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RESEARCH ARTICLE

THE GENBANK NEW DEPOSIT FOR TAXON *ASPERGILLUS SOJAE* PRK 2

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ABSTRACT

16S/18S ribosomal deoxyribonucleic acid sequencing, polymerase chain reaction and deoxyribonucleic acid sequencing has played a pivotal role in the accurate identification of bacterial isolates and the discovery of novel bacteria and fungi in explosive laden soil. Ten different Bacterial isolates and three different Actinomycetes belongs to the genera *Acinetobacter, Bacillus, Enterobacter, Enterococcus, Staphylococcus, klebsiella, Aspergillus, Coriolopsis* were isolated and identified with their 16S and 18S rDNA sequences and deposited in the The GenBank Maryland USA. All the isolates were named after the discoverer P Ravikumar, will be preserved in MTCC, India. Sanger dideoxy sequencing technology was employed and the number of base pairs, the base count of A, T, G and C was also studied. To fully utilise 16S/18S rDNA sequencing of bacteria and fungi in explosive laden soils and their bioremediation, the presence of xplA and xplB and other biodegrading gene/s were to be investigated. *Aspergillus sojae* PRK2 18S ribosomal RNA gene, with the base count 216 a 184 c 256 g 228 t partial sequence with Accession KJ938684, Version KJ938684.1 GI: 675621795 (bases 1-884) a novel strain present in the explosive laden soil of cracker industry was deposited in the The GenBank Maryland USA is discussed here.

INTRODUCTION

Aspergillus sojae is a mold species in the genus *Aspergillus*. In Japan it is used to make the ferment (Kōji) of soy sauce, the mirin and other lacto-fermented condiments like tsukemono. Soy sauce is a condiment produced by fermenting soybeans with *Aspergillus sojae*, along with water and salt. Glyceollins, molecules belonging to the Pterocarpans, are found in the soybean (*Glycine max*) and have been found to have an antifungal activity against *A. sojae* (Hyo *et. al*., 2010). Explosives are materials with high nitrogen and oxygen contents which on detonation expand to create a shock wave which exerts high pressures on the surroundings, causing an explosion and leaving toxic waste in the environment. The manufacturing, testing and use of explosive have resulted in severe contamination of both soils and groundwater (Brannon *et al*., 2005; Eisentraeger *et al*., 2007) thus necessitating their safe removal from the environment. The chemical properties and quantity of explosives waste determine their toxicity and persistence in the environment.

The net result has been bioaccumulation and bio magnifications of these explosives waste in aquatic and terrestrial organisms. The incredible versatility inherited in microbes has rendered these explosives as a part of the biogeochemical cycle. Several microbes catalyse mineralization and/or nonspecific transformation of explosive waste either by aerobic or anaerobic processes. It is likely that on-going genetic adaptation, with the recruitment of silent sequences into functional catabolic routes and evolution of substrate range by mutations in structural genes, will further enhance the catabolic potential of bacteria toward explosives and ultimately contribute to cleansing the environment of these toxic and recalcitrant chemicals (Singh *et al*., 2012). Incineration of soil to rid it of explosives can result in the exposure of workers to high levels of toxins (Esteve-Núñez *et al*., 2001). Thus, bioremediation is considered both economically feasible and environmentally sound solution. Bioremediation is the use of organisms, such as microbes or plants, to degrade or detoxify hazardous materials on the contaminated sites. Over the years, many new biological methods of bioremediation for explosive contaminated soil have been developed (Lewis *et al*., 2004). Numerous factors can affect the biodegradation processes and depends on the nature of molecules to be degraded (e.g., molecule size, charge, number and position of functional groups, solubility and toxicity) as well as the environmental conditions.

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MATERIALS AND METHODS

Study site and collection of sample

Valangaiman of Thiruvarur District of Tamilndu India (10°46′ 17.76″ N 79°38′ 12.48″ E) was selected as the sampling site for this study. Approximately 100gm of explosive laden soil from the cracker manufacturing unit and the grinding unit were collected form five different places and immediately placed into 500ml sterile air tight container, sealed to avoid contamination and transported to the laboratory for further processing.

Soil processing and isolation

3 gm of the soil samples were vigorously mixed with 3 ml of sterile distilled water and left for overnight.100 microliter of the upper surface soil liquid was then transferred into 5 ml Nutrient broth (Hi-Media M002) with a sterile micro pipette and incubated at 36° C for 48 hrs. The plates incubated for two days were visually inspected daily until typical colonies formed. The colonies were purified by further subculture on Nutrient Agar M002 to confirm the purity and preserved at -20°C until further use. The suspected isolate was further screened for large green colonies in nutrient Agar M002 medium.

Molecular confirmation

Identification with specific PCR

The colony morphologically identified *A.sojae* was further identified by PCR procedures based on amplification of 18S rDNA gene. PCR was standardised with forward and reverse PCR primers and performed in a volume of 25 microliter, the reaction mixture containing 200 mM of each dNTP, 1.5µm MgCl2, 1xPCR buffer, 10 pmol of each primer, 1U of taq DNA polymerase and 10 ng DNA. The PCR cycle protocol consists of initial denaturation at 95°C for 6 min and 30 cycles of denaturation at 95°C for 1 min, primer specific annealing for 1 min and extension at 72°C for 2 min with the final extension at 72°C for 10 min. PCR products were electrophoresed on 1% agarose gel and visualised under UV.

18S rDNA ANALYSIS

18S rDNA sequencing was used to confirm PCR identified isolate and 18S rDNA sequence of the isolate was BLAST analysed (Gee *et al*., 2003). The PCR reaction mixture for the amplification of the 18S rDNA gene consisted of 200mM of each dNTP,1.5 mM MgCl2,1x PCR buffer, 10pmol of each primer,1 U of Taq DNA polymerase and 10ng DNA. The reaction was made up to 25 microliter with sterile distilled water and the cycle consisted of initial denaturation at 95°C for 6 min and 30 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 2 min with the final extension at 72°C for 10 min. PCR products were electrophoresed on 1.0% agarose gel and visualised under UV in a gel documentation system as above. Amplified 18S rDNA PCR products were sequenced by the dideoxy chain termination method (Sanger and Coulson, 1975 & Sanger, Nicklen and Coulson, 1977) using Big dye Terminator v 3.1 sequencing kit and Big dye x Terminator Purification kit in an ABI 10 sequencer. The derived sequences were aligned using DNASTAR lasergene 9 Core Suit and BLAST analysis was with NCBI database.

RESULTS AND DISCUSSION

From the soil sample 3 different strains were isolated (Figure-1) and sub cultured successfully. One isolate was suspected and selected as possible *Aspergillus sojae* (Figure-2) based on characteristic colonial morphology. Interestingly all the environmental isolates were able grow on Nutrient broth M002 medium and agar.

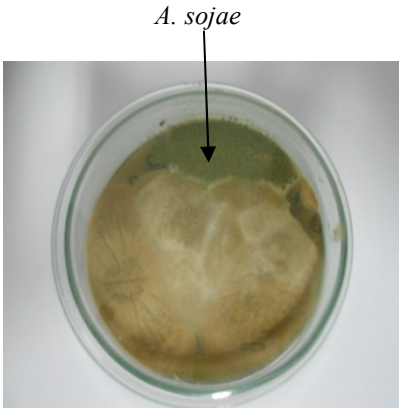


Figure 1. Showing Different Fungal Isolates in Nutrient Agar Medium With *A. sojae*

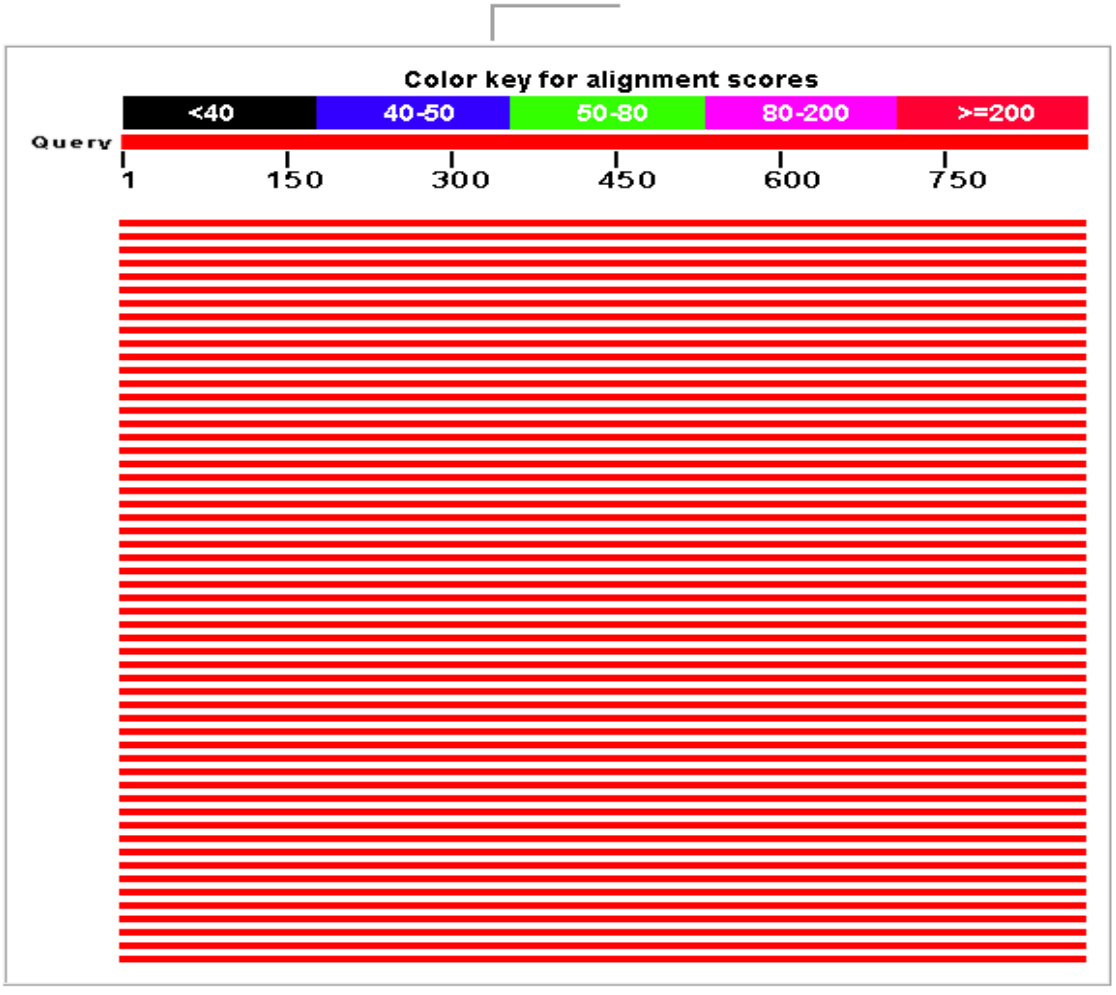
The one isolate confirmed by 18S rDNA sequencing was subjected to Sanger dideoxy sequenceing. The FASTA of *Aspergillus sojae* strain PRK2 18S ribosomal RNA gene, partial sequence Molecule type nucleic acid Query Length 884 bp is depicted below:

FASTA

```
1 tctggtgcga cgcagccgcg gtaattccag ctccaatagc gtatattaaa gttgttgcag
61 ttaaaaagct cgtagttgaa ccttgggtct ggctggccgg tccgcctcac cgcgagtact
121 ggtccggctg gacctttcct tctggggaac ctcatggcct tcactggctg tgggggaac
181 caggactttt actgtgaaaa aattagagtg ttcaaagcag gcctttgctc gaatacatta
241 gcatggaata atagaatagg acgtgcggtt ctatttttgtt ggtttctagg accgccgtaa
301 tgattaatag ggatagtcgg gggcgtcagt attcagctgt cagaggtgaa attcttggat
361 ttgctgaaga ctaactactg cgaaagcatt cgccaaggat gttttcatta atcagggaac
421 gaaagttagg ggatcgaaga cgatcagata ccgtcgtagt cttaaccata aactatgccg
481 actagggatc gggcggtgtt tctatgatga cccgctcggc accttacgag aaatcaaagt
541 ttttgggttc tgggggagt atggtcgcaa ggctgaaact taaagaaatt gacggaaggg
601 caccacaagg cgtggagcct gcggcttaat ttgactcaac acggggaaac tcaccaggtc
661 cagacaaaat aaggattgac agattgagag ctctttcttg atcttttgga tggtggtgca
721 tggccgttct tagttggtgg agtgatttgt ctgcttaatt gcgataacga acgagacctc
781 ggcccttaaa tagcccggtc cgcgtttgcg ggccgctggc ttcttagggg gactatcggc
841 tcaagccgat ggaagtgcgc ggcaataaca ggtcagtgag gccc
```

THE DISTRIBUTION OF 145 BLAST HITS ON THE QUERY SEQUENCE

An overview of the database sequences aligned to the query sequence is shown here. This graphic is an overview of database sequences aligned to the query sequence. Alignments are color-coded by score, within one of five score ranges. Multiple alignments on the same database sequence are connected by a dashed line. Mousing over an alignment shows the alignment definition and score in the box at the top. Clicking an alignment displays the alignment detail.



Sequences producing significant alignments							
Select for downloading or viewing reports	Description	Max score	Total score	Query cover	E value	Ident	Accession
1 Select seq gb|KJ938684.1|	Aspergillus sojae strain PRK-2 18S ribosomal RNA gene, partial sequence	1633	1633	100%	0.0	100%	KJ938684.1
2 Select seq gb|KM870530.1|	Aspergillus flavus strain EGY1 18S ribosomal RNA gene, partial sequence	1609	1609	100%	0.0	99%	KM870530.1
3 Select seq gb|KF175513.1|	Aspergillus sojae strain JPDA1 18S ribosomal RNA gene, partial sequence						

Aspergillus sojae strain PRK-2 18S ribosomal RNA gene, partial sequence
Sequence ID: gb|KJ938684.1|Length: 884Number of Matches: 1
Range 1: 1 to 884GenBankGraphics Next Match Previous Match First Match

Alignment statistics for match #1					
Score	Expect	Identities	Gaps	Strand	Frame
1633 bits(884)	0.0()	884/884(100%)	0/884(0%)	Plus/Plus	

Features

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Query  1    TCTGGTGCGACGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAG
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Sbjct  121  GGTCCGGCTGGACCTTTCCTTCTGGGGAACCTCATGGCCTTCACTGGCTGTGGGGGGAAC  180

Query  181  CAGGACTTTTACTGTGAAAAAATTAGAGTGTTCAAAGCAGGCCTTTGCTCGAATACATTA
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Query  421  GAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACTATGCCG
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Query  481  ACTAGGGATCGGGCGGTGTTTCTATGATGACCCGCTCGGCACCTTACGAGAAATCAAAGT
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Query  541  TTTTGGGTTCTGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGAAATTGACGGAAGGG
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            |||||||||||||||||||||||||||||||||||||||||||
Sbjct  841  TCAAGCCGATGGAAGTGCGCGGCAATAACAGGTCAGTGAGGCCC  884
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Aspergillus sojae strain PRK-2 18S ribosomal RNA gene, partial sequence

GenBank: KJ938684.1

FASTA Graphics

Go to:

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LOCUS       KJ938684 884 bp    DNA     linear   PLN 07-SEP-2014
DEFINITION Aspergillus sojae strain PRK-2 18S ribosomal RNA gene, partial Sequence.

ACCESSION   KJ938684
VERSION     KJ938684.1 GI: 675621795
KEYWORDS.

SOURCE      Aspergillus sojae
ORGANISM   Aspergillus sojae
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Eurotiomycetes; Eurotiomycetidae; Eurotiales; Aspergillaceae;
            Aspergillus.

REFERENCE  1 (bases 1 to 884)
AUTHORS    Ravikumar,P.
TITLE      Direct Submission
JOURNAL    Submitted (05-JUN-2014) Associate Professor of Botany, Government Arts
College (Autonomous), Coimbatore, Tamilnadu 641018, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology: Sanger dideoxy sequencing
            ##Assembly-Data-END##

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            /country="India"
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       61 ttaaaaagct cgtagttgaa ccttgggtct ggctggccgg tccgcctcac cgcgagtact
      121 ggtccggctg gacctttcct tctggggaac ctcatggcct tcactggctg tgggggggaac
      181 caggactttt actgtgaaaa aattagagtg ttcaaagcag gcctttgctc gaatacatta
      241 gcatggaata atagaatagg acgtgcggtt ctattttgtt ggtttctagg accgccgtaa
      301 tgattaatag ggatagtcgg gggcgtcagt attcagctgt cagaggtgaa attcttggat
      361 ttgctgaaga ctaactactg cgaaagcatt cgccaaggat gttttcatta atcagggaac
      421 gaaagttagg ggatcgaaga cgatcagata ccgtcgtagt cttaaccata aactatgccg
      481 actagggatc gggcggtgtt tctatgatga cccgctcggc accttacgag aaatcaaagt
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      721 tggccgttct tagttggtgg agtgatttgt ctgcttaatt gcgataacga acgagacctc
      781 ggcccttaaa tagcccggtc cgcgtttgcg ggccgctggc ttcttagggg gactatcggc
      841 tcaagccgat ggaagtgcgc ggcaataaca ggtcagtgag gccc
//
```

Conclusion

This research piece of work is the first report of the isolation and molecular confirmation of *Aspergillus sojae* strain PRK2 from the explosive laden soil. This isolate was initially identified by conventional morphological methods and further confirmed by advanced molecular based methods of 18S rDNA sequencing and *Aspergillus sojae* strain PRK2 specific PCR. The isolation of this important fungal species from this part of India may with their xplA, xplB, other biodegrading gene/s and catabolic gene cassette sequences for the explosives should initiate further studies on the extent of environmental bioremediation.

Acknowledgement

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