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Original Article

Molecular detection of some Gram-negative bacterial species using *folp* gene sequences

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ABSTRACT

In this study, the *folp* gene encoding DHPS was tested as a possible alternative phylogenetic marker for more closely related Gram-negative bacterial species. In this new method, 854 bp were implemented for classification instead of 1435 mostly used in 16S gene detection. Phylogenetic analysis was performed based on DNA sequences obtained from the GenBank including the most important Gram-negative bacterial species. The 16S rRNA-based tree openly portrayed three distinct clusters where cluster 1 is a mixed species cluster including *E.coli*, *Shigella* sp., *Proteus* sp., *Enterobacter* sp. *Citrobacter* sp., *Salmonella* sp., *Serratia* sp., and *Klebsiella* sp., while clusters 2 and 3 contained *P. aeruginosa* and *Haemophilus* sp. respectively. Comparatively, the *folp* gene-based tree yielded 9 clusters in which, *E.coli* and *Shigella* sp. were identified in one mixed cluster. However, other Gram-negative species such as *Klebsiella* sp., *Enterobacter* sp., *Salmonella* sp., *Citrobacter* sp., *Serratia* sp., *Proteus* sp., *Haemophilus* sp., *and P. aeruginosa* were found each in a separate cluster. In addition, DNA-DNA relatedness studies indicated high sequence divergence of *folp* gene exhibiting 47.54-98.37% interspecies homology compared to 16S rRNA with sequence similarities of 79.58-98.11%. In addition, 71.9-100 % intraspecific similarities were obtained for the *folp* gene which indicates the possibility for use of the *folp* gene as a possible efficient target for Gram-negative bacterial group taxonomic analysis. Moreover, in blind tests, this method was able for the correct identification of 10 Gram-negative bacteria isolates. In conclusion, *folp* gene sequences provide better analysis of Gram-negative bacteria.

Keywords: Gram-negative, *folp* gene, Microbial classification, DNA-DNA relatedness, 16S rRNA gene

Introduction

For studies of the bacterial genus in both the epidemiological and taxonomic fields, different old methods for bacterial identification and classification are mainly based on their metabolic activities, in addition to the morphologic appearance of the cells [1]. Activities of enzymes, carbohydrate fermentation, and reduction of chemical compounds are some examples used for phenotypic profiling of different bacterial species. In this respect, for rapid bacterial identification, commercial biochemical test kits including chemicals to test a complete set of enzyme activities can also be used [2]. Phage typing and serology are other traditional methods that have exhibited their success in bacterial classification even to their serotypes. However, these techniques can only be applied to detect cultivable bacterial pathogens. In recent years, molecular techniques have been developed to identify and classify microbes based on their genetic relatedness [3]. Sequence-based techniques could be implemented for bacterial classification even to the species level. The most used approach is based on PCR amplification fragments of different housekeeping genes, followed by sequencing. The term housekeeping genes is usually used to describe genes that code for proteins that carry out different functions for essential cellular processes.16S rRNA gene, with its species-specific variable regions, usually represents the ideal gene for designing species-specific primer [4]. In addition, a useful tool for identifying bacteria that can be suggested is the RNA polymerase β subunit gene (*rpoB*) [5].

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Moreover, the gyrase B subunit (*gyrB*) has been succeeded in the identification of bacteria to the species level. Such importance has emerged for selecting appropriate treatment of pathogenic microbes in Hospitals and clinical facilities [6]. Methodologies for practical use implements universal primers for most bacteria that can be designed to amplify the same gene by PCR across diverse genera and species. The next steps include sequencing and sequence alignment. The degree of similarity and species detection is usually calculated when DNA sequences were contrasted with a reference databank sequence and an identity score is usually given after each query process. The investigatigation of the possibility to use *folp* gene for both interspecies and interspecies DNA analysis of Gram-negative bacteria is the purpose of the study [7].

Materials and Methods

Processing of specimens

10 different bacterial isolates were obtained from different clinical sources were separated on growth media, aerobic incubation at 37°C follows with daily observation for 24 hr. Isolates were obtained from our culture collection and were identified by standard phenotypic microbiological techniques.

Processing and genomic DNA extraction

As per instructions of the manufacturer, the Genomic DNAs were extracted from different samples isolated from different sources using the QIAmp DNA Mini Kit (QIAGEN, Hilden, Germany). Nanodrop (OPTIZEN NanoQ, Mecasys) was used to determine the gDNA intensity. Purified DNAs were frozen at −80◦C.

Design and synthesis of oligonucleotide probes

A region of 854 bp targeted in this study was implemented for the identification of bacteria based on their *folp* gene sequence. Specified targets with particular primer sequences and lengths were selected, GC content, and thawing temperatures in order to be used in a single reaction at a specific temperature. The final probe sequences for species-specific oligos were compared Contrasting of all available sequences in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/) with the final probe sequences for the species-specific oligos was done to exclude any theoretical false-positive reactions caused by sequence variations and to indicate the probes that have a higher threshold sequence similarity than the required one.

PCR amplification of strain-specific genes

Table 1 lists the performed priming done for the amplification of genomic DNA. The reaction mixture was prepared to start from gDNA as a template, in a reaction mixture containing 0.5 µM of each primer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 U Taq polymerase (Thermo scientific Dream Taq Green DNA polymerase), 2 µl of template DNA and nuclease-free water was added for a total volume of 25 µl per reaction. The PCR was performed using Cycler 003 PCR Machine (A & E Lab (UK)). PCR reactions began with 5 minutes of initial denaturation at 94°C followed by 35 cycles of 94°C for 30 s, 52 for 30 s and 72°C for 30 s and a final one at 72°C for 10 min.

Table 1. Oligonucleotides used in this study

Primer name	Organism targeted	Primer Sequence	Citation
fol- F1	*Salmonella* sp.	ATGAAACTCTTCGCTCAGGG	This study
fol- F2	*E.coli*, *Shigella* sp.	ATGAAACTCTTTGCCCAGGG	This study
fol- F3	*Klebsiella* sp.	ATGAAACTTGTAGCCCAGGG	This study
fol- F4	*Enterobacter* sp.	ATGAAACTATTCGCCCAGGA	This study
fol- R1	*E.coli*, *Shigella* sp., *Enterobacter* sp.	TTACTCATAGCGTTTGTTTTCC	This study
fol- R2	*Salmonella* sp.	TTACTCATAGCGTTTGTTTCCC	This study
fol- R3	*Klebsiella* sp.	TTACTCATAACGTTTTTTT	This study

DNA sequencing

Purification of the Amplified *folp* gene fragments was done using the Gene JET PCR Purification Kit (K0691, Thermo Scientific, Waltham, MA USA). ABI PRISM® BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Bio-systems, Foster City, USA) was used for sequencing reactions. Sequencing was carried out on both strands using an ABI 3730 DNA analyzer (Applied Bio-systems, Foster City, USA). Sequences analysis was performed online in BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/).

GenBank was used to store the sequences for the study in its depository under the following accession numbers: MT281366-MT281374

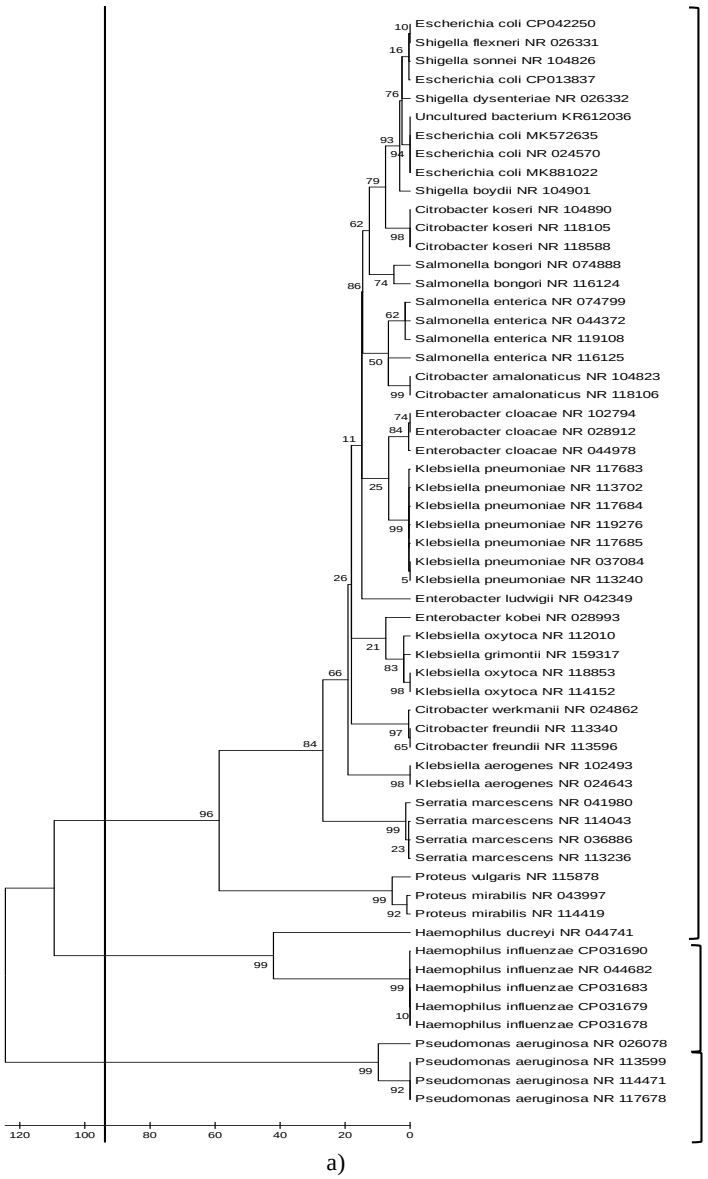
Comparative DNA sequence data

The nucleotide sequences of 16S rRNA and *folp* genes were aligned by the CLUSTAL W computer program [8]. Maximum Parsimony analysis [9] was used to reconstruct phylogenetic trees. Molecular Evolutionary Genetics Analysis package version 4.1 MEGA 4.1 software (http://www.megasoftware.net) was used to analyze the aligned sequences. The Maximum Parsimony method was employed as an evolutionary history inference [10]. The replicate trees percentage in which the associated taxa clustered together in the bootstrap test (1000 replicates) [11].

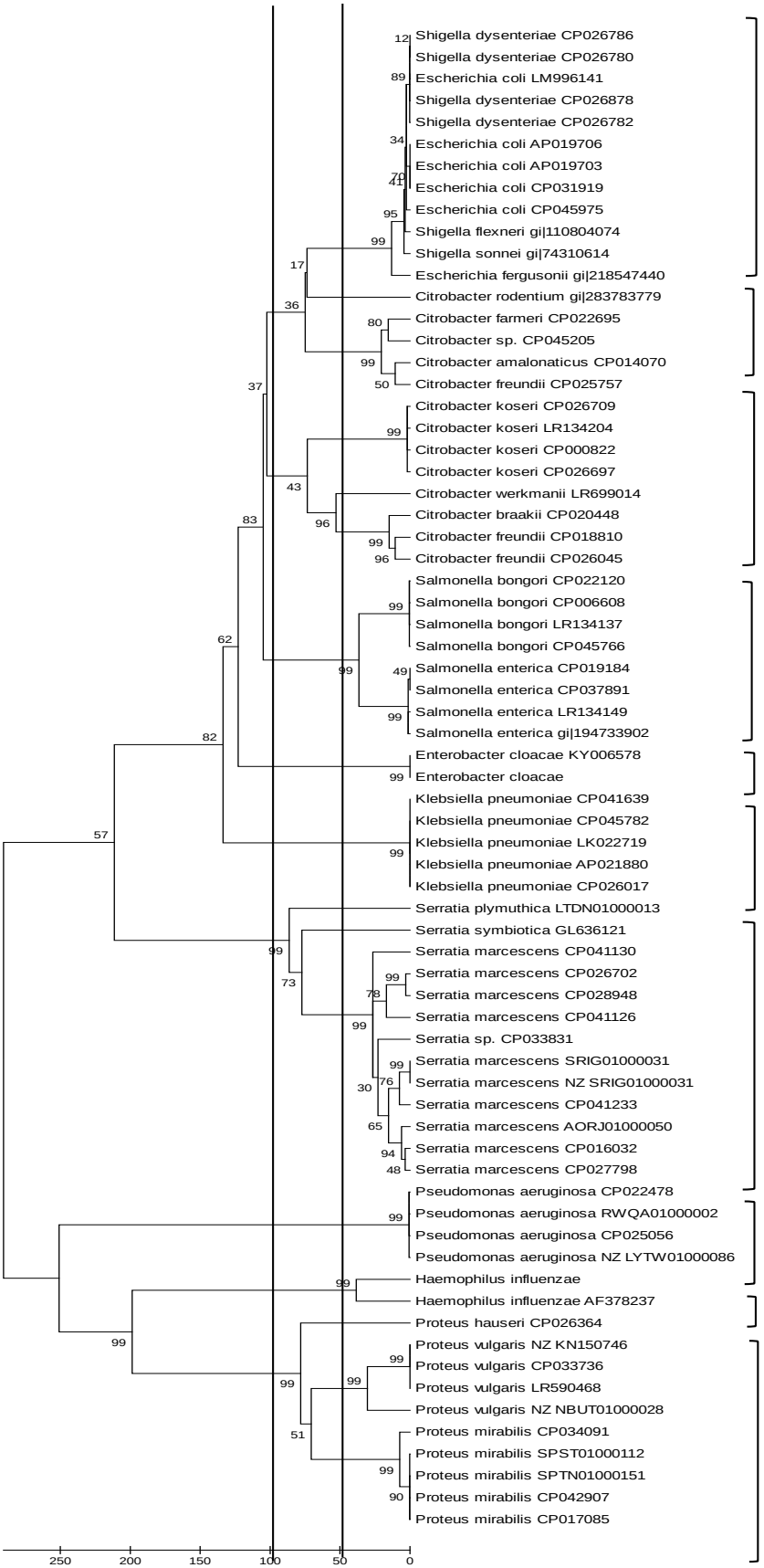
Results and Discussion

In previous studies, the conventional typing systems based on phage-typing, serotyping, and anti-biogram as phenotypes to name but a few, have been used for many years. In many cases, however, strain similarity or lack thereof is not enough but

rather the necessity is in the understanding of the relation of the isolates [12]. A frequent method for organism classification is Phylogenetic tree analysis [13]. In modern bacteria taxonomy, the standard method for the investigation of phylogenetic relationships is the 16S rRNA sequence analysis [14-18]. In bacterial identification, two types of a region in 16S rRNA gene DNA sequences are mainly implemented including the variable regions mainly used to differentiate genera and species, while the highly conserved regions are used for relationships definition among distant taxa [19, 20]. As previously documented, taxonomic problems usually emerge in members of the family Enterobacteriaceae when the 16S rRNA gene is used as a species identification marker. In addition, phylogenetic analysis cannot be easily resolved which is mainly attributed to the conservation's higher degree in closely related species [21]. Moreover, the recently identified marker *gyrB* provides an effective and fast method for bacterial species identification and phylogenetic relationships examination [22]. In addition, as previously documented, in the *gyrB* sequence comparison, compared to the 16S rDNA sequence analysis, the phylogenetic relationships at the species level are more defined, while 16S rRNA gene sequence has a higher effect in the analysis of higher orders and genera. Moreover, partial *rpoB* gene analysis and sequencing were executed to give more sensitivity [5]. However, more gene markers are required with increased specificity for more resolved phylogentic analysis. The phylogenetic trees were constructed in this study from 16S rRNA and *folp* Gram-negative bacterial species gene sequences as shown in **Figure 1**. The 16S rRNA-based tree openly delineated four distinct clusters with high bootstrap values (1000) at distance adjusted to100. Cluster 1 is a mixed species cluster in which *E.coli*, *Shigella* sp., *Klebsiella* sp., *Enterobacter* sp., *Salmonella* sp., *Citrobacter* sp., *Serratia* sp. and *Proteus* sp., while cluster 2 and cluster 3 contained *P. aeruginosa* and *Haemophilus* sp. respectively **(Figure 1)**. Comparatively, the *folp* gene-based tree yielded 9 clusters with a bootstrap value at distance adjusted to 100 and 1000 bootstrap value. *E.coli* and *Shigella* sp. were identified in one mixed cluster. However, other Gram-negative species such as *Klebsiella* sp., *Enterobacter* sp., *Salmonella* sp., *Citrobacter* sp., *Serratia* sp., *Proteus* sp., *Haemophilus* sp. *and P. aeruginosa* were found in separate clusters. In addition, at distance adjusted to 50 and 1000 bootstrap value, more resolution could be obtained even to the species level of Gram-negative bacteria.



a)



b)

Figure 1. Phylogenetic trees of Gram-negative bacteria based on 16S rRNA (a) and *folp* gene sequences (b). The evolutionary history was inferred using the Maximum Parsimony method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates).

These figures were obtained directly from the phylogenetic tree constructing program with the best possible resolution of MEGA 4 program.

Comparative DNA sequence data

At the interspecies level, DNA–DNA relatedness analyses were used for comparison of *folp* gene sequence similarities. As a result, Gram-negative species were detected in the range between 47.54- 98.37%. However, 16S rRNA gene sequence identical strains similarities were limited to 79.58-98.11%. In addition, comparative sequence analysis indicated a higher *folp* gene substitution compared to the 16S rRNA gene substitution. Interestingly, the *folp* gene sequence showed more remarkable discrimination abilities (47.54- 98.37%), which are more discriminatory than the 16S rRNA gene for species differentiation with exception of *E.coli* and *Shigella* sp. which were the most similar pair with 1.87 % sequence divergence. At the intraspecies level, high nucleotide substitution in *Proteus* sp. 22.95 %, *Serratia* sp. 27.05%, and *Citrobacter* sp. 28.10% could be detected. However 9 % or lower values could be detected in other Gram-negative bacteria **(Table 2)**. In contrast, in 16S rRNA gene results, at the interspecies level, the nucleotide substitution rates were ranged below 4 % exclusive of *Haemophilus* sp. which was 6.55 % **(Table 3)**. The *folp* gene DNA–DNA relatedness was consistent with the sequence-based phylogenetic analysis and a linear correlation was observed **(Figure 1)**. For example, *Shigella* sp. and *E.coli* which were more closely related detected in the phylogenetic analysis were detected with a divergence of 1.87 %. In addition, in *folp* gene phylogenetic analysis, *E.coli* which is highly distant from *Haemophilus* sp., *Serratia* sp., *Proteus* sp., and *P. aeruginosa* exhibiting high 44.61, 40.39, 39.81, and 42.62 % DNA–DNA *folp* gene sequence divergence respectively.

Table 2. % DNA-DNA sequence divergence between different Gram-negative bacteria based on *folp* gene sequences

	Proteus sp.	*Citrobacter* sp.	*E.coli*	*Shigella* sp.	*Salmonella* sp.	*Enterobacter* sp.	Klebsiella sp.	*Serratia* sp.	*Hemophilus* sp.	*P. aeruginosa*
Proteus sp.	22.95	44.61	39.81	39.70	41.80	40.52	40.16	46.49	46.60	52.46
Citrobacter sp	44.61	28.10	30.21	29.98	32.08	32,90	33.14	43.68	51.52	51.29
E.coli	39.81	30.21	1.17	1.87	21.19	20.37	22.13	40.39	44.61	42.62
Shigella sp.	39.70	29.98	1.87	1.41	20.84	20.37	22.13	40.28	44.61	42.74
Salmonella sp.	41.80	32.08	21.19	20.84	8.55	24.82	23.65	40.75	47.31	43.68
Enterobacter sp.	40.52	32,90	20.37	20.37	24.82	00	20.96	38.99	45.32	41.1
Klebsiella sp.	40.16	33.14	22.13	22.13	23.65	20.96	00	38.52	45.08	40.28
Serratia sp.	46.49	43.68	40.39	40.28	40.75	38.99	38.52	27.05	51.41	49.29
Hemophilus sp	46.60	51.52	44.61	44.61	47.31	45.32	45.08	51.41	8.43	48.71
P. aeruginosa	52.46	51.29	42.62	42.74	43.68	41.1	40.28	49.29	48.71	0.35

Table 3. % DNA-DNA sequence divergence between different Gram-negative bacteria based on 16S rRNA gene sequences

	Proteus sp.	*Citrobacter* sp	*E.coli*	*Shigella* sp.	*Salmonella* sp	*Enterobacter* sp	*Klebsiella* sp.	*Serratia* sp.	*Hemophilus* sp	*P. aeruginosa*
Proteus sp.	1.12	9.14	8.16	8.51	9.55	8.65	9.14	7.53	15.61	17.29
Citrobacter sp	9.14	3.71	5.04	5.32	5.53	4.62	5.39	5.37	15.19	17.50
E.coli	8.16	5.04	0.35	1.89	4.69	4.97	5.88	4.60	14.15	15.76
Shigella sp.	8.51	5.32	1.89	1.61	5.39	5.46	6.3	5.16	14.63	16.18
Salmonella sp.	9.55	5.53	4.69	5.39	3.43	5.53	6.44	6.62	15.54	16.67
Enterobacter sp.	8.65	4.62	4.97	5.46	5.53	2.1	4.2	3.97	15.05	16.31
Klebsiella sp.	9.14	5.39	5.88	6.3	6.44	4.2	3.43	4.81	15.26	16.74
Serratia sp.	7.53	5.37	4.60	5.16	6.62	3.97	4.81	0.42	14.01	15.83
Hemophilus sp	15.61	15.19	14.15	14.63	15.54	15.05	15.26	14.01	6.55	20.42
P. aeruginosa	17.29	17.50	15.76	16.18	16.67	16.31	16.74	15.83	20.42	1.46

Blind identification of some Gram-negative species

According to the newly utilized genotypic method used, the 9 isolated strains were identified as follows: 2 *Enterobacter* sp., 2 *Salmonella* sp., 2 *K.pneumonia*, 1 *Shigella* sp., and 2 *E.coli*. The distribution of different Gram-negative species in respect to the type of sepsis was illustrated in **Figure 2**.

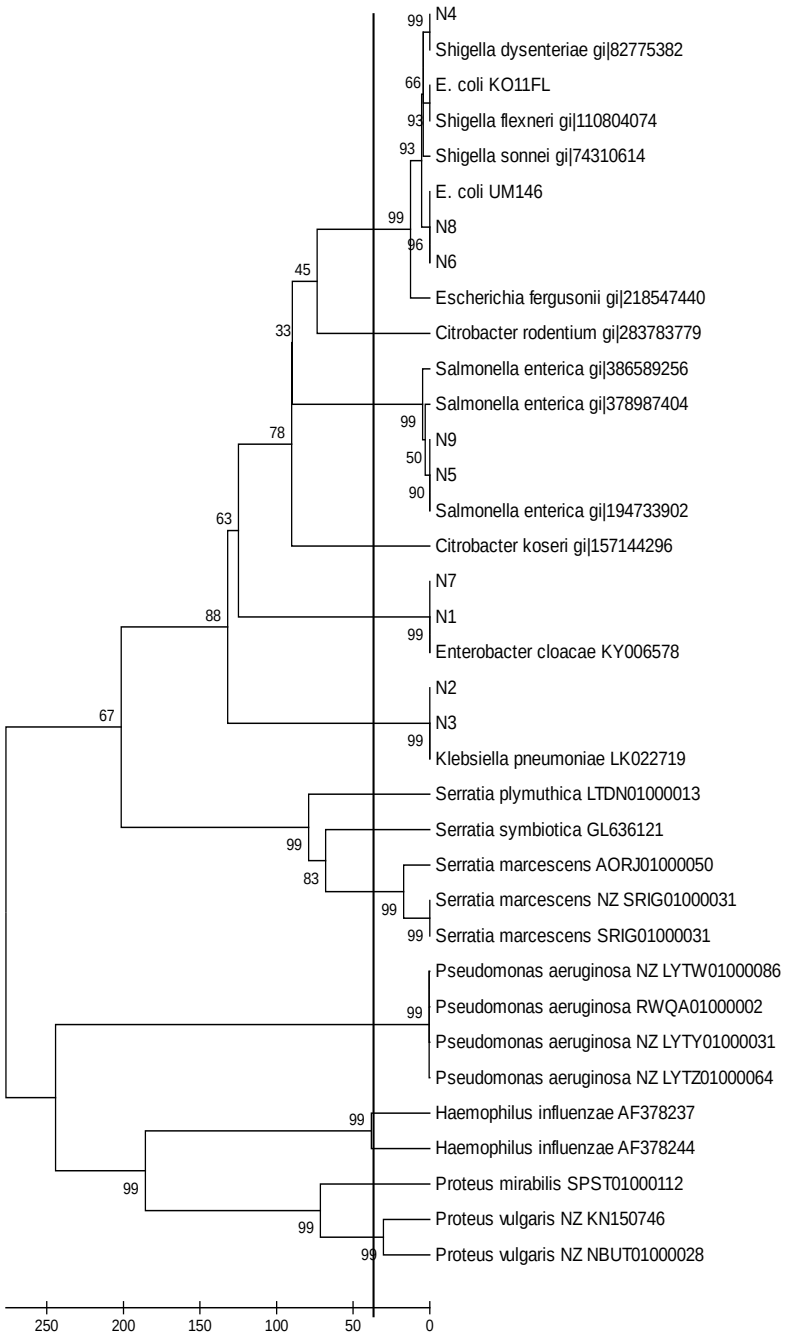


Figure 2. Blind identification of some Gram-negative species by phylogenetic analysis of *folp* gene sequences obtained in this study in addition to other gene sequences downloaded from the GenBank. All strains of this study were identified based on the closely related to reference strains installed from the GenBank

Conclusion

Gram-negative species are closely related and require more effort to be differentiated from each other. For this reason, other DNA sequencing genes are required. In this study, the *folp* gene encoding DHPS provided better resolution compared to the 16S rRNA gene sequences for both interspecies and interspecies 47.54- 98.37% and 79.58-98.11% respectively. These results indicate the possibility for implementation of *the folp* gene in DNA analysis of Gram-negative bacteria.

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Conflict of interest: None

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Ethics statement: None

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