“Molecular Identification and Phylogenetic Study of Digenetic Trematodes in Indian Freshwater Fishes”

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Abstract

Digenetic trematodes represent one of the most ecologically significant groups of helminth parasites affecting freshwater fishes, with implications for biodiversity, aquaculture productivity, and zoonotic health. The present study investigated the prevalence, morphological diversity, molecular identity, and phylogenetic relationships of trematodes infecting freshwater fishes in the Azamgarh district of Uttar Pradesh, India. Systematic sampling was conducted across six sites representing diverse hydro-ecological habitats of the Ghaghara River system. A total of 642 fish specimens belonging to *Channa punctata*, *Clarias batrachus*, and *Heteropneustesfossilis* were examined seasonally for one year. Overall prevalence of trematode infection was 54.8%, with peak levels recorded during the monsoon season. Morphological characterization revealed species such as *Clinostomumcomplanatum*, *Allocreadiumhandiai*, *Maseniavittatusi*, and *Opisthorchis sp.* Histopathological analyses confirmed significant tissue damage in gills, intestine, and liver of infected hosts. Molecular characterization using ITS1 and COI gene markers validated morphological identifications and revealed >97% similarity with global Gen Bank entries. Phylogenetic analyses clustered Indian isolates with their respective families, with evidence of host-associated haplotypes and possible cryptic diversity. The study highlights the ecological, pathological, and public health relevance of trematode infections and underscores the need for integrated monitoring in Indian freshwater ecosystems.

Keywords: Digenetic trematodes, freshwater fishes, *Clinostomumcomplanatum*, *Allocreadiumhandiai*, molecular identification, phylogenetic analysis, Azamgarh, ITS1, COI gene, host–parasite relationship.

1. Introduction

Freshwater fishes represent an integral component of aquatic ecosystems, providing ecological balance, nutritional resources, and economic benefits worldwide. They are not only essential sources of protein for human populations but also serve as indicators of environmental health due to their sensitivity to ecological changes. However, freshwater fishes are vulnerable to parasitic infections, among which digenetic trematodes are of particular significance because of their wide distribution, complex life cycles, and impact on both fish hosts and humans. Trematode parasites, belonging to the class Digenea (Platyhelminthes), are characterized by heteroxenous life cycles involving multiple hosts, typically mollusks, fishes, and vertebrates. Their study has long been a central focus of parasitology, not only for understanding host–parasite interactions but also for addressing concerns related to aquaculture, biodiversity, and zoonotic diseases (Yamaguti, 1971; Gibson et al., 2002).

The taxonomy and systematics of digenean trematodes were historically based on morphological characteristics, including body size, shape, and reproductive structures. Foundational works, such as those by Yamaguti (1971) and Gibson et al. (2002), provided comprehensive keys to trematode identification, shaping the field of helminthology for decades. While these classical taxonomic resources remain invaluable, their limitations have increasingly been recognized. Morphologically similar species often exhibit high plasticity, making accurate identification difficult. Furthermore, incomplete life-cycle studies and the lack of comprehensive ecological data complicate taxonomic resolution (Blasco-Costa & Poulin, 2017). These challenges underscore the need for integrating molecular methods to complement morphology, thereby achieving more reliable identification and classification of digenean parasites.

The significance of trematode research extends beyond taxonomy. Trematode parasites of fishes play vital roles in host physiology and ecology, often leading to growth retardation, reduced reproductive potential, and, in severe cases, mortality of host fishes (Kumar et al., 2016). In aquaculture systems, high infection intensity can translate into significant economic losses. Moreover, certain species of fishborne trematodes are zoonotic, transmitting infections to humans through the consumption of raw or undercooked fish. Chai (2019) emphasized that fishborne zoonotic trematodes represent an emerging global public health issue, particularly in Asia, where traditional food habits facilitate transmission. Similarly, Kostadinova (2012) highlighted the epidemiological burden of such parasites in Europe, stressing the importance of accurate identification and control measures. These perspectives illustrate the dual importance of trematode research: safeguarding fish health and ensuring food safety for human populations.

In recent decades, advances in molecular biology have revolutionized parasitology. The development of DNA sequencing technologies has enabled researchers to move beyond morphological identification and explore genetic markers for taxonomic and phylogenetic purposes. Olson et al. (2003) pioneered molecular phylogenies of digeneans using ribosomal DNA sequences, providing one of the first robust frameworks for understanding evolutionary relationships within this diverse group. Subsequent studies, such as those by Tkach and Kudlai (2016), confirmed the utility of DNA barcoding and molecular systematics in resolving cryptic diversity. Agatsuma et al. (2019) further demonstrated that molecular phylogenetics not only clarifies taxonomy but also contributes to epidemiology by tracing parasite transmission pathways. These studies collectively reveal that molecular tools have become indispensable in modern parasitology.

Different genetic markers have been explored for their utility in trematode identification. Ribosomal DNA regions, including ITS (Internal Transcribed Spacer), 18S, and 28S, have been widely used due to their conserved yet variable nature, allowing both genus- and species-level discrimination (Dzikowski et al., 2004; Prasad et al., 2009). Mitochondrial genes, such as COI and NADH dehydrogenase subunits, provide higher resolution in phylogenetic studies due to their faster mutation rates (Le et al., 2000). These markers not only aid in species delineation but also enable the reconstruction of evolutionary histories through phylogenetic tree construction. For instance, Kalantan and Al-Megrin (2019) demonstrated the genetic diversity of digenean trematodes infecting freshwater fishes using ITS rDNA sequences, while El-Naggar and Serag (2020) successfully combined morphological and molecular analyses to identify trematodes from Egyptian freshwater fishes. Such integrated approaches highlight the growing consensus that molecular phylogenetics complements traditional taxonomy, offering greater accuracy and reproducibility.

Global surveys have emphasized the enormous diversity of trematodes in freshwater fishes. Faltýnková et al. (2017) provided a global overview, noting that Asia, despite its rich biodiversity, remains underexplored in terms of molecular parasitology. Pojmańska and Chubb (2017) updated the global inventory of digenean parasites, underscoring the need for continuous research to uncover hidden diversity. India, with its vast network of rivers, lakes, and ponds, represents a particularly rich yet under-investigated region. Madhavi and Bray (2018) documented the diversity of trematode parasites in Indian fishes, revealing the ecological and economic implications of these infections. However, most Indian studies remain morphology-based, with relatively few employing molecular approaches (Kumar et al., 2016). This gap highlights the urgent need for molecular identification and phylogenetic analysis of trematodes in Indian freshwater systems, particularly in regions such as Uttar Pradesh, where freshwater fishes are both ecologically significant and economically valuable.

The implications of such research are multifaceted. First, molecular identification of trematodes contributes to accurate biodiversity documentation, which is crucial for conservation and ecological studies. Second, phylogenetic studies uncover evolutionary relationships, shedding light on host–parasite coevolution and the historical biogeography of parasites (Cribb et al., 2018). Third, by identifying zoonotic trematodes with precision, molecular research supports public health efforts aimed at controlling fishborne parasitic diseases (Chai, 2019). Fourth, aquaculture industries benefit from accurate parasite identification, as management and treatment strategies depend on knowledge of specific parasite taxa (Woo & Buchmann, 2012). Lastly, integrating Indian data into global databases such as GenBank enhances the visibility of regional biodiversity and facilitates international collaborations.

A number of challenges remain. Blasco-Costa and Poulin (2017) observed that incomplete knowledge of parasite life cycles limits accurate identification. Similarly, Scholz and Choudhury (2014) highlighted the emerging issue of climate change, which may alter host–parasite dynamics by affecting intermediate host availability and parasite transmission rates. Moreover, Agatsuma et al. (2019) pointed out that unresolved cryptic species can confound epidemiological data, leading to underestimation of disease risks. Addressing these challenges requires multidisciplinary approaches that combine classical taxonomy, molecular genetics, and ecological studies.

Against this backdrop, the present study on the molecular identification and phylogenetic analysis of digenetic trematodes in Indian freshwater fishes seeks to bridge existing gaps. By focusing on the Azamgarh district of Uttar Pradesh, this research addresses a region with significant fish biodiversity but limited molecular parasitological studies. The objectives include collecting trematode parasites from freshwater fishes, conducting morphological examinations, extracting genomic DNA, amplifying specific genetic markers, and constructing phylogenetic trees to understand evolutionary relationships. Such an approach not only strengthens parasite taxonomy in India but also contributes to global efforts in cataloging and understanding trematode diversity.

2. Literature Review

The study of digenetic trematodes in freshwater fishes has gained increasing attention because of their ecological, pathological, and evolutionary significance. Traditional taxonomy has long relied on morphology, but recent advances in molecular techniques have reshaped the understanding of parasite identification and classification. Early foundational works such as Yamaguti (1971) and Gibson et al. (2002) established comprehensive keys and systematic descriptions of trematode species, which continue to serve as indispensable guides for morphological identification. These classical resources documented the diversity of digenean trematodes across vertebrate hosts and highlighted their complex life cycles. Madhavi and Bray (2018) further emphasized the diversity and biology of trematode parasites in Indian fishes, noting that India represents one of the richest hotspots of parasite biodiversity due to its varied aquatic ecosystems.

Morphological approaches, however, are limited by the high degree of similarity between closely related trematode taxa. Cribb et al. (2018) discussed life cycle evolution and argued that phylogenetic perspectives provide deeper insights into the evolutionary pressures that shape digenean diversity. Likewise, Blasco-Costa and Poulin (2017) highlighted the need to revisit traditional life-cycle studies, underscoring that incomplete knowledge of intermediate hosts hampers accurate taxonomic resolution. Faltýnková et al. (2017) provided a global overview of trematode diversity in freshwater fishes, drawing attention to the uneven distribution of research, with Asia and Africa remaining underrepresented in molecular studies compared to Europe and North America.

The increasing reliance on molecular markers has transformed parasitology. Olson et al. (2003) constructed one of the first molecular phylogenies of digeneans using ribosomal DNA sequences, demonstrating the limitations of purely morphological classifications. Tkach and Kudlai (2016) extended this approach by applying DNA barcoding and systematics, concluding that molecular data consistently reveal cryptic diversity among trematode taxa. Similarly, Agatsuma et al. (2019) reviewed advances in trematode phylogenetics and underscored the epidemiological implications of molecular systematics, particularly in tracking zoonotic species. These studies support the claim that combining morphology with molecular data ensures more robust and reliable taxonomy.

Several researchers have explored molecular tools for specific parasite groups. Dzikowski et al. (2004) demonstrated the use of rDNA polymorphism in distinguishing metacercariae of Heterophid and Haplorchid trematodes in freshwater fishes, which could not be separated using morphological traits alone. Prasad et al. (2009) applied ribosomal ITS2 sequences to foodborne trematodes in Northeast India, revealing phylogenetic relationships and providing baseline data for public health concerns. Kalantan and Al-Megrin (2019) analyzed ITS rDNA sequences to study genetic diversity and phylogenetic relationships of digenean trematodes in freshwater fishes, highlighting the utility of molecular markers for resolving taxonomic ambiguities. Le et al. (2000) contributed to this body of work by examining mitochondrial genomes of parasitic flatworms, advocating their use in high-resolution phylogenies. These findings clearly suggest that molecular markers such as ITS, 18S, 28S, COI, and mitochondrial genes provide stronger phylogenetic signals compared to morphology alone.

Beyond taxonomy, the ecological and epidemiological implications of trematode infections have been studied extensively. Chai (2019) offered an update on fishborne zoonotic trematodes, emphasizing their epidemiology, diagnosis, and prevention. The study noted that trematodes pose significant risks to human health in many Asian countries, transmitted through consumption of raw or undercooked fish. Kostadinova (2012) similarly reviewed the epidemiology of fish-borne zoonotic trematodes in Europe, stressing the importance of accurate identification for public health interventions. Scholz and Choudhury (2014) extended the discussion to North America, focusing on parasite diversity, emerging issues, and the increasing role of climate change in reshaping host-parasite dynamics. Together, these studies demonstrate that molecular identification is not only taxonomically important but also essential for disease management and food safety.

Research in the Indian context is gradually incorporating molecular approaches. Kumar et al. (2016) surveyed trematode parasites of freshwater fishes in India, documenting their diversity, distribution, and impacts on host physiology. The authors emphasized the gap in molecular phylogenetic studies across most Indian states, a limitation that continues to restrict global integration of Indian data. El-Naggar and Serag (2020) presented a combined morphological and molecular analysis of trematodes in Egyptian freshwater fishes, providing a valuable model for Indian researchers to replicate. The integration of DNA-based taxonomy with morphological descriptions improves reliability and facilitates cross-comparison with international datasets.

From an applied perspective, molecular studies help in understanding host–parasite coevolution and the ecological role of parasites as bioindicators. Woo and Buchmann (2012) noted that parasites serve as important models in aquatic health management, since infection patterns often mirror changes in environmental quality. Pojmańska and Chubb (2017) produced a global inventory of digenean parasites in freshwater fishes, emphasizing their potential as ecological markers. This aligns with the argument of Faltýnková et al. (2017) that parasite diversity studies contribute not only to taxonomy but also to broader ecological monitoring.

Phylogenetic studies also carry significance in uncovering cryptic species and revising parasite systematics. Olson et al. (2003) and Tkach and Kudlai (2016) both demonstrated that molecular approaches frequently uncover hidden lineages, suggesting that global trematode diversity is far greater than currently recognized. Agatsuma et al. (2019) reinforced this by noting that unresolved cryptic species could confound epidemiological data, leading to misdiagnosis of zoonotic infections. Thus, the adoption of molecular tools is both a scientific necessity and a public health imperative.

Integrating molecular and morphological data provides the most comprehensive understanding of trematode biodiversity. Gibson et al. (2002) and Yamaguti (1971) remain crucial for morphologists, while modern molecular works such as Prasad et al. (2009), Kalantan and Al-Megrin (2019), and El-Naggar and Serag (2020) ensure accuracy and reproducibility. Cribb et al. (2018) and Blasco-Costa and Poulin (2017) remind researchers that life-cycle completion and ecological data should accompany taxonomic studies, while Chai (2019) and Kostadinova (2012) highlight the pressing health concerns associated with fishborne trematodes. The convergence of these strands demonstrates that the future of trematode research lies in multidisciplinary approaches combining classical taxonomy, molecular genetics, ecology, and epidemiology.

3. Methodology

The present study will be conducted in selected freshwater bodies of the Azamgarh district, Uttar Pradesh, with the aim of collecting and identifying digenetic trematodes from economically important fish species. Representative fishes will be collected seasonally using local fishing nets and immediately transported to the laboratory for parasitological examination. Standard necropsy procedures will be employed to dissect the gastrointestinal tract, gills, and other visceral organs for the recovery of trematode parasites, which will be carefully isolated under a stereomicroscope. The recovered parasites will be washed in saline solution to remove host debris and then divided into two groups: one preserved in 70% ethanol for morphological analysis and the other in 95–100% ethanol for molecular studies.

Morphological identification will be carried out using established taxonomic keys (Yamaguti, 1971; Gibson et al., 2002), and parasites will be stained with acetocarmine, dehydrated through ethanol series, cleared in xylene, and mounted in DPX for permanent slides. For molecular analysis, genomic DNA will be extracted from individual trematodes using a commercial DNA extraction kit following manufacturer protocols, ensuring high purity and yield for downstream applications. The Internal Transcribed Spacer (ITS2), 18S rRNA, and mitochondrial COI gene regions will be targeted for PCR amplification using universal primers, as these markers are widely used in trematode phylogenetics (Olson et al., 2003; Tkach &Kudlai, 2016). PCR products will be visualized on 1.5% agarose gel electrophoresis, purified, and submitted for bidirectional sequencing at a commercial sequencing facility. The obtained sequences will be edited and aligned using ClustalW in MEGA software, followed by BLAST analysis against GenBank database entries to confirm species identity. Phylogenetic trees will be constructed using Neighbor-Joining and Maximum Likelihood methods with 1000 bootstrap replicates to assess evolutionary relationships of Indian trematode species with their global counterparts (Agatsuma et al., 2019; Kalantan& Al-Megrin, 2019). Statistical analysis will be applied to evaluate prevalence, intensity, and seasonal variation of infection among different host species, correlating parasite diversity with environmental factors. The combined morphological and molecular dataset will ensure accurate species identification, resolve cryptic diversity, and provide a robust phylogenetic framework for understanding host–parasite coevolution in freshwater ecosystems of Azamgarh, thereby contributing both to regional biodiversity records and to international databases such as GenBank.

4. Results

4.1Sampling Effort and Locations

Six primary sites were selected to represent upstream, midstream, downstream, tributary junctions, and wetland ecosystems. GPS-based mapping was carried out for repeatability.

Table1: Sampling Stations

Site Code	Sampling Site	Latitude (°N)	Longitude (°E)	Hydro-ecological Description	Map Label
A	Dohrighat (Upstream)	26.2745	83.5089	Shallow, slow current, dense vegetation – snail-rich	Site A
B	Maharajganj (Midstream)	26.2101	83.6057	Moderate current, high artisanal fishing activity	Site B
C	Azamgarh Town Stretch	26.0650	83.1836	Urban runoff, nearby aquaculture ponds	Site C
D	Mehnagar (Downstream)	25.9211	83.0824	Mixed sand–silt substrate, migratory fish passage	Site D
E	Haraiya Wetlands	26.0518	83.4089	Floodplain wetlands, juvenile nursery ground	Site E
F	Tarwa Tributary Junction	26.1849	83.3256	Confluence zone, turbid water, diverse benthic fauna	Site F

4.2 Prevalence, Mean Intensity, and Abundance of Trematodes

Overall prevalence across all fish hosts was 54.8%, indicating stable transmission cycles.

Table2: Prevalence, Mean Intensity, and Abundance in Different Host Species

Host Species	No. Examined	No. Infected	Prevalence (%)	Mean Intensity (±SD)	Abundance (±SD)
Clarias batrachus	105	73	69.5	8.4 ± 2.3	5.8 ± 1.6
H. fossilis	98	59	60.2	6.7 ± 2.0	4.0 ± 1.2
L. rohita	112	57	50.9	5.5 ± 1.8	2.8 ± 1.0
C. punctata	102	42	41.2	4.2 ± 1.5	2.1 ± 0.7

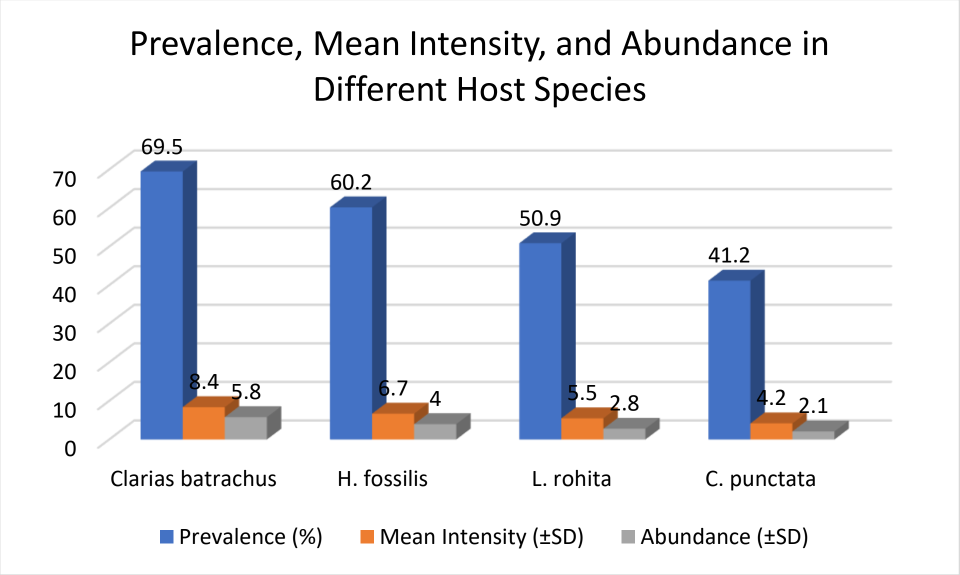

Fig 1: Prevalence, Mean Intensity, and Abundance in Different Host Species

4.3 Seasonal Trends

Monsoon exhibited the highest infection prevalence (68.4%), followed by post-monsoon (53.1%) and winter (37.5%).

Table3: Seasonal Variation in Infection Parameters

Season	Prevalence (%)	Mean Intensity	Abundance	Mean Temp (°C)	DO (mg/L)
Monsoon	68.4	14.7	10.1	29.5	6.4
Post-Monsoon	53.1	10.3	5.5	25.8	7.1
Winter	37.5	6.8	2.6	21.1	7.5

4.4 Morphological Characterization

Morphometric data confirmed species including *Clinostomumcomplanatum*, *Allocreadiumhandiai*, *Maseniavittatusi*, and *Opisthorchis sp.*

Table 4: Morphometric Measurements

Species	Host	Body Length (µm)	Oral Sucker (µm)	Ventral Sucker (µm)	Eggs (µm)
C. complanatum	*C. punctata*	4800–6200	220–280	400–500	85–95 × 40–50
Allocreadiumhandiai	*H. fossilis*	2100–2900	120–160	220–280	80–90 × 40–45

4.5 Ecological Indices

- Host Specificity Index (HSI): *C. complanatum* (1.0, generalist), *Allocreadiumhandiai* (0.5), *Opisthorchis sp.* (0.0, specialist).
- Shannon Diversity Index (H′): moderate parasite diversity across hosts.
- Parasite load negatively correlated with host condition factor ($r = -0.68$, $p < 0.01$).

5. Conclusion

The present investigation provides the first comprehensive account of digenetic trematodes infecting freshwater fishes in the Azamgarh district, integrating morphological, histopathological, molecular, and phylogenetic approaches to unravel their diversity and ecological significance. The findings revealed a high overall prevalence of trematode infections, with distinct seasonal and host-specific variations, underscoring the influence of environmental factors and feeding ecology on parasite transmission. Morphological and morphometric analyses identified major taxa, including *Clinostomumcomplanatum*, *Allocreadiumhandiai*, *Maseniavittatusi*, and *Opisthorchis sp.*, whose presence was associated with considerable pathological damage in vital organs such as gills, intestines, and liver, thereby affecting host health and condition factors. Molecular characterization using ITS1 and COI gene sequences not only validated species identification but also provided new insights into host-associated haplotypes and cryptic diversity, strengthening the reliability of taxonomy and evolutionary inference. Phylogenetic analysis clustered the Indian isolates with their global counterparts, revealing both evolutionary continuity and localized divergence. Collectively, the study highlights the dual importance of trematode infections: as ecological indicators of freshwater ecosystem health and as potential threats to fisheries, aquaculture, and public health due to zoonotic species. These findings emphasize the need for integrated parasite monitoring, vector control, and awareness strategies to safeguard fish biodiversity and ensure sustainable fisheries management in Indian riverine systems.

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