Olivier	Afrotropical (W South Africa)	Apterous	dry dung pellets/detritus	Dragging
S. (*Pachysoma*) *hippocrates* (MacLeay)	Afrotropical (W South Africa)	Apterous	dry dung pellets/detritus	Dragging
S. (*Pachysoma*) *glentoni* Harrison, Scholtz & Chown	Afrotropical (W Africa; south Olifants River)	Apterous	dry dung pellets/detritus	Dragging
S. (*Pachysoma*) *endroedyi* Harrison, Scholtz & Chown	Afrotropical (W Africa; north Olifants River)	Apterous	dry dung pellets/detritus	Dragging
S. (*Pachysoma*) *striatus* (Castelnau)	Afrotropical (W South Africa)	Apterous	dry dung pellets/detritus	Dragging
S. (*Pachysoma*) *gariepinus* (Ferreira)	Afrotropical (W Africa)	Apterous	dry dung pellets/detritus	Dragging, Rolling
S. (*Pachysoma*) *bennigseni* (Felsche)	Afrotropical (W Africa)	Apterous	dry dung pellets/detritus	Dragging
S. (*Pachysoma*) *schinzi* (Fairmaire)	Afrotropical (W Namibia)	Apterous	dry dung pellets/detritus	Dragging
S. (*Pachysoma*) *valeflorae* (Ferreira)	Afrotropical (W Namibia)	Apterous	dry dung pellets/detritus	Dragging
S. (*Pachysoma*) *fitzsimonzi* (Ferreira)	Afrotropical (W Namibia)	Apterous	dry dung pellets/detritus	Dragging
S. (*Scarabaeolus*) *rubripennis* (Boheman)	Afrotropical (Namib desert)	Macropterous	opportunistic	Rolling
Scarabaeus galenus (Westwood)	Afrotropical (Southern Africa)	Macropterous	wet dung pellets	Carrying, Tunnelling, Pushing
Scarabaeus rusticus (Boheman)	Afrotropical (South Africa)	Macropterous	wet dung	Rolling
Scarabaeus westwoodi Harold	Afrotropical (Southern + East Africa)	Macropterous	wet dung	Rolling
Scarabaeus rugosus (Hausman)	Afrotropical (SW South Africa)	Macropterous	wet dung	Rolling
Scarabaeus (*Sceliages*) *brittoni* zur Strassen	Afrotropical (W South Africa)	Macropterous	obligate necrophage	Pushing
Scarabaeus [*Drepanopodus*] *proximus* Jannsens	Afrotropical (South Africa)	Macropterous	wet dung	Rolling

*Most species of Scarabaeini are adapted to open habitats and feed on resources that are patchy. True food specialisation in the tribe is uncommon but does occur, listed above.

Table 2. Summary of the species used in this study including where the data were obtained.

Taxa	Tribe	Morphology	Molecular	Accession Numbers
S. [*Neopachysoma*] *denticollis* (Péringuey)	Scarabaeini	Harrison, 1999	Sole et al., 2005	See Text
S. [*Neopachysoma*] *rodriguesi* Ferreira	Scarabaeini	Harrison, 1999	Sole et al., 2005	See Text
S. [*Neopachysoma*] *rotundigenus* (Felsche)	Scarabaeini	Harrison, 1999	Sole et al., 2005	See Text
S. (*Pachysoma*) *aesculapius* Olivier	Scarabaeini	Harrison, 1999	Sole et al., 2005	See Text
S. (*Pachysoma*) *hippocrates* (MacLeay)	Scarabaeini	Harrison, 1999	Sole et al., 2005	See Text
S. (*Pachysoma*) *glentoni* Harrison, Scholtz & Chown	Scarabaeini	Harrison, 1999	Sole et al., 2005	See Text
S. (*Pachysoma*) *endroedyi* Harrison, Scholtz & Chown	Scarabaeini	Harrison, 1999	Sole et al., 2005	See Text
S. (*Pachysoma*) *striatus* (Castelnau)	Scarabaeini	Harrison, 1999	Sole et al., 2005	See Text
S. (*Pachysoma*) *gariepinus* (Ferreira)	Scarabaeini	Harrison, 1999	Sole et al., 2005	See Text
S. (*Pachysoma*) *bennigseni* (Felsche)	Scarabaeini	Harrison, 1999	Sole et al., 2005	See Text
S. (*Pachysoma*) *schinzi* (Fairmaire)	Scarabaeini	Harrison, 1999	Sole et al., 2005	See Text
S. (*Pachysoma*) *valeflorae* (Ferreira)	Scarabaeini	Harrison, 1999	Sole et al., 2005	See Text
S. (*Pachysoma*) *fitzsimonzi* (Ferreira)	Scarabaeini	Harrison, 1999	Sole et al., 2005	See Text
S. (*Scarabaeolus*) *rubripennis* (Boheman)	Scarabaeini	Harrison, 1999	Sole et al., 2005	AF499763
S. [*Neateuchus*] *proboscideus* (Guérin)	Scarabaeini	Harrison, 1999	Sole et al., 2005	AF499757
Scarabaeus rusticus (Boheman)	Scarabaeini	Harrison, 1999	Forgie, 2003	AF499767
Scarabaeus westwoodi Harold	Scarabaeini	Harrison, 1999	Forgie, 2003	AF499769
Scarabaeus galenus (Westwood)	Scarabaeini	Harrison, 1999	Forgie, 2003	AF499764
Scarabaeus rugosus (Hausman)	Scarabaeini	Harrison, 1999	Forgie, 2003	AF499766
Scarabaeus (*Sceliages*) *brittoni* zur Strassen	Scarabaeini	Harrison, 1999	Forgie, 2003	AF499772
Scarabaeus [*Drepanopodus*] *proximus* Jannsens	Scarabaeini	Harrison, 1999	Sole, unpubl.	AY965239

* The columns entitled molecular and morphology are data types that were used and the references in these columns indicate the source of the data

Morphological data analysis

The raw morphological data for the analyses were obtained from Harrison & Philips (2003). The morphological dataset comprised 64 characters of which 39 were external and 25 internal characters; 16 were bipolar and 48 multi-state (see Appendix 1 for characters) (For details of the morphological characters see Harrison & Philips, 2003). This morphological dataset which was originally analysed in NONA v 2.0 (Goloboff, 1997) was re-analysed in PAUP* using Parsimony analysis to determine the phylogenetic relationships between the species. The parsimony analysis was re-weighted using the re-scaled consistency index (Farris, 1969) and bootstrap analysis was used to assess the robustness of the results, using 1 000 pseudoreplicates and branch-and-bound searching. All trees were rooted and characters were coded as unordered.

Combined data analysis

A total of 21 species, comprising a single individual from each taxon, was used for the combined analysis. To compare the similarity of phylogenetic signal between different data partitions, the partition homogeneity test was calculated across and between both data partitions in PAUP*, with 1,000 replications (Farris *et al*., 1995; Creer *et al*., 2003). A Parsimony analysis was done, in PAUP*, and re-weighted using the re-scaled consistency index after which 1000 bootstrap replicates were performed (Felsenstein, 1985) with branch-and-bound searching.

TreeRot.v2 (Sorensen, 1999) was used to calculate total Bremer support (BS) values at each node (Bremer, 1988) and to determine partitioned Bremer support (PBS) (Baker & DeSalle, 1997; Baker *et al*., 1998) values for each data partition in the combined parsimony tree. Different datasets provide different amounts of support when combined. PBS, therefore, calculates the amount of support each dataset contributes towards the complete combined phylogeny. PBS values can be positive, negative or zero and their sum equals the value of the Bremer support for that node. Positive values indicate that, within a combined data framework, a given partition supports that particular node over any alternative relationships specified by the most parsimonious tree(s) without that node. Negative values indicate that, again in a combined analysis framework, the length of a partition is shorter on the topology of alternate tree(s) not containing a given node and therefore contains contradictory evidence for that node (Baker *et al*., 1998). Bremer support values were calculated using 20 unrestricted random addition sequences per node.

Bayesian Analysis

A Bayesian phylogenetic analysis for the combined and molecular datasets was performed with MrBayes 3 (Huelsenbeck & Ronquist, 2001). The Bayesian analysis approximates the posterior probability (Huelsenbeck *et al*., 2001) for a phylogenetic tree by successively altering the model parameter values in a Markov Chain Monte Carlo (MCMC) procedure. A random tree and parameter values are initially chosen and for each step in the chain a new combination of topology and parameter values are either accepted or rejected according to the Metropolis-Hastings-Green algorithm. Log-likelihood values are calculated for each topology combination and recorded, once these have reached a plateau i.e. stabilised, the frequency at which a clade appears among the sampled trees is then deemed an approximation of the posterior probability. In order to efficiently traverse the parameter space, several chains are run simultaneously at different designated theoretical temperatures. A heated chain moves more easily across a valley and thereby prevents the chain being trapped at a local optimum.

The model for Bayesian analysis was selected with the likelihood-ratio test in Modeltest. Four different analyses were run, beginning with random starting trees. For every analysis five Markov Chains (four heated (temperature = 0.05) and one cold (temperature = 1)) where run for 3 000 000 generations with trees being sampled every 100th generation. Of the four analyses, 25 000 trees were used to determine a consensus phylogeny and posterior probability of the nodes (Warren *et al*., 2003).

Results

Molecular dataset statistics

Modeltest selected the GTR model (Rodriguez *et al*., 1990) with proportion of invariable sites and gamma distribution shape parameter estimated at 0.57 and 0.88, respectively. The within-species sequence divergence ranged from as low as 0.8 % in *S. (P.) schinzi* to 5.7 %, 5.8 % and 6.3 % in *S. (P.) hippocrates*, *S. (P.) glentoni* and *S. (P.) valeflorae*, respectively (Table 3). The average nucleotide pairwise distances within *Scarabaeus (Pachysoma)* ranged from 8 % to 15.3 %, while the average amino acid pairwise distance ranged from 1.3 % to 5.5 % (Table 4).

Table 3. Average intra-specific sequence divergences for the species of *Scarabaeus (Pachysoma)*.

Species	Divergence	Std Error
S. (P.) aesculapius	0.011	0.003
S. (P.) hippocrates	0.057	0.005
S. (P.) endroedyi	0.041	0.004
S. (P.) glentoni	0.058	0.006
S. (P.) fitzsimonzi	0.016	0.003
S. (P.) gariepinus	0.027	0.003
S. (P.) bennigseni	0.024	0.004
S. (P.) rotundigenus	0.018	0.003
S. (P.) rodriguesi	0.012	0.003
S. (P.) schinzi	0.008	0.002
S. (P.) striatus	0.009	0.002
S. (P.) denticollis	0.022	0.003
S. (P.) valeflorae	0.063	0.007
Outgroups	0.118	0.006

Table 4. Average uncorrected nucleotide- (p) and amino acid- distances over all 54 individuals of *S. (Pachysoma)* analysed. The average nucleotide p-distances are indicated in the bottom left of the table while the amino acid p-distances can be found at the top right hand corner.

Species		1	2	3	4	5	6	7	8	9	10	11	12	13	14
S. (P.) aesculapius	**1**		0.029	0.039	0.027	0.033	0.034	0.033	0.044	0.041	0.030	0.041	0.037	0.043	0.051
S. (P.) hippocrates	**2**	0.123		0.043	0.013	0.040	0.037	0.042	0.043	0.043	0.039	0.051	0.040	0.049	0.053
S. (P.) endroedyi	**3**	0.123	0.110		0.043	0.041	0.039	0.041	0.045	0.055	0.044	0.049	0.042	0.046	0.056
S. (P.) glentoni	**4**	0.120	0.080	0.105		0.038	0.035	0.039	0.040	0.042	0.038	0.049	0.037	0.047	0.051
S. (P.) fitzsimonzi	**5**	0.138	0.128	0.118	0.125		0.017	0.020	0.032	0.036	0.020	0.034	0.022	0.028	0.040
S. (P.) gariepinus	**6**	0.131	0.121	0.120	0.118	0.104		0.025	0.026	0.030	0.025	0.040	0.018	0.030	0.031
S. (P.) bennigseni	**7**	0.134	0.135	0.132	0.132	0.111	0.116		0.032	0.035	0.025	0.034	0.029	0.036	0.040
S. (P.) rotundigenus	**8**	0.153	0.139	0.136	0.144	0.124	0.126	0.144		0.026	0.036	0.041	0.022	0.039	0.038
S. (P.) rodriguesi	**9**	0.140	0.127	0.128	0.137	0.124	0.125	0.137	0.110		0.035	0.048	0.031	0.045	0.044
S. (P.) schinzi	**10**	0.137	0.123	0.120	0.127	0.114	0.130	0.134	0.136	0.127		0.033	0.028	0.031	0.043
S. (P.) striatus	**11**	0.141	0.142	0.133	0.141	0.127	0.122	0.138	0.145	0.151	0.133		0.045	0.044	0.054
S. (P.) denticollis	**12**	0.151	0.140	0.134	0.139	0.129	0.129	0.144	0.111	0.109	0.135	0.149		0.032	0.036
S. (P.) valeflorae	**13**	0.127	0.109	0.109	0.119	0.117	0.116	0.132	0.137	0.121	0.095	0.131	0.132		0.046
Outgroups	**14**	0.153	0.144	0.137	0.144	0.126	0.136	0.148	0.146	0.139	0.137	0.158	0.142	0.126	

Molecular data set

Of the 474 variable sites identified, 421 sites were parsimoniously informative and 53 were singletons. The ratio of parsimoniously informative characters (421) to the number of OTU's/haplotypes (42) was very high and would have contributed to the good resolution of the MP tree. The proportion of nucleotide mutations at first, second and third base positions was 19 %, 5 % and 76 % respectively and base composition over the 1 197 base pairs was 39.2 %, 16.1 %, 30.5 % and 14.2 % for T, C, A and G respectively.

The un-weighted parsimony analysis resulted in a single tree with a length of 2177, a consistency index (CI) of 0.314, a retention index (RI) of 0.691, and a re-scaled consistency index (RC) of 0.217 (Fig 1). A single Maximum Likelihood (ML) tree was obtained assuming the GTR model with 57.2% invariant sites, a transition-transversion ratio of 1.2 and a gamma distribution shape parameter of 0.88. The un-weighted MP tree had a similar topology to those trees obtained following Neighbor Joining (NJ), Minimum Evolution (ME), ML and Bayesian analyses (results not shown) confirming that the data were not sensitive to the underlying assumptions of the different analysis methods.

The COI gene phylogeny (Fig. 1) reveals the presence of three distinct clades (labelled A, B and C). Clade A comprises 21 individuals, representing six morphological species, namely *S. (P.) hippocrates, S. (P.) glentoni, S. (P.) aesculapius*, *S. (P.) endroedyi, S. (P.) valeflorae and S. (P.) schinzi*. There is high bootstrap support (between 85 % and 100 %) for four of the six morphological species in this clade with a single individual, *S. (P.) glentoniLEIPV03,* not grouping with the other two representatives of this morphological species. Instead, a species complex comprising 11 individuals of *S. (P.) glentoni* and *S. (P.) hippocrates* (henceforth referred to as the hippocrates/glentoni complex) was recovered. Clade B supports four species, *S. (P.) fitzsimonsi, S. (P.) bennigseni, S. (P.) striatus* and *S. (P.) gariepinus* each with 100% support. Clade C (100 % support) supports three species each with 100% bootstrap support, namely *S. (P.) denticollis, S. (P.) rotundigenus* and *S. (P.) rodriguesi*.

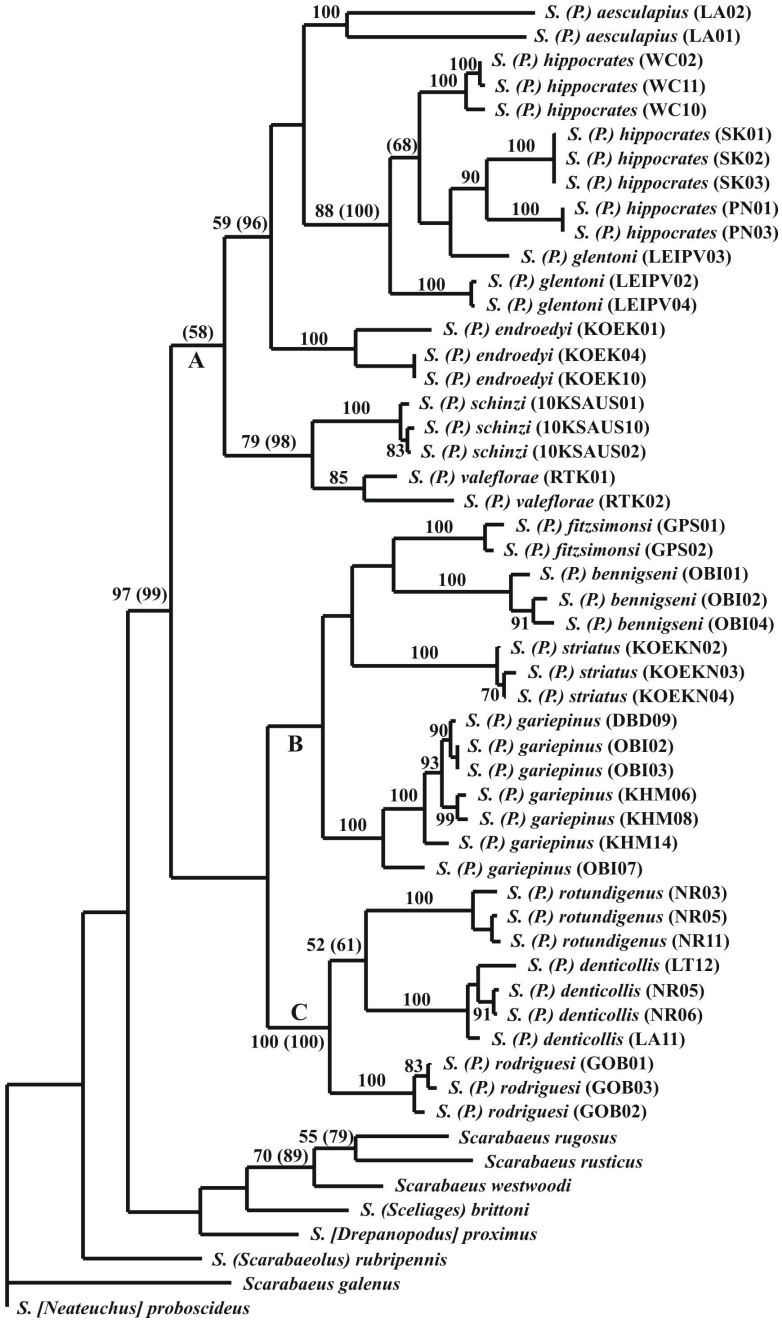

Figure 1. The single most Parsimonious tree of the COI gene phylogeny of *Scarabaeus (Pachysoma)* with bootstrap values greater than 50 % indicated next to the relevant nodes. A, B and C indicate three distinct clades within *Scarabaeus (Pachysoma)*. Maximum Likelihood bootstrap values are in brackets.

Morphological data set

Un-weighted analysis of the 64 characters (Appendix 1), of which 61 were informative, resulted in 20 most parsimonious trees (length = 244, CI = 0.51, RI = 0.74, RC = 0.38). The re-weighted parsimony analysis resulted in two most parsimonious trees (length = 77.447, CI = 0.696, RI = 0.888 and RC = 0.618), of which the strict consensus tree is shown in Figure 2. *Scarabaeus (Pachysoma)* appears monophyletic within *Scarabaeus* with 100 % bootstrap support. All the species within *Scarabaeus (Pachysoma)* appear monophyletic with relatively good bootstrap support (between 50 % and 95 %) for all 13 species. *S. (P.) schinzi* and *S. (P.) valeflorae* are sister species, with 60 % bootstrap support and appear as outliers to the other 11 species. *S. (P.) hippocrates, S. (P.) glentoni*, *S. (P.) endroedyi* and *S. (P.) aesculapius* form a distinct group (91 % bootstrap support) within the *Scarabaeus (Pachysoma)* lineage. *S. (P.) hippocrates* and *S. (P.) glentoni* form sister species, with 85 % bootstrap support.

Figure 3 shows a scanned copy of the tree taken directly out of Harrison & Philips (2003). Harrison & Philips (2003) constructed the Parsimony tree in NONA v. 2.0 (Goloboff, 1997) and for details thereof see Harrison & Philips (2003). *Scarabaeus (Pachysoma)* is clearly monophyletic within *Scarabaeus*. *S. (P.) hippocrates* and *S. (P.) endroedyi* are sister taxa *S. (P.) schinzi* and *S. (P.) valeflorae* are sister species and do not fall as outliers as in the molecular analysis. *S. (P.) fitzsimonsi*, *S. (P.) bennigseni*, *S. (P.) striatus* and *S. (P.) gariepinus* group together and are central within the *Scarabaeus (Pachysoma)* lineage. *S. (P.) denticollis*, *S. (P.) rotundigenus* and *S. (P.) rodriguesi* are sister to each other and form a distinct clade within *Scarabaeus (Pachysoma)*.

Combined Analysis

The partition homogeneity test (Farris *et al*., 1995) on the combined data (two partitions: COI 1197 bp and 64 morphological characters) indicated that the data partitions did not differ significantly (p = 0.187 at $p \geq 0.05$) and could therefore be combined. A heuristic search produced two most parsimonious trees (length = 2058, CI = 0.34, RI = 0.40 and RC = 0.14). A single most parsimonious tree was obtained by successive weighting using the re-scaled consistency index (length = 237.107, CI = 0.541, RI = 0.742 and RC = 0.402) and is presented in Figure 4. Bootstrap, Bremer and partitioned Bremer support values are also shown on Figure 4.

The combined data analysis supports both the morphological and molecular analysis by showing that *Scarabaeus (Pachysoma)* is a monophyletic lineage within *Scarabaeus* supported by both high bootstrap and Bremer support (100 % bootstrap support and BS = 30) with both the molecular and morphological data partitions contributing. The *Scarabaeus (Pachysoma)* lineage shows three distinct clades (labelled A, B and C) as in the molecular data set analysis (Fig. 1). Clade A supports six morphological species (80 % bootstrap support), namely *S. (P.) hippocrates, S. (P.) glentoni, S. (P.) aesculapius*, *S. (P.) endroedyi, S. (P.) valeflorae* and *S. (P.) schinzi*. *S. (P.) hippocrates, S. (P.) glentoni, S. (P.) aesculapius* and *S. (P.) endroedyi* form a distinct lineage (100 % bootstrap) within this clade as in both the morphological and molecular data partition analyses. *S. (P.) hippocrates* and *S. (P.) glentoni* form sister species (100 % bootstrap and BS = 12), as do *S. (P.) valeflorae* and *S. (P.) schinzi* (99 % bootstrap and BS = 6). *S. (P.) aesculapius* and *S. (P.) endroedyi* form sister species with 93 % bootstrap support. Group B supports four monophyletic species, *S. (P.) fitzsimonsi, S. (P.) gariepinus, S. (P.) bennigseni* and *S. (P.) striatus* (85 % bootstrap support, BS = 2). Group C supports three species, *S. (P.) denticollis, S. (P.) rotundigenus* and *S. (P.) rodriguesi*, with 100% bootstrap support (BS = 17). The molecular and morphological data partitions, according to the PBS, appear in most instances to contribute equally to the whole phylogeny. There are however two instances where very large support is obtained from the molecular dataset, Clade C (PBS = 16) and for *S. (P.) hippocrates* and *S. (P.) glentoni* (PBS = 11) as sister species.

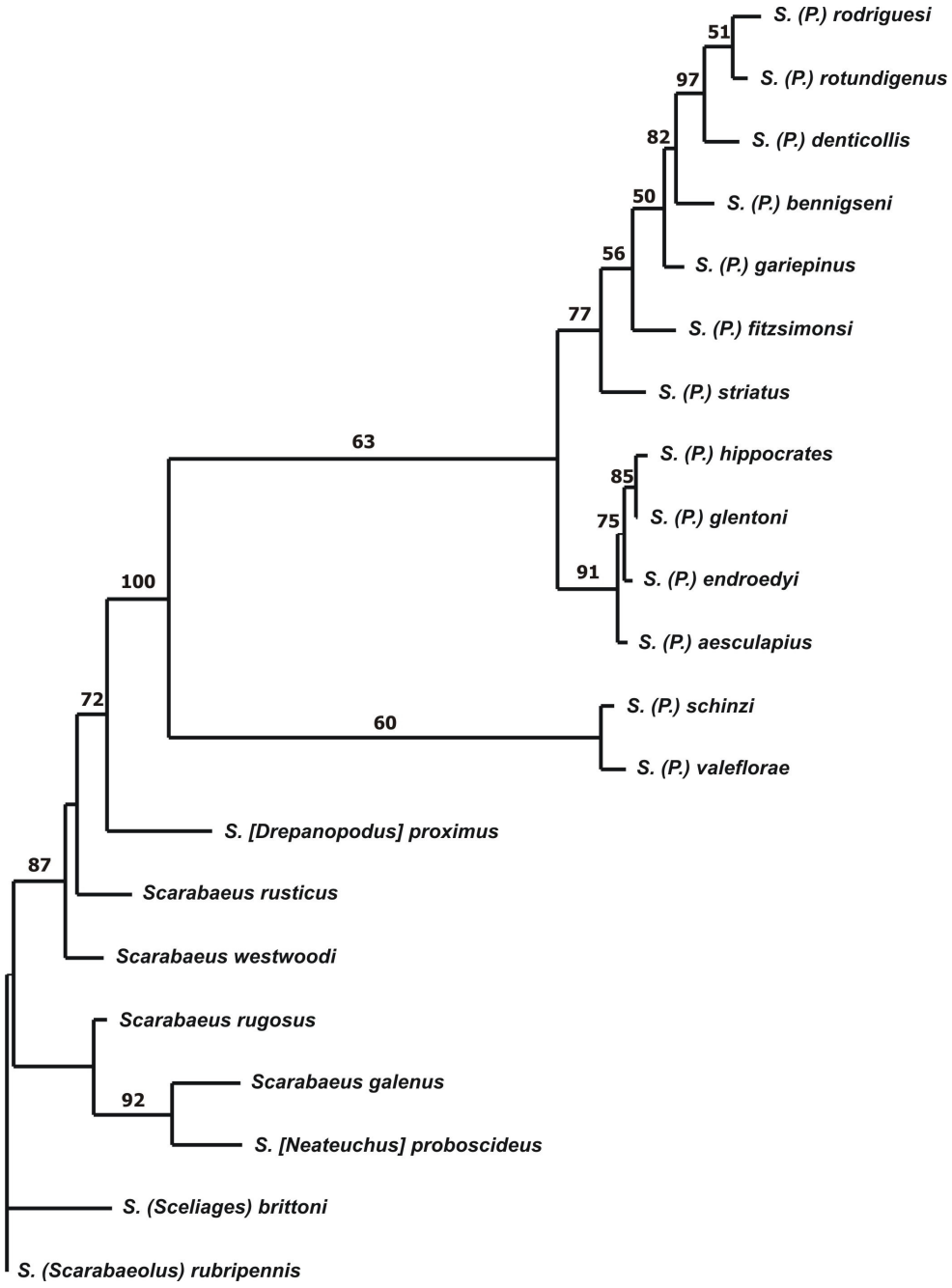

Figure. 2. The Strict consensus Parsimony tree of the morphological data partition of *Scarabaeus (Pachysoma)* with bootstrap values indicated.

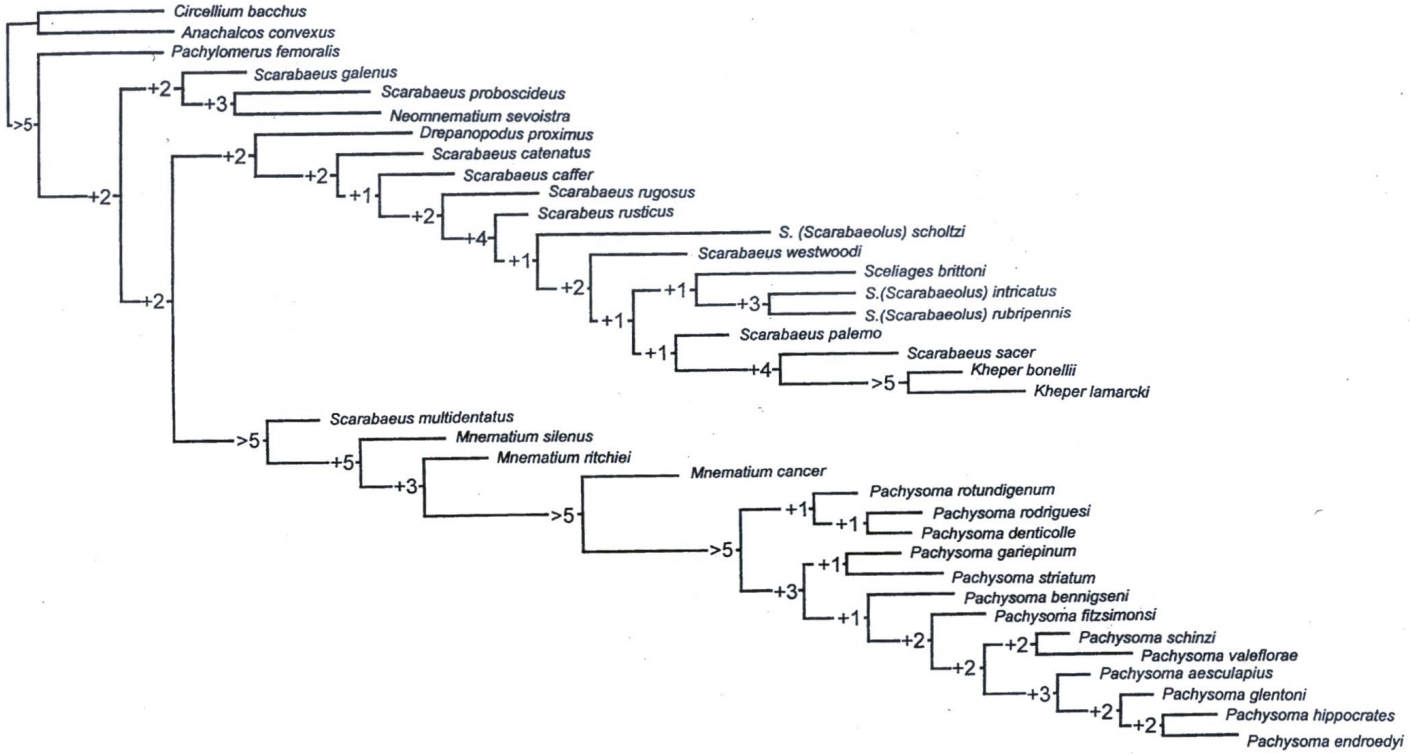

Figure 3. Cladogram depicting the relationships between '*Pachysoma*' and other winged and wingless taxa (obtained from Harrison & Philips (2003) for comparative purposes). The 823-step cladogram (CI = 0.52; RI = 0.85) was obtained after successive weighting of 37 taxa and 64 characters with NONA v 2.0. Numbers indicate decay indices i.e. number of steps needed to collapse a node.

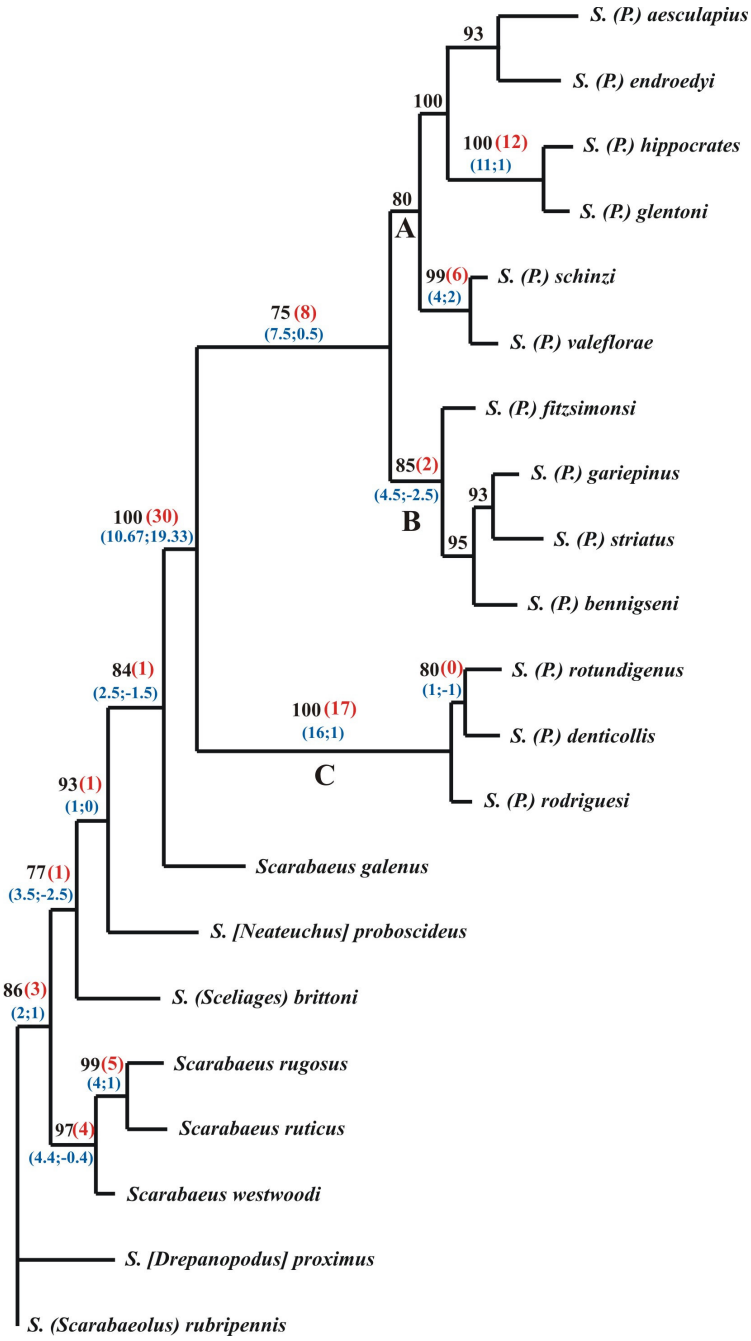

Figure 4. Parsimony tree of combined data partitions. Bootstrap values presented in bold black, Bremer support values are in red and partitioned Bremer support (PBS) values in blue. For PBS values the first value is that for the COI data partition and the second value that for the morphological data partition. (A, B and C represent three clades within the *Scarabaeus (Pachysoma)* lineage, as seen in the molecular phylogeny).

Discussion

Data analysis

Phylogenetic relationships of *Scarabaeus (Pachysoma)* were reconstructed using both molecular and morphological datasets. The expression of differences within a clade is related to its history and to the environmental parameters within which it develops. Both the morphological and COI data partitions display similar patterns between the relationships of the species and have significant phylogenetic structure. It is interesting that such different datasets provide strong phylogenetic signal as individual data partitions as well as when combined. In addition, congruence among datasets is a strong indicator of support for phylogenies based on individual datasets (Wheeler, 1995). Partitioned Bremer Support (Baker & DeSalle, 1997) provides a means of assessing the contribution of molecular and morphological data to the total support of the simultaneous analysis tree. It appeared that the COI dataset lent more support to the overall tree topology of the combined dataset analysis. However, despite the potential differences, consistent compatible trees were recovered which suggest that the models used to analyse the data were adequate for recovering the correct phylogenetic signal (Miyamoto & Fitch, 1995; Clark *et al*., 2001). Considering that the partition homogeneity test was not significant, it would indicate that the combined dataset maximises the amount of information gained by revealing the correct tree (Vogler & Pearson, 1997; Clark *et al*., 2001). Combining of datasets is under debate and a contentious issue (Bull *et al*., 1993; de Queiroz *et al*., 1995; Miyamoto & Fitch, 1995; Funk *et al*., 1995b; Huelsenbeck *et al*., 1996; Yoder *et al*., 2001). However the decision to combine datasets in this study was conservative as similar trees were obtained from both the morphological and molecular phylogenies, and the combined dataset improved resolution, lending strong support for combining good datasets. This was reflected by the robust support for clades in both the molecular and morphological data partition analyses, which was upheld by combining the data partitions.

Differences between phylogenies based on different datasets

Even though it can clearly be seen that increased or better resolution is obtained by combining datasets in this study certain differences do occur between the phylogenies. The morphological phylogeny shows *S. (P.) schinzi* and *S. (P.) valeflorae* group almost as a totally separate clade, which is a major difference between the phylogenies. The other difference noted was the relationships of the central four species, *S. (P.) fitzsimonsi, S. (P.) gariepinus, S. (P.) bennigseni* and *S. (P.) striatus,* to each other. *S. (P.) hippocrates* and *S.*

(P.) glentoni appear to be sister taxa in all our analyses but not in the phylogeny of Harrison & Philips (2003). These differences do not, however, detract from the fact that 13 good species can be identified and *Scarabaeus (Pachysoma)* is a monophyletic lineage within *Scarabaeus*. As morphological and genetic distinctiveness are not strictly correlated discrepancies are often encountered between gene trees and species trees (Vink & Paterson, 2003).

Many studies to date have included combining of datasets, some combining only different genes (i.e. mitochondrial and nuclear) while others combine genes with morphology. Both the genes and morphology of a single individual are exposed to the same environmental parameters but may respond differently in the way that these parameters are dealt with. Different data types are independent indicators of a phylogeny and by combining the unlinked data partitions one would hope to attain an overall similar picture of the relationships relating to the relevant studied taxa. Different studies based on the total evidence approach have shown that by combining different datasets as well as different combinations of the overall available data better resolved trees are more often than not obtained. For examples see Notothenioidei: Channichthyidae (Near *et al*., 2003), Aranae: Lycosidae (Vink & Paterson, 2003), Diptera: Muscidae (Savage *et al*., 2004), Coleoptera: Scarabaeidae (Cabrero-Sañudo & Zardoya, 2004) and Rodentia: Bathyergidae (Ingram *et al*., 2004). Combining data partitions provides a means to discriminate amongst alternate hypotheses posed within the group of interest.

Comparison with prior phylogenetic studies

a) Pachysoma vs. Pachysoma

The inferred trees provide robust evidence for the monophyly of *Scarabaeus (Pachysoma)*, supporting previous studies by Harrison *&* Philips (2003) and Forgie *et al*. (2005). All phylogenetic estimates in the study support the traditional morphological phylogeny by Harrison & Philips (2003). This is not surprising as *Scarabaeus (Pachysoma)* is a well-studied group of dung beetles from a taxonomic point of view (MacLeay, 1821; Ferreira, 1953; Holm & Scholtz, 1979; Mostert & Holm, 1982; Endrödy-Younga, 1989; Harrison *et al*., 2003; Sole *et al*., 2005).

Our results also generally concur with Davis's (1990) phenogram which shows three distinct groupings on the phenogram that correspond to the three clades revealed by the molecular and combined analyses within *Scarabaeus (Pachysoma),* indicated by 'A, B and C'

(Figures 1 & 4, respectively). The species groups delineated by Davis (1990) were based on 28 coded characters described by Holm & Scholtz (1979) and are as follows:

I) *S. (P.) aesculapius, S. (P.) hippocrates* and *S. (P.) schinzi* = clades labelled A on the combined and morphological phylogenies respectively

II) *S. (P.) bennigseni, S. (P.) gariepinus, S. (P.) striatus* and *S. (P.) fitzsimonsi* = clades labelled B on the combined and morphological phylogenies respectively

III) *S. (P.) rodriguesi, S. (P.) denticollis* and *S. (P.) rotundigenus* = clades labelled C on the combined and morphological phylogenies respectively.

b) mtDNA variation in Pachysoma vs. other insect orders

Intra-genic variability in evolutionary rate, at lower level taxonomy, has received little attention, but it appears that the evolutionary rates among portions of COI have remained similar throughout much of insects' evolutionary history (Lunt *et al*., 1996; Langor & Sperling, 1997). The overall A-T content of the 1 197 bp region of the partial COI gene in *Scarabaeus (Pachysoma)* is 69.7 %, which is at the lower end of the 68-76 % range reported for other insects (reviewed by Lunt *et al*., 1996). The average intra-specific COI divergences for *Scarabaeus (Pachysoma)* range between 0.8 and 6.3 % which are comparable to other species of Coleoptera for example *Pissodes* species complex (Curculionidae) (0.5 - 7.5 %; Langor & Sperling, 1997); *Ophraella* (Chrysomelidae) (3.8%; Funk *et al*., 1995a; Funk *et al*., 1995b); *Hypera postica* (Gyllenhal) (Curculionidae) (3.1 %; Erney *et al*., 1996) and *Prodontria* Broun (Scarabaeidae: Melolonthinae) (1.47 %; Emerson & Wallis, 1995). The figures are also similar in other insect orders for example *Papilio* (Lepidoptera: Papilionidae) (0 - 9 %; Sperling, 1993; Sperling & Harrison, 1994), *Apis* (Hymenoptera: Apidae) 0.15 - 1.70 %; Sittipraneed *et al*., 2001), *Drosophila* (Diptera: Drosophilidae) (1.5 - 10 %; Solignac *et al*., 1986) and *Anopheles* (Diptera: Culicidae) (0.005 - 1.2 %; Sedaghat *et al*., 2003). Comparison with these studies is cautioned, however, as the portions of mtDNA, the assessment methods used (nucleotide data/RFLP), and the degree of relatedness of the clades examined may all have differed between studies (Langor & Sperling, 1997). Recent population bottlenecks, selective sweeps of favoured haplotypes or high variance among-family reproductive success may tend to reduce mtDNA diversity within a species. Intra-specific mtDNA variation and geographic distribution of genetic variation within a species depend on both current and historical population structure as well as directional selection. Species thought to have large population sizes and/or a subdivided population structure tend

to maintain greater amounts of mtDNA variability either as nucleotide diversity within populations or as sequence divergence between populations. All of the species of *Scarabaeus (Pachysoma)*, except *S. (P.) schinzi* and *S. (P.) striatus*, show relatively large intra-specific sequence divergences. The species of *Scarabaeus (Pachysoma)* are clearly closely related and exhibit both subdivided population structure, in that some of the populations of species occur in isolated pockets within their distributional range, and others occur along continuous dune fields in relatively large population sizes.

Inter-specific divergences for *Scarabaeus (Pachysoma)* range from 8 to 16 %, which appear to be high compared with other families of Coleoptera (*Cicindela* (0.36 – 1.09 %; Cicindelidae) (Vogler et al., 1993); *Pissodes* (6.0 – 7.5 %; Curculionidae) (Langor & Sperling, 1997)) as well as when compared with other insect orders (*Heliconius erato* (3.4 %; Lepidoptera) (Brower, 1994); *Papilio* (2 - 7.7 %; Lepidoptera) (Sperling & Harrison, 1994); *Feltia* (0.5 - 4.8 %; Lepidoptera) (Sperling *et al*., 1996) and *Drosophila* (7.1 %; Diptera) (Solignac *et al*., 1986). The mtDNA lineages appear, therefore, to have diverged to a level comparable to that beyond where most sister species have attained reproductive isolation. Consequently at the inter-species level the COI gene proved to be a strong phylogenetically informative marker for distinguishing between species within *Scarabaeus (Pachysoma)* (Jones & Gibbs, 1997).

mtDNA vs. ecological divergence in the hippocrates/glentoni complex

S. (P.) hippocrates and *S. (P.) glentoni* are morphologically very similar species and can only be reliably identified based on their male genitalia and habitat preference (for details see Harrison *et al*., 2003). The two species occur sympatrically with *S. (P.) hippocrates* having wider habitat tolerance and geographic distribution - preferring vegetated soft to firm sand of coastal hummocks and hillocks, the periphery of dune systems, and river beds and banks - while *S. (P.) glentoni* is more localised and more of a habitat specialist - preferring firm vegetated sand of river banks and coastal hummocks (Harrison *et al*., 2003). These two species provide an interesting example of ecological divergence without mtDNA sequence divergence. Two reasons can be given for mtDNA showing poor resolution at the phylogenetic level. Firstly, ecological differentiation could be occurring at a faster rate than mtDNA evolution. Secondly, the flow of mtDNA is relatively free, whereas alleles for genes coding for ecological differences are anchored to local conditions. Evidence exists that indicates that relatively fast ecological divergence contributes to poor mtDNA divergence (Shapiro & Masuda, 1980; Sims, 1980; Sperling & Harrison, 1994), suggestive of the

hippocrates/glentoni complex being a recent divergent event that has yet to show distinct mtDNA divergence.

Insight into evolutionary hypotheses of Scarabaeus (Pachysoma)

The phylogenies show a strong geographic association in that the species that group together within a clade have similar distributions. *S. (P.) hippocrates, S. (P.) glentoni, S. (P.) endroedyi* and *S. (P.) aesculapius* have the most southerly distribution (Namaqualand based) within the total *Scarabaeus (Pachysoma)* distribution as well as exhibit the most plesiomorphic characters. *S. (P.) fitzsimonsi, S. (P.) bennigseni, S. (P.) striatus* and *S. (P.) gariepinus* occur in the centre of the total *Scarabaeus (Pachysoma)* distribution. The most derived species – *S. (P.) denticollis, S. (P.) rotundigenus* and *S. (P.) rodriguesi* – have the most northerly distribution and are ultra-psammophilous and therefore well adapted to the loose sand of the Namib Dune Sea.

Aridification would have placed high selection pressure on the xeric adapted winged wet dung feeders. Three main solutions can be used to deal with aridity: increasing diurnal flying efficiency by flying less and foraging faster, reducing body size and feeding on both dung and carrion (Klok, 1994, Harrison & Philips, 2003). *Scarabaeus (Pachysoma)* are flightless which would reduce water loss and energy costs and also exhibit the strategy of feeding on dry dung/detritus (Scholtz, 1989; Klok, 1994; Harrison & Philips, 2003). Dryness of the environment would result in slow rates of decay, hence insects feeding on detritus, carcasses or on persistent plant parts would find that they would persist over long periods of time (Roff, 1990; Scholtz, 2000). The combination of various factors, such as low or no competition for dry dung/detritus, the stable environment and the morphological and physiological constraints of surviving in a sandy xeric habitat may have resulted in the evolution of the present *Scarabaeus (Pachysoma)* lineage (Scholtz, 1989; Klok, 1994; Chown *et al*., 1998; Harrison & Philips, 2003).

Conclusion

Species richness, relative abundance of taxa, genetic and morphological diversity, and ecological diversity are various concepts encompassed by biodiversity. Biodiversity is a result of historical processes; therefore to study biodiversity, access to these patterns and processes is needed. A start to understanding the history behind the diversity is a good phylogeny, which was the reasoning behind this study. The major conclusion is that the combined phylogeny obtained in this study supports both the morphological and molecular

phylogenies. Results are dependant upon both the taxa sampled and the resolving ability of the datasets. In combining the information the two datasets complement each other, and the deficiencies thought to affect the overall result i.e. the small number of morphological characters being overshadowed by the large molecular dataset, are compensated for by each other. It would be advantageous in future studies to expand phylogenetic examination to include nuclear ribosomal genes like 18S or nuclear protein coding genes like elongation factor-1 α (Clark *et al*., 2001) as well as additional individuals from the hippocrates/glentoni complex. We interpret the general agreement of reconstructions between data partitions as an indicator of the validity for the combination of datasets.

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Chapter IV

Phylogeographic patterns of *Scarabaeus (Pachysoma*) (Coleoptera: Scarabaeidae) as inferred from gene genealogies and coalescent theory.

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Running title: Phylogeography of *Scarabaeus (Pachysoma)* (Scarabaeidae: Scarabaeinae).

Abstract

Mitochondrial cytochrome oxidase I (COI) sequence data were used to infer phylogeographic patterns of three species of *Scarabaeus (Pachysoma)*, a group of flightless dung beetles endemic to the arid west coast of southern Africa. Nested clade analysis in conjunction with historical demographic analysis allowed for the inference of historical bottlenecks followed by population expansion in response to climatic oscillations. All three species exhibit high overall haplotype diversity with no shared haplotypes between populations or collecting localities, refugia could, therefore, not be identified. Recent events imply human induced, environmental barriers and reduced vagility as having caused fragmentation, influencing the strong population structure seen in two of the three species. Coalescence for each species was calculated and it was estimated that all three species underwent population expansion within the Pleistocene era, in response to the formation of advective fog. The neighbor-joining trees showed *S. (P.) hippocrates* as having four distinct populations, *S. (P.) gariepinus* having three populations, two in South Africa and one in Namibia while *S. (P.) denticollis* comprised a single population along a dune field continuum in Namibia. AMOVA analysis confirmed the phylogenetic partitioning. Analysis of gene flow revealed a strong degree of south-north movement, consistent with the unidirectional wind regime, with some movement occurring in a southerly direction.

Keywords *Scarabaeus*, mitochondrial DNA, cytochrome oxidase I, Phylogeography, Namaqualand, Namib Desert

Introduction

Species consist of geographically structured populations, many of which have experienced little or no genetic contact for long periods of time due to the limited dispersal abilities of the individuals and/or as a result of habitat discontinuities (Carisio *et al*., 2004). In addition to selective forces, factors that contribute to these associations are past events such as colonisation history and current demography (Juan *et al*., 1998). By examining the variation among populations, their historical associations and the processes of genetic restructuring that may have lead to speciation, can often be revealed (Wright, 1931). Species complexes among geographically isolated populations of polytypic species have great potential for historic inferences (Kirchman *et al*., 2000). These geographically isolated populations represent the extreme of spatial patterning and are therefore of particular interest (Kirchman *et al*., 2000).

Phylogeography is the study of the principles and processes governing the geographical distributions of genealogical lineages, especially those within and amongst closely related species (Avise, 2000). Elucidating the phylogeographic patterns within the species of *Scarabaeus* (*Pachysoma)* will enable us to infer their evolutionary history, re-construct colonisation routes and identify possible refugia. Furthermore, it will be possible to identify and delineate genetically meaningful conservation units, evolutionary significant units (ESU's) and management units (MU's) (Moritz, 1994a; b), within the different species. This information will be useful for developing conservation management recommendations for preserving species of *Scarabaeus (Pachysoma).*

Scarabaeus (*Pachysoma)* represents an excellent group to study the effects of geographic isolation within species. The species are geographically isolated, occurring in pockets of discontinuous populations on coastal sands from Cape Town (33°56'S – 18°28'E) in South Africa to Walvis Bay (22°58'S – 14°30'E) in Namibia. Some of these areas are currently under threat of both anthropogenic and environmental factors. Threats to the habitat of *Scarabaeus* (*Pachysoma)* species come from the removal of the natural vegetation for large scale wheat farming in the south western Cape, commercial development on the west coast for holiday and recreational purposes e.g. Lambert's Bay and Strandfontein, mining for diamonds and other minerals as well as from exotic plant invaders modifying dune systems e.g. Port Jackson (*Acacia saligna*) and Rooikrans (*Acacia cyclops*). Furthermore, some of the species of *Scarabaeus* (*Pachysoma)* are potentially threatened as they are sought after by collectors (Harrison, 1999).

The species of *Scarabaeus (Pachysoma)* range from 2 – 5 cm in length, with *S. (P.) denticollis* and *S. (P.) rodriguesi*, representing the smallest and largest extremes in body size,

respectively. Three species, *S. (P.) hippocrates*, *S. (P.) gariepinus* and *S. (P.) denticollis* where selected for population based analyses as these species exhibited distinct south-north morphological clinal variation (Harrison, 1999).

Cytochrome oxidase I (COI) sequence data are used in the present study for comparisons within and between *S. (P.) hippocrates*, *S. (P.) gariepinus* and *S. (P.) denticollis*. We addressed the following questions: Firstly, to what degree has geographic isolation led to the genetic restructuring between populations of the same species? Secondly, what is the extent of gene flow between populations of the same species and does it correlate with patterns of geographic proximity? Thirdly, from which geographic location did the group originate and how are the populations of each species related to one another? Finally, what are the effective/actual population sizes of the species in question?

Materials and Methods

Population sampling, amplification and sequencing

For all three of the species, we sampled, where possible, a minimum of 10 individuals per designated population. Each of the species was divided into populations based on morphological and distributional data (Table 1). Total genomic DNA was extracted from 176 individuals from the thoracic muscle tissue and amplified using TL2–N-3014 and C1–J–1718 (Simon *et al*., 1994) targeting a 1345 base pair (bp) fragment. Thermal cycling parameters comprised an initial denaturation for 90 seconds at 94°C followed by 35 cycles at 94°C for 22 seconds, 48°C for 30 seconds and 72°C for 90 seconds with a final elongation step at 72°C for 1 min. Amplified COI products were purified from the tube using the High Pure PCR Product Purification Kit (Roche) according to manufacturer specifications. An analysis of the 1197 bp region generated for 46 individuals of the 13 morphological species of *Scarabaeus (Pachysoma)* (Sole *et al*., 2005) revealed that the 5' end of the COI gene was the most parsimoniously informative. Based on this we used only 960 bp from the 5' end of the partial COI gene for the present study. For this reason, each amplicon was sequenced with C1-J-1718 and C1-J-2183 (Simon *et al*., 1994). Sequencing reactions were performed at an annealing temperature of 48ºC with version 3.1 of the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer). Nucleotide sequences were determined through a capillary system on an ABI 3100 automated sequencer (Perkin-Elmer). Sequence chromatograms were visualised and edited in Sequence Navigator (Perkin-Elmer).

Analysis

Phylogenetic Analysis

Hierarchical likelihood ratio tests were performed using Modeltest 3.5 to determine the model of sequence evolution that best fit the data at hand (Posada & Crandall, 1998). Parameters such as the proportion of invariable sites and α parameter of the gamma distribution of rate variation among sites (Yang *et al*., 1994) were calculated in Modeltest. MEGA version 2.1 (Kumar *et al*., 2001) was used to calculate the transition/transversion (ti/tv) ratio. Maximum Parsimony (MP) (Kluge & Farris, 1969) and Maximum Likelihood (ML) (Felesenstein, 1973, 1981) trees were obtained using PAUP* v. 4.08b (Swofford, 1998). For MP trees, we used heuristic searches with tree-bisection-reconnection (TBR) as the branch-swapping algorithm and the nucleotides were treated as unordered characters. The starting tree was obtained via stepwise addition with random addition of sequences with 10 replicates. ML analysis was performed as above, using the values obtained from Model Test but given the computational time required no replicates were performed. The neighbor-joining (NJ) (Satou & Nei, 1987) option in the computer program MEGA was used to re-construct relationships between the populations within the three species with the model selected from Modeltest. Support for all relationships was estimated using 1000 bootstrap replicates (Felsenstein, 1985). Population assemblages within species were ascertained from mid-point rooted trees. As all tree topologies from the different tree drawing options were similar only the NJ trees are presented.

Molecular diversity

Mean nucleotide diversities within each population/assemblage were calculated with Arlequin 2.000 (Schneider *et al*., 2000). Hierarchical structuring of genetic variation was determined using AMOVA (Excoffier *et al*., 1992 new version: Schneider *et al*., 2000), which produces Φ - statistics similar to the F - statistics of Wright (1951; 1965). Φct describes the regional apportionment of genetic variation with respect to all haplotypes, Φsc describes the apportionment of variation within the populations of a given region and Φst characterises the variation between haplotypes in a single population relative to all haplotypes (Barber, 1999). Analyses were performed independently of groupings designated by the authors as well as on the assemblages identified from each neighbor-joining tree, to determine the hypothesis that best fit the data. Levels of significance of Φst - statistics were determined through 10,000 random permutation replicates (Schneider *et al*., 2000).

Historical Population Dynamics

Distance-based methods (mismatch distribution in Arlequin 2.000, Schneider *et al*., 2000), coalescence and maximum likelihood methods (LAMARC version 1.2.2 Kuhner *et al*., 2004; MIGRATE version 1.7.6.1 Beerli & Felsenstein, 1999; 2001) were used to estimate effective population size, exponential growth or shrinkage, time and rate of expansion and migration rates within and among populations/assemblages.

Population growth/decline based on two models

Stepwise Expansion Model

The program Arlequin was used to calculate the mismatch distribution (frequency of pairwise differences) between the haplotypes of a population. This evaluates the hypothesis of recent population growth (Rogers & Harpending, 1992) with the underlying assumption that population growth or decline leaves distinctive signatures on the DNA sequences compared with constant population size. Recent growth should generate a unimodal distribution of pairwise differences, but the exact mode of growth (exponential, stepwise or logistic) cannot be distinguished (Rogers & Harpending, 1992). This distribution is then compared with that expected under a model of population expansion (Rogers, 1995) calculating the estimator expansion time (τ) and the mutation parameter (θ) (Schneider & Excoffier, 1999). A non-linear least squares (Schneider & Excoffier, 1999) approach is used to estimate parameters for the stepwise growth model: $\theta_0 = 2\mu N_0$ (before expansion), $\theta_1 = 2\mu N_1$ (after expansion) and $t = \tau / 2v$ (time of expansion, note $v = m_T\mu$ which is the mutation rate for the entire DNA sequence under study where m_T is the number of nucleotides and μ is the mutation rate). N_0 and N_1 are the effective population sizes of females before and after population expansion respectively.

For the COI data we estimated the mutation rate by following the procedure in Rooney *et al*. (2001). Firstly, the number of nucleotide substitutions per site was estimated by comparing the in-group (classified as one of the three species within this study) with its sister taxa, (which was obtained from the phylogeny found in Sole *et al*., 2005; chapter 2) using the formula $d = (Tv + TvR)/m_T$, where Tv is the number of tranversions between the focal and sister species, R is the ratio of transitions to transversions within the focal species and m_T is the length of the investigated DNA sequence. Secondly, the rate of nucleotide substitution (γ) per site, per lineage, per year was estimated by $\gamma = d/2T$, where T stands for the divergence time of the two compared species (this was estimated in MEGA using Brower, 1994; 2.3 %

pairwise divergence per million years) - (Divergence estimates are under debate and surrounded by much contention. However, the decision to use the molecular clock to estimate divergence times in this study was conservative and used with caution (Graur & Martin, 2004)). Thirdly, it was possible to estimate mutation rate per nucleotide site, per generation (μ) by solving the equation $\mu = \gamma t_{g,}$ where t_g is generation time in years, which in this case was taken to be a single generation per year. The mutation rate per haplotype (v) was calculated by $v = m_T\mu$. Finally, the coalescence time (time to expansion) in generations was calculated by $t = \tau/2v$ (Rogers & Harpending, 1992) and the coalescence time in years was estimated by multiplying t with generation time.

Arlequin estimates approximate confidence intervals for θ_0, θ_1 and expansion time (τ) - which are substituted into the above equations to solve for $N_{0,}$ N_1 and t, respectively - by parametric bootstrapping of 10,000 replicates**.** If population growth applies, the validity of the stepwise expansion model is tested using the same bootstrap approach by a goodness of fit statistic (P), representing the probability that the variance in the simulated dataset is equal to or greater than that seen in the observed dataset. We also computed Harpending's Raggedness Index - R - and its significance in the same manner (Harpending, 1994).

Tajima's (1983) estimate of θ, was estimated in Arlequin, while Fu's (1994a, b) UPBLUE estimate of θ, was estimated by Fu's phylogenetic estimator of θ on line (http://hgc.sph.uth.tmc.edu/cgi-bin/upblue.pl). Tajima's estimate is based on the calculation of the mean number of pairwise differences of the sequences, while Fu's UPBLUE estimate is calculated by incorporating the genealogical information of the sequences (Su *et al*., 2001). Fu's UPBLUE estimate puts emphasis on recent mutations, revealing recent population processes, while Tajima's estimate puts more weight on ancient mutations, reflecting past population trends (Su *et al*., 2001). As $\theta = 2N\mu$ for the mitochondrial genome, the ratio of population size change is correlated with θ given a constant mutation rate (μ). Comparing Tajima's with Fu's UPBLUE estimate will give an idea of population size change in recent time. In addition, Fu's (1997) *F*s test of neutrality was carried out in Arlequin. Although the *F*s was originally designed as a test of neutrality, it has utility as an estimator of population growth (Smith & Farrell, 2005). The *F*s value tends to be negative if there is an excess of recent mutations (i.e. mutations that occur in a small number of individuals). A large negative value indicates an excess of recent mutations an outcome that can be caused by either population growth and/or selection (Su *et al*., 2001).

Exponential Expansion Model

The program LAMARC version 1.2.2 was used to estimate population parameters based on coalescence and maximum likelihood methods. LAMARC uses the Markov Chain Monte Carlo (MCMC) approach with the Metropolis-Hastings genealogy sampler to search through genealogies of which samples are taken at intervals from which to calculate a maximum likelihood of theta (θ). LAMARC calculates estimates of population exponential growth or decline, population size, recombination and migration rates. Ten short chains of 500 steps each, which were followed by 2 long chains of 10,000 steps, sampled every 20^{th} step and 4 heated chains were run at temperatures of 1, 1.3, 1.6 and 2. Each run was repeated 4 times to ensure consistency of results. We used LAMARC to estimate theta ($\theta = 2N_f(\mu)$) and growth, given as g ($\theta_{now} = \theta_t^{(-gt)}$) (or decline) rates for populations of the three species.

Migration

In order to evaluate the relationships among populations within species we used the program MIGRATE version 1.7.6.1 to estimate both effective population sizes ($\theta = 2\mu N_f$) and migration rates ($M = 2mN_f$) (Beerli & Felesenstein, 1999; 2001). MIGRATE searches through genealogy space using a likelihood ratio test and coalescent theory to estimate these parameters. It makes use of the MCMC approach with the Metropolis Hastings Green algorithm. It assumes constant effective population sizes for each population, but allows various effective population sizes for different populations (Zheng *et al*., 2003). Values of theta (θ) were estimated from the Fst-calculation. When using MIGRATE on our data we used the population subdivisions from Table 1 for each species running each analysis separately. Ten short chains with 1000 sampled genealogies and three long chains with 1,000,000 sampled genealogies each, were run. Heating was set to be active with four heated chains at temperatures of 1.00, 1.33, 1.66 and 2.00. Five runs were repeated for each species' dataset to check for consistency.

Network Estimation and Nested Clade Analysis

A haplotype network was estimated using statistical parsimony (Templeton *et al*., 1992) in TCS version 1.18 (Clement *et al.*, 2000). The method links haplotypes with smallest number of differences as defined by a 95 % confidence limit. Loops (= reticulations) in the network, which result from homoplasy in the data, were broken in accordance with the predictions derived from coalescent theory: i) common haplotypes are more likely to be found at interior

nodes of a cladogram, and rare haplotypes at the tips; ii) haplotypes represented by a single individual are more likely to connect to haplotypes from the same population than to haplotypes from different populations (Crandall & Templeton, 1993; Posada & Crandall, 2001).

Nested Clade Phylogeographical Analysis (NCPA; Templeton et al., 1995) was used to infer population history in each of the three species. NCPA first tries to reject the null hypothesis of no association between haplotype variation and geography and then attempts to interpret the significant associations (Crandall & Templeton, 1993; Templeton *et al*., 1995). The NCPA was constructed by hand, based on the parsimony network following the rules given in Templeton & Singh (1993). Such a nested design treats haplotypes as "0-step clades," groups of haplotypes separated by a single mutation as "1-step clades," groups of "1-step clades" separated by a single mutation as a "2-step clade" and so on.

GEODIS v2.0 (Posada *et al*., 2000) was used to calculate NCPA distance measures and their statistical significance. This method uses geographical distances between sampled populations to calculate two basic statistics: Dc (clade distance) and D*n* (nested clade distance). Dc measures the average distance of all clade members from the geographical centre of distribution. D*n* measures how widespread a clade is in relation to the distribution of its sister clades within the same nesting group. Random geographical distribution in coalescent theory allows one to distinguish between tip (with one connection to the remaining network) and interior (with two or more connections) clades by permutational tests of which we performed 10,000. We used Templeton's (2004) updated inference key to deduce the cause of significant associations between haplotypes. This would allow us to distinguish between historical (fragmentation, range expansion) and current (gene flow, genetic drift, system of mating) processes responsible for the observed patterns of genetic variation (Templeton *et al*., 1995).

The Mantel test (Mantel, 1967) was used to determine significant associations between genetic distances obtained in MEGA and geographic distances between the designated populations from Table 1. One thousand randomised permutations were performed using Mantel version 2.0 (Liedlof, 1999).

Table 1. Total number of individuals for each population and subpopulation. The populations were designated according to geographic and morphological differences.

Species	Population location	Population individuals	Subpopulation individuals
S. (P.) denticollis	**Population - Koichab Pan (KP)**	**13**	
	Koichab Pan		11
	Luderitz		2
	Population - Namib Rand (NR)	**8**	
	Tok Tokkie Trails		8
	Population - Gobabeb (GB)	**11**	
	Gobabeb - 5km SE Homeb		11
S. (P.) gariepinus	**Population - Langhoogte to Kommagas Road (LK)**	**12**	
	Langhoogte to Kommagas Road		12
	Population - Holgat River (HR)	**17**	
	40km N Port Nolloth - Holgat River		17
	Population (Namibia) - Hohenfells (HF)	**13**	
	Hohenfells Dunes		13
	Population (Namibia) - Daberas/Obib Dunes (DO)	**11**	
	Road from Daberas to Obib Dunes		11
	Population (Namibia) - Klinghardts Mountains (KM)	**14**	
	Klinghardts Mountains		14
S. (P.) hippocrates	**Population - Cape Town/Lamberts Bay (LA)**	**13**	
	10km West of Leipoldtville		13
	Population - Olifants/Green River (BV/KK)	**9**	
	Kommandokraal Farm (KK)		5
	Koekenaap (BV)		4
	Population - Green/Buffels River (SK)	**20**	
	Kleinsee - Sandkop		20
	Population - Buffels River/Port Nolloth (PN)	**11**	
	1km North of Port Nolloth		11

Chapter IV (a)

Genetic structure, phylogeography and demography of *S. (P.) hippocrates* based on inferences from Cytochrome Oxidase I

Introduction

The long-term persistence of many species is threatened by the loss of their natural habitat caused by human activities. Remaining habitats are often small and isolated from each other by less suitable habitat e.g. settlement areas, agriculture and roads. Isolation of local populations and reduction of suitable habitat are potential negative effects of a fragmented landscape. Causal factors of fragmentation may be human induced or environmental. Isolation is of particular significance when considering taxa with limited dispersal ability as they face an increased risk of extinction due to demographic and genetic factors.

Scarabaeus (Pachysoma) hippocrates represents a good species to evaluate the effects of geographic isolation caused in some instances by natural barriers and in others by human activities. *S. (P.) hippocrates* is one of the larger species of the flightless *Scarabaeus (Pachysoma)* occurring from Bloubergstrand (S33°48' – E18°27'), Cape Town, to Port Nolloth (S29°15' – E16°53') in Namaqualand. *S. (P.) hippocrates* prefer vegetated soft to firm sand of coastal hummocks and hillocks, the periphery of dune systems, riverbeds and banks (Harrison, 1999). The species is shown to exhibit a gradual morphological cline along this distribution with populations isolated by both natural and non-natural barriers. Habitat modification threatens certain populations of *S. (P.) hippocrates*, specifically those occurring at Port Nolloth, where the town is expanding into their habitat, and areas around Leipoldtville and Kommandokraal, where farming communities exist.

Materials and Methods

See body of Chapter 4. The localities where *S. (P.) hippocrates* were collected for this study are represented in Figure 1.

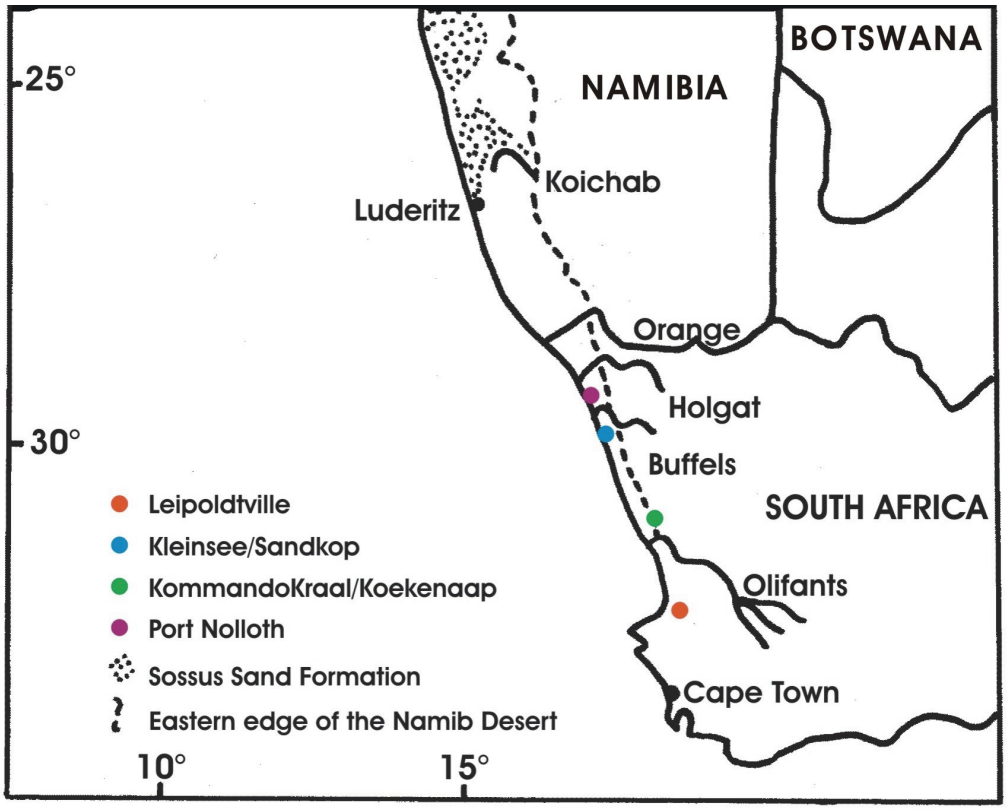


Figure 1. Collecting sites/localities, within South Africa, of *S. (P.) hippocrates* used in this study.

Results

Phylogenetic and molecular diversity

Population statistics

Overall we collected 53 individuals for molecular characterisation (GenBank Accession Numbers AY965154 – AY965206: Appendix 2). The sequences exhibited an overall A/T bias of 69.5 %. Un-corrected pairwise distances ranged from 1 to 12.3 % (data not shown). The model best fitting the data was the Transversional model with a gamma distribution of 0.0012 (TvM assuming unequal base frequencies and different transition and transversion rates, (Posada & Crandall, 1998; Nahum *et al.*, 2003). Individual LAPH13 was initially removed from the data analysis as it appeared as an outlier/out-group to *S. (P.) hippocrates* (tree not illustrated). However, as removal of the individual did not alter the relationships within the neighbor-joining tree it was included in all subsequent analyses. It may have appeared as an out-group as there is a known species complex between *S. (P.) hippocrates* and *S. (P.) glentoni* (Sole *et al.*, 2005; Chapter 2). The neighbor-joining tree can be seen in

Figure 2 (drawn in MEGA using the TvM model), indicates four distinct groups into which each population designated from Table 1 could be grouped. Parsimony and Maximum Likelihood trees exhibited similar topologies to the neighbor-joining tree with and without LAPH13 (results not shown).

Table 2 shows molecular diversity statistics obtained within each population as well as an overall estimate for the species as a whole. Mean nucleotide diversity was high for the Leipoldtville population and an order of magnitude lower in the other two populations where estimates were obtained. Port Nolloth had a single haplotype therefore estimates for this population were 0. Thirty-one haplotypes were identified among the four populations with each population having its own unique set of haplotypes specific to each geographic region. Accordingly, haplotype diversity expressed over the complete sample was relatively high (H = 0.948 +/- 0.004).

Genetic differentiation among populations

An analysis of molecular variance (AMOVA) was performed to estimate the fixation index using the optimal model of sequence evolution identified above. Analyses were performed with the species as a single group with each population defined as a region. The results of AMOVA revealed that 84.88 % of the variance resulted from among population differences while 15.12 % of the variation could be attributed to within population variation. The fixation index was high and significant at 0.849 ($p < 0.001$) indicating strong genetic differentiation between the four populations (Table 3).

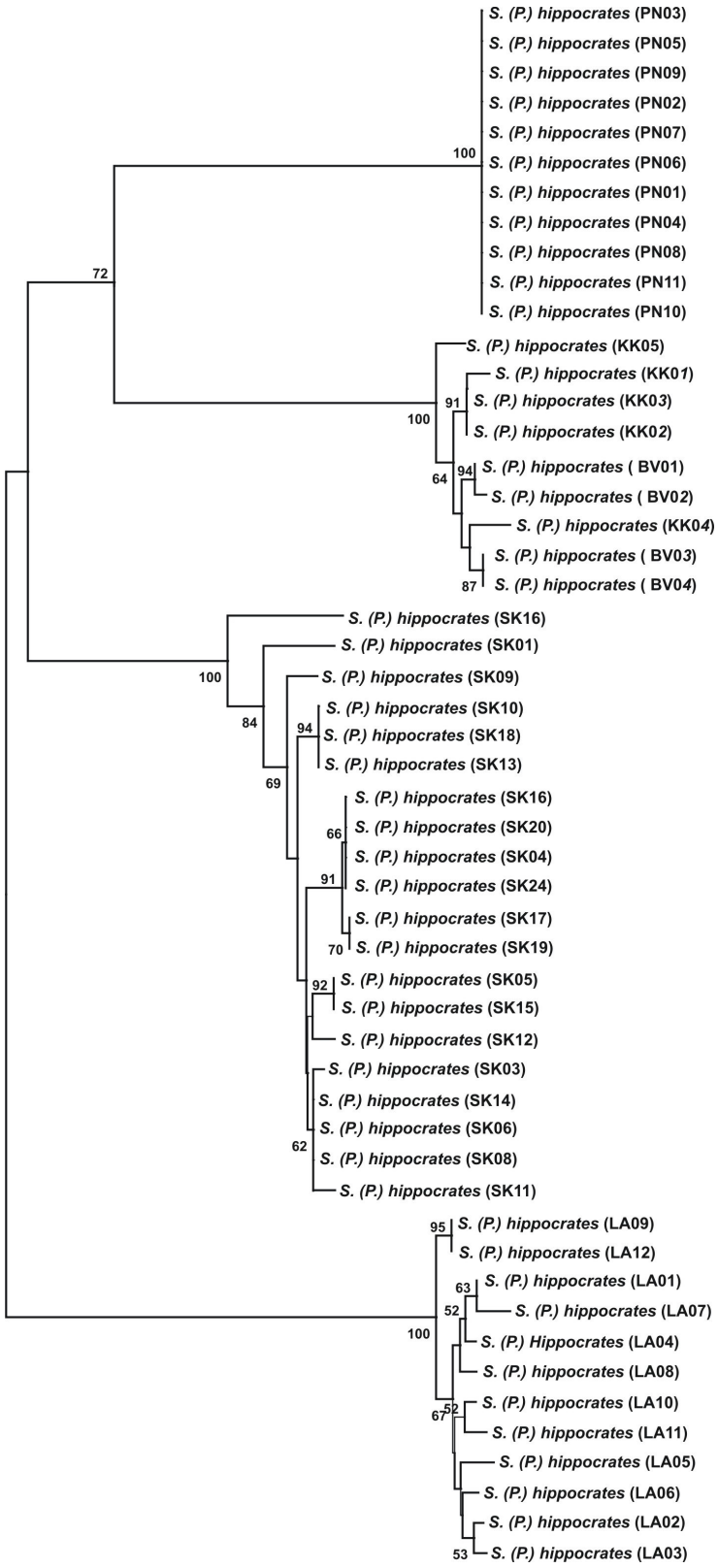


Figure 2. Mid-point rooted neighbor-joining tree for COI sequence data of *S. (P.) hippocrates*. Bootstrap values below 50 % were removed

Table 2. Summary of general nucleotide diversity statistics over the 960 bp region of *S. (P.) hippocrates*

Species	Assemblage	*N*	No. of haplotypes	Haplotype diversity	Nucleotide diversity	% Pairwise divergence	Variable Sites (V)	Parsimoniously Informative Sites (PI)	Singletons (S)
S. (P.) hippocrates	Leipoldtville	13	12	0.987 (0.035)	0.056 (0.029)	0.002 - 0.008			
	KommadoKraal/Koekenaap	9	7	0.944 (0.070)	0.007 (0.004)	0.001 - 0.008			
	Kleinsee - Sandkop	20	11	0.926 (0.034)	0.008 (0.004)	0.001 - 0.024			
	Port Nolloth	11	1	0	0	0			
Total		53	31	0.948 (0.021)	0.058 (0.003)	0.01 - 0.123	200 (20.83%)	133 (13.85%)	67 (6.98%)

[$] V, PI and S were only estimated for the overall dataset

Table 3. Summary of Fst statistics produced by AMOVA (Excoffier *et al*., 1992) for *S. (P.) hippocrates.*

Species		Φst	%	P
S. (P.) hippocrates	Among populations		84.88	<0.001
	Within populations		15.12	<0.001
	Fixation index	0.849		

[b] P values were determined from 10000 random permutations.

Demographic patterns based on the Stepwise and Exponential Expansion Models

Stepwise expansion model

Both tree topology (Fig. 2) and the mismatch distribution (Fig. 3) indicate recent sudden demographic expansion for each of the three populations. The branches of the tree within each population are small and similar in length suggesting a recent expansion in population size and geographic range. The haplotypic data (mismatch distributions) showed similar uni-model curves as expected in accordance with historically expanding populations. Both the variance (sum of the squared deviation (SSD)) and Harpendings Raggedness Index (HRI) suggested that the curves did not differ significantly under a model of population expansion.

Time of divergence

Using the 960 bp of the COI sequence we calculated the average number of nucleotide substitutions per site (*d*) and obtained a value of $d = 0.073$. The divergence time between *S. (P.) hippocrates* and *S. (P.) endroedyi,* which were shown to be sister taxa (see Sole *et al*., 2005; Chapter 2), was estimated to have occurred 2.3 million years ago. This gives the estimate of nucleotide substitutions per site per lineage per year (λ) to be $0.073/(2 \times 2{,}300{,}000) = 1.5 \times 10^{-8}$. The mutation rate per nucleotide site per generation (μ) would be 1.5×10^{-8}. The coalescence time was calculated from the (τ) values in Table 4a, in generations for each population (see below), using a mutation rate per haplotype (v) of 1.4×10^{-5}.

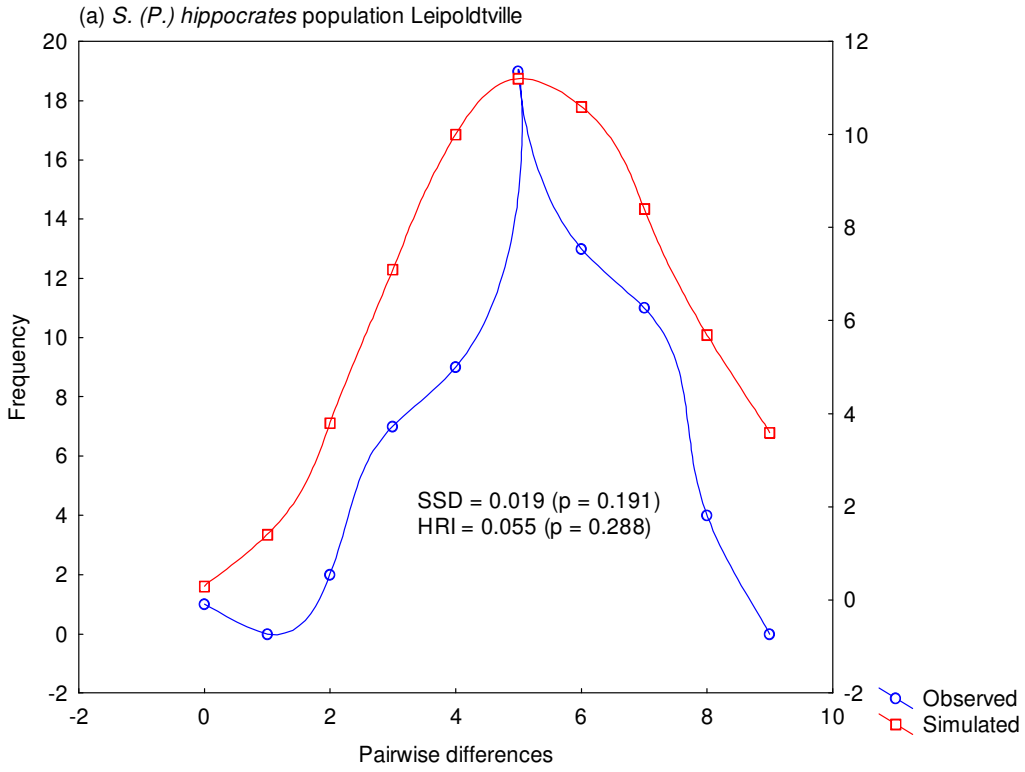
Based on the above calculated mutation rate and τ values of 5.655, 5.653 and 6.188 (Table 4a) the expansion of the Kommandokraal/Koekenaap and the Leipoldtville populations appeared to have been around 202,000 generations/years ago, while the Kleinsee - Sandkop population appeared to have undergone expansion at around 221,000 years/generations ago. Estimated effective female population size after expansion (N_1) was an order of magnitude higher than before expansion (N_0) for all three populations with the Kommandokraal/Koekenaap population having the lowest N_0 of approximately 100,000 individuals.

Exponential expansion model

The exponential expansion model indicates rapid growth for two of the populations namely, Kommandokraal/Koekenaap and Kleinsee - Sandkop having positive 'g' values (Table 4b). The Leipoldtville population, however, shows a strong negative 'g' value indicating overall population decline. Effective female population size estimated from theta (θ) did not show

large differences between populations but showed an overall tendency towards increasing population numbers.

The UPBLUE estimates for all the populations are an order of magnitude smaller than the Tajima's estimates (UPBLUE/Tajima; Table 5), except in the KommandoKraal/Koekenaap population, indicating that the Kleinsee - Sandkop and Leipoldtville populations are declining, whilst KommandoKraal/Koekenaap has a stable population. This is supported by Fu's *F*s statistics (Table 5) which are negative, but not significantly so for all the populations, indicating that the populations are experiencing minimal to no recent expansion.



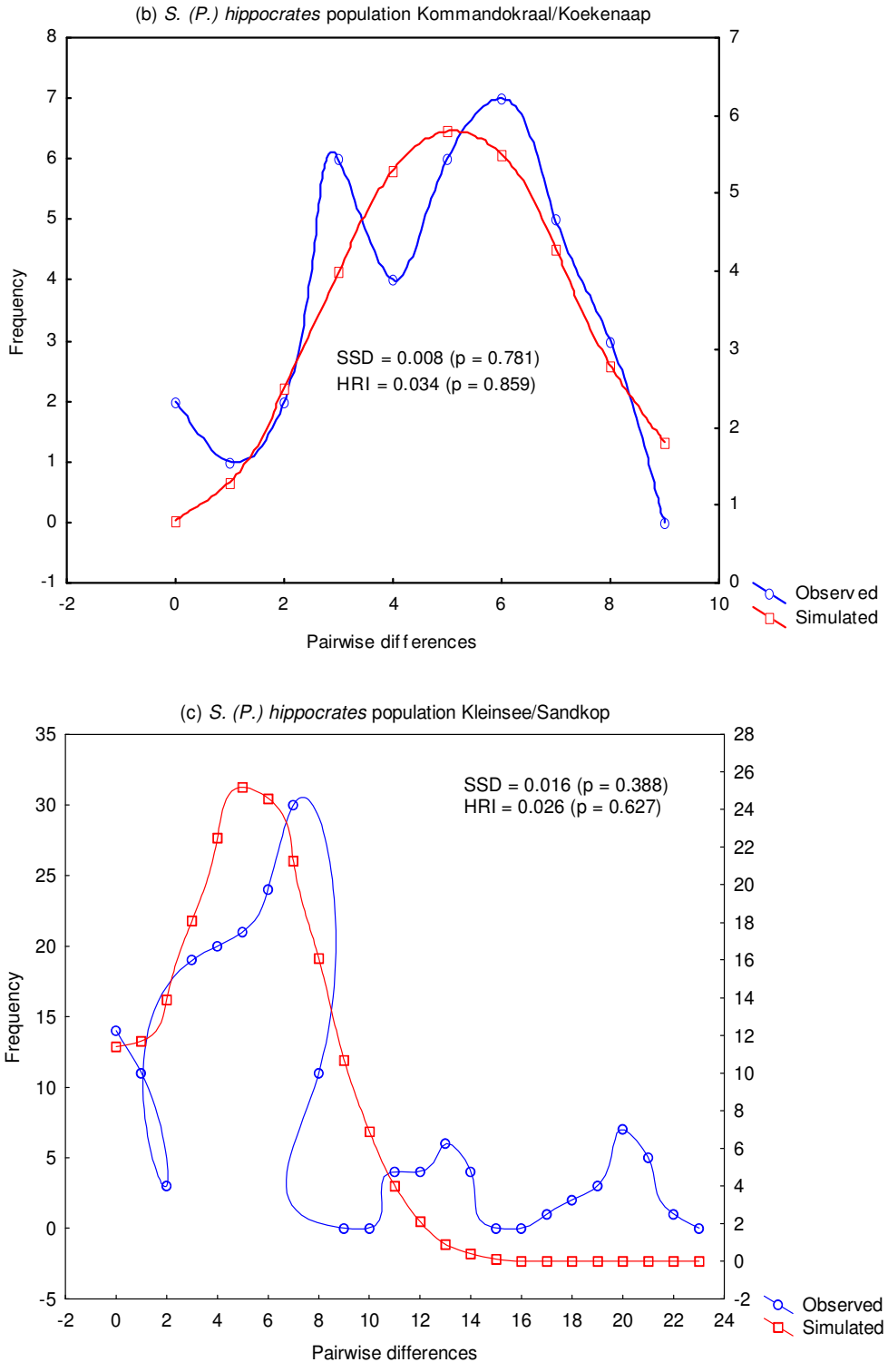


Figure 3(a–c). Mismatch frequency distributions of pairwise nucleotide differences for three of the four populations of *S. (P.) hippocrates*, with sum of the squared deviation (SSD) and Harpendings Raggedness Index (HRI) represented on the graphs.

Table 4. Estimated parameters for (a) Stepwise and (b) Exponential Expansion models of *S. (P.) hippocrates*

a) Stepwise Expansion Model

		Stepwise Expansion Model			
Species	**Populaion**	**τ**	**$\theta_0 = 2\mu N_0$**	**$\theta_1 = 2\mu N_1$**	**$t = \tau/2v$**
S. (P.) hippocrates	Leipoldtville	5.655	0	6656.250	202,000
	KommadoKraal/Koekenaap	5.653	0.003	52.598	201,900
	Kleinsee - Sandkop	6.188	0.084	16.449	221,000
	Port Nolloth	0	0	0	0

b) Exponential Expansion Model

		Exponential Expansion Model		
Species	**Population**	**$\theta = 2\mu Nf$**	**g**	**N_f**
S. (P.) hippocrates	Leipoldtville	0.0252	-19.439	840,000
	KommadoKraal/Koekenaap	0.0293	765.972	980,000
	Kleinsee - Sandkop	0.0169	74.623	564,000
	Port Nolloth	0	0	0

Table 5. Summary of estimations of Tajima's estimate, Fu's UPBLUE and Fu's *F*s statistic of *S. (P.) hippocrates*

Species		**Leipoldtville**	**KommadoKraal/Koekenaap**	**Kleinsee - Sandkop**	**Port Nolloth**
S. (P.) hippocrates	Tajima's estimate	40.078	5.476	9.839	0
	Fu's UPBLUE	0.291	5.267	0.020	0
	UPBLUE/Tajima	0.007	0.962	0.002	0
	Fu's *F*s	-1.236 (ns)	-1.366 (ns)	-0.604 (ns)	0

[$] **ns = non-significant**

Migration

The Port Nolloth population was removed from the migrate analysis after a number of runs as there appeared to be confusion as to where movement of the individuals was occurring. This was thought to be due to the fact that the Port Nolloth population consisted of a single haplotype. Once removed, MIGRATE showed movement from the southern populations (Leipoldtville and Kommandokraal/Koekenaap) into the northern Kleinsee - Sandkop population (Fig. 4).

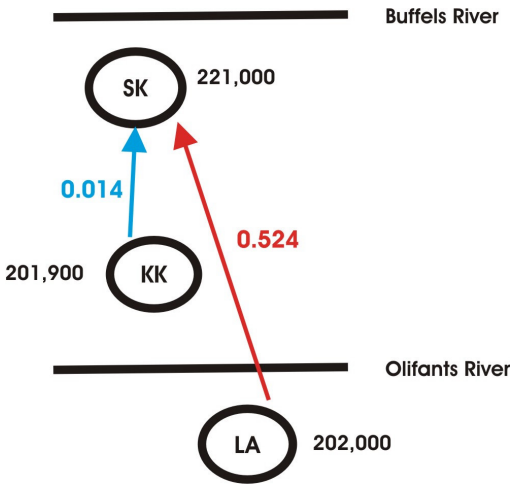


Figure 4. A schematic representation of the migration of individuals between populations of *S. (P.) hippocrates*. The coloured arrows indicate the direction of movement while the numbers in the same colour represent an approximation of the number of individuals moving/generation. The numbers in black represent coalescent times of the populations. (Abbreviations are as follows: LA = Leipoldtville, KK = KommandoKraal/Koekenaap, SK = Kleinsee - Sandkop)

Phylogenetic haplotype relationships

Using statistical parsimony the maximum number of mutational steps between two haplotypes, not including homoplasious changes and based on a probability of 95 %, was 13 mutational steps (as seen in Figure 4). The parsimony network was resolved except for the presence of two reticulations, which were broken. Haplotypes were collapsed for the drawing of the cladogram but not in the total nesting structure, which was used to calculate the contingency test values. It was not possible to determine a single root for the entire cladogram. There were three disjointed portions that could not be linked with 95 %

confidence, namely clades 6-1, 6-2 and 11-1. Furthermore, haplotypes LA13, PN01 and SK16 were separated from all other haplotypes by many mutational steps (14 - 109). Clades 6-1 and 11-1 were linked by 60 mutational steps while haplotype PN01 linked to these two clades by 64 mutational steps. Haplotype SK16 and clade 6-2 linked into the entire network with 65 mutational steps. Haplotype LA13 linked into the network with 109 steps. The network confirms the results seen in previous analyses in that there are relatively high divergence levels between the four populations, as the three disjointed portions represent the haplotypes from a single population (Table 1) with PN01 being representative of the single haplotype from 11 individuals from the Port Nolloth population.

Nested clade analysis did not reject the null hypothesis at the lower nesting levels, due to the fact that these nesting levels did not have haplotypes from different geographic regions in them. However, the contingency test showed significant geographical association of genotypes contained within haplotype groups 12-1, 13-1 and 14-1 (Table 6). All the inference events correspond to allopatric fragmentation of the populations, which would appear true as the populations are isolated by natural barriers (rivers), human habitation, farming areas and mining activities. There is no support for secondary contact between the populations as no shared haplotypes are contained between the populations.

The Mantel test revealed no significant association between genetic and geographic distances ($g = 1.282$, $r = 0.458$, $p > 0.05$). This indicated that an increase in geographic distance did not necessarily correlate with a greater degree of genetic distinction.

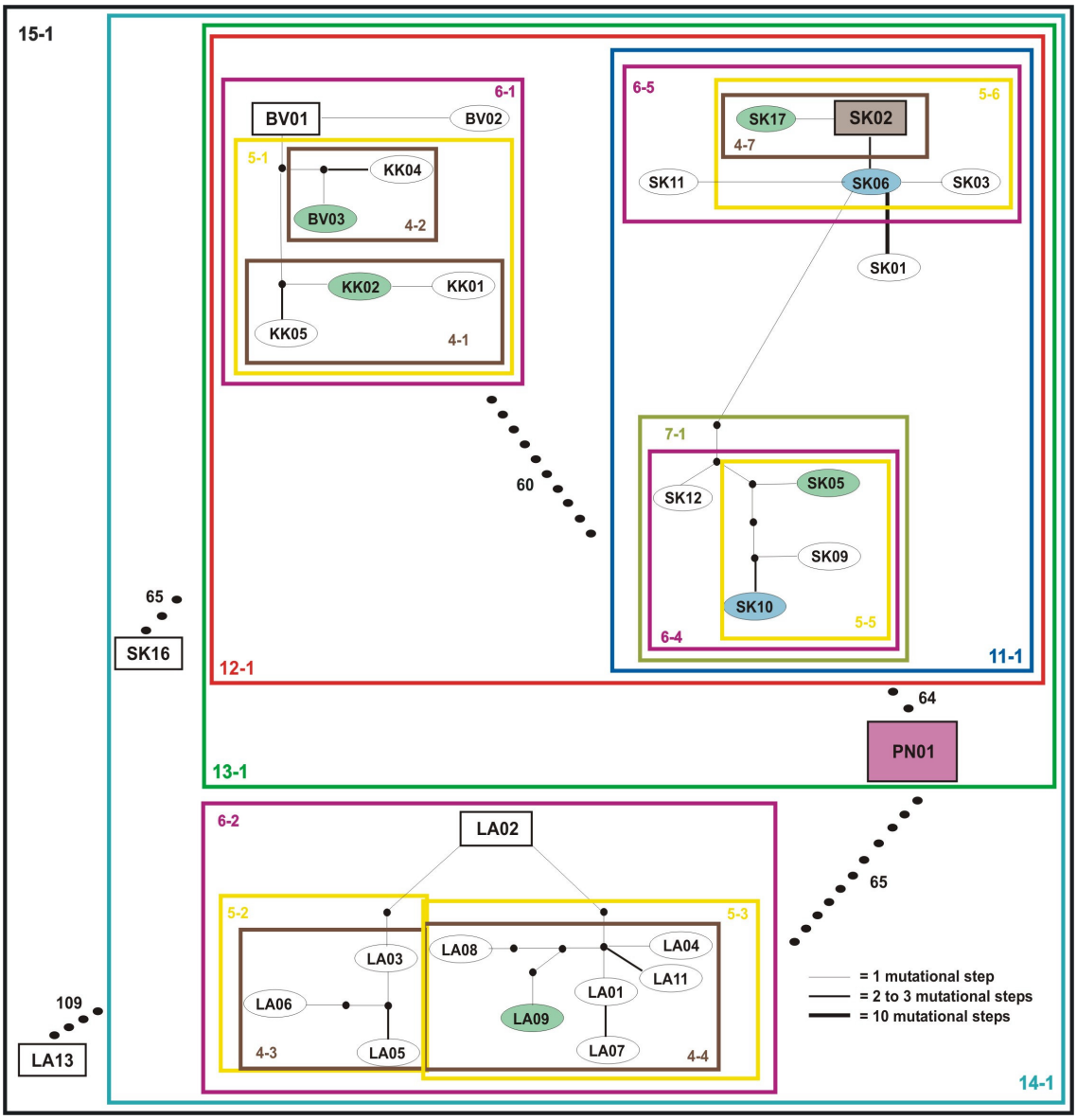


Figure 5. Statistical parsimony network and associated network design. Haplotypes are designated by letters, which represent the population from where the haplotype came (as seen in Table 1) and numbers representing the individual sequenced. The thickness of the connecting line corresponds to the number of mutational steps. Ancestral haplotypes are represented by squares. The relative frequency at which the different haplotypes occurred is indicated by different background colours, haplotypes with pink background occur 11 times, the grey background four times, a blue background occurred three times and a green background occur twice. The nested clades are represented from 4 - step clades upwards, as the mutations were only collapsed for drawing of the cladogram and not for calculation of the nested clade. Dotted lines represent alternative ambiguous connections.

Table 6. Geographical nested clade analysis for *S. (P.) hippocrates* as inferred from Templeton (2004).

Haplotype group	χ^2	P-value	Inference chain	
Clades within 12-1	28.000	<0.001	1-19-No	Allopatric fragmentation - also observed at high clade level
Clades within 13-1	39.000	<0.001	1-19-No	Allopatric fragmentation - also observed at high clade level
Clades within 14-1	53.333	<0.001	1-19-No	Allopatric fragmentation - also observed at high clade level

Discussion

Demographic patterns

S. (P.) hippocrates exhibits a high degree of genetic polymorphism as seen by the high overall haplotype diversity. Both the Stepwise and Exponential Expansion models show a strong inclination for population growth from an ancestral bottlenecked population. As expected, the four populations show appreciable mtDNA divergence in that strong support for all four of the designated populations was obtained. Strong genetic structure between the populations is clear in the highly significant fixation index value, as well as the high maximum pairwise divergence value of 12.3 % (Table 2), indicative of a relatively long historical separation.

The Mantel test shows no association between geographic and genetic distances hence distance could be ruled out as the defining factor for the distinct genetic isolation present. The most probable cause of genetic discontinuities displaying geographic disorientation could therefore be attributed to extrinsic barriers to gene flow. Extrinsic factors contributing to the genetic structure seen in *S. (P.) hippocrates* would be the Olifants and Holgat Rivers, Port Nolloth (the town), large tracts of areas on which farming occurs, as seen in the Leipoldtville and Kommandokraal areas, and mining at Kleinsee - Sandkop. This is clearly illustrated by the fact that each of the factors mentioned above relates to a specifically identified population. NCPA supports this by inferring allopatric fragmentation at all significant levels, indicating that this species may be on its way to speciation.

Previous studies dealing with other insect taxa have shown that speciation events have occurred in the sandy accumulations of river mouths in Namaqualand e.g. Thysanura (Irish, 1990). Harrison (1999) showed quite clearly that *S. (P.) endroedyi* and *S. (P.) glentoni* speciated around the Olifants River mouth with *S. (P.) endroedyi* occurring north of the Olifants River and *S. (P.) glentoni* south of the Olifants River. Both these species occur sympatrically with, and are sister to, *S. (P.) hippocrates* (Harrison & Philips, 2003; Sole *et al*., 2005). The Holgat River has also been shown to be the boundary of the northern distribution of *S. (P.) hippocrates* (Harrison *et al*., 2003). Anthropogenic influences can clearly be seen in the Port Nolloth population where the town is encroaching into the coastal dunes and destroying much of the available habitat. It would appear that anthropogenic factors, occurring over the last 100 years, affect Leipoldtville and Kommandokraal/Koekenaap populations in that individuals occur on disturbed farmland while the Kleinsee - Sandkop population is affected by mining in the area.

Rates of migration were estimated in an attempt to infer historical movements of species. Movement appeared to be in a south-north direction consistent with the unidirectional wind regime along the west coast. The Kleinsee - Sandkop population appears to have undergone expansion earlier than the other two populations, which is in contrast to the migration estimates as these show the Kleinsee - Sandkop population to be receiving individuals from the other two populations as opposed to movement from the Kleinsee - Sandkop population. However, as the expansion times between the populations appear small with the difference between the earliest and the latest coalescent events being as little as 18,000 years ago it would appear that fragmentation of these populations occurred over a similar time period after movement in a northerly direction. The coalescent events appear, therefore to, have occurred at approximately 200,000 years ago thus dating back to the late Pleistocene.

The modern semi-arid environment and winter rainfall within the south-western Cape seen today dates back to the Pliocene. Fossil pollen studies indicate that two invasions of temperate rain forest, and two wet intervals occurred between 33,000 and 45,000 years ago (van Zinderen Bakker, 1975). These climatic oscillations could have caused the historical population expansions seen in the genetic signal. The wet intervals were considered colder periods and the transitional Namib, the area between the Olifants and Orange Rivers, and the southern Namib, presently covered by gigantic dunes, would therefore have received more rain during this period. Increased rainfall would have resulted in stream rejuvenation causing an increase in the sediment source and giving rise to dune plumes occurring at the mouths of many ephemeral rivers along the west coast (van Zinderen Bakker, 1975). This highlights the importance of river mouths as barriers to gene flow and areas that could be designated as refugia for population expansion events of flightless species. These Namaqualand dunes, previously stabilised by vegetation, are extremely long and narrow and presently show signs of being overridden by shifting un-vegetated barchanoid dunes, fed by the erosion of the older coastal dunes (Tankard & Rogers, 1978).

Actual areas of refugia are difficult to identify, as there are no shared haplotypes between populations indicative of a possible ancestral population. Ancestral haplotypes are thought to be shared between, and widespread among populations (Avise *et al.*, 1987).

Current population trends

Scarabaeus (Pachysoma) hippocrates occupy the southernmost area of the total *Scarabaeus (Pachysoma)* distribution. Namaqualand is a winter-rainfall desert covering some 50,000km^2

- characterised by predictably low rainfall, and mild seasonal temperature changes (Cowling *et al*., 1999; Colville *et al*., 2002) - and exhibits a high degree of endemism in both fauna and flora. Namaqualand has not only recently been subject to extensive commercial and subsistence grazing, resulting in significant vegetation change, soil erosion and the loss of primary productivity (Colville *et al*., 2002), but also over past geological time major climatic oscillations have occurred. A combination of these factors could, therefore, have structured this species. Population fragmentation over geological time, leads to genetic divergence of populations, while human induced fragmentation acts over a short period of time eroding genetic diversity. The entire population has been fragmented by environmental factors over time which have not decreased the genetic divergence. However, within the same species, the Port Nolloth population shows a distinct loss of genetic diversity through human induced fragmentation. This indicates that both population- and human- induced fragmentation have affected this species as a whole.

Although both the stepwise and exponential expansion models indicate strong population growth these increases in population sizes may be misleading. There is no available census data for this species but considering current trends of species records and habitat destruction it is far easier to infer that population numbers are declining (Moya *et al*., 2004) and that *S. (P.) hippcrates* may be extinct in areas where it once occurred. This is supported to some extend by the UPBLUE/Tajima estimates that show that two out of four populations are in fact declining. However, it should be borne in mind that genetic signatures of population growth can be misleading (Lavery *et al*., 1996). Fu's UPBLUE estimator of θ and *F*s statistics may be an indicator that significant population growth is not occurring but recent demographic events may be masked by earlier events. The genetic signal of growth could, therefore, be an artefact of past demographic population increase as would have occurred during the Pleistocene (Lavery *et al*., 1996). It has been shown that after rapid population growth, subsequent periods of decline would have no great effect on the initial pattern of growth unless there is a major prolonged bottleneck or until equilibrium is approached (Lavery *et al*., 1996). As many species are not in equilibrium due to past demographic events and the more recent events are undetectable, the results we obtain may be misleading (Lavery *et al*., 1996).

Summary statistics show that *S. (P.) hippocrates* is a genetically and geographically well structured species. Migration estimates show gene flow to be unidirectional from the south to the north. Genetic diversity indicates that distinct genetic variability exists within

and between the populations. Genetic patterns can be related to both past geological events as well as recent fragmentation events.

In this case the anthropogenic and environmental forces discussed above are not mutually exclusive and the combination thereof provides a plausible explanation for the complex population demographic structure seen today.

Chapter IV (b)

Genetic structure, phylogeography and demography of *S. (P.) gariepinus* based on inferences from Cytochrome Oxidase I

Introduction

Scarabaeus (Pachysoma) gariepinus are distributed from the Buffels River (S29°33' – E17°24') in South Africa to the Agub Mountain (S26°59' – E15°58') in Namibia. Interestingly, they occur both south and north of the Orange River with their distribution covering two distinct biomes, Namaqualand, south of the Orange River, and the Namib Desert, north of the Orange River. Many ecological factors are found to influence distributional patterns of arthropods. These include temperature, rainfall, sand characteristics and availability of food among others. Limited vagility and narrow ecological and physiological tolerances may have promoted the present day distribution of *S. (P.) gariepinus*. The presence of this endemic species in the southwest arid regions, the fact that they exhibit south to north morphological clinal variation, are flightless with unique biology, and occur on either side of the Orange River warrants investigation into their biogeography.

Size, elytral sculpture, indument and size of the mesepisternal protuberance were found to vary within and between localities (Harrison, 1999). The populations south of the Orange River are characterized by smaller body size and red indument, while the Namibian populations are generally larger with their indument stained white to grey.

Materials and Methods

See general introduction to chapter. Details of specimen collection sites can be seen in Figure 6.

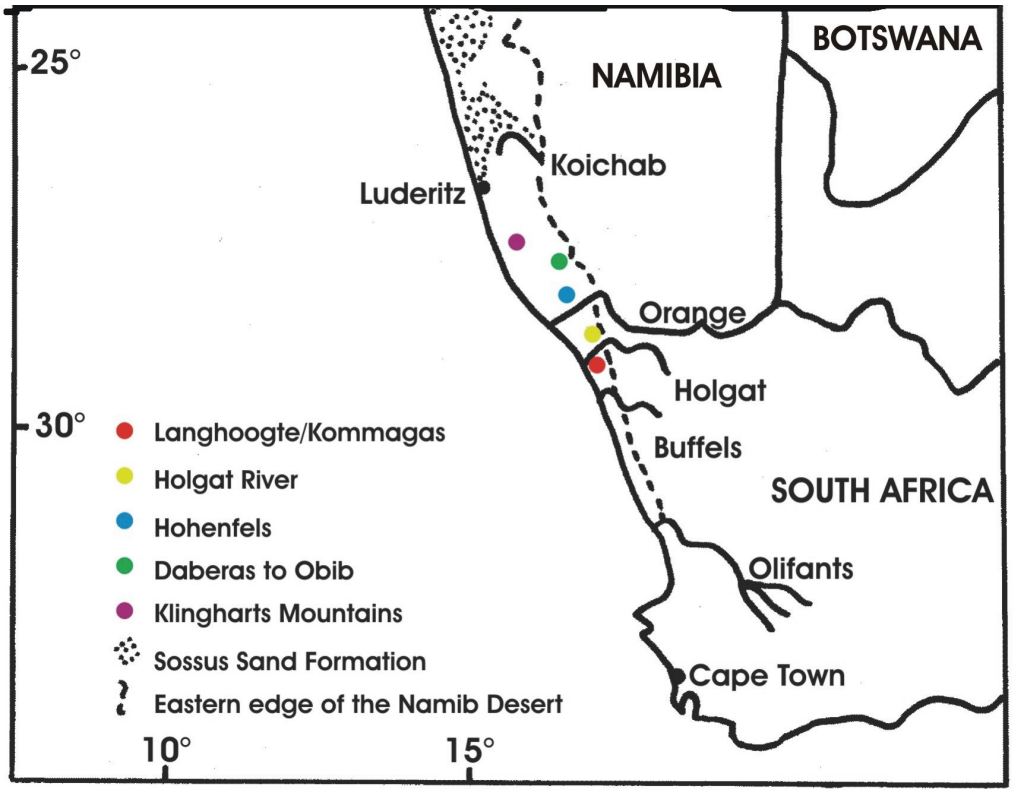


Figure 6. Localities in both Namibia and South Africa where *S. (P.) gariepinus* was collected for this study

Results

Phylogenetic and Molecular diversity

Population statistics

A total of 67 individuals were used for molecular characterisation (GenBank Accession numbers AY965087 – AY965153). The sequences exhibited an overall A/T bias of 69.10 %. Gamma distribution for the data was estimated at 1.0041 with the proportion of invariable sites being 0.7627, the transition/transversion ratio was 6.6 and the model best fitting the data selected by Modeltest was Tamura-Nei. The neighbor-joining tree of 67 individuals (Fig. 7) revealed three distinct assemblages. The first assemblage (labelled N; Fig. 7) had 100 % bootstrap support and consisted exclusively of individuals from the three populations in Namibia (Table 1). The second and third assemblages (labelled LK and HR; Fig. 7) had 100 % and 99 % bootstrap support, respectively and consisted of individuals from each of their respective populations, Langhoogte/Kommagas and Holgat River (Table 1). These three assemblages where each treated as distinct populations in further analyses. (Both the

Parsimony and Maximum Likelihood trees exhibited similar topologies to the neighbor-joining tree; data not presented).

Table 7 shows the molecular diversity statistics for each designated population from Table 1, the population designations from the neighbor-joining tree and the species as a whole. The sequenced COI fragment defined 62 unique haplotypes among the 67 individuals investigated. Accordingly, haplotype diversity expressed over the complete sample was very high (H = 0.997 +/- 0.004) (Table 7). All three assemblages contained numerous different haplotypes and no evidence was found for certain haplotypes being specific to a geographic region.

Genetic variation among populations

Mean nucleotide diversity was calculated across the three populations of the neighbor-joining tree (Table 8). The results of AMOVA revealed that differences among the three defined groups accounted for 49.6 % of the variance ($\Phi ct = 0.496$; $p = 0.001$). A high and significant Φst value of 0.704 ($p = 0{,}001$) indicated strong genetic structure between the three designated populations. Pairwise comparisons between the Φst therefore clearly support the distinctiveness of three populations. The remaining variation could be attributed to $\Phi sc = 0.415$ (among group within population variation) which was significant ($p = 0.001$), accounting for 20.9 % of the overall variation.

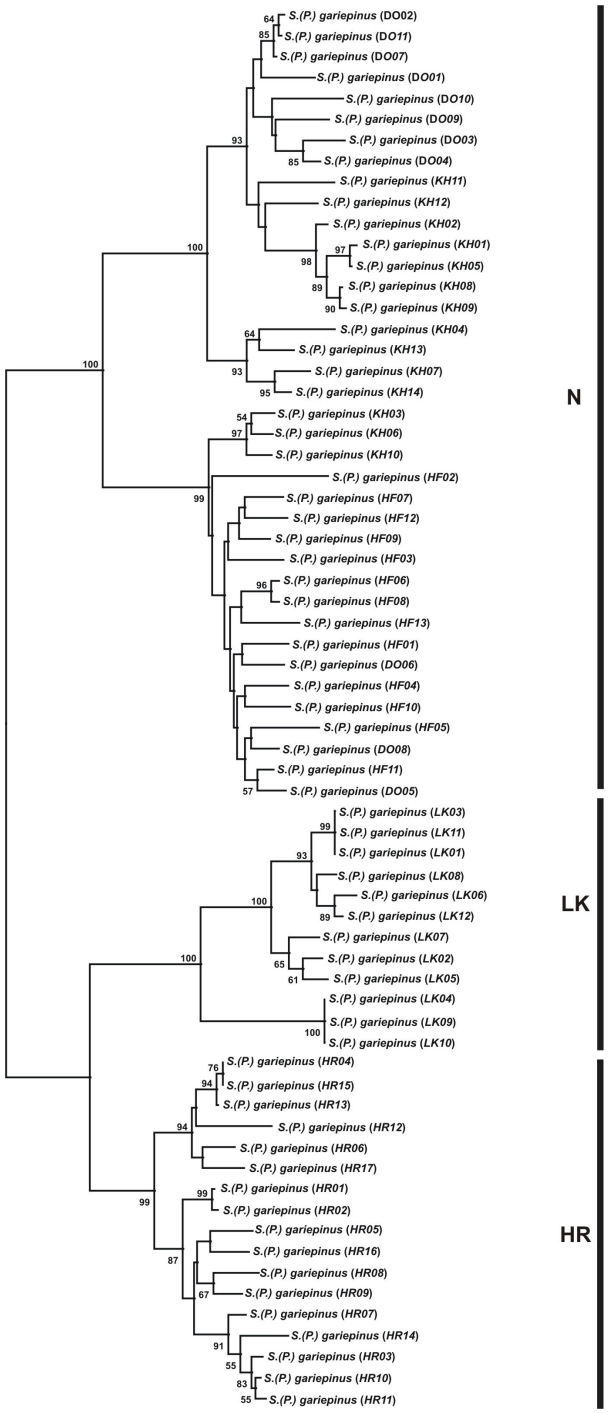


Figure 7. Mid-point rooted neighbor-joining tree for the COI sequence data of *S. (P.) gariepinus*. Bootstrap values below 50 % were removed.

Table 7. Summary of general diversity statistics of *S. (P.) gariepinus*

Species	Assemblage	*N*	No. of haplotypes	Haplotype diversity	Nucleotide diversity	% Pairwise divergence	Variable sites (V)	Parsimoniously Informative Sites (PI)	Singletons (S)
S. (P.) gariepinus	Langhoogte/Kommagas	12	8	0.909 (0.065)	0.026 (0.014)	0.004 - 0.039			
	Holgat River	17	16	0.993 (0.023)	0.023 (0.012)	0.001 - 0.035			
	Hohenfels	13	13	1.000 (0.030)	0.022 (0.012)	0.002 - 0.031			
	Daberas to Obib	11	11	1.000 (0.039)	0.038 (0.020)	0.001 - 0.063			
	Klingharts Mountains	14	14	1.000 (0.027)	0.042 (0.022)	0.001 - 0.065			
	Namibia assemblage	38	38	1.000 (0.000)	0.043 (0.021)	0.001 - 0.069			
Total		67	62	0.997 (0.004)	0.057 (0.001)	0.001 - 0.103	64 (6.67%)	30 (3.13%)	34 (3.54%)

[$] V, PI and S were only estimated for the overall dataset

Table 8. Summary of Fst statistics calculated by AMOVA (Excoffier *et al*., 1992) for *S. (P.) gariepinus*

Species		Φst	%	P
S. (P.) gariepinus	Among groups	Φct 0.496	49.6	<0.001
	Among groups within populations	Φsc 0.415	20.9	<0.001
	Within populations	Φst 0.705	29.5	<0.001

[b] P values were determined from 10000 random permutations.

Historical population dynamics based on the Stepwise and Exponential Expansion Models

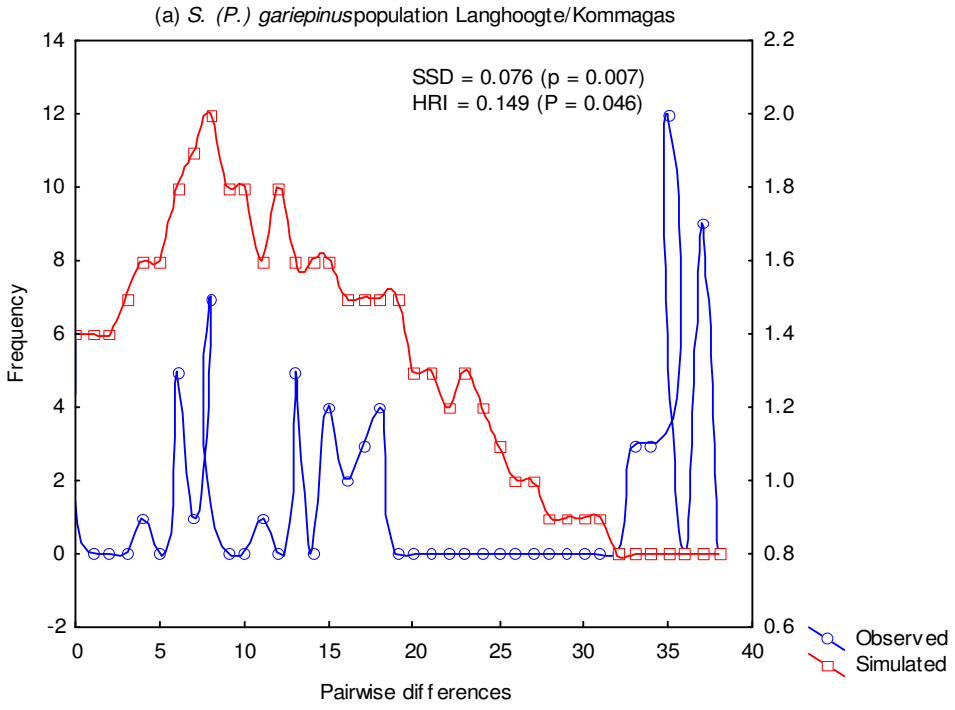
Stepwise Expansion Model

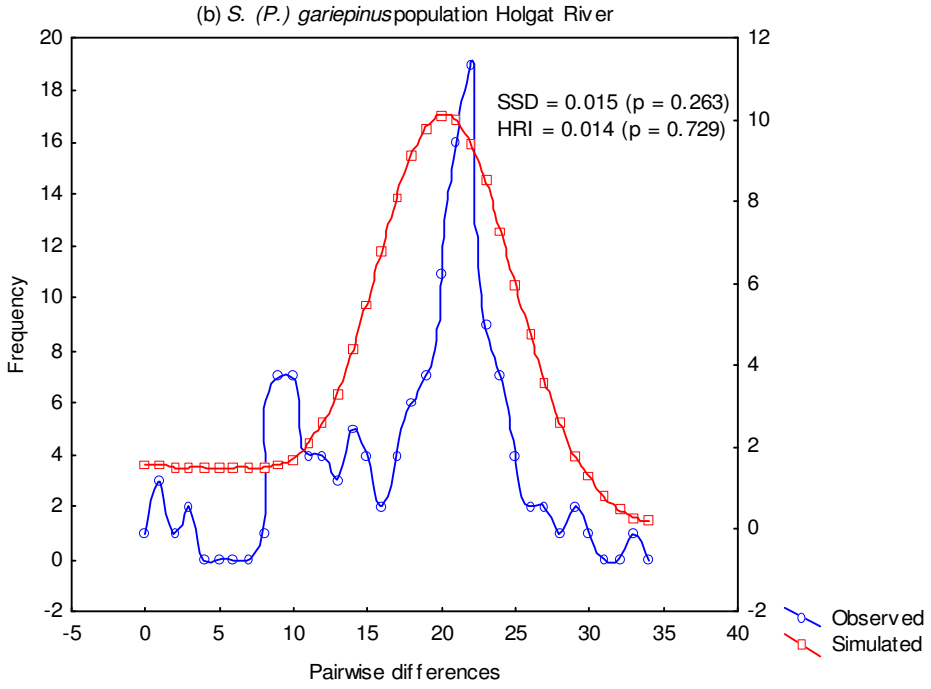
The frequency distribution for the pairwise nucleotide differences was investigated for the five populations separately (Table 1) as well as for the three populations indicated by the neighbor-joining tree. As the three populations within Namibia showed population expansion only the mismatch distribution for the Namibia population is presented. The tree topology (Fig. 7) with branches that are small and of similar length indicates recent sudden expansion. The mismatch distributions (Fig. 8) show similar uni-model curves as expected with a historically expanding population. Both the variance (Sum of the Squared Deviation - SSD) and Harpendings Raggedness Index (HRI) suggest that the simulated and expected curves do not differ significantly under a model of expansion.

Time of divergence

Using the 960 bp of the COI sequence we calculated the average number of nucleotide substitutions per site (d) and obtained a value of 0.08. The divergence time between *S. (P.) gariepinus* and *S. (P.) bennigseni* (Sole *et al.*, 2005; Chapter 2) was estimated to have occurred 2.8 million years ago. This gives the estimate of nucleotide substitutions per site, per lineage, per year (γ) to be $0.08/(2 \times 2{,}800{,}000) = 1.4 \times 10^{-8}$. The mutation rate per nucleotide site, per generation (μ) was therefore 1.4×10^{-8}. The coalescence time in generations for each population was calculated based on the τ values in Table 9a and a haplotype mutation rate (v) of 1.34×10^{-5}

The expansion of the Langhoogte/Kommagas population was estimated at around 231,000 generations/years ago. This appears to be the most recent expansion event while the Holgat River and Klingharts populations appeared to have undergone expansion much earlier at around 800,000 and 961,000 years ago, respectively. Estimated effective population size after expansion (N_1) was an order of magnitude higher than before expansion (N_0) in all populations.


(a) S. (P.) gariepinus population Langhoogte/Kommagas
SSD = 0.076 (p = 0.007)
HRI = 0.149 (P = 0.046)
Frequency
Pairwise differences
Observed
Simulated


(b) S. (P.) gariepinus population Holgat River
SSD = 0.015 (p = 0.263)
HRI = 0.014 (p = 0.729)
Frequency
Pairwise differences
Observed
Simulated

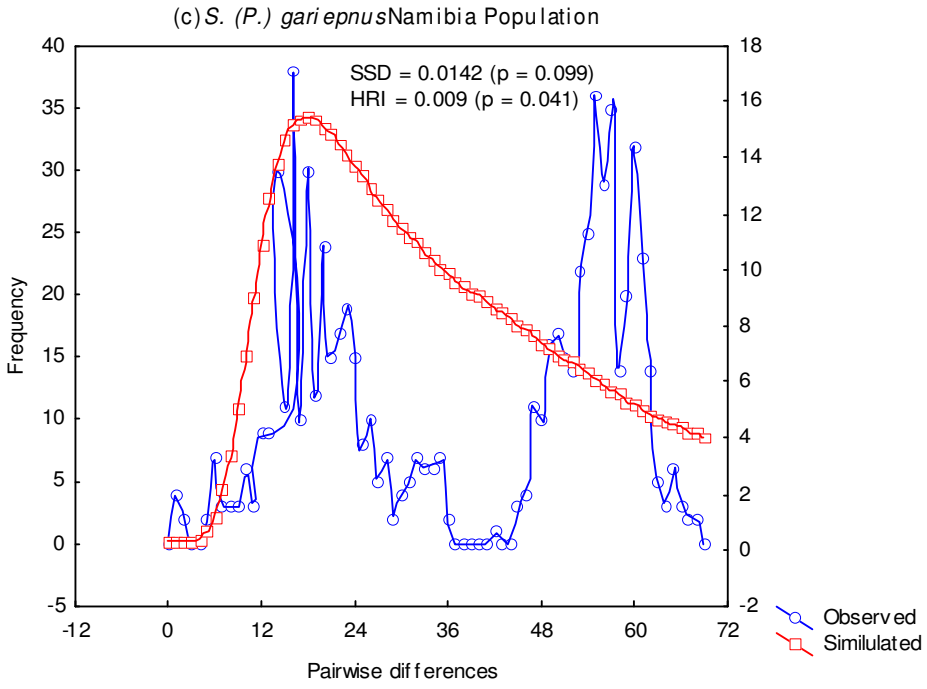


Figure 8 (a – c). Mismatch frequency distributions of pairwise nucleotide differences for the three population assemblages of *S. (P.) gariepinus*, with sum of the squared deviation (SSD) and Harpendings Raggedness Index (HRI) represented on the graphs.

Exponential Population Expansion

The exponential expansion model also indicated a rapid increase in effective population size (positive 'g' value; Table 9b) for all populations. Effective female population size estimated from θ differed markedly between populations with the Langhoogte/Kommagas population having the smallest effective female population size. These values, however, showed a consistent increase in population sizes.

The UPBLUE/Tajima estimate does not show a major recent increase in population size for any of the populations (Table 10). The Holgat River has a slightly significant negative *F*s estimate, indicating a small possible increase in population size while the Langhoogte/Kommagas population has a positive value for the *F*s estimate, indicating no recent increase in population size (Table 10). The Namibian population has a highly significant negative value indicating recent mutations leading to population growth, which is in contrast to the UPBLUE estimate.

Table 9. Estimated parameters for (a) Stepwise and (b) Exponential Expansion Models for *S. (P.) gariepinus*.

(a) Stepwise Expansion Model

		Stepwise Expansion Model			
Species	**Population**	**τ**	**$\theta_0 = 2\mu N_0$**	**$\theta_1 = 2\mu N_1$**	**$t = \tau/2\nu$**
S. (P.) gariepinus	Langhoogte/Kommagas	6.202	25.039	45.274	231,000
	Holgat River	21.371	0	85.156	800,000
	Hohenfels	16.294	0	5156.25	608,000
	Daberas to Obib	8.438	23.414	157.812	315,000
	Klingharts Mountains	26.75	28.812	79.023	961,000
	Namibia Assemblage	11.789	37.853	2710	440,000

(b) Exponential Expansion Model

		Exponential Expansion Model		
Species	**Population**	**$\theta = 2\mu Nf$**	**g**	**N_f**
S. (P.) gariepinus	Langhoogte/Kommagas	0.0279	51.045	996,000
	Holgat River	0.1371	239.111	4,900,000
	Hohenfels	1.7982	504.436	6,400,000
	Daberas to Obib	0.0722	60.816	2,600,000
	Klingharts Mountains	0.0956	76.029	3,400,000
	Namibia assemblage	0.4300	158.565	15,000,000

Table 10. Summary of estimations of Tajima's estimate, Fu's UPBLUE and Fu's *F*s statistic of *S. (P.) gariepinus*

Species		**Langhoogte/Kommagas**	**Holgat River**	**Namibia assemblage**
S. (P.) gariepinus	Tajima's estimate	17.553	18.160	31.179
	Fu's UPBLUE	20.895	1.248	1.172
	UPBLUE/Tajima	1.17	0.038	0.069
	Fu's *F*s	2.654 (ns)	-3.765 (*)	-14.857 (***)

$ ns = non-significant, * = $p < 0.05$, *** = $p < 0.001$

Migration

Migrate showed overall population movement in both a northerly as well as in a southerly direction. Within the Namibian population north and south migration between all the populations appeared to be occurring consistently (Fig. 9).

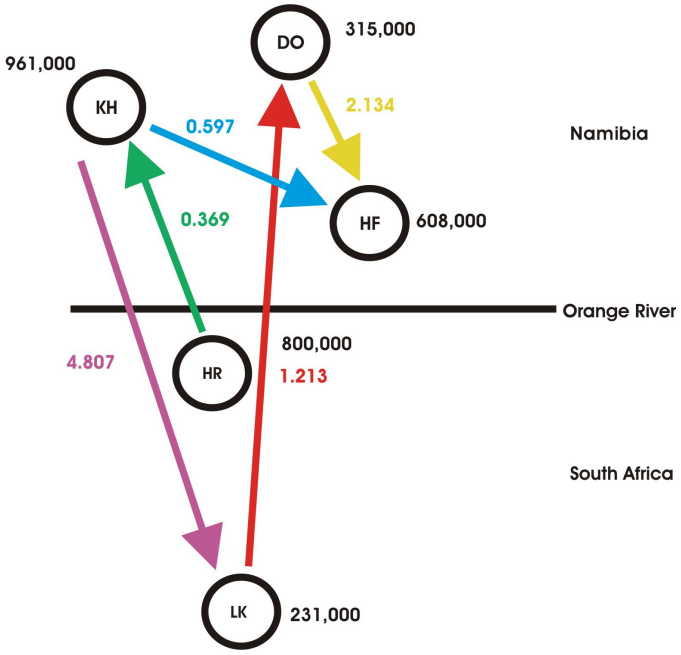


Figure 9. Schematic representation of the migration of individuals between populations of *S. (P.) gariepinus*. The coloured arrows indicate the direction of movement while the numbers in the same colour represent an approximation of the number of individuals moving/generation. The numbers in black represent coalescent times of the populations. (Abbreviations are as follows: LK = Langhoogte/Kommagas, HR = Holgat River, HF = Hohenfels, DO = Daberas to Obib, KH = Klingharts).

Phylogenetic Haplotype Relationships

Using statistical parsimony the maximum number of mutational steps between two haplotypes, excluding homoplasious changes (with a 95 % confidence), was 13 mutational steps. Given these constraints, a minimum spanning tree was constructed (Fig.10). The parsimony network was resolved except for the presence of three reticulations, which were broken. It was not possible to determine a single root for the entire cladogram. There were four major disjointed portions that could not be linked with 95 % confidence, clades 7-1, 9-2, 11-1 and 13-1. Furthermore, haplotypes DO09, DO10, LK04, KH04, KH11, KH12 and HF02

were separated from all other haplotypes by many mutational steps (from 14 to 32). Haplotypes DO09 and DO10 linked to clade 7-1 in 14 mutational steps while KH04 and KH12 linked to clades 2-9 and 8-1 with 15 and 16 mutational steps, respectively. Clade 9-2 and haplotype KH11 linked to clade 9-1 with 18 mutational steps. Haplotypes HF02 and LK04 link to clades 19-1 and 3-1 with 30 and 32 mutational steps, respectively. Clades 11-1 and 13-1 link with 45 mutational steps and the entire network links in 58 mutational steps.

The network shows two distinct clades, 14-1 and 12-1, indicating geographical distinction and supporting the neighbor-joining tree. Clade 14-1 represents the South African populations of *S. (P.) gariepinus*, with 13-1 representing the individuals from Langhoogte/Kommagas and 11-1 those individuals from Holgat River. Clade 12-1 groups all the individuals from the Namibia population together.

The contingency test showed strong geographical association of haplotypes between clades 5-1, 9-1, 10-1, 14-1 and 15-1 (Table 11). The inference chain indicated restricted gene flow with isolation by distance for clade 5-1. Both clades 9-1 and 10-1 indicate inadequate geographic sampling which made it difficult to distinguish between continuous range expansion, long distance colonisation and past fragmentation. Both clades 14-1 and 15-1 indicated allopatric fragmentation as the process responsible for the observed separation between the two South African populations and the populations in South Africa and Namibia. Secondary contact between the populations appears not to have occurred as no shared haplotypes occur between the populations.

The Mantel test revealed a significant association between geographical and genetic distances ($g = 2.055$, $r = 0.6206$, $p < 0.025$), indicating that an increase in distance was related to an increase in the genetic distinctness of the populations.

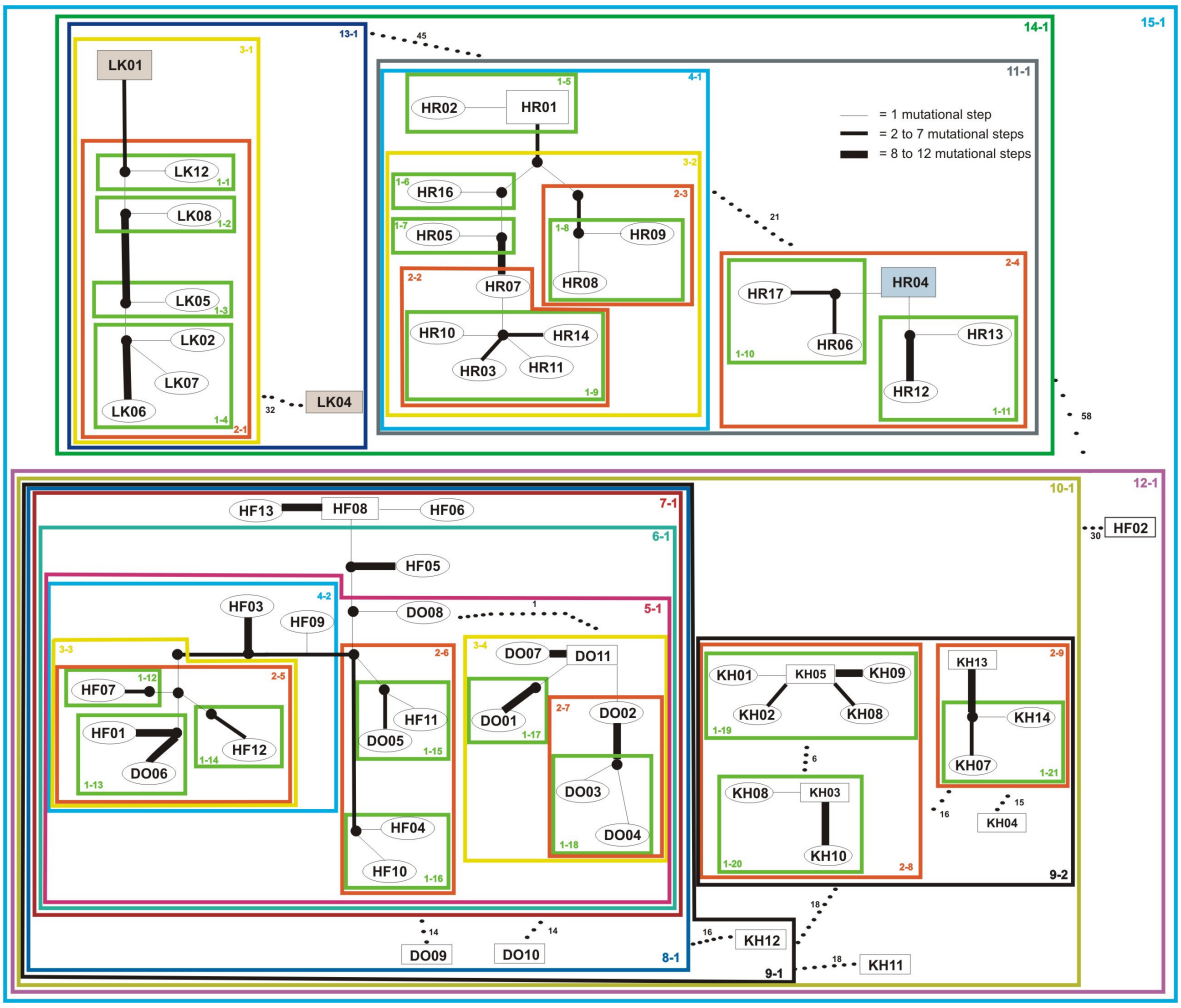


Figure 10. *S. (P.) gariepinus* statistical parsimony network and associated design. Haplotypes are designated by letters representing which population the haplotype came from (as seen in Table 1) and the numbers represent the individual sequenced. The thickness of the connection line indicates the number of mutational steps. Dotted lines represent alternative ambiguous connections. Ancestral haplotypes are represented by squares. Haplotypes with grey background are those that occurred three times and those with a blue background occurred twice.

Table 11. Results of the geographical nested clade analysis for *S. (P.) gariepinus*, inferences based on Templeton (2004)

Haplotype group	χ^2	P-value	Inference chain	
Clades within 5-1	10.644	<0.05	1-no-2-no-11-no-17-yes-4-no	Restricted gene flow with isolation by distance - applicable to lower clade levels only
Clades within 9-1	24.000	<0.05	1-yes-19-yes-20-no	Inadequate geographic sampling
Clades within 10-1	32.926	<0.000	1-no-2-no-11-yes-12-yes-13-no-14-yes	Sampling design inadequate to discriminate between contiguous range expansion, long distance colonisation and past fragmentation
Clades within 14-1	29.000	<0.000	1-yes-19-no	Allopatric fragmentation
Clades within 15-1	67.000	<0.000	1-yes-19-no	Allopatric fragmentation

Discussion

Demographic patterns

Scarabaeus (Pachysoma) gariepinus exhibits a high degree of genetic polymorphism as can be seen by the overall high haplotype diversity. Mitochondrial DNA divergence of *S. (P.) gariepinus* shows strong support for three distinct populations correlating to distinct geographic areas, a Namibian population and two populations in South Africa. The three-population hypothesis shows strong genetic structure as seen by the high Φst value of AMOVA as well as by the overall high sequence divergence (maximum of 10.3 %; Table 7). This is indicative of a long historical separation and could be due to both extrinsic and intrinsic factors.

Extrinsic factors such as environmental barriers contributing to the population structure seen in the present study could be the Orange and Holgat Rivers, which may have separated the populations in South Africa (Holgat River) from each other as well as from the Namibian population (Orange River). Previous studies indicate that sandy pockets at river mouths in Namaqualand (Endrödy-Younga, 1982a), sand accumulations in the lower Orange River (Endrödy-Younga, 1982a; Penrith, 1984) and coastal/littoral dunes (Endrödy-Younga, 1978), specifically in the western Cape (Penrith, 1986) could act as possible areas of origin for various psammophilous taxa. Nested clade analysis distinctly indicates that allopatric fragmentation is the defining factor for the fragmentation between the Langhoogte/Kommagas and Holgat River populations (clade 14-1) as well as between the South African populations and the Namibian population (clade 15-1).

Alternatively, the population structure of *S. (P.) gariepinus* could be maintained by intrinsic factors such as flightlessness, which results in reduced vagility. The Mantel test shows a strong association between geographic and genetic distances, indicating distance as an important factor for influencing the population structure. This is clearly supported by the nested clade analysis, clade 5-1, which includes individuals from two Namibian populations, namely Daberas/Obib and Hohenfels Dunes, where there is restricted gene flow due to isolation by distance. Since the Namibian population occurs on a known dune field continuum, clades 9-1 and 10-1 indicate inadequate sampling hence the need to increase sampling along the complete dune system as opposed to discrete points as was done here. This is important for understanding the apparent lack of structure within the Namibian population.

Both the mismatch distributions and the exponential expansion model indicate a sudden historical increase in all population sizes. Effective female population size differed markedly between the Stepwise and Exponential Expansion Models indicating the importance of using these values as relative indicators and not precise estimates. However, the trends identified across the historical estimation procedures were the same. Fu's *F*s statistics show strong support within the Namibian population for overall recent population growth but the UPBLUE estimate contradicts this. The UPBLUE/Tajima estimate shows the Langhoogte/Kommagas and Holgat River populations to be stable with slight growth in the Langhoogte/Kommagas population. Fu's *F*s statistic indicates a similar trend in the Langhoogte/Kommagas population, in that there is no growth. In contrast to this the *F*s statistic for the Holgat River population is significantly negative indicating recent growth. However, the contrast between the Stepwise and Exponential Expansion Models and Fu's UPBLUE/Tajima estimate and the *F*s statistic is only apparent in that the two expansion models infer population growth from historical/ancient processes whereas Fu's methods infer population parameters based on more recent mutational events.

Migration rates between populations were estimated in an attempt to infer historical movements of the species. Overall it appeared there had been a large amount of movement between populations. Movement occurred in a south-north direction, which is consistent with previous hypotheses that indicate movement with the unidirectional wind regime (Endrödy-Younga, 1982a; Sole *et al*., 2005) as well as in a north-south direction. Two populations appear to have undergone expansion earlier than the others, the Klingharts Mountains and Holgat River populations, 800,000 and 961,000 years ago, respectively. It would, therefore, appear that the ancestor of what is seen today would have invaded the Namib by simply moving from east to west or by remaining at a locality and adapting to the changing climate, thereafter moving and radiating into other favourable habitats (Irish, 1990). The addition of an extra locus (microsatellites/nuclear gene) would possibly allow for a clearer picture. The amount of movement between populations appeared high for a group of flightless individuals. This may show that under conditions of extreme environmental pressure or very favourable periods the beetles will move over long distances. Contradictory to this is the fact that each population has its own set of unique haplotypes indicating that individuals appear to remain in a certain locality.

Species boundaries and conservation issues

One of the most fundamental urges of mankind is to identify and name things (Mayr & Ashlock, 1991). It has been suggested that the taxonomy of a group should be consistent with its evolutionary history (Wiley, 1981; Frost & Hillis, 1990). Every species taxon in nature consists of numerous local populations, which raises the problem of how to treat them taxonomically. Adding dimensions of geography and time poses numerous additional problems (Mayr & Ashlock, 1991). The mitochondrial DNA of *S. (P.) gariepinus* reveals three distinct genetically isolated assemblages, which reflect different demographic histories. All three assemblages are unique in the fact that they do not share haplotypes and have probably been isolated for more than 200,000 years and may therefore warrant distinct taxonomic status under various species concepts. In principle the three assemblages could be defined as separate species based on the Phylogenetic species concept (Nixon & Wheeler, 1990) and Templeton's cohesion species concept (Templeton, 2001), as it appears that there has been no recent gene flow.

Moritz (1994a; b) identified units or targets for conservation by applying the principle of conserving ecological and evolutionary processes in an attempt to conserve biogeography (Moritz, 1999). It may be optimistic to attempt to conserve all the populations of a species therefore one would ideally like to target the populations that will ensure a species remains viable and able to survive in the short-term and diversify in the long-term. Moritz (1994a; 1999) describes these units or targets as Evolutionary Significant Units (ESUs) and Management Units (MUs). ESUs he defines as having to be reciprocally monophyletic for mtDNA alleles and to have shown significant divergence of allele frequencies at nuclear loci. MUs are recognised as populations with significant divergence of allele frequencies at nuclear or mitochondrial loci, regardless of phylogenetic distinctiveness of the alleles and are the units used for population monitoring and demographic based studies (Moritz, 1994a). According to these definitions the populations of *S. (P.) gariepinus* could be described as MUs. These populations are connected by low levels of historical gene flow but are functionally independent and would therefore need to be managed as individual entities, forming part of an inclusive species.

Chapter IV (c)

Genetic structure, phylogeography and demography of *S. (P.) denticollis* based on inferences from Cytochrome Oxidase I

Introduction

Phylogeographic structure is important for organisms with extensive ranges and complex geographical patterns. When integrated with data on geographical distribution of morphology and ecological variation such inferences can be used to test hypotheses of speciation processes (Nice *et al*., 2005).

Many formerly continuous areas of natural habitats have been subdivided into smaller habitat islands surrounded by human-altered environments (van Dongen *et al*., 1998). Artificially divided populations often have a limited number of individuals interchanging between sub-populations. The fewer individuals moving between populations the greater the effect of genetic drift in that genetic diversity decreases within and increases between sub-populations (van Dongen et al., 1998; Driscoll & Hardy, 2005). One would intuitively assume that a species occurring within fragmented habitats would show decreased genetic variation as opposed to those occurring over a continuous habitat.

Scarabaeus (Pachysoma) denticollis are restricted to the coastal and inland dunes of the central Namib dune sea (see Figure 1), and are conserved within the Namib Naukluft Park. The species occurs from Luderitz (S26°41' - E15°15') to Walvis Bay (S22°55' – E14°28') and populations of this species exhibit individuals with elytral colours ranging from orange to black with some showing a mix of the two colours (Scholtz, pers. obs.). The individuals with the black elytra were previously described as a subspecies of *S. (P.) denticollis, P. denticollis penrithae* (Harrison *et al*., 2003), but were synonomised, based on morphology, with *S. (P.) denticollis sensu stricto* by Holm & Scholtz (1979).

Materials and Methods

See main body of chapter. Details of specimen collecting sites can be seen in Figure 11.

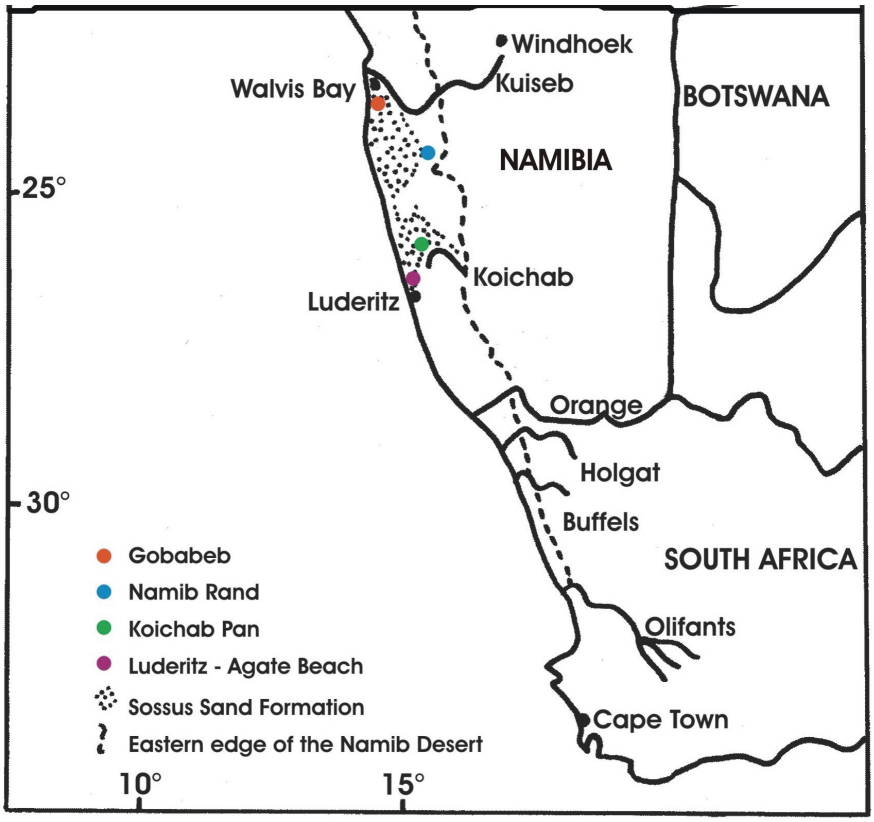


Figure 11. Localities in Namibia where *S. (P.) denticollis* were collected for this study.

Results

Phylogenetic and Molecular Diversity

Population Statistics

Thirty-two individuals were used for molecular characterisation (Genbank Accession numbers AY965207 – AY965238). The sequences exhibited an A/T bias of 69 %, which is the same as that observed for the other two species investigated in this chapter. Of the 32 individuals studied 29 represented unique haplotypes with no evidence found for a haplotype being specific to any geographic region (Table 12; which includes general molecular diversity statistics). Accordingly, overall haplotype diversity expressed was high (H = 0.992 +/- 0.011), and intermediate to that within *S. (P.) hippocrates* and *S. (P.) gariepinus*.

Gamma distribution for the data was estimated at 0.3413 (which is intermediate to that of the previous two species) with the proportion of invariable sites being 0.6791. The transition/transversion ratio was 1.9 (estimated in MEGA) and the model best fitting the data selected by Modeltest was Tamura-Nei. The neighbor-joining tree of 32 individuals can be seen in Figure 12 and indicates two assemblages, namely 1 and 2. Assemblage 1 consists of

individuals from all four localities and even though it appears that individuals from collecting localities group together, the support thereof is poor allowing for no distinct population designation within this assemblage. Assemblage 2 shows strong support for individuals from two localities (namely Koichab Pan and Agate Beach). However, individuals from these two localities also occur within assemblage 1. This as well as the fact that there are no shared haplotypes between the collecting sites of *S. (P.) denticollis* may indicate that incomplete lineage sorting has occurred i.e. speciation is in the process of occurring. *S. (P.) denticollis* is therefore treated as a single population throughout this sub-chapter (Parsimony and Maximum Likelihood trees exhibited similar topologies, data not shown).

Genetic differentiation among populations

Mean nucleotide diversity was calculated across the three collecting sites (Table 13). The results of AMOVA revealed that 22.16 % of the variance resulted from the differences among the three collecting sites, while 77.85 % of the variance resulted from differences within collecting sites. The fixation index value ($\Phi st = 0.222$) was low but significantly so ($p < 0.001$) indicating weak genetic structure between collecting sites.

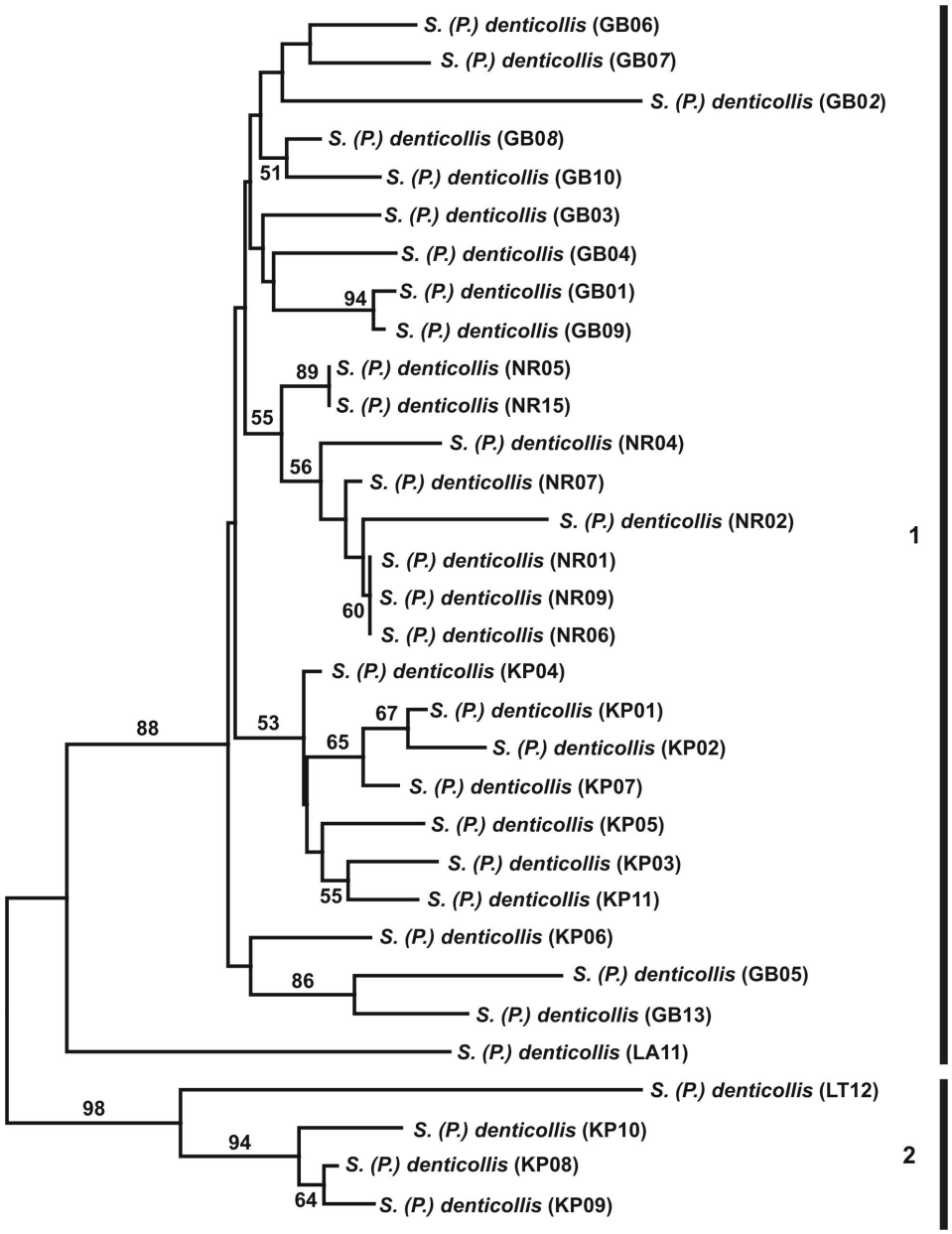


Figure 12. Mid-point rooted Neighbor-joining tree for the COI sequences of *S. (P.) denticollis*. Bootstrap values below 50 % were removed.

Table 12. Summary statistics of general nucleotide diversity over the 960 bp of *S. (P.) denticollis*

Species	Assemblage	*N*	Number of haplotypes	Haplotype diversity	Nucleotide diversity	% Pairwise divergence	Variable sites (V)	Parsimoniously Informative Sites (PI)	Singletons (S)
S. (P.) denticollis	Koichab Pan	13	13	1.000 (0.030)	0.020 (0.011)	0.003 - 0.035			
	Namib Rand	8	5	0.857 (0.108)	0.005 (0.003)	0.001 - 0.011			
	Gobabeb	11	11	1.000 (0.039)	0.013 (0.007)	0.004 - 0.024			
Total		32	29	0.992 (0.011)	0.016 (0.008)	0.001 - 0.019	90 (9.375%)	48 (5%)	42 (4.375%)

[$] V, PI and S were only estimated for the overall dataset

Table 13. Summary of Fst statistics calculated by AMOVA (Excoffier *et al*., 1992) for *S. (P.) denticollis*

Species		Φst	%	P
S. (P.) denticollis	Among collecting site variation		22.16	< 0.001
	Within collecting site variation		77.84	< 0.001
	Fixation index	0.222		< 0.001

[b] P values were determined from 10000 random permutations.

Demographic patterns based on the Stepwise and Exponential Expansion Models

Stepwise Expansion Model

The tree topology (Fig. 12) and the mismatch distributions (Fig. 13), for the individual collecting sites as well as for the entire population, indicate recent sudden demographic expansion for the species as a whole, hence only the mismatch distribution for the population as a whole is presented. These patterns suggest recent expansion in population size and geographic range. The variance (sum of the squared deviation - SSD) and Harpendings Raggedness Index (HRI) for the mismatch distributions were not significant. Under a model of population expansion, the observed and simulated curves were not significantly different from one another.

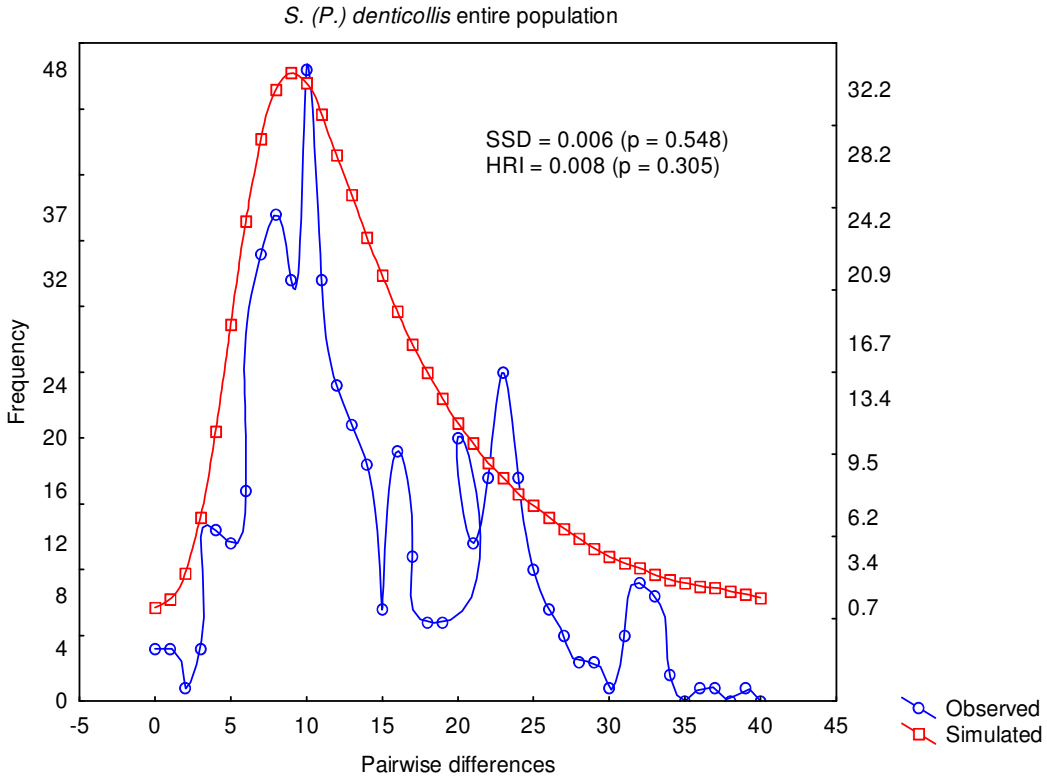


Figure 13. Mismatch frequency distribution of pairwise nucleotide differences for *S. (P.) denticollis* as a single population with sum of the squared deviation (SSD) and Harpendings Raggedness Index (HRI) represented on the graph.

Time of divergence

The average number of nucleotide substitutions per site (*d*) was calculated to be 0.06. Divergence time between *S. (P.) denticollis* and its sister species, *S. (P.) rotundigenus* (Sole *et al*., 2005; chapter 2) was estimated at approximately 2.6 million years ago. This gives the estimate of nucleotide substitutions per site, per lineage, per year (γ) to be 0.06/(2 x 2,600,000) = 1.1 x 10^{-8} with the mutation rate per nucleotide site, per generation (μ) being 1.1 x 10^{-8} and the mutation rate per haplotype (v) being 1.05 x 10^{-5}. The coalescence time in generations, for each population, was calculated using the τ values estimated by Arlequin (Table 14a).

The earliest expansion event appeared to have occurred at the Gobabeb collecting site, which was estimated at around 343,000 generations/years ago while the Koichab Pan site appeared to have undergone recent expansion approximately 144,000 generations/years ago. This is based on a τ value of 7.235 and 3.054 (Table 14a), respectively, and a mutation rate per haplotype (v) of 1.06 x 10^{-5} per COI (as calculated above). Estimated effective population size after expansion (N_1) was an order of magnitude higher than before expansion (N_0).

Exponential Expansion Model

All three collecting sites have high and positive 'g' values for the Exponential Expansion Model. Koichab Pan and Gobabeb have the highest 'g' values while Namib Rand is slightly lower. However, they all indicate historical population expansion. The effective female population sizes were large (in the millions) for both Koichab Pan and Gobabeb while an order of magnitude smaller for the Namib Rand collecting site (Table 14b)

Table 14. Estimated parameters for (a) Stepwise and (b) Exponential Expansion Models for *S. (P.) denticollis*.

(a) Stepwise Expansion Model

		Stepwise Expansion Model			
Species	**Collecting sites**	τ	$\theta_0 = 2\mu N_0$	$\theta_1 = 2\mu N_1$	$t = \tau/2\nu$
S. (P.) denticollis	Koichab Pan	3.054	21.785	4645.000	144,000
	Namib Rand	5.961	0	10.013	281,000
	Gobabeb	7.235	4.175	5712.500	343,000
	Population as a whole	6.462	8.674	810.625	306,000

(b) Exponential Expansion Model

		Exponential Expansion Model		
Species	**Collecting sites**	$\theta = 2\mu Nf$	**g**	N_f
S. (P.) denticollis	Koichab Pan	0.0873	180.209	4,000,000
	Namib Rand	0.0123	405.484	560,000
	Gobabeb	0.3837	638.549	17,000,000
	Population as a whole	0.229	301.807	10,000,000

The UPBLUE/Tajima estimate shows a two-fold increase in recent population size (Table 15). Fu's *Fs* statistic for the population as a whole was significantly negative, indicating that the population is undergoing expansion, which is in direct contrast to the other two species (Table 15).

Table 15. Summary of estimations of Tajima's estimate, Fu's UPBLUE and Fu's *Fs* statistic of *S. (P.) denticollis*

Species		Complete population
S. (P.) denticollis	Tajima's estimate	22.422
	Fu's UPBLUE	60.695
	UPBLUE/Tajima	2.718
	Fu's *Fs*	-7.315 (**)

$ ** = p < 0.01

Migration

MIGRATE indicated ancestral movement was strongly in a northerly direction with no evident movement to the south (Fig. 14).

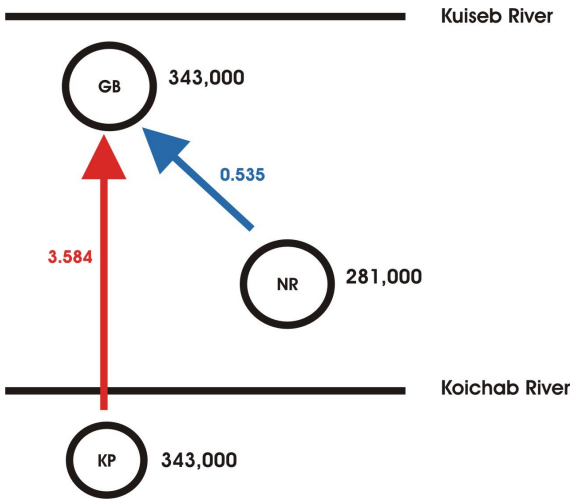


Figure 14. A schematic representation of the migration of individuals between collecting sites of *S. (P.) denticollis*. The coloured arrows indicate the direction of movement while the numbers in the same colour represent an approximation of the number of individuals moving/generation. The numbers in black represent coalescent times of the populations. (Abbreviations are as follows: KP = Koichab Pan, NR = Namib Rand, GB = Gobabeb).

Phylogenetic haplotype relationships

A 95 % parsimony cladogram was estimated for the mtDNA COI data (Fig 15) of *S. (P.) denticollis*, with the maximum number of mutational steps between two haplotypes being 13. The parsimony network was resolved except for the presence of four reticulations, which were broken. There was, however, one disjoint portion within the network, represented by clade 3-1 that could not be linked with 95 % confidence. In addition, haplotypes GO02, LA11 and LT12 were separated from the network by 14, 19 and 20 mutational steps respectively. To simplify the nested diagram not all nesting levels were represented. Haplotypes were collapsed for drawing the cladogram but not in the nesting structure that was used to calculate the contingency test values. GO02 linked to clade 14-1 in 14 mutational steps and LA11 and LT12 linked to clades 19-1 and 20-1 in 19 and 20 mutational steps, respectively. Clade 3-1 was linked by 25 mutational steps to clade 25-1 i.e. the entire cladogram.

The network shows two distinct clades in 3-1 and 25-1. Clade 3-1 consists of three individuals from Koichab Pan, while clade 25-1 consists of the balance of the individuals from all the sites sampled, including others from Koichab Pan. This supports the neighbor-joining tree in that *S. (P.) denticollis* appears as a single population on a dune continuum. The contingency test showed significant geographical association of haplotypes within clades 22-1, 25-1 and 26-1. The inference chain (Table 16) indicates restricted gene flow due to isolation by distance for clades 22-1 and 25-1. The inference for clade 26-1 indicates that the population structure could be attributed to restricted gene flow or dispersal, with some long distance dispersal over intermediate areas not occupied presently by the species, or that there was past gene flow which has been followed by the extinction of intermediate populations.

Due to the small number of populations the Mantel test could not calculate whether there were significant differences or not between the geographic and genetic distances (Liedloff pers. comm.).

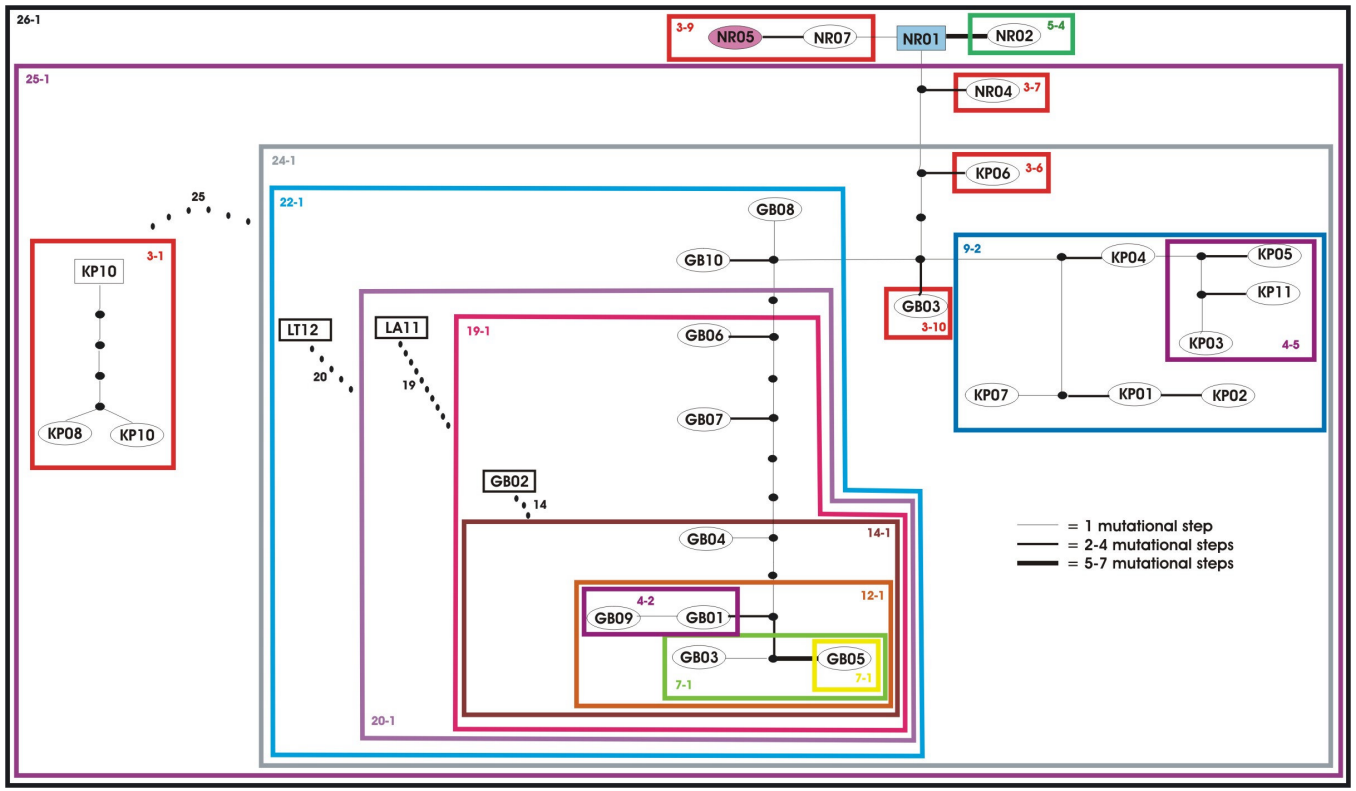


Figure 15. *S. (P.) denticollis* statistical parsimony network and associated design. Haplotypes are designated by letters, which represent the population from where the haplotype came (as seen in Table 1) and numbers representing the individual sequenced. The thickness of the connection line indicates the number of mutational steps. Dotted lines represent alternative ambiguous connections. Ancestral haplotypes are represented by squares. Haplotypes with blue background occurred three times and those with a pink background occurred twice.

Table 16. Geographical nested clade analysis of *S. (P.) denticollis*, inferences based on Templeton (2004)

Haplotype group	χ^2	P-value	Inference chain	
Clades within 22-1	20.303	<0.001	1-19-yes-20-yes-2-yes-3-no-4-no	Restricted gene flow with isolation by distance
Clades within 25-1	29.221	<0.05	1-2-yes-3-no-4-no	Restricted gene flow with isolation by distance
Clades within 26-1	26.880	<0.05	1-no-2-yes-3-yes-5-no-6-no-7-no-8-yes	Restricted gene flow/dispersal but with some long distance dispersal over intermediate areas not occupied by the species; or past gene flow followed by extinction of intermediate populations

Discussion

Demographic patterns of S. (P.) denticollis as compared with S. (P.) hippocrates and S. (P.) gariepinus

The partial mtDNA COI gene phylogeny of *S. (P.) denticollis* shows weak support for the designation of distinct populations. Poor population structure is supported by a low fixation index value, low within population variation as well as low range of within- and between-population percent pairwise distances (Table 12; 0.4% – 2.3 %). This is in contrast *to S (P.) hippocrates* and *S. (P.) gariepinus* which show strong population structure and have a larger percent pairwise divergence range (1 – 12.3 %: Table 3 Chapter 4a and 0.1 – 10.3 % Table 7 Chapter 4b) as well as higher overall nucleotide diversity. Comparison of the percent pairwise divergence and nucleotide diversity between the Namibian population of *S. (P.) gariepinus* and *S. (P.) denticollis,* which both represent a dune field continuum population, reveals that *S. (P.) denticollis* has much lower values for both parameters which is counter-intuitive. Therefore, does landscape fragmentation increase genetic differentiation due to an isolation effect? This study as well as previous ones indicates that rivers, towns, agricultural fields, roads etc. present barriers to species movement by thinning the inhabited area, causing isolation by distance. This study clearly demonstrates that genetic differentiation is higher in species occurring within fragmented landscapes as opposed to those within a continuous landscape. These results are in qualitative agreement with population genetics theory and support the results seen by Knutsen *et al.* (2000) (Order: Coleoptera – Tenebrionidae), Driscoll & Hardy (2005) (Order: Squamata – Agamid Lizards) and van Dongen *et al.* (1998) (Order Lepidoptera – Geometridae). Population fragmentation affects the genetic structure of a species and represents a potential threat to those species with reduced dispersal capabilities. However, it has been argued that some degree of genetic isolation may be advantageous for the conservation of genetic variation and that genetic diversity may be maintained if a population is subdivided into sub-populations. Within a sub-population genetic variation will decrease due to genetic drift but overall population genetic variation will be maintained as different alleles will be preserved in different sub-populations (van Dongen *et al.*, 1998). Although elevated genetic variation is observed in two of the species there are implications in that with reduced local and global populations effective population sizes and loss of advantageous alleles or fixation of disadvantageous alleles could result in the ultimate extinction of a species. This highlights the importance of understanding the patterns and processes acting on and within a species. Both demographic and fine-scale genetic factors need to be examined to reveal likely evolutionary processes acting on a population or sub-

population and will provide a strong guide for conservation management decisions (Driscoll & Hardy, 2005).

Recent versus historical population trends

S. (P.) denticollis appears on a continuum of dune fields from Koichab Pan all the way up the west coast of Namibia to Gobabeb, with both recent and historical estimates showing an increase in population size. There is historical movement in a northerly direction with the Gobabeb collecting site having received individuals from both the Namib Rand as well as the Koichab Pan collecting sites. This provides support for the hypothesis that individuals within a species are moving with their substratum, the barchan dune, in conjunction with the unidirectional wind regime. The Namibian assemblage of *S. (P.) gariepinus* shows similar trends. The nested clade analysis indicates two processes that may have shaped the *S. (P.) denticollis* population. Firstly it appears that *S. (P.) denticollis* has experienced restricted gene flow due to isolation by distance, indicating that there is a minimum amount of recent gene flow between collecting sites. The fact that this species is flightless coupled with their large distributional range (extending over 400 km) would support the fact that their movement between suitable habitats has been at a minimum. Absence of shared haplotypes between the collecting sites may also be an indication that reduced gene flow is occurring. In contrast to this, large population sizes, as indicated by both the Exponential and Stepwise Expansion Models, could have reduced the probability of collecting individuals with overlapping haplotypes. Secondly, it appears that when looking at the entire population, extinction of intermediate populations may have occurred. Extinction of intermediate populations could possibly be attributed to sub-standard habitat quality in intermediate areas, environmental barriers and human induced changes occurring within their habitat. The Agate Beach collecting site, which occurs near Luderitz, is clearly affected by recent mining activities and an encroaching town. The genetic patterns of *S. (P.) denticollis* suggest that the species is still expanding into new or formally occupied habitats - as seen in both Fu's *F*s and UPBLUE statistics - followed by a period of stasis, during which isolation by distance and intermediate population extinction are the causal factors attributed to species phylogeography.

Incomplete lineage sorting vs. clinal variation

Slight morphological differences are visible between the individuals occurring in the most southern and northern distribution of *S. (P.) denticollis* (Harrison *et al*., 2003). Elytral colour

differentiation is also noted within this species, with some individuals having black elytra, others a mix between black and orange and some individuals have orange elytra (Harrison *et al*., 2003). The colour and morphological variation is clinal and is probably a response to a selective environmental gradient (Barrowclough *et al*., 2005). The mtDNA variation within *S. (P.) denticollis* is therefore inconsistent with the morphological clinal variation. Concordance among different datasets often occurs over a long period of time. However, where rapid and recent divergence (within the last 200,000 years for *S. (P.) denticollis*) has occurred retardation of lineage sorting (i.e. incomplete lineage sorting) leading to the identification of ESU's or MU's (Moritz, 1994a & b; Nice *et al*., 2005) becomes difficult. If the traits used to define clinal variation were under selection, surveys of neutral variation would fail to detect distinctive evolutionary lineages where adaptive differences already exist. Differences in life history traits, ecological requirements, morphology and demographic characters would constitute evolutionary significance of the individuals from the different collecting sites. Therefore, ecological non-exchangeability could provide sufficient evidence for the designation of the individual collecting sites as distinct evolutionary units under the strategy posed by Crandall *et al*. (2000).

Implications for conservation of Scarabaeus (Pachysoma) species

Three distinct species of *Scarabaeus (Pachysoma)* have been studied here, all exhibiting different population demographics with population demographic overlap seen in areas of geographic similarity (as seen in the Namibian population of *S. (P.) gariepinus* and *S. (P.) denticollis* as well as the South African populations of *S. (P.) gariepinus* and *S. (P.) hippocrates*). The patterns of gene flow within the presented phylogeographic regions suggest that the three species were each a single continuous population, possessing a relatively high level of dispersal capabilities. This pattern suggests that the observed phylogeographic patterns were probably due to the extinction of intermediate populations causing fragmentation of the entire population. Extinction of intermediate populations could have been caused by anthropogenic and environmental factors, as mentioned throughout the sub-chapters. Physical barriers appear to have had an increased effect on the population structure seen in *S. (P.) gariepinus* while anthropogenic factors appear to be affecting *S. (P.) hippocrates* to a greater degree.

All three of these species were chosen as they exhibit south-north morphological clinal variation and it has clearly been shown that extensive genetic variation occurs within two of the species, *S. (P.) hippocrates* and *S. (P.) gariepinus*. There is strong evidence to

suggest that selective changes are taking place and having an effect on population structure as a whole.

The spatial scale of genetic differences indicates the scale at which conservation should occur. If the species decline over areas spanning the distance between the populations substantial genetic variation will be lost. A series of adequately sized reserves spanning the collecting sites of each population would sample most of the genetic structure. However, would these reserves sustain population perpetuity? In addition to conserving genetic diversity, an important goal of conservation should be to maintain evolutionary processes. Evolutionary processes, within all three species, appear to have been maintained by population and range expansions followed by isolation and fragmentation leading to subsequent divergence. A carefully maintained meta-population strategy may be required to prevent biodiversity loss (Driscoll & Hardy, 2005).

Summary

All three species reported on in this chapter allow for contrasting as well as similar inferences to be made. *S. (P.) hippocrates* and *S. (P.) gariepinus* exhibit strong population structure, supported by AMOVA and high sequence divergence. *S. (P.) denticollis* shows poor phylogenetic structure, as seen by the significantly low AMOVA and the low sequence divergence. All three species exhibit high haplotype diversities with no overlap of haplotypes between populations or collecting sites. Areas of refugia could therefore not be speculated upon.

All three species show strong historical population expansion as seen by the Stepwise and Exponential Expansion Models. Fu's UPBLUE and *F*s statistic indicates that *S. (P.) hippocrates* and *S. (P.) gariepinus* are not undergoing present day expansion which is in line with current species trends in that overall numbers are declining. However, as no present day census data are available, this is difficult to substantiate. Recent events are therefore masked by past events and the genetic signal observed could be misleading. *S. (P.) denticollis* shows a strong trend towards recent range expansions after which a period of stasis occurred.

Extrinsic factors such as rivers and anthropogenic influences have affected all the species in some way. The major rivers act as barriers causing fragmentation leading to allopatric fragmentation with strong support obtained from the NPCA. Anthropogenic factors affecting population structure include agriculture, town encroachment and mining activities which all remove large tracts of suitable habitat leading to fragmentation of a species and in some instances extinction of a population. Since *Scarabaeus (Pachysoma)* species are

flightless and therefore exhibit reduced vagility this appears to have contributed to species structure. NPCA analysis indicates that isolation by distance is a factor contributing to species structure and this could be directly related to low or poor vagility. In contrast to this the results of *S. (P.) gariepinus* and *S. (P.) denticollis* indicate that individuals have historically moved between populations or collecting localities. The fact that the beetles have clearly been shown to move in a south-north direction with the barchan dunes may be the factor underlying the strong movement over large geographic distances. Coalescence of the species is shown to have occurred during the Pleistocene era coincident with the onset of hyper-aridity and the formation of advective fog, which is wind blown up to 50 km inland. The formation of the fog would have allowed for a consistent source of water permitting the species to inhabit previously inhospitable areas.

S. (P.) hippocrates and *S. (P.) gariepinus* show far higher genetic divergence as opposed to *S. (P.) denticollis*. However counter-intuitive this may appear it is in line with genetic theory, in that fragmentation of a landscape, and in turn a species' populations increases genetic variation. This has implications for conservation strategies being implemented; as the variation in populations represents genetic material which if lost could result in imminent extinction.

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Chapter V

Isolation of microsatellite markers from *Scarabaeus (Pachysoma)* MacLeay (Scarabaeidae: Scarabaeinae)

Abstract

In this section of the study the isolation of polymorphic microsatellite loci for the genus *Scarabaeus* was attempted using the FIASCO protocol. The FIASCO protocol is an enrichment protocol based on the ability to recover microsatellite DNA by PCR amplification, after selective hybridisation. High quality genomic DNA is fragmented using a restriction enzyme (*MseI*) and then ligated to a known adaptor (*MseI* AFLP). Following the fragmentation-ligation step the DNA is then hybridised to specific selected 5' biotinylated probes and bound to streptavidin coated beads. After the hybridisation step and several washes to remove DNA that has bound non-specifically, the DNA is eluted and recovered by PCR. Enriched DNA fragments are then cloned into a plasmid vector using a restriction site on the known flanking regions. The resultant recombinant clones are in turn screened for microsatellite repeats by directly sequencing them using primers specific for the vector. Following the identification of clones containing repeats, primers are designed for marker optimisation. This protocol was optimised for *Scarabaeus* and four out of six potential loci were identified as being polymorphic with one being monomorphic and the other exhibiting unstable amplification reactions.

Introduction

"Microsatellite DNA" is the term used to describe tandem repeats of short sequence motifs between two (di-nucleotide) and six (hexa-nucleotides) bases in length. These repeats are arranged head to tail without interruption by any other base or motif and their functional significance is unknown. Microsatellites have been found in every organism studied so far and since they may be highly polymorphic are useful genetic markers. Adenine (A) and thymine (T) di-nucleotide repeats are the most common microsatellites in all genomes; however, they do show subtle differences in frequency distributions. Rates of mutation in microsatellites are high compared to rates of point mutations in non-repetitive DNA regions. High mutation rates in microsatellites are said to be due to either slipped strand mis-pairing during replication, which is thought to be the predominant mechanism, or recombination between DNA molecules (Hancock, 1999; Toth *et al.*, 2000).

Slippage/slipped strand mis-pairing occurs during replication when the nascent DNA strand dissociates from the template strand. When non-repetitive sequences are being replicated there is only one way in which the nascent strand can re-anneal precisely to the template strand before replication is recommenced. If the replicated sequence is repetitive the nascent strand may re-anneal out of phase with the template strand. When replication is continued after such a mis-annealing, the eventual nascent strand will be either shorter or longer than the template strand (Hancock, 1999).

Recombination could potentially alter the length of microsatellites in two ways, by unequal crossing-over or by gene conversion. Unequal crossing over results from crossover between misaligned chromosome strands. Unequal crossover gives rise to a deletion in one molecule and an insertion in the other. Gene conversion, which involves unidirectional transfer of information by recombination, probably as a response to DNA damage, can transfer sequence in an out of phase manner from one allele to another (Hancock, 1999).

The genetic architecture of a species can be interpreted as the result of historical biogeographic factors as well as contemporary ecologies and behaviours of the organism under investigation (Avise *et al.*, 1987; Avise, 2000). Phylogeography is the study of the principles and processes governing the geographical distributions of genealogical lineages, especially those within and amongst closely related species. Phylogeography therefore deals with historical and phylogenetic components of the spatial distributions of gene lineages. Time and space are joint axes of phylogeography (Avise, 2000). The majority of phylogeographic studies so far have employed mitochondrial DNA as the marker of choice, but recent developments in the field recommend the synchronous usage of nuclear-based

microsatellite markers (Avise, 1998) in order to check for congruence across several unlinked loci with different genealogical pathways.

Different protocols exist for microsatellite isolation. Traditionally microsatellites were isolated from partial genomic libraries of the species of interest which involved screening several thousand clones through colony hybridisation with repeat containing probes (Rassman *et al*., 1991). Although a simple method it can be tedious and very inefficient for species with low microsatellite frequencies. While many remain faithful to the traditional means of isolating microsatellites several other methods are being used with increased frequency in an attempt to decrease the time invested in the isolation process while increasing the yield of microsatellites. To avoid library construction and screening it was proposed to modify the randomly amplified polymorphic DNA (RAPD: Williams *et al*., 1990) approach by using either repeat-anchored random primers (Wu *et al*., 1994) or RAPD primers and subsequent Southern hybridisation of the PCR bands with microsatellite probes (Richardson *et al*., 1995). A second strategy, based on primer extension was also proposed for the production of libraries rich in microsatellite repeats. This method involves a high number of steps explaining the limited application and use by the scientific community (Ostrander *et al*., 1992). A third class of isolation was based on selective hybridisation, which appeared to be a very popular method for microsatellite isolation (Kijas *et al*., 1994). If the enrichment was successful sequencing recombinant clones alone could then identify microsatellites. Time is required to get the enrichment protocols running efficiently, but they are advantageous in that they are fast, efficient, require only basic skills in molecular biology and limited laboratory equipment as compared to that for traditional methods of microsatellite screening (for added details of these procedures see Zane *et al*., 2002 and references therein). With this in mind, and the fact that the selective hybridisation procedure had been used successfully in the Genetics Department at the University of Pretoria, it was decided to use this protocol, to isolate microsatellites, in preference to traditional or any other methods.

This part of the study was aimed at isolating microsatellites for the genus *Scarabaeus*. To examine the genetic variation between the populations of the different species both mitochondrial DNA and microsatellites need to be employed. The mitochondrial COI gene reveals relatively older genetic structuring whereas the microsatellites are thought to reveal more recent dynamics. The employment and use of DNA microsatellites is therefore expected to reveal patterns of variation that would be undetectable by other molecular markers (Kirchman *et al*., 2000). The primary goal of the molecular comparisons is to characterize genetic diversity within and between populations of the same species.

Methodology and Results

Taxonomic Samples

A single species, *Scarabaeus (Pachysoma) gariepinus*, was chosen from which to isolate microsatellites, as it is found on both sides of the Orange River and it represented populations that were more comprehensively sampled than the other species. Individuals were collected from five designated populations (see Chapter 4; Table 1), two populations in South Africa, Langhoogte/Kommagas and Holgat River, and three in Namibia, Hohenfels, Daberas/Obib and Klingharts Mountains. A minimum of 10 individuals per population, where possible, were collected otherwise as many individuals as possible were collected and stored in 99.8 % ethanol.

Isolation of Microsatellites

Isolation of microsatellites was performed following Zane *et al*., (2002), the Fast Isolation by AFLP of Sequences Containing Repeats (FIASCO protocol). This method is fast and simple and relies on the efficient digestion-ligation reaction of the amplified fragment length polymorphism (AFLP) procedure as described by Vos *et al*. (1995).

Total genomic DNA was extracted using the Roche High Pure PCR Template Preparation Kit (Roche Diagnostics). The extracted genomic DNA was digested with *MseI* and simultaneously ligated to *MseI* AFLP adaptor (5' TAC TCA GGA CTC AT 3'/5' GAC GAT GAG TCC TGA G 3'). Digestion-ligation was performed on a Hybaid Multiblock for 3 hours at 37°C in a total volume of 25µl containing 25 – 250 ng of DNA, 10 x NEB 2 buffer, 25 mM DTT, 10 x BSA, 5 mM ATP, 10 U/µl *MseI*, 2000 U/µl NEB T4 DNA ligase and 50 µM *MseI* adaptor.

The digestion-ligation mixture was subsequently diluted 10 fold- with SABAX water and amplified in a 20 µl reaction with adaptor specific primers (5' CAT GAG TCC TGA GTA AN 3') henceforth referred to as *MseI*-N where 'N' equals A, C, G and T. The 20 µl Polymerase Chain Reaction (PCR) contained 10 pmol *MseI*-N primer, 1.5 mM $MgCL_2$, 1 X *Taq* DNA polymerase buffer, 10 mM dNTP's (200 µM) in the presence of 0.4 units *Taq* DNA polymerase. Following optimisation, final thermal cycling parameters for *S. (P.) gariepinus* comprised an initial denaturation for 2 minutes at 94°C followed by 22 cycles of 94°C for 30 seconds, 53°C for 60 seconds and 72°C for 60 seconds with a final elongation step at 72°C for 7 minutes (Vos *et al*., 1995). A small amount of the PCR product (3 µl) was electrophoresed on a 1.5 % agarose gel (Fig. 1). Clear DNA smears (lanes 1 & 2) indicated that *MseI* cut the genomic DNA in fragments ranging from 250 to 1 200 base pairs (bp) in

length. Figure 1 also shows an over-representation of fragments as seen by the dark distinct band approximately 500 bp in size. This over-represented band is not ideal, as it is believed to represent multi-copy sequences in the original genome.

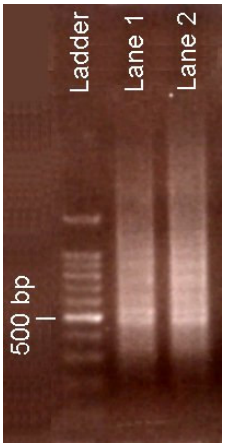

Figure 1. Electrophoresis of the PCR products amplified with the *MseI*-N primers following the digestion-ligation step. Lanes 1 & 2 contain 3 µl of PCR product. Product sizes range from 250 – 1200 bp in length.

In the AFLP protocol the *MseI*-N primer has a selective nucleotide at the 3' end, which matches the first nucleotide beyond the original restriction site, allowing for pre-selective amplification. For this protocol all four primers are mixed (*N* = A, C, G, T) allowing for the amplification of all fragments flanked by *MseI* sites, providing they have an appropriate size for PCR. The advantage of this is that if undesired bands appear within the PCR, one can go back one step and try different combinations of the selective primers, i.e. remove one of the bases 'A, C, G, T from the primer mix, and re-amplify, and in doing so preclude amplification of the unwanted bands. Over representation of bands which probably correspond to multi-copy sequences in the original genome are not favourable as they tend to be carried over during enrichment and when cross-hybridised with the biotinylated probes will represent a significant fraction of the obtained recombinant clones (Zane *et al*., 2002). A single base (either A, C, G or T) from the *MseI*-N primer was systematically removed and four different amplification reactions were preformed, the results of which can be seen in Figure 2. The reaction lacking an over represented band can be seen in reaction 4 - lanes 3 &

4 - (Fig. 2), which lacked the base 'C'. All subsequent PCR's using the *MseI*-N primer, were therefore performed without the base 'C'.

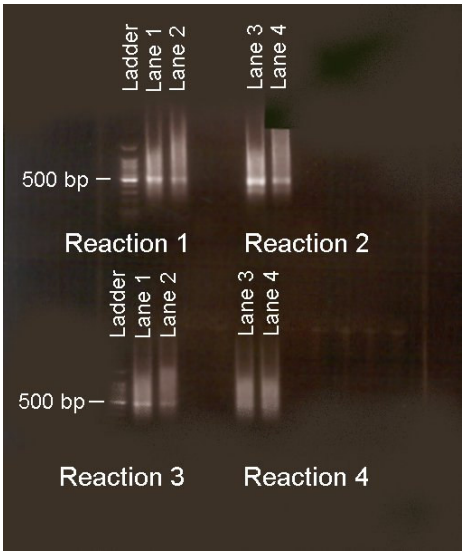

Figure 2. The four experimental amplification reactions (labelled from the top left) after the removal of a single base A (reaction 1), T (reaction 2), G (reaction 3) and C (reaction 4).

The DNA from the PCR was then used as template for hybridisation to selected biotinylated probes using the Travis Glen Protocol (http://129.252.89.20/Msats/Microsatellites.html). The DNA-probe hybrid molecules were prepared in the following way: 250 – 500 ng of the amplified DNA, 50 – 80 pmol of the selected biotinylated probe made up to a total volume of 100 µl with 4.2 X SSC, 0.7 % SDS. Probes were denatured at 95°C for 3 minutes followed by annealing at room temperature for 15 minutes. Three hundred µl of TEN_{100} were added to the prepared DNA-probe hybrid molecules.

Table 1 shows the probes used in different combinations to isolate microsatellites. Different probes can be combined in a single hybridisation reaction only if the designated probes have the same length i.e. only di-nucleotide or only tri-nucleotide probes can be combined in a single reaction.

Table 1. Biotinylated probes used to isolate microsatellites.

Probes	Length
$(ca)_{15}$	30mer
$(ct)_{8}$	16mer
$(gc)_{8}$	16mer
$(tg)_{15}$	30mer
$(ata)_{8}$	24mer
(gtg)5	15mer
$(caa)_{5}$	15mer
$(aca)_{5}$	15mer
$(cga)_{5}$	15mer
$(cca)_{5}$	15mer
$(cat)_{5}$	15mer
$(cac)_{5}$	15mer
$(ccagt)_{10}$	40mer
$(gaaa)_{6}$	24mer
$(tgtc)_{6}$	24mer
$(tatc)_{6}$	24mer
$(gata)_{6}$	24mer
$(cagc)_{6}$	24mer
$(tcca)_{6}$	24mer

The DNA molecules attached to the biotinylated probes were captured using Streptavidin coated beads (Streptavidin Magnetic Particles, Boehringer Mannheim) (Kandpal *et al*., 1994;Kijas *et al*., 1994; Mcrae *et al*., 2005). Firstly, the beads were prepared by washing 1 mg of beads three times in 500 µl TEN_{100} (10mM Tris-HCL, 1 mM EDTA, 100 mM NaCL at pH 7.5) after which they were re-suspended in 40 µl of $TEN_{100.}$ Ten µl of an unrelated PCR product were added to the prepared beads so as to prevent non-specific binding. The DNA-probe hybrid molecules were then added to the prepared beads and allowed to incubate for 30 minutes at room temperature with constant gentle agitation.

The non-specific DNA was removed by 7 non-stringency and 7 stringency washes. Non-stringency washes were performed by adding 400 µl of TEN_{1000} (10 mM Tris-HCL, 1 mM EDTA, 1M NaCL, at pH 7.5). The stringency wash was performed by adding 400 µl 0.2 SSC, 0.1 % SDS to the DNA. All washes were carried out at room temperature for 5 minutes, recovering the DNA with magnetic field separation, except for the last stringency wash which

was left at 40°C for 5 minutes. The last non-stringency and stringency washes were kept for further amplification.

The DNA was then separated from the beads-probe complex by means of two denaturation steps. The first step involved adding 50 µl of TE (10 mM Tris-HCL, 1 mM EDTA at pH 8) prior to incubation at 95°C for 5 minutes, after which the supernatant was removed and stored. The second step involved treating the beads with 12 µl of 0.15 M NaOH, removing the supernatant and neutralizing it with 12µl of 0.1667 M acetic acid. TE was then added to a final volume of 50 µl. The last non-stringency, stringency and two elutions from the denaturation step should contain an increasing amount of DNA fragments containing the repeat and should carry the *MseI*-N primer target at each end.

The DNA was then precipitated from the washing and denaturation steps by adding 1 volume of ethanol and sodium acetate (0.15 M final concentration) and then re-suspending in 50µl of water. The precipitated DNA was amplified in a 50 µl reaction containing 10 pmol *MseI*-N primer, 2 mM $MgCL_2$, 1 X *Taq* DNA polymerase buffer, 10mM dNTP's (200 µM) in the presence of 0.4 units *Taq* DNA polymerase. Thermal cycling parameters comprised an initial denaturation for 2 minutes at 94°C followed by 35 cycles of 94°C for 30 seconds, 53°C for 60 seconds and 72°C for 60 seconds with a final elongation step at 72°C for 7 minutes.

The PCR products from the washing and denaturation steps yielded a product with smears above 200 bp with the last stringency wash yielding an order of magnitude less product showing the distinct removal of non-specifically bound DNA. Figure 3 clearly indicates that the more non-stringency and stringency washes done, the more non-specifically bound DNA is removed. Figure 3 shows the effect of eight washes with lane 8 having the least amount of DNA.

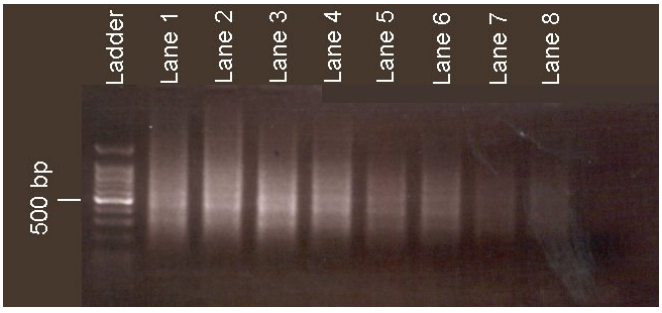

Figure 3. Amplification products of eight stringent wash steps, loaded in lanes 1-8. Lane 8 represents the last stringency wash clearly indicating that the more washes done the more non-specifically bound DNA was removed.

Cloning and optimisation

Cloning of the PCR amplicons was carried out using the TOPO TA Cloning Kit for Sequencing (Invitrogen). The PCR products from the two-denaturation steps were used as they were considered the best candidates for producing a highly enriched library.

Pre-mixed agar media containing Ampicillin (Invitrogen) was used to prepare the agar plates on which to grow up colonies. Pouring of the plates was done in a laminar flow cabinet after which the plates were allowed to cool for 30 minutes. Colonies were plated out using 50 µl and 100 µl from each transformation and incubated upside down at 37°C for 12 – 16 hours. Colonies were then cultured for 16 – 24 hours in 1000 µl of LB Broth (Invitrogen Corporation), containing 100 µg/ml of Ampicillin at 37°C, with constant agitation. Once the colonies had been cultured 10 µl of the colony was added to 10 µl of SABAX water and denatured at 96°C for 7 minutes. The balance (850 µl) of the cultured colony was added to 150 µl of glycerol, creating a 15 % glycerol solution for long-term storage at -80°C. One µl of the denatured colony was amplified in a 50 µl reaction containing the following 10 pmol of each of the T3 and T7 universal primers, 2 mM $MgCL_2$, 1 X *Taq* DNA polymerase buffer, 10 mM dNTP's (200 µM) in the presence of 0.4 units *Taq* DNA polymerase. Thermal cycling parameters were the same as mentioned above. The colony PCR products when electrophoresed on a 1.5 % agarose gel showed PCR products of different sizes (Fig. 4).

Different sized PCR products confirmed that vectors had different sized cloned products incorporated into them. A total of 260 colonies were picked all of which were sequenced. Sequencing reactions were performed at an annealing temperature of 53°C with version 3.1 of the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer). Each amplicon was sequenced with the T3 primer (Invitrogen) to screen for microsatellite (Msat) repeats. Primer pairs flanking the microsatellite repeats were then designed using Primer Designer 4 (Scientific and Educational Software). Of the 260 sequences a total of 15 possible repeat sequences were identified. These were narrowed down to six possibilities due to either poor repeats or the inability to design primers suitable for amplifying the repeat region (see Table 2 for microsatellite repeats).

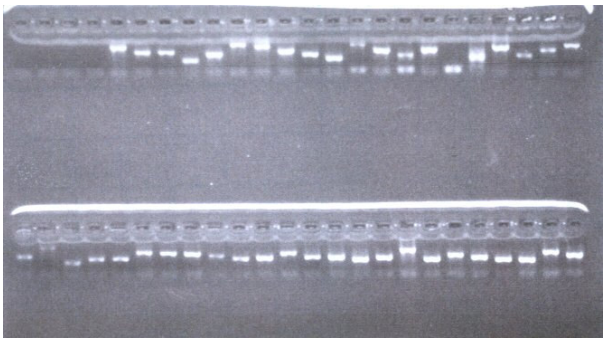

Figure 4. Electrophoresis of the size-discrete PCR products obtained from the colonies grown in LB broth containing potential Msat repeats.

To determine the optimal PCR conditions at which the primer pairs amplify the repeat DNA region, a set of 'cold tests' were performed under varying annealing temperatures, Mg-concentration and DNA-extract concentrations on a Hybaid Multiblock. Amplifications were performed in 7 µl final volume containing approximately 30-50 ng of DNA, 1-2.5 mM $MgCL_2$, 0.2 mM of each dNTP, 0.35 units of Expand High Fidelity *Taq* (Roche), 1 X buffer and 4 pmol of each primer. For each primer pair an optimal annealing temperature was obtained and cycling conditions were optimised as comprising an initial denaturation for 3 minutes at 94°C followed by 35 cycles of 94°C for 30 seconds, variable primer annealing temperatures (ranging from 44.8ºC to 53.4ºC – see Table 2) for 60 seconds and 72°C for 30 seconds with a final elongation step at 72°C for 45 minutes. The full 7 µl of PCR product was electrophoresed on an 8 % polyacrylamide gel, at room temperature overnight, against a 100 bp ladder (Promega), to test for polymorphism.

Table 2. Potential microsatellites and primers designed to determine whether the loci were polymorphic or not.

Probes	MSAT repeats	Ta	Clone	Primers					Polymorphic
				Name	Sequence	Tm	Pos	L	
1. (ca)15(ct)8	(tg)24	53.4°C	27	tg36_f	atg agt ggg tgt gtg tcg tg	61°C	36	20	Unreliable
				tg257_r	tct tcc ttg gtc ttt att ttg g	57°C	257	22	Unreliable
2. (ata)8(gtg)5									
	(cta)12	51.3°C	31	ap42_f	ggt cac gct tta gga cta ga	60°C	83	20	√
3. (caa)5(aca)5(cga)5				ap42_r	ggt tga taa ggt aga tgc cc	59°C	340	20	√
	(tg)8	44.8°C	23	ap35_f1	gcc tct tcg agt att gtg	56°C	277	18	√
				ap35_r1	cgt taa caa gga gct gca	60°C	399	18	√
	(ca)5 cg (ca)7	62°C	13	ap25_f	cgt gaa tcg acg acg tga aa	62°C	116	20	No
				ap25_r	gtg tat gta tgt gcg ggt gt	62°C	181	20	No
	(ca)3 ta (ca)6	46.8°C	15	ap27_f	cgt tat cac gcg ctc gca ca	68°C	254	20	√
				ap27_r	ccg tat ggt gcc gct tcc tt	67°C	334	20	√
	(gggtt)3 gtgatgtgtt (gggtt)2	50°C	6	ap19_f	cgt cag aga ggg tat gta ac	58°C	29	20	√
				ap19_r	cct atc ttg tag aca ggt gc	59°C	337	20	√

[£] Ticks in the polymorphic column indicate that the locus was polymorphic

Discussion

Microsatellites are found everywhere in both prokaryote and eukaryote genomes and are present within coding and non-coding regions. Their distribution, however, is not homogenous within a single genome due to different constraints on coding vs. non-coding DNA (Toth *et al*., 2000), historical processes (Wilder & Hollocher, 2001) and possible different functional roles of the repeats (Valle, 1993). The frequency of microsatellites also varies across taxa, in terms of both absolute numbers of the loci and repeats (Hancock, 1999). As microsatellites have a high mutability they are thought to play an important role in genome evolution by creating and maintaining quantitative genetic variation (Toth *et al*., 2000). The aim of this part of the study was to create a microsatellite library for *Scarabaeus.* However, due to time constraints and technical problems encountered this goal was not satisfactorily achieved.

Technical problems resulting in low yield of polymorphic loci were two-fold. Firstly, problems were experienced with cloning the DNA fragments into the vector cells. This can be seen by the small number of colonies that were available to select for growth (namely 260 across 10 different attempts). This contrasts markedly with the more than 400 colonies obtained by P. Bloomer after three attempts with Avian DNA under the same laboratory conditions and using the same reagents (pers. comm.). Probes based on the microsatellites isolated from other families of Coleoptera were taken into account and used first when attempting to increase the cloning success rate but this did not seem to solve the problem. Different agar was tried in case the vector cells were sensitive to certain agar media. The TOPO cloning manual suggested leaving the cloning reaction for different lengths of time to allow for maximising the PCR products. Different combinations of these times were tried but this did not improve the cloning reaction. The competent cells from the same kit were tested using bird and mammal DNA to see whether the competent cells were of a poor quality. However, both these sets of DNA produced a magnitude more clones on the agar as opposed to beetle DNA. After trying different combinations of times, probes, agar and testing the competent cells for competency it is concluded that *Scarabaeus* may be comparatively poor in microsatellite repeats.

According to Zane *et al*. (2002) arthropod DNA does not appear to be microsatellite rich and the general trend appears to be that the success rate for isolating positive repeats is approximately 2 %. This is exceedingly low, indicating that the success rate achieved in this study was not unrealistic particularly as the six good repeats obtained out of a total of 260 clones sequenced, works out at a 2.3 % success rate. Furthermore, approximately 50 % of

positive clones (i.e. those containing repeat motifs) are eventually discarded due to a lack of suitable sequence for primer design, or the absence of the repeat in the amplification product, or due to unreliable amplification (Zane *et al*., 2002). It has been confirmed that four of the six loci were polymorphic, a single one ($(tg)_{24}$ repeat) exhibited unreliable amplification while the last one was monomorphic. An additional consideration is that the expected frequency of tri- and tetra- nucleotide repeats is below 1 % of any clone analysed across all taxa (Zane *et al*., 2002).

The second problem we encountered was with the amplification step of a single locus, the $(tg)_{24}$ repeat, once the primers had been designed. In the 'cold tests' primer-specific annealing temperatures, DNA and $MgCl_2$ concentrations were identified, which gave the best amplification of genomic DNA. In some instances, however, the PCR would give double bands in one PCR and not in another using the same DNA and reagents, and under the same cycling parameters. If one knows the length of the fragment amplified then should any double bands occur that differ significantly in size, it could still in theory be possible to identify and select the correct band containing the repeat, based on size. However, in most cases the bands were too close together to permit adequate separation, hence it was not possible to ascertain whether the repeat was polymorphic or not. The reason for temperamental PCR's was unknown. Different types of *Taq* DNA polymerase (i.e. High Fidelity, Supertherm, Supertherm Gold and Biotools) were evaluated, between-thermal cycler variation was avoided by using the same PCR machine for optimisation as for amplification, new reagents were tried for PCR reactions and different individuals of the species were amplified but the problem still persisted. As a last resort new primers were designed for this locus but this did not seem to solve the problem.

In many cases obtaining 'well behaved' microsatellites requires considerable time and effort and even then some microsatellites may still have null alleles or single primer pairs that amplify more than a single locus (Meglécz *et al*., 2004). Difficulties arise during isolation and characterisation of microsatellites leading to few well-resolved loci (Nève & Meglécz, 2000). Problems appear during the design of primers and setting up PCR conditions. Reasons for the presence of null alleles and varying amplification intensities between individuals has been largely speculated upon but suggestions are that the flanking regions of microsatellites may be variable (Meglécz *et al*., 2004). A frequently observed problem is the amplification of more than two bands with a single primer pair (Meglécz *et al*., 2004). Two possible reasons primarily given are (i) the duplication or multiplication of microsatellite containing regions or (ii) that microsatellites lie within a minisatellite repeat unit and have microsatellite length

variations between the minisatellite repeat units. These problems appear to be common within the order Lepidoptera (Meglécz *et al*., 2004), but we have experienced them within *Scarabaeus* i.e. order Coleoptera, indicating that they may be more common across unrelated taxa. However, as failed attempts at microsatellite isolation are generally not published, the extent of the problem can only be speculated upon.

One of the major drawbacks of microsatellites is that they need to be isolated *de novo* from most species being examined for the first time. Most microsatellites are found in non-coding regions were the substitution rate is higher than in coding regions (Hancock, 1995). To design 'universal primers' matching conserved regions is therefore often problematic (Zane *et al*., 2002). Different taxa exhibit different preferences for microsatellite repeat types (Lagercrantz *et al*., 1993) hence attempting amplification across the generic, familial or order level in many instances proves fruitless.

The task of microsatellite isolation involves a large amount of effort in the time and money invested in isolation, compared to the results obtained. One has to screen genomic libraries with many appropriate probes, optimise amplification reactions at numerous steps, design primers and eventually gene scan individuals from the respective species. The number of positive clones (those containing microsatellite repeats) ranges from 12 % to as low as 0.04 % (Zane *et al*., 2002). Such isolation strategies will therefore only be successful in a limited time in taxa with high numbers of repeats e.g. fish or if a low number of microsatellite loci are needed.

Microsatellites are inherently unstable and undergo constant mutation. The abundance of certain repeat types varies with the genomic region and their distribution is often dependent on the taxonomic group examined (Hancock, 1996; Toth *et al*., 2000). Mean density for microsatellites within a species varies widely for reasons unknown, and therefore resulting in no *a priori* rule that can be forged for their predictability (Jarne & Lagoda, 1996). Moreover, overall microsatellite content within a genome is often correlated to genome size of the organism (Hancock, 1996). After taking all the above points into account the 18 months of laboratory work required to obtain four polymorphic loci exemplifies the amount of the labour involved as well as the poor success rate in the isolation process. The number of loci scored, degree of polymorphism of each locus and sample size are of paramount importance for the statistical power of microsatellites to be effective (Zane *et al*., 2002). With this in mind the process of optimising the loci will continue until such time as a minimum of five or more good polymorphic loci are obtained with which to work.

Microsatellite markers are excellent for population structure studies as they are highly variable, more likely to be neutral than other genetic markers and the results are reproducible (Jarne & Lagoda, 1996). These advantages tend to outweigh the long and expensive isolation process and establishment of appropriate amplification conditions. Even though the problems experienced, such as low microsatellite frequency and frequent PCR failure, do not appear to be unique to Coleoptera i.e. they are seen in at least one other order of insects, Lepidoptera, (Meglécz & Solignac, 1998), we are positive that with perseverance, repetition and patience, successful isolation of microsatellite loci will be obtained that will provide the desired statistical power to answer the original questions posed.

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Chapter VI

Concluding comments on the phylogeny and phylogeographic patterns of *Scarabaeus (Pachysoma)* MacLeay (Scarabaeidae: Scarabaeinae).

Throughout the preceding chapters I have attempted to identify species relationships at both a morphological and molecular level and key patterns and processes in phylogeographic history that may have shaped the population structure within *Scarabaeus (Pachysoma)* seen today. I have also attempted to highlight conservation concerns based on some of the analyses done and questions posed. In this chapter I therefore attempt to summarise the key findings of each chapter and bring together the ideas and theories to identify the most important processes affecting or having affected *Scarabaeus (Pachysoma).*

Phylogenetic history

Much contention has surrounded the taxonomy of *Scarabaeus (Pachysoma)* and related taxa over the last 50 years. One of the primary aims of this study was therefore to produce an estimate of *Scarabaeus (Pachysoma)* phylogeny using both molecular and morphological data as individual datasets and then combined, in order to address whether the group was monophyletic, how *Pachysoma* was related to *Scarabaeus*, whether *Neopachysoma* was a valid genus and whether there were 13 good species within *Pachysoma*. The phylogenetic analysis was conducted using 64 morphological characters (obtained from Harrison and Philips (2003) and 1197 bp of the Cytochrome Oxidase I (COI) gene (Sole *et al*., 2005)).

Scarabaeus (Pachysoma) was found to be a monophyletic clade within *Scarabaeus* and was therefore classified as a derived subgenus thereof (Harrison *et al*., 2003; Forgie *et al*., 2005; Sole, 2005, Chapter 3). The synonymy of *Neopachysoma* with *Pachysoma* is supported even though it is clearly a distinct lineage within *Scarabaeus (Pachysoma)* (Sole *et al*., 2005; Forgie *et al*., 2005). Morphologically there were 13 good species within *Scarabaeus (Pachysoma)*. At a molecular level strong resolution was found for 11 of the 13 species with *S. (P.) hippocrates* and *S. (P.) glentoni* forming a species complex called the hippocrates/glentoni complex. The phylogenetic tree produced from the combined dataset showed strong support for all 13 species. The morphological and molecular data partition phylogenies showed congruence with the combined phylogeny, lending strong support for combining datasets using total evidence. Phylogenetic trees based on combined data

partitions were relatively more resolved than those based on the individual data analyses. Both the data partitions contributed to the overall combined phylogeny without the morphological data being overshadowed by the large molecular dataset, indicating that both the gene chosen as well as the characters had good resolving ability and were adequate for the level of phylogenetic information required.

Phylogeographic history

Biogeographic inferences could be made due to a recent comprehensive history of the geology and palaeo-climate of the Namib Desert being available (Pickford & Senut, 1999). Speciation events and divergence times were estimated by applying a molecular clock, which was based on Brower's (1994) 2.3 % divergence per million years, to the molecular data. The use of molecular data allowed for the relation of species age to past geological and climatic events rendering a base from which to infer phylogeographic history of the species of *Scarabaeus (Pachysoma)*.

Scarabaeus (Pachysoma) is estimated to have arisen about 2.9 million years ago, which appears to be young when compared with the age of the Namib Desert - dating back to the Miocene (*ca* 15 Ma). A consistent and reliable source of water in the form of advective fog (Nicolson, 1990), which is blown up to 50 km inland, can be associated with the radiation of *Scarabaeus (Pachysoma)* into inhospitable areas along the west coast of southern Africa (Logon, 1960; Seely & Louw, 1980; Nicolson, 1990; Pickford & Senut, 1999). Clear south-north evolutionary gradients can be seen within the species of *Scarabaeus (Pachysoma)*, that are consistent with the unidirectional wind regime, indicating that the psammophilous taxa disperse with their substratum and habitat the barchan dune (Penrith, 1979; Endrödy-Younga, 1982; Prendini, 2001). Major ancient rivers such as the Orange, Buffels and Holgat appear to be gene barriers to certain species of '*Pachysoma*' as well as areas of origin of speciation events (Irish, 1990).

Strong geographic association can be seen within the phylogenies where species that group together within the clades share similar distributions along the total *Scarabaeus (Pachysoma)* distribution. Species with a suite of mostly plesiomorphic characters have a southerly distribution while their derived psammophilous relatives have central to northern Namib distributions.

Population demographics

Three species of *Scarabaeus (Pachysoma)* were selected for detailed population studies, based on the fact that they exhibited distinct south-north morphological clinal variation as seen in the study by Harrison and Philips (2003). Using distance methods, basic population analyses methods and coalescent theories (Schneider *et al*., 2000; Beerli & Felsenstein, 1999; 2001; Kuhner *et al*., 2004) an attempt was made to answer questions aimed at assessing factors that could have contributed to the population structure exhibited by these species of *Scarabaeus (Pachysoma)*.

Three distinct species within *Scarabaeus (Pachysoma)* have been studied here, all exhibiting very different population demographics with overlap seen in areas of geographic similarity. *S. (P.) hippocrates* was shown to have four distinct populations in South Africa; *S. (P.) gariepinus* had three populations, two in South Africa and one in Namibia and *S. (P.) denticollis* was identified as a single population along a dune field continuum in Namibia. The phylogeographic partitioning seen in the three species was supported by the AMOVA analysis. All three species exhibit high overall haplotype diversity. Both the Stepwise (Mismatch distributions) and Exponential (LAMARC) Expansion Models indicate strong historical population expansion. Fu's UPBLUE and *F*s statistic values, indicative of recent population parameters were not always significant for all populations throughout the three species which may be an indicator that the present populations may not be undergoing population expansion but instead are in a slight decline or are maintaining population numbers. As recent events are shown to be masked by past trends giving rise to conflicting results; species census data collected over a number of years should be conducted in order to resolve this. Application of nested clade analysis (NCPA) (Templeton *et al*., 1995) indicated allopatric speciation for those populations separated by environmental and anthropogenic barriers – such as rivers and towns – while for the Namibia population of *S. (P.) gariepinus* and the species *S. (P.) denticollis* isolation by distance and continuous range expansion could be inferred as defining population structure.

Coalescence for each species was calculated and it was estimated that all three species underwent population expansion within the late Pleistocene era. Analysis of gene flow revealed a strong degree of south-north movement, consistent with the unidirectional wind regime. Large numbers of individuals were shown to have moved between populations. A high degree of historical gene flow indicates that the species were originally continuous populations within the geographic region but extinction of the intermediate populations most

likely occurred through both natural and human factors. Recent events therefore indicate that human induced, environmental barriers and reduced vagility have had a major influence on the population structure seen within these three species.

Conservation recommendations

Populations that show gradual geographic and individual variation at both a molecular and morphological level make defining species delimitations problematic (Drotz & Saura, 2001). Extensive molecular and morphological variation occurs across all three species. However to delimit added species or sub-species based on molecular data would not be desirable and may pose problems with regard to taxonomic concerns. It is clear that selective changes are occurring within the populations and that sufficient mitochondrial divergence has occurred, affecting overall population structure. If these changes are to continue to be observed and the species conserved, conserving authorities need be made aware of the circumstances and each population should be delineated as a Management Unit (Moritz, 1994a, b). Each population is connected by low levels of gene flow and are functionally independent and therefore should be managed as individual entities. To conserve every living creature is beyond our reach but an effort needs to be made where we are aware of changes and threats occurring within species and populations of species.

Isolation of Microsatellite markers

The aim behind this part of the project was to have a nuclear marker with which to compare the mitochondrial COI sequences because, by combining and comparing the same analyses on different genes a better overall picture could be obtained of the population demographics of *Scarabaeus (Pachysoma).* A second objective behind isolating microsatellites was that as these markers are often genus specific it would be interesting to use these powerful loci on different species of the large and variable genus *Scarabaeus,* to answer additional taxonomic and demographic questions that were posed throughout this thesis.

The FIASCO protocol was chosen over other methods of microsatellite isolation as it is fast, efficient, requires only basic skills in molecular biology and limited laboratory equipment as compared to that for traditional methods of microsatellite screening. The FIASCO protocol is an enrichment protocol based on the ability to recover microsatellite DNA by PCR amplification, after selective hybridisation (Zane *et al.*, 2002). As microsatellites need be isolated *de novo* this turned out to be a daunting and labour intensive process and problems resulting in a low yield of polymorphic loci were two-fold. The first

problem encountered was with cloning the DNA fragments into the vector cells for colony growth. Probes were selected based on previous studies of Coleoptera where microsatellites were isolated but this did not improve the cloning procedure. Different agar media were tried in case the competent cells were sensitive to the agar, which they were not. Different time combinations as suggested by the TOPO cloning manual were used and lastly the competent cells were tested on both Avian and Mammalian DNA to test whether they were of poor quality, which they were not. The second problem was encountered during optimisation of a locus where consistent conflicting PCR results were obtained. In some instances the PCR's contained single bands while under the same conditions using the same reagents double bands where obtained in a separate amplification reaction. These two problems were identified in both the orders Coleoptera and Lepidoptera, indicating that they may be common across unrelated taxa (Meglécz *et al*., 2004). However, as failed attempts at microsatellite isolation are generally not published, the underlying cause of the problems experienced can only be speculated upon. Despite these difficulties the FIASCO protocol was optimised for *Scarabaeus* and four polymorphic microsatellite loci were successfully isolated. However, for the analyses to be statistically powerful this is too few to constructively work with, at least one extra locus is needed for the completion of this part of the study.

Future research

Many possibilities for future research can be suggested from this study. I include only those which will most enhance the research done and may be of particular interest.

The resolution of the hippocrates/glentoni complex has been an issue that needs to be resolved. Morphologically these two species are very similar and can reliably be identified based on male genitalia. By increasing the number of specimens and analysing a different gene better phylogenetic resolution at a molecular level, should be obtained for these two species.

An addition of a nuclear gene or genes such as a ribosomal gene - 18S/16S - or a protein-coding gene - elongation factor-1 α - would be of interest to be sequenced for the population study, as this would support or refute the slightly conflicting results regarding the biogeographic history of the group presented here. An added microsatellite locus needs to be isolated to have at least five polymorphic loci so as to ensure the statistical power of the

analyses is sufficient. The microsatellite data should be analysed and published in conjunction with the mitochondrial COI data, so as to ascertain whether overlying patterns exist between the two types of DNA. Once the microsatellite section of this study has been completed these loci can and will hopefully be successfully used within other species of *Scarabaeus* for similar and more detailed studies to elucidate phylogeographic and demographic patterns.

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Appendix 1. Character states, as defined by Harrison & Philips (2003), of the taxa used in the morphological and combined Parsimony analysis. 0 primitive; 1-5 derived (in sequence);? unknown character state; - not applicable character state.

TAXA	**Characters**						
	0	**1**	**2**	**3**	**4**	**5**	**6**
	01234567890	**1234567890**	**1234567890**	**1234567890**	**1234567890**	**1234567890**	**123**
S. (Pachysoma) rodriguesi	01301100233	1022211230	3102401511	2003230111	0323141111	1131121211	111
S. (Pachysoma) rotundigenus	01301100233	1022211030	2102301011	0003230111	1313141111	1131121211	111
S. (Pachysoma) denticollis	01301100233	1022212230	3102301011	2003230111	0323141111	1131121211	111
S. (Pachysoma) bennigseni	01301100233	0011012032	0102300011	2003230111	0223141111	1131120211	111
S. (Pachysoma) gariepinus	02301100333	0001212031	0002300011	1013230011	0323141111	1131121211	111
S. (Pachysoma) striatus	0130-211333	0002210030	0002300001	1013230011	0323141111	1131121211	111
S. (Pachysoma) fitzsimonsi	02301100313	0001012032	0002301001	1103230011	0223141111	1131121211	111
S. (Pachysoma) schinzi	01321000111	0101020032	0002301001	0003230211	0223141111	1131121211	111
S. (Pachysoma) valeflorae	01331000111	0101021332	0102301001	0003230211	0113141111	1131121211	110
S. (Pachysoma) hippocrates	0131-211311	0122010130	0102400101	0203230211	0113141111	1131121211	111
S. (Pachysoma) endroedyi	0031-211311	0021010032	0002300111	0213230211	0103141111	1131121211	111

S. (Pachysoma) glentoni	0131-211311 0022010130 0002301101 0203230211 0113141111 1131121211 111
S. (Pachysoma) aesculapius	0131-211311 0100010032 0002301101 0203230211 0223141111 1131121211 111
S. (Scarabaeolus) rubripennis	00000001011 1011110111 2002100200 2001001000 1000020000 0010010000 000
S. [Neatechus] proboscideus	32001001010 1010221120 2002100210 0002012011 1322120000 0000001000 010
Scarabaeus rugosus	11001001011 1110210010 0002200200 0001002111 1302120000 0010010000 000
Scarabaeus galenus	42001101010 2102210110 2002100200 0102012111 0101130000 0010011000 010
Scarabaeus westwoodi	40010001111 1110210310 0002100000 0001001001 0102120000 0010011000 000
Scarabaeus rusticus	11000001111 111?210110 0002200300 0001000101 0100120000 0010010000 000
Sceliages brittoni	0000-211011 1110100101 0000300010 0001201201 0110000000 0000010000 000
Scarabaeus (Drepanopodus) proximus	10000001111 0011210230 2012214320 2001002100 0002130000 0020111000 000

Appendix 2. Summary of 140 individuals of three species of *S. (P.) Pachysoma* characterised in Chapter 4

Species	Specimen ID	Locality	Co-ordinates	GenBank Accession No.
S. (P.) hippocrates	BVPH01	Brakvlei - Koekenaap	S31°25'27.3" - E18°01'38.1"	AY965154
	BVPH02	Brakvlei - Koekenaap	S31°25'27.3" - E18°01'38.1"	AY965155
	BVPH03	Brakvlei - Koekenaap	S31°25'27.3" - E18°01'38.1"	AY965156
	BVPH04	Brakvlei - Koekenaap	S31°25'27.3" - E18°01'38.1"	AY965157
	KKPH01	Kommandokraal Farm	S31°29'58.4" - E18°12'29.2"	AY965158
	KKPH02	Kommandokraal Farm	S31°29'58.4" - E18°12'29.2"	AY965159
	KKPH03	Kommandokraal Farm	S31°29'58.4" - E18°12'29.2"	AY965160
	KKPH04	Kommandokraal Farm	S31°29'58.4" - E18°12'29.2"	AY965161
	KKPH05	Kommandokraal Farm	S31°29'58.4" - E18°12'29.2"	AY965162
	LAPH01	10km W Leipoldtville	S32°13'06.3" - E18°26'06.8"	AY965163
	LAPH02	10km W Leipoldtville	S32°13'06.3" - E18°26'06.8"	AY965164
	LAPH03	10km W Leipoldtville	S32°13'06.3" - E18°26'06.8"	AY965165
	LAPH04	10km W Leipoldtville	S32°13'06.3" - E18°26'06.8"	AY965166
	LAPH05	10km W Leipoldtville	S32°13'06.3" - E18°26'06.8"	AY965167
	LAPH06	10km W Leipoldtville	S32°13'06.3" - E18°26'06.8"	AY965168
	LAPH07	10km W Leipoldtville	S32°13'06.3" - E18°26'06.8"	AY965169
	LAPH08	10km W Leipoldtville	S32°13'06.3" - E18°26'06.8"	AY965170
	LAPH09	10km W Leipoldtville	S32°13'06.3" - E18°26'06.8"	AY965171
	LAPH10	10km W Leipoldtville	S32°13'06.3" - E18°26'06.8"	AY965172
	LAPH11	10km W Leipoldtville	S32°13'06.3" - E18°26'06.8"	AY965173
	LAPH12	10km W Leipoldtville	S32°13'06.3" - E18°26'06.8"	AY965174
	LAPH13	10km W Leipoldtville	S32°13'06.3" - E18°26'06.8"	AY965175
	PNPH01	Port Nolloth	S29°14'12.9" - E16°52'01"	AY965176
	PNPH02	Port Nolloth	S29°14'12.9" - E16°52'01"	AY965177
	PNPH03	Port Nolloth	S29°14'12.9" - E16°52'01"	AY965178
	PNPH04	Port Nolloth	S29°14'12.9" - E16°52'01"	AY965179
	PNPH05	Port Nolloth	S29°14'12.9" - E16°52'01"	AY965180
	PNPH06	Port Nolloth	S29°14'12.9" - E16°52'01"	AY965181

	PNPH07	Port Nolloth	S29°14'12.9" - E16°52'01"	AY965182
	PNPH08	Port Nolloth	S29°14'12.9" - E16°52'01"	AY965183
	PNPH09	Port Nolloth	S29°14'12.9" - E16°52'01"	AY965184
	PNPH10	Port Nolloth	S29°14'12.9" - E16°52'01"	AY965185
	PNPH01	Port Nolloth	S29°14'12.9" - E16°52'01"	AY965186
	SKPH01	Sandkop	S29°40'03" - E17°12"13.2"	AY965187
	SKPH02	Sandkop	S29°40'03" - E17°12"13.2"	AY965188
	SKPH03	Sandkop	S29°40'03" - E17°12"13.2"	AY965189
	SKPH04	Sandkop	S29°40'03" - E17°12"13.2"	AY965190
	SKPH05	Sandkop	S29°40'03" - E17°12"13.2"	AY965191
	SKPH06	Sandkop	S29°40'03" - E17°12"13.2"	AY965192
	SKPH08	Sandkop	S29°40'03" - E17°12"13.2"	AY965193
	SKPH09	Sandkop	S29°40'03" - E17°12"13.2"	AY965194
	SKPH10	Sandkop	S29°40'03" - E17°12"13.2"	AY965195
	SKPH11	Sandkop	S29°40'03" - E17°12"13.2"	AY965196
	SKPH12	Sandkop	S29°40'03" - E17°12"13.2"	AY965197
	SKPH13	Sandkop	S29°40'03" - E17°12"13.2"	AY965198
	SKPH14	Sandkop	S29°40'03" - E17°12"13.2"	AY965199
	SKPH15	Sandkop	S29°40'03" - E17°12"13.2"	AY965200
	SKPH16	Sandkop	S29°40'03" - E17°12"13.2"	AY965201
	SKPH17	Sandkop	S29°40'03" - E17°12"13.2"	AY965202
	SKPH18	Sandkop	S29°40'03" - E17°12"13.2"	AY965203
	SKPH19	Sandkop	S29°40'03" - E17°12"13.2"	AY965204
	SKPH20	Sandkop	S29°40'03" - E17°12"13.2"	AY965205
	SKPH24	Sandkop	S29°40'03" - E17°12"13.2"	AY965206
S. (P.) gariepinus	LKPG01	Langhoogte/Kommgas	S29°34'03.5" - E17°24"19.7"	AY965087
	LKPG02	Langhoogte/Kommgas	S29°34'03.5" - E17°24"19.7"	AY965088
	LKPG03	Langhoogte/Kommgas	S29°34'03.5" - E17°24"19.7"	AY965089
	LKPG04	Langhoogte/Kommgas	S29°34'03.5" - E17°24"19.7"	AY965090
	LKPG05	Langhoogte/Kommgas	S29°34'03.5" - E17°24"19.7"	AY965091
	LKPG06	Langhoogte/Kommgas	S29°34'03.5" - E17°24"19.7"	AY965092
	LKPG07	Langhoogte/Kommgas	S29°34'03.5" - E17°24"19.7"	AY965093

LKPG08	Langhoogte/Kommgas	S29°34'03.5" - E17°24"19.7"	AY965094
LKPG09	Langhoogte/Kommgas	S29°34'03.5" - E17°24"19.7"	AY965095
LKPG10	Langhoogte/Kommgas	S29°34'03.5" - E17°24"19.7"	AY965096
LKPG11	Langhoogte/Kommgas	S29°34'03.5" - E17°24"19.7"	AY965097
LKPG12	Langhoogte/Kommgas	S29°34'03.5" - E17°24"19.7"	AY965098
HRPG01	Holgat River	S28°56'15.2" - E16°46"55.4"	AY965113
HRPG02	Holgat River	S28°56'15.2" - E16°46"55.4"	AY965114
HRPG03	Holgat River	S28°56'15.2" - E16°46"55.4"	AY965115
HRPG04	Holgat River	S28°56'15.2" - E16°46"55.4"	AY965116
HRPG05	Holgat River	S28°56'15.2" - E16°46"55.4"	AY965117
HRPG06	Holgat River	S28°56'15.2" - E16°46"55.4"	AY965118
HRPG07	Holgat River	S28°56'15.2" - E16°46"55.4"	AY965119
HRPG08	Holgat River	S28°56'15.2" - E16°46"55.4"	AY965120
HRPG09	Holgat River	S28°56'15.2" - E16°46"55.4"	AY965121
HRPG10	Holgat River	S28°56'15.2" - E16°46"55.4"	AY965122
HRPG11	Holgat River	S28°56'15.2" - E16°46"55.4"	AY965123
HRPG12	Holgat River	S28°56'15.2" - E16°46"55.4"	AY965124
HRPG13	Holgat River	S28°56'15.2" - E16°46"55.4"	AY965125
HRPG14	Holgat River	S28°56'15.2" - E16°46"55.4"	AY965126
HRPG15	Holgat River	S28°56'15.2" - E16°46"55.4"	AY965127
HRPG16	Holgat River	S28°56'15.2" - E16°46"55.4"	AY965128
HRPG17	Holgat River	S28°56'15.2" - E16°46"55.4"	AY965129
HFPG01	Hohenfels	S28°30'29" - E16°36"58"	AY965130
HFPG02	Hohenfels	S28°30'29" - E16°36"58"	AY965131
HFPG03	Hohenfels	S28°30'29" - E16°36"58"	AY965132
HFPG04	Hohenfels	S28°30'29" - E16°36"58"	AY965133
HFPG05	Hohenfels	S28°30'29" - E16°36"58"	AY965134
HFPG06	Hohenfels	S28°30'29" - E16°36"58"	AY965135
HFPG07	Hohenfels	S28°30'29" - E16°36"58"	AY965136
HFPG08	Hohenfels	S28°30'29" - E16°36"58"	AY965137
HFPG09	Hohenfels	S28°30'29" - E16°36"58"	AY965138
HFPG10	Hohenfels	S28°30'29" - E16°36"58"	AY965139

	HFPG11	Hohenfels	S28°30'29" - E16°36"58"	AY965140
	HFPG12	Hohenfels	S28°30'29" - E16°36"58"	AY965141
	HFPG13	Hohenfels	S28°30'29" - E16°36"58"	AY965142
	DOPG01	Daberas to Obib Dunes	S28°08'44" - E16°44"45"	AY965143
	DOPG02	Daberas to Obib Dunes	S28°08'44" - E16°44"45"	AY965144
	DOPG03	Daberas to Obib Dunes	S28°08'44" - E16°44"45"	AY965145
	DOPG04	Daberas to Obib Dunes	S28°08'44" - E16°44"45"	AY965146
	DOPG05	Daberas to Obib Dunes	S28°08'44" - E16°44"45"	AY965147
	DOPG06	Daberas to Obib Dunes	S28°08'44" - E16°44"45"	AY965148
	DOPG07	Daberas to Obib Dunes	S28°08'44" - E16°44"45"	AY965149
	DOPG08	Daberas to Obib Dunes	S28°08'44" - E16°44"45"	AY965150
	DOPG09	Daberas to Obib Dunes	S28°08'44" - E16°44"45"	AY965151
	DOPG10	Daberas to Obib Dunes	S28°08'44" - E16°44"45"	AY965152
	DOPG11	Daberas to Obib Dunes	S28°08'44" - E16°44"45"	AY965153
	KHPG01	Klingharts Mountains	S27°24'18" - E15°37"26"	AY965099
	KHPG02	Klingharts Mountains	S27°24'18" - E15°37"26"	AY965100
	KHPG03	Klingharts Mountains	S27°24'18" - E15°37"26"	AY965101
	KHPG04	Klingharts Mountains	S27°24'18" - E15°37"26"	AY965102
	KHPG05	Klingharts Mountains	S27°24'18" - E15°37"26"	AY965103
	KHPG06	Klingharts Mountains	S27°24'18" - E15°37"26"	AY965104
	KHPG07	Klingharts Mountains	S27°24'18" - E15°37"26"	AY965105
	KHPG08	Klingharts Mountains	S27°24'18" - E15°37"26"	AY965106
	KHPG09	Klingharts Mountains	S27°24'18" - E15°37"26"	AY965107
	KHPG10	Klingharts Mountains	S27°24'18" - E15°37"26"	AY965108
	KHPG11	Klingharts Mountains	S27°24'18" - E15°37"26"	AY965109
	KHPG12	Klingharts Mountains	S27°24'18" - E15°37"26"	AY965110
	KHPG13	Klingharts Mountains	S27°24'18" - E15°37"26"	AY965111
	KHPG14	Klingharts Mountains	S27°24'18" - E15°37"26"	AY965112
S. (P.) denticollis	LAPD11	Luderitz - Agate Beach	S26°41'17.1" - E15°15'50.1"	AY258254
	LTPD12	Luderitz - Agate Beach	S26°41'17.1" - E15°15'50.1"	AY258253
	KPPD01	Koichab Pan	S26°13'02.4" - E15°57"52.9"	AY965218
	KPPD02	Koichab Pan	S26°13'02.4" - E15°57"52.9"	AY965219

KPPD03	Koichab Pan	S26°13'02.4" - E15°57"52.9"	AY965220
KPPD04	Koichab Pan	S26°13'02.4" - E15°57"52.9"	AY965221
KPPD05	Koichab Pan	S26°13'02.4" - E15°57"52.9"	AY965222
KPPD06	Koichab Pan	S26°13'02.4" - E15°57"52.9"	AY965223
KPPD07	Koichab Pan	S26°13'02.4" - E15°57"52.9"	AY965224
KPPD08	Koichab Pan	S26°13'02.4" - E15°57"52.9"	AY965225
KPPD09	Koichab Pan	S26°13'02.4" - E15°57"52.9"	AY965226
KPPD10	Koichab Pan	S26°13'02.4" - E15°57"52.9"	AY965227
KPPD11	Koichab Pan	S26°13'02.4" - E15°57"52.9"	AY965228
NRPD01	Namib Rand	S25°12'52.5" - E16°01'10"	AY965229
NRPD02	Namib Rand	S25°12'52.5" - E16°01'10"	AY965230
NRPD04	Namib Rand	S25°12'52.5" - E16°01'10"	AY965231
NRPD05	Namib Rand	S25°12'52.5" - E16°01'10"	AY258255
NRPD06	Namib Rand	S25°12'52.5" - E16°01'10"	AY258256
NRPD07	Namib Rand	S25°12'52.5" - E16°01'10"	AY965233
NRPD09	Namib Rand	S25°12'52.5" - E16°01'10"	AY965234
NRPD15	Namib Rand	S25°12'52.5" - E16°01'10"	AY965237