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Molecular Phylogenetic Study on *Pseudomonas stutzeri* Isolated from Currency Notes in Khartoum State, Sudan and Identified Via16s rRNA Gene Sequence Analysis

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Abstract: *Pseudomonas stutzeri* is a valuable bacteria for understanding of the taxonomical and the phylogenetic relationships. The study of genetic relationships between organisms or genes is carried out by molecular phylogeny. We aim to study the relationships according to16S rRNA gene sequences between our samples and the closest strains from other countries over the world. To our knowledge, the phylogenetic studies between strains from Sudan with strains from other countries were not done before. A total of 140 currency notes in different denominations were collected randomly from several locations including hospitals, food sellers and transporters. From the collected notes, a total of 135 bacterial colonies were isolated and from them 14 isolates were identified as a *pseudomonas stutzeri*. In the study, streaking plate method was used for the isolation of pure bacterial culture, Chelex 100 method was used for DNA extraction, conventional PCR was used for amplification of the targeted gene, agarose gel electrophoresis and various bioinformatics tools were used for nucleotide sequence analysis. The PCR products were sent for Macrogen Company-Netherlands for purification and nucleotide sequencing. After sequencing 3 samples were noisy, hence they were excluded. According to phylogenetic analysis, we found that the samples were closely related to strains from south-east Asia (Indonesia), east Asia (China), south-central Asia (Bangladesh), south Asia (India), north Africa (Tunisia) and south Europe (Italy and Greece). Despite the samples were from the same source (currency notes), we found that there is broad sequence variation between them.

Keywords: *Pseudomonas stutzeri*, Currency Notes, Molecular Phylogenetic, Khartoum, Sudan

1. Introduction

Pseudomonas stutzeri is a gram negative straight rod [5] belonging to gamma-Proteobacteria [6].

Strains of *P. stutzeri* exhibit broad genotypic diversity [7], [8] consequently, *P. stutzeri* is a valuable bacteria for understanding of the taxonomical and the phylogenetic relationships [9].

The study of genetic relationships between organisms or genes is carried out by molecular phylogeny via the

comparing of protein or homologous DNA sequences such as ribosomal RNA and mitochondrial genes. Ribosomal RNA consists of highly conserved and variable regions as well as it is universal, consequently, is a valuable target for the phylogenetic studies [10].

P. stutzeri strains are characterized by their ability to grow in broad range of temperature, anaerobically, and organotrophically (via utilization of many organic substrates) as well as the resistance to heavy metals. Therefore, they were isolated from various environments including soil, groundwater, rhizosphere marine water, sediment, wastewater [5], clinical materials [11] and currency notes [12]. Many studies on the genotypic identification and phylogenetic analysis of *P. stutzeri* were performed via several highly specific and sensitive molecular markers including 16S rRNA gene [5]. In Sudan, it was isolated and identified via conventional methods [13], [14], [15] as well as 16S rRNA gene [16].

After the Isolation and Identification of bacteria, we aim to study the relationships according to 16S rRNA gene sequences between our samples and the closest strains from other countries over the world. Within the Ribosomal RNA genes, 16S rDNA is the most universal and conservative, therefore it is the most valuable in phylogenetic [17], hence it was selected for the study. To our knowledge, the phylogenetic studies between strains from Sudan with strains from other countries were not done before.

2. Materials and Methods

This study was conducted in Faculty of pharmacy, Omdurman Islamic University in collaboration with the Applied Bioinformatics Center, Africa City of Technology and the Department of Epidemiology, Tropical Medicine Research Institute, National Center for Research, Khartoum, Sudan between May 2016 and July 2016. A total of 140 currency notes in 5, 10, 20 and 50 denominations were collected randomly from different locations including hospitals, food sellers and transporters. The collected notes were placed into sterile plastic petri dishes and transferred to the laboratory.

2.1. Microbiological Methods

Firstly, the collected currency notes were moistened with sterile distilled water and swabbed at both sides cotton tipped swabs. The swabs were directly inoculated in 5% blood agar plates, incubated aerobically for 24 hours at 37°C, sub cultured in MacConkey and Cetrimide agars and incubated for 24 hours at 37°C. After that, the single colonies were isolated, cultured into nutrient agar plates and identified phenotypically (gram staining and biochemical tests) according to protocols described in Monica Cheesbrough [18]. Several biochemical tests were performed including Oxidase, Catalase, Indole, Glucose and Lactose fermentation, Citrate, Urease and Motility tests. The results of biochemical tests are listed in Table 2. A total of 135 bacterial colonies were isolated and from them 21 isolates were identified as a *pseudomonas* species.

2.2. Molecular Methods

At first, streaking plate method was used for the isolation of pure bacterial culture [19]. Next, 200µl from 1X phosphate buffer saline were taken and transferred to sterile 1.5ml eppendorf tube by micropipette. Then, the distinctive colonies from the pure bacterial culture were isolated and inoculated into the eppendorf tubes. After that, Chelex 100 method was used for DNA extraction [20], conventional PCR was carried out via Alpha Unit Block Assembly DNA Thermo cycler from Bio-RAD Company by using Maxime PCR premix kit (i-Taq, for 20 µl reaction) from INtRON Biotechnology with the universal primers 27F and 1495R that have the sequences (5′-AGAGTTTGGATCMTGGCTCAG-3) and (5′-CTACGGCTACCTTGTTACGA-3′) respectively [21].

The PCR reaction volumes are listed below in Table 3 and temperature cycles involved initial denaturation cycle at 94°C for 5 minutes, followed by 35 amplification cycles (1 minute at 94°C, 1 minute at 58°C and 2 minute at 72°C) as well as final extension cycles at 72°C for 10 minutes [21]. After that, agarose gel electrophoresis was carried out against the obtained PCR amplicons via Amersham gel electrophoresis device according to protocol described by Lee. P. Y et al. [22] and the PCR products wre sent to Macrogen Company-Netherlands for purification and nucleotide sequencing of both strands.

2.3. Bioinformatics Analysis

After obtaining the sequences, firstly they were checked and corrected them manually via Finch TV software [23]. From the received sequences, 14 sequences were identified as *P. stutzeri* via nucleotide BLAST tool at NCBI [24], but 3 sequences of them were noisy, consequently they were excluded. After that, various bioinformatics tools were used including GenBank database [25] to obtain the sequences of previously identified genomovars and strains with higher identity.

In addition to that, Clustal W algorithm was used for multiple sequence alignment [26] and BioEdit software [1] for the visualization of multiple sequence alignment. Moreover, MEGA 6.06 [4] was used for phylogenetic analysis and Unipro UGENE software [27] was used for the calculation of simple identity in percent. The closest strains (sequences with higher identity in BLAST search) that we used for phylogenetic analysis with their accession numbers are listed table 1.

Nucleotide Sequence Accession Numbers

The identified nucleotide sequences were deposited in the GenBank database [25] under the accession numbers KY039354 to KY039364 that listed in Table 4.

Table 1. *Nucleotide sequences of stains with higher identity in BLAST search [19] obtained from GenBank database [20].*

Strain	Country	Accession number
VKM B-97	USA	NR_116489.1
0511MAR14N1	Spain	LN774555.1
APB6	China	KP768391.1
1005	India	KU749990.1
SL-02	Indonesia	KX082892.1
SC-04	Indonesia	KX082841.1
SP-09	Indonesia	KX082842.1
SR-23	Indonesia	KX082843.1
TRA27A	Spain	JQ782508.1
K-2-7	Iran	JQ963329.1
F1	China	HQ292192.1
A10	Tunisia	KU180229.1
EGY-SCM1	Egypt	KJ545584.1
OOYW-9	Italy	KJ534280.1
ME-1	Pakistan	KF975434.1
A160/74	Spain	HF571089.1
MH004	Egypt	KU855015.1
Gr45	Greece	FR667889.1
Gr17	Greece	FN813477.1
B11	China	KT380516.1
W45	China	KT380587.1
BD-2.2.1	Vietnam	LC125170.1
B15	China	KT380520.1
MN1	India	KU708859.1
NA3	India	KU708861.1
Bon_a1	United Kingdom	FN397901.1
Xmb018	China	KT986148.1
KG-2 NRB-DRDO MP	India	KX344913.1
NB-03	Pakistan	KX262874.1
SP-10	Indonesia	KX082893.1
40/D/Mac2	Bangladesh	KT716345.1
Bd8	Sudan	KJ801394.1
N55	China	KJ004621.1
I-A-E-25	Poland	KT922026.1
ARO3	Brazil	KP744123.1
W12	China	KT380558.1
W1	China	KT380544.1

3. Results and Discussion

The nucleotide sequence chromatogram, nucleotide BLAST search results, multiple sequence alignment of samples with strains of higher identity obtained from GenBank database [25] are shown in Figure 1, 2 and 3 respectively.

P. stutzeri strains have almost a universal environmental distribution [5], hence it is not strange to be isolated from currency notes. In the same manner, Kalita M et al. isolated *P. stutzeri* from currency notes [12].

After isolation, identification of bacteria and the phylogenetic analysis, we found that sample 1 clustered with strain SC-04 from Indonesia in the same phylogenetic sub branch (100% sequence identity), sample 2 was closely related to strain N55 from China (100% sequence identity) and Bd8 from Sudan (99% sequence identity). Also, sample 3 clustered with strain OOYW-9 from Italy (95% sequence identity) and sample 4 was closely related to strain SR-23 (100% sequence identity) from Indonesia and Gr45 from Greece (100% sequence identity). In addition, sample 5 was closely related to strain PIGB61 from India (100% sequence identity) and 40/D/Mac2 from Bangladesh (100% sequence identity), sample 6, 7, 11 was closely related to strain APB6 from China (100% sequence identity) and sample 8, 9, 10 was closely related to strain CB44 from China and A10 from Tunisia (99-100% sequence identity) (Figure 5 and 6).

In contrast, we found that other strains from China (B11, W1, W12, and W45) are closely related to other strains from Egypt (MH004 and EGY-SCM1), Iran (K-2-7), Poland (I-A-E-25), Brazil (ARO3), Indonesia (SP-10) and Greece (Gr17). Moreover, other strains from India (NB-03, NA3, KG-2 NRB-DRDO MP and 1005) are closely related to other strains from Pakistan (ME-1 and NB-03), Bangladesh (40/D/Mac2) and Spain (A160/74) (Figure 5). This finding reflects the geographical variation in habitats of *P. stutzeri* strains as the samples were closely related to strains from south-east Asia (Indonesia), east Asia (China), south-central Asia (Bangladesh), south Asia (India), north Africa (Tunisia) and south Europe (Italy and Greece[28]. Unfortunately, currency notes are widely circulating agents making the identification of the exact location from which our samples were isolated is difficult.

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Pseudomonas stutzeri strain SL-04 16S ribosomal RNA gene, partial sequence	2584	2584	98%	0.0	100%	KX082884.1
Pseudomonas stutzeri strain SP-09 16S ribosomal RNA gene, partial sequence	2584	2584	98%	0.0	100%	KX082842.1
Pseudomonas stutzeri strain SC-04 16S ribosomal RNA gene, partial sequence	2584	2584	98%	0.0	100%	KX082841.1
Pseudomonas stutzeri strain 15.1' 16S ribosomal RNA gene, partial sequence	2584	2584	98%	0.0	100%	KU236477.1
Pseudomonas stutzeri gene for 16S ribosomal RNA, partial sequence, strain: BD-2.2.1	2584	2584	98%	0.0	100%	LC125170.1
Pseudomonas stutzeri strain B11 16S ribosomal RNA gene, partial sequence	2584	2584	98%	0.0	100%	KT380516.1
Pseudomonas sp. JS-C54 16S ribosomal RNA gene, partial sequence	2584	2584	98%	0.0	100%	KJ921735.1
Pseudomonas sp. 2(2013) 16S ribosomal RNA gene, partial sequence	2584	2584	98%	0.0	100%	KF171339.1
Pseudomonas stutzeri strain K-2-7 16S ribosomal RNA gene, partial sequence	2584	2584	98%	0.0	100%	JQ963329.1
Pseudomonas stutzeri strain RA10 16S ribosomal RNA gene, partial sequence	2584	2584	98%	0.0	100%	JN585674.1
Pseudomonas sp. AFB-9 16S ribosomal RNA gene, partial sequence	2584	2584	98%	0.0	100%	HQ848269.1
Pseudomonas sp. JS-C55 16S ribosomal RNA gene, partial sequence	2582	2582	98%	0.0	100%	KJ921736.1
Pseudomonas stutzeri strain Bmen1 16S ribosomal RNA gene, partial sequence	2582	2582	98%	0.0	100%	KC702822.1
Pseudomonas stutzeri strain IHBB 9574 16S ribosomal RNA gene, partial sequence	2579	2579	98%	0.0	99%	KU921576.1
Pseudomonas stutzeri strain 1005 16S ribosomal RNA gene, partial sequence	2579	2579	98%	0.0	99%	KU749990.1
Pseudomonas stutzeri strain NA3 16S ribosomal RNA gene, partial sequence	2579	2579	98%	0.0	99%	KU708861.1
Pseudomonas stutzeri strain MN1 16S ribosomal RNA gene, partial sequence	2579	2579	98%	0.0	99%	KU708859.1

Figure 1. *Nucleotide sequence chromatogram.*

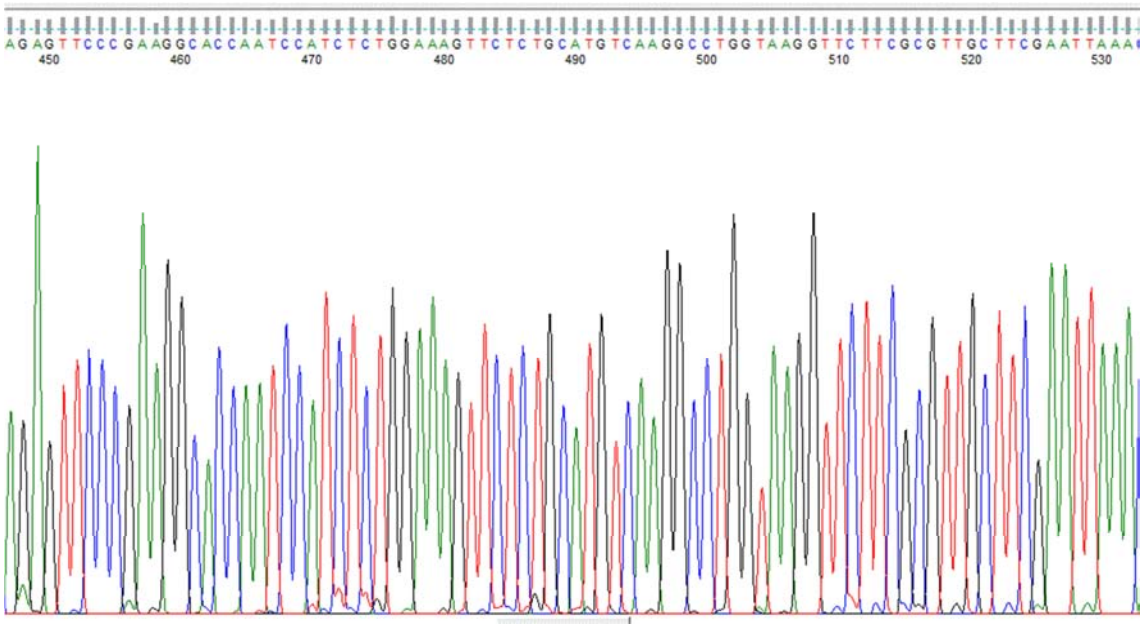

Figure 2. *Nucleotide BLAST [20] search Results.*

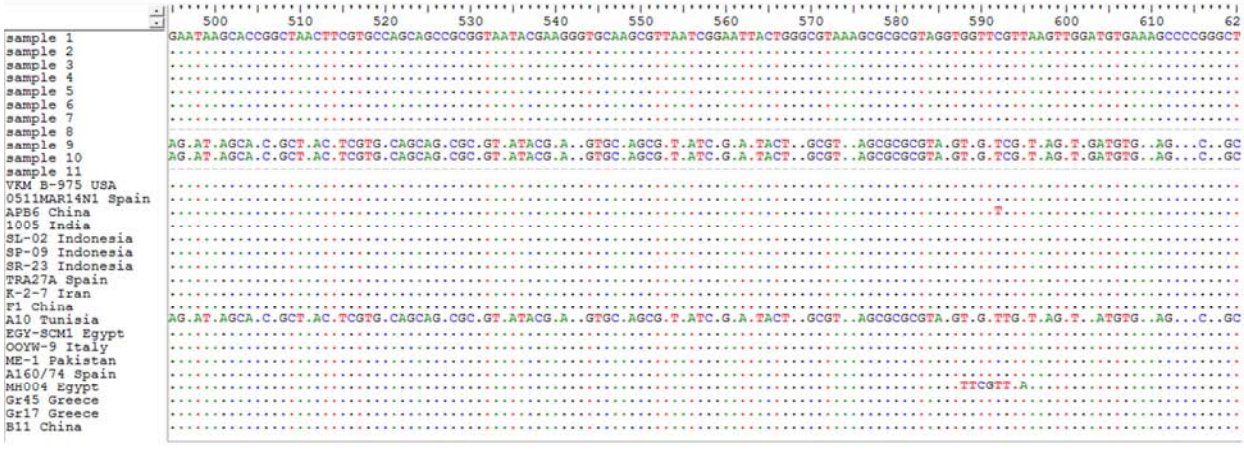

Figure 3. *Multiple Sequence Alignment of samples with strains of higher identity obtained from GenBank database [20] carried out via Clustal W multiple alignment algorithm [21] on MEGA 6.06 [22] and visualized via BioEdit software [1].*

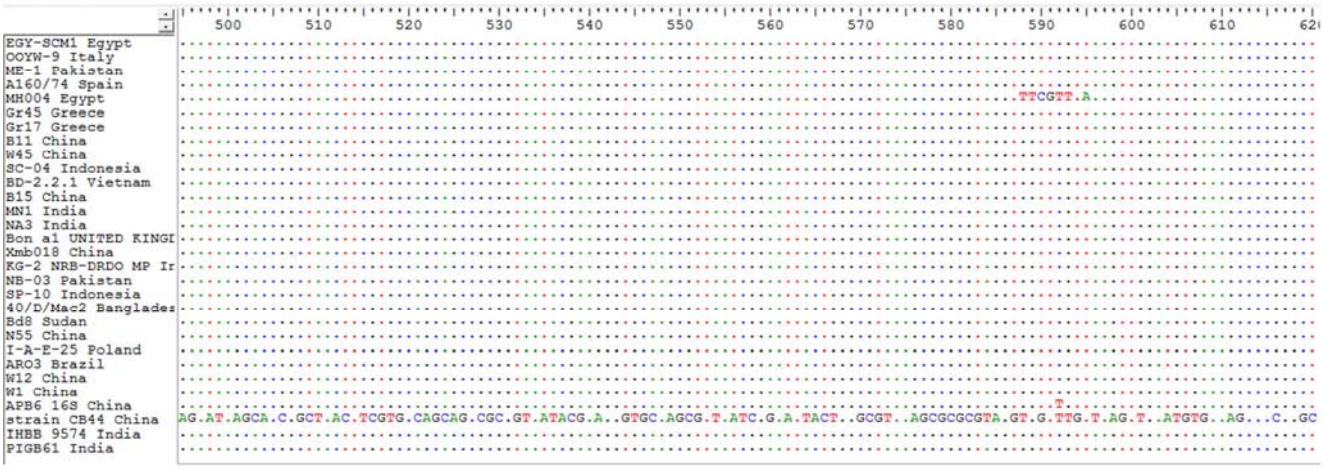

Figure 4. *Multiple Sequence Alignment of samples with strains of higher identity obtained from GenBank database [20] carried out via Clustal W multiple alignment algorithm [21] on MEGA 6.06 [22] and visualized via BioEdit software [1].*

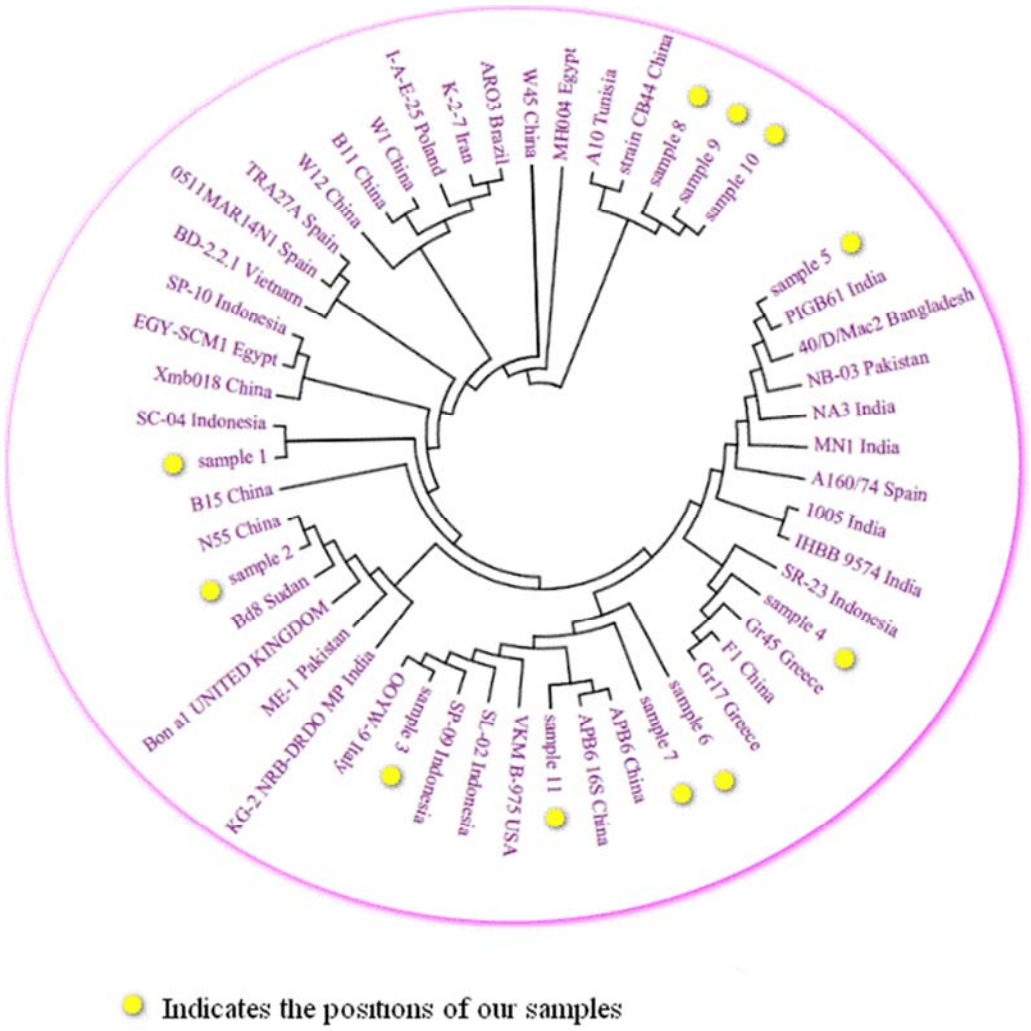

Figure 5. *Molecular Phylogenetic Analysis on samples and strains with higher identity via 16S rRNA gene sequences.*

The evolutionary history was inferred via the Maximum Likelihood method based on the Jukes-Cantor model [2]. The bootstrap consensus tree inferred from 2000 replicates [3] was taken to represent the evolutionary history of the analyzed samples [3]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. Initial tree for the heuristic search was achieved by applying the Neighbor-Joining method to a matrix of pairwise distances estimated via the Maximum Composite Likelihood (MCL) approach. The analysis involved 52 nucleotide sequences. There were a total of

1519 positions in the final dataset. The evolutionary analyses were conducted in MEGA6 [4].

	sample 1	sample 2	sample 3	sample 4	sample 5	sample 6	sample 7	sample 8	sample 9	sample 10	sample 11	VKM B-975 USA	0511MAR14N1 Spain	APB6 China	1005 India	SL-02 Indonesia	SP-09 Indonesia	SR-23 Indonesia	TRA27A Spain	K-2-7 Iran	F1 China	A10 Tunisia	EGY-SCM1 Egypt	OOYW-9 Italy	ME-1 Pakistan
sample 1	100%	96%	99%	100%	100%	100%	100%	100%	88%	89%	100%	99%	98%	98%	99%	100%	100%	100%	99%	99%	99%	69%	99%	100%	100%
sample 2	96%	100%	98%	96%	100%	100%	96%	77%	76%	76%	85%	96%	99%	96%	96%	96%	96%	96%	97%	96%	96%	64%	96%	93%	94%
sample 3	99%	98%	100%	98%	100%	100%	99%	57%	62%	62%	66%	98%	98%	98%	98%	99%	99%	98%	98%	98%	98%	62%	98%	95%	96%
sample 4	100%	96%	98%	100%	100%	100%	100%	98%	88%	89%	100%	100%	98%	100%	100%	100%	100%	100%	100%	100%	100%	70%	100%	100%	100%
sample 5	100%	100%	100%	100%	100%	100%	100%	27%	45%	45%	39%	100%	100%	99%	100%	100%	100%	100%	100%	100%	100%	56%	100%	99%	100%
sample 6	100%	100%	100%	100%	100%	100%	100%	30%	45%	44%	42%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	55%	100%	96%	97%
sample 7	100%	96%	99%	100%	100%	100%	100%	95%	88%	89%	100%	100%	98%	100%	100%	100%	100%	100%	100%	100%	100%	69%	100%	98%	98%
sample 8	100%	77%	57%	98%	27%	30%	95%	100%	95%	95%	93%	100%	43%	100%	100%	100%	100%	100%	100%	100%	99%	100%	100%	98%	98%
sample 9	88%	76%	62%	88%	45%	45%	88%	95%	100%	99%	100%	88%	53%	88%	88%	88%	88%	88%	88%	89%	88%	99%	88%	88%	88%
sample 10	89%	76%	62%	89%	45%	44%	89%	95%	99%	100%	100%	89%	53%	89%	89%	89%	89%	89%	89%	89%	89%	99%	89%	89%	89%
sample 11	100%	85%	66%	100%	39%	42%	100%	93%	100%	100%	100%	100%	53%	100%	100%	100%	100%	100%	100%	100%	99%	100%	100%	100%	100%

	A160/74 Spain	MH004 Egypt	Gr45 Greece	Gr17 Greece	B11 China	W45 China	SC-04 Indonesia	BD-2.2.1 Vietnam	B15 China	MN1 India	NA3 India	Bon a1 UNITED KINGDOM	Xmb018 China	KG-2 NRB-DRDO MP India	NB-03 Pakistan	SP-10 Indonesia	40/D/Mac2 Bangladesh	Bd8 Sudan	N55 China	I-A-E-25 Poland	ARO3 Brazil	W12 China	W1 China	APB6 16S China	strain CB44 China	IHBB 9574 India	PIGB61 India
sample 1	100%	98%	99%	99%	99%	98%	100%	99%	98%	99%	99%	100%	98%	99%	98%	100%	100%	99%	100%	99%	99%	99%	99%	98%	69%	99%	100%
sample 2	96%	95%	96%	96%	96%	96%	96%	97%	97%	97%	97%	96%	96%	97%	96%	95%	96%	99%	100%	96%	96%	96%	96%	96%	64%	96%	96%
sample 3	98%	97%	98%	98%	98%	99%	98%	98%	98%	98%	98%	98%	98%	99%	98%	97%	98%	99%	100%	98%	98%	99%	98%	98%	62%	98%	98%
sample 4	100%	99%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99%	99%	100%	99%	100%	100%	99%	100%	100%	100%	70%	100%	100%
sample 5	100%	98%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99%	100%	100%	99%	100%	100%	100%	100%	100%	99%	56%	100%	100%
sample 6	100%	98%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99%	100%	100%	99%	98%	100%	99%	100%	100%	100%	100%	100%	100%	55%	100%	99%
sample 7	100%	99%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99%	99%	100%	99%	100%	100%	100%	100%	100%	100%	69%	100%	100%
sample 8	99%	100%	99%	99%	100%	100%	100%	100%	100%	100%	100%	98%	100%	100%	100%	100%	99%	0%	37%	100%	98%	100%	100%	100%	100%	100%	99%
sample 9	88%	89%	88%	88%	88%	88%	88%	89%	88%	88%	88%	89%	88%	88%	88%	88%	88%	20%	48%	89%	89%	88%	88%	88%	99%	88%	88%
sample 10	89%	89%	89%	89%	89%	89%	89%	89%	89%	89%	89%	89%	89%	89%	89%	89%	89%	19%	48%	89%	89%	89%	89%	89%	99%	89%	89%
sample 11	100%	100%	99%	99%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	1%	48%	100%	100%	100%	100%	100%	100%	100%	100%

Figure 6. *Simple Identity in 16S rRNA gene between samples and strains with higher identity after they were aligned via Clustal W multiple alignment algorithm [3] on MEGA 6.06 [4].*

Despite the samples from the same source (currency notes), according to the results of multiple sequence alignment results (Figure 3 and 4), we found that there is a broad sequence variation between them in the nucleotide positions 495-620. In these positions, sample 1, 2, 3, 4, 5, 6 and 7 are identical (group A) as well as sample 9 and are identical (group B) with strain A10 from Tunisia and CB44 from China. In contrast, the sequences of sample 8 and 11 were not involving these nucleotide positions because they were partial sequences; hence they were represented by dashes.

The genotypic variation between *P. stutzeri* strains can be interpreted by the natural transformation [29] and rearrangements in their genomic organization (not conserved) [30] that predominantly may be as a result of adaptation to specific environment [31]. In this respect, Sikorski et al. [32] found that the transformability is common among environmental *P. stutzeri* strains.

As the Strains of *P. stutzeri* exhibit broad genotypic diversity, they were sub classified into DNA-DNA similarity groups termed as genomovars [7], [8]. In order to predict the genomovars that the samples belong, we carried out phylogenetic analysis between our samples and other strains from previously identified genomovars. We found that our samples were closely related to strains from genomovar 1 and 5 (Figure 7), but in order to confirm this result, further identification with other housekeeping genes beside 16S rDNA must be carried out.

In fact, currency notes from several countries over the world were found to be contaminated with many resistant microorganisms. The isolation of bacteria from currency notes is alarm that needs attention and health education especially in the development countries in order to prevent the risks and health hazards facing individuals be in contact with contaminated currency notes [33].

16S rDNA is characterized by its slow rate of evolution, consequently it have been used broadly for the phylogenetic reconstruction [34]. We found that 16S rDNA sequence analysis is a valuable method for the study, because it provides considerable results that achieved the objective.

The sample size was small and other genes beside the 16S rRNA gene were not used in the study, consequently the authors recommend the avoiding of these limitations for better identification and phylogenetic analysis.

4. Conclusion

P. stutzeri was identified in currency notes via 16S rDNA sequence analysis. According to the phylogenetic analysis, we found that the samples are closely related to strains from Indonesia (SC-04 and SR-23), China (N55, CB44 and APB6), Bangladesh (PIGB61), India (40/D/Mac2), Tunisia (A10), Greece (Gr45), Italy (OOYW-9) and other strain from Sudan (Bd8).

Conflict of Interest

The Authors declare that they have no conflict of interest.

Appendix

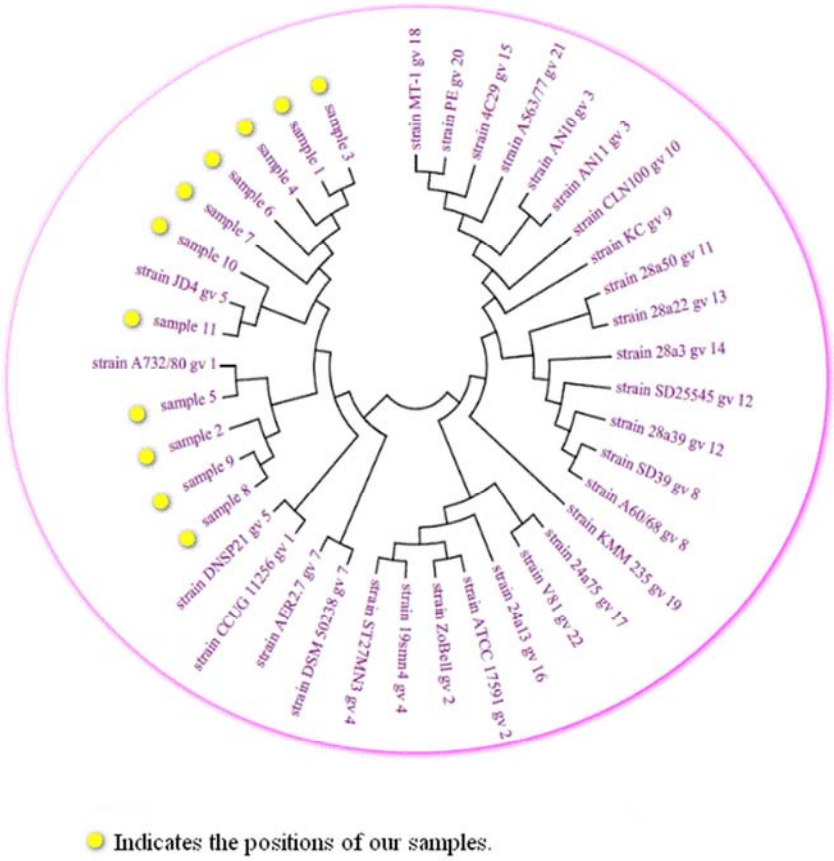

Figure 7. *Molecular Phylogenetic analysis on our samples and strains from various genomovars via 16S rDNA sequences.*

The evolutionary history was inferred via the Maximum Likelihood method based on the Jukes-Cantor model [2]. The bootstrap consensus tree inferred from 2000 replicates [3] was taken to represent the evolutionary history of the analyzed samples [3]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. Initial tree for the heuristic search was achieved by applying the Neighbor-Joining method to a matrix of pairwise distances estimated via the Maximum Composite Likelihood (MCL) approach. The analysis involved 40 nucleotide sequences. There were a total of 1516 positions in the final dataset. The evolutionary analysis was conducted in MEGA6 [4].

gv. is an abbreviation for genomovar.

Table 2. *Results of the Biochemical Tests.*

Biochemical test	result
Oxidase	positive
Catalase	positive
Indole	negative
Glucose fermentation	positive
Lactose fermentation	positive
Citrate	positive
Urease	negative
Motility	positive

Table 3. *PCR reaction volumes according to INtRON Biotechnology company instructions.*

PCR reaction mixture	Volume (µl)
DNA extract	5
Forward Primer (10pmol/µl)	1
Reverse Primer (10pmol/µl)	1
Distilled water	13
Maxime PCR preMix (20µl reaction)	5
Total volume	25

Table 4. *Accession numbers of our samples.*

Sample number	location	Accession number
1	transporters	KY039354
2	Khartoum Teaching Hospital	KY039355
3	Khartoum Teaching Hospital	KY039356
4	food sellers	KY039357
5	food sellers	KY039358
6	food sellers	KY039359
7	transporters	KY039360
8	food sellers	KY039361
9	transporters	KY039362
10	Omdurman Teaching Hospital	KY039363
11	Omdurman Teaching Hospital	KY039364

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