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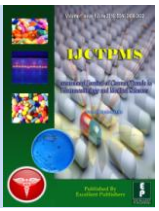

Original Research Article

Proteome Analysis of Four Immunologically Important Proteins of *Plasmodium* Species

S. Karthik[1] and S. Arumugam[2]*

[1]*Department of Biotechnology, Nandha Arts and Science College, Erode– 638 052, Tamil Nadu, India*
[2]*Department of Botany, Government Arts College (Autonomous), Salem-636 007, Tamil Nadu, India*

Corresponding author.

Abstract

In humans, malaria is mainly caused by the four species of the genus *Plasmodium* (*P. falciparum, P. vivax, P. ovale, and P. malariae*). The parasite's primary hosts and transmission vectors are female mosquitos of genus *Anopheles*, humans act as intermediate hosts. Resistance of the malaria parasite to anti-malarial drugs is increasing and becoming more widespread. The four important drug target site get resistance to various drugs. There are many proteins in *Plasmodium*; some of them are Dihydrofolate reductase (DHFR), *Plasmodium falciparum* Chloroquine Resistance Transporter (PFCRT), *Plasmodium falciparum* multi drug resistance (PFMDR) and Merozoite surface protein (MSP).The sequences of the four proteins were retrieved from NCBI Protein Data Base. The primary structure through which the amino acid composition molecular weight, isoelectric point, hydrophilic, hydrophobic, aromatic, polar and non-polar residues of proteins was identified from tools Protparam and Colorseq. The secondary structures of the proteins were identified by using the tool SOPMA which gave the helices, turns, coils and strands. The protein Sequence was then uploaded to a similarity search tool BLAST to get the similar hits from different species of *Plasmodium*. The Sequences of these hits were retrieved and uploaded to Clustal W, a multiple sequence alignment tool through which the identical and similar residues were found. In the Clustal W submission page the parameter in the tree type is changed to Phylip and the Phylip format is opened in a phylogenetic analysis tool PHYLODRAW. From PHYLODRAW by referring to pair distances, the closely related other species of *Plasmodium* to *Plasmodium falciparum* was identified.

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Abbrevations: DHFR, Dihydrofolate reductase; PFCRT, *Plasmodium falciparum* Chloroquine Resistance Transporter; PFMDR, *Plasmodium falciparum* multi drug resistance; MSP, Merozoite surface protein; SOPMA, Self-Optimized Prediction Method with Alignment.

Introduction

Dr. Ronald Ross, a British Medical Officer in Hyderabad, India discovered that mosquitoes transmitted malaria (Ito et al., 2002). Malaria remains one of the most serious tropical diseases in many parts of the world. Resistance of the malaria parasite to anti-malarial drugs is increasing and becoming more widespread. The incidence of travel-related malaria is increasing, especially in visitors to endemic African countries. Today, Malaria in the tropical world is estimated to involve 300–500 million episodes of acute illness and

more than one million deaths per year, mainly in African children (Carter and Mendis, 2002; WHO, 1999).

Causatives

Malaria is caused by protozoan parasites of the genus *Plasmodium* (Phylum: Apicomplexa). In human, four species of the *Plasmodium* parasite namely *Plasmodium falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* are responsible for malaria. In South Africa about 90-95% of the locally contracted cases are due to *P. falciparum*.

Symptoms

Symptoms of malaria include fever, shivering, arthralgia (joint pain), vomiting, anemia caused by hemolysis, hemoglobinuria, and convulsions. There may be the feeling of tingling in the skin, particularly with malaria caused by *P. falciparum*. Consequences of infection with malaria include coma and death if untreated, young children and pregnant women are especially vulnerable. Splenomegaly (enlarged spleen), severe headache, cerebral ischemia and hemoglobinuria with renal failure may occur (Pattanasin et al., 2003).

Malaria is endemic in parts of Asia, Africa, Central and South America and certain Caribbean islands; characterized by extreme exhaustion associated with paroxysms of high fever, sweating, shaking chills, and anemia. An infective disease caused by protozoan parasites that are transmitted through the bite of an infected *Anopheles* mosquito; marked by paroxysms of chills and fever (Pattanasin et al., 2003).

The blood stages of infection are responsible for all of the clinical symptoms and pathologies associated with malaria. These stages are our main focus of interest. The parasite has a complex life. When a mosquito bites a human host, sporozoites are released from the salivary glands of the mosquito into the bloodstream. Within a few minutes, the injected sporozoites invade the hepatocyte cells in the liver and after about one week they multiply into thousands of merozoites by asexual multiplication. The intermediate stage is called a trophozoite. A single merozoite gives rise to approximately 16 daughter cells, which then re-infected red cells and thereby maintain the asexual cycle. The length of the cycle determines the periodicity of the fevers and chills associated with malaria. In *Plasmodium falciparum* malaria, the parasite development in the red cell takes 48 hours. Fever occurs concomitant with release of merozoites into the blood stream, every two days (Langhorne and Anthony, 1998).

Mosquitoes

Protozoan disease, malaria is transmitted by bite of an infected female mosquito of genus *Anopheles*. The parasites primary host and transmission vector are female mosquitoes; a human acts as an intermediate. Only female mosquitoes feed on blood, thus males do not transmit the disease. Young mosquitoes first ingest the malaria parasite by feeding on a human carrier. Infected female *Anopheles* mosquitoes carry *Plasmodium* sporozoites in their salivary glands. The *Plasmodium* genus of protozoal parasites (mainly *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*) has a life cycle which is split between a vertebrate host and an insect vector. The mosquito is always the vector, and is always an *Anopheline* mosquito, although, out of the 380 species of *Anopheline* mosquito, only 60 can transmit malaria. Sexual development of *Plasmodium* begins as the merozoites invade the erythrocytes after their release from the liver. Within the erythrocyte, shizogony occurs to produce either more merozoites (taking 22 1/2 hrs in the case of *P. berghei*), or the sexual micro and macro gametocytes (taking 26 hrs). In *P. falciparum*, erythrocytic schizogony takes 48 hours and gametocytosis takes 10-12 days. Normally, a variable number of cycles of asexual erythrocytic shizogony occur before any gametocytes are produced. The immune system may produce antibodies to the gametocytes at this stage. A biting mosquito transfers about 10% of its sporozoite load. Some are destroyed by macrophages, or by antigen-specific antibodies in immune individuals, but in non-immune individuals, they reach the hepatocyte and initiate schizogeny or become hypnozoites depending on their delay trigger. All sporozoites have left the peripheral circulation system within 45 minutes.

Pathogenesis

The parasite is relatively protected from attack by the body's immune system because for most of its human life cycle it resides within the liver and blood cells and is relatively invisible to immune surveillance. The *Plasmodium falciparum* parasite displays adhesive proteins on the surface of the infected blood cells, causing the blood cells to stick to the walls of small blood vessels, thereby sequestering the parasite from passage through the general circulation and the spleen.

Diagnosis

The Preferred and most reliable diagnosis of malaria is microscopic examination of blood films, because each of the four major parasite species has distinguishing characteristics. Species identification is always based on several trophozoites.

Treatment

Chloroquine was the anti-malarial drug of choice for many years in most parts of the world. Resistance of *Plasmodium falciparum* to chloroquine has spread recently from Asia to Africa, making the drug ineffective against the most dangerous *Plasmodium* strain in many affected regions of the world. *Plasmodium falciparum* chloroquine resistance is a major cause of worldwide increases in malaria mortality and morbidity. Tests using genetic engineering techniques have isolated the part of the protein which changes to protect itself against pyrimethamine - a chemical used in anti-malarial drugs. Quadruple mutations in the *Plasmodium falciparum* DHFR enzyme give rise to the highest level of pyrimethamine resistance leading to treatment failures. *pfcrt* K76T mutation and haplotype (amino acids 72–76) and the *P. falciparum* multidrug resistance 1 mutation (N86Y) were analyzed as markers of chloroquine resistance in the DNAs of blood samples from patients with *P. falciparum* malaria in India (Vathsala et al., 2004).

Single nucleotide polymorphisms of *pfmdr1*, *pfcrt* and *P falciparum* Ca2+ ATPase gene (*pfATP6*) were assessed by PCR-restriction fragment length polymorphism (Price et al., 2004). MSP-1 has been considered as a malaria vaccine candidate. It is processed during the *Plasmodium falciparum* invasion process of red blood cells. A conserved MSP-1 C-terminal peptide was identified as a high-activity erythrocyte-binding peptide (HAEBP) termed 1585. Since conserved HAEBPs are neither antigenic nor immunogenic and decided to assess the significance of a single peptide bond replacement in 1585 (Lozano et al., 2003).

Materials and methods

NCBI

The NCBI on November 4, 1988, as a division of the National Library of Medicine (NLM) at the National Institutes of Health (NIH). NLM was chosen for its experience in creating and maintaining biomedical database. NCBI has been charged with creating automated systems for storing and analyzing knowledge about molecular biology, biochemistry, and genetics. NCBI is used to retrieve the protein sequence of DHFTR, PFCRT, PFMDR and MSP of *P. falciparum* species.

Primary structure

ProtParam: The ProtParam program predicts the various physical and chemical properties required for designing experiments for further protein analysis. It minimizes the number of experimental trials. A stable protein's instability index is smaller than 40. The relative volume of a protein occupied by the aliphatic side chains (Ala, Val, Ile and Leu) is calculated to measure the aliphatic index. The thermo stability of the protein increases with increasing aliphatic index. GRAVY, or knowledge of hydropathicity, helps in understanding the solubility characteristics of the protein.

Protein solubility helps in predicting protein structure. The primary structure prediction tool gives the physical and chemical properties of the protein. Sequences of DHFR, MSP, PFCRT and PFMDR of *Plasmodium falciparum* species are uploaded in to Protparam tool to know their consecutive molecular weight, amino acid composition and isoelectric points.

Colorseq: A primary structure analysis tool, which highlights the hydrophilic, hydrophobic, positive, negative and aromatic amino acids in red color from the query sequence. The primary structure prediction tool and the sequences of DHFR, PFCRT, PFMDR and MSP of *Plasmodium falciparum* species are uploaded to Colorseq tool.

Secondary structure

SOPMA: SOPMA is based on the homologue method of Levin et al. (1986). The improvement takes place in the fact that SOPMA takes into account information from an alignment of sequences belonging to the same family (Geourjon and Deleage, 1995). SOPMA, a secondary structure prediction tool where the sequences of DHFR, PFCRT, PFMDR and MSP of *Plasmodium falciparum* species are uploaded to know which amino acid bases tends to form helices, coils, turns and sheets in the uploaded protein sequences. It also gives the percentage of helices, turns, coils and sheets in the sequence.

Phylogenetic analysis

BLAST: BLAST is a service of the NCBI. A nucleotide or protein sequence sent to the BLAST server is compared against databases at the NCBI and a summary of matches is returned to the user. The basic BLAST algorithm can be implemented in DNA and protein sequence database searches, motif searches, gene identification searches, and in the analysis of multiple regions of similarity in long DNA sequences.

Basic Local Alignment Search Tool, which is used for similarity search. The sequences of DHTR, PFCRT, PFMDR and MSP of *Plasmodium falciparum* are uploaded to find the similar sequences that are present in the different plasmodium species and the sequences were collected to find the evolutionary relationship.

CLUSTAL W: Clustal W is a multiple sequence alignment program for DNA or protein. This is useful in comparing sequences from different sources; e.g. studying differences and similarities between sequences of the same type from different organisms, resulting in the identification of regions that are conserved from species to species. Clustal W does a pair wise comparison of every sequence first and then starts the multiple alignments with the pair of sequences that is most similar. Sequences are added one by one to the alignment based on their similarities to the starting pair.

The popularity of the programs depends on the accuracy of the results which led to robustness, portability and user-friendliness of the programs. New features include NEXUS and FASTA format output, printing range numbers and faster tree calculation. A multiple sequence alignment tool in which the similar sequences of different *Plasmodium* species have been uploaded and the tree type is changed to Phylip to get the Phylip format.

PHYLODRAW: PhyloDraw is a drawing tool for creating phylogenetic trees. PhyloDraw supports various kinds of multi-alignment programs (Dialign2, Clustal-W, Phylip format, and pair wise distance matrix) and visualizes various kinds of tree diagrams, e.g. rectangular cladogram, slanted cladogram, phylogram, free tree, and radial tree. The program can export the final tree layout to BMP (bitmap image format) and Postscript.

A phylogenetic analysis tool where the Phylip format is opened and the different forms of tree structures are viewed and the pair distance of the plasmodium species can be known from which we can know which species of plasmodium is closely related to *P. falciparum*.

Results and discussion

The present work had been focused on the immunologically important proteins of *P. falciparum*, which is one of the protozoan species responsible for malaria. The four proteins are DHFR, PFCRT, PFMDR, and MSP. The sequences of the proteins DHFR, PFMDR, PFCRT, and MSP were retrieved from NCBI protein database.

From Expasy tools, primary structure of these proteins are analyzed by using tools Protparam and Colorseq, which gives the amino acid composition, molecular weight, hydrophilic, hydrophobic, aromatic, positive, negative and hydroxyl amino acids in the sequence.

Secondary structure of these proteins are analyzed by using SOPMA tool which shows the helices, strands, coils and turns in the sequence and its composition.

Each protein was taken and performed BLAST to find the similar gene sequences that are present in different *Plasmodium* species. The similar gene sequences were then collected and performed multiple sequence alignment through Clustal W. The tree type alignment, which was obtained from Clustal W, was then changed to Phylip tree format using PHYLODRAW. The pair distance was considered to identify the closely related other *Plasmodium* species to *Plasmodium falciparum.*

Primary structure analysis of proteins

In the primary structure analysis, molecular weight and percentage of amino acids were studied (Fig. 1, Tables 1-4). The molecular weight of proteins DHFR, PFCRT, PFMDR, and MSP were found to be 71817.2, 160082.2, 48675.2 and 193719.8 respectively.

In DHFR protein, high percentage (10.5 and 10) of aspargine and lysine were recorded (Table 1). The PFCRT protein has high composition of isoleucine, leucine and phenylalanine with 10.8, 10.8 and 9% respectively (Table 2). Isoleucine (9.9%) and leucine (9.8%) were present in high composition in PFMDR (Table 3). In MSP, leucine (11.1%) and lysine (12.5) were found to be high (Table 4).

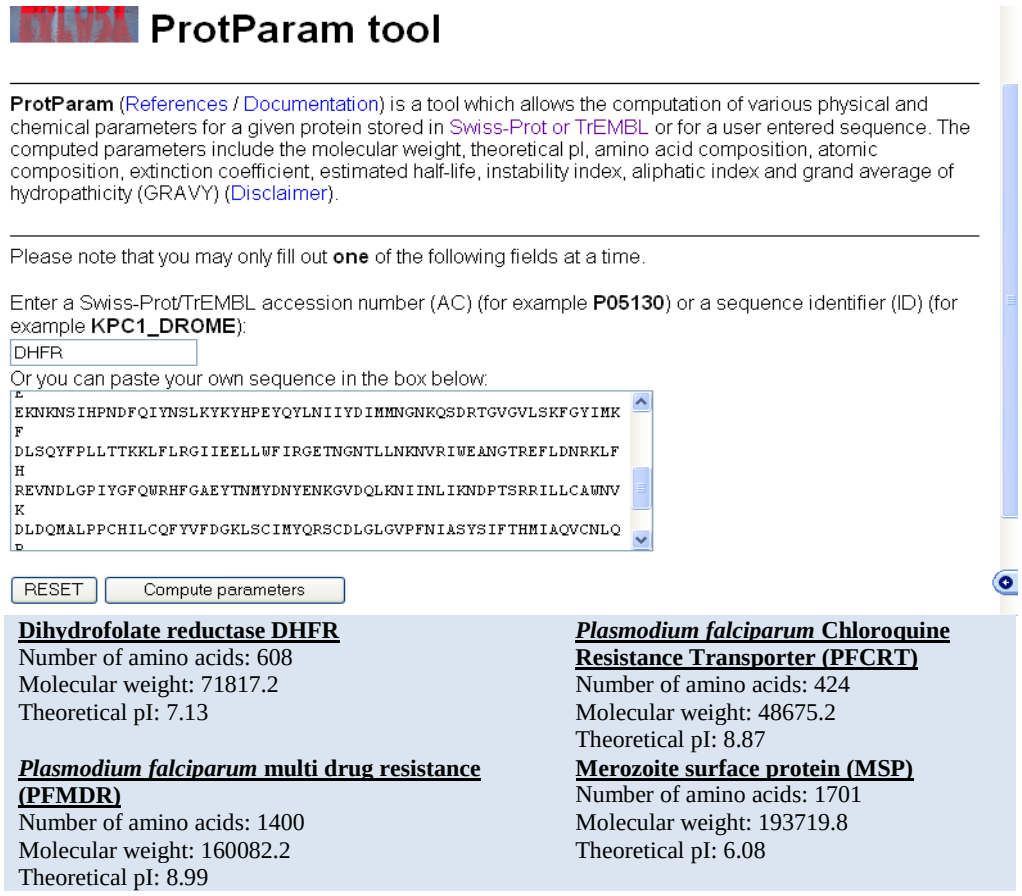

Fig. 1: The Protparam submission form in which the sequence of DHFR is uploaded for primary structure**.**

Table 1. Amino acid composition in the sequence of DHFR.

Amino acid	Single letter code	Composition in numbers	Composition percentage
Ala	A	13	2.1
Arg	R	19	3.1
Asn	N	64	10.5
Asp	D	41	6.7
Cys	C	16	2.6
Gln	Q	19	3.1
Glu	E	39	6.4
Gly	G	25	4.1
His	H	12	2.0
Ile	I	49	8.1
Leu	L	50	8.2
Lys	K	61	10.0
Met	M	17	2.8
Phe	F	34	5.6
Pro	P	19	3.1
Ser	S	25	4.1
Thr	T	26	4.3
Trp	W	6	1.0
Tyr	Y	38	6.2
Val	V	35	5.8
Total number of negatively charged residues (Asp + Glu): 80			
Total number of positively charged residues (Arg + Lys): 80			

Table 2. Amino acid composition in the sequence of PFCRT.

Amino acid	Single letter code	Composition in numbers	Composition percentage
Ala	A	18	4.2
Arg`	R	17	4.0
Asn	N	32	7.5
Asp	D	14	3.3
Cys	C	14	3,3
Gln	Q	10	2.4
Glu	E	20	4.7
Gly	G	23	5.4
His	H	5	1.2
Ile	I	46	10.8
Leu	L	46	10.8
Lys	K	27	6.4
Met	M	12	2.8
Phe	F	38	9.0
Pro	P	7	1.7
Ser	S	29	6.8
Thr	T	21	5.0
Trp	W	2	0.5
Tyr	Y	18	4.2
Val	V	25	5.9
Total number of negatively charged residues (Asp + Glu): 34 Total number of positively charged residues (Arg + Lys): 44			

Table 3. Amino acid composition in the sequence of PFMDR.

Amino acid	Single letter code	Composition in numbers	Composition percentage
Ala	A	60	4.3
Arg	R	51	3.6
Asn	N	128	9.1
Asp	D	78	5.6
Cys	C	14	1.0
Gln	Q	28	2.0
Glu	E	77	5.5
Gly	G	68	4.9
His	H	18	1.3
Ile	I	138	9.9
Leu	L	137	9.8
Lys	K	129	9.2
Met	M	34	2.4
Phe	F	83	5.9
Pro	P	28	2.0
Ser	S	123	8.8
Thr	T	65	4.6
Trp	W	6	0.4
Tyr	Y	64	4.6
Val	V	71	5.1
Total number of negatively charged residues (Asp + Glu): 155 Total number of positively charged residues (Arg + Lys): 180			

Table 4. Amino acid composition in the sequence of MSP.

Amino acid	Single letter code	Composition in numbers	Composition percentage
Ala	A	55	3.2
Arg	R	22	1.3
Asn	N	146	8.6
Asp	D	101	5.9
Cys	C	19	1.1
Gln	Q	61	3.6
Glu	E	150	8.8
Gly	G	63	3.7
His	H	29	1.7
Ile	I	111	6.5
Leu	L	188	11.1
Lys	K	212	12.5
Met	M	18	1.1
Phe	F	60	3.5
Pro	P	60	3.5
Ser	S	139	8.2
Thr	T	108	6.3
Trp	W	0	0.0
Tyr	Y	71	4.2
Val	V	88	5.2
Total number of negatively charged residues (Asp + Glu): 251			
Total number of positively charged residues (Arg + Lys): 234			

Secondary structure analysis of proteins

Using SOPMA tool, coils, helices and sheets were analyzed. In DHFR protein 38.22 % random coil and 31.09 % of alpha-helix structures were recorded (Fig. 2), whereas the PFCRT contain 30.66 % and 37.74 % of coils and helices respectively (Fig. 3). The PFMDR showed 27.43 % and 49.93 % of coils and helices respectively (Fig. 4), whereas MSP contain 31.86 % of coils and 52.67 % of helices (Fig. 5).

(blue color-helix; red color-sheet; green color-turn; magenta color-coil); **Sequence length : 608 (**Alpha helix (Hh): 189 is 31.09%; 3_{10} helix (Gg): 0 is 0.00%; Pi helix (Ii): 0 is 0.00%; Beta bridge (Bb): 0 is 0.00%; Extended strand (Ee): 125 is 20.56%; Beta turn (Tt): 61 is 10.03%; Bend region (Ss): 0 is 0.00%; Random coil (Cc): 233 is 38.32%).

Fig 2: Secondary structure analysis of DHFR protein.

(blue color-helix; red color-sheet; green color-turn; magenta color-coil); **Sequence length: 424** (Alpha helix (Hh): 60 is 37.74%; 3_{10} helix (Gg): 0 is 0.00%; Pi helix (Ii): 0 is 0.00%; Beta bridge (Bb): 0 is 0.00%; Extended strand (Ee): 107 is 25.24%; Beta turn (Tt): 27 is 6.37%; Bend region (Ss): 0 is 0.00%; Random coil (Cc): 130 is 30.66%; Ambigous states (?) : 0 is 0.00%; Other states: 0 is 0.00%.

Fig. 3: Secondary structure analysis of PFCRT protein.

(blue color-helix; red color-sheet; green color-turn; magenta color-coil); **Sequence length: 1400** (Alpha helix (Hh): 699 is 49.93%; 3_{10} helix (Gg): 0 is 0.00%; Pi helix (Ii): 0 is 0.00%; Beta bridge (Bb): 0 is 0.00%; Extended strand (Ee): 240 is 17.14%; Beta turn (Tt): 77 is 5.50%; Bend region (Ss) : 0 is 0.00%; Random coil (Cc): 384 is 27.43%).

Fig. 4: Secondary structure analysis of PFMDR protein.

(blue color-helix; red color-sheet; green color-turn; magenta color-coil); **Sequence length: 1400** (Alpha helix (Hh): 699 is 49.93%; 3_{10} helix (Gg): 0 is 0.00%; Pi helix (Ii): 0 is 0.00%; Beta bridge (Bb): 0 is 0.00%; Extended strand (Ee): 240 is 17.14%; Beta turn (Tt): 77 is 5.50%; Bend region (Ss) : 0 is 0.00%; Random coil (Cc): 384 is 27.43%).

Fig. 5: Secondary structure analysis of MSP protein.

Phylogenetic analysis

In phylogenetic analysis, the species that are closely related were identified (Figs. 6, 6a, 7, 7a, 8, 8a, 9 and 9a). In DHFR protein, the pair distance recorded was found to be high from *P. falciparum* to *P. gallinaceum* (0.99); it was recorded low in the case of *P. falciparum* to *P. yoelii* (0.96) where as it was 0.98 and 0.97 in case of *P. falciparum* to *P. berghei* and *P. falciparum* to *P. vivax* respectively (Figs. 6 and 6a). In PFCRT protein, the pair distance between *P. falciparum* to *P. chabaudi* was recorded high (0.952) and low (0.932) from *P. falciparum* to *P. yoelii*. The distance was 0.943 and 0.939 between *P. falciparum* to *P. knowlesi* and *P. falciparum* to *P. berghei* respectively (Figs. 7 and 7a). It was recorded in PFMDR protein as 0.964 from *P. falciparum* to *P. vivax*, where as it was 0.956 and 0.954 in case of *P. falciparum* to *P. chabaudi* and *P. falciparum* to *P. yoelii* respectively. The pair distance was found to be 0.948 from *P. falciparum* to *P. berghei* (Figs. 8 and 8a). In case of MSP protein, the pair distance recorded from *P. falciparum* to *P. reichenowi* was 0.9617, and 0.9612 in case of *P. falciparum* to *P. vivax* (Figs. 9 and 9a).

It is concluded that there are differences in amino acid composition and molecular weights of four proteins. Further in evolutionary aspect, they are found to be more related to other species of *Plasmodium.*

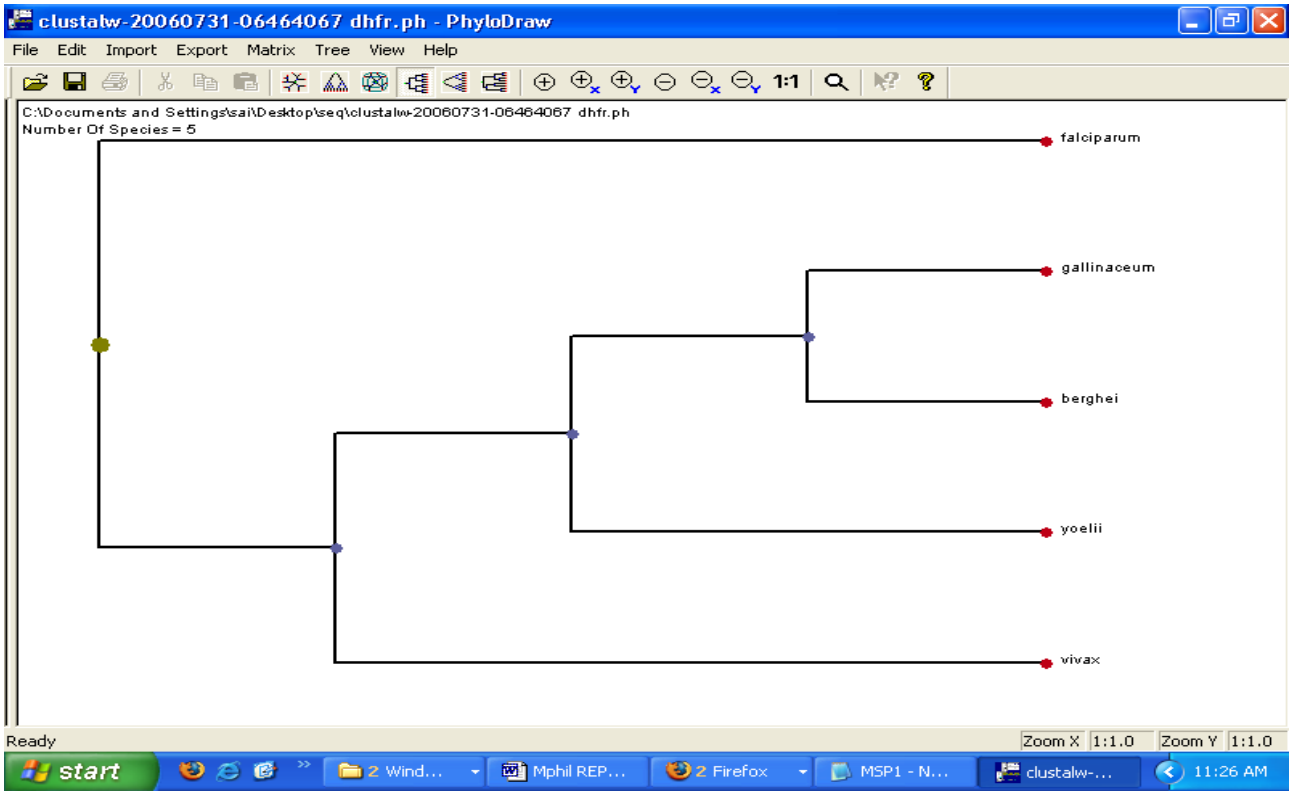

Fig. 6: The Phylip format in PhyloDraw for analysis of the tree structure viewed along with pair distances.

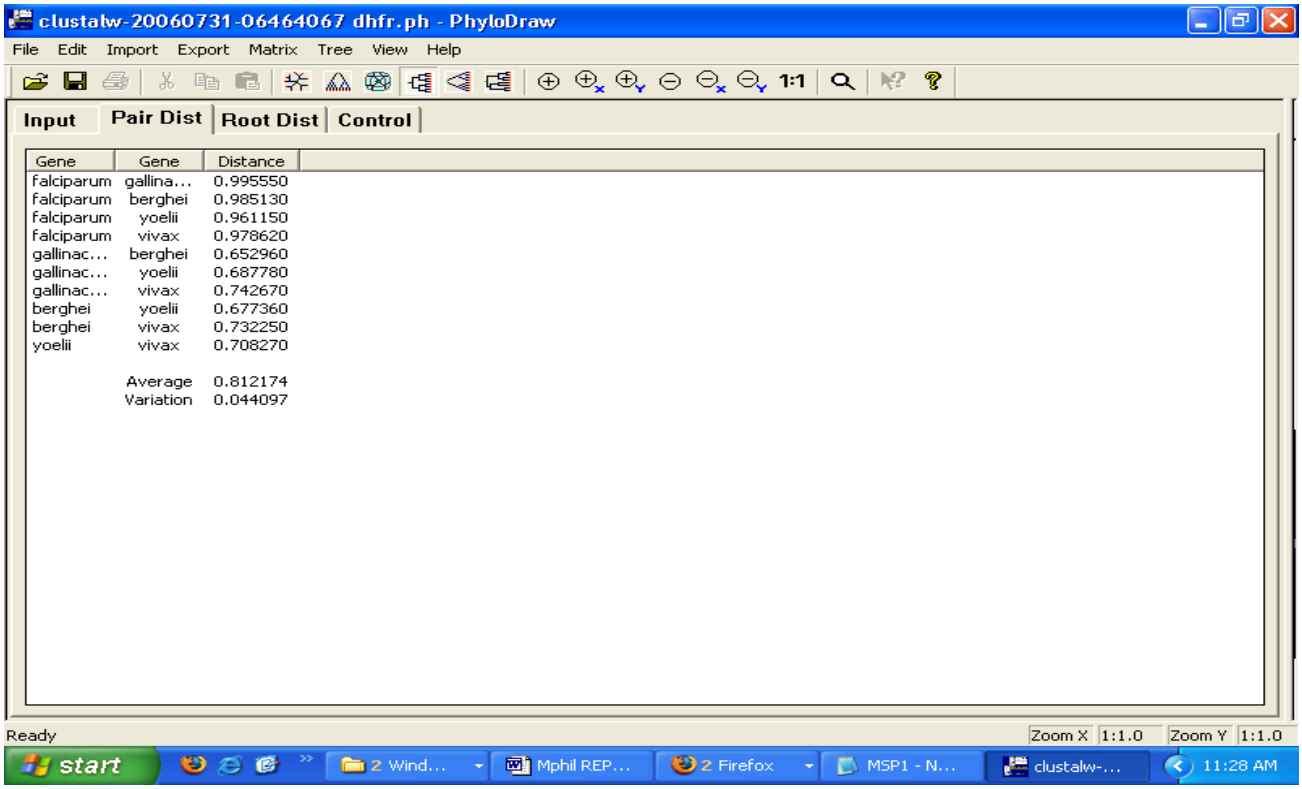

Fig. 6a: Relative pair distance.

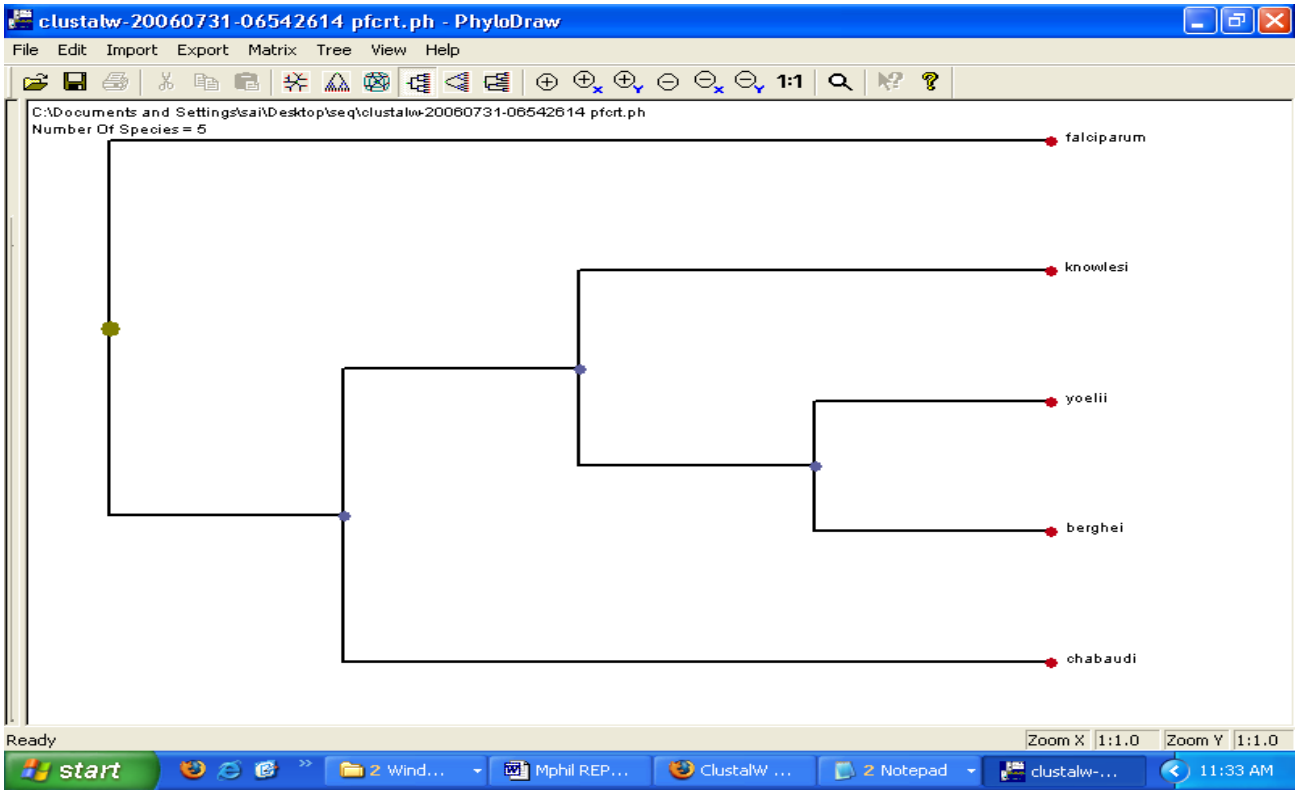

Fig. 7: The PFCRT sequence with similar sequences from different organisms got from BLAST and uploaded to ClustalW; the Phylip format is opened in the PhyloDraw to view the tree structure and pair distance.

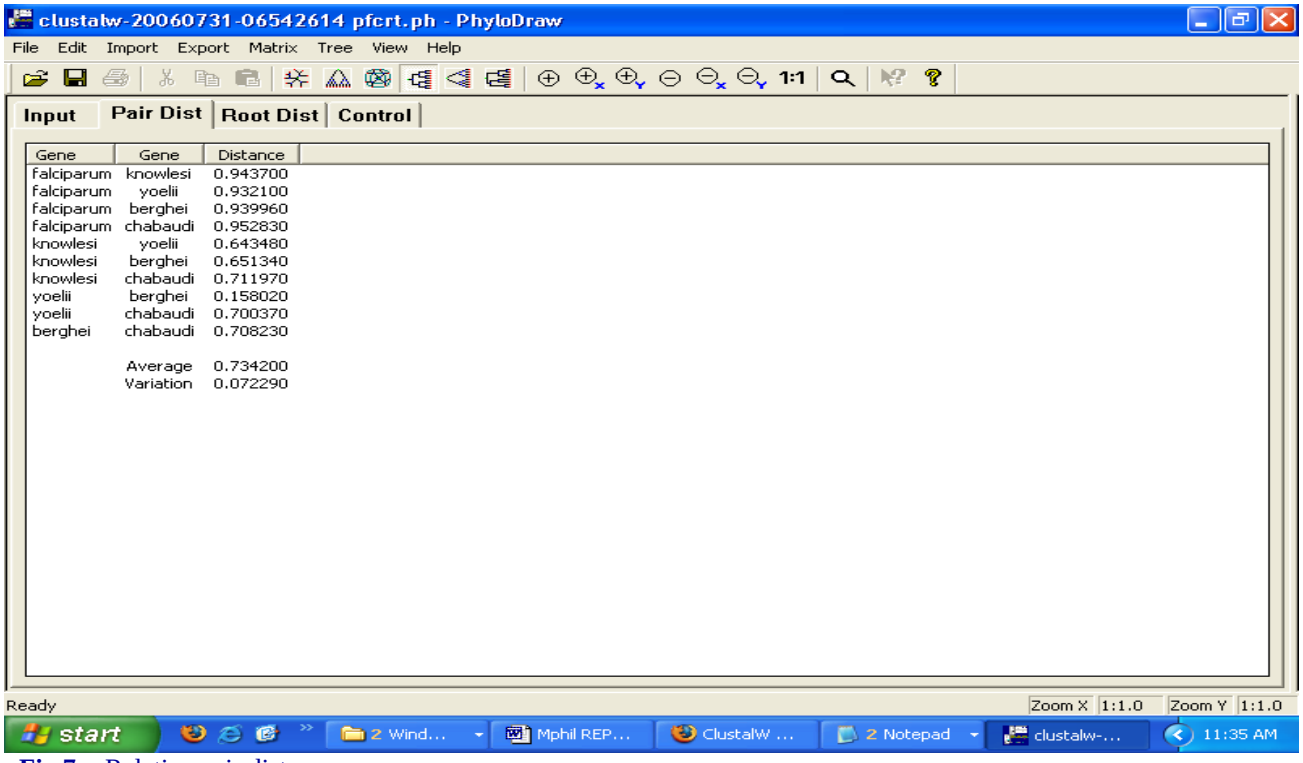

Fig 7a: Relative pair distance.

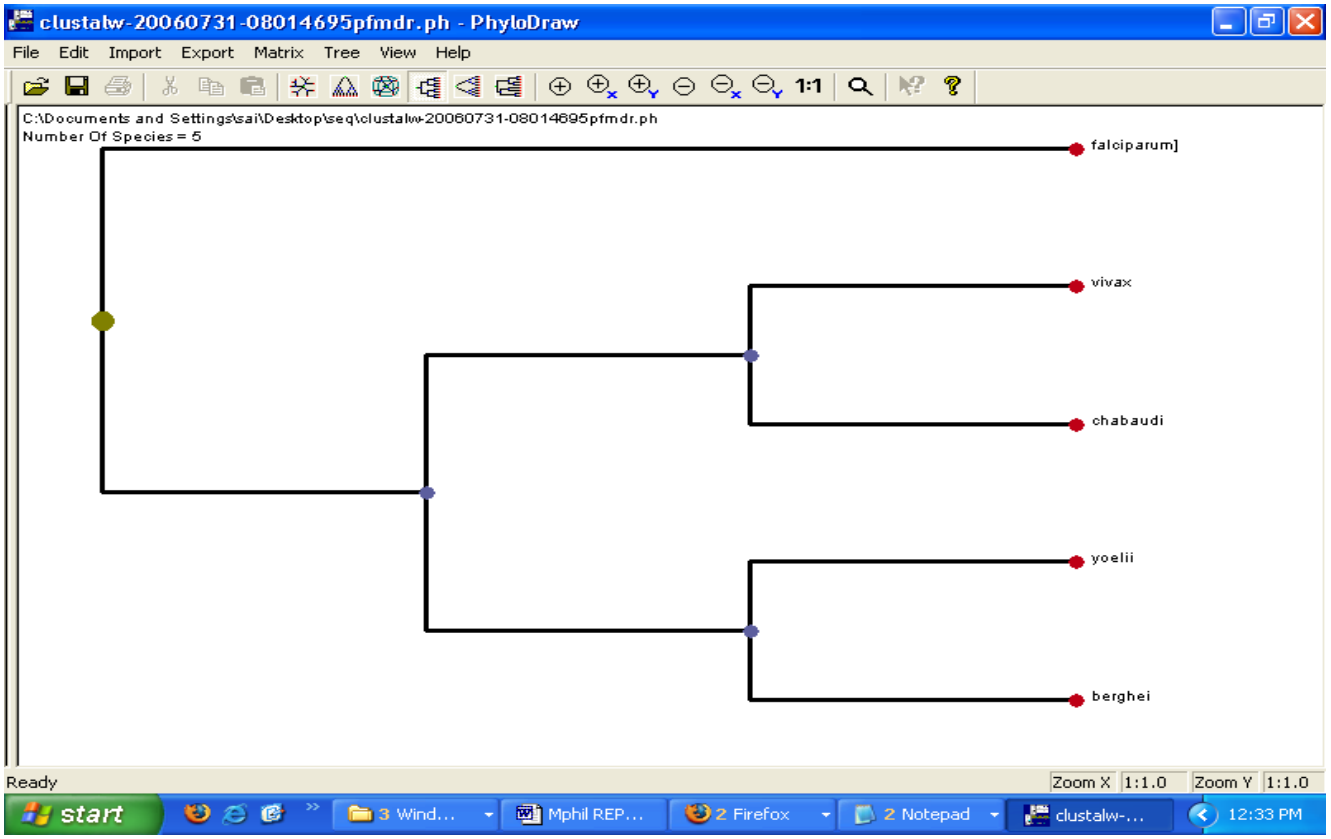

Fig. 8: The PFMDR sequence with similar sequences from different organisms got from BLAST and uploaded to Clustal W; the Phylip format is opened in the PhyloDraw to view the tree structure and pair distance.

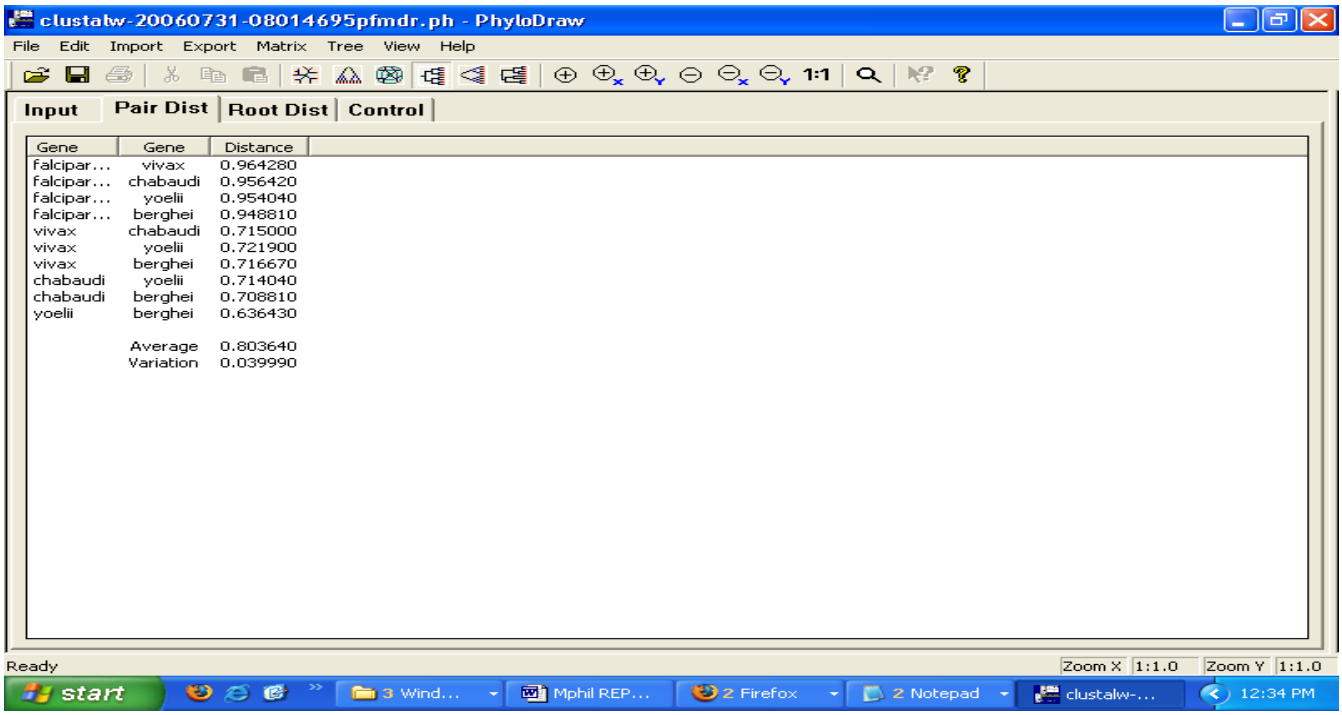

Fig 8a: Relative pair distance.

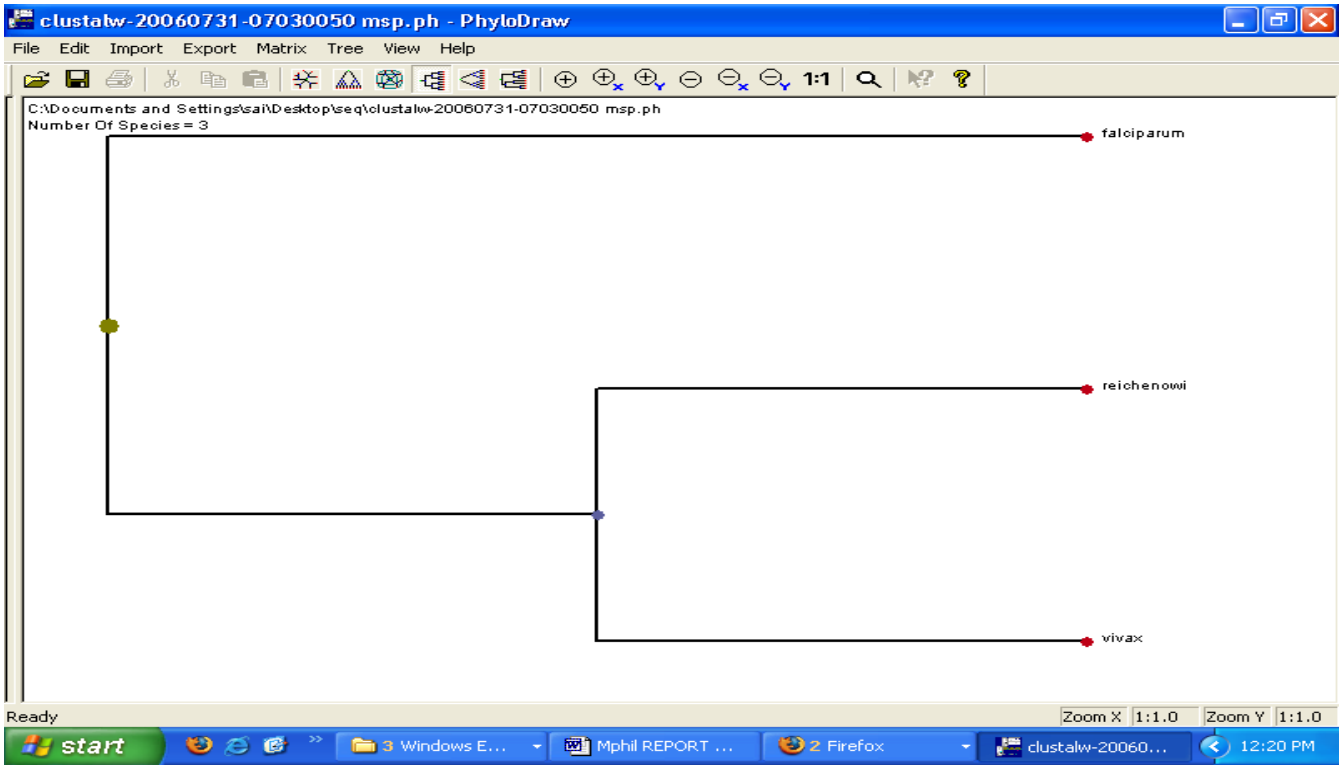

Fig 9: The MSP sequ ence with similar sequences from different organisms got from BLAST and uploaded to Clustal W; the Phylip format is opened in the PhyloDraw to view the tree structure and pair distance.

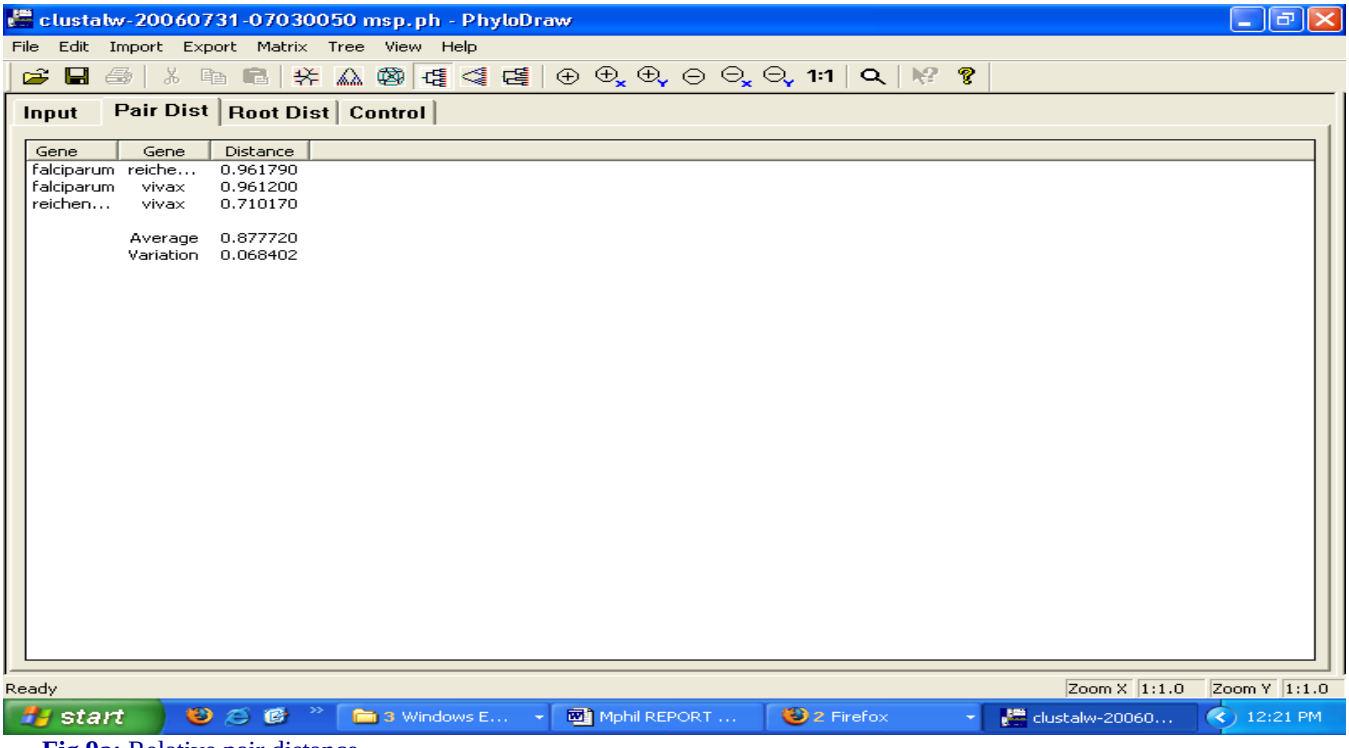

Fig 9a: Relative pair distance.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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