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THE ENIGMATIC SUBTERRANEAN AMPHIPOD GENUS (*STERNOPHYSINX*: STERNOPHYSINGIDAE): CONSERVATION AND SYSTEMATICS

A thesis submitted in fulfilment of the requirements for the degree of Master of Science

At

Rhodes University

By

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20M6695

2021

Abstract

Sternophysingidae Holsinger, 1992 is a monotypic subterranean amphipod family endemic to Namibia and South Africa. It currently has eight described species under the genus *Sternophysinx*. The species in this family are considered narrow endemics, having only been identified using morphological characters. However, the apparent wide distribution of certain species, reported in several subterranean waters in different provinces, suggests that our current taxonomic understanding of these species needs to be completed, thus needing revision. Further, detailed knowledge of the distribution and phylogenetic relationship between species of this taxon still needs to be improved. This study aimed to use integrative taxonomy to analyse both morphology and molecular biology to study hidden diversity and to further our understanding of the distribution in this taxon. The available morphological keys were used to identify species, and 12S rRNA was also used to investigate the relationships of *Sternophysinx* species and delimit species boundaries. The study primarily aimed to use mitochondrial cytochrome c oxidase subunit I (COI). However, due to technical issues such as getting the correct primers, this study's aims were altered, and 12S was used instead of CO1. However, the process of getting CO1 sequences is still ongoing, and the results will be made available elsewhere. The current study aimed to visit all the type localities of the seven species of *Sternophysinx* reported in South Africa, including all known localities in which these amphipods have been recorded in the past. Nevertheless, this study did not get samples from Nelspruit, De Hoop Nature Reserve, and Nash Nature Reserve, which cover type localities of *S. travaleensis*, Matlapitse and Chaos cave that harbours *S. calceola*. Instead, the current study added five new localities Graaff-Reinet, Beaufort West, Hogsback, Makhambathi Nature Reserve and Ntsikeni Nature Reserve, for the distribution, including two new provinces (i.e., Eastern Cape and KwaZulu-Natal) that had records of this genus in the country. Three delimitation methods, Assemble Species by Automatic Partitioning (ASAP), Bayesian implementation of the Poison Tree Processor (bPTP) and Automatic Barcode Gap Discovery (ABGD), were used to identify cryptic species within *Sternophysinx*. Phylogenetic analyses and three delimitation methods (ASAP, bPTP and ABGD) revealed considerable hidden cryptic diversity within this *Sternophysinx*. Preliminary morphological examination revealed at least two species as undescribed from Bakwena and Sterkfontein caves, and several that were morphologically identified using the current taxonomic keys did not match with the molecular analysis. For example, *S. basilobata* from three different systems, Boesmansgat cave (Northern Cape), Beaufort West (Western Cape) and Potchefstroom (North West), was in genetically different putative species. The same is true for *S. robertsi* and *S. alca* in Ficus and Peppercorn cave in Limpopo. Overall, the results of the current study demonstrate the need for a comprehensive integrative taxonomic revision of *Sternophysinx*. These findings suggest that *S. basilobata* is a complex of distinct lineages and requires further study. On the other hand, species delimitation methods suggested that there are 12- 14 candidate species (Figure 2.5). Lastly, this study investigated the threat posed by the invasive koi fish introduced to an important cave, Boesmansgat cave in the Northern Cape, which is a type locality of two endemic species (*S. megacheles* and *S. basilobata*). Stable isotope ($\delta^{13}C$ and $\delta^{15}N$) and gut content analyses of the fish were investigated to establish whether this invasive fish feed on these critical creatures, thus endangering their long-term survival. Stable isotope signatures and the stomach gut content proved to be helpful in determining the diet of the koi fish, revealing it to be an omnivorous feeder with high invertebrate preference, including amphipods. These results indicate that the introduction of koi fish in the Boesmansgat cave threatens the long-term sustainability of these amphipods and other invertebrate assemblages in this cave.

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Acknowledgements

Many thanks go to my supervisors, Mr. Musa Mlambo, Dr Samuel Motitsoe, Prof. Charles Griffiths, and Fatima Parker, for trusting me and giving me an opportunity to work on the problematic taxon of subterranean amphipods. I am grateful for your guidance, support, and patience throughout my research project.

I am grateful to the South African National Biodiversity Institute -Global Biodiversity Information Facility (SANBI-GBIF) for providing funds for my research project. I want to thank the South African Institute for Aquatic Biodiversity (SAIAB) for allowing me to conduct my lab work on their premises. Gwynneth Matcher, Taryn Bodill, and Shereese Govender are thanked for all their help and expertise in the molecular lab.

Thank you to the Sterkfontein panel Paballo Mohafa, Cebisa Mdekazi, and Andrew Pheiffer in the Cradle of Humankind for granting me a permit to sample as well as organising Sello Lucas Sekowe to escort me to the cave. The Mount Carmel farmer owner is thanked for allowing me to sample the Boesmansgat cave, and Heinie Kohl from Albany Museum is also thanked for assisting with my fieldwork and driving me to some of my sites. Wouter Jooste and Francois Ellis are thanked for risking their lives and dive the third deepest freshwater cave that has already claimed lives to catch the invasive koi fish for me. The farm owner from Beaufort West, Dalton Gibbs, is thanked for sending me the specimen. Thanks to my colleagues Nkibi Esethu, Nonkazimulo Mdidimba, Sive Kolisi, Busisiwe Moyo, Esmier Virvier Johannes, Lulutho Mancunga and Kaylee Campbell, who accompanied me on many of my cave visits. I would also thank Alungile Jeme, Abigail, Samuel Motimele, Dr Zolile Maseko, Dr Dumisani Khosa, Dr Clarke van Steenderen, Dr Pedro Braganca and Dr Albert Chakona for assisting with data analysis. I would like to thank Tapiwa Mushore and Sydney Tshilingalinga for organising my thesis document.

Lastly, I would like to thank my moms and dad, Tumeka Mgqibi, Sibongile Tshungu and Mthuthuzeli Mlungu, for your prayers, never-ending support, love and motivation. To my siblings, Nozie Mlungu and Zolani Mgqibi, thank you for keeping me sane with your jokes, motivation, and support. Thank Luvo Tsala, for constantly checking on me and sending love and support now and then. Amvuyele Ndiliyothando Mlungu and Endinako Somila Mlungu, thank you for being my pillar of strength and pushing me to be a better version of myself. Most importantly, I thank

God for establishing me and for being my guide throughout my master's journey. None of this would be possible without God's presence.

"Trust in the Lord with all your heart and lean not on your own understanding; in all your ways acknowledge him, and he will make your paths straight" Proverbs 3:5-6

Declaration

I declare that this thesis hereby submitted by me Zizile Mlungu, to the Rhodes University (Makhanda, Eastern Cape) for the degree of Masters Science in Zoology and Entomology, and that this is my own original work.

Zizile Mlungu

Date: 13/09/2023 Signature: Mlungu Location: Grahamstown.

CHAPTER 1

The global overview of Amphipoda with a specific focus on the South Africa subterranean family Sternophysingidae

This thesis aims to conduct a taxonomic review of the South African subterranean monotypic amphipods' genus *Sternophysinx* Holsinger & Straškraba 1973, using both morphological and molecular techniques 1973. South Africa is home to two freshwater amphipod families, namely Paramelitidae Bousfield, 1977, which is mostly epigean and was studied extensively in the 1990s and Sternophysingidae Holsinger, 1992, which is mostly subterranean and has not been studied in great detail. The taxonomic identity of many of the species in *Sternophysinx* is poorly resolved, and no significant taxonomic and systematics evaluation has been done since its elevation to family-level status. This project aims to fill this gap for the first time by incorporating molecular and morphological data to study the subterranean family of South African amphipods.

1. Introduction

1.1 Intra-relationship of amphipods

Crustacea is a diverse paraphyletic taxon that occurs in aquatic (i.e., marine and freshwater) and terrestrial environments with close to 100000 described species (Webber et al. 2010; Thorp et al. 2015). Crustaceans emerged during the Cambrian radiation, with fossils recorded suggesting they descended from a marine ancestor (Schram 1982; Hobbs 2000). There are currently five extant classes represented in crustacea: Branchiopoda Latreille, 1817; Maxillopoda Dahl, 1956; Cephalocarida Sacanders (1955); Remipedia Yager 1981 and Malacostraca Latreille, 1802. Branchiopoda comprise species in temporary waterbodies such as fairy shrimps, tadpole shrimps, and smooth clam shrimps (Brendonck et al. 2008; Roger 2009). Maxillopoda is the most diverse class of crustaceans that inhabit both freshwater and marine habitat and comprise ostracods, copepods and barnacles. Cephalocarida (commonly known as horseshoe shrimps) and Remipedia are small primitive taxa with nine and ten described species, respectively (Regier et al. 2010; Aiken et al. 2013). Malacostraca class includes peracaridan order, such as Amphipoda, which forms the basis of this project.

The class Malacostraca is diverse, with over 40 000 known species, such as isopods, crabs, lobsters, and copepods, exhibiting a significant variation in morphology (Richter & Scholtz 2001; Huertos 2020). It is characterised by three jointed body regions the head with five segments, the thorax with eight segments, and the abdomen with six to seven segments (Richter & Scholtz 2001). Malacostraca is divided into five superorders: Peracarida Calman 1904, Eucarida Calman 1904, Hoplocarida Calman 1904, Bathynellacea Schminke (1973) and Syncarida Packard 1885 (Covich et al. 2010). Peracarida is the most speciose superorder comprising nine extant orders, namely; Amphipoda Latreille, 1816; Cumacea Krøyer, 1846; Isopoda Latreille, 1817; Mysidacea Haworth, 1825; Tanaidacea Dana, 1849; Mictacea Bowman et al. 1985; Spelaeogriphacea Gordon, 1957; Lophogastrida Sacars, 1870 and Thermosbaenacea Monod, 1927 (Martin & Davis 2001).

Amphipoda is a monophyletic order whose phylogenetic relationship with other peracarids has been controversial (Richter & Scholtz 2001; Poore 2005; Spears et al. 2005). For example, Poore (2005) demonstrated the phylogenetic positions of isopods and amphipods as sister groups. However, this contradicts Spears et al. (2005), who concluded the opposite, isopod and amphipod are not closely related. Instead, Richter & Scholtz (2001) posited a close relationship between amphipods and Spelaeogriphacea, not amphipods and isopods or even mysids. Even though the Spelaeogriphacea - Amphipoda relationship was not strongly supported morphologically, phylogenetic analysis revealed a sister-group relationship between the two (Poore 2005; Spears et al. 2005). Interestingly, the intra-relationships within Amphipoda have also been revised recently (see 1.3)

1.2 The order Amphipoda

The name "amphipod" comes from Greek origin, meaning "different feet", while isopod means "same feet" (Chapman 2007). Amphipoda is one of the largest malacostracan taxa, with over 10 000 described species in the sea, land, lakes, rivers, sand beaches, and subterranean (Lowry & Myers 2013; Horton et al. 2021). Amphipods mostly inhabit aquatic habitats, with about 80% occurring in marine and 20% occurring in freshwater habitats (Väinölä et al. 2007; Lowry & Myers 2017). Amphipods are brooding malacostracan crustaceans (like mysids and isopods), whose young undergo direct development until they reach an advanced stage, with no independent larval dispersal stage (Covich et al. 2010, Wellborn et al. 2015). This order comprises mostly active swimmers usually found hiding in gravel and aquatic plants and may occur in dense population sizes. Ecologically, amphipods are an essential food source for fish species (Grabowski et al. 2007; Martín et al. 2013); this will be explained in detail in Chapter 3.

Unlike the rest of the malacostracan taxa, amphipods possess three pairs of pleopods and three pairs of uropods, while the other taxa have only one pair of uropods. Amphipods generally possess eight pairs of thoracic appendages (sometimes called thoracopods or pereiopods); the first five are used for walking, and the other three pairs are generally modified for swimming (Poore 2002). Within Malacostraca Latreille 1802, amphipods are morphologically mistakenly with isopods (Covich et al. 2010). However, isopods are dorso-ventrally flattened, whereas amphipods are laterally compressed; as a result, they swim sideways and are generally called side-swimmers.

1.2.1 Freshwater amphipods

Globally, more than 1900 described amphipod species occur in freshwater habitats (Väinölä et al. 2007; Wellborn & Cothran 2015). Amphipods are highly endemic to specific regions and principally occur in highly oxygenated freshwater habitats with exception to subterranean taxa the strive in oxygen deprived conditions (Covich et al. 2010). Consequently, they have been chiefly used as bioindicators of water quality, especially the genera *Gammarus* and *Niphargus* (Karaman & Pinkster 1977; Altermatt et al. 2014). For example, a study on the presence of per-and-polyfluorinated compounds (PFC) in the freshwater system was investigated using freshwater amphipods of the genus *Gammarus* Fabricius, 1775 and organs of brown trout (Windisch et al. 2020). This study found traces of PFC chemicals in both species and concluded that amphipods were a more reliable indicator for PFC contamination than fish species. Plénet (1999) investigated the resistance of the accumulation of Zn and Cu on the *G. fossarum* (Koch, 1836) and *N. rhenorhodanensis* and found that *N. rhenorhodanensis* is more resistant to Zn and Cu. The study consequently concluded that both amphipods' species are good indicators of water quality and, as such, can be used to assess ecological risk associated with anthropogenetic activities.

Amphipods are also significant in food webs as they are food sources for some living organisms such as fish (Sieg et al. 2009; Raz-Guzmán & Villegas 2018) and bats (Moyo & Jacobs 2020). They play a vital role in maintaining aquatic community structure, trophic interactions and nutrient recycling (Raz-Guzmán & Villegas 2018; Altermatt et al. 2014). For example, *Gammarus fossarum* is an essential decomposer that breaks down carbon sources (i.e., organic and inorganic matter), nitrogen, phosphorous and other associated nutrients in freshwater systems (MacNeil et al. 1997). Similar feeding behaviour was observed in *G. pulex* (Linnaeus, 1758), where the species is well adapted for feeding on organic material and fungi using the foregut and hindgut, respectively (Agrawal 1965). Studies have shown the importance of amphipods in aquatic communities by cycling organic matter (Piscart et al. 2007, 2009, 2011).

Amphipods are diverse in temperate regions, especially in the Northern Hemisphere occurring in permanent water bodies (Väinölä et al. 2007; Rodrigues et al. 2014). For instance, the genera *Gammarus* and *Niphargus* Schiødte, 1847, are the most speciose amphipods in epigean and subterranean habitats, respectively (Barnard & Barnard, 1983; Fišer et al. 2005; Väinölä et al. 2007). However, few species have been recorded in temporary water systems, and their survival strategies in such habitats are not well known (Covich et al. 2010). For example, *Crangonyx pseudogracilis* Bousfield, 1958 is a surface occurring species observed in temporary water pools with no connection to permanent water (Harris et al. 2002). Surface freshwater amphipods usually hide in vegetated areas, some under small rocks, and prefer slow-flowing water (Copilaș-Ciocianu et al. 2014; MacNeil & Dick 2014). Most freshwater amphipods occur in surface water, with genera such as *Gammarus* and *Hyalella* Smith, 1874, highly abundant in this habitat (Rodrigues et al. 2014). In addition, studies conducted in the Northern Hemisphere, specifically on Lake Baikal, showed that this lake is home to diverse species that are endemic to this ancient lake (Kamaltynov 1999; Macdonald et al. 2005).

The Southern hemisphere, on the contrary, appears to have less freshwater amphipod diversity, with few known taxa. Moreover, our understanding of the distribution of freshwater amphipods is still lagging. Meanwhile, continents such as Europe have well-documented taxonomic knowledge of amphipods (Fišer et al. 2005; Copilaș-Ciocianu et al. 2015; Copilaş-Ciocianu & Petrusek 2015). South Africa has two crangonyctoid families; Paramelitidae Bousfield, 1977, is endemic in Western Cape and Sternophysingidae Holsinger, 1992, is exclusively subterranean taxon (Griffiths 1991; Holsinger 1992; Griffiths & Stewart 1996, 2001; Väinölä et al. 2007; Lowry & Myers 2013). Paramelitidae is the most species-rich family in South Africa, with 26 described species (Griffiths & Stewart 2001). This family is only endemic to the southwestern Cape region in South Africa and comprises mainly epigean species (Griffiths & Stewart 2001). Meanwhile, Sternophysingidae is almost exclusively a subterranean family, found in springs, caves and karsts with eight known species, seven occurring in South Africa and one in Central Namibia (Griffiths & Stewart 2001). However, the knowledge about the distribution and diversity of subterranean amphipods in South Africa is lacking, and this issue will be explained further in Chapter 2.

South Africa, along with Madagascar, Morocco, and Algeria, has the highest recorded diversity of freshwater amphipods in Africa (Kayo et al. 2012). Studies of freshwater amphipods in Africa are scant, as represented by only a few historical records (Ruffo 1984; Messouli et al. 2001; Kayo et al. 2012; Piscart et al. 2013). Nevertheless, African amphipods are quite diverse and constitute about 24, 5% of freshwater amphipods represented by 13 families, of which one of the families is endemic to the region, i.e., Sternophysingidae (Väinölä et al., 2008; Kayo et al. 2012). About 72 amphipod species have been described in Africa; however, Kayo et al. (2012) argue that more species are occurring in this region, and due to the difficulties in sampling and insufficient data about this order hinder the correct estimation of African amphipod diversity. However, with improved surveys, new species have been identified. Piscart et al. (2013) survey at Mount EL Hamra in northeastern Algeria led to the identification of a new species *Echinogammarus haraktis* Piscart et al. 2013 from the genus *Echinogammarus* Stebbing, 1899. Numerous researchers' continued identification of amphipod species indicates insufficient sampling methods (Rewicz et al. 2014; Ayati et al. 2018; Hadjab et al. 2020). However, Africa continues to have insufficient amphipod diversity as most studies have concentrated on surface amphipods leaving subterranean realms less explored. For instance, *Bogidiella nubica* Ruffo, 1984 was known to occur in the subterranean waters in South Africa and Somalia. Ruffo (1984) study consequently identified *B. nubica* for the first time in Sudan. Similarly, *Sternophysinx hibernica* Griffiths, 1991 has been only recorded in the subterranean waters of Central Namibia. These finding clearly shows that more data and sampling are needed to identify the diversity of subterranean habitats.

1.3. Systematic classification of amphipods

In aquatic or terrestrial habitats, amphipods have been taxonomically challenging; however, in the last decade, the order has been revised with new suborders and infraorders (Lowry & Myers 2013, 2017; Myers & Lowry 2018). Four suborders historically represented Amphipoda: Gammaridea Latreille, 1802; Caprellidea, Leach 1814; Hyperiidea Milne Edwards, 1830 and Ingolfiellidea Hansen, 1903 (Chapman 2007; Lowry & Myers 2013). Caprellidea and Hyperiidea are exclusively marine amphipods (Chapman 2007). Caprellidea, also called skeleton shrimp, is only found in the marine environment in algae, sponges, seagrasses and other environment (Chapman 2007; Friend & Richardson 1986). Hyperiidea, differentiated from other suborders by enlarged eyes, is composed mostly of parasitic species, with only a minority that is free-living (Vinogradov et al. 1996). Ingolfiellidea is the smallest suborder, with only 44 described species occurring in the deep sea, freshwater, subterranean and brackish environment (Vonk & Schram 2003; Martín et al. 2013). Gammaridea is the most species-rich suborder with 8 300 described species, and its species mostly occur in cold and temperate regions (Chapman 2007; Martín et al. 2013; Martin & Davis 2001). The latter suborder comprises species occurring in both aquatic (marine and freshwater environment) and terrestrial environment. The above classification was mainly based on morphological similarities between species without considering the shared traits that explain more about the species' common ancestor (Vader 1983; Poore 2002). Lowry and Myers (2013) re-examined Amphipoda by investigating 115 synapomorphy characters and revised the phylogenetic classification to fill this gap.

A new classification was proposed by Lowry & Myers (2017) elevated the Ingolfiellidae to a status of an order level on their own, removing it from Amphipoda. Even though amphipods and ingofiellids share traits such as no carapace and coxal gills morphologically, ingofiellids are not considered as part of amphipods due to their different characters (Lowry & Myers 2017). Further, Myers & Lowry (2018) proposed six new Amphipoda suborders: Amphilochidea Boeck, 1871; Colomastigidea Stebbing, 1899; Hyperiidea Milne Edwards, 1830; Hyperiopsidea Birstein & Vinogradov, 1955; Pseudingolfiellidea Lowry & Myers, 2012 and Senticaudata Lowry & Myers, 2013. All these suborders share about 115 common evolutionary ancestral traits, with the possession of robust setae on the apices of uropod 1 and 2 regarded as the important character state.

Senticaudata is the newly proposed Amphipoda suborder, which comprises about 100 families that were previously included in Gammaridea (Myers & Lowry 2018). All these families had shared traits such as coxal gills (unstalked or stalked), sternal simple gills (absent or present) and other character traits. Six infraorders in the suborder Senticaudata were also proposed, including Carangoliopsida Bousfield, 1977; Talitrida Rafinesque, 1815 (Serejo, 2004); Hadziida Karaman, 1943; Corophiida Leach, 1814 (Myers & Lowry, 2003); Bogidiellida Hertzog, 1936 and Gammarida Latreille, 1802. Senticaudata is the largest and most diverse suborder of Amphipoda, which incorporates all freshwater amphipods, including the Sternophysingidae, which will be the focus of this thesis. However, the phylogenetic position of Sternophysingidae within this suborder is unknown, as no molecular data is currently available. Similarly, the inter-species relationships of this genus are not currently known for the same reason as no molecular data is available (but see Murray 2020).

1.4. Sternophysingidae Holsinger, 1992

Sternophysingidae is characterised by enlarged gnathopod 1 and the absence of coxal gill on pereiopod 7 (Holsinger 1992; Griffiths 1991). This family is comprised of a monotypic genus, *Sternophysinx* bousHolsinger & Straškraba, 1973, established by Holsinger & Straskraba (1973) (Bousfiled 1977; 1979). Sternophysingidae is a subterranean family compromising of species occurring in caves and karst systems. The genus *Sternophysinx* has a complicated taxonomic history, having been assigned to the family Bogidielloidae (Bousfield 1977), then to the family group *Pseudocrangonyx-sternophysinx* Bousfield (1978), then to the family Paramelitadae. Furthermore, this genus was removed from the family Paramelitidae without being assigned to any family (Holsinger and Straskraba 1973). It was then assigned to a new family Sternophysingidae (Holsinger 1992). *Sternophysinx robertsi* was the first species described in it but was named *Eucrangonyx robertsi* Methuen, 1911. *Eucrangonyx robertsi* was moved to a new genus *Crangonyx* Bate, 1859 which was also known as *Eucrangonyx*; the newly assigned genus *Crangonyx* created questions about its occurrence in the Southern Hemisphere. *Crangonyx* is a Northern Hemisphere genus endemic to the Holarctic region (Holsinger & Straskraba 1973; Holsinger 1992), and its occurrence in the Southern Hemisphere was questionable. Consequently, Holsinger & Straskraba (1973) conducted a study on the occurrence of *Crangonyx* in South Africa and described a new genus *Sternophysinx*. *Eucrangonyx robertsi* was then renamed as *Sternophysinx robertsi* (Methuen, 1911). This genus comprises seven known species in the South African subterranean environment (Griffiths & Stewart 2001; Milne & Griffiths 2013). In the last 20 to 30 years, there has been increased observations of this genus and more questions have arise about their distribution and systematic classification.

`1.4.1 Distribution of the genus *Sternophysinx* in South Africa

In South Africa, *Sternophysinx* spp. have been recorded in several caves such as Sterkfontein, Nash Nature Reserve, Koelenhof cave and Bakwena caves in Gauteng province; Matlapitse cave, Ficus and Peppercorn cave in Limpopo; Boesmansgat cave in Northern Cape; De Hoop Nature Reserve in Western Cape and Chaos cave in the North West Province (Holsinger & Straskraba 1973; Holsinger 1992; Griffiths & Stewart 1996; Tasaki 2006; Milne & Griffiths 2013). Generally, members of this genus were thought to be narrow endemics, as they were mostly known from single locations, but comprehensive compilation of all the records from the literature shows that is mostly not true (see Table 1). Indeed, several species are still known from only one location, such as *Sternophysinx alca* and *S. megacheles*, but several more present a wide distribution, like *S. travaalensis*, *S. filaris* and *S. calceola*. So, it is not clear whether this pattern is artefact of more sampling, or whether this is true reflection of the status question. However, experiences elsewhere have shown that species that were thought to be widely distributed have turned out to be harbouring cryptic diversity (Finston et al 2004; Copilas-Ciocianu & Petrusek 2017; Delić et al. 2017a). This study will aim to contribute to figuring out whether *Sternophysinx* spp. are naturally widely distributed or not.

Sternophysinx robertsi (Methuen, 1911) was the first species to be described and its population is recorded from two nearby caves in Makapan's Valley, i.e., Peppercorns and Ficus cave in Limpopo (Table 1), and the second population in Bakwena cave (previously called Irene cave) in Gauteng (Holsinger & Straskraba 1973). These sites are approximately 250 km apart, and by looking at the geographical distribution of this species, such distribution range is interesting for species that inhabit caves (Holsinger & Straskraba 1973; Tasaki 2006). It is an active swimmer that co-occurs with the less active *S. alca*. This species is differentiated from *S. tranvaalensis* and *S. alca* by possessing the expanded article 2 of pereiopods 5-7.

The second species to be described was *Sternophysinx filaris* Holsinger & Straškraba, 1973 from Sterkfontein Caves, Gauteng. This species has also been recorded in several caves, such as Koelenhof Cave, in the same geographic area as Sterkfontein cave and Chaos Cave near Potchefstroom in North West province (Table 1). This species is distinguished by thread-like hairs on pereopods 5 and 6 (Holsinger and Straskraba 1973; Milne & Griffiths 2013). It is usually found swimming on the surface and has been regarded as an active swimmer and a close relative of the less abundant *Sternophysinx megacheles* Griffiths & Stewart, 1996 (Holsinger 1992, Griffiths & Stewart 1996). It has also been reported from Bakwena cave, which is more than 60 km from Sterkfontein (Durand et al. 2012). This species has also been reported to occur in two provinces, Gauteng and North West, making it one of the species with an interesting wide distribution and a potential taxonomic challenge that requires further investigations.

Sternophysinx transvaalensis Holsinger & Straškraba, 1973 was the third *Sternophysinx* species to be described in this genus. It was initially reported from the spring in Nelspruit, Mpumalanga Province (Holsinger & Straskraba 1973; Griffiths 1981). Compared to the other species, *S. transvaalensis* has a small body size, with females (5.2mm) larger than males (4.5mm), a reduced article 2 of pereiopods 5-7 and short setae (Holsinger & Straskraba 1973; Griffiths 1981). This species has subsequently been reported in in several locations: including, De Hoop Nature Reserve, Western Cape, Nelspruit, Mpumalanga Province, and Nash Nature Reserve in Gauteng (see Table 1).

Sternophysinx alca Griffiths, 1981 is the fourth species described in this genus, occurring from Peppercorn's cave and Ficus cave in Limpopo. This species is distinguished by the excavation and tooth on the palm of gnathopod 1 and 2 (Griffiths 1981). It has been recorded alongside *S. robertsi* and is a poor swimmer (Stewart & Griffiths 1996). *Sternophysinx alca* has been recorded only from its type locality (Griffiths 1981).

Sternophysinx basilobata Griffiths, 1991 was initially described from specimens collected from Boesmansgat cave near Kuruman in Northern Cape Province (Stewart & Griffiths 1996). The second population was collected from the Kogelbeen cave (Irish & Marais 2002). It is characterised by anterior expansion of article 2 of pereopods 3 and 4 (Griffiths 1991). Morphologically, *S. basilobata* resembles *S. filaris* by possessing setose on article 6 and spinules on pereopods 3-7 (Stewart & Griffiths 1996). It is one of the amphipods that occurs in more than one location (Table 1) and is found in the same locality as *S. megacheles* in Boesmansgat cave (Milne & Griffiths 2013; Stewart & Griffiths 1996).

Sternophysinx calceola Holsinger, 1992 was initially recorded from Matlapitse cave near Trichardtsdal (Holsinger 1992). This species is characterised by the possession of paddle-shaped calceoli on antenna 2 and is closely related to *S. alca* and *S. transvaalensis* (Holsinger 1992). This species is also distinguished by its large body size, which ranges between 9.5 to 18 mm, with females larger than males. It has been reported to occur in more than one location covering three provinces Gauteng (Bakwena, Koelenhof cave and Sterkfontein cave), North West (Chaos cave) and Limpopo (Matlapitse cave) (Table1). In all localities except Matlapitse cave, *S. calceola* co-occurs with *S. filaris*.

Sternophysinx megacheles was described from Boesmansgat cave. This species shares common traits with *S. filaris*, such as notched telson expanded article 2 of pereopods 5-7 and setal brushes on pereopods 5 and 7 (Griffiths & Stewart 1996). It co-exists with *S. basilobata*. Of all the seven known species, only four are widely distributed: *S. filaris. S calceola, S. basilobata* and *S. transvaalensis*. Their distribution in the South African region is recorded in the table below.

Table 1.1: Checklist of the distribution of *Sternophysinx* in South Africa

Species	**Type locality**	**Museum where types are deposited**	**Collected**	**Additional localities where the species has been recorded**	**Provinces**	**References**
Sternophysinx alca Griffiths, 1981	Peppercorns Cave[1,8]	South African Museum	June 1979	Ficus cave[1,7,8]	LP	Griffiths 1981; Milne & Griffiths 2013; Durand & Peinken 2010
Sternophysinx filaris Holsinger & Straskraba, 1973	Sterkfontein Cave[2,6,7,8]	Museo Civico Di Storia Naturale	Sep 1954	Koelenhof Cave[8], Chaos Cave[7,8], Bakwena cave[2,6,7,8,9] Grobler’s Cave[11]	GP, NW	Holsinger & Straskraba 1973; Tasaki 2006; Milne & Griffiths 2013; Durand & Peinken 2010; Durand et al. 2012; Murray 2020
Sternophysinx robertsi (Methuen, 1911)	Makapan’s cave[2,6,7,8]	South African Museum	Feb 1911	Peppercorn cave[2,7,8], Ficus[2,6,7,8]	LP	Holsinger & Straskraba 1973; Tasaki 2006; Milne & Griffiths 2013; Durand & Peinken 2010
Sternophysinx basilobata Griffiths, 1991	Boesmans Gat cave[3,7]	South African Museum	Dec 1988	Kogelbeen[10]	NC, WC	Griffiths 1991; Milne & Griffiths 2013; Irish & Marais 2002
Sternophysinx calceola Holsinger, 1992	Matlapitse cave[5]	Ditsong National Museum, of Natural History	Dec 1985	Koelenhof Cave[6,8], Chaos Cave[7] Bakwena cave[9] Grobler’s Cave[11]	LP, NW, GP	Holsinger 1992; Tasaki 2006; Milne & Griffiths 2013 ; Durand & Peinken 2010; Durand et al. 2012, Murray 2020
Sternophysinx transvaalensis Holsinger & Straskraba, 1973	Near Nelspriut[2,6,8] S 25^0 33’ 20¨ E30^0 47˒ 40¨	Museo Civico Di Storia Naturale	May 1955	De Hoop Nature Reserve[7], Nash Nature Reserve[6,8]	MP, WC, GP	Holsinger & Straskraba 1973; Tasaki 2006; Milne & Griffiths 2013; Durand & Peinken 2010

Sternophysinx megacheles Griffiths & Stewart, 1996	Boesmansgat cave[4,7]	South African Museum	Aug 1991		NC	Griffiths & Steward 1996; Milne & Griffiths 2013

Limpopo - LP, Gauteng- GP, Mpumalanga- MP, North West- NW, Kwa-Zulu Natal-KZN, Western Cape- WC, Northern Cape-NC

1.5 Problem statement

Studies on subterranean amphipods with wide distribution have shown that such species are either cryptic or complex and have been studied intensively in other regions. Currently, the taxonomic work of this family in a subterranean environment is poorly known, and there has been no subsequent work on this family since the 1990s (Griffiths & Stewart 2001). Milne & Griffiths (2013) compiled known distribution records of members of this genus at the time, and the current study (Table 1) further builds up on that work, greatly improving our understanding of the distributional record. The existence of species exhibiting wide distribution is intriguing because these subterranean species were historically thought to be range restricted to just one cave. The current taxonomic identification of these subterranean amphipods needs revision to accommodate this new observation of the potentially wide occurrence of species in this taxon. There are two possibilities at play here. Firstly, these species showing unusually expansive distribution might be part of the species complex harbouring cryptic diversity. Secondly, the observed distribution might represent the natural state scientists are only uncovering now, courtesy of increased sampling efforts. If there are hidden cryptic diversity and new species are discovered, there will be a need to revise the taxonomic keys. Integrative taxonomy, whereby both morphological and molecular datasets are used to solve taxonomically challenging taxa, will be needed here (Finston et al. 2004, 2007).

1.6 Aims

This project has two overarching aims:

i. To conduct an integrated taxonomic review, using both morphological and molecular assessments, including the DNA barcoding of South African sternophysingid amphipods: Chapter 2.

ii. To investigate the impact of an introduced koi fish on the Boesmansgat cave amphipod population: Chapter 3.

1.7 Objectives

i. Perform DNA barcoding for all the currently known species by sampling topotypic material from all the known locations, plus looking for new localities.
ii. Conduct description of any new species found during the step above.
iii. Use stable isotope analysis to assess the feeding of an introduced koi fish at an important cave, Boesmansgat cave, that houses two endemic subterranean amphipods (i.e., *S. basilobata* and *S. megacheles*) to assess if they endangered by this fish.

CHAPTER 2

Integrating morphological analysis and mitochondrial genes (12S) to examine the diversity and phylogenetic relationship between *Sternophysinx* species

Sternophysinx is a genus of subterranean amphipods endemic to Namibia and South Africa (Griffiths 1991). However, the taxonomic classification of this taxon appears to be in flux, and the available taxonomic keys need to be updated, requiring revision. Additionally, recent studies on subterranean amphipods in other continents have shown a high level of morphological variation in subterranean amphipod species and have highlighted the need to incorporate other efficient ways of identifying species (Finston et al. 2004; Finston et al. 2007). In South Africa, detailed knowledge of the distribution and phylogenetic relationship between subterranean amphipods still needs to be improved. This study has used morphological and molecular analysis to re-evaluate the distribution and to understand the phylogenetic relationship amongst subterranean amphipods for the genus *Sternophysinx*. Available morphological keys (Griffiths & Stewart 2001) were used to identify collected amphipod specimens from Boesmansgat cave, Bakwena cave, Sterkfontein cave, Beaufort West, Ficus cave, Peppercorn cave, and North West. Additionally, the 12s ribosomal RNA gene was also used to complement the morphological identification and investigate the relativeness of *Sternophynsix* species.

2. Introduction

Based on the global distribution findings of freshwater amphipods, Barnard & Barnard (1983) and Väinölä et al. (2008) have proposed that the northern hemisphere region is the most species-rich as compared to other regions. For example, 45% of the Northern Hemisphere subterranean amphipod diversity is in the families Niphargidae Bousfield, 1977 and Crangonyctidae Bousfield, 1973 (Väinölä et al. 2008). On the other hand, the southern hemisphere lineages have diversified from the Gondwanaland prior to the fragmentation during the Jurassic period (Barr 1968). The Southern hemisphere includes 33 described freshwater amphipod families, with 18 being endemic to the region (Väinölä et al. 2008). Sternophysingidae is one of the 33 subterranean amphipod families distributed in the Southern Hemisphere in the Gondwana region (Väinölä et al. 2008).

As discussed briefly in Chapter 1, South African *Sternophysinx* spp. consists of several widely disturbed species, namely *S. filaris, S. calceola, S. basilobata* and *S. tranvaalensis* (Holsinger & Straskraba 1973; Holsinger 1992; Griffiths & Stewart 1996; Tasaki 2006; Durand et al. 2010). On the other hand, it also includes narrow endemics *Sternophysinx megacheles*, *S. alca* and *S. robertsi*, and so far, they have only been recorded from a single location. Previous case studies (see Holsinger & Straskraba 1973; Griffiths 1981; Griffiths & Stewart 1996) on this genus have been mainly morphologically based, and no molecular work has been done. The systematics remains unresolved, and taxonomy is in flux. Most work conducted on subterranean amphipods has found high levels of cryptic diversity, i.e., species that are genetically different but have been identified as one due to morphological similarities (Hou et al. 2007; 2011; Copilas-Ciocianu & Petrusek, 2015; 2019; Finston et al. 2004, 2017; Fišer & Zagmajster 2009). In South Africa, studies to elucidate potential cryptic diversity in freshwater amphipods have not yet been forthcoming, a gap the current study aims to contribute towards.

2.1. Subterranean organisms and their adaptation

2.1.1 Subterranean environment

Subterranean environments are home to many important organisms and are the primary source for freshwater systems (Mammola et al. 2019). Waters in springs, wells and boreholes are outflows of subterranean waters. Compared to surface species, subterranean species description and biodiversity assessment is still lagging far behind (Gibert et al. 1994). However, thousands of subterranean species, both stygofauna (aquatic species occurring in caves, springs and karst systems) and troglofaunal (air-breathing terrestrial species that inhabit caves systems), have been identified (Gibert et al. 2009). As a result of the extreme conditions in the subterranean environment, such as food scarcity, low oxygen and species that morphologically look similar (Trontelj et al. 2009), researchers have been exploring this environment. Gibert & Culver (2009) review of the diversity of subterranean species indicated that Europe holds the record with over 2000 described species compared to other continents, and Africa has the least diversity with 335 known species. Most inhabitants of the subterranean environment are crustaceans such as isopods, amphipods, copepods and other stygofauna, however, few vertebrates, nematodes, and arachnids have been occasionally observed (Galassi 2001; Culver et al. 2010; Culver & Pipan 2015).

The subterranean environment is distinguished by reduced sunlight exposure and, in some cases, complete darkness. As such, stygofauna and troglofaunal exhibit unique traits such as reduced sight, depigmentation, and elongated appendages (Abrahams 2012; Kralj-Fišer et al. 2017, 2020). These unique features make these species vulnerable to habitat alteration as their habitat permit slow population growth (Humphreys 2000). Unfortunately, increased human demand for water puts these stygofauna species under threat. In South Africa, subterranean environments are not well studied and are under threat by human activities such as mining, farming, fragmentation, destruction and pollution (Sharratt et al. 2000; Durand et al. 2012; Du Preez 2014; Ferreira et al. 2020). Despite this, subterranean species are still understudied (Gibert et al. 2009), this is also true for South Africa where the faunal diversity of this habitat is not well studied (Durand 2008).

Due to their paleontological importance, subterranean environments, particularly in the Gauteng region of South Africa, have been of interest in studying the origin of humans, for example, Sterkfontein caves (Durand 2008, 2012; Durand et al. 2012). Other ecosystems like the Bakwena Cave, Grobler's Cave and Kemp's Cave are a few caves that are home to endemic bats species such as *Miniopterus natalensis* Smith, 1833, *Myotus tricolor* Temminck, 1832 and *Rhinolophus clivosus* Cretzschmar, 1826 and have been explored to investigate the diversity of troglofauna and stygofauna (Durand et al. 2012). Moreover, the Bakwena cave is a home to various creatures, including; amphipods, nematodes, mites, beetles, moth flies and spiders (Durant et al. 2012; Jacobs et al. 2017; Moyo & Jacobs 2020). The troglofaunal species, such as, nematodes have been reported to have been occasionally accidentally introduced in the subterranean environment. Du Preez et al. (2015) investigated the occurrence of nematodes in the Wonderfontein Cave and reported that these species are abundant in this cave system. According to this study, Wonderfontein Cave comprised of 53 nematode genera (Du Preez et al. 2015). Northern Cape is one of the South African regions dominated by caves, and Irish & Marais (2002) showed species diversity in caves species in the Northern Cape Province. This study explored eight caves or karst systems, and both troglofauna and stygofauna specimens were collected or observed. From the surveyed caves, Kogelbeen cave was reported to be the most speciose-rich cave comprising bats, fungi, isopods, amphipods and spiders (Irish & Marais 2002).

2.1.2 Subterranean amphipods

Amphipods have been observed in subterranean habitats, either subterranean lakes, i.e., caves or karst systems or in springs (Culver et al. 2010). For years, subterranean amphipods have been characterized as highly endemic species, with narrow distributions. This fits the view of subterranean habitats as being highly fragmented, thus leading to divergent evolution in which the different species develop similar traits through convergence despite disparate geographic locations. With intensive subterranean exploration and a rise in publications about this habitat, subterranean amphipods have been reported to have a broader distribution, occurring in more than one locality. With the increased studies of subterranean amphipods, widely distributed subterranean amphipods have been frequently observed. For example, *Pseudocrangonyx* Akatsuka & Komai, 1922 is a subterranean genus diverse in springs in the east Asian region with 27 described species (Jung et al. 2020). This genus is poorly resolved with species that have been described as one. However, studies have been conducted to fill this gap (Lee et al. 2018, 2020; Jung et al. 2020).

Subterranean amphipods have energy limitations, such as reduced metabolism and movement due to the limited light. From the feeding point of view, subterranean habitats are underprivileged environments with significantly low energy sources (Hervant et al. 1997; Huppop 2000; Hervant et al. 2001; Hervant & Renault, 2002; Gibert & Deharveng 2002). The absence of light prevents the production of primary producers, promoting specialist feeding behaviour. However, chemolithotrophic bacteria are primary producers in subterranean habitats, which has been observed in deep-sea (Kumaresan et al. 2014). However, the absence of a primary producer in this habitat is advantageous to subterranean inhabitants as plants use already limited oxygen (Humphreys 2006).

Subterranean amphipods comprise a slender or slim body with short appendages to permit movement between rocks and shelter under small gravel (Kralj-Fišer et al. 2020; Väinölä et al. 2007). However, body size in subterranean amphipods also aids in migration, with amphipod species with a body size of less than 1 mm having been found occurring in interconnected cave systems (Copilaş-Ciocianu et al. 2018). For instance, *Stygobromus mackini* Hubricht 1943 and *Crangonyx antennatus* Cope & Packard, 1881 have wide distribution and have been observed in geographically close regions (Trontelj et al. 2019). Subterranean species generally have restricted distribution, with few species displaying a patchy and fragmented range in springs, karsts, boreholes and caves (Copilaş-Ciocianu et al. 2018). Studies on genera such as *Stygobromus* Cope, 1872*, Synurella* Wrześniowski, 1877*, Crangonyx* Bate, 1859*, Niphargus* Schiödte, 1849*, Hyalella* Smith, 1874 and *Gammarus* Fabricius, 1775 have shown subterranean amphipods to be broadly distributed, and many species have shown that these genera are highly cryptic due to their similar environmental conditions (Väinölä et al. 2007; Trontelj et al. 2009; 2012; Copilaş-Ciocianu et al. 2019).

2.2 Cryptic speciation of freshwater amphipods

Studies have revealed that the diversity of subterranean amphipods has been underestimated, and for years this taxon has been diagnosed through morphological-based methods. However, phenotypic conservatism and convergent evolution have weakened the use of morphologically based identification, potentially leading to the present underappreciation of diversity. This has been observed in cases where closely related and distant species are superficially similar in morphology and are identified as the same species (Bickford et al. 2007). With increasing accessibility and exploration of subterranean habitats, our understanding of their biological diversity is improving. Cryptic species not only cause misidentification of species but may hinder the inclusion of those species in conservation programs (Prié et al. 2012). The lack of appropriate taxonomic keys to capture cryptic diversity is a serious issue, hindering progress in distributional assessment and systematic research (Fišer & Zagmajster 2009; Fišer et al. 2015). Cryptic speciation is a well-documented phenomenon in both subterranean and surface amphipods species (Fišer et al. 2015). Cryptic species have created confusion in taxonomy, making conservation programmes impossible (Westram et al. 2013; Alther et al. 2017; Delić et al. 2017b). For example, the South African genus *Paramelita* comprises two species; *P. capensis* (Barnard, 1916) and *P. nigroculus*, that were originally reported to be widely distributed (Griffiths 1981; Cook 1991). However, Stewart (1992) investigated *P. capensis* by incorporating morphological and molecular analysis and revealed that this species is a complex of five species.

The subterranean environment supports cryptic diversity due to the extreme conditions of the habitat allowing for slow and limited phenotypic variation. Like most other subterranean creatures, Amphipods in this habitat are a textbook example of morphological conservatism (Westram et al. 2013). Consequently, subterranean amphipods have adopted similar traits that make it difficult to separate them morphologically. For example, *Gammarus fossarum* and *G. pulex* (Linnaeus, 1758) have been misidentified as the same species due to remarkable morphological resemblance; however, these two species belong to different species complexes (Rudolph et al. 2018). Many subterranean amphipod taxa have been found to exhibit cryptic diversity (Trontelj et al. 2007; Copilaş-Ciocianu et al. 2018; Rudolph et al. 2018). For example, Lefébure et al. (2007) conducted a study on *Niphargus rhenorhodanensis*, a widely spread subterranean amphipod found to be a species complex (comprising of cryptic species). Similar results were observed on *N. virei*, where at least three cryptic species were identified within the *N. virei* species complex (Lefébure et al. 2006). Recently, molecular analysis through DNA sequences has been used to identify and solve cryptic species. For over 20 years, most studies have incorporated both morphological keys and DNA analysis to identify species, especially cryptic species (Gaines et al. 2005).

2.3 Integrative taxonomy to solve cryptic species

Traditionally, species have been identified using only morphological keys; over time, the keys have become deficient due to convergence and morphological conservatism (Schlick-Steiner et al. 2010). Furthermore, the older classification keys in some taxa, especially invertebrates, have been disputed even though other identification techniques, such as image analyses and morphometrics, have been used. This is due to the diverse morphological similarities between phylogenetically distant species making morphology-based taxonomy not applicable to some species. Phenotypic plasticity (changes in morphology or behaviour of the same taxa due to the environmental conditions), cryptic species, and the lack of experts are the major taxonomic impediments identified by Hebert et al. (2003). To resolve this taxonomic incompleteness, the morphological method has been incorporated with genetic analysis (Pires & Marinoni 2010). However, taxonomists have criticized this method, fearing it might replace morphological-based identification (Dunn 2003; Adams et al. 2006; Pires & Marinoni 2010). Morphological taxonomy is still used simultaneously with molecular analysis (i.e., integrative taxonomy) when dealing with old specimens and cryptic taxa (Fišer et al. 2010).

Mitochondrial DNA (mtDNA) is the most utilised genome that has proven efficient when investigating closely related species and population structures. This is because it is easy to extract the mtDNA (Gaines et al. 2005; Simon et al. 2006). The mitochondrial genome consists of a double-stranded DNA which is responsible for encoding oxidative phosphorylation subunit proteins. It is also responsible for producing two 12S rRNA, 16S rRNA and 22 tRNA. DNA barcoding uses small DNA fragments of either mitochondrial or nuclear DNA sequences to identify species by comparing the DNA sequences from that available in the database (Hebert et al. 2003; Will et al. 2005; Pires & Marinoni 2010).

DNA sequences have been proven to be a more efficient and easy method of resolving issues in taxonomy through identifying cryptic species and species complexes (Schlick-Steiner et al. 2006;

Stockman & Bond 2007; Alter et al. 2017; Copilaş-Ciocianu et al. 2018). As a result, the use of DNA sequences in taxonomy has increased, and data has been made available through Zoobank, and Genbank (Hebert et al. 2003; Pavlidis & Stamatakis 2013; Fujita et al. 2012). DNA sequences have revealed that more species, especially subterranean species, had previously been misidentified as the same species due to morphological similarities (Finston et al. 2007; Fišer et al. 2009; 2018). Many studies on subterranean amphipods have shown cryptic diversity to be a common phenomenon, and more taxonomic problematic taxa have been resolved (Bickford et al. 2007; Adams et al. 2014; Murphy et al. 2013; 2019). For instance, *Niphargus* is a speciose-rich genus consisting of surface and subterranean amphipod species that superficially look similar. Since the introduction of molecular taxonomy, more species have been identified (Lefébure et al. 2006; 2007).

The mitochondrial gene, specifically cytochrome c oxidase I (COI), is a short stretch of a mitochondrial gene and has been used for establishing species relationships, diagnosis and identifying cryptic species. This gene has also been used even when investigating old, preserved specimens because it is easy to obtain than other genes, such as nuclear gene markers (Fišer et al. 2010). Lefébure et al. (2007) used two gene markers, mitochondrial genes (COI and 16S) and a nuclear gene (28S), to investigate the dispersion and cryptic species of widely distributed subterranean amphipod *N. rhenorhodanensis* Schellenberg, 1937. The gene markers revealed that there are several cryptic species within *N. rhenorhodanensis*. CO1 genes also explain or reveal the lineage divergence of new species by looking at morphological distinguishability, reproductive isolation and monophyly. A study that was conducted on the genus *Niphargus* indicated a divergent evolution of a surface amphipod species from a subterranean ancestor (Copilaş-Ciocianu et al. 2018; Mammola et al. 2019). Furthermore, a study on *G. fossarum* has shown that the taxon has more cryptic species than anticipated (Westram et al. 2011). *Niphargus fontanus* Bate, 1859, *Niphargus aquilex* Schiödte, 1855, and *Niphargus schellenbergi* are subterranean amphipods in Central Europe. These species have been identified as closely related, and using the CO1, and 28S rDNA gene marker, Hartke et al. (2011) revealed that *N. fontanus, N. aquilex* occurring in the Harz Mountains are genetically one species identified under *N. schellenbergi*.

The two ribosomal RNA (rRNA) genes, 12S rDNA and 16S rDNA have been also used in studying species phylogenetics. However, majority of studies in crustacea have mostly incorporated 12S rDNA and 16S rDNA with CO1 gene. This might be because the CO1 already has universal primers and easy to retrive, meanwhile 12S rDNA and 16S rDNA are highly conserved meaning genes do not mutate easily (Yang et al. 2014). For instance, Tomikawa et al. (2007) used molecular data CO1 and 12S rRNA to investigate the phylogenetic relativeness of two subgenera, *Jesogammarus* Morino, 1993 and *Annanogammarus* Morino, 1986 by using neighbor joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) phylogenetic trees. This study revealed that *Annanogammarus* is a monophyletic genus meanwhile, *Jesogammarus* is a paraphyletic and CO1 and 12S yielded similar results. Even though the 12S gene is rarely used solely, few studies on invertebrate and fish species successfully used this gene to investigate evolutionary events, phylogenetic relationships among, population dynamics and the genetic structure of species (Hillis & Dixon 1991; Wang et al. 2001; 2003). For example, Saijuntha et al. (2014) used a 12S gene marker to investigate the phylogenetic relationship and haplotype network of a widespread genus Oncomelania occurring in different localities in Philippines. Gouws et al. (2004) used allozyme and 12S rRNA to study the genetic variation of *Mesamphisopus capensis* (Barnard, 1914) in two regions in the Western Cape province. *M. capensis* is one of the four widely distributed isopod species in South Africa and has population from Cape Peninsula and the Hottentots Holland Mountains identified as the same species. Consequently, Gouws et al. (2004) found that isopods species from the two regions are comprise of four genetically different species that morphologically look like *M. capensis.*

This chapter aims to examine the phylogenetic relationship between *Sternophysinx* species using mitochondrial 12S rRNA and the available morphological keys to revise the topotypic material of this genus. Specifically, this study constructed a phylogeny that shows the relationship between the species of *Sternophysinx* and incorporated both morphological and molecular analysis to identify any new and cryptic species. This study hypothesized that *Sternophysinx* comprises cryptic species, and possibly more than seven species.

2.4 Materials and methods

2.4.1 Study sites

Sampling was conducted in nine sites; five caves, three springs and one borehole, from which subterranean amphipod specimens were collected. Two sites were in Gauteng (Sterkfontein and Bakwena caves), two in Limpopo (Peppercorn and Ficus cave), one in Northern Cape (Boesmansgat cave), one site in North West (borehole close to Moore River in Potchefstroom), two in Eastern Cape, EC (Hogsback and Mkhambathi Nature Reserve) and one site in KwaZulu-Natal, KZN (Ntsikeni Nature Reserve). Both the records in KZN and EC were new distributional records that were not previously known (Figure 2.4). Additionally, specimens from Graaff-Reinet and Hogsback were Albany Museum collections and are part of the new records. All the latter sites were springs that bubbled up after heavy rains and were opportunistically sampled as they are not permanently available for sampling. Similarly, the two other records in North West and Western Cape provinces were opportunistically collected as amphipods were caught in the borehole water facilities. In fact, in North West, the amphipod came up on the kitchen tap for drinking borehole-supplied water (Wouter Jooste, *personal communication*). Further, attempts were made to sample the other known sites in Western Cape (De Hoop cave), Northern Cape (Kolgelbeen cave), Limpopo (Matlapise cave) and Mpumalanga (Sudwana cave). Past and current literature (Holsinger & Straskraba 1973; Griffiths 1981; Griffiths 1991; Holsinger 1992; Griffiths & Stewart 1996; Griffiths & Stewart 2001; Irish & Marais 2002; Tasaki 2006; Milne & Griffiths 2013; Durand et al. 2010) was reviewed to determine the distribution of the subterranean amphipod species in South Africa (Table 1). Most published work shows that Sternophysingidae is abundant in the South African subterranean systems, including the Bakwena cave and Sterkfontein cave in Gauteng Province and Makapan's Valley in Limpopo Province.

a) Bakwena cave

The Bakwena cave (S25°53′53.3″ E28°13′19.8″) is situated in the Agricultural Research Council, Irene campus, in Centurion, Gauteng Province. This cave is a sinkhole, surrounded by grassy plains and a few *Celtis africana*, Burm N.L. trees that hide it from view. The circumference of the opening is approximately 10 m in diameter and accessible through vertical entrances via a rope (4 m), then a ladder (5 m) that extends to the main chamber (Rensburg 2010). The main chamber is about 15m wide and has a length (roof and scree floor) ranging from 1.5 m to 4 m. The main chamber extends into two small chambers, and the east chamber extends to a water pool (Figure 2.1 **B**). Bakwena cave is home to a few dark-adapted species, such as *Minopterus schreibersii* Kuhl, 1817, spiders, nematodes, fungi and two amphipod species *S. filaris* and *S. calceola* (Durand et al. 2012; Jacobs et al. 2017).

b) Sterkfontein cave

The Sterkfontein cave (26° 01'00.31" S 27° 44'00.86"E) is one of the South African National Heritage Site and a tourist attraction site and is well known for housing the most extensive collection of hominid fossils. This cave can easily access through the main gate entrance by going down the stair passage 15 m to the main chamber (Tasaki 2006). The main chamber ranges between 7 m by 14 m in width and 40 m in length. The main chamber extends to a water chamber surrounded by a fence and harbours two species of subterranean amphipods *S. filaris* and *S. calceola*. The water is clear with a depth that ranges from 0m to 16 m (Figure 2.1 **A**) (Tasaki 2006).

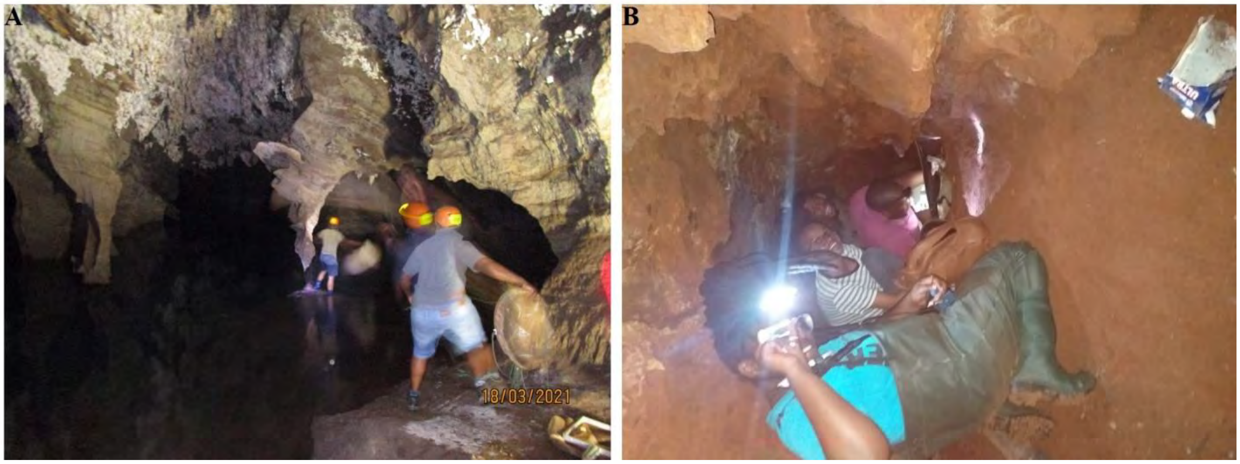

Figure 2.1: The photograph of two caves in the Gauteng Province: (a) shows Samuel and Musa sampling the deeper depth of the Sterkfontein cave using a zooplankton net (b) research team crawling down a narrow tunnel that led to the groundwater in the Bakwena cave. Pictures by Kaylee Cambell and Musa Mlambo.

c) Peppercorn and Ficus cave

Makapan Valley is Fossil Hominid Sites of South Africa World Heritage Site located in Limpopo about 15 km northeast of Mokopane. This valley comprises of paleontological and archaeological important caves which are said to have been formed during the Plio-Pleistocene period (Partridge 1966; Rayner et al. 1993). These caves include Limeworks Cave, Historic Cave, Cold Air Cave, Cave of Hearths, Peppercorn cave and Ficus cave. However, Peppercorn cave and Ficus cave are the only cave in this area that has underground water with only amphipod species recorded occurring in them.

Peppercorn cave (24°09'S 29°12'E) and Ficus cave (24°08'54.60' 'S 29°12'14.15" E) are situated in Makapan's Valley in Makopane, Limpopo. Both caves are located on the northern slopes of the Makapansgat Valley (Partridge 1966). Peppercorn and Ficus caves are situated on top of dolomite and seem to have resulted from both strike-slip faults (Partridge 1966). Peppercorn cave is easily assessable by going down a steep, dusty and rocky trail (Figure 2.2 **A, D**.). The locals also use this cave for spiritual purposes, which explains the presence of papers, candles, plastics and clothes in the cave and the water pool. Peppercorn cave harbour two co-occurring species *S. alca* and *S. robertsi*.

Meanwhile, the Ficus cave is more complex, complicated to assess, and situated on the north side of the valley on top of a hill. The entrance of the Ficus cave is reached by going up hills on a rocky trail that is covered by *Ficus burkei* Miq, 1867 tree. The entrance has a huge rock that opens up to the main chamber with *F. burkei* roots partially covering up the entrance giving the cave an ice age- look (Figure 2.2 **C**). The main chamber is dusty and has rock that may have been falling from the ceiling. This chamber has two small tunnels, with the east tunnel leading to a dead end meanwhile the west tunnel leads to the water table. The water table is reached by going down a narrow dark tunnel that is about 18 m from the main chamber. The tunnel is completely dark, dusty and has small rocks and small colony of bats was observed. Due to the presence of bat colony a Histoplasmosis causing fungi occurs in the cave and a mask is required to avoiding inhaling it. The water in Ficus cave is clearer than in Peppercorn cave, and there is no evidence of human presence in this cave (Figure 2.2 **A**, **B**). Like Peppercorn cave, Ficus cave is also a home two co-occurring species *S. alca* and *S. robertsi*.

Figure 2.2: The Makapans Valley caves sampled for subterranean amphipods: (a) and (b) the collecting amphipod from Peppercorn and Ficus cave, respectively and (c) and (d) show the entrances of Ficus cave (c) and Peppercorn cave (d).

d) Boesmansgat cave

Boesmansgat cave (27°55′18″S, 23°38′30″E), also known as Bushman's cave, in Northern Cape, South Africa is a sinkhole within the Mount Carmel game farm, about 55 km southeast of Kuruman town on the Danielskuil road. The area is at an altitude of 1 500 metres above sea level and is regarded as the third deepest freshwater sinkhole in the world, with a maximum depth of 282.6 meters deep (Beaumont and Vogel 2006). The surrounding biome is semi-arid savannah, covered mainly by grassy plains with few shrubs and trees, *Senegalia mellifera* (black thorn), *Vachellia erioloba* (camel thorn) and *Vachellia haematoxylon* (gray camel thorn) (van Rooyen et al. 2001). The sinkhole is situated 20 meters from the ground surface and reached by climbing down a steep and rocky trail (Figure 2.3 **C**), and is a well-known site for the two co-occurring endemic subterranean amphipod species, *Sternophysinx megacheles* and *S. basilobata* (Griffiths 1991; Stewart & Griffiths 1996). The water pool is 100 metres in diameter and occasionally covered by duckweed (Figure 2.3 **A, B**), with a dozen koi fish introduced in early 2010 in the sinkhole, and only two survived. The Danielskuil has extreme temperatures with hot summer (20ºC- 36ºC) and cold winter (0ºC and 20ºC) and receives an annual rainfall of 150 mm to 250 mm per annum (South African Weather Service 2021). Heavy rains are common between February and April, with dry winter between May and July. A study that looked on the koi fish feeding behaviour was conducted in Boesmansgat cave and is explained in great details in Chapter 3.

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Figure 2.3: Photographs of Boesmansgat cave in the Northern Cape, South Africa: (a) Assessing the submerged cave antrace where a bait can be placed, (b) Lowering a zooplankton net that contains a yeast bait in the cave antrace and (c) areal and a close-up view of the Boesmansgat cave)

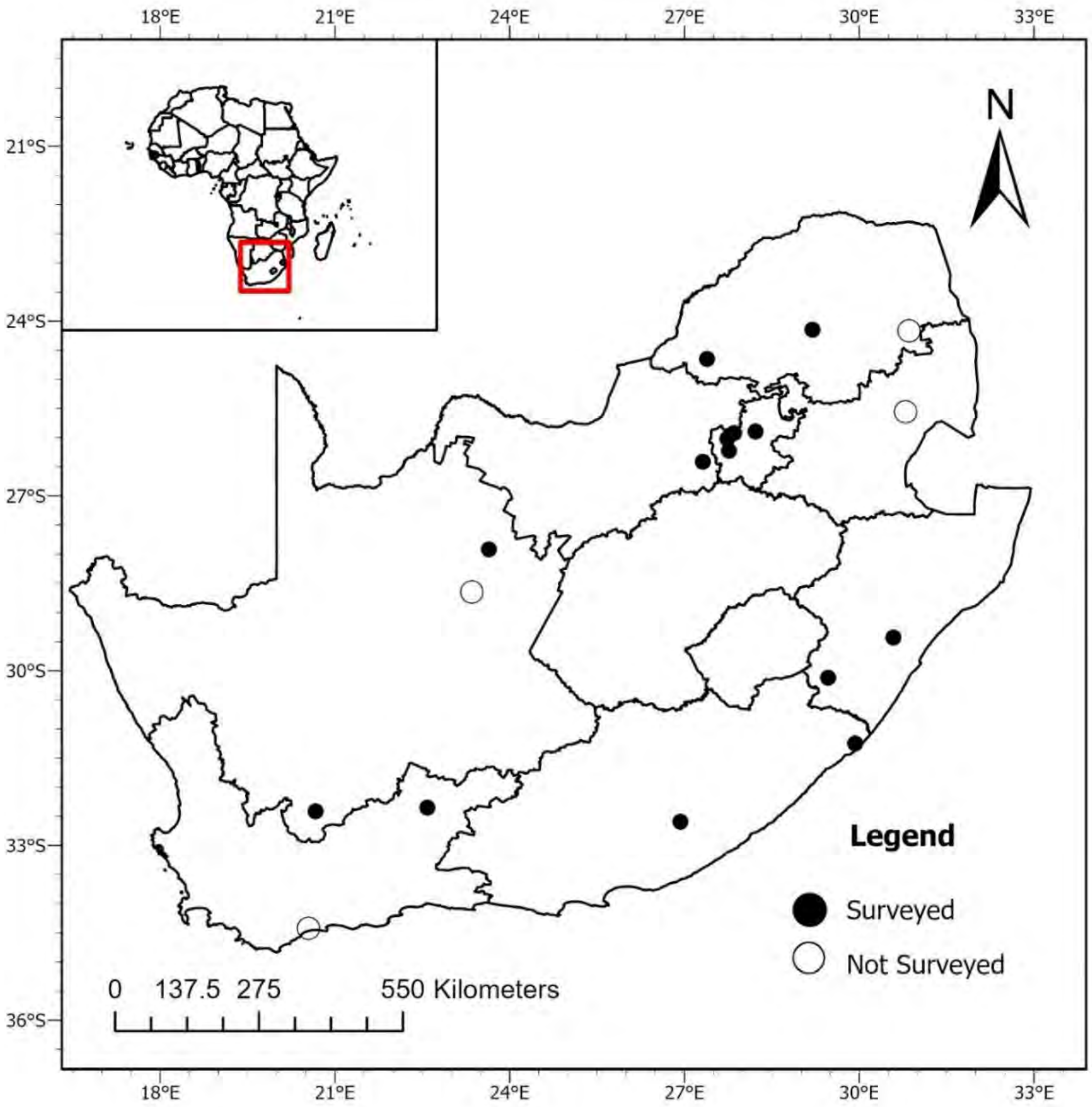

Figure 2.4: The study map includes surveyed sites presented by a shaded circle and sites that are not surveyed indicated by an open circle.

2.5 Data collection

Amphipods were collected using various techniques depending on the site's physical character from different caves using a hand net and zooplankton net in the shallow water pool. In Boesmansgat cave, a 30x30 zooplankton net attached to a 30cm rope was lowered into the submerged cave entrance and left-over night with a yeast bait inside it. Meanwhile, in the Sterkfontein, an 8cm sieve was used, and a zooplankton net was thrown into the deeper side of the water pool. After collection, specimens were immediately preserved on 70% ethanol and were kept in a cooler box until they were in the laboratory, then transferred to the refrigerator. The specimens were identified using a stereomicroscope. The morphological identification of all collected specimens was done based on Griffiths and Stewart (2001), for further DNA analysis, 2 to 3 individuals from each sampling site were processed for DNA barcoding, and the remaining specimens were conserved in 100% ethanol and accessioned at the Albany Museum, Freshwater Invertebrates department collection. All the specimens (except for specimens from Mkhambathi Nature Reserve, Ntsikeni Nature Reserve, and North West) were sent to Prof. C. Griffiths, the South African amphipod expert, for identification confirmation.

2.5.1 Morphological analysis

For morphological analysis the following keys were used to confirm the genus and species name:

1. Article 2
   i. Broad and expanded into a posterior lobe
   ii. Narrow and distally not expanded into a posterior lobe
2. Article 5 and 6
   i. With combs of setae with pereopods 5-7 with two or more accessory spines
   ii. No elongated setae with a single more accessory spine
3. Gnathopod 1
   i. With article 6 longer than palm and distally separated by an angle
   ii. With article 6 shorter than palm and separated from it
4. Antenna 2
   i. With a paddle-like calceoli
   ii. Without a paddle-like calceoli
5. Gnathopod 1 and 2
   i. Evenly convex
   ii. With a semicircular notch and tooth at midpoint

2.5.3 DNA extraction

DNA was extracted from 2 to 3 individuals from each sampling site, making up three replicates per species per site. Before extraction, the specimens were stored in distilled water overnight to hydrate samples. Then, genomic DNA was extracted following the Lysates-Mini kit (Mammalian tissue and mouse or rat tail lysate). The mitochondrial 12S rRNA gene was amplified by Polymerase Chain Reaction (PCR) using a designed forward 12S CRR 5'-AAA CCA GGA TTA GAT ACC CTA TTA T 3' and reverse primer 12S CFR 5'- GAG AGT GAC GGG CGA TAT GT-3' pairs.

For 12S rRNA gene, the polymerase chain reactions (PCR) were set following Gouws et al. (2004). The thermal cycling step involved an initial denaturing step at 94°C for 4 minutes, then 33 cycles of denaturing at 94°C for 5 minutes, followed by 35 cycles of denaturing at 94°C for 15 minutes, an annealing step 52°C for 1 minute, and extension at 72°C for 1.5 minutes. The final step was annealing for 52°C for 5 minutes and extension for 15 mins at 72°C. The PCR product success was determined by agarose gel electrophoresis, and all the products yielding clear DNA fragments were purified. The PCR product was purified using the ExoSAP purification QIAquick Kit (Qiagen, Hilden, Germany). Consequently, the ExoSAP purified products were sequenced following the Standardized protocol for Cycle Sequencing guidelines. Big Dye was used for the sequencing reactions. Further, samples were purified and precipitated using an ethanol/EDTA precipitation protocol.

2.6 Data analysis

2.6.1 *Phylogenetic analysis*

To assess the phylogenetic relativeness of the *Sternophysinx* species collected from nine subterranean systems, Bayesian Inference (BI) and Maximum Likelihood (ML) analysis were constructed based for 12S rRNA mitochondrial gene. The data set comprised 34 12S rRNA gene sequences on individuals of *Sternophysinx*. Because we could not find any closely related species to *Sternophysinx* on The National Center for Biotechnology Information (NCBI), DNA was extracted from a species of the genus *Mathamelita* Stewart & Griffiths, 1995 *Mathamelita aequicaudata* Stewart & Griffiths, 1995 and used as outgroup.

The 12S sequences were aligned and analyzed on MEGA11 v11.0.13 (Tamura et al. 2021) using Clustal W. The aligned sequences were re-aligned online using MAFFT (https://mafft.cbrc.jp/alignment/software/), then trimmed and edited on Aliview. For both phylogenies, jModelTest v2.1.10 (Posada 2008) was used to determine the best fit model. Notepad+++ v8.4.3, released on the 8th of November 2022, was used to edit sequences and create a partition for both phylogenies, and for the ML tree, the partition was run via the IQ tree web server (http://iqtree.cibiv.univie.ac.at/). The BI phylogenetic tree was constructed in MrBays v3.2.6 (Ronquist et al. 2012) with partition obtained from the jModelTest. The ML and BI phylogenies were also viewed on Figtree and edited on Inkscape v1.2 Beta, released on the 6th of April 2022.

To test whether *Sternophysinx* includes of cryptic and unidentified species three delimitation methods were employed i.e., Assemble Species by Automatic Partitioning (ASAP), Automatic Barcode Gap Discovery (ABGD), and Bayesian implementation of the Poisson Tree Processes (bPTP) (Puillandre et al. 2012; Zhang et al. 2013). The latter delineation methods are used to resolve species with single-locus data with ABGD employing pairwise distances to deliminate species and partition samples into reputed species. The distance metrix used was JukesCantor (J.C.). Similarly, to ABGD, ASAP also uses pairwise distance and 10 best partitions. However, the only difference is that ASAP uses scores to rate each partition, with the smallest score indicating the best partition. ABGD, ASAP methods were performed using the web interface https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html and https://bioinfo.mnhn.fr/abi/public/asap/ respectively. bPTP deliminates species through the use of a genetic tree for the analysis.

The bPTP is a coalescent phylogeny-based species delimitation method intended to delimit species based on single locus molecular data (Zhang et al., 2013). The method relies on the number of substitutions between species between haplotypes and assumes that more molecular variability is expected between species than within a species (Zhang et al., 2013). PopArt version 1.7 was used to visualise the mutation between haplotypes. To visualise the relationship between amphipods species sampled in different Provinces for 12S, Median-joining network was constructed in PopArt.

2.7 Results

2.7.1 Morphological identification

Specimens from Bakwena cave, Sterkfontein cave, Boesmansgat cave, Beaufort West, Ficus and Peppercorn cave were examined and identified by Prof C. Griffiths. All the specimens from the latter sites were identified following Griffiths & Stewart 2001 (page 34-37). Twenty specimens were collected from Beaufort West and were identified as *S. basilobata*. In Ficus and Peppercorn cave, 50 and 14 specimens were collected respectively, and identified as *S. alca*. In Sterkfontein and Bakwena cave 32 and 46 specimens were collected respectively. In addition, using the currently morphological diagnostic characters specimens from both caves were identified as *S. filaris* and two species were considered new and undescribed species. From North West, there was only one specimen found, and used for both morphological and molecular identification, and based on proximity to the Choas cave in that province that we could not sample, we would expect this species to be either *S. filaris* or *S. calceola*. However, due to the elongated uropod 3 and concaved posterior margin of telson we consider this species to be *S. basilobata*. In Boesmansgat cave 7 specimens (six *S. basilobata* and one *S. megacheles*) were collected and confirmed to be *S. basilobata* and *S. megacheles*.

2.7.2 Phylogenetic analysis of *Sternophysinx*

A 261-bp 12S fragment was obtained from 33 amphipod samples from nine sites sampled between 2019 - 2022 covering six South African Provinces. The BI and ML phylogenies strongly support the relationship between species of the genus *Sternophysinx* with BI posterior probability value and ML bootstrap values about 0.5 or 50% (Figure 2.1). Both phylogenies constructed by the ML and BI analyses yielded identical topologies. A phylogeny representing both BI and ML values is also shown and discussed (Figure 2.1). The BI and ML yielded seven well-supported clades with support values ranging between 1% posterior probability values and 97% to 100 % bootstrap values. In both BI and ML phylogenies, Clade I comprise North West samples that are genetically distinct from other samples collected in Makapans valley (Ficus and Peppercorn cave), Makhambathi Nature Reserve, Ntsikeni Nature Reserve, Boesmansgat cave, Bakwena, Sterkfontein cave and Beaufort West samples. Clade II is strongly supported with a 1.0 posterior probability value and 99 bootstrap values showing two closely related species from unidentified samples collected in Bakwena cave and, identified specimens from Sterkfontein and Bakwena cave that were identified as *S. filaris*. Clade III comprises samples from Bakwena cave, Sterkfontein cave and Beaufort West that forms a single species. Clade I-III shows a clear local divergence as all the species from the three different geographic regions forms genetically well supported and distinct clades. However, the clustering of *S. basilobata* (1.0 posterior probability value and 35 bootstrap values) from Beaufort west in Clade III might either suggests that this species is one of the species occurring in Bakwena and Sterkfontein cave (*S. filaris* and *S. calceola*) or a new species.

Clade IV comprises samples from Makapans Valley (Ficus and Peppercorn cave), Hogsback and Mkhambathi Nature Reserve, forming a single species. This clade is well supported (1.0 posterior probability value and 100 bootstrap values) and is closely related to Clade V, that include two specimens from Mkhambathi Nature reserve. Clade VI comprises samples from Ntsikeni Nature Reserve that forms a single species, with Clade VII comprising two cryptic species from samples collected in Boesnmansgat cave and were identified as *S. basilobata*. The former clades, i.e., Clade III to VII, share a common ancestor with Clade II. Clades V-VII are genetically and geographically distinct, with specimens from Mkhambathi Nature reserve, Ntsikeni Nature reserve and Boesmansgat cave forming well-supported clades. The occurrence of *S. basilobata* in three genetically distinct clades (Clade II, III and VII) suggests that this species is complex. Species complex, superficially similar in closely related species, is common in subterranean invertebrates.

2.7.3 Species delimitation and genetic distances

Using the three-delimitation methods ASAP, bPTP and ABGD to determine new or cryptic species, 12-14 species were identified (Table 2.1; Figure 2.1). All the delimitation methods clearly show that samples from North West (Clade I) are genetically different from the rest of samples from the six Provinces and both sequences represent a single species. ABGD and ASAP merge Clade II into a single species, whereas bPTP split this clade into two putative species (Fig.2.1). ABGD and ASAP grouped Clade III into three species, i.e., species 1 comprising of samples from Bakwena and Sterkfontein cave, species 2 with samples from Beaufort west and species 3 with samples from Sterkfontien cave while bPTP only grouped Clade III into one species. ASAP grouped all the Clade IV samples into a single species; meanwhile, ABGD and bPTP split this clade into 4-6 species, respectively. ASAP and bPTP merged Clade V into two species (species 1 from Mkhambathi Nature Reserve A, Mkhambathi Nature Reserve C and species 2 from Mkhambathi Nature Reserve D) and ABGD considered the latter samples into a single species. Similar results are shown in Clade VI, where ASAP and bPTP support splitting Ntsikeni Nature Reserves samples into two species, with ABGD merging these samples into one species. ASAP and bPTP split Clade VII into two species from samples collected in the Boesmansgat cave (*S. basilobata* C and *S. basilobata* D), while ABGD grouped all the samples into a single species.

Values for intraspecific genetic distances and the number of putative species are shown for each sampled Province in Table 2.2. The genetic distance values show clearly that amphipod species from Northern Cape, Western Cape, North West, KwaZulu Natal and Gauteng are genetically distinct. Contrary to the latter Provinces, Eastern Cape and Limpopo are genetically close or have low genetic distances (0,07%). Similarly, the bPTP and ABGD delimitation have grouped samples from Eastern Cape and Limpopo as a single species. The average genetic diversity distances between Provinces (Table 2.2) vary from 0.07% to 0.24%. Importantly, the Northern Cape population vs Western Cape, Limpopo, Eastern Cape, and North West have relatively high genetic distances (0.14- 0.24).

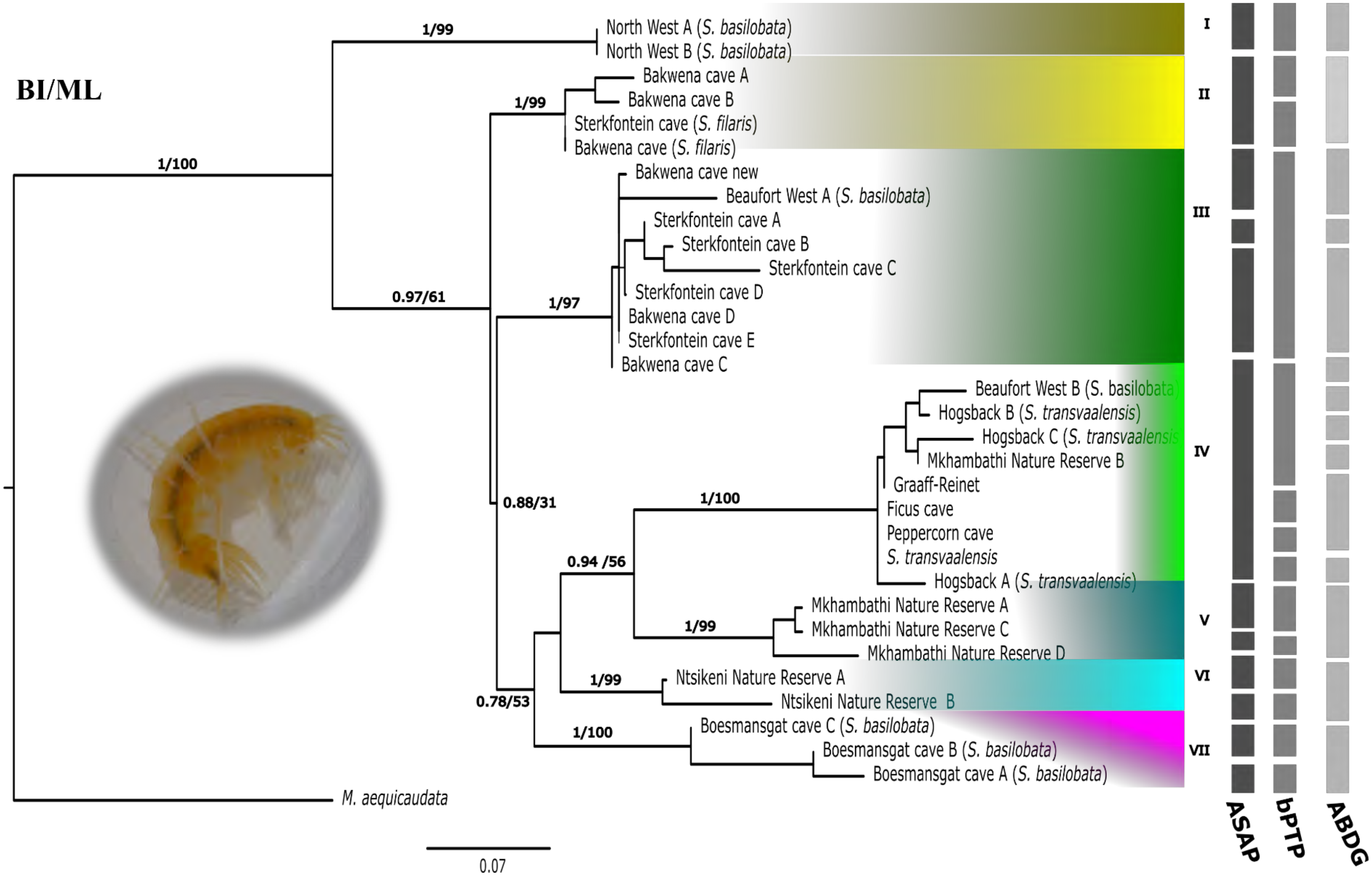

Figure 2.5: Bayesian (BI) and maximum likelihood (ML) tree showing the relationship between the species of *Sternophysinx* based on 12S gene. Each colour and roman number represent a clade thus from Clade I- VII. The bars on the right represents the three delimitation methods used (ABGD, bPTP and ASAP) with each bar column differ in colour according to the method used and number of species delimited.

Table 2.1: Shows a summary of the three-delimitation methods ASAP, bPTP and ABGD used based on the splitting in each clade.

	ASAP	**ABGD**	**bPTP**
Clade I (NW)	1	1	1
Clade II (GT)	1	1	2
Clade III (GT+ WC)	3	3	1
Clade IV (WC+ EC+ LP):	1	6	4
Clade V (EC)	2	1	2
Clade VI (EC	2	1	2
Clade VII (N C)	2	1	2
Total	**12**	**14**	**14**

Table 2.2: Genetic diversity between the seven sampled provinces Analyses were conducted using the Poisson correction.

	Northern Cape	Gauteng	Western Cape	Limpopo	Eastern Cape	North West	KwaZulu-Natal
Northern Cape							
Gauteng	0,14						
Western Cape	0,19	0,15					
Limpopo	0,19	0,19	0,14				
Eastern Cape	0,21	0,20	0,16	0,07			
North West	0,24	0,19	0,22	0,20	0,21		
KwaZulu-Natal	0,15	0,13	0,18	0,17	0,17	0,19	

2.7.4 Haplotype network and distribution of the genus *Sternophysinx*

All seven provinces clearly show that they differ in haplotype, as shown in Figure 2.6. The Gauteng haplotype is the most abundant with the least number of mutations indicating that the Gauteng haplotype is likely an older haplotype. This might be an indication that subterreanen amphipods first invaded that ground water system of the Transvaal region before spreading throughout the South African ground water. Additionally, the occurrence of two widely spread amphipod species *S. filaris* and *S. calceola* in the Gauteng province is nicely explained in both the phylogeny and in haplotype network (Figure 2.5; 2.6; 2.7)

Another important observation is the clustering of sequences from Bakwena cave and Sterkfontein cave identified as *S. filaris* within Sterkfontein cave (*S. filaris*). Similarly, sequences from Hogback (*S. transvaalensis*), Peppercorn cave and Graaf Reinet cluster with Ficus cave, and sequences from Bakwena cave D, Sterkfontein D, and E also cluster within Bakwena cave A. This clustering within Sterkfontein cave (*S. filaris*), Ficus cave and Bakwena cave means that the species that cluster within these sequences share an ancestor. The clustering of the Eastern Cape, Western Cape haplotype with Limpopo haplotype suggests that species from the two Provinces are closely related, and this is supported by both the phylogenetic tree and the delimitation methods (Figure 2.5). This might explain the co-occurrence of species in nearby cave systems, and as indicated in Figure 2.6 species from Gauteng share a common ancestor.

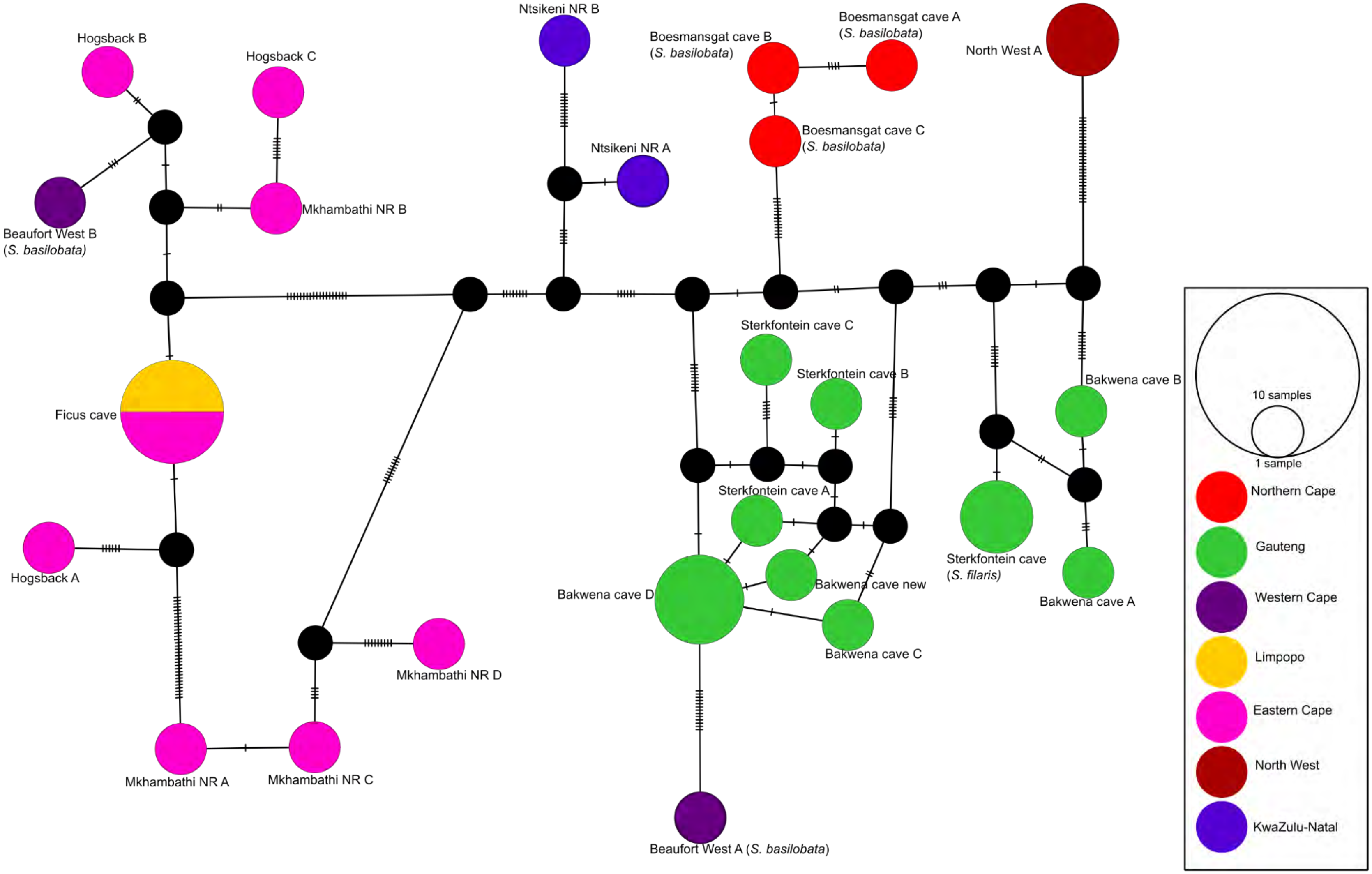

Figure 2.6: TCS PopArt network for 12S: the colourful circles represent the haplotype; the dash represents the number of mutations which are proportional to the length of lines thus few mutations are shown by short line and vise visa.

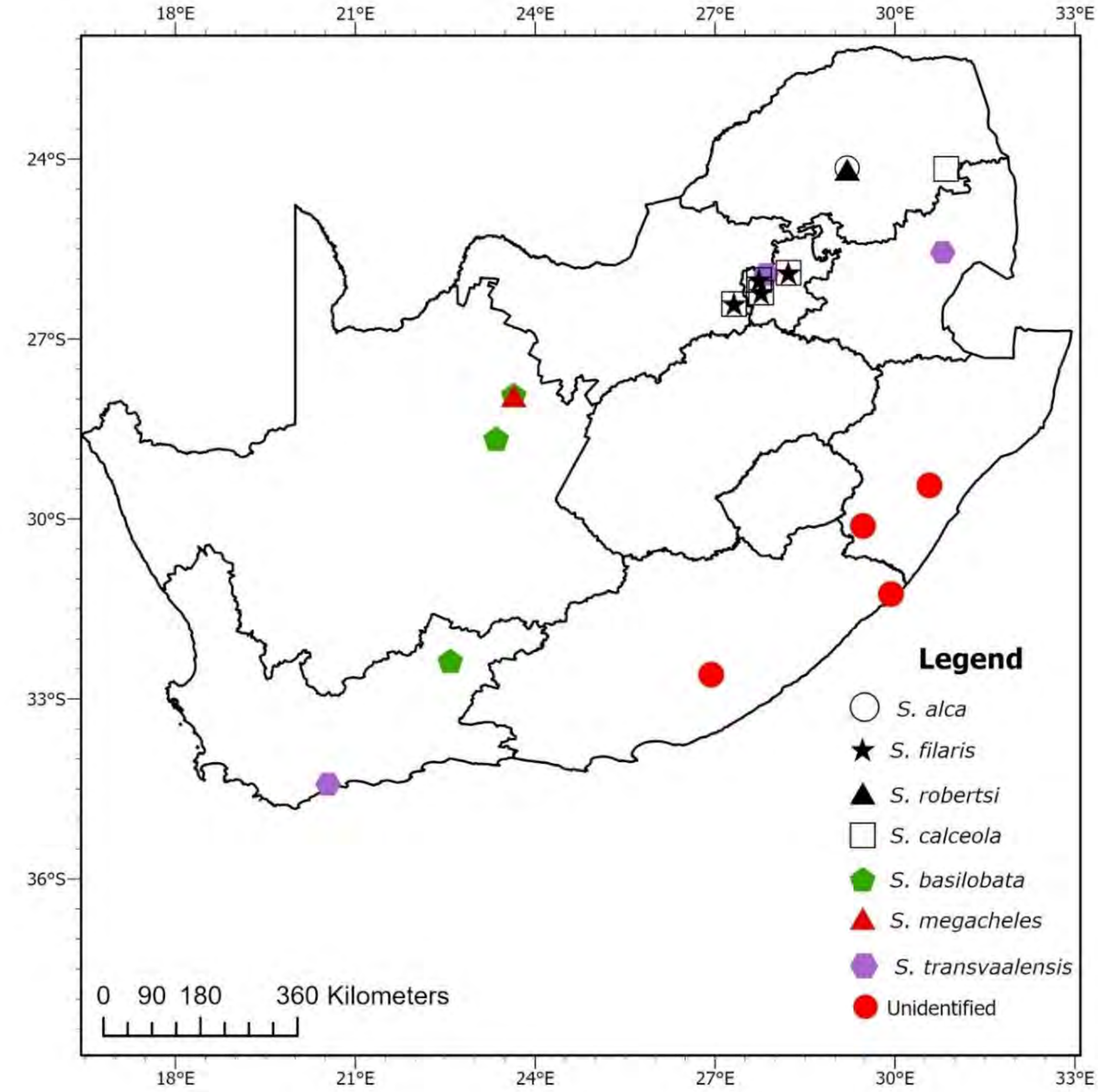

Figure 2.7: Distribution map of the *Sternophysinx* species in South Africa, including the recently sampled sites in KwaZulu Natal and Eastern Cape Province.

2.8 Discussion

This study argues that the lack of morphological variation between sternophsingids might indicate cryptic species. The observation or occurrence of the same species in different geographic regions has been proven in most cases to result in cryptic species or species complexes (Finston et al. 2004; Fišer et al. 2015). Similar to the preliminary studies on subterranean amphipods (Fišer et al. 2015), the morphological and molecular analysis has shown that *Sternophysinx* comprises cryptic species, which implies that this genus includes unidentified species. According to our morphological analysis, cryptic speciation seems to be quite frequent in co-occurring species, and this hypothesis was well explained by Cothran et al. (2013), Fišer et al. (2015) and Wellborn & Cothran (2004) whom all showed that morphological similarities are frequent in co-occurring species. These similarities are had been generally caused by the exposure to the same environmental conditions. Using three delimitation methods this study has consequently identified 14 putative species. These findings imply that *Sternophysinx* comprises of cryptic diversity and unidentified species from the known and new locations. The present study also supports the inclusion of at least seven new species from the Boesmansgat cave, North West, Ntsikeni Nature Reserve, Mkhambathi Nature Reserve, Bakwena cave and Sterkfontein cave.

Distribution of the genus Sternophynsix in South Africa:

The current distribution of the seven known species is rather complex, with the past literature arguing that the distribution of *Sternophysinx* comprises narrow endemic species that are mostly nested in Gauteng province (Tasaki 2006). Even so, most of this genus representatives have been recorded in geographically distant sites. For instance, despite the known distributions of *S. basilobata* (Boesmansgat cave and Kolgelbeen cave in Northern Cape), this study has expanded this species' distribution to Beaufort West and in a borehole in North West Province. Therefore, this study has revealed that this species is widely distributed, contradicting the present literature that supports the high endemicity of the subterranean genus *Sternophysinx* in South Africa (Holsinger & Straskraba 1973; Holsinger 1992; Griffiths & Stewart 1996). This study has consequently surveyed six new sites where *Sternophysinx* species have been found Graaf Reneit, Beaufort West (*S. basilobata*), Potchefstroom (*S. basilobata*), Mkhambathi Nature Reserve, Hogsback (*S. tranvaalensis*) and Ntsikeni Nature Reserve. Subterranean taxon with wide distribution is difficult to explain, given the isolated habitat with limited or no dispersal. Supported by previous studies, this study suggests that the South African groundwater systems are either connected or have been segregated for millions of years, resulting in widely distributed taxon (Lefébure et al. 2006; 2007; Trontelj et al. 2009). Further, present studies have highlighted that the shift in geographical and hydrological activities in groundwater systems has resulted in either isolation of close species or a connection between subterranean species (Notenboom 1991; Finston et al. 2007). The spontaneous bubbling of spring after rains in Makhambathi and Ntsikeni Nature reserve maybe be the best case that explains the underground connection, as there have been no records of *Sternophysinx* in either of these locations. Additionally, the occurrence of *S. filaris* in neighbouring sites, Sterkfontein cave and Bakwena cave, also supports an ongoing groundwater connection between some cave systems leading to the occurrence of the same species in closely located sites.

In contrast to our results, cryptic species have been observed in species with about a 200 km distribution range (Trontelj et al. 2009). However, the *S. basilobata* complex has an exceptional distributional range of over 600 km, which is unusual for subterranean species. Consequently, such a distributional range has been primarily documented in epigean amphipods (Borza et al. 2015; Copilaş-Ciocianu et al. 2017a; 2018). This distribution range raises many questions, such as, did this species complex diverge from a common ancestor or are they recent descendants or are these species a result of dispersal. In subterranean habitats, widely distributed species are hypothesized to have resulted from geographic isolation during groundwater invasion, which resulted in genetic differences in the same species (Bradbury & Williams 1997b; Humphreys 2001). Similarly, to the South African genus, two genera, *Pilbarus* and *Chydaekata*, are widely distributed and have been reported to comprise cryptic species (Finston et al. 2007).

***Morphological implication of the study's results*:**
Morphologically, the present study show that *Sternophysinx* comprises cryptic species; these findings agree with the recent work by Murray (2020), that *S. calceoal* and *S. filaris* were morphologically similar but genetically different species. This study has also found cryptic speciation from specimens collected in Ficus cave and Peppercorn cave, in the Limpopo Province of South Africa. Both caves harbour two species, *S. robertsi* and *S. alca*, yet using morphological charateristics only *S. alca* was identified from all the specimens collected in the two caves. Species exposed to or experiencing similar or same selection pressure, especially in subterranean habitats, lead to morphological similarities. These similarities might also result from shared ancestry traits, introgression and morphological plasticity (Remigio et al. 2001). As indicated in this study, most of the co-occurring species except for the population in the Boesmansgat cave (*S. basilobata* and *S. megacheles*) are similar morphologically. Using morphological analysis, this study also revealed cryptic speciation from three geographically separated sites, Boesmansgat cave, Potchefstroom (North West) and Beaufort West. Due to the possession of round lobes on article 2 of pereopods 3 and 4, longer uropod 3 and slightly concave telson which are the dignostic features of *S. basilobata* specimens from the latter sites were confirmed to be *S. basilobata*. Similar results of cryptic species, where species are superficially similar morphologically, have been observed in chiltoniid amphipods (Murphy et al. 2013). This was also the case for the problematic family of Paramelitidae, where widely distributed taxa comprise cryptic species (Finston et al. 2004; 2007). Using morphological keys, study's current findings indicates that *Sternophysinx* comprises of species with little morphological differences. This is true in S. *filaris* and *S. calceola*, in Sterkfontein and Bakwena cave, *S. alca* and *S. robertsi* in Ficus and Peppercorn cave, and *S basiloaba* complex in Boesmansgat cave and Beaufort West. looking at the morphological similarities between. These results agree with that of King et al. (2022) where *Nedsia* Barnard & Williams, 1995 comprised of morphological species.

The recent divergence hypothesis (Trontelj et al. 2009; Bravo et al. 2014; Egea et al. 2016) may be the perfect explanation for the morphological similarities in *S. basilobata*. Therefore, we argue that a dispersal or connection occurred million years ago, resulting in similar morphological feature in *S. basilobata* in different lineages. Even the haplotype of *S. basilobata* complex clearly indicates that this complex comprises of genetically different species with the North West species still undergoing a mutation. Dispersal in a subterranean environment is a phenomenon that is by far difficult to explain. However, the observations of widely distributed amphipods species have been explained by splitting landmasses, isolation, speciation and colonization of new environments (Copilaş-Ciocianu et al. 2014; 2015; 2017). *S. basilobata*, *S. filaris* and *S. calceola* are spread in five provinces, Northern Cape, Western Cape, Limpopo, North West and Gauteng province. However, *S. basilobata* is found to be more genetically diverse, comprising five species, two occurring in Boesmansgat cave, two in Beaufort West and one in Potchefstroom.

***Molecular relationship between Sternophysinx representatives*:**

In addition to the seven known species i.e., S. *alca, S. robertsi, S. filaris, S, calceala, S. megacheles, S. transvaalensis* and *S. basilobata*, the phylogenetic analysis also revealed the inclusion of specimens from North West, Mkhambathi Natue Reserve, and Ntsikeni Nature Reserve as new species. The latter results are also supported by the the three delimination methods i.e, ASAP, ABGD and bPTP with specimens from Bakwena new also identified as a possible new species. The 12S gene produced adequate results that supports the inclusion of 7 putative species with ABGD and bPTP showing similar results. This study has shown that the Boesmansgat cave, North West, and Beaufort West population identified as *S. basilobata* is a highly divergent species that forms distinct lineages and is a species complex. However, even though the latter specimens are morphological similar or identified as one species this study has shown that these species are not close relatives. Similary, Copilaş-Ciocianu et al. (2018) findings on two morphologically similar species *N. hrabei* and *N. valachicus* revealed they these species are not closely related and have invaded the surface water systems at different times. Similar to this study's findings, the wide distributed European amphipod species *Gammarus balcanicus* Schäferna, 1923 has been reported to comprise of cryptic species that occur Balkan Peninsula and the eastern Alps (Copilaş-Ciocianu & Petrusek, 2016).

South African subterranean amphipods have been understudied and with no literature on the molecular data. This study has revealed that South African subterranean amphipods comprise of hidden diversity due to morphological similarities. Following the molecular analysis, it is clear that Boesmansgat cave house three species and these findings highlights the importance of incorporating molecular analysis in taxonomy. This study has consequently noticed that species that occur in the same geographic region forms a cluster, with exception to Clade III and IV. The latter clades are rather difficult to explain genetically yet the delimitation methods do show that they represent different species. Interestingly, the 12S mtDNA haplotypes of the *S. basilobata* complex shows mutations between different haplotype. For instance, the North West complex share mutation between species from Gauteng Province. This is not a surprise as studies have shown that the Gauteng and North West have the same rock form, meaning that species in these two provinces may have been separated for years and are still undergoing a mutation.

Integrative taxonomy:

Past taxonomic studies have been based solely on morphological characters which has led to many species been either under identified or over identified. Nevertheless, when some populations evolve to different species, especially in subterranean habitat, new species tend to superficially look like other species (Trontelj et al. 2009). This makes it nearly impossible to morphologically distinguish new species from the existing one leading in species not being identified (Guzik et al. 2011; Abrams et al. 2012). However, integrating the morphological identification with molecular analysis has proven efficient in identifying new morphologically identical species (Weiss et al. 2014; Katouzian et al. 2016). For years, the South African subterranean amphipod taxon has been identified morphological with no molecular analysis to back up the morphological identification. As observed in many studies, subterranean amphipods comprise of cryptic species and integrated methods has improved the taxonomy of many understudied taxa (Murphy et al. 2013; King et al. 2022). The genus *Nedsia* has suffered the same fate as *Sternophysinx* thus has been known to comprise of narrow endemics occurring in the subterreanen habitat in the Barrow Island and the North West Cape peninsula (Halse et al. 2014). However, incorporating the molecular data with morphological character has improved the taxonomic and distributional understanding of the genus *Nedsia* (King et al. 2022). This study has out detailed taxonomic and molecular genetic studies of the family Sternophysingidae and identified 7 putative species.

Conclusion and recommendation:

Currently, this project has shown that *Sternophysinx* is widely distributed in the with three species showing cryptic speciation i.e, *S. basilobata*, *S. alca* and *S. filaris*. Consequently, two new species have been identified from Bakwena and Sterkfontein and this indicates that new taxonomic keys are needed. This project will still aim to get CO1 data, in order to get definitive confirmation of the taxonomic status of these taxa, including using a minimum 3-5 specimens per site or putative taxon, and add 16S and 28S for close inspection and confirmation of the phylogenic relationship between these taxa. Even though this was not part of this study's aim, phylogeography is an important aspect that is worth exploring as *Sternophysinx* has shown a very interesting distribution and knowing it origin and exploring if is there a connection between sites. However, the distribution of clade IV of specimens from Hogsback, Makhambathi Nature Resrve, Graaf-Reinet, Ficus and Peppercon cave remain unresolved and morphological analysis is needed confirm if these specimens are same species or different species.

CHAPTER 3

The impact of an introduced koi fish on endemic subterranean amphipod species, a case study of the Boesmansgat cave, Northern Cape South Africa

3.1 Introduction

Boesmansgat cave is a submerged freshwater cave with two co-occurring endemic subterranean amphipod species, i.e., *Sternophysinx megacheles* and *S. basilobata*. In the early 2010s, the property owner introduced several koi fish individuals to control the sporadic floating duckweed, *Lemna* sp., which covered the water surface. However, the impact of the introduced koi fish on local aquatic invertebrate species, especially the endemic subterranean amphipods, is unknown.

Lemna sp., commonly known as duckweed, is a monocotyledonous macrophyte that belongs to the family Lemnaceae. Duckweed is regarded as an opportunistic free-floating macrophyte with oval-shaped leaves and tiny roots with a 1 cm length (Haustein et al. 1990; Ceschin et al. 2018). This aquatic macrophyte occurs in still or slow-flowing water bodies, and has been used as food for fish, bioethanol production and wastewater treatment in countries such as Asia; United States, Italy and South Africa (Zirschky & Reed 1988; Azim & Wahab 2003; Pindihama et al. 2011; Yan et al. 2013; Gusain & Suthar 2017; Oyawoye 2017). However, certain species of *Lemna*., such as *L. minuta* and *L. minor*, have been declared invasive species, especially in European countries i.e., Italy, Germany, Hungary, Ireland and Belgium (Celesti-Grapow et al. 2009; Njambuya et al. 2011). In its invaded range, *Lemna* sp. form dense mats that deprive aquatic organism's oxygen, sunlight thus disrupting ecosystems processing that leads to replacement of some native aquatic plants (Janes et al. 1996; Ceschin et al. 2016). In South Africa, duckweed is not well studied, and its impact on native species is not well known. However, because of its protein-rich and rapid growth, duckweed has been exploited as food for livestock such as chickens and pigs (Marizvikuru & Francisca 2013; Gwaze & Mwale 2015).

Herbivorous biological control agents such as invertebrates, plant pathogens and fish have been introduced control duckweed in freshwater systems (Dudley et al. 1981; Mariani et al. 2020; 2021). Fish species of the family Cyprinidae and Cichliformes have been successfully used as biological control agents of nuisance and opportunistic macrophytes, i.e., duckweed. However, this phenomenon it is still debatable in South Africa whether duckweed is a threat to aquatic biodiversity or just an opportunistic cosmopolitan plant. South Africa has limited records of introduction fish species on subterranean environment, and we know less of about the fish introduction impact on subterranean aquatic species (Irish & Marais 2002; Du Preez 2014). Using stable nitrogen and carbon isotope ratios, we aim to investigate the impact of the introduced fish on the two co-occurring endemic amphipod species.

3.2 Fish species in subterranean environments

The occurrence of fish in subterranean habitats is a very intriguing observation, given that this habitat is highly restricted and arguably geographically isolated and undergoes strong selection pressure (Trajano 2001; Gibert & Deharveng 2002). Subterranean fish can be found in over 34 countries, with China being one country with 124 described subterranean species (Proudlove 2010; Niemiller et al. 2019). Proudlove (2010) argues that of the 12 000 described freshwater fish, over 1000 species may occur in subterranean habitats, but only 247 fish species are known and recorded in this habitat. South America has about 34 known subterranean fishes, with Brazil having more diversity of described species. The subterranean fish population of Brazil is dominated by the order Siluriformes (Cuvier, 1817), in which catfish predominate (Niemiller et al. 2019). About 26 described subterranean fishes occur in Mexico and the United States. Australia hosts four described subterranean fish species, with Europe being the least diverse and only two known species. Africa comprises eight described subterranean fish, with four species known to occur in Madagascar, only one species in Namibia and three in Somalia (Proudlove 2010; Niemiller et al. 2019) and none in South Africa. *Clarias cavernicola* (Trewavas, 1936), is one of the Namibian most threatened and highly endemic subterranean fish species declared critically endangered. A survey by Jacobs et al. (2021) further confirmed that *C. cavernicola* populations have declined, necessitating conservation intervention. Even though subterranean realms are considered extreme and restricted habitats, some species, such as nematodes, spiders, amphipods, and fish, have been intentionally or accidentally introduced into this habitat (Gray & Thompson 2001; Du Preez 2014; Culver & Pipan 2015).

3.3 Invasion in subterranean environments

Invasive alien species are a global problem in both aquatic and terrestrial environments. Alien species are highly destructive to their novel environment and have the ability to out-competing native species, introduce diseases, compete for resource and alter important ecosystem structure and processes (MacNeil et al. 1999; Havird et al. 2013). Fish invasions have been well documented globally, for instance, North America has the most invasive fish species, followed by Asia, Europe, South America, and Africa, with Australia having the least invasive fish species (Elvira & Almodóvar 2001; Grabowska et al. 2010; Sultana & Hashim 2015; Xia et al. 2019). However, invasive species become even more complicated and ecologically damaging when they occur in the subterranean environment due to habitat fragmentation which restricts movement. As such, rare and endemic subterranean populations are subjected to high risk of extinction.

Similar to terrestrial and aquatic environments, alien invasive have an impact on many subterranean habitats and biodiversity (Jacobs et al. 2021). Although this topic has yet to be fully explored and quantified, the limited evidence observed and borrowing from other environments, local species may be on the verge of extinction. For example, *Dikerogammarus villosus*, also called killer shrimp, is native to Ukraine surface water systems and, due to its competitive ability, it has invaded significant parts of western Europe (Pinkster et al. 1992; Nesemann et al. 1995; Mayer et al. 2008). The killer shrimp has an incredible adaptation ability, being able to invade freshwater and brackish water bodies and replace major omnivorous invertebrate species from their functional role (Bij de Vaate & Klink 1995; Dick & Platvoet 2000; Van der Velde et al. 2000). Van der Velde et al. (2000), Boets et al. (2010) and Warren et al. (2021) reported that killer shrimp has successfully replaced native species in their alien range leading to reduced aquatic biodiversity. Killer shrimp is a classic example that explains the impact of invasive species on natives, and the assumption is that the impact might be more severe, especially in subterranean environment. The *Faxonius neglectus* (Faxon, 1885), also known as ringed crayfish, is an invasive species native to North America that occurs in lentic water systems. Ringed crayfish has invaded the United States, New York, Missouri and Oregon cave systems. In the Tumbling Creek Cave, the ringed crayfish is declared a threat to the native *Antrobia culveri*, tumbling creek cavesnail (Hubricht, 1971). Mouser et al. (2018) evaluated the population demographics and morphological characteristics of *F. neglectus* in Missouri cave, where they found that the crayfish populations had a larger in body size and lived longer than those in the surface water system. This study showed that when surface species such as the ringed crayfish are introduced in caves, they have a great ability of adapting to the cave systems and might have a detrimental impact on endemic species.

Australia, Africa, South America, and Asia have recorded several introduced fish species in the subterranean environments, mainly the Cypriniformes and Siluriformes (Irish & Maris 2002; Proudlove 2010; Jacobs et al. 2021). Within the alien range, both families have successfully invaded the subterranean environment, out-competed native species and altered native species populations (Proudlove 2010; Jacobs et al. 2021). Meanwhile, in South Africa we know less about the impact of introduced species in cave systems. As such, there is a need to investigate and quantify the impact of introduced fish species in these habitats to inform conservation of specialist subterreanean species.

However, we know more about the introduction of alien fish species in freshwater habitats, especially the koi fish, which has been recorded worldwide (David et al. 2004; Koehn 2004). Koi fish (Cyprinidae) are brightly coloured domesticated Japanese breeds that have been introduced and established in many countries for ornamental purposes (Balon 1995; Pinder et al. 2005; Liu et al. 2015). *Cyprinus carpio L* (Linnaeus, 1758) and *Pseudorasbora parva* (Temminck & Schlegel, 1846) are the most invasive members of the cyprinids and have been recorded in freshwater systems in high abundance, disrupting native aquatic species (Gozlan et al. 2005). Contrarily, in Europe and Asia, koi fish are of commercial importance as food source through the aquaculture (David et al. 2004).

Koi fish have incredible traits and adaptation abilities that enable them to survive in poor water quality environments, strive in different climatic zones outside of their native range and have extended survival periods (Gehrke & Harris 2001; Miller & Crowl 2006; Stuart & Jones 2006). Continents such as South America, Europe, Asia, Africa, Australia and Oceania have been invaded by koi fish, and their impact on indigenous biodiversity differs between species. For instance, the European common carp are widely spread in Australia and Brazil. These fish species disrupt the habitat by introducing parasitic diseases to which native species are susceptible, causing trophic cascades in invaded systems and sequentially promoting their population (Koehn et al. 2000; Stuart & Jones 2006). On the other hand, *Pseudorasbora parva* (Temminck & Schlegel, 1846) is a small koi fish native to Asia and has invaded many regions, especially Europe (Gozlan et al. 2005). Britton et al. (2010) compared the feeding habits of four invasive cyprinids species *P. parva, C. carpio*, *Rutilus rutilus* (Linnaeus 1758), *Scardinius erythrophthalmus* (Linnaeus, 1758) and the ecological impact they have on native fish species. The study revealed that *P. parva* caused a trophic position shift in *S. erythrophthalmus* and native fish species, providing more evidence that *P. parva* is an aggressive pest species that can out-compete native and other invasive species as previously shown by Welcomme (1992).

South Africa is one of the African countries with no native subterranean fish, yet fish species have been recorded in subterranean habitats (Bichuette & Trajano 2010; Proudlove 2010). Irish & Maris (2002) reported *Clarias gariepensis* (Burchell, 1822) in The Eye of Kuruman cave in the Northern Cape. *Clarias gariepensis* is a surface-water fish native to South African river systems and a member of the Clariidae family and can survive in the subterranean environment for an extended period (Irish & Maris 2002; Bichuette & Trajano 2010). The fish species have been introduced to subterranean habitats in African countries such as Namibia and South Africa (Bichuette & Trajano 2010; Du Preez 2014). The introduction of *C. gariepensis* in African aquifers has led to biodiversity loss for native species such as *C. cavernicola* (Trewavas, 1936) in Namibia. *Clarias gariepensis* has been further recorded in the Wonderfontein Cave in Gauteng province, however, its ecological impacts on subterranean environment and local aquatic invertebrates are unknown (Du Preez 2014). This calls for comprehensive investigation of subterranean biology and their related threats.

3.4 Methods to quantify impacts of introduced species

Alien invasive fish species and their associated predatory behaviour has been considered to have a significant impact on native species. Traditionally, researchers have used gut content analysis to investigate alien fish species diet preference and composition (McKinney 2006; Gozlan et al. 2010). Data on feeding ecology can help us predict changes in food chains and ecosystem energy transfers. It can also help understand the interactions between predators and prey. However, the disadvantage of gut content analysis is that prey remains are usually in small pieces and not easy to identify, except in cases of sclerotized body features (i.e., head, part of the legs and elytra for water beetles) that have been used for identification. As a result, it is necessary to complement the gut content with other comprehensive and reliable methods, such as DNA barcoding and stables isotope analysis.

Stable isotopes have been widely used in ecological studies to investigate the feeding behaviour of predators, particularly fish species (Jepsen & Winemiller 2002; Davis et al. 2012). The isotopic ratios are the numerical interpretation of nature's biological and chemical processes where producers use isotopic elements and then transfer them to consumers and further higher up in the food chain (Gannes et al. 1997). Stable nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) isotopes are the overall ratios of heavy and light isotopes from the source to the sink (i.e., producers to consumers). The isotopic elements provide essential insights into trophic relationships between species (Gannes et al. 1997; Post 2002; Layman et al. 2012), and this make it possible provide an in-depth understanding of alien species ecological impact to freshwater ecosystems structure and processes (Caut et al. 2006; Hill et al. 2015; Taylor et al. 2019).

3.4.1 The impact of preservation methods used for stable isotopes biological material

Over the years, stable isotopes have become a popular and accurate tool used to study the trophic levels in species. Moreover, the efficacy of this method is limited by the preservation methods of the samples or biological materials employed in the field (Boecklen et al. 2011). Numerous studies have investigated the impact of various preservation methods of stable isotopes, with some literature still debating the use of certain methods (Barrow et al. 2008). Stable isotope preservation methods include freezing, ethanol, salt, drying, and formalin. Methods such as freezing (which includes putting the samples in ice), salting (involves dissecting and subjecting the sample to salt, which slows the rotting effect), and drying (consists of removing the sample's fluids by dissecting and using air) are in some cases not options to practice in the field and alternative methods are needed in such instances (Carabel et al. 2009). Ethanol and formalin preservation methods are the most commonly employed methods in the field (Von Endt 1994). However, the latter methods have been investigated to have a negative impact on the $\delta^{13}C$ and $\delta^{15}N$ values. For instance, a study that investigated the isotopic ratios of $\delta^{13}C$ and $\delta^{15}N$ in two different ant species used ethanol to preserve specimens in an attempt to answer the question that most researchers have: thus, can ethanol be used to store samples for isotopic research? This study found that samples stored in ethanol can be used to measure the isotopic ratio of δ15N (Kiszka 2014).

Consequently, a study that compared ethanol and frozen preserved fish samples for stable isotope analysis has found that ethanol increases the $\delta^{13}C$ isotopic ratios while $\delta^{15}N$ was much smaller than that were stored in ice (Vizza et al. 2013). This study also shows that the isotopic ratios of $\delta^{13}C$ were influenced mainly by the storage time, thus two to six months with $\delta^{15}N$ having a small change correlated with storage time. Similarly, numerous studies have shown that biological material stored in ethanol has conflicting results, with some studies showing that ethanol has no significant impact on the $\delta^{13}C$ and $\delta^{15}N$ ratios (Barrow et al. 2008). Studies that agree with the latter have incorporated storage time, and washing or cleaning the biological material with distilled water reduced the impact of ethanol on $\delta^{13}C$ and $\delta^{15}N$ ratios (Stephenson & Riley 1995; Sarakinos et al. 2002). This study reports the $\delta^{13}C$ and $\delta^{15}N$ ratios from biological material stored in 70% ethanol for a week. The second mechanism is uptake of the preservative into the tissue. Both preservatives are carbon-based chemicals with characteristic δ13C signatures. Once preserved samples are immersed, their signature may shift toward that of the preservative (Hobson et al. 1997; Gloutney and Hobson 1998; Bosley and Wainright 1999; Ponsard and Amlou 1999). In our experiment, δ13C in both fish and aquatic invertebrates (–27 to –28‰) shifted toward that of the formalin medium (–32‰) following fixation (Fig. 1b). No such shift was evi- dent for EtOH.

During an investigation to re-evaluate the taxonomy of subterranean amphipods from the genus *Sternophysinx* and understand their distribution in Boesmansgat cave Northern Cape province South Africa (see Chapter 2, pages 17-37), we observed the presence of koi fish species that have been introduced. Boesmansgat cave is a type locality of two subterranean amphipods species *S. megacheles* and *S. basilobata* with *S. megacheles* only endemic to this cave. Both species were difficult to collect as compared to other species from different sites and after several collection attempts, including leaving a baited zooplankton net over-night, it was observed that both species were most likely occupying deeper depths of the subterranean system (~30 and ~40 meters respectively) below the water surface making sampling challenging. With the presence of introduced koi fish individuals, this chapter was aimed to investigate if the presence of the introduced koi fish was responsible (predation) for the amphipod distribution. It was hypothesized that the koi fish was preying on both co-occurring amphipods and other aquatic macroinvertebrates and further altered the behaviour and abundance of *S. megacheles* and *S. basilobata* to avoid predation.

3.5 Materials and methods

3.5.1 Study site

Boesmansgat cave (27°55′18″S, 23°38′30″E), also known as Bushman cave in Northern Cape, Province, South Africa. Detailed cave description and amphipod collection method are explained in Chapter 2, page 41.

3.6 Additional data collection

3.6.1 Water chemistry

Physicochemical parameters such as pH, conductivity (EC: µS), water temperature (ºC) and dissolved oxygen concentration (DO: mg/l) were measured (n=3), per 1 metre depth (from 1 – 20 metres depth) using a water-chemistry multi-parameter (PCSTestr 35) and a DO Pen (Sper Scientific 850045) respectively. Additionally, three replicate water samples (500 ml), per 1 metre depth (from 1 – 20 metres depth) were collected to measure the water nutrients concentrations, including nitrogen (NO_3 mg/l), phosphate (PO_4^- mg/l) and ammonium (NH_4^+ mg/l) using Hanna Bench Photometer for Aquaculture– (HI83203-01) (range 0.0–30.0 mg/l).

3.6.2 Biological samples

3.6.2.1 Gut content samples and analysis

To investigate the koi fish diet composition gut content analysis was used. One koi fish individual was collected using diving spearfishing technique at around 50 metres. The individual koi fish specimen was immediately dissected to remove the internal organs, including the intestines and the stomach. These organs were kept in ice until they reached the laboratory for further analysis. The gut content analysis in interval of 30 mm, and was based only on the number of taxa (N) of prey found in the gut. All the contents within the 30 mm, were carefully transferred onto a petri dish and analyzed under a stereomicroscope were remains (i.e., legs, heads, wings and antennae parts) were identified to order or family level.

3.6.2.2 Stable isotope analysis

Aquatic organisms including primary producers (i.e., phytoplankton, periphyton, floating and submerged macrophytes and organic matter) and consumers (aquatic macroinvertebrates and vertebrates, frogs and koi fish) samples were collected using variance methods i.e., by hand and 30 × 30 cm square frame net with 1 mm mesh size aquatic hand net were used for carbon ($\delta^{13}C$) and nitrogen ($\delta^{14}N$) stable isotope analysis. The specimens were transferred into a collecting jar containing 70% ethanol (accept phytoplankton, periphyton and aquatic plants) and later identified in the laboratory.

Aquatic macroinvertebrates were identified to the lowest possible taxonomic level (Sub-Family or genus) using a stereomicroscope and Southern Africa Guides to Freshwater Invertebrates (Day et al. 2002; Day et al. 2002; 2003; de Moor et al. 2003b). Aquatic macrophytes and vertebrates, i.e., frogs and koi fish, on the other hand, were identified using Freshwater Fishes of Southern Africa (Skelton 2001), a guide to the frogs of Southern Africa (Du Preez & Carruthers 2009) and a guide to the Invasive Alien Plants in South Africa (Henderson 2001). Water samples (250 ml) were collected at each depth, and vacuum filtered through the Whatman Glass microfibre filter (GFFs) papers (GFFs, 0.7-micron pore, 47 mm diameter), using a vacuum pump (Instruvac® Rocker 300) at 20 kilopascals (kPa), and the resulting samples were used for phytoplankton. Additional, periphyton biofilm on all available submerged structures i.e., stones and aquatic plants, was scrapped off using a new toothbrush using a white tray with 500 ml distilled water. The resulting 500 ml integrated periphyton sample was divided into three sub-samples of 150 ml each and vacuum filtered onto GFFs.

For each taxon (i.e., aquatic macroinvertebrates, macrophytes, frogs and fish), three replicates of tissue samples for each individual and taxa with sufficient biomass were oven dried for 72 hrs at 50˚C. This comprised a whole specimen of aquatic invertebrates, both stems and leaves for macrophytes, toes for frogs and muscle tissue for the fish samples, all in triplicates. The dried plants and animal tissues were thereafter ground into fine powered using a mortal and pestle and the remaining coarse/fine powder was weighed into aluminium tin capsules, which was about 0.5 – 0.7 mg for plants and 1.0 – 1.2 for animal tissues. All samples including phytoplankton and periphyton GFFs filters were sent for $\delta^{13}C$ and $\delta^{15}N$ isotope analysis using a Flash EA 1112 Series coupled to a Delta V Plus stable light isotope ratio mass spectrometer via a Con-Flo IV system at the Stable Isotope Facility, University of Pretoria, South Africa.

3.7 Data analysis

All statistical analyses were performed using R version 3.6.1 (R Core Team, 2019). To test for significant differences in water chemistry parameters (i.e., pH, EC, water temperature, DO, NO_3^-, PO_4^{3-} and NH_4^+) and depth. The Shapiro-Wilk test for normality was used, and all parameters were not normally distributed ($P<0.05$) except for DO. Thus, a non-parametric test, Kruskal-Wallis was used to investigate differences between the water chemistry parameters.

Stable isotope mixture models also called mixing models are commonly used to evaluate the contribution of species diet sources through their tissue. In this study fish diet was evaluated using the mixing models which quantified the proportional contribution of sources in Boesmansgat cave. The carbon **(**$\delta^{14}C$**)** and nitrogen ($\delta^{15}N$) isotope signatures were used to investigate the importance of macroinvertebrates specifically, the two amphipod species occurring in Boesmansgat cave to the fish diet. The Bayesian mixing models used in this study included SIAR and MixSIAR (Parnell et al. 2010; Stock & Semmens 2013). Simmr is a SIAR Bayesian model package was used to investigate the source portion for stable isotope analysis. The simmr was run to investigate the mean signature of $\delta^{13}C$ and $\delta^{15}N$ values at different feeding levels. The Bayesian stable-isotope mixing model MixSIAR (Stock & Semmens 2016) was used to investigate the feeding composition of the koi fish. To investigate the trophic position, species were grouped into four groups: group 1 included all the primary producers, group 2 represented consumers, group 3 omnivores and group 4 represented predators. The Bayesian mixing models SIBER and simmr were used to examine the trophic position of the species, and an isospace was constructed. The simmr graphs were constructed with the Bayesian framework in the R (SIBER) model to verify the food web of the cave system and the feeding habits of the koi fish.

3.8 Results

3.8.1 Water chemistry

Using Shapiro-Wilk test no significant was found difference between water chemistry parameters and depth for pH (H = 5.6, P = 0.4695), EC (H = 7.9673, P = 0.2405), water temperature (H = 11.346, P = 0.07827), DO (H = 10.513, P = 0.1047), NO_3^- (H = 3.5276, P = 0.7403), PO_4^{3-} (H = 5.4097, P= 0.4924), and NH_4^+ (H = 8.259, P = 0.2197) (Table 3.1) Using one-way Kruskal Wallis, significant differences were obtained for Temp, DO and NH_4^+ between the different depths ($p < 0.05$) (Table 3.2). For water temperature, the significance was found between depths 1m and 10m, 1m and 15m ($p < 0.05$). For DO, significance was obtained only between 1m and 20m ($p < 0.05$), and for $NH_4{}^+$ the significance was between a depth of 5m and 20m ($p < 0.05$).

Table 3.1: Average ± standard deviation (SD), F-value and P-value obtained from the Kruskal test between the water chemistry parameters collected from 1m to 20m depths. Kruskal test was used to test for significant differences between different depths.

Water chemistry parameters	**Mean and standard deviation**					**Kruskal Wallis stats**		
	1m	5m	10m	15m	20m	F-value	DF	P-value
pH	7,75 (±0,12)	7,71 (±0,15)	7,47 (±0)	7,7 (±0)	7,44 (±0,32)	1.27	10/4	>0.05
EC (µS/cm)	391 (±10,58)	259,9 (±218,5)	386,3 (±2,9)	396 (±0)	390 (±7,8)	1.32	10/4	>0.05
Water temperature (°C)	22,90(±0,2)	17,19(±8,27)	21,23(±0.05)	21,2(±0,1)	21,37(±0,21)	16.26	10/4	**<0.05**
DO (mgL)	2,47(±0,15)	4,32(±2,97)	2.3(±0.3)	1,73(±0,31)	1,43(±0,15)	6.34	10/4	**<0.05**
NO_{3-} (mg/L)	1,50(±1,13)	3,89(±3,32)	1.47(±1.40)	0,37(±0,56)	4,67(±4,51)	0.87	10/4	>0.05
PO_4^{3-}(mg/L)	12,80(±11,20)	5,85(±3,34)	7.63(±9.77)	6,7(±6,80)	3,8(±6,58)	0.69	10/4	>0.05
NH_4^+(mg/L)	0,24(±0,15)	3,53(±3,61)	0.26(±0.38)	0,49(±0,44)	0,07(±0,06)	4.22	10/4	**<0.05**

3.8.2 Sweep and gut content

Comparing sweep and gut contents analyses yielded different and yet interesting results (see Table 3.2). The gut content comprises of prey items that have been recently assimilated with small unidentifiable prey items found. The gut content analysis showed two dominant aquatic taxa remains, which included Hydrophilinae and Donaccinae, with Hydrophilinae being the most abundant prey (Table 3.2). All the prey items in the gut were small hard exteriors such as legs, heads, elytra, and antennae. Furthermore, since the gut contents analyses solely relays on assimilated prey items, most on the prey items with soft tissues such as bugs and macrophytes are difficult to identify with the gut content in the intestines having completely digested items.

The results from sweep provided a more powerful insights of the trophic relations in the Boesmangat cave ecosystem. *Sternophysinx basilobata* (amphipods), Hydrophilinae/ Donaccinae (beetles), Pleidae (bugs), *Lemna* sp. (macrophytes), Glossophoniidae (leeches), and Pipidae (frogs and their juvilles) were collected and identified from the sweeping methods. Of these collected species bugs and leeches were more abundant than other species with 224 and 110 individuals collected respectively. Species such as frogs and amphipods were found in depths of 10m and 30 m explaining their scarcity in the system.

Table 3.2: The koi fish gut content prey items identified to either a family or genus level and the number of individual taxa identified compared to the macroinvertebrate species identified in sweeps in the Boesmansgat cave.

Family	Genus/Species	Sweeps	Gut content
		Taxa abundances	Count of remains
Glossophoniidae	I	10	_
Glossophoniidae	II	9	_
Glossophoniidae	III	110	_
Hydrophilinae	*Laccobius* I	8	_
Hydrophilinae	*Laccobius* II	4	_
Hydrophilinae	*Laccobius* III	_	99
Hydrophilidae	*Helochares*	3	_
Hydrophilidae larvae	_	1	_
Chironominae	_	6	_
Pleidae	_	224	_
Pipidae	*Platanna sp.*	1	_
Sternophysingidae	*Sternophysinx basilobata*	3	_
Orthocladiinae	_	3	_
Donaccinae	_	_	36

3.8.3 Stable isotopes analysis

Table 3.3 Revealed that the koi fish in the Boesmansgat cave was omnivorous and primarily fed on aquatic beetles, bugs, amphipods, leeches, frogs and tadpoles. The stable isotope analysis shows that the Boesmansgate cave is fueled by phytoplankton and organic matter with $\delta^{13}C$ values ranging between 30‰ and 20‰. Aquatic plants had the lowest $\delta^{13}C$ value of -30‰ with periphyton having the highest $\delta^{13}C$ values ranging between -15‰ and -10‰, implying that the periphyton is an essential carbon source for species that occupy the low trophic position (Figure 3.1). Organic matter appears to be the primary $\delta^{13}C$ source for midges, bugs, beetles, fish, tadpoles and frogs. The koi fish, phytoplankton, tadpoles, frogs, bugs, beetles, organic matter and midges had similar $\delta^{13}C$ values ranging from ~24‰ to ~17.5‰. Leeches and amphipods were more enriched with $\delta^{15}N$ values ranging from ~15.2‰ to 17.1‰ (Figure3.1). This studyis results have also revealed that aquatic plants had high $\delta^{13}C$ values (-26.61± 1.417‰), followed by organic matter (-25.34± 0.085‰), beetles (-23.47± 1.854‰) and midges (-23.32± 4.749‰) with periphyton having the least $\delta^{13}C$ values (-12.36± 1.651‰). High $\delta^{15}N$ values were observed in aquatic plants (14.62±0.964‰), with leeches and fish having similar $\delta^{15}N$ values of 12.88±1.701‰ and12.37±0.273‰ (Table 3.3). However, organic matter and phytoplankton had the least $\delta^{15}N$ values of 3.976±2.194‰ and 4.593±1.578‰ respectively.

The isotopic niche and carbon range for group 1 (phytoplankton, periphyton and aquatic plants) was larger than that of other groups, suggesting that group 1 served as the basal resources of the Boesmangat cave system and provided energy subsidies to higher trophic levels (group 2, 3 and 4) as expected (Figure. 3.2). Majority of the groups (1, 2 and 3) showed the similar $\delta^{15}N$ range, indicating that they were feeding in the same or similar trophic level, however their $\delta^{13}C$ range was variable, indicating variable energy resources subsidies values (Fig.3.2). Group 4 (frogs and koi fish) had the narrowest $\delta^{13}C$ range but larger $\delta^{15}N$ values indicating a possibility of specialized feeding and holding the highest trophic feeding position as it was expected.

Table 3.3: The mean and standard deviation of δ^{14}C and δ^{15}N isotope signatures (Mean ± SD) showing the prey item proportion consumered by the koi fish in the Boesmansgat cave.

Taxa	Species name	δ^{14}C (Mean ± SD)	δ^{15}N (Mean ± SD)
Phytoplankton	_	-19.89(± 1.158)	4.593(±1.578)
Periphyton	_	-12.36(± 1.651)	7.155(±0.371)
Aquatic Plants	*Lemna spp.*	-26.61(± 1.417)	14.62(±0.964)
Organic Matter	_	-25.34(± 0.085)	3.976(±2.194)
Amphipoda	*S. basilobata*	-19.42(± 0.72)	10.64(±0.190)
Tadpole	*Platanna sp.*	-22.43(±3.099)	8.11(±1.389)
Midge	_	-23.32(± 4.749)	5.477(±0.79)
Leech	Glossophoniidae	-19.53(± 1.398)	12.88(±1.701)
Beetles	*Helochares/ Laccobius*	-23.47(± 1.854)	6.327(±2.053)
Bugs	Pleidae	-21.97(± 0.128)	6.063(±0.323)

Frog	*Platanna sp.*	-21.95(± 2.79)	7.975(±1.257)
Fish	Cyprinidae	-22.64(±0.323)	12.37(±0.273)

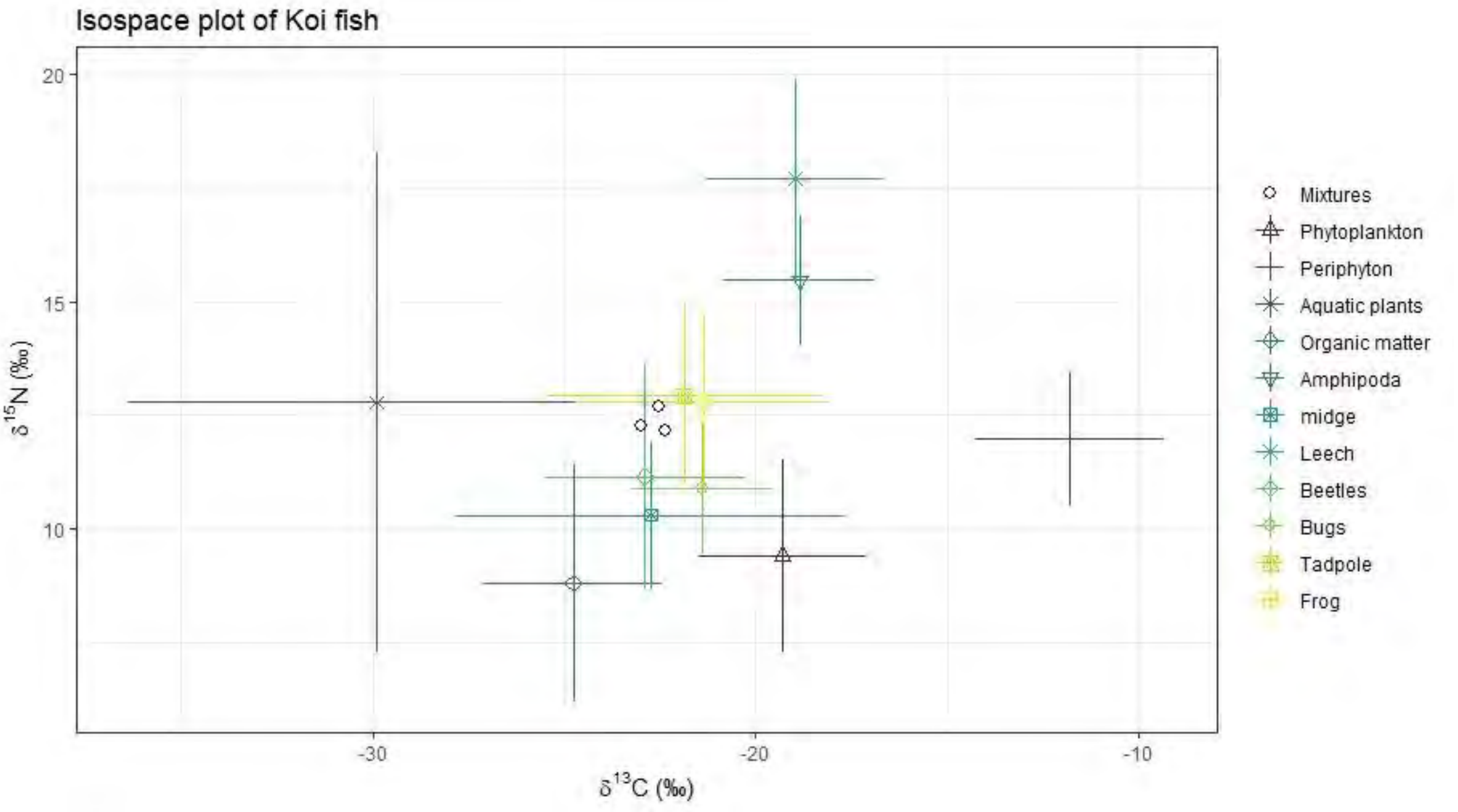

Figure 3.1: Stable isotope biplot of macroinvertebrates, koi fish species and amphibians occurring in the Boesmansgat cave. The isoplot only shows the mean values

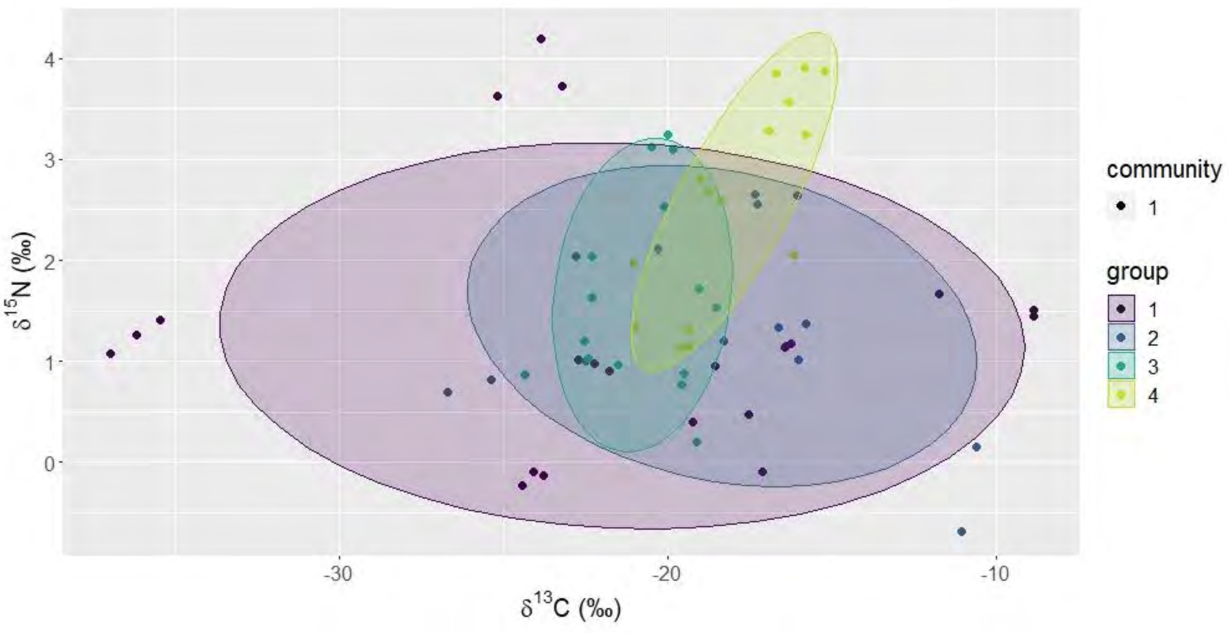

Figure 3. 2: A bi-plot graph showing the food web of the Boesmansgat cave with Group 1 presenting primary producers, Group 2 presenting consumers, Group 3 presenting omnivores and Group 4 predators

The mixing model density distribution plots estimate the importance of each prey item to the fish diet (Figure 3.3; Figure 3.4). Periphytons, followed by phytoplanktons, are narrowly distributed food sources ranging from ~0.00-25% and ~0.00-26%. This means that periphytons and phytoplanktons are the least preferred food source for the koi fish diet. Additionally, organic matter, beetles, leeches, tadpoles and frogs have high prey proportions ranging from ~0.00-40%. The latter prey items were important prey sources to the fish diet. Consequently, midges, amphipods and bugs had similar distributions to the fish diet, thus indicating the koi fish also preferred the latter food sources contributing between ~0.00-34% to the fish diet.

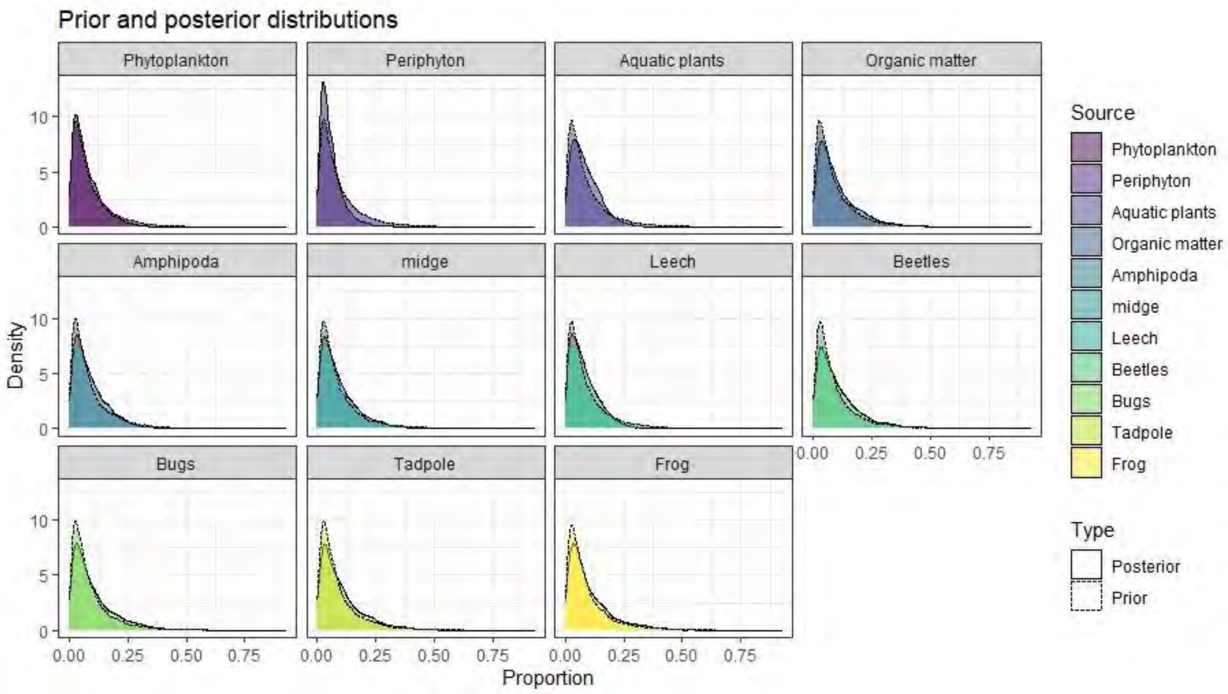

Figure 3.3: The figure shows the prior and posterior probability distributions of the contributions of all food sources to the koi fish diet.

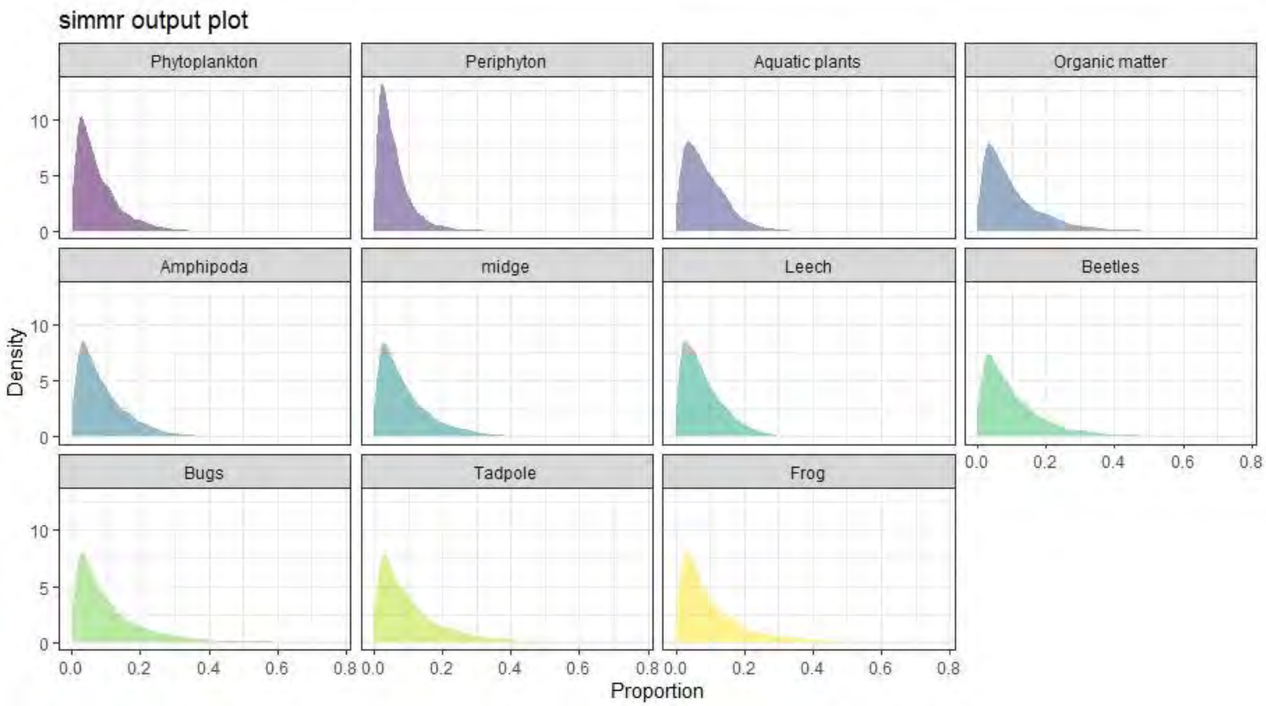

Figure 3.4: Show the koi fish food sources in densities and proportions, where amphipods were part of their preference.

3.9. Discussion

Describing the diet of fish species in an introduced environment and quantifying their impact on native species will further our understanding of the ecological impact of introduced species. Stable isotopes and gut content analyses agree with the hypothesis that the koi fish fed on the two endemic amphipod species and other aquatic invertebrates and thus could have alter the behaviour and the habitat preference of the amphipods although this assumption still needs to be further tested. These results further indicate that the two endemic amphipod species are under threat by the introduction of exotic fish in their subterranean environment and the possibility of extinction if conservation actions are not implemented.

These results are in agreement with studies by Bouffard & Hanson (1997), Zimmer et al. (2001, 2002) and Anteau & Afton (2004), that amphipods are negatively impacted by the presence of fish species in their habitat. For example, in the lakes and wetlands of the Midwest, the decline in the diversity of two amphipod species *Gammarus lacustris* Sars, 1863 and *Hyalella aztecalakes* were negatively affected by the presence of *Pimephales promelas* (Rafinesque, 1820; MacNeil et al. 1999; Anteau & Afton 2006, 2008). *Pseudorasbora parva* has been reported to have a broader macroinvertebrate preference, thus feeding on copepods, amphipods, ostracods, and cladocerans (Czerniejewski et al. 2019). However, most reports investigating alien fish species impacts on local invertebrates have been conducted in surface water systems. Interestingly, using integrative methods (i.e., gut content and stable isotope analysis), this study reported a similar feeding habit in a subterranean habitat.

One interesting observation from this study was that the two co-occurring amphipods species differ in their body size and abundance, while also occupying deeper depths of around 30-40 meters, which was different from all the other species collected from different sites (see Chapter 2, methods). *Sternophysinx megacheles* was smaller in body size (16mm) and as compared to the large-sized *S. basilobata* (26 mm), which contradicts the work of Griffiths & Stewart (1996) and Griffiths (1991). Furthermore, *S. megacheles* was less abundant than the *S. basilobata,* with 1 and 6 specimens collected respectively. The latter indicates that any further impact inflicted by the alien koi fish species might lead to the extinction of *S. megacheles* in the future if no action is taken.

Studies have shown that species of Cyprinidae are omnivores feeding on plants and animals. For example, *C. carpio var. specularis* is one fish species investigated as an omnivorous feeder (Manon & Hossain 2011). Similarly, this study found that the koi fish in Boesmansgat cave is omnivorous. Muchlisin's et al. (2015) study has yielded similar results where *Tor tambra* (Valenciennes, 1842) was an omnivorous feeder that mostly prefers green algae and earthworms. Invertebrates have been reported to be an essential food source for some fish species and have been observed in their fish gut contents. For instance, dipterans are an essential prey item for *Barbus* species, and a study by Dadebo et al. (2013) has indicated that they constituted about 62.5% of the fish diet. Like the current studies on cyprinids, the preferred food source of the koi fish in the Boesmansgat cave comprises primarily invertebrates such as beetles, bugs, leeches and frogs. The high invertebrate preference of the koi fish in the present study might be due to the lack of competition or lack of defense and limited dispersal for the invertebrates. Additionally, subterranean habitats have simple food webs dominated by invertebrates, thus the koi fish was expected to alter the trophic structure and becoming the apex predator of the system.

The results from stable isotope analysis strongly suggest that the koi fish is the top predator feeding on aquatic plants, invertebrates and amphibians. Furthermore, due to the preservation method used the isotopic results might be overinterpreted, for example, this study has low values of $\delta^{14}C$. However, studies have argued the use of ethanol for a week to not have significant effects of the $\delta^{14}C$ and $\delta^{15}N$ (Barrow et al. 2008; Javornik et al. 2019). Other studies have favoured the use of ethanol preservation due to its lipid dissolving property as lipids have been investigated to change or have significant impact on the $\delta^{14}C$ isotopic ratios (Tieszen et al. 1983). This study has also shown increase $\delta^{15}N$ values. Kiszka et al. (2014), study compared freezing and ethanol storage methods for $\delta^{14}C$ and $\delta^{15}N$ reported a –0.48 decrease in $\delta^{14}C$ value from ethanol compared to the frozen material. Nevertheless, both preservation methods thus, frozen and ethanol did not have a significant impact on the $\delta^{15}N$ values. Such literature therefore supports these studies finding that the fish species feeds on the aquatic inhabitants at the Boesmansgat cave. Species of the family Cyprinidae are known for feeding on various food sources and can shift prey items (Gosline 1973; Sibbing and Nagelkerke 2001). However, other species have been found feeding on specific food sources, for instance, Adámek et al. (2003) study on carp (*Cyprinus carpio L.*) revealed a diet rich in debris and detritus. Adámek et al. (2003) study also revealed a plant and animal feeding habit observed in some fish species (Winkelmann et al. 2011; Shelton et al. 2016). These findings are consistent with Dadebo et al. (2013) results in that *B. paludinosus* fed on both plants and invertebrates, with invertebrates and detritus more preferred at 86.3% and 93.5%.

Our study showed that gut content analysis did not observe food contents such as bugs, amphipods, duckweed and frogs, but showed more beetles remains. These findings are similar to Shelton et al. (2016) results that showed a high preference for invertebrates in the *P. burchelli* (Smith, 1841) diet. The introduced species are likely to have a negative impact on native species due to a lack of defense against the alien predator (Cox & Lima 2006). Winkelmann et al. (2011) study on the effect of benthivorous fish on the invertebrate community using gut content showed a decline in the density of *Pisidium*, *Dugesia gonocephala*, *G. pulex* and Limoniidae due to predation pressure. A similar study compared the feeding behaviour and impact of carp and African catfish on the invertebrate abundance, and the finding showed a decrease in invertebrates, periphyton, and zooplankton in treatment with carp as compared to the treatment with catfish. Gut content analysis of *Rutilus rubilio* and *Leuciscus cephalus* also showed a high invertebrate preference, with Trichoptera larvae more abundant in the gut content (Shelton et al. 2016).

The gut content is a traditional method to investigate fish feeding behaviour (Sheppard et al. 2010; McMahon & McCarthy 2016). Unfortunately, this method is limited and should be used in collaboration with other sensitive and intergrative methods, such as stable isotopes. This study observed this where prey items such as aquatic plants, midges, amphipods and leeches were not observed in the stomach gut content. In the gut content, the beetle heads, legs, antennae and elytra were the only identifiable body part. This might be because the tough exoskeleton is beetles that take time to completely digest compared to the soft tissues of frogs, bugs, duckweed, leech and amphipods which are soft bodied and need no hard exoskeleton like terrestrial insects. Gut content analysis however, is known to have limits, thus stable isotopes was able to provide a comprehensive prey preference and accounted for the misleading evidence of abundant beetles remains in the gut content. As far as this investigation is concern, there is evidence that the koi fish was omnivorous and opportunistically fed on aquatic producers (phytoplankton, periphyton and aquatic plants) which require less energy to find. Studies have also reported that gut content is inefficient in identifying prey items with soft tissues, and even the hard parts observed in the gut content are difficult to identify (Clarke et al. 2005). For example, in this study, the only prey item identified in the gut content was beetles. By comparing the results from the swept and stable isotopes, other prey items were identified. Clarke et al. (2005) had similar views or difficulties regarding the stomach gut content where small prey items were not identified and concluded that integrating stable isotopes and swept showed a broader feeding preference of fish species. Incorporating carbon and nitrogen stable isotopes and swept techniques provide a better understanding of the feeding habits of opportunistic feeders (Fisher et al. 2001). These integrated methods have been used to investigate the diet composition of fish and crayfish (Hollows et al. 2002; Stenroth et al. 2006; Brush et al. 2012). The koi fish in this study showed destructive feeding behaviour similar to that of exotic carp species more so in isolated and fragile subterranean environment like the Boesmangat cave.

Chapter 4

General Discussion and Conclusion

Due to morphological similarities which does not account for cryptic species and speciation, there have been uncertainties or inaccuracies in the systematic of most subterranean amphipod species more so that they are understudied. In South Africa, this has not been evaluated, and there is a lack of updated information about the taxonomy and distribution of subterranean amphipods. On the other hand, subterranean habitat is under threat by increasing human activities (i.e., introduction of exotic species), which may lead to the extinction of species that only occur in this habitat (Marmonier et al. 2018; Di Lorenzo et al. 2019; Griebler et al. 2019). Subterranean amphipods remain the least taxonomically studied taxon, with the available morphological keys not adequate to resolve certain species. The morphological similarities between distant species and the complex subterranean environment makes this subject even more challenging to study. However, in developed continents such as Europe, South America and Australia, the cryptic species concept in subterranean amphipods is well studied (Fišer & Zagmajster 2009; Westram et al. 2013). In contrast, developing continents such as Africa have recently explored subterranean environments but the biology is still the least studied and amphipoda are not different with limited literature about their diversity and systematics. Hence, the present study was aimed to conduct a morphological analysis using existing taxonomic keys together with molecular phylogenetic analysis using mitochondrial 12S to study the subterranean amphipod family in great detail. Additionally, this study further investigated threats of subterranean habitat by assessing the impact of koi fish on subterranean aquatic macroinvertebrates in the Boesmansgat cave through gut content and stable isotope analysis.

More than 20 years after the seminal work of Holsinger & Straskraba (1973), Griffiths (1981; 1991), Holsinger (1992) and Griffiths & Steward (1996), South African subterranean amphipods have only been identified using morphological keys and have been reported to be restricted to few locations. Compared to other published work on the hidden diversity of subterranean amphipods and the use of DNA markers, the taxonomy and systematics of the South African subterranean amphipods still needs to be improved. Chapter 2 of this thesis was aimed to assess the phylogenetic relationship of the South African subterranean amphipod genus. This study is the first to investigate the taxonomy subterranean genus *Sternophysinx* through integrative taxonomy. This study has morphologically identified two new species from Bakwena cave and Sterkfontein cave, indicating the need to produce new taxonomic keys.

Further, results also found cryptic speciation in amphipod populations from Peppercorn cave, Ficus cave, Bakwena cave and Sterkfontein cave. Cryptic speciation has been observed in co-occurring species, which has been reported in other studies. In most cases, widely distributed subterranean amphipods have been reported to be cryptic species or species complexes. This study has shown that the morphological keys alone are not reliable to identify cryptic species and that additionally analysis i.e., DNA barcoding is necessary. For instance, without the 12S rRNA data, *S. basilobata* complexes could have remained unidentified as this species have been recorded in two caves (Boesmansgat and Kolgelbeen cave) in the Northern Cape Province. Other studies have also shown that morphological keys alone can not resolve a problematic taxon more especially subterranean taxon. For example, Gouws (2004) study on widely distributed isopods species revealed that South African isopods comprises of cryptic diversity. These results were only obtained through the use of morphological keys and DNA genes, and presently new isopods species have been identified thus improving taxonomic literature of the South African isopods. In the present study, phylogenetic analysis revealed that *S. basilobata* is a species complex of three genetically distinct species, and using molecular techniques three new species within this complex have been identified. Stewart (2001) reported similar results where *P. capensis* and *P. granulus* were regarded as widely distributed species, however the study later revealed that these species are actually a complex of genetically different species. This study further adds to the growth of literature that morphological identification alone is not sufficient to study underrepresented taxa in freshwater ecosystems.

Chapter 3 was aimed to assess the impact of an introduced surface koi fish species on two co-occurring and endemic subterranean amphipods in the Boesmansgat cave. This study has shown that the introduced koi fish exhibit omnivorous feeding behaviour with a wide preference to aquatic invertebrates including the endemic amphipod which is a conservation concern. For example, Taylor et al. (2019) studied the integrated stomach gut content and ^{15}N, ^{13}C stable isotopes to assess the feeding habit of *Micropterus salmoides* (Lacepède, 1802) in two South African dams. This study revealed an opportunistic feeding style where in Mankazana Dam, the fish mostly preferred invertebrates and showed a shift in prey items, thus feeding on small fish species, Meanwhile, in Wriggleswade Dam the fish solely fed on *Gilchristella aestuaria* (Gilchrist, 1913), as expected in both dams, *M. salmoides* was at the top of the food chain. Even though this study assessed the feeding behaviour of the koi fish in one system but a similar situation was observed where the koi fish showed an opportunistic and wide feeding preferences between producers and primary and secondary consumers. This chapter, further highlight the importance of conservation and how subterranean habitats are neglected in terms of research and general conservation actions. These systems have long been under threat by mining and agriculture, and reports have been tabled on the introduction of alien species fish species in these systems, but no follow-ups have been conducted (Irish & Marais 2002). This chapter is the first to assess the impact of the alien species on subterranean taxa, especially on the least studied taxon endemic and specialist subterranean amphipods. This is an ecological concern as the Boesmansgat cave houses two endemic species, *S. megacheles* and *S. basilobata*, with *S. megacheles* only restricted to this cave and not found anywhere else and already showing limited abundances in the system.

4.1 Problems encountered

This project primary aim was to use the cytochrome oxidase subunit 1 (CO1) to understand the phylogenetic relationship of the monotypic subterranean genus *Sternophysinx*. This mitochondrial gene has been mostly utilised to investigate cryptic species, species delimitation and genetic structure of species (Finston et al. 2007; Murphy et al. 2013; King et al. 2022). However, due to the pandemic that hit South Africa and the covid19 regulation, our laboratory work was delayed. The South African Institute for Aquatic Biodiversity (SAIAB) had limited space and only final year students were prioritized for lab work. Another problem was getting a suitable primer of which we tried two primers that could not work i.e, LCO1490, HCO2198, CrustDF1 and CrustDR1. Due to this we run low of rare specimens such as *S. basilobata* and *S. megacheles* from Boesmansgat cave and few of the two undescribed species from Bakwena cave and Sterkfontein cave. As such, we had to use 2-3 specimens each site or putative taxon instead of 3-5 for amplifying the 12S rRNA gene.

There are several sites that we could not sample due to the Covid19 pandemic, and with other sites not having correct GPS coordinated thus making it nearly impossible to locate them. These sites include two sites in the Cradle of Humankind, i.e., Koelenhof cave, and springs in John Nash Nature Reserve, one cave in Limpopo province, Matlapitse cave, a cave in De Hoop Nature

Reserve is located in Western Cape Province and Kolgelbeen cave in Northern Cape. Koelenhof cave is located in Kromdraai on a private property and this cave is a home for two co-occurring amphipods species *S. filaris* and *S. calceola*. Meanwhile John Nash Nature Reserve is located in the Cradle of Humankind just few kilometers from Sterkfontein cave (9.5 km). The springs in this farm are homes for one amphipods species *S. transvaalensis*. Both sites are located in private properties and getting access during pandemic was difficult. On the other hand, Matlapitse cave is situated about 46 west of Trichardtsdal and is the type locality of *S. calceaola* (Holsinger 1992). De Hoop Nature Reserve is located in Western Cape and because of heavy rain fall that covered the entrance with water the sampling was cancelled. Kolgelbeen cave is situated in a private farm in Griekwastad and houses *S. basilobata* with cave along with Chaos cave situated in North West are very complex cave. To sample the latter caves, a cave specialist was needed to assist with sampling and locating the groundwater table. With the current results thus identification of new species and species complex, getting topotype material from these caves would add greatly to the taxonomony of *Sternophysinx*.

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