Taxonomy of the rectal endociliates of *Xenopus laevis* (Anura)

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Abstract

Amphibians are hosts to a variety of parasite groups which include protozoans, cestodes, nematodes, acanthocephalans, monogenetic flukes, digenetic flukes, and mites. Nevertheless, of these parasite groups infecting amphibians, the protozoan gut ciliates are perhaps the most abundant, yet under-studied, group.

Even in one of the world's most widely distributed amphibian invaders and well-known model, the African Clawed Frog *Xenopus laevis* (Daudin, 1802) (Anura: Pipidae), the study of protozoan parasites has fallen behind that of its metazoan counterparts. Former studies that documented intestinal ciliates of amphibians from South Africa are limited and most took place decades ago. Furthermore, these studies primarily dealt with the systematics of ciliate groups whereas most did not even identify ciliates beyond the genus level. Consequently, difficulties arise when rectal endociliate assemblages are compared among populations of the host, in their native range as well as elsewhere.

Therefore, the aim of this study was to document the gut ciliate diversity of *X. laevis,* from localities in both South Africa and France, by using an integrated taxonomic approach. Hence, for the first time, the ciliates of *X. laevis* were identified both morphologically and molecularly through modern as well as classic techniques. These techniques included differential interference contrast advanced microscopy, scanning electron microscopy, confocal microscopy, silver impregnation, haematoxylin staining and polymerase chain-reaction tests.

The gut protozoans of *X*. *laevis* from South Africa were documented as four species namely: 1) *Balantidium kirbyi*, 2) *Balantidium* sp. 1, 3) *Balantidium* sp. 2 and 4) *Protoopalina* sp. 1. Correspondingly, the gut protozoans of *X. laevis* from France were documented as: 1) *B. kirbyi*, 2) *Balantidium* sp. 3, *Balantidium* sp. 4 and 4) *Protoopalina* sp. 2.

Not only were molecular data of endociliates of frogs from South Africa obtained for the first time but the foundation for a working method of ciliate identification were laid by this study. Accordingly, this integrated approach will ease future ciliate identification in other anuran hosts and furthermore allow a better understanding of the host-parasite relationship, ecological role, and diversity of ciliates.

Keywords: Protozoa, Ciliata, *Xenopus*, rectal endociliates, diversity

Table of contents

Chapter 1:

Introduction

1.1 Frogs and their parasites

Amphibians were the first vertebrates to colonise the land and today they constitute a very successful group of animals with a near global distribution. However, amphibian numbers are declining to the point that this group is nowadays seen as the world's most endangered vertebrate group (Stuart *et al.*, 2004). There are currently 8588 amphibian species known to science (Frost, 2023), and of them 177 are known from Southern Africa (Du Preez & Carruthers, 2022). Despite South Africa's rich amphibian diversity, amphibian numbers are also declining in South Africa (Measey, 2011).

Amphibian decline appears to be primarily a consequence of habitat deterioration. Thereafter, *inter alia* diseases, parasites, pollution and poor water quality threaten amphibians' survival (Beebee & Griffiths, 2005). Given the critical role of amphibians in ecosystems and their spectacular diversity, their conservation is critical (West, 2018). Amphibians mainly contribute to ecosystems by direct and indirect alteration of ecosystem functions which include bioturbation, nutrient dynamics, and food web effects (Hocking & Babbitt, 2014). Furthermore, tadpoles are known to have a considerable effect on biomass and algal or periphyton community structures (Alford and Wilbur 1985; Morin 1987; Altig *et al*., 2007). Moreover, amphibians can alter disease transmission and pest outbreaks. For example, predatory amphibians can help reduce the spread of insect-borne illnesses through predation (Valencia-Aguilar *et al*., 2013).

Like all other animals, amphibians host a variety of parasites covering all major parasite groups (Hoff *et al*., 1984). Since South Africa flaunts with a rich amphibian diversity, it is ideally positioned for a study of the drivers behind amphibian declines, specifically in relation to the association between amphibian hosts and their parasites and pathogens. If parasite diversity correlates with amphibian diversity, parasites can be use as proxy or bioindicators to explore the possible drivers behind declining amphibian numbers. Bioindicators are used to assess the quality of a changing environment over time and may include communities, species, or biological processes. The parasites of amphibians especially the commensal protozoan amphibian gut flora can be possible bioindicators when investigating amphibian declines. Nevertheless, in order to use them as bioindicators, their taxonomy should first be resolved.

Amphibians act as hosts to a variety of parasite groups, including protozoans, cestodes, nematodes, acanthocephalans, monogenetic flukes, digenetic flukes and acari. Of all parasite groups infecting amphibians, the protozoan gut ciliates are perhaps the most abundant, yet understudied group. Ciliates are probably the largest and most specialised group of protozoans. Nevertheless, there are several unknowns regarding the taxonomy of ciliates. Like other protozoans, their small size inflicts logistical difficulties, especially in the former part of the 20th century when there was no convention for depositing type specimens of ciliates. Even though more modern methods for ciliate visualisation and sequencing were introduced over recent years and several studies (Foissner, 2014; Warren *et al*., 2017) provide guides and information on these methods, no formal standards for the application of these methods to taxonomic identification or descriptions of ciliates exist. the difficulties regarding ciliate identification pose a critical knowledge gap, since ciliates can act as valuable environmental indicators as a result of their short life cycles and sensitive cell membranes (Cairns *et al*., 1972). Moreover, Collymore *et al.* (2013) found that ciliate numbers seem to correlate with the body condition of their hosts. In addition, ciliates may be valuable informers of changing environments causing amphibian declines.

1.2 Ciliates and their history

Protozoans are defined as single or colony-forming one-celled organisms able to perform coordinated processes of life within the boundaries of a single cell. In the seventeenth century, Anton van Leeuwenhoek was probably the first person to observe ciliates as single-celled organisms through a microscope. In those days, ciliates were known as *infusoria* since they were visible in both freshwater and marine habitats, similar to algal blooms. Taxonomic research was expanded in the nineteenth century by Bütschli, Claparède, Dujardin, Kent, Lachmann, Maupas and Stein. The term *infusoria* was used until the early twentieth century, when it changed to Ciliophora (Doflein, 1901), which is still used today.

Lynn (2008) divided the history of ciliate systematics into five periods, namely: i) the Discovery Age; ii) the Exploitation Age; iii) the Infraciliature Age; iv) the Ultrastructure Age; and v) the Refinement Age. Across all these ages, several techniques and technologies contributed to unveiling the diversity of ciliates that are known to science today.

In the early ages, ciliates could only be observed in an unstained, living state using light microscopes. Later, during the Age of Infraciliature, silver staining techniques, like the “Chatton-Lwoff” technique, revealed further ciliate characteristics such as basal bodies. The Chatton-Lwoff technique provided information for Fauré-Fremiet’s next revision of ciliate classification (Fauré-Fremiet, 1950) and was formalised by Corliss (1956). In the age of Ultrastructure, electron microscopy aided in the formulation of the structural conservatism hypothesis. The structural conservatism hypothesis states that the maintenance of a structure, which, in case of ciliates, are the unit organelles and kinetodesma, is inversely related to the level of its biological organisation through time. In addition, the examination of structure variations and patterns of microtubular parts supports the structural conservatism of the ciliate cortex. Thus, depending on the role of the structure, the microtubular structures’ variability decreases, or the conservation of the structure increases, as one move from the top to lower levels of biological organization (Lynn, 2008). Later, a radically new classification system forming the base of revised classifications was presented by Lynn and Small (2002). Finally, the present Age of Refinement is characterised by gene sequencing (Lynn, 2008).

1.3 Endociliates

1.3.1 State of knowledge on endo-ciliates: symbiotic/parasitic.

Protozoa are part of the biota of diverse ecosystems, playing an important role in soil and water as well as inside animal hosts. Some protozoans are grazers of phytoplankton in marine habitats, other balance bacterial populations by predation. Heterotrophic protists can be a valuable food source and, finally, protozoans that spend parts of their life cycles in animal hosts can aid in nutrient cycling (Dopheide, *et al*., 2009). The latter are commonly known as endociliates and are found in a wide range of animal groups, including insects (Vďačný, 2020), fish (Duncan, 1977), amphibians, (Wilbert & Schmeier, 1982; Senler & Yildiz, 2000; Li *et al*., 2013) and reptiles (Rataj *et al*., 2011). In a study by Fokam *et al*. (2014), commensal ciliates of the genus *Nyctotherus* were described from the digestive tract of a terrestrial earthworm from Cameroon.

Although many free-living ciliates exist, more than 140 families of ciliates (Alveolata, Ciliophora) are known to be in a symbiotic relationship with animals (Mayén-Estrada *et al*., 2021). Corliss (2000) stated that 2600 ciliate species belonging to eight classes which are Armophorea, Heterotrichea, Litostomatea, Nassophorea, Oligohymenophorea, Plagiopylea, Phyllopharyngea and Spirotrichea have been described as symbionts. Symbiosis is defined as a sustained relationship between two or more individuals from different species. This relationship is transmitted from one generation to the next or acquired de novo in each generation (Oulhen *et al*., 2016). A wide diversity of symbiotic relationships exists in ciliates which include, commensalism, epibiosis, mutualism and parasitism.

Rumen ciliates, like Litostomatea and sub-class Trichostomatia, live as endosymbionts in the intestine of ruminants (Verni & Gualtier, 1997). These ciliates feed on bacteria and non-living particles and make use of cellulose, hemicellulose, starch, sugars, and lipids as energy sources (Verni & Gualtier,1997). Although the role of ciliate protozoa in ruminants are not quite known yet, it seems that the presence thereof benefits the rest of the microbial community and subsequently the ruminant itself. The presence of ciliates results in a more stable and degrative complex ruminal fermentation which improve community resilience (Coleman, 1986). The anaerobic degradation also benefits from rumen ciliates scavenging on the oxygen entering the rumen (Ellis *et al*., 1989). Furthermore, rumen ciliates can contribute in transferring bacterial nitrogen to higher trophic levels as well as balanced bacterial populations (Coleman, 1989; Bonhomme, 1990;

Williams & Coleman, 1997). In addition, better liquid volume turnover rate of ruminal contents is observed when compared with fauna-free ruminants (Veira, 1986; Ushida, 2018). Nevertheless, the presence of ciliates in the rumen is not regard as crucial for the survival ruminants (Williams & Coleman, 1997).

Then again, ciliates can be pathogenic — for example, the intestinal mammalian pathogen, *Balantidium coli,* can also infect humans (Schuster & Ramirez-Avila, 2008), causing persistent diarrhoea and dysentery. Moreover, the fish ciliate pathogen *Ichthyophthirius multifiliis*, also known as “white spot disease”, causes great losses in the fishing industry (Dickerson, 2006).

1.3.2 Current knowledge about ciliates in amphibians

The rectal protozoan fauna of amphibians is known to be particularly species-rich (Fantham, 1922; Wilbert & Schmeier, 1982; Delviniquier *et al*., 1995). This protozoan fauna composes of different genera which, amongst others, include *Balantidium*, *Cepedea*, *Opalina*, *Nyctotherus* and *Protoopalina*. Of these, *Nyctotherus* and *Balantidium* are both classified in the phylum Ciliophora, whereas *Cepedea*, *Opalina* and *Protoopalina* are found within the phylum Bigyra. In addition, the latter mentioned are not considered to be true ciliates, but rather flagellates (Adl *et al*., 2019). Nevertheless, in this study “endociliates” will refer to all protozoan fauna, both true-ciliates and ciliate-like organisms.

The genus *Balantidium* described by Claparède & Lachmann (1858), is currently classified below the supergroup SAR, superphylum Alveolata (Figure 1) (Adl *et al*., 2019). *Balantidium* species are known to possess a specialised field of somatic cilia to the right of the vestibulum or dextoral location called the “Villeneuve-Brachon’s” field. Villeneuve-Brachon (1940) was the first to describe a large special field of somatic kineties to the right of the vestibulum in the type species *Balantidium entozoon* Clapare`de and Lachmann, 1858 (*Bursaria entozoon* Ehr, 1838), found in a frog host. Later, Fernández-Galiano (1952) named this somatic kinetal area the Villeneuve–Brachon’s (V–B) field. *Balantidium* differs from genera of the three families of the Order,Trichostomatia in regard to the absence of concrement vacuoles and the presence of an oral cavity in the anterior part of the body (Lynn & Small, 2002). Numerous *Balantidium* species have been reported from freshwater, marine and terrestrial habitats, especially from fish (Li *et al*., 2007; Li *et al*., 2009; Li *et al*., 2011; Li *et al*., 2012) and amphibians (Li *et al*., 2008; Grim & Buonanno, 2009; Chistyakova *et al*., 2014; Li *et al*., 2014).

Known species of the genus *Balantidium* infecting amphibians include 31 species: *B. entozoon* Ehrenberg, 1838; *B. duodeni* (Stein,1867); *B. elongatum* (Stein,1867); *B. helenae* (Bezzenberger, 1903); *B. gracile* (Bezzenberger, 1903); *B. rotundum* (Bezzenberger, 1903); *B. giganteum* (1904); *B. gracile* (1904)*; B. falciformis* (Walker, 1909); *B. ovale* (Dobell, 1910); *B. hyalinum* (Dobell, 1910); *B*. *ranarum* (Ghosh,1921); *B*. *bicavata* (Bhatia and Gulati, 1927); *B*. *amygdalli* (Bhatia and Gulati,1927); *B*. *amblystomatis* (Jírovec, 1930); *B*. *sushilii* (Ray, 1932); *B*. *sinenesis* (Nie,1935); *B*. *kirbyi* (Rodriguez, 1939); *B*. *xenopi* (Du Puytorac and Grain, 1965); *B*. *rayi* (Pal and Dasgupta, 1978); *B*. *tylototritonis* (Pal and Dasgupta, 1978); *B*. *tigrinae*, (Shete and Krishnamurthy, 1948); *B. aurangabadensis* (Shete and Krishnamurthy, 1948); *B. ranae* (Shete and Krishnamurthy, 1948); *B. megastomae* (Shete and Krishnamurthy, 1948); *B. cyanophlycti* (Shete and Krishnamurthy, 1948); *B. corlissi* (Shete and Krishnamurthy, 1948); *B. mininucleatum* (Shete and Krishnamurthy, 1948); *B. ganapatii* (Shete and Krishnamurthy, 1948); B. *singaporensis* (Khan and Ip, 1986); *B. claperedei* (Mahoon & Khan 1986); *B. vanensis* (Senler & Yildiz, 2000).

Amongst these, *B*. *kirbyi* and *B*. *xenopi* were the only known species reported from the family Pipidae. Furthermore, unknown *Balantidium* sp. was also noticed by several authors (Sandon, 1941; Mohr,1942; Thurston, 1970) in *Xenopus* sp. from Africa.

Early taxonomists placed the opalinids, discovered by Von Leeuwenhoek in 1683, with the well-known astomatous ciliates also known to be exclusively symbiotic organisms. The generic name *Opalina* was created 152 years later by Purkinje & Valentin, 1835. Although protozoologists removed the Opalinidae from the holotrichous ciliates, Calkins, 1933 believed otherwise. The recognized authority on the group, Metcalf, 1909 separated the Opalinidae from the "regular" astomes and established the category "Protociliata" apart from all other ciliates which were referred to as "Euciliata" (Metcalf,1918). All biologists widely accepted the previous placement of the Opalinidae by Metcalf (1918). Nevertheless, the taxonomic allocation of this group has always been ambiguous. Later, the opalinids showed affiliations with the Mastigophora due to their monomorphic nuclei, fusion of gametes during life-cycles and longitudinal type of fission (Amaro, 1967). Currently they are placed in super phylum Stramenopiles (Adl *et al*. 2019) below the supergroup SAR (Figure 1) and present in the superclass Opalinata, within the subphylum Opalozoa (Adl *et al*., 2019).

Known species of the genus *Protoopalina* to be found in frogs include: *P. africana* (Metcalf, 1923); *P. appendiculata* Fantham, 1929; *P. brevis* (Boisson,1959); P. cacosterni (Fantham, 1929); *P*. [*capensis*] (Metcalf,1940); *P*. *caudata* (Zeller, 1877*)* Metcalf, 1923; *P. caudata discoglossi* (Metcalf, 1923); *P. cucurbitacea* (Boisson,1959); *P. cylindroides* (Tuzet & Knoepffler, *1968); P. dakariensis* Boisson, 1959*; P. daloalensis* (Tuzet *&* Zuber-Vogeli,1954); *P. drachi* (Tuzet & Knoepflter, 1968); *P. fasciata* (Fantham, 1931); *P. gariepensis* (Fantham, 1931); *P*. *globulata* (Boisson, 1959)*; P heleophrynes* (Fantham,1931); *P. intestinalis* (Ehrenberg, 1838); Metcalf, 1923; *P. lamottei* Tuzet & Knoepflller, 1968; *P. macronucleata* Tuzet & Knoepffier,1968; *P. meridionalis* Fantham & Robertson,1928; *P. mossambicensis* Metcalf, 1923; *P. nutti* Metcalf, 1923; *P. octomixa* Fantham, 1930; *P. ovalis* Fantham, 1929; *P. peguyi* Tuzet & Knoepflller, 1968; *P. petiti* Tuzet & Knoepflller, 1968; *P*. *primordialis* (Awerinzew, 1913) Metcalf, 1918; *P. pseudonutti* (Sandon, 1976); *P. regularis* (Metcalf, 1923); *P. senegalensis* (Boisson, 1959); *P. stevensoni* (Stevenson, 1911) Metcalf, 1923; *P. transvaalensis* (Fantham, 1923); *P. viridis* Fantham, 1931; *P. xenopodos* (Metcalf, 1923); *Protoopalina* sp. of Tuzet & Knoepflller, 1968; *P*. *boycotti* (Delvinquier *et al*., 1995); *P*. *pattersoni* (Delvinquier *et al*., 1995). In addition to these, several unidentified *P.* species were reported in African Anura. These include a single nucleated opaline, found in *Bufo regularis.,* placed in the genus *Protoopalina* (Metcalf, 1918) probably *P. regularis (Metcalf, 1923)*. Both Sandon (1941) and McArthur (1955) mentioned a *Protoopalina* sp. from *X. laevis* adult frogs. McArthur (1955) also mentioned finding this *Protoopalina* sp. in *X. laevis* tadpoles. Finally, Affa'a (1992) also mentioned an unspecified *Protoopalina* sp. in several frog species including *Xenopus fraseri* (Pipidae) from Cameroon.

Of these *Protoopalina* species, the following were mentioned in frogs from South Africa *P*. *boycotti*; (Delvinquier,1995); *P*. *pattersoni* (Delvinquier, 1995); *P. brevis* Boisson, 1959; *P. drachi* Tuzet & Knoepffler; *P. nutti*, Metcalf, 1923; *P. primordialis*; *P. appendiculata*, Fantham, 1929; *P. cacosterni*, Fantham, 1929; *Protoopalina* (*capensis*) of Metcalf (1940); *P. fasciata*, Fantham, 1931; *P. gariepensis*, Fantham, 1931; *P. heleophrynes*, Fantham, 1931*,* although Earl (1974) suggested the suppression of this species due to inadequate description. *Protoopalina. meridionalis* (Fantham & Robertson, 1928); *P. mossambicensis* Metcalf, 1923 Fantham's (1923). While Metcalf (1940), disagreed that Fantham may not have seen *P. mossambicensis* due to the shape of the nuclei. *Protoopalina octomixa,* Fantham, 1930; *P. ovalis Fantham, 1929; P. stevensoni* (Stevenson, 1911) Metcalf,1923; *P. transvaalensis,* Fantham, 1923; *P. viridis,* Fantham, 1931; *P. xenopodos,* Metcalf, 1923.

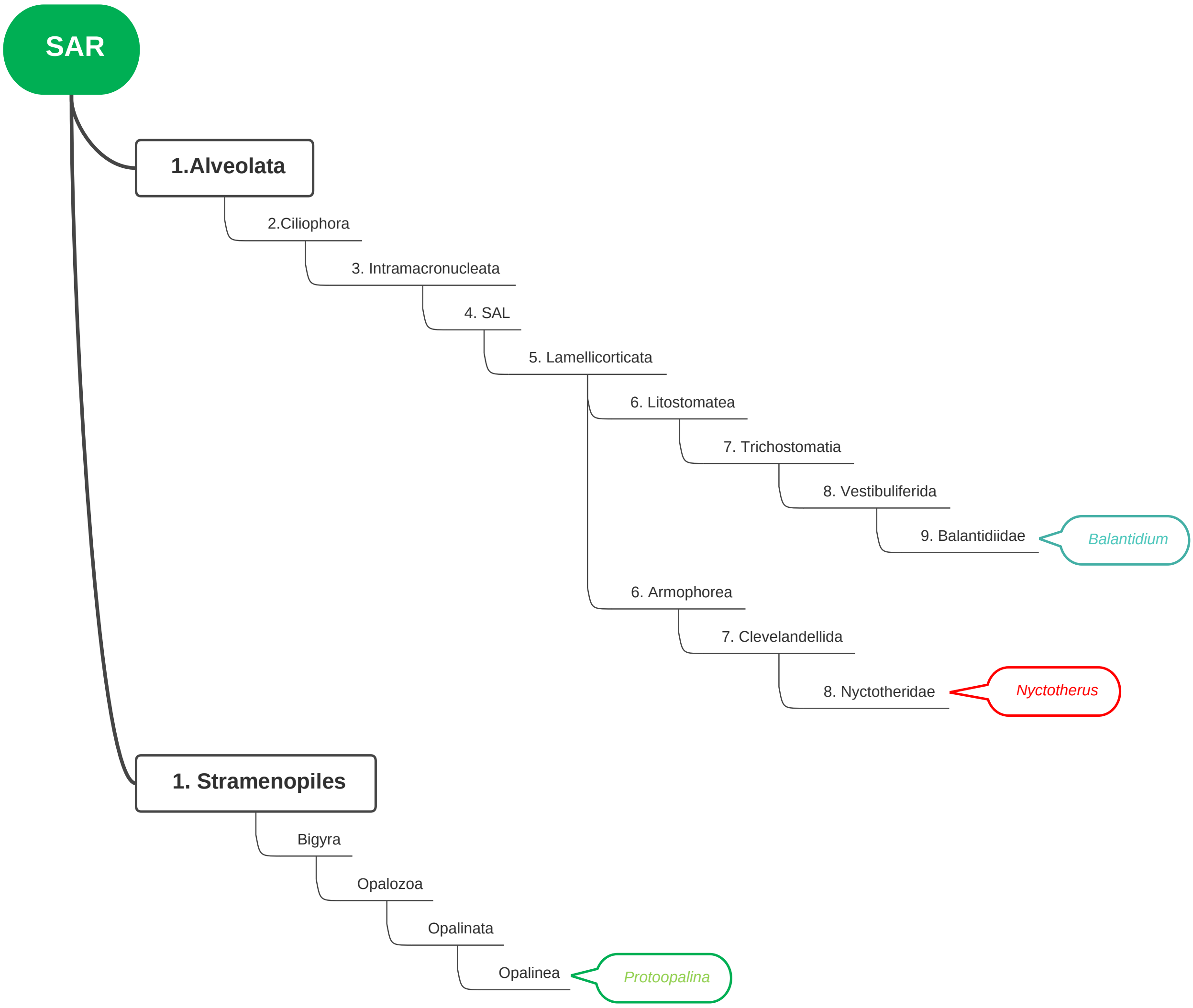

Figure 1: SAR Classification of *Balantidium*, *Nyctotherus* and *Protoopalina*

Although the protozoan fauna may differ among amphibian hosts species, both opalinids and ciliates are commonly found inhabiting anuran amphibians (Metcalf, 1918, Sandon, 1976; Delvinquier, *et al*., 1995). Due to their microscopic hair structures, opalinids might superficially resemble ciliates although they lack oral structures and differentiating micro- and macronuclei (Kostka, 2017). Interestingly, these endociliates seems to live as commensals rather than parasites in their anuran hosts. For example, Metcalf (1923) noted that they feed on the metabolic waste of the frog host found in the rectum. Furthermore, the endociliates do not seem to cause any harm to their anuran hosts (Metcalf, 1923).

Several species of *Protoopalina*, namely *P. ovalis, P. cacosterni* and *P. appendiculata*, have been observed in frog hosts from South Africa that include *Rana fuscigula* and *Cacosternum boettgeri* (Fantham, 1929). Likewise, various *Balantidium* ciliate species have been found in frogs around the world (Kuperman *et al*., 2004; Grim & Buonanno, 2009), also in South Africa (Rodriguez, 1939; Mohr,1942).

1.4 The name says it all: Ciliate characteristics

1.4.1 Morphology

Both ciliates and opalinids are single-celled organisms with complex cell structures because of the presence of more than one nucleus, cilia for Ciliophora or flagella for Opalinidae and an elaborate cytoskeleton. Ciliates' distinctive microscopic hair-like organelles, which gave rise to their name, is derived from the Latin "cilia" (plural form of "cilium"), referring to the endpoint of the eyelashes. The cilia and their basal bodies (Figure 1.1) can be found singly (monokinetids) or in pairs (dikinetids). The basal bodies may sometimes be non-ciliated (Foissner & Foissner, 1988). The basal body, together with the fibres and cilia, are called the kinetosome. Kinetosomes are arranged in rows referred to as kineties. Furthermore, ciliates possess most of the basic organelles found in protozoans. These include a cytostome and cytoproct, infundibulum, micronucleus, macronucleus and several vacuoles (food, waste, digestive and contractile) (Figure 1.2). The macronucleus performs the functional processes in the cell whereas the micronucleus contains the genetic material. Some ciliates also have extrusomes, which aid in predation, defence, and cyst formation.

Although members of the family Opalinidae are multinuclear and superficially resemble ciliates, they do not have differentiated macro- and micronuclei. Furthermore, Opalinidae are mouthless, osmotrophic flagellated protozoans. In addition they do not have cilia but rather flagella. Furthermore the Opalinata is known for possessing evenly spaced cortical ridges underlain by microtubules which range from singlets to ribbons (Adl *et al*., 2019). Based on the shape of the cell body and the number of nuclei, the family Opalinidae can be divided into two subfamilies, namely the Opalininae and the Protoopalininae (Kostka, 2017). Protoopalininae contains the genera *Protoopalina* Metcalf, 1918 and *Zelleriella* Metcalf, 1920.

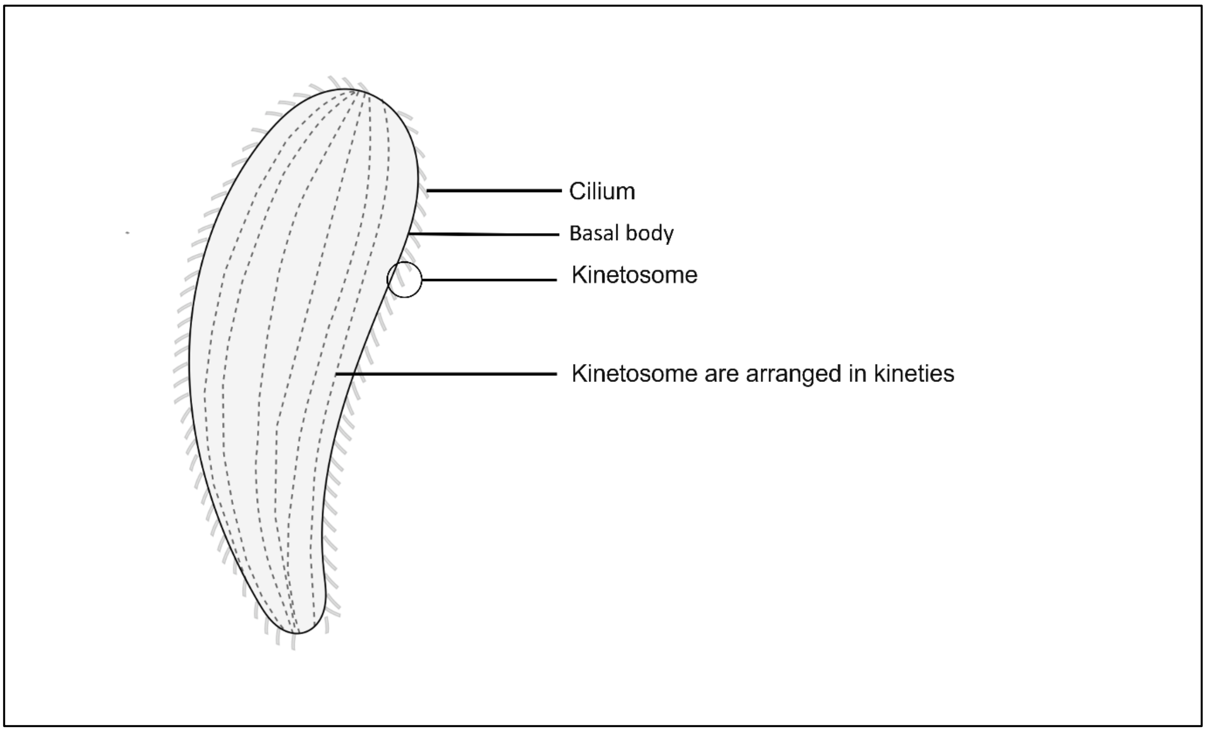

Figure 1.1: Basic external body structure of a ciliate

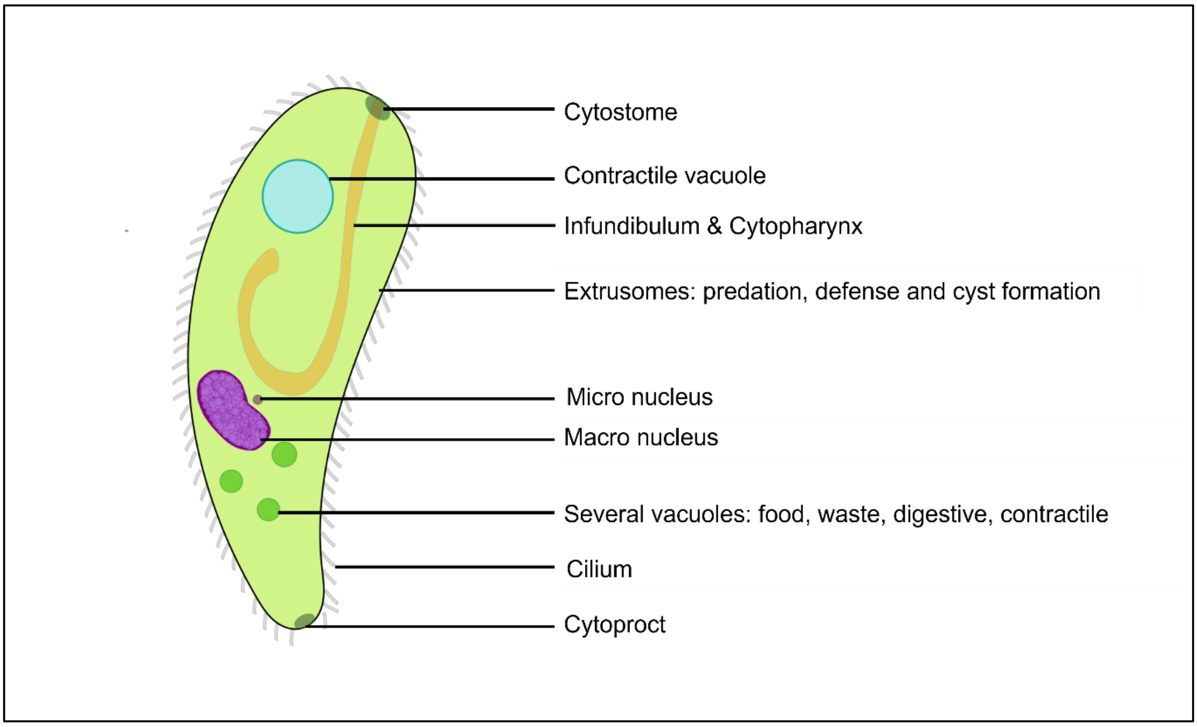

Figure 1.2: Basic internal body structure of a ciliate

1.4.2 Feeding habits

Ciliates have developed a wide range of feeding processes in response to different environments and food availability. Ciliates are known to play a considerable role as grazers of phytoplankton. Bacterivorous ciliates are seen as opportunists, since they exploit spatially and temporally variable resources (Fenchel, 1980). Degrading organic matter leads to the rapid growth of bacteria which are in turn reduced by bacterivorous ciliates. Ciliates are adapted to a temporary life because of their mobility, short generation time, ability to encyst when nutrients are limited and their ability to grow into large cells to postpone division (Fenchel,1980).

In addition, ciliates have structural features adapted for ingestion of solid foods and the seeking thereof (Metcalf, 1923). On the other hand, opalinids have lost all characteristics associated with ingestion except for the locomotor organs. Metcalf (1923) found that there was no regular pulsation of the posterior enlargement (excretory bladder) but rather an irregular contraction expulsing the granulose posterior enlargement of the excretory tube contents through the excretory pore of opalinids.

1.4.3 Life cycles & reproduction

Although some ciliates have complicated life cycles, the majority have rather simple life cycles. When nutrients are present, the ciliate population increases by cell growth and reproduction (binary fission). When nutrients are scarce, ciliates begin to starve. In many species, starvation initiates sexual reproduction. Ciliates reproduce by conjugation or autogamy when no partner is available. For some species, cysts form after conjugation or autogamy.

The endociliates and opalinids of frogs can always be found in the intestine and rectum of their hosts where they occasionally divide through longitudinal and transverse fission as described by Metcalf (1923). Within the reproductive season of the host, opalinids seem to divide with greater frequency, resulting in a rapid increase in numbers as opposed to cellular growth (Metcalf,1923). The newly divided ciliates are minute with separated nuclei. Metcalf (1923) suggested that the separation of the nuclei aids in cyst formation in the individuals that are excreted with the faeces of the host into the waterbody where the host breeds. These encysted forms descend to the bottom of the waterbody, remaining there for several days. Ultimately, the feeding tadpoles ingest the cysts, where they hatch in the rectal region of their host tadpole in the same condition as that which they were in prior to cyst formation (Metcalf, 1923).

1.5 *Xenopus laevis* as host choice for current study

The widely distributed African clawed frog, *Xenopus laevis* (Daudin, 1802) (Anura: Pipidae), native to Southern Africa, is known as a global invader (Measey *et al*. 2012). The present-day wide distribution of this anuran can be ascribed to its use as a biological pregnancy assay and model research animal since the 1930's (Gurdon & Hopwood, 2000). In more recent years, the pet trade further contributed to its human-mediated range expansion (Gurdon & Hopwood 2000; Weldon *et al.* 2007; van Sittert & Measey 2016). *Xenopus laevis* mainly appears in areas with a Mediterranean climate (Lobos & Measey 2002; Measey *et al*. 2012) and, at this time, its invasive range extends across four continents (Lobos & Measey 2002; Measey *et al*. 2012). Moreover, this global invader shows recurring range expansion — of a non-human mediated nature — within its native range due to overland movement or via artificial waterways (Measey & Davies 2011; Measey *et al*. 2012; Measey, 2016; Measey *et al*. 2017).

The genus *Xenopus* (Wagler, 1827) has a unique evolutionary position. Although different species of *Xenopus* are known to share habitats, most parasite infections are species-specific (Tinsley, 1981). Similar to their host, the parasites of *Xenopus* species or symbionts are apomorphic. Consequently, studying the ciliates of *Xenopus* species, could contribute unique information, especially from understudied localities, to better understand the evolution, phylogeny and morphology of this interesting group of protozoans.

Xenopus laevis has an extraordinarily rich parasite fauna comprising both metazoan and protozoan parasite species. Nevertheless, the study of protozoan parasites has fallen behind that of its metazoan counterparts. In contrast to the over 30 metazoan parasite species that have been described in association within this amphibian in its native southern Africa (Southwell & Kirshner, 1937; Pritchard, 1964; Thurston, 1970; Macnae *et al.*, 1973; Tinsley, 1996; Schoeman *et al.*, 2020), much less attention was given to this amphibian's protozoan fauna, with only three species formally described: *Trichodina xenopodos* (Fantham, 1924) infecting the bladder and *Protoopalina xenopodis* and *Balantidium xenopi* (Sandon,1941) in the rectum.

1.6 Ciliates of *Xenopus laevis*

Previous studies that documented intestinal ciliates and opalinids of *X. laevis* from South Africa are limited and took place decades ago (Metcalf, 1923; Rodriguez, 1939; Sandon, 1941; Mohr, 1942; Du Puytorac & Grain, 1965; Thurston, 1970; Cosgrove & Jared, 1974; Delvinquier *et al*., 1991; Delviniquier *et al*., 1995). These studies mostly dealt with the taxonomy and distribution of the ciliates of various *Xenopus* spp. across Africa, and in captive *X. laevis* imported from South Africa (Metcalf, 1923; Rodriguez, 1939; Sandon, 1941; Mohr, 1942; Du Puytorac & Grain, 1965;

Thurston, 1970; Cosgrove & Jared, 1974 Delvinquier *et al*., 1991 Delviniquier *et al*., 1995). Often, in general surveys of the parasites of *X. laevis*, the protozoan parasite fauna was either ignored (Mbokane et al., 2020;), not identified beyond genus level (Mohr, 1942; Kuperman *et al*., 2004), not identified at all (Cosgrove & Jared, 1974; Schoeman *et al*., 2019), or reported without any justification for their identification (Sandon,1941; Thurston, 1970; Lafferty & Page, 1997). Consequently, difficulties arise when rectal endociliate assemblages are compared among populations of the host, in their native range as well as elsewhere (Kuperman *et al*., 2004; Schoeman *et al*., 2019).

1.6.1 ***Balantidium xenopi*** **Du Puytorac** ***et*** **Grain, 1965. Synonyms:** ***Paranyctotherus (Balantidium) kirbyi*** **(Rodriguez,1939) Sandon, 1941*, Balantidium xenopodis*** **Kuperman, Matey, Fisher, Erwin, Warburton, Bakhireva** ***et*** **Lehman, 2004**

The largest and most abundant ciliate species in *X. laevis* was described by Rodriguez (1939) and Sandon (1941), with a similar ciliate described by Du Puytorac and Grain (1965) from *Xenopus fraseri* from Makokou (Gabon). All three descriptions were under different names. The first mention of a large ciliate that occured in the intestine of *X. laevis* was by Rodriguez (1939). This was from material obtained from frogs from the Western Cape, South Africa (specifically near Tradauw Pass and Stellenbosch), Rodriguez (1939) described *Balantidium kirbyi,* although he did not disclose why he placed the species in that particular genus. When comparing it to other *Balantidium* spp. known at the time, he remarked on *B. kirby*i's unique cytopharynx and a group of spines on the anterior dorsal surface. Two years later, Sandon (1941) concluded that this species, which he also found in the rectum of *X. laevis* from South Africa, did not belong to the genus *Balantidium*. The author regarded it as a close relative of the genus *Nyctotherus* based on its body shape, morphology of the cytopharynx and peristome, distribution of the cilia on the body (Sandon, 1941). However, the presence of a row of stout, plate-like membranellae on the right side of the peristome that extended along the whole length of the cytopharynx, resulted in the author to placing this species in the newly erected genus *Paranyctotherus* (Sandon, 1941). This ciliate, under the name *Paranyctotherus (Balantidium) kirbyi* was also reported in abundance in the rectum of *X. laevis* from a captive population in California, after six years of captivity (Mohr, 1942). Seemingly unaware of the previous descriptions, Du Puytorac and Grain (1965) described a new species *Balantidium xenopi* from *X. fraseri* outside South Africa. However, these species were synonymised by Thurston (1970) under the name *Balantidium xenopi*, who observed "a large ciliate with a narrow parallel-sided vestibulum which frequent coils in the vicinity of the macronucleus of just posterior to it" from the rectum of most of the *Xenopus* spp. she surveyed, including *X. laevis* from South Africa. However, Thurston provided no reason for the taxonomic reshuffling and also did not provide a redescription. Subsequent literature referred to this large ciliate as *B. xenopi* (Tinsley, 1981, 1996) or *B. xenopodis* (Kuperman *et al*., 2004).

In summary, it is clear from previous studies that *X. laevis* commonly harbours a large *Balantidium*-like ciliate in its rectum. However, a redescription with modern techniques is necessary to clear up the taxonomic confusion surrounding this species.

1.6.2. ***Balantidium*** **sp.**

Mohr (1942) found another *Balantidium* sp. in the material of Rodriguez (1939) from the Tradauw Pass, Western Cape, South Africa. This species was smaller than *B. xenopi* and was presumed to belong to a different species, probably the same as the one described by Fatham (1919) as "*Balantidium entozoon*". The same species, presumably, was found by a range of other investigators: after six years of captivity in *X. laevis* in California (Mohr, 1942), alongside *B. xenopi* in *X. laevis* from Cape Town (Sandon, 1941) and in most of the adult *Xenopus* spp. examined by Thurston (1970), including *X. laevis* from East Africa, Nigeria and the former Rhodesia (now Zimbabwe). Sandon (1941) mentioned that a small *Balantidium* sp. frequently fed on erythrocytes in the saline during dissection. To date, there is no published description of the smaller *Balantidium* sp. from *X. laevis*.

1.6.3 ***Protoopalina xenopodus*** **Metcalf, 1923. Synonyms:** ***Protoopalina xenopodos*** **(Fantham,1923) (Fantham, 1929)**

When looking at the other protozoan species found in the hindgut of the clawed frog, the literature gives vague descriptions, of which many are based on speculations. Of these, the genus *Protoopalina* are frequently mentioned. *Protoopalina* species were described from several members of the family Pipidae which include *X. calcaratus*, *X. laevis*, *X. tropicalis, X. victorianus* and *X. muelleri.* The original description of *P. xenopodus* was made from specimens collected from *Xenopus calcaratus* from the Congo (Metcalf, 1923). A species which closely resembles *Protoopalina xenopodus* was also observed in X. *muelleri* and *X. laevis* in a study screening the cloaca of several southern African anura (Delvinquier *et al*., 1995). Several other studies mention *Protoopalina xenopodus* in the gut of *Xenopus* species (Fantham, 1923; Fantham, 1929; Wessenberg, 1961, Thurston, 1970; Kuperman *et al*., 2004). Interestingly Thurston (1970) described *P. xenopodus* from *Xenopus* tadpoles, probably *X. victorianus*, from Kajansi. Kuperman *et al*. (2004) also mentioned *P. xenopodus* in Xenopus *laevis* from three localities in southern California, USA.

Moreover, Delvinquier *et al*. (1995) also mentioned to have found another *Protoopalina*, P. *primordialis* in three tadpoles and two juvenile *X. laevis.* In several studies, protozoans found in the gut of different *Xenopus* sp. were only identified as *Protoopalina* sp. (Sandon,1941; Mohr,1942; Affa'a, 1992; McArthur, 1995). A *Protoopalina* sp. was mentioned by Sandon (1941) in adult *X. laevis* from South Africa and Affa'a (1992) in *X. laevis* from Cameroon. Mohr (1942) also mentioned a *Protoopalina* sp., possibly *P. xenopodus*, from adult and tadpole *X. laevis* from Tradauw Pass, Western Cape. These were reported as the same specimens as those present in Rodriguez's (1939) slides. McArthur (1995) found *Protoopalina* sp. in both parent and offspring stock of *X. laevis* at the Zoology Laboratory of the State University of Iowa in the United States of America. Hallinger *et al*. (2020) found an opalinid in captive *X. laevis* from Germany.

1.6.4 Unidentified species

Numerous studies also mentioned unidentified *Nyctotherus* spp. in *Xenopus* spp., including *X. laevis* from South Africa, California and Germany (Thurston,1970 & Lafferty & Page, 1997, Kuperman *et al*., 2004; Hallinger, *et al*., 2020). Thurston (1970) briefly described a *Nyctotherus* sp. from adult and tadpole *Xenopus* sp. from Kajansi, Uganda, and Nairobi, Kenya, mentioning that it resembled *Nyctotherus. cordiformes*. Kuperman *et al*. (2004) described a *Nyctotherus* sp. in *X. laevis* from California similar to those described by Thurston (1970) from Africa, although the authors stated that further study was required to determine the species identity. Lafferty and Page (1997) found *Nyctotherus* sp. in abundance in *X. laevis* adults from the Santa Clara River in California. Cosgrove and Jared (1974) found a variety of ciliates in the faeces of *X. laevis* imported from South Africa, although no attempts were made to identify them. Interestingly other protozoan species were also reported. Fantham (1922) identified a small number of *Chilomastix caulleryi* in the intestine from adult *X. laevis* in Johannesburg, South Africa. A rectal flagellate, *Hexamita intestinalis* Dujardin, 1841, in *Xenopus* spp. from Kajansi, Lake Mutanda, Uganda and Nairobi, Kenya, were described, from both tadpoles and adult *Xenopus* sp., probably *X. fraseri* (Thurston, 1970). Green *et al*. (2003) reported a *Cryptosporidium*-infected laboratory-reared female adult *X. laevis* from South Africa.

1.7 Techniques used in ciliate studies

I. Past Techniques

In the past, permanent preparations were made by making use of Carnoy's fluid, Hollande's fluid, Schaudinn's fluid, Bouin's and Zenker's fluids as well as formalin vapour (Fantham, 1929; Rodriguez, 1939; Sandon, 1941; Cosgrove & Jared 1974; Kuperman *et al*., 2004). Furthermore, stains for observing ciliates included iron haematoxylin, Mallory's iron-haematoxylin, Delafield's haematoxylin, Heiden-Hain's haematoxylin and acetic carmine (Metcalf, 1923; Fantham,1929; Rodriguez, 1941; Sandon,1941; Thurston,1970; Cosgrove & Jared, 1974; Kuperman *et al*., 2004;).

Metcalf (1923) found that Delafield’s haematoxylin is the stain giving the best results for revealing ciliate structures. Several other stains, including Borax-carmine, alum-carmine and Lyon’s, also deliver satisfying results (Metcalf, 1923). Furthermore, iron haematoxylin gives clear stains for thin species and any cytoplasmic spherules (Metcalf, 1923).

II. Present Techniques

More modern methods of fixation for protozoans include phosphate-buffered 4% formaldehyde and Karnovsky’s for scanning electron microscopy (Kuperman *et al*., 2004; Grim & Buonanno, 2009). Although numerous molecular studies on ciliate communities do exist, molecular studies on amphibian ciliates, especially for *Protoopalina* sp. are limited. There are some exceptions (Kostka, 2004; Nishi *et al*., 2005; Grim & Buonanno, 2009; Zhao *et al*., 2018), however no molecular data exist for the ciliates of South African frogs.

Even though we are presently in the refinement age of ciliate systematics, ciliates in anurans, especially those in African anurans, have not received much attention. Although previous frog ciliate studies do exist (Metcalf, 1923; Rodriguez, 1939; Sandon, 1941; Mohr, 1942; Du Puytorac & Grain, 1965; Thurston, 1970; Cosgrove & Jared, 1974 Delvinquier *et al*., 1991 Delviniquier *et al*., 1995) they are — as mentioned earlier — limited and done with outdated techniques. Therefore, anuran ciliates can be classified as an understudied taxon. Foissner (2014) suggested a procedure for reliable differentiation of mono- and dikinetids and of ciliated and non-ciliated basal bodies. Firstly, cells should carefully be studied in vivo with interference contrast. Secondly, cells should be over-stained with protargol and/or silver carbonate. When cells are over-impregnated, cilia are easy to recognise. Thirdly, non-ciliated basal bodies and/or parasomal sacs can be identified through size since they are smaller than ciliated basal bodies. Lastly, the electron microscopic literature related to the species or group of species investigated should be studied to correlate stained structures with ultrastructural features.

III. Method integration

A single method that can reveal all the taxonomically important ciliate characteristics does not exist (Foissner, 2014). Over and above, *X. laevis*’ ciliates’ descriptions date from decades ago and need clarification as well as redescription. Thus, documenting the ciliates of this anuran species with multiple modern techniques will be of immeasurable value. Nevertheless, for a proper integrated approach and to facilitate comparison with previous studies, it is necessary to evaluate the classic methods used in former ciliate identification in relation with modern techniques.

Integrative taxonomy can be defined as the science aiming to distinguish among units of diversity by making use of multiple and complementary perspectives (Dayrat, 2005). These perspectives may include morphology, genetics, ecology, and ethology. Although integrative taxonomy is relatively a new term in science, it has been used in several studies for revised descriptions and classifications (Garraffoni, *et al*., 2019; Stec, 2022)

Although integrative taxonomy is a relatively new term in science, it has been used in several studies for revised descriptions and classifications to date (Garraffoni *et al*., 2019; Madi-Ravazzi *et al*., 2021). Integrative taxonomy can be a powerful tool to delineate multivariance in species for example (Obert & Vďačný, 2019). Since ciliates are so diverse and have a complex intracellular morphology, numerous taxonomic concepts have evolved for these protozoans over the years. Although the morphological species concept is the standard for ciliate taxonomy, the recent increase in molecular data of ciliates brings inconsistent results to the table (Vďačný, 2017). Thus, an integrative approach is needed for a critical evaluation of both the molecular and morphological outcomes.

To successfully apply the integrated approach to the taxonomy of the rectal endociliates of *X. laevis*, it is necessary to evaluate the classical methods used in former ciliate identification in relation with modern techniques. Classical techniques for ciliate identification include supravital staining, haematoxylin staining and silver impregnation, whereas modern techniques include live observations, scanning electron microscopy (SEM) and molecular analyses (Foissner, 2014).

1.7.1 The integrated approach

To compare the descriptions of the present study with former taxonomic studies of ciliates of *X. laevis* (Rodriguez, 1939; Sandon, 1941; Mohr, 1942; Du Puytorac & Grain, 1965; Thurston, 1970; Cosgrove & Jared, 1974; Delviniquier *et al*., 1995; Lafferty & Page 1997; Kuperman *et al*., 2004), it was necessary to evaluate and apply the classical methods used for ciliate identification in the past. Traditionally, ciliate taxonomy relied on staining methods which expose the distinctive nuclear components and patterns in the cilia on the surface of the cell body. Staining can be defined as the visual labelling of a biological component by attaching or depositing a colouring agent to the surrounding area of the component (Bancroft & Layton, 2019).

1.7.1.1 Classical Methods

(I) Silver impregnation

Silver impregnation is a method used to reveal the infraciliature, also known as the ciliary pattern, which is the organisation of the kinetids, fibrils and microtubules at the base of the cilia, called basal bodies, and the cortical organelles such as the extrusomes and the cytopyge. Of these, the most taxonomically important characteristic revealed by silver impregnation is the kinetids. The kinetids can be arranged as monokinetids (one kinetid), dikinetids (two) and polykinetids (more than two), forming silverline patterns which are used in ciliate taxonomic identification. The cilia themselves are usually not revealed by this method. Several silver staining methods exist that are usually modified for each different taxa of ciliates. However, there are two basic silver staining methods crucial for studying most ciliates, namely the ‘dry’ and the ‘wet’ silver nitrate staining methods.

A) The ‘dry’ silver nitrate staining method.

This method is called the ‘dry’ method since the ciliates are air-dried before the silver nitrate treatment. Klein (1958) described a basic ‘dry’ silver nitrate method, which was improved upon by Gelei (1934), Ruzicka (1966) and Foissner (1976). This method provides basic information on the somatic and oral infraciliature and is the best at revealing the silverline pattern.

B) The ‘wet’ silver nitrate staining method.

In the ‘wet’ method, the ciliates are chemically fixed with Champy’s fluid before silver nitrate treatment. The ‘wet’ methods give valuable information on both the somatic and oral infraciliature. Moreover, this method preserves the cell’s shape well. However, the silverlines are usually not clearly stained by this method.

Nevertheless, ciliates cannot only be described from the morphological characteristics revealed by the silver impregnation, since some species’ characteristics, like contractile vacuoles and nuclei, cannot be seen in silvered specimens (Foissner, 2014). Thus, species descriptions based exclusively on silver slides are regarded as incomplete.

(II) Supravital staining with methyl green-pyronin

This method uses methyl green-pyronin to instantly reveal the mucocysts of many species of ciliates. Mucocysts are membrane-bound structures which discharge a mucus-like mass to the outside of the cell, possibly important in cyst formation. Mucocysts stain selectively blue or red and can be observed in various stages of swelling. This simple method proceeds as follows (Foissner, 2014): A drop of the ciliate specimens, in the original gut fluid, are placed in an equally sized drop of methyl green-pyronin in the centre of a microscope slide. Thereafter, a coverslip with corners covered in petroleum jelly is gently placed on top of the specimens, squeezing them lightly. The ciliates are then observed immediately since the cells die within minutes. The nuclear apparatus also stains in adequately flattened cells. This staining method is only temporary since cells become darkly stained within five to ten minutes.

(III) Haematoxylin staining

The combination of haematoxylin and eosin as staining agents is a well-known, simple method in histology. Haematoxylin reveals intranuclear detail by staining the cell nuclei blue-black, whereas eosin is used to stain the cell cytoplasm and connective fibres with a variety of red, pink and orange shades and intensities (Layton *et al*., 2019). Eosin is a fluorescent, xanthene dye with the ability to distinguish between the cytoplasm of different types of cells, connective tissue fibres and matrices. This dye binds to positive charged eosinophilic compounds in salts (Bancroft & Layton, 2019).

Haematoxylin is obtained from the heartwood of the tree *Hematoxylon campechianum* and contains a natural dye called hematin. The latter acts as the major oxidation product which is responsible for the staining of certain cell components like vacuoles, nuclei and the infundubulum. Haematoxylin can be produced from hematin by 1) natural oxidation, for example Ehrlich's and Delafield's haematoxylins; and 2) chemical oxidation, for example the sodium iodate used in Mayer's haematoxylin and mercuric oxide used in Harris's haematoxylin (Humason, 1979). Chemical oxidizing agents instantly convert haematoxylin to hematein. In addition, naturally oxidized haematoxylins ought to have a longer shelf life than chemically oxidized haematoxylins because of the continuing oxidation process in air and light. Furthermore, hematin is anionic and needs the presence of a metal cation-containing mordant to result in a net positively charged dye-mordant complex. Consequently, this positively charged dye-mordant complex can bind to anionic tissues like nuclear chromatin. The type of mordant used affects the type of tissue stained as well as the final colour thereof. Thus, haematoxylin solutions are classified according to their mordant — aluminium (alum) haematoxylins, iron haematoxylins, tungsten haematoxylins, molybdenum haematoxylins, lead haematoxylins and haematoxylins without mordant (Bancroft & Layton, 2019).

For the present study alum haematoxylins for ciliate identification was employed. Alum haematoxylins are the most often used haematoxylin for nuclear staining, for example Ehrlich's, Mayer's, Harris's, Gill's, Cole's and Delafield's haematoxylins (Bancroft & Layton, 2019).

(IV) Live observation

In addition to the classical methods, live observations play a crucial part in ciliate identification (Foissner, 2014). Not only can ciliates be examined by looking at their size, shape, ciliation and oral placement, but live observation can also reveal ciliate behaviour. In addition, other characteristics that may be observed in living ciliates includes feeding habits, pigmentation, endosymbionts, distribution of vacuoles and extrusomes. Therefore, live observations are a crucial aspect in ciliate identification especially when it is paired with modern differential interference microscopy (Lynn, 2008).

1.7.1.2 Modern methods

Modern technology proposes endless possibilities for ciliate identification. The digital revolution has greatly improved photographic documentation. For instance, photos and videos of the host and habitat can be taken with a smartphone in the field. Secondly, differential interference contrast microscopy enables high resolution images with excellent contrast in unstained specimens. The sharpness of micrographs can be further improved since graphical stacking can overcome the problem of low focal depth. In addition, graphics software enables editing to remove unwanted artefacts and artificial colouring to emphasise certain structures.

Information previously unknown or unstudied are now revealed by not only DNA barcoding, but also scanning electron microscopy. The previously mentioned classic method of live observation were also improved with modern microscopy. The ability to obtain and sequence DNA material from even the smallest organisms, such as ciliates, has opened up whole new avenues of study.

(I) DNA Barcoding

The DNA barcoding system is designed to provide accurate and rapid species identifications by using short gene regions (Hebert & Gregory, 2005). DNA barcoding may be of great value in the discovery, sorting and assigning of specimens and all together support a more accessible taxonomic system, overcoming some of the limitations of morphological diagnosis, such as genetic variability, phenotypic plasticity, morphological cryptic taxa and variable life stages

(Herbert *et al*., 2003). Nevertheless, DNA barcoding is not always comfortably accepted in by all taxonomists (Hebert & Gregory, 2005).

DNA barcoding can be applied to ciliate taxonomy through evolutionary taxonomy. Evolutionary taxonomy is seen as the classification of organisms according to their phylogenetic relationship (Mayr, 1981). DNA barcoding can be applied to ciliate taxonomy by split spectrum analyses, phylogenetic networks, likelihood mapping, and finally reconstruction of ancestral morphologies (Vďačný, 2017). Nevertheless, one can most certainly not rely on DNA barcoding alone. One limitation of DNA barcoding is that it relies on a reference library for taxonomic identification. This reference library could easily be deceiving since sequences are not always reliable or well identified. Moreover, microscopy holds several advantages over molecular methods when studying ciliates. For example, observed proportions of different genera may be skewed by copy number variation of ribosomal RNA genes across the different genera or growth conditions in a sample (Medinger *et al*., 2010). To add, when 18s rRNA gene surveys are done, an apparent higher diversity of ciliates than estimated by conventional morphological methods are revealed (Moon-van der Staay *et al*., 2014). Finally, limitations exist when closely related species should be distinguished through DNA barcoding, depending upon the gene marker chosen for barcoding (Parmentier *et al*., 2013).

(II) Scanning electron microscopy

Electron microscopes were introduced in the 1930s. Since then, this microscopy method has developed into a critical tool in numerous different research fields. For the ciliated protozoans, electron microscopy aided in the understanding of the levels of organisation below the cell level and laid the foundation for the structural conservatism hypothesis in the ultrastructure age (Lynn, 2008).

1.7.1.3 Experimental

(I) Deciliation

Deciliation is a method which is used to remove the cilia from ciliates. By removing the cilia, deciliation is an alternative method to silver impregnation for revealing ciliate kinetids. Methods for deciliation are usually only applicable to certain species. The methods can be complex and species-specific, although some simple deciliation techniques involve liquid soap (Foissner, 2014).

(II) Culturing

Culturing is a method used to multiply micro-organisms under controlled laboratory conditions. These conditions are predetermined to accompany the organism's specific environmental preferences. Microbial cultures can be seen as a fundamental research tool when practicing molecular biology.

For this study, pure cultures of each ciliate morphospecies found in the gut of *X. laevis* would be of great value, especially for molecular identification. A basic protozoan culture medium consists of organic food substances added to water. Nutrient substances previously used in protozoan cultures include hay, wheat, egg albumin and pond water (Hyman, 1925). The medium is sterilised through high heat and then inoculated with the protozoans of interest. The ciliates feed on the putrefying food substances. For the successful cultivation of protozoan cultures, Hyman (1925) recommended the following:

- The food type to be used in cultures is unimportant; the food quantity relative to the amount of water is of far greater importance.
- Protozoan species flourish at their own specific food substance putrefaction rate.
- Culture succession rates are directly proportional to the food substance putrefaction rate.
- Protozoan species will be abundant in cultures with a high putrefaction rate but will disappear quickly, whereas protozoan species will be less abundant in cultures with a low putrefaction rate, although they will live longer.
- Cultures are time-bound and become unsuitable for protozoans after a period, depending on the rate of fermentation.

(III) Counting ciliates

Several methods for counting ciliates have been proposed (Schumaker, 1931; Atchley & Swezey, 1934). In some studies, counting chambers of standard design like haemocytometers were used to count rumen ciliates (Johnson *et al*., 1944; Moir, 1951; Weller & Gray, 1954). Nevertheless, haemocytometers are not effective for counting cells as large 200 µm. Rather, the MacMaster chamber enables a better quantitative measure of the ciliates in the rectum of frogs (Boyne *et al*. 1957). In this technique, a counting chamber is used, which enables the count of a known volume of faecal suspension through the microscope.

1.8 Problem statement

Modern-day microscopy and molecular techniques pose endless possibilities for ciliate identification (Foissner, 2014; Warren *et al*., 2017). With the help of species descriptions that are accompanied with accurate drawings and micrographs, live observations and DNA barcodes, the confusion surrounding the taxonomy of *X. laevis'* ciliates can be solved. Clear identification of the symbionts of this host is crucial in the light of its role as a parasite taxi (Schoeman *et al*., 2020) and its invasive status (Measey *et al*. 2012, Schoeman *et al*., 2019). Moreover, this will be the first application of modern integrated taxonomic methods to the gut ciliates of an African amphibian, which will not only provide future investigators with a clear workflow, but also provide DNA barcodes for a globally underrepresented taxonomic group.

Thus, the overarching aim for this study was to document the gut ciliate diversity of *X. laevis* by using an integrated taxonomic approach. In addition, the objectives were to: 1) collect rectal ciliates from *X. laevis* specimens both in its native range of South Africa and its invasive range in France; 2) apply and evaluate both morphological and molecular tools to confirm species identifications; and, ultimately, 3) develop a working method for the study of amphibian ciliates.

Chapter 2:

Materials and Methods

2.1 Materials and Methods Introduction

Both classic and modern techniques for ciliate identification were applied in this study. This allowed for an integration of molecular and morphological techniques. The methods primarily included DNA analysis, live observations, supravital staining, haematoxylin staining, silver impregnation, scanning electron microscopy and deciliation. Furthermore, quantitative techniques such as ciliate culturing and counting were experimented with. A general ciliate identification workflow designed during this study is described in Figure 2.1.

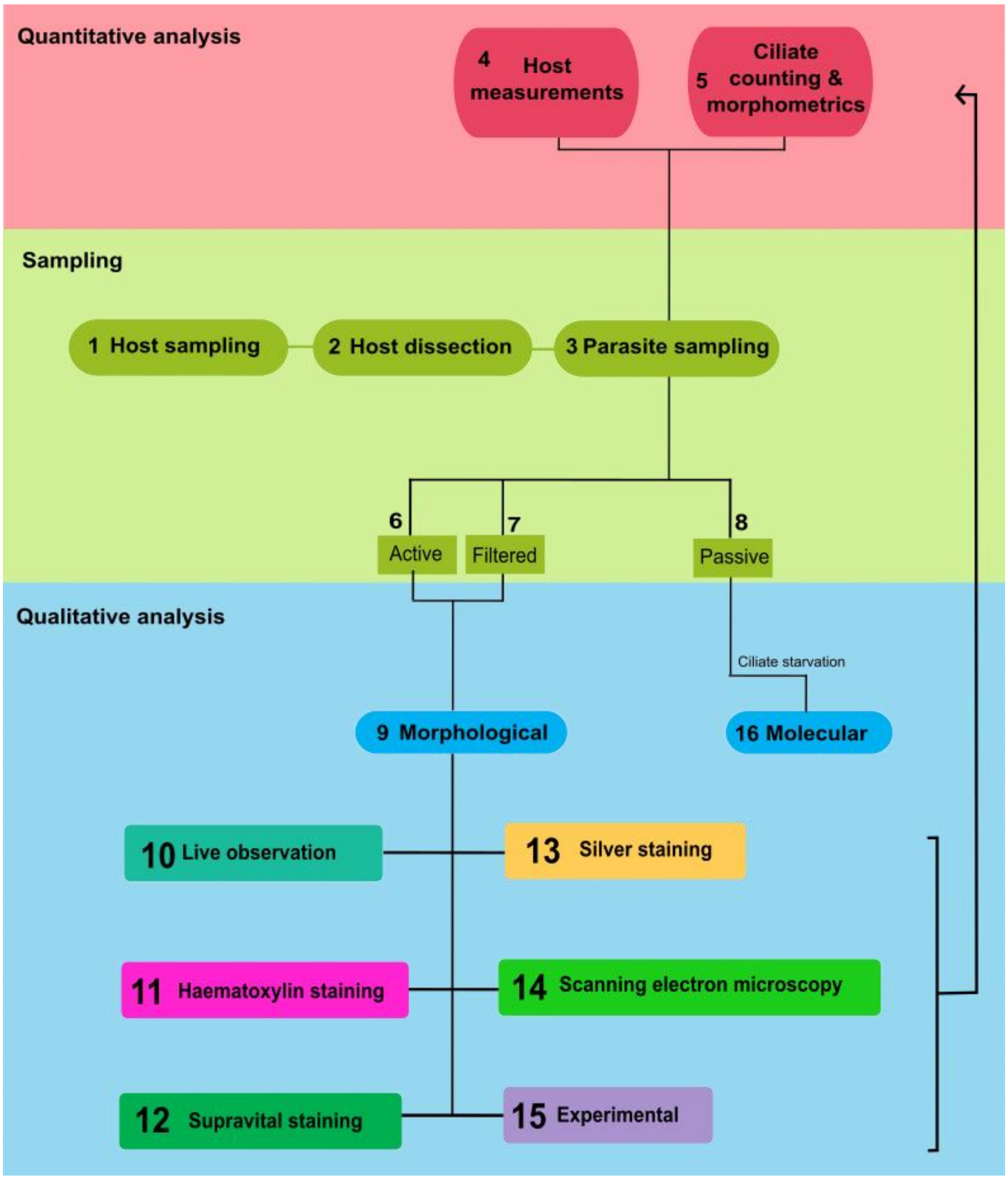

Figure 2.1: Ciliate identification workflow: *Xenopus laevis* were sampled and screened for parasites (1–3). Ciliates were sampled from the rectum of hosts (6–8). Thereafter, hosts and ciliates were analysed quantitively (4–5). Qualitative analyses were only performed on ciliates, not hosts and entailed both morphological (9–15) and molecular methods (16).

2.2 Data collection

2.2.1 Host sampling and dissection

A collection of 335 adult *X. laevis* was retrieved and screened for endociliates, which were studied using various methods. The frogs were sampled throughout all seasons from randomly selected localities in the native range in South Africa and the invasive range in France (Appendix A), caught by making use of liver-baited bucket traps. Until dissections were performed, the frogs were kept in water from their original source, replaced by dechlorinated water when necessary.

The frogs were euthanised according to internationally and institutionally accepted standard operating procedures (SOPs) by using 6% ethyl-3-aminobenzoate methanesulfonate (MS222) (Sigma-Aldrich Co., St. Louis, Missouri, United States) followed by pithing to comply with the requirement of double euthanasia (SOP: NWU-00492-16-A5). Thereafter, the frogs were sexed, weighed and measured in terms of snout-vent length (SVL) and head width. All procedures were carried out in accordance with the North-West University's AnimCare ethics guidelines under ethics number NWU-00426-21-A5 (AppendixB).

3.1.2 Ciliate sampling

The alimentary tract of each frog was removed, where after the rectum was screened for ciliates using a stereomicroscope. Three methods of ciliate isolation were evaluated, namely: a) filtered sampling; b) passive sampling; and c) active sampling.

I. Filtered sampling

Sandon (1941) developed an apparatus to concentrate and clean ciliates from rectal contents, which was adapted for this study. The apparatus comprises three 15 ml conical centrifuge tubes that fit into each other. In the apparatus developed by Sandon (1941), the inner two tubes' lower ends were cut off and replaced by membranes of bolting silk. In this study, we used ordinary gauze instead. For the top tube, two layers of gauze were used and three layers gauze for the lower. The apparatus was then filled with 0.6% amphibian saline prepared according to Ringer's method (Presnell *et al*., 1997) until the lower part of the top tube was covered. The rectal contents, containing both debris, saline and ciliates, were then poured into the top tube. After about 20 minutes, the ciliates gathered in the lowest tube as a white mass (Figure 2.2). In addition to filtered sampling, we also evaluated the use of different microns of mesh sieves (Fritsch GmbH, Idar-Oberstein, Germany) to concentrate the ciliates according to the same principle as above. By making use of a Pasteur pipette, rectal fluid containing both ciliates and debris were placed on a 150 µm sieve with a petri dish as collection container. Thereafter, the process was repeated with the filtrate using 106 µm and 53 µm mesh sifts, with a concentrated mass of ciliates in the petri dish.

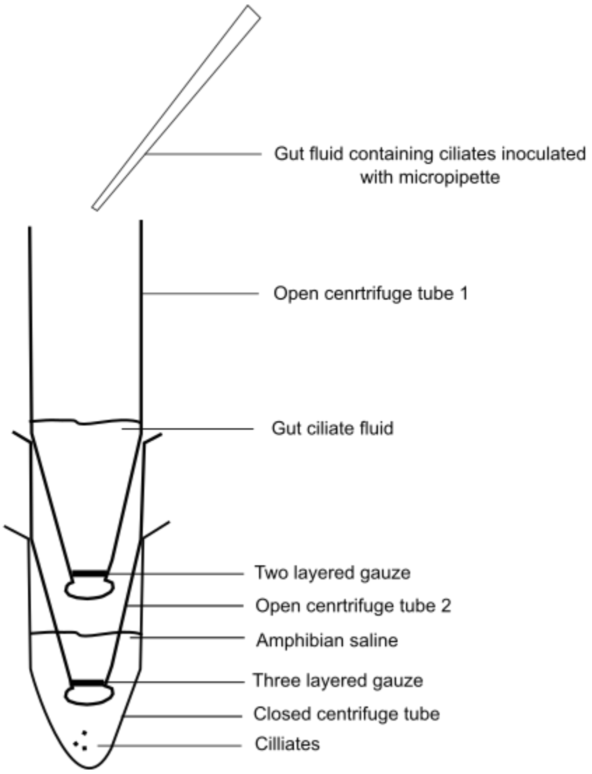

Figure 2.2: Sandon's modified apparatus for concentrating ciliates.

II. Passive sampling

The unopened rectum with its well-formed faecal matter was placed in a cavity block containing 0.6% amphibian saline. After a few seconds, ciliates began to exit from the anterior open end of the rectum. The rectum was left submerged in saline for a few minutes and ciliates could be isolated in this way without contamination with rectal debris.

III. Active sampling

For this method, the alimentary tract was placed in 0.6% amphibian saline and carefully opened in the posterior to anterior direction. Ciliates were individually collected with a micropipette. After sampling, the ciliates were washed with saline. Thereafter, the ciliates were fixed in different fixatives depending on the downstream techniques to be used. Fixatives included: 1) 100% ethanol for molecular identification; 2) 2.5% glutaraldehyde for scanning electron microscopy; and 3) Bouin’s solution and 4) 10% neutral buffered formalin for morphological identification purposes.

3.1.3 Morphometrics and general techniques

I. Morphometrics

All ciliate measurements in this study were measured in µm and done on Haematoxylin fixed organisms, except when otherwise stated. Values are given as range with mean in paranthesis.

II. Egg Albumin

Egg albumin is commonly used in histopathology as adhesive medium. Mayer's egg albumin was prepared according to protocol (Presnell et al. 1997). Albumin was stored for at least 24 h before use. To prepare albumin slides, a thin uniform layer of albumin was spread on microscope slides and left to air dry.

III. Storage

Ciliates in different fixatives were stored in cryovials at 4˚C for further use. Techniques were then applied according to each different protocol.

IV. Replacement of fixative

Fixatives were replaced by making use of a cavity block and micropipette. The ciliates were pipetted with a micropipette and sterile tips from the cryovial into the cavity block. This transfer was done with the least amount of fixative being replaced as possible. The cavity block was then filled to the top by the replacing fixative. Next the replacing fixative was decanted by using a micropipette with sterile tips while making sure that ciliate specimens stay behind in the cavity block, whereafter the process was repeated three times from the second step. Importantly this whole procedure was carried out under a stereo microscope.

3.2 Morphological observations

After removal from the host, ciliate specimens were prepared according to various methods to visualise and measure various morphological characteristics. These included live observations of unstained specimens under a light microscope, the fixation and staining of non-living specimens for morphometric measurements and the preparation of mounts for morphometric observations under a scanning electron microscope. All measurements obtained during this study were performed on stained specimens and recorded in µm, except where otherwise mentioned.

3.2.1 Live observations

Two different methods were employed and evaluated to conduct the live observations. To easily isolate and handle the sampled ciliates, the rectal fluid was placed in a cavity block, from which ciliates were removed for further observation, according to the methods described by Sandon (1941) and Foissner (2014).

Following the method of Sandon (1941), live individuals were trapped between a microscope slide and coverslip in a thin layer of petroleum jelly. Petroleum jelly was applied on both the coverslip and the slide, whereafter fine lines were drawn in the jelly with a pointed instrument. The live individuals were placed inside the lines on the microscope slide with a pipette and covered with the coverslip positioned so that its lines corresponded to that of the microscope slide. Ciliates were observed for several hours after removal from the host under a Zeiss Stemi 305 dissecting microscope and a Zeiss Axio Imager M1 compound microscope equipped with a digital imaging system (Zeiss, Oberkochen, Germany).

The second method, following Foissner (2014), was to prepare coverslips with equally sized petroleum jelly dots (approximately 2 mm in diameter) on all four corners. A drop of rectal fluid with live ciliates was placed on a microscope slide. The prepared coverslip was carefully placed on the microscope slide with the jellied corners facing down and the ciliates were observed for several minutes after removal from the host.

3.2.2 Haematoxylin staining

Ciliates fixed in 10% neutral buffered formalin, 70% ethanol and Bouin's solution were stained with two different haematoxylin stains to enhance the visibility of the nuclei, which is important for the proper identification of ciliates. Prior to staining, the ciliate specimens of interest were placed on three to five albumin-covered slides, prepared as described above in section 3.3.3. For all haematoxylin staining methods used, from the first submersion in the haematoxylin working solution to the final mounting in the synthetic medium, the microscope smears were never allowed to dry. Eosine counterstain, usually used with haematoxylin stains, were not used to improve results.

I) Mayer's haematoxylin staining (wet smears):

Ciliates fixed in Bouin's solution were replaced with 70% ethanol and directly stained from ethanol by submerging the prepared microscope slides in Mayer's haematoxylin working solution, prepared according to Presnell *et al*. (1997) for 10 minutes. The slides were then washed under slowly running tap water for 3 minutes. Then, the slides were submerged in Scott's solution for 3 minutes, followed by another wash under slowly running tap water for 3 minutes. The stained specimens were dehydrated by submerging the slides in a series of 70% ethanol for 1 minutes, 96% ethanol for 2 minutes, 100% ethanol for 6 minutes and xylene for 3 minutes. Finally, the ciliates were mounted in Eukitt mounting medium.

II) Harris' haematoxylin staining (wet smears):

Ciliates fixed in 10% neutral buffered formalin were replaced by 70% ethanol and directly stained from the ethanol in Harris' working solution, prepared according to Presnell *et al*. (1997), for 10–20 minutes. The slides were dipped twice in distilled or double distilled water. Thereafter, the slides were washed under slowly running tap water. After this treatment, the slides with specimens were dehydrated in increasing ethanol series, consisting of submerging the slide in 30%, 50%, 70%, 90%, 96% and 100% ethanol concentrations for 2 minutes each, and then in 100% ethanol for 3 minutes, followed by Xylene for 2 minutes. Finally, the ciliates were mounted in Eukitt ® synthetic mounting medium.

3.2.3 Supravital staining

Certain morphological features were also observed in non-living, fixed and stained ciliate specimens. One such method involved supravital staining with methyl green-pyronin (Foissner, 1979). The methyl green-pyronin solution was prepared with 1 g of methyl green-pyronin (CHROMA, Germany) and 100 ml of distilled water. The target ciliates, in a drop of original gut fluid, were placed in an equally sized drop of methyl green-pyronin in the centre of a microscope slide. Thereafter, a coverslip with petroleum jelly-covered dots was gently placed on top, with the jellied side facing down. This caused specimens to be lightly squeezed, which revealed the nuclear apparatus. However, most of the time it was not necessary to squeeze the specimens, since ciliates are rather thin.

3.2.4 $AgNO_3$ impregnation

A thin, uniform layer of albumin (prepared at least 24 h before) was applied to the surface of cleaned microscope slides and allowed to dry. For optimal results, the albumin-covered slides were prepared at least 24 hours before use. A drop of rectal fluid containing the target ciliates was then placed on the albumised slide and spread thinly over the slide with a needle without touching the albumin layer. The fluid was allowed to dry at room temperature. The ciliate specimens were then fixed with Bouin's solution for 30 minutes by placing the slide upside down on two horizontally positioned matches. The slide with specimens was submerged in 70% ethanol for 2 minutes, followed by submersion in 10% silver nitrate ($AgNO_3$) for 20 minutes. The slide was placed, right side up, in a petri dish and submerged in tap water. This setup was placed at 2 cm underneath a blacklight for 20 minutes. After this treatment, the slide with specimens was dehydrated with an increasing ethanol series, consisting of submerging the slide in 70%, 80%, 90%, 96% and 100% ethanol concentrations for 4 minutes each. Finally, the slide was submerged in xylene for 3 s and mounted with Eukitt ® synthetic quick hardening mounting medium (Merck, Darmstadt, Germany).

3.2.5 Scanning electron microscopy

Isolated ciliates, fixed in freshly made 2.5% glutaraldehyde and stored at 4 °C for a maximum of 48 hours, were further prepared for scanning electron microscopy by a process of chemical dehydration. As a first step, the fixing medium was replaced with freshly made phosphate buffer for 30 minutes. Phosphate buffer were made up of 80% Buffer A (35.814g/L $Na_2HPO_4.12H_2O$) and 20% of Buffer B (13.610 g/L KH_2PO_4). The specimens were then dehydrated with a series of increasing ethanol concentrations, which consisted of 30%, 50%, 70%, 80%, 96%, 99 and another batch of 99% ethanol for 30 minutes each. This treatment was followed by dehydration in an increasing hexamethyldisilazane solution series (Sigma Aldrich, Saint Louis MO, USA) of 30%, 70%, 100% and 100% for 5 minutes each. The specimens were left to dry in a desiccator for 24 hours, mounted on 12 mm aluminium stubs and sputter-coated for 2 minutes in argon gas at a pressure of 2 atm, with a gold palladium alloy. (SPI-ModuleTM Sputter Coater, SPI Supplies, West Chester, PA, USA). Thereafter specimen stubs were observed with a scanning electron microscope at 10 kV (Phenom PRO Desktop SEM, Phenom-World B., Eindhoven, Netherlands).

3.3 Experimentation with alternative methods

3.3.1 Heat fixation

A drop of gut fluid containing the target ciliates were placed on a microscope slide. A coverslip with petroleum dots were placed on top of the microscope slide. Thereafter the slide was heat fixed from beneath with a Bunsen burner flame for 3 seconds and observed under the compound microscope.

3.3.2 Iodine staining

The target ciliates, in a drop of original gut fluid, were placed in an equally sized drop of iodine in the centre of a microscope slide. Subsequently, a coverslip with petroleum jelly-covered dots was placed on top whereafter examined under the compound microscope.

3.3.3 Deciliation

A drop of rectal fluid containing the target ciliates were placed in a cavity block. An equally sized drop of deciliation reagent was placed in the cavity block and homogenised. The deciliation reagents that were evaluated included liquid soap (Sunlight, Unilever, Durban, South Africa), washing powder (OMO, Unilever, Durban, South Africa), lemon juice, lime juice, proteinase K (Thermo Fisher, Lithuania) and hand soap (Lux, Unilever, Durban, South Africa). The ciliates in the cavity block were observed with a stereomicroscope. The time of exposure of the ciliate specimens to the deciliation fluid was a minimum of 5 minutes, with the maximum exposure time depending on the reagents used since the difference in osmotic concentrations could cause the ciliates to burst. After exposure to the deciliation fluid, ciliates were placed in either 2.5% freshly prepared glutaraldehyde solution or Flemming's fluid, prepared according to du Preez & van Wyk (2007).

3.3.4 Culturing

Since food quantity seems to be an important factor when culturing protozoans (Hyman, 1925), culture mediums for each of the ciliate species were prepared according three concentrations, that is diluted, semi-concentrated and concentrated. In the diluted culture medium, the relative quantity of the hay infusion was less than the water. In the semi-concentrated culture, the relative quantity of the hay infusion was equal to the water. In the concentrated culture medium, the relative quantity of the hay infusion was more than the water. Furthermore, two types of plants were used as food sources in the culture mediums, namely an infusion of *Phleum pratense* or *Medicago sativa.*

The infusions were prepared by boiling 10 g of *Phleum pratense* or *Medicago sativa* in 1 L tap water for 30 minutes. The resulting mixture was filtered through cheesecloth and allowed to cool. Two drops of 1 M sodium hydroxide (table salt) and a pinch of black pot soil were added. Dilutions were made with tap water (30%, 60%; 90%) and 60 mL of each dilution was poured in the culturing dishes. The dishes were left uncovered for 24 hours. Dried lettuce shreds or wheat seeds (approximately 0.3 g) were added to each dish.The prepared culturing dishes were inoculated with the target ciliate specimens and left at room temperature. The cultures were observed regularly with a stereoscope to monitor population levels.

3.3.5 Counting ciliates

To count the ciliates of the South African localities, the alimentary tract was removed intact from the host, cutting it at the top of the oesophagus and the tip of the rectum. The rectum, with all its contents, as well as the intestine, was dissected from the rest of the alimentary tract and weighed separately. The rectal content was placed in a cavity block and washed with 3–20 mL (depending on the infection levels) 70% ethanol. The rectal content and ciliates were homogenised with a pipette and two aliquots of equal volume were injected into each chamber (Figure 2.3) of the McMaster counting slide (Chalex LLC, USA). The ciliates were counted using the grids of the two chambers. The ciliate count was determined by multiplying the sensitivity factor, as calculated in equation 1, by the total number of ciliates counted, as demonstrated in equation 2.

$$sensitivity = \frac{total\ volume\ ethanol\ (ml)}{volume\ of\ chambers \times mass\ of\ rectal\ content} \quad \text{(Equation 1)}$$

$$count\ of\ ciliates\ per\ gram\ of\ rectal\ contents = sensitivity\ \times total\ count\ in\ both\ chambers$$

(Equation 2)

Figure 2.3: Two chambered McMaster slide used to count ciliates (Chalex LLC, USA)

From the French localities, infection intensities of the rectal endociliates were recorded on a scale. This scale ranged from “no”, “mild” to “severe” infections. For all ciliate counts, ciliates were not counted according to morphospecies since they are polymorphic.

3.4 Molecular and phylogenetic analyses

3.4.1 Isolation and extraction

Different morphospecies were isolated by making use of a micropipette and sterile tips, as explained above. Since ciliates usually have food vacuoles with food particles, a starvation period is necessary to prevent DNA contamination. The starvation period was conducted by leaving the different morphospecies in sterilised saline. After starvation, the ciliates were fixed in 100% molecular-grade ethanol. For DNA extraction, the Quick-DNA miniprep plus extraction kit (Zymo Research, USA) was used according to the solid tissue protocol. Polymerase chain reactions

(PCRs) for DNA amplification were implemented with different primers and PCR protocols to find the best method for the barcoding of the ciliates of *X. laevis*. The optimised method is described below.

3.4.2 PCR and sequencing

Eukaryote-specific primers were used to amplify the 18S rDNA sequence, namely 'MedlinA' (5'-CGT GTT GAT CCT GCC AG-3') and 'MedlinB' (5'-TGA TCC TTC TGC AGG TTC ACC TAC-3') (Medlin et al., 1988). In addition, we also evaluated the eukaryote specific primers 'HepR900' (5'-CAAATCTAAGAATTTC ACCTCTGAC -3') (Ujvari *et al*., 2004) and 'HAM' (5'- GCCAGTAGTCA TATGCTTGTC -3') (Criado-Fornelio *et al*., 2006). The PCR protocol was implemented as follows for all primer pairs: initial denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 30 s, amplification at 61°C for 30 s and extension at 72°C for 2 minutes, ending in a final extension at 72°C for 10 minutes. PCR products were sent to a commercial company (Inqaba Biotec, Pretoria, South Africa) for purification using the ExoSAP protocol (New England Biolabs Lty., Massachusetts, United States). All sequencing was performed via BigDye® Terminator version 3.1 Cycle Sequencing, using the corresponding primer pairs necessary for the final PCR reaction, on an ABI3500XL analyser (Applied Biosystems™). Editing software, Geneious Prime (Saint Joseph, Missouri, United States) were used to assemble and edit contiguous sequences.

3.4.3 Phylogenetic analyses

In addition to the sequences obtained from the rectal endociliates of *X. laevis*, 18S rDNA sequences of ciliates from various amphibian and other animal hosts were obtained from GenBank for phylogenetic analyses (Appendix C, Table 2.1). The 18S rDNA sequence of the ciliate found in the intestinal tract of cockroaches, *Nyctotherus ovalis* (Ciliophora: Nyctotheridae) (accession number AJ009705; van Hoek et al., 1999) was included as outgroup. The sequences were aligned with the Clustal W algorithm under default settings (Sievers & Higgins, 2018) implemented in MegaX (Kumar *et al*., 2018). The sequence alignment was trimmed manually to the length of the shortest sequence for downstream analyses. The optimal model of evolution for the gene alignment was selected based on the Bayesian Information Criterion with the ModelFinder selection routine (Kalyaanamoorthy et al. 2017) implemented in W-IQ-TREE version 1.6.12 (Trifinopoulos et al. 2016). The selection routine identified the best-fit model as GTR + I + G, which is the general time reversible (GTR) model of base substitution (Tavare, 1986) allowing for a proportion of invariant sites and a discrete Gamma model of rate heterogeneity across sites (Gu *et al*. 1995).

The phylogenetic analysis was performed with the IQ-TREE algorithm (Nguyen *et al*. 2015) implemented in W-IQ-TREE version 1.6.12 (Trifinipoulos *et al*. 2016). Branch support was assessed through ultrafast bootstrapping (UFboot2; Hoang *et al.,* 2018) and the Shimodaira-Hasegawa approximate likelihood ratio test (SH-aLRT; Guindon *et al*., 2010), each with 10000 replicates. Phylogenetic trees were visualised using Figtree version 1.3.1 (Rambaut, 2010) and edited by making use of a graphic editor.

Chapter 3:

Results

3.1 Method evaluation

The use of different methods through the integrated approach gave valuable information. Some methods worked just as expected, whereas other methods did not present with the results that were anticipated. In this chapter the methods that were used were evaluated according to the following symbols, adapted from Foissner (2014), namely: ++, Exceptional; +, Good; +/–, Works sometimes; –, Did not work (Table 3.1; Figure 3.1). The decisions for symbol classification were based on the duration as well as the number of efforts needed for success.

Table 3.1. Method evaluation according to structures revelated. Table adapted from Foissner (2014).

++, Exceptional; +, good; +/–, works sometimes; –, did not work.

Structures revealed	Live observation	Dry/Wet silver nitrate staining	Methyl-green pyronin (Supravitalstaining)	SEM	Heamatoxylin staining	Heat fixation	Iodine
Shape	++	+/-	-	++	+	+	+
Nuclei	-	+/-	++	-	++	+	+
Contractile vacuole	+	-	++	-	++	+	+
Cytopyge	+/-	-	+/-	++	-	-	-
Colour	++	-	-	-	-	-	-
Mucocysts	-	-	-	-	-	-	-
Cortical granules	+/-	+/-	++	-	++	+	+
Infraciliature (basal bodies & cilia)	+/-	+/-	-	+	-	+/-	-
Silveline pattern	-	+/-	-	-	-	-	-
Cortical fibres	-	-	-	-	-	-	-
Surface ornamentation	+/-	-	-	++	-	-	-

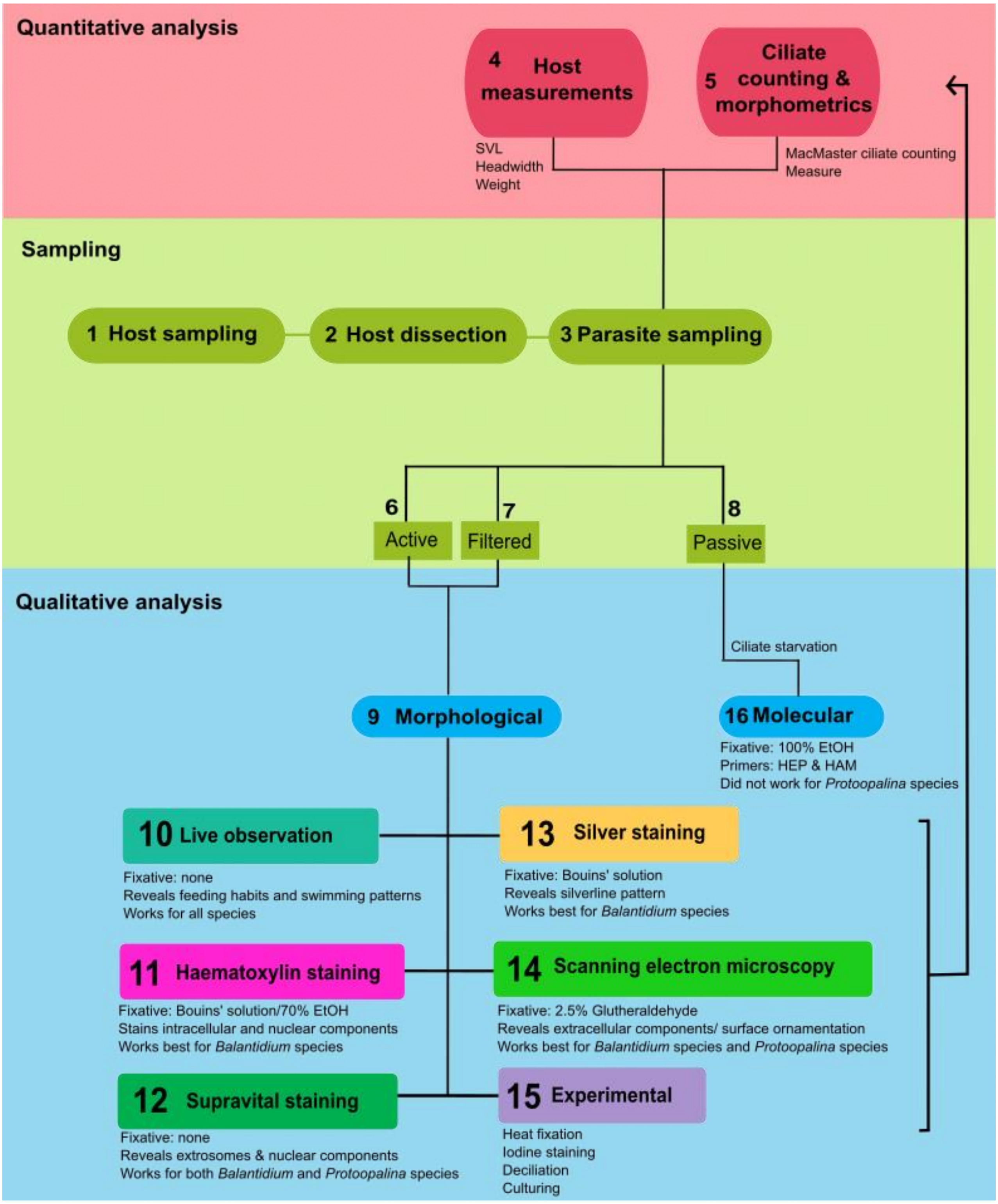

Figure 3.1: Evaluation of the ciliate identification workflow: *Xenopus laevis* were sampled and screened for parasites (1–3). Ciliates were sampled from the rectum of hosts (6–8). Thereafter, hosts and ciliates were analysed quantitively (4–5). Qualitative analyses were only performed on ciliates, not hosts, and entailed both morphological (9–15) and molecular (16) methods.

3.1.1 Sampling

Filtered sampling: The mesh sifts showed better results than the other sampling methods to concentrate and clean the ciliates, Similarly Sandon's apparatus also resulted in good concentration and is readily available.

Passive sampling: This method was found to be time-saving and less intensive than active sampling. The method worked particularly well if the specimens were used for downstream molecular analysis.

Active sampling: This method resulted in high ciliate concentrations; however, samples contained large amounts of debris. Cleaning samples manually was labour-intensive. This method worked best for downstream haematoxylin staining, silver impregnation and supravital staining.

After numerous attempts to successfully isolate ciliates from the gut, the best working method was found to be passive sampling. Vast numbers of ciliates swam out of the rectum, which eased sample cleaning, especially for molecular study. If more ciliates were needed, the rectum was gently opened and a micropipette was used to isolate the remaining ciliate morphospecies from each other and the debris.

3.1.2 Live observations (Table 3.1)

The best live observations were made by the method describe by Foissner (2014), where petroleum dots were used to separate the cover glass from the microscope slide (Figure 3.2A). Observation of swimming patterns assisted in identifying different morphospecies and different size cohorts of a specific morphospecies.For optimised downstream analyses, it was important to sort morphospecies while alive, since this will save time and ease visual classification.

3.1.3 Haematoxylin (wet smears) (Table 3.1)

The best staining results were obtained from Mayer's haematoxylin stain (Figure 3.2B). Not only did the nuclear components stain exceptionally well with this stain, but all the intercellular components, such as the infundibulum, food vacuoles and contractile vacuoles, were also revealed. This was particularly true for the *Balantidium* species.

Although Harris' haematoxylin also stained the nuclear components, it was not as distinctive as specimens stained by Mayer's haematoxylin.

3.1.4 Supravital staining (Table 3.1)

This method failed to stain mucocysts. However, the methyl-green pyronin worked exceptionally well for a quick and easy nuclear stain, especially for *Protoopalina* sp. Unfortunately, methyl-green pyronin impacts and changes the shape of the ciliates due to osmotic pressure (Figure 3.2C). As a result, this stain can not be used to characterise ciliate shapes for morphological study.

In spite of various attempts and alterations to the method of dry silver impregnation of Foissner (2012), this method did not work in the present study. However, the wet silver impregnation method worked to some extent. Kineties were vaguely visible (Figure 3.2D). For this method to work, it was: i) crucial that specimens were never allowed to dry out; and ii) important to use tap water or water of slightly higher pH than 7. Since silver impregnation was only partially successful, kineties could not be counted.

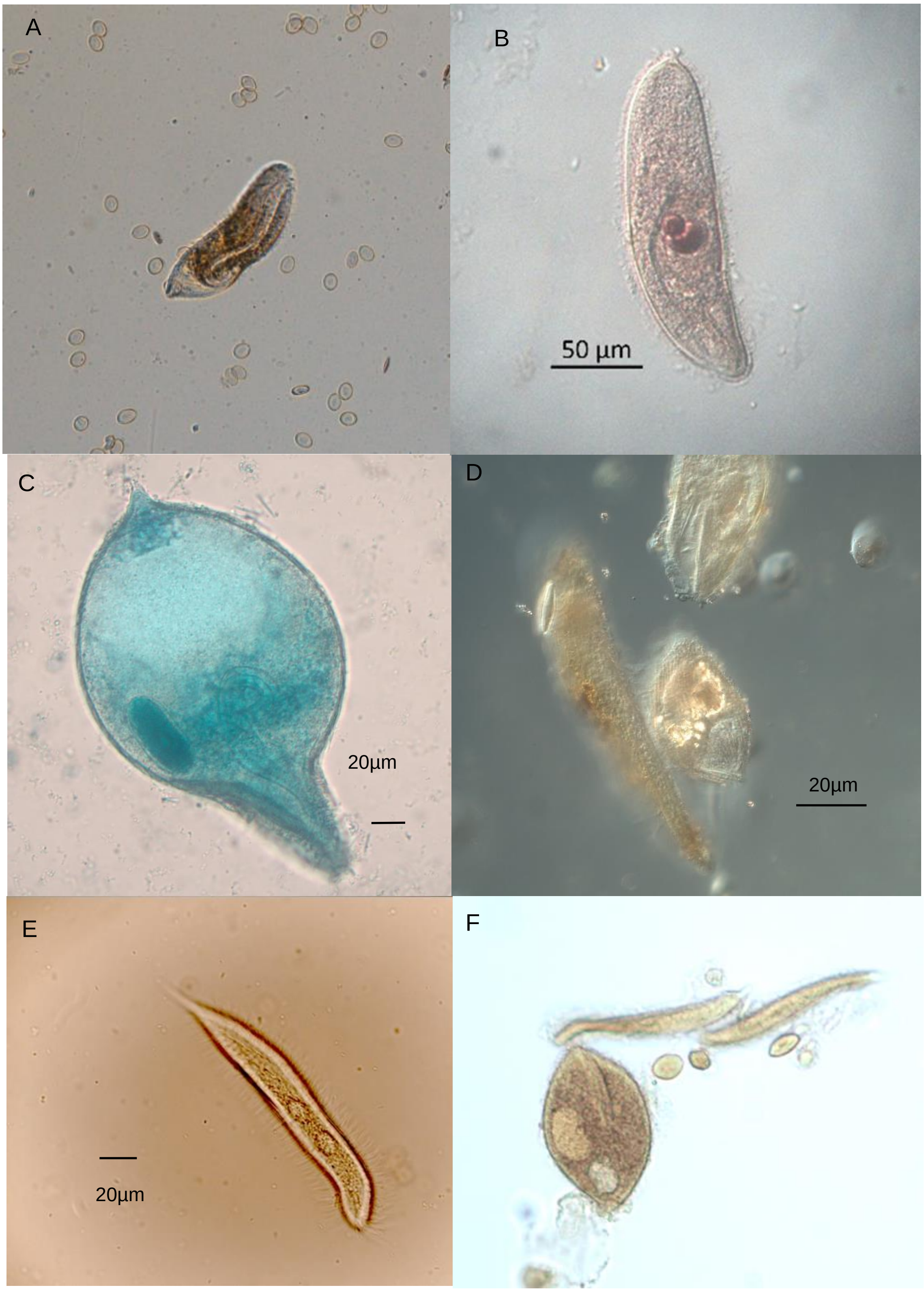

Figure 3.2: A **–** Live observation of *Balantidium kirby* collected from *Xenopus laevis* from Potchefstroom; B – *B. kirbyi* stained with Mayer's haematoxylin; C – *B. kirbyi* swollen due to osmotic pressure of methyl-green supravital staining; D – Endociliates stained with silver staining; E – Heat-killed *Protoopalina* sp.; F – Endociliates stained with iodine.

3.1.5 Silver impregnation (Table 3.1)

Silver impregnation did not work to full extent. Although the silverline pattern could be seen vaguely, this method needs to be revised for obtimal identification purposes in future studies.

3.1.5 Scanning electron microscopy (SEM) (Table 3.1)

Scanning electron microscopy aided in visualisation and description of structures impossible to see with an ordinary light microscope. By examining the gut ciliates of *X. laevis* using SEM, previous morphological uncertainties from the prior literature could be solved. As for the fixatives evaluated, glutaraldehyde worked better than Flemming as a fixative. It was found that using fresh glutaraldehyde — not older than 48 hours — was vital .

3.1.6 Molecular analysis (Table 3.1)

Passive sampling for molecular analysis delivered the best results. Importantly, sterile tips should be used when isolating different morphospecies for molecular analysis. After the morphospecies were isolated, the saline fluid was replaced three times with 100% ethanol before extractions were done. When isolating ciliates for extraction, 10 indivuduals of each morphospecies delivered the best results for a single extraction.

The most successful extractions were made by placing ciliates in distilled water instead of ethanol. This eased the extraction process by skipping the ethanol drying phase, limiting the potential of possible lower yields. The exact amount of nuclease-free water (according to extraction protocol) can also be used to prevent the loss of specimens. When water is used, it is important that extractions are carried out the same day as ciliate sampling.

For the PCR, forward primer HAM and reverse primer HEP gave the most satisfying results for *Balantidium* species. Unfortunately, *Protoopalina*-specific primers, BA and PKrc, were not sufficient for obtaining reads.

3.1.7 Experimental

3.1.7.1 Heat fixation (Table 3.1)

Heat fixation gave very positive results (Figure 3.2E). This method was quick and easy and could be used to reveal the nuclear components, the infundibulum and food vacuoles. However, this method did not yield permanent mounts.

3.1.7.2 Iodine (Table 3.1)

Although iodine did not stain the ciliature as was expected, it stained the nucleus successfully (Figure 3.2F).

3.1.7.3 Deciliation

Although deciliation did not work to the full extent, the best results were obtained from deciliation fluids, such as a commercial soap detergent powder (OMO, Unilever, Durban, South Africa) and ordinary hand soap. Kineties were revealed to some extent, although not all could be seen clearly (Figure 3.3). For all specimens, more information about the infraciliature could contribute to more conclusive

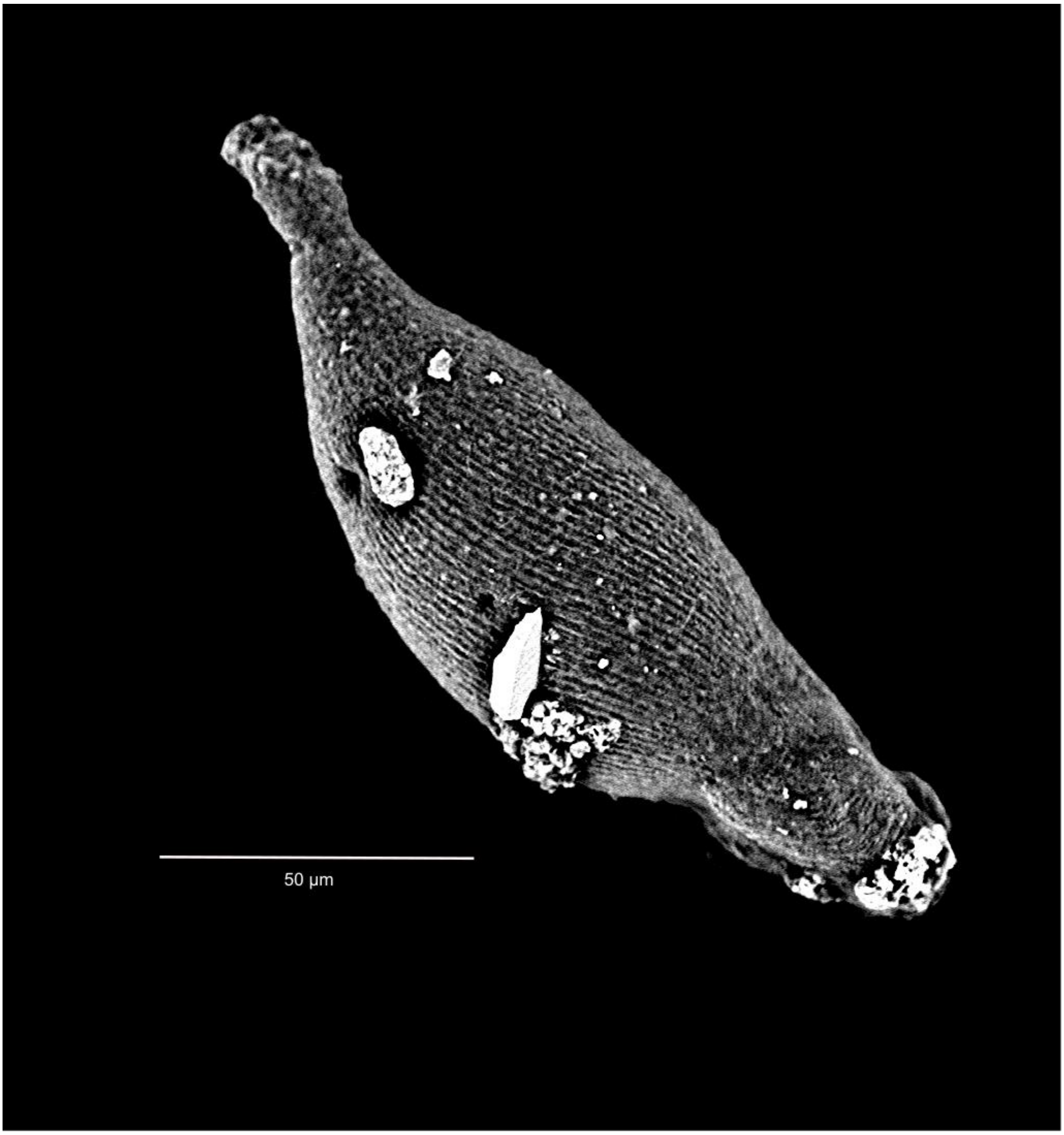

Figure 3.3: Scanning electron micrograph of *B. kirbyi* collected from *Xenopus laevis* from Dullstroom, South Africa deciliated with laundry detergent and hand soap.

descriptions. Unfortunately, due to the unavailability of Protargol and difficulty with the silver staining process, we suggest future studies to look at *Xenopus'* ciliates using transmission electron micriscopy (TEM) or deciliation.

3.1.7.4 Culturing

Despite several attempts and modifications of methods, culturing these ciliates was not successful. Specimens survived a maximum of 10 hours. Nevertheless, it was found that if leaving an unopened rectum submerged in 0.6% amphibian saline at room temperature, ciliates survived more than 48 hours in a covered cavity block. When the rectum was opened, the survival period was reduced to about 24 hours.

3.1.7.5 Ciliate counting

The MacMaster was a valuable tool for counting the protozoan specimens (Table 3.1). Since the entire rectum content was used, it was not necessary to use floatation fluids for separation. Due to high osmolarity, floatation fluids cause ciliates to rupture and are not usable with ciliates in any case. For heavy infections, weaker dilutions were made to enable counting of ciliates. Due to the variance in shape and size (polymorphism) of ciliates, one can only do a total ciliate count rather than counting the respective morphospecies. The 70% ethanol worked well as the dilution fluid, since ciliates can still be used afterwards for qualitative analysis.

The infection scale used to count the ciliates from France were well aimed. Differentiation between these infection levels were not vague, hosts either had non, a few or numerous ciliates (Table 3.2).

Table 3.1: Ciliate counts for 11 *Xenopus laevis* caught from two localities in South Africa, Dullstroom & Welverdiend.

	Mean	Range	Standard deviaton
Host mass	22.21	(3.10–76.70)	24.54
Rectum initial (g)	0.27	(0.01–0.92)	0.33
Rectum empty (g)	0.12	(0.01–0.83)	0.24
Rectum content	0.16	(0.01–0.69)	0.24
Dilution (ml)	10.73	(3.00–20.00)	8.88
Total ciliate count in chamber	1155.27	(66.00–3635.00)	1460.58
Count per g	1128184.29	(14347.83–8802413.27)	2569504.57
Total ciliate count in rectum	67441.51	660.00–242333.33)	103047.82

Table 3.2: Ciliate intensity for 113 *Xenopus laevis* caught from ten localities in France.

Locality	**Ciliate intensity per host count**		
	none	**mild**	**severe**
Ambillou	4	5	2
Bouillé-Saint-Paul		1	12
Brézé			10
Cersay 1	1	4	5
Cersay 2			8
Chalonnes		3	4
Coudray-Macouard		5	6
Epieds			12
La Butte		1	6
Massais	1	1	14
Saint-Martin		1	7
Total	6	21	86

3.2 Taxonomic description of the four morphospecies

At first glance, the ciliate community inside the rectum of *X. laevis* from South Africa revealed four morphospecies. Morphospecies 1 (RSA) = *B. kirbyi* was the largest, most frequently occurring ciliate. Morphospecies 2 = *Balantidium* sp. 1 was a reddish, small, round ciliate. Morphospecies 3 = *Balantidium* sp. 2 was a smaller round ciliate of which only molecular data was obtained. Morphospecies 4 = *Protoopalina* sp. 1 was an elongated taper opalinid. The ciliate community of *X. laevis* from France revealed morphologically comparable species to the three documented morphospecies found in South Africa. These included Morphospecies 1 (FRA) = *B. kirbyi* (Figure 3.4 A), Morphospecies 2 (FRA) = *Balantidium* sp. 3 (Figure 3.4 B), and Morphospecies 3 (FRA) = *Protoopalina* sp. 2 (Figure 3.4 C). Although the morphospecies from France were similar to those of South Africa, only the specimens from South Africa were measured (Table 3.3).

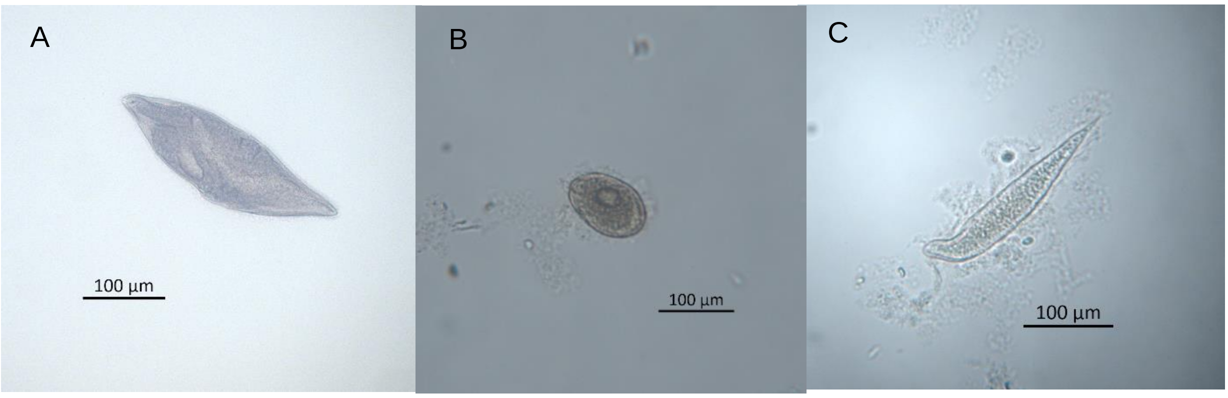

Figure 3.4: Light micrographs of A– *Balantidium kirbyi* from France (haematoxylin stained), *B– Balantidium* sp.3 (haematoxylin stained) from France, *C– Protoopalina* sp. 2. from France (haematoxylin stained).

Table 3.3: Morphometric data of *Balantidium. kirbyi, Balantidium* sp. 1 and *Protoopalina* sp. 1 from South Africa. Values are given as range with mean in paranthesis. Measurements in µm.

Characteristics	*B. kirbyi* (24 individuals)	*Balantidium* sp. 1 (6 individuals)	*Protoopalina* sp. 1 (7 individuals)
Body length after haematoxylin staining	36.1–257.2 (139.2)	36.1– 80.7 (52.3)	90.9–133.2 (110.5)
Body width after haematoxylin staining	13.4–103.2 (47.8)	26.8–45.3 (32.8)	13.0–18.0 (14.0)
Body shape & characteristics	elongated	round, reddish	long, thin
Macronucleus size	6.3–43.3 (22.4) length, 3.3–21.0 (11.8) width	9.8–21.1 (12) length, x 5.2–10.8 (7.2) width	6.1–8.1 (7.4) lemgth, x 5.2–8.5 (7.0) width
Micronucleus size	1.3–10.2 (3.5) length, x 1.0–11.9 (3) width	1.5–2.6 (1.8) length x1.6–2.4 (1.9) width	Same as macronucleus
Vestibulum	7.9–90.0 (37.3)	11.6–12.3 (11.7)	n/a
Infundibulum	23.7–234.5 (120,8)	n/a	n/a
Food vacuole	14.9–37.4 (25.9) x 13.9–29.6 (23.3)	not measured	n/a
Food vacuole count	3 to 6	not distinct	n/a
Kineties	not measured	not measured	not measured

All measurements in µm

Kineties, no measurements could be made since silver staining method did not work to full extent.

3.2.1 *Balantidium xenopi* Du Puytorac *et* Grain, 1965. Synonyms: *Paranyctotherus (Balantidium) kirbyi* (Rodriguez,1939) Sandon, 1941*, Balantidium xenopodis* Kuperman, Matey, Fisher, Erwin, Warburton, Bakhireva *et* Lehman, 2004

(Figures 3.5 – 3.7; Table 3.3)

Measurements were made from 24 specimens stained with Mayer's haematoxylin

Host: *Xenopus laevis*

Locality: South Africa: Mpumalanga, Free State, Gauteng, Northern Cape, Western Cape, Eastern Cape, North West, Limpopo; France

Site of infection: Rectum

General description. Size and Shape. Large, body elongated, round anterior, narrowing posteriorly. Convex oral side with sigmoid aboral side (Figures 3.5; 3.6A). Variable shape, well–nourished individuals more circular in mid-region. Starved specimens transparent, flattened. Highly variable in size (Figure 3.7A), body 36.0–257.2 (139.2) long, 13.4–103.2 (47.8) wide. Maximum width at midsection of body. ''Villeneuve-Brachon's'' field present (Figure 3.6D, 3.7A). **Vestibulum & Infundibulum.** Vestibulum a narrow groove, starting from largest anterior part of the body extending posteriorly, parallel with body, approximately 1/3 of body length, 7.9–90.0 (37.3) in length (Figures 3.5; 3.6B). Infundibulum (I) 23.7–234.5 (120.7) a continuous tube, extending posteriorly, coiling back anteriorly (Figures 3.5; 3.7B). Occasionally straight, extending posteriorly, no coiling (Figure 3.7C). **Nuclei**. Bi-nucleated with no karyophore; macronucleus (Ma), 6.3-43.3 (22.5) in length, 3.3–21 (11.8) in width, position variable, kidney-shaped, granular (Figures 3.5; 3.7B). Micronucleus (Mi), 1.27–10.22 (3.5) in length, 1.0–11.872 (3) in width, granular, located in depression of macronucleus (Figures 3.5; 3.7B), sometimes indistinct. **Cytoplasmic structures.** Distinct pellicle (P) (1.8) (Figure 3.7B). Food vacuoles 3–6 in number, 14.9–37.6 (mean) long, 13.9–29.6 (23.4) wide (Figures 3.5; 3.7B). Excretory vacuole (EV), at posterior third of body (Figures 3.5; 3.7B). Cytoproct (C), conic, tube-like, at most posterior region of body (Figures 3.5; 3.7B). Contractile vacuole (CV) present (Figures 3.5; 3.7B, C)., Greenish-brown granular cytoplasm (Figure 3.7C). Contractile vacuole pore (CVP), usually more than one, one anterior, one posterior (Figures 3.5; 3.6D, E). **Ciliature:** Uniform, densely ciliated (c) (Figures 3.5; 3.6 A, B, D; 3.7C). Monokinetid, kineties (k) orientated parallel to longitudinal axis (Figures 3.5; 3.6D). Cilia originating from inside of superficial cortical grooves. Multiple cortical grooves (g) arranged alternately with cortical ridges (r), also parallel to longitudinal axis of body (Figure 3.6D).

Remarks:

The somatic monokinetids and oral ciliature as simple kinetids are characteristic of the class Litostomatea. The densely ciliated oral cavity and absent toxicysts are key identifiers of the subclass Trichostomatia. Furthermore, Trichostomatia are typically known to be anaerobic endosymbionts in vertebrates. This species further belongs to the order Vestibuliferida because of the presence of a densely ciliated vestibulum. In addition, this species is classified as belonging to the genus *Balantidium* because of the presence of two nuclei, of which the macronucleus is kidney-shaped. Furthermore, *Balantidium* species possess contractile and food vacuoles, which is rare in parasitic Protozoa. This species is identified as *B. kirbyi* in accordance with previous literature describing several morphological similiarities of a *Balantidium* sp. found in *Xenopus* frogs hosts (Rodriguez, 1939; Sandon, 1941; Du Puytorac & Grain, 1965). These similarities include size, swimming patterns, colour, intracellular organelles like nuclei, contractile vacuoles, food vacuoles, vestibulum as well as the infundubulum.

In comparison with other frog *Balantidium* species, *B. kirbyi* possesses a specialised ‘’Villeneuve-Brachon’s’’ field of somatic cilia to the right of the vestibulum, similar to the ‘’Villeneuve-Brachon’s’’ field of the *Balantidium* type species *B. entozoon* (Grim & Buonanno, 2009). Moreover, *B. kirbyi* differs from *B. entozoon* / *B. elongatum* (Kornilova *et al.,* 2016), as well as *B*. *grimi* (Zhao *et al*., 2018), *B*. *honghuensis* (Li, *et al.*, 2013) and *B. duodeni* (Chistyakova *et al*., 2014), in terms of body shape, body measurements (larger vestibulum and body length) and its elongated infundubulum. Furthermore, the presence of food vacuoles distinguish *B. kirbyi* from *B. grimi* and *B*. *honghuensis.*

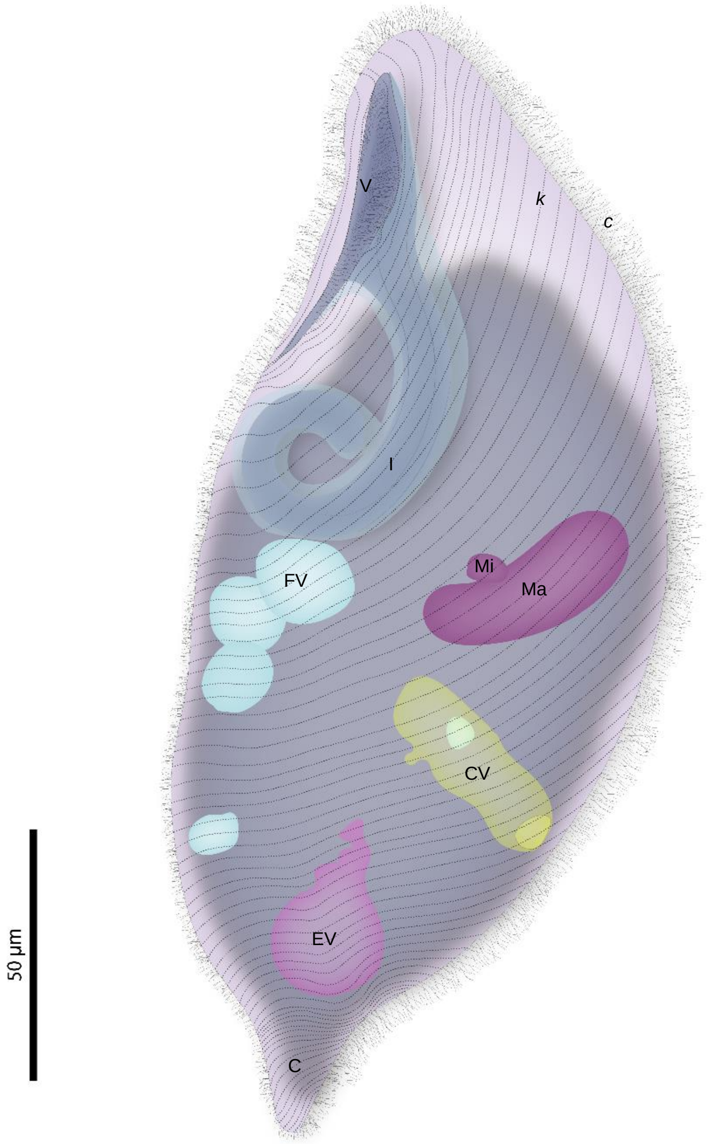

Figure 3.5: Line drawing showing intra- and extracellular features of *Balantidium kirbyi* collected from *Xenopus laevis* from South Africa. C –cytoproct, CV – contractile vacuole, Mi – micronucleus; Ma – macronucleus, k – kineties, c – cilia; I – Infundubulum; V–vestibulum.

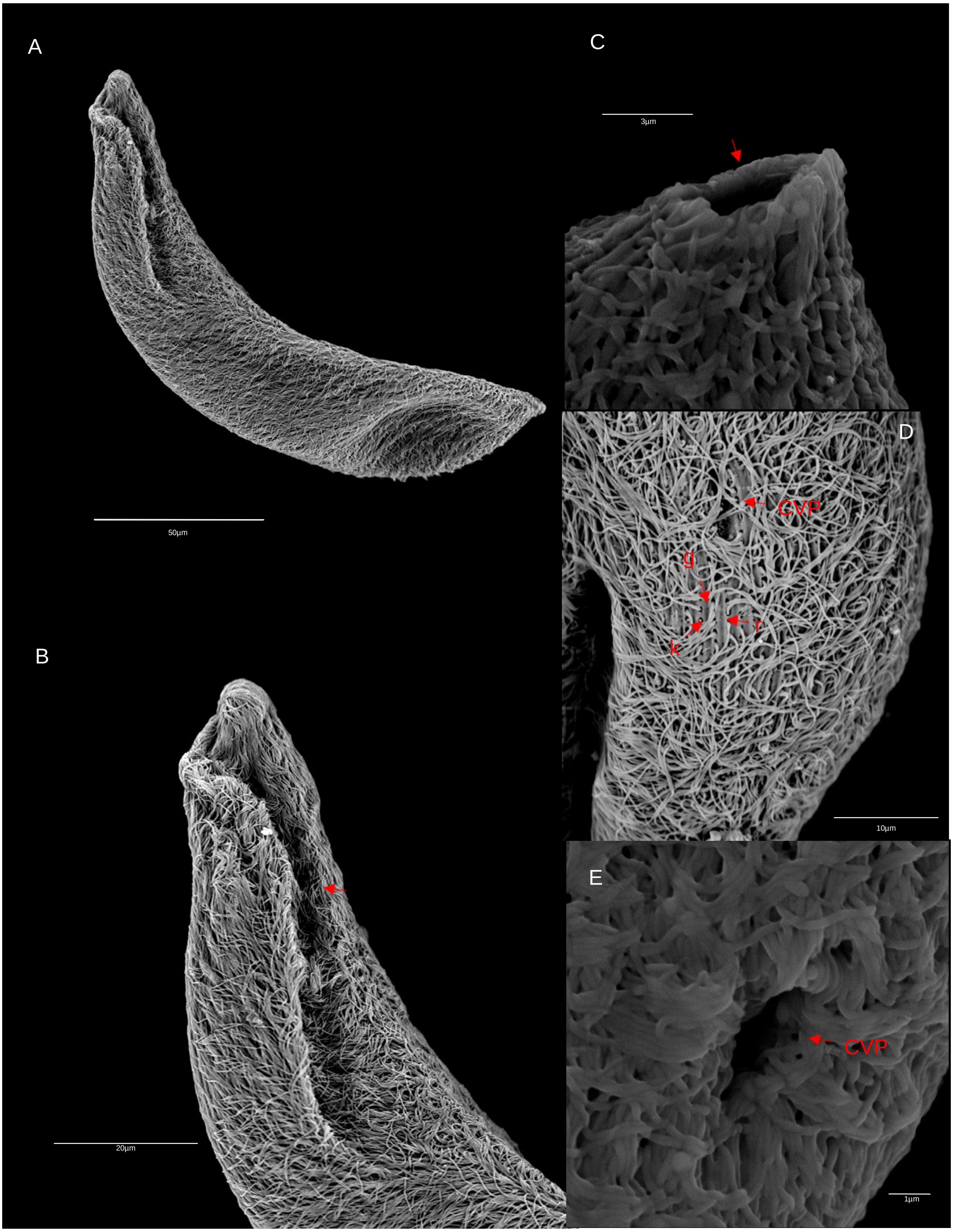

Figure 3.6: Scanning electron micrographs of *Balantidium kirbyi* collected from *Xenopus laevis* from South Africa: A – convex oral side with sigmoid aboral side, B – vestibulum; C – cytoproct (external), D – ''Villeneuve-Brachon's'' field with monokinetids (k), cortical ridges (r) and cortical grooves (g), E – contractile vacuole pore (CVP).

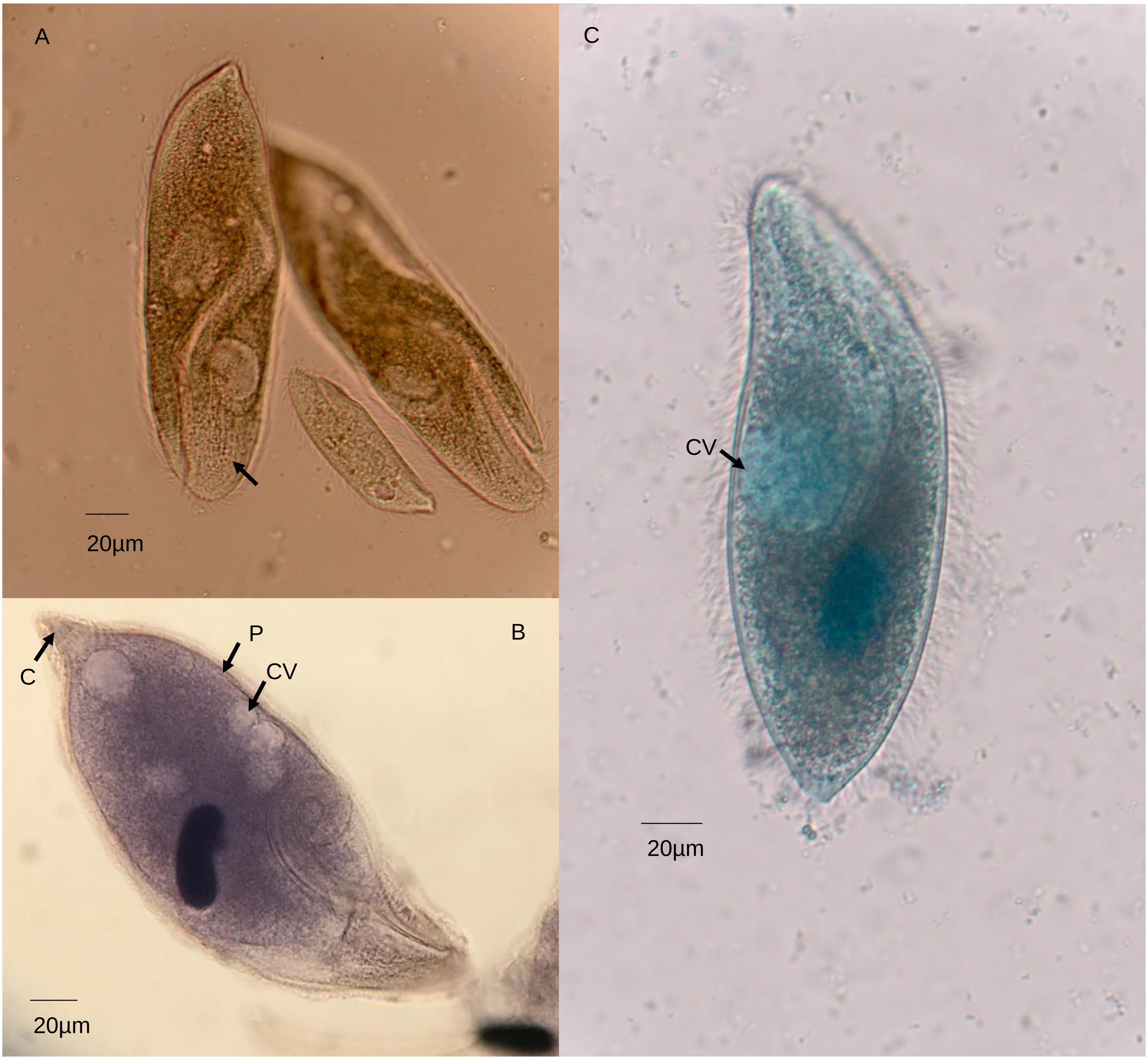

Figure 3.7: Light micrographs of *Balantidium kirbyi* colllected from Xenopus laevis from South Africa: A – variance in size, arrow showing ‘‘Villeneuve-Brachon’s’’ field; B – macronucleus (Ma), micronucleus (Mi), vestibulum (V), excretory vacuoles (EV), infundibulum (I), cytoproct (C), pellicle (P); C – straight infundubulum, swollen contractile vaculole (CV).

3.2.2 *Balantidium* sp. 1 (Figure 3.8 - 3.10; Table 3.3)

Measurements were made from six specimens stained with Mayer's haematoxylin.

Host: *Xenopus laevis*

Locality: South Africa: Mpumalanga, Free State, Northern Cape, Western Cape, Eastern Cape, North West, Limpopo

Site of infection: Rectum

General description. **Size and shape.** Small, measuring 36.1–80.7 (52.3) in length and 26.8–45.3 (32.8) in width. Maximum width in mid-section of body. Round to oval shaped body, somewhat flattened (Figures 3.8; 3.9A). Spine-like, unidentified structure (Figure 3.9C). **Infundibulum and vestibulum.** Infundibulum short. Vestibulum approximately 1/3 of body length 11.6–12.4 (11.7), more elongated, V-shaped in larger specimens (Figures 3.8; 3.9B; 3.10C). **Nuclei.** Bi-nucleated**,** macronucleus 9.785–21.06 (12) in length, 5.2–10.8 (7.2) in width, oval-shaped, usually positioned posterior to the middle region of the body (Figures 3.8; 3.10A). Micronucleus positioned close to Ma, 1.5–2.6 (1.8) in length and 1.6–2.4 (2) in width (Figure 3.8; 3.10C). No distinct food vacuoles but granulose cytoplasm, normally transparent, occasionally reddish in colour (Figure 3.10B). Kineties orientated parallel to longitudinal axis (Figure 3.9A). **Cytoproct & contractile vacuoles.** Contractile vacuole positioned in posterior region (Figure 3.10C). Cytoproct at posterior end (Figures 3.8; 3.9B). **Ciliature.** Moderately ciliated, cilia originating from inside of cortical grooves (Figure 3.9C). Deep cortical grooves arranged alternately with cortical ridges, parallel to longitudinal axis of body.

Remarks:

This species was identified as a *Balantidium* species owing to its densely cilliated oral cavity and absence of toxicysts. Furthermore, this species is classified as belonging to the genus *Balantidium* due to the presence of two nuclei, of which the macronucleus is kidney-shaped, and the presence of contractile and food vacuoles. In addition, this species clustered with of other *Balantidium* sp. from frogs in the phylogenetic analysis (Figure 3.18).

To a certain extent, *Balantidium* sp. 1 resembles the *Balantidium* species found in other anurans. These include *B. grimi*, *B. entozoon*, *B. duodeni* and *B. helenae*, although it is quite unique when one considers its characteristic red colour. Like *B. grimi, Balantidium* sp. 1 has a conspicuous narrow vestibulum that distinguishes it from other *Balantidium* species such as *B. entozoon* and *B. duodeni.* In terms of body measurements, one can discriminate *Balantidium* sp. 1 from *B. kirbyi* and *B. duodeni* by it being much smaller. *Balantidium* sp. 1 differs from *B. helenae* as the latter species possesses a remarkable “knob” at the posterior end (Senler & Yildiz, 2000). In the gut of *X. laevis, Balantidium* sp. 1 can easily be discriminated from *B. kirby* in size and shape.

The unidentified structure looks like a spinal component giving structure to the body of *Balantidium* sp. 1.

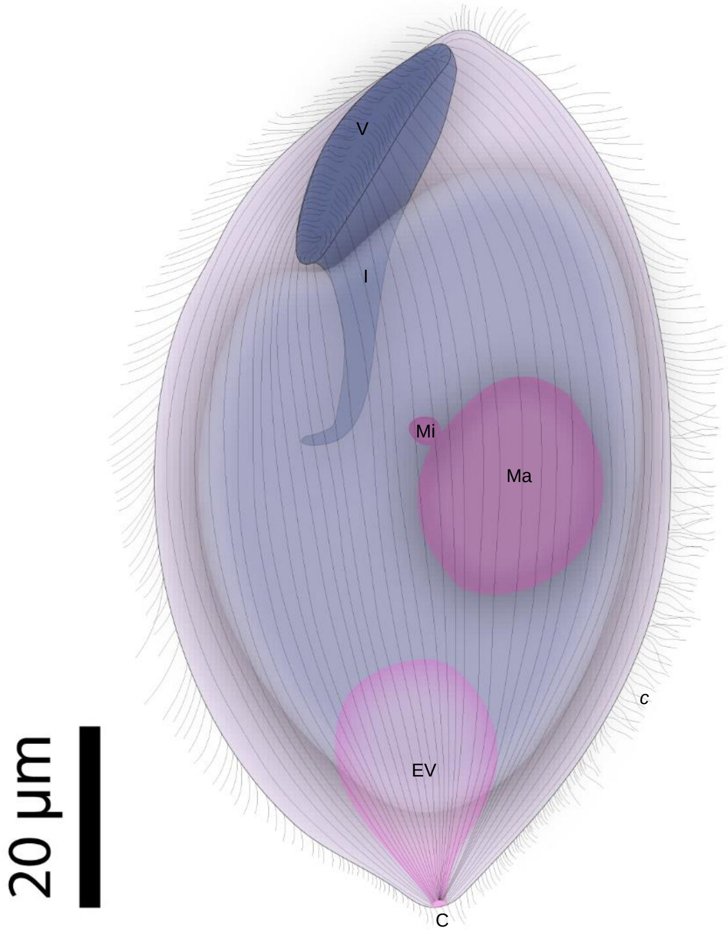

Figure 3.8: Line drawing of of *Balantidium* sp. 1 collected from *Xenopus laevis* from South Africa. V – vestibulum, Mi – micronucleus, Ma – macronucleus, I – infundibulum, EV – excretory vacuole, C – cytoproct, c – cilia.

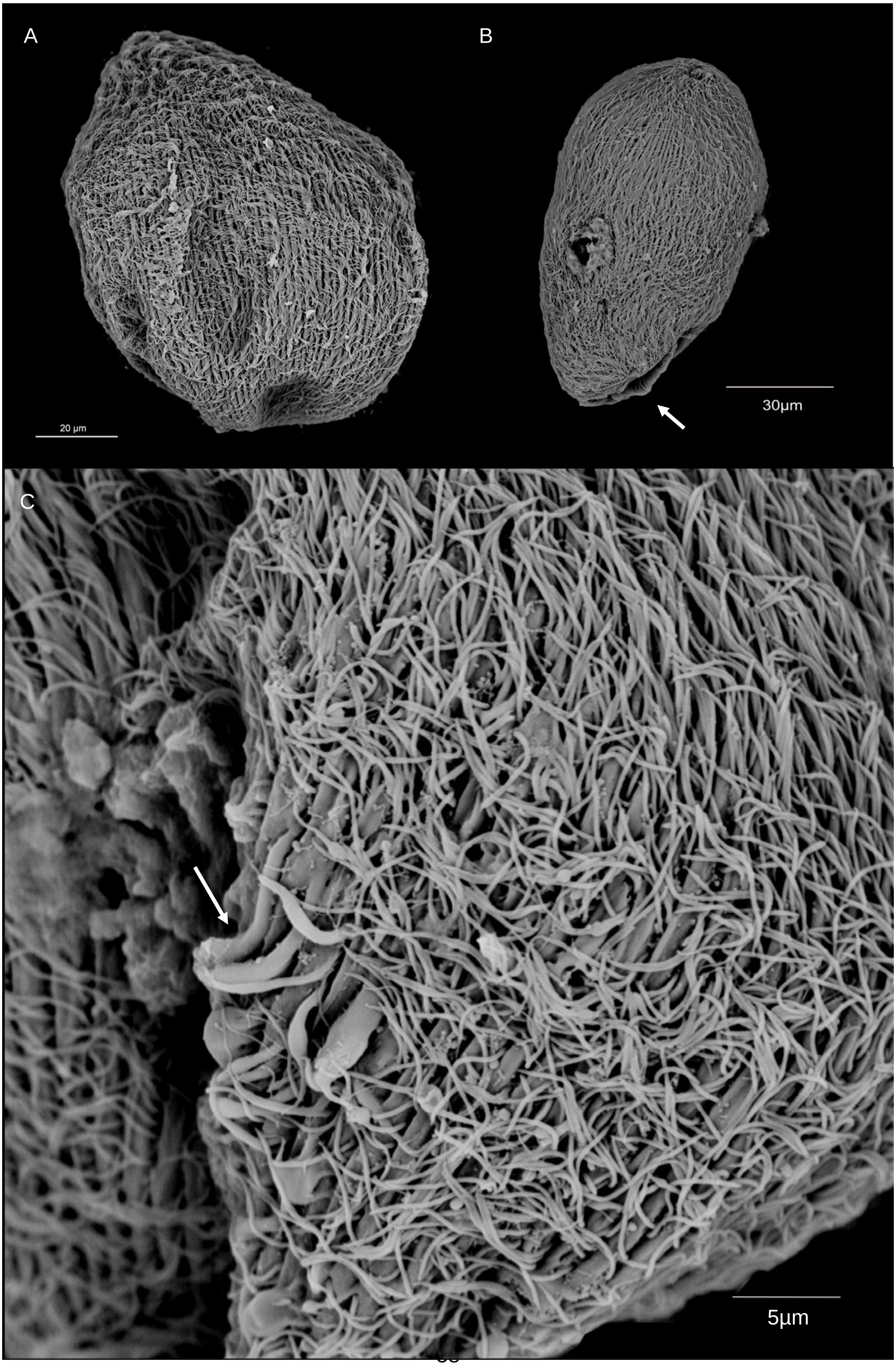

Figure 3.9: Scanning electron micrograph of *Balantidium* sp. 1 collected from *Xenopus laevis* from South Africa revealed by SEM, A – round to oval shaped body, B – vestibulum (extracellular), C – unidentified structure.

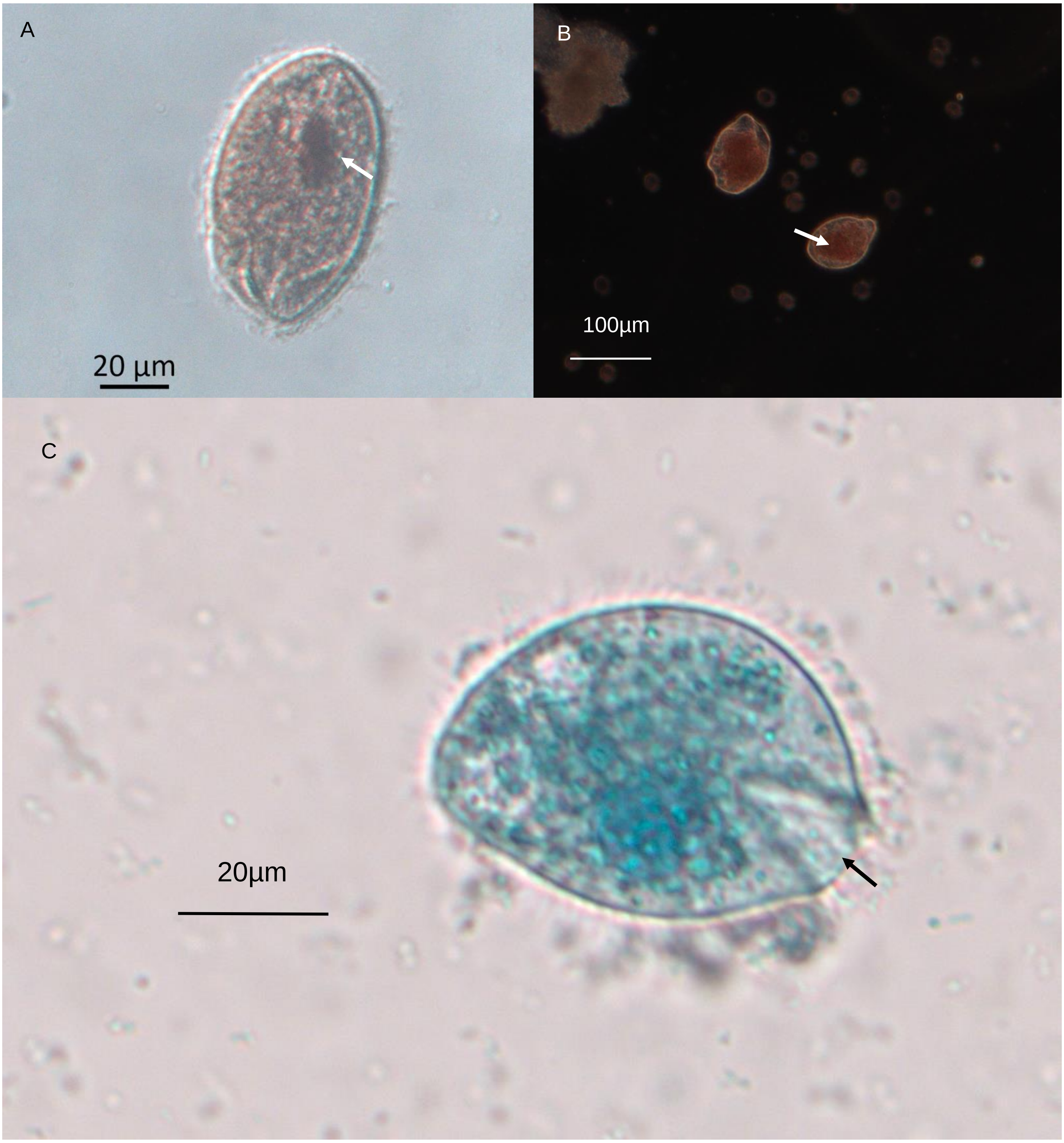

Figure 3.10: Light micrographs of *Balantidium* sp.1 collected from *Xenopus laevis* from South Africa, A– Mayer's Haematoxylin staining of nucleus, B– reddish color of granulose cytoplasm, C– V–shaped vestibulum (Intracellular) –supravital stain.

3.2.3 *Protoopalina* sp. 1 (Figures 3.11 – 3.13; Table 3.1)

Measurements were made using seven larger individuals due to difficulty of haematoxylin staining. The *Protoopalina* is vulnerable with a tendance to dry out and burst, making specimens unusable.

Host: *Xenopus laevis*

Locality: South Africa: Mpumalanga, Free State, Northern Cape, Western Cape, Eastern Cape, North West, Limpopo

Site of infection: Rectum

General description. Size and shape: Elongated, blunt anterior, tapered posterior (Figures 3.11; 3.12A). Variable size, 90.85–133.18 (110.51) long, 12.96–18.00 (14.00) wide. **Flagella and ridges**. Falx present, moderately to densely flagellated from posterior to anterior, bare caudal tip (Figures 3.11; 3.12B). Spindle-like filaments from anterior to posterior (Figure 3.12B). Flagella arranged perpendicular to ridges. Small ridges (10–13) between kineties (Figure 3.12C). **Nuclei.** Binucleated, similar in size, 6.12–8.15 (7.4) in length, 5.23–8.53) (7.0) in width (Figures 3.11; 3.13A, B), oval-shaped, arranged parallel to longitudinal axis, occasionally teardropshaped, connected by thread positioned in middle region of body (Figure 3.13B). **Cytoplasmic structures.** Granulose cytoplasm (Figure 3.13B).

Remarks:

Characteristic to Opalinata, these organisms are pluriciliated without tubular hairs. Moreover, the evenly spaced cortical ridges are also characteristic to this group. This organism's falx (no infundubulum) and flagella are typical of the genus *Protoopalina*. Furthermore, the description agrees with descriptions of *Protoopalina* species found in other anurans from around the world (Metcalf,1923; Delvinquier *et al*., 1995). The measurements of *Protoopalina* sp. 1 corresponds with the measurements of *P*. *xenopodus* (Metcalf, 1923; Wessenberg, 1961; Thurston,*1970;* Delvinquier *et al.,* 1995; Kuperman *et al*., 2004). As in *P. xenopodus* (Thurston, 1940), considerable variation in width and length occurred in *Protoopalina* sp. 1. *Protoopalina* sp. 1 closely resembles descriptions of *P*. *africana* and *P*. *primordialis* (Metcalf, 1923; Delvinquier *et al*., 1995). *Protoopalina* sp. 1 differs from *P. primordialis* by means of a less rounded anterior. The tapered posterior end of *Protoopalina* sp. 1 resembles *P*. *africana* but is not as elongated. *Protoopalina* sp. 1 corresponds to the unknown *Protoopalina* species mentioned in *Xenopus* species from previous studies in terms of size (Delvinquier *et al*., 1995), shape and nuclei (Sandon,1941; Mohr, 1942; Delvinquier *et al*., 1995).

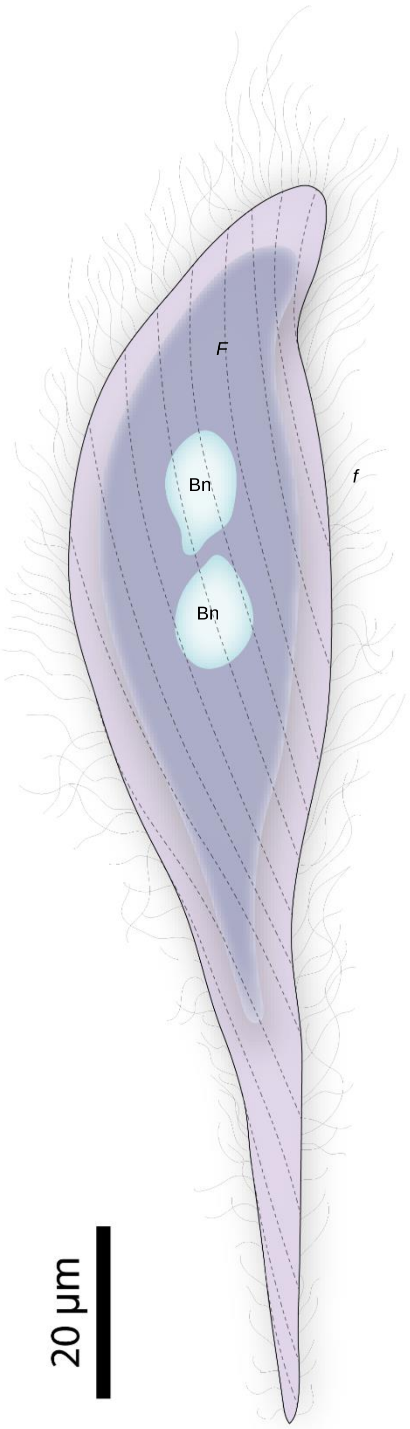

Figure 3.11: Line drawing of *Protoopalina* sp. 1 collected from *Xenopus laevis* from South Africa: Bn – Binucleus; F – Spindle-like filaments; f – flagella

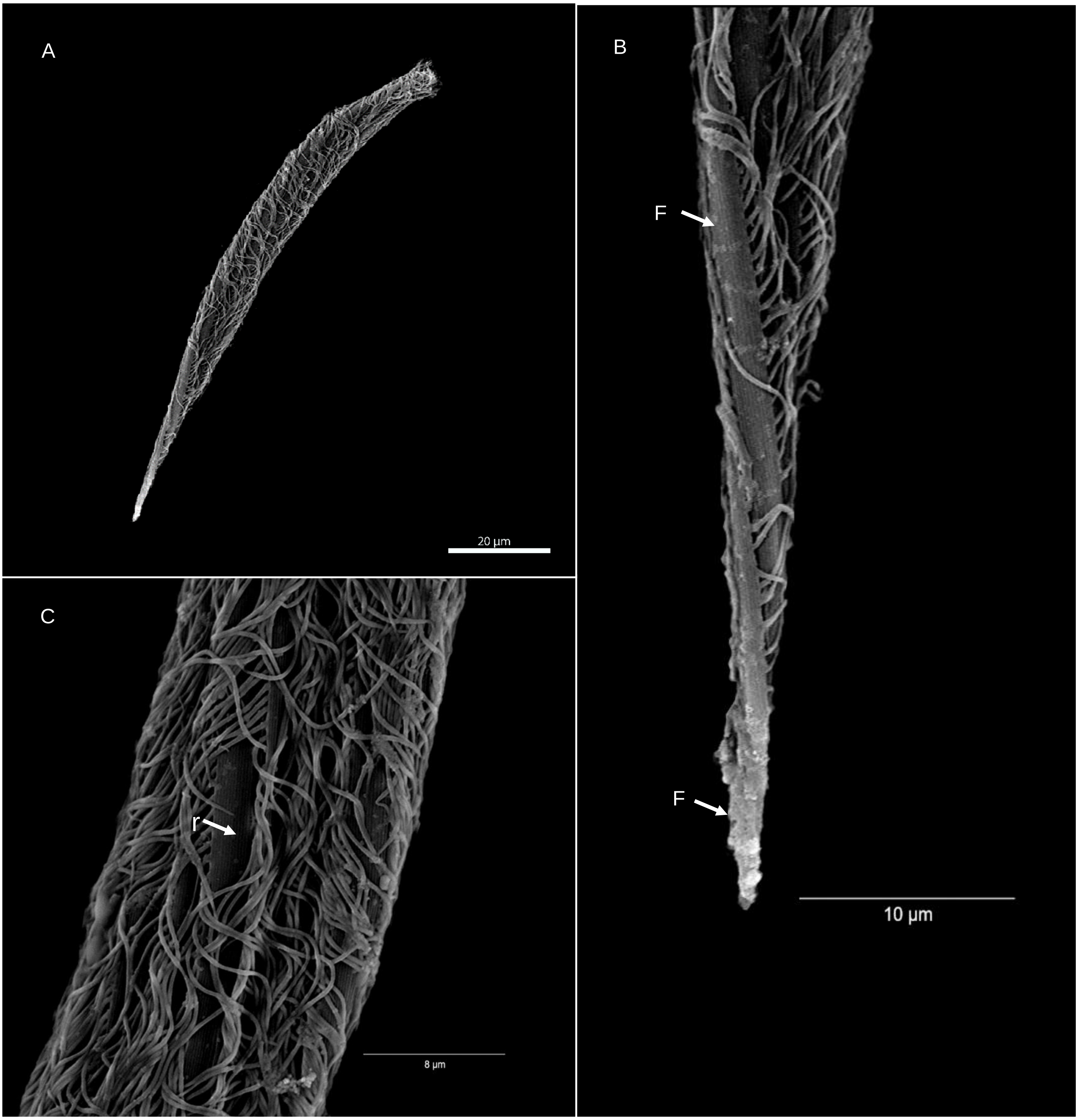

Figure 3.12: Scanning electron micrographs of *Protoopalina* sp. 1 collected from *Xenopus laevis* from South Africa. A– elongated body, blunt anterior, tapered posterior, B – bare caudal tip, spindle-like filaments (F), C – small ridges (r)

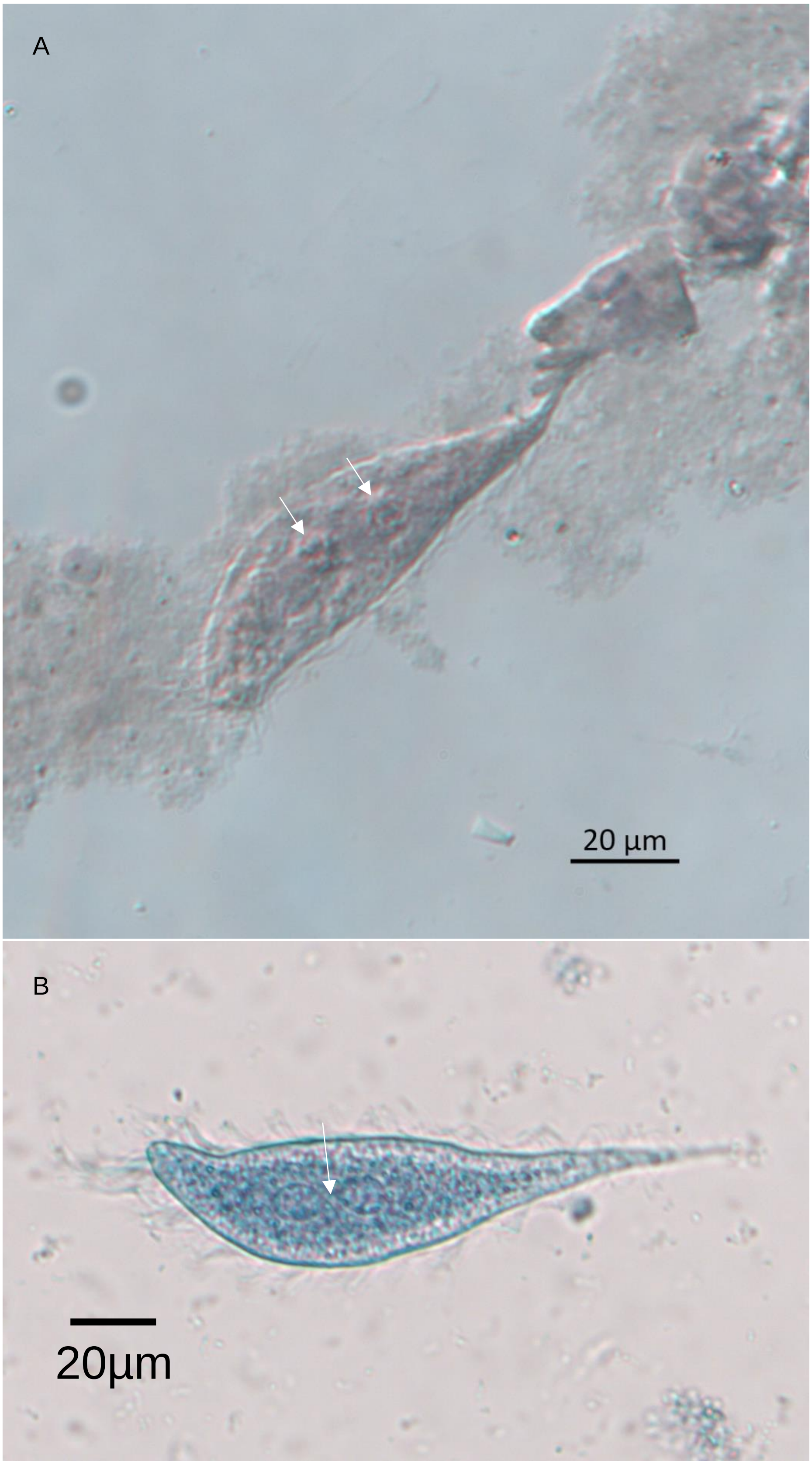

Figure 3.13: Light micrographs of *Protoopalina* sp. 1 collected from *Xenopus laevis* from South Africa A – binucleation, B – nuclei connected by thread (indicated by arrow), granulose cytoplasm.

3.3 Infection levels, ecology, population dynamics and distribution

3.3.1 Infection levels

South Africa: Of the 222 *X. laevis* examined from South Africa (Appendix A, Table 1.1), 197 (prevalence 88.74%) frogs were infected with at least one ciliate species and 25 (11.26%) frogs had no ciliates. Ciliate samples from 137 of these hosts were screened and five rectal protozoan species were identified as follows: 1) *Balantidium kirbyi* (prevalence 80.29%); 2) *Balantidium* sp*.* 1 (40.16%); 3) *Balantidium* sp. 2 (no prevalence data); 4*) Protoopalina* sp. 1 (prevalence 37.23%) and 5) an extremely rare rectal flagellate, possibly belonging to the genus *Hexamita*. Unfortunately, the prevalence of the *Hexamita* could not be determined since it was too small to perceive with ordinary microscopes and was only visible under SEM.

France: Of the 113 *X. laevis* from France (Appendix A, Table 1.1), 107 (prevalence 94.69%) frogs were infected with at least one ciliate species and 6 (5.3%) frogs had no ciliates. Ciliate samples from 75 of these hosts were screened and four rectal protozoan species were identified as follows: 1) *Balantidium kirbyi* (prevalence 89.33%); 2) *Balantidium* sp*.* 3 (60%); 3) *Balantidium* sp. 4 (no prevalence data); 4*) Protoopalina* sp. 2 (prevalence 34.67%).

3.3.2 Ecology

I. Single infections of South African and French ciliates

For *Balantidium kirbyi,* single infections occurred in 44 (32.11%) *X. laevis* from South Africa and 27 (36%) *X. laevis* from France. Interestingly for both *Balantidium* sp. 1 from South Africa and *Balantidium* sp. 3 from France no single infection occurred, these were also found with other ciliates. For *Protoopalina.* sp.1, 2/137 (1.45%) single infections occurred in *X. laevis* from South Africa, whereas 2/75 (2.67%) single infections of *Protoopalina.* sp. 2 occurred in *X. laevis from* France.

II. Endociliate distribution & occurrence

Balantidium kirbyi was found at all 34 South African localities; *Balantidium* sp. 1 was found at 24/34 localities and *Protoopalina* sp.1 was found at 22/34 localities (Table 3.4). Similarly, *B. kirbyi* and *Balantidium* sp. 3 was found at all French localities (10/10) whereas *Protoopalina* sp. 2 was found at 8/10 French localities (Table 3.5). At least one morphospecies of endociliates were present at all localities for both South Africa and France (Figure 3.14).

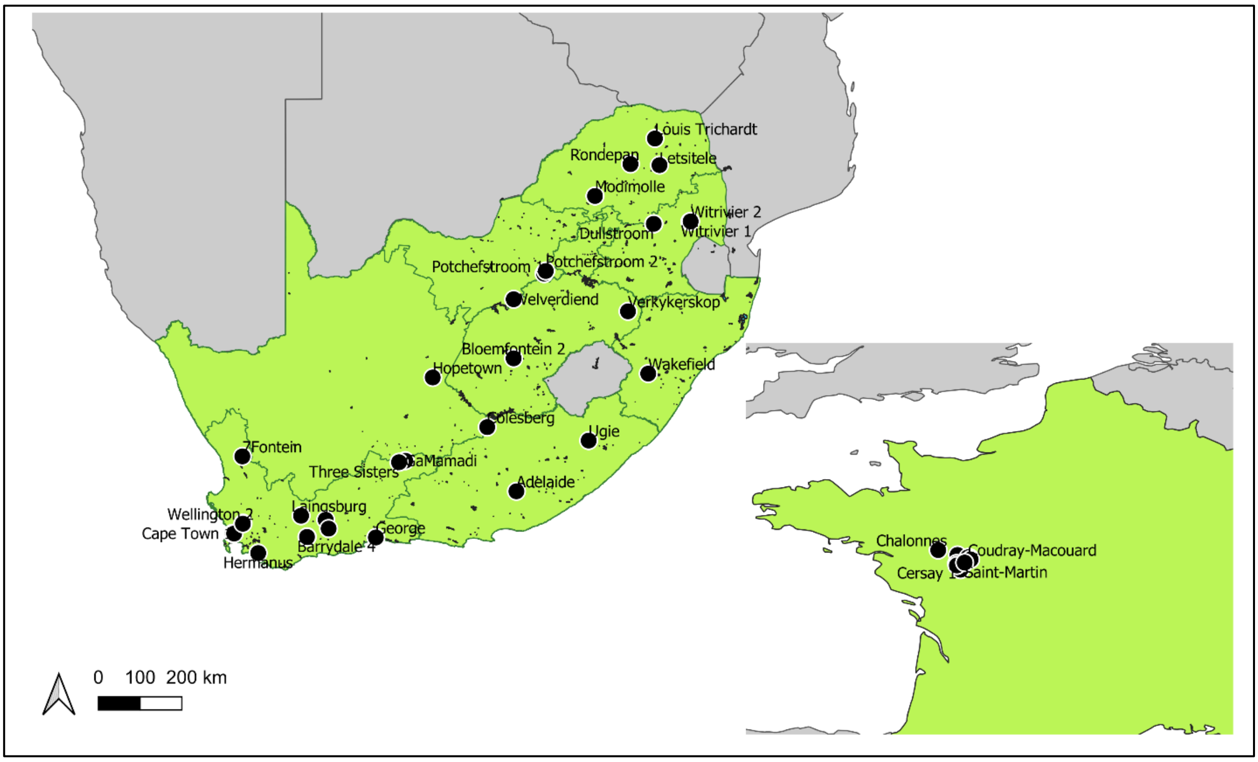

Figure 3.14: Endociliate distribution in South Africa and France. Black dots indicate the presence of at least one ciliate morphospecies. Maps were created in QGIS 3.10.2-A Coruña, based upon public domain shapefiles.

Table 3.4: Occurrence of different morphospecies of endociliates collected from *Xenopus laevis* at localities in South Africa

Locality	**Latitude**	**Longitude**	***Balantidium kirbyi***	***Balantidium* sp.1**	***Protoopalina* sp.**
7Fontein	-31.7360	18.8254	Present	Present	Present
Adelaide	-32.6879	26.2951	Present	Present	Absent
Barrydale 1	-33.9263	20.5917	Present	Absent	Absent
Barrydale 2	-33.9283	20.5915	Present	Present	Present
Barrydale 4	-33.9312	20.5873	Present	Present	Present
Bloemfontein University	29.1109	26.1794	Present	Absent	Present
Cape Town	-33.8355	18.5528	Present	Present	Present
Colesberg	-30.9384	25.5002	Present	Present	Present
Dullstroom	-25.3981	30.0380	Present	Present	Present
GaMamadi	-31.8526	23.2645	Present	Present	Absent
George	-33.9455	22.4636	Present	Present	Absent
Hermanus	-34.3701	19.2570	Present	Present	Present
Hopetown	-29.5847	24.0143	Present	Present	Present
Ladismith	-33.4637	21.0969	Present	Present	Absent
Laingsburg	-33.3527	20.4255	Present	Present	Present
Letsitele	-23.7987	30.1951	Present	Present	Present
Louis Trichardt	-23.0728	30.0710	Present	Absent	Present
Placidus	-24.6383	28.4369	Present	Present	Present
Potchefstroom 1	-26.7554	27.0505	Present	Present	Present
Potchefstoom 2 Botanical garden	-26.6828	27.0957	Present	Present	Present
Riversdale	-33.7033	21.1761	Present	Present	Present
Rondepan	-23.7693	29.4046	Present	Absent	Absent
Sewe Damme, Bloemfontein	-29.0636	26.2188	Present	Absent	Absent
Three Sisters	-31.8950	23.0956	Present	Present	Absent
Ugie	-31.3049	28.2646	Present	Absent	Present
Verkykerskop	-27.7832	29.3362	Present	Present	Present
Wakefield	-29.4797	29.8876	Present	Absent	Absent
Welgemoed	-33.8392	18.6002	Present	Present	Absent
Wellington 1	-33.5560	18.8436	Present	Present	Present
Wellington 2	-33.5713	18.8401	Present	Present	Absent
Welverdiend	-27.4541	26.2204	Present	Present	Present
Witrivier 1	-25.3390	31.0225	Present	Absent	Present
Witrivier 2	-25.3319	31.0432	Present	Absent	Present

Table 3.5: Occurrence of three different morphospecies collected from *Xenopus laevis* at localities in France

Locality	Latitude	Longitude	*Balantidium kirbyi*	*Balantidium* sp.3	*Protoopalina* sp. 2
Ambillou	47.2385	-0.3445	Present	Present	Absent
Bouillé-Saint-Paul	47.0272	-0.3442	Present	Present	Absent
Brézé	47.1725	-0.0728	Present	Present	Present
Cersay 1	47.0531	-0.3648	Present	Present	Present
Chalonnes	47.3439	-0.7635	Present	Present	Present
Coudray-Macouard	47.1872	-0.1151	Present	Present	Present
Epieds	47.1413	-0.0606	Present	Present	Present
La Butte	46.9185	-0.2803	Present	Present	Present
Massais	47.0106	-0.3581	Present	Present	Present
Saint-Martin	47.0729	-0.1898	Present	Present	Present

3.3.3 Population dynamics

I. Behavioural observations

In vivo, *B. kirbyi* were sometimes found in clusters somewhat attached to the wall of the rectum with high infections (Figure 3.15A). Nevertheless, as Sandon, 1941 observed, no indications of harm seemed to be done to the lumen wall. We suggest that the ciliates rather take on a resting siphoning state when they are close to the wall of the lumen. Endociliates were usually present as mixed infections and seem to live in harmony with each other since no cannibalistic or preying behaviour were observed (Figure 3.15B).

II. Division

Numerous specimens were observed at the starting stage of nuclear division (Figure 3.15 C). Since ciliates did not survive long enough, the whole process of division could never be observed.

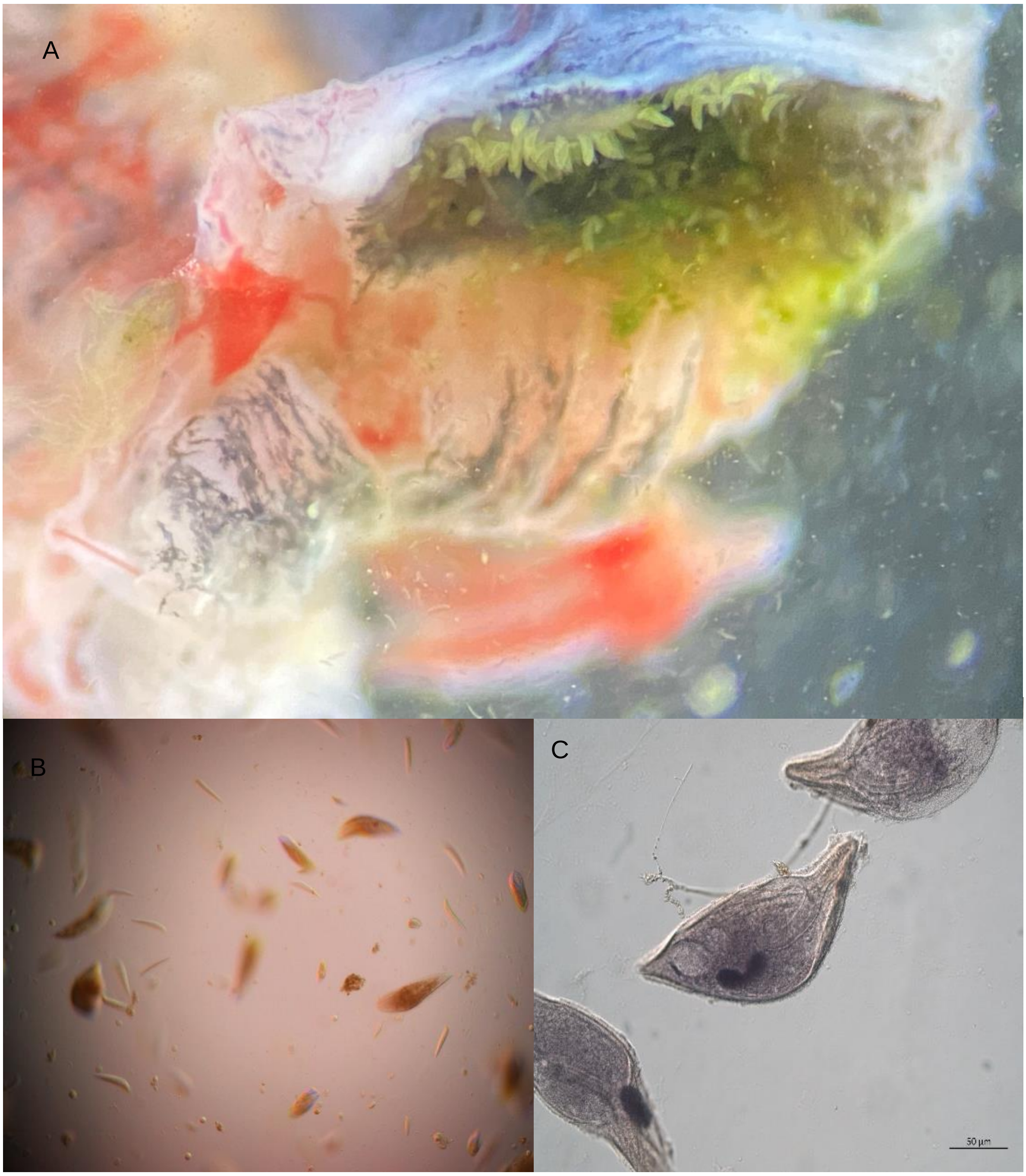

Figure 3.15: A– *Balantidium kirbyi* sometimes found in clusters, B– A frequently observed ciliate mixed infection, C– starting stage of nuclear division.

III. Movements

In *B. kirby* two general movements were observed through live observations. The first is a gliding movement with a sudden stop and aboral turn (Figure 3.16A) whereas the second movement was a more rapid movement, turning around the longitudinal axis (Figure 3.16B). The whole body of *B. kirby* practices the movement around the longitudinal axis (Figures 3.16C, D). The *Protoopalina* species shows a similar movement like the second of *B. kirby,* turning around the longitudinal axis of the body while moving forward (Figure 3.16E).

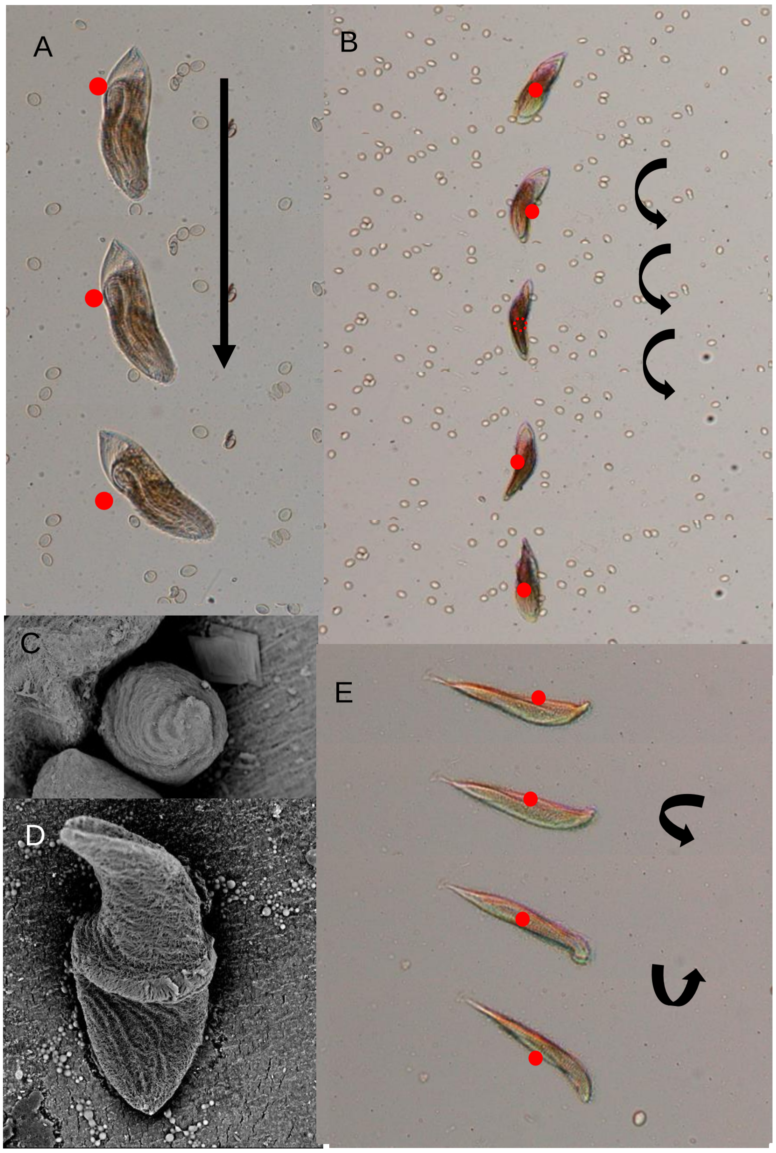

Figure 3.16: A– *Balantidium kirbyi* practicing gliding movement, B– with turn around the longitudinal axis, C– movement around the longitudinal axis (transverse view), D– movement around the longitudinal axis (lateral view), E– movement around the longitudinal axis of *Protoopalina* sp. 1.

IV Co-invasion

Interestingly, a scarce diplomonad flagellate, *Hexamita intestinalis* (Figure 3.17) from a *X. laevis* caught in Dullstroom observed under the SEM. Diplomonads are characterised by the possession of two haploid nuclei each accompanied with four flagella (Lloyed & Williams, 2014). No further molecular or morphological staining could be done due to lack of material.

Since diplomonads are sometimes opportunistic parasites (Lloyed & Williams, 2014), we suggest that the *Hexamita* sp. observed in this study is an opportunist parasite of *X. laevis.* Subsequently we support the statement that specimens of *X. laevis* are parasite reservoirs (Schoeman *et al*., 2020) which could have implications for its role as vector. Thus *X. laevis* may contribute to parasite invasions due to its status as global and domestic invader (Measey & Davies, 2011).

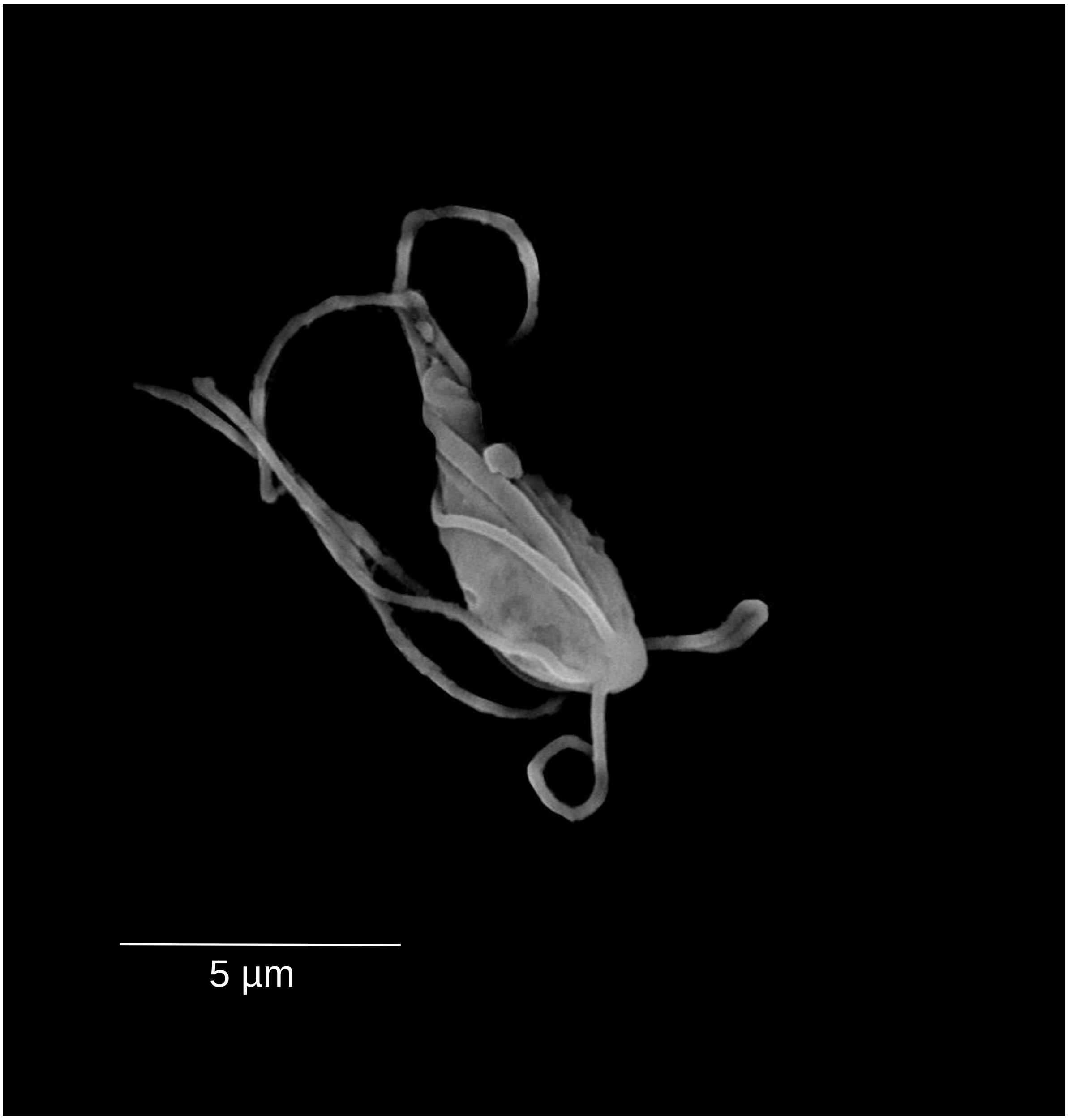

Figure 3.17: *Hexamita* sp. found in a *Xenopus laevis* specimen from Dullstroom, RSA.

3.4 Molecular and phylogenetic analysis

Molecular isolation and PCR were done on all morphospecies found from both South Africa and France. Only *Balantidium* spp. were sequenced successfully, no *Protoopalina* spp. sequences of the partial 18S rDNA were obtained for three *Balantidium* species collected in South Africa and one from France which include *B. kirbyi* (RSA), *B. kirbyi* (FRA), *Balantidium* sp. 1 and *Balantidium* sp. 2.

Interestingly, two genetic variants of B. kirbyi occurred, indicated as *B. kirbyi* (A) and *B. kirbyi* (B) in the phylogenetic tree (Fig 3.18). Variant A occurred in both South Africa and France wheras variant B only occurred in South Africa.

The phylogenetic analysis revealed that species belonging to the family Balantidiidae formed two clades where the 18S rDNA sequences obtained during the present study — those of *B. kirby* from both South Africa and France*,* *Balantidium* sp. 1 from RSA and *Balantidium* sp. 2 from RSA, grouped together with *B. duodeni*, *B. entozoon* and *B. grimi*, all from other anuran amphibians from China (Zhao *et al*., 2018), Italy (Grim & Buonanno, 2009) and Russia (Chistyakova, *et al*.,2014) (Figure 3.18). The second group consisted of two isolates of *B. coli*, reported from many ostriches and pig hosts from Spain (Ponce-Gordo *et al*., 2008) and *Buxtonella sulcata,* reported from cattle (Ito, et al., 2014). *B. kirbyi* (B) *Balantidium* sp. 1 clustered together with high support of 98 (UFboot2; Hoang *et al.,* 2018) and formed a sister clade (support 99) to all other *Balantidium* sp. from frog hosts, including *Balantidium* sp. 2 (UFboot2; Hoang *et al.,* 2018).

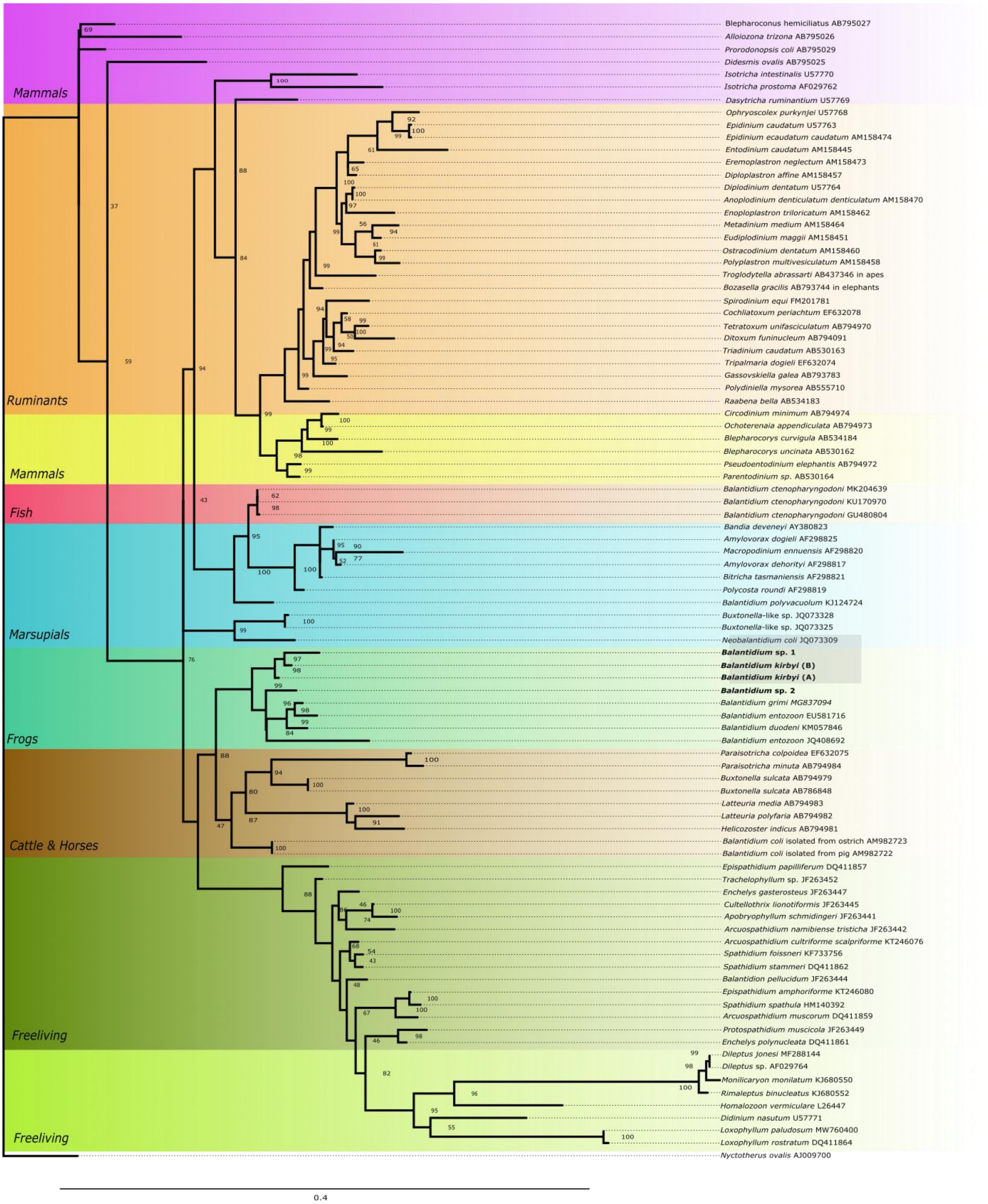

Figure 3.18. Phylogenetic tree of ciliate genera from different hosts (Appendix C: Table 3.1), performed with the IQ-TREE algorithm (Nguyen et al. 2015), assessed branch support through ultrafast bootstrapping (UFboot2; Hoang et al., 2018) and the Shimodaira-Hasegawa approximate likelihood ratio test (SH-aLRT; Guindon et al., 2010), sequences of *Balantidium* spp. marked in bold, the tree were rooted using the sequence of *Nyctotherus* ovalis as the outgroup taxa, different coloured clades represent majority animal hosts in each.

Chapter 4:

Discussion

Xenopus laevis is probably the best studied amphibian species around the globe and can be regarded a model research animal (Gurdon & Hopwood, 2000). On top of that, it is a globally invasive species (Measey *et al*., 2012). Due to its aquatic environment that facilitates parasite transfer, this frog is regarded as the perfect host for more than 25 parasite genera, representing all major parasitic groups but for the acanthocephalans (Cosgrove & Jared, 1974; Macnae *et al*., 1973; Pritchard, 1964; Southwell & Kirshner, 1937; Thurston, 1970; Tinsley, 1996; Vercammen-Grandjean, 1960). With such extensive focus on the parasites of *X. laevis*, it is surprising that the ciliate diversity is poorly known.

However, during the course of the present study, it soon became apparent why so little is known about the gut ciliates of *X. laevis* and a study that was envisaged as a straightforward taxonomic study came with its fair number of challenges.

4.1 Diversity of gut ciliates of *Xenopus laevis*

Within the *X. laevis* that we studied from South Africa, three gut ciliate species were identified, namely *B. kirbyi*, *Balantidium* sp. 1, *Balantidium* sp. 2 and an opalinid, namely *Protoopalina* sp. Specimens from the same two genera which are *Balantidium* and *Protoopalina* were morphologically identified in the *X. laevis* collected from France. However, at this stage, only *B. kirbyi* was successfully isolated and sequenced from both countries and confirmed as being the same species in *X. laevis* For the other species, molecular analysis is needed to confirm whether they are, in fact, the same species in both countries. Since both *Balantidium* and *Protoopalina* are cosmopolitan genera, the possibility exists that the species in France are not the same as those found in Africa. On the other hand, *X. laevis* is a known parasite taxi, with a number of co-introduced metazoan parasites across the world (Schoeman *et al*., 2019).

4.1.1 Species 1: *Balantidium kirbyi* Rodriguez, 1941

This species was found at all localities examined in this study from both South Africa and France. The main characteristic of this species is its high prevalence, large size and coiled infundibulum. Nevertheless, more information on the infraciliature of *B. kirbyi* is still lacking.

Size and shape

Similar to the specimens found in this study, Rodriguez (1939), Sandon (1941) and Du Puytorac and Grain (1965) described the body shape of *B. kirbyi* as elongated, roundly anterior and narrowing posteriorly. The measurements of *B. kirby* reported in this study correlates well with those reported by Rodriguez (1939) and Sandon (1941), although smaller specimens than those reported in these studies were also measured in the present study. The minimum body length reported in the various studies are 149 µm (Rodriguez, 1939), 150 µm (Sandon, 1941), 230µm (Du Puytorac & Grain, 1965) and 36.1µm in the present study. Of all of the previous studies, Du Puytorac & Grain (1965) measured a larger size range of specimens from the gut of *X. fraseri* from Gabon, with the largest being 570 µm long, compared to a maximum body length of 345 µm reported by Rodriguez (1939), 250 µm by Sandon (1941) and 257.2 µm in the present study. Moreover, the same movements described by Sandon (1941) were observed during live observation of *B. kirbyi* in the present study. In contrast to our findings, Sandon (1941) did not observe any food vacuoles in *B. kirbyi.* Additionally, Sandon (1941) described syngamy and division of *B. kirbyi* which were not noted during this study.

Vestibulum and infundibulum

The description of the vestibulum and infundibulum in this study corresponds to those reported by Sandon (1941) and Rodriguez (1939). Du Puytorac and Grain (1965) did a more in-depth description of the vestibular kineties. In the present study, limited success in the descriptions of kineties acted as a limitation and thus hampered a direct comparison.

Nuclei

The shape and the size, as well as the number of nuclei, seemed to correspond with the various sizes of specimens described in the previously mentioned studies on *B. kirbyi* (Rodriguez, 1939; Du Puytorac & Grain, 1965), namely a kidney shaped macronucelus and small round micronucleus.

Cytoplasmic structures

It is not clear from Sandon's (1941) description whether vacuoles are present in *B. kirbyi*. However, food vacuoles, contractile vacuoles and excretory vacuoles were observed in high numbers in the present study, a characteristic consistent with both Rodriguez (1939) and de Du Puytorac & Grain (1965). In accordance with Rodriguez (1939), emptying of the cytophage was also observed during live observations of the present student. Excretions were observed as a small cytoplasm-like mass being discharged from the cytophage.

Ciliature

Since silver impregnation did not work to its full extent, the present study cannot contribute much infraciliature information**.** Nonetheless, SEM confirms the presence of numerous kineties on the right and left sides of the vestibule, as well as some information regarding the direction of these structures (Figure 3.9 A-C; 3.16 C, D). Moreover, visible kineties were clearly monokinetic, supporting that this sDecies belongs to the genus *Balantidium* according to the classification by Adl *et al*. (2019). Furthermore, it is presumed that the dorsal spines described by both Sandon (1941) and Rodriguez (1939) are in fact the kineties with ridges. Although Sandon (1941) did not describe the basal apparatus in much detail, Rodriguez (1939) described the cilia to be attached to basal granules while perforating the pellicle. The latter was observed in this study by staining with Heidenain's haematoxylin and Tuan's method of destaining. Furthermore, Rodriguez (1939) mentioned that the granules are interconnected by fibrils, although he did not state by which method it was observed. Du Puytorac and Grain (1965) focused in depth on the infraciliature, especially by using transmission electron microscopy (TEM). Correspondingly, it was found that the infraciliature and oral morphogenesis, especially the anterior ends of the somatic kineties and construction of ciliature, supports placing *B. kirbyi* in the Trichostomatia. The following are known to be characteristics of Trichostomatia (Adl *et al*., 2019) and should be examined in future studies on *B. kirbyi*: *1)* are any somatic dikinetids present and do they form a clavate field?; 2) is skeletal material present?; 3) are mitochondria and hydrogenosomes present?; 4) what is the structure of the microfilamentous layer between the ecto- and endoplasm?; and 5) what is the structure of the ''Villeneuve-Brachon's'' field of somatic cilia? Thus, we suggest that TEM as well as another method of silverline staining — probably silver-carbonate staining or protargol staining — should be done to complete the description of *B. kirbyi* found in *X. laevis*. Unfortunately, due to the costs involved in protargol staining, which require access to gold-chlorite, this technique was not available in the present study.

Taxonomic classification of *B. kirbyi*

According to Sandon (1941), *B. kirbyi*, as first described by Rodriguez (1939), displayed certain characteristics that merited its inclusion in a newly erected genus *Paranyctotherus*. This genus was regarded as a close relative of *Nyctotherus*, hence the name. The distinguishing characteristics of the new genus included the finer structure of the infundibulum and peristome. In later literature, the largest ciliate of *X. laevis* was referred to as both *B. kirbyi*, *Pa. kirbyi and B. xenopi* (Thurston, 1970; Mohr, 1942; Du Puytorac and Grain, 1965; Kuperman *et al*., 2004). The present study provides both genetic and morphological evidence that this largest ciliate in the gut of *X. laevis* is unquestionably a member of the genus *Balantidium* and corresponds to both the descriptions of *B. kirbyi* (Rodriguez, 1939) and *P. kirbyi* (Sandon, 1941). We propose that *Paranyctotherus*, as described by Sandon (1941), is not a valid genus and that *P. kirbyi* is a synonym of *B. kirbyi*. As for *B. xenopi* and *B. xenopodis*, mentioned by (Du Puytorac and Grain, 1965; Kuperman, *et al*., 2004), it would seem as if this species, described by Du Puytorac and Grain (1965) from *X. fraseri* from Gabon, actually refers to *B. kirbyi* when found in *X. laevis* from southern Africa. However, more morphological and molecular studies on specimens from both hosts and localities are needed to confirm.

4.1.2 Species 2: *Balantidium* sp. 1

Although their occurrence is mentioned quite often in the literature (Sandon 1941; Mohr, 1942; Thurston, 1970), little is known about the other *Balantidium* species that parasitise *X. laevis*. The measurements of *Balantidium* sp. 1 in the present study corresponds with the unidentified *Balantidium* sp. of Thurston (1970), with minimum length of 44 µm and maximum length of 125 µm. As noted by both Thurston (1970) and Sandon (1941), this species was frequently found feeding on red blood cells released during dissection in the present study, which gave them a reddish colour.

This study has been the first to apply SEM on the other *Balantidium* spp. found in *X. laevis*, which revealed new ultrastructure information. The unidentified structure (Figure 3.9C) has, to our knowledge, never been observed or described in ciliate literature before. We propose that this structure is a component of the kineties, possibly part of the “Villeneuve-Brachon’s” field of somatic cilia, although further studies are required to investigate this phenomenon. Moreover, we propose further investigation thereof in terms of its presence in other specimens of *Balantidium* sp. 1, as well as other *Balantidium* species. Furthermore, we suggest further studies on this species should focus on TEM and silver staining, possibly silver carbonate staining or protargol staining. These additional methods will help to properly characterise the infraciliature, especially the kineties, and aid in the examination of the unknown structure, which will allow classification to the species level.

4.1.3 Species 3: *Balantidium* sp. 2

The small *Balantidium* sp*.* 2, of which only molecular data was obtained in this study was sequenced from a *X. laevis* from Potchefstroom in South Africa. Unfortunately, staining and SEM procedures were unsuccessful and no specimens were successfully sampled again.

4.1.4 Paraphyly of *Balantidium*

This study was the first to provide genetic material — 18S rDNA — of *Balantidium* species from frogs from Africa. Prior to the current study, the 18S rDNA of only four other *Balantidium* species from frogs were available, namely B. *duodeni* from the European common brown frog *Rana temporaria* (Linnaeus, 1758) from Russia (Chistyakova *et al*., 2014), *B. entozoon* from frog host, *Rana esculenta* (Linnaeus, 1758) from Italy (Grim & Buonanno, 2009) and *B. grimi* from *Quasipaa spinosa* (David, 1875) from China (Zhao *et al*., 2018). In the phylogenetic analysis of free-living and symbiotic ciliates of the Litostomatea, the *Balantidium* species from frog hosts, including *B. kirbyi* from South Africa and France, *Balantidium* sp. 1 and *Balantidium* sp. 2 from South Africa, clustered together in a single clade. However, the *Balantidium* species from mammalian hosts, currently known as *B. coli* did not fall in the same clade, rendering the genus paraphyletic. This paraphyly was noted by Grim & Buonanno (2009); Chistyakova *et al*., (2014) and Zhao *et al*. (2018). Since the type species of *Balantidium* is a frog parasite, namely *B. duodeni*, the generic allocation of *B. kirbyi* and other *Balantidium* species in the anuran clade is correct and the taxonomic classification of *B. coli* and other *Balantidium* species from the mammalian clade needs to be revised.

4.1.5 Species 4: *Protoopalina* sp. 1.

Protoopalina sp. 1 was found in 37.23% of South African localities examined in this study. The main characteristics of this species includes it similar sized binucleus as well as the absence of internal organelles. There are uncertainties whether this species is *P. xenopodus* since the original description was made on *X. calcaratus* from the Congo (Metcalf, 1923).

Size and shape

The measurements of *Protoopalina* sp. 1 described in this study correspond with the measurements made by Metcalf (1923) from *P. xenopodos*, although Metcalf (1923) also measured larger specimens, with the largest being 218 µm in length. The measurements of Thurston’s (1970) *P. xenopodus* correspond with those of the current study, although smaller specimens of 45 µm were also measured.

Nuclei

The measurements and position of the nuclei of *Protoopalina* sp. 1 from *X. laevis* were found to be smaller than the *P. xenopodos* measured by Metcalf (1923). Chromatin masses could unfortunately not be counted in the present study as was described by Metcalf (1923), although he did state that specimens were too poorly preserved to be definite.

Flagella

All other descriptions of *Protoopalina* sp. 1 found in this study, especially those comments on the ultrastructure and flagella, are new information regarding the *Protoopalina* species found in *X. laevis.* Moreover, this study was the first to use SEM on *Protoopalina* sp. found in *Xenopus* species.

Taxonomic classification of *Protoopalina* sp. 1

Metcalf (1923) mentioned seven *Protoopalina* species, namely *P. africana* (Cameroon), *P. mossambiccucis* (Mozambique), *P. nutti* ("British East Africa"), *P. primordialis* ("German East Africa"), *P. regularis* (Gold Coast, "British East Africa"), *P. stevensoni* (Sudan) and *P. xenopodus*

(Belgian Congo), from tropical and southern Africa. The type species of *P. xenopodus* was described from *X. calcaratus* from six specimens from the Niapu and Avakubi, Belgian Congo. All specimens were described from poorly fixed specimens. It was unclear whether *P. xenopodus* from Kajansi described by Thurston (1970) were from *X*. *laevis* or *X*. *muelleri*, probably *X. victorianus,* although *P. xenopodus* was found in 10 *X. laevis* specimens from Zimbabwe (Thurston 1970). Since descriptions were made from poorly fixed specimens, we suggest that *P. xenopodus* species should be revised in terms of molecular and morphological data. This should be done before it can be decided whether *Protoopalina* sp. 1 is *P*. *xenopodus*.

Delvinquier *et al*. (1995) mentioned *P. primordialis* (Awerinzew, 1913) from three *X. laevis* tadpoles from Pietersburg district (South Africa) and two *X. laevis* juveniles from Vrede (South Africa). Specimens from the present study could not be compared with this species, since all specimens of *P. primordialis* were found in a stage of mitotic division. In addition, the type description was made from specimens collected from *Amietia nutti* (Boulenger, 1896), and Awerinzew (1913) did not illustrate any of his descriptions, therefore it is not possible to make satisfactory taxonomic comparisons with *P. primordialis.*

Taking in account the *Protoopalina* spp. found in anuran hosts, as well as the high numbers of *Xenopus* spp. found in Africa, we propose that molecular analysis should be done to confirm whether *Protoopalina* sp. 1 from South Africa in the present study is indeed *P*. *xenopodus*, similar to those from the Congo described by Metcalf (1923). Furthermore, opalinids from different *Xenopus* spp. should also be examined both molecularly and morphologically to confirm species identifications. Unfortunately, no molecular data could be obtained from *Protoopalina* sp. 1 recovered from South Africa in the present study.

4.2 The integrated method

4.2.1 Sampling

Sampling ciliates and identifying them turned out not to be an easy task. It is important to remember that ciliates are polymorphic and that species cannot be recognised on only one form, especially when different methods of fixation and staining are applied. The easiest way to compare species between the different methods is to isolate samples, fix each respectively in all necessary fixatives and proceed with the various methods knowing that the only specimens present are that of the same morphospecies. Unfortunately, with all methods and procedures, ciliates can easily be lost due to air drying, ciliates staying behind in pipette tips and getting lost due to debris removal. In addition, smaller ciliates and especially those that are less prevalent should be sampled with great care. When taking the latter into consideration, always sample as many of a morphospecies as possible. Equally importantly, ciliates should be covered when placed in a cavity block, since liquid evaporates very quickly, causing a loss in specimens.

When the method of filtered sampling is used, caution should be taken when copper sieves are used since Boyne *et al*. (1957) found that holotrichs rapidly disintegrate when copper, brass or metal gauze were used in a study counting rumen Protozoa. This method is only recommended when large numbers of ciliates are present.

4.2.2 Morphological methods

Protoopalina species do not stain easily with haematoxylin, thus nuclei can be better observed when stained with methyl-green pyronin vital staining. Unfortunately, this stain alters the size of the ciliates, and even then, it only is temporarily successful, since the ciliates and opalinids, especially *Prootopalina* specimens, burst due to an increase in osmotic pressure.

Although Mayer's haematoxylin gave decent information on the intracellular components of *B. kirbyi,* these methods proved less effective for the other protozoans. Therefore, it is suggested that a better evaluation of a larger range of haematoxylin stains, as well as adhesive mediums, should be done in future studies. Metcalf (1923) found that Delafield's haematoxylin was the easiest and most accurate stain, giving the best results, whereas Rodriguez (1939) proposed Heidenain's haematoxylin and Tuan's method of destaining. Furthermore, Metcalf (1923) also mentioned that many other stains, including Borax-carmine, alum-carmine and Lyon's also delivered satisfying results. Metcalf (1923) further stated that iron haematoxylin gives clear stains for thin species and any cytoplasmic spherules.

Scanning electron microscopy makes it possible to catch sight of all protozoan species present in the gut that are not visible to the unaided eye. For example, it permitted the observation of the *Hexamita* species in this study. In addition, it is possible that other protozoan species — including ciliate species in the gut of *X. laevis* — were not all discovered in this study.

4.2.3 Molecular analysis

This study is the first to reveal genetic data from not only *X. laevis'* rectum ciliate community, but also the first to report genetic data from amphibian gut ciliates in South Africa. Of the 31 *Balantidium* species known globally, only four sequences are available on GenBank, namely *B*. *entozoon*, *B*. *duodeni* and *B*. *grimi* (Grim & Buonanno, 2009; Chistyakova,*et al*., 2014; Zhao *et al*., 2018). This study will contribute a further three *Balantidium* sequences from frog hosts. Representatives of the genus *Balantidium* are known to have shared morphological features

(Lynn, 2008). In a study by Kornilova *et al*. (2016), two species of frog *Balantidium, B. entozoon* and *B. elongatum*, showed both similar and different morphometrics, although molecular analysis showed no difference between these two *Balantidium* species. Consequently, it was suggested that *B. elongatum* is a junior synonym of *B*. *entozoon.* Therefore, we posit that the addition of more genetic data will indicate that many of the different *Balantidium* species described worldwide could possibly be synonyms, since balantidia are known to be polymorphic (Ponce-Gordo, 2008).

The present toolkit used in this study, consisting of SEM, Haematoxylin staining and DNA barcoding, is a combination of accessible and modern techniques. Currently, it identified the endociliates of *X. laevis* in South Africa as *Balantidium kirbyi*, *Balantidium* sp. 1, *Protoopalina* sp.1 and *Balantidium* sp. 2. It also revealed a possible case of co-invasion by *B. kirbyi* from South Africa to France. Nevertheless, we suggest that the ultimate toolkit for integrated protozoan identification also entails a method for proper revealing of kineties and infraciliature. Infraciliature could be revealed by protargol impregnation, TEM or a working silver staining method.

However, revelation of infraciliature is easier said than done. Protargol is not only very expensive, but also currently commercially unavailable, and more recently developed silver impregnation methods, also in other ciliate studies (Foissner, 2014), are not reliably successful. We suggest that the method of deciliation could be the answer to this quandary. Not only can kineties be seen in deciliated specimens, but other external ciliate characteristics, usually hidden by cilia, are also revealed.

Finally, all these methods can be simplified through a working ciliate culture method. Nevertheless, the reason for the unsuccessful ciliate cultures in this study is still unexplained. In addition, it was difficult to isolate ciliates, examine division and understand the life cycles of the endociliates in this study. Cultures will provide isolated single-species samples. Single-species samples will contribute to more efficient molecular analysis since it will save the time and effort needed to manually sort specimens and accordingly result in purer extractions. Furthermore, ciliate cultures can provide insights into the nature and behaviour of ciliates. For example, more in-depth studies on life cycles, polymorphism and proliferation can be studied by using cultures.

4.3 Geographic distribution, host-parasite interactions and ecology

Prevalence and abundance of protozoan species in *X. laevis*

Balantidium kirbyi was the most prevalent rectal protozoan in *X. laevis*, present at 100% of both the South African and France localities. Both smaller *Balantidium* spp. were second most prevalent in South Africa (40.16%) and France (60%). The *Protoopalina* spp. was the least prevalent present in localities from both South Africa (37.23%) and France (34.67%). In both South Africa and France, *B. kirbyi* was also the most abundant protozoan in the gut of *X. laevis*, and was present in much higher numbers than the other species. This agrees with the findings of other studies (Sandon, 1941; Thurston, 1970). Due to its high abundance and prevalence, *B. kirbyi* is probably the best species to use as a bioindicator of host and ecosystem health for both invasive and native *X. laevis*.

Co-introduced protozoan species of *X. laevis*

Based on genetic evidence that revealed *B. kirbyi*'s presence in both South African and French frog hosts, we suggest that *B. kirbyi* was co-introduced with its globally invasive host. This species now joins the metazoan parasites *Protopolystoma xenopodis* (Monogenea) (Price, 1943) and *Cephalochlamys namaquensis* (Cestoda) (Cohn, 1908) as co-introduced parasites from Africa that parasitise the French population of *X. laevis*. French *X. laevis* was introduced into the wild after many generations of laboratory cultivation (Measey et al. 2012; van Sittert & Measey 2016; Weldon et al. 2007). Therefore, this co-invasion means that *B. kirbyi* should be able to survive for long periods in captive hosts.

The flagellate of the genus *Hexamita* that was found, is a genus of parasitic diplomonads known to parasitise several animals including avian, terrestrial, and aquatic animals (Uldal & Buchmann, 1996; Evans & Lester, 2001; Cooper, *et al*., 2004). In addition, three species of *Hexamita* which are, *H. intestinalis*, *H*. *salmonis*, H truttae have been associated with disease in fish (Floyed & Reed, 1994). Moreover, *H. intestinalis* have also been reported in several frog studies (Thurston, 1970; Lank, 1971). In addition we suggest that the gut flora of *X. laevis* could still hold many more unidentified species of ecological importance.

4.4 Inaccuracies in ciliate studies and limitations in methods

Unfortunately, mistakes could easily exist in ciliate studies. According to Sandon (1976), these mistakes are primarily observational or interpretational related. Since most amphibian ciliate descriptions were done decades ago, as described earlier, the available visualisation and microscopy techniques could not adequately visualise all structures for study, with molecular data being almost non-existent. Furthermore, interpretational mistakes could easily occur due to poor fixation methods, which makes it difficult to distinguish between difference due to variable fixation or ciliate polymorphism.

Polymorphism can be described as the presence of several different forms or types of individuals among individuals of a single species due to a discontinued genetic variation. Variance in ciliate shapes and sizes has a great influence on the identification of ciliate species. As mentioned earlier, Kornilova *et al*. (2016) studied *B. entozoon* and *B. elongatum* from the gut of the European common frog *Rana temporaria* (Linnaeus, 1758). In this study, both modern electron microscopic techniques and molecular phylogenetic analysis supported that *B. elongatum* and *B. entozoon* is the same species. Thus, in accordance with Kornilova *et al*. (2016), we propose that some of the 31 amphibian ciliate species of the genus *Balantidium* described may be synonyms. Subsequently, we suggest that a survey consisting of both morphological and molecular analyses should be done on all amphibian ciliates to review their classification. The presence of the ''Villeneuve-Brachon's'' field in *X. laevis*' ciliates should be examined by use of TEM. In addition, several other species descriptions of the genus *Balantidium* need to be supplied by details of the ultrastructure.

Are the ciliates of *Xenopus laevis* mutualistic or commensal symbionts rather than parasitic?

Symbiotic systems between animal hosts and ciliates exist broadly across the animal kingdom. A few examples of hosts include elephants (Gürelli & Ito 2014); fish (Corrêa *et al*., 2019); insects (Tanahashi *et al*., 2017); molluscs (Fenchel, 1965) and even humans (Bel & Couret, 1910). Symbiotic relationships in ciliates include commensalism, epibiosis, mutualism and parasitism (Mayén-Estrada *et al*., 2021). Since this diversity of symbioses exist, a system to classify symbiotic associations was developed (Paracer & Ahmadjian, 2000). This classification is based on several aspects which include: i) dependence, being either obligate or facultative; ii) symbiont specificity; iii) nutrient obtainment; and iv) symbiont location.

In this study, it was found that, in terms of environment and food availability, *X. laevis*' endociliates are highly dependent on their host for survival. For symbiont specificity, we suggest that ciliates are host-specific since the same specific species of protozoans were found at localities widely distributed over South Africa. Furthermore, in the study by Mohr (1942), the infection of four ciliate species persisted for more than six years in *X. laevis* from South Africa. In line with this, the molecular proof that the 18S rDNA sequence of *B. kirbyi* found in France is the same as the 18S rDNA sequence of *B. kirbyi from South Africa.*

To add, given the high prevalence of *B. kirbyi*, we believe that the endociliates of *X. laevis* may rather be commensal symbionts of *X. laevis*.

No physical harm to the host caused by the endociliates was observed during this study. Since parasitism, which is less common in ciliates, usually causes harm, we oppose the statement that *X. laevi*s' endociliates are parasites. Trichostomatic ciliates are known to be symbionts of vertebrates in ruminants as well as foregut fermenters (Lynn, 2008). Interestingly, a high number of species reported as endocommensals exist for the genus *Balantidium* (Li *et al*., 2014). However, we speculate that *X. laevis and* its endociliates may rather have a mutualistic relationship in terms of nutrition. Rumen ciliates are believed to contribute to gut function by 1) shaping and regulating prokaryotic populations through their grazing behaviour (Nenninger, 1948; Oulhen *et al*., 2016); 2) adding degradative complexity (Paracer & Ahmadjian, 2000); and 3) producing hydrogenosomes providing ideal conditions for hydrogenotrophic methanogens (Sprague & Couch, 1971). The same could be true for *X. laevis* and its ciliates to a certain extent. By the same token, the ciliates may contribute to the degradation of undigested plant material or proteins in the frog intestine. We propose that since *X. laevis* is primarily an aquatic organism, this makes the clawed frog a suitable habitat for its symbiotic ciliate community. Consequently, a healthy balance is maintained in the gut of *X. laevis* by its endociliates, while the ciliates live in the protected and nutrient-rich environment offered by the host.

Conclusion

The gut protozoans of *X*. *laevis* from South Africa were documented as four species namely: 1) *B. kirbyi*, 2) *Balantidium* sp. 1, 3) *Balantidium* sp. 2 and 4) *Protoopalina* sp. 1. Correspondingly, the gut protozoans of *X. laevis* from France were documented as: 1) *B. kirbyi*, 2) *Balantidium* sp. 3, *Balantidium* sp. 4 and 4) *Protoopalina* sp. 2. Molecular analysis provided evidence that *B. kirbyi* exists in *X. laevis* from both South Africa and France. It remains to be seen whether the unidentified *Balantidium* and *Protoopalina* species from South African and French populations of *X. laevis* are, in fact, the same or different species.

Both morphological and molecular tools were applied and evaluated to confirm the protozoan species identifications. It was found that multiple methods are key to the proper identification of ciliates. In addition, we support Foissner (2014) in agreeing that it is impossible to identify ciliates by only making use of one method. Subsequently, a workflow was developed in this study to ease the identification of gut protozoans. This workflow should ideally entail functional ciliate cultures, molecular analysis, live observations, haematoxylin staining, supravital staining, silver staining, SEM, TEM and protargol impregnation or deciliation.

To a certain extent, this study clarified the gut protozoan fauna of *X. laevis* by updating the literature with a modern approach. Specifically, the taxonomic classification of *B. kirbyi* from South Africa and France was elucidated, with *B. kirbyi* from France being synonymised with *B. kirbyi* from South Africa Secondly, this study highlighted the importance of using both molecular and morphological methods for species identification. The molecular approach aided in confirming the presence of *B. kirbyi* in populations of *X. laevis* in France as a co-introduced symbiont. This study therefore laid the foundation for future ciliate identification as well as new species discoveries in other anuran hosts.

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Appendixes:

A: Table 1.1: Table of *X. laevis* dissections in France and South Africa

Dissection number	Date collected	Date dissected	Locality	Habitat
2	3/11/2017	3/16/2017	Potchefstroom 1	Vegetated natural pond
5	3/11/2017	3/24/2017	Potchefstroom 1	Vegetated natural pond
7	3/11/2017	3/28/2017	Potchefstroom 1	Vegetated natural pond
10	4/8/2017	4/8/2017	Rondepan	Uitloopsel van groter pan
17	4/9/2017	4/20/2017	Modimolle	Ornamental garden pond
19	4/9/2017	4/24/2017	Modimolle	Ornamental garden pond
20	4/9/2017	4/25/2017	Modimolle	Ornamental garden pond
23	4/12/2017	4/27/2017	Witrivier 1	Vegetated shallow dam
24	4/12/2017	4/28/2017	Witrivier 1	Vegetated shallow dam
26	4/12/2017	4/30/2017	Witrivier 1	Vegetated shallow dam
31	4/12/2017	5/1/2017	Witrivier 2	Ornamental garden pond
38	4/14/2017	5/4/2017	Cape Town 2	Vegetated dams from mountain stream
39	4/14/2017	5/4/2017	Cape Town 2	Vegetated dams from mountain stream
47	5/6/2017	5/29/2017	Letsitele	Ornamental garden pond
48	5/6/2017	5/31/2017	Letsitele	Ornamental garden pond
51	5/6/2017	6/4/2017	Letsitele	Ornamental garden pond
58	6/18/2017	6/22/2017	Hermanus	Urban recreational dam
59	6/18/2017	6/22/2017	Hermanus	Urban recreational dam
63	6/18/2017	7/3/2017	Hermanus	Urban recreational dam
66	6/26/2017	7/3/2017	Cape Town	Urban recreational dam
67	6/26/2017	7/4/2017	Cape Town	Urban recreational dam
68	6/26/2017	7/4/2017	Cape Town	Urban recreational dam
72	6/26/2017	7/18/2017	Cape Town	Urban recreational dam
73	6/26/2017	7/18/2017	Cape Town	Urban recreational dam
78	7/12/2017	7/17/2017	Welgemoed	Urban recreational dam
81	7/12/2017	7/17/2017	Welgemoed	Urban recreational dam
82	7/12/2017	7/18/2017	Welgemoed	Urban recreational dam
126	11/4/2017	11/4/2017	7Fontein	Rocky pools in mountain streams
131	11/4/2017	11/5/2017	7Fontein	Rocky pools in mountain streams
133	11/7/2017	11/7/2017	Wellington 2	Plaasdam
134	11/7/2017	11/7/2017	Wellington 1	Plaasdam
135	11/7/2017	11/7/2017	Wellington 2	Plaasdam
136	11/7/2017	11/7/2017	Wellington 1	Plaasdam
138	11/7/2017	11/14/2017	Wellington 2	Plaasdam
139	11/7/2017	11/14/2017	Wellington 2	Plaasdam
141	11/12/2017	11/15/2017	Laingsburg	Murky pool in mountain stream
143	11/13/2017	11/17/2017	Barrydale 1	Stagnant pool in river
145	11/13/2017	11/18/2017	Barrydale 4	Plaasdam

146	11/13/2017	11/18/2017	Barrydale 4	Plaasdam
147	11/13/2017	11/18/2017	Barrydale 4	Plaasdam
148	11/13/2017	11/18/2017	Barrydale 4	Plaasdam
151	11/13/2017	11/20/2017	Barrydale 2	Plaasdam
152	11/20/2017	11/20/2017	Ladismith	Vegetated dam in mountain stream
153	11/20/2017	11/20/2017	Ladismith	Vegetated dam in mountain stream
156	11/20/2017	11/21/2017	Ladismith	Vegetated dam in mountain stream
160	11/21/2017	11/24/2017	Riversdale	Deep pools in river
161	11/21/2017	11/26/2017	Riversdale	Deep pools in river
162	11/21/2017	11/26/2017	Riversdale	Deep pools in river
165	11/26/2017	11/28/2017	Three Sisters	Ornamental garden pond
166	11/27/2017	11/28/2017	Three Sisters	Ornamental garden pond
168	11/27/2017	11/28/2017	GaMamadi	Stagnant pool in river
169	11/27/2017	11/30/2017	GaMamadi	Stagnant pool in river
170	11/28/2017	12/2/2017	Adelaide	Vegetated dams
171	11/28/2017	12/2/2017	Adelaide	Vegetated dams
174	12/1/2017	12/2/2017	Ugie	Vegetated dam at fountain source
178	12/1/2017	12/4/2017	Ugie	Vegetated dam at fountain source
180	12/4/2017	12/6/2017	Colesberg	Swimming pool
185	12/6/2017	12/9/2017	Hopetown	Dam in leivoor
186	12/6/2017	12/9/2017	Hopetown	Dam in leivoor
188	12/6/2017	12/9/2017	Hopetown	Dam in leivoor
189	12/6/2017	12/10/2017	Hopetown	Dam in leivoor
190	12/6/2017	12/10/2017	Hopetown	Dam in leivoor
194	1/1/2018	1/3/2018	Verkykerskop	Ornamental garden pond
197	1/1/2018	1/18/2018	Verkykerskop	Ornamental garden pond
206	2/4/2018	2/7/2018	Wakefield	Pools in marshland
207	2/4/2018	2/12/2018	Wakefield	Pools in marshland
209	2/4/2018	2/14/2018	Wakefield	Pools in marshland
212	2/23/2018	3/2/2018	George	Vegetated dam
213	2/23/2018	3/5/2018	George	Vegetated dam
218	2/4/2019	2/12/2019	Louis Trichardt	Stagnant pool in river on farm
220	2/4/2019	2/16/2019	Louis Trichardt	Stagnant pool in river on farm
223	2/4/2019	2/23/2019	Louis Trichardt	Stagnant pool in river on farm
226	5/23/2019	5/24/2019	La Butte	
228	5/23/2019	5/24/2019	La Butte	
230	5/23/2019	5/27/2019	La Butte	
231	5/23/2019	5/27/2019	La Butte	
232	5/23/2019	5/27/2019	La Butte	
233	5/23/2019	5/27/2019	Cersay 1	
235	5/23/2019	5/28/2019	Cersay 1	
237	5/23/2019	5/28/2019	Cersay 1	
240	5/23/2019	5/28/2019	Cersay 1	

243	5/23/2019	5/29/2019	Cersay 1	
244	5/29/2019	5/29/2019	Coudray-Macouard	
245	5/23/2019	5/31/2019	Saint-Martin	
246	5/23/2019	6/3/2019	Saint-Martin	
247	5/23/2019	6/3/2019	Saint-Martin	
248	5/23/2019	6/3/2019	Saint-Martin	
250	5/23/2019	6/4/2019	Saint-Martin	
251	5/23/2019	6/4/2019	Saint-Martin	
252	5/23/2019	6/4/2019	Saint-Martin	
253	5/29/2019	6/4/2019	Coudray-Macouard	
254	5/29/2019	6/4/2019	Coudray-Macouard	
256	5/29/2019	6/5/2019	Brézé	
257	5/29/2019	6/5/2019	Brézé	
258	5/29/2019	6/5/2019	Brézé	
261	5/29/2019	6/6/2019	Brézé	
262	5/29/2019	6/6/2019	Coudray-Macouard	
266	6/7/2019	6/7/2019	Chalonnes	
270	5/29/2019	6/11/2019	Coudray-Macouard	
271	5/29/2019	6/11/2019	Brézé	
272	5/29/2019	6/11/2019	Brézé	
274	5/29/2019	6/11/2019	Brézé	
275	5/29/2019	6/12/2019	Brézé	
280	6/6/2019	6/12/2019	Ambillou	
281	6/6/2019	6/13/2019	Ambillou	
284	6/6/2019	6/13/2019	Ambillou	
286	6/7/2019	6/13/2019	Chalonnes	
287	6/7/2019	6/14/2019	Chalonnes	
288	6/7/2019	6/14/2019	Chalonnes	
291	6/18/2019	6/19/2019	Bouillé-Saint-Paul	
292	6/18/2019	6/19/2019	Bouillé-Saint-Paul	
294	6/18/2019	6/20/2019	Bouillé-Saint-Paul	
295	6/18/2019	6/20/2019	Bouillé-Saint-Paul	
297	6/20/2019	6/21/2019	Massais	
298	6/20/2019	6/21/2019	Epieds	
299	6/20/2019	6/21/2019	Epieds	
300	6/20/2019	6/24/2019	Massais	
302	6/20/2019	6/24/2019	Epieds	
304	6/18/2019	6/24/2019	Bouillé-Saint-Paul	
306	6/20/2019	6/25/2019	Epieds	
307	6/20/2019	6/25/2019	Epieds	
308	6/20/2019	6/25/2019	Massais	
309	6/20/2019	6/25/2019	Massais	
311	6/25/2019	6/26/2019	Cersay 2	
312	6/25/2019	6/26/2019	Cersay 2	
313	6/25/2019	6/26/2019	Cersay 2	

314	6/20/2019	6/26/2019	Epieds	
315	6/18/2019	6/26/2019	Bouillé-Saint-Paul	
316	6/18/2019	6/27/2019	Bouillé-Saint-Paul	
317	6/20/2019	6/27/2019	Epieds	
318	6/25/2019	6/27/2019	Cersay 2	
319	6/20/2019	6/27/2019	Massais	
322	6/18/2019	6/28/2019	Bouillé-Saint-Paul	
328	6/20/2019	7/2/2019	Epieds	
329	6/20/2019	7/2/2019	Epieds	
330	6/25/2019	7/2/2019	Cersay 2	
331	6/25/2019	7/2/2019	Cersay 2	
332	6/25/2019	7/2/2019	Cersay 2	
334	6/20/2019	7/2/2019	Massais	
337	6/20/2019	7/3/2019	Massais	
338	6/20/2019	7/3/2019	Massais	
341	11/1/2019	11/3/2019	Sewe Damme, Bloemfontein	Dam in peri-urban area
A1	16/04/21	4/5/2021	Bloemfontein 7 Damme	Vegetated natural pond
A11	30/04/21	13/5/2021	Modimolle	Ornamental garden pond
A12	30/04/22	14/05/2021	Modimolle	Ornamental garden pond
A13	30/04/23	14/05/2021	Modimolle	Ornamental garden pond
A14	30/04/24	14/05/2021	Modimolle	Ornamental garden pond
A15	30/04/25	14/05/2021	Modimolle	Ornamental garden pond
A16	30/04/26	14/05/2021	Modimolle	Ornamental garden pond
A17	30/04/27	18/05/2021	Modimolle	Ornamental garden pond
A18	30/04/28	18/05/2021	Modimolle	Ornamental garden pond
A19	30/04/29	19/08/2021	Modimolle	Ornamental garden pond
A20	30/04/30	19/08/2021	Modimolle	Ornamental garden pond
A21	30/04/31	2/9/2021	Modimolle	Ornamental garden pond
A22	30/04/32	2/9/2021	Modimolle	Ornamental garden pond
A23	23/07/21	6/9/2021	Modimolle	Ornamental garden pond
A24	16/04/21	18/05/21	Bloemfontein 7 Universiteit	Vegetated natural pond
A25	20/04/21	18/05/21	Bloemfontein 7 Universiteit	Vegetated natural pond
A28	26/04/23	26/04/2022	Potchefstroom Botanical gardens	Vegetated natural pond
A29	26/04/24	26/04/2022	Potchefstroom Botanical gardens	Vegetated natural pond
A30	26/04/25	26/04/2022	Potchefstroom Botanical gardens	Vegetated natural pond
A31	26/04/26	26/04/2022	Potchefstroom Botanical gardens	Vegetated natural pond
A32	26/04/27	26/04/2022	Potchefstroom Botanical gardens	Vegetated natural pond
A33	26/04/28	26/04/2022	Potchefstroom Botanical gardens	Vegetated natural pond
A34	1/5/2022	23/05/2022	Welverdiend	Ornamental garden pond
A35	1/5/2022	23/05/2022	Welverdiend	Ornamental garden pond
A36	1/5/2022	31/05/2022	Welverdiend	Ornamental garden pond
A37	1/5/2022	31/05/2022	Welverdiend	Ornamental garden pond

A38	1/5/2022	13/06/2022	Welverdiend	Ornamental garden pond
A39	1/5/2022	13/06/2022	Welverdiend	Ornamental garden pond
A40	26/04/22	13/06/22	Potchefstoom Botaniese tuin	
A41	1/5/2022	23/06/2022	Welverdiend	Ornamental garden pond
A42	1/5/2022	23/06/2022	Welverdiend	Ornamental garden pond
A43	1/5/2022	24/06/2022	Welverdiend	Ornamental garden pond
A44	1/5/2022	24/06/2022	Welverdiend	Ornamental garden pond
A45	15/09/2021	3/6/2022	Dullstroom	Vegetated dams from mountain stream
A46	15/09/2022	29/07/2022	Dullstroom	Vegetated dams from mountain stream
A47	15/09/2023	29/07/2022	Dullstroom	Vegetated dams from mountain stream
A49	15/09/2024	9/8/2022	Dullstroom	Vegetated dams from mountain stream
A50	12/8/2022	2/9/2022	Welverdiend	Ornamental garden pond
A51	12/8/2022	2/9/2022	Welverdiend	Ornamental garden pond

Appendix B: Ethics approval document, NWU-00426-21-A5.

Private Bag X1290, Potchefstroom
South Africa 2520

Tel: 086 016 9698
Web: http://www.nwu.ac.za/

North-West
University Animal Care, Health and
Safety Research Ethics Committee (NWUAnimCareREC)

Tel: 018 299-1208
Email: Ethics-AnimCare@nwu.ac.za (for animal studies)

28 September 2021

ETHICS APPROVAL LETTER OF STUDY

Based on approval by the North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC) on 28 September 2021, the NWU-AnimCareREC hereby approves your study as indicated below. This implies that the NWU-AnimCareREC grants its permission that, provided the general conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: An integrated approach to the taxonomy of the rectal endociliates of the globally invasive amphibian *Xenopus laevis*
Principal Investigator/Study Supervisor/Researcher: Prof LH du Preez Student:
A Jacobs - 28496698

Ethics number:

N	W	U	-	0	0	4	2	6	-	2	1	-	A	5

Institution
Study Number Year Status
Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Application Type: Single study
Commencement date: 28/09/2021 **Risk:** **Category 0**
Expiry date: 30/09/2022
Approval of the study is provided for a year, after which continuation of the study is dependent on receipt and review of an annual monitoring report and the concomitant issuing of a letter of continuation. A monitoring report is required at the end of September annually until completion of the study.

In process requirements: None

General conditions:

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

- *The principal investigator/study supervisor/researcher must report in the prescribed format to the NWU-AnimCareREC:*
  - *annually on the monitoring of the study, whereby a letter of continuation will be provided annually, and upon completion of the study; and*
  - *without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.*
- *The approval applies strictly to the proposal as stipulated in the application form. Should any amendments to the proposal be deemed necessary during the course of the study, the principal investigator/study supervisor/researcher must apply for approval of these amendments at the NWU-AnimCareREC, prior to implementation. Should there be any deviations from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.*
- *Annually a number of studies may be randomly selected for active monitoring.*
- *The date of approval indicates the first date that the study may be started.*
- *In the interest of ethical responsibility, the NWU-AnimCareREC reserves the right to:*
  - *request access to any information or data at any time during the course or after completion of the study;*
  - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process; - withdraw or postpone approval if:*
    - *· any unethical principles or practices of the study are revealed or suspected;*
    - *· it becomes apparent that any relevant information was withheld from the NWU-AnimCareREC or that information has been false or misrepresented;*
    - *· submission of the annual monitoring report, the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and/or*
    - *· new institutional rules, national legislation or international conventions deem it necessary.*
- *NWU-AnimCareREC can be contacted for further information via Ethics-AnimCare@nwu.ac.za or 018 299 1208*

Special conditions of the research approval due to the COVID-19 pandemic:

Please note: Due to the nature of the study i.e. (laboratory work involving the molecular analysis of rectal endociliates of a specific frog species), this study will be able to proceed during the current alert level, following receipt of the approval letter. No additional COVID-19 restrictions have been placed on the study except that the researcher must ensure that before proceeding with the study that all research team members have reviewed the North-West University COVID-19 Occupational Health and Safety Standard Operating Procedure.

NWU-AnimCareREC would like to remain at your service and wishes you well with your study. Please do not hesitate to contact the NWU-AnimCareREC for any further enquiries or requests for assistance.

Current details:(22654704) G:\My Drive\[1]. Research and Postgraduate Education\9.1.5.4 Templates\9.1.5.4.2_NWU-AC_EAL.docm
15 September 2021 File Reference: 9.1.5.4.2

Appendix C: Table 3.1 Ciliate genera sequences and accession numbers obtained from GenBank

Organism	Accession number	*Organism*	Accession number
Blepharoconus hemiciliatus	AB795027	*Bandia deveneyi*	AY380823
Alloiozona trizona	AB795026	*Amylovorax dogiel*	AF298825
Prorodonopsis coli	AB795029	*Macropodinium ennuensis*	AF298820
Didesmis ovalis	AB795025	*Amylovorax dehority*	AF298817
Buxtonella-like	JQ073328	*Bitricha tasmaniensis*	AF298821
Buxtonella-like	JQ073325	*Polycosta roundi*	AF298819
Neobalantidium coli	JQ073309	*Balantidium polyvacuolum*	KJ124724
Isoticha intestinalis	U57770	*Balantidium grim*	MG837094
Isotricha prostoma	AF029762	*Balantidium entozoon*	EU581716
Dasytricha ruminantium	U57769	*Balantidium duodeni*	KM057846
Ophryoscolex purkynjei	U57768	*Balantidium entozoon*	JQ408692
Epidinium caudatum	U57763	*Paraisotricha colpoidea*	EF632075
Epidinium ecaudatum caudatum	AM158474	*Paraisotricha minuta*	AB794984
Entodinium caudatum	AM158445	*Buxtonella sulcata*	AB794979
Metadinium medium	AM158464	*Buxtonella sulcata*	AB786848
Eudiplodinium maggi	AM158451	*Latteuria media*	AB794983
Eremoplastron neglectum	AM158473	*Latteuria polyfaria*	AB794982
Diploplastron affine	AM158457	*Helicozoster indicus*	AB794981
Diplodinium dentatum	U57764	*Balantidium coli*	AM982723
Anoplodinium denticulatum denticulatum	AM158470	*Balantidium coli*	AM982722
Enoploplastron triloricatum	AM158462	*Epispathidium papilliferum*	DQ411857
Polyplastron multivesiculatum	U57767	*Epispathidium amphoriforme*	KT246080
Polyplastron multivesiculatum	AM158458	*Spathidium spathula*	HM140392
Ostracodinium dentatum	AM158460	*Arcuospathidium muscorum*	DQ411859
Troglodytella abrassart	AB437346	*Balantidion pellucidum*	JF263444
Bozasella gracilis	AB793744	*Arcuospathidium cultriforme scalpriforme*	KT246076
Spirodinium equi	FM201781	*Spathidium stammer*	DQ411862
Cochliatoxum periachtum	EF632078	*Spathidium foissner*	KF733756
Tetratoxum unifasciculatum	AB794970	*Cultellothrix lionotiformis*	JF263445
Ditoxum funinucleum	AB794091	*Apobryophyllum schmidingeri*	JF263441
Triadinium caudatum	AB530163	*Arcuospathidium namibiense tristicha*	JF263442
Tripalmaria dogieli	EF632074	*Enchelys gasterosteus*	JF263447
Gassovskiella galea	AB793783	*Trachelophyllum sp*	JF263452
Polydiniella mysorea	AB555710	*Protospathidium muscicola*	JF263449
Raabena bella	AB534183	*Enchelys polynucleata*	DQ411861
Circodinium minimum	AB794974	*Dileptus jonesi*	MF288144
Ochoterenaia appendiculata	AB794973	*Dileptus sp.*	AF029764
Blepharocorys curvigula	AB534184	*Monilicaryon monilatum*	KJ680550

Table 3.1: Ciliate genera sequences and accession numbers

Organism	Accession number	*Organism*	Accession number
Blepharocorys uncinata	AB530162	*Rimaleptus binucleatus*	KJ680552
Pseudoentodinium elephantis	AB794972	*Homalozoon vermiculare*	L26447
Parentodinium sp	AB530164	*Didinium nasutum*	U57771
Balantidium ctenopharyngodon	MK204639	*Loxophyllum paludosum*	MW760400
Balantidium ctenopharyngodon	KU170970	*Loxophyllum rostratum*	DQ411864
Balantidium ctenopharyngodoni	GU480804	*Cochliatoxum periachtum*	EF632078