3.1 STUDY AREA

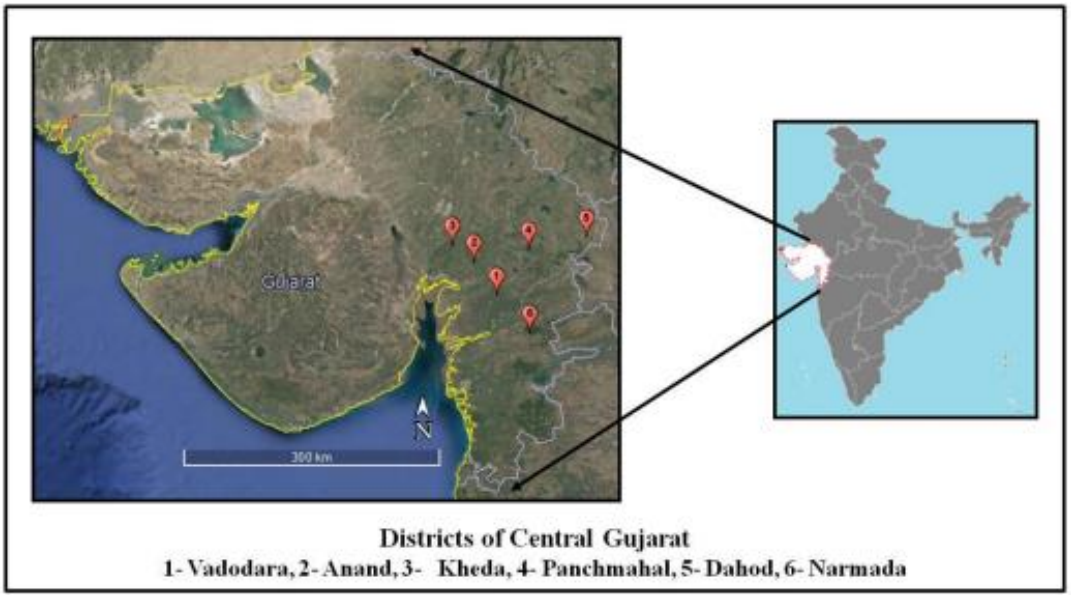

Plate 2. Study area Map

Representative freshwater sheets from the Central Gujarat area were selected for sampling and study. For sample collection, six districts including Vadodara, Anand, Kheda, Nadiad, Panchmahal, Dahod and Narmada were selected largely covering the area between Mahi and Narmada river as shown in Plate 2.

1. **Anand**

   Anand is located at 22.57°N and 72.93°E. It has an average elevation of 39 m (128 ft). The city has an area of 47.89 sq. km. A total of 145 ponds were sampled based on feasibility (Appendix I).

2. **Kheda**

   Kheda is located at 22.75nad, 72.68°E. It has an average elevation of 21 meters (68 feet). Kheda is on the banks of the Vatrak and Shedhi rivers. A total of 16 ponds were surveyed from the Nadiad area, as they were feasible (Appendix I).

3. **Vadodara**

   Vadodara district, is located in the eastern part of Gujarat, in Western India. The district covers an area of 4,312 square kilometres and is situated in the central part of mainland Gujarat, between 22°33’02” North latitude and 73°19’58” East longitude. A total 156 ponds were studied for their aquaculture status and also fish samples were collected. A data sheet/ table was created for the record (Appendix I).

4. **Panchmahal**

Panchmahal District is located in the northern region of Gujarat and is enclosed by latitudes 20.30 to 23.30 in the north and longitudes 73.15 to 74.03. Its total area covers 5210 sq. km. A total of 64 ponds were studied and sampled for their aquaculture status (Appendix I).

5. **Dahod**

Dahod is a city located on the banks of the Dudhimati River in Dahod District, Gujarat, India. 3642 km². Dahod is situated 214 kilometers (133 mi.) from Ahmedabad and 159 kilometers (99 mi) from Vadodara. The city was previously known as Dohad, which means "two boundaries", as the borders of the states of Rajasthan and Madhya Pradesh are nearby. A total of four large-sized ponds were recorded. The number of ponds is declining due to the development of housing areas (Appendix I).

6. **Narmada**

The district occupies an area of 2,755 km². The Sardar Sarovar Project (SSP) is one of the largest interstate water resource projects, spanning four major states: Maharashtra, Madhya Pradesh, Gujarat, and Rajasthan. The canal network transports water from the river Narmada to various sections of Gujarat via main canals, branch canals, sub-canals, and pipelines. A total of five ponds were selected for sampling (Appendix I).

3.1.1 Frequency of Visit

All study sites were visited to collect data for their geolocation, aquaculture status, and sampling. Additionally, further visits were carried out during ongoing fishing activity to collect fresh fish samples along with parasitic investigation. Other than this the sites were also visited during the Pre-monsoon period for the parasitic data collection, which was considered a favorable time for disease occurring.

3.1.2 Data Analysis

An excel data sheet about the pond was prepared containing the details about its geo-location, aquaculture status, and Fishery. The data was then dispersed according to the different district files for further interpretation and Maps preparation.

3.1.3 Fishing methodology

Casting nets and seine nets are useful tools for fishing and are commonly used by farmers. The most common method used was Cast net fishing for aquaculture purposes.

Plate 3. The image shows Cast netting

A casting net, also known as a throw net, is a circular net with small weights around its perimeter. It is thrown by hand so that it stretches out in the air before sinking into the water as shown in Plate 3. This method is called net casting, and fish are caught as the net is hauled back in (Burnley, E. B., 2005). A seine net is an extremely long net, with or without a bag in the centre, that is set up from the shore or a boat to encircle a specific area. Two long ropes are used to haul and herd fish. These nets are lightweight and highly efficient, and very few fish escape during hauls (Gabriel, et al., 2005). The appropriate size of the net used for catching fish is determined by the size and type of fish being targeted. Different species of fish require different sizes of nets.

3.1.4 On-site observation

The fresh fish samples were collected from the sampling sites during the ongoing fishing activity. Firstly, the species were photographed and examined for the parasites. The samples were then taken to the laboratory by storing it with Ice-packs in Thermocol box to prevent them from degradation, during transportation.

3.1.5 Laboratory work

At the laboratory, firstly the morphology and morphometry of each specimen were noted. The Morphological characters were observed, whereas the Morphometric characters were measured and recorded, generating the fish Formula for species identification, as shown in Plate 4. The identification of species was done using Day's volumes (Day Francis, 1994), other reference literature, and online resources (FishBase, 2023; FishBase 2024).

Plate 4. Shows the standard identification method.

Then further this data was documented in fish Fact format, for each species with their IUCN Red List status. Then the samples were numbered according to the given tag for easy identification called voucher preparation. Then the samples were preserved for further molecular identification in a -20 ℃ fridge (Sarma and Mankodi, 2017).

3.1.6 Specimen Preservation

For scientific studies, it is often necessary to preserve samples as specimens other than tissue which can be further used for different analysis.

Plate 5. This image shows the Preservation process.

A specimen as a voucher is preserved in Formalin, which cannot be used for molecular or phylogenetic studies. In these cases, a different preservation method is required, such as freezing the fresh samples directly under a -20 ℃ fridge or keeping them in 70 % Ethanol (Valdez-Moreno et al., 2009) as shown in Plate 5.

Modern research is incomplete without molecular identification is considered more important.

3.2 DNA Extraction

Later on, the frozen samples were thawed and tissue collection was done for the DNA Extraction process. The extraction was done manually with classic DNA isolation procedure i.e., Phenol-Chloroform described by Barker in 1998, which is as follows and shown in Plate 6.

Reagents

1. **Stock EDTA Solution [250 mM, pH 8.0]**
   Dissolve 4.6 g of EDTA*$2H_2O$ in 25 ml distilled water. Adjust pH 8.0 with 1N NaOH or Conc. NaOH and make final volume to 50 ml distilled water and Autoclave it.
2. **Tris buffer Stock solution [1M, pH 8.0]**
   Dissolve 12.114 g of Tris HCl in 50 ml distilled water. Adjust pH 8.0 with 1N Conc. 1N HCl. Make the volume to 100 ml distilled water and Autoclave it.
3. **Lysis buffer [TEN Buffer; 50 mM Tris HCl (pH 8.0); 10 mM EDTA; 100 mM NaCl]**
   Mix 6.25 ml of 1 M Tris, 5 ml EDTA (250 mM) and 0.73 g NaCl in 100 ml distilled water and make final volume upto 125 ml by adding distilled water. Autoclave it.
4. **TE Buffer [10 mM Trisbase and 1 Mm EDTA, pH 8.0]**
   Add 1.25 ml of 1 M Tris to 0.5 ml EDTA and make the volume upto 125 ml by adding distilled water.
5. **10 % SDS (Sodium Dodecyl Sulphate)**
   Dissolve 10 g of SDS in 100 ml of distilled water.
6. **70 % Ethanol**
   Mix 70 ml Ethanol with 30 ml distilled water.
7. **Chloroform: Isoamyl alcohol (24:1)**
   Mix 96 ml of chloroform with 4 ml of Isoamyl alcohol.
8. **Phenol: Chloroform (25:24:1)**
   Mix 25 ml Phenol, 24 ml Chloroform and 1 ml Isoamyl Alcohol.
9. **Proteinase K**
   20 mg/ml in autoclaved distilled water. Store at – 20 °C.

PROCEDURE

- Samples for DNA extraction were taken from the frozen fish, mainly the muscle tissue is used but here we have used Gill and Fin tissue for precision.
- A tissue sample of 100-200 mg (0.1-0.2 gm) was taken and placed in a 2 ml centrifuge tube. The samples were stored in the dehydrating agent (70 % Ethanol).
- The tissue sample was then placed in 1% Phosphate buffered saline (PBS) solution to remove any excess ethanol.
- After that 200 µl of lysis buffer was added for homogenizing the tissue using liquid nitrogen to break the hard tissue.
- After homogenizing the mixture, add 300 µl of lysis buffer, 50 µl of SDS, and 5 µl of Proteinase K. Gently mix and incubate in a water bath at 56 ℃ overnight.
- The next day, add an equal volume of Tris-Saturated Phenol to the tube and stir gently. The tubes were centrifuged at 10,000 rpm for 10 minutes at 4°C.
- Following centrifugation, the top phase was collected and transferred to a fresh 1.5 ml tube. An equal proportion of phenol, chloroform, and isoamyl alcohol (25:24:1) was added and gently mixed. The tubes were centrifuged again at 10,000 rpm for 10 minutes at 4°C.
- The upper aqueous phase was transferred to a new 1.5 mL tube. An equal volume of chloroform and isoamyl alcohol (24:1) was added and centrifuged at 10,000 rpm for 10 minutes at 4 ℃.
- Collect the upper aqueous phase in a new 1.5 ml tube (if contamination occurs, repeat to the previous step).
- Then, add 600 µl (0.6 ml) of cold absolute ethanol, mix gently, and centrifuge at 12,000 rpm for 10 minutes at 4 ℃. Incubate the mixture at -20 ℃ for 15-20 minutes for optimal results (optional step).
- The supernatant was discarded, while the pellets were collected and dried.
- Add 30-50 µl of 1X Tris buffer (TE buffer) to dissolve the pellets thoroughly.
- Store at -20 degrees Celsius for a long time.

Plate 6. Image depicting the process of DNA extraction

3.2.1 Quantification of genomic DNA:

After extracting genomic DNA, we quantified it using Sambrook *et al.* (1989). 10 µL of extracted DNA was dissolved in 30 µl of Tris buffer (pH 8) and O.D. was taken at 260 and 280 nm (Powerwave HT Microplate Spectrophotometer, BioTek). The quality was assessed by taking the (O.D. at 260nm)/ (O.D. at 280nm). Only samples that demonstrated an O.D. 260nm/O.D. 280nm ratio between 1.6 and 1.8 were selected for further experiments.

3.2.2 DNA Amplification

The amplification of the DNA samples was carried out in a Thermo-cycler. The Polymerase Chain Reaction (PCR) was done using the “Universal" primers LCO 1490 and HCO 2198. Two primers are used: one for each complementary single strand of DNA released after denaturation. Other than Universal primers, standard fish primers Fish BCL-BCH were also tried for some of the species.

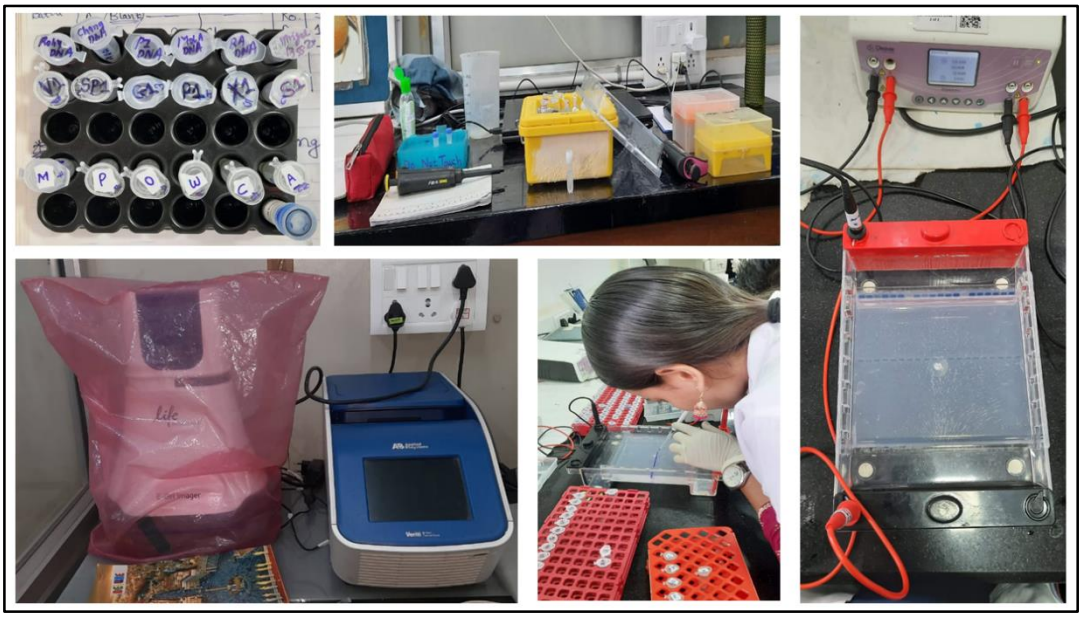

Plate 7. Image depicting the PCR process.

Polymerase Chain Reaction (PCR)

PCR was carried out using DreamTaq Green PCR Master Mix (2X) (Thermofisher) and, template DNA (25 ng/µl). The reaction was carried out in Thermal cycler (Applied Biosystems Veriti®) as shown in Plate 7.

The final concentration of PCR reagents in the reaction mixture (20 µl)

- 2X DreamTaq Green PCR Master Mix (10 µl)
- 10 pmol of each primer (1 µl)
- 50-100 ng template DNA (1 µl)

3.2.3 Cytochrome C Oxidase subunit I gene (COI) amplification

COI gene in Fish was amplified in a volume of 20 µl containing 10 µl Taq PCR reaction mix, 10 pmol forward primer, 10 pmol reverse primer, 50 ng template DNA and sterile ion-free water (to make up the final desired volume). Amplification was carried out in a Thermal cycler (Applied Biosystems Veriti®). Reactions were amplified through 35 cycles with the following conditions (Folmer *et al.*, 1994):

Table 1. PCR Conditions for Fish samples

Stage 1 **1 x cycle**	**Stage 2** **35 x cycle**	**Stage 3** **1 x cycle**
Denaturation	**Annealing**	**Extension**
Initial Denaturation 95 ℃ for 5 min	Denaturation 95 ℃ for 30 sec	Final Extension 72 ℃ for 4 min
	Annealing 52 ℃ for 60 sec	
	Extension 72 ℃ for 45 sec	

Table. 2 The list of primers with sequence (For Fish samples)

Sr. No.	**Primers used**	**Sequence (5'- 3')**	**Reference**
1.	LCO1490 (Forward) HCO2198 (Reverse)	FP- GGTCAACAAATCATAAAGATATTGG RP-TAAACTTCAGGGTGACCAAAAAATCA	**(Folmer *et al.*,1994)**
2.	FISH-BCL (Forward) FISH-BCH (Reverse)	FP-TCAACYAATCAYAAAGATATYGGCAC RP- ACTTCYGGGTGRCCRAARAATCA	**(Baldwin *et al.*, 2009)**

3.2.4 Agarose Gel Electrophoresis

- **Tris HCL EDTA Buffer or Running buffer** (50X Tris Acetate EDTA buffer also known as TAE buffer)

  Preparation: 242 gm Tris base, 57.1 ml Glacial Acetic acid, 100 ml of 500 mM EDTA dissolve in a final volume of 1 Litre

- **Ethidium bromide** (10 mg/ ml)

  Preparation: A 10 mg/ ml stock solution was prepared using distilled water and stored in a dark (Brown) colored vial.

- **DNA loading Dye**

  Preparation: Mix 3 ml glycerol (30%), 25 mg Bromophenol blue (0.25%), and distilled water and make a volume up to 10 ml.

Plate preparation and casting the gels:

To avoid contamination, a clean agarose gel casting cassette and comb were cleansed with ethanol or spirit, and the tray's open sides were taped shut. The comb was inserted into the designated slits on the plate. A calculated amount of agarose in TAE buffer was mixed to prepare a 0.8% (for DNA) or 1.8-2% (for PCR) solution. Then dissolve the agarose completely in the buffer until the solution becomes transparent by heating it in microwave at 70- 80 ℃ in oven and cooled to 50 ℃. Ethidium bromide was added in a final concentration of 0.2 mg/ml and thoroughly mixed. The liquid was then put into the casting tray and left to solidify. The comb and tapes were removed after complete solidification of agarose gel.

Plate 8. Image depicting the Gel Electrophoresis process.

3.2.5 Preparation of samples and scanning of gels:

Amplified DNA samples (5 µl) were combined with 2 µl gel loading dye and carefully put into wells using gel-loading tips. 100 bp marker electrophoresis was performed at 180V, as indicated in Plate 8. The gel photos were captured in JPEG or TIF format utilizing the gel documentation system (Biorad, USA). The gels were examined using Image Lab 3.0 software (Biorad, USA).

3.2.6 Purification of amplified PCR products:

Purification of COI gene amplified products were done using GenElute™ PCR Clean-up kit using following protocol.

- GenElute plasmid micro spin column was built in a 2.0 ml collecting tube included with the package. The columns received 0.5 ml of column preparation solution and were centrifuged at 12,000 g for 30 seconds.

- Combine 20 µl of PCR product with 100 µl binding solution. Following proper mixing, the solution was delivered to the binding column. Columns were centrifuged at 16000 g for one minute. Flow-through was discarded.
- The binding column was placed in a collecting tube, then 0.5 ml of wash solution was added and centrifuged at maximum speed for 1 minute. This step was repeated to eliminate the contaminants. To dry the binding membrane completely and to prevent the alcohol contamination in preceding reactions columns were centrifuged at maximum speed for 3 minutes.
- Columns were moved to a new 2 ml collection tube and 50 µl of elution solution was added to the centre of each.
- DNA was eluted at full speed for one minute.
- Eluted DNA (PCR product) was kept at -20ºC.

3.2.7 Cycle Sequencing

Sequencing was performed with the BigDye® Terminator v 3.1 Cycle sequencing kit. The BigDye Terminator v3.1 Cycle Sequencing Kit includes all of the necessary reagents for the sequencing reaction in a ready-to-use, pre-mixed package. Cycle sequencing was done in 10 µl volume. The reaction mixture was prepared as follows. Two reaction tubes were prepared for forward sequencing primers and reverse sequencing primers. In this experiment, COI amplification primers (LCO1490 and HCO2198) served as sequencing primers.

Table 3: Quantity of reagents used for Cycle Sequencing.

Reagent Quantity
Terminator Ready Reaction mix v 3.1 4 µl
Big dye Sequencing buffer 1 µl
Template 150 - 300 ng
Primer 10 pmole
Deionized water To make the volume up to 10 µl

Before going to the amplification reaction mixture was mixed well in an individual tube and spun down briefly. Amplification was carried out in a Thermal cycler (Applied Biosystems Veriti®). Reactions were amplified through 35 cycles with the following conditions: Initial denaturation at 96°C for 2 min, followed by 30 cycles of 96°C for 30 sec, annealing at 50°C

for 15 sec, and extension at 60°C for 4 min, followed by indefinite hold at 4°C (Ivanova et al., 2007).

3.2.8 Purification of Cycle Sequencing Products

To achieve optimal results, complete the removal of unincorporated dye terminators before performing capillary electrophoresis. Excess dye terminators in sequencing products can obscure data in the early part of the sequence and can interfere with base calling. Purification was done using the BigDyeXTerminator® Purification Kit. Kit contains SAM™ Solution and BigDye® XTerminator™ Solution.

Following purification protocol was followed:

- Cycle sequencing reaction plate was spun at 100 g for 1 minute.
- Purification reaction premix was prepared by adding 10 µl of BigDyeXTerminator® to 45 µl of SAM™ solution. Total reaction had 55 µl for each well.
- After removing seal of the 96-well plate 55 µL of SAM™ Solution/XTerminator® Solution premix was added to each well.
- Plate was sealed using MicroAmp® Clear Adhesive Films and subjected to vortex for 30 minutes at 2000 rpm on IKA® Vortex 4 digital.
- After vortexing plate was centrifuged at 1000 rpm for 2 minutes.
- Plate kept at room temperature (25ºC) before going to capillary electrophoresis.

3.2.9 Capillary electrophoresis

- Capillary electrophoresis of cycle-sequenced products was performed on the 3500 XL platform (Applied Biosystems).
- The instrument was controlled using Instrument Software 3500. Dye Set Z and the Sequencing Install Standard, BigDye® Terminator v3.1 Kit were used to generate BigDye® Direct spectral calibration information for use with the data.
- The BigDye® Cycle Sequencing Kit v3.1 utilized mobility and calibration files to optimize base calling. Plate containing cycle sequenced products was loaded on Position A/ Position B.
- Sample information was loaded on plate preparation mode.
- Capillaries were filled with POP-7™ polymer. The approximate run time for 24 samples was 2 hours and 30 minutes.

3.2.10 Analysis of sequence and submission of Barcodes to the National Centre for Biotechnology Information (NCBI)

The raw sequences were analyzed by Chromas and BioEdit. Gene sequences were aligned with forward and reverse primers to obtain consensus sequences. These sequences were compared using the Basic Local Alignment Search Tool (BLAST) or the Barcode of Life Data System (BOLD) for analytical purposes. Sequences were then uploaded to NCBI to get an accession number. Following that, further phylogenetic analysis was performed to determine species relatedness.

3.2.11 Phylogeny analysis

The phylogenetic tree provides insights into these fish species' genetic relationships and evolutionary history. It helps to understand how closely or distantly related these species are, which can be useful for various biological and ecological studies, including those related to fish parasitology and management.

3.3 Analysis for Parasitic data

Fish samples were carefully collected and inspected for external parasites (Ectoparasites). The samples were then taken to a laboratory for further examination.

Plate 9. This image depicts the collection of Ectoparasites

The external surfaces of the fish, including scales, skin, fins, gills, buccal cavity, opercula, and eyes, were thoroughly examined for parasites using a stereomicroscope as shown in Plate 9.

Plate 10. Image depicting the Endoparasites examination

The internal organs' external surfaces were tested for parasites, which were then examined separately. The digestive tract was dissected and examined longitudinally to examine for endoparasites, as shown in Plate 9. The collected Ectoparasites were preserved for further species identification by placing some in 70% alcohol and a few in 10% formalin. For small parasitic species, the whole organism was used as tissue for molecular identification.

3.3.1 Parasitic Species Identification

The DNA extraction was carried out using the standard Phenol: Chloroform method with minor changes as suggested by Warda *et al.,* 2020.

The conditions for amplification were

- Denaturation at 94 ℃ for 1 min,
- Annealing: Denaturation at 94 ℃ allows 5 cycles for 30 sec,
  Annealing at 49 ℃ for 40 sec,
  Extension at 72 ℃ for 1 min,
- Final extension at 72 ℃ for 10 min.

PCR amplification was done using Universal primers for the *Argulus* and Isopod species, whereas 28S rDNA was used for the *Lernaea* species respectively.

Table 4. The list of primers with sequence (For parasitic samples)

Sr. No.	Primers	Primer sequence (5'- 3')	Reference
1.	LCO 1490 (Forward primer) HCO 2198 (Reverse primer)	FP- GGTCAACAAATCATAAAGATATTGG RP- TAAACTTCAGGGTGACCAAAAAATCA	**Folmer *et al.*, 1994**
2.	28S rDNA (28 SF) 28S rDNA (28 SR)	FP- ACAACTGTGATGCCCTTAG RP- TGGTCCGTGTTTCAAGACG	**Song *et al.*, 2008**

Infections parameters that have been utilized are those proposed by Bush et al., 1997, that is, prevalence (% infected) and mean intensity of infection (number of parasites per infected fish).

3.3.2 Pathogenic Analysis

Events of fish diseases or mortality were documented, and samples were collected for pathogen assessment. Various methods were used for pathogen collection. For diseases like Branchiomycosis, fungal samples of *S. demigrams* were taken from the fish gill tissue, while for SVC and White Spot diseases, pathogen samples were swabbed from the fish body surface. Prevention and treatment procedures were studied, and treatments were administered wherever possible. The Ectoparasites were identified using specific identification keys and literature reviews.