Assessing genetic diversity of springtails (Collembola) across the Namib Desert and the potential role of environmental parameters in driving this diversity

by

Janine Rose Baxter

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Supervisor: Prof Don Cowan

Co-supervisors: Prof Ian Hogg

Abstract

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Janine Rose Baxter

Supervisor: Prof. D A Cowan

Department: Genetics

University: University of Pretoria

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Desert environments are characterised by harsh conditions and possess low biodiversity largely caused by abiotic factors such as; low precipitation, soil organic matter, high temperatures, high levels of evapo-transpiration, pH and salinity. These factors significantly reduce primary production, which influences the availability of food resources for deserts organisms. The diversity and the drivers of diversity for below ground invertebrates including Collembola (springtails) are relatively unknown in the Namib Desert. Previous morphological studies have found only five species on the basis of traditional taxonomy. This study assesses the diversity of Namib Desert Collembola and determines the effect of environmental parameters on this diversity,

The diversity of Namib Desert Collembola was assessed using DNA Barcoding. The

sequence information of the 178 Collembola specimens, taken from mitochondrial barcoding using the *Cytochrome-c* oxidase subunit I (COI) gene, was analyzed and Molecular Operational Taxonomic Units (MOTUs) were defined. Collembola community responses to soil physicochemical properties were investigated by using Redundancy Analysis (RDA). MOTUs were successfully indentified to family level (Isotomidae, Neanuridae and Sminthuridae). The researcher found a total of 30 MOTUs, most of which showed limited geographical localisation. The mtDNA COI (barcode) locus revealed high levels of previously unreported genetic diversity of Collembola in the Namib Desert. The RDA indicated that none of the soil physicochemical properties significantly drove variation in Collembola community composition. However, total soil nitrogen was shown to be a strong but not significant driver of variation in community composition ($p<0,054$). The taxonomic identification of the Collembola specimens was also attempted using traditional morphological analysis. A total of 23 individuals, collected from pitfall traps or extracted from soil samples, were selected for identification. Available European keys were used for identification to genus level where possible. A total eight of specimens were identified to genus level (*Folsomides* sp), 14 to family level (Entomobryidae) and one to order level (Symphypleona). Both Symphypleona and Entomobryidae were previously unreported from the Namib Desert. The *Folsomides* genus and the family Entomobryidae were the most abundant groups.

This research suggests that soil dwelling Collembola in the Namib Desert have a much higher level of diversity than previously known. However, the study also highlighted the need for a more comprehensive database for Namib Collembola that includes COI sequence data as well as the morphological identification of species.

Declaration

I, Janine Rose Baxter, declare that this thesis/dissertation 'Assessing genetic diversity of springtails (Collembola) across the Namib Desert and the potential role of environmental parameters in driving this diversity' which I hereby submit for the degree Magister Scientiae (M.Sc.) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any tertiary institution, and all the sources I have used or quoted have been indicated and acknowledged by complete references throughout.

_ _

Janine Rose Baxter

M.Sc. candidate
May 2018

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List of Abbreviations

Abbreviation	Definition
AIC	Akaike information criterion
$NH4^+$	Ammonium
BINs	Barcode Index Numbers
BOLD	Barcode of Life Database
bp	Base pair
CCDB	Canadian Centre for DNA Barcoding
C	Carbon
C/N	Carbon Nitrogen ratio
χ	Chi
Cl^-	Chlorine
COI	*Cytochrome c oxidase* subunit 1
DF	Degrees of Freedom
DNA	Deoxyribonucleic acid
ID	Identity
K2P	Kimura 2-parameter
Mg^{2+}	Magnesium
ML	Maximum likelihood
mtDNA	Mitochondrial DNA
MOTUs	Molecular Operational Taxonomic Units
NJ	Neighbour- Joining
$NO3^-$	Nitrate
N	Nitrogen
P	Phosphorous
K^+	Potassium
KCl	Potassium Chloride
ETP	Potential Evapotranspiration
pH	Potential of Hydrogen
PCA	Principal Component Analysis
RDA	Redundancy Analysis
RESL	REfined Single Linkage algorithm
Na^+	Sodium

sp	Species
S.E.	Standard Error
$SO4^{2-}$	Sulfate
UV	Ultra Violet

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Chapter 1: Dissertation Introduction

Springtails (Collembola) are globally one of the most abundant arthropod groups, they occur across a broad range of habitats, including extreme environments such as deserts and polar regions. Due to their ubiquitous nature and their ecological role as decomposers, springtails are an important functional group in soil (HOPKIN 1997). There are over 8000 described species worldwide belonging to 18 families (BELLINGER *et al.* 2018). This number is likely to represent only a fraction of the actual diversity. Springtails in many regions of the world, such as the tropics, remain understudied due to a lack of expertise and resource limitations. As a consequence the actual number of springtail species worldwide is undoubtedly greater than estimated (DEHARVENG 2004).

Namib Desert fauna are dominated by arthropods. The desert arthropods are highly diversified and abundant. Collembola may play a vital functional role in the Namib Desert soil system, due to their role in soil decomposition and influence on fungal species. They effect decomposition through feeding on fungal hyphae. Grazing of mycorrhizae on plant roots stimulates the growth of the symbiont and improves plant growth (HOPKIN 1997).

The diversity of below ground invertebrates, including Collembola, is relatively unknown in the Namib Desert. Early field studies of Namib arthropods are few and were focused on species distribution and behaviour. These studies included surveys of the micro-arthropods living in the coastal sand dunes (COINEAU AND SEELY 1983) and arthropods associated with *Welwitschia mirabilis* (MARSH 1987). Previous springtail studies listed and described five Collembolan species: *Willemia namibae, Folsomides angularis, Folsomides parvulus*, a Friesea species (THIBAUD AND MASSOUD 1988) and *Cyphoderus colurus* (BÖRNER 1913).

Due to the taxonomic impediment, many springtails remain to be described. DNA barcoding attempts to overcome this gap in knowledge by giving non-experts the tools to identify species. DNA barcoding is a molecular method that uses standardized gene regions to rapidly and accurately assign unknown individual specimens to a species (HEBERT *et al.* 2005). The Cytochrome-*c* oxidase subunit 1 (CO1) gene is the standard gene used for DNA barcoding analysis of invertebrates (HEBERT *et al.* 2005) and has been successfully used for Collembola (HOGG AND HEBERT 2004). In Chapter 3 of this dissertation, we will examine the diversity for Collembola along an environmental transect in the Namib Desert and to

determine which environmental factors drive this diversity. Specifically, we will assess the diversity of Collembola using the mtDNA COI (barcode) locus and then test the hypothesis that certain soil physicochemical factors, such as Carbon Nitrogen ratio (C/N) influences the distribution of taxa along a coastal to inland gradient using a redundancy analysis (RDA).

Previous morphological studies in the Namib Desert have found only five species on the basis of traditional taxonomy. Previous work done since to describe and list species for the region have been scarce. Therefore in Chapter 4 of this dissertation, the research will address this gap in knowledge by presenting a list of Collembola sampled from the gravel plains in the Namib Desert and indentified using morphological characteristics.

The final Chapter (Chapter 5) summarises the findings and suggests future research ideas that could build upon the current body of knowledge gained from this dissertation.

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Chapter 2: Literature Review

2.1 Deserts

Deserts are diverse landscapes, which cover one-third of the earth's land surface (Post *et al.* 1985; Parsons and Abrahams 1994). One of the most defining characteristics of a desert is aridity. Water is integral to the survival of all organisms. The Aridity Index is the measure of aridity, it is calculated as the ratio between mean annual precipitation (P) and mean annual potential evapo-transpiration (PET). PET is the combined loss of water from the water-saturated soil through plant transpiration and evaporation of water from the ground (Thornthwaite 1948). The four main classes of aridity include: the hyperarid zone (P/Etp< 0.03), the arid zone (0.03 < P/Etp<0.20), the semi-arid zone (0.20 < P/Etp< 0.50) and the sub humid zone (0.50 < P/Etp< 0.75) (Parsons and Abrahams 1994). The desert biome can be defined climatologically, biologically and physically. Climatologically a desert is a global region that includes all the arid and hyper-arid areas of the earth (Figure 1.1a). Biologically a desert is the eco-regions that include all desert adapted plants and animals (Figure 1.1b). Physically a desert is large areas of bare soil and low plant cover (Figure 1.1c) (Ezcurra 2006).

Deserts support considerable amounts of plant and animal biodiversity (Polis 1991b). The plants and animals of the desert must adapt and evolve to widespread abiotic pressures characteristic of desert regions. These abiotic factors include UV radiation exposure, extreme temperature fluctuations, xeric conditions, high summer ground surface temperature and irregular rainfall (Cary *et al.* 2010; Jones and Lennon 2010).

Most deserts occur away from coastal areas and in areas where moisture from the oceans seldom reach. However, there are some coastal deserts located on the west coast of continents (Meigs 1973). The Namib Desert on the coast of Southern Africa and the Peruvian-Chilean deserts (Atacama) are coastal fog-deserts, the aridity of these deserts are a result of cold oceanic currents. These deserts form some of the driest ecosystems on earth (Ezcurra 2006). Despite the moist air, rainfall is sporadic and highly localised, but condensation takes place every night. In the Namib Desert, the fog contributes to 150 mm of moisture per year. This condensation is an important source of moisture for living organisms in the Namib Desert, for example the fog-basking beetle (Sømme 1995).

The deserts of the world occur in six Global bio-geographical realms (EZCURRA *et al.* 2006):

- Afrotropic deserts: Sub-saharan Africa
- Australasia deserts: Australia
- The Indo-Malay region
- The Nearctic deserts: North America
- The Neotropic deserts: South America
- Palearctic realm

Deserts have developed in relative isolation. Despite this they are linked to non-desert environments. For example, deserts provide migratory corridors to many species (WEILER 2010). Deserts are important ecosystems for the study of convergent evolution (ORIANS AND SOLBRIG 1977). Convergent evolution is the process whereby distantly related organisms independently evolve similar features, as a result of having similar adaptions to specific environments or niches (STAYTON 2015). An example of convergent evolution occurring in desert organisms is the North American cacti and the African Euphorbia. Both plants possess thick, succulent stems for water storage and spines for protection. These plants may look similar but belong to different families. These two desert plants are well-adapted to the arid conditions of the desert (WIENS 1978). The extreme conditions of deserts have resulted in the evolution of a complex set of relations among desert organisms, mostly positive interactions (e.g. facilitation in plant-plant interaction) (CALLAWAY AND PUGNAIRE 1999; BRUNO et al. 2003). As a result of the extremely arid conditions, the biotic community is relatively simple and processes are more readily identified than in more temperate areas (WALL AND VIRGINIA 1999). Desert shrubs and woody legumes create a microhabitat which is crucial for the survival of other species (SØMME 1995; ANDRÉ *et al.* 1997).

About 2.1 billion people live in desert areas and 5.2 billion hectares of desert land is used for agricultural purposes. Deserts contain 27% of global soil organic carbon reserves and desertification decreases soil carbon sequestering capacity. Increased understanding of desert systems is critical for sustainable land management practices, conservation efforts and water resource planning in regions that are susceptible to continued and future desertification (EZCURRA *et al.* 2006)

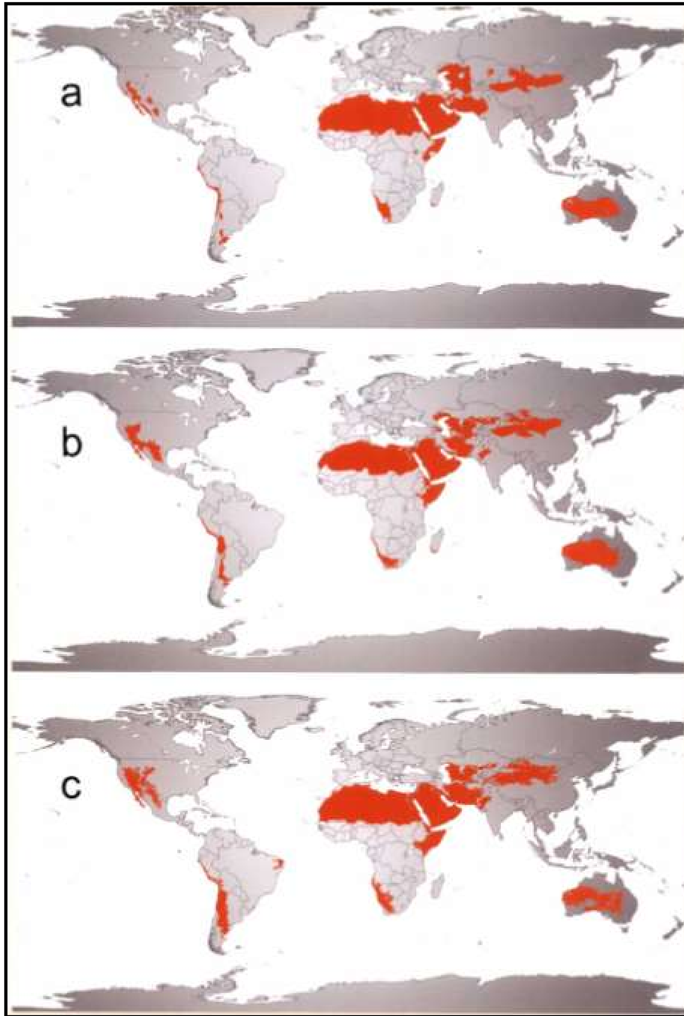

Figure 1.1. The desert biome as defined by: (a) arid and hyper-arid areas of the globe (MIDDLETON AND THOMAS 1992), (b) eco-regions that contain all desert adapted plants and animals (OLSON *et al.* 2001) and (c) as large areas of bare soil and low plant cover (USGS 2005)

2.2 The Namib Desert

The Namib Desert is a juxtaposition of three diverse habitats: The dunes, river and plains and a steep east-west environmental gradient. The Central Namib Desert covers some 30,000 km^2 and is one of the driest deserts in the world. The central Namib Desert occupies an area of gravel plains and sand dunes traversed from east to west by ephemeral watercourses (Figure 1.2.1). The central Namib is covered by gypsum crusts, calcrete and desert pavement (GOUDIE 1970). It is interspersed with weathered rock outcrops and numerous dry riverbeds.

2.2.1 Geography

The Namib Desert is a 2000km long coastal desert along the south-western coast of Africa that covers over 130 000km^2 (Figure 1.2.1). It is thought to be one of the oldest deserts on the planet (WARD *et al.* 1983). The region has unusual geomorphology and climatology and is associated with unique fauna and flora. The Namib Desert soil is dominated by gypsisols (Figure 1.2.2) and has been the focus of biological research since the turn of the century (SEELY AND UNIT 1990).

Figure 1.2.1. A map of Africa. The area coloured red represents Namibia.

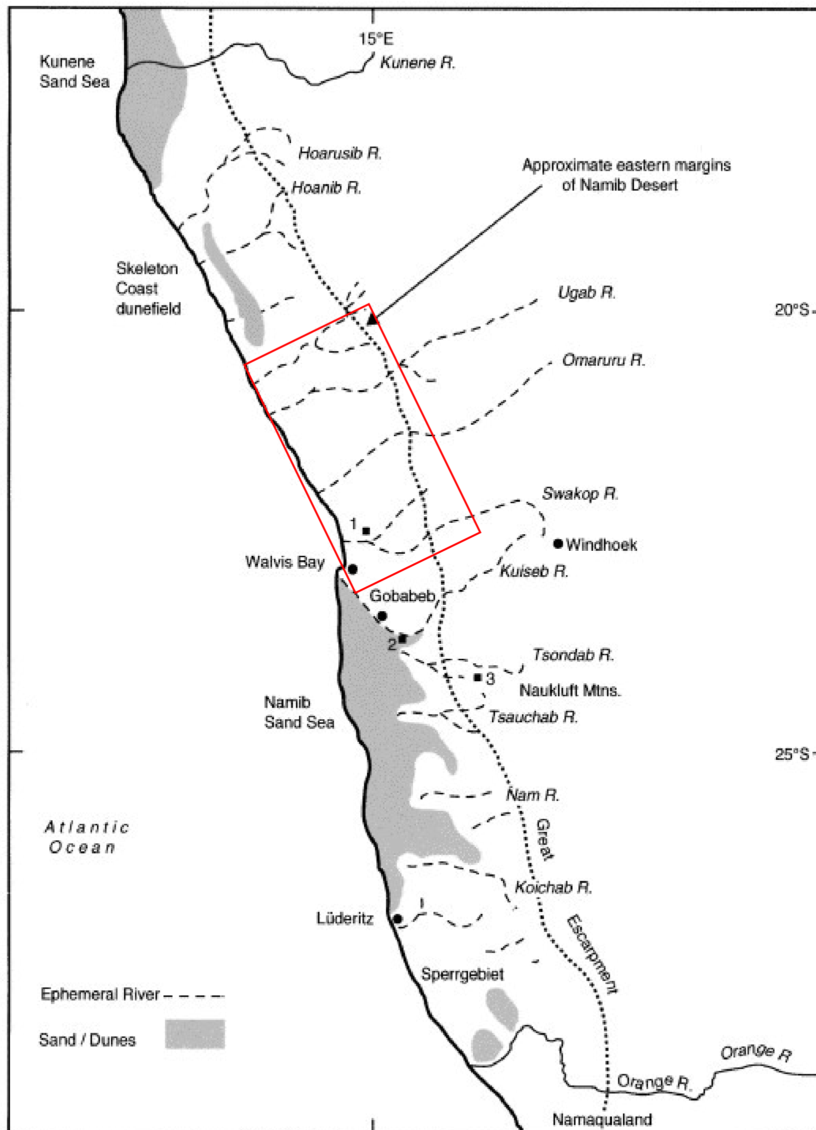

Figure 1.2.2. A map of the Namib Desert. The area within in the red box represents the central Namib. The dashed line indicates the eastern boundary (LANCASTER 2002)

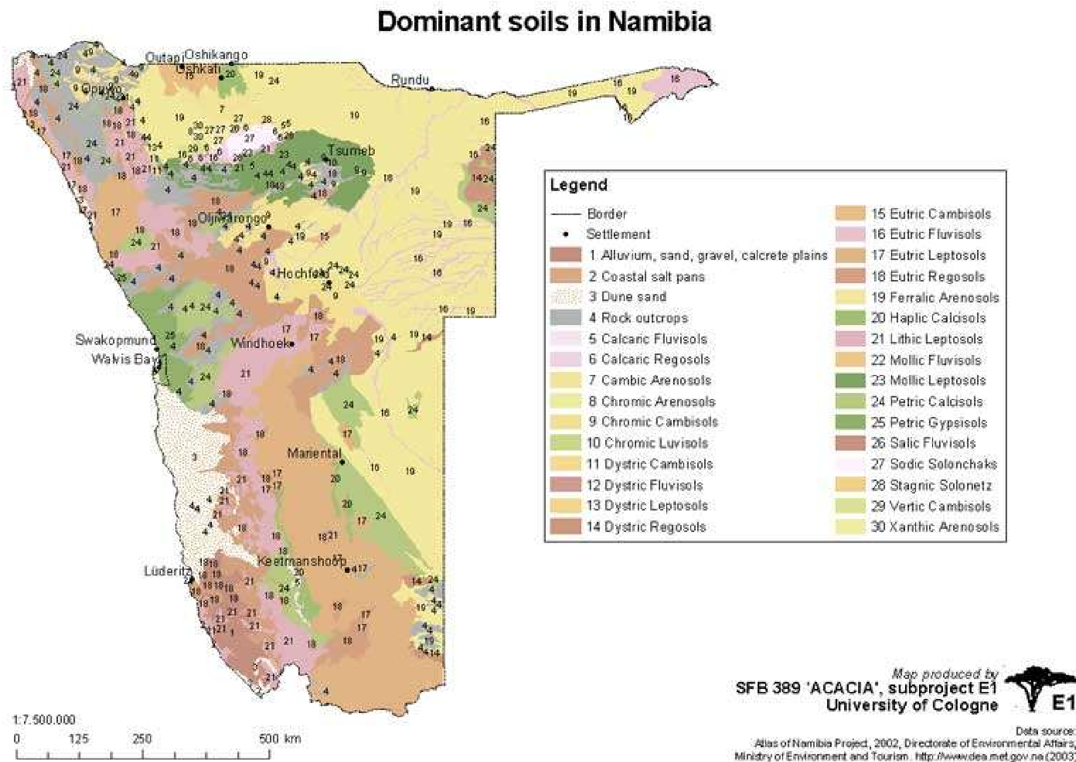

Figure 1.2.3. The major soil types of Namibia. The Namib Desert soil is dominated by petric gypsisols (ATLAS OF NAMIBIA PROJECT 2002)

2.2.2 Climate

The Namib Desert is among the most arid deserts in the world and physical conditions impose severe constraints on the living organisms in the environment. The present hyper-arid conditions of the Namib are thought to have been in place since the upwelling of the Beguela current began 15 million years ago (VAN ZINDEREN BAKKER 1975). The Desert has two sources of moisture that sustains life in the Namib, namely rainfall and fog. The Namib Desert receives an annual rainfall of 25 mm (Figure 1.2.3c) (ECKARDT *et al.* 2013). The central Namib is situated on the south-western edge of the summer rainfall zone and most rain falls during the months January to April (SCHULZE 1969). Rainfall is often sporadic and highly localised and usually occurs as convective showers (SHARON 1981).

Fog-water precipitation is the dominant moisture source over the western parts of the Desert, adjacent to and inland from the coast. It is a distinctive diagnostic characteristic of the climate

of the central Namib. The effects of fog reach 100 km inland. Namib fauna and flora show adaption to the use of fog water, for example the fog-basking beetle (SEELY 1979). Fog-water precipitation increases from the coast inland to a distance of about 35 to 60 km from the sea and then decreases further inland (LANCASTER 1984).

The Namib region shows a similarity in average monthly and yearly temperatures (GOUDIE 1970). Soil temperatures at 120 cm depth were almost consistent throughout the day in every month, whilst the temperatures closer to the surface fluctuate greatly (LANCASTER 1984).

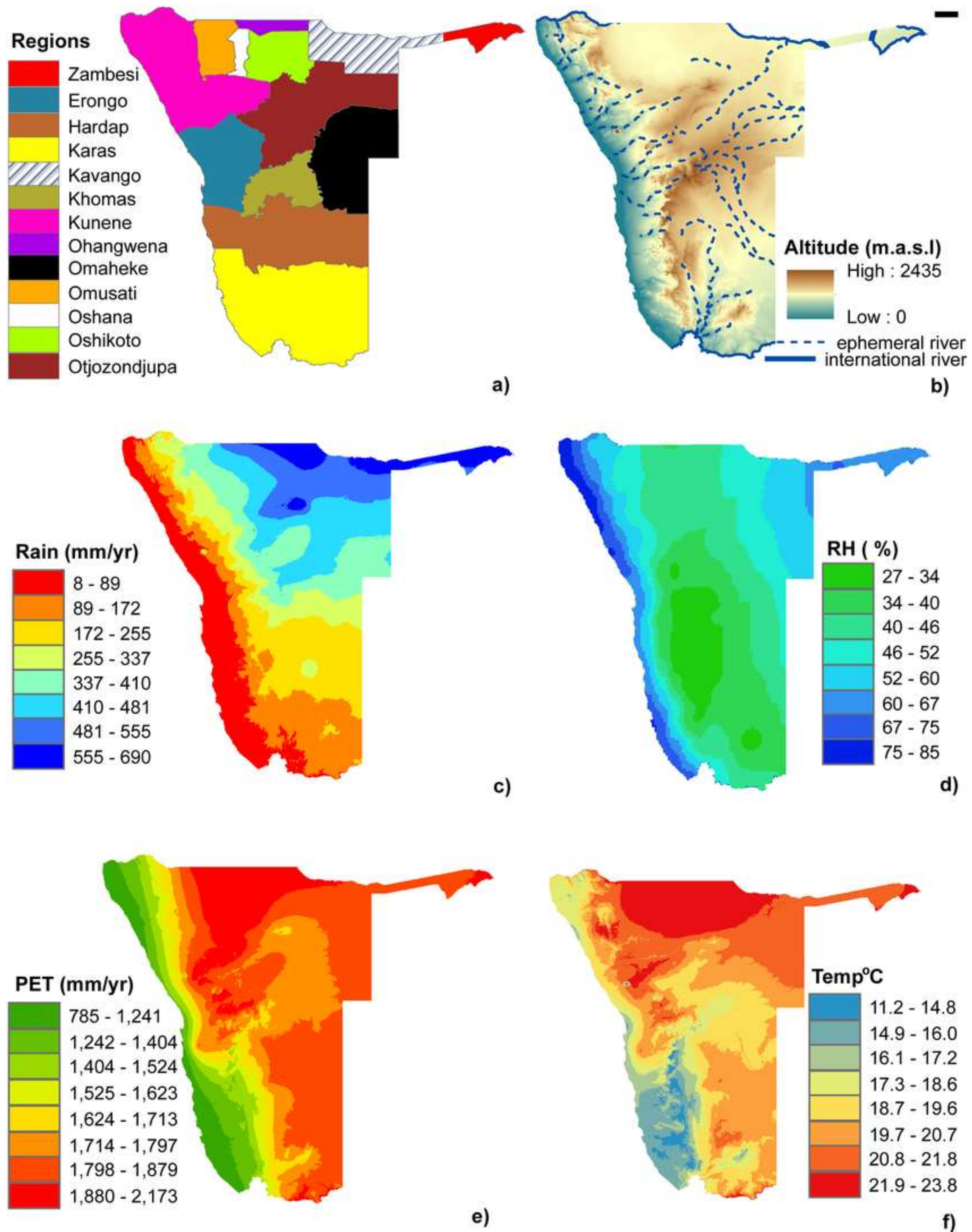

Figure 1.2.4. Namibia's geographical and meteorological data (a) administrative regions, (b) digital elevation model (DEM), (c) mean annual rainfall, (d) mean annual relative humidity (RH%), (e) mean annual potential evapotranspiration (PET) and (f) mean annual temperature. (KASEKE *et al.* 2016)

2.2.3 Vegetation

Namibia's vegetation is strongly influenced by rainfall. In the desert vegetation is sparse and short, characterised by shrubs and grassland (figure 1.2.4).

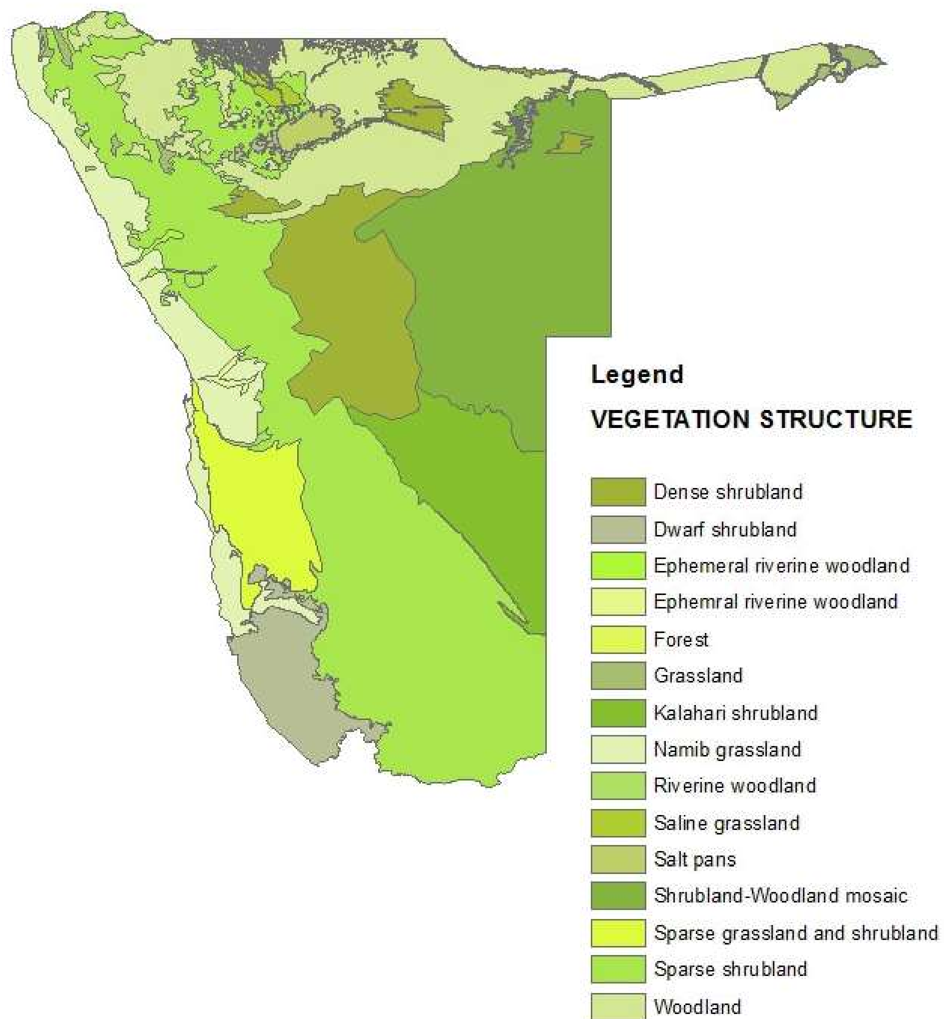

Figure 1.2.5. The vegetation structure of Namibia (ATLAS OF NAMIBIA PROJECT 2002)

2.2.4 Microclimate

In an environment where food is spatially and temporally patchy, the ability to use diverse food types is clearly advantageous. Unpredictable environmental extremes may affect food availability and offspring survival of many desert invertebrates. Plants modify the environment in which they grow by creating shade and accumulating windblown debris, silt and sand. In addition to the litter they themselves produce shade and litter, for example. *Welwitschi mirabilis* (MARSH 1990). Microhabitats associated with desert plants often provide favourable conditions for arthropod activity (CHARLEY AND WEST 1977; SANTOS *et al*. 1978). Microarthropod communities flourish in the soil mound below *Welwitschia* species. Because of the presence of rich food resources (litter), microarthopod communities are able to persist in favourable microclimates (SØMME 1995)

The microclimate is very important in desert environments, it makes it possible for living organisms to seek out refuges to survive the harsh conditions of the desert (HEATHCOTE 1983). Rocks and plants offer shade and conserve humidity forming microhabitats. Desert arthropods inhabit the microhabitats by day when the temperature is high and come out early morning or during the night. In many species, the emergence is timed by circadian rhythms (CLOUDSLEY-THOMPSON 2012). They may also exhibit inter-annual fluctuations in surface activity during favourable seasons or after rainfall, when many desert arthropods become active on the surface (SØMME 1995)

2.3 Soil organisms

Soil organisms play a critical role in ecosystem processes and services (LAVELLE *et al*. 2006). The study of soil organisms is important for conservation research as soil organisms are sensitive to changes in the environment. Changes in their diversity levels could indicate changes in soil quality and have possible implications for the continued functioning of terrestrial ecosystems (DECAËNS *et al*. 2006). Soil fauna accounts for approximately 23% (DECAËNS *et al*. 2006) of the 1.5 million described species on Earth (COSTELLO *et al*. 2013) Many arthropod groups remain to be described and there is expected to be an estimated 6.8 million terrestrial arthropod species (STORK *et al*. 2015). Despite their importance in the soil ecosystem, below ground diversity of soil organisms receive less attention than above ground diversity (DECAËNS *et al*. 2006). The lack of taxonomic identification of these groups is exacerbated by small body size which is common among soil dwelling organisms. As a result the number of formally described soil dwelling species is underestimated (DECAËNS *et al*. 2006; DECAËNS 2010).

In the Namib Desert, springtails occur in significant numbers. The diversity of belowground invertebrates and arthropods, including springtails, is relatively unknown in the Namib Desert.

2.4 Springtails (Collembola)

2.4.1. Introduction

Springtails (Collembola) are recognized as one the most abundant terrestrial arthropods on earth (HOPKIN 1997; DEHARVENG 2004). They are among the oldest and most widespread invertebrates found on the planet (GREENSLADE AND WHALLEY 1986). There is an estimated 8 500 described species worldwide, belonging to 18 families (BELLINGER *et al.* 2018) but the number of species is estimated to be 65 000 species (DEHARVENG 2004; PORCO *et al.* 2014)..

The most characteristic feature of the springtail is the jumping organ or furca. This structure is used to evade predators, but has limited dispersal capabilities (CHRISTIAN 1978; BRACKENBURY AND HUNT 1993; HOPKIN 1997). Soil-dwelling springtails have reduced or absent furca to aid movement through the soil. Springtails occur in a wide range of habitats.

They are predominately soil and leaf litter dwellers and also occur on trees, in forest canopies and on the surfaces of fresh water bodies. Their diet consists mainly of fungal hyphae or decaying plant matter. Springtails play a critical role in soil decomposition and even influence the growth of mycorrhizae and control fungal diseases of certain plants (HOPKIN 1997; RUSEK 1998). Effect on decomposition occurs through Springtails feeding of fungal hyphae. Grazing of mycorrhizae on roots stimulates the growth of the symbiont and improves plant growth.

2.4.2 Classification

Springtails form the largest of the three lineages of modern hexapods. The other two are the Protura and Diplura. Springtails belong to the class Entognatha because they have internal mouthparts (HOPKIN 1997).

The classification is a follows (HOPKIN 1997):

Kingdom: Animalia

Phylum: Arthropoda

Subphylum: Hexapoda

Class: Collembola

2.4.3 Dispersal and migration

Dispersal of springtails can occur by biotic and abiotic means. Springtails can propel themselves by using their furca or by walking around (HOPKIN 1997). Springtails may also be dispersed by using the wind, birds, human activities and even the ocean currents (CRACRAFT 1994). Springtails migrate to seek refuge in microclimates. In the desert, springtails show diurnal movement between the cool surface at night and the leaf litter during the heat of day (HOPKIN 1997).

2.4.4 Distribution and abundance

Springtails have a global distribution, occurring on every continent including Antarctica. Harsh environments (e.g. Namib Desert and Antarctica) are inhabited by few species of springtails, but these niches support a diverse population (HOPKIN 1997). Distribution is not uniform throughout the environment; most species are capable of lateral and vertical movement. Species may also undergo seasonal fluctuations (HALE 1966; BADEJO AND VAN STRAALEN 1993). Habitat preference and distribution is influenced by vegetation type, especially with vegetation in extreme environments. Distribution may also be driven by factors including soil temperature, moisture content, pH, the absence of a leaf litter layer and characteristics of the fungal community (CASSAGNE *et al.* 2003; PALACIOS-VARGAS AND CASTAÑO-MENESES 2003; ROBSON *et al.* 2009; NIELSEN *et al.* 2010; RASCHMANOVÁ *et al.* 2013). Average springtail densities in soils are usually between 10,000 and 30,000 per m^2, but can reach up to several million individuals per cubic meter of soil (RUSEK 1998). Most soils contain millions of Collembola fecal pellets, and nutrients are released from the fecal pellets due to microbe action (VERMA AND PALIWAL 2010)

2.4.5 Interaction with abiotic environment

Springtails are able to occupy these extreme niches because they are capable of utilising physiological and behavioural avoidance strategies. Physiological avoidance strategies include: chemicals in the blood that prevent freezing and making the cuticle less permeable to water, thus reducing transpiration (WORLAND AND BLOCK 2003). Springtails have a certain amount of resistance to desiccation; they are able to reduce the rate of water loss (BELGNAOUI AND BARRA 1989). Resistance to desiccation is regulated by behavioural and physiological means, such as morphological and physiological adaptions, behaviour changes, anhydrobiosis (SØMME 1995) and ecomorphosis (LAURA-REYREL 1990). Distribution is influenced by abiotic factor such as temperature, water and oxygen availability (HOPKIN 1997).

The cuticle of Collembola is composed of hexagonal granules that render it water-repellent and able to float on water (Figure 2.1). The cuticle is covered by a hydrophobic waxy layer and the unusual cuticle pattern allows the Collembola to resist getting wet at elevated pressures and getting wet by different organic liquids (HELBIG *et al.* 2011).

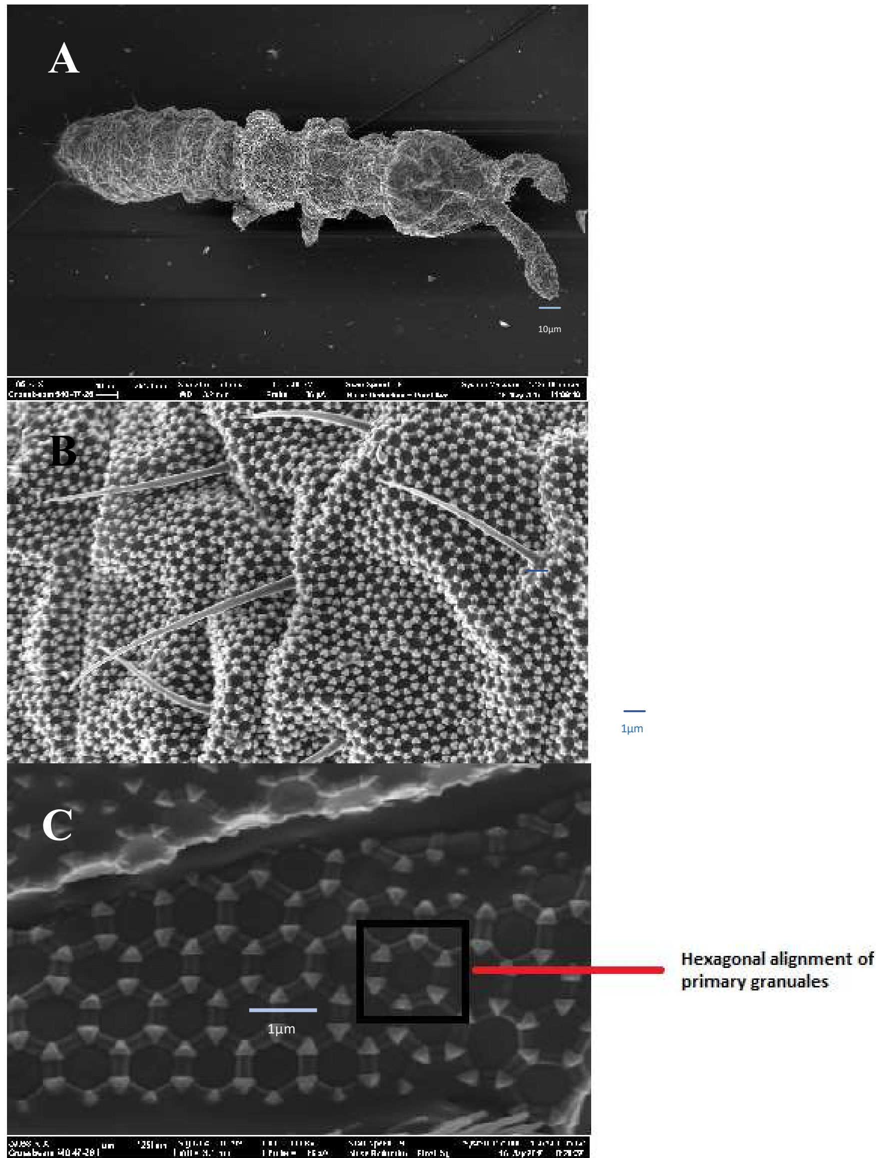

Figure. 2.1. Dorsal SEM image of *Folsomides* sp. (Collembola, Entomobryomorpha, Isotomidae) (A) hexagonal structure on the cuticle, with several sensory setae (B) the unique nanoscopic comb pattern (C). Specimens were viewed with a Zeiss (Carl Zeiss Microscopy, GmbH) CrossBeam 540 Field Emission Scanning Election Microscope.

2.4.6 Roles in decomposition processes

The decomposition process in the soil is influenced by microbes, fungi, protozoa and animals which live in soil and leaf litter habitats (VAN DER HEIJDEN *et al.* 2008). Springtails consume dead vegetation and leave behind partially decomposed faecal pellets. Soil is dense with faecal pellets, it increases the surface area and suitability of material for microbial and fungal attack. Direct grazing of hyphae may inhibit or stimulate fungal growth and may influence the distribution of a particular species (HOPKIN 1997; EISENBEIS AND WICHARD 2012).

2.4.7 Interaction with the biotic environment

Springtails play a role in the redistribution of plant material, fungal spores and bacteria (BEARE *et al.* 1992; LUSSENHOP 1992). Springtails interact with the environment both directly and indirectly. Springtails directly feed on dead vegetation and fungal hyphae, but indirectly stimulate microbes involved in the decomposition process. Few species, such as *Sminthurus viridis*, also known as the 'Lucerne flea', that feeds on plant material of clover fields are considered pests (DAVIDSON 1934). The main predator of Springtails is other arthropods. Interaction occurs between springtails. They can secrete hormones for aggregation at a rich food sources and to attract the opposite sex. Springtails can aggregate to create their own microclimate and it also makes them less prone to desiccation. (HOPKIN 1997).

2.4.8 Morphology and Identification

2.4.8.1 Morphology and Life History

Traditional species identification is challenging especially with the current taxonomic impediment. Species identification based on comparative external morphology may be complicated by character variability. In some species the distribution of pigment is species specific. However in preserved specimens the pigmentation may fade. Pigmentation also correlates with altitude and latitude. Pigmentation darkens as you approach the poles or ascend tall mountains. The darker pigmentation allows for the springtails to warm up faster and become active sooner. Pigmentation also serves as camouflage, protection from UV rays

and correlates with pollution. Individuals of the same species may differ in pigmentation depending on the circumstance. One of the main methods of taxonomic identification is 'chaetotaxy', which involves mapping the distribution of setae on the surface of the cuticle (Figure 2.2) (HOPKIN 1997; HOPKIN 2007).

Defining Springtail species boundaries has many issues: external morphology is a product of genes and environment (e.g. pigmentation of individuals within the same species may differ because of a certain diet, climate conditions or phosphate pollution). Identification is complicated by poorly preserved or damaged specimens and the cost of the expensive equipment, such as the phase contrast microscope, needed for identification. Species of Springtails also undergo epitoky, cyclomorphosis and ecomorphosis (BOURGEOIS 1973; CASSAGNAU AND RAYNAL 1964; CULIK AND NAJT 1986; FJELLBERG 1977 and TAKEDA 1985). Human error also plays a role in misidentification.

Other approaches used to identify Springtail species includes: analysis of chromosome banding patterns (provides information on geographic variation within species and genera) (Figure 2.3) (HOPKIN 1997), allozyme electrophoresis (RICHARDSON et al. 2012), and molecular techniques such as DNA barcoding (HOGG AND HEBERT 2004). Springtail groups with a flexible life history thrive in a wider range of habitats and have a larger geographical range.

Morphology of different Collembolan families may differ in structure. The arrangement of setae on the furca, as well as the shape and number of teeth on the murco (see Figure 2.2), may be used for taxonomic identification. Many more morphological characteristics are used other than the two previously mentioned, such as the arrangement of chaetae on the thorax or abdomen. Characters chosen for species identification may differ widely depending on specimen's order, family or genera. The cuticle also contains small setae, sensory structures and large spines which may also contain smaller spines, the positions of which can be used to separate species (chaetotaxy) (HOPKIN 1997; HOPKIN 2007).

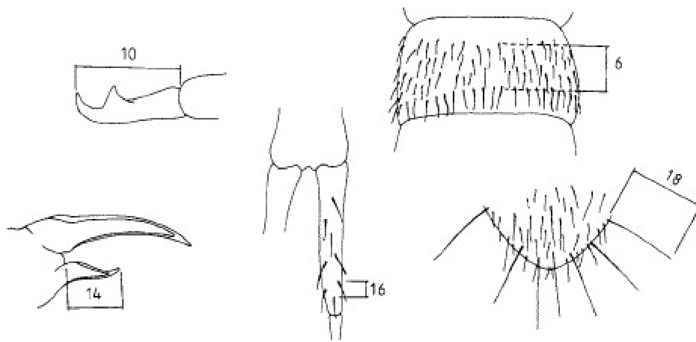

Figure 2.2 Chaetotaxy. An example of useful characteristic used to clarify species boundaries (HOPKIN 1997). Abdominal segment 3 length (6), murco length (10), empodium length on third leg (14), distance between terminal and outer subterminal dental setae (16), abdominal segment 6 terminal macroseta length (18) (WETTON 1987).

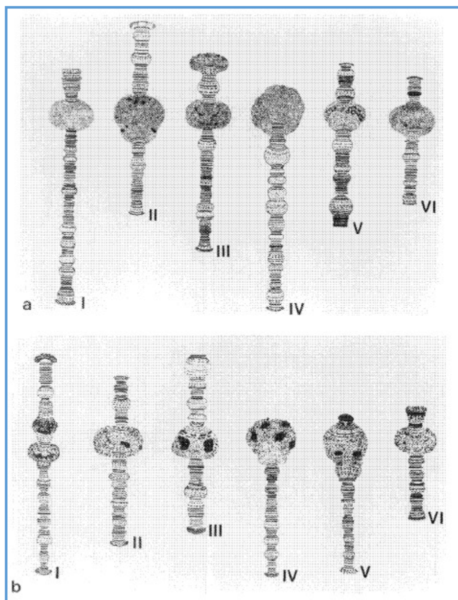

Figure 2.3 Diagram of kayotype I (a) and karyotype II (b) of chromosomes of the salivary glands of Neauridae species (HOPKIN 1997)

2.4.8.2 DNA Barcoding

Assessing biodiversity is important for ecological studies and determining areas of key importance for conservation (WILSON 1985; HEBERT *et al.* 2003). Until recently we have relied on morphological identifications to assess diversity, but this has become an issue since assessing diversity on a global scale is not feasible, due to the time and effort required to identify species using morphological characteristics (VALENTINI *et al.* 2009).

To address this issue, molecular techniques for assessing diversity have been suggested as a solution. One such technique, DNA barcoding, is a molecular method that uses standardized gene regions to rapidly and accurately assign unknown individual specimens to a species (HEBERT *et al.* 2003; HEBERT *et al.* 2005). It uses a 658bp fragment of the mitochondrial Cytochrome-*c* oxidase subunit I (COI) gene. These gene regions are variable enough to distinguish between species and conserved enough to detect members of the same species. This method allows routine identification of organisms by non experts (VALENTINI *et al.* 2009).

The Barcode of Life Database (BOLD) only has about 170,000 formally recognised animal species. The addition of new species to the database is outpacing the addition of classically named species to the database. As a result the queried sequences may not always be able to be identified to species level. DNA sequences submitted to BOLD may be clustered algorithmically to barcode index numbers (BINs), to generate Molecular Operation Taxonomic Units (MOTUs), which act as putative species (RATNASINGHAM AND HEBERT 2007).

Species descriptions for springtails are constrained by the difficulties in defining species boundaries; for example pigmentation of individuals within the same species may differ because of diet or climate. Springtail species also undergo epitoky, cyclomorphosis and ecomorphosis (HOPKIN 1997). Due the taxonomic impediment, many springtails remain to be described. DNA barcoding attempts to overcome this gap in knowledge by giving non-experts the tools to identify species.

2.5 Conclusion

The literature above highlights the high diversity of Collembola worldwide and the fact that Collembola in the Namib Desert are greatly understudied. It is therefore important for a rapid and efficient method of diversity assessment and species identification. Traditional morphological techniques of identification are often slow and difficult. Therefore there has been an increase in the use of molecular-based approaches as an alternative to traditional taxonomy for the delineation of life (HEBERT *et al.* 2003).

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Chapter 3: The Environmental Parameters That Drive the Genetic Diversity of Collembola (Springtails) Across an East-West Namib Desert Transect

3.1 Abstract

Desert environments are characterised by harsh conditions generally thought to possess low biodiversity largely driven by abiotic factors. Low precipitation and soil organic matter, as well as high temperatures, evapo-transpiration, pH and salinity, significantly reduced primary production, which in turn influences the availability of food resources for deserts organisms. The diversity and the drivers of diversity for below ground invertebrates including Collembola (Springtails) are relatively unknown in the Namib Desert. Previous morphological studies have found only five species on the basis of traditional taxonomy. Knowledge of the effects of soil physicochemical properties on Collembola community composition in the Namib Desert is also limited.

Collembola were sampled and soil physicochemical data collected along an east to west environmental gradient. The sequence information of the 178 Collembola specimens using the mitochondrial barcoding COI (Cytochrome-*c* oxidase subunit I) gene was analyzed and Molecular Operational Taxonomic Units (MOTUs) were delineated. Collembola community responses to soil physicochemical properties were investigated by using Redundancy Analysis (RDA). MOTUs were only successfully identified to family level (Isotomidae, Neanuridae and Sminthuridae) due to the fact that there was insufficient data in the BOLD database regarding Collembola in the Namib Desert. We found a total of 30 MOTUs and most MOTUs were geographically localised. The mtDNA COI (barcode) locus revealed high levels of previously unknown genetic diversity of Collembola in the Namib Desert, as well as limited dispersal of MOTUs. None of the soil factors were significant drivers of Collembola diversity along the Namib Desert transect.

3.2 Introduction

The class Collembola are globally one of the most abundant arthropod groups, they occur across a broad range of habitats, including extreme environments such as deserts and polar regions (SØMME 2012). Due to their ubiquitous nature and their ecological role as decomposers, springtails are a vital functional group in soil (HOPKIN 1997). There are over 8000 described species worldwide belonging to 18 families (BELLINGER *et al.* 2018). However, this number is likely to represent only a fraction of the actual diversity. Springtails in many regions of the world, such as the tropics, remain under-collected due to expertise and resource limitations. As a consequence the actual number of springtail species worldwide is undoubtedly greater than estimated (DEHARVENG 2004). Porco *et al.* (2014) estimated the level of global diversity to be approximately 65000 species. This number was determined by applying the ratio of described and unknown species of the whole animal kingdom (MORA *et al*. 2011).

Deserts form the largest terrestrial ecosystem and hot deserts cover more than ¼ of the world's terrestrial surface (EZCURRA 2006). Deserts are characterised by low precipitation and soil organic matter, as well as high temperatures evapo-transpiration, pH and salinity (WALL AND VIRGINIA 1999). Low soil water content and soil N, C and P limit primary productivity in arid systems (WEST AND SKUJINS 1978; LAJTHA AND SCHLESINGER 1988). These inhospitable conditions result in significantly reduced primary production, which influences the availability of food resources for deserts organisms. Due to these harsh conditions it is generally thought that deserts possess low biodiversity largely driven by abiotic factors (SØMME 1995) although it has now been shown that deserts support unexpected diverse fauna and flora, including a large number of microbial taxa (POLIS 1991). Collembola cope with these extreme conditions, such as low water availability and low plant cover, by seeking out refuges in microhabitats (ANDRÉ *et al.* 1997). One of the most commonly studied of these microhabitats is the hypolithic environment under translucent rocks (quartz). These rocks offer a more stable environment that offers increased water availability and protection from UV flux (POINTING *et al.* 2007; COWAN *et al.* 2010; WONG *et al.* 2010). Hypolithic communities in extreme desert environments are sites of primary productivity (TRACY *et al.* 2010) and are major contributors of nitrogen and carbon input (COCKELL AND STOKES 2004; COWAN *et al.* 2011). Microhabitats associated with desert

plants, such as shrubs, provide favourable environments for Collembola by creating shade and the accumulation of leaf litter, which is a rich food resource (MARSH 1990).

The Namib Desert is characterised by unusual geomorphology and climatology transversed by a well-established and documented east to west fog-rain gradient (SEELY 1990b). Rain occurs as convective summer rain of the eastern part of the desert, while fog provides moisture over the western part of the desert (SEELY 1990a). Namib Desert fauna are dominated by arthropods; the desert soil arthropods are highly diversified and abundant (SØMME 2012). Collembola play an important functional role in the Namib Desert soil system, due to their role in soil decomposition and influence on fungal species. They effect decomposition through feeding of fungal hyphae. Grazing of mycorrhizae on plant roots stimulates the growth of the symbiont and improves plant growth (HOPKIN 1997).

Early fields studies of the Namib are few and were focused on species distribution and behaviour. These studies included surveys of the micro-arthropods living in the coastal sand dunes (COINEAU AND SEELY 1983a) and arthropods associated with *Welwitschia mirabilis* (MARSH 1987a). A previous springtail study listed and described five collembolan species: *Willemia namibae; Folsomides angularis; Folsomides parvulus* a *Friesea species* (THIBAUD AND MASSOUD 1988) and *Cyphoderus colurus* (BÖRNER 1913).

Species descriptions for springtails are complicated by the difficulties in defining species boundaries; for example pigmentation of individuals within the same species may differ because of diet or climate. Juveniles also may differ from adults in chaetotaxy and in the morphology of certain characteristics. Species of Springtails also undergo epitoky, cyclomorphosis and ecomorphosis (HOPKIN 1997). DNA barcoding is thus a benefit for Collembola indentification, as it does not rely on these morphological characteristics for species description. Due to the taxonomic impediment, many springtails remain to be described. DNA barcoding attempts to overcome this gap in knowledge by giving non-experts the tools to identify species. DNA barcoding is a molecular method that uses standardized gene regions to rapidly and accurately assign unknown individual specimens to a species (HEBERT *et al*. 2005). It is not restricted by the limitations intrinsic in morphology based identification systems, such as phenotypic plasticity and genetic variability, and morphological cryptic taxa (HEBERT *et al*. 2003). The Cytochrome-*c* oxidase subunit 1 (CO1) gene is the standard gene used for DNA barcoding analysis of invertebrates (HEBERT

et al. 2005). By choosing a standard DNA fragment, various research groups can cooperate and construct a comprehensive library of DNA sequences (CATERINO *et al.* 2000).

Collembola are abundant and almost ubiquitous in soils, because of this they have been proposed as soil quality indicators (CORTET *et al.* 1999; SANTAMARÍA *et al.* 2012). Collembola are particularly sensitive to environmental changes, such as heavy metal contamination and soil physicochemical properties (ANDRÉ *et al.* 1997; LOCK *et al.* 2003). However, the knowledge about the effects of soil physicochemical properties on Collembola diversity in the Namib Desert is limited. Previous studies conducted in the Namib Desert on soil arthropods (predominantly mites and Collembola) inhabiting three habitats: the river banks and bed, the dunes and the gravel plain, has shown that soil properties explained less than 40% of variation in density, with three important factors explaining micro-arthopod richness, C/N ratio and K and Na cat-ion concentration. The K and Na cat-ion concentrations explained 69% of mite richness (ANDRÉ *et al*. 1997).

The overall aim of this study was to provide an assessment of the diversity for Collembola along an environmental transect in the Namib Desert and to determine which environmental factors drive this diversity. Specifically, we assessed diversity of Collembola using the mtDNA COI (barcode) locus and then tested the hypothesis that soil physicochemical factors influenced the distribution of taxa along a coastal to inland gradient using a redundancy analysis (RDA).

3.3 Materials and methods

3.3.1 Study area

The study was performed in the Namib Desert gravel plains along a 120km East-West transect (Figure 1a and Figure 1b). The gravel plains soil is classified as ultra-fine sand particles 50-100µm (14-30%) and 100-200µm (27-42%) (ANDRÉ *et al.* 1997). The study site is characterised by sparse vegetation cover of grassland and shrub-land. The study area encompassed three regions across latitude S23 00.064 –S 23 14.685 and longitude E14 40.2995 – E 16 08.563. These regions were classified as a fog-dominant region in the west (Sites 2-6), a central hyper-arid region (Site 8-14) and a rain region in the East (Site 16-20). Even though the moisture gradient across the transect was not significant, it provided the

opportunity to investigate patterns of Collembola diversity across the Namib Desert and whether this diversity is driven by soil physicochemical factors.

3.3.2 Sample Collection

Soil samples were collected on 12 April 2016, across a 120km east - west transect in the Namib Desert, for a total of 10 sites (Table S1). At each site approximately three sub-surface soil samples were collected (no more than 15cm deep) from around and under shrubs or quartz rocks. In some cases the whole shrub was included with the soil sample. Samples were kept in sealed plastic bags and stored at -20°C and processed within a week of collection via floatation extraction (RAW 1955). Specimen collection was done under a Stemi 2000 ZEISS stereo microscope, as specimens are very small. Images of each specimen were taken using an AxioERc5s ZEISS camera attached to a Stemi 2000 ZEISS stereo microscope. Images were used to provisionally designate springtail specimens to family level and confirmed based on the results from the BOLD ID engine. All specimen images, sequence data and collection data were uploaded to the Barcode of Life Data Systems (BOLD) system (http://v4.boldsystems.com) under the project name, Arthropods of the Namib Desert (project code: NAMIB). Individual springtails were placed in 96-well plates and preserved in 99% Ethanol. The plates were sent to the Canadian Centre for DNA Barcoding (CCDB), University of Guelph for sequencing of the COI barcode region.

3.3.3 mtDNA analysis

DNA extraction, amplification and sequencing was carried using standardized protocols (IVANOVA *et al.* 2005). A 658bp region of the mtDNA COI gene was PCR amplified using primer pairs C_LepFolF and C_LepFolR or LepF and LepR (RATNASINGHAM AND HEBERT 2007). COI sequences of greater than 631bp were generated. Sequences were verified as belonging to Collembola by using the BOLD ID engine and BLAST search engine. Only barcode compliant sequences were used for analysis. The sequences were aligned in Geneious v8.0.5 using the MUSCLE algorithm (KEARSE *et al.* 2012) and were trimmed to 590bp. The sequences were trimmed to remove data from the ends of the sequences that were bad quality and to ensure that all the sequences in the final alignment were of the same length. Sequences were compared to sequences in GenBank and the BOLD database to identify species.

The sequences were aligned to a reference mitochondrial genome *of Cryptopygus antarcticus* (GenBank accession number NC_010533.1) and translated to check for stop codons and indels. Unique sequences were verified manually by individually checking each trace file. χ^2 tests were employed in PAUP4 (SWOFFORD 2002) to determine whether base frequencies were equal for all sites, parsimony informative sites and for 3rd codon positions.

Duplicate sequences were removed from the alignment. The remaining 91 sequences were used for phylogenetic analysis. The sequence divergence (K2P) for the COI gene was calculated within and between putative species (MOTUS) using MEGA7 (KIMURA 1980). Although p-distances are preferred for DNA Barcoding studies, K2P and p-distances generate similar values and the more commonly used K2P distance values were used for comparison to other studies (COLLINS *et al.* 2012; SRIVATHSAN AND MEIER 2012). A simple Neighbour-Joining (NJ) tree was created in order to visualise the patterning of divergences among species (SAITOU AND NEI 1987) (Figure S1). The appropriate substitution model of evolution was estimated in Jmodeltest and selected based on the Akaike Information Criterion (AIC) (POSADA 2008). A Maximum Likelihood (ML) tree was also constructed in MEGA7. Bootstrap replicates (n=1000) were performed to assess support for the phylogenies estimated by both NJ and ML analysis.

GenBank and BOLD were searched for COI sequences of springtail species that had previously been reported from the Namib Desert. A single *Folsomides parvulus* (JN981069) sequence was found in the NCBI database and used in the analysis. No sequences for *Willemia namibae* sequences were found. Three sequences from the NCBI database (KF641935, KJ155514 and KR115812) and one sequence from the BOLD database (BOLD:AAN6560) representing five families (Neanuridae, Sminthuridae, Isotomidae, Dicyrtomidae, Tomoceridae and Hypogastruridae) were used as outgroups to root topology.

The BOLD ID engine was used to identify sequences to species level. Sequences did not closely match any previously available on BOLD (<85% similarity in all cases). DNA barcoding was instead used to delineate MOTUs (FLOYD *et al.* 2002; BLAXTER 2004). Sequences were assigned to BINs using the REfined Single Linkage algorithm (RESL). It has been shown that there is a close congruence between Barcode Index Numbers (BINs) and traditional morphological taxonomy (RATNASINGHAM AND HEBERT 2013). Throughout the rest of this study, BINs will be referred to as MOTUs.

Figure 3.1 a) Namibia, b) and the location of the sampling areas (sites 2-20) in the Namib Desert

3.3.4 Chemical analysis

The soil physicochemical data was consistent over the three sampling years and this data was used for the current study. Soil analyses were performed at the Soil Science Laboratory of the University of Pretoria. Eleven soilparameters were measured (Total N, pH, NH4, NO3, P, Na, K, Ca, Mg, Cl and SO4).

Soil pH was measured in 10g slurries (1:2.5 soil/deionized water) with a pH meter (Crison basic 20, Barcelona, Spain; ECKERT AND SIMS 1995). Soil nitrate (NO_3) and ammonium (NH^{+4}) were determined by extraction (2M KCl) and steam distillation with subsequent

titration performed as described by KEENEY AND NELSON (1982). Soil total carbon percentage (C) was measured by oxidizing the organic material with chromic acid and titrating the excess dichromate (WALKLEY 1935; NELSON AND SOMMERS 1982). A subsample was ground to pass through an 80 mesh screen for organic C (OC) and total N analysis by dry combustion (NELSON AND SOMMERS 1982). Ammonium acetate extraction was also used to measure salt concentrations (Na, K, Mg, and Ca) using inductively coupled plasma atomic emission spectroscopy (ICP-OES) (Spectro Genesis, Spectro Analytical Instruments GmbH, Germany). The Bray-1 method (BRAY AND KURTZ 1945) was used to quantify extractable phosphorous (P) (RONCA *et al.* 2015).

3.3.5 Statistical analyses

A Redundancy Analysis (RDA) was used to determine whether soil physicochemical properties could explain variation in Collembola community composition. RDA combines regression and principal component analysis (PCA). RDA was performed in R (TEAM 2015) using the "vegan" package (OKSANEN *et al.* 2007). Molecular Operational Taxonomic Units (MOTUs) were used in the place of species, since this study lacked species-level identification. The RESL algorithm was used to determine Molecular Operational Taxonomic Units (MOTUs) (RATNASINGHAM AND HEBERT 2013). An MOTU abundance matrix was compiled. The matrix is a table where the columns represent MOTUs and rows represent sampling sites. The presence or absence and abundance of each MOTU was recorded for each site. Soil chemistry data was log-transformed and MOTU abundance matrix was Hellinger transformed to normalize data prior to analysis (LEGENDRE AND GALLAGHER 2001).

3.4 Results

A total of 241 specimens collected from soil samples along the Namib east-west transect were sent for COI sequencing. 178 sequences were including in the analysis (Table S2, Supporting Information). Sequences that were flagged (contaminated) or were not barcode compliant were excluded from the analysis. No insertion or deletions were detected. No springtails were found in soil samples collected from Site 4 (C14-04); only one springtail

specimen was collected from Site 2 (C14-02). More springtails were collected at sites 14 and 20 (42 and 49 samples respectively) than the other sites. Thirteen specimens were collected in the fog zone (site 2-6), 87 specimens from the hyper-arid zone (site 8-14) and 78 specimens from the rain zone (site 16-20). The final alignment contained 178 sequences trimmed to 590bp representing three families of Collembola (Table S2). The nucleotide composition of the COI gene averaged across 178 sequences shows an A-T bias, which is common for insect mitochondria (Table 3.1) (BROWN 1985). Base frequencies were homogenous across all sites (χ^2=219, DF=531, P=1.00), as well as at the 1st codon position (χ^2= 55.530614, DF=531, P=1.00) and 2nd codon position (χ^2= 3.055100, DF=531, P<0.001). However, homogeneous base frequencies were rejected at the 3rd codon position (χ^2= 760.933290, DF=531, P=1.00).The 590bp alignment consisted of 247 parsimonious informative sites, 22 variable characters which are parsimony informative and 322 constant characters.

Table 3.1. The nucleotide base composition averages (%) of 91 unique Collembola sequences

	Nucleotides				A+T content	G+C
	A(%)	C (%)	G(%)	T (%)	(%)	(%)
All sites	28,1	19,6	17,5	34,8	62,9	37,1

Ninety-one haplotypes were designated for the 178 sequenced individuals and all haplotypes were assigned a BIN designation (see supplementary Table 1 for BIN designations). Haplotypes ranged from 1 to 49 per location and each haplotype was specific to a single population with no sites sharing haplotypes. Haplotype divergence ranged from 0.2% to 35.9%, with an average divergence of 18.3%, resulting in 39 amino acid changes (Table S3). The most divergent haplotype is hap 3 (Neanuridae) found at site 18. The most abundant haplotype was hap15 (Isotomidae) which contained 15 individuals collected from site 20.

The appropriate model of evolution selected was HKY+I+G (-InL=6633.5792 (AICc), Ti/Tv = 5.6586, I= 0.505, Gamma shape, 0.6920, with base frequencies at A= 0.3498 C= 0.1788 G= 0.1049 T= 0.3666).

The BIN algorithm resolved 30 MOTUs from the 178 sequences that were genetically distinct from all others, but lacked species-level identification. These MOTUs were treated as putative species (defined as MOTU 1-30 in Table S2). Sampling sites 6, 8, 10, 12 shared three MOTUs (MOTU 1, 3 and 14). MOTU 14 was shared between sites 6, 8 and 10. MOTU 3 and 14 were shared between sites 8 and 12 (refer to Figure. 2). The MOTUs were only successfully identified to family level, due to the fact that there was insufficient data in the BOLD database regarding Collembola of the Namib Desert and BLAST in GenBank could not narrow down any close relationship to genera. This can be resolved by the presence of a comprehensive library of DNA barcode sequences linked to taxonomically identified voucher specimens. Three individuals were identified as Neanuridae (haplotypes 1-3), one individual was identified as Smithuridae (haplotype 4) and the rest were identified as Isotomidae.

Sequence divergence was calculated within (Table S4a) and between MOTUs (Table S4b). Within-MOTU COI sequence divergence ranged from 0 – 1.74% with a mean (±SE) of 1.03% ± 0.13%. Between-MOTU levels of divergence ranged from 1.6 – 35.8% with a mean (±SE) of 18.8% ±0.3%.

The most divergent MOTU was MOTU 30 which had a mean sequence divergence > 28.5% (range 28.5-33.9%) from other MOTUs. Most of the MOTUs displayed an inter-specific divergence level more than 2% sequence divergence. The exceptions included three MOTU pairs, MOTU 13-16, MOTU 13-15 and MOTU 14-15, which showed divergence range of 1,6%-1,9%. In all cases the MOTU pairs occurred in closely related groups.

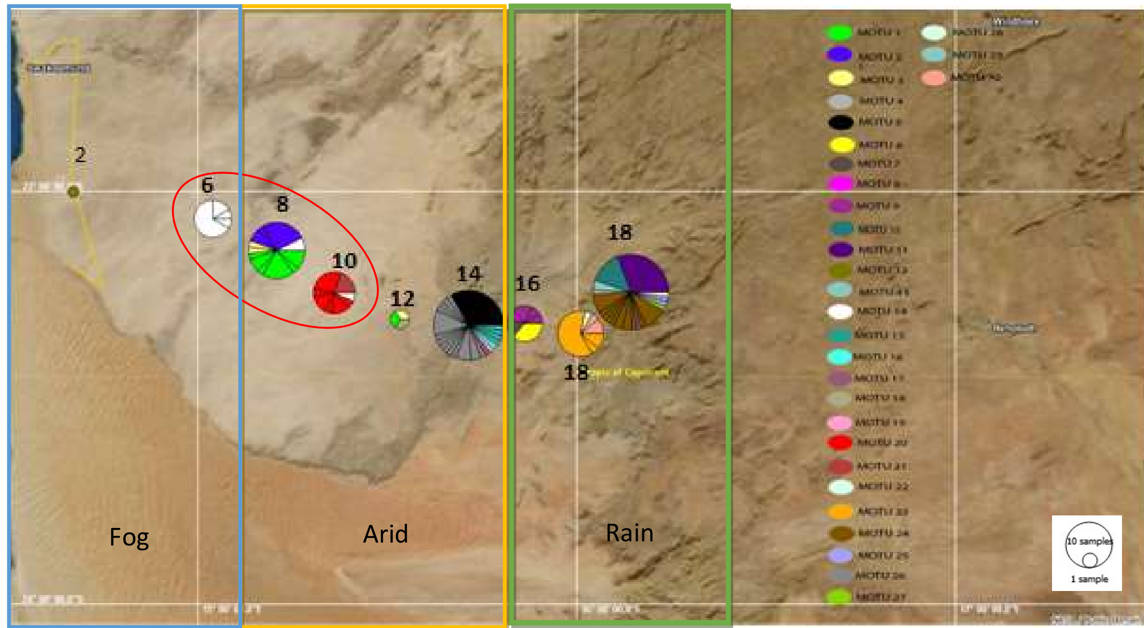

Figure 3.2. A geographical representation of the sampling localities, the occurrence and abundance of each MOTU at each of the sites (Site 2- 20). The size of the circles are proportional to the amount of individuals at each sampling site. The red oval encompasses the sites that had shared MOTUs

The NJ (Figure S1.) and ML (Figure 3.) trees were mostly consistent in the placement of into seven divergent groups. The ML tree representing the whole data set is provided by Figure 3. The only disagreement was in the placement haplotype 7 (hap7), which belonged to MOTU 27. Only one sequence was available for MOTU 27. It is likely that the inconsistencies of its placement within the phylogenetic trees were as a result of insufficient data. The phylogenetic tree showed shallow intraspecific divergence and deep interspecific divergence and showed no indication of possible cryptic speciation; intraspecific divergence levels never exceeded 2%. The NJ and ML analysis separated the haplotypes into seven major groups (A-G). Group A contained MOTU1-9, Group B contained MOTU 10-11, Group C MOTU 12-14, Group D MOTU 15-20, Group E MOTU 21-22, Group F MOTU 23-26 and Group G MOTU 29-30. The main portion of the dataset is comprised of Isotomidae, which cluster separate from the three Neanuridae individuals hap 1-3 (MOTU29 and 30), one Smithuridae hap 4 (MOTU28) and one Isotomidae (hap 7) individual. There was strong bootstrap support (>74%) for the groups, except Group C which had low support (25,9%), possibly due to the placement of the highly divergent hap 48. There was strong bootstrap support (100%) for the placement of the Neanuridae individuals as basal to all other individuals. Group G which contained the Neanuridae individuals, hap 1, 2 and 3, which had the same unique 24 amino acid changes (Table S3).

Sequence divergence was calculated within (Table S5a) and between (Table S5b) groups. Within-group sequence divergence ranged from 3,7%-19,1% with a mean (±S.E.) of 8,77% ± 0.942%. Between-group divergence levels ranged from 16,6%-33,8% with a mean (±S.E.) of 23,91% ±1,89%. Group E had the most within group divergence (19,11%). Group G was the most divergent group from all other groups, Group G was also the most distal group.

For the defined environmental regions (fog, rain and arid), within-region divergence ranged from 7,7%-18,6%, with a mean (±S.E.) of 14,13% ± 1,03%. The rain region had the most within-region divergence. Between-region ranged from 19,4-23,4% with a mean (±S.E.) of 21,13% ± 1,43%. The fog region was the most divergent region from the other regions.

The groups were not region specific (fog, arid or rain region). For example Group B is a mixture of individuals from the fog, rain and hyper arid regions. The remainder of the groups are a mixture of individuals from rain and hyper arid region. This possibly shows that the

species in this study are mostly generalist species, able to survive in a wide range of environmental conditions and use a variety of different resources.

The analysis yielded eight canonical axes, eigenvalues labelled RDA1-RDA8 and one additional unconstrained axis for residuals (PC1). The first 2 axes explained 33,94% (18,91% and 15, 03% respectively) of variation. The first axis was positively correlated with pH, P, Ca and SO4. The first axis was negatively correlated with N, NH4, NO3, Na, K, Mg and Cl. The RDA indicated that none of the soil physicochemical properties significantly drove variation in Collembola community composition. However, total soil N was shown to be a strong but not significant driver of variation in community composition ($p<0,054$). MOTU1-MOTU6, MOTU10-MOTU12, MOTU14-MOTU18, MOTU20, MOTU21, MOTU23-26 and MOTU28-30 were positively correlated with N. MOTU7-9, MOTU27 were negatively correlated with N. MOTU28 belongs to the family Sminthuridae and MOTU29-30 belong to the family Neanuridae. The rest of the MOTUs belong to the family Isotomidae (see appendix 2).

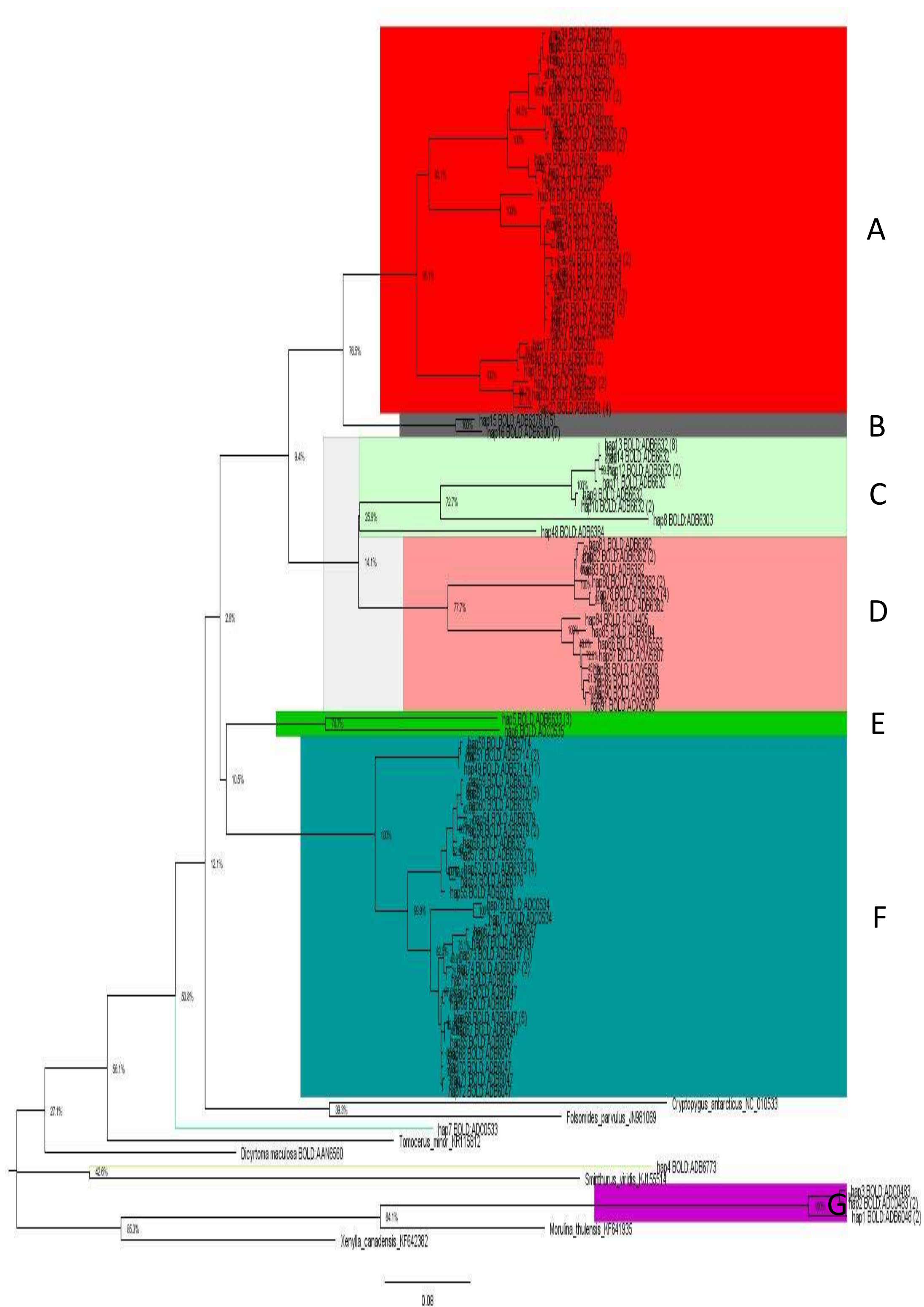

Figure 3.3. A ML tree based on 591bp COI sequences including 178 specimens. The amount of individuals for each haplotype is shown in parentheses. Bootstrap values are shown next to the tree nodes. Major clades are represented by groups A-G

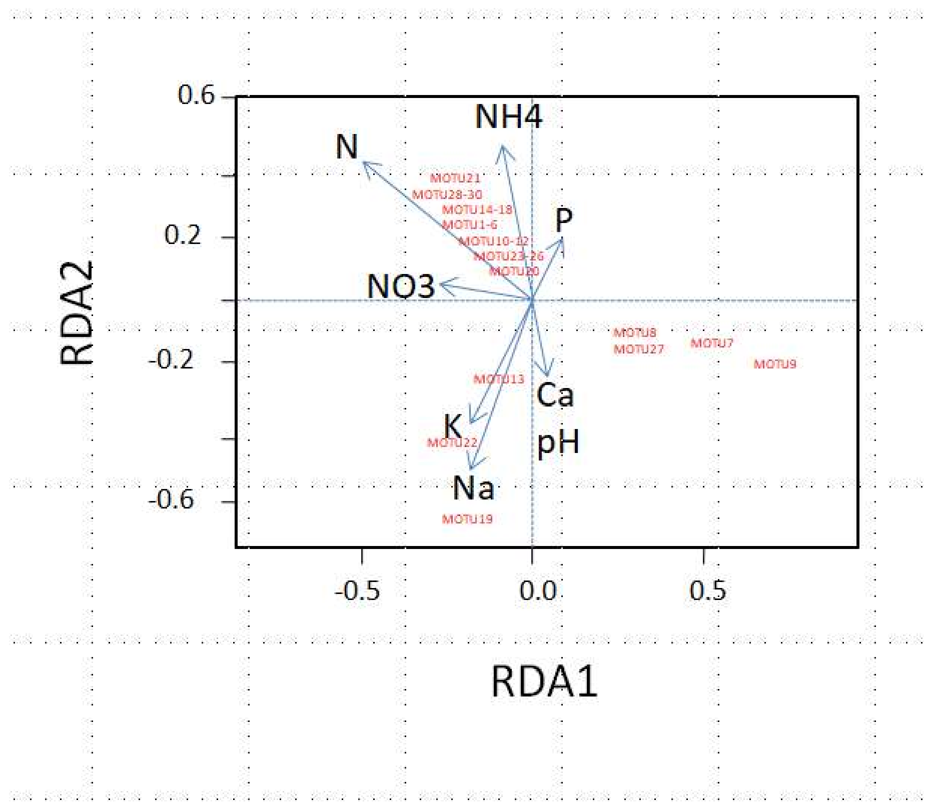

Figure 3.4 RDA with environmental variables (explanatory variables) and MOTUs. The environmental parameters are pH, P: potassium, Ca: calcium, $SO4^{2-}$: sulphate, N: nitrogen, $NH4^{+}$: ammonium, $NO3^{-}$: nitrate, Na^{+}: sodium, K^{+}: potassium, Mg^{2+}: magnesium and Cl^{-}: chloride. The direction of the arrow indicates the direction of maximum change of the variable and the length of the arrow is proportional to the rate of change

3.5 Discussion

This study assessed Collembola diversity in the Namib Desert using mitochondrial DNA (COI) sequences. Previous field studies of Collembola morphologically identified five species: *Willemia namibae; Folsomides angularis; Folsomides parvulus; Friesea* sp and *Cyphoderus colurus* (COINEAU AND SEELY 1983b; MARSH 1987b; THIBAUD AND MASSOUD 1988; BÖRNER 1913). This study revealed an unexpectedly high number of MOTUs and a high level of interspecific sequences divergence separating these MOTUs (average 18.8%).

We found high levels of MOTU richness along the Namib Desert transect. It is clear that past Collembola studies in the region have been underestimated. Thirty MOTUs were delineated and compared to previous studies that had only identified five species, this study shows that the number of springtail species is greater than previously estimated (DEHARVENG 2004). This is not surprising as it has now been shown that deserts are capable of supporting highly diverse and abundant fauna and flora, in spite of severe climatic condition and low soil organic matter content (POLIS 1991).

Most of the MOTUs were geographically restricted. There were however three MOTUs that were shared among four sampling sites. The overlapping geographic ranges could be explained by limited dispersal among sites. These soil dwelling Collembola must be capable of limited dispersal by active (walking and jumping) or passive dispersal (wind, animal and rainfall events) (FREEMAN 1952; FARROW AND GREENSLADE 1992; HOPKIN 1997; DUNGER *et al.* 2002).

The sampling done is likely not extensive enough to cover the full Collembola diversity of the sampling transect. Soil microarthropods exhibit diel periodicity with the highest populations occurring near the surface in the early morning (WHITFORD *et al.* 1981). This study did not carry out sampling early morning. Collembola are also capable of vertical migration through the soil. Collembola communities collected at deeper layers of soil, top soil and at the surface in the leaf litter may differ widely (SANTOS AND WHITFORD 1983). It is likely that this study is still underestimating Collembola diversity and more extensive sampling is required.

The sequences were only successfully identified to family level and all the sequences obtained from this study were new, with no close matches (<85%) found in either GenBank or BOLD. *Folsomides* cf. *angularis* which has previously been found in the gravel plains of the Namib Desert (THIBAUD AND MASSOUD 1988; ANDRÉ *et al.* 1997) but was not identified among the sequences in this study. There were no *Folsomides* cf. *angularis* COI sequences available for comparison. There was no *Cyphoderus colurus* COI sequence available for comparison either (BÖRNER 1913).

The family Isotomidae represented the bulk of our data, most species of this family live in the soil, in crevices on the sea shore and/or associated with fresh water. Members of this family are found throughout the world including extreme environments. Isotomidae is the most abundant soil dwelling Collembola family in Namib Desert. The Isotomidae genus *Folsomides* has a broad distribution with 60 named species (BELLINGER *et al.* 2018). One Sminthuridae individual was found in the rain region of the transect. This might be because individuals of the family live predominately on the surface of fresh water, on low vegetation, on the surface of leaf litter and on trees and our collection methods could not sample more individuals of this family. Neanuridae members are predominately found under bark and stone, leaf litter and soil (HOPKIN 1997) Despite being known to occur in soil only three individuals were found in this study, this may be because Namib Desert. Neanuridae are more likely to be found in leaf litter which was not sampled.

The Namib Desert is characterised by harsh conditions, such as low water availability and low vegetation cover. Habitats with such extreme conditions can only support few species of Collembola. However, Collembola may seek spatial refuges where temperature and moisture is sufficient for survival (CALLAGHAN *et al.* 2004). These microhabitats are patchily distributed in arid environments and separated by stretches of bare soil not suitable for arthropod survival (ANDRÉ *et al.* 1997; WALL AND VIRGINIA 1999). The mtDNA COI (barcode) locus revealed high levels of previously unknown genetic diversity of Collembola in the Namib Desert. The phylogenetic tree showed shallow intraspecific divergence (0 – 1.74%) and deep interspecific divergence (1.6 – 35.8%), which shows that the Namib springtail communities are genetically distinct. We speculate that these high levels of diversity are a result of the Collembola being restricted to their microhabitats and being unable to persist in the extreme conditions of the bare soil between these microhabitats (NOSIL 2012). As a result, their geographic ranges become fragmented and ability to disperse limited, this restricts population range and gene flow. These communities also became

isolated because of the age of their habitat. The Namib Desert is old (>180 million years) and has been isolated for a long time (GOUDIE AND ECKARDT 1999). Geographic restriction and isolation likely led to the accumulation of mutations in these communities, leading to allopatric speciation (STEVENS AND HOGG 2003; NOLAN *et al.* 2006).

Using redundancy analysis (RDA), we assessed the relative importance of soil physicochemical factors in driving variation in Collembola communities. The RDA analysis illustrated that the 11 environmental variables together explained only 33.94% of variation in Collembola community composition. However, none of the individual variables had a statistically significant influence on Collembola community structure. These results are not consistent with other studies that suggested abiotic factors, such as soil pH (SALAMON *et al.* 2008), K, P, Na and Mg (RAZO-GONZÁLEZ *et al.* 2014) and nitrogen, have a strong influence on driving variation of Collembola communities. Three factors in this study that had no significant influence on community composition (concentrations of potassium and phosphorus in the soil and soil pH), has previously been observed to shape biodiversity of Collembola in urban green space communities in Warsaw (RZESZOWSKI *et al.* 2017).

Variation in Collembola community composition was not significantly shaped by C/N ratio, and K and Na cat-ion concentration. However, these three factors have been shown to explain microarthopod richness in the Namib Desert (ANDRÉ *et al.* 1997). The results indicate that the below ground Collembola community could be shaped by the action of more than just the 11 soil physicochemical properties provided in this study, such factors include soil texture, soil structure, soil moisture and temperature. Below ground community structure might be shaped by the simultaneous action of multiple factors, with the effect of a single factor possibly being masked by the other factors (PFLUG AND WOLTERS 2001). Total soil nitrogen (N), though not a significant driver of variation in community composition, had a strong influence on variation. Nitrogen (N) was positively correlated with majority of the MOTUs. Collembola were sampled around and under microhabitats. These microhabitats, particularly shrubs in arid regions, can act as a resource sink (PETERSON *et al.* 2001; DOBLAS-MIRANDA *et al.* 2009). Collembola may be associated with high levels of soil N because soil nutrients (e.g. N) accumulate within these microhabitats.

However, it may be that N does not influence Collembola communities and, instead that Collembola have an influence on N. The positive correlation with N is likely because of

Collembola affecting N mineralisation. Nitrogen mineralisation is the process by which organic N is converted to plant-available inorganic forms. Collembola enhance N mineralization (INESON *et al.* 1982; TEUBEN 1991; CRAGG AND BARDGETT 2001) directly through their faecal pellets and indirectly by interacting with microorganisms (MCGONIGLE 1995). Bacterial and fungal grazing by Collembola increases N mineralization by increasing the respiratory, reproductive and metabolic activities of microorganisms (KANEDA AND KANEKO 2011). Collembola faecal pellets may also contribute to soil N. The soil terrestrial system is usually dense with Collembola faecal pellets (HOPKIN 1997). The concentration of mineral N in the faeces of Collembola is higher than that in their food. Therefore, Collembola make a direct contribution to nutrient availability through their faecal nutrient content (TEUBEN AND VERHOEF 1992). It is estimated that the contribution of soil fauna to total net N mineralization was between 10% and 49% (MOORE *et al.* 1988).

3.6 Conclusion

This study highlighted the need for a more comprehensive database for Namib springtails that include COI sequence data as well as the morphological identification of species. Even though our study found 30 putative species, it is likely that the number of Collembola species is still being underestimated. Further sampling is required in additional microhabitats (e.g. in leaf litter). Also sampling at different times (especially early morning) and the collection of deeper soil samples is recommended to take into account vertical migration of Collembola species.

This study showed that despite the harsh conditions of the Namib Desert, soil dwelling Collembola had much higher levels of diversity and abundance than previously known. The diversity of Collembola are not driven by the soil properties in this study. However, other abiotic factors such as soil texture, soil temperature and moisture, may drive diversity.

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Chapter 4: Morphological identification of Collembola from the Namib Desert

4.1 Abstract

The diversity of below ground invertebrates including Collembola (springtails) is relatively unknown in the Namib Desert. Previous morphological studies in the Namib Desert have found only four Collembola species based on traditional taxonomy. Here, we re-evaluated the morphological diversity of Collembola from the gravel plains of the Namib Desert for identification. Specimens were collected from pitfall traps or extracted from soil samples. A total of 23 individuals were selected for identification. Available European keys were used for identification to genus level where possible. A total of 8 specimens were identified to genus level (*Folsomides* sp), 14 to family level (Entomobryidae) and one to order level (Symphypleona). Both Symphypleona and Entomobryidae were previously unreported from the Namib Desert. The *Folsomides* genus and the family Entomobryidae were the most abundant groups. The results highlight that a combination of molecular and morphological data is needed for species level identification of Namib Collembola.

4.2 Introduction

Assessing areas of high biodiversity is important for determining areas of key importance for conservation. For invertebrate groups, the assessment of biodiversity is constrained due to the taxonomic impediment. The assessment of invertebrate diversity is particularly constrained by this impediment (GODFRAY 2002; DE CARVALHO *et al.* 2007). The diversity of belowground invertebrates, particularly springtails (Collembola) is relatively unknown in the Namib Desert (HOPKIN 1997).

The class Collembola are globally one of the most abundant arthropod groups, they occur across a broad range of habitats, including extreme environments. Due to their ubiquitous nature and their ecological role as decomposers, springtails are an important functional group in soil (HOPKIN 1997). The method of assessing Collembola diversity via molecular methods has become popular (HOGG AND HEBERT 2004; ROUGERIE *et al.* 2009). Despite this, Collembola diversity remains poorly known in the Namib Desert. In comparison, studies were more thoroughly conducted in Western Europe, North America and Antarctica (RATNASINGHAM AND HEBERT 2007).

Previous morphological studies identifying five species were undertaken by Thibaud and Massoud (1988) identifying the following species: *Willemia namibia* (Thibaud and Massoud, 1988), *Folsomides angularis* (Axelson 1905), *Folsomides parvulus* (Stach 1912), *Cyphoderus colurus* (Börner 1913) and *Friesea* sp, that occur in Namibia. Little other work has been done since to describe and list species for the region.

To address this gap in knowledge of Collembola in the Namib, DNA barcoding had been recently used to assess diversity. However, with the lack of available information for Namib Desert Collembola on the Barcode of Life Database (BOLD), specimens were only successfully identified to family level (see chapter 3). In this study we presented a list of Collembola sampled from the gravel plains in the Namib Desert and identified using morphological characteristics.

4.3 Methods and Materials

The survey was conducted in the gravel plains of the Namib Desert (see Figure 1). Collection of soil samples took place on 11 to 12 April 2016 and 24 April 2017. Pitfall traps were placed at the sampling sites on 24 April 2017 and collected on 25 April 2017. The pitfall traps consisted of 50ml plastic falcon tubes filled with 99% ethanol and buried to the lip in the ground. Pitfall traps were placed near micro-habitats, e.g. under the shade of rocks or shrubs and near leaf litter. Springtail specimens from the soil and pitfall traps were placed in PCR tubes, preserved in 99% Ethanol, labelled and stored at -20°C.

Specimen identification was carried out by Dr. Charlene Janion-Scheepers, Iziko Museum, Cape Town. Specimens were cleared in lactic acid and mounted on microscope slides using AH, Andre's modification of the original Hoyer's mounting fluid recipe (CUNNINGHAM 1972). No identification keys for Collembola of Namibia were available, thus available European keys were used for identification to genus level where possible (FJELLBERG 2007; HOPKIN 1999; HOPKIN 2007; POTAPOV *et al.* 2011).

4.4 Results

A total of 23 Collembola specimens were collected for identification. The Collembola specimens collected from the soil samples were identified as Entomobryidae (n=3), *Folsomides* (n=6) specimens and *Folsomides parvulus* (n=1). The specimens collected from the pitfall trap were identified as Symphypleona (n=1) and Entomobryidae (n=11) (see Table 4.1).

The most abundant specimens from the soil samples were *Folsomides*. These specimens were characterised by a very small body size, no pigmentation and with reduced eyes. The most abundant Collembola collected from the pitfall traps were of the family Entomobryidae.

Table 4.1. Collembola specimens recorded in the gravel plains of the Namib Desert. Collembola were identified to genus level. The number of individuals, number of possible species and life stage are shown in parenthesis

Field Code	**Identification**	**Sample from**	**Elevation (m)**	**Lat (S)**	**Long (E)**	**Date Collected**
NB17-006	*Folsomides* sp.	Soil	742	23,54011	15,41429	11/04/2016
NB17-014	Entomobryidae	Soil	487	23,2397	15,03844	11/04/2016
NB17-006	*Folsomides* sp.	Soil	742	23,54011	15,41429	11/04/2016
NB17-007	*Folsomides* sp.(perhaps 2 species?)	Soil	746	23,54	15,41435	11/04/2016
NB16-045	*Folsomides* sp.	Soil	1268	23,2453	16,14354	12/04/2016
NB16-042	*Folsomides* sp.	Soil	1097	23,34627	16,00951	12/04/2016
NB16-043	*Folsomides* sp.	Soil	1269	23,24533	16014363	12/04/2016
NB17-002T	Entomobryidae	Soil	771	23,45624	15,37921	24/04/2017
NB17-001	Entomobryidae	Soil	815	23,4565	15,37907	24/04/2017
NB17-001	Entomobryidae (3)	Pitfall trap	815	23,4565	15,37907	24/04/2017
NB17-001	Symphypleona (1)	Pitfall trap	815	23,4565	15,37907	24/04/2017
NB17-002T	Entomobryidae (2 adults, 4 juveniles)	Pitfall trap	771	23.45624	15,37921	24/04/2017
NB17-002T	*Folsomides* (cf. *parvulus*)	Soil	771	23,45624	15,37921	24/04/2017
NB17-002T	Entomobryidae (2 species?)	Pitfall trap	771	24,45624	16,37921	24/04/2017

Figure 4.1. A geographical representation of the sampling localities (Google Earth)

4.5 Discussion

Entomobryidae was the most diverse family of Collembola that can be found throughout the world (BELLINGER *et al.* 2018). They were found in a wide range of habitats, but most live in leaf litter, on the soil surface, on and under bark of trees, in the forest canopy, or in caves (HOPKIN 1997). Entomobryidae in the Namib Desert most likely occur in the leaf litter of desert shrubs. They are dominately surface-dwelling springtails, which is why they abundant in the pitfall traps. This study also provided the first morphological identifications of Symphypleona and Entomobryidae from the Namib Desert.

The order Symphypleona is one of the three main groups of springtails. The Symphypleona are very round animals, almost spherical, and some have long antennae (HOPKIN 1997). Sminthuridae, has previously been recorded from the Namib Desert using DNA Barcoding (see Chapter 3). Members of this order occur in leaf litter.

The most abundant Collembola found in the soil was from the genus *Folsomides*. *Folsomides angularis* (AXELSON 1905) and *Folsomides parvulus* (STACH 1922) have previously been found in the Namib Desert. In Chapter 3, the majority of the COI sequences belonged to the

family Isotomidae. In this chapter we were able to identify soil-dwelling specimens using morphological characteristics. The majority of specimens identified belonged to the genus *Folsomides*, which belongs to the family Isotomidae. From this we can infer that the COI sequences identified as Isotomidae in Chapter 3, most likely belong to the genera *Folsomides.* Therefore *Folsomides* appears to be a very abundant soil-dwelling springtail in the Namib Desert. The level of *Folsomides* abundance could be attributed to their small size (GREENSLADE 1981) and due to the fact that *Folsomides* species are capable of survival in harsh conditions (HOPKIN 1997). Many species in this genus have a cosmopolitan distribution (BELLINGER *et al*. 2018) and are well-known for their ability to withstand desiccation and can survive for long periods in a cryptobiotic state. Cryptobiosis is a physiological state that results in a reduced metabolic state, induced by removal of water from an animal by evaporation. Organisms can maintain this state for minutes or decades. This state is reversed with the introduction of free water (MERRIFIELD AND ROYCE 2002). *Folsomides angularis* cannot regulate its water loss well, but once desiccated can survive at relatively high temperatures (BELGNAOUI AND BARRA 1989).

4.6 Conclusion

Extrapolating from the results, this study suggests that *Folsomides* represents the dominant Collembola group living in the soil and Entomobryidae represents the dominant group of surface-dwelling Collembola. The Isotomidae specimens identified in Chapter 3 are most likely *Folsomides* specimens. There have been recent attempts to assess Collembola diversity in the Namib Desert using DNA Barcoding. However, existing sequence databases are limited because of the lack of species-level reference sequences. Comprehensive morphological identification of Collembola in the Namib Desert is still needed in conjunction with molecular methods.

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Chapter 5: Conclusion

5.1 Introduction

In this dissertation, the researcher analysed the genetic diversity of Namib Desert Collembola across an east-west transect. The researcher also assessed whether environmental parameters shaped this diversity. High levels of diversity were found among Namib Desert Collembola species. The environmental parameters tested did not significantly shape the diversity of Collembola across the transect (see Chapter 3). Morphological identification of Collembola specimens suggested that soil-dwelling Collembola in the Namib Desert are dominated by the genus *Folsomides*. Together these results show that there are unexpectedly high levels of genetic diversity among soil-dwelling Collembola in the Namib Desert and that these communities are dominated by cosmopolitan species belonging to the genus *Folsomides.*

In Chapter 3, the sequence information of the 178 Collembola specimens, from the mitochondrial barcoding COI (Cytochrome-c oxidase subunit I) gene was analyzed and Molecular Operational Taxonomic Units (MOTUs) were delineated. Collembola community responses to soil physicochemical properties were investigated by using a Redundancy Analysis (RDA).

Using the RESL algorithm (RATNASINGHAM AND HEBERT 2013) 30 MOTUs were delineated. The sequences were only successfully identified to family level and all the sequences obtained from this study were new, with no close matches (<85%) found in either GenBank or BOLD. Isotomidae is the most abundant soil dwelling Collembola family in Namib Desert. Most MOTUs were geographically restricted, indicating limited dispersal abilities. There was a high level of interspecific sequences divergence separating these MOTUs (average 18.8%). The redundancy analysis indicated that none of the soil physicochemical properties tested significantly drove variation in Collembola community composition. However, total soil Nitrogen was shown to be a strong driver of variation in community composition ($p<0,054$). The results suggest that the below ground Collembola community could be shaped by the action of more than just the 11 soil physicochemical properties provided in this study, including factors such as soil texture, soil structure and temperature.

We speculate that these high levels of diversity are as a result of the Collembola being restricted to their microhabitats. This restricts population range and gene flow. These communities have also become isolated because of the age of their habitat. The Namib Desert is old (>180 million years) and has been isolated for a long time (GOUDIE AND ECKARDT 1999). Geographic restriction and isolation likely led to the accumulation of mutations in these communities, leading to allopatric speciation (STEVENS AND HOGG 2003; NOLAN *et al.* 2006).

In Chapter 4, we morphologically identified surface-dwelling and soil-dwelling Collembola specimens in the Namib Desert gravel plains. No identification keys for Collembola of Namibia were available, thus available European keys were used and specimens were only identified up to genus level.

The soil-dwelling Collembola specimens collected consisted of three Entomobryidae specimens, eight *Folsomides* specimens and one of which is possibly *Folsomides parvulus.* The specimens from the pitfall trap consisted of one Symphypleona specimen and 12 Entomobryidae specimens. The most abundant soil dwelling Collembola were *Folsomides* sp and most abundant Collembola collected from the pitfall traps were of the family Entomobryidae. *Folsomides* and Entomobryidae are likely the most abundant groups because they have cosmopolitan distributions (BELLINGER *et al.* 2018).

This study showed that soil dwelling Collembola in the Namib Desert had much higher levels of diversity and abundance than previously known. The environmental factors that significantly shape Collembola diversity, remains unknown and should be studied further. The morphological identification, suggests that Isotomidae specimens, identified using DNA barcoding and that forms the bulk of the sequence data in chapter 3, belongs to the genus *Folsomides*. This study highlighted the need for a more comprehensive database for Namib springtails that include COI sequence data as well as the morphological identification of species.

5.2 Future Research

All of the Collembola COI sequences remain unidentified and the morphologically identified specimens were only identified up to genus level. In order to get a more precise and complete diversity estimate of Collembola in the Namib Desert, identifying unknown specimens would be beneficial. We need a more comprehensive database for Namib springtails that include COI sequence data as well as taxonomic identifications. Future studies should continue to contribute to the BOLD database with the collaboration of taxonomists.

Since we could not show that our environmental factors had a significant impact on Collembola diversity, future work should be done to determine which abiotic factors are responsible for driving variation in Collembola communities. The influence of soil moisture, temperature and texture on Collembola should be studied. This study did not take into account which Collembola communities were sampled from which microhabitat. In the future this should be taken into account as it has been shown that Collembola abundance varies greatly depending on the microhabitat (ANDRÉ *et al.* 1997).

The sampling procedure in this study was basic due to time constraints and it is likely because of this that the researcher did not get a full representation of species present in the Namib Desert. Soil microarthropods exhibit diel periodicity with the highest populations occurring near the surface in the early morning (WHITFORD *et al.* 1981). Collembola are also capable of vertical migration and sampling was limited to the top layers of soil. Future studies should sample at different times of the day and at different levels in the soil (SANTOS AND WHITFORD 1983).

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6. Appendices

Appendix 1 GPS co-ordinates of the 10 established sampling sites from along a Namib Desert transect

Sampling site	Latitude	Longitude
site2	-23.00106666666667	14.67158333333333
site6	-23.06641666666667	15.03976666666667
site8	-23.14323333333334	15.20855
site10	-23.24626666666667	15.36033333333333
site12	-23.31098333333333	15.5339
site14	-23.32545	15.71455
site16	-23.32073333333333	15.86228333333333
site18	-23.34538333333334	16.0106
site20	-23.24475	16.14271666666667

Appendix 2: Collembola BIN designation deposited in BOLD. COI BIN designations for 178 sequences representing 91 haplotypes

Haplotype	Process ID, Family name, sample ID, GPS, Gene, BIN	
hap 1	NAMIB602-16,Neanuridae,NAMIB412,-23.245,16.144,,COI-5P,BOLD:ADB6048	MOTU 29
hap 1	NAMIB608-16,Neanuridae,NAMIB418,-23.245,16.144,,COI-5P,BOLD:ADB6048	
hap 2	NAMIB510-16,Neanuridae,NAMIB320,-23.346,16.01,,COI-5P,BOLD:ADC0483	MOTU 30
hap 2	NAMIB514-16,Neanuridae,NAMIB324,-23.346,16.01,,COI-5P,BOLD:ADC0483	
hap 3	NAMIB511-16,Neanuridae,NAMIB321,-23.346,16.01,,COI-5P,BOLD:ADC0483	
hap 4	NAMIB654-16,Sminthuridae,NAMIB464,-23.346,16.01,,COI-5P,BOLD:ADB6773	MOTU 28
hap 5	NAMIB254-16,Isotomidae,NAMIB159,-23.244,15.358,,COI-5P,BOLD:ADB6633	MOTU 21
hap 5	NAMIB257-16,Isotomidae,NAMIB162,-23.244,15.358,,COI-5P,BOLD:ADB6633	
hap 5	NAMIB258-16,Isotomidae,NAMIB163,-23.244,15.358,,COI-5P,BOLD:ADB6633	
hap 6	NAMIB556-16,Isotomidae,NAMIB366,-23.245,16.144,,COI-5P,BOLD:ADC0535	MOTU 22
hap 7	NAMIB553-16,Isotomidae,NAMIB363,-23.245,16.144,,COI-5P,BOLD:ADC0533	MOTU 27
hap 8	NAMIB607-16,Isotomidae,NAMIB417,-23.245,16.144,,COI-5P,BOLD:ADB6303	MOTU 13
hap 9	NAMIB570-16,Isotomidae,NAMIB380,-23.244,15.358,,COI-5P,BOLD:ADB6632	MOTU 14
hap 10	NAMIB572-16,Isotomidae,NAMIB382,-23.143,15.207,,COI-5P,BOLD:ADB6632	
hap 10	NAMIB573-16,Isotomidae,NAMIB383,-23.143,15.207,,COI-	

	5P,BOLD:ADB6632	
hap 11	NAMIB277-16,Isotomidae,NAMIB182,-23.066,15.04,,COI-5P,BOLD:ADB6632	
hap 12	NAMIB280-16,Isotomidae,NAMIB185,-23.066,15.04,,COI-5P,BOLD:ADB6632	
hap 12	NAMIB290-16,Isotomidae,NAMIB195,-23.066,15.04,,COI-5P,BOLD:ADB6632	
hap 13	NAMIB276-16,Isotomidae,NAMIB181,-23.066,15.04,,COI-5P,BOLD:ADB6632	
hap 13	NAMIB278-16,Isotomidae,NAMIB183,-23.066,15.04,,COI-5P,BOLD:ADB6632	
hap 13	NAMIB279-16,Isotomidae,NAMIB184,-23.066,15.04,,COI-5P,BOLD:ADB6632	
hap 13	NAMIB282-16,Isotomidae,NAMIB187,-23.066,15.04,,COI-5P,BOLD:ADB6632	
hap 13	NAMIB284-16,Isotomidae,NAMIB189,-23.066,15.04,,COI-5P,BOLD:ADB6632	
hap 13	NAMIB285-16,Isotomidae,NAMIB190,-23.066,15.04,,COI-5P,BOLD:ADB6632	
hap 13	NAMIB287-16,Isotomidae,NAMIB192,-23.066,15.04,,COI-5P,BOLD:ADB6632	
hap 13	NAMIB288-16,Isotomidae,NAMIB193,-23.066,15.04,,COI-5P,BOLD:ADB6632	
hap 14	NAMIB281-16,Isotomidae,NAMIB186,-23.066,15.04,,COI-5P,BOLD:ADB6632	
hap 15	NAMIB500-16,Isotomidae,NAMIB310,-23.245,16.144,,COI-5P,BOLD:ADB6378	MOTU 11
hap 15	NAMIB502-16,Isotomidae,NAMIB312,-23.245,16.144,,COI-5P,BOLD:ADB6378	
hap 15	NAMIB503-16,Isotomidae,NAMIB313,-23.245,16.144,,COI-5P,BOLD:ADB6378	
hap 15	NAMIB549-16,Isotomidae,NAMIB359,-23.245,16.144,,COI-	

	5P,BOLD:ADB6378	
hap 15	NAMIB597-16,Isotomidae,NAMIB407,-23.245,16.144,,COI-5P,BOLD:ADB6378	
hap 15	NAMIB600-16,Isotomidae,NAMIB410,-23.245,16.144,,COI-5P,BOLD:ADB6378	
hap 15	NAMIB610-16,Isotomidae,NAMIB420,-23.245,16.144,,COI-5P,BOLD:ADB6378	
hap 15	NAMIB612-16,Isotomidae,NAMIB422,-23.245,16.144,,COI-5P,BOLD:ADB6378	
hap 15	NAMIB618-16,Isotomidae,NAMIB428,-23.245,16.144,,COI-5P,BOLD:ADB6378	
hap 15	NAMIB620-16,Isotomidae,NAMIB430,-23.245,16.144,,COI-5P,BOLD:ADB6378	
hap 15	NAMIB621-16,Isotomidae,NAMIB431,-23.245,16.144,,COI-5P,BOLD:ADB6378	
hap 15	NAMIB625-16,Isotomidae,NAMIB435,-23.245,16.144,,COI-5P,BOLD:ADB6378	
hap 15	NAMIB629-16,Isotomidae,NAMIB439,-23.245,16.144,,COI-5P,BOLD:ADB6378	
hap 15	NAMIB630-16,Isotomidae,NAMIB440,-23.245,16.144,,COI-5P,BOLD:ADB6378	
hap 15	NAMIB637-16,Isotomidae,NAMIB447,-23.245,16.144,,COI-5P,BOLD:ADB6378	
hap 16	NAMIB507-16,Isotomidae,NAMIB317,-23.245,16.144,,COI-5P,BOLD:ADB6300	MOTU 10
hap 16	NAMIB619-16,Isotomidae,NAMIB429,-23.245,16.144,,COI-5P,BOLD:ADB6300	
hap 16	NAMIB627-16,Isotomidae,NAMIB437,-23.245,16.144,,COI-5P,BOLD:ADB6300	
hap 16	NAMIB636-16,Isotomidae,NAMIB446,-23.245,16.144,,COI-5P,BOLD:ADB6300	
hap 16	NAMIB638-16,Isotomidae,NAMIB448,-23.245,16.144,,COI-	

	5P,BOLD:ADB6300	
hap 16	NAMIB639-16,Isotomidae,NAMIB449,-23.245,16.144,,COI-5P,BOLD:ADB6300	
hap 16	NAMIB640-16,Isotomidae,NAMIB450,-23.245,16.144,,COI-5P,BOLD:ADB6300	
hap 17	NAMIB658-16,Isotomidae,NAMIB468,-23.321,15.862,,COI-5P,BOLD:ADB6302	
hap 17	NAMIB518-16,Isotomidae,NAMIB328,-23.321,15.862,,COI-5P,BOLD:ADB6302	
hap 18	NAMIB520-16,Isotomidae,NAMIB330,-23.321,15.862,,COI-5P,BOLD:ADB6302	
hap 19	NAMIB656-16,Isotomidae,NAMIB466,-23.321,15.862,,COI-5P,BOLD:ADB6302	MOTU 6
hap 20	NAMIB657-16,Isotomidae,NAMIB467,-23.321,15.862,,COI-5P,BOLD:ADB6555	MOTU 8
hap 20	NAMIB519-16,Isotomidae,NAMIB329,-23.321,15.862,,COI-5P,BOLD:ADB6299	
hap 21	NAMIB660-16,Isotomidae,NAMIB470,-23.321,15.862,,COI-5P,BOLD:ADB6299	MOTU 7
hap 22	NAMIB523-16,Isotomidae,NAMIB333,-23.321,15.862,,COI-5P,BOLD:ADB6301	MOTU 9
hap 22	NAMIB524-16,Isotomidae,NAMIB334,-23.321,15.862,,COI-5P,BOLD:ADB6301	
hap 22	NAMIB655-16,Isotomidae,NAMIB465,-23.321,15.862,,COI-5P,BOLD:ADB6301	
hap 22	NAMIB659-16,Isotomidae,NAMIB469,-23.321,15.862,,COI-5P,BOLD:ADB6301	
hap 23	NAMIB242-16,Isotomidae,NAMIB147,-23.143,15.207,,COI-5P,BOLD:ADB6305	MOTU 2
hap 23	NAMIB244-16,Isotomidae,NAMIB149,-23.143,15.207,,COI-5P,BOLD:ADB6305	
hap 23	NAMIB246-16,Isotomidae,NAMIB151,-23.143,15.207,,COI-	

	5P,BOLD:ADB6305	
hap 23	NAMIB247-16,Isotomidae,NAMIB152,-23.143,15.207,,COI-5P,BOLD:ADB6305	
hap 23	NAMIB292-16,Isotomidae,NAMIB197,-23.143,15.207,,COI-5P,BOLD:ADB6305	
hap 23	NAMIB293-16,Isotomidae,NAMIB198,-23.143,15.207,,COI-5P,BOLD:ADB6305	
hap 23	NAMIB309-16,Isotomidae,NAMIB214,-23.143,15.207,,COI-5P,BOLD:ADB6305	
hap 23	NAMIB245-16,Isotomidae,NAMIB150,-23.143,15.207,,COI-5P,BOLD:ADB6305	
hap 24	NAMIB241-16,Isotomidae,NAMIB146,-23.143,15.207,,COI-5P,BOLD:ADB6305	
hap 25	NAMIB308-16,Isotomidae,NAMIB213,-23.143,15.207,,COI-5P,BOLD:ADB6305	
hap 25	NAMIB299-16,Isotomidae,NAMIB204,-23.143,15.207,,COI-5P,BOLD:ADB6383	
hap 26	NAMIB687-16,Isotomidae,NAMIB497,-23.31,15.535,,COI-5P,BOLD:ADB6383	
hap 27	NAMIB306-16,Isotomidae,NAMIB211,-23.143,15.207,,COI-5P,BOLD:ADB6383	MOTU 3
hap 28	NAMIB685-16,Isotomidae,NAMIB495,-23.31,15.535,,COI-5P,BOLD:ADB5701	
hap 29	NAMIB240-16,Isotomidae,NAMIB145,-23.143,15.207,,COI-5P,BOLD:ADB5701	
hap 30	NAMIB291-16,Isotomidae,NAMIB196,-23.143,15.207,,COI-5P,BOLD:ADB5701	
hap 31	NAMIB297-16,Isotomidae,NAMIB202,-23.143,15.207,,COI-5P,BOLD:ADB5701	
hap 31	NAMIB294-16,Isotomidae,NAMIB199,-23.143,15.207,,COI-5P,BOLD:ADB5701	
hap 32	NAMIB296-16,Isotomidae,NAMIB201,-23.143,15.207,,COI-	MOTU 1

	5P,BOLD:ADB5701	
hap 33	NAMIB298-16,Isotomidae,NAMIB203,-23.143,15.207,,COI-5P,BOLD:ADB5701	
hap 33	NAMIB301-16,Isotomidae,NAMIB206,-23.143,15.207,,COI-5P,BOLD:ADB5701	
hap 33	NAMIB302-16,Isotomidae,NAMIB207,-23.143,15.207,,COI-5P,BOLD:ADB5701	
hap 33	NAMIB307-16,Isotomidae,NAMIB212,-23.143,15.207,,COI-5P,BOLD:ADB5701	
hap 33	NAMIB303-16,Isotomidae,NAMIB208,-23.143,15.207,,COI-5P,BOLD:ADB5701	
hap 34	NAMIB295-16,Isotomidae,NAMIB200,-23.143,15.207,,COI-5P,BOLD:ADB5701	
hap 35	NAMIB300-16,Isotomidae,NAMIB205,-23.143,15.207,,COI-5P,BOLD:ADB5701	
hap35	NAMIB304-16,Isotomidae,NAMIB209,-23.143,15.207,,COI-5P,BOLD:ADB5701	
hap36	NAMIB581-16,Isotomidae,NAMIB391,-23.245,16.144,,COI-5P,BOLD:ADC0536	MOTU 4
hap37	NAMIB544-16,Isotomidae,NAMIB354,-23.324,15.714,,COI-5P,BOLD:ACU5054	
hap38	NAMIB665-16,Isotomidae,NAMIB475,-23.324,15.714,,COI-5P,BOLD:ACU5054	
hap39	NAMIB583-16,Isotomidae,NAMIB393,-23.325,15.714,,COI-5P,BOLD:ACU5054	
hap 40	NAMIB539-16,Isotomidae,NAMIB349,-23.324,15.714,,COI-5P,BOLD:ACU5054	
hap 40	NAMIB666-16,Isotomidae,NAMIB476,-23.324,15.714,,COI-5P,BOLD:ACU5054	
hap 41	NAMIB536-16,Isotomidae,NAMIB346,-23.324,15.714,,COI-5P,BOLD:ACU5054	
hap 42	NAMIB546-16,Isotomidae,NAMIB356,-23.324,15.714,,COI-	MOTU 5

	5P,BOLD:ACU5054	
hap 43	NAMIB541-16,Isotomidae,NAMIB351,-23.324,15.714,,COI-5P,BOLD:ACU5054	
hap 44	NAMIB537-16,Isotomidae,NAMIB347,-23.324,15.714,,COI-5P,BOLD:ACU5054	
hap 44	NAMIB664-16,Isotomidae,NAMIB474,-23.324,15.714,,COI-5P,BOLD:ACU5054	
hap 45	NAMIB542-16,Isotomidae,NAMIB352,-23.324,15.714,,COI-5P,BOLD:ACU5054	
hap 45	NAMIB587-16,Isotomidae,NAMIB397,-23.325,15.714,,COI-5P,BOLD:ACU5054	
hap 46	NAMIB545-16,Isotomidae,NAMIB355,-23.324,15.714,,COI-5P,BOLD:ACU5054	
hap 47	NAMIB593-16,Isotomidae,NAMIB403,-23.325,15.714,,COI-5P,BOLD:ACU5054	
hap 48	NAMIB253-16,Isotomidae,NAMIB158,-23.002,14.67,,COI-5P,BOLD:ADB6384	MOTU 12
hap 49	NAMIB508-16,Isotomidae,NAMIB318,-23.346,16.01,,COI-5P,BOLD:ADB5714	
hap 49	NAMIB513-16,Isotomidae,NAMIB323,-23.346,16.01,,COI-5P,BOLD:ADB5714	
hap 49	NAMIB515-16,Isotomidae,NAMIB325,-23.346,16.01,,COI-5P,BOLD:ADB5714	
hap 49	NAMIB643-16,Isotomidae,NAMIB453,-23.346,16.01,,COI-5P,BOLD:ADB5714	
hap 49	NAMIB644-16,Isotomidae,NAMIB454,-23.346,16.01,,COI-5P,BOLD:ADB5714	
hap 49	NAMIB646-16,Isotomidae,NAMIB456,-23.346,16.01,,COI-5P,BOLD:ADB5714	
hap 49	NAMIB647-16,Isotomidae,NAMIB457,-23.346,16.01,,COI-5P,BOLD:ADB5714	MOTU 23
hap 49	NAMIB648-16,Isotomidae,NAMIB458,-23.346,16.01,,COI-	

	5P,BOLD:ADB5714	
hap 49	NAMIB649-16,Isotomidae,NAMIB459,-23.346,16.01,,COI-5P,BOLD:ADB5714	
hap 49	NAMIB652-16,Isotomidae,NAMIB462,-23.346,16.01,,COI-5P,BOLD:ADB5714	
hap 49	NAMIB653-16,Isotomidae,NAMIB463,-23.346,16.01,,COI-5P,BOLD:ADB5714	
hap 50	NAMIB645-16,Isotomidae,NAMIB455,-23.346,16.01,,COI-5P,BOLD:ADB5714	
hap 51	NAMIB509-16,Isotomidae,NAMIB319,-23.346,16.01,,COI-5P,BOLD:ADB5714	
hap 51	NAMIB650-16,Isotomidae,NAMIB460,-23.346,16.01,,COI-5P,BOLD:ADB5714	
hap 52	NAMIB606-16,Isotomidae,NAMIB416,-23.245,16.144,,COI-5P,BOLD:ADB6379	
hap 52	NAMIB615-16,Isotomidae,NAMIB425,-23.245,16.144,,COI-5P,BOLD:ADB6379	
hap 52	NAMIB623-16,Isotomidae,NAMIB433,-23.245,16.144,,COI-5P,BOLD:ADB6379	
hap 52	NAMIB631-16,Isotomidae,NAMIB441,-23.245,16.144,,COI-5P,BOLD:ADB6379	
hap 53	NAMIB626-16,Isotomidae,NAMIB436,-23.245,16.144,,COI-5P,BOLD:ADB6379	
hap 54	NAMIB613-16,Isotomidae,NAMIB423,-23.245,16.144,,COI-5P,BOLD:ADB6379	
hap 55	NAMIB616-16,Isotomidae,NAMIB426,-23.245,16.144,,COI-5P,BOLD:ADB6379	
hap 56	NAMIB611-16,Isotomidae,NAMIB421,-23.245,16.144,,COI-5P,BOLD:ADB6379	
hap 57	NAMIB622-16,Isotomidae,NAMIB432,-23.245,16.144,,COI-5P,BOLD:ADB6379	MOTU 24
hap 57	NAMIB635-16,Isotomidae,NAMIB445,-23.245,16.144,,COI-	

	5P,BOLD:ADB6379	
hap 58	NAMIB604-16,Isotomidae,NAMIB414,-23.245,16.144,,COI-5P,BOLD:ADB6379	
hap58	NAMIB624-16,Isotomidae,NAMIB434,-23.245,16.144,,COI-5P,BOLD:ADB6379	
hap59	NAMIB554-16,Isotomidae,NAMIB364,-23.245,16.144,,COI-5P,BOLD:ADB6379	
hap60	NAMIB609-16,Isotomidae,NAMIB419,-23.245,16.144,,COI-5P,BOLD:ADB6379	
hap 61	NAMIB601-16,Isotomidae,NAMIB411,-23.245,16.144,,COI-5P,BOLD:ADB6379	
hap 61	NAMIB614-16,Isotomidae,NAMIB424,-23.245,16.144,,COI-5P,BOLD:ADB6379	
hap 61	NAMIB617-16,Isotomidae,NAMIB427,-23.245,16.144,,COI-5P,BOLD:ADB6379	
hap 61	NAMIB628-16,Isotomidae,NAMIB438,-23.245,16.144,,COI-5P,BOLD:ADB6379	
hap 61	NAMIB632-16,Isotomidae,NAMIB442,-23.245,16.144,,COI-5P,BOLD:ADB6379	
hap 62	NAMIB669-16,Isotomidae,NAMIB479,-23.324,15.714,,COI-5P,BOLD:ADB6047	
hap 63	NAMIB678-16,Isotomidae,NAMIB488,-23.324,15.714,,COI-5P,BOLD:ADB6047	
hap 64	NAMIB527-16,Isotomidae,NAMIB337,-23.323,15.714,,COI-5P,BOLD:ADB6047	
hap 65	NAMIB682-16,Isotomidae,NAMIB492,-23.324,15.714,,COI-5P,BOLD:ADB6047	
hap 66	NAMIB528-16,Isotomidae,NAMIB338,-23.323,15.714,,COI-5P,BOLD:ADB6047	
hap 66	NAMIB667-16,Isotomidae,NAMIB477,-23.324,15.714,,COI-5P,BOLD:ADB6047	MOTU 26
hap 66	NAMIB673-16,Isotomidae,NAMIB483,-23.324,15.714,,COI-	

	5P,BOLD:ADB6047	
hap 66	NAMIB676-16,Isotomidae,NAMIB486,-23.324,15.714,,COI-5P,BOLD:ADB6047	
hap 66	NAMIB679-16,Isotomidae,NAMIB489,-23.324,15.714,,COI-5P,BOLD:ADB6047	
hap 67	NAMIB677-16,Isotomidae,NAMIB487,-23.324,15.714,,COI-5P,BOLD:ADB6047	
hap 68	NAMIB671-16,Isotomidae,NAMIB481,-23.324,15.714,,COI-5P,BOLD:ADB6047	
hap 69	NAMIB530-16,Isotomidae,NAMIB340,-23.323,15.714,,COI-5P,BOLD:ADB6047	
hap 70	NAMIB680-16,Isotomidae,NAMIB490,-23.324,15.714,,COI-5P,BOLD:ADB6047	
hap 71	NAMIB533-16,Isotomidae,NAMIB343,-23.323,15.714,,COI-5P,BOLD:ADB6047	
hap 72	NAMIB672-16,Isotomidae,NAMIB482,-23.324,15.714,,COI-5P,BOLD:ADB6047	
hap 73	NAMIB529-16,Isotomidae,NAMIB339,-23.323,15.714,,COI-5P,BOLD:ADB6047	
hap 73	NAMIB670-16,Isotomidae,NAMIB480,-23.324,15.714,,COI-5P,BOLD:ADB6047	
hap 73	NAMIB681-16,Isotomidae,NAMIB491,-23.324,15.714,,COI-5P,BOLD:ADB6047	
hap 74	NAMIB535-16,Isotomidae,NAMIB345,-23.323,15.714,,COI-5P,BOLD:ADB6047	
hap74	NAMIB668-16,Isotomidae,NAMIB478,-23.324,15.714,,COI-5P,BOLD:ADB6047	
hap 75	NAMIB683-16,Isotomidae,NAMIB493,-23.324,15.714,,COI-5P,BOLD:ADB6047	
hap 76	NAMIB550-16,Isotomidae,NAMIB360,-23.245,16.144,,COI-5P,BOLD:ADC0534	MOTU 25
hap 77	NAMIB558-16,Isotomidae,NAMIB368,-23.245,16.144,,COI-	

	5P,BOLD:ADC0534	
hap 78	NAMIB259-16,Isotomidae,NAMIB164,-23.244,15.358,,COI-5P,BOLD:ADB6382	MOTU 20
hap 78	NAMIB561-16,Isotomidae,NAMIB371,-23.244,15.358,,COI-5P,BOLD:ADB6382	
hap 78	NAMIB565-16,Isotomidae,NAMIB375,-23.244,15.358,,COI-5P,BOLD:ADB6382	
hap 78	NAMIB568-16,Isotomidae,NAMIB378,-23.244,15.358,,COI-5P,BOLD:ADB6382	
hap 79	NAMIB569-16,Isotomidae,NAMIB379,-23.244,15.358,,COI-5P,BOLD:ADB6382	
hap 80	NAMIB560-16,Isotomidae,NAMIB370,-23.244,15.358,,COI-5P,BOLD:ADB6382	
hap 80	NAMIB562-16,Isotomidae,NAMIB372,-23.244,15.358,,COI-5P,BOLD:ADB6382	
hap 81	NAMIB567-16,Isotomidae,NAMIB377,F8,-23.244,15.358,,COI-5P,BOLD:ADB6382	
hap 82	NAMIB563-16,Isotomidae,NAMIB373,-23.244,15.358,,COI-5P,BOLD:ADB6382	
hap 82	NAMIB571-16,Isotomidae,NAMIB381,-23.244,15.358,,COI-5P,BOLD:ADB6382	
hap 83	NAMIB566-16,Isotomidae,NAMIB376,-23.244,15.358,,COI-5P,BOLD:ADB6382	
hap 84	NAMIB686-16,Isotomidae,NAMIB496,-23.31,15.535,,COI-5P,BOLD:ACU4405	MOTU 18
hap 85	NAMIB591-16,Isotomidae,NAMIB401,-23.325,15.714,,COI-5P,BOLD:ADB9904	MOTU 19
hap 86	NAMIB589-16,Isotomidae,NAMIB399,-23.325,15.714,,COI-5P,BOLD:ACW5553	MOTU 17
hap 87	NAMIB588-16,Isotomidae,NAMIB398,-23.325,15.714,,COI-5P,BOLD:ACW5607	MOTU 16
hap 88	NAMIB592-16,Isotomidae,NAMIB402,-23.325,15.714,,COI-	MOTU

	5P,BOLD:ACW5608	15
hap 89	NAMIB590-16,Isotomidae,NAMIB400,-23.325,15.714,,COI-5P,BOLD:ACW5608	
hap 90	NAMIB585-16,Isotomidae,NAMIB395,-23.325,15.714,,COI-5P,BOLD:ACW5608	
hap 91	NAMIB586-16,Isotomidae,NAMIB396,23.325,15.714,,COI-5P,BOLD:ACW5608	

Appendix 3 List of Amino Acid Substitutions between different haplotypes

Amino Acids

Haplotypes	1	2	3	4	7	10	11	18	22	25	28	38	39	50	84	85	89	90	91	92	97	109	110	111	112	114	116	118	121	137	148	149	151	152	155	156	165	190	193	Site
hap15A15	A	A	M	V	S	V	L	Q	F	D	I	F	V	I	L	I	T	G	G	L	A	A	G	I	A	A	A	I	S	V	T	A	M	T	R	T	L	L	T	
hap1A2	.	S	F	T	.	I	I	S	.	E	T	L	I	.	.	T	A	.	S	M	.	S	N	L	.	S	S	V	.	.	P	.	L	.	Q	I	.	.	S	20
hap2A2	.	S	F	T	.	I	I	S	.	E	T	L	I	.	.	T	A	.	S	M	.	S	N	L	.	S	S	V	.	.	P	.	L	.	Q	I	.	.	S	
hap3	.	S	F	T	.	I	I	S	.	E	T	L	I	.	.	T	A	.	S	M	.	S	N	L	.	S	S	V	.	.	P	.	L	.	Q	I	.	.	S	
hap4	S	.	.	L	A	M	I	.	I	.	.	.	I	.	I	S	M	S	.	.	V	S	N	.	S	.	S	V	A	I	N	P	.	E	.	V	.	.	S	18
hap5A3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	10
hap6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	V	.	.	.	S	.	.	.	.	.	.	.	
hap7	.	.	.	A	.	.	.	.	.	.	.	L	I	.	.	.	A	.	.	.	.	.	.	.	.	.	.	V	.	.	.	I	.	.	.	.	I	.	.	
hap8	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	20
hap9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	10
hap10A2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	8
hap11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
hap12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	6

A

2

ha

p1

3

A

8 . . . .

ha

p1

4 . . . .

ha

p1

6

A 2

7 0

ha

p1

7 . . . . I . . . . V . . . .

ha

p1

8 . . . . I . . . . V . . . .

ha

p1

9

A

2 . . . . I . . . . V . . . .

ha

p2

0 . . . . I . . . . V . . . .

ha

p2

1

A

2 . . . . V . . . .

ha

p2

2

A 1

4 . . . . I . . . . V 6

ha

p2

3

A

7 . . . . I . . . . T . . . .

ha

p2

4 . . . . I . . . . T . . . .

ha

p2

5 . . . . I . . . . T 8

A
2
ha
p2
6 . . . . I . . . . T . . . .
ha
p2 1
7 . . . . I . . . . T 2
ha
p2
8 . . . . I . . . . T 8
ha
p2 1
9 . . . . I . . . . T 2
ha
p3
0 . . . . I . . . . T . . . .
ha
p3
1
A
2 . . . . I . . . . T . . . .
ha
p3
2 . . . . I . . . . T . . . .
ha
pp
33
A
5 . . . . I . . . . T . . . .
ha
p3
4 . . . . I . . . . T . . . .
ha
p3
5
A
2 . . . . I . . . . T 8
ha
p3 2
6 . . . . I . . . . V 0
ha
p3
7 . . . . I . . . . V . . . .
ha
p3
8 . . . . I . . . . V . . . .
ha
p3 1
9 . . . . I . . . . V 4

ha
p4
0
A
2 . . . . I . . . . V . . . .
ha
p4
1 . . . . I . . . . V . . . .
ha
p4
2 . . . . I . . . . V . . . .
ha
p4
3 . . . . I . . . . V . . . .
ha
p4
4
A
2 . . . . I . . . . V . . . .
ha
p4
5
A
2 . . . . I . . . . V . . . .
ha
p4
6 . . . . I . . . . V . . . .
ha
p4
7 . . . . I . . . . V . . . .
ha
p4
8 . . . . V 2
ha
p4
9
A
11 . . . . V . . . .
ha
p5
0 . . . . V . . . .
ha
p5
1
A 1
2 . . . . V 8
ha
p5
2 2
A . . . . V 0

4
ha
p5
3 . . . . V . . . .
ha
p5
4 . . . . V . . . .
ha
p5
5 . . . . V . . . .
ha
p5
6 . . . . V . . . .
ha
p5
7
A
2 . . . . V . . . .
ha
p5
8
A
2 . . . . I . . . . V . . . .
ha
p5
9 . . . . . M . . . . V . . . .
ha
p6
0 . . . . V . . . .
ha
p6
1
A
5 . . . . V . . . .
ha
p6
2 . . . . V . . . .
ha
p6
3 . . . . V . . . .
ha
p6
4 . . . . V . . . .
ha
p6
5 . . . . V . . . .
ha
p6
6 1
A . . . . V 4

5
ha
p6
7 . . . V . . .
ha
p6
8 . . . V . . .
ha
p6
9 . . . V . . .
ha
p7
0 . . . V . . .
ha
p7
1 . . . T . . . V . . .
ha
p7
2 . . . V . . .
ha
p7
3
A
3 . . . V . . .
ha
p7
4
A
2 . . . V . . .
ha
p7
5 . . . V . . .
ha
p7
6 . . . V . . .
ha
p7 2
7 . . . V . . . 0
ha
p7
8
A
4 . . .
ha
p7
9 . . . V . . .
ha
p8
0 1
A . . . I . 0

2
ha
p8
1 . . . .
ha
p8
2
A
2 . . . .
ha
p8
3 . . . .
ha
p8 1
4 2
ha
p8
5 . . . .
ha
p8
6 . . . .
ha
p8
7 . . . .
ha
p8
8 . . . .
ha
p8
9 . . . .
ha
p9
0 . . . .
ha
p9 1
1 4

Appendix 4 Collembola intraspecific genetic distance (K2P) based on mtDNA COI (591bp) sequence variation among MOTUs

MOTU	Sequence Divergence (K2P%)
MOTU14	0,017409633
MOTU11	0
MOTU10	0
MOTU6	0,010258759
MOTU29	0
MOTU8	0
MOTU7	0
MOTU9	0
MOTU2	0,002261811
MOTU3	0,008524464
MOTU1	0,01028665
MOTU30	0,00853263
MOTU4	0
MOTU5	0,00801057
MOTU28	0
MOTU12	0
MOTU23	0,002261811
MOTU24	0,013888548
MOTU21	0
MOTU22	0
MOTU26	0,013578949
MOTU27	0
MOTU25	0,01546515
MOTU20	0,015384586
MOTU13	0
MOTU18	0
MOTU19	0
MOTU17	0
MOTU16	0
MOTU15	0,008253153
Average	1,03%
Range	0,23%-1,74%

Collembola interspecific genetic distance (K2P) based on mtDNA COI (591bp) sequence variation between MOTUs

	MOTU14	MOTU11	MOTU10	MOTU6	MOTU29	MOTU8	MOTU7	MOTU9	MOTU2	MOTU3	MOTU1	MOTU30	MOTU4	MOTU5	MOTU28	MOTU12	MOTU23	MOTU24	MOTU21	MOTU22	MOTU26	MOTU27	MOTU25	MOTU20	MOTU13	MOTU18	MOTU19	MOTU17	MOTU16
MOTU14																													
MOTU11	0,202																												
MOTU10	0,201	0,037																											
MOTU6	0,229	0,143	0,157																										
MOTU29	0,321	0,298	0,311	0,314																									
MOTU8	0,222	0,145	0,162	0,066	0,299																								
MOTU7	0,222	0,151	0,158	0,074	0,296	0,024																							
MOTU9	0,234	0,149	0,160	0,076	0,294	0,028	0,028																						
MOTU2	0,198	0,159	0,163	0,148	0,324	0,138	0,153	0,148																					
MOTU3	0,203	0,149	0,158	0,135	0,297	0,135	0,137	0,142	0,052																				
MOTU1	0,209	0,164	0,171	0,133	0,310	0,132	0,134	0,136	0,055	0,051																			
MOTU30	0,320	0,312	0,320	0,319	0,062	0,307	0,302	0,302	0,345	0,310	0,309																		
MOTU4	0,228	0,173	0,172	0,127	0,311	0,120	0,120	0,132	0,132	0,121	0,125	0,314																	
MOTU5	0,237	0,174	0,182	0,134	0,328	0,131	0,139	0,146	0,145	0,133	0,129	0,332	0,060																
MOTU28	0,310	0,318	0,321	0,285	0,340	0,281	0,273	0,283	0,285	0,262	0,294	0,358	0,300	0,294															
MOTU12	0,188	0,188	0,179	0,215	0,329	0,203	0,196	0,219	0,189	0,193	0,187	0,328	0,187	0,198	0,318														
MOTU23	0,216	0,188	0,190	0,223	0,292	0,204	0,190	0,206	0,223	0,214	0,207	0,294	0,200	0,206	0,294	0,190													
MOTU24	0,234	0,189	0,196	0,198	0,304	0,194	0,185	0,197	0,221	0,206	0,207	0,324	0,189	0,189	0,268	0,206	0,109												
MOTU21	0,227	0,217	0,210	0,211	0,349	0,215	0,211	0,218	0,226	0,218	0,238	0,356	0,228	0,228	0,310	0,229	0,219	0,211											
MOTU22	0,223	0,207	0,223	0,225	0,334	0,218	0,218	0,218	0,212	0,201	0,212	0,323	0,216	0,228	0,303	0,250	0,198	0,203	0,191										
MOTU26	0,223	0,183	0,187	0,208	0,318	0,195	0,191	0,206	0,218	0,202	0,209	0,317	0,188	0,191	0,273	0,197	0,109	0,062	0,217	0,199									
MOTU27	0,224	0,214	0,214	0,230	0,284	0,230	0,238	0,237	0,202	0,196	0,209	0,285	0,236	0,243	0,309	0,205	0,198	0,209	0,244	0,232	0,215								
MOTU25	0,235	0,184	0,189	0,210	0,304	0,200	0,184	0,198	0,227	0,197	0,212	0,306	0,193	0,205	0,291	0,220	0,110	0,091	0,221	0,191	0,055	0,213							
MOTU20	0,197	0,213	0,228	0,220	0,319	0,217	0,222	0,228	0,209	0,211	0,227	0,339	0,213	0,214	0,280	0,200	0,189	0,185	0,229	0,215	0,179	0,228	0,194						
MOTU13	0,190	0,190	0,194	0,202	0,337	0,205	0,212	0,210	0,206	0,201	0,207	0,330	0,204	0,214	0,305	0,212	0,205	0,230	0,232	0,244	0,234	0,235	0,245	0,225					
MOTU18	0,207	0,199	0,207	0,202	0,286	0,219	0,221	0,228	0,204	0,206	0,207	0,317	0,217	0,211	0,303	0,192	0,218	0,221	0,244	0,246	0,215	0,225	0,226	0,157	0,218				
MOTU19	0,199	0,199	0,204	0,206	0,273	0,210	0,207	0,224	0,190	0,195	0,191	0,300	0,205	0,201	0,295	0,182	0,212	0,209	0,255	0,255	0,200	0,221	0,216	0,156	0,212	0,035			
MOTU17	0,207	0,193	0,200	0,202	0,278	0,206	0,203	0,217	0,186	0,189	0,189	0,311	0,203	0,208	0,292	0,182	0,200	0,206	0,258	0,250	0,198	0,225	0,207	0,150	0,212	0,042	0,028		
MOTU16	0,202	0,204	0,211	0,204	0,276	0,200	0,207	0,214	0,191	0,198	0,193	0,309	0,200	0,208	0,298	0,185	0,203	0,205	0,256	0,243	0,198	0,226	0,209	0,156	0,219	0,044	0,030	0,019	
MOTU15	0,195	0,193	0,200	0,205	0,274	0,206	0,208	0,220	0,188	0,194	0,187	0,306	0,204	0,205	0,296	0,176	0,206	0,205	0,258	0,238	0,200	0,226	0,210	0,158	0,211	0,036	0,022	0,017	0,016

Appendix 5 Collembola genetic distance (K2P) based on mtDNA COI (591bp) sequence variation within groups

	d	S.E.
G	0,044033117	0,007191542
E	0,191113408	0,019790556
C	0,097919731	0,008243255
B	0,036700268	0,007894823
A	0,098096802	0,00836969
F	0,054611842	0,005939953
D	0,090749088	0,009287951

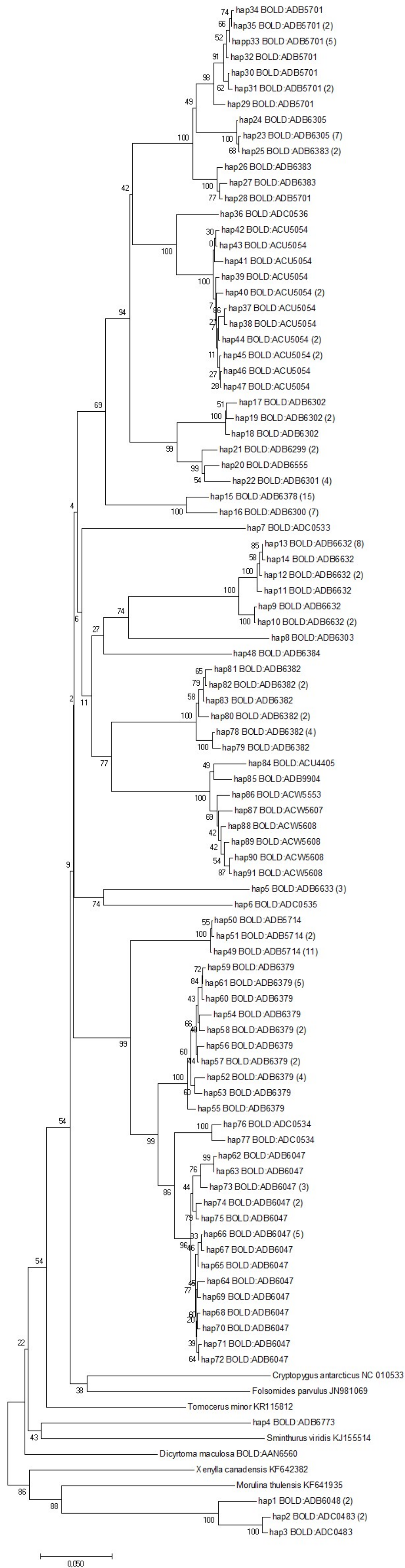
*d – distance

*S.E. – standard error

Collembola genetic distance (K2P) based on mtDNA COI (591bp) sequence variation between groups

	G	E	C	B	A	F	D
G							
E	0,338						
C	0,324	0,226					
B	0,313	0,212	0,198				
A	0,319	0,221	0,216	0,166			
F	0,313	0,207	0,223	0,187	0,201		
D	0,312	0,237	0,199	0,208	0,207	0,195	

Appendix 6 A NJ tree based on 591bp COI sequences including 178 specimens. MOTUs were assigned in BOLD (http://www.v4.boldsystems.org). The amount of individuals for each haplotype is shown in parentheses. Bootstrap values are shown next to the tree nodes



hap34 BOLD:ADB5701
hap35 BOLD:ADB5701 (2)
happ33 BOLD:ADB5701 (5)
hap32 BOLD:ADB5701
hap30 BOLD:ADB5701
hap31 BOLD:ADB5701 (2)
hap29 BOLD:ADB5701
hap24 BOLD:ADB6305
hap23 BOLD:ADB6305 (7)
hap25 BOLD:ADB6383 (2)
hap26 BOLD:ADB6383
hap27 BOLD:ADB6383
hap28 BOLD:ADB5701
hap36 BOLD:ADC0536
hap42 BOLD:ACU5054
hap43 BOLD:ACU5054
hap41 BOLD:ACU5054
hap39 BOLD:ACU5054
hap40 BOLD:ACU5054 (2)
hap37 BOLD:ACU5054
hap38 BOLD:ACU5054
hap44 BOLD:ACU5054 (2)
hap45 BOLD:ACU5054 (2)
hap46 BOLD:ACU5054
hap47 BOLD:ACU5054
hap17 BOLD:ADB6302
hap19 BOLD:ADB6302 (2)
hap18 BOLD:ADB6302
hap21 BOLD:ADB6299 (2)
hap20 BOLD:ADB6555
hap22 BOLD:ADB6301 (4)
hap15 BOLD:ADB6378 (15)
hap16 BOLD:ADB6300 (7)
hap7 BOLD:ADC0533
hap13 BOLD:ADB6632 (8)
hap14 BOLD:ADB6632
hap12 BOLD:ADB6632 (2)
hap11 BOLD:ADB6632
hap9 BOLD:ADB6632
hap10 BOLD:ADB6632 (2)
hap8 BOLD:ADB6303
hap48 BOLD:ADB6384
hap81 BOLD:ADB6382
hap82 BOLD:ADB6382 (2)
hap83 BOLD:ADB6382
hap80 BOLD:ADB6382 (2)
hap78 BOLD:ADB6382 (4)
hap79 BOLD:ADB6382
hap84 BOLD:ACU4405
hap85 BOLD:ADB9904
hap86 BOLD:ACW5553
hap87 BOLD:ACW5607
hap88 BOLD:ACW5608
hap89 BOLD:ACW5608
hap90 BOLD:ACW5608
hap91 BOLD:ACW5608
hap5 BOLD:ADB6633 (3)
hap6 BOLD:ADC0535
hap50 BOLD:ADB5714
hap51 BOLD:ADB5714 (2)
hap49 BOLD:ADB5714 (11)
hap59 BOLD:ADB6379
hap61 BOLD:ADB6379 (5)
hap60 BOLD:ADB6379
hap54 BOLD:ADB6379
hap58 BOLD:ADB6379 (2)
hap56 BOLD:ADB6379
hap57 BOLD:ADB6379 (2)
hap52 BOLD:ADB6379 (4)
hap53 BOLD:ADB6379
hap55 BOLD:ADB6379
hap76 BOLD:ADC0534
hap77 BOLD:ADC0534
hap62 BOLD:ADB6047
hap63 BOLD:ADB6047
hap73 BOLD:ADB6047 (3)
hap74 BOLD:ADB6047 (2)
hap75 BOLD:ADB6047
hap66 BOLD:ADB6047 (5)
hap67 BOLD:ADB6047
hap65 BOLD:ADB6047
hap64 BOLD:ADB6047
hap69 BOLD:ADB6047
hap68 BOLD:ADB6047
hap70 BOLD:ADB6047
hap71 BOLD:ADB6047
hap72 BOLD:ADB6047
Cryptopygus antarcticus NC 010533
Folsomides parvulus JN981069
Tomocerus minor KR115812
hap4 BOLD:ADB6773
Sminthurus viridis KJ155514
Dicyrtoma maculosa BOLD:AAN6560
Xenylla canadensis KF642382
Morulina thulensis KF641935
hap1 BOLD:ADB6048 (2)
hap2 BOLD:ADC0483 (2)
hap3 BOLD:ADC0483
0,050