# *Ichthyobodo* infections on farmed and wild fish

- Methods for detection and identification of *Ichthyobodo* spp.

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# List of papers

**Paper A.** Isaksen, T. E., Karlsbakk, E. and Nylund, A. (2007). *Ichthyobodo hippoglossi* n. sp. *(Kinetoplastea:Prokinetoplastida: Ichthyobodonidae* fam. nov.*),* an ectoparasitic flagellate infecting farmed Atlantic halibut *Hippoglossus hippoglossus*. *Diseases of Aquatic Organisms,* **73**, 207-217.

**Paper B.** Isaksen, T. E., Karlsbakk, E., Sundnes, G. A. and Nylund, A. (2010). Patterns of *Ichthyobodo necator* sensu stricto infections on hatchery reared salmon (*Salmo salar* L.) in Norway. *Diseases of Aquatic Organisms,* **88**(3), 207-214

**Paper C.** Isaksen, T. E., Karlsbakk, E., Watanabe, K. and Nylund, A. (2011). *Ichthyobodo salmonis* sp. n. (Ichthyobodonidae, Kinetoplastida), an euryhaline ectoparasite infecting Atlantic salmon (*Salmo salar* L.). *Parasitology,* **138**(9), 1164-1175.

**Paper D.** Isaksen, T. E., Karlsbakk, E., Repstad, O. and Nylund, A. (2012). Molecular tools for the detection and identification of *Ichthyobodo* spp. (Kinetoplasitda), important fish parasites. Parasitology International, **61**(4), 675-683.

# CONTENTS

# ABSTRACT

Ichthyobodosis is an important parasitic disease that has caused severe loss among ornamental and farmed fish world wide for more than a century. The disease is caused by heavy infections on skin and gills by parasitic flagellates belonging to the genus *Ichthyobodo.* In the past, infections worldwide have commonly been identified as due to a single variable species, *I. necator*. However, recent molecular studies have revealed that the genus *Ichthyobodo* consist of several different species and is far more complex than previously believed. The overall aim of this PhD project has been to identify and characterise *Ichthyobodo* species with emphasis on those that are known from Norwegian aquaculture. Therefore, effective and sensitive molecular methods (PCR techniques) for detection and identification of *Ichthyobodo* spp. have been developed and validated. With the aid of such methods several new *Ichthyobodo* genotypes have been detected from both farmed and wild fish. Working with molecularly identified *Ichthyobodo* genotypes has led to improved knowledge of character variations in the genus. Also, novel morphological characters have been identified, aiding species discrimination. Hence both molecular and morphological tools are provided that may facilitate the future description of other *Ichthyobodo* spp. Morphological descriptions of three *Ichthyobodo* species is presented, so far the only valid species within genus *Ichthyobodo* that have also been characterized through their DNA sequences. All presently valid *Ichthyobodo* species have been detected on both farmed and wild caught hosts in Norway:

1. *Ichthyobodo necator*; until recently the only species in the genus, is redescribed. It has been detected on Atlantic salmon, brown trout, rainbow trout and three-spined sticklebacks in freshwater.

2. *Ichthyobodo salmonis*; an euryhaline species able to infect Atlantic salmon in both freshwater and seawater.

3. *Ichthyobodo hippoglossi*; a marine species infecting Atlantic halibut.

# INTRODUCTION

*Genus Ichthyobodo* contains unicellular flagellate parasites that infect the external surface of aquatic hosts (skin, fins, gills). These parasites have been recorded from different fish hosts worldwide for more than a century, and severe *Ichthyobodo* infections are associated with disease (ichthyobodosis) and mortality among farmed fish (Robertson, 1985).

## History – The first descriptions of the parasite and disease

Louis-Félix Henneguy (1883) was probably the first to describe ectoparasitic flagellates from fish. He studied a disease outbreak with increased mortality among hatchery reared brown trout fry (*Salmo trutta* L.) at the College de France in Paris. The causative agent for the disease was an ectoparasitic flagellate representing a new undescribed parasite. His observations may be summarized as follows (Henneguy1883, 1884).

*The parasitic disease occurred among the fish during early stages of first feeding (three weeks after hatching, in early February), some of the fish had not yet fully absorbed their yolk sac. Clinical signs were lethargic behaviour and increased mortality. At most, hundreds of dying or dead fry had to be removed daily in a period from February till May. Attempts to treat the trout fry using salt (20-30%), alcohol or iodine failed. Consequently, the entire population of trout hatched in 1883 was lost due to this parasitic disease. The pathogenecity of the parasite was tested by introducing some infected fish to groups with healthy fish. After only two days all fry were infected with high mortality. Consequently the observed flagellate parasites were concluded to be the causative agent for the disease and mortality. The presence of large numbers of the flagellates on skin was assumed to affect the fry through severe skin irritation. In addition, gill infection was assumed to reduce respiration.*

Henneguy (1884) suggested that these flagellates were obligate parasites. That is, they were not able to survive and proliferate without susceptible hosts. Through light microscopy, he revealed that the flagellates occurred as both free swimming forms and attached non-motile forms on epidermal cells. The attached form was pear-shaped and measured 0.01 x 0.02 mm. Cellular structures such as nucleus, vacuoles and a flagellar groove was described from osmic fixed flagellates stained with carmine and methyl green.

In these stained preparations the centrally located nucleus was clearly visible and contained a bright central mass surrounded by a ring of refracting substance. Henneguy defined the thickest, most dense part of the cell as posterior. A contractile vacuole (Vc; Figure 1, p. 9) could sometimes be detected in this area.

A longitudinal flagellar groove is clearly visible in the attached form with one long flagellum emerging from the groove (see sketch 2 and 3 in Figure 1, p. 9). The parasites were observed to leave the host cell they were attached to. In this process the attached form gradually became more rounded in shape, transforming into the free swimming form. The free swimming form was described as having three flagella; one long and two shorter. The swimming pattern appeared as series of short-lived bursts of movement with turns around the longitudinal axis of the parasite.

Henneguy's (1884) original drawings of *Ichthyobodo necator* is shown in Figure 1 (p. 9).

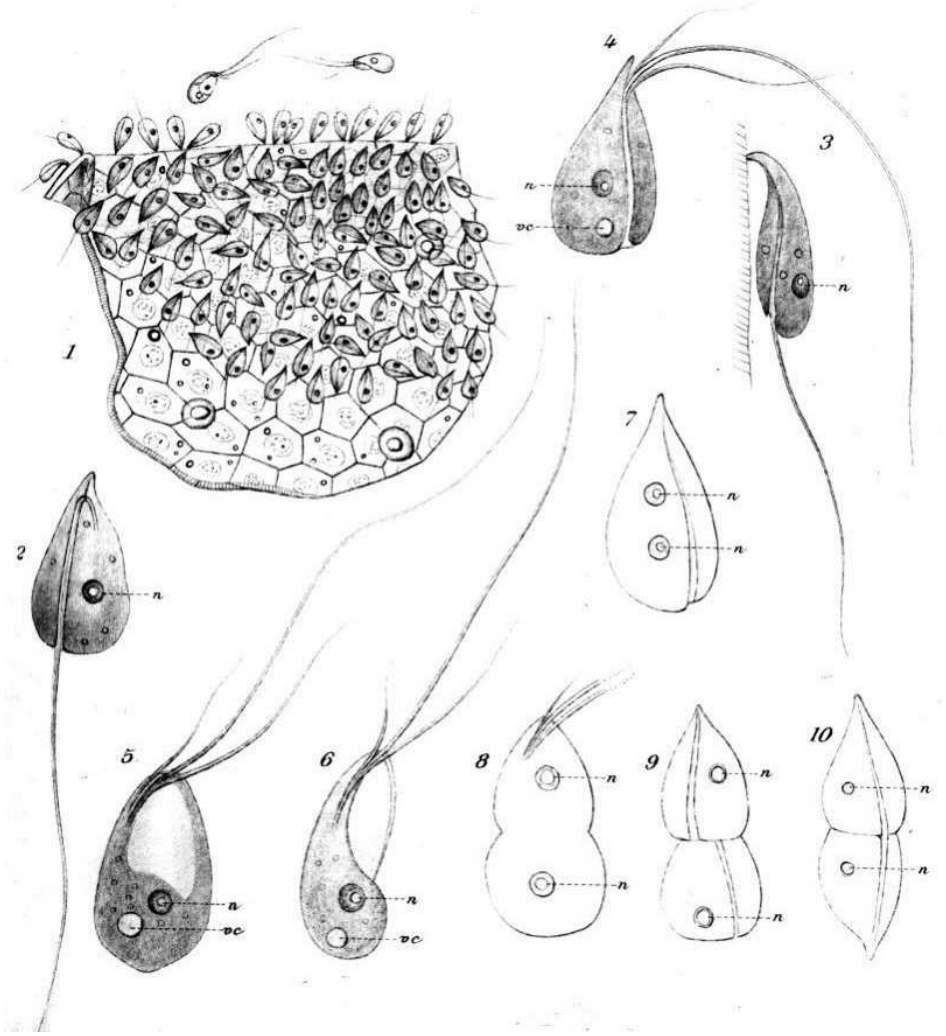


**Figure 1**. *Bodo necator*, Henneguy, 1884 (original figure). Characteristics shown are three flagella (one long and two shorter), longitudinal flagella groove and a dorso-ventral flattened cell shape. Nucleus (n) and a single contractile vacuole (vc). Two forms; free-swimming and attached. **1**: High density of infection, the parasites attached to goblet cells of the epidermis from a skin sample of brown trout fry. The parasites sometimes detach from the skin, transferring to free-swimming forms. **2**: Attached form, dorsal view, **3:** Attached form, viewed from the side. **4**: The two shorter flagella became visible during transfer from attached form to free-form. **5**: Free swimming forms, ventral view. **6**: Free swimming form, viewed from the side. **7-10**: Multiplication by transverse cell division.

## Morphology and nomenclature

Henneguy (1883) considered the novel trout parasite most similar to members of the genus *Bodo* Stein 1878, and named it *Bodo necator* (Latin *necator*; murderer, killer). However, other members of genus *Bodo* had been described with one or two flagella while *B. necator* showed three flagella. Therefore, *B. necator* was transferred to a novel genus *Costia* and named *Costia necatrix* by Leclerq (1890).

Weltner in Nietsche & Weltner (1894) found flagellates with four flagella that infected the skin of goldfish (*Carassius auratus*). These flagellates (Figure 2, p. 10) differed from the descriptions given by Henneguy (1883, 1884), not only in numbers of flagella, but also in being much smaller (attached form 5.1 µm x 13.6 µm), lacking a longitudinal groove and in showing a different type of locomotion. Hence the flagellates were considered to represent a novel species in the genus *Tetramitus*, named *Tetramitus nitschei* Weltner, 1894. However, Moroff (1904) found similar parasites with four flagella infecting the gills of brown trout (Figure 3, p. 11), and considered *T. nitschei* and *C. necatrix* as likely synonyms based on drawings and descriptions given by Weltner.

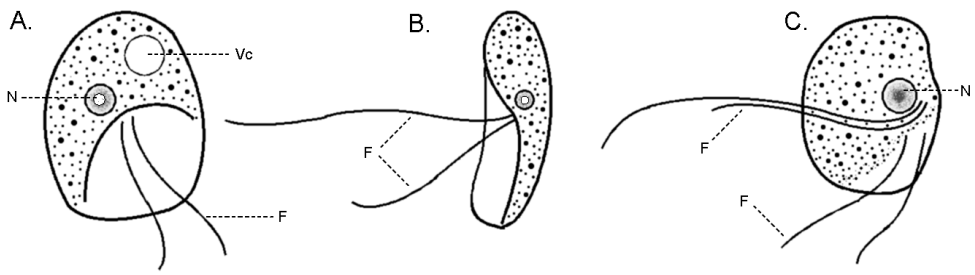


**Figure 2.** *Tetramitus nitchei* (from Nietsche and Weltner, 1894). Modified sketches. Two or four flagella (F) visible. Nucleus (N) and a single contractile vacuole (Vc). **A**: Viewed from the flattened side of the cell; **B:** Viewed from the side; **C**: Four-flagellated individual.

A study performed by G. Entz (1913, unpublished work) was published by Hartmann (1917). He found the parasite to harbour both two and four flagella and the four-flagellated forms were suggested to represent pre-division stages. Using G. Entz original material (smears and photos), Andai (1933) performed a more comprehensive study of the parasite with emphasis on morphology and the occurrences of two and four flagellated forms (Figure 4, p. 12). He described the free form of the flagellate as oval and dorso-ventrally compressed. When attached to a host cell, the parasite was more pyriform in shape. Typically, a large vacuole occurred positioned between the nucleus and the end of the flagellar pocket. A longitudinal groove extending more than half the cell length was often clearly visible on the ventral side of the cell. Andai (1933) provided accurate measurements demonstrating variation in cell size, and also showed that *Costia* cells with four flagella were larger than those with two flagella (bi-flagellated). Approximately 6% of the individuals examined, appeared to be quadriflagellate (four-flagellated). In agreement with Hartmann (1917), Andai (1933) concluded that *Costia necatrix* is a biflagellate and that quadriflagellated forms are pre-division stages. Subsequent studies on *Costia necatrix* from cyprinids (Benisch, 1936) and from hatchery reared salmonids (Fish, 1940), confirmed this, but the genus *Costia* was not abandoned.

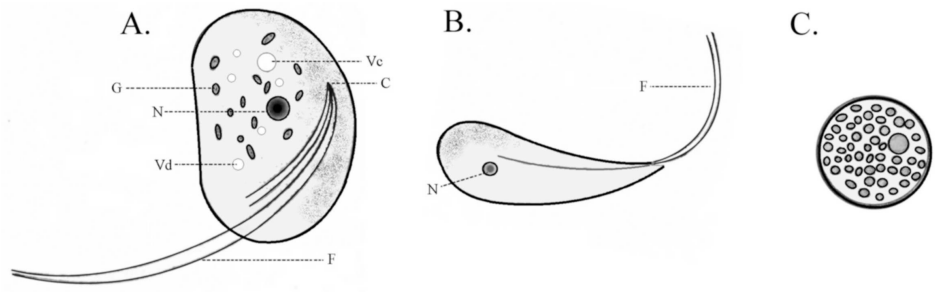


**Figure 3.** *Costia necatrix* (Moroff, 1904). Modified sketches. **A**: Ventral view; four flagella (F); two short and two long arising from a mouth pit, cytostome (C). Central nucleus (N) and vacuoles; a contractile (Vc) and smaller digestive vacuoles (Vd). Several 'randomly distributed granules' (G) in the cytoplasm. Cell size: 8-10 x 15-20 µm. **B**: Side view, cell flattened and pyriform. **C**: Cyst (7-10 µm) containing refractile granules.

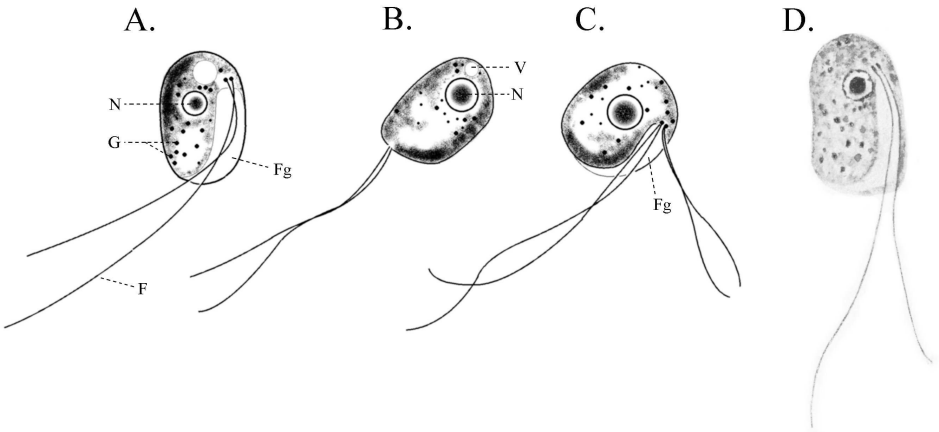


**Figure 4**. Free forms of *Costia necatrix* sensu Andai (1933). Both two and four flagellated individuals. Mean cell size was found to be 7.5 x 10.7 µm (N=100). Nucleus (N). Darkly stained granules with variable size and shape are visible in cytoplasma. Large, flagellar groove visible on the ventral side. A large, single vacuole often visible close to the origin of the flagella. **A**: Ventral view. Bi-flagellated. A large single vacuole above nucleus. Several dark, stained granules (G) visible.; **B**: Dorsal view. Flagellar groove not visible.; **C**: Ventral view, four flagellated cell. **D**. Original sketch by G. Entz (Hartmann, 1917). The figures A - C are re-drawns from Andai (1933), figure D copied from Hartmann (1917).

Davis (1943) observed a flagellated parasite infecting gills and skin of juvenile rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*) in a hatchery in West Virginia (USA). The parasites were assumed to be a *Costia* species, but differed greatly in appearance from *C. necatrix* as described by Henneguy (1883, 1884). The flagellates were pear-shaped (pyriform) with a spiral longitudinal groove. Two pairs of flagella, one short and the other pair longer than the cell body, arose from the rounded anterior part of the cells. Occasionally, only one pair of unequal length was observed. The flagellates were closely attached to the epithelium, and the free swimming form showed spiral movement. No disease, clinical signs or mortality were described.

Despite differences in morphology and movements compared to earlier descriptions of *Costia*, Davis (1943) suggested that the observed flagellates represented a new *Costia* species for which the name *Costia pyriformis* was proposed (Figure 5, p. 13). However, Tavolga and Nigrelli (1947) argued that characters such as cell size and the swimming movement are too variable and that the validity of *C. pyriformis* was dubious. Tavolga and Nigrelli (1947) studied the morphology of *C. necatrix* from different species of ornamental fish. They described the parasites as pleomorph cells harbouring four flagella (two short and two longer; Figure 6, p. 14), and suggested that two-flagellated cells (as described by Andai (1933) and others) were artefacts.

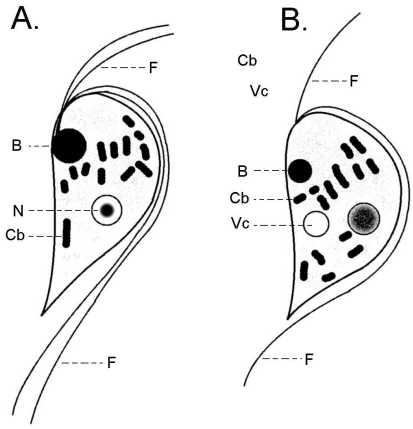


**Figure 5.** *Costia pyriformis* Davis, 1943. Free forms of the parasites as they appear in stained smears. Cell size 9-14 x 5-8 µm. Dark stained blepharoplast (B) and chromatoid bodies (Cb). The figures are modified from sketches made by Davis (1943). **A**. Lateral view. Four flagellated; Two flagella (F) mostly free from the cell body, two longer flagella alongside the cell body pointing in a different direction. Nucleus with a deeply stained karyosome. **B**. Lateral view. A single contractile vacuole (Vc).

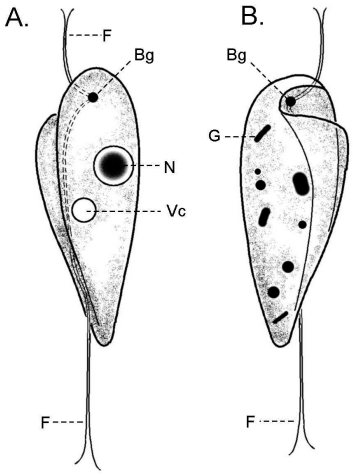


**Figure 6.** *Costia necatrix* sensu Tavolga and Nigrelli (1947). Modified sketches. Flagella have been added to B (as a mirror image of A). Four flagella, two short and two long pointing in different directions. The flagella (F) attached at the end of the groove, arising from a basal granule (Bg). **A**. Left side view (Tavolga and Nigrelli, 1947). Nucleus with darkly stained mass (N) and a contractile vacuole (Vc). **B**. Right side view (Tavolga and Nigrelli, 1947). Cell inclusions are shown as dark, rod-shaped granules (G). Nucleus and vacuole are not visible.

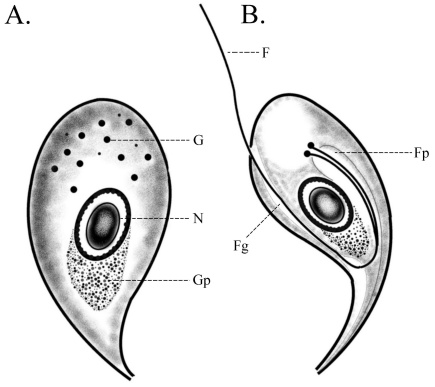


**Figure 7.** *Ichthyobodo necatrix* (syn. *Costia necatrix*) sensu Hollande in Grassé (1952). Redrawn from illustrations made by A. Hollande published in Grassé (1952). **A**. Dorsal view. Several densely stained granules (G) visible in the cell. A mass of granular plasma (Gp) shown below the nucleus (N). **B**. Ventral view. Two flagella (F); one short flagellum hidden in the pocket (Fp) or ventral groove (Fg) and one long flagellum that extend the cell length.

Grassé (1952) concurred with Tavolga and Nigrelli (1947), and considered *C. pyriformis* a synonym of *C. necatrix*. Most authors have accepted this, but Wood (1979) distinguished *Costia necatrix* and *Costia pyriformis* infections in North American salmonids (Wood, 1979). According to Joyon & Lom (1969), trophozoites (attached, parasitic form of *Ichthyobodo*) may wrongly have been described as free forms in the early descriptions, which might explain the atypical pyriform shape of free-swimming forms of *Ichthyobodo* spp. as illustrated by A. Hollande (shown in Grassé, 1952), Davis (1943) and Tavolga & Nigrelli (1947).

The generic name *Costia* Leclerq 1890, proved a junior homonym of *Costia* Kirscner 1867 (Insecta, Hymenoptera). Consequently, a new genus *Ichthyobodo* Pinto 1928, was erected for genus *Costia* Leclerq 1890. Grassé (1952) was the first to review previous descriptions and systematically summarising these parasites using the name *Ichthyobodo necatrix*[1] (Figure 7, p. 14). The parasite was re-named *Ichtyobodo*[2] *necator* by Joyon & Lom (1969) using the genus proposed by Pinto (1928) and the species name *necator* (sensu Henneguy, 1883) amended from *necatrix*.

The binomen *Ichthyobodo necator* is accepted as valid in present nomenclature. However 'Costia' is in widespread use as a common name for these flagellates.

[1] The species name 'necatrix' is a feminine form of 'necator' (Latin noun; murderer, killer)

[2] The name "*Ichthyobodo*" is derived from greece greek "*Ichthyo-*" meaning fish in a combining form, hence the genus name should be spelled *Ichthyobodo* in agreement with Pinto (1928) and Grasse (1952) and not *Ichtyobodo* as given by Joyon & Lom (1969).

# Taxonomy

The taxonomic status of genus *Ichthyobodo* Pinto 1928 syn. *Costia* Leclerq 1890 have varied during time due to available characters obtained by different methods; light microscope, electron microscope and molecular methods. The classification of genus *Ichthyobodo* is summarised in Table 1 (p. 19).

## The early studies: Light microscopical characters

Early systematics and classification of the zooflagellates was based on cell characteristics observed with the use of light microscope. As indicated above, there has been disagreement on the typical number of flagella harboured by these flagellates. Since this character have been given high emphasis, those who consider them biflagellated has placed *Ichthyobodo* Pinto 1928 (named as *Costia* Leclerq 1890) in the family Bodonidae (e.g. Hartmann, 1917; Andai, 1933), while those considering them quadriflagellated assign the genus to the Tetramitidae (e.g. Doflein, 1916; Minchin, 1922; Hall, 1953).

In the classification of the phylum Protozoa by Kudo (1966), the flagellates were placed in class Mastigophora Diesing 1865. The flagellates were further divided into subclasses Phytomastigia (pigmented, chromatophores present) and Zoomastigia (no pigments, chromatophores absent). The genus *Costia* Leclerq 1890 was placed in Zoomastigia among flagellates that possess three or more flagella; Order Polymastigida Blochmann 1884 and further among the quadriflagellates in family Tetramidae Bütschli 1884. Genus *Costia* Leclerq 1890 was diagnosed as ovoid or pyriform flagellates with two short and two long flagella, a central nucleus and a contractile vacuole located posterior in the cells. Kudo (1966) listed two species in the genus; *Costia necatrix* and *C. pyriformis*, both ectoparasites of freshwater fish.

## In the age of electron microscopy

In the middle of the 20th century, transmission electron microscopy (TEM) became available. The first ultrastructural studies of *Ichthyobodo necator* revealed individuals with both two and four flagella (Joyon & Lom, 1966, 1969; Schubert, 1966), hence substantiating previous suggestions that *Ichthyobodo* species are biflagellates and that quadriflagellates are pre-division forms (e.g. Andai, 1933).

In a revised classification of phylum Protozoa (Honigberg *et al.*, 1964), flagellates with kinetoplast were grouped in the order Kinetoplastida Honigberg 1963. The kinetoplast is defined as an argentophobic and Feulgen-positive self-replicating organelle with mitochondrial affinities. Additional characters detected in ultrastructural studies led Vickerman (1976) to revise the classification of the kinetoplastid flagellates. These new characters are only visible in electron microscopy and could not be detected in the early studies with use of light microscope. Important ultrastructural characters of order Kinetoplastida included flagellar structure as axoneme and paraxial rods. Furthermore, an elongated, single mitochondrion containing the kinetoplast may appear as a thread or network of threads in the cell.

Order Kinetoplastida (Honigberg 1963 emend. Vickerman 1976) contained the suborders Trypanosomatina Kent 1880 and Bodonina Hollande 1952. The bodonine flagellates were further subdivided in the families Bodonidae Bütschli 1887 (flagellum free from body surface and cytostome present) and Cryptobiidae (recurrent flagellum attached to the cell body). Family Bodonidae comprised three genera; *Bodo*, *Rhynchomonas* and *Ichthyobodo*. Details regarding ultrastructural characters of the genus *Ichthyobodo* are given in the next chapter (“Ultrastructure”).

## Introduction of molecular characteristics

Molecular methods have made it possible to distinguish different species by their gene sequences and to study phylogenetic relationships among different groups of organisms. Based on molecular phylogenetic studies of small subunit ribosomal RNA (SSU rRNA) and heat shock protein 90 (Hsp90) genes, a revised classification for the class Kinetoplastea has been proposed (Moreira *et al.*, 2004). The kinetoplastids are placed in phylum Euglenozoa Cavalier-Smith 1981, which together with at least 13 other phyla constitute kingdom Protozoa.

Vickerman (in Moreira *et al.*, 2004) subdivided class Kinetoplastida into the two new subclasses Metakinetoplastida and Prokinetoplastida (Figure 8, p. 20). Also, the old concept of family Bodonidae was abandoned and new groups introduced to fit the phylogenetic model. Prokinetoplastina with its single order Prokinetoplastida contain only two genera; *Ichthyobodo* and *Perkinsela*. Genus *Perkinsela* contain one species, *Perkinsela amoebae* Hollande 1980, which is an endosymbiont in amoebae of the genera *Paramoeba* and *Janickina.* However*,* several *Perkinsela amoeba*-like organisms ('PLOs') have recently been detected in other amoebae, *Neoparamoeba* spp. (Dyková *et al.,* 2000; Dyková *et al.,* 2003; Dyková *et al.,* 2008). These 'PLOs' have also been referred to as *Ichthyobodo* related organisms (IRO)(Caraguel *et al.*, 2007).

**Table 1.** Classification of *Ichthyobodo* (syn. *Costia*). The systematic position based on morphological characteristics with use of light microscope (Kudo, 1966), ultrastructure (Vickermann, 1976) and molecular phylogeny (Moreira *et al.*, 2004).

| | **Kudo 1966** | **Vickerman 1976** | **Vickerman in Moreira *et al.*, 2004** |
|---|---|---|---|
| **Phylum** | Protozoa<br>Goldfuss 1818 | Protozoa<br>Goldfuss 1818 | Euglenozoa<br>Cavalier-Smith 1981 |
| **Class** | Mastigophora<br>Diesing 1865 | Kinetoplastea<br>Honigberg, 1963 emend.<br>Vickerman 1976 | Kinetoplastea<br>Honigberg, 1963 emend.<br>Vickerman 1976 |
| **Subclass** | Zoomastigia<br>Doflein 1916 | - | Prokinetoplastina<br>Vickerman 2004 |
| **Order** | Polymastigida<br>Blochmann 1895 | Kinetoplastida<br>Honigberg 1963 emend.<br>Vickerman 1976 | Prokinetoplastida<br>Vickerman 2004 |
| **Suborder** | - | Bodonina<br>Hollande 1952 | - |
| **Family** | Tetramitidae<br>Bütschli 1887 | Bodonidae<br>Bütschli 1887 | - |
| **Genus** | ***Costia***<br>Leclerq 1890 | ***Ichthyobodo***<br>Pinto 1928 | ***Ichthyobodo***<br>Pinto 1928 |

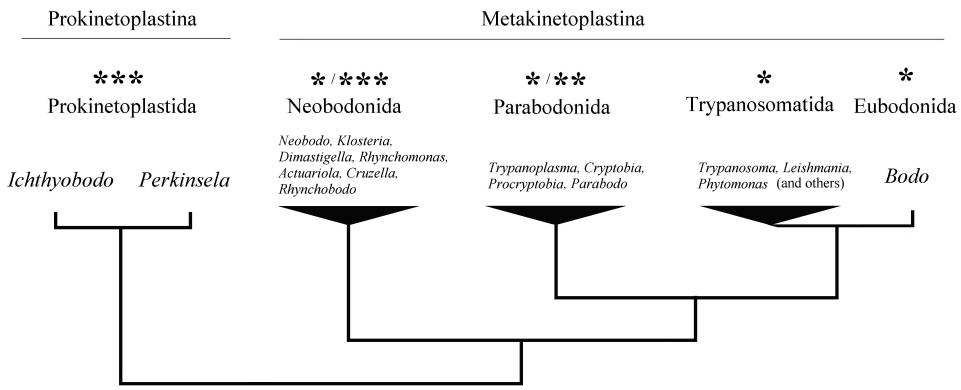


**Figure 8**. Cladogram of the phylogeny of the kinetoplastids, Class Kinetoplastea, after Moreira *et al.* (2004) and Stoeck *et al.* (2005). The original phylogenetic analyses were based on SSU rDNA sequences. Two subclasses; Prokinetoplastina (Order Prokinetoplastida) and Metakinetoplastina (Orders Neobodonida, Parabodonida, Trypanosomatida, Eubodonida). Kinetoplast type is a characteristic feature (Vickerman, 1990), shown as *eukinetoplastic, **pankinetoplastic and ***polykinetoplastic. The genus *Perkinsela* contains a single kinetoplast but with a kinetoplast DNA structure that resembles poly-kinetoplast DNA similar to *Ichthyobodo* spp (Dyková *et al.*, 2003).

## Ultrastructure

The first and most comprehensive ultrastructural studies of flagellates in the genus *Ichthyobodo* were performed by Schubert (1966) on samples from infected ornamental fish (*Carassius auratus*, *Xiphophorus helleri*) from a zoological garden in Stuttgart (Germany) and by Joyon & Lom (1966, 1969) on samples from infected carp (*Cyprinus carpio*) alevins from a fish farm in South Bohemia (Czech republic). The following descriptions of structures and cell organelles are mainly based on these studies.

Both bi-flagellated and occasionally quadriflagellated individuals are evident in TEM images of *Ichthyobodo* trophozoites (Schubert, 1966; Joyon & Lom, 1969). In transverse sections, the flagella contain an axoneme with the normal "9 + 2" microtubule structure. In addition, each flagellum contains a characteristic paraflagellar rod structure (PFR; syn. paraxial rod, paraxonemal rod) that runs alongside the axoneme. Joyon & Lom (1969) showed that the PFR structure is larger in the dorsal flagellum than in the ventral one. This pattern is also apparent in quadriflagellated cells; the two dorsally located flagella show more prominent PFR profiles than the two ventral flagella, which show a relatively contracted PFR area.

Each flagellum originates in a basal body, a kinetosome (Schubert, 1966; Joyon & Lom, 1969). These structures are short and cylindrical with cross-sections showing characteristic structures with triplets of nine fibrils (9 x 3) (Pitelka (1963, pp. 40-41). The kinetosome has previously been referred to as blepharoplast (e.g. Joyon & Lom, 1966) and has also been confused with kinetoplasts (see Vickerman & Preston, 1976).

Kinetoplasts (cf. kinetoplast – mithochondrion, Schubert 1966) appear as ovoid, DNA rich structures within a single elongated mitochondrion, and contain nucleoids with DNA fibrils (Vickerman & Preston, 1976; Lukes *et al.*, 2002). The presence of DNA makes kinetoplasts easily distinguishable from kinetosomes (no DNA content) through staining techniques for light microscopy (Dolan, 2000). The kinetoplasts in both free- and attached forms of

*Ichthyobodo* spp. are clearly visible by light microscope as densely Feulgen or Giemsa stained grains dispersed in the cell. Distribution pattern and morphology of kinetoplasts are important characteristics in the kinetoplastids, used in classification (Moreira *et al.*, 2004; see also Figure 8, p. 20). Different types of kinetoplast structure may be discerned (Vickerman, 1990). The eukinetoplastic type contains a dense mass close to the basal part of the flagella, while pankinetoplastic appear as a more diffuse mass with a more or less clustered distribution. The term polykinetoplastic is used when the kinetoplast is represented by several similarly sized granules, which is characteristic for e.g. genus *Ichthyobodo*.

The kinetoplasts DNA is composed of a network of DNA rings termed maxicircles (molecule numbers in tens) and minicircles (molecule numbers in thousands). The gene expressions of the maxicircles concern the energy metabolism in the cell, homologs to mitochondrial DNAs in higher eukaryotes, while the minicircles DNA contain genes that are encoding the guide RNA which is important for RNA editing (see Lukes *et al.*, 2010). The fine structures of kDNA in different groups of kinetoplastids are morphological distinguishable. These structures have been studied by light microscopic methods of cells stained with DNA dyes (e.g. DAPI, Giemsa) and by TEM (Lukes *et al*., 2002). The different structures have been termed as pro-kDNA, poly-kDNA, pan-kDNA and mega-kDNA. Ultrastructural studies of the polykinetoplastic genus *Ichthyobodo* have revealed kinetoplasts that appear to have poly-kDNA structures (see Joyon & Lom, 1969). The function and structure of kinetoplast DNA (kDNA) are most extensively studied among trypanosomatids in Metakinetoplastina (which includes important human parasites), while knowledge regarding the function and molecular structure of the kinetoplast are scarce for species within Prokinetoplastina; the genera *Ichthyobodo* and *Perkinsela* (Lukes *et al.*, 2010).

The nucleus is rounded and located centrally in the cell. Ultrastructurally the nucleus show a large central nucleolus (Schubert, 1966; Joyon & Lom 1969) and peripheral heterochromatin patches (Joyon & Lom, 1969). Joyon & Lom

(1966, 1969) observed a contractile vacuole located close to the flagellar pocket on the right side of the cell, posterior to the nucleus. They assumed that the contractile vacuole empty its content in the pocket. Another prominent vacuole, or several small, was commonly observed posterior in the cell. These were assumed to represent digestive vacuoles. A Golgi apparatus, recognizable by its dictyosome, is found posterior in the cell, in the area between the contractile vacuole and the putative digestive vacuole(s). Elongated tubular vesicles throughout the cytoplasma are recognized as endoplasmatic reticulum (ER, most commonly granular; rough ER) by Schubert (1966). Large lipid vesicles often appear in contact with the ER (shown in Figure 9, Joyon & Lom, 1969).

Schubert (1966) described the attachment apparatus of *Ichthyobodo* sp. from ornamental fish as finger-like processes that penetrate the host cell. Joyon & Lom (1966, 1969) termed this structure as the cytostome, being part of a cytostomeal complex of tubular fibrils. A cytostome tube extends to the posterior part of the cell and is assumed to function as a sucking organelle and also as a supporting structure of the cell (Joyon & Lom, 1969). The pellicle around the cytostome forms an attachment disc. This cytostomeal complex is referred to in the diagnosis of genus *Ichthyobodo* by Vickerman (1976) as a rostrum like structure surrounding the cytostome. The cytostomeal complex is only apparent in the attached, parasitic forms.

## Hosts and geographical range of *Ichthyobodo* spp.

*Ichthyobodo* spp. have been identified and reported from different host worldwide for more than a century. Most reports predate recent molecular data suggesting that several *Ichthyobodo* spp. exist, and identify their parasites with the then assumed cosmopolitian species, *Ichthyobodo necator* (see Lom & Dyková 1992). Genus *Ichthyobodo* was also assumed to be restricted to freshwater fish (Vickerman, 1976), since the infections known by then were from cultured salmonids, cypriniforms and from different ornamental fish species (eg. poeciliids).

The first record of *Ichthyobodo* sp. infections in fish from the marine environment was from young Chinook salmon (*Oncorhynchus tshawytscha*) in USA (Wood 1968, cited in Ellis & Wooten 1978) and Atlantic salmon (*Salmo salar*) in Scotland (Ellis & Wootten, 1978; Needham & Wootten, 1978). According to Ellis & Wootten (1978), the *Ichthyobodo* species from seawater reared salmon were morphologically identical to *I. necator*; hence they assumed that the salmon had contracted the infections in the hatchery. Consequently the parasites had to be able to survive the seawater transfer, showing a wide salinity tolerance (euryhaline). Subsequent observations of *Ichthyobodo* sp. infections in salmonids in seawater also assumed a freshwater origin (Poppe & Håstein, 1982; Urawa & Kusakari, 1990).

*Ichthyobodo* species from strictly marine fish were first detected on the skin of wild caught flatfish; plaice (*Pleuronectes platessa*) from coastal areas of Scotland (Bullock & Robertson, 1982) and winter flounder (*Pleuronectes americanus*) from bays in Newfoundland (Cone & Wiles, 1984). The parasites were identified as *I. necator.* These flatfish were regarded as possible marine reservoir hosts, a source of *I. necator* infecting farmed salmonids in the sea. In addition, it was suggested that euryhaline flounders such as the winter flounder could acquire *I. necator* from freshwater sources in estuaries. The freshwater origin of *Ichthyobodo 'necator'* infections in marine fish was challenged by Morrison & Cone (1986), who detected *Ichthyobodo* sp. on the

gills of haddock (*Melanogrammus aeglefinus*) caught 120 km offshore from Nova Scotia. Cell shape and size of the attached forms (trophozoites) were similar to the descriptions of *I. necator* from seawater (cf. Cone & Wiles, 1984; Ellis & Wootten, 1978). However, the great distance from freshwater habitats limited the possibility of acquiring *I. necator* from such an environment and from euryhaline hosts. Also, Diamant (1987) described *Ichthyobodo* sp. infections in common dab (*Limanda limanda*) from the North Sea. *Ichthyobodo* sp. detected on strict marine fish such as haddock and common dab was considered evidence for the existence of a true marine form; a likely marine *Ichthyobodo* species (Morrison & Cone, 1986; Diamant, 1987).

*Ichthyobodo* sp. infections on strictly marine hosts have also been observed on farmed fish in Norway; in turbot (*Scophthalmus maximus*), Atlantic cod (*Gadus morhua*), halibut (*Hippoglossus hippoglossus*) and spotted wolffish (*Anarhichas minor*) (Brøderud & Poppe, 1986; Grøntvedt, 2003 cited in Foss *et al.* 2004; Hjeltnes *et al.*, 1989; Rødseth, 1995; Todal *et al.*, 2004). An extended list with records of *Ichthyobodo* infections from marine fish worldwide was provided by Urawa *et al.* (1998). A more updated list is presented in Table 2 (pp. 28-33).

The apparent seawater tolerance of *I. necator* as described by Ellis & Wotten (1978) and the possible existence of a marine *Ichthyobodo* species (Diamant, 1987; Morrison & Cone, 1986) were tested by Urawa & Kusakari (1990). In an experimental study they showed that parasites identified as *I. necator* on chum salmon (*Oncorhynchus keta*) survived seawater transfer and proliferated on this host in the sea. However, a cross-infection challenge test with *Ichthyobodo necator* from chum salmon and a morphological similar *Ichthyobodo* sp. from a strict marine host, the Japanese flounder (*Paralichthys olivaceus*), suggested that these hosts were not susceptible to the other parasite. Hence, Urawa and Kusakari (1990) were the first to experimentally demonstrate the likely occurrence of two distinct *Ichthyobodo* species, differing in their host preferences.

More recent studies based on ribosomal RNA gene sequences have revealed a higher complexity of species within genus *Ichthyobodo* (Todal, *et al.*, 2004; Callahan, *et al.*, 2005). A genotype referred to as *Ichthyobodo* sp. I from Atlantic salmon and three-spined sticklebacks (*Gasterosteus aculeatus*) in freshwater in Norway was assumed to represent *Ichthyobodo necator* sensu Henneguy (1883); described from brown trout in fresh water. A clearly different genotype considered a separate species, designated *Ichthyobodo* sp. II, appeared to be euryhaline since it was detected in the gills of salmon from both freshwater, brackish and seawater.

The hosts of *Ichthyobodo* spp. (mostly recorded as *I. necator*) from both freshwater and seawater worldwide were reviewed by Robertson (1985) and Urawa *et al.*, (1998), but there are many later records. Infections by members of genus *Ichthyobodo* have so far been reported from more than 60 different host species in both freshwater and seawater (Table 2, pp. 28-33). Most records concern juvenile or adult fish, but infections by *I. necator* on fish eggs have also been observed. Hlond (1963) observed the parasite on eggs of carp, and Houghton & Bennett (1982) reported infections on rainbow trout eggs. Several studies also report infections on yolksac larve of carp and salmonids (Bauer, 1959; Henneguy, 1883; Hlond, 1963, Houghton & Bennett, 1982; Skrudland, 1987). Houghton & Bennet (1982) considered stripped broodstock as the source of such infection. Survival of the parasites on eggs are leading to *Ichthyobodo* infection of yolksac larvae and later among fry.

Among vertebrates, fish hosts clearly dominate, but *I. necator* infections have also been recorded from amphibian tadpoles (anurans and salamanders) (Bauer, 1959; Vickerman, 1976; Becker, 1977). In addition, *Ichthyobodo*-like flagellates have been detected on invertebrates; octopuses (Forsythe *et al.*, 1991) and as a hypersymbiont (identified as *I. necator*) on the tegument of the monogenean *Gyrodactylus salaris* from an *I. necator* infected Atlantic salmon parr (Bakke *et al.*, 2006).

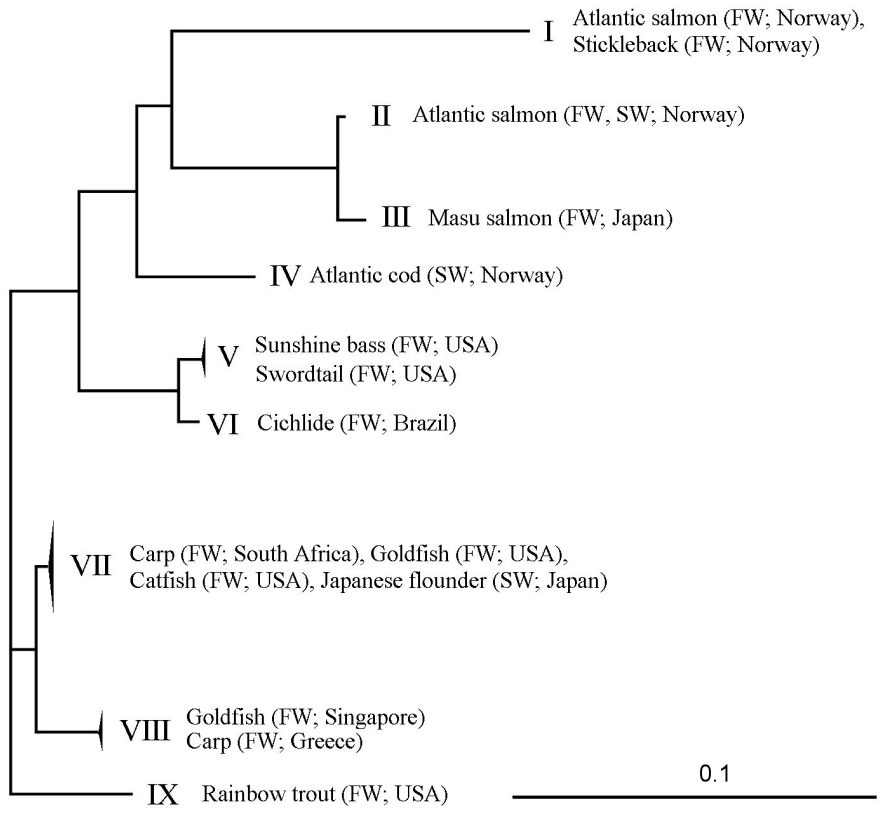


**Figure 9.** Phylogram of Prokinetoplastida, genus *Ichthyobodo*. Unrooted tree constructed with use of Bayesian method (redrawn and modified from Callahan *et al.*, 2005). The branch lengths indicate the relative evolutionary distance between *Ichthyobodo* isolates based on SSU rDNA sequences. The nine different genotypes or species of *Ichthyobodo* included designated as I – IX. Origins of the isolates are shown as common name of the host, habitat (FW, freshwater; SW, seawater) and country. Scale bar represent 0.1 nucleotide substitution per site.

**Table 2.** Fish hosts and geographical distribution of *Ichthyobodo* spp. (syn. *Costia*). Host habitat given as fresh- (FW), brackish- (BW) and seawater (SW).

| COUNTRY | HOST | COMMON NAME | WATER | SYMBIONT | REFERENCES |
|---|---|---|---|---|---|
| **AFRICA** | | | | | |
| Nigeria | **Siluriformes** | | | | |
| | *Heterobranchus longifilis* | Sampa | FW | *Ichthyobodo* sp. | Omeji *et al.*, 2010 |
| | *Clarias gariepinus* | Catfish | FW | *Ichthyobodo* sp. | Omeji *et al.*, 2011 |
| South Africa | **Cypriniformes** | | | | |
| | *Cyprinus carpio* | Common carp | FW | *Ichthyobodo* sp. | Todal *et al.*, 2004 |
| Uganda | **Perciformes** | | | | |
| | *Oreochromis niloticus* | Nile Tilapia | FW | *Ichthyobodo* sp. | Akoll *et al.*, 2012 |
| | **Siluriformes** | | | | |
| | *Clarias gariepinus* | Catfish | FW | *Ichthyobodo* sp. | Isaksen *et al.* (unpubl.) |
| **AMERICA** | | | | | |
| Brazil | **Perciformes** | | | | |
| | *Apistogramma* sp. | Cichlids | FW | *Ichthyobodo* sp. | Todal *et al.*, 2004 |
| | **Osteoglossiformes** | | | | |
| | *Arapaima gigas* | Arapaima | FW | *Ichthyobodo* sp. | Araujo *et al.*, 2009 |
| Canada | **Gadiformes** | | | | |
| | *Melanogrammus aeglefinus* | Haddock | SW | *Ichthyobodo* sp. | Morrison & Cone, 1986 |
| | **Pleuronectiformes** | | | | |
| | *Pleuronectes americanus* | Winter flounder | SW | *I. necator* | Cone & Wiles, 1984 |
| | **Salmoniformes** | | | | |
| | *Oncorhynchus mykiss* | Rainbow trout | FW | *I. necatrix* | Ostland & Byrne, 1995 |
| | *Salvelinus fontinalis* | Brook trout | FW | *Costia necatrix* | Savage, 1935 |
| | *Salmo salar* | Atlantic salmon | SW | *I. necator* | Speare, 2003 |
| Uruguay | **Mugiliformes** | | | | |
| | *Mugil platanus* | Mullet | SW | *I. necator* | Carnevia & Speranza, 2003 |
| USA | **Cypriniformes** | | | | |
| | *Cyprinus carpio* | Common carp | FW | *Ichthyobodo* sp. | Callahan *et al.*, 2005 |
| | *Carassius auratus* | Goldfish | FW | *Ichthyobodo* sp. | Callahan *et al.*, 2005 |
| | **Cyprinodontiformes** | | | | |
| | *Fundulus seminolis* | Seminole killifish | FW | *Ichthyobodo* sp. | DiMaggio *et al.*, 2008 |
| | *Xiphophorus hellerii* | Green swordtail | FW | *Ichthyobodo* sp. | Callahan *et al.*, 2005 |
| | *Poecilia reticulata* | Guppy | FW | *Costia necatrix* | Tavolga & Nigrelli, 1947 |
| | *Poecilia latipinna* | Sailfin molly | FW | *Ichthyobodo* sp. | Tobler *et al.*, 2005 |
| | *Poecilia formosa* | Amazon molly | FW | *Ichthyobodo* sp. | Tobler *et al.*, 2005 |
| | *Xiphophorus maculatus* | Platyfish | FW | *Costia necatrix* | Tavolga &Nigrelli, 1947 |

**Table 2** (continued)

| COUNTRY | HOST | COMMON NAME | WATER | SYMBIONT | REFERENCES |
|---|---|---|---|---|---|
| USA | **Perciformes** | | | | |
| | *Morone* spp. hybrid | Sunshine bass | FW | *I. necator* | Callahan *et al.*, 2002 |
| | *Sarotherodon melanotheron* | Blackchin tilapia | FW | *Costia necatrix* | Tavolga & Nigrelli, 1947 |
| | *Rachycentron canadum* | Cobia | SW | *Ichthyobodo* sp. | Bunkley-Williams & Williams, 2006 |
| | *Chaetodipterus faber* | Atlantic spadefish | SW | *Ichthyobodo*-like | Beck *et al.*, 1996 |
| | *Mugil cephalus* | Flathead grey mullet | SW | *Ichthyobodo* sp. | Urawa *et al.*, 1998 |
| | **Pleuronectiformes** | | | | |
| | *Paralichthys olivaceus* | Japanese flounder | SW | *Ichthyobodo* sp. | Brock *et al.*, 1993 |
| | **Salmoniformes** | | | | |
| | *Oncorhynchus mykiss* | Rainbow trout | FW | *Ichthyobodo* sp. | Callahan *et al.*, 2005 |
| | *Oncorhynchus mykiss* | Rainbow trout | FW | *Costia pyriformis* | Davis, 1943 |
| | *Salvelinus fontinalis* | Brook trout | FW | *Costia pyriformis* | Davis, 1943 |
| | *Oncorhynchus aquabonita* | Golden trout | FW | *Costia pyriformis* | Heckman, 1974 |
| | *Oncorhynchus mykiss* | Rainbow trout | FW | *I. necator* | Schisler *et al.*, 1999 |
| | *Oncorhynchus tshawytscha* | Chinook salmon | FW | *Ichthyobodo* sp. | Meyers, 2007 |
| | *Salmo trutta* | Brown trout | FW | *I. necator* | Schisler *et al.*, 1999 |
| | **Siluriformes** | | | | |
| | *Ictalurus punctatus* | Channel catfish | FW | *Ichthyobodo* sp. | Callahan *et al.*, 2005 |
| | *Ictalurus punctatus* | Channel catfish | FW | *I. necator* | Miyazaki *et al.*, 1986 |
| **ASIA** | | | | | |
| Iran | **Perciformes** | | | | |
| | *Astronotus ocellatus* | Oscar | FW | Ichthybodo sp. | Mohammadi *et al*. 2012 |
| | *Symphysodon discus* | Red discus | FW | Ichthybodo sp. | Mohammadi *et al*. 2012 |
| Japan | **Dactylopteriformes** | | | | |
| | *Sebastes schlegelii* | Korean rockfish | SW | *Ichthyobodo* sp. | Urawa *et al.*, 1998 |
| | **Perciformes** | | | | |
| | *Oplegnathus punctatus* | Spotted knifejaw | SW | *Ichthyobodo* sp. | Urawa *et al.*, 1998 |
| | **Pleuronectiformes** | | | | |
| | *Paralichthys olivaceus* | Japanese flounder | SW | *Ichthyobodo* sp. | Urawa & Kusakari, 1990 |
| | **Salmoniformes** | | | | |
| | *Oncorhynchus masou* | Masu salmon | FW | *Ichthyobodo* sp. | Todal *et al.*, 2004 |
| | *Oncorhynchus keta* | Chum salmon | FW | *I. necator* | Urawa & Kusakari, 1990 |
| | *Oncorhynchus keta* | Chum salmon | SW | *I. necator* | Urawa & Kusakari, 1990 |
| | *Oncorhynchus gorbuscha* | Pink salmon | FW | *I. necator* | Urawa & Awakura, 1994 |

**Table 2** (continued)

| COUNTRY | HOST | COMMON NAME | WATER | SYMBIONT | REFERENCES |
|---|---|---|---|---|---|
| Japan | **Salmoniformes** | | | | |
| | *Oncorhynchus nerka* | Sockeye salmon | FW | *I. necator* | Urawa & Awakura, 1994 |
| | *Oncorhynchus masou* | Masu salmon | FW | *I. necator* | Urawa & Awakura, 1994 |
| | **Tetraodontiformes** | | | | |
| | *Takifugu rubripes* | Japanese pufferfish | SW | *Ichthyobodo* sp. | Urawa *et al.*, 1998 |
| Philippines | **Cypriniformes** | | | | |
| | *Hypophthalmichthys nobilis* | Bighead carp | FW | *Ichthyobodo* sp. | Lumanlan *et al.*, 1992 |
| | *Mylopharyngodon piceus* | Black carp | FW | *Ichthyobodo* sp. | Lumanlan *et al.*, 1992 |
| | *Carassius auratus* | Goldfish | FW | *Ichthyobodo* sp. | Lumanlan *et al.*, 1992 |
| Singapore | **Cypriniformes** | | | | |
| | *Carassius auratus* | Goldfish | FW | *Ichthyobodo* sp. | Todal *et al.*, 2004 |
| Sri Lanka | **Cypriniformes** | | | | |
| | *Capeota and Puntius spp* | Barbs | FW | *I. necator* | Thilakaratne *et al.*, 2003 |
| | *Cyprinus carpio* | Common carp | FW | *I. necator* | Thilakaratne *et al.*, 2003 |
| | **Cyprinodontiformes** | | | | |
| | *Poecilia reticulata* | Guppy | FW | *I. necator* | Thilakaratne *et al.*, 2003 |
| | *Poecilia sphenops* | Molly | FW | *I. necator* | Thilakaratne *et al.*, 2003 |
| **AUSTRALIA** | | | | | |
| Australia | **Perciformes** | | | | |
| | *Bidyanus bidyanus* | Silver perch | FW | *I. necator* | Read *et al.*, 2007 |
| | **Clupeiformes** | | | | |
| | *Sardinella lemuru* | Scaly mackerel | SW | *I. necator* | Humphrey, 1995 |
| **EUROPE** | | | | | |
| Austria | **Salmoniformes** | | | | |
| | *Salvelinus fontinalis* | Brook trout | FW | *Costia necatrix* | Franke, 1908 |
| | *Salmo salar* | Atlantic salmon | FW | *Costia necatrix* | Franke, 1908 |
| | *Salmo trutta* | Brown trout | FW | *Costia necatrix* | Franke, 1908 |
| Austria | **Salmoniformes** | | | | |
| | *Oncorhynchus mykiss* | Rainbow trout | FW | *Costia necatrix* | Rydlo, 1984 |
| Belgium | **Perciformes** | | | | |
| | *Perca fluviatilis* | European perch | FW | *I. necator* | Grignard *et al.*, 1996 |
| Bosna and Herzegovina | **Salmoniformes** | | | | |
| | *Oncorhynchus mykiss* | Rainbow trout | FW | *Costia necatrix* | Zitnan & Cankovic, 1970 |
| Czech | **Cypriniformes** | | | | |
| | *Cyprinus carpio* | Common carp | FW | *I. necator* | Joyon & Lom, 1969 |
| | *Tinca tinca* | Tench | FW | *I. necator* | Svobodova & Kolarova, 2004 |

**Table 2** (continued)

| COUNTRY | HOST | COMMON NAME | WATER | SYMBIONT | REFERENCES |
|---|---|---|---|---|---|
| Denmark | **Salmoniformes** | | | | |
| | *Oncorhynchus mykiss* | Rainbow trout | FW | *I. necator* | Buchmann &Bresciani, 1997 |
| Finland | **Perciformes** | | | | |
| | *Stizostedion lucioperca* | Pike-perch | FW | *I. necator* | Rahkonen, 1994 |
| | *Perca fluviatilis* | European perch | FW | *I. necator* | Marcogliese *et al.*, 2011 |
| | **Salmoniformes** | | | | |
| | *Salmo salar* | Atlantic salmon | FW | I.necator | Rintamaki-Kinnunen, 1997 |
| | *Salmo trutta* | Brown trout | FW | I.necator | Rintamaki-Kinnunen, 1997 |
| France | **Salmoniformes** | | | | |
| | *Salmo trutta* | Brown trout | FW | *Bodo necator* | Henneguy, 1883 |
| Germany | **Cypriniformes** | | | | |
| | *Tinca tinca* | Tench | FW | *Costia necatrix* | Hofer, 1904 |
| | *Cyprinus carpio* | Common carp | FW | *Costia necatrix* | Benisch, 1936 |
| | *Carassius auratus* | Goldfish | FW | *Tetramitus nitschei* | Nietsche & Weltner, 1894 |
| | *Carassius auratus* | Goldfish | FW | *Costia necatrix* | Schubert, 1966 |
| | **Cyprinodontiformes** | | | | |
| | *Xiphophorus hellerii* | Green swordtail | FW | *Costia necatrix* | Schubert, 1966 |
| | **Salmoniformes** | | | | |
| | *Oncorhynchus mykiss* | Rainbow trout | FW | *Costia necatrix* | Doflein, 1916 |
| | *Salmo trutta* | Brown trout | FW | *Costia necatrix* | Moroff, 1904 |
| Greece | **Cypriniformes** | | | | |
| | *Cyprinus carpio* | Common carp | FW | *Ichthyobodo* sp. | Callahan *et al.*, 2005 |
| Hungary | **Acipenseriformes** | | | | |
| | *Acipenser ruthenus* | Sterlet sturgeon | FW | *I. necator* | Baska, 1999 |
| Ireland | **Salmoniformes** | | | | |
| | *Salmo salar* | Atlantic salmon | SW | *Ichthyobodo* sp. | Rodger *et al.*, 2011 |
| | *Salmo salar* | Atlantic salmon | | *Ichthyobodo*-like | Bermingham & Mulcahy, 2006 |
| Iceland | **Gadiformes** | | | | |
| | *Gadus morhua* | Atlantic cod | SW | *Ichthyobodo* sp. | Kristmundsson *et al.*, 2004 |
| Norway | **Gadiformes** | | | | |
| | *Gadus morhua* | Atlantic cod | SW | *I. necator* | Hjeltnes *et al.*, 1989 |
| | *Gadus morhua* | Atlantic cod | SW | *Ichthyobodo* sp. | Todal *et al.*, 2004 |
| | **Gasterosteiformes** | | | | |
| | *Gasterosteus aculeatus* | Three-spined stickleback | FW | *I. necator* | Bristow, 1993 |

**Table 2** (continued)

| COUNTRY | HOST | COMMON NAME | WATER | SYMBIONT | REFERENCES |
|---|---|---|---|---|---|
| Norway | **Perciformes** | | | | |
| | *Gobiusculus flavescens* | Two-spotted goby | SW | *Ichthyobodo* sp. | Urawa *et al.*, 1998 |
| | *Anarhichas minor* | Spotted wolffish | SW | *I. necator* | Foss *et al.*, 2004 |
| | *Labrus bergylta* | Ballan wrasse | SW | *Ichthyobodo* sp. | Askeland & Karlsbakk, 1999 |
| | **Pleuronectiformes** | | | | |
| | *Scophthalmus maximus* | Turbot | SW | *I. necator* | Brøderud &Poppe, 1986 |
| | *Hippoglossus hippoglossus* | Atlantic halibut | SW | *Ichthyobodo* sp. | Rødseth, 1995 |
| | **Salmoniformes** | | | | |
| | *Salvelinus alpinus* | Char | FW | *I. necator* | Brun & Bornø, 2010 |
| | *Salmo salar* | Atlantic salmon | SW | *I. necator* | Poppe & Håstein, 1982 |
| | *Salmo salar* | Atlantic salmon | FW | *I. necator* | Todal *et al.*, 2004 |
| | *Salmo salar* | Atlantic salmon | FW | *Ichthyobodo* sp. | Todal *et al.*, 2004 |
| | *Salmo salar* | Atlantic salmon | BW | *Ichthyobodo* sp. | Todal *et al.*, 2004 |
| | *Salmo salar* | Atlantic salmon | SW | *Ichthyobodo* sp. | Todal *et al.*, 2004 |
| Poland | **Cypriniformes** | | | | |
| | *Cyprinus carpio* | Common carp | FW | *Costia necatrix* | Hlond, 1963 |
| Portugal | **Perciformes** | | | | |
| | *Coris julis* | Rainbow wrasse | SW | *Ichthyobodo* sp. | Menezes, 1992 |
| | *Dicentrarchus labrax* | European seabass | SW | *Ichthyobodo* sp. | Santos, 1996 |
| UK | **Anguilliformes** | | | | |
| | *Anguilla anguilla* | European eel | FW | *I. necator* | McGuigan & Sommerville, 1985 |
| | **Gadiformes** | | | | |
| | *Melanogrammus aeglefinus* | Haddock | SW | *Ichthyobodo* sp. | Treasurerer, 2007 |
| | **Perciformes** | | | | |
| | *Centrolabrus exoletus* | Rock cook | SW | *Ichthyobodo* sp. | Costello *et al.*, 1996 |
| | *Symphodus melops* | Corkwing | SW | *Ichthyobodo* sp. | Costello *et al.*, 1996 |
| | *Ctenolabrus rupestris* | Goldsinny-wrasse | SW | Costia sp. | Treasurer, 1997 |
| | *Centrolabrus exoletus* | Rock cook | SW | Costia sp. | Treasurer, 1997 |
| | *Symphodus melops* | Corkwing | SW | Costia sp. | Treasurer, 1997 |
| | *Labrus bergylta* | Ballan wrasse | SW | Costia sp. | Treasurer, 1997 |
| | *Labrus mixtus* | Cuckoo wrasse | SW | Costia sp. | Treasurer, 1997 |
| | **Pleuronectiformes** | | | | |
| | *Pleuronectes platessa* | European plaice | SW | *I. necator* | Bullock & Robertson, 1982 |
| | *Limanda limanda* | Common dab | SW | *Ichthyobodo* sp. | Diamant, 1987 |

**Table 2** (continued)

| COUNTRY | HOST | COMMON NAME | WATER | SYMBIONT | REFERENCES |
|---|---|---|---|---|---|
| Norway | **Salmoniformes** | | | | |
| | *Salmo trutta* | Brown trout | FW | *I. necator* | Bruno, 1992 |
| | *Salmo salar* | Atlantic salmon | SW | *Ichthyobodo* sp. | Bruno, 1992 |
| | *Salmo salar* | Atlantic salmon | SW | *I. necator* | Ellis & Wootten, 1978 |
| | *Salmo salar* | Atlantic salmon | FW | *I. necator* | Robertson, 1979 |
| | *Oncorhynchus mykiss* | Rainbow trout | FW | *I. necator* | Robertson, 1979 |
| Spain | **Perciformes** | | | | |
| | *Sparus aurata* | Gilthead Sea bream | SW | *Ichthyobodo* sp. | Alvarez - Pellitero *et al.*, 1995 |
| | **Salmoniformes** | | | | |
| | *Salmo trutta* | Brown trout | FW | *I. necator* | Castillo *et al.*, 1991 |
| Sweden | **Salmoniformes** | | | | |
| | *Salmo salar* | Atlantic salmon | FW | *Costia necatrix* | Johansson, 1978 |
| | *Salmo trutta* | Brown trout | FW | *Costia necatrix* | Johansson, 1978 |
| Turkey | **Salmoniformes** | | | | |
| | *Oncorhynchus mykiss* | Rainbow trout | FW | *I. necator* | Balta *et al.*, 2008 |
| | *Salmo trutta* | Brown trout | FW | *I. necator* | Balta *et al.*, 2008 |
| | *Salvelinus fontinalis* | Brook trout | FW | *I. necator* | Balta *et al.*, 2008 |

## The biology of *Ichthyobodo* spp.

*Ichthyobodo* spp. are considered obligate ectoparasites (Bauer, 1959; Becker, 1977). That is, they cannot subsist or multiply without an appropriate host. The parasites disappear from a dead host (Henneguy, 1883) and have been reported to die after 30 - 60 minutes in the free-swimming form outside a host (Henneguy, 1883; Hofer, 1904; Amlacher, 1970). However, there are reports that describe survival of *Ichthyobodo* sp. in sediments for several days (Houghton & Bennett, 1982) or on dead hosts for more than 30 hours (Benisch, 1936). Tavolga and Nigrelli (1947) observed that the flagellates were able to survive and multiply in sediment, while feeding on decaying fish tissue. Hence a saprophagous phase in the life history of *Ichthyobodo* sp. was suggested. Houghton & Bennet (1982) observed that *Ichthyobodo* sp. reproduced by longitudinal cell division on both host (*Oncorhynchus mykiss*) and in sediments. A process of encystment in 3-4 days old sediments at a high water temperature (21°C) was also noted.

Cyst stages are common among kinetoplastids. Occurrence and the formation have been described for species within Neobodonidae, Parabodonidae and Trypanosomatida (Brooker & Ogden, 1972; Vickerman, 1978; Almeida Takata *et al.*, 1996; Gómez *et al.*, 2010). The first description of possible *Ichthyobodo* cyst was given by Moroff (1904). He described the encystment and suggested that such cysts might be a source for re-infection at a later moment. Robertson (1985) presented photos of possible, oval shaped *Ichthyobodo* cysts. However, evidence is scant and inconsistent, and Robertson (1985) concluded that further studies are required to confirm the ability of members in genus *Ichthyobodo* to produce cysts. Vickerman (1990) included encystment as a diagnostic character for the different genera of kinetoplastids, and placed genus *Ichthyobodo* among kinetoplastids with no cyst formation.

It is now generally accepted that the basic form of *Ichthyobodo* spp. is bi-flagellated and that the flagellates occurs in two forms; a free-swimming form and an attached parasitic form (trophozoite) (Lom & Dykova, 1992). The life-

cycle of *Ichthyobodo* spp. is simple and the free-swimming forms are able to infect new hosts by direct transmission. Mechanisms for recombination are unknown in genus *Ichthyobodo*. The parasites multiply by binary fission and the appearance of specimens with four flagella is considered a pre-division stage (Andai, 1933; Lom & Dyková, 1992). Only asexual reproduction has been suggested (Bauer, 1959).

*Ichthyobodo 'necator'* have been reported to survive and multiply on different hosts in a wide range of pH levels (4.5 – 7.5) and temperatures (2-38°C) (see Robertson, 1985). However, these observations now must be interpreted with caution, since they are likely to refer to many different *Ichthyobodo* spp. Different species may well show particular restrictions in their environmental preferences.

The free forms of the flagellates are easy to detect in fresh smears with use of light microscope (magnification x400, personal observation). The cells appear flattened with rounded or oval shape. Their movements are impulsively rapid with turns and constantly changes in directions. The free-swimming form is important for spread and colonization of new hosts. It has been suggested that *Ichthyobodo* spp. attach to new host cells with their flagella (Schubert, 1966). When attached to the uppermost epidermal cells of skin or gills of a host, the flagellates are motionless and the cell shape is more elongated and pyriform than in the free form. This transformation from a free to an attached feeding form (trophozoite) is completed within few seconds (Lom & Dyková, 1992). The parasite penetrates the surface of the host cell with the cytostome process and feed on cytoplasmic content (Schubert, 1966; Joyon & Lom, 1969; Roubal *et al.*, 1987).

## Ichthyobodosis

During the last decades, there has been almost an exponential growth in fish farming industry worldwide. In farmed fish from most regions, *Ichthyobodo* spp. infections have been found responsible for disease and mortalities (Robertson, 1985; Urawa, 1995b; Urawa *et al.*, 1991; 1998, Woo, 2006; Mitchell & Rodger, 2011) contributing to economical loss and reduced fish welfare. Ichthyobodosis is regarded as one of the most damaging parasitic diseases among farmed salmon and is probably the major cause of mortality among salmonid fry (Robertson, 1985; Sterud, 1999).

Studies have shown that *Ichthyobodo* spp. spread rapidly between hosts in fish farms, most likely by both direct contact or through free-swimming parasites (Urawa, 1996). *Ichthyobodo* infections commonly show fluctuations in flagellate abundance in hatchery reared salmonids. The most prominent peak of infection usually occurs among first-feeders (fry), indicating that younger fish are more susceptible to infection than older fish (Franke, 1908; Robertson, 1979; Wootten & Smith, 1980; Rintamaki-Kinnunen & Valtonen, 1997).

Heavy infections may occur when conditions favour the parasites. Poor rearing conditions such as low water flow and high crowding densities are considered particularly important (Schäperclaus, 1992; Urawa, 1995a;). Several *Ichthyobodo* trophozoites may attach to a single epithelium cell and a density of 30 000 parasites per $mm^2$ have been estimated on skin and fins of heavily infected juvenile tiger puffer, *Takifugu rubripes* (Urawa *et al.*, 1998). Massive infections on skin and gills can cause epithelial hyperplasia or hypertrophy and may result in severe or fatal osmoregulatory or respiratory problems (see reviews: Lom & Dyková, 1992; Urawa *et al.* 1998).

There have also been described several non-specific clinical sign of severe and prolonged *Ichthyobodo* spp. infections, including “flashing”, lethargic behaviour, listlessness, loss of appetite and increased mortality (Poppe & Håstein, 1982; Robertson, 1985; Miyazaki *et al.*, 1986; Woo & Poynton, 1995).

A “flashing” behaviour is common among pen- or tank reared fish with heavy *Ichthyobodo* infection on the skin. The “flashing” is produced by fish with silvery sides when rubbing against solid surfaces, due to the irritation caused by the infection. A common clinical sign of ichthyobodosis is also discoloration of the fish skin, which appear as a greyish layer that cover a large area of the external surface of the fish (example shown in Figure 10, p. 37). Such discoloration is a clinical sign that also has been described for other ectoparasitic infections (e.g. *Trichodina* spp. infections; Khan, 1991). The greyish layer is a result of cellular destruction and excessive mucus production (Robertson *et al.* 1981; Roubal *et al.* 1987).

Sodium chloride (Moroff, 1904) and formalin (Leger, 1909) have been used in treatment for *ichthyobodo*sis for more than a century. Formalin is still the most common and effective therapeutic used as treatment of *Ichthyobodo* infections in hatchery reared salmonids and farmed marine fish (Tojo *et al.*, 1994; Bergh *et al.*, 2001).

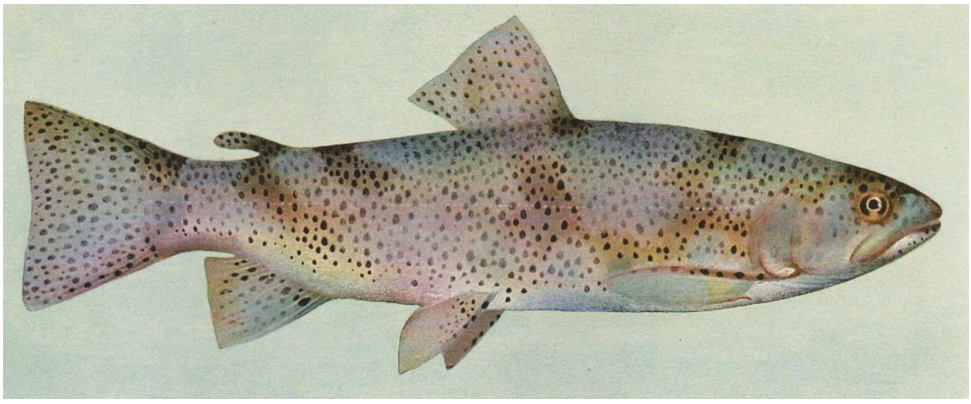

**Figure 10.** Rainbow trout with ichthyobodosis, showing typical blue-grey turbid covering on the skin (from Hofer 1904).

## Norwegian fish farming

The aquaculture industry in Norway produced more than 1 000 000 metric tons (round weight) in 2010 and the production volume is expected to increase. Total value of slaughtered farmed fish during 2010 was approximately 31 billion NOK. The most important farmed fish in Norway are salmonids (994 000 tons); Atlantic salmon (*Salmo salar*; 95 %), rainbow trout (*Oncorhynchus mykiss*; 5%), trout (*Salmo trutta*; < 1‰) and Arctic char (*Salvelinus alpinus*; < 1‰). Total production of marine fish during 2010 were 23 000 tons. The marine species include Atlantic cod (*Gadus morhua*; 92%), Atlantic halibut (*Hippoglossus hippoglossus*; 7%) and others (1%); spotted wolfish (*Anarhichas minor*) and turbot (*Scophthalmus maximus*). The statistics are obtained from Directorate of fisheries (www.fiskeridir.no) with use of official data from December 2011.

The aquaculture industries in Norway suffer losses due to several infectious diseases. According to “Fish Health Reports” by Norwegian Veterinary Institute (NVI Fish Health Report; www.vetinst.no), *Ichthyobodo* spp. are among the most common parasites in Norwegian fish production. Despite that flagellates from genus *Ichthyobodo* have been reported as causative agents of disease and mortalities, infections with these parasites among cultured fish in Norway are only occasionally reported due to ineffective methods for detection (NVI Fish Health Report; www.vetinst.no).

*Salmonids*

Heavy *Ichthyobodo* spp. on farmed salmonids has been reported as problem in both hatcheries and seawater pens. *Ichthyobodo* infections in salmonid production occur in all of Norway, but are more frequently reported from Northern Norway (see NVI Farmed Fish Health Report, 2009; www.vetinst.no).

Gill diseases in seawater reared Atlantic salmon have become an increasing problem in Norwegian aquaculture. Several different infectious agents (including *Ichthyobodo* sp.) have been associated with gill pathology such as epidermal hypertrophy, hyperplasia, inflammation and necrosis of gill tissues

(Poppe & Håstein, 1982; Steinum *et al.*, 2010; Nylund *et al.*, 2011; Rodger *et al.* 2011). Heavy *Ichthyobodo* sp. infections on the gills of seawater reared Atlantic salmon in Norway was first recorded by Poppe & Håstein (1982). Peak mortality occurred among post-smolt during summer and autumn, 10-12 weeks after sea transfer. Histological examination of gill tissues from diseased fish revealed gill inflammation and heavy infections of *Ichthyobodo* sp., hence the disease was diagnosed as ichthyobodosis. However since proliferative gill inflammation (PGI) has also been observed without heavy *Ichthyobodo* sp. infections, it has not been proven that infections with these flagellates are responsible for the pathology and disease (Steinum *et al.* 2010; Nylund *et al.* 2011).

*Marine fish*

*Ichthyobodo* infections have also been associated with gill disease in Norwegian marine fish production (NVI Fish Health Report; www.vetinst.no).

*Ichthyobodo* infections on farmed cod in Norway were first described by Hjeltnes *et al.* (1989). The flagellates were detected on the gills of weakened pen reared cod and later on the gills of juveniles suffering high mortality. Since the parasites occurred in large numbers they were assumed to contribute to the poor condition and mortality of the fish.

Parasitic diseases have been reported as the most severe problem in spotted wolfish production, and most common are *Ichthyobodo* sp. and *Trichodina* sp. infections on skin and gills of juvenile fish (Foss *et al.*, 2004). However the effects of *Ichthyobodo* sp. infections in juvenile wolfish have so far not been studied.

The first reports of *Ichthyobodo* infection among turbot (*Scophthalmus maximus*) in Norway was described by Brøderud & Poppe (1986) from tank reared fish. Heavy *Ichthyobodo* infection was detected on the gills of juvenile turbot. However, histological examination of the gill tissue could not reveal any epidermal changes like hypertrophia or hyperplasia.

# AIMS OF STUDY

Two species have been assumed to cause ichthyobodosis; *Ichthyobodo necator* among freshwater fish and an undescribed *Ichthyobodo* sp. among marine fish and salmonids in seawater (Lom & Dyková, 1992). Recent studies have indicated a higher complexity of genus *Ichthyobodo* (Todal *et al.*, 2004; Callahan *et al.*, 2005), and it is likely that previous reports of these parasites actually involved several different *Ichthyobodo* species.

The overall aim of this project is to identify and characterize different *Ichthyobodo* species and to develop effective and sensitive methods for detection and identification of the parasites.

The principal aims:

- Identify and describe *Ichthyobodo* spp. from farmed fish
- Develop effective and sensitive molecular assays for detection and identification of *Ichthyobodo* spp.
- Identify the *Ichthyobodo* spp. responsible for ichthyobodosis in the production of Atlantic salmon, and to describe the pattern of infection.

# SUMMARY OF PAPERS

**Paper A:** *Ichthyobodo hippoglossi* n. sp. *(Kinetoplastea:Prokinetoplastida: Ichthyobodonidae* fam. nov.*),* an ectoparasitic flagellate infecting farmed Atlantic halibut *Hippoglossus hippoglossus*.

A morphological comparative study of two geneticaly distinct *Ichthyobodo* species from infected Atlantic salmon (*Salmo salar*) and halibut (*Hippoglossus hippoglossus*), respectively. This study launch morphological methods that may be used to distinguish different *Ichthyobodo* species. Based on the morphological differences (fenotype) and the differences in SSU rDNA sequences (genotype), a new species *Ichthyobodo hippoglossi* is described infecting Atlantic halibut in seawater. *Ichthyobodo necator* is redescribed from Atlantic salmon parr reared in frewshwater in Norway (*I. necator* sensu stricto), in order to delimit the morphological conception of that species to a particular genotype. A new family Ichthyobodonidae is proposed.

**Paper B:** Patterns of *Ichthyobodo necator* sensu stricto infections on hatchery reared salmon (*Salmo salar* L.) in Norway.

This is the first study of the infection dynamics of *Ichthyobodo necator* sensu stricto. A cohort of salmon was followed from the egg-stage to presmolts in salmon hatchery in Norway. In order to verify that a single species was responsible for the studied infections, diagnostic PCR tests were developed that detect *I. necator* and a second species known only by its SSU rDNA sequences. Only *I necator* was detected among the studied juvenile salmon. The survey revealed peaks of infections among fry during first feeding and later among parr and pre-smolt during summer and autumn. Examination of wild caught fish in the lake that supply the hatchery with water revealed *I necator* infections in brown trout (*Salmo trutta*) and three-spined sticklebacks (*Gasterosteus aculeatus*). Hence, these hosts likely act as natural reservoirs for *I. necator* entering the hatchery.

**Paper C:** *Ichthyobodo salmonis* sp. n. (*Ichthyobod*onidae, Kinetoplastida), an euryhaline ectoparasite infecting Atlantic salmon (Salmo salar L.).

Ribosomal DNA (rDNA) sequence studies have shown that there are two clearly different *Ichthyobodo* genotypes, considered different species, that infect Atlantic salmon (*Salmo salar*) in Norway. One of these (*I. necator* s.s.) is only known from freshwater hosts, while the other (*Ichthyobodo* sp. II) is a euryhaline species able to infect salmon in both freshwater and in the marine environment. Samples of *Ichthyobodo* sp. II from the gills of salmon reared in fresh-, brackish- and seawater were studied. SSU rDNA sequence comparisons showed that the *Ichthyobodo* sp. II isolates were 100% identical with each other, but less than 93% similar with *I. necator* s.s. from salmonids in freshwater. Morphological characters that distinguish the euryhaline *Ichthyobodo* sp. II from *I. necator* include size, shape and several ultrastructural features. Based on genetical and morphological differences from other *Ichthyobodo* spp., a new species *Ichthyobodo salmonis* is proposed for *Ichthyobodo* sp. II.

**Paper D:** Molecular tools for the detection and identification of *Ichthyobodo* spp. (Kinetoplastida), important fish parasites.

A real-time PCR assay (“Costia-assay”) targeting SSU rDNA of *Ichthyobodo* spp. is presented. Calibration curves for quantification have been made, which makes it possible to estimate numbers of *I. salmonis* cells or numbers of target copies of *Ichthyobodo* spp. in a sample. The Costia-assay show high sensitivity with an experimental limit of detection that equals c. 12-18 target copies (SSU rDNA) in the tested samples. Several novel primer sets have also been designed for identification of *Ichthyobodo* spp. with use of PCR and sequencing. The use of Costia-assay for detection and the PCR primers for identification have been demonstrated. The validation tests led to the discovery of new *Ichthyobodo* genotypes from different fish hosts.

# SYNTHESIS

## Major achievements of the study

This PhD work has introduced new methods for morphological descriptions of *Ichthyobodo* spp. A standardized scheme for morphometric analyses have been used in comparative studies of different *Ichthyobodo* species. Molecular and morphological methods have been applied to re-describe the freshwater species *Ichthyobodo necator* sensu stricto (s.s.) and also to describe a novel marine and an euryhaline species, *I. hippoglossi* and *I. salmonis,* respectively. These three species represent all the valid species within genus *Ichthyobodo* so far. They can be identified using the SSU rRNA gene as a signature sequence (Paper A and C).

Dynamics of *I. necator* s.s. infection among fish in a salmon hatchery have been studied, and this survey is the very first of its kind where the involved *Ichthyobodo* parasites have been identified to the species level (Paper B).

Effective methods for detection and identification of *Ichthyobodo* spp. have been developed and new genotypes that may represent strains or species within genus *Ichthyobodo* have been detected (Paper A and D).

The results from this project are compared to previous works and implications and possible practical applications are discussed.

## Species complex in the genus *Ichthyobodo*

SSU rDNA sequencing suggests that flagellates previously considered *I. necator* are likely to represent several species (Todal, *et al.*, 2004; Callahan, *et al.*, 2005; Paper A and D). Hence it is difficult to relate biological observations on *I. necator* in the 'old sense' (sensu lato) to the now discerned genotypes which appears to represent several undescribed species. The species concept for microorganisms like protists has been a problem and topic for discussions in decades. Methodological advances starting with morphological studies using light microscope to ultrastructural studies and the most recent molecular methods have revealed an increasing level in the diversity among protists. These different methodological approaches have complicated the microbiological concepts of species due to the difficulties in choose characters that are most appropriate to distinguish species. Different species may be delineated based on distinguishing phenotypic or genotypic features, but a combination of both is preferred (Boenigk *et al.* 2012).

### Nomenclature

Two old nominal species have previously been considered synonyms of *I. necator*. *Ichthyobodo nitzchei* from goldfish (*Carassius auratus*), described as *Tetramitus nitzchei* by Nietche & Weltner (1894), may represent a valid species. Two distinct genotypes that are considered likely to be separate species, have been found to infect goldfish. These are *Ichthyobodo* sp. VII (Callahan *et al.*, 2005) and *Ichthyobodo* sp. VIII (Todal *et al.*, 2004). Morphological and genetic characterization of these and other species inferred from sequences are necessary in order to consider the possible reinstatement of *I. nitzchei*.

Davis (1943) described and named a new *Ichthyobodo* species from salmonid hosts; *Ichthyobodo pyriformis* (named *Costia pyriformis*). *I. pyriformis* was rejected by Tavolga & Nigrelli (1947) and Grassé (1952) as a valid species due to the poor description of the parasite. Indeed Isaksen *et al.* (Paper A) remarked that *I. pyriformis* may not be a species of *Ichthyobodo* at all, since it

was figured in the original description with an entire kinetoplast and the longer flagellum adhering to the cell, characters occurring in e.g. *Cryptobia* spp. For this reason *I. pyriformis* needs a re-description and a genetic characterization.

The combined use of molecular and microscopic methods makes it possible to search for characteristics (apomorphies) that may be suitable to distinguish different species in the genus *Ichthyobodo*. Two genetically distinct *Ichthyobodo* spp. detected from freshwater fish and salmonids in Norway has been re-described and described morphologically and named as *I. necator* sensu stricto and *I. salmonis* (syn. *Ichthyobodo* sp. II) respectively (Paper A and C). In addition, a strict marine *Ichthyobodo* species from Atlantic halibut (*Hippoglossus hippoglossus*) have also been described and named; *I. hippoglossi* (Paper A). These are so far the only genetically identified *Ichthyobodo* species that have been morphologically described and named.

## Morphology

The long lasting, prevailing notion that genus *Ichthyobodo* only comprised one valid species (*Ichthyobodo necator* sensu Henneguy, 1883) reflected the difficulties in finding unique morphological features that are useful in distinguishing different species at the light microscope level. However, comprehensive morphological and morphometric studies of *Ichthyobodo* cells have shown intraspecific variation and several distinguishable characters (Paper A and C).

### *Morphometrics by light microscope*

Reported measurements of *Ichthyobodo* spp. show a wide variation in cell sizes (Table 3, p. 46). The apparent variation in dimensions and shape reported may reflect both intra- and interspecific variation. However, different methods used such as fixatives may also influence dimensions (Benisch, 1936). Different stains may vary in ability to reveal important characters such as the kinetoplasts, nucleus, karyosome or the free part of the flagella (Foissner, 1991; Bruno *et al.*, 2006).

**Table 3.** Measurements of *Ichthyobodo* spp. (syn. *Costia*). Cell dimensions (size, µm) given as range (min – max) or mean, the values have been rounded of when given with decimals. Fixation and staining methods. Fixatives: Osmic acid (O), Schaudinn's fluid (S), Formalin (F), buffered formalin (Fb), air-dried (A), methanol (Me); Dyes: Carmine (C), methyl green (M), Haematoxylin (H), eosin (E), Giemsa (G), Diff-Quick (DQ). Freshwater and seawater (*) hosts. Measurements of free forms are shown in the upper part of the table, measurements of trophozoites[§] in the lower part.

| Host | Name | Size (µm) | Fix | Dye | References |
|---|---|---|---|---|---|
| Brown trout | *Bodo necator* | 10 x 20 | O, S | S, C, M | Henneguy, 1884 |
| Unknown | *Costia necatrix* | 3-15 x 5-15 | S | H | Andai, 1933 |
| Salmonid | *Costia necatrix* | 5 x 8 | S | H | Fish, 1940 |
| Carp | *Costia necatrix* | 5-8 x 7-14 | O, S | S | Benisch, 1936 |
| Carp | *I. necator* s.l. | 6-10 x 8-12 | F, O | G | Joyon & Lom, 1969 |
| Chum salmon | *I. necator* s.l. | 8-12 x 9-13 | A, Me | G | Urawa & Kusakari, 1990 |
| Ornamental fish | *Costia necatrix* | 2-8 x 5-18 | F, S | H | Tavolga & Nigrelli, 1947 |
| Salmonids | *Costia pyriformis* | 5-8 x 9-14 | O | H | Davis, 1943 |
| Salmon | *I. necator* s.s. | 8-14 x 10-16 | A, Me | DQ | Paper A |
| Salmon | *I. salmonis* | 9-14 x 7-13 | A, Me | DQ | Paper C |
| *Chum salmon | *I. necator* s.l. | 6-10 x 9-12 | A, Me | G | Urawa & Kusakari, 1990 |
| *Japanese flounder | *Ichthyobodo* sp. | 6-11 x 8-13 | A, Me | G | Urawa & Kusakari, 1990 |
| *Salmon | *I. necator* s.l. | 3-6 x 6-10 | F | H,E | Ellis & Wootten, 1978 |
| *Common dab | *Ichthyobodo* sp. | 4-6 x 9-12 | - | H | Diamant, 1987 |
| *Tiger puffer | *Ichthyobodo* sp. | 6-10 x 10-14 | - | G | Urawa *et al*. 1998 |
| *Salmon | *I. salmonis* | 8-13 x 7-12 | A, Me | DQ | Paper C |
| *Halibut | *I. hippoglossi* | 9-14 x 9-14 | A, Me | DQ | Paper A |
| Salmonids | [§]*I. necator* s.l. | 2-3 x 9-11 | Fb | H, E | Bruno, 1992 |
| Salmon | [§]*I. necator* s.s. | 6-11 x 12-18 | A, Me | DQ | Paper A |
| Salmon | [§]*I. salmonis* | 5-9 x 9-15 | A, Me | DQ | Paper C |
| *Salmon | [§]*Ichthyobodo* sp | 1-3 x 5-7 | Fb | H,E | Bruno, 1992 |
| *Halibut | [§]*I. hippoglossi* | 7-12 x 10-16 | A, Me | DQ | Paper A |
| *Salmon | [§]*I. salmonis* | 5-10 x 10-15 | A, Me | DQ | Paper C |

Cell shape of the free forms of *Ichthyobodo* spp. is often described as rounded or oval. Cell dimensions are commonly measured as minimum and maximum length of the cell. However, it is important for description and comparative studies of *Ichthyobodo* spp. dimensions that a starting point for measurements and orientation of the cell is defined. Andai (1933) presented a scheme for measuring free forms of *Ichthyobodo* sp. (as *Costia necatrix*), but the cell width and cell length is not clearly defined in this description.

The present work has introduced an alternative scheme for measurements of both free and attached forms of *Ichthyobodo* spp. with use of light microscope (Paper A). An appropriate starting point for the measurements is the border of the flagellar pocket, which correspond to the end of the cytostomeal canal and is defined as the anterior part of the cell (Paper A). The relationship between cell length (L1) and width (L2) indicate shape of the cell, and such a cell shape index (Ci; L1/L2) has proved useful in distinguishing species (Paper A; example shown in Figure 11, p. 47). Also, the axes L1 and L2 may act as a 'xy-coordinate' system standardizing other measurements providing a position of the nucleus and a relative extent of the flagellar pocket.

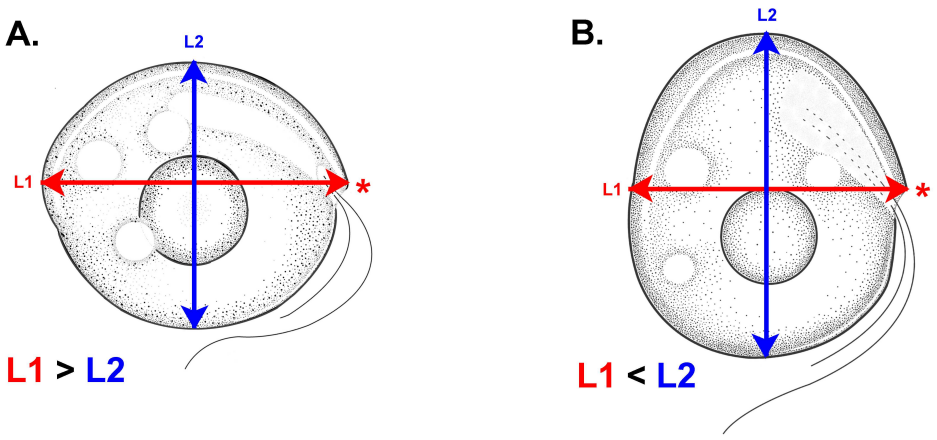


**Figure 11.** Cell shape of *Ichthyobodo* spp. The relationship between cell length (L1) and cell width (L2) for *Ichthyobodo salmonis* (A) and *I. necator* (B). The anterior part of the cell is marked with an asteric*.

Use of such standardised methods makes it possible to perform morphometric comparative studies of different species, including measurements of *Ichthyobodo* cells obtained by other researches. Cell width and cell length (as defined in Paper A) may correspond to minimum and maximum size of *Ichthyobodo* cells as presented in other studies (see Table 3, p. 46) or with 'left – right' and 'proximal – distal' as given by Andai (1933).

Molecular studies on *Ichthyobodo* spp. suggests that a high number of species may exist. Hence it may become increasingly difficult to reveal good morphological distinguishing features (apomorphies). Statistical treatments of morphometric data such as discriminant analyses may be used, but requires a standardised scheme of measurement, such as proposed here. Applications of a measurement scheme for both free forms and trophozoites have been demonstrated in the descriptions of *I. necator*, *I. hippoglossi* and *I. salmonis* (Paper A and C).

### *Intraspecific variations*

The quadriflagellated *Ichthyobodo* cells have been assumed to be pre-dividing stages (Andai, 1933; Woo & Poynton, 1995) and a larger mean size compared to biflagellated cells support this (Andai, 1933; Paper A and C). The relative amount of quadriflagellates may correspond to the proliferation rates of the parasites, which in turn may depend on the susceptibility of the host, rearing conditions in the fish farms (e.g. stock density, water flow) (Paper B). A variable ratio of biflagellates to quadriflagellates in samples may affect the morphometric means and levels of variation if these different forms are not discerned and treated separately.

Among euryhaline *Ichthyobodo* species, intraspecific variations may also relate to the different macrohabitats (freshwater, seawater). Differences in the surrounding osmotic pressure apparently affect the ability to detect contractile vacuoles, since these often appear absent in seawater (Urawa & Kusakari, 1990; Paper C). Large contractile vacuoles may also contribute to an increased size or affect shape of *I. salmonis* (Paper C).

Due to the intraspecific variations as listed above, differences in cell dimensions cannot be trusted in distinguishing *Ichthyobodo* spp. at a light microscope level; hence additional qualitative characters are needed in comparative studies.

*Characteristic structures of Ichthyobodo spp.*

Morphological characters for the studied *Ichthyobodo* spp. have been described and discussed in Paper A and Paper C. The following chapter will mainly concern important characters that include kinetoplasts, flagellar apparatus and cytostomeal complex. These structures are discussed below with supplementary TEM images used for illustrations.

Kinetoplasts. An important character of genus *Ichthyobodo* is the kinetoplasts. These structures are visible in stained smears by light microscopy as densely stained grains scattered in the cytoplasma. They are seen in both free-swimming forms and trophozoites (Paper A and C). There are apparently no regular pattern in their distribution, but numbers and size of these 'grains' may be characteristic features that distinguish different *Ichthyobodo* species. Previous studies have also noted these structures, in early studies referred to as densely stained granules (Moroff, 1904; Andai, 1933; Tavolga & Nigrelli, 1947; Joyon & Lom, 1969). However, there may be a wide range in the numbers of visible kinetoplasts in the *Ichthyobodo* spp. cells. Among *I. necator* s.s., the number of visible kinetoplasts correlate positively with cell size (Paper A). Hence, some of the variations observed in *Ichthyobodo* spp. may correspond to the cell cycle and growth in agreement with the observations described by Joyon & Lom (1969). They found a recurring pattern of pairwise or apparently partly clustered kinetoplasts suggested to represent duplication of the structure in a pre-division stage of the *Ichthyobodo* cell (Joyon & Lom, 1969).

The number and size of the kinetoplasts appears to be a distinguishing character when *I. necator* and *I. hippoglossi* are compared (Paper A). However, numbers and shape of kinetoplasts may be too uncertain and

variable to be used in discerning other *Ichthyobodo* species (e.g. comparisons of *I. necator* and *I. salmonis*; Paper C). Still, a marked polykinetoplasty is a character that distinguish genus *Ichthyobodo* from other kinetoplastids that are ectoparasitic on fish (e.g. *Cryptobia* spp.). Kinetoplast structures (nucleoids) are easily recognized in TEM images of *Ichthyobodo* spp. (see Figures 3, 6-7 in Paper C; Figure 12, p. 52).

Flagellar apparatus. The total length of the flagella is the distance from the basal bodies in the flagellar pocket to the tips (Schubert 1966, Joyon & Lom 1969), but only the part of the flagella outside this pocket may be visible and readily measurable in preparations stained with normal hematoxylin and eosin (HE) or metachromatic stains (Paper A and C). Also, in light microscopy on *Ichthyobodo* spp. in stained smears, kinetosomes (basal bodies; see TEM image in Figure 13, p. 53) could not be detected. Hence. flagella lengths is not readily obtainable from normal preparations, particular staining methods must be used (see Joyon & Lom 1969).

The paraflagellar rod (PFR) is an extra-axonemal structure of flagella restricted to species within Kinetoplastida and Euglenida (Portman & Gull, 2010). This feature is an ultrastructural character that may show variation in size and structure between groups or species. For instance, in some members of order Trypanosomatida the PFR is significantly reduced or lacking (Gadelha *et al.*, 2005; Portman & Gull, 2010). PFR is assumed to be necessary for the function of the flagella such as motility, and interspecific variations in PFR structure have been detected among trypanosomatids (Portman & Gull, 2010). Few studies have addressed the ultrastructure of the PFR in *Ichthyobodo* spp. (Schubert, 1966; Joyon & Lom, 1969). Joyon & Lom (1969) noted that cross-section of the dorsal, recurrent flagellum had a more well-developed PFR (often pear-shaped) compared to the ventral flagellum. Furthermore, Joyon & Lom (1969) assumed that the dorsal and ventral flagella represented the short and long flagella respectively. TEM images of *I. salmonis* have also revealed similar differences in PFR structure between the dorsal and ventral flagella in a cell (Paper C; Figure 14, p. 54). However, it is at present unclear if these

characters may aid in distinguishing species in the genus *Ichthyobodo* due to the few available observations.

Cytostomeal complex. Among the characteristic features used in classification of the order Kinetoplastida is the presence of a rostrum (see Table 4, Callahan 2003). Rostrum is part of the cytostomal apparatus and is most prominent among free-living kinetoplastids within families Bodonidae and Cryptobiidae (Vickerman, 1990). In smears of *Ichthyobodo* spp. studied in the present work, this structure is small and appears as a 'nose-like' protrusion (Paper A and C). The protrusion contains the end of the axostyle, a light microscopical character seen as a bent rod like structure in the cell (e.g. Figure 5C in Paper A). Ultrastructural studies suggest that this structure correspond to a set of microtubules associated with the cytostomeal tube or canal as described by Joyon & Lom (1969). The microtubules and cytostomeal tube constitutes the cytostome process that extends from the attachment disc into the host cell. The cytostomeal canal probably function as a 'sucking' organelle (Joyon & Lom, 1969), but the exact mechanism for this function is not known. The cytostome process and the cytostomeal canal is shown in TEM images of *I. salmonis* trophozoites. See Figure 12, 13 and 15 (pp. 52 - 53 and 55).

Ultrastructural studies of the attachment disc have revealed the interface with the host cell to be either smooth or covered with ridge-like structures extending also along the cytostome process. These different structures of the attachment disc were related to macrohabitat by Roubal & Bullock (1987), who found smooth attachment discs of *I. necator* cells on the gills of salmonids in seawater, and ridged on those from freshwater. It has now been shown that *I. necator* sensu stricto has ridged attachment discs, while *I. salmonis* has smooth, hence the observations by Roubal & Bullock (1987) most likely relate to different *Ichthyobodo* species (Paper C). Variations in the structure of the attachment disc appear to be valuable characters that deserve attention in future ultrastructural studies and descriptions of *Ichthyobodo* spp. Todal *et al.* (2004) noted that *I. necator* (clade A in a phylogenetic analysis) likely had ridged attachment disc, an assumption now confirmed in Paper C. Other

*Ichthyobodo* spp. studied ultrastructurally had smooth disc, and all other species examined by sequencing belong to another clade (clade B). They therefore speculated that this trait, attachment disc structure, could be a characteristic for these two clades.

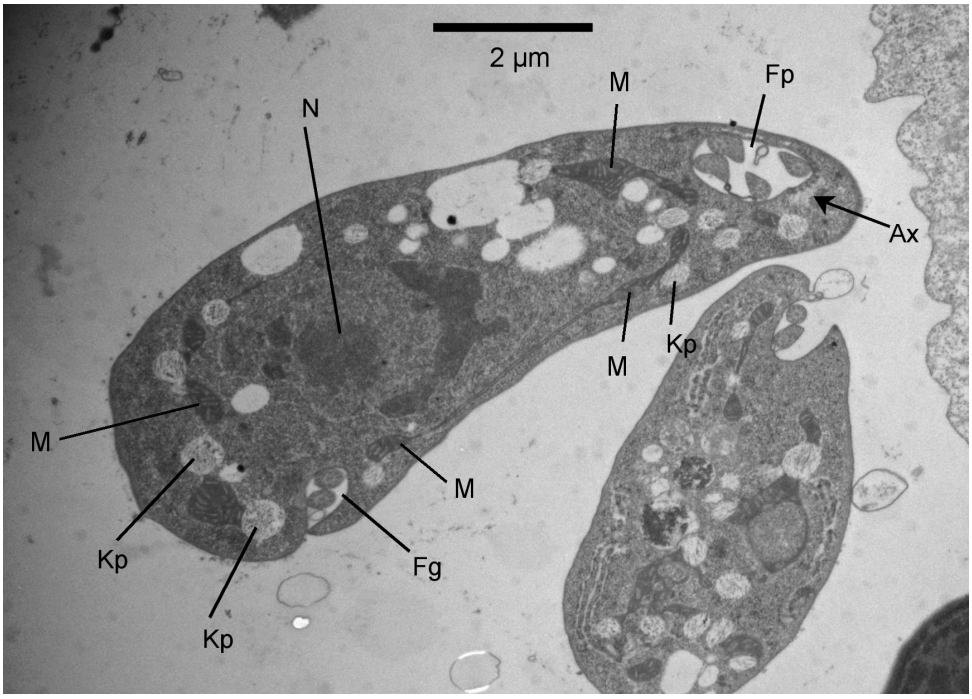


**Figure 12**. Transmission electron microscopy (TEM) image of *Ichthyobodo salmonis*. Original figure (Photo: K. Watanabe). Four flagella visible in the flagellar pocket (Fp), two flagella in the flagella groove (Fg). Kinetoplasts (Kp) randomly distributed in cytoplasma. Elongated mitochondrion (M). A relative large nucleus with nucleolus in center (N). Cross section of the axostyle (Ax) located close to the flagellar pocket (Photo: K. Watanabe).

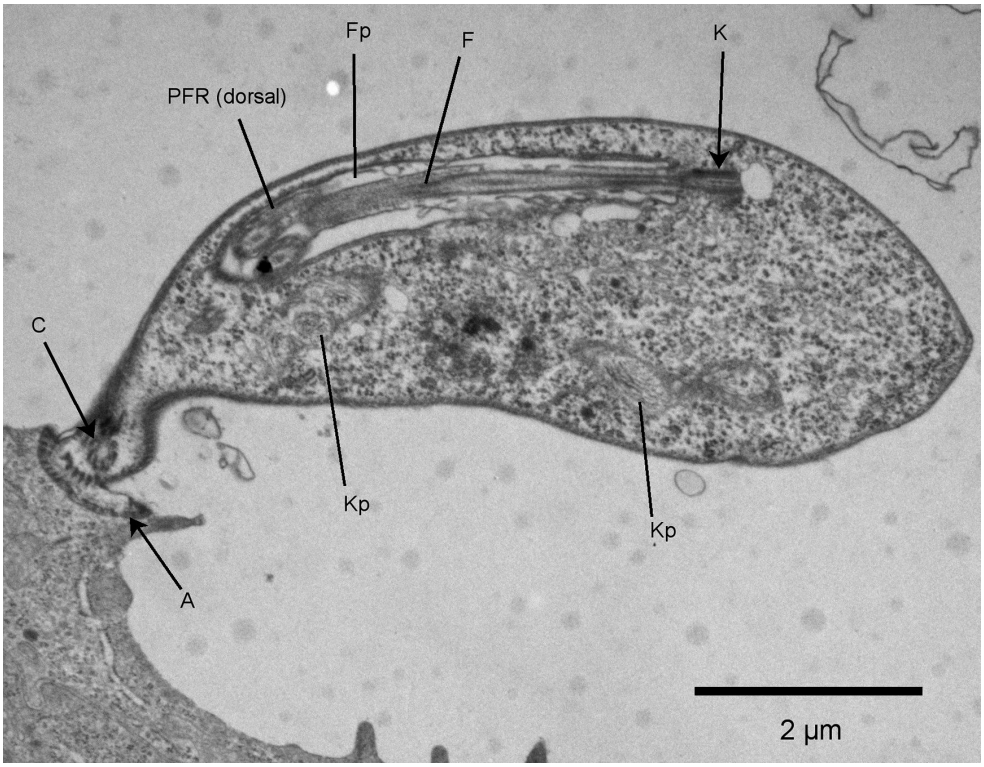


**Figure 13**. Transmission electron microscopy (TEM) image of *Ichthyobodo salmonis* attached to gills of seawater reared salmon (*Salmo salar*). Original figure (Photo: K. Watanabe). K: Kinetosome; Kp: Kinetoplast; Fp: Flagellar pocket; PFR: Paraflagellar rod; C: Cross section of the cytostome process; A: Attachment disc.

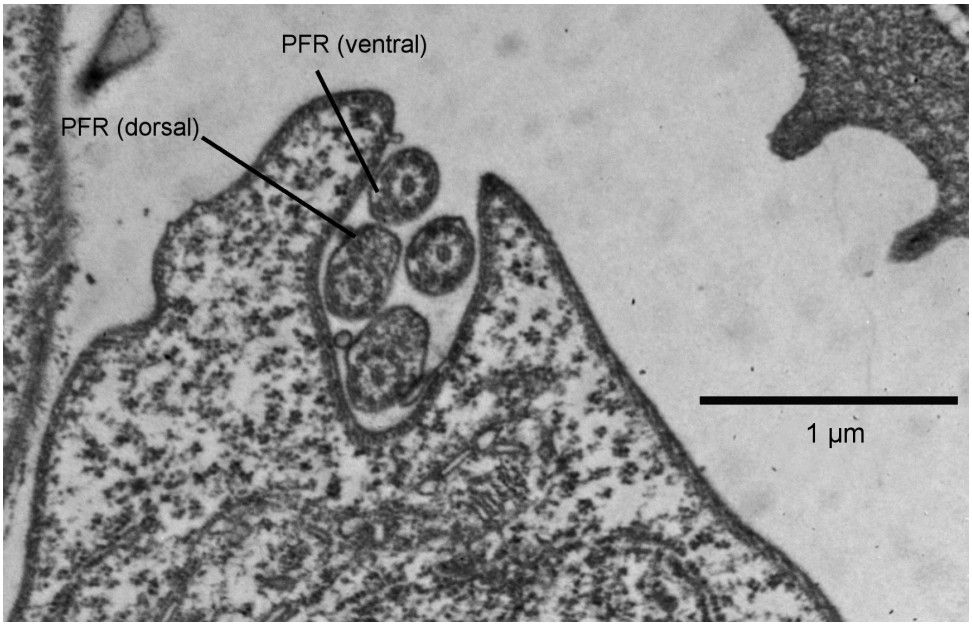


**Figure 14**. Transmission electron microscopy (TEM) image of *Ichthyobodo* sp. (likely *I. salmonis*) from gills of seawater reared salmon (*Salmo salar*). Original figure (Photo: K. Watanabe). Four flagella in a flagellar groove. Flagella structure showing axonema with paraflagellar rod (PFR).

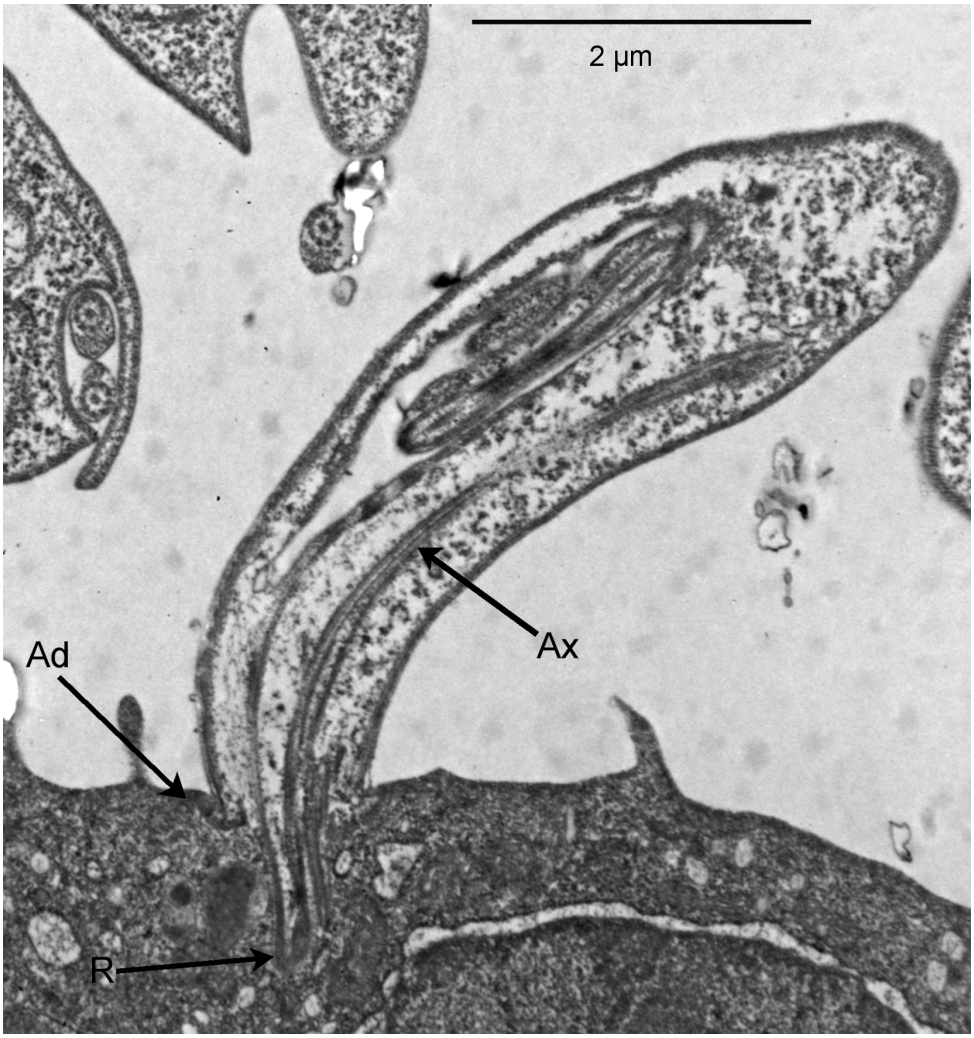


**Figure 15**. Transmission electron microscopy (TEM) image of *Ichthyobodo* sp. (unidentified, likely *I. salmonis*) attached to gills of seawater reared salmon (*Salmo salar*). Original figure (Photo: K. Watanabe). Ad: Smooth attachment disc; R: Rostrum, penetrating host cell; Ax: Axostyle, extending from rostrum (anterior) towards the dorsal part of the trophozoite.

## SSU rDNA diversity of *Ichthyobodo* spp.

Ribosomes are made of RNA (small and large subunits; SSU and LSU) and protein and are abundant in all cells with active protein synthesis (i.e. all living organisms). The small subunit ribosomal DNA (SSU rRNA gene) is evolutionary highly conserved and is the most frequently used gene in studies of kinetoplastid diversity and genetic relationship among *Ichthyobodo* spp. (Callahan *et al.*, 2002; Moreira *et al.*, 2004; Todal *et al.*, 2004; Callahan *et al.*, 2005; von der Heyden & Cavalier-Smith, 2005). SSU rRNA has also been used as a target gene for detection and identification of *Ichthyobodo* spp. in the present work (Paper A –D).

An average SSU rDNA sequence divergence larger than 1.3% between well-defined morphological species of kinetoplastids (*Trypanoplasma* spp.) have been taken as suggestive of interspecific divergence in this group (Dolezel *et al.*, 2000; Maslov *et al.*, 2001; Callahan *et al.*, 2002; Todal *et al.*, 2004). However, such a level in divergence in the SSU rRNA gene is not a consensus approach for delimiting species in protists, and it is recommended that complementary distinguishing features (morphological or molecular) support the delineating of species at low divergence for a single gene as described above (Broenick et al. 2012).

Based on SSU rDNA sequences, it has been suggested that genus *Ichthyobodo* include several species from a wide range of hosts from both freshwater and seawater (Todal *et al*. 2004; Callahan *et al*. 2005). More recently, five new genotypes that may represent different species or strains of *Ichthyobodo* spp. from fish in freshwater, brackish water and seawater in Norway have been detected (Paper D). In addition, a new sequence of an apparently new species have also been obtained from the gills of juvenile African sharptooth catfish (*Clarias gariepinus*) and juvenile Nile tilapia (*Oreochromis niloticus*) from a fish farm in Kajansi in Uganda (Isaksen *et al.* unpublished). In all, a total of 15 different *Ichthyobodo* genotypes have so far been detected. Among these, only three genotypes have been morphologically

described and identified as different species, namely *I. necator* s.s., *I. salmonis* and *I. hippoglossi* (Paper A and C). These species show a SSU rDNA sequence divergence ranging from 6% to 9% in pairwise comparisons (Table 4, p. 61). The phylogenetic relationships between the different *Ichthyobodo* spp. are shown in Figure 16 (p. 60). Similarity (%) for SSU rDNA sequences between the different genotypes is shown in Table 4 (p. 61). The phylogram and the comparisons of sequences are based on the same alignment of SSU rDNA sequences and is discussed below.

The phylogram (Figure 16, p. 60) differentiate genus *Ichthyobodo* into two major lineages; A and B. Clade A is represented by a single species, *I. necator* s.s. from different freshwater hosts in Norway. Some of the most robust descendant clades within lineage B have been denoted as $B_1$-$B_4$ (support values >80%).

Clade $B_1$ represents *I. salmonis* from farmed and wild caught Atlantic salmon (*S. salar*) in Norway and *Ichthyobodo* sp. III from hatchery reared Masu salmon (*Oncorhynchus masou*) from Japan. According to Todal *et al.* (2004), *Ichthyobodo* from Masu salmon showed a divergence of 1.5% to 1.6% at SSU rRNA gene level compared to the most closely related sequences, now identified with *I. salmonis*. However, the SSU rDNA sequence of *Ichthyobodo* sp. III (GenBank accession no. AY224689) have recently been updated by submitter (02 Feb. 2011). Pairwise comparisons of isolates representing *I. salmonis* (sp. II) and the updated *Ichthyobodo* sp. III reveal a higher nucleotide (SSU rDNA) similarity with a divergence of only 0.5% to 0.6% (Table 4, p. 61). Hence *I. salmonis* and *Ichthyobodo* sp. III are more closely related than inferred by Todal *et al.* (2004), and the Japanese *Ichthyobodo* sp. III may prove a regional variant of *I. salmonis*. The salinity tolerance of *Ichthyobodo* sp. III from masu salmon is unknown. However, *Ichthyobodo* from Japanese chum salmon (*Oncorhynchus keta*) was shown to be euryhaline (Urawa & Kusakari, 1990), hence at present the weight of evidence suggests that a single euryhaline parasite, *I. salmonis*, infects North Pacific and North Atlantic anadromous salmonids. Further studies are needed to verify this. An

interesting prospect is the correlation of the evolution and zoogeography of the salmonid hosts and their euryhaline *Ichthyobodo* symbionts.

Clade $B_2$ is a "marine" clade, represented by 3 different *Ichthyobodo* genotypes (IV, X, XI; similarity ranges from 93% to 96%) isolated from skin or gills of strict marine fish hosts from Norway; Atlantic cod (*Gadus morhua*), pollack (*Pollachius pollachius*) and Atlantic halibut (*H. hippoglossus*) (Todal *et al.*, 2004; Paper A and D). This clade include one described and named species; *I. hippoglossi*. This species has so far only been detected on Atlantic halibut, identified from both farmed and wild caught halibut in Western and Northern Norway respectively (Paper A and D). The two different genotypes of *Ichthyobodo* sp. from gadid hosts (genotypes IV and XI; Figure 16 p. 60 and Table 4 p. 61) shows only 95% similarity and may represent different species.

The clade $B_3$ represents 4 different genotypes (VII, VIII, IX, XIII; similarity ranges from 94% to 98%) of *Ichthyobodo* spp. from different hosts and with a wide geographical distribution. All but one has been isolated from freshwater fish. The exception is an *Ichthyobodo* sp. VII sequence that has been obtained from the strict marine Japanese flounder (*Paralichthys olivaceus;* see Callahan *et al.*, 2005). This sequence ($VII_c$; Table 4, p. 61), obtained from formalin fixed flounder tissues, is very similar to sequences obtained from cyprinids ($VII_a$; divergence 0.2%) and channel catfish ($VII_b$; divergence 0.3%) in the same study. It is strange that apparently same *Ichthyobodo* species identified from different freshwater fish in the USA also occur on a marine fish in Japan, hence these results needs verification.

All known *Ichthyobodo* sequences isolated from cyprinid hosts (genotypes VII, VIII and XIII) are grouped within clade $B_3$. They appear to represent 3 separate species, of which *Ichthyobodo* sp. VII is known to infect unrelated hosts (*Ictalurus punctatus*, cyprinids and *P. olivaceus*, see above).

Clade $B_4$ represents *Ichthyobodo* sequence isolates occurring on warm-water freshwater fishes, including ornamental fish (genotypes V, VI). The sequences

of *Ichthyobodo* genotypes V and VI revealed high similarity (99.0-99.2). These genotypes have been detected on hosts from USA (V; swordtail and sunshine bass) and an ornamental fish from Brazil (VI; cichlid), hence so far only from the Americas (Todal *et al.*, 2004; Callahan *et al.*, 2005). Poeciliids (swordtail) and cichlids are tropical fish commonly kept in warm-water aquaria, while *Morone* spp. hybrid (sunshine bass) has commercial value in fish farming as food fish in temperate and subtropics regions of North America (Hodson, 1989).

Sequence analyses suggest that some *Ichthyobodo* spp. are able to infect several unrelated host species (*Ichthyobodo* spp. genotypes: V, VII, VIII, XII, XV and *I. necator* s.s.). Some species also appears to occur over a wide geographical range (genotypes VII and VIII) and are able to infect hosts in both freshwater or seawater (sp. VII, *I. salmonis*). The genotype XII represents an *Ichthyobodo* species that infects estuarine fish hosts (*Gasterosteus aculeatus*, *Pomatoscistus microps*; Paper D), with a wide temperature and salinity tolerance. As these hosts, *Ichthyobodo* sp XII may prove adapted to survival in intertidal pools and estuaries with large variations in salinity and temperature. *Ichthyobodo* sp. XIV isolated from the marine fish black goby (*Gobius niger;* Paper D) in Norway represents a new lineage. This divergent genotype shows a similarity less than 92% compared to the closest relatives, and is therefore of particular interest in future morphological studies of *Ichthyobodo* spp. The black goby parasite may provide important information on character variation within genus *Ichthyobodo*.

The phylogenetic analyses based on SSU rDNA sequences indicates that the different genotypes (I to XV) represent at least 13 distinct species. Two groups are controversial, clade $B_1$ with that may contain euryhaline *Ichthyobodo*-isolates from salmonids (genotypes II and III, divergence 0.5-0.6%) and clade $B_4$ (divergence 0.8-1.0%). More extensive sampling, morphological characterization and multiple gene analyses may provide a much needed insight in the degree of SSU rDNA divergence both within and between species in genus *Ichthyobodo*.

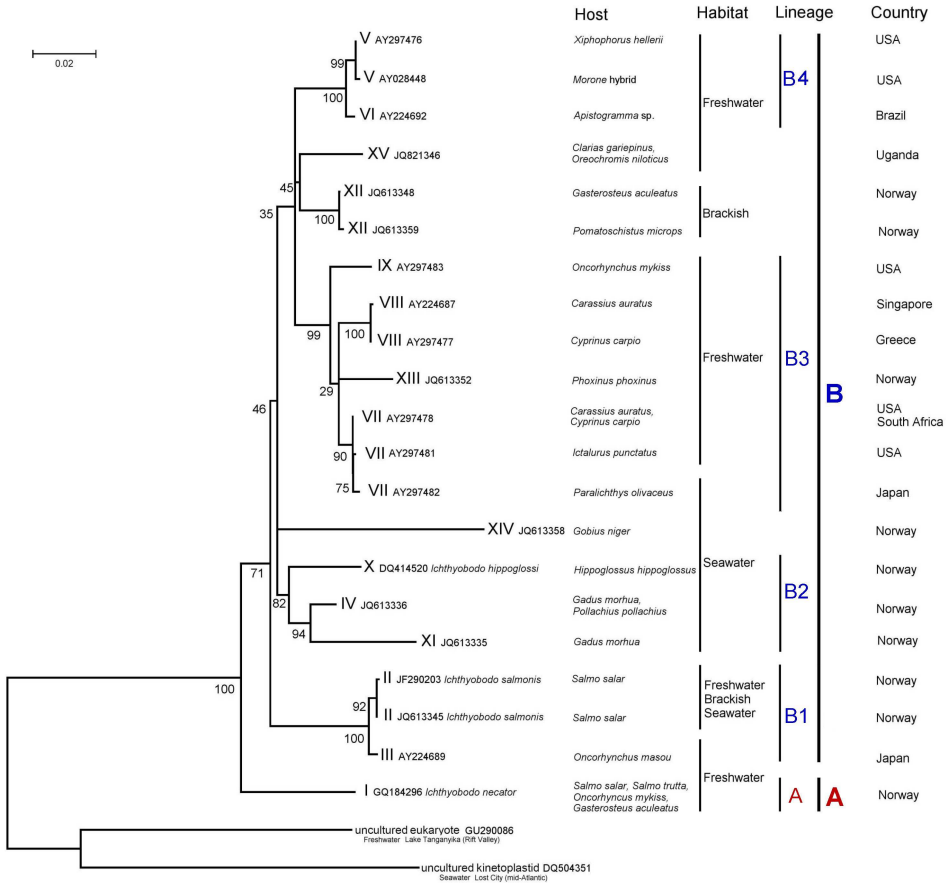


**Figure 16.** Phylogenetic analysis of genus *Ichthyobodo* by maximum likelihood (ML) method. Analyses conducted in MEGA5. The percentage of replicate trees in which the associated isolates clustered together in the bootstrap test (1000 replicates) are shown next to the branches. ML substitution model used is GTR (G+I). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis was based on an alignment of 23 SSU rDNA sequences that constitute 15 different major genotypes (I – XV). All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 1428 positions in the final dataset. Major clades of genus *Ichthyobodo* are indicated by lineages A and B ($B_1$-$B_4$ marks the most robust descendent lineages). The environmental eukaryotes represent the closest relatives (SSU rDNA) to genus *Ichthyobodo* and are used as an outgroup taxa. GenBank accession numbers are given for the representative *Ichthyobodo* sequences. Host, environment and country origin for the different genotypes (I-XV) are given (Todal *et al.*, 2004, Callahan *et al.*, 2005, Paper A, B, C and D).

**Table 4**. Pairwise comparisons of sequences. Aligned SSU rDNA sequences of *Ichthyobodo* sp. **Upper right**: Analyses conducted in Vector NTI (AlignX). Similarity (%) for all possible sequence pairs used in the alignment; **Diagonal in bold**: Analyses conducted in GeneDoc. Numbers of nucleotides without gaps; **Down left**: Analyses conducted in MEGA5. Pairwise distances. Sequence lengths with gaps included are 1529. The number of base differences per sequence from between sequences is shown. Duplicate sequences (differences = 0) have been eliminated from the alignment. The analysis involved 21 sequences that constitute 15 different *Ichthyobodo* genotypes (I – XV). GenBank accession numbers are given for the representative *Ichthyobodo* sequences. All ambiguous positions were removed for each sequence pair.

| GenBank | Sp. | I | IIa | IIb | III | IV | Va | Vb | VI | VIIa | VIIb | VIIc | VIIIa | VIIIb | IX | X | XI | XIIa | XIIb | XIII | XIV | XV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GQ184296 | **I** | **1487** | 92 | 92 | 92 | 93 | 92 | 92 | 92 | 92 | 92 | 92 | 91 | 91 | 90 | 92 | 91 | 93 | 93 | 91 | 88 | 92 |
| JF290203 | **II**a | 107 | **1477** | 100 | 99 | 94 | 93 | 93 | 93 | 94 | 94 | 93 | 93 | 93 | 91 | 93 | 92 | 94 | 93 | 92 | 91 | 94 |
| JQ613345 | **II**b | 108 | 1 | **1477** | 99 | 94 | 93 | 93 | 93 | 94 | 94 | 93 | 93 | 93 | 91 | 93 | 92 | 93 | 93 | 92 | 90 | 94 |
| AY224689 | **III** | 111 | 8 | 9 | **1479** | 94 | 93 | 93 | 93 | 94 | 94 | 93 | 93 | 93 | 91 | 93 | 91 | 94 | 93 | 93 | 91 | 94 |
| JQ613336 | **IV** | 101 | 78 | 79 | 81 | **1481** | 94 | 94 | 94 | 94 | 94 | 94 | 94 | 94 | 92 | 96 | 95 | 96 | 95 | 94 | 91 | 94 |
| AY028448 | **V**a | 98 | 84 | 85 | 86 | 66 | **1468** | 100 | 99 | 94 | 94 | 94 | 94 | 93 | 92 | 94 | 92 | 95 | 95 | 93 | 91 | 94 |
| AY297476 | **V**b | 95 | 81 | 82 | 85 | 65 | 2 | **1467** | 99 | 94 | 94 | 94 | 94 | 94 | 92 | 94 | 92 | 95 | 95 | 94 | 91 | 94 |
| AY224692 | **VI** | 98 | 83 | 84 | 85 | 67 | 14 | 12 | **1468** | 94 | 94 | 94 | 94 | 93 | 92 | 94 | 92 | 95 | 95 | 93 | 91 | 94 |
| AY297478 | **VII**a | 115 | 84 | 85 | 86 | 74 | 64 | 59 | 60 | **1482** | 100 | 100 | 98 | 98 | 96 | 94 | 92 | 96 | 96 | 97 | 91 | 95 |
| AY297481 | **VII**b | 116 | 85 | 86 | 87 | 75 | 65 | 60 | 61 | 1 | **1482** | 100 | 98 | 98 | 96 | 94 | 92 | 96 | 96 | 97 | 91 | 95 |
| AY297482 | **VII**c | 118 | 87 | 88 | 89 | 77 | 67 | 62 | 63 | 3 | 4 | **1482** | 98 | 97 | 96 | 94 | 92 | 96 | 95 | 97 | 90 | 95 |
| AY297477 | **VIII**a | 117 | 89 | 90 | 92 | 79 | 70 | 67 | 70 | 29 | 30 | 32 | **1484** | 100 | 95 | 94 | 91 | 95 | 95 | 96 | 90 | 95 |
| AY224687 | **VIII**b | 118 | 90 | 91 | 93 | 80 | 71 | 68 | 71 | 30 | 31 | 33 | 1 | **1484** | 95 | 93 | 91 | 95 | 95 | 96 | 90 | 94 |
| AY297483 | **IX** | 112 | 93 | 94 | 94 | 84 | 65 | 61 | 64 | 33 | 34 | 36 | 47 | 48 | **1514** | 92 | 90 | 94 | 94 | 94 | 88 | 93 |
| DQ414520 | **X** | 101 | 88 | 89 | 91 | 59 | 78 | 75 | 71 | 78 | 79 | 81 | 82 | 83 | 86 | **1476** | 93 | 95 | 95 | 94 | 90 | 94 |
| JQ613335 | **XI** | 120 | 107 | 108 | 112 | 66 | 98 | 95 | 97 | 94 | 95 | 97 | 105 | 106 | 103 | 88 | **1479** | 93 | 93 | 92 | 90 | 92 |
| JQ613348 | **XII**a | 100 | 89 | 90 | 93 | 65 | 53 | 48 | 50 | 56 | 57 | 59 | 64 | 65 | 61 | 65 | 96 | **1479** | 100 | 95 | 91 | 96 |
| JQ613359 | **XII**b | 102 | 91 | 92 | 95 | 67 | 55 | 50 | 52 | 58 | 59 | 61 | 66 | 67 | 61 | 66 | 98 | 2 | **1479** | 95 | 91 | 96 |
| JQ613352 | **XIII** | 127 | 102 | 103 | 104 | 90 | 79 | 75 | 77 | 40 | 41 | 43 | 48 | 49 | 56 | 86 | 106 | 71 | 73 | **1483** | 90 | 94 |
| JQ613358 | **XIV** | 150 | 113 | 114 | 113 | 111 | 107 | 106 | 105 | 111 | 112 | 114 | 118 | 118 | 126 | 123 | 129 | 116 | 117 | 122 | **1473** | 90 |
| JQ821346 | **XV** | 114 | 79 | 78 | 83 | 77 | 66 | 62 | 65 | 61 | 62 | 64 | 67 | 68 | 78 | 80 | 105 | 56 | 58 | 74 | 111 | **1484** |

## Epizootiology

Patterns of *Ichthyobodo* infections among hatchery reared European salmonids have been studied on several occasions by light microscopy (Robertson, 1979; Wootten & Smith, 1980; Rintamaki-Kinnunen & Valtonen, 1997; Paper B). Among these epizootiological studies, the most recent (Paper B) is the only survey that has used molecular methods to ascertain the *Ichthyobodo* species involved. Only the freshwater parasite *I. necator* s.s. was present in the studied farm and watercourse.

Recognizing *Ichthyobodo* spp. infections in a light microscope require experience, since trophozoites may be difficult to detect and other ectosymbionts as well as free living protists may resemble *Ichthyobodo*. Quantifying infections with this method is extremely time-consuming if accurate density or intensity estimates are sought. Usually a small subsample from a defined site is examined, and the parasite density scored on an arbitrary scale (Urawa, 1993; Rahkonen, 1994; Rintamaki-Kinnunen & Valtonen, 1997; Paper B). This method is fast if densities are high, but time consuming if prevalence and densities are low. PCR methods are far more sensitive and useful in epizootiological studies for identification and quantification of the involved pathogens (Paper D). In addition, the use of real-time PCR assays makes it is easy to screen for a range of other pathogens in for example gill diseases which is associated with a range of different agents and may be multifactorial (Mitchell & Rodger, 2011; Nylund *et al.* 2011).

*Diseae agents or secondary infections?*

Amlacher (1970) described *I. necator* (s.l.) as a “debility” parasite since occurrence in healthy fish is common and heavy infection and ichthyobodosis most often occur secondarily among weakened fish. However, it is not always obvious whether ichthyobodosis is a primary or represent a secondary infection with *Ichthyobodo* parasites in fish weakened by other infections. A

primary infection refers to an infection that affects and significantly weakens the host, while secondary infections occur because the host has been weakened.

*Ichthyobodo* parasites have been suggested as a primary pathogen and a severe stressor among salmonid fry (Pottinger & Mosuwe, 1994). However, stressors or infections with other pathogens may weaken the immune system of the host (Woo, 1992; Barton 2002) leading to increased infections with opportunistic parasites like *Ichthyobodo* spp. Healthy skin and gill surface is the most important "first line defence" against ectoparasites, hence epidermal damage makes the host susceptible to secondary infections (Urawa *et al.*, 1998). Scale losses and skin ulcers in salmon fingerlings may be associated with *I. necator* s.s. infections (Paper B). However it is not readily clear how this association arise; there are several ways such a pattern may appear:

i) Massive flagellate proliferation cause epidermal changes leading to lesions (Urawa, 1992).

ii) Flagellate infections on the skin cause itching, and rubbing against hard structures lead to scale loss and lesions (Woo & Poynton, 1995).

iii) Epidermal lesions due to other causes are colonized and exploited by *Ichthyobodo necator* (Benisch, 1936).

Skin lesions may be considered primary if such lesions are result of *Ichthyobodo* infections (i and ii). Secondary lesions are those that make the host susceptible to *Ichthyobodo* infection as described (iii). All these listed patterns (i, ii, iii) has been considered in heavy *I. necator* infections among fingerlings in a salmon hatchery, but it could not be ascertained which one was most important (Paper B). A controlled laboratory challenge may be a necessity to reveal the effect of *Ichthyobodo* infection in weakened and in healthy fish.

The importance of *Ichthyobodo* spp. as aetiological agent in diseases often depends on the developmental stage of the host. Smaller fish are more susceptible to *I. necator* infection than larger fish among hatchery reared salmonids and the mortalities are often higher among fry compared to fingerlings (Robertson 1979, Paper B). The host susceptibility also depends on the *Ichthyobodo* species involved and severity of disease may vary due to pathogenicity of the particular parasite. Most reports regarding ichthyobodosis have not identified the flagellates according to recent revisions identifying distinct genotypes; hence the knowledge of the pathology of different *Ichthyobodo* species is scarce.

*Source of infection*

The freshwater parasite *I. necator* s.s. is able to infect salmonids and sticklebacks. Naïve fish species in lakes and rivers serve as a natural reservoirs of *I. necator* and may cause infections among salmonids in hatcheries that are supplied with water from such watercourses (see Paper B).

Co-infection of *I. necator* s.s. and *I. salmonis* has recently been detected on salmon parr in a hatchery that used untreated water from a river containing sea trout and salmon (code F7 in table 5 and 8, Paper D). Co-infections by the same two *Ichthyobodo* spp. have also been detected among adult wild salmon caught in different rivers in Norway during spawning the season (code W7 and W8, Paper D). In the hatchery both salmon and trout were reared, but unlike the salmon the trout were only infected by *I. necator*. Hence, *I. salmonis* have so far only been found to infect Atlantic salmon, and may be host specific (Paper C and D). Hence so far, feral and wild Atlantic salmon are the only known natural reservoirs for *I. salmonis* infections in Norwegian salmon production.

The natural reservoirs for *Ichthyobodo* infections in marine finfish production are not well known. So far, the *Ichthyobodo* spp. genotypes from marine fish

in Norway appears to show a relatively high level of host specificity compared to species identified from freshwater hosts worldwide. *Ichthyobodo* sp. IV and sp. XI infections have so far only been detected on gadid hosts (Paper D). Both species have been found to infect farmed Atlantic cod, and sp. IV also on the gills of wild caught cod and pollack (*Pollachius pollachius*). Hence, the host range is wider than genus *Gadus* and may prove to be family Gadidae. *Ichthyobodo hippoglossi* have been detected on hatchery reared halibut larvae (*Hippoglossus hippoglossus*) in Western Norway, and later on wild caught adult halibut from Northern Norway (Paper A and D). Other hosts for *I. hippoglossi* are not known, but *Ichthyobodo* sp. infections have been reported from other Atlantic flatfish e.g. common dab (*Limanda limanda*), plaice (*Pleuronectes platessa*) and winter flounder (*Pleuronectes americanus*)(Bullock & Robertson, 1982; Cone & Wiles, 1984; Diamant, 1987). SSU rDNA sequences of *Ichthyobodo* sp. infections from these and other pleuronectids are particularly relevant in a search for further reservoir hosts of *I. hippoglossi*.

*Ichthyobodo* sp. XII have been detected on two unrelated hosts in brackish water estuaries, a goby (*P. microps*) and the three-spine stickleback (*G. aculeatus*), while *Ichthyobodo* sp. XIV is only known from the black goby (*Gobius niger*). So far, very few marine hosts have been examined. A high diversity of *Ichthyobodo* spp. in marine hosts seems likely based on the diversity observed in the few hosts examined so far. Still the identity of *Ichthyobodo* species infecting important commercial aquaculture species is unknown. This includes the infections on farmed turbot (*Scophthalmus maximus*), sea bass (*Dicentrarchus labrax*) and wolfish (*Anarhichas minor*) (see Table 2, pp. 28-33). A pressing lack of knowledge concerns the *Ichthyobodo* genotypes infecting wild caught wrasse species, which are often infected with *Ichthyobodo* sp. (e.g. Costello *et al*., 1996; Treasurer, 1997; Askeland & Karlsbakk, 1999). The wrasse is used as cleaner fish in salmon sea pens, where they remove salmon lice from the salmon. Concerns have been raised of the possible transfer of diseases between wrasse and salmon

(Treasurer, 2012). The infection risk to salmon may be predicted when these *Ichthyobodo* species or genotypes are known, albeit an experimental examination of the host range of *I. salmonis* would be preferable. In paper D, molecular tools that should aid further research in the field is provided.

## Conclusion and future aspects

The morphological studies of *Ichthyobodo* spp. in the present study have proposed and used novel morphometric methods in describing the parasites, and revealed several useful distinguishing characteristics. However, extensive morphological descriptions of genetically identified *Ichthyobodo* spp. are limited (only 3 species; *I. necator* s.s., *I. salmonis* and *I. hippoglossi*). A few apomorphic traits were discovered among these *Ichthyobodo* species at light- and electron microscopic level, for example size and structure of kinetoplasts in *I. hippoglossi* and the surface-spines seen in SEM images of *I. salmonis*. Comprehensive ultrastructural studies of separate genotypes of *Ichthyobodo* may provide further characters suitable for delineating species. Still, it is likely that *Ichthyobodo* species (i.e. distinct genotypes or groups of closely related genotypes) will be discovered where suitable morphological distinguishing charactes are found. Hence, molecular methods will be very important in the characterisation and identification of *Ichthyobodo* spp. in the future.

Specific PCR methods (Real-time PCR assay and PCR primers for sequencing) have been developed in the present work and are designed for targeting SSU rDNA of all known species within genus *Ichthyobodo*. The qPCR assay (“Costia-assay”) has proved to be sensitive and effective in detection of *Ichthyobodo* spp. and may be an important tool in monitoring fish farms for infections. The application of this method has also aided the detection and subsequent characterisation of new species or genotypes

isolated from a wide range of hosts. Overall, the PCR methods developed have contributed significantly to increased knowledge regarding the diversity and geographical range of *Ichthyobodo* spp.

Further sequencing of *Ichthyobodo* sp(p). from different hosts will likely lead to the detection of further genotypes that may represent new species or strains of *Ichthyobodo* spp., contributing to increased knowledge regarding the geographical distribution and host specificity of the parasite. The list of reported *Ichthyobodo* infections (see Table 2, pp. 28-33) is long and increasing and information of host susceptibility is needed for our understanding of the epizootiology of different *Ichthyobodo* species. According to Boenigk *et al.* (2012), a single gene sequence (e.g. SSU rRNA gene) is not always sufficient in delineate species. Hence, genotypic identification that distinguishes different species or strains should involve multi-gene analyses, for instance by using internal transcribed spacers (ITS) rRNA gene regions or cytoplasmic heat shock protein 90 (hsp90) as additional target genes (cf. Simpson *et al.*, 2002; Callahan *et al.*, 2005).

Many aspects of *Ichthyobodo* spp. biology including interactions with their fish hosts could be more readily examined with a steady supply of living parasites. It is known that infections can be sustained in tanks with unfavourable conditions such as crowding and other types of stress. However, due to ethical aspects *in vitro* cultivation of *Ichthyobodo* spp. is preferable. Cultivation trials may be performed on various epithelial cell lines. If such methods became available, genomic studies would be easier to perform. Also life cycle aspects such as cyst formation could be studied. Genomic studies would reveal important knowledge on the relationship between genus *Ichthyobodo* and the other Kinetoplastida, and would greatly expand the genetic character repertoire useful for delineating species in the family Ichthybodonidae.

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