Iraqi Journal of Biotechnology, 2015, Vol. 14, No. 2 , 47-54

Study of Phylogenetic Tree and Morphology of *Aporrectodea* Based on Mitochondrial Marker (16S rRNA gene) in Some Area South of Baghdad/ Iraq

Najwa Sh. Ahmed[1], Nebrass Faleh Chacain[2], Falih Hamzah Edan[3],Saad M. Nada[1], Anas Noori Ibraheem[1]

[1]Biotechnology Research Center, AL-Nahrain University, Baghdad

[2] Biology dep., College of Science, AL-Mustansiriyah University

[3] Research and Development Directorate

Received: April 19, 2015 / **Accepted**: October 11, 2015

Abstract: This study aimed to show the phylogenetic structure of *Aporrectodea* genus in order to verify its cladistics nature and its taxonomic validity. In this work, collection of *Aporrectodea* genus from three locations from South of Baghdad, (AL-Karrada, AL-Zafranya and New Baghdad) are studied. First, we used usual morphological characteristics to identify each species than molecular phylogenetic analyses are based on the sequences of mitochondrial 16S rRNA gene regions and used software MEGA6 and Raptorx software. Rresults of the two methods (MEGA 6 and Raptorx software) were cluster groups (organisms of 8 sample from Group1A and Group3) in one group and with distance equal to 0.006, clustering of group 2 as a single group, and reached the highest value between group 2 and group 1(B) with distance equal to 0.272 and to move away genetic traits, Raptorx software, conformation of protein for 16S*rRNA* appeared as a result of the similarity of Mega6. The marker mitochondrial *16S rRNA* gene is a powerful tool for identifying species of earthworms and provides a useful complement to traditional morphological taxonomy.

Key words: *16S rRNA*, *Aporrectodea*, Raptorx software.

Corresponding author: should be addressed (Email:Najwaun@yahoo.com)

Introduction

The identification of adult lumbricids is principally based on the type of prostomium, arrangement of the setae, position and form of the clitellum, tubercula pubertatis, and some internal organs such as the seminal vesicles and the spermathecae. The earthworms of the family lumbricidae are essential components of the soil biological community. They play a major role in the biogeochemical cycles of terrestrial ecosystems because of their influence on microbial activity, carbon and nitrogen cycles and alteration of soil (1 and 2). *Aporrectodea rosea* (Oligochaeta: Lumbricidae) known for

its important role within the functioning of an ecosystem, and its diversity can be used as an indicator of ecosystem health (3). Many of researches identification of adult earthworms by many methods like dissection of male genitalia (4and 5), the number and location of male pores because these characteristics are related to copulation and reproduction (6 and 7), all these methods are very difficult and limited because they are found to vary among families and even with a single family. Mitochondrial DNA (mt DNA) has been widely used in studies of animals because it evolves much more rapidly than nuclear DNA, thereby resulting in accumulation of differences between closely related species (8), sequence divergence is much higher among species than within species, and mtDNA genealogies generally capture the biological discontinuities recognized by taxonomists as species. However DNA can be performed at any life stage indeed reliable identification of juvenile or even partial (9). In this study we used DNA to identified earthworm because they play a good role in soil food webs and taxonomy of earthworm has studied extensively for at least two centuries.

Materials and Methods

Samples and DNA Extraction

Earthworm were collected from three locations from South of Baghdad {AL-Karrada (Group1), AL-Zafranya (Group2) and New Baghdad (Gruop3)}, by using hand sorting method (2). The local earthworms simply obtained by digging up the soil, they placed in the collecting jars along with some of the same soil, they were obtained from approximately 10-15 earthworms were required (10). They were carefully washed with water to remove any dirt, then it was placed in a Petri dish contain 10% ethanol for 10 min. Then placed in another Petri dish contains 70% ethanol for 15 min. It was then observed under the microscope to exam some morphological studies like: Colour, Length of the body, Number the segments of Clitellum, Colour of Peristomum, Distance between the Peristomum and Clitellum and TP (Tuberculata pubertatis), (11). All *Aporrectodea* in this study were identified following the taxonomic key (12). Earthworm was collected into an EDTA- tube, DNA was extracted from the samples by DNA extraction kit (Wizard® Genomic DNA Purification Kit, Promega, Madison, WI, USA) according to the manufacturer's protocol.

Amplification of *16SrRNA* by Using PCR

Detection of *16SrRNA* gene was conducted by using primers for amplification of *16SrRNA* gene. A fragment 525 bp of *16SrRNA* was amplified using forward and reverse primers5′-CCGGTCTGAACTCAGATCACGT-3′and5′CGCCTGTTTAACAAAAACAT-3′, respectively (13), (Primers set supplied by alpha DNA Company, Canada). The PCR amplification was performed in a total volume of 25µl containing 1.5µl DNA, 12.5 µl Go Taq green master mix 2X (Promega corporation, USA), 1µl of each primer (10 pmol) then the volume was completed with 25µl of nucleases free water. The thermal cycling conditions were done as follows: Denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 35sec, 58°C for 30sec and

72 °C for 35sec with final incubation at 72 °C for 7 min using a thermal cycler (Gene Amp, PCR system 9700; Applied Biosystem). Sequencing of *16SrRNA* gene was performed by Macro gen company, USA. Homology search was conducted using Basic Local Alignment Search Tool (BLAST) program which is available at the National Center Biotechnology Information (NCBI) online at (http:// www.ncbi.nlm.nih.gov) and BioEdit program. The results were compared with data obtained from Gene Bank and Raptorx software: (http: reptox. uchicago. eDu / predict) for drawing structure protein (helixes, B-Sheets and coils) of amino acid.

Results and Discussion

Morphological Study

The colour of the body was Grayish, the length of body> 8 cm., 25- 32 number of the segments of clitellum and its shape flared while the colour of the nose was pink, the distance between Peristomum and Clitellum is <2 cm. TP present, small and continuous on segment 29-31. We can investigate from all these morphological characteristics the species *Aprrectodea rosea* (Figure 1A).

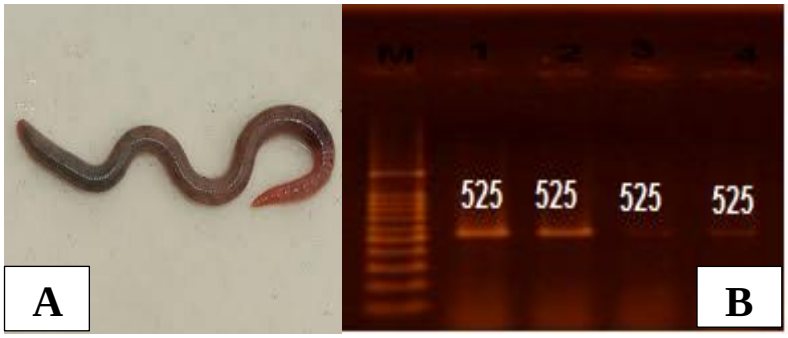

Figure (1): *(A): Aprrectodea rosea*. (B): Agarose gel electrophoresis for detection of amplified 16S ribosomal RNA gene. Bands were fractionated by electrophoresis on a 1.5 % agarose gel (2 h., 5V/cm, 1X Tris-acetic buffer) and visualized under U.V. light after staining with ethidium bromide. Lane:1 (M:100bp ladder); Lane: 1,2,3,4, (PCR product)

16SrRNA gene was successfully amplified using specific PCR primers for gene. Figure (1B) showed PCR amplification of the *16SrRNA where* a specific product at 525 bp was observed. Sequencing of this gene was performed to detect of genotype of Earthworm. Sequences alignment using BLAST and BioEdit divided Earthworm which were collected from three locations from South of Baghdad, to three group depended on similarity with wild type of the *16SrRNA* gene of *Aporrectodea* from the Gene Bank. 8 samples from group 1 and 10 sample from group 3 similarity 97% with *Aporrectodea rosea* 16S ribosomal RNA gene (Sequence ID: gb|JN794591.1|). However, 2 samples from group 1 similarity 87% with *Aporrectodea rosea* 16S ribosomal RNA gene, Sequence ID:

gb|JN794591.1|, and group 2 similarity 100 % with *Aporrectodea sp.* 3-AMVK-2006 16S ribosomal RNA gene, Sequence ID: gb|DQ533735.1| (Figure 2).

A: Group 1(A) (8 sample) and Group 3 (10 sample)

Aporrectodea rosea 16S ribosomal RNA gene, Sequence ID: gb|JN794591.1|

Score	Expect	Identities	Gaps	Strand	Frame
760 bits(411)	0.0()	444/460(97%)	2/460(0%)	Plus/Minus	

```
Query  41   ACTTTTGCGCCGCATAGATTCCCTAAGCCAACATCGAGGTGCCAACCCTCACTATTGATA  100
            ||||||||||| ||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  458  ACTTTTGCGCCACATAGATTCCCTAAGCCAACATCGAGGTGCCAACCCCCACTATTGATA  399

Query  101  AGGACTCTTTAGTGAGATTAGCCTGTTATCCCTAAGGTAGCTTGTTTTGTTGATCTTGAT  160
            |||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||  |
Sbjct  398  AGGACTCTTTAGTGAGATTAGCCTGTTATCCCTAAGGTAGCTTGTTTTGGTGATCTTGGT  339

Query  161  TAAGGGTCATAATTTAGATTAATTAATCTTTTGTAAGGGGATGATTGGTGATTTTCCCCG  220
            |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  338  TAAGGGTCATAATTTAGATTAATTAATCTTTTGTAAGGGGATGATTAGTGATTTTCCCCG  279

Query  221  GTCGCCCCAACCGAATTTTATTAAAAATTTTGAACTTTTATTAAAATAAAGCTCTATAGG  280
            |||||||||||||||||| || |||| |||| ||||||||||||||||||||||||||
Sbjct  278  GTCGCCCCAACCGAATTTTGTT-AAAA-CTTGAGTTTTTATTAAAATAAAGCTCTATAGG  221

Query  281  GTCTTCTTGTCCTTCAATGATATCTAACAGTCTTCAGTTAGAGATTAATTTTTATTAGGC  340
            |||||||||||||||||||| ||||||||||||||||||||||||||||||||| |||
Sbjct  220  GTCTTCTTGTCCTTCAATGAAATCTAACAGTCTTCAGTTAGAGATTAATTTTTATTGGGC  161
```

B: Group 1(B) (2 sample)

Aporrectodea rosea 16S ribosomal RNA gene, Sequence ID: gb|JN794591.1|

Score	Expect	Identities	Gaps	Strand	Frame
409 bits(221)	2e-110()	316/362(87%)	6/362(1%)	Plus/Minus	

Features:

```
Query  38   TAAGCC-ACATCGAGGTGCCAACCCTCAATATTGATAAGGACTCTTTAGTGAGATTAGCC  96
            |||||| |||||||||||||||| || |||||||||||||||||||||||||||||||
Sbjct  435  TAAGCCAACATCGAGGTGCCAACCCCCACTATTGATAAGGACTCTTTAGTGAGATTAGCC  376

Query  97   AGTTATCCCTAAGGCAGCTTGTTTTGTTGATCTTGATTAAGGGTCCCAATTTACATTAAT  156
            | |||||||||||| |||||||||| |||||||| |||||||| |||||| ||||||
Sbjct  375  TGTTATCCCTAAGGTAGCTTGTTTTGGTGATCTTGGTTAAGGGTCATAATTTAGATTAAT  316

Query  157  TAATCTTTTGTAAGGGGATGATTGGTGATTTTCCCCGGTCCCCCCAACCGAATTTTATTA  216
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||
Sbjct  315  TAATCTTTTGTAAGGGGATGATTAGTGATTTTCCCCGGTCGCCCCAACCGAATTTTGTTA  256

Query  217  AAATTTTTTGAAATTTTATTAAAATAAAGCTCTATAGGGTCTTCTTGTCCTTCC-TGACCT  275
            |||  |||| ||||||||||||||||||||||||||||||||||||||||||| |||  |
Sbjct  255  AAA--CTTGAGTTTTTATTAAAATAAAGCTCTATAGGGTCTTCTTGTCCTTCAATGAAAT  198
```

C: Group 2 (10 sample)

Aporrectodea sp. 3-AMVK-2006 16S ribosomal RNA gene, Sequence ID: gb|DQ533735.1|

Score	Expect	Identities	Gaps	Strand	Frame
869 bits(470)	0.0()	470/470(100%)	0/470(0%)	Plus/Minus	

```
Query  13   CATCTTTATGTGACTTTTGCGCCACATAGATTCCCTAAGCCAACATCGAGGTGCCAACCC  72
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  475  CATCTTTATGTGACTTTTGCGCCACATAGATTCCCTAAGCCAACATCGAGGTGCCAACCC  416

Query  73   CCACTATTAATAAGGACTCTTTAGTGGGATTAGCCTGTTATCCCTAAGGTAGCTTGATTT  132
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  415  CCACTATTAATAAGGACTCTTTAGTGGGATTAGCCTGTTATCCCTAAGGTAGCTTGATTT  356

Query  133  GTTGATCTTGTTTAAGGGTCATAGTTTAGATTAAGTTATCTACTTATCTGGGATGATTTA  192
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  355  GTTGATCTTGTTTAAGGGTCATAGTTTAGATTAAGTTATCTACTTATCTGGGATGATTTA  296

Query  193  TATTCCCTGGTCGCCCCAACCGAATTTTATTAAAAATTTTCTGGCTTTTGTTAAAATAAA  252
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  295  TATTCCCTGGTCGCCCCAACCGAATTTTATTAAAAATTTTCTGGCTTTTGTTAAAATAAA  236
```

Figure2:Sequencing of sense flanking the partial 16S*rRNA* gene compared with wild type obtained from Gene Bank. A: Group 1(A) (8 sample) and Group 3 (10 sample) represent of *Aporrectodea rosea* 16S ribosomal RNA gene; B: Group 1(B) (2 sample) represent of *Aporrectodea rosea* 16S ribosomal RNA gene; C: Group 2 (10 sample) represent of *Aporrectodea sp*. 3-AMVK-2006 16S ribosomal RNA gene. Group 1 from AL-Karrada, Group 2 from AL-Zafranya and Group 3 from New Baghdad

Using Hierarchical MEGA6 analysis for classify and built a phylogenetic tree species of 16S*rRNA* amino acid to three clusters. Each group did include similar number of organism; they were identical with each other. This was a result of a similar chain peptide multiple for 16S*rRNA*. Results of the method were cluster organisms of 8 samples from group1A and 10 sample from Group3 in one cluster, with distance equal to 0.006, and clustering of group 2 as a single group and reached the highest value between group 2 and group 1(B) with distance equal to 0.272 and to move away genetic traits, (Figure 3 and Table1).

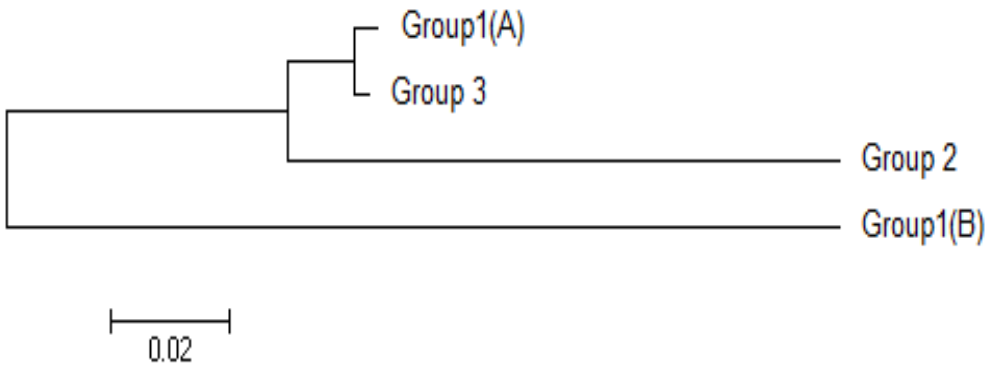

Figure 3: Phylogenetic tree of sequencing of amino acid of 16S*rRNA* gene of 3 group of earthworm, hierarchical cluster analysis determine the following clusters: Cluster one: group 1 (A) and group 3; Cluster two: group 1(B), Cluster three: group 2

Table (1): Estimates of evolutionary divergence between sequences

Species 1	Species 2	Dist	Std. Err
Group1(A)	Group1(B)	0.195	0.022
Group1(A)	Group 2	0.106	0.016
Group1(B)	Group 2	0.272	0.026
Group1(A)	Group 3	0.006	0.004
Group1(B)	Group 3	0.195	0.022
Group 2	Group 3	0.106	0.016

Translation of *16SrRNA* gene of all groups to an amino acid sequence, used Raptorx software (http:reptox.uchicago.eDu /predict) for drawing protein structure of all groups, confirm the result of protein 16S*rRNA* for group (1) A and group (3), the similarity conformation of protein, shown in figure (4), and lack of similarity of the second group with the first group conformation of protein and showed that the protein have two domain (Domain 1 from 22 amino acid to 84 and Domain 2 from 85 to 165 amino acid) as in Figure (4), and separated Group (3) as a group individually and this result compatibility with phylogenetic tree, using Maga6 software as in Figure (3).

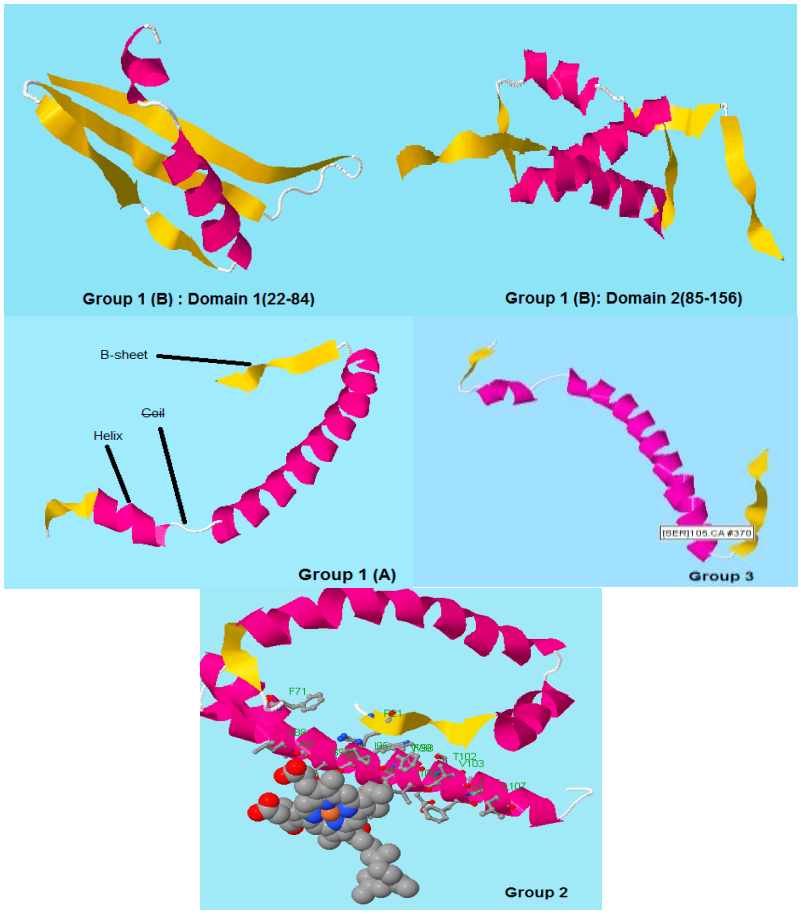

Figure (4): Confirmation of protein 16S*rRNA* for *Aprrectodea* from 3 locations from AL-Karrada (Group 1), AL-Zafranya (Group 2) and New Baghdad (Group 3), by using Raptoxt Software

DNA sequences will be analyzed using maximum likelihood, maximum parsimony, and Bayesian approaches of phylogenetic inference. Resulting trees will be then combined with morphological, ecological and other genomic evidence to determine species boundaries. DNA taxonomy and associated molecular tools are a good way to reveal the true level of biodiversity (14). However, the use of molecular tools to study earthworm taxonomy has only recently started. Molecular markers, mitochondrial and nuclear, are thus developed (15). Lumbricids represent a taxonomical diversified group of terrestrial Oligochaeta. Among the five families within Lumbricoidea, Lumbricidae family counts around 30 genera; some of them provide very diverse species based on their ecological functions. Previous studies have shown that cytochrome c oxidase I (COI) barcode phylogenies are informative at the intrageneric level for selected Lumbricidae genera (15 and 16), used DNA sequences of CO11, 12S, 16S, ND1 and tRNA mitochondrial region for 85 european earthworms

(Aporrectodea caliginosa species) from 27 different location for phylogenetic inference.
(17). Taxonomy of 3000 species earthworm from three provinces in china (Sichuan, Hebei, and Beijing) has been done by using cytochrom-c oxidase 1 (CO1) gene. The conclusion MEGA 6 and Raptorx software methods were able to give more precise details of the method from morphological characteristics through the identification and clustering of new groups with genetic dimension for the other group.

References

1- Lavelle, P. and Spain, A.V. (2001). Soil Ecology. Kluwer Academic Publishers, Dordrecht. The Netherlands.
2- Edwards, C.A. (2004). Earthworm ecology, second ed. CRC Press, Boca Raton, Florida.
3- Bienert, F.; De Danieli, S.; Miquel, C.; Coissac, E.; Poillot , C.; Brun , J. and Taberlet, P. (2012) Tracking earthworm communities from soil DNA. Molecular Ecology, 21:2017 – 2030.
4- Shen , H.P.; Tsai , C.F. and Tsai , S.C. (2003). Six new earthworm of genus *Amynthas* (Oligochaeta: Megascolecidae) from central Taiwan. Zool.Stud.,42:479 – 490.
5- Tsai, C.F.; Tsai, S.C. and Liaw, G.J. (2000). Tow new species of *Protandric pheretimiod* earthworm belonging to the genus *Metaphire* (Megascolecidae: Oligochaeta) from Taiwan. J.Nat.Hist., 34:1731 – 1741.
6- Barnes, R.D. (aNational workshop.Tasmania. (1974) Phylum Annelida, invertebrate Zoology, 3rd Ed.Toppan company, LTD.Tokyo, Japan., 233-316.
7- Bauer, R .and Christian , E. (1995). The effects of enchytraeid faces and mucus on the food choice of *Porcellio scaber* (Isopoda). Eur. J. Soil Boil.
8- Mindell, D.P.; Sorenson, M.D.; Huddleston, C.; Miranda, H.C. and Knight, A. (1997). Phylogenetic relationships among and within select avian orders based on mitochondrial DNA. In: Mindell, D.P. (Ed), Avian Molecular Evolution and Systematrics. New York, Academic Press , pp.214- 217.
9- Symondson, W.O. (2002). Molecular identification of prey in predator diets. Mol.Ecol.,11:627-641.
10- Ansari , A. A. and Saywack, P. (2011). Identification and Classification of Earthworm Species in Guyana.,7:93-99.
11- Online. (2000). Key to Reproductively Mature Earthworms found in Canada .www.attentionnature.ca/English/wormwash /about/Key/intro-huml 23K.1/9/2013.
12- Blakemore, R. J. (2006). A Series of Searchable Texts on Earthworm Biodiversity, Ecology and Systematics from Various Regions of the World 2nd Edition and Supplement (2006). In: N. KANEKO & M.T. ITO (Eds). CD-ROM Publication. COE Soil Ecology Research Group, Yokohama National University, Japan. Online: http://bioeco.eis.ynu.ac.jp/eng/database/earthworm.
13- Simon, C.; Frati, F.; Beckenbach, A.; Crespi, B.; Liu, H. and Flook, P. (1994). Evolution weighting and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Ann. Entomol. Soc. Am., 87:651–701.
14- Chang, C. H.; Rougerie, R. and Chen, J. H. (2009). Identifying earthworms through DNA barcodes: Pitfalls and promise. Pedobiologia, 52: 171-180.
15- Pérez-Losada, M.; Ricoy, M.; Marshall, J. and Jorge Dominguez, J. (2009). Phylogenetic assessment of the earthworm Aporrectodea caliginosa species complex (Oligochaeta: Lumbricidae) based on mitochondrial and nuclear DNA sequences. Molecular Phylogenetics and Evolution., 52 :293–302.
16- Pérez-Losada, M.; Bloch, R.; Breinholt, J.; Pfenninger, M. and Domínguez, J.(2012) . Taxonomic assessment of Lumbricidae (Oligochaeta) earthworm genera using DNA barcodes. European Journal of Soil Biology., 48: 41–4.
17- Huang, J.; Xu, Q.; Sun, Z.; Tang, G., and Su, Z. (2007). Identifying earthworms through DNA barcodes. Pedobiologia, 51 (4): 301-309.