INTRODUCTION

_Nippostrongylus_ Lane, 1923, a heligmonellid genus, is commonly parasitic in the digestive tract of murines. Geographically, _Nippostrongylus_ has been known for a wide range throughout the world [1-5]. _Nippostrongylus brasiliensis_ Travassos, 1914, a gastrointestinal nematode, is a cosmopolitan parasite of a commensal mouse _Mus musculus_. Our present knowledge of identification of _N. brasiliensis_ nematode is still fragmented in India. Till date, no molecular studies have been reported in India. Recently, mitochondrial genes have been successfully employed as a molecular marker for accurate identification of nematodes [6-8]. The mitochondrial cytochrome c oxidase subunit 1 (_cox1_) gene has been widely used for identification and phylogenetic studies, and enabled the discrimination of closely related species in nematode phyla [7-9]. The mitochondrial _cox1_ gene (also known as mtCO1) is a key enzyme of aerobic metabolism, which is located in the inner mitochondrial membrane and a major site for regulation of mitochondrial oxidative phosphorylation. Little information is available regarding parasitic nematodes protein structures and their comparison with isolates of same or closely related species.

During a general survey of the nematode fauna of _Mus musculus_ in the Meerut, U.P., India, several nematodes belonging to _Nippostrongylus_ were collected from the gastrointestinal tract. Their examination using light and scanning electron microscopy revealed that these parasites represented the species _N. brasiliensis_. Moreover, the specimens were also characterized by using molecular approaches. The mitochondrial _cox1_ gene was sequenced and analyzed in order to molecularly identify and estimate the validity of _N. brasiliensis_ from Indian region. We also summarized the identification and prediction of _cox1_ protein structures with comparison of isolates for taxonomic identification with a special focus on the structural aspects through bioinformatics approach.

MATERIALS AND METHODS

A total of 20 _M. musculus_ caught from Meerut (29°01’ N, 77°45’ E), U.P., India were examined for parasitic infections after dissection under chloroform or ether anesthesia. Their gastrointestinal tracts were removed and examined under a stereomicroscope. Total 20 male and 5 female nematodes were recovered from the intestine of _M. musculus_. They were washed
in saline (0.6%) and then fixed in 70% ethanol and stored until studied. For the light microscopy study, the nematodes were mounted in glycerin. A light microscope (Motíc SMZ-168, Xia- men, China) equipped with digital image analysis system (Motíc Image Plus 2.0 for Windows) and drawing attachment was used for line drawings and morphometric analysis. For scanning electron microscopic studies, parasites were fixed in 70% ethanol, dried by critical point-drier, mounted on SEM stud, and finally coated with a thin layer of gold before being examined with a JOEL Neoscope JCM5000 SEM (Nikon Instruments, Melville, New York, USA) at an accelerating voltage of 10 kV. Prepared slides of male and female N. brasiliensis were deposited in the Museum, Department of Zoology, Chaudhary Charan Singh University, Meerut, (U.P.), India, under the voucher no. Nem/2015/01. Measurements are given in Table 1.

For molecular analysis, genomic DNA was extracted using a DNeasy™ Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. Eluted DNA was kept at -20°C until further use. The partial mitochondrial cox1 gene was amplified by PCR using the primers LCO1490 (5’-GGTCAA- CAAATCATAAAGAATGG-3’) and HC02198 (5’-TAAACT TCAGGGTGACCAAAAAATCA-3’) [10] with cycling profile described previously [10]. PCR products were checked on ethidium bromide stained 1% TAE buffer gel and purified by the Purelink™ Quick Gel Extraction and PCR Purification Combo kit (Invitrogen, Carlsbad, California, USA) following the manufacturer’s instruction. Sequencing was carried out by the same primers using an ABI Big Dye Terminator version 3.1 cycle sequencing kit with an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, California, USA). Sequences were aligned using Clustal W [11] and manually adjusted. Using the BLASTn algorithm, the obtained sequence was compared with those available in the NCBI database (National Centre for Biotechnology Information; http://www.ncbi.nlm.nih.gov).

The phylogenetic tree was built using the maximum likelihood (ML) and Bayesian inference (BI) analyses. DNA pairwise distances were calculated using the Kimura 2 parameter model with the MEGA 6 software [12]. For ML analysis, GTR + G + I model was chosen based on the best fitting substitution model using the Akaike Information Criterion in MEGA 6 [12]. The tree topology was tested by using bootstrapping over 1,000 replications. TOPALi 2.5 [13] was used to construct the tree for BI analysis. For BI analysis, substitution model was tested by the Bayesian Information Criterion and GTR+I+G was chosen. BI analysis was run for 1,000,000 generations, sampling every 100th tree and discarding ‘burn in’ first 25% of the sampled tree. Oesophagostomum columbianum (KC715827) was used as an outgroup for analysis.

For study of protein sequence, a primary sequence analysis of the N. brasiliensis isolates was performed using the Prot-Param [14]. The cox1 protein secondary structure analysis of the N. brasiliensis isolates was obtained using the program SOPMA [15]. The cox1 protein sequence of N. brasiliensis and most related isolate sequence alignment were generated by ESPript 3.0 [16]. To carry out the cox1 homology search for N. brasiliensis against Protein Data Bank (PDB) was performed by using SWISS-MODEL [17]. The same model was employed to generate the 3D structure of the N. brasiliensis for cox1. The model with high score was validated by the Phyre 2 [18] and I-TASSER [19]. The model was refined by energy minimization using the NAMD package [20] and subjected to quality evaluation. MEMSAT-SVM and MEMPACK in PSIPRED workbench [21] were used for the prediction of transmembrane helices and topology of N. brasiliensis protein sequence. RAMPAGE [22] was used for quantitative protein structure evaluation of N. brasiliensis, and the Ramachandran plot was utilized for geometric assessment. To evaluate the quality of the model and study the energy of residue–residue interactions using a distance-based pair potential ProSA program [23] was employed. TM-align [24] was used for the superimposition between Indian and USA isolates of N. brasiliensis protein sequences for comparison.

### RESULTS

The male and female nematodes collected in this study were

| Table 1. Morphometric data of N. brasiliensis parasitising Mus musculus |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Body length | Body width | Buccal cavity length | Buccal cavity width | Esophagus length |
| Male | 4.11 (4.05-4.16) | 2.88 (2.85-2.90) | 0.15 (0.12-0.18) | 0.6 (0.4-0.9) |
| Female | 5.44 (5.30-5.60) | 0.11 (0.10-0.12) | 0.11 (0.10-0.12) | 0.4 (0.3-0.6) |
| Spicule length | Anus length from posterior end | Tail length |
| Male | 1.20 (1.0-1.5) | 0.54 (0.53-0.55) | - |
| Female | 1.74 (1.6-1.9) | 0.74 (0.60-0.90) | 0.52 (0.40-0.60) |

All measurements are in millimeter (mm).
diagnosed on the basis of morphological characteristics and preliminarily identified as *N. brasiliensis*. The male’s bursa with asymmetrical lateral and small dorsal lobes, each branch ending in out of the 3 tips, spicules almost equal, filiform and gubernaculum present (Fig. 1A). Female’s tail conical, pointed, vulva present near anus, and oviparous (Fig. 1B). Measurements were taken and presented in the table (Table 1). SEM images were also provided for the topological view of *N. brasiliensis* (Fig. 2).

The sequence obtained for the mitochondrial *cox1* region was 705 bp in length and deposited in the GenBank database under the accession no. KX146839. There was no *N. brasiliensis* sequence registered from India till date, thus, comparison was possible with 3 *N. brasiliensis* sequence (nos. U57035, AF096235, and AF263480) available in GenBank. The available 3 isolates of *N. brasiliensis* showed a pairwise comparison, 0.14% (U57035), 1.75% (AF096235), and 1.97% (AF263480) nucleotide difference with Indian isolate, respectively. Sequence analysis showed that in all, an isolate from USA (no. U57035) was found the closest to Indian isolate based on the *cox1* sequence. Surprisingly, phylogenetic analysis of data showed that 2 isolates of *N. brasiliensis* (nos. KX146839 and U57035) were located on the same
clade, whereas, the other 2 from Korea and UK (nos. AF096235 and AF263480) formed a separate major clade (Fig. 3). This result demonstrated the paraphyly of *Nippostrongylus* as its isolates were segregated into 2 lineages. According to the phylogenetic tree, *N. brasiliensis* formed a clade along with *Trichostrongylus* and *Heligmosomoides* as the sister group. In addition, all the species that formed the tree belonged to the superfamily Trichostrongyoidea based on mitochondrial cox1 analysis (Fig. 3).

Further, we studied the cox1 protein sequence to predict the 3D structure and comparative modeling to show the well-conserved structural elements. Results of primary and secondary sequence analysis of cox1 protein sequences of *N. brasiliensis* isolates available on the NCBI database from various geographical regions are shown in Tables 1 and 2. Sequence alignment and tertiary structure analysis of protein sequence of *N. brasiliensis* Indian isolate showed the closest similarity with an isolate from USA (Table 3), and template sample (PDB Id: 3abm) against PDB search was presented in Figs. 4 and 5. In Fig. 4A, sequence alignment comparison of the isolate from India and USA with the template sequence is shown. Fig. 4B specifically represents the sequence comparison between Indian and USA isolates of *N. brasiliensis* with template sequence along with the consensus sequence where except few sites, the nucleotide sequence from Indian isolate showed the highest similarity with USA isolate (Fig. 4C). Fig. 4D depicts about Indian and USA isolate with secondary structure elements on top of the sequences as helices with squiggles, arrows present β-strands, and TT letters show the turns. Below the sequence solvent accessibility is provided by a bar (blue is accessible, cyan is intermediate, white is buried) (Fig. 4D).

The resulting 3D model of the cox1 protein structure was sorted according to the scores evaluated by protein energy, and the validation of the model was checked by assessing the quality of protein backbone conformation by RAMPAGE for reli-
ability. Rampage $\phi$ and $\psi$ regions were used for validation of experimental protein structures. The obtained Ramachandran plot (Psi-Phi) pairs for Indian isolate had 96.6% of residues in most favored regions, while 3.4% residues in generously allowed regions (Fig. 5) whereas for USA isolate was 96.2%. The 3D model of cox1 protein sequence of isolate from India and USA along with sample template (PDB Id: 3abm) were built (Fig. 6). The ProsA analysis showed the overall interaction energy of the model was -3.71 kcal/mol, which is relatively similar to the USA isolate Z score -3.61 kcal/mol. Hydropathy analysis of cox1 protein sequence of isolate from India by MEMSAT-SVM and MEMPACK suggested the presence of 5 transmembrane (TM) helix (Fig. 7). Comparative analysis of cox1 protein structures of isolates from India and USA was performed using TM-align; it compared the 3D structures of proteins and computation structural alignments between 2 protein structures showing the structural similarity. Fig. 8 shows

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**Table 3.** Results of secondary sequence analysis of cox1 data of *N. brasiliensis* isolates available in the NCBI database from various geographical regions

| N. brasiliensis | $\alpha$-helix (%) | Extended strand (%) | $\beta$-turn (%) | Random coil (%) |
|----------------|-------------------|-------------------|-----------------|----------------|
| India          | 32.77             | 27.66             | 14.47           | 25.11          |
| USA            | 37.67             | 26.51             | 11.16           | 24.65          |
| Korea          | 31.29             | 35.37             | 10.88           | 22.45          |
| UK             | 35.88             | 37.40             | 6.87            | 19.85          |

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Fig. 4. Alignment of the cox1 protein sequence. (A) *N. brasiliensis* from India and its isolates available on Genbank database from other regions. (B) *N. brasiliensis* isolate from India and USA along with the template search against the PDB database. (C) *N. brasiliensis* isolate from India and USA and their consensus sequence (D) showing the *N. brasiliensis* isolate from India and USA with secondary structure elements presented on top.

Fig. 5. The Ramachandran plot of the structure of *N. brasiliensis* isolate from India showing residue predicted by RAMPAGE in favored and allowed outer regions.
the superimposition of Indian and USA isolates; the 2 isolates were almost similar except few bases that might be due to different geographical regions. The results shown above gave sufficient information of structurally conserved regions in a predicted protein structure.

**DISCUSSION**

The present study adds to our knowledge and provides the strong molecular based evidence for the existence of *N. brasiliensis* from India parasitizing *M. musculus* as a common parasite of rodents. Male and female parasites were identified based on morphological diagnostic characteristics but molecular data supplemented more confirmation and validation to the study. However, sometimes species identification based only on morphology cannot be considered reliable especially in the cases where species are morphologically indistinguishable. In India, little attention has been paid to molecular studies of nematodes relatively to morphology that represents an obstacle in identification. We report for the first time the use of mitochondrial *cox1* gene for molecular identification of *N. brasiliensis* from India. Mitochondrial DNA evolves very quickly in genomes that is why differences in very closely related
species can be easily identified among closely related/within species [25,26]. Phylogenetic analysis clearly stated that in all isolates of N. brasilensis available on the database had closest homology with the isolate from USA if compared with isolates from Korea and UK as they had very short cox1 protein sequences. All N. brasilensis isolates did not form a single clade and showed 2 lineages, this might be due to the paraphyly of this genus as also reported earlier, so the results were consistent with these studies [27-29]. Mitochondrial cox1 proteins have been relatively consistent functional and structural contexts over evolutionary time [30] and have been well conserved in their structures as also proved in the results of this study for determining specific relationships. The protein of isolates from India and USA confirmed 5 transmembrane helices at the same position indicating structural similarity. The outcome of RAMPAGE showed the reliability, and strong acceptance of the predicted structures of both isolates confirmed their structural similarities and the validation of Indian isolate.

In India, there is a need of attempts to integrate molecular analyses as well as morphology that can provide more effective means of characterization for nematodes. The present study added important details and indicated that the analysis of protein structural components was an important technique for obtaining the 3D structure that will help in studying phylogeny and identification of closely related isolates. The present research offered a backbone to understand the functional and structural insight of cox1 protein of N. brasilensis that can be used for future molecular studies.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

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