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GENOTYPING OF THE GENUS *PROTEUS* BY 16SRRNA AND *RPOB* SEQUENCE ANALYSIS

MOHAMMED GJ[1*], HINDI AK[1], WANG Y[2], AND AL-TAEI IH[1]

1: Biology Department of Science College, Babylon University, Babil, Iraq

2: Department of Biomedical Sciences, College of Medicine, Florida State University, Tallahassee, Florida

***Corresponding Author: E Mail:ghaidaam@ymail.com**

ABSTRACT

Comparison of the sequences of conserved genes, most commonly those encoding 16SrRNA is used for bacterial genotypic identification, but it has the disadvantages that it is often multiple-copy, has little resolution below the species level. So, in this study we also investigated the usefulness of the single-copy, protein-coding *rpoB* (RNA polymerase beta-subunit) marker as an alternative tool for bacterial genotypic identification at the species level by amplifying and sequencing. We generated a database of partial 16SrRNA gene for three *Proteus* species and partial *rpoB gene* for five *Proteus* strains and then assessed the inter and intra species divergence between the 16SrRNA and *rpoB* genes by pairwise comparison. The 16SrRNA gene sequence similarity observed among three *Proteus* species ranged from 92 to 97 %.Whereas, the *rpoB* gene sequence similarity among five *Proteus* strains was ranging from 87-98%. We found that levels of divergence between the *rpoB* sequences between different strains were markedly higher than those between their 16SrRNA genes. The results obtained in our phylogenetic analysis suggested that the *rpoB* gene can be used as an alternative molecular marker for genetic relatedness in the genus *Prote*us and a powerful tool identification, which may be useful for universal bacterial identification.

Keywords: *Proteus*, Genetic Analysis, 16SrRNA Sequencing, *rpoB* Sequencing

INTRODUCTION

The small subunit ribosomal RNA gene is the recognized gold standard for estimating the phylogenetic diversity in microbial communities **[1, 2]**. This marker gene is universally present and has the advantage of containing both highly conserved fragments, facilitating the design of PCR primers targeting all members of a community, and more variable regions that allow for the discrimination of different microbial taxa. However, the 16SrRNA gene is not without potential drawbacks, where members of the family *Enterobacteriaceae* have not been subjected to extensive phylogenetic 16SrRNA gene sequence analysis because the high degree of conservation in closely related species leaves many taxonomic problems unresolved **[3,4]**. In particular, the genetic relationships between closely related species *Proteus vulgaris and Proteus penneri* could not be clearly resolved by this method **[5]**. So, the use of alternative markers has been proposed, including the beta subunit of RNA polymerase, *rpoB* **[6-8]**. Among the core bacterial genes, the *rpoB* gene has been identified as one of the few potential candidates suitable for bacterial phylogenetic analyses and has been proposed as a powerful tool for universal bacterial genetic identification **[8, 9]**.

The use of the *rpoB* gene offers various potential advantages over standard 16SrRNA gene-based approaches. First, since most bacterial genomes contain multiple copies of the 16SrRNA gene, and copy number varies per species, extrapolation of relative abundances from gene recovery frequencies is seriously impaired. This is further complicated by the fact that sequence variation between the different 16SrRNA gene copies present exists in some genomes **[10, 11]**. *rpoB* typically occurs in a single copy **[12]**. Second, the *rpoB* gene is a highly conserved housekeeping gene and one copy is present in all bacteria because of its essential role in cellular metabolism. Primers and probes targeting the *rpoB* gene have been used for the specific detection and phylogenetic analysis of several bacterial groups belonging to the family *Enterobacteriaceae,*the high level of conservation across 16SrRNA genes can obscure most intraspecific, and sometimes interspecificvariation **[13]**. In contrast, the higher resolution *rpoB* marker is capable of revealing molecular variation down to the population level **[8, 14-17]**. Third, genetic divergence of *rpoB* correlates better with overall genomic divergence and provides better bootstrap support for phylogenetic

reconstruction **[8]**. Fourth, given the fact that *rpoB* is a protein-encoding gene, the data generated from this marker is more readily interpreted in an evolutionary framework. So, this study aimed to test the performance of the16SrRNA gene as important phylogenetic tool for bacterial identification between *Proteus* species and the use of *rpoB* gene as a marker for genetic analysis of *proteus strains.*

MATERIAL S AND METHODS

Bacterial Strains

A total of 5 *Proteus* strains belonging to the species *P. penneri* (1 strain), *P. vulgaris* (3 strains), and *P.mirabilis* (1 strain) were studied. Reference strains were obtained from the American Type Culture Collection (ATCC), Florida, Unitied States. Clinical isolates were identified by standard biochemical methods at the Department of Biomedical Science, University of Florida State.

DNA Extraction and Genes Amplification

Genomic DNA was extracted by a commercial nucleic acid extraction kit (Illustra bacteria genomic Prep. Mini Spin Kit, VWR-USA) according to the manufacturer's instructions. PCR amplification was performed in a 25µl reaction volume as shown in **Table 1**.

Table 2 clarifys the primers sequences and PCR conditions which used to amplify a portion of the coding region of 16SrRNA and the *rpoB* genes.

New primers have been designed for *P. vulgaris* 16SrRNA on the basis of *P.mirabilis* and *P. penneri* 16SrRNA gene alignment by manual searching for areas of sequence conservation across two species **(Table 3)**. Given that no regions of sufficient length with 100% complementarity exist in the alignment, primers were designed to maximize complementarily at the 3' end. Primers were designed with annealing temperatures of between 50 and 60°C **[21]**.

Detection of Amplified Products by Agarose Gel Electrophoresis

Successful PCR amplification was confirmed by agarose gel electrophoresis, The PCR products were assessed by electrophoresis (Amercham Biosciences, USA) in 1% agarose gel with 0.5% ethidium bromide (Alfa Aesar, USA). Agarose gel was prepared by dissolving 0.45gm of agarose powder in 40ml of TBE buffer (pH:8) in Microwave(Kenmore, USA), allowed to cool to 50^{o}C and then ethidium bromide at the concentration of 0.5mg/ml was added **[22]**.

The comb was fixed at one end of the tray for making wells used for loading DNA sample.

The agarose was poured gently into the tray, and allowed to solidify at room temperature for 30 min. The comb was then removed

gently from the tray. The tray was fixed in an electrophoresis chamber filled with TBE buffer that covered the surface of the gel, 5µl of DNA sample mixed with Gel Loading Dye Blue (Biolabs, USA)was transferred into the wells in agarose gel, and in one well we put 1µl of DNA ladder (Biolabs, USA).

The electric current was allowed at 110 volt for 60min. UV Trans-illuminater (San. Gabriel, USA) was used for the observation of DNA bands, and then gel was photographed using aGel Documentation System with a digital camera (Bio Rad, USA).

Sequencing And Phylogenetic Analysis

Partial 16SrRNA(740-1295bp) and *rpoB* (400-641bp) gene nucleotide sequences were obtained from the PCR amplicons generated with the same primers mentioned above in **Table 2** for *P.mirabilis and P.penneri*and primers in **Table 3** for *P.vulgaris* after diluted them to make a 3.2pmol/µl working solution .The PCR products of 16SrRNA and *rpoB* were sequencedin duplicates by Genome Express using the standard Big Dye Terminator v3.1 protocol. We used Big Dye Xterminator (Applied Biosystems, USA) for clean-up and the reactions were run on the ABI 3730 Genetic Analyzer (Applied Biosystems, USA). DNA sequencing chemistries are performed using Big-Dye terminator chemistry on double or single stranded DNA and PCR products [9]. Percentage nucleotide identity and pair wise uncorrected (p-distances were calculated for 16SrRNA and *rpoB* gene sequences of clinical strains of the *Proteus* examined by using Sequence alignments Explorer CLUSTAL **[23]**. Phylogenetic analysis was carried out using the software MEGA version 4.1[24] using the neighbour-joining and maximum-parsimony methods to reconstruct the phylogenetic tree in MEGA 4.1 package, with the option of complete deletion of gaps **[23, 24]**. The statistical significance of the *rpoB*phylogenies inferred was estimated by bootstrap analysis with Bootstrap (500 replicates; seed=31332).

RESULTS

Sequence homology of 16SrRNA gene between *Proteus species*

To obtain the taxology group, the PCR product of 16SrRNA gene was sequenced. The band size of 16SrRNA genesequence was 1295bp and 887bp,1141bp and 740bp,and 1059bp for *P.penneri* ATCC 33519, *P.vulgaris* ATCC 6380 and *P.mirabilis* ATCC56283712 respectively as shown in **Figure (1a)**.

Percentage nucleotide identity and pairwise uncorrected (p-) distances were calculated for 16SrRNA gene sequences of clinical strains of the 3 species examined by using

alignments Explorer CLUSTAL **[23]** in Microsoft Mega 4.1 package. The sequence of *P.vulgaris* 16SrRNA geneshowed 644/702(92%) homology with the sequence of *P.mirabilis* 16SrRNA gene and 1065/1096(97%) with 16SrRNA gene of *P.penneri*. Whereas, the identity between *P.mirabilis* and *P.penneri* 16SrRNA genes was 846/914 (93%).

Evolutionary tree, based on 16SrRNA gene sequences, were constructed by the neighbour-joining, parsimony and maximum-likelihood method in MEGA 4.1 package as shown in **Figure (1b)**. Also we used the relative rate test (Tajima's test) in MEGA4 to detect the evolutionary rate between *Proteus* species 16SrRNA gene sequences showed in **(Table 4)**.

Constitution of *rpoB* Database in *Proteus species*

The presence of alternating conserved and variable regions within bacterial RNA polymerase β subunit **[26, 27]** allowed to Christophe *et al*., **[28]** to design primers within conserved flanking the sequence encoding the variable poly peptide region 4 **[29]**. The *rpoB* region chosen For comparison corresponded to codon 500-670 of the 1342 amino acid coding region in *E.coli* **[27, 28]**.

A portion of the coding region of the *rpoB* gene was amplified with forward primer CM7 (5'-AACCAGTTCCGCGTTGGCCTGG-3') and reverse primers CM31b (5'-CCTGAACAACACGCTCGGA-3') and CM32b (5'-CGGAACGGCCTGACGTTGCAT-3') accordingto the technique described by Mollet *et al*., **[9]**. The PCR amplicons generated were sequenced with the same primers to obtain partial *rpoB* gene sequences. The band size for the *rpoB* gene sequences of the strains used in this study are shown in **Figure 2a**. The bands size of *rpoB* gene sequence purified from DNA product of *P. vulgaris* ATCC 6898 was 555bp, 487bp and 400bp, 510bp for *P. penneri* ATCC 33519, *P. mirabilis* ATCC 56283712 band size was 518bp, 641bp and 590bp for *P .vulgaris* ATCC 29905 and it was 523bp for *P. vulgaris* ATCC 6380.

Sequence alignment and phylogenetic analysis was carried outon 500nt fragments using the Alignment Explorer/ CLUSTAL and MEGA software version 4.1 **[24]**. To ensure the stability and reliability of phylogenetic relationships among strains used in this study, phylogenetic tree was reconstructed by using the neighbour-joining and maximum-parsimony methods in MEGA 4.1 package, with the option of complete deletion of gaps **[23]**. The statistical significance of the phylogenies inferred was

estimated by bootstrap analysis with Bootstrap (500 replicates; seed=31332).

The examination of *Proteus* strains by *rpoB* sequencing showed between 5 and 13% nt differences in the *rpoB* 500 bp region analysed. On the basis of *rpoB* sequence divergence, thetype strain of *P. mirabilis* ATCC 56283712was diverged by 7%,6%,5%,and 8% from *P. vulgaris*ATCC 6380,*P. vulgaris* ATCC 29905, *P. vulgaris* ATCC 6898 and *P.penneri* ATCC 33915 respectively.*P. penneri* differed by 6-13% from all *P.vulgaris* strains. Whereas,*P. vulgaris*strains were differing from each other by 2-8% of their nucleotides.

The phylogenetic tree derived from partial *rpoB* gene sequences of clinical strains of 3 species of the genus *Proteus* showed in **(Figure 2b)**.

Table 1: Contents of the Reaction Mixture

No.	Contents of reaction	Volume
1.	Accustart ™ Taq PCR SuperMix(VWR-USA)	12.5 µl
2.	Upstream primer(IDT-USA)	1µl
3.	Downstream primer(IDT-USA)	1 µl
4.	DNA template	2 µl
5.	Nuclease free water(ddH2o)	8.5 µl
Total volume		25 µl

Table 2: Primers Sequences and PCR Conditions for 16SrRNA and *rpoB genes*

Genes	Primer sequence (5′-3′)	Size of product bp	PCR condition	Reference
*27*F* 1492R*	AGAGTTTGATCMTGGCTCAG ACG GCT ACC TTG TTA CGA CTT	1400 bp	94°C 5min 1x 94°C 1min 53°C 1min 35x 72°C 1:30min 72°C 10min 1x	[18]
27 F1522R	AGAGTTTGATCMTGGCTCAG AAGGAGGTTATCCANCCRCA	1382 bp	94°C 5min 1x 94°C 1min 57°C 1min 35x 72°C 1:30min 72°C 10min 1x	[19,20]
rpoBF CM7 R(CM31b)	AACCAGTTCCGCGTTGGCCTGG CCTGAACAACACGCTCGGA	1088bp	94°C 5min 1x 94°C 1min 46°C 1min 40x 72°C 1:30min 72°C 10min 1x	[9]
rpoB CM7 R(CM32b)	AACCAGTTCCGCGTTGGCCTGG CGGAACGGCCTGACGTTGCAT	930bp	94°C 5min 1x 94°C 1min 53°C 1min 40x 72°C 1:30min 72°C 10min 1x	[9]

NOTE: *F: Forward Primer, R: Reverse Primer

Table 3: Designed Primers Sequences and PCR Conditions for 16SrRNA and *rpoB genes* [21]

Genes	Primer sequence (5′-3′)	Size of product	PCR condition
F1 R1	CCG AAG GTT AAG CTA CCT AC CCA TGT GTA GCG GTG AAA TG	1400bp	94°C 5min 1x 94°C 1min 47°C 1min 35x 72°C 1:30min 72°C 10min 1x
F2 R2	CTA GCG ATT CCG ACT TCA TG CGG TCG ATT TAA CGC GTT AG	800bp	94°C 5min 1x 94°C 1min 48°C 1min 35x 72°C 1:30min 72°C 10min 1x

Table 4: Results from the Tajima test for 3 Sequences [25]	
Configuration	**Count**
Identical sites in all three sequences (m_{iii})	481
Divergent sites in all three sequences (m_{ijk})	13
Unique differences in Sequence A (m_{ijj})	8
Unique differences in Sequence B (m_{iji})	15
Unique differences in Sequence C (m_{iij})	507
Note: The equality of evolutionary rate between sequence (A);*Proteus penneri*16SrRNAgene and sequence (B);*Proteus vulgaris*16SrRNA gene is tested using sequence (C);*Proteus mirabilis* 16SrRNA gene as an outgroup in Tajima's relative rate test in MEGA4[24]. The χ^2 test statistic was 2.13 (P = 0.14440 with 1 degree[s] of freedom). P-value less than 0.05 is often used to reject the null hypothesis of equal rates between lineages	

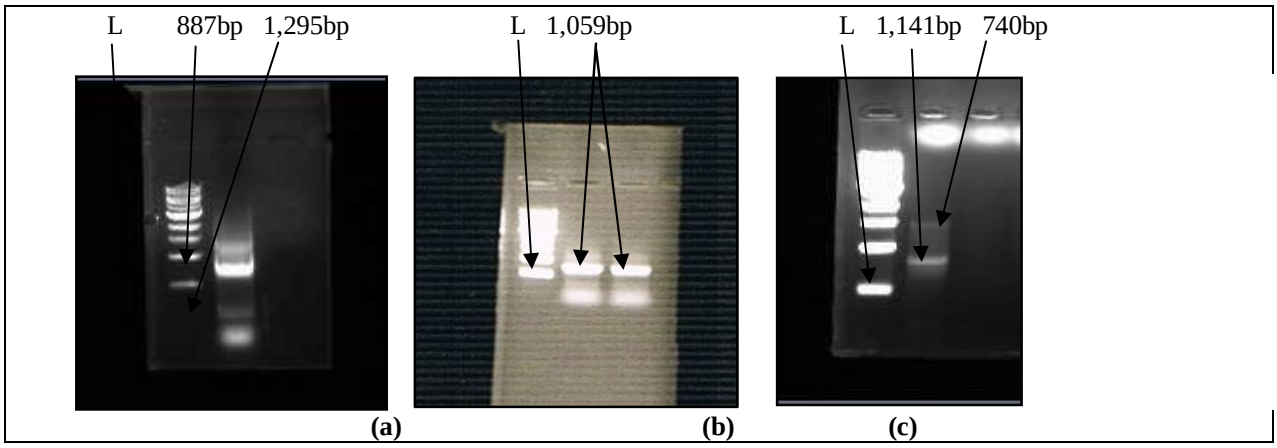

Figure (1a): Results of 16SrRNA gene and PCR product of *Proteus* species on 1% agarose gel electrophoresis. L: DNA Ladder, 1000bp (a); *Proteus penneri*ATCC 33519(b); *Proteus mirabilis*ATCC 56283712 and (c); *Proteus vulgaris* ATCC 6380

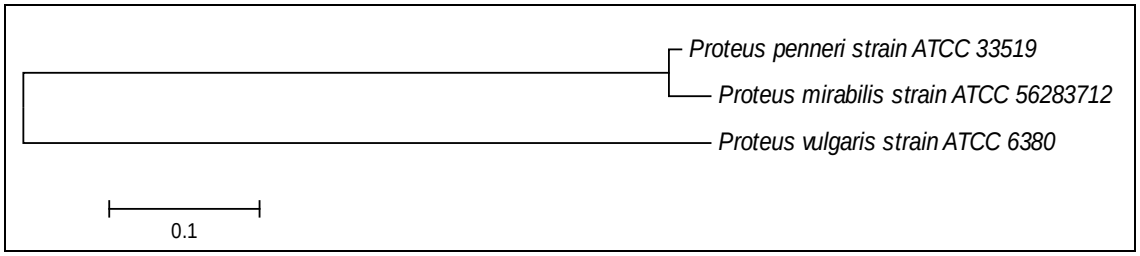

Figure (1b): Phylogenetic tree of *Proteus species* based on 16SrRNAgene sequence analysis. The tree was constructed with the similarity and neighbor joining by MEGA 4.0.Bar (0.1)represents sequence divergence.

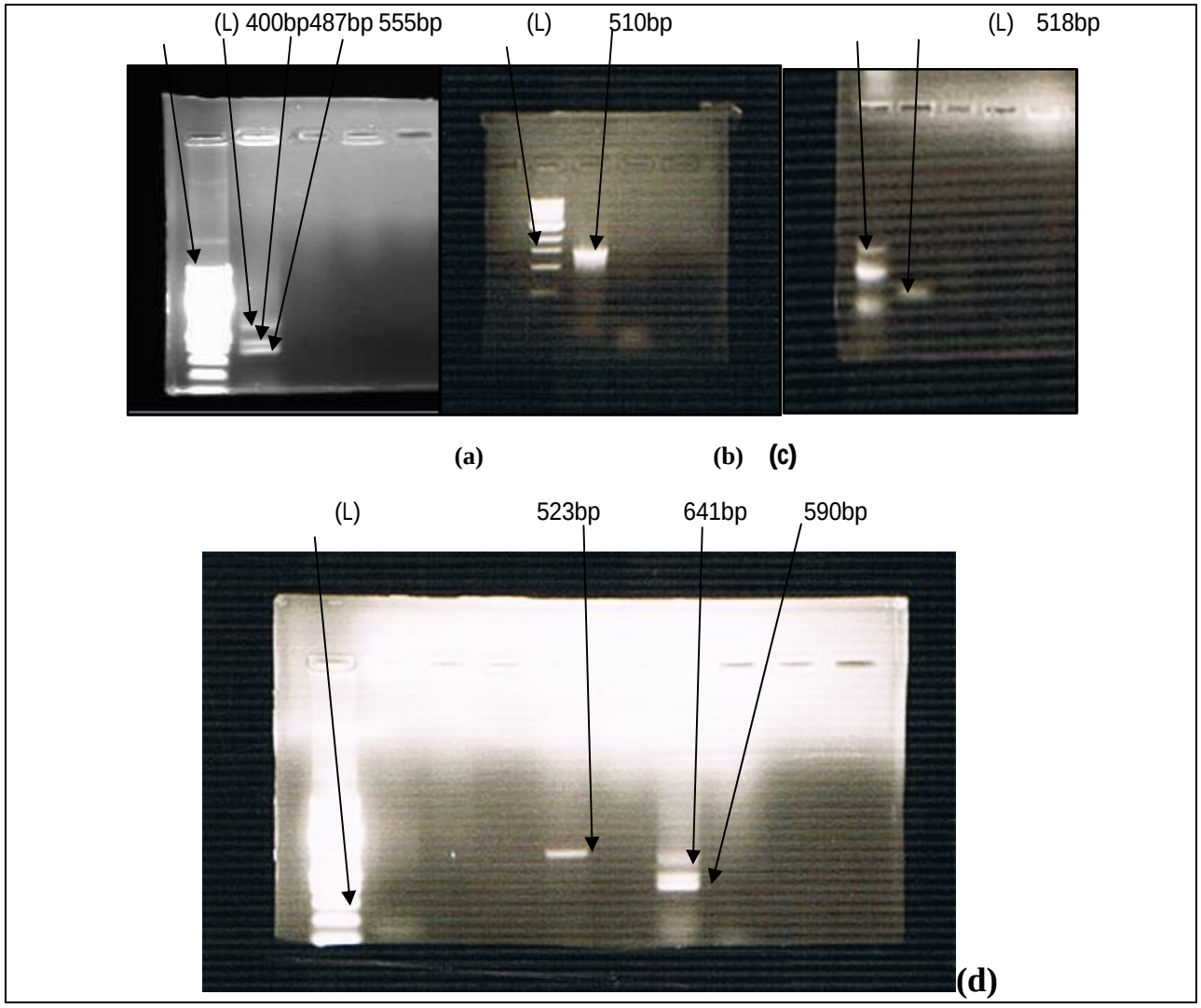

Figure (2a): Results of *rpoB*gene and PCR product of *Proteus* strains on 1% agarose gel electrophoresis. L:DNA Ladder,100bp (a); *P.vulgaris* ATCC 6898(b); *P.penneri* ATCC 33519, (c); *P.mirabilis* ATCC 56283712,(d); *P.vulgaris* ATCC 6380 and *P.vulgaris* ATCC 29905

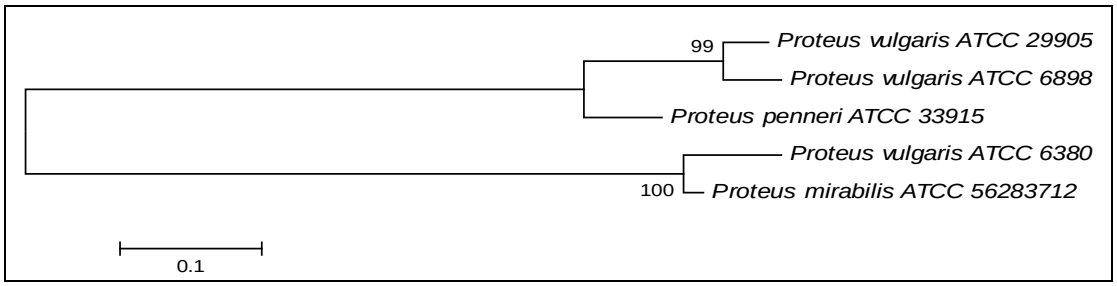

Figure 2b: Phylogenetic analysis of partial nucleotide sequences of the *rpoB* gene of type and clinical strains of *Proteus* genus. The tree was generated using the neighbour-joining method. Bootstrap values >50% (based on 500 replicate datasets). Bar, 0.1 substitutions per nucleotide position

DISCUSSION

It has been confirmed that sequencing only a part of the 16SrRNA gene can be sufficient to establish phylogenetic relationships **[12, 30]**. In this study, the phylogenetic tree of 16SrRNA gene sequences for three strains of the genus *Proteus*was constructed as shown in **(Figure1b)**. We have found that type strain of *P. penneri* ATCC 33519 clustered with *P.mirabilis* strain ATCC 56283712 within the same branch of the tree, while *P. vulgaris* type strain ATCC 6380 stood apart in the other branch although there was low degree of divergence in the 16SrRNA gene sequences between clinical strains of *P.vulgaris and P.penneri*. So, phylogeny estimated from a single gene should be treated with caution **[31, 32]**. The low degree of divergence in the 16SrRNA gene sequence which obtained in this study was similar to that reported between closely related members of other genera within the family *Enterobacteriaceae*, such as sequence divergences of 2.4–1.9% were observed when a 5' 978nt 16SrRNA gene fragment from the type strain of *Proteus myxofaciens* was compared to that of other type strains belonging to the same genus and 16SrRNA phylogenetic analysis could not clearly separate it from the other species of the genus *Proteus* **[27]** and other study between *Escherichia coli* and members of the genera *Shigella* and *Salmonella* (95.3–99.6% similarity) and between members of the genera *Enterobacter* and *Klebsiella*(97.2–98.3% similarity) **[33]**. This fact has already suggested the existence of phylogenetically conserved regions among bacteria.

Recently the 16SrRNA gene sequence with the size of 1.5 Kb was considered and widely used in bacterial taxology because it contains high conservation region which have variable nucleic acid region in different species **[34]**. Furthermore, the most importance is that 16SrRNA gene can be sequenced easily. By combining the molecular phylogeny with traditional approaches, such as

morphological, physiological and biochemical characteristics, bacteria identification can be carried out more accurately **[18, 35 and 36]**.

The comparison of *rpoB* gene sequence divergences among clinical strains revealed that they were generally closely related to their respective type strain.So,to study the phylogenetic relationships among the three species of the genus *Proteus*, a phylogenetic tree was derived from *rpoB* sequencing results. As shown above in **Figure 2b**, five *rpoB* groups supported by >50% bootstrap values could be described. *P. penneri* strain, and two type strains of *P. vulgaris* clustered in two separate groups within the same branch of the tree, while *P. vulgaris* type strain ATCC 6898 stood apart along with the *P. mirabilis* strain.

As already shown by the phylogenetic analysis, distinct *rpoB* gene sequence types could be recognized in *Proteus vulgaris* ATCC 6898. In particular, two clades, corresponding to the *Proteus vulgarisrpoB* clades observed in the phylogenetic tree, *P.vulgaris* (type strain ATCC 29905 and clinical strain 6380) and *P.vulgaris* (Type strain 6898) showed sequence divergences of 2–8% from each other but both were equally distant from the other species of the *Proteus* genus. Attempts have been made to define a cut-off for *rpoB* gene sequence-based identification of bacteria. Mollet *et al.*, **[9]** found an intraspecies similarity range of 98–100%when they analysed a 512bp fragment of the *rpoB* gene inclinical isolates of enteric strains. For 600–825 bp gene fragments,a *rpoB* sequence similarity of at least 96–97%seems to be the threshold for correct species identification **[37, 38 and 39]**. According to these limits, the 8% sequence divergence detected between strains of *Proteus vulgaris* could be sufficient to suggest possible further taxonomic adjustments within this species.

Our results indicate that 16SrRNA and *rpoB* gene sequence comparison seems to be an appropriate method for inferring genetic relationships within the *Proteus* species and species strains and suggest the need for taxonomic adjustments.

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