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Molecular characterization of veterinary important trematode and cestode species in the mithun *Bos frontalis* from north-east India

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Abstract

Helminth infections in the mithun *Bos frontalis*, including the liver fluke *Fasciola gigantica*, hepato-gastric amphistomes *Explanatum explanatum*, *Paramphistomum epiclitum* and *Calicophoron calicophorum*, and the cestodes *Echinococcus granulosus* and *E. ortleppi* were studied in north-east India over a 2-year period from 2012 to 2014. Cystic echinococcosis caused by *E. granulosus* and *E. ortleppi* was found to be highly prevalent in the mithun, with *E. ortleppi* being reported for the first time. Molecular markers, including the internal transcribed spacer 2 (ITS-2), 28S rDNA and mitochondrial NADH dehydrogenase sub-unit1 (*nad*1) were used to confirm the identification of the trematode and cestode species.

Introduction

The rare bovine species known as the mithun *Bos frontalis* is part of a rich biodiversity in the forests of north-east India and in parts of Bhutan, Myanmar, China and Bangladesh (fig. 1). The mithun is generally found at an altitude of 1000–3000 m above mean sea level and is well adapted to this environment (Chaurasia *et al.*, 2010). It plays an important role in the socio-economic and cultural life of the tribal population of this region, being used primarily as a beef animal. The mithun is raised under free-range conditions in its natural habitat and, as a result, no systematic methodology is adopted for the field diagnosis of the sub-clinical parasitic infections in the animal. Furthermore, the aetiological agents of the parasitic diseases have not been adequately recorded. No systematic studies on the epidemiology, genetic characterization and diagnosis of the helminths affecting the mithun have been conducted. Sporadic studies done on the prevalence of different parasites could not lead to the formulation of strategic control measures on parasitic diseases of this rare species. Also, identification of the helminths has been based on conventional methods of morphometry, and characterization based on molecular tools like ribosomal DNA, internal transcribed spacers and mitochondrial genes, providing reliable genetic markers for differentiation of the parasite species/strains, has not been done. The present study focuses on the occurrence and identification of amphistomes, liver flukes and cystic echinococcosis prevalent in the mithun, using 28S ribosomal DNA, internal transcribed spacer 2 (ITS-2) and mitochondrial *nad*1 as molecular markers.

Materials and methods

Collection and examination of helminths

Post-mortem examinations were conducted on 90 mithun slaughtered at various tribal rituals and ceremonies in Arunachal Pradesh and Nagaland, where infections with the trematodes *Fasciola gigantica*, *Paramphistomum epiclitum, Calicophoron calicophorum* and *Explanatum explanatum* and cestodes *Echinococcus granulosus* and *E. ortleppi* were recorded. The majority of these

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Fig. 1. The distribution of the mithun *Bos frontalis* (shaded area) in the north-east region of India.

helminths were fixed in 70% ethanol, washed in distilled water, stained with borax-carmine (SD Fine Chemicals, Mumbai, India), dehydrated in an ethanol series and mounted in DPX (SD Fine Chemicals). In addition, identification to species level was compared by sequencing with molecular markers.

Molecular and phylogenetic analyses

ITS-2, 28S rDNA and mitochondrial *nad*1 sequences were used as markers for the species identification of *Fasciola* flukes and for studying the polymorphisms in these sequences. Up to 22 *F. gigantica* were recovered from the mithun from Itanagar and Doimukh areas of Arunachal Pradesh and were used in the characterization of the parasite. The flukes were washed with normal saline, preserved in 70% ethanol and stored at −20°C until DNA extraction. Genomic DNA was extracted from the individual worms using a commercial DNA extraction kit (QIAGEN GmbH, Germany) and polymerase chain reaction (PCR) amplification of the ITS-2 region was carried out. Primers were designed on 5.8S and 28S rDNA sequences flanking the ITS-2 region to amplify a fragment of 550 bp (Marcilla *et al.*, 2002) (table 1).

Likewise, the partial sequences of 28S rDNA corresponding to the 16–633 bp (618 bp) region and 343 bp of the *nad*1 gene were PCR amplified using forward and reverse primers (accession nos AJ440785 and LC012910; table 1). The PCR products were sequenced and Basic Local Alignment Search Tool (BLAST), NCBI (National Center for Biotechnology Information, USA) was used to confirm the identity of the sequences. The phylogenetic relationship of the *nad*1 sequence of the *F. gigantica* flukes was carried out by neighbour-joining tree in MEGA 6.0 (http://www.megasoftware.net), with GenBank accession numbers given for each *F. gigantica* haplotype, where available. Bootstrap analysis of 1000 replicates was applied and values given at relevant nodes of the constructed tree.

Genomic DNA of 14 *P. epiclitum*, 14 *C. calicophorum* and 17 *E. explanatum* adult flukes was extracted using a commercial DNA isolation kit (Qiagen). The ITS-2 sequences of *P. epiclitum* (290 bp), *C. calicophorum* (480 bp) and *E. explanatum* (442 bp) were PCR amplified with species-specific primers (table 1). These PCR products were sequenced and analysed using BLAST.

Thirty-seven hydatid cysts were recovered over a period of 1 year from the slaughtered mithun in Arunachal Pradesh. The DNA was extracted from the germinal membrane of ten hydatid cysts using a commercial DNA isolation kit (QIAGEN) and a partial sequence of 377 bp of the *nad*1 gene was PCR amplified from these cysts. The PCR amplicons were purified, sequenced and analysed using BLAST. The phylogenetic analysis was done to compare these sequences with known strains/species of *Echinococcus* circulating globally. The phylogenetic tree was constructed using a neighbour-joining tree in MEGA 6.0, with GenBank accession numbers and the genotype given for each *Echinococcus* isolate when available. Bootstrap analysis of 1000 replicates was applied and values given at relevant nodes of the constructed tree.

Table 1. Primers used for PCR amplification of marker sequences of *F. gigantica*, hydatid and three amphistomes of mithun.

Parasite species	Primer name	Annealing temp. (°C)	Primer sequence
F. gigantica	ITS-2 forward and reverse	55	F-5′-GGTGGATCACTGGGCTCGTG-3′ R-5′-TATGCTTAAATTCAGCGGGT-3′
F. gigantica	28S-rDNA forward and reverse	53	F-5′-ACGTGATTACCCGCTGAACT-3′ R-5′-CTGAGAAAGTGCACTGGCAAG-3′
F. gigantica	Nad1 forward and reverse	55	F-5′-TGTTTTTGAAATTTCTTTATTTG-3′ R-5′-CACATACCACCAAAC CCCCTAA-3′
P. epiclitum	ITS-2 forward and reverse	55	F-5′-CTTATAAACTATCACGACGCCCA-3′ R-5′-TAGAACACCACAGTAGGTGATCGTC-3′
E. explanatum	ITS-2 forward and reverse	55	F-5′-TGTGTCGATGAAGAGCGCAG-3′ R-5′-TGGTTAGTTTCT TTTCCTCCGC-3′
C. calicophorum	ITS-2 forward and reverse	55	F-5′-TGTGTCGATGAAGAGCGCAG-3′ R-5′-TGGTTAGTTTCTTTTCCTCCGC-3′.
Echinococcus spp.	Nad1 forward and reverse	50	F-5′-TGAAGTTAGTAATTAAGTTTAA-3′ R-5′-AATCAAATGGAGTACGATTA-3′

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Results and discussion

Of 90 mithun examined, the prevalences of infection ranged from 16.6% for cystic echinococcosis, with all cysts sterile; to 7.8% for *F. gigantica*; 8.8% for *P. epiclitum*; 4.4% for *E. explanatum* and 2.2% for *C. calicophorum*. Intensities of infection with *F. gigantica* ranged from 10 to 300 flukes, where some of the livers showed signs of traumatic hepatitis and fibrosis. Intensities of infection with amphistomes ranged from 34 to 107 flukes per animal.

Twenty-two *Fasciola* flukes (mithun isolate) were PCR amplified for the ITS-2 region and the sequence results confirmed the species as *F. gigantica.* The sequence differed from *F. hepatica* ITS-2 at six nucleotide positions: 207, 231, 270, 276, 327 and 334, with one distinguishing deletion of a T nucleotide occurring at position 327. The substitution of T → C at positions 207 and 231, C → T at 270 and 276, and G → A at position 334 clearly differentiated the species as *F. gigantica*. None of the specimens sequenced for ITS-2 matched with *F. hepatica*. However, some of the ITS-2 sequences from these flukes showed several nucleotide variations differing from the typical six nucleotide substitutions used for distinguishing between *F. gigantica* and *F. hepatica.* A partial sequence of 618 bp of 28S rDNA was also generated from these adult flukes, which showed a 100% match with 28S rDNA of *F. gigantica*. Each sequence revealed a consistent single nucleotide polymorphism (SNP) at position 284, with a transition of G → A in all the specimens sequenced.

A partial sequence of 343 bp of the *nad*1 gene of 12 *F. gigantica* flukes was generated. Of these 12 flukes sequenced, 10 showed a nucleotide sequence identical to the haplotypes ND1-E1 and ND1-E3 previously reported from *Fasciola* flukes in north-east India (Hayashi *et al.*, 2015) and neighbouring countries (Peng *et al.,* 2009; Ichikawa *et al.,* 2010, 2011; Mohanta *et al.*, 2014). However, the *nad*1 nucleotide sequence of the remaining two flukes showed many substitutions differing from the others. The relationship of these flukes is depicted in the phylogenetic tree, where the two flukes are placed distantly from other published haplotypes of the parasite (fig. 2). The frequency of other *Fasciola nad*1 haplotypes from the mithun could not be determined due to the small number of specimens being sequenced.

ITS-2 regions of three trematode species, *E. explanatum* (442 bp), *P. epiclitum* (290 bp) and *C. calicophorum* (480 bp), were amplified by PCR (fig. 3). The sequence analysis of ITS-2 regions of 17 *E. explanatum* and 14 *P. epiclitum*, respectively, showed no nucleotide changes in the sequences compared to the isolates reported from domestic ruminants in north-east India (accession numbers JX678258, JX678260 and JX678261). Likewise, sequence analysis of the ITS-2 region of 14 *C. calicophorum* showed 100% identity with isolates from domestic ruminants from north-east India (accession no. GU133057). These data indicated that ITS-2 is a suitable marker for identification of the above amphistome species and also confirm that amphistomes isolated from the host mithun were identical to the isolates of these parasites infecting cattle and buffaloes.

All the 37 hydatid cysts recovered from the mithun were sterile and DNA isolated from the germinal membrane of ten cysts was PCR amplified for the *nad*1 gene fragment. BLAST alignment of the 377-bp partial

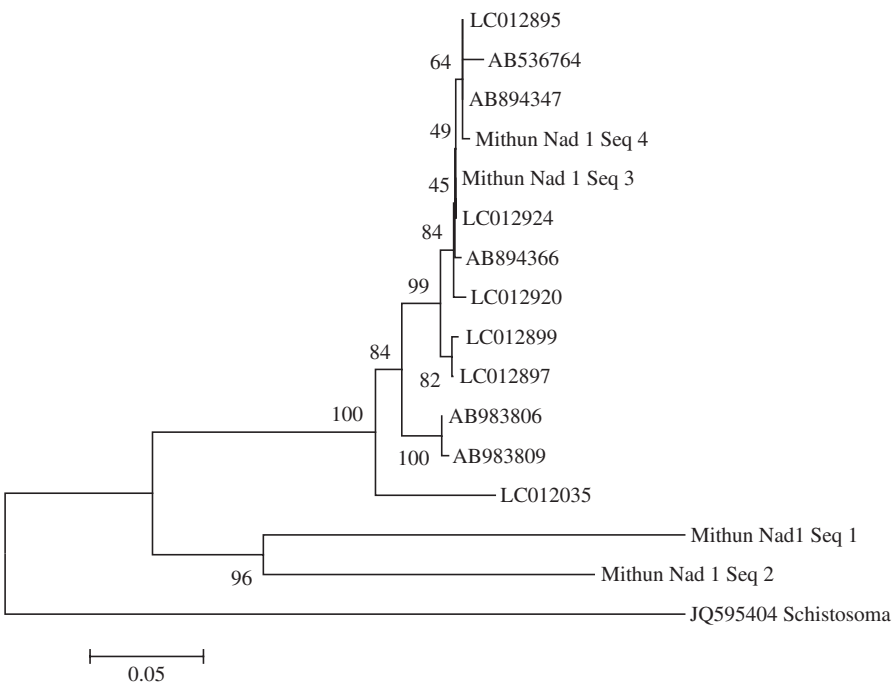

Fig. 2. The phylogenetic tree of the partial DNA sequences of *nad*1 from *F. gigantica* in mithun and *F. gigantica nad*1 sequences submitted to GenBank. The neighbour-joining tree was constructed using a p-distance model with bootstrap analysis of 1000 replicates.

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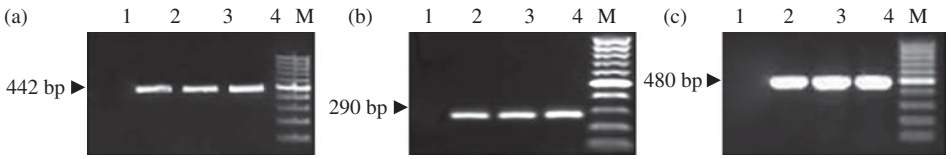

Fig. 3. PCR amplification of ITS-2 regions of the trematode species (a) *Explanatum explanatum* (442 bp); (b) *Paramphistomum epiclitum* (290 bp); and (c) *Calicophoron calicophorum* (480 bp). Lanes: M, 100-bp DNA marker; 2–4, ITS-2 sequences of each species; 1, negative controls.

sequence of the *nad*1 gene showed 100% identity with *E. granulosus* in 8 out of 10 *Echinococcus* isolates. The other two isolates showed 99% identity with *E. ortleppi*. The phylogenetic relationship of these ten *Echinococcus* isolates showed that eight (80%) *Echinococcus* isolates from the mithun clustered with *E. granulosus* (genotypes G1–3) with strong bootstrap (1000) replicates. However, the other two isolates (20%) clustered with *E. ortleppi* (genotype 5) (fig. 4). *Nad*1 gene sequences of *E. granulosus* and *E. ortleppi* (mithun isolates) were submitted to GenBank with accession numbers KM884826 and KM884827.

Parasitic gastroenteritis is one of the major causes of pathogenic conditions in the mithun directly or indirectly associated with their morbidity and mortality. Helminths are the causative agents of immature amphistomosis, calfhood mortality and production losses in these animals (Chakraborty *et al.*, 2001; Rajkhowa *et al.*, 2003). Fasciolosis caused by *F. gigantica* has been reported previously in the mithun (Chamuah, 2005) but studies on the molecular characterization of the fluke were not carried out earlier. The ITS-2, 28S rDNA and *nad*1 sequences have been shown as the specific markers for differentiating *F. gigantica* from *F. hepatica* (Marcilla *et al.*, 2002; Huang *et al.*, 2004; Itagaki *et al.*, 2005; Ichikawa *et al.*, 2011; Raina *et al.*, 2015). Using these markers in the present study, the liver fluke prevalent in the mithun was confirmed as *F. gigantica*. The partial sequence of *nad*1 of ten *F. gigantica* flukes was identical to the haplotypes ND1-E1 and ND1-E3 previously reported from *Fasciola* flukes in north-east India (Hayashi *et al.*, 2015). However, the other two flukes differed from known haplotypes. The frequency of other haplotypes prevalent in the mithun could not be determined in the present study due to the small number of specimens sequenced. Identification of the

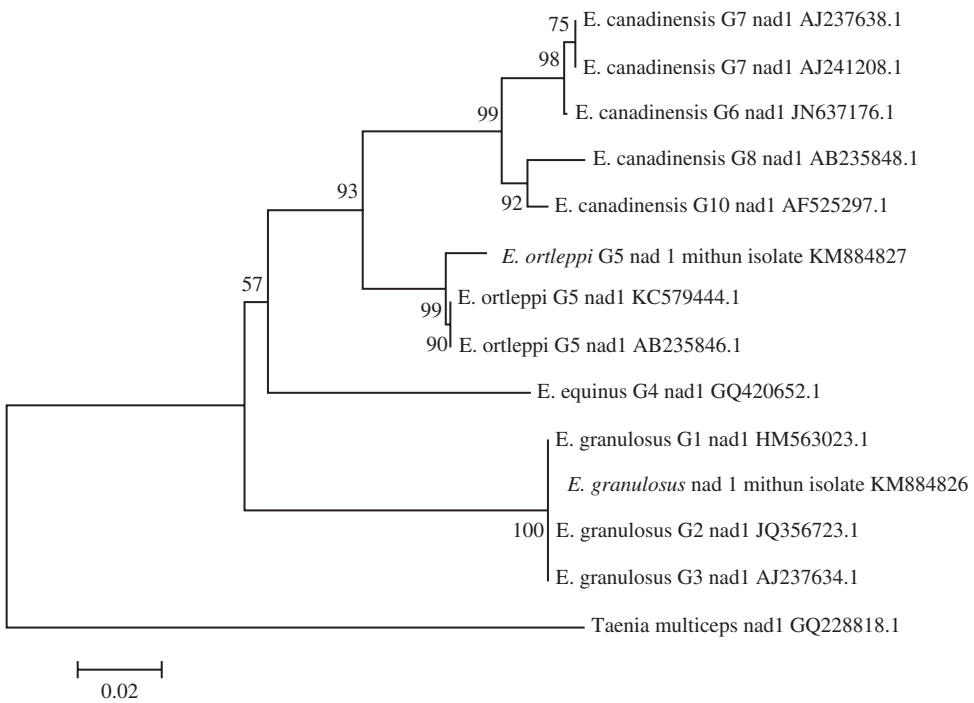

Fig. 4. The phylogenetic tree of the partial DNA sequences of *nad*1 from *Echinococcus* species in mithun and *nad*1 sequences of *Echinococcus* species submitted to GenBank. The neighbour-joining tree was constructed using a p-distance model with bootstrap analysis of 1000 replicates.

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Fasciola species prevalent in different hosts in this country, using molecular tools, has gained relevance because of the prevalence of *F. hepatica* reported in sheep in the north-western Himalayan region (Sharma *et al.*, 1989) and *F. gigantica* in cattle and buffaloes in the north-eastern region of the country (Prasad *et al.*, 2009, 2011; Hayashi *et al.*, 2015). The geographical co-existence, and even the presence of hybrid forms that have both nuclear and mitochondrial sequences of these two species, have been described in several countries (Huang *et al.*, 2004; Itagaki *et al.*, 2005; Peng *et al.*, 2009) but not in India. Therefore, molecular characterization of the *Fasciola* species prevalent in the different hosts in this country is required.

Hydatidosis has been reported previously in the mithun, affecting a large animal population (Chamuah, 2005). In the present study about 16.6% of mithun were found to be infected. The *nad*1 sequence of ten isolates confirmed that both *E. ortleppi* and *E. granulosus* are prevalent in the mithun but that *E. granulosus* is the predominant species. The same results have been reported previously, with *E. granulosus* being the predominant species infecting domestic ruminants in the plains of India (Bhattacharya *et al.*, 2007; Pednekar *et al.*, 2009; Sharma *et al.*, 2013). Nevertheless, the present study also indicated that *E. ortleppi* should be considered equally as an infectious form of *Echinococcus* in the mithun. This result provides evidence for the circulation of *E. ortleppi* in this host for the first time. Therefore, *E. ortleppi* should be considered during epidemiological surveys for this important parasitic infection in domestic ruminants and mithun. As more sequencing data become available, our understanding of the epidemiology and transmission pattern of *Echinococcus* strains/species between the mithun, domestic ruminants, humans and canids in this region will be enhanced. There is the possibility of maintaining a sylvatic cycle, with wild carnivores such as the fox, jackal and wolf acting as reservoirs of infection in this region.

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Conflict of interest

None.

References

Bhattacharya, D., Bera, A.K., Bera, B.C., Maity, A. & Das, S.K. (2007) Genotypic characterization of Indian cattle, buffalo and sheep isolates of *Echinococcus granulosus*. *Veterinary Parasitology* **143**, 371–374.

Chakraborty, A. (2001) Pathology of *Fasciola gigantica* infection in captive wild herbivores with special reference to SEM and EDAX study. *Indian Veterinary Journal* **78**, 43–47.

Chamuah, J.K. (2005) Studies on some aspects of parasites of mithun (*Bos frontalis*). M.V.Sc. thesis submitted to Assam Agricultural University, Khanapara, Guwahati, Assam, India.

Chaurasia, R.K., Mech, A., Dhali, A., Vidyarthi, V.K., Rajkhowa, C. & Sharma, V.B. (2010) Effect of altitude and season on rectal temperature, pulse rate and respiration rate in mithun (*Bos frontalis*). *Indian Journal of Hill Farming* **23**, 25–29.

Hayashi, K., Ichikawa-Seki, M., Mohanta, U.K., Singh, T.S., Hiromu, T.S. & Itagaki, S.T. (2015) Molecular phylogenetic analysis of *Fasciola* flukes from eastern India. *Parasitology International* **64**, 334–338.

Huang, W.Y., He, B., Wang, C.R. & Zhu, X.Q. (2004) Characterization of *Fasciola* species from Mainland China by ITS-2 ribosomal DNA sequence. *Veterinary Parasitology* **120**, 75–83.

Ichikawa, M., Iwata, N. & Itagaki, T. (2010) DNA types of aspermic *Fasciola* species in Japan. *Journal of Veterinary Medical Science* **72**, 1371–1374.

Ichikawa, M., Bawn, S., Maw, N.N., Htun, L.L., Thein, M., Gyi, A., Sunn, K., Katakura, K. & Itagaki, T. (2011) Characterization of *Fasciola* spp. in Myanmar on the basis of spermatogenesis status and nuclear and mitochondrial DNA markers. *Parasitology International* **60**, 474–479.

Itagaki, T., Kikawa, M., Terasaki, K., Shibahara, T. & Fukuda, K. (2005) Molecular characterization of parthenogenic *Fasciola* species in Korea on the basis of DNA sequences of ribosomal ITS1 and mitochondrial NDI gene. *Journal of Veterinary Medical Science* **67**, 1115–1118.

Marcilla, A., Bargues, M.D. & Mas-Coma, S.A. (2002) PCR-RFLP assay for the distinction between *Fasciola hepatica* and *Fasciola gigantica*. *Molecular and Cellular Probes* **16**, 327–333.

Mohanta, U.K., Ichikawa-Seki, M., Shoriki, T., Katakura, K. & Itagaki, T. (2014) Characteristics and molecular phylogeny of *Fasciola* flukes from Bangladesh, determined based on spermatogenesis and nuclear and mitochondrial DNA analysis. *Parasitology Research* **113**, 2493–2501.

Pednekar, R.P., Gatne, M.L., Thompson, R.C. & Traub, R.J. (2009) Molecular and morphological characterisation of *Echinococcus* from food producing animals in India. *Veterinary Parasitology* **165**, 58–65.

Peng, M., Ichinomiya, M., Ohtori, M., Ichikawa, M., Shibahara, T. & Itagaki, T. (2009) Molecular characterization of *Fasciola hepatica*, *Fasciola gigantica* and aspermic *Fasciola* sp. in China based on nuclear and mitochondrial DNA. *Parasitology Research* **105**, 809–815.

Prasad, P.K., Tandon, V., Biswal, D.K., Goswami, L.M. & Chatterjee, A. (2009) Use of sequence motifs as barcodes and secondary structures of internal transcribed spacer 2 (ITS2, rDNA) for identification of the Indian liver fluke, *Fasciola* (Trematoda: Fasciolidae). *Bioinformation* **3**, 314–320.

Prasad, P.K., Goswami, L.M., Tandon, V. & Chatterjee, A. (2011) PCR-based molecular characterization and *in-silico* analysis of food-borne trematode parasites *Paragonimus westermani*, *Fasciolopsis buski* and *Fasciola gigantica* from north-east India using ITS2 rDNA. *Bioinformation* **6**, 64–68.

Raina, O.K., Jacob, S.S., Sankar, M., Bhattacharya, D., Bandyopadyay, S., Varghese, A., Chamuah, J.K. & Lalrinkima, H. (2015) Genetic characterization of *Fasciola gigantica* from different geographical regions of India by ribosomal DNA markers. *Journal of Parasitic Diseases* **39**, 27–32.

Rajkhowa, S., Bujarbaruah, K.M., Rajkhowa, C. & Thong, K. (2003) Haemato-biochemical changes in mithun with gastrointestinal nematodiasis. *Indian Veterinary Journal* **80**, 1064–1066.

Sharma, M., Fomda, B.A., Mazta, S., Sehgal, R., Singh, B.B. & Malla, N. (2013) Genetic diversity and population genetic structure analysis of *Echinococcus granulosus* sensu stricto complex based on mitochondrial DNA signature. *PLOS One* **8**, e82904.

Sharma, R.L., Dhar, D.N. & Raina, O.K. (1989) Studies on the prevalence and laboratory transmission of fascioliasis in animals in the Kashmir valley. *British Veterinary Journal* **145**, 57–61.