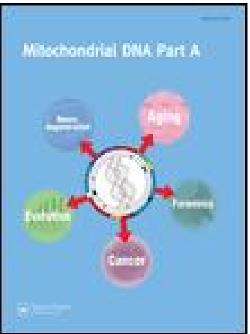

Mitochondrial DNA Part A

DNA Mapping, Sequencing, and Analysis

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RESEARCH ARTICLE

Genetic assessment of leech species from yak (*Bos grunniens*) in the tract of Northeast India

Nilkantha Chatterjee[a*], Bishal Dhar[a*], Debasis Bhattarcharya[b], Sourabh Deori[b], Juwar Doley[b], Joken Bam[b], Pranab J. Das[b], Asit K. Bera[b], Sitangshu M. Deb[b], Ningthoujam Neelima Devi[a], Rajesh Paul[a], Sorokhaibam Malvika[a] and Sankar Kumar Ghosh[a]

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ABSTRACT
Yak is an iconic symbol of Tibet and high altitudes of Northeast India. It is highly cherished for milk, meat, and skin. However, yaks suffer drastic change in milk production, weight loss, etc, when infested by parasites. Among them, infestation by leeches is a serious problem in the Himalayan belt of Northeast India. The parasite feeds on blood externally or from body orifices, like nasopharynx, oral, rectum, etc. But there has been limited data about the leech species infesting the yak in that region because of the difficulties in morphological identification due to plasticity of the body, changes in shape, and surface structure and thus, warrants for the molecular characterization of leech. In anticipation, this study would be influential in proper identification of leech species infesting yak track and also helpful in inventorying of leech species in Northeast India. Here, we investigated, through combined approach of molecular markers and morphological parameters for the identification of leech species infesting yak. The DNA sequences of *COI* barcode fragment, *18S* and *28S rDNA,* were analyzed for species identification. The generated sequences were subjected to similarity match in global database and analyzed further through Neighbour-Joining, K2P distance based as well as ML approach. Among the three markers, only *COI* was successful in delineating species whereas the *18S* and *28S* failed to delineate the species. Our study confirmed the presence of the species from genus *Hirudinaria, Haemadipsa*, *Whitmania,* and one species *Myxobdella annandalae,* which has not been previously reported from this region.

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COI; Neighbour-Joining (NJ); K2P distance; 18 and 28S; leech; yak

Introduction

Leeches are segmented worms under Phylum Annelida. The distinguishing properties of leeches from other members of Annelids are the existence of anterior and posterior suckers, invariable number of body segments, and body cavity largely filled with muscles and connective tissue (Smyth & Wakelin 1994). These leeches are commonly found to occur in terrestrial habitats, lakes, ponds, springs, small streams, and pools of water. Leeches nourish on blood of various animals to which they connect themselves and drop off after having engorged (Soulsby 1982; Boden 1998). The parasites get into the mouth when the animals drink contaminated water or feed on grass and plant parts contaminated with leech, and attaches to oral and laryngeal mucus membrane by means of their terminal sucker and feed on blood and may cause severe anaemia in the host (Rajaei et al. 2014). Hirudiniasis is also reported in human, a good number people figure out that they are parasitized by a leech in the lead finding the worm attached to their body parts. Occasionally, the organism enter human orifices – a condition known as mucosal, orifical, vesical, or internal hirudiniasis depending on the localization of the leech. While most leeches feed as ectoparasites for short periods of time, some of them that feed on mucous membranes have been known to stay in an orifice for days or weeks on end (Harding 1927; Phillips et al. 2010). Cases of invasive leeches are also reported where the organism found to thrive in the nasopharyngeal region, urethra, vagina, or rectum (Almallah 1968), depending upon the actual site of the bite, the symptoms may include haemorrhaging, haemoptysis, dysphonia, coughing, or severe anaemia (Turner 1969). Internal haemorrhage from leeches bite in the oral, nasopharynge, urethra, or in the urinary bladder, also poses a serious problem in which, clot formation is inhibited by urine flow (Alam et al. 2008; Phillips et al. 2010). These conditions, may lead to secondary bacterial infections that can be life threatening very rapidly (Cundall et al. 1986; Kose et al. 2008).

Like human, domestic, and wild animals mainly the livestock like cow, buffalo, sheep, etc. are also at the great risk for orificial hirudiniasis in relation to the amount of time such animals spend at leech-inhabited lands, water bodies or wetlands (Harding 1927). Leeches from Himadipsidae often attaches to the animal on the skin during grazing and causes

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heavy bleeding, which is difficult to heal, while leeches from Praobdellidae are more likely to afflict animals with respiratory problems by causing bleeding in nasal tract, difficulties in swallowing (Oral infestation) as different from other leeches, anaemia by excessive haemorrhage (Lai et al. 2011; Bahmani et al. 2014).

The present study focuses on the leech infestation of yaks in the Himalayan belt of Northeastern India. Yak is a paradigmatic symbol of Tibet and high altitudes of Arunachal Pradesh (part of Himalayan belt of Northeastern India). This long-haired bovid found throughout the Himalayan region of South Central Asia, the Tibetan Plateau Mongolia, and Russia. Yak have many anatomical and physiological traits that enable them live at high altitude, including high metabolism, acute senses, impressive foraging ability, enlarged hearts and lungs, and a lack of blood vessel constriction in the lungs when faced with relatively low oxygen conditions. There are a number of different phenotypic categories among Indian yak. The 'common' yak resemble medium size hill cattle in conformation; 'Bisonian' yak are bigger animals; 'Bare-back' yak have a long body, and little hair on their backs (Pal et al. 1994). Almost everything from the yak is used for sustainability of highlanders of yak tracts and is highly valued for its high quality milk, meat, skin, etc and thus this animal has become the only source of income for the upland people.

However, yaks suffer drastic change in milk production, weight loss, etc. when infested by parasites. Among them, infestations by leeches are a serious problem in this region and have gained a fearsome reputation for feeding externally on blood, often from yaks. Morphological identification of leech faced with impediments because of plasticity of the body and changes in shape and surface structure. Moreover, exclusive documentation of leech diversity from the yak tract of north-eastern region of India has been lagging behind due to the lack of quantitative data and shortage of taxonomical intervention. Hence, there is ample need of inventorying leech species inhabiting this region and in this scenario molecular characterization employing genetic sequence assessment of leech species would be helpful to achieve this goal. In recent years, DNA Barcode technology proved to be effective molecular tools for species identification (Hebert et al. 2003; Hajibabaei, Singer, Clare, et al. 2007; Hajibabaei, Singer, Hebert, et al. 2007) even in case of any uncertainty in identification of the specimens and studying biodiversity, like Fish (April et al. 2011), Diptera (Pramual et al. 2011), Lepidoptera (Hausmann et al. 2011), Mirids (Rebijith et al. 2012), Butterfly (Hebert et al. 2004), Coral reef fish (Hubert et al. 2010), Medicinal plants (Chen et al. 2010), spruce budworm food web (Smith et al. 2011), Parasitoid flies (Diptera) (Smith et al. 2006), leeches (Sket & Trontelj 2008; Phillips et al. 2010; Tubtimon et al. 2014).

Here, we employed the combined approach to identify the leeches collected from yak (*Bos grunniens*) in different parts of the breeding tracts from Arunachal Pradesh of North-eastern region of India. The morpho-taxonomy and species-specific DNA sequences of Mitochondrial Cytochrome c oxidase subunit 1 (*COI*) barcode sequence and nuclear genes of *18S* and *28S* rDNA are analyzed with basic bioinformatics tools. Due to the plasticity in body structure and shape of leeches, it is very perplexing to identify them based on morphology. In anticipation, this study would be helpful in inventorying leech species and subsequently assessment of the presence of new or overlook species infested yak of this region.

Materials and methods

Sample collection

The leech specimens from yak tract of Northeast India were collected from different localities within the regions. A total of eight representative specimens of the commonly occurring leeches were collected from the grazing field as well as from the body of the host, near the nose, while some were collected near the facial skin. However, one specimen was found to be attached on the hoof of the animal. Isolated organisms were washed three times in phosphate buffer saline (PBS) (pH 7.2), morphologically characterized, and finally preserved in 70% alcohol (v/v) till further use. The details of the sample specimens with voucher IDs and geographic location are given in the Table 1.

Isolation of genomic DNA and amplification of COI, 18S, and 28S rDNA

The Genomic DNA was extracted from the tissue samples collected aseptically from each of the specimens in TES buffer (50 mM Tris-HCl, 25 mM EDTA, and 150 mM NaCl) and DNA was extracted by Phenol-Chloroform-Isoamyl alcohol method (Sambrook & Russell 2006). To amplify specific gene, published primers were used (Phillips et al. 2010) (Supplementary Table S1). The amplification reaction was carried out in 20 µl containing DNA template, 10 mM each dNTPs mix, 20 pmol of each primer (both Forward and Reverse) (Sigma Aldrich) and 2 µL 10× PCR buffer supplied by the manufacturer along with 1 unit of *Hifidelity* DNA polymerase (Thermo Fisher Scientific, Waltham, MA).

For amplification of *COI* and *18S,* PCR reaction comprised of 35 cycles that comprised of denaturation at 94 °C for 1 min, annealing at 49 °C for 30 s and elongation was done at 68 °C for 2 min followed by final extension at 68 °C for 5 min. On the contrary, amplification of *28S* was done by initial denaturation at 94 °C for 1 min followed by 35cycles, which comprised of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, and elongation was done at 68 °C for 2 min. Final elongation was done for 68 °C for 5 min.

Sequencing of PCR amplicons

The amplified products were purified and sequenced bi-directionally using automated DNA Sequencer (ABI 3500 Genetic Analyzer; Applied Biosystem Inc., Foster City, CA). The sequencing reaction was performed using 1 µl of BDTv3.1 (Applied Biosystems, Foster City, CA), 1.5 µl of 5× Sequencing buffer; a final concentration of 0.5 pmol of each of the primers was maintained in separated reaction, template DNA 70 ng/µl. The Chain termination reaction was carried out as per manufacturer instructions and re-suspended in Hi-Di formamide prior to load in the capillary.

Table 1. Leech samples collected from different region of yak tract of Northeast India along with their voucher ID, Genbank Accession number and geographical location (GPS Coordinates).

Voucher ID	Gene	Genbank accession no.	Identified as	Latitude/Longitude
SGNC-HM1	*18S*	KU646498	*Hirudinaria manillensis*	27.20 N 92.24 E
	28S	KU646505		
	CO1	KT693106		
SGNC-HM2	*18S*	KU646499	*Hirudinaria manillensis*	27.20 N 92.24 E
	28S	KU646506		
	CO1	KT693107		
SGNC-Hg1	*CO1*	KT693108	*Hirudinaria manillensis*	27.20 N 92.24 E
SGNC-HMO1	*18S*	KU646500	*Haemadipsa montana*	27.30 N 92.65 E
	28S	KU646507		
	CO1	KT693109		
SGNC-HJ1(D3f)	*18S*	KU646501	*Myxobdella annandalei*	27.21 N 92.14 E
	28S	KU646508		
	CO1	KT693110		
SGNC-HJ2 (D4f)	*18S*	KU646502	*Myxobdella annandalei*	27.21 N 92.14 E
	28S	KU646509		
	CO1	KT693111		
SGNC-Wl1	*18S*	KU646503	*Whitmania laevis*	27.25 N 92.72 E
	28S	KU646510		
	CO1	KT693112		
SGNC-Wl2	*18S*	KU646504	*Whitmania laevis*	27.30 N 93.49 E
	28S	KU646511		
	CO1	KT693113		

Bioinformatics analysis

The generated sequence chromatograms were checked by Sequence Scanner v1 and Seq Scape v2.7 (Applied Biosystems, Foster City, CA) and further analyzed by BLASTN (Altschul et al. 1990) search tool at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast) to check percentage similarity of the developed sequences with the database sequences. Comparison of the developed sequences with the database *COI* barcode sequences without any indels and coherent amino acid codes with a partial fragment of mitochondrial *COI* gene confirmed the sequences being correct and no NUMT being amplified (Zhang & Hewitt 1996). The sequences were also aligned by using CLUSTALX.

The identification of the specimens was mainly done by three approaches. First, the sequences were subjected to similarity match in the BOLD species identification system (BOLD-IDS, www.barcodinglife.org) (Ratnasingham & Hebert 2007) as well as GenBank database through the MEGABLAST program for identification of the samples at the species level (Bhattacharjee et al. 2012; Dhar & Ghosh 2015). In this study, the similarities in the range of 97–100% of the query sequence with E-value lower than the cutoff and considered to categorize the query sequences into their respective species. Second, conventional method of Neighbour-Joining (NJ) clustering (with 1000 bootstrap support) and distance matrices were performed using the Kimura 2-Parameter model (Hebert et al. 2003) through MEGA5.2 (Tamura et al. 2011) by taking the related sequences from the database as replicates if available. Third, morphological parameters were extensively used for some cases, which showed ambiguity in similarity approach. Finally, ML tree was constructed for further confirmation of the species and its delineation with respect to other related species.

Morphology of the specimens

The specimens which could not be resolved by similarity match approach were categorized based on the taxonomic characters that were available from the original description and subsequent re-description. For identification, the meristic counts and morphology keys are recorded as per the standard literature.

Results

DNA sequence annotation and submission

The sequencing results were obtained in the course of two chromatograms for each sample; one for the forward strand and another for the reverse strand. The software SeqScanner (Applied Biosystems) outputted the chromatograms in the shape of the original sequence. The quality for basecall of each of the sequences were checked with SeqScanner and found to be in the score of 40–50 QV in all the cases, which confirmed that the sequences being 99.99% accurate. The chromatograms were checked and found without any background noise, which was then confirmed with the reference library dataset in terms of query coverage, ORF, etc. All the analyzed sequences were deposited in GenBank through the BankIt sequence submission tool (http://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank) and received valid accession numbers (Table 1). The sequences were also submitted in BOLD following BOLD sequence submission protocol (BOLD management paper) and received valid process ID's.

Species identification by preexisting sequences in the database

The sequences after submission were subjected to analysis by similarity match approaches using preexisting sequences of the database for species level identification. The sequences of *COI*, *18SrDNA*, *28SrDNA* from the studied specimen were subjected to similarity match in the Genbank, where *COI* from the eight specimen showed little significant match (94–89%) with the available species sequences except for two

Table 2. Similarity match approach of the generated sequences of collected specimens with the database sequences of Genbank and BOLD-IDS for Species identification.

Sl NO	Sample ID	Genbank accn no.	Gene	Similarity match global data base		Closest match in NCBI
				Genbank at NCBI (%)	BOLD-IDS	
1	SGNC-HM1	KT693106	*COI*	*Hirudinaria manillensis* 94%	No records	GQ368747, GQ368748, JN412849,
2	SGNC-HM2	KT693107	*COI*	*Hirudinaria manillensis* 94%		GQ368747, GQ368748, JN412849,
3	SGNC-Hg1	KT693108	*COI*	*Hirudinaria manillensis* 94%		GQ368747, GQ368748, JN412849,
4	SGNC-HMO1	KT693109	*COI*	*Haemadipsa montana* 89%		HQ203182
5	SGNC- D3f	KT693110	*COI*	*Myxobdella annandalei* 97%		GU394014
6	SGNC-D4f	KT693111	*COI*	*Myxobdella annandalei* 98%		GU394014
7	SGNC-Wl1	KT693112	*COI*	*Whitmania laevis* 89%		KM655839
8	SGNC-Wl2	KT693113	*COI*	*Whitmania laevis* 89%		KM655839

specimens, which showed significant similarity in the range of 97–98% with reference sequences of *Myxobdella annandalei* (Table 2). But, the *18SrDNA, 28SrDNA* failed to distinguish them into respective species or any taxonomic ranks. All those sequences showed similarity in the range of 98–88% with the multiple species in the database and hence remain inconclusive.

Furthermore, the *COI* sequences were further subjected to similarity match using Barcode of life data systems (BOLD-IDS) for species identification. The DNA barcode database provides a system of species identification based upon the finding of the closest match of the query sequences with database sequences (Species Level Barcode Records as well as Public Record Barcode Database). The BOLD-IDS revealed no match with the reference datasets and thus it was inconclusive with the similarity match approach (Table 2).

Identification of the leeches with the morphological parameter

The species which cannot be confirmed from similarity match approach were further analyzed though conventional morpho-taxonomy for first hand identification of the species. Live colour of dorsal and ventral surfaces of the leeches was recorded. The species were identified as *Haemadipsa montana*, *Hirudinaria manillensis,* and *Whitmania laevis* on the basis of their colour, specific marking pattern on the dorsum, lateral bands, number, and arrangement eyes, shape, and size.

Haemadipsa montana

Body length 14–37 mm, maximum body width 2.5–5.3 mm, up to 10.5 mm in specimen filled with blood. Body elongated, slender cylindrical, with dorsal moderately depressed from the end of the body to the head, venter more or less flat in relaxed specimen. The dorsal side appears chocolate brown in colour with dark stripes (Raj & Gladstone 1981; Nesemann & Sharma 2001). Dorsally, there are six longitudinal wavy lines situated in paramedian position bordering a yellow central stripe (Supplementary Figure 1A). These lines impart a beaded appearance of the dorsal surface. The ventral surface is orange red in colour; marginally there is an orange stripe which is bounded dorsally by brown sub marginal stripe (Supplementary Figure 1A).

Hirudinaria manillensis

It is commonly known as the paddy field leech or buffalo leech (Harding & Moore 1927). These leeches are large robust in size, sometimes adults measures more than 150 mm. The colour of live leech varies from light olive-green to olive-brown. While not in extension, the dorsal surface appears to have a black coloured median line, however, on extension the line appears broken (Nesemann & Sharma 2001; Lai & H 2005; Kutschera & Roth 2006). Located at the broken end of the midline and lateral to each side of it are two small broken lines (Supplementary Figure 1B). In young leeches, the black patterns are most prominent, while with the increment age and size of the leech the makings become less prominent. The ventral surface is reddish brown in colour with broad black sub-marginal stripes. The margins have a narrow sharply defined yellow or orange stripe (Supplementary Figure 1B). This leech inhabits the rice field, ponds, swamps, sluggish streams, and springs. It attacks cattle, buffalo, and man; and occasionally it also attacks frogs, snakes, and turtles.

Whitmania laevis

Large thick-bodied leech of nearly uniform width for most of the length with abruptly tapered anterior end, head is small; mid-body is cylindrical. Eyes are five pairs, positioned in a curvilinear space or arch. Suckers are of medium size. Very sluggish in nature and measures up to 120 mm when fully extended. It is brownish olive to olive yellow in colour with five brown or blackish stripes, a median and a pair each on the paramedian fields (Nesemann & Sharma 2001; Lai & H 2005). Typically each of these five stripes bears a series of pale yellowish oval or quadrangular spots. The ventral surface is pale-brown in colour with numerous black spots arranged irregularly (Supplementary Figure 1C).

Neighbour-Joining cluster and K2P genetic distance

For the *COI* barcode sequences, the species sequences generated in this study was taken along with the database sequences of the same species. The sequences from the Genbank with which our sequences showed closest match were downloaded as barcode replicates for the study. All the sequences taken for the analysis were trimmed from both the ends to

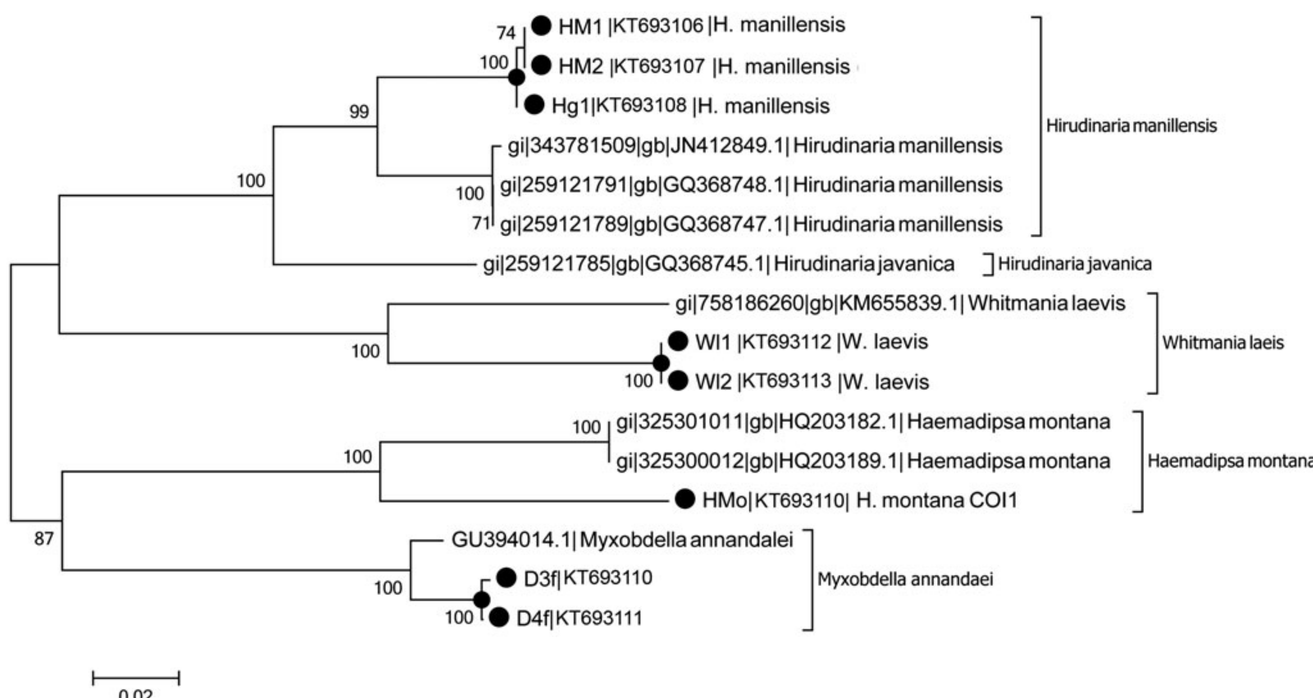

Figure 1. NJ tree based on *COI* sequences showed clear delineation of the species. The black dot in the tree represents the generated sequences while rests are from Genbank as barcode replicates.

make the database sequences and the sequences generated of consensus size. The NJ tree was created as per standard barcoding protocol using 1000 bootstrap replicates. The NJ tree showed distinct clustering of the species from one another with a strong bootstrap support. However, the generated sequences show a deep divergence from the available database sequences as shown in Figure 1. Similarly, the genetic distance based on K2P model was calculated to identify the species boundary. Within the cluster, the mean K2P distance was found to be 0.055 ± 0.008 and maximum K2P distance was 0.0802. While, between the clusters, mean K2P distance was observed as 0.231 ± 0.021 with Minimum K2P distance of 0.102. For the two species, *H. montana* and *W. laevis,* the K2P distance was quite higher than the other species which is 0.08 and 0.086, respectively.

For *18S* and *28S* sequences, the species sequences generated in this study was taken along with the database sequences of the species with which it showed similarity. The NJ tree was created by K2P model using 1000 bootstrap replicates for *18S* and *28S* individually. The NJ tree showed cohesive clustering of the multiple species with a strong bootstrap support Figure 2. Similarly, the genetic distance based on K2P model was calculated to identify the species boundary. Within the cluster, the mean K2P distance for *18S* was found to be 0.0014 ± 0.0006 with maximum distance of 0.003. While, between the clusters, mean K2P distance was observed as 0.018 ± 0.003 with minimum K2P distance of 0.001. Likewise, within the cluster the mean K2P distance for *28S* was found to be 0.003 ± 0.002 with maximum K2P distance was 0.003. While, between the clusters, mean K2P distance was observed as 0.044 ± 0.013 with minimum K2P distance of 0.0101.

Maximum likelihood approach

The Maximum Likelihood analysis was carried out by taking into account the *COI* with the best fit model, T92 + G for phylogenetic analysis through Model test in MEGA 6.2 considering lowest scores of the Bayesian Information Criterion (BIC) with and without assuming the existence of evolutionary rates among sites modelled by discrete gamma distribution (+ G) and allowance of the presence if invariant sites (+ I) as detailed in Supplementary Table S2. The *18S* and *28S rDNA* was not taken into account as it did not work well with species demarcation due to lower evolutionary rates.

The Maximum likelihood tree (ML) tree showed a distinct delineation of the species as it was in case of NJ tree using *COI*. All the congeners diverged from a common node or in other words all the species clustered distinctly and the species from genus diverged from a common ancestral node as shown in Figure 3. However, there exist deep divergences within the conspecific sequences with a well bootstrap support, which gives a signal of recently diverged or overlooked species in the taxa. However, a detailed study is still necessary with covering maximum number of species per genus.

Discussion

Yak regarded as one of the remarkable domestic animal as its products are important components of their daily diet and livelihood. Domestication of yak in particular has led to the progress, prosperity and economic advancement for the group of people in the Himalayan belt of Northeastern India because of the value of the yak as a packing animal and its product as food resources. In general, temperature is the single most important factor determining the distribution,

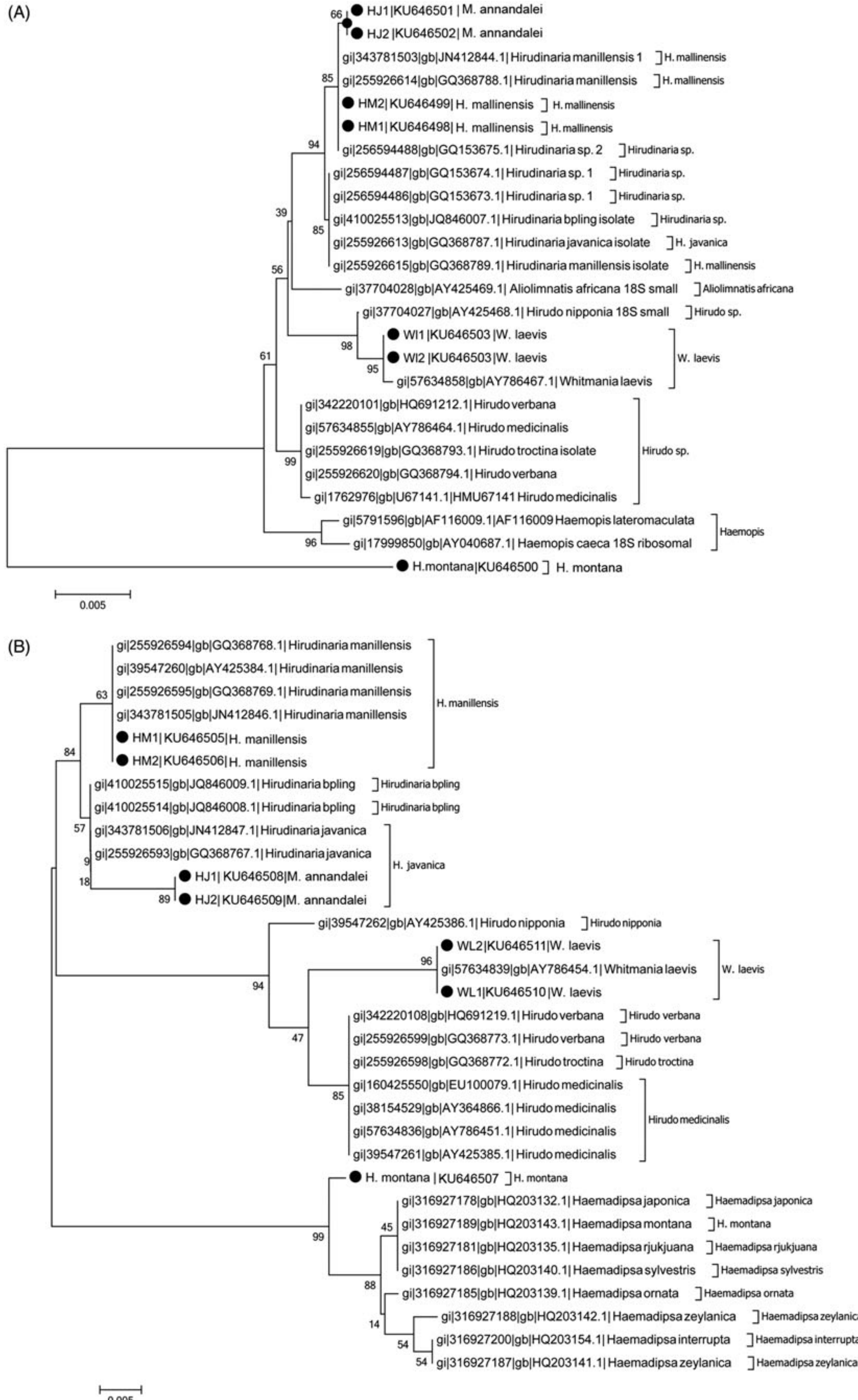

Figure 2. (A) NJ tree based on *18S* sequences of the leech specimens. (B) NJ tree based on *28S* sequences of the leech specimens. Black dots represents the generated sequences whereas rests from database as replicates.

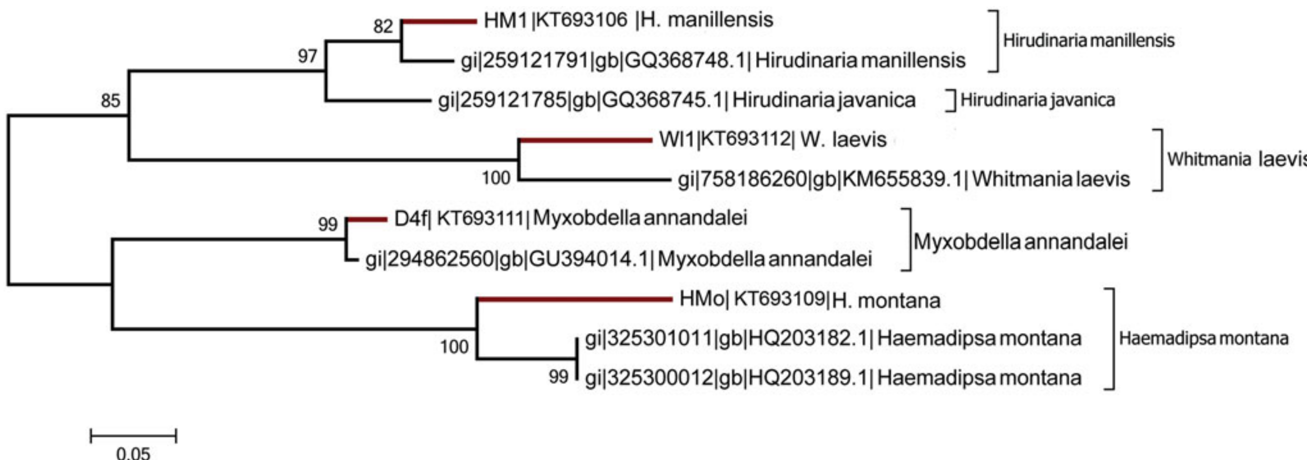

Figure 3. Maximum Likelihood (ML) tree of the leech samples using *COI* sequences. The tree was reconstructed based on Model selection following Baysian Criteria for phylogenetic reconstruction.

stocking density and, indirectly, the growth rate of yak. Yak survives and performs adequately if the annual mean temperature is below 5 °C and the average in the hottest month is not above 13 °C. In Arunachal Pradesh, particularly above 2438 m yak survives within this temperature range, distributed in high altitudes West Kameng district. Infestation of yak by leeches is a serious health problem in the migratory tracts (region or sector of land) and grazing grounds of yak. The parasites get into the mouth and attaches to oropharyngeal and laryngeal mucus membrane when the animals drink infested water or attaches to body parts when walk through the dense forest by means of powerful terminal sucker. Leeches are important predators which affect other animals like amphibians, reptiles, fishes and annelids as well. This has been already documented in the literature that leech has been used for medicinal purpose (*H. medicinalis*), which belongs to the genus *Hirudo* i.e. (Orevi et al. 1992). Since inception, the first description of *Hirudo complanata* by Linnaeus (1758) (now under the genus Glossopora), its status and morphological ranges have been disputed. The underline cause of such dispute lies with the fact that the external morphology, colour pattern, etc. appears to be variable within each morphological types. Morphological identification of leech is different because of plasticity of the body and changes in shape and surface structure of the specimen during preservation (Verovnik et al. 1999).

The need for molecular based characterization of leeches along with convention taxonomic analysis for proper identification and validation of the leech species is indispensible, where traditional taxonomy fails to identify due to morphological perplexity. For preliminary identification, leeches were collected from host as well as from the grazing field. Here in this study, we employed combined approach of molecular identification as well as conventional morpho-taxonomy to identify eight collected specimens into their respective species viz., *Hirudinaria manillensis, Haemadipsa montana, Whitmania laevis, and Myxobdella annandalei*. Among the species identified, Whitmania laevis was found to be attached with the yak hoof. There was no indication of it as parasite. It might have attached to the animal from the forest during grazing. The other specimen of *Myxobdella annandale, a known species* that was newly identified to be present in Northeast India from this study and was not reported earlier to be present in yak tracts of Northeast India.

The sequences generated from all the specimens were mitochondrial *COI* as species specific genetic marker (Hebert et al. 2003; Bely & Weisblat 2006; Lai, Nakano & Chen 2011) as well as *18S* and *28S rDNA* from nuclear origin to assess the leech diversity. However, *18S* and *28S* sequences, due to its slow evolution rate showed poor performance in distinguishing species or its diversity. It lacked the capacity to delineate species due to less variability in the sequences and mostly the sequences showed homology with multiple species under the same or different genus of leech in similarity match with preexisting sequences of the database. This was further evident in the genetic distance estimation, where there is an overlap in the genetic distance of similar species and its nearest neighbour (Figure 4). In other words, the *18S* as well as *28S* failed to estimate the boundary between the species of same or different genus and showed cohesive clustering of multiple species in NJ tree (Figure 2). To overcome this discrepancy and identify the collected species, some morphological parameters were considered to confirm our species and helped us to identify the respective species.

On the other hand, the barcode region of *COI* sequence of mtDNA showed promising results (Table 3) in delineating species as concordant with the previous reported studies (April, Mayden, Hanner & Bernatchez 2011; Dhar & Ghosh 2015). Here, also it successfully demarcates species based on genetic distance as calculated as per standard protocol of DNA barcoding as well as NJ clustering where all the species clustered distinctly (Phillips et al. 2010). The genetic distance showed high within species divergence which is also congruent with the NJ tree where there exists a deep divergence within species as evident from similarity match approach where most of the collected species showed moderate similarly with the database published sequences. Same trend was also observed in ML phylogeny using *COI* sequences where all the species clustered distinctly but there also existed deep divergence within species. This occurs due to the possibility

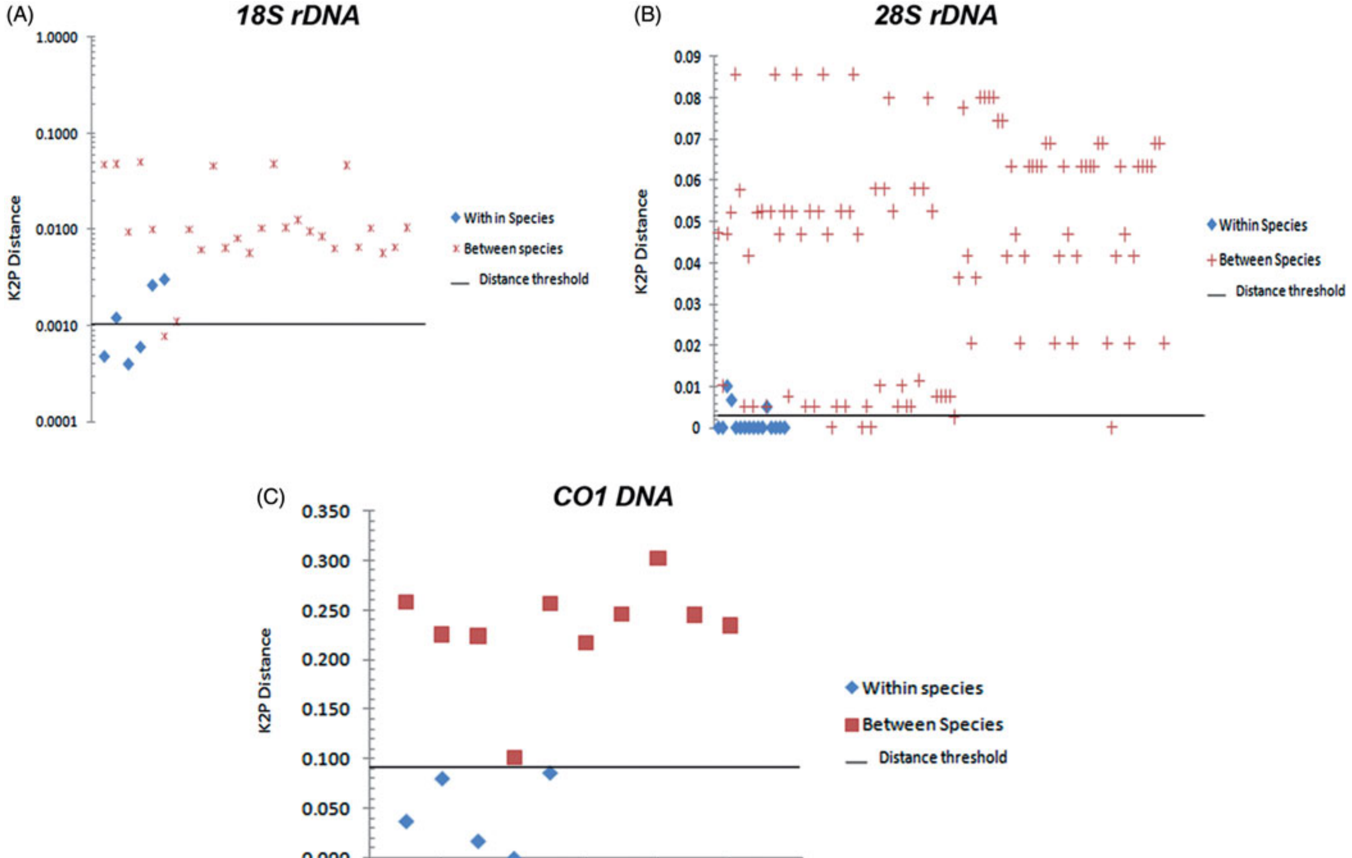

Figure 4. Scatter plot representation of the K2P genetic distance based on (A) *18S rDNA*, (B) *28S rDNA* and (C) *COI* of the leeches collected from Arunachal Pradesh. A threshold taken a minimum distance between species represented by a straight line.

Table 3. Selection of *CO1* for species level identification of leech and phylogenetic analysis.

Locus/Gene	Origin	Within species max distance	Distance with nearest neighbour	Remarks
18S rDNA	Nuclear	0.003	0.001	Failed to delineate species
28S rDNA	Nuclear	0.0101	0.003	Failed to delineate species
COI	Mitochondrial	0.0802	0.102	Successfully delineate species

of haplotype diversity because of the presence of geographically isolated species (Jamaluddin et al. 2011), as most of the sequences were from Thailand, USA, etc. Sometime such divergence may exist due to the presence of overlooked or cryptic diversity in the respective species sequences (Hebert et al. 2004; Witt et al. 2006; Chakraborty & Ghosh 2014), which is again difficult to distinguish by morphology and require immense taxonomic expertise. However, a detailed study covering the entire taxa is further required. In anticipation, this study undertaken would be significant in identification of leech species infesting yak tract in Arunachal Pradesh and also helpful in inventorying of leech species in Arunachal Pradesh.

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Disclosure statement

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