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Molecular characterization of *Bertiella studeri* infecting a primate in South India

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Summary

Bertiella spp. is a mite-borne cestode parasite that inhabits the small intestine of wide range of mammals, including non-human primates. In the present study, the morphological and molecular analysis of *Bertiella studeri* recovered from the small intestine of a bonnet macaque (*Macaca radiata*) from Wayanad, Kerala (South India) was performed. Acetic alum carmine staining identified the cestode morphologically based on the characters like broader proglottids, which contain irregularly alternating genital pores, single set of reproductive organs, 280 testes and a tubular transverse uterus. Molecular characterization was done using 18SrRNA, ITS1-5.8S and COX1 genes. Phylogenetic trees were constructed using MEGA X based on the Maximum Likelihood (ML) method (Hasegawa-Kishino-Yano (HKY) model). Cytochrome oxidase I gene could detect the existence of genetic variation in the parasite from two different hosts viz., monkey (Kerala, Argentina, and Kenya) and human (Sri Lanka). A minimum spanning network of haplotypes was generated by the haplotype networking with the above sequences using the popARTv1.7. Haplotype analysis based on COX1 revealed that the parasite haplotype was different in each country with highest population frequency in Sri Lanka.

Keywords: Cestode; 18SrRNA; ITS1-5.8S; COX1; Phylogeny; Haplotypes

Introduction

The genus *Bertiella* (Cestoda: Anoplocephalidae) includes cestode parasites, prevalent in a wide range of mammals, primates, flying lemurs, rodents, and marsupials of Africa, Asia, South America, and other regions (Denegri & Perez-Serrano, 1997). This cestode is common in Old World as well as New World monkeys (Kuntz, 1982). Out of 40 species identified under the genus *Bertiella,* nearly ten are known to infect primates (Galan-Puchades *et al.,* 2000). Blanchard (1891) described the type species, *B. studeri*, from the common chimpanzee (*Pan troglodytes*). *Bertiella mucronata* and *Bertiella satyri* are the other valid species which are zoonotic (Bhagwant, 2004; Foitova *et al.,* 2011). *Bertiella studeri* parasitises the small intestine of primates, the natural hosts of the parasite (Gallella *et al.,* 2011). Oribatid mites, the intermediate host of the parasite are often present in the soil fauna while the definitive hosts acquire the infection by the accidental ingestion of mites containing the cysticercoids (Denegri & Perez-Serrano, 1997).

Majority of human infections caused by *Bertiella* spp. were described in individuals who had some interaction or association with non-human primates, either as pets or in zoos (Servian *et al.*, 2020). The deforestation and urbanisation due to human invasion to newer habitats resulted in the reduction in the natural habitat of the primates (Sharma *et al.*, 2018). Although the illness is fre-

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quently asymptomatic, it can produce anorexia, gastroenteritis, intermittent diarrhoea, and abdominal pain (Acha & Szyfres, 2003). More than 50 cases of human bertiellosis were reported in 29 nations of which majority were due to *B. studeri* (Servian *et al.,* 2020). The most complete description of *B. studeri* of nonhuman origin was made based on the specimens collected from Indian monkeys (Galan-Puchades *et al.,* 2000). However, the species level identification is difficult through morphology. When morphological information is insufficient or confusing, the molecular markers are helpful additional tools for the identification of the parasite and genotyping (Dolezalova *et al.,* 2015).

In the present study, we provide the morphological and molecular analysis of *B. studeri* tapeworm recovered from the small intestine of a bonnet macaque (*Macaca radiata*) from Wayanad, Kerala. The molecular targets used in the present study included nuclear markers like 18S ribosomal RNA (18S rRNA), internal transcribed spacer subunit 5.8S (ITS1-5.8S) and mitochondrial marker like mitochondrial cytochrome c oxidase subunit I (COX1) genes.

Materials and Methods

Sample collection and morphological identification

A single specimen of cestode was collected from the small intestine of a bonnet macaque (*Macaca radiata*) found dead in roadside at Bavali, Mananthavady, Wayanad on 09-09-21. The collected sample was placed in a labelled clean container. The cestode was transported on the same day to the Department of Veterinary Parasitology, College of Veterinary and Animal Sciences, Pookode, Kerala, India, for further processing and identification. The specimens were flattened, fixed and later stained using Acetic alum carmine and mounted using DPX. They were examined using a stereo zoom microscope (Leica M 205C, Germany). The remaining portion of the sample was stored in a −20 °C deep freezer until further processing.

Genomic DNA isolation and polymerase chain reaction (PCR)

The genomic DNA was isolated from the cestode collected from the small intestine of the bonnet macaque using DNeasy ®blood and tissue kit (M/s Qiagen, Hilden, Germany) according to the manufacturer's protocol. After the extraction, the DNA sample was quantified using a Nanodrop 2000C spectrophotometer (M/s. Thermo Scientific Massachusetts, USA). The isolated DNA was used for the amplification of 18S ribosomal RNA (18SrRNA), internal transcribed spacer subunit 1-5.8S (ITS1-5.8S) and mitochondrial cytochrome c oxidase subunit1 (COX1) genes (Table 1).

All the PCRs were performed in an automated thermal cycler with a heated lid (Eppendorf, Germany). The PCRs were set up in a total volume of 25 µL using 10X buffer (Thermo Scientific, USA), dNTP mix (0.2 mM, Thermo Scientific, USA), 0.5 U *Taq* DNA Polymerase (Thermo Scientific, USA), 10 pmol each of forward and reverse primers, 50 – 100 ng of template DNA and nuclease-free water.

Table 1. Details of PCR primers used in this study.

Organism	Gene	Primers	Amplification conditions	Amplicon length (bp)	References
B. studeri	18SrRNA	F 5'AACCTGGTTGATCCTGCCAGT3' R 5'TGATCCTTCTGCAGGTTCACCTAC3'	denaturation at 94ºC for 3 min; 30 cycles of 94ºC for 30 s; 55ºC for 1min; 72ºC for 1 min; final extension at 72ºC for 10 min	412	Medlin *et al.,* 1988
B. studeri	COX1	F 5'TGGTTTTTTGTGCATCCTGAGGTTTA3' R 5'AGAAAGAACGTAATGAAAATGAGCAAC3'	denaturation at 94ºC for 1 min 30 s; 30 cycles of 94ºC for 50 s; 45ºC for 1 min 30 s; 72ºC of 1 min 30 s; final extension at 72ºC for 7 min	448	Okamoto *et al.,* 1997
B. studeri	ITS1-5.8S	F 5'GCGGAAGGATCATTACACGTTC3' R 5'GCTCGACTCTTCATCGATCCACG3'	denaturation at 94ºC for 2 min; followed by first cycle; 94°C for 2 min; 63°C for 2 min; 72°C for 1 min; 34 cycles of 94°C for 20 s; 63°C 20 s; 72°C 45 s; final extension at 72ºC for 7 min	806	MacNish *et al.,* 2002

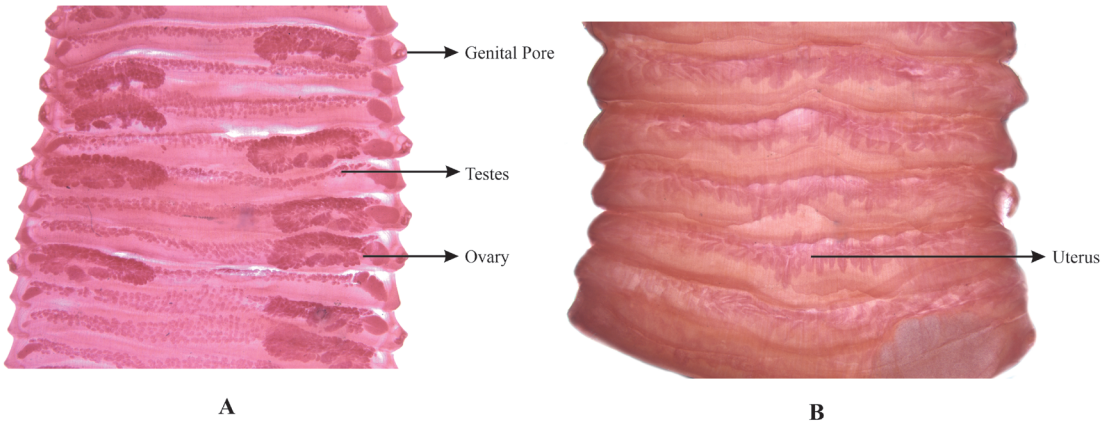

Fig. 1. Proglottids of *B. studeri*.
A – mature segment of *B. studeri*, B – gravid segment of *B. studeri*

Phylogenetic and haplotype network analysis

The amplicons of PCRs targeting 18S rRNA, ITS1-5.8S and COX1 were sent for sequencing in both forward and reverse directions to M/s AgriGenome Labs Private Ltd, Cochin, Kerala. The nucleotide sequences were then edited using the BioEdit programme by comparing the chromatogram obtained from the sequencing reactions using the forward and reverse primers. The nucleotide sequences were analysed for their identity using National Centre for Biotechnology Information - Basic Local Alignment Search Tool (NCBI-BLAST) (www.ncbi.nlm.nih.gov/BLAST).

The multiple sequence alignment was performed with the previously published sequences from GenBank using the ClustalW programme. The aligned sequences were trimmed to the same length (with gaps) from which phylogenetic trees were constructed based on the Maximum Likelihood (ML) method, using the MEGA X programme with the suitable models. The reliability of the topologies was tested by bootstrapping with 1000 replications [18SrRNA, ITS1-5.8S and COX1 Hasegawa-Kishino-Yano (HKY) model]. The intraspecific and interspecific genetic distances were calculated. A minimum spanning network of haplotypes was generated by the haplotype networking with the above sequences using the popARTv1.7.

Ethical Approval and/or Informed Consent

All procedures performed in studies were in accordance with the ethical standards. Permission was obtained for the conducting post-mortem of the monkey carcass as per DCWYD/262/2019-DSC1

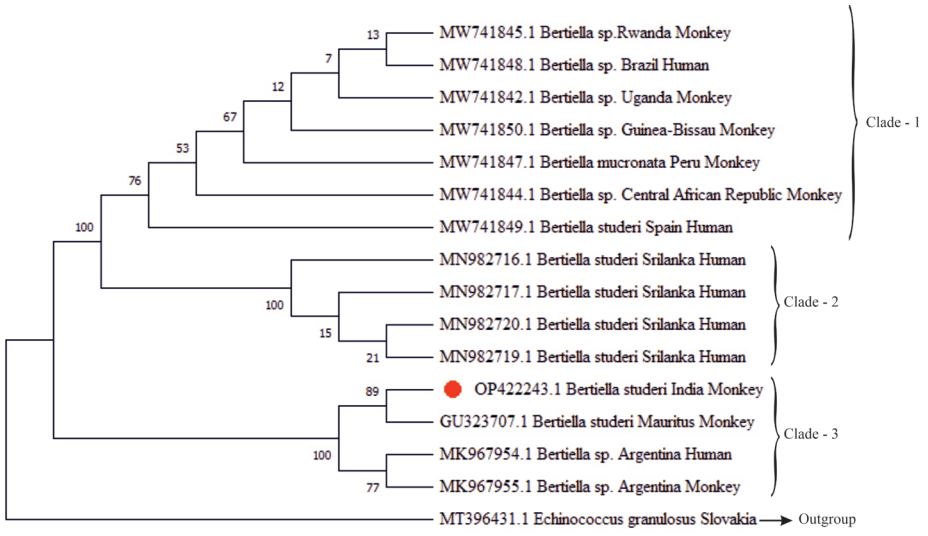

Fig. 2. Phylogenetic tree constructed using 18SrRNA gene sequence of *B. studeri*.
The evolutionary history was inferred by using the Maximum Likelihood method and Hasegawa-Kishino-Yano model. The tree with the highest log likelihood (-1247.04) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. This analysis involved 16 nucleotide sequences. There was a total of 315 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

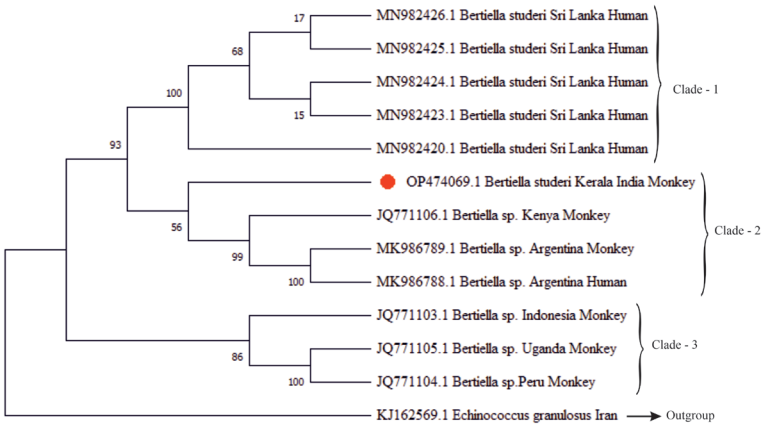

Fig. 3. Phylogenetic tree constructed using COX1 gene sequence of *B. studeri*.

The evolutionary history of *B. studeri* was inferred by using the Maximum Likelihood method and Hasegawa-Kishino-Yano model. The tree with the highest log likelihood (-1017.95) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. This analysis involved 13 nucleotide sequences.

Dt. 29-04-2020 of District Collector, Wayanad and KVASU/DAR/R3/32296/18 Dt. 01-05-2020 of DAR, KVASU.

Results

Proglottids were much broader than long (length = 1.688 mm, width = 15.905 mm of the stained specimen). Irregularly alternating genital pores with single set of reproductive organs were noticed in each proglottid. Testes were approximately 280 per proglottid, occupying dorsal anterior portion of proglottid. Thick-walled small cirrus sac was present. Transverse uterus was found extending in tubular stage across middle of proglottid (Fig 1).

The PCRs using the genus/species specific primers targeting 18SrRNA, COX1 and ITS1-5.8S genes amplified ~412 bp, ~452 bp, ~806 bp products respectively. The sequences were submitted to the GenBank (NCBI) and accession numbers (OP422243, OP474069, OP477441) were assigned.

When the sequences of 18SrRNA were used for the phylogenetic analysis using Maximum Likelihood method, the isolates of *Bertiella* spp. from different parts of the world formed three clades (Fig. 2). The isolates from Kerala, South India occupied clade 3 with other monkey isolates from Mauritius and Argentina and human isolates from Argentina. However, human and monkey isolates of different countries could not be differentiated. The phylogenetic tree constructed using COX1 gene differentiated the isolates based on their hosts. The monkey isolate of *B. studeri* targeting the COX1 gene from Kerala, South India formed clade-2 sharing with monkey isolates from Argentina, and Kenya (Fig. 3). The monkey isolates of *Bertiella* spp. from different countries were grouped in clade 2 and 3, while human isolates were grouped in clade 1. When the ITS1-5.8S was used for phylogenetic analysis, monkey isolates from Kerala, South India were grouped together with monkey/human isolates from Japan and Argentina as clade-1 (Fig. 4). The other human *Bertiella* spp. isolates from Uganda were grouped

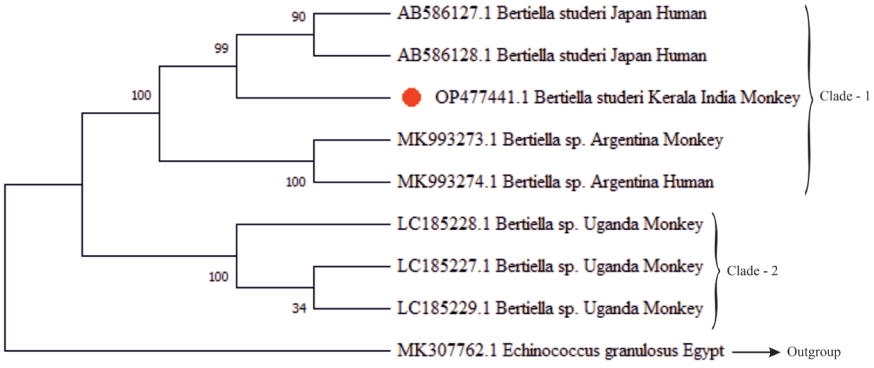

Fig.4. Phylogenetic tree constructed using ITS1-5.8S gene sequence of *B. studeri*.

The evolutionary history was inferred by using the Maximum Likelihood method and Hasegawa-Kishino-Yano model. The tree with the highest log likelihood (-1819.29) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. This analysis involved 9 nucleotide sequences. There was a total of 604 positions in the final dataset.

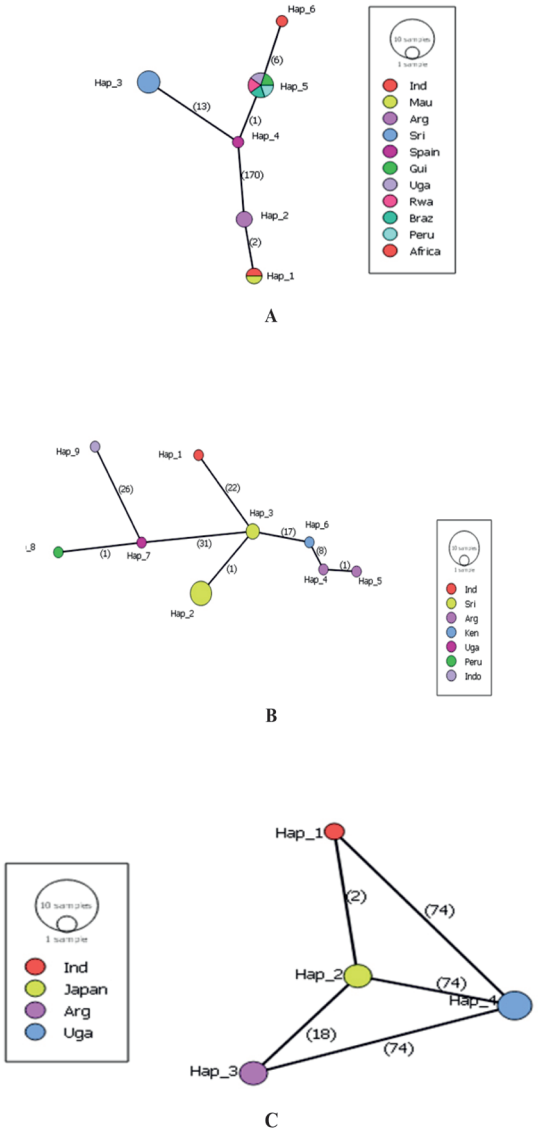

Fig.5. Minimum spanning network of *B. studeri* species complex determined by A) 18SrRNA, B) COX1, C) ITS1-5.8S genes using POPART program. The size of a circle indicates the relative frequency of sample such as *B. studeri* and *Bertiella* spp. Hatch marks (numbers) along the branches indicate the numbers of mutations. Each colour indicates a different geographic area.

separately in clade-2. The calculated intraspecific distance among *Bertiella* spp. isolates ranged between 0.00 – 0.540 for 18S rRNA gene, and 0.00 – 0.0120 for COX1 gene and 0.00 – 0.004 for ITS1-5.8S. Interspecific divergence between *B. studeri* and other *Bertiella* spp. isolates ranged from 0.007 – 0.542 (18SrRNA) gene, and 0.063 – 0.124 (COX1) and 0.004 – 0.14 (ITS1-5.8S).

Overall, six haplotypes of *Bertiella* spp. (Table 2, Fig. 5) were identified based on 18SrRNA gene. Out of six haplotypes, haplotype 5 was widespread with their existence in Brazil, Peru, Guinea, Rwanda and Uganda. Haplotype 1 was observed in India and Mauritius. Remaining four haplotypes were unique to different countries. Nine haplotypes of *Bertiella* spp. identified based on COX1 gene, were unique to each country. The frequency of haplotypes 2 and 3 from Sri Lanka was high. The haplotype analysis using ITS1 5.8S sequences could identify four haplotypes in accordance with the variable site distribution. A high level of frequency of population was documented in haplotype 4 from Uganda.

Discussion

The genus *Bertiella* includes well-known intestinal cestodes of the Anoplocephalidae, infecting both humans and nonhuman primates (Beveridge, 1994). In the present study, the parasite recovered from the intestine of a dead bonnet macaque was identified as *B. studeri* based on the morphological characters of proglottid (Stunkard, 1940; Galan-Puchades *et al.,* 2000; Sharma *et al.,* 2018).

Table 2. Frequency of haplotypes of *Bertiella* spp. in different countries.

18SrRNA			COX1			ITS1-5.8 S		
Haplotype	Frequency	Country	Haplotype	Frequency	Country	Haplotype	Frequency	Country
Hap_1	2	India, Mauritius,	Hap_1	1	India	Hap_1	1	India
Hap_2	2	Argentina	Hap_2	5	Sri Lanka	Hap_2	2	Japan
Hap_3	4	Sri Lanka	Hap_3	2	Sri Lanka	Hap_3	2	Argentina
Hap_4	1	Spain	Hap_4	1	Argentina	Hap_4	3	Uganda
Hap_5	5	Guinea-Bissau, Uganda, Rwanda, Brazil, Peru	Hap_5	1	Argentina			
Hap_6	1	Central African Republic	Hap_6	1	Kenya			
			Hap_7	1	Uganda			
			Hap_8	1	Peru			
			Hap_9	1	Indonesia			

In the evolutionary analysis using 18S rRNA, the monkey isolates from India, Argentina and Mauritius under clade 3 were clustered together, along with human isolate from Argentina. The genetic distance between clade 2 and 3 was 0.54. Bhagwant (2004) reported that the human infection caused by *B. studeri* in Mauritius might have been accidentally introduced onto the island of Mauritius from the Southeast Asia along with monkeys in the seventeenth century. *Bertiella studeri* human isolate from Argentina was recovered from the caretaker who was in close contact with the howler monkey (Servian *et al.,* 2020). The monkey/human isolates from Rwanda, Brazil, Uganda, Guinea-Bissau, and Peru were in single clade (clade 1) based on 18SrRNA sequences and these isolates were genetically closer to *B. mucronata*. Previously, Dolezalova *et al.* (2015) also reported similar findings. The *B. studeri* human isolate from Spain in the same clade might have resulted from a wrong identification.

The monkey isolates from Wayanad (Kerala, India), Kenya and Argentina were separated from the human isolates of *B. studeri* from Sri Lanka with a genetic distance of 0.121 indicating the genetic differentiation of *B. studeri* of human and monkey origins when COX1 was used for genotyping. Hence, mitochondrial DNA revealed larger level of species-to-species divergence, during genotyping (Servian *et al.,* 2020).

Unavailability of sufficient numbers of sequences for *B. studeri* ITS1-5.8S sequences in the GenBank was a constraint during the phylogenetic analysis. The *B. studeri* sequences from India, Japan and Argentina occupied a single clade away from the isolates of Africa**.** The human and monkey isolates could not be differentiated when phylogenetic analysis was performed using ITS1-5.8S sequences.

Haplotype analysis based on COX1 revealed single haplotype unique to each country with an exception, Sri Lanka and Argentina where two haplotypes were observed**.** This high population frequency in Sri Lanka, may be due to the presence of a greater number of isolates used for the study compared to other countries. Single haplotype formation unique to each country may sometimes indicate the emergence of subspecies of the parasite as a result of the evolution (Selcuk *et al.,* 2022).

There are very few studies on the occurrence of human bertiellosis from India (Malik *et al*., 2013; Sharma *et al*., 2018). The present study forms the first molecular characterization of the monkey isolate of *B. studeri*. The drastic increase in the human population in the current scenario and encroachment of the animal habitats especially the primate habitats might have increased the emergence of zoonotic pathogens (Kowalewski *et al*., 2011). Since Wayanad district of Kerala, South India is a major part of Western Ghats, the possibility of human and primate interaction is more. Hence, more studies on the diagnosis, zoonotic potential and control of zoonotic pathogens like *Bertiella* spp. are essential in this region**.**

Conclusion

The present study forms the first report molecular confirmation and haplotype analysis of *B. studeri* using nuclear (18SrRNA, ITS1-5.8S) and mitochondrial (COX1) genes from Wayanad, Kerala, South India. Host differentiation (human Vs monkey) was possible with COX1 gene. However, ITS1-5.8S could detect the genetic variation of *Bertiella* spp. in different continents.

Recommendation

The sample size should be increased from different areas to perform elaborate studies on the genetic variation of the *Bertiella* spp. Samples from humans and monkeys should be included in the study to understand the zoonotic importance of the parasite.
Sequencing and phylogenetic analysis may be performed using more samples to better understand the genetic diversity of the parasite.

Conflicts of Interests

The authors declare that they have no conflicts of interest in relation to this article.

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Author contribution

P.F.S. and C.K.D. wrote the main manuscript text, A.V. and K.G.A.K. prepared figures 1 – 5, A.J. and A.I. prepared tables 1 – 2, A.R. and G.C. analysed the data and R.R. conceived the study. All authors reviewed the manuscript.

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