NOTE *Parasitology*

Molecular characterization and phylogenetic analysis of *Explanatum explanatum* in India based on nucleotide sequences of ribosomal ITS2 and the mitochondrial gene *nad1*

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ABSTRACT. The aim of this study was to analyze the phylogenetic relationship between *Explanatum explanatum* populations in India and other countries of the Indian subcontinent. Seventy liver amphistomes collected from four localities in India were identified as *E. explanatum* based on the nucleotide sequences of ribosomal ITS2. The flukes were then analyzed phylogenetically based on the nucleotide sequence of the mitochondrial gene *nad1* in comparison with flukes from Bangladesh and Nepal. In the resulting phylogenetic tree, the *nad1* haplotypes from India were divided into four clades, and the flukes showing the haplotypes of clades A and C were predominant in India. The haplotypes of the clades A and C have also been detected in Bangladesh and Nepal, and therefore, it seems they occur commonly throughout the Indian subcontinent. The results of AMOVA suggested that gene flow was likely to occur between *E. explanatum* populations in these countries. These countries are geographically close and have been historically and culturally connected to each other, and therefore, the movements of host ruminants among these countries might have been involved in the migration of the flukes and their gene flow.
KEY WORDS: *Explanatum explanatum*, India, ITS2, *nad1*, phylogeny

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The family Paramphistomidae includes 19 genera and over 70 species, and its members are well-known parasites, mainly of livestock [11]. Immature flukes of almost all species of the family cause intestinal paramphistomiasis in ruminant hosts during their migration, while adult flukes commonly parasitize in the rumen and cause less damage to the host. On the other hand, adult amphistomes of the genus *Explanatum* inhabit the bile duct and cause granulomatous lesions and thickening of the duct by attaching to the epithelium of the duct using the acetabulum (ventral sucker) [6, 13]. *Explanatum* infection causes economic losses to the livestock industry by decreasing daily product and growth rates. *Explanatum explanatum*, which is the type species of the genus, is distributed mainly in Asia and Africa and detected commonly in domestic ruminants in the Indian subcontinent [1, 6, 18]. In India, *E. explanatum* is widely distributed and can be commonly detected in the bile duct of buffaloes [3, 6, 16, 19].

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Since the morphological identification of adult amphistomes requires specialized knowledge and techniques, molecular methods are used to precisely discriminate amphistomes [2, 17]. Recently, molecular identification methods based on the nucleotide sequence of the ribosomal internal transcribed spacer 2 (ITS2) have been developed for the precise identification of amphistomes, including *E. explanatum* [8, 9]. In addition, the nucleotide sequence of mitochondrial NADH dehydrogenase subunit 1 (*nad1*) has been used for intraspecific phylogenetic analysis in many helminth species [7, 12, 15]. However, no information about these molecular markers of *E. explanatum* in India has been reported yet. In this study, based on the ITS2 sequences, we identified liver amphistomes from India as *E. explanatum* and analyzed the intraspecific variation and phylogenetic relationship between the species from India and other countries of the Indian subcontinent on the basis of *nad1* sequences.

Seventy liver amphistomes were collected from the bile ducts of 30 buffaloes and 4 cattle at slaughterhouses and meat markets from Delhi, Mumbai, Gangtok and Imphal in India, from February to December 2014 (Table 1). The flukes were fixed in 70% ethanol and transported to the laboratory. Total DNA was extracted from each fluke with a High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions and stored at −20°C until use. The ITS2 region, including partial 5.8S and 28S

Table 1. The profiles of *E. explanatum* analyzed in this study

Locality	Host code	Specimen code	Mitochondrial *nad1*		
			Haplotype	Accession no.	Clade
Delhi	Buffalo#1	EE-1	ND1-IN1	LC128866	A
		EE-2	ND1-IN14	LC128879	C
	Buffalo#2	EE-3	ND1-IN18	LC128883	C
		EE-4	ND1-IN10	LC128875	A
	Buffalo#3	EE-5	ND1-IN1	LC128866	A
		EE-6	ND1-IN25	LC128890	A
	Buffalo#4	EE-7	ND1-IN1	LC128866	A
		EE-8	ND1-IN2	LC128867	A
	Buffalo#5	EE-9	ND1-IN1	LC128866	A
		EE-10	ND1-IN17	LC128882	C
	Buffalo#6	EE-11	ND1-IN1	LC128866	A
		EE-12	ND1-IN1	LC128866	A
	Buffalo#7	EE-13	ND1-IN4	LC128869	A
		EE-14	ND1-IN12	LC128877	B
	Buffalo#8	EE-15	ND1-IN3	LC128868	A
		EE-16	ND1-IN1	LC128866	A
	Buffalo#9	EE-17	ND1-IN1	LC128866	A
		EE-18	ND1-IN1	LC128866	A
	Buffalo#10	EE-19	ND1-IN1	LC128866	A
		EE-20	ND1-IN1	LC128866	A
	Buffalo#11	EE-21	ND1-IN1	LC128866	A
		EE-22	ND1-IN19	LC128884	C
	Buffalo#12	EE-23	ND1-IN4	LC128869	A
		EE-24	ND1-IN17	LC128882	C
	Buffalo#13	EE-25	ND1-IN11	LC128876	A
		EE-26	ND1-IN11	LC128876	A
	Buffalo#14	EE-27	ND1-IN16	LC128881	C
		EE-28	ND1-IN13	LC128878	C
	Buffalo#15	EE-29	ND1-IN1	LC128866	A
		EE-30	ND1-IN1	LC128866	A
	Buffalo#16	EE-31	ND1-IN1	LC128866	A
		EE-32	ND1-IN10	LC128875	A
	Buffalo#17	EE-33	ND1-IN10	LC128875	A
		EE-34	ND1-IN1	LC128866	A
	Buffalo#18	EE-35	ND1-IN8	LC128873	A
		EE-36	ND1-IN13	LC128878	C
	Buffalo#19	EE-37	ND1-IN2	LC128867	A
		EE-38	ND1-IN1	LC128866	A
	Buffalo#20	EE-39	ND1-IN1	LC128866	A
		EE-40	ND1-IN1	LC128866	A
	Buffalo#21	EE-41	ND1-IN1	LC128866	A
		EE-42	ND1-IN5	LC128870	A
	Buffalo#22	EE-43	ND1-IN1	LC128866	A
		EE-44	ND1-IN1	LC128866	A
	Buffalo#23	EE-45	ND1-IN15	LC128880	C
		EE-46	ND1-IN1	LC128866	A
	Buffalo#24	EE-47	ND1-IN1	LC128866	A
		EE-48	ND1-IN17	LC128882	C
	Buffalo#25	EE-49	ND1-IN7	LC128872	A
		EE-50	ND1-IN10	LC128875	A
	Buffalo#26	EE-51	ND1-IN9	LC128874	A
		EE-52	ND1-IN10	LC128875	A
	Buffalo#27	EE-53	ND1-IN1	LC128866	A
		EE-54	ND1-IN1	LC128866	A
Mumbai	Buffalo#28	EE-55	ND1-IN1	LC128866	A
		EE-56	ND1-IN1	LC128866	A
		EE-57	ND1-IN1	LC128866	A
		EE-58	ND1-IN1	LC128866	A
		EE-59	ND1-IN1	LC128866	A
Gangtok	Buffalo#29	EE-60	ND1-IN22	LC128887	C
		EE-61	ND1-IN20	LC128885	A
		EE-62	ND1-IN14	LC128879	C
		EE-63	ND1-IN21	LC128886	A
		EE-64	ND1-IN23	LC128888	C
Imphal	Buffalo#30	EE-65	ND1-IN26	LC128891	A
		EE-66	ND1-IN1	LC128866	A
	Cattle#31	EE-67	ND1-IN28	LC128893	E
		EE-68	ND1-IN27	LC128892	E
	Cattle#32	EE-69	ND1-IN6	LC128871	A
		EE-70	ND1-IN24	LC128889	A

(442 bp), was amplified with the ITS2-F and ITS2-R primer set [10], and the *nad1* fragment (657 bp) was amplified with the Pc-nad1-F1 (5′-CAGATTCGGAAGGGGCCTAA-3′) and Pc-nad1-R1 (5′-ACGTAGCACGAGCCCAAATA-3′) primers [15]. The ITS2 and *nad1* amplicons were directly sequenced in both directions with a BigDye Terminator v3.1 Cycle Sequence Kit (Applied Biosystems, Foster City, CA, U.S.A.), using the same primers as those for PCR on an ABI 3500 Genetic Analyzer (Applied Biosystems). The resultant sequences were initially assembled using ATGC ver. 6.0.3 (Genetyx Co., Tokyo, Japan), and the haplotypes were distinguished by GENETYX ver. 10 (Genetyx Co.). In addition to the *nad1* sequences identified in this study, reference sequences of *E. explanatum* from Bogra, Khulna, Sylhet and Mymensingh in Bangladesh (haplotype codes: Bd1 to Bd30, Genbank accession nos.: LC101685–LC101714) and Chitwan in Nepal (N1 to N15, LC101715–LC101729) [15], as well as outgroup sequences of *Paramphistomum cervi* (KT198987) and *Fasciola hepatica* (AF216697), were used for phylogenetic analysis. The sequences were aligned, and a phylogenetic tree was constructed by the neighbor-joining method in MEGA version 6.06 [20], using the Tamura and Nei model with gamma distribution, which was selected with the maximum likelihood test. Node support was assessed with 1,000 bootstrap replicates. The analysis of molecular variance (AMOVA) [4] of genetic structure among the populations from Delhi, Mumbai, Gangtok and Imphal in India, Bogra, Khulna, Sylhet and Mymensingh in Bangladesh, and Chitwan in Nepal was performed using Arlequin ver. 3.5.1.2 [5].

There was no diversity among the ITS2 sequences of the 70 flukes, and they were completely identical to the sequences of *E. explanatum* from Myanmar (AB743577) [10], Bangladesh (LC101682) [15] and Nepal (LC101684) [15], indicating the flukes were molecularly identified as *E. explanatum*, according to the previous report [8]. The result suggests that the ITS2 sequence of *E. explanatum* is highly

conserved and rarely shows intraspecific variation. Further, the ITS2 sequence differed at 7 nucleotide sites from the most closely related species (*Paramphistomum leydeni*) and was clearly distinguished from that of other amphistome species [8, 9]; therefore, the ITS2 sequence is considered to be a suitable marker for discriminating *E. explanatum* from other amphistome species.

The *nad1* sequences showed 60 substitution sites, yielding 28 haplotypes, ND1-IN1 to ND1-IN28 (LC128866–LC128893) (Table 1). In the neighbor-joining tree, the *nad1* haplotypes of *E. explanatum* from India were divided into four clades (clades A, B, C and E) (Fig. 1). Haplotypes of clades A and C were predominant (67/70) in India, while clades B and E were remarkably limited in number and locality. Further, the haplotypes of the clades A and C have also been detected in Bangladesh and Nepal, so it seems they occur commonly throughout the Indian subcontinent. In addition, the haplotypes of different clades were found in flukes from single hosts; e.g., ND1-IN1 (A) and ND1-IN14 (C) were found in the flukes from Buffalo#1 (Table 1). The fixation index among the countries (*F*ct) was not significant in AMOVA, indicating no genetic difference in *E. explanatum* populations among the countries. On the other hand, the fixation index among localities within countries (*F*sc) and that within localities (*F*st) were significant, suggesting that there are significant genetic differences in *E. explanatum* populations among localities within countries and within localities. Then, the percentage of variation within localities (72.41%) was extremely higher than that among localities within countries (18.28%). These results indicate that gene flow was likely to occur among the *E. explanatum* populations in India, Bangladesh and Nepal (Table 2). These countries are geographically close and have been historically and culturally connected to each other, and therefore, the movements of host ruminants among these countries might have been involved in the migration of the flukes and their gene flow [14]. Similarly, the populations of *Fasciola gigantica*, a ruminant parasite, have also shown high genetic similarity in these three countries [7]. However, further studies using additional flukes collected from many localities are required to elucidate a detailed phylogenetic relationship of *E. explanatum* in the Indian subcontinent.

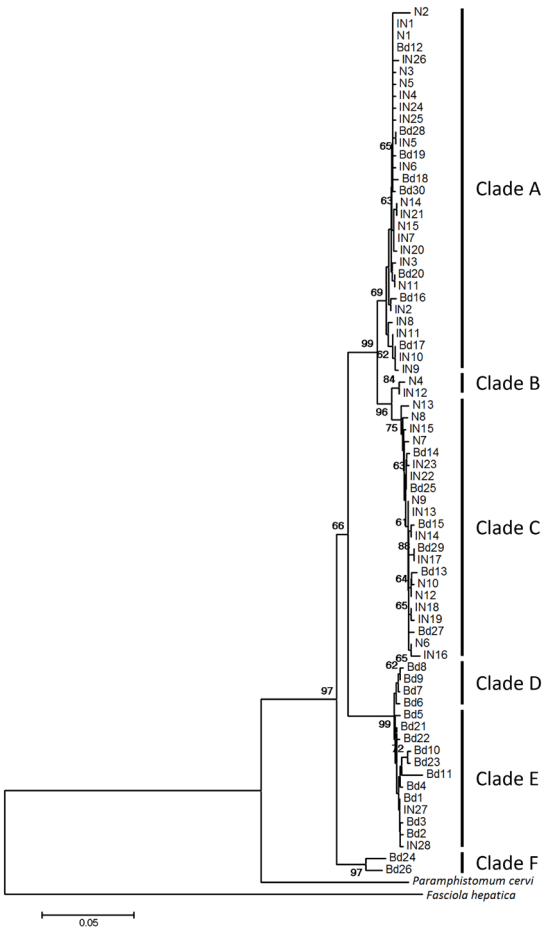

Fig. 1. Phylogenetic tree of *E. explanatum* on the basis of partial sequences of the *nad1* gene. The tree was constructed by the neighbor-joining method using the Tamura and Nei with gamma distribution. The node support was calculated with 1,000 bootstrap replicates. Six clades were divided based on over 95% bootstrap replicates. IN1 to IN28 represent the haplotype codes of ND1-IN1 to ND1-IN28 (LC128866-LC128893). Bd1 to Bd30 (LC101685-LC101714) and N1 to N15 (LC101715-LC101729) were used as reference haplotypes detected in Bangladesh and Nepal, respectively.

Table 2. Analysis of molecular variance (AMOVA) of genetic structure among populations of *E. explanatum* from India, Bangladesh and Nepal

Source of variation	d.f.	Sum of squares	Variation components	Percentage of variation	Fixation index
Among countries	2	211.414	1.59807 Va	18.28	*F*ct=0.18279
Among localities within countries	6	87.871	0.81383 Vb	9.31	*F*sc=0.11391*
Within localities	147	930.651	6.33096 Vc	72.41	*F*st=0.27587*
Total	155	1,229.936	8.74286		

Countries: India, Bangladesh and Nepal. Localities: Delhi, Mumbai, Gangtok and Imphal in India, Bogra, Khulna, Sylhet and Mymensingh in Bangladesh, and Chitwan in Nepal. d.f. degrees of freedom. * Significant (P<0.05).

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