Phylogenetic Analysis of Rust Causing Fungi Based on Selected Loci of Coding Region.

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In

Plant Genetics and Genomics

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DECLARATION

I hereby declare that this thesis is my own work and effort and that it has not been submitted anywhere for any award. Where other source of information has been used, they have been acknowledged.

Rimsha Ashraf

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List of Abbreviation

Code	Abbreviation
IMMYT	International Maize and Wheat improvement centre
STS	Sequence tagged site
CAPS	Cleaved amplified polymorphic sequence
RFLP	Restriction fragment length polymorphism
SCAR	Sequence characterized amplified region
SSR	Single Sequence Repeats microsatellites
PCR	Polymerase Chain Reaction
Pm	Pico mole
Taq.	*Thermus aquaticus*
$MgCl_2$	Magnesium Chloride
DNTPs	Deoxynucleotide Triphosphates
CTAB	Cetyltrimethyl ammonium bromide
SDS	Sodium Dodecyl Sulphate
EDTA	Ethylene diamine tetra acetate
T.E	Tris borate
T.B.E	Tris borate EDTA
rpm	Revolution per minute
˚C	Centigrade

µl	Microlitre
O.D	Optical density
ng	Nano gram
mM	milli Molar
BT	Beta tubulin
TEF	Translation Elongation factor
Phyre2	**P**rotein **H**omology/Analog**Y** **R**ecognition **E**ngine
ExPASy	Expert Protein Analysis System
BLAST	Basic Local Alignment Search Tool
NCBI	The National Center for Biotechnology Information

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Abstract

In present study rust species from different regions of Pakistan were comparatively screened for beta tubulin and translation elongation factor in order to analyze the phylogeny of *Puccinia* spp. Beta tubulin and translation elongation factor are the polymorphic sequences of genes, which help in determination of genetic variation. This study revealed that point mutation is the major sequence variation including the heterokaryotic variation within the species and homokaryotic variation among the species. The phylogenetic analysis of these rust species illustrated high similarity with globally distributed varieties present in NCBI. The amplified products were further characterized through nucleotide sequence assay. Multiple sequence alignment revealed significant genetic diversity in rust loci. The protein sequences of beta tubulin and translation elongation factor genes demonstrated 3-D structures and functional helical domains verifying the stability of proteins and showing differences in disordered factor (unstability) and alpha helix (stability). Furthermore, the Protparam and Pepstats (Expasy) demonstrated differences in physiochemical properties of these proteins. Similarly, the molecular weight and isoelectric values pointed to the possible antimicrobial activity. The present study further suggested that the coding regions are more efficient in identification of rust species compared to non-coding regions. As the coding regions are conserved regions with least error rate and these conserved regions play a vital role in phylogenetic analysis. In future, advance computational assay will help to identify the mutational sites in these species and will assist to overcome rust associated problems in the field of agriculture.

INTRODUCTION

1.0 Rust Fungi

The rust fungi (phytopathogens) belong to order uredinales which is a monophyletic group. The term rust has been derived from the colour as indicator of infection due to these fungi. Economically, rust fungi cause havoc in crops as well as many plants which act as secondary hosts to these pathogens (McMullen et al., 2010). Rusts are biotrophic and obligate parasites. These get nutrients from plants and in course complete their life cycle. From ferns to advanced monocots and dicots, rust fungi comprising 7000 species parasitic to plants. Three types diploid, haploid and dikaryotic nuclear conditions play important role in formation of two to five types of spores during reproductive cycle of rusts.

1.1 Puccinia

Puccinia belongs to the division Basidiomycota. Members of this group are obligate parasites to crops, cereals and plants, causing remarkable yield loses (Alexopoulos and Mims, 1996). *Puccinia* among uredinales is a well-known group of fungi. These filamentous hyphae forming fungi reproduce sexually by the formation of basidiospores, and asexually by the formation of urediniospores (Schumann and Leonard, 2000). *Puccinia* is a large group of fungi with as many as 4000 known species. *Puccinia* are obligate parasites to plants and also termed as rust fungi. Rust fungi follow a very complex and difficult series of events in its life cycle (Petersen, 1974). *Puccinia* requires living hosts or tissue for its survival and reproduction. Generally, these organisms require primary as well as an alternative host during the course its life cycle

1.2 History of rust fungi

Aristotle (384-322Bc) writes due to warm vapours rusts produced which causes heavy destruction of grains, cereals and crops. Theophrastus (371-287Bc) has written effect of rust on cereals (Roelfs et al. 1992). Roman festival of Robigalia celebrated on 25 April to pray to Robigo rust God to reduce loss of wheat from rust (Chester 1946). Fontana and tozzetti considered rust parasitic plants for grain crops. In 1767 rust were

found as biological entities. Economically, rust fungi cause a great loss in production of crops.

1.3 Global dispersal of rust fungi

Rust fungi are diverse and show great assortment in the tropics. Rusts are most pervasive obligate parasites to certain vascular plants and demonstrate properties nearly on all kinds of plants i.e. gymnosperms and various nascent, innovative families of monocots and dicots (Laundon, 1965; Hennen & Buritica, 1980). At the same phase, rust species host assortments are slender and explicit (Cummins and Hiratsuka, 2003).

Moderate and near temperate areas are rich with enormous number of rust fungi with the decrease of temperature number of microcyclic species decreasing, whether this may be instigated by latitude or elevation. Uredinoid aecia of rust fungi are common stove areas but peridermoid aecia are common in cool regions (Cummins, 1959). Rust spates on grain crops have been testified on wheat and barley. Nevertheless, a lot of vegetables like beans, peas, garlic, carrots, and lettuce are also badly affected. Rust pathogens are also origins of severe destruction of and lianas like pear, grapes, peach, plum, apple and fruit trees if these are not meticulous (Bacigolova *et al.,* 1998; Muller-Scharer *et al.,* 1998).

1.4 Total number of Rust species

The rust species number fluctuated from 5000 (Cummins and Hiratsuka, 2003) to 7000 species (Hawksworth *et al*., 199; Maier *et al.,* 2003; Ono and Aime, 2006). Rust fungi are globally well known and number of species is more than 7000 engaged in 14 families and 163 genera (Kirk *et al.,* 2001; Anonymous, 2005). Rust fungi are mobile plant pathogens due to their wide distribution, diversity and typically wind dispersed spores (Brown *et al.,* 2002).

1.5 Rust Flora of Pakistan

Pakistan rust flora for the first time accumulated by Butler and Bisby (1933) then, trailed by others (Arthur & Cummins 1933; Sydow 1939; Cummins 1943; Malik & Khan 1944; Hasnain *et al.,* 1959; Jorstad & S. H. Iqbal, 1967). Rust fungi from west area of Pakistan containing 250 species of rusts fungi are collected and defined

(Ahmad 1956 a,b; Ahamd *et al.*, 1997). Almost 400 species in 21 genera on about 350 host plant species are reported from Pakistan (Ahmad *et al*., 1997).

1.6 Classification of rust fungi based on life cycle

There are five types of spores in rust fungi.Those comprised of five divergent spore phases are called **Macrocyclic rust.**On other hand uredial stage absent from life cycle are characterized as **Demicyclic rust taxa**.In rust both aecial and uredial stages are absent, are termed as **Microcyclic rust**. Pycnia stage is absent in such type of life cycles (Cummins and Hiratsuka, 2003). Moreover, rust fungi require two different plant hosts to accomplish their life cycle (**heteroecious fungi**) and rust species which required single host species (**autoecious fungi**). In the life cycle of rust fungi three different nuclear phases have been defined i.e. the diploid dikaryon and haploid monokaryon (Petersen, 1974).

Life cycle exclusively of compound rust fungi (**Fig. 1.1**) starts at the end of the developing season by manufacturing two-celled teliospore with two nuclei in each cell (Leonard and Szabo, 2005). In several genera lactospores are formed instead of teliospores they frequently have thinner walls and propagate without a latent period (Petersen, 1974). Dark pustules are formed by teliospore (telia) on dead host tissue,typically have thick walls and assist as overwintering and sleeping stage (Kolmer *et al.*, 2009). Two nuclei cells which are formed singly on stalks are called urediniospores (Kolmer *et al.*, 2009). Wind is the basic source to spread the urediniospore and work as re-infecting agent of the gramineous host (Petersen, 1974; Leonard and Szabo, 2005). The modification in the type of spore production is occurring when uredinial host starts getting old uredinium contains teliospores as it is a second type of spores followed by urediospores production (Mendgen, 1984). The uredium converts into telium or telial sorus (Mendgen, 1984).

Telia can have dissimilar forms and localities. In various genera (*Uredinipsos*) teliospores endure on telia fenced with the host plant cuticle; a monolayer is formed in Melampsora teliospores whereas in *Phakopsora* they can be found in more than one rank (Petersen, 1974). According to Anikster (1986) teliospores with basidia are two of the most imperative phases in the life series of uredinales. Teliospores are essential for overwintering for almost half of all *Uredinales.* Frequently is an after-ripening

period of the teliospores required for supporting propagation (Cumminis and Hiratsuka, 1983).

Heteroecius rusts have developed basidiospores which are actively discharged into the air by wind to their other hosts (Leonard and Szabo, 2005). Basidiospores have germ tube, infect their hosts by penetrating their germ tubes via intact wall of the epidermal cell(Longo *et al.,* 2006). Vesicles and infection hypha are the structures which are formed after infection, they develop under the epidermis of the plant tissue (Bushnell and Roelfs, 1984). Apparantely older plants are not infected by basidiospore because a thick cuticle will not allow the germ tube to enter into the leaf tissue (Leonard and Szabo, 2005). Basidiospores are brittle and cannot endure dry conditions. not infected by basidiospore because a thick cuticle will not allow the germ tube to enter into the leaf tissue (Leonard and Szabo, 2005). Basidiospores are brittle and cannot endure dry conditions and are released during nights as well as during moisture periods (Kolmer *et al.,* 2009). Flask-shaped pycnia arise by the infection of leaf tissue which gives haploid hyphal colonies (Leonard and Szabo, 2005; Kolmer *et al.,* 2009; Bushnell and Roelfs, 1984).

Tiny pycniospores are produced within the pycnium.Cap of Insect attracting nectar is enclosed by pycnia (Leonard and Szabo, 2005; Kolmer *et al.,* 2009).Insects and rain drops are the major source of scattering the pycniospores among pycnia of surrounding plants (Kolmer *et al.,* 2009). In rusts life cycle at this phase, fertilization takes place between pycniospores of mating type (+) and (-) mating mating type (Anikster, 1999). In this process, pycniospores represent male gametes are represented by pycniospores and female gametes are represented byflexuous hypha that develops from the top of the pycnia (Leonard and Szabo, 2005).For successful mating e.g. assembly of aecia, production of pycniospore caps is crucial (Anikster, 1999). The main component of this cap consists of a protein that possibly functions as fungal mating-type specified pheromones (Anikster, 1999).

On the leaf surface aecium is present having dikaryotic cup shape enclosing numerous aecia (Bushnell and Roelfs, 1984). The location of the pustule may vary in different rust species. The pycnial clusters appear in on the opposite side of the leaf in certain cereal ruts whereas in other rust species aecia can appear at the edge of pycnia

or even in the same place where the pycnia were fashioned (Kolmer *et al.*, 2009). Sato and Sato (1985) distinguish 14 morphological types of aecia: eridermia, roestelia, aecidium, 3 types of peridermium, 6 types of caeoma and 3 types of uraecium. This classification is based on a number of morphological characteristics such as hymenium, spore ontogeny, peridium, position of aecia and aeciospores morphology in the leaf tissue, and some others. (Cummins and Hiratsuka, 2003) Certain dikaryotic aeciospores are developed inner side of aecium.

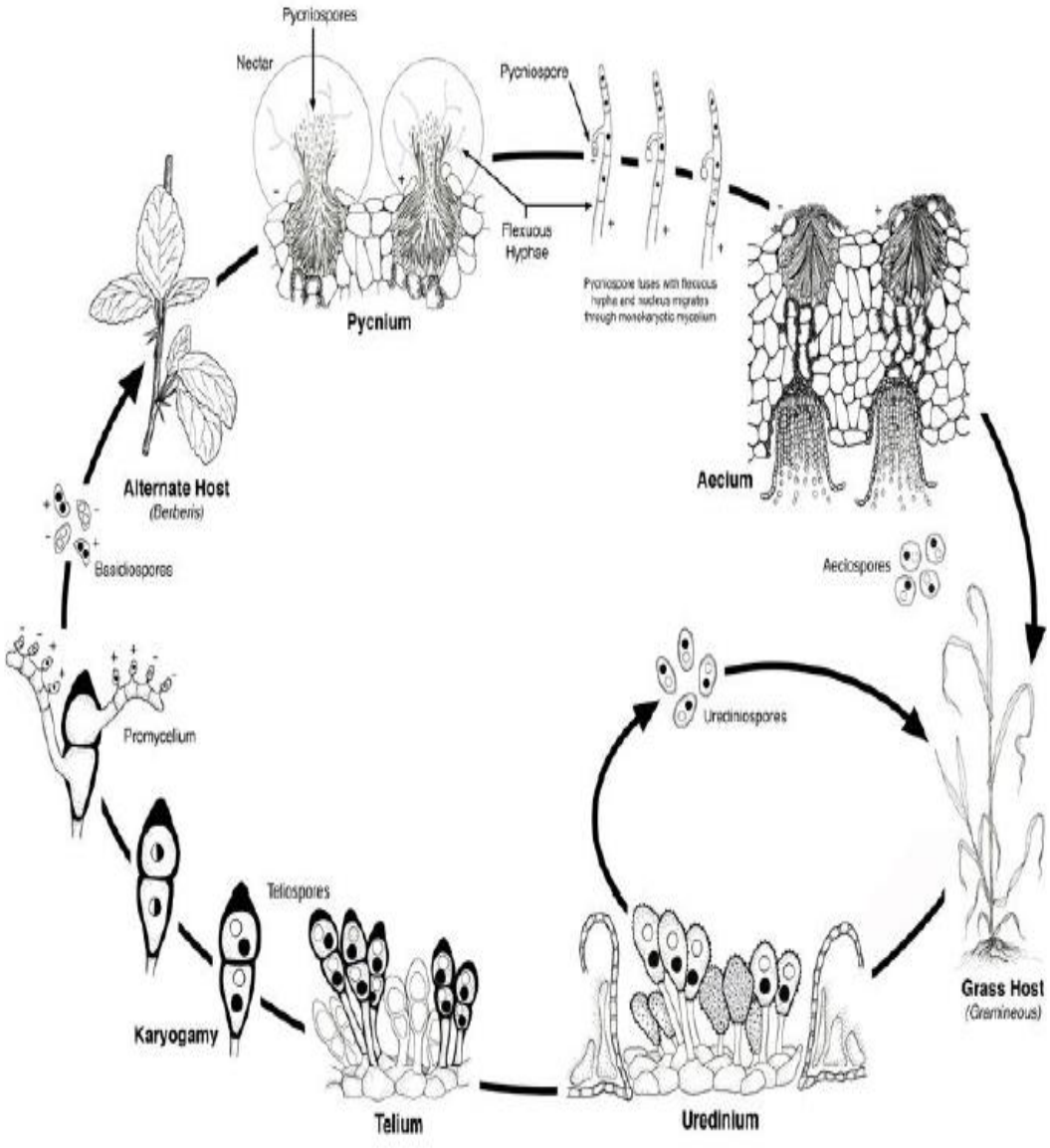

Figure 1.1: *Typical rust species life cycle (Leonard and Szabo., 2005).*

1.7 Long distance spread of Rust Spores

Long-distance spreads of rust fungi spores are responsible for the damage that rust diseases induce (Nagarajan and Singh, 1990). In the USA spore movements from the southwest to the northwest according to prevailing winds. Peanut rust *(P. arachidis* Speg) was restricted to tropical America and China until 1969 and then, suddenly appeared in southern Asia and Oceania during the early 1970s on the Ivory Coast in 1976, and in Senegal in 1980 (Savary *et a1.*, 1988). *Puccinia mefanicephala,* the sugar

cane rust, was distributed in scattered form in Africa and Asia and was of less significance for many years. In 1978, sugar cane rust attacked the Dominican Republic and one year later it has spread in all sugarcane growing areas in the Americas.

According to mesoscale meteorology analysis and report of total one month of 1978, it was found that a Saharan dust cloud traveled through the Atlantic trade winds from the coast of West Africa to the Caribbean area within 5 days in June 1978 (Purdy *et al.,* 1985). Suppose a terminal deposition velocity of 12.2 mms-1, a urediospore at an altitude of 3000 m could travel for 2.3 days. With a uniform wind speed of 10mSi, horizontal transport of 2000 km would occur during 2.3 days. In the Caribbean region, the spores met the highly susceptible cane cultivar B 4362, which had been grown in the region for more than 20 years.

The rust spread through Southeast Asia in the following years and finally reached Bahia, Brazil, early in 1970. Two years later, the rust fungi invaded Paraguay and Argentina. It reached Nicaragua in 1976, Peru in 1979, and was detected in Ecuador and Mexico during 1981. Wind alone was not always responsible for distribution of this disease. It could not have spread this way from Brasilia to Nicaragua because of prevailing natural geographical barriers. The introduction into Nicaragua constitutes a good example of spread by migrant workers, who carried seedlings and thus seem to have imported the disease (Schrieber and Zentmyr, 1984). Altogether, it took coffee rust about 120 years to travel around the world.

1.8 Economic values of rust fungi

Rust fungi are economically very significant because they are playing a vital role in destruction of crops. Agricultural crops are badly attacked by rust fungi. Decrease in biomass addition as well reproduction and devastation of plants are caused by rust fungi.rust fungi are the basic source of destruction of crops and as a result gives negative social and economic influences (Leonard *et al.,* 2005 & Stokstad, 2007).

Cereal crops are also infected by rust on wheat (*Puccinia triticina* Erikss., *Puccinia graminis* f.sp. *tritici* Erikss. and Henn., and *Puccinia striiformis* f.sp. *tritici* Erikss.), oats (*Puccinia graminis* f.sp. *avenae* Erikss. and Henn., *Puccinia coronata*

f.sp. *avenae* P. Syd & Syd), barley (*Puccinia graminis* f.sp. *tritici* Erikss. and Henn., *Puccinia hordei* G. H. Otth, *Puccinia striiformis* f. sp. *hordei* Erikss., *Puccinia coronata* Corda), rye (*Puccinia recondita* Roberge), and corn (*P. sorghi* Schwein).

Rusts attack fruits and ornamental species.According to research rust fungi attacks on 46%of European *Rosaceae*(Helfer, 2005) including peach (Tranzschelia discolor (Fuckel) Tranzschel & 1991, Michailides & Kable, 1985) ,leaf rust on plum (*Tranzschelia pruni-spinosa* (Pers.) Dietel var. *discolor*), rusts on roses (*Phragmidium tuberculatum* Jul. Müll., *Phragmidium mucronatum* (Pers.) Schltdl., and *Phragmidium rosae-multiflorae* Dietel), and cedar-apple rust (*Gymnosporangium juniperi-virginianae* Schwein).

The most important rust pathogens on conifers are rusts of pine needle (*Coleosporium asterum* Dietel.), white western gall rust (*Endocronartium harknessii* J.P. Moore), pine blister rust (*Cronartium ribicola* Fisch.), pine gall rust (*Cronartium quercuum* f.sp. *fusiforme* Hedgc. & Hunt; Burdsall & Snow), spruce chrysomyxa rust *Chrysomyxa ledi* (Alb. & Schwein.), and spruce needle rust (*Chrysomyxa ledicola* Lagerh.)

According to Arthur (1924) ferns have the most ancient lineage of rust fungi, and there are only three fern families *(Osmundaceae, Polypodiaceae* and *Schizaeaceae)* that may be infected by rusts. On the fern species from the family *Osmundaceae,* only one rust species occurs **(***Uredinopsis osmundae* Magn.), on *Polypodiaceae* spp. *Hyalopsora aspidiotus* (Magn.) Magn., *Milesia magnusiana* (Jaap) Faull, *Calidion lindsaeae* (Henn.) Syd. & P. Syd. and *Calidion dumontii* Buriticá occur, and *Schizaeaceae* spp. are bearing rust species from the genus *Dicaeoma* (Arthur, 1924).

1.9 Economic losses incurred by rust infestation.

Wheat, cereals and crops all are badly infected by rust. Severity of rust disease is very high, its severity is 100%(6.7infection/cm^2) to destroy the tillers of adult wheat (Rowell and Roelfs et al., 1976).Severity of disease directly decrease the growth of plants by reducing their photosynthetic area, causing loss of water and nutrients as a result transport system disrupted (Roelfs et al., 1985).Lesions of rust occupy larger area of plants tissues. Flag leaves, peduncle, glumes and awns are the basic parts of

the plants which helps in transport of nutrients and water, these parts are mostly infected from rust diseases (Roelfs et al., 1985). Pathogen requires water and nutrients from host plant to produce larger amount of urediospores. An infected plant has less growth rate due to imbalance in water and nutrients. Infected plants are more susceptible to winterkill, small head and decreased spikelet fertility. Rupture of plant tissues by fungus block the transport system as a result root death occurs (Bushnell and Rowell et al., 1968). Disruption of nutrients transport to filling grains causes shriveled kernels (Calpouzos et al., 1976). Severity of infection is very high, if inoculum density is high, environment is ideal for fungal infection resistant cultivars can fail (Roelfs et al., 1972).

1.10 Control methods of rust fungi

Rust fungi is well known group of fungi because of their significant complex life cycles and economic importance. Identification of barberry (*Berberis* spp.) is secondary host for *P. striiformis* (Jin *et al.*, 2010). During Middle Ages peoples knew that Barberry plants were associated with cereal rusts and a law of eradication of barberry plants was proposed by many countries (Zadoks and Bouwman, 1985). Barberry plants in Sweden were also eradicated until 1994, when the law was abolished**.** Uredinial and telial stages are the most responsible and economically important stages in the life cycle of uredinales (Leonard and Szabo, 2005), other stages as aecial stage are of minor importance. Sato and Sato in 1985, for example, were morphologically described the aecial stages of some rust fungi and study of grass fungi that have *Berberis* and *Mahonia* spp. as uredinial and aecail host was made by Cummins and Greene in 1966.

The first effective eradication law near grain fields to control rusts was established by Rouen, France, since 1660. The Connecticut barberry law of 1726 and other recently established barberry laws in other places and countries are proofs of farmer's observation. This method of control is still used today (Roelfs, 1982), but only for *Puccinia graminis.*

Today two methods chemical control and breeding for resistance are used. Buchenauer (1982) evaluated the use of the more established fungicides such as dithiocarbamates, and compared them with carboxylic acid anilides, morpholine

derivates, Indar, and inhibitors of ergosterol biosynthesis. Some fungicides as Azole triazole showed high biological activity at low application rates (Kuck and Scheinpflug, 1986; Sauter *et al.,* 1996). Rowell (1985) described the differences in vitro tests for fungicides, such as spore germination and mycelial growth tests, or in vivo tests, such as seedling assays and field evaluation. In Western Europe, high cropping systems, high yield and high grain prices permit economic use of chemical control of rusts. However, genetic resistance is important under any circumstance.

Most rust resistant cereal cultivars have remained so for 5 years or more, Showed the approximate duration of better breeding policy (Roelfs *et al.,* 1992). Still further cultivars found resistant for a number of years. A discussion of this problem can be found in several reviews (Robbelen and Sharp, 1978; Dyck and Kerber, 1970; Parlevliet, 1985; Knott, 1989). Recent progress in the establishment of durable resistance was explained by Steffensen (1992) and Mclntosh (1992). An overview on genes for resistance against wheat rusts is available (Mclntosh *et al.,* 1995). As an alternative to the pure line cultivars, intraspecific mixtures of different genotypes or interfield diversity together with a regional deployment of resistance genes was discussed (Mundt and Browning, 1985). Many rust resistance genes as Sr genes have been destroyed with the evolution of a super race capable of suppressing such genes (Gates and Loegering, 1991). Also, cultural methods such as early sowing to avoid stem rust or the induction of resistance to rust by avirulent races (Van Asch *et al.,* 1992) should not be forgotten. The detection of chemically induced resistance in wheat opens new dimensions to rust control (Gorlach *et al.,* 1996).

Many efforts have been made to control rusts biologically especially with hyperparasitic effects (Kranz, 1981), but it is still in infancy. Out of 24 species of fungi in 12 genera screened for their hyperparasitic activity towards *Puccinia coronata* on oat seedlings, 7 *Verticillium* spp. and *Acremonium implicatllm* were able to colonize uredial sori (Leinhos and Buchenauer, 1992). In addition, *Erwinia* spp., *Bacillus* spp., and *Trichoderma* spp. have been recommended for rust control (Govindasamy and Balasubramanian, 1989; Kempf and Wolf, 1989; Rytter *et al.,* 1989). However, because of the high humidity requirements of hyperparasitic fungi, such methods are not influential in arid or temperate conditions (Grabski and

Mendgen, 1986). In the tropics, good results may be obtained (Saksiriat and Hoppe, 1990).

1.11 Molecular work of rust fungi

Molecular markers are use in unlimited quantity as well without the influence of environment; they are simple, fast and targeted. Genetically plant pathogens are assorted and under this consequence study of phytopathogens at DNA level is important for study of population. Molecular identification techniques of phytopathogens have beaten taxonomy. The unremarkable nature of fungal life is joined with the lack of realistic morphological information for distinction and identification of species have delayed a descriptive concept of the fungal kingdom and DNA sequence data have been created of fungi (Blackwell et al., 2006; James et al., 2006).

Since from the last 20 years molecular markers based on DNA have been used for the identification of cereal rust populations,and are proved helpful to get information about origin and dispersal of rust genotypes. In North America genetic markers were developed and used in the studies of *Puccinia triticina* populations. These types included amplified fragment length polymorphism (AFLP) (Kolmer, 2001; Mebrate et al., 2006), random amplified DNA polymorphism (RAPD) (Kolmer & Liu, 2000; Kosman et al., 2004), and simple sequence repeat (SSR) polymorphism (Zane et al., 2002). Another region for phylogenetic analysis is ITS region related to ribosomes which have been used successfully for some of the rust species of *Uromyces, Puccinia* (Kropp *et al*., 1997; Zambino and Szabo, 1993; Roy *et al*., 1998; Pfunder *et al., 2001*), and the genus *Cronartium* (*Vogler and Bruns, 1998*). ITS region in combination with β-tubulin and EF1-α gene were successfully used in phylogenetic studies of rust fungi (van der Merve *et al*., 2008; Liu & Hambelton, 2010).

1.12 Evolutionary linkage of rust fungi

According to Evolutionary point of view macrocyclic rust species are ascendant of the microcyclic rust species (Shattock and Preece, 2000). Replication of telia occurs in microcyclic rust species the aecia of the parental macrocyclic rust species and present on the aecial host initially inhabited by the inherited heteroecious species (Shattock and Preece, 2000). This was used in the 'Tranzschel" method of

identifying the alternate host of the rust anticipated to be heteroecious. According to this method one should look for aecial stages of microcyclic species that have morphologically similar telia and teliospores to the suspected heteroecious rust. Those species that demonstrate similar telia and teliospores morphology, and share a communal host but with different life cycles are called "interrelated species" (Cummins and Hiratsuka, 1983). First time phylogenetic study of fungus was done by using 5.8S rRNA (Gottschalk and Blanz, 1984). 18S rDNA is also useful for fern rusts and certain rust fungi (Maier *et al*., 2003). EST-SSR markers developed by Dracatos *et al*., (2006) for the *P. coronate f. sp. lolii* which is useful for allies (*Puccinia spp*.). Molecular pylogenetic analyses of other rust species have already been performed (Kolmer, 2001; Hovmoller et al., 2002; Justesen et al., 2002).

1.12.1 Beta tubulin

Sequences have also been use for phylogenetic study of fungi and these analyses have examined evolutionary relationship of fungi from kingdom to specie level (Baldauf and Palmer et al., 1993, Thon and Royse et al., 1999). Beta tubulin genes found in eukaryotes that's why they accurately reflect the phylogenetic lineages of eukaryotes, while some proteins remain conserved but a few deletion and insertion also occur (Edlind et al., 1996, Thon and Royse 1999). Phylogenetic analysis of rust species have been undertaken, using morphological characters, rRNA sequences and isozyme analysis for comparison (Swann and Taylor 1993, Zambino and Szabo 1993).After structural study of rust species, microtubules are essential for various activities and beta-tubulin plays a key role in formation of these tubules (James A.Kolmer et al., 2009). Beta-tubulin helps in study of Molecular mechanism which gives functional diversification in fungal tubulins as well as evolution (zhongtao zhao et.al 2014). Distinct orthologous and paralogous clades are formed due to certain independent duplication and losses of beta tubulin in different fungal lineages (Michael AYLIFFE et al., 2001). Beta-tubulin involves in many of cellular processes like flagellar motility, intracellular transport and cell division in eukaryotic organisms (xianming chen et al., 2012). Two beta-tubulin genes are playing important role in fungicides resistance and also in hyphal growth, they have been characterized in wheat scab rust (Jin-Rong Xu et al., 2014).

1.12.2 Translation elongation factor

TEF has been used for phylogenetic study of rust species (Gentile et al., 2015 Kristensen et al., 2005).EF is characterized by high discrimination level at species level and have been used as genetic marker for phylogenetic study of several fungal species for their accurate distinctions i.e, Fusarium species (O´Donnell et al., 2000).TEF is very suitable for phylogenetic analysis of rust species because of highly variable introns (van der Merwe et al., 2007). Translation elongation factor has been useful for many multi-locus phylogenetic studies (Chaverri et al., 2003; Reeb et al., 2004; Cai et al., 2005). The main advantage of these protein coding genes (BT and TEF) is they are single copies in fungi and avoid pitfalls of paralogous comparisons. During planta development translation elongation factor is useful for quantification of active mycelium and calibration of RNA accumulation analysis of differentially expressed fungal genes (Marion K.Seier et al., 1996).TEF invovles in structure, growth, amino acid metabolism, cell defense, cell cycle, cell signaling, energy cycle, lipid, and nucleotide metabolism (L.P.N.M Kroon et al., 2004). This gene is important in protein modification, ribosomal protein complex, sugar metabolism, transcription factor, transport metabolism and virulence in pathogen(LI.Ming-Ju et al., 2014).

1.13 Significance of house-keeping genes

TEF, beta-tubulin, these are called house-keeping genes because they occur in the form of cluster and are basically having complete family of genes (Chen et al., 2015) . These genes play a vital role in origin, evolution, identification, migration, and pathogenic mechanisms of plants (Ming-Ju et al., 2014)). In molecular study of house -keeping genes we have come to know that each gene has 6-8 polymorphic loci including 3-4 phylogenetic important loci (Lious et al., 2006). They also play vital role in study of genetic diversity of certain rust species (Chen et al., 2015).Following are the implications of TEF and beta-tubulin. Beta tubulin and translation elongation factor, these three genes are called housekeeping genes. These genes cause point mutation which gives certain major sequence variation, including heterokaryotic and homokaryotic variations among isolates (xianming chen et al., 2015).Polymorphic base pairs are present in these three genes which are used to develop single polymorphism (SNP) markers (Maneu et al., 1996). Housekeeping genes are useful to

develop cleaved amplified polymorphic sequence (CAPS) markers. Polymorphic sequences of these genes are useful in study of genetic variation among isolates of rust pathogen. Importance and mechanism of heterokaryosis in pathogen evolution is also studied on base of housekeeping genes. Sequence of these housekeeping genes are compared and polymorphic region is identified which causes point mutation (Chen et al., 2015)**.**

1.14 Aims and objectives

- To study *Puccinia graminis*, *Puccinia striiformis*, *Puccinia striiformoides*, and *Puccinia triticina* on base of selected loci of coding region (BT & TEF) of Pakistani isolates.
- To study BT and TEF at protein level using computational modelling.
- To scrutinize the level of stability in proteins, how they play an important role in genetic variation and conservation of these genes.
- Phylogenetic analysis of *Puccinia graminis*, *Puccinia striiformis*, *Puccinia striiformoides* and *Puccinia triticina* based on selected loci of coding region.

Material and Methods Chapter 2

This research project was performed during 2015 in the experimental lab of Plant Genetics and Genomics at Quaid-i-Azam University Islamabad. This project has two major parts i.e. phylogenetic analysis of different *Puccinia species* and their protein analysis.

2.1 Collection of rust species (*Puccinia)*

According to the climate change rust and host plants collection was done during May and June season 2015.And the areas of collection we selected were mostly hilly areas of Pakistan because rust attacks on plants mostly in temperate areas. Plants infected with the rust fungi were collected from: Murree (Ghora Gali, Barian, Bhurban, Bansra Gali & Sunny Bank), Azad Jammu & Kashmir (Kotli & Rawalakot), Swat District of Khyber Pakhtunkhwa (KPK), Islamabad (Pir Sohawa, Rumley) and Hazara Division (HariPur) were broadly surveyed during October-November, 2014 and April-June, 2015. DNA of *Puccinia triticina*, *Puccinia striiformoides*, *Puccinia tritici* and *Puccinia graminis* were extracted in Genetics and Genomics Lab (QAU).

Table 2.1: Identified *Puccinia* species through their ITS accession numbers

Sr. No.	Codes	*Puccinia* spp.
01	RR-1	*Puccinia graminis* (BT)
02	RR-2	*Puccinia graminis* (TEF)
03	RR-3	*Puccinia striiformis* (BT)
04	RR-4	*Puccinia striiformoides* (BT)
05	RR-5	*Puccinia triticina* (BT)
06	RR-6	*Puccinia triticina* (TEF)

2.2 Molecular Assessment

2.2.1 DNA extraction from urediniospores, aeciospores and teliospores of different rust species

DNA was isolated from urediniospores, aeciospores and teliospores of infected leaves and twigs of different rust fungi species according to Barnes and Szabo (2007) with slight modifications. Approximately 15mg spores excised from single spot of infected leaves were taken in 2ml eppendorf tube and ground via liquid nitrogen and vortex using 2mm silica glass beads at high speed. Then, 600 µl CTAB buffer was added to each tube, and the solution was incubated for 1 hour at 65 ^{o}C with gentle shaking, after each 15 minutes all tubes were cooled at room temperature for 5 minutes. Then, 6 µl marcapto and 4 µl proteinase K were added to each tube carefully. For denaturation of proteins Proteinase K was added. And removed by adding 600 µl chloroform/iso-amyle alcohol (24:1, v/v), gently shaked 50 times. Centrifugation was performed at 13000 rpm for 10 minutes, the aqueous phase removed and remaining supernatant phase were transfers to new tubes. Then, supernatant were transferred into new tubes and added 50 µl sodium acetate (5.2) and 500 µl pure ethanol, than centrifuged at 13000 rpm for 10 minutes.the DNA appear in the form of pellet at the bottom of tube. The DNA was was washed by 70% ethanol precipitation and dried for 2 hours. 50 ml PCR distilled water was added and kept all tubes overnight. In order to remove RNA, DNA was treated with 2 µl RNase. All samples were incubated at 37 ^{o}C for 1hr. An aliquot of the extracted DNA was checked by using 0.8% agarose gel for further quantification and visualization.

2.2.2 Gel electrophoresis for DNA quality

Gel electrophoresis technique was followed in order to check quality of DNA for further analysis. Gel was prepared by dissolving 0.8 g of agarose in 80ml 5X TBE (Tris boric acid EDTA) buffer and 20ml of distilled water. This solution was melted in microwave oven for approximately 2 minutes. Ethidium bromide 2µl was added into melted gel and then dissolved melted gel was kept at room temperature 25C°. Gel was loaded into the casting tray and cooled to solidify the gel for approximately 10 minutes. Total DNA samples were mixed with bromophenol blue dye and then loaded on wells of agarose gel. Temperature of the gel tank was 90C°. After about 30 minutes run, the gel

was checked via UV gel documentation system (BIO-RAD). The gel image showed that highly concentrated form of DNA is present which was reflected by bright bands and images were saved.

2.2.3 PCR amplification of beta-tubulin of rust species

BAF6-f and BAF2-r are forward and reverse primers (GeneBank accession number S56922) which were used to amplify DNA of all rust species (Fraaije et al., 2001) The sequences of primers are as follows:

Table 2.2: Universal primers for beta-tubulin

BAF6-f Forward Primer	BAF2-r Reverse Primer
5'ACCCACAACCGCCAACATGCG TGA -3'	5'CGTACCGGGCTCGAGATCGAGATCGAC GAG-3'

A total reaction mixture was 20 µl comprised of: 10.6 µl PCR water, 2.5 µl 10X Taq buffer, 2ul $MgCl_2$, 0.4µl dNTPs, 0.5µl Taq Polymerase, 1µl of forward and 1µl of reverse primers of beta tubulin (BAF6-f, BAF2-r) respectively, 2ul of DNA (30 ng/ µl). PCR profile was optimized as given below;

Table 2.3: PCR profile of touch down 60 C°(BT)

Steps	Touch down 60 C°	Time
Initial denaturation	95 C°	5 minutes
Denaturation	94 C°	30 seconds.
Annealing	46 C°	30 seconds
Extension	70 °C 2-35 cycles	90 seconds
Final extension	72 C°°	7 minutes

2.2.4 Gel electrophoresis

The amplified product of 4µl was mixed with loading dye bromophenol blue and resolved on 0.8% agarose gel. DNA ladder of 1kb was also loaded for our product size. Gel was observed by getting photographic image under UV in gel documentation system (BIO-RAD).

2.2.5 PCR amplification of translation elongation factor of *Puccinia* species

Forward oliFmk3-f and **Reverse oliFmk4-**r (Marion K. SEIER et al., 2009) are forward and reverse primers were used to amplify DNA of all rust species. The sequences of primers are as follows:

Table 2.4: Universal primers for Translation Elongation Factor

Forward oliFmk3-f	Reverse oliFmk4-r
5'GGAAGTTTGAAACCCCCAAGTACT-3	5'TTGGAGGTTTCCTTGACGATTTC-3'

A total reaction mixture was 20 µl comprised of: 10.6 µl PCR water, 2.5 µl 10X Taq .buffer, 2ul $MgCl_2$, 0.4µl dNTPs, 0.5µl Taq. Polymerase. 1µl of forward and 1µl of reverse primers of translation elongation factor respectively, 2ul of DNA (30 ng/ µl). PCR profile was optimized as given below;

Table 2.5: PCR profile of touch down 60 C° (TEF)

Steps	Touch down 60 C°	Time
Initial denaturation	95 C°	5 minutes
Denaturation	94 C°	30 seconds.
Annealing	57 C°	30 seconds
Extension	70 °C 2-35 cycles	90 seconds
Final extension	72 C°°	7 minutes

2.2.6 Gel electrophoresis

The amplified product of 4µl was mixed with loading dye bromophenol blue and resolved on 0.8% agarose gel. DNA ladder of 1kb was also loaded for our product size. Gel was observed by getting photographic image under UV in gel documentation system (BIO-RAD).

2.2.7 Purification of PCR products

The PCR samples were purified using Gene Jet PCR purification kit (Thermo Scientific Lithuania, Europe). 20µl of binding buffer was added in 20µl of PCR product and mixed thoroughly. After mixing PCR product with binding buffer was transferred to purification column and centrifuged at 12,000rpm for 1min. After centrifugation 700µl washing buffer was added and again centrifuged at 12,000rpm for 1min. Filtrate was discarded and again the column with filtered PCR product was centrifuged at 12,000rpm for 1 min. Columns were shifted to new Eppendorf tubes and 20µl eluent buffer was added. After adding eluent buffer PCR product was left at room temperature for one minute and centrifuged at 12,000 rpm for 1 min. The purified PCR products were checked on 2% agarose gel by taking 3µl of each sample with 2µl of loading dye and again the image was taken through gel documentation to confirm the purity of PCR products.

2.2.8 BT and TEF sequencing

PCR product of each spp. of 30µl was selected for sequencing (Beirn, Moy et al. 2011). All the samples were purified using Gene Jet PCR purification kit according to the manufacturer instructions (Thermo Scientific Lithuania, Europe). All samples were partially sequenced commercially from Europhins mwg/operon, using Sanger's method (www.eurofinsdna.com).

2.3 Computational Analysis

On the basis of computational technology sequence analysis was managed with the assistance of bioinformatic tools used for RNA, DNA or protein sequences to ascertain and compare with already sequenced samples through sequence alignment and different sequence databases (Durbin, 1998)

2.4 NCBI Analysis

To confirm the identity of *P. triticina, P. graminis, P. striiformoides* and *P.striiformis* their partial sequences of BT and TEF were blast on NCBI http://blast.ncbi.nlm.nih.gov/Blast.cgi with somewhat similar sequences to find out the similarities of these sequences with their relevant *Puccinia* sps. Partial sequences of above mentioned *Puccinia* sps. subbmitted to NCBI with different codes.

2.5 Basic local alignment search tool (BLAST)

Basic local alignment search tool is a sequence comparison algorithm (a fixed procedure embodied in a computer program) used to find regions of sequence similarity and compare nucleotide and or protein sequences from the same or different organisms. BLAST results assist to infer functional and evolutionary clues about the structure and function of newly sequenced genes. Nucleotide BLAST (nBLAST) and Protein BLAST (pBLAST) were used to find the similarity of sequences with already reported sequences present in different NCBI databases *(http://blast.ncbi.nlm.nih.gov/Blast.cgi).*

2.6 TRANSLATE

For decoding of nucleic acid sequences **(DNA/RNA)** to their analogous peptide sequence according to genetic codes **EXPASY** program is used. Expasy has been used for translation of nucleotides into proteins. Nucleotide sequences of beta tubulin and translation elongation factor were converted into amino acid sequences

according to standard genetic code for **3D** structural analysis by using **PHYRE2** and protein molecular mass was predicted.

2.7 Phyre2 - Protein Homology/ Analogy Recognition Engine

Phyre2 predicts the 3D structure of a protein sequence based on HMM-HMM alignment techniques http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index. For a given sequence, it detects known homologues based on PSI-Blast, constructs a hidden Markov model (HMM) of the sequence based on the detected homologues and scans this HMM against a database of HMMs of known protein structures. The database is based on SCOP and latest depositions in PDB. The top 20 highest scoring matches of the query to known template structures are used to construct 3D models of the query. Here, the subset of the top 20 matches with confidence > 50% is being converted to a unified annotation. The list is traversed in decreasing order of confidence and non-overlapping templates are added in the annotation. Matches that have significant overlap (> 30 residues or > 50% of the length of the smallest template) with previously selected matches are discarded. Therefore, Phyre2 results as presented here are not necessarily in agreement with the original results.

2.8 Molecular evolutionary genetic analysis version 6.0 (MEGA 6.0)

MEGA 6.0 an integrated tool employed for phylogenetic trees, assesses rates of molecular evolution, inferring ancestral sequences and testing evolutionary hypothesis (Zhenyue Lin et al., 2015; Tamura et al., 2011). The bootstrap data set was used directly for constructing the phylogenetic tree using MEGA 6.0 programs for calculating multiple distance matrixes. The multiple distance matrixes were uploaded to construct phylogenetic tree using neighbor-joining algorithm 9NJ) method.Phylogenetic analysis of *P. triticina, , P. graminis,* and *P. striiformoides and P.striiformis* was performed with the help of MEGA 6.0 by removing gaps in sequences.

CHAPTER 3

Results

3.1 DNA extraction and visualization

The genomic DNA extracted from the urediniospores and aeciospores was assessed on 1% agarose gel. Purified DNA samples were selected for PCR amplification of Beta-tubulin and Translation elongation factor regions and shown as amplification in Fig. 3.1.

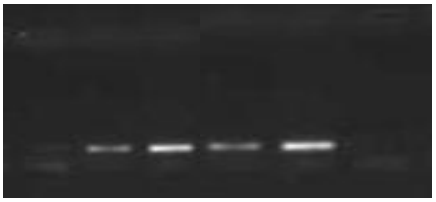

Fig 3.1 DNA bands of *Puccinia* spp.; well#2: *Puccinia graminis*, well#3: *Puccinia striiformis*, well#4: *Puccinia striiformoides*, well#5: *Puccinia triticina*

3.2 PCR amplification of beta-tubulin and translation elongation factor of *Puccinia* spp.

The BT and TEF loci of *P. graminis*, *P. striiformis*, *P. striiformoides* and *P. triticina* were amplified. The 800bp to 1000bp bands have been resolved on 1% agarose gel (Fig. 3.2).

Fig. 3.2: The 500bp BT and TEF products on 1% agarose gel.Well#1,2 from right side: *P. graminis* BT and TEF , well#3: *P.striiformis* , well#4: *P. striiformoides* and well#5, 6: *P. triticina* BT&TEF

3.3 Sequence analysis of Beta tubulin and translation elongation factor

All PCR purified products of beta tubulin and translation elongation factor were sequenced. The sequence of *Puccinia* species showed range of 800bp to 1000bp length of nucleotides. Sequences were analysed through blast to assess the homology.

All sequences matched with the already reported sequences on NCBI with almost 85% identity, and their respective identities were determined.

Beta tubulin in *Puccinia graminis*

GTCGTTGAGCCTTACAACGCGACTTTGTCGGTCCACCAACTGGYCGAAAACTCCGATGAAACCTTTTGTATCGA
CAACGAGGCCCTTTACGACATCTGCTTCCGTACCCTGAAGTTGGCCACACCGACCTACGGTGACCTCAATCACT
TGGTCTCGATCGTGATGAGTGGTATCACCACTTGTCTTCGATTCCCCGGTCAACTGAACTCGGACCTCCGTAAA
CTGGCTGTCAACATGGTTCCTTTCCCTCGATTGCACTTCTTCATGGTCGGATTCGCTCCGCTTACCGCTCGTGG
AAGCCAACAGTACCGAGCCATCACCGTCCCCGAGTTGACCTCGCAAATGTTTGATGCCAAGAACATGATGGCC
GCCTCAGACCCCAGACACGGCCGATACTTGACCGTCGCTGCTTACTTCCGTGGTAAGGTCTCCATGAAAGAGG
TCGAAGAGAACATGCTGTCCGTCCAGAGCAAAACTCGAACTACTTTGTTGAGTGGAGTAAGTCCAATTTTGCCT
CAGGTGGCTATTTTTAACCATGTCGACGTAGTTGACTCACAACACTGGTCTCATGATACCCTTGTAGTTCCAAA
CAACGTTCAAACCGCCCATTGTGACATCGCTCCTCGTGCACACAAGATGTCCGTGACTTTCATCGGTAACTCGA
CCGCTATCCAAGACTTGTTCAAGCGGGTGGCCGATCAATTCACGGCCATGTTCAGGCGTAAAGCTTTCCTGCAT
TGGTACACTGGAGAAGGGTGCGTCCGGATATCCTCAG

Beta –tubulin in *Puccinia striiformis*

GTGGCCACCTTCTCCGTTGTCCCCTCGCCCAAGGTGTCTGATACCGTTGTCGAGCCTTACAACGCCACCTTGTCG
GTTCATCAACTGGTCGAAAACTCGGACGAAACCTTTTGTATCGATAACGAGGCACTTTACGACATCTGCTTCCGC
ACCCTGAAATTGGCTACACCTACCTACGGTGACCTCAATCACTTGGTCTCGATCGTCATGAGTGGGATCACCACC
TGTCTTCGATTCCCCGGTCAGCTCAACTCTGATCTCCGTAAACTAGCTGTCAACATGGTTCCTTTCCCTCGATTGC
ACTTCTTCATGGTCGGATTCGCTCCGCTTACCGCTCGTGGAAGCCAACAATACCGGGCAATCACCGTCCCAGAGT
TGACATCGCAAATGTTTGATGCCAAGAACATGATGGCCGCTTCTGACCCGAGACACGGCCGATACTTGACTGTTG
CCGCTTACTTCCGTGGAAAGGTTCCATGAAAGAAGTCGAAGAGAACATGCTGTCCGTTCAAAGCAAGAACTCCAA
CTACTTTGTTGAGTGGAGTAAGTCCGGATTTCGTCTGTTTCTCTCATGTCGGCTTATGATTATGTTACTCAAATCT
GCGAACACTTGGTATAATCTCAGTTCCGAACAACGTCCAAACCGCCCACTGTGACATTGCTCCACGTGGCCACAA
GATGTCGGTGACATTTATCGGAAACTC

Beta-tubulin in *Puccinia striiformoides*

GGTGTCTGATACCGTTGTTGAGCCTTACAACGCCACCTTGTCGGTCCATCAACTGGTCGAAAACTCGGACGAA
ACCTTTTGTATCGACAACGAGGCACTTTACGATATCTGCTTCCGCACCCTGAAATTGGCTACACCTACCTACGG
TGACCTCAATCACTTGGTCTCGATCGTCATGAGTGGGATCACCACCTGTCTTCGATTCCCCGGTCAGCTTAACT
CTGACCTCCGTAAACTAGCTGTCAACATGGTTCCTTTCCCTCGATTGCACTTCTTCATGGTCGGATTTGCTCCG
CTTACCGCTCGTGGAAGCCAACAATACCGCGCAATCACCGTCCCAGAGTTGACATCGCAAATGTTTGATGCCA
AGAACATGATGGCCGCTTCTGACCCGAGACACGGCCGATACTTGACCGTTGCCGCTTACTTCCGTGGAAAGGT
TTCCATGAAAGAAGTTGAAGAGAACATGCTGTCCGTTCAAAGCAAGAACTCCAACTACTTTGTTGAGTGGAGTA
AGTCCGGATCTCATTCGTTTCTCCCATGTCGGCTTATGATCATGATACTCATATCTGTGGCCACTTGGTATAATT
TTAGTTCCAAACAACGTCCAAACCGCCCACTGTGACATTGCTCCACGTGGCCACAAGATGTCCGTGACATTTAT
CGGAAA

Beta-tubulin in *Puccinia triticina*

GTCGTTGAGCCCTACAACGCGACCTTGTCGGTCCACCAACTGGTCGAAAACTCTGACGAGACCTTTTGTATCGA
CAACGAGGCCCTCTACGACATCTGTTTCCGCACCCTGAAGTTGGCCACACCCACCTACGGTGACCTCAACCACT
TGGTCTCGATCGTCATGAGTGGTATCACCACCTGTCTCCGATTCCCCGGTCAGCTGAACTCCGACTTGCGTAAA
CTGGCCGTCAACATGGTCCCCTTCCCCCGATTGCATTTCTTCATGGTCGGATTTGCTCCACTTACCGCCCGTGG
GAGCCAGCAGTACCGTGCGATCACCGTGCCTGAGTTGACCTCGCAAATGTTCGATGCCAAGAACATGATGGCT
GCCTCAGACCCGAGACACGGCCGATACTTGACCGTCGCCGCCTACTTCCGTGGTAAGGTTTCGATGAAAGAAG
TCGAAGAAAACATGYTGTCAGTCCAAAGCAAAACTCGAACTACTTTGTTGAGTGGAGTAAGTCAYATTTTGCGC
AGATGACTATCCCTTTACGCCTCTCGGTGTACTCTACTGATCGCGGTCCTCTCTTTATCTCATGGCAGTTCCAA

ACAACGTCCAAACCGCTCATTGTGACATCGCACCTCGTGCACACAAGATGTCTGTGACATTTATCGGTAACTCG
ACTGCCATCCAAGATTTGTTCAAGCGGGTGGCCGATCAATTCACGGCCATGTTCAGACGTAAAGCTTTCCTGCA
TTGGTACACTGGAGAAGGGTACGTCTAGGTATTCCTCAGATGTTTTGTATGTCAACGAGTTGGCTC

Translation Elongation Factor in *Puccinia graminis*

GGATCCTTCAAGTGAGCTTTATCTTCTGCTTCCTATCTTGGACCTCCAGGTGTTCGTCTACTGAGATGGTCTAT
CATAGGTACGCATGGGTGCTTGACAAGCTGAAAGCCGAGCGTGAGCGTGGTATCACCATCGACATTGCCGTGA
GTCGATCCTGAGCTTGTGAGGACTCTCATGCGGCCTGCTGACCATTCCCACTACAGTTGTGGAAGTTTGAAACC
CCCAAGTACTACGTCACCGTCATTGATGCCCCCGGACATCGTGATTTCATCAAAAACATGATCACTGGTACCTC
CCAAGCCGATTGTGCTATCCTCATCATTGCCGCCGGTACTGGTGAATTCGAAGCTGGTATCTCCAAGGATGGC
CAGACTCGTGAACACGCCCTCCTAGCCTTCACCCTTGGTGTCCGACAACTCATCGTTGCCATCAACAAGATGGA
CACCACCAAATGGTCCGAGCAGAGGTTTGTTTTTTATCCCCTCTTCACTCCTTGCCCCTCTCATCTAACTTATCT
TCTGCCCCCGATACGAGGAAATCGTCAAGGAAACCTCCAACTTCGTCAAGAAGGTCGGGTACAACCCCAAATC

Translation Elongation Factor in *Puccinia triticina*

GGATCCTTCAAGTAAGCTCCCTCCCATCATCCACTCGTCCGACACCTCGTCTCAGTACTTATCCTGTACTATAG
GTACGCATGGGTACTTGACAAACTGAAAGCTGAGCGTGAGCGTGGTATCACCATCGATATCGCTGTAAGTTCC
CCCCTCACACCTACCGGGCATGCAACGTATTACTGACCGCTATTACTGTAGCTGTGGAAGTTCGAAACCCCCAA
GTACTACGTTACTGTCATCGATGCCCCCGGACATCGTGATTTCATCAAGAACATGATCACCGGTACCTCGCAAG
CCGATTGTGCTATCCTCATCATTGCTGCCGGTACGGTGAATTCGAAGCTGGTATCTCCAAGGACGGCCAAACTC
GTGAACACGCCCTCCTCGCCTTCACCCTTGGGGTGCGACAACTGATTGTTGCCATCAACAAGATGGACACCAC
TAAATGGTCCGAGCAGAGGTGTGTTGCTCCCCATGTCATTGTGTTGATTCTCTTCATCTGGCTCACTGCTTGCC
TCAAGGTACGACGAAATTGTCAAGGAAACCTCTAACTTCGTCAAGAAGGTTGGATACCAGCCCAAGTGCATCC
CATTCGTCCCTATCTCTGGATGGCACGGTGACAACATGTTGGAAGAATCCACCAACATGGGCTGGTTCAAGGG
ATGGACCA

3.4 Protein analysis

Nucleotide sequences of the respectives genes were converted to proteins/amino acids using Expasy tool. The nucleotide sequence consisted of six open reading frames and each one of the Open Reading Frame (ORF) was selected to determine the number of amino acids. In ORF "M" denotes the start codon. Following were the products of translate tool.

Beta tubulin in *Puccinia graminis*

MSGITTCLRFPGQLNSDLRKLAVNMVPFPRLHFFMVGFAPLTARGSQQYRAI
TVPELTSQMFDAKNMAASDPRHGRYLTVAAYFRGKVSMKEVEENMLSVQS
KNSNYFVEWSKSNFASGGYF

Beta tubulin in *Puccinia striiformis*

MSGITTCLRFPGQLNSDLRKLAVN**M**VPFPRLHFF**M**VGFAPLTARGSQQYRAITV
PELTSQMFDAKNMAASDPRHGRYLTVAAYFRGKVSMKEVEENMLSVQSKNSN
YFVEWSKSGFLLSCRLMIMLLKSANTWYNLSSEQRPNRPL

Beta tubulin in *Puccinia striiformoides*

M**SGITTCLRFPGQLNSDLRKLAVN**M**VPFPRLHFF**M**VGFAPLTARGSQQYRA
ITVPELTSQ**M**FDAKN**M**AASDPRHGRYLTVAAYFRGKVSMKEVEEN**M**LSVQ
SKNSNYFVEWSKSGSHSFLPCRLI**M**ILISVATWYNFSSKQRPNRPL

Beta tubulin in *Puccinia triticina*

MSGITTCLRFPGQLNSDLRKLAVN**M**VPFPRLHFF**M**VGFAPLTARGSQQYRAITV
PELTSQ**M**FDAKN**M**AASDPRHGRYLTVAAYFRGKVS**M**KEVEEN**M**XSVQSKNS
NYFVEWSKSXFAQMTIPLRLSVYSTDRGPLFISWQFQTTSKPLIVTSHLVHTRCL

Translation Elongation Factor in *Puccinia triticina*

MITGTSQADCAILIIAAGTGEFEAGISKDGQTREHALLAFTLGVRQLIVAINKMD
TTKWSEQRCVAPHVIVLILFIWLTACLKVRRNCQGNL

Translation Elongation Factor in *Puccinia graminis*

MITGTSQADCAILIIAAGTGEFEAGISKDGQTREHALLAFTLGVRQLIVAINK**M**D
TTKWSEQRFVFYPLFTPCPSHLTYLLPPIRGNRQGNLQLRQEGRVQPQI

3.5 Physiochemical Analysis

The protein sequences were used for computational modelling revealing the conservation and their phylogenetic relationship with other species. Different physiochemical properties e.g. Molecular weight, Isoelectric point, Charge, Average residue weight, Aliphatic index, Extinction coefficient and Estimated half-life etc. have been observed for **Beta-tubulin** and **Translation elongation factor** of different *Puccinia* spp. via **Protparam** and **Pepstats**. The physiochemical analyses of beta tubulin and translation elongation factor have been described in Table 3.1 and 3.2. Atomic composition of proteins gives the basic molecular formula of proteins through which types of amino acids can determine by checking their functional groups. **Pepstats** and **Protparam** are used to determine the composition of proteins of **Beta tubulin** and

Translation elongation factor genes of *Puccinia sps*. Given table shows the atomic composition of proteins (Table 3.2).

Table 3.1: Assessment of physiochemical properties of proteins

***Puccinia* species**	**Molecular weight**	**Theoretical pI**	**Extinction coefficient**	**Aliphatic index**	**Grand average of hydropathicity (GRAVY)**
Puccinia graminis (BT)	3838.2	4.94	84.80	41.21	-0.664
Puccinia graminis (TEF)	11556.3	8.77	86.05	93.95	-0.091
Puccinia striiformis (BT)	17100.9	10.14	18450	73.96	-0.204
Puccinia striiformoides (BT)	17008.8	10.14	18475	73.29	-0.152
Puccinia triticina (BT)	18344.7	10.01	18450	73.85	-0.123
Puccinia triticina (TEF)	10054.8	8.48	11000	114.57	0.404

Table 3.3: Molecular formula of proteins (*Puccinia* spp) and charged residues

Sr. No.	Species	Formulas of proteins	Atoms	Charged residues of amino acids			
01	*P.graminis BT*	$C_{171}H_{250}N_{42}O_{55}S_2$	520	(Asp+Glu)	-4	(Arg+Lys)	+3
02	*P. graminis* (TEF)	$C_{516}H_{825}N_{145}O_{148}S_4$	1638	(Asp+Glu)	-8	(Arg+Lys)	+10
03	*P. striiformis* (BT)	$C_{763}H_{1199}N_{213}O_{210}S_{12}$	2397	(Asp+Glu)	-9	(Arg+Lys)	+19
04	*P. striiformoides* (BT)	$C_{761}H_{1191}N_{211}O_{208}S_{12}$	2383	(Asp+Glu)	-8	(Arg+Lys)	+18
05	*P. triticina* (BT)	Ambiguous		(Asp+Glu)	-9	(Arg+Lys)	+18
06	*P. triticina* (TEF)	$C_{446}H_{733}N_{125}O_{126}S_6$	1436	(Asp+Glu)	-7	(Arg+Lys)	+9

Table 3.4: Percentage and number of different Amino acids in Beta tubulin and Translation elongation factor of *P.graminis, P.striiformis, P.striiformoides and P.triticina.*

Sr. No	Amino acids	*P. graminis* (BT).		*P. graminis* (TEF)		*P. striiformis* (BT)		*P. striiformoides* (BT)		*P. triticina* (BT)		*P. triticina* (TEF).	
		Per.%	No.	Per.%	No.	Per.%	No.	Per.%	No.	Per.%	No.	Per.%	No.
01	Ala (A)	3.0	1	7.7	8	6.7	10	6.7	1	6.2	10	10.9	10
02	Arg (R)	0.0	0	6.7	7	8.1	12	7.4	0	6.8	11	5.4	5
03	Asn (N)	12.1	4	2.9	3	6.0	9	5.4	4	3.7	6	3.3	3
04	Asp (D)	0.0	0	2.9	3	2.0	3	2.0	0	2.5	4	3.3	3
05	Cys (C)	0.0	0	1.9	2	1.3	2	1.3	0	1.2	2	4.3	4
06	Gln (Q)	3.0	1	8.7	9	4.0	6	4.0	1	5.0	8	5.4	5
07	Glu (E)	12.1	4	4.8	5	4.0	6	3.4	4	3.1	5	4.3	4
08	Gly (G)	6.1	2	8.7	9	4.7	7	4.7	2	4.3	7	7.6	7
09	His (H)	0.0	0	1.9	2	1.3	2	2.0	0	2.5	4	2.2	2
10	Ile (I)	0.0	0	8.7	9	2.0	3	3.4	0	3.1	5	10.9	10
11	Leu (L)	3	1	10.6	11	10.7	16	8.7	1	8.7	14	10.9	10
12	Lys (K)	9.1	3	2.9	3	4.7	7	4.7	3	4.3	7	4.3	4
13	Met (M)	6.1	2	1.9	2	6.7	10	6.7	2	5.6	9	2.2	2
14	Phe (F)	9.1	3	4.8	5	6.7	10	6.7	3	6.8	11	3.3	3
15	Pro (P)	0.0	0	5.8	6	5.4	8	6.0	0	5.6	9	1.1	1
16	Ser (S)	18.2	6	3.8	4	10.1	15	10.7	6	9.0	16	3.3	3
17	Thr (T)	0.0	0	8.7	9	4.7	7	4.7	0	7.5	12	8.7	8
18	Trp (W)	3.0	1	1	1	1.3	2	1.3	1	1.2	2	2.2	2
19	Tyr (Y)	6.1	2	1.9	2	3.4	5	3.4	2	3.1	5	0.0	0
20	Val (V)	9.1	3	3.8	4	6.0	9	6.7	3	7.5	12	6.5	6
21	Pyl (O)	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0
22	Sec (U)	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0

The percentages and number of amino acids have been tabulated for different *Puccinia* species (Table 3.4). The data suggest higher percentages of amino acid in each of the *Puccinia* species (Fig. 3.4). For each of the species for instance *Puccinia graminis* BT has a higher percentage of Ser (S) i.e. 18.2%., *Puccinia graminis* TEF has 10.6% of Leu (L), *Puccinia striiformis* BT has 10.7% Leu (L), *Puccinia striiformoides* BT 10.7% Ser (S), *Puccinia triticina* BT contains 9.0% Ser (S) and *Puccinia triticina* has equal percentage of Ala (A), Leu (L) i.e. 10.9%. The graph showed amino acids percentages which were commonly higher among species of *Puccinia*, in *Puccinia graminis* these were significant but in *Puccinia triticina* showed the non significant value (Figure 3.4).

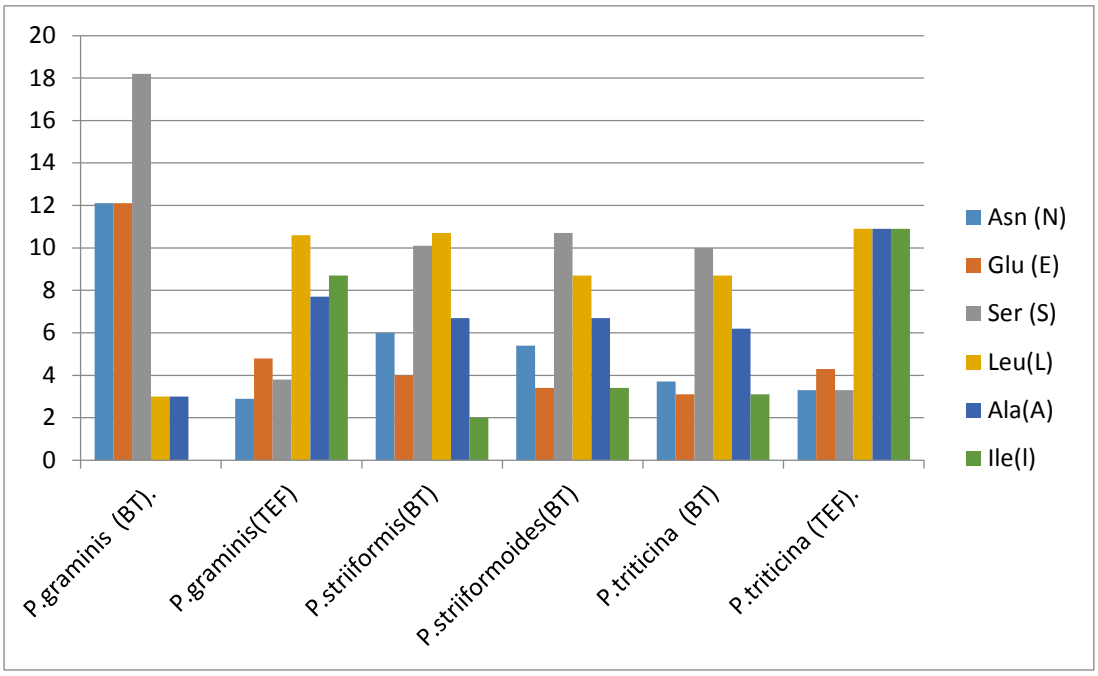

Fig. 3.4: Percentages of amino acids in different Puccinia sps.

3.6 Protein Intensive Models

3.6.1 *Puccinia graminis* BT

Protein homology/analogy was computed with the help of phyre2 tools. The depicted protein structure of *P. graminis BT* represented the presence of beta–tubulin in *Puccinia graminis* (Figure 3.5). Highly conservative secondary structure of beta tubulin appeared in red alpha helix strand as shown in the key to figure and signified the conservation of protein while the alpha-helix strand shown in blue signified the low confidence ratio.

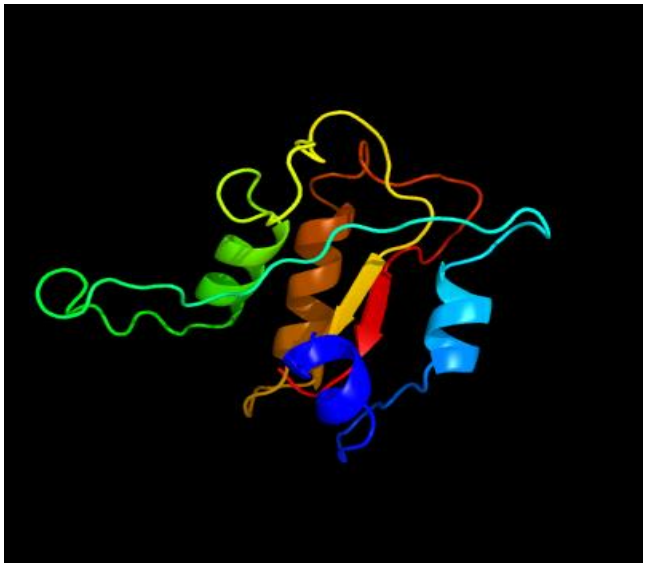

Fig. 3.5: *Puccinia graminis* BT 3D structure and domain analysis

In beta tubulin of *P. graminis*, the predicted presence of alpha-helices, beta-strands and disordered regions were shown graphically together with a color-coded confidence bar (Fig.3.6). Beta strands determined disordered proteins especially at pathological state, therefore the percentages of disordered and beta strand were quite close in value. The high percentage of alpha-helix demonstrate the stability of protein. Hence, sample RR-1 predicts that *Puccinia graminis* BT product is a stable protein.

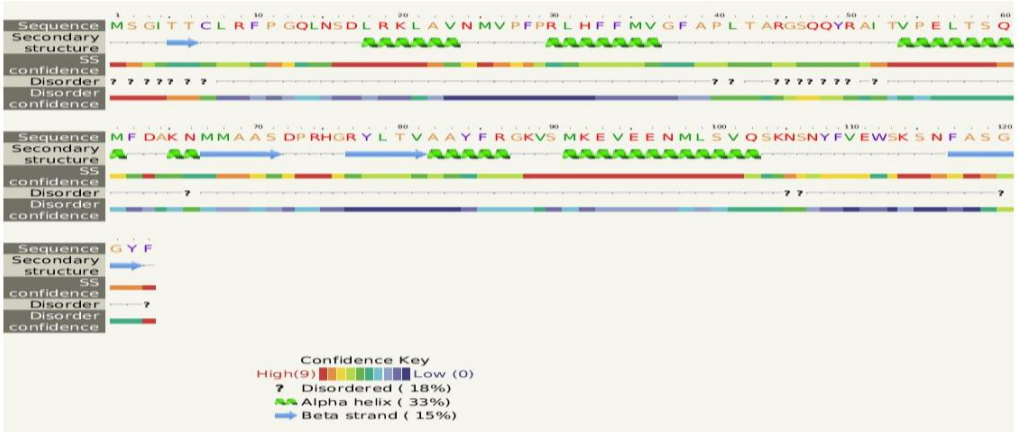

Figure 3.6: Domain analysis of *Puccinia graminis* BT

Puccinia graminis BT showed high hydrophobicity on Protscale kyte-Doolittle scale (Fig. 3.7). The amino acid sequence was positioned along x-axis whereas y-axis depicted the degree of hydrophobicity. Amino acids with high hydrophobicity indicated that these residues were in contact with solvent. Therefore, they were likely to reside on the outer surface of the protein.

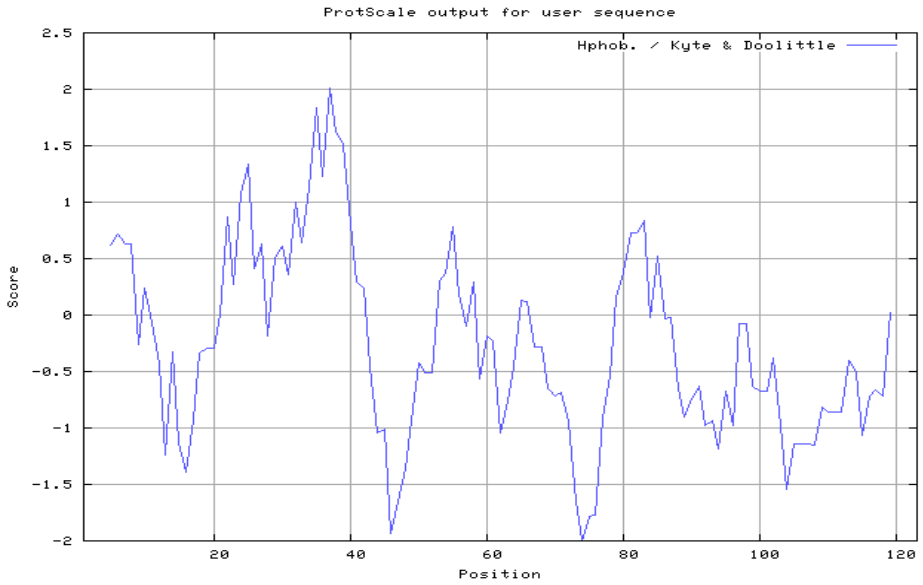

Fig.3.7: Protscale of *Puccinia graminis* (beta tubulin)

The analysis depicted the percentage and number of amino acids in *Puccinia graminis BT* (Fig. 3.8). The percentage and number of Leu (L) was high in *beta-tubulin* of *Puccinia gramins* while Cys (C), His (H) and Trp (W) were present at low percentage.

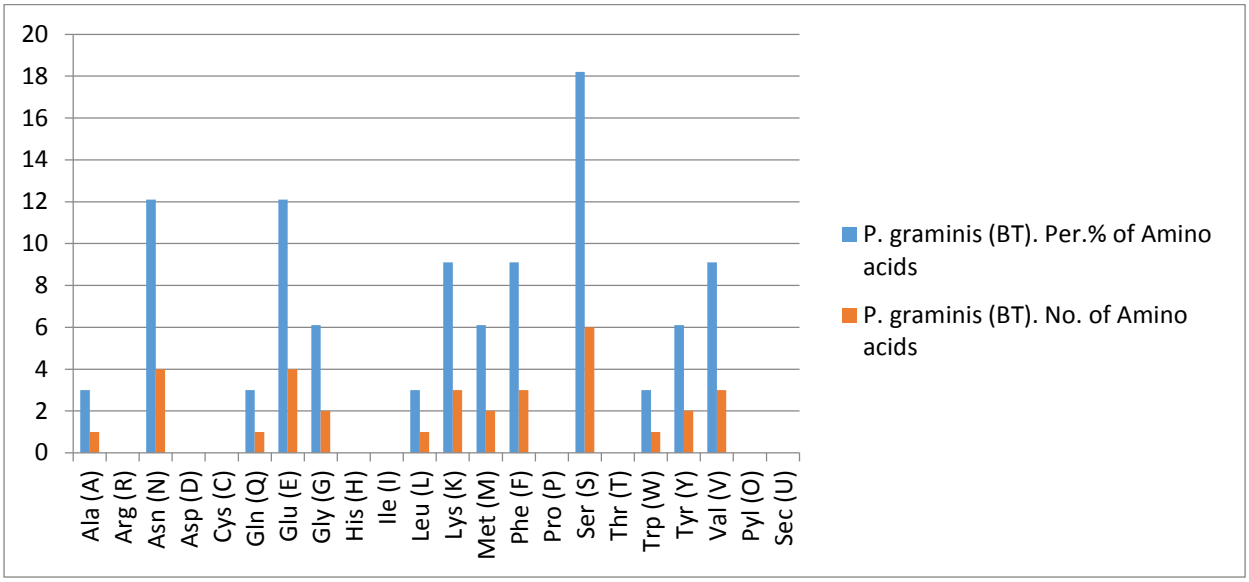

Fig. 3.8: The number and percentage of amino acids (*P. graminis* BT)

3.6.2 Puccinia graminis TEF

The protein structure of *Puccinia graminis* TEF, represented the presence of beta tubulin in *Puccinia graminis* (Fig. 3.9). Highly conserved secondary structure of Translation

Elongation Factor was shown in red alpha helix strand (Fig. 3.9) while alpha-helix strand that was blue in colour showed low confidence ratio.

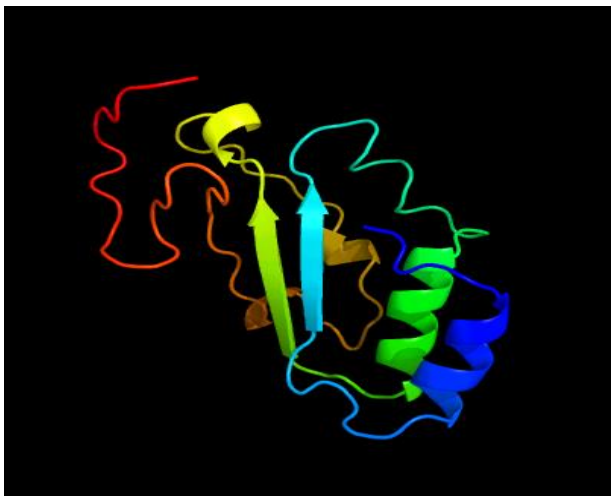

Fig. 3.9: *Puccinia graminis* TEF 3D structure and domain analysis

In translation elongation factor of *P. graminis* predicted presence of alpha-helices, beta-strands and disordered regions (Fig. 3.10). The Beta strand indicated disordered proteins especially at pathological state, While a higher percentage of alpha-helix depicted more stability in protein structurally than the disordernace. Hence, sample RR-2 (representing *Puccinia graminis* TEF) predicted a highly stable protein.

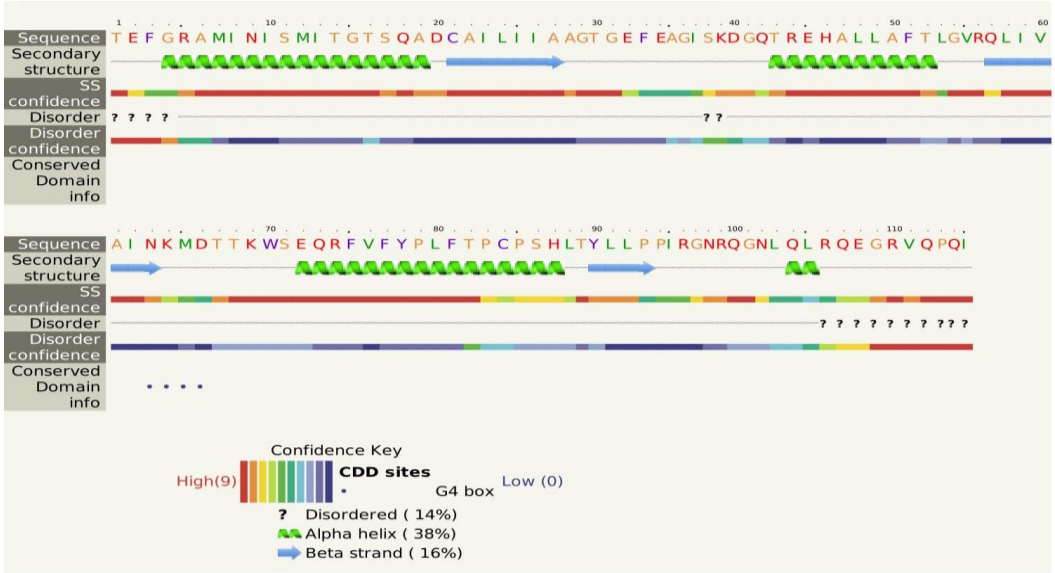

Figure 3.10: Domain analysis of *Puccinia graminis* TEF

Puccinia graminis TEF showed high hydrophobicity on Protscale kyte-Doolittle scale. The amino acids with high hydrophobicity indicated that these residues were in contact with solvent. Therefore, they were likely to reside on the outer surface of the protein.

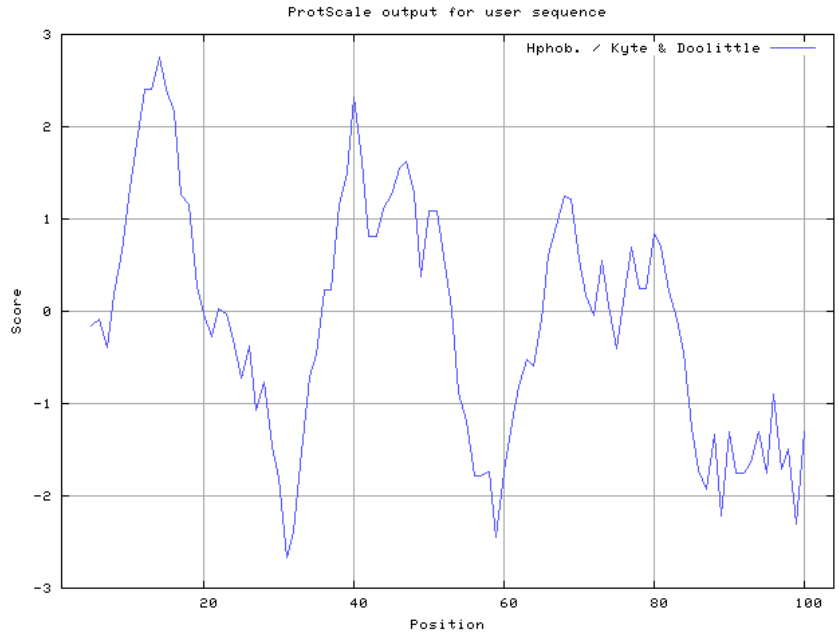

Fig. 3.11: Protscale of *Puccinia graminis* (translation elongation factor)

The percentage and number of amino acids in *Puccinia graminis TEF*. Percentage and number of Leu (L) was found higher in *Puccinia graminis* TEF while Cys (C), His (H) and Trp (W) were present in low percentages and number (Fig. 3.12).

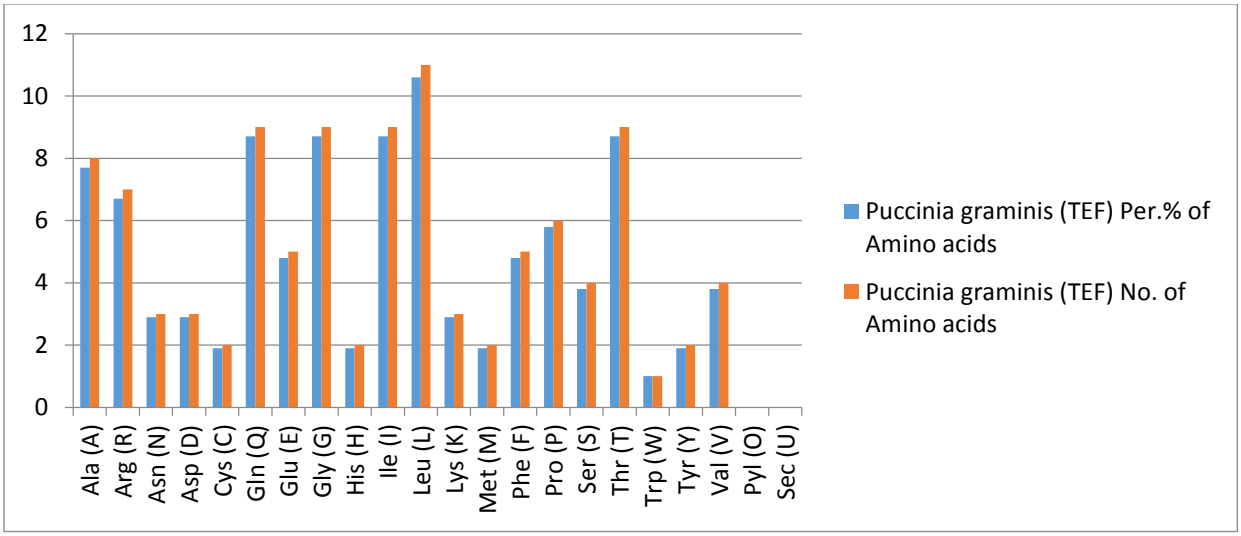

Fig. 3.12: The number and percentage of amino acids (*Puccinia graminis* TEF)

3.6.3 Batch processing of BT & TEF in *Puccinia graminis*

Fig. 3.13 showed that the Protein structures of *Puccinia graminis* consisted of two coding regions Beta-tubulin and Translation elongation factor. Both coding regions represented highly conservative secondary structure of proteins. The alpha-helix showed in blue depicted low confidence ratio in the presence of proteins due to disorder factor.

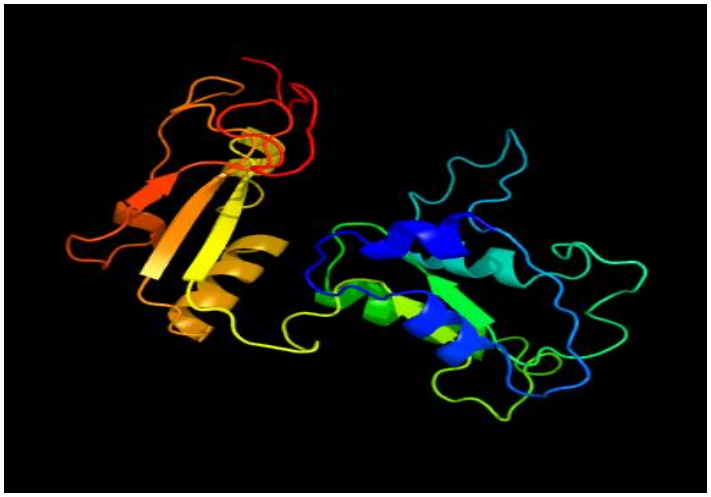

Fig.3.13: *Puccinia graminis* (BT & TEF) 3D structure and domain analysis

The Beta-tubulin and Translation elongation factor were both present in *Puccinia graminis* andpredicted presence of alpha-helices, beta-strands and disordered regions (Fig. 3.14). Alpha-helix was 39% which indicated the stability in depicted protein structure while disordered percentage was low. Alpha-helix showed stability in fungus because BT and TEF were conserved regions.

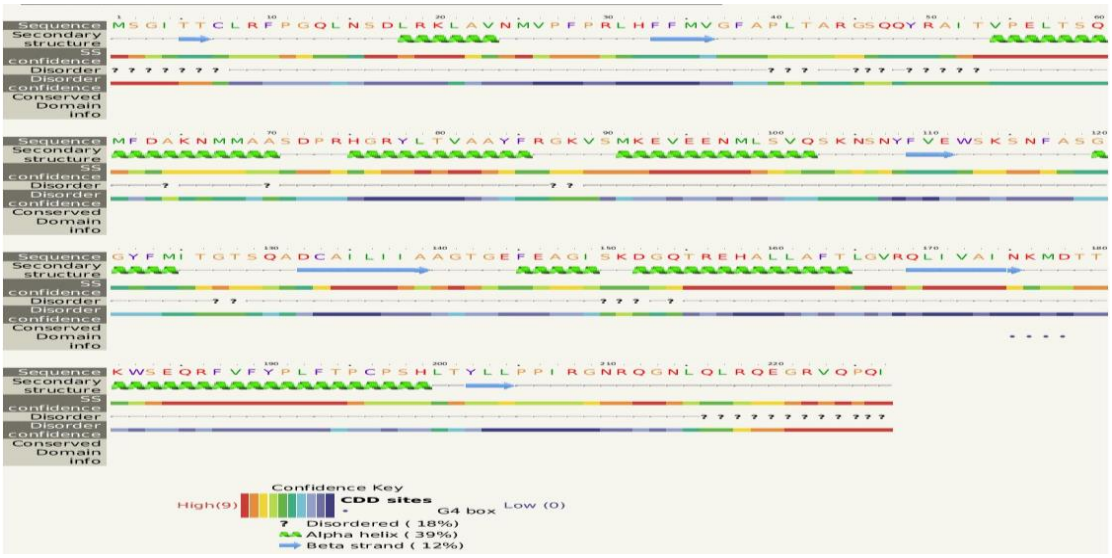

Figure 3.14: Domain analysis of *Puccinia graminis* BT & TEF batch processing

Puccinia graminis BT and TEF showed high hydrophobicity on Protscale kyte-Doolittle scale (Fig. 3.15). The amino acid showed high hydrophobicity suggesting that these residues were in contact with solvent. Therefore, they were likely to reside on the outer surface of the protein.

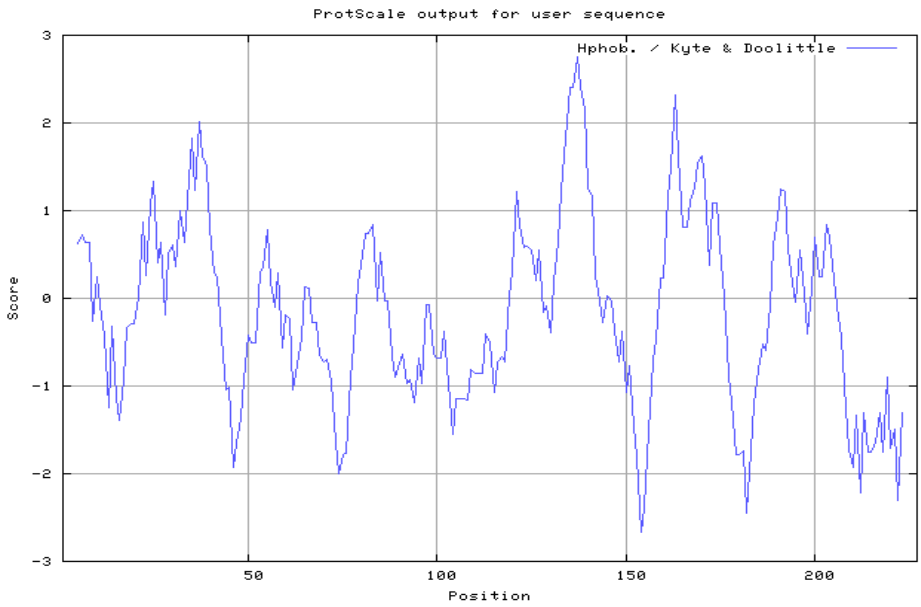

Fig. 3.15: Protscale (To determine the position of amino acids in 3D structure and their score of hydrophobicity) of *Puccinia graminis* (Beta-tubulin and translation elongation factor).

3.6.4 *Puccinia striiformis* (BT): The protein structure of *Puccinia striiformis* BT, represented presence of beta tubulin in *Puccinia striiformis*. Highly conservative secondary structure of beta tubulin were shown in red alpha helix strand according to confidential key (shows conservation of protein) while alpha-helix strand depicted in blue showed low confidence ratio. Generally the structure of beta-tubulin in *Puccinia striiformis* showed a conserved pattern of protein.

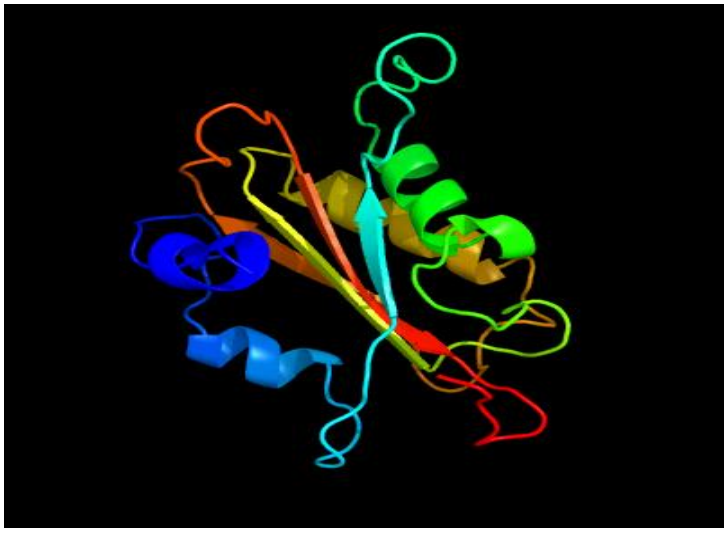

Fig. 3.16: *Puccinia striiformis* (BT) 3D structure and domain analysis

The analysis of beta-tubulin of *Puccinia striiformis* predicted the presence of alpha-helices, beta-strands and disordered regions (Fig. 3.17). The beta strands showed disordered proteins especially at pathological state. The data further showed equal percentage of Alpha-helix (stability) and disorder (unstability).

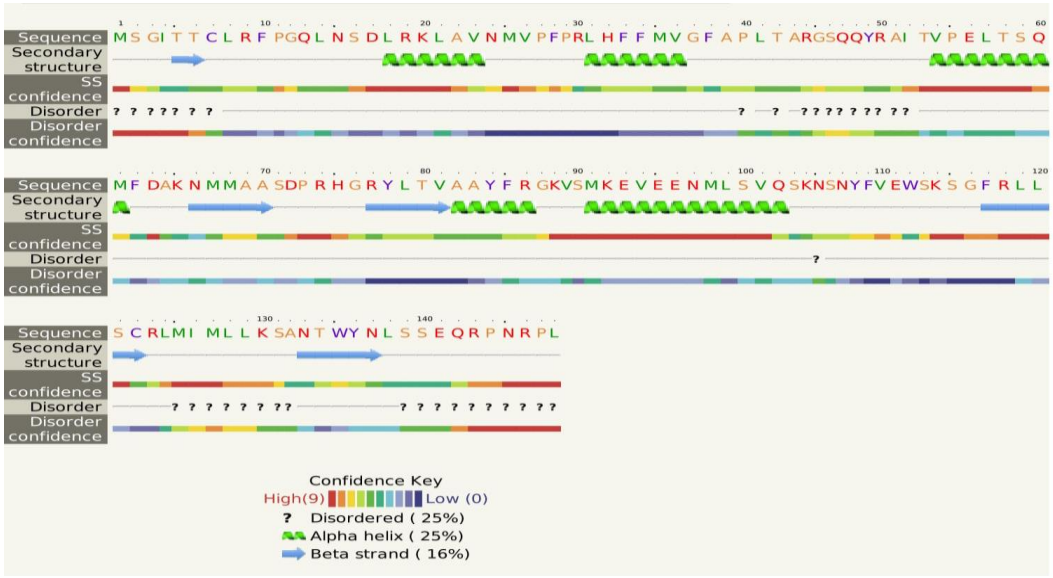

Figure 3.17: Domain analysis of *Puccinia striiformis* BT

The *Puccinia striiformis BT* showed high hydrophobicity on kyte-Doolittle Protscale (fig. 3.18). The high hydrophobicity in amino acid sequences indicated that these residues were in contact with solvent. Therefore, they were likely to reside on the outer surface of the protein.

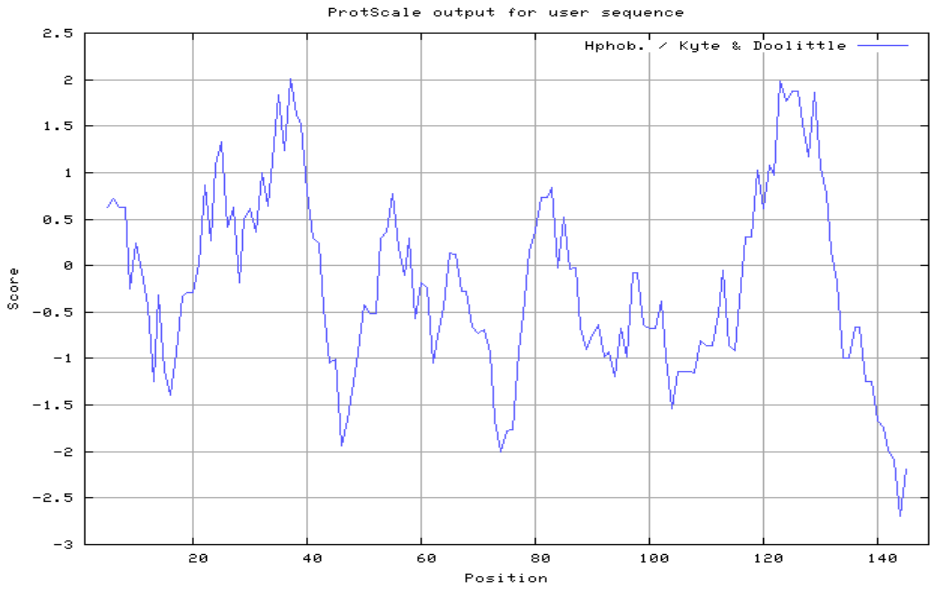

Fig. 3.18: Protscale of *Puccinia striiformis* (Beta-tubulin)

The number and percentages of amino acids in *Puccinia striiformis BT* (fig. 3.19) especially of Leu (L) was high in beta tubulin of *Puccinia striiformis* while Cys (C), His (H) and Trp (W) were found in low percentages.

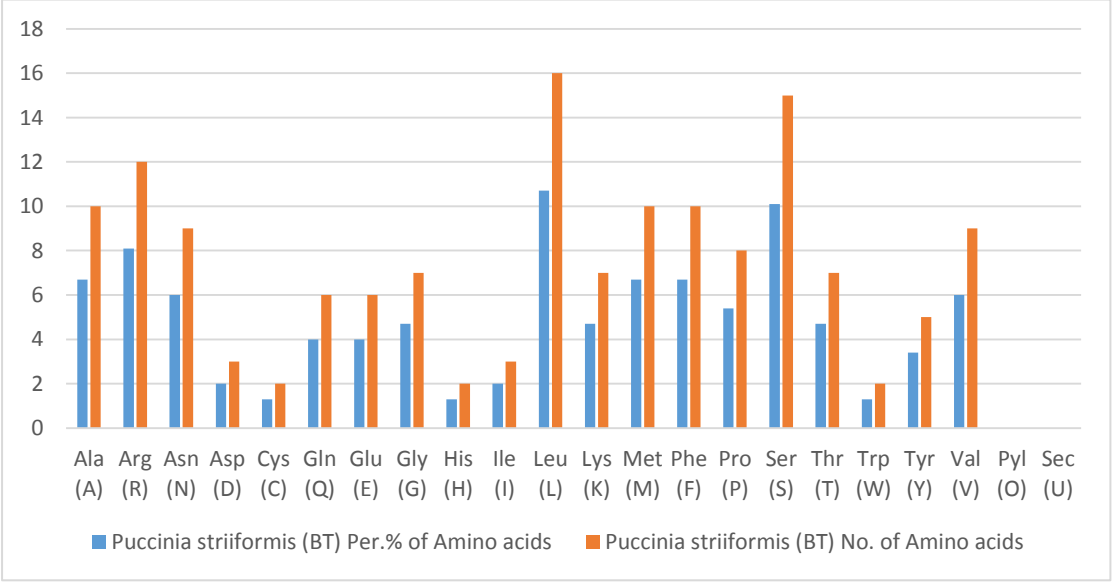

Fig. 3.19: The number and percentage of amino acids (*P. striiformis* BT).

3.6.5 Puccinia striiformoides (BT)

The results showed protein structure of *Puccinia striiformoides* BT, represented the presence of beta tubulin in *Puccinia striiformoides* (Figure 3.19). Highly conserved secondary structure of beta tubulin (shown in red strand) and alpha-helix strand shown

in blue depicted low confidence ratio. Hence the 3D-structure of beta-tubulin in *Puccinia striiformoides* show conserved protein.

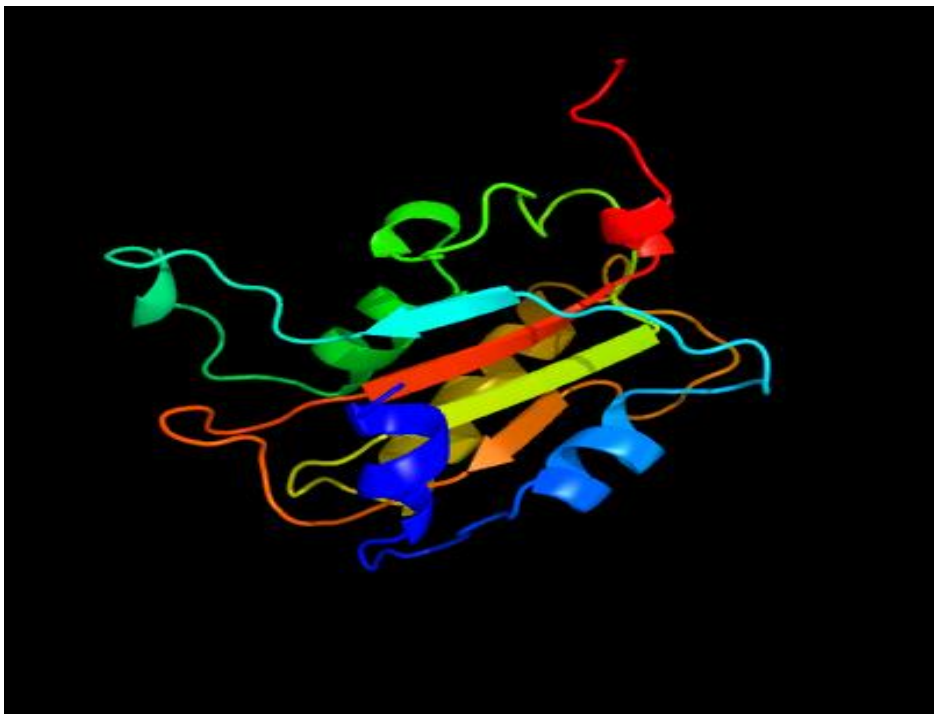

Fig. 3.20: *Puccinia striiformoides* (BT) 3D structure and domain analysis

In RR-4 sample (*Puccinia striiformoides*) proteins of beta-tubulin gene was highly destructive and unstable which could badly infect host plants due to unstable proteins (Fig. 3.21).

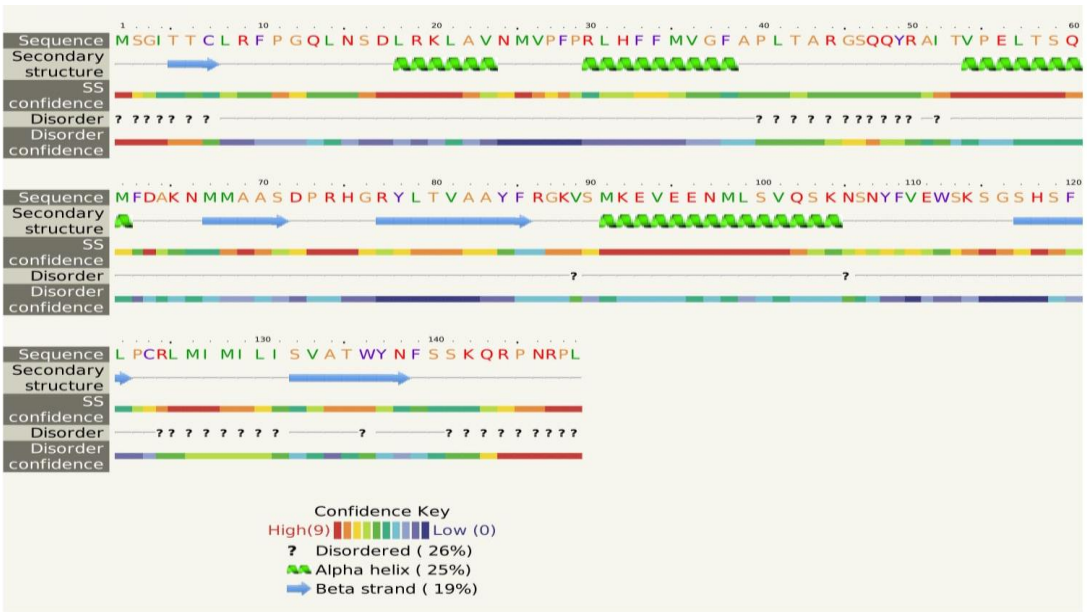

Figure 3.21: Domain analysis of *Puccinia striiformoides* BT

Puccinia striiformoides BT showed high hydrophobicity on protscale kyte-Doolittle scale (Fig. 3.22). Amino acids with high hydrophobicity indicated that these residues were in contact with solvent. Therefore, they were likely to reside on the outer surface of the protein.

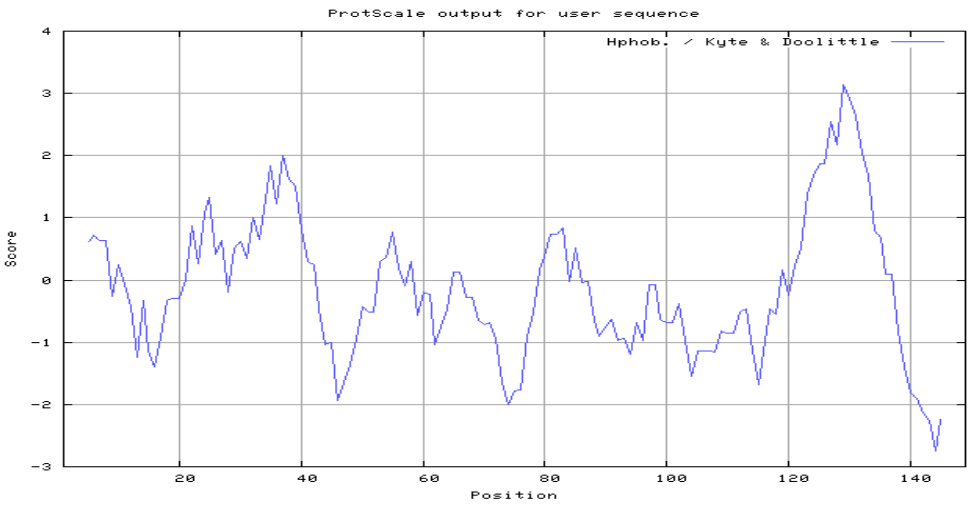

Fig. 3.22: Protscale (To determine the position of amino acids in 3D structure and their score) of *Puccinia striiformoides* (Beta-tubulin)

Percentage and numbers of amino acids in *Puccinia striiformoides BT* showed a higher percentage of Ser (S) high in *Beta-tubulin* of *Puccinia striiformoides* while Cys (C), Asp (D) and Trp (W) were present in low percentage.

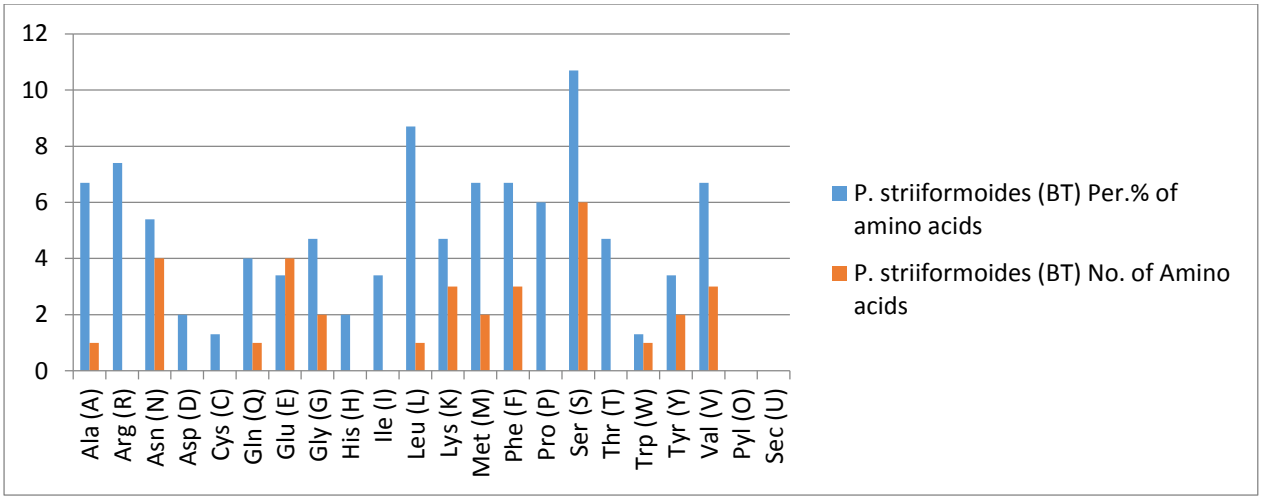

Fig. 3.23: The number and percentage of amino acids (*Puccinia striiformoides* BT)

3.6.6 *Puccinia triticina* (BT)

The protein structure of *Puccinia triticina* BT, showed the presence of beta tubulin. Highly conservative secondary structure of beta tubulin shown in red (shows conservation of protein) while the alpha-helix strand in blue show low confidence ratio (Fig. 3.24).

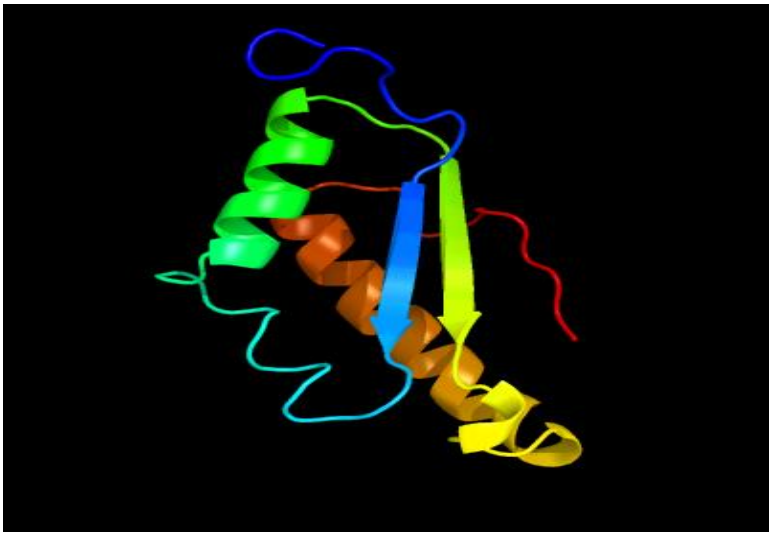

Fig. 3.24: *Puccinia triticina* (BT) 3D structure and domain analysis

of the beta-tubulin of *Puccinia triticina* predicted the presence of alpha-helices, beta-strands and disordered regions (Fig. 3.25). Beta strand showed disorder protein especially at pathological state, therefore in the percentages of disordered and beta strand were almost close (Fig. 3.25) and a high percentage of Alpha-helix. The later predicted the stability in depicted protein though disordered factor was also present. Therefore, the RR-5 sample pertaining to *Puccinia triticina* BT revealed a stable protein with sequence conservation.

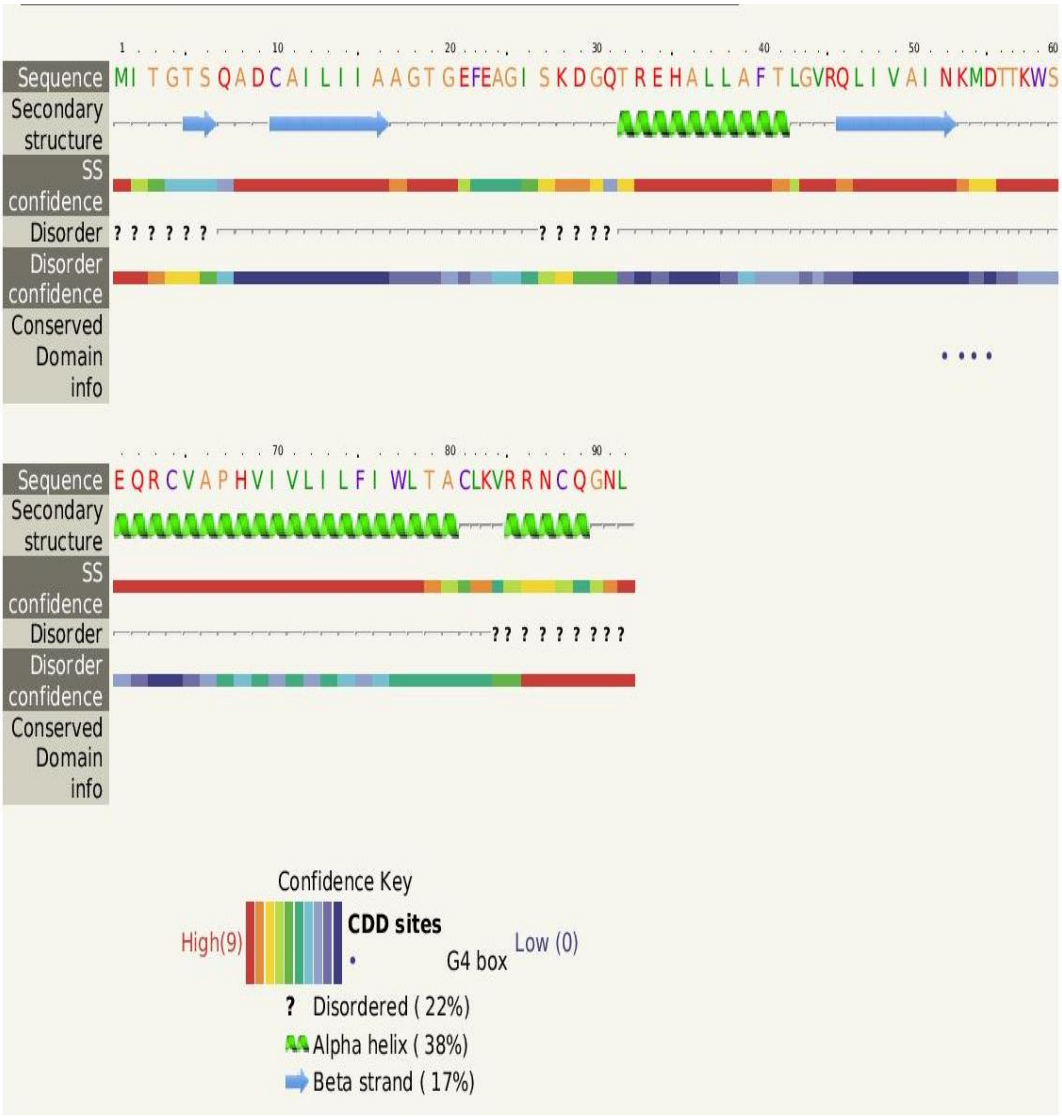

Figure 3.25: Domain analysis of *Puccinia triticina* BT

Puccinia triticina BT showed high hydrophobicity as shown on Protscale Kyte-Doolittle scale (Fig. 3.26). The amino acids sequence depicted the degree of hydrophobicity. Amino acids with high hydrophobicity indicated that these residues were in contact with solvent. Therefore, these were likely to reside on the outer surface of the protein.

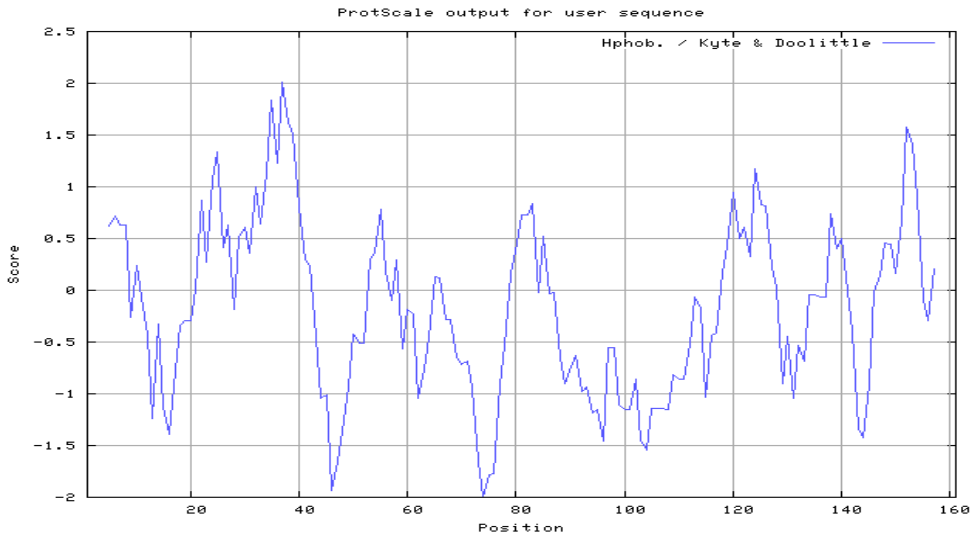

Fig. 3.26: Protscale (to determine the position of amino acids in 3D structure and their score) of *Puccinia triticina* (beta-tubulin).

Fig. 3.27 show the percentage and numbers of amino acids in *Puccinia triticina BT*. Percentage and number of Ser(S) was highest value in Beta-tubulin while those of Cys (C), Asp (D) and Trp (W) were present with low percentage and number.

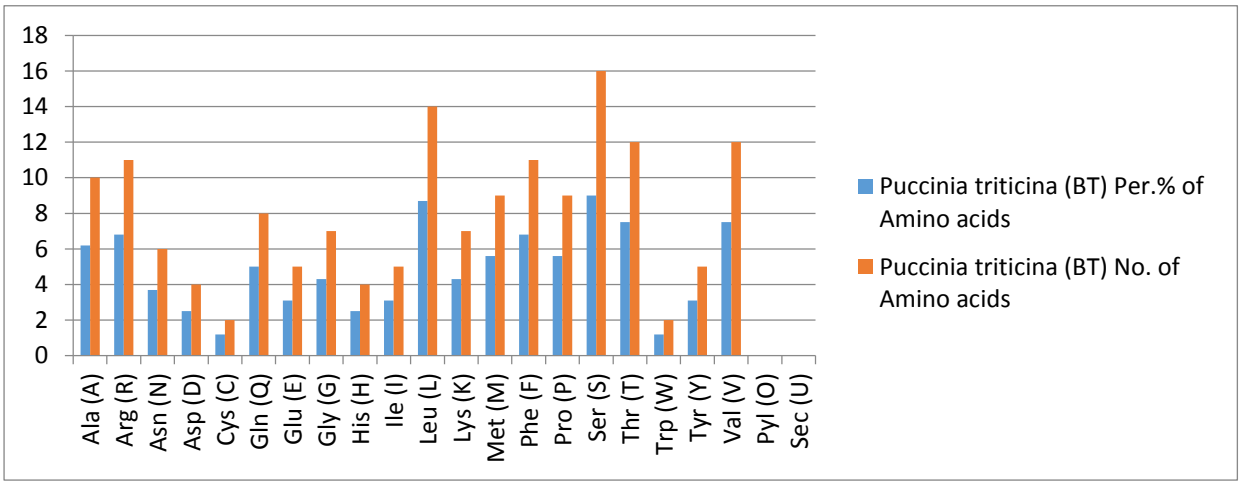

Fig. 3.27: The number and percentage of amino acids depicted for *Puccinia triticina* BT.

3.6.7 *Puccinia triticina* (TEF)

The structure of *Puccinia triticina* TEF depicted a highly conserved secondary structure of beta tubulin as shown in red strand (Fig. 3.28) while the alpha-helix strand shown in blue revealed a a low confidence ratio. Overall the 3D-structure of TEF in showed conservation of protein.

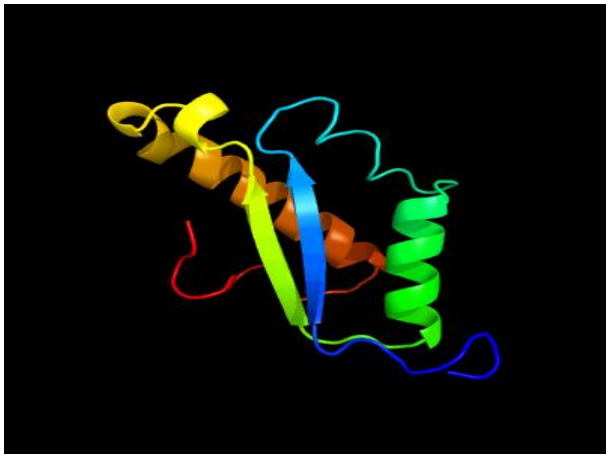

Fig. 3.28: *Puccinia triticina* (TEF) 3D intensive structure and domain analysis

The translation elongation factor of *Puccinia triticina* predicted presence of alpha-helices, beta-strands and disordered regions (Fig. 3.29) . The beta strands showed disordered protein . The high percentage of alpha-helix provided stability to protein although disorder was also present. Hence the sample RR-6 (*Puccinia triticina* TEF) was highly stable and conserved.

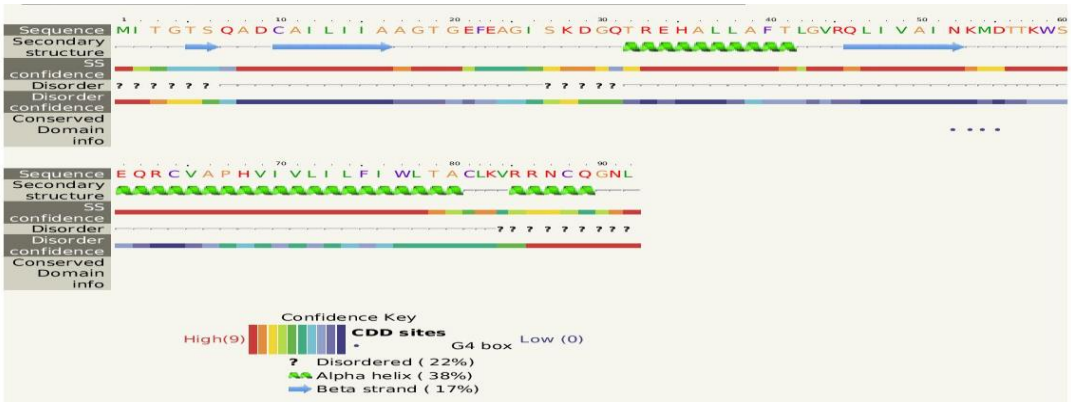

Figure 3.29: Domain analysis of *Puccinia triticina* TEF

Puccinia triticina TEF showed high hydrophobicity (Fig. 3.30). Amino acids sequence was positioned along x-axis whereas y-axis depicted the degree of hydrophobicity. Amino acids with high hydrophobicity indicated that these residues were in contact with solvent. Therefore, these were likely to reside on the outer surface of the depicted protein.

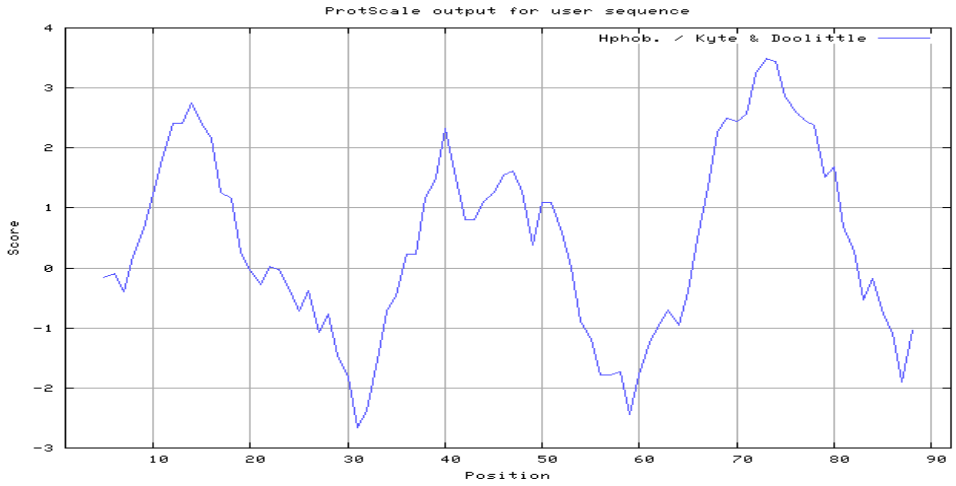

Fig. 3.30: Protscale (To determine the position of amino acids in 3D structure and their score) of *Puccinia triticina* (TEF)

The data (Fig. 3.31) represented percentage and numbers of amino acids in *Puccinia triticina* TEF. The percentages and number of Ala (A), Ile (l) and Leu (L) were high in Translation elongation factor of *Puccinia triticina* while Pro(P) and Trp(W) were low in percentage and number**.**

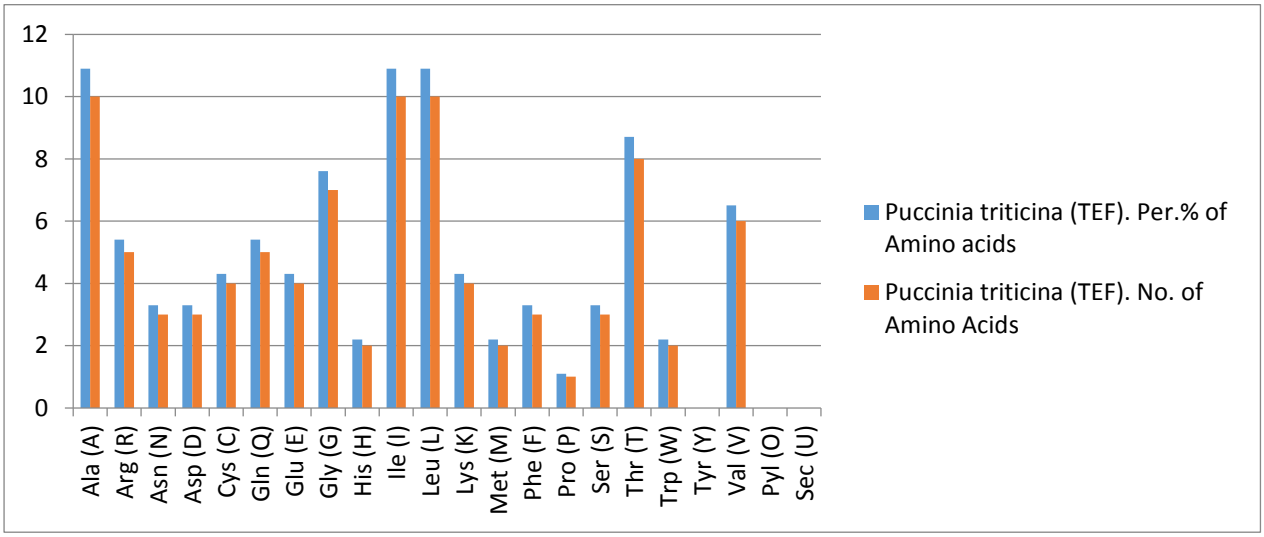

Fig. 3.31: The percentage and number of amino acids (*Puccinia triticina* TEF).

3.6.8 *Puccinia triticina* (BT &TEF)

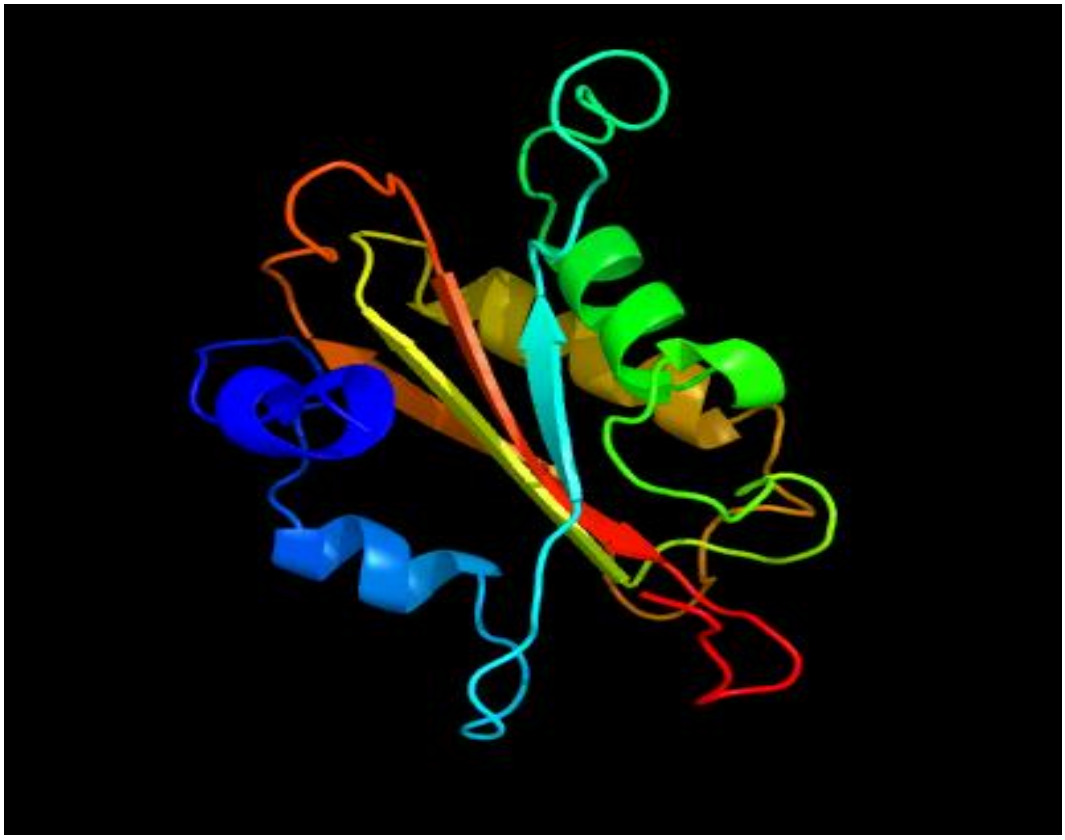

Fig. 3.32: *Puccinia triticina* (BT &TEF) 3D structure and domain analysis

Beta-tubulin and Translation elongation factor of *Puccinia triticina* predicted presence of alpha-helix, beta-strands and disordered regions (Fig. 3.32). The Beta-tubulin and Translation elongation factor strands showed presence of disorder protein , the percentages of disordered and beta strand were similar. The high percentage of alpha-helix showed stability to protein although disorder was present too.

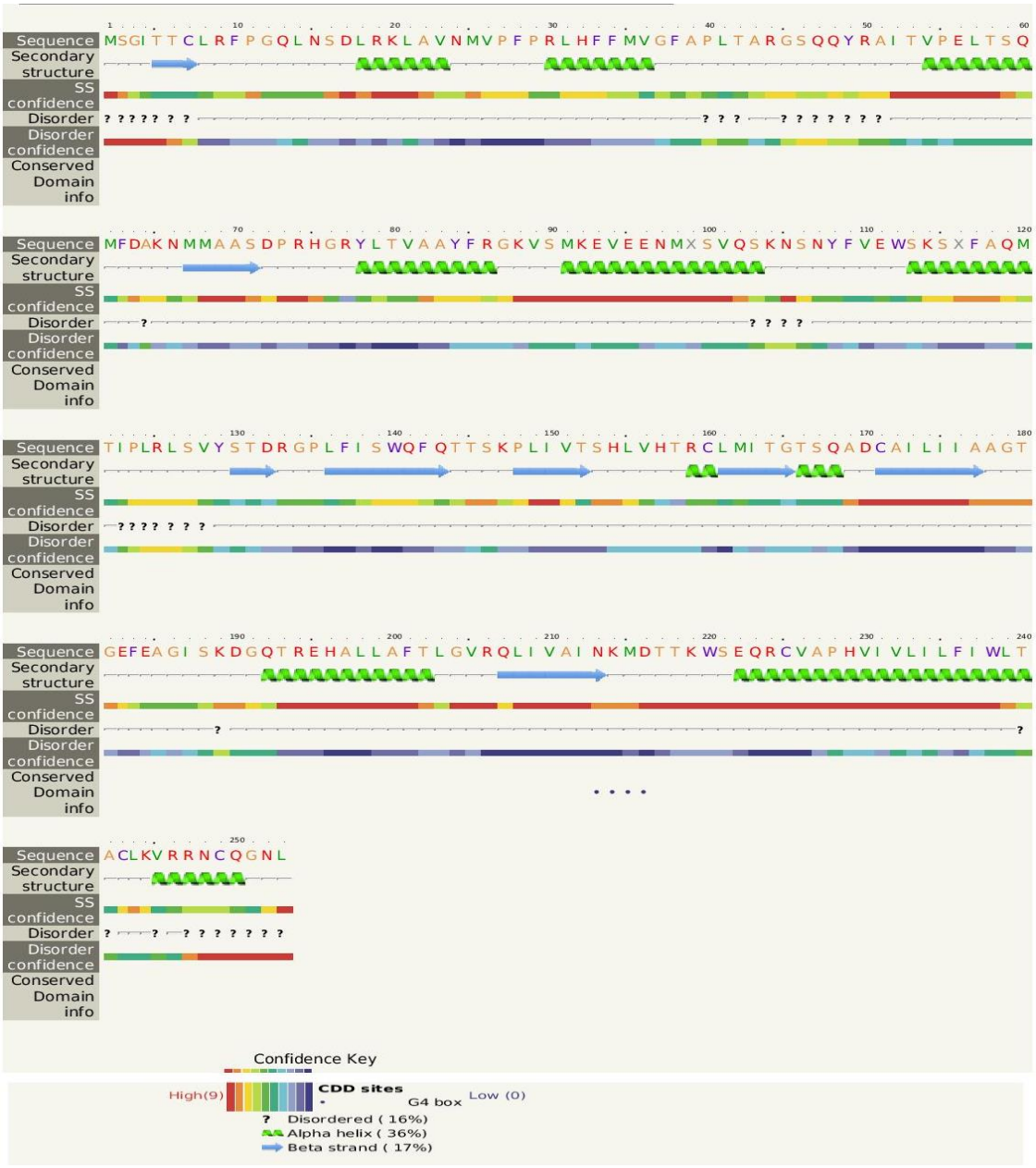

Figure 3.32: Domain analysis of *Puccinia triticina* BT & TEF batch processing

Puccinia triticina BT and *TEF* showed high hydrophobicity on Protscale kyte-Doolittle scale (fig. 3.33). Amino acids sequence with high hydrophobicity indicated that these residues were in contact with solvent. Therefore, they were likely to reside on the outer surface of the protein.

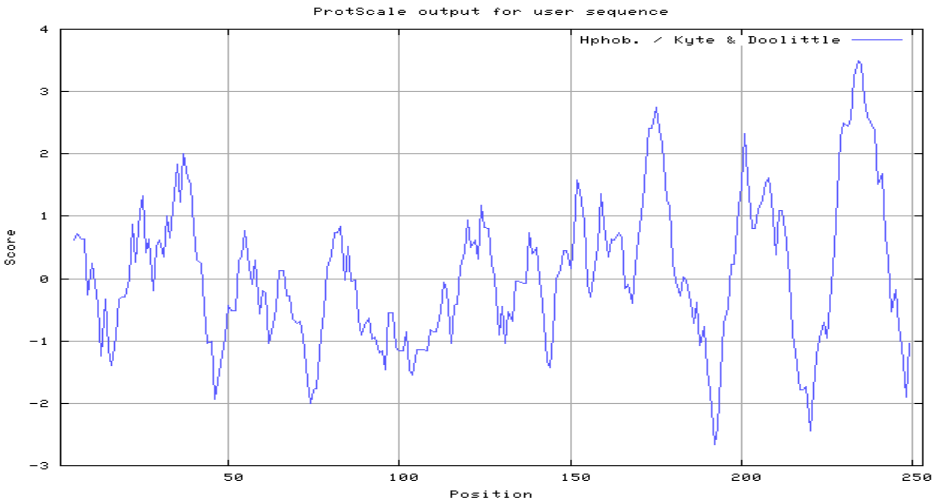

Fig. 3.33: Protscale showing the position of amino acids and their score as depicted for *Puccinia triticina* (BT &TEF).

3.7 Gene Docking

Multiple sequence alignment

Nucleotide sequences of all six samples for four species have been aligned and compared with already reported sequences of *Puccinia graminis* f. *tritici*. Data revealed that shaded regions have significant similary level with *Puccinia graminis* f. *tritici* elucidated in Fig. 3.34. Beta-tubulin and Translation Elongation factor both genes were highly conserved in *Puccinia* species. Therefore, the isolates were used for gene docking to extract highly conserved regions. The highly conserved regions were shown in black strips while those in light grey colour showed the non-significant similarities. Variable amino acid residue distribution was indicated in non-shading bars. The diversity and genetic modifications caused variations in sequence in the respective varities .

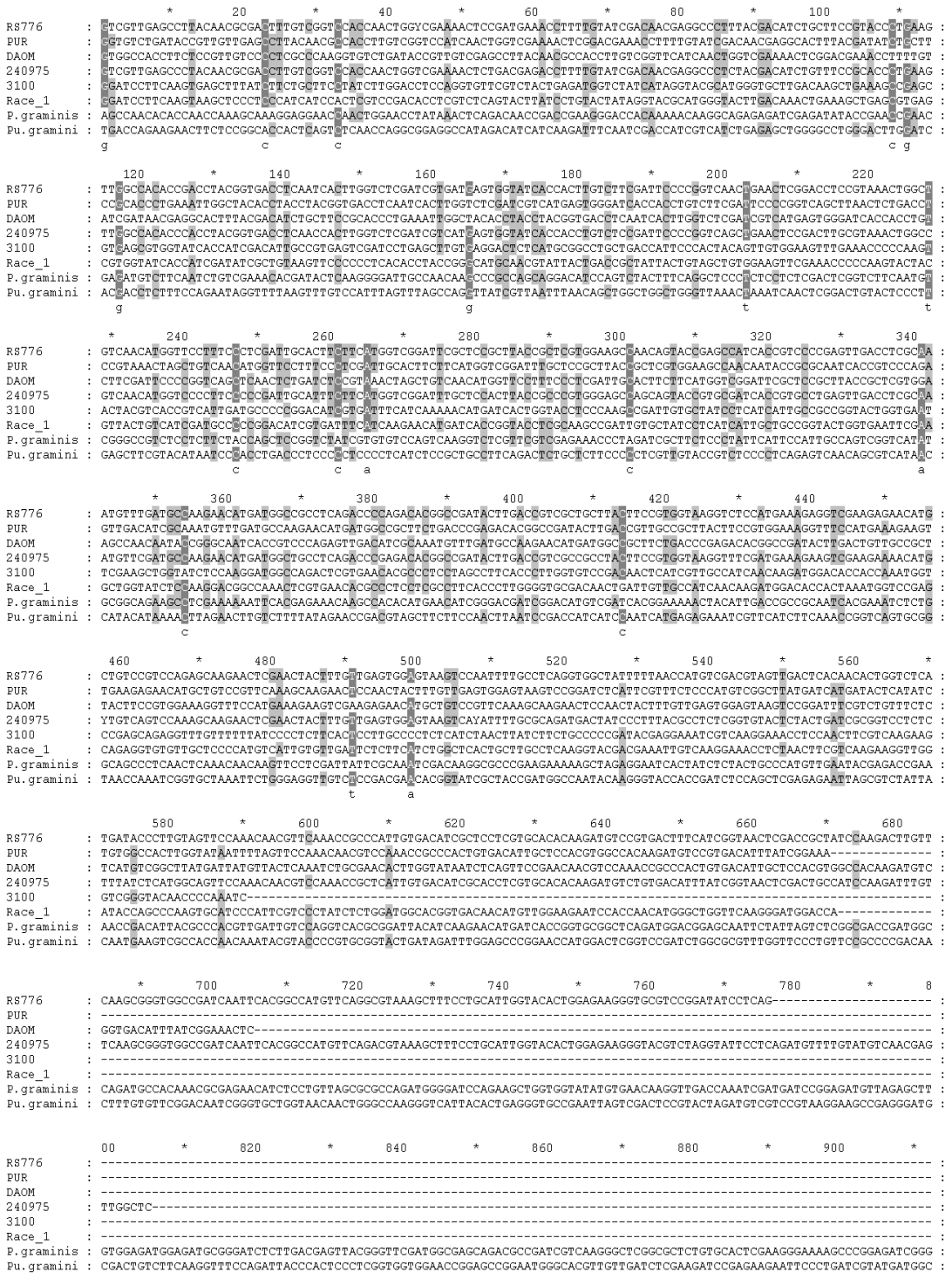

Fig. 3.34: Gene docking of *Puccinia* spp. using *Puccinia graminis* f. sp. *tritici* as a reference sequence**.**

3.8 Phylogenetics of *Puccinia* spp.

3.8.1 *Puccinia graminis* beta tubulin (RR-1) *Puccinia graminis* beta-tubulin (RR-1) showed 100% homology with *Puccinia triticina*. Isolate RR-1 also showed close resemblance within the group and across groups for example *Uromyces*. Phylogenetic tree depicted homology of beta-tubulin region in *Puccinia graminis* RR-1 sample with *Aecidium, Uredo* sps. and some *Puccinia* species (Table 3.5).

Table 3.5: Top nine matches of *Puccinia graminis* BT (RR-1) on NCBI

Serial number	Species	Query coverage	Identity	Accession
01	*Puccinia graminis*	100%	99%	HQ317589.1
02	*Puccinia triticina*	100%	90%	HQ317596.1
03	*Puccinia graminis* f. *tritici*	97%	98%	DQ983221.1
04	*Puccinia albulens*	96%	87%	EF570795.1
05	*Aecidium eburneum*	97%	90%	EF570866.1
06	*Uromyces gaubae*	96%	90%	EF570869.1
07	*Uredo* sp.	96%	88%	EF570871.1
08	*Uromyces dactylidis*	97%	90%	EF570854.1
09	*Uromyces gageae*	96%	90%	DQ983219.1

Puccinia graminis (RR-1) showed 99% Identity with the reported sequence of *Puccinia graminis* (HQ317589.1) and this group showed close resemblance with *Puccinia albulens*, *Puccinia graminis* f.sp.*tritici*, *Uromyces dactylidis* and *Uromyces gageae*. This group showed 94% homology with *Aecidium eburneum* and 95% homology with *Uredo* sp. as well 73% with *Uromyces gaubae*. Phylogenetic analysis also depicted species diversity among *Puccinia* and other groups. *Puccinia triticina* was found highly diverged from all species as depicted through homology percentage.

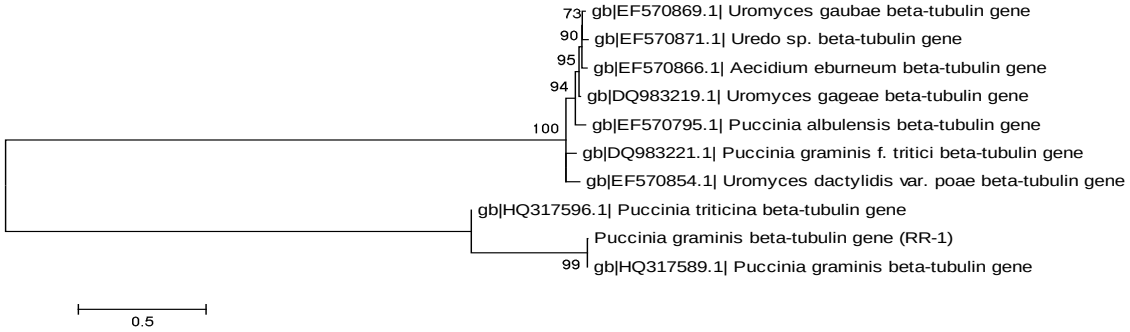

Fig. 3.35: Phylogenetic analysis of *Puccinia graminis* BT (RR-1)

3.8.2 *Puccinia graminis* TEF (RR-2)

The sample RR-6 (*Puccinia graminis* TEF) showed homology with different species of *Puccinia* and *Dietelia* and *Alternaria*. Table 3.6 presents homology of *Puccinia graminis* translation elongation factor with other species of *Puccinia group* (*Puccinia xanthii*), *Dietelia portoricens* and *Alternaria smyrnii* with different query coverage and identity. The data depicted the conservation of translation elongation factor in *Puccinia graminis.*

Table 3.6: Top nine matches of *Puccinia graminis* TEF (RR-2) on NCBI

Species	Query cover	Identity	Accession no
Puccinia graminis	100%	100%	KC853390.1
Puccinia melampodii	84%	90%	EU882268.1
Puccinia xanthii	87%	91%	EU682270.1
Puccinia graminis* f. *tritici	100%	98%	DQ925267.1
Dietelia portoricens	84%	85%	DQ925241.1
Puccinia pazschkei	85%	85%	DQ925278.1
Puccinia arenariae	85%	85%	DQ925257.1
Puccinia poae-nemoralis	87%	85%	DQ925279.1
Aletrnaria smyrnii	83%	91%	JQ672454.1

The sequence of *Puccinia graminis* TEF (RR-2) showed different homology percentages (Fig. 3.36) with *Puccinia* sp, *Dietelia portoricens and Alternaria smyrnii.* RR-2 showed 43% identity with certain *Puccinia* sp. While *Dietelia portoricens* showed 61% homology with *Puccinia pazschkei*. It was clear from this tree that all species showed homology with each other but only *Alternaria smyrnii* is out-group specie due to lack of homology with other species.

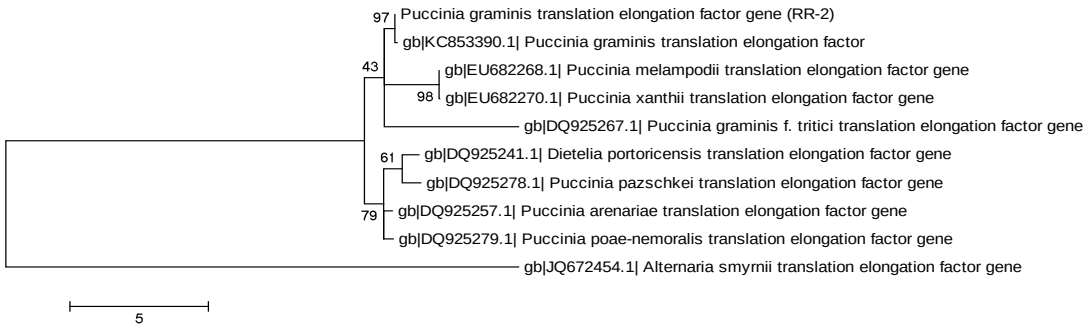

Fig. 3.36: Phylogenetic Analysis of *Puccinia graminis* TEF (RR-2)

3.8.3 *Puccinia striiformis* beta-tubulin (RR-3).

Puccinia striiformis beta-tubulin (RR-3) showed 99% homology with *Puccinia striiformis*. Isolate RR-3 also showed (Table 3.7) close resemblance within the group and with *Uromyces*. Phylogenetic tree showed homology of *Puccinia striiformis* beta tubulin with *Uromyces gageae* and *Puccinia* sp.

Table 3.7: Top ten matches of *Puccinia striiformis* Beta-tubulin (RR-3) on NCBI

Sr.no.	Species	Query coverage	Identity	Accession number
01	*Puccinia striiformis*	100%	99%	HM067989.1
02	*Puccinia striiformoides*	95%	96%	HM067983.1
03	*Puccinia gansensis*	90%	95%	HM067986.1
04	*Puccinia pseudostriiformis*	80%	96%	HM067996.1
05	*Uromyces gageae*	99%	90%	DQ983219.1
06	*Puccinia drabae*	99%	88%	EF570805.1
07	*Puccinia saxifragae*	99%	87%	EF570836.1
08	*Puccinia chrysosplenii*	99%	87%	EF570837.1
09	*Puccinia vaginatae*	80%	88%	EF570846.1
10	*Puccinia hordei*	80%	90%	EF570814.1

Puccinia striiformis BT (RR-3) showed 30% homology with *Puccinia striiformoides*and 92% homology with other *Puccinia* sp. All species were showing (Fig. 3.37) homology but diversity of these species across the group was quite high.

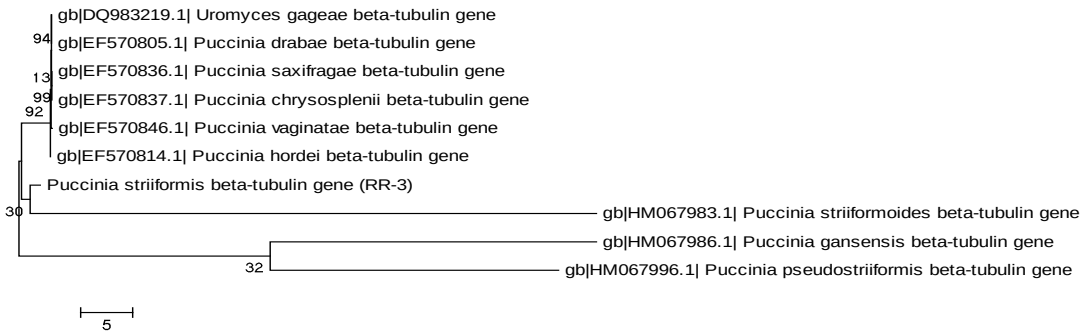

Fig. 3.37: Phylogenetic depiction of *Puccinia striiformis* BT (RR-3)

3.8.4 *Puccinia striiformoides* beta-tubulin (RR-4)

When sequence of sample RR-4 (*Puccinia striiformoides BT*) was blast at NCBI, it showed homology with different species of *Puccinia* and certain other species across the *Puccinia* group. Table 3.8 show homology of *Puccinia striiformoides* beta tubulin with other species of *Puccinia* (i.e. *Puccinia striiformis*) and *Uromyces* with different query coverage and identity.

Table 3.8: Top ten matches of *Puccinia striiformoides BT (RR-4)* on NCBI

Sr.No.	Species	Query coverage	Identity	Accession
01	*Puccinia striiformoides*	92%	100%	HM067999.1
02	*Puccinia striiformis*	100%	97%	EF570843.1
03	*Puccinia gansensis*	93%	96%	HM067986.1
04	*Puccinia striiformis* f. sp. *tritici*	90%	97%	FJ612005.1
05	*Puccinia pseudostriiformis*	85%	96%	HM067996.1
06	*Uromyces gageae*	100%	89%	DQ983219.1
07	*Puccinia drabae*	100%	90%	EF570805.1
08	*Uromyces ehrhartae*	100%	88%	EF570867.1
09	*Puccinia morthieri*	99%	87%	EF570823.1
10	*Uromyces* cf. *viciae-fabae*	90%	87%	HM147313.1

Given data represented the ratio of conservation of Beta-tubulin in *Puccinia striiformoides.* Phylogenetic tree showed (Fig. 3.37) highly diverse groups of species.

Puccinia striiformoides BT (RR-4) showed divergence with certain species of *Puccinia and Uromyces* by showing less homology 37% with already reported species i.e., *Puccinia pseudostriiformis* etc.

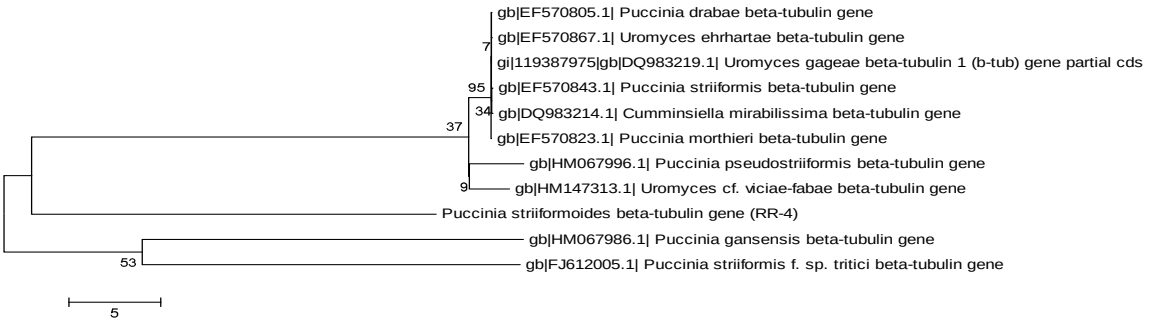

Fig. 3.37: Phylogenetic depiction of *Puccinia striiformoides* BT (RR-4)

3.8.5 *Puccinia triticina* beta tubulin (RR-5)

When sequence of sample RR-5 (*Puccinia triticina BT)* was blast at NCBI, it showed homology with different species of *Puccinia* and certain other species across the *Puccinia* group. Table 3.9 represented homology of *Puccinia triticina* beta-tubulin with other species of *Puccinia* group (i.e. *Puccinia recondite*), *Uromyces poae* and Aecidium brachycomes with different query coverage and identity. Given data represented the ratio of conservation of beta tubulin gene in *Puccinia triticina.*

Table 3.9: Top ten matches of *Puccinia triticina* beta tubulin (RR-5) on NCBI

Sr.no.	Species	Query cover	identity	Accession
01	*Puccinia triticina*	100%	99%	HQ317596.1
02	*Puccinia recondita*	94%	98%	HM068008.1
03	*Puccinia agropyrina*	93%	98%	EF570831.1
04	*Puccinia actaeae*	93%	98%	DQ983222.1
05	*Uromyces poae*	93%	91%	DQ983224.1
06	*Puccinia digraphidis*	93%	90%	EF570839.1
07	*Puccinia graminis*	97%	90%	HQ317611.1
08	*Puccinia pseudostriiformis*	80%	90%	HM067996.1
09	*Puccinia graminis* f. sp. *tritici*	93%	90%	DQ983221.1
10	*Aecidium brachycomes*	94%	85%	EF570791.1

After blast on NCBI *Puccinia triticina* BT (RR-5) it give high diverse ratio with other species but showed homology with *Puccinia graminis* that was already reported. This group showed 43% homology with *Puccinia*, *Aecidium* and *Uromyces groups.* While *Puccinia pseudostriiformis* showed (Fig. 3.38) diversity with other species and acting as an out group.

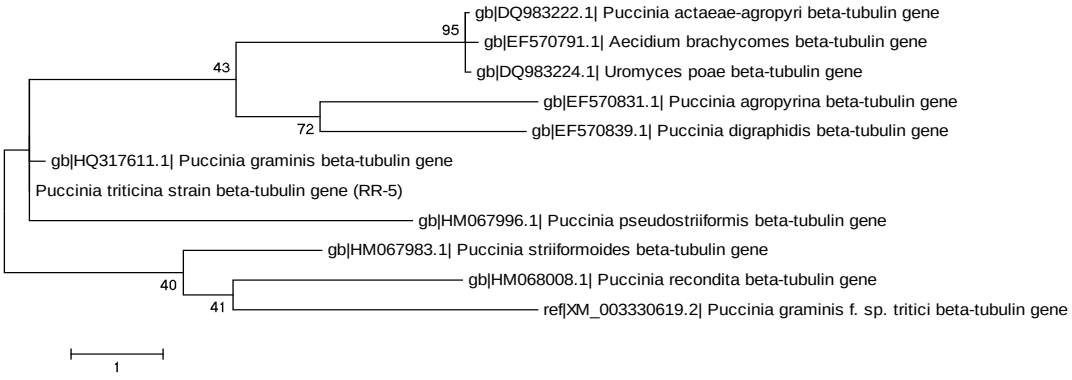

Fig. 3.38: Phylogenetic Analysis of *Puccinia triticina* BT (RR-5)

3.8.6 *Puccinia triticina* Translation elongation factor

When sequence of sample RR-6 (*Puccinia triticina TEF*) was blast at NCBI, it showed homology with different species of *Puccinia* and certain other species across the *Puccinia* group. Below table 3.10 represented homology of *Puccinia triticina* translation elongation factor with other species of *Puccinia* group (i.e, *Puccinia agropyrina*, *Mycotypha microspora*, *Ochropsora ariae and Uredo* sps.with different query coverage and identity. Given data represented the ratio of conservation of translation elongation factor in *Puccinia triticina.*

Table 3.10: Top ten matches of *Puccinia triticina* TEF (RR-6) on NCBI

Sr. No.	Species	Query coverage	Identity	Accession
01	*Puccinia triticina*	100%	99%	JN592753.1
02	*Puccinia agropyrina*	87%	98%	DQ925248.1
03	*Puccinia actaeae-agropyri*	89%	96%	DQ925247.1
04	*Puccinia persistens*	80%	99%	JX533466.1
05	*Puccinia recondita*	80%	94%	JX533489.1
06	*Puccinia subalpina*	82%	94%	JX533486.1
07	*Puccinia xanthii*	75%	90%	EU682270.1
08	*Mycotypha microspora*	78%	88%	AF157272.1
09	*Ochropsora ariae*	76%	80%	DQ925243.1
10	*Uredo* sp.	77%	84%	EF560589.1

Below Phylogenetic tree (Fig. 3.39) represented diversity of *Puccinia triticina* TEF (RR-6) with in the species and across the species, when it was blast on NCBI. While *Ochropsora ariae* behaved like an out group of specie by giving no homology with other species of *Puccinia, Mycotypha microspore and Uredo* sp.

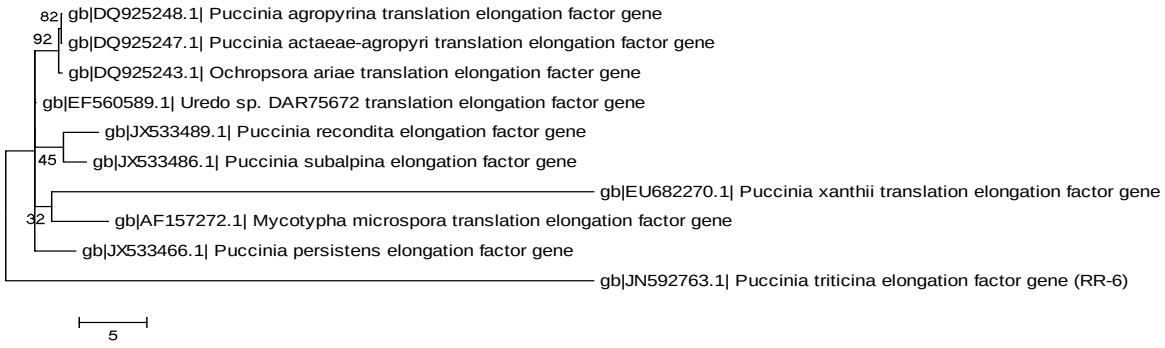

Fig. 3.39: Phylogenetic analysis of *Puccinia triticina* TEF (RR-6)

Chapter 4

Discussion

The causal agents for wheat rusts are *Puccinia* species. Wheat rusts are important to study because they cause world wheat production constraint (Sarri and Prescott, 1985). During present study aeciospores of *P. graminis, P. striiformis, P. striiformoides* and *P. triticina* are used for identification and phylogenetic study of rust species based on selected coding regions (BT and TEF), their sequences analysis and phylogenetic relationship among the species. Two different partial genes (β-tubulin and EF1-α) were used. This study revealed that the size of obtained sequences vary between genes: such that the sequences were obtained for Translation Elongation factor gene and the length of sequences that of β-tubulin partial genes were longer.

4.1 DNA sequence analysis and species identification

The study focused on DNA sequence analysis and phylogenetic relationship between different species. In general, it is more difficult to obtain sequences by using beta-tubulin and translation elongation factor genes**.** The tendency of sequences using coding regions is very low. In the taxonomic study of *P. striiformis*, Liu and Hambilton (2010) successfully amplified 48 sequences from ITS; 33 from beta-tubulin. Similarly, in the study about evolutionary relationship among *Uromycetes* and *Puccinia* species (Van der Merwe et al., 2007) 66 from TEF and 31 from Beta-tubulin were amplified. Since, amplification ratio of beta-tubulin is lower as compare to other genetic markers, the results presented here agree with the previous studies made on these loci.

Generally, it is assumed that EF1-α gene is suitable for studies of closely related species because of its highly variable introns (van der Merwe et al. 2007). But in this study the identification ratio of *Puccinia* spp. by using beta-tubulin is 4 while, ratio of identification of TEF is 2. As, this study reveals high percentage of identification of *Puccinia* species based on Beta-tubulin data. Several other studies also reported ITS marker for barcoding of plants (Kress et al., 2005, Edwards et al., 2007). But other studies indicate the inherent difficulties associated with ITS, e.g. low PCR success (Chase et al., 2007, Kress et al., 2004)**.**

4.2 Translation of nucleotides into proteins

This study focused on protein sequences which were further used for bioinformatics computational modelling revealing the accuracy of conservation and their phylogenetic relationship with other species. *Puccinia triticina* has high molecular weight while all other species were showing lower molecular weight as compare to *Puccinia triticina* while number of amino acids in *Puccinia striiformis* (BT) was larger as compare to other species by giving a longer conserved region. Extinction- coefficient phenomena was observed by Gill (1989) indicated the absorption of light in particular protein at specific wavelengths is highest for *P. striiformoides* BT. Aliphatic index predicts thermostability (Ika, 1980) of particular protein is highest in *P. triticina* (TEF). Estimated half-life indicates time, in which half of proteins disappear after synthesis in a cell. Kharrazi and Bobojonov, (2012) has also assessed these physiochemical parametres in wheat, triticale and rye. Atomic composition of *Puccinia* spp. shows number of carbon and sulphur atoms in *P. striiformis* BT and *P. striiformoides* BT are greater as compare to other *Puccinia* species. Proteins of all six species (*Puccinia*) are stable and their stability index is 24-39. Numbers and percentage of amino acids in these 6 samples of *Puccinia* spp. (Only those amino acids which are commonly high in 6 isolates) Ser(S) is high in *P. graminis* BT as compare to other five isolates. Asn, Glu, Leu, Ala, Ile, and Ser these are commonly high in percentage and numbers in *P. graminis* BT, *P. graminis* TEF, *P. striiformis* BT, *P. striiformoides* BT, *P. triticina* BT and *P. triticina* TEF. *P. graminis* BT is rich with amino acid Asn, Glu and Ser, which are low in number and percentage as compared to other species.

4.3 Computational modeling of proteins

Proteins homology and analogy assessed as 3D structures of proteins of *P. graminis* BT & TEF, *P. striiformis* BT, *P. striiformoides* BT and *P. triticina* BT&TEF. Domain-analysis of these structures has determined the percentage of alpha-helix, which helps in stability of proteins, Beta-sheet and disordered percentage. Beta sheet and disordered percentage are directly linked with each other because they give unstability to the proteins at pathological level. Batch analysis of *P. graminis* (BT&TEF) and *P. triticina* (BT and TEF) alpha-helix percentage is high as

compare to *P. striiformis* and *P. striiformoides* which determines the stability of proteins. Protscale kyte-Doolittle scale (1982), proteins of all *Puccinia* spp. have shown hydrophobicity due to amino acids contact with solvent. The validated protein model proposed in this study can be used further to dock with possible co-factors or relevant protein interactors to understand the potential mechanism of anti-stress and defense properties of these proteins. Because the multiple sequence alignment by gene docking revealed conserved regions which play a key role in phylogenetic studies of *Puccinia* spp. Beta tubulin and translation elongation factor are the polymorphic sequences of genes, which help in determination of genetic variation (Chen et al., 2012). The computational assay described point mutation is the major sequence variation at specific level (Kelley et al., 2015).

4.4 Phylogenetic analysis

The phylogenetic relationships of *Puccinia* species revealed that mutation is the major evolutionary mechanism to create heterokaryotic and genetic variation (Chen et al., 2012). Accuracy of Phylogenetics is directly linked with exon (coding regions) instead of introns (non-coding regions). Exons are highly homologous whereas introns are highly conserved sequences (Maneu et al., 1996). Fungal phylogenetics has been revolutionized with advances in molecular biology leading to phylogenetic algorithms and multigene datasets. Phylogenomics and multigene give relationship of species at very high level (James et al., 2006; Spatafora et al., 2006). Beta- tubulin and Translation elongation factor are used as a maker for phylogenetic study at species level (Walker et al., 2012).

The phylogenetic relationship of *Puccinia graminis* BT with *P. triticina, P. graminis* f. sp. *tritici, P. albulens, Uromyces, Uredo spp*. and *Aecidium eburneum* with different identity (above 87%) and different query coverage (above 96%) has been determined (Chen et al., 2012). *P. graminis* BT (RR-1) closely related with other species of *Puccinia* spp. *Uromyces* and *Aecidium species* has been determined. Hence by using Beta-tubulin gene as a phylogenetic marker, this study reveals that *Puccinia graminis* is closely related with other groups. This study shows phylogenetic relationship of *P. graminis* with other groups and within the group by using genetic marker (Translation elongation factor). *Puccinia graminis* and Puccinia triticina do not have any relation with each other according to ITS (Chen et al., 2012). This study also reveals that

Puccinia graminis and *Puccinia triticina* have no relation with each other. *Puccinia graminis* f. *tritici* is an out-group which does not link with any other species except *P. graminis, P. graminis* shows 43% homology with other species in phylogenetic tree (Walker et al., 2012).

According to ITS study four *Puccinia striiformis, Puccinia striiformoides, Puccinia pseudostriiformis* and *Puccinia gansensis* have monophyletic origin (Walker et al., 2012). This study also shows similar relation of *Puccinia striiformis, Puccinia striiformoides, Puccinia pseudostriiformis* and *Puccinia gansensis* has monophyletic origin. *Puccinia striiformis* causes heterokaryosis which is involved in pathogenic variation. Mechanism of heterokaryosis in evolution of pathogen has been studied using three genes MAPK, Beta-tubulin and Translation elongation factor genes (Chen et al., 2012). This study also shows that *P. striiformis* (BT) causes heterokaryosis as in phylogenetic tree sample RR-3 shows quite divergence from other members of *Puccinia* as well as *Uromyces* (30% homology). Since, heterokaryosis causes genetic variation in species which has been clear from this study. *P. striiformoides* (BT) shows 53% homology with *P. striiformis* and 73% homology with *Uromyces* as well as with *Puccinia* species, it shows diverse behavior of phylogenetic linkage. This study reflects *P. triticina* (BT) act as outgroup by showing 43% homology with *Puccinia* and other species of group. Phylogenetic analysis of *P. triticina* TEF (RR-6) by showing 0% homology with *Puccinia* species but very close to *Puccinia persistens*. Since, it shows greater diversity in *Puccinia* group by acting as an out group.

Polymorphic sequences of genes are very useful in determination of genetic variation among *Puccinia* species (Chen. et al., 2012). The study further revealed that genetic variation exists in *Puccinia* species as they are studied on basis of polymorphic sequences. The study revealed that point mutation is the major sequence variation including the heterokaryotic variation within the species and homokaryotic variation among the species.

Conclusion

- Based on these coding regions the identification and characterization of *Puccinia* spp. are done suggesting their usefulness.
- Computational modelling of *P. graminis, P. striiformis, P. striiformoides and P. triticina* are done with 3D structures and domain analysis, which play a pivotal role in genetic variation.
- The assessment of physiochemical parameters signified their role in stability and conservation of proteins in Beta-tubulin and Translation elongation factor of *Puccinia* spp.
- Furthermore, in phylogenetic analysis BT and TEF of selected *Puccinia* species proved high level of conservation.

Future perspectives

- Genetic variation can be controlled by using bioinformatics computational tools.
- These bioinformatics tools can apply on pathogenic causing genes for comprehensive study of pathogens.
- Mutational changes can be assessed within pathogenicity causing genes by applying different chemicals.
- These genetic markers can be used for barcoding of specific pathogen.

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