e-ISSN:2321-6190
p-ISSN:2347-2294

Research & Reviews: Journal of Zoological Sciences

A Simple Protocol to extract DNA from earthworm tissue for Molecular studies

Kushwaha T, Vishwakarma A, Rahul Paliwal[1], Sashidhar Burla[2] and Shweta Yadav[3]*

[1]Zoological Survey of India, High Altitude Regional Centre, Solan (HP), India

[2]The Energy Research Institute, New Delhi, India

[3]School of Biological Sciences, Dr HS Gour Central University, Sagar (MP), Pin Code-470003, India

Research Article

Received date: 12/11/2015

Accepted date: 21/12/2015

Published date: 25/12/2015

***For Correspondence**

Associate Professor in Zoology, School of Biological Sciences, Dr H S Gour Central University, Sagar (MP), Pin Code-470003, India, Tel: 09479983812

E-mail: kmshweta3@yahoo.com

Keywords: Organic matter, Genotoxicity

ABSTRACT

A simple protocol has been developed to extract high quality of DNA from different species of earthworms of family Moniligastridae (*Drawida travancorensis D.pellucida*, *D.parambikimala, D.modesta*); Megascolecidae (*Perionyx sansibaricus)* Octochaetidae (*Travoscolides chengannures*)*;* Lumbricidae (*Octolasion cyaneum,Eisenia andrei, E.fetida*) *Eudrilidae* (*Eudrilus eugeniae*) collected from western Ghats of India. A good quantity (261.6 to 151 µg/µl) of DNA was obtained. With present modified protocol. Ratio of A260/A280 was also in range of 1.91-1.98 while, routine protocol was recorded 1.48-2.24 µg/µl. Extracted DNA was used to amplify 683 bp of the cytochrome oxidase I (COI) gene with LCO1490 and HCO2198 primers. All sequences of amplified gene were aligned, edited and analyzed using Maximum Likelihood method to characterize different species of earthworm. It appears that modified protocol used in present study was efficient and easy to extract good quality of DNA from different species of earthworms.

INTRODUCTION

An important aspect of biodiversity is the relative importance of species in the functioning of ecosystems; this is particularly so far the soil biota which regulates organic matter and nutrient dynamics in soil. Soil bio resources have been recognized as the foundation for sustainable livelihood and food security. The importance of earthworms cannot be ignored because they have enormous potential to improve soil condition on a sustainable basis. Earthworms are ancient organism since they have been on our planet for 600 million years. They have survived through the mass extinctions, and helped life to sustain on the earth and human civilization by ploughing and fertilizing the soil. As far back as 1881, Charles Darwin, the great naturalist, observed "It may be doubted whether there are many other animals which have played so important a part in the history of the world as have these lonely organized creatures and every inch of soil on the entire planet had surely passed through the belly of earthworms several times".

Approximately, 3700 species of earthworms are known in the world but number of estimated species may be as high as 8000[1]. In India, 418 species and subspecies belonging to 69 genera have been reorganized on the basis of morphological characteristics[2]. This number is expected to rise to about 800 with extensive surveys of large unexplored areas. High earthworm diversity in India is primarily due to its geographical location with a wide latitudinal range (between 8.4˚N and 37.6˚N and longitudinal range 68.7˚E and 97.25˚E), complex topography, varied climate (ranging from temperate to arctic in the Himalaya to tropical in the peninsular India) and past geological history that is linked to ancient super continent of Gondwana land from which it separated in the late Jurassic and drifted to collide with the Asian mainland in the Eocene[3].

Earthworm are also used as significant model organism in ecotoxicology, physiology, biochemical and genetic studies[4,5] "The Genetic makeup of different strains in relation to environment also influences their effectiveness in bioconversion process and fertility management of soils"[6-10]. Species identification of adult earthworms is possible only by dissection of the anterior end. However, this method is labor intensive, time consuming and very difficult for non-specialists, particularly when dealing with field collections consisting of several different earthworm species. Furthermore, identification is limited to adult worms as most

life stages are unidentifiable, many morphological and anatomical characteristics of earthworms are variable[3]. Consequently the degree of variability can differ and features can overlap the taxa[11]. Presently, molecular marker including DNA barcode may be promising approach to resolve this dilemma of taxonomy[12]. However, this couldn't replace traditional taxonomy but may be a powerful tool for identifying species of earthworms.

In general, a molecular technique requires isolation of Genomic DNA for characterization and also to quantify genotoxicity of pollutant-induced DNA modifications. Therefore, isolation of high quality of DNA is an essential process for molecular studies. Several protocols have been established to isolate pure and integral DNA from plant tissues, bacteria, blood and many other animals including insects and mammalian tissues[13-21]. But for earthworms studies are still limited. In present study we have developed a simple protocol to isolate high quality of DNA from different species of earthworms in short period. Which is modified CTAB (Cetyltrimethyl ammonium bromide) method, described by Murray and Thompson (1980) generally used for extracting plant DNA[22]. In general, the CTAB genomic DNA extraction method involves a cell lysis along with Chloroform: Isoamyl alcohol and use of proteinase K. Results were compared with commercially available kits and Original CTAB Method (Doyle & Doyle, 1987)[23].

MATERIALS AND METHODS

Different species (*Drawida travancorensis, D.pellucida*, *D.modesta, D.parambikulamana;Travoscolides chengannures*; *Perionyx sansibaricus*; *Octolasion cyaneum*; *Eisenia andrei; Eisenia fetida; Eudrilus eugeniae*) of earthworms(2-5 in numbers) were collected from Western Ghats (Wayanad forest, Kerala), India and preserved in 100% ethanol. Extraction of DNA and their amplification for *coi-* I *gene* was carried out at The Energy Resources Institute, New Delhi, India.

1 M Tris HCl (pH-8.0); 5M Sodium chloride (NaCl); 0.5 M Ethylene diaminetetra acetic acid (EDTA) pH-8.0; CTAB (cetyltrimethyl ammonium bromide); Chloroform: isoamylalcohol (24:1); Ethanol; 1xTE buffer (pH-8.0); 1 M Tris Hcl, 0.5 M EDTA; Agarose (molecular grade) were used for extraction of DNA. The universal primers LCO1490 and HCO2198 (Folmer *et al*. 1994) were used to amplify 683 bp of cytochrome oxidase I (COI) gene. PCR reactions consisted of 0.3 µl (± 30 ng) DNA template, 12.5 µl PCR Master Mix (Xceleris), 11 µl nuclease free water and 10 pmol (1 µl) of each of the primer. PCR cycling comprised an initial denaturation step at 94°C for 4 min followed by 35 cycles at 94°C for 1 min, 45°C for 1 min and 72°C for 1 min. A final extension step at 72°C for 10 min followed by 4°C for 10 min completed the reactions. The PCR amplified products were ran on 1% agarose gel electrophoresis containing ethidium bromide, DNA staining dye and the photograph was taken under transilluminator emitting UV light. All the sequences were aligned, edited and analysed in MEGS v5.The steps followed in extraction of DNA are as under:

- Earthworm tissues of different species up to 1 cm were taken in clean & dry eppendrof tube.
- Added 200 µl of 1xTE buffer (1 M Tris Hcl, 0.5 M EDTA bring upto volume to 1 L with ddH_2O as suggested by Doyle & Doyle, 1987).
- Added 500 µl CTAB buffer (1 M Tris Hcl, 5 M NaCl, 0.5 M EDTA 20% Cetyltrimethyl ammonium bromide (CTAB) bring total volume to with dd H_2O followed Doyle & Doyle, 1987).
- 3-5 µl Proteinase K (20 mg/ml) added.
- Vigorously vortex.
- Added RNase (2 µl) (10 mg/ml) and incubate at -20°C for 1 hour or overnight. (RNase was also added to prevent RNA contamination).
- Incubated at 55°C for 1 hour.
- Mixed Intermittently.
- Added equal volume of Chloroform: Isoamylalcohol (24:1).
- Centrifuged at 14000 rcf (Relative centrifugal force) for 7 minute.
- Transferred aqueous phase to a fresh centrifuge tube and above step repeated twice.
- Added 35 µl of chilled 7.5 M ammonium acetate, 200 µl isopropanol (0.5 volumes). And left DNA to settle down for 20 minute.
- Centrifuge at 14000 rcf for 10 minute.
- Supernatant was discarded.
- Added 500 µl 70% ethanol.
- Centrifuge at 14000 rcf for 3 minute.
- Discarded the supernatant.
- Added equal volume of the 100% ethanol.

e-ISSN:2321-6190
p-ISSN:2347-2294

- Centrifuge at 14000 rcf for 3 minute.
- Discarded the supernatant.
- The tube was kept for drying at room temperature.
- Dry pellet or DNA dissolve in 30 µl 1X TE (10 mM Tris.HCl and 1 mM EDTA) buffer and incubated for 2 hour at room temperature.
- Stored at -20°C for further use.

RESULTS AND DISCUSSION

DNA was quantified by measuring optical density (O.D) at A260 and A280 with a nanodrop. Samples were subjected to electrophoresis in 1X TE buffer for 30 minute at 110v. 2 µl6x loading dye with 3 µl of the isolated genomic DNA was loaded on 1% agarose gel stained with Ethidium bromide to check quality of DNA .The gels were photographed **(Figure 1)** on gel documentation system (protein simple Fluor chem. M).The average yield of total nucleic acid ranges 261.6 µg/µl to 1517.6 µg/µl **(Table 1).**

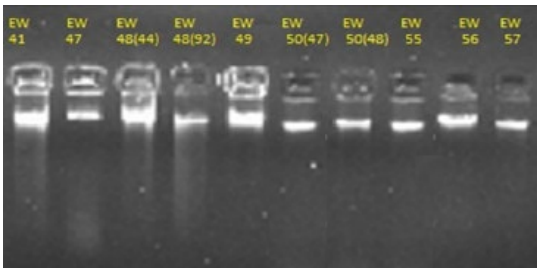

Figure 1. High molecular weight total DNA extracted with modified protocol from different species of earthworms and were ran on 1% agarose gel electrophoresis containing Ethidium bromide, DNA staining dye and the photograph was taken under tranilluminator emitting UV light.

Table 1. Quantitative estimates of DNA concentration (µg/µl) observed under NanoDrop 2000c/Uv-vis spectrophotometer.

SNo	Sample Code	With CTAB method (µg/µl)	With modified protocol	A260/A280 (with CTAB Method)	A260/A280 (with modified protocol)
1.	EW41-A43	6.8	731.3	2.24	1.98
2.	EW47-A42	20	556	1.64	1.96
3.	EW41-A36	20.1	1047.0	1.99	1.93
4.	EW49-A46	30.7	1517.6	1.98	1.94
5.	EW74A-A28	63.2	455	1.68	1.91
6.	EW48-A92	84.8	311.5	1.97	1.96
7.	EW33-A7	25.1	284.5	1.74	1.94
8.	EW65-A13	24.2	261.6	1.95	1.98
9.	EW34-6	12.5	642.6	1.48	1.94
10.	EW71-A25	65.2	465	1.68	1.91

This is much higher than those obtained with commercial kits and other available methods. The A260/A280 ratio was in the range of 1.91 to 1.98 which indicated the purity of the nucleic acid. Whole procedure completed within 340 minutes however others take about 430 minutes. The DNA Extracted by this method yielded reproducible bands proving its appropriateness for PCR amplification **(Figure 2)** using gel extraction. Sequencing chromatogram was showed clear peaks without mixed/uninterrupted reads **(Figure 3).**

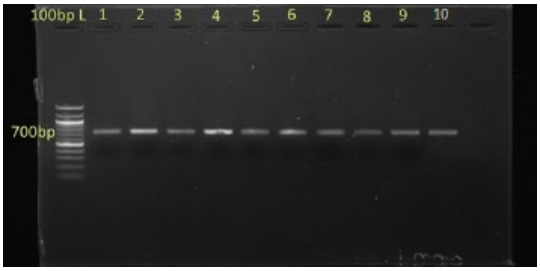

Figure 2. PCR amplification of mitochondrial cytochrome c oxidase I (COI) gene using specific primers
The partial sequences of mitochondrial cytochrome c oxidase I (COI) gene has been amplified using LCO1490 FP and HCO_2 198 gene specific primers at an annealing temperature of 45°C. The PCR amplified products were ran on 1 % agarose gel electrophoresis containing Ethidium bromide, DNA staining dye and the photograph was taken under tranilluminator emitting UV light.

e-ISSN:2321-6190
p-ISSN:2347-2294

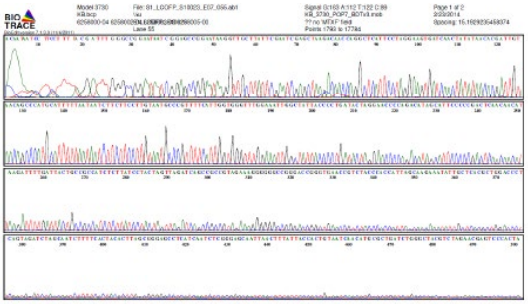

Figure 3. Sequencing chromatogram of EW sample using LCO1490 forward primer showing clear peaks without mixed/uninterrupted reads. GenBank accession descriptions of the sequences from earlier studies are shown in the phylogenetic tree. Out group used here is Sepia aculeata COI gene, cuttlefishes (Mollusca). The value at the branches shows the ML bootstrapping values given in percentages. Bar 1 changes/10 characters.

GenBank accession description of the sequences from earlier studies is shown in the phylogenetic tree **(Figure 4).** Out group used here is *Diplocardia komaerki* COI gene gi.82400071. The value at the branches shows the ML bootstrapping values given in percentages. Bar 5 changes/100 characters. The maximum likelihood tree inferred from *coi* –I gene DNA sequences showing the phylogenetic position of Sample EW-41-A34,EW-47-A42, EW49-A46 and EW-41-A36 of *Drawida travancorensis, D.pellucida* , *D.modesta, D.parambikulamana* respectively; EW-74A-A28 for *Travoscolides chengannure*s; EW-48-A46 for *Perionyx sansibaricus* ; EW-33-7 *for Octolasion cyaneum*; EW-65-A13 *Eisenia andrei; EW-34-6 Eisenia fetida;* EW-71-A25 of *Eudrilus eugeniae*. Study confirms modified protocol may helpful to extract high yield of DNA for different species of earthworms available at different ecological niches. This may be used to identifying earthworms and also for other applied studies.

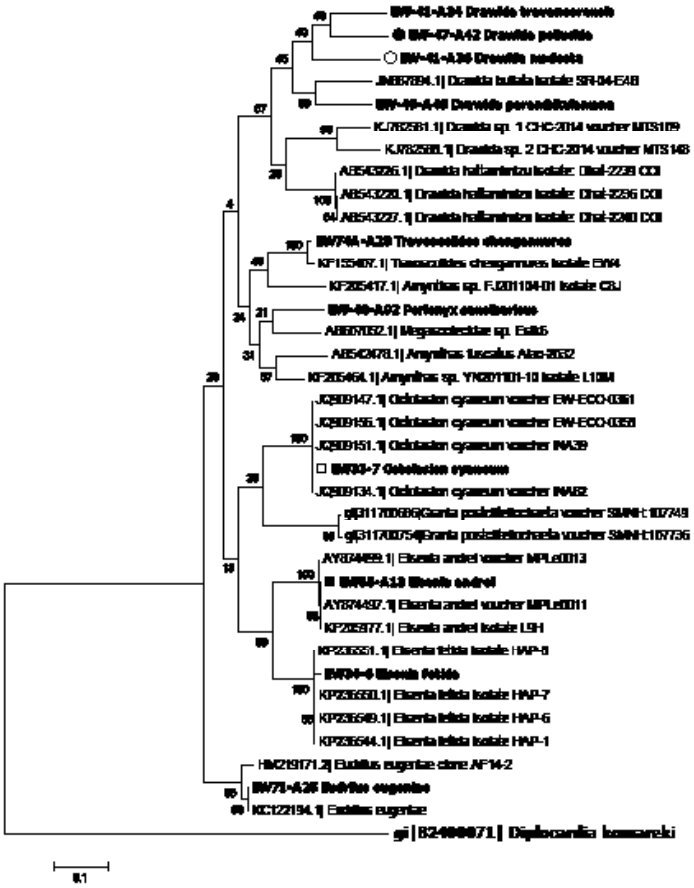

Figure 4. Maximum likelihood phylogenetic analysis Perionyx sansibaricus (EW 48-A92) GenBank accession description of the sequences from earlier studies are shown in the phylogenetic tree. Out group used here is Sepia aculeata COI gene, cuttlefishes (Mollusca). The value at the branches shows the ML bootstrapping values given in percentages. Bar 1 changes/10 characters.

CONCLUSIONS

The present modified CTAB protocol with reduction in number of handling steps, and elimination of long incubations yielded good quality of DNA from different species of earthworms of different ecological niches in short period. This may be helpful to study earthworms for molecular and other purposes. Absence of toxic chemicals makes a noticeable alternative for the extraction of high quality and yield of nucleic acid from different species of earthworms in general.

e-ISSN:2321-6190
p-ISSN:2347-2294

ACKNOWLEDGEMENT

We acknowledge the financial support of the Department of Biotechnology, Ministry of Science and Technology, Govt. of India, New Delhi, to carry out this study.

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