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Molecular barcode analysis and phylogeny of parasitic helminthes: A laboratory manual

Sushil Kumar Upadhyay
Dept. of Biotechnology, M.M. Engineering College, Maharishi Markandeshwar Deemed University Mullana, Ambala-133 207, Haryana, India
upadhyay.k.sushil@gmail.com

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Abstract

As all eukaryotic organisms, helminthes also have Deoxyribonucleic acid (DNA) as hereditary genetic material. The DNA mostly located in the nucleus of cell (nuclear DNA), but a little quantity of DNA can also be found in mitochondria (mitochondrial DNA or mtDNA). DNA of organisms is a unique feature with encoded information which describes all phenotypic and physiological characteristics of bearing animals like barcode of a physical object. Paul Hebert, researcher at the University of Guelph in Ontario, Canada, proposed "DNA barcoding" as a contrivance to identify biological species in 2003. Until now, organisms were identified using morphological characters like shape, size and color of body parts. There was a skilled researcher could make routine observations and microscopically identify specimens using morphological "keys", but in several cases a learned and eminent taxonomist was considered necessary. When a specimen was mechanically or physically broken or if in an immature stage of development then even experts might not be able to made proper identifications. The validation of taxa was performed to remove ambiguity in identification of cestodes and nematodes by phylogenetic analysis, which was earlier studied by using morphotaxometric analyses. DNA barcoding resolves these ambiguities of morphological identifications because even sub-proficient worker can acquire barcodes from a little amount of tissue. Author wish to explore by the present work about ambiguities in traditionally identified organisms, which can be evaluated and validated by barcoding, phylogeny and molecular heterogeneity, so that individuals may place at appropriate taxonomic status.

Keywords: DNA barcoding, Phylogeny, Mtcoi, 18S rRNA, ITS-1&2, PCR, AGE.

Introduction

Barcoding of an organism utilizes a tiny genetic sequence from a definite and conserved part of the genome, as a supermarket scanner differentiates products using black stripes of Universal Product Code (UPC). Molecular barcoding can provide dual purpose as a decent and reliable contrivance in taxonomist's toolbox supplementing their knowledge as well as being an innovative appliance for non-experts who need to make a swift identification. The techniques of molecular taxonomy[1] employed genomic DNA extraction[2] from specimens according to the provided protocol. The conserved gene region which is being used as standard barcode for almost all animal groups is *mtcoi,* ITS-1, ITS-2 and 18S rRNA gene. The barcoding projects have four components such as first biological specimens, second well equipped and standardized laboratory, third databases for public reference library and fourth data analysis to find closest matching reference record in the database (Figure-1).

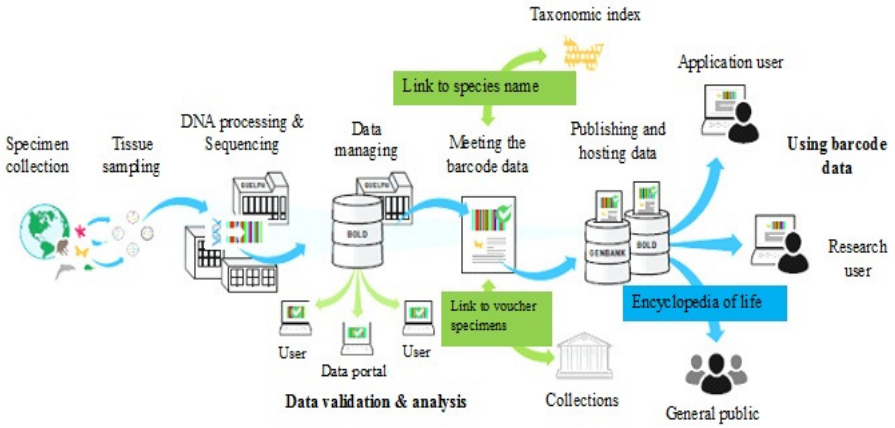

Figure-1: An outline for tools and techniques employed in DNA barcoding.

Material and Methods

Protocol for the genomic DNA extraction by using TNEUS (Tris-HCl, NaCl, EDTA, Urea, SDS) buffer: Reagents required: The chemicals (Tris-hydrochloride (mw 121.14); Sodium chloride (mw58.44); Proteinase-K (PK); Isoamyl alcohol: Phenol: Chloroform (1:25:24v/v); Isoamyl alcohol: Chloroform (1:24); Sodium acetate; Sodium Dodecyl Sulfate (SDS); Dehydrated ethanol; Isopropyl alcohol; Tris-EDTA (TE) buffer (10X); Urea (mw60.06) and EDTA (mw372.24)) required for extraction of genomic DNA were very sensitive and molecular grades purchased from authorized and licensed dealer.

Preparation TNEUS buffer: An aqueous solution of 10mM Tris-HCl (pH7.5), 125mM Sodium chloride, 10mM Ethylenediaminetetraacetic acid (EDTA), 8M Urea, and 1% SDS (w/v) was prepared.

Procedure[3]: The genomic DNA was extracted from either freshly collected or preserved tissue of model parasitic helminthes. For same taken out fresh or alcohol preserved tissue (10-25mg) of helminthes parasite and dried it on sterilized tissue paper until all alcoholic contents were evaporated from tissue. These air dried tissues were transferred in micro centrifuge tube (1.5ml) containing 250 µl of TNEUS buffer. Homogenization of tissues were performed by manual homogenizer or alternatively cut tissues in small pieces by sterilized scissor. Vortexing of solution was performed vigorously after adding 7.5µl of Proteinase-K (15mg/ml). The tube containing buffer, tissue homogenate and protienase-K was incubated in water bath or heating block at 54°C for 3-5 hours or overnight or until tissue dissolved completely meanwhile solution was mixed vigorously several times after an interval 2-3 hours. The sample was cooled at room temperature before proceeding for next step. An equal volume of IPC solution (isoamyl alcohol: phenol: chloroform : : 1:25:24v/v) was added and mixed well by vigorous vortexing for 2-3 minutes followed by centrifugation of content at 13000xg for 10 minutes. The supernatant was collected in fresh centrifuge tube and equal amount of isoamyl alcohol: chloroform (1:24v/v) was added and proceeded by centrifugation at 15000xg for 10 minutes. The supernatant was again collected in fresh micro centrifuge tube and doubled volume of isopropyl alcohol mixed gently till white flakes appeared. The centrifuge tube was placed in deep freezer at -20°C to -50°C for 30-60 minutes. The tube was taken out to bring sample at room temperature (RT) and centrifuged at 15000xg for 10 minutes. The supernatant was carefully decanted by slanting micro centrifuge over tissue paper or by micro-pipetting at 45° angles. Chilled 70% dehydrated ethanol (300µl) was added and centrifuged at 15000xg for minutes. Supernatant was decanted carefully again for washing of DNA pellets. It was followed by addition of chilled absolute ethyl alcohol and centrifuged at 15000xg for 10 minutes for final washing of DNA pellets. The supernatant was decanted carefully again and DNA pellets were lyophilized or DNA pellets were completely air dried at RT in laminar flow for 4-5 hours. Finally pellets were dissolved in DNA rehydration solution or in TE buffer (1X) and stored at 4°C. It was genomic DNA sample or elute.

Modified methods for isolation of genomic DNA (Promega Cat. A1120)[3]: Taken out the fresh or alcohol preserved tissue of parasitic helminthes (10-25mg) and preservatives were removed completely using sterilized tissue paper. The tissues were transferred in micro centrifuge tube (1.5ml) containing 60µl of 0.5M Ethylenediaminetetraacetic acid solution (pH8.0) and 250 µl of NLS (Nuclei lysis solution). The micro-centrifuge tube containing tissues and solution was cooled in ice bucket. The solution in micro-centrifuge was turned cloudy when chilled. Chilled tissue was grounded in liquid nitrogen using a mortar-pestle and 7µl of PK solution (10mg/ml) was added followed by vigorous vortexing for 2-3 minutes. Solution was incubated overnight at 45°C with gentle shaking in water bath and ensures helminthes tissue was digested perfectly (optional: 1.5µl of RNase was added to nuclear lysate and mixed by vortexing followed by incubation for 20-25 minutes at 37°C). Sample was allowed to cool at RT for 5 minutes before further proceeding. The sample was vortexed and 100µl of protein precipitation solution added and again vortexed vigorously at high speed for 1-2 minutes. The sample was chilled in ice bucket to 15-20 minutes and centrifuged for 10 minutes at 16000xg.

The supernatant containing DNA (precipitate discarded) was carefully transferred in to a clean micro centrifuge tube (1.5ml) containing 300 µl of isopropyl alcohol (Note: Leave interphase liquid in original tube to avoid contamination of DNA solution with precipitated protein). The solution was mixed gently by inversion until white thread like strands of DNA appeared, after appearance of threads; it was centrifuged for 10 minutes at 16000xg at 18°C. The debris containing DNA as small white flakes were collected and supernatant discarded carefully, thereafter, 300 µl of 70% ethanol (RT) added and centrifuged at cooling temperature for 10 minutes at 16000xg. Supernatant was carefully decanted to remove ethanol (at 45° angle) and continued air drying of DNA flakes for 2-3 hours under laminar flow or may be kept it for overnight (Note: The DNA pellet was unfastened at that time so that avoid aspiring of pellet into pipette). The DNA flakes were rehydrated by 25-50 µl of DNA rehydration solution or TE buffer (1X) and stored elutes (containing genomic DNA) at 4°C for further applications.

Qualitative detection of genomic DNA by Agarose Gel Electrophoresis[3]: **Requirements:** The presence of genomic DNA in elute was tested by agarose gel electrophoresis (AGE) required several high quality molecular grade sophisticated chemicals like agarose to cast gel as stationary medium, bromophelol blue (BPB) as tracking dye, ethidium bromide (EtBr) as DNA binding UV luminescent dye, running buffer TAE (50X) as electrolyte and sample supposed to containing genomic DNA.

Procedure: The 1% agarose suspension was prepared in 50 ml of TAE (1X) buffer by gentle heating on hot plate or in microwave. The content was allowed to cool (55°C) and 1µl of ethidium bromide mixed properly. (Note: Always wear gloves while working with this dye because it is mutagenic). Horizontal electrophoresis apparatus equipped smartly and well casting comb inserted at 3mm away from terminal wall and bottom of tray. Agarose suspension with Etbr poured into gel casting tank. Care should be taken to avoid air bubbles in gel. Now kept setup for about 30-40 minutes after that TAE buffer (1X) buffer was poured in electrophoresis chamber containing gel may get immersed about 3-5 mm. The well casting comb was taken out carefully and sample supposed to containing DNA (2µl) with loading dye (2µl) loaded into the slots of submerged gel (Note: Mix DNA sample and loading dye very well before use). The whole setup connected with electric lead in such a way that negative terminal was at the end where samples have been loaded. Electrophoresis was performed at 50-100V for 10-15 minutes and after that agarose gel was taken out from electrophoresis chamber and observed under UV trans-illuminator or gel-doc (Note: Avoid breakage of gel). The genomic DNA were appeared as bands fluoresced under UV light which confirmed presence positively (Figures-2,3).

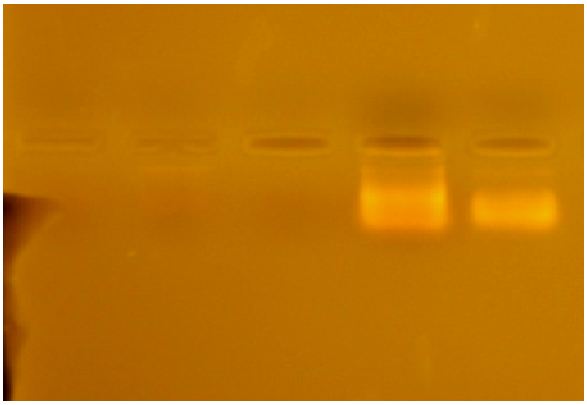

Figure-2: Genomic DNA of parasitic tapeworms after agarose gel electrophoresis.

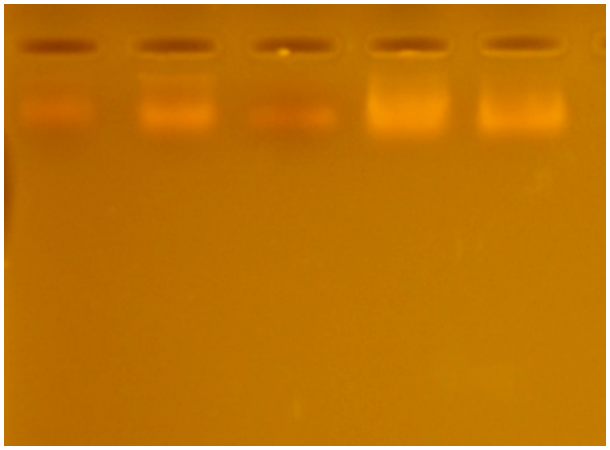

Figure-3: Genomic DNA of parasitic roundworms after agarose gel electrophoresis.

Quantitative estimation of genomic DNA: By UV spectrophotometer: Requirement: The quantitative estimation of genomic DNA by UV spectrophotometer required sample supposed to containing DNA and sterile distilled water (mq) or TE buffer.

Procedure: Switched on spectrophotometer and allowed it to warm up about 10-15 minutes proceeded by calibration at 260 nm wave length thereafter, switched on UV lamp of spectrophotometer. The instrument was calibrated at zero absorbance by using distilled water or Tris-EDTA buffer. The first cubed tube taken out, dried by tissue paper, filled by diluted DNA sample and again placed in spectrophotometer and ran the system.

The absorbance reading at 260 nm noted down in practical register. The concentration of DNA (ng/µl) was calculated by multiplying 50 with absorbance reading at 260 nm and dilution factor.

By Nanodrop (ND1000): Procedure: At first switched on system, thereafter, file of software opened by author and clicked on nucleic acid by adjusting cursor. The sterilized distilled water (1.5µl) was loaded on electrode plate of Nanodrop and pressed OK button. Electrode tip wiped or dried very well and further 1.5 µl of blank (TE 1X) loaded on electrode then press OK after that, cursor or sample window displayed automatically in which the sample name was filled. Electrode tip wiped again and 1.5 µl of DNA sample loaded then pressed on measure. The readings displayed on screen noted down as concentration of DNA in ng/µl. Electrode wiped again and pressed next button proceeded by reports button and saved file in system continuously clicked on reblank button and 1.5µl of TE (1X) buffer loaded on electrode again, pressed button of measure which reflected 0.0 reading on the screen then pressed on exit button (Note: File automatically saved in excel file format as per by default setting of software).

Polymerase Chain Reaction (PCR): The *mtcoi*, Internal Transcribed Spacers (ITS-1 and 2) and 18S rRNA gene were amplified by Polymerase Chain Reaction (PCR) technique[4] using "Perkin Elmer Thermo Cycler". The primers used for amplification of nematode *coi* and 18S rRNA gene were LCO1490 (F), HCO2198 (R)[5] and Nem18SF, Nem18SR[6] respectively. However, primers used for cestode *coi* and 18S rRNA gene were COI-A (F), COI-B (R) and JLR24 (F), JLR25 (R) respectively. The primers for ITS-1 (SS1 & NC13R) and ITS-2 (SS2 and NC2) were common for both tapeworms and roundworms.

Reaction mixture (20 µl): A 20µl reaction mixture of various sophisticated molecular grade chemicals were prepared by adding 14.488µl Triple distilled water, 2.4µl 10X PCR buffer, 0.48µl 2.5 mM dNTPs, 0.96µl $MgCl_2$, 0.24µl Primers (F), 0.24 µl Primers (R), 0.192µl Taq DNA Polymerase and 1.0µl sample containing genomic DNA in a PCR tube.

Procedure: PCR tube containing reaction mixture was placed in PCR machine and standardized program created in thermocycler. The program for amplification included initial denaturation at 94°C for 3 minutes followed by 30 cycles of denaturation at 94°C for 1 minute, annealing temperature of primers at 48°C for 2 minutes and extension at 72°C for 2 minutes and final extension at 72^0C for 8 minutes.

Qualitative estimation of amplified gene: PCR reaction mixture (2µl) was analyzed by AGE in 1.8% agarose gel containing ethidium bromide (Figures-4,5). A total of 50ng concentration of amplified PCR product was used for sequencing.

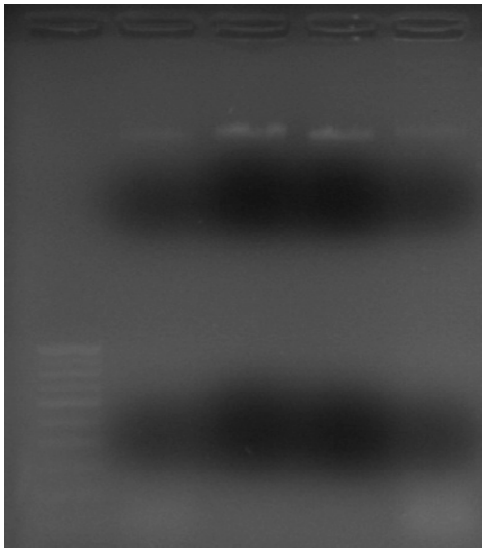

Figure-4: PCR product of target genes of tapeworms after agarose gel electrophoresis.

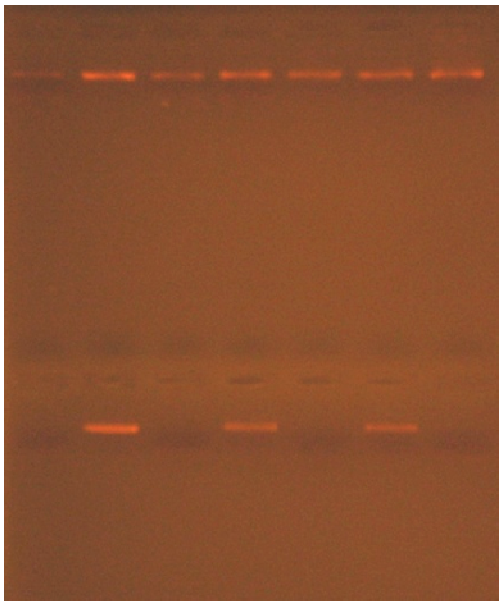

Figure-5: PCR product of target genes of roundworms after agarose gel electrophoresis.

Barcode analysis: The PCR products were analyzed, purified and transformed[7] for further phylogenetic study. The purified PCR product was sequenced by ABI 3730 autoseqencer. The obtained consensus sequences of target gene was compared by using computational technique, BLAST (Basic Local Alignment Search Tool)[8] with related species sequences available in database, GenBank of NCBI (National Center for Biotechnology Information). The phylogenetic tree, neighbor joining dendrogram[9] was generated using MEGA (Molecular Evolutionary Genetic Analysis)[10] and Clustal-w computer program[11] simultaneously evolutionary distances were computed by Kimura's two parameter method[12]. DNA sequences were edited in BioEdit[13] and tree was evaluated by Bootstrap test values[14] based on 1000 replications. If values of such parameters were obtained statistically significant then organisms were validated as new form[15,16].

Results

Results of this study are shown are give Figures-6,7.

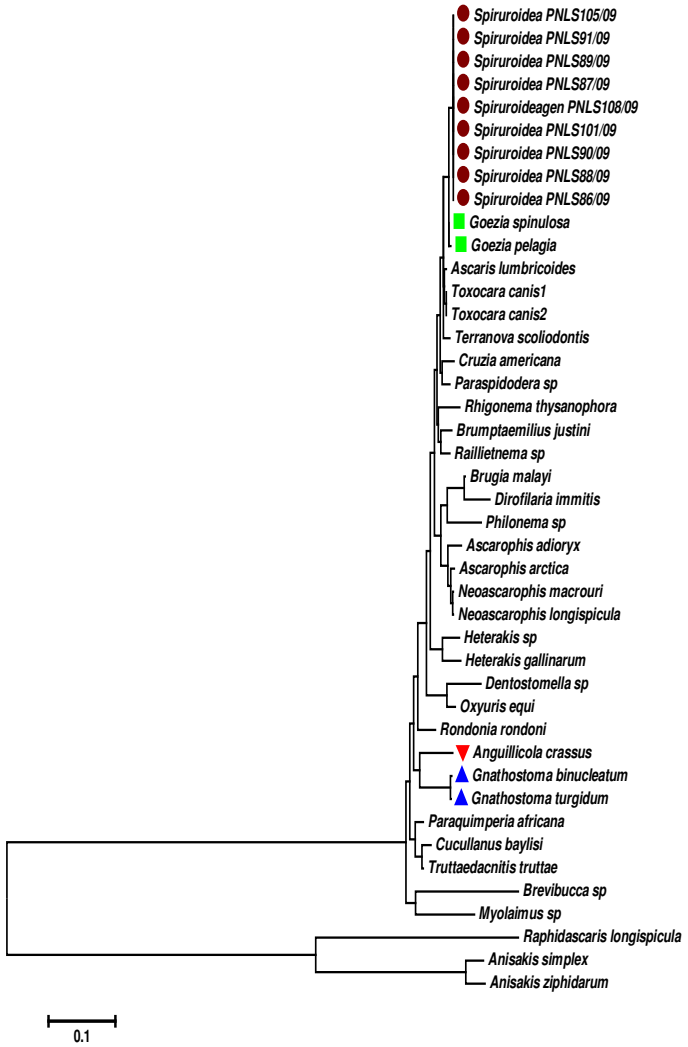

Figure-6: Molecular phylogenetic analysis by ML method through *in silico* advanced software MEGA5.1.1.

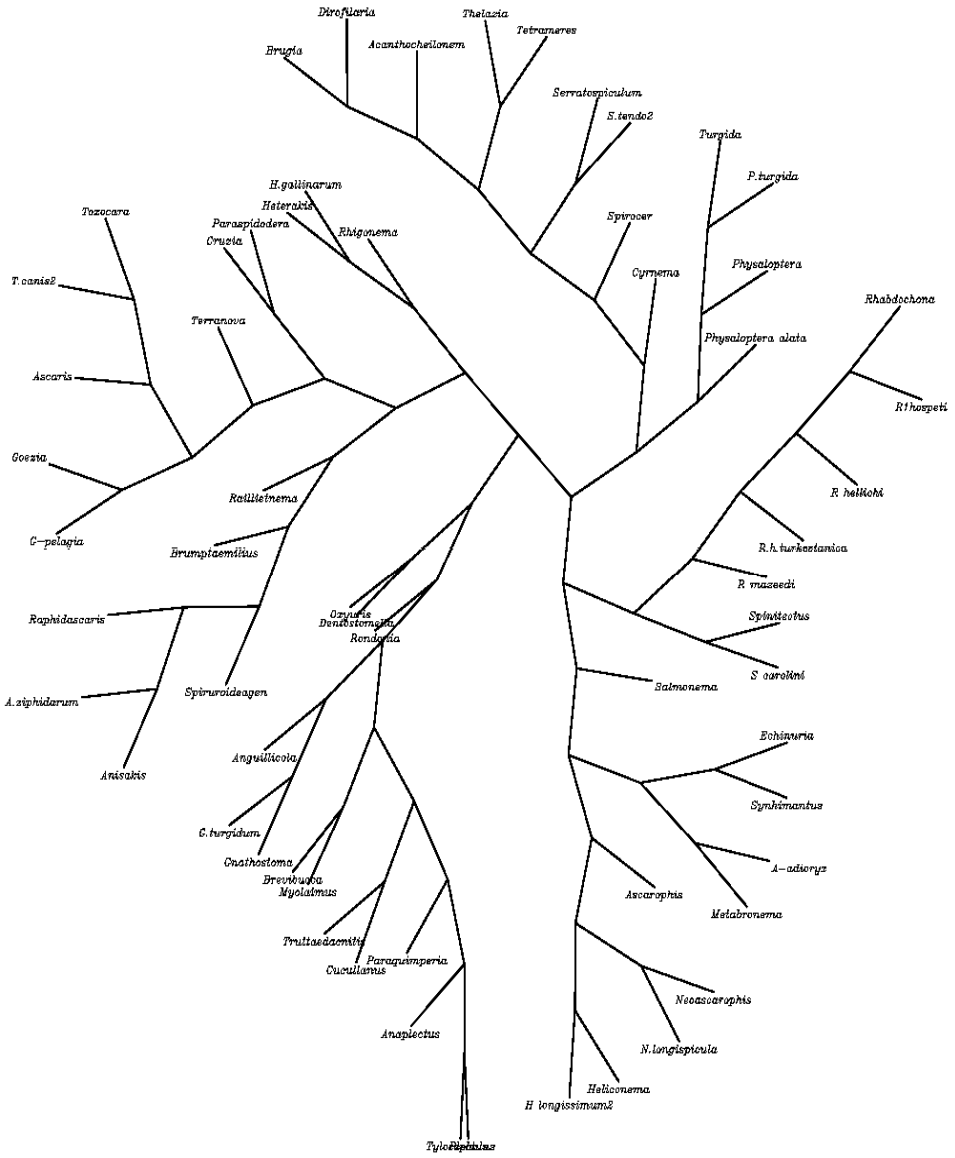

Figure-7: Molecular phylogenetic analysis by un-rooted NJ (Clustal W) *in silico* generated through software MEGA5.1.1.

Conclusion

The findings were concluded by the existence of all sequences derived from individual specimens of collected worms from several host of same species forming single clade as *Spiruroidea* in close proximity of *Goezia*. The molecular analysis was corroborated to morphotaxometric analysis and numerical taxonomy. Therefore, on the basis of aforementioned differences with closely related existing genera and species and formation of different unified clade, it is proposed to accommodate new form in the taxonomic hierarchy.

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