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Original paper

Documentation of *Radix acuminata* as the intermediate host for *Fasciola gigantica* in Meghalaya, Northeast India

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ABSTRACT. Fasciolosis, caused by *F. gigantica*, is a significant parasitic disease affecting livestock and humans. The study investigates the presence of *Radix acuminata* in local water bodies and its potential to harbor *F. gigantica* cercariae larvae. The study aims to elucidate the role of *Radix acuminata* in the transmission cycle of *Fasciola gigantica*. The taxonomic complexity of lymnaeids poses challenges, prompting molecular and morphological analysis for accurate species identification. The findings shed light on the transmission dynamics of fasciolosis in this region and emphasize the importance of understanding intermediate hosts for effective control measures. This research contributes to the broader field of parasitology and informs strategies for mitigating the impact of fasciolosis in Meghalaya, Northeast India.

Keywords: fasciolosis, cercariae, lymnaeids, *Radix acuminata*, Northeast India

Introduction

Fasciolosis is a food-borne trematodosis caused by *F. gigantica* and *F. hepatica*. *F. gigantica* is distributed in Asia and Africa, while *F. hepatica* is prevalent in Europe, the Americas, and Australia [1]. Both of the species are closely related in terms of morphology, genetics, and anatomical structure. They have a significant impact on livestock worldwide, leading to economic losses due to death, decreased host productivity, increased susceptibility to other illnesses, and reduced milk, meat, and wool production [2]. In addition to these two species, an intermediate or hybrid form has been reported in Asia and Africa [3].

The family Lymnaeidae holds immense parasitological significance as it serves as an intermediate host for various helminth species, and causing zoonotic diseases such as fasciolosis. The life cycle of these flukes begins with the release of immature eggs into the biliary ducts, which are then passed into the stool. In water, these eggs develop within two weeks, releasing miracidia that invade lymnaeid snails. Inside the snail, various developmental stages occur, ultimately leading to the release of cercariae. These cercariae encyst as metacercariae on aquatic plants. When humans or animals consume these contaminated plants, they become infected. The metacercariae penetrate the intestinal wall, migrate to the peritoneal cavity, travel through the liver into the biliary ducts, reach adulthood, and begin laying eggs [4].

In general, *F. hepatica* is transmitted via *Galba*/*Fossaria* lymnaeids, while the *Radix* group serves as the intermediate hosts for *F. gigantica* [5]. In India, *R. auricularia* [6,7] and *R. acuminata* [8,9] have been identified as intermediate hosts for *F. gigantica*. Lymnaeids, as a group, present taxonomic challenges, making it difficult to differentiate among lymnaeid species involved in the transmission of fasciolosis. Morphological examination of many lymnaeids is challenging due to their wide variety of shell morphologies while sharing similar anatomical traits [10]. Molecular assessment, coupled with morphological analysis, has significantly improved the accuracy and reliability of animal species identification.

This comprehensive approach, incorporating

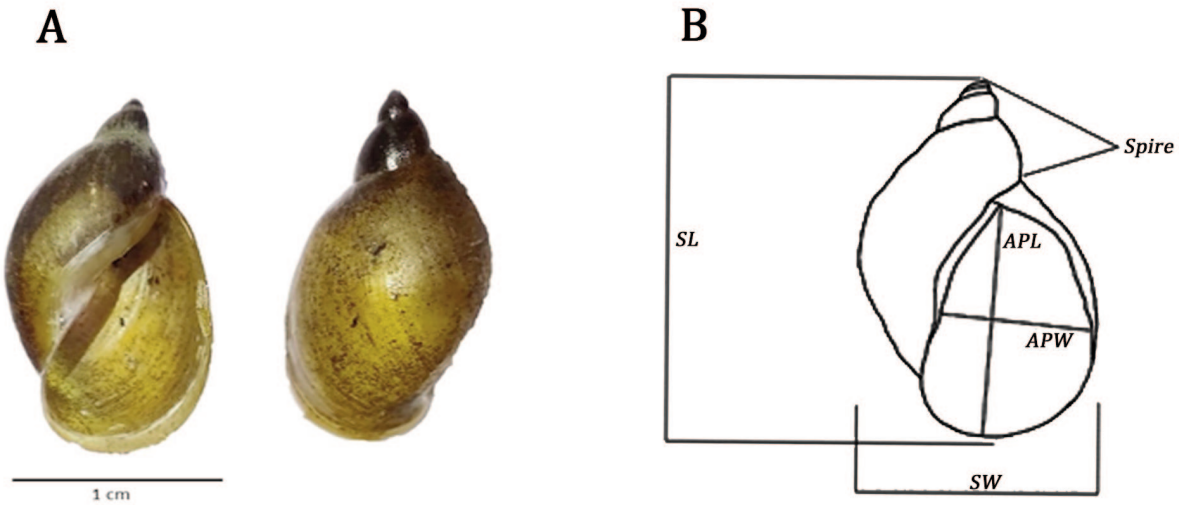

Figure 1. Morphology of snail (A) and morphometric parameters (B)

genetic and physical characteristics, resolves taxonomic uncertainties, distinguishes cryptic species, and reveals evolutionary relationships among closely related species [11]. Genetic markers like the ribosomal Internal Transcribed Spacer 2 (ITS2) and the mitochondrial Cytochrome Oxidase 1 (mtCO1) have been widely used for species delimitation [12,13]. The ribosomal Internal Transcribed Spacer 2 (ITS2) has played a significant role in successfully delineating species within lymnaeids [14–17]. The use of the ITS2 region and the mitochondrial Cytochrome Oxidase 1 (mtCO1) gene for species delimitation within the *Fasciola* genus has been well-documented [18,19].

Fasciolosis in humans and animals is emerging as a significant infection in South Asia, particularly in India [20,21]. Notably, human fasciolosis has also been reported from patients in Northeast India, indicating a potentially high prevalence of the infection in the region [20,22,23]. Transmission pattern studies of fasciolosis are lacking in Northeast India, and owing to the presence of intermediate species, the identification of the intermediate host is crucial. Hence, the present study is essential for comprehending fasciolosis and devising effective control strategies.

Materials and Methods

Sample collection and preliminary analysis

The snails were collected from Erpakon (25.824001, 91.882465), Ri-Bhoi district of Meghalaya, and brought to the laboratory for further analysis. Morphometric analysis was conducted to quantify and assess the morphological characters of the snail and was compared with the available Indian mollusc literature data [24,25]. A total of 40 snails were used in the morphometric analysis, and six measurable parameters were recorded, which include SL: shell length, SW: shell width, APL: aperture length, APW: aperture width, SPL: spire length, and SPW: spire width (Fig. 1). Statistical analysis was performed using Excel 2016. DNA extraction was carried out on two snails, while the

Table 1. List of the PCR primers, sequences, including references used in the study

Sl No.	Organism	Primers	Sequences	Reference
			ITS2	
1	Snail and cercariae	3S (forward)	5′-GGTACCGGTGGATCACTCGGCTCGTG-3′	[27]
		A28 (reverse)	5′-GGATCCTGGTTAGTTTCTTTTCCTCCGC-3′	
			CO1	
2	Cercariae	JB3 (forward)	5’-TTTTTTGGGCATCCTGAGGTTTAT-3’	[28]
		JB4.5 (reverse)	5’-TAAAGAAAGAACATAATGAAAATG-3’	

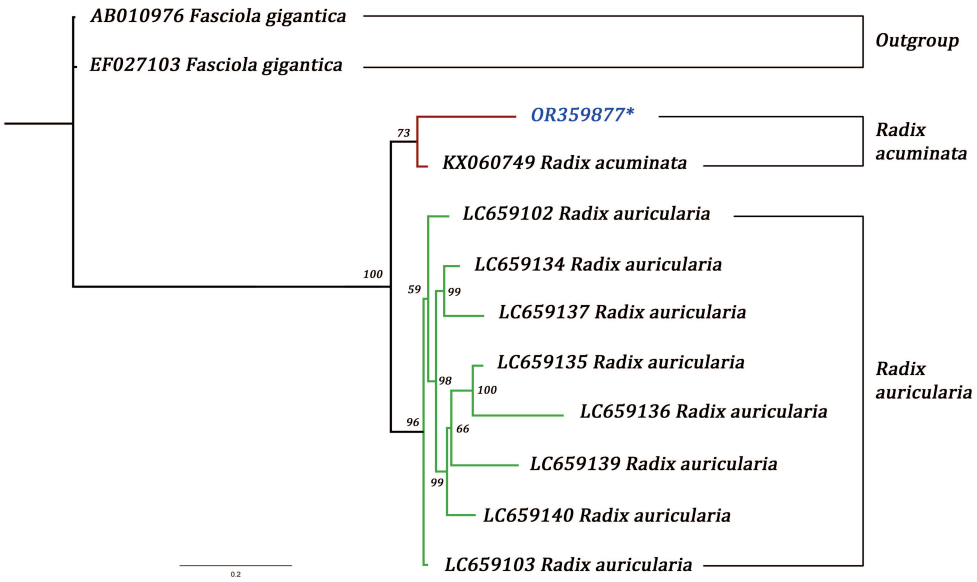

Figure 2. Phylogenetic relationships among *R. acuminata* and *R. auricularia* species inferred from Bayesian analysis of the ITS2 spacer region, with numbers at the nodes representing posterior probability values; *sequence generated for the study

remaining snail population was kept in an aquatic environment overnight to induce cercaria shedding.

DNA extraction, PCR amplification and sequencing

DNA was extracted from the foot of the snail following the phenol-chloroform precipitation technique [26]. PCR amplification was done for ITS2 using 3S and A28 primers [27]. The cercariae (17 numbers) were pooled onto a 1.5 ml Eppendorf tube, and DNA was extracted using the Qiagen DNA Kit. Cercarial DNA was subsequently amplified for mtCO1 using JB3 and JB4.5 primers [28] and ITS2 using A28 and 3S primers [27]. The details of the PCR primers used and their sequences are outlined in Table 1. The extracted DNA was then purified and sent to Apical Scientific Sequencing Services in Malaysia. The generated sequences were then submitted to NCBI-Genbank, and accession numbers were obtained.

Sequence and phylogenetic analysis

The generated sequences, along with related sequences for snails and *Fasciola*, were analysed, and phylogenetic analysis was performed. The sequences were aligned using MUSCLE, which is integrated within MEGAX [29], and then subjected to jModeltest v2.1.5 [30] to determine the best fit model for the different alignments. Species tree reconstruction was performed for the various alignments using Bayesian inference with MrBayes v. 3.2.2 [31]. Bayesian posterior probabilities were computed for the various datasets using Metropolis-coupled Markov Chain (MCMC), and the analyses were run with four chains for 1,00,00,000 generations, and sampled every 1000 generations, with the first 25% of the trees discarded as the 'burn-in' phase. Finally, FigTree v1.4 (http://tree.bio.ed.ac.uk/), was used for viewing the trees.

Results

Preliminary analysis

The snails had brown-colored shells (Fig. 1), and live specimens exhibited triangular tentacles with eye spots at their base [32]. The oblong, slender shells had a short acuminate spire, a large aperture, and an inflated body whorl with a slightly angular top [24,25]. The morphometric measurements of the snails are presented in Table 2. The shell length ranged from 13 to 24 mm, with an average length of 18.07 ± 2.37 mm, while the shell width ranged from 5 to 11 mm, with an average width of 8.40 ± 1.53 mm. There were four whorls, with the last whorl being large and inflated. The spire was short, and the apex was blunt, with a length ranging from 2 to 8 mm and a width ranging from 4 to 6 mm, with averages of 5.72 ± 1.39 mm and 5.00 ± 0.75 mm, respectively. The aperture was large, extended, and moderately expanded, with a length ranging from 9 to 17 mm and a width ranging from 4 to 8 mm, with averages of 12.37 ± 1.47 mm and 6.57 ± 1.05 mm,

Table 2. Morphometric measurements (mean ± SD) of the shell of freshwater lymnaeid snail collected from Meghalaya

Parameters	Minimum (mm)	Maximum (mm)	Mean ± SD
Shell length	13	24	18.07 ± 2.37
Shell width	5	11	8.4 0 ± 1.53
Aperture length	9	17	12.37 ± 1.47
Aperture width	4	8	6.57 ± 1.05
Spire length	2	8	5.72 ± 1.39
Spire width	4	6	5.00 ± 0.75

respectively. Cercarial shedding was spotted from 3 snails, and cercariae possessing a single tail, which is typical for *Fasciola* species, were observed.

Molecular and phylogenetic analysis

Complete rDNA ITS2 sequences were generated for both snails and cercariae, while partial mtCO1 amplification was achieved for the cercariae. Subsequently, these sequences were submitted to GenBank, and accession numbers were acquired.

Based on our morphological analysis, we inferred that it is *R. acuminata*. Therefore, for species tree reconstruction, our collected isolate was analyzed alongside *R. acuminata* and the closely related *R. auricularia* species. In the context of ITS2 sequences for *R. acuminata*, we utilized the only available sequence from the NCBI database in our analysis, in contrast to *R. auricularia* (Tab. 3). The ITS2 species tree reconstructed for snails shows the presence of distinct *R. auricularia* and *R. acuminata* clusters, respectively. Our isolate forms a clade with *R. acuminata* and exhibits good posterior probability values (Fig. 2). *Fasciola gigantica* sequences were used as outgroups for the phylogenetic analysis mentioned above.

In the case of *Fasciola* cercariae, *Paragonimus westermanii* species were chosen as outgroups for phylogenetic analysis, both for ITS2 and mtCO1. The complete ITS2 sequence data generated were compared with representatives of *F. gigantica*, *F. hepatica*, and the ”intermediate” *Fasciola* sp. The species tree reconstruction places our sequence

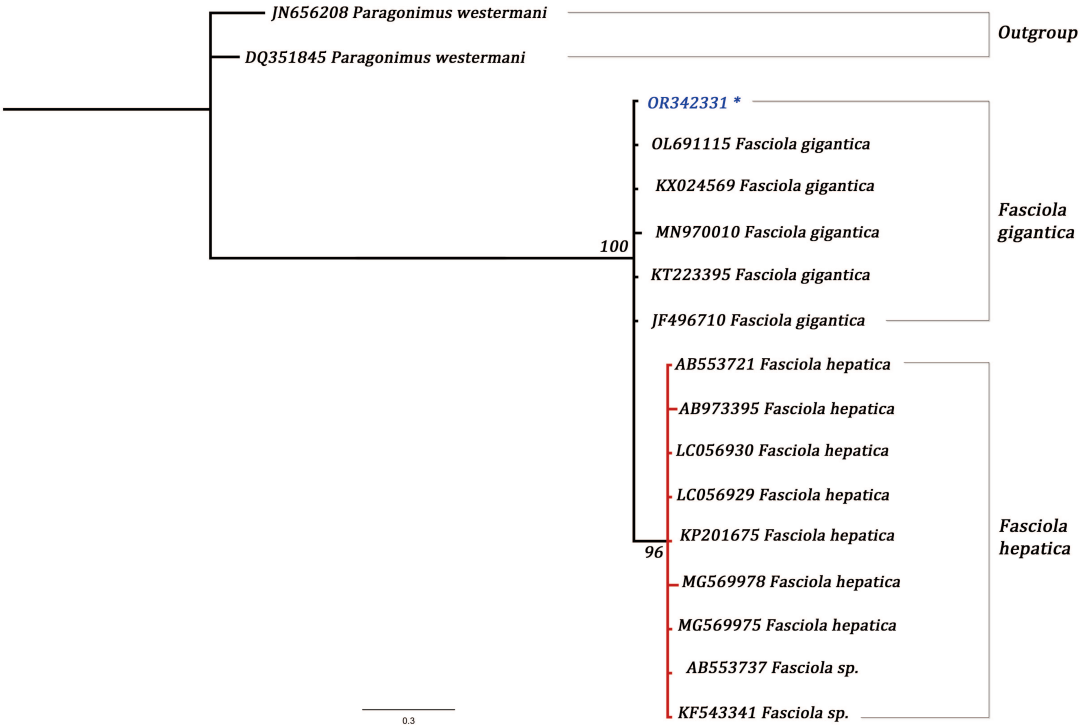

Figure 3. Phylogenetic relationships among *Fasciola* species inferred from Bayesian analysis of the ITS2 spacer region, with numbers at the nodes representing posterior probability values; *sequence generated for the study

Table 3. List of *Radix* and *Fasciola* species, including geographical location and GenBank accession number, used in the study; *sequences generated for the study

Snail species	Location	Country	Accession Number	
			ITS2	
Radix acuminata	Meghalaya	India	OR359877*	
	Andhra Pradesh	India	KX060749	
Radix auricularia	Primorsky Krai	Russia	LC659102	
			LC659103	
	Hokkaido	Japan	LC659134	
			LC659135	
			LC659136	
			LC659137	
	Ovorkhangai	Mongolia	LC659139	
	Irkutsk	Russia	LC659140	
Liver fluke species	Location	Country	Accession Number	
			ITS2	**mtCO1**
Fasciola gigantica	Meghalaya	India	OR342331*	OR364052*
	West Bengal	India	OL691115	
	Thua Thien-Hue	Vietnam	MN970010	
	Iranshahr	Iran	KT223395	
		China	JF496710	MH621335
				OR123716
	Mizoram	India	KX024569	
	Basrah	Iraq		OR063931
		Algeria		MN913873
	Cairo	Egypt	AB553721	
		Ireland	AB973395	
Fasciola hepatica		Ecuador	LC056929	
	Cotopaxi Province	Ecuador	LC056930	
		Ireland	KP201675	
	El Alto	Bolivia	MG569978	
	Toluca	Mexico	MG569975	
		Iran		KT893725
				MK447938
		Australia		AF216697
				NC002546
Fasciola sp.	Cairo	Egypt	AB553737	
		China	KF543341	OP837076
	Assam	India		KR560071

within the same clade as *F. gigantica* (Fig. 3). Similarly, the partial mtCO1 species tree also shows our isolate grouping together with *F. gigantica* (Fig. 4), indicating that our cercaria belongs to *F. gigantica*.

Discussion

Fasciolosis in India is primarily caused by *F. gigantica*, and while *F. hepatica* has been described, uncertainties arise as these assessments rely solely on morphological criteria [33]. Notably, the presence of the intermediate hybrid *Fasciola* sp. in Northeast India has been documented using both

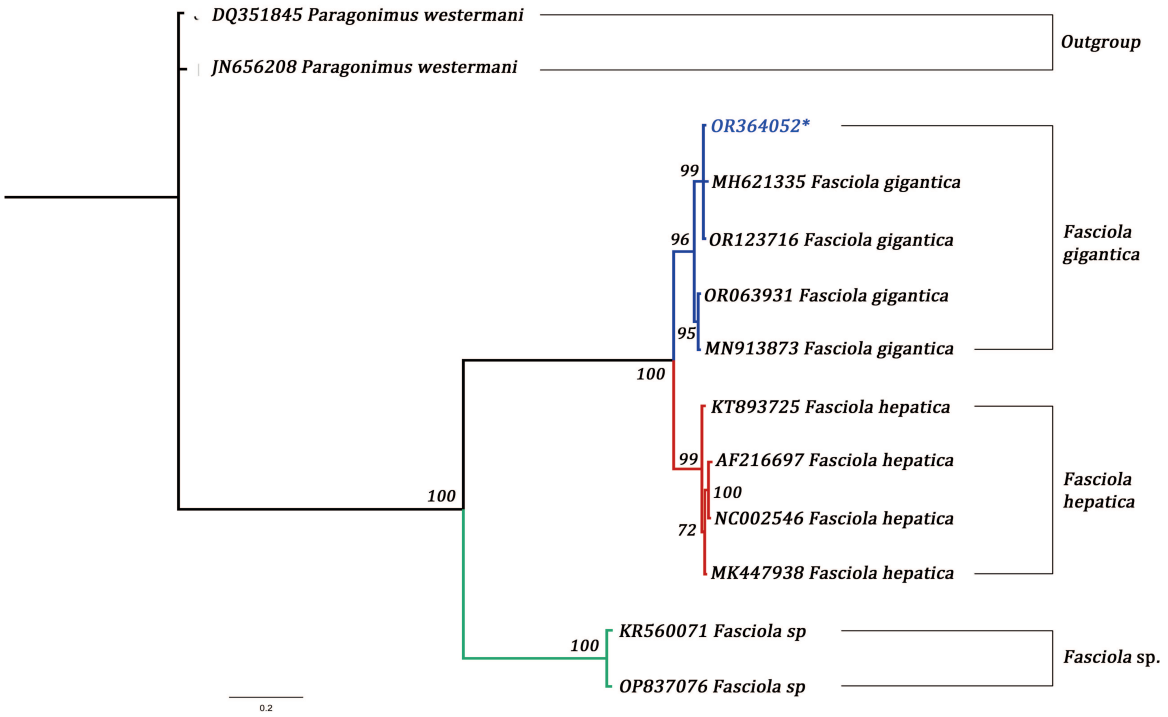

Figure 4. Phylogenetic relationships among *Fasciola* species inferred from Bayesian analysis of the mtCO1 gene, with numbers at the nodes representing posterior probability values; *sequence generated for the study

morphological and molecular data, as detailed in the study conducted by Lyngdoh et al. [21]. This discovery bears significant implications for our understanding of fasciolosis in Northeast India.

In India, both *R. auricularia* and *R. acuminata* serve as lymnaeid intermediate hosts responsible for fasciolosis. *R. auricularia* has been reported to be associated with *F. gigantica* in North, South, and Central India [32,34], but there have been no reports from Northeast India. In fact, experimental data have confirmed the potential transmission of *Fasciola* species via *R. auricularia*, suggesting it as a likely intermediate host [35]. On the other hand, *R. acuminata* has been documented in various parts of the country, including Northeast India [25]. *R. acuminata* is known for its adaptability to a wide range of temperatures [36], making it quite common in the climatic conditions of Northeast India. The collected lymnaeids share morphological similarities with *R. acuminata*, as indicated by shell morphometric data. Furthermore, our assertion that the isolated snail is *R. acuminata* is supported by the reconstructed ITS2 species tree, where our isolate groups with another *R. acuminata* sample from Andhra Pradesh, India. This is the first report of the involvement of *R. acuminata* as the intermediate host for *F. gigantica* transmission in Northeast India. This information is important as Northeast India harbours cases of both animal and human fasciolosis, and this study is crucial in formulating control measures against fasciolosis in Northeast India. Previous research has already explored transmission control strategies for fasciolosis in the country with *R. acuminata* as the intermediate host species [9]. Therefore, identifying the intermediate host responsible for fasciolosis in Northeast India is essential for understanding its transmission dynamics in the region.

While examining ITS2 and mtCO1 data from *Fasciola* larval forms in NCBI, we observed a limited number of reports. This study contributes to the molecular sequence repository for *Fasciola* species' larval forms and provides valuable insights for future research. It also demonstrates the reliability and effectiveness of ITS2 and mtCO1 primers in characterizing cercaria larval forms of *Fasciola* worms molecularly. The reconstructed species tree based on ITS2 sequences clearly illustrates the strong connection between our isolate to *F. gigantica*. Both the ITS2 and the mtCO1 tree infer similar results as shown in figures 3 and 4. Our cercaria isolate formed a distinct clade alongside the *F. gigantica* group. The data from both ribosomal ITS2 and mtCO1 sequences provide conclusive

evidence that the collected cercaria belongs to the *F. gigantica* species. Additionally, it demonstrates that it is not a representative of the intermediate *Fasciola* species found in Northeast India. It is well recognized that the predominant *Fasciola* fluke in Meghalaya, Northeast India, is largely acknowledged to be *F. gigantica* [37]. Therefore, this finding validates the occurrence of *F. gigantica* within this geographical area. Although the intermediate species has been reported in Northeast India, there has been no documentation of its presence in Meghalaya. Geographically, Northeast India is characterized by the presence of the mighty Brahmaputra River, a natural boundary that separates the region into two biodiversity hotspots: The Eastern Himalayan Hotspot and the Indo-Myanmar Hotspot. Major rivers are known to act as geographical barriers, limiting species' ability to traverse them and leading to restricted gene flow, which can result in species isolation [38]. In this context, intermediate *Fasciola* species, morphologically resembling *F. hepatica*, have been documented in regions north of the Brahmaputra River, primarily within semi-wild ruminants like yak and mithun. These semi-wild ruminants are uniquely adapted to the rugged mountainous terrain and hold significant cultural, economic, and practical value for local communities. It has been suggested that the presence of these intermediates at high altitudes is due to human-mediated animal movement within the Eastern Himalayan region [39]. However, this evidence presents only one aspect of the situation without considering the intermediate host's role in maintaining the *Fasciola* flukes' life cycle in the environment. Therefore, it is essential to identify the specific molluscan host responsible for transmitting and sustaining these intermediate flukes. Doing so would not only enhance our understanding of transmission studies but also provide a comprehensive overview of fasciolosis in Northeast India.

Owing to its geography and the role it plays in transmission biology, India requires comprehensive research on the intermediate hosts utilized by *Fasciola* to comprehend the transmission dynamics of this parasite within the country. India, specifically Northeast India, exhibits a notable prevalence of human helminth infections, including fasciolosis [23], attributable to factors like food habits, socioeconomic conditions, hygiene practices, and restricted healthcare accessibility [40]. Hence, the current study holds significant importance in providing fundamental and crucial information about the molluscan species associated with fasciolosis in Northeast India. In conclusion, the overall information gathered from this study is the confirmation of the presence of *R. acuminata* and its role as the intermediate host of *F. gigantica* in Meghalaya, Northeast India. This study is the first ever description of its kind in establishing the intermediate host responsible for fasciolosis from the entire region. As there has never been any transmission work with regards to fasciolosis in Northeast India, therefore this study holds valuable information.

Acknowledgements

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