International Journal of Plant Pathology and Microbiology

E-ISSN: 2789-3073
P-ISSN: 2789-3065
IJPPM 2023; 3(2): 35-40
www.plantpathologyjournal.com
Received: 16-06-2023
Accepted: 18-07-2023

Bamidele Helen Olaide
University of Ilorin, Kwara State, Nigeria

Fawole Oluyemisi Bolajoko
Department of Agronomy, University of Ilorin Kwara State Nigeria

Balogun Olusegun Samuel
Department of Crop Protection, University of Ilorin Kwara State Nigeria

Correspondence Author;
Bamidele Helen Olaide
University of Ilorin, Kwara State, Nigeria

Molecular characterization of bacteria associated with deterioration of some fruits and vegetables

Bamidele Helen Olaide, Fawole Oluyemisi Bolajoko and Balogun Olusegun Samuel

Abstract
This study was to carry out the molecular characterization of isolated bacterial from some deteriorated fruits and vegetables. Common bacterial affecting selected fruits and vegetables were isolated. They include: *Pseudomonas aeruginosa, Salmonella enterica, Enterococcus faecium* and *Lactobacillus plantarum*. They were molecularly characterized using 16SrDNA sequence analyses. DNA extraction was carried out using Zymo DNA mini kit prep and the primer used were 16SF and 16SR. The 16SrDNA regions of these pathogens were amplified by polymerase chain reaction (PCR) with Gene Amp 9700 thermocycler and then sequenced using Applied Biosystem 3130X gene analyser. Nucleotide sequences were deciphered using nBLAST. Alignment and construction of phylogenetic tree were performed using Neighbour Joining method of MEGA 7 software together with the sequence of related strains which were downloaded from GenBank. The results of the DNA yield and purity were within the range of 23 to 215.2 ng/µl and 1.84 to 1.93. The number of nucleotide for all the organisms isolated varies from 490 to 1563, BLAST searches revealed that the organisms identified were within the range of 93-100% identical. The results demonstrated that good yield and pure DNA extracts were obtained from the selected fruits and vegetables and the primers used are useful for bacterial identification.

Keywords: DNA extraction, primers (16 SF and 16 SR), gel electrophoresis, polymerase chain reaction, Sequencing and Phylogenetic analysis

Introduction
Fruits and vegetables have high dietary and nutritional values and due to these qualities they are very important (Barth *et al*., 2009) [2]. Consumption of fruits and vegetables has been promoted because of their vitamins, minerals, antioxidants, and fiber content. Consuming them also has the ability in reducing the risk of some specific diseases (Oguntibeju *et al*., 2013) [20] and due to these, consuming fruits and vegetables has dramatically increased by more than 30% during the past few decades (Barth *et al*., 2009) [2].
Fruits and vegetables deteriorate fast because they are perishable in nature and cannot withstand too high or low temperature and this deterioration is mainly caused by two main activities which are natural degradation due to activities of enzymes and growth of microorganisms (bacteria, molds and yeasts). Bacteria and fungi may also produce waste products which act as poisons or toxins, thus causing an unplanned and unpleasant effect (Bakri *et al*., 2010) [1]. Deoxyribonucleic acid (DNA) extraction is the process by which nucleic acids in a cell is separated away from proteins, membranes, and other cellular material contained in the cell from which it is recovered. The ability to extract and purify DNA is often the key starting point for a variety of downstream experimental procedure like polymerase chain reaction (PCR). Pure DNA extraction give good polymerase chain reaction (PCR) products compared to DNA with lower purity which requires several dilutions before it will give a good PCR product (Turaki *et al*., 2017) [29]. Kumari *et al*., (2012) [16] also reported that quality and overall yield are important for molecular techniques in plant molecular biology, genetic materials conservation and crop improvement. The purpose of this study was to carry out the molecular characterization of isolated bacterial from deteriorated fruits and vegetables.

Materials and Methods
The bacterial isolates were preserved by growing them on Nutrient Broth and stored in MC-Cartney bottle until they were taken to International Institute of Tropical Agriculture (IITA) Bioscience Centre Laboratory for further analysis. The DNA extraction and purification were prepared according to the manual of Zymo Research Corp. (ZR Plant/Seed DNA Mini Prep Kit)

Assessment of DNA quantity and purity

The quantity of the extracted DNA was compared by measuring the concentration and purity using a UV Spectrophotometer (NanoDrop™ 2000, Thermo Scientific). DNA extracts were quantified by measuring the absorbance at 260 nm (A260). DNA purities were estimated by calculating the A260/A280 ratios. Samples calculated to have A260/A280 ratios of 1.7–2.0 were assumed to be pure, free from protein, and other contaminants (Cawthorn *et al*., 2011) [3]. Every sample was measured three times. Measurements were taken at room temperature following sufficient mixing of all samples.

Agarose gel electrophoresis and spectrophotometry

Agarose gel (1.5%) was prepared, microwaved at 100 ºC for 3 minutes and allowed to cool. Five microliter (5 µl) of ethidium bromide (EtBr) was added to 150 ml agarose gel, poured into the agarose gel tank (Compact L/XL Biometra by Analytik Jena Company) and allowed to solidify. Subsequently, 3 µl of loading dye was added to 5 µl each of the samples. Samples were resolved, loaded and allowed to run at 100 V for 45 min before viewing on UV light source and photographed.

Polymerase Chain Reaction amplification and sequencing

PCR Cocktail mix for bacteria isolates

The DNA of the selected isolated bacteria were subjected to the following cocktail mix and condition for the PCR. The PCR cocktail mixture contained 1.0 µl of 10× PCR buffer reactions, 1.0 µl of 25 mM MgCl2 and 0.5 µl of each 5pMol of forward (16 SF) and reverse (16SR) primers. Addition of 1.0 µl of DMSO, 0.8 µl of 2.5 Mm DNTPs, 0.1 µl of Taq 5 µ/µl, 2.0 µl of 10 ng/µl DNA and 3.1µl of H20 per 10 µl reaction mixture (Table 1).

Table 1: PCR Cocktail mix for bacteria

Component	Concentration
10× PCR buffer	1.0
25mM $MgCl_2$	1.0
5pMol forward primer	0.5
5pMol reverse primer	0.5
DMSO	1.0
2.5 Mm DNTPs	0.8
Taq 5µ/µl	0.1
10 ng/µl DNA	2.0
H_2O	3.1
	10µl

The primers used for the 16S rDNA molecular characterisation of the selected associated bacteria were: 16SF: GTGCCAGCAGCCGCGCTAA 16SR: AGACCCGGGAACGTATTCAC (Mauti *et al*., 2013) [18]

PCR condition

The following thermocycling parameters are typically utilized by initially denaturing at 94 °C for 5 min, followed by 36 cycles of 94 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 45 sec. This was followed with a final extension step of 72 °C for 7 min and later left at 10 °C as the hold tempt for further used. This procedure was carried out using a modified method of Schoch *et al*., (2012) [26] and Promputtha and Miller (2010) [22]. The amplicon from the reaction was loaded on 1.5% agarose gel tank (Compact L/XL Biometra by Analytik Jena Company). The ladder used is hyper ladder 1 from Bioloine. (Table 2).

Table 2: PCR condition

Initial den.	Den.	Ann. tempt	Extension	No. of circles	Final extension	Hold tempt
94 °C	94 °C	56 °C	72 °C	36	72 °C	10 °C
5 min	30 sec	30 sec	45 sec		7 min	∞

PCR product purification

The PCR was purified with the following protocol 2 vol (20 µl) of absolute ethanol was added to the PCR product. It was incubated at room temperature for 15 minutes, spinned down at 10000 rpm for 15 minutes before decanting the supernatant and then spin down at 10000 rpm for 15 minutes and 2 vol (40 µl) of 70% ethanol was added before decanting supernatant then it was air dried. About 10µl of ultrapure water was added and then checked for amplicon on 1.5% agarose using gel documentation system (UV Trans illuminator by Aplegen). The PCR product was used for sequencing reaction.

Sequencing of selected bacterial isolates

The sequencing reaction was purified by using Sanger sequencing of the purified PCR products which is performed using Big Dye Terminator v3.1 cycle sequencing with 3µl of DNA template and 0.5 µl of 2 µM of each primer. Both strands are sequenced (bi directionally) using a combination of the following primers: 16SF, and 16SR. The product from the purification process was loaded and the sequence are generated on the ABI 3130 genetic analyser from Applied Biosystems (Gene Amp PCR system 9700 by Applied Biosystems).

Sequence data view and alignment

The sequences data was viewed and analyzed with Mega 7 and Bioedit software and were compared with sequences in National Centre for Biotechnology Information (NCBI), USA which was done by using nucleotide Basic Local Alignment Search Tool (BLAST). Sequence alignments were performed using Clustal W (Thompson *et al*., 1994) [28] and Alignments were subsequently adjusted manually using Bioedit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) (Hall, 1999) [9] and MEGA 7 (Kumar *et al*., 2016) [31]. The consensus sequence of the 16S and ITS region were submitted for a BLAST using the NCBI GenBank database to obtain species-level information (El-Elimat *et al*., 2014) [7]

Phylogenetic analyzes

The evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei, 1987) [23]. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016) [31]. Moreover, this method has been reported as fast and accurate both for examining the relationships among species and also to assign unidentified samples to known species (Hebert *et al*., 2003) [10]. This method was also compared with Clustal Omega to construct the trees

Maximum Likelihood

Sequences were edited using Bio-edit and Mega 7 software. Each sequence was subjected to an individual BLAST to verify its identity in GenBank. The newly obtained sequences were aligned with highly similar, homologous sequences from GenBank using the multiple sequence alignment program MUSCLE (Edgar, 2004) [6], Clusta Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) with default parameters. The final alignment was optimized by eye and

manually corrected. Maximum likelihood methods were used in phylogenetic analyses for all genes.

Results

Quantitative estimation of DNA by Nano-drop Spectrophotometer

High quantity and good yield of DNA was obtained during extraction from all the selected fruits and vegetables. The concentration of DNA (nucleic acid) obtained from the samples measured in nano-gram per microlitre (ng/µl) and the absorbance ratios of DNA purity were shown in the Table 3. The results of the agarose gel electrophoresis of the isolated bacteria (Plate 1) are marker (M), *Pseudomonas aeruginosa* (a), *Salmonella enterica* (b), *Lactobacillus plantarum* (c) and *Enterococcus faecium* (d).

Table 3: Quantity and purity of Nucleic acids extracted from selected microorganisms

Sample ID	Nucleic Acid (ng/µl)	A 260/A 280 ratio
Pseudomonas aeruginosa	215.2	1.89
Salmonella enterica	23	1.93
Lactobacillus plantarum	34.4	1.84
Enterococcus faecium	42.2	1.85

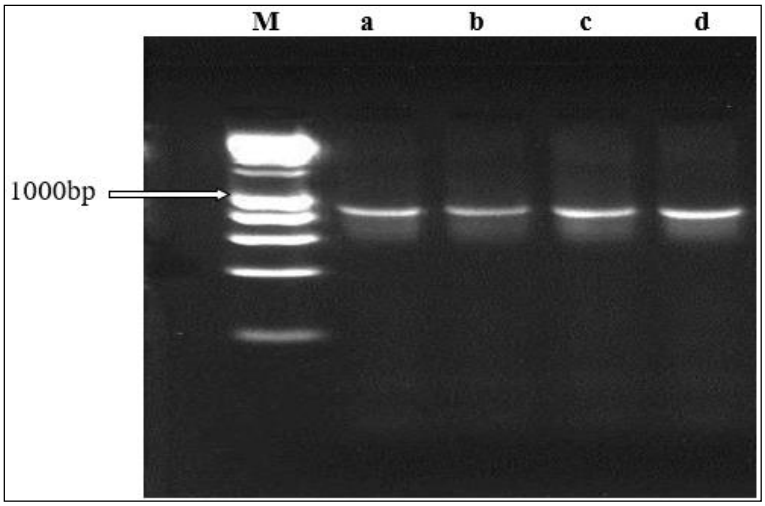

Plate 1: Agarose gel electrophoresis of products from PCR performed with primers 16SF and 16SR. *Pseudomonas aeruginosa* (a), *Salmonella enterica* (b), *Lactobacillus plantarum* (c) and *Enterococcus faecium* (d)

Amplification, sequencing and data alignment of selected bacteria isolates

The primer pairs successfully amplified all the bacterial isolated which signified their uniform effectiveness in amplifying intense bands. The 93-100% amplification rate of selected bacterial isolates was obtained in the present study. Based on the sequence of GenBank reference strains and pathogenic strains, the phylogenetic tree was generated for bacteria species. The organisms, number of nucleotide based on the query lengths of amplified fragments using the primers 16SF and 16SR for bacteria range from 490 to 1526 are shown in Table 4. The accession number, query covered and the percentage of identity are *Pseudomonas aeruginosa* (NR117678.1) with 100% query cover and 99% identity, *Salmonella enterica* (CP 022663.1) with 100% query cover and 93% identity, *Lactobacillus plantarum* (MK 347022.1), *Enterococcus faecium* (KT 626391.1) with 100% query cover and 100% identity.

Table 4: Nucleotide sequence amplification

No of nucleotide sequence amplified					
Organisms	**Accession no.**	**16SF**	**16SR**	**Percentage of query cover**	**Percentage of Identity**
Pseudomonas aeruginosa	NR117678.1	847	1526	100	99
Salmonella enterica	CP022663.1	490	1275	100	93
Lactobacillus plantarum	MK347022.1	1439	764	100	100
Enterococcus faecium	KT626391.1	869	540	100	100

Phylogenetic analysis

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987) [23]. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) [27] and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016) [31]. The phylogenetic tree analyzed are shown in Figure 1-4.

Nucleotide composition for amplified bacteria and fungi

Table 5 shows the nucleotide frequencies and all the frequencies are given in percentage. The average total for T (U), C, A and G are 21.7, 22.8, 23.7 and 29.7 with the sum total of 1221.8. The table also shows the total of each accession number with the total ranging from 490 to 2239 based on each accession number.

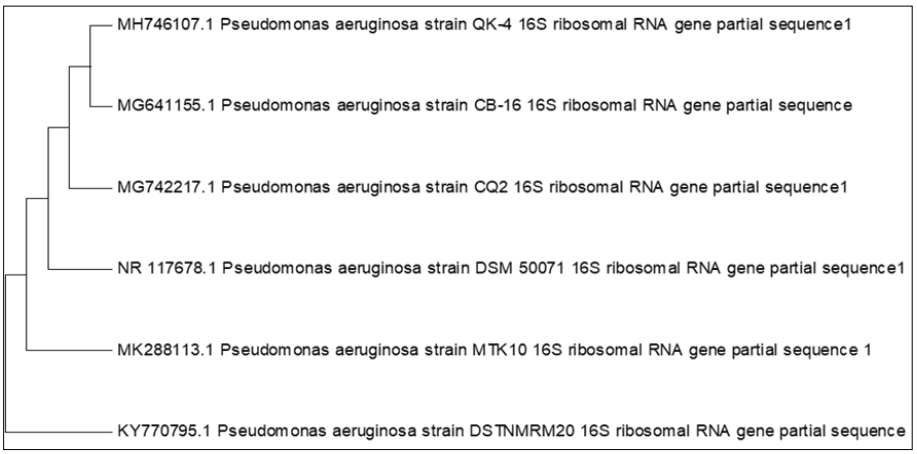

Fig 1: Phylogenetic tree of *Pseudomonas aeruginosa* sequence

The optimal tree of *Pseudomonas aeruginosa* with the sum of branch length 0.02013175 and there were a total of 828 positions in the final dataset.

Fig 2: Phylogenetic tree of *Salmonella enterica* sequence

The optimal tree of *Salmonella enterica* is with the sum of branch length 1.34350770 and there were a total of 418 positions in the final dataset.

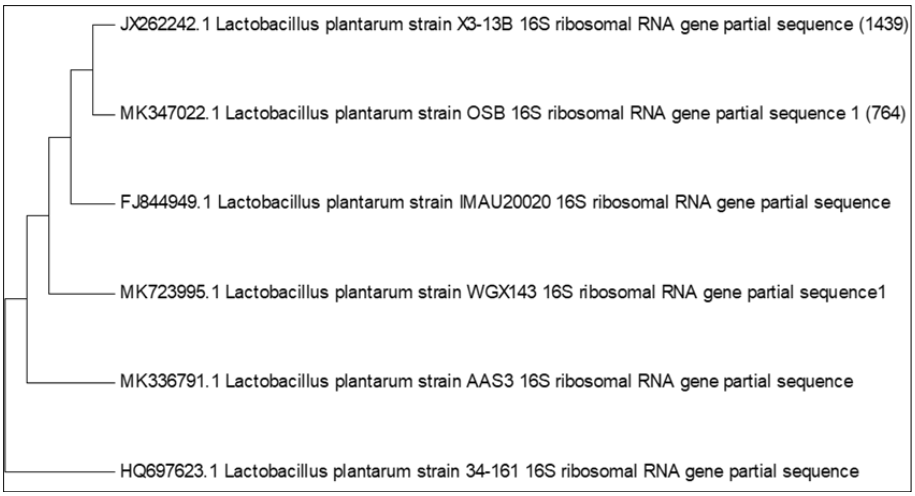

Fig 3: Phylogenetic tree of *Lactobacillus plantarum* sequence

The optimal tree of *Lactobacillus plantarum* is with the sum of branch length 0.00434595 and there were a total of 463 positions in the final dataset

.

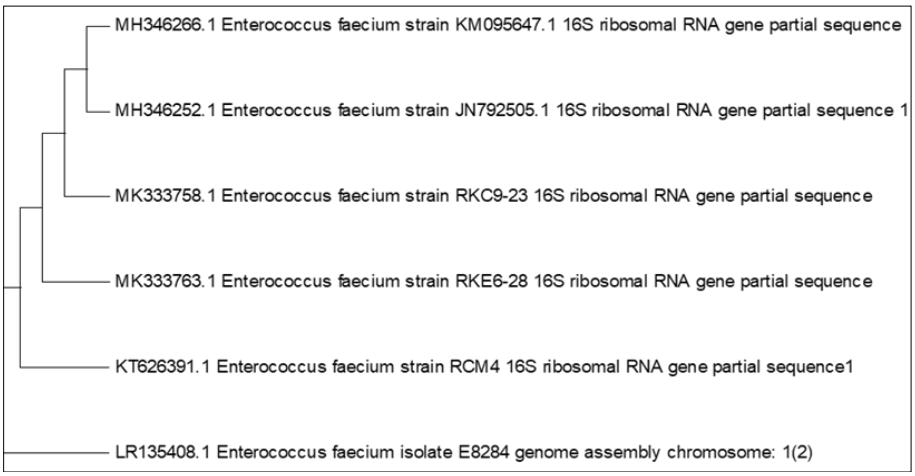

Fig 4: Phylogenetic tree of *Enterococcus faecium* sequence

The optimal tree of *Enterococcus faecium* is with the sum of branch length 0.56871898 and there were a total of 452 positions in the final dataset.

Table 5: Nucleotide composition for amplified bacteria sequence

S/N	Accession no	T(U)	C	A	G	Total
1	NR 117678.1	20.6	22.7	25.2	31.6	1526.0
2	MK288113.1	20.8	23.7	24.9	30.6	847.0
3	MG742217.1	20.6	22.7	25.2	31.5	1360.0
4	MH746107.1	20.1	22.7	25.5	31.7	1431.0
5	MG641155.1	20.5	22.9	25.2	31.4	1431.0
6	KY770795.1	20.7	23.5	25.8	30.0	966.0
7	CPO28311.1	22.3	26.3	25.0	26.4	1566.0
8	LR134233.1	21.9	25.5	24.5	28.1	1378.8
9	LR134232.1	25.5	21.8	21.0	31.6	490.0
10	CP029862.1	22.2	26.1	23.9	27.9	2239.0
11	JQ315897.1	19.9	23.5	24.6	32.0	1470.0
12	CP022663.1	22.1	25.8	26.1	26.0	1275.0
13	MK347022.1	21.9	20.3	27.6	30.2	764.0
14	JX262242.1	22.3	22.0	26.6	29.0	1439.0
15	MK723995.1	22.3	22.0	26.2	29.4	1563.0
16	FJ844949.1	22.1	21.9	26.5	29.5	1492.0
17	HQ697623.1	22.1	21.8	26.6	29.5	1440.0
18	MK336791.1	22.1	21.7	26.7	29.4	1208.5
19	MK333758.1	20.6	22.1	26.1	31.2	869.0
20	MH346252.1	21.0	22.3	25.9	30.8	902.0
21	MH346266.1	21.2	22.2	25.9	30.7	910.0
22	KT626391.1	20.9	21.1	25.9	32.0	540.0
23	LR135408.1	25.8	19.5	33.5	21.2	1285.0
24	MK333763.1	21.8	22.5	25.4	30.3	930.0
	Average	21.7	22.8	23.7	29.7	1221.8

Discussion

Good quality DNA was extracted from the isolated bacteria which showed that contaminants were not present. This collaborated the report of Mirbahar *et al*., (2014) [19] that good quality DNA isolation is necessary and important. Pich and Schubert (1993) [21] described DNA purity in the range of 1.6 - 1.7 in which shows the absence of contaminants. This study showed that the 16SrDNA primer used are good for phylogenetic analysis. Schloss (2010) [25] showed that the quality of 16S sequences are essential to accurate phylogenetic placement and taxonomic classification (Jumpstart Consortium Human Microbiome Project Data Generation Working Group, 2012). The percentages of identification of the isolated microorganisms are within the range of 93-100 percent sequence similarity of the genome as *P. aeruginosa* had 99 percent, *L. plantarum* had 100 percent, *E. faecium* is within 99-100 percent and *S. enterica* is within 93 percent. This is in disagreement with most of the investigations reporting the amplification rates of 92-100% (Kress *et al*., 2005; Kress and Erickson, 2007; Fazekas *et al*., 2008; Hollingsworth *et al*., 2009; Kress *et al*., 2009; CBOL Plant Working Group 2009; Ebihara *et al*., 2010) [15, 13, 8, 11, 14, 4, 5].

Conclusion

In conclusion, good quality DNA was extracted from the isolated bacteria which showed that contaminants were not present. The 16SrDNA primer proved to be useful in identification and characterization of the isolated microorganisms as they provide a more reliable method for identification of species than morphological characters.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

1. Bakri Y, Masson M, Thonart P. Isolation and identification of two new fungal strains for xylanase production. Applied Biochemistry and Biotechnology. 2010;162:1626-1634.oi: 10.1007/s12010-010-8944
2. Barth M, Hankinson TR, Zhuang H, Breidt F. Microbiological Spoilage of Fruits and Vegetables. W.H. Sperber, M.P. Doyle (eds.), Compendium of the Microbiological Spoilage of Foods and Beverages, Food Microbiology and Food Safety. C Springer Science Business Media, LLC; c2009. p. 135-183.
3. Cawthorn D, Steinman HA, Witthuhn RC. Comparative study of different methods for the extraction of DNA from fish species commercially available in South Africa. Food Control. 2011;22:231-244.
4. Taia WK, Mahdy RA, Bassiouni EM. Pollen morphological study in some Bauhinia L. species and their phylogenetic indications. Int. J. Bot. Stud. 2022;7(2):515-26.
5. Ebihara A, Nitta LH, Ito M. Molecular species identification with rich floristic sampling: DNA barcoding the Pteridophyte flora of Japan. Plos One. 2010;5:e15136.

6. Edgar RC. Muscle multiple sequence alignment with high accuracy and high throughout. Nucleic Acids Research. 2004;32:1792-179710.1093/nar/gkh340.
7. El-Elimat T, Raja HA, Figueroa M, Falkinham JO, Oberlies NH. Greensporones: Resorcyclic acid lactones from an aquatic *Halenospora* sp. Phytochemistry. 2014;104:114-120. 10.1016/j.phytochem.2014.04.006.
8. Fazekas AJ, Burgess KS, Kesanakurti PR, Graham SW, Newmaster SG, Husband BC, *et al*. Multiple multilocus DNA barcodes from the plastid genome discriminate plant species equally well. Plos One. 2008;3(7):1-12.
9. Hall TA. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series. 1999;41:95-98.
10. Hebert PON, Cywinska A, Ball SL, DeWaard JR. Biological identification through DNA barcodes. Proceedings of the Royal Society of London Series B. 2003;270:313-321.
11. Hollingsworth ML, Clark A, Forrest LL, Richardson J, Pennington RT, Long DG, *et al*. Selecting barcoding loci for plants: evaluation of seven candidate loci with species- level sampling in three divergent groups of land plants. Molecular Ecology Resources. 2009;9:439-457.
12. Jumpstart Consortium Human Microbiome Project Data Generation Working Group. Evaluation of 16S rDNA-based community profiling for human microbiome research. Plos One. 2012;7:e39315. PMID: 22720093
13. Kress WJ, Erickson DL. A two-locus global DNA barcode for land plants: The Coding Rbcl Gene complements the non-coding trnHpsbA Spacer Region. Plos One. 2007;2:508.
14. Kress WJ, Erickson DL, Jones FA, Swenson NG, Perez R, Sanjur O, *et al*. Plant DNA barcodes and a community phylogeny of a tropical forest dynamics plot in Panama. Proceedings of the National Academy of Sciences, USA. 2009;106:18621-18626.
15. Kress WJ, Wurdack KJ, Zimmer EA, Weigt LA, Janzen DH. Use of DNA barcodes to identify flowering plants. Proceedings of the National Academy of Sciences, USA. 2005;102:8369-8374.
16. Kumari V, Bansal A, Aminedi R, Taneja D, Das N. Simplified extraction of good quality genomic DNA from a variety of plant materials. African Journal of Biotechnology. 2012;11(24):6420-6427, http://www.academicjournals.org/AJB, DOI: 10.5897/AJB11.2366
17. Wahab A, Abaseen N, Hayat M, Khan B, Luqman M. Advances in understanding the DNA-repair mechanism activated by CRISPR/Cas9. Int. J Biol. Sci. 2022;4(2):01-10.
DOI: 10.33545/26649926.2022.v4.i2a.68
18. Mauti EM, Mauti GO, Ouno GA, Mabeya BM, Kiprono SJ. Molecular identification of soil bacteria by 16srDNA sequence. Journal of Natural Sciences Research. 2013;3(14):52-53.
19. Mirbahar AA, Khan S, Saeed R, Kauser N, Jahan B, Markhand GS. DNA extraction and optimization from fibrous leaves of some date palm cultivars from Pakistan. FUUAST Journal of Biology. 2014;4(2):119-122.
20. Oguntibeju OO, Truter EJ, Esterhuyse AJ. The Role of Fruit and Vegetable Consumption in Human Health and Disease Prevention, Diabetes Mellitus-Insights and Perspectives; c2013. DOI: 10.5772/50109.
21. Pich U, Schubert I. Midiprep method for isolation of DNA from plants with a high content of polyphenolics. Nucleic Acid Research. 1993;21:3328.
22. Promputtha I, Miller AN. Three new species of Acanthostigma (Tubeufiaceae, Dothideomycetes) from Great Smoky Mountains National park. Mycologia. 2010;102(3):574-587. https://doi.org/10.3852/09-051
23. Saitou N, Nei M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution. 1987;4:406-425.
24. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Sciences. 197774:5463-5467.
25. Schloss PD. The effects of alignment quality, distance calculation method, sequence filtering, and region on the analysis of 16S rRNA gene-based studies. PLoS Journal of Computational Biology. 2010;6:e1000844. Pmid: 20628621
26. Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, *et al*. Fungal Barcoding, C.; Fungal Barcoding Consortium Author, L. Proceedings of the National Academy of Sciences, U.S.A. 2012;109:6241-6246. [PubMed]
27. Tamura K, Nei M, Kumar S. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences (USA). 2004;101:11030-11035.
28. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research. 1994;22:4673-4680.
29. Turaki AA, Ahmad B, Magaji UF, Abdulrazak UK, Yusuf BA, Hamza AB. Optimised cetyltrimethylammonium bromide (CTAB) DNA extraction method of plant leaf with high polysaccharide and polyphenolic compounds for downstream reliable molecular analyses. African Journal of Biotechnology. 2017;16(24):1354-1365. DOI: 10.5897/AJB2017.15942
30. CBOL Plant Working Group. A DNA barcode for land plants. Proceedings of the National Academy of Sciences USA. 2009;106:12794-12797.
31. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for bigger datasets. Molecular Biology and Evolution. 2016;33:1870-1874.