MOLECULAR CLONING AND CHARACTERIZATION OF PFR1 AND PFR2 GENES OF *Trypanosoma evansi*

Thesis

Submitted in partial fulfilment of the requirement for the degree

of

MASTER OF VETERINARY SCIENCE

in

VETERINARY PARASITOLOGY

By

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To
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Dated: 14th **July, 2009**

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Certified that the research work embodied in this thesis entitled **"Molecular cloning and characterization of PFR1 and PFR2 genes of *Trypanosoma evansi*"** *submitted by* **Dr. Biswa Ranjan Maharana, Roll No. 4596,** *for the award of* **Master of Veterinary Science** *degree in* **Veterinary Parasitology** *at Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate himself under my supervision and guidance.*

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Biswa Ranjan Maharana.

(Biswa Ranjan Maharana)

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Abbreviations

APS	:	Ammonium Per Sulphate
bp	:	Base pairs
DNA	:	Deoxy-ribonucleic acid
dNTPs	:	Deoxy-nucleotide triphosphates
EDTA	:	Ethylenediamine tetra-acetic acid
Fig.	:	Figure
gm	:	Gram
IPTG	:	Isopropyl-b-D-thiogalactopyranoside
IU	:	International Unit
kb	:	Kilo base
kDa	:	Kilo Dalton
LB	:	Luria Bertani
mA	:	Milli ampere
MCS	:	Multiple cloning sites
$MgCl_2$	:	Magnesium chloride
$MgSO_4$	:	Magnesium sulphate
ml	:	Milliliter
NaCl	:	Sodium chloride
NSS	:	Normal Saline Solution
OD	:	Optical density
OMP	:	Outer membarane Protein
PAGE	:	Polyacrylamide gel electrophoresis
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
PFR	:	Paraflagellar rod.
pH	:	-Log hydrogen ion concentration
RE	:	Restriction endonuclease
RNase	:	Ribonuclease
rpm	:	Revolutions per minute
SDS	:	Sodium dodecyl sulphate
TAE	:	Tris-acetate-EDTA
TBE	:	Tris borate EDTA

TE	:	Tris-EDTA
TEMED	:	n,n,n',n'- Tetramethylethylenediamine
Tris	:	Tris-hydroxy methyl aminoethane
UV	:	Ultraviolet
V	:	Volts
Viz.	:	Namely
X-Gal	:	5-bromo-4-chloro-3-indolyl-b-D- galactopyranoside

UNITS OF MEASUREMENT

µg	:	Microgram
µl	:	Microlitre
%	:	Percent
^{o}C	:	Degree Celsius
g	:	Cetrifugal force equal to gravitational acceleration
gm	:	Gram
hr	:	Hours
M	:	Molar
mg	:	Milligram
min	:	Minutes
ml	:	Milliliter
mM	:	Milli Molar
ng	:	Nano gram
N	:	Normallity
pmol	:	Picomoles
Sec	:	Second (s)
U	:	Unit(s)

Contents

Chapter I

Introduction

Trypanosomosis is one of the most important haemoprotozoan diseases of animals and human beings caused by *Trypanosoma* spp. These parasites occur principally in blood and tissue fluids of their hosts as intercellular parasites and cause a wide range of clinical manifestations. Trypanosomosis or Surra caused by *Trypanosoma evansi* is an economically important arthropod-borne disease and is widely distributed among the different livestock species in Asia, Africa, Central and South America.

Trypanosoma evansi identified by Griffith Evans (1880) while investigating Surra in camels was shown to be the first trypanosome pathogenic in mammals. It belongs to the blood and tissue-dwelling group of trypanosome species and its geographical predisposition is three times greater than the tse-tse borne trypanosome group (Woo, 1977). Unlike African trypanosomes, *T. evansi* does not undergo cyclical development within the arthropod vector and is mechanically transmitted by members of two important biting flies of the families, Tabanidae and Muscidae (Subfamily–Stomoxynae) with *Tabanus* spp. are implicated as the principal vector of the parasite. The occurrence of disease is often correlated to seasonal peaks resulting in outbreaks during the rainy season also known as "Surra seasons" and its preponderance in damp areas described as "Surra zones".

Trypanosoma evansi affects a wide range of hosts including both domestic livestock and wild animals such as cattle, buffalo, horse and camel inflicting significant economic losses (Luckins, 1908). *T. evansi* comprises a large number of morphologically identical populations that differ significantly in terms of biological characteristics, such as host range, virulence or pathogenicity and drug sensitivity (Boid *et al*., 1996). Accordingly, the clinical disease varies from host to host and region to region. The disease assumes importance in horses and camels

besides cattle, buffaloes, donkeys, mules, sheep, goats, pigs and dogs in Asia and Africa. In Indo-China, horses are mainly affected followed by camels, bovines and buffaloes (Luckins, 1998; Mahmoud and Gray, 1980); whereas in erstwhile Soviet and Middle Asia, camels are the main hosts infected followed by horses. In Africa, (Somalia, Kenya, Ethiopia, Sudan, Chad, Nigeria and French West Africa), camels are mostly affected. In Central and South America, horses are mainly affected followed by cattle (Mahmoud and Gray, 1980). According to Davila *et al*. (2003) *T. evansi* affects mainly horses and dogs in the New World. During May 2000, thirteen tigers (Pantheratigris Linn.) including twelve white tigers died in Nandankanan Biological Park,Bhubaneswar, due to an epidemic of trypanosomosis. These unprecedented deaths of wild felids due to *T.evansi* infection have attracted global attention.

Surra in India is most common in areas where the environment for breeding of the transmitting agents like tabanid flies is conducive. The incidence is higher in areas following monsoon, rains, floods and inundations. The worst affected areas in northern India are Punjab, Gujarat and Rajasthan and Uttar Pradesh. (Gill, 1991). Incidence has been reported from Andhra Pradesh, Bengal, Assam, Maharastra and Madhya Pradesh. In other states, the incidence is low. According to one estimate, the losses incidental to cameline surra in the state of Rajasthan alone account for 250 million rupees annually (Mathur, 1979). The disease is characterized by fever, anaemia, loss of body condition with consequent reduction in milk yield and capacity to work, and high rate of abortion in pregnant animals (Schillinger and Rottcher, 1986). The fatality rate in untreated horses and camels is nearly hundred percent, but the same is considerably lower in cattle and buffaloes. The survivors become carriers of the parasite for the susceptible animals. Indigenous cattle and buffaloes may act as reservoirs of infection for horses and camels.

Surra in equines is an acute and fatal disease and classified under OIE (Office Internationale des Epizooties) listed priority diseases, whereas in camels, the disease often assumes a chronic course leading to loss of body condition, abortion and agalactia (Rottcher *et al*., 1987; Faye, 1997)., Although *T. evansi* is not an important pathogen of pigs, reproductive disorders and loss of immune responsiveness to vaccine immunogens in the host are well documented (Bajyana Songa *et al*., 1987; Holland *et al*., 2003). The most significant impact possibly comes from the chronic form of the disease where abortion, infertility, reduced milk yield and weight gain and lower work output are the common hallmarks of infection.

The zoonotic potential of *T. evansi* has been largely presumptuous and overlooked. The report of two clinical cases of *T. evansi* infection in human beings in Maharashtra and West Bengal added a new dimension to the epidemiology of *T. evansi* infection (OIE, 2006). Trypanosomosis is a proven fatal disease of human beings with the death of the infected person recorded in West Bengal, India, drawing a direct correlation with "nagana" caused by *T. congolense* in Africa.(Report of the Meeting of the OIE Ad-hoc Group on non-tse-tse transmitted animal trypanosomoses [NTTAT] Paris, 22 may, 2005)

There is competition between the various variant antigenic types (VATs) in a given host to establish themselves in different microenvironments and niches, represented by different species of hosts (Seed *et al*., 1984). This leads to a characteristic latent or "cryptic" infection, which may be a specific strategy of the parasite to evade the host immune responses or may be due to the selection pressure. These cryptic infections in enzootic form are responsible for production losses in dairy herds (Otte *et al*., 1994). Semi-intensive nature of animal husbandry practices in India with scattered animal population in the form of unorganized herds pose a threat to other susceptible species (horses and camels) reared in the vicinity.

The diagnosis of *T. evansi* infection is usually based on the history of the prevalence of *T. evansi* infection and of biting flies especially tabanids; clinical symptoms; laboratory examination of blood and body fluids by direct examination, animal inoculation tests and immunodiagnostic tests. As the general clinical signs of the various forms of *T. evansi* infection are not pathognomonic, laboratory diagnostic methods are essential for confirmation of infection. Detection of chronic carrier or reservoir cases is quite challenging. Haematological and biochemical tests are not specific for *T. evansi* infection, but they reveal pathological consequences of infection. Standard trypanosome detection methods including parasite concentration and inoculation of laboratory animals are widely employed with varying degree of efficacy. Serology is an important adjunct to laboratory diagnosis for detection of specific antibody responses to infection and the antigen detection tests compliment the diagnostic techniques for active surveillance of the disease. The immunological methods based on antibody and antigen detection, however, do not have absolute predictive value. Estimates of predictive value of different serological tests indicate that enzyme- linked immunosorbent assay (ELISA) for detecting IgG antibodies is more likely to classify correctly uninfected animals and card agglutination test (CATT) is more likely classify correctly truly the infected animals. There are considerable antigenic similarities among different species of pathogenic trypanosomes making

the current serology based detection a suspect in tse-tse transmitted areas. Use of recombinant antigens, it is premised may offer precision to diagnosis in terms of specificity and sensitivity.

The DNA based detection procedures eliminate cross reactions and increase specificity and sensitivity (McLaughlin *et al*., 1996). A wide range of DNA based techniques are in use for trypanosome detection (Boid *et al*., 1996; Desquesnes and Davila, 2002), including polymerase chain reaction (PCR), random amplified polymorphic DNA (RAPD) analysis, kinetoplast DNA analysis, minicircle DNA analysis, minisatellite DNA analysis and DNA hybridization using repetitive DNA sequences. PCR and nucleic acid hybridization both offer specificity and sensitivity as applicable to large scale analysis of trypanosome samples (Hide and Tait, 1991). A simple and rapid method for detection of *T. evansi* in dromedary camels using nested PCR has also been described (Tewari, 2003; Aradaib and Majid, 2006).

The haemoprotozoan assumes a greater importance to biologists in terms of their very unique ability of evading the host immune responses by a phenomenon of antigenic variation; the molecular basis of which is not completely understood since the organism was discovered in 1880. Trypanosomes are also known as elusive trypanosomes because of their ability to escape the host immuno-evasion tactics (Borst and Rudenko, 1994; Donelson, 2003).The ability to keep one-step ahead of the host immune responses is central to their survival strategy and thereby poses serious limitations in exploitation of the conventional vaccine approaches for control of the disease. However, Shapiro (1989) provided the most comprehensive description of the theoretical and the empirical targets which may provide some rational approaches to disease management in future. Consequently, trypanosomes constitute an important model for cell biologists and veterinary parasitologists to elucidate the potential invariant antigens as futuristic vaccine targets for designing better ways of management of the disease. Therefore, the quest for potent parasite molecules to use as vaccine targets for these organisms goes unabated world over.

One of the approaches premised by Shapiro(1989) for more sustainable control of these notorious organisms is based on anti-disease approach i.e reducing the pathological effects caused by the parasite derived pathogenic molecules which may aid in moderating the disease significantly. Among other molecules, parasite derived proteases also assume importance as potential targets.Initial immunization trials using this molecule as an antigen against *T.congolense* suggest a role of "congopain"(cysteine proteinase) in immunosupression and reducing the pathogenic effect.

One of the most unique structural features of the trypanosome flagellum is the presence of a large para-crystalline filament, the paraflagellar rod (PFR), which extends alongside the axoneme from the flagellar pocket to the flagellum tip. The PFR was first described 45years ago (Vickerman, 1962b).Unlike the axoneme, which is broadly conserved among the eukaryotes, the PFR is restricted to kinetoplastids, euglenoids and dinoflagellates. PFR is vital for trypanosome motility (Bastin *et al*., 1998) and is unique among the kinetoplastids as their heteropolymers provide the building block of flagellum (Abdille *et al*., 2008).The PFR is necessary for motility and provides support for metabolic regulators that may influence flagellar movement /beating (Gadelha *et al*., 2005). This PFR is an elegant and stable lattice-like arrangement of protein filaments which is composed of two major and related proteins PFR1 and PFR2. The nucleotide sequence of PFR2 gene of *T. evansi* showed 100% homology with the sequence of the PFR2 gene of *T. brucei* and 83.4% and 76.6% similarity with that of *T. cruzi* and *Leishmania mexicana*, respectively (Adbille *et al*., 2008).

The PFR1 gene of *T. evansi* is also known to be similar to PFR1 genes *of T. brucei* and *Trypanosoma cruzi*. The expressed protein from the PFR1 gene was 68.4% homologous to the PFR2 protein of *T. evansi*. This unique protein also showed 99.8%, 87%, 77.9% and 77.5% similarity to the PFR1 protein of *T. brucei, T. cruzi, Leishmania mexicana and Leishmania major,* respectively (Adbille *et al*., 2008). It is likely that the conserved domain among various PFR genes present in kinetoplastids could be used as a target for the development of vaccine against multiple trypanosome species and calls for experimental testing. There is no work reported on the conserved PFR region of *T.evansi* from the Indian sub-continent.

With the above background information, the present investigation was undertaken with the following objectives:

1. **Molecular cloning of major components of PFR such as PFR1 and PFR2 genes of *Trypanosoma evansi* in a suitable vector host system.**
2. **Characterization of PFR1 and PFR2 genes of *Trypanosoma evansi* by restriction enzyme digestion and nucleotide sequence analysis as well as by applying bio-informatics.**

Chapter II

Review of Literature

Brief History

Trypanosomes are haemoprotozoa that occur in vertebrates, principally in their blood and tissue fluids and the disease caused by them is termed as trypanosomosis. In the Indian sub –continent, trypanosomosis is more popularly known as surra, which is a typical haemolytic disease of a wide range of warm-blooded animals caused by *Trypanosoma evansi*. The disease is a serious constraint for both livestock agriculture and human health. *T. evansi* was first isolated from the blood of infected camels and horses in India by Griffith Evans, a British veterinarian in 1880. The disease is known by various local names as per the clinical manifestation of the disease.

The genus is grouped into two divisions 'Stercoraria' and 'Salivaria' (Hoare, 1964), which is essentially based on its life cycle pattern and course of its development inside the vectors. The salivarian trypanosomes, also called African trypanosomes are unicellular eukaryotes which infect both animals and humans. The major trypanosome species of livestock in Africa are *T. congolense, T. vivax* and *T. brucei* in cattles, sheep and goats; *T. simiae* in pigs and *T. evansi* in camels. *T. vivax* assumes importance among cattle population of South America, while *T. evansi* affects camels and pigs in Asia and horses, cattle and domestic buffaloes in South America, India and South East Asia. *T. brucei gambiense* and *T. brucei rhodesiense* are the causative agents of human African trypanosomosis. The primary importance of *T. cruzi* is as a pathogen of human beings causing Chagas' disease in South America, although this stercorarian trypanosome can also cause disease in dogs.

Trypanosoma evansi is transmitted mechanically by various species of haematophagous flies, most important of which is *Tabanus* sp. and does not undergo a biological developmental

phase inside the intermediate host. An essential feature of this mechanical transmission in this organism is the interrupted nature of feeding of flies which visits from one host to the other quickly and repletes, as parasites do not survive in the proboscis of the flies for more than 10-15 minutes.

Trypanosoma evansi affects a wide variety of domestic animals primarily horse and camel as well as cattle, buffalo, sheep, goat, pig and dog (Hoare, 1972). Various wild animals such as elephant, tiger, deer, fox, jackal, etc. are equally susceptible to infection by *T. evansi* (Gill, 1991). The experimental host range includes mice, rat, guinea pig, rabbit, chicken, duck, hamster, badger, monkey, jerboa, bat, mongoose, cat, wood cat, squirrel, Indonesian Black hare, etc. (Gill, 1991).

In Africa these parasites are usually transmitted by tse-tse flies, which infest an area of about 10 million square kilometers, where the impact of the disease is greater. Outside this tse-tse area, these organisms are transmitted mechanically by blood sucking flies belonging to the genera *Tabanus, Stomoxys, Hippobosca, Lyperosia* and *Chrysops* (Soulsby, 1982; Gill, 1991). The literature on trypanosomes is exhaustive and hence, only information relevant to the present area of investigation is reviewed.

Maintenance of *T. evansi* isolates

Trypanosomes can be maintained through serial *in vivo* passage in a wide range of laboratory animal hosts and also by cryo-preservation (Lumsden *et al*., 1973). Mostly, the inbred strains of mice are preferred, as many strains do not sustain relapsing infection (Morrison and Murray, 1979). Luckins *et al*. (1978) established that the pre-patent period in mice is dose-dependent and directly related to host resistance.

Isolation and purification of *T. evansi*

Laboratory investigation on trypanosomes requires isolation of host cell-free organisms. The various methods used for separation of trypanosomes from host blood components involve use of hypotonic lysis or glycerol lysis of host blood cells, haemagglutination and differential centrifugation (Simmons *et al*., 1964), sucrose gradients (Williamson and Cover, 1966), agglutination of mice red blood cells using dog sera (Jatkar and Purohit, 1977), isopycnic isolation of trypanosomes in Percoll gradients (Grab and Bwayo, 1982) and phytohaemagglutination (Ahuja *et al*., 1985).

Purification of trypanosomes using anion exchangers (DEAE-cellulose) by chromatography (Lanham, 1968; Lanham and Godfrey, 1970; Lonsdale-Elccles and Grab, 1987) showed that this method of purification of trypanosomes has marked effects on the ATP levels within parasites and there is an increased release of variable surface glycoprotein, peptidase and phospholipase from parasites, while retaining their morphology and motility.

Antigens of trypanosomes

The feasibility of developing effective immunological control strategies for animal trypanosomosis has been investigated for many years, but no practical approach has yet emerged. Most of the serological tests for animal trypanosomosis in current use suffer from lack of specificity, well defined antigens or antibodies necessary for designing simple, accurate tests that can be easily adapted for field use (Nantulya *et al*., 1987). The tests designed for detection of trypanosomal antibodies cannot differentiate between an active infection and the past infection (Voller, 1977; Van Meirvenne and Le Ray, 1985). Some of the serological tests are not sufficiently specific to detect unequivocally the species-specific identity of the trypanosomes (Nantulya *et al*., 1987).

The antigens of trypanosomes may be broadly classified as antigens derived from coat, plasma membrane, flagellar pocket, cytosolic and nuclear regions. The variant surface antigens are highly immunogenic and present in substantial amount as variable surface glycoproteins (VSG). It is theorized that the VSG coat acts as a "smoke screen" shielding parasite membrane antigens from stimulating an immune response. The invariant surface proteins are concealed beneath the outer variant surface glycoproteins and thus remain unavailable for stimulating the host immune response, although molecules may be presented only after death/ disintegration of the organism (Overath *et al*., 1994).

The variant surface proteins or the variable surface glycoproteins undergo the process of "antigenic variation", and thereby instability is observed in the "serological characteristics" during the course of an African trypanosome population (Shapiro, 1989). The potential number of distinct variable surface glycoproteins expressed on any given trypanosome is estimated at about 100 by the currently available serological studies (Capbern *et al*., 1977) and nearly about a 1000 on the basis of molecular studies (Van der Ploeg *et al*., 1982). The molecular basis of antigenic variation as it is understood today may be thus summarized as a typical

variation resulting from switching of one of the VSG genes from a large repertoire of such genes present in the genome which is selectively transcribed and translated in the entire population of trypanosomes in a defined period of time.

The gene encoding paraflagellar rod protein 2 (PFR2) was first identified in *T. cruzi* by pre-embedding immunoelectronmicroscopy with a gold-tagged secondary antibody (Saborio *et al*., 1989), and it is believed to be a member of a multi-gene family of nearly 30 protozoan parasites. The protein with a highly organized three-dimensional structure has been found only in kinetoplastids, euglenoids, and some dinoflagellates (Clark *et al*., 2005; Bastin *et al*., 1998). Studies revealed that recombinant PFR2 from *L. mexicana* (Saravia *et al*., 2004) and *T. cruzi* (Luhrs *et al*., 2003) was strongly immunogenic in rodent models and is responsible for parasite cell motility (Clark *et al*., 2005). This striking conservation of PFR2 proteins in these parasite strains and species suggest that the PFR2 protein could be used as a common vaccine antigen, which could be effective against not only different strains within one trypanosome species but also in other species of the same genus.

Although intracellular antigens can serve as protective epitopes against various parasitic diseases (Dessein *et al*., 1988; Certa *et al*., 1988; Sher *et al*., 1989; Wain., 1994), the mechanism of how antibody mediated response against sub-cellular or sub-pellicular antigens induce protective immunity is still not clear (Li *et al*., 2007). Lubega *et al*. (2002) considered that some sub-cellular antigens in near pure form could induce a strong and protective immune response. Although the immune cells may not readily access the internal antigens, the antibodies they induce can be internalized by a mechanism not yet understood properly (Balaban *et al*., 1995).

Recombinant proteins of trypanosomes: molecular cloning and expression

Various target sequences have been cloned and expressed and the resulting recombinant proteins have been studied for their structure-function relationship in detail over last few years. Li *et al*. (2007) reported the cloning and prokaryotic expression of beta tubulin of *Trypanosoma evansi* and subsequently used the same for immunisation studies. Molecular expression and biochemical characterization of a protein of cathepsin B-like protease family of *Trypanosoma*

congolense was reported by Mendoza-Palomares *et al*. (2008). The workers studied the therapeutic and immunoprophylactic potential of this molecule. Further, the recombinant protein was used for specific diagnosis of the infection.

Katzenback *et al*. (2008) expressed beta-tubulin protein *T. danilewskyi* in prokaryotic system and used it for generating immunity to infection in gold fish. Cloning and characterization of the homologue of cyclophilin A (CypA) from *Trypanosoma brucei brucei, Trypanosoma congolense, Trypanosoma evansi* and *Trypanosoma vivax* was reported by Pellé *et al*. (2002).

Allen and Ullman (1993) cloned, characterized and expressed hypoxanthine-guanine phosphoribosyltransferase gene from *Trypanosoma brucei*.

Cloning, expression and characterization of the acyl-CoA-binding protein of different African trypanosomes were carried out (Milne and Ferguson, 2000) to study the enzyme's role in lipid metabolism. Pamer *et al* (1991) reported the prokaryotic expression and analysis of the *Trypanosoma brucei rhodesiense* full and truncated cysteine protease. Milner and Hajduk (1999) reported the cloning, expression and characterization studies of serum resistance associated protein in *Trypanosoma brucei rhodesiense*. Roggy and Bangs (1999) reported molecular cloning and biochemical characterization of the 'ATPases associated with a variety of cellular activities (AAA)' family member VCP- homologue in African trypanosomes.

Suzuki *et al*. (2004) cloned and characterized alternative oxidase (AOX) gene of *Trypanosoma vivax* and evaluated its importance in drug targeting. Phillips *et al* (1988) expressed *Trypanosoma brucei* ornithine decarboxylase in prokaryotic system to study its properties as an effective drug target. Rasooly and Balaban (2004) reported expression of trypanosome microtubule-associated protein p15 in a prokaryotic system and studied its immunogenecity potential against African sleeping sickness.

Fang *et al*. (2007) reported molecular cloning, prokaryotic expression, purification, and characterization of the *Trypanosoma cruzi* exopolyphosphatase (TcPPX) to study the biochemical functions of this enzyme. Augustine and coworkers (2006) reported molecular cloning of a *Trypanosoma cruzi* cell surface casein kinase II substrate, Tc-1 for analyzing its role in various cellular functions. Molecular cloning and characterization of the protein kinase-

a regulatory subunit of *T. cruzi* was reported by Huang *et al.* (2006). *Trypanosoma cruzi* surface molecule gp82 was expressed heterologously to study its apoptotic properties in melanoma cells (Atayde *et al.*, 2008)

Vaccination against trypanosomosis

Antigenic variation exhibited by the organism is the basis for their escape from the host defense systems (Borst and Rudenko, 1994; Donelson, 2003), and because of this, the prospects of an effective vaccine have been considered poor (Li *et al*, 2007). However, the most effective and sustainable way of controlling trypanosomosis should focus on development of a safe and cost-effective vaccine (Lubega *et al*., 2002).

Surface antigens that can be used as a vaccine against trypanosomosis include two glycoproteins namely the variable surface glycoprotein (VSG) of the blood stream forms and the procyclin of the procyclic forms (Pays and Nolan, 1998). Due to the variability of surface glycoprotein, a vaccine based on it is considered as not a feasible preposition (Lubega *et al*., 2002); likewise, procyclin has also not attracted much attention (Lubega *et al*., 2002).

The receptors for absorptive endocytosis are a class of non-varying molecules exposed on the parasite surface.Receptor mediated endocytosis by clathrin (a lattice coat of protein)coated vesicles occur in blood stream form of trypanosomes (Goldstein *et al.*, 1979).Coated vesicle receptors are usually present in very small amounts on any cell surface (Pastan and Willingham, 1981), and they are formed only in small region of the flagellar pocket of trypanosomes (Langreth and Balber, 1975). Although, these molecules may not normally induce strong protective immune response, if the isolated proteins are used as immunogens, a strong immune response is expected to be elicited.

It has been suggested that atleast some of the effects of disease, i.e. anaemia, cachexia and immunosuppression could be caused by parasite released or secreted molecules or toxins, *viz*., haemolysins, lymphocyte mitogens, inflammatory factors and hepatotoxins. When such purified/or defined molecules are used as immunogens the pathological effects of the disease would be lower and the less debilitated animal is expected to mount a stronger immune response and moderate the disease significantly against the parasite, a true anti-disease approach.

Some enzymes of African trypanosomes, *viz.*, aldolase,hexokinase (Risby and Seed, 1969,1970) and phosphohexoisomerase failed to protect mice from challenge infection (Seed, 1974) when used as immunogens. However, there is a need for concerted research attention on these enzymes as futuristic targets.

Proteases are enzymes which catalyze the hydrolysis of proteins or peptide bonds. They are present in a wide range of protozoa including trypanosomatids. In this group of flagellates, these enzymes have been implicated in host parasite interactions, intracellular survival and escape and in the processing of host proteins to fulfill nutritional requirements (North, 1982, 1991; North *et al*., 1983).Of the four proteinase types,*viz*. cysteine-,metallo-,aspratate- and serine proteases, the cysteine and metalloproteases are predominant with various activities in these group of organisms. Cysteine proteinase activities are well known in *Trypanosoma cruzi* (Bontempi *et al*., 1984), *Leishmania* sp. and *T.brucei* where they are mainly responsible for liposomal protein degradation. Although a metalloproteinase activity has been detected in *T.cruzi* (Bontempi *et al*., 1984), it is unclear whether this activity is related to the leishmanial gp 63 (Medina-Acosta *et al*., 1994). Cysteine proteinases of *Trypanosoma* sp. (trypanopain) are lysosomally located (Mbawa *et al*., 1991) and presumed to be involved in intracellular digestive and catabolic proteolysis. Additionally, the enzyme also play a role in opsonization by degrading internalysed antibody-variant surface glycoprotein complexes (Russo *et al.*, 1994). Enzymes released in the blood stream have also been proposed to contribute to pathogenesis by degrading various host proteins. A 33 kDa protein of *Trypanosoma congolense* has been found to be a major protein in infected cattle and the production of antibody to this antigen appeared to correlate with enhanced resistance to trypanosomosis. Authie *et al*. (2001) concluded that 33kDa antigen of *T.congolense* and the cysteine protease are the same molecule.

The parasite derived proteases have been identified as potential targets. Initial immunization trials, using this molecule as an antigen against *T. congolense* suggest the role of 'cangopain' (cysteine proteinase) in immunosuppression and reducing the pathogenic effects. Further protective response using formalin inactivated *T. evansi* as immunogen was demonstrated in both laboratory animals as well as experimental calves against both homologous and heterlogous challenge (Tewari, 2003).

There is growing optimism with the use of intracellular antigens as protective epitopes against parasitic diseases (Dessein *et al*, 1988; Certa *et al*, 1988; Sher *et al*, 1989; Wain, 1994). However, the question of how antibodies to subcellular or subpellicular antigens induce

protective immunity still remains to be answered (Li *et al*, 2007). Lubega *et al*. (2002) considered that some sub-cellular antigens in near pure form could induce strong and protective immune responses,and this hypothesis was later proved by immunization with native tubulin with encouraging results. Studies on the nematode *Brugia pahangi*, showed that the viability of these worms was significantly reduced by the use of anti beta-tubulin antibodies (Bughio *et al*., 1993). Complete protection has been reported using the flagellar fraction from *Trypanosoma brucei* that consisted of microtubule associated protein (MAP 52) and two glycosomal enzymes (Balban *et al*., 1995). Although the immune cells may not access the internal antigens as such, the antibodies they induce can be internalized by a mechanism not yet well understood (Balaban *et al*., 1995). There is ample evidence for the internalization theory in many works conducted on African trypanosomes (Liu *et al*, 2000; Gull, 2002; Lubega *et al*, 2002), as well as for the involvement of flagellar pocket in this process (Mkunza *et al*., 1995).

Vaccine development against animal trypanosomosis based on variant surface glycoprotein no longer holds any promise (Donelson *et al*., 1998). This has prompted researchers to investigate the alternative novel invariant proteins like paraflagellar rod proteins present in the kinetoplastid flagellum. This is a unique structure of trypanosoma flagellum due to presence of this paracrystaline structure (PFR).

Structurally, it extends alongside of the axoneme from the flagellar pocket to the flagellum tip. This PFR is an elegant and stable lattice-like arrangement of protein filaments which is composed of two major and related proteins PFR1 and PFR2.The first step towards understanding the function of the PFR was made by the demonstration that ablation of the PFR structure in *Leishmania mexicana* or procyclic *T. brucei* produced cells that were fully viable and proliferation competent but that exhibited either abnormal flagellar movement patterns (Maga *et al.,* 1999) or paralyzed flagella (Bastin *et al.*, 2000).This work demonstrated that the PFR is essential for flagellar motility. These proteins have been isolated from *T. cruzi, T. brucei* and *Leishmania* species (Beard *et al*., 1992; Deflorin *et al.*, 1994; Moore *et al*., 1996; Fouts *et al*., 1998; Gadelha *et al*., 2005).The paraflagellar rod protein 2 (PFR2) gene was first identified in *T. cruzi* by pre-embedding immunoelectronmicroscopy with a gold-tagged secondary antibody (Saborio *et al*., 1989), and it is believed to be a member of a multi-gene family of nearly 30 protozoan parasites.

This protein is responsible for parasite cell motility (Clark *et al*., 2005; Bastin *et al.*, 1998) and provides support for metabolic regulators that may influence flagellar beating. Studies revealed that the recombinant PFR2 from *L. mexicana* (Saravia *et al.,* 2004) and *T. cruzi* (Luhrs *et al.,* 2003) elicited strong immunogenicity in rodent models. Pure paraflagellar rod-like protein protected mice against *Trypanosome cruzi* infection (Ruth *et al*., 1995).

Now many ultrastuctural proteins of PFR like ADK-A (Pullen *et al*., 2004), ADK-B (Ginger *et al*., 2005), PDEB1 and PDEB2 (Oberholzer et *al., 2007*), PFR1 (Durand-Dubief *et al*., 2003), PFR2 (Bastin *et al.,* 1998), Tryp-CaM (Ridgley *et al*., 2000) have been identified out of which PFR1 and PFR2 are considered as of major significance.

PFR indirectly helps in cell morphogenesis, parasite motility, cell division and immune evasion as an important part of flagellum. Interestingly, the electron-dense plaques formed by trypanosomatids upon attachment to invertebrate epithelia contain filaments that appear similar to those of the PFR and originate in the PFR itself, leading to a testable hypothesis that the PFR may be one of the critical organelle mediating attachment to vector cell surfaces (Gadelha *et al.,* 2005).

The 100% identity of the PFR2 protein of *T. evansi* to that of *T. brucei* only reaffirms the earlier reported findings, which showed 90.2% and 83.3% amino acid sequence homology of the *T. cruzi* PFR2 protein to that of *T. brucei* and *L. mexicana*, respectively (Clark *et al*., 2005). Similarly, 100% identity between *T. b. brucei* and *T. b. gambiense* PFR2 proteins was reported within species (Gadelha *et al*., 2004). The expressed protein from the PFR1 gene was 68.4% homologous to the PFR2 protein of *T. evansi*, and showed 99.8%, 87%, 77.9% and 77.5% similarity to the PFR1 protein of *T. brucei, T. cruzi, Leishmania mexicana and Leishmania major*, respectively (Adbille *et al*., 2008).This striking conservation of PFR1 and PFR2 proteins in these parasite strains and species suggests that these could be used as a common vaccine antigen and may be effective not only against different strains within one trypanosome species but also other species of the same genus.

Chapter III

Materials and Methods

3.1 Materials

3.1.1 Culture media and antibiotics

Luria Bertani broth (Hi-Media), Luria Bertani agar (Amresco). All the antibiotics, *viz.,* ampicillin,chloramphenicol and kanamycin were purchased from DUCHEFA.

3.1.2 Reagents for DNA and RNA studies

Taq polymerase, T4 DNA ligase, DNA 100bp ladder plus ruler, RNase A, isopropyl thio β-D-galactopyranoside , 6X gel loading dye (MBI Fermentas); X-gal (Promega); calcium chloride, chloroform (Amresco); saturated phenol, EDTA (SRL, India), ethidium bromide (Hi Media), agarose (Biogene), TRIZOL (Invitrogen) ethanol, diethyl pirocarbonate (DEPC), DEPC treated NFW, chloroform, primers, MuMLV reverse transcriptase (Fermentas), RT buffer, oligo dT/ hexamer primer, RNase inhibitor (Fermentas)

3.1.3 Reagents for protein purification and analysis

Nitriloaceticacid agarose (QIAGEN); lysozyme (Roche Diagnostic GmbH); acrylamide, bis-acrylamide, ponceau S (SIGMA); TEMED, ammonium persulfate, coomasie brilliant blue R-250, sodium dodecyl sulphate (SRL, India); protein molecular weight marker, prestained molecular weight marker (MBI Fermentas); protein molecular weight marker (Bangalore Genei); dialysis sacks (HiMedia); β-mercaptoethanol (SD Fine chemicals); NiNTA agarose, imidazole (QIAGEN), Ni-NTA conjugate(Qiagen).

3.1.4 Kits

Gel extraction kit, PCR purification kit, Plasmid purification miniprep kit (Qiagen, Germany.); Blood DNA isolation kit (Qiagen, Germany).

3.1.5 Other general chemicals

Tris base, sodium dodecyl sulphate, glycine, boric acid, glacial acetic acid (SRL, India); sodium hydroxide, hydrochloric acid, citric acid, sodium chloride (SD Fine chemicals); sodium dihydrogen phosphate, disodium hydrogen phosphate, potassium dihydrogen phosphate (Merck); sodium carbonate, sodium bicarbonate (HiMedia); methanol, glycerol (Qualigens), potassium chloride (BDH), glutathione - oxidized and reduced (SRL).

3.1.6 Prokaryotic Host

$DH_5\alpha$ and BL-21(DE3) strains of *E. coli* (Invitrogen) were used for the study.

3.1.7 Vectors

pET-32a expression vector (Qiagen); pGEM-T cloning vector (Promega), pDRIVE cloning vector(Invitrogen).

3.1.8 Parasite

Izatnagar isolate of *Trypanosoma evansi* (horse strain), maintained as cryostocks in the repository of the laboratory, was used for the study.

3.1.9 Software Used

DNA STAR, Gene tool, CLC workbench 3.0, LOOPP (CBSU).

3.1.10 Glasswares and Plasticwares

The glasswares and plasticwares, used for the present study, were purchased from Borosil, Schott Duran, Axygen, Tarson, Prolab, Nalgene, Nunc, Greiner, and Eppendorff.

3.1.11 Equipment

Universal 32R centrifugation (Hettich- Zentrifugen); Soniprep 150 sonication (Sanyo); Thermo NESLAB RTE 7 circulatory water bath; Shake Temp SW22 rocking waterbath (JULABO); PTC-200 thermal cycler (MJ Research); SYNGENE gel documentation system; BD FACSCalibur machine, Lambda 25 UV/Vis spectrometer (Perkin Elmer); Olympus CH30 compound microscope; Sartorius BP 2215 weighing balance; SPINWIN microfuge spin apparatus, Horizontal gel electrophoresis apparatus and Vertical gel electrophoresis apparatus (Bangalore Genei); ELISA reader (Biorad) Thermo Orion (Model 420) pH meter, Millipore

membrane filters (0.45µm pore size; Millipore), Micropipettes (Eppendorf), Deep freezer (Vest frost), Liquid nitrogen (LN_2) container (MSA, USA), Neubauer's haemocytometer, Spectrophotometer (Nanodrop® ND 1000, USA).

3.1.12 Mice

Inbred Swiss albino mice of either sex of 6-12 weeks of age, weighing about 25-30 g, were procured from the Laboratory Animal Research (LAR) Section, IVRI. The mice were maintained under standard rearing conditions in the divisional laboratory animal shed.

3.2 Methods

3.2.1 General precautions

The following safety precautions were undertaken during the course of the present study. All equipments used in handling of the organism were washed thoroughly with water and subsequently swabbed with alcohol. All infected laboratory animals were maintained in an isolated room, with no access to mechanical vectors. All the mice cages were thoroughly washed with 30% ammonia solution before placement of subsequent batches of animals. Dead laboratory animals were immersed in 10% formalin before disposal. Sacrificed experimental animals were incinerated.

3.2.2 Maintenance of *T. evansi* infection

3.2.2.1 *In vivo* propagation in mice

A convenient infective dose and passage interval for rodents were arrived at by infectivity titration of *T. evansi* (Lumsden *et al*., 1973). This involved assessing the degree of parasitemia, and determining the working dose for routine passage by dilution of *T. evansi* infected blood obtained through tail bleeds in PBS to yield microscopically appreciable number of parasites. The passage intervals were arrived at after careful titration of the dose of the inoculum of animal infective trypanosomes.

3.2.2.2 Cryopreservation of *T. evansi*

Stabilates containing viable *Trypanosoma evansi* were set up in cryovials and stored in LN_2. *Trypanosoma evansi* infected blood was collected using heparinised syringe from mouse at teeming level of parasitemia, by cardiac puncture under chloroform anaesthesia. Freshly collected blood was mixed with equal volume of trypanosome dilution buffer containing

glycerol (TBDG-20), avoiding frothing. Approximately 0.5 -1ml aliquots of stabilate were collected in each cryovial and placed at -85^{0}C overnight following a brief cooling for 30 minutes at 0^{0}C. The material was finally transferred to $LN_{2.}$

3.2.3 Separation of host cell- free trypanosomes.

As separation of trypanosomes from host blood components is mandatory for various immunological and molecular biology applications, it was accomplished by a combination of centrifugation and anion exchange chromatography using DEAE cellulose.

Host cell-free trypanosomes were obtained by DEAE cellulose chromatography of infected blood following the method of Lanham and Godfrey (1970) with minor modifications. For activation of the net positive charge on the bead surface, the dried DEAE cellulose beads were treated with alkali and acid in close succession. Initially, the DEAE cellulose beads were swollen in N/10 NaOH solution for one hour with occasional stirring. The swollen beads were then washed with several changes of double distilled water till the pH reached to physiological one. The beads were subsequently subjected to treatment with N/10 HCL (1 g in 10 ml) for an hour with occasional stirring. The slurry was washed repeatedly with double distilled water until the pH rose above 6.0 and kept in refrigerator in a closed bottle until use.

For chromatography, the activated DEAE cellulose slurry was packed carefully into a sintered glass column (30x2.5 cm) up to a height of 8-10 cm and then equilibrated with phosphate saline glucose buffer (PSG, pH 8). The flow rate of PSG running buffer was maintained at 3-5 ml per hour.

The infected blood, collected in anticoagulant (heparin, 10 IU per ml), was diluted to a ratio of 1:3 with chilled PSG buffer (pH 8) before chromatographic separation and kept at 4^{0}C for about an hour prior to application to the column. The diluted blood was then carefully layered over the packed bead surface along the wall of the column. As the blood cells retained in the column due to electrostatic interaction between the positively charged beads and the net negative charge expressed by the cells at this pH, the organisms passed through the column and collected in the washing. The washing buffer was examined under the high power of microscope to spot the actively moving trypanosomes. The chromatography was performed at an ambient temperature of 20^{0}C and the fractionated trypanosomes were pelletted by

centrifugation at 3000 rpm for 20 min. The pelletted trypanosomes were suspended in PBS (pH 7.2) and used freshly for RNA isolation or stored at -20^{0}C for whole cell antigen preparation.

3.2.4 Enumeration of trypanosomes

The trypanosomes were enumerated following the method of Dacie and Lewis (1963). The trypanosome suspension was diluted 1:50 in Kolmer's reagent. The organisms were counted in the four corner squares of the Neubauers counting chamber at 400x magnification following loading the diluted suspension. The concentration of the trypanosomes was determined using the following formula:

Trypanosomes per ml= Total organisms in the four corner squares X dilution factor X $10^4/4$

Values were considered only if those of the two dilutions were concordant.

3.2.5 Antigen preparation

3.2.5.1 Preparation of whole cell lysate antigen

A purified suspension of trypanosome in PBS (pH 7.4) was used for preparing the whole cell lysate antigen. The suspension was subjected to rapid freezing and thawing for 5 times in liquid nitrogen, followed by 10 cycles of ultrasonication (Soniprep, Japan) at 15 amplitude each for 30 sec on ice. An interval of 30 sec was given between each cycle. Phenyl methyl sulfonyl fluoride (PMSF) was added to a final concentration of 0.1mM to the cell suspension before sonication in order to avoid proteolytic denaturation of proteins. The product was centrifuged at 13,000 rpm at 4^{0}C for 30 min. The supernatant was carefully aspirated and the protein concentration was estimated following the method of Lowrey *et al*. (1951).

3.2.6 Raising hyperimmune serum against whole cell antigen

Two New Zealand White rabbits, weighing approximately 1.5 kg each, were injected with *T. evansi* whole cell lysate antigen at the dose rate of 250 µg per animal.

The whole cell-lysate trypanosome antigen was emulsified in Freund's complete adjuvant (FCA) and used for primary immunization. Two successive booster doses were given along with Freund's incomplete adjuvant, 15days apart. Rabbits were bled after seven days of the 2nd booster; serum was collected and stored at -20^0 C for further use.

3.2.7 Isolation of total RNA of *Trypanosoma evansi*

Total RNA was extracted directly from the host cell-free trypanosomes using Trizol reagent as per manufacturer's (Gibco BRL) recommendations. One ml of Trizol reagent was added with 5-10 X 10^6 numbers of trypanosomes and lysed by repetitive pipetting. The mixture was incubated at 15-30°C for 5 min for complete dissociation of nucleoprotein complexes. This was vigorously shaken for 15 sec after adding 0.2 ml of chloroform. The mixture was centrifuged at 12,000x g for 15 min at 2-8°C which permitted separation of the phases into lower organic phenol-chloroform phase and an upper aqueous phase. The aqueous phase was transferred to a fresh tube and RNA was precipitated by keeping the tube at 15-30°C for 10 min following addition of 0.5 ml of isopropyl alcohol. Then it was centrifuged at 12,000x g for 10 min at 2-8°C. The supernatant was discarded and the RNA pellet was washed once with 1 ml of 75% ethanol prepared using diethylpyrocarbonate (DEPC @ 0.01%) treated water. The sample was mixed by vortexing and centrifuged at 7500 x g for 5 min at 2-8°C and then the RNA pellet was air-dried and redissolved in 20µl of DEPC treated nuclease-free water and stored at -70°C until further use.

3.2.8 Synthesis of *T. evansi* complimentary DNA (cDNA) by reverse transcription

Complimentary DNA (cDNA) was synthesised from the total trypanosome RNA using oligo dT primer following the standard protocol.

A 50µl RT-PCR Reaction with RNA was set up as follows:

Template RNA	15µl	(4.5 µg)
Oligo dT	2µl	(100 pM)

The mixture was heated at 70°C for 5 min and snap cooled on ice and the following was added:

RT buffer (5X)	10 µl
dNTPs (10mM)	5 µl
RNase inhibitor (40U/µl)	0.25 µl
MuMLV RT (200U/µl)	2 µl
DEPC treated NFW	15.75 µl
Total reaction volume	50 µl

The mixture was then incubated at 42°C for 1h followed by 70°C for 10 min to inactivate the RT.

The cDNA thus synthesized was quantified using spectrophotometer (Nanodrop®, USA)

3.2.9. Cloning strategy of PFR1 gene in pGEM-T cloning vector

3.2.9.1. Polymerase chain reaction (PCR) based amplification of PFR1

For PCR based amplification of the entire ORF of PFR1 gene of *T. evansi*, the following pair of primers was used:

PFR1 forward primer (FPFR) : 5' ATG GCC GCA GTT GAC GAT G 3'
(19mer, GC content 57.8%)

PFR1 reverse primer (RPFR) : 5' CTA TTC GAG GCG TGC CGG T 3'
(19mer, GC content 63.1%)

The PCR reaction was carried out in a standard 25µl reaction volume using the following reagents (Fermentas) in a 0.2 µl PCR tube (Axygen, USA).

cDNA(36 ng/ µl)	1µl
FPFR1 (20 pM/ µl)	1 µl
RPFR1 R(20 pM/µl)	1 µl
Taq polymerase buffer (10X)	2.5µl
$MgCl_2$ (25 mM)	1.0 µl
dNTP (10mM)	0.5 µl
Taq polymerase	0.25 µl
NFW Water up to	25 µl

The cycling conditions used for amplification of the PFR1 gene were as follows:

Step 1: Denaturation	94^0C for 4 min
Step 2: Denaturation	94^0C for 45 seconds
Step 3: Annealing	57^0C for 45 seconds
Step 4: Elongation	72^0c for 2 min
Step 5: Final elongation	72^0C for 15 min

The positive amplification was visualized by electrophoresis of the product on 1% agarose gel in a submarine horizontal electrophoresis apparatus (Bangalore Genei).

3.2.9.2. Agarose gel electrophoresis

Agarose gel (1%) was prepared by boiling the agarose (low EEO, Bangalore Genei) in an appropriate volume of 1X TAE. It was then allowed to cool to about 50C and ethidium bromide (10 µg /ml) was added to a final concentration of 0.5 µg/ml. About 30 ml of agarose gel was poured into the casting tray and was allowed to solidify at RT for 20 min following insertion of the comb. Prior to electrophoresis, 2 µl of 6X loading buffer (MBI Fermentas) (containing 0.25% w/v bromophenol blue, 0.25% W/V xylene cyanol FF and 40% W/V sucrose in water) was added to 10 µl of PCR product. Twelve micro-liters of the samples were loaded in each well of the gel along with a 100 bp DNA ladder plus molecular weight marker (MBI Fermentas) in a designated well. Electrophoresis was performed at 2 - 3 volts / cm^2 or at 40 V). After sufficient migration (3- 4 h), the gel was examined in a gel documentation system and the image was stored or thermal print was obtained.

3.2.9.3. Purification of the PCR product (gene coding for PFR1)

The PCR product was purified using Qiagen Mini elute gel extraction kit: QIAGEN [QIAGEN GmbH, Hilden, Germany] following manufacturer's protocol. Approximately, 150µl of PCR product was run in 1% low melting point (LMP) agarose (Sigma-Aldrich, St. Louis, USA) gel in TAE buffer along with molecular weight DNA ladder. With a clean, sharp razor blade the gel slice containing the desired DNA fragment was excised on a UV transilluminator with minimum UV exposure. The slice was then cut into small pieces and was placed into a sterile 1.5 ml microcentrifuge tube. About 0.6 ml QG buffer (supplied with the kit) was added to it and incubated at 50°C in a heated water bath for 10 min with intermediate vortexing until the gel was completely dissolved. After dissolving, the entire volume was transferred to the spin column provided and centrifuged for 1 min at 13,000 rpm. The column was washed once with 750 µl of wash buffer (PE) and centrifuged for another 1 min at 13,000 rpm. In order to remove all the traces of ethanol from the column, the empty column was centrifuged for 1 min, thereafter the DNA was eluted with 30 µl of elution buffer (EB). The quantification of the purified PCR product was done spectrophotometrically (Nanodrop®., USA). The PCR product was further confirmed by restriction enzyme analysis using Hindlll enzyme as follows.

PCR pdt	10µl
*Hind*III	1µl
Buffer Red	2 µl
Water to	20 µl

The digestion reaction was carried out at 37^0 C for 4h.

The DNA sequence coding for PFR1 gene, thus purified, was used for cloning into pGEM-T cloning vector (Qiagen, Germany) (Fig. 1).

3.2.9.4. Preparation of *Escherichia coli* DH5α competent cells

Fresh competent cells were prepared following the protocol of Sambrook *et al*. (1989). *E. coli* $DH_5\alpha$ (glycerol stock) cells were streaked on a LB agar plate and incubated overnight at 37^0C. A single colony was picked up from the plate and inoculated into 10 ml LB broth and incubated with constant shaking for overnight at 37^0C. One hundred microlitres of the overnight culture was inoculated to a fresh 10 ml LB broth and was grown until mid log phase. The culture was kept on ice for 1 h and the cells were pelleted by centrifugation at 6,000 rpm for 10 min. The cell pellet was resuspended in 1 / 3 vol of 100 mM calcium chloride and was incubated on ice for 30 min. The cells were harvested by centrifugation at 6,000 rpm and again resuspended in 2 ml of 100 mM calcium chloride. Fifteen percent glycerol was added to the cell suspension before aliquoting in a sterile 1.5 ml microfuge tube. The competent cells thus prepared were stored at - 70^0 C until use.

3.2.9.5. Ligation

Ligation reaction for cloning into pGEM-T cloning vector was carried out as follows:

pGEM®-T cloning vector	1 µl	(50 ng)
Purified PCR product (PFR1)	3 µl	(115 ng)
2X ligation buffer	5 µl	
T4 DNA ligase	1 µl	

For proper ligation, the reaction was allowed to continue at 4^0C for 16 h.

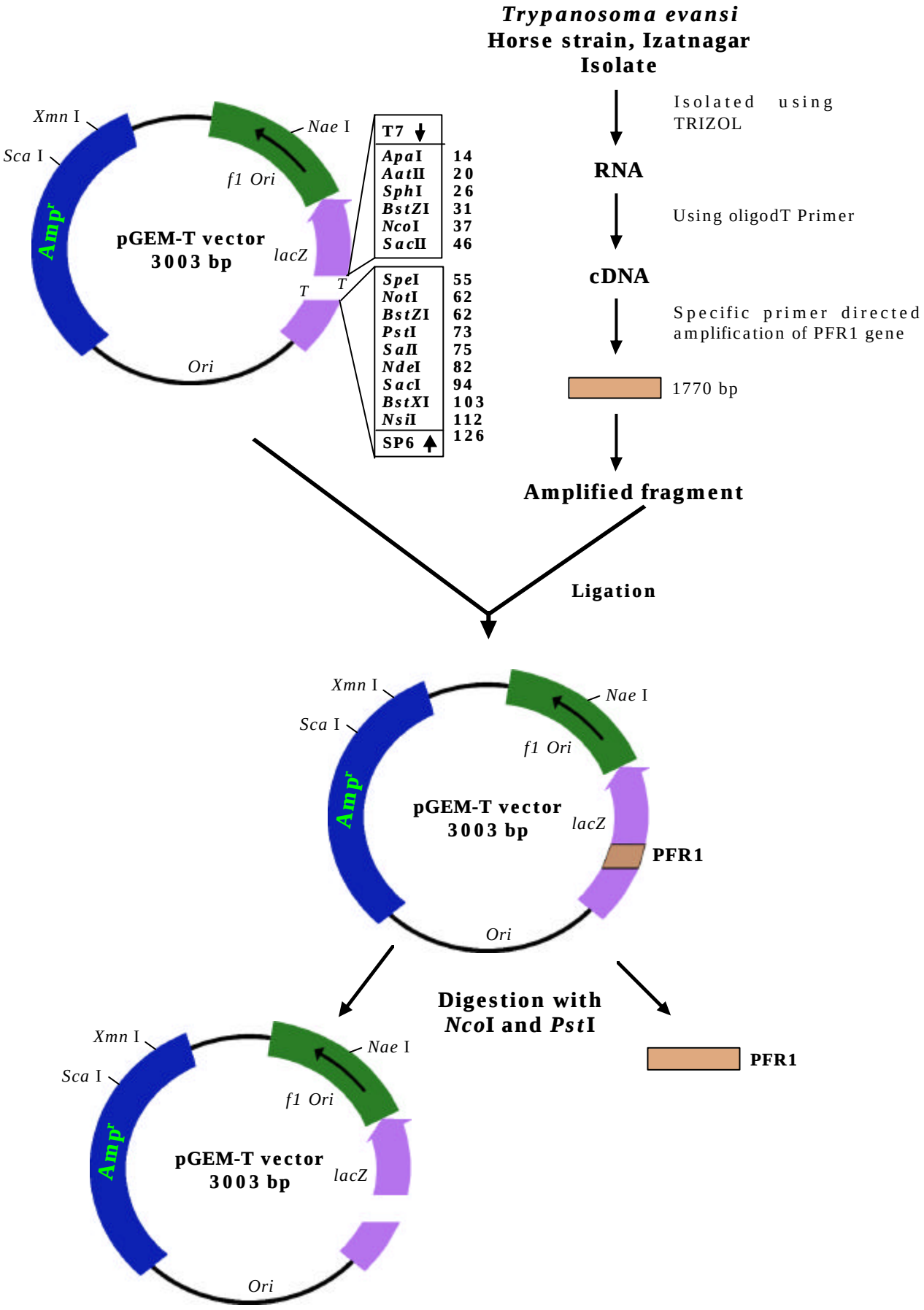


Fig.1 : Schematic diagram showing cloning of PFR1 gene

3.2.9.6. Transformation of DH_5a cells

An aliquote (250 µl) of the frozen competent cells was thawed on ice for 30 min. To this 5 µl of the ligation mixture was added and mixed gently and then further incubated on ice for 15-20min. The cells were then subjected to heat shock at 42^0 C for 90 sec, and immediately chilled on ice for 10 min. The cells were supplemented with 800 µl of fresh autoclaved LB broth and incubated for 1 h at 37^0 C. Two hundred microlitres of the transformed cells were then plated on LB agar plates supplemented with ampicillin (100 µg / ml), X-gal (20 µg / ml) and IPTG (500 µM). The plates were then allowed to dry under laminar flow and incubated at 37^0C for 12 -14 h for the development of blue and white colonies.

3.2.9.7. Colony screening for identification of positive clones

In order to identify the positive clones (containing the inserts), plasmids were isolated form the white colonies and digested with restriction enzymes (REs).

Plasmid DNA from the transformed $DH_5\alpha$ cells was isolated following standard protocol (Sambrook *et al.*, 1989). Three white colonies were picked up randomly with sterile toothpicks and were inoculated in 10 ml LB broth containing 100 µg/ ml ampicillin and grown overnight at 37^0C with constant shaking at 140 rpm. From the overnight culture, 1.5 ml was transferred to a microfuge tube and the cells were pelleted by centrifuging at 13,000 rpm for 1 min. The media was drained off completely and the pellet was resuspended in 150 µl of buffer P_1 (refer appendix). The resuspended cells were lysed gently after adding 150 µl of buffer P_2 (refer appendix) by inverting the tube 4 – 5 times. The proteins and genomic DNA were precipitated by adding 150 µl of buffer P_3 (refer appendix) and incubating on ice for 5 min. The entire suspension was centrifuged for 10 min at 4^0 C. The supernatant was then transferred to a fresh tube and equal volume of phenol: chloroform: isoamyl alcohol in the ratio of 25:24:1 was added. The tube was centrifuged for 10 min and the upper aqueous phase was transferred to a fresh tube. The DNA was precipitated by addition of 2 volumes of ethanol and incubated at -20^0 C for 2 h. The precipitated DNA was pelleted by centrifugation at 13,000 rpm for 10 min after which the pellet was washed with 70 % alcohol. The pellet was then air- dried and finally dissolved in 30 µl of TE buffer (pH 8.0) and stored at -20^0 C until use.

For confirmation of the insert in the clones, a restriction digestion reaction using *Nco*I *pst*I restriction enzymes was set up as follows:

Plasmid DNA	10µl	
Nco1	1µl	(30 IU)
Pst1	1 µl	(30 IU)
2X buffer Tango	4 µl	
Water to	20 µl	

The digestion reaction was carried out at 37^0 C overnight.

3.2.10. Characterization of PFR1 gene of *Trypanosoma evansi*

A subculture of positive clone harbouring the desired PFR1 gene was sent for custom DNA sequencing to Delhi University Dept. of Biochemistry. The sequence information received was analyzed using DNA Star and Gene Tool softwares.

3.2.11. Cloning strategy of PFR2 gene in PDRIVE cloning vector

3.2.11.1 Polymerase chain reaction (PCR) based amplification of PFR2

For amplification of the PFR2 gene the forward primer containing restriction site for *Eco*RI (TEPFR2F) and reverse primer containing *HindIII* restriction site (TEPFR2R) at the 5' were used.

TEPFR2F: 5'GGA ATT CAT GAG CGG AAA GGA AGT TGA AG 3'
TEPFR2R: 5' CCC AAG CTT CTG AGT GAT CTG CGG CAT CGT G 3'

The composition of PCR reaction mixture was as follows:

cDNA(36 ng/ µl)	1µl
TEPFR2F (20 pM/ µl)	1 µl
TEPFR2R (20 pM /µl)	1 µl
Taq polymerase buffer (10X)	2.5µl
$MgCl_2$ (25 mM)	1.5 µl
dNTP (10mM)	0.5 µl
Taq polymerase	0.25 µl
NF Water up to	25 µl

The cycling conditions were as follows:

Step 1: Denaturation	94^0C for4 min
Step 2: Denaturation	94^0C for 45 sec
Step 3: Annealing	57^0C for 40sec
Step 4: Elongation	72^0c for 2 min
Step 5: Final elongation	72^0C for 15 min

The reaction was allowed to continue for 35 cycles following which the amplified product was electrophoresed in 1% agarose gel and the confirmation of the product was done based on its size in the gel.

3.2.11.2 Purification of the PCR product

Purification of the PCR product was done using Qiagen gel extraction Kit, as per the method described in the previous section (Section 3.2.9.3).

The PCR product was further confirmed by restriction enzyme analysis using *SspI* enzyme as follows.

PCR pdt	10µl
SspI	1µl
Buffer Green	2 µl
Water to	20 µl

The digestion reaction was carried out at 37^0 C for 4h. The DNA sequence coding for PFR2 gene, thus purified, was used for cloning into pDRIVE cloning vector (Fig. 2).

3.2.11.3 Ligation

Ligation reaction for cloning into pDRIVE cloning vector was carried out as follows:

pDRIVE cloning vector	1 µl	(50 ng)
Purified PCR product (PFR2)	3 µl	(115 ng)
2X ligation buffer	5 µl	
T4 DNA ligase	1 µl	

For proper ligation, the reaction was allowed to continue at 4^0C for 16 h.

Transformation in $DH_5\alpha$ and colony selection was done, as described earlier.

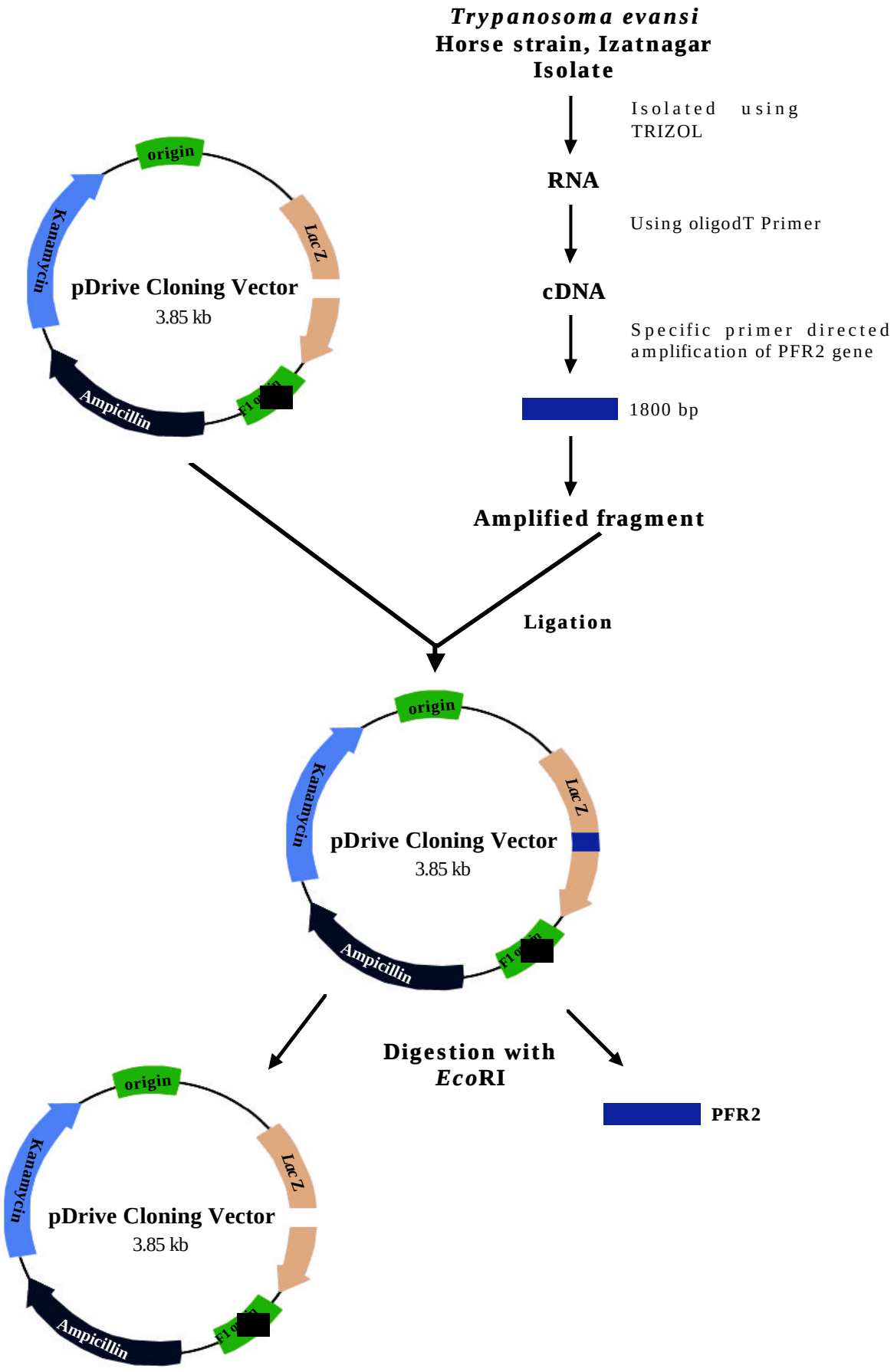


Fig.2 : Schematic diagram showing cloning of PFR2 gene

3.2.11.4 Amplification of both PFR1 and PFR2 form whole genomic DNA of *T. evani*

Amplification of both PFR1 and PFR2 were also carriedout from whole genomic DNA of *T. evansi* using specific primers with the protocols as described above in order to know the presence or absence of introns with these genes.

3.2.12 Expression of *Trypanosoma evansi* PFR2 gene in prokaryotic system

For expression of the gene in a prokaryotic expression system, pET-32a (Novagen) vector was chosen (Fig. 3). In order to clone the gene into the expression vector, the PFR2 gene was double digested with *EcoRI* and *HindIII* restriction enzymes simultaneously. The reaction mixture is constituted as follows:

Plasmid DNA	20 µl
EcoR1	2 µl
Hindlll	2µl
10X Buffer Red	4 µl
Water to	40 µl

In order to facilitate directional cloning of the gene, the expression vector was also double digested with the same enzymes. The reaction mixture contained:

pET-32a vector	20 l
EcoRI	2µl
HindIII	2µl
Buffer Red	4µl
Water to	40 µl

The reaction was carried out at 37^0C for 4 h and the digestion was checked by electrophoresis in 1% agarose gel. Following digestion, both the expression vector and the PFR2 gene were purified using Qiagen gel extraction kit, as described previously.

Directional cloning of the PFR2 gene into the pET-32a expression vector was carried out by setting up a ligation reaction as:

pET-32a vector	72 ng (4 µl)
(Double digested with *EcoRI*& *Hind*III)	

PFR2 gene (Double digested *EcoRI* & *Hind*III)	3µl
T_4DNA Ligase	1 µl
10X ligation buffer	1µl
NFW up to	10µl

The ligation reaction was carried out by incubating the reaction mixture for 16 h at 4^0C.

The transformation protocol followed was same, as described earlier with the exception that BL21 strain of *E coli* cells was used as host instead of $DH_5\alpha$ cells. The culture plate containing the colonies was used for preparing a master plate. Colonies were selected randomly and streaked on this master plate and were allowed to grow by incubating the plate overnight at 37^0C. This master plate, thus prepared, was stored safely for further screening of clones for the insert. Confirmation of the recombinant clones was done by PCR and restriction enzyme analysis.

For PCR confirmation of the clones, a direct colony PCR was performed. Half of a single colony was picked up with a sterile loop and transferred to a microfuge tube containing 30 µl of nuclease- free water. It was boiled for 10 min and immediately placed on ice for 5 min. After spinning at 13,000 rpm for 1 min to pellet the cell debris, 5 µl of the clear supernatant was used as template for the PCR reaction using TEPFR2F and TEPFR2R primers.

Restriction enzyme analysis was carried out by digesting the plasmid DNA, obtained as described earlier, with *EcoRI* and *HindIII* restriction enzymes.

Plasmid DNA	10 µl	(30 µg)
EcoRI	1 µl	
*Hind*III	1 µl	
10X Buffer Red	2 µl	
Water to make	20 µl	

The reaction was carried out by incubating the mixture at 37^0C for 4 h.

3.2.12.1 Induction of expression of the positive clones

Five positive colonies, harbouring the desired PFR2 gene, were chosen from the master plate for induction. The colonies were picked up with a sterile toothpick and inoculated in 5 ml of LB broth and kept overnight at 37^0C with constant shaking for at 140 rpm for 12- 15 h. Ten milliliters of fresh LB broth was then inoculated with 100 µl of the overnight culture and further incubated at 37^0 C with constant shaking until mid-log phase. One milliliter of the culture was collected from each tube and kept as an uninduced control. To the rest of the culture, IPTG was added at a final concentration of 1mM and kept at 37^0 C, with constant shaking at 140 rpm. One milliliter of the induced culture was collected every hour starting from 3 h onwards. All the cultures collected were pelleted by centrifugation at 13,000 rpm and kept at -20^0 C till further use.

3.2.12.2 SDS-PAGE analysis

The induced recombinant BL21 pellets collected at different hourly intervals and the controls were resuspended in 50µl of SDS-PAGE sample buffer (2X). The volume was made to 100µl by addition of autoclaved distilled water to the samples.The pellets were boiled for 10 min in a water bath in order to lyse and denature the bacterial proteins. Samples were then spun at 12,000rpm to pellet the cellular debris following which 40µl of the supernatant was analyzed on 12% SDS-PAGE (Laemmli, 1970) under denaturing conditions at 100V for 2-3 h. The gel was stained using Coomasie Brilliant Blue R-250.

3.2.12.3 Purification of PFR2 gene

The cells from 100 ml of the induced culture was pelleted and the pellet was resuspended with 5 ml of purification lysis buffer containing 8M urea (Buffer A, see appendix). The cell suspension was kept at room temperature for 1-2 h with intermittent vortexing. Subsequently, the debris was pelleted by centrifugation at 13,000 rpm for 30 min and the clear supernatant was transferred to a clean tube. Then 800 µl of Ni-NTA agarose slurry containing 2.5 mM imidazole (37.5 µl) and 20 mM β-mercaptoethanol (5 µl) was mixed thoroughly with the supernatant and kept on a rotatory shaker for 1 h with intermittent mixing. The lysate-resin mixture was then loaded on to an empty 5 ml polypropylene column (Qiagen) equilibrated

with 1X Tris-phosphate buffer (pH 8.0). The flow-through passed through the column was collected in the tube and the column was washed with 15 ml of wash buffer (pH 7.0) to which 2.5 mM imidazole (9 µl , pH 7.0) was added and finally eluted as 500 µl fractions with 4 ml of elution buffer (pH 4.2-4.5).

Later larger cultures of 1-2 liters were set up for production of bulk quantity of the recombinant proteins.

3.2.12.4 Renaturation of the protein

Renaturation of the denatured recombinant protein (in 8M urea) was achieved by dialysis of the eluted product using a dialysis bag (10 kDa cut-off) against decreasing molar concentration of urea as 6M, 4M and 2M in tris-saline-EDTA (TSE) buffer (pH 7.2) for 3 h interval each in 6M and 4M concentration with periodic mixing and overnight in 2M urea concentration at 4°C. Thereafter, it was kept in TSE buffer for 3 h at 4°C followed by keeping finally in Tris-saline for 3 h . Any debris formed during the renaturation was removed by centrifugation at 10,000 rpm for 10 min in refrigerated centrifuge. The purity of the protein was checked by electrophoresis on SDS-PAGE using 12% gel, as described earlier. The protein was aliquoted in 0.5 ml aliquots and stored at 4°C till use.

3.2.12.5 Concentration of renatured protein

The protein was concentrated to about 50% of the initial volume using a vacuum freeze drier.

3.2.12.6 Western blot analysis of the recombinant PFR2 gene

The specific reactivity of the recombinant PFR2 protein thus expressed was checked by Western blotting. About 500 ng of the purified recombinant protein was loaded and analysed on SDS-PAGE. The resolved protein was subsequently transferred to a nitrocellulose membrane using a blotting apparatus (Bio-Rad) in 50nm Tris base, 380mM glycine, 0.1% SDS, 20%methanol at 100mA constant current for 3 h. Successful transfer of the protein to the membrane was confirmed by staining the membrane with Ponceau's stain. The stain was removed by washing the membrane 2-3 times with TBS buffer. The unbound surface of the membrane was blocked overnight with 3% skimmed milk in TBS, at 4°C. Following washing

with TBS-Tween (0.05%) 3 times for ten minutes, Ni-NTA anti-histidine HRPase conjugate (Qiagen) was added at 1:1000 dilution and incubated at 37^0C for 1 h. The membrane was washed 3 times with TBS-Tween and subsequently developed with DAB solution (Bangalore Genei).

3.2.12.7 Analysis of protein concentration

Following purification, the concentration of the recombinant protein was assayed by modified Lowry protein assay kit (Pierce, USA) according to the manufacturer's protocol. Five different dilutions of albumin standards (*viz*, 25, 125, 250, 500 and 750 µg/ml) were prepared from the stock bovine serum albumin (concentration 2.5 mg/ml)with PBS, pH 7.4. About 40 µl of each standard along with the dialyzed recombinant PFR2 and blank standard (PBS) in replicate were placed into the wells of a microtitre plate. Immediately 200 µl of modified Lowry's reagent was added to all the wells and the plate was incubated for 10 min at RT under cover. Twenty microlitres of 1X Folin & Ciocalteu's phenol reagent was added to each well, the contents were mixed properly and the plate was incubated for 30 min at RT. Absorbance was measured at 750 nm. Average OD of the blank standard replicates was subtracted from the other individual standards as well as the recombinant PFR2 protein. Standard curve was prepared by plotting the average blank corrected values for each BSA standard versus its concentration in µg/ml. Concentration of the recombinant protein was determined from the standard curve by placing its OD value.

3.2.12.8 Dot-blot analysis of the recombinant PFR2 protein

Strips of nitrocellulose membrane (0.45 µm) were used to dot-blot the PFR2 recombinant protein of *T. evansi*. About 5 µl of antigen was dot-blotted per well. Antigen dotted strips were air-dried and placed in a clean petri dish (Axygen). All steps were performed at room temperature. Three percent non-fat milk powder in PBS was used as blocking agent and the strips were incubated at RT for 2 h, washed thrice with PBS-T. Hyperimmune serum raised in rabbit was used in 1:100 and 1:200 dilutions for checking the reactivity of the protein. The strips were incubated for 1 h at 37°C followed by which washing, as described above. Subsequently, the membrane strip was washed three times with PBS-T and incubated with

anti-rabbit HRPO conjugate (1: 10000 dilution) (Sigma) at 37oC for another 1 h. The membrane was given a final wash with PBS-T thrice and subsequently developed with DAB solution (Biomatik) in dark. The strips were checked at 1 min interval and the reaction was stopped by dipping strips in distilled water as soon as the colour developed. The immunoreactivity was also checked with Ni-NTA anti-histidine HRPase conjugate (Qiagen), as described previously.

Chapter IV

Results

Maintenance of *T. evansi* in mice and its harvesting

The passage dose and interval for rodents was determined by infectivity titration of *T. evansi* (Lumsden *et al*., 1973). The method consisted of microscopic examination of peripheral wet blood film at magnification of 400X following experimental infection in mice and assessing the degree of parasitaemia. The working dose for routine passage was arrived at by dilution of *T. evansi* infected blood through tail bleeds in PBS that yielded 11-20 parasites per microscopic field; so as to obtain at least 10^4- 10^5 organisms for intraperitoneal inoculation of naïve mice. The passage interval determined was 3-4 days. Within 3 to 4 days post- infection (PI), all the infected mice showed symptoms of trypanosome infection. The signs included severe inanition with anorexia. Mice were used for harvesting the organisms once the teeming level of parasitaemia was confirmed by wet blood film examination.

The blood collected by cardiac puncture of infected mice was then used for obtaining cell-free trypanosomes by DEAE-cellulose chromatography (Fig. 4).

Isolation of total RNA from *T. evansi* and cDNA synthesis

Total RNA from *T. evansi* was isolated from the trypomastigotes by Trizol reagent based extraction method (Fig.5). The final concentration of total RNA was determined as 0.3µg/µl by machine UV spectrum assay using Nanodrop®. Complementary DNA (cDNA) was synthesized from the total RNA using reverse transcriptase. The integrity of cDNA was checked by agarose gel electrophoresis. Based on UV spectrum absorbance at 260 nm, quantification of the cDNA was done which was calculated as 36 ng / µl.

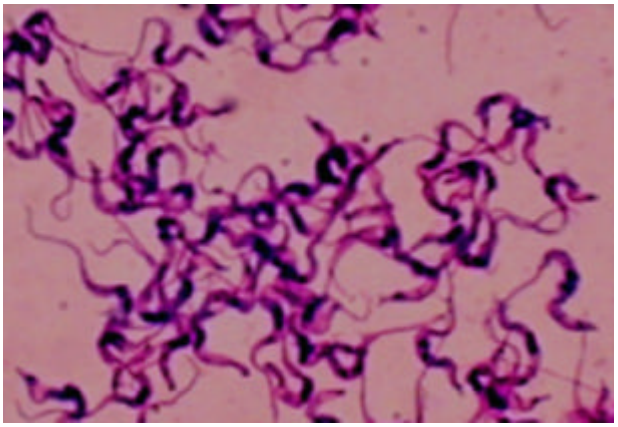


Fig.4 : Cell free trypanosomes

Fig.5 : RNA of *T. evansi*

Cloning of PFR1 gene and its characterization

The coding sequence of *T. evansi* PFR1 gene was retrieved from GenBank (Accession No: EU366960). The entire ORF of PFR1 gene was amplified from the Izatnagar isolate of *T. evansi* using specific forward and reverse primers. The specificity and size of the amplified product was checked by agarose gel electrophoresis and a single band of 1770 bp was resolved (Fig. 6). The amplified product was purified from the PCR contaminants using a commercial gel extraction kit (Qiagen, Germany). The purity of the eluted product was re-checked by electrophoresis on 1% agarose gel. The PCR product was further confirmed through restriction analysis with *HindIII* enzyme. After digestion two bands were obtained at 1003 bp and 767 bp as it contains the *HindIII* site at 1003 bp as per the analysed result of published sequence using DNA STAR and GENE TOOL soft ware (Fig. 7). The concentration of the purified PCR amplicon was measured as 35.2 ng/µl. This purified product of 1770 bp was used for ligation in a T/A cloning vector to facilitate sequencing and characterization. For this, pGEM-T T/A cloning vector, having an MCS (Multiple cloning sites) incorporated into a LacZ a peptide coding region, was chosen for easy selection of recombinant clones in pGEM-T cloning vector. Selection of *E. coli* DH5α positive colonies was done by blue-white colony screening method (Fig. 8).

Plasmids were isolated from the overnight grown cells of the positive clone and were subjected to double restriction digestion using *NcoI* and *Pst*I enzymes. The release of the insert of desired length was checked by agarose gel electrophoresis of the enzyme digested product (Fig.9).The size of the insert was 1806 instead of 1770 as *NcoI* and *Pst*I digest the vector at 37bp and 73 bp, respectively. Colony PCR further confirmed the positive clone (Fig.10). The positive clone was custom sequenced for nucleotides. The nucleotide sequence revealed 99.9% homology and only one nucleotide change at 867bp of PFR1 ORF between the Izatnagar and China isolates. The nucleotide sequence also showed 99.8%, 82.3% and 79.8% homology with *T.brucei, T.cruzi* and *C.fasciculata,* respectively (Fig.11, 12, 13). The full coding sequence of *T. evansi* PFR1 is presented in Table. 1**.** The mature PFR1 protein comprised of 590 amino acids. The deduced amino acid sequence of *T. evansi* PFR1

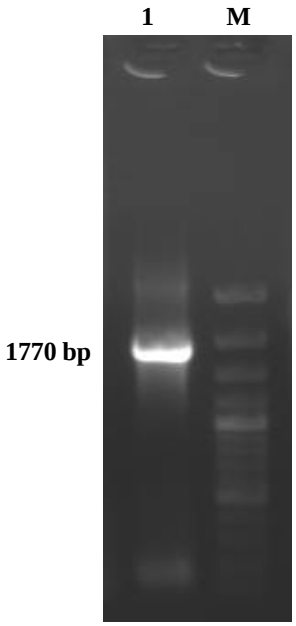


Fig.6 : Specific PCR amplification of PFR1 gene of *T. evansi* using cDNA as template

Lane M : 100 bp DNA ladder plus

Lane 1 : Amplicon of 1770 bp from cDNA template

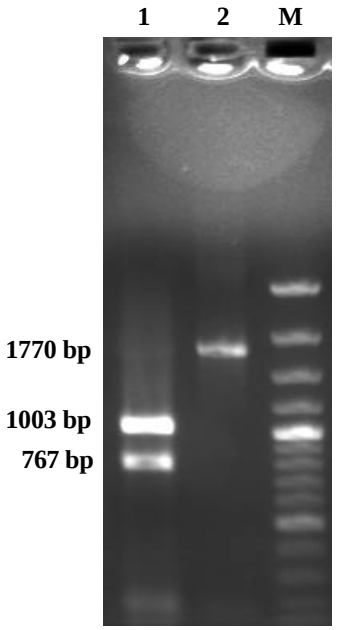


Fig.7 : RE analysis for confirming PFR1 gene

Lane M : 100 bp DNA ladder plus

Lane 1 : *Hind* III digested PCR pdt of *T. evansi*

Lane 2 : Uncut PCR pdt of *T. evansi*

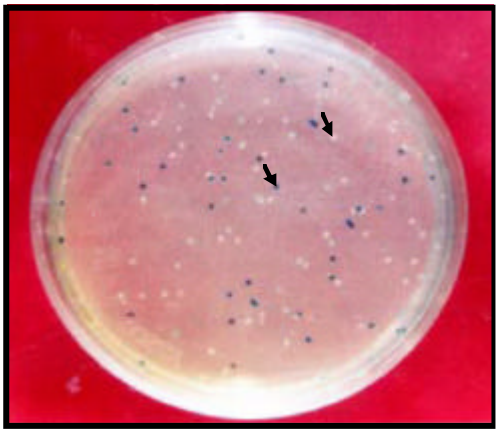


Fig.8 : Blue white screening of colonies

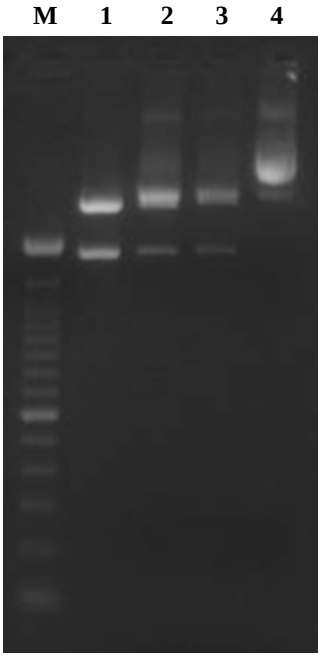


Fig.9 : RE analysis for confirming the presence of PFR1 gene in p^{GEM-T} cloning vector

Lane M : 100 bp DNA ladder plus
Lane 1,2,3 : Insert release by *Nco*I and *Pst*I
Lane 4 : Recombinant plasmid undigested

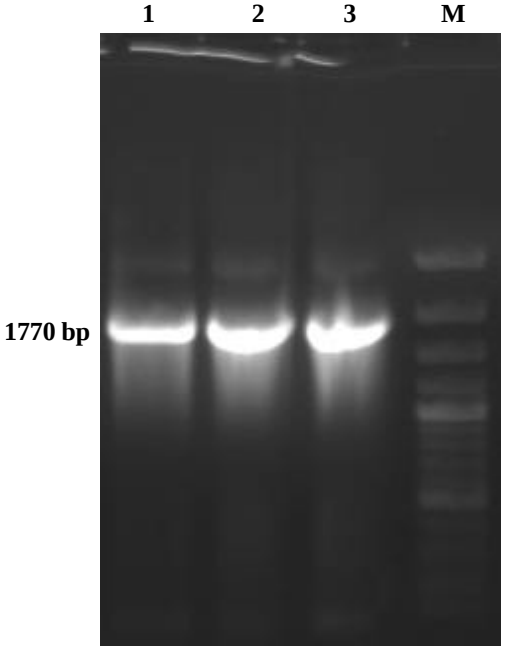
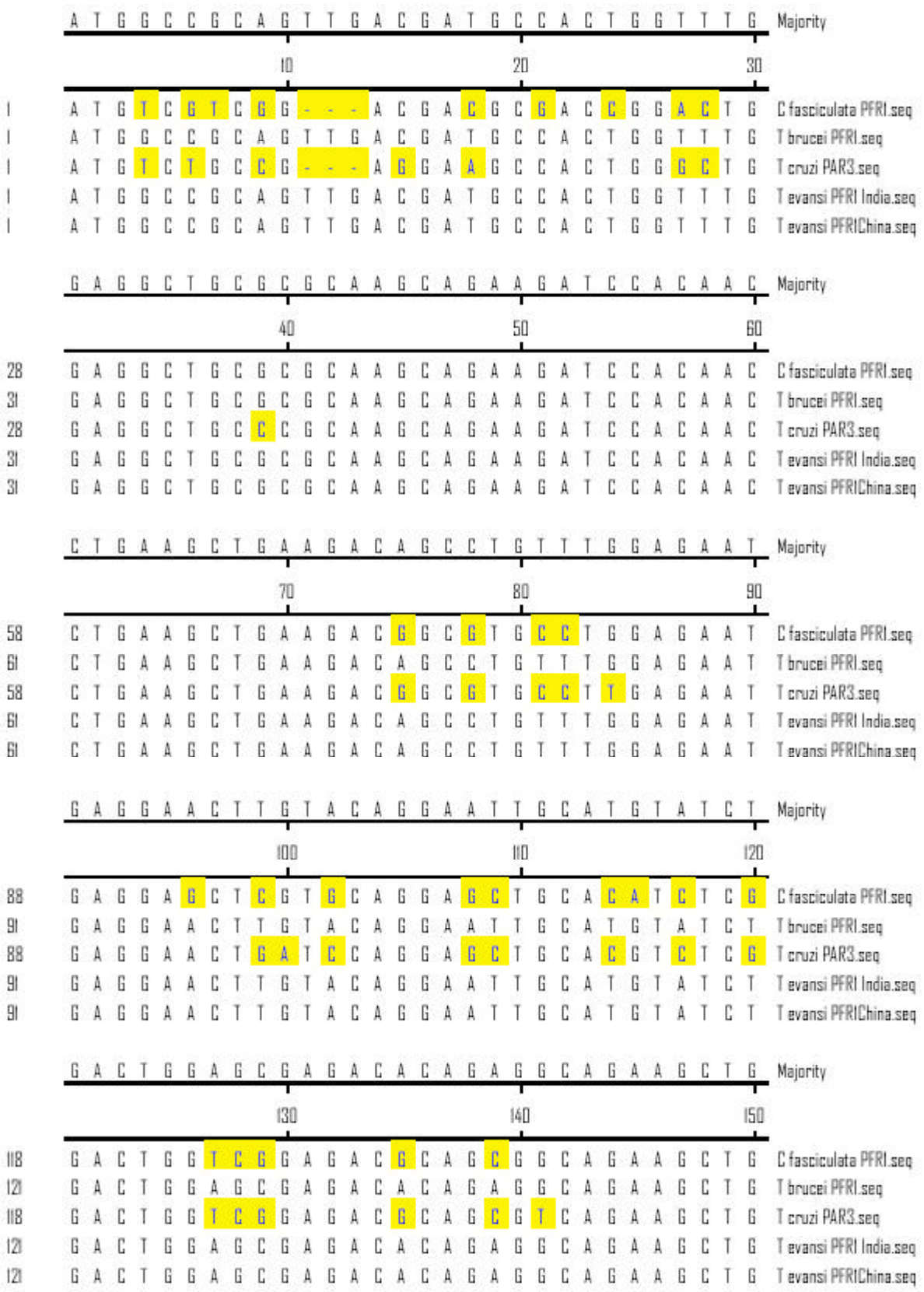


Fig.10 : Colony PCR from transformed colonies

Lane M : 100 bp DNA ladder plus
Lane 1,2&3 : PFR1 amplicons from colony PCR

Fig. 11 : Alignment of PFR1 nucleotide sequence



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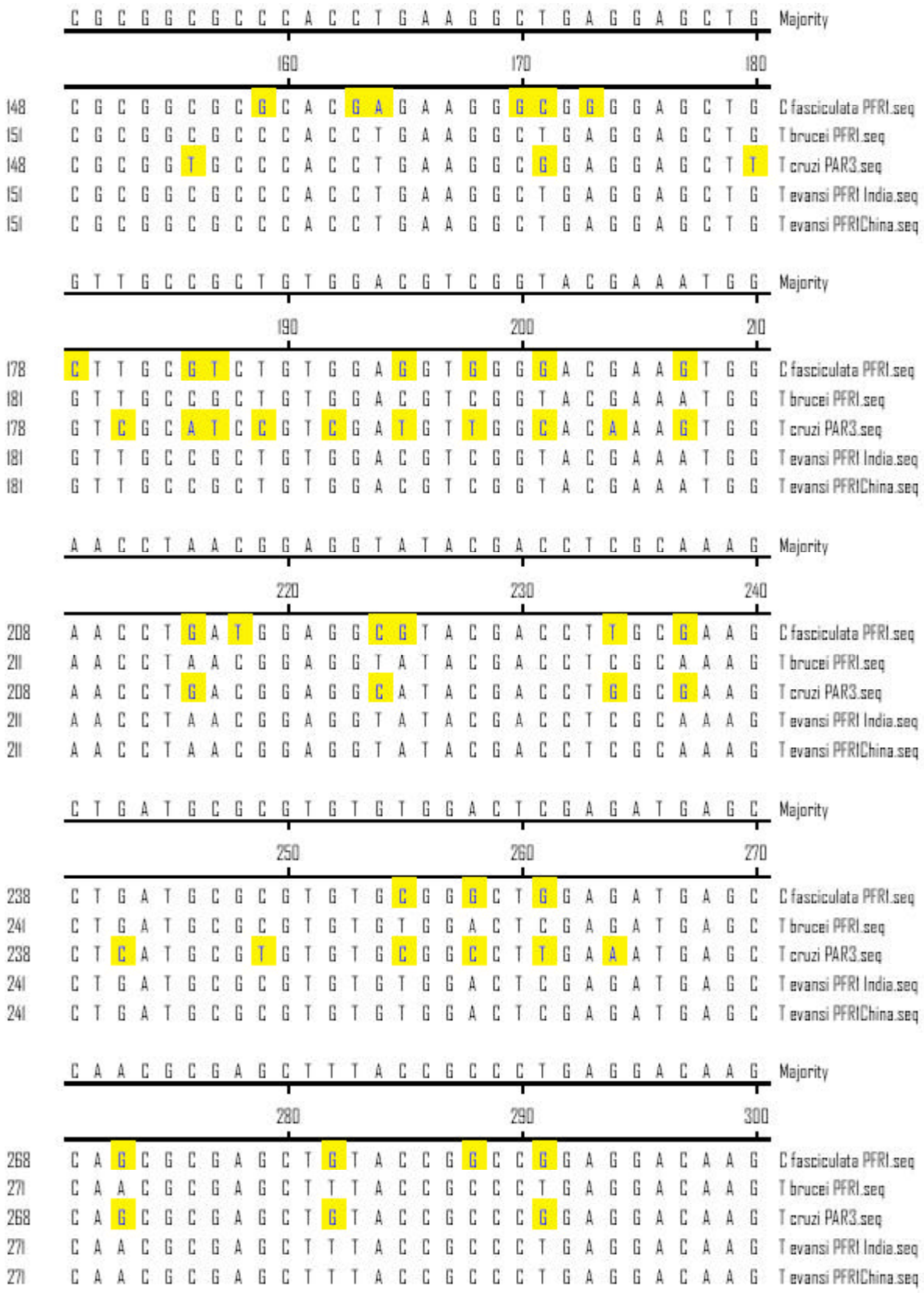
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148 C G C G G T G C C C A C C T G A A G G C G G A G G A G C T T T cruzi PAR3.seq
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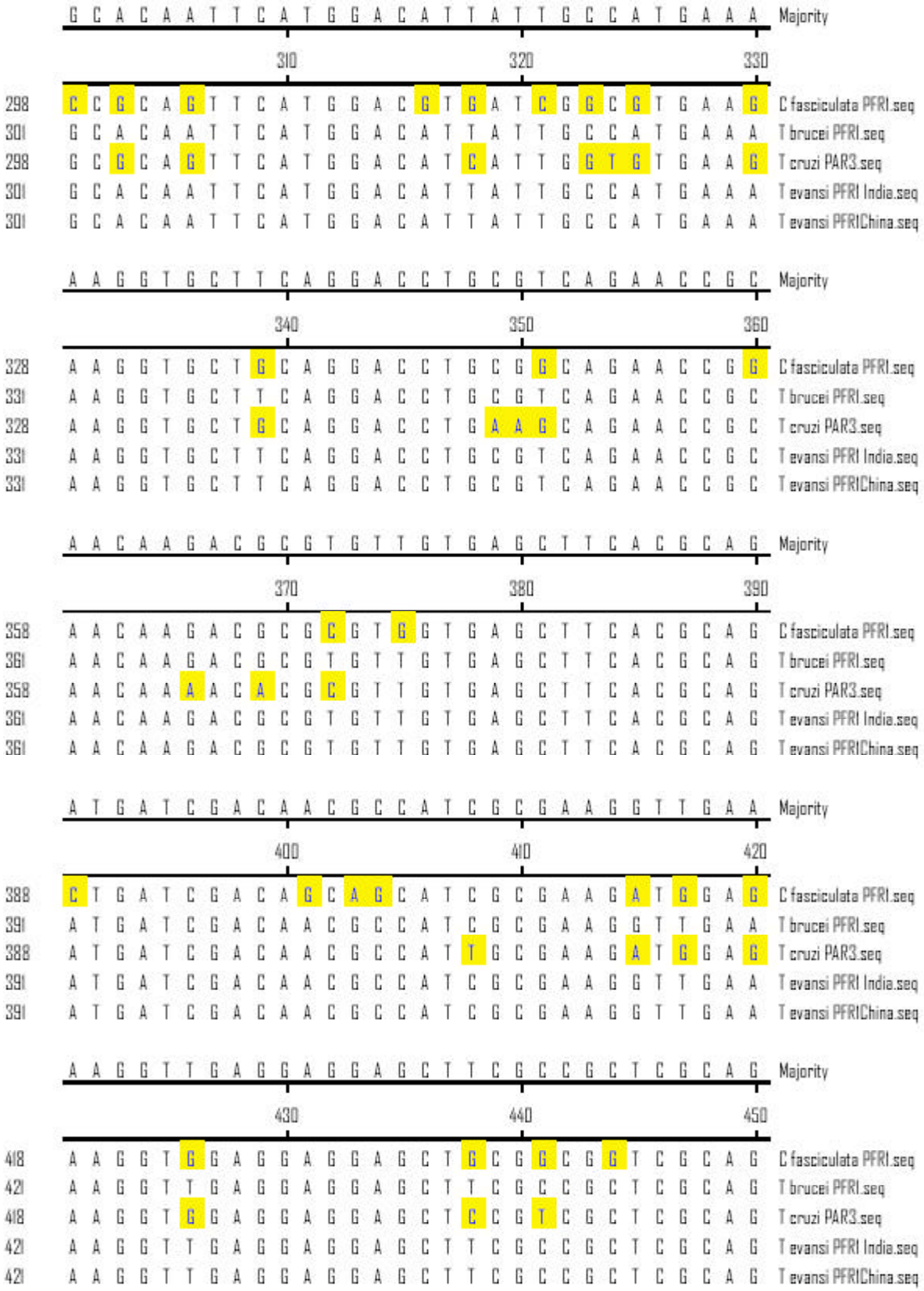
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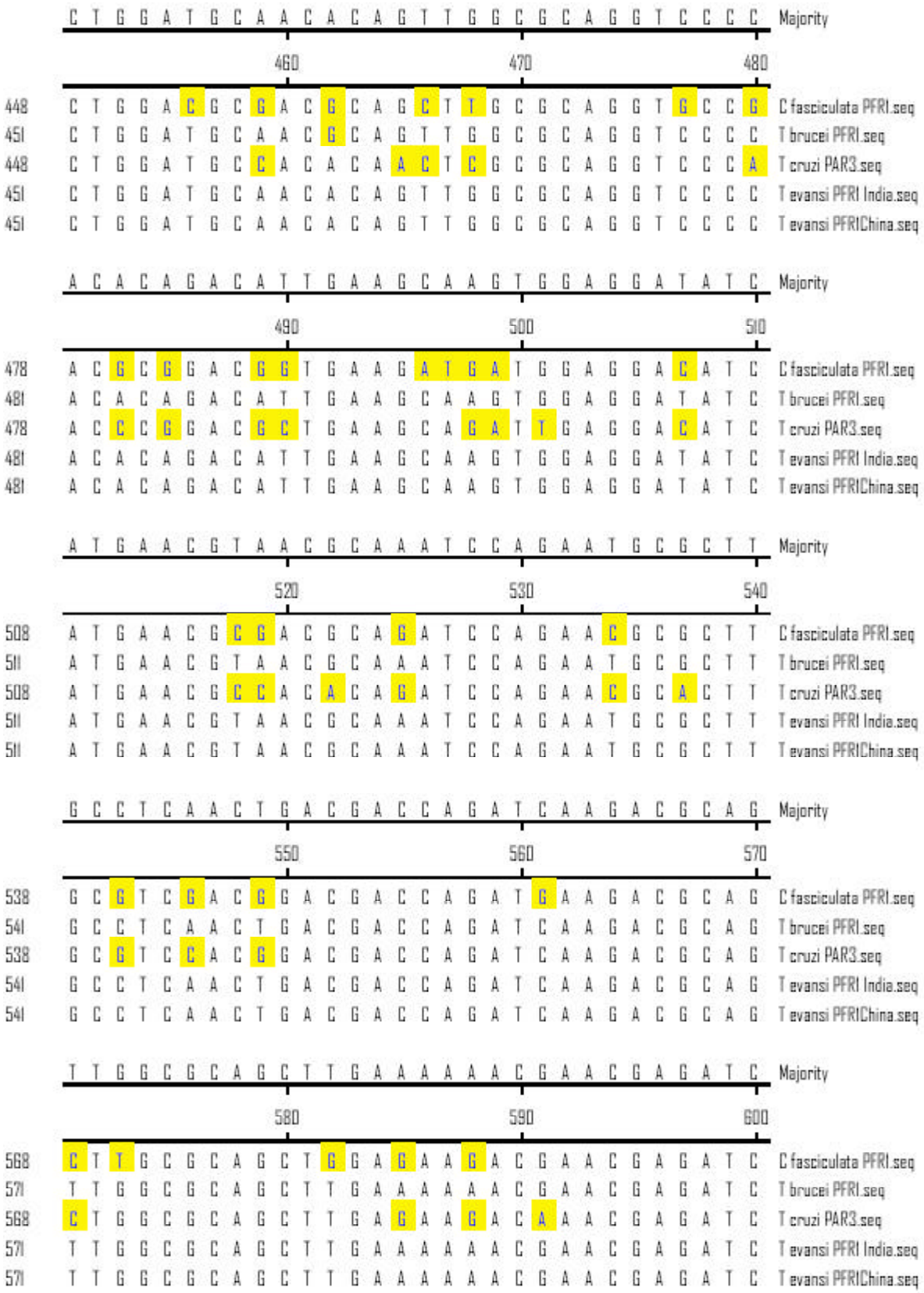
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508 A T G A A C G C C A C A C A G A T C C A G A A C G C A C T T T cruzi PAR3.seq
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511 A T G A A C G T A A C G C A A A T C C A G A A T G C G C T T T evansi PFRIChina.seq
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550
560
570
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541 G C C T C A A C T G A C G A C C A G A T C A A G A C G C A G T brucei PFRI.seq
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590
600
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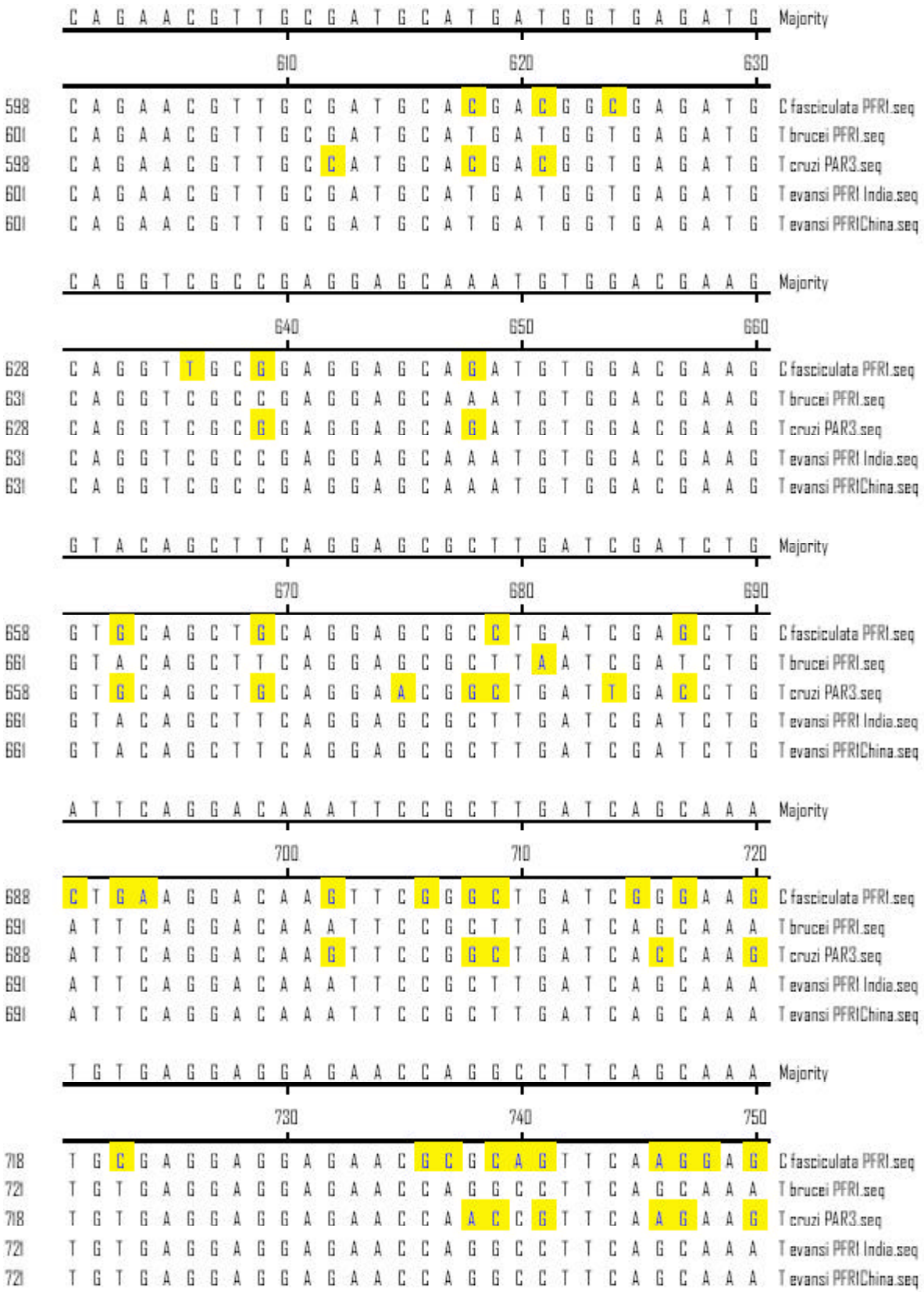
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620
630
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Majority
640
650
660
628 C fasciculata PFRI.seq
631 T brucei PFRI.seq
628 T cruzi PAR3.seq
631 T evansi PFRI India.seq
631 T evansi PFRIChina.seq
Majority
670
680
690
658 C fasciculata PFRI.seq
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658 T cruzi PAR3.seq
661 T evansi PFRI India.seq
661 T evansi PFRIChina.seq
Majority
700
710
720
688 C fasciculata PFRI.seq
691 T brucei PFRI.seq
688 T cruzi PAR3.seq
691 T evansi PFRI India.seq
691 T evansi PFRIChina.seq
Majority
730
740
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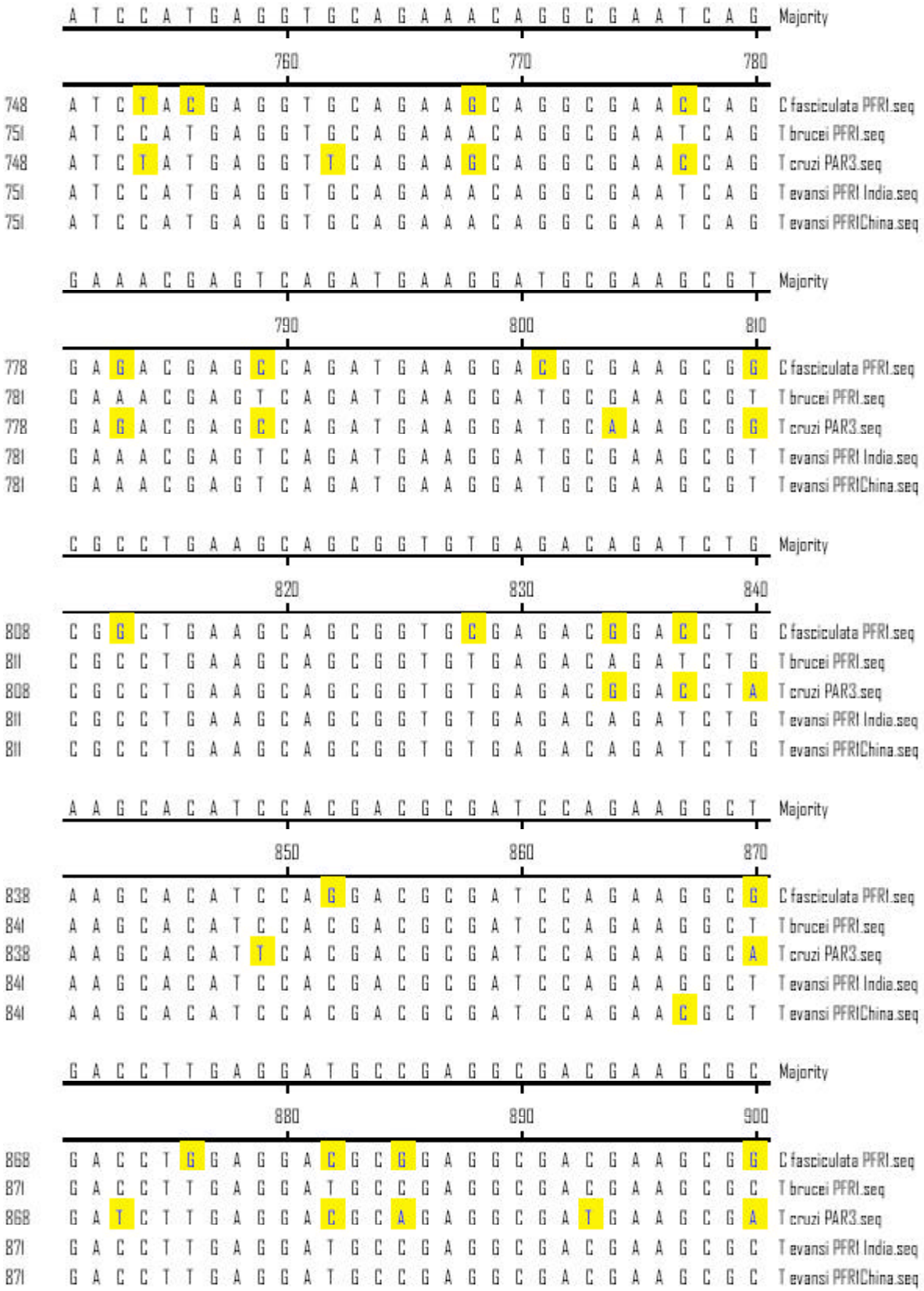
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748 A T C T A T G A G G T T C A G A A G C A G G C G A A C C A G T cruzi PAR3.seq
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751 A T C C A T G A G G T G C A G A A A C A G G C G A A T C A G T evansi PFRIChina.seq
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781 G A A A C G A G T C A G A T G A A G G A T G C G A A G C G T T evansi PFRIChina.seq
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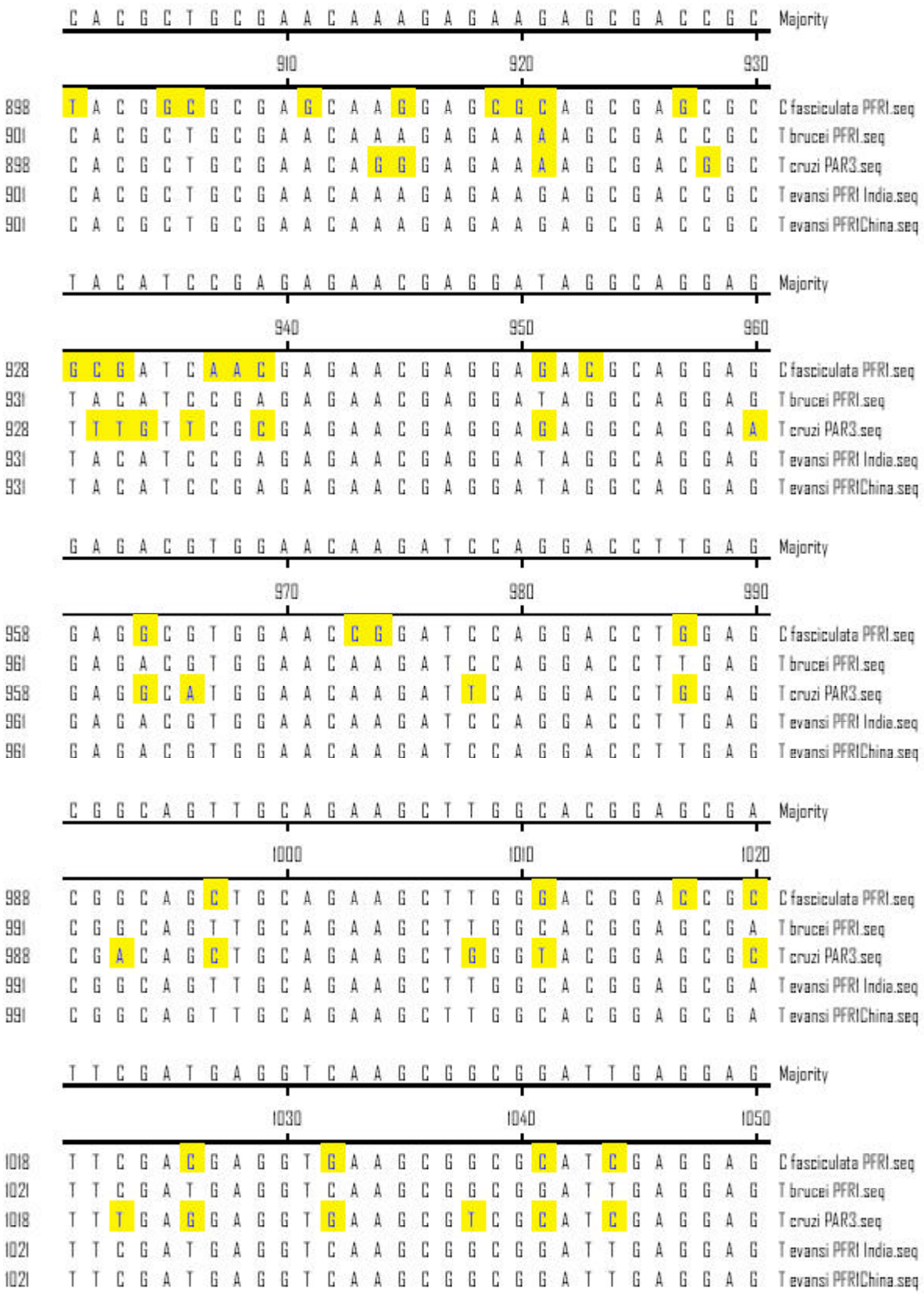
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931 T A C A T C C G A G A G A A A C G A G G A T A G G C A G G A G T evansi PFRI India.seq
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961 G A G A C G T G G A A C A A G A T C C A G G A C C T T G A G T evansi PFRI India.seq
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1000 1010 1020
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988 C G A C A G C T G C A G A A G C T G G G T A C G G A G C G C T cruzi PAR3.seq
991 C G G C A G T T G C A G A A G C T T G G C A C G G A G C G A T evansi PFRI India.seq
991 C G G C A G T T G C A G A A G C T T G G C A C G G A G C G A T evansi PFRIChina.seq
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1021 T T C G A T G A G G T C A A G C G G C G G A T T G A G G A G T brucei PFRI.seq
1018 T T T G A G G A G G T G A A G C G T C G C A T C G A G G A G T cruzi PAR3.seq
1021 T T C G A T G A G G T C A A G C G G C G G A T T G A G G A G T evansi PFRI India.seq
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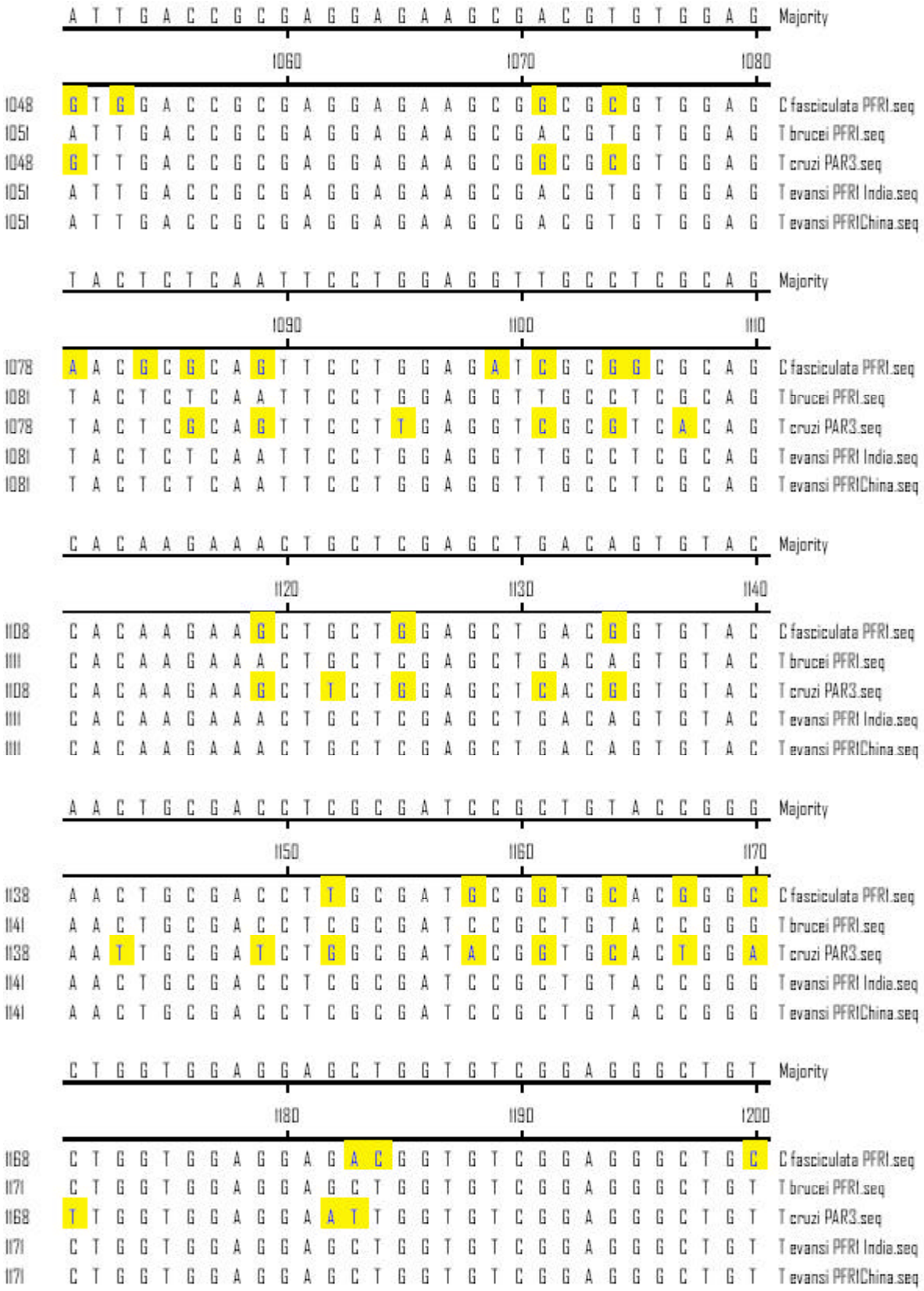
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1060
1070
1080
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1051 T brucei PFRI.seq
1048 T cruzi PAR3.seq
1051 T evansi PFRI India.seq
1051 T evansi PFRIChina.seq
Majority
1090
1100
1110
1078 C fasciculata PFRI.seq
1081 T brucei PFRI.seq
1078 T cruzi PAR3.seq
1081 T evansi PFRI India.seq
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Majority
1120
1130
1140
1108 C fasciculata PFRI.seq
1111 T brucei PFRI.seq
1108 T cruzi PAR3.seq
1111 T evansi PFRI India.seq
1111 T evansi PFRIChina.seq
Majority
1150
1160
1170
1138 C fasciculata PFRI.seq
1141 T brucei PFRI.seq
1138 T cruzi PAR3.seq
1141 T evansi PFRI India.seq
1141 T evansi PFRIChina.seq
Majority
1180
1190
1200
1168 C fasciculata PFRI.seq
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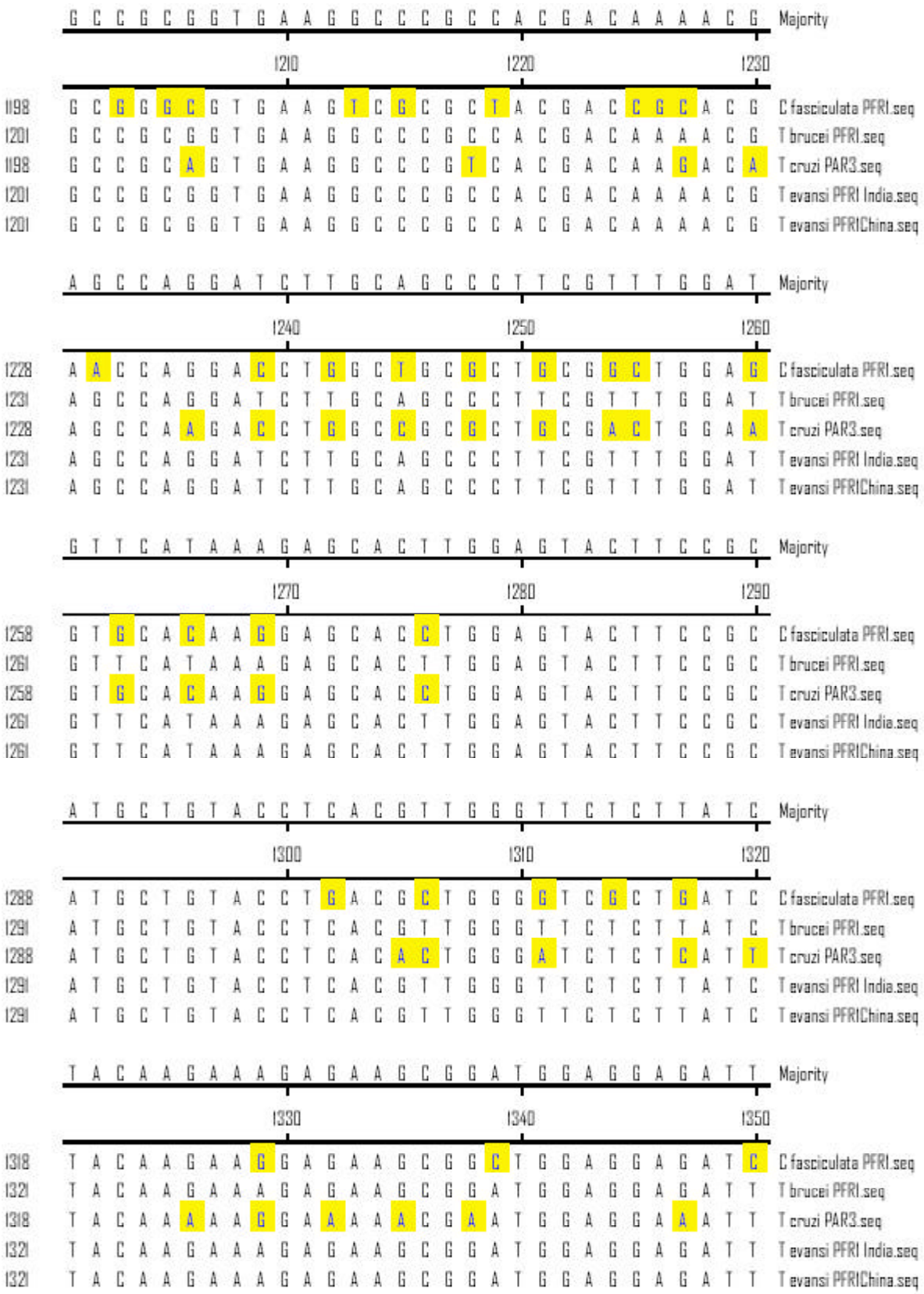
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1230
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1201 T evansi PFRI India.seq
1201 T evansi PFRIChina.seq
Majority
1240
1250
1260
1228 C fasciculata PFRI.seq
1231 T brucei PFRI.seq
1228 T cruzi PAR3.seq
1231 T evansi PFRI India.seq
1231 T evansi PFRIChina.seq
Majority
1270
1280
1290
1258 C fasciculata PFRI.seq
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Majority
1300
1310
1320
1288 C fasciculata PFRI.seq
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1288 T cruzi PAR3.seq
1291 T evansi PFRI India.seq
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Majority
1330
1340
1350
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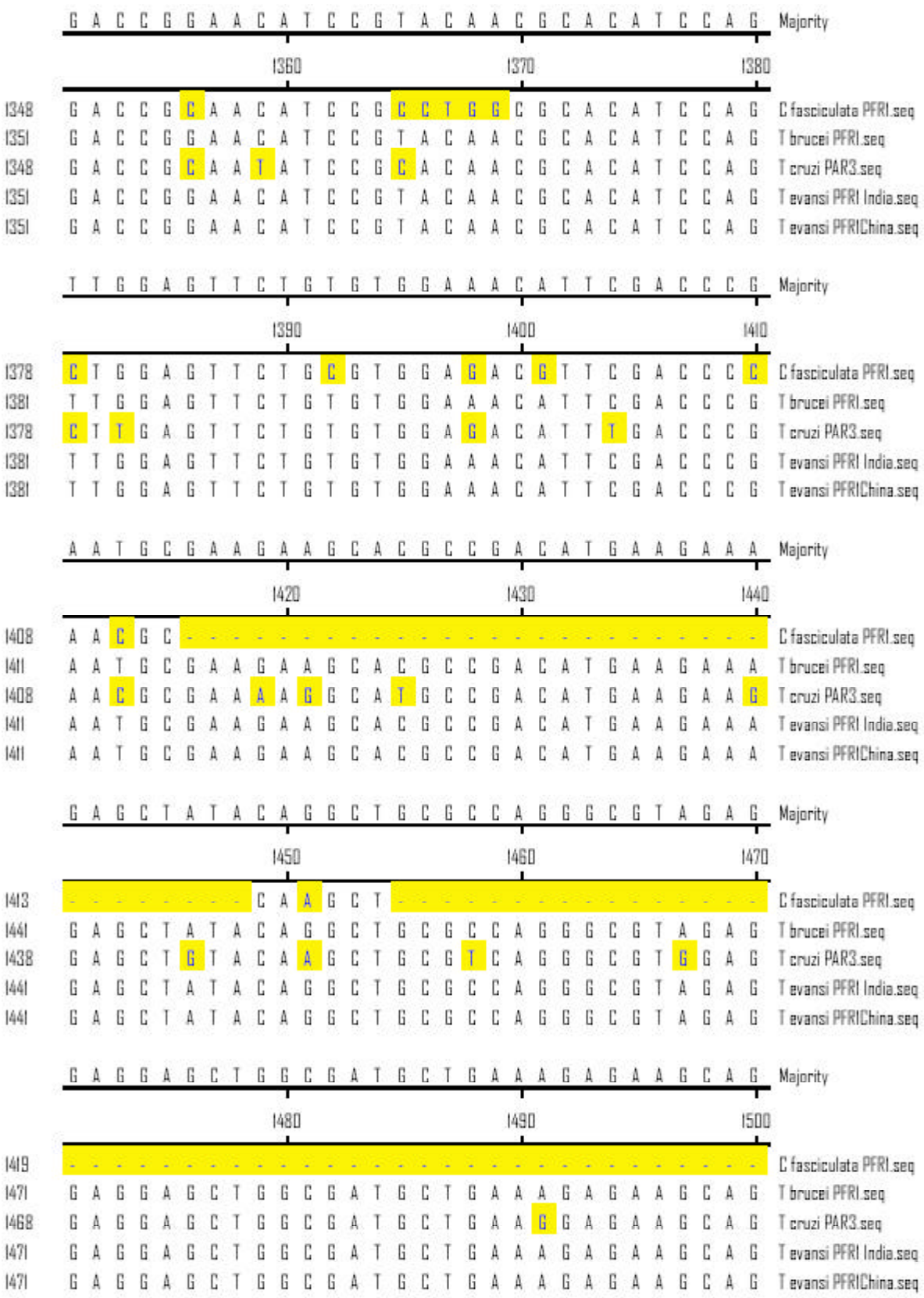
Majority
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1380
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Majority
1390
1400
1410
1378 C fasciculata PFRI.seq
1381 T brucei PFRI.seq
1378 T cruzi PAR3.seq
1381 T evansi PFRI India.seq
1381 T evansi PFRIChina.seq
Majority
1420
1430
1440
1408 C fasciculata PFRI.seq
1411 T brucei PFRI.seq
1408 T cruzi PAR3.seq
1411 T evansi PFRI India.seq
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Majority
1450
1460
1470
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1441 T evansi PFRI India.seq
1441 T evansi PFRIChina.seq
Majority
1480
1490
1500
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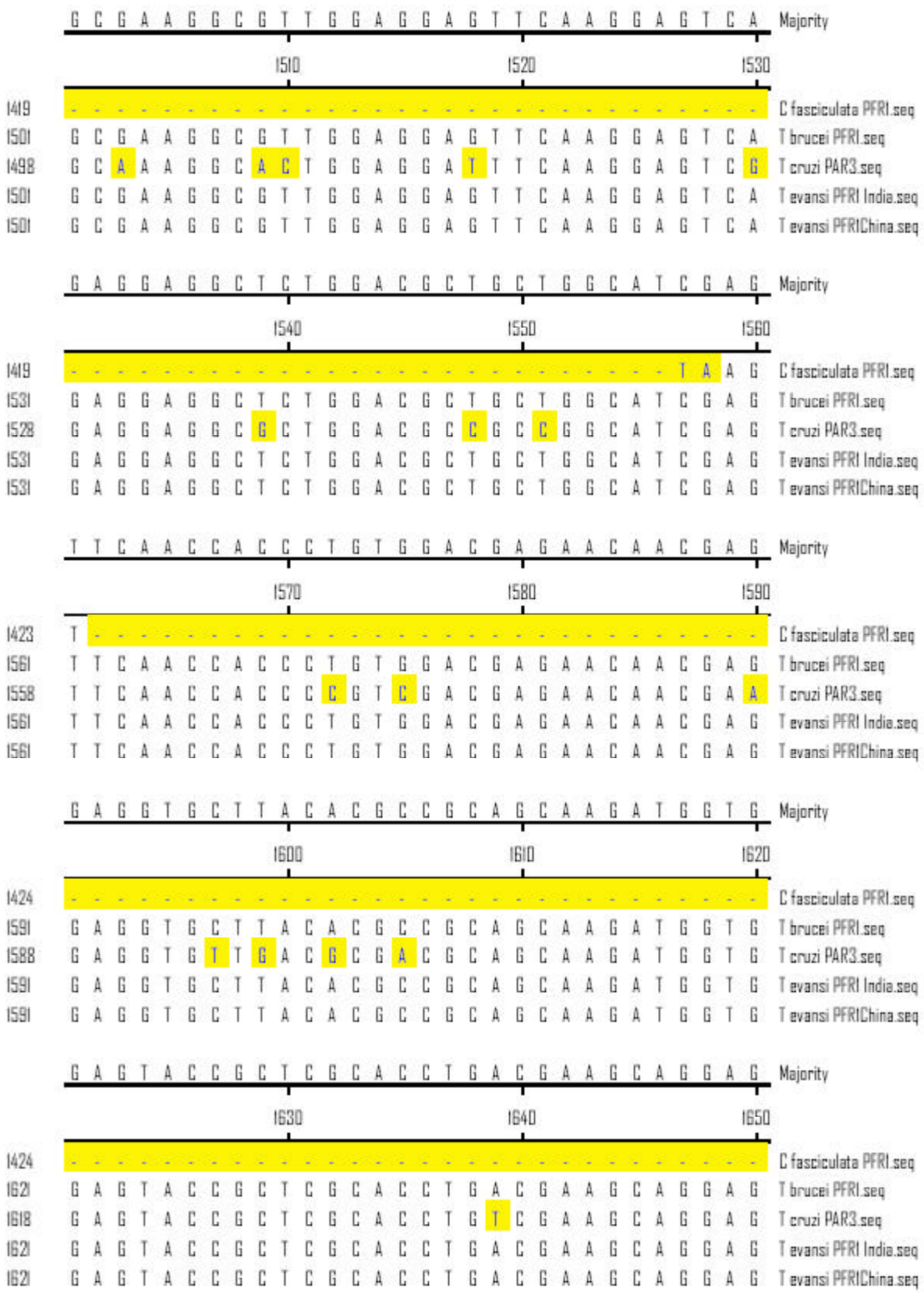
Majority
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1520
1530
1419 C fasciculata PFRI.seq
1501 T brucei PFRI.seq
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1501 T evansi PFRIChina.seq
Majority
1540
1550
1560
1419 C fasciculata PFRI.seq
1531 T brucei PFRI.seq
1528 T cruzi PAR3.seq
1531 T evansi PFRI India.seq
1531 T evansi PFRIChina.seq
Majority
1570
1580
1590
1423 C fasciculata PFRI.seq
1561 T brucei PFRI.seq
1558 T cruzi PAR3.seq
1561 T evansi PFRI India.seq
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Majority
1600
1610
1620
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1591 T brucei PFRI.seq
1588 T cruzi PAR3.seq
1591 T evansi PFRI India.seq
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Majority
1630
1640
1650
1424 C fasciculata PFRI.seq
1621 T brucei PFRI.seq
1618 T cruzi PAR3.seq
1621 T evansi PFRI India.seq
1621 T evansi PFRIChina.seq

contd.....

Decoration 'Decoration #1': Shade (with solid bright yellow) residues that differ from the Consensus.

Percent Identity

	1	2	3	4	5	
1		79.7	84.0	79.8	79.7	1
2	20.5		82.1	99.8	99.8	2
3	16.2	18.1		82.3	82.2	3
4	20.5	0.2	18.1		99.9	4
5	20.6	0.2	18.1	0.1		5
	1	2	3	4	5	

Divergence

1 C fasciculata PFR1.seq
2 T brucei PFR1.seq
3 T cruzi PAR3.seq
4 T evansi PFR1 India.seq
5 T evansi PFR1China.seq

Fig.12 : Sequence pair wise distances of PFR1 (nucleotide sequence)

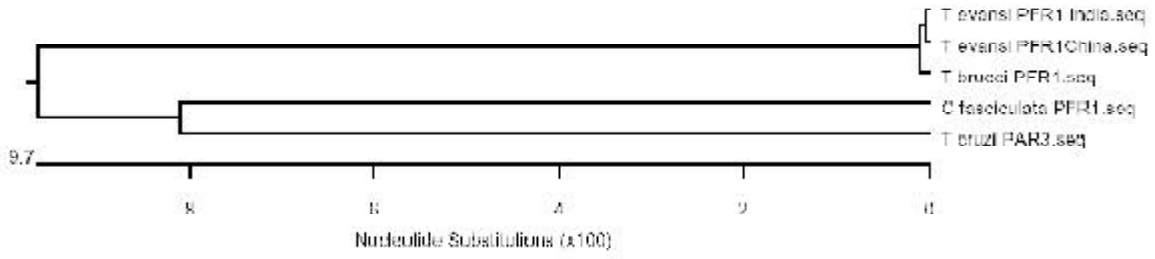


Fig.13 : Phylogenetic tree analysis of PFR1 nucleotide sequence

Percent Identity

Divergence

	1	2	3	4	5	
1		85.9	87.1	85.9	85.7	1
2	15.2		92.9	99.8	99.7	2
3	13.9	6.2		92.9	92.7	3
4	15.2	0.0	6.2		99.7	4
5	15.5	0.2	6.4	0.2		5
	1	2	3	4	5	

1 C fasciculata PFR1.pro
2 T brucei PFR1.pro
3 T cruzi PAR3.pro
4 T evansi PFR1 India.pro
5 T evansi PFR1China.pro

Fig.14 : Sequence pair wise distances of PFR1 (Deduced amino acid sequence)

revealed 99.7% homology between Izatnagar and China isolate. It also showed 99.8%, 92.9%and 85.9% homology with *T.brucei*, *T.cruzi* and *C.fasciculata*, respectively (Fig.14).

The computer simulated recombinant PFR1 three dimensional model has a single domain. The protein conformation consisted of beta sheets with intermittent alpha chains. The template is monomer in nature. A brief outline of various characteristics, viz. the titration curve (Fig.15), the antigenicity plot (Fig. 16), hydrophobicity plot, and the two-dimensional and three-dimensional structure of the recombinant PFR1 protein (Fig.17,18) is presented here under.

Cloning of PFR2 gene and its characterization

The coding sequence of *T. evansi* PFR2 gene was retrieved from GenBank (Accession No: EU258755). The entire ORF of PFR2 gene was amplified from the Izatnagar isolate of *T. evansi* using specific forward and reverse primers. The specificity and size of the amplified product was checked by agarose gel electrophoresis and a single band of 1800 bp was resolved (Fig.19). The amplified product was purified from the PCR contaminants using a commercial gel extraction kit (Qiagen, Germany). The purity of the eluted product was re-checked by electrophoresis on 1% agarose gel. The concentration of the purified PCR amplicon was measured as 35 ng / µl. The PCR product was further confirmed through restriction analysis with *SspI* enzyme. After digestion two bands were obtained at 1248 bp and 552 bp as it contains the *SspI* site at 552 bp as per the analysed result of published sequence using DNA STAR and GENE TOOL (Fig.20). This purified product of 1800 bp was used for ligation in a T/A cloning vector to facilitate sequencing and characterization. For this, pDRIVE T/A cloning vector, having an MCS (Multiple cloning sites) incorporated into a LacZ a peptide coding region, was chosen for easy selection of recombinant clones in pDRIVE cloning vector. The selection of *E. coli* DH5α positive colonies was done by blue-white colony screening method.

Plasmids were isolated from the overnight grown cells of the positive clone and were subjected to restriction digestion using *EcoRI* enzyme. The release of the insert of desired length was checked by agarose gel electrophoresis of the enzyme digested product (Fig.21).

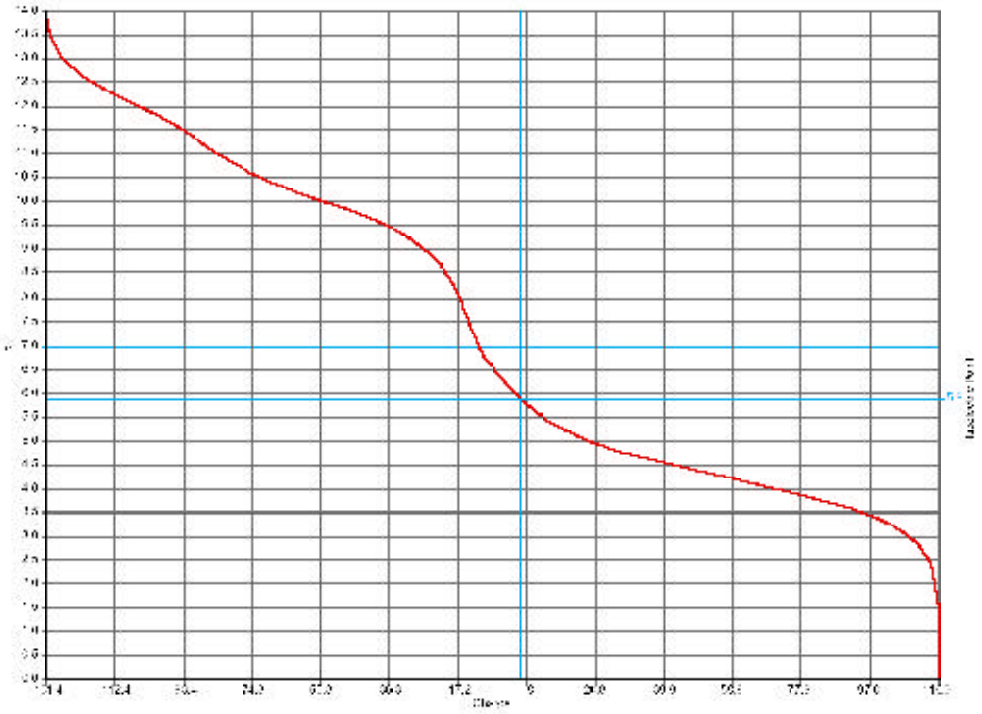


Fig.15 : Titration curve of PFR1 of *T. evansi*

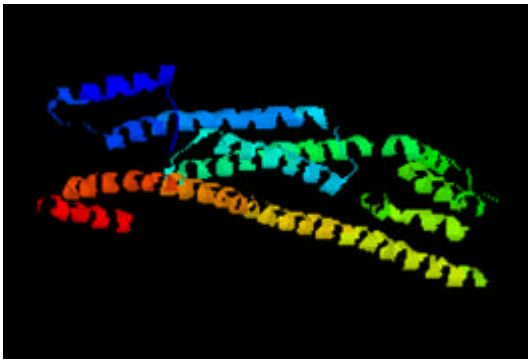


Fig.17 : 3D Ribbon model of PFR1 protein

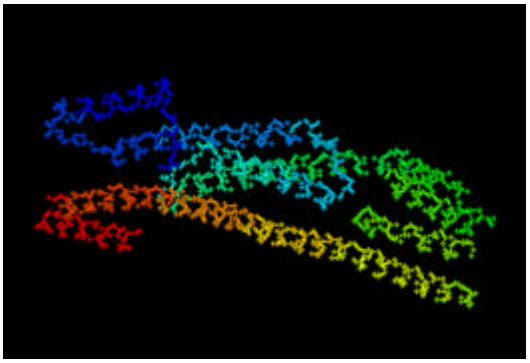


Fig.18 : 3D Ball stick model of PFR1 protein

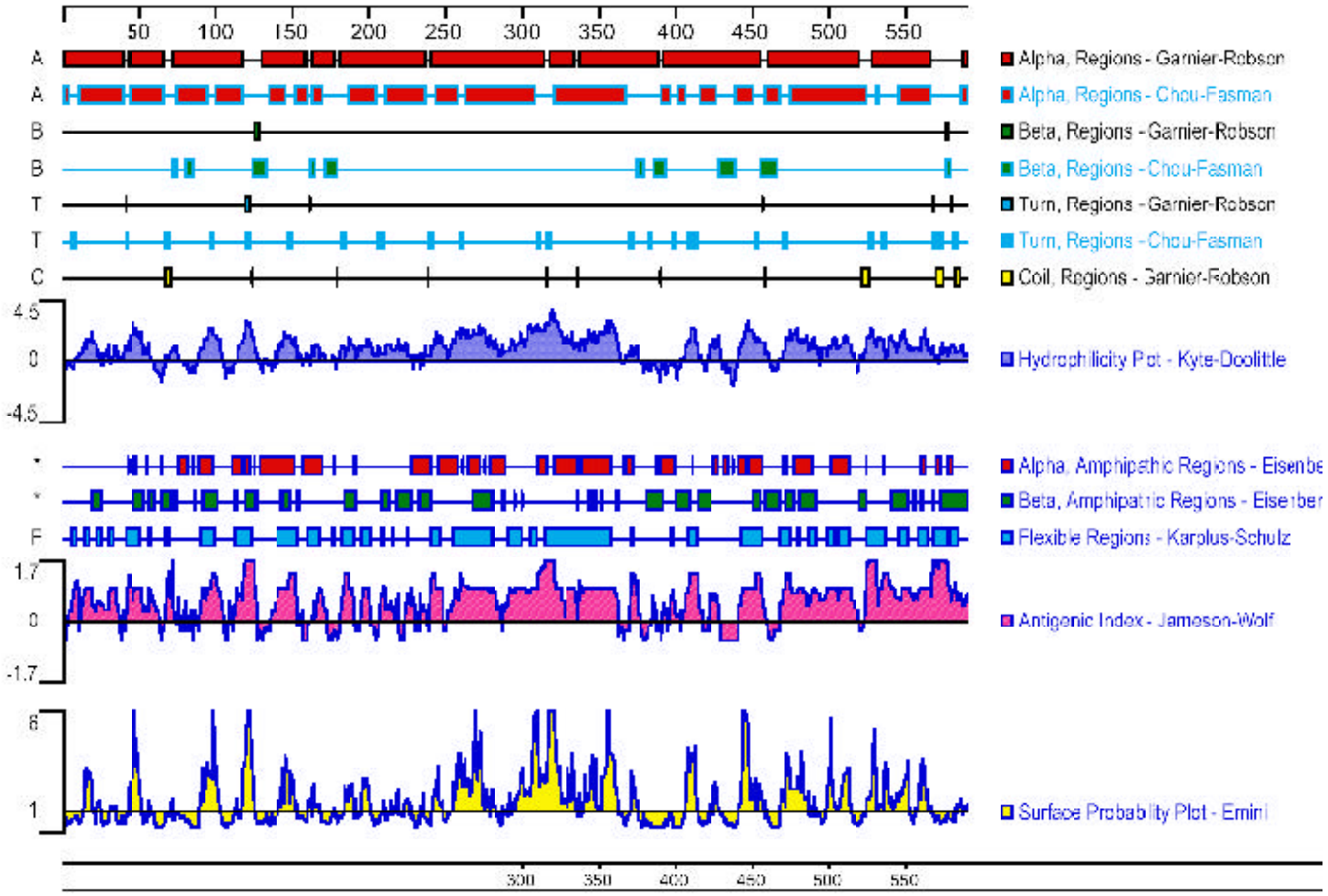


Fig.16 : Antigenic analysis of PFR1 protein using protean programme of DNA star software to predict the antigenic

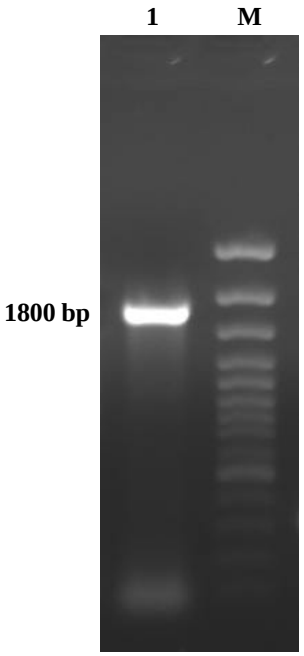


Fig.19 : Specific PCR amplification of PFR2 gene

Lane M : 100 bp DNA ladder plus
Lane 1 : Amplicon of 1800 bp from cDNA template

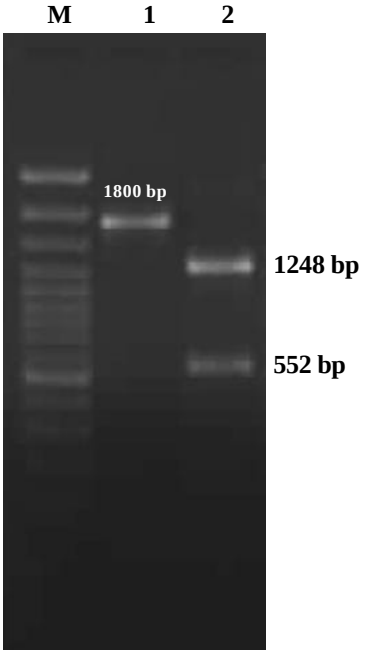


Fig.20 : RE analysis for confirming PFR2 gene

Lane M : 100 bp DNA ladder plus
Lane 1 : Uncut PCR pdt. of *T. evansi*
Lane 2 : *SSP*I digested PCR pdt. of *T. evansi*

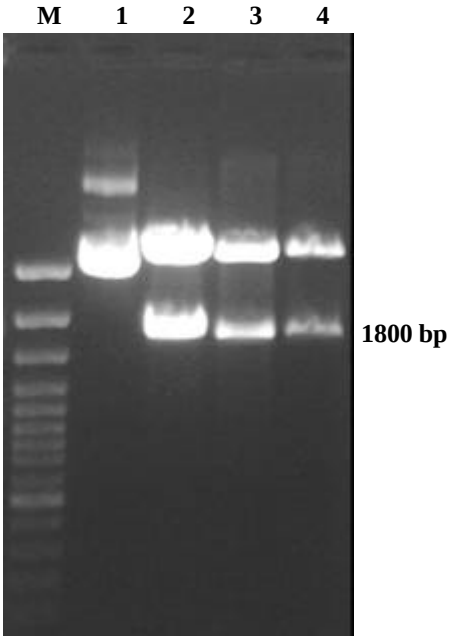


Fig.21 : RE analysis for confirming the presence of PFR2 gene in pDRIVE cloning vector

Lane M : 100 bp DNA ladder plus
Lane 1 : Recombinant plasmid undigestd
Lane 2,3,4 : Recombinant plasmid digested with *Eco*RI releasing insert of 1800 bp

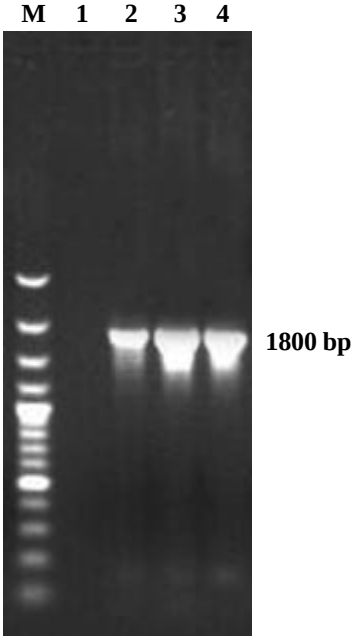


Fig.22 : Colony PCR from transformed colonies

Lane M : 100 bp plus DNA marker
Lane 1 : Negative control
Lane 2,3,4 : PFR2 amplicons from colony PCR

Colony PCR further confirmed the positive clone (Fig.22).The positive clone was custom sequenced for nucleotides. The nucleotide sequence revealed 99.9% homology and only one nucleotide change at 928bp of PFR2 ORF between the Izatnagar and China isolates. The nucleotide sequence also showed 99.9%, 82.4% and 74.8% homology with *T.brucei*, *T.cruzi* and *C.fasciculata*, respectively (Fig. 23, 24, 25). The mature PFR2 protein comprised of 600 amino acids. The deduced amino acid sequence of *T. evansi* PFR2 revealed 99.7% homology between Izatnagar and china isolate. It also showed 99.7%, 89.9% and 82.5% homology with *T.brucei, T.cruzi and C.fasciculata,* respectively(Fig.26).

Amplification of both PFR1 and PFR2 from whole genomic DNA of *T. evansi*

Similar sized amplicons for both PFR1 and PFR2 genes only confirmed the intronless nature of these genes (Fig. 27).

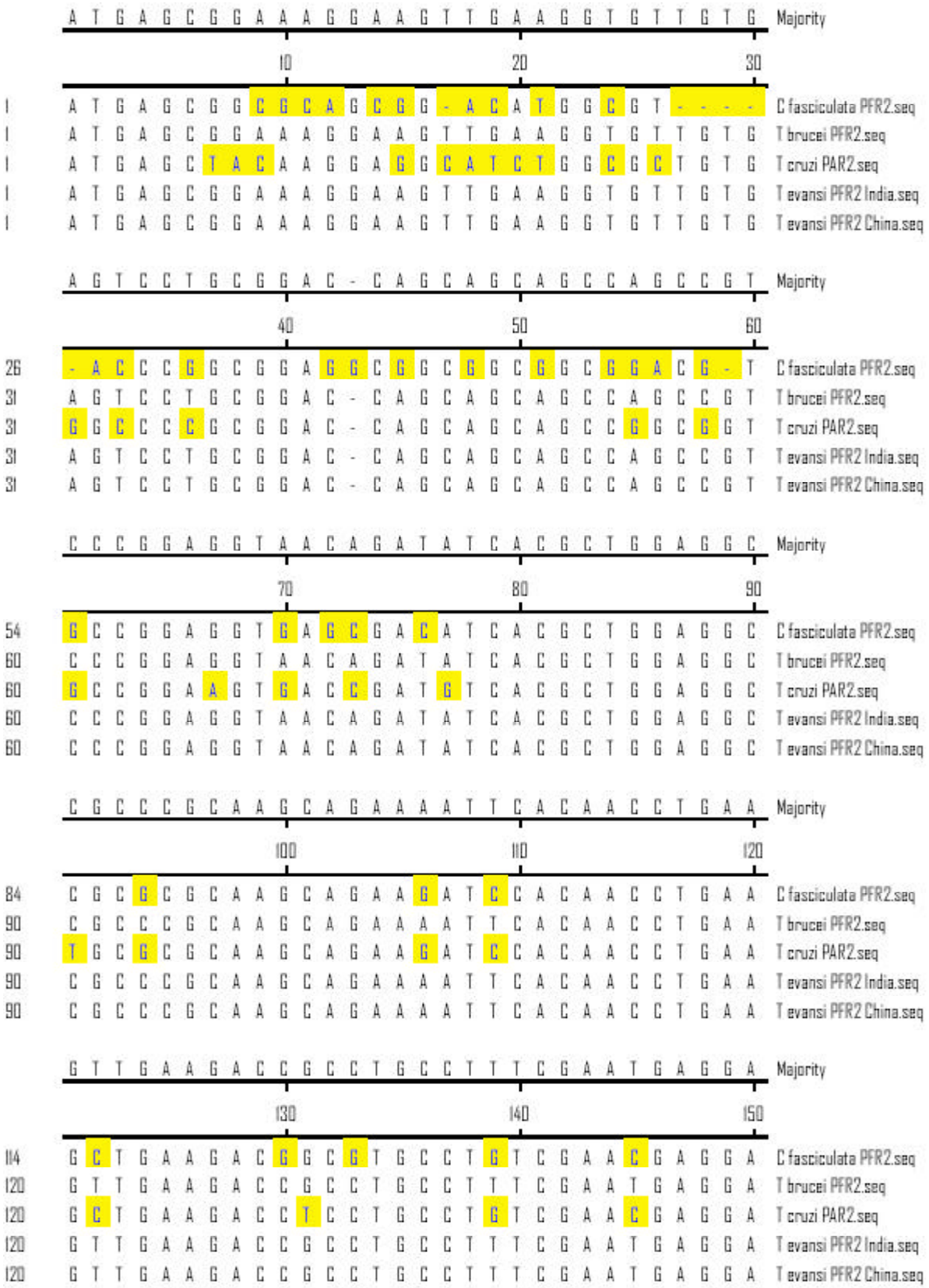
Cloning of PFR2 gene for expression

The full ORF of PFR2 (from start codon to 1800 bp) was PCR amplified from the Izatnagar isolate of *T. evansi* using primers TEPFRF and TEPFRR. The amplified product was checked and purified. The purified product was double digested with *EcoRI* and *HindIII* at 37^0C for 4-5 h. This double digested product was purified and eluted with 40 µl elution buffer and the DNA concentration was quantified. From this double digested product, 200ng was used for ligation.

Expression of recombinant PFR2 protein using prokaryotic system

The expression vector, ET-32a was also double digested with the same set of restriction enzyme, *viz*. *EcoRI* and *HindIII* and eluted in 40 µl of final volume. The concentration of double stranded DNA was estimated as 18.2 ng/ µl. From this preparation 72 ng DNA was used for overnight ligation at 4^oC. BL21 cells were transformed with the ligated plasmids and plated in the presence of ampicillin and chloramphenicol. Following an overnight incubation, the white colonies were selectively isolated.

Fig.23 : Alignment of PFR2 nucleotide sequence



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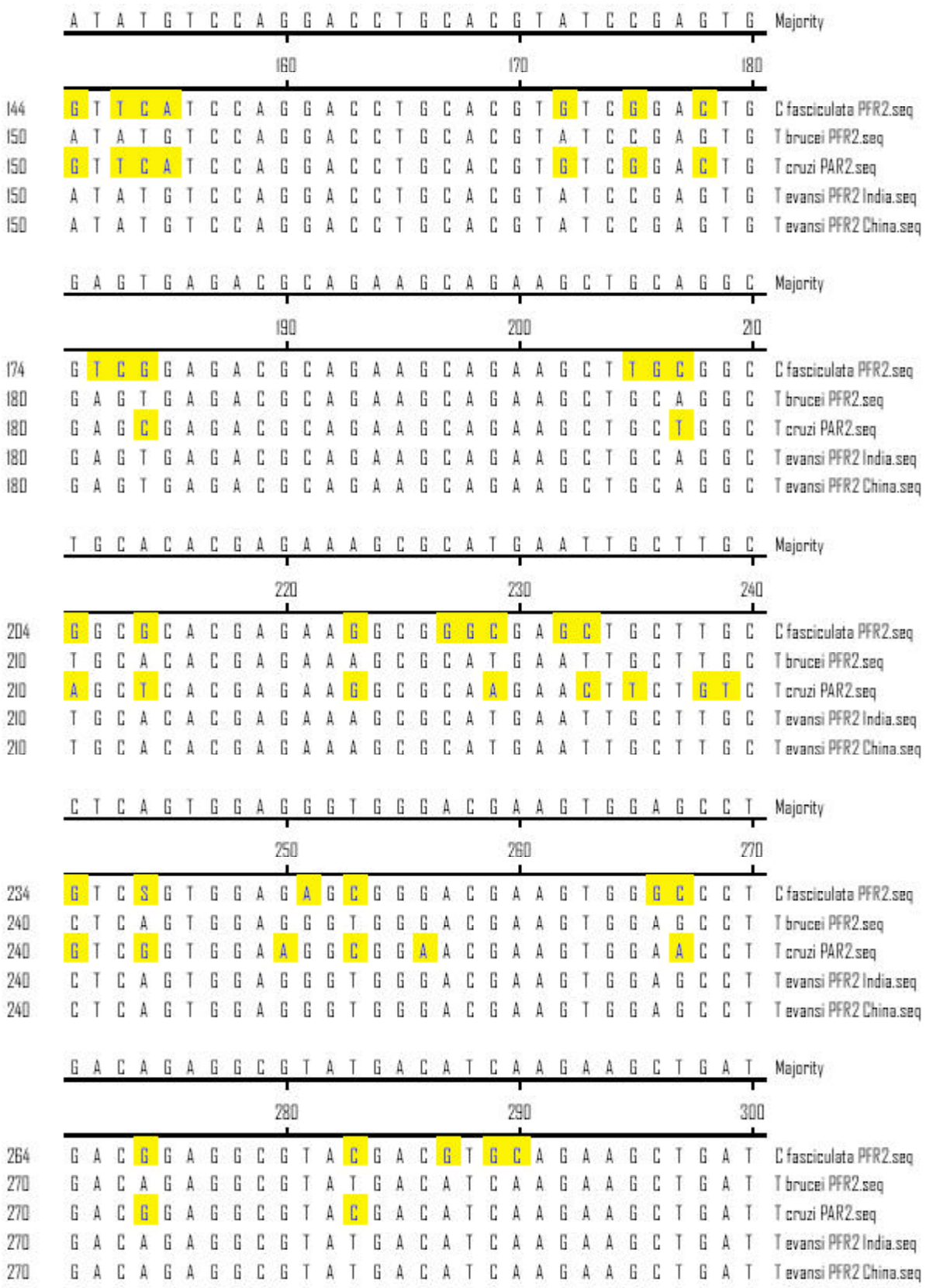
Majority
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170
180
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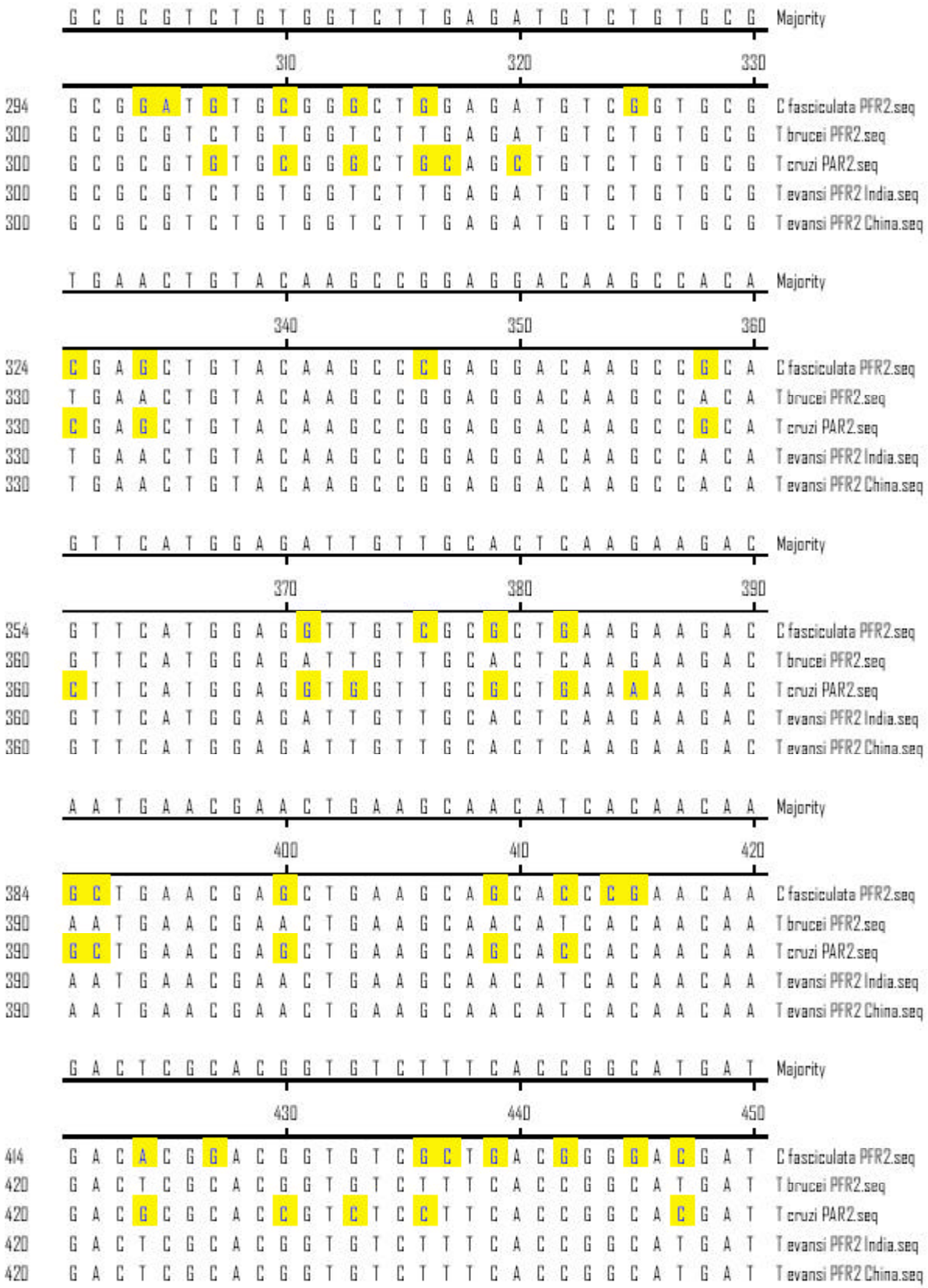
150 T brucei PFR2.seq
150 T cruzi PAR2.seq
150 T evansi PFR2 India.seq
150 T evansi PFR2 China.seq
Majority
190
200
210
174 C fasciculata PFR2.seq
180 T brucei PFR2.seq
180 T cruzi PAR2.seq
180 T evansi PFR2 India.seq
180 T evansi PFR2 China.seq
Majority
220
230
240
204 C fasciculata PFR2.seq
210 T brucei PFR2.seq
210 T cruzi PAR2.seq
210 T evansi PFR2 India.seq
210 T evansi PFR2 China.seq
Majority
250
260
270
234 C fasciculata PFR2.seq
240 T brucei PFR2.seq
240 T cruzi PAR2.seq
240 T evansi PFR2 India.seq
240 T evansi PFR2 China.seq
Majority
280
290
300
264 C fasciculata PFR2.seq
270 T brucei PFR2.seq
270 T cruzi PAR2.seq
270 T evansi PFR2 India.seq
270 T evansi PFR2 China.seq

contd.....


Majority
310
320
330
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300 T brucei PFR2.seq
300 T cruzi PAR2.seq
300 T evansi PFR2 India.seq
300 T evansi PFR2 China.seq
Majority
340
350
360
324 C fasciculata PFR2.seq
330 T brucei PFR2.seq
330 T cruzi PAR2.seq
330 T evansi PFR2 India.seq
330 T evansi PFR2 China.seq
Majority
370
380
390
354 C fasciculata PFR2.seq
360 T brucei PFR2.seq
360 T cruzi PAR2.seq
360 T evansi PFR2 India.seq
360 T evansi PFR2 China.seq
Majority
400
410
420
384 C fasciculata PFR2.seq
390 T brucei PFR2.seq
390 T cruzi PAR2.seq
390 T evansi PFR2 India.seq
390 T evansi PFR2 China.seq
Majority
430
440
450
414 C fasciculata PFR2.seq
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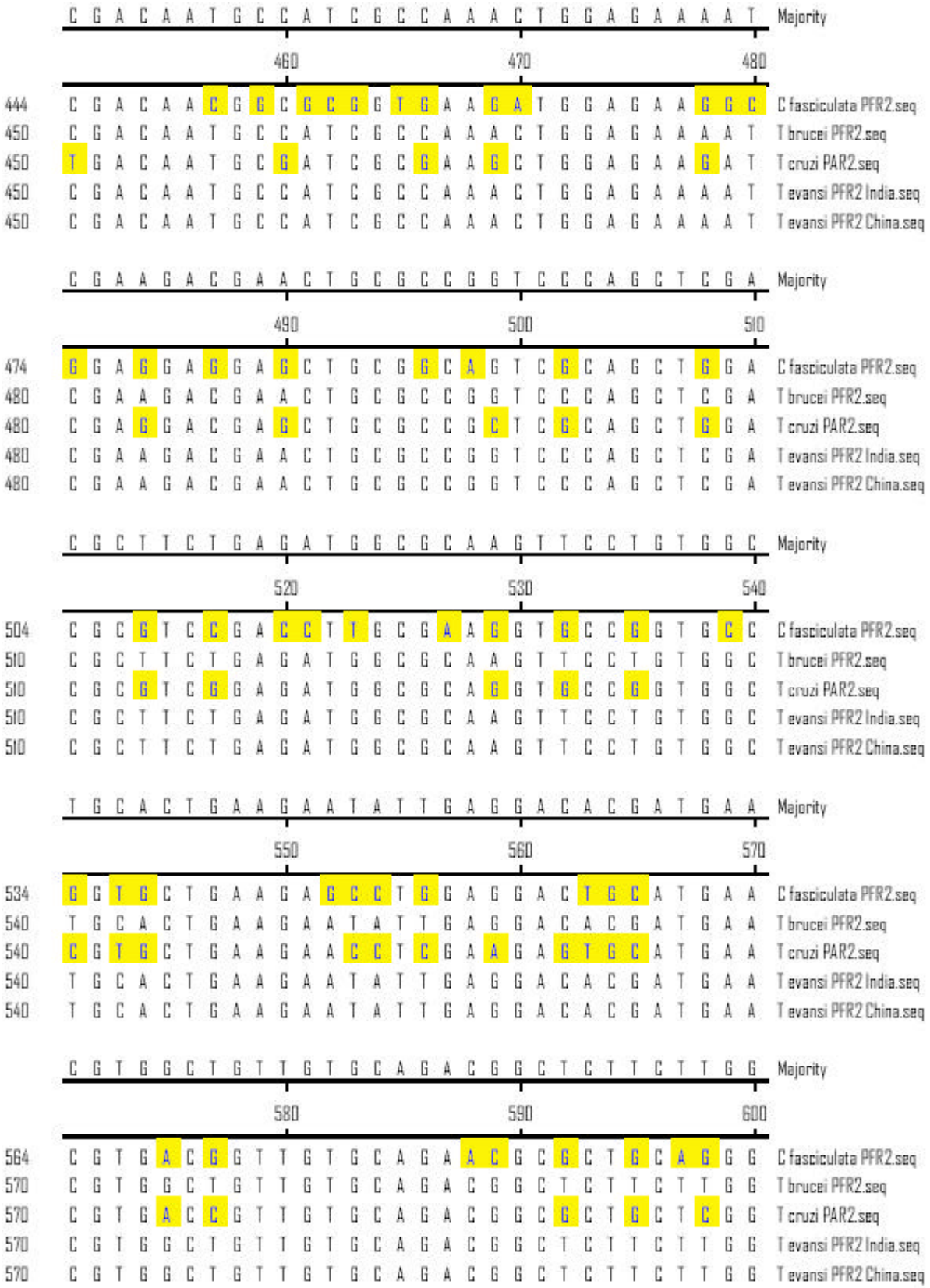
420 T cruzi PAR2.seq
420 T evansi PFR2 India.seq
420 T evansi PFR2 China.seq

contd.....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470
480
444 C fasciculata PFR2.seq
450 T brucei PFR2.seq
450 T cruzi PAR2.seq
450 T evansi PFR2 India.seq
450 T evansi PFR2 China.seq
Majority
490
500
510
474 C fasciculata PFR2.seq
480 T brucei PFR2.seq
480 T cruzi PAR2.seq
480 T evansi PFR2 India.seq
480 T evansi PFR2 China.seq
Majority
520
530
540
504 C fasciculata PFR2.seq
510 T brucei PFR2.seq
510 T cruzi PAR2.seq
510 T evansi PFR2 India.seq
510 T evansi PFR2 China.seq
Majority
550
560
570
534 C fasciculata PFR2.seq
540 T brucei PFR2.seq
540 T cruzi PAR2.seq
540 T evansi PFR2 India.seq
540 T evansi PFR2 China.seq
Majority
580
590
600
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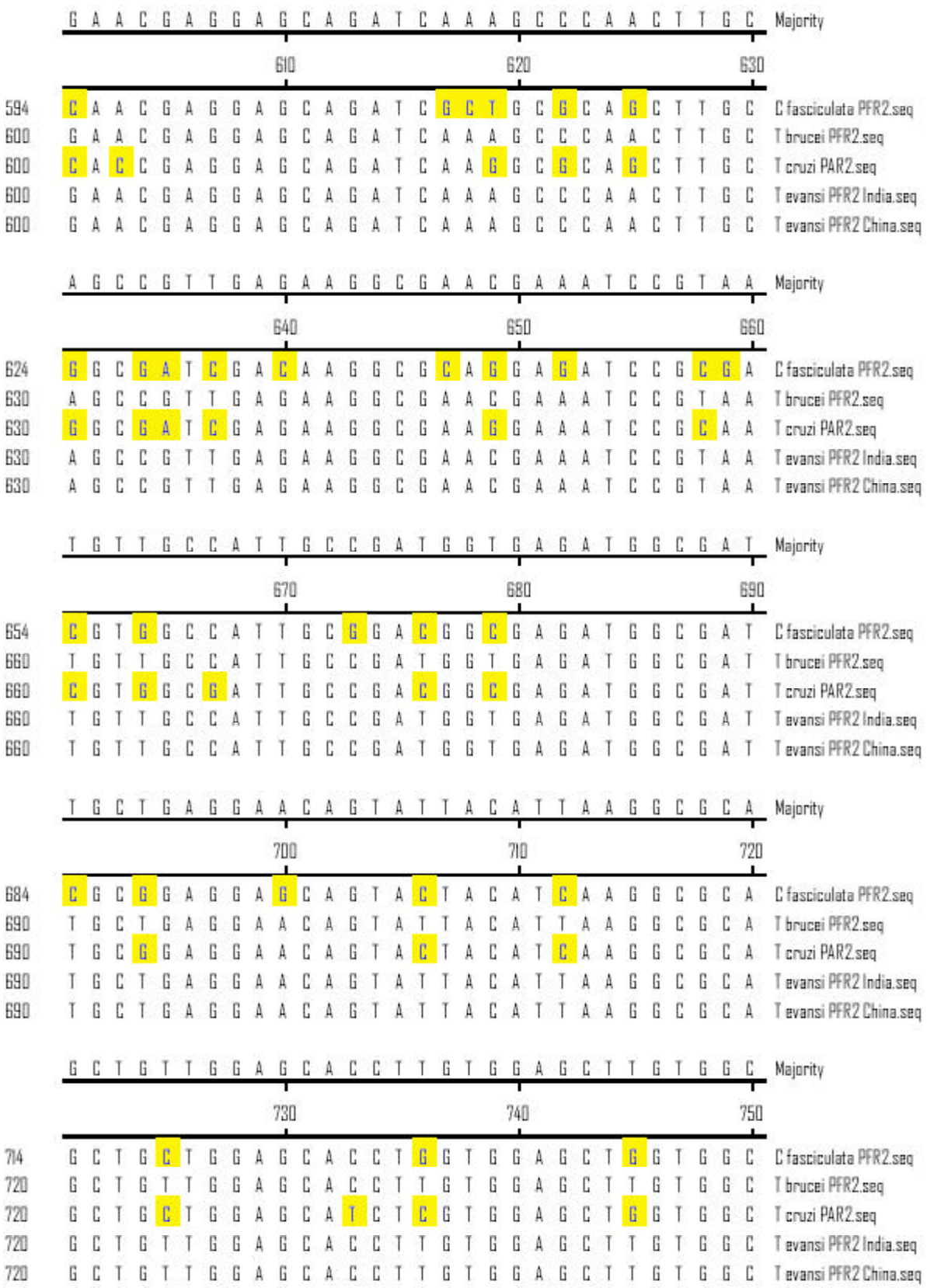
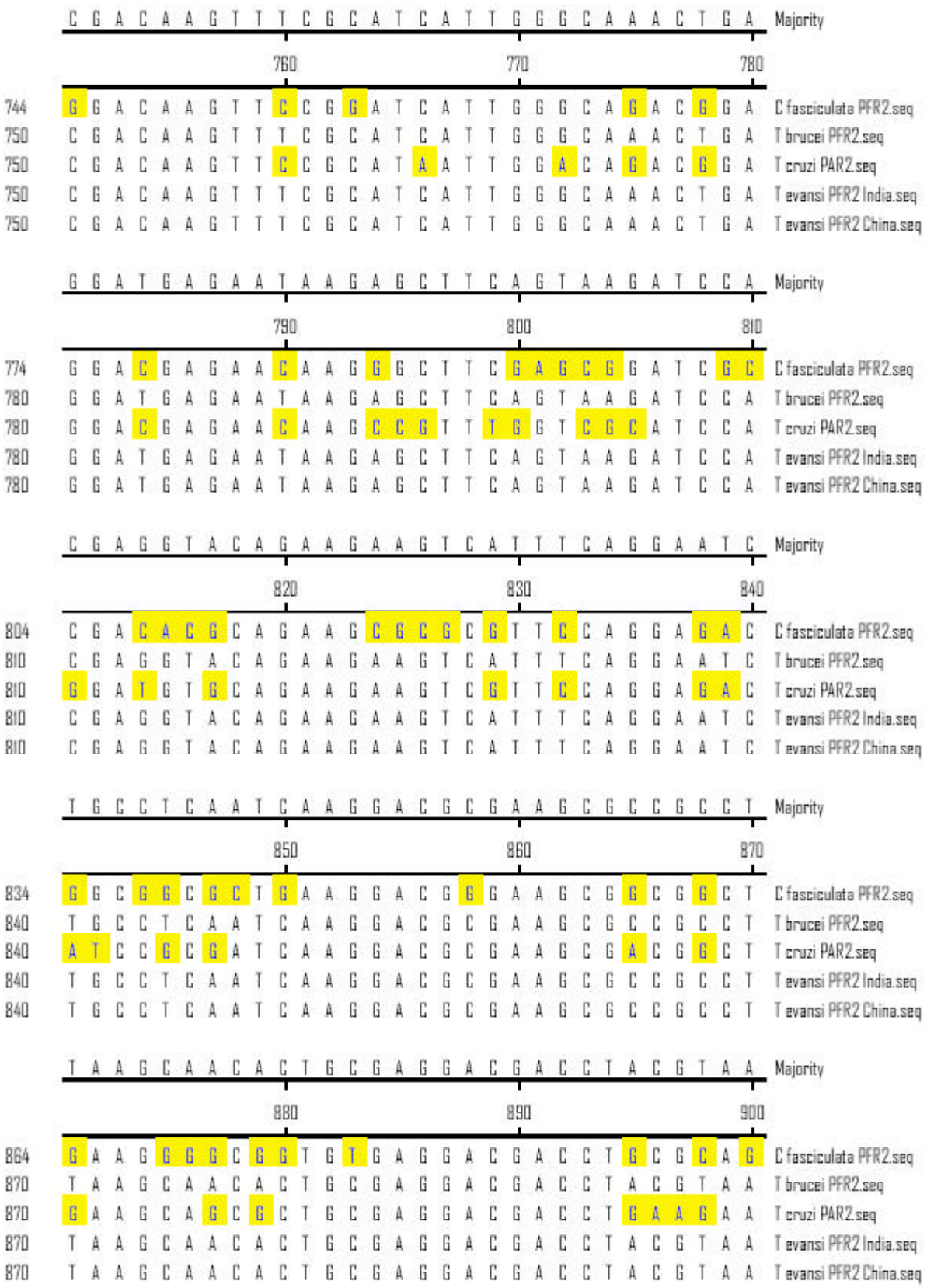
570 T brucei PFR2.seq
570 T cruzi PAR2.seq
570 T evansi PFR2 India.seq
570 T evansi PFR2 China.seq


Majority
610
620
630
594 C fasciculata PFR2.seq
600 T brucei PFR2.seq
600 T cruzi PAR2.seq
600 T evansi PFR2 India.seq
600 T evansi PFR2 China.seq
Majority
640
650
660
624 C fasciculata PFR2.seq
630 T brucei PFR2.seq
630 T cruzi PAR2.seq
630 T evansi PFR2 India.seq
630 T evansi PFR2 China.seq
Majority
670
680
690
654 C fasciculata PFR2.seq
660 T brucei PFR2.seq
660 T cruzi PAR2.seq
660 T evansi PFR2 India.seq
660 T evansi PFR2 China.seq
Majority
700
710
720
684 C fasciculata PFR2.seq
690 T brucei PFR2.seq
690 T cruzi PAR2.seq
690 T evansi PFR2 India.seq
690 T evansi PFR2 China.seq
Majority
730
740
750
714 C fasciculata PFR2.seq
720 T brucei PFR2.seq
720 T cruzi PAR2.seq
720 T evansi PFR2 India.seq
720 T evansi PFR2 China.seq

contd.....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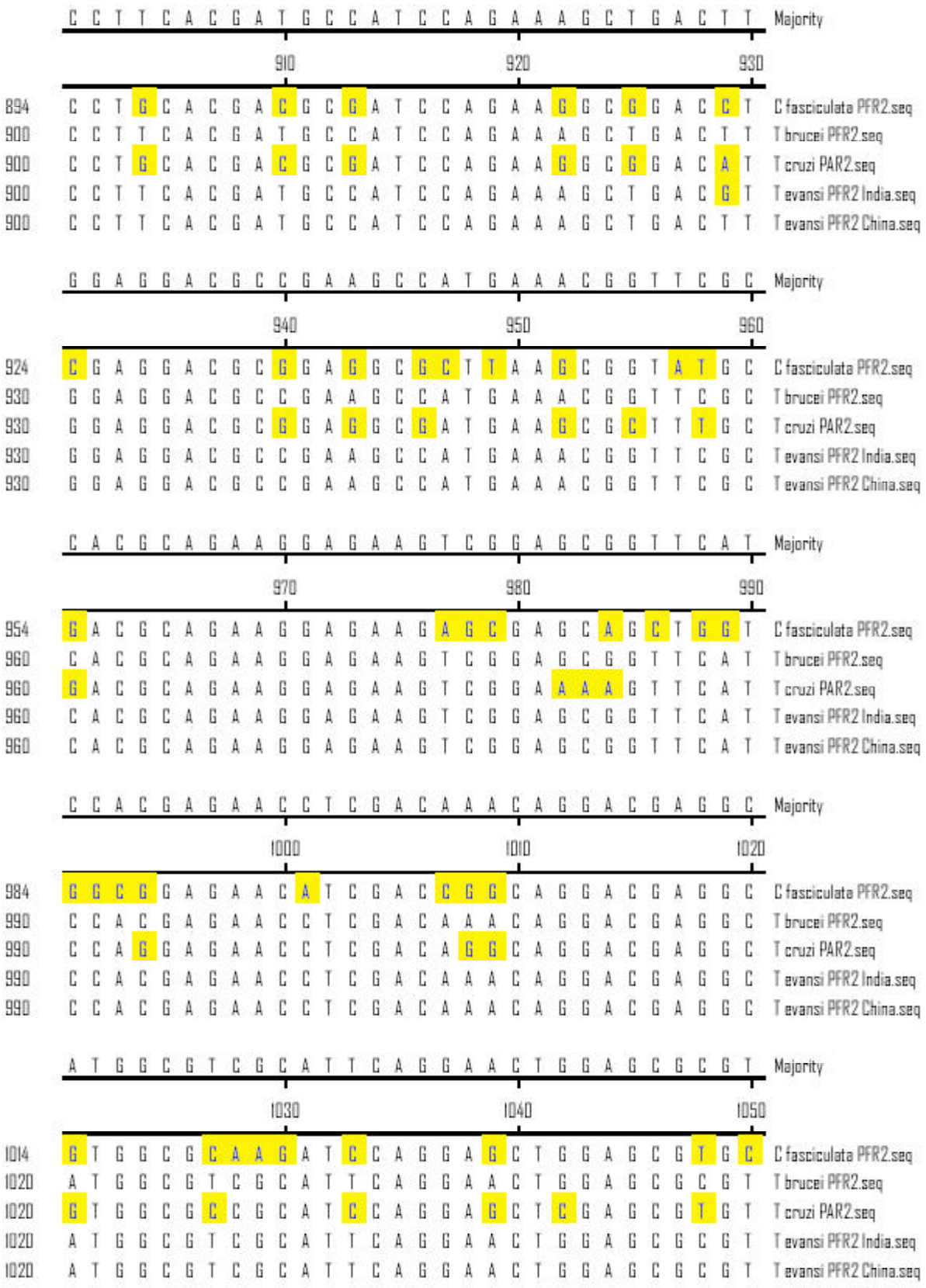
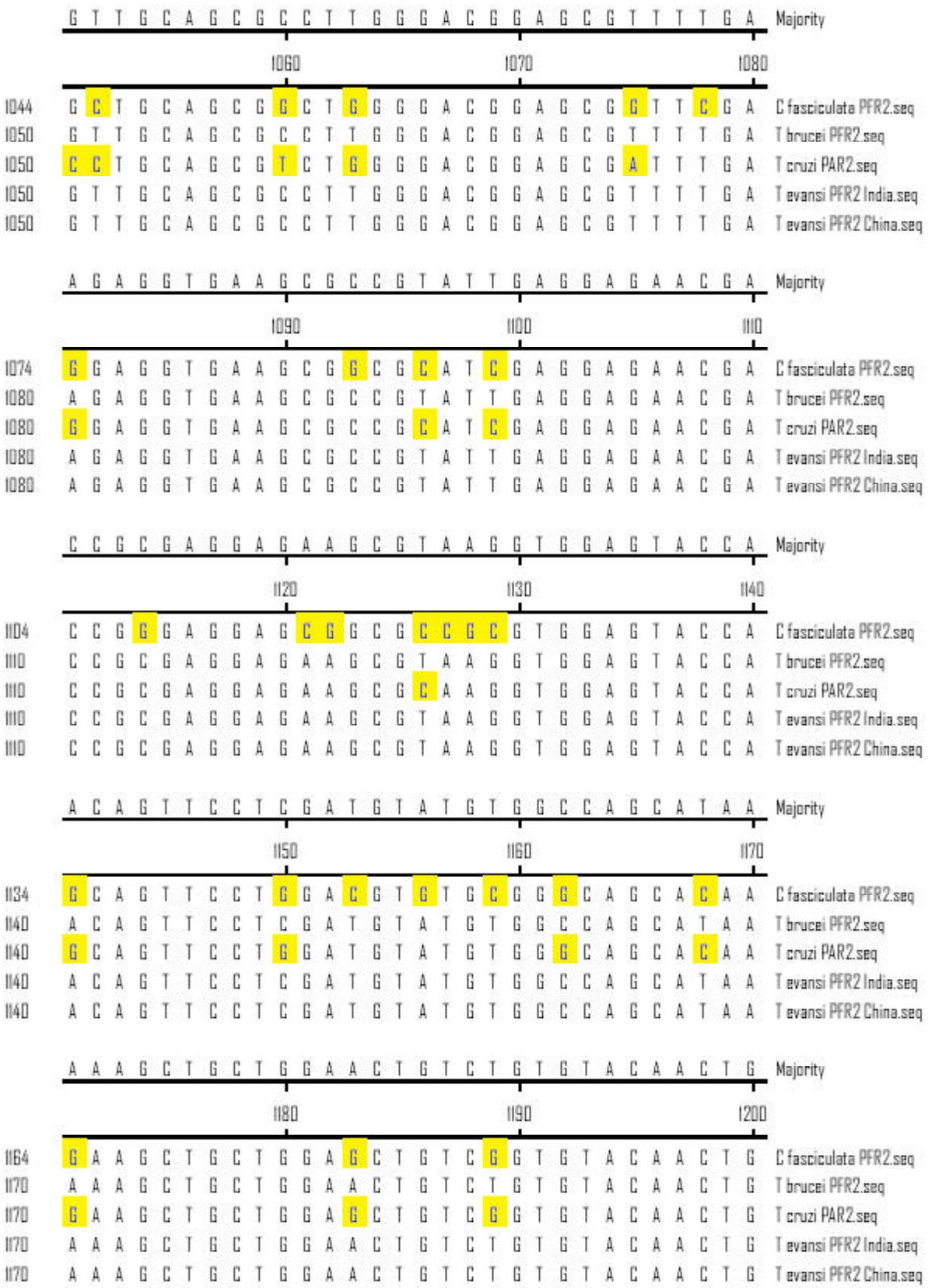
760
770
780
744 G G A C A A G T T C C G G A T C A T T G G G C A G A C G G A C fasciculata PFR2.seq
750 C G A C A A G T T T C G C A T C A T T G G G C A A A C T G A T brucei PFR2.seq
750 C G A C A A G T T C C G C A T A A T T G G A C A G A C G G A T cruzi PAR2.seq
750 C G A C A A G T T T C G C A T C A T T G G G C A A A C T G A T evansi PFR2 India.seq
750 C G A C A A G T T T C G C A T C A T T G G G C A A A C T G A T evansi PFR2 China.seq
G G A T G A G A A T A A G A G C T T C A G T A A G A T C C A Majority
790
800
810
774 G G A C G A G A A C A A G G G C T T C G A G C G G A T C G C C fasciculata PFR2.seq
780 G G A T G A G A A T A A G A G C T T C A G T A A G A T C C A T brucei PFR2.seq
780 G G A C G A G A A C A A G C C G T T T G G T C G C A T C C A T cruzi PAR2.seq
780 G G A T G A G A A T A A G A G C T T C A G T A A G A T C C A T evansi PFR2 India.seq
780 G G A T G A G A A T A A G A G C T T C A G T A A G A T C C A T evansi PFR2 China.seq
C G A G G T A C A G A A G A A G T C A T T T C A G G A A T C Majority
820
830
840
804 C G A C A C G C A G A A G C G C G C G T T C C A G G A G A C C fasciculata PFR2.seq
810 C G A G G T A C A G A A G A A G T C A T T T C A G G A A T C T brucei PFR2.seq
810 G G A T G T G C A G A A G A A G T C G T T C C A G G A G A C T cruzi PAR2.seq
810 C G A G G T A C A G A A G A A G T C A T T T C A G G A A T C T evansi PFR2 India.seq
810 C G A G G T A C A G A A G A A G T C A T T T C A G G A A T C T evansi PFR2 China.seq
T G C C T C A A T C A A G G A C G C G A A G C G C C G C C T Majority
850
860
870
834 G G C G G C G C T G A A G G A C G G G A A G C G G C G G C T C fasciculata PFR2.seq
840 T G C C T C A A T C A A G G A C G C G A A G C G C C G C C T T brucei PFR2.seq
840 A T C C G C G A T C A A G G A C G C G A A G C G A C G G C T T cruzi PAR2.seq
840 T G C C T C A A T C A A G G A C G C G A A G C G C C G C C T T evansi PFR2 India.seq
840 T G C C T C A A T C A A G G A C G C G A A G C G C C G C C T T evansi PFR2 China.seq
T A A G C A A C A C T G C G A G G A C G A C C T A C G T A A Majority
880
890
900
864 G A A G G G G C G G T G T G A G G A C G A C C T G C G C A G C fasciculata PFR2.seq
870 T A A G C A A C A C T G C G A G G A C G A C C T A C G T A A T brucei PFR2.seq
870 G A A G C A G C G C T G C G A G G A C G A C C T G A A G A A T cruzi PAR2.seq
870 T A A G C A A C A C T G C G A G G A C G A C C T A C G T A A T evansi PFR2 India.seq
870 T A A G C A A C A C T G C G A G G A C G A C C T A C G T A A T evansi PFR2 China.seq


Majority
910
920
930
894 C fasciculata PFR2.seq
900 T brucei PFR2.seq
900 T cruzi PAR2.seq
900 T evansi PFR2 India.seq
900 T evansi PFR2 China.seq
Majority
940
950
960
924 C fasciculata PFR2.seq
930 T brucei PFR2.seq
930 T cruzi PAR2.seq
930 T evansi PFR2 India.seq
930 T evansi PFR2 China.seq
Majority
970
980
990
954 C fasciculata PFR2.seq
960 T brucei PFR2.seq
960 T cruzi PAR2.seq
960 T evansi PFR2 India.seq
960 T evansi PFR2 China.seq
Majority
1000
1010
1020
984 C fasciculata PFR2.seq
990 T brucei PFR2.seq
990 T cruzi PAR2.seq
990 T evansi PFR2 India.seq
990 T evansi PFR2 China.seq
Majority
1030
1040
1050
1014 C fasciculata PFR2.seq
1020 T brucei PFR2.seq
1020 T cruzi PAR2.seq
1020 T evansi PFR2 India.seq
1020 T evansi PFR2 China.seq

contd.....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1070
1080
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1050 T cruzi PAR2.seq
1050 T evansi PFR2 India.seq
1050 T evansi PFR2 China.seq
Majority
1090
1100
1110
1074 C fasciculata PFR2.seq
1080 T brucei PFR2.seq
1080 T cruzi PAR2.seq
1080 T evansi PFR2 India.seq
1080 T evansi PFR2 China.seq
Majority
1120
1130
1140
1104 C fasciculata PFR2.seq
1110 T brucei PFR2.seq
1110 T cruzi PAR2.seq
1110 T evansi PFR2 India.seq
1110 T evansi PFR2 China.seq
Majority
1150
1160
1170
1134 C fasciculata PFR2.seq
1140 T brucei PFR2.seq
1140 T cruzi PAR2.seq
1140 T evansi PFR2 India.seq
1140 T evansi PFR2 China.seq
Majority
1180
1190
1200
1164 C fasciculata PFR2.seq
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1170 T cruzi PAR2.seq
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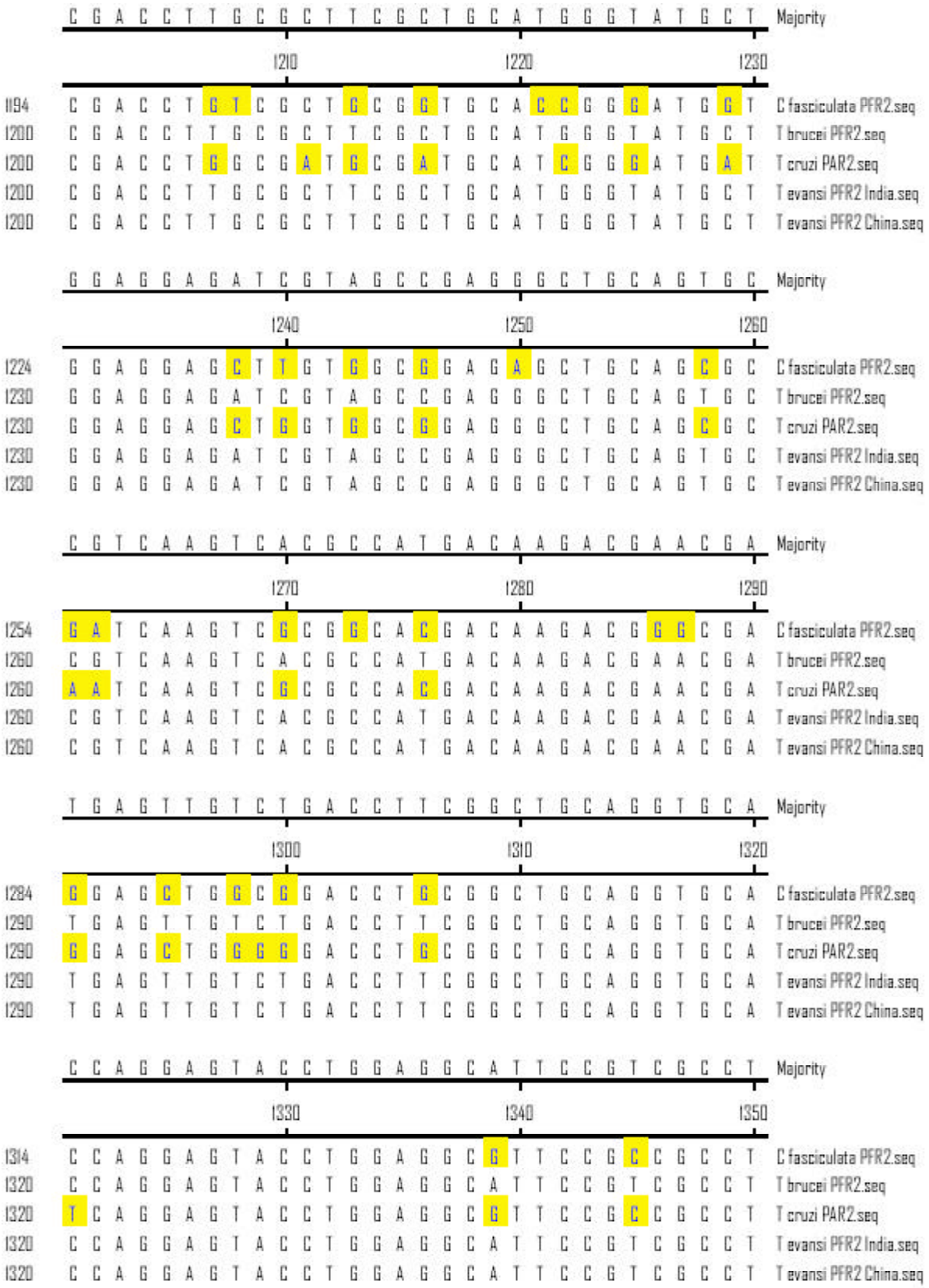
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1230
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1200 T brucei PFR2.seq
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1200 T evansi PFR2 India.seq
1200 T evansi PFR2 China.seq
Majority
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1250
1260
1224 C fasciculata PFR2.seq
1230 T brucei PFR2.seq
1230 T cruzi PAR2.seq
1230 T evansi PFR2 India.seq
1230 T evansi PFR2 China.seq
Majority
1270
1280
1290
1254 C fasciculata PFR2.seq
1260 T brucei PFR2.seq
1260 T cruzi PAR2.seq
1260 T evansi PFR2 India.seq
1260 T evansi PFR2 China.seq
Majority
1300
1310
1320
1284 C fasciculata PFR2.seq
1290 T brucei PFR2.seq
1290 T cruzi PAR2.seq
1290 T evansi PFR2 India.seq
1290 T evansi PFR2 China.seq
Majority
1330
1340
1350
1314 C fasciculata PFR2.seq
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1320 T cruzi PAR2.seq
1320 T evansi PFR2 India.seq
1320 T evansi PFR2 China.seq

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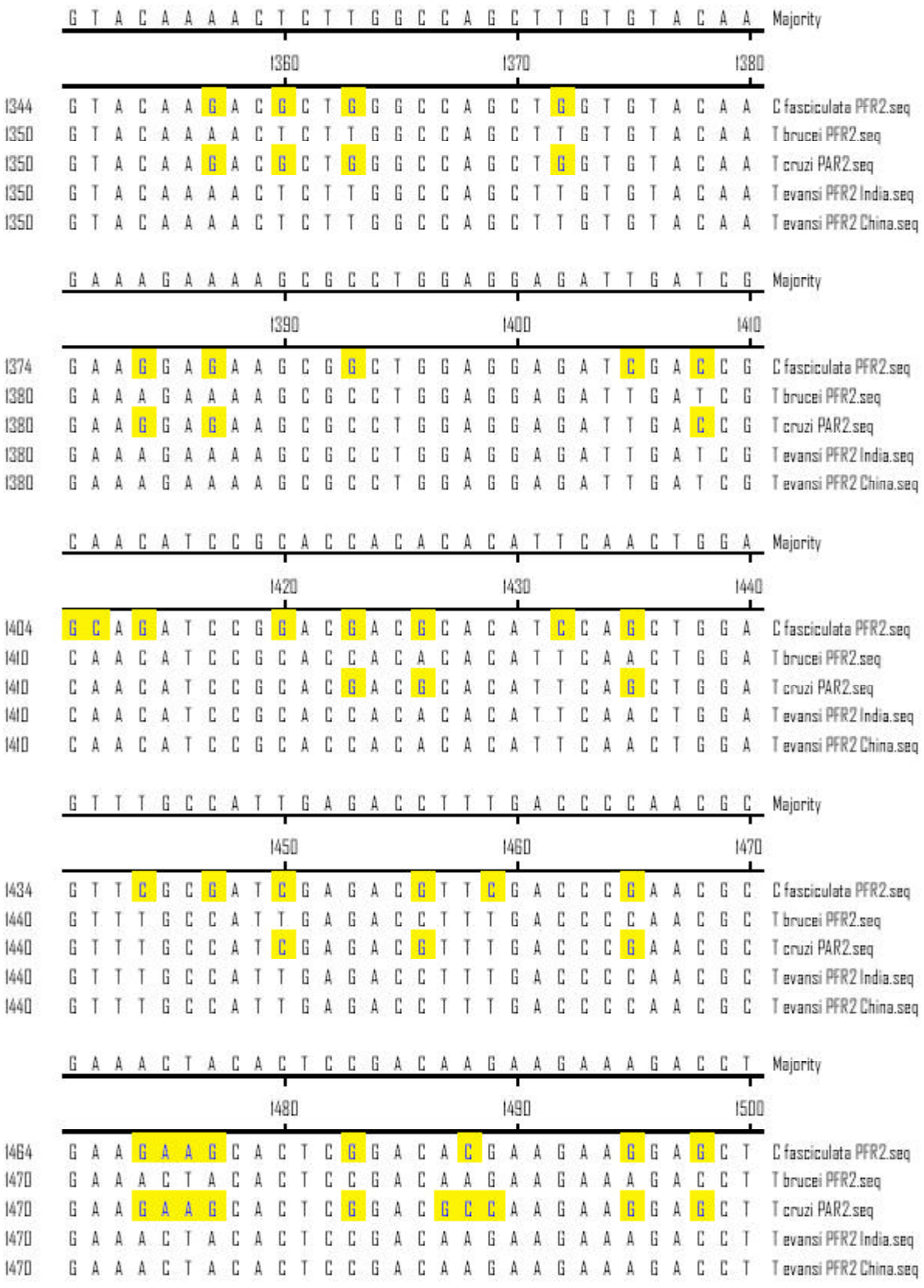
Majority
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1370
1380
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1350 T evansi PFR2 India.seq
1350 T evansi PFR2 China.seq
Majority
1390
1400
1410
1374 C fasciculata PFR2.seq
1380 T brucei PFR2.seq
1380 T cruzi PAR2.seq
1380 T evansi PFR2 India.seq
1380 T evansi PFR2 China.seq
Majority
1420
1430
1440
1404 C fasciculata PFR2.seq
1410 T brucei PFR2.seq
1410 T cruzi PAR2.seq
1410 T evansi PFR2 India.seq
1410 T evansi PFR2 China.seq
Majority
1450
1460
1470
1434 C fasciculata PFR2.seq
1440 T brucei PFR2.seq
1440 T cruzi PAR2.seq
1440 T evansi PFR2 India.seq
1440 T evansi PFR2 China.seq
Majority
1480
1490
1500
1464 C fasciculata PFR2.seq
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1470 T cruzi PAR2.seq
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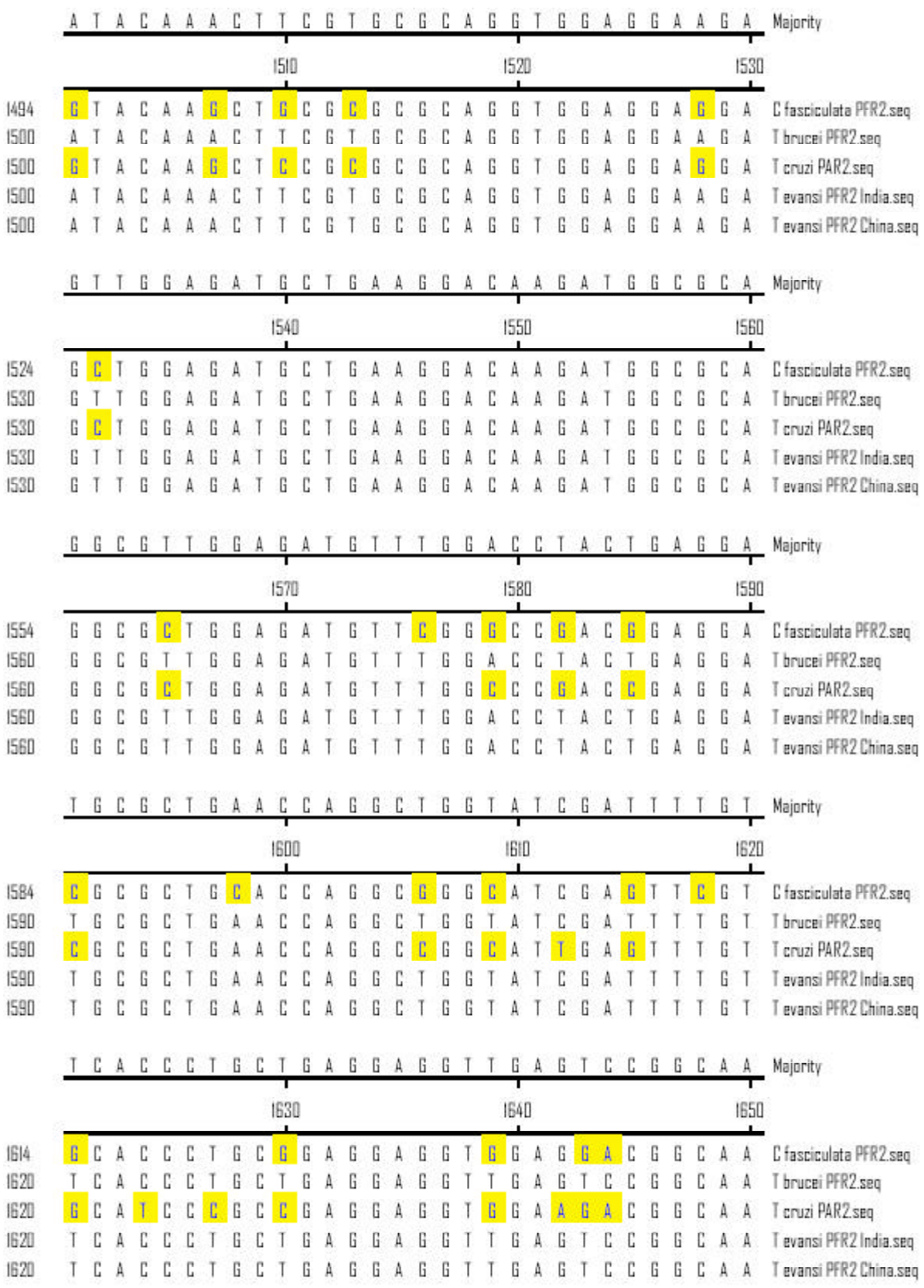
1470 T evansi PFR2 China.seq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1500 A T A C A A A C T T C G T G C G C A G G T G G A G G A A G A T brucei PFR2.seq
1500 G T A C A A G C T C C G C G C A G G T G G A G G A G G A T cruzi PAR2.seq
1500 A T A C A A A C T T C G T G C G C A G G T G G A G G A A G A T evansi PFR2 India.seq
1500 A T A C A A A C T T C G T G C G C A G G T G G A G G A A G A T evansi PFR2 China.seq
G T T G G A G A T G C T G A A G G A C A A G A T G G C G C A Majority
1540 1550 1560
1524 G C T G G A G A T G C T G A A G G A C A A G A T G G C G C A C fasciculata PFR2.seq
1530 G T T G G A G A T G C T G A A G G A C A A G A T G G C G C A T brucei PFR2.seq
1530 G C T G G A G A T G C T G A A G G A C A A G A T G G C G C A T cruzi PAR2.seq
1530 G T T G G A G A T G C T G A A G G A C A A G A T G G C G C A T evansi PFR2 India.seq
1530 G T T G G A G A T G C T G A A G G A C A A G A T G G C G C A T evansi PFR2 China.seq
G G C G T T G G A G A T G T T T G G A C C T A C T G A G G A Majority
1570 1580 1590
1554 G G C G C T G G A G A T G T T C G G G C C G A C G G A G G A C fasciculata PFR2.seq
1560 G G C G T T G G A G A T G T T T G G A C C T A C T G A G G A T brucei PFR2.seq
1560 G G C G C T G G A G A T G T T T G G C C G A C C G A G G A T cruzi PAR2.seq
1560 G G C G T T G G A G A T G T T T G G A C C T A C T G A G G A T evansi PFR2 India.seq
1560 G G C G T T G G A G A T G T T T G G A C C T A C T G A G G A T evansi PFR2 China.seq
T G C G C T G A A C C A G G C T G G T A T C G A T T T T G T Majority
1600 1610 1620
1584 C G C G C T G C A C C A G G C G G C A T C G A G T T C G T C fasciculata PFR2.seq
1590 T G C G C T G A A C C A G G C T G G T A T C G A T T T T G T T brucei PFR2.seq
1590 C G C G C T G A A C C A G G C C G G C A T T G A G T T T G T T cruzi PAR2.seq
1590 T G C G C T G A A C C A G G C T G G T A T C G A T T T T G T T evansi PFR2 India.seq
1590 T G C G C T G A A C C A G G C T G G T A T C G A T T T T G T T evansi PFR2 China.seq
T C A C C C T G C T G A G G A G G T T G A G T C C G G C A A Majority
1630 1640 1650
1614 G C A C C C T G C G G A G G A G G T G G A G G A G C G G C A A C fasciculata PFR2.seq
1620 T C A C C C T G C T G A G G A G G T T G A G T C C G G C A A T brucei PFR2.seq
1620 G C A T C C C G C C G A G G A G G T G G A A G A C G G C A A T cruzi PAR2.seq
1620 T C A C C C T G C T G A G G A G G T T G A G T C C G G C A A T evansi PFR2 India.seq
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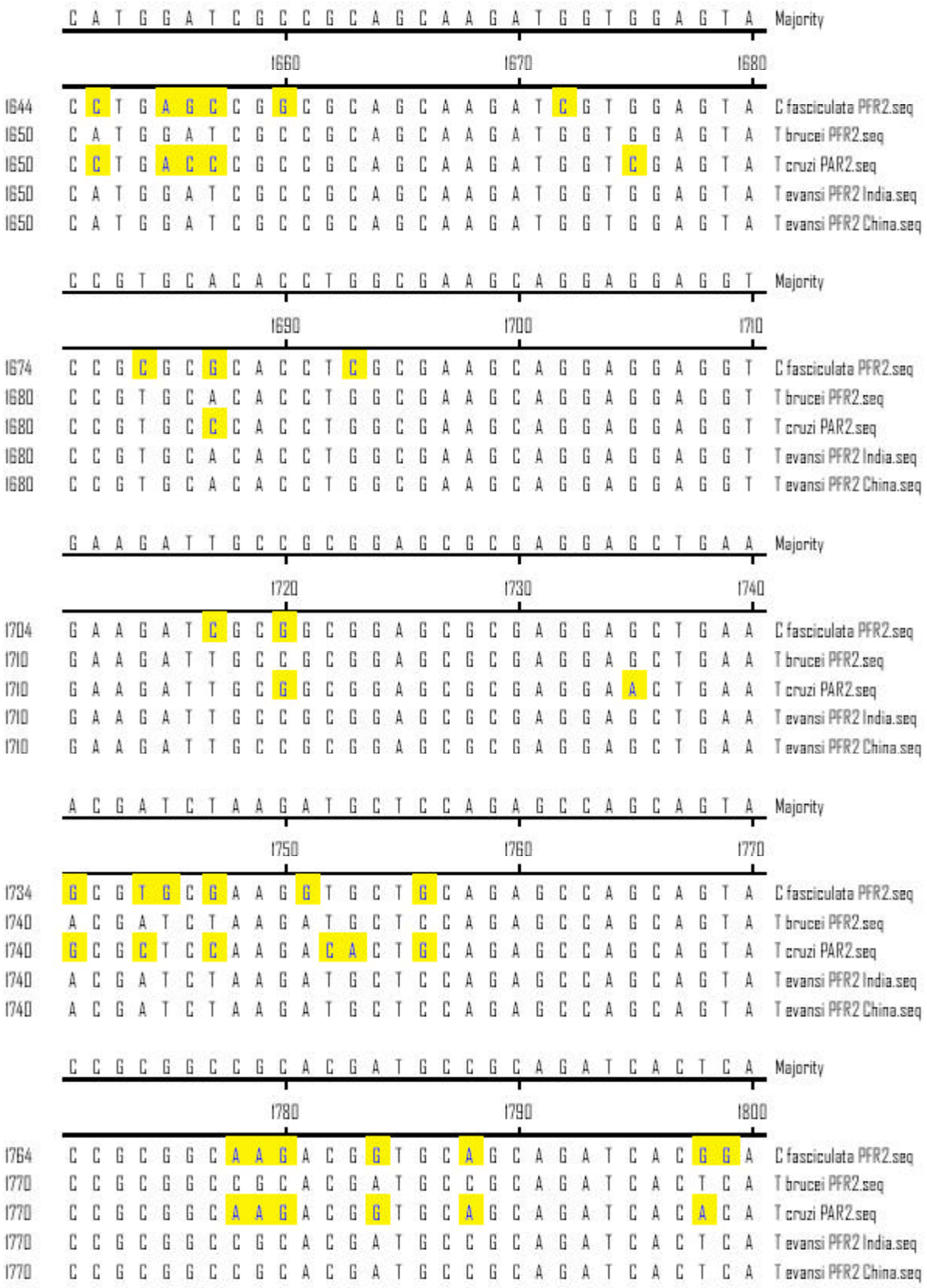
contd.....


Majority
1660
1670
1680
1644 C fasciculata PFR2.seq
1650 T brucei PFR2.seq
1650 T cruzi PAR2.seq
1650 T evansi PFR2 India.seq
1650 T evansi PFR2 China.seq
Majority
1690
1700
1710
1674 C fasciculata PFR2.seq
1680 T brucei PFR2.seq
1680 T cruzi PAR2.seq
1680 T evansi PFR2 India.seq
1680 T evansi PFR2 China.seq
Majority
1720
1730
1740
1704 C fasciculata PFR2.seq
1710 T brucei PFR2.seq
1710 T cruzi PAR2.seq
1710 T evansi PFR2 India.seq
1710 T evansi PFR2 China.seq
Majority
1750
1760
1770
1734 C fasciculata PFR2.seq
1740 T brucei PFR2.seq
1740 T cruzi PAR2.seq
1740 T evansi PFR2 India.seq
1740 T evansi PFR2 China.seq
Majority
1780
1790
1800
1764 C fasciculata PFR2.seq
1770 T brucei PFR2.seq
1770 T cruzi PAR2.seq
1770 T evansi PFR2 India.seq
1770 T evansi PFR2 China.seq

contd.....

	G T A G	Majority
1794	G T A G	C fasciculata PFR2.seq
1800	G T A G	T brucei PFR2.seq
1800	G T A A	T cruzi PAR2.seq
1800	G	T evansi PFR2 India.seq
1800	G T A G	T evansi PFR2 China.seq

Decoration 'Decoration #1': Shade (with solid bright yellow) residues that differ from the Consensus.

Percent Identity

Divergence

	1	2	3	4	5	
1		75.0	84.1	74.8	75.0	1
2	25.7		82.4	99.9	100.0	2
3	15.0	19.0		82.4	82.4	3
4	25.8	0.1	19.0		99.9	4
5	25.7	0.0	19.0	0.1		5
	1	2	3	4	5	

C fasciculata PFR2.seq
T brucei PFR2.seq
T cruzi PAR2.seq
T evansi PFR2 India.seq
T evansi PFR2 China.seq

Fig.24 : Sequence pair wise distances of PFR2 (Nucleotide sequence)

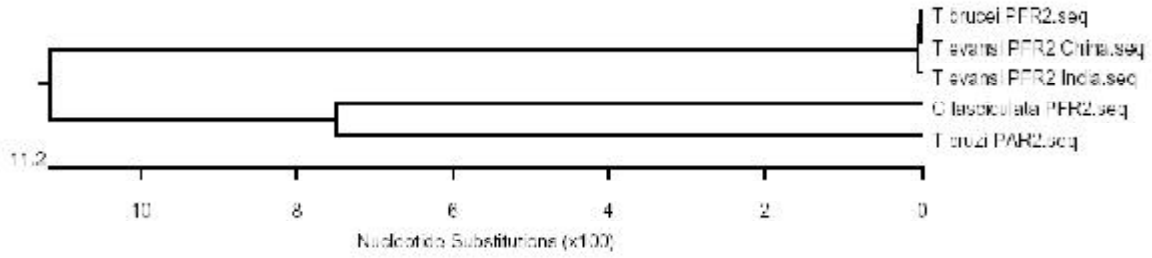


Fig.25 : Phylogenetic tree analysis of PFR2 (Nucleotide sequence)

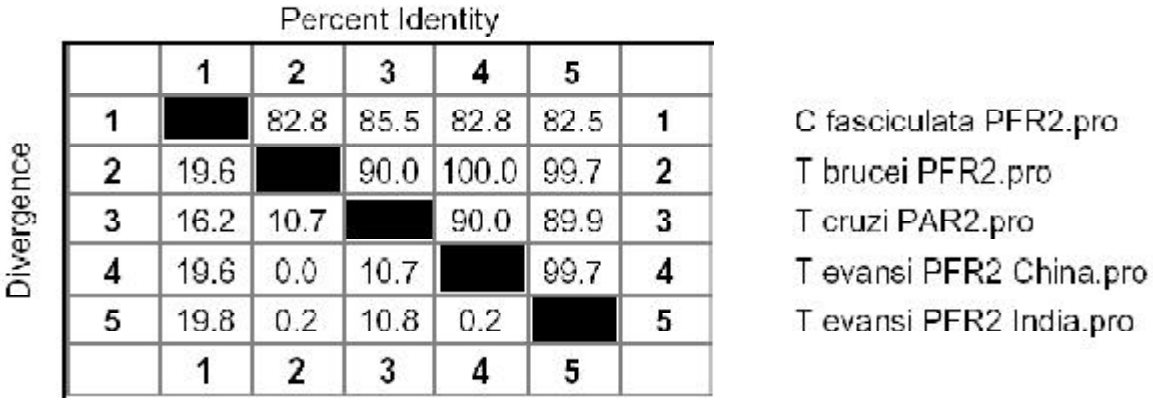


Fig.26 : Sequence pair wise distances of PFR2 (Deduced amino acid sequence)

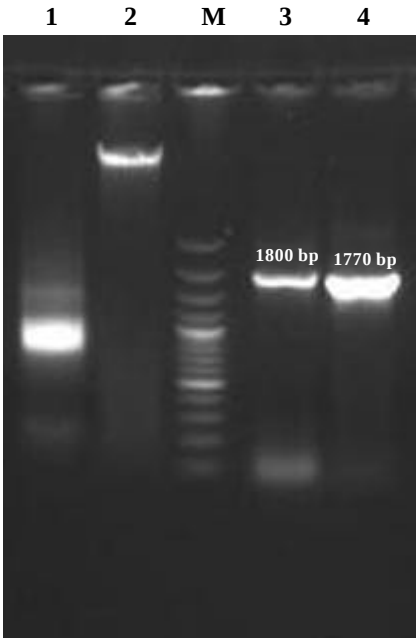


Fig.27 : Specific PCR amplification from DNA of *T. evansi*

Lane M : 100 bp plus DNA ladder plus
Lane 1 : RNA of *T. evansi*
Lane 2 : DNA of *T. evansi*
Lane 3 : Amplification of PFR2 from *T. evansi* DNA
Lane 4 : Amplification of PFR1 from *T. evansi* DNA

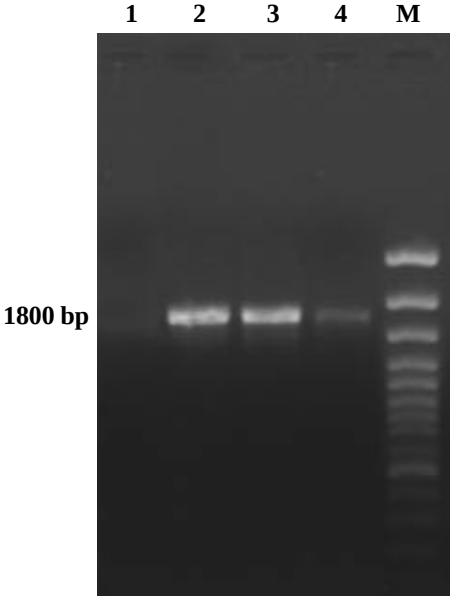


Fig.28 : Colony PCR amplification of PFR2 gene from transformed colonies

Lane M : 100 bp DNA ladder plus
Lane 1 : Control
Lane 2,3&4 : PFR2 amplicon from colony PCR

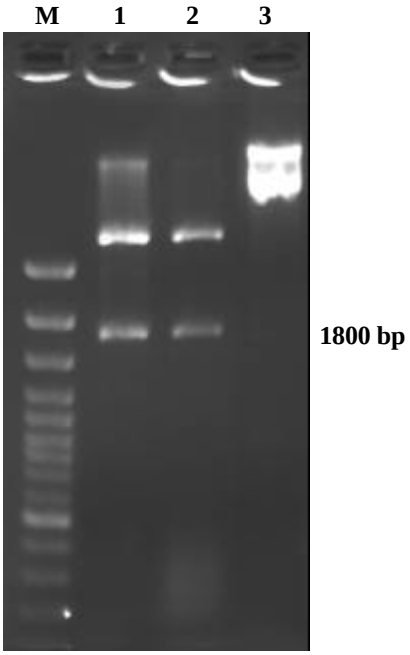


Fig.29 : RE analysis for confirming the presence of PFR2 gene in pET32a expression vector

Lane M : 100 bp DNA ladder plus
Lane 1,2 : Recombinant pET32a digested with *Eco*RI and *Hind*III
Lane 3 : Recombinant pET32a undigested

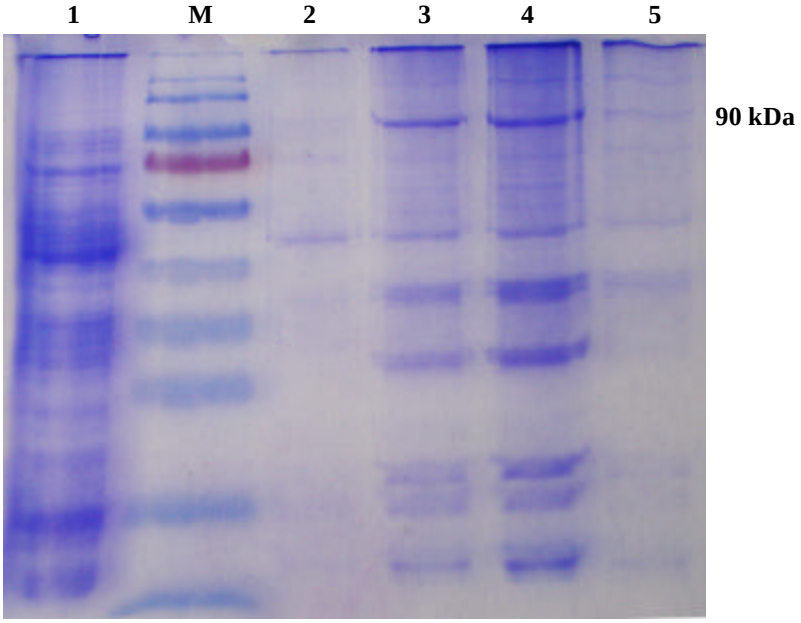


Fig. 30 : SDS-PAGE analysis of PFR2 expression

Lane M : Prestained protein ladder (Fermentas)
Lane 1 : Uninduced control
Lane 2 : 2h post induction
Lane 3 : 4h post induction
Lane 4 : 6h post induction
Lane 5 : 8h post induction

Colony PCR (Fig.28) and restriction enzyme analysis confirmed the presence of PFR2 gene specific 1800 product in gel electrophoresis (Fig.29). For expression of the protein, the positive colonies were induced with 1mM IPTG at the log growth phase of the culture when the OD reached to 0.6. Following induction, the culture was grown and the samples were collected after 4 h, 6 h, 8 h and 10 h of induction along with the control, uninduced culture for analyzing the level of expression of the target protein. SDS-PAGE analysis revealed a high level of expression of recombinant PFR2 of the molecular size of 90 kDa at 4 h of incubation (Fig 30).

Purification of recombinant PFR2

Upon centrifugation, nearly 450-500 mg of pellet was obtained from each 100 ml of culture. The pellet was resuspended in 5 ml of lysis buffer containing 8M urea. Ni-NTA agarose slurry containing 2.5 mM imidazole and 20 mM β-mercaptoethanol along with wash buffer containing 2.5 mM imidazole yielded pure recombinant PFR2 protein devoid of any bacterial protein contamination

The purity of the fusion protein was checked by SDS-PAGE analysis. The protein was resolved at 90 kDa (Fig.31). Further, the purified protein was dialysed against decreasing concentration of urea for 3 to 4 h and finally against Tris-saline pH 7.4 for 24 h at 4^0C for proper refolding.

Structure and properties of mature recombinant PFR2

The computer simulated recombinant PFR2 three dimensional model has a single domain. The protein conformation consisted of beta sheets with intermittent alpha chains. The template is monomer in nature. A brief outline of various characteristics, viz. three-dimensional structure of the expressed recombinant PFR2 protein (Fig.32, 33), the charge index , titration curve (Fig.34) the antigenicity plot, hydrophobicity plot , and the two (Fig. 35) and composition (Fig. 36) is presented here under.

Western blot analysis of expressed histidine-tagged recombinant PFR2 protein

The specific reactivity of the recombinant proteins, thus expressed, was checked by western blotting. About 500 ng of the each purified recombinant PFR2 protein was loaded for

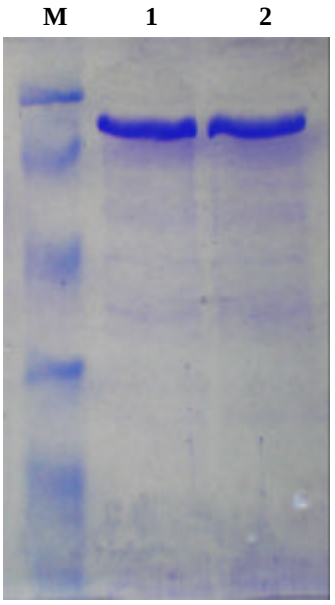


Fig.31 : SDS-PAGE analysis of purified PFR2 fusion protein

Lane M : Prestained protein molecular weight marker

Lane 1,2 : Elute 1 & 2 from Ni-NTA agaraose column

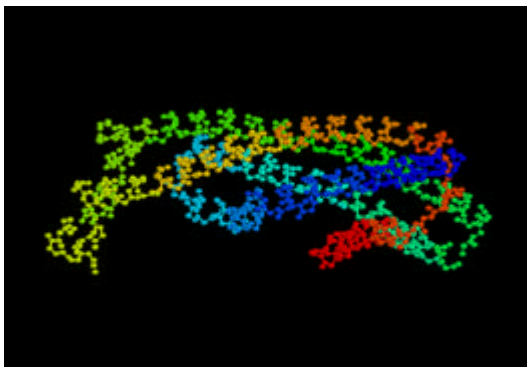


Fig.32 : 3D Ball stick model of PFR2 protein

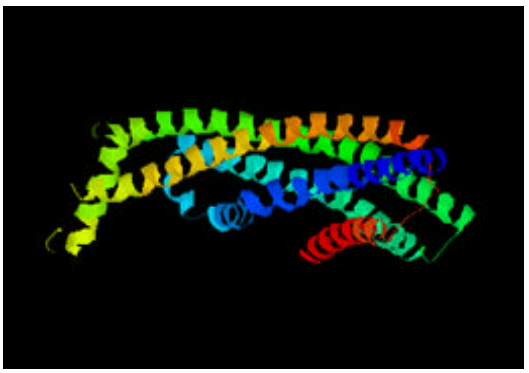


Fig.33 : 3D Ribbon model of PFR2 protein

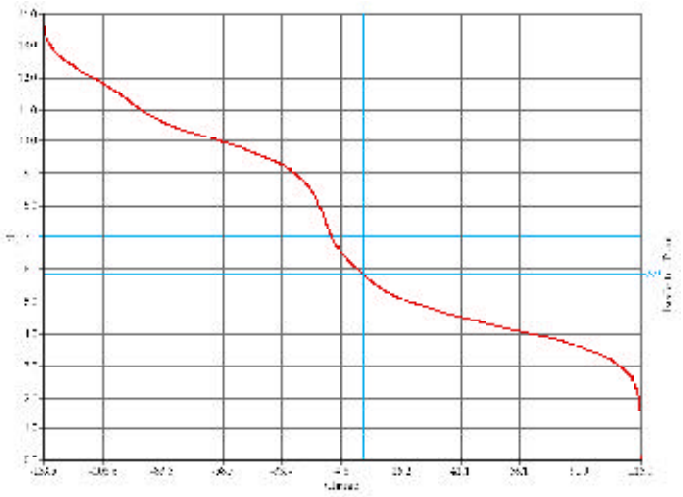


Fig.34 : Titration curve of PFR2 of *T. evansi*

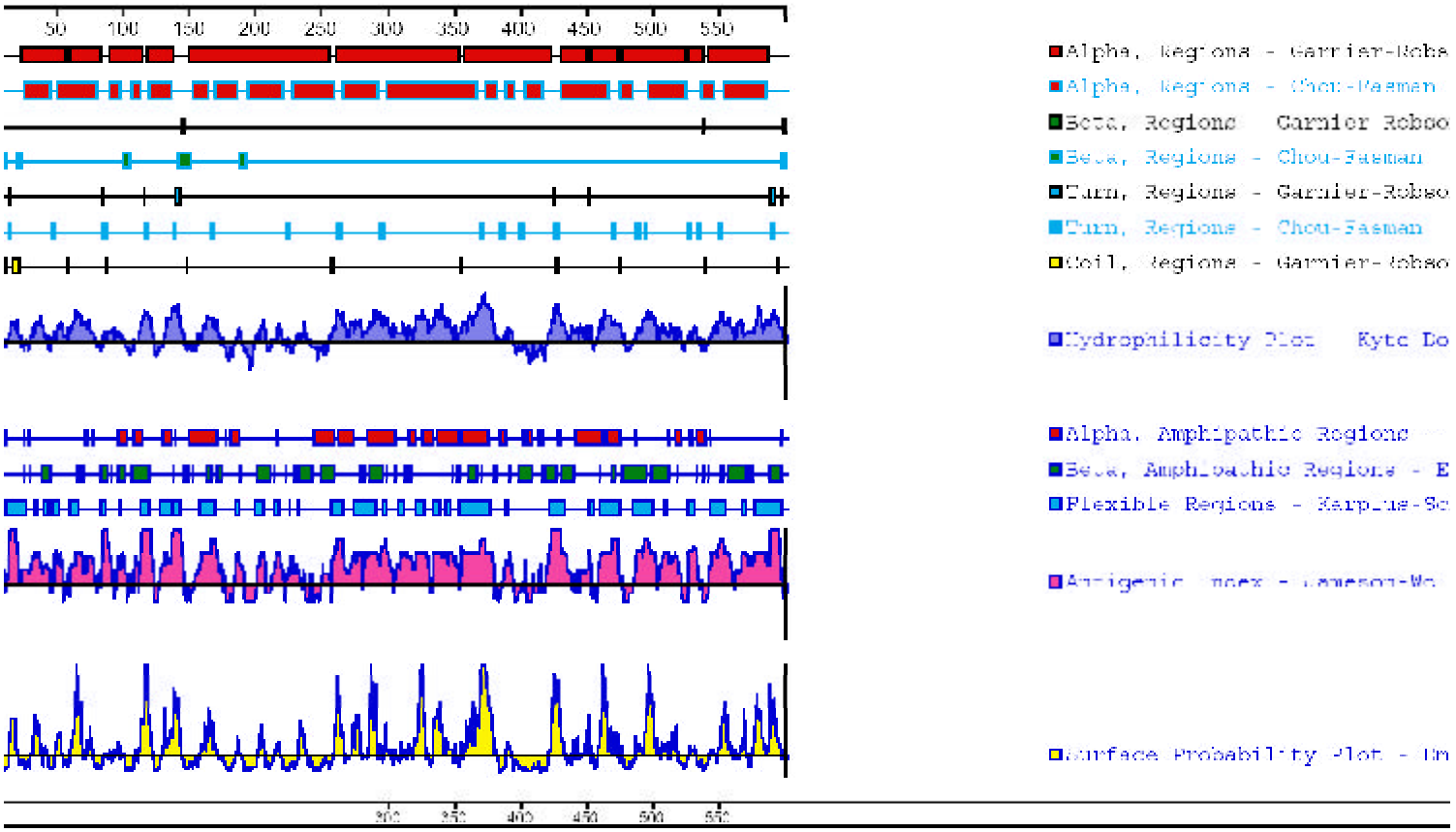


Fig.35 : Antigenic analysis of PFR2 protein using protean programme of DNA star software to predict the antigenic sites

Fig.36 : Composition of PFR2 protein

Analysis	Whole Protein
Molecular Weight	69583.26 m.w.
Length	600
1 microgram =	14.371 pMoles
Molar Extinction coefficient	34550=5%
1 A(280) =	2.01 mg/ml
Isoelectric Point	5.87
Charge at pH 7	-13.16

Whole Protein Composition Analysis

Amino Acid(s)	Number count	% by weight	% by frequency
Charged (RKHYCDE)	248	47.31	41.33
Acidic (DE)	113	20.28	18.83
Basic (KR)	97	19.40	16.17
Polar (NCQSTY)	131	21.70	21.83
Hydrophobic (AILFWV)	194	28.60	32.33
A Ala	53	5.41	8.83
C Cys	7	1.04	1.17
D Asp	34	5.62	5.67
E Glu	79	14.66	13.17
F Phe	14	2.96	2.33
G Gly	18	1.48	3.00
H His	18	3.55	3.00
I Ile	29	4.72	4.83
K Lys	59	10.87	9.83
L Leu	58	9.43	9.67
M Met	19	3.58	3.17
N Asn	20	3.28	3.33
P Pro	10	1.40	1.67
Q Gln	42	7.73	7.00
R Arg	38	8.53	6.33
S Ser	26	3.25	4.33
T Thr	23	3.34	3.83
V Val	37	5.27	6.17
W Trp	3	0.80	0.50
Y Tyr	13	3.05	2.17
B Asx	0	0.00	0.00
Z Glx	0	0.00	0.00
X Xxx	0	0.00	0.00
. Ter	0	0.00	0.00

electrophoresis on SDS-PAGE. The separated proteins were subsequently electro-transferred to nitrocellulose membranes . Subsequently, Ni-NTA anti-histidine-HRPase conjugate (Qiagen) was used for specific reactivity to the recombinant His- tagged protein, which confirmed the presence of the histidine tagged protein. The membrane was developed with DAB solution (Sigma).

The immunoreactivity at the unique 90 kDa region specific for the PFR2 on the nitrocellulose membrane confirmed the presence and purity of the recombinant proteins (Fig. 37). Similar pattern of reactivity was observed when the respective proteins were incubated with hyperimmune and known positive serum (Fig.38) and also when the respective proteins were blotted on to nitrocellulose membrane strips (Fig.39).

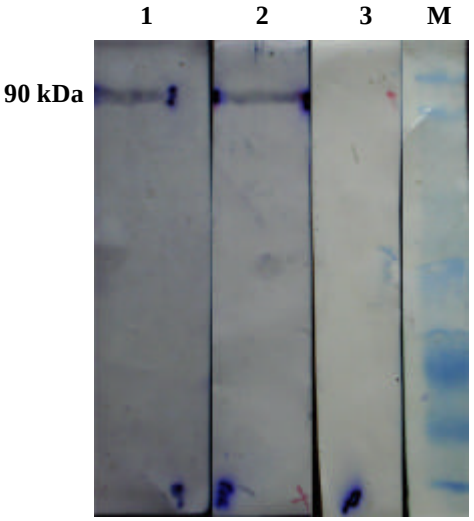


Fig.37 : Western blot analysis PFR2 protein using NINTA HRPO conjugate

Lane M : Prestained molecular weight protein marker
Lane 1 : Purified PFR2 protein
Lane 2 : Purified PFR2 protein
Lane 3 : Control

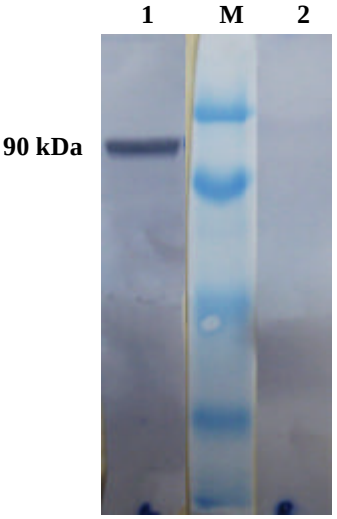


Fig.38 : Western blot analysis of purified PFR2 protein of *T. evansi* using hyperimmune sera

Lane M : Prestained molecular protein weight marker
Lane 1 : Purified PFR2 protein
Lane 2 : Negative control

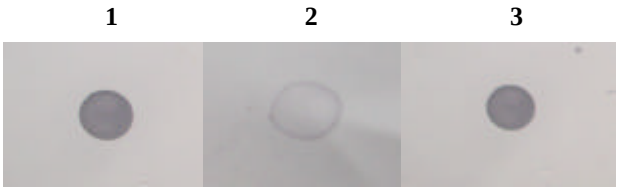


Fig.39 : Dot blot analysis of PFR 2 protein with Ni-NTA HRPO conjugate

1 : Purified PFR2 protein
2 : Control
3 : Purified PFR2 protein

Chapter VI

Discussion

Surra, caused by *Trypanosoma evansi*, is an economically important disease of a wide range of domestic and wild animals and is the most widely distributed of all animal trypanosomes. Hitherto, the little understood zoonotic nature of this organism came to light since 2005. Although the evidence for *T.evansi* as a zoonotic pathogen of humans is equivocal, there has been a notable change in the epidemiology of this protozoan infection observed in various hosts in different endemic regions. During May 2000, thirteen tigers (Pantheratigris Linn.) including twelve white tigers died in Nandankanan Biological Park,Bhubaneswar, due to an epidemic of trypanosomosis. These unprecedented deaths of wild felids due to *T.evansi* infection have attracted global attention.

Trypanosoma evansi has been the centre of intrigue to the scientific community not only as an inimitable model organism to study the life cycle and metabolic pathways in their molecular details but also for the greater practical need of developing a protective vaccine against this elusive organism. The notorious antigenic variation phenomenon is so unique that it attracts the attention of veterinary parasitologists, cell biologists and molecular biologists alike today. Significant inroads have been made over the decades towards understanding the development and multiplication of the organism as well as mechanisms of immune evasion and its patterns of persistence in the host, while the prospects for development of a successful vaccine for control of the organism still remain a distant dream. Although some effective epidemiological tools have been developed in the recent past, there are only few drugs available for effective chemotherapeutic application against the disease. The need for a safe and protective vaccine to control this widely prevalent infection needs no reiteration. Therefore, the quest for

the vaccine against trypanosomosis continues in this millenium with new insights into parasite biology and understanding the molecular basis of the pathogens and parasite survival strategies. In the absence of information about putative vaccine candidates, the prospects of potential immunoprophylactic control of *T.evansi* which is known to have limited antigenic diversity are greater than other members of trypanosoma by exploring novel targets conceptualized for the organism.

The present extends this perception by cloning and characterization of novel invariant proteins like paraflagellar-rod like protein present in the kinetoplastid flagellum. This is a unique structure of trypanosome flagellum due to presence of this paracrystaline structure (PFR).

Structurally, it extends alongside of the axoneme from the flagellar pocket to the flagellum tip. This PFR is an elegant and stable lattice-like arrangement of protein filaments which is composed of two major and related proteins PFR1 and PFR2. The protein with a highly organized three-dimensional structure has been found only in kinetoplastids, euglenoids, and some dinoflagellates. It is responsible for parasite cell motility (Clark *et al*., 2005; Bastin *et al*., 1998) and provides support for metabolic regulators that may influence flagellar beating (Catarina *et al*., 2005). For the first time, cloning and characterization of both PFR1 and PFR2 in *Trypanosoma evansi* has been carried out from India.

The present investigation describes molecular cloning of major flagellar constituent proteins like PFR1 and PFR2 and expression of recombinant PFR2, in order to study its efficiency as a target protein for development of immunity in future.

Virulent horse isolate of *T. evansi*, maintained in the Division was used in the study. Blood was collected in heparin from experimentally infected mice at teeming level of parasitaemia. The organisms were separated from the blood by ion-exchange chromatography using DEAE cellulose.

Complementary DNA (cDNA) was synthesized by reverse- transcription following the total RNA isolation from *T. evansi* using oligo dT primer. The specific primer-directed PCR amplification of the full length gene sequence (1770bp) encoding PFR1 was achieved using cDNA as template.The amplicon was purified following standard protocol. This purified product of 1770 bp was used for ligation in a T/A cloning vector to facilitate sequencing and

characterization. For this, pGEM-T T/A cloning vector, having an multiple cloning sites (MCS) incorporated into a LacZ a peptide coding region, was chosen for easy selection of recombinant clones in pGEM-T cloning vector. This is the first information on the sequence generated from an Indian isolate of *T. evansi* and submitted to GenBank from India (Accession No. FJ968743). The nucleotide sequence revealed 99.9% homology and only one nucleotide variation at 867th position where guanine (G) was found to replace cytosine (C) of PFR1 ORF between the Izatnagar and China isolates. Change in one aminoacid with lysine in place of aspargine indicates that these two isolates donot show any discernible polymorphism though stands geographically and distinctly separated. The nucleotide sequence also showed 99.8%, 82.3% and 79.8% homology with *T. brucei, T. cruzi and C. fasciculata,* respectively. This indicates that the PFR1 sequence is not only conserved among different strains within one trypanosome species but also in other species of the same genus.

Similarly, a PCR product of 1800 bp specific for PFR2 of *T. evansi* was amplified using gene-specific forward and reverse primers. The amplicon was purified following the standard protocol. The full length PFR2 sequence was cloned in pDRIVE cloning vector and the sequence information was submitted to GenBank (Accession No.FJ901341). This is the first information on the sequence generated from an Indian isolate of *T. evansi* and submitted to GenBank from India. The nucleotide sequence revealed 99.9% homology and variation with only one nucleotide at position 928 was observed where thymine (T) is replaced by guanine (G) of PFR2 ORF between the Izatnagar and China isolates. Variation in one aminoacid with valine instead of leucine was observed. The nucleotide sequence also showed 99.9%, 82.4% and 74.8% homology with *T. brucei, T. cruzi and C. fasciculata,* respectively.

In vitro expression of PFR2 protein was carried out using pET32a vector, as His- tagged fusion protein incorporating *EcoRI* and *HindIII* restriction sites in the expression primers. The *EcoRI* and *HindIII* restriction enzymes were incorporated in the expression primers since the sequence information of PFR2 gene revealed the absence of the sites for either of these restriction enzymes.

The BL-21 strain of *E. coli*, used as the host cell, has plasmid conferring resistance to chloramphenicol incorporated into it. Since, the pET32a vector plasmid has ampicillin resistance gene engineered into it, the transformed BL-21 cell showed resistance to both of the antibiotics,

which ensured the growth of the transformed cells in a medium containing the standard inhibitory concentrations of the antibiotics ampicillin (@ 100ìg/ml) and chloramphenicol (@ 34ìg/ml). The recombinant clones were analysed by colony PCR and insert release analysis by restriction digestion using *EcoRI* and *HindIII* for confirmation of the desired insert. The amplicon was cloned into pET32a vector and expressed in the prokaryotic system, using BL21 strain of *E. coli*. A high level of expression PFR2 was noted following six hours of induction of the culture with 1mM IPTG. Adbille *et al*. (2008) used pET 21a vector and expressed PFR2 of *T. evansi* in BL21 (DE3) pLysS bacteria. The workers reported maximum yield of the protein at 6 hours post-induction. The mature PFR2 protein comprised of 600 amino acids with a deduced molecular weight of 69kDa (Adbille *et al*., 2008). But in the present study, pET32a vector has been used for expression. As this vector contains HisTag, TrxTag and S Tag, it adds about 20 kDa length of the recombinant PFR2 protein. However, the expressed fusion protein was resolved at 89 kDa in SDS-PAGE. The deduced amino acid sequence of *T. evansi* PFR2 revealed 99.7% homology between Izatnagar and China isolates. There is variation in one aminoacid with valine instead of leucine. It also showed 99.7%, 89.9% and 82.5% homology with *T.brucei, T.cruzi and C.fasciculata*, respectively, indicating that the PFR2 gene is highly conserved in the kinetoplastid species.

The full length PFR2 proteins were purified using Ni-NTA agarose beads, where the 6x His-tag binds to the Ni-NTA column and the proteins were eluted by imidazole mediated competitive recovery at low pH. Purification was done under denaturing conditions using 8M urea to lyse and recover the cytoplasmic contents into the lysis buffer supernatent. The refolding of the eluted protein was achieved by dialysis against Tris-saline (pH 7.4). The immuno-blot analysis using specific Ni-NTA HRP conjugate, that binds specifically with the histidine tagged protein confirmed that the recombinant proteins were expressed and purified efficiently. Similarly, the hyper-immune and known positive and negative sera were used to confirm the recombinant protein.

Eukaryotic genes expressed in a prokaryotic host system using *E. coli,* often appear as insoluble inclusion body, especially when the expression level is high. As reported previously, it has also been observed that following solubilization with a denaturing reagent (8M urea), the purified expression product may tend to lose its immunoreactivity because of its random coil conformation following denaturation. Therefore, it was necessary to get the protein refolded to

its native conformation state to ensure the desired level of immunoreactivity. In our study, the inclusion bodies were purified from the cell lysates by treating with denaturating lysis buffer containing urea and subjected to centrifugation. Following solubilization, the soluble proteins were bound to Ni-NTA agarose containing imidazole and b-mercaptoethanol to lessen the non-specific binding and break any bonding of the recombinant protein with bacterial proteins. It was observed that dialysis of the recombinant denatured PFR2 against the decreasing molar solutions of urea in TSE buffer yielded satisfactory result.

In the present study, PFR2 was expressed as a fusion protein in *E.coli*, a prokaryotic expression system. It is well documented that several post- translational modifications precede before integrating the 600 aminoacid long pro-PFR2 peptide to the flagellar constituent. This post-translational modification of the protein may not have been achieved by expressing this protein in *E.coli* and the lack of these post-translational modifications on the biological activity and immunoprophylactic potential of this protein needs to be ascertained through further biological experiments.

The principal finding of this investigation therefore, was the identification of constituents of the paraflagellar rod-like protein i.e PFR1 and PFR2 gene in *T.evansi* in horse strain of Izatnagar isolate. The sequence analysis of both PFR1 and PFR2 revealed that these genes are highly conserved in kinetoplastid species. Another important finding was the intron less genes. To study the intron-less nature of both the genes, amplification of the target DNA coding for PFR1 and PFR2 was also done from the whole genomic DNA template of *T. evansi* (horse strain). Similar sized amplicons for both PFR1 and PFR2 genes only confirmed the intron-less nature of these genes. This PFR is necessary for proper parasitic motility and viability and further known to play a key role in the life cycle of this fascinating organism. PFR proteins have been demonstrated to be immunogenic when PFR2 was used alone (Saravia *et al*., 2004) and/or co-administered together with PFR1 and PFR2 against *T.cruzi* infection in mice (Luhrs *et al*., 2003). More over, it is interesting to note that they bear no homology to any human and livestock animal proteins (Clark *et al*., 2005). Till date, there is no report of vaccination study on this target molecule in *T.evansi*. The above research findings together with the reported homology only further reaffirm the notion that vaccination with PFR molecule(s) could be effective not only in different strains within a trypanosome species but also against

other species of the same genus. It is also likely that a PFR DNA or protein subunit vaccine with multiple epitopes of PFR proteins could be more immunogenic and effective against trypanosome infections than a monovalent PFR vaccine. Due to the limited duration of the study, these interesting biological questions could not be answered and were beyond the purview of the present investigation.The present study has standardized the protocol of molecular cloning and expression of the PFR protein(s) for evaluation by other investigators in the laboratory. The immunogenic and protective effects of paraflagellar rod protein of *T.evansi* need to be explored in laboratory (rodent) and large experimental animal models to understand their practical value. Further studies are therefore warranted to find answers to the role of this novel molecules of trypanosomal flagellum and their morphogenesis and biological value.

Chapter VII

Summary

Surra, caused by *Trypanosoma evansi*, is an economically important disease of a wide range of domestic and wild animals and is the most widely distributed of all animal trypanosomes. These parasites occur principally in blood and tissue fluids of their hosts as intercellular parasites and cause a wide range of clinical manifestations. The report of two clinical cases of *T. evansi* infection in human beings in Maharashtra and West Bengal recreates interest in the changing epidemiology of *T. evansi* infection.

. Trypanosomes achieved the dubious distinction as elusive trypanosomes because of their host immuno-evasion tactics by a little understood phenomenon of antigenic variation. The ability to keep one-step ahead of the host immune responses is central to their survival strategy which poses serious limitations in exploitation of the conventional vaccine approaches for control of the disease. Although various target molecules have been tested for their immunoprophylactic efficacy against trypanosomosis, it is still a distant dream due to the notorious antigenic variation displayed by the organism. Vaccine development against animal trypanosomosis based on variant surface glycoprotein no longer holds any promise. This has prompted researchers to investigate the alternative novel invariant proteins like paraflagellar rod proteins present in the kinetoplastid flagellum. This is a unique structure of trypanosome flagellum due to presence of this paracrystaline structure (PFR).

Structurally, it extends alongside of the axoneme from the flagellar pocket to the flagellum tip. This PFR is an elegant and stable lattice-like arrangement of protein filaments which is composed of two major and related proteins PFR1 and PFR2. PFR is vital for trypanosome motility and cell morphogenesis. It is unique among the kinetoplastids as their heteropolymers

provide the building block of flagellum. Unlike the axoneme, which is broadly conserved among the eukaryotes, the PFR is restricted to kinetoplastids, euglenoids and dinoflagellates. It is likely that the conserved domain among various PFR genes present in kinetoplastids could be used as a target for the development of vaccine against multiple trypanosome species and calls for experimental testing.

The present investigation describes molecular cloning of major flagellar constituent proteins like PFR1 and PFR2 and expression of recombinant PFR2, in order to study its efficiency as a target protein for development of immunity in future.

Virulent *T. evansi*, horse isolate in the Division was used in the study. Blood was collected in heparin from experimentally infected mice at teeming level of parasitaemia. The organisms were separated from the blood by ion-exchange chromatography using DEAE cellulose.

Total RNA was isolated from 5 x 10^5 host cell-free trypanosomes and cDNA was synthesized using oligo-dT primers. A PCR product of 1770 bp specific for PFR1 of *T. evansi* was amplified using self-designed gene specific primers. The amplicon was purified following standard protocol.The full length PFR1 sequence was cloned in pGEMÒ-T cloning vector and the sequence information was submitted to GenBank (Accession No. FJ968743). This is the first information on the sequence generated from an Indian isolate of *T. evansi* and submitted to GenBank from India. The nucleotide sequence revealed 99.9% homology and only one nucleotide change at 867bp of PFR1 ORF between the Izatnagar and China isolates. The nucleotide sequence also showed 99.8%, 82.3% and 79.8% homology with *T. brucei*, *T. cruzi* and *C. fasciculata*, respectively.

Similarly a PCR product of 1800 bp specific for PFR2 of *T. evansi* was amplified using self-designed gene specific primers. The amplicon was purified following the standard protocol. The full length PFR2 sequence was cloned in pDRIVE cloning vector and the sequence information was submitted to GenBank (Accession No.FJ901341). This is the first information on the sequence generated from an Indian isolate of *T. evansi* and submitted to GenBank from India. The nucleotide sequence revealed 99.9% homology and only one nucleotide change at 928bp of PFR2 ORF between the Izatnagar and China isolates. The nucleotide sequence also

showed 99.9%, 82.4% and 74.8% homology with *T. brucei*, *T. cruzi* and *C. fasciculata*, respectively..

The *in vitro* expression of the PFR2 protein was carried out using pET32a vector, as His- tagged fusion protein incorporating *EcoRI* and *HindIII* restriction sites in the expression primers. The amplicon was cloned into pET32a vector and expressed in the prokaryotic system, using BL21 strain of *E. coli*. The mature PFR2 protein comprised of 600 amino acids. The deduced amino acid sequence of *T. evansi* PFR2 revealed 99.7% homology between Izatnagar and China isolate. It also showed 99.7%, 89.9% and 82.5% homology with *T.brucei, T.cruzi and C.fasciculata,* respectively

The PFR2 protein was purified using Ni-NTA agarose beads under denaturing conditions and was later renatured by dialysis aginst tris-saline (pH 7.4). Western blot analysis of expressed histidine-tagged recombinant proteins confirmed their identity. The immunoreactivity at the unique 90 kDa region specific for the PFR2 on the nitrocellulose membrane confirmed the presence and purity of the recombinant proteins. Similar pattern of reactivity was observed when the respective proteins were incubated with hyperimmune serum also when the respective proteins were blotted on to nitrocellulose membrane strips.

Chapter VII

Mini Abstract

Surra, caused by *Trypanosoma evansi*, is an economically important disease of a wide range of domestic and wild animals and is most widely distributed. These parasites occur principally in blood and tissue fluids of their hosts as intercellular parasites and cause a wide range of clinical manifestations. Since trypanosomes can effectively evade the host immune response by displaying an array of variable surface glycoproteins, attempts for developing a protective immunogen has not been met with success. Nevertheless, various target molecules have been tested with varying degree of success. PFR is one of the major constituent proteins of the flagella and structurally, it extends alongside of the axoneme from the flagellar pocket to the flagellum tip. This PFR is an elegant and stable lattice-like arrangement of protein filaments which is composed of two major and related proteins PFR1 and PFR2. PFR is vital for trypanosome motility and cell morphogenesis. Unlike the axoneme, which is broadly conserved among the eukaryotes, the PFR is restricted to kinetoplastids, euglenoids and dinoflagellates. So it has been considered as a vaccine target owing to its strategic location and invariable nature. In the present study, molecular cloning of PFR1 was carried out using pGEM®-T vector and the nucleotide sequence revealed 99.9% homology and only one nucleotide change at 867bp of PFR1 ORF between the Izatnagar and China isolates. The nucleotide sequence also showed 99.8%, 82.3% and 79.8% homology with *T. brucei*, *T. cruzi* and *C. fasciculate*, respectively. Similarly, PFR2 gene was cloned using pDRIVE vector and the nucleotide sequence revealed 99.9% homology and only one nucleotide change at 928bp of PFR2 ORF between the Izatnagar and China isolates. The nucleotide sequence also showed 99.9%, 82.4% and 74.8% homology with *T. brucei*, *T. cruzi* and *C. fasciculata*, respectively. The prokaryotic expression of PFR2 protein was done using pET32a vector, as His- tagged fusion protein in BL21 strain of *E. coli*. The PFR2 protein was purified using Ni-NTA agarose beads under denaturing conditions and was later renatured by dialysis aginst tris-saline (pH 7.4). The recombinant protein was confirmed by Western blot and dot blot analysis, using hyperimmune sera as well as Ni-NTA HRP conjugate.

Chapter VIII

लघु सारांश

ट्रिपैनोसामा इवेन्साइ द्वारा उत्पन्न सर्रा आर्थिक दृष्टि से एक मुख्यरोग है, जो पालतू एवं अन्य पशुओं में विस्तृत रूप से पाया जाता है। यह परजीवी मुख्य रूप से पोषक पशुओं के रक्त एवं उतक द्रव में अन्तरकोशीय परजीवी के रूप में पाया जाता है तथा विभिन्न प्रकार के लाक्षणिक प्रभाव उत्पन्न करता है। चूंकि ट्रिपैनोसोमा पोषक पशु के प्रतिरक्षा प्रभावों का उद्‌भेदन विभिन्न प्रकार के उतपरिर्वतनकारी सतही ग्लाइको प्रोटीन के कारण प्रभावशाली रूप से कर लेते हैं, इस कारणवश एक प्रतिरक्षक प्रतिजन के विकास में सफलता प्राप्त नहीं हुई है। पी.एफ.आर., कशाभ से सम्बद्ध एक मुख्य प्रोटीन है तथा संरचनात्मक रूप से यह एक्सोनीम के साथ–साथ कशाभक कोष से कशाभ के शीर्ष तक उपस्थित होता है। पी.एफ.आर. एक भव्य स्थाई लैटिस स्वरूप में प्रोटीन तन्तुओं के रूप में होता है तथा दो मुख्य एवं संबंधित प्रोटीन पी.एफ.आर.1 और पी.एफ.आर.2 से बनता है। पी.एफ.आर. ट्रीपैनोसोमा की गतिशीलता तथा कोशिका के संरचनाविकास के लिए अत्यावश्यक है एकजोनीम से भिन्न जो यूकैरियोट में संरक्षित है, पी.एफ.आर. केवल काइनेटोप्लास्टिड, यूग्लिना तथा द्विकशाभकों में ही पाया जाता है। अतः इसकी उपस्थिति तथा अपरिवर्तनशीलता के कारण इसे टीके के लक्ष्य के रूप में उपयुक्त पाया गया है। प्रस्तुत अध्ययन में पी.एफ.आर.1 का आण्विक पूर्वजन पी.जी.ई.एम–टी. वाहक की सहायता से किया गया तथा पी.एफ.आर. ओ.आर.एफ. के न्यूक्लियोटाइड क्रम में इज्जतनगर एवम् चीनी आइसोलेट के बीच 867 बी.पी. में भिन्नता पाई गई जबकि समरूपता 99.9 प्रतिशत थी। यह समरूपता ट्रिपैनोसोमा इवेन्साई, ट्रिपैनोसोमा क्रुजाई तथा क्रीथीडिया फैसिकुलाटा के साथ क्रमशः 99.8, 82.3 प्रतिशत तथा 79.8 प्रतिशत थी। इसी प्रकार पी.एफ.आर.2 का पूर्वजन, पी. ड्राइव वाहक की सहायता से किया गया तथा इज्जतनगर एवम् चीनी आइसोलेट के बीच न्यूक्लोटाइड समरूपता 99.9 प्रतिशत पाई गई। केवल एक 928 बी.पी. न्यूक्लियोटाइड का अन्तर पी.एफ.आर.2 ओ.आर. एफ इज्जतनगर एवं चीनी आइसोलेट के बीच पाया गया। टी. ब्रुसाई, टी. क्रुजाई एवं सी. फैसीकुलाटा के साथ न्यूक्लियोटाइड क्रम में समरूपता 99.9 प्रतिशत, 82.4 प्रतिशत तथा 74.8 प्रतिशत क्रमशः पाई गई। पी.एफ.आर.2 प्रोटीन की पूर्वकेन्द्रक में अभिव्यक्तता पी ई टी 32ए वाहक की सहायता से एवं हीस बन्धित संयुक्त प्रोटीन का ई. कोलाई बी.एल.21 स्ट्रेन में किया गया। पी.एफ. आर.2 प्रोटीन का शुद्धिकरण एनआई–एन टी ए एगरोज के दानों के प्रयोग से विकृतिकृत अवस्था में किया गया एवं इसके बाद ट्रीस सेलाइन (पी एच–7.4) के विरूद्ध अपोहन द्वारा इसका पुनः प्राकृतिकरण किया गया। पुनर्योगित प्रोटीन की उपस्थिति वेस्टर्न ब्लॉट तथा डॉट ब्लॉट विश्लेषण द्वारा सुनिश्चित की गई जिसमें अतिप्रतिरक्षित सीरम एवं एनआई–एनटीए एचआरपीओ संयुग्मी का उपयोग किया गया।

Chapter IX

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Appendix I

5X TBE buffer

Tris base	54 g
Boric Acid	27.5 g
0.5M EDTA (pH 8.0)	20 ml
Add distilled water to make up volume to 1000ml	

Tris EDTA (TE) buffer 1M (pH 8.0)

Tris-Hcl (pH 8.0)	10 mM
EDTA (pH 8.0)	1 mM

Gel Loading Dye (6X)

Tris-HCl (pH 7.6)	10 mM
Bromophenol blue	0.03%
Xylene cyanol FF	0.03%
Glycerol	60%
EDTA	60 mM

Ethidium Bromide (10mg/ml)

Ethidium bromide	10 mg
Distilled Water	1 ml

Phosphate Buffered Saline

Sodium Chloride	8 g
Potassium Chloride	0.2 g
Disodium Hydrogen Phosphate (hydrated)	1.15 g
Potassium Dihydrogen phosphate	0.20 g
Add distilled water to make up volume to 1000ml	
Adjust pH to 7.2 or 7.4	

Phosphate Saline Glucose Buffer (PSG)

Solution A:	
$Na_2HPO_4.2H_20$	13.4 g
NaH_2PO_4	0.78 g
NaCl	4.25 g
Add distilled water to make up volume to 1000 ml	
Solution B:	
Glucose	10 g
Add distilled water to make up volume to 400 ml	

Just before use, 6 parts of Solution A is mixed with 4 parts of solution B to make PSG (pH 8.0) of 1% glucose

PMSF stock (100 mM)

Phenyl methyl sulfonyl fluoride	17.4 mg
Isopropanol	1 ml

Store at -20 ^{0}C

REAGENTS FOR PLASMID ISOLATION

Resuspension buffer (P1)

Tris .Cl (pH. 8.0)	50 mM
EDTA	10 mM

Add RNase 100µg / ml and store at 4^{0}C

Lysis buffer (P2)

NaOH	200 mM
SDS	1%

Neutralization buffer (P3)

Potassium acetate	3 M

Adjust pH to 5.5 by adding glacial acetic acid

REAGENTS FOR CLONING & EXPRESSION

Luria Bertani (LB) Broth

Bacto Tryptone	1 g
NaCl	1 g
Yeast Extract	0.5 g

Add distilled water to make up volume to 100 ml

Adjust pH to 7.5 by NaOH. Sterilize by autoclaving

Luria Bertani (LB) Agar

LB Agar was prepared by adding 1.5% agarose to LB medium

X-gal (5'bromo-4-chloro-3-indolyl β-D-galactopyroside)

X-gal	25.0 mg
Dimethyl Formamide	1 ml

IPTG (Isopropylthio β-D-galactoside) (1 M)

IPTG	238.3mg
Distilled Water	1 ml

Filter, sterilize and store at -20^{0}C

Ampicillin

Ampicillin powder	100 mg
Sterile distilled water	1 ml

Filter sterilize and store at 4^0C

Chloramphenicol

Chloramphenicol powder	34 mg
Ethanol	1 ml

Filter sterilize and store at 4^0C

EDTA (Ethylene diamine tetra acetate) (0.5M, pH8.0)

EDTA	18.61 gm
Distilled water	80 ml

Stir vigorously on magnetic stirrer to mix. Adjust pH to 8.0 and make the volume up to 100ml. Autoclave and store at room temperature.

1M Calcium Chloride Solution

$CaCl_2.6H_2O$	54 g
Distilled Water	200 ml

Sterilize by passing through a 0.22μm filter.

REAGENTS FOR SDS-PAGE

30% Acrylamide stock

Acrylamide	29.0 g
N'-N bis methylene Acrylamide	1 g

Dissolve to make a final volume of 100ml. Filter and store at 4^0C in dark bottles.

Separating Gel Buffer (1.5M Tris pH 8.8)

Tris base	18.18 g
Distilled water	75 ml

Dissolve Tris and adjust the pH to 8.8 using concentrated HCl. Make up the volume to 100ml with distilled water.

Stacking Gel Buffer (1.0M Tris pH 6.8)

Tris base	12.1g
Distilled water	75 ml

Dissolve Tris and adjust the pH to 6.8 using concentrated HCl. Make up the volume to 100ml with distilled water.

10% Sodium Dodecyl Sulphate solution

SDS	1 g
Distilled water	10 ml

10% Ammonium per sulphate

APS	100 mg
Distilled water	1 ml

1X SDS PAGE Electrophoresis Buffer (pH 8.3)

Tris base (25mM)	3.025 g
Glycine (250mM)	18.75 g
SDS(0.1%)	1.0 g

Dissolve in distilled water to make up the final volume to 1000 ml

Sample Solubilizing Buffer

Glycerol (10%)	2 ml
2 Mercaptoethanol (5%)	1 ml
SDS (1%)	200 mg
0.5M Tris pH 6.8	1.7 ml
Bromophenlo blue	0.1%

Adjust the volume to 10ml with distilled water

Staining Solution

Coomasie brilliant blue R-250	200 mg
Methanol	40%
Acetic acid	10%

Distilled water to make up to 100ml

Destaining Solution

Methanol	40%
Acetic acid	7%

Distilled water to make up to 100ml

Reagents for Western Blotting

Transfer buffer

Tris base	14.4g
Glycine	3.0 g
SDS	1 g
Methanol	20%

Distilled water to make up to 1000ml

Tris buffered saline, TBS (25mM Tris)

Sodium Chloride	8 g
Potassium Chloride	0.2 g
Tris base	3 g
Distilled water	800 ml

Adjust pH to 8.0 with HCl and then make up the volume to 1000ml. Sterilize by autoclaving.

TBST

TBS	1000 ml
Tween 20	500 µl

Blocking buffer

Bovine Serum Albumin (BSA)	3ml
TBS	97 ml

Substrate buffer for development

DAB	6 g
8% Nickel chloride	50 µl
Hydrogen Peroxide	10 µl

Add PBS to make up volume to 10ml

Reagents for purification of recombinant protein

Lysis buffer

Sodium dihydrogen phosphate	50 mM
Sodium chloride	300 mM
Urea	8 M
Imidazole	10 mM

Dissolve in distilled water and adjust the pH to 8.0

Wash buffer

Sodium dihydrogen phosphate	50 mM
Sodium chloride	300 mM
Urea	8 M
Imidazole	10 mM

Dissolve in distilled water and adjust the pH to 7.0

Elution buffer

Sodium dihydrogen phosphate	50 mM
Sodium chloride	300 mM
Urea	8 M

Dissolve in distilled water and adjust the pH to 4.5

Imidazole (1M)

Imidazole powder	3.3 g

Make volume to 50 ml with distilled water and adjust pH to 7.0

Tris saline EDTA buffer

Tris (pH 7.2)	25 mM
NaCl	25 mM
EDTA	2 mM

Vitae

Dr Biswa Ranjan Maharana was born on July 11, 1981 in Nayagarh, Orissa, India. He did his schooling at Brajendra High school, Nayagarh, Orissa, where he grew up. He received his B.V.Sc. & A.H degree at College of Veterinary science and Animal Husbandry, Orissa, in August 2006.

AWARDS/HONOURS/FELLOWSHIPS:

1. Awarded certificate of excellence for **Essay writing** at School level.
2. Awarded certificate of proficiency for securing **A Grade in computer science and Applications.**
3. Awarded certificate of participation in the **7th All India Conference of the Association of Public Health veterinarians and National symposium on Prospective role of Veterinary Public Health in Integrated Rural Development** Organised by College of Veterinary Science and Animal Husbandry,O.U.A.T,Bhubaneswar.
4. University **scholarship** during B.V.Sc. & A.H programme for academic excellence.
5. **Indian Council of Agricultural Research- Junior Research Fellowship** (ICAR-JRF) during M.V.Sc. programme after competing at National level.
6. Awarded various certificates for **academic excellence**,**attending conferences** and for **participating in various extra curricular activities** at School and University level.

PROFESSIONAL MEMBERSHIPS

1. Lifetime Member of Orissa Veterinary Council.
2. Lifetime Member of Indian Association For Advancement of Veterinary Parasitology.

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