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Molecular characterization of *Lampito mauritii* kinberg treated with tannery sludge

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Abstract: **DNA barcoding is a powerful tool used for species identification by the expanding exploitation of mtDNA gene cytochrome c oxidase I (COI), as a genetic marker. In the present study, COI gene based DNA barcoding was employed to identify the anecic species *Lampito mauriti*. Molecular characterization of *Lampito mauriti*. was performed using Polymerase Chain Reaction (PCR) for COI gene. Phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA7) software for the obtained sequences. The evolutionary divergences between its closely related species were also performed to disclose its amendments that occurred during evolution.**

Keywords: Lampito mauriti, **Polymerase Chain Reaction (PCR), cytochrome c oxidase I (COI), MEGA7.**

1. INDRODUCTION

DNA Barcoding is a molecular and bioinformatics tool that helps to identify biological species (Hebert *et al*., 2003). DNA Barcoding applied systematically to all metazoans, by the use of one or few (Mitochondrial markers). It is a taxonomic method which uses, genetic marker in an organisms DNA to identify it, whether it is belonging to particular species (Meyer and Paulay, 2005). It differs from molecular phylogeny in that not to determine the patterns of relationship , but to identify an unknown sample in terms of pre-existing classification. The most commonly used barcode region for animals is a segment of approximately 600 base pairs of the mitochondrial gene cytochrome oxidase 1 (COI). One of the major advantage of DNA Barcode is the possibility to associate life history stages and genders or to identify organisms from part / pieces , and it provides 95% resolution of the species – level recognition among mammals. DNA Barcoding can be performed at any stage of life of an organism whether live or dead.

Earthworms constitute upto 90% of the soil invertebrate biomass and are important ecosystem engineers (Tischler, 1965; Edwards, 2004). Species identification of adult earthworms is possible by dissection of the male genetalia (Tsai *et al*., 2000 ; Shen *et al*., 2003). This method is considered as labour intensive, time consuming particularly dealing with field collections consisting of several different earthworm species. Identification is limited to adult worms , as most of the life stages are unidentifiable. The morphological and anatomical characteristics of earthworms are variable and the degree of variability can differ and also the features can overlap between taxa (Pop *et al*., 2003). Mitochondrial DNA has been widely used in molecular taxonomy of animals, because it evolves more rapidly than nuclear DNA ,and also it has been used to identify the differences between closely related species (Hebert *et al*., 2003 a, b). The culture of earthworms on a large scale is in higher demand for the production of protein and biofertilizer. In every region of the world , many species of earthworm cultured namely *Eisenia fetida, Lumbricus terrestris, Perionyx excavates, Eudrilus eugeniae* and *Lampito mauritii. Lampito mauritii* and Eudrilus eugeniae were used in the bio management of papermill sludge. The potentiality of earthworms was also assessed in terms of their efficiency and sustainability of vermicomposting. Identification of earthworm species is performed at an adult stage by dissection of the male genital organ, but that process is quite tedious,

labor intensive and time consuming. Moreover, the other life stage worms remain unidentifiable with this method of identification. Hence, DNA barcoding is preferred as it applies to all life forms. Keeping in this view the present was undertaken to elucidate the Molecular characterization of *Lampito mauritii* kinberg treated with tannery sludge using cytochrome c oxidase I (COI).

2. MATERIALS AND METHODS

COLLECTION OF THE SAMPLE

Lampito mauritii (Kinberg) was used in the present study for the degradation of the tannery sludge. Earthworm was collected from Ramancherry, Chennai Tamil nadu, India in plastic containers with soil and cowdung, transferred carefully to the laboratory with adequate moisture. They were handsorted and the species were identified on naked morphological observations (Nagavelamma *et al*., 2004). The biologically treated tannery sludge was collected from Pallavaram Tanners Association,Government Effluent treatment plant, Chennai Tamil Nadu ,India.

EXPERIMENTAL SETUP OF VERMI REACTORS

A Laboratory scale setup of vermi reactors was made up of plastic material in the form of open box provided with wire like holes at the bottom for drainage and ventilation. The reactors consisted of a basal layer of pebbles followed by coarse sand and a layer of garden soil and with a layer of dried cattle dung. These reactors were incubated with earthworms. The experimental units were maintained in triplicates and the control units were maintained five in numbers. In triplicates a total number of 36 experimental units were maintained to analyse the parameters at an interval of 12 days. The soil samples were collected from the triplicate experimental and control units of vermi composting reactors at an interval of 12 days. The soil samples and earthworm samples were subjected to physio-chemical parameters DNA barcoding and sequencing.

ISOLATION OF GENOMIC DNA

Isolation of genomic DNA was carried out by phenol chloroform extraction method (Navot *et al*., 1991). The pellet obtained in the last step was washed with 70% ethanol, air-dried completely, suspended in Tris-EDTA buffer and stored at -20ºC until further use. The isolated DNA was assessed qualitatively and quantitatively by agarose gel electrophoresis and spectrophotometric method respectively.

PCR AND DNA SEQUENCING

The polymerase chain reaction was carried out for amplification of the isolated DNA. The reaction volume was 20µl and the mixture consisted of forward and reverse primers, deoxyribonucleotide triphosphates (dATP, dCTP, dGTP and dTTP), template DNA sample and TaqDNA polymerase. Internal regions of COI gene were amplified using the following primers: COI-F(5'-GGTCAACAAATCATAAAGATATTGG-3') and COI-R(5'-TAAACTTCAGGGT GACCAAAAAA TCA-3'). The reaction mixture was subjected to initial denaturation step at 94°C for 3minutes, followed by 35 cycles of denaturation at 94°C for 45 seconds, primer annealing at 47°C for 1minute, extension at 72°C for 1 minute and 20 seconds with the final extension at 72°C for 7 minutes. The PCR product underwent purification and DNA sequencing was performed by sanger sequencing (Applied biosytems 3500).

SEQUENCE ANALYSIS

Nucleotide blast (BLASTn) using BLAST program and Genbank nucleotide database with default parameters was performed to determine the identity and the closest known relatives of the obtained sequences. Phylogenetic tree was constructed using maximum likelihood method in MEGA version-5 (Molecular Evolutionary Genetics Analysis). The Distance Matrix Explorer, an action menu of MEGA5 was used to compute the pair wise difference between the obtained target sequence to its maximum aligned sequence.

3. RESULTS

The present elucidated the molecular characterization and phylogenetic relationship of *Lampito mauritii* kinberg treated with tannery sludge using cytochrome c oxidase I (COI). DNA was isolated from the *Lampito marutii* and its purity was quantified by the A260/A280 ratio in a spectrophotometer. DNA concentration along with the purity details are given in Table 1. Amplicons after PCR amplification were of 600bp for COI gene (Figs 1 -4). Present study was conceded to determine the evolutionary relationship of *Lampito marutii*. FASTA alignment of *Lampito marutii* and *Lampito marutii*

treated with tannery sludge was presented. The sequence obtained from the purified PCR product was subjected to comparison with the nucleotide database BLASTn and was found to have maximum identity to *Amynthas sp*, belonging to the family *Megascolecidae* (Figs. 5 -6) Construction of relationships between sequences is achieved using phylogenetic analysis that is aimed at identifying species and sequences were observed (Figs 7 -8) Target, *Lampito marutii* branches to *Amynthas sp*, indicating the closest relationship, while it is closely related to Megascolecidae species (Figs 9-10).

4. DISCUSSION

Earthworms are one of the most important and beneficial macro fauna used extensively in organic farming. Currently more than 7,245 species of earthworms have been classified at global level, of which 4000 earthworms species are described (Fragosa, 2001; Reynolds, 1998) Morphologically earthworm species can be distinguished on the basis of several characteristics like growth, number of segments, length, and position of clitellum. Although, morphological characters of these organism depends on environmental conditions and organic matter which forms part of their diet (Curry and Schmidt, 2007). Molecular markers are essential to understand its population biology as well as the underlying selective processes such as those imposed by temperature gradients or change, migration rates, population isolation due to habitat fragmentation, historical events (e.g. bottlenecks, range expansions), and also mating behaviour (Avise, 1994). Molecular markers based on the DNA sequence are more varied and reliable. The PCR-based molecular marker approach requires very less DNA, and is technically simple and cheaper. Molecular markers are considered to provide the best estimates of genetic diversity.

The highly conserved mitochondrial enzyme cytochrome oxidase c is coded by multiple genes containing regions that evolved at different rates. These markers were exploited as DNA barcodes because of their potential to identify putative regulatory elements, as they possess sufficient sequence diversity, individually or in combination, to discriminate among species. The usual and recommended method of DNA barcoding involves polymerase chain reaction (PCR) amplification of suitable regions of the genome, sequence analysis of the amplicons, and alignment of the analysed sequence with reference sequences (Lunt *et al*., 2006; Sass *et al*., 2007; Amendt *et al*., 2004).

Maximum likelihood tree constructed for the target *Lampito marutii* using MEGA7 software (Figs. 8 -10). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (-6600.24) is shown. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. 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. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 595 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Tamura and Nei, 1993; Felsenstein , 1985 AND Tamura *et al*., 2016)

Construction of relationships between sequences is achieved using phylogenetic analysis that is aimed at identifying species and sequences were observed (Figs 7 -8) Target, *Lampito marutii* branches to *Amynthas sp*, indicating the closest relationship, while it is closely related to Megascolecidae species (Figs 9-10). Similarly, Sharma *et al*. (2011) analysed the genetic diversity in earthworms using DNA markers also suggested the molecular markers are a good choice for diversity analysis of earthworm individuals. Likewise, Pérez-Losada *et al*. (2012) who observed the taxonomic assessment of Lumbricidae (Oligochaeta) earthworm genera using DNA barcodes. Our results also confirm that COI barcodes are a good proxy for estimating intrageneric phylogenetic diversity and relationships in earthworms.

5. CONCLUSION

Earthworms are one of the most important and beneficial macrofauna, and are used extensively in organic farming. Earthworms mediate soil biological regulation systems, and produce biogenic structures. They help to maintain soil structure, water infiltration, and regulate the availability of nutrients assimilated by plants. The present investigation showed the molecular characterization and phylogenetic relationship of *Lampito marutii* using, COI gene based DNA barcoding. The results of this study indicate that COI gene based DNA barcoding offer a reliable and effective means of assessing genetic variation in earthworm also DNA barcoding will help conflicting taxonomy and further exploration of species diversity in India.

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APPENDICES

List of Table:

Table 1 Purity of the DNA observed in a spectrophotometer by calculating A260/A280 ratio.

Sample	Absorbance at 260nm(A260)	Absorbance at 280nm(A280)	Concentration (ng/µl)	Purity (A260/A280)
Sample 1	0.225	0.124	11900	1.81
Sample 2	0.369	0.198	18450	1.86

List of Figure:

Fig. 1 Genomic DNA isolated from *Lampito marutii*

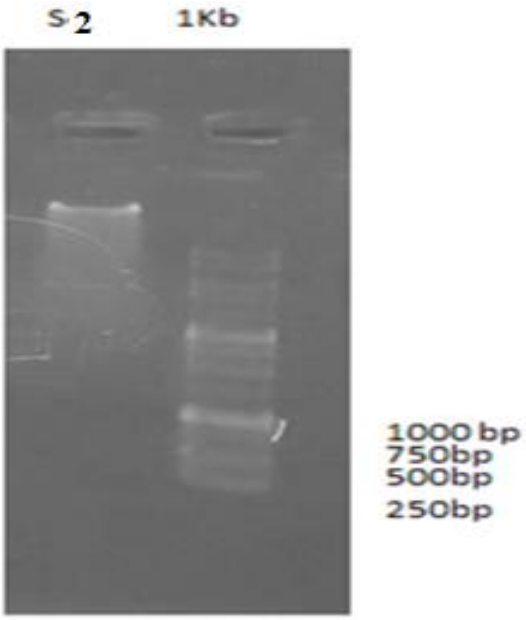

Fig. 2 Genomic DNA isolated from *Lampito marutii* treated with tannery sludge

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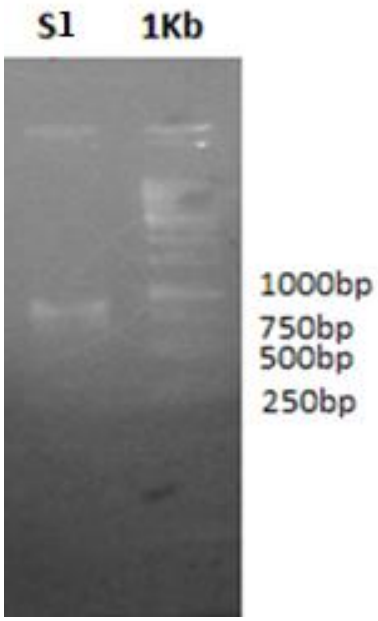

Fig. 3 PCR amplicon of *COI* gene (*Lampito marutii*)

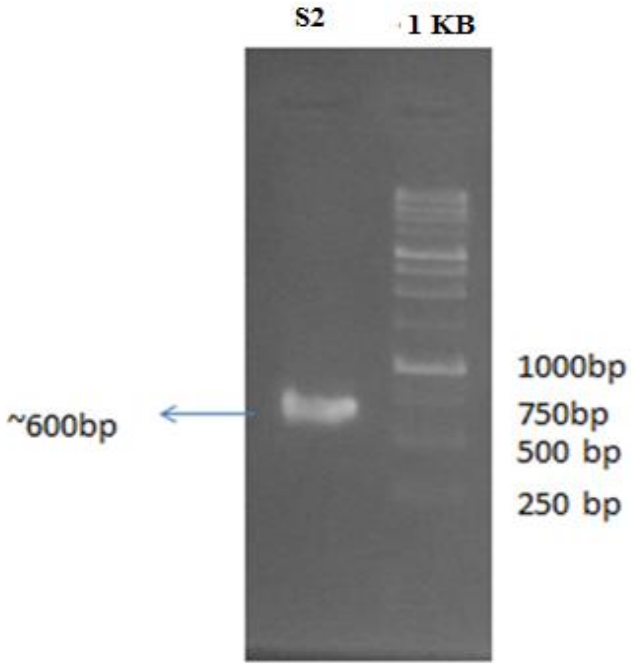

Fig. 4 PCR amplicon of *COI* gene (*Lampito marutii* treated with tannery sludge)

FASTA format of *Lampito marutii*

**AGAGGGGGTCTCCCCCTCCAGCAGGGTCAAAAAACGAGGTATTTAAGTTTCGGTCTGTGATTAATATGGTAATTG
CACCAGCCAATACAGGTAGTGATAGTAGGAGGAGTACTACTGTAATAACTACGGCTCATACAAATAGGGGAATTC
GCTCTAATCGTAATCCTGCTCATCGTATGTTAATGACTGTTGTGATGAAGTTAATTGCACCAAGAATTGAGGAGG
CTCCAGCTAAATGTAGGGAGAAAATTGCAAGGTCTACAGATGGTCCAGAGTGTGCAATATTTCTTGCTAGGGGCG
GATAGACAGTCCATCCTGTACCGGCACCTTTTTCTACGGCAGCTGATGAAACTAGTAAAATAAGCGACGGAGGTA
GTAGTCAAAATCTTATGTTGTTTAGACGGGGAAATGCTATGTCTGGTGCGCCCAGTATTAGTGGGAGAAGTCAGT
TTCCAAACCCGCCAATAAATACTGGTATTACAAGAAAGAAAATTATCAGAAAGGCGTGAGCGGTTACAATTGTAT
TATATAATTGGTCCCTTCCGAGGAAGGCACCTGGTTGTCTTAGTTCGATTCGAATGAG**

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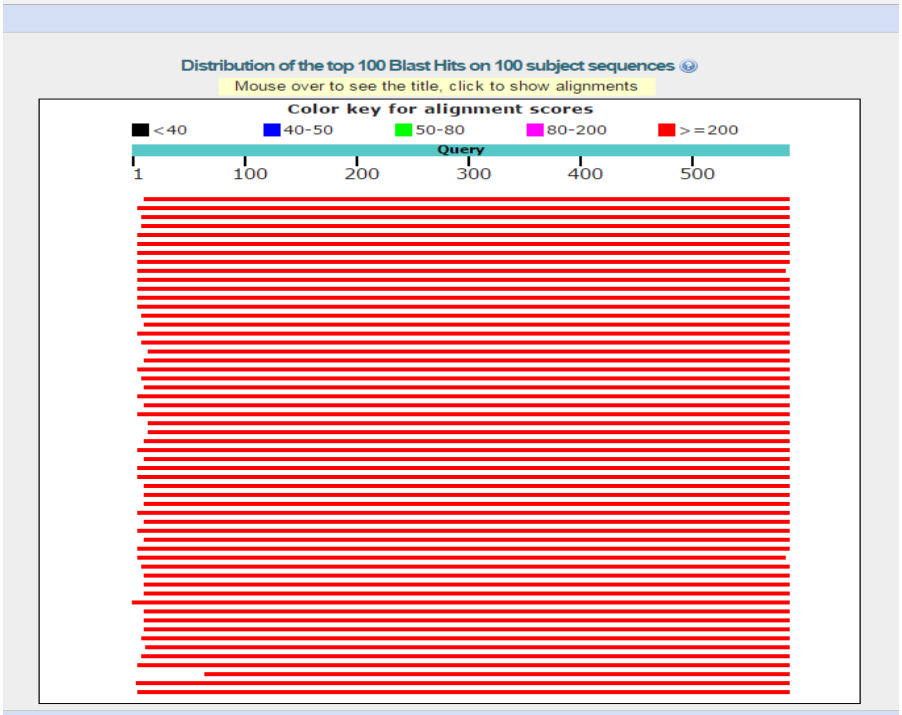

Fig. 5 BlAST results of *Lampito marutii*

FASTA format of *Lampito marutii* treated with tannery sludge

**AGAGGGGGTCTCCCCCTCCAGCAGGGTCAAAAAACGAGGTATTTAAGTTTCGGTCTGTGATTAATATGGTAATTG
CACCAGCCAATACAGGTAGTGATAGTAGGAGGAGTACTACTGTAATAACTACGGCTCATACAAATAGGGGAATTC
GCTCTAATCGTAATCCTGCTCATCGTATGTTAATGACTGTTGTGATGAAGTTAATTGCACCAAGAATTGAGGAGG
CTCCAGCTAAATGTAGGGAGAAAATTGCAAGGTCTACAGATGGTCCAGAGTGTGCAATATTTCTTGCTAGGGGCG
GATAGACAGTCCATCCTGTACCGGCACCTTTTTCTACGGCAGCTGATGAAACTAGTAAAATAAGCGACGGAGGTA
GTAGTCAAAATCTTATGTTGTTTAGACGGGGAAATGCTATGTCTGGTGCGCCCAGTATTAGTGGGAGAAGTCAGT
TTCCAAACCCGCCAATAAATACTGGTATTACAAGAAAGAAAATTATCAGAAAGGCGTGA**

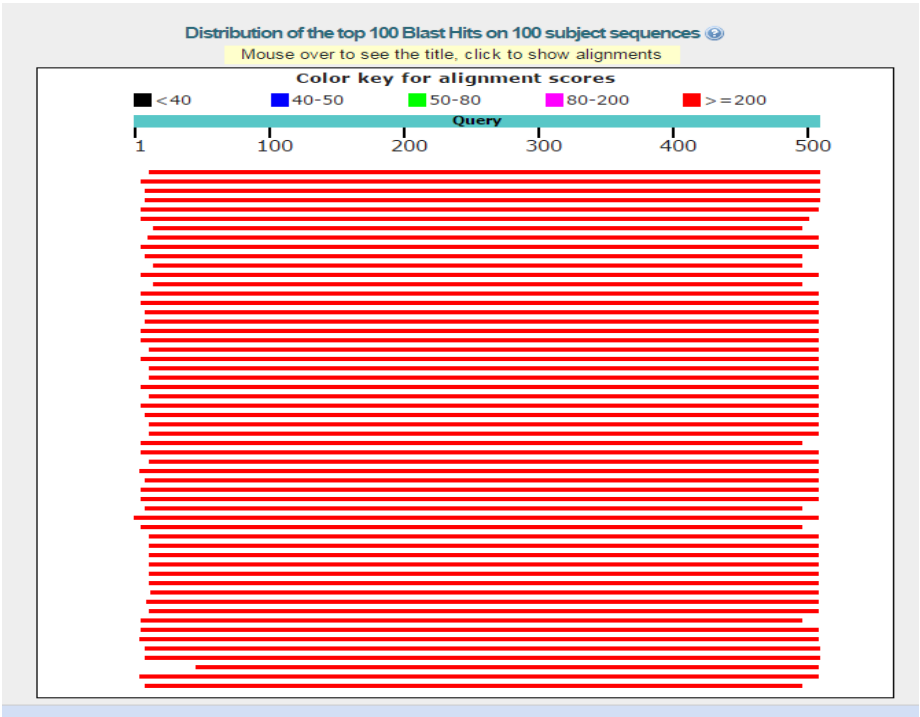

Fig. 6 BlAST results of *Lampito marutii* treated with tannery sludge

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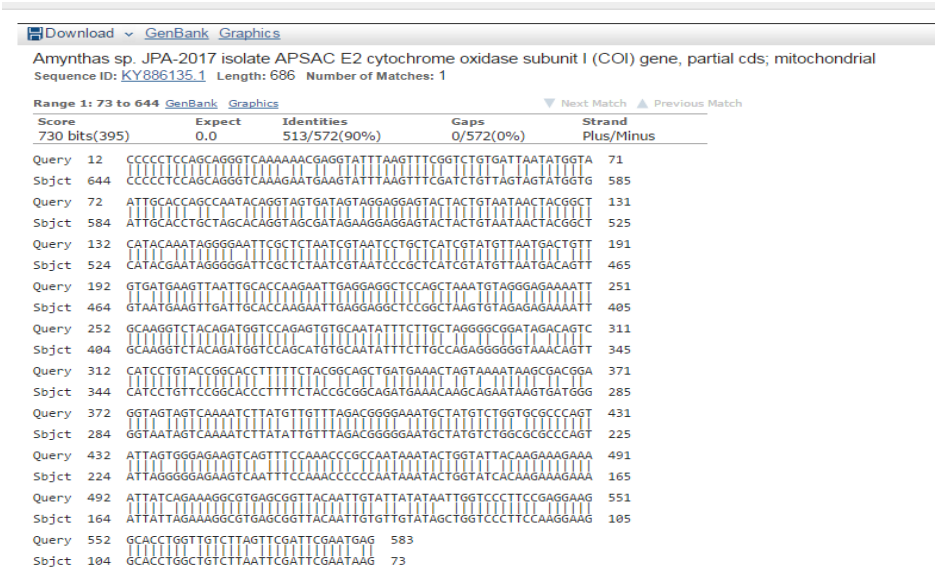

Fig. 7 Alignment summary of *Lampito marutii* using BLASTn

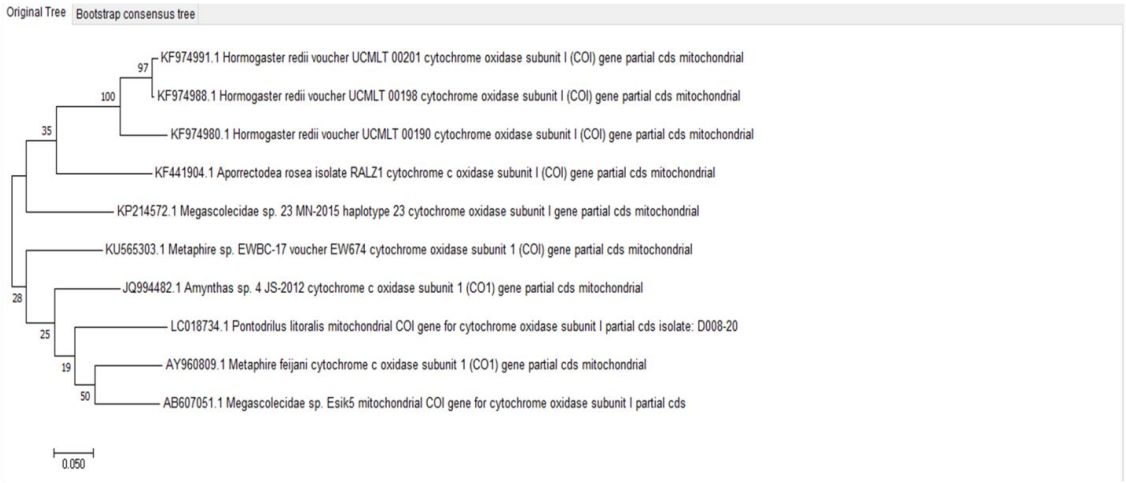

Fig. 8: Molecular Evolutionary Genetics Analysis of target *Lampito marutii* with sequence producing significant alignment from databasefor COI region.

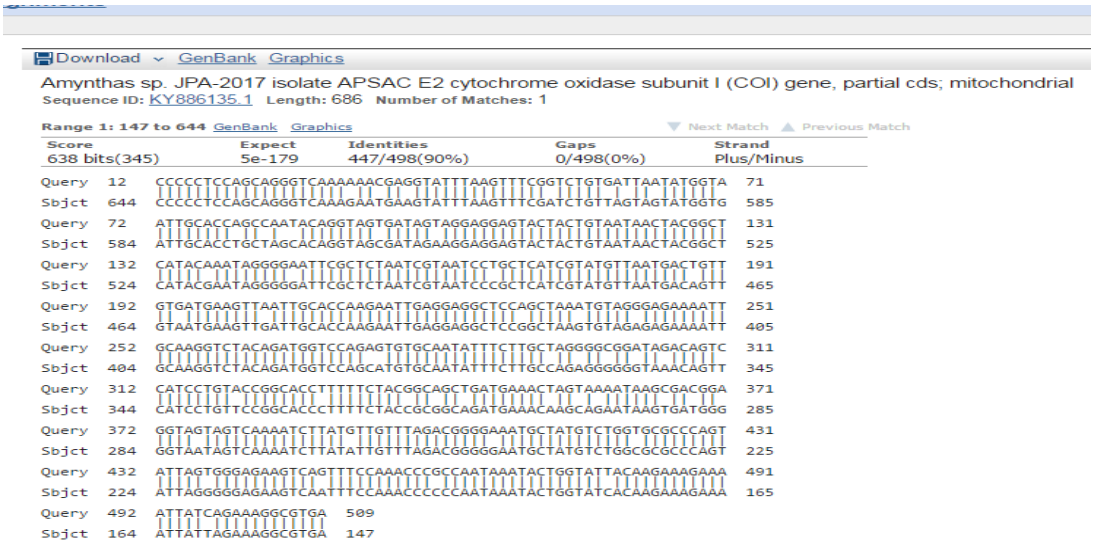

Fig. 9 Alignment summary of *Lampito marutii* using treated with tannery sludge BLASTn

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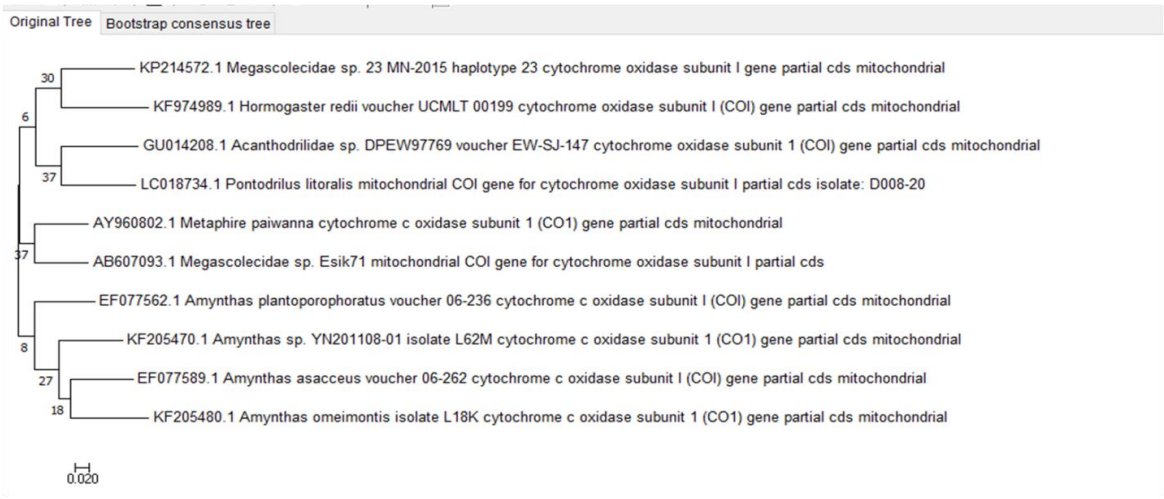

Fig. 10 Molecular Evolutionary Genetics Analysis of target *Lampito marutii* treated with tannery sludge with sequence producing significant alignment from database for COI region.