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Molecular Characteristics of *Eisenia fetida* (Haplotaxid; Lumbricidae) and Electrophoretic Pattern of Glycolipoprotein Complex of G-90

Somayeh Zare Pourshob[1], Behnam Meshgi[1*], Parviz Shayan[1], Gholam Reza Hassanpour[2]

[1.] *Department of Parasitology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran*

[2] *Center for Research of Endemic Parasites of Iran, Tehran University of Medical Sciences, Tehran, Iran*

Abstract

BACKGROUND: A large part of our country's waste is organic matter, so researchers are studying fertilizer production from organic waste in various ways, including compost and vermicompost. On the other hand, glycolipoprotein extract of *Eisenia fetida* (G-90) has been defined to show numerous biological activities, e.g., anticoagulation, fibrinolysis, and anti-oxidative, etc.

OBJECTIVES: The purpose of the present study was to determine phylogenetic relationship of the Iranian isolate of *Eisenia fetida (E. fetida*) with the other available taxa and to determine the G-90 protein complex for evaluation of its biological activities.

METHODS: A piece of 1×1cm clitellum was separated and used for DNA extraction after homogenate preparation (by two different methods). The second internal transcribed spacer of the nuclear ribosomal DNA (rDNA-ITS2) and cytochrome C oxidase subunit 1 of the mitochondrial DNA (mtDNA-COX1) from adult *E. fetida* were amplified using polymerase chain reaction (PCR). Subsequently, PCR products were sequenced and their phylogenetic relationships were determined. Furthermore, The G-90 protein complex was extracted from adult worms and electrophoretic pattern of proteins was obtained by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

RESULTS: The electrophoretic pattern of glycolipoprotein G-90, a protein complex, showed 10 bands with molecular weights of 14-130 kDa. ITS2 and COX1 sequences were 516 bp and 277 bp long, respectively. The amplified DNA sequences from both ribosomal and mitochondrial sequences had 88%-99% and 99% similarity to relevant sequences in GenBank, respectively.

CONCLUSIONS: mtDNA-COX1 showed no considerable sequence variations as compared to other isolates in GenBank, while rDNA-ITS2 exhibited more variations, in comparison with other isolates, indicating more variations among some of these isolates. Our results revealed that rDNA-ITS2 of our *E. fetida* was in a separate subclade, showing the greatest similarity with EF534709.1 isolate (99%) provided in GenBank, followed by JX531618.1 (94%), and KU708469.1 (88%). Our COX1 sequence demonstrated the high similarity of 99% when compared with five isolates from GenBank (MH475674.1, MH475673.1, MH475672.1, MH475670.1, and MH475666.1). The G-90 glycolipoprotein showed different proteins that can be assessed for their potential biological activities in medicinal properties.

KEYWORDS: Earthworm, *Eisenia fetida*, G-90, Molecular characteristics

Correspondence
Behnam Meshgi, Department of Parasitology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
Tel: +98 (021) 61117070, Fax: +98 (021) 66933222, Email: Bmeshgi@ut.ac.ir

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Introduction

Earthworms belong to the phylum of Annelida and near 700 genera of them have been recognized up to now, but estimated number of earthworm species may reach up to 7000 species (Pechenik, 2009). These worms have a cylindrical body comprised of many segments (80-190). There are specific loops at the anterior part of their body called clitellum which has a particular role in sexual reproduction of some annelids. Earthworms are regarded as one of the most important types of living creatures in the soil and play a key role in vermicompost production (Karmegam *et al.*, 2019; Sharma and Garg, 2019). This process consists of oxidating and stabilizing organic wastes through routine activities of earthworms and other microorganisms.

Eisenia fetida (Phylum Annelida, Family Lumbricidae) is regarded as one of the most important species of earthworms and used in vermicompost process, fish feed (Musyoka *et al.*, 2019) and poultry (Gunya *et al.*, 2019) as well as in medicine. The protein complex of G-90 is a mixture of macromolecules with glycolipoprotein extracted from tissues of the mature worm (Grdisa and Herzenjak, 2007).

G-90 can be used for the treatment of dermal wounds due to its component involving in accelerating the cellular proliferation (Deng *et al.*, 2018; Song *et al.*, 2015) and enhancing the synthesis of different growth factors (e.g., fibroblast growth factor [FGF] and epidermal growth factor [EGF] in cell cultures). In fact, EGF plays its roles in this process by stimulating epithelial cells proliferation, and FGF through veins elimination, and increasing fibroblasts growth (Grdisa *et al.*, 2004). G-90 has many bioeffects such as insulin-like protein activities accompanied with mitogenic effects (DiCicco-Bloom and Black, 1988), anti-oxidative activities, hemostasis regulation, hemolytic effects (Wang *et al.*, 2019), bacteriostatic and bacteriolytic activities (Tutar and Karaman, 2017), anti-inflammatory and antipyretic effects (Balamurugana *et al.*, 2009), fibrinolytic and anti-coagulant activities (Akazawa *et al.*, 2018; Popovic *et al.*, 2001), therapeutic effects on tumor cells (Chen *et al.*, 2007; Cooper *et al.*, 2004), proliferative effect on cells (Permana et al ., 2018; Herzenjak *et al.*, 1993), and enhancing cell adhesion (Popovic *et al.*, 1998) as well as stimulating the regeneration of peripheral nerves through Schwann cells migration (Chang *et al.*, 2009; Bhambri *et al.,* 2018; Moon and Kim, 2018). Lumbrokinases are a group of enzymes with molecular weights of 25-32 kDa isolated from coelomic fluid and intestinal tissue of *E. fetida* (Mihara *et al.*, 1991), which has been used as a thrombolytic drug in patients with acute and chronic thrombotic disorders.

The current study aimed to identify adult worms using molecular techniques according to the second internal transcribed spacer of the nuclear ribosomal DNA (rDNA-ITS۲) and cytochrome C oxidase subunit ۱ of the mitochondrial DNA (mtDNA-COX۱) from adult *E. fetida* and to determine electrophoretic pattern of glycolipoprotein extract (G-۹۰) for assessment of their potential biological activities in near future.

Materials and Methods

1. Preparation of G-90

The fresh *E. fetida* earthworms were collected from an earthworm farm in Karaj, Iran. They were kept under optimal conditions in the laboratory to breed (Lowe *et al.*, 2014). Ten *E. fetida* earthworms were washed with 0.6% sodium chloride solution, to cleanse their intestine and body surface. They were cut into pieces of about 1-2 cm long and then homogenized with the homogenizer. Samples were transferred to a beaker containing methanol-chloroform solution in a 1:1 ratio and left at 4℃ overnight. Then distilled water was added until it reached the total volume of 20 ml. The mixture was centrifuged at 4000 rpm for at least 15

min. Three clearly visible layers were obtained. The upper layer was removed and methanol evaporated almost completely. The remaining sample containing G-90 was stored at -20℃ until use.

2. Electrophoretic Pattern of G-90

The extracted G-90 was loaded onto SDS-PAGE gels and ran according to Laemmli (1970). Electrophoretic patterns of the samples with different concentrations were screened under reducing conditions by discontinuous buffer system in a Mini-protean III cell apparatus (Bio-Rad) at 110v constant voltage for 60 min. SDS-PAGE was performed with a 12% resolving gel and a 5% stacking gel. A protein marker covering a wide range of molecular weights from 10-200 kDa (Fermentas, SM 0661) was used to determine the molecular weights of the proteins. The gels were stained with coomassie blue and then photographed.

3. DNA Extraction and PCR

DNA was extracted from clitellum of a single mature *E. fetida* using a DNA extraction kit (MBST, Iran) according to the instructions of the manufacturer. DNA was extracted using two different methods: isoamyl alcohol/chloroform and lysis techniques. The first method: isoamyl alcohol/chloroform (1:24) was added and mixed well by inversion. The contents were centrifuged at 8000 rpm for 10 min and the supernatant aqueous layer containing DNA was transferred to another centrifuge tube and 2/3rd volumes of cold isopropanol was added, mixed by inversion to precipitate the nucleic acids. All DNA samples were stored at -20℃ until further studies. DNA fragments of r-ITS2 and mt-COX1 were amplified based on the specific primer design including: EfI: (forward; 5′-CGATGAAGAGCGCAGCCAGC-′3) and (reverse; ′5- CTGAGGGAATCCTTGTTAG-′3) for ITS2, and EfC: (forward; ′5-GAGCTAAGACAACCAGGTGC-′3) and (reverse; ′5- GGCTAGGTCTACTGAGGGC-′3) for COX1. The PCR reaction was performed in a final volume of 50µl, containing 25µl of Taq master mix (Sinaclon, Iran), 2µl of each primer (10µM each), 10µl of template DNA using an automated thermocycler under the following thermal conditions: 5 min incubation at 94℃ as initial denaturation step to denature the double stranded DNA, followed by 35 cycles of 94℃ for 45s (denaturation step), 57℃ for 45s for COX1 primers and 53℃ for 45s for ITS2 primers (annealing step), and 72℃ for 45s (extension step). Lastly, the PCR was completed with a final additional extension step at 72℃ for 10 min. Samples without genomic DNA were used as negative controls and a DNA marker was used to show the length of the ITS2 and COX1 amplicons. The PCR products were electrophoresed using 1% agarose gels in 0.5x TBE buffer and thereafter visualized using Syber Safe stain (Sinaclon, Iran) on a UV illuminator. Consequently, the PCR products were purified using a quick PCR product purification kit (MBST, Iran) based on the instructions of the manufacturer.

4. Sequencing and Phylogeny

s method, genomic′According to Sanjer DNA sequencing was performed in both directions for positive PCR amplicons by the Kawsar Biotech CO. (Tehran, Iran). The samples were assessed using Basic Local Alignment Search Tool (BLAST) and Bioedit software. The sequences were aligned and compared with each other and those of *Eisenia* spp. isolates as previously submitted to GenBank. The phylogenetic relationships of all these *Eisenia* isolates were generated by Mega 6 software based on ITS2 and COX1 sequences.

Results

The results of this study are presented in two parts comprised of protein electrophoretic pattern of G-90 complex and molecular characteristics of *E. fetida*.

1. Electrophoretic Pattern of G-90

The electrophoresis of the glycolipoprotein -G The electrophoresis of the glycolipoprotein

G-90 showed 10 bands with approximate molecular weights of 14-130 kDa extracted from adult *E. fetida* (Figure 1).

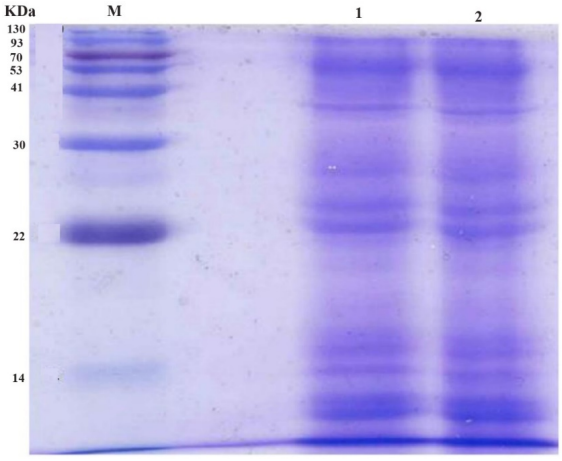

Figure 1. SDS-PAGE analysis of G-90 complex extracted from the Iranian isolate of *E. fetida*

Lane 1: Sample (Dialysis bag), Lane 2: Sample (Filter paper), M: Marker (Fermentas-SM 0661).

2. Molecular Characteristics

2.1. DNA Extraction

Genomic DNA was extracted from a single Iranian isolate of *E. fetida*. The clitellum was the best part for high quality DNA extraction. The samples were loaded onto 1% agarose gel and were run for 45 minutes at a constant voltage of 100 v. Subsequently, the gel was stained with Syber Safe to check the presence of DNA in the sample. Direct lysis was a better DNA extraction method than isoamyl alcohol/chloroform one (Fig. 2A).

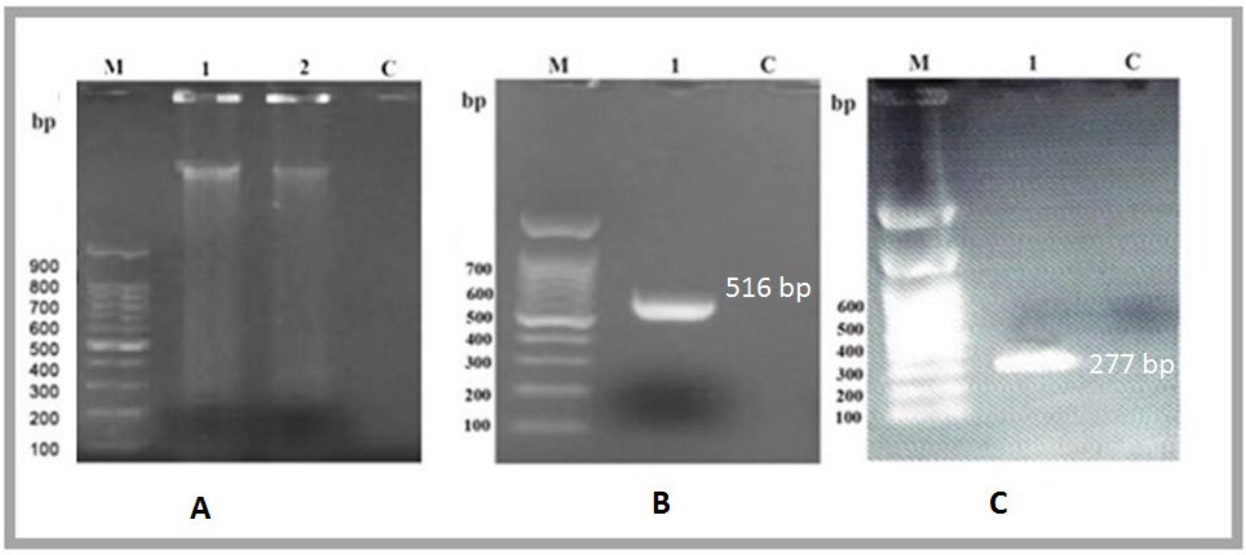

Figure 2. DNA was extracted from adult *E. fetida* and analyzed on 1% agarose gel (A). The extracted DNA was amplified using specific primers derived from COX1 (B) and ITS-2 (C) by PCR. Lane 1-2: Samples, lane C: Negative control, lane M: DNA size marker (100 bp DNA ladder).

2.2 PCR Analysis and Sequencing

PCR was performed to amplify 516 bp long ITS2 and 277 bp long COX1 fragments of *E. fetida*. The results are shown in Figure 2 B and C. Sequences obtained in the present study were recorded in GenBank with accession numbers of MN989855 and MN989928 for ITS2 and COXI, respectively. These sequences were compared with available data in GenBank for ITS2 (Accession numbers: EF534709.1, JX531618.1 and KU708469.1) and COX1 (Accession numbers: MH475674.1, MH475673.1,

MH475672.1, MH475670.1, and MH475666.1). The BLAST analysis demonstrated that *E. fetida* isolate (EF534709.1) had higher similarity with our ITS2 sequence (99%), followed by JX531618.1 (94%), and KU708469.1 (88%).

COX1 nucleotide sequence showed a high similarity of 99%, compared with five isolates from GenBank (Figures 3 and 4).

All available ITS2 and COX1 nucleotide sequences were used to generate the following phylogenetic trees (Figure 5).

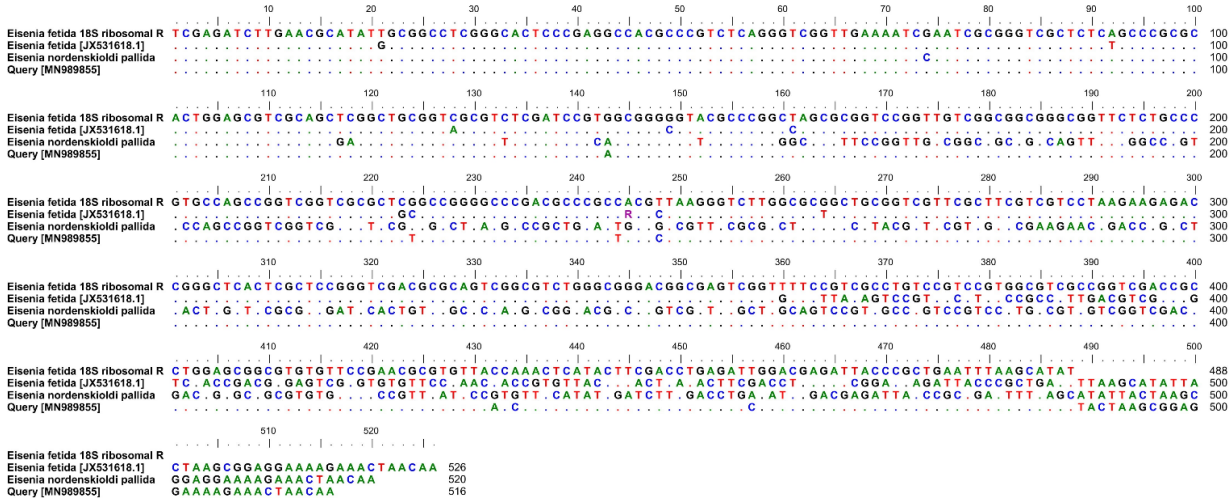

Figure 3. Alignment of the Nucleotide sequence of rDNA-ITS2 of the Iranian isolate of *E. fetida* with other sequences recorded in GenBank using reference sequences from GenBank.

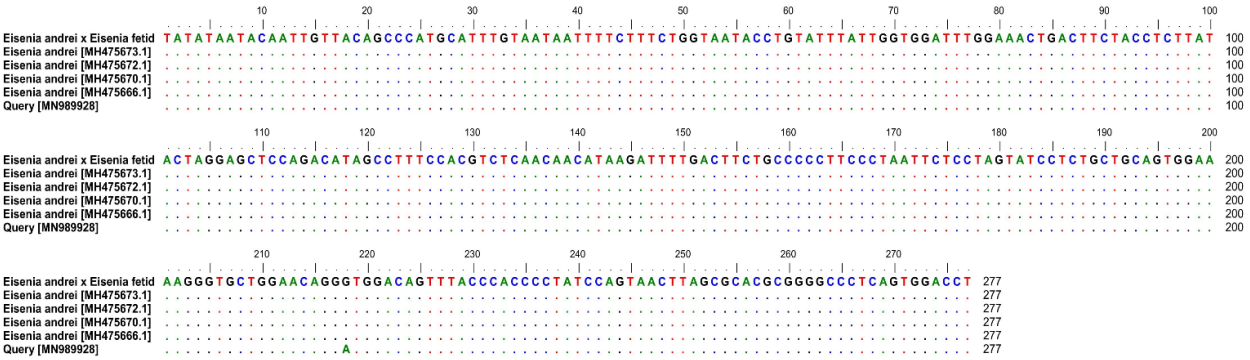

Figure 4. Alignment of the Nucleotide sequence of mtDNA-COX1 of the Iranian isolate of *E. fetida* with other sequences recorded in GenBank using reference sequences from GenBank.

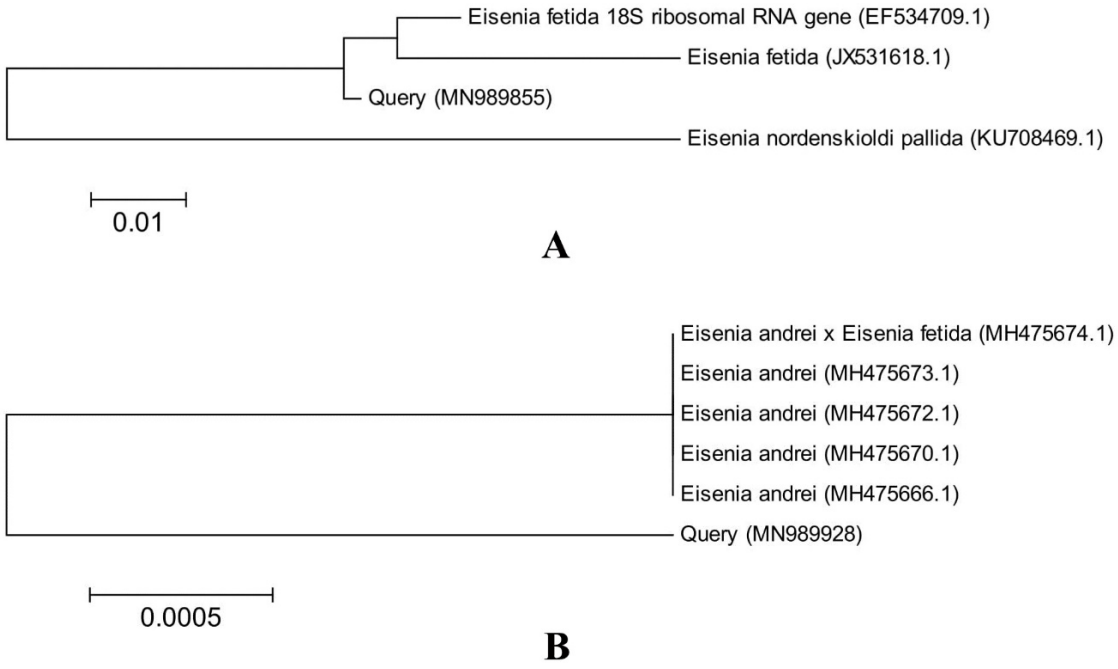

Figure 5. Phylogenetic relationships of the Iranian isolate of *E. fetida* based on ITS2 sequences (A) and COX1 (B) in comparison with other isolates.

We applied the NCBI-BLAST program, restricting searches to species of *Eisenia*, to retrieve a representative set of *Eisenia* sequences of the COX1 gene for phylogeny analysis. In the global sequence alignment, *Eisenia* isolates were found in database. We aligned short amplicon-based consensus sequences of *Eisenia* species isolates with COX1 derived isolates from our study. A tree was generated based on restricted sequence alignment (Fig. 5B), that revealed a high similarity of our isolate with *Eisenia* isolates. Sequences were assignable to identify of about 99% for each sequence, where all obtained isolates of NCBI database were included in a cluster.

Three isolates from the ITS2 nucleotide sequences, as well as our isolate (ITS2) were included in phylogeny analysis that exhibits some subclade of *Eisenia* sp. ITS2 demonstrated more differences in sequence than COX1 as seen in sequence alignment and phylogenetic tree (Fig. 5A). Additionally, two sequences from the *E fetida* isolates (EF534709.1; JX531618.1) are placed in a subclade, while our query (MN989855) was placed in another subclade. On the other hand, *Eisenia nordenskioldi pallida* isolate (KU708469.1) was also placed in a separate subclade. Similarities of sequences to our ITS2 isolate ranged from 88% to 99%.

Discussion

Earthworms have been used for many years in empirical approaches and biological sciences. Due to significant advances in various scientific fields, the use of these worms in industry and medicine is increasing exponentially due to their compost's fertilizing properties and biological activities of earthworm extracts (e.g., G-90 protein: anticoagulation, fibrinolysis, anti-oxidative, and bacteriostatic, etc.).

Two main goals of the present study were to perform phylogenetic analysis of the Iranian isolate of *E. fetida* and preparation of somatic proteins of the earthworm for the future use. DNA extraction of *E. fetida* was performed via two different techniques including lysis and

isoamyl alcohol/chloroform methods. However, lysis method showed significantly better results and the extracted DNA had higher quality. Two earthworm species were commonly used in ecotoxicology, physiology, biochemistry and genetic studies which are *E. fetida* and *E. andrei* (Albani *et al.*, 2003).

The Phylogenetic analysis of these 2 species was performed based on their nuclear and mitochondrial DNA sequences (Perez-Losada *et al.*, 2005). In the present study, the phylogenetic trees showed that nucleotide sequences of nuclear ribosome DNA had more variability as compared to that of mitochondrial DNA; therefore, our ITS2 isolate showed 88% to 99% similarity, as compared to other ITS2 sequence registered, while the COX1 sequence revealed high level of similarity (99%) as compared with other COX1 sequences as recorded previously at nucleotide sequence level and 100% similarity at amino acid level.

Earthworms have the greatest impact on soil, such as soil fertilization by digging holes in soil and mixing organic materials and nutrients including nitrogen, phosphor, calcium and potassium (Mora *et al.*, 2005). There have been many studies on earthworms in the recent years (Reynolds and Reynolds, 1972). Nevertheless, over the past two decades, their use has been increased significantly and certain macromolecules have been extracted from earthworms, in particular from *Eisenia* species, where a G-90 Glycolipoprotein complex has been known as a resource of biologically active molecules.

The effects of homogenized tissue of *E. fetida* (G-90) on hemostasis regulation have been approved invitro. The fibrinolytic and anticoagulative activities of G-90 can affect bleeding and coagulation process fundamentally, which is very similar to heparin, therefore G-90 can be considered as an appropriate source for human and veterinary medicine (Kawakami *et al.*, 2016). Various studies have shown that the coelomic fluid of earthworms can have many biological activities including bacteriostatic (Tutar and Karaman, 2017; Cooper *et al.*, 2004), proteolytic (Nakajma *et al.*, 1993), cytolytic and hemolytic activities (Popovic *et al.*, 2001; Prochazkova *et al.*, 2006; Matusic-Pisl *et al.*, 2011), as well as wound-healing (Goodarzi *et al.*, 2016; Popovic *et al.*, 2001) and anti-tumor activities (Liu *et al.*, 2017; Augustine *et al.*, 2017; Augustine *et al.*, 2018). Macromolecules of G-90 is capable of suppressing the growth of melanoma cells both in vivo and invitro (Herzenjak *et al.*, 1993) and its coelomic cytolytic factor (CCFI) can eliminate malignant tumor cells ((Liu *et al.*, 2017).

In the present study, 10 protein bands were separated from G-90 complex based on their molecular weights (14-130 kDa) for the future use through gel electrophoresis (SDS-PAGE).

Medina *et al.*, (2003) reported 16 protein bands (6.9-205 kDa) among which 4 bands had molecular weights of more than 60 kDa, 8 bands were about 29.1-70 kDa and molecular weights of the rest were 6.9 to 29.1 kDa. The clear and specified fragments were frequently visible in the range of 40-66 kDa. Immunoassay techniques proved the existence of insulin-like compounds in G-90 complex (Herzenjak *et al.*, 1993). The presence of immunoglobulin-like structures in G-90 was previously demonstrated (Popovic *et al.*, 1998). Fibrinolytic-serin proteases have been isolated from tissues of the worms belonging to the Lumbricidae family, such as *E. fetida*, *Lambricus rubellus* and *L. terrestris* (Faiz *et al.*, 2018; Mihara *et al.*, 1991). These enzymes converted plasminogen into plasmin and then directly lysed the existing fibrin and prolonged the coagulation process. These thrombolytic or fibrinolytic agents are urokinase-type plasminogen activator (u-PA) and lumbrokinase (Mihara *et al.*, 1991). They have an important role in epidermal wound-healing (Deng *et al.*, 2018). After purification of G-90 on Sepharose, 2 bands were identified: PI (34 kDa) and PII (24 kDa) both of which had

fibrinolytic, anti-coagulative and esterase activities (Grdisa and Herzenjak, 2007).

Extraction and isolation of a high quality DNA have been considered as critical processes in molecular studies. Several protocols have been reported for DNA extraction from plant, bacterial, blood and animal tissues, e.g., insects and mammals (Saghai-Maroof *et al.*, 1994), but DNA extraction methods for earthworms are very scarce. In the present study, two methods were used for DNA extraction from *E. fetida* earthworms, which were lysis and isoamyl alcohol/chloroform (1:24) methods. The DNA extracted by lysis method had a significantly higher quality in comparison to DNA extracted by isoamyl alcohol/chloroform method.

Conclusion

Our findings revealed no considerable variations in mtDNA-COX1 as compared to available isolates in GenBank, while rDNA-ITS2 exhibited considerable variations in comparison with the same sequence in other isolates. Our *E. fetida* placed in another subclade based on its rDNA-ITS2 sequence, revealing higher variability in comparison with other isolates. The G-90 glycolipoprotein showed different proteins that can be assessed for their potential biological activities in medicinal properties.

Acknowledgments

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Conflict of Interest

The authors declared that there is no conflict of interest regarding the publication of this article.

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خصوصیات مولکولی *ایزنیا فتیدا* و الگوی الکتروفورتیک کمپلکس گلیکولیپوپروتئین G-90

سمیه زارع‌پور شوب[۱]، بهنام مشگی[۱*]، پرویز شایان[۱]، غلامرضا حسن‌پور[۲]

[۱] گروه انگل‌شناسی، دانشکده دامپزشکی، دانشگاه تهران، تهران، ایران

[۲]مرکز تحقیقات انگل‌های بومی ایران، دانشگاه علوم پزشکی تهران، تهران، ایران

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زمینه مطالعه: بخش عمده‌ای از زباله‌های کشور ما از ترکیبات آلی تشکیل شده است، بنابراین محققان در حال مطالعه و اجرای تولید کودهای آلی به روش‌های مختلف از جمله کمپوست و ورمی‌کمپوست هستند. از طرف دیگر عصارهٔ گلیکولیپوپروتئین *ایزنیا فتیدا* (G-90) از فعالیت‌های زیستی بی‌شماری نظیر ضدانعقادی، فیبرینولیتیک، ضدتوموری، ضدالتهابی و باکتریواستاتیک برخورداراست.

هدف: هدف از پژوهش حاضر، تعیین هویت کرم بالغ *ایزنیا فتیدا* جدایهٔ ایران بر اساس ویژگی‌های مولکولی، تهیه و آماده‌سازی گلیکولیپوپروتئین G-90 و شناسایی الگوی الکتروفورتیک آن بوده است.

روش کار: کمپلکس پروتئینی G-90 از کرم بالغ استخراج و به‌منظور شناسایی الگوی الکتروفورتیک از روش سدیم دودسیل‌سولفات-پلی‌آکریل‌آمید (SDS-PAGE) استفاده شد. قطعه‌ای به ابعاد ۱×۱ سانتی‌متر از ناحیه کلیتلوم برای استخراج DNA(به دو روش مختلف) به کار برده شد. واکنش زنجیره‌ای پلیمراز با استفاده از DNA ریبوزومی (ITS2) و میتوکندریایی(COX1) انجام شد. نتایج حاصل از تعیین ترادف نوکلوتیدها و نیز روابط فیلوژنتیک بین آنها بررسی شد.

نتایج: الگوی الکتروفورتیک کمپلکس پروتئین G-90، تعداد۱۰ باند با وزن مولکولی۱۳۰-۱۴ کیلو دالتون را نشان داد. اندازه توالی ITS2 و COX1 به ترتیب ۵۱۶ و ۲۷۷ جفت باز بود. توالی‌های DNA ریبوزومی (با شماره دستیابی MN989855) و میتوکندریایی (با شماره دستیابی MN989928) در بررسی حاضر به ترتیب شباهت ۸۸٪ تا ۹۹٪ و ۹۹٪ را در مقایسه با ترادف‌های موجود در بانک جهانی ژن نشان دادند.

نتیجه‌گیری نهایی: بر اساس یافته‌های به‌دست آمده از بررسی حاضر، توالیCOX1-DNA در مقایسه با ۵ توالی موجود در بانک جهانی ژن، فاقد تفاوت معنی‌دار است، در حالیکه ترادف ITS2-DNA نسبت به ۳ توالی موجود از اختلاف بیشتری برخوردار است. بررسی درخت شجره‌شناسی ترادف‌های به‌دست‌آمده نیز نتایج حاصل را تایید می‌کند. گلیکولیپوپروتئین G-90 با توجه به فعالیت‌های زیستی بالقوه‌ای که دارد، می‌تواند در علوم پزشکی مورد استفاده قرار گیرد.

واژه‌های کلیدی: کرم خاکی، *ایزنیا فتیدا*، G-۹۰، ویژگی‌های مولکولی

نویسندهٔ مسئول: بهنام مشگی، گروه انگل‌شناسی، دانشکده دامپزشکی، دانشگاه تهران، تهران، ایران ایمیل: bmeshgi@ut.ac.ir