Molecular characterisation and phylogenetic relationships among the cyprinid fishes of the genus *Enteromius* Cope, 1867 and their monogenean parasites of the genus *Dactylogyrus* Diesing, 1850 within the Limpopo River System

by

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Declaration

I declare that the thesis hereby submitted to the University of Limpopo for the degree of Doctor of Philosophy in Zoology has not been previously submitted by me for a degree at this or any other university; that it is my own work in design and in execution, and that all material contained herein has been duly acknowledged.

Raphahlelo ME (Mr) **Date April 2021**

Signature: 

Dedication

This thesis is dedicated to all the loved ones I have lost along the years of my academic journey. How they never saw the completion of this piece.

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This has been one of the major endeavours in which many people have made a significant contribution to the completion of this project.

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Research outputs

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Oral presentations

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Poster presentations

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Other publications

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Abstract

The present study was conducted to evaluate the morphological and molecular characterisation of species of *Dactylogyrus* parasitising *Enteromius* spp. from the Limpopo River System, South Africa. In addition, the study was intended to establish host-parasite associations from this system. A total of 95 host specimens were collected from eight localities between 2015 and 2016 within the Limpopo River System. Fish hosts were collected using gill nets, seine nets, fyke nets, and an electric shocker. From these, three host species were identified, *E*. *afrohamiltoni*, *E*. *unitaeniatus*, and *E*. *trimaculatus* where after monogenean parasites were retrieved from the gills using stereo microscopes. Morphometric analysis of the haptoral hard parts and male copulatory organs were studied for species identification, supported by nuclear ribosomal DNA sequences of the partial 18S rDNA region and the entire ITS-1 and partial 5.8S rDNA region, and the partial 28S rDNA region. Examination of *E*. *afrohamiltoni* revealed the presence of *D*. *afrohamiltonii* which is the first record of a monogenean parasite from this host. In addition, *E*. *unitaeniatus* revealed the presence of two species of *Dactylogyrus*: *D*. *letabaensis* and *D*. *limpopoensis* which are the first record of monogenean parasites from this host. The remaining *Dactylogyrus* species were retrieved from *E*. *trimaculatus*, namely, *D*. *afrolongicornis*, *D*. *allolongionchus*, and *D*. *myersi*. *Enteromius trimaculatus* harboured five of the species retrieved. The two species, *D*. *afrolongicornis* and *D*. *allolongionchus* were the most abundant from six of the eight localities studied, followed by *D*. *myersi* abundant in five of the eight localities. *Dactylogyrus afrohamiltonii* was considered a strict specialist, while the remaining species were considered to be intermediate specialists.

Forty-one sequences of the partial 18S rDNA and the entire ITS-1 and partial 5.8S rDNA region and 19 sequences of the partial 28S rDNA region of *Dactylogyrus* species, including *Pseudodactylogyrus anguillae* were included to reconstruct the phylogenetic relationships. Based on this, molecular analysis of *D. afrolongicornis* from *Enteromius* hosts were recorded for the first time for the combined 18S rDNA and the entire ITS-1 and partial 5.8S rDNA region. The analysis revealed several groupings of *Dactylogyrus* species inferred largely from European cyprinoids and corresponded to host specificity. From the partial 28S rDNA, three clades were revealed linked to their biogeographical regions. Phylogenetic analysis from the 28S rDNA suggests that *D*. *aspili* from *E*. *macrops* and *D*. *afrolongicornis* are closely related.

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Chapter 1: Introduction

1.1 Background and rationale for the study

Over the last few decades, parasites of class Monogenea Carus, 1863 has been of great research interest and has been used as a model to study a broad range of interdisciplinary topics, including taxonomy and diversity, host-parasite interactions, population and community ecology, immunoecology, evolution and phylogeny, aquaculture and pathology, therapy and control, and equally utilised as biological indicators of ecosystem health (Mashego 1983; Buchmann 1999; Sasal et al. 1999; Harris et al. 2000; Sures 2004; Mouillot et al. 2005; Mbokane et al. 2015a; Vanhove et al. 2016; Benovics et al. 2020a).

The same can be said about their fish hosts, with most researches largely confined to their classification and phylogeny, particularly within major clades such as Cypriniformes (cyprinids), Perciformes (cichlids), and Siluriformes (catfishes), therefore, addressing several hypothetical questions relating to their taxonomic status, radiation processes, evolutionary dynamics and adaption to various aquatic habitats throughout the world (Zardoya and Doadrio 1999; Dowling et al. 2002; Durand et al. 2002; Agnese and Teugels 2005; Wang et al. 2007; He et al. 2008; Day et al. 2009; de Graaf et al. 2010; Koblmüller et al. 2010; Tsigenopoulos et al. 2010; Breman et al. 2016).

One of the persistent problems in fish parasitology occurs during species identification, either of the parasite or its host. In order to identify species correctly and equally construct their phylogenetic relationships, both morphological and molecular diagnoses are essential. These present reliable, consistent, and complementary methods in the identification and accurate classification of species. Conversely, *Dactylogyrus* Diesing, 1850 species remain poorly studied in Africa, more so on molecular phylogeny despite their enormous diversity (Gibson et al. 1996). The recent limitations of molecular data on *Dactylogyrus* species in African studies highlight the importance and an interest in investigating the phylogenetic relationships with their hosts, particularly *Enteromius* Cope, 1867 and thus expand from previous studies of traditional morphology to address phylogenetic relationships of this host-parasite system.

Identification based mainly on morphological features is often complicated and problematic, more especially in congeners, a common issue in *Dactylogyrus* species (Morand et al. 2002; Šimková et al. 2002; Jarkovský et al. 2004). Therefore, from the above discussions, the present study will attempt to study the phylogenetic relationships of this host-parasite system from the Limpopo River System by using molecular approaches.

1.2 Problem statement

Species identification within *Enteromius* hosts seems to be relatively impossible given the vast diversity and close affinities of members of this genus. The problem is further exacerbated by the nature of their small-bodied size, which makes them even more difficult to identify. This often leads to misidentified hosts, resulting in erroneous reports of parasites, particularly gill monogeneans. This is enhanced by the fact that monogeneans show a high host specificity. These problems raise numerous questions concerning the validity of the hosts and their reported parasites.

Traditionally, identifications of monogeneans based only on morphological characters have been complicated. Overtime, they have been subjected to numerous revisions and re-descriptions. Preliminary observations in Africa indicate that the parasitofauna of *Enteromius* hosts is inferred largely from morphological characteristics, and until recently, no study of molecular sequences has been performed. Correct identification, either of fish or the parasite is important to avoid erroneous taxonomic decisions. The recent lack of interest in the study of *Enteromius* hosts is mainly because of the difficulty encountered in their study.

1.3 Aim

The aim of the present study was to infer phylogenetic relationships of monogenean parasites of *Dactylogyrus* species based on molecular approaches and essentially investigate host-parasite interactions of this system from the Limpopo River System. In order to achieve the above aim, specific objectives are required.

1.4 Objectives

The main objectives of this study were to:

i. Compare the morphological characters of *Dactylogyrus* species parasitising *Enteromius* hosts from the Limpopo River System.
ii. Infer phylogenetic relationships of *Dactylogyrus* species from the Limpopo River System by amplifying the partial 18S rDNA region, the entire ITS-1 and partial 5.8S rDNA region, and the partial 28S rDNA region.

Null hypotheses

i. There are no unambiguous morphological characters that distinguish *Dactylogyrus* species parasitising *Enteromius* hosts in the Limpopo River System.
ii. There is no molecular variation that distinguishes *Dactylogyrus* species from the Limpopo River System based on the partial 18S rDNA region, the entire ITS-1 and partial 5.8S rDNA region, and the partial 28S rDNA region.

Therefore, the following are the fundamental questions posed in this study:

i. Are there unambiguous morphological characteristics that distinguish *Dactylogyrus* species associated with *Enteromius* hosts?
ii. Are the partial 18S rDNA region, the entire ITS-1 and the partial 5.8S rDNA region, and the partial 28S rDNA region reliable markers to infer phylogenetics for species of *Dactylogyrus*?

1.5 Thesis outline

This thesis is divided into five chapters that address molecular and phylogenetic studies of monogenean parasites of *Dactylogyrus* species specific to cyprinid fish of *Enteromius* hosts along the various localities of the Limpopo River System.

Chapter 1 instigates the rationale behind this thesis. The problem statement, aim, objectives, null hypotheses and questions imposed in this thesis are included.

Chapter 2 describes the sampling area and specific localities. The materials and methods for sampling strategies of the host specific to this thesis are provided.

Chapter 3 focuses on the morphological study of species of *Dactylogyrus* parasitising *Enteromius* hosts from the Limpopo River System. The materials and

methods for sampling strategies of monogenean parasites specific to this thesis are provided. Examination, fixation, preparation, and analysis of gill monogenean methodologies for morphological characterisation are outlined. The morphological aspects of monogeneans collected from the hosts are discussed. This chapter includes a manuscript attached in Appendix 3 to strengthen the novelty of this study.

Chapter 4 deals with the molecular study of species of *Dactylogyrus* parasitising *Enteromius* hosts from the Limpopo River System. Examination, fixation, preparation, and analysis of gill monogenean methodologies for molecular characterisation are described. The results and discussion are given in detail.

Chapter 5 contains a summary in the form of conclusions and recommendations for future studies and practical implications.

Chapter 2: Materials and methods

2.1 Description of study area and localities

Host specimens were collected from eight localities between 2015 and 2016 along the Limpopo River System in Limpopo Province, located in South Africa (Figure 2.1). The localities were selected to emphasise the vast distribution of the host specimens throughout this aquatic system. Table 2.1 provides a summary of hosts examined, the numbers of hosts collected and their collection sites. Moreover, the Global Positioning System (GPS) coordinates of each site were recorded.

2.1.1 Limpopo River System

The Limpopo River System is located in the southern African region, and its catchment area distribution is among Botswana (20 %), Mozambique (20 %), South Africa (45 %), and Zimbabwe (15 %), where it flows eastwards into the Indian Ocean (Boroto 2001; Maposa et al. 2014; Trambauer et al. 2014). The river covers an area of about 1.3 % of the continent (over 400 000 square kilometres), making it the second largest river basin in Africa to the Zambezi River (Chilundo et al. 2008; Silva et al. 2010). The geographical position of the Limpopo River System is demarcated by latitude between 20°S and 26°E longitude and latitude 26°S and longitude 3°E, with an average annual runoff of 5 500 thousand cubic metres per year and a mean annual rainfall of 530 millimetres per year, ranging between 200 and 1 200 millimetres per year (Boroto 2001; Sawunyama et al. 2006; Trambauer et al. 2014) predominantly characteristic of an area of low rainfall (Tshikolomo et al. 2013).

The climatic conditions of the Limpopo River System vary spatially from a semi-arid region to an arid region in the upper Kalahari Desert, making the river unreliable and vulnerable to extreme hazards such as floods and severe droughts due to its seasonal rainfall variability (Boroto 2001; Silva et al. 2010; Maposa et al. 2014; Trambauer et al. 2014). However, the river is important in the utilisation for domestic use, livestock watering, fishing and irrigation, urban supply and mining (Boroto 2001). Particularly in South Africa, the Limpopo River System is located in the north-eastern region of the country and comprises major tributaries, namely, Crocodile, Elephants, Letaba, Luvuvhu, Olifants, Shashe, Mzingwane, Mwenezi Rivers. Thus, from an ecological and evolutionary point of view, the information on

the genetic variability as well as the spatial distribution of aquatic populations must be known in the Limpopo River System.

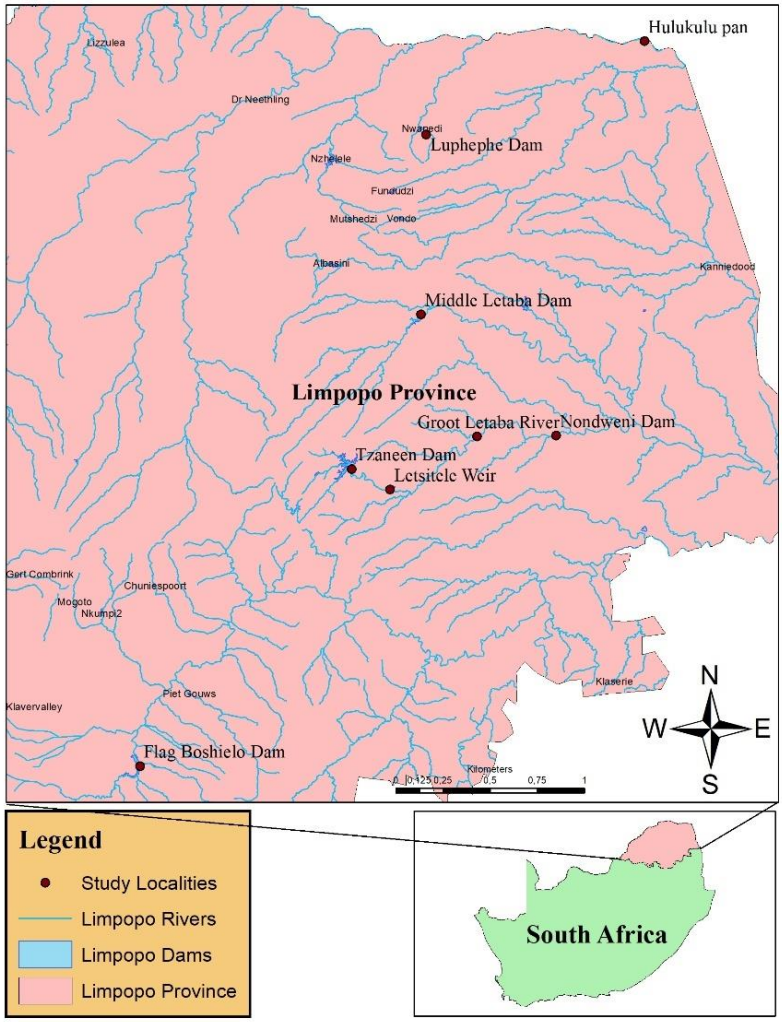

Figure 2.1: The geographical position of the study area and various locations for the sampling of *Enteromius* hosts. (Map created in ArcGIS version 10.3 by Raphahlelo ME)

Table 2.1: Specimens of *Enteromius* hosts collected during the present study.

Host specimens	N	Locality
E. afrohamiltoni	3	Hulukulu Pan
E. unitaeniatus	1	Tzaneen Dam
	13	Middle Letaba Dam
	5	Letsitele Weir
	1	Luphephe Dam
	3	Nondweni Dam
E. trimaculatus	26	Middle Letaba Dam
	7	Groot Letaba River
	4	Letsitele Weir
	10	Flag Boshielo Dam
	15	Luphephe Dam
	7	Nondweni Dam

N = number of fish collected

2.1.2 Localities

It is important to note that several localities during the present investigation comprised the presence of hippopotamus and crocodile populations and made it difficult during the sampling of host specimens.

Flag Boshielo Dam

Flag Boshielo Dam (24°46´51,46"S; 29°25´32,57"E) was initially known as the Mokgoma Matlala Dam and was later renamed Arabie Dam (Botha 2005). However, in 2001 it was further renamed after a political activist and freedom fighter Marutle Flag Boshielo (Tapela et al. 2015). It is situated about 30 kilometres north-east of the town of Marble Hall in the Limpopo Province, South Africa (Figure 2.2). The dam was constructed in 1987 to provide water for domestic, irrigation, industrial and recreational use for nearby communities (McCartney et al. 2004; Heath et al. 2010). The dam has a catchment area of 23 712 square kilometres, a surface area of 1 288 hectares, a storage capacity of 184 X 10^6 cubic metres and an average depth of 15 metres (DWAF 2003).

Figure 2.2: The middle region of the Flag Boshielo Dam. (Photograph by Raphahlelo ME)

Groot Letaba River

The Groot Letaba River (23°41'27.58"S, 30°35'45.16"E) (Figure 2.3), originates from a mountainous Haenertsburg area and flows towards lower parts of the catchment in the eastern part over a distance of about 30 kilometres (Katambara and Ndiritu 2007; Nyabeze et al. 2007; Louw et al. 2010; Sinha and Kumar 2015). More than 20 dams and weirs are located in the Groot Letaba Catchment with Tzaneen Dam on the Groot Letaba River and the Middle Letaba Dam being the two largest in Limpopo Province (Sinha and Kumar 2015; Querner et al. 2016). The gross surface water availability in the Groot Letaba sub-area is estimated at 168 million cubic metres per annum, which is derived from the yield of Tzaneen and Ebenezer Dams as well as significant run-off-river abstractions (Sinha and Kumar 2015). The upper region of the river is characterised by intensive irrigation, mixed with farming, including cattle ranching, game farming and dry land crop production (Nyabeze et al. 2007; Querner et al. 2016).

Figure 2.3: A shallow, slow flowing stream of Groot Letaba River (Elands). (Photograph by Raphahlelo ME)

Hulukulu Pan

Hulukulu Pan (22°20'22.31"S, 31°10'06.09"E) (Figure 2.4), is located approximately 728 metres southwest of the Limpopo River. The pan receives water through seepage from the river. According to Deacon (2007), silt could potentially be deposited into the pan due to back flooding of the Limpopo River which takes place every 2–3 years. The surrounding area of the pan consists of a dense canopy of trees and shrubs which could potentially provide habitat for surrounding wildlife.

Figure 2.4: A slow flowing lake of Hulukulu Pan with aquatic vegetation. (Photograph by Raphahlelo ME)

Letsitele Weir

Letaba River Catchment consists of several weirs, including the Letsitele Weir (23°52'19.60"S, 30°17'55.67"E) (Figure 2.5), which is located downstream of Tzaneen Dam. The Letsitele Weir is part of the Letsitele River, where intensive irrigation is practised upstream along the river (Ashton et al. 2001; Nyabeze et al. 2007; Sinha and Kumar 2015; Querner et al. 2016).

Figure 2.5: A fast flowing turbid stream of Letsitele Weir. (Photograph by Raphahlelo ME)

Luphephe Dam

The Luphephe Dam is situated (22°39.492'S, 30°25.342'E) in the Nwanedi Nature Reserve of the Limpopo Province, South Africa (Figure 2.6). The dam is fed by the Luphephe Stream situated in the foothills of the Venda Mountains, northern Limpopo Province. This section receives low and erratic rainfall with average annual precipitation that varies between 450 and 650 millimetres and an average annual runoff of 60 millimetres (Ashton et al. 2001), making it one of the most drought-prone areas. The construction of this dam, together with its adjoining dam, the Nwanedi Dam, commenced in 1964 by then the Department of Water Affairs. The dams are referred to as the “twin” dams due to their connection by a 2.5-metre-deep channel with a surface area of approximately 220 hectares. They are estimated to have a carrying capacity of $19.1X10^6$ cubic metres of water. The dams consist of a high diversity of fish and plant species and are referred to be pristine

due to the absence of agricultural activities, industrial and mining schemes (Mbokane et al. 2015a).

Figure 2.6: A pristine lake of Luphephe Dam. (Photograph by Raphahlelo ME)

Middle Letaba Dam

The Middle Letaba Dam (23°16'27.08"S, 30°24'16.55"E) (Figure 2.7), is located in the north-eastern area of South Africa and covers an area of 14 086 square kilometres. The dam is located within the Middle Letaba River, a part of the Letaba River Catchment which is fed by the Koedoes River, Brandboontjies River and minor streams (Tshikolomo et al. 2012), and it is one of the major dams in the catchment. The dam was constructed in 1984 to meet the constant water demand supply for nearby communities for irrigation. It has a capacity of $173X10^6$ cubic metres, with a total surface area of 1 943 hectares and a depth of 34 metres (Polling et al. 1992; Querner et al. 2016). It provides water for various purposes as well as food in the form of fish. Land use in the catchment upstream is characterised by intensive irrigation farming, including subsistence, commercial and recreational (Querner et al. 2016). The Middle Letaba Dam delivers water through a 60 kilometre long canal into the Nsami Dam with a capacity of 4 cubic metres per second (Tapela et al. 2015; Querner et al. 2016) which feeds the nearby villages with water along the canal.

Figure 2.7: A shallow stream of Middle Letaba Dam. (Photograph by Raphahlelo ME)

Nondweni Dam

Nondweni Dam (23°41'16.84"S, 30°51'57.78"E) (Figure 2.8), is situated downstream of the Tzaneen Dam in the Groot Letaba River. According to Seshoka et al. (2004), the importance of the dam, especially during hot days, allows the upstream commercial farmers to pump large amounts of water. Several purification plants supply water to three villages, namely, Selwane, Nondweni, and Mahale, through a pipeline filling reservoirs in each village.

Figure 2.8: A deep, fast flowing stream of Nondweni Dam. (Photograph by Raphahlelo ME)

Tzaneen Dam

Tzaneen Dam (23°48'07.78"S, 30°10'01.54"E) (Figure 2.9), is located on the Groot Letaba River and is one of the major dams in the Letaba River Catchment. In view of the economic importance of Tzaneen Dam, it supplies water to downstream users for domestic, irrigation for agriculture, industry and recreation and is regarded as a priority to poor communities (Nyabeze et al. 2007). The dam was constructed in 1977 with a capacity of $157.7X10^6$ cubic metres being the largest downstream reservoir across the Letaba River (Sinha and Kumar 2015). Water for irrigation along the Groot Letaba River is supplied by the upstream Tzaneen Dam (DWA 2013). The status of the dam since 2018 has experienced periods of drought that resulted in low dam levels, which had a severe effect on the aquatic populations such as fish, birds and other animals that rely on this dam for food and shelter.

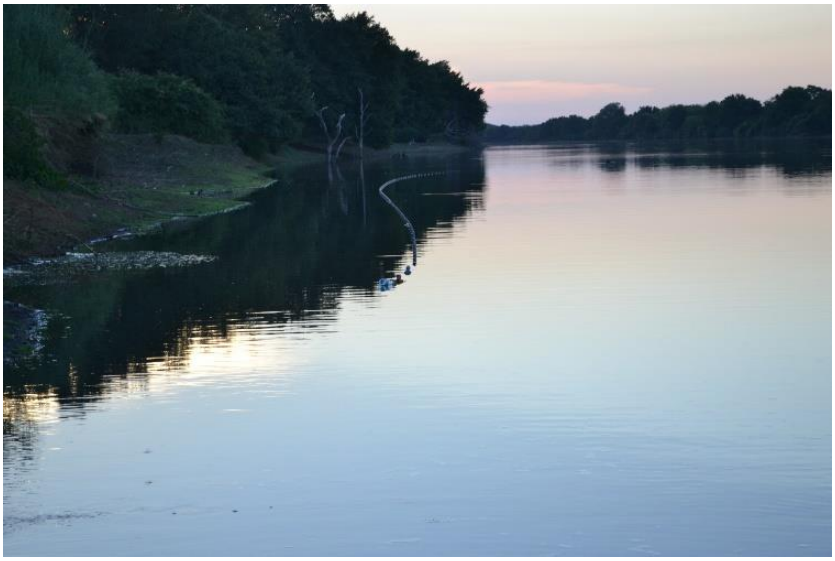

Figure 2.9: A slow flowing stream of Tzaneen Dam. (Photograph by Raphahlelo ME)

2.2 Sampling host specimens

2.2.1 Host sampling

In order to meet the objectives outlined in Chapter 1, a great amount of time and effort was spent looking for host specimens in various localities along the Limpopo River System. It needs to be mentioned that our data reflects random sampling, therefore, no ecological parameters such as water quality and seasonality were

considered during this study. It is necessary to have a basic knowledge of the host population in question to apply correct methods during sampling, depending on the locality and its habitat. Therefore, three suitable sampling methods were used to sample hosts, including gill netting, electrofishing and seine netting (Figure 2.10). The employment of these techniques was motivated by the small size ranges of the host, 20–30 millimetres standard length (Skelton 2001), and regions accessible by the methods mentioned above for maximum yield.

Figure 2.10: Different sampling methods used: A – Gill netting, B – Electrofishing and C – Seine netting. (Photographs by Raphahlelo ME)

Gill nets of stretched single mesh size of 30 millimetres were left overnight at relatively deep waters of more than 1 metre. A sample of 5–10 individuals per species were targeted during each survey as a representative number. Electrofishing, using an electric shocker (LR-24, Smith Root Company, USA), was employed for shallow and flowing areas. Both seine and fyke netting (30 millimetre mesh nylon) were used where suitable, especially in open waters.

2.2.2 Host transportation

Individual host specimens were kept in separate holding tanks to prevent the transfer of parasites (host-switch). Hosts were transported alive in constantly aerated holding tanks containing site water to the field laboratory for further identification and examination.

2.2.3 Host identification

In order to distinguish individual host species, identification was performed using methods described by Skelton (2001). In addition, the host names used in the present study are presented according to Froese and Pauly (2018) for the recent and updated scientific names. For every host specimen, information such as total length (TL), standard length (SL) and the fork length (FL) using a ruler, weight (g) using a scale, sex (male or female) were recorded. Photos of host specimens were taken, while additional photographs from fish websites were printed out to strengthen the identification.

2.2.4 *Enteromius*

The following nomenclature follows that of Froese and Pauly (2018).

Phylum: Chordata
Class: Actinopterygii
Order: Cypriniformes
Family: Cyprinidae
Subfamily: Cyprininae
Genus: *Enteromius* Cope, 1867

There are 21 *Enteromius* species known from South Africa, namely, *E*. *afrohamiltoni* (Crass, 1960), *E*. *amatolicus* (Skelton 1990), *E*. *annectens*

(Gilchrist and Thompson, 1917), *E*. *anoplus* (Weber, 1897), *E*. *argenteus* (Günther, 1868), *E*. *bifrenatus* (Fowler, 1935), *E*. *brevipinnis* (Jubb, 1966), *E*. *eutaenia* (Boulenger, 1904), *E*. *gurneyi* (Günther, 1868), *E*. *lineomaculatus* (Boulenger, 1903), *E*. *mattozi* (Guimaraes, 1884), *E*. *motebensis* (Steindachner, 1894), *E*. *neefi* (Greenwood, 1962), *E*. *pallidus* (Smith, 1841), *E*. *paludinosus* (Peters, 1852), *E*. *radiatus* (Peters, 1853), *E*. *toppini* (Boulenger, 1916), *E*. *treurensis* (Groenewald, 1958), *E*. *trimaculatus* (Peters, 1852), *E*. *unitaeniatus* (Günther, 1866) and *E*. *viviparus* (Weber, 1897) (Froese and Pauly 2018). They are typically opportunistic feeders on any small creatures, diatoms, or detritus, and in turn, form a valuable food for larger fish and predators (Skelton 2001).

Enteromius afrohamiltoni is a benthopelagic, tropical freshwater species found between 13°S and 27°S of the equator. It is commonly known as the Plump barb (Fouché 2009) or simply Hamilton's barb. It favours placid waters such as pans and large pools, typically feeds on insects and serves as bait for tigerfish (Skelton 2001). *Enteromius afrohamiltoni* is found in the Pongola, Incomati and Limpopo River Systems and northwards to the lower and middle Zambezi River System (Jubb 1966). It attains a maximum length of 175 millimetres standard length.

Enteromius trimaculatus is a potamodromous, benthopelagic, tropical freshwater species found between 9°S and 30°S of the equator. It consists of three clear spots on the side of the body, from the base of the caudal peduncle along the lateral line; for this reason, it is commonly known as the "Threespot barb". It is generally a hardy species, commonly found in various habitats, more so living among vegetation (Dejen et al. 2002). It can tolerate high temperatures between 24°C and 26°C; its diet consists primarily of insects and other aquatic organisms. It attains a maximum size of 150 millimetres standard length (Skelton 2001). Species distribution is associated with the Komati and Vaal River Systems as well as the northern tributaries of Orange River and the Umvoti River in Natal, northwards to the middle Zambezi River System (Groenewald 1958; Jubb 1966). Elsewhere, it is found at the east coast from Ruvuma, Tanzania to the Cunene and Zambian Congo Systems as well as Lake Bangweulu and its associated rivers (Skelton 2001).

Enteromius unitaeniatus is a benthopelagic, tropical freshwater species found between 10°S and 27°S of the equator (Polling et al. 1992). It is commonly known

as the Longbeard barb/Slender barb. It occurs in various habitats, especially in standing and flowing waters, typically thriving in dams and lakes and feeds on grass reeds and aquatic invertebrates (Skelton 2001). It breeds in summer after rainy months. It attains a maximum length of 140 millimetres standard length (Skelton 2001). *Enteromius unitaeniatus* is found widespread as far as the Zambian Congo System and the Cunene, Okavango, Zambezi south to the Pongola and Incomati River System (Groenewald 1958; Jubb 1966; Skelton 2001). Elsewhere, it is found in the Cuanza and Bengo in Angola (Skelton 2001).

2.2.5 Ethics approval

This study was approved by the Animal Research Ethics Committee (AREC) of the University of Limpopo prior to fish collection, ethics number AREC/05/2019: PG (Appendix 1). A permit for the collection of fish was obtained from the Department of Economic Development and Tourism, Limpopo Province, approval number ZA/LP/HO/3370 (Appendix 2). Hosts were killed humanely by severing the spinal cord with small scissors just behind the brain while immersed completely with site water in a Petri dish.

Chapter 3: Morphological study of species of *Dactylogyrus* parasitising *Enteromius* hosts from the Limpopo River System

3.1 Introduction

The study of Monogenea Carus, 1863 dates back a long way. During the 18th century, the first finding of a monogenean was described from the skin of the Atlantic halibut (*Hippoglossus hippoglossus*) (Müller 1776). Despite efforts made by Müller (1776), he mistakenly regarded the parasite as a leech and named it *Hirrudo hippoglossi*. Based on this, van Beneden (1858) restored its status as a monogenean and named it *Epipdella* (now *Entopdella*) *hippoglossi*. Indeed, considerable advancements have been made ever since this discovery, which highlights the potential of research in the field of monogeneans, along with their related biological disciplines over the next centuries.

Monogeneans belong to members of the flatworms (Phylum Platyhelminthes) of the Neodermata (Perkins et al. 2010). Within the neodermatans, there are three distinct classes of obligate parasites, namely, Cestoda, Trematoda and Monogenea (Lockyer et al. 2003). Cestoda are commonly known as tapeworms and includes Gyrocotylidea, Amphilinidea and Eucestoda. Trematoda are known as flukes that include Aspidogastrea and Digenea. Monogenea are known as gill/skin or blood flukes that include Monopisthocotylea and Polyopisthocotylea. Cestoda and Trematoda are exclusively endoparasitic, with complex indirect life cycles that require one or more intermediate and definitive hosts to complete their life cycle (Perkins et al. 2010). Monogenea on the other hand, are ectoparasitic, a few being endoparasitic inside the host body (nasal cavities, alimentary canal, ureter, bladder, and cloaca) with a simple direct life cycle. They are known to be extremely host-specific compared to other parasitic groups (Šimková et al. 2001a; Mendlová et al. 2010).

The division of Monogenea follows two subclasses, the epithelial feeding Monopisthocotylea and the blood feeding Polyopisthocotylea (Perkins et al. 2010). In turn, they are sometimes referred to as Polyonchoinea and Heteronchoinea, respectively (including the two infraclasses of Polystomatoinea and Oligonchoinea) (Boeger and Kritsky 2001) both known to parasitise various animal groups of invertebrates and vertebrates. *Isancistrum* de Beauchamp, 1912 has been

recorded on the arms and tentacles of cephalopods (squids), whilst species of *Polystoma* Zeder, 1800 have been reported from the urinary bladder of amphibians (frog) and chelonians (turtles). Specimens of *Heterocotyle* Scott, 1904 have been reported on the gills of elasmobranchs (stingrays) and *Oculotrema* Stunkard, 1924 has been recorded from the eyes of a mammal (hippopotamus). Others primarily parasitise the skin (e.g. *Gyrodactylus* von Nordmann, 1832), stomach (*Enterogyrus* Paperna, 1963), nasal cavity (*Paraquadriacanthus* Ergens, 1988), ureter (*Acolpenteron* Fischthal & Allison, 1940), urinary bladder (*Urogyrus* Bilong, Birgi & Euzet, 1994) or gills (*Dactylogyrus* Diesing, 1850) of major groups of freshwater fishes. The exceptional diversity of the latter parasite genera, a common and widespread genus (Gibson et al. 1996), is of interest to the present study.

3.2 Genus *Dactylogyrus*

Monopisthocotylea monogeneans of the genus *Dactylogyrus* are gill ectoparasites, primarily infesting cyprinid fishes, although to a lesser extent found to parasitise fish of other families, namely, Gobiidae and Nothobranchiidae (Khalil and Polling 1997; Řehulková et al. 2018). They are oviparous, typically laying their eggs into the water, which hatch into a free-swimming ciliated larva called oncomiracidium that must attach to the fish host to complete the life cycle (Paperna 1996). *Dactylogyrus* represents the most diversified species group among monogeneans with more than 900 nominal species (Gibson et al. 1996). It is followed by members of genus *Gyrodactylus* with 409 nominal species (Harris et al. 2004) and *Cichlidogyrus* Paperna, 1960 with more than 100 nominal species (Kmentová et al. 2016). There is strict host specificity i.e. strict specialists (species-specific to a single host) in most *Dactylogyrus* species with their cyprinid hosts. Others may infest phylogenetically closely related hosts (congeneric hosts) known as intermediate specialists or may infest non-congeneric but phylogenetically closely related host species (intermediate generalists) (Šimková et al. 2013a, 2017). *Dactylogyrus* offers an excellent model for evolutionary studies because of its high-species richness, their diverse speciation mechanisms and their ecological dominance in aquatic ecosystems (Benovics et al. 2018, 2020a) and their associated cyprinid fishes.

Several studies specifically focused on *Dactylogyrus* species in Asia and Europe (Morand et al. 2002; Šimková and Morand 2008; Šimková et al. 2000, 2001a,

2001b, 2001c, 2002, 2003, 2004, 2006a, 2007; Jarkovský et al. 2004; Mouillot et al. 2005; Nitta and Nagasawa 2016; Ling et al. 2016). These studies have revealed diverse populations with high ecological implications of either intra/interspecific events through investigating morphological and molecular data of sclerotised structures, which demonstrate important evolutionary implications with their hosts.

For example, Šimková et al. (2001a) investigated the patterns of specificity in *Dactylogyrus* species of cyprinid fishes. They noted that specialist parasites in relation to total length and base length of their haptor correlated positively with host body size, thus suggesting a strong link between the haptor's adaptive processes to their hosts. However, there was no significance for generalist parasites. In addition, Šimková et al. (2002) investigated the co-existence of congeneric monogeneans and their relationship to the haptor and copulatory organ morphology for nine *Dactylogyrus* species from the gill of roach. They noted that these parasite species have similar morphology of the haptor but differ in the morphology or size of their male copulatory organ (MCO) for reinforcement of reproductive barriers. Furthermore, Morand et al. (2002) investigated large *Dactylogyrus* species and found no significant differences between the morphology of the haptor and copulatory organs from cyprinid hosts of a given region. Moreover, Jarkovský et al. (2004) investigated the morphological characteristics of the attachment apparatus (haptor) and MCO for 52 *Dactylogyrus* species parasitising 17 species of cyprinid fishes. They noted that specialist parasites possess more similarity in attachment apparatus than within generalist parasites attributed to speciation with their hosts.

3.2.1 Status of *Dactylogyrus* in Africa

Many *Dactylogyrus* species in Africa have been described by Paperna (1973, 1979) in eastern and western Africa from mainly cyprinid fishes (Khalil and Polling 1997). To date, 104 species of *Dactylogyrus* have been described from Africa (Řehulková et al. 2018), mainly from Cameroon (14 spp.), Kenya (4 spp.), Gabon (5 spp.), Ghana (9 spp.), Guinea (9 spp.), Mali (9 spp.), Morocco (17 spp.), Senegal (3 spp.), Sierra Leone (1 spp.), South Africa (14 spp.), Tanzania (5 spp.), Tunisia (2 spp.) and Uganda (12 spp.). Of these, 39 species have been reported from *Enteromius* hosts (Řehulková et al. 2018; Mashego and Matlou 2018; Raphahlelo et al. 2020).

In southern Africa, the genus *Dactylogyrus* was first mentioned by Price et al. (1969a), who described two species, *Dactylogyrus jubbstrema* Price, Korach & McPott, 1969 from the gills of *Glossogobius giuris* (Hamilton, 1822) and *Dactylogyrus pienaari* Price, Korach & McPott, 1969 from the gills of *Labeo rosae* Steindachner, 1894 from KwaZulu-Natal. In the same period, Price et al. (1969b) described two further species, *Dactylogyrus myersi* Price, McClellan, Druckenmiller & Jacobs, 1969 from the gills of *E. trimaculatus* and *Dactylogyrus varicorhini* Bychowski, 1958 from the gills of *Labeobarbus kimberleyensis* (Gilchrist & Thompson, 1913) from Lydenburg. Subsequent taxonomic studies of this genus followed, which culminated into 22 species primarily from cyprinid fishes (Table 3.1) (Mashego 1983; Olivier et al. 2009; Crafford et al. 2012, 2014; Mbokane et al. 2015a; Truter et al. 2016; Mashego and Matlou 2018; Raphahlelo et al. 2020).

As already mentioned, 21 *Enteromius* species are known from South Africa; of these, 11 species have been studied for monogenean as well as other parasites, including, acanthocephalans, cestodes, crustaceans, trematodes, nematodes, protists and myxozoans. These include *E*. *afrohamiltoni*, *E*. *argenteus*, *E*. *eutaenia*, *E*. *lineomaculatus*, *E*. *mattozi*, *E*. *neefi*, *E*. *pallidus*, *E*. *paludinosus*, *E*. *radiatus*, *E*. *trimaculatus* and *E*. *unitaeniatus* (van As and Basson 1984; Saayman et al. 1991; Khalil and Polling 1997; Mashego 2000; Olivier et al. 2009; Mbokane et al. 2015b).

Table 3.1: *Dactylogyrus* species known to parasitise cyprinids in different water bodies from South Africa.

Parasite species	Host	Locality	
1. *D*. *afrohamiltonii* Raphahlelo, Přikrylová & Matla, 2020	*Enteromius afrohamiltoni* (Crass, 1960)	Hulukulu Pan	(1)
2. *D*. *afrolongicornis* Paperna, 1973	*Enteromius trimaculatus* (Peters, 1852)	Seshego Dam	(2)
		Piet Gouws Dam	
		Mohlapitse River	
		Nwanedi-Luphephe Dams	(3)
		Middle Letaba Dam	(4; 1)
		Groot Letaba River	
		Letsitele Weir	
		Flag Boshielo Dam	
		Luphephe Dam	
		Nondweni Dam	
3. *D*. *afrolongicornis alberti* Paperna, 1973	*Enteromius trimaculatus* (Peters, 1852)	Seshego Dam	(2)
		Middle Letaba Dam	(4)
		Nwanedi-Luphephe Dams	(3)
4. *D*. *afrosclerovaginus* Paperna, 1973	*Enteromius paludinosus* (Peters, 1852)	Seshego Dam	(2)
		Middle Letaba Dam	(4)
5. *D*. *allolongionchus* Paperna, 1973	*Enteromius trimaculatus* (Peters, 1852)	Seshego Dam	(2)
		Middle Letaba Dam	(4; 1)
		Groot Letaba River	(1)
		Letsitele Weir	
		Flag Boshielo Dam	
		Luphephe Dam	
		Nondweni Dam	
6. *D*. *dominici* Mashego, 1983	*Enteromius paludinosus* (Peters, 1852)	Turfloop Dam	(2)
		Middle Letaba Dam	(4)
7. *D*. *enidae* Mashego, 1983	*Enteromius neefi* (Greenwood, 1962)	Lingwe River	(2)

8. *D*. *extensus* Mueller & Van Cleave, 1932	*Cyprinus carpio* Linnaeus, 1758	Vaal Dam	(5)
9. *D*. *iwani* Crafford, Luus-Powell & Avenant-Oldewage, 2012	*Labeo capensis* (Smith, 1841) *Labeo umbratus* (Smith, 1841)	Vaal Dam	(6)
10. *D*. *jubbstrema* Price, Korach & McPott, 1969	*Glossogobius giuris* (Hamilton, 1822)	KwaZulu-Natal Natal	(7) (8)
11. *D*. *lamellatus* Achmerow, 1952	*Ctenopharyngodon idella* (Valenciennes, 1844)	Vaal Dam	(5)
12. *D*. *larindae* Crafford, Luus-Powell & Avenant-Oldewage, 2012	*Labeo umbratus* (Smith, 1841) *Labeo capensis* (Smith, 1841)	Vaal Dam	(6)
13. *D*. *letabaensis* Raphahlelo, Přikrylová & Matla, 2020	*Enteromius unitaeniatus* (Günther, 1866)	Middle Letaba Dam Letsitele Weir Luphephe Dam Nondweni Dam	(1)
14. *D*. *limpopoensis* Raphahlelo, Přikrylová & Matla, 2020	*Enteromius unitaeniatus* (Günther, 1866) *Enteromius trimaculatus* (Peters, 1852)	Tzaneen Dam Letsitele Weir Groot Letaba River Flag Boshielo Dam	(1)
15. *D*. *mattozii* Mashego & Matlou, 2018	*Enteromius mattozi* (Guimaraes, 1884)	Piet Gouws Dam	(9)
16. *D*. *minutus* Kulviec, 1927	*Cyprinus carpio* Linnaeus, 1758	Vaal Dam	(5)
17. *D*. *myersi* Price, McClellan, Druckenmiller & Jacobs, 1969	*Enteromius trimaculatus* (Peters, 1852)	Lydenburg Natal Seshego Dam Middle Letaba Dam Groot Letaba River Letsitele Weir Flag Boshielo Dam Nondweni Dam	(10) (8) (2) (4; 1)
18. *D*. *nicolettae* Crafford, Luus-Powell & Avenant-Oldewage, 2012	*Labeo capensis* (Smith, 1841)	Vaal Dam	(6)
19. *D*. *pienaari* Price, Korach & McPott, 1969	*Labeo rosae* Steindachner, 1894	KwaZulu-Natal	(7)

		Natal	(8)
20. *D*. s*pinicirrus* Paperna & Thurston, 1969	*Labeobarbus marequensis* (Smith, 1841)	Luphephe Dam	(2)
		Nwanedi Dam	
		Middle Letaba Dam	(4)
	Labeobarbus marequensis (Smith, 1841) *Enteromius trimaculatus* (Peters, 1852) *Enteromius radiatus* (Peters, 1853)	Nwanedi-Luphephe Dams	(3)
21. *D*. *teresae* Mashego, 1983	*Enteromius paludinosus* (Peters, 1852)	Seshego Dam	(2)
		Middle Letaba Dam	(4)
		Ramsar wetland	(11)
22. *D*. *varicorhini* Bychowski, 1958	*Labeobarbus kimberleyensis* (Gilchrist & Thompson, 1913)	Lydenburg	(10)
		Pongola River	(8)

Reference: 1 = Raphahlelo et al. (2020); 2 = Mashego (1983); 3 = Mbokane et al. (2015a); 4 = Olivier et al. (2009); 5 = Crafford et al. (2014); 6 = Crafford et al. (2012); 7 = Price, Korach and McPott (1969a); 8 = Paperna (1979); 9 = Mashego and Matlou (2018); 10 = Price, McClellan, Druckenmiller and Jacobs (1969b); 11 = Truter et al. (2016)

3.3 Materials and methods

3.3.1 Examination of gills for monogeneans

The gills were carefully and gently examined using fine needles by scraping through the gill lamellae. Monogeneans were carefully detached from the gills, counted, and isolated on a slide with a drop of water observed under a dissecting stereo microscope (Figure 3.1).

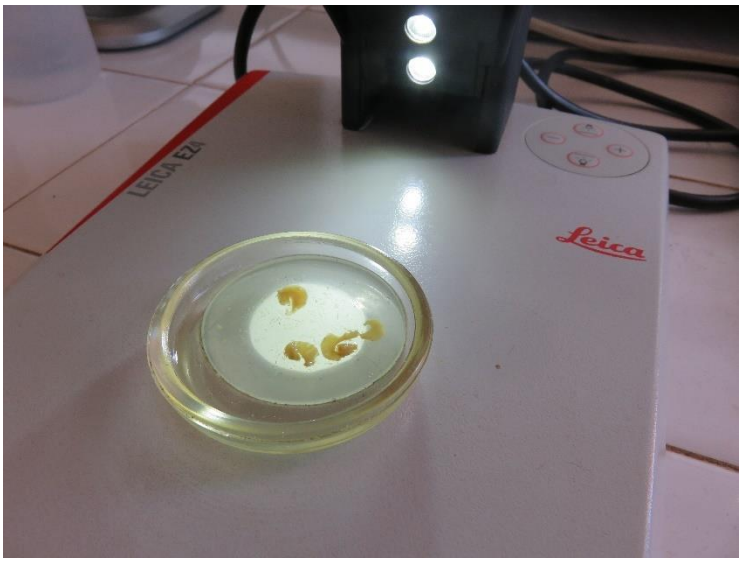

Figure 3.1: Examination of gill monogeneans with the aid of a stereo microscope (Leica EZ4). (Photograph by Raphahlelo ME)

3.3.2 Fixing and preparation of monogeneans on slides

Fixing was done quickly to avoid drying of water on the slide as the worm can be lost or disintegrate. For the fixing of a semi-permanent medium ammonium picrate-glycerine solution (GAP) (Malmberg 1957) was used. For permanent preparations, the slides were remounted in Canada balsam following the protocol of Ergens (1969).

3.3.3 Identification of monogeneans using sclerotised structures

For correct identification and description of species, important features of monogeneans were observed on completely flattened specimens. They were identified using sclerotised structures of the attachment apparatus (haptor) and reproductive system, male copulatory organ (MCO) and vaginal armament. Measurements (in micrometres) were taken directly from the slide under a light

microscope Olympus BX50, equipped with an imaging software (Soft Imaging System GHBM 1986 version 1.5.1), a drawing tube, a digital camera, and a calibrated eye piece (Figure 3.2). Twelve characters were measured on sclerotised structures [anchors, transverse bar, vestigial ventral bar, hooks and MCO]. Other characteristic includes, among others, the size of the body (length, width); shape, number, and arrangement of haptoral structures; structure and size of the MCO, vaginal armament (length, width). All measurements were performed according to Gussev in Bychovskaya-Pavlovskaya et al. (1962), except the tube trace length (copulatory tube) was measured according to Musilová et al. (2009). All measurements are presented as the mean with the range in parentheses.

Figure 3.2: Olympus BX50 microscope used in morphological characterisation. (Photograph by Raphahlelo ME)

3.3.4 Deposition of permanent materials

Holotype, paratypes and voucher specimens were deposited in the parasitological collection museums in the National Museum, Bloemfontein, South Africa, the Royal Museum for Central Africa, Tervuren, Belgium and the helminthological collection held at the Institute of Parasitology, Biology Centre, Academy of Sciences of the Czech Republic, České Budějovice, Czech Republic.

3.3.5 Ecological analysis

Ecological parameters for prevalence, mean intensity and mean abundance of infestation are given according to Bush et al. (1997). Prevalence is the number of

individuals of the hosts infested with a particular parasite species divided by the number of hosts examined (expressed as a percentage); Mean intensity is the total number of individuals of all parasites found in a sample of particular host species divided by the number of hosts infested with that parasite. Mean abundance is the total number of individuals of a particular parasite species in a sample of particular host species divided by the total number of hosts of that species examined.

3.4. Results and discussion

The following publication emanates from the present study and forms part of Chapter 3 results (see Appendix 3).

Raphahlelo ME, Přikrylová I, Matla MM. 2020. *Dactylogyrus* spp. (Monogenea, Dactylogyridae) from the gills of *Enteromius* spp. (Cypriniformes, Cyprinidae) from the Limpopo Province, South Africa with descriptions of three new species. *Acta Parasitologica* 65: 396–412.

Based on the morphometric analyses of the sclerotised structures of the haptor and the reproductive system, six species of *Dactylogyrus* were found on three *Enteromius* host populations within the Limpopo River System, South Africa. *Dactylogyrus afrohamiltonii* Raphahlelo, Přikrylová & Matla, 2020 (Figure 3.3 A) was found on *Enteromius afrohamiltoni* (Crass, 1960), can be differentiated from other known species based on the presence of stout anchors and pairs I and II hooks (heavily sclerotised and largest). *Dactylogyrus limpopoensis* Raphahlelo, Přikrylová & Matla, 2020 (Figure 3.3 B) was found on *Enteromius unitaeniatus* (Günther, 1866), differs in the male copulatory organ morphology (distally articulated accessory piece), pair II hook (markedly curved) and in the presence of a small vestigial ventral bar. *Dactylogyrus letabaensis* Raphahlelo, Přikrylová & Matla, 2020 (Figure 3.3 C) was retrieved from *E*. *unitaeniatus* and can be identified based on having a M-shaped copulatory tube, distally termination to U-shape and the presence of a long vestigial ventral bar. *Dactylogyrus afrolongicornis* Paperna, 1973 (Figure 3.4 A) was found on *Enteromius trimaculatus* (Peters, 1852) and can be distinguished by the presence/absence of a soft to weakly sclerotised or even non-sclerotised bar membrane and/or heavy and thick bar plates. *Dactylogyrus allolongionchus* Paperna, 1973 (Figure 3.4 B) was retrieved from *E*. *trimaculatus* and can be differentiated in having a rounded vagina armed with spindle-shaped

sclerites. *Dactylogyrus myersi* Price, McClellan, Druckenmiller & Jacobs, 1969 (Figure 3.4 C) was found on *E*. *trimaculatus*, is characterised by its huge, long anchors which are about one-third of the entire body length.

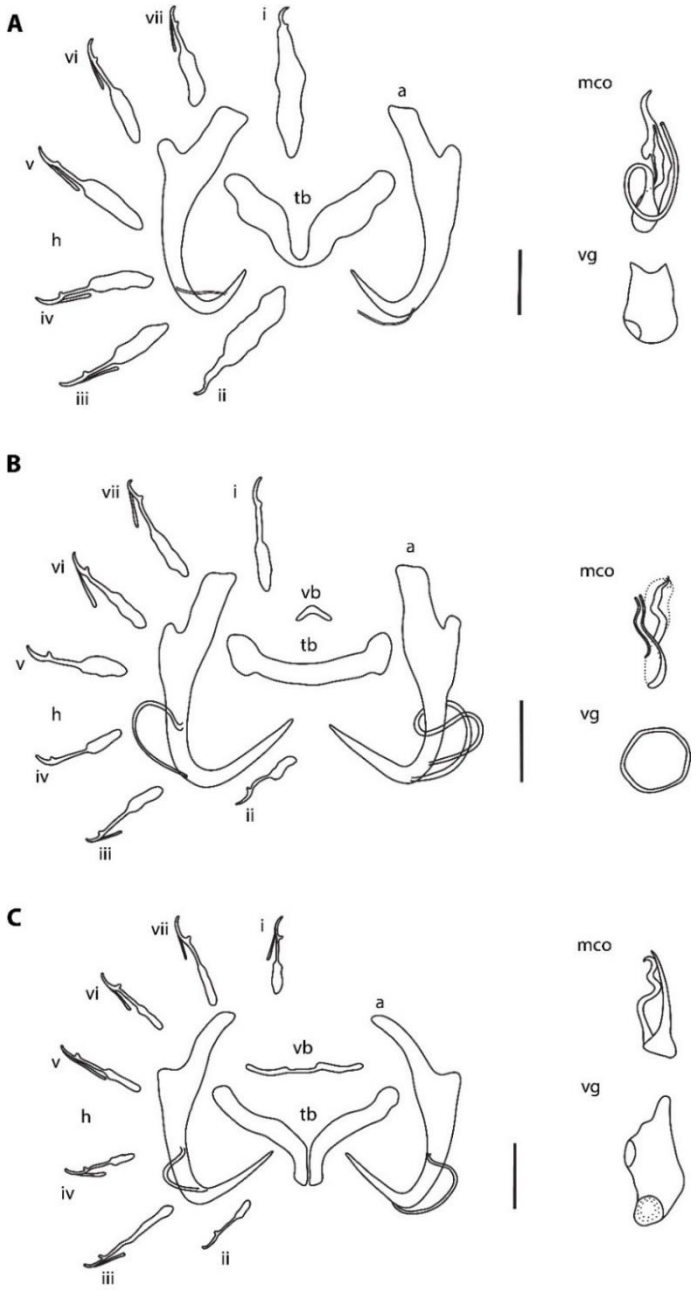

Figure 3.3: Diagrammatic drawings of A, *Dactylogyrus afrohamiltonii* Raphahlelo, Přikrylová & Matla, 2020. B, *Dactylogyrus limpopoensis* Raphahlelo, Přikrylová & Matla, 2020. C, *Dactylogyrus letabaensis* Raphahlelo, Přikrylová & Matla, 2020, a. anchor; vb. vestigial ventral bar; tb. transverse bar; h. hook (pairs i–vii); mco. male copulatory organ; vg. vagina. Scale bar=10 *μ*m. (Taken from Raphahlelo et al. 2020)

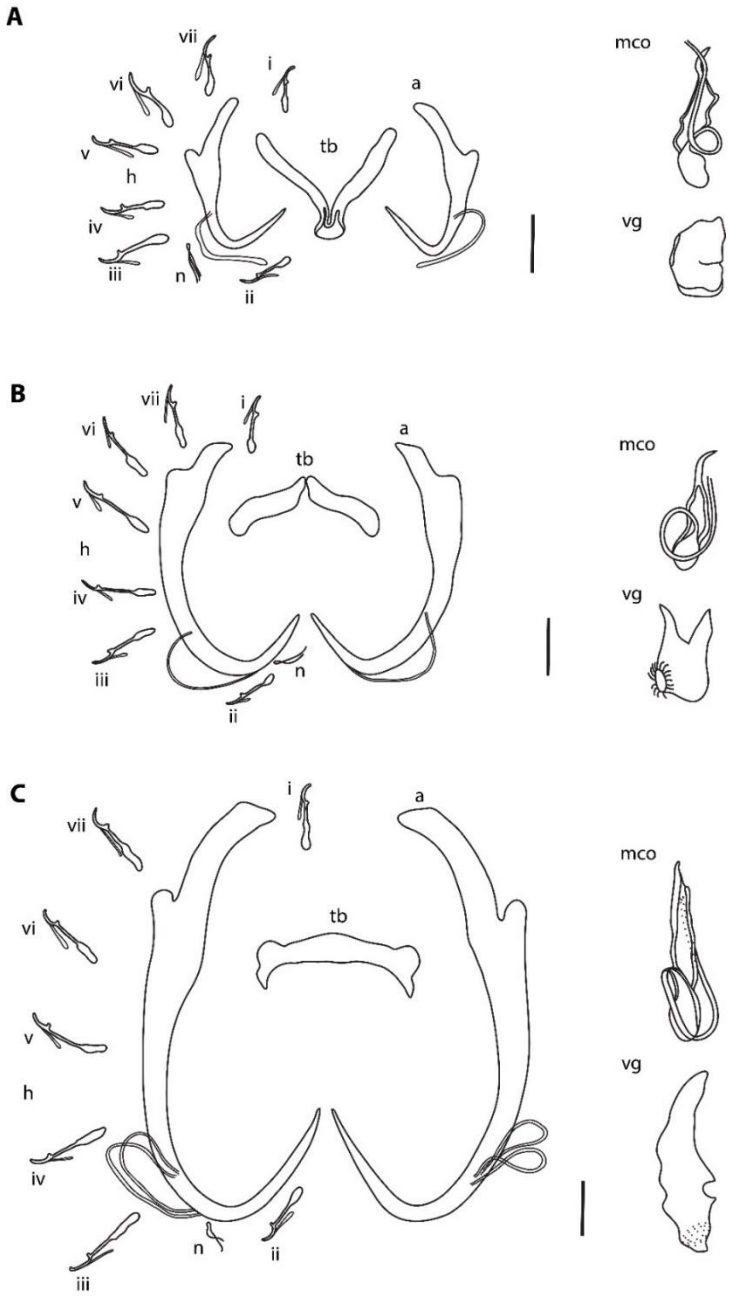

Figure 3.4: Diagrammatic drawings of A, *Dactylogyrus afrolongicornis* Paperna, 1973. B, *Dactylogyrus allolongionchus* Paperna, 1973. C, *Dactylogyrus myersi* Price, McClellan, Druckenmiller & Jacobs, 1969, a. anchor; tb. transverse bar; h. hook (pairs i–vii); n. needle; mco. male copulatory organ; vg. vagina. Scale bar=10 *µ*m. (Taken from Raphahlelo et al. 2020)

A detailed morphological description of *Dactylogyrus* species from the current study as well as their measurements are outlined in the above mentioned publication. The prevalence, mean intensity and mean abundance are given in Table 3.2. *Dactylogyrus afrohamiltonii* was found to be host-specific to *E*. *afrohamiltoni* and represents the first monogenean record on this host. *Dactylogyrus afrolongicornis*, *D*. *allolongionchus*, *D*. *letabaensis*, *D*. *limpopoensis* and *D*. *myersi* were found to infest either *E*. *trimaculatus* or *E*. *unitaeniatus*. *Dactylogyrus afrolongicornis* and *D*. *allolongionchus* were the most abundant parasites from *E*. *trimaculatus* in six of the eight localities, followed by *D*. *myersi* from the same host, abundant in five of the eight localities. Furthermore, *E*. *trimaculatus* hosted five *Dactylogyrus* species (*D*. *afrolongicornis*, *D*. *allolongionchus*, *D*. *letabaensis*, *D*. *limpopoensis* and *D*. *myersi*) and was a common host in six of the eight localities studied.

To date, 12 species of *Dactylogyrus* (excluding *D*. *afrolongicornis alberti*) have been reported to parasitise *Enteromius* hosts from South Africa (Table 3.2). The additional ten species were from other fish genera. Among the 12 species of *Dactylogyrus* known to parasitise *Enteromius* hosts from South Africa, five are strict specialists, namely, *D*. *afrohamiltonii* Raphahlelo, Přikrylová & Matla, 2020; *D*. *dominici* Mashego, 1983; *D*. *enidae* Mashego, 1983; *D*. *mattozii* Mashego & Matlou, 2018 and *D*. *teresae* Mashego, 1983. Six are intermediate specialists, namely, *D*. *afrolongicornis* Paperna, 1973; *D*. *afrosclerovaginus* Paperna, 1973; *D*. *allolongionchus* Paperna, 1973; *D*. *letabaensis* Raphahlelo, Přikrylová & Matla, 2020; *D*. *limpopoensis* Raphahlelo, Přikrylová & Matla, 2020 and *D*. *myersi* Price, McClellan, Druckenmiller & Jacobs, 1969. Lastly, only one is an intermediate generalist, namely, *D*. *spinicirrus* Paperna & Thurston, 1969 (Řehulková et al. 2018; Mashego and Matlou 2018; Raphahlelo et al. 2020). Many *Dactylogyrus* species can co-exist on the same host. Šimková et al. (2000) found nine species of *Dactylogyrus* from the gills of the roach *Rutilus rutilus* (Linnaeus, 1758). From the present study, *D*. *letabaensis* co-existed with *D*. *limpopoensis* from *E*. *unitaeniatus* from Letsitele Weir, while *D*. *afrolongicornis*, *D*. *allolongionchus* and *D*. *myersi* co-existed on *E*. *trimaculatus* from most of the localities studied.

Table 3.2: Infestation statistics of *Dactylogyrus* species infesting *Enteromius* hosts at the various locations of the Limpopo River System.

	D. afrohamiltonii P; MI; MA	*D. afrolongicornis* P; MI; MA	*D. allolongionchus* P; MI; MA	*D. letabaensis* P; MI; MA	*D. limpopoensis* P; MI; MA	*D. myersi* P; MI; MA
FBD	-	80.0; 1.1; 0.9	80.0; 0.3; 0.2	-	10.0; 1.0; 0.1	80.0; 0.9; 0.7
GLR	-	100; 2.4; 2.4	100; 0.4; 0.4	-	14.3; 1.0; 0.1	100; 0.6; 0.6
Hulukulu Pan	100; 6.7; 6.7	-	-	-	-	-
Letsitele Weir	-	75.0; 4.3; 3.3	75.0; 1.0; 0.8	100; 2.2; 2.2	100; 3.2; 3.2	75.0; 1.3; 1.0
Luphephe	-	86.7; 1.1; 0.9	86.7; 0.2; 0.1	100; 4.0; 4.0	-	-
MLD	-	76.9; 1.4; 1.1	76.9; 0.4; 0.3	69.2; 5.2; 3.6	-	76.9; 0.7; 0.5
Nondweni	-	57.1; 1.8; 1.0	57.1; 1.0; 0.6	66.7; 3.5; 2.3	-	57.1; 0.8; 0.4
Tzaneen	-	-	-	-	100; 5.0; 5.0	-

P (%) = prevalence; MI = mean intensity; MA = mean abundance

FBD = Flag Boshielo Dam; GLR = Groot Letaba River; MLD = Middle Letaba Dam

The morphological discrepancy of the subspecies *D*. *afrolongicornis alberti* were reported in Raphahlelo et al. (2020) and were considered to unambiguously resemble *D*. *afrolongicornis*. Our results support the morphological evidence that formally synonymises *D*. *afrolongicornis* and *D*. *afrolongicornis* a*lberti* to be a single species based on poor morphological differentiation characters such as the presence of heavy and thick bar plates of the transverse bar and the soft to weakly bar membrane (Raphahlelo et al. 2020).

Chapter 4: Molecular study of species of *Dactylogyrus* parasitising *Enteromius* hosts from the Limpopo River System

4.1 Introduction

Over the past 10–15 years, the employment of genetics in parasitological studies has been of a significant development. Prior to that, parasite identification used to be based almost exclusively on morphological characters. It is now routine to use DNA sequences to identify parasites and achieve a higher level of discrimination among morphologically similar species (Poulin and Keeney 2008). Consequently, the use of molecular data has increased our understanding of the diversity and phylogenetic relationships of parasites and, in particular, of the genus *Dactylogyrus* (Benovics et al. 2018, 2020a, b; Rahmouni et al. 2017). It has proven to be a powerful and rapid method in population implications and evolutionary studies (Šimková et al. 2004). The latter point is recognised to be significant as the majority of *Dactylogyrus* species seem to be genetically characterised in Europe and Asia. Thus molecular data of *Dactylogyrus* species have been more efficient for inferring species description and phylogenetic characterisation (Šimková et al. 2004) and has been proposed to be the most reliable source for strong species identification and phylogenetic inference to sufficiently discriminate between closely related species and populations.

The identification and classification of *Dactylogyrus* species based on the partial 18S rDNA and the entire ITS-1 and partial 5.8S and/or the partial 28S rDNA gene sequences are a common procedure in phylogenetics. These genes have been used in molecular identification of different monogenean genera and continue to be valuable genetic markers (Desdevises et al. 2000, 2002; Zięţara and Lumme 2002; Plaisance et al. 2005; Pouyaud et al. 2006; Wu et al. 2007; Mendlová et al. 2010, 2012; Vignon et al. 2011; Justine et al. 2013; Řehulková et al. 2013; Šimková et al. 2013b; Chiary et al. 2014; Madanire-Moyo and Avenant-Oldewage 2014; Sarabeev and Desdevises 2014; Messu Mandeng et al. 2015; Khang et al. 2016; Raphahlelo et al. 2016; Benovics et al. 2017, 2018, 2020a, b; Chaudhary et al. 2017; Rahmouni et al. 2017; Šimková et al. 2017; Verma et al. 2017).

These molecular markers have also been used in other parasitic groups infesting fish i.e. endohelminths (Koubková et al. 2008; de Chambrier et al. 2009, 2011; Mašová et

al. 2010; Schaeffner et al. 2011; Scholz et al. 2011; Chibwana et al. 2013) and protozoans (Lom and Nilsen 2003; Dyková et al. 2005; Bartošová-Sojková et al. 2015) supported by molecular techniques to infer phylogenetic relationships of closely related species, demonstrating the importance of these set of gene sequences.

Essentially, this has resulted in a greater interest to use these genes to study the phylogenetic relationship of monogenean parasites in southern Africa, with subsequent attention focused on *Enteromius* hosts and *Dactylogyrus* parasites for this purpose. In this study, we report on nucleotide sequences of partial 18S, the entire ITS-1 and partial 5.8S, and partial 28S rDNA sequences of *Dactylogyrus* species from *Enteromius* (Cyprinidae) hosts.

4.2 Materials and methods

4.2.1 Fixing of monogeneans for DNA extraction

The worms were bisected using needles, where after the anterior part containing the male copulatory organ was completely flattened under the cover slip and fixed by GAP for species identification. The posterior part containing the haptor was fixed in a 1.5 millilitres Eppendorf tube filled with 96 % molecular grade ethanol. The samples were stored in a refrigerator for further isolation.

4.2.2 DNA extraction

Prior to DNA extraction, the microtubes with individual monogeneans fixed in 96 % ethanol were left on a heat block at 65°C for four hours to evaporate residual ethanol from a microtube. Thereafter, total genomic DNA was extracted using a GeneJet Genomic DNA Purification kit (number 0722, for 250 preps, lot 00285037, Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer's recommended protocols (Figure 4.1 A).

Figure 4.1: A – DNA extraction, B – Measuring DNA concentration using the Thermo Scientific NanoDrop™ version 2000, C – PCR amplification and D – Preparation of gel loading dye. (Photographs by Mangena T)

4.2.3 Quantification of DNA concentration

Total DNA concentration was measured using a Thermo Scientific NanoDrop™ version 2000. Prior to DNA quantification, a blank was established onto the bottom pedestal using a 1 *µ*l Elusion Buffer (Figure 4.1 B). Two microlitres of the nucleic acid were quantified by measuring absorbance at 260 (A260) and 280 (A280) nm of each sample. The purity of the samples was confirmed by checking the optical density (OD) ratio of absorbance at 260:280 and 260:230, with ratios above 1.0 and nucleic acid concentrations highest were acceptable.

4.2.4 Polymerase chain reaction (PCR)

The partial 18S rDNA together with the entire internal transcribed spacer 1 (ITS-1) and partial 5.8S region of the rDNA of monogenean parasites were amplified by PCR (Figure 4.1 C), in one round using the forward primer S1 (5'–ATTCCGATAACGAACGAGACT–3') (Sinnappah et al. 2001) and the reverse primer IR8 (5'–GCTAGCTGCGTTCTTCATCGA–3') which anneal to the 18S and 5.8S rDNA regions, respectively (Šimková et al. 2003). The partial 28S rDNA was amplified using the forward primer C1 (5'–ACCCGCTGAATTTAAGCAT–3') and the reverse primer D2 (5'–TGGTCCGTGTTTCAAGAC–3') (Hassouna et al. 1984; Mendlová et al. 2012). Subsequently, PCR mixture concentrated to the final volume of 20 *µ*l containing, 1 *µ*l

of distilled water, the forward and reverse primers of 1.5 *µ*l, respectively, 10 *µ*l of a One *Taq* Master mix (with standard buffer), 6 *µ*l template DNA was achieved. PCR cycling protocol and parameters were those reported in Šimková et al. (2003, 2004) as follows: an initial denaturation step of (94°C for 2 minutes), followed by 39 cycles of amplification (94°C for 20 seconds, 56°C for 30 seconds and 72°C for 10 minutes) and followed by final 4 minutes extension hold at 72°C using a MiniOpticon real-time PCR system, applied on Bio-Rad CFX Manager software, version 3.1 (Figure 4.2).

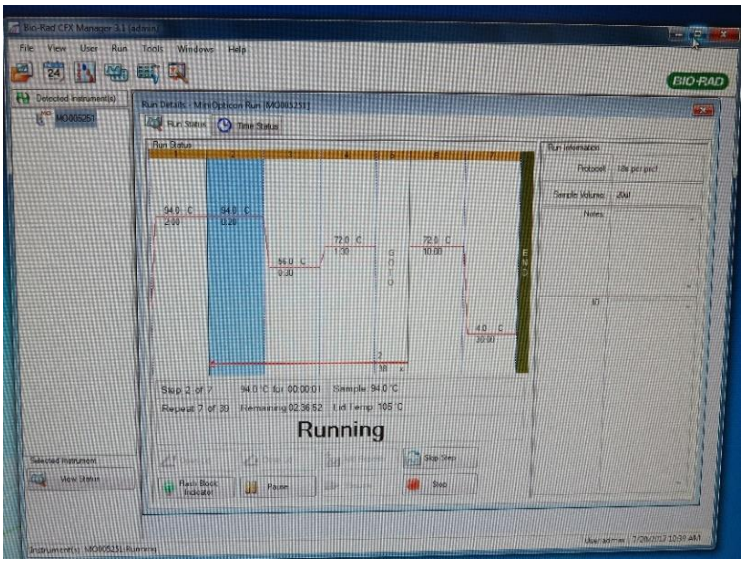

Figure 4.2: Digital image analysis of the PCR cycling protocols, applied on Bio-Rad CFX Manager software. (Photograph by Raphahlelo ME)

4.2.5 Electrophoresis

The 5 *µ*l of PCR products were visualised and photographed under an ultraviolet transilluminator (Herolab UVT-20 M) (Figure 4.3), examined on 1 % agarose-TAE gel, stained with ethidium bromide (Šimková et al. 2004), and run at 150 V for ± 10 minutes for PCR product visualisation (Figure 4.1 D). The obtained PCR products were purified using GeneJet Purification Kit (number 0702, for 250 preps, lot 00333157, Thermo Fisher Scientific, Massachusetts, USA) following the manufacturer's recommended protocols. All PCR products were sent to Inqaba Biotechnical Industries (Pty) Ltd laboratory in Pretoria for the final purification and sequencing.

Figure 4.3: An ultraviolet transilluminator (Herolab UVT-20 M) used to visualise PCR products. (Photograph by Raphahlelo ME)

4.2.6 BLAST analysis

The sequences of the partial 18S rDNA, the entire ITS-1 and partial 5.8S rDNA, and the partial 28S rDNA regions from *Dactylogyrus* specimens, extracted from *Enteromius* hosts within the Limpopo River System, were compared with those for the same genes of *Dactylogyrus* from cyprinoid hosts for any matches or closely related species in GenBank using BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) electronic software in June 2020. For the 18S, the BLAST hit from GenBank revealed the first 100 species with >96 % similarity. Subsequently, the BLAST hit for the 28S revealed the first 100 species and were considered based on >85 % similarity. The selected sequences were considered based on the species with high query coverage, maximum scores, and high percent identity. In total, 58 species of *Dactylogyrus* were downloaded as ingroup taxa, including outgroup taxa *Pseudodactylogyrus anguillae* (Yin & Sproston, 1948), a monogenean parasite of anguillid eel *Anguilla anguilla* (Linnaeus, 1758), for the 18S rDNA (AJ490162) and 28S rDNA (AJ969950), following Šimková et al. (2003, 2004) and Šimková et al. (2006a), respectively (Table 4.1).

Table 4.1: *Dactylogyrus* species used for sequencing the combined 18S rDNA, entire ITS-1 and partial 5.8S rDNA, and partial 28S rDNA with their cyprinoid hosts, sampling localities and GenBank accession numbers. Sequences not available are indicated by -.

			GenBank accession numbers	
Parasite species	Host	Country	18S rDNA + ITS-1–5.8S rDNA	28S rDNA
D. alatus	*Alburnus arborella*	Italy	-	MK434946
D. aspili	*Enteromius macrops*	Senegal	-	KY629359
D. auriculatus	*Abramis brama*	Czech Republic	MG792838	-
D. balkanicus	*Barbus plebejus*	Croatia	MG792861	-
D. bicorniculus	*Rhodeus atremius atremius*	Japan	-	LC093099
D. caballeroi	*Rutilus ohridanus*	Albania	MG792902	-
D. chondrostomi	*Chondrostoma nasus*	Czech Republic	AJ564116	-
D. chranilowi	*Abramis ballerus*	Czech Republic	AJ564117	-
D. claviformis	*Hemiculter leucisculus*	China	-	MK353162
D. crivellius	*Barbus peloponnesius*	Greece	KY629339	-
D. crucifer	*Rutilus lacustris*	Greece	MG792898	-
D. difformis	*Scardinius plotizza*	Bosnia and Herzegovina	MG792908	-
D. difformoides	*Scardinius plotizza*	Bosnia and Herzegovina	MG792909	-
D. dirigerus	*Chondrostoma nasus*	Greece	MG792873	-
D. distinguendus	*Blicca bjoerkna*	Czech Republic	AJ564125	-
D. dyki	*Barbus peloponnesius*	Greece	MG792858	-
D. ergensi	*Chondrostoma knerii*	Bosnia and Herzegovina	MG792870	-
D. erhardovae	*Rutilus ohridanus*	Albania	-	MK434952
D. falcatus	*Abramis brama*	Czech Republic	AJ564130	-
D. fallax	*Rutilus rutilus*	Czech Republic	MG792906	-
D. folkmanovae	*Squalius vardarensis*	Greece	MG792935	-
D. hemiamphibothrium	*Gymnocephalus cernuus*	Czech Republic	-	AJ969946
D. ivanovici	*Pachychilon pictum*	Greece	MG792883	-
D. izjumovae	*Scardinius dergle*	Croatia	MG792907	-
D. latituba	*Hemiculter leucisculus*	China	-	MK353163
D. martinovici	*Pachychilon pictum*	Greece	MG792885	-
D. marocanus	*Carasobarbus fritschii*	Morocco	-	KY629355

D. nanoides	*Squalius squalus*	Bosnia and Herzegovina	MG792929	-
D. nanus	*Sarmarutilus rubilio*	Italy	-	MK434953
D. parvus	*Alburnus alburnus*	Czech Republic	AJ564146	-
D. petenyi	*Barbus balcanicus*	Bulgaria	EF582621	-
D. petkovici	*Pachychilon pictum*	Greece	MG792887	-
D. primarius	-	China	-	KX812457
D. propinquus	*Abramis sapa*	Czech Republic	AJ564147	-
D. prostae	*Squalius cephalus*	Czech Republic	MG792914	-
D. pseudogobii	-	China	-	KX812458
D. ramulosus	*Aspius aspius*	Czech Republic	AJ564149	-
D. rarissimus	*Rutilus ohridanus*	Albania	MG792903	-
D. rarissimus	*Telestes fontinalis*	Croatia	-	MG792997
D. rutili	*Rutilus lacustris*	Greece	MG792900	-
D. rysavyi	*Alburnoides thessalicus*	Greece	MG792851	-
D. sekulovici	*Pachychilon pictum*	Greece	MG792889	-
D. soufii	*Telestes montenigrinus*	Albania	MG792946	-
Dactylogyrus sp. 1	*Luciobarbus albanicus*	Greece	KY201100	-
Dactylogyrus sp. 2	*Tropidophoxinellus spartiaticus*	Greece	MG792950	-
Dactylogyrus sp. 3	*Parachondrostoma turiense*	Spain	MN365687	-
Dactylogyrus sp. 4	*Squalius torgalensis*	Portugal	MN365696	-
Dactylogyrus sp. 5	*Enteromius niokoloensis*	Senegal	-	KY629358
Dactylogyrus sp. 6	*Hypophthalmichthys molitrix*	China	-	MN567975
D. squameus	-	China	-	KX812459
D. suecicus	*Rutilus lacustris*	Greece	MG792901	-
D. tissensis	*Alburnoides thessalicus*	Greece	MG792852	-
D. tuba	*Leuciscus idus*	Czech Republic	AJ564157	-
D. vastator	*Abbottina rivularis*	China	-	MH790263
D. vistulae	*Telestes montenegrinus*	Albania	-	MG793063
D. wunderi	*Abramis brama*	China	KJ605444	-
D. zandti	*Abramis brama*	Czech Republic	MG792839	MG792953
P. anguillae	*Anguilla anguilla*	Austria	AJ490162	-
P. anguillae	*Anguilla anguilla*	Slovakia	-	AJ969950

4.2.7 Sequence alignment and phylogenetic analysis

Consensus sequence alignments of the partial 18S, the entire ITS-1 and partial 5.8S, and the partial 28S rDNA regions were aligned using ClustalX Multiple Alignment version 2.1 (Thompson et al. 1997) using default settings. Sequences were manually edited virtually in BioEdit Sequence Alignment Editor version 7.2.5 (Hall 1999) to remove gaps/deletions. Prior to complete alignment, sequences were trimmed to match with the lengths of those obtained from GenBank. An evolutionary model was selected by Molecular Evolutionary Genetics Analysis (MEGA) version X (Kumar et al. 2018) using the Bayesian Information Criterion (BIC) model in accordance to Nei and Kumar (2000). According to the BIC, models with the lowest scores are considered to describe the substitution pattern the best. The general time reversible model (GTR) with gamma distribution rates (G/Γ) and some sites invariable (I) was selected as the appropriate nucleotide substitution model of evolution for ML tree in MEGAX.

Phylogenetic comparison applying Maximum Likelihood (ML) (Nei and Kumar 2000), Neighbour-Joining (NJ) (Saitou and Nei 1987) and Maximum Parsimony (MP) (Nei and Kumar 2000) statistics were implemented in MEGAX. Support for branches were assessed using the bootstrap resampling procedure with 1000 replicates (Felsenstein 1985). Positions with less than 95 % site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). The pairwise genetic distances between the selected *Dactylogyrus* species for the combined 18S rDNA and the entire ITS-1 and partial 5.8S, and the partial 28S rDNA were computed in MEGAX.

Bayesian Inference (BI), also using the GTR+Γ+I model, was implemented in MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003). Posterior probabilities were calculated over 6 000 000 generations, sampling the Metropolis coupling Markov Chain Monte Carlo (MCMC) every 100 generations. Four simultaneous MCMC steps were performed for each data partition with one cold and three incrementally heated chains. A minimum of two independent runs (replicates) were conducted to check the similarity of the likelihood plateau and verify the consistency of the results. One-fourth of the samples were discarded as “burn-in” (~25 %), while the remaining trees were used to construct a 50 % majority rule consensus tree based on the average standard deviation of the split frequency value (<0.01) to ensure convergence in tree search.

According to Ronquist and Huelsenbeck (2003), the average standard deviation of the split frequency value between 0.01 and 0.05 may be adequate for the purpose of well-supported parts of the tree. All trees were visualised in FigTree version 1.3.1 (Rambaut 2008).

Values for posterior probabilities (PP) and bootstrap (BS) were considered according to Wahlberg et al. (2003) and Yang et al. (2006) as follows: weak support 50–63 %/0.5–0.69, moderate support 64–75 %/0.7–0.84, good support 76–88 %/0.85–0.94, and strong support 89–100 %/0.95–1.00. Therefore, only the PP and BS values were displayed on the BI phylogram.

4.3 Results

DNA were successfully extracted from four specimens of *D*. *afrolongicornis* (Figure 4.4). Based on the BIC model, the K2+G+I for 18S rDNA and HKY+G for 28S were chosen as the optimal model of sequence evolution. These models were substituted by the GTR+G+I model as the closest to the optimal models since the selected models cannot be implemented in MrBayes 3.1.2 for Bayesian Inference (BI) analyses for each alignment, as outlined above. Since phylograms yielded similar trees with very similar branching patterns, only the BI and the ML values are shown on the BI phylogram. The remaining phylograms are presented separately.

Combined 18S rDNA and ITS-1–5.8S rDNA

Phylogenetic analyses were undertaken on the newly sequenced *D. afrolongicornis,* 40 species of *Dactylogyrus*, and one outgroup species of *P. anguillae* from GenBank. Sequences were selected based on the criteria as already mentioned above. The total length was determined to be 1149 bp long. From the BIC (Table I, Appendix 4), the best fit model of molecular evolution obtained from ModelTest based on the likelihood ratio test was the K2+G+I model, with a maximum likelihood value (-In*L* 7635.260), with invariable sites (I = 0.330) among site-rate heterogeneity approximated by gamma distribution (α = 0.455).

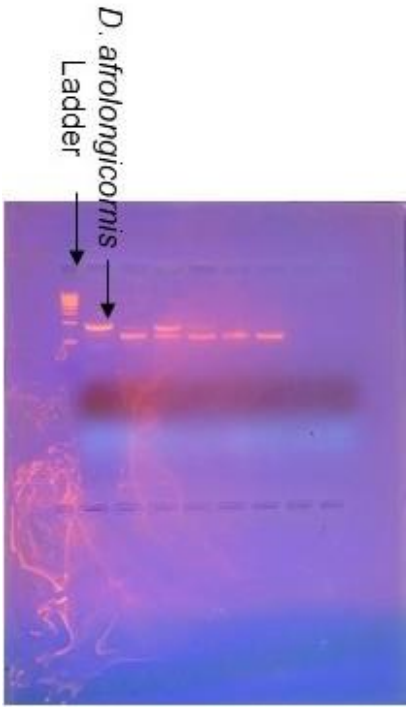

Figure 4.4: PCR products visualised under ultraviolet transilluminator analysed on 1 % agarose gel electrophoresis and marked with 1 kb DNA ladder loaded in the first well.

The highest log likelihood value (-In*L* 7599.41) resulting from ML analysis with a discrete gamma distribution (α = 0.377) was achieved. The optimal tree for NJ had a sum of branch length = 1.643 (Figure 4.5). The MP analysis provided the most parsimonious tree with tree length = 1367 steps, consistency index (CI) = 0.388 and retention index (RI) = 0.478 (Figure 4.6). The phylogenetic analyses placed the newly sequenced *D*. *afrolongicornis* at the basal position of the ingroup taxa. The estimated pairwise sequence divergence between *D*. *afrolongicornis* and the ingroup taxa ranged from 0–26.1 %. The closest *Dactylogyrus* species to *D*. *afrolongicornis* estimated from un-corrected pairwise genetic distance was *Dactylogyrus folkmanovae* Ergens, 1956 with 22.9 % while the most distant species from the former species was *Dactylogyrus ivanovici* Ergens, 1970 with 26.1 % (Table 4.2). The genetic variation between *D*. *afrolongicornis* and *P*. *anguillae* was found to be 31.3 %. For the BI analysis, the average standard deviation of the split frequencies stabilised at 0.017. Through mid-point rooting, the phylogram contained several groupings that correspond to host specificity (Figure 4.7).

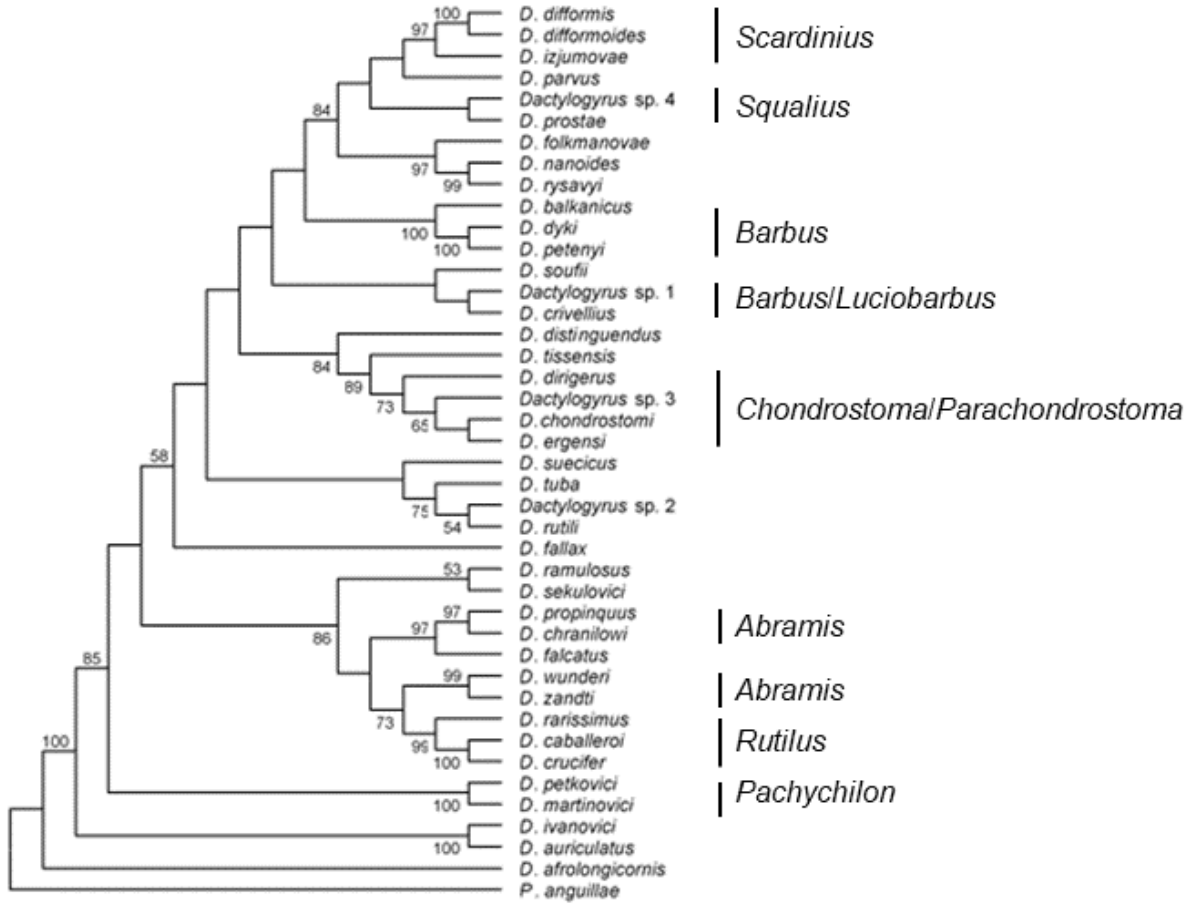

Figure 4.5: A phylogenetic tree of 42 nucleotide sequences, inferred from the combined 18S rDNA and ITS-1–5.8S rDNA constructed from Neighbour-Joining method. There were a total of 1149 positions in the final dataset. Numbers below the nodes indicate bootstrap (BS) values (1000 replicates).

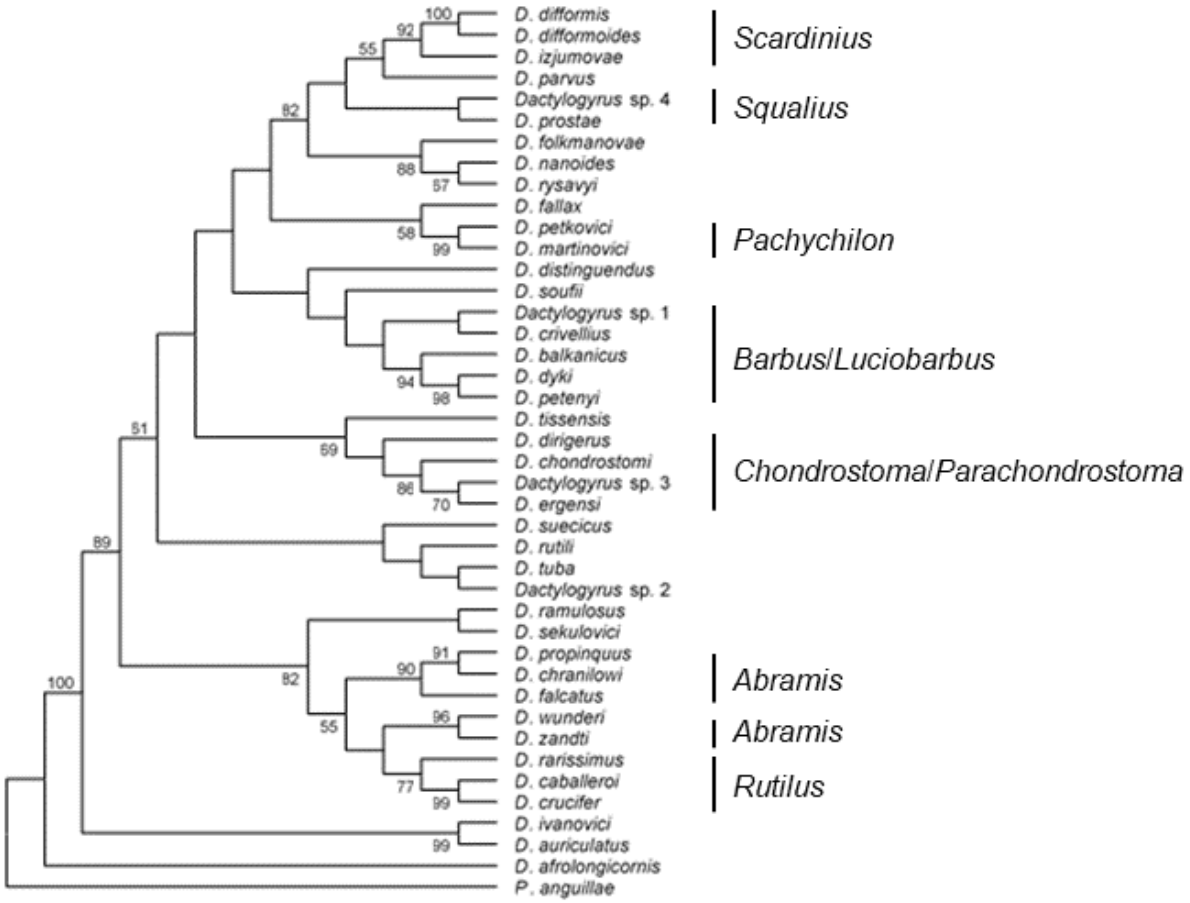

Figure 4.6: A phylogenetic tree of 42 nucleotide sequences, inferred from the combined 18S rDNA and ITS-1–5.8S rDNA constructed from Maximum Parsimony method. There were a total of 1149 positions in the final dataset. Numbers below the nodes indicate bootstrap (BS) values (1000 replicates).

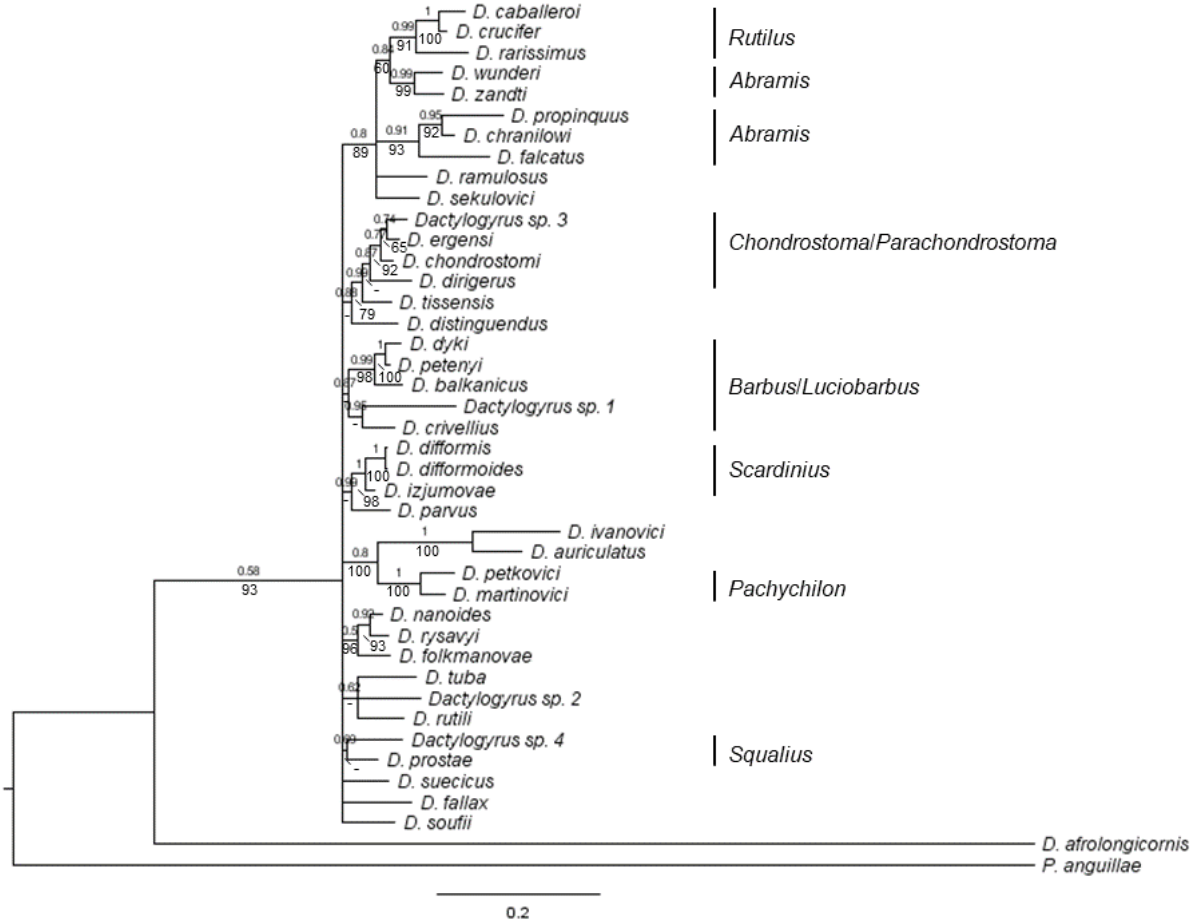

Figure 4.7: Phylogenetic tree of 41 *Dactylogyrus* species inferred from the combined 18S rDNA and ITS-1–5.8S rDNA, with *P. anguillae* as the outgroup. Numbers above the nodes indicate posterior probability (PP) values resulting from BI analysis. Numbers below the nodes indicate bootstrap (BS) values for ML analysis. No PP and BS values below 50 % are displayed, indicated by -.

Table 4.2: Gamma un-corrected pairwise genetic distances between 41 *Dactylogyrus* species, for the alignment of combined 18S rDNA and ITS-1–5.8S rDNA.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
1 *D. wunderi*																																									
2 *D. propinquus*	0,104																																									
3 *D. nanoides*	0,101	0,105																																								
4 *D. suecicus*	0,078	0,098	0,057																																							
5 *D. tuba*	0,107	0,120	0,087	0,067																																						
6 *Dactylogyrus* sp. 1	0,124	0,124	0,096	0,076	0,105																																					
7 *D. rarissimus*	0,086	0,110	0,103	0,091	0,111	0,135																																				
8 *D. caballeroi*	0,081	0,109	0,107	0,091	0,103	0,120	0,064																																			
9 *D. difformis*	0,093	0,110	0,059	0,062	0,079	0,088	0,098	0,099																																		
10 *D. difformoides*	0,093	0,110	0,059	0,062	0,079	0,088	0,098	0,099	0,000																																	
11 *Dactylogyrus* sp. 3	0,088	0,102	0,071	0,060	0,082	0,093	0,098	0,097	0,069	0,069																																
12 *D. distinguendus*	0,094	0,111	0,075	0,064	0,081	0,092	0,107	0,105	0,070	0,070	0,065																															
13 *D. tissensis*	0,095	0,111	0,072	0,055	0,084	0,095	0,105	0,099	0,064	0,064	0,048	0,061																														
14 *D. crucifer*	0,068	0,103	0,098	0,084	0,098	0,117	0,061	0,027	0,089	0,089	0,090	0,098	0,086																													
15 *D. chondrostomi*	0,085	0,103	0,074	0,046	0,072	0,088	0,102	0,100	0,076	0,076	0,026	0,056	0,041	0,093																												
16 *D. ivanovici*	0,132	0,156	0,139	0,115	0,142	0,149	0,134	0,128	0,132	0,132	0,121	0,139	0,129	0,129	0,125																											
17 *D. ramulosus*	0,075	0,100	0,084	0,070	0,093	0,113	0,089	0,088	0,087	0,087	0,077	0,089	0,086	0,076	0,078	0,129																										
18 *D. balkanicus*	0,101	0,107	0,073	0,073	0,093	0,093	0,105	0,102	0,069	0,069	0,075	0,077	0,081	0,098	0,083	0,142	0,090																									
19 *D. rysavyi*	0,111	0,115	0,023	0,068	0,093	0,097	0,115	0,116	0,066	0,066	0,072	0,075	0,076	0,110	0,075	0,147	0,092	0,081																								
20 *D. zandti*	0,044	0,096	0,087	0,075	0,097	0,120	0,084	0,077	0,083	0,083	0,089	0,095	0,091	0,067	0,094	0,131	0,076	0,100	0,101																							
21 *D. izjumovae*	0,099	0,107	0,050	0,066	0,081	0,090	0,102	0,101	0,026	0,026	0,067	0,070	0,064	0,095	0,076	0,131	0,088	0,064	0,056	0,084																					
22 *D. chranilowi*	0,078	0,053	0,092	0,070	0,102	0,109	0,088	0,086	0,097	0,097	0,080	0,095	0,088	0,077	0,078	0,139	0,080	0,097	0,102	0,074	0,099																				
23 *D. parvus*	0,100	0,107	0,059	0,069	0,082	0,102	0,094	0,097	0,052	0,052	0,072	0,069	0,075	0,095	0,076	0,125	0,088	0,082	0,061	0,093	0,043	0,097																			
24 *D. sekulovici*	0,073	0,101	0,089	0,072	0,097	0,108	0,088	0,092	0,080	0,080	0,069	0,085	0,079	0,079	0,075	0,129	0,067	0,086	0,097	0,073	0,084	0,074	0,087																		
25 *Dactylogyrus* sp. 4	0,116	0,127	0,077	0,079	0,100	0,101	0,123	0,127	0,073	0,073	0,084	0,094	0,082	0,115	0,090	0,156	0,109	0,074	0,074	0,113	0,066	0,105	0,068	0,101																	
26 *Dactylogyrus* sp. 2	0,112	0,127	0,091	0,076	0,082	0,120	0,108	0,104	0,086	0,086	0,095	0,092	0,089	0,103	0,093	0,150	0,108	0,099	0,098	0,104	0,087	0,112	0,087	0,105	0,113																
27 *D. ergensi*	0,092	0,102	0,076	0,053	0,083	0,090	0,107	0,101	0,079	0,079	0,027	0,057	0,045	0,092	0,025	0,129	0,076	0,080	0,075	0,094	0,077	0,085	0,081	0,080	0,094	0,099															
28 *D. dirigerus*	0,098	0,112	0,079	0,054	0,093	0,099	0,117	0,114	0,079	0,079	0,056	0,065	0,054	0,107	0,048	0,140	0,083	0,095	0,082	0,102	0,081	0,093	0,086	0,087	0,097	0,107	0,038														
29 *D. fallax*	0,112	0,121	0,088	0,069	0,092	0,107	0,117	0,111	0,091	0,091	0,083	0,091	0,089	0,107	0,080	0,138	0,093	0,088	0,093	0,110	0,087	0,105	0,091	0,101	0,103	0,097	0,082	0,097													
30 *D. dyki*	0,103	0,111	0,062	0,072	0,093	0,091	0,115	0,102	0,069	0,069	0,069	0,082	0,077	0,097	0,076	0,138	0,098	0,042	0,075	0,098	0,068	0,093	0,079	0,090	0,082	0,093	0,077	0,092	0,088												
31 *D. soufii*	0,107	0,121	0,081	0,065	0,093	0,090	0,122	0,108	0,083	0,083	0,076	0,079	0,067	0,105	0,072	0,140	0,093	0,077	0,083	0,106	0,078	0,097	0,086	0,090	0,086	0,095	0,075	0,084	0,091	0,076											
32 *D. folkmanovae*	0,114	0,119	0,046	0,070	0,098	0,100	0,113	0,119	0,072	0,072	0,080	0,087	0,080	0,109	0,085	0,138	0,108	0,081	0,053	0,099	0,061	0,105	0,063	0,102	0,081	0,105	0,083	0,093	0,100	0,078	0,084										
33 *D. petenyi*	0,097	0,108	0,062	0,065	0,090	0,089	0,110	0,099	0,067	0,067	0,066	0,081	0,073	0,094	0,071	0,131	0,093	0,033	0,072	0,093	0,064	0,089	0,075	0,086	0,074	0,090	0,075	0,088	0,081	0,017	0,071	0,073									
34 *D. petkovici*	0,114	0,130	0,092	0,082	0,111	0,114	0,114	0,112	0,091	0,091	0,098	0,091	0,099	0,115	0,099	0,136	0,112	0,098	0,094	0,106	0,090	0,115	0,090	0,107	0,103	0,102	0,103	0,105	0,098	0,102	0,107	0,101	0,092								
35 *D. falcatus*	0,103	0,090	0,105	0,095	0,118	0,141	0,100	0,094	0,109	0,109	0,109	0,110	0,103	0,093	0,102	0,149	0,098	0,123	0,118	0,094	0,107	0,069	0,101	0,094	0,131	0,123	0,113	0,111	0,122	0,117	0,116	0,117	0,114	0,121							
36 ***D. afrolongicornis***	**0,245**	**0,252**	**0,231**	**0,235**	**0,253**	**0,246**	**0,253**	**0,251**	**0,245**	**0,245**	**0,238**	**0,251**	**0,239**	**0,247**	**0,239**	**0,261**	**0,243**	**0,245**	**0,233**	**0,242**	**0,242**	**0,243**	**0,245**	**0,237**	**0,231**	**0,253**	**0,242**	**0,246**	**0,243**	**0,241**	**0,245**	**0,229**	**0,235**	**0,251**	**0,252**						
37 *D. martinovici*	0,105	0,133	0,094	0,085	0,111	0,118	0,118	0,112	0,096	0,096	0,093	0,095	0,095	0,112	0,097	0,132	0,106	0,094	0,098	0,101	0,094	0,109	0,099	0,105	0,111	0,109	0,099	0,109	0,093	0,098	0,102	0,104	0,089	0,044	0,120	0,249					
38 *D. crivellius*	0,104	0,113	0,069	0,058	0,088	0,081	0,112	0,108	0,071	0,071	0,070	0,076	0,069	0,099	0,073	0,131	0,088	0,068	0,072	0,103	0,069	0,096	0,079	0,091	0,085	0,097	0,070	0,073	0,082	0,064	0,069	0,078	0,061	0,095	0,118	0,245	0,093				
39 *D. auriculatus*	0,129	0,147	0,129	0,110	0,139	0,138	0,128	0,130	0,124	0,124	0,113	0,126	0,121	0,130	0,116	0,093	0,124	0,130	0,132	0,127	0,127	0,129	0,119	0,126	0,141	0,140	0,121	0,124	0,129	0,131	0,136	0,136	0,122	0,115	0,136	0,258	0,113	0,124			
40 *D. prostae*	0,095	0,109	0,057	0,066	0,086	0,094	0,107	0,107	0,053	0,053	0,074	0,075	0,072	0,101	0,074	0,137	0,095	0,076	0,058	0,089	0,042	0,094	0,058	0,094	0,064	0,092	0,078	0,084	0,090	0,069	0,084	0,062	0,064	0,091	0,111	0,241	0,092	0,073	0,123		
41 *D. rutili*	0,108	0,116	0,083	0,058	0,080	0,110	0,103	0,100	0,084	0,084	0,078	0,084	0,085	0,101	0,077	0,138	0,096	0,088	0,092	0,102	0,079	0,103	0,083	0,105	0,103	0,079	0,073	0,089	0,085	0,087	0,085	0,094	0,085	0,106	0,122	0,242	0,108	0,085	0,132	0,082	
42 *P. anguillae*	0,253	0,259	0,279	0,272	0,263	0,279	0,271	0,266	0,277	0,277	0,265	0,258	0,268	0,265	0,254	0,282	0,270	0,270	0,280	0,261	0,277	0,257	0,271	0,264	0,272	0,271	0,266	0,275	0,264	0,270	0,275	0,268	0,271	0,261	0,258	0,313	0,268	0,279	0,273	0,274	0,284

From the phylogram, the ingroup taxa had a weak BI support (PP = 0.58) with a strongly supported ML (BS = 93). Within the ingroup taxa, the first grouping had a moderate BI support (PP = 0.8) with a strong ML support (BS = 89), comprised mostly common parasites of *Rutilus* and *Abramis* spp. *Rutilus* and *Abramis* spp. formed sister groups and had a moderately supported BI (PP = 0.84) and a weakly supported ML (BS = 60). The *Rutilus* spp. group was strongly supported (PP = 0.99, BS = 91). This group was formed by *Dactylogyrus rarissimus* Gussev, 1966, which formed a sister group with *Dactylogyrus caballeroi* Prost, 1960 and *Dactylogyrus crucifer* Wagener, 1857. Both *D*. *caballeroi* and *D*. *crucifer* were placed as the sister species and were strongly supported (PP = 1, BS = 100). The *Abramis* spp. group were strongly supported (PP = 0.99, BS = 99) and was formed by *Dactylogyrus wunderi* Bychowsky, 1931 and *Dactylogyrus zandti* Bychowsky, 1933 which were placed as sister species. The second *Abramis* spp. group had a good BI support (PP = 0.91) and a strongly supported ML (BS = 93), comprised of *Dactylogyrus falcatus* (Wedl, 1857), which formed a sister group with *Dactylogyrus propinquus* Bychowsky, 1931 and *Dactylogyrus chranilowi* Bychowsky, 1931. Both *D*. *propinquus* and *D*. *falcatus* were placed as the sister species and were strongly supported (PP = 0.95, BS = 92).

The second grouping had a good BI support (PP = 0.88) with a weakly supported ML, with mostly *Dactylogyrus* species parasitising *Chondrostoma*/*Parachondrostoma* spp. The third grouping had a good BI support (PP = 0.87) with a weakly supported ML, comprised common parasites of *Barbus*/*Luciobarbus* spp. The fourth grouping was strongly supported by BI (PP = 0.99), however, had a weakly supported ML, comprised mostly common parasites of *Scardinius* spp. The fifth grouping was moderately supported by BI (PP = 0.8), however, had a strongly supported ML (BS = 100). This group comprised of *Dactylogyrus petkovici* Ergens, 1970 and *Dactylogyrus martinovici* Ergens, 1970 which formed a sister group with *D*. *ivanovici* and *Dactylogyrus auriculatus* (Nordmann, 1832). *Dactylogyrus ivanovici* and *D*. *auriculatus* formed sister species and were strongly supported (PP = 1, BS = 100). The two sister species, *D*. *petkovici* and *D*. *martinovici* from *Pachychilon pictum* (Heckel & Kner, 1858), were strongly supported (PP = 1, BS = 100). *Dactylogyrus folkmanovae* formed a sister group with *Dactylogyrus nanoides* Gussev, 1966 and *Dactylogyrus rysavyi* Ergens, 1970 and were weakly supported by BI (PP = 0.5), however, had a strongly supported

ML (BS = 96). The two sister species, *D*. *nanoides* and *D*. *rysavyi* had a good BI support (PP = 0.92) with a strongly supported ML (BS = 93).

28S rDNA

The phylogenetic relationship of *D*. *afrolongicornis* was assessed, including 18 ingroup *Dactylogyrus* species and one outgroup species of *P*. *anguillae* retrieved from GenBank (Table 4.1). The total length of the partial 28S rDNA gene was determined to be 930 bp long. Evaluation of the ModelTest revealed the best fit model of molecular evolution based on the BIC method (Table II, Appendix 4), the likelihood ratio (-In*L* 4655.541) using the HKY+G model of nucleotide substitution, with no invariable sites and among site heterogeneity by gamma distribution (α = 0.346). The highest log likelihood value (-In*L* 4632.97) resulting from ML analysis with a discrete gamma distribution (α = 0.521) was achieved. The optimal tree for NJ had a sum of branch length = 1.034 (Figure 4.8). The MP analysis provided the most parsimonious trees with tree length = 812 steps, consistency index (CI) = 0.546 and retention index (RI) = 0.660 (Figure 4.9). The phylogenetic analyses placed *D*. *afrolongicornis* within the African group. The genetic variation between *D*. *afrolongicornis* and *Dactylogyrus aspili* Birgi & Lambert, 1987 was found to be 8.6 %, while a genetic variation of 12.1 % and 20.6 % was found between *Dactylogyrus* sp. 5 and *Dactylogyrus marocanus* El Gharbi, Birgi & Lambert, 1994, respectively, indicating that they are different species within the African lineage estimated from sequence divergence (Table 4.3). The most distant *Dactylogyrus* species to *D*. *afrolongicornis* was found to be *Dactylogyrus* sp. 6 with 21.8 %. The genetic variation between *D*. *afrolongicornis* and *P*. *anguillae* was found to be 28.5 %. The estimated pairwise sequence divergence within the Asian lineage ranged from 0.9–23.5 %, while the European lineage ranged from 3.5–22.5 %.

The average standard deviation of split frequencies stabilised at 0.011 for the BI analysis. Through mid-point rooting, the estimated ML tree contained three clearly identified major clades based on the biogeographical regions, the Asian lineage, the European lineage, and the African lineage clustered (Figure 4.10).

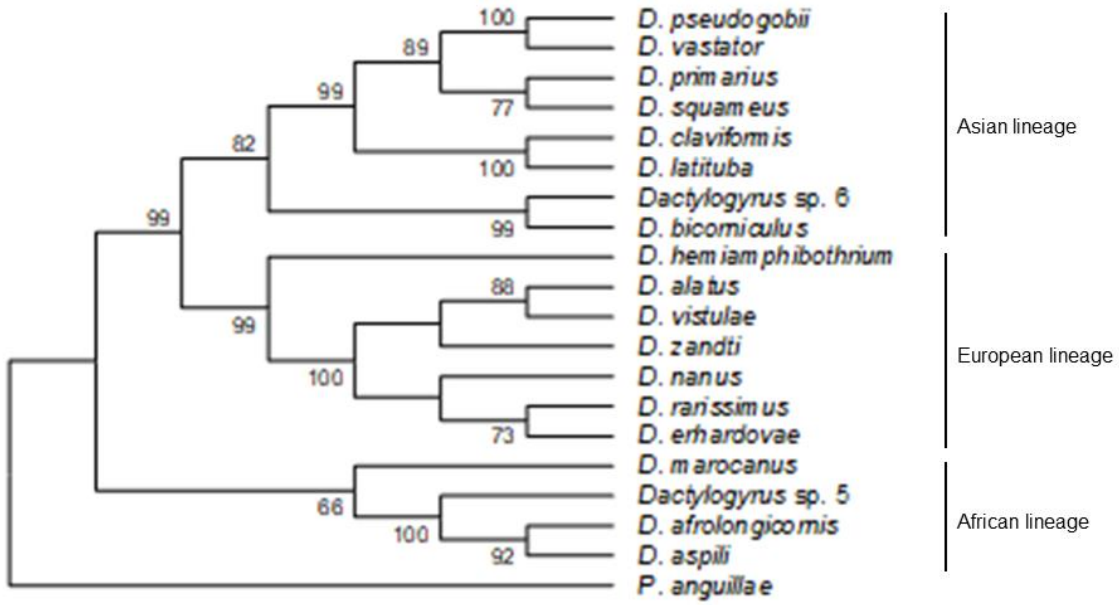

Figure 4.8: A phylogenetic tree of 20 nucleotide sequences, inferred from 28S rDNA constructed from Neighbour-Joining method. There were a total of 930 positions in the final dataset. Numbers below the nodes indicate bootstrap (BS) values (1000 replicates).

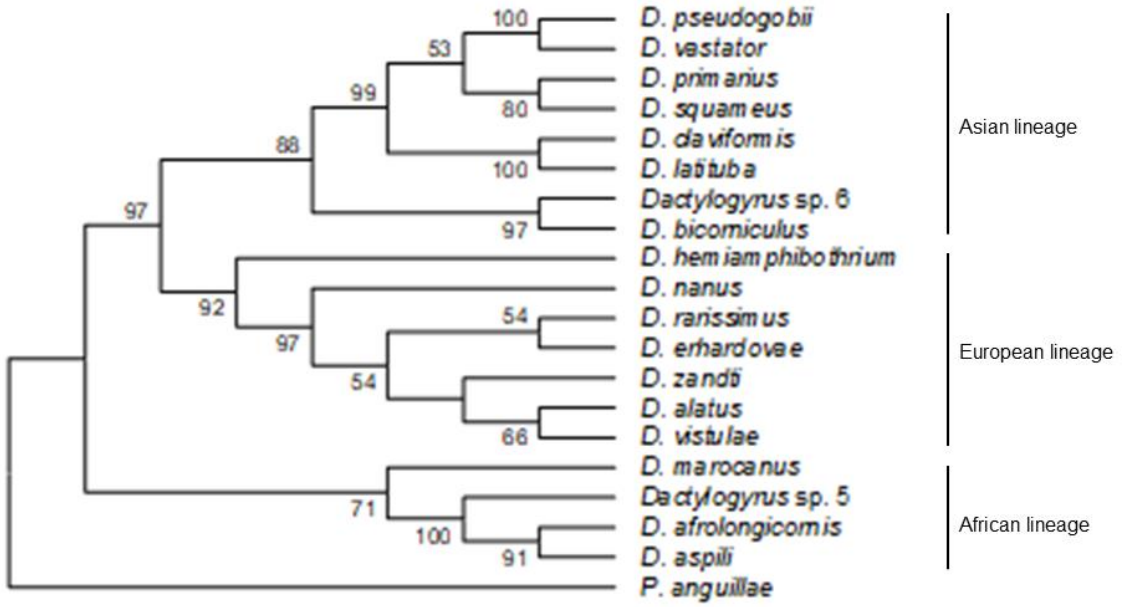

Figure 4.9: A phylogenetic tree of 20 nucleotide sequences, inferred from 28S rDNA constructed from Maximum Parsimony method. There were a total of 930 positions in the final dataset. Numbers below the nodes indicate bootstrap (BS) values (1000 replicates).

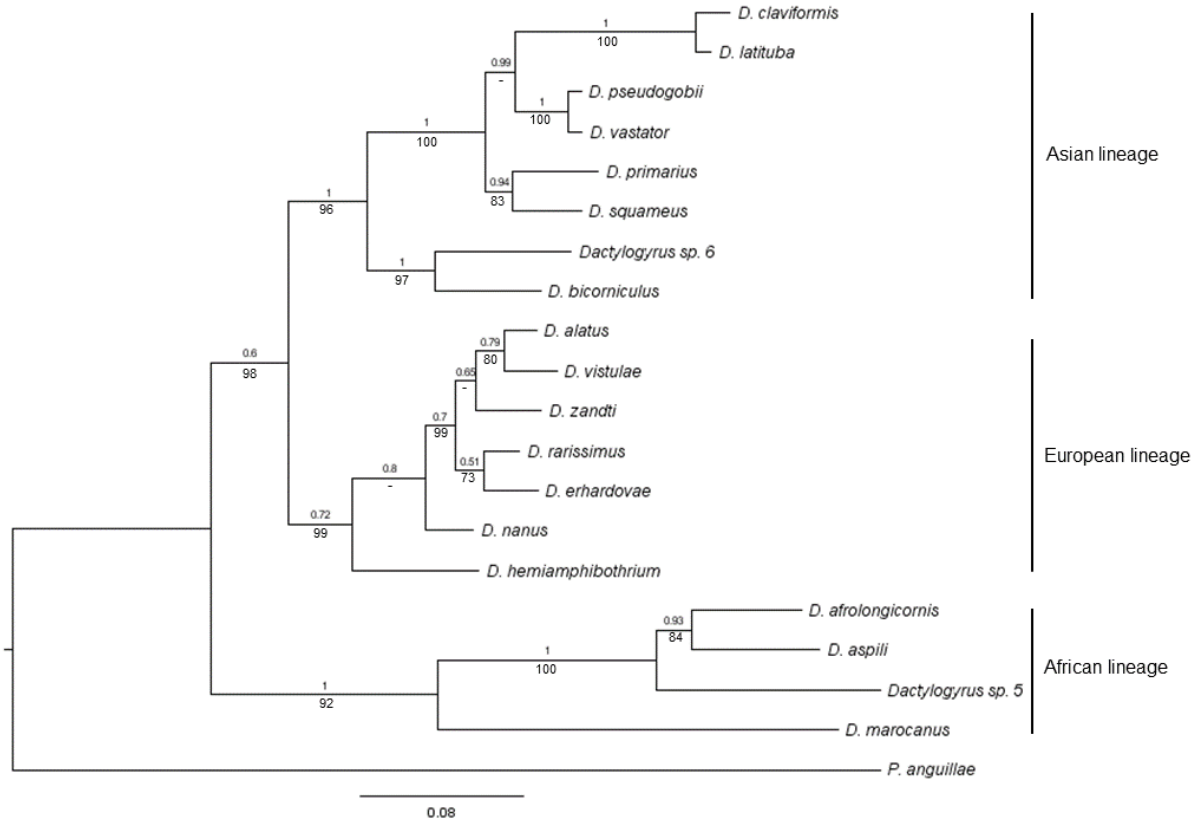

Figure 4.10: Phylogenetic tree of 19 *Dactylogyrus* species inferred from 28S rDNA, with *Pseudodactylogyrus anguillae* as the outgroup. Numbers above the nodes indicate posterior probability (PP) values resulting from BI analysis. Numbers below the nodes indicate bootstrap (BS) values for ML analysis. No PP and BS values below 50 % are displayed, indicated by -.

Table 4.3: Gamma un-corrected pairwise genetic distances between 19 *Dactylogyrus* species, for the alignment of partial 28S.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1 *D. afrolongicornis*																			
2 *Dactylogyrus* sp. 5	0,121																		
3 *D. claviformis*	0,212	0,225																	
4 *D. zandti*	0,200	0,218	0,147																
5 *D. rarissimus*	0,202	0,225	0,151	0,053															
6 *Dactylogyrus* sp. 6	0,218	0,235	0,147	0,125	0,122														
7 *D. aspili*	0,086	0,124	0,205	0,187	0,188	0,210													
8 *D. pseudogobii*	0,209	0,208	0,096	0,145	0,143	0,129	0,211												
9 *D. vastator*	0,213	0,208	0,091	0,142	0,141	0,117	0,213	0,009											
10 *D. nanus*	0,195	0,201	0,134	0,048	0,036	0,119	0,176	0,130	0,126										
11 *D. alatus*	0,189	0,207	0,131	0,040	0,044	0,117	0,177	0,130	0,126	0,051									
12 *D. marocanus*	0,206	0,219	0,216	0,209	0,223	0,199	0,205	0,211	0,208	0,215	0,211								
13 *D. latituba*	0,201	0,215	0,019	0,143	0,152	0,145	0,197	0,094	0,089	0,132	0,134	0,218							
14 *D. erhardovae*	0,190	0,208	0,144	0,050	0,035	0,132	0,186	0,144	0,140	0,056	0,052	0,223	0,144						
15 *D. vistulae*	0,204	0,219	0,157	0,059	0,053	0,129	0,188	0,148	0,146	0,054	0,035	0,220	0,153	0,058					
16 *D. hemiamphibothrium*	0,197	0,200	0,130	0,092	0,087	0,129	0,178	0,128	0,122	0,085	0,083	0,208	0,129	0,091	0,098				
17 *D. primarius*	0,205	0,207	0,102	0,145	0,136	0,126	0,201	0,072	0,072	0,132	0,128	0,213	0,098	0,134	0,141	0,128			
18 *D. bicorniculus*	0,213	0,225	0,135	0,139	0,128	0,084	0,210	0,119	0,114	0,123	0,121	0,204	0,132	0,138	0,133	0,122	0,116		
19 *D. squameus*	0,217	0,221	0,097	0,136	0,137	0,119	0,212	0,069	0,071	0,123	0,123	0,217	0,095	0,137	0,142	0,133	0,061	0,106	
20 *P. anguillae*	0,285	0,279	0,275	0,250	0,259	0,255	0,254	0,273	0,268	0,253	0,247	0,275	0,261	0,268	0,246	0,247	0,280	0,270	0,264

The African group formed the basal lineage and comprised the newly sequenced *D*. *afrolongicornis* which was found to be the closest related with *D*. *aspili* and were moderately supported (PP = 0.93, BS = 84). *Dactylogyrus* sp. 5 formed a sister species with *D*. *afrolongicornis* and *D*. *aspili* cluster and were strongly supported (PP = 1, BS = 100). All three species were retrieved from *Enteromius* hosts. *Dactylogyrus marocanus* from *Carasobarbus fritschii* (Günther, 1874) was placed at the basal position within the lineage and was strongly supported (PP = 1, BS = 92).

The Asian group was strongly supported (PP = 1, BS = 96) and formed four separate lineages, the most basal formed by *Dactylogyrus bicorniculus* Nitta & Nagasawa, 2016 and *Dactylogyrus* sp. 6 and were strongly supported (PP = 1, BS = 97). *Dactylogyrus claviformis* Mizelle & Klucka, 1953, *Dactylogyrus latituba* Gussev, 1955, *Dactylogyrus pseudogobii* Akhmerov, 1952 and *Dactylogyrus vastator* Nybelin, 1924 formed sister groups and had a strong BI support (PP = 0.99), however, with poorly supported ML. Both *D*. *claviformis*/*D*. *latituba* (parasites of *Hemiculter leucisculus* (Basilewsky, 1855)) and *D*. *pseudogobii*/*D*. *vastator* group were strongly supported (PP = 1, BS = 100) and (PP = 1, BS = 100), respectively. Furthermore, *Dactylogyrus primarius* Gussev, 1955 and *Dactylogyrus squameus* Gussev, 1955 (both from unknown hosts) formed sister groups with *D*. *claviformis*, *D*. *latituba*, *D*. *pseudogobii*, and *D*. *vastator* and were strongly supported (PP = 1, BS = 100). Both *D*. *primarius* and *D*. *squameus* formed sister species and had a good BI and ML support (PP = 0.94, BS = 83).

The European group had the most basal species *Dactylogyrus hemiamphibothrium* Ergens, 1956 and was moderately supported by BI (PP = 0.72) but strongly supported by ML (BS = 99). Within this lineage, *D*. *zandti* formed a sister group with *Dactylogyrus alatus* Linstow, 1878 and *Dactylogyrus vistulae* Prost, 1957 and were weakly supported by both BI (PP = 0.65) and ML. Both *D*. *alatus* and *D*. *vistulae* formed sister species and were moderately supported by BI (PP = 0.79) with good ML support (BS = 80). Both *D*. *rarissimus* and *Dactylogyrus erhardovae* Ergens, 1970 formed a sister group with *D*. *alatus*, *D*. *vistulae*, and *D*. *zandti* and were moderately supported by BI (PP = 0.7), however, had a strong ML support (BS = 99). Furthermore, *D*. *rarissimus* and *D*. *erhardovae* formed sister species and were weakly supported by BI (PP = 0.84) with moderate ML support (BS = 73). Lastly, *Dactylogyrus nanus* Dogiel & Bychowsky, 1934 was moderately supported by BI (PP = 0.8) but had a poorly resolved ML,

forming a sister group with *D*. *alatus*, *D*. *vistulae*, *D*. *zandti*, *D*. r*arissimus*, and *D*. *erhardovae*.

4.4 Discussion

The genus *Dactylogyrus* is one of the largest monogenean genera in the world, with more than 900 nominal species (Gibson et al. 1996). Despite the high diversity displayed by this genus, molecular phylogenetic analyses of African dactylogyrids remain poorly understood. Furthermore, the lack of phylogenetic studies on African cyprinids has resulted in a limited understanding of *Dactylogyrus* relationships in Africa. Data on molecular characterisation of *Dactylogyrus* species in Africa have only been reported recently (Rahmouni et al. 2017; Šimková et al. 2017) with an intention to understand host-parasite interaction with their biogeographical environments.

Rahmouni et al. (2017) published the insight of *Dactylogyrus* species parasitising three *Luciobarbus* Heckel, 1843 species collected from various basins in northern Morocco and ended up describing four new *Dactylogyrus* species (i.e. *Dactylogyrus scorpius* Rahmouni, Řehulková & Šimková, 2017, *Dactylogyrus benhoussai* Rahmouni, Řehulková & Šimková, 2017, *Dactylogyrus varius* Rahmouni, Řehulková & Šimková, 2017 and *Dactylogyrus falsiphallus* Rahmouni, Řehulková & Šimková, 2017. Rahmouni et al. (2017) highlighted further divergence of their Moroccan *Dactylogyrus* species as very distinct from those recorded from Europe characterised by molecular data. Furthermore, Šimková et al. (2017) demonstrated a northern route dispersion of *Dactylogyrus* species parasitising Northwest African *Luciobarbus* hosts instead of the southern route. Thus, *Dactylogyrus* species provide a model system to study how species appear to cross geographical boundaries (Šimková et al. 2006a). Šimková et al. (2006b) noted that *Dactylogyrus* species with similar morphological attachment of the haptor cluster together as a result of adaptions of the sclerotised hard parts throughout its evolution.

Combined 18S rDNA and ITS-1–5.8S rDNA

From our results, the phylogenetic analyses inferred from combined 18S rDNA and ITS-1–5.8S rDNA revealed several groupings that correspond to host specificity (mostly inferred from European cyprinoids). The first groupings included the species *D*. *caballeroi*, *D*. *crucifer*, and *D*. *rarissimus* (common parasites of *Rutilus* spp.) and

D. *wunderi*, *D*. *zandti*, *D*. *falcatus*, *D*. *propinquus*, and *D*. *chranilowi* (common parasites of *Abramis* spp.). The groupings of *D*. *caballeroi* and *D*. *crucifer* support the association between *Rutilus* hosts (Benovics et al. 2020a). Furthermore, the sister species between *D*. *caballeroi*, *D*. *crucifer*, and *D*. *rarissimus* from the present study were congruent with the findings of Šimková et al. (2004) infesting *Rutilus* hosts. *Dactylogyrus falcatus*, *D*. *wunderi,* and *D*. *zandti* are common parasites of *Abramis brama* (Linnaeus, 1758), although the latter two species were collected from different biogeographical regions. These species share similar haptoral hard parts but differ in their MCO and vaginal armament.

The second grouping comprised *D*. *distinguendus* from *Blicca bjoerkna* (Linnaeus, 1758), *D*. *tissensis* from *Alburnoides thessalicus* Stephanidis, 1950, *D*. *dirigerus* and *D*. *chondrostomi* both from *Chondrostoma nasus* (Linnaeus, 1758), *D*. *ergensi* from *Chondrostoma knerii* Heckel, 1843, and *Dactylogyrus* sp. 3 from *Parachondrostoma turiense* (Elvira, 1987). This group was not resolved and is congruent with Benovics et al. (2020a). This is consistent with Šimková et al. (2006b) who noted similar adaptions of the sclerotised hard parts of the haptor from *Dactylogyrus* species infesting phylogenetically related host species. These species have similar ‘massive’ anchors. The third grouping comprised *D*. *dyki*, *D*. *petenyi*, *D*. *balkanicus*, and *D*. *crivellius* (common parasites of *Barbus* spp.) and *Dactylogyrus* sp. 1 from *Luciobarbus albanicus* (Steindachner, 1870).

The fourth grouping comprised *D*. *parvus* from *Alburnus alburnus* (Linnaeus, 1758), *D*. *izjumovae* from *Scardinius dergle* Heckel & Kner, 1858, *D*. *difformis*, and *D*. *difformoides* (from *Scardinius plotizza* Heckel & Kner, 1858). *Dactylogyrus izjumovae* from *S*. *dergle*, *D*. *difformis* and *D*. *difformoides* from *S*. *plotizza* were strongly supported. This is congruent with Benovics et al. (2018, 2020a). From the study of Šimková et al. (2004), our analyses revealed congruence between sister species *D*. *difformis* and *D*. *difformoides* with the exception of *D*. *izjumovae*. The fifth grouping comprised *D*. *petkovici* and *D*. *martinovici*, common parasites of *P*. *pictum*. *Dactylogyrus petkovici* and *D*. *martinovici* share similar types of thin anchor hooks and a ventral bar with five extremities but differ in the shapes of their copulatory organ (Benovics et al. 2018). Our analyses are congruent with Benovics et al. (2018).

Dactylogyrus ivanovici was found on *A. brama* while *D*. *auriculatus* was found from *P. pictum*.

While *D*. *folkmanovae* is known to parasitise *Squalius* spp. (Benovics et al. 2018), it can be found on other cyprinoids (Jarkovský et al. 2004). This species appeared to cluster with *D*. *nanoides* from *Squalius squalus* (Bonaparte, 1837) and *D*. *rysavyi* from *A. thessalicus*. *Dactylogyrus tuba* from *Leuciscus idus* (Linnaeus, 1758), *Dactylogyrus* sp. 2 from *Tropidophoxinellus spartiaticus* (Schmidt-Ries, 1943) and *D*. *rutili* from *Rutilus lacustris* (Pallas, 1814) appeared to show polytomy. According to Benovics et al. (2018, 2020a), *D*. *rutili*, *D*. *nanus*, and *D*. *suecicus* are common parasites of *Rutilus* spp. *Dactylogyrus prostae* from *Squalius cephalus* (Linnaeus, 1758) and *Dactylogyrus* sp. 4 from *Squalius torgalensis* (Coelho, Bogutskaya, Rodrigues & Collares-Pereira, 1998) forming sister species. The phylogenetic analyses of the newly sequenced *D*. *afrolongicornis* formed a sister group with the ingroup taxa and appeared as the basal species through a midpoint rooting technique indicating the only species inferred from Africa from *E. trimaculatus*. This is in accordance with the 28S rDNA gene (from the present study), which placed *D*. *afrolongicornis* with the basally positioned African group (retrieved from *Enteromius* hosts). Currently, there are no sequences for species of *Dactylogyrus* from *Enteromius* hosts for the 18S rDNA in the nucleotide database from GenBank.

Specimens of the genus *Enteromius* Cope, 1867 are small diploid smiliogastrins exclusively distributed from the African continent. Previously, these small barbs were placed in the artificial *Barbus sensu lato* assemblage. Yang et al. (2015) revised the classification of ‘barbs’ using five mitochondrial genes. They elevated the genus *Enteromius* to accommodate all African diploid ‘*Barbus*’, as *Enteromius,* arguing that it’s the oldest available genus-group name of these fishes. However, there was a proposal not to consider these changes as valid (Schmidt and Bart 2015), but the proposed changes have been acknowledged by the African fish specialist Paul Skelton in 2016.

28S rDNA

Phylogenetic analysis of the partial 28S rDNA revealed three well supported clades linked to their biogeographical regions. These species clustered together with respect

to their similarity in morphological hard parts (Šimková et al. (2006b). The strongly supported Asian *Dactylogyrus* lineage comprised well supported sister species *D*. *claviformis* and *D*. *latituba* parasites of *H*. *leucisculus*, *D*. *pseudogobii* (from an unnamed host), *D*. *vastator* from *Abbottina rivularis*, *D*. *primarius*, and *D*. *squameus* (both from unnamed hosts), the basally placed *D*. *bicorniculus* from *Rhodeus atremius atremius* (Jordan & Thompson, 1914), *Dactylogyrus* sp. 6 from *Hypophthalmichthys molitrix* (Valenciennes, 1844). Most species were recovered from China, except *D*. *bicorniculus* which was recovered from Japan (Nitta and Nagasawa 2016).

The European lineage comprised *D*. *alatus* from *Alburnus arborella* (Bonaparte, 1841), *D*. *vistulae* from *Telestes montenegrinus* (Vukovic, 1963), *D*. *zandti* from *A. brama*, *D*. *erhardovae* from *Rutilus ohridanus* (Karaman, 1924), *D*. *rarissimus* from *Telestes fontinalis* (Karaman, 1972), *D*. *nanus* from *Sarmarutilus rubilio* (Bonaparte, 1837) and *D*. *hemiamphibothrium* from *Gymnocephalus cernuus* (Linnaeus, 1758). These species were retrieved from different host specimens from different biogeographical regions. They may parasitise other cyprinoids, however, the occurrence of *D*. *nanus* *D*. *vistulae* and *D*. *zandti* on other fish genera may be a result of accidental occurrence or an error in identification. Lastly, the strongly supported African lineage comprised the newly sequenced *D*. *afrolongicornis* from *E. trimaculatus* together with *D*. *aspili* from *Enteromius macrops* (Boulenger, 1911), *Dactylogyrus* sp. 5 from *Enteromius niokoloensis* (Daget, 1959), and *D*. *marocanus* from *C*. *fritschii*.

Šimková et al. (2017) noted that West African cyprinids and their co-evolving *Dactylogyrus* species originated from Asia which is in accordance with the origins of African cyprinid fauna. Furthermore, the *Dactylogyrus* species lineage parasitising *Enteromius* species in their study was not supported. Our results provide clear support for this clade comprising species of *Dactylogyrus* specific to *Enteromius* hosts. *Dactylogyrus aspili* was clearly placed as the sister species of *D*. *afrolongicornis* and was strongly supported.

There is no denying the importance of morphological characteristics of the attachment apparatus and reproductive system in discriminating between *Dactylogyrus* species (Šimková et al. 2004), however, supplementary molecular data are essential to clearly

clarify and verify the separation or relationship of the different species lineages/or divergence among different geographical populations.

Chapter 5: Summary

5.1 Morphological characterisation

Members of the genus *Dactylogyrus* are fish ectoparasites primarily infesting the gills of cyprinoids. *Dactylogyrus* species are the most diverse group among monogeneans and consist of more than 900 nominal species (Gibson et al. 1996). In this study, a total of six *Dactylogyrus* species were found from three *Enteromius* host populations within the Limpopo River System, South Africa. Three species, *D*. *afrohamiltonii*, *D*. *letabaensis*, and *D*. *limpopoensis* were described as new to science while three were known, *D*. *afrolongicornis*, *D*. *allolongionchus*, and *D*. *myersi*. *Dactylogyrus afrohamiltonii* represents the first monogenean record from *E*. *afrohamiltoni*. In addition, *D*. *letabaensis* and *D*. *limpopoensis* represent the first monogenean records from *E*. *unitaeniatus*. Our data revealed that *D*. *afrolongicornis* and *D*. *allolongionchus* were the most abundant parasite species in the six localities from the eight studied, followed by *D*. *myersi*, abundant in five of the eight localities. *Enteromius trimaculatus* was a common host in six of the eight localities studied and harboured five *Dactylogyrus* species (*D*. *afrolongicornis*, *D*. *allolongionchus*, *D*. *letabaensis*, *D*. *limpopoensis*, and *D*. *myersi*). The present study synonymised *D*. *afrolongicornis alberti* with *D*. *afrolongicornis* as reported by Raphahlelo et al. (2020) who argued that *D*. *afrolongicornis alberti* is, in fact, *D. afrolongicornis*.

Currently, only 12 *Dactylogyrus* species are known from seven *Enteromius* hosts in South Africa. It is clear that the species richness of *Dactylogyrus* on African *Enteromius* hosts may be higher than originally anticipated (Truter et al. 2016), considering that only 21 out of 213 valid species of *Enteromius* have been found to harbour *Dactylogyrus* spp. (Raphahlelo et al. 2020). Recently, Mashego and Matlou (2018) described a new *Dactylogyrus* species from *E*. *mattozii* from Piet Gouws Dam, representing the first monogenean record from this host. These studies mentioned above opened opportunities to investigate and explore *Enteromius* hosts in Africa for possible *Dactylogyrus* species in the near future. The identification of three new species from the present study indicates the existing potential that the species richness of these parasites on African cyprinoid fish from other genera may also be higher than is currently known, which is corroborated by the studies of Crafford et al. (2012), who described three species of *Dactylogyrus* from *L*. *capensis* and *L*. *umbratus*

from the Vaal Dam. Musilová et al. (2009) described three species of *Dactylogyrus* from *L*. *coubie* from West Africa, Senegal. Finally, Rahmouni et al. (2017) described four species of *Dactylogyrus* from *L. rifensis*, *L*. *moulouyensis* and *L*. *maghrebensis* collected from various basins in northern Morocco.

5.2 Molecular characterisation

There have been numerous studies on the phylogenetic relationships of *Dactylogyrus* spp. from Asia and Europe, however, very little is known about their phylogeny in Africa. The studies of the phylogenies are important to understand the patterns of evolutionary relationships amongst species. Šimková et al. (2006b) proposed that *Dactylogyrus* species with similar morphological attachment of the haptor cluster together as a result of adaptions of the sclerotised hard parts throughout its evolution. From the present study, the phylogenetic relationships among *Dactylogyrus* species were assessed using the partial 18S rDNA, entire ITS-1 rDNA, and partial 28S rDNA genes. A total of 59 *Dactylogyrus* species, including the outgroup taxa *Pseudodactylogyrus anguillae* were used to reconstruct the phylogenetic analyses. From the combined 18S rDNA and the entire ITS-1 and partial 5.8S rDNA, the phylogenetic analysis revealed several groupings of *Dactylogyrus* species inferred largely from European cyprinoids and correspond to host specificity. However, three groupings had poorly resolved ML support, namely, the group that comprised mostly *Dactylogyrus* species commonly parasitising *Chondrostoma*/*Parachondrostoma* spp., the *Barbus*/*Luciobarbus* spp. group, and the *Scardinius* spp. group. The newly sequenced *D*. *afrolongicornis* from *E. trimaculatus* was revealed as the only species originating from Africa.

The partial 28S rDNA revealed three well supported clades linked to their biogeographical regions. The phylogenetic position of *D*. *afrolongicornis* was nested within the African *Dactylogyrus* lineage and suggests that *D*. *aspili* and *D*. *afrolongicornis* are sister species. The clustering of *D*. *aspili*, *D*. *afrolongicornis*, and *Dactylogyrus* sp. 5 correspond to host associations of the small diploid African *Enteromius* spp. (Smiliogastrini). Our results are congruent with previous studies by Šimková et al. (2004) and Benovics et al. (2018, 2020a) that confirm most of the phylogenetic relationships of species of *Dactylogyrus* in their studies.

Benovics et al. (2018) focussed on 53 *Dactylogyrus* species parasitising endemic cyprinoids in the Balkans, extensively focusing on cophylogenetic relationships of the cyprinoid fish from the region and their specific parasites on a wider range. The study revealed that most of the endemic cyprinoids harboured *Dactylogyrus* species of different origins which probably is attributed to multiple host switching. Additionally, Benovics et al. (2020a) focused on 49 *Dactylogyrus* species from 62 endemic cyprinoid fish from Balkan and Apennine Peninsulas, investigating cophylogenetic relationships between their endemic cyprinoids. Their analyses suggest that host switch played a major role in the evolutionary history of *Dactylogyrus* species parasitising endemic cyprinoids. Šimková et al. (2004) focused on 51 *Dactylogyrus* species parasitising central European cyprinoids. Their study revealed that intra-host duplication was most important in the diversification process of *Dactylogyrus* species than host switch.

Much can be learned from the above mentioned studies as they highlight areas of particular interest to investigate host-parasite systems and cophylogenetic relationships. For future strategies, more *Enteromius* hosts should be collected and examined for *Dactylogyrus* species. From these, additional *Dactylogyrus* sequences are needed to provide conclusive outcomes.

Unfortunately, some of the DNA samples failed to amplify and hence were not included in the current study. The failed sequences include *D. afrohamiltonii*, *D. allolongionchus*, *D. letabaensis*, *D. limpopoensis*, and *D myersi*. This could probably be due to the usage of the same lab that is utilised to extract multiple groups of parasitological species and increases the risk of cross-contamination and/or due to insufficient DNA samples for specimens (Øines and Schram 2008; Pereira et al. 2008). Nonetheless, utmost care in surface/workbench sterilisation using absolute ethanol, fixing, and preservation conditions during DNA extraction were appropriately practised in the present study.

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Appendix 1

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ANIMAL RESEARCH ETHICS COMMITTEE CLEARANCE CERTIFICATE

MEETING:	**02 April 2019**
PROJECT NUMBER:	**AREC/05/2019: PG**
PROJECT:	
Title:	Molecular Characterisation and phylogenetic relationships among the cyprinid fishes of the Genus Barbus Cuvier & Cloquet, 1816 and their Monogenean Parasites of Dactylogyridae within the Limpopo and Olifants River Systems.
Researchers:	ME Raphahlelo
Supervisor:	Dr MM Matla
Co-Supervisor/s:	Dr I Přikrylová
School:	Molecular and Life Sciences
Degree:	PhD

PROF JW NGAMBI
CHAIRPERSON: ANIMAL RESEARCH ETHICS COMMITTEE

The Animal Research Ethics Committee (AREC) is registered with the National Health Research Ethics Council, Registration Number: **AREC-290914-017**

Note:
i) **i) Should any departure be contemplated from the research procedure as approved, the researcher(s) must re-submit the protocol to the committee.**
ii) **ii) The budget for the research will be considered separately from the protocol.**
iii) **PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES.**

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Appendix 2

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DEPARTMENT OF
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DO SCIENTIFIC RESEARCH ON AQUATIC BIOTA

(Issued in terms of the provisions of the Limpopo Environmental Management Act 2003, Act no. 7 of 2003)
In terms of and subject to the provisions of the abovementioned legistation and the regulation framed thereunder, the holder of this permit is here by authorized to catch and/or collect the species and number of acquatic biota specified on the table below for scientfic purpose in the property mentioned on this permit.

Permit Holder

Full Name:	Prof. W.J. Luus-Powell	ID No.:	7105290132083
Postal Address:	Private Bag X1106	Physical Address	University of Limpopo 34 Palm Street
Postal Suburb:	Sovenga	Physical Suburb:	Polokwane
Postal State:	Limpopo	Physical State:	Limpopo
Postal Country:	South Africa	Physical Country	South Africa
Postal Code:	0727	Postal Code:	

Permit Details

Permit No:	ZA/LP/HO/3370	Stamp :	CITES & PERMIT MANAGEMENT ENVIRONMENTAL AFFAIRS LIMPOPO PROVINCE
Effective Date:	2015/11/10		
Valid Until:	2018/11/10		
Paid: (ZAR)	52.00		
Receipt No.:	1081143		

Farm Name / Organization	District	Province	Country
		Limpopo	South Africa

Species Name	Scientific Name	Quantity	Note
		0	Collection of various fish species

Issued by : Makgata RJ — 2015/11/10 Issue Date — Signature Of Permit Holder

I acknowledge, accept and understand fully the permit conditions as described.

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Appendix 3

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ORIGINAL PAPER

Dactylogyrus spp. (Monogenea, Dactylogyridae) from the Gills of *Enteromius* spp. (Cypriniformes, Cyprinidae) from the Limpopo Province, South Africa with Descriptions of Three New Species

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Abstract

Background Monogenean parasites of the genus *Dactylogyrus* Diesing, 1850 parasitize mostly gills of cyprinoids hosts. Of 100 species currently known from African continent, approximately 35 have been described from *Enteromius* spp. Results of recent studies indicate that there are still many undescribed species of the genus *Dactylogyrus* in South Africa and systematic surveys can bring many new findings.

Methods During the period April 2015–May 2016, three species of the genus *Enteromius* were sampled from eight localities across Limpopo Province. Monogenean parasites were collected from the gills of the hosts using stereomicroscopes. Morphometric analysis of the hard parts of the attachment organ and male copulatory organs were performed to confirm species identity.

Results Presence of three new and three previously described *Dactylogyrus* species is reported. Newly described species include: *Dactylogyrus afrohamiltoni* sp. nov. from *Enteromius afrohamiltoni*; *Dactylogyrus limpopoensis* sp. nov. and *Dactylogyrus letabaensis* sp. nov. from *Enteromius unitaeniatus*. In addition, *Dactylogyrus afrolongicornis*, *Dactylogyrus allolongionchus* and *Dactylogyrus myersi* were identified from *Enteromius trimaculatus*. Newly identified species possess morphometric characters based on which they can be clearly identified from currently known species.

Conclusion Present results show that small barbs, especially those not previously studied for monogenean parasites, are potentially very interesting target to study to recover new species of the genus *Dactylogyrus* and to bring new contribution to the knowledge of the diversity of African parasites.

Keywords Monogeneans · *Dactylogyrus* · Cyprinidae · Ectoparasites

Introduction

Fish of Cyprinodei are known to be parasitized by gill monogeneans of Diplozoidae Palombi, 1949 and Dactylogyridae Bychowsky, 1933, of which members of the latter family, *Dactylogyrus* Diesing, 1850 are the most diverse [5]. Using morphological criteria of the sclerotized structures, African *Dactylogyrus* can be divided into three distinctive species groups: *D. afrobarbae*-like group, *D. pseudanchoratus*-like group and *D. varicorhini*-like group [18, 19]. To date, species of the *D. afrobarbae*-like and *D. pseudanchoratus*-like groups are known only from African species of *Enteromius* Cope, 1867 and *Labeo* Cuvier, 1817. Members of the *D. varicorhini*-like group are common on *Labeobarbus* Rüppëll, 1836 (included syn. *Varicorhinus*) in Africa and on some European and Asian genera [11].

In Africa, recently 100 *Dactylogyrus* species have been listed to parasitize freshwater hosts with 35 species being recorded from *Enteromius* spp. [21]. From this host genus, *Enteromius kerstenii* (Peters, 1868) is known to host the highest numbers of *Dactylogyrus* spp, eight and from all known African *Dactylogyrus* spp., *Dactylogyrus brevicirrus* Paperna, 1973 is a parasite recorded from the highest number of hosts, nine species of four genera [21]. Ten

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species of *Dactylogyrus* have been reported to parasitize *Enteromius* spp. from South Africa of which five species have been described ibidem (Table 1) [11, 21]. Recently, several studies reported the presence of *Dactylogyrus* species in South Africa from various species of *Enteromius*, *Labeo* and *Labeobarbus* [1–3, 11, 12, 14, 17, 23, 28]. From Limpopo Province, Olivier et al. [17] recorded 20 species of the genus *Dactylogyrus* from an indigenous fish community of 11 cyprinoid species from the Middle Letaba Dam of which only eight species of *Dactylogyrus* are known to science. Furthermore, Crafford et al. [2, 3] reported on monogenean parasites from *Labeo* spp. in the Vaal Dam, with a description of three new species *Dactylogyrus iwani* Crafford, Luus-Powell et Avenant-Oldewage, 2012, *Dactylogyrus larindae* Crafford, Luus-Powell et Avenant-Oldewage, 2012 and *Dactylogyrus nicolettae* Crafford, Luus-Powell et Avenant-Oldewage, 2012 together with the records of three previously known species *Dactylogyrus extensus* Mueller et Van Cleave, 1932, *Dactylogyrus minutus* Kulviec, 1927 and *Dactylogyrus lamellatus* Achmerow, 1952 and the presence of another unidentified *Dactylogyrus* species belonging to *D. varicorhini*-like species group.

The present study gives new insight on the species richness of *Dactylogyrus* species parasitizing *Enteromius* spp. in South Africa and presents the descriptions of three new dactylogyrid species. A morphometric description of each species is presented.

Materials and Methods

Host and Parasite Collection

Gill nets of stretched single mesh size of 30 mm, seine nets, fyke nets and an electric shocker (LR-24, Smith Root Company, USA) were used to sample fish hosts from various localities from the Limpopo River System (Table 2). A permit for the collection of fish was obtained from the

Table 1 *Dactylogyrus* species know to parasitize *Enteromius* spp. in different water bodies from South Africa

Parasite species	Host	Locality	References
1. *Dactylogyrus afrolongicornis* Paperna, 1973	*Enteromius trimaculatus* (Peters, 1852)	Seshego Dam	Mashego [10]
		Piet Gouws Dam	
		Mohlapitse River	
		Middle Letaba Dam	Olivier et al. [17]
		Nwanedi-Luphephe dams	Mbokane et al. [14]
2. *Dactylogyrus afrolongicornis alberti* Paperna, 1973	*Enteromius trimaculatus* (Peters, 1852)	Seshego Dam	Mashego [10]
		Middle Letaba Dam	Olivier et al. [17]
		Nwanedi-Luphephe dams	Mbokane et al. [14]
3. *Dactylogyrus afrosclerovaginus* Paperna, 1973	*Enteromius paludinosus* (Peters, 1852)	Seshego Dam	Mashego [10]
	Enteromius trimaculatus (Peters, 1852)	Middle Letaba Dam	Olivier et al. [17]
4. *Dactylogyrus allolongionchus* Paperna, 1973	*Enteromius trimaculatus* (Peters, 1852)	Seshego Dam	Mashego [10]
		Middle Letaba Dam	Olivier et al. [17]
5. *Dactylogyrus dominici* Mashego, 1983	*Enteromius paludinosus* (Peters, 1852)	Turfloop Dam	Mashego [10]
		Middle Letaba Dam	Olivier et al. [17]
6. *Dactylogyrus enidae* Mashego, 1983	*Enteromius neefi* (Greenwood, 1962)	Lingwe River	Mashego [10]
7. *Dactylogyrus mattozii* Mashego et Matlou, 2018	*Enteromius mattozi* (Guimaraes, 1884)	Piet Gouws Dam	Mashego and Matlou [11]
8. *Dactylogyrus myersi* Price, McClellan, Druckenmiller et Jacobs, 1969	*Enteromius trimaculatus* (Peters, 1852)	Lydenburg	Price et al. [20]
		Natal	Paperna [19]
		Seshego Dam	Mashego [10]
		Middle Letaba Dam	Olivier et al. [17]
9. *Dactylogyrus spinicirrus* Paperna et Thurston, 1969	*Enteromius trimaculatus* (Peters, 1852)	Nwanedi-Luphephe dams	Mbokane et al. [14]
	Enteromius radiatus (Peters, 1853)		
10. *Dactylogyrus teresae* Mashego, 1983	*Enteromius paludinosus* (Peters, 1852)	Seshego Dam	Mashego [10]
		Middle Letaba Dam	Olivier et al. [17]
		Barberspan Wetland	Truter et al. [28]

Table 2 An overview of collected host species with indication of the number of collected fish per locality and the *Dactylogyrus* species identified, including the geographical co-ordinates in the present study from the Limpopo River System

Host species	*N*	Locality	*Dactylogyrus* spp.	Geographical co-ordinates
E. afrohamiltoni	3	Hulukulu pan	AF	22° 20′ 22.31″ S, 31° 10′ 06.09″ E
E. unitaeniatus	1	Tzaneen Dam	LI	23° 48′ 07.78″ S, 30° 10′ 01.54″ E
	13	Middle Letaba Dam	LE	23° 16′ 27.08″ S, 30° 24′ 16.55″ E
	5	Letsitele Weir	LI, LE	23° 52′ 19.60″ S, 30° 17′ 55.67″ E
	1	Luphephe Dam	LE	22° 39.492′ S, 30° 25.342′ E
	3	Nondweni Dam	LE	23° 41′ 16.84″ S, 30° 51′ 57.78″ E
E. trimaculatus	26	Middle Letaba Dam	AFR, AL, MY	23° 16′ 27.08″ S, 30° 24′ 16.55″ E
	7	Groot Letaba River	LI, AFR, AL, MY	23° 41′ 27.58″ S, 30° 35′ 45.16″ E
	4	Letsitele Weir	AFR, AL, MY	23° 52′ 19.60″ S, 30° 17′ 55.67″ E
	10	Flag Boshielo Dam	LI, AFR, AL, MY	24° 49′ 05″ S, 029° 26′ 39″ E
	15	Luphephe Dam	AFR, AL	22° 39.492′ S, 30° 25.342′ E
	7	Nondweni Dam	LE, AFR, AL, MY	23° 41′ 16.84″ S, 30° 51′ 57.78″ E

N number of collected fish, *AF D. afrohamiltonii* sp. nov., *LI D. limpopoensis* sp. nov., *LE D. letabaensis* sp. nov., *AFR D. afrolongicornis*, *AL D. allolongionchus*, *MY D. myersi*

Department of Economic Development and Tourism, Limpopo Province, approval number ZA/LP/HO/3370. Fish were separated per species and immediately transferred to containers and transported live to the field laboratory. Fish were identified according to Skelton [22] and were killed by severing the spinal cord from the head. Gills were removed and placed into Petri dishes filled with dam water and examined for the presence of monogenean parasites using a stereomicroscope (Leica EZ4, Leica Microsystems, Germany). Parasites were removed, recorded and mounted in ammonium picrate–glycerine solution (GAP) [9].

Parasite Identification

For the identification, specimens were studied and measured using an Olympus BX50 Nomarski Differential Contrast microscope fitted with a camera and imaging software (Soft Imaging System GmbH 1986 version 1.5.1) and a drawing apparatus. Drawings of hard parts were digitized and arranged using Adobe Photoshop CS6 and Adobe Illustrator CS6 version 13.0. A total of 12 characteristics were measured on sclerotized structures [anchors, transverse bar, vestigial ventral bar, hooks and male copulatory organ (MCO)]. The arrangement of the hooks follows numbering according to Mizelle [15]. All measurements were performed according to Gussev in Bychovskaya-Pavlovskaya et al. [7], except the following: the tube trace length (copulatory tube) of the MCO was measured according to Musilová et al. [16]. All measurements are expressed in micrometres (µm) and presented as the mean ± standard deviation with the range in parentheses.

For comparative analyses of haptoral sclerotized structures and MCO, the type specimen of *D. afrolongicornis*, Syntypes M.T. 35.923 and *D. afrolongicornis alberti*, Type M.T. 35.934 were obtained from Africa Museum, Tervuren, Belgium. Prior to depositing in museum collections, the specimens in GAP were transferred into Canada balsam following the procedure proposed by Ergens [4].

Selected specimens were deposited as holotype, paratypes or voucher in the parasitological collection in the National Museum, Bloemfontein, South Africa (NMBP), in the Africa Museum, Tervuren, Belgium (M.T.) and in the helminthological collection held at the Institute of Parasitology, Academy of Sciences of the Czech Republic, České Budějovice, Czech Republic (IPCAS). Note that the remaining paratypes from each species described are kept in the Department of Biodiversity, University of Limpopo for future reference.

Results

A total number of 95 specimens of three *Enteromius* species were examined from eight localities within the Limpopo River System. Based on the morphometric analyses of the sclerotized structures, the presence of three new and three previously described *Dactylogyrus* species were confirmed. Details on numbers of collected hosts from all localities and their coordinates are given in Table 2. Morphological descriptions of all six species are provided below.

Dactylogyridae Bychowsky, 1933

Dactylogyrus Diesing, 1850

Dactylogyrus afrohamiltoni sp. nov. (*Figs.* 1, 2*; Table* 3) Body length 337.4 ± 70.4 (245.4–493.6) long; 48.5 ± 9.1 (28.5–64.0) wide (at maximum width, usually across the

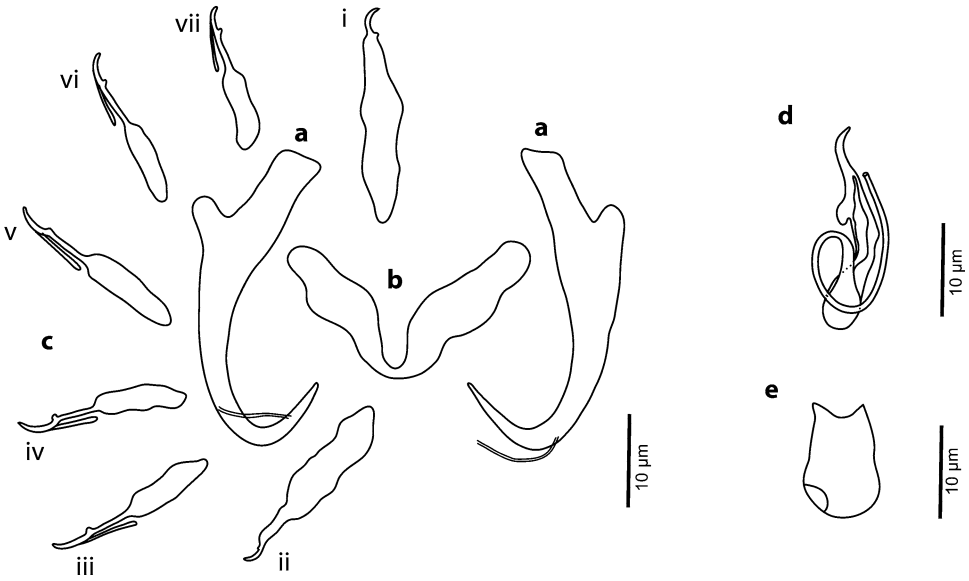

Fig. 1 Drawings of sclerotized structures of *Dactylogyrus afrohamiltoni* sp. nov. from *Enteromius afrohamiltoni* (Crass, 1960) collected from Hulukulu pan, Limpopo Province, South Africa. a Anchor. b Transverse bar. c Hook (pairs i–vii). d Male copulatory organ. e Vagina. Scale bar = 10 µm

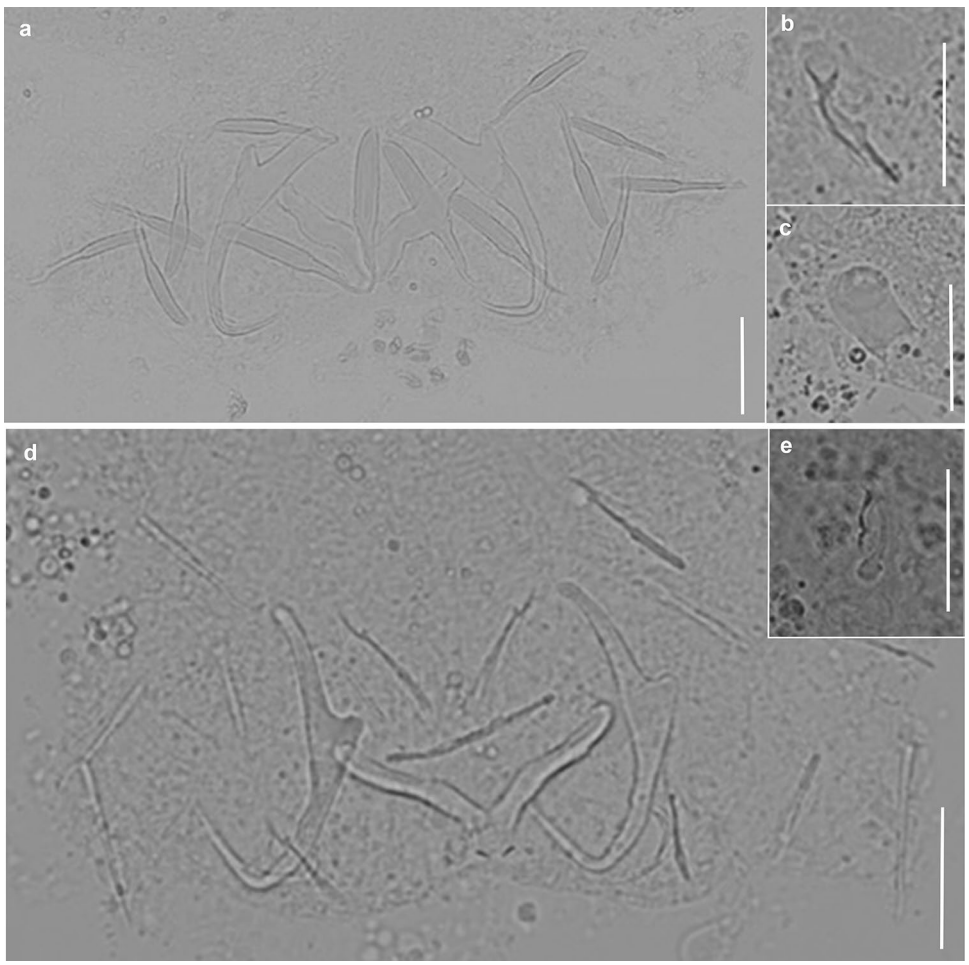

Fig. 2 Microphotographs of sclerotized structures of *Dactylogyrus afrohamiltonii* sp. nov (**a**–**c**) and *Dactylogyrus letabaensis* sp. nov. (**d**, **e**). **a**, **d** Haptoral structures. **b**, **e** Male copulatory organ. **c** Vagina. Scale bars = 20 µm

MCO). Single pair of stout anchors, with relatively short inner root: total length 43.6 ± 1.3 (40.1–45.5) long; shaft 33.7 ± 0.9 (31.1–35.1) long; tip 11.3 ± 0.7 (9.8–12.5) long; inner root 12.8 ± 0.9 (10.4–14.0) long; outer root 4.1 ± 0.8 (2.6–5.9) long. A single thick transverse bar divided into heavy, wide plates by a median constriction, typically

Table 3 Comparative morphometric data for *Dactylogyrus* species (*D. afrohamiltonii* sp. nov., *D. limpopoensis* sp. nov. and *D. letabaensis* sp. nov.) analysed in the present study collected form *Enteromius afrohamiltoni*, *Enteromius unitaeniatus* and *Enteromius trimaculatus* from the Limpopo River System

Type host	*D. afrohamiltonii* sp. nov	*Dactylogyrus*. sp. 3	*D. afroruahae*	*D. limpopoensis* sp. nov	*D. longiphallus*	*D. letabaensis* sp. nov	*D. parviphallus*
	E. afrohamiltoni	*E. afrohamiltoni*	*Enteromius* sp.	*E. unitaeniatus*	*L. victorianus*	*E. unitaeniatus*	*E. kerstenii*,
	Present study	Swanepoel [23]	Paperna [19]	Present study	Paperna [19]	Present study	Paperna [19]
Site	Gills	Gills	Gills	Gills	Gills	Gills	Gills
Type locality	Hulukulu pan, South Africa	Pongola Floodplains, South Africa	Ruaha River, Tanzania	Tzaneen Dam, South Africa	Lake Victoria, Uganda	Middle Letaba Dam, South Africa	South Kyoga, Uganda
Material studied	20	12	3	15	22	30	10
Body							
Length	337.4 ± 70.4 (245.4–493.6)	316.8 ± 61.5 (251.2–477.1)	350–400	313.3 ± 56.6 (231.4–403.2)	120–330	400.6 ± 80.3 (272.6–602.7)	160–210
Width	48.5 ± 9.1 (28.5–64.0)	52.7 ± 10.6 (36.7–70.5)	70–100	75.3 ± 17.6 (41.4–102.4)	35–100	77.5 ± 14.7 (46.5–103.9)	50–80
Anchors							
Total length	43.6 ± 1.3 (40.1–45.5)	47.9 ± 1.4 (45.7–50.3)	36–41	35.9 ± 1.9 (30.5–38.4)	31–44	40.3 ± 1.1 (38.2–43.0)	30–37
Shaft	33.7 ± 0.9 (31.1–35.1)	36.8 ± 1.1 (35.5–38.8)	25–32	26.4 ± 1.3 (24.5–28.1)	20–25	27.2 ± 0.7 (25.3–28.8)	20–27
Tip	11.3 ± 0.7 (9.8–12.5)	11.5 ± 0.5 (10.8–12.8)	11–12	15.7 ± 1.5 (11.0–17.2)	7–17	14.4 ± 0.6 (13.4–16.0)	6–10
Inner root	12.8 ± 0.9 (10.4–14.0)	15.2 ± 2.0 (12.1–18.7)	14–15	11.4 ± 1.5 (7.5–13.6)	11–25	17.4 ± 1.3 (14.2–19.7)	8–17
Outer root	4.1 ± 0.8 (2.6–5.9)	4.8 ± 0.8 (3.9–6.6)	2	2.8 ± 0.5 (1.9–3.5)	1–5	1.8 ± 0.4 (1.0–2.8)	1–4
Transverse bar							
Total length	26.3 ± 1.8 (23.7–29.5)	31.7 ± 3.9 (25.9–38.3)	43–46	28.0 ± 2.5 (22.6–32.7)	15–24	28.9 ± 1.8 (22.6–31.8)	29–40
Width	5.2 ± 0.7 (4.0–6.8)	1.7 ± 0.5 (1.0–2.5)	–	2.9 ± 0.3 (2.1–3.2)	–	3.2 ± 0.4 (2.3–4.0)	–
Vestigial bar							
Total length	–	–	–	6.7 ± 1.4 (4.5–8.5)	–	26.4 ± 1.8 (22.7–29.7)	14–19
Width	–	–	–	0.9 ± 0.2 (0.5–1.2)	–	1.3 ± 0.2 (1.1–1.8)	–
Hooks			18–30		10–24		15–25
I	32.8 ± 1.5 (30.7–36.1)	29.2 ± 4.9 (19.9–34.7)	–	16.7 ± 1.8 (12.3–19.7)	–	17.8 ± 1.4 (14.2–19.4)	–
II	31.5 ± 1.4 (29.4–35.2)	30.3 ± 3.6 (23.1–34.5)	–	17.0 ± 1.7 (13.5–19.7)	–	16.8 ± 2.1 (13.5–23.2)	–
III	25.3 ± 2.0 (20.4–28.5)	24.4 ± 2.7 (17.6–28.0)	–	16.0 ± 1.7 (13.1–19.0)	–	16.9 ± 1.9 (14.3–23.4)	–
IV	25.5 ± 1.7 (21.5–28.8)	24.2 ± 5.5 (8.2–29.3)	–	16.4 ± 3.1 (11.1–21.3)	–	23.2 ± 2.2 (16.4–26.2)	–
V	25.6 ± 1.7 (20.4–28.4)	26.0 ± 3.9 (18.1–30.8)	–	18.3 ± 1.9 (13.5–21.5)	–	18.6 ± 1.4 (16.0–21.7)	–
VI	25.3 ± 1.3 (21.9–27.1)	26.6 ± 2.1 (21.9–28.8)	–	17.9 ± 1.6 (14.0–20.5)	–	18.8 ± 1.8 (15.4–22.3)	–
VII	24.2 ± 1.6 (21.1–27.7)	26.6 ± 2.5 (22.4–29.5)	–	18.4 ± 0.9 (16.9–20.2)	–	20.8 ± 2.1 (14.9–24.2)	–
MCO							
Copulatory tube	30.9 ± 2.4 (25.8–35.2)		25–29	23.1 ± 1.7 (19.8–25.5)	39 (35–63)	14.1 ± 1.4 (11.8–16.6)	9–10

Table 3 (continued)

Type host	*D. afrohamiltonii* sp. nov	*Dactylogyrus*. sp. 3	*D. afroruahae*	*D. limpopoensis* sp. nov	*D. longiphallus*	*D. letabaensis* sp. nov	*D. parviphallus*
	E. afrohamiltoni	*E. afrohamiltoni*	*Enteromius* sp.	*E. unitaeniatus*	*L. victorianus*	*E. unitaeniatus*	*E. kerstenii*,
	Present study	Swanepoel [23]	Paperna [19]	Present study	Paperna [19]	Present study	Paperna [19]
Accessory piece	18.2 ± 1.1 (16.1–19.9)	17.2 ± 1.9 (13.4–21.1)	15–19	16.1 ± 1.1 (13.8–17.7)	22–40	15.1 ± 1.4 (12.6–17.8)	8
Vagina							
Length	12.3 ± 1.9 (6.9–16.1)	–	15–17	17.3 ± 7.2 (5.5–24.9)	5–8	11.6 ± 2.5 (7.1–14.3)	–
Width	7.9 ± 1.3 (5.5–10.2)	–	9–10	18.5 ± 2.4 (16.2–22.1)	4–9	9.2 ± 1.2 (8.0–12.2)	–

For each characteristic (in µm), the mean ± standard deviation with the range in parentheses are given

V-shaped: 26.3 ± 1.8 (23.7–29.5) long; width 5.2 ± 0.7 (4.0–6.8). Hooks stout with thick handles, in 7 pairs, dissimilar in size: pairs I and II heavily sclerotized and largest in comparisons with other pairs; hook lengths; pair I 32.8 ± 1.5 (30.7–36.1); pair II 31.5 ± 1.4 (29.4–35.2); pair III 25.3 ± 2.0 (20.4–28.5); pair IV 25.5 ± 1.7 (21.5–28.8); pair V 25.6 ± 1.7 (20.4–28.4); pair VI 25.3 ± 1.3 (21.9–27.1); pair VII 24.2 ± 1.6 (21.1–27.7). MCO complex composed of copulatory tube, loosely coiled once following a U-shaped path that terminates poorly distally and an elongated accessory piece that encloses the base of copulatory tube to form a capsule like structure and terminates distally in a curved spike: copulatory tube 30.9 ± 2.4 (25.8–35.2) long; accessory piece 18.2 ± 1.1 (16.1–19.9). Vagina proximally rounded or with two pointed projections, poorly sclerotized consisting of a vaginal pore on the dextral posterior section: 12.3 ± 1.9 (6.9–16.1) long; 7.9 ± 1.3 (5.5–10.2) wide.

Taxonomic Summary

Type Host

Enteromius afrohamiltoni (Crass, 1960) (Cyprinidae). Collection date: April 2015.

Type Locality

Hulukulu pan (22° 20′ 22.31″ S, 31° 10′ 06.09″ E), Limpopo Province, South Africa.

Type Material

Holotype NMBP 447; paratype NMBP 448; voucher M.T. 38224; two vouchers IPCAS M-669.

Site

Gill lamellae.

Etymology

The species epithet is proposed after the host *E. afrohamiltoni*.

Remarks

Although Swanepoel [23] reported *Dactylogyrus* sp. 3 from *Enteromius afrohamiltoni* (Crass, 1960) (syn. *B. afrohamiltoni*) from Pongola Floodplains, Nyamiti Pan Outlet, it has not been identified to species level. The present material was compared to drawings of Swanepoel [23] material and the two specimens were found to be unambiguously morphologically similar based on sclerotized haptoral structures and MCO, additionally, their measurements fall within similar size ranges. *Dactylogyrus afrohamiltoni* sp. nov. closely resembles *Dactylogyrus afroruahae* Paperna, 1973, described from an unknown small *Enteromius* sp. in Ruaha River, Tanzania. Although Paperna [19] measured the overall hook pairs, this species possesses similarly shaped haptoral hard parts, but differs from the former in the size of the hooks, particularly pairs I and II. In addition, these hook pairs were noted to be longer than pairs III–VII (Table 3), which is in contrast with most dactylogyrids where these sclerites are the smallest. The MCO in the new species bears a notch just before the spike terminates from the accessory piece, while *D. afroruahae* has a simple base. Vagina nearly similar, with the exception of the size, longer in *D. afroruahae* and vaginal pore dextrally posteriorly positioned in the present material. The finding of *D. afrohamiltoni* sp. nov. represents the first record of monogenean parasites for *E. hamiltoni*.

Dactylogyrus letabaensis sp. nov. (Figs. 2, 3; Table 3)

Body length 400.6 ± 80.3 (272.6–602.7) long; width 77.5 ± 14.7 (46.5–103.9) usually across the MCO. Single pair of anchors: 40.3 ± 1.1 (38.2–43.0) long; shaft 27.2 ± 0.7

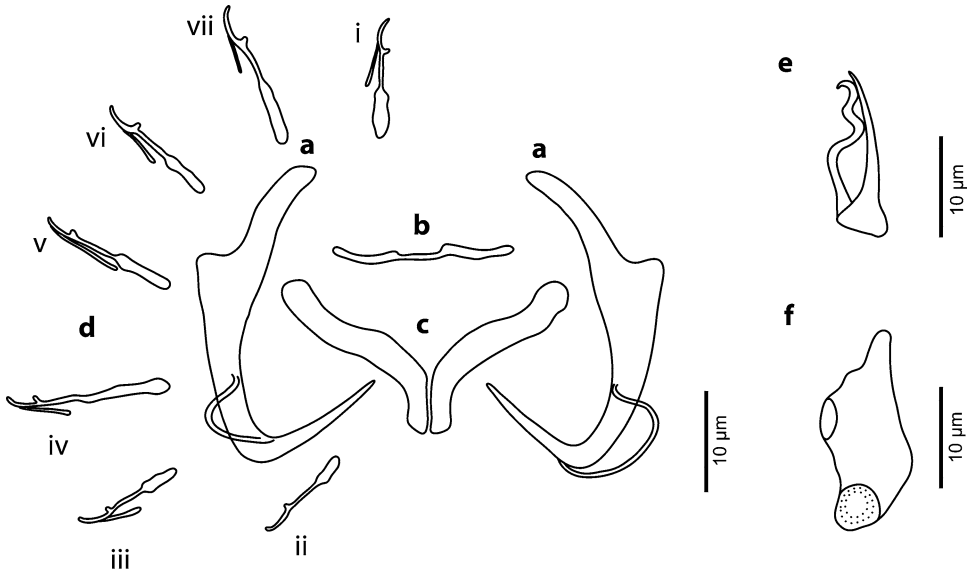

Fig. 3 Drawings of sclerotized structures of *Dactylogyrus letabaensis* sp. nov. from *Enteromius unitaeniatus* (Günther, 1866) collected from Tzaneen Dam, Limpopo Province, South Africa. a Anchor. b Vestigial ventral bar. c Transverse bar. d hook (pairs i–vii). Scale bar = 10 µm. e Male copulatory organ. f Vagina. Scale bar = 20 µm

(25.3–28.8) long; tip 14.4 ± 0.6 (13.4–16.0) long; inner root 17.4 ± 1.3 (14.2–19.7) long; outer root 1.8 ± 0.4 (1.0–2.8) long. Two bars: transverse bar large and thick, broadly Y-shaped bending sharply towards mid-point, 28.9 ± 1.8 (22.6–31.8) long, 3.2 ± 0.4 (2.3–4.0) wide; small vestigial ventral bar anterior to the first, very thin and long with narrowed median part, 26.4 ± 1.8 (22.7–29.7) long, 1.3 ± 0.2 (1.1–1.8) wide. Hooks in seven pairs, dissimilar in size: pair IV longer compared with other pairs; hook lengths; pair I 17.8 ± 1.4 (14.2–19.4) long; pair II 16.8 ± 2.1 (13.5–23.2) long; pair III 16.9 ± 1.9 (14.3–23.4) long; pair IV 23.2 ± 2.2 (16.4–26.2) long; pair V 18.6 ± 1.4 (16.0–21.7) long; pair VI 18.8 ± 1.8 (15.4–22.3) long; pair VII 20.8 ± 2.1 (14.9–24.2) long. MCO complex consisting of a M-shaped copulatory tube, distally termination to U-shape and thick accessory piece: copulatory tube 14.1 ± 1.4 (11.8–16.6) long; accessory piece 15.1 ± 1.4 (12.6–17.8). Vagina armed with spindle-shaped sclerites, weakly sclerotized and inconspicuous: 11.6 ± 2.5 (7.1–14.3) long; 9.2 ± 1.2 (8.0–12.2) wide.

Taxonomic Summary

Type Host

Enteromius unitaeniatus (Günther, 1866) (Cyprinidae). Collection date: November 2015.

Type Locality

Middle Letaba Dam (23° 16′ 27.08″ S, 30° 24′ 16.55″ E), Limpopo Province, South Africa.

Other Localities

Letsitele Weir (23° 52′ 19.60″ S, 30° 17′ 55.67″ E), Luphephe Dam (22° 39′ S, 30° 25′ E) and Nondweni Dam (23° 41′ 16.84″ S, 30° 51′ 57.78″ E), Limpopo Province, South Africa.

Type Material

Holotype NMBP 452; two paratypes NMBP 453–4; three vouchers M.T. 38227–9; two vouchers IPCAS M-671.

Site

Gill lamellae.

Etymology

The specific epithet is proposed after the type locality, Middle Letaba Dam.

Remarks

Dactylogyrus letabaensis sp. nov. is similar to *Dactylogyrus parviphallus* Paperna, 1973 in the shape of the transverse bar, presence of long vestigial ventral bar and hooks. But *D. letabaensis* sp. nov. differs from *D. parviphallus* in the dimension and the morphology of the haptoral hard parts. Anchor total length, anchor tip, vestigial bar total length, MCO copulatory tube and accessory piece are longer at *D. letabanensis* sp. nov. than at *D. parviphallus,* for details see Table 3. The shape of the accessory piece of the latter species is simple and not bending while at *D. letabaensis* sp. nov. it turns in an open spiral.

Dactylogyrus limpopoensis sp. nov. (Figs. 4, 5; Table 3)

Body length 313.3 ± 56.6 (231.4–403.2) long; width 75.3 ± 17.6 (41.4–102.4) usually across the MCO. Single pair of anchors: 35.9 ± 1.9 (30.5–38.4) long; shaft 26.4 ± 1.3 (24.5–28.1) long; tip 15.7 ± 1.5 (11.0–17.2) long; inner root 11.4 ± 1.5 (7.5–13.6) long; outer root 2.8 ± 0.5 (1.9–3.5) long. Two bars observed, transverse bar widest at each extremity, 28.0 ± 2.5 (22.6–32.7) long, 2.9 ± 0.3 (2.1–3.2) wide; small vestigial ventral bar, broadly V-shaped, reduced in size, 6.7 ± 1.4 (4.5–8.5) long, 0.9 ± 0.2 (0.5–1.2) wide. Hooks in 7 pairs, dissimilar in size and shape: pair II markedly curved, and pairs I–IV similar in size when compared with other pairs; hook lengths; pair I 16.7 ± 1.8 (12.3–19.7); pair II 17.0 ± 1.7 (13.5–19.7); pair III 16.0 ± 1.7 (13.1–19.0); pair IV 16.4 ± 3.1 (11.1–21.3); pair V 18.3 ± 1.9 (13.5–21.5); pair VI 17.9 ± 1.6 (14.0–20.5); pair VII 18.4 ± 0.9 (16.9–20.2). MCO complex with a non-coiling copulatory tube and elongated accessory piece, articulated distally: copulatory tube 23.1 ± 1.7 (19.8–25.5) long; accessory piece 16.1 ± 1.1 (13.8–17.7). Vagina non-sclerotized and inconspicuous: 17.3 ± 7.2 (5.5–24.9) long; 18.5 ± 2.4 (16.2–22.1) wide.

Taxonomic Summary

Type Host

Enteromius unitaeniatus (Günther, 1866) (Cyprinidae). Collection date: November 2015.

Type Locality

Tzaneen Dam (23° 48′ 07.78″ S, 30° 10′ 01.54″ E), Limpopo Province, South Africa.

Other Host and Localities

Enteromius unitaeniatus, Letsitele Weir (23° 52′ 19.60″ S, 30° 17′ 55.67″ E); *Enteromius trimaculatus* (Peters, 1852), Groot Letaba River (23° 41′ 27.58″ S, 30° 35′ 45.16″ E) and Flag Boshielo Dam (24° 49′ 05″ S, 029° 26′ 39″ E), Limpopo Province, South Africa.

Type Material

Holotype NMBP 449; two paratypes NMBP 450 and 451; two vouchers M.T. 38225–6; three vouchers IPCASM-670.

Site

Gill lamellae.

Etymology

The specific name is suggested after its first finding in the Limpopo Province.

Remarks

In Olivier et al. [17] *Dactylogyrus* sp. 1 was recorded from *Enteromius unitaeniatus* (Günther, 1866) from Middle Letaba Dam, but no drawings, measurements or photos of sclerotized structures were provided for comparison with the present material. *Dactylogyrus limpopoensis* sp. nov. resembles

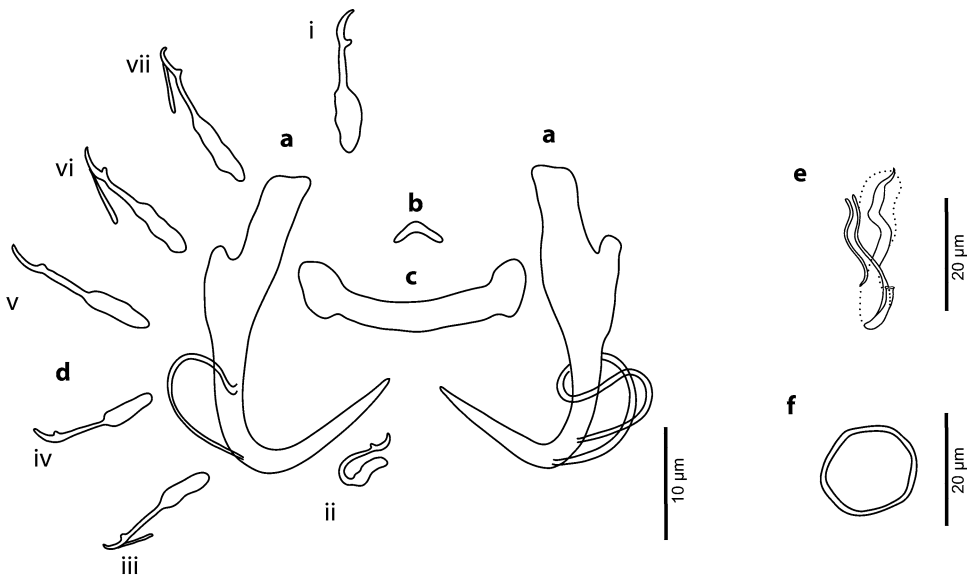

Fig. 4 Drawings of sclerotized structures of *Dactylogyrus limpopoensis* sp. nov. from *Enteromius unitaeniatus* (Günther, 1866) collected from Middle Letaba Dam, Limpopo Province, South Africa. a Anchor. b Vestigial ventral bar. c Transverse bar. d Hook (pairs i–vii). e Male copulatory organ. f Vagina. Scale bar = 10 µm

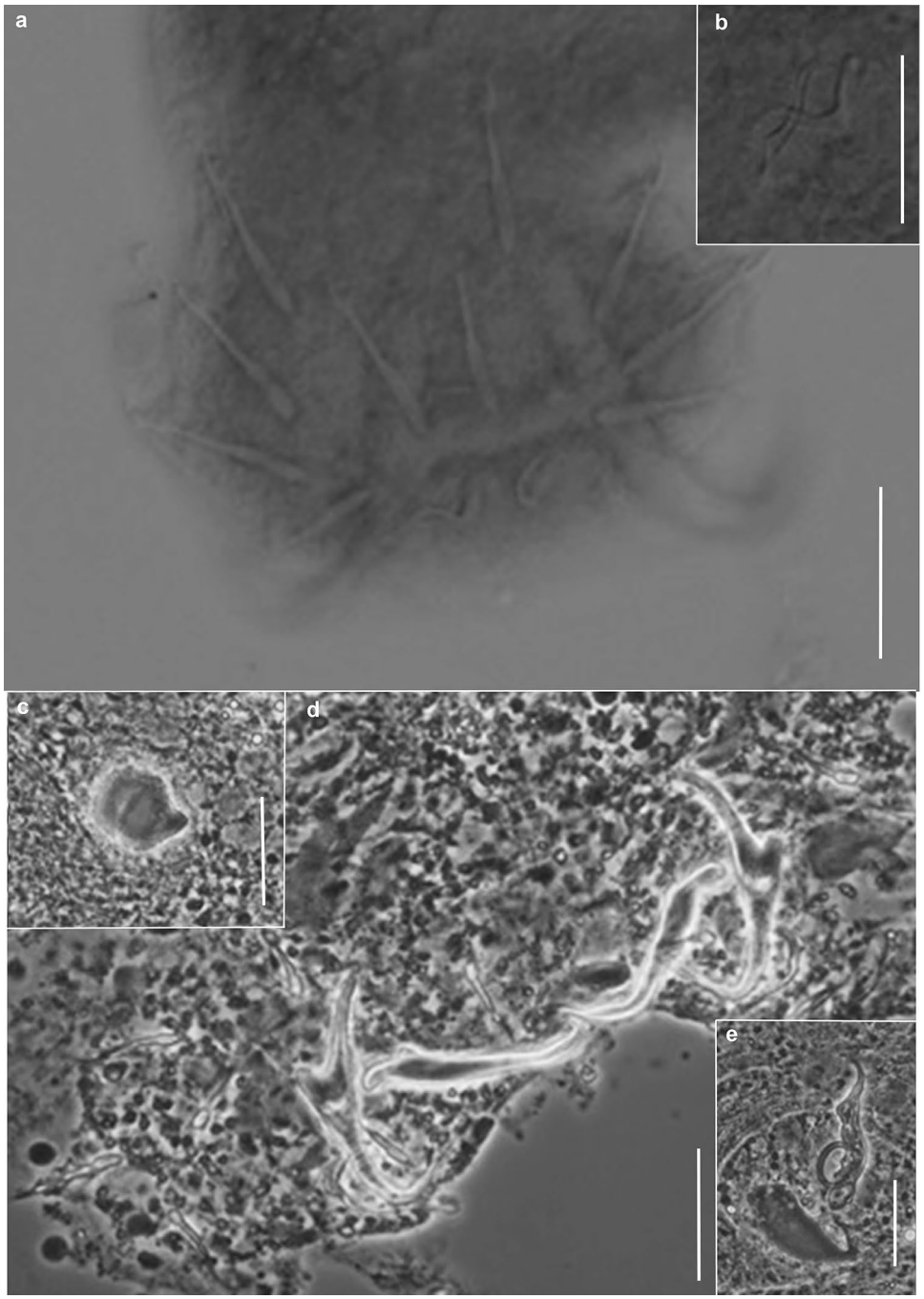

Fig. 5 Microphotographs of sclerotized structures of *Dactylogyrus limpopoensis* sp. nov (a, b) and *Dactylogyrus afrolongicornis* (b–e). a, d Haptoral structures. b, e Male copulatory organ. c Vagina. Scale bars = 20 µm

Dactylogyrus longiphallus Paperna, 1973 collected from *Labeo victorianus* Boulenger, 1901 in Uganda and Kenya [19]. *Dactylogyrus limpopoensis* sp. nov. differs from the latter by the presence of a pair II hooks being curved and having distally articulating accessory piece. These two species clearly differ by the dimension of the MCO complex being smaller at *D. limpopoensis* sp. nov. (MCO copulatory tube 19.8–25.5; accessory piece 13.8–17.7) than at *D. longiphallus* (MCO copulatory tube 35–63; accessory piece 22–40). Guégan and Lambert [6] re-described *D. longiphallus* providing new metrical data what shows that total length of transverse bar of *D. limpopoesnis* sp. now. is longer than that one of *D. longiphallus* (Table 3).

Dactylogyrus afrolongicornis Paperna, 1973 (Figs. 5, 6; Table 4)

Body length 459.9 ± 106.6 (297.0–727.5) long; 62.7 ± 14.3 (42.8–90.5) wide usually across the MCO. Single pair of anchors, delicate with long inner root: total length 42.6 ± 2.5 (35.3–46.8); shaft 28.9 ± 1.6

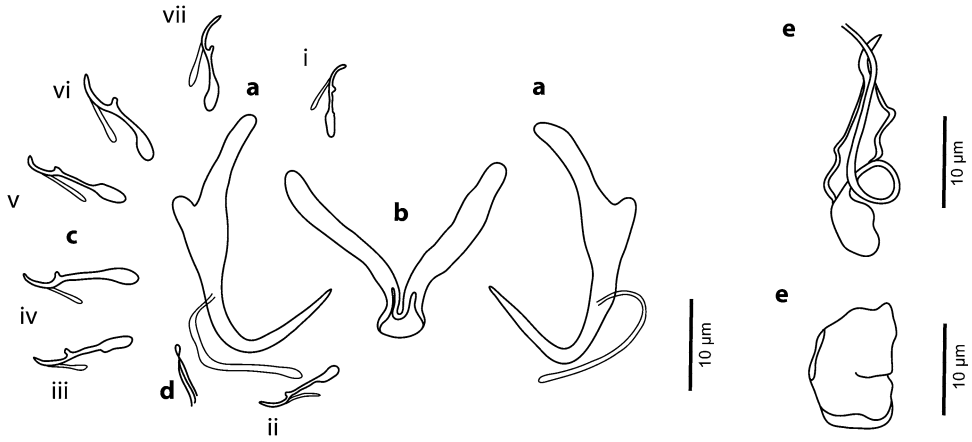

Fig. 6 Drawings of sclerotized structures of *Dactylogyrus afrolongicornis* from *Enteromius trimaculatus* (Peters, 1852) collected from Middle Letaba Dam, Limpopo Province, South Africa. a Anchor. b Transverse bar. c Hook (pairs i–vii). d Needle. e Male copulatory organ. f Vagina. Scale bar = 10 µm

(25.4–31.7) long; tip 16.0 ± 1.2 (13.9–18.6) long; inner root 17.6 ± 1.6 (14.6–21.1) long; outer root 2.7 ± 0.6 (1.6–3.9) long. Transverse bar broadly V-shaped with a soft poorly sclerotized or even non-sclerotized mid portion: 36.1 ± 3.3 (28.7–42.2) long; width 4.9 ± 0.8 (3.5–7.0). Hooks in seven pairs, dissimilar in size: pair IV longer compared with other pairs; hook lengths; pair I 16.2 ± 1.7 (12.2–18.8); pair II 17.1 ± 1.4 (14.1–19.3); pair III 15.8 ± 2.0 (12.5–21.6); pair IV 18.7 ± 1.5 (16.1–21.7); pair V 17.7 ± 1.4 (14.5–20.2); pair VI 17.9 ± 1.8 (14.0–20.7); pair VII 16.3 ± 1.1 (14.2–18.1). Pair of needles located between pairs II and III. MCO complex composed of coiled (one complete ring) copulatory tube and an elongated accessory piece that terminates in a spike: copulatory tube 39.7 ± 4.0 (35.4–47.8) long; accessory piece 25.2 ± 2.1 (20.8–29.8). Vagina exhibits various shapes armed with a large club-shaped vaginal pore: 11.7 ± 3.6 (4.8–19.4) long; 10.9 ± 1.4 (8.5–13.2) wide.

Taxonomic Summary

Type Host

Enteromius cf. *kersteni* (Cyprinidae).

Type Locality

Mobuku River, Uganda.

Other Records

Enteromius cf. *kersteni* and *Enteromius* cf. *perince*, Rwempum River, Uganda [19]; *Enteromius trimaculatus*, Piet Gouws Dam, Seshego Dam, Mohlapitse River [10]; Middle Letaba Dam [17] and Nwanedi-Luphephe dams [14].

Present Host and Localities

Enteromius trimaculatus, Middle Letaba Dam (23° 16′ 27.08″ S, 30° 24′ 16.55″ E), Groot Letaba River (23° 41′ 27.58″ S, 30° 35′ 45.16″ E), Letsitele Weir (23° 52′ 19.60″ S, 30° 17′ 55.67″ E), Flag Boshielo Dam (24° 49′ 05″ S, 029° 26′ 39″ E), Luphephe Dam (22° 39′ S, 30° 25′ E) and Nondweni Dam (23° 41′ 16.84″ S, 30° 51′ 57.78″ E), Limpopo Province, South Africa.

Deposited Material

Three vouchers IPCAS M-672; three vouchers M.T. 38230–2; three vouchers NMBP 455–7.

Remarks

This parasite was originally described from *Enteromius* cf. *kersteni* [18] and later recorded from *Enteromius* cf. *perince* in Uganda [19]. Other records of this species are those of Mashego [10], Olivier et al. [17] and Mbokane et al. [14] from *E. trimaculatus*. In the present study, the parasites were retrieved from the gills of *E. trimaculatus*. In comparison with those materials in previous studies [10, 19], they are morphologically identical and their measurements fall within similar size ranges (Table 4). Paperna [18] described *D. afrolongicornis* together with its subspecies *D. afrolongicornis alberti*. Based on his observations, the subspecies was separated from the former by the presence of heavy and thick bar plates of the transverse bar [19].

Although Paperna [19] reported another *D.* cf. *afrolongicornis* from *E.* cf. *kersteni*, Mobuku River in Uganda, it probably belongs to *D. afrolongicornis*, taking into account the same type-host and locality. From studying considerable material in the present study, we are convinced that subspecies *D. afrolongicornis alberti* is not valid as no evident feature to differentiate it from *D. afrolongicornis* was observable during studying present material. In fact, all specimens

Table 4 Comparative morphometric data (in µm) used in this study for known *Dactylogyrus* species (*D. afrolongicornis*, *D. allolongionchus* and *D. myersi*) reported from *Enteromius* species and their type localities

Parasite species	Price et al. [20]	Paperna [19]			Present study		
	D. myersi	*D. afrolongicornis*	*D. allolongionchus*	*D. myersi*	*D. afrolongicornis*	*D. allolongionchus*	*D. myersi*
Type host	*E. trimaculatus*	*E.* cf. *kersteni*	*E. perince*	*E. perince*	*E. trimaculatus*	*E. trimaculatus*	*E. trimaculatus*
Site	Gills	Gills	Gills	Gills	Gills	Gills	Gills
Type locality	Lydenburg, South Africa	Mobuku River, Uganda	Lake Albert, Uganda	Lake Albert, Uganda	South Africa	South Africa	South Africa
Material studied	12	10	5	5	23	7	13
Body							
Length	323 (298–339)	180–440	200–310	180–270	459.9 ± 106.6 (297.0–727.5)	313.3 ± 30.8 (283.8–373.7)	4845.0 ± 99.4 (366.1–662.6)
Width	94 (86–102)	60–100	80–160	60–120	62.7 ± 14.3 (42.8–90.5)	60.2 ± 7.6 (47.4–68.1)	75.4 ± 11.8 (48.9–90.9)
Anchors							
Total length	107 (100–112)	43–49	57–62	89–114	42.6 ± 2.5 (35.3–46.8)	58.7 ± 1.5 (56.0–59.9)	101.9 ± 3.8 (95.3–107.8)
Shaft	–	27–30	42–54	70–82	28.9 ± 1.6 (25.4–31.7)	54.1 ± 2.0 (50.4–56.5)	77.7 ± 4.0 (66.6–81.8)
Tip	–	14–16	16–20	25–35	16.0 ± 1.2 (13.9–18.6)	17.6 ± 1.4 (15.3–19.3)	34.4 ± 2.5 (31.3–38.7)
Inner root	–	20–23	11–19	18–40	17.6 ± 1.6 (14.6–21.1)	7.8 ± 1.5 (6.2–10.5)	32.3 ± 1.6 (29.5–35.2)
Outer root	–	4–6	3–6	3–7	2.7 ± 0.6 (1.6–3.9)	1.9 ± 0.3 (1.5–2.5)	3.2 ± 0.7 (1.9–3.9)
Transverse bar							
Total length	47 (43–52)	60–82	36–51	39–41	36.1 ± 3.3 (28.7–42.2)	21.5 ± 2.4 (17.5–23.4)	39.6 ± 1.5 (37.2–42.5)
Width	–	3–5	3–7	–	4.9 ± 0.8 (3.5–7.0)	3.9 ± 0.6 (3.2–4.9)	5.4 ± 1.2 (4.1–8.4)
Hooks	20–24	16–25	15–21	16–20			
I	–	–	–	–	16.2 ± 1.7 (12.2–18.8)	16.3 ± 2.4 (13.0–20.8)	15.8 ± 1.9 (13.2–20.0)
II	–	–	–	–	17.1 ± 1.4 (14.1–19.3)	16.0 ± 2.1 (11.6–17.5)	16.3 ± 2.5 (10.8–19.8)
III	–	–	–	–	15.8 ± 2.0 (12.5–21.6)	20.2 ± 1.3 (17.6–21.7)	19.8 ± 3.2 (14.7–24.6)
IV	–	–	–	–	18.7 ± 1.5 (16.1–21.7)	20.5 ± 1.0 (19.2–22.1)	22.8 ± 2.1 (19.5–26.2)
V	–	–	–	–	17.7 ± 1.4 (14.5–20.2)	19.4 ± 1.3 (17.6–21.1)	20.6 ± 2.3 (16.6–24.4)
VI	–	–	–	–	17.9 ± 1.8 (14.0–20.7)	19.7 ± 1.3 (17.7–21.9)	18.8 ± 1.7 (16.5–22.2)
VII	–	–	–	–	16.3 ± 1.1 (14.2–18.1)	19.2 ± 1.0 (17.8–20.6)	18.8 ± 1.5 (16.5–21.8)
MCO							
Copulatory tube	–	26–29	22–25	24–32	39.7 ± 4.0 (35.4–47.8)	29.6 ± 2.5 (24.8–32.6)	43.4 ± 2.1 (37.5–45.9)
Accessory piece	28 (25–31)	17–22	17–22	17–22	25.2 ± 2.1 (20.8–29.8)	17.3 ± 1.9 (14.9–20.2)	23.5 ± 1.1 (22.0–25.9)
Vagina							
Length	–	24–28	–	–	11.7 ± 3.6 (4.8–19.4)	10.2 ± 2.1 (8.3–14.5)	36.3 ± 3.3 (30.0–40.4)

Table 4 (continued)

Parasite species	Price et al. [20]	Paperna [19]			Present study		
	D. myersi	*D. afrolongicornis*	*D. allolongionchus*	*D. myersi*	*D. afrolongicornis*	*D. allolongionchus*	*D. myersi*
Type host	*E. trimaculatus*	*E.* cf. *kersteni*	*E. perince*	*E. perince*	*E. trimaculatus*	*E. trimaculatus*	*E. trimaculatus*
Width	–	11–13	–	–	10.9 ± 1.4 (8.5–13.2)	8.7 ± 2.3 (5.6–12.8)	7.4 ± 1.8 (4.9–11.7)

should be identified as *D. afrolongicornis* indicating that the transverse bar can vary in the shape and does not hold merit as a diagnostic feature for species identification. Moreover, these two species have never occurred together [14]. Such observation supports our suggestion to consider this species as not valid considering co-infection of *Dactylogyrus* species infecting a single host or a number of closely related species (host-specificity hypothesis) [8, 27]. Such observation also supports the study of Mbokane [13] who discussed the difficulties of differentiating between the two subspecies.

Dactylogyrus allolongionchus Paperna, 1973 (Figs. 7, 8; Table 4).

Body length 313.3 ± 30.8 (283.8–373.7) long; 60.2 ± 7.6 (47.4–68.1) wide across the MCO. Single pair of large anchors: total length 58.7 ± 1.5 (56.0–59.9); shaft 54.1 ± 2.0 (50.4–56.5) long; tip 17.6 ± 1.4 (15.3–19.3) long; inner root 7.8 ± 1.5 (6.2–10.5) long; outer root 1.9 ± 0.3 (1.5–2.5) long. Transverse bar short, separated into two distinct haves and broadly inverted V-shaped: 21.5 ± 2.4 (17.5–23.4) long; width 3.9 ± 0.6 (3.2–4.9). Hooks in seven pairs, dissimilar in size: pairs III and IV longer in size compared with other pairs; hook lengths; pair I 16.3 ± 2.4 (13.0–20.8); pair II 16.0 ± 2.1 (11.6–17.5); pair III 20.2 ± 1.3 (17.6–21.7); pair IV 20.5 ± 1.0 (19.2–22.1); pair V 19.4 ± 1.3 (17.6–21.1); pair VI 19.7 ± 1.3 (17.7–21.9); pair VII 19.2 ± 1.0 (17.8–20.6). Pair of needles located between pairs II and III. MCO complex composed of one complete coiled copulatory tube and an elongated accessory piece that terminates in a spike: copulatory tube 29.6 ± 2.5 (24.8–32.6) long; accessory piece 17.3 ± 1.9 (14.9–20.2). Vagina rounded or with two pointed projections; consisting of a pore, armed with spindle-shaped sclerites: 10.2 ± 2.1 (8.3–14.5) long; 8.7 ± 2.3 (5.6–12.8) wide.

Taxonomic Summary

Type Host

Enteromius perince (Rüppell, 1835) (Cyprinidae).

Type Locality

Lake Albert, Uganda.

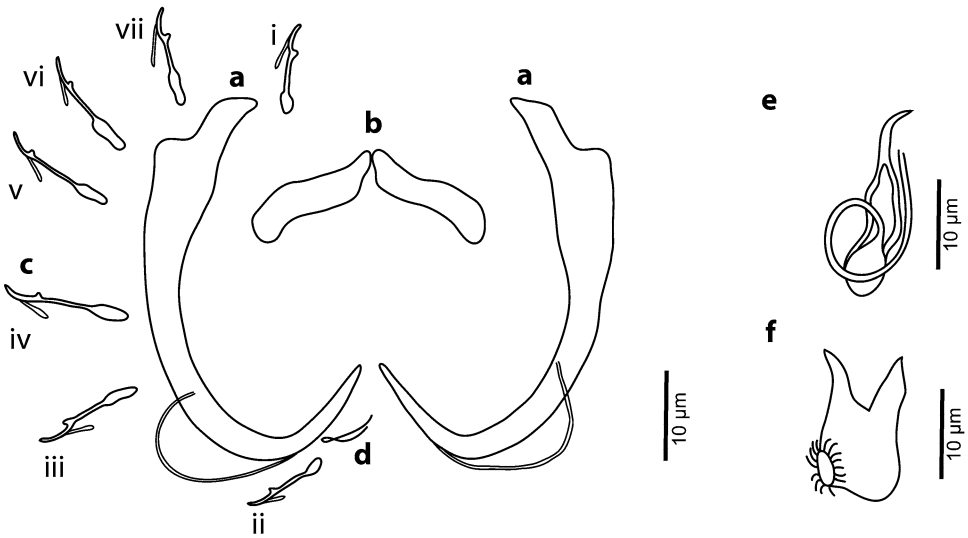

Fig. 7 Drawings of sclerotized structures of *Dactylogyrus allolongionchus* from *Enteromius trimaculatus* (Peters, 1852) collected from Groot Letaba River, Limpopo Province, South Africa. a Anchor. b Transverse bar. c Hook (pairs i–vii). d Needle. e Male copulatory organ. f Vagina. Scale bar = 10 µm

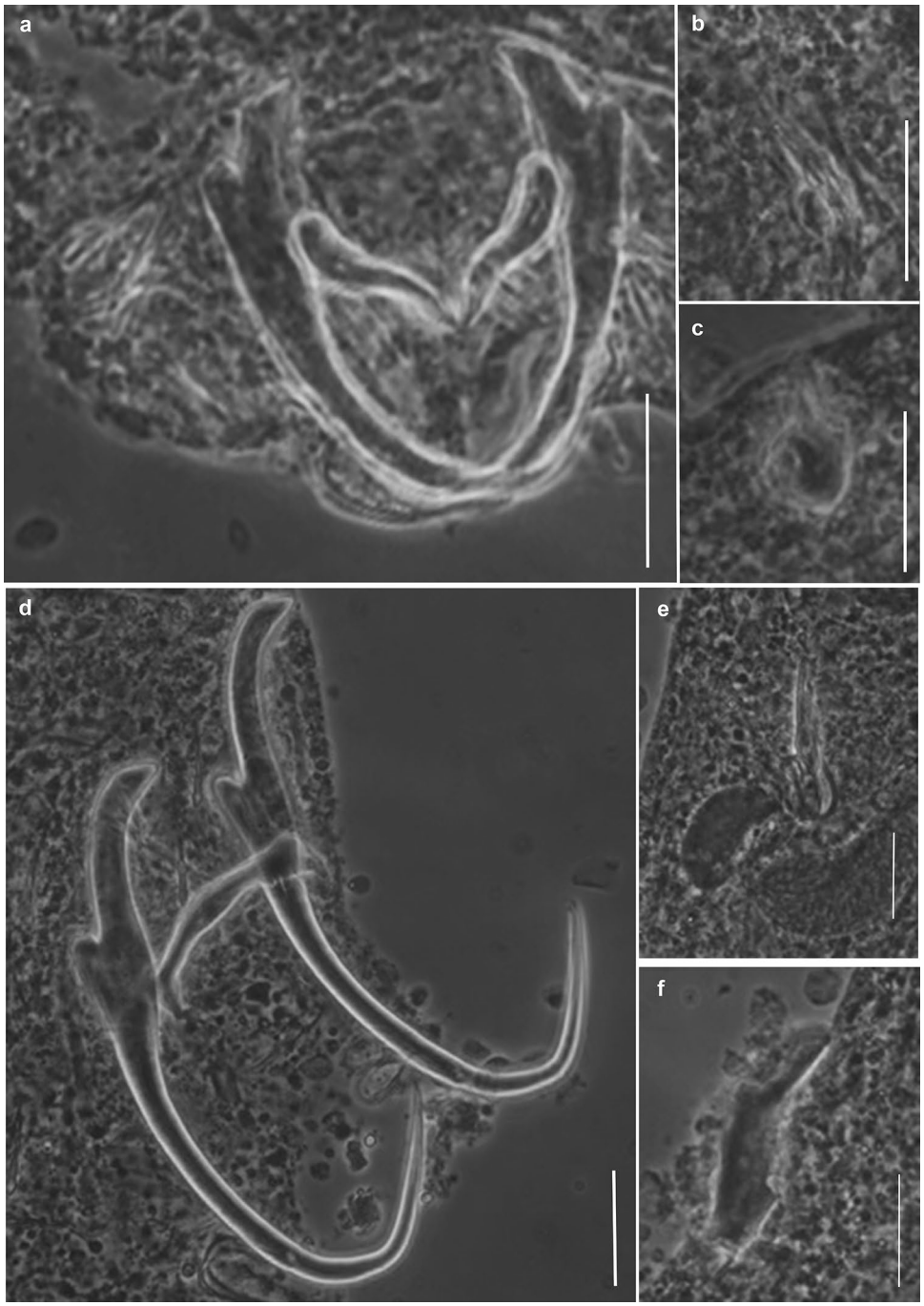

Fig. 8 Microphotographs of sclerotized structures of *Dactylogyrus allolongionchus* (a–c) and *Dactylogyrus myersi* (d–f). a, d Haptoral structures. b, e Male copulatory organ. c, f Vagina. Scale bars = 20 µm

Other Records

Enteromius perince, Lake Albert, Uganda [19]; *Enteromius trimaculatus*, Seshego Dam, Piet Gouws Dam, Mohlapitse River [10] and Middle Letaba Dam [17], Limpopo Province, South Africa.

Present Host and Localities

Enteromius trimaculatus, Middle Letaba Dam (23° 16′ 27.08″ S, 30° 24′ 16.55″ E), Groot Letaba River (23° 41′ 27.58″ S, 30° 35′ 45.16″ E), Letsitele Weir (23° 52′ 19.60″ S, 30° 17′ 55.67″ E), Flag Boshielo Dam (24° 49′ 05″ S, 029° 26′ 39″ E), Luphephe Dam (22° 39′ S, 30° 25′ E) and

Nondweni Dam (23° 41′ 16.84″ S, 30° 51′ 57.78″ E), Limpopo Province, South Africa.

Deposited Material

Three vouchers IPCAS M-673; two vouchers M.T. 38232–3; two vouchers NMBP 458–9.

Site

Gill lamellae.

Comments

This parasite was described from *E. perince* in Uganda [18] and later recorded from the same host in Uganda [19]. The other records of this species are those of Mashego [10] and Olivier et al. [17]. In the present study, this parasite was retrieved from the gills of *E. trimaculatus*. The shapes of the haptoral sclerites and reproductive organs of present specimens are identical with those given in the original description and their measurements fall within size ranges from the previous findings [10, 19] (Table 4).

Dactylogyrus myersi Price, McClellan, Druckenmiller et Jacobs, 1969 (Figs. 8 and 9; Table 4).

Body length 4845.0 ± 99.4 (366.1–662.6) long; 75.4 ± 11.8 (48.9–90.9) wide usually across the MCO. Single pair of long anchors, about one-third the body length at times: total length 101.9 ± 3.8 (95.3–107.8); shaft 77.7 ± 4.0 (66.6–81.8) long; tip 34.4 ± 2.5 (31.3–38.7) long; inner root 32.3 ± 1.6 (29.5–35.2) long; outer root 3.2 ± 0.7 (1.9–3.9) long. Transverse bar characterised by spiny projections at each extremity: 39.6 ± 1.5 (37.2–42.5) long; width 5.4 ± 1.2 (4.1–8.4). Hooks in 7 pairs, dissimilar in size: pair IV longer in size compared with other pairs; hook lengths; pair I 15.8 ± 1.9 (13.2–20.0); pair II 16.3 ± 2.5 (10.8–19.8); pair III 19.8 ± 3.2 (14.7–24.6); pair IV 22.8 ± 2.1 (19.5–26.2); pair V 20.6 ± 2.3 (16.6–24.4); pair VI 18.8 ± 1.7 (16.5–22.2); pair VII 18.8 ± 1.5 (16.5–21.8). Pair of needles located between pairs II and III. MCO complex composed of coiled copulatory tube and an elongated accessory piece that terminates in a spike: copulatory tube 43.4 ± 2.1 (37.5–45.9) long; accessory piece 23.5 ± 1.1 (22.0–25.9). Vagina elongated, armed with spindle-shaped denticulate sclerites posteriorly: 36.3 ± 3.3 (30.0–40.4) long; 7.4 ± 1.8 (4.9–11.7) wide.

Taxonimic Summary

Type Host

Enteromius trimaculatus Cyprinidae).

Type Locality

Pongola River, Lydenburg, South Africa.

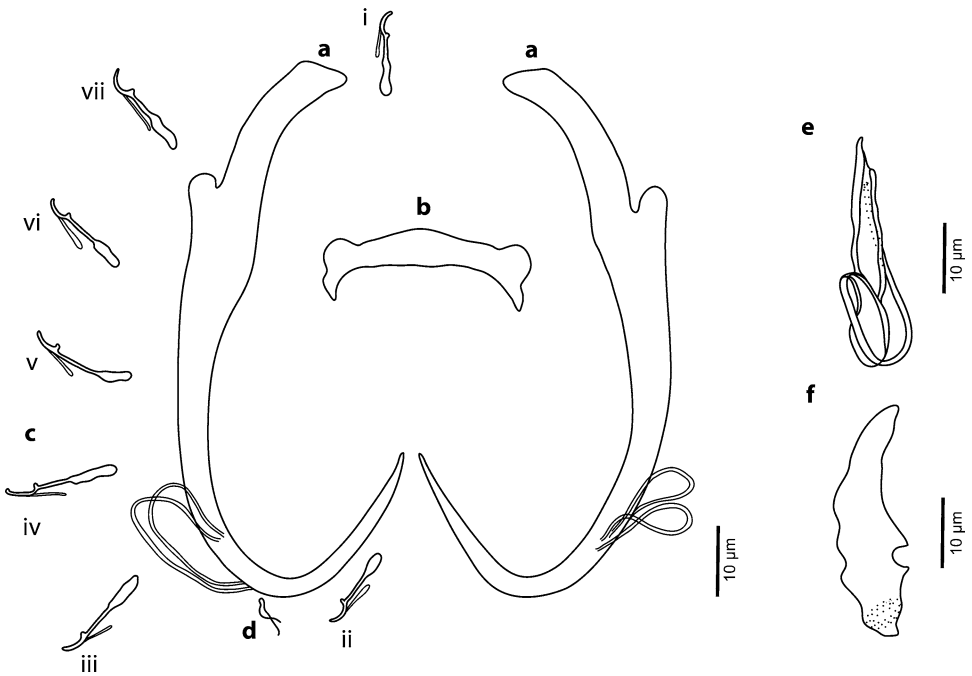

Fig. 9 Drawings of sclerotized structures of *Dactylogyrus myersi* from *Enteromius trimaculatus* (Peters, 1852) collected from Letsitele Weir, Limpopo Province, South Africa. a Anchor. b Transverse bar. c Hook (pairs i–vii). d Needle. e male copulatory organ. f Vagina. Scale bar = 10 µm

Other Records

Enteromius perince, Lake Albert, Uganda and *E. trimaculatus*, Pongola River, Natal, South Africa [19]; *E. trimaculatus*, Seshego Dam [10] and Middle Letaba Dam [17], Limpopo Province, South Africa.

Present Host and Localities

Enteromius trimaculatus, Middle Letaba Dam (23° 16′ 27.08″ S, 30° 24′ 16.55″ E), Groot Letaba River (23° 41′ 27.58″ S, 30° 35′ 45.16″ E), Letsitele Weir (23° 52′ 19.60″ S, 30° 17′ 55.67″ E), Flag Boshielo Dam (24° 49′ 05″ S, 029° 26′ 39″ E) and Nondweni Dam (23° 41′ 16.84″ S, 30° 51′ 57.78″ E), Limpopo Province, South Africa.

Deposited Material

Three vouchers IPCAS M-674; two vouchers M.T. 38235–6; three vouchers NMBP 460–2.

Site

Gill lamellae.

Remarks

This parasite was first described from *E. trimaculatus* in Lydenburg, South Africa [20] and later recorded from *E. perince* in Uganda and *E. trimaculatus* in South Africa [19]. The other southern African records of this species are those of Mashego [10] and Olivier et al. [17] from the gills of *E. trimaculatus*. In this study the parasite was retrieved from the gills of the type host. The shapes and dimensions of the sclerotized structures of the haptor and reproductive system correspond with previous studies and were found to be within similar ranges (Table 4).

Discussion

Amongst monogeneans, Dactylogyridae is the most speciose family mainly occurring on the gills of cyprinoid fishes [5], with the genus *Dactylogyrus* being one of the most species rich within the family [26].

The description of three new *Dactylogyrus* species found from the gills of two *Enteromius* species in the present study results in a total number of 13 valid species of *Dactylogyrus* known in South Africa. The finding of *D. afrohamiltoni* sp. nov. represents the first record of monogenean on *E. afrohamiltoni*. The strict specialists, one parasite species being known from a only one host species, are not found often. From all 35 *Dactylogyrus* spp. which have been found on *Enteromius* hosts, only *Dactylogyrus mawli* Paperna, 1969 and *Dactylogyrus enidae* Mashego, 1983 have been described from a single host, *Enteromius macrops* (Boulenger, 1911) and *Entromius neefi* (Greenwood, 1962), respectively [21]. Additional 20 out of 35 *Dactylogyrus* species parasitizing these small barbs have been reported from a single host species but they can co-exist on their host with other/s species. *Dactylogyrus limpopoensis* sp. nov. and *D. letabaensis* sp. nov. were found to co-exist on *E. unitaeniatus* which represent inter-specific associations, previously observed from European leuciscids [24, 25]. Olivier et al. [17] reported one *Dactylogyrus* species from *E. unitaeniatus* from the Middle Letaba Dam, which has not been identified to species level, but theoretically it could be one of the species being described in the present study.

Dactylogyrus afrolongicornis alberti was originally described from *E. perince* [18] and later recorded from *E.* cf. *kersteni* in Uganda [19]. It has been proposed to be distinct from *D. afrolongicornis afrolongicornis* in having heavy and thicker bar plates only. Otherwise both subspecies are identical in the shape of haptoral structure and MCO [19]. Mbokane [13] argued that *D. afrolongicornis alberti* is morphologically indistinguishable from *D. afrolongicornis afrolongicornis*. Comparing specimens from the present study, however, validates our specimens as *D. afrolongicornis* only, and we have to state that both subspecies are indistinguishable by not having stable characters on which the identification could be based on. As part of the present study, it was observed that due to this soft to weakly sclerotized or non-sclerotized membrane in some instances, the bar may vary depending on the position or mounting alterations of the worm. Moreover, these two subspecies have never been observed together [14], and this supports our suggestion to consider these two subspecies as not valid and keep *D. afrolongicornis* only.

Triplet of species, *D. afrolongicornis*, *D. allolongionchus* and *D. myersi* were found to co-occurre on the gills of *E. trimaculatus*. This finding represents the first report of such co-occurence for these particular three species. The co-existence of several *Dactylogyrus* species on their leuciscids host is common phenomena [24, 25]. Out of 22 *Enteromius* spp., which are currently known as hosts for *Dactylogyrus* parasites, 13 fish species have been recorded to have two or more parasite species [21]. Hosts with the most species of *Dactylogyrus* reported are *E. kersteni* and *E. perince* with eight and seven, respectively [21].

The identification of three new species of *Dactylogyrus* indicates an existing potential that the species richness of these parasites on fish from African continent itself can be much higher than is currently known. In South Africa Crafford et al. [2] described three new *Dactylogyrus* species on two barb species of *Labeo* or study of Musilová et al. [16],

who described three species of the genus from *Labeo coubie* Rüppell, 1832 from Senegal, West Africa. Moreover, short communication by Truter et al. [28] suggests there might be many more unknown *Dactylogyrus* species on African *Enteromius,* especially taking into account the parasites' great diversity together with high diversity of their potential hosts and it does not really matter if they have been previously studied for parasites or not. In this moment, the search in fish database results in 213 valid *Enteromius* species of which only 21 have been recorded to host *Dactylogyrus* spp. These numbers clearly indicate that the potential for finding many new dactylogyrid species is enormous taking into consideration other fish genera that can be parasitized by this group of Platyhelminthes.

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Compliance with Ethical Standards

Conflict of Interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Appendix 4

Table I: Maximum Likelihood fits of 24 different nucleotide substitution models for the combined 18S rDNA and ITS-1–5.8S rDNA

Model	#Param	BIC	AICc	lnL	Invariant	Gamma	R	Freq A	Freq T	Freq C	Freq G	A=>T	A=>C	A=>G	T=>A	T=>C	T=>G	C=>A	C=>T	C=>G	G=>A	G=>T	G=>C
K2+G+I	84	16154,84616	15438,90385	-7635,260249	0,329515042	0,454633306	2,0500764	0,25	0,25	0,25	0,25	0,04	0,04	0,17	0,04	0,17	0,04	0,04	0,17	0,04	0,17	0,04	0,04
T92+G+I	85	16159,73252	15435,27166	-7632,439581	0,327883246	0,451412395	2,05219847	0,253394938	0,253394938	0,246605062	0,246605062	0,04	0,04	0,17	0,04	0,17	0,04	0,04	0,17	0,04	0,17	0,04	0,04
K2+G	83	16166,1123	15458,68865	-7646,157162	n/a	0,231495432	2,01068724	0,25	0,25	0,25	0,25	0,04	0,04	0,17	0,04	0,17	0,04	0,04	0,17	0,04	0,17	0,04	0,04
HKY+G+I	87	16170,34226	15428,84463	-7627,216768	0,32946986	0,452033751	2,05644429	0,233802062	0,272987813	0,233453864	0,259756261	0,04	0,04	0,18	0,04	0,16	0,04	0,04	0,18	0,04	0,16	0,04	0,04
T92+G	84	16170,76069	15454,81838	-7643,217514	n/a	0,231338386	2,01254623	0,253394938	0,253394938	0,246605062	0,246605062	0,04	0,04	0,16	0,04	0,16	0,04	0,04	0,17	0,04	0,17	0,04	0,04
GTR+G+I	91	16171,65444	15396,08456	-7606,817486	0,334188688	0,472359782	1,84986979	0,233802062	0,272987813	0,233453864	0,259756261	0,07	0,04	0,16	0,06	0,16	0,03	0,04	0,19	0,03	0,14	0,04	0,03
TN93+G+I	88	16175,96211	15425,94625	-7624,762849	0,325386961	0,450708206	2,04561996	0,233802062	0,272987813	0,233453864	0,259756261	0,04	0,04	0,15	0,04	0,18	0,04	0,04	0,2	0,04	0,14	0,04	0,04
HKY+G	86	16180,67743	15447,69813	-7637,648194	n/a	0,22940534	2,01755931	0,233802062	0,272987813	0,233453864	0,259756261	0,05	0,04	0,17	0,04	0,16	0,04	0,04	0,18	0,04	0,16	0,05	0,04
TN93+G	87	16185,3781	15443,88046	-7634,734686	n/a	0,23139601	2,01111985	0,233802062	0,272987813	0,233453864	0,259756261	0,05	0,04	0,15	0,04	0,17	0,04	0,04	0,2	0,04	0,14	0,05	0,04
GTR+G	90	16187,30008	15420,2481	-7619,904148	n/a	0,232418157	1,79293336	0,233802062	0,272987813	0,233453864	0,259756261	0,07	0,04	0,16	0,06	0,16	0,03	0,04	0,19	0,04	0,14	0,04	0,03
K2+I	83	16596,15108	15888,72743	-7861,17655	0,51773765	n/a	1,8420027	0,25	0,25	0,25	0,25	0,04	0,04	0,16	0,04	0,16	0,04	0,04	0,16	0,04	0,16	0,04	0,04
T92+I	84	16603,05013	15887,10782	-7859,362232	0,517706531	n/a	1,84035688	0,253394938	0,253394938	0,246605062	0,246605062	0,04	0,04	0,16	0,04	0,16	0,04	0,04	0,16	0,04	0,16	0,04	0,04
HKY+I	86	16614,52499	15881,54569	-7854,571974	0,517734231	n/a	1,84633457	0,233802062	0,272987813	0,233453864	0,259756261	0,05	0,04	0,17	0,04	0,15	0,05	0,04	0,18	0,05	0,15	0,05	0,04
GTR+I	90	16615,04614	15847,99416	-7833,777179	0,517216184	n/a	1,56938799	0,233802062	0,272987813	0,233453864	0,259756261	0,08	0,04	0,16	0,07	0,14	0,04	0,04	0,17	0,04	0,14	0,04	0,04
TN93+I	87	16622,70409	15881,20646	-7853,397683	0,517371307	n/a	1,83328271	0,233802062	0,272987813	0,233453864	0,259756261	0,05	0,04	0,16	0,04	0,16	0,05	0,04	0,19	0,05	0,14	0,05	0,04
JC+G+I	83	16627,61312	15920,18946	-7876,907568	0,299876114	0,4377855	0,5	0,25	0,25	0,25	0,25	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08
JC+G	82	16635,20727	15936,30238	-7885,968488	n/a	0,237211178	0,5	0,25	0,25	0,25	0,25	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08
JC+I	82	17043,90836	16345,00347	-8090,319034	0,516685081	n/a	0,5	0,25	0,25	0,25	0,25	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08
K2	82	17704,7124	17005,80751	-8420,721053	n/a	n/a	1,61317248	0,25	0,25	0,25	0,25	0,05	0,05	0,15	0,05	0,15	0,05	0,05	0,15	0,05	0,15	0,05	0,05
T92	83	17710,6993	17003,27564	-8418,450659	n/a	n/a	1,61291943	0,253394938	0,253394938	0,246605062	0,246605062	0,05	0,05	0,15	0,05	0,15	0,05	0,05	0,16	0,05	0,16	0,05	0,05
TN93	86	17714,99558	16982,01628	-8404,80727	n/a	n/a	1,61663827	0,233802062	0,272987813	0,233453864	0,259756261	0,05	0,04	0,13	0,04	0,17	0,05	0,04	0,2	0,05	0,12	0,05	0,04
GTR	89	17721,4374	16962,90343	-8392,236652	n/a	n/a	1,40968842	0,233802062	0,272987813	0,233453864	0,259756261	0,08	0,04	0,13	0,07	0,15	0,05	0,04	0,18	0,05	0,12	0,05	0,04
HKY	85	17728,17458	17003,71372	-8416,660612	n/a	n/a	1,61372912	0,233802062	0,272987813	0,233453864	0,259756261	0,05	0,04	0,16	0,04	0,14	0,05	0,04	0,17	0,05	0,14	0,05	0,04
JC	81	18116,81257	17426,42655	-8632,034983	n/a	n/a	0,5	0,25	0,25	0,25	0,25	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08

Table II: Maximum Likelihood fits of 24 different nucleotide substitution models for 28S rDNA

Model	#Param	BIC	AICc	lnL	Invariant	Gamma	R	Freq A	Freq T	Freq C	Freq G	A=>T	A=>C	A=>G	T=>A	T=>C	T=>G	C=>A	C=>T	C=>G	G=>A	G=>T	G=>C
HKY+G	42	9712,82417	9395,336294	-4655,541125	n/a	0,346471366	2,17761519	0,222526124	0,277158286	0,208850551	0,29146504	0,04	0,03	0,2	0,03	0,14	0,05	0,03	0,19	0,05	0,15	0,04	0,03
GTR+G+I	47	9715,510428	9360,259545	-4632,971044	0,285362055	0,729522622	1,76703335	0,222526124	0,277158286	0,208850551	0,29146504	0,08	0,02	0,18	0,06	0,14	0,05	0,03	0,19	0,03	0,14	0,05	0,02
TN93+G	43	9716,377843	9391,336799	-4652,53532	n/a	0,353998878	2,13597941	0,222526124	0,277158286	0,208850551	0,29146504	0,04	0,03	0,17	0,03	0,17	0,05	0,03	0,22	0,05	0,13	0,04	0,03
GTR+G	46	9716,622137	9368,923289	-4638,309541	n/a	0,360824167	1,70033933	0,222526124	0,277158286	0,208850551	0,29146504	0,08	0,03	0,18	0,06	0,14	0,05	0,03	0,18	0,04	0,14	0,05	0,03
HKY+G+I	43	9719,833835	9394,792791	-4654,263316	0,272729554	0,664902012	2,18033752	0,222526124	0,277158286	0,208850551	0,29146504	0,04	0,03	0,2	0,03	0,14	0,05	0,03	0,19	0,05	0,15	0,04	0,03
TN93+G+I	44	9724,003411	9391,409483	-4651,565462	0,241717497	0,615752181	2,13867072	0,222526124	0,277158286	0,208850551	0,29146504	0,04	0,03	0,17	0,03	0,17	0,05	0,03	0,22	0,05	0,13	0,04	0,03
K2+G	39	9730,165151	9435,338476	-4678,559541	n/a	0,357674915	2,08338255	0,25	0,25	0,25	0,25	0,04	0,04	0,17	0,04	0,17	0,04	0,04	0,17	0,04	0,17	0,04	0,04
K2+G+I	40	9737,141346	9434,760654	-4677,264997	0,271248266	0,685536509	2,08554682	0,25	0,25	0,25	0,25	0,04	0,04	0,17	0,04	0,17	0,04	0,04	0,17	0,04	0,17	0,04	0,04
T92+G	40	9739,822782	9437,442091	-4678,605715	n/a	0,357666228	2,08347324	0,249842205	0,249842205	0,250157795	0,250157795	0,04	0,04	0,17	0,04	0,17	0,04	0,04	0,17	0,04	0,17	0,04	0,04
T92+G+I	41	9746,795518	9436,861092	-4677,309441	0,271366462	0,685787157	2,08565875	0,249842205	0,249842205	0,250157795	0,250157795	0,04	0,04	0,17	0,04	0,17	0,04	0,04	0,17	0,04	0,17	0,04	0,04
HKY+I	42	9795,854477	9478,366601	-4697,056278	0,503245666	n/a	1,97675174	0,222526124	0,277158286	0,208850551	0,29146504	0,05	0,03	0,19	0,04	0,14	0,05	0,04	0,19	0,05	0,15	0,05	0,03
GTR+I	46	9802,185648	9454,486799	-4681,091296	0,502386489	n/a	1,55455862	0,222526124	0,277158286	0,208850551	0,29146504	0,08	0,03	0,18	0,06	0,13	0,06	0,03	0,17	0,04	0,14	0,06	0,03
TN93+I	43	9803,318758	9478,277715	-4696,005777	0,50141328	n/a	1,94560102	0,222526124	0,277158286	0,208850551	0,29146504	0,05	0,03	0,18	0,04	0,15	0,05	0,04	0,2	0,05	0,14	0,05	0,03
K2+I	39	9810,366017	9515,539342	-4718,659974	0,50211803	n/a	1,90952487	0,25	0,25	0,25	0,25	0,04	0,04	0,16	0,04	0,16	0,04	0,04	0,16	0,04	0,16	0,04	0,04
T92+I	40	9820,009225	9517,628533	-4718,698936	0,50212351	n/a	1,9096511	0,249842205	0,249842205	0,250157795	0,250157795	0,04	0,04	0,16	0,04	0,16	0,04	0,04	0,16	0,04	0,16	0,04	0,04
JC+G	38	10020,38197	9733,109594	-4828,450592	n/a	0,386308567	0,5	0,25	0,25	0,25	0,25	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08
JC+G+I	39	10027,01264	9732,185968	-4826,983287	0,294394391	0,821259119	0,5	0,25	0,25	0,25	0,25	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08
JC+I	38	10086,21396	9798,941586	-4861,366588	0,499311238	n/a	0,5	0,25	0,25	0,25	0,25	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08
GTR	45	10251,91298	9911,766447	-4910,737603	n/a	n/a	1,45124426	0,222526124	0,277158286	0,208850551	0,29146504	0,07	0,04	0,14	0,06	0,15	0,07	0,04	0,2	0,03	0,11	0,07	0,02
TN93	42	10253,9477	9936,459822	-4926,102889	n/a	n/a	1,68979075	0,222526124	0,277158286	0,208850551	0,29146504	0,05	0,04	0,14	0,04	0,17	0,05	0,04	0,22	0,05	0,11	0,05	0,04
K2	38	10270,49233	9983,219958	-4953,505774	n/a	n/a	1,68407721	0,25	0,25	0,25	0,25	0,05	0,05	0,16	0,05	0,16	0,05	0,05	0,16	0,05	0,16	0,05	0,05
HKY	41	10274,38484	9964,450416	-4941,104103	n/a	n/a	1,68794521	0,222526124	0,277158286	0,208850551	0,29146504	0,05	0,04	0,18	0,04	0,13	0,05	0,04	0,18	0,05	0,14	0,05	0,04
T92	39	10280,1707	9985,344025	-4953,562315	n/a	n/a	1,68409779	0,249842205	0,249842205	0,250157795	0,250157795	0,05	0,05	0,16	0,05	0,16	0,05	0,05	0,16	0,05	0,16	0,05	0,05
JC	37	10527,19063	10247,47284	-5086,637565	n/a	n/a	0,5	0,25	0,25	0,25	0,25	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08