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Research Article

Phylogenetic and genetic diversity analysis in *Leptospira* species based on the sequence homology pattern of 16S rRNA gene

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ABSTRACT

Leptospirosis is a bacterial zoonosis, caused by pathogenic spirochete which belongs to the genus *Leptospira*. It exists in diverse ecological habitats and affects almost all the mammals including humans. Several online databases like NCBI etc will provide the complete genomic sequence data of various *Leptospira* species. However, the Phylogenetic and genetic diversity Analysis in *Leptospira* species based on 16S rRNA gene has not studied in detail. Therefore the present study was conducted. Sequences of various species related to genus *Leptospira* obtained from the NCBI database etc and aligned (CLUSTAL_X). Two Phylogenetic trees were constructed (MEGA-5) in which the first one is related to various serovars of *L. interrogans* and the other is related to various species of *Leptospira.* The Phylogenetic trees revealed the relationship and genetic diversity of various serovars of *L. interrogans* and the other *Leptospira* species, with their nearest phylogenetic relatives. In the first tree, two major clades were observed which were named as A and B, whereas in the second tree, three major clades were observed and named as A, B and C respectively. *Aquifex pyrophilus* strain has been used for out grouping in both the trees. The genetic distance between the species in the phylogenetic tree is presented by a bar which represents 0.5 nucleotide substitutions per alignment position in the 16S rRNA gene sequence among the various serovars of *L. interrogans* while 0.05 nucleotide substitutions in case of various species related to the genus *Leptospira*. Thus, the findings from the above study confirm that the genus *Leptospira* exhibits genetic diversity in the 16S rRNA gene.

Keywords: Leptospira, 16S rRNA gene, Phylogenetic analysis, Genetic diversity analysis

INTRODUCTION

Globally Leptospirosis is the most important public health concern. It is a bacterial zoonosis, caused by pathogenic spirochete which belongs to the genus *Leptospira*, which affects almost all the mammals including humans.[1] It is cosmoplotialy distributed and exists in diverse ecological habitats which includes from water to soil and in the tissues of various mammalians and its prevalence is high in tropics. So many environmental factors play a key role in transmission of this disease, which occurs especially in flood associated urban outbreaks, in rural endemics and also with other occupational and recreational exposure. It may also occurs either direct contact with rodents or pet animals or polluted water, infected urine, venereal and placental transfer, bite wounds or ingestion, whereas the indirect transmission takes place either with contaminated food, water or bedding.[2] Infection is facilitated with Leptospires which penetrate in to mucous membranes and multiply rapidly upon entering the blood system, spreading to other tissues including the kidneys, liver,

spleen, nervous system, eyes and genital tract, finally turns into a serious disease. Symptoms of acute *Leptospira* infections include fever, shivering, muscle tenderness, vomiting, rapid dehydration, meningitis, pneumonitis, hepatitis, nephritis, pancreatitis, erythema nodosum and death.[3]

Under reporting is a major issue in analyzing the actual incidence of leptospirosis in many Asian countries.[4] In India, it is directly related to either monsoons or poor sanitary conditions, with multiple epidemics reported.[5-9] In southern coastal belt during the monsoons Leptospirosis has become a common cause for febrile illness, especially in the states of Orissa, Andhra Pradesh and Tamil Nadu. Till today there is no concrete epidemiological data available from these regions, even though numerous cases have been diagnosed. Earlier studies revealed that it is quite common in Tamil Nadu, Kerala and the Andamans.[5] Recent study states that it has just started appearing in various other states either as outbreaks or as sporadic cases with significant incidence[2]. Its prevalence also reported from the states like west Bengal, Uttar Pradesh, Pondicherry, Chennai, Pune, Maharashtra, Bangalore and Manipal region in Karnataka, Cochin region in Kerala, Ranchi and other coal mining areas of Bihar and Gujarat.[5-10]

Modern advancements in molecular biology, led to the introduction of polymerase chain reaction (PCR), found to be promising for the diagnosis of leptospirosis. Several assays of PCR have been applied to various clinical specimens include urine, blood, cerebrospinal fluids and semen etc to detect Leptospiral DNA.[11] Recent studies on DNA-DNA hybridization and 16S rRNA sequence analysis have led for the classification of *Leptospira* species and so far 20 species has been identified from this genus, which includes i.e., 9 pathogenic species (*L. interrogans*, *L. kirschneri*, *L. kmetyi*, *L. borgpetersenii*, *L. santarosai*, *L. noguchii*, *L. weilii*, *L. alexanderi*, and *L.alstoni*) which are being considered as pathogenic agents of leptospirosis (Levett et al., 2006), along with other 6 saprophytic species (*L. biflexa*, *L. wolbachii*, *L. meyeri*, *L. vanthielii*, *L. terpstrae*, and *L. yanagawae*), and 5 intermediate species (*L. inadai*, *L. broomii*, *L. fainei*, *L. wolffii*, and *L. licerasiae*), which are species of unclear pathogenicity segregated into a distinct group from pathogens and saprophytes.[11] However, the Phylogenetic and genetic diversity Analysis in *Leptospira* species based on 16S rRNA gene has not studied in detail. Therefore the present study is undertaken to assess the sequence homology of 16S rRNA gene from various species of the genus *Leptospira* and elucidate the genetic distance based on the phylogenetic analysis.

METHODS

In the present study all the 16S rRNA gene sequences of various species of the genus *Leptospira* has been obtained from the online databases like NCBI (National Center for Biotechnology Information), EzTaxon (server 2.1) and ribosomal data project. The BLAST (basic local alignment search tool) algorithm has been preformed to calculate the percentage of similarity between known sequences.[12] The 16S rRNA gene sequences obtained from the database was subjected to alignment using the CLUSTAL_X program.[13] The software Molecular Evolutionary Genetics Analysis version 5.05 (MEGA5) was used for construction of Phylogenetic trees and statistical analyses.[14] The Phylogenetic trees were constructed based on the 16S rRNA gene sequences using tree construction programmed algorithms like maximum likelihood (ML) and Neighbour-joining (NJ) methods as described.[13-15] The bootstrap confidence levels were evaluated by generating one thousand bootstrap trees. These node values indicate the level of degree of confidence in inferring that the nodes do indeed occur at those locations. However the bootstrap values at nodes are shown in the tree. The gene sequences used in this study were documented in the table 1 and 2, with their respective Gene bank IDs and they can be retrieved from the Gen Bank nucleotide sequence database.

RESULTS

The 16s rRNA gene sequences related to 35 strains of various serrovars of *L. interrogans* were obtained from the online database like NCBI etc (Table 1). Sequences of these serrovars of *L. interrogans* were aligned using the CLUSTAL_X. Phylogenetic analysis was performed to elucidate the 16s rRNA gene polymorphism among these serovars. Hence, phylogenetic tree was constructed based on the 16s rRNA gene sequences using the software mega-5. In the phylogenetic tree, the strain *Aquifex pyrophilus* (NR02172.1) has been used for out grouping. The phylogenetic tree also reveals that the serrovars of *L. interrogans* separated into two distinct clades, A and B (Figure 1). The clade A is comprised of 32 serrovars, which were grouped together and formed in to a single cluster which has been depicted in the figure 1. The second clade B consists of only 3 serovars, which were formed into a cluster (DQ 522197.1; DQ522227.1; DQ522194.1) (Figure 1). These results reveal that there is a no significant variation observed in the sequence homology of 16S rRNA gene among the various serovars of *L. interrogans* in the first clade. Hence they all segregated and formed into a cluster. In contrast, the second clade B exhibited slight variation in the sequence homology of 16S rRNA gene. The genetic distance between the various serovars in the phylogenetic tree is presented with a bar. This bar represents the changes in the nucleotide positions. The phylogenetic tree data represents that 0.5 nucleotide substitutions per alignment position in the sequence homology of 16S rRNA gene has been noticed, which indicates variation in the sequence homology of 16S rRNA gene among the various serovars of *Leptospira interrogans*. However the level of variation in the sequence homology of 16S rRNA gene is very less.

Tabel 1: The various serovars of *Leptospira interrogans* with accession numbers (Gene bank IDS) used in this study.

S. No.	Various Serovars of *Leptospira interrogans*	Gene bank ID
1	*Aquifex pyrophilus strain* Kol5a (Out group strain)	NR 029172.1
2	*Leptospira interrogans*	EF536989.1
3	*Leptospira interrogans*	M71241.1
4	*Leptospira interrogans*	X17547.1
5	*Leptospira interrogans* 56601	AE010300
6	*Leptospira interrogans* ATCC 23581T	AY631894
7	*Leptospira interrogans* FPW1024	EF536986.1
8	*Leptospira interrogans serovar Australis strain Ballico*	AY996794.1
9	*Leptospira interrogans serovar Australis strain Swart*	FJ154557.1
10	*Leptospira interrogans serovar Autumnalis strain Akiyami*	AY996791.1
11	*Leptospira interrogans serovar Bataviae*	EF536993.1
12	*Leptospira interrogans serovar Bratislava strain*	FJ154547.1
13	*Leptospira interrogans serovar Bulgarica strain*	AY996792.1
14	*Leptospira interrogans serovar Canicola strain*	FJ154561.1
15	*Leptospira interrogans serovar Copenhageni strain*	AY996790.2
16	*Leptospira interrogans serovar Copenhageni strain*	NR 074524.1
17	*Leptospira interrogans serovar Grippotyphosa*	EF536975.1
18	*Leptospira interrogans serovar Hardjo strain*	AY996796.1
19	*Leptospira interrogans serovar Hardjo strain*	AY996797.1
20	*Leptospira interrogans serovar Hardjo-prajitno strain*	FJ154553.1
21	*Leptospira interrogans serovar Icterohaemorrhagiae strain*	EU581713.1
22	*Leptospira interrogans serovar Icterohaemorrhagiae strain*	AY631894.1
23	*Leptospira interrogans serovar Lai strain* 56601	NR 074481.1
24	*Leptospira interrogans serovar Manilae strain*	FJ154545.1
25	*Leptospira interrogans serovar Muelleri strain*	FJ154568.1
26	*Leptospira interrogans serovar Muenchen*	EF537000.1
27	*Leptospira interrogans serovar Muenchen strain*	FJ154565.1
28	*Leptospira interrogans serovar Mujunkunmi*	EF537002.1
29	*Leptospira interrogans serovar Pyrogenes strain*	AY996793.1
30	*Leptospira interrogans serovar Sejroe strain*	FJ154558.1
31	*Leptospira interrogans strain* BEL039	DQ522232.1
32	*Leptospira interrogans strain* CEH006	DQ522194.1
33	*Leptospira interrogans strain Coppenhageni* DB36	JQ988857.1
34	*Leptospira interrogans strain* H0775	JQ906650.1
35	*Leptospira interrogans strain* HAI029	DQ522197.1
36	*Leptospira interrogans strain* VAR010	DQ522227.1

Tabel 2: The various species of the genus *Leptospira* with accession numbers (Gene bank IDS) used in this study.

S. No.	*Leptospira* species	Gene bank ID
1	*Aquifex pyrophilus strain* Kol5a (Out group strain)	NR 029172.1
2	*Leptospira alexanderi* ATCC 700520^T	AY631880
3	*Leptospira alexanderi serovar Manhao* 3 *strain*	NR 043047.1
4	*Leptospira alexanderi serovar Nanding strain*	AY996804.1
5	*Leptospira alstoni serovar Pingchang*	DQ991480.1
6	*Leptospira alstoni serovar Sichuan*	AY631881.1
7	*Leptospira biflexa Patoc IT*	CP000786
8	*Leptospira borgpetersenii serovar Ballum strain*	HM776722.1
9	*Leptospira borgpetersenii serovar Hardjo-bovis strain*	NR 074526.1
10	*Leptospira borgpetersenii serovar Javanica strain*	FJ154600.1
11	*Leptospira borgpetersenii serovar Mini strain*	FJ154592.1
12	*Leptospira borgpetersenii Veldrat Bataviae* 46^T	Z21630
13	*Leptospira broomii* ATCC BAA-1107^T	AY796065
14	*Leptospira fainei* BUT 6^T	AY631885
15	*Leptospira inadai* ATCC 43289^T	AY631896
16	*Leptospira kirschneri* ATCC 49945^T	AY631895
17	*Leptospira kirschneri serovar Cynopteri strain*	NR 043051.1
18	*Leptospira kirschneri serovar Kambale*	FJ154562.1
19	*Leptospira kirschneri serovar Pomona strain*	FJ154559.1
20	*Leptospira kmetyi* Bejo-Iso9^T	AB279549
21	*Leptospira kmetyi serovar Malaysia strain*	NR 041544.1
22	*Leptospira kmetyi serovar Malaysia*	AB279549.1
23	*Leptospira licerasiae* VAR010^T	EF612284
24	*Leptospira meyeri Iowa City* Frog^T	Z21648
25	*Leptospira noguchi* Fort Bragg	LNU12671
26	*Leptospira noguchii* CZ 214^T	Z21635
27	*Leptospira noguchii serovar Panama strain*	NR 043050.1
28	*Leptospira santarosai serovar Alexi strain*	FJ154585.1
29	*Leptospira santarosai serovar Bakeri strain*	FJ154589.1
30	*Leptospira santarosai serovar Trinidad strain*	FJ154598.1
31	*Leptospira santarosai shermani* 1342K^T	Z21649
32	*Leptospira weilii* ATCC 43285^T	U12676
33	*Leptospira weilii Cellidoni*	U12676.1
34	*Leptospira wolbachii* CDCT	Z21638
35	*Leptospira wolffii Khorat*-H2^T	EF025496

Next, we assessed the 16S rRNA gene sequence homology of various *Leptospira* species by phylogenetic analysis to elucidate the genetic distance. 69 strains of various *Leptospira* species have been selected for this study and *Aquifex pyrophilus* strain has been used for out grouping (Table 1 and 2). The phylogenetic tree results clearly indicate that the various species of the genus *Leptospira* were segregated and formed into different clades (Figure 2). Three major clades were observed in the phylogenetic tree, which were named as A, B and C respectively. The various serovars of *L. interrogans* strains were segregated and formed in to a single cluster in the clade A. Similarly, the other species like *L. kirschneri* also share the same clade of *L. interrogans* (n=32). The various serovars of *L. noguchii* (n=3), *L. meyeri* (n=1), *L. alstoni* (n=2), *L. kmetyi* (n=3), *L. alexanderi* (n=3), *L. weilii* (n=2), *L. borgpetersenii* (n=5), and *L. santarosai* (n=4) were also segregated into individual small clusters in the same axis of *L. interrogans*. These results clearly represent that the various *Leptospira* species strains which are segregated in the Clade A are almost near in terms of their genetic distance with slight variation (Figure 2).

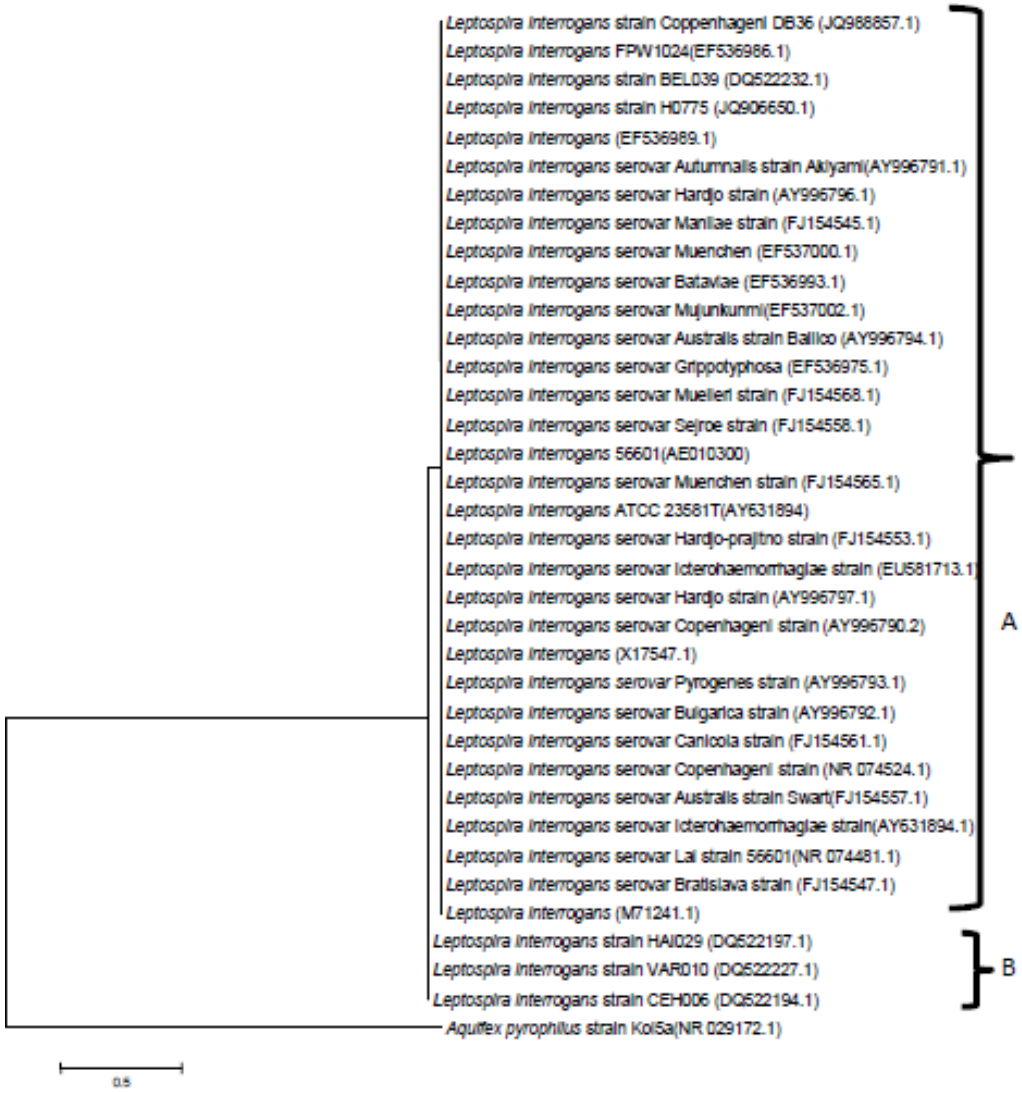

Figure 1: The findings from the Phylogenetic tree (based on 16S rRNA gene sequences obtained from the NCBI database) reveals the relationship and genetic diversity of various serovars of *Leptospira interrogans* , with their nearest phylogenetic relatives. Phylogenetic trees were constructed by Maximum likelihood and Neighbour-joining method. Majorly two dominant clades were observed in the phylogenetic tree , which were named as A and B. *Aquifex pyrophilus* strain (NR 029172.1) has been used for out grouping. The bar represents 0.5 nucleotide substitutions per alignment position.

Two major clusters have been observed in the second clade B. The individual strains like *L. inadai, L. broomii,* and *L. Fainei* were grouped together and formed a first cluster whereas in the second cluster the serrovars of *L. interrogans* (n=3), were grouped together with individual strains like *L. wolffii*, and *L. licerasiae.* These results indicate that there is marked deviation in their genetic distance. In the final clade C, the individual strains like *L. biflex* and *L. wolbachii* were out grouped together, exhibiting maximum, variability in their genetic distance when compared to serovars of *L. interrogans*. The genetic distance between the various species in the phylogenetic tree is represented by a bar which represents 0.05 nucleotide substitutions per alignment position among the various species of the genus *Leptospira.* These findings indicate a clear cut deviation in the sequence homology of 16S rRNA gene among various species of the genus *Leptospira*. Thus the above study clearly demonstrates variations in the sequence homology of 16s rRNA gene which reflects in genetic diversity of the genus *Leptospira*.

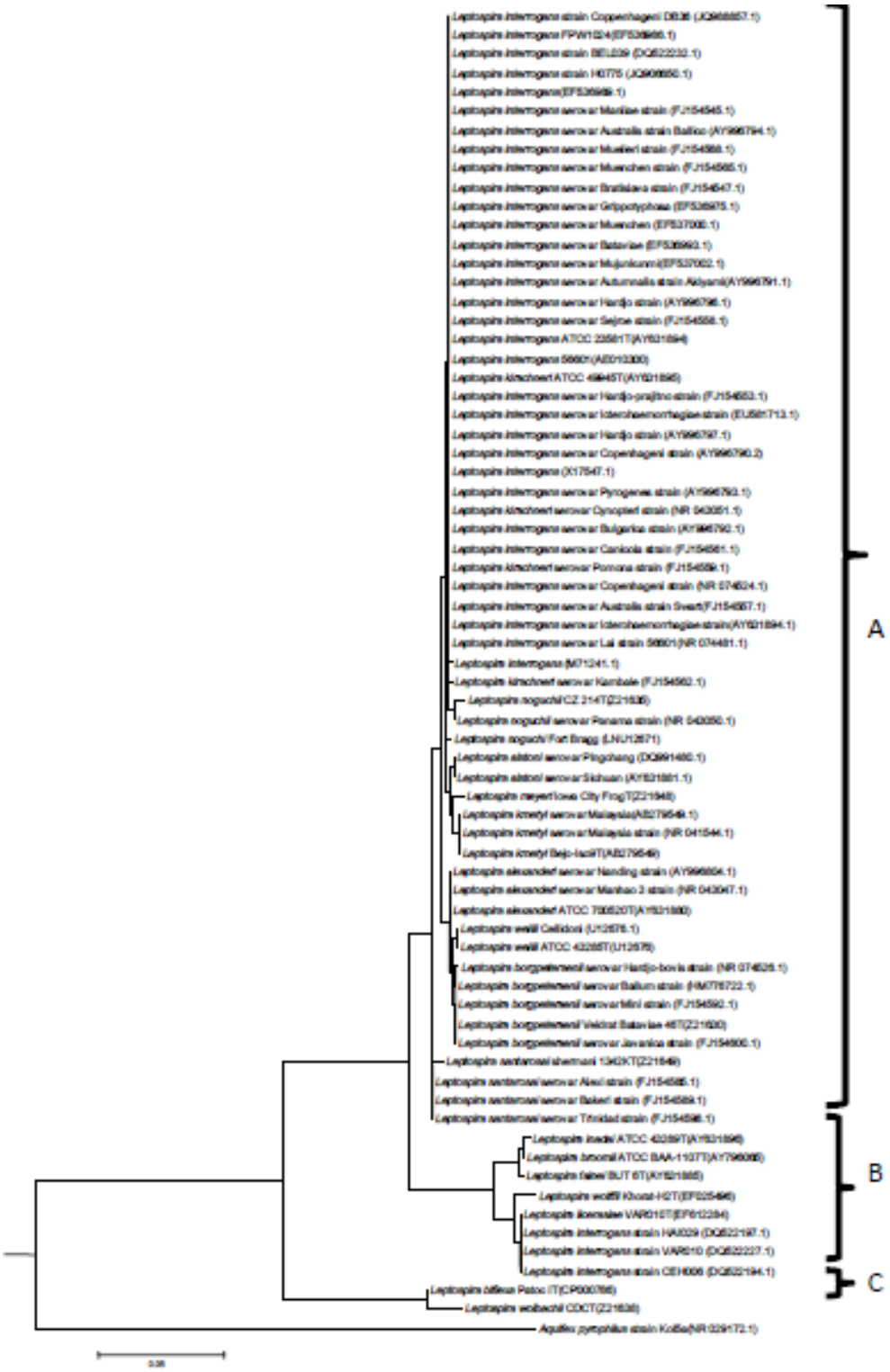

Figure 2: The findings from the Phylogenetic tree (based on 16S rRNA gene sequences obtained from the NCBI database) reveals the relationship and genetic diversity of various Species of *Leptospira* genus, with their nearest phylogenetic relatives. Phylogenetic trees were constructed by Maximum likelihood and Neighbour-joining method. Majorly three dominant clades were observed in the phylogenetic tree, which were named as A, B and C respectively. *Aquifex pyrophilus* strain (NR 029172.1) has been used for out grouping. The bar represents 0.05 nucleotide substitutions per alignment position.

DISCUSSION

Leptospirosis is the major health concern, caused by pathogenic spirochete which belongs to the genus *Leptospira*. Various molecular tools were applied for the classification of *Leptospira* species include PFGE (Pulsed field gel electrophoresis), RFLP (restricted fragment polymorphism), arbitrarily primed PCR, fluorescent amplified fragment length polymorphism, variable number tandem repeats and 16S rRNA gene analysis.[16] In the genus *Leptospira*, 20 species have been identified based on the above mentioned methods that include both saprophytic and pathogenic species.[17-19] Several serological methods further classified these *Leptospira* species in to various serovars. Nearly 300 serovars have been identified, out of which 200 are being considered as pathogenic.[18,20-21] The complete genomic sequence data of various *Leptospira* species around the world are available through various online data bases like NCBI etc for the pathogenic *Leptospira* strains like, *L. interrogans* serovars Lai[22] and Copenhageni,[23] *L. borgpetersenii* serovar Hardjo strains L550 and JB197,[24] and the saprophyte strains like *L. biflexa* serovar Patoc I [25] etc. However, the Phylogenetic and genetic diversity Analysis in *Leptospira* species based on the sequence homology pattern of 16S rRNA gene has not studied in detail. Hence the present investigation is carried in an aim to assess the sequence homology of 16S rRNA gene from various species of the genus *Leptospira* and elucidate the genetic distance based on the phylogenetic Analysis.

The species *L. interrogans* is one of the dominant pathogen majorly found in the most infections related to Leptospirosis when compared to other pathogenic species like *Leptospira borgpetersenii, Leptospira santarosai, Leptospira noguchii, Leptospira weilii, Leptospira kirschneri and Leptospira alexanderi* are considered to be the most prominent agents of Leptospirosis.[18] Cerqueira et al, 2009 demonstrated that the *L. interrogans species* is associated with severe human Leptospirosis, while the other strains like *L. santarosai* have shown their association with pigs and cattle.[16] Hence, we first analyzed the various serovars occurring around the world related to species *L. interrogans.* The 16s rRNA gene sequences of 35 strains of various serrovars of *L. interrogans* has been obtained from the online database like NCBI (Table 1) and phylogenetic analysis was performed to demonstrate 16s rRNA gene polymorphism among the serovars. For out grouping *Aquifex pyrophilus* (NR02172.1) strain has been used. The findings from the phylogenetic tree indicate that the serrovars of *L. interrogans* segregated into two distinct clades (Figure 1). Various *L. interrogans* serrovars up to 32 serrovars were segregated in to a single cluster in the first clade, whereas in the second only 3 serovars were grouped and formed the cluster. This segregation may be due to fact that these species has been isolated from the same source.[26] From the above results it is clearly evident that there is no significant variation observed in the sequence homology of 16S rRNA gene among the various serovars of *L. interrogans* in the first clade. Hence they all segregated and formed into a cluster. However in the second clade there is slight variation in the sequence homology of 16S rRNA gene. The genetic distance between the species in the phylogenetic tree is represented by a bar which represents 0.5 nucleotide substitutions per alignment position in the sequence homology of 16S rRNA gene among the various serovars of *Leptospira interrogans* which indicates variation in the sequence homology of 16S rRNA gene. However the level of variation in the sequence homology of 16S rRNA gene is very less. Thus our findings were correlated with other studies which demonstrated that 16S rRNA gene will exhibit a low degree of polymorphism.[27]

Next we studied the 16S rRNA gene sequence homology of various *Leptospira* species by phylogenetic analysis to elucidate the genetic distance. For this study a total of 70 strains have been selected, out of this *Aquifex pyrophilus* strain has been used for out grouping (Table 1 and 2). The findings from the phylogenetic tree clearly indicate that segregation of various *Leptospira* species into different clades (Figure 2). Majorly three dominant clades were observed in the phylogenetic tree, which were named as A, B and C respectively. The distribution of various serovars of *L. interrogans* strains was found to be predominant in the Clade A. All the *L. interrogans* strains of various serovars were segregated and formed in to a single cluster. Similarly, the other species like *L. kirschneri* also share the same clade of *L. interrogans* (n=32). The various serovars of *L. noguchii* (n=3), *L. meyeri* (n=1), *L. alstoni* (n=2), *L. kmetyi* (n=3), *L. alexanderi* (n=3), *L. weilii* (n=2), *L. borgpetersenii* (n=5), and *L. santarosai* (n=4) were also segregated into individual small clusters in the same axis of *L. interrogans*. These findings indicate that the above mentioned various *Leptospira* species strains are almost near in terms of their genetic distance with slight variation (Figure 2).

In the second clade B, two major clusters have been observed. In the first cluster individual strains like *L. inadai, L. broomii,* and *L. Fainei* were grouped together whereas in the second cluster the serrovars of *L. interrogans* (n=3), were grouped with individual strains like *L. wolffii,* and *L. licerasiae.* These results indicate that there is marked deviation in their genetic distance. In the final clade C, the individual strains like *L. biflex* and *L. wolbachii* were out grouped together, exhibiting maximum, variability in their genetic distance when compared to *L. interrogans* serovars which was segregated in the first Clade A. The genetic distance between the species in the phylogenetic tree is represented by a bar which represents 0.05 nucleotide substitutions per alignment position among the various

species of the genus *Leptospira* which indicates clear cut deviation in the sequence homology of 16S rRNA gene. Thus the above study clearly demonstrates variations in the sequence homology of 16s rRNA gene of various *Leptospira* species. Our results were correlated with the earlier study conducted by Ganoza et al., 2006.[26]

CONCLUSION

The results obtained from the above study confirm that the genus *Leptospira* exhibits genetic diversity in the 16S rRNA gene. The Phylogenetic trees revealed the relationship and genetic diversity of various serovars of *L. interrogans* and the other *Leptospira* species, with their nearest phylogenetic relatives. In the first tree, two major clades were observed which were named as A and B, whereas in the second, three major clades were observed and named as A, B and C respectively. *Aquifex pyrophilus* strain has been used for out grouping in both the trees. The genetic distance between the species in the phylogenetic tree is presented by a bar which represents 0.5 nucleotide substitutions per alignment position in the 16S rRNA gene sequence among the various serovars of *L. interrogans* while 0.05 nucleotide substitutions in case of various species related to the genus *Leptospira*.

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