World Applied Sciences Journal 16 (3): 427-432, 2012
ISSN 1818-4952

16S rDNA Based Genomic Analysis of a Bacterium Isolated from Earthworm (*Lennogaster pusillus,* Stephenson) Midden

Suruchi Kumari, Priyanka Saha and M.P. Sinha

Department of Zoology, Ranchi University, Ranchi-834008, Jharkhand, India

Abstract: The present study dealt with the identification of a bacterium having dominance in earthworm (*Lennogaster pusillus*) midden by 16S rDNA based genomic analysis. The bacterium identified was *Aeromonas punctata* strain JM10 (Genbank Accession No: GU205197.1). Isolated DNA of the bacterium showed amplicon band of 1500 bp, when resolved on agarose gel. Amplification of the 16S rDNA gene from the *Aeromonas* sp. was done with forward primer having 814 bp and reverse primer having 913 bp, which further provide consensus sequence of 1418 bp. The query sequence of 1418 bp matched the alignments scores of >= 200 in the distribution of 100 BLAST hits. About 10 types of homolog strains of *Aeromonas punctata* were found as its closest relatives on the basis of sequence producing significant alignments.

Key words: BLAST · Aeromonas Punctata · Amplicon Band · Strain

INTRODUCTION

Earthworms have been recognized as important organisms contributing to healthy soils [1]. Earthworms make a major contribution to decomposition in ecosystems where they are present mainly in the drilosphere i.e. lining of the burrows and in casts and middens. They promote macro aggregate formation by depositing middens rich in glycoproteins, polysaccharides, bacteria and clay [2-5]. Earthworm middens are hot spots of microbial activity, nutrient dynamics and represent a suitable model for studying earthworm mediated influence on soil microbial communities by alteration of the patch structure of the microbial environment [6]. Around 56% of the ecosystem's gross productivity is metabolized by soil microflora and fauna [7]. Major constituents of decomposers are bacteria and among them there are many ubiquitous species which need to be detected. Bacterial communities of middens have not been studied in detail in tropical conditions. There is a paucity of information on their identification and molecular characterization. To meet the objective of characterization, dominant bacteria from earthworm (*Lennogaster pusillus*) midden was isolated. DNA was extracted and two distinct prokaryotic repetitive elements consensus oligonucleotide were used in polymerase chain reaction (PCR) amplification. Oligonucleotide produced a band of DNA in agarose - gel electrophoresis. These bands provided unambiquous DNA finger prints of different eubacterial species and strains. Widespread distribution of these repetitive DNA in the genomes of various micro-organisms enables rapid identification of bacterial species and strains [8]. For further bioinformatic characterization BLAST was used which is one of the most widely used bioinformatics programmes [9, 10]. The determination and analysis of complete genome sequence have recently enabled many major advances to be made in the area of microbial evolutionary biology [11]. The present study dealt with the identification of a bacterium having dominance in earthworm middens by genomic analysis based on 16S rDNA.

MATERIALS AND METHODS

Soil and Earthworms Sampling: Soil and earthworms were collected from the grassland ecosystem of Ranchi, Jharkhand, India. Both the soil and earthworms were brought to laboratory and earthworms were cultured in plastic container containing sampled soil under sterilized, moist and oxygenated conditions. The characteristics of soil analysis are given in Table 1. Earthworms collected from the sampling sites were identified as *Lennogaster pusillus*, Stephenson. The middens produced by cultured earthworms were collected with sterilized aluminum foil and enumeration of bacterial population was done.

Corresponding Author: M.P. Sinha, Department of Zoology, Ranchi University, Ranchi- 834008, Jharkhand, India.

Table 1: Edaphic profile of the collected soil samples

Characteristics	Value (M ±S.D. n=3)
pH	5.81 ±0.07
Organic carbon (mg C g^{-1} Soil)	6.52 ± 0.11
Nitrogen (mg N g^{-1} Soil)	0.78 ± 0.01
Phosphorous (kg P $hec.^{-1}$)	27.9 ± 0.62
Potassium (kg K $hec.^{-1}$)	148.0 ±0.49

Soil Bacteria Culture: Dilution plate method [12] was used for estimating the bacterial population of collected middens. 1mL inoculum of $1:10^7$ dilution of the primary suspension was taken and Czapek dox agar media was used for culture. Out of the several colonies, the most dominant (80%; punctiform, entire margin, cream in the color with flat elevation) was isolated and pure cultured by streak plate method and slant was prepared for genomic analysis.

DNA Extraction and Purification: DNA was isolated from the culture [13]. Its quality was evaluated on 1.2% agarose gel.

Amplification of 16S rDNA Gene and Sequencing: Fragment of 16S rDNA gene was amplified by PCR from the above isolated DNA. The PCR amplicon was purified to remove contaminants by using a QIA quick purification kit (Qiagen, Hidden, Germany) after seaken GTG (FMC) agarose gel electrophoresis (1 X Trisacetate EDTA). Forward and reverse DNA sequencing was carried out with 8F and 1492R primers using BTD v 3.1 cycle sequencing kit on ABI 3730 X 1 genetic analyzer and consensus sequence of DNA was generated by aligner software. The 16S rDNA gene sequence was used to carry out BLAST with nr database of NCBI genbank database [14, 15].

RESULTS AND DISCUSSION

The bacterium, on the basis of genomic analysis and molecular characterization, was identified as A*eromonas punctata* strain JM10 (Genbank Accession No: GU205197.1) based on nucleotide homology. The taxonomy of the genus *Aeromonas* is, however, confusing and controversial [16]. Till now the number of valid species in the genus has grown to 14 with a new family Aeromonadaceae established to house the genus *Aeromonas*. Despite this explosion in number of new genomospecies only five (*A. hydrophila, A. caviae, A. veronii, A. jondae* and *A. schutoertii*) are currently recognized as human pathogens. Convincing evidence suggested that some aeromonads to cause gastroenteritis, but it is presently unclear whether many of the strains isolated from feces are involved in diarrheal disease. Many questions regarding this genus *Aeromonas* remain unanswered. So that genomic study of this genus was necessary.

Aeromonas species are recognized as etiological agents of a wide spectrum of disease in man and animals. In developing countries potentially pathogenic *Aeromonas* sp. is very common in drinking water and in different types of foods. Significant association of *Aeromonas* sp. with diarrhea in children has been reported from several countries [17].

Aeromaonadaceae falls between the family Vibronaceae and Enterobacteriaceae [18] on the basis of a collection of molecular genetic data and its phylogenetic relationship. Aeromonas are autochthonous to aquatic environment and useful micro-biota of fish, amphibians and other animals [19]. The family Aeromonadaceae is represented by four genera in which *Aeromonas* is the largest genera containing about 20 species and 12 sub species [20]. In the present research communication, we report the genotypic characterization of *Aeromonas punctata* species.

The microbial populations in the earthworm midden were high. The bacteria were identified as Gram -ve bacteria. It is thought that the Gram -ve envelope provides a structure better suited to support the life in nutrition enrich environment than Gram +ve bacteria cell wall [21].

Aeromonas was found in the isolates from the cast but not from the soil. It appeared that the entire genus found in the cast isolates were also found in the soil isolates except *Aeromonas* [22]. *Aeromonas* spp. are typically associated with fish and aquatic habitats. They have been previously identified as dominant isolates in the midden of the earthworm *E. foetida* [23] and have been associated with the gut of *Phertima* sp. [24]. The midden of *Lennogaster pusillus*, the species of earthworm taken for the present study was dominated by *Aeromonas punctata*. The present study is in conformity with the previous report of Toyota and Kimura [23, 24] even in tropical Indian conditions.

DNA was isolated and sequenced using 16S rDNA, a single discrete PCR amplicon band of 1500bp was observed when resolved on agarose gel (Fig. 1). Amplification of the 16S rDNA gene from the *Aeromonas* sp. was done with forward primer 8F having 814bp and reverse primer 1492R having 913 bp (Table 2 and 3). Consensus sequence of 1418 bp rDNA gene was generated from forward and reverse sequence data using aligner software (Table 4) to characterize the species.

Table 2: Forward sequence: 814 bp of A*eromonas punctata* strain JM10

CATGCAAGTCGAGCGGCAGCGGGAAAGTAGCTTGCTACTTTTGCCGGCGAGCGGCGGACGGGTGAGTAATGCCTGGGAAATTGCCCAGTCGAGGGG
GATAACAGTTGGAAACGACTGCTAATACCGCATACGCCCTACGGGGGAAAGCAGGGGACCTTCGGGCCTTGCGCGATTGGATATGCCCAGGTGGGA
TTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGGA
CTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGC
ACTTTCAGCGAGGAGGAAAGGTCAGTAGCTAATATCTGCTGGCTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGCG
GTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACCTG
GGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGT
GGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGA
TGTCGATTTGGAGGCTGTGTCCTTGAGACGTGGCTTCCGGAGCTAA

Table 3: Reverse sequence: 913 bp of A*eromonas punctata* strain JM10

GTGGTAACGCCCTCCCGAAGGTTAAGCTATCTACTTCTGGTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCAC
CGCAACATTCTGATTTGCGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGACGCGCTTTTTGGGATTCGCTCAC
TATCGCTAGCTTGCAGCCCTCTGTACGCGCCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCGGTT
TATCACCGGCAGTCTCCCTTGAGTTCCCACCATTACGTGCTGGCAACAAAGGACAGGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACA
CGAGCTGACGACAGCCATGCAGCACCTGTGTTCTGATTCCCGAAGGCACTCCCGTATCTCTACAGGATTCCAGACATGTCAAGGCCAGGTAAGGTTCT
TCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCG
ATTTAACGCGTTAGCTCCGGAAGCCACGTCTCAAGGACACAGCCTCCAAATCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGC
TCCCCACGCTTTCGCACCTGAGCGTCAGTCTTTGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCATTTCACCGCTACACCTGG
AATTCTACCCCCCTCTACAAGACTCTAGCTGGACAGTTTTAAATGCAATTCCCAGGTTGAGCCCGGGGCTTTCACATCTAACTTATCCAACCGCCTGCG
TGCGCTTTACGCCCAGTAATTCC

Table 4: Consensus Sequence of 16s rDNA gene of A*eromonas punctata* strain JM10 (1418 bp)

CATGCAAGTCGAGCGGCAGCGGGAAAGTAGCTTGCTACTTTTGCCGGCGAGCGGCGGACGGGTGAGTAATGCCTGGGAAATTGCCCAGTCGAGGGGG
ATAACAGTTGGAAACGACTGCTAATACCGCATACGCCCTACGGGGGAAAGCAGGGGACCTTCGGGCCTTGCGCGATTGGATATGCCCAGGTGGGATT
AGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCC
TACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTT
CAGCGAGGAGGAAAGGTCAGTAGCTAATATCTGCTGGCTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC
GAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTG
CATTTAAAACTGTCCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAG
GCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATT
TGGGCTGTGTCCTTGAGACGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGG
GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGCCTTGACATGTCTGGAATCCTGTAGAGATACGGGAGT
GCCTTTCGGGAATCAGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGTCCTTT
GTTGCCAGCACGTAATGGTGGGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCA
GGGCTACACACGTGCTACAATGGCGCGTACAGAGGGCTGCAAGCTAGCGATAGTGAGCGAATCCCAAAAAGCGCGTCGTAGTCCGGATTGGAGTCTG
CAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCAAATCAGAATGTTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCAT
GGGAGTGGGTTGCACCAGAAGTAGATAGCTTAACCTTCGGGAGGGCGTTACCAC

Table 5: Close homologs of *Aeromonas punctata* strain JM10

Accession	Description	Max. score	Total score	Query coverage	E Value	Max. ident
EU862311.1	Uncultured bacterium clone Niu10	2617	2617	99%	0.0	100%
GQ259885.2	*Aeromonas punctata* strain 159	2614	2614	100%	0.0	99%
GU205197.1	*Aeromonas punctata* strain JM10	2614	2614	100%	0.0	99%
GU205195.1	*Aeromonas punctata* strain JW04	2614	2614	100%	0.0	99%
FJ494901.1	*Aeromonas* sp. B27	2614	2614	100%	0.0	99%
FJ168776.1	*Aeromonas punctata* strain 219c	2614	2614	100%	0.0	99%
FJ168775.1	*Aeromonas punctata* strain 176c	2614	2614	100%	0.0	99%
FJ168774.1	*Aeromonas punctata* strain 360c	2614	2614	100%	0.0	99%
DQ979324.1	*Aeromonas punctata* strain MPT4	2614	2614	100%	0.0	99%
AY987761.1	*Aeromonas punctata* strain RK 65541	2614	2614	100%	0.0	99%

Max. score = Maximum score; E value = Expected value; Max. ident. = Maximum identification

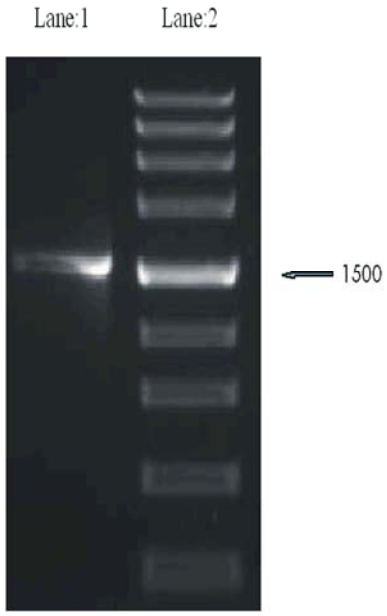

Fig. 1: Gel image of 16S rDNA amplicon.
Lane 1: 16S rDNA amplicon band; Lane 2: DNA marker

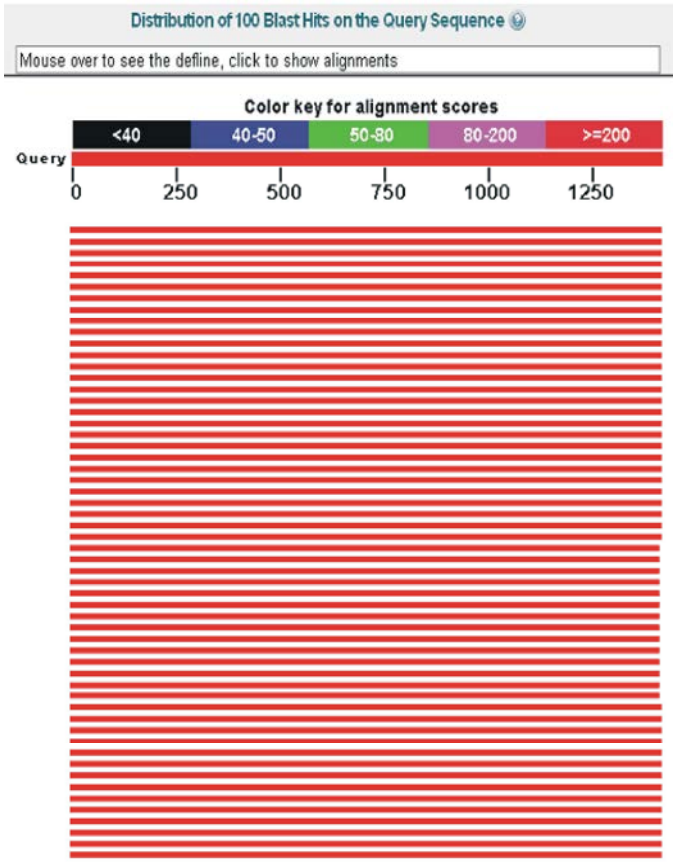

Fig. 2: BLAST DATA: (Alignment view using combination of NCBI GeneBank); Sequence Producing Significant Alignments

Along with the DNA sequence of *Aeromonas punctata* strain JM 10 sp. 16S rDNA gene from the gene bank was used as an input sequence for the identification of regulatory elements analysis because this strain has maximum similarity (100% identity) during BLAST (Fig. 2) on the query sequence of 1418 bp matched the alignments scores of >=200 in the distribution of 100 BLAST hits.

Almost complete 16S rDNA gene sequence of strain of *Aeromonas punctata* was used as the query to search for homologous sequence in the gene bank database. Sequence analysis reveled that its closest relative with 99% similarity is *Aeromonas punctata* strain 159, which was followed by *Aeromonas punctata* strain JW04, *Aeromonas* sp. B27, *A. punctata* strain 219C, *A. punctata* strain 176C, *A. punctata* strain 360 C, *A. punctata* strain MPT4 and *A. punctata* strain RK 65541 having the same confidence level and 100% similarity with uncultured bacterium clone Niu 10 (Table 5).

About 10 types of strains of *Aeromonas punctata* were found as its closet relative on the basis of sequence producing significant alignments. The uncultured bacterium close Niu 10 scored maximum score of 2617, which is equal to the total score and other type strain of *Aeromonas punctata* socred maximum score of 2614, which is also equal to total score. This shows the 100% sequence similarity in query coverage of amino acids. The expectation value (E) of all these *Aeromonas punctata* strain was 0.0, which showed that all the sequence of 10 different strains are homogenous in comparison to *Aeromonas punctata* strain JM10. A good criterion for homogeneity E≤ 0.001 or 0.01 for protein searched against a protein database. Thus the E value of *Aeromonas punctata* strain JM10 is more significant to match.

Results were most informative in which, closely related species and *Aeromonas punctata* strain JM10 were not closely related to the genomospecies *Aeromonas hydrophila*, *Aeromonas caviae*, *A. veronii*, *A. jondai* and *A. schutoertii*. Thus it is not pathogenic in nature and it is predominantly found in earthworm midden and might be a cause for more fertility in agro-ecosystem.

REFERENCES

1. Roming, D.E., M.J. Garlynd, R.F. Harris and K. Mcseeney, 1995. How farmers assess soil health and quality. J. Soil and Water Conservation, 50: 229-236.
2. Foster, R.C., 1988. Microenvironments of soil microorganisms. Bid. Fertil. Soils, 6: 189-203.
3. Daniel, O. and J.M. Anderson, 1992. Microfial biomass and activity in contrasting soil materials after passage through the gut of the earthworm *Lumbricus rubellus hoffmeister*. Soil. Biol. Biochem., 24: 465-470.
4. Tomlin, A.D., M.J. Shipitalo, W.M. Edwards and R. Protz, 1995. Earthworms and their influence on Soil Structure and infiltration. In: Earthworms ecology and biogeography, Ed., Hendrix, P.F., Lewis Publishers. Chelsea, Mich. pp: 159-184.
5. Edwards, C.A. and P.J. Bohlen, 1996. Biology of earthworms, Chapman and Hall, London, United Kingdom.
6. Aira, M., N. Mc Namara, T. Piearce and J.J. Dominguez, 2009. Microbial communities of Lumbricus terrestris L. middens, Strictire, activity and changes through time in relation to earthworm presence. J. Soil and Sediments, 9(1): 54-61.
7. Macfadyen, T., 1993. The contribution of the microfauna to total soil metabolism, Soil Organisms, pp: 3-16.
8. Versalovic, J., T. Koeuth and R. Lupski, 1991. Distribution of repetitive DNA sequence in enbacteria and application of finger printing of bacterial genomes. Nucleic Acid Res., 19(24): 6823-6831.
9. Altschul, S.F., W. Gish, W. Miller, E.W. Myers and D.J. Lipman, 1990. Basic local aligment search tool. J. Mol. Biol., pp: 403-410.
10. Casey, R.M., 2005. BLAST sequences Aid in Genomics and prohemics, Business Intelligence Network.
11. Elisen, J.A., 2000. Assessing Evolutionary relationships among microbes frame whole gemone analysis. Current Opinion in Microbial., 3(5): 475-800.
12. Thornton, H.G., 1922. On the development of standardized agar medium for counting soil bacteria with special regard to the repression of spreading colonies. Ann. Applied Biol., 2: 241-274.
13. Thangaraj, K., M.B. Joshi, A.G. Reddy, N.J. Gupta, B. Chakravarty and L. Singh, 2002. CAG repeat expansion in the androgen receptor gene is not associated with male infertility in Indian populations. J. Androl., 23: 815-818.
14. Marchler-Bauer, A., A.R. Panchenko, B.A. Shoemaker, P.A. Thissen, L.Y. Geer and S.H. Bryant, 2000. CDD. A database of conserved domain aligments with links to domain three-dimensional structure. Nucleic Acids Res., 30: 281-283.

15. Pruitt, K.D., T. Tatusova and D.R. Maglott, 2005. NCBI ref. seq. a curaled non-redundant sequence database of genomes, transcripts and proteins. Nucleic Acids Res., 33: D501-D504.
16. Carnaham, A.M., M. Altewegg, P.J. Gosling and S.W. Joseph, 1995. (Ed.) Taxonomy of the genus Aeromonas, John Wiley and sons Ltd. Chichesler, England. pp: 1-38.
17. Ghenghesh, K.S., S.F. Ahmad, A.E. Rania, A. Alef and K. John, 2008. Aeromonas associated infections in developing countries. J. Infect Dev. Ctries, 2: 81-98.
18. Colwell, R.R., M.R. MacDonell and J. Deley, J. 1986. Proposal to recognize the family Aeromonasdaceae fam. Nov. International J. Systemic Bacteriol., 78: 231-238.
19. Minnana Galbis, D., M. Farfan, C. Fushe and J.G. Lorein, 2004. Aeromonas molluscorum sp. Nov. isolated from bivalve mollusks. Int. J. Syst. Evol. Microbial., 54: 2073-2078.
20. http://www.bacterio.cict.fr/index/html.
21. Arajo, R.M., R.M. Arrivfas and R. Pres, 1991. Distribution of Aeromonas species in waters with different levels of pollution. J. Appl. Bacterial, 71: 182-186.
22. Furlong, M.A., D.R. Singleton, C.C. David and W.B. Whitman, 2002. Molecular and culture based analyses of prokaryotic communities from an Agricultural soil and the Burrows and Casts of the Earthworm Lumbricus tubellus. Appl. Environ. Microbial., 68: 1265-1279.
23. Toyota, K. and M. Kimura, 2000. Microbial community indigenous to the earthworm. E. foetida, Biol, Fertil. Soils, 31: 187-190.
24. Toyota, K. and M. Kimura, 1994. Earthworms disseminate a soil borne plant pathogen. Fusarium oxyporum sp. raphani. Biol. Fertile. Soils, 18: 32-36.