

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

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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 × *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.

Protozoan parasites cause a number of diseases that can seriously impact aquaculture and wild fish populations (Hoffmann 1999; Valtonen and Koskivaara 1994). 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 (Becker 1977; Cone and Wiles 1984; Ellis and Wootton 1978; Forsythe et al. 1991; Morrison and Cone 1986; Urawa and Kusakari 1990). 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 (Post 1987; Robertson 1979). As a result, *I. ne-*

cator is considered one of the most dangerous parasites of cultured fish (Buchmann et al. 1995; Hoffmann 1999; Post 1987; Rintamaki-Kinnunen and Valtonen 1997).

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 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 (Atkins et al. 2000; Foissner 1991; Zhukov 1991). Most species are free-living heterotrophs that selectively graze on bacteria and are considered important members of the microbial food web (Eccleston-Parry and Leadbeater 1995; Lavrentyev et al. 1997; van Hatten et al. 1999; Zhukova and Kharlamenko 1999). However, a number of species within the genera *Ichthyobodo*, *Cryptobia*, and *Trypanoplasma* have adapted to a parasitic lifestyle (Joyon and 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 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 endosymbi-

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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 sorokinii* (AF208888), *Bodo* sp. (cf. *uncinatus*) (AF208884), *Bodo* sp. ATCC50149 (AY028449), *Crithidia oncopelti* (L29264), *Cruzella marina* (AF208878), *Cryptobia bullocki* (AF080224), *Cryptobia catastomi* (AF080226), *Cryptobia heliciis* (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).

Table 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
genera <i>Bodo</i>	genera <i>Bodo</i>	subfamily Bodoninae
<i>Ichthyobodo</i>	<i>Ichthyobodo</i>	genera <i>Bodo</i>
<i>Parabodo</i>	<i>Parabodo</i>	<i>Ichthyobodo</i>
<i>Rhynchobodo</i> (<i>Phyllomitus</i>)	<i>Rhynchobodo</i>	<i>Parabodo</i>
	<i>Rhynchomonas</i>	<i>Rhynchobodo</i>
	<i>Cryptobia</i>	<i>Rhynchomonas</i>
	<i>Dimastigella</i>	<i>Pleuromonas</i>
	<i>Procryptobia</i>	
	<i>Trypanoplasma</i>	
	<i>Cephalothamnium</i>	
family Cryptobidae		subfamily <i>Cryptobiinae</i>
genera <i>Cryptobia</i>		genera <i>Cryptobia</i>
<i>Dimastigella</i>		<i>Dimastigella</i>
<i>Procryptobia</i>		<i>Procryptobia</i>
<i>Trypanoplasma</i>		
<i>Cephalothamnium</i>		
		family Cephalothamniidae
		genus <i>Cephalothamnium</i>

onts (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 (Larsen and Patterson 1990; Woo 1987). 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 × *M. chrysops* female) farmed in North Carolina aquaculture facilities commonly harbor subclinical infections of *I. necator*, which can reach epidemic proportions during spring and late fall. Epidemics routinely produce heavy parasite loads on the skin and gills (> 1,000 parasites/mm²). 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 °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 °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 g for 10 min at 14 °C. *Bodo caudatus*, *B. saltans*, *Bodo* sp. and *D. trypaniformis* were inoculated and grown for 3 d at 22 °C. Population densities on day three reached 10⁵–10⁸ cells/ml, depending upon the species (the density of organisms in most cultures started declining 4 d after inoculation). Cell suspensions were filtered to remove particulates and pelleted at 22 °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 µl) 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 2). Approximately 10 ng of genomic DNA was combined with 1× PCR buffer (PromegaTM), 100 ng of KinSSUF1, 100 ng KinSSUR1, 0.10 mM dNTPs, and 5 units of *Taq* Polymerase (PromegaTM). The PCR reactions for *I. necator* were amplified using 1.0 mM MgCl₂ (PromegaTM) and an annealing temperature of 50 °C. All other templates were amplified using 1.5 mM MgCl₂ and annealing temperatures of 55–60 °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'-GACTA-CGTCCCCGCCATTG-3'), which accounts for the lower an-

Table 2. Conserved primers used for sequencing both strands of the kinetoplastid 18S rRNA gene.

Coding Strand Specific Primers:	
M13 Forward	5'GTTTTTCCCGTACAGAC'3
KinSSUF1	5'GGTTGATTCTGCCAGTAGTC'3
SSUseqF1	5'GGAGCCTGAGAAATAGCTAC'3
SSUseqF2	5'TGGTTAAAGGTTCTCGTAGTTG'3
SSUseqF3	5'AACACAGCGAAGGCATTC'3
SSUseqF4	5'TGGTCGGTGGAGTGATTTG'3
Non-coding Strand Specific Primers:	
KinSSUR1	5'CAAATGGCAGGACGTAATC'3
SSUseqR1	5'CTATTTCTCAGGCTCCCTC'3
SSUseqR2	5'TCAACTACGAAACCTTTAAC'3
SSUseqR3	5'GCCTTCGCTGTAGTTCCGTC'3
SSUseqR4	5'AACAAATCACTCCACCGAC'3
M13 Reverse	5'CAGGAAACAGCTATGAC'3

nealing temperature 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 (InvitrogenTM). Plasmid DNA from positive clones was isolated using the QIAprep Spin Mini Prep Kit (QiagenTM). 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 DeoxyTM Terminator Cycle Sequencing Kit (Applied Biosystems-ABI)(Table 2). Sequencing primers were located approximately 600–650 bp apart.

To confirm that the sequences obtained from samples containing *I. nicator* 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 *Sau*3 A I endonucleases (PromegaTM) (data not shown). Contaminants consisting of other bodonids and protozoa were easily distinguished from *I. nicator* 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 (Martinez 1983; Needleman and Wunsch 1970). Consensus sequences were aligned relative to other kinetoplastid species from the GenBankTM database using pileup (Genetics Computer Group 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

Table 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

of increasing complexity (Posada and 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; number of rate categories (i.e. number of segments of the 18S 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 and 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. 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. nicator*, 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 nucleotide difference between species in each sublineage, plus or minus one standard deviation, was then calculated (Table 3A). An estimate of the genetic variation between sublineages (putative genera) was obtained by first determining a consensus sequence for each sub-

Table 3B. Percent (%) similarity matrix between consensus sequences of kinetoplastid sublineages. Similarities were determined using paired difference from PAUP* (Swofford, 1999).

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. 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* (Eyen 1977), *Bodo edax* (Bullis, R.A., unpubl. data), *Bodo saliens* (Larsen and Patterson 1990), *Bodo saltans* (Brooker 1971), *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 salmositica* (Paterson and Woo 1983), *Cyrtithia 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* (Mylinikov 1986a), *Rhynchobodo necator* (Joyon and Lom 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 Hanne 1999).

Sublin.	Species	Family	Flag. num.	Rostrum	Kinetoplast structure	Flag. attach. to body	Lifestyle	Bacterial Endo-symbionts	Masti-gonemes	Resting Cyst
suborder Bodonina										
	<i>I. necator</i>	Bodonidae	2	partially-developed	polykinetoplastic	no	ectoparasite	no	no	unknown
1	<i>B. sorokini</i>	Bodonidae	2	short	eukinetoplastic	no	free-living	unknown	unknown	unknown
2	<i>C. salmositica</i>	Cryptobiidae	2	not present	eukinetoplastic	yes	ecto/haemoparasite	no	unknown	no
2	<i>C. bullocki</i>	Cryptobiidae	2	not present	eukinetoplastic	yes	ecto/haemoparasite	no	no	no
2	<i>C. catastomi</i>	Cryptobiidae	2	not present	eukinetoplastic	yes	haemoparasite	no	no	no
2	<i>T. borreli</i>	Cryptobiidae	2	not present	eukinetoplastic	yes	haemoparasite	no	no	no
3	<i>C. helicis</i>	Cryptobiidae	2	not present	eukinetoplastic	yes	parasite	no	no	no
3	<i>P. nitrophilus</i>	Bodonidae	2	short	eukinetoplastic	no	free-living	no	no	unknown
3	<i>B. caudatus</i>	Bodonidae	2	short	eukinetoplastic	no	free-living	no	no	yes
4	<i>B. edax</i>	Bodonidae	2	short	eukinetoplastic	no	free-living	yes	no	no
4	<i>Bodo</i> sp. (<i>uncinatus</i>)	Bodonidae	2	short	eukinetoplastic	no	free-living	unknown	unknown	unknown
4	<i>B. saltans</i>	Bodonidae	2	short	eukinetoplastic	no	free-living	yes	yes	no
4	<i>Bodo</i> sp.	Bodonidae	2	short	eukinetoplastic	no	free-living	no	no	yes
suborder Trypanosomatina										
	<i>L. tarentolae</i>	Trypanosomatidae	1	not present	eukinetoplastic	no	parasite	no	no	no
	<i>C. oncopelti</i>	Trypanosomatidae	1	not present	eukinetoplastic	no	parasite	yes	no	no
	<i>T. brucei</i>	Trypanosomatidae	1	not present	eukinetoplastic	yes	haemoparasite	no	no	no
	<i>T. simiae</i>	Trypanosomatidae	1	not present	eukinetoplastic	yes	haemoparasite	no	no	no
	<i>T. cruzi</i>	Trypanosomatidae	1	not present	eukinetoplastic	yes	haemoparasite	no	no	no
	<i>T. avium</i>	Trypanosomatidae	1	not present	eukinetoplastic	yes	haemoparasite	no	no	no
suborder Bodonina										
5	<i>Rhynchobodo</i>	Bodonidae	2	well-developed	polykinetoplastic	no	free-living	no	no	unknown
6	Kinetoplastid flagellate	Bodonidae	2	unknown	unknown	no	free-living	unknown	unknown	unknown
6	<i>Cruzella marina</i>	unknown	2	unknown	unknown	unknown	free-living	unknown	unknown	unknown
6	unidentified eukaryote	Bodonidae	2	short	unknown	no	free-living	unknown	unknown	unknown
6	<i>B. saliens</i>	Bodonidae	2	short	unknown	no	free-living	unknown	unknown	unknown
6	<i>B. designis</i>	Bodonidae	2	short	eukinetoplastic	no	free-living	yes	no	no
7	<i>D. trypaniformis</i>	Cryptobiidae	2	well-developed	polykinetoplastic	yes	free-living	no	no	yes
7	<i>D. mimosa</i>	Cryptobiidae	2	well-developed	polykinetoplastic	yes	free-living	no	no	yes
8	<i>R. nasuti</i>	Bodonidae	2	well-developed	eukinetoplastic	no	free-living	yes	yes	unknown

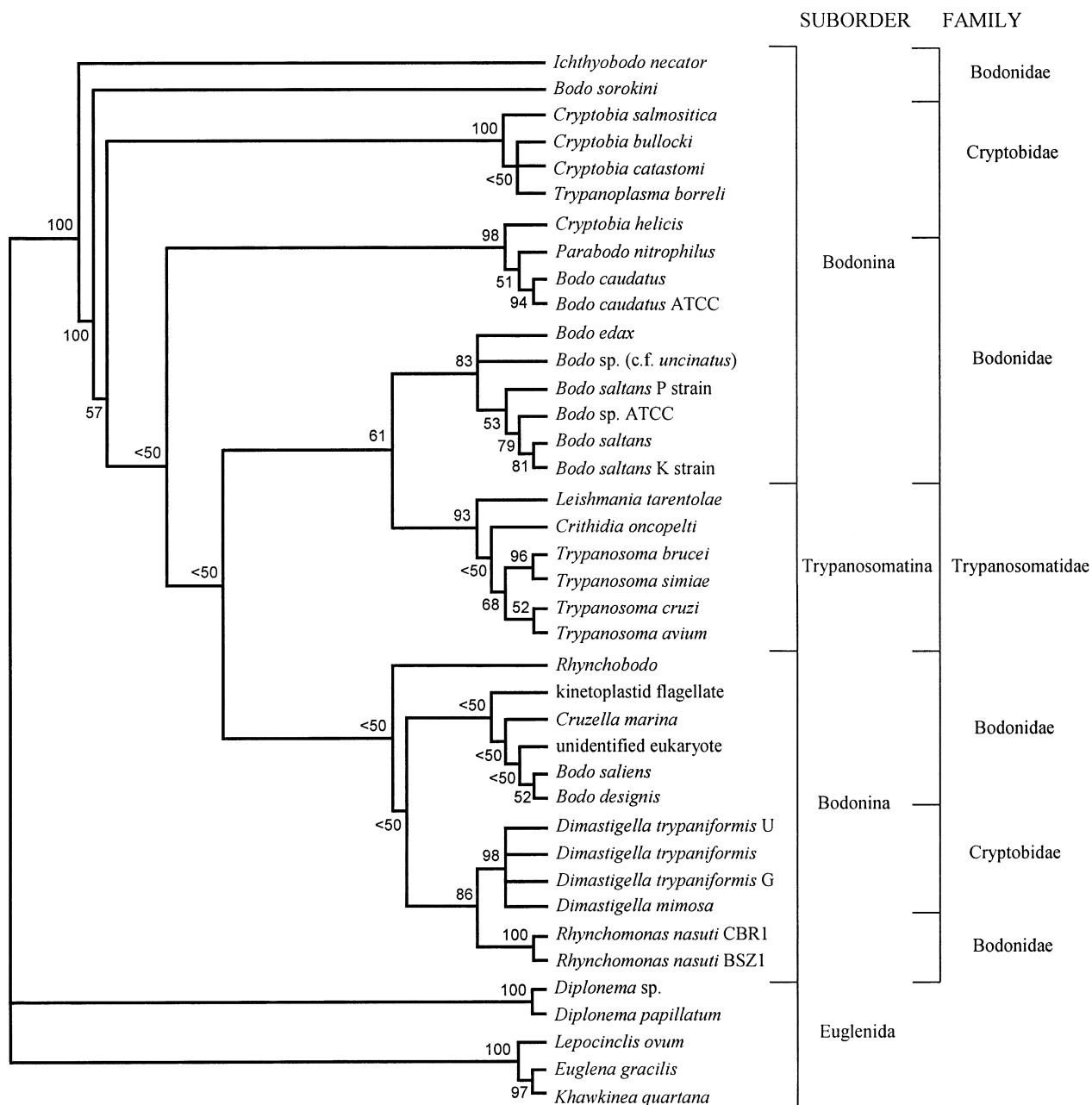


Fig. 1. Maximum-likelihood (ML) tree based on 34 kinetoplastid and 5 euglenoid 18S ribosomal RNA gene sequences. Tree was constructed, using PAUP* (Swofford, 1999) from a conservative secondary structure alignment and the ML model was estimated with MODELTEST (Posada and Crandall 1998). The major taxonomic 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.

lineage 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 sublineages were then estimated using PAUP* as described above (Table 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). 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. 1, 2).

RESULTS

Our results strongly supported the order Kinetoplastida as a monophyletic group which consisted of two major lineages

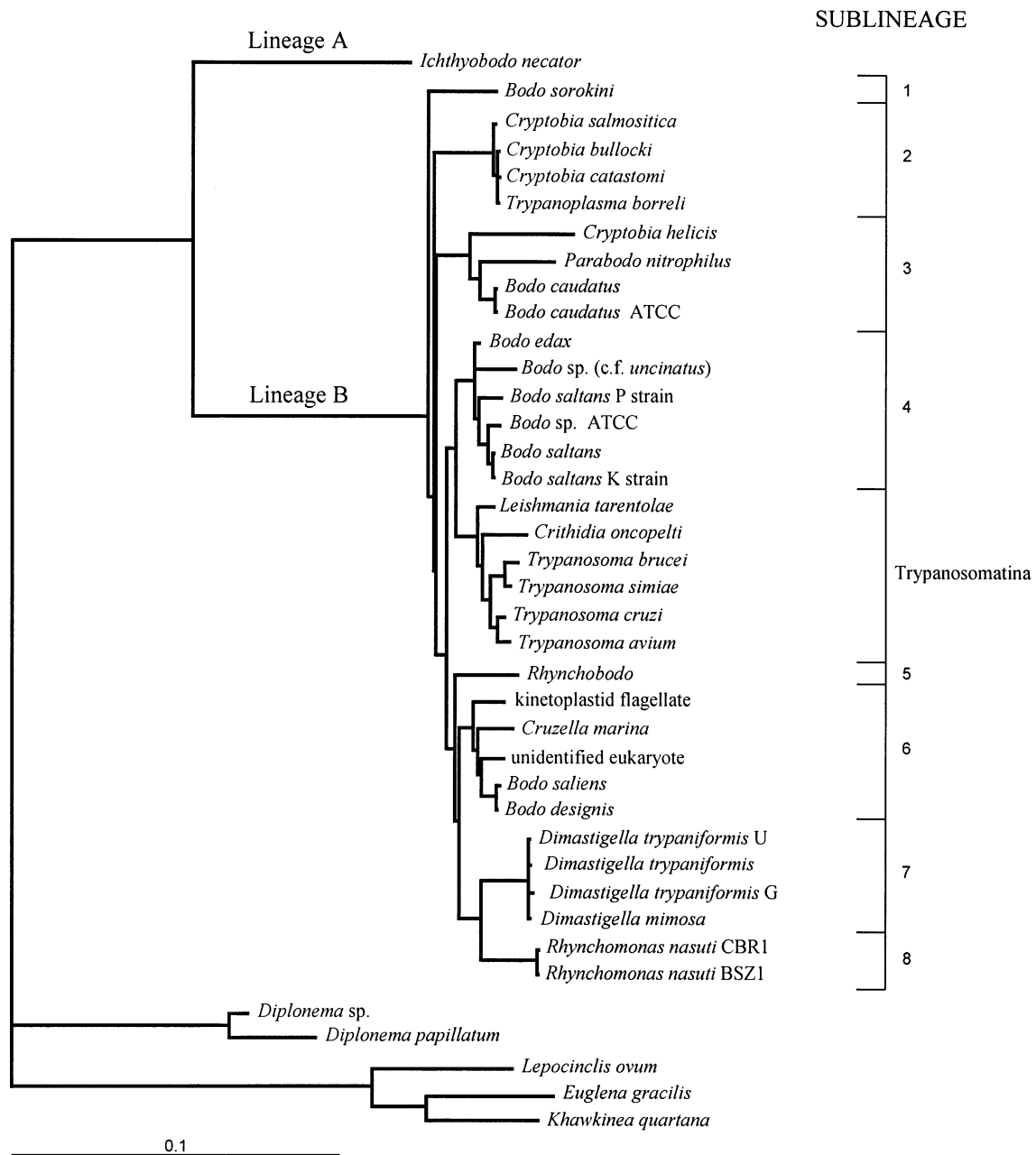


Fig. 2. Phylogram of the maximum-likelihood (ML) tree showing relative branch lengths. Tree was constructed using PAUP* (Swofford, 1999) from a conservative secondary structure alignment and the ML model was estimated with MODELTEST (Posada and Crandall 1998). Sublineages 1–8 for the suborder Bodonina 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*.

(Fig. 1, 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. 1, 2). Additionally, the low bootstrap support of the deeper branches within lineage B indicated that these evo-

lutionary 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. 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. 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 Cryptobiidae (Fig. 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 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 to 8 ranged from 5.2–11.7% (Table 3B, Fig. 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 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 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 3A).

The morphological characteristics traditionally used for classifying these organisms was not in absolute concordance with the 18S rRNA phylogeny (Table 4, Fig. 1, 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 sublineage 3, was clearly a genetically separate organism from the hemoparasitic species of sublineage 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; Larsen and Patterson 1990; Zhukov 1975). Behavioral characteristics, such as movement and feeding behavior, are also commonly used to classify organisms (Eyden 1977; Karpov and 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 (Alleman et al. 1990; Faria-e-Silva et al. 2000; Fernandes et al. 1993; Maclean and Amiro 1973).

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 1, 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/trypanosomatid group (B) all have low bootstrap support, making it difficult to assess suborder assignments within this lineage (Fig. 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 Cryptobiidae, nor the family Bodonidae into the subfamilies Bodoninae and Cryptobiinae. All of these taxonomic schemes are based on flagellar attachment (Table 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 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. 2). Species in these genera are among the easiest to identify because of their kinetoplast structure and well-developed rostra (Table 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 3A, B). The remaining *Bodo* species, however, were distributed among sublineages 1, 3, and 6. This supports previous studies that concluded *Bodo* to be polyphyletic (Dolezel et al. 2000; Wright et al. 1998). Recent evidence indicates that many *Bodo* species are adapted to consuming specific types of bacteria (Cochran-Stafira and von Ende 1998; Simek et al. 1997; Zhukova and 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 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 ancestral 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. 1).

Comparison of the molecular and classical phylogeny at the species level. The sublineages in this study that are best supported by classical 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 3A). The smallest difference (1.3%) is between the *Cryptobia/Trypanoplasma* 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 criterion 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 (Atkins et al. 2000; Hajduk et al. 1986).

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 (Berchtold et al. 1994; Breunig et al. 1993; 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 and 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., unpubl. 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 (Buchmann and Bresciani 1997; Valtonen and Koskivaara 1994). 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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