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DEVELOPMENT OF NOVEL MARKERS, USING COMPUTATIONALLY EXTRACTED CLASSI TYPE EST-SSRS, IN WHEAT LEAF RUST FUNGUS *Puccinia triticina*

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This study focused on the development of EST-simple sequence repeats markers and the detection of their transferability and their utility for evaluating wheat leaf rust pathogen diversity. A total of 44,407 publicly available EST sequences derived from *Puccinia triticina* were computationally mined. Di-nucleotide repeat density covered the vast majority of assembled ESTs (45%). The tri-repeat motif (TCT) and penta-repeat motif (TTCTT) were the most repeated motif. A set of 103 Class I type sequences containing simple sequence repeats were further analyzed by BLASTX similarity. Nineteen primer pairs flanking regions of EST-SSRs were designed. Of the 19 primer pairs tested, 10 successfully amplified fragments. Their polymorphism was evaluated with 8 *Puccinia triticina* (*Pt*) single-uredinal isolates collected from the different regions of Turkey. These newly developed EST-SSR primer pairs can be implicated as stable markers for pathogen diversity analysis. It was also shown that some leaf rust EST-SSR markers were capable of cross-amplification in *P. graminis* f. sp. *tritici*.

Key words: Puccinia triticina, EST, simple sequence repeats

INTRODUCTION

Cereal rust pathogens have flexible genomes and tend to develop a large number of different virulence phenotypes. Due to this genome behavior, plant rust pathogens have ranked in the first three threatening biotic factors that limit crop growth and yield (HUERTA-ESPINO *et al.*,

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2011). *Puccinia triticina* Eriks, a macrocyclic rust fungus with five spore stages on two different hosts (ANIKSTER *et al.,* 2005), is a serious causative of wheat leaf rust and has a relatively large genome size, determined as 135 Mb (FELLERS *et al.,* 2013). Wheat leaf rust is one of the widespread diseases. Analysis of geographic diversity of *P. triticina* in Europe (KOLMER *et al.,* 2013b), Iran (DADREZAIE *et al.,* 2013), Russia (GULTYAEVA *et al.,* 2012), Turkey (KOLMER *et al.,* 2013a) and China (KOLMER, 2015) showed clear evidences of the spread of this fungus at global level. Leaf rust has, in recent years, become increasingly found in the West Asia region, Central and Eastern Europe (MORGOUNOV *et al.,* 2012). In Turkey, leaf rust often infects wheat-planted in the fall, which is grown in the humid coastal areas, and viable infections can overwinter most years (KOLMER *et al.,* 2013a).

Exact identification of leaf rust pathotypes carries significance for pathogen-specific resistant crop development. Also, race-borne epidemics differs the breeding process due to the dynamic nature of the host-pathogen relationship. This challenge is under the continuous control of rust pathogen surveys (MCINTOSH, 2007). To date, 34 different races belonging to *P. triticina* have been reported (http://www.uniprot.org). Cyclical pathotype movement between alternative host plant and cultivated wheat has been very effective in *Puccinia* diversity. The pathogenicity of urediniospores is more effective than aceiospores due to the host-based fractionation. In addition, identification of pathogen-specific virulence genes is an important step for the development of improving resistant wheat cultivar. Studies performed on diversity of the population of *Puccinia triticina* have proved the transformation capacity of races (KOLMER *et al.,* 2013a,b). GIRAUD *et al.* (2008) emphasized the importance of molecular marker types for the dissection of plant pathogens. AFLP and RAPD markers have been used for diversity studies in international (KOLMER and LIU, 2000) and Canada-originated *P. triticina* populations (KOLMER, 2001). MEBRATE et al. (2006) studied *P. triticina* isolates from Ethiopia and Germany using the AFLP marker system. Simple Sequence Repeats (SSR) markers are useful for tagging the most polymorphic sites of the *Puccinia* genome (DUAN *et al.,* 2003; SZABO and KOLMER, 2007). These markers have been used for the identification of barley leaf rust (KARAOGLU and PARK, 2014) and wheat leaf rust (KOLMER, 2015; TEREFE *et al.,* 2014). Recently, an expressed sequence tag (EST) database has been developed from several lifecycle stages of *P. triticina* (HU *et al.,* 2007; XU *et al.,* 2011). A number of EST-based SSR markers are available for leaf rust (WANG *et al.,* 2010).
The objectives of this study are (1) to determine the abundance and distribution of EST-SSRs in the *P. triticina* genome, (2) to develop and characterize the EST-SSR markers, and (3) to reveal pathogenic variability and genetic relationships within *P. triticina* isolates in Turkey using these markers and their potential to transfer to *Puccinia graminis* f. sp. *tritici*.

MATERIALS AND METHODS

Puccinia triticina isolates

Eight isolates of *P. triticina* were obtained from different farmer fields in the 2013 growing season in Turkey were used (Table 1).

Table 1. Sample genotypes used for diversity analysis

Isolate	Species	Region of collection	Virulence phenotype	Virulence (*Lr* genes)
TUR 17-1	*Puccinia triticina*	Edirne	FHPTQ	2c,3,16,26,3ka,17,30,B,10,14a,18,3bg,14b
TUR 18-1	*Puccinia triticina*	Sakarya	PBFSQ	1,2c,3,17,30,B,10,14a,3bg,14b
TUR 5-2	*Puccinia triticina*	Çanakkale	MBDSS	1,3,17,B,10,14a,3bg,14b,20
TUR 15-2	*Puccinia triticina*	Şanlıurfa	PBFSS	1,2c,3,17,30,B,10,14a,3bg,14b,20
TUR 3-3	*Puccinia triticina*	Diyarbakır	FHPSQ	2c,3,16,26,3ka,17,30,B,10,14a,3bg,14b
TUR 13-3	*Puccinia triticina*	Sakarya	FCPTQ	2c,3,26,3ka,17,30,B,10,14a,18,3bg,14b
TUR 18-2	*Puccinia triticina*	Sakarya	PCFSQ	1,2c,3,26,17,30,B,10,14a,3bg,14b
TUR 11-2	*Puccinia triticina*	Sakarya	FHPTQ	2c, 3,16,26,3ka,17,30,B,10,14a,18,3bg,14b
TUR 24-1	*Puccinia graminis* f. sp. *tritici*	Ankara	TKTTC	

Virulence phenotypes

Single uredinial isolates of *P. triticina* were derived from the leaves following the same procedures as described in KOLMER et al. (2010). The single uredinial isolates were tested for virulence on 20 lines of Thatcher wheat in differential genotypes for single leaf rust resistance genes. The Thatcher lines were divided into five sets containing four differential sets: set 1, Lr1 (isogenic line RL6003), Lr2a (RL6000), Lr2c (RL6047), Lr3a (RL6002); set 2, Lr9 (RL6010), Lr16 (RL6005), Lr24 (RL 6064), Lr26 (RL6078); set 3, Lr3ka (RL6007), Lr11 (RL6053), Lr17a (RL6008), Lr30 (RL6049); set 4, LrB (RL6047), Lr10 (RL6004), Lr14a (RL6013), Lr18 (RL6009); set 5, Lr3bg (RL6042), Lr14b (RL6006), Lr20 (RL 6092), and Lr28 (RL6079). Thatcher was used as a susceptible control. Urediniospores of each isolate were inoculated to 8–9 day-old plants in differential sets. Virulence phenotypes were determined 10–12 days after inoculation for each isolate on each Thatcher differential line using a 0–4 scale (LONG and KOLMER, 1989). According to infection types, (IT) 0– 2 were classified as avirulent and IT 3–4 were classified as virulent. Each isolate was named with a five-letter code based on virulence/avirulence as adapted from LONG and KOLMER (1989).

Single uredinial isolate of *P. graminis* f. sp. *tritici was* derived from infected stems in Ankara using the same procedures as MERT et al. (2012).

EST-SSR mining, characterization and primer design

A total of 44,407 *P. triticina* EST sequences deposited in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) were aligned and assembled by SeqmanPro software (DNA Star; DNASTAR, Madison, WI, USA). For the tracking of perfect and imperfect repeats in assembled ESTs, eTRA1.0 was run with an algorithm reported in Bilgen *et al*. (2004). Contig and singleton sequences were separately processed for repeat motif structures, frequencies of Class I and Class II SSRs. The criteria for minimum length of motifs in SSRs was selected as 18 for di- and tri-repeats, 20 for tetra- and penta-repeats, and 24 for hexa-repeats. Class I type repeat sequences were selected according to the least number of total repeat length 20 bp. In order to develop Class I type EST SSR markers, qualified repeats and their BLASTX scores were compared and 19 Class I type SSRs were used in validation studies. Primer sets were designed on the Batchprimer 3.0 program (YOU *et al.,* 2008) and listed in Table 2.

DNA extraction and EST-SSR genotyping analysis

Genomic DNA was extracted using the OmniPrep kit for Fungus (Geno Tech ®, USA) according to the manufacturer's instructions. DNA concentration was determined using NanoDrop ND-1000 Spectrophotometer (ThermoScientific-®, USA). Polymerase Chain Reaction and amplification were carried out as previously described (KARAOĞLU *et al.,* 2013) with some minor modifications. PCR was carried out in a 25 µl reaction volume containing 1x PCR buffer, 1.5-3 mM $MgCl_2$, 0.2 mM dNTPs, 0.8 µM of each primer, 0.5 U Taq polymerase and 20 ng sample DNA. PCR amplifications were performed in a thermal cycler with an initial denaturation step at 94 °C for 4 minutes, then 30 cycles at 94 °C for 30 s, 50-55°C (depending on primer Tm) for 30 s and 72 °C for 30 s, followed by a 7 min final extension at 72 °C. Exact band sizes were detected with an ABI3730XL genetic analyzer®.

Data analysis

The alleles of each EST-SSR locus were scored based on the absence (0) or presence (1) of alleles. The genetic and virulence similarity was determined using the complement of Dice-coefficient. The virulence and genetic dissimilarity dendrograms were constructed using the unweighted pair group method of arithmetic averages (UPGMA) (SHAN program, NTSYS version 2.1) (ROHLF, 1998). The correlation between virulence and EST-SSR genotypes was investigated by analyzing the similarity matrices from virulence and EST-SSRs using MXCOMP module in NTSYS. Number of alleles, observed heterozygosity (Ho), and expected heterozygosity (He) were calculated with POPGENE version 1.32 (YEH *et al.,* 1999)

Polymorphic information content (PIC) values were calculated for each primer according to the formula: $PIC = 1 - \sum(Pij)^2$, where Pij is the frequency of the i^{th} pattern revealed by the j^{th} primer summed across all patterns revealed by the primers (ANDERSON *et al.,* 1993).

RESULTS AND DISCUSSION

In *P. triticina*-derived ESTs, five different motifs were detected. Di-nucleotide repeats was the most abundant class (45%) followed by tri- to hexa- repeats at very low levels (0.58-0.02%). The average percentages of Class I and Class II type SSRs calculated as 5.7% and 42.6%, respectively. Significant repeat index was almost equal for contig and singleton sequences (Table 2).

A total of 103 Class I type EST-SSRs were computationally extracted from the 44,407 *Puccinia triticina*-derived EST libraries. The number of mined *Puccinia triticina* ESTs was higher than those reported in CHEN *et al.* (2012) and WANG *et al.* (2010). On the other hand, ZHONG et al. (2009) screened three cDNA libraries covering 60,579 sequences and developed 72 SSR markers. Class I type EST-SSRs contained 14 different tri-nucleotide repeats, 11 different di-nucleotide repeats and 10 different tetra-nucleotide repeat motifs. Similarly, the previous studies indicated that di and tri nucleotides are predominantly placed (KARAOGLU *et al.,* 2009), tetra nucleotide repeat bins were found at low frequency rates in genomes (GROVER *et al.,* 2007; WANG *et al.,* 2010).

Nineteen primer pairs were designed to amplify the regions containing EST-SSRs (Table 3). Ten primer pairs gave amplification products. The remaining primers gave less or more extensive or no PCR amplification. As stated by (WANG *et al.,* 2010), the failure in amplifications might be attributed to the presence of introns, insertion or deletion in binding sites of PCR primers.

Table 2. Numerical values of Puccinia triticina EST sequences deposited in NCBI Genebank and percentages of subsequent in silico analysis

Total EST number	44,407
Contig number (assembled EST number)	8346
Singleton number	9566
Contig repeat index	1.468
Class I microsatellites (contig based)	426
Class II microsatellites (contig based)	5210
Singleton repeat index	1.519
Class I microsatellites (singleton based)	594
Class II microsatellites (singleton based)	2415
Di-nucleotid-contig/singleton	5573/2496
Tri-nucleotid- contig/singleton	49/55
Tetra-nucleotide contig/singleton	6/5
Penta-nucleotide contig/singleton	6/2
Hexa-nucleotide-contig/singleton	2/1
Average Class I SSR percentage	5,7%
Average Class II SSR percentage	42,6%
Imperfect repeat percentage	17.6%

Allelic variation of 10 EST-SSRs were estimated in eight *P. triticina* isolates collected from fields in Turkey and five primer pairs were found to be polymorphic, four of them had low PIC values. The most significant PIC score (0.56) was obtained from the TGT repeat that was carried out on the Contig2837 sequence. The allele numbers varied from one to three alleles per locus and the range of allelic size observed was between 122 and 232 bp. There is no direct relationship between the repeats length and polymorphism. Percentage of polymorphic EST-SSRs is 50% and higher than the percentage (10.2%) reported by WANG *et al*. (2010). Chi-squared tests for Hardy-Weinberg equilibrium were conducted for marker loci. None of the loci fitted the Hardy-Weinberg equilibrium. This result was expected because the leaf rust population is asexually reproduced and clonal.

Newly generated EST-SSR markers can provide informative data for a race-specific classification and can serve as exact molecular evidences in the detection of pathogen diversity. In this study, 10 EST-SSR markers gave discriminative results in the isolates collected from Turkey. In other previous research analyzing the population structure of 20 *P. striiformis* isolates, by CHEN *et al*. (2012), 17 of 46 EST-SSRs were found to be useful to assess the amount of polymorphism among the isolates.

Cross-amplification for loci Contig3949, Contig686 and GR489455.1 was found in *P. graminis tritici*, all of them being monomorphic. Previous studies have shown that there are shared sequence similarities among different *Puccinia* species. *P. graminis*-specific primers showed cross-amplification in *P. triticina*, *P. striitiformis,* and coronate isolates (KARAOGLU *et al.* 2013). Similarly, WANG *et al.* (2010), who used 204 EST-SSR primer pairs for the identification of *P. triticina*, *P. coronate,* and *P. graminis,* found eight cross-amplified primer pairs. Likewise, DRACATOS *et al.* (2006) reported 12 EST SSR markers that exhibited cross-amplification products in closely related *Puccinia* species.

Table 3. Ten EST-SSR markers for Puccinia tritici, their primer sequences, repeat motifs, amplification conditions, product size, number of alleles, observed (Ho) and expected (He) heterozygosity, polymorphic information content (PIC), cross-species amplification.

Locus	Primer sequence (5'–3')	Repeat motif	Tm (◦C)	Size range (bp)	Number of alleles	Ho	He	PIC	Cross amplification *P. graminis* f. sp. *tritici*
Contig3949	F:CTCTTTCTCTTCGTCTTCTCC R:CACACAAAATCGCAAAATAA	(TC)12	55	165-166	2	0.125	0.125	0.06	+
Contig3308	F:TATAAGCGACTTTGTGTCGAG R:TTCATGTGGAAACAGTCATAA	(TGT)8	55	199-202-203	3	0.500	0.542	0.03	-
Contig2837	F: AGGCTCTGTGGGTATGGA R: TCAAACTCTGCTGGGCTAC	(TGT)7	56	189-204	2	0.750	0.500	0.56	-
Contig686	F: ACAAGAAAAACAGCCAAGG R: AGCCATCACACCACTATCA	(GAT)8	55	229-232	2	0.125	0.125	0.02	+
GR493131.1	F: CAACAAGATGACAAGAAGCA R: CCATACCGAACGACTCAC	(AAAG)5	54	122-130	2	0.125	0.125	0.02	-
Contig3881	F: GTGGTTGATTGGGTGTGT R: GAGTGTGGGTTGTATTTTTGT	(GA)23	54	180	1				-
Contig5340	F: CCTCTCTTTCTCCTTTATTCG R: TTTGATGACGAAAAGATC	(TA)10	55	147	1				-
GR489069.1	F: CCTCTACTCAACCCTCAACA R: CATTGCTGCTTCTGCTTC	(CAG)9	55	129	1				+
GR489455.1	F: CCAATACTTCACCCCAAC R: CTCTTCCACGCAATCTTC	(GAG)8	53	214	1				-
Contig3304	F: GACTCGCCTTTCACGACT R: TAGGAGGAGCAGCAATAGA	(TCTT)5	56	197	1				-

The isolates TUR 11-2; TUR 18-1; TUR 17-1, TUR 18-1, TUR 15-2, TUR 13-3, TUR 18-2, TUR 11-2; TUR 18-1, TUR 5-2, TUR 15-2, TUR 13-3; TUR 11-2 presented two bands at polymorphic loci contig3949, contig686, contig2837, contig3308, GR493131.1, respectively, indicating heterozgosity of the dikaryotic urediniospores. The isolate TUR 11-2 from Sakarya was discriminated from the other isolates by two loci (contig 3649 and GR493131.1) with unique alleles.

The cluster of virulence showed that 8 *P. triticina* isolates from Turkey were grouped into three (Fig 1). On the basis of 10 EST-SSR loci analyzed, the cluster analysis indicated that *P. triticina* isolates were clustered into four clades (I, II, III, IV) (Fig 2). Seven distinct genotypes were identified. Two isolates from Edirne and Sakarya provinces were found to be exactly the same as each other. According to both dendrograms (Fig. 1-2), there are clear differences among isolates collected from Sakarya.

When the dissimilarity matrices from EST-SSRs and that of virulence were analyzed with MXCOMP module in NTSYS, no correlation was found between virulence phenotypes and EST-SSR genotypes. The isolates TUR 17-1 and TUR 18-2 that belong to the different virulence phenotypes FHPTQ and PCFSQ, respectively, had the highest genetic identity (100%). In contrast, isolates TUR 17-1 and TUR 11-2 that belong to the same virulence phenotype (FHPTQ)

were genetically more distant (71% genetic similarity). This result was not in agreement with the previous study that indicated a strong correlation based on the dissimilarity matrices from EST-SSRs and virulence (WANG *et al.*, 2010). The authors also detected a strong correlation between virulence to some single resistance genes (*Lr2a, 2c, Lr17a*) and EST-SSR genotypes in the population of *P. triticina* in Canada. However, such absence of association between virulence phenotypes and molecular genotypes is not unique to *P. triticina* (ADMASSU *et al.*, 2010; SEMBLAT *et al.*, 2000; VALERIO *et al.*, 2005).

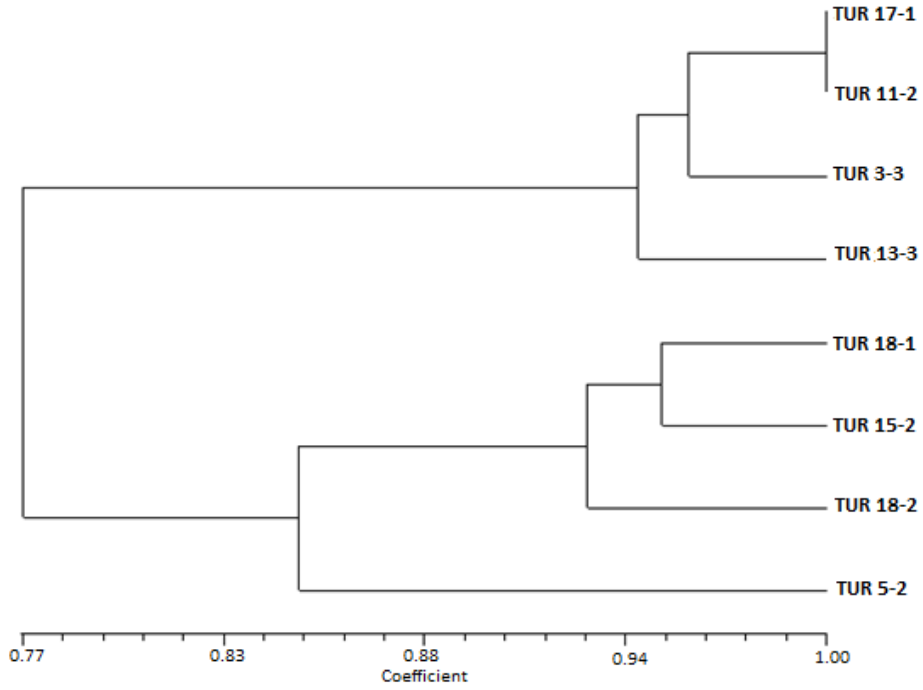

Figure 1. Virulence dissimilarity dendogram of 8 *Puccinia triticina* isolates collected from Turkey

Despite the limited number of isolates used in this study, newly developed EST-SSR markers will be beneficial in revealing the extent of genetic variability within leaf rust populations and tracing global movement of leaf rust pathogens and their evolution. Three loci are useful for other species, *P. graminis* f. sp. *tritici*. There is a need for larger sets of easy-to-use molecular marker tags during genetic structure dissection in fungi-based plant pathogens. In this regard, scanning the pathogen genome using *in silico* methods could provide alternative marker sources for new marker candidates.

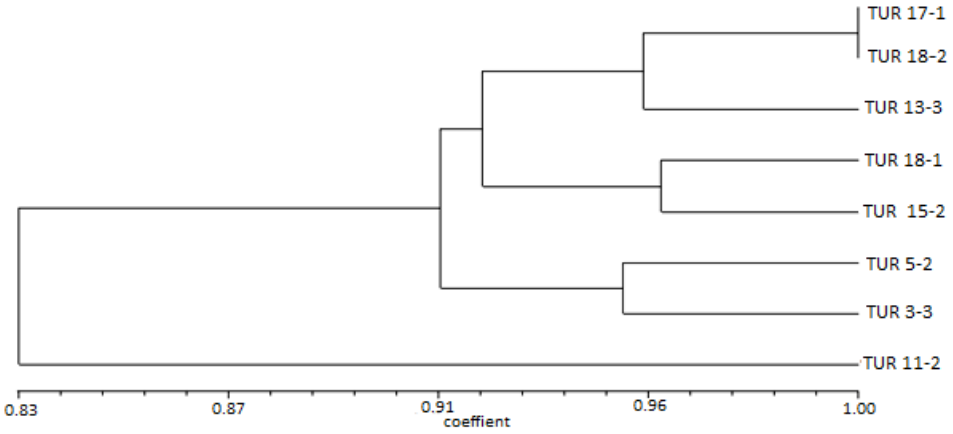

Figure 2. Genetic dissimilarity dendogram of 8 *Puccinia triticina* isolates collected from Turkey

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RAZVOJ NOVIH MARKERA KORIŠĆENJEM KOMPJUTERSKI EKSTRAHOVANIH ClassI tip EST-SSRs, KOD GLJIVE *Puccinia triticina* KOJA IZAZIVA RĐAVOST LISTA PŠENICE

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Izvod

Istraživanja su fokusirana na razvoj EST – jednostavnih ponovljivih sekvenci kao markera, detekciju njihovog transfera u genomu i njihovog korišćenja u ocenjivanju diverzieta patogena koji izazivaju rđavost lista pšenice. Korišćno je ukupno 44,407 javno dostupnih EST sekvenci poreklom iz Di – nukleotidne ponovljive sekvence, koji pokrivaju ogromnu većinu udruženih ESTs (45%). Tri-Trepetitivni motiv (TCT) i penta-repeat motiv (TTCTT) su bili najčešći repetitivni motivi. Set od 103 sekvence klase 1 sekvenci, koje sadrže jednostavne repetitivne sekvence su dalje analizirane korišćenjem BLASTX sličnosti. Devetnaest parova prajmer graničnih (flanking) regiona EST-SSRs su označeni. Od 19 analiziranh parova 10prajmera je uspešno umnožilo fragmente. Njihov polimorfizam je ocenjen korišćenjem 8 pojedinačnih *Puccinia triticina* (*Pt*) uredinal izolata sakupljenih iz različitih regiona Turske. Novi razvijeni EST-SSR parovi prajmer sekvenci mogu da se koriste kao stabilni marker u analizi diverziteta patogenosti. Takođe je pokazano da neki EST – SSR markeri rđavosti lista su bili sposobni za kros – umnožavanje kod *P. graminis* f. sp. *tritici*.

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