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MOLECULAR PHYLOGENETIC STUDY OF EARTHWORM *EUDRILUS EUGENIAE*

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ABSTRACT:

Earthworms belong to invertebrate's phylum Annelida; order Oligochaeta are very important constituents of soil. They are considered as soil rejuvenators and soil bioengineers. They sustain various environmental conditions and generally prefer the moist and damp environment. They play a major role in recycling of organic waste from various sources including agricultural waste, fruit waste; industrial organic waste. Farmyard, kitchen waste, market waste, template waste, etc. they have great capacity in increasing their population. Majority of earthworm species are exotic but acclimatized to Indian context very well. Very little attention has been paid on abundance, distribution and diversity, molecular phylogeny of the exotic earthworm breed, *Eudrilus eugeniae* especially from the drought prone Solapur region from western Maharashtra India. The present investigation has been focused to understand molecular markers and its co-relation with the help of phylogenetic studies and 18s rRNA analysis. The study also focuses to understand the sequencing of the 18s rRNA of earthworm *Eudrilus eugeniae*. Sequence analysis and phylogenetic tree building were carried out using bioinformatics tools. Multiple sequence analysis and evolutionary analysis of 18s rRNA of earthworm speices *Eudrilus eugeniae* was carried out by ClustalW tool and phylogenetic analysis using MEGA software. The *Eudrilus eugeniae* showed closest similarity to *Dichogaster saliens.* Phylogenetic analysis and multiple sequence alignment of *Eudrilus eugeniae* 18s rRNA sequence through neighbor joining was performed which showed its pattern of variations and relationship among different species.

Keywords: Earthworms, exotic species, *Eudrilus Eugenia*, 18s rRNA, sequence analysis, phylogenetic tree.

[1] INTRODUCTION

Over the last 120 years, various classification of earthworms have been proposed and debated using morphological characters, sometimes in an evolutionary context, but rarely with any explicit analysis of character data, resulting in intuitive conclusions (Michaelsen 1900; Stephenson 1930; Gates 1972). Earthworms serve as good bio indicators. They give the knowledge of the physicochemical characteristics of native habitat by enriching the soil with organic matter which in

turn facilitates improvement of soil fertility. They produce a good quality of manure by recycling organic waste. They also change the soil characters by incorporating new components and enrich the soil. The earthworms used for the production of organic eco friendly vermicompost which can be used in various gardens and agriculture. The final product produced is rich in nutrient and course material can be used conveniently. It also supports sustainable agriculture (Kale & Karmegam, 2010).

Eudrilus eugeniae (Kinberg) is a species of earthworm extensively used in organic matter recycling throughout the world (Bano *et al.,* 1987; Reinecke *et al.,* 1992; Giraddi, 2000; Chaudari *et al.,* 2001). The genetic makeup of the strains and the environment has profound influence on the efficiency of earthworms in bio-conversion process. Molecular methods have become powerful and there are precise tools for identification and analysis of genetic diversity of earthworm species. The present study is mainly focused to understand the sequencing of the earthworm *Eudrilus eugeniae* by using 18s r RNA. This analysis is also to explore the possibility of molecular markers in accordance with the relationship in phylogenetic tree. The study also explores the phylogenetic analysis and comparison of 18s rRNA gene in *Eudrilus eugeniae, Dichogaster saliens, Eisenia fetida and Pontodrilus litoralis.*

[II] MATERIALS AND METHODS

2.1 Sample collection

Earthworms of *Eudrilus eugeniae* were procured from ZARS (Zonal Agricultural Research Station Solapur). Samples were preserved in 95% ethanol solution for DNA extraction and phylogenetic analysis.

2.2 DNA extraction and quantification

DNA extraction was carried out using SDS-Ammonium acetate method modified from Moore *et al.,* 2004. 0.5X0.5cm tissue of sample was washed and ground in 0.5ml extraction buffer (50mM Tris HCl, 50mM EDTA, 250mM NaCl, 1.5%Sucrose) and 100µl of SDS (20%) was added to it. The solution was vortexed and incubated for 30 min at 65ºC, 150µl of 7.5M Ammonium acetate was then added to the above solution and mixed by inverting. The tubes were incubated at 4ºC for 15 minutes. DNA was extracted using 1 volume of chloroform: isoamyl alcohol mixture (24:1) and centrifuging at 10000rpm for 5mins. Double volume of ethanol (96-100%) was added to the aqueous phase in a new tube, inverted twice and allowed to stand at 4ºC for 30 minutes. The mixture was then centrifuged at 10000 rpm for 15 minutes. After drying for few seconds pellet was dissolved in 50µl elution buffer (10mM Tris-HCl, 1mM EDTA). The DNA was stored at 4ºC for further use. Concentration of DNA was determined using UV-1800 spectrophotometer (Schimadzu Corporation). The DNA was stored at -200C for further use.

2.3 PCR amplification

The polymerase chain reaction (PCR) is a molecular technology used to amplify a single or a few copies of a piece of DNA across numerous orders of magnitude, producing thousands of copies of a specific DNA sequence (Bartlett and Stirling, 2003). Molecular phylogeny of earthworm was determined by amplifying genomic 18s rRNA region. Two primers specific to 18s rRNA region used in this study were 18s5-F and 18s1100R in order to amplify approximately 1000bp sequence of earthworm. PCR amplification was performed using Biometra thermal cycler. The PCR mixture contained 2.5µl of 10X buffer, 1µl of each primer (diluted 10 times), 2.5µl of 2.5mM of each dNTP, 2.5 Units of Taq DNA polymerase and 1µl Template DNA. 18s rRNA amplification were 18s5F- 5' CTGGTTGATYCTGCCAGT 3' and 18s1100R - 5' CTTCGAACCTCTGACTTTCG 3' (Williams *et al.,* 2006). The PCR amplification cycle consisted of, a cycle of 5 min at 94 °C; 30 cycles

of 45 sec at 94 °C, 45 sec at 54°C, 1 min and 30 sec at 72 °C; and 1 cycle of 5 min at 72 °C.

2.4 Gel electrophoresis

Gel electrophoresis is a method for separation and analysis of macromolecules (DNA, RNA and proteins) and their fragments, based on their size and charge. Gel electrophoresis was performed using 1.0% agarose to analyze the size of amplified PCR product.

2.3 Sequencing

The small subunit (SSU) 18S rRNA gene is one of the frequently used genes in phylogenetic studies and an important marker for random target polymerase chain reaction (PCR) in environmental biodiversity screening (Meyer *et al*., 2010). Sequencing is the procedures of determining the specific order of nucleotides within a DNA molecule. The PCR product was purified using AxyPrep PCR Clean up kit (AP-PCR-50). It was further sequenced using Applied Biosystems 3730xl DNA Analyzer USA and chromatogram obtained. The primer used was 18s5F- 5' CTGGTTGATYCTGCCAGT as sequencing primer.

2.4 Local sequence alignment

BLAST (Altschul *et al*., 1990) was performed for *Eudrilus eugeniae* 18s rRNA sequence to identify its relatives in different species *using* BLASTn (nucleotide BLAST) tool of NCBI (http://www.ncbi.nlm.nih.gov). Identification of highest similarity and identity to *Eudrilus eugeniae* can be done using BLAST (Basic Local Alignment Search Tool) with a word size of 28 and expected threshold 10.

2.5 Multiple sequence analysis

Multiple sequence alignments are now one of the most widely used bioinformatics analyses. They are needed routinely as parts of more complicated analyses or analysis pipelines and there are several very widely used packages, e.g. Clustal W (Thompson *et al.*, 1994) and Clustal X (Thompson *et al.*, 1997). The ClustalW tool of EBI was used to perform multiple sequence alignment with gap open cost 10.0 and gap open extension cost 0.1 of eleven sequences of earthworm including *Eudrilus eugeniae.*

2.6 Phylogenetic analysis

The resulting alignment of eleven sequences was used for the construction of phylogenetic tree with MEGA software (Neighbor joining method) (Maddison *et al*., 1997). Phylogenetic analysis of *Eudrilus eugeniae* 18s rRNA sequence was carried out using MEGA software. Phylogenetic tree was constructed by the software showing the ancestral relationship among the sequences.

[III]RESULTS

PCR amplification studies revealed that genomic 18s rRNA region was amplified using two primers 18s5-F and 18s1100R specific to 18s rRNA region. The amplified product was used for further analysis. The size obtained was approximately 1000bp for 18srRNA region [Fig. 1]. The genomic 18s rRNA of *Eudrilus eugeniae* was sequenced using Applied Biosystems 3730xl DNA Analyzer USA and chromatogram obtained. The 18s rRNA gene was found to be of 570 base pairs which can be used for further analysis. Local sequence alignment of *Eudrilus eugeniae* showed 96% identity with *Dichogaster saliens* and 95% identity with the other nine species with a query coverage of around 88% and e value 0.0 using BLAST tool [Fig. 2]. Multiple sequence analysis of *Eudrilus eugeniae* along with its close homologs was subjected to multiple alignments to find out the identity among all the species and their evolutionary relationship. It showed 90-99% identity between all the eleven species. A phylogenetic tree with MEGA software of *Eudrilus eugeniae* and its closely related species was constructed. The tree gives different cluster showing their relationship with each other. The sequences which lie in the same cluster are closely related. The molecular phylogeny of *Eudrilus eugeniae* had been

determined by analyzing 18S rRNA gene sequence. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.16172589 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown above the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2011) and are in the units of the number of base substitutions per site. The analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 556 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Felsenstein, 1985) [Fig. 3].

[IV] DISCUSSION

Erseus and Kallersjo (2004) have studied 18s rDNA phylogeny of Clitellata (Phylum Annelida). In the 18S tree they have observed a sister group relationship to other Clitellates. They further stated that the sister group relationship between the enchytraeid earthworm clade and the lumbricid leech clade was supported. James and Davidson (2012) established relationship in the earthworm families. They analyzed the molecular data from 14 Crassiclitellata families representing 54 genera. They have shown a strong support for Metagynophora consisting of Crassysclitellata Monoligastridae with the help of 28S, 18S and 16S gene sequences. Novo *et al*., 2011 studied the biogeography of group of earthworms in Mediterranean basin and also studied phylogenetic puzzle of Homogastridae Clitellate earthworms. Considering the importance of exotic species *Eudrilus eugeniae* phylogenetic relationship, the present study advocates the use of neighbor joining method to investigate the role of earthworms that might be played by evolutionary correlation in facilitating recycling of the organic waste and accomplishment of morphological disparity in various evolutionary lineages.

[V] CONCLUSION

On the basis of position of sample sequence of *Eudrilus eugeniae* in the phylogenetic tree, it can be concluded that *Eudrilus eugeniae* showed closest similarity to *Dichogaster saliens.* Our phylogenetic analysis suggests that *Eudrilus eugeniae*, a very close relative to *Dichogaster saliens and Eisenia fetida* form a separate clade in the phylogenetic tree. Further responsibities remain open for the molecular biologists to undertake research in the field of molecular phylogenetics in accordance with complete gene prediction through bioinformatics tools are necessary.

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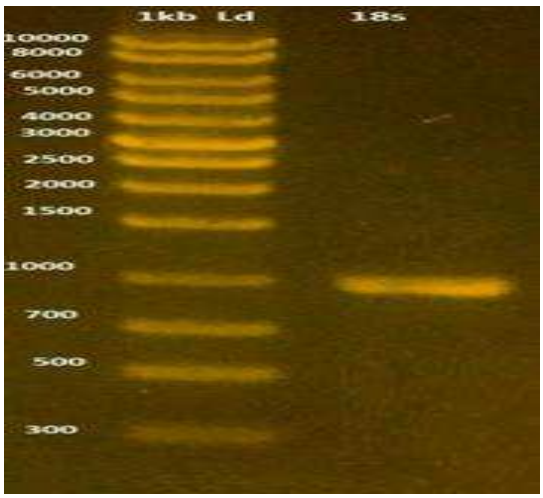

Figure. 1: 18s rRNA amplification in earthworm
Lane 1: 1Kb DNA marker (300bp-10000bp), Lane 2: 18s rRNA PCR product for earthworm

Accession	Description	Query coverage	Max ident
AY365463.1	*Dichogaster saliens* 18S ribosomal RNA gene, partial sequence	88%	96%
GU931690.1	*Pontodrilus litoralis* 18S ribosomal RNA gene, partial sequence	86%	95%
AY365462.1	*Pontodrilus litoralis* 18S ribosomal RNA gene, partial sequence	86%	95%
AJ272312.1	*Octolasium lacteum* 18S rRNA gene	88%	95%
HQ621907.1	*Hormogaster redii redii* isolate IGL1 18S ribosomal RNA gene, partial sequence	88%	95%
HQ621905.1	*Hormogaster redii redii* isolate GHI2 18S ribosomal RNA gene, partial sequence	88%	95%
HQ621895.1	*Hormogaster redii redii* isolate ALG1 18S ribosomal RNA gene, partial sequence	88%	95%
AB558505.1	*Eisenia fetida* gene for 18S ribosomal RNA, partial sequence	88%	95%
AB076887.1	*Eisenia fetida* gene for 18S rRNA, partial sequence	88%	95%
X79872.1	*Eisenia fetida* 18S rRNA gene	88%	95%

Figure. 2: Local alignment of *Eudrilus eugeniae* using BLAST tool

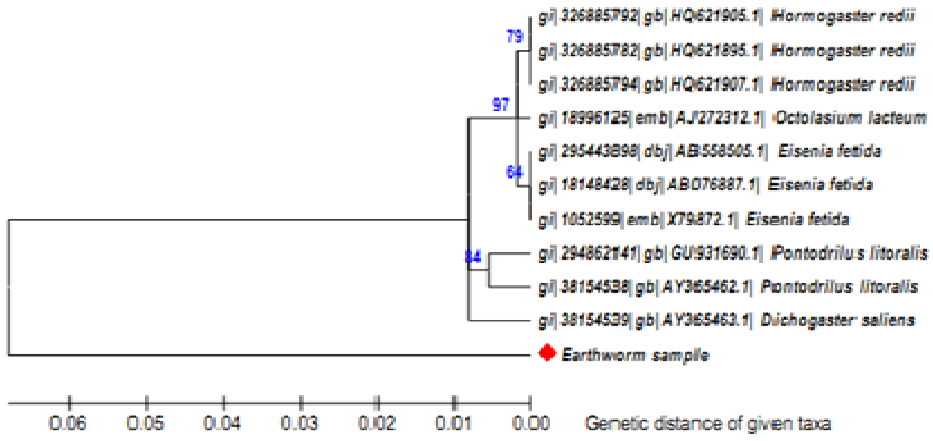

Figure 3: Evolutionary relationship of *Eudrilus eugeniae*