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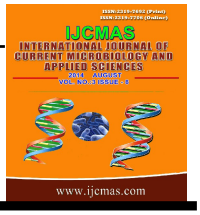

Original Research Article

An *in silico* assessment of molecular evolution at 16S rRNA of *Pseudomonas sp*.

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Keywords

Pseudomonas sp, 16S rRNA gene, Molecular evolution, MEGA5.2

A B S T R A C T

An *in silico* attempt was made to assess the molecular divergence at 16S rRNA of forty eight different species of *Pseudomonas* worldwide first time based on Molecular Evolutionary Genetics Analysis version 5.2 (MEGA5.2). Topological evaluations gave support to a common origin for native strains as well as most of the strains retrieved from NCBI database. Distinct selection pressure at different sites of nucleotide of 16S rRNA sequence was detected to estimate the mechanism underlying the evolution of all *Pseudomonas sp* strain first time. This research was a premonition report for India to restrict pathogens' attack in agriculture sectors due to high degree of nucleotide diversity and decreased population of rhizobacteria like *Pseudomonas sp* in soil rather than only climatic change itself.

Introduction

Some researchers described *Pseudomonas* species as species of the authentic *Pseudomonas* but the phylogenetic positions of several species were not demonstrated [Scarpellini et al, 2004; Kersters et al, 1996]. Then Phylogenetic affiliations of all *Pseudomonas* species were shown with the help of 16S rRNA sequences (Anzai et al, 2000). The primary phylogenetic groups based on the 16S rDNA sequences of fluorescent pseudomonads were identified (Kwon et al, 2005). An extensive study was performed to clarify the taxonomic affiliation of some fluorescent *Pseudomonas sp* from grass (Behrendt et al, 2007).

Phylogenetic analysis on the basis of 16S rDNA sequences provided better understanding in evaluation of *Pseudomonas sp* bacteria (Issar et al, 2012). Several workers had been demonstrated the affiliation of *Pseudomonas sp* through phylogenetic study but the novel approach of this study was to detection of distinct selection pressure at 16S rRNA sequences for estimation of the mechanism underlying the evolution of all *Pseudomonas sp* strain and determination of molecular divergence in terms of mean evolutionary diversity within subpopulations; inter continent population, coefficient differentiation between two continents, evolutionary

divergence over sequence pairs, average evolutionary divergence over all sequence pairs between *Pseudomonas sp* of two continents first time.

Materials and Methods

Native *Pseudomonas sp* Isolation

Native *Pseudomonas sp* was isolated in King's B medium (KB) (King et al, 1954). After 48 hrs, predominant colonies with different morphologies were individually inoculated in slant for purifying cultures. Positive fluorescent colonies were maintained in 20% glycerol at -80^0 C prior to characterization.

Analysis of Different Species of *Pseudomonas* Strain

Strains of different species of *Pseudomonas* retrieved from NCBI database as well as native *Pseudomonas aeruginosa* strain recovered from different sources were used as indicated in Table1 to make a comparative study for our investigation.

Total DNA Extraction and 16S rDNA PCR Amplification

Genomic DNA of bacterial strain was prepared according to the procedures of (Murray and Thormpson, 1980) with the exception that for Gram-negative bacteria, lysozyme was not used. The 16S rRNA gene was amplified from each isolate by PCR using the universal primers 27F (5'-AGAGTTTGATCCTGGTCAGAACGCT-3') and 1492R (5'TACGGCTACCTTGTTACGACTTCACCCC-3'), which were designed to amplify universally conserved regions of the 16S rRNA gene and amplification of an approximately 1500 bp PCR product. PCR was carried out in a thermal cycler (Eppendrof AG, Mastercycler personal-22331, Humburg, Germany). Amplification of targeted DNA was carried out in 50 µl reaction volumes, each containing 50 ng of bacterial genomic DNA, 2.5 µl 10X *Taq* polymerase assay buffer, 2 mM $MgCl_2$, 50 mM Tris-HCl, 200 µM (each) deoxynucleoside triphosphates, 0.4 µM (each) primer and 1 U of Taq polymerase (Bangalore Genei).

The PCR conditions were as follows: initial denaturation at 95 °C for 4 min followed by 32 cycles of denaturation at 94 °C for 45 s, annealing at 56 °C for 1 min and extension at 72 °C for 1.5 min followed by a final extension at 72 °C for 10 min. The presence and yield of specific PCR products (16S rRNA gene) was electrophoresed in 1% agarose (w/v) gel.

Nucleotide Sequence Accession Numbers

The 16S rDNA nucleotide sequences of native isolate obtained in this study was submitted to the GenBank database (http://www.ncbi.nlm.nih.gov) and the accession numbers are given in Table.1.

DNA Sequencing of 16S Region and Sequence Alignment, Phylogenetic, selection pressure and different molecular divergence parameter Analysis

Direct sequencing of PCR products was conducted for positive strand in forward direction using Applied Biosysterns sequencers 373A and 377. The sequence reaction was conducted using the Sanger dideoxy sequencing Sequencing kit (Applied Biosystems, Foster City) following the manufacturer's instructions. Sequence analysis of these strains was also performed using BLAST (blastn) search tool (http://www.ncbi.nlm.nih.gov) available on the NCBI homepage.

A Maximum Likelihood (ML) tree of 16S rDNA sequences native strains was also constructed with the 16S rDNA sequences of closely related members of *Pseudomonas sp* as retrieved from the NCBI GenBank (http://www.ncbi.nlm.nih.gov). Sequence data were aligned with CLUSTALW 1.6 and genetic distances were obtained using Kimura's two-parameter model by best fit substitution model method followed by evolutionary trees construction by the Maximum Likelihood method using Molecular Evolutionary Genetics Analysis (MEGA) 5.2 software (Tamura et al, 2011). A bootstrap analysis of 1000 replicates was performed to estimate the confidence of tree topologies. The mean (relative) evolutionary rates (calculated in K2 parameter model) were scaled such that the average evolutionary rate across all sites was 1 (Kimura, 1980).

Tajima's Neutrality statistical method was performed to test the diversifying selection or purifying selection (Tajima, 1989). Mean Evolutionary Diversity within Subpopulations; inter continent population, coefficient differentiation between two continents, evolutionary divergence over sequence pairs and average evolutionary divergence over all sequence pairs between India and other continent were computed to calculate the molecular divergence.

Results and Discussion

Identification of *Pseudomonas aeruginosa*

All native *Pseudomonas aeruginosa* genomic DNA was isolated and PCR amplified with 16S rDNA universal primer pair (27F/1492R). Electrophoretical analysis of PCR products obtained from the amplification of 16S rDNA genes confirmed that full length (1500bp) genes were amplified for all *Pseudomonas aeruginosa* (Figure. 1). The amplified product was sequenced and sequence of DNA fragment was compared to the sequences available in GenBank, NCBI. Sequence analysis of these isolates was also performed using BLAST (blastn) search tool (http://www.ncbi.nlm.nih.gov) available on the NCBI homepage. All native *Pseudomonas aeruginosa* strains used in the study exhibited 96 to 98% sequence similarity to the *Pseudomonas aeruginosa* available in NCBI database with lowest E-value and maximum query coverage and identity. Theses sequence data has been deposited in the GenBank (Table. 1).

Molecular Divergence and Phylogenetic Affiliation of 16S rDNA of *Pseudomonas sp*

For construct a Phylogenetic tree of forty eight 16S rDNA sequences including all native strain, firstly find out Best-Fit Substitution Model. MEGA5.2 software (Tamura et al, 2011) now contains facilities to evaluate the fit of major models of nucleotide substitutions. Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the substitution pattern the best. In this context K2 model (Kimura's two parameter) displayed lowest BIC scores with the value of 2777.5. (Table. 2) So this model can be carried out for further Phylogenetic analysis.

The multiple alignment file was then used to create phylogram using MEGA5 software using the K2 model based on bootstrap analysis of 1000 replicates was performed to estimate the confidence of tree topologies. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Tamura et al, 2011). The tree with the highest log likelihood (-2296.1600) is shown in figure 2. The percentage of trees in which

the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value.

The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 48 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 100 positions in the final dataset. In this study, all the native strains from West Bengal were grouped into same cluster where most of the Indian *Pseudomonas aeruginosa* strains were lying down. The Phylogenetic tree revealed that all the native strain fit in to an evolutionary cluster comprising members of *Pseudomonas aeruginosa* and other *Pseudomonas sp* grouped into separate cluster according to their relatedness (Figure. 2) The mean (relative) evolutionary rates (calculated in K2 parameter model) were scaled such that the average evolutionary rate across all sites was 1(Kimura, 1980). This means that sites showing a rate < 1 are evolving slower than average and those with a rate > 1 are evolving faster than average (Table. 3). The most of the sites showed a relative rate of evolution less than 1.

Whenever Tajima's Neutrality statistical method was performed to test the nucleotide mutation hypothesis, (Tajima, 1989) it was strongly exhibited the support of the result of Table 3. In this case Tajima's test statistics was become positive (3.311203) (Table. 4). Distinct selection pressure was first detected to estimate the mechanism underlying the evolution of all the sequences of *Pseudomonas aeruginosa*. The difference between non-synonymous and synonymous substitution rates (dN-dS) for the sequences of all strains were almost always positive indicating that the gene was under positive selection or diversifying selection.

In this study, comparative sequence analysis was carried out to determine molecular evolutionary divergence and classify the sequences into different continent like Asia, America, and Europe etc. In addition, nucleotide substitution was evaluated in respect of different continent to understand nucleotide divergences like diversity within the continent population, inter continent population, and coefficient differentiation between two continents. The computation of mean diversity of an entire population of different species of *Pseudomonas* from Indian origin showed highest divergence (0.971) against all the continents (Table. 5).

But it was remarkable that the species of *Pseudomonas* from America attributed the low degree of diversity (0.524) resulting the least chance of getting mutated, so that American strains could be used as good antagonists against plant pathogens to prevent economic losses in the agriculture sector. Mean Evolutionary Diversity within Subpopulations, inter continent population (Asia: 0.060, America: 0.244), coefficient differentiation between two continents (Asia: 0.66, America: 0.305), evolutionary divergence over sequence pairs, average evolutionary divergence over all sequence pairs (Asia: 0.914, America: 0.971) between India and other continent were computed to calculate the molecular divergence (Table. 5)

Table.1 List of the Strain Name with Accession No and Location

Strain name	**Accession no**	**Location**
Pseudomonas sp._Canada_1	AY275488	Canada
Pseudomonas sp._Canada_2	AY275487	Canada
Pseudomonas sp._Canada_3	AY275486	Canada
Pseudomonas sp._Canada_4	AY275484	Canada
Pseudomonas sp._USA_1	DQ904601	USA
Pseudomonas sp._USA_2	DQ904594	USA
Pseudomonas syringae _ Romania_1	FR877577	Romania
Pseudomonas syringae _ Germany _2	AM084408	Germany
Pseudomonas syringae _ Canada _3	AY275478	Canada
Pseudomonas syringae _India_ 4	HM629456	India
Pseudomonas syringae _ Isreal_ 5	KF436657	Isreal
Pseudomonas stutzeri_ Belgium _1	HF675140	Belgium
Pseudomonas stutzeri_ India _2	KF532951	India
Pseudomonas stutzeri_ India_ 3	EF633260	India
Pseudomonas stutzeri_ India_4	EF633259	India
Pseudomonas aeruginosa_Pakistan_1	DQ211696	Pakistan
Pseudomonas aeruginosa_Taiwan_2	AY337580	Taiwan
Pseudomonas aeruginosa_Egypt_3	AY074896	Egypt
Pseudomonas aeruginosa_India_4	EU167929	India
Pseudomonas aeruginosa_ India_5	AY999032	India
Pseudomonas aeruginosa_India_6	AY956935	India
Pseudomonas aeruginosa_UK_7	AM293357	UK
Pseudomonas aeruginosa_UK_8	AM263499	UK
Pseudomonas aeruginosa_UK_9	AM263498	UK
Pseudomonas fluorescens_ Japan_1	U63901	Japan
Pseudomonas fluorescens_India_2	EF159157	India
Pseudomonas fluorescens_ Japan_3	U63900	Japan
Pseudomonas putida_ Vietnam_ 1	AJ310536	Vietnam
Pseudomonas putida_ India _2	HE585987	India
Pseudomonas putida_ UK_ 3	AJ276650	UK
Pseudomonas putida_Sweetzarland_4	HG313638	Sweetzarland
Pseudomonas putida_ Japan _5	AB362881	Japan
Pseudomonas putida_ Romania_ 6	FR877564	Romania
Pseudomonas putida_ Itali _7	HF546530	Itali
Pseudomonas putida_ Austria _8	HE588047	Austria
Pseudomonas putida_ Hungari_ 9	HE681203	Hungari
Pseudomonas putida_ Germany_ 10	FR851917	Germany
Pseudomonas putida_ Spain_ 11	FN995245	Spain
Azotobacter vinelandii_1	L40329	Unknown
Azotobacter vinelandii_2	EF100155	New Zealand

Pseudomonas sp. GRTM	KC169988	India
Pseudomonas sp. GATS	KC169987	India
Pseudomonas sp. GNST	KC169994	India
Pseudomonas sp. TMGR	JX094352	India
Pseudomonas sp. GTRS	KC169990	India
Pseudomonas sp. TSSG	KC169991	India
Pseudomonas sp. GTNS	KC169989	India
Pseudomonas sp. TSGR	KC169995	India

Table. 2 Maximum Likelihood Fits of 24 different Nucleotide Substitution Models

Model	#Param	BIC	AICc	InL	Invariant	Gamma	R	Freq A	Freq T	Freq C	Freq G
K2	94	2777.5	2172.5	-990.34	n/a	n/a	1.2801	0.25	0.25	0.25	0.25
T92	95	2783.6	2172.2	-989.17	n/a	n/a	1.3007	0.217	0.217	0.283	0.283
K2+I	95	2784.8	2173.4	-989.78	0.01231	n/a	1.311	0.25	0.25	0.25	0.25
K2+G	95	2785.3	2173.9	-990.02	n/a	19.2323	1.306	0.25	0.25	0.25	0.25
JC	93	2788.3	2189.7	-1000	n/a	n/a	0.5	0.25	0.25	0.25	0.25
T92+G	96	2791.4	2173.7	-988.86	n/a	19.0399	1.3283	0.217	0.217	0.283	0.283
T92+I	96	2791.7	2173.9	-988.97	0.008194	n/a	1.3127	0.217	0.217	0.283	0.283
K2+G+I	96	2793.1	2175.3	-989.67	0.011349	31.1559	1.3255	0.25	0.25	0.25	0.25
JC+I	94	2795.8	2190.8	-999.49	0.01189	n/a	0.5	0.25	0.25	0.25	0.25
JC+G	94	2796.2	2191.2	-999.71	n/a	21.2656	0.5	0.25	0.25	0.25	0.25
T92+G+I	97	2799.8	2175.6	-988.78	0.006517	23.0461	1.3339	0.217	0.217	0.283	0.283
HKY	97	2801.4	2177.3	-989.62	n/a	n/a	1.3814	0.2279	0.206	0.2477	0.3183
JC+G+I	95	2804.1	2192.7	-999.41	0.010891	37.3343	0.5	0.25	0.25	0.25	0.25
HKY+I	98	2809	2178.4	-989.16	0.011382	n/a	1.3711	0.2279	0.206	0.2477	0.3183
HKY+G	98	2809.1	2178.5	-989.19	n/a	16.3755	1.3897	0.2279	0.206	0.2477	0.3183
TN93	98	2809.8	2179.3	-989.58	n/a	n/a	1.3661	0.2279	0.206	0.2477	0.3183
HKY+G+I	99	2817.1	2180.2	-988.97	0.009902	22.8156	1.3928	0.2279	0.206	0.2477	0.3183
TN93+I	99	2817.3	2180.4	-989.09	0.011836	n/a	1.3845	0.2279	0.206	0.2477	0.3183
TN93+G	99	2817.5	2180.5	-989.17	n/a	16.7079	1.396	0.2279	0.206	0.2477	0.3183
GTR	101	2821	2171.3	-982.45	n/a	n/a	1.3177	0.2279	0.206	0.2477	0.3183
TN93+G+I	100	2825.5	2182.1	-988.91	0.010488	23.514	1.4047	0.2279	0.206	0.2477	0.3183
GTR+I	102	2828.9	2172.8	-982.18	0.009671	n/a	1.329	0.2279	0.206	0.2477	0.3183
GTR+G	102	2829.1	2173	-982.25	n/a	24.9756	1.3337	0.2279	0.206	0.2477	0.3183
GTR+G+I	103	2837.3	2174.8	-982.11	0.008565	41.6985	1.3379	0.2279	0.206	0.2477	0.3183

Abbreviations: GTR: General Time Reversible; HKY: Hasegawa-Kishino-Yano; TN93: Tamura-Nei; T92: Tamura 3-parameter; K2: Kimura 2-parameter; JC: Jukes-Cantor, BIC: Bayesian Information Criterion, AIC: Akaike Information Criterion (corrected), InL: Maximum likelihood value, G: Gamma parameter, I: Invariant parameter, R: Transition-Transversion Bias.

Table.3 Estimation of Relative Rate of Evolution of Base Substitution

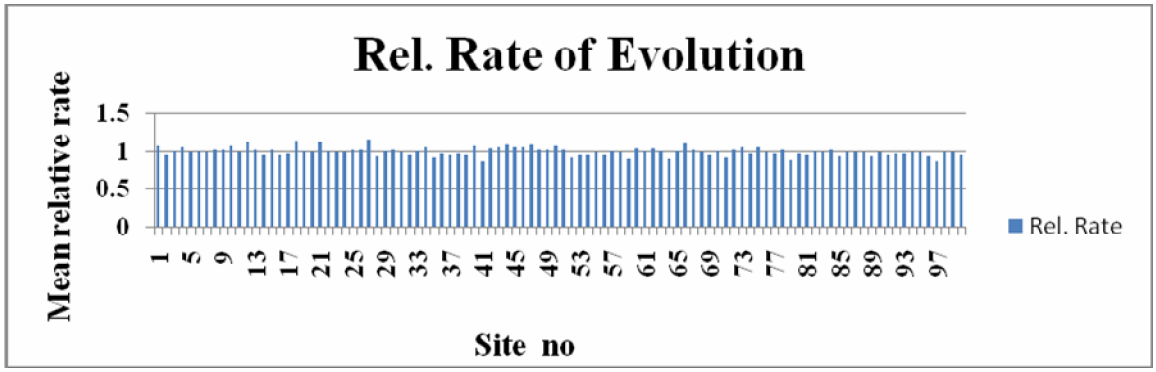


Table.4 Tajima's Neutrality Test

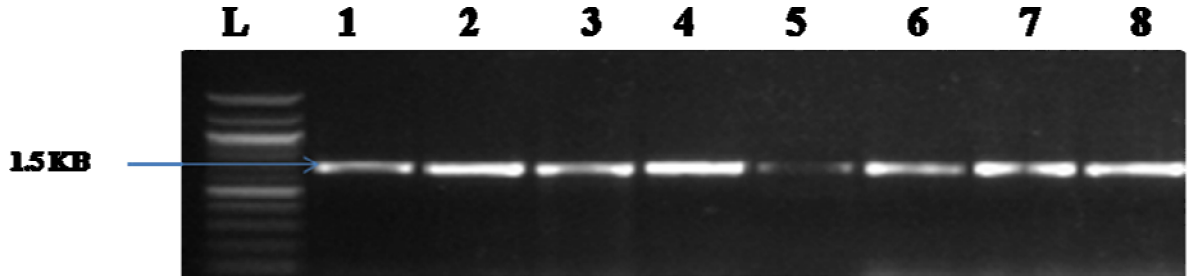
m	*S*	p_s	Θ	π	*D*
48	98	0.980000	0.220822	0.425576	3.311203

m = number of sequences, *S* = Number of segregating sites, $p_s = S/m$, $\Theta = p_s/a_1$, π = nucleotide diversity, and *D* is the Tajima test statistic.

Table.5 Estimation of different evolutionary parameters

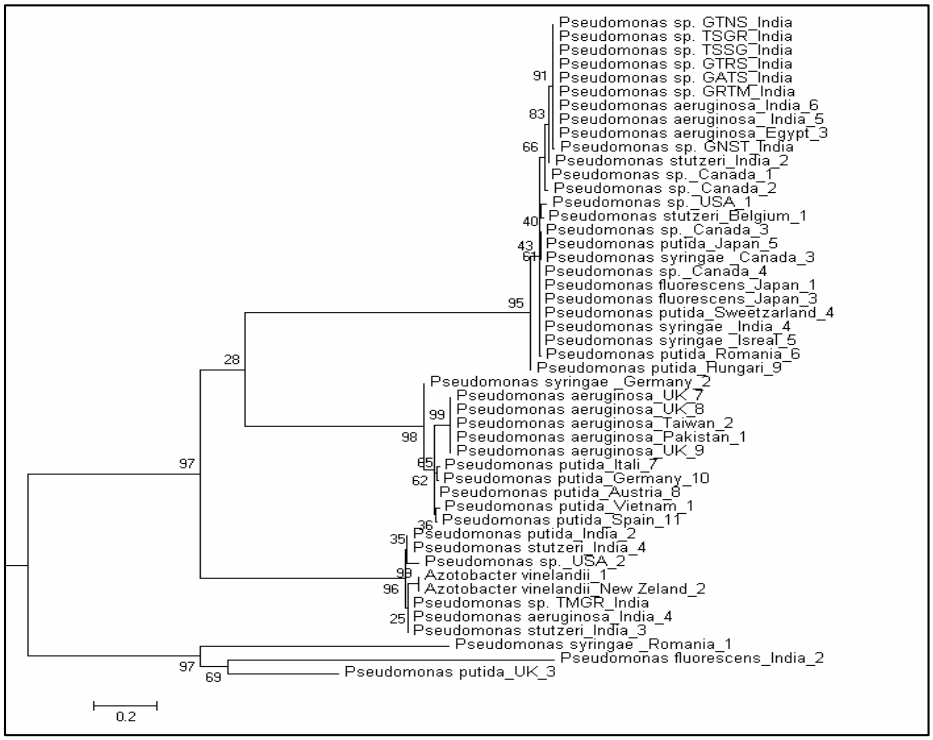
Continent/Sub-continent/*Azotobacter sp*	Diversity within a continent population	Mean Evolutionary Diversity within Subpopulations	Inter-population Diversity between two continents	Co-efficient of differentiation between two continents	Evolutionary divergence over sequence pairs between groups	Average Evolutionary divergence over all sequence pairs
India	0.971	-	-	-	-	-
America	0.524	0.836	0.244	0.305	0.879	0.971
Europe	0.857	1.103	0.094	0.085	1.179	1.001
Asia	0.871	0.854	0.060	0.066	0.901	0.914
Azotobacter sp	0.003	0.485	0.425	0.467	1.406	1.161

Figure.1 Agarose gel electrophoresis of PCR amplified *Pseudomonas sp* 1500bp 16S rDNA amplificons by 16S universal primer



Lanes were named with respective isolates; L: 100 bp molecular marker, 1: *Pseudomonas* sp. GRTM; 2: *Pseudomonas* sp. GATS; 3: *Pseudomonas* sp. GNST; 4: *Pseudomonas* sp. TMGR; 5: *Pseudomonas* sp. GTRS; 6: *Bacillus subtilis* subsp. TGNS; 7: *Enterobacter* sp. TSNG; 8: *Pseudomonas* sp. TSSG; 9: *Pseudomonas sp.* GTNS.

Figure.2 Maximum Likelihood relationships among different species of *Pseudomonas sp* in worldwide collection.



Several works had been demonstrated for considering the possible source of diversifying selection of bacteria like *Pseudomonas*. The different gene responsible for production of different protein showed diversifying selection (Smith et al, 2005; Polley and Conway, 2001; Boyd and Hart, 1998), but this study first time demonstrated diversifying selection based molecular evolution at 16S rRNA gene. Molecular evolution of Indian *Pseudomonas sp.* population was much more diverged from that of Europe and America and closely related to Asian population which strongly supported the topology of native strain.

Result also clarified American and European strains much more differ from Indian strains in respect of different molecular divergence parameter. Selection pressure supportive molecular evolution was hypothesized in this research. The selection pressure result can be explained by two possibilities either the population size might be decreasing or might have evidence for positive selection at this locus. In positive selection, mutations accumulation at different nucleotide sites was become very common in the population.

As the population of *Pseudomonas sp* had recently begun to drop off, the mutations that occur were likely to be happened and the nucleotide diversity would be created rapidly. The graphical representation of relative rate of evolution showed that a large number of site evolving faster and had tendency to be getting mutated. Therefore it is alarming in India as well as Asian country. So nationwide control measures are to be essential. The mutated ineffective strains will not be able to control the crop disease in eco-friendly manner by reducing the use of harmful chemicals, along with enhancing the productivity of different crops at reduced dose of fertilizers. Therefore it would create a new threat of pathogen infection in crop plants wherever the cultivated. In this concern, this is right time to focus our attention on the control of pathogens in West Bengal as well as India.

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References

Scarpellini M, Franzetti L, Galli A. 2004. Development of PCR assay to identify Pseudomonas fluorescens and its biotype. FEMS Microbiol Letters, 236:257–260.

Kersters K, Ludwig W, Vancanneyt M, De Vos P, Gillis M, Schleifer KH. 1996. Recent change in the classification of the pseudomonads: an overveiw. Syst Appl Microbiol., 19:465-477.

Anzai Y, Kim H, Park JY, Wakabayashi H, Oyaizu H. 2000. Phylogenetic affiliation of the pseudomonads based on 16S rRNA sequence. Int J Syst Evol Microbiol., 50:1563–1589.

Kwon SW, Kim JS, Crowley DE, Lim CK. 2005. Phylogenetic diversity of fluorescent pseudomonads in agricultural soils from Korea. Letters in Appl Microbiol., 41:417–423.

Behrendt U, Ulrich A, Schumann P, Meyer JM, Sproer C. 2007. *Pseudomonas lurida sp.* nov., a fluorescent species associated with the phyllosphere of grasses. Int J Syst Evol Microbiol., 57:979–98.

Issar S, Sharma S, Choudhary DK, Gautam HK, Gaur RK. 2012. Molecular Characterization of *Pseudomonas* spp. Isolated from Root Nodules of Various Leguminous Plants of Shekhawati Region, Rajasthan, India. Am J Plant Sci., 3:60-63.

King EO, Ward MK, Rany DE. 1954. Two simple media for the demonstration of pyocyanin and Xuorescein. J Lab Clin Med., 44:301–307.

Murray MG and Thormpson WF. 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res., 8: 4321–4326.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol Biol Evol., 28: 2731-2739.

Kimura M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol., 16:111-120.

Tajima F. 1989. Statistical methods to test for nucleotide mutation hypothesis by DNA polymorphism. Genetics, 123:585-595.

Smith EE, Elizabeth HS, Spencer DH, Rajinder K, Maynard VO. 2005. Evidence for Diversifying Selection at the Pyoverdine Locus of *Pseudomonas aeruginosa.* J Bacteriol., 187(6): 2138–2147.

Polley SD and Conway DJ. 2001. Strong diversifying selection on domains of the Plasmodium falciparum apical membrane antigen 1 gene. Genetics, 158:1505–1512.

Boyd EF and Hart DL. 1998. Diversifying selection governs sequence polymorphism in the major adhesin proteins fimA, papA, and sfaA of Escherichia coli. J Mol Evol., 47:258–267.