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Taxonomic status of *Trichodina heterodentata* Duncan, 1977 (Ciliophora: Peritrichia) using standard morphology, as well as molecular techniques

by

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DISSERTATION

submitted in fulfillment of the requirements for the degree

MAGISTER SCIENTIAE IN ZOOLOGY

Department of Zoology and Entomology
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CHAPTER 1 - INTRODUCTION

The protist microcosm is one of the richest groups in regards to their morphological variation, and exceeds that of all the other eukaryotic kingdoms (Sogin & Silberman 1998). They are predominantly unicellular organisms that inhabit a heterogenous array of environments, ranging from free living to parasitic in nature. Within this complex group, the ciliated protists, with their characteristic rows of cilia for locomotory actions are the most identifiable (Lynn 2017). According to the recently revised eukaryotic classification of Adl *et al.* (2012), where they avoid the use of traditional higher taxonomic hierarchy, the phylum Ciliophora Doflein, 1901 falls into the group Alveolata Cavalier-Smith, 1991, which in turn falls under the super-group SAR Burki *et a*l., 2007 (a cluster of the groups; Stramenopiles Patterson, 1989; Alveolata and Rhizobia Cavalier-Smith, 2002).

Trichodinids are members of the family Trichodinidae Raabe, 1959 and together with its two sister families (Urceolariidae Dujardin, 1840 and Trichodinopsidae Kent, 1881) make up the order Mobilida Kahl, 1933 (Oligohymenophorea de Puytorac *et al,* 1974: Peritrichia Stein, 1859). The largest group within this family (and order) is the genus *Trichodina* Ehrenberg, 1838 that consists of more than 300 described species to date (Tang *et al*. 2013) all of them symbiotic on or in a diverse range of hosts.

Trichodinids are usually associated with teleost fishes, especially as ectosymbionts, but have been reported from many other hosts, from invertebrates such as *T. oxystelis* Sandon, 1965 from the marine gastropod *Oxystele* Philippi, 1847 (Basson & van As 1992), *T. diaptomi* (Dogiel, 1940) Basson and van As, 1991 from the freshwater calanoid copepod, *Metadiaptomus meridianus* (van Douwe, 1912) (Basson & van As 1991), *Pallitrichodina stephani* van As and Basson, 1993 from the terrestrial Giant African Snail *Achatina panthera* (Férussac, 1821) (van As & Basson 1993) to vertebrate animals like the African Clawed Toad *Xenopus laevis laevis* (Daudin, 1802), hosting *T. xenopodos* Fantham, 1924 in its urinary bladder (Kruger *et al.* 1991), *T. rhinobatae* van As and Basson, 1996 found in the Lesser Guitarfish, *Rhinobatos annulatus* Smith, 1841 (van As & Basson 1996) and even in the reproductive tract of waterfowl (Carnaccini *et al.* 2016).

As already mentioned, trichodinids are in symbiotic relationships with their hosts, in most cases and in their natural environment, the relationships are commensalistic and/or epizooic (living externally on an organism, with no indication of symbiotic association) in nature. However, some species of ectozoic *Trichodina* have shown to be pathogenic towards their hosts in artificial conditions, especially aquaculture environments. These species have been reported to cause a disease known as trichodiniasis prevalent in fish fry and fingerlings in aquaculture that can lead to high mortality, having serious economic impacts on the fisheries industries.

Khoshnood and Khoshnood (2014) reported that when trichodinid infested hosts experience external stress factors trichodiniasis may lead to hyperplasia, lesions and necrosis of the epidermal cells, impeding respiration and feeding of especially the smaller fish hosts. Hoffman and Lom (1967) and Markiewicz and Migala (1980) both commented on *Trichodina fultoni* Davis, 1947 that may cause trichodiniasis in hatchery reared freshwater fish like *Carassius auratus* (Linnaeus, 1758) and *Ictalurus punctatus* (Rafinesque, 1818). *Trichodinella epizootica* (Raabe, 1950) Šramek-Hušek, 1953, another genus in the family Trichodinidae has also been reported to be the cause of severe damage on the gill epithelia (Nikolić *et al.* 2003).

Trichodina heterodentata Duncan, 1977 is one of the most cosmopolitan ectozoic trichodinids, with a wide variety of hosts, mostly on teleosts as part of a multispecies trichodinid infestation. With this global distribution and diversity of hosts, the morphology of *T. heterodentata* has been well documented (as illustrated and discussed in **Chapter 2**), but very little molecular analysis have been done and to date only one 18S rDNA sequence has been published by Tang *et al.* (2013). During previous parasitological surveys in the Okavango Delta, Botswana, by the Aquatic Ecology group from University of the Free State, it has been noticed that certain tadpoles seem to have a single species infestation of *T. heterodentata* on their gills and skin. This provided the perfect opportunity to identify a technique for isolating genomic DNA from this trichodinid, as there can be no contamination, due to the absence of other trichodinid species. The geographic isolation of the Okavango River System also played a very important role, as this system is

basically a closed off water body, with hitherto no known introduced aquatic fish and anuran species. The Nxamasere Floodplain creates seasonal refugia for aquatic life during the dry periods of the year, temporarily trapping fish and tadpoles that in turn become an important food source for the assorted water birds that surround the plain.

Based on this and knowledge from previous post-graduate students working on tadpoles and trichodinids (Kruger *et al.* 1993a; Kruger *et al.* 1993b; Groenewald 2013) a working hypothesis was formulated for the present study:

> **Anuran tadpoles from the Nxamasere Floodplains, Okavango Delta, Botswana, are host to a single trichodinid infestation, i.e. *Trichodina heterodentata* Duncan, 1977, also recorded as part of a multispecies infestation on various freshwater fish species.**

In order to ascertain whether this hypothesis should ultimately be accepted or rejected relies on five specific objectives that will make out the core of **Chapters 4**, **5**, **6**, **7** and **8**.

The analysis of morphological characteristics using specific stains and/or impregnations was, and still is, one of the most important taxonomic techniques used for the description of protozoans. The morphological taxonomy of mobiline ciliophorans is reliant on the silver staining method as devised by Klein (1926), i.e. impregnating the specimens with silver nitrate, then exposing the specimens to a UV light source. Lom (1958) proposed this method as the standard staining protocol for detecting differences between denticle structures for different trichodinid species. Since its introduction in 1958, this method has been very successful and is still regarded as the key morphological technique. Unfortunately silver nitrate does not impregnate equally well across the large variety of trichodinids, and the trichodinids from certain types of hosts do not impregnate well at all. The first objective, as discussed in **Chapter 4**, explores the difficulty of silver nitrate impregnation for different trichodinid groups:

I. **To develop a silver impregnation protocol for tadpole trichodinids similar to the staining results obtained for teleost hosts.**

The previous findings of a single species infestation of trichodinids, morphologically very close to *T. heterodentata,* on tadpoles from the Nxamasere floodplains, led to the next two objectives of this project, namely:

II. **To confirm that *Trichodina* species infestations on tadpoles in the Okavango Delta represent a single species infestations of *T. heterodentata*.**
III. **To determine whether ectosymbiotic trichodinids from anurans are morphometrically similar to *T. heterodentata* from fish hosts in the same locality.**

Chapter 5 deals with the results of an in-depth morphological analysis of trichodinids collected from two toad species, *Schlerophrys gutturalis* (Power, 1927) and *S. poweri* (Hewitt, 1935) using both the unified morphological characteristics technique proposed by Lom (1958) and the van As and Basson (1989) denticle description. The morphometric data were compared to 24 reports of *T. heterodentata* from various freshwater teleost and amphibian hosts from 19 countries worldwide. One teleost host, *Pseudocrenilabrus philander* (Weber, 1887) was found in the same standing pools as the *Schlerophrys* species tadpoles, and the collected *T. heterodentata* from this fish species, was also measured, described and compared to the other populations. Even though *T. heterodentata* is such a well described and recorded trichodinid, there were still some questions in the literature regarding its distribution, morphology and variation. **Chapter 6**, written as an article that will be sent in for publication, looks at two of these questions, namely: why, with its international distribution has this species never been reported in northern America and how can it be that the largest morphological measurements for body diameter are from the original species description by Brian Duncan in 1977, with no subsequent record exceeding his measurements.

Up to date only a handful of research groups have worked on the molecular taxonomy of trichodinids, while almost no work has been done on the rest of the Mobilida. Most molecular analysis and phylogenetic inferences were, and still are, done by doctors Fa Hui Tang and Yuan Jun Zhao at the Key Laboratory of Animal Biology in Chongqing, China. With the nucleus of trichodinid molecular work done in China, the few published phylogenetic trees are mostly made up of trichodinids that are found on hosts in the Chinese river systems and have almost no other representatives over the world. This unrepresentative inferences, along with the growing importance of molecular research in the protozoan research field gave rise to the fourth objective, examined in **Chapter 7**:

IV. **To determine if it is possible to extract gDNA from anuran host trichodinid material and make phylogenetic inferences using 18S SSU rRNA.**

Chapter 6 raises another important concern; whether *T. heterodentata* is actually a species complex or is indeed a single species with a very wide range of hosts and distribution. *Trichodina domerguei* Wallengren, 1897 was just such a case, originally described as one species with slight variation, described as *forma* by Dogiel (1940) but later found to be a species complex by Lom (1960). Combining the results from **Chapter 7** with the subsequent concern raised in **Chapter 6**, the fifth and final objective can be developed:

V. **Determine whether *Trichodina heterodentata* is a single non-host specific species found on both anuran and teleost hosts or a species complex.**

The five objectives of this study were all met and the results, not only confirms that *T. heterodentata* is, in fact a species complex and trichodinids found on tadpoles from the Nxamasere Floodplain is not *T. heterodentata*, but that all methodologies used and discussed can be used as a protocol for the best possible description of a new trichodinid species from anuran tadpoles.

CHAPTER 2 - LITERATURE REVIEW

THE OKAVANGO RIVER SYSTEM

The Okavango River System is one of the largest rivers in southern Africa traversing three countries, Angola, Namibia and Botswana and covering a surface area of almost 192 500km^2 (Mendelsohn & el Obeid 2004). From its humble origin, namely a series of headwater streams and small tributaries flowing from the south of the hilly Angolan highlands (Fig. 2.1), it delivers about 9.4km^3 of water per year into the Okavango Delta in north-western Botswana (Mendelsohn & el Obeid 2004).

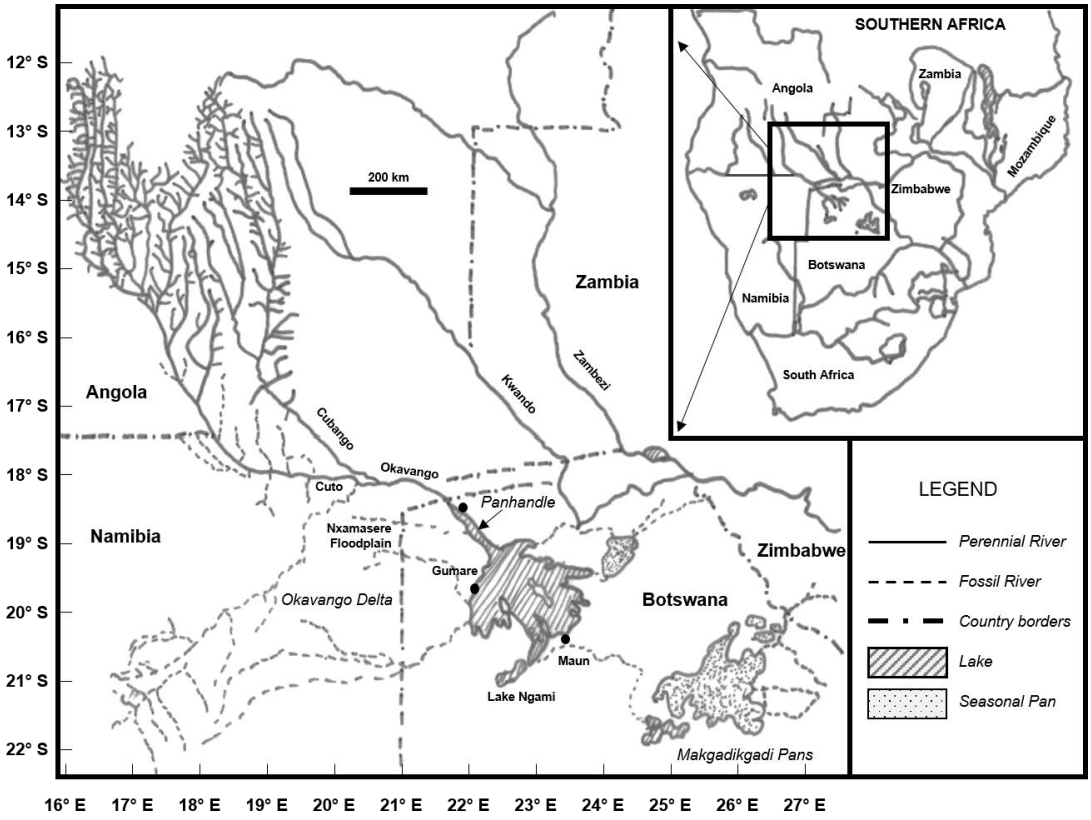


Figure 2.1: The Okavango River System in southern Africa (redrawn and adapted from West *et al.* 2015).

The average annual rainfall in the Angolan catchment area, lying 1 700m above sea level, is three times higher than that of the Okavango Delta at only 940m above sea level. The annual floodwater, which originates in Angola during November, from the tributaries is

transported very slowly across the semi-desert Kalahari by two large rivers, the Cuito and the Cubango Rivers, which combines to form the Okavango River (Fig. 2.1). The Okavango River then flows into the Okavango Panhandle and into the alluvial fan of the Okavango Delta Proper between February and March. The Cuito River transports 45% of the Angolan catchment water and the Cubango/Okavango River carries 55% down into Botswana.

Shaw (1984) states that due to evapo-transpiration and groundwater recharge almost 95% of this floodwater is lost within the Okavango Delta, whilst the balance drains towards one of the three lake basins that forms the end-sinks of the drainage system. Two of these lake basins, namely Lake Ngami and the Makgadikgadi Pans can be observed in Figure 2.1, with the Mababe Depression to the north east of the delta (Fig. 2.2.).

With the large inflow of floodwater from the Okavango River there are also six historical outflows from the Okavango Delta (Fig. 2.2); the Magwegqana Spillway which connects the Okavango to the Linyanti-Chobe Swamps; the Savuti Channel, which connects the Linyanti-Chobe Swamps with the Mababe Depression; the Mababe River which transports water to the southern boundary of the Mababe Depression; the Thamalakane River System, which is the largest outflow; the Kunyene River which flows into Lake Ngami and the Thoage River, which until the late 1800's connected the Thoage System with Lake Ngami, but is now mostly dried up due to natural and man-made blockages (Shaw 1984). The volume of these outflows varies from year to year and decade to decade as changes in the inflow, the precipitation and the hydrological conditions of the surrounding swamps plays important roles.

Other very important geological traits that contributes to the uniqueness of the Okavango River System are the fault-lines that lie directly south-east to the edge of the delta, which is the result of the collapse of a segment of the earth's crust (Hutchins *et al*. 1976).

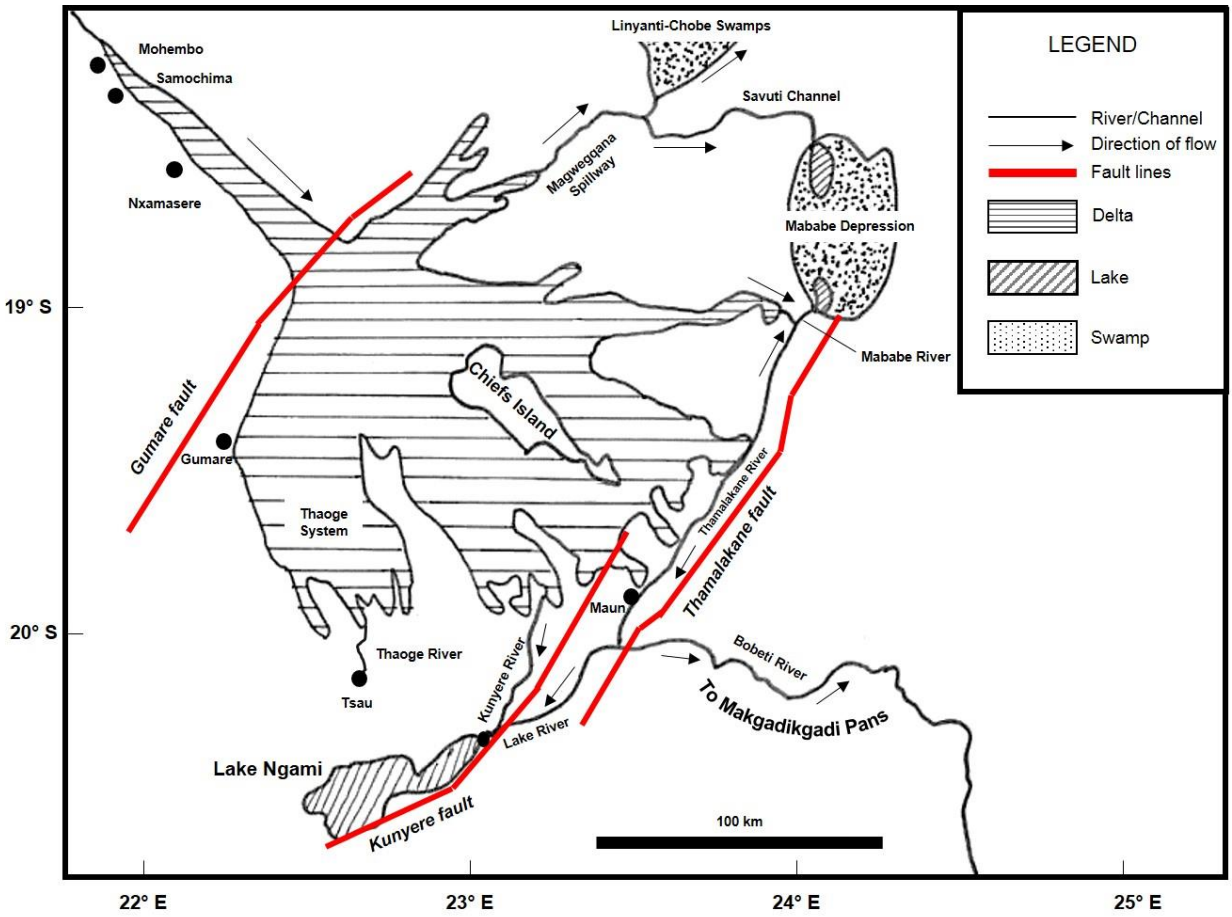


Figure 2.2: The outflows of the Okavango River System within Botswana (redrawn from Shaw 1984).

The two most important faults, the Kunyene and the Thamalakane faults, which rise with a maximum height of five meters to the south-east and dip again in the northwest, form geological barriers that prevent the delta to spread any further east (Mendelsohn & el Obeid 2004). These two faults (indicated in red in Fig. 2.2), along with another fault, the Gumare fault, separates the panhandle region from the alluvial fan.

The Okavango Delta can be viewed as a closed and seasonal ecological environment because of its unique geohistory and geological features, as there is only one inflow into this system (the Okavango River), which is regulated by seasonal rainfalls, combined with a slow drainage system of basin sinks and outflow rivers.

The Okavango Delta

According to McCarthy *et al*. (1998) the Okavango Delta can be subdivided into two main regions; the panhandle (Fig. 2.3**A**) to the northwest and the delta proper that is basically the alluvial fan to the south of the panhandle (Fig. 2.3**B**). This division is based on the duration of flooding within these two regions; the panhandle, which consists of a large wandering river bordered by permanent swamps and seasonal floodplains and has a gradient of 1:5 500, compared to the 1:3 300 gradient of the delta proper fan region (McCarthy & Ellery 1997). It is due to these almost horizontal gradients that the floodwater flows at such an incredibly slow pace once it enters the Botswana Delta System, where the water is literally pushed across the sand rather by hydraulic force than fall with the aid of gravity.

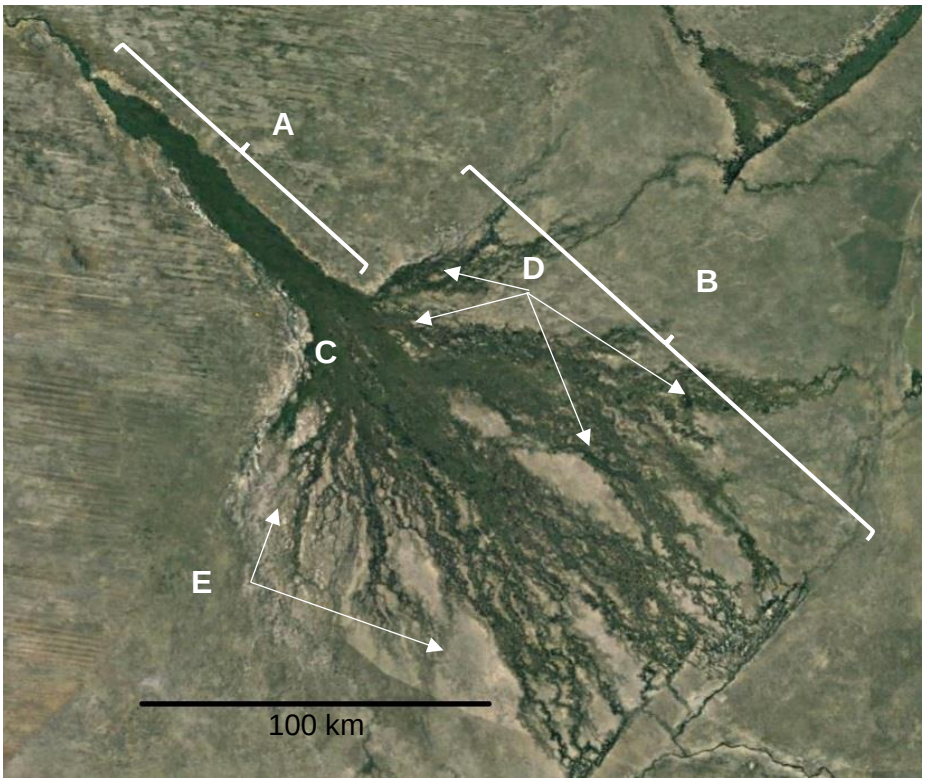


Figure 2.3: Satellite image of the Okavango Delta System consisting of **A** – Panhandle region; **B** – Delta proper; **C** – Permanent swamp; **D** - Seasonal swamp; **E** – Occasional swamp (google.com/earth/index.html, accessed on 14 June 2017 and adapted from Gumbricht *et al*. (2004)).

The Okavango Delta can also commonly be divided into four major physiographic regions (Table 2.1 and Fig. 2.3); a confined entry channel, the panhandle (Fig. 2.3**A**), the permanent swamp (Fig. 2.3**C**), the seasonal swamp (Fig. 2.3**D**) and the sand- dominated lower parts that are occasionally flooded (Fig. 2.3**E**). Even though each of these regions consists of wetter and drier areas, none of the regions are uniform in character. The wetland is surrounded by, and contains, large islands dominated by dry woodland and savannah (Gumbricht *et al*. 2004).

Table 2.1: Physiographic regions with typical wet ecoregions of the Okavango Delta (adapted from Gumbricht *et al*. (2004)).

Physiographic region	Typical wet regions
Panhandle	Permanent swamp, primarily floodplain
Permanent swamp	Permanent swamp, primarily floodplain
Seasonal swamp	Primary floodplain around the channels
Occasional swamps	Occasionally flooded grassland

During a study by Ramberg *et al*. (2006) on the biodiversity of the Okavango Delta (in an area of 28 000km^2) 1 300 species of plants, 71 species of fish, 33 species of amphibians, 64 species of reptiles, 444 species of birds and 122 species of mammals were identified. Using Rosenzweig's (1995) log/log relationship (species-area curve) Ramberg *et al*. (2006) crudely compared the biodiversity between the Okavango Delta and other countries in southern Africa. Table 2.2 shows this comparison for the six biological groups of the Okavango Delta, Botswana, Angola, Namibia and South Africa. Even though the 329 species km^{-2} in the delta is slightly higher than that of Botswana (Table 2.2), which can be expected as Botswana itself is largely an arid country, the species diversity is still relatively small compared to South Africa and the other bordering countries (Ramberg *et al.* 2006). What makes this species density so impressive is the relatively small geographical area it is concentrated in and the unique seasonal changes that occur as the floodwater slowly treacles into the delta and then dissipates again at a later stage.

Ramberg *et al*. (2006) concluded that there are no confirmed endemic species in the Okavango Delta, which might be due to a combination of geographical features and

climate variability, whereas the Okavango-Zambesi Wetland Complex has a fair number of endemic species. This highlights the uniqueness of the habitat density (not the number of habitats, but rather the fact that species have evolved to use more than one habitat) and the biological productivity caused by the historical annual flooding patterns and the mechanical flood-pulse of the Okavango Delta.

Table 2.2: Number of species in the different biological groups compared to other southern African countries (table adapted from Ramberg *et al*. (2006)).

	Okavango Delta	**Botswana**	**Angola**	**Namibia**	**South Africa**
Area 10^3 km^2	25	600	1 247	824	1 221
Plant density per km^2	210	182	400	727	1 629
Fish	71	81	268	97	220
Amphibians	33	36	80	32	95
Reptiles	64	143	150	140	301
Birds	444	569	872	640	774
Mammals	122	154	276	154	247
Total	2 034	2 983	6 646	4 222	21 397
Total species density per 1 km^2	329	285	351	346	1 761

Okavango Panhandle

The panhandle region (Fig. 2.3**A**) of the Okavango Delta lies at the north-western corner of Botswana and flows into the alluvial fan of the delta proper after encountering the Gumare fault. This broad, linear floodplain is characterised by a labyrinth of permanent deep, fast-flowing channels along the edge, lagoons, smaller annual floodplains and temporary swamps, interspersed with islands. McCarthy (2006) highlights that the islands (although fewer in the panhandle than in the delta proper) are formed mainly by termite mounds, but also many other processes, like aggradation.

Nxamasere Floodplains

The Nxamasere Valley (Fig. 2.4**A**) starts in the Kalahari dune fields in northeast Namibia and spreads to the east for 150km before joining the panhandle region of the Okavango River. It is one of many transient drainage lines that rises near the Botswana-Namibia border and flow towards the Okavango Delta (Nash *et al.* 1997). It has a semi-arid climate, which is broken by a wet season during the annual rainfalls in November, then again with the arrival of the floodwater between February and March. Judging from the level and locality of the floodwater in Fig. 2.4**A**; the satellite photo might have been taken either at the beginning of the inflow season during February or March, or when the water recedes during July or August. In Figs. 2.4**B** and **C** taken from the air flying over the Nxamasere floodplain during August of 2010, the floodwater was busy drying up and forming isolated pools. According to Sawula and Martins (1991) the mean annual temperature is 26°C with an average rainfall around 490mm $year^{-1}$ and a potential evapotranspiration value of about 1 860mm/$year^{-1}$ (Wilson & Dincer 1976).

West (2016) found that both the Nxamasere and Ngaringi Plains and the Thamalakane River, that are seasonally inundated with floodwater, constantly have water. The amount of water varies yearly and is dependent on the flooding. These frequently inundated floodplains tend to have the highest species diversity in zooplankton (West 2016), which lays the basis for a diverse and intricate food web in this seasonal ecosystem.

A unique fact of the Nxamasere Floodplain is that it is the only region in the delta where slight eutrophic conditions appear, due to the standing pools being isolated when the floodwater starts evaporating or receding. The algae in these isolated pools produce more oxygen in the water than in other parts of the delta where the aquatic plants release their oxygen above the water (West *et al.* 2015). These pools are also quite shallow, which not only produce a larger surface area for gaseous exchange to take place, but also serve as a perfect environment for lots of aquatic species that spawn in nests close to the surface, such as the African pike, or along the marginal shallow vegetation, such as the bufonid toads.

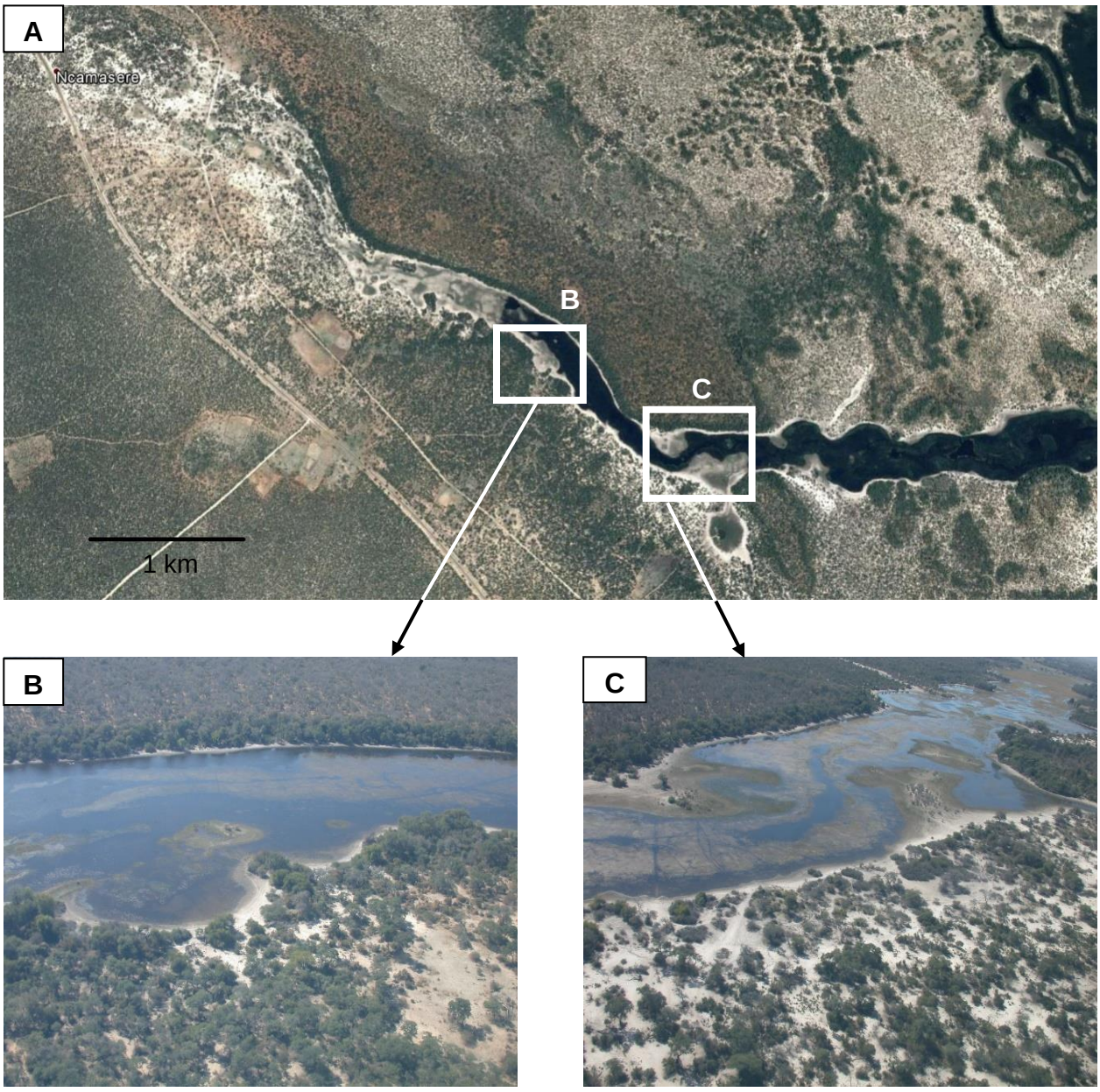


Figure 2.4: A - Satellite image of the Nxamasere Floodplains (google.com/earth/index.html, accessed and adapted on 14 June 2017) with **B** & **C** - Aerial close-up photos from two sections of the Nxamasere Floodplain with receding flood, August 2010 (photos courtesy of the Aquatic Ecology Research Group of the Zoology and Entomology Department, University of the Free State).

General Hydrobiology of the Nxamasere Floodplain

Both Mendelsohn & el Obeid (2004), and West *et al*. (2015) stated that the water flowing into and out of the Okavango Delta is extremely clear (very little turbidity during most of the year), and attribute this to the fact that the inflow system and catchment area, the Okavango River and the Okavango Panhandle meander over the Kalahari sands, which is an ancient desert, and has no to very little minerals, silt or clay in its composition (Mendelsohn & el Obeid 2004). These authors also mentioned that most of the nutrients coming down with the annual flooding are filtered out by the aquatic flora. West *et al*. (2015) also attributes the low levels of suspended chemicals in the water column, such as nitrogen and phosphorous, to the absence of geologically recent rock weathering, as there are almost no rock formations in the Okavango Delta.

The clarity of the water is of utmost importance for the sustainability of the food webs, as most of the fish species indigenous to the Okavango Delta have evolved into highly specialist feeding types, most needing an environment with high visibility and little turbidity to hunt, reproduce and survive. This is one of the most important reasons why the introduction of bottom grubbing omnivores that feed on animal and plant detritus in the bottom sediments, such as *Cyprinus carpio* Linnaeus, 1758, *Carassius auratus* (Linnaeus, 1758) or *Tinca tinca* (Linnaeus, 1758) introduced into South Africa, should never be introduced into the complex and fragile structure of the Okavango Delta.

During a recent survey of the Okavango Panhandle on the quality of the surface water by West *et al*. (2015), the following data of Nxamasere was obtained over three years between the months of July and December (Table 2.3): electroconductivity appears to increase from the Okavango Panhandle mainstream towards the fossil beds of the Nxamasere Floodplain, where the highest conductivity was observed. The same tendency was also observed for total dissolved solids. It seems that during July the highest dissolved oxygen concentrations were obtained, again with Nxamasere having the highest concentration, but during October and November the dissolved oxygen concentration at the Nxamasere Floodplain seemed to decrease, roughly having the same concentration as the adjoining regions.

Table 2.3: Physical and chemical parameters as observed by West *et* al. (2015) for the Panhandle region, Nxamasere Floodplain along with the target water quality range for aquatic ecosystems according to the South African water quality guidelines (DWAF, 1996a, 1996b) (* = arithmetic mean) (^{W} = Winter season) (^{S} = Summer season).

Physical/Chemical Parameter	Target water quality Range	Panhandle	Nxamasere Flood Plain during flooding
pH		6.09 – 7.38	7.46 – 9.21
Electric conductivity (mS m^{-1})	N/A	2.7 – 5.9	10.88 – 28.3
Total dissolved solids (mg l^{-1})	N/A	19 - 50	102 - 198
Dissolved Oxygen (%)	80 - 120	71.4* ^{W}	71.4* ^{W}
Temperature (°C)	N/A	17.13*^{W} - 29.23*^{S}	17.13*^{W} - 29.23*^{S}
Calcium (mg l^{-1})	0 – 32.0	1.28 – 5.06	6.77 – 22.76
Magnesium (mg l^{-1})	4.0 – 10.0	0.28 – 1.03	1.50 – 4.11
Sodium (mg l^{-1})	N/A	2.69 – 9.73	15.44*
Chloride (mg l^{-1})	N/A	0.59 – 3.26	4.74*^{W}
Potassium (mg l^{-1})	N/A	1.01 – 2.66	4.65 – 10.82
Bromide (mg l^{-1})	N/A	Below detection limit	
Nitrite (mg N l^{-1})	N/A	Below detection limit	
Phosphates (mg l^{-1})	N/A	Below detection limit	
Sulphate (mg l^{-1})	Less than 10.0	Less than 2.0	

For all the chemical elements measured in the waters, those found in the Nxamasere Floodplain were again the most elevated, and this may be due to high evaporation rates. When the floodplain is closed off from the broader floodplain, elements like magnesium, sodium and potassium are more concentrated. The elements that are determinant of eutrophication, such as phosphates and the major inorganic nitrogen components, are extremely low or absent in the delta, which means that these regions have extremely low eutrophication levels. As stated in the previous section (**Nxamasere Floodplains**), there is a level of eutrophication present at Nxamasere, but to the extent that it has a positive contribution to the water quality, rather than the over eutrophication that occurs in so many other isolated waterbodies outside the Okavango Delta.

SOUTHERN AFRICAN HOSTS

TELEOST HOSTS

During a 2001 assessment, Skelton (2001) identified 280 species from 105 genera and 39 families of fishes in southern Africa, of which 64% are primarily freshwater and 22% are secondary freshwater species. The remaining species (14%) are peripheral and sporadic marine species found in the lower reaches of rivers and coastal lakes.

Skelton (2001) observed that southern African freshwaters are home to 15 indigenous fish families (of these more than half are endemic), of which the Cyprinidae is by far the largest family with 88 species (40% of the total fish species) of all the primary and secondary southern African freshwater families, followed by the Cichlidae with 37 species (17% of the total fish species). There are also 24 alien fish species that were introduced to some of the southern African freshwater systems.

Skelton (2001) further stated that southern African freshwater fish species can be divided into two biogeographical groups; namely the tropical Zambezian fauna and the temperate fauna (Fig. 2.5). He also noted a general pattern in their distribution, it seems that species diversity declines from the large Zambezian fauna group in the north towards the south, with the southern fauna comprising relatively small number of species (only 36 in total), which are all entirely endemic.

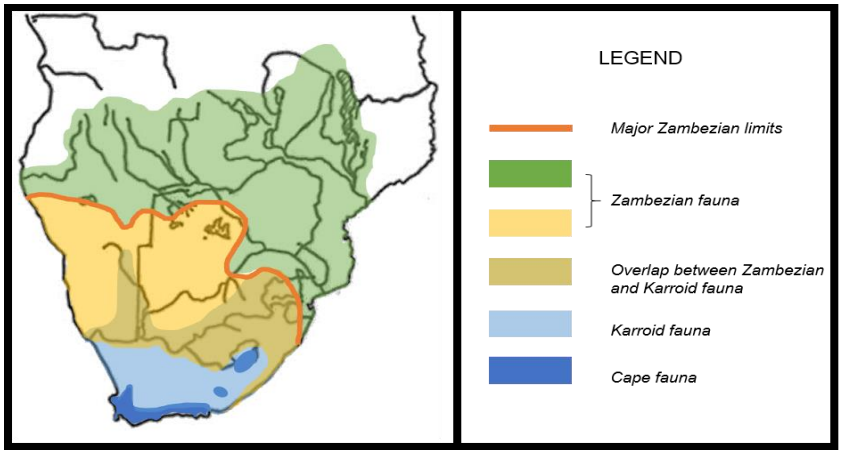


Figure 2.5: Freshwater fish fauna distribution and biogeographical zones of southern Africa (redrawn and adapted from Skelton 2001).

Fishes of the Okavango Delta

In the Botswana Okavango Delta System, there are representatives of 15 different freshwater fish families, distributed throughout the riverine floodplains, the permanent and seasonal swamps. Like the rest of southern Africa the two largest families are that of the Cyprinidae, with five genera and 25 species, and the Cichlidae, with seven genera and 18 species (Skelton *et al.* 1985) (Table 2.4).

Table 2.4: Total number of fish families, genera and species found within the Okavango System (from the Angolan Highlands through to the outflows of the Delta Proper) and the Okavango Delta (below the Popa Rapids in Northern Botswana) (Table adapted from Ramberg *et al*. (2006)).

Family	Genera	Species in the Okavango System	Species in the Okavango Delta
Mormyridae	5	6	6
Kneriidae	2	2	0
Cyprinidae	5	25	17
Distichodontidae	2	3	3
Characidae	4	4	4
Hepsetidae	1	1	1
Claroteidae	1	1	1
Amphiliidae	2	3	2
Schilbeidae	1	1	1
Clariidae	2	7	6
Mochokidae	2	8	6
Poeciliidae	1	3	3
Cichlidae	7	18	18
Anabantidae	2	2	2
Mastacembelidae	1	2	1
Total	**38**	**86**	**71**

The Okavango Delta has not yet been subjected to any introduced or translocated fish species (van As & Basson 1992), which makes it an ideal environment for parasitological studies, as none of the parasites within the system could have been introduced by host species and in most cases it can be inferred that these parasites are indigenous to the system.

Fish Parasites of the Okavango Delta

Although many studies on the aquatic life in the Okavango Delta have been done in the past, until the late 1990's almost no research was done on the parasites of fish and amphibians of this region (Basson & Van As 2002). Since then the Aquatic Parasitology Group from the department of Zoology and Entomology, University of the Free State, has done extensive research and produced some publications on the fish parasite diversity in the Okavango Delta and surrounding regions. This research is still ongoing.

Pseudocrenilabrus philander (Weber, 1887)

Although this study focusses on trichodinids from anuran hosts, a single teleost species, *Pseudocrenilabrus philander* (Weber, 1887) was collected from the Nxamasere floodplain sharing some of the ponds with the tadpoles. *Trichodina heterodentata* was found on these teleost specimens, then examined and analysed for morphological comparison with the trichodinids found on the tadpoles.

The southern mouthbrooder, *P. philander* is the only one of three species in the genus *Pseudocrenilabrus* Fowler, 1934 that occurs in southern Africa. It is a representative of the highly diverse and successful Cichlidae family which has a wide distribution in southern Africa; the Zambezi, Limpopo, upper Zaire and Orange River basins, also in the river systems of KwaZulu-Natal, southern Mozambique, the sinkholes of Namibia and the swamps of the Okavango Delta (Holden & Bruton 1994). Skelton (2001) also notes that these fish are found in the southern Congo tributaries and Lake Malawi.

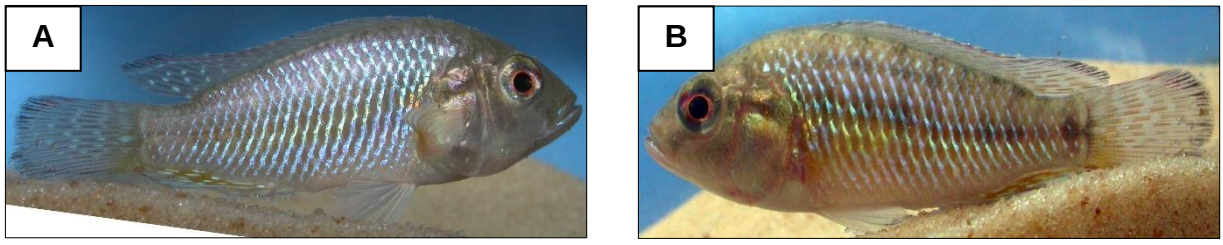


Figure 2.6: **A** and **B** – Lateral views of *Pseudocrenilabrus philander* (Weber, 1897) adult males to highlight the differences in colouration (photos taken by Kit Williams, courtesy of Prof. L. Basson).

Pseudocrenilabrus philander is a medium sized cichlid, reaching a total length of 130mm, whose colouration differs with sex, locality and breeding season. There is sexual dimorphism as all females usually have light brown bodies with black vertical bars and yellowish fins. Male bodies (Figs. 2.6**A** & **B**) are a mesh of iridescent blue and yellow with an oblique bar through the eye and an iridescent blue lower jaw. The dorsal, anal, caudal and pectoral fins have contrasting iridescent blue or red blocks on them, while the pelvic fins are black. The male colours differ with location and gets more pronounced during the breeding season.

These fish seem to prefer the marginal, shallow vegetated regions of swampy areas with slow-flowing waters, but can be found in a wide diversity of environments, from flowing rivers and lakes to isolated sinkholes, and in the case of the Okavango Delta, in seasonal standing pools (Holden & Burton 1994; Skelton 2001). According to Loiselle (1982) *P. philander* is considered to be the most ecologically versatile of the African cichlids, because of its tolerance for wide fluctuations in pH, salinity and temperature.

Like most other African cichlids, *P. philander* is a maternal mouthbrooder, where the female scoops up batches of newly lain eggs into her mouth after the male fish fertilised them and then incubates these eggs until they hatch into free swimming fry. *Pseudocrenilabrus philander* has interesting alternative reproductive behaviour strategies in both sexes; firstly, as observed by Wickler (1963), the female scoops up non-fertilised eggs along with the fertilised eggs. She then collects spermatozoa from the male of her choices' genital opening for intrabuccal fertilisation. The second alternative behavior happens with the male fishes; according to Chan and Ribbink (1990) the male fish that are not able to successfully compete for and hold territories for mating adopts "sneaking" behaviour to fertilise the eggs of the females.

ANURAN HOSTS

Southern Africa boasts 13 of the 32 families within the order Anura (phylum Chordata; class Amphibia). These 13 families, according to Du Preez & Carruthers (2009), are divided into 33 genera, all of which share a common ancestor as indicated by DNA research. Collectively Namibia, Botswana, South Africa, Zimbabwe, Swaziland, Lesotho and southern Mozambique play hosts to 157 species of frogs.

The Family Bufonidae Gray, 1825

The family Bufonidae Gray, 1825, consists of 15 genera (Channing *et al*. 2012), all of whom, according to Passmore & Carruthers (1995), are true toads. Most of the bufonids are terrestrial toads that are resistant to desiccation. Poynton (1996) states that typical southern African toads tend to live in grasslands and fynbos relatively close to open water bodies where they lay their eggs in long strings. Only two species from the genus *Schlerophrys* Tschudi, 1838 (previously *Amietophrynus* Frost, 2006)) will be discussed, as the rest of the genera and species have no direct relevance in this dissertation.

Based on geographic origin of the bufonids, Frost *et al.* (2006) removed all the African representatives from the genus *Bufo* Laurenti, 1768 and placed them under the new genus, *Amietophrynus*. In 2016, after morphometrical and morphological analysis, the South African toad species, *Schlerophrys capensis* Tschudi, 1838 was compared with five other South African toad species. Through the use of Discriminant Function Analysis, Ohler & Dubois (2016) found that by comparing 76 specimens of South African bufonids, *S. capensis* is synonymous to the currently known *Amietophrynus rangeri* (Hewitt, 1935).

Schlerophrys, which remained a valid genus until 2006, was the only nomen amongst all 33 synonyms listed by Duellman & Trueb (1986) of the generic nomen *Bufo*, to have an African type species. For the above mentioned reason, it was decided that *Schlerophrys* can be a valid nomen for an African bufonid genus if such a taxa had to be erected. This led to the moving of 43 species of bufonids, based on morphometric and morphological differences along with geographical distribution, from the genus *Amietophrynus* to the

genus *Schlerophrys* (Ohler & Dubois 2016). Included in the newly erected genus, *Schlerophrys* are both the species of interest for the present study, namely *S. gutteralis* (Power, 1927) and *S. powerii* (Hewitt, 1935).

Members of the southern genus African *Schlerophrys* are all rough-skinned terrestrial toads (Channing *et al*. 2012) that lay vast numbers of eggs; between 10 000 and 25 000 by a single female at a time (Passmore & Carruthers 1995) (Fig. 2.7**E** & **F**). These eggs are laid in gelatinous double threads, wound around vegetation in shallow to medium depth freshwater bodies. Breeding takes place twice yearly in correspondence with the seasonal rainfalls. In southern Africa, the winter-rainfall season usually occurs from July to October, while the summer rainfall is between September and January.

Although these toads breed seasonally in southern Africa, based on personal observations, they do not seem to breed seasonally in Botswana, but wherever and whenever there is enough water. This might be due to the unique seasonal flooding and minimal variation of daily temperature changes between the winter and summer seasons of the Okavango Delta floodplains. Tadpoles of both these species are generally small and black with many iridiophores all over their bodies.

The Guttural Toad, *Schlerophrys gutterali*s, is a large (140mm in length) pale ground coloured toad with dark patches over its ventral side and a distinct vertebral line along the midline of the back (Figs. 2.7**A** & **B**) (Seymour *et al*. 2001; Du Preez & Carruthers 2009) Major distinguishing marks are two symmetrical dark patches; the first behind the eyes, and the other pair on the snout (Conradie *et al.* 2006). They are distributed through Somalia, Kenya, Tanzania, the Democratic Republic of Congo, Angola, Namibia, Botswana and South Africa. The tadpoles of *S. gutteralis* naturally occur in any permanent or semi-permanent pools, usually in the shallow water during the day, moving to the deeper patches at night.

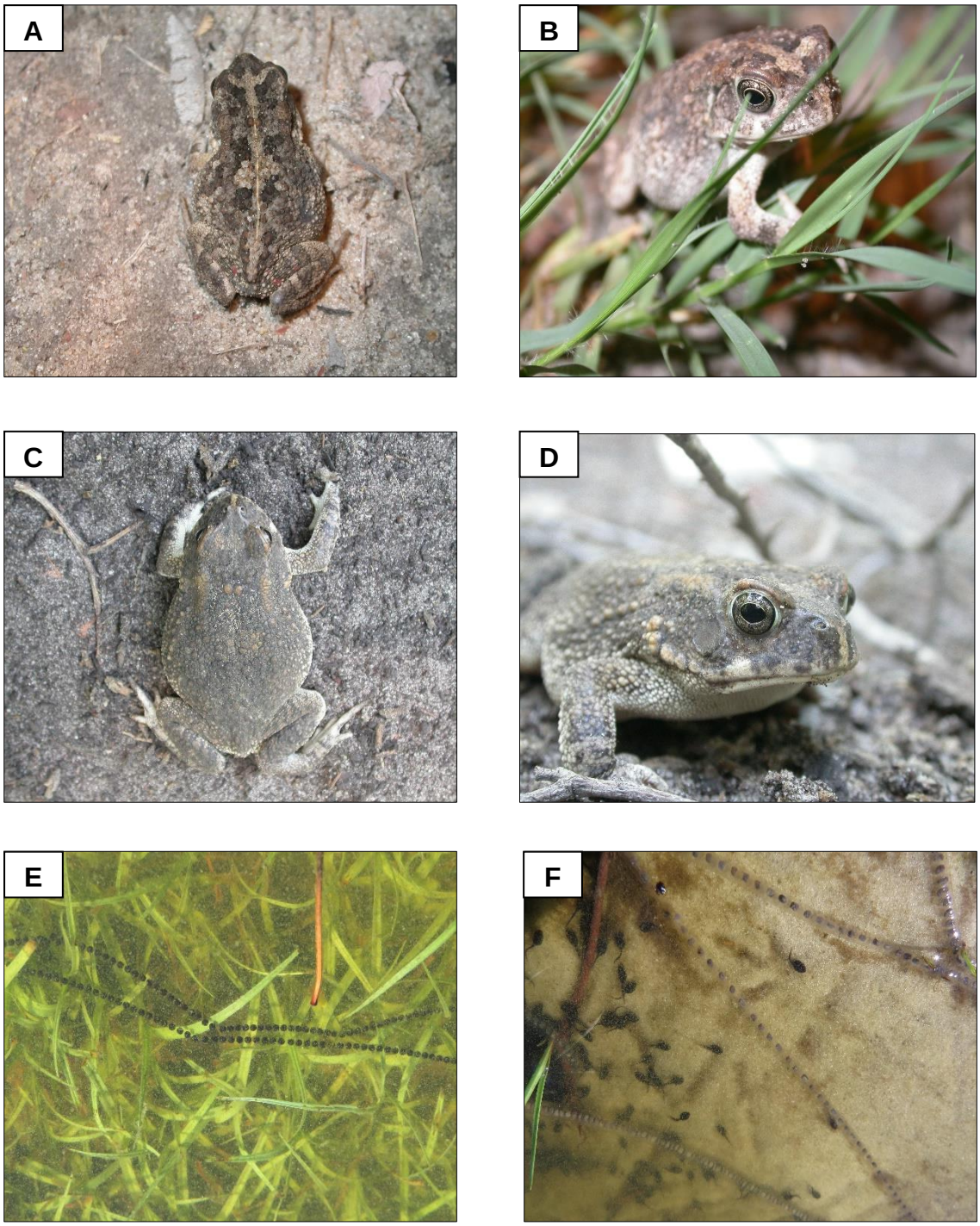


Figure 2.7: **A** – Dorsal view of *Schlerophrys gutteralis* (Power, 1927) adult; **B** – Anterior view of *Schlerophrys gutteralis* adult; **C** – Dorsal view of adult *Schlerophrys poweri*; **D** – Anterior view of adult *Schlerophrys poweri* (Hewitt, 1935)*;* **E -** Egg strings of *S. gutteralis* in the Nxamasere ponds; **F** – Egg strings and newly hatched tadpoles of *Schlerophrys* sp. (A – D photos taken by Kit Williams, courtesy of Prof. L. Basson, E & F – courtesy of Prof. L Basson.)

The tadpoles of *S. gutteralis* reach a length of 25mm with a characteristic black body dorsally, iridescent spots extend only to the ventral surface, leaving a clear throat and ventral midline (Channing *et al*. 2012). Its tail is 2.3 times its body length. *Schlerophrys gutteralis* tadpoles usually reach metamorphoses after ten weeks, when their front legs break through and their gills are completely resorbed.

The Western Olive Toad, *Schlerophrys poweri*, is a thickset and robust toad (Fig. 2.7**C** & **D**) that reaches a maximum size of 100mm. Its ventral side is easily identified by the reddish-brown patches on a yellow-brown or olive-green background. Unlike *S. gutteralis,* the patches behind the eyes are not fused together and it also has no patches on its snout (Du Preez & Carruthers 2009). This species is found in southern Angola, northern Namibia, Botswana and central South Africa (Channing *et al*. 2012).

These tadpoles reach 18mm in length with their tails being about 1.8 times the length of their bodies (Channing *et al.* 2012). *Schlerophrys poweri* prefers the peripheral edges of temporary shallow pools. Their bodies are completely black, both dorsally and ventrally. According to Channing *et al*. (2012) the whole development of *S. poweri* takes about 10 weeks from hatching to adulthood.

THE FAMILY TRICHODINIDAE RAABE, 1959

The first record of a trichodinid was already observed in 1703 by Antonie van Leeuwenhoek, while examining a *Hydra* species under his newly invented microscope. This hydra mobilid was subsequently described as *Cyclidium pediculus* by O.F. Müller in 1786 and then redescribed as *Trichodina pediculus* by Ehrenberg in 1838, also from a hydra (Raabe 1959). Hereafter *T. pediculus* has been found on various aquatic hosts, ranging from bufonid tadpoles (Zick 1926) to cyprinid fish (Kazubski 1991b), and is ironically still a valid species until today.

Having large ecological variability and being highly cosmopolitan (Antarctica being the only continent where they haven't been described…yet), more than 260 trichodinid species, representing 11 genera have been described (Basson & van As 2006; Hu 2011) Of the 11 genera, eight are associated with marine, estuarine or freshwater fish. Although the majority of trichodinids has been described from fish hosts, research has shown that they occur on and inside a wide field of hosts.

Besides teleost fishes, trichodinids have been described from amphibians (anurans, salamanders and newts), aquatic and terrestrial invertebrates (such as the genus *Pallitrichodina* van As and Basson, 1993 found on the viscous mucus of the giant land snail *Achatina* (van As & Basson 1993)), in the urinary tract of some families of chondrichthyans like the Lesser Guitarfish (van As & Basson 1996) and very recently in the reproductive tracts of waterfowl (Carnaccini *et al.* 2016).

Trichodinids are both ecto- and endosymbionts, using the tissue of their hosts as substrates to hover across or to temporarily attach to (Basson & van As 2006). As ectosymbionts they are usually found on the gills or whirling around in the mucus secretion that lines the skin, fins (of fish and tadpoles) or the moist exposed membranes in-between the toes (of young, but still fully aquatic amphibians). As endosymbionts, they are mostly found in the urinary, reproductive and digestive tracts of their vertebrate hosts.

When it pertains to host specificity, trichodinid species have varying preferences. Some species such as *Trichodinella epizootica* (Raabe, 1950) Šramek-Hušek, 1953, occurs on many different fish species and has a universal distribution (van As & Basson 1987), whereas other species can be extremely host specific, such as *T. xenopodos* Fantham, 1924 that are only found in the urinary bladder of the African clawed frog, *Xenopus laevis laevis* Duadin, 1802 (Kruger *et al*. 1991). Some trichodinids prefer certain species as hosts, but have other hosts they can infest when their preferred host is absent, an example is *T. heterodentata*, which prefers fish hosts, but seemingly readily infest tadpoles.

Trichodinids also seem to show seasonal variability with regards to occurrence/infestation and size. Studies on seasonal infestations by Kazubski and Migala (1968) found that four different trichodinid species collected from carp in Poland, displayed differences in body dimensions and number of denticles during different times of the year; *Trichodina nigra* Lom, 1960*, T. acuta* Lom, 1961*, T. pediculus* Ehrenberg, 1883 and *T. mutabilis* Kazubski and Migala*,* 1968, were found to be larger in the late autumn and early spring and had a higher number of denticles. Kazubski and Migala (1968) reasoned that a possibility for this might be due to the role temperature plays on the rate of fission.

Continuing with seasonal research, Özer (2003) found that infestation occurrences of *Trichodina domerguei* Wallengren, 1897 on *Neogobius melanostomus* (Pallas, 1814) from the Black Sea, Turkey, increase during the spring season, then decreasing again later. No studies so far have showed a statistic difference of trichodinid preference between the sexes of their host.

The history of trichodinid systematics and taxonomy have been varied and fluid since the beginning. Due to the development of taxonomic systems using morphological characteristics first introduced by Dogiel (1940), then adapted by Fauré-Fremiet (1943), standardised by Lom (1958), expanded on by Raabe (1963) and refined by van As and Basson (1989), the state of higher systematics has always been in constant flux.

With the advent of molecular taxonomy, the systematics of trichodinids are even more mutable. Recent research done by Chinese parasitologists is challenging the traditional morphological classification system, which is the main reason for the current study and will be discussed in more detail in the **History of Trichodinid Research and Descriptions** section of this chapter. For the purpose of this dissertation the taxonomic system devised Lom and de Puytorac (1994) will be used (see Table 2.5).

Table 2.5: Classification of the genus *Trichodina* Ehrenberg, 1838 as set out by Lom & de Puytorac (1994) and Adl *et al*. (2012).

	Taxon	Taxon author/s	Taxon date
Super-group	SAR	Burki *et al.*	2007
Group	Alveolata	Cavalier-Smith	1991
Phylum	Ciliophora	Doflein	1901
Class	Oligohymenophorea	De Puytorac *et al.*	1974
Subclass	Peritrichia	Stein	1859
Order	Mobilida	Kahl	1933
Family	Trichodinidae	Claus	1874
Genus	*Trichodina*	Ehrenberg	1838

GENERAL INTERNAL STRUCTURE AND ORGANELLES

Trichodinids are unicellular eukaryotic protozoan mobiline ciliophorans, but share most of the internal anatomy and physiology with the peritrichous sessile ciliophorans. Davies (1947) identified trichodinids as the most specialised of all the "parasitic" protozoans, due to their great structural complexity. All trichodinid members of the family Trichodinidae share the same basic morphology.

They all possess distinct adoral and aboral sides, where the adoral side houses the feeding/buccal apparatus and the aboral side comprises an adhesive disc surrounded by somatic ciliature. Both the adoral and aboral sides are important morphological features used in trichodinid taxonomy.

The typical mobilid internal anatomy consists of the U- or C-shaped macronucleus, centered in the middle of the trichodinid body always accompanied by the minute (and in some cases, rarely seen) micronucleus. Most of the internal body mass consists of feeding vacuoles, which are formed one by one at the cytostome at the end of the infundibulum (Fig. 2.8).

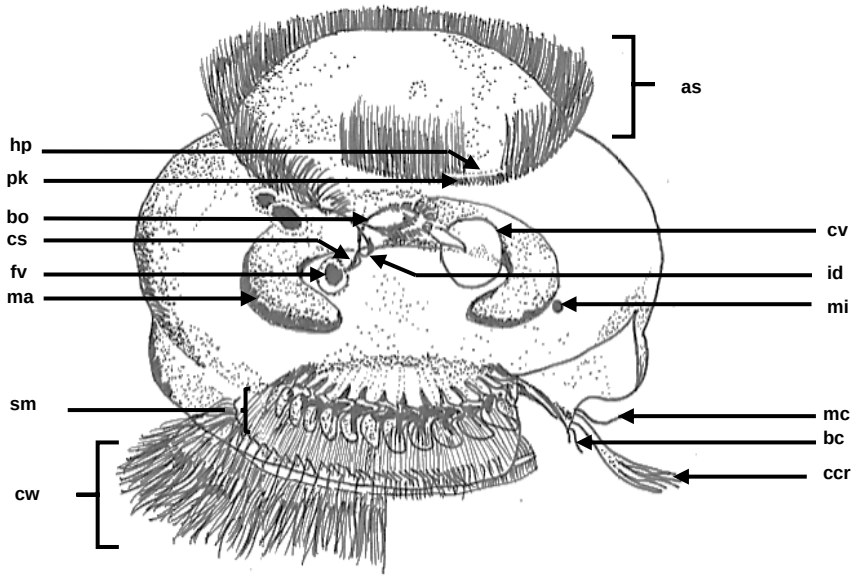


Figure 2.8: Basic internal structure based on a marine epizoic trichodinid, where; **as** – Adoral spiral; **bc** – Basal cilia; **bo** – Buccal opening; **ccr** – Compound ciliary rows; **cs** – Cytostome; **cv** – Contractile vacuole; **cw** – Compound wreath; **fv –** Feeding vacuole; **hp** – Haplokinety; **id** – Infundibulum; **ma** – Macronucleus; **mc** – Marginal cilia; **mi** – Micronucleus; **pk** - Polykinety; **sm** – Striated membrane (redrawn from Uzmann & Stickney (1954)).

Throughout the cell body there are contractile structures, consisting of protein filaments (i.e. longitudinal and circular fibers), called myonemes (Febvre 1981). Myonemes have the ability to rapidly shorten the moment it comes in contact with calcium (Shi *et al*. 2004).

Adoral morphology

According to Lom (1958) the oral side contains the cytostome aperture and oral apparatus. Two parallel rows of closely set ciliary kinetosomes make up a complex that forms the adoral spiral (Uzmann & Stickney 1954). The outside row of cilia carrying kineties is known as the polykinety, which consists of more than one row of kinetosomes, where the inside row, the haplokinety, is constructed of a single row of kinetosomes.

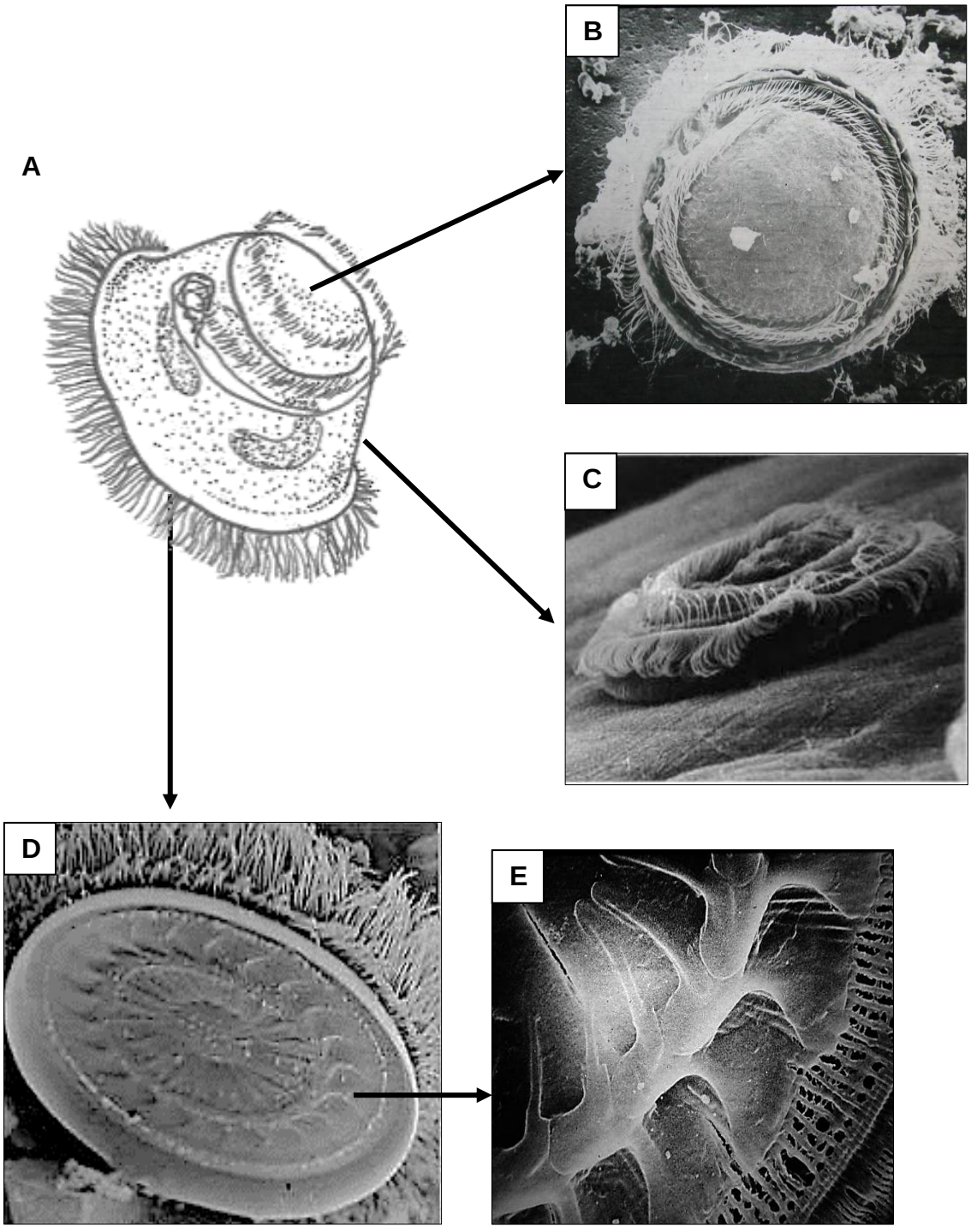


Figure 2.9: **A** – Trichodinid (redrawn from Lom, 1958); **B** – Adoral view of *T. uniforma* van As & Basson, 1989; **C** – Profile view of *T. heterodentata* Duncan, 1977 on tadpole skin, with adoral and aboral cilia prominent; **D** – aboral view of *T. heterodentata*; **E** - aboral view (detail of denticles without pellicle); (B to E micrographs courtesy of the Aquatic Parasitology Collection).

When viewed adorally, the haplo- and polykineties spirals anticlockwise up to the buccal opening (Figs. 2.9**A**, **B** & **C**).

For each of the genera there is different degree of spiraling shown by the adoral zone, for instance; in the genus with the largest number of species, *Trichodina*, this structure spirals from 360° to 540°, whereas in the genus *Vauchomia* Mueller, 1938 it completes two to three full spirals and in the genus *Semitrichodina* Kazubski, 1963 doesn't even complete half a turn. This variation in the degree of spiraling of the adoral ciliature is one of the important morphological characteristics for trichodinid taxonomy. In Table 2.6 the variations and the taxonomic importance of the degree of the adoral spiral between the genera within the family Trichodinidae are presented.

The kinetosomes of the haplo- and polykineties start diverging once they reach the buccal opening along the outer wall of an invaginated depression, spiraling into the infundibulum. At the buccal opening the haplokinety enters the infundibulum directly, while the polykinety spirals for another 180° before entering (Fig. 2.8). This out-of-sync spiraling of the two kineties lends support to the infundibulum.

Aboral morphology

The adhesive disc is a pellicle covered, concave structure with its circular outline reinforced by a skeletal complex of interlinking units known as denticles (Basson & van As 2006) (Fig. 2.9**D**). This adhesive disc consists of three rings; the denticle ring, the striated membrane, (consisting of radial pins)(Lom 1958) and the border membrane, which in turn is made up of the peripheral pins.

Each individual denticle has three separate regions (Fig. 2.10), namely the proximal ray, the central part and the distal blade (van As & Basson 1989). The shape and dimensions of the blade and ray differ greatly from species to species and between genera. The shape of the adoral spiral, as discussed previously, as well as the denticle morphology make up the two major characteristics used in morphologically differentiating the various genera.

The central part possesses a prominent, sharply tapering cone, the central conical part, that fits perfectly into the central part opening of the preceding denticle (van As & Basson 1990) (Figs. 2.10; 2.11**D** & **E**). The central conical part functions as the link connecting the denticles. Both the blade and the ray to some degree (depending on the specific species) have small protrusions (apophyses), which may limit or enable articulation of the denticles.

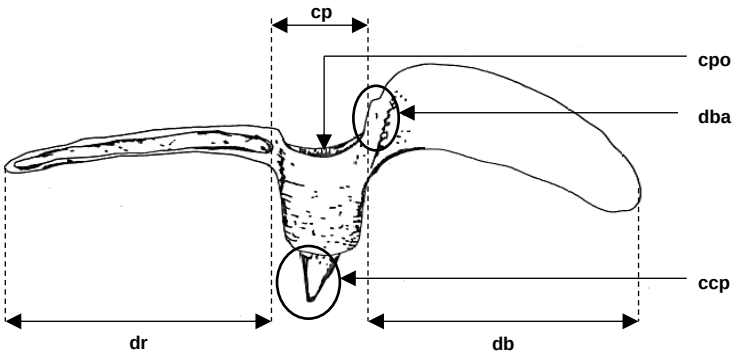


Figure 2.10: Morphology of an individual denticle with **cp** – central part; **ccp** – central conical part; **cpo** – central part opening; **db** – denticle blade; **dba** – denticle blade apophysis; **dr** – denticle ray (redrawn from Lom (1958)).

The type, shape and position of these apophyses can extend or constrict the denticle articulation. In the case of *T. heterodentata*, which is an ectosymbiont, the more prominent apophyses seem to constrict movement of the denticle ring (Figs. 2.11**A** & **B**), but in the endosymbiotic, *T. xenopodos* the smaller apophyses can increase articulated movement, producing a structure that aids the turning movement of the individual denticles. Van As and Basson (1990) compared the association of the denticles within the denticle ring to that of the vertebrae within the spinal column of vertebrates (Fig. 2.11**B**). Besides the flexible structural analogy, van As and Basson (1990) also remarked on the denticle ring providing support to the body and supplying attachment for myonemes, which, like the muscles in vertebrates, make body movements possible.

Overlapping the blade on the outer boundary of the denticles, is a circular ribbon-like structure known as the striated band (Davies 1947) (Figs. 2.9**E** & 2.11**E**). Sirgel (1983) defined the striated membrane as a series of close-fitting radial pins stretching adorally

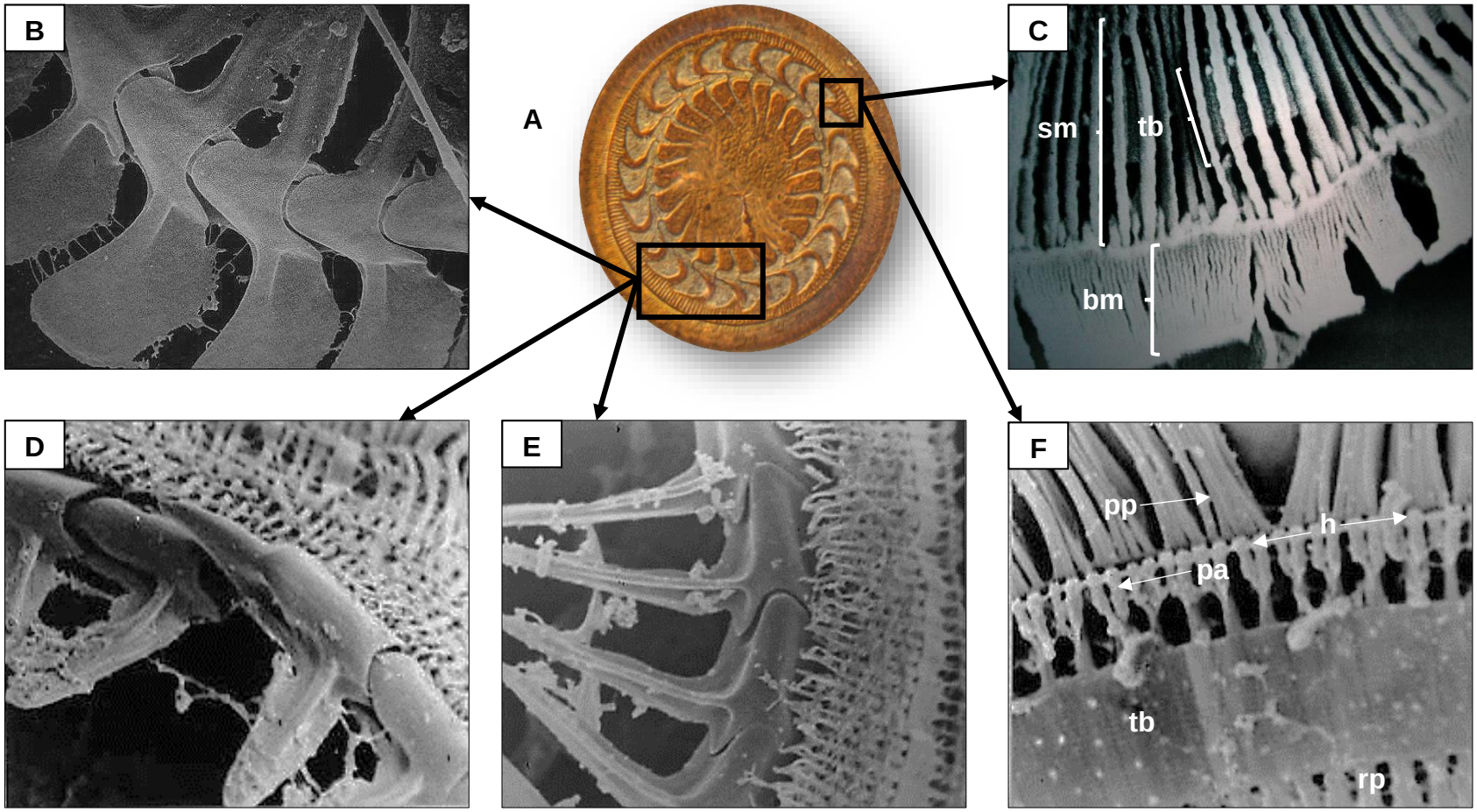


Figure 2.11: Scanning electron micrographs highlighting the different regions of the denticle apparatus from a silver nitrate impregnated trichodinid. **A** - *T. heterodentata* Duncan, 1977 (compound micrograph). **B** – Interlocking denticles from *T. dampanula* van der Bank, Basson & van As, 1990 viewed aborally; **C** – Aboral view of *T. xenopodos* Fantham, 1924 radial pins and part of border membrane with thatched band; **D** – Central conical part pulled out of the central part opening viewed adorally of *T. heterodentata*; **E –** *Trichodina heterodentata* denticles exhibiting striated band viewed adorally, **F** – Detail of attachment between border membrane and radial pins of *T. xenopodos* viewed adorally; **bm** – Border membrane; **h** – Hinge; **pa** – Pin attachment; **pp** – Peripheral pins; **rp** – Radial pins; **sm** – Striated membrane; **tb** – Thatched band (**B** to **F** scanning electron micrographs curtesy of the Aquatic Parasitology Collection).

from the denticle to the border membrane (Fig. 2.11**C**). It was later proven that the striated membrane in ectocommensals extends from the border membrane to the central part of the denticle, while in endocommensals it extends to the rays and beyond (Kruger *et al*., 1993a).

Each of these radial pins ends in small structures (in the case of *T. heterodentata* these structures are almost fork-shaped) that form hinges to which the closer spaced peripheral pins of the border membrane attach (Fig. 2.11**F**). Between the hinge and the denticles, there is a sheath-like band structure (the thatched band) on each pin that extends anteriorly and overlays several radial pins (Fig. 2.11**F**).

Trichodinid mobility

Trichodinids are the only permanently mobile group of the peritrichous ciliophorans and therefore have a locomotory organelle consisting of a compound wreath (Basson & van As 2006) of diagonally slanted ciliary rows (Figs. 2.8 & 2.9**D**). Except for the basal ring, which is separated from the rest of the circlet by a septum, the compound wreath comprises between six and ten cilia. The third ring, only present in some species, lying adorally to the second ring of cilia, is called the marginal cilia. The vast myoneme networks inside trichodinids are responsible for the movement of the cilia and denticles in the specific media that the trichodinid finds itself in, and this network also differs remarkably depending on the viscosity of the specific aqueous host medium. *Pallitrichodina* spp. myoneme networks need to be, and are, much more vast and pronounced, due to the extreme viscosity of the thick slime produced by *Achatina fulica* Bowdich, 1822 and *A. panthera* (Férussac, 1821) that the trichodinids have to effortlessly maneuver through (van As & Basson 1993).

TRICHODINID REPRODUCTION

Even though sexual reproduction through conjugation has been described in trichodinids (Davies 1947; Hirschmann & Partsch 1955), the most common means of reproduction is by asexual binary fission.

This process is initiated by the micronucleus, which starts a complex mechanism with a cascade of developments for both the nuclei (Kruger *et al*., 1995) and the organelles, especially the denticle ring. For the relevance of this dissertation it is only needed to discuss what happens to the adhesive disc after the micronucleus initiates fission.

According to Basson and van As (2006) the first evidence of division in the denticle ring is the formation of a noticeable band to the distal side of the blade, which will develop into the new denticle ring. This band is produced firstly, by a thickening of the areas on each side of the radial pins adoral to the thatched band (Fig. 2.11**F**), which then fuse together to form a platelet. During the formation of this band, the adhesive disc is separated into two semicircular structures which then closes again to form two smaller discs, one in each daughter cell.

In each daughter cell, the denticles of the newly formed adhesive disc is now reduced to half their original number, but during division, the platelets (as described above) elongates into an overlapping, interwoven mesh. Each platelet will give rise to a new denticle, with the central part developing first, followed by the blade. The number of platelets giving rise to new denticles is the same number of denticles as that of the parent individual. While the blade is still developing the ray starts to form. This denticular growth continues and simultaneously the circumference of the cell increases. The original denticle ring (from the parent individual) is then resorbed in the centre of the adhesive disc.

Together with the distal development of the new denticle blade, radial pins start extending in that direction. Before the resorption of the old denticle ring, these radial pins, although newly formed, are still extensions of the original radial pins. Shortly before complete resorption, an additional set of radial pins develops in between each of the existing radial pins. Lom (1973) suggested that the new radial pins originate from barren kinetosomes that lie between the original radial pins.

HISTORY OF TRICHODINID RESEARCH AND DESCRIPTIONS

GENERAL HISTORY OF THE GENUS *TRICHODINA* EHRENBERG, 1838

HIGHER TAXONOMY AND SYSTEMATICS

James-Clark (1866) and Wallengren (1897) penned the first attempts of an exact and sufficient description of *Trichodina* Ehrenberg, 1838 species. Up to this point, and in many cases afterwards, authors attempted to describe trichodinids, but without any standardisation, their descriptions tended to be doubtful, with many errors in their observations (Lom 1958).

Because of these inefficient descriptions combined with suspect morphological interpretations, the original family of trichodinids Urceolariidae Dujardin 1840, was a confusion of three genera; *Anhymenia* Fabre-Domergue, 1888; *Cyclocyrrha* Faber-Domergue, 1888 and *Acyclochaeta* Zick, 1926 (Lom 1958).

According to Mueller (1937), Fulton (1923) organised the family Urceolariidae into the following three genera: *Urceolaria* (with marginal cilia and an absence of blades on the denticles), *Cyclochaeta* Jackson, 1875 (with marginal cilia and blades on denticles, but adoral spiral does not complete a 360° turn of the body) and *Trichodina* (with marginal cilia, blades and the adoral spiral making a complete turn of the body). To these three genera, Mueller (1938) added the genus *Vauchomia* Mueller, 1938 that has the same traits as the latter two of the above mentioned genera, except that the adoral spiral makes two or more complete rotations around the body.

Fauré-Fremiet (1943) devised a new, more ordered system with only two genera, each with their own subgenus: *Trichodina* (subgenus *Cyclochaeta*) and *Urceolaria* (subgenus *Leiotrocha* Fabre-Domergue, 1888), discarding the genus *Vauchomia* as a synonym of *Trichodina*, in spite of the morphological differences in the adoral spiral. Uzmann and Stickney (1954) completed the Fauré-Fremiet (1943) system and reinstated *Vauchomia,*

but as a new sub-genus. The genus *Trichodinella* (Raabe, 1950) Šramek-Hušek, 1953 was added by Lom (1958) to the family Urceolariidae.

Eventually, after Lom's (1958) contributions, which helped clean up the systematics of trichodinids, the family Trichodinidae was created alongside the Urceolariidae, Leiotrochidae Johnston, 1938; Polycyclidae Poljansky, 1951 and Trichodinopsidae Kent, 1881. The new family, Trichodinidae, contained all the above genera except *Urceolaria*, which was placed within the family Urceolariidae.

A phylogenetic tree was composed by Raabe (1963), highlighting the main and best-known genera (*Trichodinella*, *Trichodina* and *Vauchomia*) together with the then newly described genera (*Dipartiella* Stein, 1961; *Tripartiella* Lom, 1959 and *Semitrichodina* Kazubski, 1963) (Fig. 2.12).

The most influential taxonomic scientists for trichodinids were Valentine Dogiel (1822-1955), Jiří Lom (1931-2010), Jo van As and Linda Basson. The contributions of these scientists will be discussed in more detail in the next section of this chapter.

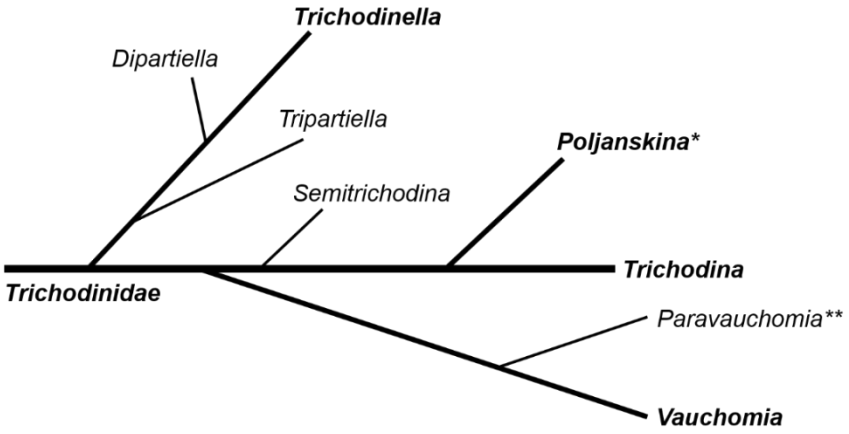


Figure 2.12: Phylogenetic tree of the family Trichodinidae Raabe, 1959 as described by Raabe (1963) based on morphological characteristics of that time. Two of these genera have been lowered to species level; **Poljanskina* Raabe, 1963 to *Trichodina oviducti* Poljansky, 1955 and ***Paravauchomia* Raabe, 1963 to *Trichodina urinaria* Dogiel, 1940. Genera in bold indicates the main lineages (redrawn from Gong *et al*. (2005)).

HISTORY OF TRICHODINID TAXONOMY AND SPECIES TAXONOMY AND DESCRIPTIONS

VALENTIN ALEXANDROVICH DOGIEL (1882 – 1955)

Figure 2.13: Professor Valentin Dogiel during his tenure as head of the Laboratory of Protozoology at the Zoological Institute of the USSR Academy of Sciences, Leningrad (photograph courtesy of the Department of Invertebrate Zoology, Faculty of Biology, St. Petersburg State University).

Valentin Dogiel (Fig. 2.13) was the first author to carefully evaluate uniform characteristics for the classification of a new species of *Trichodina* (Lom 1958). Dogiel (1940) based his findings exclusively on trichodinids found from fish, which were similar to each other. From examining the morphology of these trichodinids he constructed a list of reliable characteristics for use to correctly describe a new species. Dogiel (1940) also proposed that when the characteristic deviations were small, it was better to rather create forms from pre-existing, yet highly variable species descriptions than creating sub-species (Lom 1958).

Dogiel's (1940) 12 principle characteristics for the description of a new species within the genus *Trichodina* are as follows:

- Position of micronucleus with regards to macronucleus (Fig. 2.14**A** - **Ma**), where it is situated at terminal end of macronucleus. Position is noted as +y if micronucleus is externally to right termination of macronucleus; -y if micronucleus is left of right termination and $-y^{-1}$ if it lies internally to right termination (Fig. 2.14**A**).
- Diameter of macronucleus (Fig. 2.14**A** - **Ma**) vertical to bilateral plain.
- Length of distance between macronucleus terminations (Fig. 2.14**A** - **dt**).
- Diameter of adhesive disc (Fig. 2.14**B** – **b**), with taking note of variations when using different fixation methods.

- Number of denticles in denticle ring.
- Shape of denticles, although Dogiel (1940) found this an insufficient characteristic.
- Diameter of denticle ring (Fig. 2.14**B** – **a**).
- Number of radial pins on striated membrane between two denticles (Fig. 2.14**B** – **c**).
- Diameter of body width above adhesive disc.
- Location of contractile vacuole; central or excentric to body.
- Dogiel (1940) believed that for accuracy proportions between different measurements are important, as they are constant within the same species.
- Ratio of ray to blade of individual denticles.

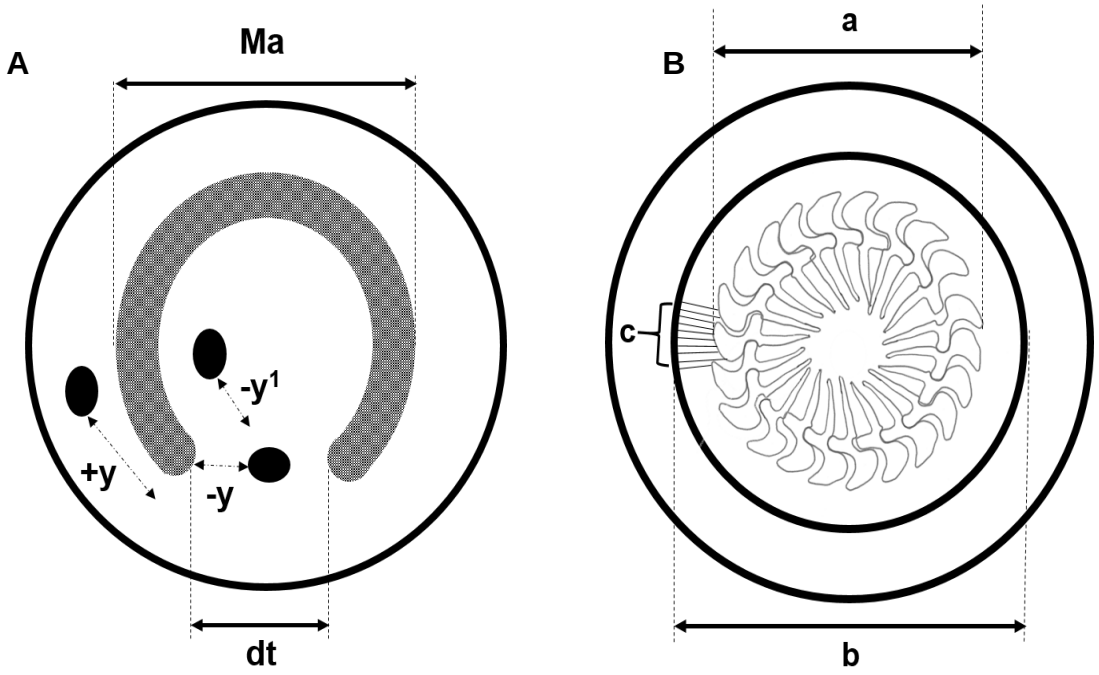


Figure 2.14: Important characteristics to be described as suggested by Dogiel (1940). **A** - Macronucleus and spatial relationship of micronucleus in regard to the right terminal end; **B** – Recommended measurements of the denticle structure by Dogiel (1940) (**Ma** – Macronucleus; **dt** - Distance between terminal ends of macronucleus; **a** – Diameter of denticle ring; **b** - Adhesive disc diameter; **c** – Number of radial pins per denticle) (redrawn and adapted from Lom (1958)).

Although Dogiel's (1940) scheme was a breakthrough for the description of new species, Lom (1958) was concerned that there was no reference to the body shape, presence of the velum, presence of marginal cilia or the adoral spiral. Lom (1958) infers that the

difference of these characteristics within trichodinids found on populations of fish hosts may have shown no or very little variation and that's why Dogiel (1940) paid no attention to these, but that these characteristics are very important when comparing species from different hosts. Dogiel (1940) used his method, especially the detailed proportions of the nucleus, primarily to distinguish between different forms in the species *T. domerguei*, but this method was later used by other Soviet authors to raise Dogiel's forms to independent species (Lom 1958).

Fauré-Fremiet (1943) used Dogiel's (1940) system, but for his own taxonomic endeavors, he hailed the number of denticles in the denticle ring as the principle characteristic to be observed for species description. Lom (1958) states that although Fauré-Fremiet took the inner and outer diameters of the denticle ring into account, he seemed to have disregarded the dimensions of the denticle ring, along with the dimensions of the individual denticles. Based on the work of Dogiel and Fauré-Fremiet, Lom constructed a unified method for a complete species description, which is still used today.

JIŘÍ LOM (1931 – 2010)

Professor Jiří Lom (Fig. 2.15) noticed that for many *Trichodina* species described, there needed to be a thorough revision. He also noted that even with Dogiel's (1940) method, the problems associated with species of *Trichodina* described from fish are only questions of systematic classification. After scrutinizing over the 12 principle morphological characteristics to unify the description of a new species proposed by Dogiel (1940) and commented on by Fauré-Fremiet (1943), Lom suggested that for any trichodinid description a very careful analysis of uniform criteria must be used for one to make the best possible comparison of individual species.

Lom (1958) utilised all the important features from the work of Dogiel (1940) and Fauré-Fremiet (1943) and insisted that a complete species description must contain information of the following 15 characteristics:

Figure 2.15: Professor Jiří Lom during his visit to South Africa (photograph courtesy of Professor L. Basson).

- Shape of body.
- Structure of adhesive disk (Fig. 2.16**A**).
- Shape of denticles.
- Dimensions of denticles (Fig. 2.16**A**).
- Number of denticles per individual trichodinid.
- Diameter of denticle ring (Fig. 2.16**A** - **drd**)
- Number of radial pins (Fig. 2.16**A** – **nrp/d**).
- Border membrane width (Fig. 2.16**A** - **bmw**).
- Velum.
- Aboral ciliary rings.
- Nuclear apparatus (Fig. 2.16**B**).
- Course of adoral zone: (Fig. 2.16**C**).
- Situation of contractile vacuole in the body (Fig. 2.16**B**),
- Host specificity.
- Other cellular organelles.

Lom (1958) stated that many previous authors gave very little attention to the shape of the trichodinids when they are in their normal physiological environment, especially their profile shape (i.e. its height.) He also noted that clearer understanding of the denticle ring, both from live and dry silver-impregnated observations must be made, especially when studying large numbers of individuals.

Lom (1958) commented on Dogiel's (1940) limited use of the shape of the denticles for taxonomic characteristics. He highlighted the importance of this trait, and insisted that Klein's (1926) "dry" silver nitrate-impregnation method must be used to describe the shape of the denticles, as it is the clearest and least complicated technique, compared to the saponin and Mallory's staining methods used by Fauré-Fremiet (1943) and the iron hæmatoxylin stain suggested by Dogiel (1940). Van As and Basson (1989) elaborated on the use of the denticle shape and dimensions as one of the most important characteristics for species descriptions of trichodinids.

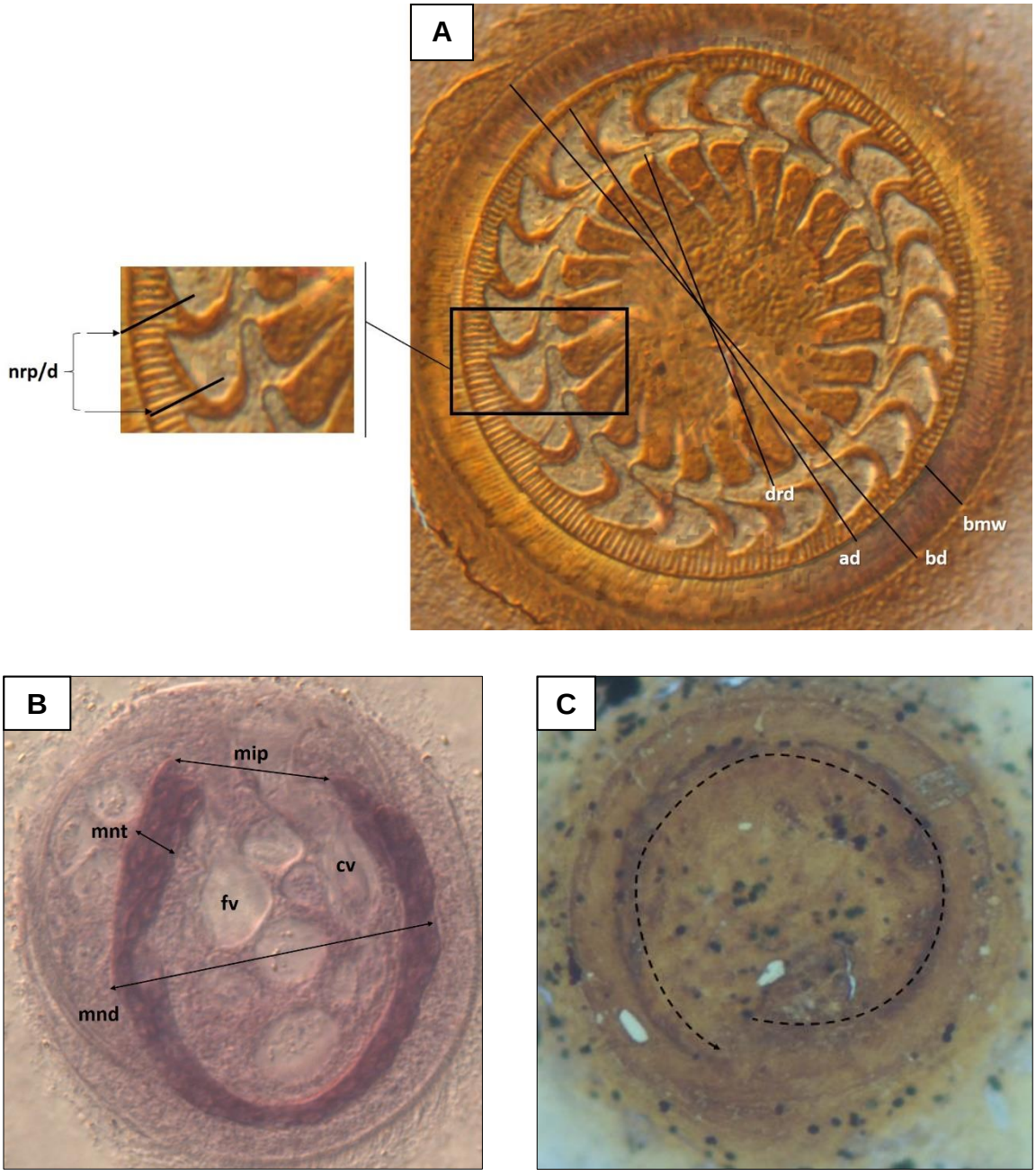


Figure 2.16: Morphological measurements of the (**A**) aboral denticle structure as standardised by Lom (1958). **bd** - Body diameter; **ad** - Adhesive disc diameter; **drd** - Denticle ring diameter; **bmw** – Border membrane width; **nrp/d** - Number of radial pins per denticle. **B** - Measurements for the nuclear apparatus (**mnd** – Macronucleus diameter; **mnt** – Macronucleus thickness; **mip** – Distance between terminal ends of macronucleus and cellular organelles (**fv** – Feeding vacuole; **cv** – Contractile vacuole). **C** – The course of the adoral zone.

One of Lom's (1958) most important, and often overlooked, characteristics is the host specificity of the trichodinids, as there are noticeable differences between the types of hosts and their trichodinids. Lom (1958) also suggested cross-infestation experiments of host types are important to find out whether some species don't show adaptive modifications under different host conditions.

Some of Lom's (1958) suggested biometric characteristics have fallen away with more defined taxonomic techniques through the years, as more trichodinids have been described. The two characteristics not used anymore, or to a lesser degree, are the pellicular border, known as the velum, which is usually absent from entozoic species, and is also unobservable when the "dry" silver-impregnation technique is used. The other characteristic is the aboral ciliary rings, which is only completely observed in live specimens and special care must be taken when focusing, and with the aid of a scanning electron microscope (SEM), both of which is extremely time consuming, and in the case of SEM, expensive.

Using these morphological characteristics, Lom and Laird (1969) devised three categories into which already described trichodinids can be placed on the validity of their systematics. Within the first group they clumped all the trichodinid species descriptions that are (thus valid) recognised and don't need to change; the W.E.S (Well Established Species) group. The second category, known as the R.U.N. (Revision Urgently Needed) group contains species that were described before Klein's (1926) "dry" silver impregnation method was used for taxonomy, but that contain some identifiable or unique characteristic. The last category they named N.D. (*nomen dubium*) where the species descriptions were so inadequate, that these could not be identified again. *Nomen dubium* should never be confused with *nomen nodum* (Ride *et al*. 1985), which has approximately the same meaning, except that in the case of the latter the taxonomic name must fail to obey certain Articles of the International Code of Zoological Nomenclature (Ride *et al*. 1985); if published before 1931 (Article 12) and after 1930 (Article 13).

VAN AS AND BASSON (1989) CONTRIBUTIONS

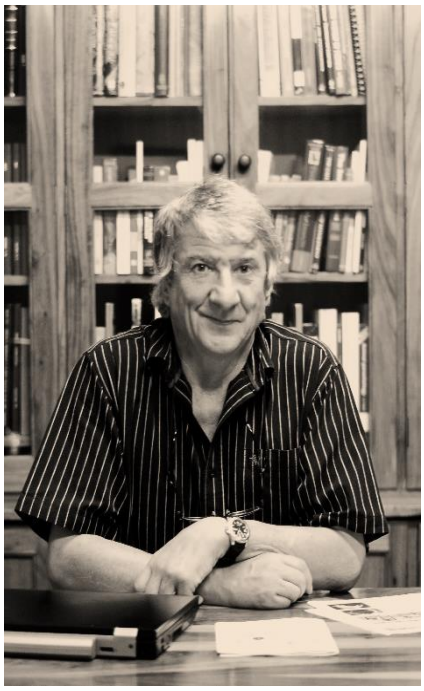


Figure 2.17: Professor Jo van As, previous Head of the Zoology and Entomology Department at the University of the Free State (photograph courtesy of Prof. L. van As).

In the late eighties trichodinid morphology was refined even more by Professors Linda Basson (Fig. 2.18) and Jo van As (Fig. 2.17). They found that the characteristics described by Lom (1958) are satisfactory for the comparison, and then description, between trichodinid species that differ substantially, but can be problematic when it comes to species that have minor morphological differences (van As & Basson 1989). They introduced additional characteristics, pertaining to the shape and form of the denticles, to add to Lom's (1958) contributions. The descriptions proposed by van As and Basson (1989) are based on silver nitrate impregnated specimens from airdried smears. Van As and Basson (1989) noted that in past descriptions too little attention was given to the shape, form and relation to each other, of the three identifiable sections of the denticle; the ray, the central part and the blade. They proposed that three consecutive denticles of individual trichodinids should be re-drawn from silver-impregnated micrographs.

Figure 2.18: Professor Linda Basson, Head of the Zoology and Entomology Department at the University of the Free State (photograph courtesy of Professor L. Basson).

A fixed point of reference should be used to relate these structures with one another. Three straight lines originating at the centre of the adhesive disc (Fig. 2.19 – **ca**), extending to the tips of three individual blades (Fig. 2.19 – **tp**) should be drawn; the line towards the middle blade, which will run horizontally between the posterior and anterior sides of the denticles will be the *y* axis, whereas the line towards the anterior side will be the *y + 1* axis and the posterior line referred to

as the *y – 1* axis (Fig. 2.19). An additional x-axis should be drawn perpendicular to the y-axis through the middle of the central area part. These axes provide lines and points of reference for the description of the shape of the denticles and their relation to each another.

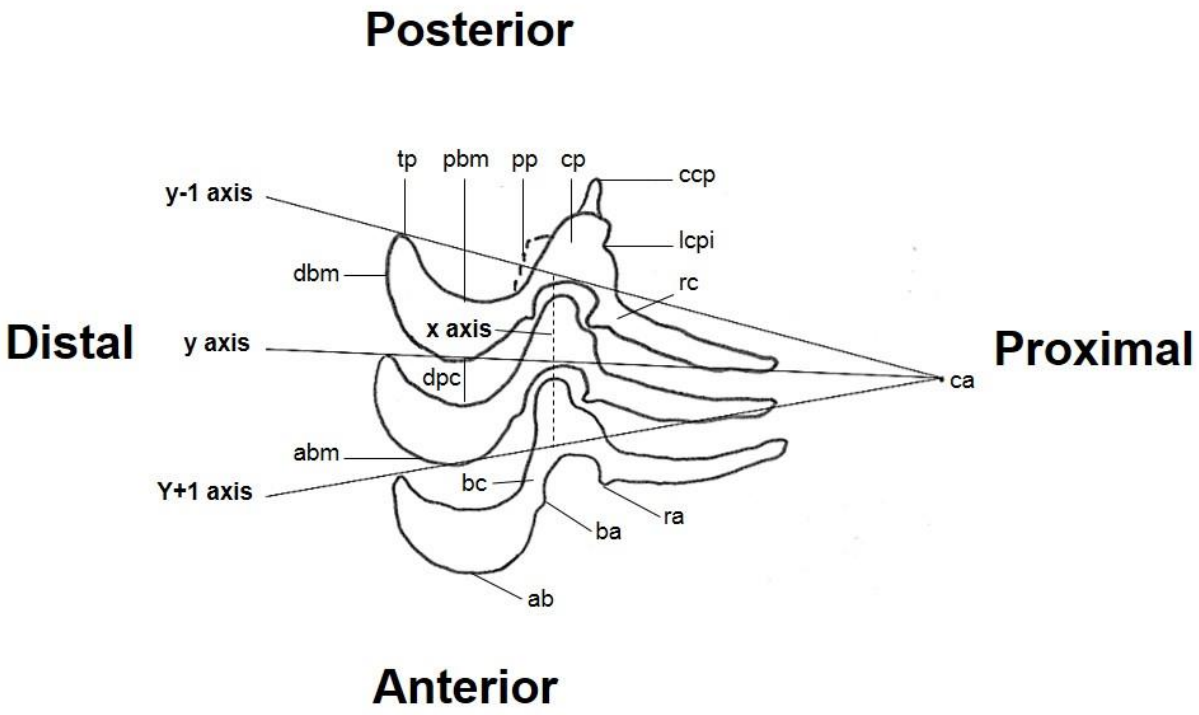


Figure 2.19: Morphological measurements of the three consecutive denticles as proposed by van As and Basson (1989); **ab** – Apex of blade; **abm** – Anterior blade margin; **ba** – Blade apophysis; **bc** – Blade connection; **ca** – Adhesive disc centre; **cp** – Central part; **ccp** – Central conical part; **dbm** – Denticle blade margin; **dpc** – Deepest point of curve relative to apex; **lcpi** – Lower central part indentation; **pbm** – Posterior blade margin; **pp** – Posterior projection; **ra** – Ray apophysis; **rc** – Ray connection; **tp** – Tangent point (redrawn from Basson & As (2002)) (not drawn to scale).

All the various regions on the denticles are indicated that must be discussed and compared for an accurate description. All the characteristics given in Fig. 2.19 must always be described in comparison to one of the axes, be it the distance from, towards or the cutting of an axis, and its direction (distal, proximal, anterior or posterior). Some structures, like the posterior projection (Fig. 2.19 – **pp**) or apophyses on the blade, central part or ray (Fig. 2.19 – **ba** & **ra**), might only be visible by scanning electron microscopy or

may completely be absent in some species. The presence or absence of these with silver nitrate impregnated structures are an important characteristic that should be mentioned.

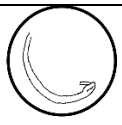
One of the advantages of this method is that absolute values are of no importance as only relative values are required. However, van As and Basson (1989) insisted that this method be used alongside that of Lom's (1958) contributions, hence accurate measurements of the denticular elements and other morphometric data are still important. To standardise the description of these morphometrical measurements, van As and Basson (1989) also proposed that all measurements in a population be given as minimum to maximum values, followed by the arithmetic mean, the standard deviation and the number of specimens measured in the population in parentheses. In the case of the number of denticles and radial pins, which are statistically discreet data, the mode, instead of the arithmetic mean must be provided. All measurements obtained for this dissertation will be given in this format, except in Table 2.10, which is a summary of all the biometrical data from the published occurrences of *T. heterodentata*, where some authors still used the arithmetic mean, instead of the mode (indicated by an asterisk (*)).

Subsequently 11 genera have been described, to date, in the family Trichodinidae by incorporating the classification systems imposed by Lom (1958) and van As and Basson (1989). The eleven genera are distinguished based on the denticle shape and the curve of the adoral zone (Table 2.6).

Table 2.6: The 11 genera of the family Trichodinidae Raabe, 1959 to date (redrawn from Basson & van As (1989), *Pallitrichodina* van As & Basson, 1993, redrawn from van As & Basson (1993) and *Heterobladetrichodina* Hu, 2011 from Hu (2011)).

Genus	Taxon Author	Locality where first described	Denticle structure	Adoral spiral
Trichodina	Ehrenberg, 1838	Germany		

Table 2.6 (cont.): The 11 genera of the family Trichodinidae Raabe, 1959 to date (redrawn from Basson & van As (1989), *Pallitrichodina* van As & Basson, 1993, redrawn from van As & Basson (1993) and *Heterobladetrichodina* Hu, 2011 from Hu (2011))

Genus	Taxon Author	Locality where first described	Denticle structure	Adoral spiral
Vauchomia	Mueller, 1938	United States of America		
Trichodinella	(Raabe, 1950) Šramek-Hušek, 1953	Poland Hungary		
Tripartiella	Lom, 1959	Czech Republic		
Dipartiella	Stein, 1961	Baltic Sea		
Paratrichodina	Lom, 1963	Czech Republic		
Semitrichodina	Kazubski, 1963	Slovakia		
Trichodoxa	Sirgel, 1983	South Africa		
Hemitrichodina	Basson & van As, 1989	South Africa		
Pallitrichodina	Van As & Basson, 1993	South Africa		
Heterobladetrichodina	Hu, 2011	China		

TRICHODINID-HOST INTERACTIONS

Most research done on the symbiotic association and relationship between trichodinids and their hosts were done on fish hosts. Basson and van As (2006) commented that trichodinids are predominantly commensals, presumably feeding on waterborne particles, bacteria or detritus particles on the surface of their hosts. In natural conditions, teleost trichodinids show an over-dispersion, thus never occurring in large numbers on all the individuals of a specific host population, which causes little or no harm to their hosts. On the other hand, when the hosts are in cultured environments, the trichodinid infestation usually increases due to environmental factors, such as water quality, over feeding or large temperature fluctuations.

These factors and the presence of other pathogens may have negative effects on their hosts. Mortalities will usually be found in fry and fingerlings of cultured populations, but in most of these cases the trichodinids are not the primary cause of death, but rather concurrently with secondary infections such as fungi, bacteria and/or viruses.

This lack of research done on the trichodinid-host association with hosts other than fish, and the trichodinid nature, makes it very difficult to categorise the specific symbiotic association. Some researchers refer to trichodinids as parasitic, while others consider them commensals, but most refer to the ciliophoran-host relationship as symbiotic, as the true association is not yet understood.

Trichodinid-amphibian/Trichodinid-anuran relationship

Not all trichodinids are host specific and within their environmental locality they can be found on different types of hosts. A prime example of this can be found in *Trichodina pediculus*. Kazubski (1965) states that *T. pediculus* primarily prefers freshwater hydras as host, but also readily occurs on fish. He further affirms that when anuran tadpoles are present within localities shared by hydras and fish, temporary populations of trichodinids are formed on the tadpoles.

In 1991 Kazubski set out to compare *T. pediculus* observed as ectosymbionts on hydras (*Hydra vulgaris*) and *Pelmathohydro oligactis* anurans (*Rana temporaria* and *Bufo bufo)* and on crucian carp (*Carassius carassius*) (Kazubski 1991a; Kazubski 1991b; Kazubski 1991c). Morphologically, Kazubski (1991c) concluded, there were slight differences between the mean values of the metric characters, but they were statistically insignificant. He noticed that the trichodinids move to fish and tadpole hosts when the hydra population is absent during certain times of the year, although they can still freely move from host type to host type when all probable hosts are present.

Lom (1961) deduced that trichodinids infesting tadpoles are not host specific and use their tadpole hosts as facultative hosts whenever their preferred fish hosts (hydra in the case of *T. pediculus*) are too few in numbers or absent. A few amphibian hosts, as fish hosts, are not restricted only to external infestations, but also to endo-trichodinid infections. The first trichodinid-amphibian interactions investigated were done mainly on endosymbiotic trichodinids.

Fulton (1923) first observed *Trichodina urinicola* in the bladders of American bufonids, various authors have described and discussed trichodinid infections from different amphibian hosts across the world (Table 2.7). Of the amphibians, the anurans are by far the most susceptible to trichodinid infection and infestations, although they have also been found in salamanders (Ibara 1931; Mueller 1938) and newts (Lom 1958).

Lom (1958) constructed his unified standardisation of trichodinid taxonomy by using his morphological descriptions to compare three forms of the same endosymbiotic species, *Trichodina urinicola* Fulton, 1923, f. *typica* Lom, 1958, *T. urinicola* Fulton, 1923, f. *bohemica* Lom, 1958 and *T. urinicola* Fulton, 1923, f. *taeniatus* Lom, 1958 and proving that these are just different morphological variations within the species *T. urinicola* from the urinary bladder of newts. Two species of anuran endotrichodinids, *T. okajimae* Ibara, 1931 and *T. urinicola* Fulton, 1923, *urinicola* Haider, 1964, fall into Lom and Laird's (1969) R.U.N. category, which mean that these species descriptions are questionable at best and need to be re-examined or placed into the *nomen dubium* category.

Table 2.7: Endosymbiotic trichodinids reported since 1923, including the locality of the host and the anuran host they infected (**Schlerophrys*) ([o] newts and salamanders) (** questionable confirmation of species).

Trichodinid species	References	Country	Anuran/Amphibian host
T. urinicola	Fulton (1923)	United States of America	*Necturus maculosus* [O]
T. xenopodos	Fantham (1924)	South Africa	*Xenopus laevis*
*T. okajimae***	Ibara (1931)	Japan	*Hynobius tokyoensis*
Vauchomia nephritica	Mueller (1938)	United States of America	*Esox masquinongy*
T. entzii	Bretschneider (1935)	France	*Rana esculenta*
Trichodina spp.	Fauré-Fremiet & Mugard (1946)	France	*R. esculenta*
T. ranae	Da Cunha (1950)	Portugal	*R. ridibunda perezi*
T. vesicola	Suzuki (1950)	Italy	*R. rugosa*
T. ranae	Capuse & Dancáu (1957)	Romania	*R. esculenta*
T. urinicola	Lom (1958)	Czech Republic	*Triturus cristatus* & *T. taeniatus* [O]
*T. urinicola urinicola***	Haider (1964)	Germany	*Triturus* species [O]
T. ranae	Kazubski (1980)	Poland	*R. esculenta*
T. ranae	Ramadan & Jobair (1950)	Saudi Arabia	*R. ridibunda*
T. dampanula	Van Der Bank *et al*. (1989)	South Africa	*Bufo** *gutteralis*

Even though the adult stages of frogs and toads do not seem to harbour any ectozoic trichodinids, except for a short time in between the webs of their toes after metamorphoses, their larval tadpole stages make an ideal location for these symbionts. Interestingly, little research was done on these ectosymbionts until the latter half of the 20th century, when interest in anuran endo-trichodinids seemed to have taken a back seat.

More research has been done in the Northern Hemisphere on the anuran-trichodinid relationship, but recently South African (Kruger *et al.* 1991; Kruger *et al*. 1993a; Kruger *et al.* 1993b; Kruger *et al*. 1995) and South American (Dias *et al*. 2009; Fernandes *et al*. 2011; Harris *et al*. 2013) parasitologists have been publishing on the relationship of both endozoic and entozoic symbionts. Table 2.8 gives a concise summary of all ecto-trichodinids described from anuran hosts from 1921 to 2013.

Table 2.8: Ectosymbiotic trichodinids reported since 1921, including the locality of the host, and the anuran host they infected (* = questionable confirmation of species, described without Lom's (1958) standardised use of silver impregnation for morphological descriptions).

***Trichodina* species**	**References**	**Country**	**Anuran/Amphibian host**
T. pediculus	Keiser (1921)	Germany	*Triton* sp. larvae
T. pediculus	Fulton (1923)	United States of America	*Necturus maculosus*
Trichodina spp.	Diller (1926)	United States of America	*Rana* spp. *Bufo* spp.
Trichodina spp.	Ariake (1929)	Japan	Unknown tadpoles
*T. pediculus**	Pai (1950)	China	*Rana* spp.
*T. bulbosa**			*Bufo* spp.
*Cyclochatea hydrae**	Suzuki (1950)	Japan	*Rana rugosa*
Trichodina sp.*			
T. pediculus	Raabe (1950)	Poland	*Rana esculenta*
T. domerguei	Raabe (1959)	Poland	*Rana terrestris* *Bufo bufo*
T. acuta *T. nigra*	Lom (1961)	Czech Republic	*Rana ridibunda* *R. esculenta* *R. temporaria* *B. bufo* *Bombina bombina* *Hyla arborea*
T. incissa	Lom (1963)	unknown	*Rana esculenta*
T. domerguei cf. *latispina**	Chen (1963)	China	*Rana* spp.
T. nobilis			
T. reticulata			*Bufo* spp.
T. nigra			
Trichodina spp.	Sandon (1965)	South Africa	*Rana grayi*
Trichodina spp.	Thurston (1970)	Uganda	*X. laevis*
*T. steini**	Kattar (1975)	Brazil	*Bufo ictericus*
*T. hypsilepis**	Arthur & Lom (1984)	Cuba	Unknown

Table 2.8 (cont.): Ectosymbiotic trichodinids reported since 1921, including the locality of the host, and the anuran host they infected (* = questionable confirmation of species, described without Lom's (1958) standardised use of silver impregnation for morphological descriptions).

***Trichodina* species**	**References**	**Country**	**Anuran/Amphibian host**
T. reticulata	Kazubski (1988)	Poland	*R. temporaria* *R. esculenta* *B. bufo*
T. pediculus	Kazubski (1991b)	Poland	*Rana nigromaculata* *B. bufo gargarizans*
T. heterodentata	Kruger *et a*l. (1993b)	Southern Africa	*Amieta fuscigula* *Kassina senegalensis* *Cacosternum boettgeri* *X. laevis*
T. heterodentata	Dias *et al*. (2009)	Brazil	*Rinella pombali*
T. heterodentata	Fernandes *et al.* (2011)	Brazil	*R. pombali*
Trichodina spp.	Harris *et al.* (2013)	Azores	*Pelophylax perezi*

Diller (1926) wrote his seminal study on the processes of trichodinid binary fission and endomixis investigating trichodinids collected from the skin and gills of various species of tadpoles, both Bufonidae and Ranidae. Some descriptions in Table 2.8 have since been regarded as suspect, due to doubtable identification or been declared as *nomen nodum*.

Both species described by Pai (1950), *T. bulbosa* and T. *pediculus* are doubtful and *T. bulbosa* from tadpoles has since been named as *nomen nodum*. *Trichodina domerguei*, as described by Chen (1963) was one of Dogiel's (1940) *formas* of *T. domerguei* (i.e. *Trichodina domerguei* f. *latispina*), which was renamed *T. domerguei* f. *acuta* and later raised to species level, namely *T. acuta*, which is a highly cosmopolitan trichodinid species. *Trichodina steinii*, described by Kattar (1975) is also doubtful, as *T. steinii* has only ever been found on fish hosts, and is another *nomen nodum*. Arthur and Lom's (1984) description of *T. hypsilepis* will be discussed in **Chapter 6**, as it may possibly be a synonym for *T. heterodentata*, as described by Wellborn (1967). According to Lom and Laird's (1969) categories there are only six pre-1988 species in Table 2.8 and *T. heterodentata* that are well established.

BACKGROUND AND REVIEW OF *TRICHODINA HETERODENTATA* DUNCAN, 1977

Trichodina heterodentata (Fig. 2.20) was first described from fish breeding farms in the Philippines by Duncan (1977) as ectoparasites from three different imported freshwater fish species; the gurami *Trichopodus trichopterus* (Pallas, 1770) and most notably from African cichlids; *Coptodon zillii* (Gervais, 1848) and *Oreochromis mossambicus* (Peters 1885). Since then *T. heterodentata* has been reported throughout the Middle East, the Far East, southern Africa, Australia and most recently from South America and Northern Botswana.

Comparing the biometrical data (in µm) of all published populations of *T. heterodentata* (Table 2.9) it is noticeable that there are variances of the denticle ring, along with the number of denticles, between and within different populations, highlighting Duncan's cunning choice of species nomenclature, *heterodentata* meaning "different teeth".

Figure 2.20: Micrograph of *Trichodina heterodentata* Duncan, 1977 from the original type material prepared by Duncan (1977) from *Oreochromis mossambicus* in the Philippines, Population A (Scalebar = 50µm) (micrographs taken at the Smithsonian Museum Invertebrate Collection, Washington, D.C., 2017).

According to Duncan's (1977) morphological measurements (Table 2.9), all three populations of *T. heterodentata* were described as a large trichodinid with a slightly convex body with a diameter ranging from 70-122µm (only the lowest and highest measurements of all three populations are given here, as no mean can be calculated without all the original material); adhesive disc diameter range between 47-81µm with a poorly to well-developed border membrane with width of 3.4-5.5µm. The

Table 2.9: Biometrical data (in µm) of all published populations of *Trichodina heterodentata* Duncan, 1977 (ADD - Adhesive disc diameter, BD - Body diameter, BMW - Border membrane width, CL – Collection locality, DBL - Denticle blade length, DCPW - Denticle central part width, DL - Denticle length, DRD - Denticle ring diameter, DRL - Denticle ray length, DS - Denticle span, HS - Host species, LoH - Location on host, n - population size, nD - Number of denticles, nRP/D - Number of radial pins per denticle) (*pertains to the mode, rather than the mean) ([a] pertains to the data as is in publication, but probably an error).

	Duncan (1977)			**Basson, van As & Paperna (1983)**	**Albaladejo & Arthur (1989)**		
CL	Philippines A	Philippines B	Philippines C	South Africa	*Taiwan A*	*Taiwan B*	*Indonesia*
LoH	Skin & Fins	Skin, Fins & Gills	Skin	Skin, Fins & Gills	Skin & Gills	Skin & Gills	Skin & Gills
HS	*Oreochromis mossambicus*	*Coptodon zillii, O. mossambicus*	*Trichopodus trichopterus*	*Pseudocrenilabrus philander*	*Hypophthalmichthys nobilis*	*Ctenopharyngodon idella*	*Cyprinus carpio*
BD	71-106 (85)	58-108 (80)	70-122 (93)	47.5-69.1 (55.3±3.8)	50.0-65.0 (57.9±3.7)	60.0-65.5 (63.7±1.7)	53.0-71.5 (57.3±3.7)
ADD	47-63 (56)	47-63 (57)	54-81 (67)	39.5-59.8 (46.9±4.1)	45.0-55.0 (48.7±3.5)	50.0-55.0 (53.3±1.6)	44.0-61.0 (52.1±5.4)
DRD	26-37 (32)	26-37 (36)	30-52 (41)	23.2-37.8 (29.3±2.9)	28.0-38.0 (32.5±2.5)	37.0-48.5 (37.1[a]±1.9)	24.5-41.2 (35.0±4.2)
DL	8	7.5-11 (9.2)	6.3	5.1-8.6 (6.6±0.8)	7.5-9.0 (8.1±0.5)	7.0-11.0 (8.8±1.0)	5.2-10.2 (8.9±1.2)
DBL	4.1	4.7-7.1 (5.7)	5.5	3.4-5.5 (4.3±0.4)	5.0-6.5 (5.6±0.5)	5.0-7.0 (5.9±0.5)	5.0-6.5 (5.6±0.5)
DRL	6.9	6.9-10.3 (8.1)	8.2	4.6-8.1 (6.3±0.9)	5.5-10.5 (7.7±1.3)	6.0-9.5 (7.3±1.2)	6.0-9.5 (8.0±0.5)
DCPW	3.4	1.4-3.4 (2.6)	4.1	1.6-3.3 (2.7±0.3)	1.0-2.0 (1.9±0.3)	2.0-3.0 (2.1±0.3)	2.2-3.5 (3.0±0.4)
DS	8	7.5-11 (9.2)	6.3	-	13.0-18.0 (15.3±1.3)	14.0-19.0 (15.4±1.5)	14.3-18.0 (16.6±1.1)
BMW	2.7	3.4-5.5 (4.7)	4.1	3.2-6.4 (4.2±0.6)	4.0-6.0 (4.9±0.4)	5.0-6.0 (5.3±0.4)	4.0-6.5 (5.3±0.6)
nD	20-27 (23)*	29-31 (26)*	18-31 (27)*	22-29 (25)*	21-25 (23.5±1.7)	22-26 (24.0±1.0)	20-24 (21.8±1.2)
nRP/D	11	6-14 (10)*	11	9-13 (10)*	12-14	11-12	11-13
n	52	25 (100 - dent)	59	51	17	10	18

Table 2.9 (cont.): Biometrical data (in µm) of all published populations of *Trichodina heterodentata* Duncan, 1977 (ADD - Adhesive disc diameter, BD - Body diameter, BMW - Border membrane width, CL – Collection locality, DBL - Denticle blade length, DCPW - Denticle central part width, DL - Denticle length, DRD - Denticle ring diameter, DRL - Denticle ray length, DS - Denticle span, HS - Host species, LoH - Location on host, n - population size, nD - Number of denticles, nRP/D - Number of radial pins per denticle) (*pertains to the mode, rather than the mean) ([a] pertains to the data as is in publication, but probably an error).

	Van As & Basson (1986)	**van As & Basson (1989)**		**Bondad-Reantoso & Arthur (1989)**	**van As & Basson (1992)**	**Kruger *et al.* (1993b)**			
	Taiwan	Israel	Venezuela	Philippines	Namibia	South Africa A	South Africa B	South Africa C	South Africa D
LoH	Skin, Fins rarely gills	Skin & Fins	Skin & Fins	Gills - rarely Skin	Skin & Fins	Skin & Gills	Skin & Gills	Skin & Gills	Skin & Gills
HS	*O. mossambicus*	*Tilapia* fry	*O. mossambicus*	*O. niloticus*	*Marcusenius macrolepidotus, Petrocephalus catostoma, P. philander*	*Xenopus laevis* tadpoles	*X. laevis* tadpoles	*X. laevis* tadpoles	*X. laevis* tadpoles
BD	62.3-95.2 (76.2±7.4)	48.1-67.3 (59.3±4.7)	52.4-78.0 (65.6±5.3)	39.2-53.0 (47.1±3.8)	45.5-52.5 (48.8±2.5)	-	-	-	-
ADD	53.0-82.6 (64.9±8.1)	39.6-56.7 (49.9±4.5)	44.3-67.0 (56.2±4.9)	32.8-45.0 (39.4±3.9)	40.0-44.0 (41.8±1.7)	39.3-58.8 (46.3±4.7)	36.6-55.6 (45.5±5.1)	41.7-56.7 (50.4±4.6)	41.1-64.3 (49.3±5.6)
DRD	31.4-51.7 (40.8±4.8)	23.9-35.6 (30.3±3.4)	26.7-43.2 (34.9±3.4)	19.2-28.4 (24.3±2.5)	23.0-25.5 (24.5±1.0)	22.2 - 35.3 (28.2±3.5)	22.3-34.7 (28.5±3.3)	25.6-35.5 (30.9±2.4)	24.4-41.2 (31.4±3.8)
DL	8.1-11.6 (9.7±1.0)	5.2-8.9 (7.5±1.1)	6.4-11.2 (8.6±1.0)	4.8-8.8 (7.0±1.0)	6.0-8.0 (7.1±0.6)	5.3-8.7 (7.3±0.9)	6.4-9.3 (7.7±0.9)	6.3-11.3 (9.2±1.1)	7.4-9.9 (8.4±0.8)
DBL	4.7-7.7 (6.1±0.7)	3.7-5.6 (4.8±0.5)	4.1-6.8 (5.5±0.6)	4.0-5.6 (4.6±0.5)	2.0-5.0 (4.1±0.9)	4.1-5.6 (4.8±0.5)	4.3-7.0 (5.4±0.7)	3.7-6.1 (4.9±0.7)	3.9-6.2 (5.1±0.6)
DRL	6.1-11.3 (9.1±1.2)	5.5-8.9 (7.4±1.0)	5.8-11.8 (8.8±1.3)	4.0-6.4 (5.6±1.2)	5.5-7.5 (6.3±0.9)	4.2-7.3 (5.9±0.9)	4.6-7.1 (6.2±0.7)	5.7-8.6 (7.1±0.8)	5.9-9.2 (7.2±0.8)
DCPW	2.7-4.9 (3.9±0.6)	1.8-3.5 (2.9±0.4)	1.8-4.7 (3.3±0.5)	1.6-2.4 (2.1±0.3)	1.5-4.0 (2.5±0.7)	1.9-3.6 (2.9±0.5)	2.6-3.5 (2.9±0.4)	2.1-3.2 (2.7±0.3)	1.8-3.3 (2.6±0.4)
DS	-	-	-	10.4-13.6 (12.6±0.9)	11.5-14.5 (12.0±0.8)	10.1-16.5 (13.6)	11.2-17.7 (14.4)	11.5-17.8 (14.6)	11.6-18.7 (14.9)
BMW	3.9-7.0 (5.4±0.7)	2.9-6.1 (4.8±0.7)	3.9-5.9 (4.9±0.5)	3.1-4.8 (2.9 [a] ±6.3)	3.0-4.5 (3.4±0.6)	1.4-5.5 (2.9)	3.9 - 6.2 (5.4)	4.0-6.3 (5.2)	3.5-6.1 (4.7)
nD	20-27 (23)*	22-25 (22)*	20-30 (24)*	21-25 (22.3±1.1)	20-22 (21)*	18-25 (20)*	19-26 (22)*	20-22 (21)*	21-25 (24)*
nRP/D	10-13 (12)*	10-12 (11)*	10-13 (11)*	10-13	11-12 (11)*	7-12 (9)*	6-10 (7)*	6-10 (10)*	7-13 (11)*
n	25	20	54	31	8	25	14	18	35

Table 2.9 (cont.): Biometrical data (in µm) of all published populations of *Trichodina heterodentata* Duncan, 1977 (ADD - Adhesive disc diameter, BD - Body diameter, BMW - Border membrane width, CL – Collection locality, DBL - Denticle blade length, DCPW - Denticle central part width, DL - Denticle length, DRD - Denticle ring diameter, DRL - Denticle ray length, DS - Denticle span, HS - Host species, LoH - Location on host, n - population size, nD - Number of denticles, nRP/D - Number of radial pins per denticle) (*pertains to the mode, rather than the mean) ([a] pertains to the data as is in publication, but probably an error).

	Basson & van As (1994)	**Al-Rasheid *et al*. (2000)**	**Dana *et al*. (2002)**			**Asmat (2004)**
CL	Taiwan	Egypt	Indonesia A	Indonesia B	Indonesia C	India
LoH	Skin, Fins & Gills	Gills	Skin	Skin	Skin	Gills
HS	*Candida barbata, C. auratus, C. carpio, H. molitrix, Sarcocheilichthys nigripinnis, Formosania lacustre, Misgurnus anguillicaudatus, Gambusia affinis, Tilapia* sp., *Rhinogobius brunneus*	*Hydrocynus forskalli*	*Oxyeleotris marmorata*	*C. carpio*	*Clarias gariepinus*	*Anabas testudineus, Puntius gelius*
BD	49.0-61.0 (53.9±3.2)	51.2-60.0 (54.6±4.1)	50.0-65.3 (60.3±5.7)	53.0-71.5 (57.9±3.7)	62.4-78.4 (68.4±4.1)	46.1-61.2 (54.6±3.3)
ADD	40.0-52.0 (44.4±3.5)	44.0-52.0 (46.2±3.2)	39.6-55.4 (50.7±5.9)	44.0-61.0 (52.1±5.4)	51.2-68.8 (58.1±4.5)	41.8-52.0 (45.6±2.8)
DRD	24.5-32.5 (27.9±2.6)	28.0-36.0 (31.6±2.7)	23.4-36.0 (32.1±4.0)	24.5-41.2 (35.0±4.21)	32.8-42.4 (36.7±2.3)	26.0-33.6 (30.4±1.7)
DL	6.0-9.0 (7.4±0.7)	8.0-10.4 (9.2±0.9)	7.2-9.0 (8.1±0.7)	5.2-10.2 (8.9±1.2)	7.2-10.0 (8.8±0.9)	7.6-9.2 (8.3±0.6)
DBL	4.0-5.5 (4.8±0.3)	5.6-7.2 (6.3±0.8)	3.2-5.4 (4.5±0.7)	5.0-6.5 (5.6±0.5)	4.4-6.4 (5.3±0.5)	4.1-7.1 (5.3±0.6)
DRL	5.5-7.0 (6.4±0.4)	6.4-8.8 (7.5±0.8)	5.0-7.7 (6.8±0.9)	6.0-9.5 (8.0±1.0)	6.4-10.0 (8.2±1.0)	5.9-8.2 (6.9±0.7)
DCPW	2.0-3.0 (2.3±0.3)	1.6-2.4 (2.2±0.3)	3.2-4.5 (3.8±0.5)	2.2-3.5 (3.0±0.4)	3.2-4.4 (3.7±0.4)	2.0-3.1 (2.8±0.4)
DS	12.0-16.0 (13.8±0.8)	12.8-16.0 (14.6±1.4)	13.5-16.7 (15.2±1.6)	14.3-18.0 (16.6±1.1)	14.4-19.2 (17.1±1.1)	13.7-17.9 (15±1.0)
BMW	4.0-5.0 (4.8[a] ±0.3)	4.0-5.0 (3.5±0.5)	3.6-5.4 (4.8±0.5)	4.0-6.5 (5.3±0.6)	3-7.2 (5.3±0.8)	3.1-5.6 (4.5±0.6)
nD	21-24 (22 & 23)*	21-24 (23)*	22-24 (22.7±1.0)	20-24 (21.8±1.2)	22-29 (24.0±1.8)	21-26 (23.1±1.2)
nRP/D	8-10 (10)*	10-13 (12)*	9-11 (9.8±0.8)	11-13	-	9-13 (10.8±1.2)
n	20	-	17	18	30	40

Table 2.9 (cont.): Biometrical data (in µm) of all published populations of *Trichodina heterodentata* Duncan, 1977 (ADD - Adhesive disc diameter, BD - Body diameter, BMW - Border membrane width, CL – Collection locality, DBL - Denticle blade length, DCPW - Denticle central part width, DL - Denticle length, DRD - Denticle ring diameter, DRL - Denticle ray length, DS - Denticle span, HS - Host species, LoH - Location on host, n - population size, nD - Number of denticles, nRP/D - Number of radial pins per denticle) (*pertains to the mode, rather than the mean) (ª pertains to the data as is in publication, but probably an error).

	Dove & O'Donoghue (2005)	**Tao *et al.* (2008)**	**Dias *et al.* (2009)**	**Martins *et al.* (2010)**	**Miranda *et al.* (2012)**	**de Pádua *et al.* (2012)**	**Öztürk & Çam (2013)**
CL	Australia	China	Brazil	Brazil	Peru	Brazil	Turkey
LoH	Skin, Fins & Gills	-	Skin	Skin & Gills	Skin, Fins & Gills	Skin, Fins & Gills	Skin, Fins & Gills
HS	*C. barbata, Glossamia aprion gilii, O. mossambicus, C. carpio, Hypseleotris compressa, H. galli, H. klunzingeri, Philypnodon grandiceps, Galaxias maculatus, G. olidus, Gerres* sp.	*C. auratus*	*Rhinella pombali*	*Ictalurus punctatus*	*Arapaima gigas*	*Piaractus mesopotamicus*	*Neogobius fluviatilis, Pomatoschistus marmoratus, Proterorhinus marmoratus*
BD	-	37.0-62.5 (49.8±3.2)	(55.1)	27.0-77.0 (59.4±8.5; 34)	47.3-76.0 (56±5.25)	45.0-58.5 (50.4±3.7)	45.0-64.0 (51.7±3.09)
ADD	41.2-89.2 (56.9)	28.0-51.0 (42.6±2.3)	38.9-60.0 (49.8±5.3)	40.0-72.0 (60.2±6.7; 42)	37.1-51.3 (45.7±3.8)	35.1-49.7 (41.2±3.3)	37.0-55.0 (43.4±2.60)
DRD	24.7-37.4 (31.2)	23.0-37.0 (28.5±2.1)	24.6-33.2 (29.2±2.1)	27.0-47.0 (38.5±4.5; 42)	20-34.7 (28.21±2.71)	19.9-30.3 (24.4±2.4)	24.0-39.0 (27.2±1.70)
DL	-	6.4-9.0 (7.4±0.8)	5.4-8.7 (6.9±0.7)	7.0-13.0 (10.3±1.2; 126)	6.31-8.6 (7.55±0.62)	6.1-9.1 (7.7±0.7)	-
DBL	4-7.4 (5.2)	4.5-5.5 (5.1±0.3)	3.0-5.2 (4.2±0.4)	4.0-8.0 (6.2±0.8; 126)	3.9-5.5 (4.46±0.59)	3.7-5.5 (4.6±0.4)	4.0-6.0 (4.8±0.22)
DRL	5.5-11.2 (7.9)	5.0-6.5 (6.1±0.7)	4.9-8.8 (6.7±0.7)	3.0-12.0 (8.5±1.7; 126)	3.9-7.8 (6.74±0.81)	5.1-9.2 (7.2±0.8)	5.0-8.0 (5.6±0.34)
DCPW	2.3-4.6 (3.0)	2.0-2.5 (2.2±0.3)	2.03-3.72 (2.7±0.3)	2.0-6.0 (3.8±0.7; 126)	1.5-3.1 (2.35±0.28)	1.2-2.7 (1.9±0.4)	1.0-3.0 (2.17±0.21)
DS	-	11.0-15.0 (13.2±1.2)	10.4-14.6 (12.5)	12.0-22.0 (18.4±2.2; 126)	7.1-15.7 (13.9±1.74)	11.6-15.5 (13.7±0.9)	11.0-17.0 (12.9±0.65)
BMW	-	3.0-5.0 (4.2±0.5)	2.9-5.1 (4.2±0.4)	3.0-7.0 (5.1±1.7; 34)	-	3.5-5.5 (4.3±0.4)	4.0-5.0 (3.3±0.25)
nD	21-26 (23)*	22-29 (25±0.8)	19-24 (22)*	23.0-28.0 (24.4±1.6; 42)	12-24 (20.7±2.6)	16-22 (20)*	20-26
nRP/D	10-13 (11)*	10-12 (11)	7-10 (7)*	5.0-15.0 (11.8±2.1; 42)	7-13 (11.8±1.6)	8-12 (10)*	7-8
n	18	25	50	different populations given after standard deviation	40	50	21

Table 2.9 (cont.): Biometrical data (in µm) of all published populations of *Trichodina heterodentata* Duncan, 1977 (ADD - Adhesive disc diameter, BD - Body diameter, BMW - Border membrane width, CL – Collection locality, DBL - Denticle blade length, DCPW - Denticle central part width, DL - Denticle length, DRD - Denticle ring diameter, DRL - Denticle ray length, DS - Denticle span, HS - Host species, LoH - Location on host, n - population size, nD - Number of denticles, nRP/D - Number of radial pins per denticle) (*pertains to the mode, rather than the mean) (ª pertains to the data as is in publication, but probably an error).

	Valladão *et al.* (2013)	**Worananthakij & Maneepitaksanti (2014)**	**Valladão *et al.* (2016)**	**Utami & Rokhmani (2016)**	**Nurrochmah & Riwidiharso (2016)**
CL	Southeast Brazil	Thailand	Brazil	Java	Java
LoH	Skin, Fins, Gills & Mouth	Skin & Gills	Skin	-	Skin
HS	*Prochilodus linaetus*	*O. niloticus x O. mossambicus*	*O. mossambicus*	-	*Osphronemus goramy*
BD	48.4-65.9 (56.9±3.6)	62.2-92.5 (76.9)	38.4-59.2 (50.5±4.1)	-	62.5-102.5 (84.3±9.4)
ADD	39.4-55.3 (47.7±3.6)	49.0-83.7 (67.1)	31.3-50.3 (42.3± 4.0)	38.0-82.0	45.6-62.5 (53.2±4.5)
DRD	23.0-37.6 (29.4±2.6)	28.7-47.5 (39.2)	12.2–32.4 (25.4 ± 3.1)	22.0-36	25-52.2 (48.5±9.4)
DL	5.8-9.3 (7.8±0.7)	7.5-10.0 (8.9)	5.6–9.7 (6.9 ± 0.6)	-	-
DBL	3.8-5.7 (4.6±0.4)	5.0-8.7 (6.9)	2.9–5.2 (4.2 ± 0.4)	-	-
DRL	6.0-9.0 (7.7±0.8)	6.2-11.2 (8.9)	(3.1–8.1) (6.1 ± 1.0)	-	-
DCPW	2.1-4.2 (3.0±0.5)	-	1.4–3.2 (2.3 ± 0.4)	-	-
DS	13.0-17.6 (15.4±1.0)	-	7.8–15.5 (12.5 ± 1.4)	-	-
BMW	2.8-5.7 (4.5±0.4)	-	3.2-5.2 (4.2 ± 4.0)	-	2.5-7.5 (4.66±1.6)
nD	20-26 (23±0.9)	24-27 (27)	11–27 (23.7 ± 2.2)	-	20-26 (24±1.6)
nRP/D	6-12 (9.8±1.2)	9-10 (10)*	7–16 (11.3 ± 1.9)	-	-
n	102	10	114	-	25

denticle ring diameter is between 26-52μm consisting of 18 to 31 denticles and 6 to 14 radial pins per denticle. The denticle blades are strong, semi-circular with a prominent apophysis on the anterior side with a length from 4.1-7.1μm, tapering off towards a sharp pointing tip. The ray of the denticle is strong, generally straight, but curved backwards near the central part connection and tapers off towards the tip, it has a length ranging from 6.9-10.3μm. The central part width is from 1.4-4.1μm and the total denticle length is between 6.3-11.0μm. Consequent descriptions from different populations across the world (Table 2.9) has found *T. heterodentata* to generally have much smaller dimensions, but this is discussed later in **Chapters 5** and **6**.

This trichodinid is not excessively host specific, while leaning toward cichlid hosts, they have been described on the fins, skin and gills of various other fish species. Similarly, they are found on the gills and on the skin of the juvenile stage (or tadpoles) of anurans, in recent descriptions from Brazil, they were also found in the mouth. To date *T. heterodentata* has only been described as ectosymbionts, and is unlikely to be found infecting their hosts internally.

The data of all published *T. heterodentata* populations reported and measured since the initial description by Duncan (1977) from the Philippines are summarised in Table 2.9. As stated in **Chapter 3** all measurements are given in μm with the arithmetic mean in parentheses, in some cases the mode was used for the number of denticles and number of radial pins per denticle, these are all annotated with an * in the table.

MOLECULAR RESEARCH

THE USE OF DNA IN MODERN TAXONOMY

According to Hillis and Dixon (1991) the field of systematic biology has undergone three significant revolutions since the early 1970's. They postulate that these are the development and refinement of systematic theory itself, the use of computers for data analysis refinement and, most recently, the use of molecular techniques. The importance of molecular systematics lies in the fact that, unlike the lack of universal homologous morphological characteristics amongst all biota, certain genes share fundamental biochemical functions, which can be found in all species (Hillis & Dixon 1991). These genes are easily extracted, aligned and sequenced to infer phylogenetic relationships within the systematics of life.

For decades geneticists have been studying ribosomal RNA (rRNA) genes and their associated spacer regions (collectively called ribosomal DNA (rDNA)) to address phylogenetic relationships from the earliest derivation of life to relatively recent evolutionary events. Ribosomes (nuclear organelles that guide protein synthesis from messenger RNA) are a combination of certain rRNAs and ribosomal proteins making out two major subunits, the small ribosomal subunit (SSU) and the large ribosomal subunit (LSU). Each of these subunits contains a specific type of rRNA and proteins. The SSU has a single type of rRNA with 30 proteins in eukaryotes, whereas the LSU has two types of rRNAs with 30 to 40 ribosomal proteins. Each ribosome has a single copy of each of these proteins, and because protein synthesis is the requirement for life, they are present in all living systems.

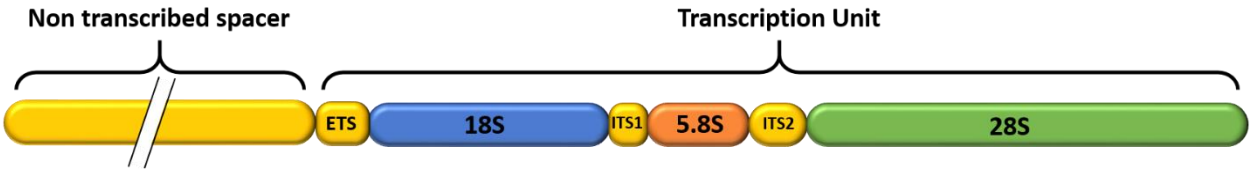


Figure 2.21: Nuclear small subunit ribosomal DNA (SSU rDNA) array of eukaryotic cells. **ETS** – external transcribed spacer; **ITS** – internal transcribed spacer (figure adapted from Hillis & Dixon (1991)).

The rDNA map (array) of the eukaryotic nuclear genome, contains hundreds of alternating repeated copies of the transcription units and the non-transcribed spacers (Fig. 2.21).

Table 2.10: Differences in nucleotide size and evolutionary lineage of the ribosomal subunits (Huysmans & de Wachter 1987; Dams *et al.* 1988; Gutell & Fox 1988; Gutell *et al.* 1990; Neefs *et al.* 1990).

Ribosomal subunit	Nucleotides	Sedimentary velocity unit	Organism and organelle
LSU	≈ 1500	16S	Vertebrate mitochondria
	≈ 2900	23S	Prokaryote genome
	>4000	28S	Eukaryote genome
SSU	≈ 900	12S	Vertebrate mitochondria
	≈ 1500	16S	Prokaryote genome
	≈ 1800	18S	Eukaryote genome

rRNAs are organised by the number of nucleotides (nt) they consist of and are characterised in sedimentation velocity units; Svedburg (S) (Hillis & Dixon 1991). Each subunit rRNA ranges in size according to where it is found in the genome and whether it is found in the nuclear genome of prokaryotes or eukaryotes or in vertebrate mitochondria (Table 2.10).

A very important, and often neglected, attribute of the repeating rDNA units is that the different regions evolve at different rates (Hillis & Dixon 1991). Ribosomal DNA regions evolved during various geological eras and periods (Fig. 2.22). It is clear that the 18S small subunit nuclear genes are amongst the slowest evolving regions, and therefore has highly conserved sequences. This is an important aspect; as “universal” primers can be constructed to sequence rRNA or rDNA from many different species. Unfortunately, because if the extremely slow rate of change of 18S rRNA, the gene is only truly successful for reconstructing phylogenetic data dating from the Precambrian. Hillis and Dixon (1991) assert that for recently evolved taxa (vertebrates, especially mammals) this gene provides almost no useful regions for comparing sequences of taxa that diverged since the Cretaceous.

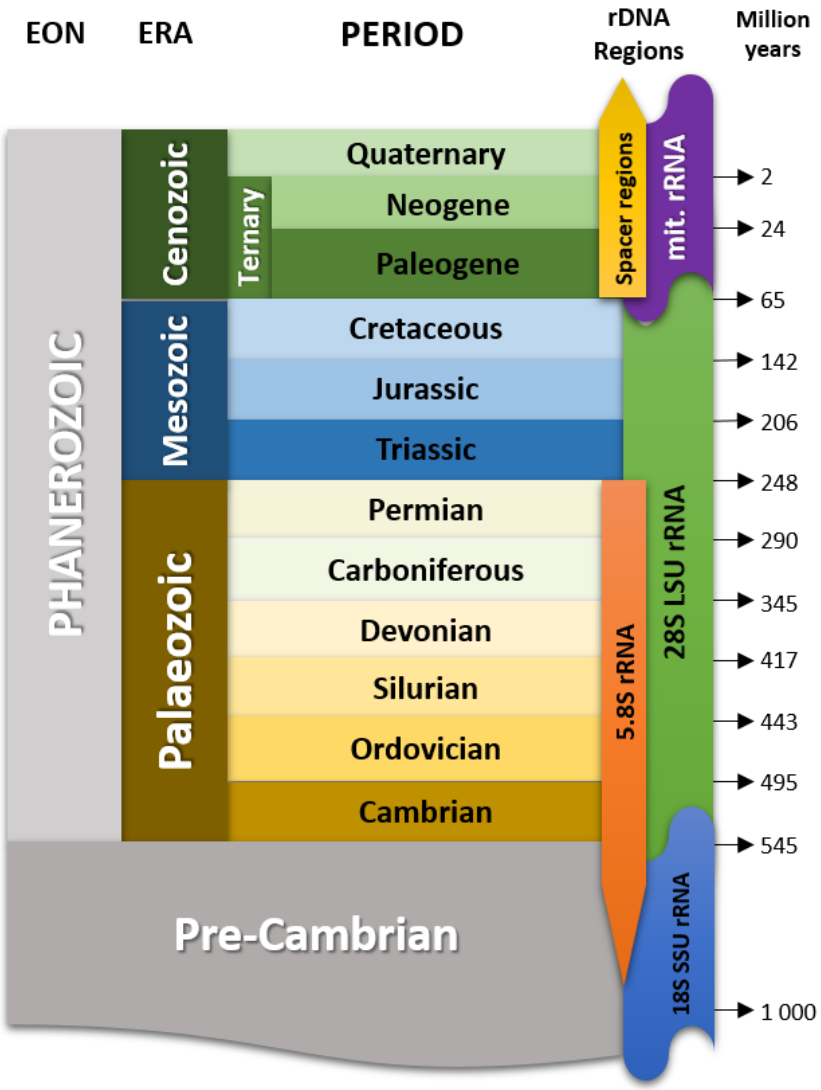


Figure 2.22: Evolutionary rate of rDNA development over time compared to the geological time scale (graphic representation adapted from data by Hillis & Dixon (1991) and McCarthy & Rubidge (2005)).

For inferring more successful phylogenetic relationships among closely related organisms, Hillis and Dixon (1991) propose using the more rapidly evolving large subunit rRNA. Unfortunately this gene has not been as extensively researched as the small subunit rRNA, but have recently gained more attention, especially in vertebrate comparisons.

Mitochondrial (and chloroplast) rDNA is used to clarify phylogenies of very closely related taxa, and have been very successful in mammals, especially inferences of the phylogeny

within marsupials, artiodactyls and primates (Hixon & Brown 1986; Miyamoto *et al*. 1989; Thomas *et al*. 1989). Although the spacer regions have been used less frequently than the other rDNA regions, recent studies have shown that these regions can be used for relatively recent (having diverged within the last 50 million years) closely related taxa. Variations within these spacer regions have also been used to study hybridisation, markers in population genetics and to distinguish between species or strains (Saghai-Maroof *et al.* 1984). Hillis and Dixon (1991) recommend using the two internal transcribed spacers (Fig. 2.21: ITS1 and ITS2) for studying closely related species.

However, Bachy *et al.* (2013) state that almost all diversity and systematic studies of protists are still based on the routine analysis of the 18S rRNA genes by means of PCR amplification, classical cloning and Sanger sequencing.

MOLECULAR WORK DONE ON TRICHODINIDS

Molecular techniques for taxonomy and systematics have only very recently reached the trichodinid research field. Except for molecular systematics research of Oligohymenophorea by Utz and Eizirik (2007), all other molecular phylogenetic inferences published for trichodinids have been done by a select research group in China.

CONTRIBUTIONS BY YING-CHUN GONG AND COLLUEAGUES

During the late 2000's, Gong *et al.* (2006) started hinting towards the possibility that peritrichs did not present a monophyletic group, due to the two orders, Sessilida and Mobilida not clustering together when comparing small subunit rRNA. Even though this theory was disputed by Utz and Eizirik (2007), this notion was quickly followed up by a rather controversial publication where Zhan *et al*. (2009) separated the mobilids from the sessilids and raised Mobilida Kahl, 1933 to subclass status. Recently Gong and company constructed a super matrix with 58 genes from 24 ciliophoran species and found that the class Oligohymenophorea is monophyletic, containing two robust clades within. One of these clades is the Peritrichia with the orders Sessilida and Mobilida maximally supported.

They also proposed that the two peritrich orders arose from the same ancestor, as the classical view, supported by morphology, has always suggested.

The first molecular work done on trichodinid taxonomy was done by Gong and Yu (2007) utilising the 18S SSU rRNA gene, based on the groundbreaking molecular research of ciliophorans by Elwood *et al.* (1985), who suggested that ciliophoran protozoans diverged from the rest of the eukaryotic line of descent at more or less the same time as the radiation of plants, animals and fungi, thus making the use of small subunit ribosomal RNA sequences excellent for inference of closely related taxa.

Gong and his fellow researchers published all their 18S sequences in the GenBank/NCBI database (all the gene sequences, their collection localities and publications, see **Chapter 7**: Table 7.3). From these gene sequences many phylogenetic inferences has been made about the systematics and taxonomy of trichodinids. This study will challenge some of these inferences, also adding more sequences to the data base, which should not only make future trichodinid phylogenetic inferences more robust, but also add new distribution data from outside China.

CHAPTER 3 - MATERIAL AND METHODS

COLLECTION OF MATERIAL

COLLECTION SITE

All material was collected in the Nxamasere Floodplain, which is found on the eastern banks of the Okavango River in the system's panhandle in northern Botswana (Fig. 3.1). Six isolated standing pools were identified for collecting within the Nxamasere Floodplain (Fig. 3.2). These pools are isolated from each other for most of the year and make ideal refuges for the surviving aquatic organisms caught in the plain after the floodwater has receded. The localities were named NX1 to NX6 (NX1 being the furthest from the Okavango river body and NX5/6 the closest).

Figure 3.1: Satellite image showing the six collection localities in the Nxamasere Floodplain (Scalebar = 900 m) (google.com/earth/index.html, accessed on 14 June 2017 and adapted).

The positions of each sampling site were recorded with a Garmin Geographical Positioning System (GPS) (Table 3.1). Due to the seasonal fluctuations in the size of the pools, general GPS coordinates are given, as these vary slightly from one collection season to the next. Collection sites 5 and 6 (NX5 & NX6) share the same coordinates, as these are two separated pools during the drier latter months of the year, and are connected during the earlier months of the year.

Table 3.1: Longitude and latitude of collection pools in the Nxamasere Floodplain for 2015 and 2016**.**

Standing pool	**LONGITUDE**	**LATITUDE**
Nxamasere 1 (NX1)	S 18° 35, 770'	E 0 22° 01, 551'
Nxamasere 2 (NX2)	S 18° 36, 007'	E 0 22° 01, 349’
Nxamasere 3 (NX3)	S 18° 35, 396'	E 0 22° 00, 766’
Nxamasere 4 (NX4)	S 18° 35, 247'	E 0 22° 00, 198'
Nxamasere 5 (NX5)	S 18° 34, 984'	E 0 22° 00, 035’
Nxamasere 6 (NX6)		

RESEARCH CAMP

During the collection and research expeditions all researchers stayed at the Leseding Research Camp (Fig. 3.3) of the Aquatic Parasitology Research Group of the University of the Free State. It was established on the grounds of the Krokavango Crocodile Farm, which is on the bank of the Samochima Lagoon, adjacent to Samochima Village. The camp includes, amongst accommodation, ablution and cooking amenities, also a fully operational and outfitted dry laboratory with microscopy and microphotographic facilities and staining equipment, an undercover outside wet laboratory for dissection, maintenance of the live collected samples and aquariums of various sizes for different experiments.

COLLECTION AND TENDING OF HOST SPECIES

All aquatic host species were collected from standing pools in the Nxamasere Floodplain (Botswana) using shrimp nets. The host tadpole species, *Schlerophrys* spp. (Figs. 3.4**A**

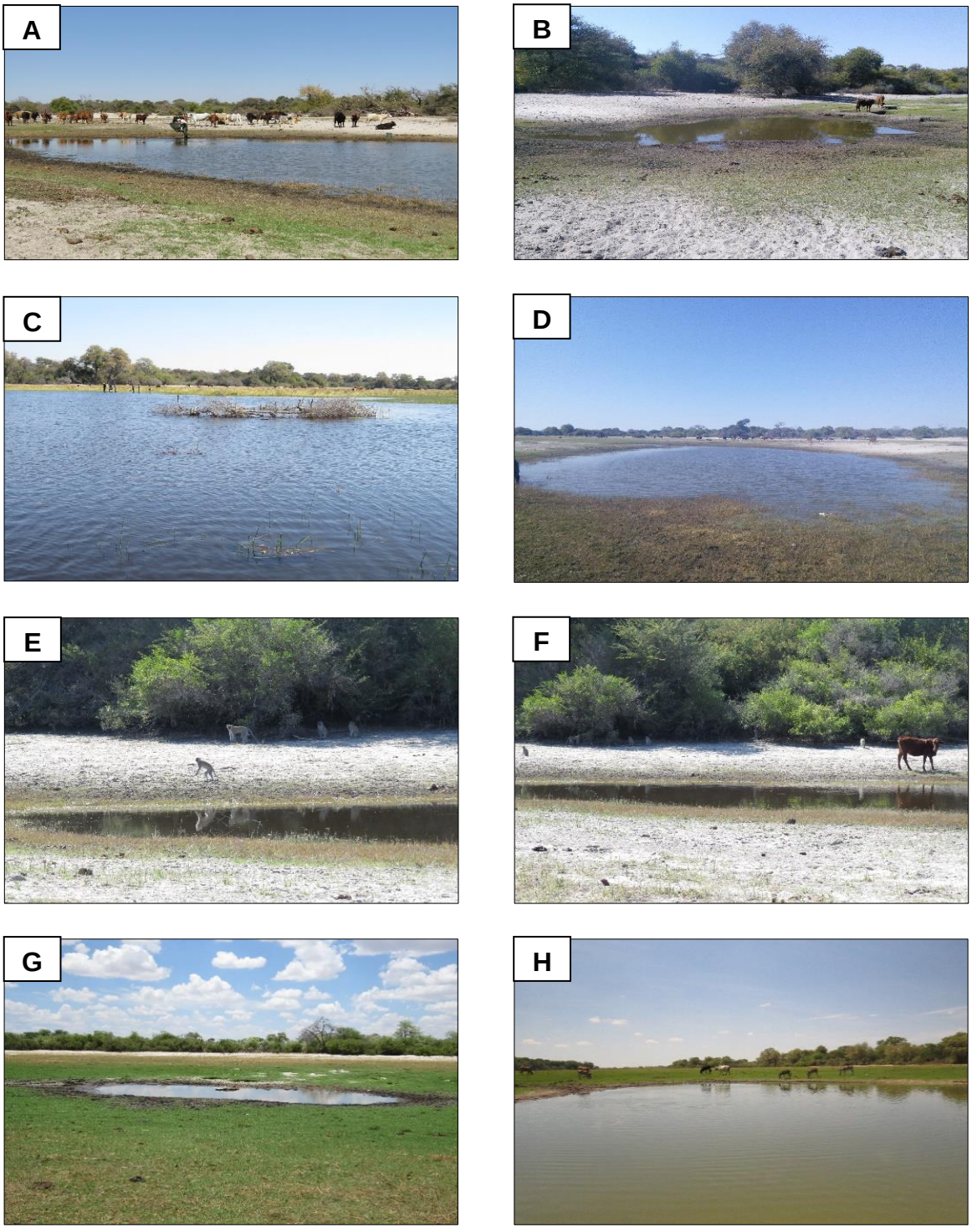


Figure 3.2: Collection localities in the Nxamasere floodplain, Botswana during winter 2016 (July/August): **A** - NX1; **B** - NX2; **C** - NX3; **D** NX4; **E** - NX5; **F** - NX6 and during summer 2016 (November/December): **G** - NX1 and **H** – NX4.

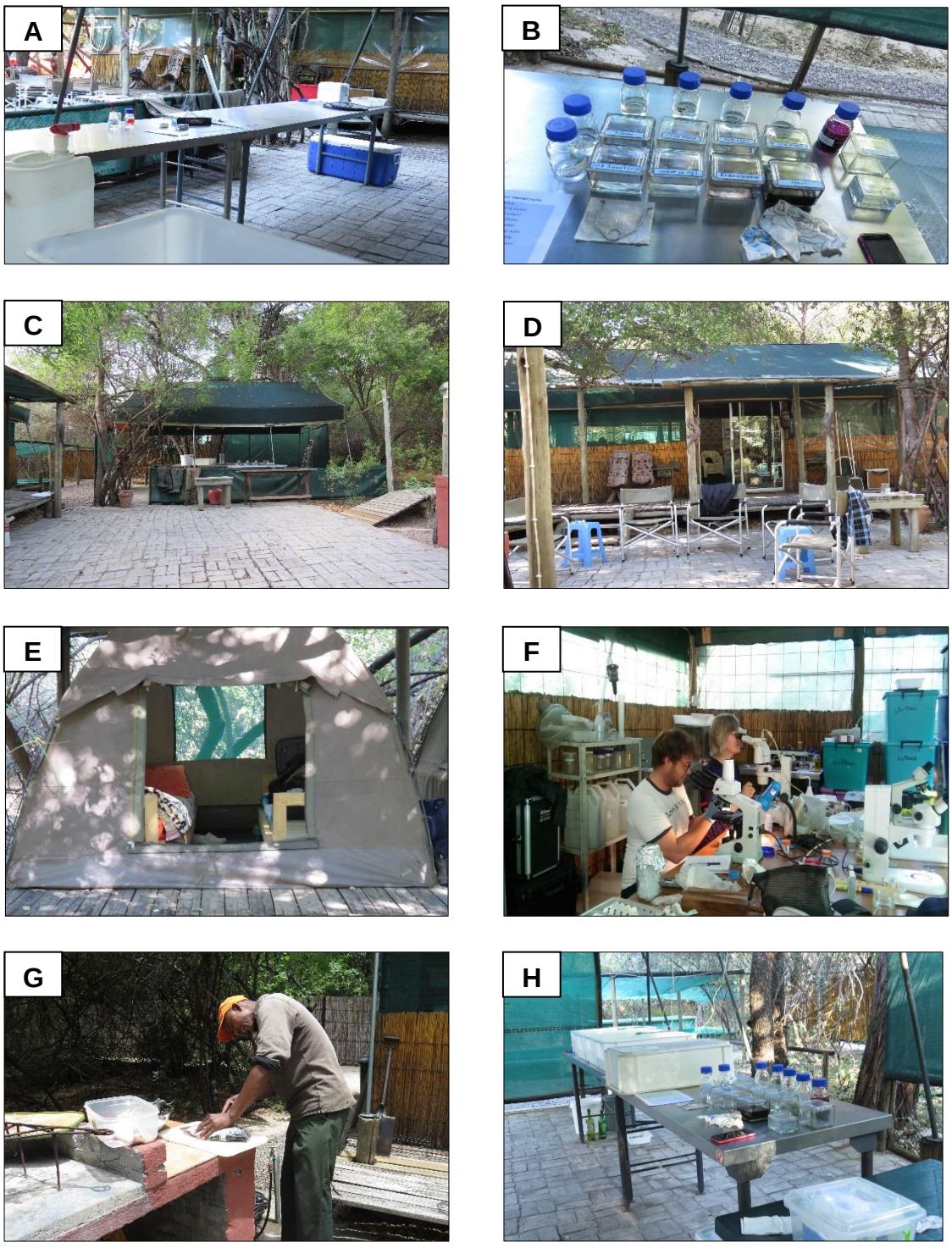


Figure 3.3: Leseding Research Camp: **A** - Dissection tables and aerated host aquaria in background in wet laboratory; **B** - Hæmatoxylin staining centre in wet lab; **C** - Wet laboratory from outside view; **D** - Dry laboratory from outside view; **E**- Raised tents that served as accommodation; **F** - Working in the dry lab; **G** - Fish dissection; **H** - Wet laboratory.

to **C**), were collected from six different pools in the floodplain, each pool being of different depth and size. *Pseudocrenilabrus philander* hosts (Fig. 3.4**E**) were collected from some of the same pools (pools 1, 2 and 6) during the 2015 expedition only.

After collection, hosts were taken back to the research facilities at Leseding where the specimens were separated per different localities into separate aerated tanks. Water used for the aquariums was collected from the collection localities, to keep the pH and conductivity the same as the natural environment.

Each tank was equipped with an oxygen pump and an aquarium heater which regulated the temperature and kept it constant as the ambient temperatures fall dramatically during the winter nights. The hosts were fed every two days: the fish fed on normal veterinary aquarium fish food, while the tadpoles were fed on bloodworms (obtained previously from a pet shop).

Fish hosts were euthanised by cerebral commotion, which is a painless procedure, resulting in immediate death for the host. Because there are no ethical protocols available in the literature for the euthanasia of tadpoles, the same procedures as for fish were followed for all tadpoles collected in the field, namely cerebral commotion. Ethical clearance was obtained from the University of the Free State Ethical Committee, with Ethics approval record number UFS-AED2017/0017 (**Appendix C**). After euthanasia of the tadpole/fish host wet smears were made of the skin and gills. Live observations were made using compound and dissecting microscopes, micrographs were taken using a digital camera setup that is compatible with both microscopes.

The majority of tadpoles collected were from the guttural toads, *Schlerophrys gutturalis* and the western olive toads, *Schlerophrys poweri*, along with a single specimen of the Angolan reed frog, *Hyperbolius parallelus* Günther, 1865 (Fig. 3.4**D**). Some of the collected tadpoles were fixed in 100% ethanol, 4% and 10% buffered formalin and sent to Mr. Werner Conradie, curator of herpetology at the Port Elizabeth National Museum for identification.

HOST NOMENCLATURE

Due to the constantly changing nomenclature of hosts associated with trichodinids, the most recent taxonomic names of all the specimens were used (Table 3.2).

Table 3.2: The common name, original and valid taxonomic or incorrectly accepted genus and species names of species nomenclature that has changed recently**.**

COMMON NAME	ORIGINAL	MOST RECENT
Redbelly Tilapia	*Tilapia zilli*	*Coptodon zilli*
Three spot Gourami	*Trichogaster trichopterus*	*Trichopodus trichopterus*
Bighead Carp	*Aristichtys nobilis*	*Hypophthalmichthys nobilis*
Tasselled-mouth Loach	*Crossostoma lacustre*	*Formosania lacustre*
Golden Barb	*Puntius gelius*	*Pethia gelius*
Meshscaled Topminnow	*Aplocheilichthys hutereaui*	*Micropanchax hutereaui*
Johnston's Topminnow	*Aplocheilichthys johnstoni*	*Micropanchax johnstoni*
Striped Topminnow	*Aplocheilichthys katangue*	*Micropanchax katangue*
Guttural Toad	*Amietophrynus gutteralis*	*Schlerophrys gutteralis*
Western Olive Toad	*Amietophrynus poweri*	*Schlerophrys poweri*

SEASONALITY

Three collection expeditions were carried out, the first of these occurred during the winter months (July to August) of 2015, followed up in 2016 during the same season. Another collection expedition was undertaken during the summer (November to December) in 2016.

The differences of the water volume in each locality between the winter and summer seasons are quite large, with much less water, thus much smaller pools, during the summer collection than during the winter collection. The seasonal differences between three of these localities from July 2016 (Figs. 3.5**A, C** and **E**) and December 2016 (Figs. 3.5**B, D** and **F**) were quite prominent.

July/August 2015

8 – 23 July 2015

A total of 160 slides for morphological and nuclear examination were prepared from 92 anuran tadpoles and 12 *P. philander* specimens. Infestation rates over time was also recorded. All six localities in the Nxamasere floodplain were sampled.

July/August 2016

19 July – 9 August 2016

During this second trip to Botswana, a total of 679 dry and wet smears were collected for morphological examination, along with 35 samples of numerous trichodinids collected in different concentrations of ethanol (70%, 96% and absolute (100%)) from the two different *Schlerophrys* hosts from different population localities for molecular analysis. All six localities in Nxamasere were sampled. *Schlerophrys gutturalis* was found in all six of the pools collected, but *S. poweri* was only found in pool 6 (population NX6).

Along with *Trichodina* specimens, in one population (NX5) the hosts showed a very high infestation of *Trichodinella* species, and these were also collected for both morphological and, molecular scrutiny. Silver nitrate experiments (refer to **Chapter 4**) for cleaner or more detailed morphological data were also conducted. Infestation rates over time were recorded.

November/December 2016

20 November – 1 December 2016

The final trip to Botswana was exclusively planned for collecting new material for testing Kazubski and Migala's (1968) seasonal morphological variability hypothesis. Fewer smears (all impregnated on site with silver nitrate) were collected; 51 smears from tadpole hosts. Tadpoles were only present in abundant numbers in pool 1 (NX1-summer), so all measurements were done for this population. Infestation rate over time was recorded.

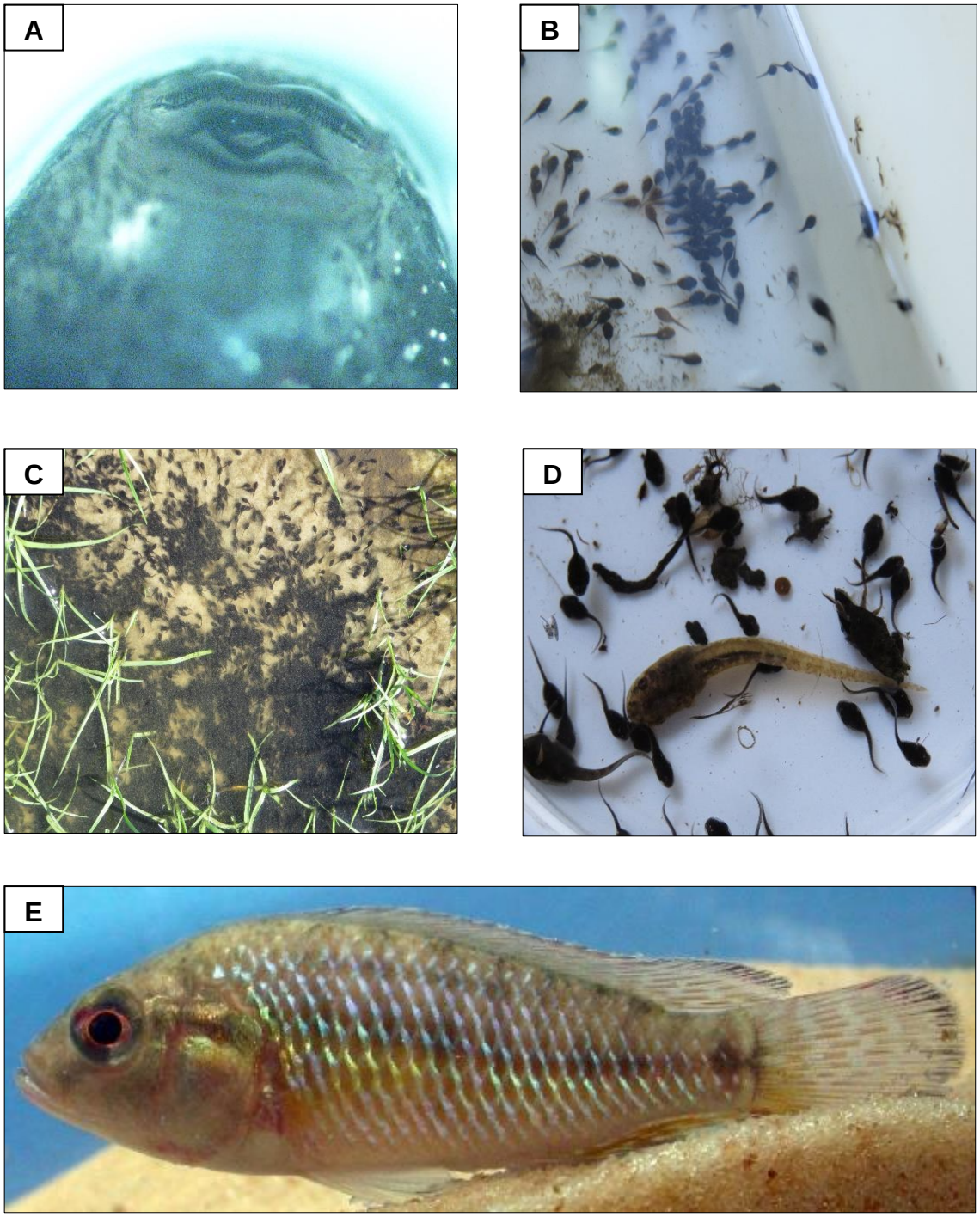


Figure 3.4: Hosts collected during 2016 from Nxamasere. **A** – Dissection microscopy of mouth parts for taxonomic identification; **B** – *Schlerophrys* sp. separated into different aquariums according to locality collected; **C** - Tadpoles in vast numbers in the Nxamasere pools; **D** – Different tadpole species; **E** – *Pseudocrenilabrus philander* (E – photo taken by Kit Williams, courtesy of Prof. L. Basson).

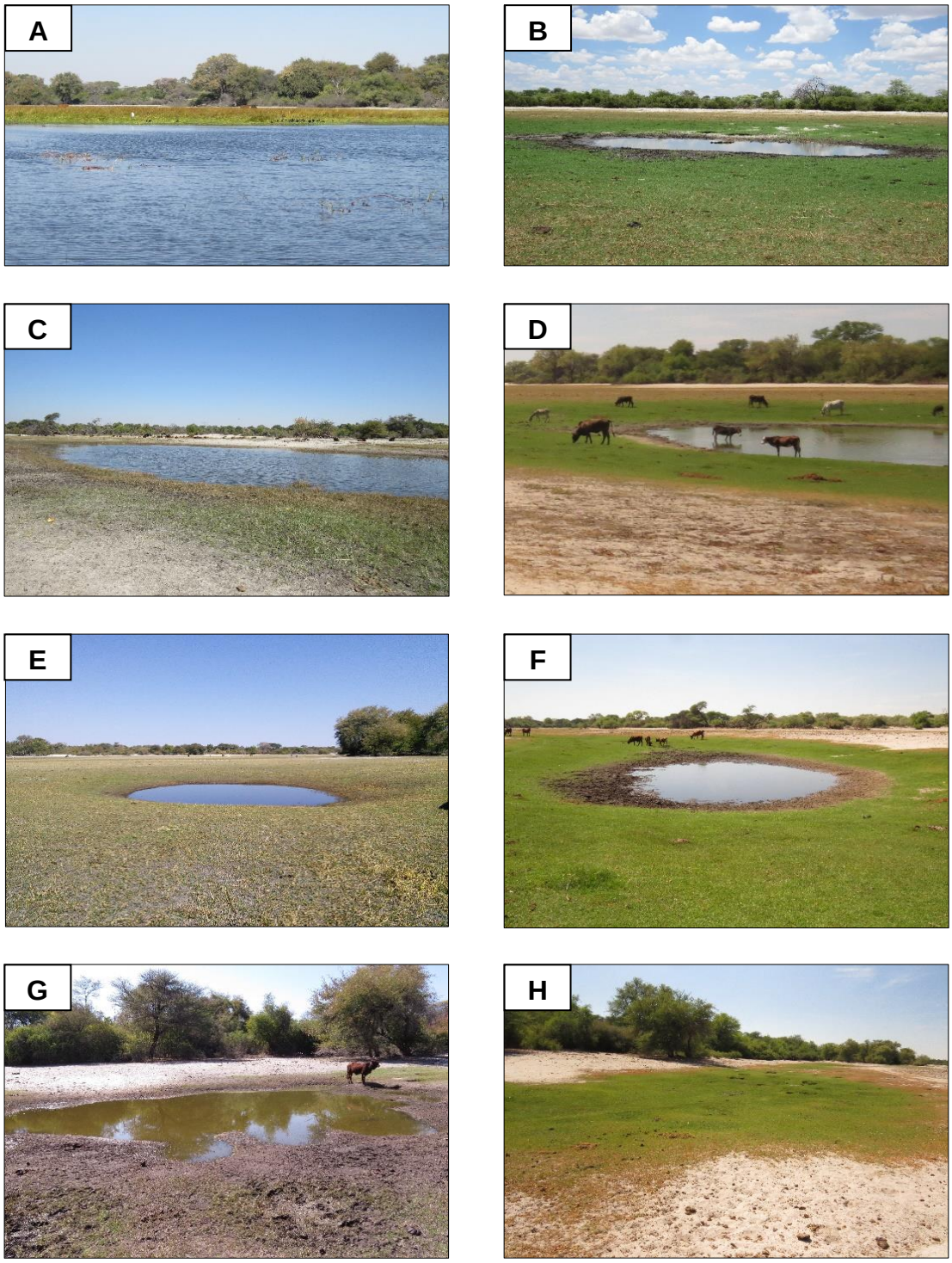


Figure 3.5: Collection localities in the Nxamasere floodplain in 2016 illustrating the differences between the winter and summer seasons: **A** - NX1 winter and **B** - NX1 summer; **C** - NX2 winter and **D** - NX2 summer, **E** - NX4 winter and **F** - NX4 summer; **G** - NX5 winter and **H** - NX5 summer.

MORPHOLOGY

SILVER IMPREGNATION

The main focus of the morphological analysis is based upon the “dry” silver impregnation method developed by Klein (1926). The technique used for this specific research is an adapted version of Klein’s technique, as described by Lom (1958) and Wellborn (1967) (Basson *et al*. 1983). The slides were submerged in a 4% silver nitrate ($AgNO_3^{-1}$) solution for 10 minutes, thereafter were washed with distilled water and finally placed under a UV light (254nm) for 20 to 30 minutes. In some cases, the slides were put in either direct sunlight or under a UV-A light (365nm).

Chapter 4 – Silver Nitrate Experiments, deals with an in-depth discussion of Klein’s methodology and the evolution of silver impregnation, along with experiments to improve impregnation of anuran trichodinids by means of different substrates. **Chapter 7 - Article** was written up in an article format, which also includes a complete methodology and results.

HÆMATOXYLIN STAINING

To stain the nuclear apparatus of *Trichodina heterodentata*, Mayer’s hæmatoxylin staining method, as suggested by Wellborn (1967) and Basson *et al*. (1983) was used. The slides were air dried on location and then stained back at the research laboratory of the Free State University, Bloemfontein in the following manner:

The slides were placed in xylene twice for three minutes each, thereafter they were rehydrated with diminishing concentrations of ethanol (100% twice for three minutes, followed by 96% and then 70%, each for two minutes). Staining with hæmatoxylin followed for eight to 11 minutes then washed with fast running tap water (pH=6) for three minutes. After three to five minutes slides were submerged in Scott’s blueing solution and once again washed with distilled water. If the nuclear material was overstained and too dark for satisfactory observations, the slides were de-stained with an acid-ethanol solution (70% ethanol and a few drops of HCl) for one minute. After the staining process

was completed, the slides were dehydrated back to 100% ethanol (starting with 70% for one minute, followed by 96% for two minutes and finally twice for three minutes at 100%). Lastly the slides were again cleared with xylene twice for three minutes, after which coverslips were put on the slides using either Eukitt® mounting medium or Canada Balsam.

MEASUREMENTS ACCORDING TO LOM (1958)

The prescribed measurements initially proposed by Lom (1958) (**Chapter 2:** Fig. 2.16): and later adapted by van As and Basson (1989) were done for all sampled populations (Fig. 3.6). After silver nitrate impregnation (back at the University of the Free State, South Africa for the 2015 collection trip and at the Leseding Research Camp, Botswana for 2016), the prepared slides were marked to indicate suitable adult trichodinids.

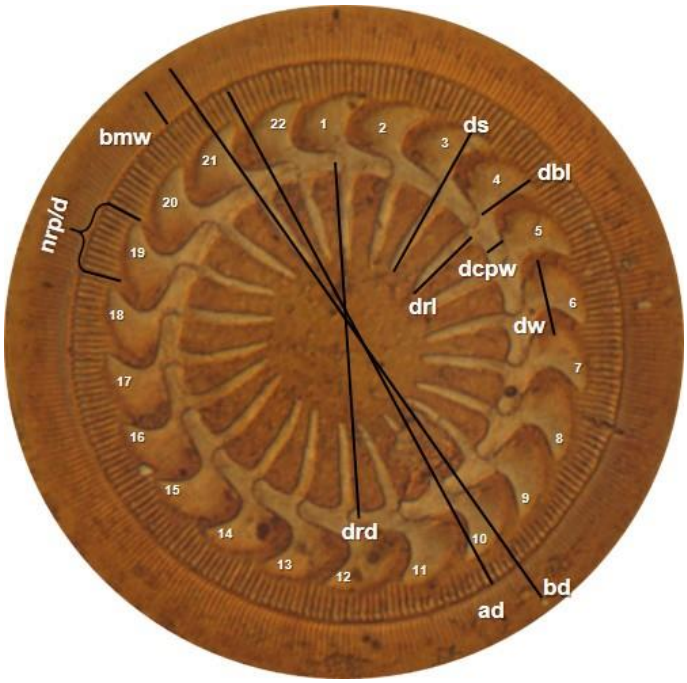


Figure 3.6: Morphological measurements of the aboral denticle structures as standardised by Lom (1958) with recent additions by contemporary taxonomists. **ad** - Adhesive disc diameter; **bd** - Body diameter; **bmw** – Border membrane width; **dbl** – Denticle blade length; **dcpw –** Denticle central part width; **drd** - Denticle ring diameter; **drl** – Denticle ray length; **ds** – Denticle span; **dw** – Denticle width; **nrp/d** - Number of radial pins per denticle. Number of denticles per individual is also determined.

Hereafter the marked specimens were photographed and measured using a Zeiss Axiophot compound microscope fitted with a Nikon DXM 1 200F digital camera. All the measurements were presented as follows:

minimum to maximum (mean ± standard deviation)

All morphological measurements are provided in µm, and all micrograph plates have a scale bar of 10µm. All biometrical data/measurements were statistically compared and then presented in comparative box-plots in **Chapter 5**.

DENTICLE DESCRIPTIONS ACCORDING TO VAN AS AND BASSON (1989)

Three consecutive denticles of individual trichodinids were re-drawn from silver-impregnated micrographs, as discussed in **Chapter 2**. Each of the descriptions is a representative individual from every population collected, and in some cases more than one individual were used to show the morphological variation within the population groups. The various axes were obtained by extrapolating a straight line from the center of the adhesive disc towards the nearest tangent point on the tip of the blade for each of the three denticles. An additional x-axis was drawn perpendicular to the y-axis. These lines were used as references for the comparative descriptions.

For each of the populations a representative, which expresses the general morphology of that specific sample group, was chosen. In some cases, the population had more than one typical morphological profile and then more than one representative were chosen to measure and compare.

The anuran individual representatives were named as follows: ***NX1a (summer)***, where ***NX*** represents Nxamasere; the number (***1***)- the pool in which the hosts were collected, the letter (***a***)– when more than one representative specimen was used for that population, followed by (***summer***) - the season in which these were collected. For the samples collected in the winter of 2015, a ***-15*** postfix is added, and those collected from *Pseudocrenilabrus philander* hosts, a P is added after NX (***NXP***). All scalebars in the results sections for the denticle description drawings are equal to 5µm.

MOLECULAR

SAMPLES, STRAINS AND PLASMIDS

Complete *Trichodina heterodentata* and *Trichodinella epizootic*a individuals along with host tissue were obtained from the field, Nxamasere Floodplain, Okavango Panhandle, Botswana. Collected tissue samples were preserved in distilled water ($_dH_2O$), 70%, 80%, 96% ethanol and absolute ethanol. All samples were kept at a maximum temperature of 4°C.

The TOP10 *Escherichia coli* strain from Invitrogen was used as host for genetic manipulation experiments. *Escherichia coli* was grown in Luria-Bertani (LB) medium (10g.l^{-1} tryptone, 5g.l^{-1} yeast extract and 5g.l^{-1} NaCl, pH 7) at 37°C with aeration (200rpm). Kanamycin (30µg.ml^{-1}) was used as selective pressure when required.

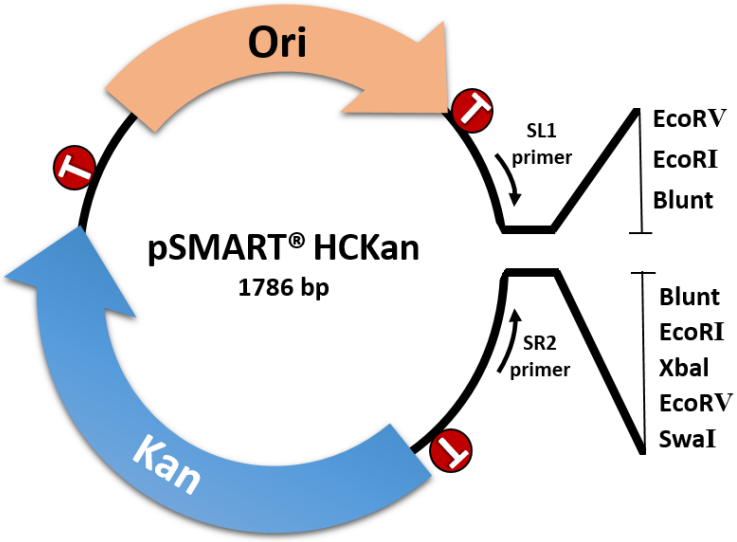


Figure 3.7: Schematic diagram of the pSMART® HCKan vector indicating the kanamycin resistance gene (KanR in blue), the origin of replication (Ori in orange), the location of the blunt-cloning site and the binding sites of the sequencing primers, SL1 and SR2 (Lucigen®) and restriction enzymes (T = Transcriptional terminators to prevent insert-driven transcripts from entering the vector).

The pSMART HCKan high-copy vector (Lucigen) was used as cloning vector (Fig. 3.7), which allowed for blunt-end cloning of the PCR amplified products. This facilitated sequencing of the target PCR products.

ISOLATION OF genomic DNA (gDNA)

Two milliliters of each of the tissue samples were centrifuged for 15 minutes (20 000*x*g; 4°C). After the alcohol/$_{d}H_2O$ supernatant was discarded, the gDNA was extracted from the remaining pellet using the REDExtract-N-Amp Tissue PCR Kit according to the manufacturers instruction (Thermo Scientific™). The pellet was dissolved in 100µl of the extraction solution and 25µl tissue preparation solution for 10 minutes and then incubated at 95°C for three minutes. Before the final mixing by vortex, 100µl neutralisation solution was added.

CLONING

Polymerase Chain Reaction (PCR) Amplification

The target regions, complete and partial 18S SSU rDNA, were PCR amplified using primers sets proposed by Tang *et al.* (2017) and Tang *et al*. (2016). Primers were designed using the Integrated DNA Technologies OligoAnalyzer 3.1 online tool (Table 3.3). The complete 18S, partial 18S and ITS1-5.8S-ITS2 genes were amplified from the gDNA of the trichodinids using primer set 1, 2 and 3, respectively.

Table 3.3: Primer sequences used for PCR amplification of the 18S and ITS1-5.8S-ITS2 regions of the SSU rDNA (using REDExtract-N-AMP Tissue Kit *Taq* polymerase).

Primer Set	Primer	Sequence	T_m (°C)	PCR Annealing Temperature (°C)
1	ERIB 1 EukA	5′-ACC TGG TTG ATC CTG CCA G-3′	58.9	56
	ERIB 10 EukA	5′-CTT CCG CAG GTT CAC CTA CGG-3′	60.2	
2	MX 5	5′-CTG CGG ACG CAG TAA ATC ACT-3′	61.0	51
	MX 3	5′-CCA GGA CTT AGG GCA TCA CAG A-3′	60.1	
3	ERIB10-v	5′-CCG TAG GTG AAC CTG CGG AAG-3′	60.1	56
	28s1r	5′-GTG TTT CAA GAC GGG TCG -3′	53.6	

Initial PCR reactions were performed using a T100-thermal cycler (Biorad Technologies) with the REDExtract-N-Amp™ Tissue PCR Kit (Sigma-Aldrich-Merck®). The PCR reaction mixture was made up to total volume of 10µl consisting of 0.4µl forward primer (5'), 0.4µl reverse primer (3'), 5µl REDExtract-N-AMP PCR reaction mix (containing the needed buffer, salts, dNTP's *Taq* polymerase, RED*taq* dye and JumpStart *Taq* antibodies), 2µl template DNA and 2.2µl MiliQ deionised water.

Conditions for the REDExtract-N-AMP™ reactions comprised of an initial denaturing step at 94°C for five minutes, followed by 30 cycles of denaturing at 94°C (40 seconds), annealing at primer specific temperatures (Table 3.3) for one minute and elongation at 72°C (1.5 minutes). A final elongation step of 10 minutes at 72°C to ensure complete elongation of amplified product.

The PCR products obtained during the first round of amplification were cleaned up from the gel and used as template DNA in a second round of amplification using the KAPA polymerase system. The PCR reaction mixture consisted of 0.3µl forward primer (5'), 0.3µl reverse primer (3'), 5µl KAPA HiFi HotStart ReadyMix (already containing 2.5Mm $MgSO_4$, dNTP (0.3mM each) and 0.5U KAPA HiFi HotStart DNA Polymerase per 25µl reaction), 1µl template DNA and 3.4µl MiliQ water. For the initial amplification of the ORFs, 100ng of gDNA was added to the reaction mixture, whereas 8ng of DNA was added in subsequent steps where plasmid DNA served as the template.

Reaction conditions consisted of an initial denaturing step at 94°C for three minutes, followed by 30 cycles of denaturing at 98°C (20 seconds), annealing at 63°C (PCR-gradient for the KAPA HotStart ReadyMix demonstrated that 63°C delivered the optimum results for all the primers) for 15 seconds and elongation at 72°C (1.3 minutes). A final elongation step of 10 minutes at 72°C to ensure complete elongation of amplified product.

For both PCR processes described above the PCR product was cleaned from the gel using the GeneJet Gel Extraction Kit (Thermo Scientific™) according to the manufacturers instruction.

Blunt-end Ligation and Plasmid Propagation

The gel purified PCR product from the second round of amplification was dried using a Speedy-Vac concentrator and dissolved in 15µl of deionised water (mH_2O). Phosphorylation of the PCR product was achieved by incubation with 1µl of T4 polynucleotide kinase (PNK, Thermo Scientific™), 2µl Buffer A and 2µL 10mM ATP in a total volume of 20µl at 37°C for 20 minutes. PNK was deactivated by incubation at

72°C for 15 minutes. The phosphorylated PCR product was ligated into the pSMART® cloning vector. Protocols for ligation was performed according to the manufacturers instruction.

Chemically competent *Escherichia coli* TOP10 (Invitrogen) cells were prepared according to the method described by Hanahan (1983) with modifications. Psi broth (5g.l^{-1} yeast extract, 20g.l^{-1} tryptone, 5g.l^{-1} magnesium sulphate, pH 7.6 with KOH) were prepared and a 100ml placed into flasks and then inoculated with 1ml of an overnight culture. The cultures were grown at 37°C until an absorbance of 0.6 at 600nm was obtained.

The cells suspensions were placed on ice for 30 minutes followed by centrifugation (3000*x*g; 10min; 4°C), hereafter the cells were resuspended in 40ml TfbI buffer (30mM potassium acetate, 100mM rubidium chloride, 10mM calcium chloride, 15% glycerol, pH 5.8) and incubated on ice for 15 minutes. The cells were collected by centrifugation (3000*x*g; 10min; 4°C) and resuspended in 4ml TfbII buffer (10mM MOPS-NaOH, 75mM calcium chloride, 10Mm rubidium chloride, 15% glycerol, pH 6.5). The suspended cells were incubated on ice for 10 minutes, aliquoted and snap frozen in liquid nitrogen followed by storage at -80°C.

The ligation reaction was performed in a total reaction volume of 10µl and consisted of 10x ligation buffer (1µl), pSMART® vector (30ng), PCR product (5.5µl), 50% PEG4000 (1µl) and T4 DNA Ligase (0.3 Weiss units). The ligation reactions were incubated overnight at 4°C.

The ligation mixture was transformed into competent *E. coli* TOP10 cells for propagation. For the transformation reactions, 50µl of chemically competent *E. coli* TOP10 cells were taken from the -80°C storage and thawed on ice. Five microliters of the ligation mixture was added to the cells and incubated on ice for 30 minutes. The cells were heat-shocked at 42°C for 40 seconds and returned to ice for two minutes. Two hundred and fifty microliters pre-heated (37°C) SOC media (20g.l^{-1} tryptone, 5g.l^{-1} yeast extract, 0.01M NaCl, 2.5mM KCl, 0.01M $MgCl_2$, 0.01M $MgSO_4$ and 0.02M glucose) was added to the transformation mixture and incubated at 37°C for one hour with gentle shaking.

The transformation mixture was plated onto LB agar plates containing 30µg.ml^{-1} kanamycin and incubated for 12 to 16 hours at 37°C. Single colonies were inoculated, using a sterile toothpick, into 5ml LB media supplemented with kanamycin (30µg.ml^{-1}) to maintain selective pressure and cultured for 12 to 16 hours at 37°C with shaking.

Plasmid isolation was done by using the lysis by boiling method as proposed by Ehrt and Schnappinger (2003). Obtained plasmids were screened for gene insertion using either one or two restriction enzymes. The pSMART®: trichodinid construct was digested with *Eco*RI (for MX, ERIB10-v and 28s1r primers) and double digested with *Eco*RI and *Hind*III (ERIB1 and ERIB10 primers) by incubating 2µl of plasmid, 1µl of 10X Buffer O (MX) or Buffer R (ERIB EukA) and deionised water to a total volume of 10µl, at 37°C for three hours.

DNA mini-preparations (extraction and clean-up) followed the 16-hour growth of the positive transformants. This was performed using the GeneJET Plasmid Miniprep Kit as per manufacturer's recommendations (Thermo Scientific™).

Analytical Techniques

DNA electrophoresis

All PCR and DNA products were analysed with the use of agarose gel electrophoresis. All the agarose gels consisted of 0.8% agarose in TAE buffer (0.04M Tris-HCl, 1mM EDTA pH 8.0 and 0.021mM glacial acetic acid) with 0.6µg.ml^{-1} ethidium bromide. DNA was separated within the agarose gels at 100V for 30 to 45 minutes. DNA was visualised under a high radiation UV source (ChemiDoc XRS Gel Documentation system; Bio-Rad Laboratories), while DNA to be isolated from agarose gels for further studies was visualised using a low radiation UV source (Darkreader, Clare Chemical Research), and was isolated using the GeneJET Gel Extraction Kit according to manufacturer's recommendation (Thermo Scientific™). DNA fragment size was estimated based on electrophoretic mobility relative to that of the GeneRuler™ DNA Ladder Mix (Thermo Scientific) during the same electrophoretic run.

DNA sequencing

To determine the nucleotide composition of the relevant genes, purified positive plasmids were used in sequencing reactions. Sequencing was preformed using a 3130*xl* Genetic Analyser (Hitachi). The BigDye® Terminator (v 3.1) Cycle Sequencing Kit (Applied Biosystems®) was used to amplify the inserts as per manufacturer's instructions, using SR1 forward and SL2 reverse primers.

Evolutionary relationships of taxa

All consensus gene sequence alignments were constructed using the Geneious 7® software package. Hereafter phylogenetic trees of sampled Botswana sequences and NCBI sequences, using the Neighbour-Joining method (Saitou & Nei 1987). The bootstrap test (1 000 replicates) was used for the percentage of replicate trees in which the associated taxa clustered together (Felsenstein 1985) The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980) and are in the units of the number of base substitutions per site. The analysis involved 44 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 1 094 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al.* 2016).

CHAPTER 4 - SILVER NITRATE EXPERIMENTS

INTRODUCTION

Trichodinids have always been notoriously constant when it comes to successful silver nitrate impregnation. Firstly; they seldom have the same constant impregnation success that can be observed with other ciliophorans. Another hindrance is that marine trichodinids produce problems because of the high salt conc entration in marine waters, as stated by Foissner (2014) about marine peritrichs. A precipitation reaction between the NaCl and $AgNO_3$ occurs which leaves an insoluble white silver chloride (AgCl) precipitate, making any impregnation observations almost impossible. The purpose of this chapter stems from former observations and preliminary research done for this project regarding silver nitrate impregnation of anuran hosts trichodinids. It has been noted that smears made from anuran host tend to yield impregnations which aren't as clear and constantly well impregnated as those of their teleost hosts. Initially an experiment was done on smears of anurans that were collected from the same tadpole hosts, at the same localities in the Okavango Delta, but between 2006 and 2007, i.e. 10 year old slides (Experiment 1). This experiment tried different methods of preparing the already smeared slides, along with altering the original impregnation "dry" silver technique as prescribed by Klein (1926) and incorporating elements of Klein's (1926) modified Chatton-Lwoff (Chatton & Lwoff 1930) silver staining technique. Professor Jǐrǐ Lom, relayed to the author via Professor Linda Basson, who worked extensively with Prof. Lom, that the optimal impregnation results were usually achieved when the specimens were impregnated no later than 48 hours after smears were made. This may be problematic as impregnation facilities aren't always at hand on expeditions, or previously smeared slides left for years before staining, could still possess valuable taxonomic or other scientific information. It was therefore important to find a method that allows for consistently high-quality impregnations of old and stored unstained smears. A question also arose whether the problematic impregnation observed with the anuran trichodinids were due to the prolonged time between the 2006/2007 smears and their impregnation, or whether the mucus from the anurans played a role. This question, and the knowledge that teleost trichodinids impregnate with less difficulties than anuran hosts, led to Experiments 2 to 5, using different substrates/fixatives.

LITERATURE AND METHODOLOGY

CHEMICAL OVERVIEW

Silver nitrate is an inorganic chemical compound with the empirical formula $AgNO_3$ (Figs. 4.1 **A** and **B**), first discovered by the alchemist Albertus Magnus in the 13th century, who noticed that the product from dissolving silver in nitric acid tends to blacken the skin (Szabadváry 1992). Besides the fact that silver nitrate is the precursor to numerous other silver products, it is also less sensitive to light than the other metal-halides, proving its essential use in manual photography.

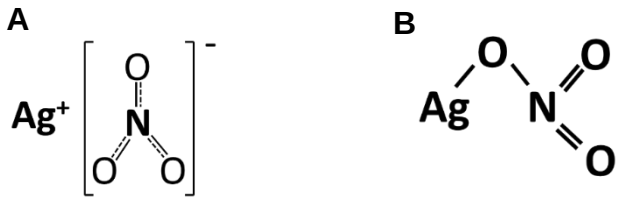


Figure 4.1: Chemical configurations of silver nitrate: **A** - Natural state and **B** - Binding state.

Silver nitrate, like all heavy metals in ionic form, has a great affinity for protein (Lavelle 1985). In biological sciences silver staining is extremely useful, as it selectively alters the appearance of target tissue for the microscopy for histological sections, especially to detect trace numbers of proteins (Kerenyi & Gallyas 1973). In biochemical techniques, like temperature gradient gel electrophoresis and polyacrylamide gels, silver nitrate is used for detecting proteins and peptides in these gels (Switzer *et al*. 1979).

Lavelle (1985) explains the basic mechanism underlying silver impregnation as the formation of insoluble metallic silver nuclei. The silver ions in the impregnation solution combine with specific tissue components, like amino acids. The spontaneous reduction, to precursor metal nuclei, depends on the redox potential of the tissue needed to be stained. During the development process additional silver (from silver ions in the solution) is deposited on the newly formed nuclei by the reducing agents in the solution. This process results in a specific staining pattern, which can be used to identify contours or morphological characteristics on the tissue which has been stained/impregnated.

Peters (1955a) mentions that during the development stage, the largest part of the unreduced silver is combined with amino acids of the tissue (especially histidine), and the rest is then reduced to form these silver nuclei. These two processes happen at different speeds; the unreduced combination is completed in under 20 minutes (dependent on pH and temperature) and the silver nuclei formation is a much lengthier process.

Peters (1955b) also concluded that the pH of the imprinting solution plays a very important role, and from experimentation he noticed that at a very low pH (4.5-5.6) there was almost no staining, and that staining gets progressively better as the pH increases, but after a pH of 10.3, however, the stained fibres became coarse and too homogeneous for any visible detail observations.

For all the good that silver nitrate impregnation has brought the world of taxonomy, it still has its problems, such as the failure of removing surplus silver stain and limited reproducibility (Meywald *et al.* 1996). Various experiments have been done to aid the impregnation process for clearer and more detailed results. Pearson and O'Neill (1946) mixed aqueous gelatin with their solution for silver impregnation of nerve fibres and found the results to show good uniform impregnations of human and mouse nerve fibres, embryos and fetuses. Moskowitz (1950) in his paper on protist staining using protein silver, re-introduced the use of a gelatin solution, as proposed by Chatton-Lwoff's (1930) technique. The use of aqueous gelatin for silver staining has also been used on the silver staining of chromosomes with positive results (Meywald *et al.* 1996; Kavalco & Pazza 2004).

HISTORIC USE IN TAXONOMY

First used by Golgi in 1873 and then incorporated into histological methods by Ranvier, Klein (1926) introduced the adapted silver-impregnation method of dry-fixation and impregnation with silver nitrate for Protozoa (Kudo 1950). Silver nitrate revealed fundamental patterns of fibrillar structures in the ectoplasm of ciliophorans (Corliss, 1953).

Klein (1926) subjected numerous ciliophoran genera and species to this method and observed that there was a fibrillar system in the ectoplasm at the level of the basal granules which could not be demonstrated by any other methods. Klein (1926) named these fibers "silver lines" and the whole complex system, the "Silverline System", which is still used today by most biologists. Protozoologists like Chatton and Lwoff (1930), Corliss (1953) and Lom (1958) all applied, and in some cases refined, the silver-impregnation method, to many other ciliophorans and confirmed Klein's observations.

Klein's (1926) "dry" silver method (so named because of the dry fixation of the specimens) was first used on trichodinids by Raabe (1950) and then promoted by Lom (1958) as the standard staining method for the trichodinid denticle structure.

THE DRY SILVER IMPREGNATION AS PROPOSED BY KLEIN (1958)

Klein's "dry" silver method of 1926 as quoted by the author in 1958 reads as follows:

"*A medium-sized drop of water (containing the ciliophorans) is spread on a degreased slide and dried at room temperature. After it is dry the smear is covered with a 2% aqueous $AgNO_3$ solution by means of an eyedropper. This silver solution is left on the smear for six to eight minutes. Thereafter the smear is thoroughly rinsed with distilled water from a dropper and the slide placed in bright daylight on a white background (a porcelain plate for example). This reduces, i.e., darkens the silver nitrate. After the reduction is completed the slide is thoroughly rinsed in tap water, dried in a vertical position, and mounted in neutral balsam."* – Klein (1958).

According to Klein (1958), two of the most important aspects of the above-mentioned method is the size of the water drop containing the ciliophorans and the reduction time. The size of the drop is important as too large an amount of water that takes too long to evaporate/dry out will raise the electrolyte concentration to lethal levels, killing the ciliophorans. Post mortem changes can then occur to the ciliophorans in the remaining water, which gives incorrect observations. For the best results, the death of the ciliophoran must correspond exactly to the drying of the water.

Concerning the reduction time; if this period is prolonged, the already impregnated Silverline System may become covered by silver precipitate, which leaves the ciliophoran obscured by a rusty-red artifact (Klein 1958).

Chatton and Lwoff (1930) along with Fauré-Fremiet and his students, redefined Klein's technique, making it somewhat more challenging and time consuming, but the results, according to Corliss (1953), seemed to be advantageous in the following four regards; an absence of distortion, uniform impregnation, higher percentage of perfect specimens and possibly using this technique for both freshwater and marine species. One of the most striking contributions from the Chatton-Lwoff method (1930) was embedding the fixated ciliophorans in a layer of saline gelatin. The use of different substrates into which the ciliophorans may be embedded is the focus of the experiments that makes up this chapter.

Lom (1958) reverted to Klein's (1926) original "dry" silver method without gelatin, and insisted that this method becomes one of the important criteria for trichodinid morphological taxonomy. This criterion was one of the main reasons why many of trichodinids described before 1958 ended up in Lom & Laird's (1969) R.U.N. or *nomen nodum* categories.

MATERIAL AND METHODS

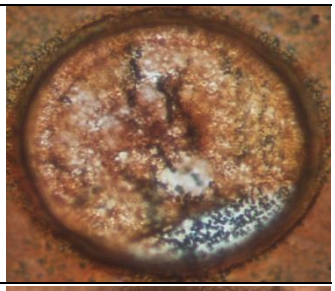
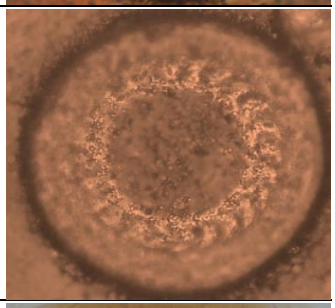
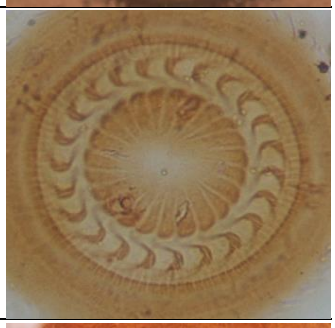
All slides used for the experiments in this chapter were of *Trichodina heterodentata,* from anuran hosts, collected from different localities in the Okavango River System, from 2006 to 2016. All slides were treated with three different substrates; albumen, gelatin (both pre-applied to the slide, or dripped on post preparation) and mucus collected from three different fish species, *Gambusia affinis* (Baird & Girard, 1853), *Cyprinus carpio* Linnaeus, 1758 (both collected and used from Loch Logan in Bloemfontein) and *Micropanchax* Myers, 1924 species (collected from the Okavango River System in 2016). The substrate treated slides were then left for different times to dry (i.e. 24, 48, 72 or 120 hours) before impregnated with silver nitrate.

Experimentation also included the kind of radiation silver impregnated slides were exposed to; UV light, UV-A (black light) and the full midday sun at the Leseding Camp in the Okavango Panhandle.

Micrographs were taken using a Nikon Eclipse E100 microscope with a Nikon DXM 1200F camera and compared with one another. Five slides per experiment were prepared, on each of these slides 10 individual impregnated trichodinids were randomly chosen and ranked according to the quality of denticle impregnation, the probability of measurements possible and the extent of descriptions conceivable. The average of these ten individuals were calculated per slide and these averages for the five slides were in turn used for a general average ranking per experiment. Table 4.1 provides a more detailed explanation of the ranking levels and their implication, 5 being the best and 1 the poorest impregnation. It must be noted that the rankings were used on a sliding scale, including rankings in between the specifications (Table 4.1).

The silver nitrate experiments were exclusively carried out for the interest of the different impregnation results, therefore all other ambient (such as humidity) and locality data were ignored and not interpreted.

Table 4.1: Ranking specifications for all silver nitrate experiments.

Rank	Quality type	Depiction of quality type	Micrograph example of quality type
1	Poor	Impregnations extremely grainy, almost no denticles visible, no measurements possible	
2	Inadequate	Denticles visible, but no measurements possible	
3	Average	Denticles visible, minimal measurements possible (mostly only number of denticles and body diameter)	
4	Good	Denticles visible, all measurements can be made, but quality not of publishable standard	
5	Excellent	Denticles visible, very high quality for photo plate publications.	

EXPERIMENT 1: SILVER NITRATE IMPREGNATION OF OLD SLIDES

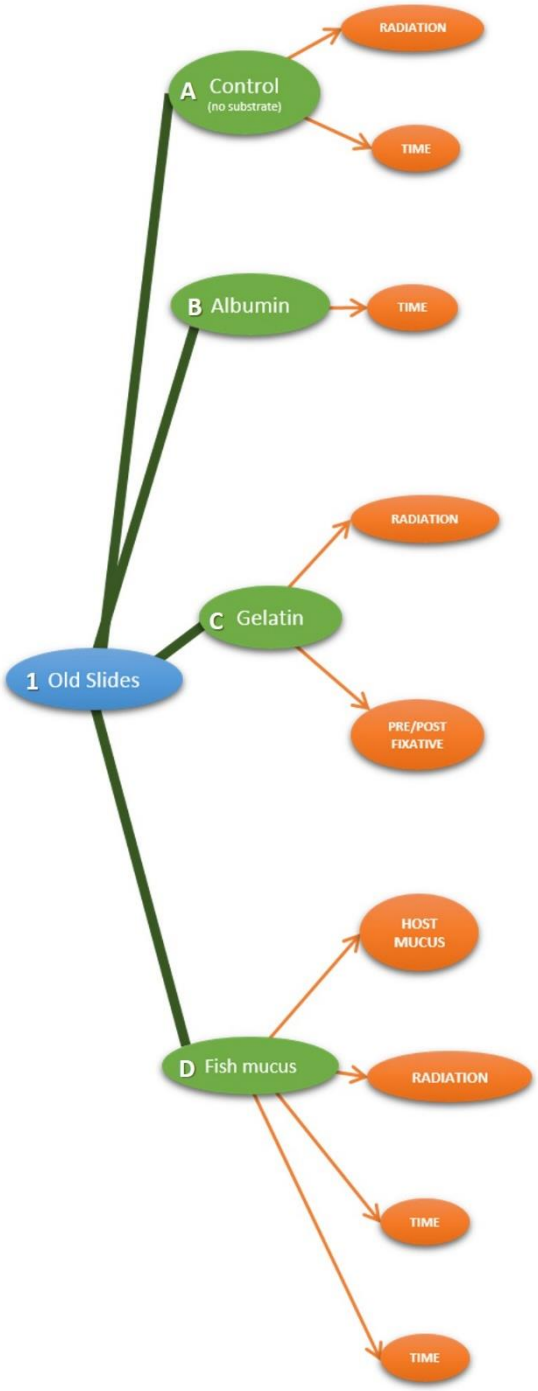


Figure 4.2: Graphic illustration of Experiment 1 (Blue - Experiment; Green - methods; Orange - Variations).

All the slides that were used for this experiment were slides smeared from the gills and skin of their anuran hosts between 2006 and 2007, also from the Okavango Delta. These slides have up to date not been stained or impregnated with silver nitrate.

This experiment used the same substrates that were used for Experiments 2 to 5, but the time between preparation of slides and impregnation was restricted to a maximum of 48 hours in the case of the latter four experiments. Two other variables were also included for Experiment 1:

- Temperature during impregnation process: the prepared slides were either impregnated at ambient room temperature (between 20°C and 25°C) or in a walk-in refrigerator constantly set at a constant 4°C.

- Type of radiation for final step of impregnation: the prepared slides were exposed for 20 minutes under a UV light or 20 minutes under a UV-A (black light). This variable was also used in some of the subsequent experiments.

Method A (Fig. 4.2) consisted of three variations of using no substrates, with differences in time and type of radiation used (control method).

Method B (Fig. 4.2): Albumin was applied to the old unstained smears and either immediately impregnated under UV light (Variation I) or left for 24 hours to dry before impregnation with UV light (Variation II).

Method C (Fig. 4.2) tested for the impregnation with an aqueous gelatin solution using either UV light or UV-A radiation.

Method D (Fig. 4.2) applied fish mucus obtained from *Gambusia affinis* and *Cyprinus carpio* caught at the Loch Logan Dam in Bloemfontein, South Africa. These slides were then tested for better impregnation by using different radiation sources (UV and UV-A), different periods before impregnating after applying the substrate and whether there is a difference between impregnating the slides submerged under distilled water or in dry air under UV/UV-A light.

During **Experiment 1**, fresh samples of unknown tadpoles from Loch Logan in Bloemfontein were also collected. These were smeared on slides pre-prepared with the same aqueous gelatin solution as for Method C, as per the Chatton-Lwoff technique (Corliss 1953). This experimental outcome led to planning the experiments on site in the Okavango Delta, and the results are given in comparison of Method C, discussed above, in Figure 4.2**B**.

EXPERIMENT 2: ALBUMEN AS SUBSTRATE

The Mayer albumen fixative as recommended by Humason (1962) was prepared as follows:

Egg white was separated from the rest of an egg yolk, the egg white was then beaten only until the white had broken up (but not till it was stiff) and then transferred into a measuring cylinder. It was left overnight until the oxygen bubbles lifted all the impurities to the top of the liquid. The topmost liquid was then discarded. Glycerol, equal in volume to the remaining egg white was then added. Finally, 1:100 parts $C_{10}H_{14}O$ (thymol) was

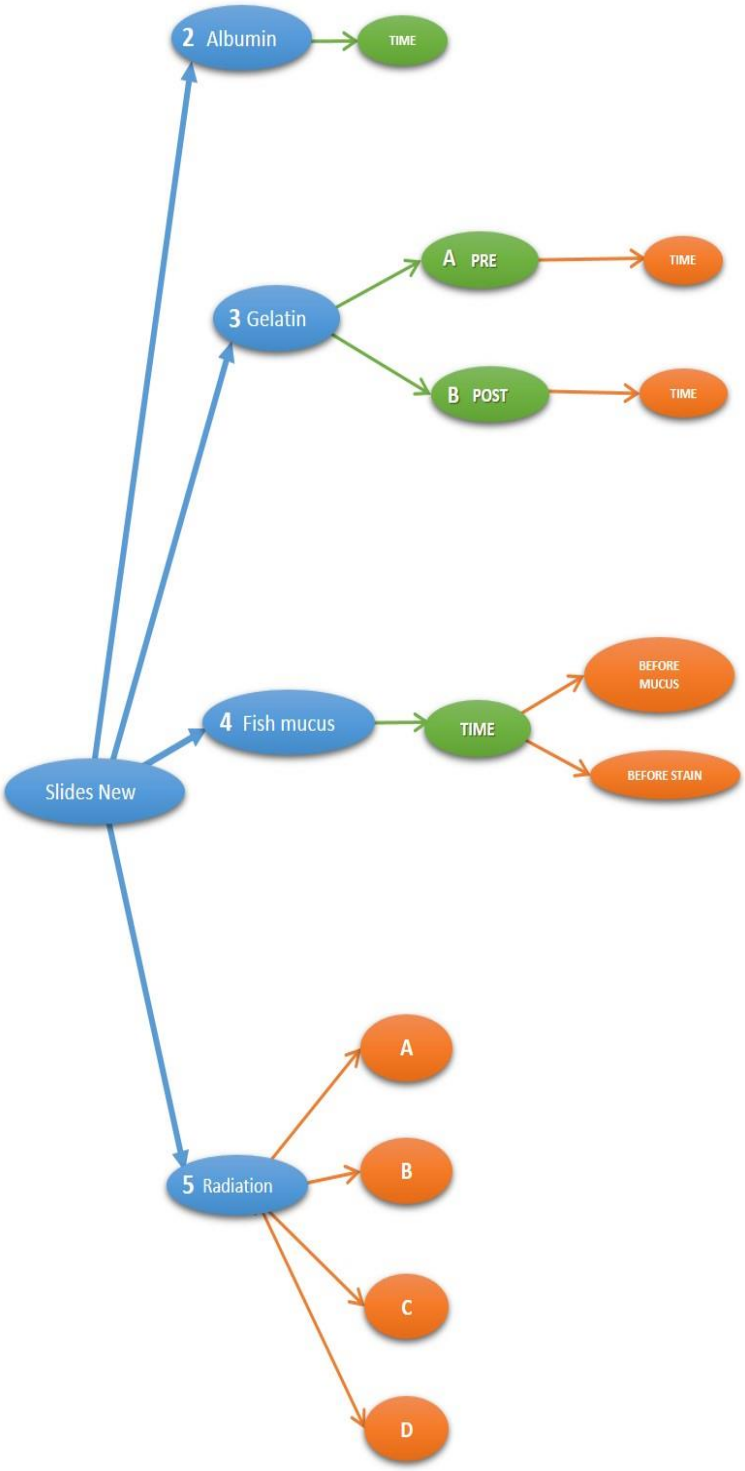


Figure 4.3: Graphic illustration of fresh slide impregnation experiments 2 to 5 (Blue - Experiments followed; Green - Methods; Orange - Variations).

added to the glycerol-albumen solution to prevent the growth of mould. The aqueous albumen solution was stored at 4°C until it was used.

All albumen treatments were done using previously prepared slides that were smeared from the host and then air-dried (Fig. 4.3). The experimental time between albumen application and silver nitrate impregnation was the same as the eight variations of the two methods for the gelatin substrate experiment (Table 4.2).

EXPERIMENT 3: GELATIN AS SUBSTRATE

In total 16 different variations were performed, eight variations (VIII - VXI) of the pre-smeared method and eight (I-VIII) for the post-smearing method. Weaver's gelatin fixative (Weaver 1955) was prepared as provided by Humason (1962), as follows:

One gram of gelatin was dissolved in 1L water by heating it up on a hotplate (microwave for two minutes in a more modern setting), and

shaken well after the first minute of heating. The dissolved solution was then left to cool down to room temperature, 0.1g $CrK(SO_4)_2$ (chromic potassium sulphate) was added and then stored at 4°C until use.

Method A (Fig. 4.3) (Pre-smearing slides): the slides were dipped in the aqueous gelatin mixture stock and left to air-dry. Hereafter the skin and gills of the hosts were smeared and left to dry on these gelatin prepared slides in a dry, dark locality for specific times of hours before silver impregnating using Klein's (1926) method as modified and discussed by Lom (1958). Variation I was impregnated with silver nitrate immediately after the wet-smeared slide was dry. Variation II was left for 24 hours before silver staining, variation III for 48 hours and so forth (Table 4.2).

Method B (Fig. 4.3) (Post-smearing slides): a drop or two of the aqueous gelatin stock mixture was immediately dripped onto the dried smeared slides. The treated slides were then left to dry in a dry, dark locality for specific times, as per experiment, before impregnated with silver nitrate (Table 4.2).

Table 4.2: Time elapsed between treatment/ host smear of slide and silver nitrate impregnation**.**

Variation number	Time left to dry before silver impregnation
I	Immediately after smears dried
II	24 hours
III	48 hours
IV	72 hours
V	96 hours
VI	120 hours
VII	1 week
VIII	2 weeks

EXPERIMENT 4: FISH MUCUS AS SUBSTRATE

Sixteen variations in total were performed. For this experiment two time frames were created, namely, time elapsed before treating the post-smeared slides with the fish mucus and time elapsed before impregnating the post-smeared slides with silver nitrate (Table 4.3) (Fig. 4.3).

The fish mucus used were obtained from three different species of fish, the first experiments (as discussed in **Experiment 1**) done at the Bloemfontein campus in South Africa, were both from imported fish species; *Cyprinus carpio*, using both the Common Carp and the Koi variant, and *Gambusia affinis;* the Mosquitofish.

Gambusia affinis was chosen because this species tends to have very few to no trichodinid infestations in its mucus, and therefore it eliminates the possibility of contaminating the prepared smears. Since these experiments yielded very positive impregnation results it was decided to use the mucus from an equivalent endemic fish species in the Okavango System to be applied at the Leseding Research Camp at Samochima.

Table 4.3: Time elapsed between host smear of slide and time before silver nitrate impregnation with fish mucus.

Variation number	Time before treated with mucus	Time before silver nitrate impregnation	Variation number	Time before treated with mucus	Time before silver nitrate impregnation
I	Immediately after drying		**IX**	48 hours	120 hours
II	24 hours	24 hours	**X**	72 hours	120 hours
III	24 hours	48 hours	**XI**	72 hours	144 hours
IV	48 hours	48 hours	**XII**	120 hours	168 hours
V	24 hours	72 hours	**XIII**	0	7 days
VI	48 hours	72 hours	**XIV**	0	192
VII	48 hours	96 hours	**XV**	0	192
VIII	24 hours	120 hours	**XVI**	0	14 days

The Okavango System is enclosed and has to date, no introduced or alien fish species. A suitable mucus donor needed to be found, preferably similar to *G. affinis*. The closest relatives to *G. affinis*, found the same order, Cyprinodontiformes in these waters were the topminnows, *Micropanchax* species. Besides being abundant in the waters of the Okavango River itself, they have, like the imported *Gambusia* species, very few to no trichodinids in their mucus.

These were caught using a seine net and kept in isolation from any other collected live material. To obtain their mucus, they were lightly smeared with a slide on the lateral sides of their bodies. Most topminnows used as mucus donors were Johnston's Topminnow, *M. johnstoni* (Günther, 1894), the Meshscaled Topminnow, *M. hutereaui* (Boulenger, 1913) and the Striped Topminnow, *M. katangue* (Boulenger, 1912).

Another aspect was also tested for the fish mucus experiments; whether there is a difference between impregnation when mucus-prepared slides were impregnated dry or submerged under distilled water, during the UV-light exposure phase of the silver nitrate technique. This was tested in variations 14 and 15, where variation 14 was left dry under the UV light and experiment 15 were submerged under distilled water. Both latter variations had a drying out period of 192 hours prior to impregnation.

As control method, the smeared slides were "dry" silver impregnated at 24-hour intervals without any substrate treatment, using a UV light.

EXPERIMENT 5: RADIATION

This experiment was constructed to challenge the pre-conception that freshly smeared slides must be air-dried in the absence of direct sunlight or heat.

To test whether there is a difference in the manner slides are prepared for impregnation and whether there is a notable difference between the radiation of the sun in the Okavango region to that of the UV light, the following four experimental methods were constructed (Fig. 4.3):

Method A: Slides were treated with $AgNO_3$, washed with distilled water (the usual silver method) and then the silver nitrate treated slide was manually dried (gently using a piece of tissue paper to absorb some of the distilled water residue left on the slide after the penultimate step in the downstream experiment) and placing the slide in the sun for 20 to 30 minutes.

Method B: The freshly prepared wet-slide was placed directly in the sun to dry before impregnation. After impregnation and washing, it was again left directly in the sun for 20 to 30 minutes.

Method C: This involved smearing the gills or skin of the host, manually drying the slide, leaving the smear in the sun for an hour, preparing the smear with fish mucus, drying the prepared slide in the sun for another hour, then impregnating the slide with silver nitrate and substituting the UV light for the sun again.

Method D: The same as method C, except that the smeared slide was not manually dried before leaving it in the sun, thus using the sun to dry the slide, before commencing with the rest of the method.

For the control group, the silver nitrate treated slides were left in the sun for 20 to 30 minutes after impregnation (replacing the UV light step of the impregnation process with the rays of Okavango sun), without manually drying the slide first and with none of the above-mentioned substrates.

RESULTS AND DISCUSSION

EXPERIMENT 1: SILVER NITRATE IMPREGNATION OF OLD SLIDES

Method A: No substrate

Variation II, using the UV-A light (Figs. 4.4**A** and 4.5**C**), yielded the best quality impregnations for Method A. A large difference between the three variants of this method was found (Figs. 4.5**A** to **D).** Variation I (Figs. 4.5**A** and **B**), in general, produced very poor-quality impregnations, which could not be of any use for taxonomic measurements. Variation III, also under UV light but after 24 hours, (Fig. 4.5**D**) produced better impregnations than Variation I, but was too grainy for measurements.

Method B: Albumen substrate

When albumin is applied and immediately radiated under UV light (Variation I), the results are of better quality and more detail in the denticles can be observed (Fig.4.4**B**) (Figs. 4.5**E** vs. 4.5**F**).

Method C: Gelatin substrates

Old slides coated with gelatin before being smeared tended to deliver better mean results under the UV light (Variation I) than under UV-A light (Variation II)**,** but the differences were statistically only marginal and implies that there is almost no difference between the averages of the two variations (Fig. 4.4**C**) More denticular detail is visible in the gelatin application developed under the UV-A light (Fig. 4.5**H**), compared to the grainy impregnation of the UV light development (Fig. 4.5**G**).

Method D: Fish mucus substrates

The mean outcomes of all the variations tested for this method is displayed in Figure 4.4**D**. The results indicate that between the two fish mucus donor species, *Cyprinus carpio* yielded the highest ranks and best impregnations (Variation VII and VIII). *Gambusia affinis* on the other hand showed very little difference between the temperature

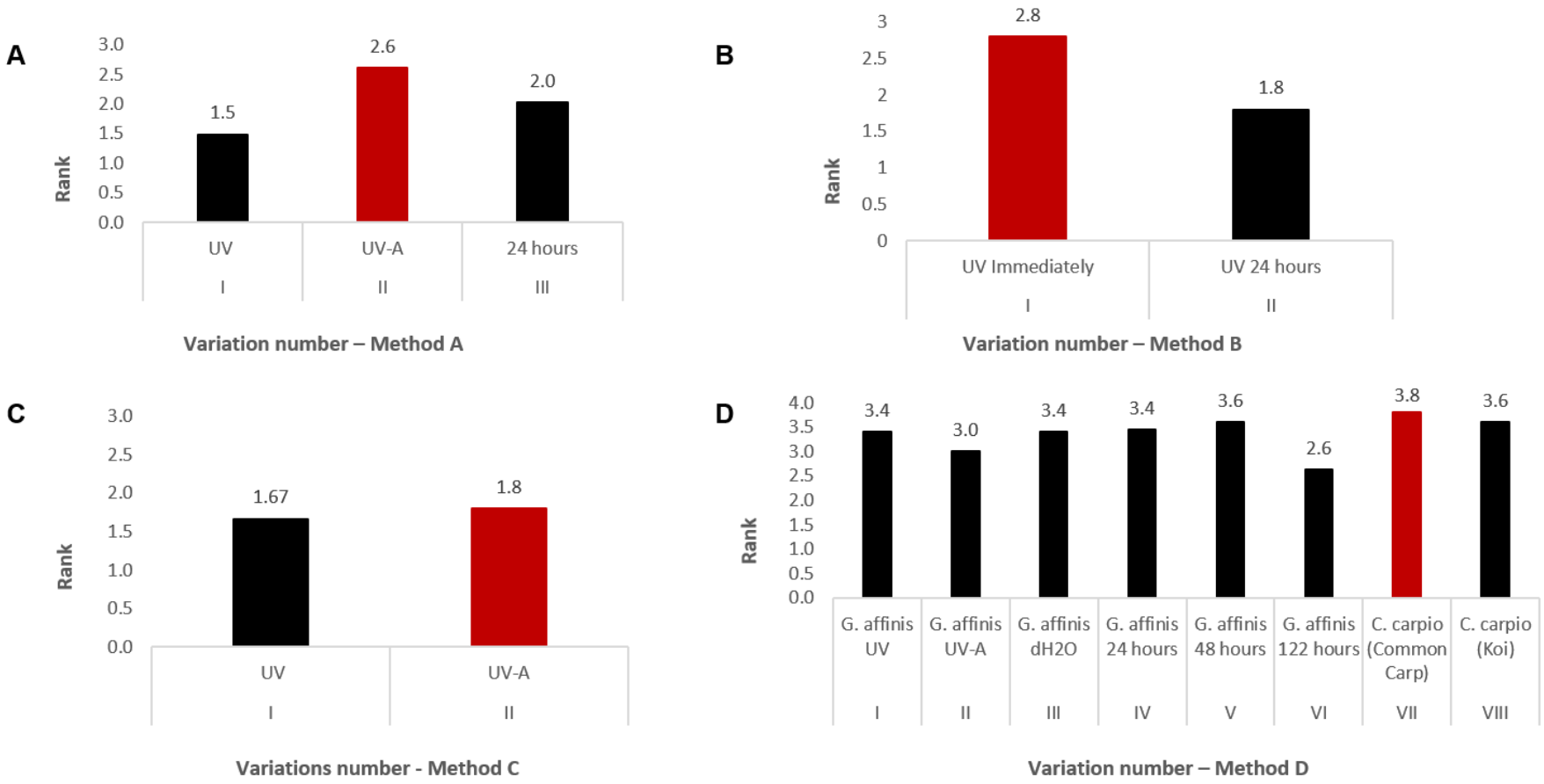


Figure 4.4: Ranking comparisons for the different silver nitrate methods for old slides. **A** – No substrate, with different UV radiation and time elapsed; **B** – Albumen substrate under UV light, at different times before impregnation; **C** - Gelatin substrate under different UV lights; **D** - Fish mucus methods under different UV lights, different species of fish mucus, different time before impregnation and ways of impregnating (dry or submerged under distilled water).

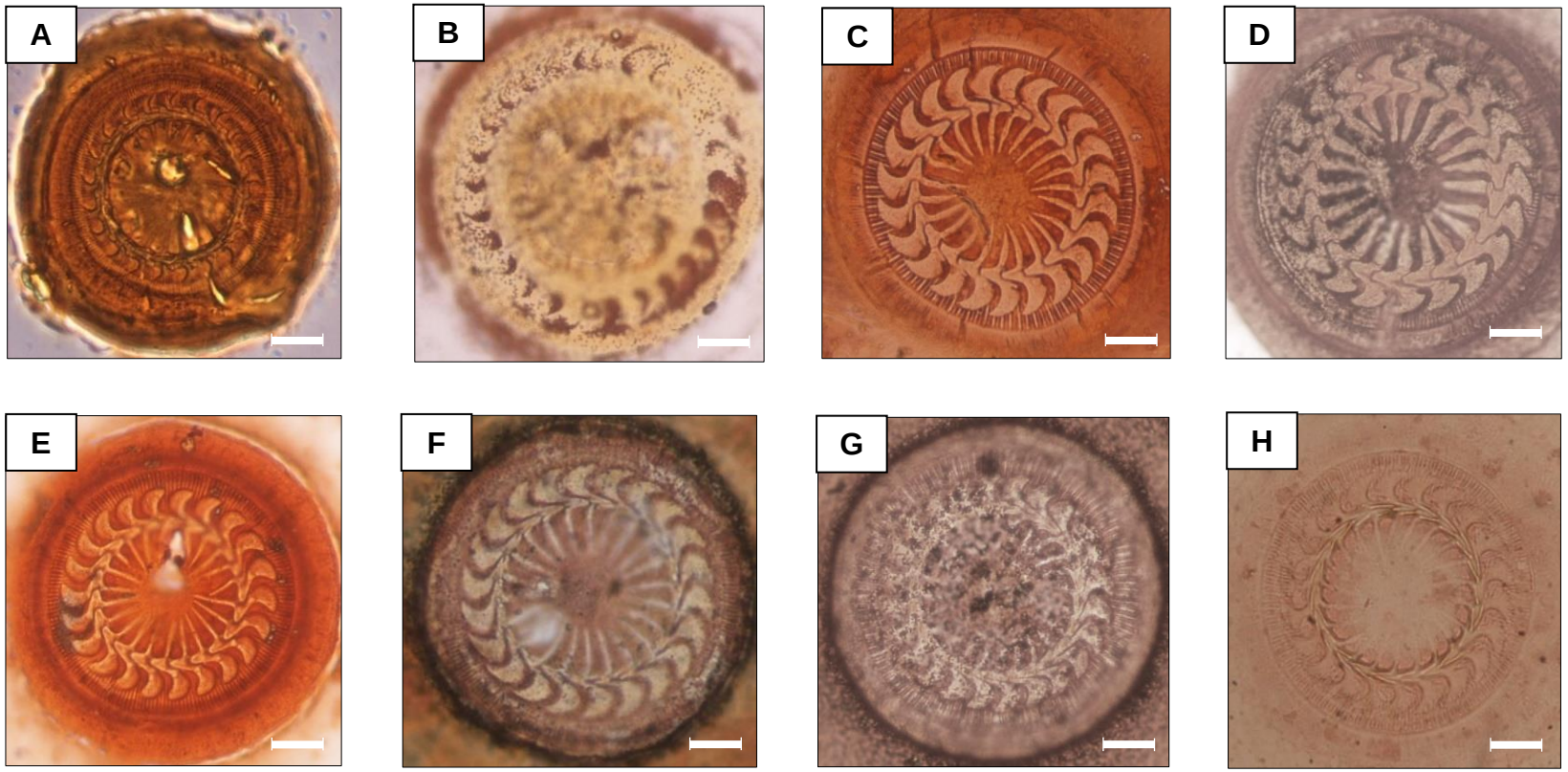


Figure 4.5: Representative micrographs of the impregnation results from the different silver nitrate substrate experiments to stain old slides. **A** and **B** – No substrate under UV light; **C** – No substrate under UV-A light; **D** – No substrate 24 hours after smear; **E** - Albumen substrate, immediate impregnation; **F** - Albumen substrate, UV, 24 hours before impregnation; **G** – Gelatin substrate, UV light; **H** - Gelatin substrate, UV-A light (scale = 10μm).

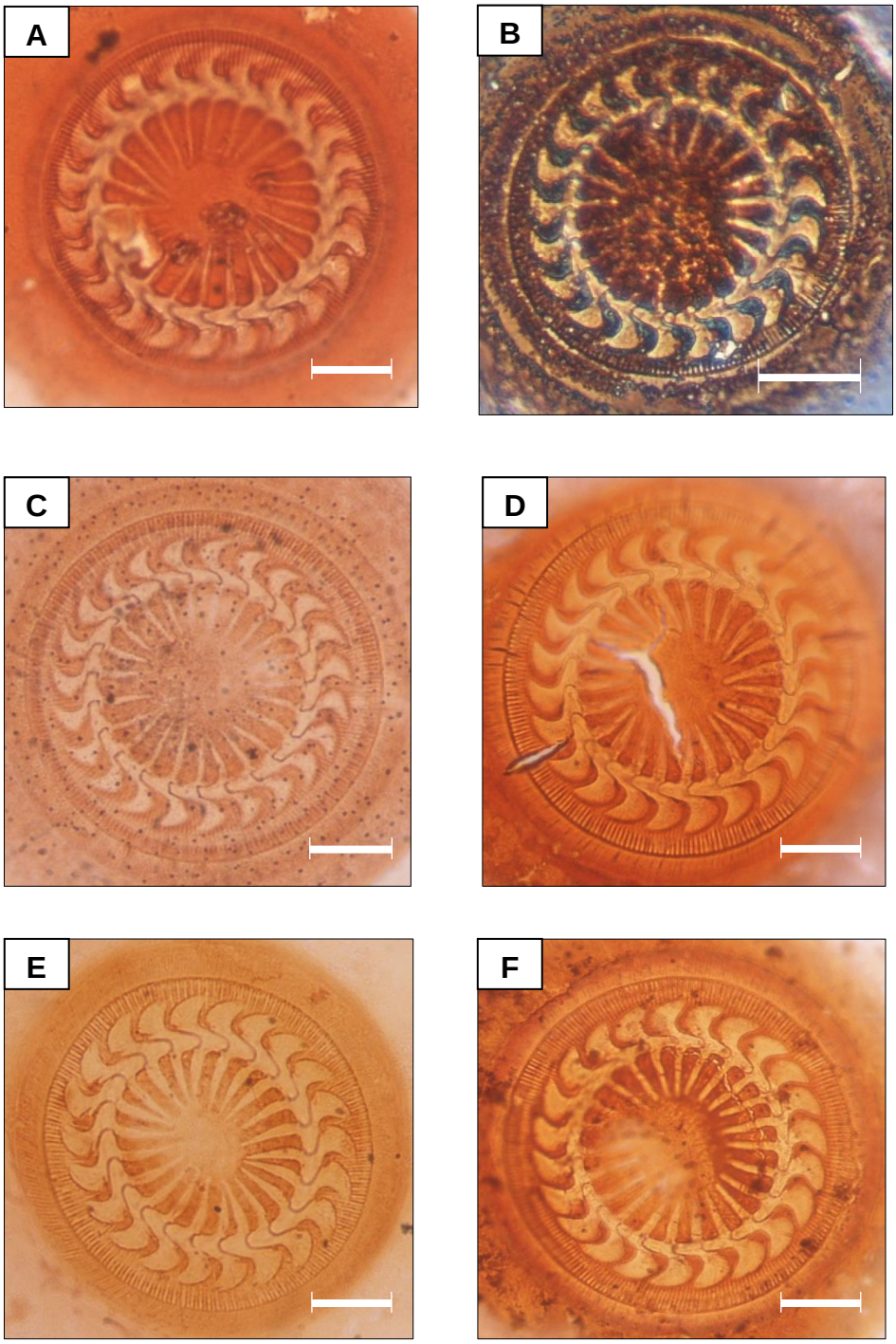


Figure 4.6: Representative micrographs of the impregnation results from the fish mucus silver nitrate substrate methods to stain old slides. **A** – *G. affinis* mucus under UV light; **B** – *G. affinis* mucus under UV-A light; **C** - Common carp (*C. carpio)* mucus under UV light; **D** - Koi (*C. carpio* variant); **E** – *G. affinis* mucus after 24 hours before staining; **F** – *G. affinis* mucus after 48 hours before staining (scale = 10μm).

and development media (under distilled water or dry). There does seem to be a marked difference between the radiation media, as Variation II, UV-A, ranked the second lowest of all the tested variations. Time also seemed to play an important role regarding *G. affinis* mucus as substrate, as the best impregnations were seen after 48 hours, but from 122 hours onwards very bad impregnations were observed. Figures 4.6**A** to **F** demonstrates the differences between fish mucus types and influence of time on the silver nitrate impregnation.

IMPREGNATION OF OLD SLIDES SUMMARY

Experiment 1 slides collected and kept in a lab, or which were not stained for more than ten years can be stained with relative ease. The best method for accomplishing this, from the above mentioned experimental methods and variations, seems to be by applying the mucus scraped from *G. affinis* fish. Type of light radiation plays an important role as the results shows that UV, rather than UV-A (black light) works the best. Figure 4.7 compared the most successful variations of the different methods used for Experiment 1.

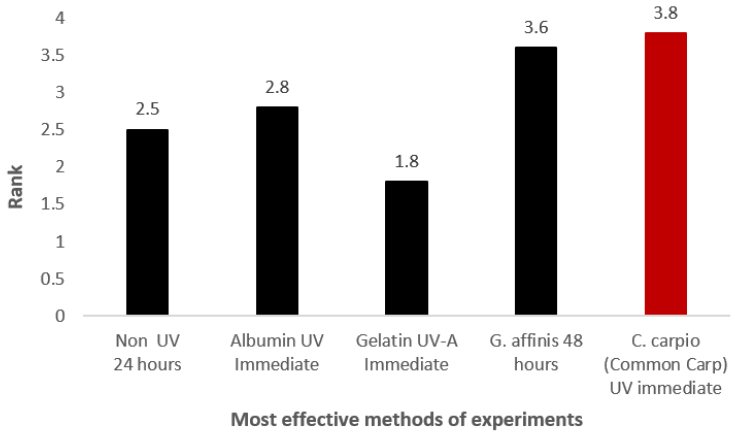


Figure 4.7: Comparisons of average ranks for all the different substrate methods for Experiment 1. The red indicating the highest ranking out of the groups of experiments.

Although *C. carpio* mucus resulted in the highest ranks observed, it must be added that members of the Cyprinidae family are known to host various ectotrichodinids on their skin and without a thorough examination of the collected mucus for possible trichodinid species contamination, erroneous inferences may be made about the species diversity of

the ciliophorans that were stained on the slides. *Gambusia affinis*, on the other hand, have very low, if any ectotrichodinids infestations on their skin and this reduces the possibility of "foreign" trichodinids marring the results.

It must be noted that in these experiments temperature didn't seem to play a significant role. Therefore; no comments can be made about its influence on impregnation and temperature was therefore omitted in the experiments prepared on location in Botswana**.**

EXPERIMENT 2: ALBUMEN SUBSTRATE

Using albumen as a substrate yielded generally low rankings which placed this method in the second "Inadequate" category of the ranking scale. The highest rank and best silver impregnation attained, was at 24 hours before staining, and the lowest at 96 hours. After one week, no to very little staining could be observed (Fig. 4.8).

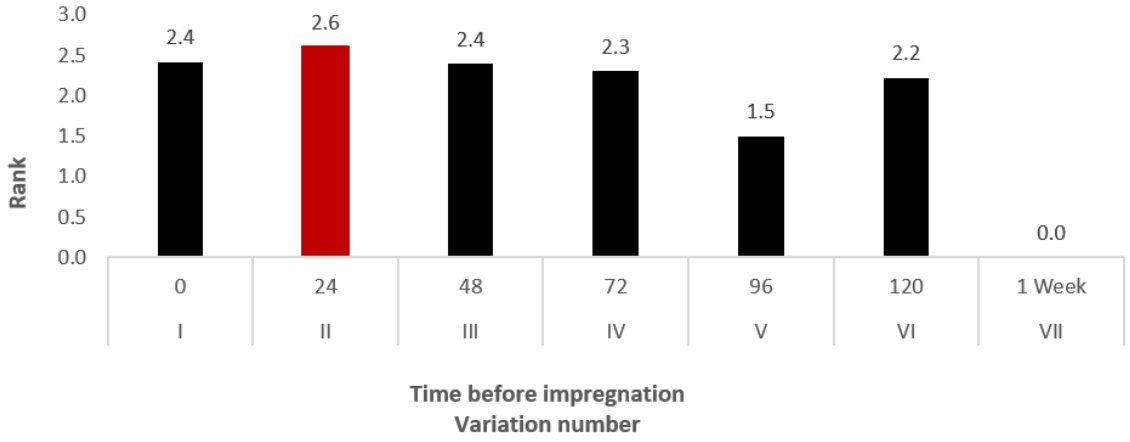


Figure 4.8: Ranking comparisons for the seven different variations of albumen as substrate for silver nitrate staining. The red indicating the highest ranking out of the groups of experimental variations.

EXPERIMENT 3: GELATIN SUBSTRATE

By comparing the average ranks of the different variations of the two gelatin methods, the method of applying gelatin to the already air-dried smears made from the host (Method B) yielded overall higher rankings (Fig. 4.9**B**) than those of Method A; preparing the slide with gelatin before smearing the skin and gills of the host (Fig. 4.9**A**).

Within each method group the time lapse differences were also noteworthy, for the post-gelatin method (Method B) the best results were yielded after leaving the treated slides 24 and 48 hours before silver nitrate impregnation (Fig. 4.14**D**). An increase in rankings as time elapsed up to 24/48 hours, then fluctuating slightly before suddenly dropping after one week was observed for Method B (Fig. 4.9B).

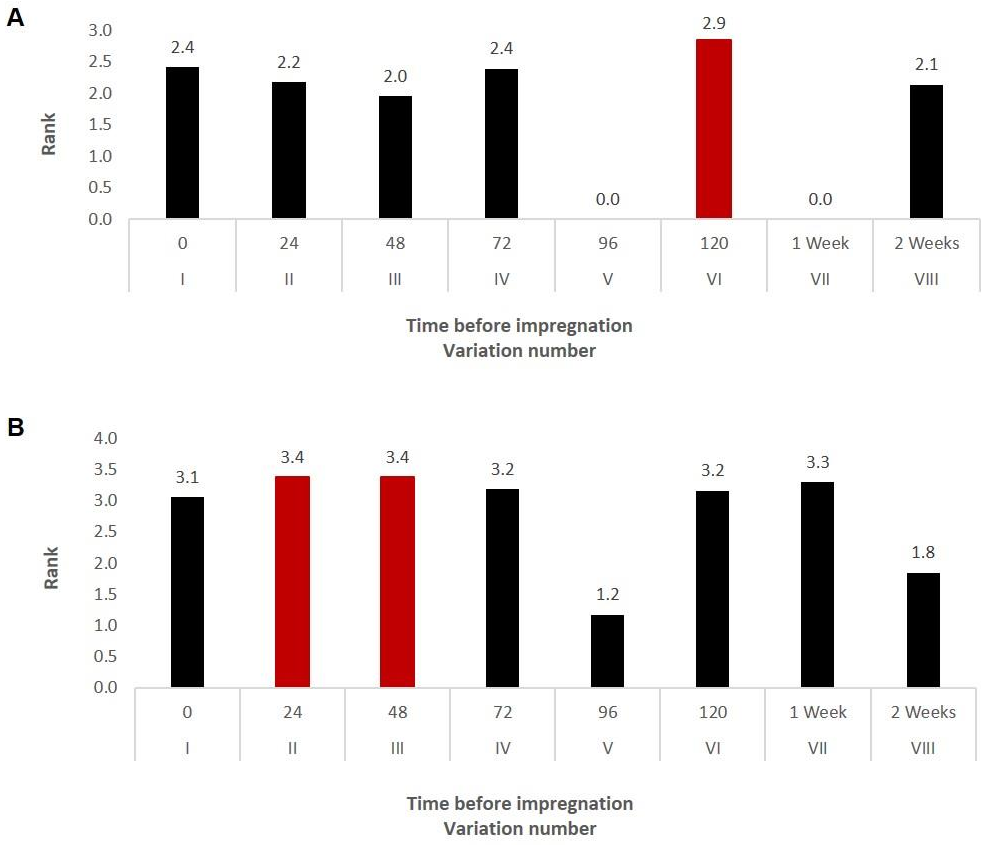


Figure 4.9: Ranking comparisons for the 16 different variations of the two gelatin methods as substrate for silver nitrate staining. **A** – Slides treated with gelatin pre wet-smear of host (Method A); **B** – Slides treaded with gelatin after wet-smear of host (Method B). In each case the red indicates the highest ranking for the group experiments.

For Method A (Fig. 4.9**A**), the opposite pattern for impregnation quality to that of Method B (Fig. 4.9**B**) was observed; the ranks steadily declined up to 48 hours before silver staining, which produced the lowest rank, then gradually increased again up to 120 hours before silver staining, which yielded the highest rank (Fig. 4.14**C**).

In both cases very bad impregnations (and no impregnation at all for Method A) were noted around 96 hours before silver staining.

EXPERIMENT 4: FISH MUCUS SUBSTRATE

Variation X yielded the highest rank from this experiment, where the smeared slide was dried for 72 hours, treated with the *Micropanchax johnstoni* mucus substrate and then left for another 48 hours (120 hours from initially smearing the host) before impregnation, while the weakest impregnation results was produced by Variation XI (72 hours/120 hours)(Fig. 4.10).

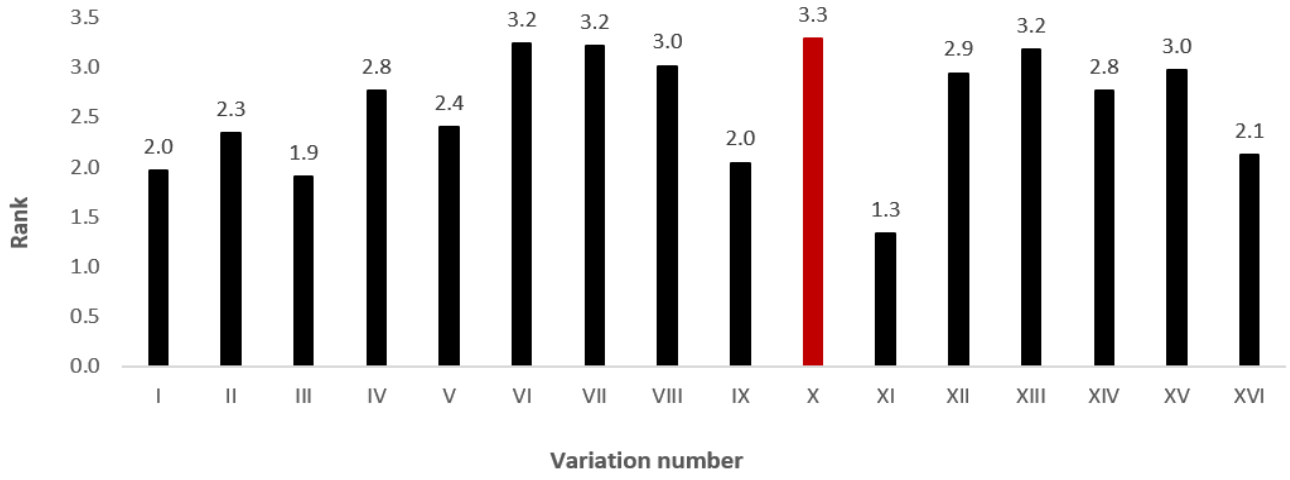


Figure 4.10: Ranking comparisons for 16 different variations of fish mucus as substrate for silver nitrate staining. The red indicating the highest ranking out of the groups of experiments.

Variations VI (48 hours/72 hours), VII (48 hours/96 hours) and XII (0 hours/7 days) also yielded high ranks, suggesting that the best time to leave the slides to dry is between 48 hours and 72 hours before being treated with fish mucus, alternatively good ranks were also observed from applying the mucus immediately and leaving the prepared slide from 1 to 1½ weeks before impregnating. A maximum time for impregnation can be noted when after two weeks the impregnations became less visible and measurements more difficult.

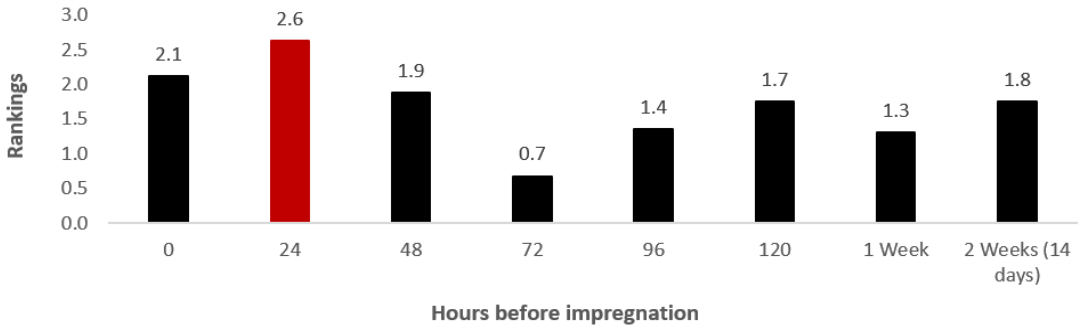


Figure 4.11: Ranking comparisons for the control group where no substrate for silver nitrate staining was used. The red indicating the highest ranking out of the groups of experiments.

Variations III (24 hours/48 hours) and XI (72 hours/144 hours) fell into the first rank category, “Poor”, and produced grainy overstaining with almost no measurements possible. The control group (Fig. 4.11) demonstrated that the best impregnation happened after 24 hours, with very little impregnation happening at 72 hours and beyond.

EXPERIMENT 5: RADIATION EXPERIMENTS

The clearest and best quality impregnations were obtained in Method C (Fig. 4.12), where the post-impregnated slide was first manually dried with a small tissue before commencing with the rest of the method. The control method delivered the most unclear impregnations, almost 50% less quality. Methods A and D provided the same rank in impregnation, but was only markedly less than Method C’s rank. Figure 4.14**F** shows a micrograph of a very successful impregnation using Method C.

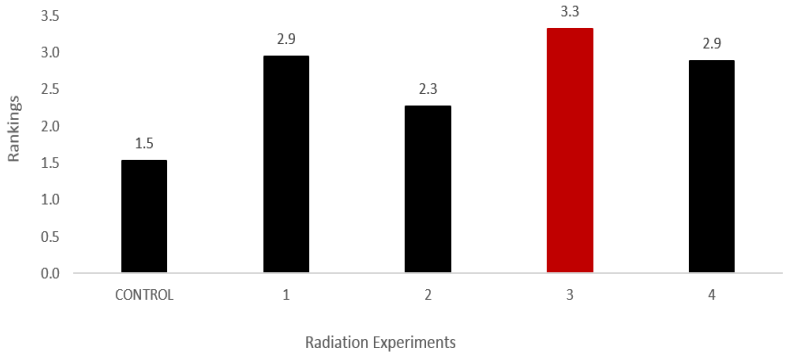


Figure 4.12:
Ranking comparisons for four different methods of radiation experiment for silver nitrate staining. The red indicating the highest ranking out of the groups of experiments.

SUMMARY OF SUBSTRATES AND RADIATION EXPERIMENTS

Comparing all the different substrate experiment methods, Experiments 2, 3 and 4 (Fig. 4.13) and their average ranks, the post-gelatin method (after both 24 and 48 hours) yielded the best impregnation results (Fig. 4.14**D**), but only marginally better than the fish mucus method (after 120 hours)(Fig. 4.14**E**). Using albumen as a substrate yielded the same clarity and usability as the control group that used no substrate at all (Fig. 4.14**B**). The difference between the albumen experiment and the control group (Fig. 4.14**A**) was that the albumen experiment tended to overstain the trichodinids to such a degree that very little was observable, whereas the control group tended to under-stain. The pre-gelatin method produced better impregnation than the control group and albumen experiment (Fig. 4.14**C**), but leaned towards causing grainier impregnation than the rest.

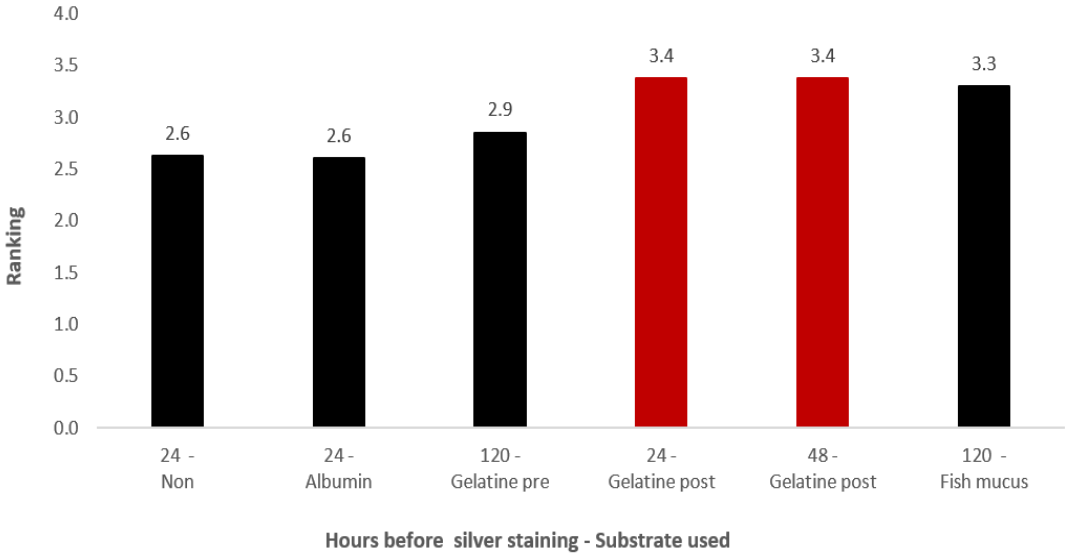


Figure 4.13: Comparisons of average ranks for all the different substrate experiments and methods for silver nitrate staining. The red indicating the highest ranking out of the groups of experiments.

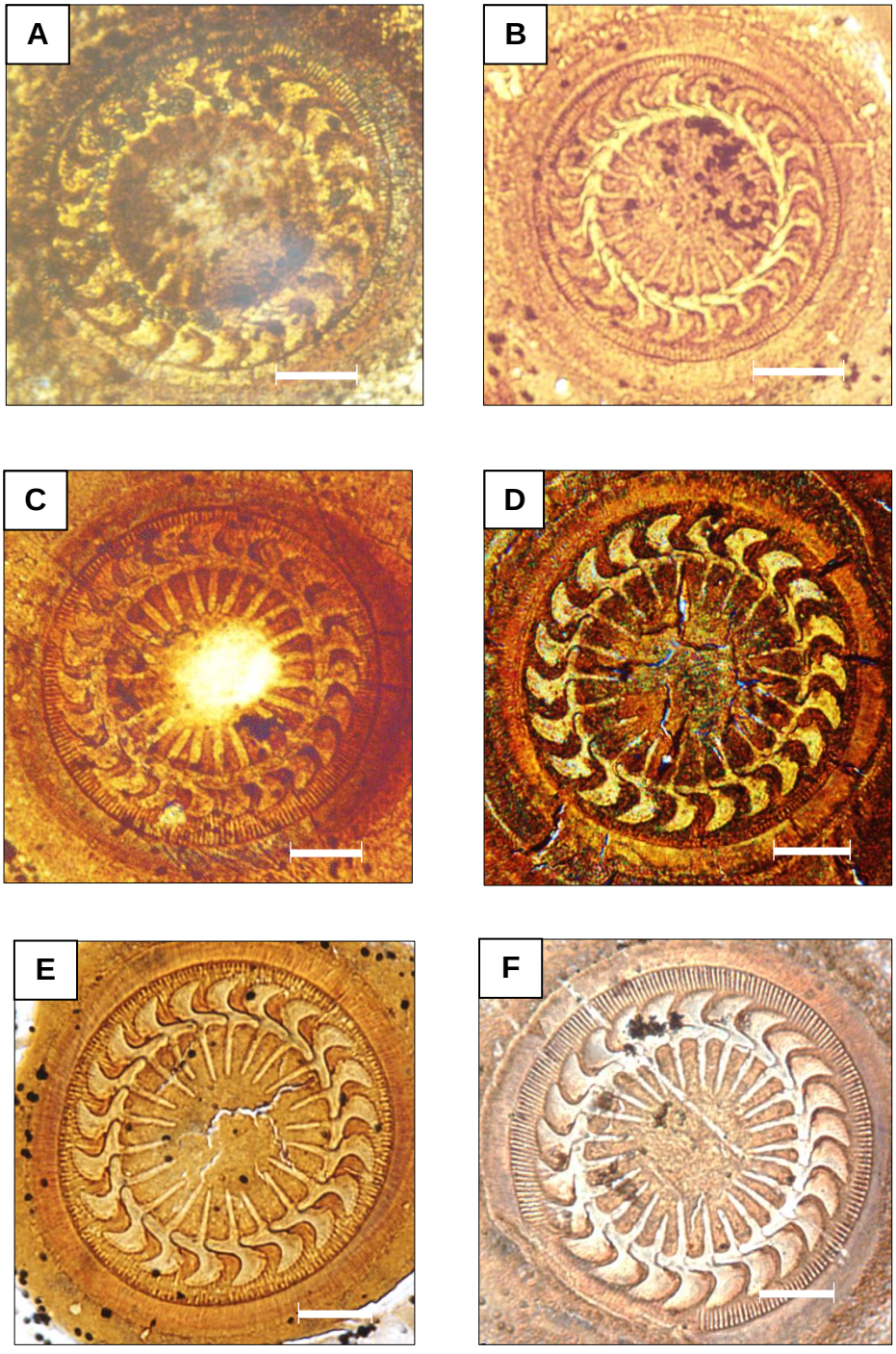


Figure 4.14: Representative micrographs of impregnation experiments 2 to 4. **A** – Control group with no substrate; **B** – Albumen experiment, 24 hours after smear; **C** - Pre-gelatin method, 24 hours after smear; **D** - Post-gelatin method, 48 hours after smear; **E** - Fish mucus methods; Variation X, 72 hours after treated with mucus and 120 hours before stained; **F** - Radiation Method C with fish mucus substrate (scale = 10 µm).

During the radiation experiments, Method C, where the excess water was gently removed, delivered the best results (Fig. 4.14**F**). This method was to be expected to yield good results, as Klein (1958) discussed the influence of high electrolyte concentrations with too much water. The drawback of this method is that high trichodinid numbers must be present on the smeared sample, as loss of material usually occurs when manually drying the slide. Although a large trichodinid sample smeared is recommended when manually drying the slide before impregnation, is not necessarily a restriction of this method; in the case of a very low infestation; extreme care when using this method, could restrict loss of material.

The fact that the radiation experiments yielded such superb results, by drying freshly smeared slides that were in direct sunlight at all times, demonstrates that the pre-conception of air-drying slides protected from bright light is doubtable at best, and in the case of material collected from anuran hosts dried under the Botswanan sun, this appears to be a fallacy.

GENERAL AND CONCLUDING REMARKS

In the past, it was seen that old material (that hasn't been stained yet), especially those collected from anuran hosts, seemed to impregnate very badly or, most of the time, not at all. Experiment 1 verified that for anuran slides left for 10 years, it is possible to still get useable to good slides, negating Lom's comment that all slides must be impregnated before 48 hours. Because of its historical success with fish symbionts when using the Chatton-Lwoff (1930) method, the inadequate results of the gelatin method was unanticipated. Albumin as a substrate gave better results than the control method (no-substrate), but fish mucus (preferably a fish order that has low trichodinid numbers) gave the best overall results, when impregnated under UV light.

When impregnating newly made slides, it was observed that the best two substrates to use were gelatin, applied after the smear has been made, and fish mucus. The difference between these two were so marginal that it is safe to state that in the field the use of fish mucus would be the chosen method, as the preparation of gelatin and the storage thereof

can be time consuming and challenging. The period before and after applying the substrate also played a crucial role, and it seems that the optimal time to let slides dry with the mucus substrate before impregnation is 48 to 72 hours. Hereafter it must be stained with silver nitrate within two weeks for optimal results. When using fish mucus, the results can then be amplified by using direct sun as radiation, rather than a UV or UV-A light.

From the above experiments, it can be confirmed that trichodinid samples collected from tadpoles in southern Africa do not appear to yield the same impregnation success as those of fish hosts. Experiments 2 to 4 indicated that by adding a substrate to the smear, better results can be obtained, implying that the natural mucus/slime of anuran hosts is not enough for successful impregnation. The following two protocols should be helpful when impregnating trichodinids collected from anuran hosts from sub-Saharan Africa:

1. Old slides:

Already smeared slides that have been left for up to 10 years must be treated with a fish mucus substrate. The fish mucus should be obtained from fish donors known to have few to no trichodinids, preferably from the order Cyprinodontiformes. If none of this order can be found, the mucus from any carp or koi may be used, but extreme care must be taken to inspect the mucus from every individual fish for fish trichodinids. After the slides have been treated with the substrate, they should be left to dry between 24 and 48 hours before impregnation with silver nitrate under UV light for 15 to 20 minutes at room temperature.

2. Newly collected slides:

Slides that were freshly smeared from the gills or skin of their anuran host must be manually dried using a tissue or soft absorbent cloth, taking great care to soak up just the excess liquid and reduce material loss. The slide should then be air-dried further for an hour in direct sunlight before being treated with the fish mucus substrate (the same

directions apply here as for the old slides). The treated slide must then be dried in the sun again for an hour, without manually drying as before. The treated smear should now be impregnated with silver nitrate, as described by Klein (1926) and left in the sun to reduce for 15 to 20 minutes.

Plate A (**Appendices**: page A) presents of the above mentioned protocols in a visual diagram incorporating Figures 4.2 and 4.3.

The above mentioned techniques will need to be tested for different localities and different trichodinid hosts. Many questions arose during the experiment that need to be addressed in future research. These questions include;

- Why does it seem that a “cut-off” period for impregnation is visible after 96 hours for gelatin and albumin substrates, but not for fish mucus?

- What exactly in the fish mucus is responsible for such good impregnations?

- Do environmental factors, such as temperature, humidity or position of the sun play a role?

- What is the influence of the ambient variants, i.e. the conductivity of the water, where the hosts are found on?

CHAPTER 5 - RESULTS – MORPHOLOGY

HÆMATOXYLIN STAINING

Hæmatoxylin staining is used as part of Lom's (1958) standardised morphological characteristics for species descriptions. Because this study deals with, what seems to be, a single species collected from tadpoles in the Nxamasere Floodplain, nuclear description and the course of the adoral spiral does not play such a big taxonomic role and thus the focus was centered around silver impregnated observations. This said, the nuclear apparatus and course of the adoral zone must still be mentioned.

NUCLEAR APPARATUS:

Adult *Trichodina heterodentata* specimens collected from tadpoles had a C-shaped macronucleus (Figs. 5.1**A** to **E)** with a mean external diameter of 31.1-57.4μm (47.0±9.5), thickness of 4.5-11.3μm (7.2±2.5) and length of area between terminations of macronucleus 3.8-38.5μm (21.1±13.7). In some specimens the macronucleus was in the process of division and the nucleus was broken up into spherical shapes (Fig. 5.1**F**). No micronucleus was observed.

ADORAL SPIRAL

The adoral spiral followed a course of 370°- 405° (visible in Fig. 5.1**F**), which falls perfectly with the variation for the genus *Trichodina*.

SILVER NITRATE IMPREGNATIONS

Biometric data results will be discussed in three sections; comparing the denticular measurements, as proposed by Lom (1958), for all six populations collected during the same season (winter of 2016), then describing the morphological differences between trichodinids collected from the same pool (Nxamasere pool 1) during different seasons (winter of 2015 along with winter and summer of 2016). The last section will be a detailed description of the individual denticles, using the van As and Basson (1989) technique.

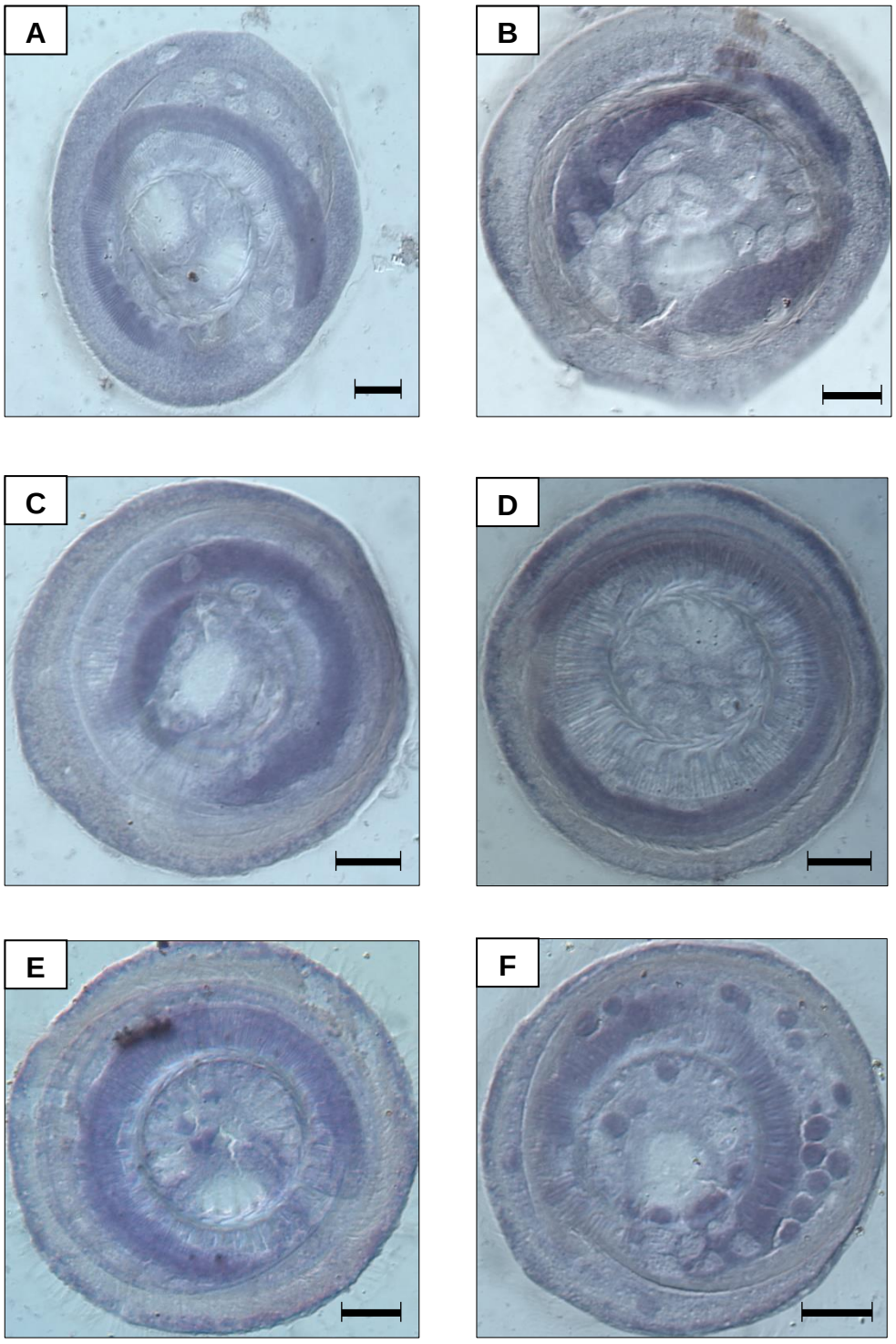


Figure 5.1: Micrographs of hæmatoxylin stained nuclear material for representatives of the different *Trichodina heterodentata* Duncan, 1977, populations of the winter 2015 collection. **A** to **E** shows the different orientations of the macronuclei clearly in the adult stages, whereas **F** illustrates a macronucleus in the process of breaking up before binary fission, with a prominent adoral spiral (scale = 10µm).

Even though the morphometrical data obtained from the teleost hosts are not of taxonomic importance for the classification of the anuran trichodinids for this dissertation, it is still important to compare the two host types' trichodinids, as *T. heterodentata* is known to occur on the fish and tadpole species. The teleost data collected during the winter of 2015 is compared to the anuran populations from Nxamasere pool 5. All morphometrics attained from the Botswana populations will be compared to those found in previous descriptions of *T. heterodentata* (Table 2.9, **Chapter 2**).

BIOMETRIC DATA FROM ANURAN HOSTS

Winter 2016

Based on the comparative morphological dimensions of all six populations, with only the minimum and maximum values given, the trichodinids had a convex body diameter ranging from 43.5-62.6μm; adhesive disc diameter between 37.3-51.2μm with a poorly to well-developed border membrane with width (2.7-5.7μm) (Table 5.1, Fig. 5.2). The denticle ring diameter is between 21.3 and 35.3μm consisting of 20 to 28 denticles and eight to 13 radial pins per denticle. The denticle blades are strong, semi-circular with a prominent apophysis on the anterior side with a length from 4.3-7.1μm, tapering off towards a sharp pointing tip. The ray of the denticle is strong, generally straight, but curved backwards near the central part connection and tapers off towards the tip, it has a length ranging from 4.5-7.4μm. The central part width is from 1.2-2.7μm and the total denticle span is between 5.9-16.0μm.

There is little biometric variation between trichodinids collected from the different pools and hosts (*S. poweri* found in Nxamasere pool 6). All the biometric data falls into the size category for *T. heterodentata* recorded from southern African and South American anuran tadpole host provided by Kruger *et al.* (1991); Kruger *et al*. (1993b); Kruger *et al*. (1995); Dias *et al*. (2009); Fernandes *et al*. (2011) and Harris *et al*. (2013).

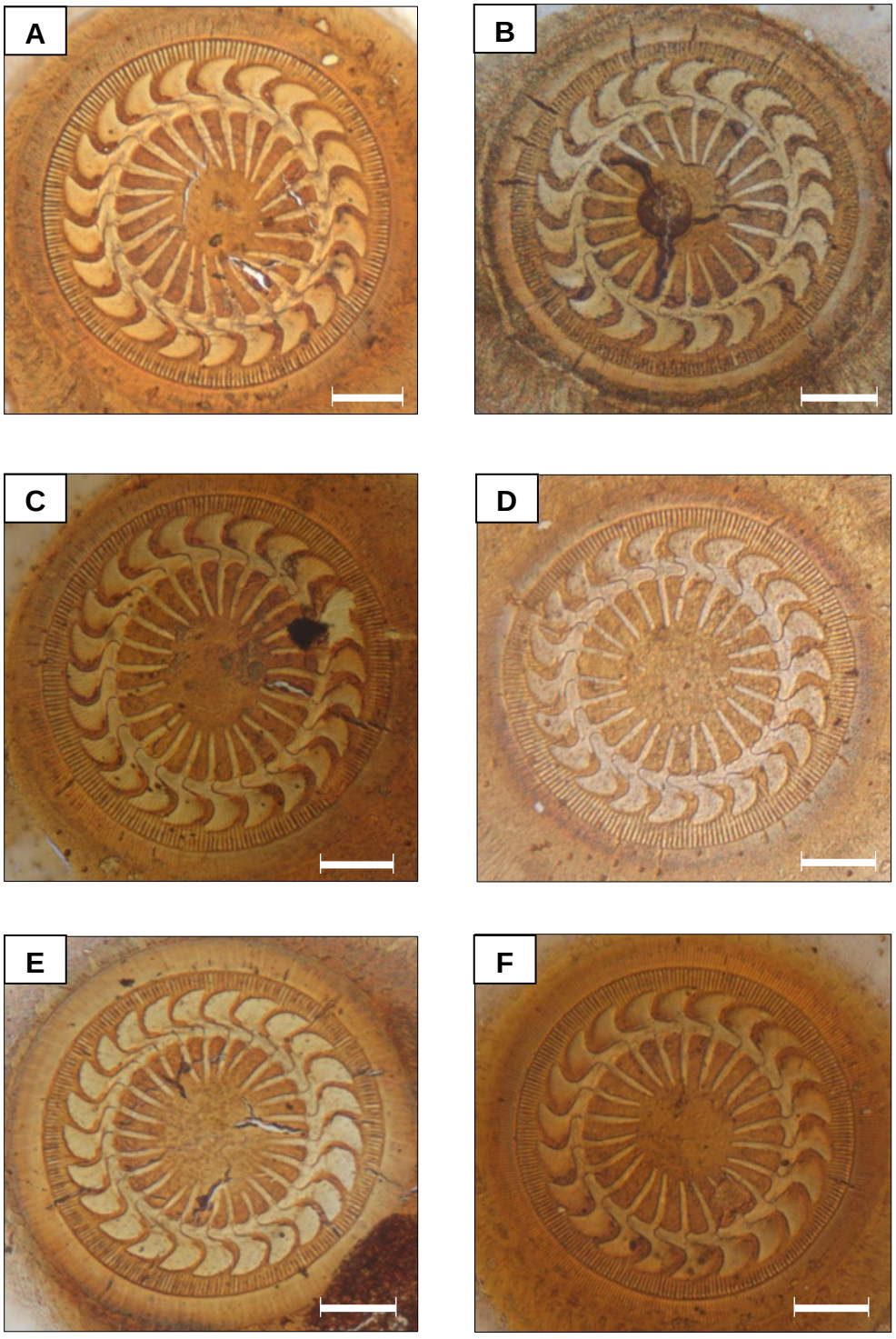


Figure 5.2: *Trichodina heterodentata* Duncan, 1977, representatives of the six populations measured from the Nxamasere Floodplain: **A -** NX1; **B -** NX2; **C -** NX3; **D -** NX4; **E -** NX5; **F -** NX6 collected from anuran hosts during the 2016 winter (July to August) expedition (scale = 10µm).

Table 5.1: Biometrical data (in µm) of the six *T. heterodentata* Duncan, 1977 populations collected from the Nxamasere floodplain during the winter of 2016 from tadpole hosts (ADD - Adhesive disc diameter, BD - Body diameter, BMW - Border membrane width, CL – Collection locality, DBL - Denticle blade length, DCPW - Denticle central part width, DL - Denticle length, DRD - Denticle ring diameter, DRL - Denticle ray length, DS - Denticle span, HS - Host species, L – Locality, LoH - Location on host, n - population size, nD - Number of denticles, nRP/D - Number of radial pins per denticle).

L	**Nxamasere 1**	**Nxamasere 2**	**Nxamasere 3**	**Nxamasere 4**	**Nxamasere 5**	**Nxamasere 6**
Year	2016	2016	2016	2016	2016	2016
LoH	Skin & Gills	Skin & Gills	Skin & Gills	Skin & Gills	Skin & Gills	Skin & Gills
HS	*Schlerophrys gutturalis*	*Schlerophrys gutturalis*	*Schlerophrys gutturalis*	*Schlerophrys gutturalis*	*Schlerophrys gutturalis*	*Schlerophrys poweri*
BD	47.6-59.1 (53.9 ± 3.3)	43.5-60.0 (51.9 ± 4.1)	47.1-60.0 (55.3 ± 3.3)	47.0-57.4 (53.3 ± 2.6)	45.9-57.9 (51.9 ± 3.2)	46.8-62.6 (54.5 ± 3.5)
ADD	38.6-48.2 (44.9 ± 2.9)	37.9-51.2 (43.4 ± 3.6)	39.3-50.9 (46.3 ± 3.1)	40.4-48.2 (44.7 ± 2.2)	37.3-48.7 (43.4 ± 3.2)	37.9-51.2 (45.5 ± 3.3)
DRD	24.6-30.9 (28.0 ± 1.8)	21.3-32.6 (26.9 ± 2.5)	25.6-32.7 (29.4 ± 1.9)	24.5-32.4 (28.0 ± 1.8)	22.4-32.9 (27.7 ± 2.6)	22.1-35.3 (28.7 ± 2.7)
DW	5.4-7.3 (6.3 ± 0.6)	5.4-7.3 (6.3 ± 0.6)	5.6-8.1 (6.7 ± 0.7)	5.7-7.8 (6.7 ± 0.5)	5.5-7.2 (6.2 ± 0.5)	5.9-7.1 (6.5 ± 0.4)
DBL	5.4-6.3 (5.9 ± 0.2)	4.3-6.5 (5.5 ± 0.5)	4.6-7.1 (5.8 ± 0.5)	5.0-6.6 (5.7 ± 0.4)	5.0-6.3 (5.6 ± 0.4)	5.0-6.4 (5.8 ± 0.4)
DRL	5.3-7.4 (6.6 ± 0.5)	5.1-8.6 (6.4 ± 0.9)	5.2-8.0 (6.9 ± 0.7)	5.3 – 8.0 (6.7 ± 0.6)	4.5-8.9 (6.5 ± 0.9)	5.4-7.9 (6.7 ± 0.6)
DCPW	1.3-2.1 (1.7 ± 0.2)	1.2-1.9 (1.5 ± 0.2)	1.4-2.7 (1.9 ± 0.3)	1.4-2.3 (1.9 ± 0.2)	1.3-2.2 (1.8 ± 0.3)	1.5-2.1 (1.8 ± 0.2)
DS	11.4-14.5 (13.2 ± 0.7)	10.6-14.5 (12.6 ± 1.2)	11.8-16.0 (13.7 ± 1.1)	12.0-14.8 (13.3 ± 0.7)	10.2-15.5 (12.8 ± 1.2)	5.9-7.1 (6.5 ± 0.4)
BMW	2.9-5.0 (4.4 ± 0.5)	2.7-5.2 (4.4 ± 0.5)	3.7-5.5 (4.6 ± 0.5)	3.1-5.7 (4.5 ± 0.7)	3.1-4.9 (4.2 ± 0.5)	2.7-5.7 (4.6 ± 0.6)
nD	20-26 (22 ± 1.4)	20-25 (22 ± 1.4)	22-28 (24 ± 2)	20-24 (22 ± 1)	22-28 (25 ± 1.5)	21-25 (23 ± 1.3)
nRP/D	9-12 (11 ±1.0)	8-12 (10 ±1)	9-13 (10 ±1)	10-12 (11 ±1)	9-13 (10 ±1)	9-12 (10 ±0.8)
n	25	25	25	25	25	25

Seasonal Measurements

Trichodinids collected from anuran hosts during the winter of 2015, winter of 2016 and summer of 2016 were silver impregnated and measured (Table 5.2, Fig. 5.3). When comparing these three different seasonal populations from the same locality (Nxamasere pool 1 (NX1)), it becomes apparent that there is very little difference between any of the morphological characteristics. Focusing on minor differences such as the adhesive disc diameter; the average in the summer is larger, but the maximum individual diameters were larger for both the winter populations.

Table 5.2: Biometrical data (in µm) of *T. heterodentata* Duncan, 1977 populations comparing three different collection expeditions, during the winter of 2015 and 2016, and summer 2016 from the same locality (ADD - Adhesive disc diameter, BD - Body diameter, BMW - Border membrane width, CL – Collection locality, DBL - Denticle blade length, DCPW - Denticle central part width, DL - Denticle length, DRD - Denticle ring diameter, DRL - Denticle ray length, DS - Denticle span, HS - Host species, L – Locality, LoH - Location on host, n - population size, nD - Number of denticles, nRP/D - Number of radial pins per denticle).

L	**Nxamasere 1**	**Nxamasere 1**	**Nxamasere 1**
Year	WINTER 2015	WINTER 2016	SUMMER 2016
LoH	Skin & Gills	Skin & Gills	Skin & Gills
HS	*Schlerophrys gutturalis*	*Schlerophrys gutturalis*	*Schlerophrys gutturalis*
BD	42.8-62.0 (50.7 ± 4.8)	46.5-63.7 (54.6 ± 4.3)	47.6-59.1 (53.9 ± 3.3)
ADD	34.8-53.2 (41.9 ± 4.5)	38.5-54.5 (41.6 ± 4.0)	38.6-48.2 (44.9 ± 2.9)
DRD	21.3-32.0 (26.5 ± 2.9)	24.1-34.5 (28.6 ± 2.8)	24.6-30.9 (28.0 ± 1.8)
DW	5.3-7.0 (6.2 ± 0.5)	5.3-7.0 (6.2 ± 0.5)	5.4-7.3 (6.3 ± 0.6)
DBL	3.6-5.6 (4.6 ± 0.5)	4.7-6.5 (5.7 ± 0.5)	5.4-6.3 (5.9 ± 0.2)
DRL	4.8-8.5 (6.2 ± 0.8, 25)	5.3-9.3 (7.0 ± 0.9)	5.3-7.4 (6.6 ± 0.5)
DCPW	1.2-2.2 (1.7 ± 0.3, 25)	1.2-2.6 (1.7 ± 0.3)	1.3-2.1 (1.7 ± 0.2)
DS	10.9-14.8 (12.3 ± 1.1, 25)	11.1-16.5 (13.6 ± 1.2)	11.4-14.5 (13.2 ± 0.7)
BMW	2.9-5.2 (4.4 ± 0.5)	2.6-5.4 (4.5 ± 0.6,)	2.9-5.0 (4.4 ± 0.5)
nD	20-29 (23 ± 2.0)	21-27 (24 ± 2.0)	20-26 (22 ± 1.4)
nRP/D	7-12 (9 ±1.0)	7-12 (10 ±1.0)	9-12 (11 ±1.0)
n	25	25	25

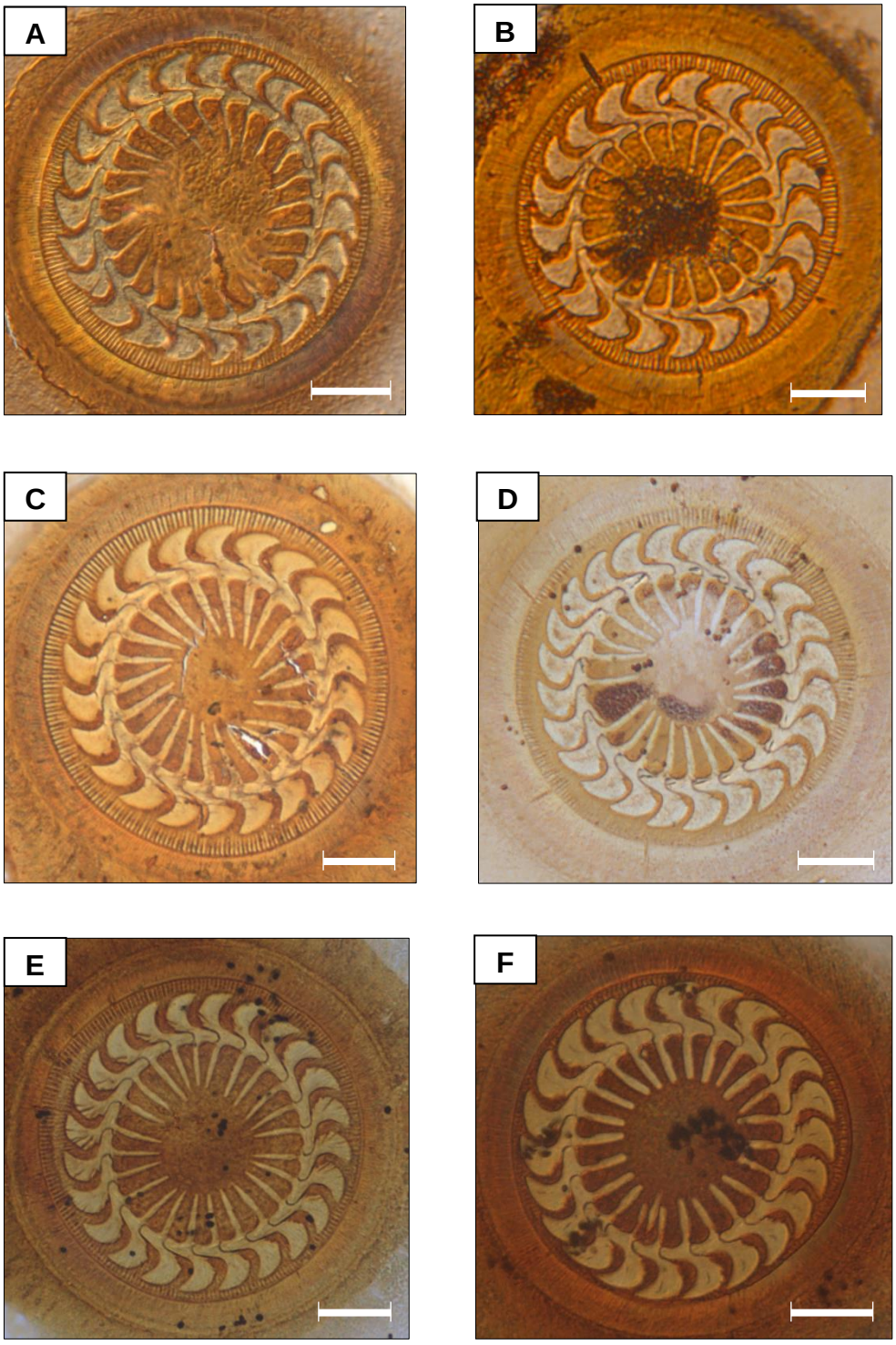


Figure 5.3: *Trichodina heterodentata* Duncan, 1977, representatives from population NX1 during winter 2015 (**A** & **B**), winter of 2016 (**C** & **D**) and summer (**E** & **F**) of 2016 (scale = 10µm).

The winter 2016 specimens (Table 5.2 and Figs. 5.3**C** & **D**) were the largest for body diameter, denticle ring diameter, border membrane width, denticle ray length, denticle span and number of denticles, compared to the winter 2015 specimens (Table 5.2 and Figs 5.3**A** & **B**) that had the smallest dimensions for most of the measurements. The summer specimens (Table 5.2 and Figs. 5.3**E** & **F**) showed the largest dimension for denticle length, denticle blade length and number of radial pins per denticle. The average of the denticle central part width was the same for all three seasons measured. There appears to be minimal seasonal variation within this environment. The small morphometric variation between the different localities (Table 5.1) and seasons (Table 5.2), even though it negates the findings of Kazubski and Migala (1968), is understandable because of the specific climate of the Okavango Delta region.

BIOMETRIC DATA FROM TELEOST HOSTS

The morphological data measured for *P. philander* trichodinids during the winter of 2015 compared to observations described from other African and international teleost *T. heterodentata* trichodinids (**Chapter 2**: Fig. 2) shows extremely similar data:

Basson *et al.* (1983) also described *T. heterodentata* from South African *P. philander* which yielded almost the same dimensions as those collected from Botswana in 2015. The Namibian *T. heterodentata* populations measured from three different fish species (again including *P. philander*) by van As and Basson (1992) and those measured from *Hydrocynus forskalli* in the Egyptian Nile (Al-Rasheid *et al.* 2000) was only slightly smaller than those from Botswana 2015 populations. International descriptions (**Chapter 2**: Table 2.10) show that the Botswanan *T. heterodentata* is the same size order as those described from populations in Taiwan (Albaladejo & Arthur 1989; Basson & van As 1994), Israel (van As & Basson 1989), India (Asmat 2004), Peru (Martins *et al.* 2010), Turkey (Öztürk & Çam 2013) and Southeast Brazil (Valladão *et al*. 2013). Measurements from populations in the Philippines (Bondad-Reantaso & Arthur 1989), China (Tao *et al.* 2008) and Brazil (de Pádua *et al*. 2012) are slightly smaller, whereas *T. heterodentata* described from the Philippines (Duncan 1977) (see **Chapter 7**), Venezuela (van As & Basson 1989), Indonesia (Dana & Hadiroseyani 2002), Australia (Dove & O'Donoghue 2005) and

Thailand (Worananthakij & Maneepitaksanti 2014) are larger. The Botswana trichodinid's morphometric measurements from *P. philander* falls perfectly within the medium sized *T. heterodentata* group as described in **Chapter 2**.

By comparing the biometric data of the teleost hosts collected in 2015 to those of the anuran hosts collected in 2016 (Table 5.3), the trichodinids from *P. philander* (Figs 5.4**B**, **D** & **F**) have larger dimensions than those from the *Schlerophrys* hosts (Figs. 5.4**A**, **C** & **E**). The number of denticles are the same for both populations, but is usually a relatively constant characteristics for *T. heterodentata* from various host types. The most striking morphometric difference is the adhesive disc diameter, which in comparison with the rest of the two data sets, is larger for the teleost trichodinids.

Table 5.3: Biometrical data (in µm) of two *Trichodina heterodentata* Duncan, 1977 populations comparing those from *Schlerophrys gutturalis* (Power, 1927) hosts and those from *Pseudocrenilabrus philander* (Weber, 1897) hosts during the winter of 2016 (ADD - Adhesive disc diameter, BD - Body diameter, BMW - Border membrane width, CL – Collection locality, DBL - Denticle blade length, DCPW - Denticle central part width, DL - Denticle length, DRD - Denticle ring diameter, DRL - Denticle ray length, DS - Denticle span, HS - Host species, L – Locality, LoH - Location on host, n - population size, nD - Number of denticles, nRP/D - Number of radial pins per denticle).

L	**Nxamasere**	**Nxamasere 5**
Year	WINTER 2015	WINTER 2016
LoH	Skin & Gills	Skin & Gills
HS	*Pseudocrenilabrus philander*	*Schlerophrys gutturalis*
BD	46.3-63.8 (56.5±4.4)	45.9-57.9 (51.9±3.2)
ADD	40.0-54.6 (47.5±4.0)	37.3-48.7 (43.4±3.2)
DRD	25.1-36.6 (30.4±2.9)	22.4-32.9 (27.7±2.6)
DW	4.9-7.4 (6.2±0.7)	5.5-7.2 (6.2±0.5)
DBL	5.1-6.9 (5.8±0.5)	5.0-6.3 (5.6±0.4)
DRL	5.9-8.9 (7.2±0.7)	4.5-8.9 (6.5±0.9)
DCPW	1.4-2.8 (2.0±0.4)	1.3.-2.2 (1.8±0.3)
DS	12.6-16.8 (14.0±1.1)	10.2-15.5 (12.8±1.2)
BMW	2.3-5.3 (4.6±0.7)	3.1-4.9 (4.2±0.5)
nD	22-28 (24±1.5)	22-28 (25±1.5)
nRP/D	8-14 (10±1.3)	9-13 (10±1)
n	25	25

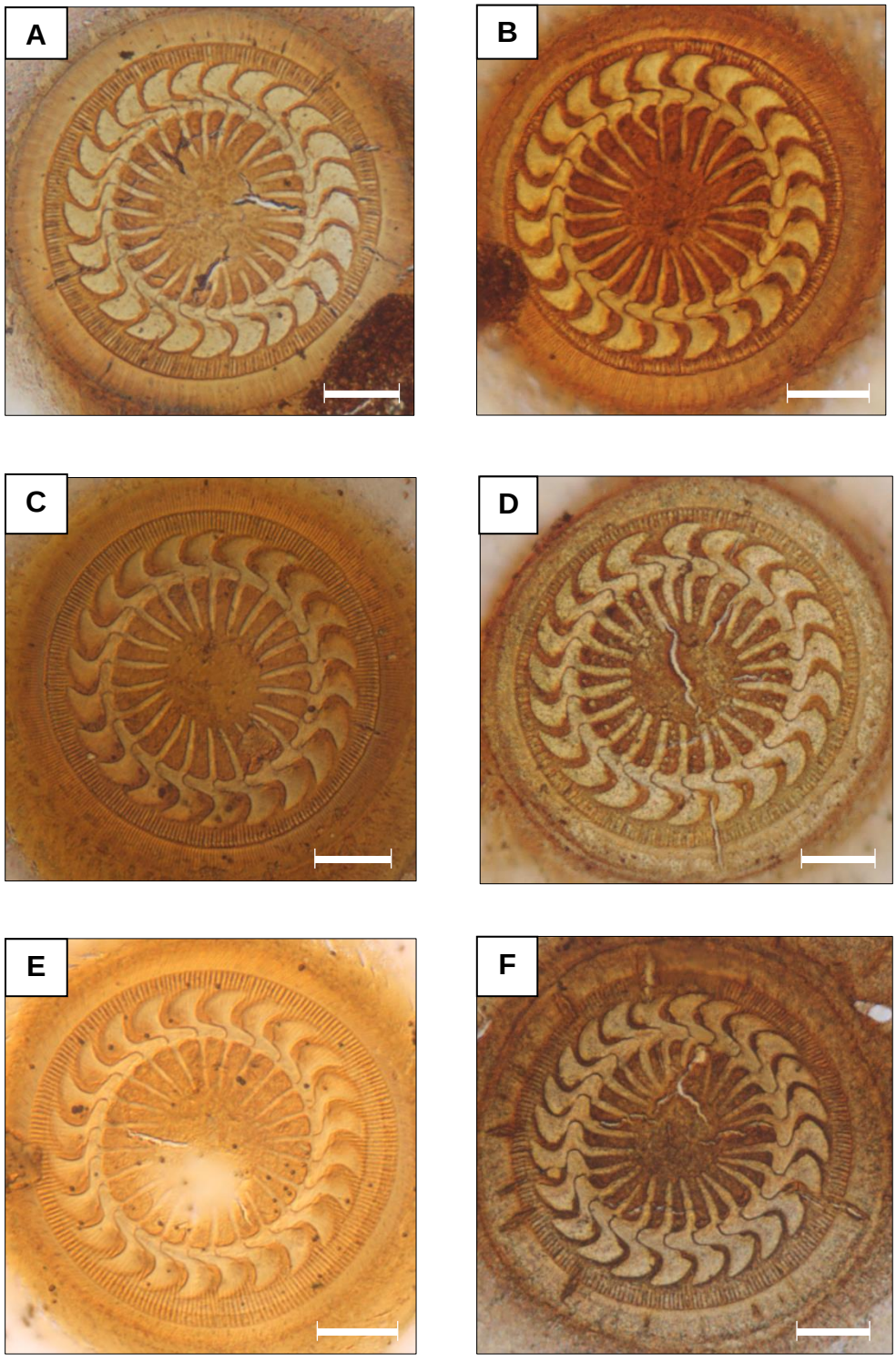


Figure 5.4: *Trichodina heterodentata* Duncan, 1977 representatives from anuran hosts from **A** - NX5; **C** - NX6; **E** - NX4 compared to those from *P. philander* (Weber, 1897) (**B**, **D** & **F**) (scale = 10µm).

COMPARISON OF *T. HETERODENTATA* DUNCAN, 1977 BIOMETRIC DATA FROM ANURAN AND TELEOST HOSTS

Not all the variables measured as proposed by Lom (1958) was used for the box-plot comparisons follow below, as some of the measurements have shown to be redundant as indicators of differences between *T. heterodentata* populations (i.e. overall body diameter, denticle span, border membrane width, number of denticles and number of radial pins per individual denticle).

Comparing all the morphometric data from the examined and measured trichodinids from both hosts (Fig. 5.5), the following is clear:

For almost all of the populations from anuran hosts, the trichodinids exhibited very insignificant statistical differences in their measured dimensions. Measurements for variables like the adhesive disc diameter (Fig. 5.5**A**), denticle ring diameter (Fig. 5.5**B**), central denticle width (Fig. 5.5**D**), ray length (Fig. 5.5**E**) and denticle width (Fig. 5.5**F**) presented negligible differences between the individual anuran populations, for both locality, i.e., the pools the hosts were found in, as well as for seasonality. The biggest seasonal difference was observed for blade length (Fig. 5.5**C**), where the 2015 winter population exhibited much smaller dimensions than those collected during the following seasons.

The biggest difference in morphometrics between the anuran and teleost host trichodinids seemed to be that the teleost hosts, except from having slightly larger dimensions (with the one exception being denticle width (Fig. 5.5**F**), where population NX3 exhibited the largest measured width), constantly displayed a much larger range between the smallest and largest individual population measurements of each of the different variables (Figs. 5.5**A** to **F**).

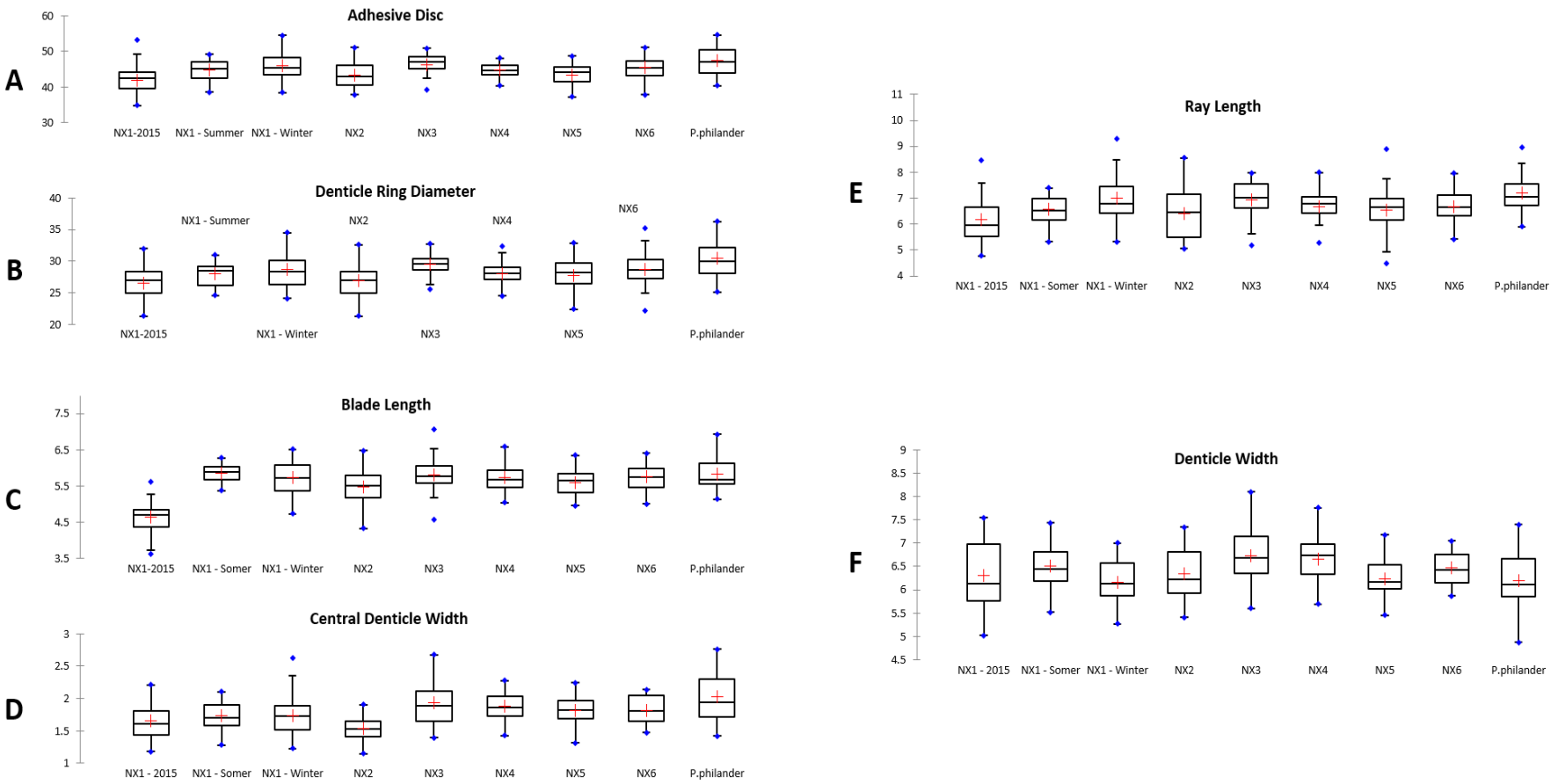


Figure 5.5: Comparative biometric statistics of all *Trichodina heterodentata* Duncan, 1977 populations collected from anurans and *Pseudocrenilabrus philander* (Weber, 1897) during the three expeditions to Nxamasere. All measurements are given in µm. NX1-2015 – 2015 winter collection from anuran hosts from Nxamasere 1; NX1-summer – 2016 summer collection from anuran hosts in pool 1; NX1-Winter – 2016 winter collection from anuran hosts from Nxamasere pool 1; NX2 to NX6 – 2016 winter collection from anuran hosts from pools 2 to 6 in Nxamasere; *P. philander* – winter 2016 collection from fish hosts in various pools from the Nxamasere Floodplain (**A** – Adhesive disc, **B** – Denticle ring diameter, **C** – Blade length, **D** – Central denticle width, **E** – Ray length and **F** – Denticle width).

DENTICLE DESCRIPTIONS FROM ANURAN HOSTS

All denticle descriptions were made according to the van As and Basson (1989) method of comparing the relationships of different aspects of three denticles per individual trichodinid. These descriptions were made on individual trichodinids that best represent their specific populations. In most cases two or more individuals were chosen as the variation in those populations was large and the selected specimens best represented these variations. Table 5.4 illustrates the most typical representatives of each of the anuran host population with reference to their host, locality and season in which they were collected, the micrograph from which the consecutive denticles were drawn, along with the corresponding figure number of the detailed descriptions that follows (Fig. 5.6).

Table 5.4: Denticle dimensions of *Trichodina heterodentata* Duncan, 1977 from anurans from the Nxamasere Floodplain as proposed by van As & Basson (1989 and 1992) for individuals representing different populations, including drawn denticles, micrograph of denticles and figure in which the denticle description will follow.

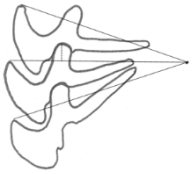
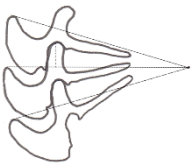
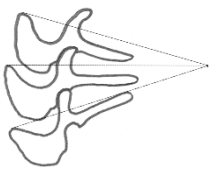
NX1 (winter) *Schlerophrys gutturalis* (Power, 1927) Nxamasere Floodplain 1 July 2016			**Fig. 5.6A**
NX2a (winter) *Schlerophrys gutturalis* Nxamasere Floodplain 2 July 2016			**Fig. 5.6B**
NX2b (winter) *Schlerophrys gutturalis* Nxamasere Floodplain 2 July 2016			**Fig. 5.6C**

Table 5.4 (cont.): Denticle dimensions of *Trichodina heterodentata* Duncan, 1977 from anurans from the Nxamasere Floodplain as proposed by van As & Basson (1989 and 1992) for individuals representing different populations, including drawn denticles, micrograph of denticles and figure in which the denticle description will follow.

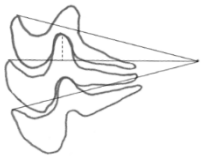
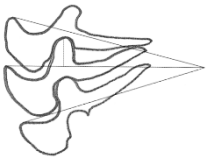
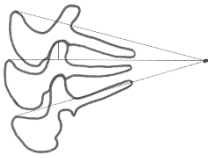
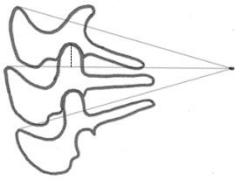
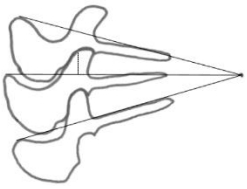
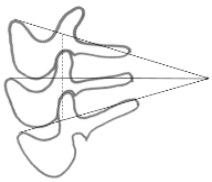
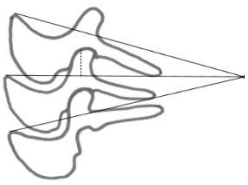
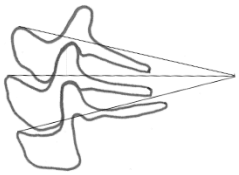
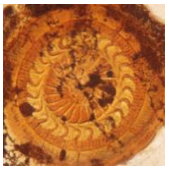
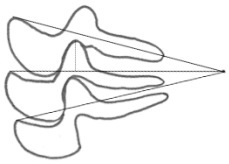
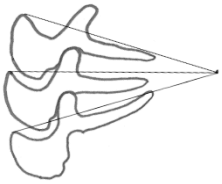
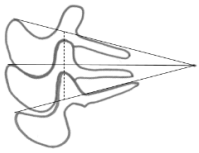
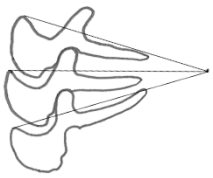
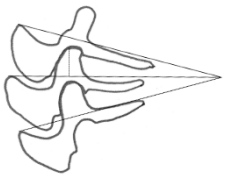
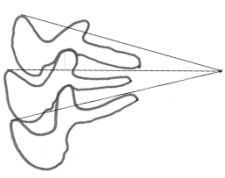
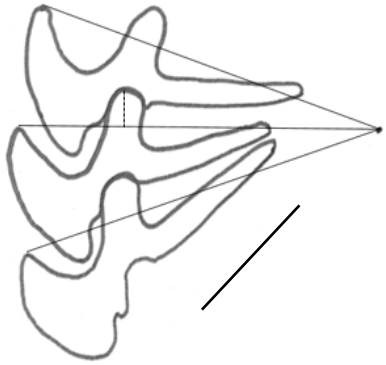
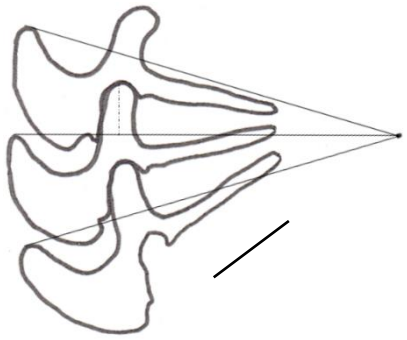
NX3a (winter) *Schlerophrys gutturalis* Nxamasere Floodplain 3 July 2016			**Fig. 5.6D**
NX3b (winter) *Schlerophrys gutturalis* Nxamasere Floodplain 3 July 2016			**Fig. 5.6E**
NX3c (winter) *Schlerophrys gutturalis* Nxamasere Floodplain 3 July 2016			**Fig. 5.6F**
NX4a (winter) *Schlerophrys gutturalis* Nxamasere Floodplain 4 July 2016			**Fig. 5.6G**
NX4b (winter) *Schlerophrys gutturalis* Nxamasere Floodplain 4 July 2016			**Fig. 5.6H**
NX4c (winter) *Schlerophrys gutturalis* Nxamasere Floodplain 5 July 2016			**Fig. 5.6I**
NX5a (winter) *Schlerophrys gutturalis* Nxamasere Floodplain 5 July 2016			**Fig. 5.6J**

Table 5.4 (cont.): Denticle dimensions of *Trichodina heterodentata* Duncan, 1977 from anurans from the Nxamasere Floodplain as proposed by van As & Basson (1989 and 1992) for individuals representing different populations, including drawn denticles, micrograph of denticles and figure in which the denticle description will follow.

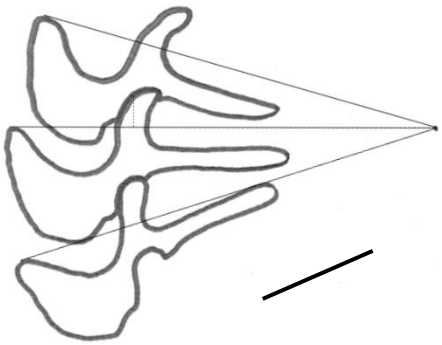
NX5b (winter) *Schlerophrys gutturalis* Nxamasere Floodplain 5 July 2016			**Fig. 5.6K**
NX6a (winter) *Schlerophrys poweri* (Hewitt, 1935) Nxamasere Floodplain 6 July 2016			**Fig. 5.6L**
NX6b (winter) *Schlerophrys powerii* Nxamasere Floodplain 6 July 2016			**Fig. 5.6M**
NX1a (summer) *Schlerophrys gutturalis* Nxamasere Floodplain 1 November 2016			**Fig. 5.6N**
NX1b (summer) *Schlerophrys gutturalis* Nxamasere Floodplain 1 November 2016			**Fig. 5.6O**
NX1a-15 *Schlerophrys gutturalis* Nxamasere Floodplain 1 July 2015			**Fig. 5.6P**
NX1b-15 *Schlerophrys gutturalis* Nxamasere Floodplain 1 July 2015			**Fig. 5.6Q**



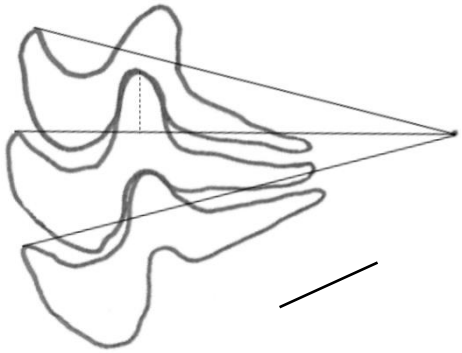
Population Number:	**NX1-winter (Fig. 5.6A)**
Host Species:	***Schlerophrys gutturalis***
Locality:	**Nxamasere Floodplain 1**
Date of Collection:	**July 2016**
Blade form:	Broad, filling large section between *y* and *y+1* axes.
Tangent point of blade:	Tangent point slightly more proximal; than distal blade margin.
Distal blade margin/surface:	Slightly rounded with straight slope towards proximal direction, running parallel to border membrane.
Post blade margin/surface:	Smooth, deeply curved, L-shaped.
Deepest point of curve relative to apex of blade:	Deepest part slightly more proximal than blade apex.
Apex of blade:	Pointed, reaches beyond y-axis.
Anterior blade apophysis:	Very prominent indentation.
Posterior projection:	Not observed.
Central part:	Robust, rounded tip, fitting tightly into preceding central part.
Lower central part indentation:	Not observed.
Central part relation to *x*-axis:	Distal surface of central part smaller and more sloping than proximal surface.
Central part relation to *y*-axis:	Extends more than halfway past y-axis.
Ray form:	Strong, straight, of equal width for whole length. Quick tapering towards blunt tip. Some curve strongly towards posterior direction, touching and extending past the *y*-axis.
Ray apophysis:	Present, directed anteriorly.
Ray connection:	Strong, same width as rest of ray.
Relationship of denticle above and below x-axis:	1 : 1.3



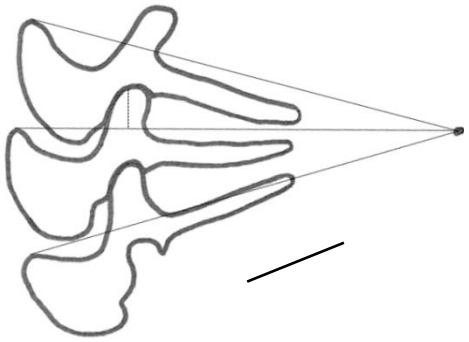
Population Number:	**NX2a-winter (Fig. 5.6B)**
Host Species:	***Schlerophrys gutturalis***
Locality:	**Nxamasere Floodplain 2**
Date of Collection:	**July 2016**
Blade form:	Broad, filling large section between *y* and *y+1* axes.
Tangent point of blade:	Slightly more proximal than distal blade margin.
Distal blade margin/surface:	Curved, running parallel to border membrane.
Post blade margin/surface:	Smooth, C-shaped.
Deepest point of curve relative to apex of blade:	Deepest point more proximal than blade apex.
Apex of blade:	Rounded, all reach slightly beyond y+1 axis.
Anterior blade apophysis:	Prominent indentation.
Posterior projection:	Not observed.
Central part:	Extended and elongated. Slightly enlarged rounded tip. Compact, fitting tightly into preceding central part.
Lower central part indentation:	Not observed.
Central part relation to *x*-axis:	Distal surface almost equal to proximal surface, with distal surface slightly larger.
Central part relation to *y*-axis:	Central part extends more than halfway past y-axis.
Ray form:	Delicate, straight, of equal width for most of ray, quick tapering towards blunt tip, slight curve towards posterior direction, touching and extending past the *y*-axis near tip.
Ray apophysis:	Very prominent, directed anteriorly.
Ray connection:	Delicate, slightly narrower than rest of ray.
Relationship of denticle above and below x-axis:	1 : 1.7



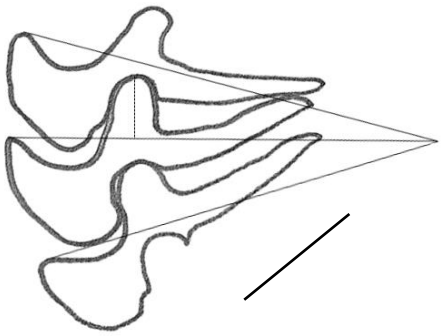
Population Number:	**NX2b-winter (Fig. 5.6C)**
Host Species:	***Schlerophrys gutturalis***
Locality:	**Nxamasere Floodplain 2**
Date of Collection:	**July 2016**
Blade form:	Broad, filling large section between *y* and *y+1* axes.
Tangent point of blade:	Slightly more proximal than distal blade margin.
Distal blade margin/surface:	Slightly curved, starts off parallel to border membrane, strong proximal slope.
Post blade margin/surface:	Smooth, L-shaped.
Deepest point of curve relative to apex of blade:	Deepest part slightly more proximal than blade apex.
Apex of blade:	Rounded, some touches y-axis, other extend beyond the y+1 axis.
Anterior blade apophysis:	Slight indentation.
Posterior projection:	Not observed.
Central part:	Extended and very elongated, tip with strong curve proximally, slightly enlarged rounded tip, fitting into preceding central part.
Lower central part indentation:	Not observed.
Central part relation to *x*-axis:	Distal surface of central part smaller and more sloping than proximal surface, extends more than halfway past x-axis.
Central part relation to *y*-axis:	Central part extends more than halfway to y-axis.
Ray form:	Delicate, straight, of equal width for whole length, quick tapering towards blunt tip; Extends towards y-axis, but does not touch it
Ray apophysis:	Prominent, directed anteriorly.
Ray connection:	Delicate, slightly narrower than rest of ray
Relationship of denticle above and below x-axis:	1 : 1.2



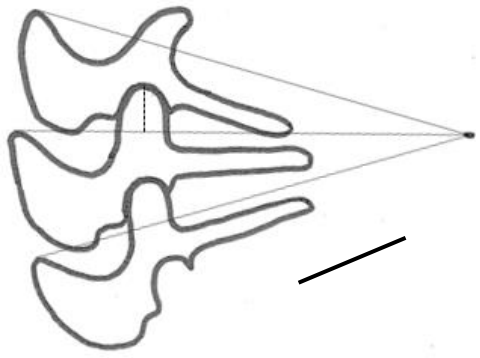
Population Number:	**NX3a-winter (Fig. 5.6D)**
Host Species:	***Schlerophrys gutturalis***
Locality:	**Nxamasere Floodplain 3**
Date of Collection:	**July 2016**
Blade form:	Broad, filling large section between *y* and *y+1* axes.
Tangent point of blade:	More proximal than distal blade margin.
Distal blade margin/surface:	Curved; gradually sloping proximally. Starts off parallel to border membrane.
Post blade margin/surface:	Smooth, moderately shallow curve, closer to *y-1*/*y+1* axis than *y* axis.
Deepest point of curve relative to apex of blade:	Deepest part very close, but slightly more proximal than apex of blade.
Apex of blade:	Mostly rounded and all extends beyond y+1. axis, while some only touches y+1 axis.
Anterior blade apophysis:	Most not observed, some has very slight indentation.
Posterior projection:	Not observed.
Central part:	Very robust, tapers off towards posterior end, compact fitting into preceding central part.
Lower central part indentation:	Not observed.
Central part relation to *x*-axis:	Distal surface is almost equal to proximal surface .
Central part relation to *y*-axis:	Central part extends less than halfway past y-axis.
Ray form:	Robust, straight, gradually thins out towards a blunt tip, no curve, extends anteriorly cutting y+1 axis near tip.
Ray apophysis:	Not observed.
Ray connection:	Robust, tapering with rest of ray with no narrowing indentation.
Relationship of denticle above and below x-axis:	1 : 1.3



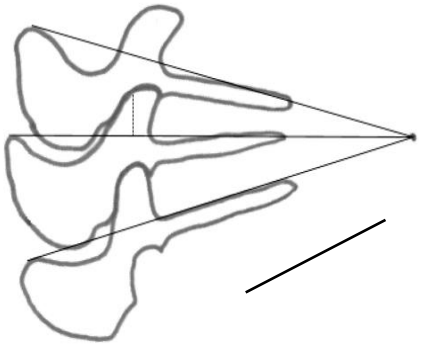
Population Number:	**NX3b-winter (Fig. 5.6E)**
Host Species:	***Schlerophrys gutturalis***
Locality:	**Nxamasere Floodplain 3**
Date of Collection:	**July 2016**
Blade form:	Broad, filling large section between *y* and *y+1* axes.
Tangent point of blade:	More proximal than distal blade margin.
Distal blade margin/surface:	Curved, gradually sloping proximally, running parallel to border membrane.
Post blade margin/surface:	Shallow L-shaped.
Deepest point of curve relative to apex of blade:	Deepest part more proximal than blade apex.
Apex of blade:	Rounded and most extends beyond y+1 axis, while some only touches y+1 axis.
Anterior blade apophysis:	Prominent indentation.
Posterior projection:	Not observed.
Central part:	Robust, slightly curve towards proximal side; rounded tip, compact fitting into preceding central part at tip only.
Lower central part indentation:	Not observed.
Central part relation to *x*-axis:	Distal surface of central part smaller than proximal surface.
Central part relation to *y*-axis:	Extends halfway past the y-axis.
Ray form:	Delicate, straight, of equal width for whole length tapering towards blunt tip, does not cut any axes.
Ray apophysis:	Very prominent, directed anteriorly.
Ray connection:	Delicate, slightly narrower than rest of ray.
Relationship of denticle above and below x-axis:	1 : 1.4



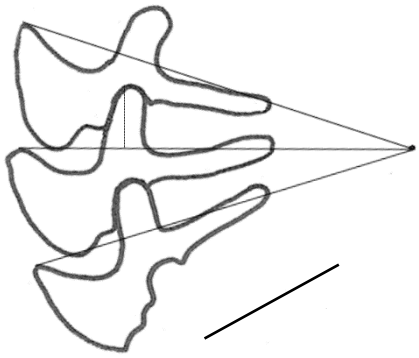
Population Number:	**NX3c-winter (Fig. 5.6F)**
Host Species:	***Schlerophrys gutturalis***
Locality:	**Nxamasere Floodplain 3**
Date of Collection:	**July 2016**
Blade form:	Broad, filling large section between *y* and *y+1* axes.
Tangent point of blade:	Slightly more proximal than distal blade margin.
Distal blade margin/surface:	Curved, sudden proximal slope, initially running parallel to border membrane.
Post blade margin/surface:	Shallow L-shaped.
Deepest point of curve relative to apex of blade:	Deepest part slightly more proximal than apex of blade, almost on same plain.
Apex of blade:	Rounded and most extends beyond y+1 axis, while some only touches y+1 axis.
Anterior blade apophysis:	Prominent indentation.
Posterior projection:	Not observed.
Central part:	Robust and squat, very slight curve towards proximal side, round posterior tip, slight space after preceding central part.
Lower central part indentation:	Not observed.
Central part relation to *x*-axis:	Distal surface of central part slightly larger than proximal surface.
Central part relation to *y*-axis:	Extends halfway past the y-axis.
Ray form:	Strong, slight curve towards posterior, tapering towards somewhat sharp tip, extends past both *y* and y-1 axes.
Ray apophysis:	Prominent, directed anteriorly.
Ray connection:	Strong, same width as rest of ray.
Relationship of denticle above and below x-axis:	1 : 1.1



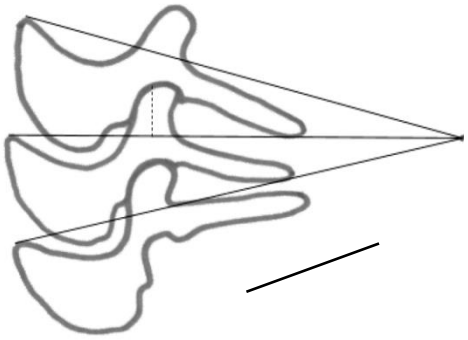
Population Number:	**NX4a-winter (Fig. 5.6G)**
Host Species:	***Schlerophrys gutturalis***
Locality:	**Nxamasere Floodplain 4**
Date of Collection:	**July 2016**
Blade form:	Broad, filling large section between *y* and *y+1* axes.
Tangent point of blade:	Slightly more proximal than distal blade margin.
Distal blade margin/surface:	Slightly curved sloping in proximal direction, posterior side parallel to border membrane.
Post blade margin/surface:	Deep L-shaped curve.
Deepest point of curve relative to apex of blade:	Deepest part far more proximal than blade apex.
Apex of blade:	Rounded, touches and some slightly extends beyond *y*-axis.
Anterior blade apophysis:	Very prominent indentation.
Posterior projection:	Not observed.
Central part:	Robust, straight towards posterior, large round posterior tip, compact fitting into preceding central part.
Lower central part indentation:	Not observed.
Central part relation to *x*-axis:	Distal surface of central part is very slightly larger than proximal surface.
Central part relation to *y*-axis:	Extends almost halfway past y-axis.
Ray form:	Delicate, straight, of equal width for whole length tapering towards blunt tip; some extends anteriorly and touches y-1 axis.
Ray apophysis:	Prominent, directed anteriorly.
Ray connection:	Delicate, very slightly narrower than rest of ray, mostly same width.
Relationship of denticle above and below x-axis:	1 : 1.2



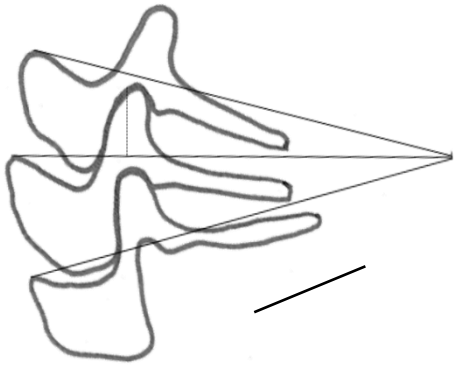
Population Number:	**NX4b-winter (Fig. 5.6H)**
Host Species:	***Schlerophrys gutturalis***
Locality:	**Nxamasere Floodplain 4**
Date of Collection:	**July 2016**
Blade form:	Broad, filling large section between *y* and *y+1* axes.
Tangent point of blade:	Slightly more distal than distal blade margin.
Distal blade margin/surface:	Curved, gradually sloping proximally, parallel to border membrane.
Post blade margin/surface:	Very shallow L-shaped curve.
Deepest point of curve relative to apex of blade:	Deepest part slightly more proximal than blade apex.
Apex of blade:	Rounded and most extends beyond y+1 axis, other just touches y+1 axis.
Anterior blade apophysis:	Slight indentation.
Posterior projection:	Not observed.
Central part:	Robust but elongated, curved towards proximal, blunt round posterior tip, compact fitting into preceding central part.
Lower central part indentation:	Not observed.
Central part relation to *x*-axis:	Distal surface of central part is slightly smaller than proximal surface.
Central part relation to *y*-axis:	Extends halfway past the proximal y-axis.
Ray form:	Delicate, straight, of equal width for whole length tapering towards blunt tip, touches y-axis.
Ray apophysis:	Present, directed anteriorly.
Ray connection:	Delicate, very slightly narrower than rest of ray, mostly same width.
Relationship of denticle above and below x-axis:	1 : 1.2



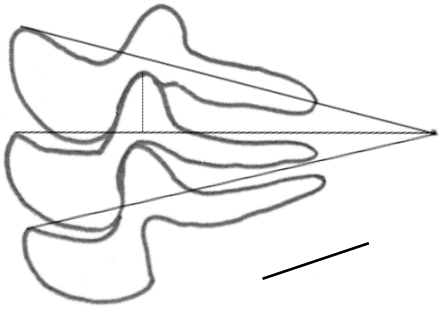
Population Number:	**NX4c-winter (Fig. 5.6l)**
Host Species:	***Schlerophrys gutturalis***
Locality:	**Nxamasere Floodplain 4**
Date of Collection:	**July 2016**
Blade form:	Broad, filling large section between *y* and *y+1* axes.
Tangent point of blade:	Pointed towards distal side with sharp rounded tip, but slightly more proximal than distal blade margin.
Distal blade margin/surface:	Slight curve with sudden straightened slope towards posterior side, near tangent point it runs parallel to border membrane.
Post blade margin/surface:	L-shaped shallow curve.
Deepest point of curve relative to apex of blade:	Deepest part far more proximal than apex of blade.
Apex of blade:	Somewhat pointed and touch, but does not extend past y+1 axis.
Anterior blade apophysis:	Prominent indentation.
Posterior projection:	Not observed.
Central part:	Robust, curved towards proximal, blunt round posterior tip, compact fitting into preceding central part.
Lower central part indentation:	Not observed.
Central part relation to *x*-axis:	Distal surface of central part slightly smaller than proximal surface.
Central part relation to *y*-axis:	Extends more than halfway past y-axis.
Ray form:	Delicate, straight, of equal width for whole length tapering towards blunt tip, touches and extends slightly past y-axis.
Ray apophysis:	Prominent, directed anteriorly.
Ray connection:	Relatively strong, slightly narrower as rest of ray.
Relationship of denticle above and below x-axis:	1 : 1



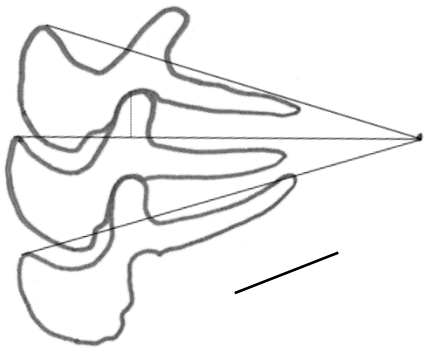
Population Number:	**NX5a-winter (Fig. 5.6J)**
Host Species:	***Schlerophrys gutturalis***
Locality:	**Nxamasere Floodplain 5**
Date of Collection:	**July 2016**
Blade form:	Broad, filling large section between *y* and *y+1* axes.
Tangent point of blade:	Pointed towards distal side with sharp rounded tip and slightly more proximal than distal blade margin.
Distal blade margin/surface:	Straightened slope towards proximal side, running parallel to border membrane.
Post blade margin/surface:	Relatively deep L-shaped curve.
Deepest point of curve relative to apex of blade:	Deepest part marginally more distal than blade apex, almost on the same plain.
Apex of blade:	Rounded and extends beyond y+1 axis.
Anterior blade apophysis:	Very prominent indentation.
Posterior projection:	Not observed.
Central part:	Robust, curved towards proximal, blunt round posterior tip, compact fitting into preceding central part.
Lower central part indentation:	Not observed.
Central part relation to *x*-axis:	Distal surface of central part smaller than proximal surface.
Central part relation to *y*-axis:	Extends more than halfway past the y-axis.
Ray form:	Delicate, straight, of equal width for whole length tapering towards blunt tip, extends anteriorly and touches the y+1 axis.
Ray apophysis:	Present, directed anteriorly.
Ray connection:	Delicate, slightly narrower than rest of ray.
Relationship of denticle above and below x-axis:	1 : 1



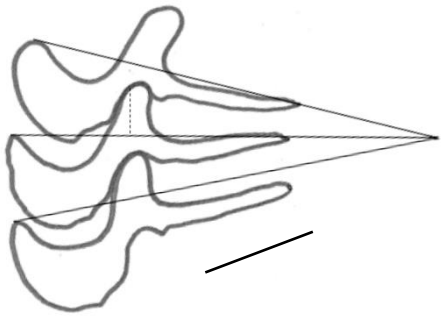
Population Number:	**NX5b-winter (Fig. 5.6K)**
Host Species:	***Schlerophrys gutturalis***
Locality:	**Nxamasere Floodplain 5**
Date of Collection:	**July 2016**
Blade form:	Broad, filling large section between *y* and *y+1* axes.
Tangent point of blade:	Pointed towards distal side with rounded tip, more proximal than distal blade margin.
Distal blade margin/surface:	Shallow curve, mostly not gradual curve, running parallel to border membrane.
Post blade margin/surface:	L-shaped with slope towards proximal, shallow curve.
Deepest point of curve relative to apex of blade:	Deepest part and apex of blade on the same plain.
Apex of blade:	Most are pointed (some flat) and extend beyond y+1 axis.
Anterior blade apophysis:	Not observed.
Posterior projection:	Not observed.
Central part:	Slender and elongated, curved towards proximal, blunt round posterior tip, compact fitting into preceding central part.
Lower central part indentation:	Not observed.
Central part relation to *x*-axis:	Distal surface of central part slightly smaller than proximal surface.
Central part relation to *y*-axis:	Extends far beyond halfway past y-axis.
Ray form:	Delicate, straight, of equal width for whole length tapering towards blunt tip, extends anteriorly but, does not touch y+1 axis.
Ray apophysis:	Present, directed anteriorly.
Ray connection:	Some specimens markedly more narrow, most same width as rest of ray.
Relationship of denticle above and below x-axis:	1 : 1.4



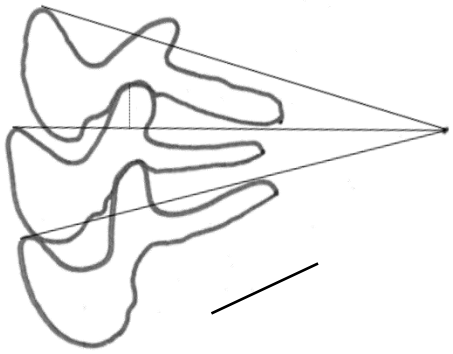
Population Number:	**NX6a-winter (Fig. 5.6L)**
Host Species:	***Schlerophrys poweri***
Locality:	**Nxamasere Floodplain 6**
Date of Collection:	**July 2016**
Blade form:	Very broad, filling almost complete section between *y* and *y+1* axes.
Tangent point of blade:	Pointed towards distal side with wide rounded tip, very slightly more proximal than distal blade margin.
Distal blade margin/surface:	Gradually curved, tangent point region running parallel to border membrane, followed by strong slope towards proximal side.
Post blade margin/surface:	Shallow curved shape with strong slope towards proximal.
Deepest point of curve relative to apex of blade:	Deepest part slightly more proximal than blade apex.
Apex of blade:	Rounded and extends beyond y+1 axis.
Anterior blade apophysis:	Not observed.
Posterior projection:	Not observed.
Central part:	Robust and squat, straight, V-shaped, blunt round posterior tip, compact fitting into preceding central part.
Lower central part indentation:	Not observed.
Central part relation to *x*-axis:	Both distal and proximal sides of equal surface.
Central part relation to *y*-axis:	Extends halfway past y-axis.
Ray form:	Strong, slightly curved towards posterior, of equal width for whole length tapering towards blunt tip, extends anteriorly and touches y+1 axis.
Ray apophysis:	Not observed.
Ray connection:	Strong, same width as rest of ray.
Relationship of denticle above and below x-axis:	1 : 1.



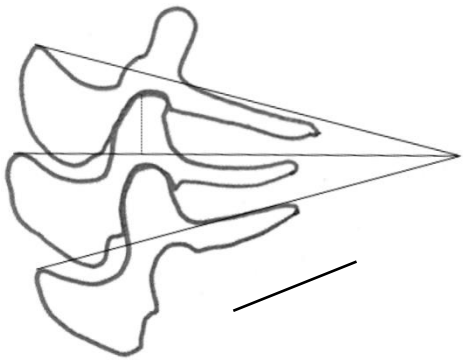
Population Number:	**NX6b-winter (Fig. 5.6M)**
Host Species:	***Schlerophrys poweri***
Locality:	**Nxamasere Floodplain 6**
Date of Collection:	**July 2016**
Blade form:	Broad, filling large section between *y* and *y+1* axes.
Tangent point of blade:	Slightly more proximal than distal blade margin.
Distal blade margin/surface:	Gradually curving slope in proximal direction, which runs parallel to border membrane.
Post blade margin/surface:	Semi-deep L-shaped curve with slope towards posterior.
Deepest point of curve relative to apex of blade:	Deepest part more proximal than apex of blade.
Apex of blade:	Slightly pointed, extends beyond y+1 axis.
Anterior blade apophysis:	Slight indentation.
Posterior projection:	Not observed.
Central part:	Slender, curved towards proximal, blunt, round posterior tip, fitting into preceding central part.
Lower central part indentation:	Not observed.
Central part relation to *x*-axis:	Distal surface of central part slightly smaller than proximal surface.
Central part relation to *y*-axis:	Extends halfway past y-axis.
Ray form:	Robust, most straight, some curve posteriorly, of equal width for whole length tapering towards blunt tip, most does not touch or extend past any axes.
Ray apophysis:	Minor, directed anteriorly.
Ray connection:	Strong, same width as rest of ray.
Relationship of denticle above and below x-axis:	1 : 2.



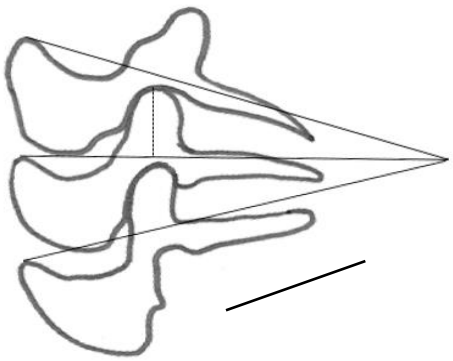
Population Number:	**NX1a-summer (Fig. 5.6N)**
Host Species:	***Schlerophrys gutturalis***
Locality:	**Nxamasere Floodplain 1**
Date of Collection:	**November 2016**
Blade form:	Filling large section between *y* and *y+1* axes.
Tangent point of blade:	Slightly more proximal than distal blade margin.
Distal blade margin/surface:	Rounded curve sloping gradually towards proximal side, running parallel to border membrane.
Post blade margin/surface:	Deep L-shaped curve.
Deepest point of curve relative to apex of blade:	Deepest part somewhat more proximal than apex of blade.
Apex of blade:	Most rounded and extends beyond y+1 axis.
Anterior blade apophysis:	Very slight indentation.
Posterior projection:	Not observed.
Central part:	Slender, leaning proximally, blunt round posterior tip, compact fitting into preceding central part.
Lower central part indentation:	Not observed.
Central part relation to *x*-axis:	Distal surface of central part is slightly smaller than proximal surface.
Central part relation to *y*-axis:	Extends halfway past the y-axis.
Ray form:	Delicate, straight, of equal width for whole length and mostly tapering towards somewhat pointed tip, extends posteriorly and touches y+1 axis.
Ray apophysis:	Present, directed anteriorly.
Ray connection:	Delicate, narrower than rest of ray.
Relationship of denticle above and below x-axis:	1 : 1



Population Number:	**NX1b-summer (Fig. 5.6O)**
Host Species:	***Schlerophrys gutturalis***
Locality:	**Nxamasere Floodplain 1**
Date of Collection:	**November 2016**
Blade form:	Broad, filling large section between *y* and *y+1* axes.
Tangent point of blade:	Most tangent points slightly more proximal than distal blade, others most distal point on blade.
Distal blade margin/surface:	Slightly curved slope in proximal direction, running parallel to border membrane.
Post blade margin/surface:	Very deep L-shaped with slope towards proximal.
Deepest point of curve relative to apex of blade:	Deepest part more proximal than blade apex.
Apex of blade:	Some pointed, most are round and extends beyond y+1 axis.
Anterior blade apophysis:	Slight indentation.
Posterior projection:	Not observed.
Central part:	Robust to elongated, leaning towards proximal, large blunt round posterior tip, compact fitting into preceding central part.
Lower central part indentation:	Not observed.
Central part relation to *x*-axis:	Distal surface of central part is smaller than proximal surface.
Central part relation to *y*-axis:	Extends halfway past the y-axis.
Ray form:	Strong, straight, of equal width for whole length tapering towards blunt tip, does not touch or extend beyond any axes.
Ray apophysis:	Not observed.
Ray connection:	Strong, marginally more narrow than rest of the ray.
Relationship of denticle above and below x-axis:	1 : 1



Population Number:	**NX1a-15 (Fig. 5.6P)**
Host Species:	***Schlerophrys gutturalis***
Locality:	**Nxamasere Floodplain 1**
Date of Collection:	**July 2015**
Blade form:	Broad, filling large section between *y* and *y+1* axes.
Tangent point of blade:	Slightly more proximal than distal blade margin.
Distal blade margin/surface:	Mostly straight curve, quickly sloping in proximal direction, only tangent point surface parallel to border membrane.
Post blade margin/surface:	Relatively deep L-shaped curve with slope towards proximal.
Deepest point of curve relative to apex of blade:	Deepest part more proximal than blade apex.
Apex of blade:	Most are pointed and extends beyond y+1 axis.
Anterior blade apophysis:	Prominent indentation.
Posterior projection:	Not observed.
Central part:	Most robust and broad, blunt round posterior tip, compact fitting into preceding central part.
Lower central part indentation:	Not observed.
Central part relation to *x*-axis:	Distal surface of central part slightly smaller than proximal surface.
Central part relation to *y*-axis:	Extends halfway past y-axis.
Ray form:	Mostly delicate, straight but some curves posteriorly, of equal width for whole length tapering towards blunt tip, extends towards but does not touch y+1 axis.
Ray apophysis:	Present, directed anteriorly.
Ray connection:	Delicate, slightly narrower than rest of ray.
Relationship of denticle above and below x-axis:	1 : 1

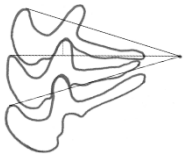
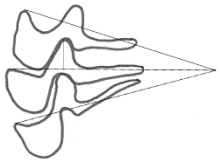
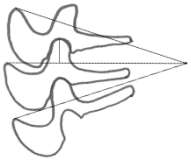


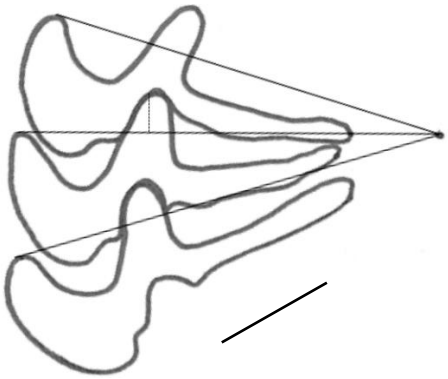
Population Number:	**NX1b-15 (Fig. 5.6Q)**
Host Species:	***Schlerophrys gutturalis***
Locality:	**Nxamasere Floodplain 1**
Date of Collection:	**July 2015**
Blade form:	Broad, filling large section between *y* and *y+1* axes.
Tangent point of blade:	Slightly more proximal than distal blade margin.
Distal blade margin/surface:	Relatively shallow curved slope towards proximal side, running parallel to border membrane.
Post blade margin/surface:	Deep L-shaped curve.
Deepest point of curve relative to apex of blade:	Deepest part more proximal than apex of blade.
Apex of blade:	Most are rounded and touches and some extends beyond y+1 axis.
Anterior blade apophysis:	Quite prominent indentation.
Posterior projection:	Not observed.
Central part:	Robust, leaning towards proximal, blunt round posterior tip, compact fitting into preceding central part.
Lower central part indentation:	Not observed.
Central part relation to *x*-axis:	Distal surface of central part slightly smaller than proximal surface.
Central part relation to *y*-axis:	Extends far beyond halfway past y-axis.
Ray form:	Delicate, most slightly curved towards anterior, of equal width for whole length and most tapering towards blunt tip, extends anteriorly, but does not touch y+1 axis.
Ray apophysis:	Present, directed anteriorly.
Ray connection:	Strong, same width as rest of ray.
Relationship of denticle above and below x-axis:	1 : 2

DENTICLE DESCRIPTIONS FROM TELEOST HOSTS

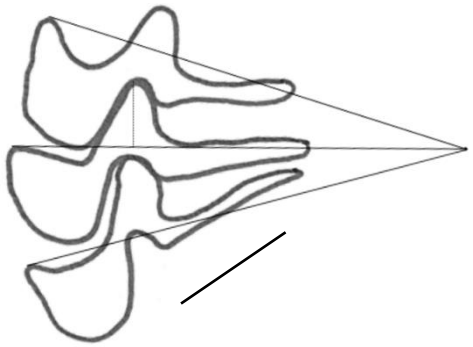
As with the denticle descriptions for the anuran hosts, the same was done for the teleost hosts collected during the winter of 2015. Table 5.5 illustrates the most typical representatives of the fish host population; the micrograph from which the consecutive denticles were drawn, the hosts and the season in which these were collected in. Figures 5.7**A** to **C** provides the detailed denticle descriptions for each of the teleost host trichodinids.

Table 5.5: Denticle dimensions as proposed by van As & Basson (1989 and 1992) for individuals representing different populations collected from *Pseudocrenilabrus philander* (Weber, 1887).

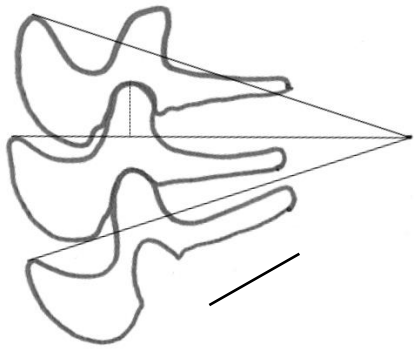
NXPa *Pseudocrenilabrus philander* Nxamasere Floodplain July 2016			**Fig. 5.7A**
NXPb *Pseudocrenilabrus philander* Nxamasere Floodplain July 2016			**Fig. 5.7B**
NXPc *Pseudocrenilabrus philander* Nxamasere Floodplain July 2016			**Fig. 5.7C**



Population Number:	**NXPa (Fig. 5.7A)**
Host Species:	***Pseudocrenilabrus philander***
Locality:	**Nxamasere Floodplain**
Date of Collection:	**July 2016**
Blade form:	Large, broad blade filling whole surface between y and y+1 axes.
Tangent point of blade:	Tangent point slightly more proximal than distal blade margin.
Distal blade margin/surface:	All curved, gradually sloping in proximal direction, running parallel to border membrane.
Post blade margin/surface:	Deep, smooth L-shaped curve.
Deepest point of curve relative to apex of blade:	Deepest point of curve proximal to blade apex.
Apex of blade:	Rounded, extends past y+1 axis.
Anterior blade apophysis:	Prominent.
Posterior projection:	Not observed.
Central part:	Very robust, slightly narrower tip, fitting tightly into preceding denticles' central part.
Lower central part indentation:	Not observed.
Central part in relation to *x*-axis:	Distal surface of central part slightly smaller and more sloping than proximal surface.
Central part in relation to *y*-axis:	Extends exactly halfway beyond y axis.
Ray form:	Very strong, straight, of equal width for whole ray, tapers off towards blunt tip.
Ray apophysis:	Quite prominent, directed anteriorly.
Ray connection:	Delicate, slightly narrower than rest of ray.
Relationship of denticle above and below x-axis:	1 : 1.4



Population Number:	**NXPb (Fig 5.7B)**
Host Species:	***Pseudocrenilabrus philander***
Locality:	**Nxamasere Floodplain**
Date of Collection:	**July 2016**
Blade form:	Large, broad, almost filing whole surface between y and y+1 axes.
Tangent point of blade:	Tangent point distal of distal blade margin in two of three denticles.
Distal blade margin/surface:	Most are straight, with sudden proximal slope on anterior side, other curved with gradual proximal slope.
Post blade margin/surface:	Very shallow L-shape, only slightly curved.
Deepest point of curve relative to apex of blade:	Deepest part of curve and apex of blade in same plain.
Apex of blade:	Rounded, but most does not touch y+1 axis.
Anterior blade apophysis:	Not observed.
Posterior projection:	Not observed.
Central part:	Very robust, relatively pointed tip, most fits tightly into preceding denticle's central part.
Lower central part indentation:	Not observed.
Central part in relation to *x*-axis:	Distal surface almost same size, but slightly smaller and more sloping than proximal surface.
Central part in relation to *y*-axis:	Extends more than halfway beyond the y axis.
Ray form:	Strong, straight, tapering off towards blunt tip, mostly of equal width as rest of ray.
Ray apophysis:	Present in only some specimens.
Ray connection:	Delicate and slightly narrower than rest or ray in most, others no visible difference.
Relationship of denticle above and below x-axis:	1 : 1.6



Population Number:	**NXPc (Fig. 5.7C)**
Host Species:	***Pseudocrenilabrus philander***
Locality:	**Nxamasere Floodplain**
Date of Collection:	**July 2016**
Blade form:	Large, broad and filling space between y and y+1 axes.
Tangent point of blade:	Tangent point slightly more proximal than distal blade margin.
Distal blade margin/surface:	Curved with gradual slope in proximal direction.
Post blade margin/surface:	Smooth rather deep L-shaped curve.
Deepest point of curve relative to apex of blade:	Deepest part of curve slightly more proximal than apex of blade.
Apex of blade:	Rounded, touches and some slightly extends past y+1 axis.
Anterior blade apophysis:	Present, not very prominent.
Posterior projection:	Not observed.
Central part:	Robust, more broadly rounded tip, fitting tightly into preceding central part.
Lower central part indentation:	Not observed.
Central part in relation to *x*-axis:	Distal surface of central part slightly smaller than proximal surface and has slight slope.
Central part in relation to *y*-axis:	Extends halfway beyond y axis.
Ray form:	Strong, equal width to rest of ray, tapering off to rounded tip.
Ray apophysis:	Present in most, always in anterior direction.
Ray connection:	Relatively strong, slightly narrower than rest of the ray.
Relationship of denticle above and below x-axis:	1 : 1.3

Denticle Description Comparison for Anuran Hosts:

Trichodina heterodentata is a species that displays an incredible amount of variation in morphological characteristics. Using van As and Basson's (1989) denticle descriptions, this variation seems more prominent at first than using only the measurements proposed by Lom (1958), but deeper investigation and comparison help to quantify the range of variation within this species.

Of the three denticle parts described for *T. heterodentata* from anuran hosts using the van As and Basson (1989) denticle descriptions, the blade region was the most constant with minor differences between the specimens examined. Almost all had large, broad blades filling a large part of the section between the y and y+1 axes with the tangent point being slightly more proximal than the distal blade margin. The distal blade margin (surface), was generally curved, gradually sloping towards the proximal direction and parallel to the border membrane, except for Figs. 5.6**I** and **P** that suddenly sloped away after the tangent point area of the blade, resulting in the distal blade margin not running parallel with the border membrane. Most of the posterior blade margins (surface) were smoothly curved in a shallow L-shape (except Fig. 5.6**C** that had a C-shape), with a few exceptions that was more deeply curved (Figs. 5.6**M**, **N**, **O** & **P**). In all the specimens the deepest point of the curve was more proximal than the apex of the blade, although some were almost on the same plain (Figs. 5.6**D**, **K** & **M**). One specimen has a single denticle that had the apex of the blade more towards the proximal direction than the deepest point of the curve (Fig. 5.6**Q**).The apexes of the blade were generally rounded, with Figs. 5.6**A**, **I**, **K**, **M**, **O** and **P** being slightly more pointed, and most of them extended past the y+1 axis, besides Fig. 5.6**Q** that only touched the y+1 axis and Figs. 5.6**G** and **I** that did not extend to the y+1 axis. The anterior blade apophysis had varying degrees of prominence, from very prominent (i.e. Fig. 5.6**A**), prominent (i.e. Fig. 5.6**B**) or slightly prominent (i.e. Fig. 5.6**C**), in most specimens, except in Figures 5.6**D**, **K** and **L** where no anterior blade apophyses were observed. No posterior projections on any specimens were observed.

For most specimens the central parts are slender and elongated, with only a few individuals that displayed strongly developed central parts (Figs. 5.6**D**, **F**, **G**, **L** & **P**).

Except for one specimen (Fig. 5.6**P**), no lower central part indentations were observed. The distal surface for most was smaller and more sloping than the proximal with exceptions (Figs. 5.6**D**, **F** & **G**) where the distal surface was larger than the proximal surface and Figures 5.6**G** and **L** where the two sides were almost of equal size. For the majority of the specimens the central part extended halfway (Figs. 5.6**A**, **C**, **E**, **G**, **H**, **M** & **O**) past or more than halfway (Figs. 5.6**B**, **D**, **F**, **I**, **J**, **L** & **M**) the y axis, three exception extended far beyond and close to the y-1 axis (Figs. 5.6**K**, **P** & **Q**).

The form of the ray is mostly delicate, some are strong (Figs. 5.6**A**,**B**,**D**, **F**, **I** & **J**) and a few are robust (Figs. 5.6**L**,**M** & **O**). Almost all rays were of straight and of equal width for the whole length, tapering off towards a blunt tip, the exceptions were Figures 5.6**F** and **Q** that are curved and Figures 5.6**F** and **N** ending in sharp tips. The majority of rays touches or even extends past the y-1 axis, while a few does not touch or extend past any axes (Figs. 5.6**C**, **E**, **G**, **M**,**O** & **P**) and the minority touches the y+1 axis (Figs. 5.6**D**, **J**, **K** & **L**). In most cases the ray apophysis was present, except for Figures 5.6**D**,**L** and **O** where it was not observed. In some specimens the ray apophyses were very prominent (Figs. 5.6**B**, **C**, **E** & **I**). The ray connections of the examined specimens were almost all marginally narrower than the width of the rest of the ray.

The relationship of the denticle above and below the x-axis fell in a range between 1:1 and a maximum of 1:7, with most of the ranges clustered around 1:2.

Denticle Description Comparison for Teleost Hosts:

Comparing the denticle descriptions of *T. heterodentata* from the *P. philander* specimens, the following was noted:

All specimens had large, broad sickle-shaped blades that filled most of the surface between the y and y+1 axes. Most of the tangent points were slightly proximal of the distal blade margin, except for Figure 5.7**B**, which were distal to the blade margin. Again, most of the distal blade margin (surface) had deep, smooth and gradually sloping L-shaped curves, which ran parallel to the border membrane, except Figure 5.7**B** that had

mostly straight distal blade margins, not running parallel to the border membrane. The deepest point of the curve was slightly more proximal than the apex of the blade, but in specimen NXPb (Fig. 5.7**B**) the blade apex and the deepest part of the curve was in the same plane. The apex blade for most specimens were rounded, touching and extending past the y+1 axes, where NXPb (Fig. 5.7**B**) did not touch the y+1 axis. The anterior blade apophysis were present in two specimens, very prominent in NXPa (Fig. 5.7**A**) and marginally prominent in NXPc (Fig. 5.7**C**), but absent for NXPb (Fig. 5.7**B**), while there was no posterior projection observed in any of the specimens.

Most central parts were very robust, with their posterior tips more pointed than rounded, except Figure 5.7**C** that was robust with a rounded posterior tip. In all the specimens the distal surfaces of the central parts were marginally smaller and more sloping than the proximal surface (Figs. 5.7**A** to **C**), and they all extended past the y-axis.

All specimen's rays were strong to very strong (i.e. Fig. 5.7**A**), mostly of equal width as rest of the ray, tapering off to a blunt or rounded tip. The anterior apophyses were present in all specimens and always directed anteriorly. Specimen NXPb (Fig. 5.7**B**) only had the apophysis visible on one denticle. The ray connections for all specimens were only slightly narrower than the rest of ray, specimens NXPb (Fig. 5.7**B**) and NXPc (Fig. 5.7**C**).

The relationship of the denticle above and below the x-axis fell in a range between 1: 1.3 (Fig. 5.7**A**) and a maximum of 1: 1.7 (Fig. 5.7**B**), with a mean of 1: 1.4.

CHAPTER 6 - Article Format

To be submitted to the Journal *Hydrobiologia*, Kluwer Academic Publishers, The Netherlands in December 2017.

TITLE: Two trichodinid species, *Trichodina heterodentata* Duncan, 1977 and *Trichodina hypsilepis* Wellborn, 1969 (Oligohymenophorea: Peritrichia) re-evaluated using historical morphological data and museum type material

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KEYWORDS: *Trichodina hypsilepis*, *T. heterodentata*, alpha taxonomy, species complex, *Oreochromis mossambicus*

THIS PAPER HAS NOT BEEN SUBMITTED ELSEWHERE IN IDENTICAL OR SIMILAR FORM, NOR WILL IT DURING THE FIRST THREE MONTHS AFTER ITS SUBMISSION TO *HYDROBIOLOGIA.*

Two trichodinid species *Trichodina heterodentata* Duncan, 1977 and *Trichodina hypsilepis* Wellborn, 1969 (Oligohymenophorea: Peritrichia) re-evaluated using historical morphological data and museum type material

Gerhard de Jager · Linda Basson · Jo van As

Abstract *Trichodina heterodentata* was first described from fish breeding farms in the Philippines by Bryan Duncan in 1977 as ectoparasites of imported cichlids, more specifically the southern African *Oreochromis mossambicus* from the Limpopo River System. This trichodinid has subsequently been described from almost every continent, bar North America. This cosmopolitan species, with a seeming preference for cichlid hosts, has unambiguous morphological features, but with distinct variances between and within populations. After reviewing previous descriptions from North American trichodinids, analysing the morphological data (both generally published information along with the original type from the Smithsonian Museum) and investigating the distribution patterns of the southern African introduced *O. mossambicus* throughout the North American water systems, it seems likely that three of the four trichodinids investigated are all synonyms of *T. heterodentata*. *Trichodina hypsilepis* Wellborn, 1967, *T. salmincola* Wellborn, 1967 and *T. vallata* Wellborn were very similar, while *T. funduli* Wellborn, 1967 differed quite substantially. This not only annihilates several North American trichodinid species, but also probably indicates an insidious African alien introduction sneaking its way into this continent.

Keywords *Trichodina hypsilepis* · *T. heterodentata* · alpha taxonomy · species complex · *Oreochromis mossambicus*

Introduction

Members of the family Trichodinidae Raabe, 1959 are all eukaryotic, protozoan, mobiline ciliophoran ecto- and endosymbionts, using the tissue of their hosts as substrates to hover across or to temporarily attach to. Trichodinids are usually associated with freshwater, estuarine or marine teleost fishes, but have been described from amphibians (Fulton, 1923; Lom, 1958; Kazubski, 1988; Kruger *et al*., 1991; Kruger *et al.,* 1993; Dias *et al*., 2009), aquatic and terrestrial invertebrates (Kazubski, 1991; Basson & van As, 1991; Basson & van As, 1992; van As & Basson, 1993), found in the urinary tract of chondrichthyans (van As & Basson, 1996) and recently in the reproductive tracts of waterfowl in Canada (Carnaccini *et al.,* 2016). Members of the Trichodinidae has a large ecological variability and is highly

cosmopolitan with more than 300 species, representing 11 genera (Basson & Van As, 1989; van As & Basson, 1993; Hu 2011) described and the genus *Trichodina,* Ehrenberg, 1830, having the greatest number of species. Representatives within the genus *Trichodina* are primarily identified on the morphological differences in the reach of their adoral spiral and the aboral denticle ring of the adhesive disc (Lom, 1958: 252).

The history of trichodinid systematics and taxonomy have been varied and fluid since the beginning. Due to the development of taxonomic systems using morphological characteristics first introduced by Dogiel (1940), then adapted by Fauré-Fremiet (1943), standardised by Lom (1958), expanded on by Raabe (1963) and refined by van As and Basson (1989) the state of higher systematics has always been in constant flux.

Even though trichodinids have been described from the United States of America (Fulton, 1923; Mueller, 1937; Hirshfield, 1949; Uzmann & Stickney, 1954; Estes *et al.*, 1997), recently there has been very few descriptions or records.

An important contribution was made by Wellborn (1967) when he described eight new species from freshwater teleost hosts from the Southeastern U.S. Of these new species, *T. hypsilepis*, from the highscale shiner shows similar denticle morphology and biometrics as those of the cosmopolitan *T. heterodentata.* The only other morphological description of *T. hypsilepis* was by Arthur and Lom (1984: 179) infesting the skin of unidentified tadpoles from a freshwater pond in Havana, Cuba.

Trichodina heterodentata is credited in having a large range of biometric variation in its denticle structure. Duncan (1977: 80) commented on this variance as reason for his nomenclature of this species. Using the large variation of body diameter for each described population, *T. heterodentata* can be clumped into three overarching groups according to body diameter size.

A topographical distribution pattern is observed as group one clusters only around Pacific Asia. Although the international descriptions from new hosts and localities subsequent to the work of Duncan, have highlighted the denticular differences, Duncan's original description still contains specimens with the largest overall dimensions.

Wellborn (1967) and Duncan (1977) used the morphological characteristics proposed by Lom (1958) in their descriptions, as the van As & Basson (1989: 161) denticle description for identifying additional information regarding the relationship of the individual denticles between closely related species, had not yet been published.

As stated earlier, *T. heterodentata* was first described from the southern African cichlid *O. mossambicus* (Peters 1852), (the Mozambique tilapia), which was probably one of the most widely distributed aquaculture fishes in the world.

The present paper compares two species of trichodinids; the relatively unknown *T. hypsilepis* and the well-studied *T. heterodentata* by incorporating alpha-taxonomy from historical data and re-investigated museum type material, commenting on the possibility of *T. heterodentata* being a species complex and the introduction of this symbiont from the southern African continent into North

America by tracing the distribution history of the cichlid fish *Oreochromis mossambicus*.

Material and methods

Photomicrographs used in this study for the re-measurements of biometric data was based on the museum type material (holo- and paratype) housed in the Protozoan and Helminth collections of the Smithsonian Institute in Washington, USA. The type material for *T. heterodentata* was collected by Duncan (1977) from three populations of freshwater teleost hosts (USNM No. 24485, USNM 24486 and USNM No. 24487) in the Philippines. Wellborn's (1967) type material was used for *Trichodina hypsilepis* (USNM No. 61651 & 61652) and *T. fultoni* (1357367 & 1357368) from Alabama, *T. salmincola* (USNM 1357379 & 1357380) from North Carolina and *T. vallata* (USNM 1357384) from Georgia. The above-mentioned material was collected from various freshwater fish hosts.

The type material was impregnated with silver nitrate using Klein's (1926) method, as recommended by Lom (1958), by the original authors. All their morphometric measurements follows the proposed uniform characteristics system by Lom's (1958) and are given in micrometers. Minimum and maximum values, followed by the arithmetic mean and standard deviation, are given where possible. For two characteristics; number of denticles and number of radial pins per denticle, the mode, rather than the arithmetic mean, is given. Denticle descriptions for all species, except *T. funduli*, from the Smithsonian micrographs were done according to the methods proposed by van As and Basson (1989).

Results

Morphometric measurements of the type material from the Smithsonian from Duncan's (1977) populations are provided and it was noted that most of population C trichodinids (Figures 1e & f) were still in their juvenile stages, therefore these were not measured as they would skew statistical results. Only populations A (Figures 1a & b) and B (Figures 1c & d) were used for the new measurements.

Denticle descriptions from the Smithsonian type material follows:

Trichodina heterodentata Duncan, 1977
(Figures 1a to d, 3a) (Table 1)

Hosts: Oreochromis mossambicus (Peters 1885) (population A) and C. *zillii* (Gervais, 1848) (population B).
Location on host: Gills, body and fins.
Locality: Both populations A and B from Freshwater Aquaculture Centre, Central Luzon State University, Muños, Nueva Ecija, Philippines.
Type-specimens: Holotype slide USNM No. 24485 (population A) and Paratype slide USNM No. 24486 (population B) (Smithsonian).
Description: Remeasured type material results and Duncan's (1977) original morphometric data are provided in Table 1.

Broad sickle-shaped blades that filled most of the area between the y and y-1 axes. A very deep smooth and gradually sloping L-shaped distal blade margin is observed, which ran parallel to the border membrane. The deepest part of the curve is more proximal than the apex of the blade, which is rounded, touching and in some cases

extending past the y+1 axis. A prominent blade apophysis is present and no posterior projection was observed.

The central part is robust and squat with the proximal surface being smaller than the distal surface and it extended almost halfway to the y axes.

The ray is strong and of equal width throughout the ray, tapering off somewhat towards a rounded tip, with a small anterior ray apophysis observed. The ray connection is somewhat narrower than the ray itself.

Trichodina hypsilepis Wellborn, 1967
(Figs. 2a to c, 3b) (Table 1)

Host: Notropis hypsilepis Suttkus and Raney, 1955, highscale shiner .
Location on host: Body and fins.
Locality: Halawakee Creek, Chambers County, Alabama and the Chattahoochee River, Chambers County, Alabama, USA.
Type-specimens: Holotype and one paratype slide USNM Helm. Coll. No. 64651 and 61652 (Smithsonian). Paratypes in the author's collection.

Description: Original morphometric data by both Wellborn (1969) and Artur & Lom (1984) are given in Table 1. Re-measured type material results were the same as the original Wellborn (1969) publication data and not included in Table 1.

The denticles were shown to have strong, semi-circular blades filling the section between the y and y+1 axes with a prominent anterior apophysis. The tangent point of the blade is located more proximal than the distal blade margin, which in itself is curved, sloping gradually in the proximal direction and not running parallel to the border membrane. The posterior blade margin forms a shallow C-shape with the deepest part more proximal than the apex of the blade that touches the y+1 axis, but in some blades, do not reach the y axis.

The central part is generally robust and strongly developed with no observable lower central part indentation. The distal area is larger than the proximal area and slopes slightly. The central part extends to touch the y-axes.

The rays of the denticle is generally delicate to strongly developed, mostly straight and of equal width for the whole length, tapering towards a sharp to rounded tip. The rays generally touch and in some cases extends beyond the y-1 axis. A small anterior ray apophysis is present and the ray connections were narrower than the width of the rest of the ray.

Trichodina salmincola Wellborn, 1967
(Figs. 2d, 3c) (Table 2)

Host: Oncorhynchus mykiss (Walbaum, 1792) rainbow trout.
Location on host: Fins and body.
Locality: Haywood county, North Carolina.
Type-specimens: Holotype USNM Helm. Coll. No. 61657 and one paratype USNM Helm. Coll. No. 61658. Paratypes in authors personal collection.
Description: Original morphometric data by Wellborn (1969) are given in Table 2.

The denticles have a strong, broad sickle-shaped blade that fill the area between the y-axes. The tangent point of the blade is small and proximal to the distal blade margin that is curved and not parallel to the border membrane, sloping proximally on the anterior side. The posterior blade surface is deep and has a smooth L-shaped curve with the deepest

point of its curve in on the same plain as the apex of the blade. The apex is rounded and touches or slightly extends past the y+1 axis. A prominent blade apophysis was observed.

The mostly slender central part was elongated and extended, curved slightly proximally. The distal area is much larger and more sloping than the proximal area, while it extends more than halfway past the y-axis. No lower central part indentation was observed.

The denticle ray is delicate, tapering off towards a pointed tip, extending towards the y+1 axis with a prominent anteriorly directed ray apophysis and a very narrow ray connection.

Trichodina vallata Wellborn, 1967
(Figs. 2e, 3d) (Table 2)

Host: Ictalurus punctatus (Rafinesque, 1818), channel catfish.
Location on host: Fins, body and gills.
Locality: National Fish Hatchery, Whitfield County, Georgia.
Type-specimens: Three holotypes in USNM Helm. Coll. No. 61662.
Description: Original morphometric data by Wellborn (1969) are given in Table 2.

Denticle blade fills most of the space between the y and y-1 axes. The tangent point of the blade is proximal to the distal blade margin that has a straight sloping curve, not parallel to the border membrane. The posterior blade margin has a very shallow (the deepest point of the curve being very close to the y axis), with a slight C-shaped curve, with the deepest point on the same level as the apex of the blade. No posterior projection was observed but the anterior blade apophysis is present.

A very robust and squat central part, almost straight with a rounded tip was observed. The distal surface is slightly smaller, proximally more sloped than the distal side and the central part extended past the y-1 axes. No lower central part indentation was detected.

The ray is very robust, of equal width for the whole length that tapers sharply towards a very rounded tip and extended past the y-1 axis. The ray apophysis is present and the ray connection, even though slightly narrower than the rest of the ray, was robust.

Trichodina funduli Wellborn, 1967
(Fig. 2f) (Table 2)

Host: Fundulus notti (Agassiz, 1854), Starhead topminnow.
Location on host: Body and fins
Locality: Swift Creek, Augauta County, Alabama.
Type-specimens: Holotype and paratype: USNM Helm. Coll. No. 61645 and 61646. Paratypes in author's personal collection.
Description: Original morphometric data by Wellborn (1969) are given in Table 2.

The silver nitrate impregnation of Wellborn's (1967) original holotype material was of such quality that denticle structures could not successfully be drawn for descriptive purposes.

Discussion

The large body diameter variation within the recorded populations of *T. heterodentata* makes it possible to clump this species into three overarching groups. Group I is the "largest body diameter" group containing

Table 1 Comparison of Duncan's (1977) original *Trichodina heterodentata* Duncan, 1977 measurements with remeasured data obtained from his paratype specimens in the Smithsonian museum, with biometric data from two descriptions of *T. hypsilepis* Wellborn, 1967 (ADD - adhesive disc diameter, BD - body diameter, BMW – border membrane width, CL – collection locality, DBL - denticle blade length, DCPW - denticle central part width, DL - denticle length, DRD - denticle ring diameter, DRL - denticle ray length, DS - denticle span, HS - host species, LoH - location on host, n - population size, nD - number of denticles, nRP/D - number of radial pins per denticle) (* pertains to the mode, rather than the mean) (** individuals measured for number of denticles) (all measurements in µm).

	Trichodina heterodentata				***Trichodina hypsilepis***	
	Duncan (1977)			Smithsonian 2017 Measurements of Duncan's paratype	Wellborn (1967)	Arthur & Lom (1984)
CL	Philippines A	Philippines B	Philippines C		Alabama, USA	Cuba
LoH	Skin & Fins	Skin, Fins & Gills	Skin		Skin & Fins	Skin
HS	*Oreochromis mossambicus*	*Coptodon zillii*, *O. mossambicus*	*Trichopodus trichopterus*		*Notropis hypsilepis*	Unknown tadpole
BD	71-106 (85)	58-108 (80)	70-122 (93)	66.1-85.8 (75.1 ± 6.0)	63-80 (70)	55.1-85.7 (67.4 ± 6.9)
ADD	47-63 (56)	47-63 (57)	54-81 (67)	57.8-79.5 (65.4 ± 6.0)	46-57 (52)	39.8-56.1 (47.6 ± 3.9)
DRD	26-37 (32)	26-37 (36)	30-52 (41)	34.5-49.8 (41.5 ± 4.4)	27-35 (32)	25.5-34.2 (29.4 ± 2.3)
DL	8	7.5-11 (9.2)	6.3	6.7-9.7 (7.9 ± 0.8)	11-13 (12)	10.2-14.8 (12.4 ± 1.1)
DBL	4.1	4.7-7.1 (5.7)	5.5	5.5-7.8 (6.3 ± 0.6)	5-6 (5.3)	4.5-5.6 (5.2 ± 0.8)
DRL	6.9	6.9-10.3 (8.1)	8.2	6.2-10.1 (8.0 ± 1.0)	7-9 (8)	6.6-11.2 (8.2 ± 0.8)
DCPW	3.4	1.4-3.4 (2.6)	4.1	1.8-4.4 (2.8 ± 0.5)	2-3 (2.6)	2-3.1 (2.5 ± 0.4)
DS	-	-	-	14.5-20.0 (17.0 ± 1.6)	-	14.3-19.4 (15.9 ± 1.0)
BMW	2.7	3.4-5.5 (4.7)	4.1	2.6-7.4 (4.8 ± 1.0)	4.0-4.5 (4.3)	4.1-6.1 (4.9 ± 0.5)
nD	20-27 (23*)	29-31 (26*)	18-31 (27*)	22-31 (28* ± 1.8)	21-24 (23*)	20-23 (21.0 ± 0.8)
nRP/D	11	6-14 (10*)	11	7-14 (12* ± 1.3)	10	9-12 (commonly 10-11)
n	52	25 (100**)	59	33	20	25

Table 2 Comparison of Wellborn's (1967) original *Trichodina salmincola* Wellborn, 1967, *T. vallata* Wellborn, 1967 and *T. funduli* Wellborn, 1967 measurements (ADD - adhesive disc diameter, BD - body diameter, BMW – border membrane width, CL – collection locality, DBL - denticle blade length, DCPW - denticle central part width, DL - denticle length, DRD - denticle ring diameter, DRL - denticle ray length, DS - denticle span, HS - host species, LoH - location on host, n - population size, nD - number of denticles, nRP/D - number of radial pins per denticle) (* pertains to the mode, rather than the mean) (all measurements in µm).

	Trichodina salmincola	***Trichodina vallata***	***Trichodina funduli***
CL	North Carolina	Georgia	Alabama
LoH	Skin & Fins	Skin, Fins & Gills	Skin & Fins
HS	*Salmo gairdneri*	*Ictalurus punctatus*	*Fundulus notti*
BD	61-85	52-80 (61)	70-104 (90)
ADD	40-59 (51)	31-49 (44)	54-65 (60)
DRD	29-39 (33)	24-29 (27)	33-41 (37)
DL	10-12 (11)	9-11 (10)	11-13 (12)
DBL	5-6 (5.8)	9-11 (10)	5-7 (6)
DRL	8-10 (8.5)	6.5-7.5 (7)	7-10 (9)
DCPW	2.0	1.5-2.0	2.0-3.0 (2.2)
DS	-	-	-
BMW	2-4 (3)	3-5 (4)	3-5 (4)
nD	21-26 (24*)	18-21 (22*)	23-27 (26*)
nRP/D	12	10	10
n	20	20	20

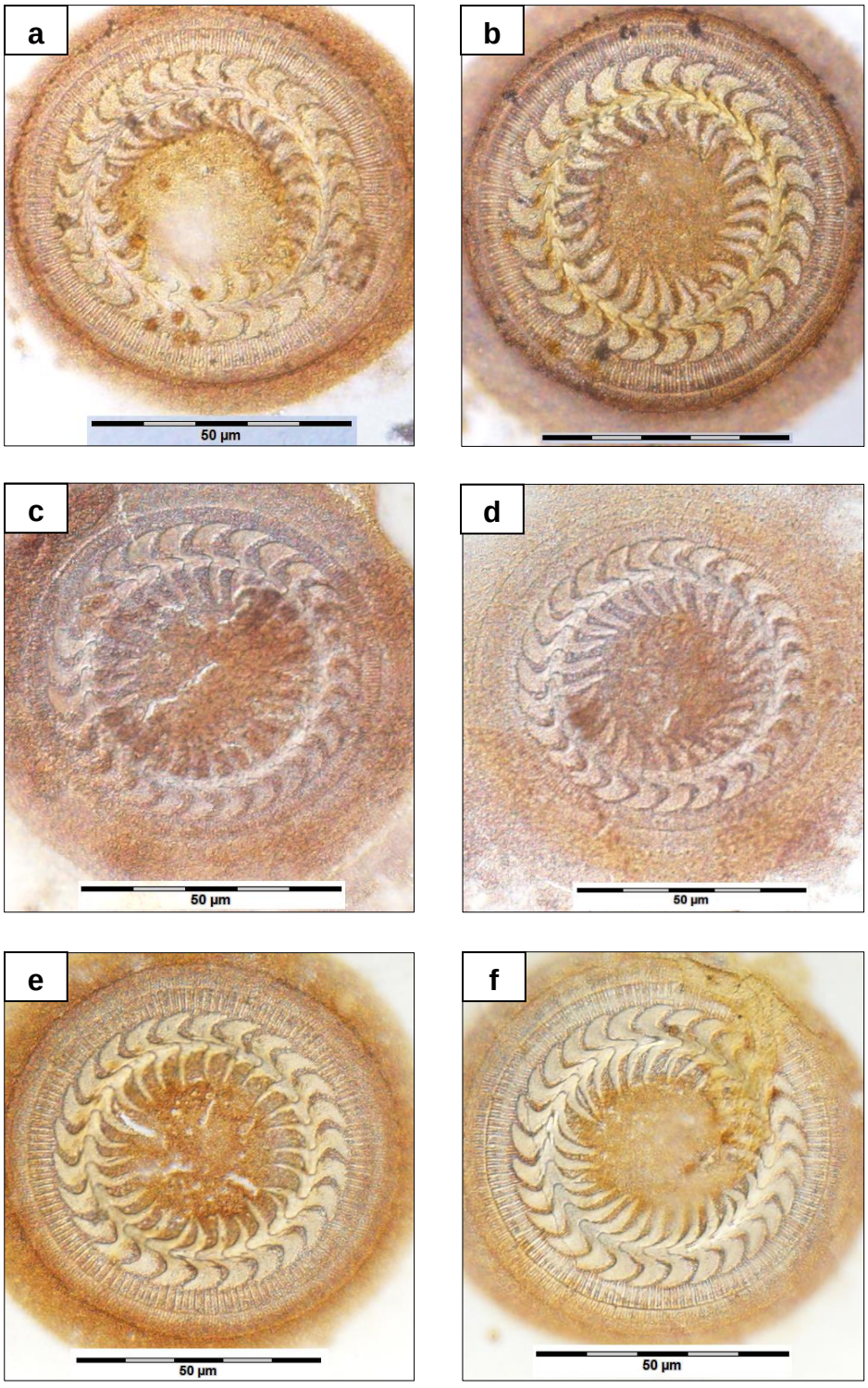

Fig. 1 Micrographs of *Trichodina heterodentata* Duncan, 1977 taken at the Smithsonian Museum from Duncan's (1977) paratype material, where **a** and **b** represent specimens from Duncan's population A from *Oreochromis mossambicus* (Peters, 1885) hosts, **c** and **d** from population B (*Coptodon zillii* (Gervais, 1884) hosts) and **e** and **f** from population C (*Trichogaster trichopterus* (Pallas, 1770) hosts) (scale is 50 µm).

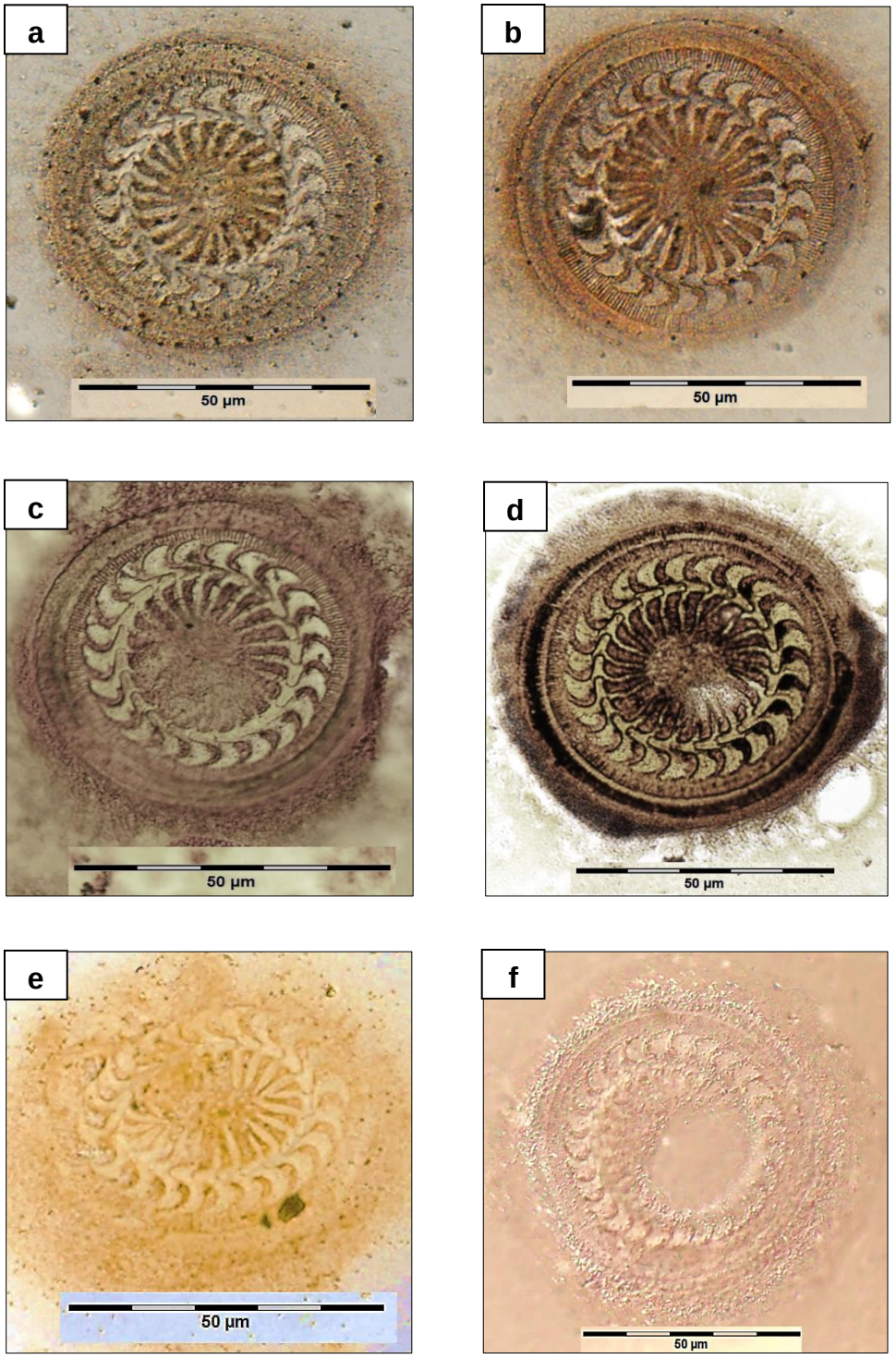

Fig. 2 Micrographs taken at the Smithsonian Museum from Wellborn's original 1967 paratype material. (**a** to **c** - *Trichodina hypsilepis* Wellborn, 1967 (host: *Notropis hypsilepis* Suttkus & Raney, 1955), **d** - *T. salmincola* Wellborn, 1967 (host*: Oncorhynchus mykiss* (Walbaum, 1792)), **e** - *T. vallata* Wellborn, 1967 (host: *Ictalurus punctatus* (Rafinesque, 1818)) and **f** – *T. funduli* Wellborn, 1967 (host: *Fundulus notti* (Agassiz, 1854) (scale = 50 µm).

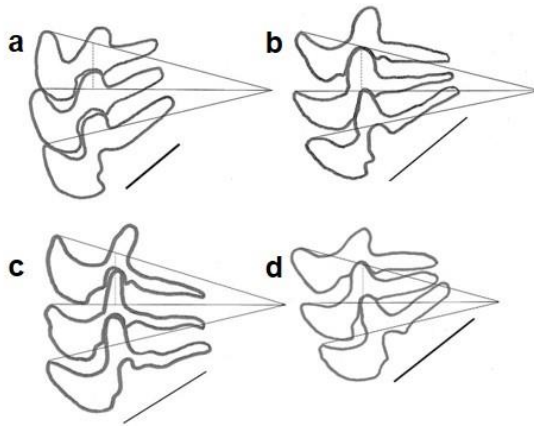

Fig. 3 Comparisons of denticles redrawn from micrographs taken of **a** *Trichodina heterodentata* Duncan, 1977, **b** *T. hypsilepis* Wellborn, 1967, **c** *T. salmincola* Wellborn, 1967 and **d** *T. vallata* Wellborn, 1967 paratypes housed at the Smithsonian Museum, USA.

populations with a mean body diameter larger than 70µm, while group II ("middle sized"), are between 55 and 69µm. Group II boasts with the most records for *T. heterodentata* and group III, with populations smaller than 54µm, are restricted to very few record, and primarily from tadpole hosts.

The morphometric measurements made from the Smithsonian micrographs and compared to Duncan's (1977) data was informative: although the re-measured data still fell into the larger-body diameter group, the measurements weren't nearly as large as those originally published by Duncan (Table 1). We believe that Duncan may have made an error with his measurements or scale conversions, as the latest data fit perfectly within the range of all the following descriptions, and supports the topographical pattern for the larger specimens in Pacific Asia.

Denticle descriptions from *T. salmincola* showed similar traits to both Wellborn' (1967) *T. hypsilepis* and *T. heterodentata*, but unfortunately there were too few useable specimens from the original holotype photomicrographs to compile a large enough population for morphometric analysis. From the denticle descriptions and Wellborn's original measurements it seems possible that *T. salmincola* might also be a synonym for *T. heterodentata*, but not enough data is available for any concrete suggestions. *Trichodina vallata*, had almost no denticular similarities with any of the other mentioned species after completing van As & Basson's (1989) denticle description, and therefore appear to be a valid species.

Comparing the van As & Basson (1989) denticle descriptions for *T. heterodentata* and *T. hypsilepis* (Figures 3a & b), similarities such as the shape of the blade with its very prominent anterior apophysis, the central part and especially the shape of the ray, were so pronounced that one can easily reason that they may be the same species, and probably belong to the same species.

If this assumption is correct, the next question to be asked will be how, and through which host, an African trichodinid got into the North American river systems? As *T. heterodentata* appears to prefer cichlid hosts, and *O. mossambicus* is endemic to southern African waters, the distribution of this host worldwide is important.

The initial export of *O. mossambicus* from Africa was to the East. The first stock, according to Guerrero (1994) was brought to Java by East African traders in 1938, from there they were exported into most of Pacific Asia and eventually to the Philippines, through Thailand during the 1950s and 60s (De Silva *et al.*, 2004). At the same time sixty Mozambican tilapias were sent from Singapore to Hawaii in 1954, and the progeny of these fish were then sent to the public aquarium in New York, who in turn, sent the offspring of those to Alabama (Rogers, 1961; Smith-Vaniz, 1968), Arizona (Hoover & St. Amant, 1970; Minckley, 1973) and California (St. Amant, 1966) for aquaculture resources or sport fishing. At this time many tilapias escaped into the natural water systems during loading and harvesting or through containment failures, resulting in the first reported Mozambique tilapia in the Alabaman River System in 1965, which is the same river system in which *T. hypsilepis* was first described in 1967. This distribution and introduction of *O. mossambicus* across the East into the southern states of North America adds more weight to the possibility that *T. hypsilepis* and *T. heterodentata* are indeed the same species.

The International code of Zoological nomenclature (Ride *et al*., 1985) states in Article 23, that the Principle of Priorities (using the oldest available name for a valid taxon) must always be applied. This paper, however, recommends that *T. hypsilepis* found on fish hosts, albeit the oldest available taxon of the two, be named a synonym of *T. heterodentata*. The reasons for this recommendation are two-fold; Wellborn (1967) described *T. hypsilepis* using a comparatively small trichodinid population, which was described from a fish host, while the only other records of this trichodinid were from tadpole hosts by Arthur and Lom (1984). *Trichodina heterodentata,* on the other hand, was described from three large trichodinid populations, all from cichlid hosts (*O. mossambicus*) by Duncan (1977) and has subsequently been described from various countries worldwide by more than 18 authors. Synonymising *T. heterodentata* to *T. hypsilepis* might cause more confusion in the already bewildering world of mobiline taxonomy.

Analysing the morphometrics and then comparing the van As & Basson (1989) denticle descriptions for the *T. heterodentata* records from tadpoles (Kruger *et al*., 1993; Dias *et al.,* 2009) with those of *T. hypsilepis* (Wellborn, 1967; Arthur & Lom, 1984) it can be observed that within the *T. heterodentata*-like grouping two noticeable categories can be distinguished; the first falling within groups I and II size order, exhibiting more robust rays, generally more than 24 denticles per individual and always associated with fish hosts (almost all fish hosted *T. heterodentata* and Wellborn's *T. hypsilepis* description). The other category fall into the group III size group, with less robust, but more delicate rays, usually more than 23 denticles per individuals and associated only with tadpole hosts (all *T. heterodentata* from tadpole hosts and *T. hypsilepis* as described by Arthur and Lom (1984)).

During recent molecular analysis of 18S SSU rDNA trichodinid gene sequences, collected from tadpoles in the Okavango Delta, soon to be published in a separate article, it was observed that these clustered into their own clade around a *T. hypsilepis* sequence, obtained from anuran hosts in China and separate from the fish host "*heterodentata"* gene sequences recorded on NCBI.

It is therefore further proposed that the species taxon "*hypsilepis"*, rather than sink into taxonomic obscurity, be kept, but used for "*heterodentata*"-like trichodinids from tadpole hosts only and the species taxon *T. heterodentata* be used for freshwater teleost hosts only. This new taxonomy will also highlight *T. hypsilepis* and *T. heterodentata* being generally more host specific than previously perceived.

This case study illustrates that the weight of almost 200 years of diligent morphological techniques cannot be ignored, especially when it comes to investigating historical data and making taxonomic inferences from it. It also illustrates that morphology cannot stand alone as the pinnacle of taxonomy, because without the understanding of parasite-host interactions and knowledge of host distribution history those inferences would be mere sweeping statements.

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Compliance with ethical standards Ethical clearance obtained.

Conflict of interest The authors declare that they have no conflict of interest.

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CHAPTER 7 - RESULTS – MOLECULAR

Collection and Extraction of gDNA

Altogether thirty-five 2 ml Eppendorf vials of tissue were collected for molecular analysis. Trichodinid samples were collected in various fixative vials, from various hosts location on the host, or host tissue itself (the tip of the tadpole tails preserved in samples 18, 25, 33 and 34) (Table 7.1). Two trichodinid genera were collected; *Trichodina* sp. from anuran hosts collected at Nxamasere pools 3, 4, 5 and 6 and *Trichodinella* sp., also from anuran hosts, from pool 5. Only *Schlerophrys* individuals from pool 5 had a high enough infestation of *Trichodinella* species for collection (samples 31, 32 to 35). Two samples from pool 6, samples 26 and 28 contains mostly *Trichodina* symbionts, but they also contained small numbers of *Trichodinella*, these were kept for analysis (see Table 7.1).

During the winter of 2015 expedition samples were fixed in absolute ethanol for molecular analysis, but were not kept at the desired temperature (-8°C) and could only be analysed a year after collection. The PCR reaction done using gDNA extracted from these samples did not give the desired product as it was concluded that the DNA was too degraded to be used in downstream amplification reactions (Fig. 7.1**A**). When gDNA extracted from the 2016 samples were used as template the desired products were observed (Fig. 7.1**B**).

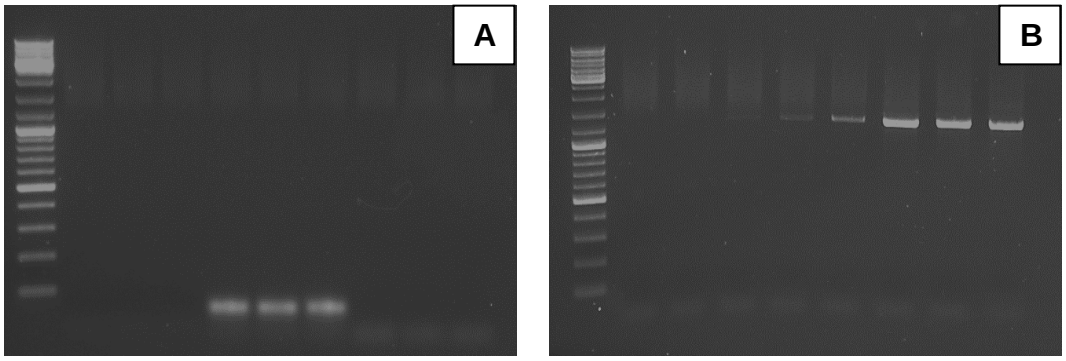


Figure 7.1: Gel electrophoresis of **A** – PCR products using gDNA extracted from the 2015 expedition samples and **B** – PCR products obtained for a temperature gradient PCR using intact gDNA from the 2016 collection and primer set MX.

Genomic DNA extracted from samples stored in absolute ethanol and 96% ethanol was used as template to evaluate the most effective storage method. Gel electrophoresis of the PCR products obtained using the three different primers showed that samples stored in water gave high intensity bands compared to band intensities observed for the 100% and 96% ethanol. Only one sample was stored in this fashion and repeatability could not be tested. For downstream amplification reactions gDNA extracted from samples stored in 96% ethanol was used as this sample also yielded excellent electrophoresis bands, in comparison with the absolute (100%) and 70% ethanol. Different samples were collected, in a variation of collection media and different concentrations (Table 7.1).

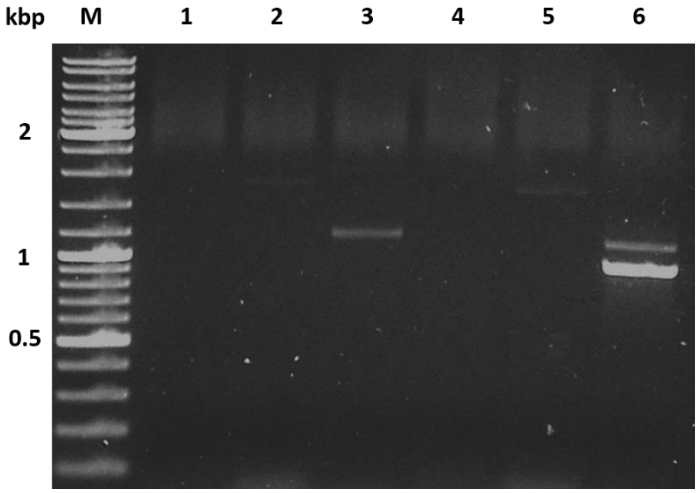


Figure 7.2: Agarose gel electrophoresis of the PCR product obtained using gDNA from two *Trichodina* samples collected in 2016, amplified using three different prime sets; MX5:MX3 (lanes 1 & 4), ERIB1:ERIB10 (lanes 2 & 5) and ERIB10-v:28s1r (lanes 3 & 6). (**M** –GeneRuler^{TM} DNA Ladder Mix). (sample in lanes 1 to 3 was fixed in absolute ethanol, while the sample in lanes 4 to 6 was preserved in 96% ethanol).

Genomic DNA was successfully extracted from all the anuran host samples as the agarose gel electrophoresis in Figure 7.2 indicates, using three different primer sets.

Genomic DNA extracted from the anuran hosts were not analysed for this dissertation, as their molecular taxonomy has not yet been published and the morphological descriptions made were sufficient.

Table 7.1: Collection data of molecular samples during the Winter 2016 collection at the Nxamasere Floodplains, Botswana.

Sample number	Host Population	Symbiont isolated	Location on Host tissue	$_dH_2O$	70% Ethanol	96% Ethanol	Absolute Ethanol
1	3	*Trichodina heterodentata*	Gills	2 mL			
2	5	*Trichodina heterodentata*	Water				2 mL
3	5	*Trichodina heterodentata*	Water				2 mL
4	4	*Trichodina heterodentata*	Gills				2 mL
5	4	*Trichodina heterodentata*	Gills				2 mL
6	4	*Trichodina heterodentata*	Gills				2 mL
7	4	*Trichodina heterodentata*	Gills				2 mL
8	4	*Trichodina heterodentata*	Gills				2 mL
9	4	*Trichodina heterodentata*	Gills			2 mL	
10	4	*Trichodina heterodentata*	Gills			2 mL	
11	4	*Trichodina heterodentata*	Gills			2 mL	
12	4	*Trichodina heterodentata*	Gills			2 mL	
13	4	*Trichodina heterodentata*	Gills		2 mL		
14	4	*Trichodina heterodentata*	Gills		2 mL		
15	4	*Trichodina heterodentata*	Gills		2 mL		
16	4	*Trichodina heterodentata*	Gills		2 mL		
17	4	*Trichodina heterodentata*	Gills		2 mL		
18	4	*Schlerophrys gutturalis*	Tip of Tail		2 mL		
19	4	*Trichodina heterodentata*	Gills		2 mL		
20	6	*Trichodina heterodentata*	Gills			2 mL	

Table 7.1 (cont.): Collection data of molecular samples during the Winter 2016 collection at the Nxamasere Floodplains, Botswana.

Sample number	Host Population	Symbiont isolated	Location on Host tissue	$_dH_2O$	70% Ethanol	96% Ethanol	Absolute Ethanol
21	6	*Trichodina heterodentata*	Gills			2 mL	
22	6	*Trichodina heterodentata*	Gills			2 mL	
23	6	*Trichodina heterodentata*	Gills			2 mL	
24	6	*Trichodina heterodentata*	Gills				
25	6	*Schlerophrys powerii*	Tip of Tail		2 mL		
26	6	*Trichodina heterodentata* & *Trichodinella* sp.	Gills		2 mL		
27	6	*Trichodina heterodentata*	Gills		2 mL		
28	6	*Trichodina heterodentata* & *Trichodinella* sp.	Gills				2 mL
29	6	*Trichodina heterodentata*	Gills				2 mL
30	5	*Schlerophrys gutturalis*	Tip of Tail		2 mL		
31	5	*Trichodinella* sp.	Gills		2 mL		
32	5	*Trichodinella* sp.	Gills			2 mL	
33	5	*Schlerophrys gutturalis*	Tip of Tail		2 mL		
34	5	*Schlerophrys gutturalis*	Tip of Tail		2 mL		
35	5	*Trichodinella* sp.	Gills				2 mL

PCR Amplification and Gel Electrophoresis

Three different primer sets were used, each of which amplifies a different part of the SSU gene (Fig. 7.3). The first primer set, EriB-EriB10 (Fig. 7.3 blue), the universal eukaryotic primer (Tang *et al*. 2013) amplified a large region of the 18S region that is between 1.6 and 1.7kbp large (Fig. 7.4). A second set of primers, MX5-MX3 (Fig. 7.3 black) that amplifies a smaller (± 1.3kbp) (Fig. 7.5), central part of the 18S region, is also used, as in some cases the universal primer pair may have a poor yield (Tang & Zhao 2016). The third set of primers, EriB10-v-28s1r (Fig. 7.3 green), used to amplify the ITS1-5.8S-ITS2 region is approximately 1.2kbp large (Fig. 7.6).

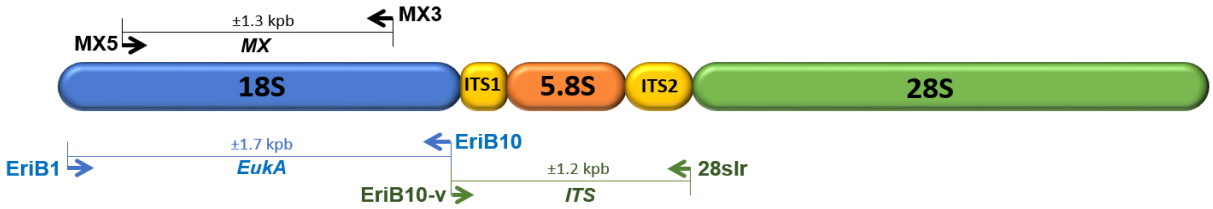


Figure 7.3: The three primer pairs used, EukA (EriB1 (forward)-EukA10 (reverse)), MX (MX5 (forward)-MX3 (reverse)) and ITS (EriB10-v (forward)-28s1r (reverse)) primer pairs, the size (in kilo base pairs (kbp)) and the regions of the 18S SSU rDNA gene these primer pairs amplifies during PCR.

PCR amplification with REDExtract® *Taq* polymerase using both the 18S region primer sets of eight samples showed the expected size of the DNA regions (Fig. 7.3). Unfortunately, it was later discovered that REDExtract® DNA *Taq* polymerase produces A-overhangs, while the chosen cloning vector required blunt ends. For this reason, the PCR products was again amplified using KAPA HiFi HotStart DNA *Taq* polymerase giving the same results.

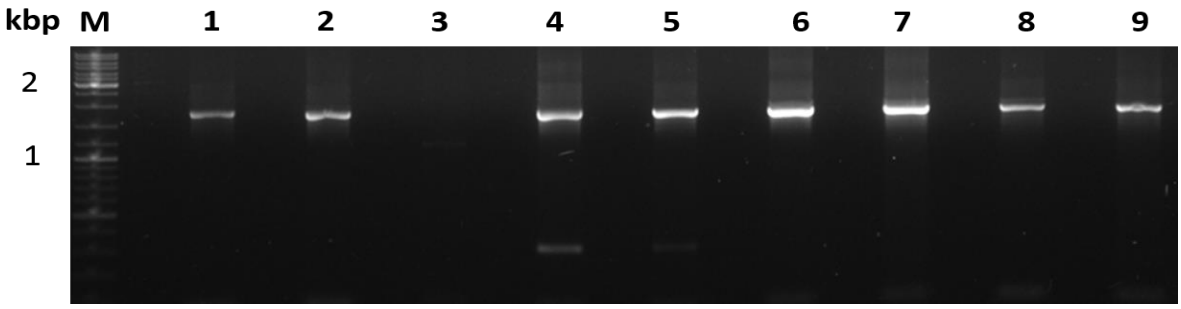


Figure 7.4: Agarose gel electrophoresis of PCR product obtained from using gDNA from collected samples amplified by the EukA primer set showing the segment size (in kbp) with M = GeneRuler™ DNA ladder.

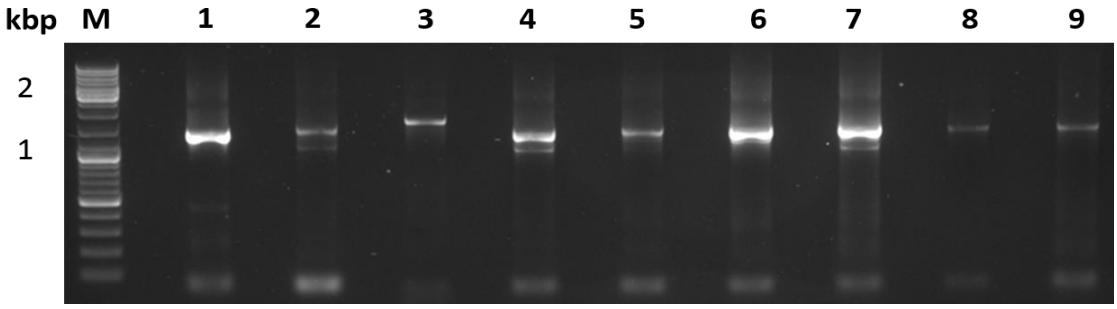


Figure 7.5: Agarose gel electrophoresis of PCR product obtained using gDNA from collected samples amplified by the MX primer set showing the segment size (in kbp) with M = GeneRuler™ DNA ladder.

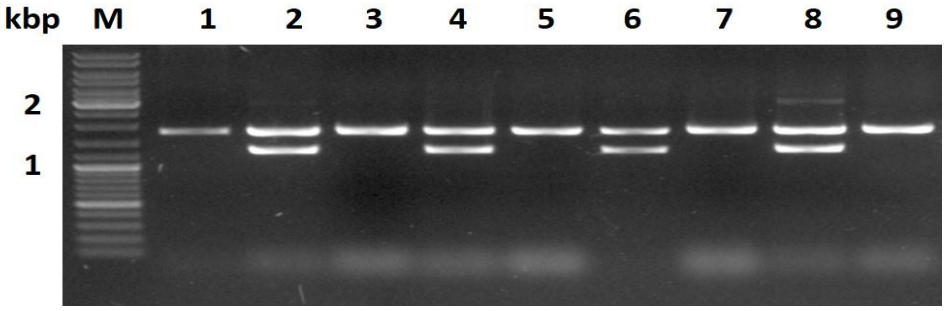


Figure 7.6: Agarose screening gel electrophoresis PCR product digested with *Eco*RI and *Hind*III restriction enzymes from collected samples amplified by the ITS primer set showing the segment sizes (in kbp) with M = GeneRuler™ DNA ladder.

Cloning and Sequencing of the 18S SSU Gene

Phosphorylated PCR products were ligated into pSMART and transformed into chemically competent *E. coli* TOP cells. The transformation culture was plated out onto LB agar plates containing 30µg.ml^{-1} kanamycin. Ten positive transformants were inoculated into 5ml LB media containing 30µg.ml^{-1} kanamycin to maintain selective pressure and grown for 16 hours at 37°C. DNA mini-preparations followed the 16 hour growth of the positive transformants. This was performed using the GeneJET plasmid extraction kit as per manufacturer's recommendations (Thermo Scientific). RFLP was used as screening method using *Eco*RI and *Hind*III.

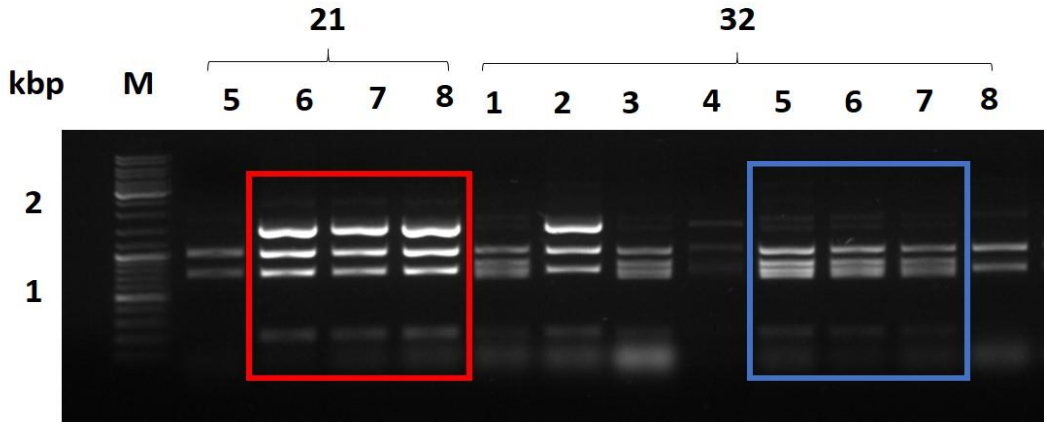


Figure 7.7: Agarose gel electrophoresis of the products from collected sample 21 (populations 6) and 32 (population 5) amplified by the universal eukaryotic primer set and digested with restriction enzymes *Eco*RI and *Hind*III with M = GeneRuler™ DNA ladder.

There are distinct differences in the digestion profiles between the 18S bands of *Trichodina* sp. that shows very clear bands corresponding to the 800, 1 000 and 1 500bp bands on the DNA ladder (Fig. 7.7 red block) and *Trichodinella* sp., whose clear bands corresponds to the 700, 800, 900 and a 1 000 bp, with light bands also at 1 300 and 1 500bp (Fig. 7.7 blue block). This difference in digestion profiles between the two trichodinid groups may be used as a preliminary screening step to elucidate if both groups are present on a host, before sequencing.

Sanger sequencing was performed on selected plasmid constructs of the complete 18S SSU rRNA region. The screening gel of the 18S SSU inserts digested with *Eco*RI and *Hind*III revealed certain patterns of bands (representing the sizes of the segments cut by the restriction enzymes) for the two types of trichodinids collected form anurans (Fig 7.6).

The 18S rDNA of 1 800bp is larger than normal for conventional sanger sequencing reads. Sequencing of the gene from both sides with the SL1 and SR2 sequencing primers yielded reads with overlapping 3' end of approximately 100bp thereby successfully sequencing the entire gene and thus negated the necessity for using the internal MX primer pair.

Sequencing results, per population collected were blasted (Table 7.2) against the NCBI database for their closest related species. All constructs that was shown to be pSMART backbone, instead of trichodinid genes were discarded. Blasting revealed that the majority of trichodinid sequences collected from *Schlerophrys* species in the Nxamasere Floodplain were more closely related to the 18S SSU gene sequences of *Trichodina hypsilepis*, rather than *T. heterodentata* in the NCBI database. Table 7.2 illustrates that in the majority of cases the Okavango sequences aligned 98 to 99% with *T. hypsilepis* and only 90% to 93% with that of *T. heterodentata*. Cloned sequences from sample 32 (population 5) corresponded with *Trichodinella* sequences from the NCBI database that was expected as this population represented the only locality where a combination of *Trichodinella* and *Trichodina* species was found on the anuran hosts. Sample 35, also from population 5 confirms the mixture of trichodinid genera found on the hosts.

The same procedures were followed for the ITS1-5.8S-ITS2 regions from collected trichodinids, but very few blasting comparisons could be made, as there is a very limited number of trichodinids whose ITS regions have been amplified and deposited in any gene sequence data base.

Table 7.2: pSMART clones of sampled 18S SSU rDNA sequences, and which population they were collected from, blasted against NCBI database, closest related species and percentage of relatedness.

Sample nr.	Population	Clone	Blasted species	%
2	4	2-7	*T. heterodentata*	90%
3	5	3-5	*T. hypsilepis*	99%
			T. heterodentata	92%
		3-6	*T. hypsilepis*	99%
			T. heterodentata	91%
		3-8	*T. hypsilepis*	98%
			T. heterodentata	90%
		4-2	*T. hypsilepis*	99%
			T. heterodentata	93%
21	6	21-6	*T. hypsilepis*	99%
			T. heterodentata	91%
		21-7	*T. hypsilepis*	99%
			T. heterodentata	91%
		21-8	*T. hypsilepis*	99%
			T. heterodentata	92%
23	6	23-3	*Trichodinella sp.*	99%
		23-8	*T. hypsilepis*	99%
			T. heterodentata	91%
26	6	26-4	*T. hypsilepis*	99%
			T. heterodentata	91%
		26-6	*T. hypsilepis*	99%
			T. heterodentata	91%
32	5	31-7	*Trichodinella epizootica*	99%
		32-2	Undescribed *Trichodinella* sp.	99%
		32-3	Undescribed *Trichodinella* sp.	98%
		32-6	Undescribed *Trichodinella* sp.	99%
35	5	35-2	*Trichodina hypsilepis*	99%
			T. heterodentata	91%

PHYLOGENY ACCORDING TO 18S SSU SEQUENCES

18S rDNA inserts were successfully sequenced and assembled (**Appendices**: Plate B, page B) and a phylogenetic tree, using the Neighbour-Joining method (Saitou & Nei 1987), was compiled using some of the sampled trichodinid sequences from the Nxamasere Floodplain and other trichodinid gene sequences obtained from the NCBI database. Included in the phylogenetic tree are all the members of the family Urceolariidae with the outgroups *Colpoda magna* (Gruber, 1879) Lynn, 1978, and *Coleps hirtus* (O.F. Müller, 1786) Nitzsch, 1827, all sequences are available in the GenBank/NCBI database and can be viewed in Table 7.4.

The phylogenetic tree of the 18S rDNA (Fig. 7.8) reveals that the Family Trichodinidae formed a monophyletic clade (with a Maximum Likelihood (ML) of 100%, supporting the recent findings of Gentekaki *et al.* (2017), while a paraphyletic assemblage can be observed for the genus *Trichodina*, with a high support (100% ML) that species from both genera *Trichodinella* (Fig. 7.8**A** and Fig. 7.9) and *Trichodina* (Fig. 7.8**B** and Fig. 7.10) fall within this assemblage (more detail will be addressed in **Chapter 8**).

The trichodinid sequences from anuran host material in the Nxamasere Floodplain from the winter 2016 collection form two very noticeable clades within the paraphyletic trichodinids assemblage. Sequences E23_8, E3_8, E3_5. E21_8, E26_6, E3_6, E2_7, E 21_6, E35_5 and E4_2 form a robust clade (100% ML) around *T. hypsilepis* and not *T. heterodentata* as expected (Fig. 7.8**B** and Fig. 7.10). The statistical p-values (genetic distances) calculated for the number of base pair differences per site on the above mentioned sequences are given in Table 7.3 and further supports that the sequenced trichodinids are closer related to *T. hypsilepis* and indeed the same species. Tang and Zhao (2016) proposed that for 18S rDNA data the minimum and maximum genetic distances (P-values) should be between 0.000 - 0.005 for intraspecific level, 0.005 - 0.15 for genus-species levels and higher than 0.15 for family level. This assumption also confirms the phylogenetic findings, as samples E3_8 and E23_8 do not make part of the *T. hypsilepis* clade, but another closely related trichodinid, as observed in the Neighbour-Joining phylogenetic tree (Table 7.3 and Fig. 7.8**A**).

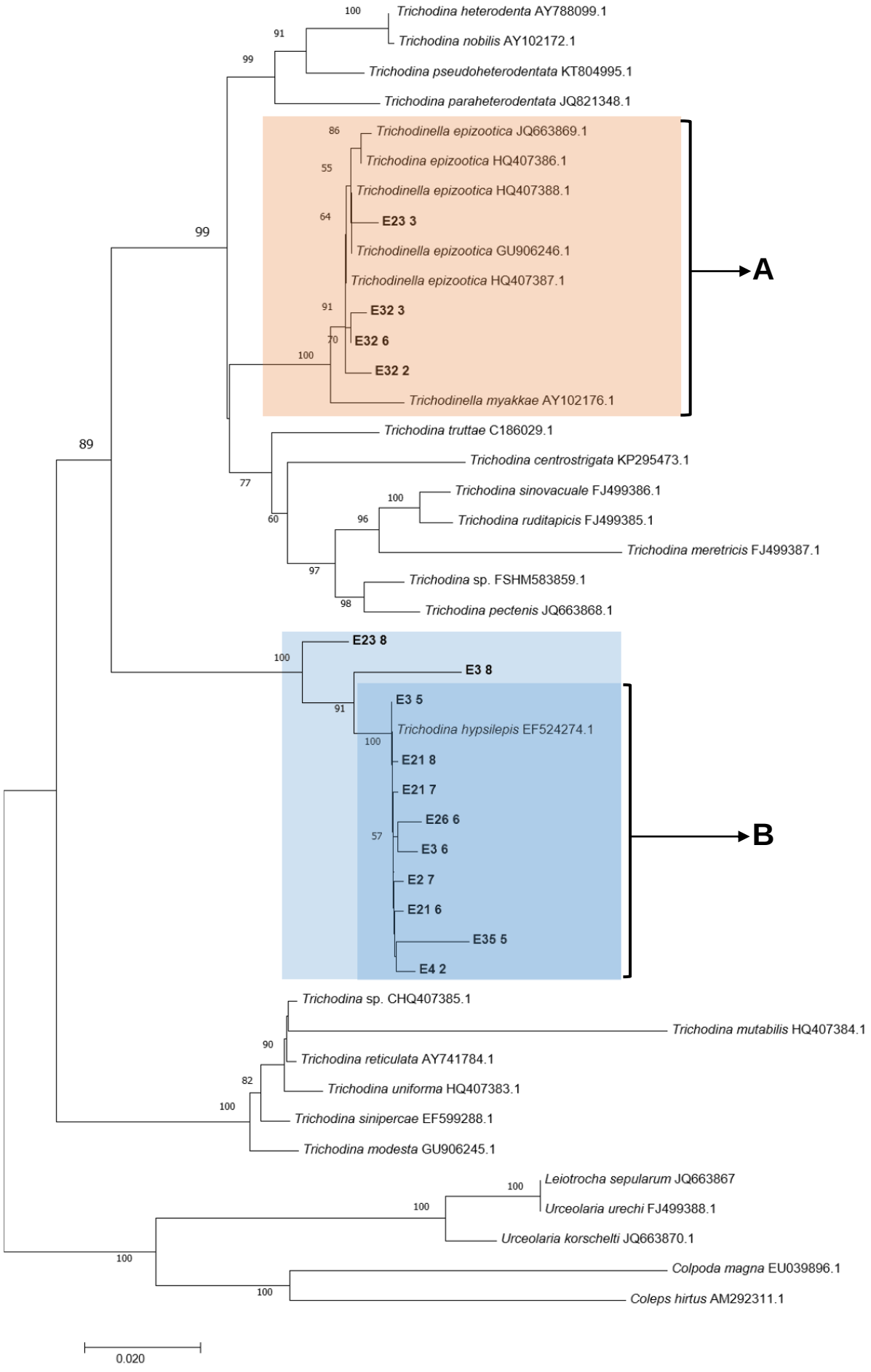
Table 7.3: 18SSU sequences from cloned inserts and their respective p-values for *Trichodina. heterodentata* Duncan, 1977 and *T. hypsilepis* Wellborn, 1967 from the NCBI database (P>0.05 is significantly different), P-values in red illustrates a significant difference.

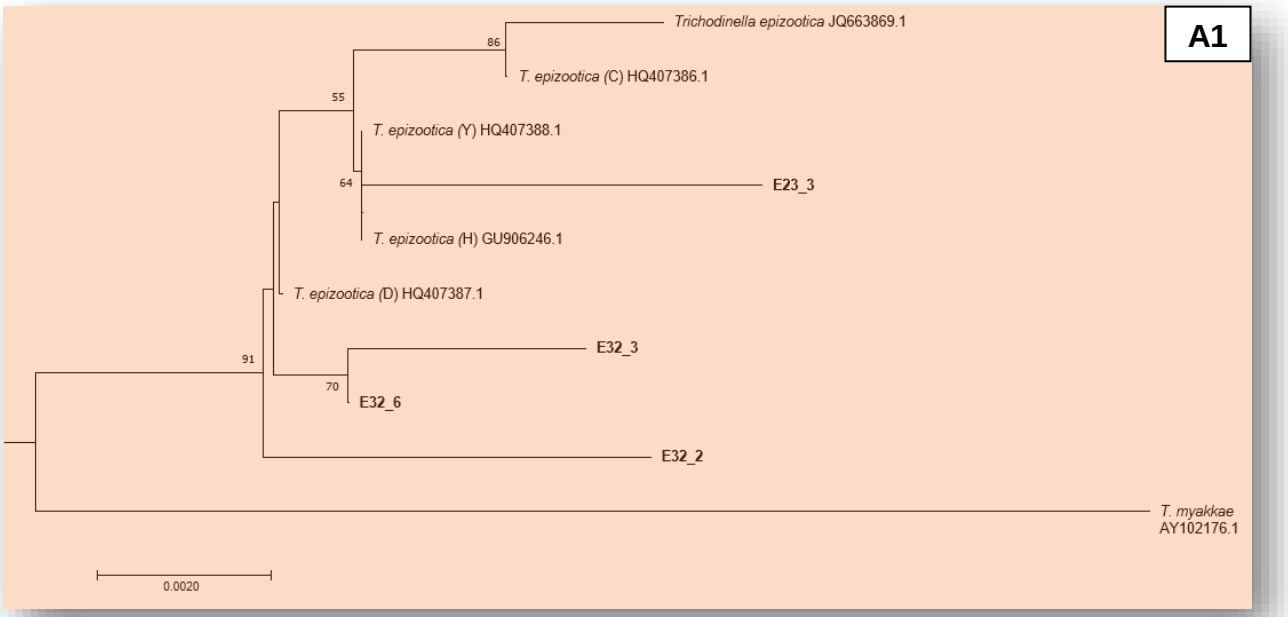
Sequence	*T. heterodentata*	*T. hypsilepis*
E35_5	0.101	0.012
E21_8	0.090	0.001
E26_6	0.094	0.005
E3_6	0.093	0.005
E3_5	0.090	0.000
E21_6	0.091	0.002
E27_7	0.090	0.001
E4_2	0.093	0.004
E2_7	0.091	0.002
E3_8	0.097	***0.025***
E23_8	0.082	***0.016***

The other types of trichodinids collected from the anuran hosts in Nxamasere, were morphologically identified as *Trichodinella* species, and all of the samples (E23_3, E32_3, E32_6 and E32_2) formed a cluster around the *Trichodinella* samples already in the NCBI/GenBank database (Fig. 7.8**A** and Fig. 7.9).

Table 7.4: List of all 18S SSU rDNA sequences used for phylogenic inference.

Species selected	Accession nr.	Author/Collector (Year)	Locality
Trichodina hypsilepis	EF524274	Gong *et al*. (unpublished)	Freshwater
T. pectenis	JQ663868	Zhan *et al.* (2013)	Marine
T. sinipercae	EF599255	Gong *et al.* (unpublished)	Freshwater
T. reticulata	AY741784	Gong *et al.* (2006)	Freshwater
T. heterodentata	AY88099	Gong *et al.* (2006)	Freshwater
T. nobilis	AY102172	Gong *et al.* (2006)	Freshwater
T. ruditapicis	FJ499385	Zhan *et al.* (2009)	Marine
T. sinonovaculae	FJ499386	Zhan *et al.* (2013)	Marine
T. meretricis	FJ499387	Zhan *et al.* (2013)	Marine
T. paraheterodentata	GU906244	Tang *et al*. (2013)	Freshwater
T. modesta	GU906245	Tang *et a*l. (2013)	Freshwater
T. uniforma	HQ407383	Tang *et al*. (2013)	Freshwater
T. mutabilis	HQ407384	Tang *et al*. (2013)	Freshwater
T. centrostrigeata	KP295473	Wang *et al*. (2015)	Freshwater
T. truttae	LC186029	Mizuno *et a*l. (unpublished)	Freshwater
T. pseudoheterodentata	JQ821348	Tang *et al***.** (2017)	Freshwater
***Trichodinella* sp.**	JQ663869	Zhan *et al.* (2013)	Freshwater
T. myakkae	AY102176	Gong *et al.* (2006)	Freshwater
***T. epizootica* (C)**	HQ407386	Tang *et al*. (2013)	Freshwater
***T. epizootica* (D)**	HQ407387	Tang *et al*. (2013)	Freshwater
***T. epizootica* (Y)**	HQ407388	Tang *et al*. (2013)	Freshwater
***T. epizootica* (H)**	GU906246	Tang *et al.* (2013)	Freshwater
***Trichodina* species**	HM583859	Nyland & Isaksen (unpublished)	Freshwater
***Trichodina* species**	HQ407385	Tang & Zhao (unpublished)	Freshwater
Leiotrocha serpularum	JQ663867	Zhan *et al*. (2013)	Marine
Urceolaria urechi	FJ499388.1	Zhan *et al*. (2009)	Marine
U. korschelti	JQ663870.1	Zhan *et al*. (2013)	Marine
Colpoda magna	EU039896.1	Dunthorn *et al.* (2008)	Marine
Coleps hirtus	AM292311.1	Barth *et al.* (2008)	Marine


Trichodina heterodenta AY788099.1
Trichodina nobilis AY102172.1
Trichodina pseudoheterodentata KT804995.1
Trichodina paraheterodentata JQ821348.1
Trichodinella epizootica JQ663869.1
Trichodina epizootica HQ407386.1
Trichodinella epizootica HQ407388.1
E23 3
Trichodinella epizootica GU906246.1
Trichodinella epizootica HQ407387.1
E32 3
E32 6
E32 2
Trichodinella myakkae AY102176.1
A
Trichodina truttae C186029.1
Trichodina centrostrigata KP295473.1
Trichodina sinovacuale FJ499386.1
Trichodina ruditapicis FJ499385.1
Trichodina meretricis FJ499387.1
Trichodina sp. FSHM583859.1
Trichodina pectenis JQ663868.1
E23 8
E3 8
E3 5
Trichodina hypsilepis EF524274.1
E21 8
E21 7
E26 6
E3 6
E2 7
E21 6
E35 5
E4 2
B
Trichodina sp. CHQ407385.1
Trichodina mutabilis HQ407384.1
Trichodina reticulata AY741784.1
Trichodina uniforma HQ407383.1
Trichodina sinipercae EF599288.1
Trichodina modesta GU906245.1
Leiotrocha sepularum JQ663867
Urceolaria urechi FJ499388.1
Urceolaria korschelti JQ663870.1
Colpoda magna EU039896.1
Coleps hirtus AM292311.1
0.020



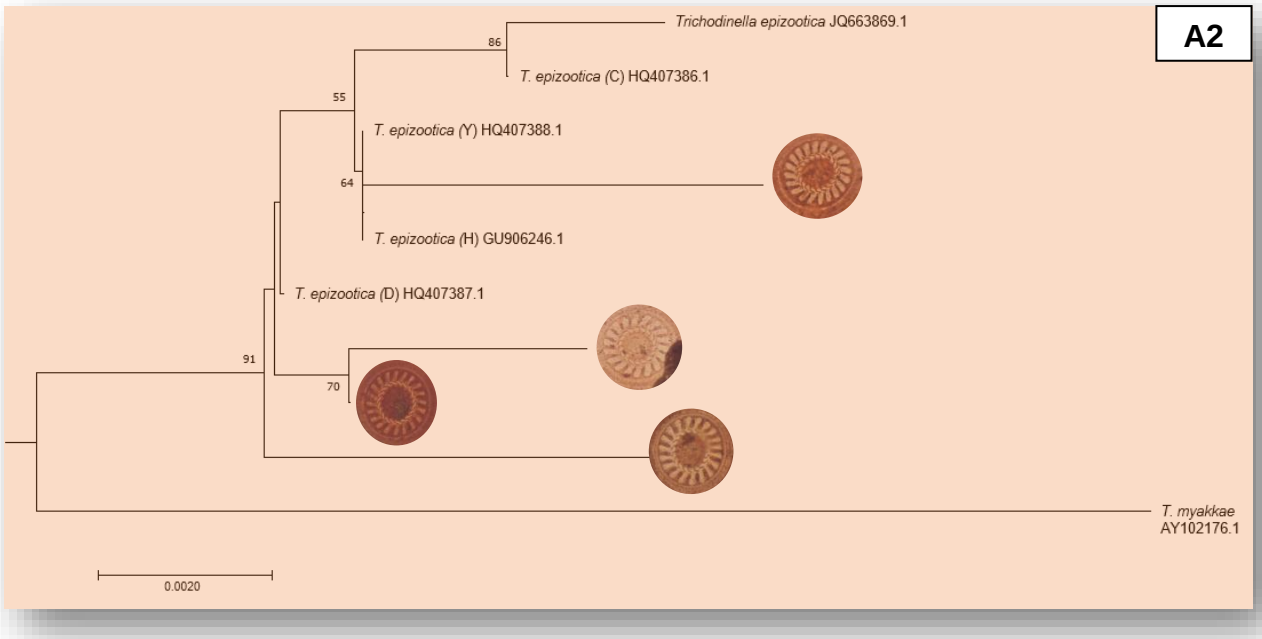


Figure 7.8: (previous page) The optimal phylogenetic tree is shown. Blue and orange highlight the clades in which the trichodinids collected in Botswana fall, the separate boxes (**A** and **B** above) show the specific clades expanded. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

Figure 7.9: (this page) **A1** – Enlargement of the Botswanan anuran *Trichodinella* sp. clade. **A2** - same as A1, but with micrographs of a representative individual from the specific population from which the sequenced sample was taken.

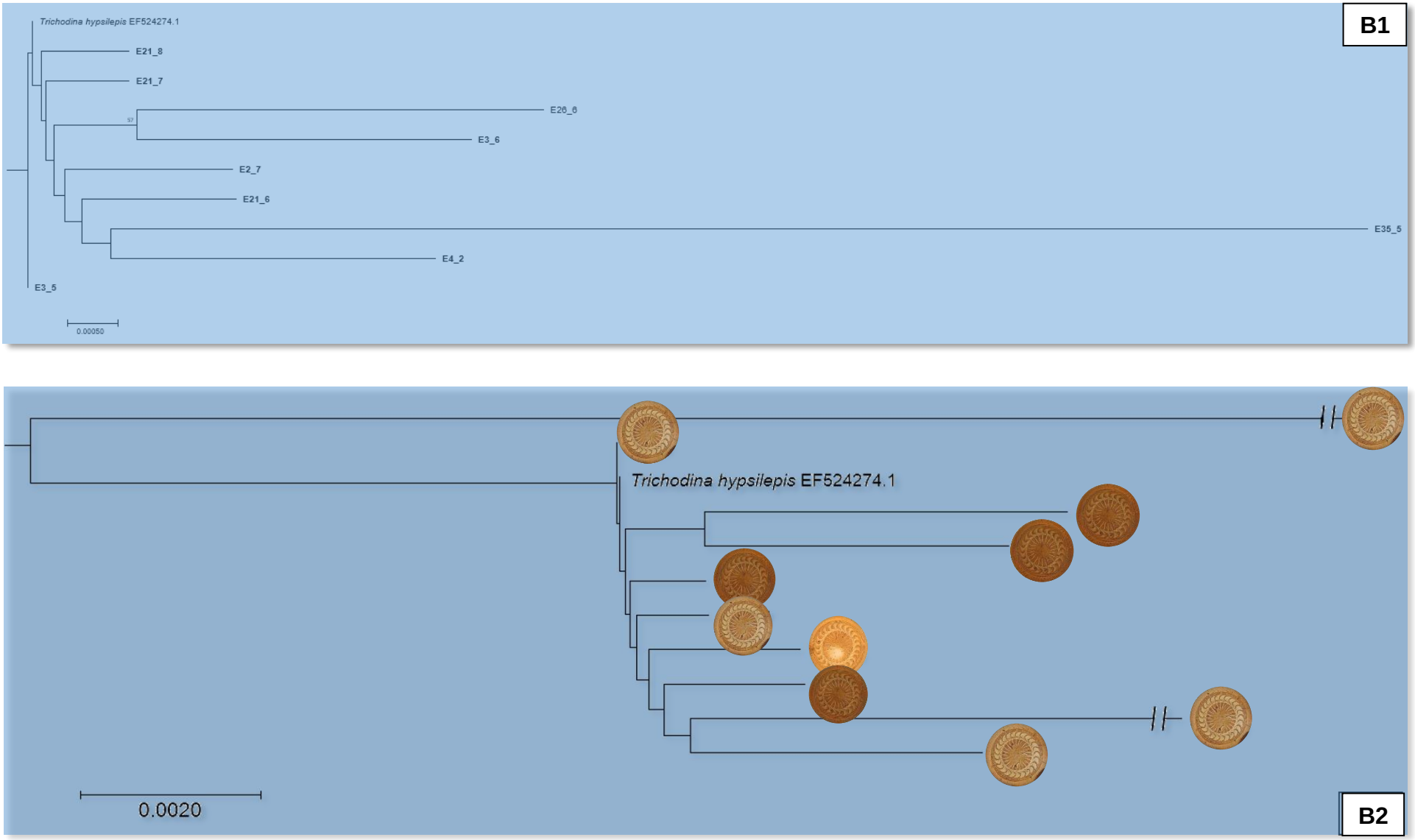


Figure 7.10: B1 – Enlargement of the Botswanan anuran *Trichodina* sp. clade. **B2** - same as B1, but with micrographs of a representative individual from the specific population from which the sequenced sample was taken (**B2** is not according to scale).

CHAPTER 8 - DISCUSSION

The initial hypothesis, as presented in **Chapter 1: Introduction** needs to be addressed first to avoid confusion with regards to the species names that will be used for the rest of this chapter:

> **Anuran tadpoles found in the Nxamasere Floodplains, Okavango Delta, Botswana, are host to a single infestation of the ecto-symbiont *Trichodina heterodentata* Duncan, 1977, that has also been recorded as part of a multispecies infestation on various freshwater fish species.**

Upon a more comprehensive examination, using the van As and Basson (1989) method of denticle descriptions of closely related species, some morphological differences, even though quite minimal at first, came to light. Research into the historical descriptions of *T. heterodentata* and *T. heterodentata*-like trichodinids leading to the work of Wellborn (1967) and Arthur & Lom (1984), challenged the status quo of the species *T. hypsilepis* and in doing so the taxon validity of *T. heterodentata*. An assumption was postulated that *T. hypsilepis* found on fish hosts be declared a synonym of *T. heterodentata* due to the already discussed motivations as highlighted in **Chapter 6**.

Molecular analysis of the 18S small subunit rRNA of the *T. heterodentata*-like specimens collected from anuran tadpoles in Nxamasere, indicated that these are genetically not as closely related as previously thought, but rather form a robust clade around a NCBI sequence of *T. hypsilepis* (deposited, but not published by Gong *et al.* (unpublished)) (**Chapter 7**: Table 7.4). Therefore, when considering the data collected and analysed for this study, the hypothesis that trichodinids from anuran tadpoles found in the Nxamasere Floodplains, Okavango Delta, Botswana, are *T. heterodentata* cannot be supported and the hypothesis should therefore be rejected.

Accepting an alternative hypothesis that trichodinids sharing the basic morphology with that of *T. heterodentata* are part of a, hitherto unknown, species complex, possibly being more host specific than anticipated, and that tadpole hosted trichodinids with the same morphological traits as *T. heterodentata* are in fact *T. hypsilepis*, as morphologically described by Arthur and Lom (1984) and molecularly determined in this dissertation. By comparing the morphological measurements of the ectozoic trichodinids found by Arthur and Lom's (1985) on unidentified Cuban tadpoles (**Chapter 6**: Table 1), *T. heterodentata* from *Rhinella pombali* (Baldissera, Caramaschi & Haddad, 2004) tadpoles from Brazil (Dias *et al*. 2009) and on *Xenopus laevis laevis* (Kruger *et al.* 1993b) (**Chapter 2**: Table 2.9), with those from the southern African *Schlerophrys* hosts, it appears that they are in fact all *T. hypsilepis*.

Therefore, hereafter all references to the species taxon *T. heterodentata* will only be applicable to freshwater teleost hosts and *T. hypsilepis* only to freshwater anuran tadpole hosts.

Comparing the substantial number of *T. heterodentata* population descriptions, the measured trichodinids can be clumped into three overarching groups (large, medium and smaller sized trichodinids) according to body diameter, which is the most striking difference. The first and larger grouping (Group I) represents all the populations with a mean body diameter above 70µm (Duncan 1977; van As & Basson 1986; Worananthakij & Maneepitaksanti 2014).

Group II including more than 60% of all the *T. heterodentata* in literature, is by far the largest number of populations, between 53µm and 70µm. In this middle group, most the body diameters are between 50µm and 60µm (Basson *et al*. 1983; Albaladejo & Arthur 1989; Basson & van As 1994; Al-Rasheid *et al*. 2000; Kruger *et al*. 1991; Dana & Hadiroseyani 2002; Asmat 2005; Dove & O'Donoghue 2005; Martins *et* al. 2010; Miranda *et al*. 2012; Öztürk & Ćam 2013; Valladão *et a*l. 2013), while a smaller number falls between 60µm and 70µm (Albaladejo & Arthur 1989; Van As & Basson 1989; Dana & Hadiroseyani 2002).

The last group (Group III) includes populations with the smallest body diameter between 40µm and 52µm (Bondad-Reantoso & Arthur 1989; van As & Basson 1992; Kruger *et al.* 1993b; Tao *et al.* 2008; Dias *et al*. 2009).

Duncan's (1977) three Philippine populations, even though they are in the largest mean dimension group, also have the largest difference (variation) of individual trichodinid body diameter, ranging from 58µm (population B) to 122µm (population C), where none of the others exceeds 99µm. Duncan's (1977) three populations, compared to all the other *T. heterodentata* described around the world, seem to be the largest in size. The only minimum sized populations that falls roughly into the rest of the globally observed measurements, falls in the lower ranges of his population B group.

From all the descriptions, only two populations were from tadpole hosts, those of Kruger *et al.* (1993b), who described *T. heterodentata* from *Xenopus laevis* and Dias *et al.* (2009) from *Rhinella pombali*. Both these populations fall into the group with the smallest dimensions.

Even though *T. heterodentata* is known to be a species with a highly variable morphology, a lot of the described variation might be due to incorrect measurements of either immature trichodinids/daughter cells, trichodinids that underwent odd mutations or artifacts arising during silver nitrate impregnation. Some trichodinids are more variable during their development (Basson, pers. comm.; van As, pers. comm.[1]) than other species, therefore extreme care must be taken to only measure adult individuals. In this study, where populations were found in large numbers, including many juvenile stages, it was possible to observe and record the complete transition from adults undergoing binary fission through the different stages of the daughter cells back to adulthood.

[1] Prof. L. Basson and Prof. J.G. van As, trichodinid specialists, Department of Zoology and Entomology, University of the Free State.

Figures 8.1**A** to **P** demonstrate a chronological succession of the ontogeny for *T. hypsilepis* sampled in Botswana, where Figure 8.1**A** indicates an adult individual in the process of binary fusion**.** In Figures 8.1**B** to **I** the denticle ring of the original daughter cell is still very prominent (steadily becoming less so as the process continues) and the new denticle ring is only visible from **D** onwards. From Figures 8.1**J** to **O** the systematic resorption of the old denticle ring is visible, while Figure 8.3**P** is an immature individual where the old denticle ring is not present anymore, however, the radial pins development is not yet completed, giving the impression of having slight spaces between each of the pins. Figures 8.2**E** and **F** are also representatives of these young immature individuals that are easily interpreted as adults and erroneously measured by some workers.

The importance of adult only populations for any morphological description of trichodinids are highlighted by some of the *T. heterodentata* records (Table 2.9). It is clear that Martins *et al.* (2010) and Miranda *et al.* (2012) measured both adult and juvenile specimens, which gave a considerably wide range of body diameter measurements, ranging from 27.0 to 77.0μm (Martins *et al.* 2010) and five to 15 radial pins per denticle (Miranda *et al*. 2012) that is impossible when using a completely adult population, but using immature individuals also distorts the biometrics.

An enigma that has been observed by Basson, van As and Kazubski (Basson, pers. comm.[2]) is the so-called “Halo” effect (Figs. 8.2**A** to **D**). This effect has not yet been studied in detail and the reason for its formation is still unsure, therefore all interpretations are based on assumptions. This effect has been noticed within trichodinid populations where hosts have been kept under laboratory conditions for a period of time (longer than three weeks). This phenomenon has not been observed from populations directly from the natural environment. A prominent band that impregnates as a “halo” becomes visible in both adults and young individuals not yet matured. It is believed that this band is linked to the peripheral band, as electron microscopy revealed that the peripheral band becomes

[2] [2] Prof. L. Basson, trichodinid specialists, Department of Zoology and Entomology, University of the Free State.

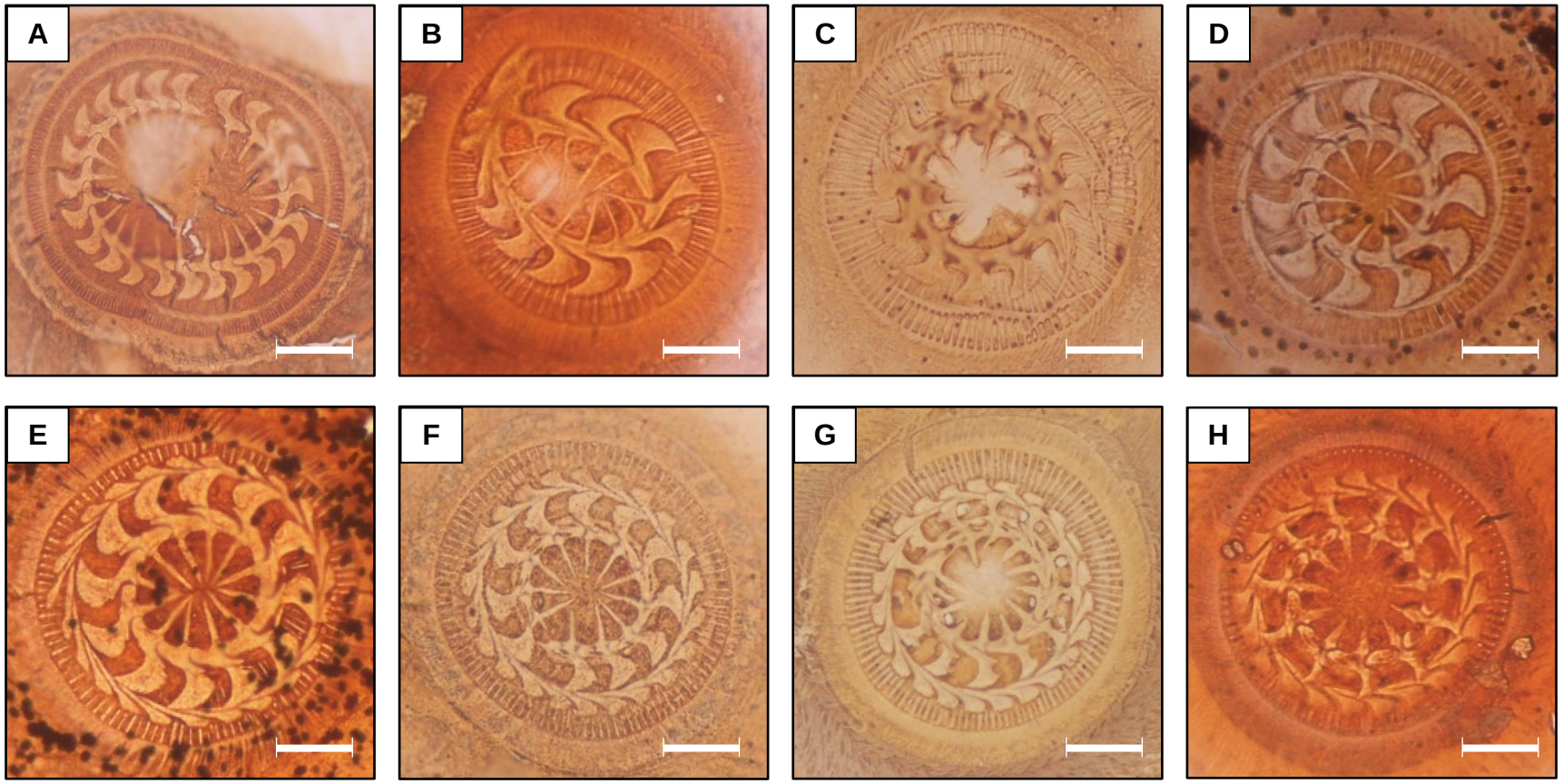


Figure 8.1: Chronologic development of *Trichodina hypsilepis* Wellborn, 1967 during binary fission (**A**) to a young individual, which is almost a measurable adult **(P**). **A** – daughter cell form; **B** through **O** – young individuals during the progressive development from daughter cells, where the old denticle ring is slowly being resorbed, to **P** – immature individual where resorption of old denticle is complete (scale = 10 µm).

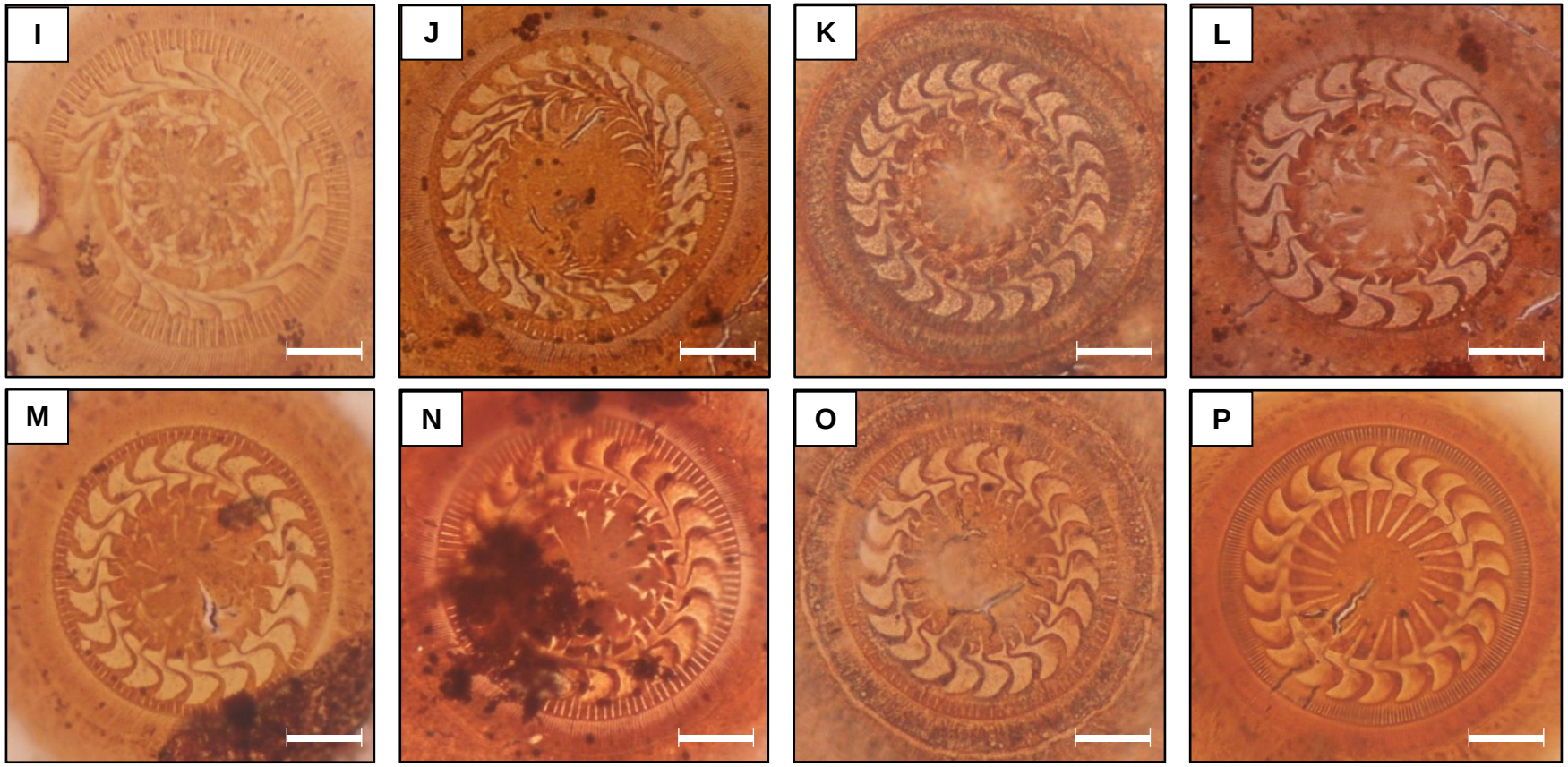


Figure 8.1 (cont.): Chronologic development of *Trichodina hypsilepis* Wellborn, 1967 during binary fission (**A**) to a young individual, which is almost a measurable adult **(P**). **A** – daughter cell form; **B** through **O** – young individuals during the progressive development from daughter cells, where the old denticle ring is slowly being resorbed, to **P** – immature individual where resorption of old denticle is complete (scale = 10 µm).

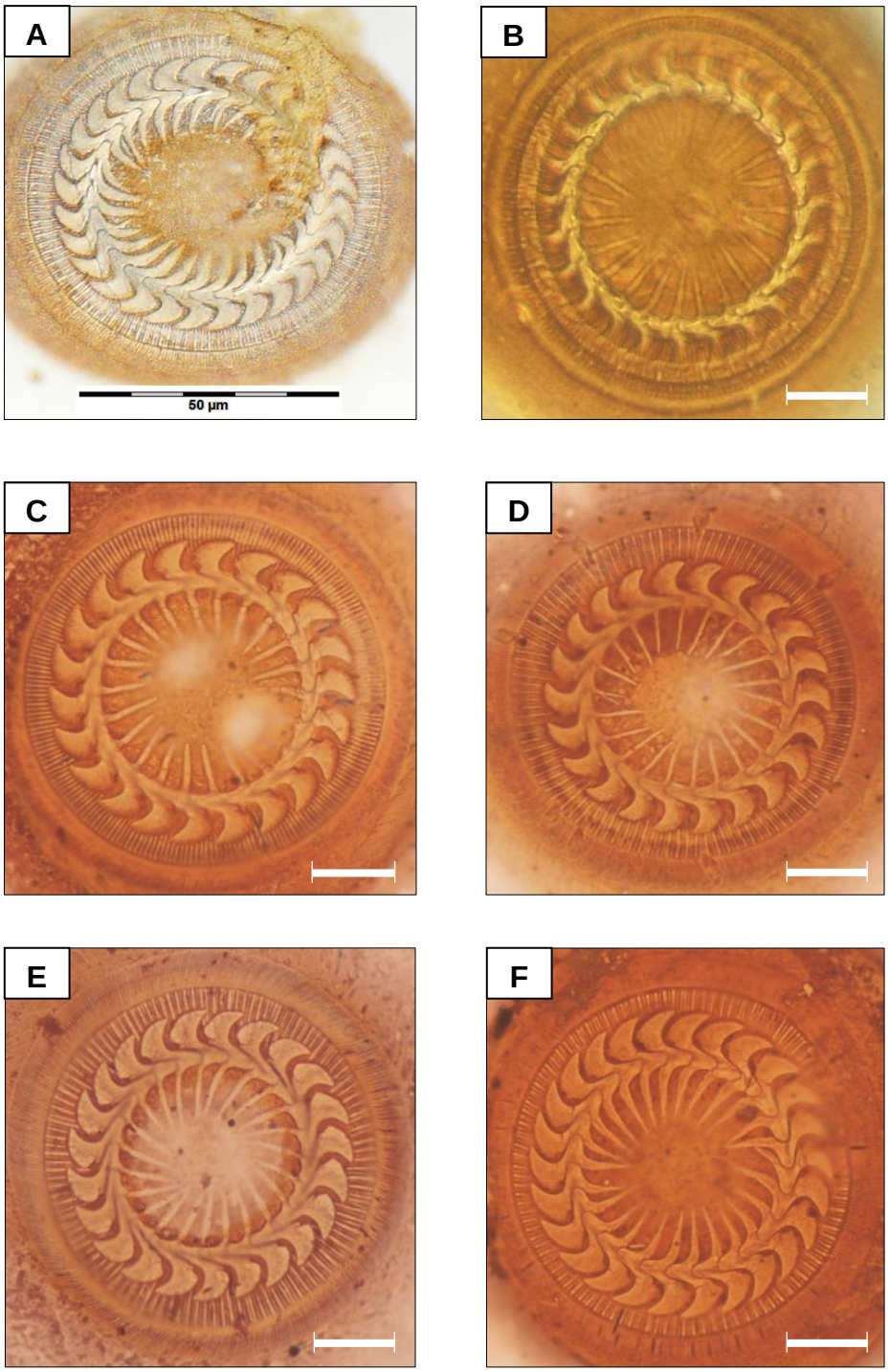


Figure 8.2: Photomicrographs of silver nitrate impregnated trichodinid specimens that should not be used for morphological description. **A** - 2017 micrograph of *Trichodina heterodentata* Duncan, 1977 population C from the Smithsonian Institute illustrating both the "halo" effect and a young individual (scale = 50µm); **B** to **F** – micrographs of *T. hypsilepis* Wellborn, 1967 from Botswana tadpole hosts illustrating the "halo" effect (**B**, **C** and **D**) and immature individuals (**D**, **E** and **F**) (scale = 10µm).

very prominent in adult specimens just before binary fission. The “halo” band is accompanied by and associated with abnormally large individuals and peculiarly shaped denticles, usually in adult individuals, but in the cases of *T. heterodentata* from Duncan’s population C and *T. hypsilepis* from Botswana it is also visible in immature individuals. We suspect that Duncan (1977) might have kept his population C *T. heterodentata* hosts in isolation for a lengthy period of time, as most of the type material micrographs obtained from this population at the Smithsonian Institute exhibits young individuals with the “halo” effect as illustrated by Figure 8.2**A**. This population C was also the biggest single population of *T. heterodentata* ever recorded. Measuring these abnormally large young individuals Duncan might have overlooked the radial pins that have not been fully developed (Duncan 1977: Fig. 7, page 79) that led to his unnaturally large body diameter measurements for his original *T. heterodentata* description (**Chapter 6**).

Silver nitrate impregnation (see **Chapter 4**) verges on alchemy and sometimes it may cause the formation of artefacts on specimens obscuring or distorting the denticles (Figs. 8.3**A**, **D** & **F**). Certain trichodinid species have also shown to be predisposed to gene-to-protein-mutations that causes malformations of the denticles (Figs. 8.3**B**, **C** & **E**). Individuals with these malformed or imperfectly impregnated adhesive discs shouldn’t be measured and also not be included in any population for biometric studies. This is an important reason why a large number of individuals should be screened before choosing measurable members for a population.

There are other obstacles that can also lead to incorrect morphological interpretations. Too small a number of individuals per population is problematic, i.e. in the case of Albaladejo and Arthur (1989) and Worananthakij and Maneepitaksanti (2014) where they based their description/record on ten individuals only, are good examples of this. Not only are these populations too small for any statistically significance testing, but with the known variation within populations, especially with species like *T. heterodentata* or *T. pediculus* it may also distort any inference.

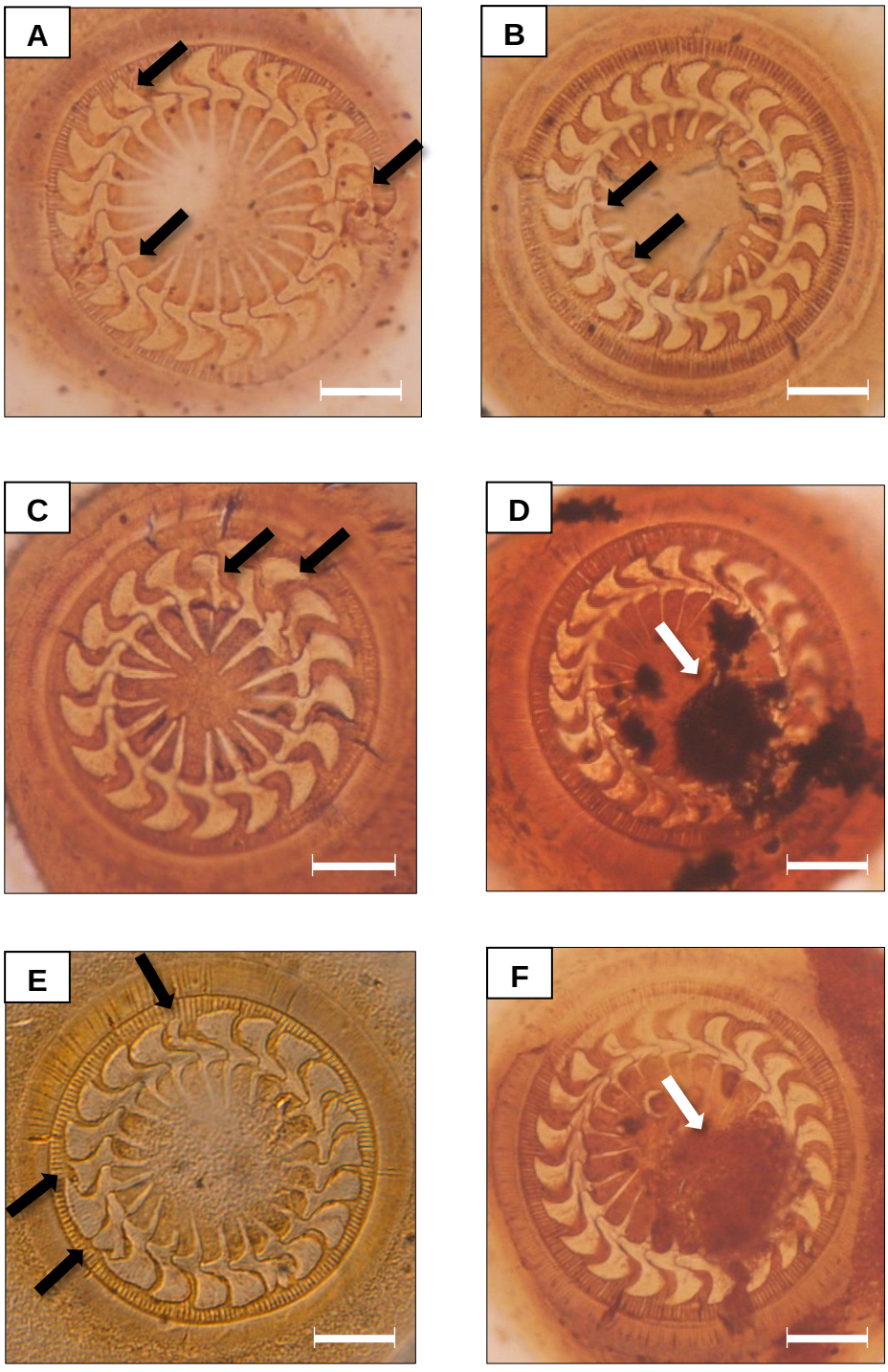


Figure 8.3: Photomicrographs of silver nitrate impregnated *T. hypsilepis* Wellborn, 1967 specimens from tadpoles in Nxamasere, Botswana with genetic mutations and or silver nitrate artifacts**:** **A** - NX1, **B** and **C** – NX2; **D** – NX3; **E** and **F** – NX6 (scale = 10µm) (Arrows indicated some of these abnormalities; White arrows = mutations, black arrows= artefacts).

Included in Table 2.9 (**Chapter 2**) are two records *of T. heterodentata*; Utami and Rohkmani (2016) and Nurrochmah and Riwidiharso (2016) that provide very little biometric data and the printed micrographs (Utami & Rokhmani 2016: Fig. 4.2) are of extremely poor quality. Even though these two examples are published conference proceedings, recently more and more journal publications also exhibit ambiguous micrographs and/or careless measurements for species descriptions, causing more confusion in the taxonomy of an already perplexing group of ciliophorans.

The standardisation of clear morphological descriptions, as proposed by Lom (1958) and van As and Basson (1989) needs to be followed meticulously and combined with or followed by standard molecular protocols along with excellent quality micrographs.

In **Chapter 9**, the conclusion, the five objectives of this study will form a guideline for a standardised protocol that should be used for the morphological and molecular description of new trichodinid species. In the case of this project, this protocol was used for the description of a new record for an ectozoic trichodinid species from the gills and skin of anuran tadpoles.

Despite the fact that *T. hypsilepis* was collected from two different host species and during alternating seasons, the specimens examined generated very little morphological variation (**Chapter 5**: Table 5.2 and Fig. 5.5). *Trichodina hypsilepis* appears to be morphologically more consistent than *T. heterodentata*, lending more weight to the suggestion that this species cannot be *T. heterodentata*, as one of *T. heterodentata*'s key features is that this is a highly variable species.

Another important feature of *T. hypsilepis* is the structure of their denticle rays that are more delicate (i.e. thinner) than those of *T. heterodentata*. Denticle ray thickness was not included by Lom (1958) as part of his 15 characteristics for morphologic description, but could conceivably be an important characteristic to examine for future morphometric analysis if working within a possible species complex or with cryptic species.

According to Kazubski and Migala (1968) and Özer (1999) large seasonal differences can be observed for both teleost and tadpole hosted trichodinids in the northern Hemisphere. Even though inspecting trichodinids for seasonal morphological differences was not one of the main objectives for this project, having the opportunity to collect from the same locality during different seasons led us to investigate this phenomenon on the morphologically constant *T. hypsilepis* in the Okavango Delta. This species exhibited, again, only minor variation in denticles proportions between the populations collected during different seasons. The most noticeable disparities were: the adhesive disc diameter and denticle blade length which were somewhat larger during the summer 2016 collection, while the body diameter, denticle ring diameter, denticle ray length, border membrane width and number of denticles per individual trichodinid were slightly, but only marginally smaller. Denticle length, denticle central part width and denticle span stayed almost constant between seasons. No temperature measurements were recorded during fieldwork, but as the gathered data indicated temperature fluctuations weren't big enough to play an important role in seasonal morphological variations of these trichodinids.

When comparing *T. heterodentata* descriptions from tadpole hosts (Kruger *et al.* 1993b; Dias *et al.* 2009) with that of *T. hypsilepis*, it is clear that these all represent the same species. When Kruger *et al.* (1993b) did their research, they noticed small differences between the trichodinids found on tadpole and teleost hosts, but could not validate these differences as molecular techniques for most protozoans weren't available yet, and *T. heterodentata* was notorious for having a wide variation. After using the Lom's (1958) unified morphological characteristics technique, Dias *et al.* (2009) came to the conclusion that *T. heterodentata*, like *T. pediculus* was not confined to one host type only. Unfortunately they did not put enough emphasis on the van As and Basson (1989) denticle descriptions, and accounted for the morphological variation (Dias *et al.* 2009: Fig. 2 page 476), like Kruger *et al.* (1993b) as being part *T. heterodentata*'s notoriety. The differences between the two species became apparent once an in-depth morphological comparison, in combination with molecular analysis, was made.

Molecular analyses of trichodinids, except for the work done by Gong *et al*. (2005; 2006; 2007) and Tang *et al*. (2013; 2016; 2017), still have a long way to go and the present study represents the first phylogenetic research into mobilids done in southern Africa.

The most challenging aspect of the molecular research was the isolation of the individual trichodinids, as their small size and sharing their host with many other ciliophorans, enhances the chances of contamination. As mentioned above, this was the main reason tadpole hosts from the Nxamasere Floodplain were chosen for this project, but that only eliminated other trichodinid contamination. Several *Epistylis* Ehrenberg, 1830 species and other free living protozoans were abundant on the hosts and in their aquatic environment, which needed to be accounted for. Different numbers of trichodinids were collected under a dissection microscope by means of a modified glass pipette and were stored in varying concentrations of ethanol, as very little information of the sampling techniques for trichodinid molecular work is ever revealed in the literature. To our surprise the better gDNA extractions from the sampled trichodinids were from 96% and 75% ethanol and not from absolute (99%) ethanol, that is usually regarded as the best for molecular results. Distilled water also delivered excellent results as preservative, but can be a problem in the field, since it needs to be kept below 4°C at all times as the DNA degenerates much quicker in water than in ethanol under ambient temperatures. It was also noted that trichodinids stored in ethanol for more than a year yielded very little usable DNA, it is therefore advised that gDNA extraction happen as soon as possible after collection as the extract can be stored for a much longer period of time.

When planning the downstream molecular process, especially when cloning is intended, it is very important to identify the type of *Taq* Polymerase that is in the commercially available kits and the cloning vectors available. In this study we realised that the REDExtract kit *Taq* Polymerase gave A-overhangs during the PCR amplification, and the pSMART HCKan high-copy vector (Lucigen) is blunt ended, pressing us to use the REDExtract-N-Amp™ Tissue PCR Kit (Sigma-Aldrich-Merck®) for extraction and initial amplification, but then to repeat the amplification process with KAPA HiFi HotStart ReadyMix, as this commercial kit's *Taq* Polymerase in turn produces blunt-ends.

The choice of which genes to amplify for phylogenetic inference, and thus which primers to use, is also very important. All of the published phylogenetic trees in the literature and most nucleotide sequences deposited in the databanks are of the 18S small subunit rRNA gene. For this reason most molecular trichodinid research is always done using this gene. As mentioned in **Chapter 2**, this gene, although successfully used, is extremely conservative and makes inferences of recent evolutionary changes, especially in protists, which evolve at a faster rate than multicellular organisms, challenging. In the case of the 18S gene, the primer set that allowed for the best and largest range of eukaryotic organisms, was the universal eukaryotic primer set, ERIB1 (forward) and ERIB10 (reverse). For trichodinids this primer set sequenced very well from both sides (with the SL1 and SR2 sequencing primers) producing overlapping 3' end reads of approximately 100bp, and it was not necessary to use any other primer sets to amplify smaller regions of the gene in case of incomplete readings.

Previous molecular chapters (2 and 7) mentioned that the ITS1-5.8S-ITS2 region was also amplified and sequenced successfully in this study, unfortunately, at the time of this study there were only 7 partial ITS region trichodinid nucleotide sequences available in the NCBI/GenBank to compare it with and none of *T. hypsilepis*. The use of these ITS genes, along with the 28S SSU rRNA genes and the, very neglected in mobilid studies, nuclear genes (COI) must be investigated, as these genes will be able to explain more of recent systematics and also give a stronger interpretation of intergenic and intraspecies taxonomy as indicated by Hillis & Dixon (1991).

The process of cloning, or rather subcloning in this case, might be time consuming, but it is very important when working with trichodinids, as most trichodinids do not occur in single infestations like *T. hypsilepis* on the southern Hemisphere tadpoles. The method of subcloning purifies isolates of the specific gene obtained from the electrophoresis gel and thereby reduces the possibility of eventually sequencing DNA that is not of the organism one is investigating, e.g. *Epistylis* spp. also present on many tadpoles in the case of the present study.

Screening for the inserts in the transformed DNA plasmids by using minipreps followed by restriction digestion can be very useful when working with multispecies infestation/infection of trichodinids. After running an agarose gel electrophoresis of the products from collected samples amplified by the universal eukaryotic primer set and digested with restriction enzymes *Eco*RI and *Hind*III, differences in the digestion profiles of *T. hypsilepis* and *Trichodinella* sp. were observed (**Chapter 7**: Fig. 7.7). Even though *Trichodinella* is still only a related genus of *Trichodina* (but not supported according to the molecular evidence of this study and other studies), this opens up the possibility of identifying different species of trichodinids molecularly from the same host.

Because *Trichodinella* is morphologically so distinct from *Trichodina* and other members of the family Trichodinidae, it has always been accepted that this group forms a genus on its own (Fig. 8.4). Recent molecular work, though, have found that this hypothesis is possibly erroneous, as Gao *et al.* (2017) and Tang *et al.* (2017) found that the *Trichodinella* that they sequenced did not make a separate genus clade, but actually fell well within the genus *Trichodina.* During the sampling and collecting of *T. hypsilepis* in Nxamasere for this study, it was found that in one pool a population of *Schlerophrys gutturalis* tadpoles were infested with *Trichodinella*, most probably a temporary population originating from teleost hosts trapped in the same pool with them. These *Trichodinella* specimens were kept for both morphological description (not included in this dissertation) and molecular analysis, to ascertain whether the technique for gDNA extraction from *T. hypsilepis* could be repeated and if the two would produce the expected genetic distance. Figures 8.4**A** to **F** are all silver nitrate impregnated *Trichodinella* specimens collected from tadpole hosts in the above mentioned pool, most probably *T. epizootica*.

When these *Trichodinella* sequences were included in the phylogenetic tree with all the available trichodinid data, it grouped perfectly with the sequences from Gao *et al.* (2017) and Tang *et al.* (2017), echoing the possibility that *Trichodinella* is not a separate genus, but actually another possible species complex within the genus *Trichodina.* If this assumption is proven to be correct, then the use of restriction enzyme digestion during

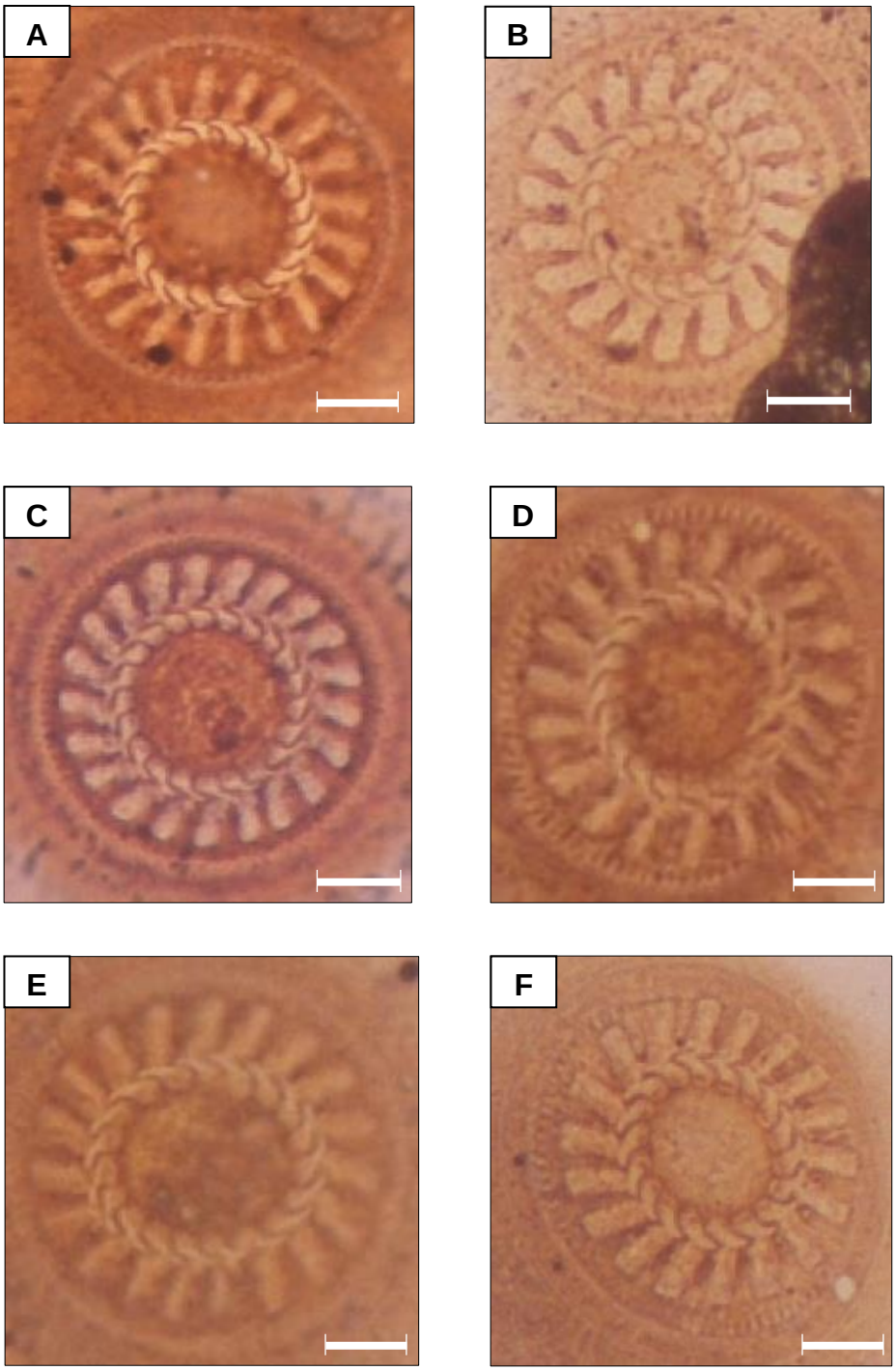


Figure 8.4: Photomicrographs of silver nitrate impregnated *Trichodinella* specimens **A** to **F** that were found on *Schlerophrys gutturalis* (Power, 1927) from population NX6, winter 2016 from Nxamasere, Botswana (scale = 1 µm)**.**

the screening process after subcloning will be another significant tool to use for multispecies trichodinid infestations on teleost hosts.

The use of molecular techniques have certainly enhanced the understanding of systematics and taxonomy, but combined with this incredible world that it has opened up, it still isn't without its problems. These problems if overlooked, as has been the case so often in the field of protist taxonomy, may have dire consequences on the manner we perceive the systematics of these organisms.

The importance of cloning, for instance, became apparent in a recent publication by Fariya *et al*. (2017) where a new species, *T. chirhinii* Fariya *et al*., 2017, from the gills of fish host *Cirrhinus mrigala* (Hamilton, 1822) was described using both morphological and molecular data. However, no silver nitrate impregnated micrographs were included in the publication, meaning accuracy could not be verified, relying heavily on the molecular analysis alone for taxonomic interpretation. Standard PCR amplification of the 18S SSU rRNA was performed, but these PCR products were directly sequenced with no cloning process to check for possible contamination, as many fish hosts usually display a multispecies trichodinid infestation. The interesting aspect of their results is that their sequence grouped along with *T. hypsilepis*, which has only been found on amphibians to date. This would be a significant finding, unfortunately both their techniques for morphometric and molecular analyses left too much room for incorrect assumption and potential contamination, leaving a vastly questionable record.

An enormous challenge of molecular taxonomy is the creditability of the sequences added into international nucleotide data banks. There are at present no compulsory taxonomic prerequisites for depositing a sequence into NCBI/GenBank. Many of the sequences in NCBI have not been published in peer reviewed journals. For the present study four unpublished sequences had to be used to enhance the robustness of the phylogenetic tree (**Chapter 7**: Table 7.4). Besides the unpublished sequences, which usually implies that no morphometrics were done to establish the validity of the description, the data bank itself also does not make any reference to host, ecto- or endozoic nature, locality of host,

environment or even seasonality. Without this important information it is virtually impossible to make any significant inferences of the species and no matter how strong the molecular research seems to be, it cannot bring anything to the taxonomic table on its own. It is therefore imperative that nucleotide data banks make it compulsory for anyone working on SAR organisms to include quality micrographs of the morphologically important characteristics of the organisms, location and environment (free living or host) for all sequences deposited. This will add more weight to the molecular data and also avoid possible misinterpretations.

This being said, we should also guard against blatantly disregarding new technological advances we are unsure of in any field of science. Sandon (1965) already warned that the need for precision in morphological descriptions was more essential than ever during his life, owed to the approaching use of systematic data handling of computers. His warning was not completely irrational, his fear that the loss of human imagination and interpretation might be replaced by pre-programmed models is understandable, and can be of even graver concern today, as taxonomists have so many different techniques to choose from for their craft. Fortunately, the advance of computer programmes has made the field of taxonomy much faster (not always easier, though), especially in statistical analysis and phylogenetic inferences. The field of biological taxonomy is currently standing at another crossroads; each leading into an inviting path of spectacular new techniques. Each of these paths have the promise of robust results which may finalise the ever-lasting questions of our search, but taxonomy, being one of the fundamental layers of biology, cannot journey down a single path only. Taxonomy and systematics need to incorporate so much more than just the morphology of the organism, or the genetic distances between similar genes; it is also influenced by geological time, host distribution, environmental conditions and so much more we have yet to discover. Like in all other sciences, taxonomy has and never will have an absolute truth, it will always strive to prove the next hypothesis to be incorrect (Cox & Forshaw 2009), and here lies the wonder that has led us to this point in time, we now need to muster the imagination to interpret the weight and attributes of all possible techniques to build the new hypotheses.

CHAPTER 9 - CONCLUSIONS/PROTOCOL

I **To develop a silver impregnation protocol for tadpole trichodinids similar to the staining results obtained for teleost hosts.**

- **Old slides**: fish mucus as substrate (preferably members of the Cyprinodontiformes uninfested with trichodinids).
- Dry mucus treaded slides for 24 to 48 hours.
- Impregnate following Klein's (1926) silver nitrate technique under UV light for 15 to 20 minutes.

- **New slides (in the field on location)**: specimen smeared slide should be manually dried (with great care) with tissue or absorbent cloth.
- Slide should be air-dried in direct sunlight for hour.
- Treat slide with fish mucus (same as above).
- Air dry treated slide again for an hour in direct sun.
- Impregnate following Klein's (1926) silver nitrate technique in direct sun (or under UV light if available) for 15 to 20 minutes.

II **To confirm that *Trichodina* sp. infestations on tadpoles in the Okavango Delta represent a single species infestation.**

- Measure the impregnated specimens under a compound microscope, using Lom's (1958) modified standardised morphological characteristics (see Table 2.9), a minimum of 25 adult individual trichodinids for each population.
- All measurements must include and be annotated as follows:
- Maximum to minimum values (mean* ± standard deviation).
- (*in the case of number of denticles and number of radial pins per denticle use the mode rather than the mean).
- High resolution micrographs must be taken of a measured representative individual, including a clear scale in µm.
- Statistically compare all the measured populations from the same host species/types.

III **To determine whether ectozoic trichodinids from anurans are morphometrically and morphologically similar to *T. heterodentata* from fish hosts in the same localities.**

- Statistically compare all the measured populations from the different host species/types., if more than one host species is present.
- Choose a representative from each population and perform the van As and Basson (1989) denticle description from a high resolution micrograph for three consecutive denticles of each chosen individual.
- If there are large noticeable variation or differences with the profile of a population, choose a representative for each of the divergent individuals.
- Compare the relationships the denticle blade, central part and ray for each of the representatives, take care to note any variation recurrence no matter how slight.

The final outcome for the current study revealed that tadpoles from the Nxamasere Floodplain, Botswana are not host to *T. heterodentata*, but *T. hypsilepis*.

IV **To determine if it is possible to extract gDNA from Botswana anuran host trichodinid material and make phylogenetic inferences using 18S SSU rRNA.**

- Extract gDNA from trichodinids collected and preserved in ethanol (75 – 85%) by using a *REDExtract-N-Amp Tissue PCR kit.*
- Amplify the required region of the gene (preferably 18S SSU, as it is currently the most sequenced for protozoan taxonomy) by using specific primers (universal eukaryotic primer set works well for ciliophorans).
- Clean up the desired PCR products from the gel for cloning in a suitable vector.
- After successfully cloning, sequence (Sanger sequencing) selected plasmid constructs of the desired gene (in this case the complete 18S SSU rRNA region).
- Align (consensus align) the sequences using Geneious, or similar programme.
- Draw a phylogenetic tree (MEGA software), using either the Neighbour Joining or Maximum Likelihood models.

V

To determine whether *Trichodina heterodentata* is a single non-host specific species found on both teleost and anuran hosts or a species complex.

- Compare all sequences (published only) of the family in NCBI/GenBank (or similar data banks).
- It is imperative that all sequences deposited in any gene data bank be accompanied by a complete morphological description and a clear micrograph of the deposited gene's species.
- All data concerning locality (country, water system, GPS coordinates), host (type, genus and species) and location on/in host MUST also be presented with each deposited sequence*.

(*Unfortunately the last two points are not yet considered important by the nucleotide databases, but it is very important that this data must be included).

- Only if all the above criteria are met, can the interpretation of the phylogenetic tree be reliable and comparison to the morphology possible.

CHAPTER 10 - REFERENCE LIST

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*Not seen in the original form.

ABSTRACT

The family Trichodinidae Raabe, 1959 (Ciliophora, Peritrichia) consists of 11 genera, of which the cosmopolitan genus *Trichodina* Ehrenberg, 1838 has the largest number of species. A substantial majority of trichodinid species are associated with marine and freshwater fish hosts, while some are also found associated with amphibians, as well as a variety of invertebrate hosts. Some trichodinid species show high levels of host specificity, whilst others occur on a range of hosts. *Trichodina heterodentata* Duncan, 1977 was first described from fish breeding farms in the Philippines as ectoparasites of imported cichlids, more specifically the Mozambique tilapia from southern Africa, *Oreochromis mossambicus* (Peters 1885), from the Limpopo River System. *Trichodina heterodentata* has subsequently been described from Europe, Australia, India, China, South America and Africa, however, it has not yet been found in North America. This cosmopolitan species, with a seeming preference for cichlid hosts, has unambiguous morphological features, but with distinct variances between and within populations. Most fish species harbouring *T. heterodentata* display multi-trichodinid infestations, however, on its amphibian host, *T. heterodentat*a occurs as a single infestation, which makes it perfect for using it as a model species. After reviewing previous descriptions from North American trichodinids, analysing the morphological data (both from literature along with type material obtained from the Smithsonian Museum) and investigating the distribution patterns of the southern African introduced *O. mossambicus* throughout North American water systems, it seems likely that *Trichodina hypsilepis* Wellborn, 1967, is a synonym of *T. heterodentata*. This not only delegates several North American trichodinid species into synonymy, but also probably indicate an insidious African alien introduction sneaking it way into this continent. Morphological analysis of *T. heterodentata*, shows that this species is highly variable in regards to its denticle structure, which leads one to wonder if it's might be a species complex, or indeed a single species. Trichodinid taxonomic work to date has primarily focused on morphological characteristics together with host and distribution records. With the advent of modern biochemical laboratory procedures, more and more emphasis is placed on molecular techniques in all fields of taxonomy, especially in parasitology. However, little molecular taxonomy work has been done on trichodinids. Trichodinid specimens were obtained from six different isolated host tadpole

(*Schlerophrys* spp.) populations on the Nxamasere Flood plains, Okavango Panhandle, Botswana. This dissertation will represent the first study in southern Africa to successfully isolate genomic DNA from mobiline symbiotic ciliophorans. Genomic DNA was isolated from *T. heterodentata* and the 18S rDNA gene was amplified by PCR. After Sanger sequencing a complete phylogenetic tree of all available trichodinid sequences were constructed. All results indicate that trichodinids collected from tadpole hosts in the Okavango Delta are not *T. heterodentata*, but rather *T. hypsilepis*, supporting the theory that *T. heterodentata*, as it is known to date, is a species complex.

KEYWORDS: *Trichodina heterodenta, Trichodina hypsilepis, Oreochromis mossambicus, Schlerophrys gutteralis, Schlerophrys poweri,* Nxamasere Floodplain, Okavango Panhandle, morphology, molecular, taxonomy

OPSOMMING

Die familie Trichodinidae Raabe, 1959 (Ciliophora, Peritrichia) bestaan uit 11 genera, waarvan die mees kosmopolitiese genus, *Trichodina* Ehrenberg, 1838 die meeste spesies huisves. Die oorgrote meerderheid trigodina-spesies word met mariene en varswater visgashere geassosieër, terwyl ander op amfibiërs en ‘n verskeidenheid ander invertebrate gevind word. Sommige trigodinas is baie gasheerspesifiek en sommige word by talle verskillende tipe gashere aangetref. *Trichodina heterodentata* Duncan, 1977 was die eerste keer vanaf visteëlplase in die Filippyne op ingevoerde vis beskryf, meer spesifiek vanaf die ingevoerde bloukurpers,*Oreochromis mossambicus* (Peters 1885) uit die suider-Afrikaanse Limpopo Rivierstelsel. *Trichodina heterodentata* is sedertdien vanuit Europa, Australië, Indië, Sjina, Suid-Amerika en Afrika beskryf, maar nog nooit in Noord-Amerika nie. Hierdie kosmopolitiese spesie, met ‘n blykbare voorkeur vir Cichlidae gashere, het unieke morfologiese eienskappe, maar daar heers ook baie duidelike variasies tussen en binne verskillende bevolkings. *Trichodina heterodentata* word op meeste visspesies aangetref as deel van ‘n multi-spesie infestasie, alhoewel, op ‘n amfibiese gasheer blyk dit om ‘n enkel spesie infestasie te wees, wat hierdie gasheer ‘n ideale modelgasheer vir die studie van *T. heterodentata* maak. Na ‘n ondersoek van Noord-Amerikaanse trigodinas (vanaf literatuur en tiepmateriaal verkry vanaf die Smithsonianmuseum) en die verspreidingspatrone van *O. mossambicus*, blyk dit moontlik dat *T. hypsilepis* Wellborn, 1967 ‘n sinoniem van *T. heterodentata* kan wees. Laasgenoemde bevindings sink nie net sommige Noord-Amerikaanse trigodina spesies nie, maar verklaar ook hoe ‘n indringerspesie vannaf Afrika die pad na die Noord-Amerikaanse kontinent kon meemaak. As gevolg van die groot variasie wat *T. heterodentata* se tandringstrukture vertoon, word die vraag, of hierdie ‘n spesieskompleks of inderdaad ‘n enkel spesie is, gevra. Tot op hede het meeste van trigodina se taksonomie op morfologiese eienskappe en gasheerverspreiding berus. Selfs met die ontwikkeling van die moderne biochemiese laboratorium prosedure, waar al hoe meer gewig op molekulêre tegnieke (veral in parasitologie), maar ook in alle velde van taksonomie, geplaas word, is daar weinig molekulêre navorsing op trigodina-taksonomie gedoen. Trigodinas is vanaf ses verskillende paddavisgasheerbevolkings in die Nxamasere-vloedvlakte in die Okavango-pypsteel, Botswana versamel. Hierdie

verhandeling sal die eerste studie in suiderlike-Afrika wees waar genomiese DNS vannaf simbiotiese siliate suksesvol geïsoleer word. Genomiese DNS is vanaf *T. heterodentata* geïsoleer en die 18S klein subeenheid rDNS deur PCR geamplifiseer. Na Sanger-verlenging is ‘n volledige filogenetiese boom saamgestel met al die beskikbare trigodina-gene. Die resultate dui aan dat die trigodinas vanaf paddavisse in die Okavango-delta nie *T. heterodentata*, soos aanvanklik gespekuleer, is nie, maar eerder *T. hypsilepis*. Dit ondersteun dus die teorie dat *T. heterodentata*, soos tans bekend is, ‘n spesies-kompleks is.

SLEUTELWOORDE: *Trichodina heterodenta, Trichodina hypsilepis, Oreochromis mossambicus, Schlerophrys gutteralis, Schlerophrys poweri,* Okavango-pypsteel, Nxamasere-vloedvlakte, morfologie, molekulêr, taksonomie.

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APPENDICES

PLATE A: Graphic representation of the silver nitrate impregnation experimental layout, with the best protocol for each type of slide highlighted in yellow.

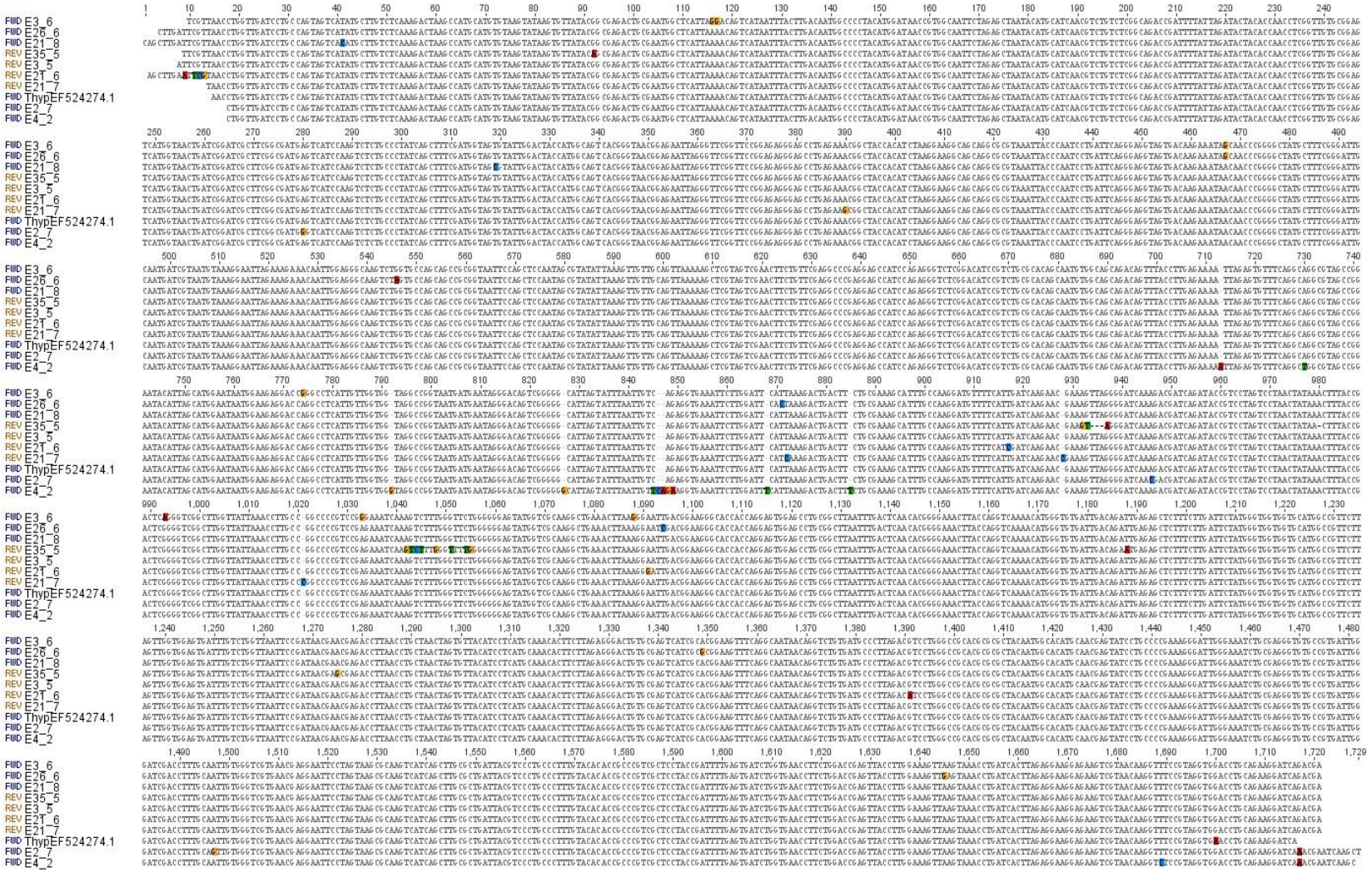

PLATE B: 18S SSU rRNA gene assemblages clustered around *T. hypsilepis* Wellborn, 1967 from the NCBI database.

ETHICAL CLEARANCE

UNIVERSITY OF THE FREE STATE
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YUNIVESITHI YA FREISTATA

UFS·UV
HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

Animal Research Ethics

09-May-2017

Dear Mr Gerhard De Jager

Student Project Number: UFS-AED2017/0017

Project Title: Assessment of Trichodina heterodentata Duncan, 1977 (Ciliophora: Peritrichia) using molecular and morphological taxonomy.

Department: Zoology and Entomology (Bloemfontein Campus)

You are hereby kindly informed that, at the meeting held on 04-May-2017, the Interfaculty Animal Ethics Committee approved the above project.

Kindly take note of the following:

1.
A progress report with regard to the above study has to be submitted Annually and on completion of the project. Reports are submitted by logging in to RIMS and completing the report as described in SOP AEC007: Submission of Protocols, Modifications, Amendments, Reports and Reporting of Adverse Events which is available on the UFS intranet.

2.
Researchers that plan to make use of the Animal Experimentation Unit must ensure to request and receive a quotation from the Head, Mr. Seb Lamprecht.

3.
Fifty (50%) of the quoted amount is payable when you receive the letter of approval.

Yours Sincerely

Derek Litthauer
2017.05.09
21:35:11
+02'00'

Prof. Derek Litthauer Chair: Animal Research Ethics Committee

D