CHAPTER 3

ISOLATION AND IDENTIFICATION OF DIFFERENT PARASITES OF FRESHWATER CARP

3.1 Introduction

The major carp farming mainly dominates fresh water aquaculture of West Bengal. The most important prerequisite of fish production is availability of healthy fish fingerlings of carps. It is evident from the available literature that parasitic diseases caused significant damage in fish industry mostly affecting the fry and fingerlings.

Some groups of parasites possess unique features that enable a diagnosis of whole organisms to be made, many of these features may not be easily observed or identified and the degree of taxonomic detail available may vary between the major groups. Generally, identification of parasites based on recognizing morphological detail and determining the life cycle of the parasites in the fish. The host specificity of particular parasite, its habitat along with water temperature and other environmental conditions, can also provide additional clues to parasite's identity. The location of the parasite in or on the host is also important, as some parasites are only found in certain organs or tissues.

3.2 Materials and Methods

3.2.1 Examination of host fishes and collection of parasites

Live host specimens namely, *Labeo bata* and *Labeo calbasu* were randomly sampled and collected from the fish farms, ponds and near by markets of Howrah, Hooghly, Purba and Paschim Medinipur, South and North 24 parganas and Nadia district. The hosts were examined immediately after collection. The parasites were collected from infected areas for gross observation and identification. At first, the external surface of the host body including scales, fins, skin, fin- base etc. were examined by collecting the mucus for ectoparasites. Gills were removed from the branchial cavity and placed on a glass slide for microscopic examination. After examination of the external surface, the organs were dissected to search out the internal parasites. To investigate the body cavity and general viscera, the body of the

host fish were dissected out for examination. The parietal peritoneal lining of the body cavity, outer surface of the visceral organs and serous membranes were examined for encysted larva.

3.2.3 Preparation of slide for myxozoan parasites

i. Myxosporidian cysts when found attached on the tissues like gills, fins, scales, and body surface, were isolated carefully.

ii. Free floating cysts found in the gill were isolated with the help of dropper.

iii. For detailed study and taxonomic identification, fresh cysts were first taken on clean grease free glass slides.

iv. The cysts were slightly ruptured on one end with a needle. The spores released from the cyst were mounted with cover glass and examined.

v. When cysts were not found gill, body, and fin smears were prepared on grease free clean slides with a drop of 0.5% NaCl solution and air-dried for delection of parasites.

vi. For detection of iodinophyllic vacoules in the sporoplasm, fresh spores sealed with cover slips, were treated with Lugol's Iodine solution and examined under oil immersion microscope.

vii. To detect any external shell envelopes surroundig the spores, Indian ink method as described by Lom and Vavra (1963) were followed.

viii. Some of the fresh spores were treated with 5% potassium hydroxide (KOH) solution or saturated aqueous solution of urea [CO(NH2)2] for the extrusion of polar filament.

ix. Permanent mounting of myxosporen parasites were done by staining with Giemsa. Airdried smears on grease free clean slides were treated with acetone free absolute methyl alcohol for about eight minutes to fix the parasites and again dried.

x. Now the stock solution of Giemsa was diluted with water in the ratio of 1: 2 and buffered at pH 7.2.

xi. The slides were then placed in the staining rack and covered with dilute stain for forty to fifty minutes.

xii. Finally, the slides were washed by pouring neutral distilled water or buffer solutions until the colour did not turn to a noticeable extent and the slides were dried in air.

xiii. The slides are now ready for examination under microscope and photographs were taken with the help of Olympus phase contrast microscope fitted with digital camera.

3.2.4 Preparation of slides for ciliophoran parasites

i. The gill arches were removed and rubbed against the surface of a clean, grease free dry microscopic slide.

ii. The smear thus produced, was allowed to dry in air for three to five minutes. Precautionary measures were taken for a minimum loss of water from the gill tissues.

iii. Mucus collected from body surface was also treated the same way to prepare body smear.

iv. The preparations were then subjected to silver impregnation after the method of Klein (1958). The slides were stained with 2% silver nitrate (AgNO3) for seven to ten minutes and rinsed three times with distilled water to remove excess stain by holding the slides in slanting position.

v. The stained slides were then transferred to petri dishes filled with distilled water up to the brim so as to immerse the slides completely.

vi. The petridishes were placed over white filter papers and kept into a small UV sterilization chamber containing UV tube and irradiated for twenty five to thirty minutes.

vii. The slides were finally air dried completely and mounted in D.P.X.

viii. During exposure to UV light, the smears turned brown as the silver in argentophilic structures was reduced. The darker their appearance, the better was the impregnation.

ix. To stain the trophonts of Ichthyophthirius, Giemsa stain was used. Trophonts were collected by scrapping the gill and body surface of infected fishes and smeared on grease free slides.

x. Dried smears were fixed in acetone free absolute methyl alcohol for ten minutes and again dried.

xi. Now the stock solution of Giemsa was diluted with water in the ratio of 1: 2 and buffered at pH 7.2.

xii. The slides were then placed on a staining rack and covered with dilute stain for forty to fifty minutes.

xiii. Finally, the slides were washed by pouring neutral distilled water or buffer solutions until the colour did not turn to a noticeable extent and the slides were dried in air.

3.2.5 Preparation of monogenean parasites

i. Parasites in the cyst form were released from the cyst and preserved.

ii. Mucus and other dirt particles attached to the parasite body were removed by vigorous irrigation with a narrow-mouthed dropper before fixation.

iii. Digeneans and monogeneans were press fixed and stored in 5% NBF and stained in Gowers carmine or Alum carmine.

iv. Properly stained specimens were dehydrated in alcohol series, cleared in creosote and mounted in Canada balsam.

3.2.6 Preparation of crustacean parasites

Crustacean parasites were fixed in 7% NBF and later on washed properly and transferred to 70% alcohol for long term preservation. For detailed study, the parasites were washed and dissected in 50% aqueous lactic acid using wooden slides (Humes and Gooding, 1994).

3.2.7 Measurement and figure

The measurements of the parasites were taken using a calibrated ocular micrometer. Photomicrographs were taken with an Olympus phase contrast microscope fitted with Olympus camera.

3.3 Results

3.3.1 Isolation and identification of myxozoan parasites

Myxosporidians constitute typical fish parasites known to produce cysts on different regions of the body and internal tissues and organs. Myxozoans are an extremely abundant and diverse group of organisms, speciated by spore shape and size. The common myxosporidians genera are *Myxoobolus, Henneguya* and *Thelohanellus* etc. Symptoms of this infestation include weakness, emaciation, raising of the scales etc.

3.3.1.1 CLASSIFICATION OF THE PHYLUM MYXOZOA

The system of classification presented here is based on that of Kent et al. (2000b) with the addition of the Class Malacosporea.

Phylum: Myxozoa Grasse, 1970

Class: Myxosporea Butschli, 1881.

Order: Bivalvulida Schulman, 1959.

Sub-order: Sphaeromyxina Lom and Noble, 1984 with one family and one genus *Sphaeromyxa*.

Sub-order: Variisporina Lom and Noble, 1984 with ten familie and thirty three genera e.g. *Myxidium*, *Ceratomyxa*, *Sphaerospora*.

Sub-order: Platysporina Kudo, 1919 with one family and thirteen genera e.g. *Myxobolus*, *Henneguya, Thelohanellus*.

Order: Multivalvulida Schulman, 1959 with six families and seven genera.

Class: Malacosporea Canning,Curry, Feist, Longshaw and Okamura, 2000.

Order: Malacovalvulida Canning, Curry, Feist, Longshaw and Okamura, 2000, with one family and two genera *Buddenbrockia* and *Tetracapsuloides.*

3.3.1.2 Myxosporidian belonging to the genus *Myxobolus* Butschli, 1882.

Description of *Myxobolus* sp.I

The species has been described from *Labeo calbasu* collected collected from Naihati, North 24 Parganas, West Bengal. These were isolated from the gills of the infected fish. Plasmodia were creamy white in colour and rounded in shape (2-3 mm in diameter). It contains both late developmental stages and mature spores. Mature spores are slightly rounded to oval shaped and anterior and posterior ends blunt semicircular. The two polar capsules are unequal in size. The polar filaments are not extruded out. Inside the polar capsules there are 8-9 coils in case of larger polar capsules and 4-5 coils were found inside the smaller polar capsules (Fig 3.1A and 3.1B).

Table3.1. Statistical analysis of measurement of the spores of *Myxobolus* sp.I

Parameters	Range (µm)	Mean (µm)	Standard Deviation (SD)	Standard Error (SE)	Coefficient of Variance (CV%)
Length of the spore (LS)	12.04-16.83	14.94	1.72	1.29	11.50
Breadth of the spore (BS)	9.08-13.36	10.88	1.53	1.12	14.10
Length of Larger Polar Capsule (LLPC)	5.20-8.06	6.55	0.95	0.84	14.50
Breadth of Larger Polar Capsule (BLPC)	3.16-5.10	4.20	0.69	0.66	16.70
Length of Smaller Polar Capsule (LSPC)	3.26-4.99	3.93	0.57	0.59	14.50
Breadth of Smaller Polar Capsule (BSPC)	2.24-3.06	2.73	0.25	0.47	9.30

Spore Index:

LS: BS = 1: 0.728
LLPC: BLPC = 1:0.642
LSPC: BSPC = 1: 0.696
LLPC: LSPC = 1: 0.599
BLPC: BSPC = 1: 0.651

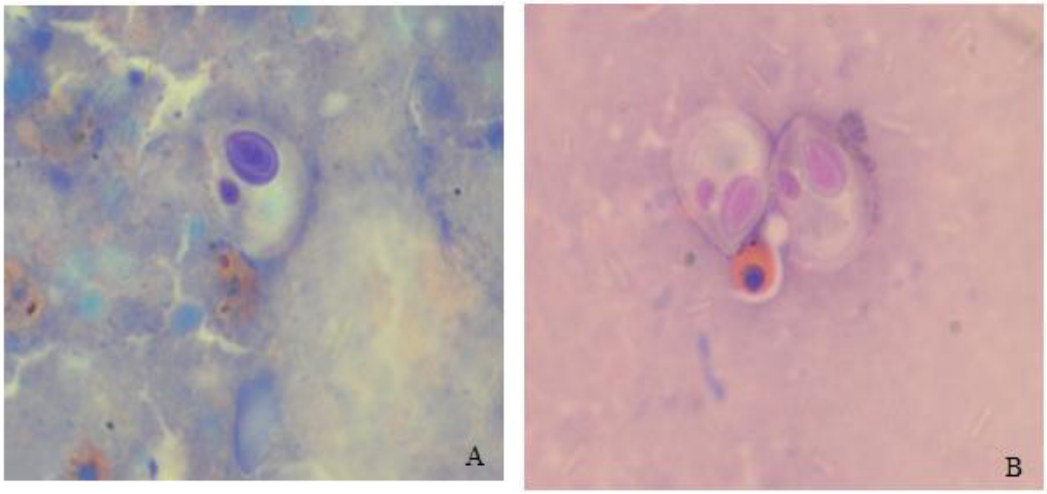

Fig 3.1A and 3.1B: *Myxobolus* sp.I

Description of *Myxobolus potaili* Lalitha Kumari, 1969

The species has been observed from the freshwater carp *Labeo calbasu* collected from Kalyani, Nadia, West Bengal. White rounded plasmodia are observed attached with the gills of the host body. Mature spores are pear shaped measuring [7.13±0.14 (6.9-7.1) X 4.69±0.44 (4.1-5.1)] with rounded posterior and blunt anterior end (Fig 3.2A and 3.2B). The shape and size of the specimen is similar to that of *Myxobolus potaili* described from *Labeo potail* skyes. The shell valves are devoid of any partietal fold or marking (Fig b). Two equal sized pyriform polar capsules measuring [3.48±0.4 (3-3.9) X 1.97±0.11 (1.8-2.1)] with greatly rounded posterior and sharply pointed anterior end converge closely. There is no mucous envelop around the spore as well as iodinophilous vacuole in the sporoplasm.

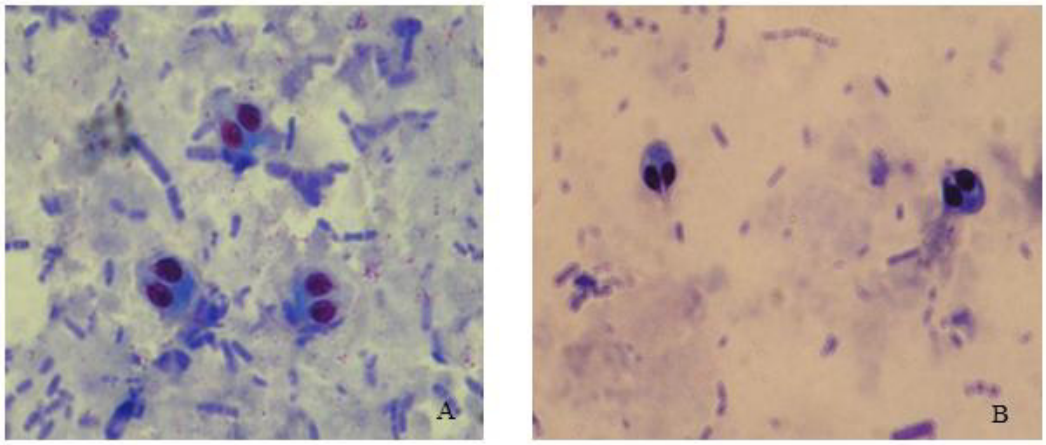

Fig 3.2A and 3.2B: *Myxobolus potaili*

Description of *Myxobolus* sp.II

The species was identified from freshwater carp *Labeo bata* (Hamilton) collected from Purba Medinipur, West Bengal. The plasmodia appeared as creamy white cyst on the gill lamallae. Mature spores are elongated and pyriform in valvular view and lenticular in sutural view. The spore size are 16.93± 0.38 (16.2-17.1) x 7.05 ± 0.48 (6.2-7.5) and are bluntly pointed anteriorly with a rounded posterior end. Two anteriorly situated pyriform polar capsules run parallel to each other (Fig3.3A and 3.3B). Two polar capsules are equal in size.

Table 3.2: Statistical analysis of measurements of the spores of *Myxobolus* sp.II

Measurements	Range(µm)	Mean(µm)	SD	SE	CV%
Length of the spore (LS)	16.2-17.1	16.93	0.38	1.8	2.3
Breadth of the spore (BS)	6.2-7.5	7.05	0.48	1.15	7.03
Length of the polar capsule (LPC)	9.8-10.9	10.52	0.49	1.4	4.8
Breadth of the polar capsule (BPC)	2.3-3.9	2.9	0.61	0.68	21.3

Spore index

LS : BS = 1:0.416
LPC : BPC = 1:0.275
LS : LPC = 1:0.621
BS : BPC = 1:0.411

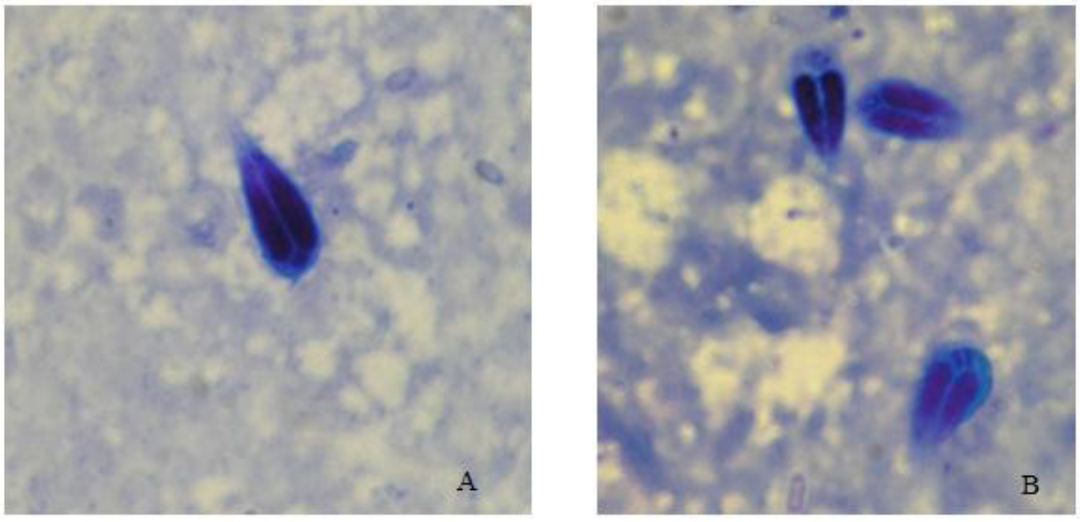

Fig 3.3A and 3.3B: *Myxobolus* sp.II

Description of *Thelohanellus* sp.

The species has been identified from the freshwater carp *Labeo calbasu* (Hamilton, 1822) collected from Naihati, West Bengal. The spores are pyriform in shape and strikingly elongate with rounded posterior and a blunt anterior proximity. There are no markings or folds on the valves. Mature spores measure 13.68±0.69µm in length and 4.32±0.36 µm in breadth.

Large sized pyriform polar capsule measuring 8.75±0.44 µm in length and 3.876±0.24 µm in breadth occupy a major portion of the spore cavity. There are about seventeen to nineteen coils of the polar filament. When completely extruded, anterior end of the long thread like polar filament measuring 95.5±0.5 (92-95.8) µm appeared to be straight line (Fig 3.4A and 3.4B).

Table 3.3: Statistical analysis of measurements of the spores of *Thelohanellus* sp.

Measurements	Range(µm)	Mean(µm)	SD	SE	CV%
Length of the spore (LS)	12.8-14.2	13.68	0.69	1.6	5.1
Breadth of the spore (BS)	3.8-4.8	4.32	0.36	0.89	8.6
Length of the polar capsule (LPC)	7.8-8.9	8.75	0.44	1.2	5.17
Breadth of the polar capsule (BPC)	3.5-4.1	3.876	0.24	0.82	6.44
Length of the polar filament (LPF)	92-95.8	95.5	0.5	1.3	

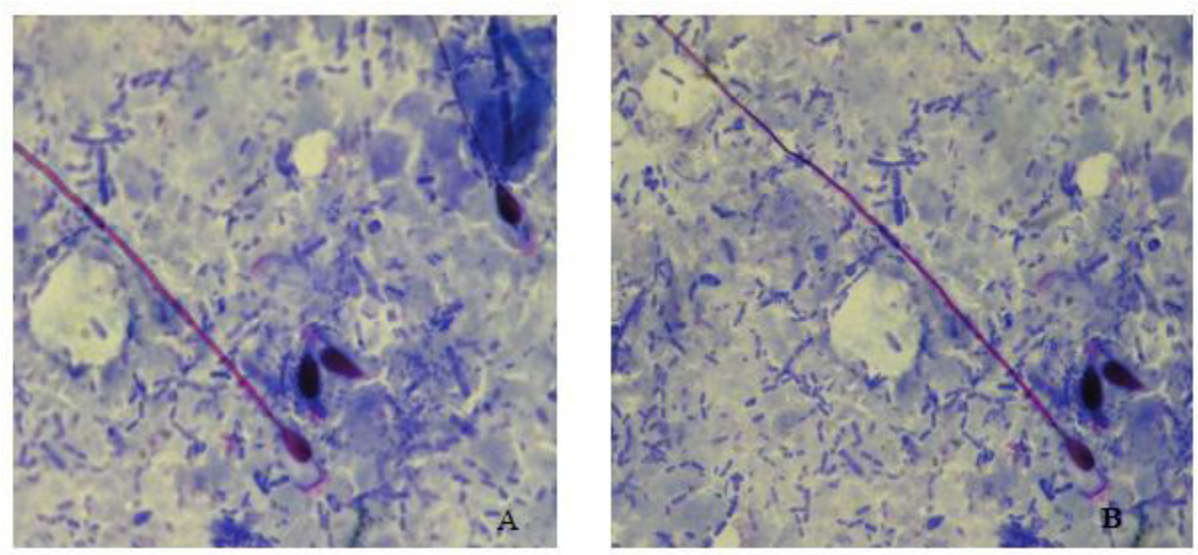

Fig 3.4A and 3.4B: *Thelohanellus* sp.

3.3.2 Isolation and identification of ciliophoran parasites

Most of the ciliate protozoans have tiny hair like structures called cilia that are used for locomotion and/or feeding. Ciliates have a direct life cycle and many are common inhabitants of pond-reared fish.

During the present study, members of three genera, viz. *Trichodina*, and *Trichodinella, Tripartiella*, have been identified from fresh water carp fishes. *Ichthyophtheirius multifilis* causing ich or white spot disease in fishes was also observed.

Trichodiniasis disease is caused by trichodinid ciliophorans. Clinically, fish usually exhibit flashing and become lethargic. The skin may develop ulcers and increase of mucus production. Though the masses of organisms are attached by the adhesive discs and denticles of exoskeleton, there is secondary hyperplasia and hypertrophy of the gill epithelium and underlying epithelial cells undergo necrosis. Ichthyophthiriasis disease is caused in carps by protozoan ciliate, *Ichthyophthirius multifilis*, which infect different regions of the body externally. Whitish cysts appear on the skin, gill and fins.

3.3.2.1 CLASSIFICATION OF THE PHYLUM CILIOPHORA

The system of classification adopted in this review is based on that of Lynn, 2003. The phylum is divided into two subphyla: Postciliodesmatophora with characteristic microtubular ribbons linking all kinetosomes in a kinety, comprising two classes; and Intramacronucleata, in which macronuclear division involves microtubules that lie inside it. This subphylum comprises nine classes with a total of nineteen subclasses. The freshwater fish inhibiting symbiotic or parasitic ciliates trichodinids are grouped under the subclass Peritrichia of the class Oligohymenophorea, one of the major taxon of the subphylum Intramacronucleata. Another important ciliate, *Ichthyophtherius* is included under the class Prostomatea.

Phylum:	Ciliophora Doflein, 1901
Subphylum:	Intramacronucleata Lynn, 1996
Class:	Oligohymenophorea de Puytorac et al, 1974
Subclass:	Peritrichia Stein, 1859
Order:	Mobilina Kahl, 1933 (e.g. *Trichodina sp.*, *Tripertiella sp., Trichodinella sp.*)
Class:	Prostomatea Schewiakoff, 1896 (e.g. *Ichthyophtherius* sp.)

3.3.2.2 Trichodinid parasites

The genus *Trichodina* Ehrenberg, 1830 is identified with its adoral ciliary spiral making a turn of 330°-540°. The denticulate ring composed of denticles with straight or curved blades, distinct rays of various shapes and lengths, and central parts lack and anteriorly directed projections. The parasites are found in gills and skins of the host fish.

Description of *Trichodina nandusi*

The species has been identified from *Labeo bata* collected from Kalyani, Nadia, West Bengal. These are medium sized trichodinids measuring 42.1-

53.0(47.1±3.4) µm in diameter and are disc shaped. Denticulate ring consists of 20.2-28.5(24.1±1.1) µm large sized denticles measuring 12.5±0.6µm in span and 5.2±0.2µm in length. There are 5-9 (6.7±0.8µm) radial pins per denticle (Fig 3.5). The species was identified by the presence of central clear area which is subdivided into many small granular structures and spatulate rays.

Fig 3.5: ***Trichodina nandusi***

Description of *Tripertiella bulbosa*

The species is identified by the elongated blade with parallel lateral margins, which are constricted at either end, and is joined at the centre by a prominent constriction at the mid length of the blade. It has been observed from *Labeo bata*. These are free moving disc shaped trichodinid with a diameter of 15.5-20.2(17.7±1.8). Around the adhesive disc measuring 12.5-18.1(15.2 ± 2.2), there is a finely starited border membrane. The central area of the disc is finely granular with a diameter of 4.1-6.5(5.5±0.8). Denticulate ring consists of 22-25(23±1.8) denticles and 3-5 radial pins per denticle (Fig3.6). The species is identified by the presence of parallel lateral margins of the spherical blade which is attached to the anterior projection through its stem-like narrow basal part.

Fig 3.6: *Tripertiella bulbosa*

Description of *Ichthyophthirius multifilis* Fouquet, 1876

The spherical to ovoid trophonts may reach 1mm or more in diameter, have short cilia covering the entire surface and have a single horseshoe-shaped macronucleus and a single round or oval micronucleus which is sometimes visible under 100 x magnification (Fig 3.7) and a smaller rounded micronuclus which is visible in stained preparation.The adult parasite moves slowly in a tumbling manner. The immature forms (tomites) are smaller, translucent, and move quickly.

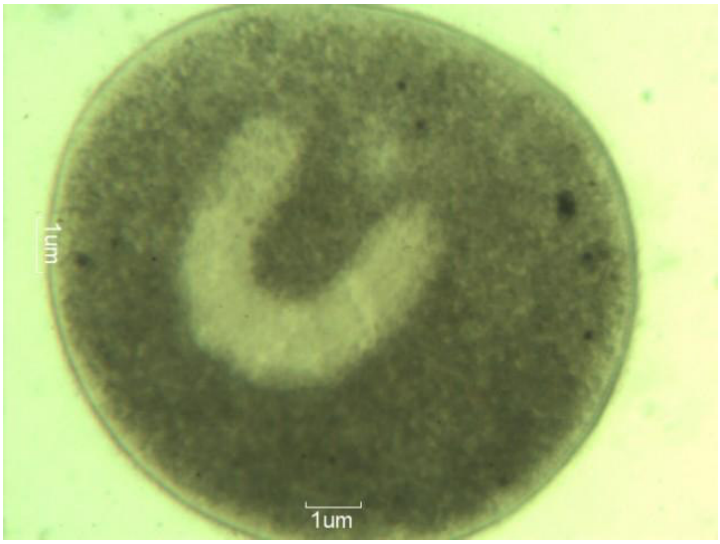

Fig 3.7: *Ichthyophthirius multifilis*

3.3.3 Isolation and identification of monogenean parasites

Monogeneans are a class of parasitic flateworms or flukes commonly invade the gills, skin and fins of fish. Monogeneans have direct life cycles (no intermediate host) and are host and site-specific. These parasites are still widespread in freshwater wildlife, farm fishes and marine habitats. *Gyrodactylus* and *Dactylogyrus* are the two most common genera of monogeneans that infect freshwater fish. They differ in their reproductive strategies and their method of attachment to the host fish.

Classification of *Dactylogyrous* sp. (According to Diesing, 1850)

Kingdom: Animalia

Phylum: Platyhelminthes

Class: Monogenea

Order: Monopisthocotylea

Family: Dactylogyridae

Genus: *Dactylogyrus*

Classification of *Gyrodactylus* sp. (According to Malmberg, 1957)

Kingdom: Animalia

Phylum: Platyhelminthes

Class: Monogenea

Order: Monopisthocotylea

Family: Gyrodactylidae

Genus: *Gyrodactylus*

Description of *Dactylogyrous* sp.

The genus *Dactylogyrus* is found on the gills of mostly cyprinid fishes. *Dactylogyrus* is recognized by a four-lobed head with four eye spots. The average length of this species is 1.2 mm and width 0.33 mm. Body is short and flattened, with uniform width throughout, but narrowing towards both anterior and posterior ends. Haptors are slightly separated. Anchors are bifid with well developed outer and inner roots and strongly recurved pointed tips. Both dorsal and ventral connecting bars present (Fig 3.8). When the worm is present in large numbers, gill hyperplasia and necrosis can result.

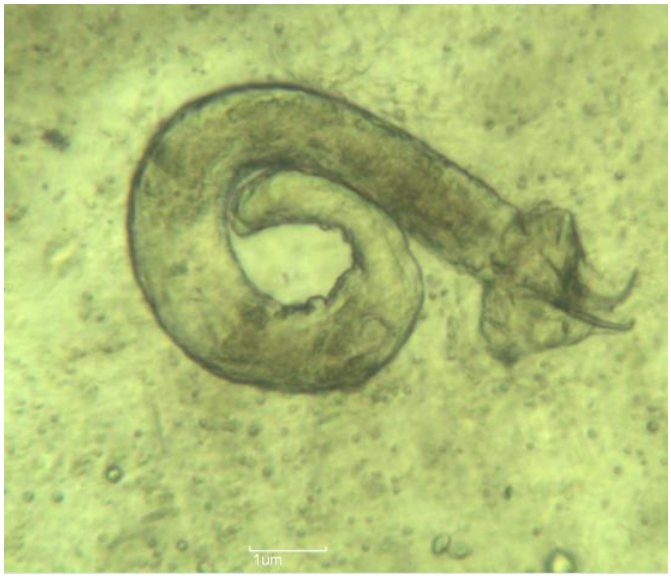

Fig 3.8: *Dactylogyrous* sp.

Description of *Gyrodactylus* sp.

The genus *Gyrodactylus* is a small monogenetic fluke attaches to gills, fins and skin epithelium using an attachment organ known as an opisthohaptor armed with a pair of large hooks and sixteen marginal hooklets. The average length of this species is 0.75 mm. The head of the worm is bilobed, lacks eyespots (3.9). Heavy infestations by the parasite can result in destruction of the gills or skin epithelium due to mechanical damage caused by the attachment organ.

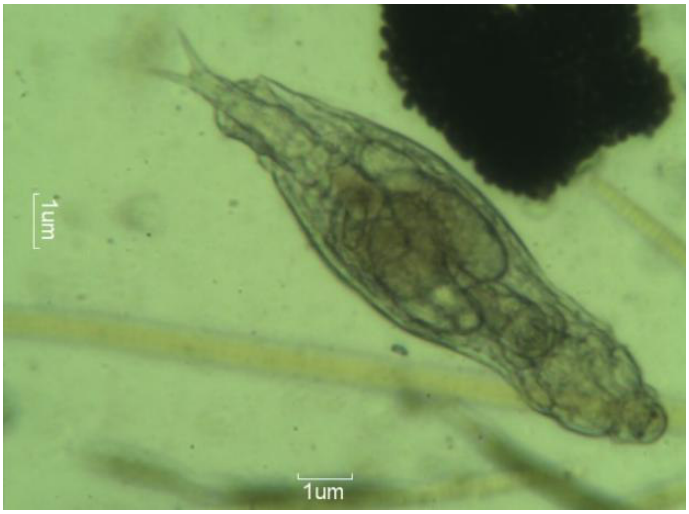

Fig 3.9: *Gyrodactylus* sp.

3.3.4 Isolation and identification of Crustacean parasites

The fish lice (Argulidae) are an important group of crustacean fish parasites. Fish lice with their dorso-ventrally flattened bodies and characteristic appendages are unmistakable. Clinical signs in infected fish include intense irritation which causes fish to rub or scrape against objects in the aquarium walls, erratic swimming, and poor growth. It causes pathological changes due to direct tissue damage and secondary infections.

Classification:

Kingdom: Animalia

Phylum: Arthropoda

Subphylum: Crustecea

Class: Maxillopoda

Subclass: Branchiura

Order: Arguloida (Yamaguti, 1963)

Family: Argulidae (Leach, 1819)

Description of *Argulus* sp.

This parasite is 1.9 mm long. It has a flattened, oval body which is almost entirely covered by a wide carapace. Compound eyes are prominent, and the mouth parts and the first pair of antennae are modified to form a hooked, spiny proboscis armed with suckers (Fig 3.10).

Fig 3.10: *Argulus* sp.

3.4 Discussion

Fish suffers from different diseases as they can carry different pathogens and parasites. During this study, heterogenecity in myxozoan and ciliophorans parasites have been observed in freshwater carp. The site of infection in most of the cases is gill though in some cases parasites have been isolated from the fins as well. Myxozoan and Ciliophoran diseases were found to be very common in all the freshwater carps throughout the year. Myxosporidians belonging to two genera namely *Myxobolus* and *Thelohanellus,* among the ciliophorans, *Trichodina, Tripertiela, Ichthyothirius multifilis*, monogeneans belonging to two genera *Dactylogyrus* and *Gyrodactylus* and Crustacean belonging to *Argulus* have been isolated and identified. Most of the species were earlier reported from different

geographical areas of India. Although they showed morphometric variations, the overall characters were similar and could easily be identified. Some new species with distinguished characteristics have also been observed. The abundance and diversity in *Myxobolus* sp. was much more than the other genus. Only a single speceis of *Thelohanellus* sp. have been observed. Numerous members of ciliates *Trichodina*, and *Tripartiella* have been observed and identified by their characteristic features during the course of study. All the species was previously described by different authors from India as well as abroad. A majority of freshwater fishes carry heavy infection of parasites which cause deterioration in the food value of fish and may even result in their mortality. These parasites use the fish for their shelter and food and destruct more or less each and every organ resulting in pathogenic effects (Lilley *et al.,* 1992). Parasites interfere with the nutrition, metabolism and secretary function of alimentary canal, damage nervous system and even upset the normal reproduction of the hosts (Rahman *et al.,* 1998a, b). The distribution of these parasites of the same host and their incidence and intensity of infestation varies from one place to another. Fish diseases are the great threat in our fish culture system. Many fish species affects by various types of diseases every year and as a result, production of fishes decreases significantly. Proper steps should be taken to prevent fish diseases and to protect these important fish species from extinction. From the study it was observed that the parasites were most important pathogen for disease outbreak.