MATERIALS
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METHODS
Fresh water major carps, *Cirrhinus mrigala* (Ham) and *Labeo rohita* (Ham) were procured from seven water bodies (ponds) viz., Arnapara talab, Bhoodha talab, Doormar talab, Kho-Kho talab, Maharajbandh, Mathpara talab and Telibandha talab of Raipur city area during the course of study. Fishes of approximately same weight (100-150 g) and size (15-20 cm) were used during the study.

Fish were collected live with the help of local fishermen, packed in separate polythene bags and transported immediately to the laboratory. They were kept in separate glass aquarium with proper aeration. The fishes thus collected were numbered, measured, weighed, sexed and subjected to thorough examination of protozoan parasites. Firstly fishes were observed superficially for any signs of disease symptoms. Their body surface was examined carefully for any visible outgrowth or cyst etc. Normally, in addition to colour change, dull spots on the body or excess mucous production, behaviours, such as whirling, rotating and swimming upside down, gulping air frequently, rubbing against firm objects or sluggishness with lack of escape reactions, identified the diseased fishes.

Externally, the skin, fin and scales were subjected to thorough microscopic examination by preparing smears on glass slides. Operculum was cut off and their inner sides were thoroughly examined. Gills from both sides were taken into separated petri dishes containing 0.5% saline. Subsequently, the body was cut through mid ventral incision and various visceral organs were collected individually in cavity block containing 0.5% saline. The body musculature was also teased and collected separately. Every care was taken to avoid contamination. Each organ/tissue was smeared on a glass slide with a drop of 0.5% saline solution, covered with a cover slip and sealed with paraffin wax and examined under light and phase contrast microscope at low and high magnification for protozoan parasites.

The cysts and plasmodia were carefully isolated from tissues, placed on a glass slide in 0.5% saline, teased with a fine needle, covered with a cover slip and observed
under oil immersion objective for spores and other developmental stages. Gall bladder contents were directly smeared on a glass slide and examined for coelozoic protozoans under a compound microscope. Adequate record of fishes found infected, sex wise, size and weight wise, in respect of each protozoan species recovered were carefully maintained throughout.

Species identification was based on morphological details using following methods and measurements of the specimen in the live stage. At times, due to very heavy infections, study of living material was time consuming. However, in such situation, fresh mounts containing spores were stored with 1% agar and cover slip sealed with paraffin wax at 4°C. Spores were viable and healthy for more than 48 hrs. Measurements were taken from large number of parasite on fresh material using a calibrated ocular micrometer and are expressed in μm unless otherwise stated. Drawings were made with the help of Camera Lucida. Photographs were taken using a micro photographic camera attachment on Carl-Zeiss binocular and Olympus trinocular microscope. The species identified are described in this dissertation. All type slide meant for new description are deposited with School of Life Sciences of Pandit Ravishankar Shukla University, Raipur. Although species naming becomes valid after publication (International code of Zoological Nomenclature, Article 7 and 8), in the present thesis, all new species were named tentatively to avoid difficulty while description.

**Hanging drop preparation:**

Spores were obtained from tissues, organs or cysts of the infected fish, as described earlier and were placed on cover slip with a drop of 0.5% saline solution. A thick layer of grease was applied around the grooves of the grooved slides. The cover slip was then inverted over the layer of grease and the entire preparation was examined under the microscope. The process enabled the spores being examined without any pressure from the cover slip.
Lugol’s Iodine preparation for iodinophilous vacuole:

For observing the nature of iodinophilous vacuole, fresh spores was treated with lugol’s iodine solution (1g iodine and 1g potassium iodide dissolved in 100 ml distill water). Few drops of solution was added to the spore suspension on glass slide and covered with a cover slip, sealed with paraffin wax and viewed under different magnification of compound microscope. The iodinophilous vacuole when present, took mahogany red stain. The size, position and structure of the vacuole were noted for the taxonomic identification of the myxozoans. The sporoplasm in fresh spore preparations showed a clear hyaline rounded area in the centre which became dark brown, when the spores were treated with lugol’s iodine solution.

Indian ink technique for mucus envelops:

The presence of mucus envelop around the myxosporean species was detected by mixing a drop of Indian ink with a drop of spore suspension taken on a glass slide and observed under a microscope. The Ink particles were excluded by the mucous envelop, which appears light against a dark background.

Extrusion of polar filament:

Polar filaments were extruded by placing fresh spores in a drop of saturated aqueous solution of urea, different concentration of potassium hydroxide (2.5%-10%) or ammonia solution. The filaments extruded within 30 seconds. The length of polar filaments was measured for taxonomic diagnosis.

Giemsa staining:

This was applied for staining of the developmental stages, mature spores and the polar filament
Reagents Used:

1. *Giemsa stain*: 0.6 g Giemsa powder was dissolved by continuous stirring in 50 ml glycerin and warmed for 3 hours at 60°C. Cooled to room temperature and 50 ml acetone free Methyl alcohol was added to it and the whole was stored in dark for a month for maturation. Diluted 1:10 with distilled water before use.

2. *Buffered water (pH 7.0)*: 0.25 g potassium dihydrogen phosphate and 1.7 g disodium hydrogen phosphate were dissolved in 950 ml distilled water. The pH was adjusted to 7.0 using dilute acid or alkaline and whole was made to 1 litre with distilled water.

Material was smeared on a glass slide with a drop of distilled water, air dried, fixed in methyl alcohol for 5 min. and stained with Giemsa stain for 1 to 2 hour, washed with buffered water, air dried and examined under the microscope.

Staining of polar filaments: Spore suspension was treated with 10% KOH solution which extruded polar filaments in most of the cases. The KOH treated slides were blotted with a filter paper and allowed to dry in air for 30 minutes. After fixing in methyl alcohol for 5-7 minutes, the smear was stained with Giemsa by the usual procedure as above. The polar filaments were brilliantly stained with Giemsa staining.

Heidenhain’s Iron alum haematoxylin:

Reagents Used

1. *Schaudinn’s fluid*: 65 ml saturated aqueous mercuric chloride; 30 ml 95% alcohol and 5 ml glacial acetic acid were mixed together.
2. *Carnoy’s fixative*: 60 ml absolute alcohol, 30 ml chloroform and 10 ml glacial acetic acid were mixed together.
3. *Mordant*: 3 g Ammonium ferric sulphate dissolved in 100 ml distilled water
4. *Heidenhain’s haematoxylin*: 1 g powder Haematoxylin was dissolved in 90 ml distilled water and whole was made to 100 ml with ethyl alcohol
For permanent preparation, the infected tissue was smeared on glass slide and fixed either in Schaudinn's fluid (20-30 min) or Carnoy's fixative (20 min). When using the Schaudinn's fixative, smears were treated with iodinated alcohol (few Iodine crystals in 70% alcohol) for removing mercuric chloride. Slide down graded in alcohol series and then treated with 3% iron alum solution (Mordant) over night. Stained with Heidenhain's haematoxylin for 30 min. and differentiated, if necessary, with 1% iron alum solution. The slides were washed thoroughly with water, upgraded in alcohol series, cleared in xylene and mounted in DPX or Canada balsam.

Seasonal incidence:

Data for one annual cycle from July 1998 to June 1999 on various myxosporean species recorded in Cirrhinus mrigala and Labeo rohita was subjected to analysis of incidence of infection sex, month, season and organ wise and is presented in this dissertation.