J. Crop Prot. 2015, 4 (3): 321-328

Research Article

PCR-based characterization of *Puccinia graminis* f. sp. *tritici* race TTSSK from Iran

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Abstract: Stem rust caused by *Puccinia graminis* f. sp. *tritici* (*Pgt*) is one of the destructive diseases of wheat in the world. The fungal pathogen can infect 365 different grass and more than 70 *Berberis* species. DNA sequences for the ribosomal RNA internal transcribed spacer (ITS) have proven suitable to explore relationships at the species and subspecies levels. An isolate of *Pgt* which was collected from Iran and designated as TTSSK was used in this study. Three samples of the isolate were used. ITS region of the samples was amplified and sequenced. Consensus tree based on Maximum Parsimony clustering method was produced by Mega 6.0. Iranian isolate of TTSSK was placed in a clade with *P. graminis* which was isolated from *Berberis* sp. and *Pgt* isolate from Tajikistan. The results showed that more than one conserved genomic regions would be informative to identify phylogenetic relationship of Iranian *Pgt* isolates and samples from different parts of the world. Complementary studies with more sequence data from other genome loci are in progress.

Keywords: ITS, maximum parsimony, phylogenetic relation, stem rust, Ug99 race group

Introduction

Stem rust (black rust), caused by *Puccinia graminis* f.sp. *tritici* (*Pgt*) is one of the most important and destructive diseases of wheat in the world. The fungal pathogen is obligate parasite and can infect wheat plants during growing season (Singh *et al.,* 2006). The urediniospores can infect 365 different grass species including economically important crops like wheat, rye and oats (Leonard and Szabo, 2005). Also more than 70 *Berberis* species and some *Mahonia* species have been reported as alternate hosts of *Pgt* (Berlin *et al.,* 2012).

Emergence of Ug99 (TTKSK) race in 1999 imposed a serious threat to wheat production worldwide due to its virulence against *Sr31* (Singh *et al.,* 2011). Ug99 had been confirmed in Uganda, Kenya, Ethiopia, Sudan, and Yemen. Occurrence of Ug99 in Yemen was a significant confirmation that it was moving toward the important wheat growing areas of the Middle East and Asia. Afterward its presence in Iran was confirmed in March 2008 and supported these predictions. Iranian Ug99 isolates were collected from Borujerd and Hamadan, in northwestern Iran (Nazari *et al.,* 2009). Also, to date it is not known to have spread beyond Iran. Unusual occurrence of stem rust in Pakistan in 2009 imposed some fears of an Ug99 incursion, but phenotyping of the samples and DNA analysis revealed the absence of Ug99 and presence of another important race (Mirza *et al.,* 2010; Singh *et al.,* 2011). Changes in pathogen populations are rapid. To date, 8 pathotypes have been reported in Ug99 lineage which each has a unique combination of virulence and they differ for

Handling Editor: Vahe Minassian

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Received: 9 June 2014, Accepted: 28 October 2014
Published online: 11 May 2015

virulence to resistance genes *Sr21, Sr24, Sr31* and *Sr36* (Jin *et al.,* 2008, 2009; Park *et al.,* 2011; Pretorius *et al*., 2010, 2012). These pathotypes have been shown to have identical fingerprints using microsatellite markers, suggesting that they have risen from a common ancestor via single-step mutation (Pretorius *et al.*, 2010).

Molecular approaches based on polymerase chain reaction (PCR) have become efficient tools for quick detection and study of the plant pathogens (Edel, 1998; Mutasa *et al.,* 1995; Ward, 1994). The internal transcribed spacer (ITS) regions are useful for targets and have been used for specific detection of numerous plant pathogens (Ge and Guo, 2003; Ghignone *et al.,* 2003; Sholberg *et al.,* 2004; Yang *et al.,* 2003; Zhao *et al.,* 2007). House-keeping genes are considered valuable for studies on population structure and macroevolution; this is in contrast with virulence/avirulence genes which are subjected to strong host selection (Taylor *et al*., 1999). In recent years, EF1α and β-tub1 genes (Van der Merwe *et al.,* 2007) and nucLSU rDNA (Maier *et al.,* 2007) sequences have been used to study phylogeny of Pucciniaceae. Although, rDNA ITS regions have proven helpful for phylogenetic studies as well (Alaei *et al.,* 2009; Abbasi *et al.,* 2004; Chatasiri *et al.,* 2006; Kropp *et al.,* 1997; Pei *et al.,* 2005; Smith *et al.,* 2004; Szabo, 2006; Weber *et al.,* 2003; Wood and Crous, 2005; White *et al*., 1990). There are advantages to use rDNA ITS regions in phylogenetic studies including, multi copy nature, inter specific diversity and availability in gene bank (Barnes and Szabo, 2007; Hamelin *et al.,* 1996; Schena *et al.,* 2004).

As study of phylogenetic relationships of Iranian isolates of *Pgt* Ug99 race group with other *Pgt* isolates have not been performed, the object of this study was phylogenetic characterization of *Pgt* TTSSK race in Iran based on molecular markers.

Materials and Methods

Stem rust isolate

An isolate of *Pgt* which was collected from Dasht-e Azadegan (Khuzestan province) was received from the Department of Cereal Research, Seed and Plant Improvement Institute Karaj, Iran. The isolate was maintained and purified on Morocco cultivar using McIntosh *et al.* (1995) method. Seven-day-old seedlings were inoculated by urediniospores. Seedlings were incubated at 18 ºC in a dew chamber in darkness for 14h. Then the inoculated plants were placed on the greenhouse bench at 25 ± 2 ºC with a photoperiod of 16h (Jin *et al.,* 2007). 14 days after inoculation single pustule was used for inoculation of healthy susceptible seedlings. Afterward single-postulated isolate was increased on Morocco cultivar. Race identification was done based on differential sets received from CIMMYT and ICARDA (Jin *et al.,* 2008) and designated as TTSSK (Mojerlou *et al.,* 2013). The difference between TTSSK and TTKSK was confirmed by virulence against *Sr36* and avirulence against *Sr17* using Combination VII (*Sr17*), LC/ Kenya Hunter (*Sr17*) and W2691SrTt-1 (*Sr36*) differential lines.

Genomic DNA extraction

Total DNA was extracted according to Roos-Amsaleg *et al.* (2002) method with some modifications. Three DNA samples were extracted from single- postulated urediniospores and were considered as Pgt1, Pgt2 and Pgt3. 1mg of non-germinated urediniospores was ground in a 1.5ml micro tube with 50µl of extraction buffer (100mM Tris-HCl, pH 9.0; 20mM EDTA, pH 8.0; 1.4mM NaCl, 2% CTAB). Then total volume reached to 600µl by adding extraction buffer. The solution was incubated for 2h at 65 ºC. Then 600µl phenol: chloroform: isoamyl alcohol (25:24:1, v/v/v/) was added to each tube. Phases were separated by 13000 rpm centrifugation. Upper phase was transferred to 1.5ml micro tube and DNA was precipitated with 700µl of cold isopropanol. DNA pellet was dried and re-suspended in 50µl sterilized deionized water (Wang *et al.,* 2009). The extracted DNA was treated with RNase A. Then quality and quantity of the isolated DNA were determined using agarose 1% (w/v) electrophoresis and spectrophotometer (Eppendorf 6131) measurement. DNA samples were adjusted to final concentration of 50 ng/ µl for PCR amplification.

ITS amplification

Three isolates of *Pgt* race TTSSK were used in PCR amplification of ITS region with universal primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') (Gardes and Bruns, 1993) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990). PCR parameters were: an initial denaturation for 5 min at 94 ºC, then 40 cycles consisting of denaturation at 94 ºC for 1 min, annealing at 46 ºC for 1 min and extension at 72 ºC for 1 min and a final extension at 72 ºC for 10 min. sterilized distilled water was used as negative control. PCR products were electrophoresed in a 1.2% (w/v) agarose gel containing ethidium bromide (0.5µg/ml) in 1X TAE buffer. Approximately 700bp of an ITS1, 5.8S and ITS2 regions were amplified using ITS1F and ITS4 primers.

Sequencing and phylogenetic analysis

PCR products were direct sequenced. Sequencing was performed by the Pishgam Biotech Company (www.pishgambc.com). Blast searching of ITS sequences was done and the sequences were aligned using Mega 6.0 software. Clustering method Maximum Parsimony (MP) was used to estimate the phylogeny with the aid of Mega 6.0. 1000 bootstrap replicates were performed. Consensus tree was produced by Mega 6.0 too.

Results and Discussion

Amplified segment (Fig. 1) for three samples of *Pgt* race TTSSK from Iran were sequenced and aligned together with related sequences obtained from Genbank. Afterward data were analyzed by Mega 6.0 software and a consensus tree was estimated. The estimated consensus tree for ITS region using MP clustering method is shown in Fig. 2.

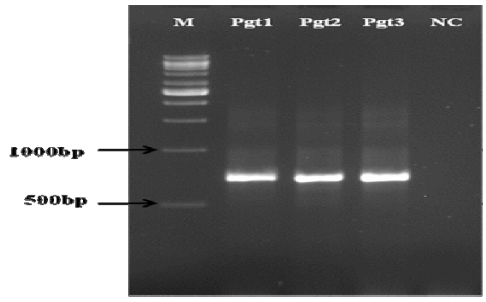

Figure 1 Ethidium bromide- stained agarose gel of Polymerase chain reaction products with universal primers ITS1F and ITS4 from 3 isolates of *Puccinia graminis* f.sp. *tritici* (*Pgt*). NC = Negative control (sterile water) and M = 1Kb DNA ladder (NL-1411Vivantis).

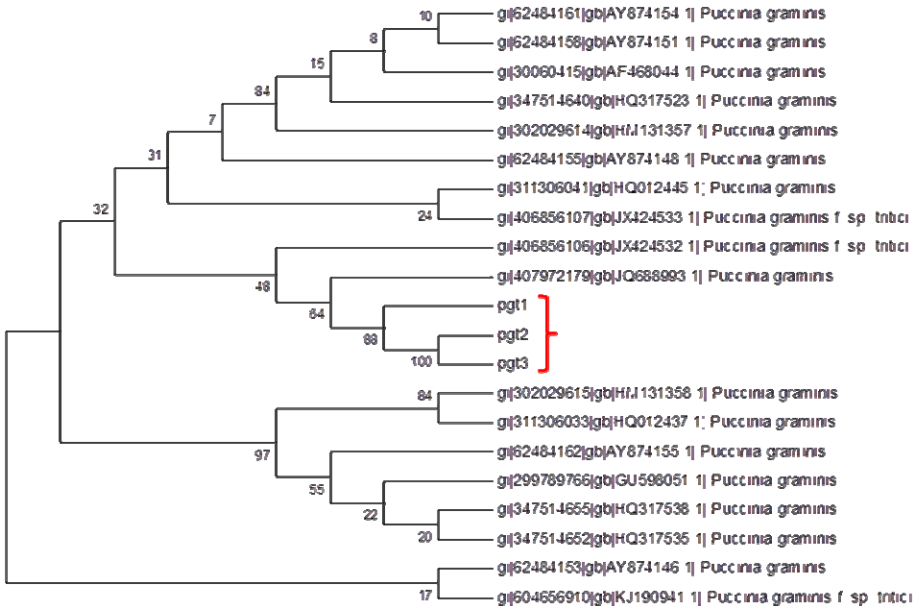

Figure 2 Phylogenetic relationships of a selection of isolates in the *Puccinia graminis* species based on Maximum Parsimony analysis of ITS sequences.

As the three DNA samples were extracted from one *Pgt* isolate, they were grouped together. Samples of Pgt2 and Pgt3 were similar and supported well (bootstrap 100). Interestingly they showed to be slightly different from Pgt1. It suggested that they may be mixed during single postulation process. The phylogenetic analysis showed a monophyletic core group of *P. graminis* divided into two main groups, A and B. Iranian isolate of TTSSK was placed in a clade with *P. graminis* (JQ688993) which was isolated from *Berberis* sp. (Berlin *et al*., 2012) and one *Pgt* isolate from Tajikistan (JX424532). To conclude whether the surface- level winds were involved in spread of *Pgt* from Iran to Tajikistan, more meteorological and weather satellite information is needed. Nagarajan *et al.* (2013) showed that the spread of Ug99 urediniospores from Africa to Lorestan and Hamadan Province, Iran, was not likely to occur due to surface-level winds and something unusual- a tropical cyclone Gonu- happened in 2007. The cyclone crossed the eastern- most tip of Oman and turned to the north-northwest and made landfall on the Makran coast of Iran. They concluded if Gonu Had enabled the spread of Ug99 -TTKSK from Ethiopia to Iran, then why other *Pgt* virulence types from Ethiopia were not recorded in Iran at that time. Therefore the North Arabian Sea tropical cyclones and western distribution were not involved with the appearance of Ug99 in Iran in 2007.

The results showed that most of the isolates which were collected from grass species hosts including *Poa* sp., *Festuca* sp., *Lolium* sp. and *Avena* sp. were grouped together in clade B and the isolates from *Triticum aestivum*, *Secale cereals*, *Agropyron repens* were grouped together in clade A. The Iranian isolate was placed in clade B. The ITS region sequences have been used for detection, identification and phylogenetic analysis of several plant pathogens including rust fungi and our results agree with those of other researchers (i.e Alaei *et al.,* 2009; Zhao *et al.,* 2007). *P. graminis* is a complex species and several methodshave been used to classify the divisions, and *formae speciales*, subspecies and varieties are representative examples of complexity in this species (Berlin *et al.,* 2012).

Abbasi *et al.* (2004) showed that the species, *Pgt,* is highly complex based on ITS sequence and that morphological and genetic characters by themselves are not useful to clarify evolutionary relationship of samples from different parts of the world. Berlin *et al.* (2012) showed the high genetic diversity within each *formae specials* of *P. graminis*. Our results showed that more than one conserved genomic region would be informative to identify phylogenetic relationship of Iranian *Pgt* isolates and samples from different parts of the world. This result is corresponding to other researchers (Abbasi *et al.,* 2004; Berlin *et al.,* 2012).

In conclusion, in this study we determined rDNA ITS sequence information from Iranian isolate of *Pgt* race TTSSK which can be a variant of Ug99 race group and it would be useful to clarify phylogenetic relationship of Iranian isolates and other isolates from different parts of the world. As *Pgt* is highly adapted to long distance migration, this information would be helpful to determine the movement route of Ug99 and show the lineage of Iranian isolates. Of course complementary studies with more isolates and more sequence data from other genomic loci are needed.

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شناسایی مولکولی نژاد TTSSK قارچ *Puccinia graminis* f. sp. *tritici* از ایران

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چکیده: بیماری زنگ ساقه که توسط عامل *Puccinia graminis* f. sp. *tritici* (*Pgt*) ایجاد می‌شود یکی از بیماری‌های مخرب گندم در سراسر دنیا می‌باشد. عامل بیماری می‌تواند ۳۶۵ گونه مختلف از گیاهان علفی و بیش از ۷۰ گونه زرشک را آلوده نماید. استفاده از توالی ناحیه ITS برای بررسی ارتباط فیلوژنی در سطح گونه و زیرگونه مناسب می‌باشد. در این پژوهش از یک جدایه *Pgt* که از ایران جمع‌آوری و به‌عنوان TTSSK نام‌گذاری شده بود، استفاده شد. سه نمونه از جدایه قارچ مورد ارزیابی قرار گرفت. ناحیه ITS نمونه‌ها تکثیر و تعیین توالی شد. درخت توافقی به‌روش ماکزیمم پارسیمونی و با استفاده از نرم‌افزار Mega 6.0 رسم شد. جدایه ایرانی TTSSK در کنار جدایه جدا شده از زرشک و جدایه تاجیکستان قرار گرفت. نتایج نشان داد که بیش از یک ناحیه حفاظت‌شده ژنومی برای شناسایی رابطه فیلوژنتیکی جدایه‌های ایرانی *Pgt* با جدایه‌های سایر مناطق دنیا مورد نیاز می‌باشد. مطالعات تکمیلی به‌کمک جدایه‌های بیشتر و توالی سایر نواحی حفاظت شده ژنوم درحال انجام می‌باشد.

واژگان کلیدی: ITS، ماکزیمم پارسیمونی، رابطه فیلوژنتیکی، زنگ ساقه، گروه نژادی Ug99