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Molecular Profiling of *Lytocestus heteropneustii* (Cestoda: Caryophyllidea) Parasitizing *Heteropneustes fossilis* (Bloch, 1794) from India

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Abstract: The majority of people worldwide are still at risk of developing chronic illnesses, deformities or dying as a result of parasite infections, which restrict economic progress. The current work was designed with the large subunit ribosomal gene of *Lytocestus heteropneustii* (Bloch, 1794) with primer design, phylogenetic analysis and motif analysis used to evaluate the molecular taxonomic status of *Lytocestus heteropneustii* and active sites to identify gene function and cell signaling. Phylogenetic studies have validated the cestode taxonomy and the designed primer set can be used in the PCR amplification process. Furthermore, identified motifs will be helpful in future medication design research, which could be crucial for improving animal health. Genomic analysis has the greatest potential for identifying new therapeutic targets, creating vaccine diagnostics, and investigating the biological mechanisms of drug resistance, antigenic diversity, infectivity and disease.

Keywords: *Lytocestus heteropneustii*, Primer design, Phylogeny, Motifs, Genomic analysis, *Heteropneustes fossilis*

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Introduction

Parasites show their presence in all animal taxa in varying degrees. Fish is one of the most favorable hosts and can be parasitized by various parasitic species. The majority of fish, both cultivated and wild, have shown parasitic infections. In addition to acting as hosts, for many parasites, they also act as carriers of numerous parasite larvae where they mature and can infect many other vertebrates, including humans with life-threatening illnesses. Parasites have amazing ways of adjusting within hosts to complete their life cycle. Numerous parasite species show varying degree of host specificity to infect their host through various biological mechanisms (Deepika *et al*., 2023). Additionally, the effects of a single parasite species on various host species may vary

greatly. The world's economy is severely handicapped by billions of people suffering from tropical parasitic diseases, especially in the 20th century when a rapid breakthrough happened in medical science (Dias, 1992). Cestode is one of the most threatened parasites among other helminth parasites (Deepika *et al*., 2024). Cohn (1908) reported *Lytocestus* from *Clarias fuscus* with its type species *L. adhaerens.* Woodland (1926) confirmed this genus, which contained the type species with four more *Lytocestus* species. The diagnosis of genus *Lytocestus* included an undifferentiated holdfast, parenchyma muscles in a ring around the testes, and a lack of post-ovarian yolk glands.

The molecular study examines parasite genomes using computational, bioinformatics and sequen-cing approaches (Srivastava *et al*., 2024). The ultimate target of this work is to understand the evolutionary link and biological signaling pathways to determine the potential sites of each parasite-encoded gene. Bioinformatics provides new insights into parasite management, allowing for better control via monitoring and drug design.

Docking, genomic analysis, transcriptome analysis, and metagenomics help to find effective target therapeutic compounds and evaluate the host-parasite association. The current work focuses on the molecular analysis of *Lytocestus heteropneustii* using 28S rDNA through an *in silico* approach that involves primer design, phylogenetic analysis and motif prediction. The designed PCR primer pairs will assist in identifying and selecting accurate primers in their molecular study of *Lytocestus heteropneustii*. The taxonomic validity of the cestode was supported by phylogenetic analysis, which revealed strong similarities with other *Lytocestus heteropneustii* rDNA sequences submitted to the NCBI under accession number PP377810. The researchers also identified distinct positions in the 28S rDNA gene of *Lytocestus heteropneustii* where various motifs were found. DNA motif analysis is fundamental for investigating any biological functioning. Fatma *et al.* (2019) identified transcription factor-binding sites to better understand gene expression regulatory mechanisms. Targeting this motif can effectively control the illness produced by *Lytocestus heteropneustii* from freshwater fish.

Materials and Methods

In this present work, the 28S rDNA gene of the *Lytocestus heteropneustii* was used.

Retrieval of the sequence: The sequence was retrieved from the NCBI database using accession number PP377810. The following studies were performed as follow:

Primer designing: Primer designing was performed using a computational tool of NCBI (https://www.ncbi.nlm.nih.gov).

Phylogenetic analysis: For the taxonomic validation of the worm, a phylogenetic tree was constructed using the Maximum likelihood and Bayesian inference method MEGA v7 (Kumar *et al.*, 2016) and the Bayesian inference (BI) probabilities method TOPAL 2.0 (McGuire *et al.*, 2000).

Motif's study: The motifs were studied using computational tools *i.e.,* Transfac.

Results

Primer designing: For analysis of primers of 28S rDNA for *Lytocestus heteropneustii*, ten pairs of primers were designed using *in silico* computational tools (Figs. 1a, b).

Phylogenetic analysis: Phylogenetic analysis inferred from 28S rDNA has shown close relationships with all the sequences available belonging to the same genus in the gene bank. (Fig. 2). The 28S gene showed a more conserved region in *Lytocestus heteropneustii* sequences. All the *Lytocestus heteropneustii* isolates formed monophyletic clades submitted from India (KC332251, KC332252 & PP377810).

Motif study: Four different motifs and their positions namely AMIDATION (70-73 and 210-213), CK2_PHOSPHO_SITE Casein kinase II (137-140, 172-175 and 216-219), MYRISTYL (104 -109) and PKC_PHOSPHO_SITE Protein kinase C (76-78, 95-97, 124-126, 164-166 and 206-208)

Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TTCAGTCGGTCAGGGTGTTG	Plus	20	56	75	59.89	55.00	2.00	0.00
Reverse primer	CACAGGCACCATCCAACTCT	Minus	20	645	626	59.96	55.00	2.00	0.00
Product length	590								

Primer pair 2

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GCCAGTCTACCTTAGCCAGC	Plus	20	253	272	60.18	60.00	3.00	2.00
Reverse primer	AGCGACACGATGATCAGACC	Minus	20	596	577	59.90	55.00	6.00	3.00
Product length	344								

Primer pair 3

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TGACTAGTCCGCCGGTAGAT	Plus	20	110	129	59.82	55.00	8.00	2.00
Reverse primer	GCTGGCTAAGGTAGACTGGC	Minus	20	272	253	60.18	60.00	3.00	2.00
Product length	163								

Primer pair 4

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CCGAGTGTTAGTCGGAGCAA	Plus	20	165	184	59.76	55.00	5.00	3.00
Reverse primer	CCATCCAACTCTAGGCCACC	Minus	20	637	618	59.82	60.00	4.00	1.00
Product length	473								

Primer pair 5

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CAAGCTGCAAGCCCAGAGTA	Plus	20	36	55	60.32	55.00	4.00	2.00
Reverse primer	CGTGCACTGAAATTGCCTGT	Minus	20	335	316	59.69	50.00	6.00	2.00
Product length	300								

Fig. 1(a): Designed primers for *Lytocestus heteropneustii.*

Primer pair 6

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TGTCCTGTAGTCGCAGTGTG	Plus	20	403	422	59.69	55.00	3.00	3.00
Reverse primer	CACCACCGGACTCCTCATTT	Minus	20	621	602	59.68	55.00	5.00	0.00
Product length	219								

Primer pair 7

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TGTGCATTCTGGGTGACTCG	Plus	20	419	438	60.32	55.00	4.00	2.00
Reverse primer	CGGCCGTCGTCAAATAACAG	Minus	20	507	488	59.63	55.00	6.00	1.00
Product length	89								

Primer pair 8

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CTGGCCGATGCACTTTCTCT	Plus	20	206	225	60.39	55.00	4.00	0.00
Reverse primer	CAAGCACCGTGCACTGAAAT	Minus	20	342	323	59.69	50.00	7.00	2.00
Product length	137								

Primer pair 9

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GCCGATGCACTTTCTCTGTG	Plus	20	209	228	59.55	55.00	4.00	1.00
Reverse primer	AGCACCGTGCACTGAAATTG	Minus	20	340	321	59.69	50.00	7.00	2.00
Product length	132								

Primer pair 10

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	ATAGAGGTAAACGGGTGGCG	Plus	20	15	34	59.54	55.00	2.00	2.00
Reverse primer	CACACTGCGACTACAGGACA	Minus	20	422	403	59.69	55.00	3.00	0.00
Product length	408								

Fig. 1(b): Designed primers for *Lytocestus heteropneustii.*

Figs. 1 (a, b): Designed primers of 28S rDNA Region of *Lytocestus heteropneustii.*

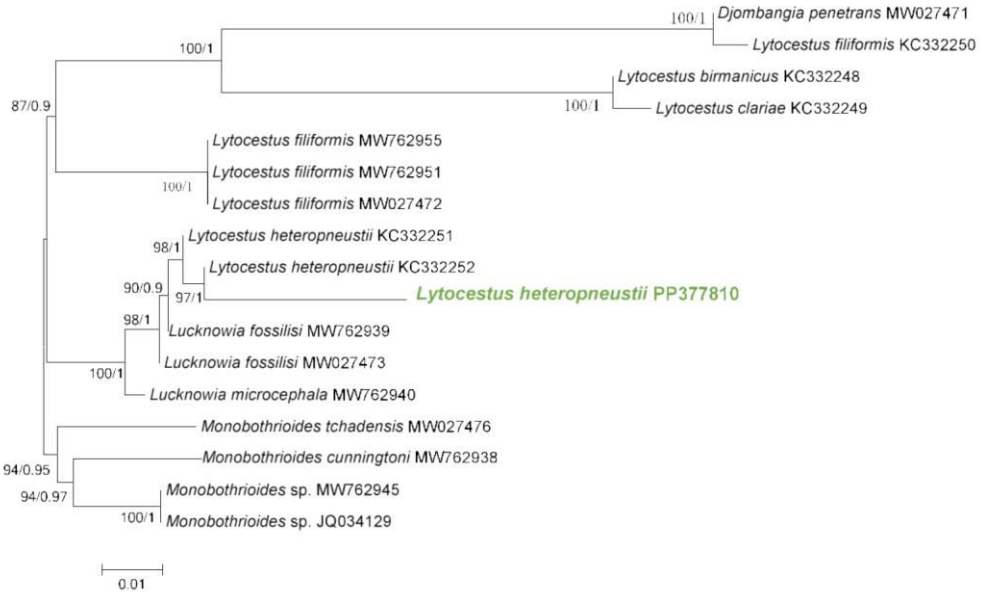

Fig. 2: Phylogenetic tree generated for *Lytocestus heteropneustii* based on the 28S rDNA using maximum likelihood analysis and Bayesianinference probabilities methods.

Fig. 3(a): Amidation motif.

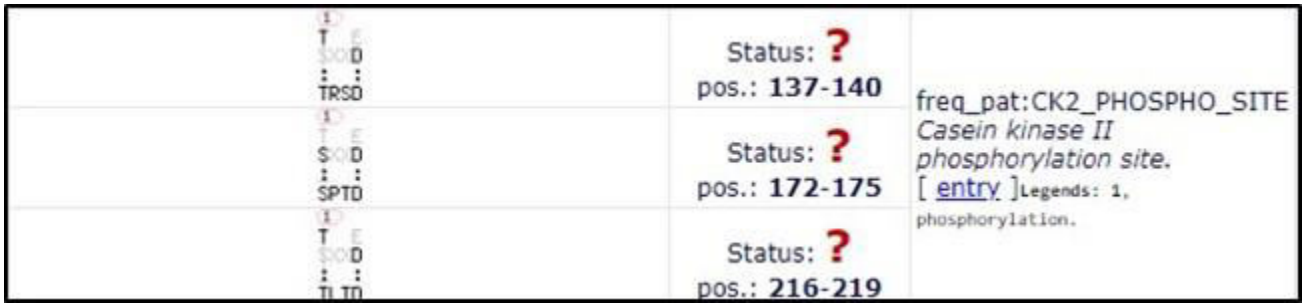

Fig. 3(b): CK2_PHOSPHO_SITE Casein kinase II motif.

Fig. 3(c): Myristyl motif.

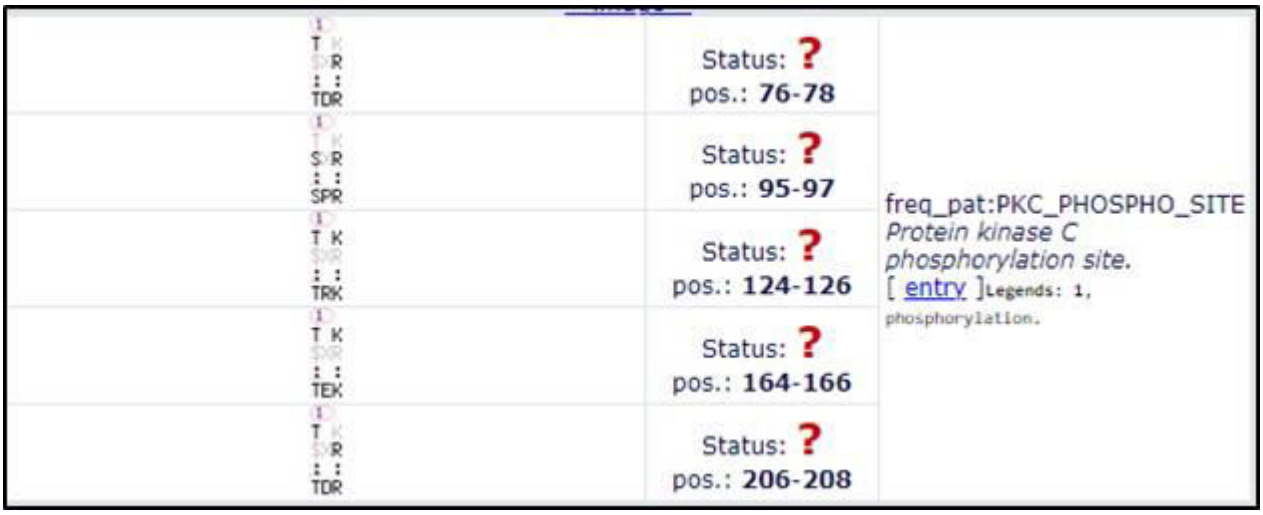

Fig. 3(d): PKC_PHOSPHO_SITE Protein kinase C motif.
Figs. 3(a, b, c, and d): Illustration of various motifs of 28S rDNA of *Lytocestusheteropneustii*

respectively (Figs. 3 a, b, c, d) were found in the 28S rDNA of *Lytocestus heteropneustii.* The role of these motifs has been widely regulated in gene expression and DNA coding.

Discussion

In the last 15 years, molecular techniques for studying parasites and diseases have advanced significantly. Polymerase chain reaction (PCR) has led to significant improvements in the field of molecular taxonomy in terms of parasitic DNA. The polymerase chain reaction technique's increased sensitivity for analysis of the alterations at the level of a single cell, which is often insufficient for parasite-related diagnosis. PCR primers are a pre-requisite to carry out any molecular studies (Apte *et al.*, 2009). Phylogenetics and DNA motif analysis significantly improve our understanding of several characteristics of annotated gene activity.

In the present study, ten primer pairs were designed (Figs. 1 a, b). The ideal parameters for selecting the best primer length are 15 to 25 nucleotides, and the primer's optimum length 19 to 21 nucleotides is considered. A primer with fewer than 19 nucleotides was not considered as it failed to anneal with the genome, whereas primers with more than 21 nucleotides result in a self-complementary situation. 47 to 60% GC content

range was selected. The GC concentration determines the binding affinity within the genome. A larger GC content demands a higher temperature for dissociation. The average temperature (Tm) was between 57 and 63 °C, a little temperature variation was employed to re-attach the forward and reverse primers to the RNA. The optimal temperature was 60°C and the primer was set kept at the same temperature. To prevent amplification, large introns were used to separate genomic DNA primers.

Molecular taxonomic studies rely on conserved regions of ribosomal DNA. 28S rDNA of *Lytocestus heteropneustii* was effectively amplified using PCR (Amin *et al.*, 2024). The 715 bp nucleotide sequence obtained from the PCR amplification product was followed by BLAST and compared to *Lytocestus* spp. sequences available on GenBank. The BLAST result showed that the newly generated sequence of *Lytocestus heteropneustii* is closer to the species of *Lytocestus* and is found to be closer within a similar identity of 99.71% similarity. Phylogenetic trees were generated by comparing *Lytocestus heteropneustii* sequences to available 28S sequences from other *Lytocestus* species using maximum likelihood analysis (ML) in MEGA v7 (Kumar *et al.*, 2016) and the Bayesian inference (BI) probabilities method TOPAL 2.0 (McGuire *et al.*, 2000) (Fig. 2), revealing similar tree topologies with substantial bootstrap support for clades. In the bootstrap test, scores of 70% or more show phylogenetic correctness and imply reliable grouping among distinct members of genus *Lytocestus*.

A DNA motif is a characterized pattern of nucleic acids with biological importance, such as locations where a transcription regulatory protein binds to a DNA element (Fatma *et al.*, 2019). The pattern typically consists of 5 to 20 base pairs (bp) and is known to repeat multiple times within a gene or in distinct genes. Structure patterns in proteins are frequently linked to DNA motifs. Finding patterns in biopolymer (nucleotide or protein) sequences helps in comprehending the structure and function of the molecules, the utmost goal of motif discovery (Lu *et al.*, 2023). Motifs can be used to find connections between the composition and operation of biological networks, among many other domains. Feedback loops, feedforward loops, and a few other tiny structures have been found by empirical investigations of networks as "motifs" that regularly appear in real-world networks and may contribute in a variety of ways to significant functions in these systems. Usually, the target protein's recognition motif is located in a short, unstructured segment. These motifs attach themselves to the comparatively flat recognition domains via a tiny groove and are found in the loop or terminal sections of the target protein (Weissensteine *et al.*, 2023).

Amidation site motif: Amidation plays a critical biological activity, including the transformation of carboxylic acids into amides. Amidating enzymes conduct this transformation and serve as a crucial process in the creation of physiologically active chemicals. The significance of amidation extends beyond fundamental biochemistry into pharmaceuticals, biotechnology, and a variety of other industries (Fig. 3a).

CK2_PHOSPHO_SITE motif: Protein kinase CK2 is involved in cell proliferation, cell differentiation, apoptosis and neoplastic transformation. CK2 has been reported to be dysregulated in many malignancies and performs a dual function in promoting cell proliferation and inhibiting apoptosis. The recent link between abnormal CK2 expression and poor prognosis in prostate cancer and acute myeloid leukemia lends credence to CK2's direct role in tumor genesis and recurrence. Indeed, CK2 is now seen as a prospective therapeutic target, facilitating the development of pharmacological inhibitors (Fig. 3b).

Myristyl site motif: Myristoylation is crucial for biological processes such as signal transduction, cellular localization, oncogenesis, protein stability and membrane localization. The N-myristoylation moiety improves protein stability, protein-protein interactions, and subcellular localization to organelles or the plasma membrane (Fig. 3c).

PKC_PHOSPHO_SITE motif: The PKC family is involved in various biological processes, including cellular differentiation, proliferation, adhesion, motility and apoptosis, as well as illnesses including cancer and inflammation. PKC isozyme-specific peptide substrates are crucial for understanding cellular processes and creating medicines that target PKC. PKC isozyme-specific peptide substrates analyze the phosphorylation sites of target proteins. PKC Phosphorylation regulates cell growth and gene expression. PKCs also regulate signal transduction pathways in the immune system, affecting both innate and adaptive immunity, which leads to the expression of critical immune genes. PKCs serve as mediators in immune cell signaling at the immunological synapse. It acts as a cytoplasmic signal transducer, mediating cell responses from the plasma membrane to the nucleus. It can transduce signals within the nucleus, unlike the cytoplasmic route (Fig. 3d).

DNA coding patterns that play functional functions in biological systems were identified by the study. Medication manufacturing procedures have been made simpler by computational techniques. The discovery of novel drug vaccines protects against helminth sickness and this can be achieved via identifying motif information. Due to the discovery's resemblance through motifs, disease control may be greatly impacted. These motif-based areas could provide good therapeutic targets for intervention. Gilbert (2011) reported that interactions between the parasite and its host can be hampered by blocking the parasite's genes from functioning. Molecular docking is a technique that inserts tiny ligands into receptors in a variety of orientations, combinations, and locations using computer-generated 3D structures. This method helps in medicinal chemistry and drug discovery by offering insights into molecular recognition. Computer-aided drug design and discovery docking (CADDD) are one of them (Azevedo *et al*., 2009). There are drawbacks to traditional docking techniques, including static or semi-flexible target/ligand management.

Docking approaches that consider protein-ligand flexibility and binding configurations have been made possible by recent developments in computers, proteomics, and genomics (Shen *et al*., 2024). More realistic representations of protein-binding interactions and more precise predictions of binding poses are made possible by receptor flexibility. Ligand: Protein flexibility can be achieved using protein ensembles or dynamic docking. The energetic and mechanistic components of drug-receptor interactions are taken into account via dynamic docking, together with the solution and entropic effects (Jakhar *et al.*, 2020). A labor-intensive method called docking is employed in quick drug development to find possible pharmacological agents in vast chemical libraries.

Conclusion

The present study depended on a large subunit of Ribosomal DNA from the *Lytocestus heteropneustii* genome to estimate the gene functioning using an *in silico* genomics approach. Designed PCR primer pairs from the 28S rDNA sequence will help to perform advanced molecular studies on the *Lytocestus heteropneustii*. The study identifies four motifs in coding DNA that play a crucial role in protein folding conformational stability, intracellular transport, antibiotic resistance, homeostasis, ligand interaction, sexual commitment, chemotaxis, DNA repair, cell cycle control and circadian rhythm regulation. The authors believe that identifying these motifs might serve as an important role in disease control by docking. The motif sites could be an attractive target of intervention for treating cestode parasites by blocking the gene functionality of the parasite and hindering its host-parasite interactions. The phylogenomic analysis of 28S rDNA regions showed nucleotide similarities with other *Lytocestus heteropneustii* sequences retrieved worldwide, which confirms its taxonomic validity. These findings open possibilities for a more in-depth analysis of *Lytocestus heteropneustii* parasite and to further our understanding of cestode biology and the

search for new drugs regarding cestode biology and designing new drugs for ceasing infection.

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