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DETECTION OF MOLECULAR POLYMORPHISM OF *PUCCINIA TRITICINA* FROM WHEAT IN ROMANIA

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Abstract

Leaf rust of wheat (Triticum aestivum L.) is caused by Puccinia triticina Ericks and is a common disease of this plant species in northwest, southern, southeast, and eastern Europe. It has been shown that the severity of leaf rust increased in the last years in many regions of Europe and highlighted the need for selection of wheat cultivars with race specific resistance genes. In order to select wheat resistant lines and develop efficient management programs for disease control, the characterization (phenotypic and molecular) of leaf rust races isolated from specific area is an important step of researches. To date, several molecular markers have been used to describe variation in P. triticina in Europe and worldwide.

The aim of this work was to study the molecular diversity of P.triticina collected from Romanian fields (Fundulea, Livada, Pitesti). Single-uredinial isolates were obtained from several populations of P.triticina and used for inoculation of detached wheat leaves cultivar F133. Genomic DNA was isolated from 20 fresh single-uredinial samples (2-10 mg spores) and from eight populations of P. triticina urediniospores (10-20 mg spores) using OmniPrep for Fungus kit. The molecular polymorphism was evaluated through RAPD (with eight decameric primers) and by using 15 SSR primers and two ISSR primers.

Results showed a reduced polymorphism at population level, but not identity, as indicated the RAPD analysis. No more than two alleles were detected in the leaf rust populations with the SSR markers used in experiments according to our separation protocol. Differences in the SSR loci were detected in the single-uredinial isolates. Similar results were obtained with ISSR primers: ISSR 17898A primer allowed the detection of the highest polymorphism among the individual isolates. Evidence for molecular variability between single urediniospores isolates will be correlated with the virulence phenotypic analysis in further studies.

Keywords*: Puccinia triticina, RAPD analysis, SSR markers*
*first and second authors have a similar contribution

INTRODUCTION

Puccinia triticina Eriks is the causal agent of wheat leaf rust, a disease that have a significant economic impact on wheat (*Triticum aestivum* L) production in Romania and worldwide. Intensive studies have been performed in order to evaluate the level of resistance in wheat cultivars and to select the most resistant ones, but the results showed that the resistance is correlated with the virulence phenotypes of the specific (local) fungal population (Kolmer et al., 2001). Nowadays, more than 70 leaf rust resistance genes in wheat have been analysed and their distribution in specific geographic areas is associated with the local *P. triticina* populations (Singh et al., 2013). The isolation and characterization of local fungal populations could offer information to understand the genetic mechanisms underlying variations in *P. triticina* and to development effective strategies to control the disease (Wang et al., 2010). Several methods have been used to identify the molecular markers in order to evaluate the genetic variations in *P. triticina* populations: amplified fragment length polymorphism - AFLP) (Kolmer et al., 2001), random amplified polymorphic DNA - RAPD (Kolmer et al., 1995; Kolmer et al., 2000) and simple sequence repeat SSR markers (Duan et al., 2003; Szabo et al., 2007; Mantovani et al., 2010; Visser et al., 2012). Most of the studies showed the genetic diversity in *P. triticina* populations from central Asia, North America and Europe

(Kolmer et al., 2000; Ordonez et al., 2007; Mantovani et al., 2010; Kolmer et al., 2011; Kolmer et al., 2012).

MATERIALS AND METHODS

1. ***P. triticina*** **isolates**: The eight *P. triticina* populations isolated from common wheat from Romanian fields (Fundulea, Livada, Albota Pitesti), as well as 20 single-uredinial isolates were used in the experiments (*Table 1*).

Table 1. Samples of *P. triticina* used in experiments

Population	*Fungal origin (location/year/source)*	*Single-uredinial isolates*
RB2012	Fundulea/2012/ population from field	
DuRes 14	Fundulea/2012/ population obtained by *in vitro* multiplication (on plantlet)	MP14/1-14/10; MP26
DuRes 17/18/25/26/27	Fundulea/2013 /population from field	MP17/1-17/10
DuRes 19	Fundulea/2012/ population from field	

Single-uredinial isolates were obtained from several populations of *P. triticina* using the susceptible wheat leaves, cultivar F133, according to detached primary leaf technique (Goyeau et al., 2012) with slight modifications (Ittu, 2013, personal communication): approximately 10-12 ml of water agar (10 g. L^{-1}) containing benzimidazole (100 mg.L^{-1}) (*Fluka*), used to retard senescence, were distributed in Petri dishes. Eight or ten of leaf sections (size 3-5 cm) were placed on agar surface and the cut ends were inserted in agar. Strips of agar were overlaid the cut edges of the leaf section, thereby delaying senescence, and to prevent contamination by other microorganisms, too. Each plate was sprayed with urediniospores suspensions in Tween 20 (0.001%) (*Calbiochem*, Germany). Then, inoculated leaves in the Petri-dish were placed into a dew chamber at 80-90% relative humidity and 15°C for 24 hours. The temperature was increased to 25°C for 16 hours photoperiod and 18°C for 8 hours in dark. Evaluation of leaf rust development was carried out based on appearance and the rate of pustule development.

2. **DNA isolation** from *P.triticina* spores was performed using two methods, with the commercial kit *OmniPrep for Fungus* (*Geno Tech*, USA) and with the CTAB method, as described by Wang et al., 2012.

2.1. Genomic DNA was isolated from fresh single-uredinial samples according to Wang et al. (2012) with slight modifications. An amount of 2-10 mg fresh uredospores were crushed in a 1.5 ml microcentrifuge tube with 300 µl of extraction buffer [100 mM Tris–HCl, pH 9.0; 20 mM EDTA, pH 8.0; 1.4 mM NaCl; 2% cetyltrimethylammonium bromide (CTAB)]. The protocol was improved by the addition of 0.1 g sterile glass bead (diameter 0.45-0.50 µm) in lysis buffer, and ground by vigorous stirring spores (2 times for 1 minute) using Minibead beater equipment (*Biospec Products*, UK). Then, 300 µl of extraction buffer was added to each tube and the mixture was supplemented with 60 µl SDS (20% sodyumdeodecylsulphate) and incubated for 2 hours at 65°C. The tubes were mixed gently every 20 minutes. Proteins were denature and removed by repeated extractions with 660µl Tris saturated phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v). Phases were separated by centrifugation, the aqueous phase removed and DNA precipitated with 700 µl of isopropanol. After 1 hour at -20°C, the phases were separated by centrifugation at 14000 rpm for 7 minutes. DNA was resuspended in 100 µl of TE buffer (10 mM TrisHCl, pH 7.6, 1 mM EDTA) and RNase 10 mg /ml (*Fermentas*, France) was added, then the mixture was incubated for 30 minutes at 37°C. DNA was pelleted by alcohol precipitation, dried and resuspended in 25 µl of Tris–EDTA buffer (10 mM Tris–HCl, pH 7.6; 1 mM EDTA).

2.2. Genomic DNA from eight populations of *P. triticina* urediniospores (10-25 mg spores) was isolated using *OmniPrep for Fungus* kit (*Geno Tech*, USA) according to the manufacturer's instructions, with some modifications: the spores were incubated with 2 µl lyticase (*Sigma*) solution (200 U final concentration), at 37^0C, for 45 minutes and mechanical disintegrated using Minibead beater equipment (*Biospec Products*, UK) for 90 seconds.

DNA concentration was determined using using NanoDrop ND-1000 Spectrophotometer (*ThermoScientific*, USA).

3. **Molecular analysis with RAPD**. RAPD-polymerase chain reaction conditions, running and scoring of gels were previously described, as well as the 10-base primers UBC 402, 450,

489, 517, 519, 521, 538, 556 (Kolmer et al., 1995). Briefly, 10 – 100 ng/µl DNA was amplified in 25 µl reaction volume using AmpliTaq mixture (*Applied Biosystems*, USA) and 2.5 µl primer (10 µM). The thermocycler was programmed at 94^0C for 3 min; followed by 40 cycles of 1 min at 94^0C, 2 min at 36^0C and 2 min at 72^0C; then 10 min at 72^0C.

4. **Molecular analysis with SSR markers**. *P. triticina* isolates were profiled with 15 SSR primer pairs (*Table 2*). The PCR reactions were performed in a volume of 50 µl, contained 1 µl ADN (10 – 100 ng µl), 2.5µl of each primer (10 µM), 4 µl dNTP Mix (200 µM dATP, dCTP, dGTP, dTTP), 4 µl of $MgCl_2$ (25mM), 1 µl of 360 GC Enhancer, AmpliTaq360 DNA Polymerase (1.25U/reaction) and tampon AmpliTaq (10X) (*Applied Biosystems*, USA). Amplification conditions were similar, the differences being the annealing temperatures (59^0C, 57^0C, and 52^0C).

Table 2. The primer sequences and characteristics of 15 *Puccinia triticina* SSR primers used in this study (Duan et al., 2003; Szabo et al., 2007; Visser et al., 2012)

Locus	*Primer sequence (5' – 3')*	*Product (pb)*	*Allele*
PtSSR 3	F: TTCAATTTGCCCCTTGACTC R: AGGTAGCATTGCCAGTGGCA	271-301	4
PtSSR 68	F: GACTCAGCCCACTGCTAA R: GATGGCGACGTATTTGGTCT	305–327/337, 347,356, 360,362	9/5
PtSSR 76	F: GGCGTCGTATTTCTCGTAGC R: TTCGGACTACTGGGTAAGCA	393402/404, 409	3/2
PtSSR92	F: CCAAGGAACAGTCCACCAAG R: GAGTCGGGTAAGCCATCTGA	242-252/234, 237,246, 249	4/4
PtSSR 151A	F: TCATCGCACTCCACTCAGAC R: ATGCTGCCCAACCTGCTC	456 –476/495	4/1
PtSSR 152	F: CTCCGTTCCTCTTTCTGTCG R: CCATCGCAACCAACAAACA	384-388/411, 416	2/2
PtSSR 154	F: ACGGTCAACAGCCAACTACC R: CCTCGTCATCCTGGTTGAGT	242,272/260, 276,279, 281,287	7/5
PtSSR 158	F: GACGACTTCGTCACTGCTGA R: GAGGAGAAGCCGTTCTGTTG	227-232/236, 241	3/2
PtSSR 164	F: GTGGAAGTGAGCGGAAGAAG R: GGAGATGGGCAGATGAGGTA	214-222/210, 218,220	3/3
PtSSR 173	F: CTCAGCGACCTCAAAGAACC R: GAGACGACGGATGTCAACAA	211-219/210, 121,217, 220	4/4
PtSSR 184	F: GGTCTGGCGAATCTTTCCTT R:CATTTTTAGTTGTGAGCCCTTG	373-569/331, 338,480, 491	5/4
PtSSR 186	F: GCCACGAGAAATACATAGAA ATAAAA R: GGTTGTTGATGGGCTTGAGT	335-347/367, 372, 376, 381	6/4
RB4	F: CAGTATTGTGGTGGTTGGATG R: ACTCAAGAATAATGGGGAACA	244, 230	2
RB10	F: TAAGATTGGTGGTATGTGGTG GA R: TTGTCTTTCATCTCATCCAGCC	218	1
RB29	F: CTCACCAAACATCAAGCACC R: GAGCCTAGCATCAGCATC	118-180	9

5**. Molecular analysis with ISSR markers**: Two ISSR primers were used in the experiments: primer 17898A that amplified the repetitive sequence $(CA)_6$ AC, and primer HB15 specific for the repetive sequence $(GTG)_3$ GC. PCR was performed into 0,2 ml tubes containing 25 µl final volume of a reaction mixture consisting of 1X buffer, 0,2mM dNTPs, 1µl of 10µM primer, 1.3U Taq (AmpliTaq 360), 2mM $MgCl_2$, 2 µl DNA and 0.5 µl enhancer solution. The thermocycler was programmed 2 min at 94°C, followed of 35 cycles of: 30 sec at 94°C, 45 sec at 44°C, 1.30 min at 72º C and 10 min at 72°C.

The PCR products have been visualized by gel electrophoresis in agarose 0.8-2%. Sizes were estimated by comparison with DNA size markers, 100 bp DNA Ladder (*Promega*, USA).

RESULTS AND DISCUSSIONS

Detached primary leaf technique has been used to obtain single-uredinial isolates of *P. triticina*. Artificial laboratory conditions (temperature, humidity) allowed germination of urediniospores from the populations and at the end of experiments 21 single-uredinial isolates were retained from *P. triticina* populations DuRes 14 and DuRes 17 (*Figure 1*).

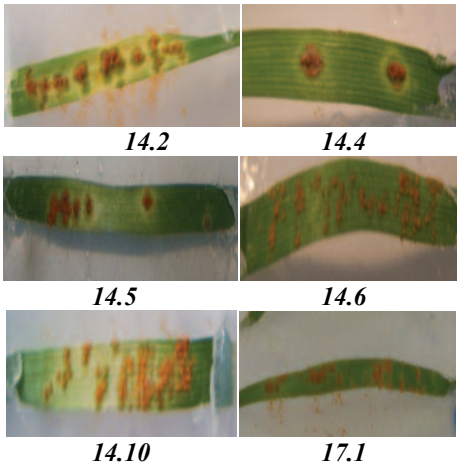

Figure 1. Morphological aspect of single-uredinial isolates of *Puccinia triticina* on F133 wheat cultivar

The urediniospores multiplied *in vitro* by detached primary leaf technique were used for DNA isolation with CTAB method, while the DNA from fungus populations was isolated with a commercial kit. The CTAB method was efficient in DNA isolation of samples with small amounts of spores (under 10 mg), while the *OmniPrep for Fungus* kit was used for samples with 10 - 20 mg of urediniospores. For the later, the extraction protocol was optimize using both mechanical and chemical treatments for spore lyses and a supplementary DNA

cleaning step (*Table 3*). The concentration and purity of DNA obtained with the commercial kit was higher than the quality of DNA obtained by CTAB method. For both methods, the grinding of the spores improved the amount of nucleic acid isolated.

Table 3. Results obtained using *OmniPrep for Fungus* kit for DNA isolation of *Puccinia triticina*

Sample	*Spores (mg)*	*DNA concentration (ng/ul)*	*DNA purity*	
			(A_{260}/ A_{280})	*(A_{260}/ A_{230})*
RB2012	18.4	238.6	2.1	2.3
DuRes14	18.4	503.4	1.73	1.88
DuRes17	16.8	50.0	1.73	0.77
DuRes 18	18.9	507.0	1.84	1.75
DuRes19	17.6	221.7	1.73	0.95
DuRes25	17.1	61.0	1.53	1.52
DuRes 26	16.7	121.5	1.86	2.08
DuRes 27	17.7	74.5	1.58	1.57

Molecular markers have been proved to be valuable tools in the evaluation of genetic diversity within and between species and populations. It has been showed that different markers might reveal different variations, their distribution throughout the genome and the extent of the DNA target in plant or fungal populations and species. The optimization of the polymerase chain reaction (PCR) favoured the development of different molecular techniques such as RAPD, AFLP, simple sequence repeats (SSR or microsatellite), sequence-tagged sites (STS), or intersimple sequence repeat polymorphic DNA (ISSR) (Le Maitre et al., 2013). The molecular analysis using ISSR markers was poorly documented for *P. triticina*, although these molecular markers have been extensively used for other *Puccinia* species (Spakman et al., 2010).

In the first step of the study we compared the RAPD profile of three populations of *P. triticina* RB2012, DuRes 14 and DuRes 18 and of the single-uredinial isolate MP 26, using 8 UBC primers. The molecular phenotypes distinguished by each primer generally varied by one to three bands. However, the RAPD profiles were identical for *P. triticina* populations DuRes 14 and DuRes 18 for the primer UBC 521. The initial results showed a reduced polymorphism at the population level. Moreover, the molecular analysis of population DuRes 14 and the single-uredinial isolate MP 26 (developed from population DuRes 14) with primers UBC 519 and UBC 538 showed a clear intrapopulation molecular differentiation (*Figure 2*). Further comparative examination of the RAPD profiles of the higher number of populations and isolates will provide information necessary for a reliable genetic analysis of the *P. triticina* isolated from Romanian fields.

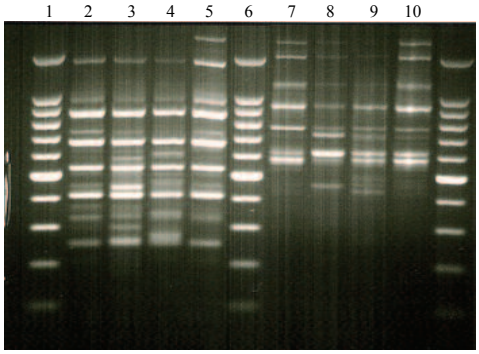

Figure 2. The RAPD analysis with UBC 519 (left) and UBC 538 (right) primers for *Puccinia triticina* DuRes 14 (lane 2, 7), DuRes 18 (lane 3, 8), RB2012 (lanes 4,9) and MP26 (lanes 5,10). Lane 1,6 - Benchop 100bp DNA Ladder (Promega)

Two types of primers were used to analyse the molecular polymorphism of *P. triticina* populations and isolates: primers for SSR and primers for ISSR markers. The selected 15 SSR markers were tested on three fungal populations (DuRes 14, DuRes 18 and RB2012) and on single-uredinial isolate MP26 (*Table* 4*)*. The results obtained with the primer PtSSR 184 showed the genetic differentiation among the populations and isolates. Thus, for this microsatellite locus the SSR profile of the populations DuRes 14 and DuRes 18 showed only one allele (~590 bp), while in the population RB 2012 were detected two alleles (~590bp an 430bp). Moreover, in single-uredinial isolate MP 26, obtained from population DuRes 14, there are two alleles, showing a clear evidence of the intrapopulation molecular polymorphism. Similar results were obtained during genetic analysis of isolates from DuRes 14 and DuRes 17 populations with ISSR primers, ISSR 17898A primer allowed

the detection of the highest polymorphism among the individual isolates.

Table 4. The results obtained with 15 SSR primers selected for genetic analysis of *Puccinia triticina*

Primer	T_a (^0C)	*Amplicon lenght on agarose gel (bp)*			
		DuRes 14	*DuRes 18*	*RB2012*	*MP 26*
PtSSR 3	57/52	-	-	-	-
PtSSR 68	57	-	-	350	350
PtSSR 76	57	400	400	390	390
PtSSR 92	57	240	240	250	240
PtSSR 151A	52	510	510	490	490
PtSSR 152	57	400	400	400	400
PtSSR 154	59	270	270	290	290
PtSSR 158	59	220	220	240	240
PtSSR 164	59	210	220	210	210
PtSSR 173	59/57	210	210	220	220
PtSSR 184	**59**	**590**	**590**	**430, 590**	**430, 590**
PtSSR 186	59/57	380	380	380	380
RB4	57/52	240	240	240	240
RB10	52	-	-	-	-
RB29	52	-	180	180	180

Additional research on the virulence and molecular polymorphism will be needed in order to better characterize the *P. triticina* populations and isolates from Romanian fields.

CONCLUSIONS

1.The detached primary leaf technique was optimized and improved to obtain single-uredinial isolates of *P. triticina*.
2.The CTAB method was efficient in DNA isolation of *P. triticina* samples with small amounts of spores, while *OmniPrep for Fungus* kit was used to obtain better quality DNA.
3.The molecular polymorphism of the *P. triticina* isolated from Romanian fields was detected using RAPD, SSR and ISSR markers and clear evidenced among the single-uredinial isolates. Results showed a reduced polymorphism at the population level, but not identity, as indicated the RAPD analysis. No more than two alleles were detected in the leaf rust populations with the SSR markers used in experiments according to our separation protocol.

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