ABSTRACT

CALLAHAN, HEATHER ANN. **Molecular Characterization of the *Ichthyobodo necator* Complex: An Important Fish Ectoparasite. (Under the direction of Edward J. Noga and Michael Levy).**

Ichthyobodo necator is a member of the Order Kinetoplastida and an important fish ectoparasite with a broad host and ecological range. When *Ichthyobodo* were exposed to the anesthetic tricaine, buffered with sodium bicarbonate, parasites remained attached to the skin of fish. When parasites were exposed to unbuffered tricaine, they detached almost completely from the skin. This finding indicated that tricaine should always be buffered when fish are to be clinically evaluated to prevent a reduction or complete loss in parasite load. Unbuffered tricaine was used to collect large numbers of parasites from the skin and gills of hybrid striped bass (*Morone saxatilis* male x *M. chrysops female*) for DNA isolation and analysis. A method for obtaining samples for DNA isolation without use of tricaine was also determined. Four preservation methods (Utermohl's solution, ethanol, formalin, freezing) and two DNA isolation methods (DNA isolation kit, crude lysate) were examined. It was concluded that ethanol-fixation was the best preservation method for DNA isolation and PCR amplification of *Ichthyobodo* DNA. Using these methods, genomic DNA was isolated from *Ichthyobodo* trophonts collected from five freshwater and one marine fish. The 18S ribosomal RNA (rRNA) gene from each isolate was PCR amplified, cloned and sequenced. The 18S rRNA gene suggested that rather than being a single species, *Ichthyobodo* represented a complex of at least four different species, each of which occupied a distinct geographical region. The 18S rRNA gene was also amplified and cloned for related kinetoplastid speceis. Phylogenetic relationships within the Order Kinetoplastida (suborders Bodonina and Trypanosomatina) were

determined. The suborder Bodonina appeared to contain at least 3 major lineages, none of which correlated with currently recognized families. *Ichthyobodo* had the most divergent sequence within the Bodonina, indicating it was the most genetically distinct bodonid currently known. The study of *Ichthyobodo* has contributed to a new understanding of phylogenetics and systematics for the Order Kinetoplastida, as well as insight into the potential for the spread of this parasite locally, nationally and internationally.

MOLECULAR CHARACTERIZATION OF THE *ICHTHYOBODO NECATOR* COMPLEX: AN IMPORTANT FISH ECTOPARASITE
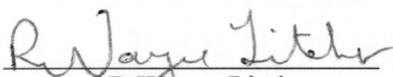
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HEATHER ANN CALLAHAN

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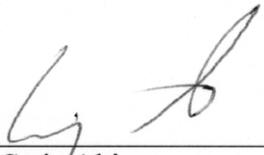
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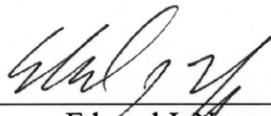2003

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PERSONAL BIOGRAPHY

Heather Ann Callahan was born on February 2, 1970 in Port Jerfferson, Long Island to

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ACKNOWLEDGEMENTS

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

GENERAL INTRODUCTION

Protozoans of the Order Kinetoplastida are a diverse and unique group of flagellates that include some of the most commonly studied organisms in the world. The order contains a number of parasitic, ectocommensal, and free-living species that occur over a wide range of habitats. They parasitize every major animal group as well as plants and arthropods, while many species can live in soil, water, and even deep ocean vents (Vickerman 1990, Atkins et al. 2000, Lopez-Garcia et al. 2003). A number of these organisms have devastating effects on human and livestock populations (i.e., *Trypanosoma*, *Leishmania*)(Roberts & Janovy 2000). As a result, certain genera have been well-studied; however, little is known of others. One such poorly studied organism is *Ichthyobodo necator*, a common pathogen of economically important cold water, temperate, warm water, and tropical fish species (Robertson 1985, Urawa et al. 1998).

Ichthyobodo necator is the smallest bi-flagellated fish ectoparasite. Individuals range from 3 to 18 μm in length and 2.5 to 6 um in width and exist as both attached and free-swimming forms (Becker 1977). Free-swimming forms are ovoid with a convex dorsal and concave ventral side and can be identified with light microscopy by the flickering motion that results from its body shape and a spiral swimming pattern that alters its refractility (Noga 1996). Attached forms appear dorsoventrally compressed and pyriform in shape and the flagella are not readily apparent. They can multiply quickly by binary fission and are easily identified by the appearance of four flagella just prior to division. Large numbers of *I. necator* trophozoites can destroy the epithelial layer of the

skin and gills, which in turn disrupts homeostasis, causing osmoregulatory stress and death (Robertson 1979, Robertson et al. 1981, Noga 1996). Clinical signs of disease include lethargy, disorientation, dark body pigmentation, clamped fins, and reddening at the base of the dorsal fin. Fish may have increased mucus production followed by extensive epidermal sloughing along with hyperplasia of epithelial cells leading to spongiosis (Roubal & Bullock 1987). Fry and young fingerlings are particularly susceptible and infections can result in high mortalities, often with little pathology (Becker 1977, Robertson 1979).

Ichthyobodo necator attaches to and penetrates epithelial cells via an attachment disc that forms around the cytostome (Shubert 1966, Joyon & Lom 1969). Parasites seem to have a preference for Malpighian cells (Bullock & Robertson 1982), but may also be present on mucus and chloride cells (Roubal & Bullock 1987). The parasite is believed to feed on the cytoplasm by the use of a cytostomic process that is inserted into the cell. There is also some speculation that *I. necator* secretes a substance that aids in the breakdown of cytoplasmic constituents due to the presence of vacuoles and vesicles in close proximity to the cytostome (Roubal & Bullock 1987).

Ichthyobodo necator is an obligate parasite with a simple life cycle and transmission is direct between hosts. It has a broad host range and has been found to infect fish throughout North America, Europe, and Asia (Urawa et al. 1998). While *I. necator* was originally considered a freshwater pathogen (Vickerman 1976), variants have also been observed on captive marine fish (Cone & Wiles 1984, Beck et al. 1996), salmon smolts that have been transferred from fresh to saltwater (Ellis & Wootten 1978),

pelagic marine fish (Morrison & Cone 1986), and even amphibians and marine invertebrates (Becker 1977, Forsythe et al. 1991).

From histologic sections and electron micrographs, all organisms described thus far closely resemble *I. necator* in morphology and pathogenicity. Differences that have been observed are attributed more to the effect of differences in the macroenvironment. This is supported by observations of the morphology of the attachment structure between freshwater versus marine variants on salmon (Roubal & Bullock 1987) and the absence of a large vacuole in a marine variant on common dab (*Limanda limanda* L.)(Diamant 1987). However, there is some evidence to support host specificity of various *Ichthyobodo* isolates. Urawa and Kusakari (1990) found that when healthy Japanese flounder (*Paralichthys olivaceus*) and chum salmon (*Oncorynchus keta*)were exposed to *Ichthyobodo* from infected fish, only individuals exposed to the same host species became infected even though the two *Ichthyobodo* isolates appeared morphologically identical.

Members of the Order Kinetoplastida, have a kinetoplast, a unique structure of concentrated mitochondrial DNA for which the order is named (Vickerman & Preston 1976). This extranuclear DNA, or kDNA, is present as circular DNAs referred to as maxicircles and minicircles that are interlocked into a large network. The maxicircles encode mitochondrial structural genes and genes for energy transduction, while the minicircles encode guide RNAs that are used to extensively edit the maxicircle products by insertion and deletion of uridine nucleotides (Shapiro & Englund 1995).

The Order Kinetoplastida consists of two suborders, the exclusively parasitic Suborder Trypanosomatina and the Suborder Bodonina (Vickerman 1994). Members of

the Order Trypanosomatina contain a single concentrated area of kDNA near the basal

bodies of the flagella (Vickerman & Preston 1976) while the members of the Suborder

Bodonina have greater dispersion of kDNA. Many bodonine species have a single

diffuse area near the basal bodies, while others organize their kDNA into multiple areas

or pools throughout the mitochondrion as is the case for *I. necator* (Joyon & Lom 1966,

Lukescaron et al. 1998), *Cryptobia vaginalis* (Vickerman 1977), *Cryptobia helicis*

(Lukescaron et al. 1998) and *Dimastigella trypaniformis* (Breunig et al. 1993). Members

of each suborder can also be differentiated by the number of flagella present;

trypanosomatines have a single anterior (locomotory) flagellum, while bodonines have

both an anterior and a posterior (recurrent) flagellum.

Kinetoplastids have additional distinguishing characteristics that differentiate

them from other flagellates. These characteristics include a unique organelle called the

glycosome that compartmentalizes the enzymes of the glycolytic chain for more efficient

energy production and glycosylphosphatidylinositol anchors of membrane proteins that

can be adapted in different ways for host evasion (Vickerman 1994). The best known

example of the latter is the surface membrane of *T. brucei* that displays antigenic

variation of surface glycoproteins which helps the parasite to evade the host's specific

immune system.

In contrast to the functionally unique features of the order, members of the Order

Kinetoplastida demonstrate a lack of distinguishing morphological characteristics for

species within genera and even between genera (Maslov et al. 2001). As a result, much

phylogenetic and disease diagnostic research has focused on using gene sequences to

distinguish between species and even subspecies or molecular variants. This has been

particularly important in regions where different variants co-exist, causing not only taxonomic confusion but different clinical manifestations of disease (Uezato et al. 1998, Mauricio et al. 2000, Robello et al. 2000, Grisard 2002). The most widely sequenced gene for the taxonomic identification of kinetoplastids has been the small-subunit ribosomal RNA (SSU rRNA or 18S rRNA). In all eukaryotes, this gene is arranged in a linear fashion along with two other subunits (the 5.8S and 28S genes) that together, along with the 5S which is transcribed separately, comprise the ribosome; these three subunits are separated by internal transcribed spacer (ITS) regions that are edited out during rRNA processing (Fig 1). Each cluster of rRNA genes is separated by a non-transcribed spacer (NTS) region.

Fig 1. Arrangement of the eukaryotic rRNA genes
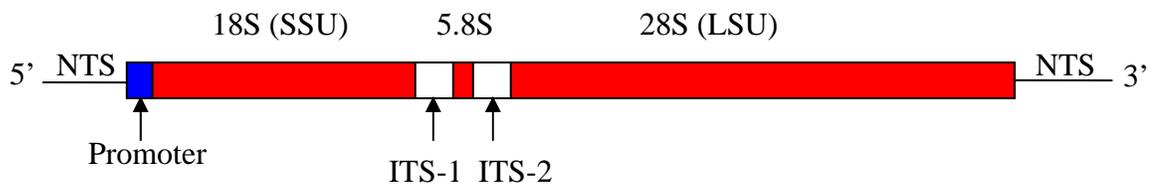
This gene has been very attractive for phylogenetic studies because it is a highly conserved structural gene that evolves slowly over time. Additionally, there are greater than 200 copies, arranged tandemly on a chromosome, making it an easy target sequence to amplify (Moran et al. 1994). The SSU rRNA gene, especially, the hypervariable regions of the gene (i.e. loop regions that are not constrained by the structural properties of the gene as a whole and thus tend to be more rapidly evolving) have been useful for

developing PCR assays that are highly discriminatory at the species level or lower taxonomic levels (van Eys et al. 1992, Noyes et al. 1997, Noyes et al. 1999, Cupolillo et al. 2000, Kawashita et al. 2001). Assays have also been developed from the internal transcribed spacers within the rRNA cistron (Cupolillo et al. 1995, Agbo et al. 2001, Mendonca et al. 2002), the non-transcribed spacers between the ribosomal gene repeats (Guevara et al. 1992, Harris et al. 1998), and maxicircle and minicircle non-transcribed spacer regions (Belli et al. 1998).

SSU rRNA sequences have suggested that the Suborder Trypanosomatina evolved from the Suborder Bodonina and that the Suborder Bodonina consists of the Family Bodonidae and the Family Cryptobiidae (Lukes et al. 1997, Wright et al. 1999). However, neither the Bodonidae nor Cryptobiidae were monophyletic in these studies; instead, species of the same genera were dispersed over multiple clades (Dolezel et al. 2000). Additionally, the order of emergence from the base of the phylogenetic tree remains unclear. Morphologically, *I. necator* resembles the bodonids and has been placed within the Family Bodonidae. However, *I. necator* is the only known parasite within the family.

The purpose of my research was to determine the phylogenetic relationship of *I. necator* to other members of the Suborder Bodonina and Family Bodonidae, and to determine if the addition of *Ichthyobodo* to the phylogenetic tree helps to clarify the relationships among the other kinetoplastids. The purpose of this research was also to investigate the possible existence of other *Ichthyobodo* species and subspecies that may form a complex of morphologically similar organisms. Crucial steps to the study of this organism have involved establishing a reliable method for the production of large

numbers of parasites, determining the best DNA isolation method, designing polymerase chain reaction (PCR) primers that are specific to the Order Kinetoplastida, Family Bodonidae, and Genus *Ichthyobodo*, and determining the best method of *Ichthyobodo* preservation to obtain isolates from other hosts and locations.

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Chapter 1

Tricaine Dramatically Reduces the Ability to Diagnose Protozoan Eectoparasite (*Ichthyobodo necator*) Infections

ABSTRACT: Tricaine (MS-222) is a widely used anaesthetic and euthanasia agent for fish. While evaluating hybrid striped bass (*Morone saxatilis* male x *M. chrysops* female) for the presence of the kinetoplastid ectoparasite, *Ichthyobodo necator* ("costia"), we discovered that unbuffered tricaine caused the rapid detachment and mortality of this parasite. Solutions of unbuffered and buffered (with sodium bicarbonate) tricaine were serially diluted (1,000, 250, 100, 50, 25, and 0 mg tricaine/L). Blind trials were conducted by placing scales, heavily infected with costia, into different treatments following a split plot design. The motility of parasites from scales was scored and statistically evaluated using ANOVA. The effect of pH of high and low unbuffered tricaine solutions (1,000 and 0 mg/L) on motility was evaluated and fish were euthanized in unbuffered or buffered tricaine (1000 mg/L). The motility of *Ichthyobodo* decreased with increasing unbuffered tricaine concentrations. Motility in buffered tricaine remained unchanged. pH of the unbuffered solutions was found to have no effect on *Ichthyobodo* and parasites remained attached to the skin of fish that were euthanized in buffered tricaine but detached almost completely from the skin of fish that were euthanized in unbuffered tricaine. Our results indicated that tricaine should always be buffered when clinically evaluating fish for ectoparasites.

KEYWORDS: anaesthetic, protozoa, MS-222, disease diagnosis

Tricaine (tricaine methanesulfonate), also known as MS-222, is a widely used anaesthetic and euthanasia agent for fish and amphibians. Tricaine is particularly effective in fish because it is highly water- and lipid-soluble and readily crosses the gill membrane (Hunn & Allen 1974, Treves-Brown 2000). Additionally, tricaine moves bidirectionally across the gills, allowing rapid removal from the body and a quick post-anaesthetic recovery (Hunn et al. 1968). For euthanasia, tricaine is often used unbuffered, but when used as an anaesthetic, tricaine is commonly buffered because it causes a significant decrease in pH at effective dosages.

Tricaine is advocated as a chemical restraint for clinical evaluation of fish (Post 1987, Brown 1993, Noga 1996) including the collection of gill and skin samples for diagnosing common ectoparasites. One of the most common ectoparasites of cultured fish is the kinetoplastid flagellate, *Ichthyobodo necator* (Henneguy 1883)(Robertson 1985, Urawa et al. 1998). *Ichthybodo necator* ("costia") primarily infects young fish, causing high mortalities if left untreated (Urawa et al. 1991, Grignard et al. 1996). While clinically evaluating fish for the presence of this parasite, it was discovered that unbuffered tricaine caused the rapid detachment and mortality of *Ichthyobodo* trophonts from the skin after 5 min of immersion. This suggested that the use of tricaine has a serious impact on the clinical evaluation of skin and gill infections for this parasite. To understand this phenomenon, the effect of varying concentrations of both buffered and unbuffered tricaine on the motility and attachment of *I. necator* was examined.

Hybrid striped bass (*Morone saxatilis* male x *M. chrysops* female), subclinically infected with *I. necator*, were obtained from a local producer. Fish were housed in 300 L aquaria at low temperature (14 $^\circ$C) and high density (1 fish, 70-85 mm length, per 5 L

water); they were fed a restricted diet to inhibit growth. Outbreaks of ichthyobodosis occurred spontaneously in the laboratory during spring and autumn, but could be induced at other times of the year by either increasing or decreasing the temperature several degrees for a 24 hour period before returning to 14 ^{o}C. All fish used for this study were approximately one year in age.

Sick fish with typical signs of ichthyobodosis (lethargic, disoriented, dark pigmentation, clamped fins, reddening at the base of the dorsal fin) were screened for severe *I. necator* infections. Infections on the body primarily occurred around the base of the dorsal fin. Therefore, samples were removed from this region for each experiment. Due to the small size of the fish, the number of scales removed with each sampling varied between 2 and 5. Infection intensity was assessed by placing scales in aquarium water on a microscope slide, covering with a plastic coverslip, and examining the sample at 100X using a Nikon inverted phase contrast microscope. Only fish with severe infections (>1000 parasites/mm^{2}) were used for experiments. Because of the variability in infectivity between fish, a split plot experimental design was employed.

Solutions of unbuffered tricaine (1000 mg/L) were made by dissolving and diluting tricaine in aquarium water (0.036 mg/L hardness, 0.054 mg/L alkalinity, pH 6.3). An equivalent stock solution of tricaine (1000 mg/L) was buffered with 2000 mg/L sodium bicarbonate and also diluted in aquarium water. Final test concentrations for both tricaine dilution series were 1000, 250, 100, 50, 25, and 0 mg tricaine/L, respectively. The diluted solutions were then coded by another investigator as either U1-U6 or B1-B6 so that observations were done blindly. Aliquots (50 Fl) of the randomly assigned

tricaine dilutions were added to the bottom of 75 cm^2 Corning™ tissue culture flasks (top side removed). Each treatment was replicated 5 times (Table 1.1).

Samples containing 2 - 5 infected scales were added to each tricaine aliquot. The scales were incubated at room temperature for 5 min and then covered with a plastic coverslip. Parasite motility (percentage of visible parasites moving) was quickly scored as follows: 1 = 0-25 % motility, 2 = 25-50 % motility, 3 = 50-75 % motility, 4 = 75-100 % motility. Half values were used when a 1 could not be distinguished from a 2, a 2 from a 3, and so on. For consistency, only the area around one, moderately infected scale was scored for parasite motility. Infected scales from 12 fish were scored, and the differences among the test dilutions for both the buffered and unbuffered tricaine dilution series were log-transformed and analyzed using analysis of variance (ANOVA).

In preliminary experiments, it was determined that as the unbuffered tricaine concentration increased, the pH decreased to as low as 3.3 at 1000 mg/L tricaine. In contrast, all buffered tricaine solutions remained between pH 6.6 - 7.5 (Fig 1.1). To determine the effect of pH on parasite motility, aquarium water was adjusted to either pH 7.5 or pH 3.3 using 0.1N HCl. Duplicate, 50 µl drops of each pH solution were placed on a flask as described before. Scales from the fish described above were added to each replicate pH solution and parasite motility was assayed as described. Parasite motility at the two pHs was compared using the Wilcoxon rank test (Steel et al. 1997). The pHs in the second set of replicate solutions were measured to confirm that they did not substantially change after the addition of scale tissue.

To determine how euthanizing fish in buffered and unbuffered tricaine affected the attachment of *I. necator* to the skin, four heavily infected hybrid striped bass were placed into a solution of either 1000 mgL tricaine with 2000 mg/L sodium bicarbonate or 1000 mg/L tricaine alone. After 10 min, the fish were fixed in 10 % neutral buffered formalin. After seven days, the fish were decalcified in EDTA for an additional seven days. All fish were sectioned at the posterior border of the dorsal fin using standard histological procedures. Slides were photographed with an Olympus VANOX AHS-3 photomicroscope using an Olympus C-35AD-4 camera.

There was a dramatic, dose-dependent decrease in *I. necator* motility with increasing unbuffered tricaine concentrations (Fig 1.1). In contrast, parasite motility in buffered tricaine solutions remained unchanged. The decreased motility with unbuffered tricaine was significantly different from that of buffered tricaine at all concentrations greater than 25 mg/L (Fig 1.1). Similarly, when fish were euthanized in 1000 mg/L buffered tricaine, *I. necator* remained attached to the skin (Fig 1.2A), but completely detached when fish were euthanized in unbuffered tricaine (Fig 1.2B). The low pH alone was not responsible for this effect, as there was no difference in the motility of *I. necator* incubated in aquarium water at pH 3.3 versus pH 7.5 (p = 0.125, n = 13).

The recommended sedative dose of tricaine for most fish ranges from 10-40 mg/L, while the anaesthetic dose is 50-250 mg/L for immersion or 1000 mg/L sprayed on the gills of large fish (Summerfelt & Smith 1990, Ross & Ross 1999). Concentrations commonly used for euthanasia range from 150 to 1000 mg/L (Noga 1996). Our results demonstrated that unbuffered tricaine concentrations as low as 50 mg/L significantly affect parasite motility and at higher doses cause *I. necator* to rapidly detach from the

19

host tissue and become immobilized. Our results also suggest that this is not directly caused by the drop in pH, but rather by a decrease in the unionized form of tricaine. Tricaine methansulfonate is a weak base with a pKa of 3.5 (Treves-Brown 2000). In unbuffered media, a much higher proportion of tricaine would be in the ionized form. As the concentration of unbuffered tricaine increases, the pH of the solution approaches the pKa, which would decrease the amount of drug that can pass through lipophilic cell membranes (Allen & Hunn 1986). As a result, tricaine uptake would need to be via an active transport mechanism (ion channel) across the membrane. It appears that *I. necator* may be able to prevent the passive transport of the lipophilic (unionized) form of the drug, but may have an active transport mechanism that allows the ionized form to enter into the cell.

Many guidelines for the collection of clinical samples from fish include appropriate tricaine concentrations and buffering requirements (Summerfelt & Smith 1990, Noga 1996, Ross & Ross 1999). However, some others do not (Post 1987, Brown 1993, Reimschuessel 1997). As a result, tricaine may be used in inappropriately large concentrations without buffering. Our findings indicate that tricaine should always be buffered when fish are to be clinically evaluated, particularly in water with low alkalinity. Buffering is less important when assaying parasites of marine fish due to the higher buffering capacity of seawater.

As with all skin and gill ectoparasites, the severity of clinical signs and outcome for recovery is directly related to the number of parasites present. Thus, it is essential that an accurate estimate of parasite load be determined during the clinical exam. If tricaine is unbuffered, ectoparasites might be reduced in number or totally lost, providing

20

an incorrect assessment of their clinical importance. To our knowledge, this is the first report that tricaine can affect protozoa. However, it has been known for some time that tricaine can narcotize metazoans (Delly 1985). Tricaine has previously been reported to anaesthetize the rotifer, *Branchionus calyciflorus* (Nogrady & Keshmirian 1986) and the nematode, *Caenorhabditis elegans* (McCarter et al. 1999). We have also narcotized leeches (*Myzobdella*) with tricaine (E. J. Noga & R. A. Bullis, Personal Communication) and have observed that a high concentration of unbuffered tricaine narcotizes *Trichodina,* another common ectoparasite of fish, although they are not completely immobilized like *I. necator* (H. A. Callahan, Personal Communication). Future work should examine how the ionized form of tricaine anaesthetizes *I. necator* and if tricaine affects other protozoan parasites as well.

ACKNOWLEDGEMENTS

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Table 1.1. The split plot design used for examining the effect of tricaine on *Ichthyobodo*

necator motility. Each tricaine dose from buffered (B) and unbuffered (U) dilution series

was replicated 5 times. Dosages were randomly assigned numbers (U1-U6 or B1-B6)

and aliquoted as indicated below.

Flask number	Treatments
1	U1,U2,U5
	U2,U5,U6
2	U1,U2,U4
	U1,U4,U6
3	U4,U5,U6
	U2,U3,U4
4	U1,U3,U5
	U3,U4,U5
5	U1,U3,U6
	U2,U3,U6
6	B1,B2,B5
	B2,B5,B6
7	B1,B2,B4
	B1,B4,B6
8	B4,B5,B6
	B2,B3,B4
9	B1,B3,B5
	B3,B4,B5
10	B1,B3,B6
	B2,B3,B6

Figure 1.1. The motility of *I. necator* after exposure to different concentrations of buffered or unbuffered tricaine solutions, where 1 = 0-25, 2 = 25-50%, 3 = 50-75%, 4 = 75-100% motility. Motility scores between the same buffered and unbuffered concentrations were compared using ANOVA. Each point on the graph represents the mean score from 12 fish. (*) denotes significance at < 0.01, (**) denotes significance at < 0.0001. The pH of the 0 mg/L and 1000 mg/L concentrations are indicated above the corresponding point.
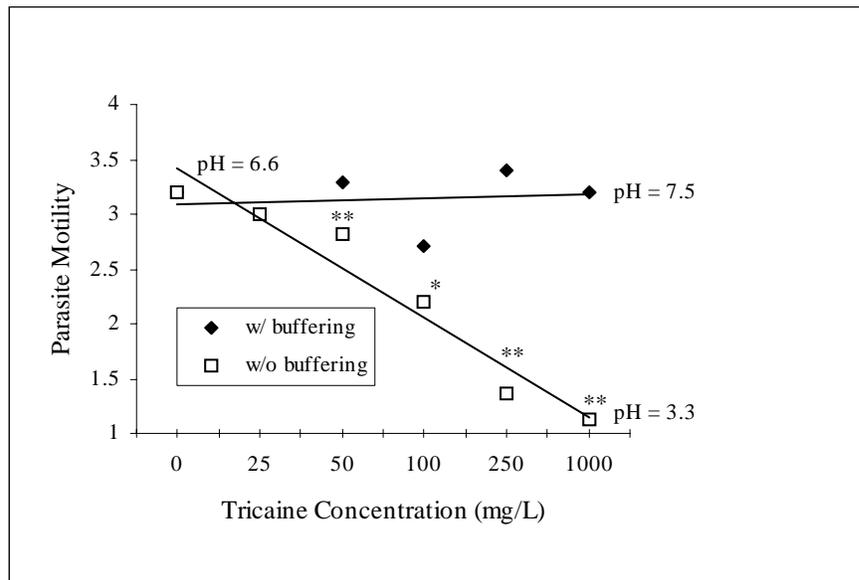
Figure 1.2. Histological cross-section of the skin of hybrid bass. (a) Attached

Ichthyobodo necator trophonts (arrow) after fish were euthanized in buffered tricaine

(1,000 + 2,000 mg/L sodium bicarbonate). The epidermis (e) and the scales (s) are

indicated. (b) The absence of *I. necator* trophonts after fish were euthanized in

unbuffered tricaine (1,000 mg/L) (H&E, Bar = 40 µm).
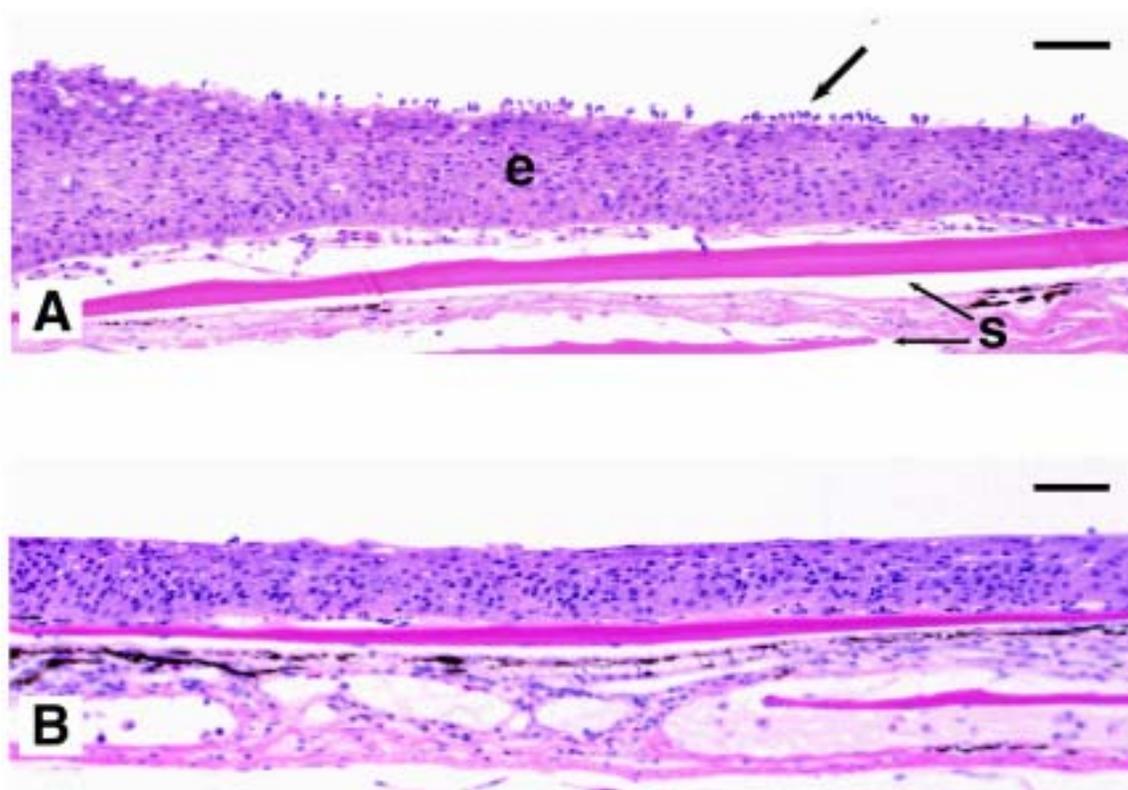
Chapter 2

Determination of the Optimal Fixation Method for DNA Isolation and

PCR Amplification of the Fish Ectoparasite, *Ichthyobodo necator*

ABSTRACT: *Ichthyobodo* is a widespread and important parasite of the skin and gills of both freshwater and marine fish. This organism has been difficult to study because *in vitro* culturing methods have not been successful. We wanted to evaluate preservation methods for DNA isolation and analysis of this important parasite. Using both skin and gill tissue from *Ichthyobodo* infected hybrid striped bass (*Morone saxatilis* male x *M. chrysops* female), we examined four preservation methods (Utermöhl's solution, ethanol, formalin, freezing without fixation at -70 °C) and two DNA isolation methods (DNA isolation kit, crude lysate). Sample preservation and DNA isolation methods were compared by amplifying segments of the rRNA genes and their internal transcribed spacers using two different primer sets that produced either a 1.2 or 3 kb product. Both skin and gill preserved in Utermöhl's solution, ethanol, and frozen generated a product 100 % of the time when DNA was isolated using the isolation kit. Amplification of the 3 kb product was attempted first and found to be variable and dependent on both the type of preservation and the length of storage time. Samples that could not initially generate the 3 kb product were able to generate the 1.2 kb product. None of the formalin-fixed tissues or crude lysate preparations generated amplification products of any length. Spiked controls of the crude lysate preparations suggested that this was not due to inhibitors in the tissue. We concluded that ethanol-fixation and DNA isolation using the QiaAmp DNA Mini Kit Protocol (Qiagen™) was the best method for obtaining PCR products greater than 1,000 bp and that this method provided a rapid and easy field preparation of protozoan DNA that can be used to study parasites of fish that are not easily cultured.

INTRODUCTION

Molecular methods for disease diagnosis are becoming increasingly prevalent in assessing the health of cultured fish (Cunningham 2002). DNA sequencing and other molecular techniques have provided evidence that many protozoan parasites previously considered a single taxon may instead represent a multispecies complex (Franco et al. 1996, Stringer 1996, Monis et al. 1999). One example of this is the important protozoan ectoparasite, *Ichthyobodo necator*. Despite the morphological evidence for only one *Ichthyobodo* species, the worldwide occurrence of *Ichthyobodo* on both marine and freshwater fish and recent cross-infection studies between a freshwater and marine isolate (Urawa & Kusakari 1990) suggest that this monotypic genus may actually consist of more than one species. *Ichthyobodo*, like almost all fish parasites, cannot be cultured *in vitro*, requiring that isolates be collected and preserved from live, infected fish.

In order to reliably assess the molecular taxonomic relationships of similar organisms, protozoa must be preserved well enough to maintain the fidelity of their DNA. We have recently sequenced the 18S ribosomal RNA (rRNA) gene from live *Ichthyobodo* collected from hybrid striped bass (*Morone saxatilis* male x *M. chrysops* female)(Callahan et al. 2002). This approach involved euthanasia of the fish to collect large numbers of trophonts (Callahan & Noga 2002); further improvements led to the isolation of *Ichthyobodo* DNA from skin and gill biopsies without risk to the fish (HC Callahan, unpublished data).

We subsequently wanted to examine the 18S rRNA gene from *Ichthyobodo* isolates of as many fish and locations as possible. Because of the difficulty in shipping live fish that may die of ichthyobodosis before arriving, we needed to evaluate the best

method for preserving *Ichthyobodo* trophonts on skin and gill biopsies for DNA isolation. In the present study, we compared four preservation methods and two DNA isolation techniques. Comparisons were based on the generation of specific products using two different primer sets.

MATERIALS AND METHODS

Cultivation of Parasites. Hybrid striped bass (*Morone saxatilis* male x *M. chrysops* female) subclinically infected with *Ichthyobodo* were maintained and infection induced as described previously (Callahan and Noga 2002). Sick fish with typical signs of ichthyobodosis (lethargic, disoriented, dark pigmentation, clamped fins, reddening at the base of the dorsal fin) were screened for severe *Ichthyobodo* infections. Infection was confirmed by removing a scale from the posterior region of the dorsal fin for each fish used in the experiment and examining at 100X using a Nikon inverted phase contrast microscope. Only fish with severe infections (>1000 parasites/mm^2) were used for the experiment.

Sample Collection. To determine the optimal method for collecting *Ichthyobodo* for DNA extraction and PCR amplification, four preservation methods were examined. For each infected fish, skin and gill samples were collected and placed in Utermöhl's solution (an iodine-based fixative)(Guillard 1973), 70 % ethanol (ETOH), 10 % neutral buffered formalin (NBF), or frozen without fixation at -70 °C. Approximately twelve scales and 25 mg of gill were placed in the appropriate tube with each tube containing 1 ml of preservative. Samples fixed in Utermöhl's solution were wrapped in foil to prevent photo

30

degradation of the fixative. For a single fish, both skin and gill tissue were collected for each preservation method, for two time points (one week and one month), and for two DNA isolation methods (Qiagen™ kit and crude lysate preparation). A total of five infected fish were used in this experiment.

DNA Isolation and PCR Amplification. After one week and again at one month, two tubes of each tissue type from each fixative were pelleted by centrifuging at 400 x g for 10 minutes at room temperature. The fixatives were then removed and the tissue was washed 2X with 200 μl of PBS (pH 7.2). One tube of each tissue type from each preservation method was resuspended in TE (pH 8.0), boiled for 15 minutes, and then stored at -20°C until testing. The second tube of each tissue type was resuspended in Buffer ATL and the DNA was isolated using the QiaAmp DNA Mini Kit Protocol (Qiagen™).

Approximately 10 ng of either purified genomic DNA or 2 μl of the crude lysate was combined with 1X Low Salt PCR buffer (Stratagene), 100 ng of KinSSUF1, 100 ng KinITSR2, 0.10 mM dNTPs, and 5 units of Taq Plus Long Polymerase (Stratagene). The primers KinSSUF1 (Callahan et al 2002) and KinITSR2 (5' ACTTTCGCTCACGCTACTTG 3') generated a 3 kb product that spanned the 18S rRNA, ITS-1, 5.8S rRNA, ITS-2, and ~350 bp of the 28S rRNA. These primers were used to generate a product for each sample. However, the 3 kb product could not be generated for every sample. As a result, we substituted KinSSUF1 for the forward primer, KinITSF1 (5' TCCCTGCCATTTGTACACAC 3')(which excluded the 18S rRNA gene and produced a fragment 1.2 kb in length). This allowed us to evaluate if

fixative-related DNA degradation still allowed significantly sized DNA fragments to be generated.

The PCR cycling conditions for all amplifications were 1 cycle at 94 $^{\circ}$C for 3 min, 33 cycles at 94 $^{\circ}$C for 1 min, 55-60 $^{\circ}$C for 1 min (decreasing the annealing temperature 0.5 $^{\circ}$C after every third cycle), and 72 $^{\circ}$C for 2 min 30 sec, followed by 72 $^{\circ}$C for 5 min. All reactions were visualized on 0.8 % agarose gels that were stained with ethidium bromide.

RESULTS AND DISCUSSION

DNA from all samples fixed in Utermöhl's or ETOH, and frozen at -70 $^{\circ}$C, that were isolated using the Qiagen™ protocol, generated amplification products. Generation of the 3 kb product was more variable and was dependent on the preservation method and the length of time the sample was stored (Table 2.1). After one week, skin fixed in Utermöhl's solution generated the 3 kb product 4 out of 5 times, but after one month, only the 1.2 kb product was generated 4 out of 5 times. For gill fixed in Utermöhl's, solution, the 1.2 kb product was generated 3 out of 4 times after one week and 4 out of 4 times after 1 month. Only one gill sample fixed in Utermöhl's generated the 3 kb product. ETOH-fixed, skin tissue generated the 3 kb product 5 out of 5 times after one week and 4 out of 5 times after one month (Table 2.1). The ETOH-fixed gill tissue also generated the 3 kb product 4 out of 4 times after one week but only 2 out of 4 times after one month. Freezing the tissue at -70°C produced mixed results. For the skin, at both one week and one month, only 3 out of 5 samples generated the 3 kb product, with the other 2 samples generating the 1.2 kb product (Table 2.1). Similarly, the gill generated

32

the 3 kb product 2 out of 4 times after one week and 1 out of 4 times after one month. All other samples generated the 1.2 kb product.

Only one lysate, a skin sample fixed for one week in Utermöhl's solution, generated a product. To assess whether this was due to inhibitors from the skin and gill tissue, 12 PCR reactions (6 gill and 6 skin samples consisting of 2 ETOH, 2 Utermöhl's, and 2 frozen samples, respectively) containing 2 ul of a crude lysate sample were spiked with *Ichthyobodo* DNA extracted using the Qiagen™ protocol. The primers KinITSF1 and KinITSR2 were used to generate the 1.2 kb product. None of the ethanol-fixed scale samples and only 1 of 2 ethanol-fixed gill samples generated products. For Utermöhl's-fixed lysates, only 1 of 2 gill samples generated a product. All other spiked lysates generated the 1.2 kb product. None of the NBF-fixed samples generated a product of either size.

Several studies have evaluated preservation methods for kinetoplastid protozoans (Uezato et al. 1998, Marques, 2001 #180, Carnevale et al. 2000). However, the organism in question has typically been a human blood pathogen, where results may not be easily extrapolated to a fish ectoparasite such as *Ichthyobodo*. Several studies have also focused on how to use tissue from a fixation method that is not conducive to DNA isolation and analysis (Dowd et al. 1998, Coombs et al. 1999). For clinical cases involving mammals or fish, the most commonly used fixative is formalin, a simple and readily available fixative. However, the usefulness of organisms preserved in formalin is limited for DNA analysis. Additionally, the use of formalin generates a toxic waste disposal problem. To isolate and amplify DNA from *Ichthyobodo* or other unculturable protozoa, a preservation method that allows the transport of samples from different hosts and

33

locations was needed. Because we were the first to sequence DNA from an *Ichthyobodo* isolate (Callahan et al. 2002), the stability of this parasite's DNA after preservation of any kind was unknown. Our evaluation of four different preservation methods and two DNA isolation techniques indicated that fixation in 70 % ethanol and DNA isolation using the QiaAmp DNA Isolation Kit repeatedly generated a 3 kb product, even after one month storage. Our results also indicated that parasites on skin tissue preserved better than those on gill tissue with all methods, including freezing. For the fixatives, this may have been a result of the amount of tissue versus fixative present in the sample. Scales are embedded in the dermis of fish and then covered by a thin layer of epithelium. This epithelium only covers the part of the scale that is not overlapped by the other scales. In contrast, the gills have a more complicated structure with a much larger surface area covered by epithelium (Roberts & Ellis 2001). It is possible that due to the larger surface area, the gill tissue may not have been adequately fixed, leading to DNA degradation and shorter amplification products.

We also assessed whether there may be inhibitors of amplification in the gill and skin tissue by evaluating crude lysates spiked with purified *Ichthyobodo* DNA. Of the 4 ethanol-fixed samples only 1 generated a product. However, with the exception of 1 Utermöhl's-fixed gill sample, all other samples generated a product suggesting that residual ethanol inhibits amplification but the tissue itself does not. These results indicate that boiling *Ichthyobodo*-infected tissue does not sufficiently lyse the protozoa for DNA amplification.

After 70 % ethanol, Utermöhl's solution was the best preservation method. Utermöhl's solution was originally developed for fixation of algae (Guillard 1973).

34

However, our results suggested that it would not be suitable for preservation and transport of *Ichthyobodo* trophonts. For the skin, only the 1.2 kb product was primarily generated after one month, indicating that the DNA had degraded during storage. Utermöhl's solution degrades quickly in light, due to the oxidation of the iodide ion. While all samples were covered in foil and stored in darkness, the fixative was still unsuitable for DNA isolation.

Surprisingly, freezing at –70 °C was not the best preservation method. Frozen tissue is often considered comparable to fresh tissue for PCR (Baszler et al. 1999, Uezato et al. 1998). However, for *Ichthyobodo*, after only one week, approximately half of the products generated were the smaller 1.2 kb sequence. Additionally, freezing may not be as feasible as other methods because some researchers may not have access to a –70 °C freezer. It is also more expensive to ship frozen samples.

The least effective preservation method was NBF. No products were generated from any formalin fixed sample even after 1 week. Formalin is never a good method for DNA preservation, as formaldehyde covalently modifies DNA, causing it to fragment (Rish et al. 1996). While our further studies of *Ichthyobodo* fixed in NBF have allowed some DNA isolation and amplification if the tissue is crushed and placed in a Qiashredder (Qiagen™) prior to tissue lysis (data not shown), that method was not evaluated in this study.

Overall, the most reliable method for preparing *Ichthyobodo* isolates for DNA isolation and PCR amplification was fixation of infected tissue in 70 % ethanol and DNA isolation using a commercial extraction and purification kit (QiaAmp DNA isolation kit). Using this method, products of up to 3 kb were reproducibly generated using PCR. This

method may also allow the collection and DNA isolation and analysis of other unculturable protozoan fish ectoparasites from both marine and freshwater environments. Molecular studies of many of these organisms would help clarify taxonomic and phylogenetic relationships. Questions such as host preferences, geographic distribution, and pathogenicity could also be addressed.

ACKNOWLEDGEMENTS

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Table 2.1. Amplification products generated from *Ichthyobodo* DNA that was preserved using four different methods. Results include only samples isolated using the QiaAmp DNA isolation kit (Qiagen™). Amplification of the 3 kb product was attempted first. Samples that did not generate this product size were then used to generate the 1.2 kb product. Skin tissue was collected from a total of 5 fish and gill tissue was collected from a total of 4 fish.

Preservation Method	Total # of fish	Tissue	Time	Product Size 3 kb	1.2 kb
70 % Ethanol	5	skin	1 week	5	ND
	5		1 month	4	1
	4	gill	1 week	4	ND
	4		1 month	2	2
Utermohl's Solution	5	skin	1 week	4	1
	5		1 month	1	4
	4	gill	1 week	1	3
	4		1 month	0	4
Freezing	5	skin	1 week	3	2
	5		1 month	3	2
	4	gill	1 week	2	2
	4		1 month	1	3
NBF	5	skin	1 week	0	0
	5		1 month	0	0
	4	gill	1 week	0	0
	4		1 month	0	0

Chapter 3

Evidence for More Than One Species of *Ichthyobodo* Based on Ribosomal RNA and ITS Differences: Implications for the Management of Aquaculture Stocks

ABSTRACT: *Ichthyobodo necator* ("costia") is a common and important parasite that infects the skin and gills of many freshwater and marine fish. Costia infections are often fatal and are a nuisance to aquaculture operations worldwide. Whether *Ichthyobodo* isolates from different hosts represent a single species or are part of a multispecies complex with differing host preferences has been a matter of debate. Knowing if multiple *I. necator* species exist, and if those species have broad or narrow host specificity has important implications for the management of costia. To address the questions of species diversity and host specificity, genomic DNA was isolated from *Ichthyobodo* trophonts collected from *Oncorhynchus mykiss* (rainbow trout), *Cyprinus carpio* (koi), *C. carpio* (mirror carp), *Carassius auratus* (goldfish), *Ictalurus punctatus* (channel catfish), *Xiphophorus helleri* (swordtail), and *Paralichthys olivaceus* (Japanese flounder). The SSU ribosomal RNA (rRNA) gene from each isolate and the internal transcribed spacers (ITS) from six isolates were PCR-amplified, cloned and sequenced. The resulting sequences were aligned and the mutational differences were compared. The SSU and ITS rRNA sequence data suggested that the *Ichthyobodo* isolates examined represented a complex of four different species, each of which occupies a distinct geographical region. The current sampling did not suggest that any of the species were host-specific, indicating that exchange of infected fish from one region to another has a high potential for spreading the disease.

KEY WORDS: aquaculture, costia, fish ectoparasite, ITS (internal transcribed spacers), LSU rRNA (Large subunit ribosomal RNA), SSU rRNA (small subunit ribosomal RNA)

INTRODUCTION

Protozoan ectoparasites of fish are a serious concern for the aquaculture industry both nationally and internationally (Schisler et al. 1999, Missen & Dobson 2000, Bergh et al. 2001, Crosby 2001). One of the most common protozoan parasites is *Ichthyobodo necator* ("Costia"). *Ichthyobodo necator* is a member of the Order Kinetoplastida, a unique group of flagellates that are recognized by one or more areas of concentrated mitochondrial DNA, termed the kinetoplast (Vickerman & Preston 1976). The Order Kinetoplastida contains a number of parasitic, ectocommensal, and free-living species, including a number of medically important genera (i.e., *Trypanosoma*, *Leishmania*) (Vickerman 1990). *Ichthyobodo necator* primarily infects young fish and can cause high mortalities in cultured fish populations if left untreated (Sommerville 1984, Urawa et al. 1991, Urawa 1995, Grignard et al. 1996).

Ichthyobodo necator trophonts feeding on the skin and gill epithelium multiply rapidly by binary fission. Epithelial damage results in osmotic stress, respiratory impairment, and secondary bacterial/fungal infections (Robertson et al. 1981). Unlike many fish parasites, *I. necator* has been found on numerous freshwater and marine hosts from a broad range of habitats (Robertson 1985, Forsythe et al. 1991, Urawa et al. 1998). To date, only two species of *Ichthyobodo* have been identified, *I. necator* and *I. pyriformis*. *Ichthyobodo pyriformis* was named based on its smaller size compared to *I. necator* (Davis 1943). In general, ultrastructural and light microscopy comparisons between *I. necator* and *I. pyriformis*, as well as isolates of other hosts, have indicated little to no morphological differentiation between these two species (Cone & Wiles 1984, Morrison & Cone 1986, Diamant 1987, Roubal & Bullock 1987, Bruno 1992).

42

Cross-infection studies between Japanese flounder (*Paralichthys olivaceus*) and chum salmon (*Onchorhynchus keta*) have indicated that naïve fish only become infected when exposed to infected fish of the same species (Urawa & Kusakari 1990). These limited cross-infection studies were consistent with there being different *Ichthyobodo* species or strains, each with a specific host affinity.

To test the hypothesis that there were different species of *Ichthyobodo* with specific host affinities, we collected samples of *Ichthyobodo*-infected tissues from freshwater and marine fish, then PCR-amplified and sequenced the SSU rRNA gene for each isolate. The SSU rRNA gene was selected because it generally diverges at a rate that allows related species to be distinguished from one another (Briones et al. 1999, Sehgal et al. 2001). The resulting SSU rRNA sequences for each isolate were aligned and putative species designations established based on nucleotide differences that typify related species in the Suborder Bodonina, the suborder to which *Ichthyobodo* belongs. As further validation of the putative species designations, the internal transcribed spacers (ITS) within the rRNA cistron were amplified and sequenced from six of the nine isolates. ITS regions diverge relatively rapidly and have been used in a number of different groups to distinguish closely related species within a genus (Cupolillo et al. 2000, Kawashita et al. 2001, Le Roux et al. 2001, Olivier et al. 2001, Berzunza-Cruz et al. 2002). The association between each putative *Ichthyobodo* species and host species, habitat, and geographic region was then examined.

MATERIALS AND METHODS

Sample collection and DNA isolation. *Ichthyobodo* trophonts were obtained from a

variety of hosts and locations. Trophonts were collected from *Oncorhynkus mykiss* (rainbow trout), and *Cyprinus carpio* (koi) in North Carolina, U.S.A., from *C. carpio* (koi), *Ictalurus punctatus* (channel catfish), and *Carassius auratus* (goldfish) in Arkansas, U.S.A., from *Xiphophorus helleri* (swordtail) in Florida, U.S.A., from *Paralichthys olivaceus* (Japanese flounder) in Japan, and from *C. carpio* (mirror carp) in Greece. All samples were submitted to our laboratory after being clinically evaluated by other clinicians and researchers for the presence of *Ichthyobodo*. All parasites from fish in North Carolina were collected directly from the body of the fish following the procedure described in Callahan et al. 2002. All other samples, consisting of both skin and gill tissue, were fixed in 70 % ethanol, except for *P. olivaceus*, which was fixed whole in 10 % neutral buffered formalin. To aid in lysis of *Ichthyobodo* cells from *P. olivaceus*, skin was crushed using the blunt end of a plastic swab and then filtered through a QIAshredder (Qiagen™). None of the samples fixed in 70 % ethanol required additional lysis methods. DNA was isolated from all samples using the QiaAmp DNA Mini Kit (Qiagen™).

PCR amplification. Approximately 10 ng of genomic DNA was combined with 1X Low Salt PCR buffer (Stratagene), 100 ng of KinSSUF1, 100 ng KinITSR2, 100 μM dNTPs, and 5 units of Taq Plus Long Polymerase (Stratagene). The primers KinSSUF1 and KinITSR2 produce a 3 kb product spanning the SSU rRNA, ITS-1, 5.8S rRNA, ITS-2, and ~350 bp of the 5' end of the LSU rRNA (Figure 3.1). These primers were used to obtain the *C. carpio* (North Carolina) isolate sequence. The DNA in some of the preserved material had degraded to such a degree that it was impossible to amplify the

desired 3 kb product. There were also several nucleotide differences at the primer sites

for some of the isolates. In this case the gene regions of interest were amplified and

cloned in sections using KinSSUF1 with ISR1, ISF1 with KinSSUR3, and ISF1 with

KinITSR2. ISF1 and ISF2 were used to amplify the region around the ISF1/ISR1

primers, which both annealed to the same DNA sequence (Fig 3.1). The *C. auratus*, *C.*

carpio (Arkansas), *O. mykiss*, and *X. helleri* isolate sequences were obtained using these

primers. For the *I. punctatus, P. olivaceus*, and *C. carpio* (Greece) isolates the DNA

could only be amplified with KinSSUF1/ISR1 and ISF1/KinSSUR3 which meant that the

sequence starting in ITS-1 through the 5' end of the LSU rRNA gene was missing.

All templates were amplified using an annealing temperature range of 55-60 $^{\circ}$C,

except for the primers ISF2/ISR2, which had a range of 50-55 $^{\circ}$C. The PCR cycling

conditions for all amplifications were 1 cycle at 94 $^{\circ}$C for 3 min, 33 cycles at 94 $^{\circ}$C for 1

min, 55-60 $^{\circ}$C for 1 min (decreasing the annealing temperature 0.5 $^{\circ}$C after every third

cycle), and 72 $^{\circ}$C for 2 min 30 sec, followed by 72 $^{\circ}$C for 5 min.

Cloning and sequencing. The fragments generated using KinSSUF1/KinITSR2 and

KinSSUF1/ISR1 were cloned using either the TOPO TA protocol (Invitrogen™) or the

pGEM-T Easy Vector Protocol (Promega™). Plasmid DNA from positive clones was

isolated using the QIAprep Spin Mini Prep Kit (Qiagen™). Three clones containing the

correct insert were diluted and sequenced in both directions on an ABI 373A automated

sequencer using conserved primers and the Taq Dye Deoxy Terminator Cycle

Sequencing Kit (Applied Biosystems-ABI). Fragments generated using ISF2 /ISR2 and

ISF/KinITSR2 did not clone efficiently. Amplification products were, therefore, purified

using the PCR Purification Kit (Qiagen™) and sequenced directly. Three separate PCR reactions were diluted and sequenced as described. Sequencing primers for the SSU rRNA are from Callahan et al. 2002. PCR primers (ISF1, ISR1, ISF2, ISR2, KinSSUR3) were also diluted and used as sequencing primers when necessary to fill in gaps in the sequence (Fig 3.1).

SSU rRNA and ITS sequence comparisons. To determine the similarity of the SSU rRNA gene among the *Ichthyobodo* isolates, the proportion of differing nucleotide sites was calculated for each pair of isolates. This analysis provided a direct comparison of mutational differences among the isolates. The sequences were aligned relative to each other based on a previously sequenced *Ichthyobodo* isolate (AY028448) from hybrid striped bass (*Morone saxatilis* male x *M. chrysops* female) and rRNA secondary structure (Van De Peer et al. 1994). Calculations were performed using the paired difference algorithm in PAUP* (Swofford 1999).

The ITS-1 and ITS-2 sequences could not be obtained for all isolates. However, these sequences were amplified and sequenced for six of the nine isolates (*Morone*, *X. helleri*, *C. auratus*, *C. carpio* (koi), and *O. mykiss*). ITS sequences were aligned using Multalin (Corpet 1988). The alignment was then used to check the validity of the SSU rRNA results. No additional analyses were done because the ITS sequences could not be aligned with confidence.

RESULTS AND DISCUSSION

Average differences between species in the Suborder Bodonina range from 1.3 –
13.8 % (Callahan et al. 2002). Nucleotide differences between *Ichthyobodo* isolates
ranged from 0 – 4.6 % (Table 3.1). Based on these differences, isolates fell into four
distinct groups with average sequence differences ranging from 1.9 – 4.5 % (Table 3.2).
These sequence divergences are well within range of the differences observed in the
Suborder Bodonina and suggest that the *Ichthyobodo* isolates represent four molecularly
distinct species (Table 3.1, 3.2). The first species consisted of the *Morone* and *X. helleri*
isolates. These isolates differed from each other by 0.2 % which corresponded to 3
nucleotide substitutions, but differed from the other species by 3.5 to 4.5 %, which
corresponded to between 65 to 83 nucleotide substitutions. The second and third species
consisted of the *O. mykiss* and *C. carpio* (mirror carp) isolates, respectively. The *O.
mykiss* isolate differed from the other species by 2.4 to 4.6 % which corresponded to
between 45 to 86 nucleotide substitutions, while the *C. carpio* (mirror carp) isolate
differed from the other species by 1.8 to 4.5 % which corresponded to between 33 to 83
nucleotide substitutions. The fourth species consisted of the *P. olivaceus*, *C. carpio* (koi),
C. auratus, and *I. punctatus* isolates; this group was the most diverse, consisting of both
freshwater and marine fish. These isolates differed from each other by 0 to 0.2 % which
corresponded to between 0 to 4 nucleotide differences, but differed from the other species
by 1.8 to 4.1 % which corresponded to between 33 and 74 nucleotide substitutions. The
majority of the sequence differences occurred in the variable regions of the SSU rRNA
gene, specifically V2, V4, and V7 (data not shown). These variable or loop regions are

not constrained by the structural properties of the gene as a whole and are therefore more prone to mutation.

The internal transcribed spacers (ITS) which separate the SSU rRNA gene from the 5.8S rRNA and LSU rRNA gene have also been used to distinguish between kinetoplastid isolates and strains (Cupolillo et al. 1995, Agbo et al. 2001, Berzunza-Cruz et al. 2002, Mendonca et al. 2002). These sequences are even more successful at distinguishing between closely related species than the SSU or LSU because of the increased divergence rates. We sequenced the ITS regions from several of the *Ichthyobodo* isolates. Unfortunately, ITS sequences from all the isolates could not be obtained. ITS sequences that were obtained, supported 3 of the 4 SSU-defined species. However, between species comparisons could not be made because the ITS-1 and ITS-2 regions were too divergent to identify homologous bases (Fig. 3.2A, 3.2B). This high divergence is further support for the fact that the different isolate groups represent true species. Furthermore, the ITS data indicated that there were several strains within each of these species groups. This was apparent by the inability to align the ITS sequences within the SSU-defined species groups but not between species groups (Fig 3.2A, 3.2B), which suggests that the isolates of species group 1 (*Morone* and *X. helleri*) are two different strains of the same species, the three members of species group 4 (*C. auratus*, *C. carpio*-NC, and *C. carpio*-AR) are a single strain of a different species, and that species group 2 (*O. mykiss*) is a different species from the members of both groups 1 and 4.

Contrary to expectations based on the limited cross-infection studies (Urawa & Kusakari 1990), our results do not provide evidence for host specificity. None of the SSU groups were defined by the host species. Additionally, three isolates from the same

48

host species fell into two different groups. Group 3, consisting of *C. carpio* (mirror carp), was separated from the two *C. carpio* (koi) isolates of group 4 by a SSU rRNA sequence difference of 1.8 % which is equivalent to 33 nucleotide differences. There were also two or more host genera within two of the four groups (Species groups 1 and 4) This suggests that it is possible for *Ichthyobodo* to move between different hosts.

Our results also do not provide evidence for ecological differences among hosts. This is demonstrated by the close similarity between *Ichthyobodo* SSU sequences obtained from *P. olivaceus*, a marine fish, and *C. carpio*, *C. auratus*, and *I. punctatus*, which are all freshwater fish. These isolates differed among each other, on average, by 0.1 % which is equivalent to 1 nucleotide difference (Table 3.1). This small difference in SSU rRNA sequence suggested that there is no ecological barrier (marine vs. fresh water) separating these *Ichthyobodo* isolates.

While there is no apparent ecological or host specificity among our nine *Ichthyobodo* isolates their molecular differences may represent the overlap of the geographic regions from which each host originated. For example, there is frequent importation of koi (*C. carpio*) to the U.S. from Japan for the pet fish market and there is the potential for transmission of pathogens among koi, channel catfish (*I. punctatus*), and goldfish (*C. auratus*) stocks in Arkansas, as farms are in close proximity to one another. Additionally, *C. carpio* (koi), *C. auratus*, and *I. punctatus* require similar environmental conditions and may be maintained together in aquaculture facilities, providing opportunity for cross-infection of *Ichthyobodo*. Finally, it has already been observed that *Ichthyobodo* can survive transfer from fresh water to a marine environment (Ellis & Wootten 1978, Urawa & Kusakari 1990). It has also been speculated that the occurrence

of *Ichthyobodo* on marine fish was a result of exposure to coastal freshwater fish (Bullock & Robertson 1982, Cone & Wiles 1984). *Paralichthys olivaceus* is native to Japan. As metamorphosing larvae, *P. olivaceous* may be found along the coast in less than 10 m of water (Koshiishi et al. 1985); therefore, it is possible *Ichthyobodo* might be transmitted between *C. carpio* (koi) and *P. olivaceus*. Unfortunately, we were unable to obtain a *C. carpio Ichthyobodo* isolate from Japan for comparison.

Geographic separation may also explain the 1.8 % difference between the *C. carpio* (mirror carp)(species group 3) isolate that was collected in Greece and the *C. carpio* (koi)(species group 4) isolates that were collected from Arkansas and North Carolina in the U.S.A. (Fig. 3.3). Species group 1, which contained the *Morone* and *X. helleri* isolates may also be related by geography (Figure 3.3). *Morone* (striped bass, white bass, and their hybrids) are commonly stocked in lakes, ponds, and rivers throughout the eastern United States (Hodson 1989). Tropical aquarium fish, such as *X. helleri*, have been released into the natural environment and have established themselves on the east coast of Florida (Courtenay et al. 1984). As a result, these fish may come into contact with each other, allowing movement of *Ichthyobodo* between them. Relocation of these fish for stocking or culturing purposes would then introduce this *Ichthyobodo* isolate to new locations.

Finally, *O. mykiss*, which is geographically separated from members of both groups 1 and 4, is a cold water fish that is found in mountainous areas (Figure 3.3). Their preference for colder water would isolate them and preclude them from overlapping with any of the other fish hosts represented in our study. However, where their ranges overlap with other fish, including other trout, the same *Ichthyobodo* isolate may occur.

For years it has been speculated that the genus *Ichthyobodo* consisted of more than one species (Diamant 1987, Bruno 1992, Urawa et al. 1998). These speculations were based on a number of factors previously mentioned, including its occurrence on both freshwater and marine fish, as well as invertebrates, and the specificity of the *O. keta* (chum salmon) and *P. olivaceus* (Japanese flounder) isolates. Despite this evidence, the existence of the two previously named *Ichthyobodo* species, *I. necator* and *I pyriformis*, has been questioned (Robertson 1985). This is because the only difference between these species is their size and *Ichthyobodo* trophonts can vary in size on the same fish, to the same degree as the size differences reported for both *Ichthybodo* species. Additionally, both species were described from the same host, trout (Davis 1943, Joyon & Lom 1969). Because there are no reliable distinguishing traits between these two species or any of the other described isolates, morphological traits cannot be used to determine species within the genus *Ichthyobodo*. Unfortunately, the existence of *I. pyriformis* cannot be denied or confirmed by our data

We can conclude from our SSU rRNA and ITS analysis of nine isolates that *Ichthyobodo* consists of at least four species that have a number of subspecies that can be differentiated by the geographical location from which each host originated. This has serious implications for both the national and international aquaculture industry. If *Ichthyobodo* can move between hosts there is potential to spread new species and strains that may be more virulent upon exposure to new hosts or resistant to management. Understanding more about species and strain differences may help determine the types of fish that can be stocked together, as well as, the risk involved with using water from local

waterways, which may provide a means to transfer *Ichthyobodo* between local and cultured fish species.

Further work should involve cross-infection studies using the isolates that have been sequenced, as well as sequencing additional isolates and genes to further define the *Ichthyobodo* complex. This would allow development of PCR-based assays that could be used to differentiate more virulent or treatment resistant species and strains prior to the importation or exportation of fish.

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53

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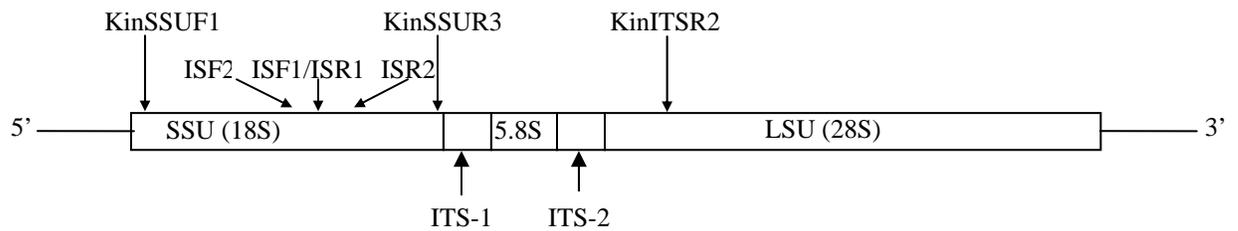
Figure 3.1. Schematic of the rRNA genes diagramming the approximate binding sites of each primer used to obtain the SSU and ITS sequences of the *Ichthyobodo* isolates. The primer sequences are as follows: KinSSUF1 (5' GGTTGATTCTGCCAGTAGTC 3'), KinSSUR3 (5' TTGTTACGACTTTTGCTTCC 3'), KinITSR2 (5' ACTTTCGCTCACGCTACTTG 3'), ISF1 (5' GGCATTCCCTGCTCCTTA 3'), ISFR1 (5' TAAGGAGCAGGGAATGCC 3'), ISF2 (5' TTACCAGTACAGGAAAACGC 3'), ISR2 (5' GTTTTTCCAGTTTACCCACC 3'). Figure is not drawn to scale.

Table 3.1. SSU rRNA gene sequence comparisons between *Ichthyobodo* isolates. Paired difference comparisons were determined using PAUP* (Swofford 1999). All values are expressed as percent difference (%). Values in parentheses indicate the number of nucleotide dfferences.

	Group 1	Group 2	Group 3	Group 4				
	X. helleri	*O. mykiss*	*C. carpio*[a]	*P. olivaceus*	*C. auratus*	*C. carpio*[b]	*C. carpio*[c]	*I. punctatus*
Morone	0.2 (3)	4.6 (86)	4.5 (83)	4.1 (74)	3.8 (70)	3.8 (70)	3.8 (70)	3.9 (71)
X. helleri		4.4 (82)	4.2 (77)	3.8 (70)	3.5 (65)	3.5 (65)	3.5 (65)	3.6 (66)
O. mykiss			3.4 (64)	2.5 (47)	2.4 (45)	2.4 (45)	2.4 (45)	2.4 (45)
C. carpio[a]				2 (36)	1.8 (33)	1.8 (33)	1.8 (33)	1.9 (34)
P. olivaceus					0.2 (4)	0.2 (4)	0.2 (4)	0.3 (4)
C. auratus						0 (0)	0 (0)	0.1 (1)
C. carpio[b]							0 (0)	0.1 (1)
C. carpio[c]								0.1 (1)

[a] mirror carp from Greece

[b] koi from Arkansas, U.S.A.

[c] koi from North Carolina, U.S.A.

Table 3.2. Mean mutational difference (%) between SSU rRNA sequences of putative species groups. Means were determined using paired difference from PAUP* (Swofford 1999). Values in parentheses indicate the mean number of nucleotide differences.

Species	2	3	4
1	4.5 (84)	4.4 (80)	3.7 (69)
2		3.4 (64)	2.4 (45)
3			1.9 (34)

```
         1        10        20        30        40        50        60        70        80        90       100       110       120       130
         |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
IM       TTGTCAAGA---TTTCATA-C-CTCTTGCAGCAA----------------AAACATCCCATAT---GCGCCC-----TC------T-TCTCT--ATTCATTCC----------------TGTGCG------T
IXh      TTGTCAAGA---WTTCATA-C-CTCTTGCAGCAA----------------AMACAATCTATAT---GCGCCC-----TCCTCTTY-TCTCT--CTACATTCC----------------TGTGCG------T
IOm      TT-TCGAGAGTTTTTCAAAACACTCTTTTGGTTATATTGTTTCTAGTGTGAAACATTG-ATATAATGCTCTCTATTTCTTATATATATAT--ATATATTAAATAGAGAAAAGAAAAATGTATGAAAAGAT
ICa      TT-TCGAGGGTTTT-CGAA-CACTCTTTT--TT----------------GAATC------TAT-----------TTTCCAATAAATATAT--ATATATT---TA--------------TGT--GAATA--T
ICcNC    TT-TCGAGGGTTTT-CGAA-CACTCTTTT--TT----------------GAATC------TAT-----------TTTCCAATAAATATAT--ATATATT---TA--------------TGT--GAATA--T
ICcAR    TT-TCGAGGGTTTT-CGAA-CACTCTTTT--TT----------------GAATC------TAT-----------TTTCCAATAAATATATGTATATATT---TA--------------TGT--GAATA--T
Consensus TT.TCgAGagtttTtCaaA.CaCTCTTtt.gtta...............gAaaCa....aTAT...gc.c.c..ttTCc.ata.aTaTaT..aTatATT...ta.............TGT..Gaa.a..T

         131      140       150       160       170       180       190       200       210       220       230       240       250       260
         |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
IM       GT-GT--------------------ATTTACTTACACATGTTCTT--C--------AGGAA--------AG-AGAGAG-AGAGAAGAGGGAAAAGTACCATATTTTGCA-ACGTGTAAAGAAT--AGAA
IXh      AT-GT---------------------A-----CTTACACATGTKCTT--C--------AGGAA--------AG-AGACAG-AGAG--GAGGGAAAAGTACCATATT--GCA-ACGTGTAAAGAAT--AGAA
IOm      GTTGTTTATAGTAGATTTCTTTTTTACTATTCTCTCTCACATTTTCTTTTCTCTCTTCTATAAACAATAT-AGTAGAGAGRAGAGAA----GATAA---------TAA-GGAGAAG---AAAGAGGGGAGAA
ICa      -TTG----------------------CGCTCTC----TTTCTTT-CTCTCTTATATGTATA-TATCAG-AGAAAGGAGAGAA-ATGGAAAAG-----ATGTAATGGAGA-G----AA-GAATTGAAAA
ICcNC    -TTG----------------------CGCTCTC----TTTCTTT-CTCTCTTATATGTATA-TATCAG-AGAAAGGAGAGAA-ATGGAAAAG-----ATGTAATGGAGA-G----AA-GAATTGAAAA
ICcAR    -TTG----------------------CGCTCTC----TTTCTTT-CTCTCTTATATGTATA-TATCAG-AGAAAGGAGAGAA-ATGGAAAAG-----ATGTAATGGAGA-G----AA-GAATTGAAAA
Consensus .TtGt...................a..c.CTctCacattTtCTTt.Ctctctt.tAtgaA.a.tat.AG.AGA.AG.AGAGaa.a.gGAaAAg....at.TaatGgAgA.G...AAaGAat.gAgAA

         261      270       280       290       300       310       320       330       340       350       360    366
         |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+-------+-----|
IM       ATATTCTTTCCTCAAGTTTTTTA-G--T----TACG-TTGTTCTG-TTG---TGTGTGTGAACATA-AC--CACA------ACGACAACGGT---AACTCTTGAACA
IXh      ATATTCTTTCCTCAAGTTTTTTA-G--T----TACG-TTGTTCTG-TTG---TGTGTGTGAWCATATACA-CRCA------ACGACAACGGT---AACTCTTGAACA
IOm      AGAGT------CT-A-CTTTTT---GCATC-ATCC-A--ATTTTAATTGCATTGTGTATTAA-ATATATATCTTATTATTTATTACAATG-TGACAATTGATACACA
ICa      AG-GTAATTACT-AACTTTTTCAAGAATCTATTCTATTATTTTAATTGCATTGGGGAAGAA-AG------TC------------ACAATG-T--CAATTTCTAAACA
ICcNC    AG-GTAATTACT-AACTTTTTCAAGAATCTATTCTATTATTTTAATTGCATTGGGGAAGAA-AG------TC------------ACAATG-T--CAATTTCTAAACA
ICcAR    AG-GTAATTACT-AACTTTTTCAAGAATCTATTCTATTATTTTAATTGCATTGGGGAAGAA-AG------TC------------ACAATG-T--CAATTTCTAAACA
Consensus AgagT..tt.CT.AacTTTTT.a.G.aTc.aT.C.attaTTtTaaTTGcatTGtGtatgAa.Ata.a..tC..a......a...ACAAtG.T..cAAtT..TaaACA
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Figure 3.2A. ITS-1 from six *Ichthyobodo* isolates representing three of the four SSU-defined groups. Group 1 consists of IM (*Ichthyobodo Morone*) and IXh (*Ichthyobodo X. helleri*), group 2 consists of IOm (*Ichthyobodo O. mykiss*), and group 4 consists of ICa (*Ichthyobodo C. auratus*), ICcNC (*Ichthyobodo C. carpio* from North Carolina, U.S.A.), ICcAR (*Ichthyobodo C. carpio* from Arkansas, U.S.A.). Sequences were aligned using Multalin (Corpet 1983).

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        1        10        20        30        40        50        60        70        80        90       100       110       120       130
        |---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
IM        ATATCATCACGAAAAGAAGACATGATGTGTGTAGAGTGTGTATGGTAGAAGGTTTACTTTCCGACCACACATATTATATATACATTGTGSATTCTTT-TGTGGGGTTGGGGACGT----A-ACAGGCCCT
IXh       ATATCATCACGAAAAGAAGACATGATGTGTGTAGAGTGTGTATAG--GAAGGTTTACTTTCCGACCACA--TATTATATATACATTGTGGATTCTTT-TGTGGGGTTGGGGACGT----A-ACAGGCCCT
IOm       ATA--A-----AAAAGAAG--------------GAATGT-TAT-----AA------------------------ATTACATATA---------TCTTTCTGTG---TTGGGGACATTTTAA-AC-GGCCCT
ICa       ATA--------AAAA-AAGAAA----------AGCATTTCTGT------------------------------TT---T------------TTCTT---GTG---TTGGAGACG----AAGAGAGGTTTT
ICcNC     ATA--------AAAA-AAGAAA----------AGCATTTCTGT------------------------------TT---T------------TTCTT---GTG---TTGGAGACG----AAGAGAGGTTTT
ICcAR     ATA--------AAAA-AAGAAA----------AGCATTTCTGT------------------------------TT---T------------TTCTT---GTG---TTGGAGACG----AAGAGAGGTTTT
Consensus ATA..a.....AAAAgAAGa.a.........aGaaTgT.TaT.....aa....................aTTa.aTata.........tTCTTt.tGTG...TTGGgGACgt...aA.AcaGGcccT

        131      140       150       160       170       180       190       200       210       220       230       240       250       260
        |---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
IM        TAAATCTCTACG-GA-GAGAG-GAGAGATGTA-ATCATCA------TTCTCTA--TGAA---------TGAGAGGA--AACTCTTACTCATGGATGGGGTGTGTTCTGTCCTTCTCTCCGGTATA----AT
IXh       TAAATCT--ACG-GA-GAGAG-GAGA--TGTG-ATCAT---------TCTCTA--TGAA---------TGGGAGGA--AACTCTTATTCATGGATGGGGT----TCTCTCTTTCTCTCCGGTATA----AT
IOm       TAAATCTATATGCGATGAGAATGTGAGTTTTTATATTATCACTACTTTTGTCTTCTTGAAAAAGAAGATGAGGGGACGAGATATAAAATAT--AT---------TCCACACATTCTCTC-GG-ATA----A-
ICa       TCCCCCT------CGA--AGGCTCTTAAATGTCTATAATC--TTCTC-TCTCTTTTTGAGTG------TGTGTGGT-GTG-TAAAAAACAAG-A-----------CACGCG--CCAGAGGAGAGAGAGGAG
ICcNC     TCCCCCT------CGA--AGGCTCTTAAATGTCTATAATC--TTCTC-TCTCTTTTTGAGTG------TGTGTGGT-GTG-TAAAAAACAAG-A-----------CACGCG--CCAGAGGAGAGAGAGGAG
ICcAR     TCCCCCT------CGA--AGGCTCTTAAATGTCTATAATC--TTCTC-TCTCTTTTTGAGTG------TGTGTGGT-GTG-TAAAAAACAAG-A-----------CASRCR--CSMGMSRRRRGAGAGRRR
Consensus TaaatCT..a.gcGA.gAGa.tgtgA.aTgT.tAT.ATc..t.ct..TcTCTt.tTGAa.........TG.G.GGa.gag.TataAaacAtg.At..........Cac.C.ttCtctc.gg.atA....a.

        261      270       280       290
        |---------+---------+---------|
IM        GATCAACC-ATGACATGTGTATTTGATATT
IXh       GATCAACC-ATGACATGTGTATTTGATATT
IOm       -ATC-------TGA-ATTTAAACTTCTCATT
ICa       AAACAAAAGAT-AAATCTGAACTT--TGTT
ICcNC     AAACAAAAGAT-AAATCTGAACTT--TGTT
ICcAR     RRASAAAMRAW-ARATMWRWMYKW--WSTT
Consensus .atcaa...atgA.AT.tgaactt..taTT
```
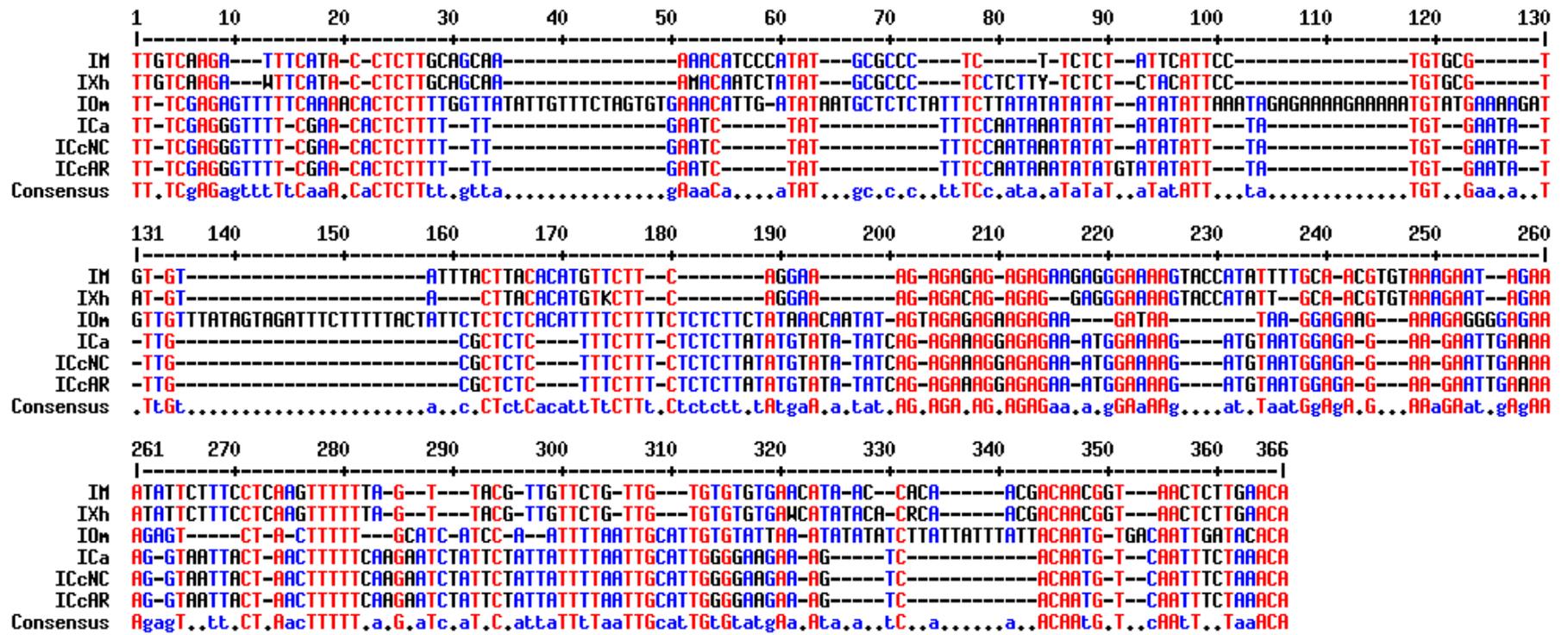
Figure 3.2B. ITS-2 from six *Ichthyobodo* isolates representing three of the four SSU-defined groups. Group 1 consisted of IM (*Ichthyobodo Morone*) and IXh (*Ichthyobodo X. helleri*), group 2 consisted of IOm (*Ichthyobodo O. mykiss*), and group 4 consisted of ICa (*Ichthyobodo C. auratus*), ICcNC (*Ichthyobodo C. carpio* from North Carolina, U.S.A.), ICcAR (*Ichthyobodo C. carpio* from Arkansas, U.S.A.). Sequences were aligned using Multalin (Corpet 1983).

Figure 3.3. World map depicting the geographical relationships of the *Ichthyobodo* isolates as determined by the SSU rRNA nucleotide differences. **Group 1** consisted of *Ichthyobodo Morone* and *Ichthyobodo X. helleri*, **group 2** consisted of *Ichthyobodo O. mykiss*, **group 3** consisted of Ichthyobodo C. carpio from Greece, and **group 4** consisted of *Ichthyobodo C. auratus*, *Ichthyobodo C. carpio* from North Carolina, U.S.A., *Ichthyobodo C. carpio* from Arkansas, U.S.A.

Chapter 4

Molecular Taxonomy of the Suborder Bodonina (Order Kinetoplastida), Including the Important Fish Parasite, *Ichthyobodo necator*

The GenBank accession numbers for all species used in the analysis are as follows; *Bodo caudatus* (X53910), *Bodo caudatus* ATCC30905 (AY028450), *Bodo designis* (AF209856)*, Bodo edax* (AY028451), *Bodo saliens* (AF174379), *Bodo saltans* strain Konstanz (K) (AF208889), *Bodo saltans* strain Petersburg (P) (AF208887), *Bodo saltans* (AY028452), *Bodo sorokini* (AF20888)*, Bodo* sp. (cf. *uncinatus*) (AF208884), *Bodo* sp. ATCC50149 (AY028449)*, Crithidia oncopelti* (L29264), *Cruzella marina* (AF208878), *Cryptobia bullocki* (AF080224), *Cryptobia catastomi* (AF080226), *Cryptobia helicis* (AF208880)*, Cryptobia salmositica* (AF080225), *Dimastigella mimosa* (AF208882)*, Dimastigella trypaniformis* Glasgow isolate (X76495) *Dimastigella trypaniformis* Ulm isolate (X76494), *Dimastigella trypaniformis* ATCC50263 (AY028447), *Diplonema* sp. (AF119812), *Diplonema papillatum* (AF119811), *Euglena gracilis* (M12677), *Ichthyobodo necator* (AY028448), *Khawkinea quartana* (U84732), kinetoplastid flagellate LFS2 (AF174380), *Leishmania tarentolae* (M84225), *Lepocinclis ovata* (AF061338), *Parabodo nitrophilus* (AF208886), *Rhynchobodo* (U67183)*, Rhynchomonas nasuti* BSZ1 isolate (AF174378), *Rhynchomonas nasuti* CBR1 isolate (AF174377), *Trypanosoma avium* (U39578), *Trypanosoma brucei* (M12676), *Trypanosoma cruzi* (M31432), *Trypanosoma simiae* (U22320), *Trypanoplasma borreli* (L14840) and unidentified eukaryote (UEU130868).

ABSTRACT: *Ichthyobodo necator* is an important fish ectoparasite with a broad host and ecological range. A novel method, involving the use of an anesthetic, allowed the collection of large numbers of parasites from the skin and gills of hybrid striped bass (*Morone saxatilis* male x *M. chrysops* female). Genomic DNA from these samples was used to amplify and clone the 18S rRNA gene. The 18S rRNA gene was similarly cloned from *Bodo caudatus, Bodo edax*, *Bodo saltans*, an unidentified *Bodo* species, and *Dimastigella trypaniformis*. The resulting sequences were aligned with other representative kinetoplastid species using pileup and similarities in secondary structure. Phylogenetic relationships within the suborder Bodonina and representatives of the suborder Trypanosomatina were determined using maximum-likelihood statistics. The phylogenetic analyses strongly supported the order Kinetoplastida as a monophyletic assemblage consisting of at least two major lineages. One lineage consisted exclusively of *I. necator,* indicating that it may represent a new suborder. The second lineage consisted of all other kinetoplastid species.   This second lineage appeared to contain at least 8 bodonine sublineages, none of which correlated with currently recognized families. For three sublineages, there was a close correspondence between the 18S phylogeny and the classical taxonomy of *Dimastigella*, *Rhynchobodo*, and *Rhynchomonas*. In contrast, *Bodo* and *Cryptobia* were polyphyletic, containing species in two or more sublineages that may represent separate genera.

KEY WORDS: *Bodo*, *Cryptobia*, morphology, phylogeny, small-subunit ribosomal RNA, *Trypanosoma,* Trypanosomatina

INTRODUCTION

Protozoan parasites cause a number of diseases that can seriously impact aquaculture and wild fish populations (Valtonen & Koskivaara 1994, Hoffmann 1999). One of the most important of these is the ectoparasite commonly referred to as costia (*Ichthyobodo necator*), which infects the skin and gills of fish (Robertson 1985). *Ichthyobodo necator* is typically considered a pathogen of freshwater fish (Vickerman 1976). However, it has been observed on salmon smolts, captive marine fish, feral marine fish, and even amphibians and marine invertebrates (Vickerman 1976, Becker 1977, Ellis & Wootten 1978, Cone & Wiles 1984, Morrison & Cone 1986, Urawa & Kusakari 1990, Forsythe et al. 1991). Epidemics among farmed fish have been reported worldwide in commercially important cold water, temperate, warm water, and tropical species (Robertson 1985, Urawa et al. 1998). Infections cause high mortality in a few days unless treated. One factor that makes these infections difficult to treat is that typically the fish show few clinical signs of distress or behavioral abnormalities before significant mortalities occur (Robertson 1979, Post 1987). As a result, *I. necator* is considered one of the most dangerous parasites of cultured fish (Post 1987, Buchmann et al. 1995, Rintamaki-Kinnunen & Valtonen 1997, Hoffmann 1999).

Ichthyobodo necator is a member of the order Kinetoplastida, a unique and diverse group of protozoans recognized by a large concentrated area of extranuclear DNA within the single mitochondrion, termed the kinetoplast (Vickerman 1976). Members of the order Kinetoplastida are divided into the exclusively parasitic suborder (s.o.) Trypanosomatina, which includes the *Trypanosoma* and *Leishmania* species, and the s.o. Bodonina, which

67

includes a variety of parasitic, ectocommensal, and free-living species (Vickerman 1994).

Trypanosomatines have a single flagellum and a small, compact kinetoplast, while the

bodonines have two flagella and a larger, more diffuse kinetoplast. Many members of the

Trypanosomatina have been well-studied due to their impact on human and domestic animal

populations. In contrast, the Bodonina have been studied little. Members of the Bodonina

are commonly found in soil, and in freshwater and marine environments (Foissner 1991,

Zhukov 1991, Atkins et al. 2000). Most species are free-living heterotrophs that selectively

graze on bacteria and are considered important members of the microbial food web

(Eccleston-Parry & Leadbeater 1995, Lavrentyev et al. 1997, van Hannen et al. 1999,

Zhukova & Kharlamenko 1999). However, a number of species within the genera

Ichthyobodo, *Cryptobia*, and *Trypanoplasma* have adapted to a parasitic lifestyle (Joyon &

Lom 1969, Woo 1987).

The family relationships within the s.o. Bodonina are controversial, with three

different morphology-based classification schemes currently in use (Table 4.1). The most

commonly employed morphological characteristics used to distinguish families, genera, and

species within the s.o. Bodonina are obtained from observations of stained cells via light

microscopy and include cell size, shape, flagellar attachment, and extent of rostral

development, as well as movement and feeding behavior. The presence or absence of an

encysted stage in the life cycle is also considered taxonomically important (Vickerman

1990). Closely related species are also frequently distinguished by ultrastructural traits,

including the position of the cytostomal opening relative to the flagellar pocket, type of

microtubular array associated with the cytopharynx, the presence or absence of mastigonemes, and the presence or absence of endosymbionts (Vickerman 1991). Unfortunately, these ultrastructural characteristics have not been determined for most Bodonina species, nor do they always differ significantly between species, limiting taxonomic utility. This lack of defining morphological variation has led to confusion, and in some cases controversy, over genus and species designations (Woo 1987, Larsen & Patterson 1990). Therefore, we decided to apply a molecular approach to evaluate the species-level assignments within the s.o Bodonina and to determine the taxonomic position of the important fish parasite, *I. necator*, which has traditionally been placed within the family Bodonidae (Vickerman 1976).

In this study, we sequenced the 18S rRNA gene for *I. necator, Bodo caudatus* (American Type Culture Collection [ATCC] 30905), *B. edax* (ATCC30903), *Bodo* sp. (ATCC50149), *Bodo saltans*, and *Dimastigella trypaniformis* (ATCC50263). These sequences, along with previously published sequences from members of both the suborders Bodonina and Trypanosomatina, were used in phylogenetic analyses. We then compared the resulting rooted tree topology to current classical taxonomic schemes.

MATERIALS AND METHODS

Propagation of *Ichthyobodo necator*. Hybrid striped bass (*Morone saxatilis* male x *M. chrysops* female) farmed in North Carolina aquaculture facilities commonly harbor subclinical infections of *I. necator*, which can reach epidemic proportions during spring and

69

late fall. Epidemics routinely produce heavy parasite loads on the skin and gills (>1000

parasites/mm^2). Diagnosis of hybrid striped bass fingerlings with *I. necator* was confirmed

by light microscopy and both scanning and transmission electron microscopy. A subclinical

infection was maintained on fingerlings by keeping fish at a low temperature (14 ^{o}C) under

crowded conditions (1 fish, 40 mm length, per 2 L water), and feeding them a restricted diet

to maintain a small body size. Outbreaks occurred spontaneously in the laboratory during

spring and fall, and could be induced at other times of the year by either increasing or

decreasing the temperature several degrees for a 24-h period before returning the

temperature to 14 ^{o}C. Fish remained susceptible to *I. necator* for approximately two years,

after which outbreaks would no longer occur.

Propagation of other kinetoplastids. *Bodo caudatus*, *B. edax*, *Bodo* sp., and

Dimastigella trypaniformis were obtained from the American Type Culture Collection

(ATCC). *Bodo saltans* was isolated in 1987 and provided by Dr. Keith Vickerman

(University of Glasgow). All *Bodo* species (except for *B. edax,* which was amplified from a

frozen stock) were cultured following ATCC protocols (ATCC 1991).

Isolation of genomic DNA. To collect *I. necator*, infected 10 month-old, 40 mm-

long hybrid striped bass were bathed in a 1 mg/ml tricaine methanesulfonate (MS-222,

Argent Chemical Lab, Redmond, WA) solution for 10 min. This caused the immediate

detachment of the parasites from the skin and gills. The solution containing the detached

parasites was then filtered through Whatman 541 paper to remove larger particulate matter. Cells in the filtrate were pelleted by centrifugation at 400 x g for 10 min at 14 ^{o}C. *Bodo caudatus*, *B. saltans*, *Bodo* sp. and *D. trypaniformis* were inoculated and grown for 3 days at 22 ^{o}C. Population densities on day three reached 10^5-10^8 cells/ml, depending upon the species (the density of organisms in most cultures started declining 4 days after inoculation). Cell suspensions were filtered to remove particulates and pelleted at 22 ^{o}C

Pellets of all protozoa (except *B. edax*) were washed twice with 2 ml of 0.07 M PBS, pH 7.4. Genomic DNA was then isolated following Saunders et al. (1997). *Bodo edax* was pelleted from frozen cultures, resuspended in Tris-EDTA (pH 8.0) and boiled for 15 min. The resulting lysate (2 ml) was then used directly in PCR reactions.

PCR amplification. Amplification and sequencing primers were synthesized to correspond with conserved regions within the 18S rRNA gene of representative kinetoplastid species (Table 4.2). Approximately 10 ng of genomic DNA was combined with 1X PCR buffer (PromegaJ), 100 ng of KinSSUF1, 100 ng KinSSUR1, 0.10 mM dNTPs, and 5 units of Taq Polymerase (PromegaJ). The PCR reactions for *I. necator* were amplified using 1.0 mM $MgCl_2$ (PromegaJ) and an annealing temperature of 50 ^{o}C. All other templates were amplified using 1.5 mM $MgCl_2$ and annealing temperatures of 55-60 ^{o}C, depending on the species. Additional sequencing of the 3'-end of the 18S gene from *I. necator* revealed a 2-base difference from the KinSSUR1 reverse primer (coding strand, 5'-GA*C*TACGTCCC*C*GCCATTTG-3'), which accounts for the lower annealing temperature

71

required to generate a product using this primer. The PCR cycling conditions for all amplifications were 1 cycle at 94 °C for 3 min, 35 cycles at 94 °C for 1 min, 50-60 °C for 1 min, and 72 °C for 1 min 30 sec, followed by 72 °C for 5 min.

Cloning and sequencing. Amplification products were cloned directly using the TOPO TA protocol (Invitrogen™). Plasmid DNA from positive clones was isolated using the QIAprep Spin Mini Prep Kit (Qiagen™). Three clones containing the correct insert were diluted and sequenced in both directions on an ABI 373A automated sequencer using conserved primers and the Taq Dye Deoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems-ABI)(Table 4.2). Sequencing primers were located approximately 600-650 bp apart.

To confirm that the sequences obtained from samples containing *I. necator* were truly from this specific organism, a number of primer combinations were used to amplify the 18S rRNA gene from infections on different fish and from different populations and generations (data not shown). All sequenced clones, when aligned relative to each other, indicated nearly identical overlapping regions. Additionally, amplified products were evaluated by RFLP analysis using *Hae*III and *Sau3*A I endonucleases (Promega™)(data not shown). Contaminants consisting of other bodonids and protozoa were easily distinguished from *I. necator* by the difference in size of the PCR products and the banding patterns generated by the digests.

Alignment of the SSU rRNA genes. Consensus sequences, based on the three clones, were determined for each species using SeqMan II (1998), which utilizes the Martinez and Needleman-Wunsch alignment algorithm (Needleman & Wunsch 1970, Martinez 1983). Consensus sequences were aligned relative to other kinetoplastid species from the GenBank™ database using pileup (GCG 1999) followed by manual alignment based on secondary structure. Secondary structure alignments for most of the s.o. Trypanosomatina members and several members of the s.o. Bodonina were available from the rRNA secondary structure database (Van De Peer et al. 1994). These species were used as templates for determining secondary structure of the species not yet mapped. Areas that could not be resolved using this method were removed from further analysis.

Phylogenetic analysis of the SSU rRNA genes. Phylogenetic analyses were similar to those described by Litaker et al. (1999) and Cunningham et al. (1998). The best maximum likelihood model for the sequence data set consisting of all 39 species was determined using MODELTEST, a program which uses the likelihood-ratio test to evaluate 56 evolutionary models of increasing complexity (Posada & Crandall 1998). The maximum-likelihood model that best fit the data was a general-time-reversible model with rate heterogeneity. The specific conditions of the model selected were as follows: nucleotide frequencies were estimated; number of substitution types (i.e., transition and transversion states) = 6; proportion of sites estimated to be invariable = 0.1962; rates for variable sites were assumed to follow a discrete gamma distribution with shape parameter = 0.6656;

73

number of rate categories (i.e., number of segments of the18S gene that have a different rate of divergence) = 4; average rate for each category was represented by the mean; molecular clock was not enforced.

MODELTEST estimates the maximum-likelihood parameters based on a starting tree constructed using the neighbor-joining (NJ) method (Saitou & Nei 1987). Therefore, we used a neighbor-joining tree with 1,000 bootstrap replicates as the starting tree for a heuristic search. Following an approach outlined by Cunningham et al. (1998) and Litaker et al. (1999), the neighbor-joining tree was used as the starting point for a nearest neighbor interchange (NNI) heuristic search. The final NNI tree was then used as a basis for a tree bisection-reconnection (TBR) analysis. Support for each of the phylogenetic groups was estimated by 25 bootstrap replicates under the maximum-likelihood model. Groups retained in 50% or more of the trees were indicated on the final maximum-likelihood tree (Fig. 4.1).

Morphological and 18S rRNA sequence comparisons among the Bodonina. The phylogenetic analysis indicated that all the members of the s.o. Bodonina, with the exception of *I. necator*, were distributed among one of eight sublineages. Three of these sublineages corresponded with well-defined genera. Hence, the sublineages were evaluated as if they represented putative genera. The amount of genetic variation (as nucleotide differences), both within and between each sublineage, was determined. For the within-sublineage (genus) estimates, the starting alignments employed in the phylogenetic analyses were analyzed using the paired difference algorithm in PAUP* (Swofford 1999) the mean percent

74

nucleotide difference between species in each sublineage, plus or minus one standard deviation, was then calculated (Table 4.3A). An estimate of the genetic variation between sublineages (putative genera) was obtained by first determining a consensus sequence for each sublineage using SeqMan II (1998). The resulting consensus sequences for each sublineage were then aligned using the secondary structure of the 18S rRNA gene as described above. Areas that could not be resolved were excluded from the analysis. The percent nucleotide differences between sublineage were then estimated using PAUP* as described above (Table 4.3B).

To further illustrate the problems in classifying species within the s.o. Bodonina on the basis of morphology alone, a summary of the most commonly used characteristics was constructed from available published literature (Table 4.4). All the species used in the molecular analyses were listed in the table as belonging to one of three families, the Bodonidae, Cryptobidae, or Trypanosomatidae, following Vickerman's (1976) amended classification scheme. Additionally, all the species in Table 4 are listed in the same order as they appeared in both trees so that both the molecular and classical taxonomic information could be more easily compared (Fig. 4.1, 4.2).

RESULTS

Our results strongly supported the order Kinetoplastida as a monophyletic group which consisted of two major lineages (Fig. 4.1, 4.2). One lineage (lineage A) consisted exclusively of the fish parasite, *I. necator*, while the other (lineage B), consisted of all the

other kinetoplastid species. Initial analyses using only the *Diplonema* species as an outgroup, did not place *I. necator* within the order Kinetoplastida (data not shown). However, the use of additional euglenoid species provided 100 % bootstrap support for *I. necator* being a kinetoplastid. Initial analyses also suggested that *I. necator* was more closely related to species associated with *Cruzella marina* and *Bodo designis*; however, this topology was not supported by the maximum-likelihood analysis (Fig. 4.1, 4.2). Additionally, the low bootstrap support of the deeper branches within lineage B indicated that these evolutionary relationships cannot be clearly resolved at this time. However, terminal branches within lineage B were better resolved, indicating possibly nine distinct sublineages of closely related organisms that might represent different genera (Fig. 4.2). Of the nine sublineages, eight belonged within the suborder Bodonina and one consisted of representatives from the suborder Trypanosomatina.

Sublineage 1 consisted exclusively of *B. sorokini*, while sublineage 2 contained species of *Cryptobia* and *Trypanoplasma*. The separation of these two sublineages was supported by low bootstrap values, suggesting that they may belong to a single sublineage: A single sublineage was also supported by initial analyses. However, initial analyses, using only *Diplonema* species as an outgroup, placed *B. sorokini* within sublineage 3 (data not shown). Sublineage 3 contained one species each from the genera *Bodo*, *Parabodo*, and *Cryptobia*. However, sublineage 4 contained only species of the genus *Bodo*: The Trypanosomatina appeared to be most closely related to sublineage 4. Sublineage 5 consisted exclusively of *Rhynchobodo,* which may represent a distinct group of organisms.

However, sublineage 6 contained the most heterogeneous species, including *C. marina*, two unidentified kinetoplastids, and two species currently placed in the genus *Bodo*. These two *Bodo* species were clearly divergent from the *Bodo* species in sublineages 3 and 4 (Fig. 4.2). Sublineages 7 and 8 were composed of the genera *Dimastigella* and *Rhynchomonas*, respectively. None of the sublineages corresponded to the traditional separation of the suborder Bodonina into the families Bodonidae and Cryptobidae (Fig. 4.1).

Sublineages 5, 7, and 8 corresponded with the accepted genera *Rhynchobodo*, *Dimastigella*, and *Rhynchomonas*. The mean percentage nucleotide differences between the *Dimastigella* and *Rhynchomonas* species were 1.7 % and 1.9 %, respectively (Table 4.3A). The mean nucleotide differences within sublineages 2, 3, 4, and 6 were variable. Nucleotide differences in sublineages 2 and 4 ranged from 1.3 - 5.1 %, whereas those in sublineages 3 and 6 varied from 11.3 - 13.8 %. The nucleotide differences among sublineages 1 - 8 ranged from 5.2 - 11.7 % (Table 4.3B, Fig. 4.2).

The general concordance between sublineages 2, 4, 7, and 8, and the data presented in Table 3, suggested that the nucleotide differences in the 18S rRNA genes among members of the same genus is on the order of 1 - 5 % (Table 4.3A). The >10 % difference observed between species within subgroups 3 and 6 strongly suggested that they contained species from more than one genus, which is consistent with their having representatives from more than one currently recognized genus. The nucleotide differences between sublineages ranged from 5 - 12 % (Table 4.3B). Sublineages 2, 3, and 4 all had values around 5 % with respect to each other. These values were close to the within-sublineage values, suggesting a

closer genetic relationship to each other than to the other sublineages (Table 4.3A).

The morphological characteristics traditionally used for classifying these organisms was not in absolute concordance with the 18S rRNA phylogeny (Table 4.4, Fig. 4.1, 4.2). Within the classically defined Bodonina, *Dimastigella*, *Ichthyobodo*, *Rhynchobodo*, and *Rhynchomonas* could be distinguished from other genera by a combination of behavior, rostral development, kinetoplast structure, and flagellar attachment to the body. The *Cryptobia* species could also be distinguished from other genera based on these traits. However, *C. helicis,* an endoparasite of gastropods, from subgroup 3, was clearly a genetically separate organism from the hemoparasitic species of subgroup 2, yet was morphologically indistinguishable from these species. The *Bodo* species are the most difficult to morphologically differentiate from one another and are also the least well-described group. However, their relationships based on the 18S rRNA gene clearly demonstrated that they are not a single group.

DISCUSSION

Within the order Kinetoplastida, taxonomic assignment to either the s.o. Bodonina or s.o. Trypanosomatina has been based on the presence of either one or two flagella. Further classification to the family level, particularly for members of the s.o. Bodonina, has been primarily based on flagellar attachment to the body (Vickerman 1976). Given the relatively few defining characteristics available, assignment to the genus and species level has traditionally been based upon a small number of light microscopic, ultrastructural, and

behavioral traits. However, almost none of these traits has been uniformly examined for all currently classified bodonine species. The inconsistent use of various traits and the overall lack of morphological variation between species, have led to confusion in the classification of species within the s.o. Bodonina. For example, the presence of mastigonemes on the flagella of *B. saltans* (Brooker 1965) has been used to help distinguish it from other *Bodo* species; however, many species have not been evaluated for this trait (Kozloff 1948, Zhukov 1975, Larsen & Patterson 1990). Behavioral characteristics, such as movement and feeding behavior, are also commonly used to classify organisms (Eyden 1977, Karpov & Zhukov 1983). Unfortunately, behavioral observations can be subjective and evaluated differently depending upon the experience of the observer. Furthermore, the environments from which organisms were collected, the culture conditions under which they were grown, and the length of time in culture can alter their behavior, ultrastructure, and morphology (Maclean & R. 1973, Alleman et al. 1990, Fernandes et al. 1993, Faria-e-Silva et al. 2000).

Given the limitations inherent in using traditional morphological characteristics to classify members of the s.o. Bodonina, we employed a molecular approach to determining taxonomic relationships within this group. As a result, our analysis suggests that morphological characteristics are inadequate for valid classification of these organisms.

Comparison of the molecular and classical phylogeny at the suborder level. The phylogenetic results from our study separate the order Kinetoplastida into two distinct lineages. These lineages, however, do not correspond with the classically accepted

taxonomic organization that separates the Kinetoplastida into the suborders Bodonina and Trypanosomatina (Table 4.1, 4.4). The first lineage consists only of *I. necator* (lineage A) and the second lineage consists of the remaining members of the s.o. Bodonina and all the members of the s.o. Trypanosomatina (lineage B). These findings indicate that *I. necator* may belong to a new suborder and that all remaining bodonids and trypanosomatids belong to one or more suborders. The deep branches within the bodonid/trypansomatid group (B) all have low bootstrap support, making it difficult to assess suborder assignments within this lineage (Fig. 4.1).

Comparison of the molecular and classical phylogeny at the family level. Our analyses also did not support the separation of the Bodonina into two families, the Bodonidae and Cryptobidae, nor the family Bodonidae into the subfamilies Bodoninae and Cryptobiinae. All of these taxonomic schemes are based on flagellar attachment (Table 4.1). Thus, as was first proposed by Vickerman (1978) flagellar attachment to the plasma membrane appears to have no phylogenetic significance. This is also true for several genera within the sister suborder Trypanosomatina, where flagellar attachment may or may not be present, depending upon the developmental stage (Vickerman 1976).

While we did not find evidence for traditional family distinctions within the s.o. Bodonina, there are clearly several sublineages within the bodonid/trypanosomatid lineage (B). The relationships between these sublineages may represent family-level associations. However, this cannot be confirmed with our molecular analysis of the 18S rRNA gene alone.

Resolution of the sublineages into families will require 1) sequencing of the 18S rRNA gene from additional species, 2) sequencing of other genes that vary in a taxonomically informative way at the family level, followed by phylogenetic analyses to determine if the results are similar to that obtained using the 18S rRNA gene, and 3) systematic examination of ultrastructural differences between all the known species to determine if any consistent family-level traits exist.

Comparison of the molecular and classical phylogeny at the genus level. By comparing the congruence between well-established genera defined by morphology and the corresponding 18S rRNA data, we evaluated when species might belong to different genera. Each bodonine sublineage within the bodonid/trypanosomatid lineage (B) may represent one or more separate genera based on the criterion of nucleotide difference (Table 4.3B). The 18S rRNA data (i.e., nucleotide differences) closely agreed with the classical taxonomy for *Rhynchobodo, Dimastigella*, and *Rhynchomonas* (sublineages 5, 7 - 8, respectively)(Fig. 4.2). Species in these genera are among the easiest to identify because of their kinetoplast structure and well-developed rostra (Table 4.4).

The *Cryptobia*/*Trypanoplasma* sublineage (2) was also fairly homogeneous, as was first reported by Wright et al. (1999). We support their view that this sublineage may represent a distinct genus. All four species within this sublineage are united by being hemoparasites of fish. However, they share morphological characteristics with the genetically distinct *C. helicis*, a monogenetic gastropod parasite, in sublineage 3. Although

the morphological similarity of *C. helicis* to sublineage 2 suggests convergent evolution, this cannot be evaluated thoroughly using the available information.

One group of *Bodo* species also formed a well-defined genus-level group (sublineage 4)(Table 4.3A, 4.3B). The remaining *Bodo* species, however, were distributed among sublineages 1, 3, and 6. This supports previous studies that concluded *Bodo* to be polyphyletic (Wright et al. 1999, Dolezel et al. 2000). Recent evidence indicates that many *Bodo* species are adapted to consuming specific types of bacteria (Simek et al. 1997, Cochran-Stafira & von Ende 1998, Zhukova & Kharlamenko 1999). Hence, over time, there may have been evolutionary divergence of a precursor of these "*Bodo*" species as they became specialized to feed on different bacterial prey. Until more data are available, the genus *Bodo* should be viewed as representing a functionally defined group of morphologically similar species.

Sublineage 3 (consisting of *Cryptobia, Parabodo*, and *Bodo* species) and sublineage 6 (consisting of *Bodo*, *Cruzella,* and 2 unidentified species) are the most heterogeneous sublineages. Both sublineages appear to consist of more than one genus. Sublineage 3 has a 11.3 % mean nucleotide difference between species and likely contains as many as 4 distinct genera. The differences in the 18S rRNA gene sequences between members of sublineage 6 are larger than any other sublineage, ranging from 12.5 % to 15.1 % (Table 4.3A). Each member of this sublineage may also represent a distinct genus, or even additional sublineages that cannot be resolved without new sequence data.

Bodo sorokini represents the most ancestral species within lineage B. This finding

conflicts that of Dolezel et al. (2000) who found the most ancentral kinetoplastid species to be *C. marina* and *B. designis*, which fall within sublineage 6 of our study. Again, because of the low bootstrap support for the deep branches, the assignment of any sublineage as being ancestral to the others cannot be made with certainty (Fig. 4.1).

Comparison of the molecular and classical phylogeny at the species level. The sublineages in this study that are best supported by classsical taxonomy are the *Cryptobia* species assemblage in sublineage 2, the *Dimastigella* species in sublineage 7, and the *Rhynchomonas* species in sublineage 8. The average 18S rRNA sequence divergence between these species is 1 - 2 % (Table 4.3A). The smallest difference (1.3 %) is between the *Cryptobia*/*Trypanosplasma* species (sublineage 2). At present, there is no controversy as to whether or not the organisms within lineage 2 represent distinct species. Therefore, an average nucleotide difference of greater than 1.3 % was assumed to indicate when two organisms might be considered separate species. This criteria suggests that the two *Rhynchomonas* isolates used in this analysis, which differ by 1.9 %, and the *B. caudatus* isolates, which differ by 1.8 %, are probably different species. However, insufficient morphological characterization has been done on these isolates to support or refute this conclusion (Hajduk et al. 1986, Atkins et al. 2000).

The largest 18S rRNA sequence difference of 6.1 % was between isolates that were thought to belong to the same species, between the *B. saltans* K and P strains. This large sequence divergence supports separating these strains into different species. However,

ultrastructural and behavioral data have not been published for either of these strains. We believe that closer examination of these strains may reveal significant morphological differences at the ultrastructural level. In contrast, our *B. saltans* isolate and the *B. saltans* K strain only differ by 0.2 %, indicating that they are the same species.

The monophyly of the *Dimastigella* species is the same as observed in previous studies (Breunig et al. 1993, Berchtold et al. 1994, Dolezel et al. 2000). However, our addition of a third *Dimastigella trypaniformis* isolate (ATCC) to the analysis clearly indicates that the Glasgow isolate is more closely related to *D. mimosa* than are the other two *D. trypaniformis* isolates. The Ulm and Glasgow strains also differ in nucleotide sequence by 2 %, suggesting that they, as well as the newly sequenced strain, are probably separate species.

The phylogenetic position of *Ichthyobodo necator*. The most significant finding of our study is the position of *I. necator* within the order Kinetoplastida. *Ichthyobodo necator* is truly a unique member of the order Kinetoplastida. Unfortunately, very little is known about *I. necator*'s life history, exact feeding mechanism, or whether it is a single taxon. One problem that has hampered study of this organism is that it is difficult to maintain infections and impossible to culture them in vitro. To address this problem, we developed a novel propagation and collection method, which enabled us to obtain large numbers of *I. necator* cells for DNA isolation and subsequent PCR amplification and sequencing of the 18S rRNA gene.

Limited experimental evidence suggests that some *Ichthyobodo* isolates are host-specific (Urawa & Kusakari 1990); however, there were no ultrastructural differences found among these isolates. Additionally, Davis (1943) described a smaller "species" of *Ichthyobodo*, *I. pyriformis*, infecting trout. It is uncertain whether this is a separate species because differentiation from *I. necator* was based on its smaller size. Size is unlikely to be a relevant taxonomic trait because *I. necator* populations can vary considerably in size (HAC., unpub. data).

Determining whether *I. necator* is a single taxon or is a complex of morphologically similar species is important for the future of fish farming. Once a fish population becomes infected, *I. necator* can persist subclinically, requiring continuous prophylactic treatments to prevent further outbreaks (Valtonen & Koskivaara 1994, Buchmann & Bresciani 1997). It is also unknown if isolates differ in pathogenicity or whether some isolates have more than one host. Therefore, our future work will determine the genetic relatedness of various *I. necator* isolates by developing PCR assays to discern isolates from different hosts and locations worldwide. Our goal is to provide information on the diversity of these organisms and to follow their introduction and spread into local and international farming facilities.

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Table 4.1. The three traditional classification schemes currently in use for the Suborder Bodonina. In schemes 1 and 3 assignment to family is based on the attachment of the posterior flagellum to the plasma membrane. In scheme 3, *Cephalothamnium* has been placed in its own family because it is the only colonial species within the suborder.

1. Vickerman, (1976)	2. Vickerman, (1978) (1990)	3. Zhukov, (1991)
family Bodonidae	family Bodonidae	family Bodonidae
		subfamily Bodoninae
genera *Bodo*	genera *Bodo*	genera *Bodo*
Ichthyobodo	*Ichthyobodo*	*Ichthyobodo*
Parabodo	*Parabodo*	*Parabodo*
Rhynchobodo (*Phyllomitus*)	*Rhynchobodo*	*Rhynchobodo*
Rhynchomonas	*Rhynchomonas*	*Rhynchomonas*
	Cryptobia	*Pleuromonas*
	Dimastigella	
Procryptobia		
	Trypanoplasma	
	Cephalothamnium	
family Cryptobidae		subfamily Cryptobiinae
genera *Cryptobia*		genera *Cryptobia*
Dimastigella		*Dimastigella*
Procryptobia		*Procryptobia*
Trypanoplasma		
Cephalothamnium		
		family Cephalothamniidae
		genus *Cephalothamnium*

Table 4.2. Conserved primers used for sequencing both strands of the kinetoplastid 18S

rRNA gene.

Coding Strand Specific Primers:

KinSSUF1	5' GGTTGATTCTGCCAGTAGTC '3
M13 Forward	5' GTTTTTCCCGTCACGAC '3
SSUseqF1	5' GGAGCCTGAGAAATAGCTAC '3
SSUseqF2	5' TGGTTAAAGGGTTCGTAGTTG '3
SSUseqF3	5' AACTACAGCGAAGGCATTC '3
SSUseqF4	5' TGGTCGGTGGAGTGATTTG '3

Non-coding Strand Specific Primers:

KinSSUR1	5' CAAATGGCAGGGACGTAATC '3
SSUseqR1	5' CTATTTCTCAGGCTCCCTC '3
SSUseqR2	5' TCAACTACGAACCCTTTAAC '3
SSUseqR3	5' GCCTTCGCTGTAGTTCGTC '3
SSUseqR4	5' AACAAATCACTCCACCGAC '3
M13 Reverse	5' CAGGAAACAGCTATGAC '3

Table 4.3A. Mean nucleotide difference (%) between species within a kinetoplastid sublineage containing more than one sequence. Means were determined using paired difference from PAUP* (Swofford 1999).

Sublineage	2	3	4	6	7	8
Mean	1.3	11.3	5.1	13.8	1.7	1.9
Std. Dev.	0.3	6.6	1.9	1.3	0.6	ND
No. of Species	4	4	6	5	4	2

Table 4.3B. Percent (%) similarity matrix between consensus sequences of kinetoplastid sublineages.

Sublineage	2	3	4	5	6	7	8
1	6.7	7.1	7.9	10.7	9.6	11.7	12.1
2		5.8	5.2	8.7	7.6	9.8	9.9
3			5.5	9.5	7.8	10.1	10.2
4				7.1	6.5	9.0	8.3
5					8.0	10.0	9.3
6						8.4	8.4
7							7.6

Table 4. 4. Morphological features used to distinguish various species within the order Kinetoplastida.   Species are listed as they appear in Fig.1, 2. Family designations follow Vickerman (1976). References used to construct the table are as follows: *Bodo caudatus* (Brooker 1971; Hajduk 1986), *Bodo designis* (Eyden 1977), *Bodo edax* (Bullis, R.A., unpubl. data), *Bodo saliens* (Larsen and Patterson 1990), *Bodo saltans* (Brooker 1971), *Bodo* sp. (RAB unpubl. data), *Bodo* sp. c.f. *uncinatus* (Blom et al. 1998), *Bodo sorokini* (Zhukov 1975), *Cruzella marina* (Dolezel et al. 2000), *Cryptobia bullocki* (Strout 1965), *Cryptobia catastomi* (Bower and Woo 1977), *Cryptobia helicis* (Kozloff 1948), *Cryptobia salmositica* (Paterson and Woo 1983), *Crithidia oncopelti* (Hollar et al. 1998; Vickerman 1976), *Dimastigella mimosa* (Frolov 1997)*, Dimastigella trypaniformis* (Breunig 1993), *Ichthyobodo necator* (Joyon and Lom 1966; Shubert, 1966), kinetoplastid flagellate (Atkins 2000), *Leishmania tarentolae* (Vickerman 1976), *Parabodo nitrophilus* (Mylinkov 1986a), *Rhynchobodo* (Brugerolle 1985; Mylnikov 1986b), *Rhynchomonas nasuti* (Swale 1973), *Trypanoplasma borreli* (Vickerman 1977), *Trypanosoma avium* (Vickerman 1976), *Trypanosoma brucei* (Vickerman 1976), *Trypanosoma cruzi* (Vickerman 1976), *Trypanosoma simiae* (Vickerman 1976), unidentified eukaryote (van Hannen 1999).

Sublin.	Species	Family	Flag. Num.	Rostrum	Kinetoplast Structure	Flag. Attach. to Body	Lifestyle	Bacterial Endosymbionts	Mastigonemes	Resting Cyst
	Suborder Bodonina									
	I. necator	Bodonidae	2	partially-developed	polykinetoplastic	no	ectoparasite	no	no	unknown
1	*B. sorokini*	Bodonidae	2	short	eukinetoplastic	no	free-living	unknown	unknown	unknown
2	*C. salmositica*	Cryptobidae	2	not present	eukinetoplastic	yes	ecto/haemoparasite	no	no	no
2	*C. bullocki*	Cryptobidae	2	not present	eukinetoplastic	yes	ecto/haemoparasite	no	no	no
2	*C. catastomi*	Cryptobidae	2	not present	eukinetoplastic	yes	haemoparasite	no	no	no
2	*T. borreli*	Cryptobidae	2	not present	eukinetoplastic	yes	haemoparasite	no	no	no
3	*C. helicis*	Cryptobidae	2	not present	eukinetoplastic	yes	parasite	no	no	no
3	*P. nitrophilus*	Bodonidae	2	short	eukinetoplastic	no	free-living	no	no	unknown
3	*B. caudatus*	Bodonidae	2	short	eukinetoplastic	no	free-living	no	no	yes
4	*B. edax*	Bodonidae	2	short	eukinetoplastic	no	free-living	yes	no	no
4	*Bodo* sp.*(uncinatus)*	Bodonidae	2	short	eukinetoplastic	no	free-living	unknown	unknown	unknown
4	*B. saltans*	Bodonidae	2	short	eukinetoplastic	no	free-living	yes	yes	no
4	*Bodo* sp.	Bodonidae	2	short	eukinetoplastic	no	free-living	no	no	yes

99

Table 4. 4 (continued)

| Sublin.	Species	Family	Flag. Num.	Rostrum	Kinetoplast Structure	Flag. Attach. to Body	Lifestyle	Bacterial Endosymbionts	Mastigonemes	Resting Cyst
Suborder Trypanosomatina										
	L. tarentolae	Trypanosomatidae	1	not present	eukinetoplastic	no	parasite	no	no	no
	C. oncopelti	Trypanosomatidae	1	not present	eukinetoplastic	no	parasite	yes	no	no
	T. brucei	Trypanosomatidae	1	not present	eukinetoplastic	yes	haemoparasite	no	no	no
	T. simiae	Trypanosomatidae	1	not present	eukinetoplastic	yes	haemoparasite	no	no	no
	T. cruzi	Trypanosomatidae	1	not present	eukinetoplastic	yes	haemoparasite	no	no	no
	T. avium	Trypanosomatidae	1	not present	eukinetoplastic	yes	haemoparasite	no	no	no
	Suborder Bodonina									
5	Rhynchobodo	Bodonidae	2	well-developed	polykinetoplastic	no	free-living	no	no	unknown
6	Kinetoplastid flagellate	Bodonidae		unknown	unknown	no	free-living	unknown	unknown	unknown
6	Cruzella marina	unknown	2	unknown	unknown	unknown	unknown	unknown	unknown	unknown
6	unidentified eukaryote	Bodonidae	2	short	unknown	unknown	free-living	unknown	unknown	unknown
6	B. saliens	Bodonidae	2	short	unknown	no	free-living	unknown	unknown	unknown
6	B. designis	Bodonidae	2	short	eukinetoplastic	no	free-living	yes	no	no
7	D. trypaniformis	Cryptobidae	2	well-developed	polykinetoplastic	yes	free-living	no	no	yes
7	D. mimosa	Cryptobidae	2	well-developed	polykinetoplastic	yes	free-living	no	no	yes
8	R. nasuti	Bodonidae	2	well-developed	eukinetoplastic	no	free-living	yes	yes	unknown
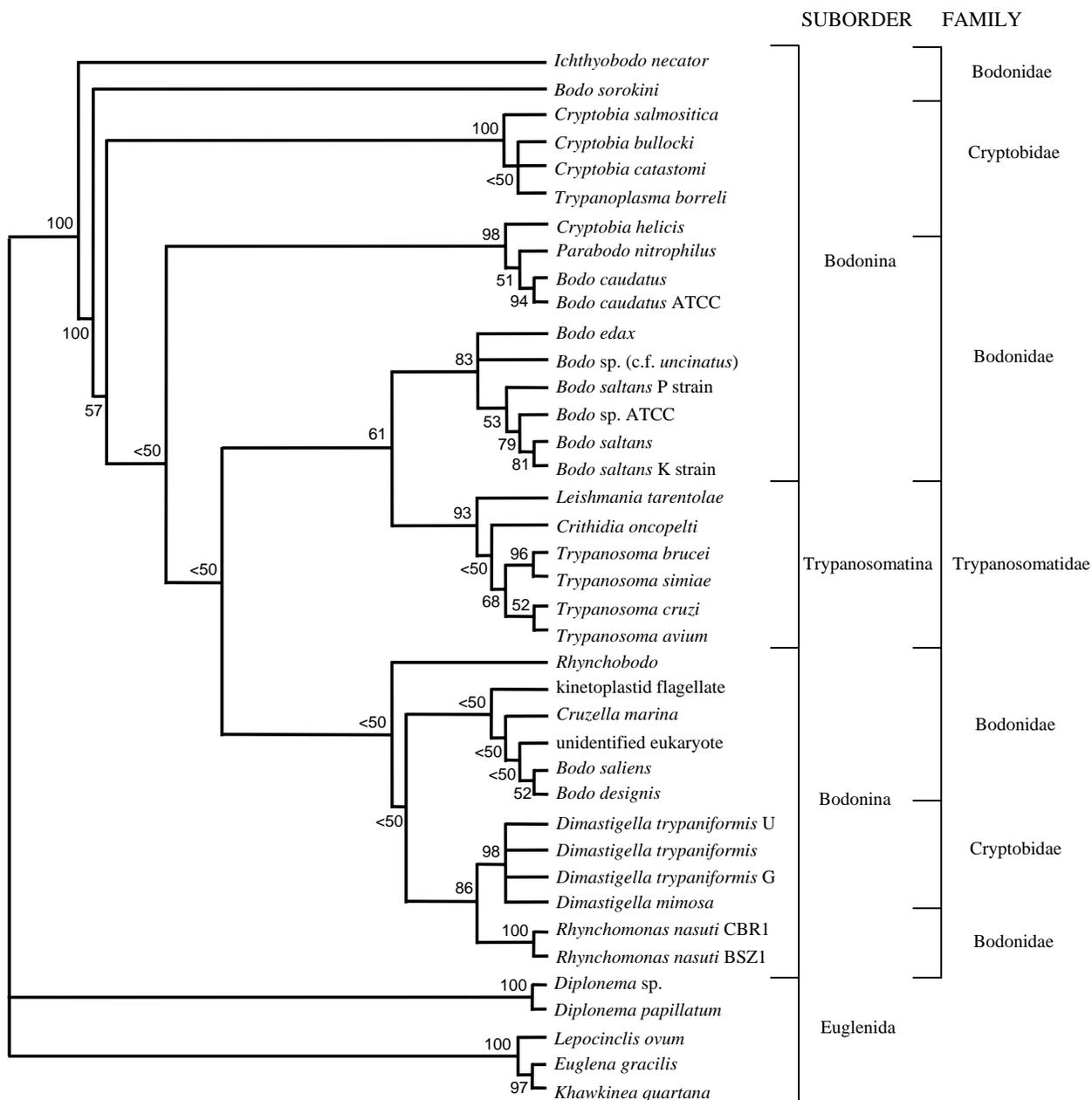
Figure 4.1. Maximum-likelihood (ML) tree based on 34 kinetoplastid and 5 euglenoid 18S ribosomal RNA gene sequences. Tree was constructed, using PAUP* (Swofford, 1999), from a conservative secondary structure alignment and ML model estimated with MODELTEST (Posada & Crandall 1998). The major taxonomic lineages from the 18S phylogenetic analysis are compared to the current family classifications as detailed by Vickerman (1976). Twenty-five bootstrap replicates for each of the branches in the tree were calculated under the maximum-likelihood model.
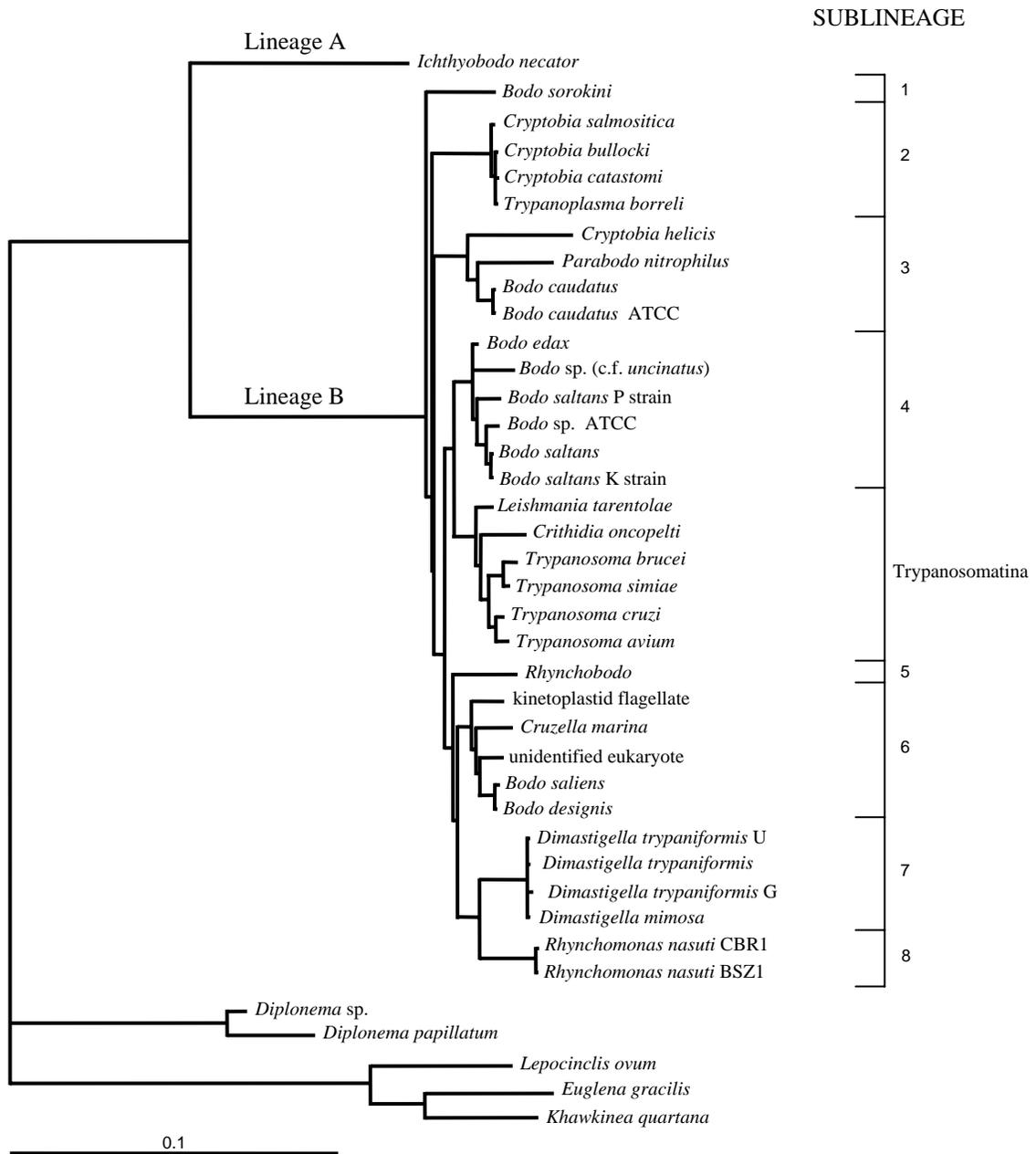
Figure 4.2. Phylogram of the maximum-likelihood (ML) tree showing relative branch lengths. Tree was constructed, using PAUP* (Swofford, 1999), from a conservative secondary structure alignment and ML model estimated with MODELTEST (Posada & Crandall 1998). Sublineages 1-8 for the suborder Bodonina are indicated. Members of the suborder Trypanosomatina are not indicated by a sublineage designation. The scale indicates the number of nucleotide substitutions per site determined by paired differences from PAUP*.