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FRESHWATER INVASIVE ALIEN CRAYFISH SPECIES IN SOUTH AFRICA: TESTING eDNA ASSESSMENT AND DNA BARCODING.

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

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By

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PREFACE

The research data contained in this thesis was completed by a MSc candidate, Nonkazimulo D. Mdidimba while based in the Department of Zoology and Entomology at Rhodes University under the supervision of Mr Musa Mlambo, Dr Samuel Motitsoe, and Dr Tsungai Zengeya.

This research study was in collaboration with the Department of Freshwater Invertebrates in Albany Museum, Department of Zoology and Entomology at Rhodes University and the South African National Biodiversity Institute (SANBI) Biological Invasions Directorate.

This research study represents the original work by the MSc candidate and has never been submitted to any University other than Rhodes University, Makhanda (Grahamstown), South Africa.

ABSTRACT

The expansion of the wild populations of the two invasive alien crayfish species, i.e., *Cherax quadricarinatus* and *Procambarus clarkii* in South Africa is of high concern. Given this, innovative and improved monitoring methods are required to be explored in order to effectively detect and manage their spread. Environmental DNA (eDNA) has shown promising and reliable results to detect and monitor alien crayfish in freshwater systems even at low densities. This research study aimed to field-test the application of eDNA for detecting and monitoring invasive alien freshwater crayfish species (IAFCs) in South African freshwater systems (Chapter 2). Further, this study also aimed to confirm the true taxonomic identity of the IAFCs in South African freshwater systems by using DNA barcoding to avoid potential misidentifications with morphological identification alone (Chapter 3). Also, ectocommensal temnocephalans and leeches attached to the crayfishes were also identified with DNA barcoding. The mitochondrial COI gene fragment of the crayfishes and the ectocommensal temnocephalans and leeches was amplified, sequenced, and used for species identification. For eDNA monitoring of *C. cainii, C. quadricarinatus* and *P. clarkii*, water samples were collected from Eastern Cape, Free State and KwaZulu Natal sites that are known to house these species. The current study did not detect the eDNA of the wild populations of both *C. quadricarinatus* and *P. clarkii*. This failure was encountered despite employing several methods, such as, end-point polymerase chain reaction (PCR), quantitative PCR (qPCR) and next-generation sequencing (NGS). The eDNA assessment to detect IAFCs in the wild populations is discussed in detail, and corrective measures suggested (Chapter 2). However, only *C. cainii* was able to be detected by eDNA. *Cherax cainii* is housed/farmed in an aquaculture facility (Vaughn Bursey's Stock Farm in Eastern Cape) with large number of individuals per pond. For this, NGS was able to detect *C. cainii* but the other two methods, end-point PCR and qPCR were not able to detect the species. Based on the results, refinement of eDNA monitoring methods for the South African freshwater systems is recommended. Evaluation of different DNA collection methods, preservation, DNA extraction kits and primer design to obtain effective eDNA detection is discussed as possible sources of error. Interestingly, the newly established population of *P. clarkii* in Mimosa Dam in Free State was infested with alien leeches, that were confirmed by DNA barcoding to be *Helobdella europaea*

and *Helobdella octatestica*. Consequently, this study reports for the first time in South Africa the presence of this alien freshwater leeches. The presence of alien leeches and *P. clarkii* suggests a double invasion in Mimosa Dam, *P. clarkii* seems to continue expanding its range in the country. However, the long-established population of *P. clarkii* in Driehoek Farm in Mpumalanga, which has been in the country for over 30 years did not have any leech or temnocephalan infestation. This could suggest several interesting ecological phenomena, including environmental filtering over time of the co-invaders, differential source populations, and post-introduction adaptation.

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CHAPTER 1
GENERAL INTRODUCTION

1.1. Biological Invasions in South Africa

South Africa (SA) has a long history of invasive alien species (IAS) introductions (van Wilgen et al. 2020), majority of which were introduced intentionally during early globalisation from Europe and America (Faulkner et al. 2017, van Wilgen and Wilson 2018). Introduction of IAS in the country were mainly for ornamental, horticulture, agriculture and forestry, aquaculture, mariculture and biological control purposes (van Wilgen 2020). However, some IAS were introduced accidentally as commodity contaminants or as stowaways on transport vectors (van Wilgen and Wilson 2018). Further, some IAS were accidentally co-introduced with their parasites and pathogens, for example, Sirex woodwasp (*Sirex noctilio*) was introduced in the country with infested timber (Picker and Griffiths 2017). Moreover, some IAS can be transported into the country with the luggage of tourists, or as contaminants of imported goods. Even though the rate of intentional introduction of high-risk species is expected to decrease due to improved regulations, it is also expected that the rate of unintentional introductions will rise due to increases in trade and tourism (van Wilgen and Wilson 2018). Several IAS have escaped from captivity including aquaculture facilities into the wild, including invasive alien freshwater crayfish species (IAFCs) in South Africa (de Moor 2002), which is the focus for this thesis.

More than half of IAS is SA (i.e., 53%) are terrestrial plants (van Wilgen et al. 2020). The impacts posed by the alien plants on South African ecosystems include threatening local biodiversity, elevating soil erosion, altering and intensifying fire regimes (van Wilgen et al. 2014). They also reduce water supply, threatening the health of humans and livestock, reduce agricultural productivity and damage rangelands (Hill et al. 2020). To increase IAS awareness, SA has committed to report on biological invasions, their impacts and management through documentation such as the National Status Report on biological invasions (Wilson et al. 2017, Zengeya and Wilson 2020) and the National Biodiversity Assessment (Driver et al 2012). The status report creates an independent assessment of the status of biological invasions and their management in SA and aims to lessen the negative impacts of IAS on ecosystems, the economy and livelihoods, and maintain some benefits of IAS where possible and necessary

(Wilson et al. 2017, van Wilgen and Wilson 2018, Zengeya et al. 2020). The mandate for these reports emanates from the National Environmental Management: Biodiversity Act (NEM: BA, Act 10 of 2004), where they must produce every five years or so. The results from the current study should contribute to the next iteration of these reports. So, the current study aimed to create information that will contribute to these important policy documents for the better conservation of SA biological diversity.

1.2 Impacts of Biological Invasions in South African Freshwater Systems

IAS is one of the major global drivers of biodiversity change, in a long list that also includes habitat destruction or degradation, overexploitation, water pollution, and global climate (Dudgeon et al. 2006, Darwall et al. 2008, Dudgeon 2010, Reid et al. 2018). Ecosystem services are the benefits provided by the environment to humans such as food, water, timber, air and water purification, waste treatment, disease regulation and pollination (Charles and Dukes 2007). Thus, the introduction of alien species has caused implications and deteriorated the ecosystem services globally (Vilà et al. 2011, Galiana et al. 2014, Doherty et al. 2016).

Freshwater systems contain the vital natural resources (Vörösmarty et al. 2010) and play an important role of providing various important goods and services such as clean water, food, building materials, water filtrations, flood and erosion control and hydropower generation (Darwall et al. 2008, Strayer and Dudgeon 2010). Freshwater systems provide habitat to approximately 9.5% of the Earth's described species (Geist et al. 2015). However, despite the significant ecological and socio-economic importance of freshwater systems, they are threatened by anthropogenic activities including habitat destruction and IAS (Darwall et al. 2008, Vörösmarty et al. 2010, Carrizo et al. 2017). Freshwater systems are susceptible to invasion by a range of taxa from aquatic plants, aquatic snails, freshwater fish and freshwater crayfish (Strayer 2010, Hill and Coetzee 2017). The presence of IAS in novel environments can threaten native aquatic biodiversity and related ecological functions and compromise some important socio-economic ecosystem services (Richardson et al. 2004, van Wilgen et al. 2008, Strayer 2010, Traveset and Richardson 2014, Kumschick et al. 2018, Egoh et al. 2020).

The water hyacinth (*Eichhornia crassipes*), for example, has completely altered many receiving South African freshwater systems, by changing water chemistry, oxygen levels,

water quality and water quantity through high evapotranspiration rate (Richardson et al. 2004, Coetzee et al. 2011, Fraser et al. 2016, Hill and Coetzee 2017). Water hyacinth and other free-floating aquatic weeds have been reported to reduce the diversity of benthic macroinvertebrate and outcompete native macrophytes (Richardson et al. 2004, Midgley et al. 2006, Coetzee et al. 2014, Coetzee et al. 2020, Motitsoe et al. 2020). It also forms dense mats that can clog waterways thus impacting transportation, recreation, hydropower stations and fisheries (Midgley et al. 2006). Due to its negative impacts, it is regarded as the most destructive and damaging alien macrophyte in freshwater ecosystems (Fraser et al. 2016, Hill and Coetzee 2017).

Other destructive invasive alien macrophytes include water lettuce (*Pistia stratiotes*), Kariba weed (*Salvinia molesta*), parrot's feather (*Myriophyllum aquaticum*) and red water fern (*Azolla filiculoides*) and are the five worst alien invasive macrophytes species present in South Africa known to have negative effects to aquatic ecosystems (Martin and Coetzee 2011). Lastly, most of research on invasive plants in SA has focused on terrestrial plants, like *Pinus spp,* Australian *Acacia spp* and *Hakea spp,* which have caused untold damage to water quantity and quality, sucking catchments dry (Le Maitre et al. 2002, Van Wilgen et al. 2008).

On the animal front, invasive alien fishes have invaded most of the major rivers in the country and are considered the major factor affecting freshwater biodiversity (Tweddle et al. 2009). There are at least 21 alien fish species that have been introduced in freshwater ecosystems in South Africa (Weyl et al. 2020). The main pathways for the introduction of alien fishes in South Africa are aquaculture, biological control, conservation translocations, inter-basin water transfer schemes (IBTs), ornamental and pet escapes, and recreational angling (Ellender and Weyl 2014). Invasive alien fish species have higher establishment rates than most other taxa, this facilitates their successful establishment in new areas and often allow them to outcompete native fish (Jeschke and Strayer 2006). For example, rainbow trout *Oncorhyncus mykiss* and largemouth bass *Microptus salmoides* have resulted in local extinctions of native small fish species such as fiery redfin *Pseudobarbus phlegethon,* Berg River redfin *P. burgi* and Maloti minnow *P. quathlambae* to name a few (Ellender and Weyl 2014, Shelton et al. 2015). *Micropterus salmoides* (largemouth bass), *Micropterus dolomieu* (smallmouth bass), *Oreochromis niloticus* (Nile tilapia), *Oncorhynchus mykiss* (rainbow trout)

and *Salmo trutta* (brown trout) are classified as destructive based on the negative impacts they posed in the introduced freshwater systems (Zengeya et al. 2017).

Finally, the global economic cost of invasive species in aquatic systems is estimated at US$345 billion per annum (Cuthbert et al. 2021). In South Africa, invasive alien plants reduce the livestock production by approximately R340 million annually and this has significantly affected the country's economy (van Wilgen et al. 2020). Also, the cost to manage freshwater invasive alien macrophytes by herbicide in South Africa between 2010 and 2018 was estimated at R42 million per year (van Wilgen et al. 2020). Taking into consideration the economic loss caused by IAS and the cost to manage them in South African waters, it is evident that there is a need for techniques that will enable early detection of invasions where costs and potential for eradication or management is still feasible (Mazza et al. 2014). Assessing environmental DNA (eDNA) has been proposed as one such method and is a target of this study.

1.3 Freshwater Crayfish Invasion

1.3.1 Freshwater Crayfish Invasion

Freshwater crayfish belong to the phylum Arthropoda, within the subphylum Crustacea and order Decapoda, infraorder Astacidea, which includes three superfamilies, Astacoidea (Northern Hemisphere crayfish) and Parastacoidea (Southern Hemisphere crayfish) (Crandall and De Grave 2017). The superfamily, Astacoidea is made of two main families, Cambaridae and Astacidae. The Cambaridae's native distribution includes the North America, east of the Rocky Mountains, north into southern Canada and south through Mexico and Asia (Crandall and De Grave 2017). The second family, Astacidae is naturally distributed on the west of the Rocky Mountains (mainly in the Pacific North-West) and in Europe (Crandall and De Grave 2017). The second superfamily, Parastacoidea consists of one family, Parastacidae (Souty-Grosset and Fetzner 2016). Parastacidae's native distribution includes Australia, New Zealand, Brazil, Chile, Uruguay and Madagascar (Crandall and Buhay 2008). At present, more than 690 described freshwater crayfish species are naturally distributed in all continents except mainland Africa and the Antarctica (Crandall and De Grave 2017).

Globalisation has resulted in freshwater crayfish species being moved around the world for various reasons. In East Africa, for example, crayfish were intentionally introduced for aquaculture, to increase food production, and as biological control of bilharzia causing snails (Hofkin et al. 1991, Mikkola 1996, Smart et al. 2002, Monde et al. 2017). Unfortunately, this experiment did not turn out well, as the crayfish cause untold damage, not only to the ecosystem structure and biodiversity, but also on the physical structure of the dams (Holdich 2002, Lodge et al. 2012). The introduction of crayfish as a biological control agent was highly critisized by Weyl et al. (2017), arguing that it is in contrast with current thinking regarding the management of biological invasion, as it can cause unexpected outcomes. Generally, introductions of crayfishes for aquaculture production, research, biological control of disease hosts, or as ornamental species in aquaria or water gardens are the main routes (Taugbøl and Skurdal 1999, Taylor et al. 2007, Gherardi 2010, Manfrin et al. 2019, Haubrock et al. 2021).

The Louisiana red swamp crayfish *Procambarus clarkii* (Girard 1852) is a widely introduced crayfish species in the world (Oficialdegui et al. 2019). *Procambarus clarkii* is native to northern Mexico, and southern and south-eastern United States (Gherardi and Acquistapace 2007, Gherardi 2010) and established in the global aquaculture industry and, as a result, has been introduced to all continents except Australia and Antarctica (Huner and Barr 1991, Oficialdegui et al. 2019).It is also the first crayfish species to be introduced in South Africa, through accidental escape from the neighbouring country, Swaziland (see de Moor 2002). Despite several attempts to eradicate it at the introductory region, e.g., Mpumalanga province, this species was re-discovered to maintain a healthy, breeding population 22 years later (Nunez et al 2017a). This is because *P. clarkii* exhibits high phenotypic plasticity which enables it to adapt and establish populations in different environments and temperatures that are far beyond its optimal thermal conditions (Gherardi 2006, Oficialdegui et al. 2019). *Procambarus clarkii* also exhibits biological and ecological traits such as rapid growth, high fecundity, early maturity, generalist and opportunistic feeding habits and resistance under unfavourable environmental conditions which allow it to be a successful invader in new areas (Gherardi 2006, Oficialdegui et al. 2019).

In addition to *P. clarkii*, four invasive alien freshwater crayfish species (IAFCs) have been regarded highly invasive globally, which include Rusty crayfish *Faxonius rusticus* (Girard 1852), Signal crayfish *Pacifastacus leniusculus* (Dana 1852), spiny-cheek crayfish *Faxonius limosus*

(Rafinesque 1817) and virile crayfish *Orconectes virilis* (Hagen 1870) (Twardochleb et al. 2013). *Faxonius rusticus* is a native to North America and has devastated the European native crayfish species through competition and spreading of diseases (Agersnap et al. 2017). *Pacifastacus leniusculus* is also native to North America (Larson et al. 2012) and is highly invasive in Europe where it displaced the native crayfish *Austropotamobius pallipes* in England and France (Dunn et al. 2009, Filipova et al. 2013) and displaced native *Pontastacus leptodactylus* in Croatia (Dragičević et al. 2020). *Pacifastacus leniusculus* is has also threatened the native noble crayfish *Astacus astacus* in majority of the European rivers (Skov et al. 2011). *Faxonius limosus* formerly known as *Orconectes limosus* is native to North America, is invasive in Europe and has the largest distribution range (Zorić et al. 2020, Bonk and Bobrek 2021). *Faxonius limosus* has been reported to occur in freshwater systems of 23 European territories (Todorov et al. 2020). *Orconectes virilis* is native to North America (Larson et al. 2010) and is invasive in United Kingdom where it outcompetes the native crustaceans including the North American *P. leniusculus* (James et al. 2015).

Other emerging IAFCs include Australian red claw crayfish *Cherax quadricarinatus* (von Martens 1868) and the Marbled crayfish *Procambarus virginalis* (Lyko 2017) as they also exhibit traits such as rapid growth and omnivore feeding habit which enable their successful establishment and successful invasion (Andriantsoa et al. 2020, Madzivanzira et al. 2020). In addition, *P. virginalis* is an obligately parthenogenetic species, i.e., it exclusively reproduces asexually where growth and development of embryos occur from an unfertilised egg (Vojkovská et al. 2014, Vogt 2021). This means that a single introduction of an individual can quickly lead to establishment and invasion of this species (Vogt 2018). *Procambarus virginalis* originates from South-eastern region of United States (Keller et al. 2014). It is invasive in Europe where it continues to spread rapidly (Ercoli et al. 2019, Hossain et al. 2020, Maciaszek et al. 2022), Asia (Zeng and Yeo 2018) and Africa (Andriantsoa et al. 2019). Moreover, *C. quadricarinatus* originates from north-eastern Australia and southern New Guinea (Patoka et al. 2018). It is invasive in Africa (Nunes et al. 2017b, Madzivanzira et al. 2020), Asia (Belle and Yeo 2010, Akmal et al. 2021), Europe (Weiperth et al. 2019, Arias Rodriguez and Burrial 2021), North America (Bortolini et al. 2007, Morningstar et al. 2020). The species' invasion in Africa is discussed in detail in sections 1.3.2 and 1.3.3.

Regardless of the primary route of crayfish introduction beyond their native range, most have ended up establishing naturalised populations in the wild outside their native range (Lodge et al. 2012). Crayfish pose threats to aquatic system trophic interactions in invaded systems due to their capability of integrating into the food web at many levels as they are omnivores (Moyle and Light 1996). The threats posed by IAFCs are one of the greatest concerns for many freshwater ecologists (Peay et al. 2010, Furse and Coughran 2011, Coughran and Furse 2012, Leland et al. 2012) with pronounced effects on freshwater populations, communities and ecosystems (Lodge et al. 2012, Twardochleb et al. 2013). For example, crayfish can alter habitat through their burrowing behaviour, increasing the rates of leaf-litter breakdown and nutrient cycling in freshwater (Aquiloni et al. 2010). They can change lakes and wetlands from clear to phytoplankton dominated turbid water by grazing and burrowing, reducing benthic algae and macrophyte cover (Aquiloni et al. 2010). Various studies have also shown their ability to reduce the biomass and richness of hydrophytes, snails, and other components of the food web, and thus affect ecosystem (dis)services (Gherardi 2007). Lastly, amphibian and fish populations can suffer through predation on eggs and larvae, and competition for shelter (Reynolds and Souty-Grosset 2011).

1.3.2 Freshwater Crayfish Invasion in Africa

The African continent which is devoid of native crayfish species (except for Madagascar) has been a recipient of nine crayfish species to date including the noble crayfish *Astacus astacus* (Linnaeus 1758), smooth marron *Cherax cainii* (Austin and Ryan 2002), yabby *Cherax destructor* (Clark 1936), Australian redclaw *C. quadricarinatus*, spiny cheek crayfish *Faxonius limosus* (Rafinesque 1817), signal crayfish *Pacifastacus leniusculus* (Dana 1852), red swamp crayfish *Procambarus clarkii* (Girard 1852), marbled crayfish *Procambarus virginalis* (Lyko 2017) and White River crayfish *Procambarus zonangulus* (Hobbs and Hobbs 1990) (Madzivanzira et al. 2020). Five of these species i.e., *A. astacus*, *C. quadricarinatus*, *F. limosus*, *P. clarkii* and *P. virginalis*, have established naturalised populations in fourteen African countries (Figure 1.1) due to escapes from captivity, intentional releases, and unaided introduction and spread (de Moor 2002).

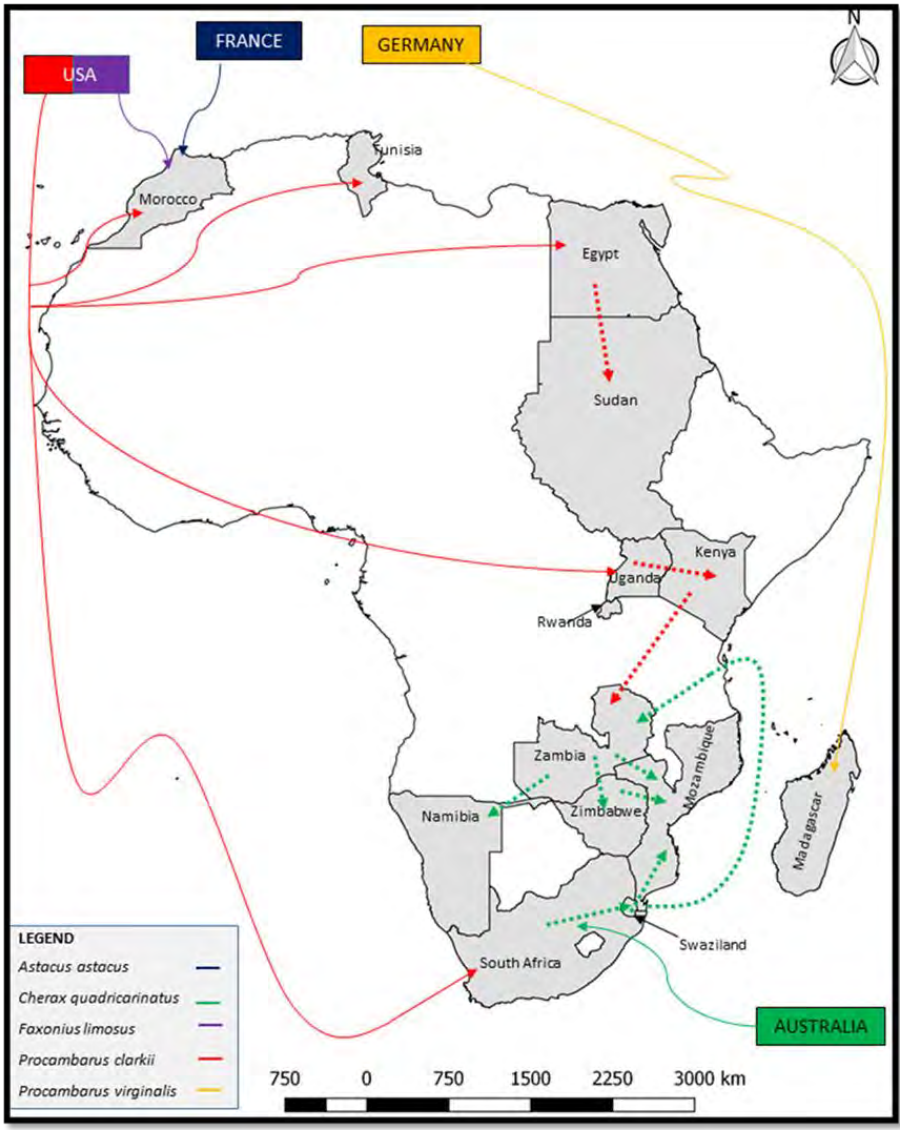


Figure 1. 1 Map showing the introduction routes of five established crayfish species in Africa (Source Madzivanzira et al. 2020).

The main driver of crayfish introductions and spread in Africa was to provide socio-economic benefits through aquaculture and fisheries development, which according to Madzivanzira et al. (2020) has had limited evidence of success. Kenya exported several hundred tonnes of the Louisiana crayfish, *P. clarkii* to European countries between 1975 and 1981 (Oficialdegui et al. 2019), however the industry collapsed due to a cholera outbreak in East Africa (Foster and Harper 2007). Other examples of failed crayfish farms are documented in Swaziland, South Africa, and Zambia (Nakayama et al. 2010, Nunes et al. 2017c, Douthwaite et al. 2018). Further, invasive crayfish can negatively affect fisheries activities through damage of fishing

gear, declining fisheries performance, and damage to agricultural water infrastructure (Madzivanzira et al. 2020).

1.3.3 Freshwater Crayfish Invasion in South Africa

Literature on crayfish invasions in SA, from the first introductions in the 1960s, is well summarised in Nunez et al. (2017c). Four crayfish species have been imported into SA: *Cherax cainii*, *Cherax destructor*, *Cherax quadricarinatus* and *Procambarus clarkii*. Pet trade in the 1980s resulted in the illegal importation of *P. clarkii* into the country (de Moor & Bruton 1988), currently only *P. clarkii* and *C. quadricarinatus* have established wild populations in the country (Nunes et al. 2017c, Madzivanzira et al. 2020). South Africa imposed restrictions on the importation and culturing of *C. quadricarinatus*, however, farmers established a *C. quadricarinatus* farm next to the Sand River Dam in neighbouring Swaziland, from where specimens later escaped and spread into South Africa (de Moor 2002; Nunes et al. 2017b; c). The first wild population of *C. quadricarinatus* in South African was recorded in 2002, in the Komati River in Mpumalanga Province, close to the Swaziland border (Nunes et al. 2017b). At present, *C. quadricarinatus* is established in the Inkomati, Mbuluzi and Usutu River basin all of which run through South Africa, Swaziland and Mozambique (Nunes et al. 2017b,c). It has also established wild populations in the Crocodile River in Kruger National Park (Nunes et al. 2017c, Petersen et al. 2017), Pongola River in Ndumo Game Reserve in northern KwaZulu-Natal (Du Preez & Smit 2013; Nunes et al. 2017a,c) which will be discussed in detail in Chapter 2.

The first record of wild population of *P. clarkii* populations in the country was recorded in Crocodile River, Mpumalanga in 1988 (Schoonbee 1993). The second record of the species was in Driehoek Farm in Mpumalanga Province in 1991 (de Moor 2002). Attempts to eradicate the *P. clarkii* populations by reducing water level and physically removing specimens proved unsuccessful Nunes et al. (2017a). In 2018, a second record of a population established in the wild for *P. clarkii* was discovered in Mimosa Dam, Free State which is more than 600 km away from previous record (Barkhuizen et al. 2021). This population is believed to have been introduced into the dam to provide stock for the pet trade (Barkhuizen et al. 2021). As this thesis was finalised, report of a third population in the wild was presumed in the Western

Cape Province, and more investigation will be conducted to determine population genetics of there now three wild populations (John Measey *pers. comm.*)

Cherax cainii, on the other hand, was introduced in SA in 1976 by a private fish farmer in KwaZulu-Natal for aquaculture purposes, although, this venture was short-lived (de Moor and Bruton 1988). Several farmers developed an interest to culture *C. cainii* and applied for permits, and several consignments of *C. cainii* were imported into SA from Western Australia (Mitchell and Kock 1988, van den Berg et al. 1990). Farming of *C. cainii* was unsuccessful and currently, only two farms are operational, and both are located in the Eastern Cape Province (Madzivanzira et al. 2020). There are currently no reports of *C. cainii* in the wild (Nunes et al. 2017c) despite unverified reports of *C. cainii* individuals being detected in the Buffalo River in the 1980s ostensibly because of aquaculture escape (de Moor and Bruton 1988). But after seeing the aquaculture practise at Vaughn Bursey's Stock Farm, needing constant aeration to keep oxygen levels and temperature favourably, it seems unlikely for this species to survive the conditions in the wild. The same could be true for *C. destructor*, which is also only reported from controlled environments in this country.

Given the difficulty of controlling crayfish populations once they are established, programmes such as early detection and rapid response become a critical element, especially if eradication is the management goal (Tobin 2018, Faulkner et al. 2020). Key to this is the development of appropriate monitoring methods (Madzivanzira et al. 2020). There are various methods for monitoring crayfish in freshwater systems ranging from traditional methods such as baited trapping (Madzivanzira et al 2019, Manfrin et al. 2019), electrofishing (Manfrin et al. 2019), diurnal and nocturnal visual surveys (Nunes et al. 2019). These traditional methods are invasive and have proven to be less effective in detecting the presence of elusive crayfishes. However, an increasing body of literature is coming out demonstrating the superiority of modern technology i.e., bioacoustics and eDNA.

1.4 Problem Statement

Freshwater crayfish invasions and their impacts on ecosystems have been extensively studied in Europe and North America, however not much has been done in Africa (Nunes et al. 2017c, Madzivanzira et al. 2020, Madzivanzira et al. 2021). In South Africa, the two species with recorded wild, feral population; *P. clarkii* and *C. quadricarinatus* are spreading and expanding their distribution of new established populations. Given the potential environmental impacts of IAFCs as discussed above, this is a major concern for aquatic ecosystems and related ecosystems services. Lastly, its observed that the introduction and spread of IAFCs in SA has resulted in the co-introduction of alien temnocephalan species (Avenant-Oldewage 1993; Du Preez & Smit 2013; Tavakol et al. 2016, Tavakol et al. 2021). With that been said, there is an urgent need to identify, monitor and map the population distribution of IAFCs in SA using reliable and high-resolution techniques such as eDNA and DNA barcoding of both crayfish species and their co-introduced temnocephalans and leeches.

South Africa can mitigate the ecological and economic impacts posed by IAFCs by employing eDNA technique to allow early detections and rapid response of alien species. This way, management of alien species can be carried out at the early stages of invasions where eradication is often feasible and avoid ecological and socio-economic costs. This is also a mandate of the National Environmental Management: Biodiversity Act (Act 10 of 2004), which aims to develop monitoring programmes that will enable early detection, and effective management (Sink et al. 2012). Moreover, IAS pose severe ecological impacts and can inflect huge economic losses (van Wilgen et al. 2020). Morphological-based identification, particularly for non-native species can be challenging to non-taxonomist more so when the species is not from the region, secondly some identifications keys are solely based on juvenile life stages or vice versa. This can be challenging when identifying specimens and can result in misidentification of alien species. DNA barcoding can be used to accurately identify the invasive alien species and their co-introduced species, and prompt preventive measures with subsequent regulatory control can be initiated. Also, knowing the true identity and having molecular signature (i.e., DNA barcodes) of populations already established could facilitate cross-checking with new populations if they come from same source, and also allow cross-check with pet-shops to establish if they are the source of new introductions.

1.5 Aims of this study:

1. **To field test the potential of eDNA for alien invasive crayfish species detection and monitoring in South African freshwater systems**

Procambarus clarkii and *Cherax quadricarinatus* are regarded burrowers, they make burrows in the littoral zone and this lifestyle sometimes compromises traditional sampling and collection methods. However, eDNA has promising results to detect alien species that are difficult to collect, for example, *P. clarkii* (see Treguier et al. 2014). This technique does not rely on physically collecting organisms but rather on genetic material released by organisms into the environment (Manfrin et al. 2019). In SA, the early detection, management, and eradication of IAS is a national priority. As such, this study will contribute to this mandate by assessing the ability of eDNA as a technique to detect and provide early warning indication, before the IAFCs population doubles and start spreading to other freshwater systems. The feasibility of eDNA was based on how sensitive and accurate the method was to detect the captive *C. cainii* and wild populations of *C. quadricarinatus* and *P. clarkii.* This aim will be achieved by comparing eDNA results with traditional sampling methods (i.e., visual surveys, aquatic net sweeping and baited traps), by re-visiting known locations for *C. quadricarinatus* (see Nunes et al. 2017c) and newly infested dam, in Free State where *P. clarkii* was reported. The 16S marker 1471 (5'CCTGTTTANCAAAAACAT3') and 1472 (5'AGATAGAAACCAACCTGG3') specific to *C. cainii* and *C. quadricarinatus* and COI marker SPY_ProCla_F (5'AACTAGGGGTATAGTTGAGAG3'), reverse primer SPY_ProCla_R (5'CAGAAGCTAAAGGAGGATAA3') and probe, SPY_ProCla_Probe (5'FAM-AGGAGTTGGAACAGGATGGACT-MBG-3') specific to *P. clarkii,* to identify the target crayfish species (Chapter 2).

2. **Barcoding of invasive alien freshwater crayfish (Cherax cainii, Cherax quadricarinatus and Procambarus clarkii) and their co-introduced or novel associated ectoparasites.**

Misidentification, which is rife with morphological identification, of IAS can affect their risk analysis assessment and management interventions because these rely on accurate identification of species, understanding their biology, behaviour and potential impacts in novel ecosystems. DNA barcoding, on the other hand, provides accurate species identification

(Hebert et al 2003). For example, in South Africa the import permit continues to be issued for both *C. tenuimanus* and *C. cainii*, even though *C. tenuimanus* is now known to be highly restricted and only found in few rivers in Australia (Austin and Ryan 2002). As such, it is not known whether the species currently occurring in SA is *C. cainii* or *C. tenimanus*. Therefore, DNA barcoding to identify and know the exact species of marron that occurs in the country. Moreover, IAFCs have been reported in SA to be carriers of alien temnocephalans (Tavakol et al. 2016, Tavakol et al. 2021). These co-introduced temnocephalans and leeches can be invasive and pose harmful effects on native species (Du Preez and Smit 2014, Tavakol et al. 2016, Tavakol et al. 2021). DNA barcoding also offers a reliable identification of the co-introduced temnocephalans and the newly recorded leeches, as this area of research has not been given enough attention in the country. This study aimed to identify the alien leeches that were observed on *P. clarkii* specimen collected in Free State*,* which is the first record of alien freshwater leech reported in SA. Identification was carried out using morphological identification keys and DNA barcoding. In addition, the study reported on the alien temnocephalan co-introduced with *C. cainii* in Vaughn Bursey Stock Farm in Eastern Cape. The last report on temnocephalan co-introduced with *C. cainii* was over two decades ago (Mitchell and Kock 1988, Avenant-Oldewage 1993). Chapter 3 contributes towards the building-up of reference library of invasive alien freshwater crayfish species occurring in SA, by barcoding *C. cainii*, *C. quadricarinatus* and *P. clarkii* and their parasites.

CHAPTER 2
Field-testing the potential of environmental DNA (eDNA) in detecting invasive alien freshwater crayfish species (IAFCs) in South Africa; a case study.

2.1 Introduction

Traditionally, invasive alien freshwater crayfish species (IAFCs) detection and management has been based on trapping and visual inspections (Nunes et al. 2017; Green et. 2018). Traditional methods can be less effective in open freshwater systems such as rivers and lakes with more biotopes for the species to hide (Rudnick et al. 2003). Further, several freshwater systems with invasive crayfishes in South Africa (SA), for example, have dangerous animals, like hippopotamus and crocodiles (e.g., Du Preez and Smit 2013), thus making trapping in such systems highly challenging. Also, biological activities of crayfishes, like being nocturnal and digging burrows make visual inspection ineffective. For the specimens to be visually spotted or attracted to the traps it is generally a result of the crayfish activities, which is the product of specimens' size and abundance, thus sites with many and large individuals likely to be ideal for traditional methods. In contrast, sites with few individuals, and perhaps smaller specimens, traditional sampling of crayfishes could be infeasible. Such a scenario is general true during the early stages of invasion, where there are still few individuals usually juveniles-sub-adult mix (Treguier et al. 2014)). This is usually a critical time to implement eradication before population numbers explode causing huge loss in ecosystems functioning (Treguier et al. 2014).

Detection of invasive alien species (IAS) in the early stages of invasion is often impractical unless invasion density exceeds a certain threshold (Myers et al. 2000). In the early stages of invasion, the frequency of introduced species or propagators is low, often making detection difficult when using traditional methods (Rees et al. 2014, Dunn et al. 2017, Xia et al. 2021). However, detection at the early stage of invasion is crucial for effectively managing biological invasions as opposed to when populations have been established (Dougherty et al. 2016, Dunn et al. 2017). Early detection of IAS at low densities assures successful eradication operations and a decrease in the costs of managing and reducing the impacts of IAS on ecosystems, the economy and livelihoods (Mehta et al. 2007, Larson et al. 2017).

Emerging techniques, such as environmental DNA (eDNA) have been shown to be effective in detecting IAS at both low densities (Dougherty et al. 2016) and in established populations (Manfrin et al. 2019).

2.1.1 What is Environmental DNA?

Environmental DNA (eDNA) refers to the DNA shed by organisms into their environment through release of hair, skin cells, gametes, fecal matter, saliva and decomposition of dead organisms (Ficetola et al. 2008, Lodge et al. 2012, Barnes & Turner 2016, Weldon et al. 2020). Environmental DNA can occur in two forms, as intracellular DNA —which makes up the bulk of detectable DNA, or as extracellular referring to DNA molecule found outside a cell (Andruszkiewicz et al. 2017). These environmental traces of DNA can be used to detect the presence of an organism (Nevers et al. 2018). The two most common eDNA capturing methods used from water samples are filtration and precipitation (Hinlo et al. 2017, Harper et al. 2019). In the filtration method, environmental water samples are filtered through filter papers, typically of pore size ranging from 0.2 µm (Spens et al. 2017), 0.45 µm (Sugawara et al. 2021), 0.8 µm (Renshaw et al. 2015, Wacker et al. 2018) to trap DNA from the water for further analysis. The filtration process allows the concentration of DNA traces from large volumes of water (Renshaw et al. 2015). The precipitation method of capturing eDNA involves the use of sodium acetate and absolute ethanol (Ficetola et al. 2008).

Following the eDNA capture method through filtration or precipitation, is the preservation method. Preservation is essential to minimize DNA degradation before further analysis. Preservation of filter papers or DNA precipitate is carried out using preservative solutions such as ethanol and Longmire's lysis buffer to minimize eDNA degradation prior to extraction (Hinlo et al. 2017). Preserved samples are recommended to be stored at -20°C in dark conditions to minimize degradation of eDNA. However, samples preserved in Longmire's lysis buffer do not require cold storage and can be stored at room temperature (Williams et al. 2016).

In the laboratory, eDNA processing of preserved filter membranes includes DNA extraction and DNA amplification methods such as endpoint Polymerase Chain Reaction (PCR) (Gargan et al. 2017), Droptlet Digital PCR (ddPCR) (Pont et al. 2018, Brys et al. 2021) and quantitative

PCR (qPCR) (Dunn et al. 2017, Przybyla-Kelly et al. 2023). For eDNA sequencing, Next Generation Sequencing (NGS), which is a massively parallel DNA sequencing technology (Fernandes et al. 2018). For example, Thalinger et al. (2019) employed both end-point PCR and ddPCR to assess the efficiency of eDNA to detect the presence and spawning migrations of Danube bleak, *Alburnus mento*, and Vimba bream, *Vimba vimba* in Austria. Both techniques (end-point PCR and ddPCR) successfully detected the presence and migration of *A. mento* and *V. vimba* in river Zeller Ache in Austria. Quantitative PCR is the commonly used technique in DNA based monitoring to detect invasive alien species. For example, it was employed by Goldberg et al. (2013) and Woodell et al. (2021) to detect invasive alien mud snails, *Potamopyrgus antipodarum* in USA. Quantitative PCR was able to detect *P. antipodarum* even at low densities where the species was not physically collected.

2.1.2 Benefits of eDNA as a monitoring method

Traditional monitoring methods can be costly and time-consuming, however eDNA has shown to have relatively more advantages in biomonitoring (Gaither et al. 2022). That is, the main advantage lies in minimized sampling time, as no physical collection of organisms is required (Goldberg et al. 2016, Valentini et al. 2016, Elbrecht et al. 2017, Geerts et al. 2018, Mauvisseau et al. 2019a). Since eDNA does not require the collection of targeted specimens, it is thus regarded as non-invasive because it minimizes habitat disruption during sampling and spillover of non-target species or by-catch (Rees et al. 2014, Deiner et al. 2015). Environmental DNA also minimizes the chance of missing species due to insufficient sampling efforts (Minamoto et al. 2016, Geerts et al. 2018). That is, eDNA allows species detection even when organisms are not physically observed or without knowledge of their presence in freshwater systems (Deiner and Altermatt 2014, Valentini et al. 2016). Due to this ability, it has appeared to be an improved and efficient tool for biological monitoring surveillance in aquatic systems (Lodge et al. 2012, Takahara et al. 2012, Pont et al. 2018).

Environmental DNA has also been shown to be sensitive in detecting rare species (Macisaac 2017). For example, eDNA detected the rare and endangered Alabama Sturgeon *Scaphirhynchus suttkusi* and Gulf Sturgeon *Acipenser oxyrinchus desotoi* in Alabama, USA (Pfleger et al. 2016). Environmental DNA can also be employed in habitats where traditional

monitoring methods have been challenging to implement due to logistic constraints such as low accessibility to the site (Valentini et al. 2016). It also holds promising benefits, such as increased speed, accuracy, and resolution of species misidentification (Valentini et al. 2009, Ji et al. 2013, Gibson et al. 2014). For example, Deiner and Altermatt (2014) detected traces of eDNA 10km downstream from a source where the organisms were physically captured. In addition, eDNA can be applied after IAS eradication as a monitoring tool to confirm if the management plan was successful (Manfrin et al. 2019). In general, there are few eDNA studies available, majority of work is done in marine ecosystem (see section 2.1.3; von der Heyden 2023) while freshwater system is lagging, at least of studies from South Africa. The current study was trying to fill that gap, by testing eDNA methods in South African freshwater system, using invasive crayfishes as the target species.

Like other biological monitoring methods, eDNA also has drawbacks, such as the influence of the laboratory methods used (Piggott 2016) and false negatives, which is the non-detection of species-specific DNA fragments in water samples (Roussel et al. 2015). false negative detections, which occur when the targeted species is not detected but present on-site (Erickson et al. 2017). These false negatives can be attributed to low DNA quantity from the collected water sample, ineffective sample preservation, insufficient sampling efforts and PCR inhibitors (Pont et al. 2018). Low DNA quantity can be due to small amounts of DNA released by the species and low DNA concentrations in the water samples due to environmental dilution (Roussel et al. 2015). Furthermore, false negative detection can result from DNA being lower the detection threshold (Roussel et al. 2015). Conversely, false positives occur when eDNA of the target species is detected, but the organism is not present (Erickson et al. 2017). The reason for eDNA to be detected even though species is not present can be due to various reasons such as eDNA transportation in the field and eDNA sample cross-contamination in the laboratory (Roussel et al. 2015). For example, Pilliod et al. 2013 found that eDNA was not able to detect the presence of giant salamanders upstream, and this was attributed to eDNA transportation. Pilliod et al. 2013 stated that giant salamanders eDNA possibly moved downstream as pulses of high concentration of DNA fragments thus resulting in false negative detection. This was further illustrated in an experimental study were where giant salamanders were detected 15 meters downstream from where it was introduced (Pilliod et al. 2014).

2.1.3 The use of eDNA to detect invasive alien crayfish.

Environmental DNA has proven to detect early invasions of crayfishes and pathogens carried by IAFCs in first world countries such as France and the USA (Tréguier et al. 2014, Dougherty et al. 2016, Larson et al. 2017, Geerts et al. 2018). However, the majority of eDNA studies in freshwater ecosystems have focused on detecting and monitoring alien invasive fish and amphibians (Dougherty et al. 2016). The application of eDNA in monitoring alien crayfish in ecological studies is continuously increasing as there are studies by Tréguier et al. (2014), Dougherty et al. (2016), Agersnap et al. (2017), Dunn et al. (2017), Larson et al. (2017), Geerts et al. (2018), Manfrin et al. (2019), Troth et al. (2020) and Porco et al. (2022). Tréguier et al. (2014) investigated the efficiency of eDNA in detecting the red swamp crayfish, *P. clarkii* by sampling ponds in France. The eDNA results complemented bait trapping and the detection was positive with successful detection of 59% (Treguier et al. 2014). Other studies on using eDNA to detect alien crayfish include that of Dougherty et al. (2016), who developed qPCR assay to detect *Orconectes rusticus* at low abundances in inland lakes in the USA. The research successfully detected the species in all the lakes with physical specimen collection through trapping. In addition, Larson et al. (2017) also showed successful results using qPCR to detect eDNA of *O. rusticus* and *Pacifastacus leniusculus* in North American. Environmental DNA was used to detect the presence or absence of *Procambarus clarkii* in Belgium and the results showed that detection was possible with the species-specific ProCla primer even at low DNA concentration (Geerts et al. 2018).

2.1.4 Environmental DNA in South Africa

South Africa is a developing country and one of the challenges encountered in ecological studies in the country is lack of funds and resources to conduct eDNA monitoring surveillance studies (von der Heyden 2023). Therefore, this results in limited studies on the use of eDNA in freshwater system biological monitoring in the country. However, eDNA has been used to detect *Syngnathus watermeyeri* and *S. temminckii* from faecal matter of a critically endangered South African estuarine pipefish, *Syngnathus watermeyeri* in estuaries in Bushmans and Kariega where the species only occur in the country (Ntshudisane et al. 2021). Environmental DNA has also been employed as a biodiversity surveillance tool in Delta Park in Johannesburg and it detected 26 phyla and 522 species (Webster et al. 2020).

Environmental DNA has also been employed to survey biodiversity in protected areas. For example, Farrell et al. (2018) used eDNA to survey bacterial diversity in waterholes in Kruger National Park (KNP). Farrel et al. (20118) identified over 3000 taxa belonging to nine genera. Authors used eDNA metabarcoding to detect alien invasive silver carp (*Hypophthalmichthys molitrix*) from 11 of 12 watersheds sampled in KNP. Farrell et al. (2018) also used eDNA metabarcoding to detect the mammals that use the watershed in KNP. The eDNA detected 86 mammal species out of the 147 that have been documented in the park. Within the KNP, Krol et al. (2019) compared traditional mosquito trapping with eDNA to identify mosquito species in waterbodies in KNP. Through traditional aerial mosquito trapping, 3918 adults were trapped, resulting in 38 mosquito species identified morphologically and 34 species identified using eDNA metabarcoding. Environmental DNA was also employed in a freshwater fish surveillance study conducted in Eastern Cape (Castañeda et al. 2020). In this study, eDNA was found to be reliable in detecting native and alien freshwater fish as it outperformed traditional methods such as visual and snorkel surveys. Environmental DNA has also shown to be a reliable surveillance tool in marine systems. For example, Rossouw et al. (2023) detected species of arthropods, ascidians, cnidarians, echinoderms, ctenophores, molluscs, polychaetes, teleosts and sponges which were never recorded in the country. Despite the success rate of eDNA based species detection, there is still a huge gap in assessing and understanding the methodological aspects of the method (Czachur et al. 2021). This shows the promising benefits that eDNA offers as a monitoring method in freshwater systems.

2.1.5 Problem statement and study objectives

Cherax quadricarinatus and *P. clarkii* have established populations in the wild (discussed in detail in Chapter 1). Given this, attempts should be made to monitor their expansion into other waterbodies and their distribution in SA. For example, *P. clarkii* has expanded its distribution and is now established in Free State. Thus, this highlights the need for methods such as eDNA to monitor its expansion in the country. The continuing spread of alien crayfish in SA can pose detrimental and socio-economic impacts (Nunes et al. 2017a, Madzivandzira et al. 2021c).

Considering the burrowing behaviour of both *C. quadricarinatus* and *P. clarkii*, traditional methods such as visual surveys and trapping can be limiting in detecting the species mainly in

the early stage of invasion. From all this, it is evident that there is a need to incorporate innovative monitoring methods such as eDNA to successfully detect alien crayfish and avoid further impacts posed by the IAFCs in South African freshwater systems. This chapter assesses whether eDNA could be a reliable and effective monitoring method for the IAFCs in SA. This was achieved by comparing eDNA analysis with the traditional crayfish baited trapping and visual survey to detect the presence of *C. cainii, C. quadricarinatus* and *P. clarkii*. For eDNA species detection, three species identification techniques were used, i.e., end-point PCR, quantitative PCR and Next Generation Sequencing. The end-point PCR and qPCR were used because they have shown to be reliable to detect target species in freshwater systems (Mauvisseau et al. 2019a). Next Generation Sequencing has been used extensively in metabarcoding studies and it has also shown to be reliable for the detection of presence/ absence of species in freshwater systems (Taberlet et al. 2012, Comtet et al. 2015, Valentini et al. 2016, Emilson et al. 2017, Estrada-Franco et al. 2020, Danziger and Frederich 2022). Based on the growing reliability of eDNA to detect target species in freshwater systems from the literature, this study sought to determine whether eDNA would effectively detect the presence of *C. cainii, C. quadricarinatus* and *P. clarkii* from South African freshwater systems.

2.2 MATERIALS AND METHODS

2.2.1 Study Sites

Study sites were selected based on the known and current occurrence of alien freshwater crayfish, *C. cainii*, *C. quadricarinatus* and *P. clarkii* in SA following studies and surveys from Du Preez and Smit (2013), Nunes et al. (2017a, b, c) and Madzivandzira et al. (2021c). Study sites included various water bodies around SA i.e., rivers, pans and dams, known for the occurrence of the wild populations of *C. quadricarinatus* (KwaZulu-Natal and Mpumalanga Provinces) and *P. clarkii* (Free State and Mpumalanga) were sampled in January 2021 and May 2022 and October 2022 (Figure 2.1). Whereas for *C. cainii* Vaughn Bursey Stock Farm (VBSF) ponds were used as field positive control. Vaughn Bursey Stock Farm in the Eastern Cape is the only known site for the occurrence of *C. cainii* in the country. *Cherax cainii,* samples were collected in November 2019 from artificial ponds in VBSF.

In total 14 water bodies were sampled, the majority were for *C. quadricarinatus*, however most of the KwaZulu Natal and Mpumalanga sites were not sampled due regional floods in January 2021 (Figure 2.1). The sites that were safe to sample during this period were Lake Mzingazi and Nundwane River in Richard's Bay, Pongola Poort Dam (downstream) in Jozini and Pongola River (upstream) near the eSwatini border and four sites in Ndumo Game Reserve (i.e., Usutu River, kuShokwe Pan, Banzi Pan and Nyamiti Pan) (Figure 2.2). For *P. clarkii,* Mimosa Dam in Odendaalrus in Free State was sampled in January 2021, which is the newly reported population in the country (Alfreds 2018, Madzivandzira et al. 2017c), revisited and sampled in June 2022 and in Driehoek Farm November 2021.

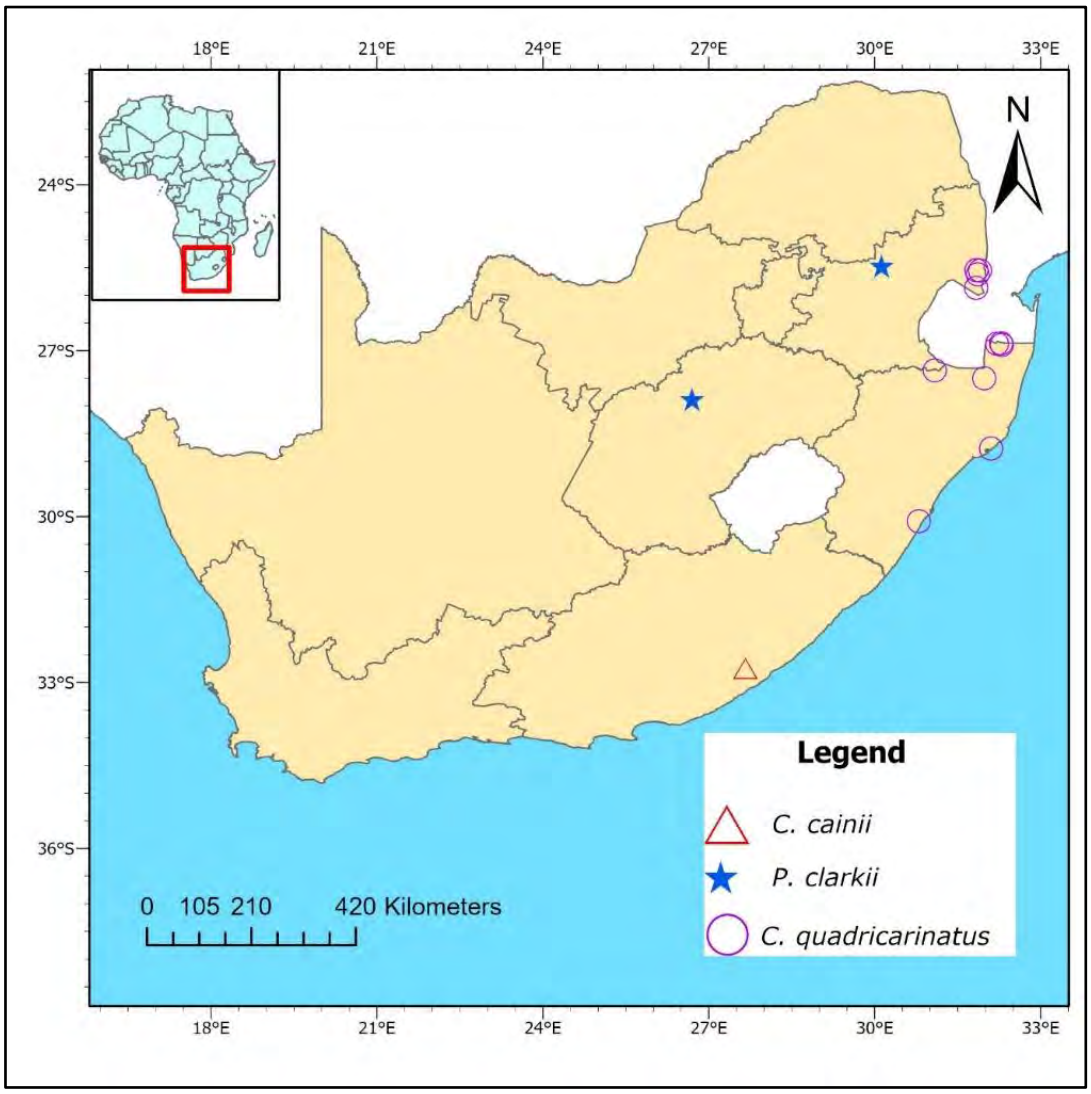


Figure 2.1 Map showing study areas and the sampling sites. *Cherax cainii* in in the Eastern Cape, *Cherax quadricarinatus* in KwaZulu Natal and Mpumalanga. *Procambarus clarkii* in Free State and Mpumalanga.

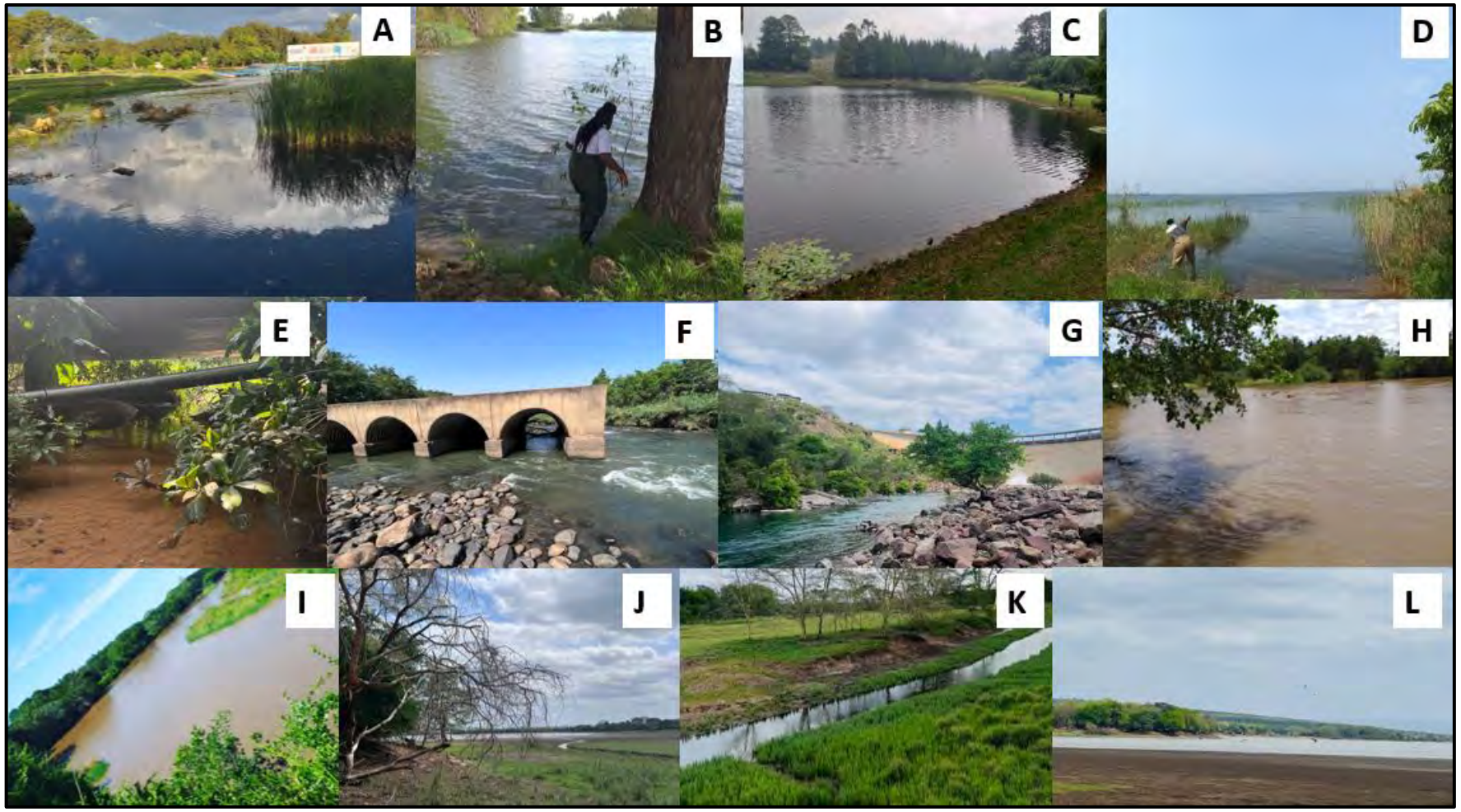


Figure 2.2 Study sites, Mimosa Dam Outside (**A**), Mimosa Dam Inside (**B**), Driehoek Farm (**C**), Lake Mzingazi (**D**), Nundwane River (**E**), Pongola River upstream (**F**), Pongola Poort Dam downstream (**G**), Banzi Pan (**H**), Usutu River (**I**), kuShokwe Pan (**J**), Nyamiti Pan (**K, L**).

2.2.2 eDNA water sampling

Water samples for eDNA were collected at each site prior *C. cainii*, *C. quadricarinatus* and *P. clarkii* collection by sweeping and trapping. Sampling was carried out following the methodology by Dougherty et al. (2016) and Larson et al. (2017). All sampling gear (waders, boots and forceps) was soaked in 10% bleach solution for a minimum of 10 minutes, rinsed with deionised water, and air-dried before sampling. The sampling gear was sprayed with 10% bleach in between sampling and a new pair of nitrile gloves was worn to avoid cross-contamination between sites. To improve the sampling coverage and maximise the chances of species detection through eDNA, three 500 ml water samples were collected at each site just below the water surface using clear polyethylene sampling containers. Water samples were immediately stored on ice in the dark to minimise eDNA degradation and within three hours of collection samples were filtered through a nitrocellulose membrane (47 mm diameter and 0.45 µm pore size) using a Nalgene™ hand-operated vacuum pump with Gauge (Figure 2.3). For the negative control, 250 ml of deionized water was used.

Handling and storing filter membranes was carried out following Pilliod et al. (2013) methodology. The filter membranes were rolled inward using sterilized forceps and placed into 2.0 ml microcentrifuge tubes filled with absolute ethanol for preservation (Figure 2.3). Each microcentrifuge tube was immediately sealed and placed in a labelled zip-lock bag. Samples were immediately stored in the dark at -20°C on ice to minimise eDNA degradation. In between sampling, the forceps were decontaminated with a 10% bleach solution and rinsed thoroughly with deionized water.

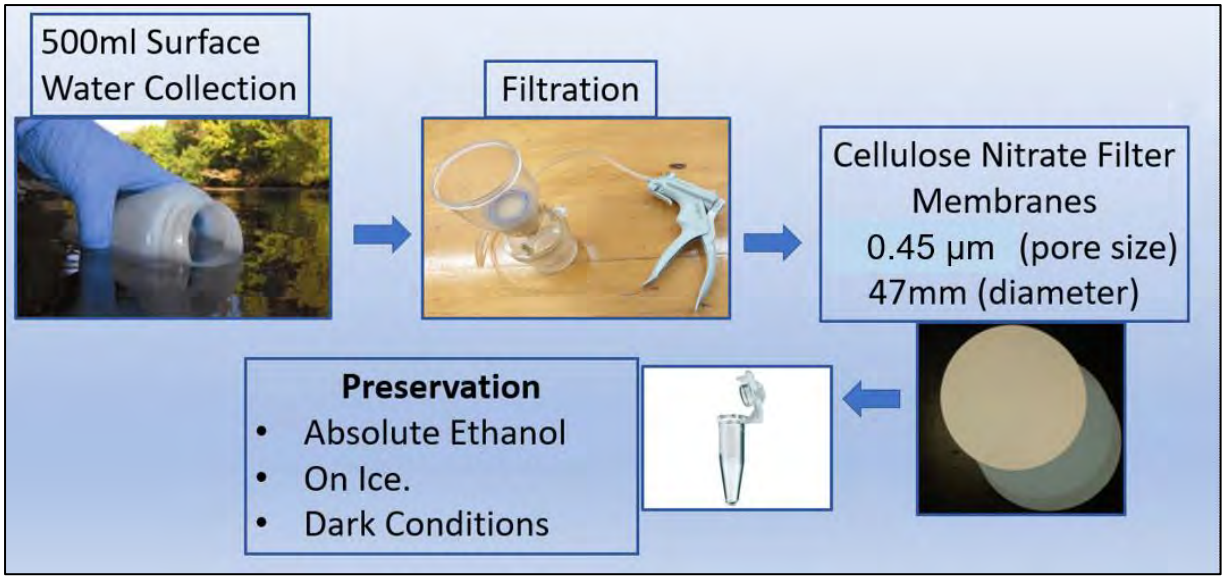


Figure 2.3 Schematic workflow eDNA sampling in the field showing eDNA collection, filtration and preservation.

2.2.4 Crayfish trapping surveillance.

Following Nunes et al. (2017b)'s procedure, traditional IAFCs was carried out, were at each site, 10-minute visual surveys were conducted along the littoral zone of the water body to look for burrows, specimens of crayfish and/or body parts residuals. A SASS net (30 × 30 cm frame, 1 mm mesh size) was used to collect crayfish specimens, additionally three Promar collapsible crayfish traps (61 x 46 x 20 cm, 10 mm mesh size), baited with 100g of dry dog food were deployed at different biotopes at each site and left overnight for 15 to 16 hours (Figure 2.4). Crayfish abundance was calculated based on catch per unit effort (CPUE). Catch per unit effort was calculated by dividing the total number of crayfish caught by the number of baited traps per night, i.e., CPUE = number of individuals/ numbers of traps/ night (g. trap. night $^{-1}$). Specimen caught were immediately euthanatized in 1 Litre containers with absolute ethanol. Specimens caught served as a positive control genomic DNA (gDNA) in end-point PCR, quantitative PCR (qPCR) and Next-Generation Sequencing (NGS).

Figure 2.4 Traditional crayfish trapping using baited Promar collapsible crayfish traps (61x46x20 cm, 10 mm mesh size).

2.2.5 Genomic DNA extraction

Genetics laboratory work was carried out at the South African Institute of Aquatic Biodiversity (SAIAB) molecular laboratory in Makhanda. Tissue from crayfish's abdomen was used for genomic DNA (gDNA) extraction using DNeasy® Blood and Tissue kit (QIAGEN) following the manufacturer's protocol with slight modifications. Tissues from targeted species, i.e., *C. cainii, C. quadricarinatus* and *P. clarkii* were placed in separate 2 ml tube. Per sample, 720 µl Buffer ATL was added, and the tubes were incubated at 56°C in a heat block for 30 min. After the incubation, 80 µl Proteinase K was added, and the tubes were placed in the shaking incubator at 56°C overnight. Thereafter, each tube was vortexed briefly for 15 seconds, and the mixture was transferred to spin column tubes (provided by the manufacturer with the kit). The tubes were centrifuged at 4000 x g for 1 minute. The supernatant in the collection tube of the spin column was discarded, and the remaining pellet was retained. A volume of 600 µl of Buffer AL and 600 µl of 70% ethanol were added, and the tubes were centrifuged at 8000 x g for 3

minutes, and the supernatant was then discarded. 500 µl of wash Buffer AW1 was added, tubes were centrifuged at 4000 x g for 1 minute, and the supernatant was again discarded. A volume of 500 µl second wash buffer (Buffer AW2) was added in each tube, and these were centrifuged at 4000 x g for 1 minute, and the supernatant was discarded. The spin column was transferred into 2.0 ml microcentrifuge tube, and 50 µl of elution buffer was added to each tube. The tubes were incubated at room temperature for 1 minute then centrifuged at 4000 x g for 1 minute. The DNA extracted was quantified, i.e., DNA concentration (ng/µl) using a NanoDrop (Thermo Fisher Scientific, Inc., Waltham, USA). The supernatant in the 2.0 ml microcentrifuge tube was the DNA samples; samples were stored at -20°C in a freezer in the laboratory before conducting end-point PCR, qPCR and NGS.

2.2.6 Environmental DNA extraction

Prior conducting DNA extraction, bench tops and hoods were decontaminated with 10% bleach solution followed by 70% ethanol (Goldberg et al. 2016). Environmental DNA samples (filter membranes) were extracted following Agersnap et al. (2017) method using the DNeasy® Blood and Tissue kit (QIAGEN). Each filter was directly subjected to DNA extraction; one half of each filter was used for DNA extraction and the other half was archived, that is, stored in -20°C in the freezer in the laboratory as a reserve. In each tube, 720 µl Buffer ATL was added and tubes were incubated at 56 °C in a heat block for 30 minutes. After the incubation, 80 µl Proteinase K was added and the tubes were at 56°C overnight in shaking incubator. After overnight incubation, each tube was vortexed briefly for 15 seconds and the mixture was transferred to spin column tubes (provided by the manufacturer). The tubes were centrifuged at 4,000 x g for 1 minute. The supernatant in the collection tube of the spin column was discarded, and the pellet was used for the remaining of the DNA extraction process. 600 µl of Buffer AL and 600 µl of 70% ethanol were added and tubes were centrifuged at 8,000 x g for 3 minutes, the supernatant was discarded. 500-µl of wash Buffer AW1 was added, tubes were at 4,000 x g for 1 minute and the supernatant was discarded. 500-µl second wash buffer, Buffer AW2 was added in each tube, the tubes were centrifuged at 4,000 x g for 1 minute and the supernatant was discarded. The spin column was transferred into 2.0 ml microcentrifuge tube and 50 µl of elution buffer was added in each tube. The tubes were incubated at room temperature for 1 minute then centrifuged at 4,000 x g for 1 minute. The supernatant in the

2.0 ml microcentrifuge tube was the DNA samples and was kept at -20°C in a freezer in the laboratory to minimize DNA degradation prior to conducting end-point PCR, qPCR, and Next Generation Sequencing.

2.2.7 End-point PCR

End-point PCR reactions were performed on a Veriti 96 well thermal cycler (Applied Biosystems, Thermo Fisher Scientific, RSA). Primer pair 1471 (5'CCTGTTTANCAAAAACAT3') and 1472 (5'AGATAGAAACCAACCTGG3') 545bp (Munasinghe et al. 2004). The primer pair targets 16S rRNA region and is specific to *Cherax* species which were used to detect the presence of *C. cainii* and *C. quadricarinatus*. PCR reactions were conducted in 25 µl volume consisting of 12.5 µl (Taq 2x Master Mix, Thermo Fisher Scientific, RSA), 3 µl of DNA, 7.5 µl of molecular grade water, 1 µl of forward and reverse primer (10 µM). The thermocycling conditions were 95°C for 5 minutes, 30 cycles of 30 seconds at 95°C, 30 seconds at 50°C, 30 seconds at 72°C, 72°C for 5 minutes and 25°C for 1 minute (Munasinghe et al. 2004). The annealing temperature of 48°C was for both *C. cainii* and *C. quadricarinatus.*

2.2.8 Agarose gel electrophoresis

A concentration of 1% agarose gel was prepared to visualise PCR products by adding 1.0 g of agarose into 100 ml of 1X TAE (45 mM Tris pH 8, 45 mM acetic acid, 1 mM ethylenediaminetetraacetic acid [EDTA]). The agarose was heated in the microwave for 2 minutes to ensure that it is completely dissolved in the TAE buffer. The solution was allowed to cool, and 5 µl of 0.5 µg/l ethidium bromide was added. The contents were slowly poured into the gel casting tray with a pre-placed comb. The gel was solidified by cooling at room temperature for 20 minutes. After polymerization, the combs were removed, and the casting tray was placed in the electrophoresis chamber and was filled with 1X TAE buffer. After addition of prepared samples into the wells, the gel was run at 120 volts for 30 minutes and then visualized using UV light (GeneGenius Bioimaging System - Syngene, USA).

2.2.9 DNA sequencing

The DNA from PCR products were purified using the Qiaex II kit (Qiagen) and sequenced in one direction (forward) using the Big Dye v.3 sequencing kit following the standard protocol. The obtained sequences of the crayfish were searched on the Basic Local Alignment Search Tool (BLAST) (Casey 2005) to find a sequence based on the 16S rRNA gene for species identification (http://www.ncbi.nlm.nih.gov).

2.2.10 Quantitative PCR

Quantitative PCR (qPCR) reactions were performed on a QuantStudio™ 5 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, RSA). The qPCR was carried out using *P. clarkii* specific assay targeting the mitochondrial COI region (Treguier et al. 2014). The standard dilution using gDNA of *P. clarkii* was prepared to create a qPCR standard curve ranging from 10^{-2} to 10^{-8} using molecular grade water. The dilutions were made to validate the sensitivity and efficiency of the primers to detect *P. clarkii.* Three replicates per eDNA sample and qPCR negative controls using molecular grade water were also included for each experiment (Figure 2.5). Quantitative PCR was performed using ProCla primers of 96 bp, specific to *P. clarkii.* Forward primer SPY_ProCla_F (5'AACTAGGGGTATAGTTGAGAG3'), reverse primer SPY_ProCla_R (5'CAGAAGCTAAAGGAGGATAA3') and probe, SPY_ProCla_Probe (5'FAM-AGGAGTTGGAACAGGATGGACT-MBG-3') designed by Treguier et al. (2014) for qPCR assay were used. Quantitative PCR reactions were performed in a total reaction volume of 25 µl, containing 12.5 µl (Taq_ Environmental Master Mix, Lasec, RSA), 3 µl of DNA, 6.5 µl of ddH2O, 1 µl of forward and reverse primer (10 µM) and 1 µl of probe (2.5µM). The thermocycling conditions were 50°C for 5 minutes and 95°C for 10 minutes, followed by 55 cycles of 95°C for 30 seconds and 56°C for 1 minute (adapted from Treguier et al. 2014). Environmental DNA laboratory was conducted under supervision of experienced laboratory technician in SAIAB, after several attempts of failed qPCR, samples were sent to Inqaba Biotec in Pretoria, however results where no different.

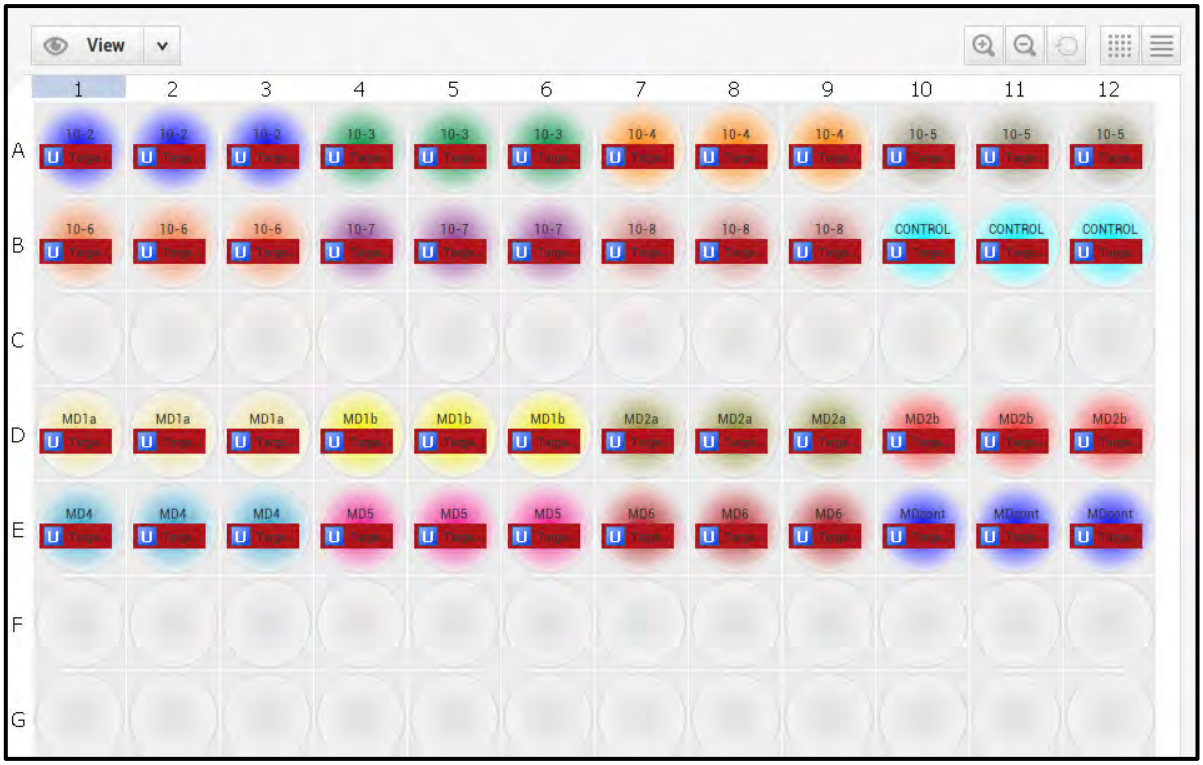


Figure 2.5 Diagram obtained from QuantStudio™ Design and Analysis Software v1.5.2 showing the qPCR experiment design illustrating genomic DNA dilutions (10^{-2} to 10^{-8}) in rows A & B and eDNA samples in rows D & E on qPCR plate for the detection of P. *clarkii*.

2.2.11 Next Generation Sequencing Metabarcoding

Next generation Sequencing (NGS) library construction and sequencing were performed at Macrogen Europe B.V., Netherlands. The NGS library was constructed for 16S rRNA Primer pair 1471 (5'CCTGTTTANCAAAAACAT3') and 1472 (5'AGATAGAAACCAACCTGG3') 545 bp for *C. cainii* and *C. quadricarinatus* and mitochondrial COI SPY_ProCla_F (5'AACTAGGGGTATAGTTGAGAG3'), reverse primer SPY_ProCla_R (5'CAGAAGCTAAAGGAGGATAA3') 96 bp for *P. clarkii*. Paired-end Metagenome Amplicon Sequencing was carried out using Sequencing - MiSeq - 300 PE following the Herculase II Fusion DNA Polymerase Nextera XT Index V2 Kit manufacturer's protocol.

Table 2.1 Forward and reverse primers, and probe sequences used for detecting *C. cainii*, *C. quadricarinatus* and *P. clarkii*.

Target species	Primer sequence 5'-3'	Method	Reference
Cherax cainii and *Cherax quadricarinatus*	**16s rRNA** 1471 (5'-CCTGTTTANCAAAAACAT-3') and 1472 (5'-AGATAGAAACCAACCTGG-3')	• End-point PCR • NGS	Munasinghe et al. (2004)
Procambarus clarkii	**COI region** SPY_ProCla (SPY_ProCla_F 5'-AACTAGGGGTATAGTTGAGAG-3') and (SPY_ProCla_R 5'-CAGAAGCTAAAGGAGGATAA-3') SPY_ProCla_Probe (SPY_ProCla_Probe 5'-FAM-AGGAGTTGGAACAGGATGGACT-MBG-3')	• qPCR	Treguier et al. (2014)

2.3 RESULTS

2.3.1 Traditional Crayfish trapping surveillance.

A total of 21 *P. clarkii* specimens were caught in Mimosa Dam, resulted in 13 males and 8 females over 1 night, with capture per unit effort (CPUE) for *P. clarkii* equivalent to 7 g/trap.night^{-1}. The average body mass of females from Mimosa Dam was 47.4 g, and that of males was 65.4 g (Figure 2.6a, Table 2.2). In Driehoek Farm, there was a total of 3 specimens caught over 1 night, that is, 1 male and two females. The average body mass was 16.8g. *Procambarus clarkii* specimens also varied in body length ranging from 95 to 231 mm, specimens from Mimosa Dam had an average body length for females being 165.5 mm and that of males was 187.4 mm. The *P. clarkii* specimens from Driehoek Farm had average body of 122.7 mm (Figure 2.6b, Table 2.2). However, traditional baited trapping was not successful in catching *C. quadricarinatus* in Nundwane River, Lake Mzingazi, Pongola River (upstream),

and Pongola Poort Dam (downstream). In Ndumo Game Reserve, one specimen was caught in Banzi Pan (Table 2.2), and no specimens were caught in Usutu River, kuShokwe Pan and Nyamiti Pan.

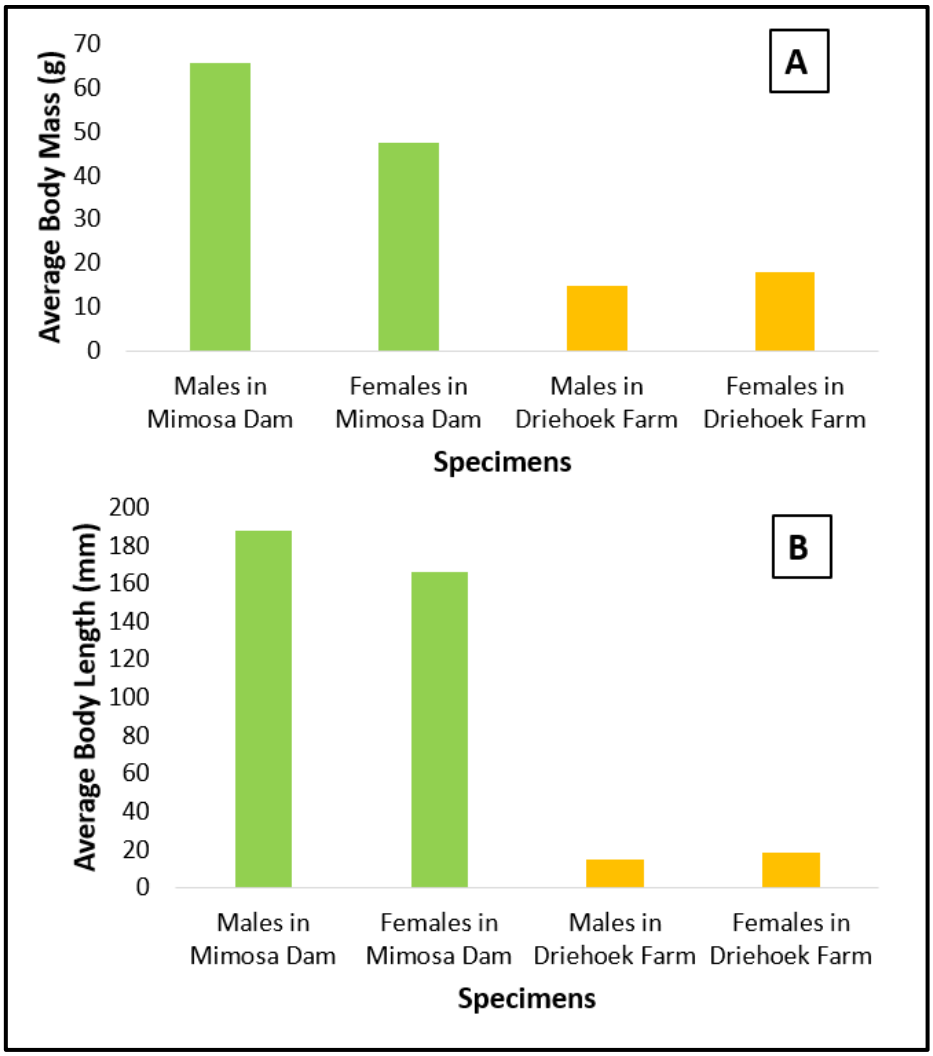


Figure 2. 6 Average body mass (**A**) and length (**B**) of collected *P. clarkii* specimens.

Table 2.2 Sites *C. cainii*, *C. quadricarinatus* and *P. clarkii* were caught. Sites, coordinates, average mass (g), average size (body length in mm), number of females (NF) and number of males (NM) of *C. cainii*, *C. quadricarinatus* and *P. clarkii* caught per site.

Site	**Coordinates**	**Target species**	**Average mass (g)**	**Average body length (mm)**	**NF**	**NM**
Vaughn Bursey Farm	27°55'46.65" S 32°71'21.67" E	*C. cainii*	52.7	96.3	1	1
Banzi Pan	26°87'78.01" S 32°29'02.87" E	*C. quadricarinatus*	54.7	138	1	0
Mimosa Dam	27°88'13.36" S 26°69'52.24" E	*P. clarkii*	58.5	177.7	8	13

2.3.2 Environmental DNA (eDNA) Surveillance

The genomic DNA was pure as confirmed by the absorbance 260/280 ratio (Table 2.4). The ratio of absorbance at 260 and 280nm (260/280) is used to determine the DNA purity. The recommended absorbance reading is between 0.1 and 2.0. However, the absorbance reading above 2.0 indicates impure DNA samples. The eDNA concentration of *C. cainii* from pond 1 (with less than 100 individual) had less than 10 ng/µl. Pond 2 (more than 500 individuals) and pod 3 (more than 1000 individuals) had more than 20 ng/µl (Table 2.4). The greatest eDNA concentration for *C. quadricarinatus* was 98.0 ng/µl yielded by sample UR1 from Usutu River and the lowest was 4.8 ng/µl from Dam 1 in Mpumalanga (Table 2.4). For *P. clarkii*, the highest eDNA concentration was 47.5 ng/µl from sample M2A sampled outside Mimosa Dam. The lowest DNA concentration was 7.4 ng/µl sampled inside Mimosa Dam (Table 2.4). For all sites, negative controls were included. Polymerase Chain Reaction failed, as it resulted in primer dimers, that is unsuccessful PCR amplification (Figure 2.7). Figure This resulted in successful PCR for the target species, *C. cainii*, *C. quadricarinatus* and *P. clarkii* and no amplification appeared in the negative controls, as expected (Figure 2.8). The obtained sequences were confirmed in NCBI BLAST (https://blast.ncbi.nlm.nih.gov). The results from gel electrophoresis showed that eDNA was amplified for C. *cainii* and *C. quadricarinatus* by an indication of DNA bands in the gel. However, there were multiple DNA bands indicating that the primer might have also amplified the non-target DNA from the water samples (Figure 2.9a). Due to this, the annealing temperature was adjusted from 56°C to 48°C to obtain clean DNA bands of appropriate size (Figure 2.9b). The eDNA samples with appropriate DNA bands failed to sequence, and several DNA sequencing attempts were made thereafter without a success.

Since the aim of this study was to field-test the reliability of eDNA to detect IAFCs in SA by comparing it with the traditional baited trapping. Traditional baited trapping was found to successful in detecting the crayfishes in the field (Table 2.3). However, quantitative PCR which was used to measure eDNA for *P. clarkii* was unsuccessful (Figure 2.10 and Figure 2.11). The Next Generation Sequencingx results were able to detect *C. cainii* from sample collected at Vaughn Bursey's Stock Farm. For *P. clarkii,* NGS was not able to detect the species. However, for *C. quadricarinatus* eDNA samples were not included in NGS due to financial constraints.

Table 2.3 Summary of where target alien freshwater crayfish were caught (traditional baited trapping and eDNA surveys), **+** indicating positive detection and **–** indicating negative detection.

Sampling site	**Target species**	**Traditional survey**	**eDNA survey**
Vaughn Bursey's Farm	*Cherax cainii*	+	-
Banzi Pan	*Cherax quadricarinatus*	+	-
Mimosa Dam	*Procambarus clarkii*	+	-

Table 2.4 Environmental DNA concentrations and purity (260/280) for *C. cainii*, *C. quadricarinatus* and *P. clarkii*.

Target Species	**Sampling site**	**Sample ID**	**eDNA concentration (ng/µl)**	**Purity 260/280**
Cherax cainii	Vaughn Bursey Farm Pond 1	VB1a	6.3	1.93
	Vaughn Bursey Farm Pond 1	VB1b	6.8	2.08
	Vaughn Bursey Farm Pond 1	VB1c	6.0	2.22
	Vaughn Bursey Farm Pond 1	**Control VB1**	1.3	1.47
	Vaughn Bursey Farm Pond 2	VB2a	36.0	2.25
	Vaughn Bursey Farm Pond 2	VB2b	28.1	1.63
	Vaughn Bursey Farm Pond 2	VB2c	25.6	1.59
	Vaughn Bursey Farm Pond 2	**Control VB2**	1.0	1.86
	Vaughn Bursey Farm Pond 3	VB3a	27.3	1.39
	Vaughn Bursey Farm Pond 3	VB3b	36.5	2.04
	Vaughn Bursey Farm Pond 3	VB3c	32.1	1.89
	Vaughn Bursey Farm Pond 3	**Control VB3**	0.9	1.50

Table 2.4 (continuation) Environmental DNA concentrations and purity (260/280) for *C. cainii*, *C. quadricarinatus* and *P. clarkii*.

Target Species	Sampling site	Sample ID	eDNA concentration (ng/µl)	Purity 260/280
Cherax quadricarinatus	Mpumalanga Dam 1	D1A	7.0	1.69
	Mpumalanga Dam 1	D1B	4.8	1.89
	Mpumalanga Dam 1	D1C	7.3	1.77
	Mpumalanga Dam 1	**Control D1**	1.9	1.53
	Mpumalanga Dam 2	D2A	10.7	1.48
	Mpumalanga Dam 2	D2B	10.3	1.62
	Mpumalanga Dam 2	D2C	27.5	1.47
	Mpumalanga Dam 2	**Control D2**	3.1	1.60
	Mpumalanga Dam 3	D3A	14.2	1.81
	Mpumalanga Dam 3	D3B	21.8	2.03
	Mpumalanga Dam 3	D3C	22.7	1.87
	Mpumalanga Dam 3	**Control D3**	3.4	1.77
	Lake Mzingazi	LM1	11.6	2.08
	Lake Mzingazi	LM2	20.5	1.90
	Lake Mzingazi	LM3	20.4	1.85
	Lake Mzingazi	**Control LM**	5.3	1.71

Table 2.4 (continuation) Environmental DNA concentrations and purity (260/280) for *C. cainii*, *C. quadricarinatus* and *P. clarkii*.

Target species	Sampling site	Sample ID	DNA concentration ng/µl	Purity 260/280
Cherax quadricarinatus	Nundwane River	NR1	48.3	1.45
	Nundwane River	NR2	35.1	1.50
	Nundwane River	NR3	10.8	1.65
	Nundwane River	**Control NR**	3.7	1.44
	Pongola Dam (downstream)	PD1	46.2	1.30
	Pongola Dam (downstream)	PD2	50.6	1.31
	Pongola Dam (downstream)	PD3	32.1	1.47
	Pongola Dam (downstream)	**Control PD**	1.0	1.41
	Pongola River (upstream)	PR1	21.4	1.68
	Pongola River (upstream)	PR2	27.1	1.42
	Pongola River (upstream)	PR3	27.6	1.34
	Pongola River (upstream)	**Control PR**	4.4	2.66
	Usutu River (Ndumo)	UR1	98.0	1.34
	Usutu River (Ndumo)	UR2	18.7	1.30
	Usutu River (Ndumo)	UR3	91.5	1.33
	Usutu River (Ndumo)	**Control UR**	3.5	1.68
	Banzi Pan (Ndumo)	BP1	81.2	1.33
	Banzi Pan (Ndumo)	BP2	83.7	1.32
	Banzi Pan (Ndumo)	BP3	85.5	1.34
	Banzi Pan (Ndumo)	**Control BP**	4.2	1.26

Table 2.4 (continuation) Environmental DNA concentrations and purity (260/280) for *C. cainii*, *C. quadricarinatus* and *P. clarkii*.

Target species	Sampling site	Sample ID	DNA concentration ng/µl	Purity 260/280
Procambarus clarkii	Mimosa Dam (Inside)	M1A	7.4	1.82
	Mimosa Dam (Inside)	M1B	11.0	1.12
	Mimosa Dam (Inside)	M1C	7.5	1.32
	Mimosa Dam (Inside)	**Control MD1**	1.0	1.09
	Mimosa Dam (Outside)	M2A	47.5	1.60
	Mimosa Dam (Outside)	M2B	27.2	1.52
	Mimosa Dam (Outside)	M3B	29.7	1.36
	Mimosa Dam (Outside)	**Control MD2**	5.2	1.32

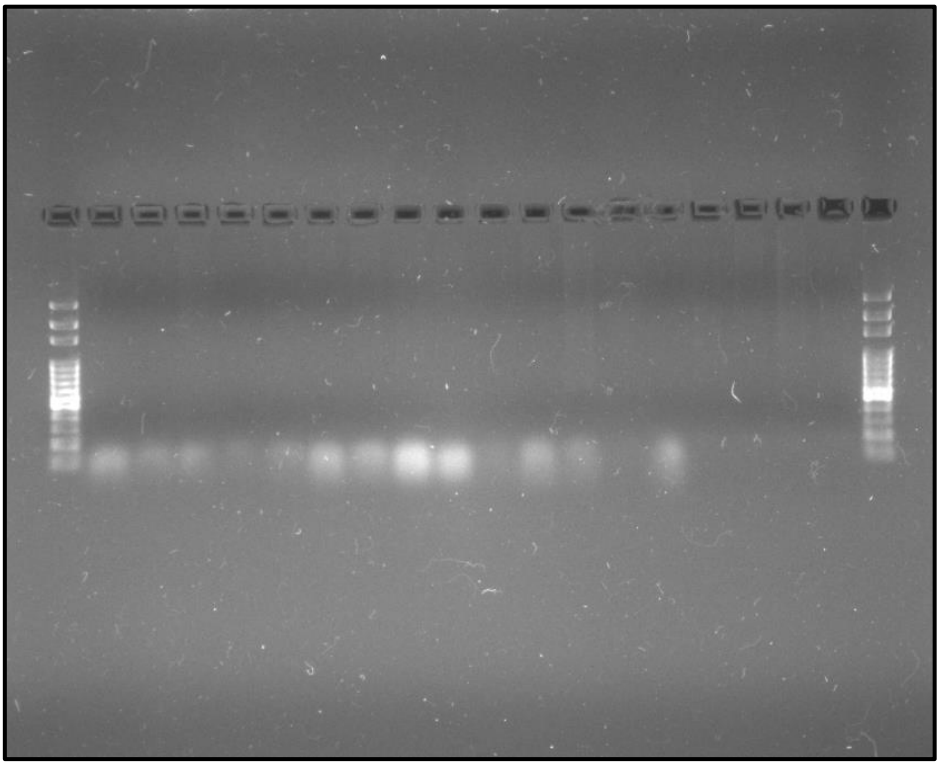


Figure 2.7 Primer dimers instead of DNA bands for *C. cainii* and *C. quadricarinatus* on 1% agarose gel from PCR products (annealing temperature 48°C) (failed PCR).

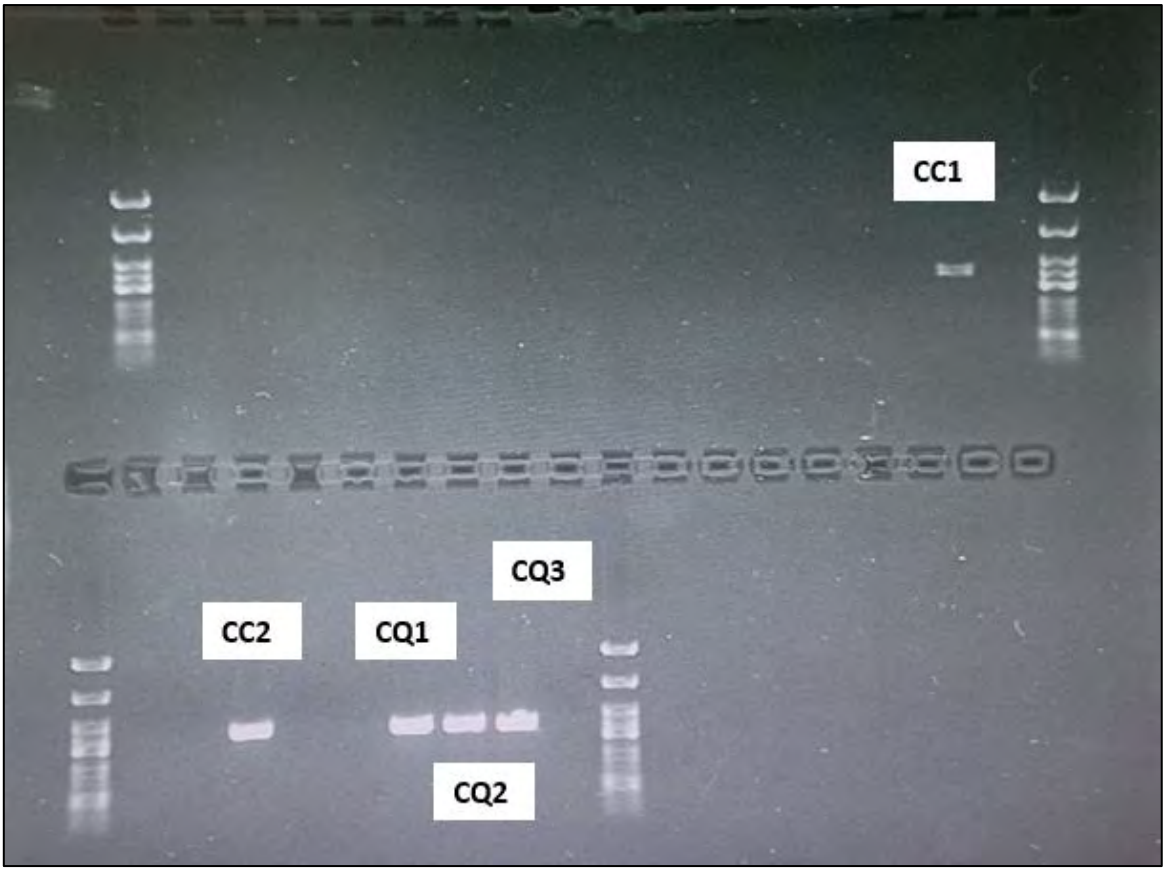


Figure 2.8 Single clear DNA bands of *C. cainii* (CC1 and CC2) and *C. quadricarinatus* (CQ1, CQ2 & CQ3) on 1% agarose gel from PCR products (annealing temperature 48°C).

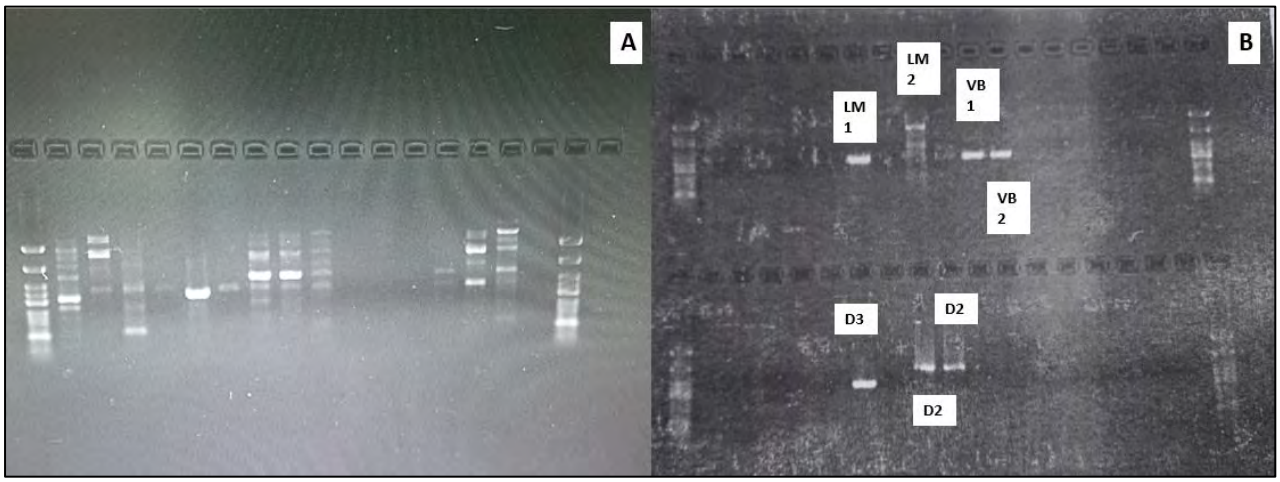
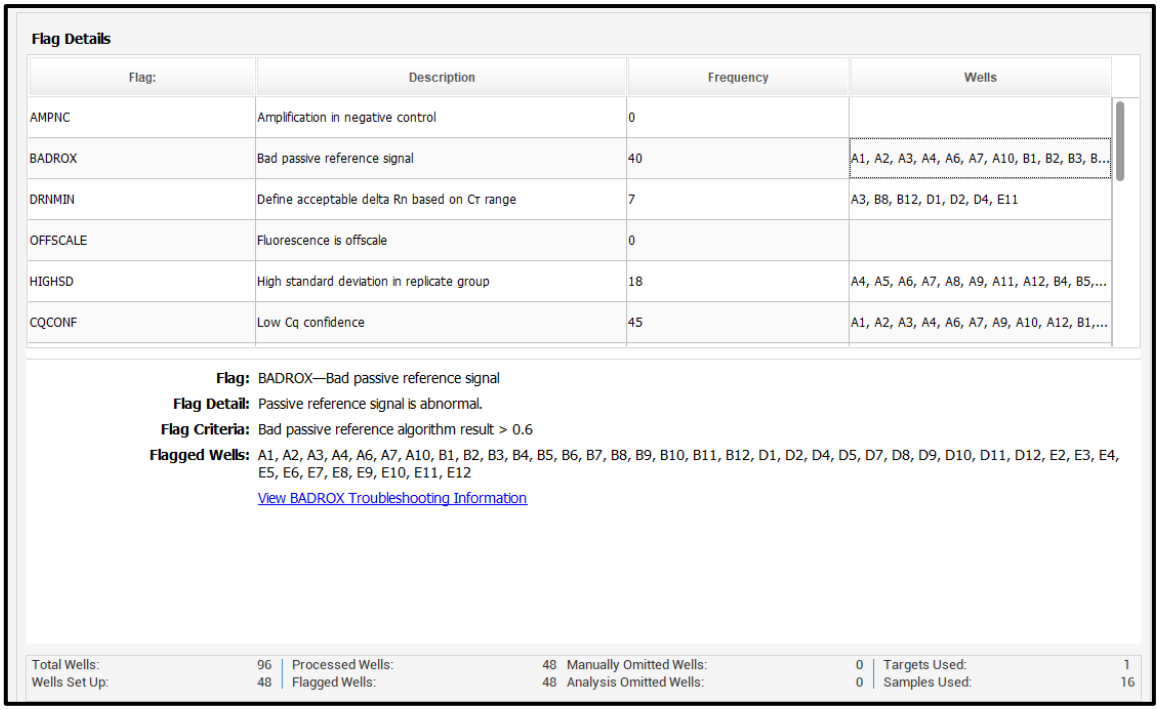


Figure 2.9 DNA bands from eDNA samples; multiple amplification including non-target DNA (annealing temperature 50°C) **(A),** DNA bands from DNA samples (annealing temperature 48°C), **(B)** Lake Mzingazi (LM1, LM2), Vaughn Bursey's Farm (VB1, VB2), Dams in Mpumalanga (Dam 2, Dam 2, Dam 3).



Flag Details

Flag:	Description	Frequency	Wells
AMPNC	Amplification in negative control	0	
BADROX	Bad passive reference signal	40	A1, A2, A3, A4, A6, A7, A10, B1, B2, B3, B...
DRNMIN	Define acceptable delta Rn based on Cт range	7	A3, B8, B12, D1, D2, D4, E11
OFFSCALE	Fluorescence is offscale	0	
HIGHSD	High standard deviation in replicate group	18	A4, A5, A6, A7, A8, A9, A11, A12, B4, B5,...
CQCONF	Low Cq confidence	45	A1, A2, A3, A4, A6, A7, A9, A10, A12, B1,...

Flag: BADROX—Bad passive reference signal
Flag Detail: Passive reference signal is abnormal.
Flag Criteria: Bad passive reference algorithm result > 0.6
Flagged Wells: A1, A2, A3, A4, A6, A7, A10, B1, B2, B3, B4, B5, B6, B7, B8, B9, B10, B11, B12, D1, D2, D4, D5, D7, D8, D9, D10, D11, D12, E2, E3, E4, E5, E6, E7, E8, E9, E10, E11, E12

View BADROX Troubleshooting Information

Total Wells:	96	Processed Wells:	48	Manually Omitted Wells:	0	Targets Used:	1
Wells Set Up:	48	Flagged Wells:	48	Analysis Omitted Wells:	0	Samples Used:	16

Figure 2.10 QC Summary for qPCR experiment obtained from the QuantStudio™ Design & Analysis Software v1.5.2 showing failed qPCR for *P. clarkii* as negative controls were amplified (wells B10-B12 and E10-12).

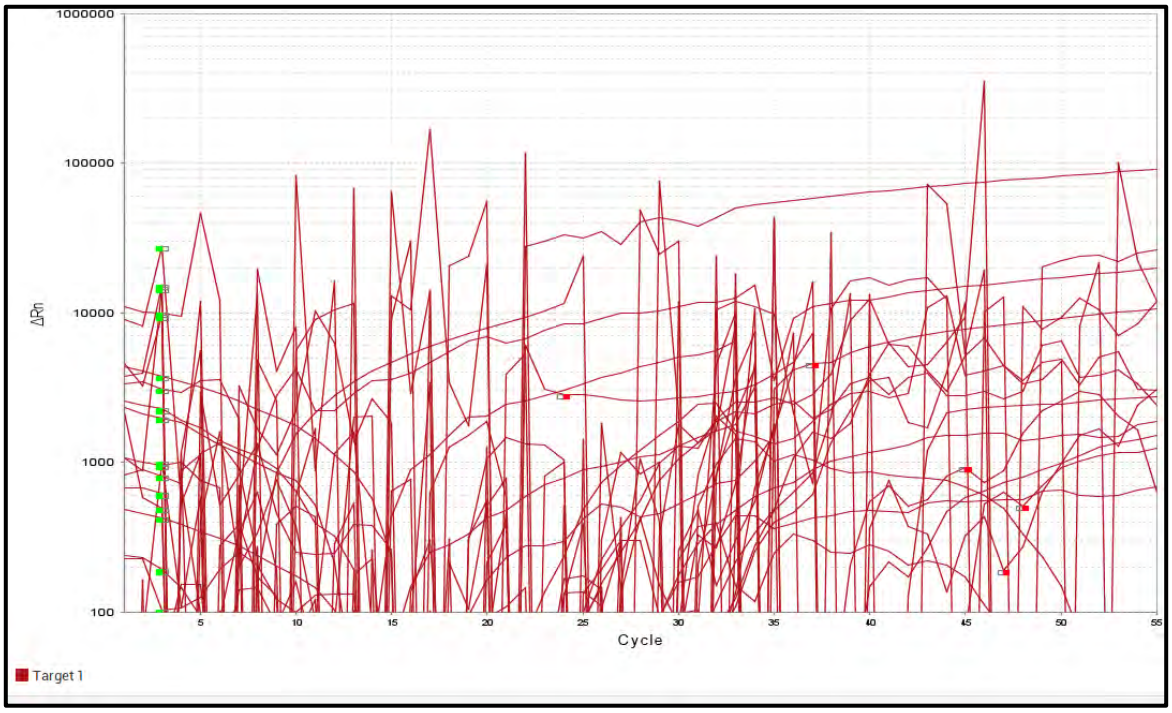


Figure 2.11 Amplification plot showing unsuccessful (failed) qPCR for the detection of *P. clarkii.*

2.4 DISCUSSION

This chapter aimed to investigate the reliability of eDNA as an effective detection and monitoring tool for IAFCs in SA. The next-generation sequencing (NGS) detected *C. cainii* due to its highly sensitive throughput sequencing. Studies have shown NGS to be able to detect the target species even at low abundance where traditional sampling was not able to (Taberlet et al. 2012, Comtet et al. 2015, Gueuning et al. 2019, Huggins et al. 2022). For example, in Yamamoto et al. (2017), NGS detected 23 species that were not detected by traditional underwater visual survey in Maizuru Bay in Japan. Similarly, NGS was also able to detect Japanese water shrew *Chimarrogale platycephala* in Japan, which was difficult to detect with traditional visual surveys and camera traps (Yonezawa et al. 2020). The failure of traditional sampling methods to not detect the Japanese water shrew was attributed to the species' small size and nocturnal behaviour (Yonezawa et al. 2020). The relatively high sensitivity of NGS to detect species in aquatic systems is due to its ability to sequence and identify multiple genes in a sample (McElroy et al. 2020).

The results for end-point PCR and qPCR showed unexpected outcomes. Instead, this section outlines the challenges and limitations observed when employing eDNA as a monitoring method for IAFCs in SA. The first challenge encountered in the field was that not all sites known for the occurrence of *C. quadricarinatus* were sampled due to flooding in January 2021. A second challenge encountered in the field was the difficulty of filtering adequate amounts of water for sites with high turbidity. Many South African waters are known for high turbidity or suspended solids (Kirk 1985). The volume of water filtered through can influence eDNA yield, eDNA quality and eDNA detection (Mauvisseau et al. 2019b). As previously mentioned, some freshwater systems in SA invaded by invasive crayfishes have crocodiles and hippos. These animals actively and regularly disturb the benthos in these sites, thus result in high turbidity, which affects the success of eDNA detection. The aquaculture ponds that housed *C. cainii* were consistently aerated and water quality was regularly monitored, which could be the reason why NGS was able to detect *C. cainii*.

Another factor that can influence eDNA detection accuracy, is the type of DNA extraction method used (Renshaw et al. 2015, Williams et al. 2016, Hinlo et al. 2017, Johnson et al. 2019). For example, the PowerWater Kit has been designed to improve eDNA yield from water

samples (Lear et al. 2018). The kit has been shown to be reliable in extraction eDNA from filtered membranes because of its built-in protein and PCR inhibitor removal step (Turner et al. 2014). As shown in Coster et al. (2021), the eDNA detection of the rusty crayfish (*Faxonius rusticus*) was more successful in samples that were preserved in Longmire's buffer and extracted with the PowerWater Kit compared to samples preserved in absolute ethanol and extracted with DNeasy Blood and Tissue Kit, which was the method used in this study. Moreover, the burrowing behaviour of *C. quadricarinatus* and *P. clarkii* could have resulted in decreased amount of eDNA in water and subsequently resulted in negative detection. This was a case in Treguier et al. (2014) where eDNA only detected *P. clarkii* in 59% sites where the species was known to occur. Since, eDNA is diluted in water and subsequently results in low eDNA concentration (Roussel et al. 2015, Sansom and Sassoubre 2017, Curtis et al. 2021) sediment samples should also be explored particularly in deep water systems as crayfish are burrowing. Moreover, it was found that eDNA performed better for detecting crayfish in small and shallow ponds than in deep ponds (Treguier et al. 2014).

The current study also encountered technical challenges with regards to sequencing eDNA PCR products to confirm *C. cainii* and *C. quadricarinatus*. This was unexpected as the primers successfully amplified and sequenced genomic DNA of both species, which illustrated that the primers 1471 and 1472 (Munasinghe et al. 2004) were able to amplify the *C. cainii* and *C. quadricarinatus*, the difficulty was with eDNA samples. The eDNA PCR products showed DNA bands on the gel electrophoresis, indicating successful DNA amplification. DNA sequencing is critical for eDNA because the presence of target species is confirmed by verifying the sequences on BLAST. Based on the end-point PCR results of the current study, the successful PCR products observed on gel electrophoresis were not enough as sequencing failed several attempts. The failed eDNA sequencing could have been due to low DNA concentration or eDNA degradation based on the preservation method used in this study (preserved filter membranes in absolute ethanol) according to Pilliod et al. (2013). The type of preservation methods can influence the eDNA degradation. For example, Copper et al. (2021) found that eDNA samples preserved in Longmire's buffer yielded more DNA concentration of Dwarf sawfish (*Pristis clavate*) than samples preserved in absolute ethanol. However, in Spens et al. (2017) there was no difference in eDNA yield and quality from samples preserved in

Longmire's buffer and absolute ethanol. Therefore, other eDNA preservation methods should also be explored for the successful detection of IAFCs in SA.

Quantitative PCR has been used for the successful detection of *P. clarkii* beyond its native range (Treguier et al. 2014, Cai et al. 2017, Riascos et al. 2018, Kawai 2022, Lo Presti et al. 2022, Ogata et al. 2022) and in these studies there were no explicit technical challenges encountered for qPCR detection of *P. clarkii.* However, failed qPCR results for the detection of *P. clarkii* in this present study might have been due to sewage pollution in Mimosa Dam as the site was heavily polluted by faecal sewage. The aspect of sewage pollution's influence on eDNA has not been explored to the best of my knowledge. Another possible reason for failed eDNA detection could be our choice of primers used which could have negatively affected eDNA detection (Valentini et al. 2016; Xia et al. 2018; Trujillo-Gonzalez et al. 2021). Geerts et al. (2018) found RodRiv primers to be more sensitive in detecting the presence of *P. clarkii* than the ProCla primer which we used in this study. Both ProCla and RodRiv were found to be successful to amplify the *P. clarkii* gDNA, however, for eDNA samples, RodRiv primers were found to be more sensitive in detecting *P. clarkii* with a success rate was 61% and that of ProCla primers was 22% (Geerts et al. 2018). Geerts et al. (2018) also used the ProCla primers that were designed by Treguier et al. (2014). Even though ProCla primers were able to successfully detect the presence of *P. clarkii* in the previous studies (Treguier et al. 2014, Geerts et al. 2018, Riascos et al. 2018), the results of this study show that the use of this primer can be limiting. Therefore, other primers and the designing of new primers should be explored to allow successful detection of IAFCs in SA.

The eDNA detection of *P. clarkii* was compared to the capture-based distribution of the species obtained from trapping (Geerts et al. 2018). Geerts et al. (2018)'s results showed that eDNA detection accuracy was 86%, which indicated that eDNA had high levels of detection. Environmental DNA has been found to be highly sensitive in detecting target species at low population abundances, which is associated with early stages of invasion (Dougherty et al. 2016, Larson et al. 2017, Sepulveda et al. 2019, Keller et al. 2022). Also, regarding the issue of primers, it will be worth exploring the use of RodRiv primers (Forward: 5'-TGGAGGCTGGAATGAATGG-3', Reverse: 5'-GGT CTTATCGTCCCTCTAA-3' and specific 6-FAM MGB labelled probe 5'-TGG ACG AGA AGG AAG CTGTC-3') designed by Baudry et al. (2021) for qPCR assay specific to *C. quadricarinatus* in future studies. These primers successfully

detected *C. quadricarinatus* in all known sites sampled in Martinique Island (Baudry et al. 2021).

From the results, negative detection by eDNA does not necessarily imply that the species is absent in the system as this was the case for *C. quadricarinatus* in Banzi Pan and *P. clarkii* in Mimosa Dam. As such, understanding the dynamics of eDNA is crucial prior to employing the method in the field. Therefore, we recommend a future study that will assess the dynamics of eDNA in a mesocosm experiment. The mesocosm experiment will provide a good opportunity to test the sensitivity of eDNA, from eDNA collection to eDNA amplification. We believe that eDNA detection can also be improved by filtering larger volumes of water since DNA concentration of target species is usually low in water samples due to dilution as proven in Minamoto et al. (2016). The results from this study illustrate that eDNA is indeed a complex technique that requires an in-depth evaluation of its technical limitations before employing it as biomonitoring method in the wild. This highlights the need for a refined and standardized eDNA protocol from collection, preservation, eDNA extraction and amplification for an improved and efficient eDNA-based crayfish monitoring in South African freshwater systems. This is of high concern because of continuous expansion and spread of *C. quadricarinatus* and *P. clarkii* in the country.

2.5 CONCLUSION

In this study, traditional baited traps and visual surveys confirmed the presence of *P. clarkii* and *C. quadricarinatus* in the wild (historical known sites for *P. clarkii* and *C. quadricarinatus*), while eDNA largely produced non-detection in those same sites. However, *C. cainii* which served as our positive field control was detected using eDNA through NGS, this was possibly due to the high sensitivity nature of the metagenomics technology. The successful detetction of *C. cainii* by NGS illustrates that the technique can offer solution when there are limitations encountered with end-point PCR and qPCR. This study builds on the very few eDNA studies conducted in South Africa. Environmental DNA holds a great promise for improving species detection in freshwater systems however, it has its limitations because it is a sensitive method that involves several steps that are highly sensitive such as eDNA collection (e.g., filtration), eDNA preservation, eDNA extraction and amplification. Any mistake or problem encountered

at any of the stages can affect the success of eDNA detection thus resulting in false negatives or unsuccessful detections (Goldberg et al. 2016). Also, primer choice can also be limiting which affect the sensitivity of eDNA to detect presence of target species (Geerts et al. 2018). As such, the use of eDNA in monitoring IAFCs in SA requires well refined methods that will improve its sensitivity and detection capability as a monitoring method in freshwater systems. It is also evident from our study that eDNA laboratory processing requires more experience in the laboratory as it involves a lot of trail-and-errors and for this experienced knowledge is critical.

CHAPTER 3

Barcoding of invasive alien freshwater crayfish (*Cherax cainii, Cherax quadricarinatus* and *Procambarus clarkii*) and their co-introduced or novel associated ectoparasites.

3.1 INTRODUCTION

3.1.1 Ectoparasites co-introduced with freshwater alien crayfish.

The spread of invasive alien freshwater crayfish species (IAFCs) to new areas beyond their native range has resulted in the co-introduction of various agents such as viruses, bacteria, fungi and metazoan parasites (Du Preez and Smit 2013, Chiesa et al. 2015, El-Moaty et al. 2016). Freshwater crayfish of families, Parastacidae, Cambaridae and Astacidae, are hosts of fungal pathogens such as *Aphanomyces astaci* (Lodge et al. 2012, Chiesa et al. 2015). *Aphanomyces astaci* is a non-host specific pathogen and can transfer to native decapods such as freshwater crabs (Chiesa et al. 2015, Strand et al. 2019). It was reported to have been co-introduced with *Procambarus clarkii* in Europe from North America in the 19th century (Strand et al. 2019) and was responsible for drastic decline in populations of the European crayfish *Astacus astacus* (Holdich et al. 2009).

Temnocephalans are the mostly reported platyhelminthes (flatworms) associated and co-introduced with IAFCs in South Africa (SA) (Du Preez and Smit 2013, Hurry et al. 2014, Sugiani et al. 2015, Tavakol et al. 2016, Ngamniyom 2020). The temnocephalans live attached to the external body surface of their host or within the branchial chamber (Sugiani et al. 2015), and they feed on small crustaceans, rotifers, immature aquatic insects and nematodes (Sugiani et al. 2015). The temnocephalans are also known to be non-host specific and can transfer to other organisms. For example, Avenant-Oldewage (1993) reported the temnocephalan, *T. chaeropsis,* to have transferred to native freshwater crabs. Since the temnocephalans are non-host specific, they can disrupt biological processes and cause mortality in native species. However, the impacts of temnocephalan on native freshwater organisms in South Africa, particularly on crabs, are still lacking (Du Preez and Smit 2013).

Currently, five alien temnocephalans have been reported in association with IAFCs in SA (Table 3.1). *Temnocephala chaeropsis* was the first temnocephalan ectoparasite reported from *Cherax cainii* on a crayfish farm in the Free State by Mitchell and Kock (1988). It was later identified in Gauteng on a crayfish farm by Avenant-Oldewage (1993). Also, on the same host, a second temnocephalan, *Diceratocephala boschmai* was identified (Avenant-Oldewage 1993). The study by Du Preez and Smit (2013) identified *D. boschmai* from *C. quadricarinatus* in Ndumo Game Reserve in KwaZulu-Natal. Also, a study by and Tavakol et al. (2016) identified two new temnocephalans species, *Craspedella pedum* and *Didymorchis sp.* from *C. quadricarinatus* in the Komati River and Masibekela Dam in Mpumalanga. Tavakol et al. (2021) barcoded ectoparasites i.e., *C. pedum* and *D. boschmai from C. quadricarinatus* (Tavakol et al. 2016) and results revealed that both *C. pedum and D. boschmai* are of Australian origin, which suggests that they were co-introduced with their host, *C. quadricarinatus* into SA (Tavakol et al. 2021).

Table 3. 1 Summary of known records of ectoparasites with their co-introduced alien freshwater crayfish in South Africa.

Ectoparasite species	**Crayfish hots species**	**Locality**	**Reference**
Temnosewellia chaeropsis	*Cherax tenuimanus*	Crayfish farm, Free State	Mitchell and Kock, 1988
T. chaeropsis	*C. cainii*	Crayfish farm, Gauteng	Avenant-Oldewage 1993
Diceratocephala boschmai	*C. quadricarinatus*	Lake Nyamiti, Ndumo Game Reserve, northern KwaZulu Natal	Du Preez and Smit 2013
Craspedella pedum, *Diceratocephala boschmai,* and *Didymorchis sp*.	*C. quadricarinatus*	Komati River, Mpumalanga	Tavakol et al. 2016
C. *pedum* and *D*. *boschmai*	*C. quadricarinatus*	Komati River, Mpumalanga	Tavakol et al. 2021

3.1.2 DNA Barcoding as a reliable tool to trace species origin and complex association.

DNA barcoding is a method for identifying taxonomically unknown specimens through sequencing a short fragment of DNA such as the mitochondrial cytochrome c oxidase subunit I (COI), 12S rRNA and 16S rRNA genes that function as unique identifiers to classify and identify species (Hebert et al . 2003, Ball et al. 2005, Hebert and Gregory 2005, Valentini et al. 2009, Abinawanto and Hamidah 2017, Elsaied et al.2021, Tsoupas et al. 2022, Rivas-Ferreiro et al. 2023, Long et al. 2023). The gene sequences are digital collections of biological data used to confirm morphology-based taxonomy (Hebert et al. 2003, Valentini et al. 2009). The gene sequences have the potential to solve and/or confirm morphology-based taxonomy (Hebert et al. 2003, Long et al. 2023).

DNA barcoding offers a supplementary method for species identification, particularly when morphology-based identification keys are challenging to use, limited or inaccessible (Ball et al. 2005, Hanzen et al. 2020, Tsoupas et al. 2022). As such, this tool makes the Linnaean taxonomic system accessible to ecologists, conservationists and various agencies that manage pests, invasive species, and food safety (Hebert and Gregory 2005). Also, DNA barcoding offers benefits such as speeding up the pace for new species been described (Hebert & Gregory 2005), understanding interactions between species (Pfenninger et al. 2007, Tedersoo et al. 2007) and to reveal the existence of cryptic species (Hebert et al. 2003, Valentini et al. 2009, Amador et al. 2021, Tsoupas et al. 2022, Takenaka et al. 2023).

Identifying cryptic species employing morphology-based taxonomy can often be challenging as these genetically different species are often morphologically identical (Govender 2017, Amador et al. 2021, Shalu et al. 2023). Lastly, the majority of morphology-based taxonomy keys are based on male genitalia, for example, Diptera and Coleoptera identification keys and this can lead to limitations in identifying female specimen (Valentini et al. 2009) (summarised in Table 3.2).

Table 3. 2 Summary of traditional morphology-based taxonomy versus DNA Barcoding.

Morphology-based taxonomy	DNA Barcoding
Requires a high level of taxonomy expertise to identify species (Valentini et al. 2009).	Offers accurate species identifications (Valentini et al. 2009, Abinawanto and Hamidah 201).
Often exists for certain life stages and can lead to misidentifications of species (Fleck et al. 2006, Valentini et al. 2009).	Allows juveniles and adults to be linked to provide accurate species identifications (Fleck et al. 2006).
Identification of species is based on morphological characters. This can be challenging when identifying cryptic species which are not easy to distinguish morphologically (Govender 2017, Amador et al. 2021).	Has the ability to accurately identify cryptic species (Amador et al. 2021, Tsoupas et al. 2022).
Species can employ phenotypic plasticity and species can be identified as new species, whereas they have just acquired different morphological traits from the original species because of exposure to different environmental conditions (Hebert et al. 2003).	DNA barcoding can resolve this problem by accurately identifying species (Hebert et al. 2003).
The majority are based on male genitalia and identifying females using such taxonomy keys can be challenging (Valentini et al. 2009).	Can resolve this problem by accurately identifying species regardless of gender (Valentini et al. 2009).

3.1.3 Co-introduced crayfish temnocephalans and leeches in South Africa: detection, identification and barcoding

The downside of co-introduced temnocephalans is that they can affect native species and humans in the introduced range (Gherardi 2007). This highlights the need for studies looking at detecting, identifying, and understanding the biology of co-introduced temnocephalans and leeches in association with their hosts (IAFCs) to assist in managing biological invasions. The recent studies by Du Preez & Smit (2013) and Tavakol et al. (2016, 2021) have shown a significant increase in the number of the alien temnocephalans co-introduced with crayfish in freshwater systems in SA. In addition, there is also a need for studies to investigate further different alien species co-existing, in this case, *C. cainii* and Temnocephalan in Vaughn

Bursey's Stock Farm, leeches and *P. clarkii* in Mimosa Dam as this aspect is understudied in the country. The co-introduced temnocephalans are often challenging to detect, particularly in the early stages of their infestation and another challenge includes the difficult to identify morphologically as they require taxonomy expertise, alternative they require other molecular techniques such as DNA barcoding (Du Preez and Smit 2013).

The studies using DNA barcoding for taxonomy of crustaceans and other ecological research is limited compared to other taxa, such as fish (Bezeng and van der Bank 2019). This gap highlights the need to incorporate DNA barcoding in different taxa and research niches including biological invasion. This will help build the DNA barcoding reference library, particularly for alien crustaceans in SA as this group is understudied in the country, yet they also pose negative impacts socio-economically and environmentally globally, as highlighted by Bezeng and van der Bank (2019). Currently, there is only one study by Tavakol et al. (2021) that have barcoded alien species that have been co-introduced with IAFCs in the country. Tavakol et al. (2021) identified alien temnocephalan species, *Craspedella pedum* and *Diceratocephala boschmai* from *C. quadricarinatus* caught from Komati River in Mpumalanga.

3.2 AIM OF THE STUDY

The current study aimed to (i) barcode IAFCs (*C. cainii, C. quadricarinatus* and *P. clarkii*) occurring in South Africa, together with their associated ectocommensal temnocephalans and leeches. (ii) Contribute towards the build-up of the reference library on invasive alien species. This was achieved by using morphological identification keys and DNA barcoding to identify specimens to species level.

3.3 MATERIALS AND METHODS

3.3.1 Study sites

Invasive alien crayfish specimens and associated parasites were collected from Vaughn Bursey's Farm in Kei Road, Eastern Cape (27°55'46.65" S; 32°71'21.67" E), Banzi Pan in Ndumo Game Reserve, KwaZulu Natal (26°87'78.01" S; 32°29'02.87" E), Mimosa Dam in Odendaalrus, Free State (27°88'13.36" S; 26°69'52.24" E), and Driehoek Farm in Dullstroom, Mpumalanga

(25°12’44.8” S; 30°05’84.0” E) between November 2020 and January 2021 (Figures 3.1 and 3.2). These sites were selected on the basis that they were known to have existing population of alien freshwater crayfish, *C. cainii, C. quadricarinatus*, *P. clarkii* (Du Preez and Smit 2013, Nunes et al. 2017b, Alfreds 2018, Madzivanzira et al. 2020). *Cherax cainii* was collected from Vaughn Bursey’s Farm (see Chapter 2), *C. qudaricrainatus* was collected in Banzi Pan, whereas *P. clarkii* was collected from Mimosa Dam and Driehoek Farm Dam.

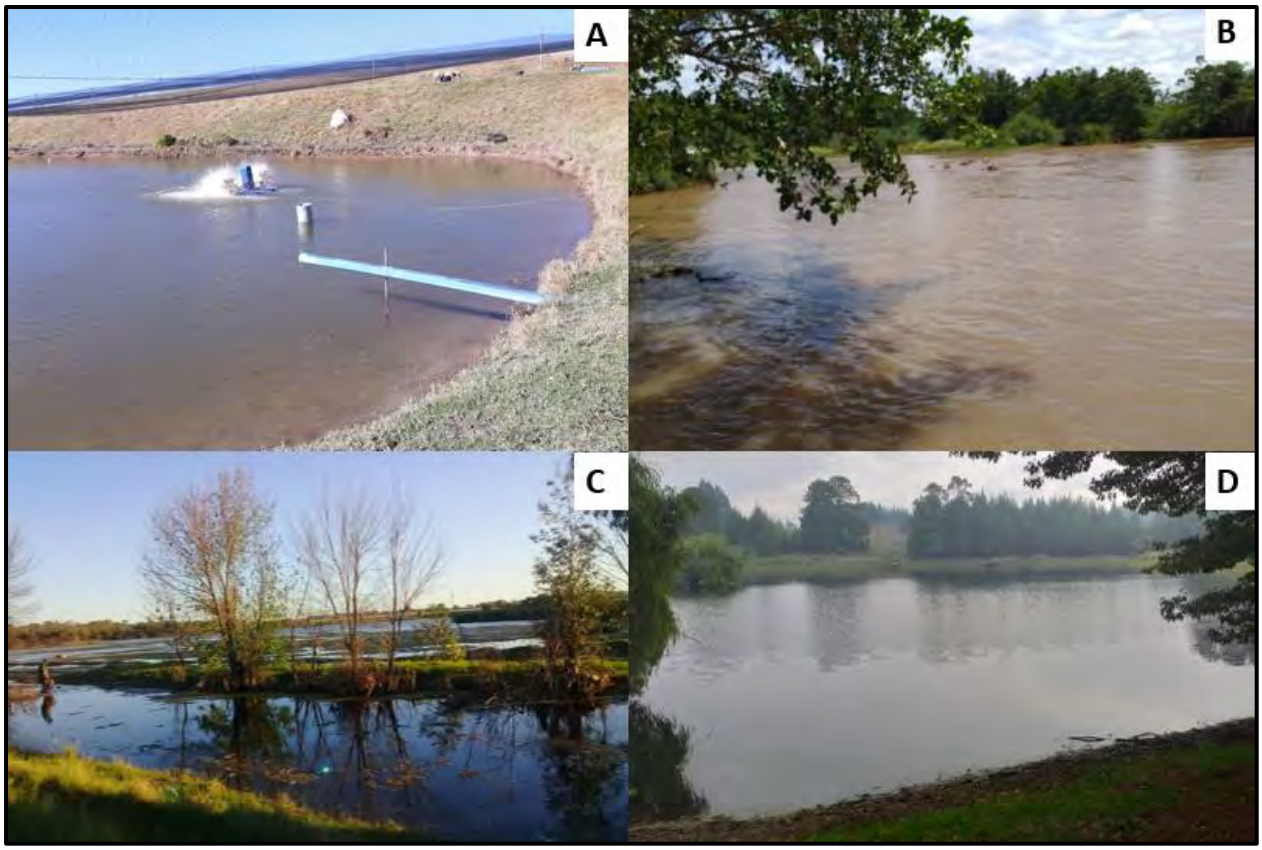


Figure 3. 1 Study sites, pond in Vaughn Bursey’s Stock Farm (**A**), Banzi Pan in Ndumo Game Reserve (**B**), Mimosa Dam in Odendaalrus (**C**), Dam in Driehoek Farm in Dullstroom (**D**).

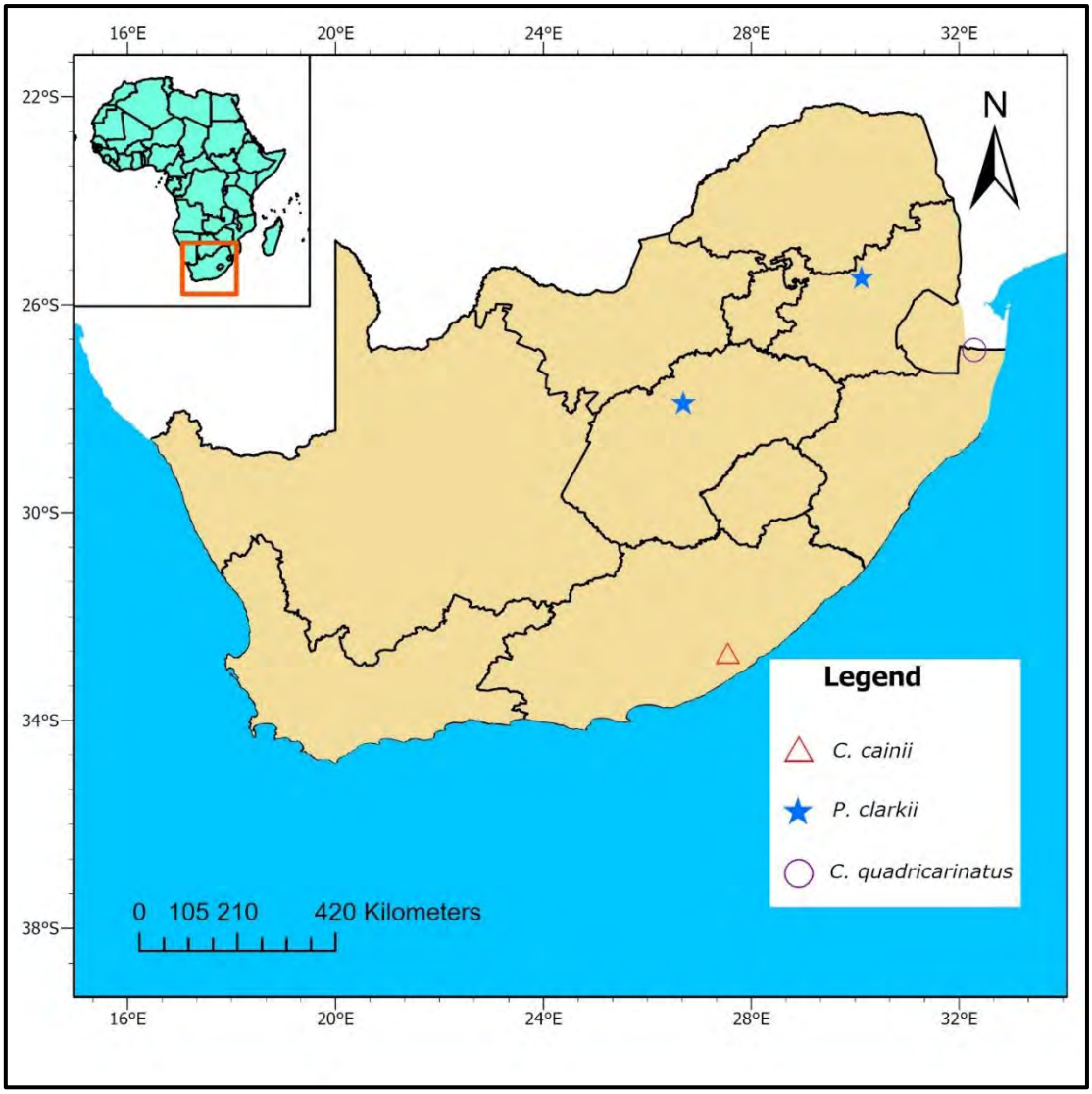


Figure 3.2 Map showing study sites for the three target alien freshwater crayfish, that is, *C. cainii* in Vaughn Bursey's Stock Farm, *C. quadricarinatus* in Banzi Pan in Ndumo Game Reserve, *P. clarkii* Mimosa Dam in Odendaalrus, Free State and Driehoek Farm in Dullstroom, Mpumalanga.

3.3.2 Biological data collection

Crayfish Sampling

Prior conducting sampling, crayfish visual surveys, (i.e., looking for molted exoskeletons and crayfish burrows alongside waterbodies) were carried out for 10 to 20 minutes along the littoral zone. Thereafter, a 1-meter-long hand-held SASS net (30 × 30 cm frame, 1 mm mesh size) was used to collect crayfish samples by sweeping vegetated habitats (< 1-meter depth) along the littoral zone at each site. Then crayfish baited trapping was carried out following Nunes et al. (2017a), following the steps mentioned in Section 2.2.4. In Vaughn Bursey's Stock

Farm baited trapping and visual survey were not done since the site is a known crayfish farm, instead the farmer donated two crayfish specimens from one of the ponds which were caught by hand. Upon arrival in the laboratory, each crayfish specimen was measured for body length in centimetres and weighed to get body weight in grams. All crayfish specimens were morphologically identified using available identification keys including Austin and Ryan (2002), Barki et al. (2006) and Larson (2007).

Examination for the associated parasites

Crayfish specimens were examined following Du Preez and Smit (2013) protocol. The *Cherax cainii, C. quadricarinatus* and *P. clarkii* body parts such as cheliped, rostrum, abdomen, legs, and in between joints were examined under a Zeiss Stemi 508 stereomicroscope for the presence of the associated parasites. The examination of *C. cainii* specimens revealed whitish spots on the carapace and the specimens were gently scraped with a toothbrush to transfer the parasites onto a glass Petri dish containing 80% ethanol. The parasites from *P. clarkii* specimens were removed using forceps and immediately transferred into 12 ml glass vials containing 80% ethanol. Collected parasites specimens were thereafter photographed using a Zeiss Stemi 508 stereomicroscope with an Axiocam ERc5s camera attached. Specimens were thereafter preserved and stored as part of the collection in the Freshwater Invertebrates Department at Albany Museum, Makhanda Eastern Cape.

The flatworms collected from *C. cainii* (Vaughn Bursey's Farm) were identified using Cannon and Sewell (1995) description and the leeches from *P. clarkii* (Mimosa Dam) were identified following Lai et al. (2009)'s morphological identification key. Selected specimens of ectoparasites were further processed for DNA barcoding done at the Genetics Laboratory at the South African Institute for Aquatic Biodiversity (SAIAB), Makhanda.

3.3.3 Molecular analysis

DNA extraction for crayfish and the associated flatworms and leeches

Invasive alien crayfish samples (*C. cainii, C. quadricarinatus* and *P. clarkii*) and their associated flatworms and leeches' genomic DNA was extracted using DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's instructions (similar DNA extraction protocol in Chapter 2, section 2.2, page 23). The tissue from the crayfish was extracted from the abdomen (Camma et al. 2010, Sieber et al. 2022) and a whole ectoparasite specimen was used. The DNA concentration (ng/µl) was measured using a Thermo Scientific™ NanoDrop 2000 spectrophotometer.

Polymerase Chain Reaction (PCR)

The mitochondrial cytochrome *c* oxidase subunit I gene (COI) region of IAFCs, alien leeches and temnocephalans was amplified by standard polymerase chain reaction (PCR) using the universal primer pair LC01490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HC02198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al. 1994). *Cherax cainii* and C*. quadricarinatus* were also amplified in the 16S rRNA gene using the oligonucleotide primer pair 1471 (5'-CCTGTTTANCAAAAACAT-3') and 1472 (5'-AGATAGAAACCAACCTGG-3') (Munasinghe et al. 2004). DNA amplification was carried out in a total reaction volume of 25 µl containing 3 µl of DNA, 12.5 µl of Taq polymerase, 6.5 µl ddH2O, 1 µl of forward primer (10 µM) and 1 µl of reverse primer (10 µM). The negative control (used molecular grade water in place of DNA) was included for each species in the PCR reaction. Thermal cycling conditions consisted of an initial denaturation of 95°C for 5 minutes followed by 35 cycles of 95°C for 30 seconds, annealing at 48°C for 30 seconds and extension at 72°C for 45 seconds with a final extension step at 72°C for 5 minutes. The PCR products were electrophoresed on a 1% agarose gel stained with ethidium bromide and visualized on a UV transilluminator. The DNA from PCR products were purified using the Qiaex II kit (Qiagen) and sequenced in one direction (forward) using the Big Dye v.3 sequencing kit following the standard protocol. The obtained sequence of the crayfish and ectoparasites were searched on the Basic Local Alignment Search Tool (BLAST) to find a sequence based on the mitochondrial COI gene for species identification (http://www.ncbi.nlm.nih.gov).

Sequence alignment and analysis

The obtained sequences of invasive alien crayfish, *C. cainii, C. quadricarinatus* and *P. clarkii*, and their ectoparasites were aligned using MAFFT version 7 (Rozewicki et al. 2019), a multiple sequence alignment program. The aligned sequences were viewed and trimmed in AliView version 1.28 (Larsson 2014). The sequences were saved in Fasta format on Notepad++ version 8.2. The sequences were deposited on BLAST (BLAST: Basic Local Alignment Search Tool (nih.gov) (Altschul et al. 1990) to compare and match our obtained gene sequences to the entire database sequences to identify our specimens to species level.

Phylogenetic tree construction

Using the COI sequences of IAFCs generated from Sanger sequencing for this present study and sequence matches from NCBI BLAST (Altschul et al. 1990 (Table 3.3). A maximum likelihood (ML) phylogenetic tree was constructed using IQ Tree (Nguyen et al. 2015) on the web server (http://iqtree.cibiv.univie.ac.at/) for *C. cainii, C. quadricarinatus*, *P. clarkii* and the leeches. The ML phylogenetic tree was constructed in relation to the sequence matches from NCBI BLAST, and the specimens are listed in Table 3.2. Unfortunately, COI gene for temnocephalans failed several times to amplify and sequence at both SAIAB genomics laboratory and Inqaba Biotec. The outgroups for the ML phylogenetic tree selected were *Astacopsis franklinii* (accession: MW220050.1) for *Cherax* species and *Faxonius virilis* (ON103573.1) for *P. clarkii*. For the leeches, *Placobdelloides siamensis* was selected as an outgroup. The obtained phylogenetic trees were viewed and edited on FigTree v1.3.2 (Yu et al. 2017).

Table 3.2 List of crayfish specimens that were used for genetic analysis based on mitochondrial gene cytochrome c oxidase subunit I (COI) match on NCBI BLAST.

Species	Location	GenBank Accession
Cherax cainii	**Vaughn Bursey Stock Farm, South Africa**	**OP013264.1**
Cherax cainii	Australia, Oceania	KM039098.1
Cherax cainii	Australia, Oceania	HG942366.1

Cherax cainii	Australia, Oceania	KF649849.1
Cherax tenuimanus	Australia, Oceania	AF510187.1
Cherax tenuimanus	Australia, Oceania	AF510186.1
Cherax tenuimanus	Australia, Oceania	AF510185.1
Cherax tenuimanus	Australia, Oceania	AF510184.1
Cherax tenuimanus	Australia, Oceania	AF510183.1
Cherax tenuimanus	Australia, Oceania	AF510182.1
Cherax tenuimanus	Australia, Oceania	AF510181.1
Cherax quadricarinatus	**Banzi Pan, Ndumo Game Reserve, South Africa**	**OQ255654.1**
Cherax quadricarinatus	Hungary, Central Europe	KY745779.1
Cherax quadricarinatus	Indonesia, Southeast Asia	KX377348.1
Cherax quadricarinatus	USA, North America	DQ006294.1
Cherax quadricarinatus	China, East Asia	MT966753.1
Cherax quadricarinatus	China, East Asia	MT966737.1
Procambarus clarkii	**Mimosa Dam, Odendaalrus, South Africa**	**OP013265.1**
Procambarus clarkii	**Driehoek Farm, Mpumalanga, South Africa**	**OP013266.1**
Procambarus clarkii	Prague, Central Europe	MK439900.1
Procambarus clarkii	Spain, Southwestern Europe	MK026733.1
Procambarus clarkii	Spain, Southwestern Europe	MK026732.1
Procambarus clarkii	USA, North America	MK308033.1
Procambarus clarkii	France, Western Europe	KY112712.1
Procambarus clarkii	Malaysia, Southeast Asia	KY112709.1
Procambarus clarkii	China, East Asia	KX268742.1
Procambarus clarkii	USA, North America	JX441375.1
Procambarus clarkii	USA, North America	KJ645848.1

Table 3.3 List of leeches found on invasive alien *P. clarkii* from Mimosa Dam, that were used for genetic analysis based on mitochondrial gene cytochrome c oxidase subunit I (COI) match on NCBI BLAST.

Species	Location	Genbank Accession
Helobdella sp.	**Mimosa Dam, South Africa**	**OQ255710.1**
Helobdella sp.	**Mimosa Dam, South Africa**	**OQ255711**
Helobdella adiastola	Mexico, North America	MK354120.1
Helobdella adiastola	Mexico, North America	MK354119.1
Helobdella adiastola	Mexico, North America	MK354139.1
Helobdella adiastola	Mexico, North America	MK354139.1
Helobdella adiastola	Mexico, North America	MK354143.1
Helobdella octatestisaca	Portugal, Southern Europe	MN335876.1
Helobdella octatestisaca	USA, North America	MH729329.1
Helobdella octatestisaca	USA, North America	KY498613.1
Helobdella octatestisaca	Taiwan, East Asia	FJ000342.1
Helobdella stagnalis	USA, North America	HQ179860.1
Helobdella europaea	**Mimosa Dam, South Africa**	OQ255707
Helobdella europaea	Portugal, Southern Europe	MN335875.1
Helobdella europaea	USA, North America	MF804537.1
Helobdella europaea	Spain, Southwestern Europe	KC904242.1
Helobdella europaea	Taiwan, East Asia	FJ000349.1
Helobdella europaea	USA, North America	DQ995304.1
Helobdella europaea	New Zealand, Oceania	AY856049.1
Helobdella europaea	Ukraine, Eastern Europe	MT258557.1
Helobdella europaea	Australia, Oceania	AF329052.1
Helobdella europaea	Germany, Western Europe	AY576008.1
Helobdella europaea	Mexico, North America	AY856047.1

3.4 RESULTS

3.4.1 Morphological identification of crayfish and their associated ectoparasites

Cherax cainii (smooth marron)

Crayfish specimens from Vaughn Bursey's Farm were identified as *Cherax cainii* based on the median carina extending continuously, without depression, to the cervical groove (Austin and Ryan 2002). The specimens were also identified based on the presence of five keels on the dorsal surface of their heads and two small spines on their telson (Figure 3.3A) (Prastowo 2017). The presence of five keels along the head spines on the telson is *C. cainii*'s distinguishable features from other *Cherax* species as they have four keels along the head (Figure 3.3A) and two small spines on the telson (Figure 3.3B) (Prastowo 2017).

Figure 3.3 A showing the presence of keels on the head (morphological characteristic indicated by red arrow) that were used to *Cherax cainii* specimens caught in Vaughn Bursey's Farm, Kei Road, Eastern Cape.

Figure 3.3 B showing the presence of (circled in red) that were used to morphologically identify *Cherax cainii* specimens caught in Vaughn Bursey's Farm, Kei Road, Eastern Cape.

An average of 19 temnocephalans were observed from *C. cainii* specimens collected from Vaughn Bursey's Stock Farm. The temnocephalan specimens lacked body pigment, i.e., their bodies were translucent, ovoid in shape and bared five anterior tentacles (Figure 3.4A, B).

Based on these morphological characters, the temnocephalans were identified as *Craspedella pedum* following identification key by Cannon and Sewell (1995). The sites of infection on *C. cainii* specimens were rostrum, carapace, chelipeds and walking legs.

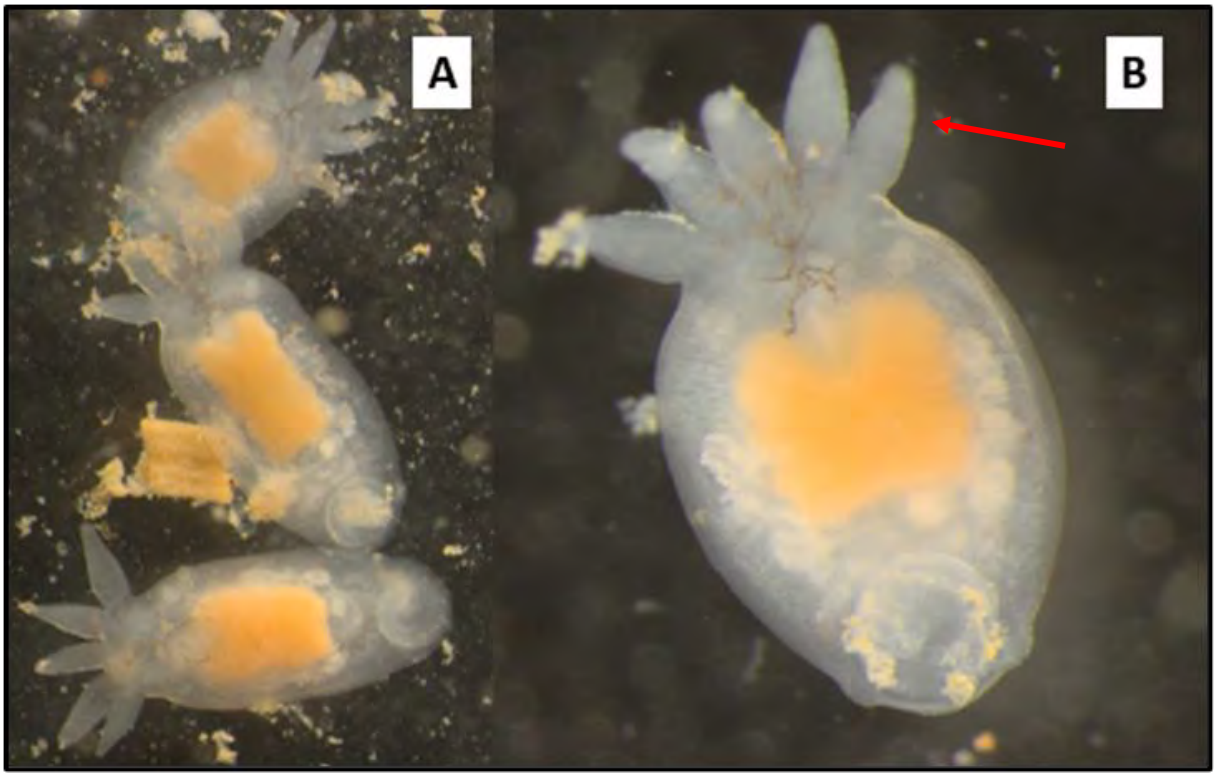


Figure 3.4 Temnocephalan, *Craspedella pedum* collecetd from *Cherax cainii* in Vaughn Bursey Stock Farm, Kei Road, Eastern Cape (**A**). A red arrow indicating the distinctive character of the *C. pedum*- 5 anterior tentacles (**B**).

Cherax quadricarinatus (Australian red claw crayfish)

Cherax quadricarinatus specimen were caught during the surveillance at Banzi Pan in Ndumo Game Reserve. The crayfish specimen was identified based on the dark green body colour and the presence of four distinct anterior ridges (carinae) on the carapace (Barki et al. 2006) (Figure 3.5). The dark green colour of the specimen is washed off by ethanol that the specimen was preserved in (80% ethanol). Since sampling was carried out immediately after major flooding in the area, only one specimen was caught and no ectocommensal temnocephalans were observed.

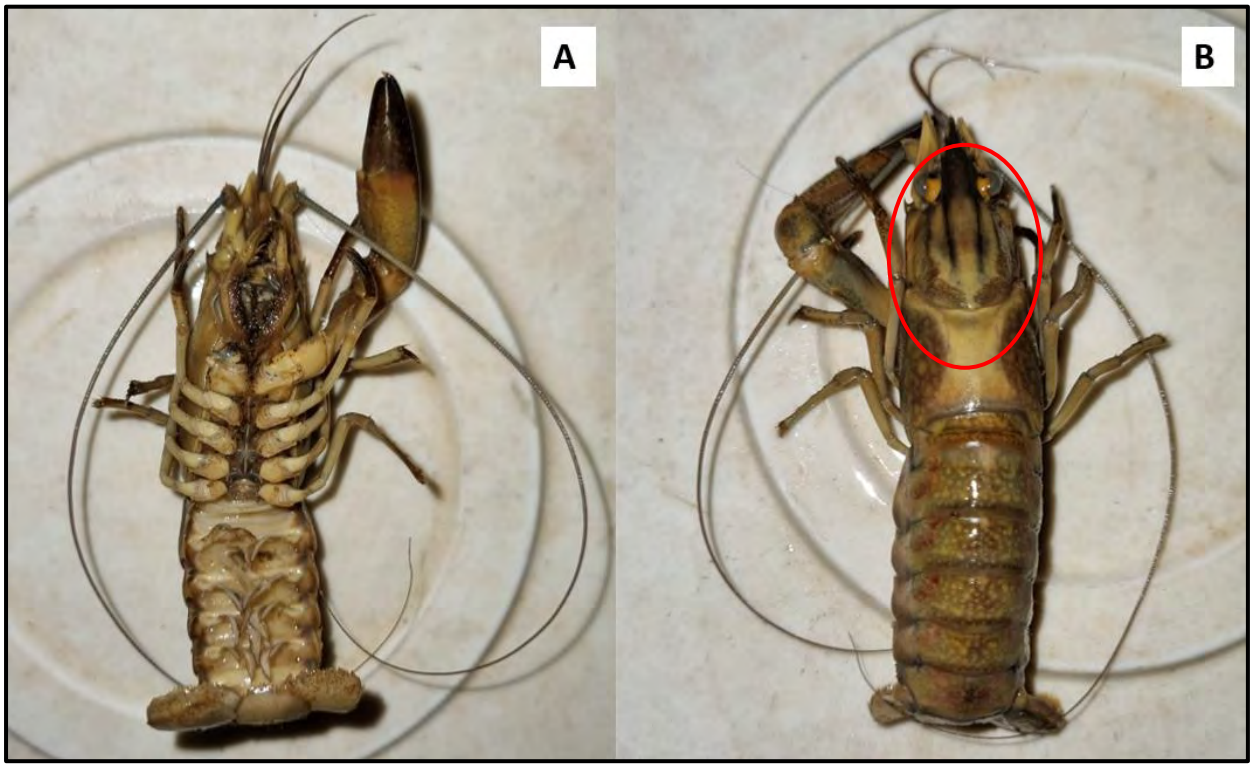


Figure 3.5 *Cherax quadricarinatus* specimen caught from Banzi Pan in Ndumo Game Reserve, KwaZulu Natal. Ventral side of species (**A**). Four distinctive anterior ridges which is distinctive feature of the species circled in (**B**).

Procambarus clarkii (red swamp crayfish)

In Mimosa Dam, twenty-one *P. clarkii* specimens were caught and four specimens in Driehoek Farm Dam (summarised in Table 3.3). All crayfish specimens from both Mimosa Dam and Driehoek Farm Dam were identified as *Procambarus clarkii* based on the presence of long and narrow pincers, bumps on chelae, black wedge pattern on the dorsal side of the tail (Larson 2007). *Procambarus clarkii* specimens from the two populations, i.e., Free State and Mpumalanga, exhibited a few morphological variations such as colour and size. *Procambarus clarkii* adult specimens from the Free State were larger in size, with an average body length of 17.3 cm, than the Mpumalanga specimens which were 12.4 cm (Table 3.3). Two juveniles were collected, they were light green in colour which is a typical colour for juveniles. They had an average body length of 6.5 cm, indicating a smaller body length than the adults. The adult specimens from Free State were bright red in colour (Figure 3.6B), the ones from Mpumalanga were black with slight, greenish-coloured bodies and red limbs (Figure 3.6A).

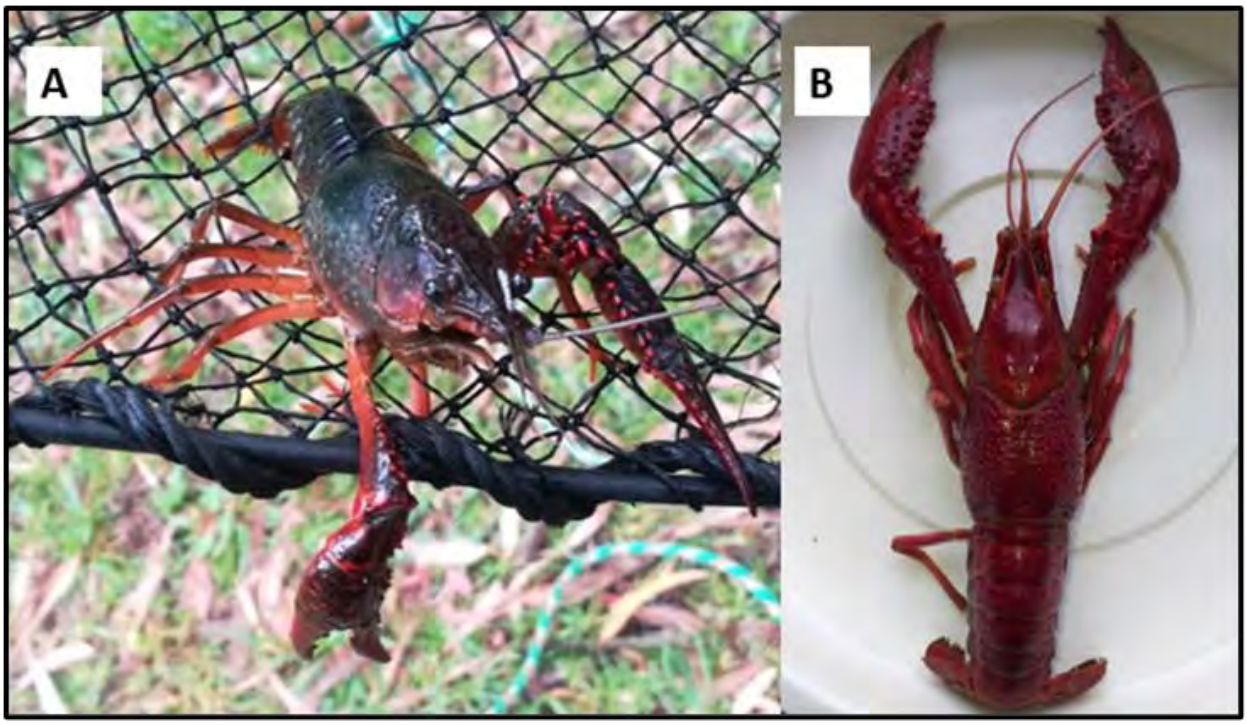


Figure 3.6 *Procambarus clarkii* specimen caught in Driehoek Farm, Mpumalanga (**A**), specimen caught in Mimosa Dam, Odendaalrus, Free State (**B**).

No ectosymbionts were found on the *P. clarkii* specimens caught in Driehoek Farm Dam. However, the *P. clarkii* specimens from Mimosa Dam were heavily infested with leeches. Fifteen (71%) *P. clarkii* specimens were infested with leeches from the twenty-one specimens collected. Three *P. clarkii* specimens were heavily infested by more than 100 parasite count. The average number of leeches from *P. clarkii* specimens in Mimosa Dam was 39.9 (Table 3.3). The sites of infestation on *P. clarkii* specimens were the rostrum, cheliped, chela, and abdomen, in between walking legs and joints (Figure 3.7A, B, C, D). Based on the leeches' morphological characters they were identified as two species. The leeches that bore rows of papillae on the dorsal face were identified as *Helobdella europaea* (Figure 3.8 A, B, C, D). Those that lacked papillae on the dorsal face were identified only up to genus level as *Helobdella* sp. (Figure 3.9A, B, C). In addition, several adult leeches had juveniles attached to their ventral side (Figure 3.10).

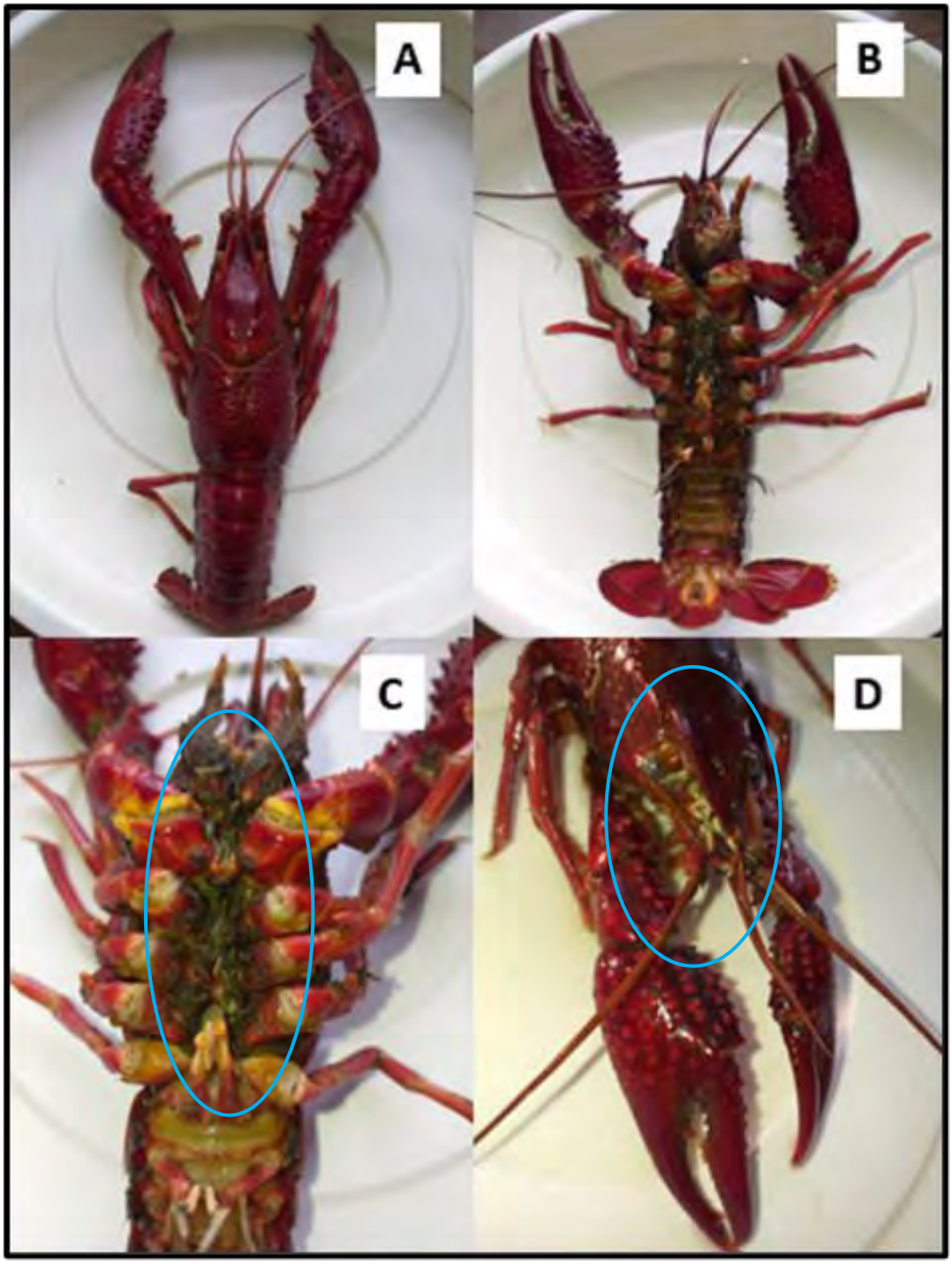


Figure 3.7 *Procambarus clarkii* dorsal view (**A**), ventral view (**B**), sites of parasite infection, i.e., abdomen, joints and in between walking legs (**C**), infested rostrum (**D**).

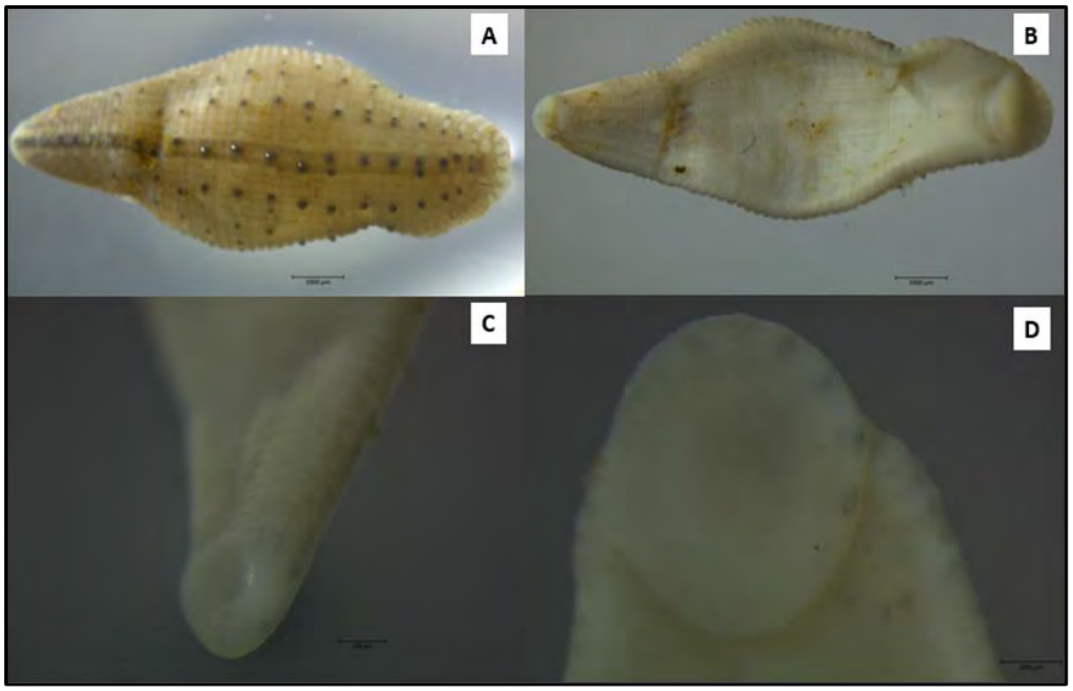


Figure 3.8 *Helobdella europaea* parasite from *P. clarkii,* dorsal view (**A**), ventral view (**B**), mouth (**C**), posterior sucker (**D**).

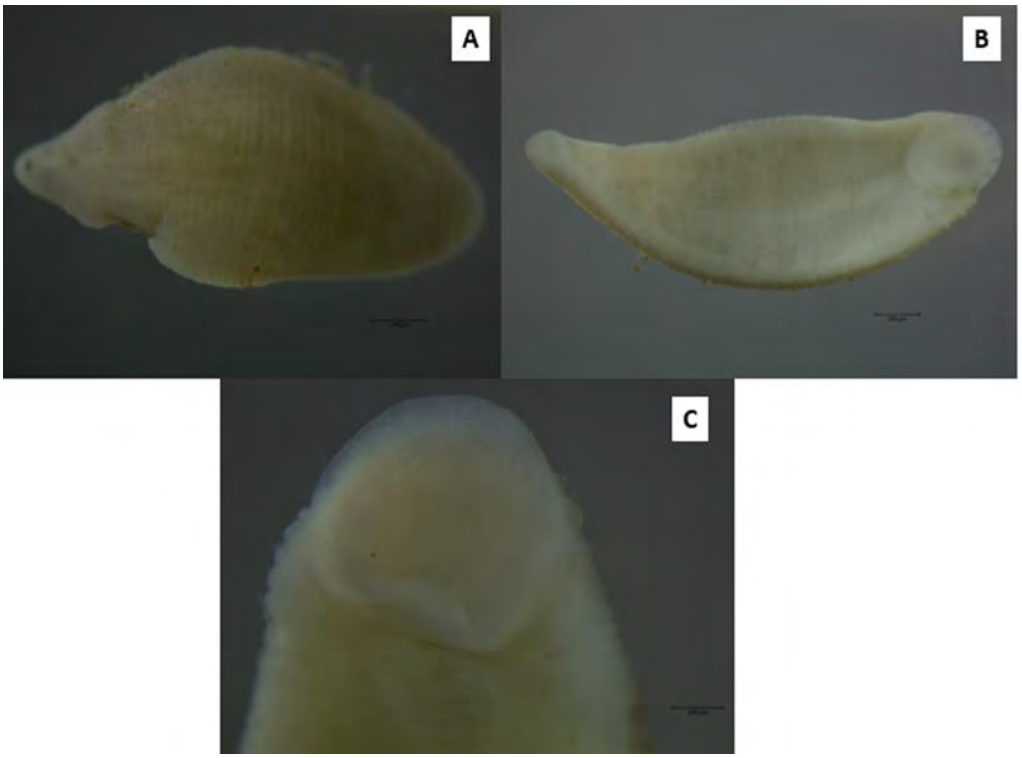


Figure 3.9 *Helobdella* sp. ectoparasitic leech from *P. clarkii,* dorsal view (**A**), ventral view (**B**), mouth (**C**).

Figure 3. 10 Adult *Helobdella* sp. with juveniles attached to its abdomen (ventral surface).

Table 3.3 Summary of temnocephalans caught from *C. cainii* and C*. quadricarinatus* specimens, and leeches caught from *P. clarkii* specimens, indicating the crayfish life stages and their average body weight (in grams) and body length (in centimeters).

Site	**Crayfish species**	**Average count (n) of associated leeches for *P. clarkii* and temnocephalans for *C. cainii* and *C. quadricarinatus***	**Juvenile crayfish (n)**	**Adult crayfish (n)**	**Average crayfish weight (g)**	**Average crayfish length (cm)**
Vaughn Bursey's Farm	*Cherax tenuimanus*	19	0	2	87.9	20.6
Banzi Pan	*Cherax quadricarinatus*	0	0	1	29.1	13.8
Driehoek Farm	*Procambarus clarkii*	0	0	4	16.9	12.4
Mimosa Dam	*Procambarus clarkii*	39.9	2	19	58.5	17.3

3.4.2 Phylogenetic Analysis

This study used the COI mitochondrial DNA marker to identify *C. cainii*, *C. quadricarinatus*, *P. clarkii* and alien *Helobdella* leeches. Species identification based on the sequence similarity approach was carried out using BLAST, GenBank database (Table 3.1). The patterns of similarity for alien crayfish and the leeches based on the COI sequences and their relatives are shown in the ML phylogenetic tree (Figures 3.11, 3.12, 3.13 and 3.14). The sequence matches for the *C. cainii* from Vaughn Bursey Farm were both *Cherax cainii* and *Cherax tenuimanus*. This was further analysed in ML phylogenetic, were the *C. cainii* specimen was monophyletic to *Cherax cainii* specimens with 83% bootstrap (BS) support. This indicates that the specimen found in Vaughn Bursey Farm is *C. cainii* (Figure 3.11). The sequence match for *C. quadricarinatus* specimen caught in Banzi Pan was *C. quadricarinatus* specimen with 94% BS support (Figure 3.11). *Cherax cainii* was further analysed using 16S rRNA primer pair to fully confirm its species identification (Figure 3.12). The sequences from specimens caught in Vaughn Bursey's Stock Farm clustered together with *Cherax cainii* with 99% bootstrap support value, indicating that the species found in Vaughn Bursey's Stock Farm is indeed *Cherax cainii.* The BS support values for *P. clarkii* specimens from Mimosa Dam were 41% and 66%. For Driehoek Farm specimens it was 39% and 45% (Figure 3.13).

The morphologically identified specimens, *H. europaea* had *H. europaea* matches on BLAST. *Helobdella sp*. had *Helobdella adiastola, Helobdella octatestisaca and Helobdella stagnalis* on BOLD (Table 3.2). This was further analyzed in ML tree, and our *H. europaea* had 43% BS value in relation to matching *H. europaea* sequences (Figure 3.14). *Helobdella octatestisaca* and *Helobdella* sp. specimens had 65% BS value in relation to *Helobdella octatestisaca gene* match (Figure 3.14).

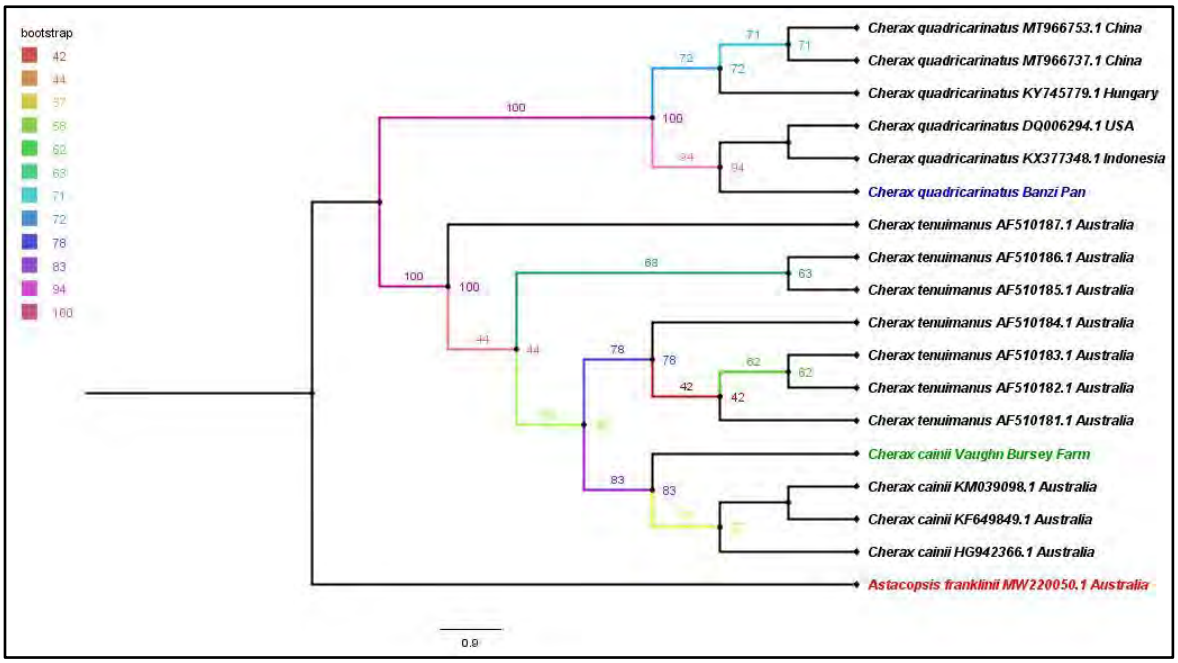


Figure 3.11 Maximum Likelihood phylogenetic relationship based on mitochondrial COI gene marker among *Cherax* specimens caught in Vaughn Bursey Farm and Banzi Pan, South Africa in relation to *Cherax* species globally. The numbers at each node represent the bootstrap proportions based on 1000 replications.

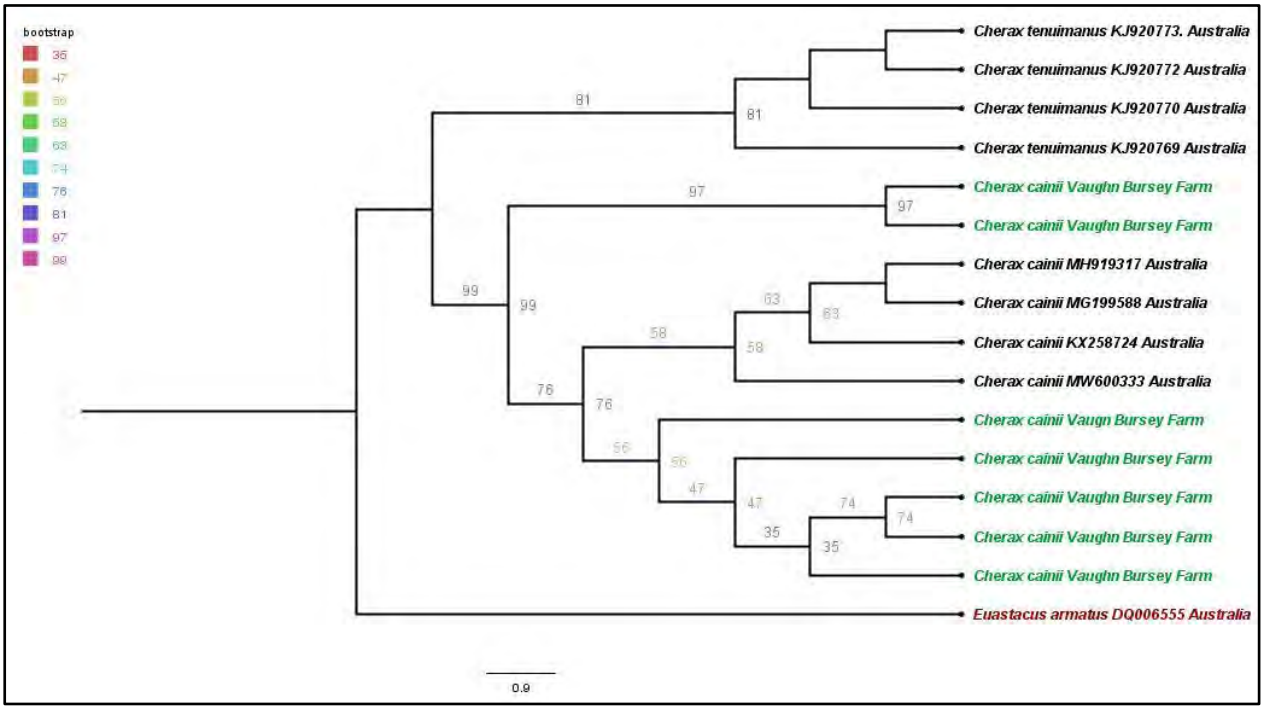


Figure 3.12 Maximum Likelihood phylogenetic relationship based on mitochondrial 16S ribosomal DNA gene marker among *Cherax cainii* specimens caught in Vaughn Bursey Farm in relation to *Cherax* species globally. The numbers at each node represent the bootstrap proportions based on 1000 replications.

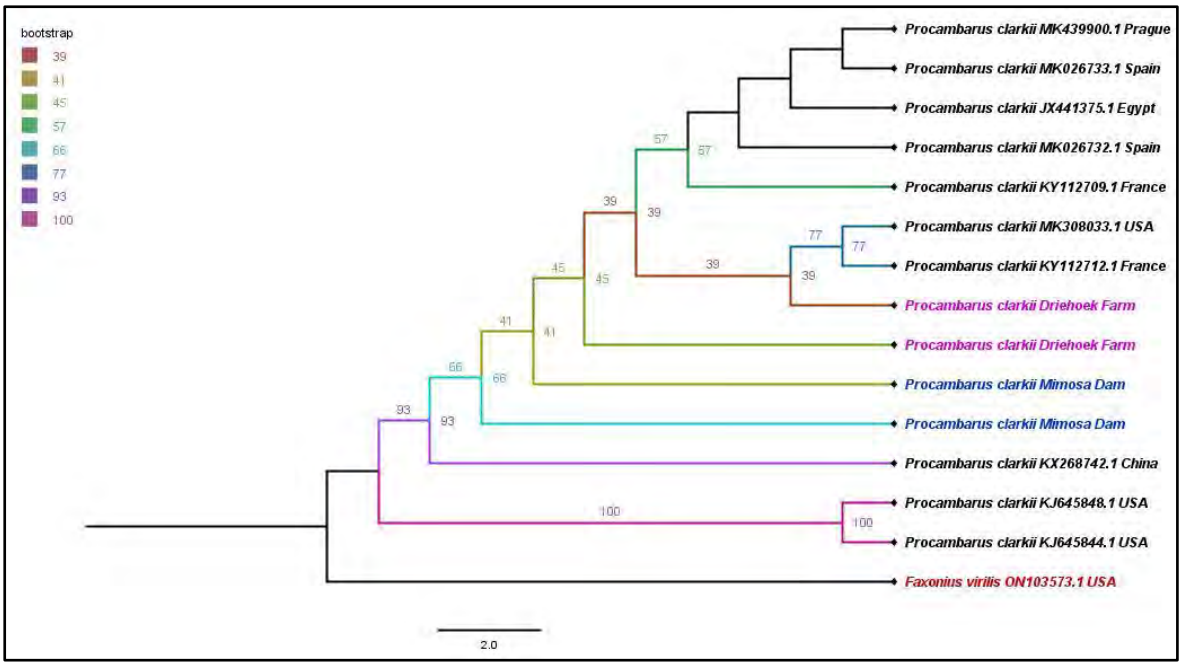


Figure 3.13 Maximum Likelihood phylogenetic relationship based on mitochondrial COI gene marker among *Procambarus clarkii* specimens caught in Mimosa Dam, South Africa in relation to *P. clarkii* species globally. The numbers at each node represent the bootstrap proportions based on 1000 replications.

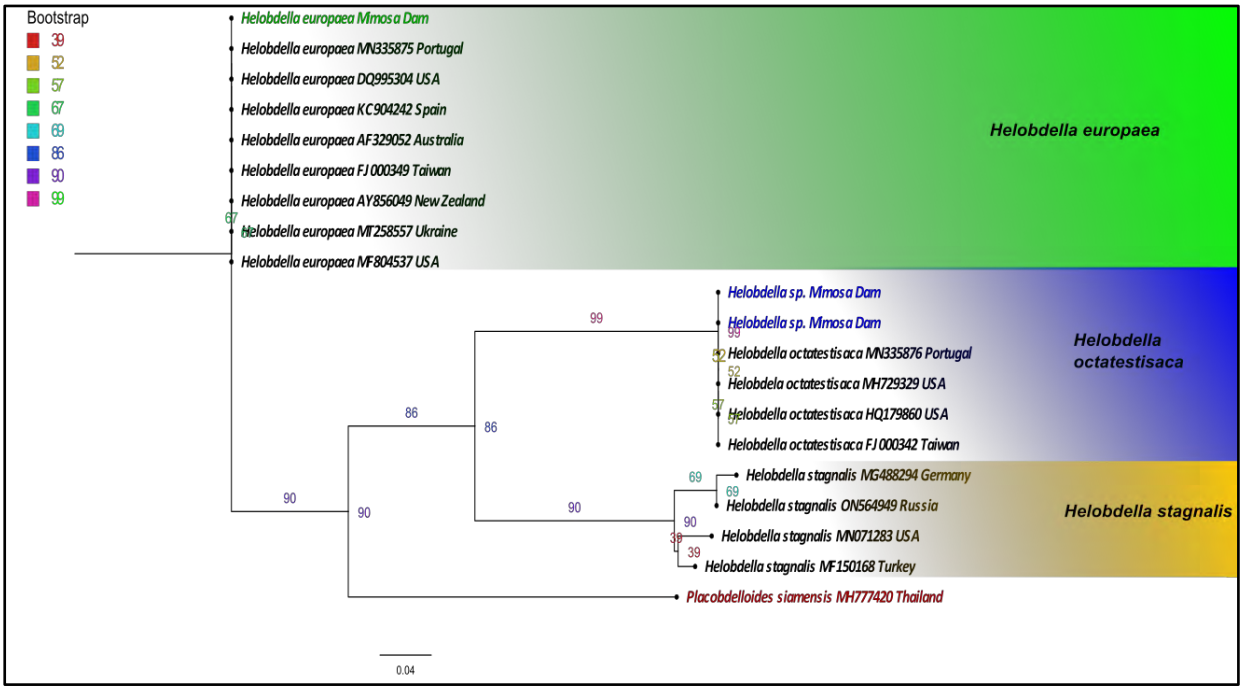


Figure 3.14 Maximum Likelihood phylogenetic relationship based on mitochondrial COI gene marker among *Helobdella* leeches caught from *P. clarkii* specimens in Mimosa Dam, South Africa in relation to *Helobdella* species globally. The numbers at each node represent the bootstrap proportions based on 1000 replications.

3.5 DISCUSSION

DNA barcoding confirmed the species identification of the three crayfish species sampled in this in this study: *C. cainii, C. quadricarinatus* and P. *clarkii*. DNA barcoding also confirmed the species identification of the *Helobdella* leeches collected from the *P. clarkii* specimens. To date, no study in South Africa have reported barcodes of alien freshwater crayfish in South Africa. The present study barcoded three alien crayfish species (i.e., *Cherax cainii, Cherax quadricarinatus* and *Procambarus clarkii*) and their associated ectocommensal temnocephalans and leeches currently occurring in freshwater systems in South Africa. This is an addition information to the known temnocephalans recorded from *C. quadricarinatus* by Du Preez and Smith (2013) and Tavakol et al. (2016). The temnocephalans from *C. quadricarinatus* later barcoded by Tavakol et al. (2021). This study further recorded alien freshwater leeches caught from *P. clarkii* in Mimosa Dam. The leeches were morphologically identified, and species identification was verified by DNA barcoding. The molecular results complemented the morphological identification of the alien crayfish species and revealed two new alien freshwater leeches *Helobdella europaea* and *Helobdella octatestisaca* that were associated with the invasive alien *P. clarkii* in Mimosa Dam. Moreover, the identification of *Cherax cainii* from Vaughn Bursey's Farm through DNA barcoding further confirmed that the alien marron species occurring in South Africa is Cherax *cainii* (Nunes et al. 2017a) and not *Cherax tenuimanus* (De Moor 2002) as previously thought.

3.5.1 Cherax cainii

Cherax cainii has been translocated from its native range, south-west Australia to new areas such as Africa, Chile, China, Europe, North America, and South America due to its importance in recreational fishing, aquaculture industry and pet trade (Morrissy et al. 1990, Lawrence et al. 1995, Alderman and Wickins 1996, Molony et al. 2006, Nunes et al. 2017a, Madzivanzira et al. 2020). *Cherax cainii* is known to be still in captivity, for example, Vaughn Bursey's Stock Farm on Kei Road in the Eastern Cape. As such, the species accurate identification is important for a better understand of its biology for an early detection and management response in the future in case it would escape into the wild. With regards to understanding its biology, *C. cainii* prefers clear, deep waters and sandy biotopes (Mosig 1998, Molony et al. 2004), and is

intolerant to low dissolved oxygen (Bryant and Papas 2007) with an optimum water temperature between 12°C and 30°C (Pattikawa and Wenno 2014). Thus, this provides an indication that there would be minimal chances for the species to become invasive in the country due to its habitat preference and water chemistry requirements. Another reason for the *C. cainii* to struggle establishment in the wild and become invasive is because the average winter temperatures in South Africa are between -2°C and 26°C (ZaR, 2022), which is out of range for the species' optimal water temperature (12°C). This was also evident in Vaughn Bursey's Stock Farm were the farmer encountered a massive stock loss of smooth marrons during winter when he initially started farm *C. cainii.* The farmer regularly monitored and modified water temperatures and dissolved oxygen to best suit the crayfish for survival. This can be the reason why the species has not been invasive in the country since its introduction in the early 1980s (De Moor 2002, Nunes et al. 2017a).

This study confirms the presence of alien temnocephalan, *Craspedella pedum* from the only known existing populations of *C. cainii* in the country occurring in Vaughn Bursey Stock Farm. *Craspedella pedum* is a Platyhelminthes (flatworm) of the order Temnocephalida (Cannon and Sewell 1995) and native to Australia (Cannon and Sewell 1995, Tavakol et al. 2016, Sibraa et al. 2021, Tavakol et al. 2021). We believe that *C. pedum* was co-introduced with *C. cainii* from Australia since both species are native to this region. *Craspedella pedum* was first recorded in the country by Tavakol et al. (2016) from *C. quadricarinatus* caught in Komati River in Mpumalanga. Its species identification was later confirmed by Tavakol et al. (2021) through DNA barcoding. *Craspedella pedum* is not host specific as it has been recorded on other species such as *C. quadricarinatus* (Tavakol et al. 2016, Tavakol et al. 2021) and *P. clarkii* in Thailand (Ngamniyom 2020). From this it is likely that *C. pedum* can transfer to our native crustaceans. The association of temnocephalans and crayfish is an ancient relationship that is important for biological conservation (Hoyal Cuthill et al. 2016). For example, temnocephalans *Temnocephala rouxii*, *Notodactylus handschini* and *Diceratocephala boschmai* were recorded associated with *Cherax quadricarinatus* in the native region Northern Australia (Cannon 1991). A study by Jones and Lester (1992) studied the commensal relationship between the temnocephalan *Diceratocephala boschmai* and *C. quadricarinatus.* The crayfish provided transportation for the temnocephalans since they are sedentary and

provided surface for oviposition (surface for the temnocephalans to lay eggs on) (Jones and Lester 1992).

3.5.2 Cherax quadricarinatus

Cherax quadricarinatus is commonly known as the red claw crayfish and belongs to the Parastacidae family (Curtis and Jones 1995). It is native to Australia in north-eastern Queensland, eastern parts of Northern Australia and south-eastern Papua New Guinea (Nunes et al. 2017b; Madzivanzira et al. 2020, Madzivanzira et al. 2021). This species is gonochoristic, meaning that it displays sexually dimorphic growth patterns where males grow rapidly and larger in size that females (Masser and Rouse 1997, Ghanawi and Saoud 2012). The specimen caught from Banzi Pan in Ndumo Game Reserve did not have any infestations of temnocephalans or leeches. This does not necessarily mean that the specimens are free from temnocephalan infestations since only one specimen was caught. However, regular monitoring of *C. quadricarinatus* in the country is crucial to manage the expansion of the species and the subsequent spread of alien temnocephalans.

3.5.3 Procambarus clarkii

Procambarus clarkii is the most widely distributed alien freshwater crayfish of them all as it has been successfully introduced in all continents except in the Antarctica from its native regions, Northern Mexico and southeast of USA (Cruz and Rebelo 2007, Larson 2007, Crandall 2010, Lodge et al. 2012, Nunes et al. 2017a, Ngamniyom 2020, Madzivanzira et al. 2020). The two *P. clarkii* populations found in South Africa exhibited variation in body colouration, which might have been due to different environmental conditions since they were collected in in two different geographic areas, i.e., Free State and Mpumalanga Provinces. Due to the geographic separation, they cannot interbreed and are exposed to different environmental conditions. *Procambarus clarkii* can have dark body colouration in clear and acid-stained water and light in opaque and muddy waters (Huner and Barr 1991, Vogt 2002). This was true for this study, because *P. clarkii* specimens from Mimosa Dam were bright in colour compared to specimens from Driehoek Farm. Mimosa Dam was muddy and swampy, and Driehoek Farm Dam had clear water, which might have resulted in variation in colouration between

specimens from these two populations. The *P. clarkii* population in Mimosa Dam in Free State is newly reported population in South Africa (Alfreds 2018).

Procambarus clarkii can live in various habitats such rivers, lakes, ponds, streams, canals, temporary pools, marshes and ditches with mud or sand bottoms (Huner and Barr 1991) and this is what allowed the species to establish its population in Mimosa Dam. *Procambarus clarkii* can be able to survive in water temperatures from as low as 5°C and high as 35°C which are beyond its optimum water temperature range (Powell and Watts 2006). In addition, Maciaszek et al. (2019) reported a newly established population of *P. clarkii* in Żerań Canal in Warsaw in Poland where average annual temperatures are between 6°C and 8°C (Maciaszek et al. 2019). This shows that *P. clarkii* can survive in colder water temperatures in the wild and this can be the reason why the species has been able to establish its population in both Free State and Mpumalanga where water temperatures are usually below its optimum water temperatures during winter season where average minimum temperatures are -8°C (Guiding SA, 2022) and 7°C (Weather Spark, 2022) respectively.

This study also reports on the association between two alien species in Mimosa Dam, i.e., the highly invasive *P. clarkii* and the two alien freshwater leeches, *Helobdella europaea* and *Helobdella octatestisaca. Helobdella* Blanchard, 1896 leeches are of the family Glossiphoniidae and are characterized by the presence of a small chitinous scute on the dorsal side and a protrusible proboscis which is used for feeding (Pfeiffer et al. 2004, Siddall and Budinof 2005). *Helobdella europaea* was first described as the German leech, *Helobdella striata* Kutschera, 1985 and was later renamed as *Helobdella europaea* Kutschera, 1987. Sidall et al. (2005) showed that *Helobdella triseralis* is of the South American *Helobdella triserialis* species complex and is therefore a South American species (Siddall and Budinoff 2005, Malnas et al. 2016, Perera et al. 2019, Morhun et al. 2021). *Helobdella* leeches are known as non-blood feeding freshwater leeches (Oceguera-Figueroa et al. 2010, Malnas et al. 2016, Perera et al. 2019, Morhun et al. 2021).

The *Helobdella* leeches are predaceous and feed on invertebrates such as chironomids, oligochaets, freshwater snails and freshwater insect larvae by sucking body fluids with a proboscis (Pfeiffer et al. 2004). *Helobdella* leeches are also scavengers and feed on the dead bodies of aquatic vertebrates such fish and amphibians (Kutschera 2004). *Helobdella* leeches brood their young and their young are attached on their abdomen (Siddall and Budinoff 2005).

Based on the feeding of *Helobdella europaea* and *Helobdella octatestisaca,* and no literature evidence that the leeches cause harm on crayfish as their host, therefore their association is commensalism. Commensalism is defined as a relationship between two organisms where one organism benefits without causing harm to the other one (Hulme-Beaman et al. 2016). *Procambarus clarkii* probably serves the leeches for mode of movement and translocation and increase the leeches' chance of acquiring prey for feeding. As seen in Perera et al. (2019) *H. europaea* and *H. stagnalis* were reported to be attached to shells of aquatic turtles in Spain (Perera et al. 2019). The aquatic turtles provided a perfect medium for the leeches to reproduce without harming the turtles and this association was commensalism (Perera et al. 2019). A closely related species, *Helobdella stagnalis,* was found attached on amphibians (*Bombina variegate*) in Bosnia and Herzegovina, southeast Europe and the amphibian was believed to provide a mode of translocation for the leeches (Zimic 2015). A similar case of two or more alien species having a commensal association in an introduced area, is that of James et al. (2015) where alien North American Branchiobdellidan species (*Xironogiton victoriensis* and *Cambarincola* aff. *Okadai*) were association with North American signal crayfish (*Pacifastacus lenisuculus*) in Wales, Europe. Moreover, the continuing spread of *P. clarkii* in South Africa (Nunes et al. 2017a, Madzivanzira et al. 2020) can facilitate the spread and expansion of *H. europaea* and *H. octatestica* in South African freshwater systems.

The current distribution records of *Helobdella europaea* are Australia, Europe, Hawaii, South Africa, and Taiwan (Siddall and Budinoff 2005, Lai et al. 2009, Perera et al. 2019). In South Africa, *H. europaea* was first reported in Gwalagwala near Hoedspruit in Limpopo in 2001 by Siddall and Budinof (2005). Siddall and Budinof (2005) collected *H. europaea* specimens under rocks in slow-flowing water. However, this report since Siddal and Budinof (2005) study, this study is the first to report *H. europaea* and *H. octatestisaca* from live specimens as they were previously found in natural biotope such as attached on stones, wood logs in water, ditches, ponds, and slow-flowing streams (Perera et al. 2019, Morhun et al. 2021) and dead animals (Kutschera 2004).

The introduction pathways of *H. europaea* and *H. octatestisaca* into SA is unclear, however there are two possibilities. *Helobdella* leeches might have been introduced into the country via aquarium trade, the introduction of aquatic plants such as *Salvinia molesta* and *Pistia stratiotes,* or snails (Lai et al. 2009, Perera et al. 2019) as *H. europeae* was first reported in the

country near Hoedspruit in Limpopo by Siddal and Budinof (2005). It is possible that the species might have been co-introduced with *Salvinia molesta* into SA, as there are reports of *S. molesta* in Limpopo that were confirmed by Martin et al. (2018). Also, since there are no reports on *S. molesta* in the Free State as confirmed by Martin et al. (2018). The second possibility could be that *H. europaea* and *H. octatestisaca* might have been co-introduced with the *P. clarkii* in Mimosa since both the leeches (*H. europaea* and *H. octatestisaca*) and their host (*P. clarkii*) originate from South America.

Based on the high number of infestations of *H. europaea* and *H. octatestisaca* from the crayfish specimens collected in Mimosa Dam and their non-host specificity it is possible that the alien leeches are also associated with South African native organisms. The Helobdella leeches have been reported on various hosts such as fish (Kutschera 2004), amphibians (Zimic 2015) and aquatic turtles (Perera et al. 2019), and this indicates a high chance of the leeches switching host from crayfish to native organisms. The *Helobdella* leeches prey on aquatic invertebrates such as chironomids, oligochaetes, and mollusks, and displace native freshwater leech species (Pfeiffer et al. 2004, Siddall and Budinoff 2005, Lai et al. 2009) and based on this they can affect the diversity of native freshwater invertebrates. As such, a follow-up study in Mimosa Dam is crucial as it will allow determining the extent of invasion of the alien *H. europaea* and *H. octatestica* leeches. The follow-up study will also help us understand whether the alien leeches have spread to native species, since sampling was only focused on the crayfish for this study.

3.6 CONCLUSION

This study confirms the accurate species identification of IAFCs occurring in SA, C*. cainii, C. quadricarinatus a*nd *P. clarkii* through use of DNA barcoding. This was important particularly for *C. cainii* as there are reports of both *C. tenuimanus* and *C. cainii* occurring in the country. This study also confirmed the presence of *Craspedella pedum* from *Cherax cainii* in Vaughn Stock Farm, as current studies have reported the commensal temnocephalan from *C. quadricarinatus* (Tavakol et al. 2016, Tavakol et al. 2021). Furthermore, this study reported for the first time in the country, alien freshwater leeches, *Helobdella europaea* and *Helobdella octatestica* from *Procambarus clarkii* in Mimosa Dam. This report on *H. europaea* and *H.*

octatestica from the highly invasive *P. clarkii* will contribute towards updating the list of alien Annelida occurring in SA. There is currently one alien Annelida recorded in the country, i.e*., Eukerria saltensis*, a freshwater earthworm which is native to South America (Christoffersen 2008, Weyl et al. 2020). The limitations of this study include the low number of *C. quadricarinatus* caught in Banzi Pan in Ndumo Game Reserve, which might have been due low sampling effort as sampling was carried out just after the flooding event occurred in the region in January 2021 and once off over-night traps in Richard's Bay, Pongola and Ndumo Game Reserve in October 2023.

CHAPTER 4

GENERAL CONCLUSION

This study explored and tested the potential of eDNA to detect the presence of invasive alien freshwater crayfish species (IAFCs), i.e., *Cherax cainii, Cherax quadricarinatus* and *Procambarus clarkii* in South Africa (SA). This was achieved by comparing eDNA monitoring for presence or absence of IAFCs with traditional baited traps and visual surveys. The traditional baited traps outcompeted eDNA in detecting the wild populations of *C. quadricarinatus* and *P. clarkii*. However, there was an exception for *C. cainii* where eDNA was detected the species in Vaughn Bursey's Stock Farm. The struggles and limitations of eDNA experienced in the laboratory illustrate that eDNA is not a straight-forward, quick and easy to use as it has been indicated in literature (Deiner et al. 2015, Goldberg et al. 2016, Valentini et al. 2016, Elbrecht et al. 2017, Mauvisseau et al. 2019b). Therefore, it requires genomic experts both in the field and in the laboratory as it is a sensitive method. Our study shows that eDNA needs to be refined and standardized to be effective and suitable in detecting IAFCs in SA. Environmental DNA for IAFCs monitoring should be further assessed, that is, different filtering, preservation, eDNA extraction kits and different primers should be explored. For instance, this study used only ProCla primers for the detection of *P. clarkii* (Treguier et al. 2014), 1471/1472 primers for the detection of *C. cainii* and *C. quadricarinatus* (Munasinghe et al. 2004). New primers should be designed and tested for effective eDNA monitoring for IAFCs. Therefore, eDNA should not be considered as a method to replace traditional monitoring method but as a complimentary tool to be employed with traditional methods for effective monitoring of IAFCs in SA.

This study reports and confirms the presence of three IAFCs currently present in SA, i.e., *C. cainii, C. quadricarinatus* and *P. clarkii* through use of DNA barcoding. DNA barcoding is crucial in invasion biology for correct species identification which is essential for effective management of invasive alien species and conservation strategies for native species (Keskin et al. 2013). *Cherax quadricarinatus* and *P. clarkii* have feral populations in the country, while *C. cainii* is still in captivity facility, i.e., Vaughn Bursey's Stock Farm in Eastern Cape. This study also reports on the alien leeches, *Helobdella europaea* and *Helobdella octatestisaca* associated with *P. clarkii*, which this type of association has never been reported in South

Africa. The association of *P. clarkii* and alien leeches highlights a need for improved methods to monitor *P. clarkii*'s expansion and subsequently, spread of *Helobdella europaea* and *Helobdella octatestisaca* in the country. The temnocephalan, *Craspedella pedum* from *C. cainii* specimens serves as the first report from the *C. cainii* as it has been reported from Tavakol et al. (2016) and Tavakol et al. (2021).

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