

# **The distribution of aquatic molluscs and associated trematodes within the Limpopo Lowveld ecoregion, South Africa**

**MH le Roux**

 **orcid.org 0000-0001-5939-5359**

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| Supervisor:    | Prof NJ Smit   |
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## Abstract

The continued rise in global temperature under the current climate change predictions is placing immense pressure on natural freshwater ecosystems, with thousands of freshwater species known to be either threatened or extinct, due to the added pressure on freshwater ecosystems. Freshwater gastropods have an important ecological role as primary consumers and vectors for various medical and veterinary parasitic trematode infections. Shifts in precipitation patterns, a rise in global temperature, habitat availability, water quality and the presence of invasive species can affect the distribution and abundance of native freshwater snail species. The movement of native and invasive species to new ecological niches can extend the current ranges of medically and economically important parasitic trematode species. Southern Africa's rich freshwater snail diversity constitutes approximately 101 native and 14 invasive species although this is based on historical data, with the current status being unclear.

To accurately identify freshwater molluscan species from the Limpopo Lowveld region, morphological traits and molecular analysis were used to determine the current distribution of freshwater molluscan species in the region compared to historical data derived from the National Freshwater Snail Collection (NFSC) database. The current distribution was used to indicate how molluscan populations may have changed over an extended period. Thus, providing critical information to model future trends through scientific methods and aid in the development and implementation of management programs to preserve and protect local freshwater ecosystems. When compared to historical data, it was found that the native freshwater snail diversity of South Africa has seen a notable decline and more than 50% of the snails collected are considered invasive species, in contrast to the 14% of invasive species in the historical dataset. This provides clear support that one of the most critical threats to the native freshwater snails of the Limpopo system is the presence of invasive species. Furthermore, these invasive species not only pose a threat to native snail biodiversity but the diversity of the parasitic trematodes that use native snails as intermediate hosts. One of the invasive snail species collected during the current study was *Tarebia granifera*, a prolific and successful invader from Southeast Asia. The ability of *T. granifera* to rapidly spread through the aquatic ecosystem, resist unfavourable conditions that are detrimental to native molluscan species, and lack natural predators is concerning for native species. To date, no parasitic infections have been found in *T. granifera* in South Africa and it is thus not a known vector for any digenean parasites in South Africa. In contrast, native species such as *Bulinus* and *Biomphalaria* are vectors for various digenean parasites including *Schistosoma* sp. that transmits schistosomiasis.

The use of morphological and molecular analysis of the COI (Cytochrome c oxidase subunit 1) and ITS2 (Internal transcribed spacer 2) genetic sequences aided in correctly identifying freshwater molluscs to species level. The use of 28S rRNA genetic sequences with specialized primers that amplify trematode rRNA was used to establish prepatent trematode infections within molluscan species. The use of the same extracted DNA sample for both mollusc identification and identification of prepatent trematode infections reduced the cost and increased sample capacity. The combination of morphological and molecular identification provided new insight into the genetic diversity within the same species and genetic mutations between sites of collection. Molluscs that were morphologically identified as *Radix natalensis* (Krauss, 1848) formed four genetically diverse groups located at different sites. Thus, indicating some form of transportation between sites or a vector transporting the molluscan species between the various locations. Comparing the current distribution to the historically collected specimens in the NFSC database suggested that native genera have critically decreased in spatial distribution and abundance. The combined effect of increased environmental and anthropogenic pressures on native species is of concern to the future health of the natural ecosystem function within the north-eastern provinces of South Africa.

The reduction in native freshwater snail vectors could indirectly affect the presence of parasitic trematodes. The most effective method to assess patent infections is by examining snail tissue through dissection. The second method is through by cercarial shedding, where freshwater snail specimens naturally shed cercarial trematodes when exposed to artificial or natural light. This occurs when the sporocyst within the snail reaches maturity and the unsuccessful cercarial shedding tests could indicate that molluscan species may not have a trematode infection or have an immature or early-stage infection. Early-stage trematode infections require molecular monitoring to detect the prepatent trematode infections within specimens collected. In the present study 227 snails were screened for patent trematode infections. No patent infections were detected. However, molecular analysis for the prepatent infections indicated the presence of three different trematode infections within five different molluscan specimens. Trematode species detected were identified as *Neofibricola smiti*, *Orientocreadium batrachoides*, *Petasiger variospinosus*.

The current study successfully identified molluscan species at sites of historical significance, thereby allowing to establish the changes in molluscan diversity from historically sampled areas and establish that the native freshwater snail diversity is in a dramatic decline from historically recorded diversity. The decline in native known vectors of medically and economically important parasitic trematodes, could negatively affect the presence and abundance of trematode species. This was evident with none of the molluscan vectors indicating any signs of cercarial shedding.

However, prepatent infections of three parasitic trematodes were detected and the current study detected the first reported infection of *Neofibricola smiti* Achatz, Martens, Kudlai, Junker, Boe & Tkach, 2022 and *Orientocradium batrachoides* Tubanguui, 1931 within molluscan vectors. These findings from the current study indicate the lack of information regarding the life cycle of parasitic trematodes and the need to further examine the relationship between trematode species and their molluscan vectors.

Key terms: freshwater snails, invasive molluscs, trematodes, parasites, morphology, molecular identification, *Orientocradium*, *Neofibricola*.

## Abbreviations

| Full term                                                            | Abbreviation |
|----------------------------------------------------------------------|--------------|
| Analysis Of Similarities                                             | ANOSIM       |
| Basic Local Alignment Search Tool                                    | BLAST        |
| <i>Biomphalaria</i>                                                  | <i>B.</i>    |
| <i>Bulinus</i>                                                       | <i>B.</i>    |
| Canonical-Correlation Analysis                                       | CCA          |
| Deoxyribonucleic acid                                                | DNA          |
| Department of Water Affairs and Forestry, South Africa               | DWAF         |
| Free State                                                           | FS           |
| Free State Department of Tourism, Environmental and Economic Affairs | FSDTEEA      |
| KwaZulu-Natal                                                        | KZN          |
| Limpopo high flow                                                    | LHF          |
| Limpopo low flow                                                     | LLF          |
| Multiple Sequence Comparison by Log-Expectation                      | MUSCLE       |
| National Centre for Biotechnology Information                        | NCBI         |
| National Freshwater Snail Collection                                 | NFSC         |
| Neglected Tropical Disease                                           | NTD          |
| North-West University                                                | NWU          |
| <i>Petasiger</i>                                                     | <i>P.</i>    |
| <i>Physella</i>                                                      | <i>P.</i>    |
| <i>Pseudosuccinea</i>                                                | <i>P.</i>    |
| ribosomal Ribonucleic acid                                           | rRNA         |
| South African Scoring System                                         | SASS         |
| World Health Organization                                            | WHO          |

# TABLE OF CONTENTS

|                                                                                                     |           |
|-----------------------------------------------------------------------------------------------------|-----------|
| <b>ACKNOWLEDGEMENTS .....</b>                                                                       | <b>I</b>  |
| <b>ABSTRACT .....</b>                                                                               | <b>II</b> |
| <b>ABBREVIATIONS.....</b>                                                                           | <b>V</b>  |
| <b>CHAPTER 1 GENERAL INTRODUCTION.....</b>                                                          | <b>1</b>  |
| 1.1 Interactions between freshwater molluscs and the environment .....                              | 1         |
| 1.2 Identification of freshwater molluscs and their role as vectors for<br>digenean trematodes..... | 2         |
| 1.3 Control measures .....                                                                          | 5         |
| 1.4 Problem statement .....                                                                         | 7         |
| 1.5 Chapter layout of the dissertation.....                                                         | 8         |
| <b>CHAPTER 2 STUDY AREA, MATERIALS AND METHODS .....</b>                                            | <b>9</b>  |
| 2.1 Study region.....                                                                               | 9         |
| 2.1.2 Limpopo province .....                                                                        | 14        |
| 2.1.1 North West province .....                                                                     | 15        |
| 2.1.2 Free State province .....                                                                     | 19        |
| 2.2 Methods.....                                                                                    | 21        |
| 2.2.1 Water quality .....                                                                           | 21        |
| 2.2.2 Habitat assessment .....                                                                      | 21        |
| 2.2.3 Freshwater mollusc collection and morphological identification .....                          | 22        |
| 2.2.4 Molecular analyses.....                                                                       | 23        |

|       |                    |    |
|-------|--------------------|----|
| 2.2.5 | Data analyses..... | 27 |
|-------|--------------------|----|

|                                                                                                                                                                         |                                   |  |           |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------|--|-----------|
| <b>CHAPTER 3 IDENTIFYING MOLLUSCAN DIVERSITY WITHIN THE NORTH-EASTERN PROVINCES OF SOUTH AFRICA, USING A COMBINATION OF MORPHOLOGICAL AND MOLECULAR TECHNIQUES.....</b> |                                   |  | <b>28</b> |
| <b>3.1</b>                                                                                                                                                              | <b>Introduction .....</b>         |  | <b>28</b> |
| <b>3.2</b>                                                                                                                                                              | <b>Problem statement .....</b>    |  | <b>29</b> |
| <b>3.3</b>                                                                                                                                                              | <b>Aims and objectives.....</b>   |  | <b>29</b> |
| <b>3.4</b>                                                                                                                                                              | <b>Materials and methods.....</b> |  | <b>29</b> |
| <b>3.5</b>                                                                                                                                                              | <b>Results .....</b>              |  | <b>30</b> |
| 3.5.1                                                                                                                                                                   | Bulinidae.....                    |  | 30        |
| 3.5.2                                                                                                                                                                   | Planorbidae .....                 |  | 38        |
| 3.5.3                                                                                                                                                                   | Lymnaeidae.....                   |  | 45        |
| 3.5.4                                                                                                                                                                   | Physidae.....                     |  | 52        |
| <b>3.6</b>                                                                                                                                                              | <b>Discussion .....</b>           |  | <b>55</b> |
| 3.6.1                                                                                                                                                                   | Bulinidae.....                    |  | 55        |
| 3.6.2                                                                                                                                                                   | Planorbidae .....                 |  | 57        |
| 3.6.3                                                                                                                                                                   | Lymnaeidae.....                   |  | 57        |
| 3.6.4                                                                                                                                                                   | Physidae.....                     |  | 58        |
| <b>3.7</b>                                                                                                                                                              | <b>Conclusion.....</b>            |  | <b>59</b> |

|                                                                                                                                             |                              |
|---------------------------------------------------------------------------------------------------------------------------------------------|------------------------------|
| <b>CHAPTER 4: DECLINE IN THE NATIVE FRESHWATER SNAIL DIVERSITY OF THE NORTH-EASTERN PROVINCES OF SOUTH AFRICA: A CAUSE FOR CONCERN.....</b> | <b>60</b>                    |
| <b>4.1 Introduction .....</b>                                                                                                               | <b>60</b>                    |
| 4.1.1 Problem statement .....                                                                                                               | 63                           |
| 4.1.2 Aims and Objectives .....                                                                                                             | 63                           |
| <b>4.2 Materials and Methods .....</b>                                                                                                      | <b>63</b>                    |
| <b>4.3 Results .....</b>                                                                                                                    | <b>64</b>                    |
| 4.3.1 Water quality analysis.....                                                                                                           | 64                           |
| <b>4.4 Molluscan species identification .....</b>                                                                                           | <b>68</b>                    |
| 4.4.1 Historic molluscan distribution compared to currently collected species.....                                                          | 68                           |
| 4.4.2 High flow season sampling .....                                                                                                       | 70                           |
| 4.4.3 Low flow season sampling .....                                                                                                        | 70                           |
| <b>4.5 Comparison of high flow and low flow sampling sites .....</b>                                                                        | <b>73</b>                    |
| <b>4.6 Discussion .....</b>                                                                                                                 | <b>77</b>                    |
| 4.6.1 Current molluscan distribution and the threat of invasive molluscan species ....                                                      | 77                           |
| 4.6.2 Environmental factors and water chemistry effects on native molluscs .....                                                            | <b>Error!</b>                |
|                                                                                                                                             | <b>Bookmark not defined.</b> |
| 4.6.3 Water chemistry.....                                                                                                                  | 78                           |
| <b>4.7 Conclusion.....</b>                                                                                                                  | <b>79</b>                    |

|                                                                                                                                                                |            |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| <b>CHAPTER 5 PARASITIC TREMATODE DIVERSITY WITHIN FRESHWATER MOLLUSCAN VECTORS COLLECTED IN THE NORTH-EASTERN PROVINCES OF SOUTH AFRICA.</b>                   | <b>80</b>  |
| <b>5.1 Introduction</b>                                                                                                                                        | <b>80</b>  |
| <b>5.2 Aims and Objectives</b>                                                                                                                                 | <b>81</b>  |
| <b>5.3 Materials and methods</b>                                                                                                                               | <b>81</b>  |
| <b>5.4 Results</b>                                                                                                                                             | <b>82</b>  |
| 5.4.1 Cercarial shedding                                                                                                                                       | 82         |
| 5.4.2 <i>Neofibricola smiti</i> Achatz, Martens, Kudlai, Junker, Boe & Tkach, 2022                                                                             | 83         |
| 5.4.3 <i>Petasiger variospinosus</i> (Odhner, 1910) Yamaguti, 1933                                                                                             | 84         |
| 5.4.4 <i>Orientocreadium batrachoides</i> Tubanguui, 1931                                                                                                      | 86         |
| <b>5.5 Discussion</b>                                                                                                                                          | <b>88</b>  |
| <b>5.6 Conclusion</b>                                                                                                                                          | <b>90</b>  |
| <b>CHAPTER 6 CONCLUSIONS AND RECOMMENDATIONS</b>                                                                                                               | <b>91</b>  |
| <b>6.1 Molluscan species identification (Chapter 3)</b>                                                                                                        | <b>91</b>  |
| <b>6.2 The current distribution of molluscan species compared to the historical data available for the Limpopo Lowveld ecoregion, South Africa (Chapter 4)</b> | <b>92</b>  |
| <b>6.3 Parasitic trematode diversity within molluscan vectors collected (Chapter 5)</b>                                                                        | <b>93</b>  |
| <b>6.4 Recommendations</b>                                                                                                                                     | <b>94</b>  |
| <b>REFERENCE LIST</b>                                                                                                                                          | <b>95</b>  |
| <b>APPENDIX A:</b>                                                                                                                                             | <b>A-1</b> |
| A-A. Appendix one                                                                                                                                              | A-1        |

## LIST OF TABLES

|                                                                                                                                                                                                                                                                                                                                                                                                                                            |    |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Table 2.1: Site names, date of sampling, site coordinates and site classification for all of the selected sampling sites in the Limpopo, North West and Free State provinces. FS-Free State; LHF-Limpopo high flow; LLF-Limpopo low flow. ....                                                                                                                                                                                             | 13 |
| Table 2.2: Genetic markers used during polymerase chain reaction (PCR) amplification of COI gene and ITS2 region of molluscan species. Table adapted from Schols <i>et al.</i> (2019). ....                                                                                                                                                                                                                                                | 25 |
| Table 2.3: Genetic markers used in the Infection RD-PCR and <i>Schistosoma</i> RD-PCR. Adapted from Schols <i>et al.</i> (2019) .....                                                                                                                                                                                                                                                                                                      | 26 |
| Table 3.1: Table indicating base pair differences between members of the <i>Bulinus africanus/globosus</i> group. Above the diagonal are the percentage of identical base pairs, and below the diagonal are base pair differences of the ITS2-region. Genetic sequences generated during this study are coloured blue to match the phylogenetic tree generated (Figure 3.2). ....                                                          | 34 |
| Table 3.2: Indicates base pair differences between members of the <i>Bulinus truncatus/tropicus</i> group. Above the diagonal are the percentage of identical base pairs, and below the diagonal are base pair differences of the ITS2-region. Genetic sequences generated during this study are coloured orange to match the phylogenetic tree generated (Figure 3.2). ....                                                               | 34 |
| Table 3.3: Table indicating base pair differences between members of the <i>Bulinus truncatus/tropicus</i> group and closest neighbouring branches of the phylogenetic tree (Figure 3.3). Above the diagonal is the percentage of identical base pairs, and below the diagonal are base pair differences of the COI-gene. Genetic sequences generated during this study are coloured orange to match the phylogenetic tree generated. .... | 36 |

|                                                                                                                                                                                                                                                                                                                                                                           |    |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Table 3.4: Table indicating base pair differences between members of the <i>Biomphalaria pfeifferi</i> . Above the diagonal are the percentage of identical base pairs, and below the diagonal are base pair differences of the ITS2 region. Genetic sequences generated during this study are coloured blue to match the phylogenetic tree generated (Figure 3.6). ..... | 41 |
| Table 3.5: Table indicating base pair differences between members of the <i>Gyraulus</i> species. Above the diagonal are the percentage of identical base pairs, and below the diagonal are base pair differences of the ITS2 region. Genetic sequences generated during this study are coloured orange to match the phylogenetic tree generated (Figure 3.6). .....      | 42 |
| Table 3.6: Table indicating base pair differences between members of the <i>Biomphalaria</i> above the diagonal are the percentage identical base pairs, and below the diagonal are base pair differences of the COI-gene. Genetic sequences generated during this study are coloured blue to match the phylogenetic tree generated (Figure 3.7).....                     | 43 |
| Table 3.7: Table indicating base pair differences between members of the <i>Gyraulus</i> above the diagonal are the percentage identical base pairs, and below the diagonal are base pair differences of the COI-gene. Genetic sequences generated during this study are coloured orange to match the phylogenetic tree generated (Figure 3.7).....                       | 44 |
| Table 3.8: Table indicating base pair differences between members of the <i>Radix</i> . Above the diagonal are the percentage of identical base pairs, and below the diagonal are base pair differences of the ITS2 region. Genetic sequences generated during this study are coloured grey to match the phylogenetic tree generated (Figure 3.9).....                    | 47 |
| Table 3.9: Table indicating base pair differences between members of the <i>Pseudosuccinea</i> above the diagonal are the percentage identical base pairs, and below the diagonal are base pair differences of the ITS2 region. Genetic sequences generated during this study are coloured green to match the phylogenetic tree generated (Figure 3.9). .....             | 47 |

|                                                                                                                                                                                                                                                                                                                                                                                                                                                              |    |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Table 3.10: Table indicating base pair differences of <i>Radix natalensis</i> below the diagonal and percentage identical base pairs of the COI-gene above the diagonal. Grouped and colour coded based on phylogenetic grouping as seen in (Figure 3.10). .....                                                                                                                                                                                             | 49 |
| Table 3.11: Table indicating base pair differences between members of the <i>Pseudosuccinea</i> . Above the diagonal are the percentage identical base pairs, and below the diagonal are base pair differences of the COI-gene. Genetic sequences generated during this study are coloured green to match the phylogenetic tree generated (Figure 3.9). .....                                                                                                | 49 |
| Table 3.12: Table indicating base pair differences between members of <i>Physella acuta</i> from the Africa clade 3. Above the diagonal are the percentage identical base pairs, and below the diagonal are base pair differences of the COI-gene. Genetic sequences generated during this study are coloured blue to match the phylogenetic tree generated (Figure 3.13). .....                                                                             | 53 |
| Table 4.1: Molluscan specimens collected in the north-eastern provinces of South Africa during surveys conducted in the 1960-1980 period and housed within the NFSC database. The listed species include native and invasive species* collected during this period and adapted from (De Kock <i>et al.</i> , 2004).....                                                                                                                                      | 61 |
| Table 4.2: The <i>in situ</i> water quality parameters measured in the field and chemical water quality analysis obtained during the high flow sampling (April-May 2021) and low flow sampling (August 2021) respectively at the 25 selected sites within the North-eastern provinces. The standard deviation between all the sites is indicated in the last row labelled SD*. The target water quality requirement (TWQR) is included in the last row. .... | 66 |
| Table 4.3: Total nitrogen and total phosphorus ratio between the 25 sites sampled within the north-eastern provinces. Total Nitrogen is calculated by adding the concentrations of Ammonium (NH <sub>4</sub> <sup>+</sup> ), Nitrate (NO <sub>3</sub> <sup>-</sup> ) and Nitrite (NO <sub>2</sub> ) together. Mean and standard deviation (SD) indicated in last row for both high flow and low flow sampling sites. * HF-High flow; LF-Low flow. ....       | 67 |

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |    |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Table 4.4: Species historically identified within the selected study are by data available from the NFSC database from the NWU-Potchefstroom campus, compared to species collected during this study and morphologically and molecularly identified from the sites located in the Limpopo Lowveld ecoregion. Presence indicated by (X).....                                                                                                                                                                                          | 69 |
| Table 5.1: Nucleotide genetic divergence among <i>Neofibricola</i> included in the molecular phylogenetic analyses of the 28S rDNA. Values above the diagonal are expressed in the percentage pairwise genetic distance (p-distance), while values below the diagonal represent the number of base pair differences. The analysis involved seven nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1209 positions in the final dataset.....                                   | 84 |
| Table 5.2: Nucleotide genetic divergence among <i>Petasiger</i> , <i>Echinostoma</i> and <i>Hypoderaeum</i> taxa included in the molecular phylogenetic analyses of the 28S rDNA. Values above the diagonal are expressed in the percentage pairwise genetic distance (p-distance), while values below the diagonal represent the number of differences. The analyses involved 14 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1014 positions in the final dataset. .... | 86 |
| Table 5.3: Nucleotide genetic divergence among Orientocreadiidae and Leptophaliidae taxa included in the molecular phylogenetic analyses of the 28S rDNA. Values above the diagonal are expressed in the percentage pairwise genetic distance (p-distance), while values below the diagonal represent the number of differences. The analyses involved 11 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1014 positions in the final dataset. ....                         | 88 |

## LIST OF FIGURES

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |    |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 1.1: The life cycle and different stages of <i>Schistosoma</i> infection, indicating the hosts and mediums used to move between vectors and finally depending on <i>Schistosoma</i> species present the definitive host is infected (Janoušková <i>et al.</i> , 2022). .....                                                                                                                                                                                    | 5  |
| Figure 1.2: The estimated distribution of schistosomiasis causing trematodes based on the historical collection of molluscan vectors. The shaded area was established as native distribution during the 1930s, and the area within the black border was assessed during the 1960s-1980s during the collection of specimens for the National Freshwater Snail Collection (NFSC) database. Map adapted from Magaisa <i>et al.</i> (2015). .....                          | 6  |
| Figure 2.1: Map indicating all sites sampled during this study. A) indicates sites located within the Limpopo and North West provinces, and B) indicate sites sampled within the Seekoeivlei Nature Reserve, Free State province. Sites indicated on the map with red site markers were sampled during what is regarded as the high flow season, and sites with black site markers were sampled during what is considered the low flow season. ....                    | 10 |
| Figure 2.2: Map indicating locations sampled during the high flow (HF) and low flow (LF) seasons in 2021 in the North West And Limpopo provinces, South Africa. The high flow sites are indicated in red (sampled April-May 2021), and the low flow sites are shown in black (sampled August 2021). L- Limpopo; HF-high flow, LF-low flow. ....                                                                                                                        | 11 |
| Figure 2.3: Map indicating selected sites sampled on the Klip River, Wasgoedspruit and Pampoenspruit located near the Seekoeivlei Nature Reserve, Memel, Free State province, South Africa. FS-Free State.....                                                                                                                                                                                                                                                         | 12 |
| Figure 2.4: Photographs of the sites sampled during the high flow sampling period. The first three sites located on the Limpopo River, (A) Site LHF1, (B) Site LHF2, (C) Site LHF3, (D) Site LHF4 is located on the Olifants River, (E) Site LHF5 is located on the Shingwedzi River, (F) Site LHF6 located on the Luvuvhu River, (G) Site LHF7 located on the Groot Letaba River, (H) Site LHF8 located on the Letaba River and Site LHF9 on the Olifants River. .... | 16 |

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |    |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 2.5: Photographs of the sites sampled during the low flow sampling period. (A) Site LLF1 Isolated stream near the Hex River, (B) Site LLF2 Farm Dam near the Crocodile River, (C) Site LLF3 is located Crocodile River, (D) Site LLF4 located on the Buffelspruit, (E) Site LLF5 is located at the Donkerpoort Dam, (F) Site LLF6 located on the Klein Sand River and (G) Site LLF7 located on the Mokolo River.....                                                                                                                                    | 17 |
| Figure 2.6: Photographs of the sites sampled during the low flow sampling period. (A) Site LLF8 is on Bloed River (B) Site LLF9 located at the Stanford Lake, (C) Site LLF10 is located at theTzaneen Dam, (D) Site LLF11 located at the Mothomeng Dam, (E) Site LLF12 is located at the Middle-Letaba River, (F) Site LLF13 located on Luvuvhu River and (G) Site LLF14 located on the Magic Dam near Tshivhazwaulu town, (H) Site LLF15 located at a Unnamed Dam near Valdezia town and (I) Site LLF16 located below Albasini Dam on the Luvuvhu River. .... | 18 |
| Figure 2.7: Photographs of the Seekoeivlei Nature Reserve indicating the (A) the thick vegetation cover surrounding all of the sites sampled along the Klip River, (B) The oxbows formed by the Klip River, (C) Flooding of the perimeter fence of the Seekoeivlei Nature Reserve and (C) the high flow velocity of the Klip River as it flows out of the Seekoeivlei Nature Reserve .....                                                                                                                                                                     | 20 |
| Figure 2.8: Example of the datasheet used during the qualitative habitat assessment. ....                                                                                                                                                                                                                                                                                                                                                                                                                                                                      | 22 |
| Figure 2.9: Photographs illustrating the different methods of collection used. (A and B) the use of a snail sampler. (C) screening each sample taken with the snail sampler for the presence of molluscs. (D) hand-picking from vegetation present within the sampling area.....                                                                                                                                                                                                                                                                               | 23 |
| Figure 3.1: Bulinidae specimens collected during the current study. Morphologically identified as <i>Bulinus africanus</i> (A-B). (A) the ventral view of <i>Bulinus africanus</i> and (B) the dorsal view. (C-D) Identified as <i>Bulinus depressus</i> . (C) the ventral view of <i>Bulinus depressus</i> and (D) the dorsal view. (A-D) Scale bar = 5 mm.....                                                                                                                                                                                               | 31 |

- Figure 3.2: Bayesian inference phylogram of *Bulinus* genus based on partial sequences of the ITS2 rDNA region. New sequences are in bold. Posterior probability followed by bootstrap support values are indicated next to the branches (posterior probability < 70 and bootstrap < 0.80 not shown). *Biomphalaria grablata* was used as an outgroup. The branch length scale bar indicates the number of substitutions per site. Sequences generated during the current study are represented in bold. .... 33
- Figure 3.3 Bayesian inference phylogram of *Bulinus* genus based on partial sequences of the COI mtDNA gene. New sequences are in bold. Posterior probability followed by bootstrap support values is indicated next to the branches (posterior probability < 70 and bootstrap < 0.80 not shown). *Biomphalaria grablata* was used as an outgroup. The branch length scale bar indicates the number of substitutions per site. Sequences generated during the current study are represented in bold. .... 35
- Figure 3.4: Map indicating locations where *Bulinus* specimens were collected and molecularly identified. *Bulinus africanus/globosus* group is indicated with a blue circle on the map, and *B. truncatus/tropicus* group is indicated with an orange circle. Sites where both groups were collected are indicated with a blue and orange circle..... 37
- Figure 3.5: Planorbidae specimens of *Biomphalaria* and *Gyraulus* species collected during the current study. Morphologically identified as *Biomphalaria pfeifferi* (A-B). (A) The ventral view of *Biomphalaria pfeifferi* and (B) The dorsal view. (C-D) Identified as *Gyraulus costulatus*. (C) the ventral view of *Gyraulus costulatus* and (D) the dorsal view. (E-F) Identified as *Gyraulus connollyi*. (E) The ventral view of *G. connollyi* and (F) the dorsal view. (A-F) Scale bar = 5 mm..... 39
- Figure 3.6: Bayesian inference phylogram of species of the genus *Biomphalaria* and *Gyraulus* based on partial sequences of the ITS2 rDNA region. New sequences are in bold. Posterior probability followed by bootstrap support values is indicated next to the branches (posterior probability < 70 and bootstrap < 0.80 not shown). *Bulinus africanus* was used as an outgroup. The branch length scale bar indicates the number of substitutions per site. Sequences generated during the current study are represented in bold. .... 41

Figure 3.7: Bayesian inference phylogram of species of *Biomphalaria* and *Gyraulus* based on partial sequences of the COI mtDNA gene. New sequences are in bold. Posterior probability followed by bootstrap support values is indicated next to the branches (posterior probability < 70 and bootstrap < 0.80 not shown). *Bulinus globosus* was used as an outgroup. The branch length scale bar indicates the number of substitutions per site. Sequences generated during the current study are represented in bold. .... 43

Figure 3.8: Map indicating sites where specimens belonging to the *Biomphalaria pfeifferi* (indicated by the blue circles), *Gyraulus connollyi* (indicated by the orange circles) and *Gyraulus constulatus* (indicated by the orange diamond-shaped icon). The area within the red square is the site sampled within the Seekoeivlei Nature Reserve. Enlargement of the area within the red square indicates the three sites sampled within Seekoeivlei Nature Reserve, where specimens of *G. connollyi* were collected. .... 44

Figure 3.9: Bayesian inference phylogram of species of *Radix* and *Pseudosuccinea* based on partial sequences of the ITS2 region. New sequences are labeled PC and RN. Posterior probability followed by bootstrap support values is indicated next to the branches (posterior probability < 70 and bootstrap < 0.80 not shown). *Bulinus forskalii* was used as an outgroup. The branch length scale bar indicates the number of substitutions per site. .... 46

Figure 3.10: Bayesian inference phylogram of species of *Radix* and *Pseudosuccinea* based on partial sequences of the COI mtDNA gene. New sequences are labeled PC and RN. Posterior probability followed by bootstrap support values are indicated next to the branches (posterior probability < 70 and bootstrap < 0.80 not shown). *Bulinus forskalii* was used as an outgroup. The branch length scale bar indicates the number of substitutions per site. .... 48

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |    |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 3.11: Map indicating the sites with <i>Radix natalensis</i> that phylogenetically grouped between locations based on the COI-gene mutations. A yellow circle characterises areas with monophyletic group 1 present, pink for group 2, green for group 3 and purple for group 4. The only site with two of the monophyletic groups present was site LLF13 with a green and pink circle icon on the map indicating that monophyletic groups 2 and 3 were present. .... | 50 |
| Figure 3.12: Geographical locations where specimens identified as <i>Pseudosuccinea columella</i> were collected. ....                                                                                                                                                                                                                                                                                                                                                      | 51 |
| Figure 3.13: Bayesian inference phylogram of species of <i>Physella</i> based on partial sequences of the COI rDNA gene. New sequences are in bold. Posterior probability followed by bootstrap support values is indicated next to the branches (posterior probability < 70 and bootstrap < 0.80 not shown). <i>Bulinus africanus</i> was used as an outgroup. The branch length scale bar indicates the number of substitutions per site. ....                            | 53 |
| Figure 3.14: Map indicating site LLF4 where the specimen identified as <i>Physella acuta</i> used for genetic analyses was collected. ....                                                                                                                                                                                                                                                                                                                                  | 54 |
| Figure 4.1 Graph indicating (A) the total number of genera historically collected at each site (retrieved from the NFSC), compared to the current number of genera collected during this study and (B) the number of individuals collected during the high flow sampling (LHF) (April-May 2021) and low flow sampling (LLF) (August 2021) respectively at the 25 selected sites within the north-eastern provinces, South Africa. ....                                      | 72 |
| Figure 4.2: Non-Metric Multidimensional Scaling (nMDS) indicating, differences in molluscan species diversity and abundance during the high flow sampling (LHF) (April-May 2021) and low flow sampling (LLF) (August 2021), respectively, at the 25 selected sites within the north-eastern provinces. ....                                                                                                                                                                 | 74 |

- Figure 4.3: Canonical correspondence analysis (CCA) indicating differences between sites sampled during the high flow sampling (LHF) (April-May 2021) and low flow sampling (LLF) (August 2021) respectively, at the 25 selected sites within the north-eastern provinces in terms of snail diversity and select water quality variables. Green circles and lines indicate sites sampled during the high flow season. Red squares and lines indicate the sites sampled during the low flow season. Blue arrows indicate water quality parameters, and black triangles indicate molluscan species collected. The CCA explains 44.06% of data variation, with axis 1 explaining 26.91% and axis 2 explaining 17.15%. .... 75
- Figure 4.4: Non-Metric Multidimensional Scaling (NMDS) indicating the presence of the invasive *T. granifera* and their effect on species distribution between sites sampled during the high flow sampling (LHF) (April-May 2021) and low flow sampling (LLF) (August 2021) respectively at the 25 selected sites within the north-eastern provinces. .... 76
- Figure 5.1: Bayesian inference phylogram of selected *Neofibricola* taxa based on partial sequences of the 28S rDNA gene. Newly sequenced species are highlighted in bold. The host/vector species are indicated in brackets, and the hosts class is given. The Posterior probability followed by bootstrap support values are indicated next to the branches (posterior bootstrap < 70 and probability < 0.85 not shown). *Trichobilharzia* sp. was used as an outgroup. The branch length scale bar indicates the number of substitutions per site. .... 83
- Figure 5.2: Bayesian inference phylogram of selected *Petasiger*, *Echinostoma* and *Hypodermaeum* taxa based on partial sequences of the 28S rDNA gene. Newly sequenced species are highlighted in bold. The host/vector species are indicated in brackets, and the hosts class is given. Posterior probability followed by bootstrap support values is indicated next to the branches (posterior bootstrap < 70 and probability < 0.85 not shown). *Fasciola hepatica* was used as an outgroup. The branch length scale bar indicates the number of substitutions per site. .... 85

Figure 5.3: Bayesian inference phylogram of selected Orientocreadiidae and Leptophaliidae taxa based on partial sequences of the 28S rDNA gene. Newly sequenced species are highlighted in bold. The host/vector species are indicated in brackets, and the hosts class is given. Posterior probability followed by bootstrap support values is indicated next to the branches (posterior bootstrap < 70 and probability < 0.85 not shown). *Magnivitellinum simplex* was used as an outgroup. The branch length scale bar indicates the number of substitutions per site. .... 87

# Chapter 1 General introduction

## 1.1 Interactions between freshwater molluscs and the environment

Freshwater ecosystems are regarded as the most endangered ecosystems in the world, with thousands of species known to be either threatened or already extinct (Thomsen *et al.*, 2012). The one of the several key indicator of the status of a freshwater ecosystem is the biodiversity thereof, as it provides information regarding habitat complexity and diversity as well as resource availability (Nwoko *et al.*, 2022). Numerous studies have been conducted on freshwater ecosystems, and several aspects have been identified as essential factors that affect species diversity and abundance within an ecosystem. These include the overexploitation of freshwater habitats, pollution, flow modification of river systems, destruction of freshwater habitats for residential, agricultural, and industrial use, the invasion of non-native species, and climate change (Dudgeon *et al.*, 2006; Nwoko *et al.*, 2022). Population growth, industrial development, and increased agricultural and mining activities within a study area increases anthropogenic pressures on the natural freshwater environment and negatively affect the region's ecology (Ubisi *et al.*, 2017).

The effects of climate change have a substantial adverse impact on freshwater ecosystems, as changes in temperature and precipitation lead to alterations in the hydrological regimes of these habitats (Markovic *et al.*, 2014). Changes in the water level directly affect water temperature, which affects evaporation and, in turn, affects salinity and nutrients such as nitrates and phosphates within the water column (Früh *et al.*, 2016). Such changes in water quality have the potential to alter freshwater molluscan species distribution by decreasing the available instream habitat and water quality, therefore increasing competition between molluscan species (Quayle *et al.*, 2010). The effects of increases in temperature on molluscan species is well documented based on their biological evolution and reproduction (De Kock *et al.*, 2004; De Kock & Wolmarans, 2005a, 2005b, 2006a, 2006b, 2007, 2009). However, the effects of climate change is not limited to a general rise in temperature and require further investigation to accurately determine the influence of climate change on specific molluscan species (Markovic *et al.*, 2014).

Freshwater molluscs are soft-bodied, unsegmented animals with a shell enclosure wherein they live, belonging to the class Gastropoda and can be divided into two groups, Prosobranch and Pulmonata (Day & de Moor, 2002; Brown *et al.*, 2010). Prosobranch molluscan species are generally either male or female, but some species have the ability to change sex as they grow and are either male or female when they reach maturity (Day & de Moor, 2002; Brown *et al.*, 2010). This group reproduces through one of three methods: viviparity, ovoviviparity, or oviparity.

Viviparous and ovoviviparous species give birth to live offspring that, in part, develop in the uterus. Oviparous species lay eggs in clusters from which the offspring hatch (Day & de Moor, 2002; Brown *et al.*, 2010). Pulmonate members of freshwater molluscs are oviparous hermaphrodites, indicating that these members have both male and female sex organs present (Brown *et al.*, 2010). This group includes species known to be intermediate hosts (vectors) for medically, veterinary and economically important trematode species (Day & de Moor, 2002).

This may alter species' geographical distribution and introduce invasive and endemic species into new geographical areas where they were previously not present. Selbach & Poulin (2020) further showed that temperature increases positively affected trematode cercarial transmission between molluscan hosts within the freshwater environment. However, the impact of the increase in temperature varied between different trematode species (Selbach & Poulin, 2020). The diversity of freshwater molluscan species is vast, with approximately 7000 freshwater snail species identified worldwide (Lydeard *et al.*, 2004). Of those, 400 species have been identified from the African continent, and an estimated 111 have been collected in the southern African region (Day & de Moor, 2002; Nwoko *et al.*, 2022) although the exact number of native and invasive species present within South Africa is still a topic of debate (Day & de Moor, 2002). The distribution and abundance of specific genera of freshwater molluscs such as *Bulinus* (O.F. Müller, 1781), *Biomphalaria* (Preston, 1910), *Radix* (Montfort, 1810), *Pseudosuccinea* (F. C. Baker, 1908), *Physella* (Haldeman, 1843) and *Tarebia* (H. Adams & A. Adams, 1854) are well documented in South Africa (De Kock *et al.*, 2004; De Kock & Wolmarans, 2005a, 2005b, 2006a, 2006b, 2007, 2009). However, all of the distribution data from the numerous articles by De Kock and co-authors are based on specimens collected during the 1960s-1980s for the National Freshwater Snail Collection (NFSC) housed at the North-West University. Therefore, the current distribution data of molluscan species is outdated and needs reassessment.

## **1.2 Identification of freshwater molluscs and their role as vectors for digenean trematodes**

Taxonomic identification of freshwater molluscs can be challenging and may require specialized training to identify species based on shell morphology and internal anatomy (Pfenninger *et al.*, 2006). This is especially evident within species that are closely related. One example is the identification of species of *Bulinus* to the lowest taxonomical level. The morphological similarities between members makes *Bulinus* spp. one of the most difficult to identify accurately (Day & de Moor, 2002; Brown *et al.*, 2010). Therefore, identification with general morphological traits are sufficient to group specimens of this genus into one of four morphological groups. Three of these

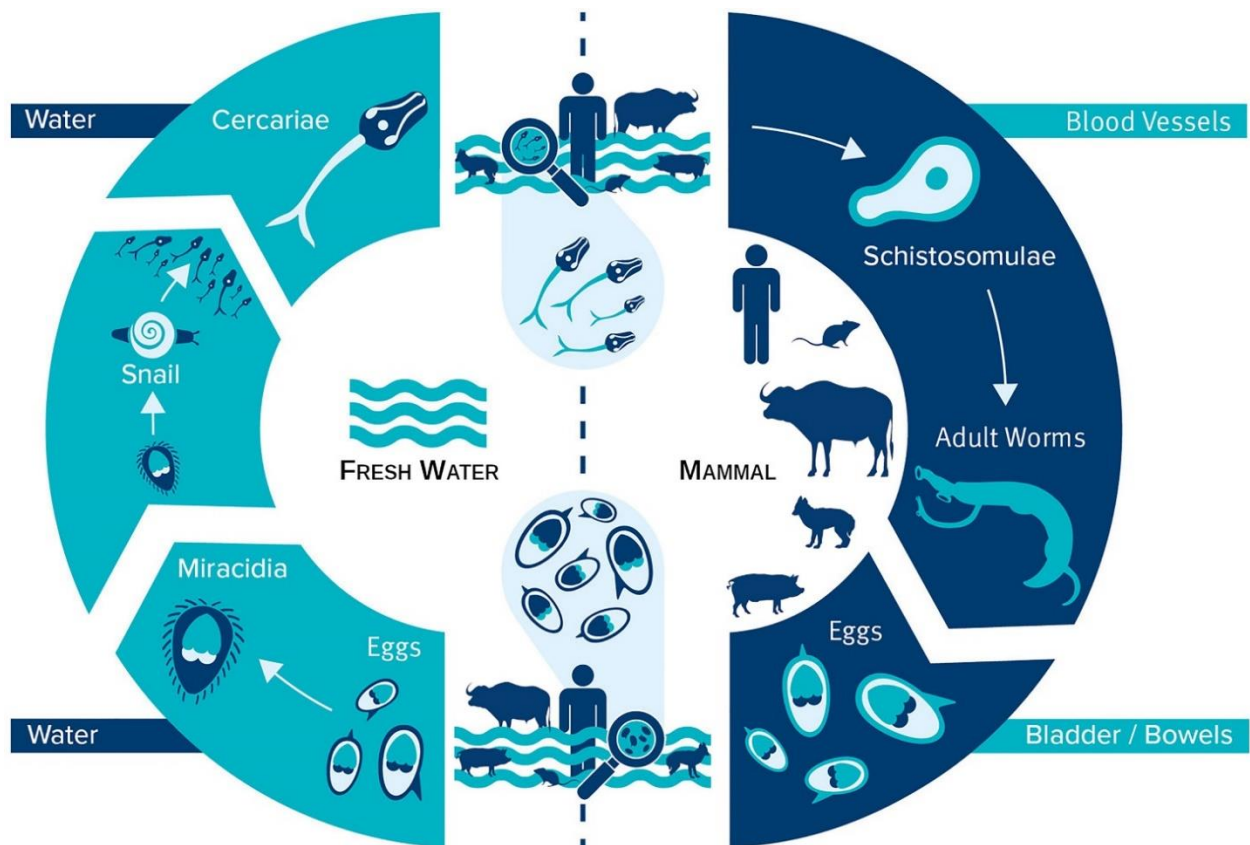
groups are known to be present in South Africa, namely *Bulinus africanus/globosus*, *Bulinus truncatus/tropicus* and the *Bulinus forskalii* group (Day & de Moor, 2002; Brown *et al.*, 2010). Incorrect identification causes problems when assessing the population dynamics of molluscan species within a specific area (Lawton *et al.*, 2015). Secondly, the incorrect identification of molluscan vectors of medical and veterinary importance will also result in inaccurate parasitological studies of the life cycle analysis of trematodes (Pfenninger *et al.*, 2006). Therefore, combining morphological and molecular analyses may offer a solution to the challenge of identifying freshwater molluscs (Pfenninger *et al.*, 2006; Lawton *et al.*, 2015). The assessment of the internal transcribed spacer 2 nuclear ribosomal DNA (ITS2) and cytochrome c oxidase subunit 1 mitochondrial gene (COI) genetic regions as species identification markers indicate a higher phylogenetic resolution. The COI genetic region also provides new insight into the evolutionary relationships of molluscan species (Lawton *et al.*, 2015). Therefore, the use of morphological identification and molecular analysis of the ITS2 and COI genetic regions is necessary to accurately identify molluscan species and contribute to the continued debate regarding the evolutionary relationships of molluscan species.

The distribution of different parasitic trematode species depends on the ecology and distribution of the intermediate snail host species (Boissier *et al.*, 2019). Most parasitic trematodes have an indirect life cycle that requires the presence of both the intermediate- and definitive mammalian host (Giannelli *et al.*, 2016). Humans are considered the definitive hosts for medically important trematode species, such as *Schistosoma* and *Fasciola* species, although other vertebrates are infected with the parasitic trematode and can function as reservoirs for a wide range of disease-causing trematodes (Bogitsh *et al.*, 2019; Boissier *et al.*, 2019). Understanding the life cycle of trematode species and parasite movement between intermediate and definitive hosts is fundamental for controlling and eliminating trematode-borne diseases (Colley *et al.*, 2014; Nelwan, 2019). One of the most notable and relatively well-documented trematode parasitic infection is the neglected tropical disease (NTD) schistosomiasis caused by trematode members of the *Schistosoma* genus (Colley *et al.*, 2014). In 1851 Theodore Bilharz described a parasitic infection, termed bilharzia, and later renamed schistosomiasis. The main parasitic trematodes causing schistosomiasis are *Schistosoma mansoni*, transmitted by snail species of the genus *Biomphalaria*. *Schistosoma haematobium* is transmitted by the snail species of the genus *Bulinus* genus and *Schistosoma japonicum* is transmitted by the snails of the genus *Oncomelania* (Gryseels *et al.*, 2006). Schistosomiasis is roughly estimated to affect more than 230 million people worldwide and is considered the second most significant tropical disease globally, based on the human health impact, second only to malaria (Colley *et al.*, 2014; Magaisa *et al.*, 2015). Transmission of parasites and subsequent infection is common globally in human populations and even more so in underdeveloped rural areas. Schistosomiasis is regarded as endemic to 74

countries worldwide, with intestinal schistosomiasis caused by *Schistosoma mansoni* regarded as the most important and broadly distributed *Schistosoma* species to human health, and is distributed within 54 countries across the globe (Boissier *et al.*, 2019). Transmission of parasites and subsequent infection is a common occurrence globally (Figure 1.1) in human populations and even more so in underdeveloped rural areas. Tropical diseases and parasitic infections have gained new traction in the public health discussion. Recent immunology studies have indicated a connection between chronic parasitic infections and the impairment of protective responses to acute bacterial and viral infections and may include the impairment of protective responses of childhood vaccines (King, 2010; Hotez *et al.*, 2014).

The life cycle of schistosomiasis provides a good example regarding the intricate life cycle of trematodes. Adult trematode worms live in the veins of the definitive host, where the female and male worms mate and produce eggs (Figure 1.1). *Schistosoma mansoni* individuals/pairs are estimated to produce approximately 350 eggs per day (Cheever *et al.*, 1994; Colley *et al.*, 2014). Eggs are excreted to the environment through faeces or urine, depending on the *Schistosoma* species that the definitive host is infected by. Eggs that reach freshwater will hatch and release free-swimming ciliated miracidia that will, in turn, infect the appropriate intermediate host molluscan species. The miracidia will penetrate the intermediate host and undergo asexual replication and eventually release thousands of cercariae. This process requires approximately 4-6 weeks before infectious cercariae are mature enough to be released (shed) by the intermediate host snail into the freshwater (Colley *et al.*, 2014; Nelwan, 2019). Cercariae can remain infective in freshwater for 1-3 days after release, but their energy reserves are typically depleted within a few hours after release. The cercariae find and penetrate the skin of the definite human or mammalian host. In the case of infection of *Schistosoma*, the schistosomula (maturing *Schistosoma* larva) requires approximately 5-7 weeks to develop and start producing eggs. These intermittent intervals of the miracidia, where the release of cercariae and production of eggs cannot be detected, but infection is ongoing, are termed the prepatent periods (Colley *et al.*, 2014). Identifying prepatent infections within molluscan vectors is challenging due to the lack of cercarial released by molluscan vectors. It, therefore, requires molecular techniques to identify the infection within the molluscan vector positively.

I would suggest adding a paragraph on the pathology and role of the disease in humans. How is the disease characterized, what causes it, what are its impacts?



Adapted from graphic development by SCI foundation, originally adapted from illustration by Genome Research Limited.

Figure 1.1: The life cycle and different stages of *Schistosoma* infection, indicating the hosts and mediums used to move between vectors and finally depending on *Schistosoma* species present the definitive host is infected (Janoušková *et al.*, 2022).

### 1.3 Distribution and Control measures in South Africa

The status of medically important parasitic trematodes and their associated vectors within South Africa is based on national prevalence estimates. Without reliable and sound scientific data, epidemiological data is used to determine the possible distribution and range of infection of medically important infections. Parasitic trematode infections are not considered a severe public health issue in South Africa. However, in the case of schistosomiasis, it is estimated to infect more than 4-5 million people within South Africa and has numerous adverse effects on the local economy (Chitsulo *et al.*, 2000; Craik *et al.*, 2023). In South Africa, historical areas of high trematode transmission and infection of schistosomiasis causing trematodes were based on information from a study conducted in 1938 and then reviewed by De Kock & Wolmarans (2005a, 2005b, 2005c) and De Kock *et al.* (2004) as indicated in Figure 1.2, during De Kock and Wolmarans study intermediate host snails *Biomphalaria* and *Bulinus* species were used to identify possible areas of high transmission of the medically important *Schistosoma* trematode species. Settlements in these areas are primarily informal without appropriate treated water supply and

sanitation (King, 2010). These circumstances force the local population to rely on natural water sources for domestic and recreational use, increasing the chance of a parasitic trematode infection due to the lack of access to a clean water source (Wolmarans *et al.*, 2002). The process of effective control includes accurate identification and the implementation of reliable water supply and sanitation treatment, environmental management, snail control, and education of the local population on appropriate procedures to prevent infection (WHO, 2018).

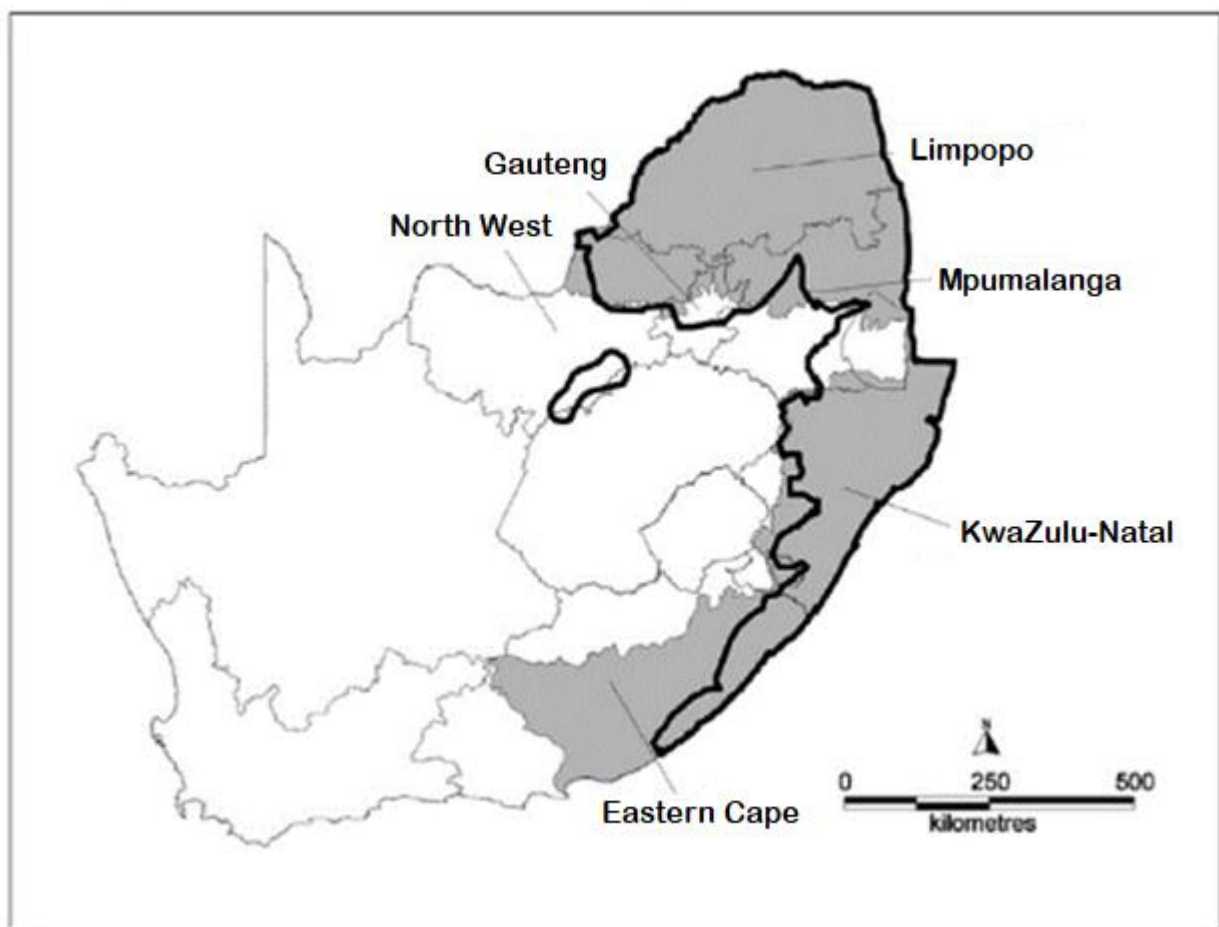


Figure 1.2: The estimated distribution of schistosomiasis causing trematodes based on the historical collection of molluscan vectors. The shaded area was established as native distribution during the 1930s, and the area within the black border was assessed during the 1960s-1980s during the collection of specimens for the National Freshwater Snail Collection (NFSC) database. Map adapted from Magaisa *et al.* (2015).

## 1.4 Problem statement

The previous data collected to establish areas within South Africa that are prone to parasitic trematode outbreaks were previously assessed during the 1930s and then reviewed in the 1980s. The distribution of parasitic trematodes was estimated based on the presence of the vector species. Rural populations in South Africa have since increased by an estimated 20 million within areas prone to high trematode parasitic infections (Macrotrends, 2021). Population increases in rural areas and sub-par infrastructure in poor communities are contributing factors to the increased transmission of parasitic trematode infections, and environment-to-human transmission increases with the consumption of infected water sources (King, 2010; Ciddio *et al.*, 2017).

The lack of information regarding the status of accurate molluscan vector identification and distribution of native and invasive species within South Africa inhibits the effective estimation of status and control of parasitic trematode infections. Therefore, the aspects that need to be implemented in South Africa for effective control and prevention of parasitic infection include a full assessment of the current species that are endemic to South Africa based on historic distribution statistics and investigation of possible new areas of transmission. In the light of the above the main aims of this study were to:

1. Accurately identify molluscan species collected during the current study to the lowest taxonomic level possible through the use of multiple lines of evidence.
2. Investigate the current distribution of freshwater molluscan species within the north-eastern region of South Africa.
3. Determine the role of environmental variables and invasive species affecting native freshwater molluscan species diversity and abundance in the studied region.
4. Identify parasitic trematode infections present in freshwater molluscan species collected and assess the parasitic load within specimens.

To achieve the above aims the following objectives for this study were set:

- Conduct field collection surveys for freshwater gastropods in regions of historical importance identified from the NFSC database.
- Accurately identify molluscan specimens using morphological and molecular analysis by assessing characteristics and distribution of species from published literature. The molecular analysis of the ITS2 and COI genetic regions by phylogenetic comparison to published genetic sequences.

- Use historical records of molluscan species collected in the study region, to assess the change in species diversity.
- Determine the roles of water quality, habitat structure and invasive species in determining freshwater gastropod diversity, abundance, and distribution by conducting qualitative habitat assessment and water sample collection for nutrient analysis.
- Identify parasitic infections and calculate the parasitic load in snail host species using cercarial shedding experiments and molecular methods to assess prepatent trematode infections supported by relevant literature.

## **1.5 Chapter layout of the dissertation**

**Chapter 1:** Provides background on the literature available on freshwater molluscs native to South Africa. Identifying the current gap in accurate knowledge regarding freshwater molluscan species and their associated parasitic infections. The present study's aims and objectives are also provided.

**Chapter 2:** Provides information regarding the study area and detailed information on all the materials and methods used during the current study.

**Chapter 3:** Focuses on identifying freshwater molluscs collected by using a combined approach of morphology and molecular analyses, as well as investigates the genetic diversity between molluscan species collected with the aid of molecular and phylogenetic analyses.

**Chapter 4:** Assesses the current distribution of molluscan species collected and compare the current distribution to the historical distribution of native molluscan species.

**Chapter 5:** Investigates the parasitic trematode community within the molluscan specimens collected during this study, using cercarial shedding and molecular analyses of the snail tissue to detect prepatent infections.

**Chapter 6:** Provides concluding remarks on the main research findings concerning the study, aims and objectives. It also highlights the limitations and provides recommendations for future research.

## Chapter 2 Study area, Materials and Methods

### 2.1 Study region

The north-eastern provinces of South Africa are delineated by two distinct freshwater ecoregions, namely the Southern temperate highveld ecoregion and the Zambezan lowveld ecoregion (Scott, 2019). The North West, Mpumalanga, Free State and southern parts of the Limpopo provinces are located within the Southern temperate highveld ecoregion, defined by Darwall *et al.* (2009). The Southern temperate highveld is situated in the interior of South Africa. It extends from south of the Magaliesberg-Pilanesberg range up to the Soutpansberg and foot slopes of the Drakensberg Mountains (Cowan, 1995). The topography of the Southern temperate highveld ecoregion is known for its rolling grassy plateau with an elevation of 900-1900 meters above sea level (Cowan, 1995). South Africa borders the Great Escarpment to the west and the Mozambique coastal plain to the east. The Zambezan lowveld ecoregion includes the northern parts of the Limpopo province and the eastern parts of the Mpumalanga and Kwazulu-Natal provinces within South Africa (Scott, 2019). This ecoregion comprises mainly of low-lying regions, floodplains, and seasonally flooded wetlands, and the Zambezan lowveld ecoregion is where the Limpopo River is located. The two ecoregions primarily have a sub-tropical to tropical climate and fall within South Africa's tropical summer-rainfall region (Austral summer, December to March). Agriculture and human settlements have mainly altered the ecoregions, and resource-use has heavily impacted the remaining habitat (Martin & Burgess, 2018). The study area for this project covers both ecoregions, and most of the sites were concentrated in the Limpopo province. Therefore, the study area is collectively referred to as the Limpopo Lowveld ecoregion.

During this study, 35 sites were selected, with 25 sites located in the North West and the Limpopo provinces and ten located in the Free State province in the Seekoeivlei Nature Reserve (Figure 2.1). Site selection was based on the availability of historical data from the NFSC database. The final selection of 35 sites was based on the accessibility of the selected sites. Site coordinates and relevant information is listed in Table 2.1. Nine of these designated sites were located on the Limpopo River catchment (Labelled: LHF1-9; Figure 2.2) and were all found on rivers. These sites were sampled during the post-wet season in austral autumn (April to May 2021) and considered as the high flow period. The second sampling of the selected sites took place during the austral winter period (June to August), and this is considered to be the low flow period of the Limpopo River catchment (LLF1-16; Figure 2.2).

Ten sampling sites were within the Seekoeivlei Nature Reserve at the southern limit of the north-eastern provinces (FS1-10; Figures 2.1 and 2.3). Site information is included in Table 2.1. These

sites were sampled for additional genetic identification and determination of potential parasitic infections in molluscan vectors.

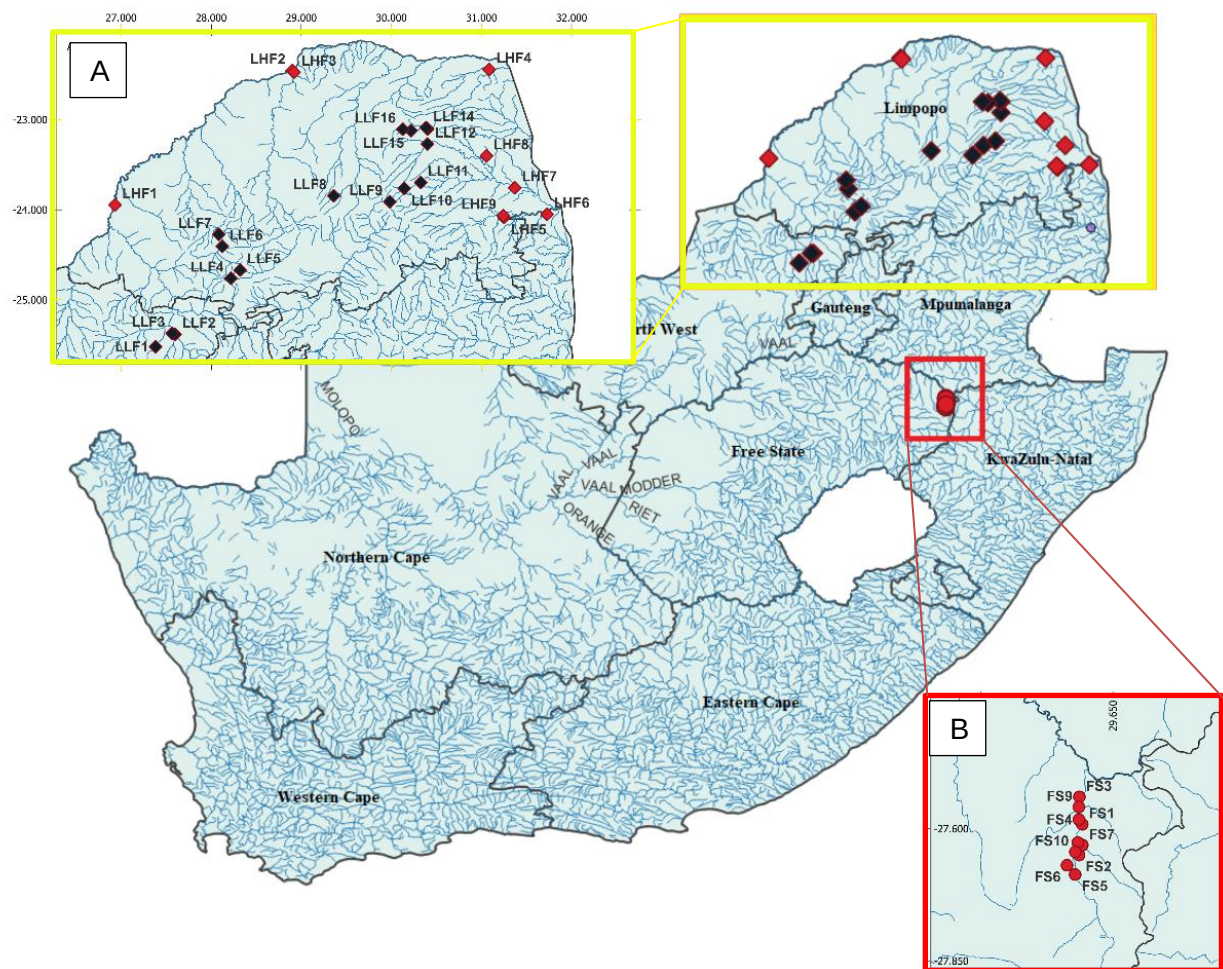


Figure 2.1: Map indicating all sites sampled during this study. A) indicates sites located within the Limpopo and North West provinces, and B) indicate sites sampled within the Seekoeivlei Nature Reserve, Free State province. Sites indicated on the map with red site markers were sampled during what is regarded as the high flow season, and sites with black site markers were sampled during what is considered the low flow season.

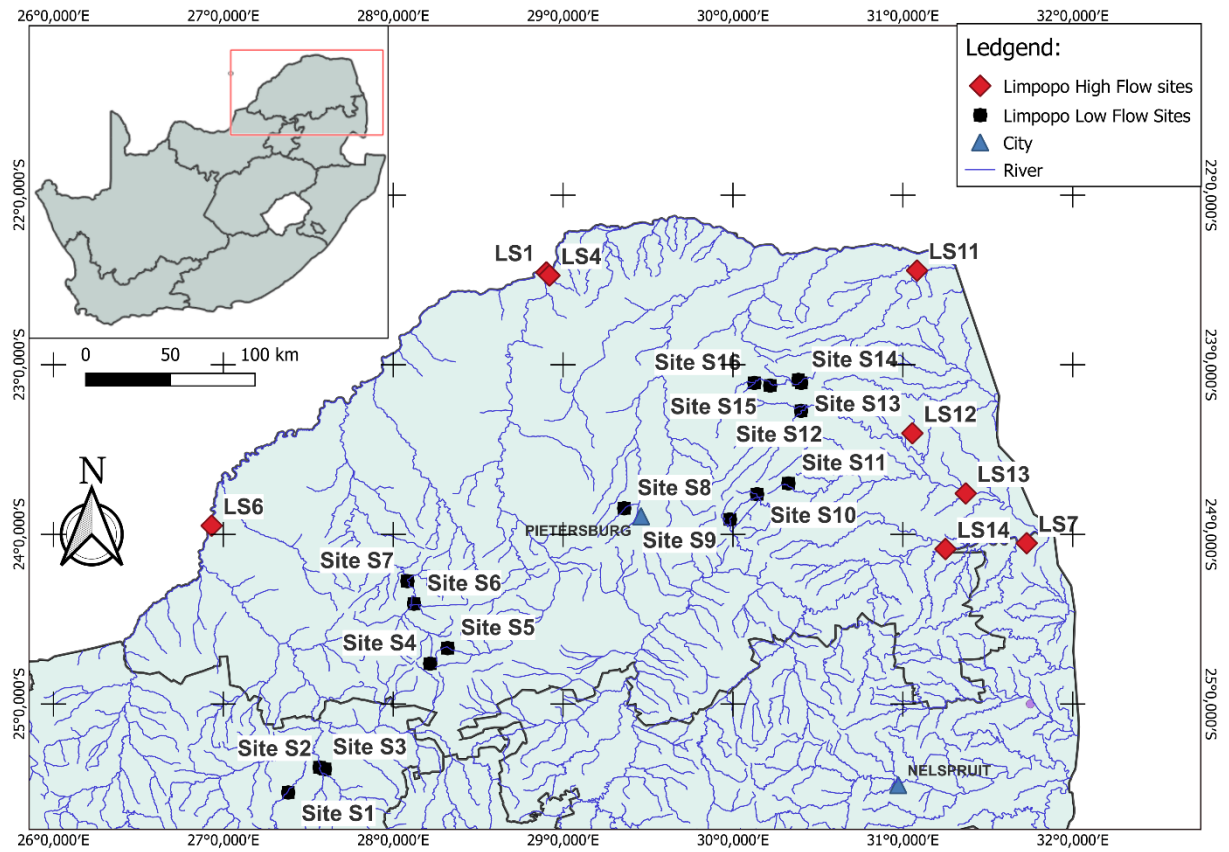


Figure 2.2: Map indicating locations sampled during the high flow (HF) and low flow (LF) seasons in 2021 in the North West And Limpopo provinces, South Africa. The high flow sites are indicated in red (sampled April-May 2021), and the low flow sites are shown in black (sampled August 2021). L-Limpopo; HF-high flow, LF-low flow.

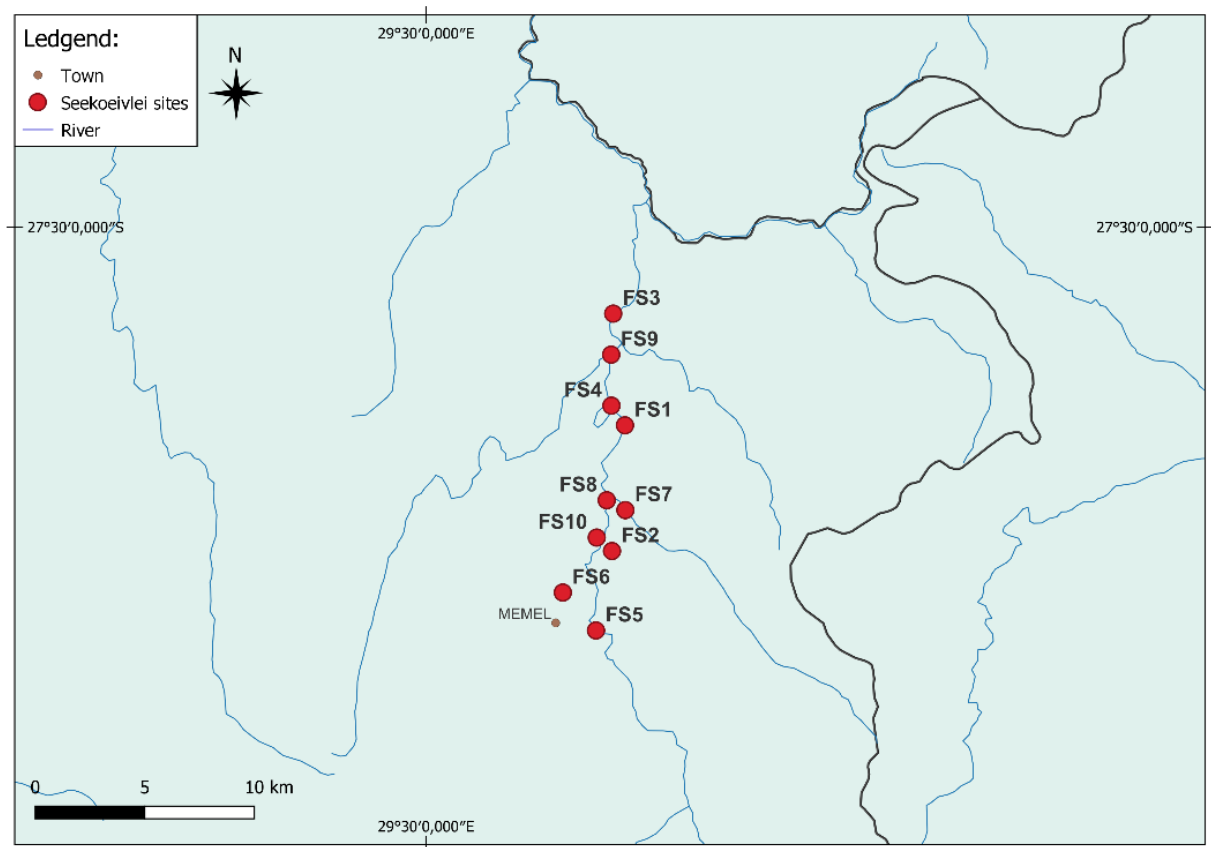


Figure 2.3: Map indicating selected sites sampled on the Klip River, Wasgoedspruit and Pampoenspruit located near the Seekoeivlei Nature Reserve, Memel, Free State province, South Africa. FS-Free State.

Table 2.1: Site names, date of sampling, site coordinates and site classification for all of the selected sampling sites in the Limpopo, North West and Free State provinces. FS-Free State; LHF-Limpopo high flow; LLF-Limpopo low flow.

| Site Code                        | Sampling Date | Coordinates                        | Classification                |
|----------------------------------|---------------|------------------------------------|-------------------------------|
| <b>high flow sampling sites</b>  |               |                                    |                               |
| LHF1                             | 2021/04/22    | 22°26'47.9" S; 28°52'44.6" E       | Limpopo River                 |
| LHF2                             | 2021/04/25    | 22°28'54.5" S; 28°55'07.1" E       | Limpopo River                 |
| LHF3                             | 2021/04/26    | 23°56'40.9" S; 26°55'50.9" E       | Limpopo River                 |
| LHF4                             | 2021/04/29    | 24°03'14.7" S; 31°43'35.1" E       | Olifants River                |
| LHF5                             | 2021/05/03    | 23°13'15.8" S; 31°33'18.4" E       | Shingwedzi River              |
| LHF6                             | 2021/05/04    | 22°25'45.4" S; 31°15'27.4" E       | Luvuvhu River                 |
| LHF7                             | 2021/05/05    | 23°40'39.1" S; 31°05'55.1" E       | Groot Letaba River            |
| LHF8                             | 2021/05/06    | 23°56'36.2" S; 31°44'06.4" E       | Letaba River                  |
| LHF9                             | 2021/05/07    | 24°04'00.2" S; 31°14'33.0" E       | Olifants River                |
| <b>low flow sampling sites</b>   |               |                                    |                               |
| LLF1                             | 2021/08/23    | 25°31'18,85" S; 27°22'55,3" E      | Hex River                     |
| LLF2                             | 2021/08/23    | 25°23'0.6936" S; 27°35'54.8052" E  | Roodekopjes Dam               |
| LLF3                             | 2021/08/23    | 25°22'25,3" S; 27°33'59.6"E        | Crocodile River               |
| LLF4                             | 2021/08/24    | 24°45'43.848" S; 28°13'3.0324" E   | Buffels Spruit                |
| LLF5                             | 2021/08/24    | 24°40'14.2716" S; 28°19'12.8604" E | Donkerpoort Dam               |
| LLF6                             | 2021/08/24    | 24°24'31.4208" S; 28°7'20.2224" E  | Klein sand River              |
| LLF7                             | 2021/08/24    | 24°16'32.3724" S; 28°5'0.9708" E   | Mokolo River                  |
| LLF8                             | 2021/08/25    | 23°50'49.56" S; 29°21'37.9008" E   | Bloed River                   |
| LLF9                             | 2021/08/25    | 23°54'41.22" S; 29°58'53.4216" E   | Stanford Lake                 |
| LLF10                            | 2021/08/25    | 23°45'47.6172" S; 30°8'30.3612" E  | Tzaneen Dam                   |
| LLF11                            | 2021/08/25    | 23°41'57.2712" S; 30°19'35.5296" E | Mothomeng Dam                 |
| LLF12                            | 2021/08/26    | 23°16'34.2084" S; 30°23'51.1044" E | Middle Letaba Dam             |
| LLF13                            | 2021/08/26    | 23°6'24.408" S; 30°24'4.1184" E    | Luvuvhu River                 |
| LLF14                            | 2021/08/26    | 23°5'20.7132" S; 30°23'1.6836" E   | Magic Dam                     |
| LLF15                            | 2021/08/26    | 23°7'22.0152" S; 30°13'7.6116" E   | Dam, Valdezia                 |
| LLF16                            | 2021/08/26    | 23°6'25.0416" S; 30°7'32.8044" E   | Luvuvhu River                 |
| <b>Free State sampling sites</b> |               |                                    |                               |
| FS1                              | 2022/02/10    | 27°35'30,6" S; 29°35'31,7" E       | Klip River                    |
| FS2                              | 2022/02/10    | 27°39'00,3" S; 29°35'10,1" E       | Klip River                    |
| FS3                              | 2022/02/11    | 27°32'24,35" S; 29°35'12,13E       | Klip River                    |
| FS4                              | 2022/02/11    | 27°34'57,8" S; 29°35'09,1" E       | Klip River                    |
| FS5                              | 2022/02/11    | 27°41'12,8" S; 29°34'43,4" E       | Klip River                    |
| FS6                              | 2022/02/11    | 27°40'9,5" S; 29°33'48,15" E       | Pampoenspruit; Natural Spring |
| FS7                              | 2022/02/11    | 27°37'52,05" S; 29°35'32,4" E      | Wasgoedspruit; Natural Spring |
| FS8                              | 2022/02/11    | 27°37'35,59" S; 29°35'1,29" E      | Klip River                    |
| FS9                              | 2022/02/12    | 27°33'32,79" S; 29°35'8,76" E      | Klip River                    |
| FS10                             | 2022/02/12    | 27°38'37,87" S; 29°34'44,66" E     | Klip River                    |

### 2.1.2 Limpopo province

The Limpopo province is the most north-eastern province of South Africa, bordering Zimbabwe, Botswana, and Mozambique. The Limpopo River forms the border between the Limpopo province and the neighbouring countries of Zimbabwe and Botswana and flows through Mozambique to the east coast of southern Africa (Maponya *et al.*, 2017; Scott, 2019). The Limpopo River is the third largest in Southern Africa based on its length of 1750 km, with a drainage area covering approximately 415000 km<sup>2</sup> (Mosase & Ahiablame, 2018; Martin, 2022). The mean annual rainfall of the Limpopo River basin ranges from 160-1100 mm, with yearly temperature averages ranging from 0°C in the winter to 36°C in the summer (Mosase & Ahiablame, 2018). Tributary rivers of the Limpopo River in South Africa sampled during this study included the Crocodile-, Mogalakwena-, Olifants-, Luvuvhu-, Shingwedzi- and Letaba Rivers.

Population growth, industrial development, and increased agricultural and mining activities within the Limpopo province increase anthropogenic pressures on the natural freshwater environment and negatively affect the region's ecology. The population is estimated to be nearing 23 million people in primarily rural settlements within the Limpopo province, which relies heavily on the basin's natural water resources (Mosase & Ahiablame, 2018). The increased pressure on the region's natural resources already places a great deal of stress on this ecosystem (Ubisi *et al.*, 2017). Research further indicates that climate change will likely lead to rises in temperature and changes in rainfall patterns across the country that could have a detrimental effect on aquatic ecosystems, increasing the frequency of flooding and drought events (Mwenge Kahinda *et al.*, 2016; Mosase & Ahiablame, 2018).

The sites sampled during the high flow period were all located in the north to north-eastern parts of the Limpopo province, labelled LHF1 to LHF9 (Figure 2.4). All the sites were located within the Limpopo River catchment with little to no marginal and emergent vegetation and a primarily sandy substrate with exposed bedrock. In some cases, no floating vegetation was present during this survey. The sites sampled during the low flow sampling period were located more inland than those sampled during the high flow within the Limpopo River catchment and had a greater diversity of different habitat types present with varying substrates and vegetation present. Photographs taken on the sampling day are shown below, LLF4-7 (Figure 2.5; D-G) and Sites LLF8-16 (Figure 2.6; A-I).

### **2.1.1 North West province**

The North West province is situated on the flat interior plateau of South Africa, bordering Botswana to the north and stretching to the Vaal River in the south. The North West province falls in the austral summer rainfall season with annual precipitation of 300 mm-500 mm. The temperature ranges from an average of 17°C to a high of 30°C during the summer season and an average of 3°C and a high of 21°C during the winter season within the North West province (Winde & Erasmus., 2011). The area falls within the Southern temperate highveld ecoregion with predominantly savanna and thornveld biomes and temporary pans (Galvin *et al.*, 2008).

Two economically important industries within the North West province are agriculture and mining, with the latter being one of the largest employers within the region (Yager *et al.*, 2012). The sites sampled during the current study were located close to Rustenburg, one of the largest cities within the province, with the mining industry dominating the local landscape. The water used within the mining industry is predominantly discharged into the surface waters of surrounding natural rivers and dams, potentially harming the aquatic ecosystem within the discharge area (Erasmus *et al.*, 2020). Sites LLF1-3 were sampled within the northern parts of the North West province at the Hex River (Figure 2.5; A), Roodekopjes Dam (Figure 2.5; B) and the Crocodile River (Figure 2.5; C).

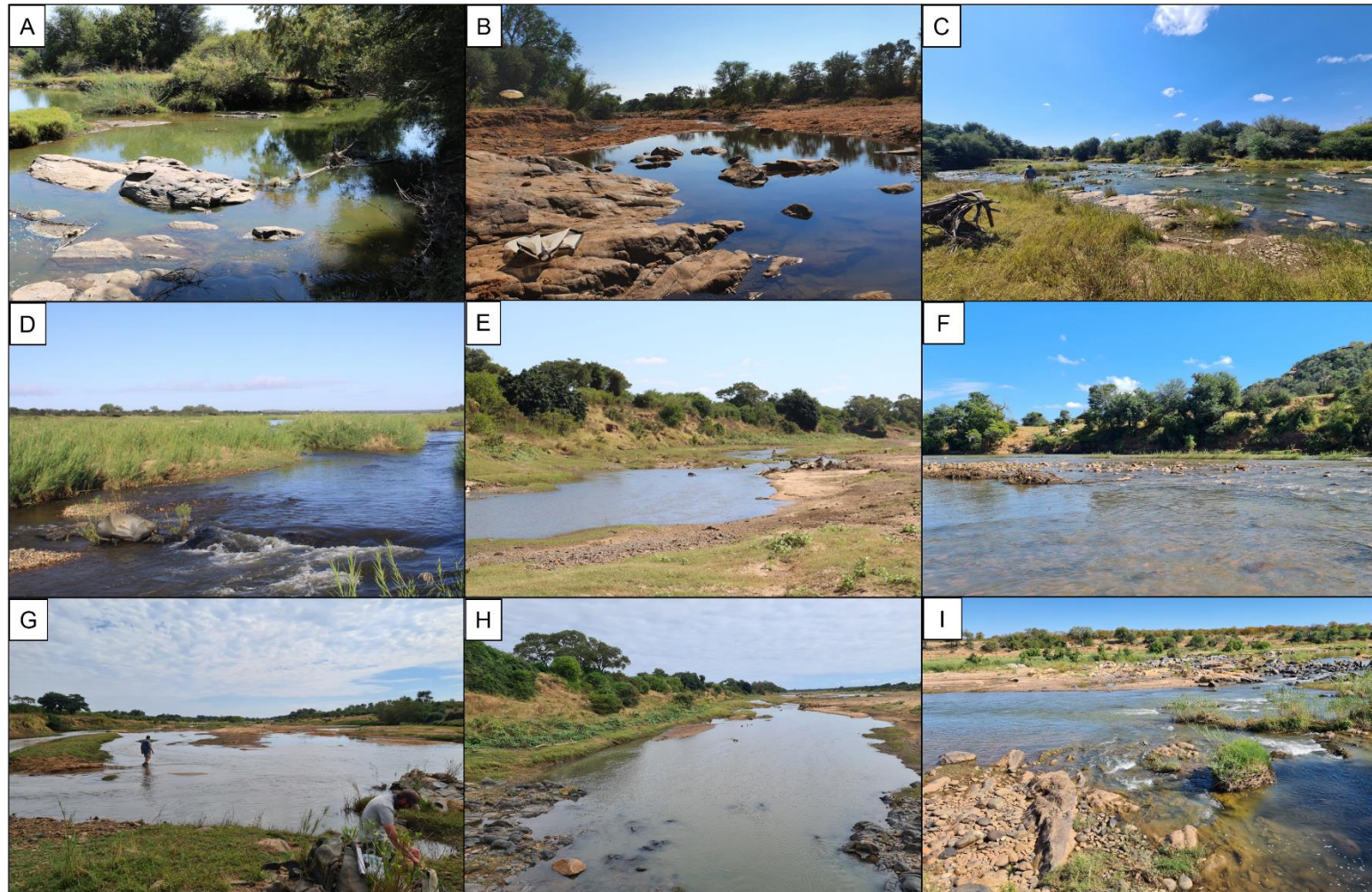


Figure 2.4: Photographs of the sites sampled during the high flow sampling period. The first three sites located on the Limpopo River, (A) Site LHF1, (B) Site LHF2, (C) Site LHF3, (D) Site LHF4 is located on the Olifants River, (E) Site LHF5 is located on the Shingwedzi River, (F) Site LHF6 located on the Luvuvhu River, (G) Site LHF7 located on the Groot Letaba River, (H) Site LHF8 located on the Letaba River and Site LHF9 on the Olifants River.

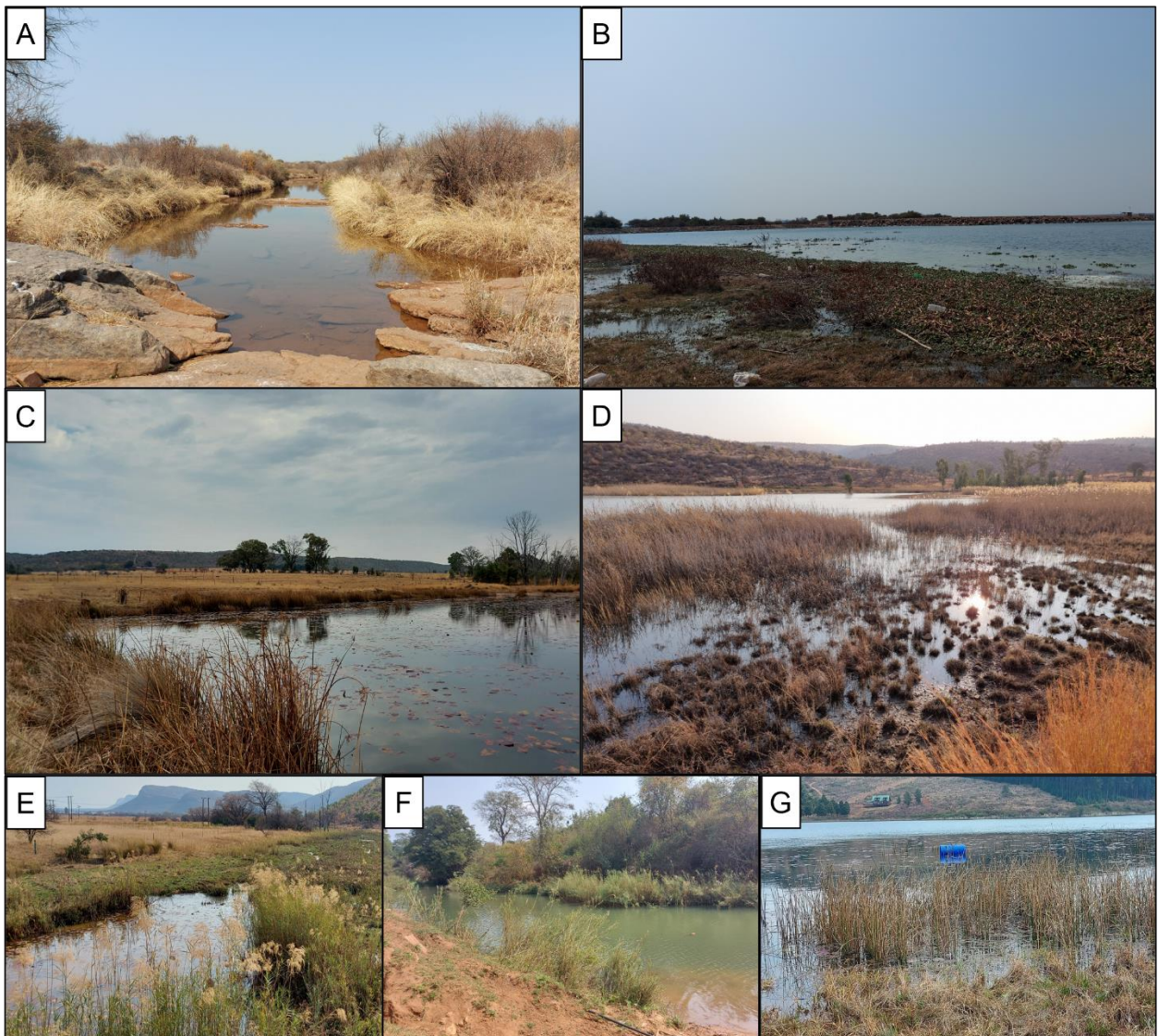


Figure 2.5: Photographs of the sites sampled during the low flow sampling period. (A) Site LLF1 Isolated stream near the Hex River, (B) Site LLF2 Farm Dam near the Crocodile River, (C) Site LLF3 is located at the Crocodile River, (D) Site LLF4 located on the Buffelspruit, (E) Site LLF5 is located at the Donkerpoort Dam, (F) Site LLF6 located on the Klein Sand River and (G) Site LLF7 located on the Mokolo River.

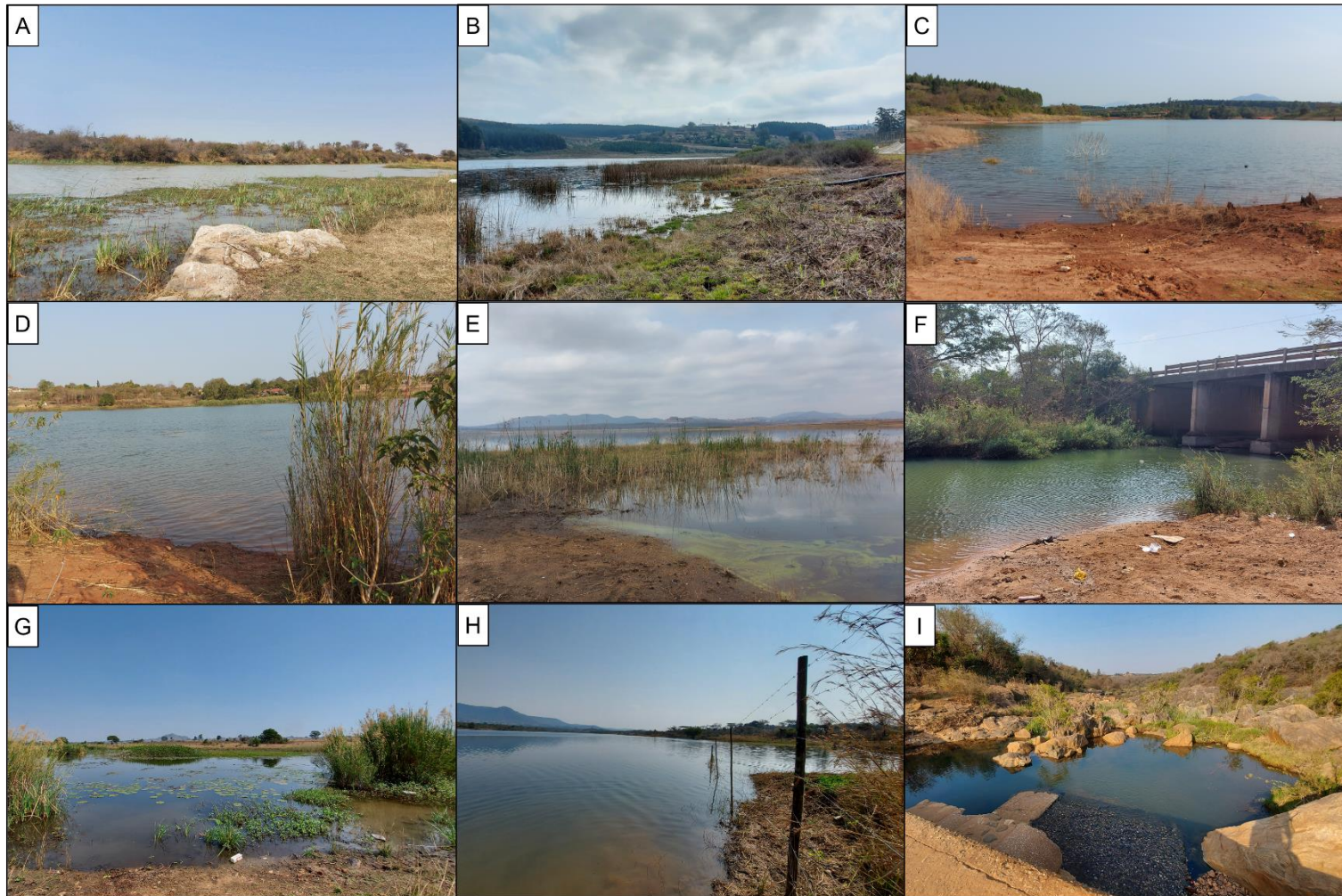


Figure 2.6: Photographs of the sites sampled during the low flow sampling period. (A) Site LLF8 is on Bloed River (B) Site LLF9 located at the Stanford Lake, (C) Site LLF10 is located at the Tzaneen Dam, (D) Site LLF11 located at the Mothomeng Dam, (E) Site LLF12 is located at the Middle-Letaba River, (F) Site LLF13 located on Luvuvhu River and (G) Site LLF14 located on the Magic Dam near Tshivhazwaulu town, (H) Site LLF15 located at a Unnamed Dam near Valdezia town and (I) Site LLF16 located below Albasini Dam on the Luvuvhu River.

### 2.1.2 Free State province

The Seekoeivlei Nature Reserve (Figure 2.3; Figure 2.7 A-D) was declared a nature reserve in 1978 and is listed as a Ramsar wetland of international importance, located near the small town of Memel, Free State province, South Africa and is found within the more extensive Seekoeivlei wetland and covers an estimated 4754 ha (FSDTEEA, 2005). The Seekoeivlei wetland is an estimated 12 000 ha and consists of over 200 oxbows that have formed over centuries by the meandering flow of the Klip River (Tooth *et al.*, 2002; McCarthy *et al.*, 2010) and are regarded as a floodplain depression providing essential water purification and flood mitigation functions (McCarthy *et al.*, 2010; Ollis *et al.*, 2015). The Klip River flows through the nature reserve and forms part of the upper Vaal River catchment and is considered one of the important tributaries supplying approximately 46% of the surface water flow in the upper Vaal River catchment (DWAF, 2004). The Seekoeivlei Nature Reserve is the only protected area in the Free State province that covers the Amersfoort Highveld Clay Grassland and the Eastern Temperate Freshwater Wetland veld type (McCarthy *et al.*, 2010). The annual precipitation of this area is measured to be between 700 mm-1200 mm, with most of the yearly rainfall occurring from November to March. Annual mean temperature ranges from 13°C to 25°C during the summer season and -1°C and 15°C during the winter season (McCarthy *et al.*, 2010).

The Free State province sites (Figure 2.7 A-D) were all located within and surrounding the Seekoeivlei Nature Reserve. All the sites had extensive amounts of vegetation present (marginal, emergent, and floating vegetation), as seen in Figure 2.7 (A), limiting the accessibility to the selected sites. Due to the proximity of the sites and the lack of differences in habitat, only selected images are included in Figure 2.7 (A-D).



Figure 2.7: Photographs of the Seekoeivlei Nature Reserve indicating the (A) the thick vegetation cover surrounding all of the sites sampled along the Klip River, (B) The oxbows formed by the Klip River, (C) Flooding of the perimeter fence of the Seekoeivlei Nature Reserve and (C) the high flow velocity of the Klip River as it flows out of the Seekoeivlei Nature Reserve

## **2.2 Methods**

### **2.2.1 Water quality**

Water quality parameters were measured as well as the total Phosphorus and Nitrogen of each site was calculated and compared to the Department of Water Affairs (DWAF, 1996) target water quality guidelines for aquatic ecosystems to determine the trophic state of the selected site and suitability for freshwater molluscan species.

*In situ* water quality variables were measured at each site with an ExStik II EC 500 instrument. Measurements were made in triplicate, and variables included total dissolved solids (TDS; mg/L), temperature (°C), pH, conductivity (µS/cm) and dissolved oxygen (DO% and mg/l). Water samples were also collected in clean 500 mL plastic bottles, frozen at -4 °C on the day of sampling and transported back to the North-West University's Potchefstroom campus, where the samples were frozen and stored until further water nutrient tests could be conducted.

Unfiltered water samples were allowed to thaw and come to room temperature (~20°C). The following chemical variables were analysed: turbidity (FAU), ammonium (NH<sub>4</sub><sup>+</sup>), sulphates (SO<sub>4</sub><sup>2-</sup>), nitrites (NO<sub>2</sub><sup>-</sup>), nitrates (NO<sub>3</sub><sup>-</sup>), chlorides (Cl<sup>-</sup>), phosphates (PO<sub>4</sub><sup>3-</sup>) and total hardness using chemical-specific test kits and standardized protocols from supplier Merck chemicals ([www.merck-chemicals.com](http://www.merck-chemicals.com)), (Nitrate test [109713], Phosphate test [114848], Nitrite test [114776], Chloride test [114897], Ammonium test [1114752], Phosphate test [HC574967], Sulphate test [114791]; Total hardness test [100961]) and absorbances determined using a Merck Spectroquant® Pharo 300 water quality spectrophotometer with.

### **2.2.2 Habitat assessment**

A qualitative habitat assessment of the riparian and aquatic vegetation was performed at each site to determine the habitat suitability for freshwater snail species (Figure 2.8). The habitat type (river, wetland, isolated pool), as well as the presence of marginal-, floating-, emergent-, and submerged vegetation, were determined along with algae presence; the weather conditions at the time of sampling, and the site coordinates were recorded on the day of collection. Any anthropogenic activity within or near the collection area was also noted, as this could impact the habitat suitability for the freshwater molluscan species (Appleton *et al.*, 2015a, 2015b).

|                      |            |                                             |  |
|----------------------|------------|---------------------------------------------|--|
| Data sheet for Site: |            |                                             |  |
| Date:                |            | Weather:                                    |  |
| Time:                |            | Habitat type<br>(e.g. pool, wetland, river) |  |
| Habitat assessment   |            |                                             |  |
|                      | In current | Out of current                              |  |
| Marginal vegetation  |            |                                             |  |
| Floating vegetation  |            |                                             |  |
| Emergent vegetation  |            |                                             |  |
| Submerged vegetation |            |                                             |  |
| Algae                |            |                                             |  |
| Stones               |            |                                             |  |
| GSM                  |            |                                             |  |
| Additional notes:    |            |                                             |  |

Figure 2.8: Example of the datasheet used during the qualitative habitat assessment.

### 2.2.3 Freshwater mollusc collection and morphological identification

Molluscs were sampled and subsequently collected from all 35 sites in the North West, Limpopo and Free State provinces of South Africa. Each site was sampled for a maximum of 30 minutes. Samples were collected with the aid of a snail sampler (20cm by 20cm), standard SASS (South African Scoring System) net, and hand-picking from vegetation and rocks where possible (Figure 2.8). All specimens were identified morphologically in the field with the aid of Day & de Moor (2002), "Guides to the Freshwater Invertebrates of Southern Africa Volume 6: Arachnida & Mollusca". In the laboratory, the intact molluscan specimens of the molluscs genera *Bulinus*, *Biomphalaria*, and *Gyraulus* with the least amount of shell damage were also photographed using a Nikon AZ100M microscope with the aid of a Nikon digital sight DS-F12 camera and associated NIS-Elements software (Nikon Instruments, Tokyo Japan) and edited using Adobe Photoshop (Adobe Inc., 2019). Dissection microscopy with the aid of a Nikon C-LEDS dissection microscope was done for further morphological identification to a lower taxonomic level, as these genera are particularly difficult to identify based on shell morphology.

All collected samples were screened for parasitic infection using the cercarial shedding method (Webber *et al.*, 1986). This method entails alternating eight-hour periods of dark and light intervals over the course of 48 hours. Light exposure is done to induce shedding of the cercarial life stage of trematodes, potentially infecting the sampled freshwater molluscs (Wolmarans *et al.*, 2002). Shedding examination was conducted by placing individual molluscan specimens in containers filled with distilled water (ddH<sub>2</sub>O) under an artificial light source, out of direct sunlight, to induce a stress response within the host molluscan species and artificially stimulate cercarial shedding.

During the eight-hour window of light exposure, samples were examined at 20-minute intervals with the aid of a Nikon stereomicroscope (Nikon Instruments, Tokyo, Japan) for signs of cercarial shedding. Afterwards, the specimens were placed in containers that prevent light penetration, thus simulating night-time conditions and reducing stress (Wolmarans *et al.*, 2002; Schwelm *et al.*, 2020). Feeding was withheld during the light exposure and restored afterward. When the 48-hour shedding experiment was completed, specimens were euthanized, given a specific sample code and preserved in separate Eppendorf tubes with 98% molecular-grade ethanol. Samples were placed in a freezer (-4°C) for further laboratory analyses at NWU, Potchefstroom



Figure 2.9: Photographs illustrating the different methods of collection used. (A and B) the use of a snail sampler. (C) screening each sample taken with the snail sampler for the presence of molluscs. (D) hand-picking from vegetation present within the sampling area.

#### 2.2.4 Molecular analyses

To validate the morphological identification of collected molluscan species, molecular characterization, identification, and potential trematode infections were conducted, particularly for *Bulinus* species, which are known to be difficult to identify morphologically (Day & de Moor, 2002). The mollusc was carefully removed from its shell using forceps and small tongs, with the shell being crushed and the tissue removed if the specimen could not be extracted without damaging the shell or soft tissue. Subsequently, a piece of hepatopancreas was cut with a sterile scalpel

blade and placed in a newly labelled Eppendorf tube with 98% molecular-grade ethanol. The specimen was then preserved in a freezer at -20°C until further analyses. Cross-contamination was prevented by sterilizing all forceps and tongs using bleach and 98% ethanol between specimens (Hoogendoorn *et al.*, 2019; Schols *et al.*, 2019).

Identification of potential trematode infection was established using molecular identification by extracting the trematode 28S rRNA gene from the molluscan tissue. The larger samples of each molluscan species identified were utilized to test for prepatent trematode infections molecularly. Larger, more mature molluscan specimens have a higher chance of trematode infection (Levri *et al.*, 2005; Giannelli *et al.*, 2016). Therefore, larger specimens were used for molecular analyses.

Molecular analyses were conducted by extracting DNA from the stored sample using a combination of hepatopancreas and tissue collected from the foot of the snail specimen. After removing as much ethanol as possible, the residual ethanol within the Eppendorf tube was evaporated by placing the open tube in a heat block at 56°C. Total genomic DNA was extracted from the snail tissue and hepatopancreas using the Chelex®100 method and following the protocol developed by Walsh *et al.* (1991) for DNA extraction. The supernatant of each extraction was transferred to sterile tubes and frozen (-20°C) to prevent DNA degradation.

The DNA samples were amplified using the appropriate primers by polymerase chain reaction (PCR) protocols for the target gene fragments. Primers were chosen based on the preliminary identification of molluscan species and recommendations from previous studies (Folmer *et al.*, 1994; Tkach *et al.*, 2001; Veeravechskij *et al.*, 2018; Hoogendoorn *et al.*, 2019; Schols, 2019; Schols *et al.*, 2019). All the primers used in this study are summarized in Table 2.2. A PCR master mix created containing the appropriate PCR reagents was prepared to reduce pipetting, cross-contamination risk, and possible mixing errors (West & Sawyer, 2006; Hoogendoorn *et al.*, 2019; Schols *et al.*, 2019). The polymerase chain reaction amplifications were visualized by 1% agarose gel electrophoresis, and impurified samples were sent to Inqaba Biotechnical Industries (Pty) Ltd. in Pretoria, South Africa, for purification and sequencing.

Geneious v.11 bioinformatics software (Biomatters, Auckland, New Zealand) was used to assemble the newly generated sequences. Sequences from the present study were compared with relevant sequences of trematode species available in GenBank (NCBI, <https://blast.ncbi.nlm.nih.gov/Blast.cgi>) by using the BLAST tool to determine genetic similarities to identify trematode species present. Sequences were aligned using MUSCLE (Edgar, 2004) as implemented in Geneious v. 11, under the default parameter values. Aligned sequences were analysed with the aid of the jModeltest v. 2.1.2 software (Posada, 2008) to determine the best substitution model according to each dataset's Akaike information criterion (AIC). Bayesian

inference was performed by the MrBayes plugin (Huelsenbeck *et al.*, 2001; Ronquist & Huesenbeck, 2003) for Geneious v. 11, and maximum likelihood analysis was carried out with the aid of MEGA11 (Tamura *et al.*, 2021). The nodal support (for maximum likelihood and Bayesian inference analysis) was validated by applying 1000 bootstrap pseudo replicates. The phylogenetic tree was visualized using the MrBayes plugin within Geneious v.11. Pairwise genetic distance matrices as a percentage difference (p-distance) and the number of base pair (bp) differences between sequences used for the phylogenetic analyses, were calculated with Geneious v. 11 software.

Table 2.2: Genetic markers used during polymerase chain reaction (PCR) amplification of COI gene and ITS2 region of molluscan species. Table adapted from Schols *et al.* (2019).

| Genetic marker   | Primer  | Primer Sequence (5'–3')        | Reference                     |
|------------------|---------|--------------------------------|-------------------------------|
| <b>28S rDNA</b>  | DigI2   | AAG CAT ATC ACT AAG CGG        | (Tkach <i>et al.</i> , 2001)  |
|                  | 1500R   | GCT ATC CTG AGG GAA ACT TCG    | (Tkach <i>et al.</i> , 2001)  |
|                  | ECD2    | CTT GGT CCG TGT TTC AAG ACG GG | (Tkach <i>et al.</i> , 2001)  |
|                  | 300F    | CAA GTA CCG TGA GGG AAA GTT G  | (Tkach <i>et al.</i> , 2001)  |
| <b>ITS2 rDNA</b> | 3S      | GGTACCGGTGGATCACGTGGCTAGTG     | (Bowles <i>et al.</i> , 1993) |
|                  | ITS2.2  | CCTGGTTAGTTTCTTTTCCT CGC       | (Cribb <i>et al.</i> , 1998)  |
| <b>COI mtDNA</b> | LCO1490 | GGTCAACAAATCATAAAGATATTGG      | (Folmer <i>et al.</i> , 1994) |
|                  | HCO2198 | TAAACTTCAGGGTGACCAAAAAATCA     | (Folmer <i>et al.</i> , 1994) |

Infection RD-PCR and *Schistosoma* RD-PCR for *Schistosoma* species detection and identification were used to simultaneously evaluate for schistosomiasis infection within molluscan species known to be an intermediate host for the neglected tropical diseases (De Kock, 1995; Wolmarans *et al.*, 2002; De Kock *et al.*, 2004; Schols *et al.*, 2019). Molluscan DNA already extracted by the Chelex®100 method was used for the *Schistosoma* Multiplex-PCR protocol as described by Schols *et al.* (2019). The list of primers used for the Multiplex-PCR for specific *Schistosoma* detection and identification is described in Table 2.3.

Table 2.3: Genetic markers used in the Infection RD-PCR and *Schistosoma* RD-PCR. Adapted from Schols *et al.* (2019)

| Genetic marker            | Primer         | Primer Sequence (5'–3')       | Reference                             |
|---------------------------|----------------|-------------------------------|---------------------------------------|
| <b>Infection RD-PCR</b>   | 18S_SNAIL_F    | AGTATGGTTGCAAAGCTGAAACTT<br>A | (Carolus <i>et al.</i> , 2019)        |
|                           | 18S_SNAIL_R    | TACAAAGGGCAGGGACGTAAT         | (Carolus <i>et al.</i> , 2019)        |
|                           | 18S_Digenea_F  | CAGCTATGGTTCCTTAGATCRTA       | (Schols <i>et al.</i> , 2019)         |
|                           | 18S_Digenea_R  | TATTTTTCGTCACCTCCCCGT         | (Schols <i>et al.</i> , 2019)         |
|                           | ITS2_Schisto_F | GGAAACCAATGTATGGGATTATTG      | (Gryseels <i>et al.</i> , 2006)       |
|                           | ITS2_Schisto_R | ATTAAGCCACGACTCGAGCA          | (Gryseels <i>et al.</i> , 2006)       |
| <b>Schistosoma RD-PCR</b> | Asmit1         | TTTTTTGGTCATCCTGAGGTGTAT      | (Littlewood <i>et al.</i> , 1997)     |
|                           | Sh.R           | TGATAATCAATGACCCTGCAATAA      | (Webster <i>et al.</i> , 2010)        |
|                           | Sman.R         | TGCAGATAAAGCCACCCCTGTG        | (Van den Broeck <i>et al.</i> , 2011) |
|                           | Smat.R         | CACCAGTTACACCACCAACAGA        | (Schols <i>et al.</i> , 2019)         |
|                           | Sb.R           | CACAGGATCAGACAAACGAGTAC<br>C  | (Webster <i>et al.</i> , 2010)        |

### 2.2.5 Data analyses

Data regarding the historical distribution of freshwater snails in the study regions were obtained from the NFSC to compare the historical distribution and diversity of snail species in the sampling areas to those collected during the current study. These data could only be compared qualitatively as the historical data only indicates the species at sampling sites with no quantitative information available.

Data collected from the 25 sites sampled in the Limpopo and North West provinces were analysed as individual data points. The number of individuals and the number of taxa were determined for the various sites using untransformed data. Molluscan species data were then transformed by way of square root ( $\sqrt{x}$ ) to mitigate the effect of dominant taxa (de Necker *et al.*, 2021). Collected environmental data, excluding pH, were log-transformed [ $y = \log(x + 1)$ ] to improve data normality, before further parametric tests. The number of taxa collected at each site was compared between the two sampling surveys using "Flow" as the *a priori* factor to test the impact of seasonal flow for both. Differences between the sampling sites were also assessed based on the presence or absence of the invasive mollusc *Tarebia granifera* using the *a priori* factor "*Tarebia granifera*." Results were visualized using a non-metric multidimensional scaling (NMDS) plot and dendrogram was created in Primer v7 using a Bray-Curtis similarity matrix (Clarke *et al.*, 2014; Clarke & Gorley, 2015; de Necker *et al.*, 2021). The factors "Flow" and *Tarebia granifera* presence or absence were tested for significant differences using the Analysis of Similarity (ANOSIM). Two values are produced by the ANOSIM, an R-value (from 0 to 1) and a significance-level percentage value. The closer the R-value is to 1, the more critical the chosen factor is. The significance level indicates whether the element being tested is significant ( $\leq 5\%$ ) or not significant ( $\geq 5\%$ ; (Clarke & Warwick, 1994; Clarke & Gorley, 2015). To determine potential differences in molluscan abundance and diversity and water quality parameters between sampling sites and seasons, a canonical correspondence analysis (CCA) was created using Canoco v5. This is a multivariate analysis technique that uses axes to transform data in a linear combination of predictors to extract gradients that drive the composition of ecological communities with Canoco v5 (ter Braak & Šmilauer, 2012). Trematode infections were calculated based on prevalence of infections and secondly the parasitic load within a specimen based on total number of cercaria released per specimen.

## **Chapter 3 Identifying molluscan diversity within the north-eastern provinces of South Africa, using a combination of morphological and molecular techniques.**

### **3.1 Introduction**

Aquatic molluscs are crucial primary consumers in freshwater ecosystems, serving as the foundation of the food web (Dudgeon *et al.*, 2006). With the Phylum Mollusca being second in number of species only to Arthropoda, identifying shared traits among its many taxa is daunting (Telford & Budd, 2011). Taxonomic identification of freshwater molluscs can be challenging and may require specialized training to identify species based on shell morphology and internal anatomy (Pfenninger *et al.*, 2006). Combining morphological and molecular analyses may offer a solution to the challenge of identifying freshwater molluscs. Failure to correctly identify native, medically important freshwater molluscan species is a cause for concern (Pfenninger *et al.*, 2006). The incorrect identification of medically important molluscan species inhibit the effective assessment and implementation of control measures based in sound scientific knowledge (Hastein *et al.*, 2005). Morphological identification of *Bulinus* species, for instance, can be challenging as these species are typically classified into one of four morphological groups, with three of these groups being found within southern Africa (Day & de Moor, 2002). Therefore, Identification with general morphological traits is sufficient to group specimens into one of the four morphological groups. However, Identification of most molluscan species can only be done by examining the reproductive organs of gastropod species and requires specialised training (Day & de Moor, 2002). The incorrect identification causes problems when assessing the population dynamics of molluscan species within a specific area (Lawton *et al.*, 2015). Secondly, the incorrect identification of molluscan vectors of medical and veterinary importance will result in inaccurate parasitological studies of the life cycle analyses of trematodes (Pfenninger *et al.*, 2006). To achieve a higher phylogenetic resolution and accurately distinguish between species the employing ITS2 and COI genetic regions as genetic markers for species identification is used.

### **3.2 Problem statement**

Inaccurate identification of freshwater mollusc species is hindering efforts to establish the distribution and status of native species, as well as to determine the impact of invasive species on native species.

### **3.3 Aims and objectives.**

This chapter aims to accurately identify freshwater mollusc species collected during this study by using a combination of morphological identification from published literature and molecular analyses of the conserved genetic regions.

### **3.4 Materials and methods**

All the materials and methods used for morphological and molecular identification used in this chapter are provided in full in Chapter 2.

### 3.5 Results

In total 3099 molluscan specimens were collected from 35 sites in South Africa's Limpopo, North West and Free State provinces (Figure 2.1) and morphologically identified to genus level and, where possible, to species level in the field. Molluscan specimens collected were identified as species of the genera *Bulinus* spp., *Biomphalaria* spp., *Gyraulus* spp., *Radix* sp., *Pseudosuccinea* sp. and *Physella* sp. Larger specimens of the different taxa collected were used for molecular analysis resulting in 73 novel COI and ITS2 genetic sequences generated. Specimens identified as *T. granifera* were also collected during this study, however, this data formed part of another study that used molecular and morphological methods to identify the invasive *T. granifera* that, together with the current research, collectively formed part of a larger, more extensive study reassessing the status of schistosomiasis-causing vectors within South Africa. The identification and molecular characterisation of *Tarebia granifera* was made by JJ Pearson, and all genetic analyses are provided in his MSc entitled: "The role of *Tarebia granifera* as an invasive in freshwater ecosystems of southern Africa" (Pearson, 2022).

#### 3.5.1 Bulinidae

Species of the family Bulinidae belonging to the genus *Bulinus* are morphologically grouped into four groups, and these three are known from within South Africa. These three groups are, firstly, the *B. africanus/globosus* group containing members of *B. globosus*, *B. africanus* and *B. nasutus*. The second group is the *B. truncatus/tropicus* group containing members of *B. truncatus* and *B. tropicus*, and in some cases, *B. natalensis* and *B. depressus* are included within this group. The third group is the *B. forskalii* group. Members of this group are easily distinguishable from the other Bulinidae groups due to their elongated and ribbed shell. Similar morphological traits are used to ease the identification of *Bulinus* species and group them in the field, as well as determine their medical importance, as trematodes associated with some *Bulinus* specimens are species-specific and historically used to determine the possible spread of these trematodes.

A total of 225 specimens were collected and morphologically identified to be members of the Bulinidae, specifically species of *Bulinus*. It is not easy to distinguish between *Bulinus* species due to the many overlapping morphological similarities between species of this genera. The *B. africanus/globosus* group and the *B. truncatus/tropicus* group are the two groups native to South Africa that have the most overlapping morphological features, as both groups have a globose shape with a short spire. Specimens belonging to *B. africanus/globosus* were identified by their globose shape, short spire, and large basal whorl. Both *B. africanus* and *B. globosus* have a fold on the ventral surface, a distinguishing characteristic of this group. The *B. truncatus/tropicus* group is identifiable by having a globose shape shell, moderately developed spire, and rounded

columella. A total of 115 specimens collected belonged to the *B. africanus/globosus* group (Figure 3.1; A-B), and 110 specimens were identified to belong to the *B. truncatus/tropicus* group (Figure 3.1).

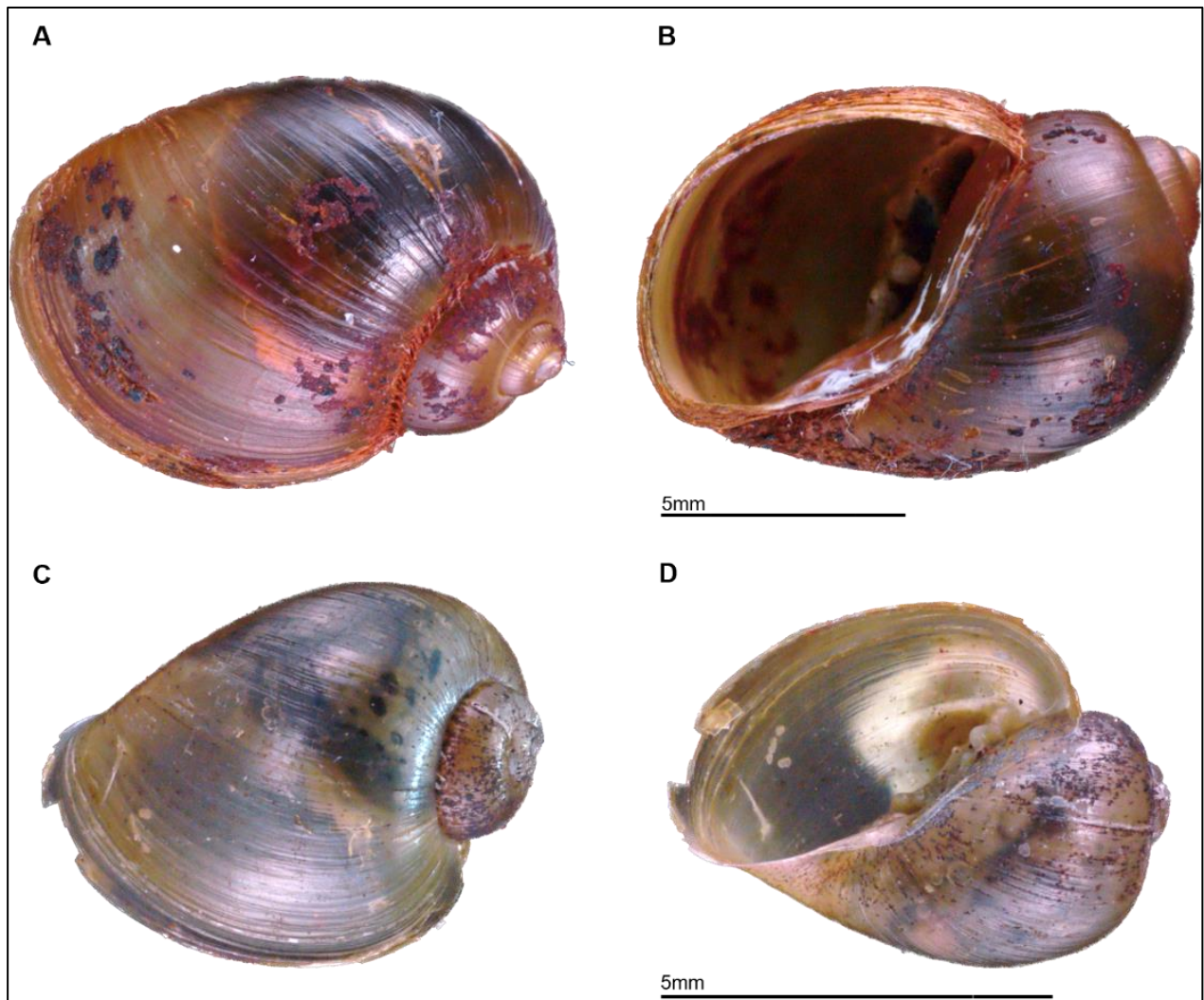


Figure 3.1: Bulinidae specimens collected during the current study. Morphologically identified as *Bulinus africanus* (A-B). (A) the ventral view of *Bulinus africanus* and (B) the dorsal view. (C-D) Identified as *Bulinus depressus*. (C) the ventral view of *Bulinus depressus* and (D) the dorsal view. (A-D) Scale bar = 5 mm.

The molecular analyses resulted in 24 novel sequences. Phylogenetic trees were generated based on the ITS2 (601 base pairs; Figure 3.2) and COI (491 base pairs; Figure 4.4).

Phylogenetic analyses of the novel ITS2 sequences (Figure 3.2; Table 3.1; Table 3.2) showed that the specimens from this study fall within two distinct clades. The first group of six sequences formed a clade with available sequences of the *B. africanus/globosus* group and ten sequences grouped with the *B. truncatus/tropicus* group. The six sequences all grouped with published *B. africanus* sequences retrieved from GenBank (AM921970), confirming the morphological identification of these species as *B. africanus*. The ten sequences in the *B. truncatus/tropicus* group were similar to available sequences of *B. truncatus*, providing a positive molecular identification of these specimens.

The COI mtDNA analyses (Figure 3.3; Table 3.3) indicated a separate phylogenetic group within the *B. truncatus/tropicus* group. Morphologically these species were identified in the field as *B. tropicus* and some as *B. depressus*. It is clear from the phylogenetic analyses that these novel genetic sequences did not clade with *B. tropicus* or *B. truncatus*, forming a separate clade. The separation but the close phylogenetic relationship between *B. tropicus* and the distinct clade formed by the samples generated during this study were identified as *B. depressus*.

All the Bulinidae species collected were within the Limpopo province, South Africa. As seen in Figure 3.4, members of the *B. africanus/globosus* group were collected at more sites than *B. truncatus/tropicus*. Only four locations where both *Bulinus* groups were collected had more than one of the two groups present (LLF7, LLF8, LLF11, LLF14). Morphological identification was accurate to the specific *Bulinus* group. However, the addition of morphological analyses and phylogenetic analyses supported the designation of *B. africanus* and *B. depressus*.

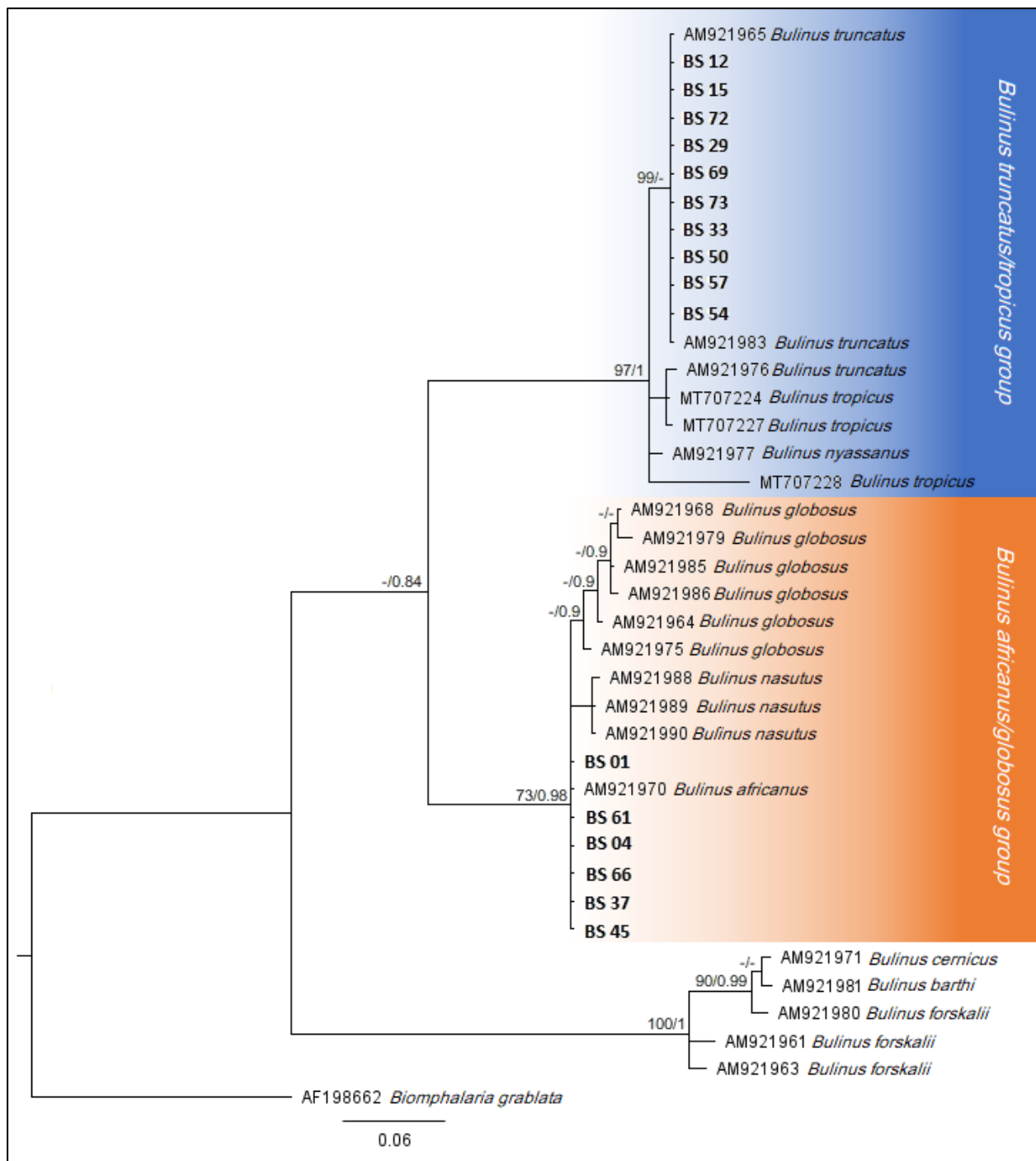


Figure 3.2: Bayesian inference phylogram of *Bulinus* genus based on partial sequences of the ITS2 rDNA region. New sequences are in bold. Posterior probability followed by bootstrap support values are indicated next to the branches (posterior probability < 70 and bootstrap < 0.80 not shown). *Biomphalaria grablata* was used as an outgroup. The branch length scale bar indicates the number of substitutions per site. Sequences generated during the current study are represented in bold.

Table 3.1: Table indicating base pair differences between members of the *Bulinus africanus/globosus* group. Above the diagonal are the percentage of identical base pairs, and below the diagonal are base pair differences of the ITS2-region. Genetic sequences generated during this study are coloured blue to match the phylogenetic tree generated (Figure 3.2).

|   | Species name             | Sequence number | 1  | 2   | 3   | 4   | 5   | 6   | 7   | 8  |
|---|--------------------------|-----------------|----|-----|-----|-----|-----|-----|-----|----|
| 1 | <i>Bulinus africanus</i> | BS01            |    | 100 | 100 | 100 | 100 | 100 | 100 | 97 |
| 2 | <i>Bulinus africanus</i> | AM921970        | 0  |     | 100 | 100 | 100 | 100 | 100 | 97 |
| 3 | <i>Bulinus africanus</i> | BS61            | 0  | 0   |     | 100 | 100 | 100 | 100 | 97 |
| 4 | <i>Bulinus africanus</i> | BS04            | 0  | 0   | 0   |     | 100 | 100 | 100 | 97 |
| 5 | <i>Bulinus africanus</i> | BS66            | 0  | 0   | 0   | 0   |     | 100 | 100 | 97 |
| 6 | <i>Bulinus africanus</i> | BS37            | 0  | 0   | 0   | 0   | 0   |     | 100 | 97 |
| 7 | <i>Bulinus africanus</i> | BS45            | 1  | 1   | 1   | 1   | 1   | 1   |     | 97 |
| 8 | <i>Bulinus globosus</i>  | AM921975        | 13 | 13  | 13  | 13  | 13  | 13  | 12  |    |

Table 3.2: Indicates base pair differences between members of the *Bulinus truncatus/tropicus* group. Above the diagonal are the percentage of identical base pairs, and below the diagonal are base pair differences of the ITS2-region. Genetic sequences generated during this study are coloured orange to match the phylogenetic tree generated (Figure 3.2).

|    | Species name              | Sequence number | 1  | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12 | 13 | 14 | 15 | 16 |
|----|---------------------------|-----------------|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|----|----|----|----|----|
| 1  | <i>Bulinus truncatus</i>  | AM921965        |    | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 99 | 86 | 86 | 86 | 82 |
| 2  | <i>Bulinus depressus</i>  | BS12            | 0  |     | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 99 | 86 | 86 | 86 | 82 |
| 3  | <i>Bulinus depressus</i>  | BS15            | 0  | 0   |     | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 99 | 86 | 86 | 86 | 82 |
| 4  | <i>Bulinus depressus</i>  | BS72            | 0  | 0   | 0   |     | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 99 | 86 | 86 | 86 | 82 |
| 5  | <i>Bulinus depressus</i>  | BS29            | 0  | 0   | 0   | 0   |     | 100 | 100 | 100 | 100 | 100 | 100 | 99 | 86 | 86 | 86 | 82 |
| 6  | <i>Bulinus depressus</i>  | BS69            | 0  | 0   | 0   | 0   | 0   |     | 100 | 100 | 100 | 100 | 100 | 99 | 86 | 86 | 86 | 82 |
| 7  | <i>Bulinus depressus</i>  | BS73            | 0  | 0   | 0   | 0   | 0   | 0   |     | 100 | 100 | 100 | 100 | 99 | 86 | 86 | 86 | 82 |
| 8  | <i>Bulinus depressus</i>  | BS33            | 0  | 0   | 0   | 0   | 0   | 0   | 0   |     | 100 | 100 | 100 | 99 | 86 | 86 | 86 | 82 |
| 9  | <i>Bulinus depressus</i>  | BS50            | 0  | 0   | 0   | 0   | 0   | 0   | 0   | 0   |     | 100 | 100 | 99 | 86 | 86 | 86 | 82 |
| 10 | <i>Bulinus depressus</i>  | BS57            | 0  | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |     | 100 | 99 | 86 | 86 | 86 | 82 |
| 11 | <i>Bulinus depressus</i>  | BS54            | 0  | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |     | 99 | 86 | 86 | 86 | 82 |
| 12 | <i>Bulinus truncatus</i>  | AM921983        | 2  | 2   | 2   | 2   | 2   | 2   | 2   | 2   | 2   | 2   | 2   |    | 86 | 86 | 85 | 81 |
| 13 | <i>Bulinus natalensis</i> | AM921976        | 60 | 60  | 60  | 60  | 60  | 60  | 60  | 60  | 60  | 60  | 60  | 62 |    | 99 | 98 | 88 |
| 14 | <i>Bulinus tropicus</i>   | MT707224        | 59 | 59  | 59  | 59  | 59  | 59  | 59  | 59  | 59  | 59  | 59  | 61 | 5  |    | 99 | 89 |
| 15 | <i>Bulinus tropicus</i>   | MT707227        | 61 | 61  | 61  | 61  | 61  | 61  | 61  | 61  | 61  | 61  | 61  | 63 | 8  | 3  |    | 88 |
| 16 | <i>Bulinus tropicus</i>   | MT707228        | 79 | 79  | 79  | 79  | 79  | 79  | 79  | 79  | 79  | 79  | 79  | 81 | 51 | 48 | 51 |    |

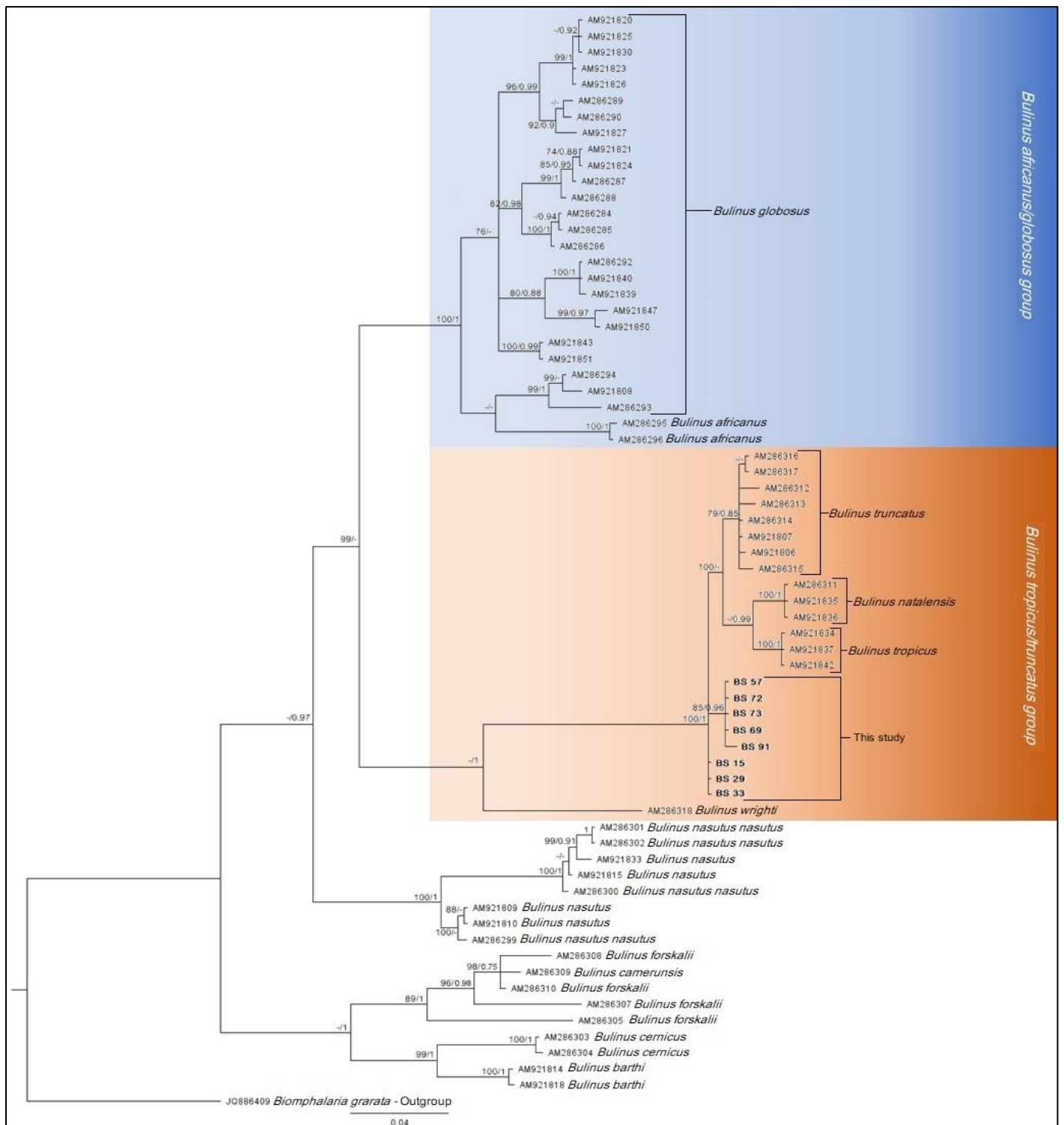


Figure 3.3 Bayesian inference phylogram of *Bulinus* genus based on partial sequences of the COI mtDNA gene. New sequences are in bold. Posterior probability followed by bootstrap support values is indicated next to the branches (posterior probability < 70 and bootstrap < 0.80 not shown). *Biomphalaria grarata* was used as an outgroup. The branch length scale bar indicates the number of substitutions per site. Sequences generated during the current study are represented in bold.

Table 3.3: Table indicating base pair differences between members of the *Bulinus truncatus/tropicus* group and closest neighbouring branches of the phylogenetic tree (Figure 3.3). Above the diagonal is the percentage of identical base pairs, and below the diagonal are base pair differences of the COI-gene. Genetic sequences generated during this study are coloured orange to match the phylogenetic tree generated.

|    | Species name              | Sequence number | 1  | 2   | 3   | 4  | 5   | 6   | 7  | 8  | 9  | 10  | 11  | 12  | 13  | 14 | 15 | 16  | 17  | 18 | 19  | 20  | 21  | 22  |
|----|---------------------------|-----------------|----|-----|-----|----|-----|-----|----|----|----|-----|-----|-----|-----|----|----|-----|-----|----|-----|-----|-----|-----|
| 1  | <i>Bulinus natalensis</i> | AM286311        |    | 100 | 100 | 98 | 98  | 98  | 96 | 97 | 97 | 97  | 97  | 97  | 97  | 97 | 97 | 97  | 97  | 97 | 97  | 97  | 97  | 97  |
| 2  | <i>Bulinus natalensis</i> | AM921835        | 0  |     | 100 | 98 | 98  | 98  | 96 | 97 | 97 | 97  | 97  | 97  | 97  | 97 | 97 | 97  | 97  | 97 | 97  | 97  | 97  | 97  |
| 3  | <i>Bulinus natalensis</i> | AM921836        | 0  | 0   |     | 98 | 98  | 98  | 96 | 97 | 97 | 97  | 97  | 97  | 97  | 97 | 97 | 97  | 97  | 97 | 97  | 97  | 97  | 97  |
| 4  | <i>Bulinus tropicus</i>   | AM921834        | 11 | 11  | 11  |    | 100 | 100 | 97 | 97 | 97 | 97  | 97  | 97  | 97  | 97 | 98 | 98  | 98  | 97 | 97  | 97  | 97  | 97  |
| 5  | <i>Bulinus tropicus</i>   | AM921837        | 11 | 11  | 11  | 0  |     | 100 | 97 | 97 | 97 | 97  | 97  | 97  | 97  | 97 | 98 | 98  | 98  | 97 | 97  | 97  | 97  | 97  |
| 6  | <i>Bulinus tropicus</i>   | AM921842        | 11 | 11  | 11  | 0  | 0   |     | 97 | 97 | 97 | 97  | 97  | 97  | 97  | 97 | 98 | 98  | 98  | 97 | 97  | 97  | 97  | 97  |
| 7  | <i>Bulinus truncatus</i>  | AM286312        | 19 | 19  | 19  | 15 | 15  | 15  |    | 99 | 99 | 99  | 99  | 99  | 99  | 99 | 98 | 98  | 98  | 98 | 98  | 98  | 98  | 98  |
| 8  | <i>Bulinus truncatus</i>  | AM286313        | 18 | 18  | 18  | 16 | 16  | 16  | 7  |    | 99 | 99  | 99  | 99  | 99  | 99 | 98 | 98  | 98  | 98 | 98  | 98  | 98  | 98  |
| 9  | <i>Bulinus truncatus</i>  | AM286314        | 14 | 14  | 14  | 14 | 14  | 14  | 5  | 4  |    | 100 | 100 | 100 | 100 | 99 | 99 | 99  | 99  | 99 | 99  | 99  | 99  | 98  |
| 10 | <i>Bulinus truncatus</i>  | AM921807        | 14 | 14  | 14  | 14 | 14  | 14  | 5  | 4  | 0  |     | 100 | 100 | 100 | 99 | 99 | 99  | 99  | 99 | 99  | 99  | 99  | 98  |
| 11 | <i>Bulinus truncatus</i>  | AM286316        | 15 | 15  | 15  | 14 | 14  | 14  | 6  | 5  | 1  | 1   |     | 100 | 100 | 99 | 99 | 99  | 99  | 99 | 99  | 99  | 99  | 98  |
| 12 | <i>Bulinus truncatus</i>  | AM286317        | 15 | 15  | 15  | 14 | 14  | 14  | 6  | 5  | 1  | 1   | 0   |     | 100 | 99 | 99 | 99  | 99  | 99 | 99  | 99  | 99  | 98  |
| 13 | <i>Bulinus truncatus</i>  | AM921806        | 15 | 15  | 15  | 15 | 15  | 15  | 4  | 5  | 1  | 1   | 2   | 2   |     | 99 | 99 | 99  | 99  | 99 | 99  | 99  | 99  | 99  |
| 14 | <i>Bulinus truncatus</i>  | AM286315        | 18 | 18  | 18  | 15 | 15  | 15  | 7  | 6  | 4  | 4   | 3   | 3   | 5   |    | 98 | 98  | 98  | 98 | 98  | 98  | 98  | 98  |
| 15 | <i>Bulinus depressus</i>  | BS15            | 14 | 14  | 14  | 13 | 13  | 13  | 10 | 9  | 7  | 7   | 8   | 8   | 8   | 9  |    | 100 | 100 | 99 | 99  | 99  | 99  | 99  |
| 16 | <i>Bulinus depressus</i>  | BS29            | 14 | 14  | 14  | 13 | 13  | 13  | 10 | 9  | 7  | 7   | 8   | 8   | 8   | 9  | 0  |     | 100 | 99 | 99  | 99  | 99  | 99  |
| 17 | <i>Bulinus depressus</i>  | BS33            | 14 | 14  | 14  | 13 | 13  | 13  | 10 | 9  | 7  | 7   | 8   | 8   | 8   | 9  | 0  | 0   |     | 99 | 99  | 99  | 99  | 99  |
| 18 | <i>Bulinus depressus</i>  | BS57            | 13 | 13  | 13  | 14 | 14  | 14  | 11 | 10 | 6  | 6   | 7   | 7   | 7   | 10 | 4  | 4   | 4   |    | 100 | 100 | 100 | 100 |
| 19 | <i>Bulinus depressus</i>  | BS72            | 13 | 13  | 13  | 14 | 14  | 14  | 11 | 10 | 6  | 6   | 7   | 7   | 7   | 10 | 4  | 4   | 4   | 0  |     | 100 | 100 | 100 |
| 20 | <i>Bulinus depressus</i>  | BS73            | 13 | 13  | 13  | 14 | 14  | 14  | 11 | 10 | 6  | 6   | 7   | 7   | 7   | 10 | 4  | 4   | 4   | 0  | 0   |     | 100 | 100 |
| 21 | <i>Bulinus depressus</i>  | BS69            | 14 | 14  | 14  | 15 | 15  | 15  | 12 | 11 | 7  | 7   | 8   | 8   | 8   | 11 | 4  | 4   | 4   | 0  | 0   | 0   |     | 100 |
| 22 | <i>Bulinus depressus</i>  | BS91            | 16 | 16  | 16  | 15 | 15  | 15  | 10 | 13 | 9  | 9   | 10  | 10  | 8   | 13 | 6  | 6   | 6   | 2  | 2   | 2   | 2   |     |

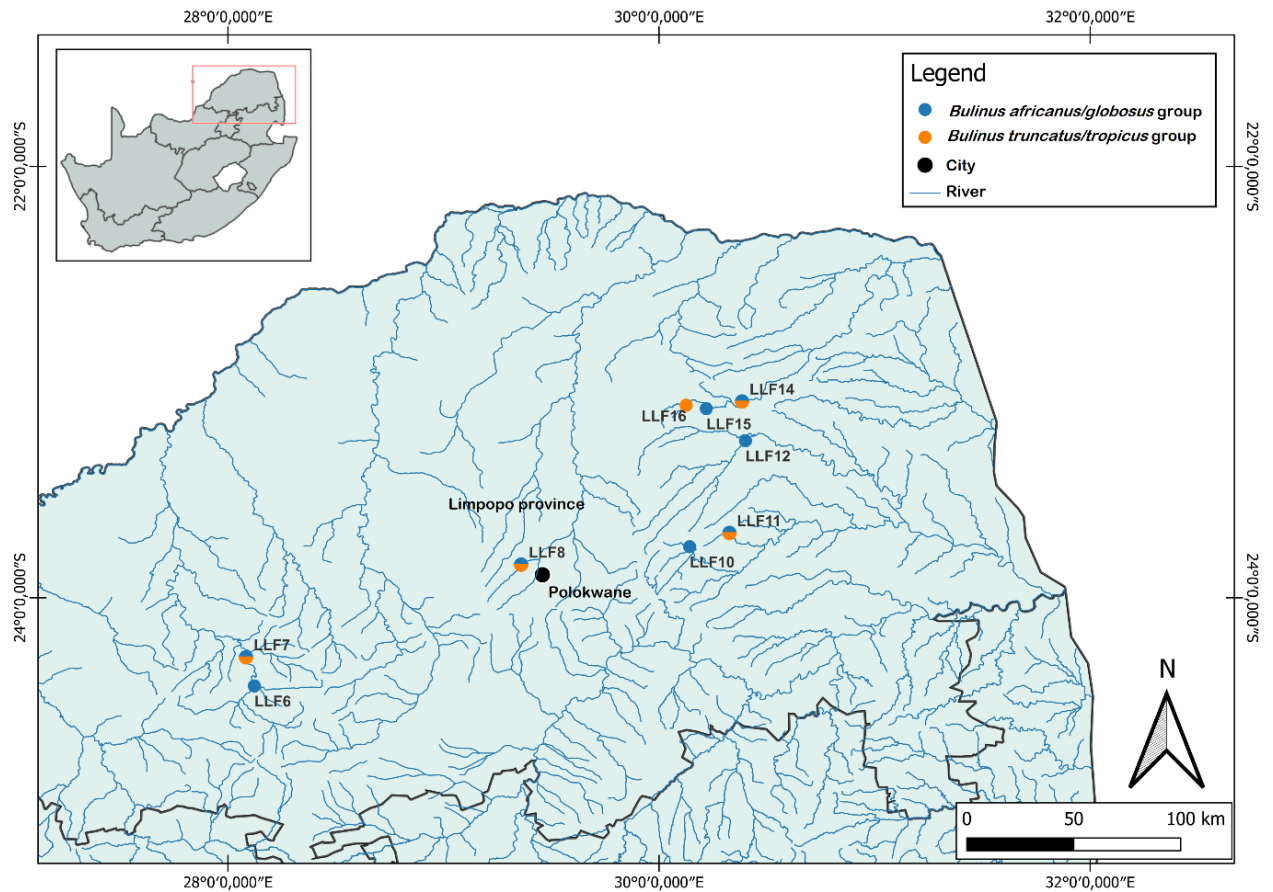


Figure 3.4: Map indicating locations where *Bulinus* specimens were collected and molecularly identified. *Bulinus africanus/globosus* group is indicated with a blue circle on the map, and *B. truncatus/tropicus* group is indicated with an orange circle. Sites where both groups were collected are indicated with a blue and orange circle.

### 3.5.2 Planorbidae

A total of 294 specimens were collected in this study and were morphologically identified, based on their shell morphology, as members of the Planorbidae family. Seven species representing the Planorbidae genera are known from southern Africa (Day & de Moor, 2002). Specimens collected during this study were identified as species of two of these, the *Biomphalaria* and *Gyraulus*. Originally, based on morphological characteristics, the specimens were identified in the field as *Biomphalaria pfeifferi* based on the circular shape of the umbilicated shell and the even rounded basal whorl (Figure 3.5; A-B). A second species of *Biomphalaria*, *Biomphalaria grablata*, was identified morphologically in the field. However, in the laboratory, all these specimens were again analysed and re-identified as *Gyraulus costulatus*. The morphological similarities of *B. grablata* and *G. costulatus* resulted in the mistakenly identified specimens since both species have an angular basal whorl and are almost similar in size. Further identification under a dissection microscope confirmed the identification of *G. costulatus* with the observation of strong, regularly spaced transverse ribs (Figure 3.5; C-D). The third Planorbidae species collected was identified as a member of the *Gyraulus* genus in the field and confirmed to be *G. connollyi* (Figure 3.5; E-F) in the laboratory by observing the presence of fine growth lines, the rounded shoulder of the basal whorl and the aperture deflecting downwards that are characteristic of this species.

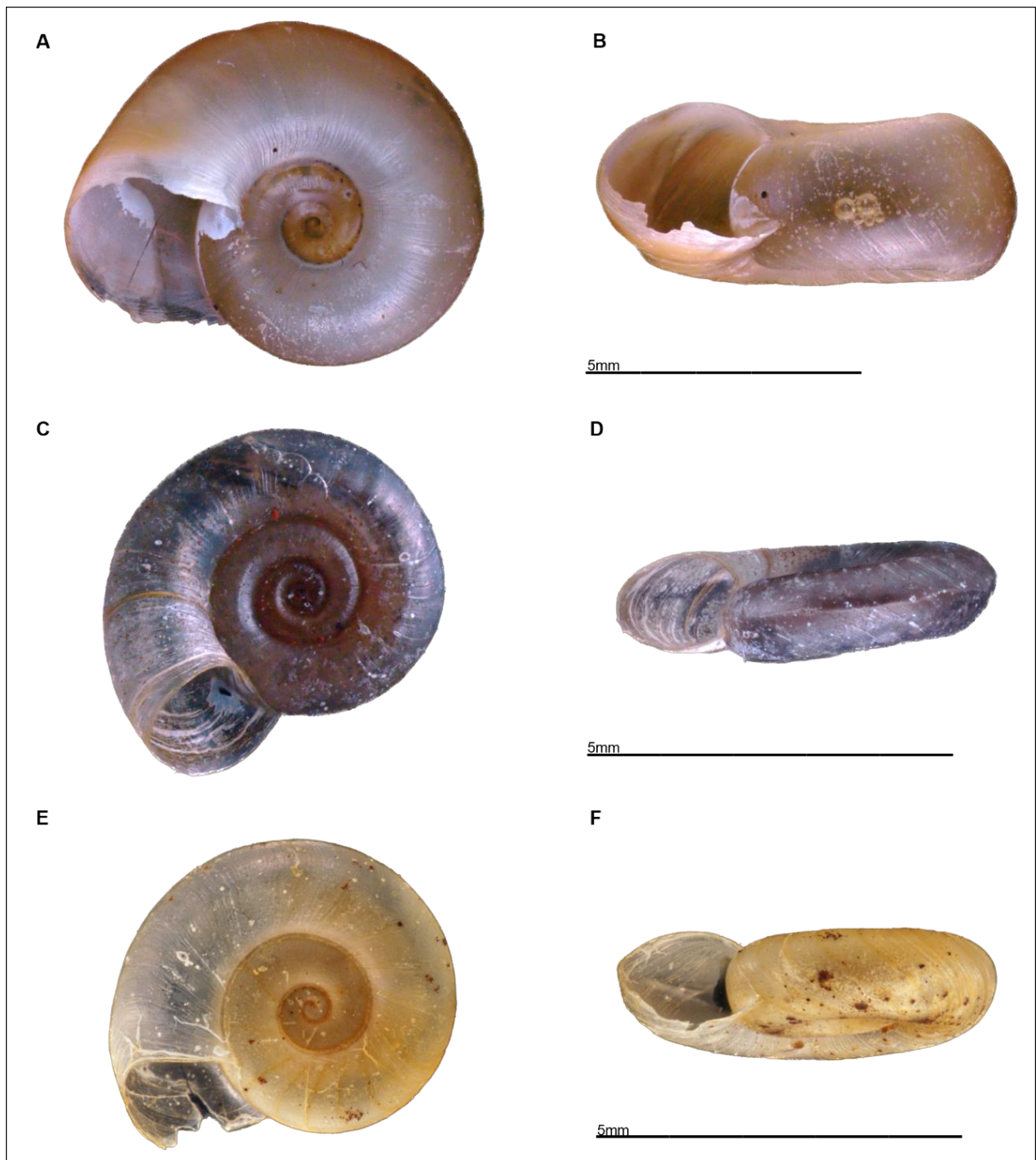


Figure 3.5: Planorbidae specimens of *Biomphalaria* and *Gyraulus* species collected during the current study. Morphologically identified as *Biomphalaria pfeifferi* (A-B). (A) The ventral view of *Biomphalaria pfeifferi* and (B) The dorsal view. (C-D) Identified as *Gyraulus costulatus*. (C) the ventral view of *Gyraulus costulatus* and (D) the dorsal view. (E-F) Identified as *Gyraulus connollyi*. (E) The ventral view of *G. connollyi* and (F) the dorsal view. (A-F) Scale bar = 5 mm.

The molecular analyses resulted in 12 novel sequences for these species of Planorbidae. Phylogenetic trees were generated using sequences of ITS2 (544 bp; Figure 4.5) and COI (527 bp; Figure 4.6) genetic regions. The ITS2-rDNA gene phylogenetic tree (Figure 3.6) indicated the five novel sequences morphologically identified to belong to *B. pfeifferi* were confirmed by the phylogenetic analyses with good nodal support. The base pair differences (Table 3.4) between the sequence generated during this study and the sequences of *B. pfeifferi* published on GenBank, indicated zero to one base pair difference. The interspecific variation for *Biomphalaria* species in this dataset is 0% (0–1 nt).

*Gyraulus* sp. (BP11 and BP19) collected in the North West province, South Africa, grouped with good support with *G. convexiusculus*. The identification of *G. convexiusculus* is improbable as this species is primarily found in the Middle East and, to date, has not been recorded in South Africa or the African continent. Thus, indicating a close evolutionary relationship between these two species. The base pair differences (Table 3.5) between the sequence generated during this study and the sequences of *Gyraulus* published on GenBank, indicated 10 to 11 base pair differences. This dataset's interspecific variation for *Gyraulus* species is 0-2% (10-11 nt). No sequences of the ITS2 region were available on GenBank (NCBI, <https://blast.ncbi.nlm.nih.gov/Blast.cgi>) for *G. costulatus* to compare the sequences generated during this study. Samples collected in the Seekoeivlei Nature Reserve were only used for COI-genetic analyses.

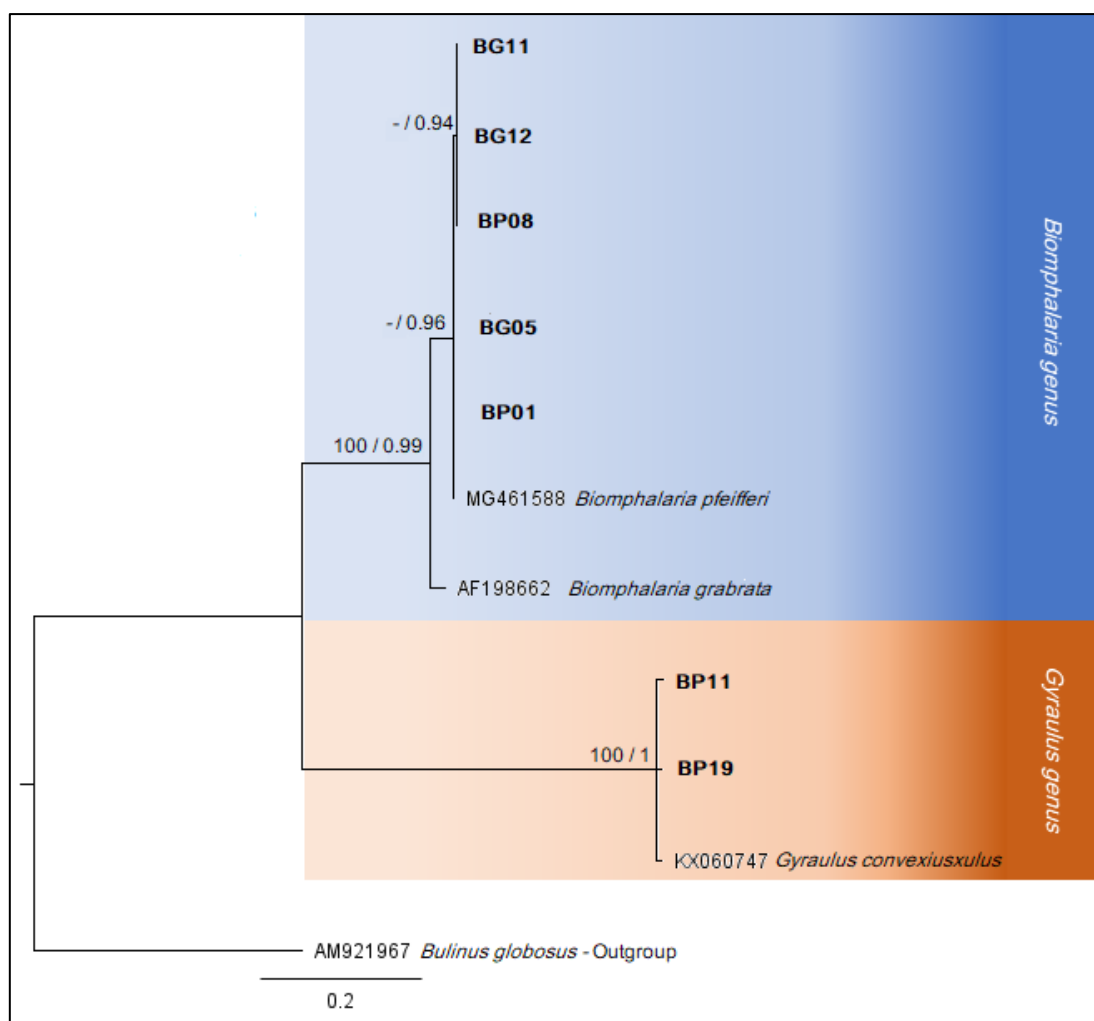


Figure 3.6: Bayesian inference phylogram of species of the genus *Biomphalaria* and *Gyraulus* based on partial sequences of the ITS2 rDNA region. New sequences are in bold. Posterior probability followed by bootstrap support values is indicated next to the branches (posterior probability < 70 and bootstrap < 0.80 not shown). *Bulinus africanus* was used as an outgroup. The branch length scale bar indicates the number of substitutions per site. Sequences generated during the current study are represented in bold.

Table 3.4: Table indicating base pair differences between members of the *Biomphalaria pfeifferi*. Above the diagonal are the percentage of identical base pairs, and below the diagonal are base pair differences of the ITS2 region. Genetic sequences generated during this study are coloured blue to match the phylogenetic tree generated (Figure 3.6).

|   | Species name                  | Sequence number | 1  | 2   | 3   | 4   | 5   | 6   | 7  |
|---|-------------------------------|-----------------|----|-----|-----|-----|-----|-----|----|
| 1 | <i>Biomphalaria pfeifferi</i> | MG461588        |    | 100 | 100 | 100 | 100 | 100 | 93 |
| 2 | <i>Biomphalaria pfeifferi</i> | BP08            | 1  |     | 100 | 100 | 100 | 100 | 93 |
| 3 | <i>Biomphalaria pfeifferi</i> | BG12            | 1  | 0   |     | 100 | 100 | 100 | 93 |
| 4 | <i>Biomphalaria pfeifferi</i> | BG11            | 1  | 0   | 0   |     | 100 | 100 | 93 |
| 5 | <i>Biomphalaria pfeifferi</i> | BG05            | 0  | 1   | 1   | 1   |     | 100 | 93 |
| 6 | <i>Biomphalaria pfeifferi</i> | BP01            | 0  | 1   | 1   | 1   | 0   |     | 93 |
| 7 | <i>Biomphalaria glabrata</i>  | AF198662        | 29 | 30  | 30  | 30  | 29  | 29  |    |

Table 3.5: Table indicating base pair differences between members of the *Gyraulus* species. Above the diagonal are the percentage of identical base pairs, and below the diagonal are base pair differences of the ITS2 region. Genetic sequences generated during this study are coloured orange to match the phylogenetic tree generated (Figure 3.6).

|   | Species name                   | Sequence number | 1  | 2  | 3  |
|---|--------------------------------|-----------------|----|----|----|
| 1 | <i>Gyraulus costulatus</i>     | BP11            |    | 99 | 98 |
| 2 | <i>Gyraulus costulatus</i>     | BP19            | 6  |    | 98 |
| 3 | <i>Gyraulus convexiusculus</i> | KX060747        | 11 | 10 |    |

The phylogenetic tree for the COI gene (Figure 3.7) supports the morphological identification of *Biomphalaria pfeifferi* with the base pair differences (Table 3.6) between the sequence generated during this study and the sequences of *B. pfeifferi* published on GenBank, indicated one to six base pair differences. This dataset's interspecific variation for *Biomphalaria* species is 0-7% (1-7 nt). Molecular analysis in the case of *Gyraulus* was only able to positively confirm these specimens to genus level and confirm that two different species are present. The two species of *Gyraulus* collected during the current study indicated considerable interspecific variation based on the base pair differences and difference in percentage identity (Table 3.7) between species collected in the North West province (BP19) separated phylogenetically from the specimens (SX1,15,17, JX12) collected in the Seekoeivlei Nature Reserve within the Free State province, South Africa. The samples collected at the Seekoeivlei Nature Reserve grouped phylogenetically with a published sample of *G. chinensis* (AF199086). As there are no available sequences of these two species in GenBank and the morphological identification confirmed (Figure 3.5) them as *G. costulatus* and *G. connollyi*, respectively, this study constitutes the first genetic data for these two species.

The distribution of *B. pfeifferi* (Figure 3.8) is expected in the north-eastern parts of South Africa, as confirmed during the current study. *Gyraulus costulatus* was collected at the sites sampled in the Limpopo province of South Africa (Figure 3.8), and *G. connollyi* was identified at sites sampled at the Seekoeivlei Nature Reserve within the Free State province of South Africa (Figure 3.8).

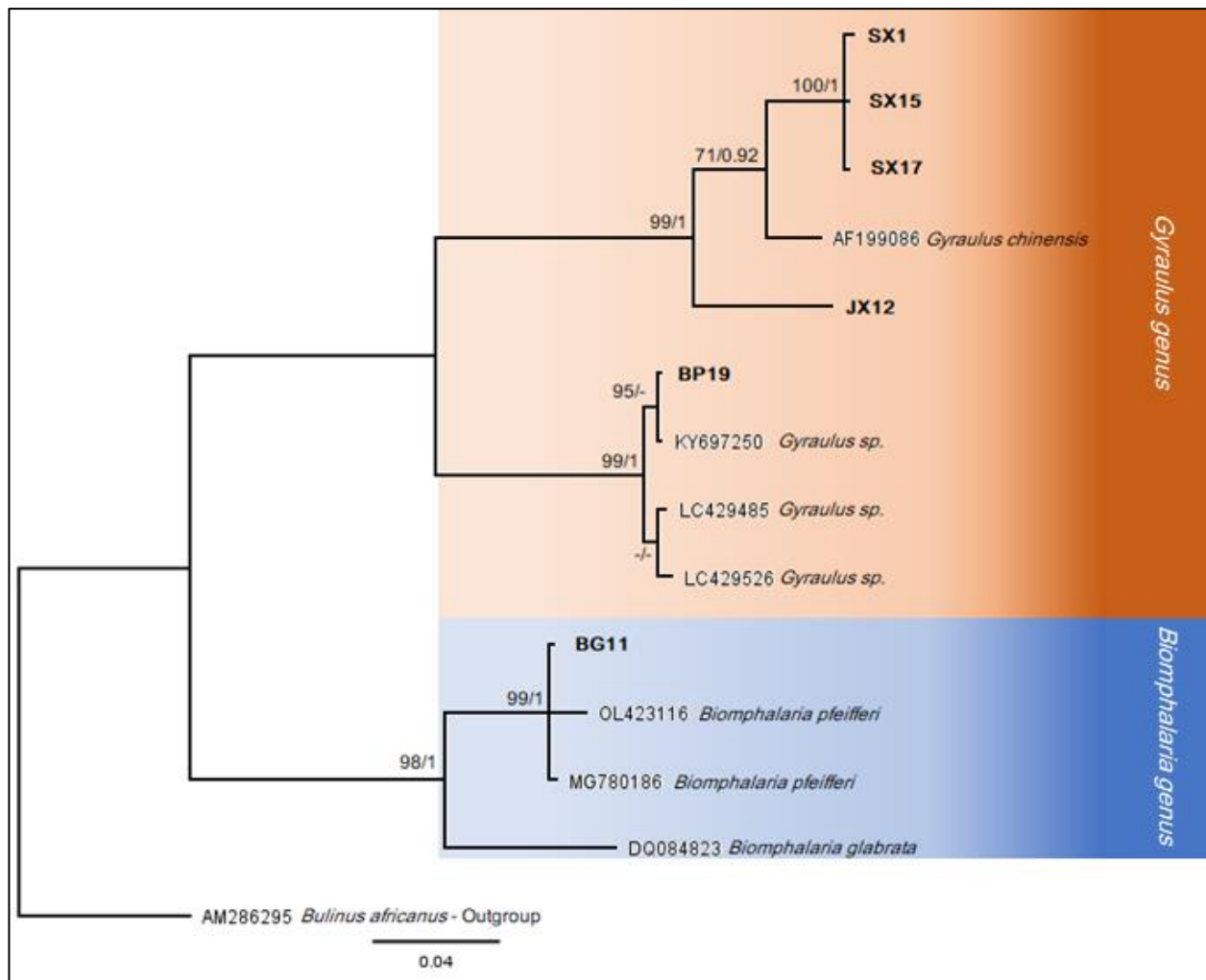


Figure 3.7: Bayesian inference phylogram of species of *Biomphalaria* and *Gyraulus* based on partial sequences of the COI mtDNA gene. New sequences are in bold. Posterior probability followed by bootstrap support values is indicated next to the branches (posterior probability < 70 and bootstrap < 0.80 not shown). *Bulinus globosus* was used as an outgroup. The branch length scale bar indicates the number of substitutions per site. Sequences generated during the current study are represented in bold.

Table 3.6: Table indicating base pair differences between members of the *Biomphalaria* above the diagonal are the percentage identical base pairs, and below the diagonal are base pair differences of the COI-gene. Genetic sequences generated during this study are coloured blue to match the phylogenetic tree generated (Figure 3.7).

|   | Species name                  | Sequence number | 1  | 2  | 3   | 4  |
|---|-------------------------------|-----------------|----|----|-----|----|
| 1 | <i>Biomphalaria pfeifferi</i> | BG11            |    | 99 | 100 | 93 |
| 2 | <i>Biomphalaria pfeifferi</i> | OL423116        | 6  |    | 99  | 93 |
| 3 | <i>Biomphalaria pfeifferi</i> | MG780186        | 1  | 7  |     | 93 |
| 4 | <i>Biomphalaria glabrata</i>  | DQ084823        | 37 | 39 | 36  |    |

Table 3.7: Table indicating base pair differences between members of the *Gyraulus* above the diagonal are the percentage identical base pairs, and below the diagonal are base pair differences of the COI-gene. Genetic sequences generated during this study are coloured orange to match the phylogenetic tree generated (Figure 3.7).

|   | Species name               | Sequence number | 1  | 2   | 3   | 4  | 5  | 6  | 7  | 8   | 9  |
|---|----------------------------|-----------------|----|-----|-----|----|----|----|----|-----|----|
| 1 | <i>Gyraulus connollyi</i>  | SX1             |    | 100 | 100 | 93 | 96 | 87 | 87 | 87  | 88 |
| 2 | <i>Gyraulus connollyi</i>  | SX15            | 1  |     | 100 | 93 | 96 | 88 | 88 | 88  | 88 |
| 3 | <i>Gyraulus connollyi</i>  | SX17            | 1  | 0   |     | 93 | 96 | 88 | 88 | 88  | 88 |
| 4 | <i>Gyraulus connollyi</i>  | JX12            | 37 | 36  | 36  |    | 93 | 87 | 87 | 87  | 87 |
| 5 | <i>Gyraulus chinensis</i>  | AF199086        | 20 | 19  | 19  | 36 |    | 88 | 88 | 88  | 88 |
| 6 | <i>Gyraulus</i> sp.        | KY697250        | 66 | 65  | 65  | 68 | 63 |    | 99 | 100 | 99 |
| 7 | <i>Gyraulus</i> sp.        | LC429485        | 66 | 65  | 65  | 68 | 64 | 3  |    | 99  | 99 |
| 8 | <i>Gyraulus costulatus</i> | BP19            | 66 | 65  | 65  | 68 | 63 | 0  | 3  |     | 99 |
| 9 | <i>Gyraulus</i> sp.        | LC429526        | 65 | 64  | 64  | 67 | 62 | 4  | 3  | 4   |    |

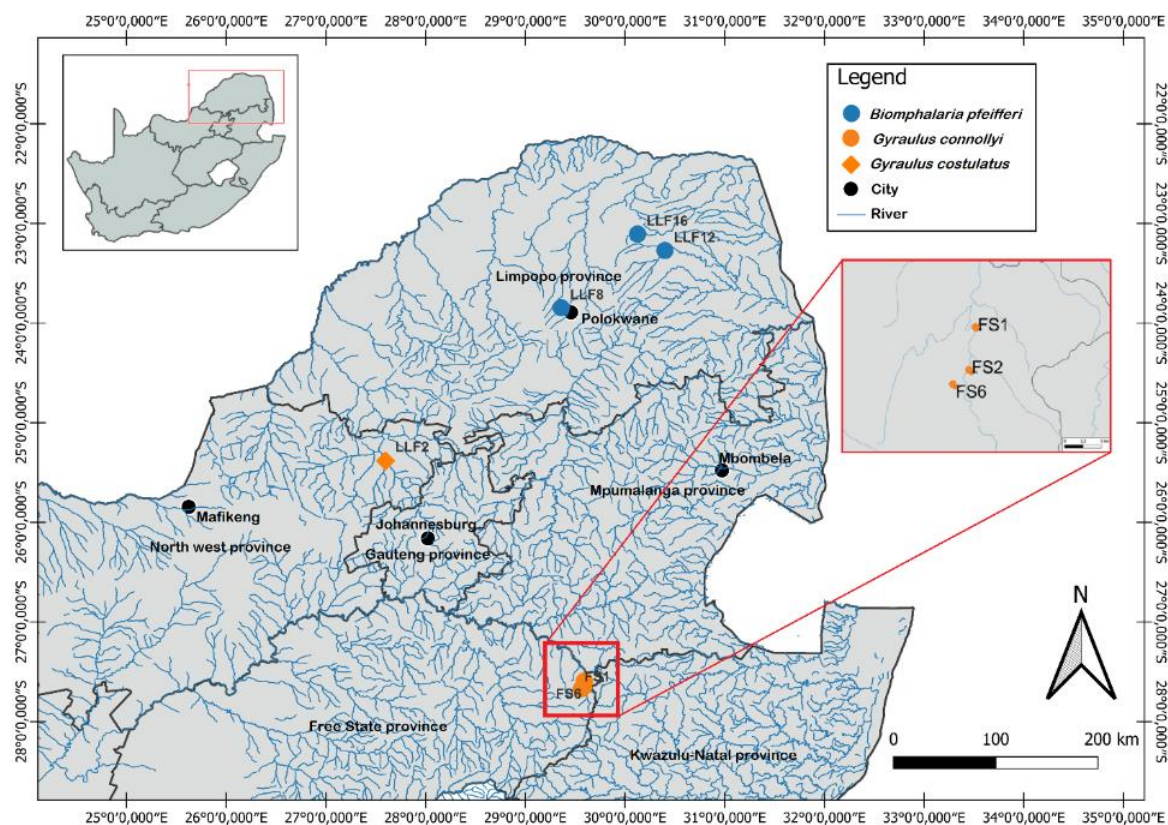


Figure 3.8: Map indicating sites where specimens belonging to the *Biomphalaria pfeifferi* (indicated by the blue circles), *Gyraulus connollyi* (indicated by the orange circles) and *Gyraulus costulatus* (indicated by the orange diamond-shaped icon). The area within the red square is the site sampled within the Seekoeivlei Nature Reserve. Enlargement of the area within the red square indicates the three sites sampled within Seekoeivlei Nature Reserve, where specimens of *G. connollyi* were collected.

### 3.5.3 Lymnaeidae

In total 264 molluscan specimens were collected from 13 sites in South Africa's Limpopo and North West provinces and easily morphologically identified as members of the Lymnaeidae family. Specimens were morphologically identified to be species of *Radix* and *Pseudosuccinea*. A total of 221 of the specimens were morphologically identified to be *Radix natalensis* based on the remarkably swollen basal whorl and the distinctly visible growth lines. The remaining 43 specimens were morphologically identified as *Pseudosuccinea columella* based on the swollen basal whorl, but less swollen than the basal whorl of *R. natalensis* and the presence of growth and ridge lines.

The molecular analyses resulted in 36 novel sequences of *Radix natalensis* and *Pseudosuccinea columella*. Phylogenetic trees were generated using sequences of ITS2 (477 bp; Figure 3.9) and COI genetic regions (426 bp; Figure 3.10).

The ITS2 analyses confirmed the morphological identification of *R. natalensis* with good nodal support (Figure 3.9). The base pair differences (Table 3.8) between the sequence generated during this study and the sequences of *R. natalensis* published on GenBank, indicated zero to two base pair differences, except for specimens RN58 and RN02 collected at sites LLF14 and LLF04 during the current study. The sequences showed a 16 to 18 base pair difference between all of the *R. natalensis* sequences published, as well as the sequences generated during the current study when compared. However, the phylogenetic tree generated (Figure 3.9) visually indicates that these two specimens clade with the *R. natalensis* species. This dataset's interspecific variation for *Radix* species is 6% (0–18 nt).

The genetic analyses of the ITS2 region confirmed the identification of *Pseudosuccinea columella* clades with good nodal support (Figure 3.9). The base pair differences (Table 3.9) between the sequence generated during this study and the sequences of *P. columella* published on GenBank, indicated mostly zero base pair difference, with one exception of one sequence (JN614470) collected at the Rio Negro Colombia by Correa *et al.*, (2011), that indicated a three base pair difference from the other sequences in the dataset. The interspecific variation for *Pseudosuccinea* species in this dataset is 1% (0–3 nt).

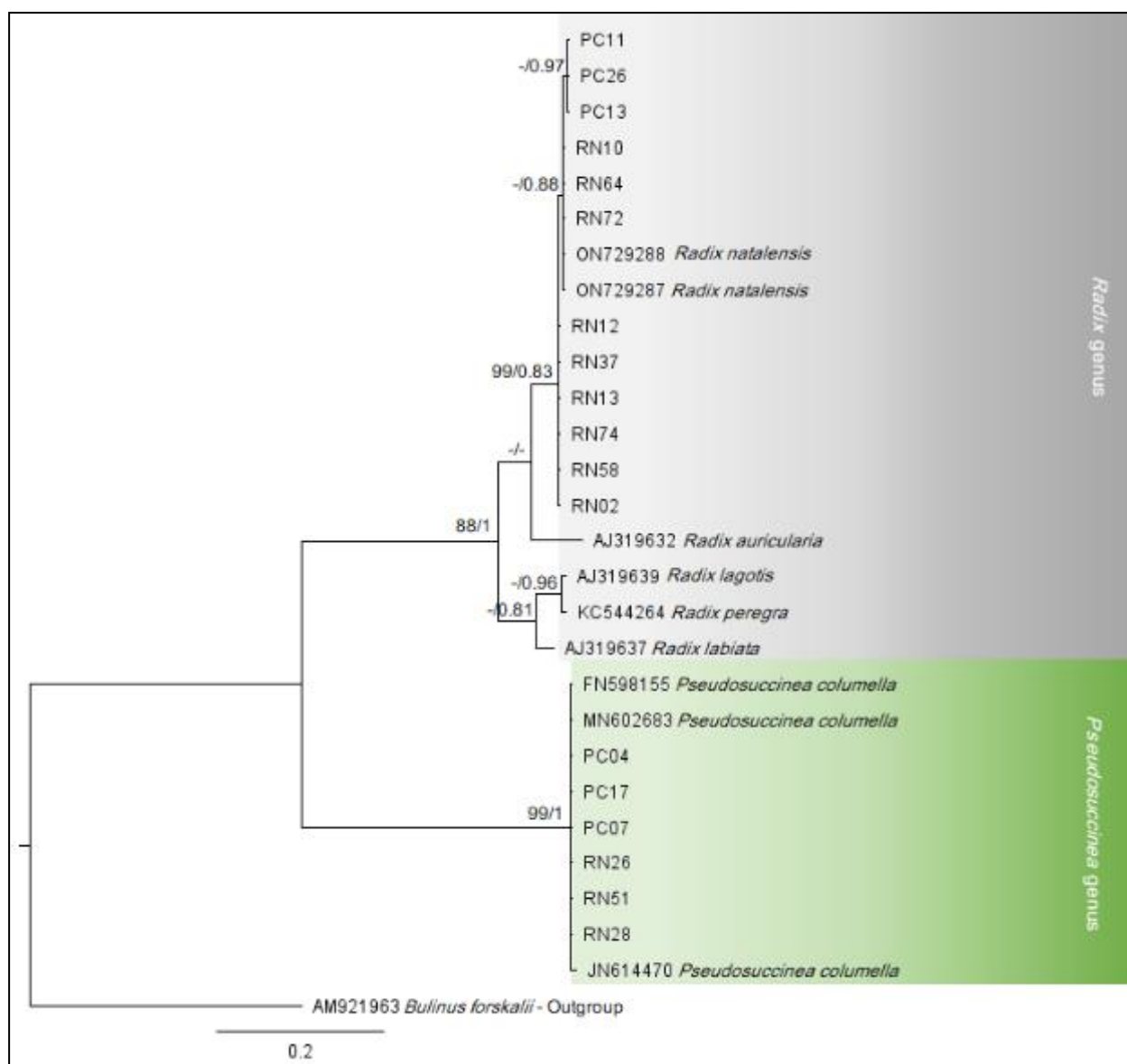


Figure 3.9: Bayesian inference phylogram of species of *Radix* and *Pseudosuccinea* based on partial sequences of the ITS2 region. New sequences are ladled PC and RN. Posterior probability followed by bootstrap support values is indicated next to the branches (posterior probability < 70 and bootstrap < 0.80 not shown). *Bulinus forskalii* was used as an outgroup. The branch length scale bar indicates the number of substitutions per site.

Table 3.8: Table indicating base pair differences between members of the *Radix*. Above the diagonal are the percentage of identical base pairs, and below the diagonal are base pair differences of the ITS2 region. Genetic sequences generated during this study are coloured grey to match the phylogenetic tree generated (Figure 3.9).

|    | Species name            | Sequence number | 1  | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11 | 12  | 13  | 14  |
|----|-------------------------|-----------------|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|----|-----|-----|-----|
| 1  | <i>Radix natalensis</i> | RN10            |    | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 95 | 95  | 100 | 100 |
| 2  | <i>Radix natalensis</i> | RN64            | 0  |     | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 95 | 95  | 100 | 100 |
| 3  | <i>Radix natalensis</i> | PC11            | 1  | 1   |     | 100 | 99  | 99  | 99  | 99  | 100 | 100 | 94 | 94  | 100 | 99  |
| 4  | <i>Radix natalensis</i> | PC26            | 1  | 1   | 0   |     | 99  | 99  | 99  | 99  | 100 | 100 | 94 | 94  | 100 | 99  |
| 5  | <i>Radix natalensis</i> | RN12            | 1  | 1   | 2   | 2   |     | 100 | 100 | 100 | 99  | 100 | 95 | 95  | 100 | 99  |
| 6  | <i>Radix natalensis</i> | RN37            | 1  | 1   | 2   | 2   | 0   |     | 100 | 100 | 99  | 100 | 95 | 95  | 100 | 99  |
| 7  | <i>Radix natalensis</i> | RN13            | 1  | 1   | 2   | 2   | 0   | 0   |     | 100 | 99  | 100 | 95 | 95  | 100 | 99  |
| 8  | <i>Radix natalensis</i> | RN74            | 1  | 1   | 2   | 2   | 0   | 0   | 0   |     | 99  | 100 | 95 | 95  | 100 | 99  |
| 9  | <i>Radix natalensis</i> | PC13            | 1  | 1   | 0   | 0   | 2   | 2   | 2   | 2   |     | 100 | 94 | 94  | 100 | 99  |
| 10 | <i>Radix natalensis</i> | RN72            | 0  | 0   | 1   | 1   | 1   | 1   | 1   | 1   | 1   |     | 95 | 95  | 100 | 100 |
| 11 | <i>Radix natalensis</i> | RN58            | 17 | 17  | 18  | 18  | 16  | 16  | 16  | 16  | 18  | 17  |    | 100 | 95  | 94  |
| 12 | <i>Radix natalensis</i> | RN02            | 17 | 17  | 18  | 18  | 16  | 16  | 16  | 16  | 18  | 17  | 0  |     | 95  | 94  |
| 13 | <i>Radix natalensis</i> | ON729288        | 0  | 0   | 1   | 1   | 1   | 1   | 1   | 1   | 1   | 0   | 17 | 17  |     | 100 |
| 14 | <i>Radix natalensis</i> | ON729287        | 1  | 1   | 2   | 2   | 2   | 2   | 2   | 2   | 2   | 1   | 18 | 18  | 1   |     |

Table 3.9: Table indicating base pair differences between members of the *Pseudosuccinea* above the diagonal are the percentage identical base pairs, and below the diagonal are base pair differences of the ITS2 region. Genetic sequences generated during this study are coloured green to match the phylogenetic tree generated (Figure 3.9).

|   | Species name                    | Sequence number | 1 | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9  |
|---|---------------------------------|-----------------|---|-----|-----|-----|-----|-----|-----|-----|----|
| 1 | <i>Pseudosuccinea columella</i> | FN598155        |   | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 99 |
| 2 | <i>Pseudosuccinea columella</i> | MN602683        | 0 |     | 100 | 100 | 100 | 100 | 100 | 100 | 99 |
| 3 | <i>Pseudosuccinea columella</i> | PC04            | 0 | 0   |     | 100 | 100 | 100 | 100 | 100 | 99 |
| 4 | <i>Pseudosuccinea columella</i> | PC17            | 0 | 0   | 0   |     | 100 | 100 | 100 | 100 | 99 |
| 5 | <i>Pseudosuccinea columella</i> | PC07            | 0 | 0   | 0   | 0   |     | 100 | 100 | 100 | 99 |
| 6 | <i>Pseudosuccinea columella</i> | RN26            | 0 | 0   | 0   | 0   | 0   |     | 100 | 100 | 99 |
| 7 | <i>Pseudosuccinea columella</i> | RN51            | 0 | 0   | 0   | 0   | 0   | 0   |     | 100 | 99 |
| 8 | <i>Pseudosuccinea columella</i> | RN28            | 0 | 0   | 0   | 0   | 0   | 0   | 0   |     | 99 |
| 9 | <i>Pseudosuccinea columella</i> | JN614470        | 3 | 3   | 3   | 3   | 3   | 3   | 3   | 3   |    |

The COI genetic analyses of *R. natalensis* indicated that the species phylogenetically were grouped into four monophyletic clades within the *R. natalensis* species group. Genetically the base pair variation between COI-gene of the novel sequences was limited, with only 2-17 base pair differences between *R. natalensis* sequences (Table 3.10). The groups identified by the phylogenetic analyses were geographically isolated (Figure 3.11), and the sites are colour coded to relate to Table 3.10. The locations where different monophyletic clades were collected are separated from each other, as seen on the map (Figure 3.11), and the sites where the same monophyletic clades were collected are not in close proximity. The only location with an overlap of species is site LLF13 that contained species of both groups 2 and 3.

The COI-genetic analyses confirmed the identification of *P. columella*. It indicated no genetic variance between *P. columella* specimens based on the *Pseudosuccinea* dataset, with all sequences matching 100% with each other and zero base pair differences. The collection of *P. columella* was mainly in the Limpopo province of South Africa, as indicated in Figure 3.12.

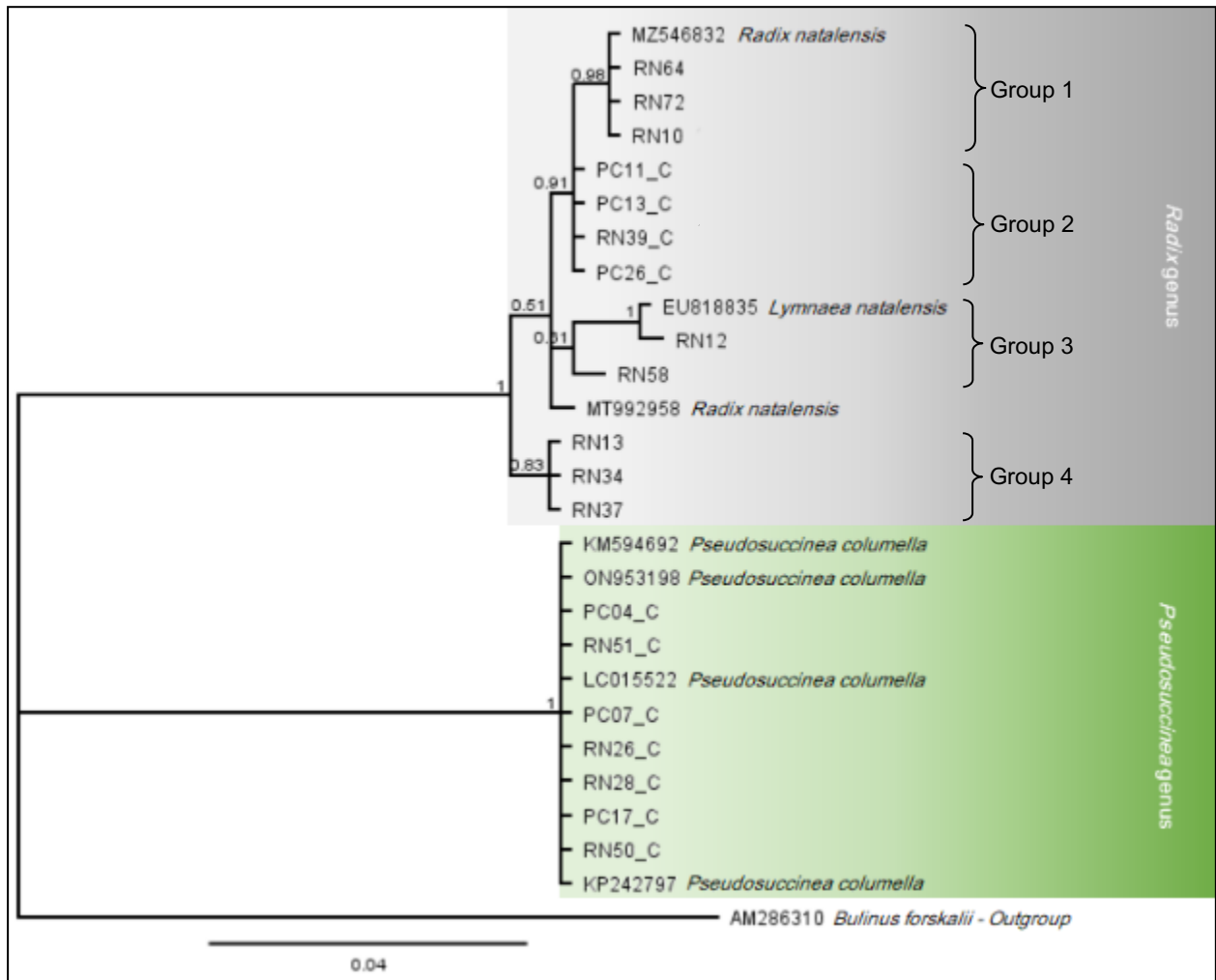


Figure 3.10: Bayesian inference phylogram of species of *Radix* and *Pseudosuccinea* based on partial sequences of the COI mtDNA gene. New sequences are labelled PC and RN. Posterior probability followed by bootstrap support values are indicated next to the branches (posterior probability < 70 and bootstrap < 0.80 not shown). *Bulinus forskalii* was used as an outgroup. The branch length scale bar indicates the number of substitutions per site.

Table 3.10: Table indicating base pair differences of *Radix natalensis* below the diagonal and percentage identical base pairs of the COI-gene above the diagonal. Grouped and colour coded based on phylogenetic grouping as seen in (Figure 3.11).

|    | Sample code | Site collected | 1  | 2   | 3   | 4   | 5   | 6   | 7   | 8  | 9  | 10  | 11  | 12  |
|----|-------------|----------------|----|-----|-----|-----|-----|-----|-----|----|----|-----|-----|-----|
| 1  | RN64        | LLF3           |    | 100 | 100 | 100 | 99  | 99  | 100 | 98 | 98 | 99% | 99  | 99  |
| 2  | RN72        | LLF 3          | 0  |     | 100 | 100 | 100 | 100 | 100 | 98 | 99 | 99% | 99  | 99  |
| 3  | RN10        | LLF 11         | 0  | 0   |     | 100 | 100 | 100 | 100 | 98 | 99 | 99% | 99  | 99  |
| 4  | PC11        | LLF 13         | 2  | 2   | 2   |     | 100 | 100 | 100 | 99 | 99 | 99% | 99  | 99  |
| 5  | PC13        | LLF 13         | 5  | 2   | 2   | 0   |     | 100 | 100 | 99 | 98 | 99% | 99  | 99  |
| 6  | RN39        | LLF 6          | 5  | 2   | 2   | 0   | 0   |     | 100 | 99 | 98 | 99% | 99  | 99  |
| 7  | PC26        | LLF 9          | 2  | 2   | 2   | 0   | 0   | 0   |     | 99 | 99 | 99  | 9   | 99  |
| 8  | RN12        | LLF 13         | 13 | 10  | 10  | 7   | 8   | 8   | 8   |    | 97 | 98  | 98  | 98  |
| 9  | RN58        | LLF 4          | 14 | 6   | 6   | 4   | 13  | 13  | 4   | 17 |    | 98  | 99  | 99  |
| 10 | RN13        | LLF 10         | 8  | 7   | 6   | 4   | 7   | 7   | 5   | 13 | 14 |     | 100 | 100 |
| 11 | RN34        | LLF 8          | 6  | 6   | 6   | 4   | 4   | 4   | 4   | 9  | 6  | 0   |     | 100 |
| 12 | RN37        | LLF 8          | 6  | 6   | 6   | 4   | 4   | 4   | 4   | 9  | 6  | 0   | 0   |     |

Table 3.11: Table indicating base pair differences between members of the *Pseudosuccinea*. Above the diagonal are the percentage identical base pairs, and below the diagonal are base pair differences of the COI-gene. Genetic sequences generated during this study are coloured green to match the phylogenetic tree generated (Figure 3.10).

|    | Species name                    | Sequence number | 1 | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  |
|----|---------------------------------|-----------------|---|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 1  | <i>Pseudosuccinea columella</i> | KM594692        |   | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 2  | <i>Pseudosuccinea columella</i> | ON953198        | 0 |     | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 3  | <i>Pseudosuccinea columella</i> | PC04            | 0 | 0   |     | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 4  | <i>Pseudosuccinea columella</i> | RN51            | 0 | 0   | 0   |     | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 5  | <i>Pseudosuccinea columella</i> | LC015522        | 0 | 0   | 0   | 0   |     | 100 | 100 | 100 | 100 | 100 | 100 |
| 6  | <i>Pseudosuccinea columella</i> | PC07            | 0 | 0   | 0   | 0   | 0   |     | 100 | 100 | 100 | 100 | 100 |
| 7  | <i>Pseudosuccinea columella</i> | RN26            | 0 | 0   | 0   | 0   | 0   | 0   |     | 100 | 100 | 100 | 100 |
| 8  | <i>Pseudosuccinea columella</i> | RN28            | 0 | 0   | 0   | 0   | 0   | 0   | 0   |     | 100 | 100 | 100 |
| 9  | <i>Pseudosuccinea columella</i> | PC17            | 0 | 0   | 0   | 0   | 0   | 0   | 0   | 0   |     | 100 | 100 |
| 10 | <i>Pseudosuccinea columella</i> | RN50            | 0 | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |     | 100 |
| 11 | <i>Pseudosuccinea columella</i> | KP242797        | 0 | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |     |

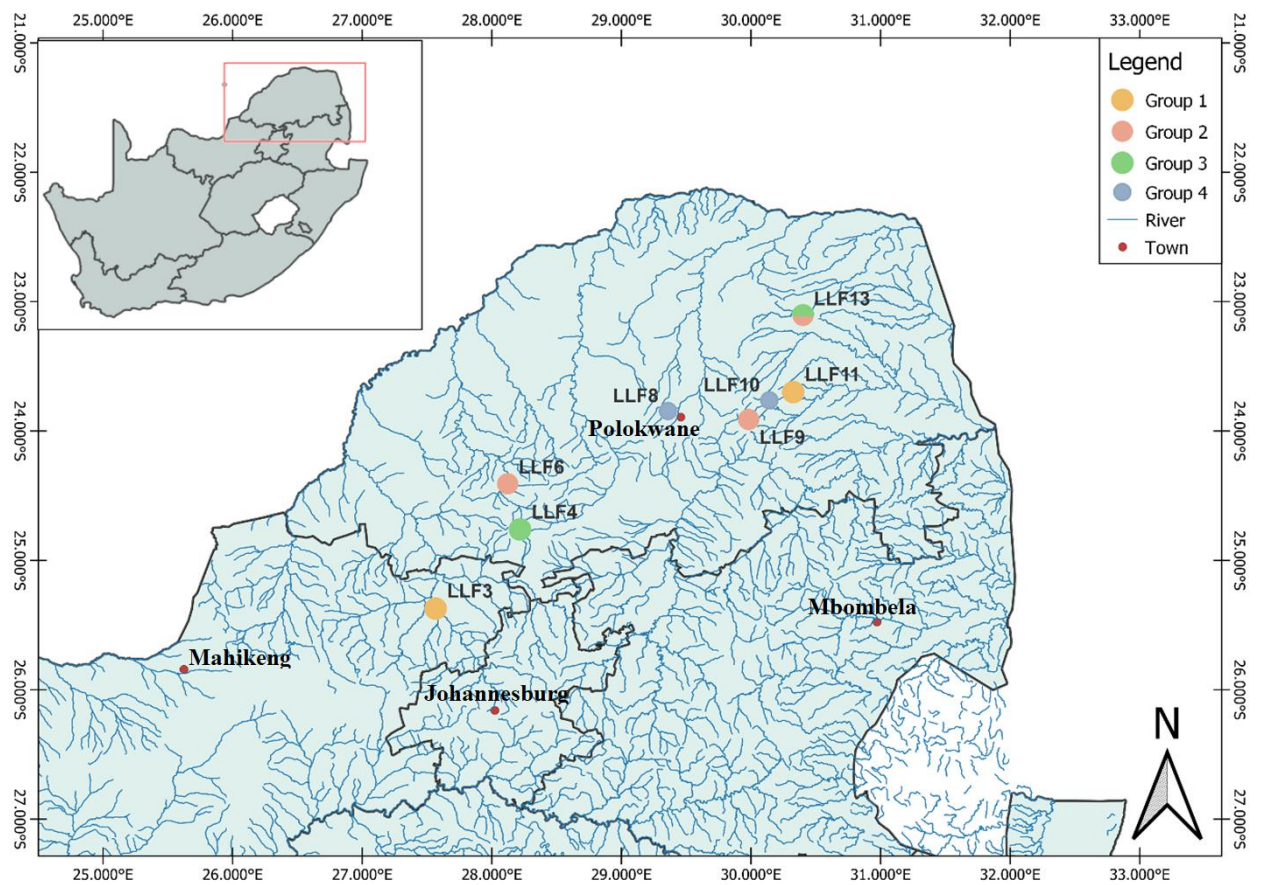


Figure 3.11: Map indicating the sites with *Radix natalensis* that phylogenetically grouped between locations based on the COI-gene mutations. A yellow circle characterises areas with monophyletic group 1 present, pink for group 2, green for group 3 and purple for group 4. The only site with two of the monophyletic groups present was site LLF13 with a green and pink circle icon on the map indicating that monophyletic groups 2 and 3 were present.

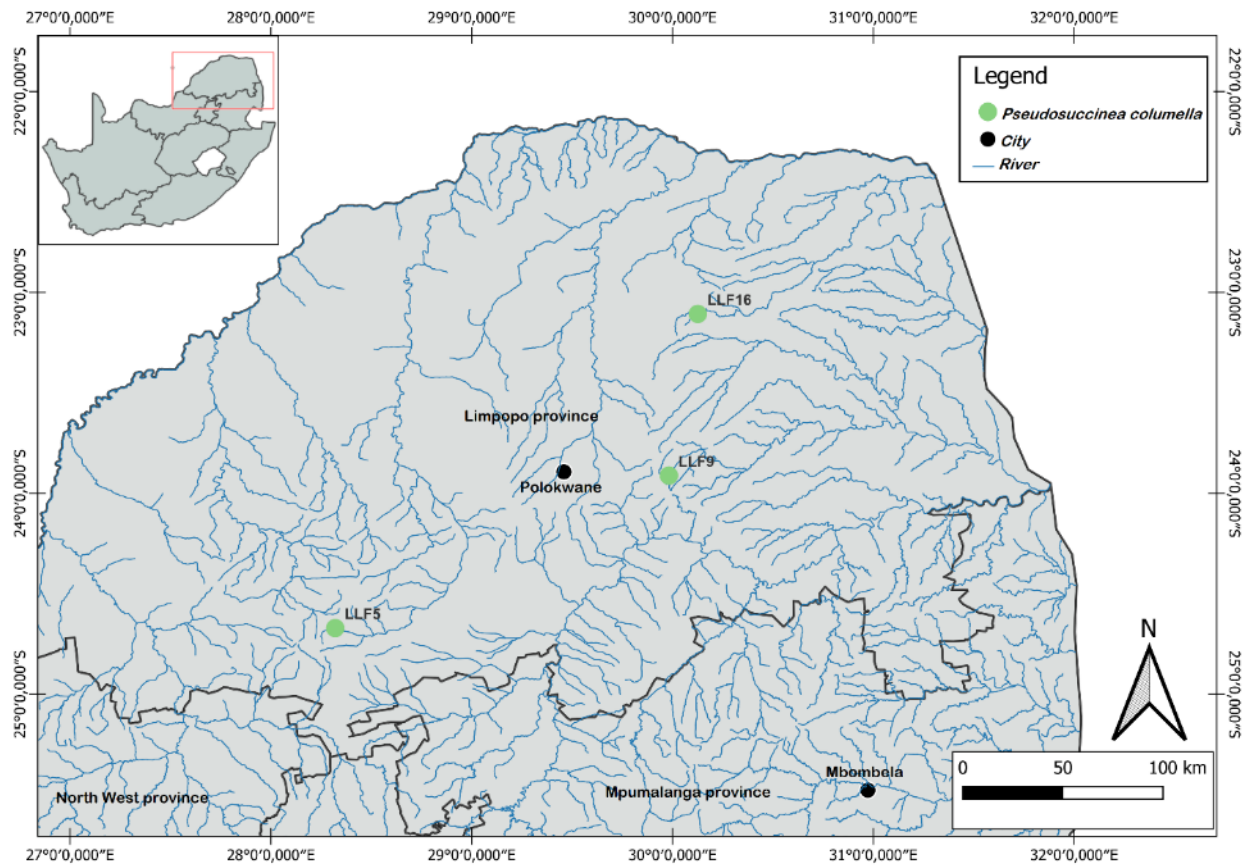


Figure 3.12: Geographical locations where specimens identified as *Pseudosuccinea columella* were collected.

### 3.5.4 Physidae

A total of 140 specimens belonging to the Physidae were collected at three sites within the Limpopo province, South Africa. The specimens collected were morphologically identified as *Physella acuta*. The morphological identification was based on the characteristics of the ovate shell shape, the spire being sharply pointed and the glossy texture of the shell.

Molecular analyses were conducted, and a single novel sequence of *P. acuta* was generated from the specimens collected. Phylogenetic analyses were generated based on sequences of the *Physella* COI genetic dataset (Figure 3.13). The *Physella* specimen collected was identified as belonging to Africa clade 3 as defined by Lawton *et al.*, (2018). The specimen used for genetic analysis was collected at site LLF4, as indicated on the map below (Figure 3.14). The sequences showed a zero to 15 base pair difference between *P. acuta* published sequences as well as the sequences generated during the current study when compared. In this dataset, the interspecific variation for *P. acuta* species within Africa clade 3 is 0-2% (0-15 nt).

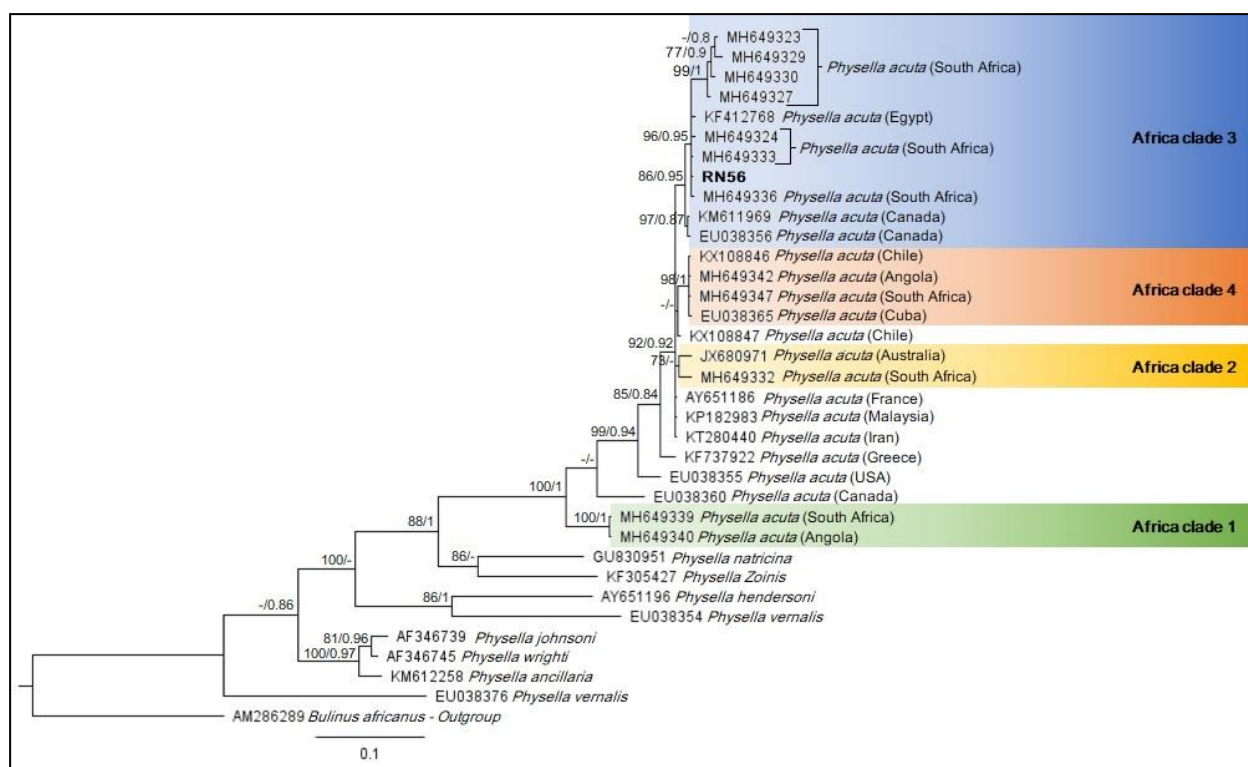


Figure 3.13: Bayesian inference phylogram of species of *Physella* based on partial sequences of the COI rDNA gene. New sequences are in bold. Posterior probability followed by bootstrap support values is indicated next to the branches (posterior probability < 70 and bootstrap < 0.80 not shown). *Bulinus africanus* was used as an outgroup. The branch length scale bar indicates the number of substitutions per site.

Table 3.12: Table indicating base pair differences between members of *Physella acuta* from the Africa clade 3. Above the diagonal are the percentage identical base pairs, and below the diagonal are base pair differences of the COI-gene. Genetic sequences generated during this study are coloured blue to match the phylogenetic tree generated (Figure 3.13).

|    | Species ID                            | GenBank     | 1  | 2  | 3   | 4   | 5  | 6   | 7   | 8   | 9   | 10 | 11  |
|----|---------------------------------------|-------------|----|----|-----|-----|----|-----|-----|-----|-----|----|-----|
| 1  | <i>Physella acuta</i> (South Africa)  | MH649323    |    | 99 | 100 | 100 | 98 | 98  | 98  | 98  | 98  | 98 | 98  |
| 2  | <i>Physella acuta</i> (South Africa)  | MH649329    | 3  |    | 99  | 99  | 97 | 98  | 98  | 98  | 98  | 97 | 97  |
| 3  | <i>Physella acuta</i> (South Africa)  | MH649330    | 2  | 3  |     | 100 | 98 | 98  | 98  | 98  | 98  | 98 | 98  |
| 4  | <i>Physella acuta</i> (South Africa)  | MH649327    | 2  | 5  | 2   |     | 98 | 98  | 98  | 98  | 99  | 98 | 98  |
| 5  | <i>Physella acuta</i> (Egypt)         | KF412768    | 11 | 14 | 11  | 9   |    | 100 | 100 | 100 | 100 | 99 | 99  |
| 6  | <i>Physella acuta</i> (South Africa)  | MH649324    | 9  | 12 | 9   | 9   | 2  |     | 100 | 100 | 100 | 99 | 99  |
| 7  | <i>Physella acuta</i> (South Africa)  | MH649333    | 10 | 13 | 10  | 8   | 1  | 1   |     | 100 | 100 | 99 | 99  |
| 8  | <i>Physella acuta</i> (present study) | <b>RN56</b> | 10 | 13 | 10  | 8   | 1  | 1   | 0   |     | 100 | 99 | 99  |
| 9  | <i>Physella acuta</i> (South Africa)  | MH649336    | 9  | 12 | 9   | 7   | 2  | 2   | 1   | 1   |     | 99 | 99  |
| 10 | <i>Physella acuta</i> (Canada)        | KM611969    | 12 | 15 | 12  | 10  | 5  | 5   | 4   | 4   | 5   |    | 100 |
| 11 | <i>Physella acuta</i> (Canada)        | EU038356    | 12 | 15 | 12  | 10  | 5  | 5   | 4   | 4   | 5   | 0  |     |

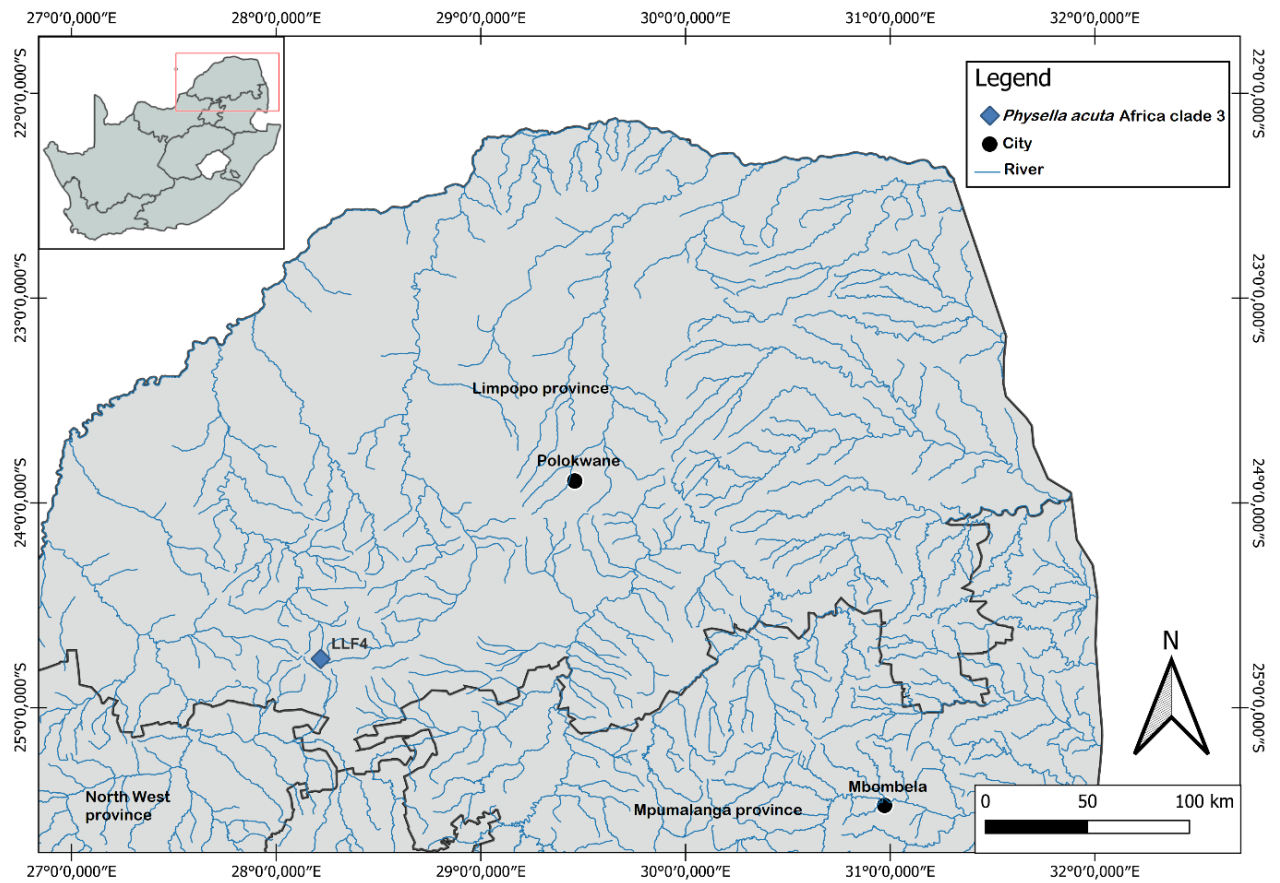


Figure 3.14: Geographical location of site LLF4, where the specimen identified as *Physella acuta* used for genetic analyses was collected and identified to belong to Africa clade 3.

### 3.6 Discussion

The importance of freshwater molluscs as primary consumers and their essential role in the nutrient cycle is well known. However, the study of freshwater molluscan species cannot be established without accurately identifying specimens at the species level (Dudgeon *et al.*, 2006; Pfenninger *et al.*, 2006). Molluscs are also important as some species are essential for the transmission of parasitic infections of economic and medical importance for humans and livestock. Bringing order to the molluscan chaos' (Telford & Budd, 2011) is one of the most significant challenges that limit the resolution of molluscan associations (Schrödl & Stöger, 2014; Wanninger & Wollesen, 2019).

#### 3.6.1 Bulinidae

*Bulinus* specimens are difficult to morphologically identify to species level due to their shell structures being similar. Thus, morphological identification requires the observation of shell micro-sculpture morphology and the shape and size of reproductive organs (Raahauge & Kristensen, 2000; Jørgensen *et al.*, 2007). The latter can only distinguish between certain species within the *B. africanus/globosus* group, namely the *B. africanus*, *B. globosus* and *B. nasutus* members (Raahauge & Kristensen, 2000). However, to achieve this, specialized training is required. *Bulinus africanus* and *B. nasutus* can be inaccurately identified due to the lack of significant differences in these two species' shell morphology and reproductive organs (Raahauge & Kristensen, 2000). Therefore, a combined approach of shell morphology and genetic analysis is an efficient method of establishing an accurate identification at species level (Jørgensen *et al.*, 2007). Results from the morphological identification indicated that two of the four species groups of *Bulinus* that have been identified globally (Raahauge & Kristensen, 2000; Day & de Moor, 2002; Jørgensen *et al.*, 2007) and the three groups that have been found in southern Africa were collected during the current study. The two groups identified are the *B. africanus/globosus* group and the *B. tropicus/truncatus* group (Raahauge & Kristensen, 2000; Day & de Moor, 2002; Jørgensen *et al.*, 2007).

Genetic analyses were conducted using the ribosomal ITS2 region and mitochondrial COI gene to distinguish between different *Bulinus* members of the specimens collected. (Kane *et al.*, 2008; Jørgensen *et al.*, 2011). The phylogenetic relationships between members of the four species groups of Bulinidae remain obscure. Therefore, the need to use a combination of conservative genes was used to resolve the inconsistencies within these species groups and explain the evolutionary origin.

The ITS2 analyses of the *B. africanus/globosus* group confirmed that the specimens collected belong to this group and species level with all six of the novel sequences generated grouping with good nodal support to the genetic sequence *B. africanus* (AM921970) published on GenBank.

The ITS2 analyses of the *B. truncatus/tropicus* group indicated that specimens that were morphologically identified to belong to the *B. truncatus/tropicus* group were genetically identified as *B. truncatus* specimens. However, the COI genetic analyses contradicted the results obtained from the ITS2 analysis. Indicating that the group still grouped within the *B. truncatus/tropicus* group but not with the *B. truncatus*, *B. tropicus* or *B. natalensis* sequences published on GenBank (NCBI, <https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Thus, this indicates that in the case of the *B. truncatus/tropicus* group, the ITS2 genetic region cannot accurately identify specimens to species level and should be used to complement the results of the phylogenetic analysis of the COI genetic region (Yao *et al.*, 2010). Therefore, the use of morphological traits and multiple genetic markers (ITS2 region and COI gene), especially the COI gene, is used to accurately identify species of Bulinidae (Jørgensen *et al.*, 2007; Kane *et al.*, 2008).

Based on morphological traits and the phylogenetic analysis, these specimens were positively identified to be members of *B. depressus*. However, there is still some dispute regarding the latter clade as *B. depressus*. In some cases, *B. depressus* is not included within the *B. tropicus/truncatus* group (Day & de Moor, 2002; De Kock & Wolmarans, 2005b), but Brown (1994) considered that *B. depressus* should be a part of the *B. tropicus/truncatus* group. Still, with the similarities between *B. depressus* and *B. natalensis*, one can easily argue that *B. natalensis* should also be included within the *B. tropicus/truncatus* group (Brown *et al.*, 1991; Jørgensen *et al.*, 2007; Jørgensen *et al.*, 2011)

Using multiple lines of evidence, the results of the ITS2 dataset confirm the morphological identification of several specimens (BS15, -29, -33, -57, -72, -73, -91) to the *B. truncatus/tropicus* group. The COI gene further identifies that phylogenetically specimens formed a separate clade within the *B. truncatus/tropicus* group. However, the identified specimens did not group with published sequences of *B. truncatus*, *B. tropicus* or *B. natalensis*. Therefore, these sequences can be positively identified as *B. depressus*. The novel sequences generated during this study topologically cladded within the *B. truncatus/tropicus* group and the sister group *B. wright* (Jørgensen *et al.*, 2011). The results of the phylogenetic analyses of the COI-mtDNA gene presented here support the idea that *B. depressus* does indeed belong within the *B. tropicus/truncatus* group. The geographical area of collected specimens during this study is within the historically collected ranges of *B. africanus* and *B. depressus* (De Kock & Wolmarans., 2005a, 2006b), corroborating the identification of *B. africanus* and *B. depressus*.

### 3.6.2 Planorbidae

Specimens collected from the Planorbidae family were successfully morphologically identified as *B. pfeifferi* by their umbilicated (depressed navel) and evenly rounded periphery region (Day & de Moor, 2002; De Kock *et al.*, 2004). The phylogenetic analysis, with the aid of the amplification of the ITS2 and COI genetic regions, supported the morphological identification of these specimens.

Two species of *Gyraulus* known to be native to South Africa are *G. costulatus* and *G. connollyi* (Day *et al.*, 2002). According to Brown *et al.* (1969), the morphological differentiation between the *Gyraulus* species collected was established by viewing the conchological characteristics. However, this was not the case, as *G. costulatus* and the invasive species *Biomphalaria grablata* share similarities when it comes to in-field identification. The ITS2 phylogenetic analyses of *Gyraulus* species collected in the North West province indicated that species were correctly identified as belonging to the *Gyraulus* genus. The newly generated sequences grouped with good nodal support to a published sequence of *G. convexiusculus*. However, *G. convexiusculus* has only been identified in the Middle East and Western parts of India (Ayyagari & Sreerama, 2020). The phylogenetic identification of *Gyraulus* species collected was more challenging, with *G. connollyi* and *G. costulatus* not having genetic sequences present on GenBank. The COI phylogenetic analysis indicated a concerning result with specimens collected at the Seekoeivlei Nature Reserve grouping with a published sequence of *G. chinensis*, a known invasive that has been reported at a facility supplying fish and aquatic plants for the aquarium trade in the Kwazulu-Natal province, South Africa (Appleton *et al.*, 2015b). However, the specimens were morphologically identified to not be *G. chinensis* but rather the native species *G. costulatus*. All *Gyraulus* specimens were identified utilizing their presence within South Africa by reviewing published literature and morphological traits of the shell morphology (Day & de Moor, 2002).

### 3.6.3 Lymnaeidae

The morphological identification of *R. natalensis* was confirmed with molecular analyses and morphological characterisation, but the formation of monophyletic groups within the study area with similarities between isolated populations was of interest. The identification of *Radix* species using shell morphology is regarded as inaccurate by Pfenninger *et al.* (2006) due to the variability of largely overlapping morphological traits between species and phenotypical plasticity to environmental conditions. This is confirmed by the map indicating the localities of the phylogenetically grouped specimens and assuming that the environmental conditions to which these specimens were exposed were identical (Pfenninger *et al.*, 2006). The other possibility for the isolated population of genetically similar species may be from lateral dispersal between water bodies with the aid of migratory avian species, which has been previously reported (Kappes &

Haase, 2012). The dispersal of molluscan species depends on a wide range of factors (geographical, vector used and climate). To test this observation, more rigorous field surveys will be required.

The identification of the invasive Lymnaeidae *P. columella* was confirmed by morphological identification and supported by the phylogenetic analysis of the ITS2 and COI genetic regions. All the sequences generated by this study and published sequences from GenBank (NCBI, <https://blast.ncbi.nlm.nih.gov/Blast.cgi>) indicated no genetic variation. This result suggests that the method *P. columella* primarily uses to reproduce is self-fertilization (Alba *et al.*, 2019). This suggests that in the Limpopo province, South Africa, the population of the invasive *P. columella* is from a single invasion or multiple invasions from the same region (Nicot *et al.*, 2008).

#### **3.6.4 Physidae**

The invasive *P. acuta* has been reported in several freshwater habitats across South Africa and is considered the second most widespread invasive molluscan species (De Kock & Wolmarans, 2007; Lawton *et al.*, 2018). *Physella acuta* has not been identified as any vector for snail-borne diseases within South Africa (Lawton *et al.*, 2018). However, the rapid dispersal of *P. acuta* throughout a freshwater ecosystem can harm the native species of freshwater molluscan species present (Dobson, 2004). Studies have indicated that in locations where the invasive *Physella* species were present, the native populations of medically important molluscan vectors have decreased. Therefore, suggesting that *Physella* could be used as a potential biological control to reduce the risk of infection in locations identified as high transmission areas for medically important digenetic trematodes (Dobson, 2004). The use of *P. acuta* as a possible biological control measure must be accompanied by accurate identification and strict monitoring to determine the ecological impact and mitigate any undesired impacts on the local ecology. According to Lawton *et al.* (2018), the use of morphological and molecular analysis of the COI genetic sequence has been widely adapted to identify *P. acuta* at the species level and can be used to determine the geographical origin of the invasion. In a study conducted by Lawton *et al.* (2018), four distinct African clades were identified within samples collected from the Vaal River in South Africa, Angola and Burundi. The four African clades indicated that *P. acuta* resulted from multiple invasions (Lawton *et al.*, 2018). During the current study, the application of COI genetic analysis accurately identified the invasive *Physella acuta* from populations sampled within South Africa and published on GenBank (NCBI, <https://blast.ncbi.nlm.nih.gov/Blast.cgi>) was identified to be a member of the African clade 3. This clade contains haplotypes from Mexico, the USA, Greece, Egypt, Iran, Macedonia and South Africa (Lawton *et al.*, 2018). The identification of species belonging to all 4 clades within South Africa was collected by Lawton *et al.*, (2018), were within the Vaal River catchment in the North West province. Thus, indicating that *P. acuta* from

different African clades can be located within the same area (Lawton *et al.*, 2018). Therefore, suggesting that it is highly plausible that more than one of the *P. acuta* African clades could be present at a single site during the current study. However, this will require further research.

### **3.7 Conclusion**

The study aimed to accurately identify freshwater molluscan species collected during this study to the lowest possible taxonomic level. Due to the shared morphological traits between members of the same genera collected, multiple lines of evidence were required to identify molluscan specimens to species level accurately. Therefore, identification was determined by using morphological traits and molecular analysis of the ITS2 and COI genetic regions and compared to published sequences available by using phylogenetic analysis.

The molecular analysis of the ITS2 and COI genetic regions supported in large the morphological identification. Identifying the presence of *B. africanus*, *B. depressus*, *B. pfeifferi*, *G. costulatus*, *G. connollyi*, *R. natalensis*, *P. columella*, *P. acuta*. The identification of *B. depressus*, *G. costulatus* and *G. connollyi* resulted in additional sequences of the ITS2 and COI sequences generated for these species. Molecular analysis also indicated that *R. natalensis* formed four genetically diverse groups located at different isolated sites. This means some form of locomotion between sites or vectors transporting molluscan species between the selected areas of investigation. The molecular analysis of the invasive *P. columella* indicated that all specimens collected at various sites were genetically identical, with no base pair differences of the ITS2 and COI genes. Therefore, indicating that the invasion of *P. columella* was the result of a single specimen or multiple invasions from a single location.

## **Chapter 4: Decline in the native freshwater snail diversity of the north-eastern provinces of South Africa: a cause for concern.**

### **4.1 Introduction**

Freshwater molluscs are regarded as nature's ultimate grazers and fulfil an essential role in the food web of freshwater ecosystems as primary consumers feeding on peri- and epiphytic aquatic biota (Strong *et al.*, 2007). Making them a necessary part of the natural nutrient cycle and energy transfer to higher trophic levels within the freshwater ecosystem (Strong *et al.*, 2007). Therefore, the changes in flow velocity, habitat availability, water chemistry, presence of predators and the invasive organisms a major factor affecting the diversity and abundance of native molluscs within aquatic systems (Brown *et al.*, 2010; Covich, 2010). Many of these single-valved species spend their entire lives in a few square meters of freshwater systems, making them vulnerable to environmental changes. The presence of emergent and floating vegetation is a determining factor for the presence of native and medically important molluscan species (Wood *et al.*, 2019). Freshwater molluscs are therefore regarded as reliable indicators of the present ecological status of freshwater systems based on their presence or absence (Hubendick, 1958; Brown, 1978; Selbach & Poulin, 2020). Southern Africa, including South Africa, has a rich freshwater mollusc diversity, with approximately 101 species native to the country and an additional 14 invasive species. (Day & de Moor, 2002; Manyangadze *et al.*, 2021). Molluscs native to South Africa are an essential food source for aquatic and terrestrial predators and are vital in maintaining ecosystem function (Brown *et al.*, 2010). Several of these molluscan species also have secondary roles as intermediate hosts (vectors) for a range of parasitic infections that threaten the health of humans and animals (Chitsulo *et al.*, 2000; Adenowo *et al.*, 2015). Thus there is a need to continuously monitor the distribution and abundance of freshwater molluscs in South Africa (Wolmarans *et al.*, 2002; King, 2010; Adekiya *et al.*, 2019). Historically identified species within the north-eastern provinces of South Africa during the previous assessment of schistosomiasis vectors during the 1960s-1980s were identified and assembled into the National Freshwater Snail Collection (NFSC) database of South Africa by De Kock *et al.*, (2004); De Kock & Wolmarans (2005a, 2005c, 2005b, 2006a, 2006b, 2009) from samples collected from 1960-1980 surveys. All the species collected during this survey are housed at the North-West University (NWU) in Potchefstroom, South Africa and listed in the NFSC.

Table 4.1: Molluscan specimens collected in the north-eastern provinces of South Africa during surveys conducted in the 1960-1980 period and housed within the NFSC database. The listed species include native and invasive species\* collected during this period and adapted from (De Kock *et al.*, 2004).

| Nr | Specie name (Authority)                                   | Family      | Nr | Specie name (Authority)                                                     | Family        |
|----|-----------------------------------------------------------|-------------|----|-----------------------------------------------------------------------------|---------------|
| 1  | <i>Bellamya capillata</i> (Frauenfeld, 1865)              | Viviparidae | 15 | <i>Melanoides tuberculata</i> (O. F. Müller, 1774)                          | Thiaridae     |
| 2  | <i>Biomphalaria pfeifferi</i> (Krauss, 1848)              | Viviparidae | 16 | <i>Melanoides victoriae</i> (Dohrn, 1865)                                   | Thiaridae     |
| 3  | <i>Bulinus africanus</i> (Krauss, 1848)                   | Bulinidae   | 19 | <i>Physella acuta</i> * (Draparnaud, 1805)                                  | Physidae      |
| 4  | <i>Bulinus depressus</i> (F. Haas, 1936)                  | Bulinidae   | 20 | <i>Pseudosuccinea columella</i> * (Say, 1817)                               | Lymnaeidae    |
| 5  | <i>Bulinus forskalii</i> (Ehrenberg, 1831)                | Bulinidae   | 21 | <i>Radix natalensis</i> (Krauss, 1848)                                      | Lymnaeidae    |
| 6  | <i>Bulinus globosus</i> (Morelet, 1866)                   | Bulinidae   | 22 | <i>Segmentorbis angustus</i> *(Jickeli, 1874)                               | Planorbidae   |
| 7  | <i>Bulinus natalensis</i> (Kuster, 1841)                  | Bulinidae   | 23 | <i>Segmentorbis kanisaensis</i> * (Preston, 1914)                           | Planorbidae   |
| 8  | <i>Bulinus tropicus</i> (Krauss, 1848)                    | Bulinidae   | 24 | <i>Segmentorbis planodiscus</i> (Melvill & Ponsonby, 1897)                  | Planorbidae   |
| 9  | <i>Bulinus wrighti</i> (Mandahl-Barth, 1965)              | Bulinidae   | 25 | <i>Stenophysa marmorata</i> * (Guilding, 1828)                              | Physidae      |
| 10 | <i>Cleopatra ferruginea</i> (I. Lea & H. C. Lea, 1851)    | Paludomidae | 26 | <i>Thiara amarula</i> * (Linnaeus, 1758)                                    | Thiaridae     |
| 11 | <i>Galba truncatula</i> * (O. F. Müller, 1774)            | Lymnaeidae  | 27 | <i>Vittina natalensis</i> * (Reeve, 1855)                                   | Neritidae     |
| 12 | <i>Gyraulus connollyi</i> (D. S. Brown & Van Eeden, 1969) | Planorbidae | 28 | <i>Lanistes ovum</i> (Troschel, 1845)                                       | Ampullariidae |
| 13 | <i>Gyraulus costulatus</i> (Krauss, 1848)                 | Planorbidae | 29 | <i>Lentorbis carringtoni</i> * (Azevedo, de Medeiros & Da Costa Faro, 1961) | Planorbidae   |
| 14 | <i>Lentorbis junodi</i> (Connolly, 1922)                  | Planorbidae |    |                                                                             |               |

Environmental and climate change is expected to profoundly affect freshwater ecosystems and change the characteristics of freshwater habitats, resulting in a shift in molluscan species diversity and abundance (Cordellier *et al.*, 2012). Abrupt changes in flow rate, temperature and water nutrients are expected to alter the abundance of parasitic and vector species present by affecting vector species diversity and abundance as well as introducing invasive species to new ecological areas where environmental factors were previously unsuited (Pedersen *et al.*, 2017; Selbach & Poulin, 2020). Changes in water nutrients due to climate change add a level of pressure on species currently inhabiting a specific geographical niche (Cordellier *et al.*, 2012; Selbach & Poulin, 2020). Water nutrient factors such as phosphates and ammonia nitrate concentrations (N:P ratio), pH and dissolved oxygen concentration directly affect the abundance of molluscan species within aquatic ecosystems. pH and dissolved oxygen concentration mainly affect molluscan species richness and diversity (Jurkiewicz-Karnkowska, 2011). Flow regulation of rivers through a dam and weir construction causes water levels to increase upstream and decrease downstream of the in-stream water management infrastructure. Changes in the water level directly affect water temperature, which affects evaporation and, in turn, affects salinity and

nutrients such as nitrates and phosphates within the water column (Früh *et al.*, 2016). Such changes in water quality have the potential to alter freshwater molluscan species distribution by decreasing the available instream habitat and oxygen saturation, therefore increasing competition between molluscan species for available food and reducing suitable habitat for egg deposits (Quayle *et al.*, 2010; Chimbari *et al.*, 2020). Most of South Africa's rivers have been regulated, to some degree, to aid the growing population and ensure adequate access to water (Quayle *et al.*, 2010). The north-eastern provinces of South Africa are of high economic importance, particularly concerning the mining and agricultural sectors that fuel expanding rural settlements and cause the population to grow. The effect of anthropogenic water use by rural communities, flow regulation and climate change decreases the natural habitat of molluscan species native to South African river systems and increases competition between native and invasive molluscan species for the remaining suitable habitat (Zhu & Ringler, 2012; Kemp *et al.*, 2016).

The interaction between native and invasive species is key to understanding their effect on native species populations, yet this is not always measured (Riley *et al.*, 2008). Invasive molluscan species have a detrimental effect on the function and services of an ecosystem they are introduced to (Appleton, 2003). The lack of natural predators, reproductive success and, often, the lack of parasitic burden likely allows invasive species to outcompete native species for the same ecological niche (Riley *et al.*, 2008). This disrupts the food web and can lead to a bottom-up effect on the aquatic system where invasive species are present by not being palatable to predators or being better at evasion than native species. In South Africa, four invasive freshwater molluscs were identified in the north-eastern provinces that require monitoring and have the potential to become a threat to native species, including *Pseudosuccinea columella*, *Physella acuta*, *Aplexa marmorata* and *Tarebia granifera* (Appleton, 2003; De Kock *et al.*, 2008; Weyl *et al.*, 2020). *Tarebia granifera* is the most notorious of these invasive species as it has numerous adaptations to make it the most successful invader, including having a shell that is exponentially harder than that of most native species, making it inedible for most predators (Strayer, 1999; Appleton *et al.*, 2009; Covich, 2010). In a study conducted in 2008 within the Kruger National Park (KNP) by De Kock & Wolmarans (2008), *T. granifera* was found in high abundance. Worryingly, this indicates not only the high level of success this invasive mollusc has accomplished from its first collection in 1999 from KwaZulu-Natal (De Kock & Wolmarans, 2008) but also that conservation areas are at great risk of being invaded. The exponential spread of invasive molluscan species most likely harms native species diversity and causes numerous negative consequences for aquatic ecosystems that become invaded (De Kock & Wolmarans, 2008; Riley, Dybdahl & Hall, 2008). Therefore, continuous monitoring of native species and the spread of invasive species is of great importance in assessing the current status of South Africa's freshwater molluscs. Assessing the current ecological state of the area of interest, by examining the physical,

chemical water quality and biological habitat. As well as assessing the limiting factor(s) affecting freshwater molluscan diversity can also be established (Joubert *et al.*, 2015; Kemp *et al.*, 2016).

#### **4.1.1 Problem statement**

The current distribution status of native freshwater gastropods and the effect of naturogenic, anthropogenic and invasive species on native freshwater gastropods in the north-eastern provinces of South Africa is regarded as an understudied field, with the last full assessment conducted during the 1960s to 1980s on the distribution of native species. There is thus a need to reassess the status of native freshwater gastropods in the north-eastern provinces of South Africa.

#### **4.1.2 Aims and Objectives**

The central aim of this section of the study is to assess the current distribution of native and invasive freshwater gastropod species within the north-eastern provinces of South Africa, based on sites of historical importance from the NFSC database of South Africa. This was achieved by conducting field collection surveys for freshwater gastropods in regions of historical importance from the NFSC database and comparing it to historical records of molluscan species collected in the study region. Another aim was to determine the roles of water quality, habitat structure and invasive species in determining freshwater gastropod diversity, abundance, and distribution by conducting qualitative habitat assessment and water sample collection for nutrient analysis.

#### **4.2 Materials and Methods**

The materials and methods used in this chapter, as well as the study area and necessary permit details, were provided in Chapter 2.

## 4.3 Results

### 4.3.1 Water quality analysis

Water quality variables obtained from all the sampling sites indicated that the lowest measured temperature was at site LLF4 (Figure 2.1; Figure 2.2) (15.1°C) during the low flow season, while the highest temperature was measured at LHF4 (26°C) in the high flow season.

The *in situ* measurements taken at each site indicated that pH remained similar between all the sites with a mean of 8.3 ( $\pm 0.52$ ) during the high flow sampling period and a mean of 8.4 ( $\pm 0.56$ ) in the low flow season. The highest pH measured was 9.27 at site LLF4 during the low flow season and the lowest pH was measured at site LHF3 during the high flow season with a value of 7.5. The highest EC and TDS measured were during the high flow at site LHF3 (2415  $\mu\text{S}/\text{cm}$  EC and 1665 mg/L TDS, respectively) compared to the lowest measurements during the low flow at site LLF9 (30.6  $\mu\text{S}/\text{cm}$  and 35.7 mg/L). Electrical conductivity at sites LLF 1, 3, 5 and 6 was excluded due to technical difficulties with the ExStik II EC 500 instrument. These water samples were only analysed in the laboratory.

Dissolved oxygen saturation between all the sites sampled was within the target concentration of 80% to >120% dissolved oxygen saturation, with a single site (LLF 4) with oxygen saturation of 63.7% / 6.79 mg/L indicating a sub-lethal concentration of  $\text{O}_2$  within the water column.

Chemical analysis (Table 4.2) of unfiltered water samples collected during the high- and low flow season indicated remarkably varied differences between seasons. The concentration of  $\text{Cl}^-$ ,  $\text{PO}_4^{3-}$  and  $\text{NH}_4^+$  varied remarkably between high flow and low flow seasons. High levels of Chlorine ( $\text{Cl}^-$ ) ranged from 603 mg/L at site LHF 2 to 13.37 mg/L at site LHF 6 during the high flow season, and the concentration of chlorine-ions was lower during the low flow season with an average of 11.88 mg/L with a standard deviation of 6.14 mg/L. The  $\text{PO}_4^{3-}$  and  $\text{NH}_4^+$  varied remarkably between the high flow sites, from 0.1 mg/L  $\text{PO}_4^{3-}$  at site LHF1 to a low of <0.1 mg/L  $\text{PO}_4^{3-}$  measured at site LHF9, below the detectable limit of the Phosphate test. The  $\text{NH}_4^+$  concentration varied less between high flow sites compared to the  $\text{PO}_4^{3-}$ , with site LHF4 with the highest concentration at 0.14 mg/L  $\text{NH}_4^+$  and site LHF9 with the lowest concentration at 0.01 mg/L  $\text{NH}_4^+$ . However, the low flow sites concentrations of  $\text{PO}_4^{3-}$  was remarkably higher with an average concentration of 3.9 mg/L with a standard deviation of 2.21 mg/L and  $\text{NH}_4^+$  concentrations being lower with an average concentration of 0.01 mg/L and a standard deviation of 0.01 mg/L between all of the sites.

Nitrate concentrations varied between sites sampled during the low flow and high flow seasons. The low flow sites differed, with five sites (LLF 5,6,14,15,16) with  $\text{NO}_3^-$  concentrations below 0.5

mg/L. One of the low-flow sites (LLF 10) with  $\text{NO}_3$ -concentration of 6.9 mg/L. The remaining ten sites sampled during the low flow season had no definitive differences in nitrate concentration within the 0.5 – 2.5 mg/L range. Nitrite concentrations of low flow sampling sites were all very low (<0.01 mg/L). The concentration of phosphorus differed between the high flow and low flow seasons. All the sites sampled during the low flow season had phosphorus concentrations above the 0.25 mg/L threshold, with the highest detected at site LLF14 (8.88 mg/L) and the lowest concentration at site LHF9 (0.01 mg/L).

Table 4.2: The *in situ* water quality parameters measured in the field and chemical water quality analysis obtained during the high flow sampling (April-May 2021) and low flow sampling (August 2021) respectively at the 25 selected sites within the North-eastern provinces. The standard deviation between all the sites is indicated in the last row labelled SD\*. The target water quality requirement (TWQR) is included in the last row.

| Date            | Site Code | In situ measurements |             |                      |                          |                 |                      | Chemical Water quality              |                                     |                        |                                      |                        |                                      |
|-----------------|-----------|----------------------|-------------|----------------------|--------------------------|-----------------|----------------------|-------------------------------------|-------------------------------------|------------------------|--------------------------------------|------------------------|--------------------------------------|
|                 |           | Temperature (°C)     | pH          | Dissolved Oxygen (%) | Oxygen saturation (mg/L) | Turbidity (FAU) | Conductivity (us/cm) | NH <sub>4</sub> <sup>+</sup> (mg/L) | NO <sub>3</sub> <sup>-</sup> (mg/L) | NO <sub>2</sub> (mg/L) | PO <sub>4</sub> <sup>3-</sup> (mg/L) | Cl <sup>-</sup> (mg/L) | SO <sub>4</sub> <sup>2-</sup> (mg/L) |
| 22-Apr-21       | LHF1      | 22.40                | 9.20        | 121.4                | 10.8                     | 9.67            | 781.50               | 0.06                                | 1.77                                | 0.08                   | 0.24                                 | 53.00                  | 14.00                                |
| 25-Apr-21       | LHF2      | 21.60                | 7.60        | 106.8                | 9.4                      | 8.00            | 857.00               | 0.14                                | 0.93                                | 0.00                   | 0.16                                 | 603.00                 | 216.67                               |
| 26-Apr-21       | LHF3      | 23.60                | 7.50        | 154                  | 12.8                     | 20.00           | 2415.00              | 0.04                                | 1.33                                | 0.02                   | 0.04                                 | 81.33                  | 58.00                                |
| 29-Apr-21       | LHF4      | 26.00                | 8.80        | 131.9                | 11.4                     | 12.00           | 422.00               | 0.12                                | 1.97                                | 0.01                   | 0.11                                 | 32.67                  | 62.67                                |
| 03-May-21       | LHF5      | 24.90                | 8.30        | 118.5                | 10                       | 17.67           | 381.00               | 0.18                                | 0.87                                | 0.01                   | 0.05                                 | 87.33                  | 12.00                                |
| 04-May-21       | LHF6      | 20.30                | 8.60        | 109.5                | 10.2                     | 8.00            | 547.00               | 0.10                                | 0.41                                | 0.00                   | 0.10                                 | 13.37                  | 4.00                                 |
| 05-May-21       | LHF7      | 22.70                | 8.40        | 113.2                | 9.7                      | 11.67           | 804.50               | 0.05                                | 0.20                                | 0.01                   | 0.03                                 | 19.33                  | 14.67                                |
| 06-May-21       | LHF8      | 22.70                | 8.80        | 125.5                | 11                       | 12.67           | 1285.50              | 0.02                                | 0.27                                | 0.01                   | 0.02                                 | 28.33                  | 13.00                                |
| 06-April-May-21 | LHF9      | 22.30                | 8.30        | 115.2                | 10.2                     | 13.00           | 1007.00              | 0.01                                | 1.00                                | 0.01                   | 0.01                                 | 26.00                  | 69.67                                |
| Mean ±SD*       | -         | 22.94 ± 0.52         | 8.39 ±13.57 | 121.78 ±0.98         | 10.61 ±3.84              | 12.52 ±3.84     | 944.50 ±585.47       | 0.08 ±0.05                          | 0.97 ±0.59                          | 0.02 ±0.02             | 0.08 ±0.07                           | 104.93 ±177.85         | 51.63 ±63.1                          |
| 23-Aug-21       | LLF1      | 18.30                | 9.25        | 122.60               | 11.72                    | 14.00           | -                    | 0.03                                | 2.00                                | 0.01                   | 3.74                                 | 33.00                  | 48.00                                |
| 23-Aug-21       | LLF2      | 18.70                | 9.09        | 145.40               | 13.38                    | 31.00           | 665.00               | 0.02                                | 1.20                                | 0.01                   | 5.37                                 | 21.00                  | 68.00                                |
| 23-Aug-21       | LLF3      | 19.40                | 9.04        | 114.30               | 10.45                    | 13.00           | -                    | 0.01                                | 1.60                                | 0.01                   | 6.15                                 | 10.00                  | 91.00                                |
| 24-Aug-21       | LLF4      | 15.10                | 9.27        | 63.70                | 6.79                     | 7.00            | 916.00               | 0.01                                | 0.70                                | 0.01                   | 3.19                                 | 10.00                  | 8.00                                 |
| 24-Aug-21       | LLF5      | 17.50                | 9.08        | 85.00                | 8.14                     | 7.00            | -                    | 0.01                                | 0.50                                | 0.01                   | 7.11                                 | 10.00                  | 7.00                                 |
| 24-Aug-21       | LLF6      | 23.00                | 8.48        | 117.70               | 9.11                     | 20.00           | -                    | 0.01                                | 0.50                                | 0.01                   | 3.32                                 | 10.00                  | 4.00                                 |
| 24-Aug-21       | LLF7      | 21.60                | 8.57        | 91.20                | 8.75                     | 5.00            | 208.00               | 0.01                                | 0.50                                | 0.01                   | 0.82                                 | 10.00                  | 10.00                                |
| 25-Aug-21       | LLF8      | 17.50                | 7.93        | 97.10                | 9.07                     | 17.00           | 278.00               | 0.01                                | 1.20                                | 0.01                   | 5.31                                 | 7.00                   | 304.00                               |
| 25-Aug-21       | LLF9      | 18.20                | 8.10        | 88.80                | 8.57                     | 7.00            | 30.60                | 0.01                                | 1.80                                | 0.01                   | 3.57                                 | 10.00                  | 4.00                                 |
| 25-Aug-21       | LLF10     | 21.90                | 7.93        | 124.40               | 10.59                    | 8.00            | 48.50                | 0.01                                | 6.90                                | 0.01                   | 4.62                                 | 10.00                  | 40.00                                |
| 25-Aug-21       | LLF11     | 23.60                | 7.72        | 123.30               | 10.48                    | 12.00           | 171.20               | 0.01                                | 1.70                                | 0.01                   | 3.12                                 | 9.00                   | 37.00                                |
| 26-Aug-21       | LLF12     | 19.40                | 7.77        | 109.70               | 10.19                    | 19.00           | 214.00               | 0.01                                | 1.60                                | 0.01                   | 0.97                                 | 10.00                  | 21.00                                |
| 26-Aug-21       | LLF13     | 18.50                | 7.76        | 110.30               | 10.49                    | 10.00           | 394.00               | 0.01                                | 2.40                                | 0.01                   | 0.81                                 | 10.00                  | 42.00                                |
| 26-Aug-21       | LLF14     | 24.20                | 7.63        | 101.90               | 9.30                     | 16.00           | 151.60               | 0.01                                | 0.50                                | 0.01                   | 8.88                                 | 10.00                  | 17.00                                |
| 26-Aug-21       | LLF15     | 20.70                | 7.82        | 97.20                | 8.98                     | 14.00           | 184.60               | 0.01                                | 0.50                                | 0.01                   | 1.99                                 | 10.00                  | 2.50                                 |
| 26-Aug-21       | LLF16     | 21.90                | 7.73        | 106.10               | 9.61                     | 10.00           | 897.00               | 0.01                                | 0.50                                | 0.01                   | 3.49                                 | 10.00                  | 2.00                                 |
| Mean ±SD*       | -         | 19.97 ± 2.49         | 8.3 ± 0.61  | 106.17 ± 18.74       | 9.73 ± 1.48              | 13.13 ± 6.38    | 346.54 ± 296.65      | 0.01 ± 0.01                         | 1.51 ±1.52                          | 0.01 ± 0.00            | 3.9 ± 2.21                           | 11.88 ± 6.14           | 44.09 ± 71.69                        |
| TWQR            | -         | -                    | -           | 80–120 %             | 9.09–12.77               | <65             | -                    | ≤0.007                              | 0.5-2.5                             | 0.5-2.5                | 0.005-0.025                          | -                      | -                                    |

\*SD-Standard deviation

Table 4.3: Total nitrogen and total phosphorus ratio between the 25 sites sampled within the north-eastern provinces. Total Nitrogen is calculated by adding the concentrations of Ammonium ( $\text{NH}_4^+$ ), Nitrate ( $\text{NO}_3^-$ ) and Nitrite ( $\text{NO}_2$ ) together. Mean and standard deviation (SD) indicated in last row for both high flow and low flow sampling sites. \* HF-High flow; LF-Low flow.

| Site Code | Total Nitrogen (T-N): Total Phosphorus (T-P) ratio |                        |                      |                           |                |           |
|-----------|----------------------------------------------------|------------------------|----------------------|---------------------------|----------------|-----------|
|           | $\text{NH}_4^+$ (mg/L)                             | $\text{NO}_3^-$ (mg/L) | $\text{NO}_2$ (mg/L) | $\text{PO}_4^{3-}$ (mg/L) | Total N (mg/L) | N:P Ratio |
| LHF1      | 0.06                                               | 1.77                   | 0.08                 | 0.24                      | 1.91           | 8:1       |
| LHF2      | 0.14                                               | 0.93                   | 0                    | 0.16                      | 1.07           | 6.7:1     |
| LHF3      | 0.04                                               | 1.33                   | 0.02                 | 0.04                      | 1.39           | 35:1      |
| LHF4      | 0.12                                               | 1.97                   | 0.01                 | 0.11                      | 2.1            | 19:1      |
| LHF5      | 0.18                                               | 0.87                   | 0.01                 | 0.05                      | 1.06           | 21:1      |
| LHF6      | 0.1                                                | 0.41                   | 0                    | 0.1                       | 0.51           | 5:1       |
| LHF7      | 0.05                                               | 0.2                    | 0.01                 | 0.03                      | 0.26           | 9:1       |
| LHF8      | 0.02                                               | 0.27                   | 0.01                 | 0.02                      | 0.3            | 15:1      |
| LHF9      | 0.01                                               | 1                      | 0.01                 | 0.01                      | 1.02           | 102:1     |
| LLF1      | 0.03                                               | 2                      | 0.01                 | 3.74                      | 2.04           | 1:1       |
| LLF2      | 0.02                                               | 1.2                    | 0.01                 | 5.37                      | 1.23           | 0.2:1     |
| LLF3      | 0.01                                               | 1.6                    | 0.01                 | 6.15                      | 1.62           | 0.3:1     |
| LLF4      | 0.01                                               | 0.7                    | 0.01                 | 3.19                      | 0.72           | 0.2:1     |
| LLF5      | 0.01                                               | 0.5                    | 0.01                 | 7.11                      | 0.52           | 0.1:1     |
| LLF6      | 0.01                                               | 0.5                    | 0.01                 | 3.32                      | 0.52           | 0.2:1     |
| LLF7      | 0.01                                               | 0.5                    | 0.01                 | 0.82                      | 0.52           | 0.6:1     |
| LLF8      | 0.01                                               | 1.2                    | 0.01                 | 5.31                      | 1.22           | 0.2:1     |
| LLF9      | 0.01                                               | 1.8                    | 0.01                 | 3.57                      | 1.82           | 0.5:1     |
| LLF10     | 0.01                                               | 6.9                    | 0.01                 | 4.62                      | 6.92           | 1.5:1     |
| LLF11     | 0.01                                               | 1.7                    | 0.01                 | 3.12                      | 1.72           | 0.6:1     |
| LLF12     | 0.01                                               | 1.6                    | 0.01                 | 0.97                      | 1.62           | 1.7:1     |
| LLF13     | 0.01                                               | 2.4                    | 0.01                 | 0.81                      | 2.42           | 3:1       |
| LLF14     | 0.01                                               | 0.5                    | 0.01                 | 8.88                      | 0.52           | 0.1:1     |
| LLF15     | 0.01                                               | 0.5                    | 0.01                 | 1.99                      | 0.52           | 0.3:1     |
| LLF16     | 0.01                                               | 0.5                    | 0.01                 | 3.49                      | 0.52           | 0.1:1     |
| SD*       | 0.04±0.05                                          | 1.314±1.32             | 0.01±0.01            | 2.53±2.6                  | 1.36±1.32      |           |

\*SD-Standard deviation

#### **4.4 Molluscan species identification**

##### **4.4.1 Historic molluscan distribution compared to currently collected species.**

The number of species collected decreased from historically collected specimens, with nine different species collected during the current study compared to the 14 species historically collected at the same sites sampled in the current study. The number of individuals indicated that a total of 4557 invasive freshwater snail individuals were collected, and only 1629 native freshwater snail species were collected during the Limpopo lowveld sampling (Table A2, Table A3). Historically, a maximum of seven different species were identified at four of the sites sampled during the current study (LLF13-16). The current diversity of species collected across sites LLF 13-16 was only four different species collected at LLF16. Figure 4.1 indicates the decline in species diversity between historically sampled sites and the number of species collected during the current study.

The current molluscan diversity has remarkably declined from the historically recorded species within the study area (Figure 4.1). The historic molluscan diversity within the same study area was compared to the current molluscan species collected and identified (Chapter 3). Historical data was retrieved from the NFSC database housed at the NWU- Potchefstroom campus (Table 4.4). The results indicated that the diversity of molluscan species declined from 15 different species historically collected with 3 invasive species present to 8 species collected during the current study with 3 invasive species present, when compared to the historically collected specimens. However, there was an new invasive species present that was identified as *T. granifera* and was not historically collected within the study area but was collected during the current study.

Table 4.4: Species historically identified within the selected study are by data available from the NFSC database from the NWU-Potchefstroom campus, compared to species collected during this study and morphologically and molecularly identified from the sites located in the Limpopo Lowveld ecoregion. Presence indicated by (X)

| Species:                        | Species historically collected in the NFSC | Species collected during the current study |
|---------------------------------|--------------------------------------------|--------------------------------------------|
| Bulinidae                       |                                            |                                            |
| <i>Bulinus africanus</i>        | X                                          | X                                          |
| <i>Bulinus depressus</i>        | X                                          | X                                          |
| <i>Bulinus forskalii</i>        | X                                          |                                            |
| <i>Bulinus globosus</i>         | X                                          |                                            |
| <i>Bulinus natalensis</i>       | X                                          |                                            |
| <i>Bulinus tropicus</i>         | X                                          |                                            |
| Lymnaeidae                      |                                            |                                            |
| <i>Pseudosuccinea columella</i> | X                                          | X                                          |
| <i>Radix natalensis</i>         | X                                          | X                                          |
| Physidae                        |                                            |                                            |
| <i>Physella acuta</i>           | X                                          | X                                          |
| Planorbidae                     |                                            |                                            |
| <i>Biomphalaria grablata</i>    | X                                          |                                            |
| <i>Biomphalaria pfeifferi</i>   | X                                          | X                                          |
| <i>Gyraulus connollyi</i>       | X                                          |                                            |
| <i>Gyraulus costulatus</i>      | X                                          | X                                          |
| Thiaridae                       |                                            |                                            |
| <i>Melanoides tuberculata</i>   | X                                          | X                                          |
| <i>Melanoides victoriae</i>     | X                                          |                                            |
| <i>Tarebia granifera</i>        |                                            | X                                          |

#### 4.4.2 High flow season sampling

During the high flow sampling period, 2993 molluscan individuals from two genera were collected from eight sites within the Limpopo, North West and Mpumalanga provinces of South Africa. The most abundant species present was the invasive *T. granifera* (n=2059 individuals, totalling 68.8% of specimens collected during the high flow sampling period) collected at all the high flow sites. The invasive species *P. acuta* (n=2, 0,1%) was only found at one of the high flow sites (site LHF7), and *Melanoides tuberculata* (n=932, 31.1%) was collected at a single site (LHF3) where few *T. granifera* were present (only five individuals). The high flow sites were all river sites with little to no marginal-, emergent, submerged vegetation present. Each site had a single dominant taxa present, with six of the eight high flow sites (LHF1,-2,-4,-5,-6,-8;), only having one molluscan taxon present, namely *T. granifera*. The LHF3 and LHF7 sites had a second taxa present, namely the native species *M. tuberculata* or the invasive *P. acuta*, in addition to *T. granifera*. Where a second taxon was collected during the high flow sampling period, one of the two taxa was always at a lower abundance and the other in a high abundance. At site LHF3, two species were collected: *M. tuberculata* was in high abundance, with 932 individuals collected and *T. granifera* in low abundance, with five individuals collected; at site LHF7, the opposite was found: with *T. granifera* in high abundance, with 139 individuals collected and *P. acuta* in low abundance, with two individuals collected.

#### 4.4.3 Low flow season sampling

A total of 910 molluscan individuals from six different genera were collected during the low flow sampling period. Species were identified with the aid of morphological and molecular methods (Discussed in chapter 3) and were identified to be members of the *B. africanus* group (containing *B. globosus* and *B. africanus*), *B. tropicus/truncatus* group (containing *B. tropicus*, *B. truncatus*, *B. natalensis* and *B. depressus*), *P. acuta*, *B. pfeifferi*, *R. natalensis*, *P. columella*, *G. costulatus* and *T. granifera* species. Of these, species from three genera were considered invasive (*P. acuta*, *P. columella* and *T. granifera*) and five were considered native (*B. africanus*, *B. depressus*, *G. costulatus*, *B. pfeifferi*, *R. natalensis*). The most abundant native molluscan species collected across all the low flow sites were identified to be *Radix natalensis* (n=221 individuals, 24% of total specimens collected in the low flow sampling period) collected at sites LLF1,-3,-4,-5,-6,-7,-8,-9,-10,-11,-14 with *Bulinus* spp. (n=110, 12% of total specimens collected in the low flow sampling period) being the least abundant native molluscan species collected in the same period at sites LLF 6,-7,-11,-12,-13,-14. The site LLF16 had the highest species diversity, with four different genera collected. Two are known invasive species (*P. columella* and *P. acuta*) and two native species (*Bulinus* spp. and *G. costulatus*). Site LLF1 had the lowest species diversity, with only two individuals of *R. natalensis* present. Sites LLF3, -4, and -5 also only had one molluscan genus

present, namely *R. natalensis*, but in higher abundance than site LLF1, with an average of 34 specimens at the three low flow sites. The highest number of individuals was collected at site LLF2, with 121 individuals of *P. acuta* and 111 individuals of *B. pfeifferi*.

The invasive molluscan species *T. granifera* was only collected at two sites (LLF12, -13) during the low flow sampling period, with only 16 individuals found at each site. Generally, during the low flow sampling period, sites with *T. granifera* indicated lower species diversity and abundance. In addition to *T. granifera*, two other genera were present at each site. At LLF12 *B. africanus* and *G. costulatus* were present (24 and 80 individuals respectively); at LLF13, *B. africanus* and *P. columella* was present (one and six individuals respectively).

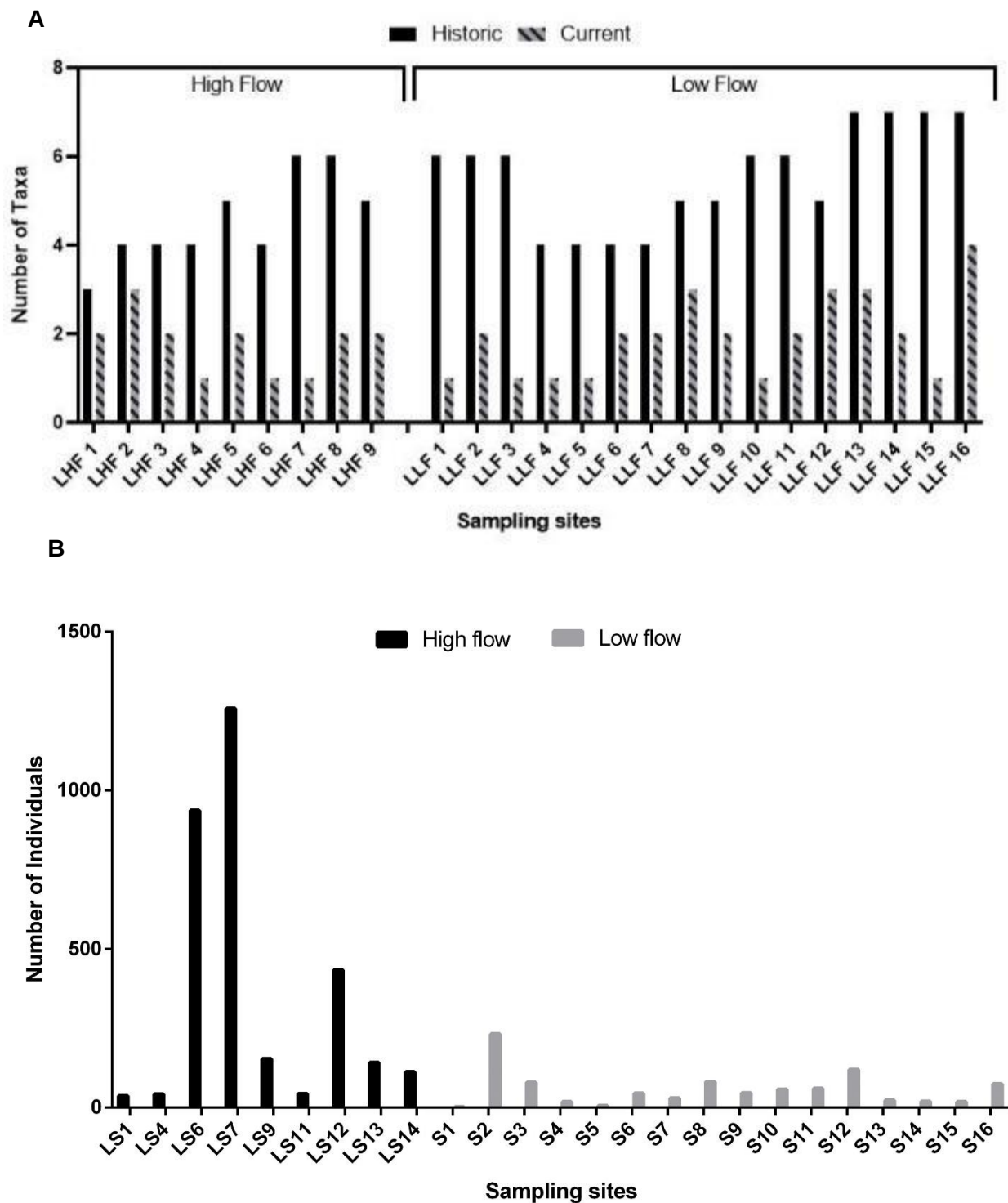


Figure 4.1 Graph indicating (A) the total number of genera historically collected at each site (retrieved from the NFSC), compared to the current number of genera collected during this study and (B) the number of individuals collected during the high flow sampling (LHF) (April-May 2021) and low flow sampling (LLF) (August 2021) respectively at the 25 selected sites within the north-eastern provinces, South Africa.

#### 4.5 Comparison of high flow and low flow sampling sites

The non-metric Multidimensional scaling (nMDS) plot indicated a separation between the high flow sites and the sites sampled during the low flow period (Figure 4.2). The analysis of similarities (ANOSIM) further determined that there was a substantial difference between these groups based on the *a priori* factor “Flow” (R-value = 0.625) and that these differences were significant (significance level percentage = 0.1%).

The canonical correspondence analysis (CCA) indicated a clear separation between the sites sampled during the high flow season compared to those sampled during the low flow season (Figure 4.3). The CCA explained 44.06% of data variation, with 26.91% explained on the first axis and 17.15% explained on the second axis. Indicating sites sampled during the high flow grouped together and apart from sites sampled in the low flow and separation between the sampling seasons resulted from differences in molluscan diversity, water chemistry and habitat. The variables driving the separation between sites were the overwhelming presence of the invasive species *T. granifera* and the absence of native species during the high flow period. As well as the differences in phosphate, nitrate, temperature and oxygen saturation between the high- and low flow sites. Species diversity during the high flow sampling season was lower, with only three genera collected, namely *T. granifera*, *M. tuberculata* and *P. acuta*, of which two, namely *T. granifera* and *P. acuta*, are considered invasive to South Africa, and *M. tuberculata* is regarded as native to South Africa. The invasive *T. granifera* was also extremely dominant during the high flow sampling period, reaching an average of up to 241 individuals per site during this period. The native *M. tuberculata* was only collected during the high flow season at one site LHF3 in high abundance (932 individuals), the only site sampled during the high flow sampling period where *T. granifera* was collected at the lowest abundance (five individuals).

At the sites sampled during the low flow sampling period, six different genera were collected, ranging from an average of 58 individuals at each of the sites sampled during the low flow sampling period. There was no clear dominance of one species with the four invasive species (*P. acuta*, *P. columella*, *G. costulatus* and *T. granifera*) and three native species (*Bulinus spp.*, *G. costulatus*, *R. natalensis* and *B. pfeifferi*) collected during the low flow sampling period. The highest number of individuals collected at one site was 121 individuals of *P. acuta* at site LLF2. *Radix natalensis* was the most commonly found between the 16 sites sampled during this period (LLF1,-3,-4,-5,-6,-7,-8,-9,-10,-12,-14).

Qualitative habitat assessment of substrate and aquatic vegetation indicated that sites sampled during the study had substantial differences in aquatic vegetation present (see Appendix, Table A1). Sites sampled during the high flow sampling period had less vegetation present, and the

substrate was predominantly sand. No floating and submerged vegetation were present at the high flow sites. Algae were only observed at high flow sites LHF1 and LHF 7. In contrast, the assessment of sites sampled during the low flow had a higher presence of aquatic vegetation. Floating vegetation, predominantly *Pontederia crassipes* (water hyacinth), was present at 12 of the 16 low flow sampling sites, with sites LLF1,-5,-10 and LLF13 having an absence of floating vegetation. Algae were present at 14 of the 16 sites sampled during this period, with only LLF1 and LLF3 being absent of algae.

There was a distinct separation between the sampling sites based on the presence or absence of the invasive snail species *T. granifera*, with two groups forming on the nMDS (Figure 4.4). The analysis of similarities (ANOSIM) indicated that these groups were substantially different (R-value = 0.764) and significant (significance level percentage = 0.1%). Sites where *T. granifera* were present, distinctly separated from those where *T. granifera* were absent, regardless of sampling season. This is evident as two sites from the low flow period (LLF12 and LLF13) were the only two sites in the low flow where *T. granifera* was collected, grouped with all the high flow sampling sites where *T. granifera* was present.

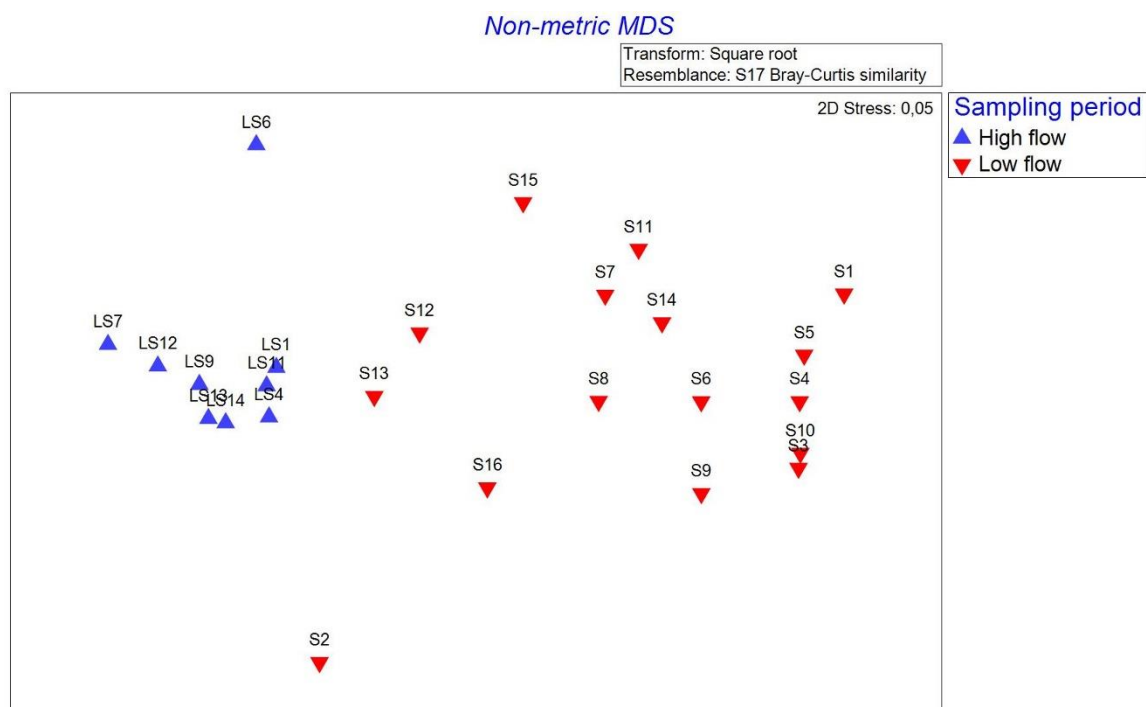


Figure 4.2: Non-Metric Multidimensional Scaling (nMDS) indicating, differences in molluscan species diversity and abundance during the high flow sampling (LHF) (April-May 2021) and low flow sampling (LLF) (August 2021), respectively, at the 25 selected sites within the north-eastern provinces.

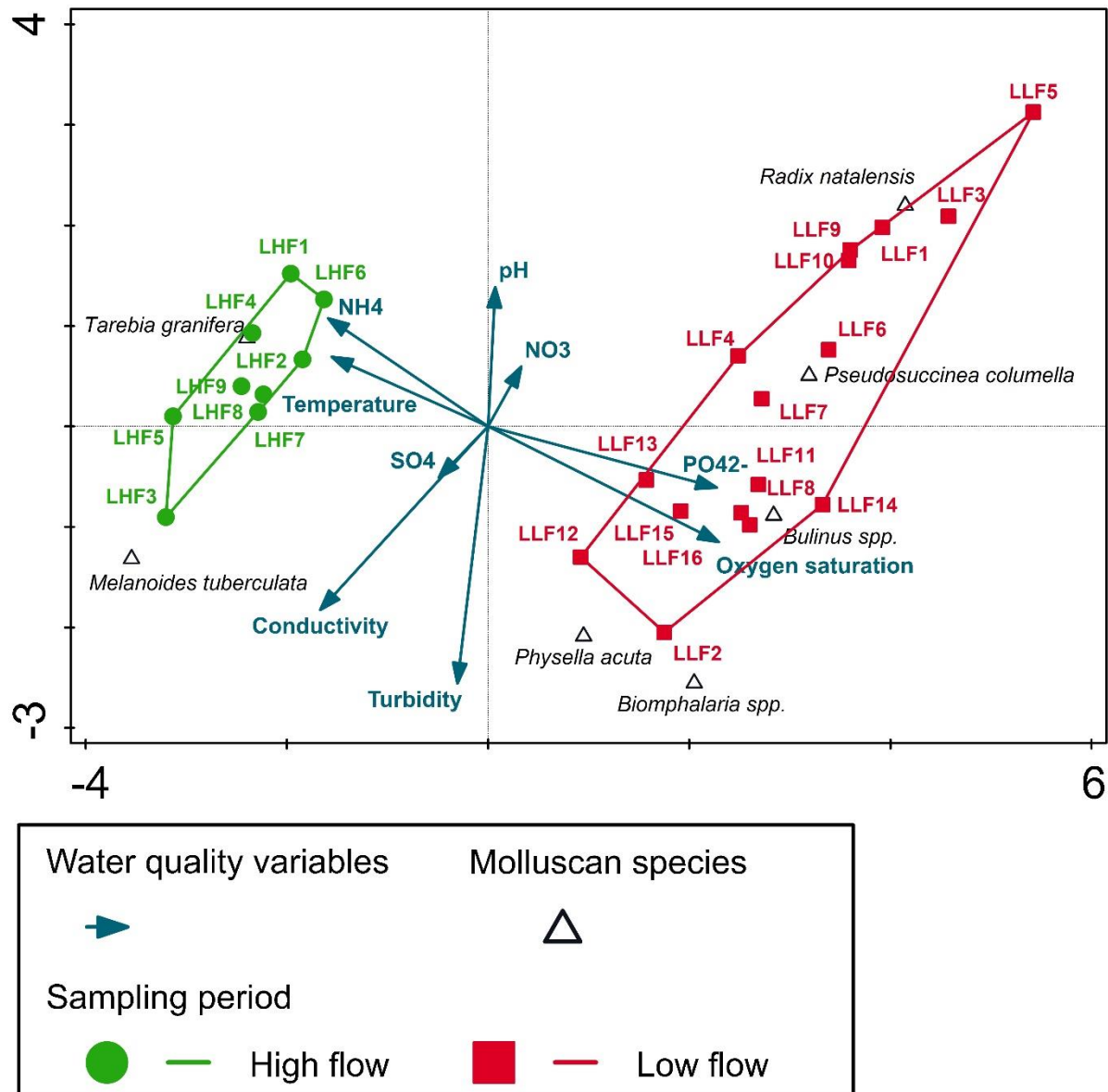


Figure 4.3: Canonical correspondence analysis (CCA) indicating differences between sites sampled during the high flow sampling (LHF) (April-May 2021) and low flow sampling (LLF) (August 2021) respectively, at the 25 selected sites within the north-eastern provinces in terms of snail diversity and select water quality variables. Green circles and lines indicate sites sampled during the high flow season. Red squares and lines indicate the sites sampled during the low flow season. Blue arrows indicate water quality parameters, and black triangles indicate molluscan species collected. The CCA explains 44.06% of data variation, with axis 1 explaining 26.91% and axis 2 explaining 17.15%.

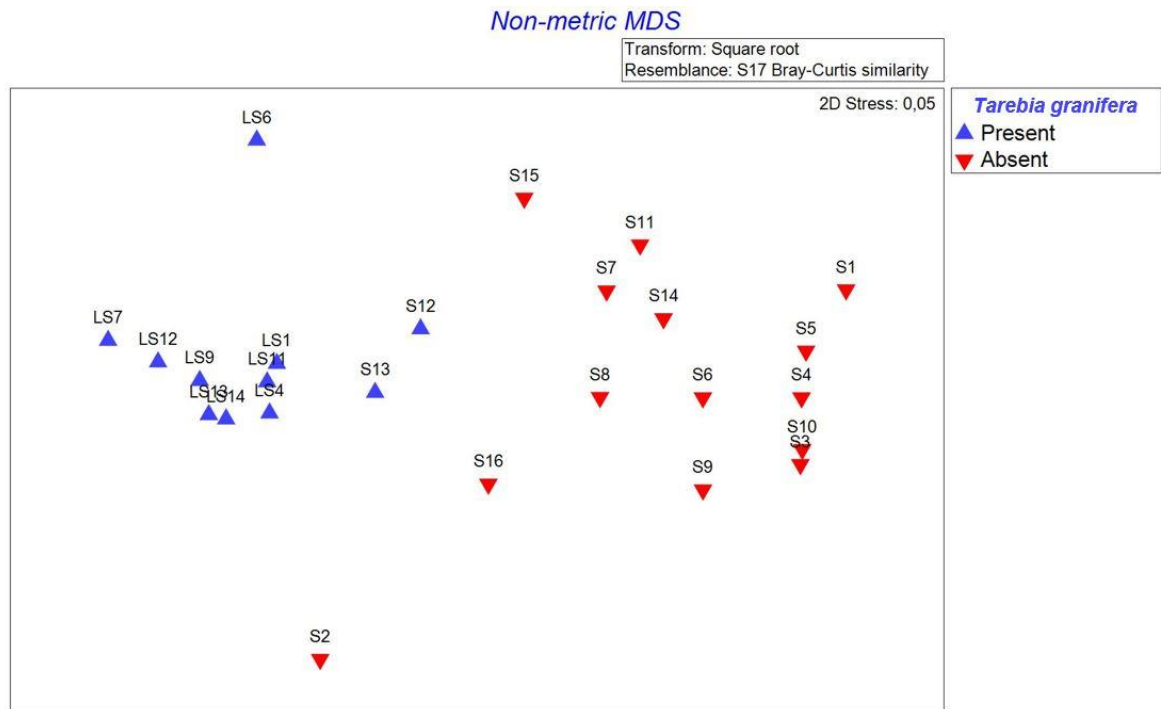


Figure 4.4: Non-Metric Multidimensional Scaling (NMDS) indicating the presence of the invasive *T. granifera* and their effect on species distribution between sites sampled during the high flow sampling (LHF) (April-May 2021) and low flow sampling (LLF) (August 2021) respectively at the 25 selected sites within the north-eastern provinces.

## 4.6 Discussion

The aim of this study was to investigate the current distribution of freshwater molluscan species within the north-eastern region of South Africa and compare historical data available from the NFSC database of species collected during the 1960s-1980s. Findings from this study indicated that there has been a decrease in molluscan diversity since historical sampling took place. Results further indicated that seasonal differences and water chemistry significantly affected molluscan species diversity between sampling seasons. Additionally, one of the most critical drivers identified to affect molluscan species diversity and abundance during the current study was the invasive species *T. granifera*.

### 4.6.1 Current molluscan distribution and the threat of invasive molluscan species

The current molluscan species distribution within the north-eastern provinces collected during this study was eight taxa of molluscan specimens and included *Physella*, *Tarebia*, *Melanoides*, *Radix*, *Pseudosuccinea*, *Bulinus*, *Gyraulus* and *Biomphalaria*. Molluscs native to South Africa and the north-eastern provinces that were historically recorded but absent in the recent study are included in Table 4.4 (De Kock & Wolmarans, 2005, 2009; Foord & Fouché, 2010). The expected species that were historically collected within the area of the selected sites of the current study were, to a great extent, absent. One invasive species collected in the present study that was not historically present is *T. granifera*. The invasive species *T. granifera* was first reported at a concrete-lined reservoir in KwaZulu-Natal, South Africa, in 1999. However, the actual date of introduction is theorized to be earlier than 1996 (Appleton *et al.*, 2009). The impact of this invasive species is believed to be detrimental to native species diversity and abundance and has a bottom-up effect on the natural food web (Appleton *et al.*, 2009). Using *T. granifera* as determining factor indicated that all the sites where *T. granifera* was present were grouped together, signifying that the presence or absence of this invasive snail likely has a negative ecological effect on the native species diversity and abundance, reducing and, in some cases eliminating native species (Appleton, 2003; De Kock *et al.*, 2008; Appleton *et al.*, 2009; Appleton *et al.*, 2015a). In all the sites where *T. granifera* was sampled, the invasive was in exponentially higher abundance.

Similarly, Hill *et al.* (2015) noted that in habitats where *T. granifera* was present together with endemic species, the invasive (*T. granifera*) was numerically higher in abundance than the native species. A paper by Pointier (1999) reported the presence of the invasive mollusc *T. granifera* has resulted in decreased populations and, in some cases, the disappearance of the endemic molluscan species *B. grablata* in habitats affected by *T. granifera* in Puerto Rico and Venezuela. The effect of *T. granifera* numerically dominating endemic taxa and the absence of natural predators could have a top-down influence on the food web within systems where they are present

and affect resource competition between native and invasive molluscan species (Appleton *et al.*, 2009; Hill *et al.*, 2015).

#### **4.6.2 Environmental factors affecting native molluscan distribution**

The cumulative effect of habitat loss due to the anthropogenic effects of a growing population, increased water use and climate change is affecting molluscan distribution (Dudgeon *et al.*, 2006; Jurkiewicz-Karnkowska, 2011). This was evident in the current study by the decrease in native molluscan species from the historically recorded molluscan distribution. The qualitative habitat assessment (Table A1) indicated that aquatic vegetation, a food source for grazing aquatic molluscan species, was largely absent during the high flow sampling period, possibly contributing to reduced species diversity as observed during this season. This was similar to the results obtained by Jurkiewicz-Karnkowska (2009; 2011), that identified a large decrease in water flow during the seasonal transitioning period resulted in early stages of succession, that negatively affected species composition (Jurkiewicz-Karnkowska, 2009). Increased sediment cover has also been shown to negatively affect macroinvertebrate communities, thus indicating that the removal or loss of aquatic vegetation negatively affects the diversity and abundance of aquatic macroinvertebrates, including molluscs, due to the loss of vegetation, reducing the available suitable habitat for molluscan species (Davis *et al.*, 2018).

#### **4.6.3 Water chemistry**

Sites sampled during the high flow seasons' N:P ratio indicated that most of the high flow sites were in an oligotrophic state, with only three sites being slightly impacted according to the N:P ratio (DWAF, 1996). The oligotrophic state is associated with low levels of nutrients and algae. The opposite was observed during the low flow season, with most of the sites indicating all the sites being in a mesotrophic state based on the total nitrogen within the water column. According to Jurkiewicz-Karnkowska (2011), an aquatic ecosystem's nitrogen, phosphate and pH concentrations remarkably influence species abundance. Although the temperature and pH of the sites were within the optimal range for native species to be present in high abundance, this was not the case as a low abundance of native species was identified, particularly at the high flow sites. Temperature and salinity ranges for non-native species are, in some instances, higher for certain non-native species than for more sensitive endemic species (Verbrugge *et al.*, 2012). This was evident during the current study, where salinity was exponentially higher across all the sites sampled during the high flow season. Only one native molluscan species was present during the high flow period, *M. tuberculata*, and two invasive species were present, namely *T. granifera*, observed in high abundance (average of 242 individuals per site), and *P. acuta*, present in low abundance (two individuals) at only one of the high flow sites. Therefore, suggesting that invasive

species can better adapt to adverse environmental conditions and this allows them to out compete native species and spread faster through the ecosystem (Pearson, 2022).

#### **4.7 Conclusion**

This study aimed to first assess the current distribution of both native and invasive freshwater gastropod species within the north-eastern provinces of South Africa, based on sites of historical importance from the NFSC database of South Africa. The second aim was to determine the impact of naturogenic and anthropogenic factors on freshwater gastropod diversity, abundance, and distribution by conducting a qualitative habitat assessment and water nutrient analysis. Comparing the current distribution to the historically recorded specimens in the NFSC database suggested that native genera have decreased in spatial distribution and abundance. This is possibly due to habitat disruption due to anthropogenic factors and increased water use, causing changes in water quality and the presence of invasive molluscan species competing for the same ecological niche or a combination of multiple factors.

The combined effect of increased environmental and anthropogenic pressures on native species is of concern to the future health of the natural ecosystem function within the north-eastern provinces. The detrimental effects of a bottom-up alteration to the food web and the ecological and economic implications will be irreparable. The ability of *T. granifera* to rapidly spread through the aquatic ecosystem, resist unfavourable conditions that are detrimental to native molluscan species, the lack natural predators is of concern for the native species. Without intervention, this invasive species has the potential to completely displace many of the native freshwater snail species in the Lowveld ecoregion of southern Africa. The continuation of this trend will most likely result in the extinction of the native species. The possibility of invasive species disrupting the local food web remains a constant concern that needs to be thoroughly investigated. However, the loss of native snail species also has potential positive effects on the ecosystem. To date, no parasitic infections have been found in *T. granifera* in South Africa; it is thus not a known vector for any digenean parasitic species in South Africa and may therefore reduce the presence of medically important trematode diseases such as schistosomiasis. This may have a positive effect on the human health aspect in the area of invasion. However, the ecological effect of the removal of native freshwater snail species is not known and may have a bottom-up effect on the ecosystem function and food-web.

## **Chapter 5 Parasitic trematode diversity within freshwater molluscan vectors collected in the north-eastern provinces of South Africa.**

### **5.1 Introduction**

Freshwater molluscs function as an important intermediate host (vector) for most parasitic trematodes affecting human and animal health worldwide (Schols *et al.*, 2020). Parasitic trematodes mostly have a multi-stage life cycle that includes the presence of a suitable molluscan vector species and the presence of a suitable definitive human or vertebrate host (Schols, 2019). Many of these trematode species are of veterinary, economical and medical importance, substantially affecting the local population and economic stability of the region where they are located (King, 2010; Schols, 2019). Trematodes of medical and veterinary importance include members of the genera *Schistosoma*, *Fasciola* and *Echinostoma*, which infect humans and livestock. Until recently, a low infection of trematode parasites was thought to have little to no effect on the health of their hosts and did not require medical treatment. Recent studies, however, have indicated that the presence and intensity of parasitic infection increase the risk of morbidity due to the increased risk of chronic disease development (King, 2010; Lacorcia *et al.*, 2018).

Moreover, immunological research has indicated a correlation between chronic trematode infection and reduced protective responses against unrelated bacterial and viral infections (Malhortra *et al.*, 1999; LaBeaud *et al.*, 2009; King, 2010; Lacorcia & Prazeres da Costa, 2018). Therefore, undiagnosed trematode infections directly affect food security and decrease the quality of life and life expectancy of populations near the affected area (King, 2010; Lacorcia & Prazeres da Costa, 2018). For this reason, research has focussed on the above-mentioned three trematode species because they cause such severe diseases in humans and animals, while there has been a lack of research on other parasitic species that are found in aquatic ecosystems (Colley *et al.*, 2014; Adenowo *et al.*, 2015).

It is thus important to analyse the current distribution of parasitic trematodes that infect molluscan species, as climate change and anthropogenic factors can alter the availability of (1) suitable vectors and (2) allow vectors to spread to areas that historically were unsuitable for the vectors and associated trematode species. However, as no cercarial shedding was observed (see results below) and no visible cyst was found in dissected snails, the objectives were adjusted to only focus on the molecular screening of the molluscan species for trematode infections.

## **5.2 Aims and Objectives**

This chapter aimed to assess the parasitic trematode species and parasitic load within molluscan species collected from the north-eastern provinces of South Africa. The first objectives of this section of the study was to assess the parasitic load of select freshwater molluscs by examining the number of cercariae shed as well as the prevalence of infection within molluscan members during the cercarial shedding experiment. The second objective was to identify digenean trematode infections through morphological and molecular analyses of the cercarial output by molluscan species. The third objective was to molecularly assess molluscan tissue taken from the foot of the molluscan species and hepatopancreas were to molecularly analysed for the presence of prepatent digenean trematode infections.

## **5.3 Materials and methods**

Throughout this chapter, shedding experimentation as well as molecular analyses were conducted following methods described by Schols (2019), Schols *et al.* (2019) and Hoogendoorn *et al.* (2019). All analyses were done using the methodology explained in Chapter 2.

## 5.4 Results

### 5.4.1 Cercarial shedding

In total 910 specimens of freshwater molluscan members of the *Bulinus*, *Biomphalaria*, *Radix* and *Pseudosuccinea* genera were used to screen for signs of cercarial shedding. No cercarial shedding was observed during a 48-hour light and dark exposure test. After the negative results were obtained from the cercarial shedding experiment, specimens were euthanised and dissected, and the molluscan species' soft tissue was inspected for signs of infection. No visible cysts were observed during the inspection of soft tissue. Samples were therefore prepared for molecular analyses to determine if prepatent trematode infection(s) were present.

Molecular analyses of the tissue of representatives of *Biomphalaria* and *Bulinus* were negative for *Schistosoma* infection. The presence of *Fasciola hepatica* was not detected within species of *Pseudosuccinea* and *Radix*, its known molluscan intermediate hosts, collected during this study. Due to none of the known vectors indicating signs of infection with the cercarial shedding, nor could these species be detected by molecular methods. However, three trematode infections were detected within samples of *Bulinus* spp. *Neofibricola smiti* was molecularly detected in one specimen of *B. globosus* collected at site LLF14. Whereas, two infections *Petasiger variospinosus* was detected in two specimens of *B. depressus* collected at site LLF6 and LLF7. In addition, *Orientocreadium batrachoides* was molecularly detected in two specimens of *Radix natalensis* collected at site LLF6.

#### 5.4.2 *Neofibricola smiti* Achatz, Martens, Kudlai, Junker, Boe & Tkach, 2022

The 28S rRNA sequence isolated from the *B. africanus* specimen collected at site LLF 14 (Figure 2.1) was identical to that of *N. smiti* when compared to sequences published on GenBank. The phylogeny of the *Neofibricola* dataset (Figure 5.1) was validated by good nodal support with Bayesian inference of 0.85 out of a maximum score of 1 and a maximum likelihood of 96 out of a maximum score of 100.

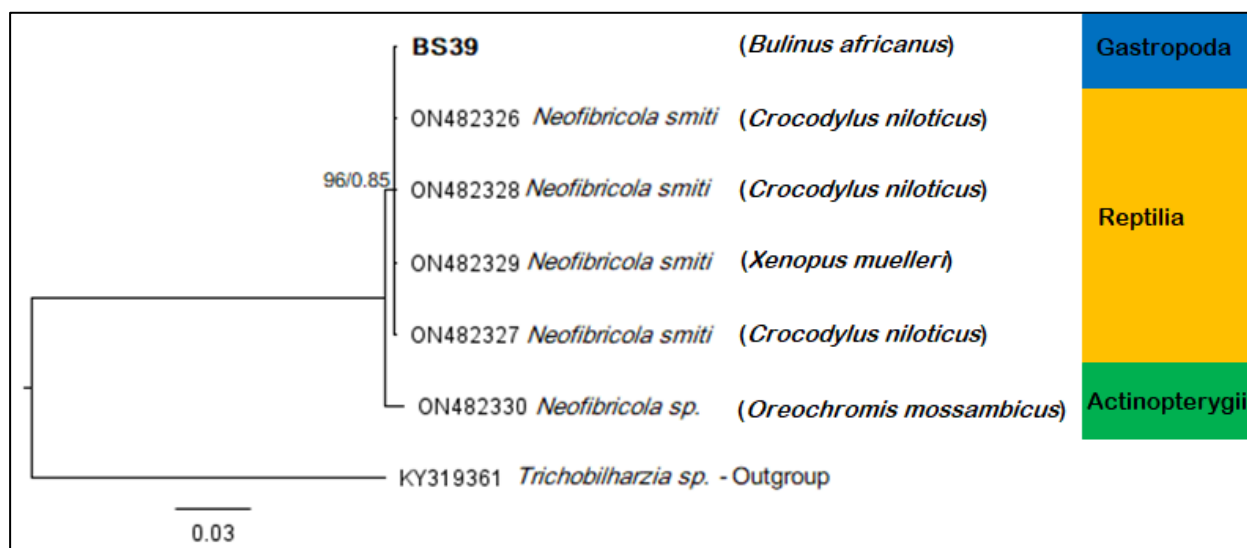


Figure 5.1: Bayesian inference phylogram of selected *Neofibricola* taxa based on partial sequences of the 28S rDNA gene. Newly sequenced species are highlighted in bold. The host/vector species are indicated in brackets, and the hosts class is given. The Posterior probability followed by bootstrap support values are indicated next to the branches (posterior bootstrap < 70 and probability < 0.85 not shown). *Trichobilharzia sp.* was used as an outgroup. The branch length scale bar indicates the number of substitutions per site.

The identification of *N. smiti* was supported by the pairwise genetic distance (p-distance) of 0% pairwise difference indicated in Table 5.1. The base pair differences between the sequence generated during this study and the sequences of *N. smiti* published on GenBank indicated zero base pair differences. The interspecific variation for *Neofibricola* species in this dataset is 0–1% (0–12 nt).

Table 5.1: Nucleotide genetic divergence among *Neofibricola* included in the molecular phylogenetic analyses of the 28S rDNA. Values above the diagonal are expressed in the percentage pairwise genetic distance (p-distance), while values below the diagonal represent the number of base pair differences. The analysis involved seven nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1209 positions in the final dataset.

| Species name |                            | Sequence number | 1   | 2   | 3   | 4   | 5   | 6   | 7   |
|--------------|----------------------------|-----------------|-----|-----|-----|-----|-----|-----|-----|
| 1            | <i>Neofibricola smiti</i>  | <b>BS39</b>     |     | 0%  | 0%  | 0%  | 0%  | 1%  | 28% |
| 2            | <i>Neofibricola smiti</i>  | ON482326        | 0   |     | 0%  | 0%  | 0%  | 1%  | 28% |
| 3            | <i>Neofibricola smiti</i>  | ON482328        | 0   | 0   |     | 0%  | 0%  | 1%  | 28% |
| 4            | <i>Neofibricola smiti</i>  | ON482329        | 0   | 0   | 0   |     | 0%  | 1%  | 28% |
| 5            | <i>Neofibricola smiti</i>  | ON482327        | 0   | 0   | 0   | 0   |     | 1%  | 28% |
| 6            | <i>Neofibricola sp.</i>    | ON482330        | 12  | 12  | 12  | 12  | 12  |     | 29% |
| 7            | <i>Trichobilharzia sp.</i> | KY319361        | 320 | 320 | 320 | 320 | 320 | 322 |     |

#### 5.4.3 *Petasiger variospinosus* (Odhner, 1910) Yamaguti, 1933

The 28S rRNA sequence isolated from the *B. depressus* specimens collected at sites LLF 6 (sample BS62) and LLF 7 (Sample BS92) was identical to that of *Petasiger* sp.5 when compared to sequences published on GenBank (NCBI, <https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Following its submission to GenBank, *Petasiger* sp. 5 was morphologically identified to be *Petasiger variospinosus* ((Odhner, 1910) Yamaguti, 1933) by Hammoud *et al.* (2022). The phylogeny of *P. variospinosus* dataset (Table 5.2) indicates good nodal support for Bayesian inference and maximum likelihood analyses, with Bayesian inference of 1 out of a maximum score of 1 and a maximum likelihood of 99 out of a maximum score of 100.

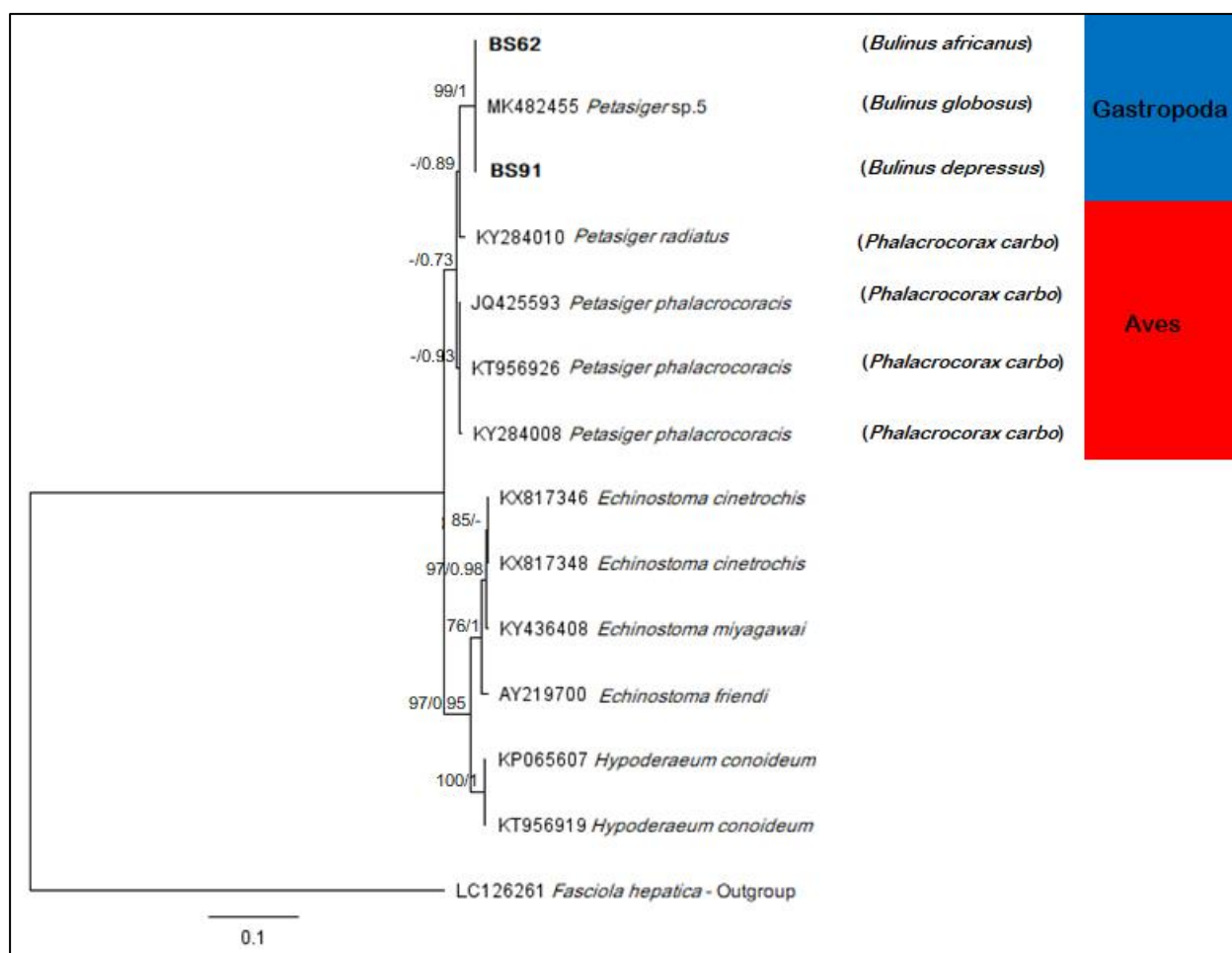


Figure 5.2: Bayesian inference phylogram of selected *Petasiger*, *Echinostoma* and *Hypoderaeum* taxa based on partial sequences of the 28S rDNA gene. Newly sequenced species are highlighted in bold. The host/vector species are indicated in brackets, and the hosts class is given. Posterior probability followed by bootstrap support values is indicated next to the branches (posterior bootstrap < 70 and probability < 0.85 not shown). *Fasciola hepatica* was used as an outgroup. The branch length scale bar indicates the number of substitutions per site.

The identification of *Petasiger variospinosus* was supported by the pairwise genetic distance (p-distance) of 0% pairwise difference indicated in Table 5.3. The analyses indicate zero base pair differences between specimen BS62 collected at site LLF6 and *Petasiger* sp. 5, and only one base pair difference between specimen BS91 collected at site LLF7 and *Petasiger* sp. 5. The newly generated sequences differed by one base pair. The overall interspecific variation of *Petasiger* species is 0–3–% (0–26 nt).

Table 5.2: Nucleotide genetic divergence among *Petasiger*, *Echinostoma* and *Hypoderaeum* taxa included in the molecular phylogenetic analyses of the 28S rDNA. Values above the diagonal are expressed in the percentage pairwise genetic distance (p-distance), while values below the diagonal represent the number of differences. The analyses involved 14 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1014 positions in the final dataset.

| Species name |                                  | Sequence number | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  | 13  | 14  |
|--------------|----------------------------------|-----------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 1            | <i>Echinostoma friedi</i>        | AY219700        |     | 1%  | 1%  | 1%  | 4%  | 4%  | 9%  | 9%  | 9%  | 7%  | 7%  | 7%  | 7%  | 97% |
| 2            | <i>Echinostoma cinetorchis</i>   | KX817346        | 7   |     | 0%  | 0%  | 4%  | 4%  | 9%  | 9%  | 9%  | 7%  | 7%  | 7%  | 7%  | 97% |
| 3            | <i>Echinostoma cinetorchis</i>   | KX817348        | 7   | 0   |     | 0%  | 4%  | 4%  | 9%  | 9%  | 9%  | 7%  | 7%  | 7%  | 7%  | 97% |
| 4            | <i>Echinostoma cinetorchis</i>   | KY436408        | 6   | 2   | 2   |     | 4%  | 4%  | 9%  | 9%  | 9%  | 7%  | 7%  | 7%  | 7%  | 97% |
| 5            | <i>Hypoderaeum conoideum</i>     | KP065607        | 18  | 32  | 32  | 32  |     | 0%  | 8%  | 8%  | 9%  | 7%  | 7%  | 7%  | 7%  | 97% |
| 6            | <i>Hypoderaeum conoideum</i>     | KT956919        | 18  | 32  | 32  | 32  | 0   |     | 8%  | 8%  | 9%  | 7%  | 7%  | 7%  | 7%  | 97% |
| 7            | <i>Petasiger variospinosus</i>   | <b>BS62</b>     | 40  | 72  | 72  | 72  | 68  | 68  |     | 0%  | 0%  | 3%  | 3%  | 3%  | 2%  | 96% |
| 8            | <i>Petasiger</i> sp. 5           | MK482455        | 40  | 72  | 72  | 72  | 68  | 68  | 0   |     | 0%  | 3%  | 3%  | 3%  | 2%  | 96% |
| 9            | <i>Petasiger variospinosus</i>   | <b>BS91</b>     | 40  | 73  | 73  | 73  | 69  | 69  | 1   | 1   |     | 3%  | 3%  | 3%  | 2%  | 96% |
| 10           | <i>Petasiger phalacrocoracis</i> | JQ425593        | 34  | 63  | 63  | 63  | 62  | 62  | 24  | 24  | 25  |     | 0%  | 0%  | 1%  | 94% |
| 11           | <i>Petasiger phalacrocoracis</i> | KT956926        | 34  | 63  | 63  | 63  | 62  | 62  | 24  | 24  | 25  | 0   |     | 0%  | 1%  | 94% |
| 12           | <i>Petasiger phalacrocoracis</i> | KY284008        | 34  | 65  | 65  | 65  | 64  | 64  | 26  | 26  | 27  | 2   | 2   |     | 1%  | 94% |
| 13           | <i>Petasiger phalacrocoracis</i> | KY284010        | 35  | 66  | 66  | 66  | 65  | 65  | 21  | 21  | 22  | 11  | 11  | 13  |     | 94% |
| 14           | <i>Fasciola hepatica</i>         | LC126261        | 369 | 786 | 786 | 788 | 784 | 784 | 777 | 777 | 776 | 776 | 776 | 776 | 775 |     |

#### 5.4.4 *Orientocreadium batrachoides* Tubanguí, 1931

The phylogenetic analyses of the 28S rRNA sequence isolated from the *Radix natalensis* specimens collected at site LLF 6 (Figure 2.1) was identified as *Orientocreadium batrachoides* based on comparisons with sequences published on GenBank. The phylogenetic analysis of the *O. batrachoides* dataset (Table 5.3) indicated good nodal support for Bayesian inference and maximum likelihood analysis, with Bayesian inference of 0.85 out of a maximum score of 1 and a maximum likelihood of 96 out of a maximum score of 100.

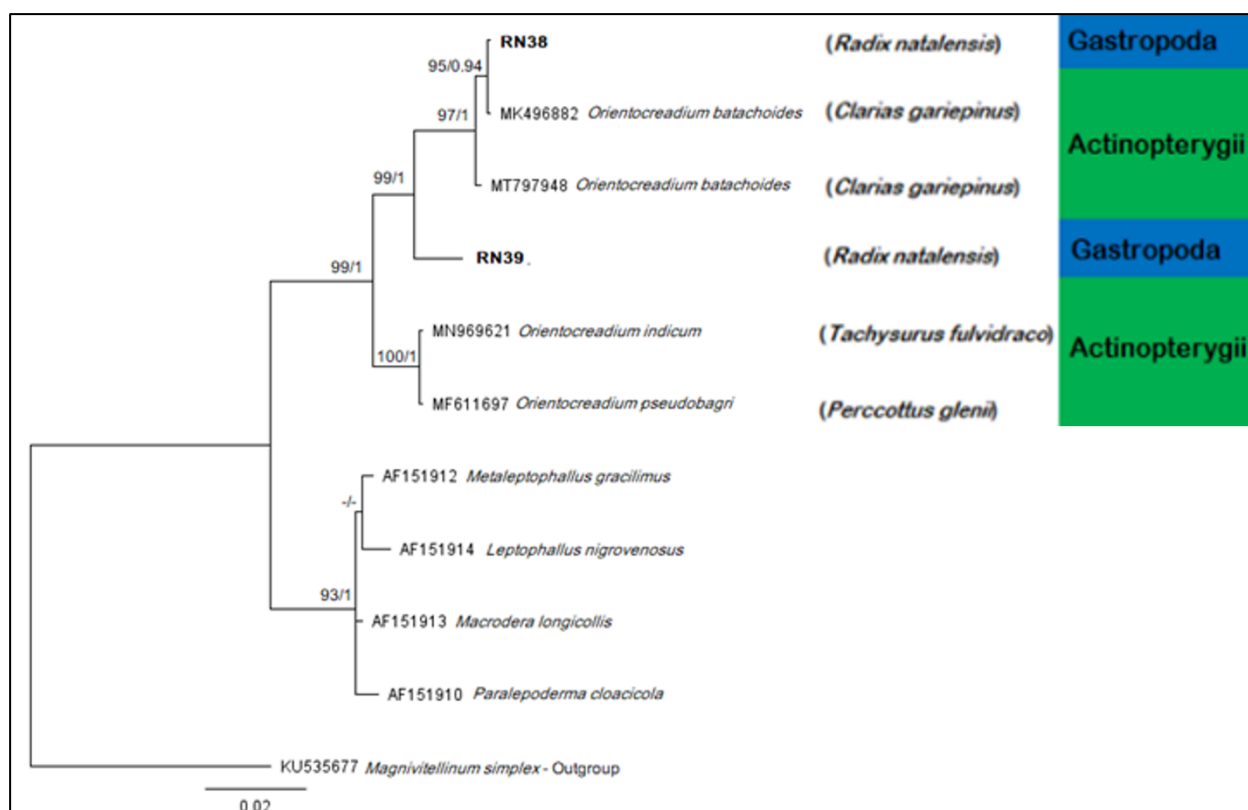


Figure 5.3: Bayesian inference phylogram of selected Orientocreadiidae and Leptophaliidae taxa based on partial sequences of the 28S rDNA gene. Newly sequenced species are highlighted in bold. The host/vector species are indicated in brackets, and the hosts class is given. Posterior probability followed by bootstrap support values is indicated next to the branches (posterior bootstrap < 70 and probability < 0.85 not shown). *Magnivittellum simplex* was used as an outgroup. The branch length scale bar indicates the number of substitutions per site.

The identification of *O. batrachoides* was supported by the pairwise genetic distance (p-distance) of 0% pairwise difference indicated in Table 5.3 above the diagonal for the specimen RN38 collected at site LLF4, identifying it as a sample of *O. batrachoides*. The molecular 28S rRNA sequences generated matched and phylogenetically grouped with sequences published by Dumbo *et al.* (2019) with acceptable branch supports of maximum likelihood and Bayesian inference. Specimen RN39 collected at site LLF6 had a 2-3% percentage pairwise genetic distance to the published sample of *O. batrachoides* (MK496882; MT797948) and 26-27 base pair differences between the samples of *O. batrachoides*.

Table 5.3: Nucleotide genetic divergence among Orientocreadiidae and Leptophaliidae taxa included in the molecular phylogenetic analyses of the 28S rDNA. Values above the diagonal are expressed in the percentage pairwise genetic distance (p-distance), while values below the diagonal represent the number of differences. The analyses involved 11 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1014 positions in the final dataset.

| Species Name |                                     | Sequence number | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  |
|--------------|-------------------------------------|-----------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 1            | <i>Orientocreadium indicum</i>      | <b>RN38</b>     |     | 3%  | 0%  | 0%  | 3%  | 3%  | 6%  | 7%  | 6%  | 7%  | 14% |
| 2            | <i>Orientocreadium indicum</i>      | <b>RN39</b>     | 26  |     | 3%  | 2%  | 3%  | 3%  | 6%  | 6%  | 6%  | 6%  | 13% |
| 3            | <i>Orientocreadium batrachoides</i> | MK496882        | 0   | 26  |     | 0%  | 3%  | 3%  | 6%  | 7%  | 6%  | 7%  | 14% |
| 4            | <i>Orientocreadium batrachoides</i> | MT797948        | 6   | 27  | 6   |     | 3%  | 3%  | 6%  | 7%  | 6%  | 6%  | 14% |
| 5            | <i>Orientocreadium indicum</i>      | MN969621        | 38  | 31  | 38  | 32  |     | 0%  | 5%  | 5%  | 5%  | 5%  | 13% |
| 6            | <i>Orientocreadium pseudobagri</i>  | MF611697        | 38  | 31  | 38  | 32  | 0   |     | 5%  | 5%  | 5%  | 5%  | 13% |
| 7            | <i>Metaleptophallus gracillimus</i> | AF151912        | 71  | 61  | 71  | 65  | 53  | 53  |     | 1%  | 1%  | 1%  | 12% |
| 8            | <i>Leptophallus nigrovenosus</i>    | AF151914        | 70  | 62  | 70  | 65  | 55  | 55  | 8   |     | 1%  | 1%  | 12% |
| 9            | <i>Macrodera longicollis</i>        | AF151913        | 70  | 60  | 70  | 65  | 54  | 54  | 5   | 9   |     | 1%  | 11% |
| 10           | <i>Paralepoderma cloacicola</i>     | AF151910        | 72  | 60  | 72  | 67  | 57  | 57  | 8   | 12  | 7   |     | 12% |
| 11           | <i>Magnivitellinum simplex</i>      | KU535677        | 137 | 128 | 137 | 132 | 122 | 122 | 111 | 116 | 113 | 114 |     |

## 5.5 Discussion

Molluscan species subjected to shedding experiments were unsuccessful and indicated no signs of cercarial shedding of parasitic digenean trematodes. The complex life cycle of species-specific trematodes that are native to the north-eastern South African region that is known to infect molluscan species could be absent from molluscan vectors due to their complex life cycle (Sacolo-Gwebu *et al.*, 2019). It is known that the neglected tropical disease schistosomiasis, commonly known as bilharzia, is caused by members of the genus *Schistosoma* and is transmitted by the species-specific intermediate hosts (vectors) of the genera *Bulinus* and *Biomphalaria* (De Kock *et al.*, 2004; De Kock & Wolmarans, 2005a). As mentioned in Chapter 4, a total of 476 members of the *Bulinus* and *Biomphalaria* genera were collected during the Limpopo low-flow (LLF) sampling during the August 2021 field collections with no signs of *Schistosoma* infection detected by both molecular methods and shedding experiments. An experiment conducted by De Kock (1973) measuring the ideal temperature for reproduction habits and survival of *B. africanus* under different environmental conditions indicated that survival and reproduction rates of *B. africanus*

were decreased in freshwater ecosystems with an ambient water temperature below 20°C. This concurs with the negative results of this study regarding the infection prevalence of *Schistosoma* intermediate host species during the August 2021 sampling at the end of the South African winter season. This is supported by the low prevalence of *Schistosoma* infected *B. africanus* and *B. globosus* molluscan samples collected during a recent study conducted in the KwaZulu-Natal province, South Africa (Sacolo-Gwebu *et al.*, 2019). The prevalence of *Schistosoma* infections of *Bulinus* species collected in KwaZulu-Natal by Sacolo-Gwebu *et al.* (2019) resulted in a very low prevalence of 1% and 0,9% of *S. haematobium* and *S. mansoni*, respectively, during the shedding experiment. Monitoring of trematode transmission infections becomes less effective as infection rates decrease among definitive hosts in contact with the selected area of study (Abbasi *et al.*, 2010). Therefore, the additional use of molecular PCR tests was used to detect the presence of immature infections, resulting in the detection of three trematode species: *Neofibricola smiti* ex *B. africanus*, *Petasisiger variospinosus* ex *B. depressus*, *O. batrachoides* ex *R. natalensis*.

*Neofibricola smiti* is a digenean trematode of which the adult life stage has been collected from the Nile crocodile (*Crocodylus niloticus* (Laurenti, 1768)) and metacercariae have been collected from the African clawed frog (*Xenopus muelleri* (Peters, 1844)) (Achatz *et al.*, 2022). The identification of *N. smiti* within *B. depressus* is the first report of the infection of this trematode species within a freshwater mollusc, thus providing valuable information on the life cycle and hosts of this recently described species. This study also provides the first molecular characterisation of the life stages of *N. smiti* from freshwater molluscan specimens of the genus *Bulinus*.

Another trematode species collected from the Limpopo Lowveld ecoregion was molecularly identified as *P. variospinosus*, which was detected in samples of *B. depressus*. As mentioned, the sequences of what was labelled as *Petasisiger* sp. 5 on GenBank were morphologically identified to be *P. variospinosus* ((Odhner, 1910) Yamaguti, 1933) by Hammoud *et al.* (2022), infecting *B. globosus*, *B. ugandael* and *B. truncatus* collected from Kenya (Laidemitt *et al.*, 2019; Hammoud *et al.*, 2022). It was now also molecularly detected in the current study in *B. depressus* and *B. africanus* from the Limpopo Lowveld ecoregion of South Africa. The life cycle of *P. variospinosus* was experimentally completed by King & Van As (2000). During that study, it was identified that trematode cercariae of *P. variospinosus* infect the molluscan host as the first intermediate host and cercariae released by the molluscan vector infected larvae of *X. laevis* as second intermediate host, before infecting of the definitive avian host.

The third trematode species molecularly identified from snails collected during the study, is *O. batrachoides* from *R. natalensis*. *Orientocreadium batrachoides* is known to use the African sharp-tooth catfish (*Clarias gariepinus*; (Burchell, 1822)) as a definitive host. Little information is

available on the life cycle of *O. batrachoides*. This is the first known molecular characterisation of larval *O. batrachoides* from the freshwater molluscs *B. depressus*

## 5.6 Conclusion

This chapter aimed to examine freshwater molluscan vectors for infections of digenean trematodes and determine the parasitic prevalence within vectors infected with digenean trematodes. This was partially successful with positive parasitic trematodes identified using molecular analyses of the molluscan tissue (hepatopancreas). The cercarial shedding experiments were unsuccessful indicating that molluscan species, which tested positive for the presence of digenean trematodes, had an immature or early-stage infection. The declining survival and reproduction rate of native molluscan species during colder seasons and the life cycle complexity of the *Schistosoma* and *Fasciola* digenean trematodes are possible reasons that these species were not present within the vectors collected.

Positive results of *N. smiti* within specimens of *Bulinus* is the first detection of this digenean trematode within a molluscan host, indicating *B. depressus* as a possible vector for *N. smiti* and producing new insight into the life cycle of *N. smiti*. *Petasisger variospinosus* detected within specimens of *B. depressus* was expected as *B. depressus* forms part of the *B. truncatus/tropicus* group. Therefore, suggesting that *P. variospinosus* is not host-specific within the *B. truncatus/tropicus* group. The addition of the newly generated data on these parasites gives a broader insight into the geographical range of digenean trematodes as well as much-needed insight into the complex life cycles of these parasitic species. The detection of *O. batrachoides* within specimens of *R. natalensis* is also the first report of this digenean trematode within a freshwater molluscan species.

## Chapter 6 Conclusions and recommendations

The accurate assessment of the current status of native molluscan species relies on the accurate identification of molluscan species to the lowest taxonomic level. Currently, the identification of species that share common morphological traits is the subject of a large debate between freshwater ecologists. Therefore, multiple complementary identification methods are required to accurately identify molluscan species to the lowest taxonomic level possible. Broadening the current knowledge and aiding in accurately determining the current status of native species distribution and their associated trematodes. Numerous studies have suggested the threat invasive molluscan species pose to the native molluscan species and the possible benefits of reducing native molluscan species that function as vectors for medically and economically important parasitic trematodes. Therefore, the main aim of this study was to accurately identify molluscan species collected and determine if trematode infections were present within the collected populations, as well as determine factors affecting the current molluscan distribution when compared to historical data collected. This chapter will provide concluding remarks on the main research findings concerning this study's aim and recommendations for future research.

### 6.1 Molluscan species identification (Chapter 3)

The morphological identification of all the *Bulinidae* specimens indicated the presence of two of the four *Bulinus* groups, namely the *B. africanus/globosus* group and the *B. truncatus/tropicus* group. However, further identification based on morphology indicated conflicting results based on the shared morphological traits between species of the same group. Therefore, the molecular analyses of the ITS2 region indicated that specimens belonging to the *B. africanus/globosus* group were identified as *B. africanus* by the ITS2 genetic sequence generated. Unfortunately, the generation of COI genetic sequences from specimens collected during this study belonging to the *B. africanus/globosus* group was unsuccessful.

The molecular analyses of the ITS2 and COI genetic regions of specimens supported the identification of specimens belonging to the *B. truncatus/tropicus* group. However, further analyses of the COI genetic region and phylogenetic analyses indicated that specimens collected did not clade with published sequences of *Bulinus* known to be a part of the *B. truncatus/tropicus* group. Thus, the morphological identification of *B. depressus* was supported by the molecular analyses of the ITS2 region to belong to the *B. truncatus/tropicus* group and further supported by the COI gene phylogenetic analyses by forming a separate clade within the *B. truncatus/tropicus* group and not matching with *B. truncatus*, *B. tropicus* or *B. natalensis*. Thereby confirming the identification of *B. depressus* and generating the first COI gene sequences of *B. depressus*.

Two genera of Planorbidea were morphologically identified to be members of the *Biomphalaria* and *Gyraulus*. The morphological identification of *B. pfeifferi* was successfully confirmed with the molecular analyses of the ITS2 and COI genetic regions. However, the morphological identification of *G. costulatus* was more intricate as *G. costulatus* and *B. grablata* share morphological traits. The small size of the specimens further increased the difficulty of morphological identification. Further investigation identified the specimens in question as *G. costulatus* and was supported by the ITS2 genetic analyses. The COI genetic analyses of *Gyraulus* specimens collected in the North West provinces and during the sampling of the Seekoeivlei Nature Reserve separated genetically between the two sampling areas, indicating the presence of two different species of *Gyraulus* were collected. Due to the absence of published ITS2 and COI genetic sequences for *G. costulatus* and *G. connollyi*, the two species of *Gyraulus* native to South Africa, the molecular analyses were only used to confirm the presence of two different species and was able to confirm the identity of the two species to genus level. Therefore, the identification to species level was based on the morphology of the two different species and successful in identifying the specimens collected at the Seekoeivlei Nature Reserve to be *G. connollyi* and specimens collected at the North West site to be *G. costulatus*. Therefore, the ITS2 and COI genetic analyses resulted in the first sequences generated for both the species identified as *G. connollyi* and *G. costulatus*.

Two species from the Lymnaeidae group were successfully identified as *R. natalensis* and *P. columella* from samples collected. The phylogenetic analyses of the COI gene indicated that *R. natalensis* formed four genetically distinct groups within the *R. natalensis* species clade. When comparing the four groups that formed within the *R. natalensis* species clade to the geographical location of collection, it indicated that similar groups were collected at different sites. Therefore, suggesting that the molluscan specimens of *R. natalensis* possibly used a human or molluscan vector to move to the different locations where the genetically identical groups were collected. The second species collected were identified as *P. columella* and were genetically identical to other specimens collected. Thus, indicating the possibility of the historical invasion of a single specimen or multiple invasions from the same area.

*Physella acuta* was identified to belong to the Africa clade 3, indicating that the specimen is a descendent of the invasion that originated from Northern America.

## **6.2 The current distribution of molluscan species compared to the historical data available for the Limpopo Lowveld ecoregion, South Africa (Chapter 4)**

The current molluscan distribution was compared to the historical data from the NFSC database collected during the 1960s-1980s. The present diversity of molluscan species within the Limpopo

Lowveld ecoregion indicated a drastic decline in species diversity, with an increased presence of invasive molluscan species and the identification of *T. granifera*. Historical data indicated that *T. granifera* was not present in the current area. The rapid spread of *T. granifera* from the date the invasive was first reported in the Kwazulu-Natal province in 1999, to the current identification of this invasive in the northern parts of South Africa is a cause for concern. The results indicated that sites, where *T. granifera* was present had low molluscan diversity and abundant native species. Suggesting that *T. granifera* outcompetes the native molluscan species for the same ecological niche. The cumulative effect of habitat loss due to flooding events and anthropogenic activities affecting the freshwater environment was a contributing factor affecting species diversity and availability. However, *T. granifera* is the molluscan species with the highest abundance during the high flow season. Indicating the ability of this invasive species to rapidly adjust to changing environmental factors and exploit the suitable habitat available. During the low flow season, *T. granifera* were present in lower abundance, possibly indicating that the lack of adverse weather events, such as floods or droughts, reduces the ability of *T. granifera* to out-compete endemic molluscan species. Indicating that the presence of native molluscan species possibly increases the difficulty of invasive species colonising new ecological areas, but the sensitivity of the native molluscan species to adverse ecological events is the main ecological driver that aids in the successful invasion of non-native freshwater molluscan species. The continuation of this trend of invasive species outcompeting native species, reducing diversity and abundance, could lead to the removal of the native molluscan population and have adverse effects on the local ecosystem and food web.

### **6.3 Parasitic trematode diversity within molluscan vectors collected (Chapter 5)**

All molluscan species were screened for parasitic trematode infections using two methods. Firstly, molluscan specimens were placed in separate wells under artificial light for 48 hours to examine specimens for signs of cercarial shedding. Secondly, specimens were dissected and morphologically analysed for prepatent trematode infections that have not reached the appropriate maturity to produce metacercariae. When tested, the cercarial shedding indicated negative results, with none of the molluscan specimens collected showing signs of cercarial shedding. All specimens of *Bulinus* and *Biomphalaria* are known hosts of trematode species causing schistosomiasis and tested negative for the presence of trematodes belonging to *Schistosoma*. However, three species of digenean trematode were identified through molecular analyses of the 28S rDNA gene, indicating the presence of a prepatent infection. The species identified were genetically identified to be *N. smiti*, *P. variospinosus* and *O. batrachoides*. The first identification of prepatent infections of *N. smiti* and *P. variospinosus* within the molluscan

vectors collected provides new insight and the need for further research to be conducted assessing the relationship between digenean trematodes and the molluscan vectors they infect.

#### **6.4 Recommendations**

From the results and findings of this study it is clear that there are scope and potential for future research to further our knowledge on South African freshwater molluscs, the environmental and biological factors that impact on their diversity and distribution and the potential medical and veterinary important trematodes they host. Future work could potentially include the following:

- Conduct more regular sampling of selected sites during different seasons and over a more extended period, for the collection of specimens and to get a better understanding of the ecosystem functions during different seasons. The study over an extended period will give greater insight into invasive molluscan species' impact on the native molluscan species.
- Increase the molluscan species use for identification by a combined approach of morphological and molecular methods to aid in solving the highly debated identification of molluscan species to the lowest taxonomic level and aid in the design of a haplotype network to better understand the relationships among their DNA sequences on a local and international scale.
- Many of the molluscan species collected during this study are known vectors for parasitic trematode species, which were not detected within the current populations collected. The increase of regular sampling across multiple seasons accounts for seasonal changes, will increase the chances of finding infected molluscan species and will give additional insight regarding the parasitic infection of molluscan species as some parasitic trematode species may be sensitive to seasonal changes.
- The formation of genetically identifiable groups of *R. natalensis* between different localities sampled needs further research to determine the factors influencing the genetic mutations visualized by the phylogenetic analyses.

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## Appendix A:

Table A1: Qualitative habitat assessment observed at the Limpopo high and low flow sites collected during 2021 sampling.

| Limpopo high flow sites |           |                                    |                           |                    |                    |          |          |           |       |       |     |
|-------------------------|-----------|------------------------------------|---------------------------|--------------------|--------------------|----------|----------|-----------|-------|-------|-----|
| Date                    | Site code | GPS coordinates                    | Weather                   | Habitat Type       | Habitat Assessment |          |          |           |       |       |     |
|                         |           |                                    |                           |                    | Marginal           | Floating | Emergent | Submerged | Algae | Stone | GSM |
| 2021/04/22              | LHF1      | 22°26'47.9"S<br>28°52'44.6"E       | Sunny, Warm               | Limpopo River      | Yes                | No       | Yes      | No        | Yes   | Yes   | Yes |
| 2021/04/25              | LHF2      | 22°28'54.5"S<br>28°55'07.1"E       | Sunny, Warm               | Mogalakwena        | Yes                | No       | No       | No        | No    | Yes   | Yes |
| 2021/04/26              | LHF3      | 23°56'40.9"S<br>26°55'50.9"E       | Sunny, Warm               | Limpopo River      | No                 | No       | No       | No        | No    | No    | Yes |
| 2021/04/29              | LHF4      | 24°03'14.7"S<br>31°43'35.1"E       | Sunny, Warm               | Olifants River     | Yes                | No       | Yes      | No        | No    | Yes   | No  |
| 2021/05/03              | LHF5      | 23°13'15.8"S<br>31°33'18.4"E       | Sunny, Warm               | Shinqwetsi River   | Yes                | No       | Yes      | No        | No    | No    | Yes |
| 2021/05/04              | LHF6      | 22°25'45.4"S<br>31°15'27.4"E       | Sunny, Warm               | Luvuvhu River      | No                 | No       | No       | No        | No    | Yes   | Yes |
| 2021/05/05              | LHF7      | 23°40'39.1"S<br>31°05'55.1"E       | Sunny, Warm               | Groot Letaba River | Yes                | No       | Yes      | No        | Yes   | Yes   | Yes |
| 2021/05/06              | LHF8      | 23°56'36.2"S<br>31°44'06.4"E       | Sunny, Warm               | Letaba River       | Yes                | No       | Yes      | No        | No    | Yes   | Yes |
| 2021/05/07              | LHF9      | 24°04'00.2"S<br>31°14'33.0"E       | Sunny, Warm               | Olifants River     | Yes                | No       | Yes      | No        | No    | Yes   | Yes |
| Limpopo low flow sites  |           |                                    |                           |                    |                    |          |          |           |       |       |     |
| Date                    | Site code | GPS Coordinates                    | Weather                   | Habitat Type       | Habitat Assessment |          |          |           |       |       |     |
|                         |           |                                    |                           |                    | Marginal           | Floating | Emergent | Submerged | Algae | Stone | GSM |
| 2021/08/23              | LLF1      | 25°31'18.85"S<br>27°22'55.3"E      | Sunny, Warm               | Hex River          | Yes                | No       | No       | Yes       | No    | Yes   | Yes |
| 2021/08/23              | LLF2      | 25°23'0.6936"S<br>27°35'54.8052"E  | Sunny, Warm               | Roodekopjes Dam    | Yes                | Yes      | Yes      | Yes       | Yes   | No    | Yes |
| 2021/08/23              | LLF3      | 25°22'25.3"S<br>27°33'59.6"E       | Sunny with a light breeze | Crocodile River    | Yes                | Yes      | No       | No        | No    | No    | Yes |
| 2021/08/23              | LLF4      | 24°45'43.848"S<br>28°13'3.0324"E   | Sunny, Warm               | Buffelspruit       | Yes                | Yes      | Yes      | Yes       | Yes   | No    | Yes |
| 2021/08/24              | LLF5      | 24°40'14.2716"S<br>28°19'12.8604"E | Overcast & Cool           | Donkerpoort Dam    | Yes                | No       | Yes      | Yes       | Yes   | Yes   | Yes |
| 2021/08/24              | LLF6      | 24°24'31.4208"S<br>28°7'20.2224"E  | Overcast & Cool           | Klein sand River   | Yes                | Yes      | No       | Yes       | Yes   | Yes   | Yes |
| Date                    | Site code | GPS Coordinates                    | Weather                   | Habitat Type       | Habitat Assessment |          |          |           |       |       |     |
|                         |           |                                    |                           |                    | Marginal           | Floating | Emergent | Submerged | Algae | Stone | GSM |
| 2021/08/24              | LLF7      | 24°16'32.3724"S<br>28°5'0.9708"E   | Sunny, Warm               | Mokolo River       | Yes                | Yes      | No       | Yes       | Yes   | No    | Yes |
| 2021/08/24              | LLF8      | 23°50'49.56"S<br>29°21'37.9008"E   | Sunny, Warm               | Bloed River        | Yes                | Yes      | Yes      | Yes       | Yes   | No    | Yes |
| 2021/08/25              | LLF9      | 23°54'41.22"S<br>29°58'53.4216"E   | Sunny, Windy (cool)       | Stanford Lake      | Yes                | Yes      | Yes      | None      | Yes   | No    | yes |
| 2021/08/25              | LLF10     | 23°45'47.6172"S<br>30°8'30.3612"E  | Overcast & windy          | Tzaneen Dam        | No                 | No       | Yes      | Yes       | Yes   | No    | Yes |
| 2021/08/25              | LLF11     | 23°41'57.2712"S<br>30°19'35.5296"E | Sunny with a light breeze | Mothomeng Dam      | Yes                | Yes      | Yes      | No        | Yes   | Yes   | Yes |
| 2021/08/25              | LLF12     | 23°16'34.2084"S<br>30°23'51.1044"E | Sunny, Warm               | Middle Letaba Dam  | Yes                | No       | Yes      | Yes       | Yes   | No    | Yes |
| 2021/08/26              | LLF13     | 23°6'24.408"S<br>30°24'4.1184"E    | Sunny, partly cloudy      | Luvuvhu River      | Yes                | No       | No       | No        | Yes   | No    | Yes |
| 2021/08/26              | LLF14     | 23°52'0.7132"S<br>30°23'1.6836"E   | Sunny, Warm               | Magic Dam          | Yes                | Yes      | Yes      | Yes       | Yes   | Yes   | Yes |
| 2021/08/26              | LLF15     | 23°7'22.0152"S<br>30°13'7.6116"E   | Sunny, Warm               | Dam, Valdezia      | Yes                | Yes      | No       | Yes       | Yes   | No    | Yes |
| 2021/08/26              | LLF16     | 23°6'25.0416"S<br>30°7'32.8044"E   | Sunny, Warm               | Luvuvhu River      | No                 | No       | No       | No        | No    | Yes   | Yes |

Table A2: Number of individuals collected at each of the sites in the Limpopo lowveld ecoregion sampled during the high flow period.

| Limpopo high flow species collection |           |                          |                               |                       |
|--------------------------------------|-----------|--------------------------|-------------------------------|-----------------------|
| Site description                     | Site code | <i>Tarebia granifera</i> | <i>Melanoides tuberculata</i> | <i>Physella acuta</i> |
| Limpopo River                        | LHF1      | 36                       |                               |                       |
| Limpopo River                        | LHF2      | 37                       |                               |                       |
| Limpopo River                        | LHF3      | 5                        | 932                           |                       |
| Olifants River                       | LHF4      | 1258                     |                               |                       |
| Shingwedzi River                     | LHF5      | 43                       |                               |                       |
| Luvuvhu River                        | LHF6      | 434                      |                               |                       |
| Groot Letaba River                   | LHF7      | 139                      |                               | 2                     |
| Letaba River                         | LHF8      | 107                      |                               |                       |
| Olifants River                       | LHF9      | 112                      |                               |                       |
| Total individuals collected          |           | 2171                     | 932                           | 2                     |

Table A3: Number of individuals collected at each of the sites in the Limpopo lowveld ecoregion sampled during the low flow period.

| Limpopo low flow species collection |           |                       |                      |                               |                            |                         |                                 |                          |
|-------------------------------------|-----------|-----------------------|----------------------|-------------------------------|----------------------------|-------------------------|---------------------------------|--------------------------|
| Site description                    | Site code | <i>Physella acuta</i> | <i>Bulinus</i> group | <i>Biomphalaria pfeifferi</i> | <i>Gyraulus costulatus</i> | <i>Radix natalensis</i> | <i>Pseudosuccinea columella</i> | <i>Tarebia granifera</i> |
| Hex River                           | LLF1      |                       |                      |                               |                            | 2                       |                                 |                          |
| Roodekopjes Dam                     | LLF2      | 121                   |                      | 111                           |                            |                         |                                 |                          |
| Crocodile River                     | LLF3      |                       |                      |                               |                            | 79                      |                                 |                          |
| Buffels Spruit                      | LLF4      |                       |                      |                               |                            | 18                      |                                 |                          |
| Donkerpoort Dam                     | LLF5      |                       |                      |                               |                            | 6                       |                                 |                          |
| Klein sand River                    | LLF6      |                       | 28                   |                               |                            | 17                      |                                 |                          |
| Mokolo River                        | LLF7      |                       | 29                   |                               |                            | 1                       |                                 |                          |
| Bloed River                         | LLF8      |                       | 52                   |                               | 22                         | 7                       |                                 |                          |
| Stanford Lake                       | LLF9      |                       |                      |                               |                            | 24                      | 22                              |                          |
| Tzaneen Dam                         | LLF10     |                       |                      |                               |                            | 57                      |                                 |                          |
| Mothomeng Dam                       | LLF11     |                       | 55                   |                               |                            | 5                       |                                 |                          |
| Middle Letaba Dam                   | LLF12     |                       | 24                   |                               | 80                         |                         |                                 | 16                       |
| Luvuvhu River                       | LLF13     |                       | 1                    |                               |                            |                         | 6                               | 16                       |
| Magic Dam                           | LLF14     |                       | 14                   |                               |                            | 5                       |                                 |                          |
| Dam, Valdezia                       | LLF15     |                       | 18                   |                               |                            |                         |                                 |                          |
| Luvuvhu River                       | LLF16     | 17                    | 4                    |                               | 38                         |                         | 15                              |                          |
| Total individuals collected         |           | 138                   | 225                  | 111                           | 140                        | 221                     | 43                              | 32                       |

## ETHICS APPROVAL LETTER OF STUDY

Based on the review by the **Faculty of Natural and Agricultural Sciences Ethics Committee (FNASREC)**, the Committee hereby clears your study as no ethical risk. This implies that the FNASREC grants permission that, provided the general conditions specified below are met, the study may be initiated, using the ethics number below.

**Study title: Digenean trematode diversity in vectors transmitting schistosomiasis within fresh-water ecosystems of southern Africa**

**Study Leader/Supervisor: Prof NJ Smit**

**Student: MH Le Roux**

**Ethics number:**                      **N W U - 0 1 3 5 2 - 2 2 - A 9**  
                                                 Institution                      Study Number                      Year                      Status

*Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation*

**Application type: Single**

**Risk Category:**

**No Risk**

**Commencement date: 25/08/2022**

**Expiry date: 30/11/2023**

### General conditions:

*The following general terms and conditions apply:*

- *The commencement date indicates the date when the study may be started.*
- *In the interest of ethical responsibility, the NWU-SCRE and FNASREC reserves the right to:*
  - *request access to any information or data at any time during the course or after completion of the study;*
  - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process;*
  - *withdraw or postpone approval if:*
    - \* *any unethical principles or practices of the study are revealed or suspected;*
    - \* *it becomes apparent that any relevant information was withheld from the FNASREC or that information has been false or misrepresented;*
    - \* *submission of the annual (or otherwise stipulated) monitoring report, the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and / or*
    - \* *new institutional rules, national legislation or international conventions deem it necessary.*
- *FNASREC can be contacted for further information or any report templates via [Roelof.Burger@nwu.ac.za](mailto:Roelof.Burger@nwu.ac.za) 018 299 4269*

The FNASREC would like to remain at your service as scientist and researcher, and wishes you well with your study. Please do not hesitate to contact the FNASREC or the NWU-SCRE for any further enquiries or requests for assistance.

Yours sincerely,



Prof Roelof Burger

Chairperson Faculty of Natural and Agricultural Sciences Ethics Committee (FNASREC)

14 September 2021

## ETHICS APPROVAL LETTER OF STUDY

Based on approval by the North-West University Health Research Ethics Committee (NWU-HREC) on 14/09/2021, the NWU-HREC hereby approves your study as indicated below. This implies that the NWU-HREC grants its permission that, provided the general conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

**Study title:** Current status and future predicted distribution patterns of bilharzia transmitting snails under climate change and implications for vector-borne diseases in South Africa

**Principal Investigator/Study Supervisor/Researcher:** Dr L de Necker

**Student:** -

**Ethics number:**

|   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| N | W | U | - | 0 | 0 | 4 | 5 | 2 | - | 2 | 0 | - | A | 1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

Institution Study Number Year Status

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation;  
A = Authorisation

**Application Type:** Larger study

**Commencement date:** 14/09/2021

**Expiry date:** 30/09/2022

**Risk:**

**Minimal**

Approval of the study is provided for a year, after which continuation of the study is dependent on receipt and review of an annual monitoring report and the concomitant issuing of a letter of continuation. A monitoring report is due at the end of September annually until completion of the study.

### General conditions:

*While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, the following general terms and conditions will apply:*

- *The principal investigator/study supervisor/researcher must report in the prescribed format to the NWU-HREC:*
  - *Annually on the monitoring of the study, whereby a letter of continuation will be provided annually, and upon completion of the study; and*
  - *without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.*
- *The approval applies strictly to the proposal as stipulated in the application form. Should any amendments to the proposal be deemed necessary during the course of the study, the principal investigator/study supervisor/researcher must apply for approval of these amendments at the NWU-HREC, prior to implementation. Should there be any deviations from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.*
- *Annually a number of studies may be randomly selected for active monitoring.*
- *The date of approval indicates the first date that the study may be started.*
- *In the interest of ethical responsibility, the NWU-HREC reserves the right to:*
  - *request access to any information or data at any time during the course or after completion of the study;*
  - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process;*

