# TEST REPORT

**Workflow**

- Genomic DNA was isolated from the sample provided by the scientist.
- The ~1.5 kbp, 16s-rDNA fragment was amplified using high–fidelity PCR polymerase. The PCR product was sequenced Bi-directionally.
- The sequence data was aligned and analyzed to identify the Bacteria and its closest neighbors

**Protocol:**

DNA Extraction:

- The sample was picked up and placed in a mortar and homogenized with 1 ml of extraction buffer and the homogenate was transferred to a 2 ml-microfuge tube.
- An equal volume of Phenol: Chloroform: Isoamlyalcohol (25:24:1) was added to the tubes and mixed well by gently Shaking the tubes.
- The tubes were centrifuged at room temperature for 15 min at 14,000 rpm.
- The upper aqueous phase was collected in a new tube and an equal volume of Chloroform: Isoamly alcohol (24:1) was added and mixed.
- The upper aqueous phase obtained after centrifuging at room temperature for 10 min at 14,000 rpm was transferred to a new tube.
- The DNA was precipitated from the solution by adding 0.1 volume of 3 M Sodium acetate pH 7.0 and 0.7 volume of Isopropanol.
- After 15 min of incubation at room temperature the tubes were centrifuged at 4ºC for 15 min at 14,000 rpm.
- The DNA pellet was washed twice with 70% ethanol and then very briefly with 100% ethanol and air dried.
- The DNA was dissolved in TE (Tris-Cl 10 mM pH 8.0, EDTA 1 mM).
- To remove RNA 5 µl of DNAse free RNAse A (10 mg/ml) was added to the DNA.

**DNA Quantification:**

| S.No | Sample ID | DNA (ng/µl) |
|---|---|---|
| 1 | NSW1 | 101 |

**PCR Conditions**

**PCR Amplification of 16S Gene:**
101 ng of Extracted DNA (NSW1) was used for amplification along with 10Pm of each primer

**Composition of TAQ Master MIX:**

1) High-Fidelity DNA Polymerase
2) 0.5mM dNTPs
3) 3.2mM MgCl2
4) PCR Enzyme Buffer

| **Cycling Conditions** | | |
|---|---|---|
| Initial Denaturation | 3 minutes at 94°C | |
| Denaturation | 1 minutes at 94°C | 30 Cycles |
| Annealing | 1 minutes 50°C | |
| Extension | 2 minutes at 72°C | |
| Final Extension | 7 minutes at 72°C | |

| **PCR Amplification conditions** | **Volume** |
|---|---|
| DNA | 1 ul |
| 16s Forward Primer | 2 ul |
| 16s Reverse Primer | 2 ul |
| dNTPs (2.5mM each) | 4 ul |
| 10X Taq DNA polymerase Assay Buffer | 10 ul |
| Taq DNA Polymerase Enzyme (3U/ ml) | 1 ul |
| Water | 30 ul |
| Total reaction volume | 50 ul |

**Primer Details -** The PCR product size ~1.5 kb

| N | Oligo Name | Sequence (5`à 3`) | Tm | GC-content |
|---|---|---|---|---|
| 1 | 16s Forward | GGATGAGCCCGCGGCCTA | 55.5 | 72.2 |
| 2 | 16s Reverse | CGGTGTGTACAAGGCCCGG | 52.3 | 65.0 |

## Sample 1: S1

Aligned Sequence Data of Sample – NSW1 (1470)

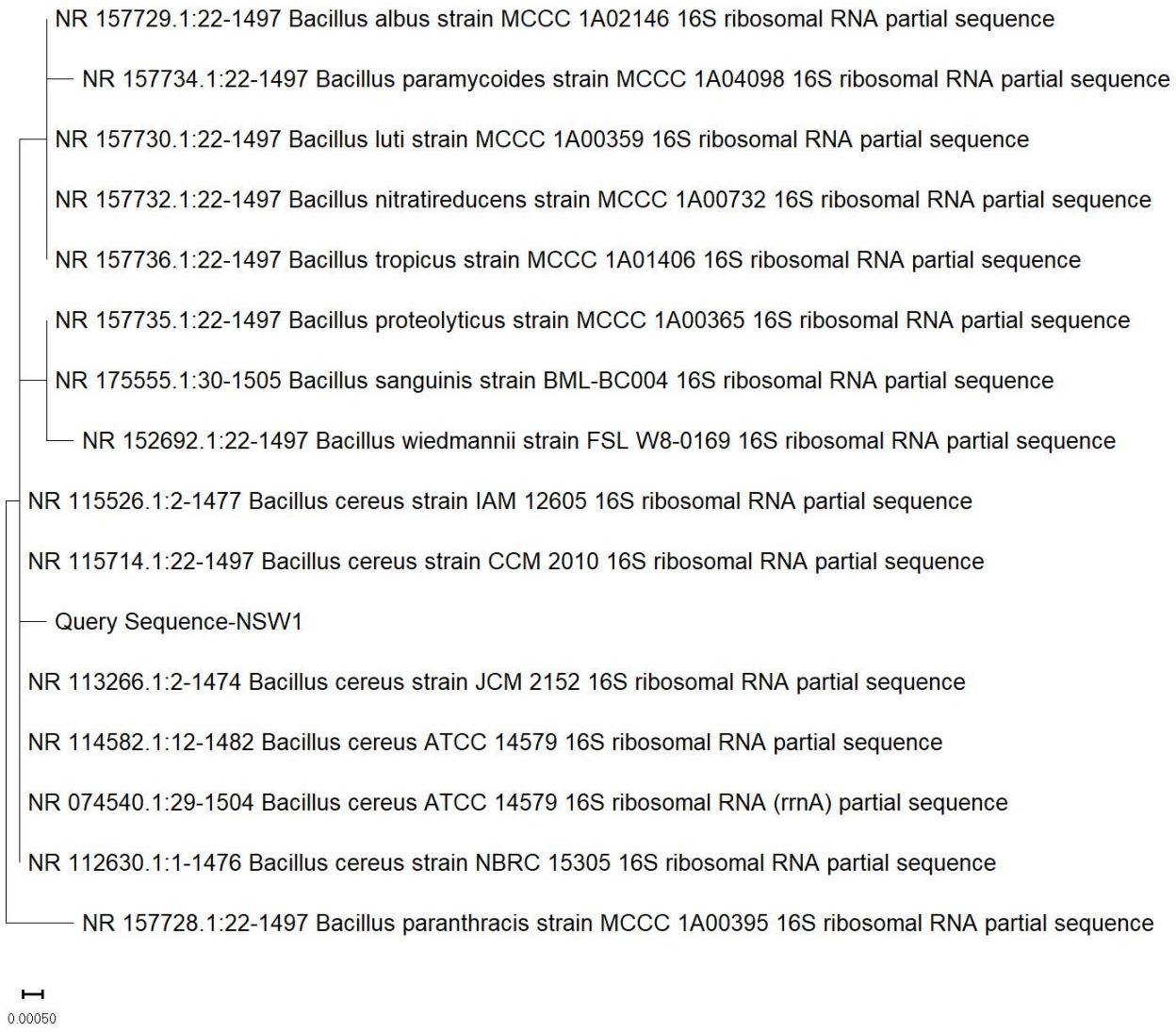
>NSW1

ATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGT
AACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGT
CACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCTACAC
TGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGA
AGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACT
ACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGC
CCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGG
AACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGT
AAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACT
CAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACCC
TAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCGAGCGCAACC
CTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTAT
GACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGG
CTGCAACTCGCCTACATAGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACAG
CACGAGAGTTTGTAACACCCGAAGTCGGTGGGGTAACCTTTTTGGAGCCAGCCGCCTAAGGTGGGACAGATGATTGGGGTGAAG

Sample: NSW1

- The Microbe was identified as Bacillus cereus as it showed highest similarity of 99.26 % with Bacillus cereus ATCC 14579 16S ribosomal RNA (rrnA), *partial sequence* with accession no. NR_074540.1
- Tree of top 10 closely related species is shown here

Phylogenetic Tree




## Evolutionary relationships of taxa

The evolutionary history was inferred using the Neighbor-Joining method [1]. The optimal tree is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [2] and are in the units of the number of base substitutions per site. This analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1503 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 [3]


1. Saitou N. and Nei M. (**1987**). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**:406-425.
2. Tamura K., Nei M., and Kumar S. (**2004**). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences* (*USA*) **101**:11030-11035.
3. Tamura K., Stecher G., and Kumar S. (**2021**). MEGA 11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology and Evolution* https://doi.org/10.1093/molbev/msab120.

# BLAST DATA

RID: 5BEN8AKN013
Job Title:NWS1
Program: BLASTN
Database: rRNA_typestrains/16S_ribosomal_RNA 16S ribosomal RNA (Bacteria and Archaea type strains)
Query #1:  Query ID: lcl|Query_7759965 Length: 1470

Sequences producing significant alignments:

| Description | Scientific Name | Common Name | Taxid | Max Score | Total Score | Query cover | E Value | Per. Ident | Acc. Len | Accession |
|---|---|---|---|---|---|---|---|---|---|---|
| Bacillus cereus ATCC 14579 16S ribosomal RNA (rrnA), partial... | Bacillus cer... | NA | 226900 | 2660 | 2660 | 100% | 0.0 | 99.26 | 1512 | NR_074540.1 |
| Bacillus cereus strain IAM 12605 16S ribosomal RNA, partial... | Bacillus cereus | NA | 1396 | 2660 | 2660 | 100% | 0.0 | 99.26 | 1486 | NR_115526.1 |
| Bacillus cereus strain CCM 2010 16S ribosomal RNA, partial... | Bacillus cereus | NA | 1396 | 2660 | 2660 | 100% | 0.0 | 99.26 | 1535 | NR_115714.1 |
| Bacillus cereus strain NBRC 15305 16S ribosomal RNA, partial... | Bacillus cereus | NA | 1396 | 2660 | 2660 | 100% | 0.0 | 99.26 | 1476 | NR_112630.1 |
| Bacillus tropicus strain MCCC 1A01406 16S ribosomal RNA, parti... | Bacillus tro... | NA | 2026188 | 2654 | 2654 | 100% | 0.0 | 99.19 | 1509 | NR_157736.1 |
| Bacillus proteolyticus strain MCCC 1A00365 16S ribosomal RNA,... | Bacillus pro... | NA | 2026192 | 2654 | 2654 | 100% | 0.0 | 99.19 | 1509 | NR_157735.1 |
| Bacillus nitratireducens strain MCCC 1A00732 16S ribosomal RNA... | Bacillus nit... | NA | 2026193 | 2654 | 2654 | 100% | 0.0 | 99.19 | 1509 | NR_157732.1 |
| Bacillus luti strain MCCC 1A00359 16S ribosomal RNA, partial... | Bacillus luti | NA | 2026191 | 2654 | 2654 | 100% | 0.0 | 99.19 | 1509 | NR_157730.1 |
| Bacillus albus strain MCCC 1A02146 16S ribosomal RNA, partial... | Bacillus albus | NA | 2026189 | 2654 | 2654 | 100% | 0.0 | 99.19 | 1509 | NR_157729.1 |
| Bacillus sanguinis strain BML-BC004 16S ribosomal RNA, partial... | Bacillus san... | NA | 2817476 | 2654 | 2654 | 100% | 0.0 | 99.19 | 1555 | NR_175555.1 |
| Bacillus cereus strain JCM 2152 16S ribosomal RNA, partial... | Bacillus cereus | NA | 1396 | 2654 | 2654 | 99% | 0.0 | 99.25 | 1474 | NR_113266.1 |
| Bacillus cereus ATCC 14579 16S ribosomal RNA, partial sequence | Bacillus cer... | NA | 226900 | 2651 | 2651 | 99% | 0.0 | 99.25 | 1482 | NR_114582.1 |
| Bacillus wiedmannii strain FSL W8-0169 16S ribosomal RNA,... | Bacillus wie... | NA | 1890302 | 2649 | 2649 | 100% | 0.0 | 99.12 | 1540 | NR_152692.1 |
| Bacillus paramycoides strain MCCC 1A04098 16S ribosomal RNA,... | Bacillus par... | NA | 2026194 | 2649 | 2649 | 100% | 0.0 | 99.12 | 1509 | NR_157734.1 |
| Bacillus paranthracis strain MCCC 1A00395 16S ribosomal RNA,... | Bacillus par... | NA | 2026186 | 2643 | 2643 | 100% | 0.0 | 99.05 | 1509 | NR_157728.1 |
| Bacillus fungorum strain 17-SMS-01 16S ribosomal RNA, partial... | Bacillus fun... | NA | 2039284 | 2643 | 2643 | 100% | 0.0 | 99.05 | 1576 | NR_170494.1 |
| Bacillus pacificus strain MCCC 1A06182 16S ribosomal RNA,... | Bacillus pac... | NA | 2026187 | 2638 | 2638 | 100% | 0.0 | 98.99 | 1509 | NR_157733.1 |
| Bacillus thuringiensis strain IAM 12077 16S ribosomal RNA,... | Bacillus thu... | NA | 1428 | 2638 | 2638 | 100% | 0.0 | 98.99 | 1486 | NR_043403.1 |
| Bacillus thuringiensis strain NBRC 101235 16S ribosomal RNA,... | Bacillus thu... | NA | 1428 | 2634 | 2634 | 100% | 0.0 | 98.92 | 1477 | NR_112780.1 |
| Bacillus clarus strain ATCC 21929 16S ribosomal RNA, complete... | Bacillus clarus | NA | 2338372 | 2632 | 2632 | 100% | 0.0 | 98.92 | 1552 | NR_180213.1 |
| Bacillus toyonensis strain BCT-7112 16S ribosomal RNA, partial... | Bacillus toy... | NA | 155322 | 2632 | 2632 | 100% | 0.0 | 98.92 | 1544 | NR_121761.1 |
| Bacillus thuringiensis strain ATCC 10792 16S ribosomal RNA,... | Bacillus thu... | NA | 1428 | 2628 | 2628 | 99% | 0.0 | 98.98 | 1482 | NR_114581.1 |
| Bacillus mobilis strain MCCC 1A05942 16S ribosomal RNA, partia... | Bacillus mob... | NA | 2026190 | 2627 | 2627 | 100% | 0.0 | 98.85 | 1509 | NR_157731.1 |
| Bacillus pseudomycoides strain NBRC 101232 16S ribosomal RNA,... | Bacillus pse... | NA | 64104 | 2627 | 2627 | 100% | 0.0 | 98.85 | 1477 | NR_113991.1 |
| Bacillus mycoides strain NBRC 101238 16S ribosomal RNA, partia... | Bacillus myc... | NA | 1405 | 2615 | 2615 | 100% | 0.0 | 98.71 | 1477 | NR_113996.1 |
| Bacillus mycoides strain NBRC 101228 16S ribosomal RNA, partia... | Bacillus myc... | NA | 1405 | 2615 | 2615 | 100% | 0.0 | 98.71 | 1477 | NR_113990.1 |
| Bacillus mycoides strain DSM 11821 16S ribosomal RNA, partial... | Bacillus myc... | NA | 1405 | 2615 | 2615 | 100% | 0.0 | 98.71 | 1531 | NR_024697.1 |
| Bacillus mycoides strain 273 16S ribosomal RNA, partial sequence | Bacillus myc... | NA | 1405 | 2610 | 2610 | 99% | 0.0 | 98.71 | 1513 | NR_036880.1 |
| Bacillus paramobilis strain BML-BC017 16S ribosomal RNA, parti... | Bacillus par... | NA | 2817477 | 2593 | 2593 | 98% | 0.0 | 98.90 | 1503 | NR_175556.1 |
| Bacillus hominis strain BML-BC059 16S ribosomal RNA, partial... | Bacillus hom... | NA | 2817478 | 2580 | 2580 | 98% | 0.0 | 98.70 | 1504 | NR_175557.1 |
| Bacillus mycoides strain ATCC 6462 16S ribosomal RNA, partial... | Bacillus myc... | NA | 1405 | 2573 | 2573 | 98% | 0.0 | 98.69 | 1461 | NR_115993.1 |
| Bacillus bingmayongensis strain FJAT-13831 16S ribosomal RNA,... | Bacillus bin... | NA | 1150157 | 2536 | 2536 | 97% | 0.0 | 98.41 | 1443 | NR_148248.1 |
| Bacillus pseudomycoides 16S ribosomal RNA, partial sequence | Bacillus pse... | NA | 64104 | 2516 | 2516 | 100% | 0.0 | 97.23 | 1532 | NR_114422.1 |
| Bacillus cytotoxicus strain NVH 391-98 16S ribosomal RNA,... | Bacillus cyt... | NA | 580165 | 2497 | 2497 | 100% | 0.0 | 97.29 | 1544 | NR_074914.1 |
| Bacillus rhizoplanae strain JJ-63 16S ribosomal RNA, partial... | Bacillus rhi... | NA | 2880966 | 2459 | 2459 | 97% | 0.0 | 97.70 | 1454 | NR_181926.1 |
| Bacillus manliponensis strain BL4-6 16S ribosomal RNA, partial... | Bacillus man... | NA | 574376 | 2377 | 2377 | 94% | 0.0 | 97.43 | 1400 | NR_125530.1 |
| Bacillus gaemokensis strain BL3-6 16S ribosomal RNA, partial... | Bacillus gae... | NA | 574375 | 2346 | 2346 | 91% | 0.0 | 98.08 | 1351 | NR_116644.1 |
| Bacillus anthracis strain ATCC 14578 16S ribosomal RNA, partia... | Bacillus ant... | NA | 1392 | 2342 | 2342 | 88% | 0.0 | 99.08 | 1306 | NR_041248.1 |
| Rossellomorea marisflavi strain TF-11 16S ribosomal RNA, parti... | Rossellomore... | NA | 189381 | 2289 | 2289 | 99% | 0.0 | 94.80 | 1506 | NR_025240.1 |
| Heyndrickxia acidicola strain 105-2 16S ribosomal RNA, partial... | Heyndrickxia... | NA | 209389 | 2287 | 2287 | 99% | 0.0 | 94.73 | 1548 | NR_041942.1 |
| Bacillus tianshenii strain YIM M13235 16S ribosomal RNA,... | Bacillus tia... | NA | 1463404 | 2281 | 2281 | 100% | 0.0 | 94.67 | 1550 | NR_133704.2 |
| Mangrovibacillus cuniculi strain R1DC41 16S ribosomal RNA,... | Mangrovibaci... | NA | 2593652 | 2272 | 2272 | 99% | 0.0 | 94.61 | 1549 | NR_181118.1 |

**The Sequencing mix Composition and PCR Conditions are as follows:**10µl Sequencing Reaction
Big Dye Terminator Ready Reaction Mix: 4µlTemplate
(100ng/ul): 1µl
Primer (10pmol/λ): 2µl
Milli Q Water : 3µl

**PCR Conditions: (25 cycles)**
Initial Denaturation : 96°C for 5
min Denaturation : 96°C for 30
sec Hybridization : 50 °C for 30
sec Elongation : 60 °C for
1.30 min

**Instrument and Chemistry Details**
Sequencing Machine: ABI 3130 Genetic Analyzer
Chemistry Cycle sequencing kit: Big Dye Terminator version 3.1"
Polymer & Capillary Array: POP_7 pol Capillary Array.Analysis
protocol: BDTv3-KB-Denovo_v 5.2
Data Analysis: Seq Scape_ v 5.2
Software Reaction Plate: Applied Biosystem Micro Amp
Optical 96-Well Reaction plate

**Identification software details:**

Phylogentic Tree Builder uses sequences aligned with System Software aligner. A distance matrixis generated using the Jukes-Cantor corrected distance model. When generating the distance matrix, only alignment model positions are used, alignment inserts are ignored and the minimum comparable position is 200. The tree is created using Weighbor with alphabet size 4 and length size 1000.

Weighbor Tree: Weighbor is a weighted version of Neighbor Joining that gives significantly less weight to the longer distances in the distance matrix. The weights are based on variances and covariances expected in a simple Jukes-Cantor model.

Jukes-Cantor Correction: The Jukes-Cantor distance correction is a model which considers that as two sequences diverge, the probability of a second substitution at any nucleotide site increases. For distance-based trees such as Weighbor, the difference in nucleotides is considered for the distance, therefore, second substitutions will not be counted and the distance will be underestimated. Jukes and Cantor createad a formula that calculates the distance taking into account more than just the individual differences (1969; Evol.of Protein Molecules, Academic Press)

Bootstrap: Bootstrapping is a statistical method for estimating the sampling distribution by resampling with replacement from the original sample. In making phylogenetic trees, the approach is to create a pseudoalignment by taking random positions of the original alignment. Some columns of the alignment could be selected more than once or not selected at all. The pseudoalignment will be as long as the original alignment and will be used to create a distance matrix and a tree. The process is repeated 100 times and a majority consensus tree is displayed S3owing the number (or percentage) of times a particular group was on each side of a branch without concerning the subgrouping.

Reference:

William J. Bruno, Nicholas D. Socci, and Aaron L. Halpern (2000). Weighted Neighbor Joining: A Likelihood-Based Approach to Distance-Based Phylogeny Reconstruction, Mol.Biol.Evol.DDSKVG13(1): 189-197. 2E. O. Wiley, D. R. Brooks, D. Siegel-Causey, V. A. Funk (1991). The Compleat Cladist: A Primer of Phylogenetic Procedures. Freely available at http://taxonomy.zoology.gla.ac.uk/teaching/CompleatCladist.pdf

# Results

## Genomic DNA (Gdna)


*Fig 1S: gDNA loaded on 1% Agarose gel*

PCR amplification with 16S primers

**Lane Description:** 1-DNA Marker(100bp) 2. Sample

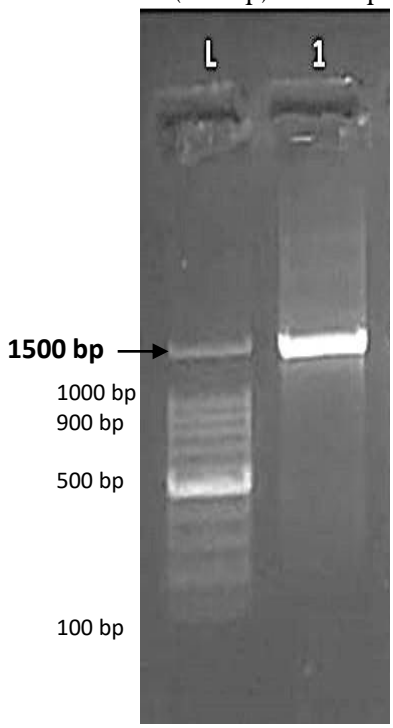



*Fig 2S: PCR amplified product loaded on 1.2 % Agarose gel*