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The variability of a *Pyrenophora tritici-repentis* population as revealed by inter-retrotransposon amplified polymorphism with regard to the *Ptr ToxA* gene

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Pyrenophora tritici-repentis (PTR) is one of the pathogens causing leaf spots in wheat. It occurs everywhere wheat is grown and forms populations with a high genetic variability. The aim of this study was to find a key to explain the relation between PTR population variability and its race spectrum. We studied the variability of a *P. tritici-repentis* population by analysing the retroelements Pyggy, TfoI, and MITE using two approaches: SSAP and IRAP. By analysing all 122 *Pyrenophora* spp. with four SSAP primer combinations and two IRAP markers, 186 polymorphic bands were detected. Cluster analysis based on molecular data showed that the variability of *P. tritici-repentis* isolates established by retrotransposon analysis cannot be explained by the race spectrum, except for race 4, or by geographic origin. A significant correlation was found, with two SSAP, three TfoI, and two MITE markers, between the presence or absence of the marker and the presence or absence of the *Ptr ToxA* gene, which is considered to be the main pathogenicity factor of this fungus. We found retrotransposons a powerful tool for the study of fungal population-genetic variability.

Key words: *Pyrenophora tritici-repentis*, *Ptr ToxA* gene, retrotransposons.

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Pyrenophora tritici-repentis je jedním z původců listových skvrnitostí pšenice. Vyskytuje se všude tam, kde se pěstuje pšenice a tvoří značně variabilní populaci. Hlavním cílem práce bylo vysvětlit vztah mezi variabilitou populace *P. tritici-repentis* a jejím rasovým spektrem. Variabilitu populace *P. tritici-repentis* jsme studovali pomocí dvou přístupů analýzy retrotransposonů Pyggy, TfoI a MITE: SSAP a IRAP. Analýzou 122 izolátů *Pyrenophora* spp. pomocí čtyř SSAP primerových kombinací a dvou IRAP markerů jsme detekovali 186 polymorfních signálů. Klastrová analýza získaných dat ukázala, že variabilitu izolátů *P. tritici-repentis* není možné vysvětlit rasovým spektrem izolátů s výjimkou rasy 4 ani místem sběru izolátů. Statisticky významná korelace byla nalezena u dvou SSAP, tří TfoI a dvou MITE markerů mezi přítomností či nepřítomností markeru a přítomností či nepřítomností genu pro Toxin A, který je považován za hlavní faktor patogenity u tohoto druhu houby. Retrotransposony se ukázaly být účinným nástrojem ke studiu genetické variability populací hub.

INTRODUCTION

Tan spot of wheat (*Triticum aestivum*) has been identified in most wheat growing areas throughout the world and causes significant economic losses (Ciuffetti and Tuori 1999).

The fungus *Pyrenophora tritici-repentis* (Died.) Drechsler, anamorph *Drechslera tritici-repentis* (Died.) Shoemaker, is a homothallic ascomycete responsible for a foliar disease – tan spot of wheat (*Triticum* spp.). Its race structure is derived from the fact that *P. tritici-repentis* isolates produce multiple host-selective toxins causing different symptoms on host plant leaves (Strelkov and Lamari 2003). Currently, at least eight races of *P. tritici-repentis* have been characterised. Each race has its typical virulence pattern in three effective wheat-differential cultivars (Strelkov and Lamari 2003). A compatible interaction between the race and its corresponding susceptible host-differential cultivars was found to be mediated by a host-specific toxin. Ptr ToxA is one of three toxins characterized to date and found in races 1 and 2 of the fungus. It is a protein and the direct product of a single gene (Ciuffetti et al. 1997). The transformation of the *ToxA* gene into a non-toxin producing isolate resulted in both toxin production and pathogenicity, therefore it is considered to be a main pathogenicity factor in *Ptr ToxA*$^{+}$ isolates (Ciuffetti et al. 1997).

The variability of the *P. tritici-repentis* population studied by using molecular markers showed no correlation between variability and race structure (Friesen et al. 2005, Singh and Hughes 2006, Leisova et al. 2008). From a morphological point of view, the *P. tritici-repentis* population seemed to be uniform. The only difference between the races was the presence of toxin genes, their expression, and their participation in pathogenesis. However, recently a new possibility to study *P. tritici-repentis* population variability arose when several families of retrotransposons were found in the proximity of the *Ptr ToxA* gene (Lichter et al. 2002).

Retrotransposons represent a molecular marker system that has been used to study diversity within closely related species (Kemken and Kück 1998). They comprise a ubiquitous class of repetitive elements in all eukaryotic genomes. Unlike transposons, retrotransposons do not excise, but are transcribed and by reverse transcription inserted into DNA as copies of the mother element. The developed retrotransposon analysis methods are based on the joint between retrotransposon and genomic DNA that is formed during the integration process. These joints can be detected with PCR amplification, between a primer corresponding to the retrotransposon and a primer matching a nearby motif, in the genome (Schulman et al. 2004). In Inter-Retrotransposon Amplified Polymorphism (IRAP), segments between two nearby elements are amplified and length polymorphism of the PCR product is detected (Kalendar et al. 1999). The

Sequence-Specific Amplified Polymorphism (SSAP) method is derived from Amplified Fragment Length Polymorphism (AFLP) (Waugh et al. 1997). This method was used to study the diversity within *Pyrenophora teres* and *P. graminea* populations (Taylor et al. 2004).

The aim of this study was to determine if any differences could be identified between different races of *P. tritici-repentis*. For this purpose, the analysis of retrotransposons was used and the correlation between retrotransposon markers and the presence of the *Ptr ToxA* gene in *P. tritici-repentis* genome was tested.

MATERIAL AND METHODS

Fungal isolates and race classification. One hundred and twenty isolates of *P. tritici-repentis* (PTR) were evaluated (Tab. 1). Two *Pyrenophora teres* isolates (PTT – *P. teres* f. sp. *teres*, PTM – *P. teres* f. sp. *maculata*) were added into the analyses as outliers (Tab. 1). Most PTR isolates were collected from wheat from different regions of the Czech Republic. Five isolates of PTR originated from the Slovak Republic, six from Argentina, two from the USA, two from Canada and three from Russia.

Infected leaves were incubated in wet chambers at 18 °C under UV light for two days to induce conidiophore production, followed by one day in the dark to induce conidia production. Single spore isolates were cultivated on potato-carrot agar (PCA).

To determine PTR races, four different wheat cultivars (Glenlea, 6B662, 6B365 and Salamouni) (Lamari et al. 1998) were used. Conidial suspensions (3×10^3 spores/ml) of the isolates were sprayed on seedlings in the two-leaf stage. Three pots with approximately 10 plants per wheat cultivar were used for each fungal isolate. The reaction of the cultivars was measured between 7 and 10 days after inoculation, using a 1 to 5 rating scale (Lamari et al. 1989).

DNA extraction. DNA was extracted from mycelia according to the optimised protocol by Leisova et al. (2005) using an extraction buffer (0.35 M sorbitol, 0.1 M Tris, 5 mM ethylenediaminetetraacetic acid (EDTA), pH 7.5), lysis buffer (2 M NaCl, 0.2 M Tris, 50 mM EDTA, 2% cetyltetramethyammonium bromide (CTAB), pH 7.5) and a 5% solution of CTAB in the medium of a high concentration of NaCl (minimum 0.5 M). DNA was precipitated by one volume of absolute ethanol and diluted in an appropriate volume of TE buffer. DNA was run in a 0.8% agarose gel to verify the quality and the concentration. λ HindIII (Fermentas, Vilnius, Lithuania) was used to determine the size and concentration of DNA.

Tab. 1. Fungal isolates used in the study. CZE: Czech Republic.

DNA	Isolate	Geographic origin	Host, cultivar	Year of collection	Race	Cluster	Ptr ToxA +
PTR-001	*P. tritici-repentis*	CZE, Přerov	*Triticum aestivum*		1	I	1
PTR-003	*P. tritici-repentis*	CZE, Prostějov	*Triticum aestivum*, Alka	2000	1	IV	1
PTR-004	*P. tritici-repentis*	CZE, Znojmo	*Triticum aestivum*, Corsaire	2003	2	IV	1
PTR-005	*P. tritici-repentis*	CZE, Přerov	*Triticum aestivum*, Hana	2000	1	I	1
PTR-006	*P. tritici-repentis*	CZE, Ústí nad Orlicí	*Triticum aestivum*, Zuzana	2003	1	I	1
PTR-007	*P. tritici-repentis*	CZE, Chrudim	*Triticum aestivum*	2001	1	I	1
PTR-008	*P. tritici-repentis*	CZE, Uherské Hradiště	*Triticum aestivum*	2003	1	I	1
PTR-009	*P. tritici-repentis*	CZE, Přerov	*Triticum aestivum*		1	I	1
PTR-011	*P. tritici-repentis*	CZE, Praha-Ruzyně	*Triticum aestivum*	2002	1	I	1
PTR-012	*P. tritici-repentis*	CZE, Svitavy	*Triticum aestivum*, Samanta	2003	1	I	1
PTR-013	*P. tritici-repentis*	CZE, Praha-Stupice	*Triticum aestivum*, Zuzana	2003	1	I	1
PTR-014	*P. tritici-repentis*	CZE, Praha-Stupice	*Triticum aestivum*, Petrus	2003	1	I	1
PTR-015	*P. tritici-repentis*	CZE, Plzeň-Lužany	*Triticum aestivum*, Complet	2002	1	I	1
PTR-016	*P. tritici-repentis*	CZE, Pelhřimov	*Triticum aestivum*, Ritmo	2000	1	I	1
PTR-017	*P. tritici-repentis*	CZE, Chrudim	*Triticum aestivum*, Alana	2001	1	I	1
PTR-018	*P. tritici-repentis*	CZE, Praha-Ruzyně	*Triticum aestivum*	2002	1	I	1
PTR-020	*P. tritici-repentis*	CZE, Opava	*Triticum aestivum*, Versailles	2000	1	IV	1
PTR-021	*P. tritici-repentis*	CZE, Praha-Ruzyně	*Triticum aestivum,* Clever	2002	1	IV	1
PTR-022	*P. tritici-repentis*	CZE, Praha-Ruzyně	*Triticum aestivum*, Banquet	2002	1	I	1
PTR-023	*P. tritici-repentis*	CZE, Brno	*Triticum aestivum*, Ebi	2002	1	I	1
PTR-024	*P. tritici-repentis*	CZE, Nymburk	*Triticum aestivum*, Elpa	2002	1	I	1
PTR-025	*P. tritici-repentis*	CZE, Kladno	*Triticum aestivum*	2000	1	I	1
PTR-026	*P. tritici-repentis*	CZE, Plzeň-Lužany	*Triticum aestivum*, Rialto	2002	1	I	1
PTR-027	*P. tritici-repentis*	CZE, Kroměříž	*Triticum aestivum*, Versailles	1998	1	IV	1
PTR-028	*P. tritici-repentis*	CZE, Plzeň-Lužany	*Triticum aestivum*, Samanta	2002	1	IV	1
PTR-029	*P. tritici-repentis*	CZE, Třebíč	*Triticum aestivum*, Banquet	2002	1	IV	1
PTR-030	*P. tritici-repentis*	CZE, Prostějov	*Triticum aestivum*	2002	1	I	1
PTR-031	*P. tritici-repentis*	Russia	*Triticum aestivum*		1	IV	1
PTR-032	*P. tritici-repentis*	CZE, Nymburk	*Triticum aestivum*, Versailles	2002	1	I	1
PTR-033	*P. tritici-repentis*	CZE, Náchod	*Triticum aestivum*	2003	1	I	1
PTR-034	*P. tritici-repentis*	CZE, Ústí nad Orlicí	*Triticum aestivum,* Record	2002	1	I	1
PTR-035	*P. tritici-repentis*	CZE, Beroun	*Triticum aestivum*	2000	1	IV	1
PTR-036	*P. tritici-repentis*	CZE, Přerov	*Triticum aestivum*, Bruta	2000	1	IV	1
PTR-037	*P. tritici-repentis*	CZE, Kroměříž	*Triticum aestivum*	1998	1	I	1
PTR-039	*P. tritici-repentis*	CZE, Chrudim	*Triticum aestivum*, Šárka	2001	1	I	1
PTR-041	*P. tritici-repentis*	CZE, Chrudim	*Triticum aestivum*, Šárka	2001	4	III	0
PTR-042	*P. tritici-repentis*	CZE, Benešov	*Triticum aestivum*	1998	1	IV	1
PTR-043	*P. tritici-repentis*	CZE, Praha-Stupice	*Triticum aestivum*	2001	1	IV	1

DNA	Isolate	Geographic origin	Host, cultivar	Year of collection	Race	Cluster	Ptr ToxA +
PTR-044	*P. tritici-repentis*	CZE, Kladno	*Triticum aestivum*	2000	1	IV	1
PTR-045	*P. tritici-repentis*	CZE, Přerov	*Triticum aestivum*, Sulamit	2000	1	I	1
PTR-046	*P. tritici-repentis*	CZE, Nymburk	*Triticum aestivum*	2001	1	IV	1
PTR-047	*P. tritici-repentis*	Russia	*Triticum aestivum*		1	I	1
PTR-048	*P. tritici-repentis*	CZE, Chrlice	*Triticum aestivum*, Corso	2003	1	I	1
PTR-049	*P. tritici-repentis*	CZE, Litoměřice	*Triticum aestivum*	2003	1	I	1
PTR-050	*P. tritici-repentis*	CZE, Plzeň-Lužany	*Triticum aestivum*, Rialto	2003	1	I	0
PTR-051	*P. tritici-repentis*	CZE, Ústí nad Orlicí	*Triticum aestivum*, Corso	2003	1	III	1
PTR-052	*P. tritici-repentis*	CZE, Plzeň-Lužany	*Triticum aestivum*, Samanta	2003	1	IV	1
PTR-053	*P. tritici-repentis*	CZE, Pelhřimov	*Triticale*	2003	1	I	1
PTR-054	*P. tritici-repentis*	CZE, Klatovy	*Triticum aestivum*, Sulamit	2003	1	IV	1
PTR-055	*P. tritici-repentis*	CZE, Kroměříž	*Triticum aestivum*, Versailles	1998	1	I	1
PTR-056	*P. tritici-repentis*	CZE, Kutná Hora	Triticum aestivum	2000	1	I	1
PTR-058	*P. tritici-repentis*	CZE, Praha-Ruzyně	*Triticum aestivum*	2000	1	IV	0
PTR-060	*P. tritici-repentis*	CZE, Benešov	*Triticum aestivum*	1998	1	I	1
PTR-061	*P. tritici-repentis*	CZE, Chrudim	*Triticum aestivum*, Versailles	2001	1	IV	1
PTR-062	*P. tritici-repentis*	CZE, Kroměříž	*Triticum aestivum*, Charger	1998	1	IV	1
PTR-063	*P. tritici-repentis*	CZE, Litoměřice	*Triticum aestivum*	2003	1	I	1
PTR-064	*P. tritici-repentis*	CZE, Kladno	*Triticum aestivum*	2000	1	I	1
PTR-065	*P. tritici-repentis*	CZE, Chrudim	*Triticum aestivum*, Versailles	2001	1	I	0
PTR-066	*P. tritici-repentis*	Slovak Republic	*Triticum aestivum*	2000	1	I	1
PTR-067	*P. tritici-repentis*	CZE, Chrudim	*Triticum aestivum*	2001	1	I	1
PTR-068	*P. tritici-repentis*	CZE, Opava	*Triticum aestivum*, Samanta	2001	1	IV	1
PTR-069	*P. tritici-repentis*	Russia	*Triticum aestivum*		1	IV	1
PTR-070	*P. tritici-repentis*	CZE, Pelhřimov	*Triticum aestivum*, Versailles	2003	1	I	1
PTR-071	*P. tritici-repentis*	CZE, Pelhřimov	*Triticum aestivum,* Grandis	2004	1	I	1
PTR-072	*P. tritici-repentis*	CZE, Svitavy	*Triticum aestivum*	2005	1	I	1
PTR-074	*P. tritici-repentis*	Argentina	*Triticum aestivum*	2005	1	IV	1
PTR-075	*P. tritici-repentis*	Argentina	*Triticum aestivum*	2005	1	IV	1
PTR-076	*P. tritici-repentis*	USA (DW2)			5	IV	0
PTR-077	*P. tritici-repentis*	Argentina	*Triticum aestivum*	2005	3	IV	0
PTR-078	*P. tritici-repentis*	Slovak Republic	*Triticum aestivum*	2004	3	IV	0
PTR-079	*P. tritici-repentis*	CZE, Plzeň-Lužany	*Triticum aestivum,* Saxana	2003	1	I	1
PTR-080	*P. tritici-repentis*	Slovak Republic	*Triticum aestivum*, Velta	2004	1	I	1
PTR-081	*P. tritici-repentis*	CZE, Plzeň-Lužany	*Triticum aestivum*	2002	1	IV	1
PTR-082	*P. tritici-repentis*	Slovak Republic	*Triticum aestivum*	2004	1	IV	1
PTR-083	*P. tritici-repentis*	CZE, Plzeň-Lužany	*Triticum aestivum*	2002	1	IV	1
PTR-084	*P. tritici-repentis*	CZE, Pelhřimov	*Triticum aestivum*, Floret	2005	1	IV	1
PTR-085	*P. tritici-repentis*	CZE, Litoměřice	*Triticum aestivum,* Ebi	2004	1	IV	1
PTR-086	*P. tritici-repentis*	Argentina	*Triticum aestivum*	2005	3	IV	0

DNA	Isolate	Geographic origin	Host, cultivar	Year of collection	Race	Cluster	Ptr ToxA +
PTR-087	*P. tritici-repentis*	CZE, Benešov	*Triticum aestivum,* Akteur	2004	8	IV	1
PTR-088	*P. tritici-repentis*	CZE, Svitavy	*Triticum aestivum,* Sulamit	2003	8	I	1
PTR-089	*P. tritici-repentis*	CZE, Třebíč	*Triticum aestivum*, Sandra	2005	1	IV	1
PTR-090	*P. tritici-repentis*	CZE, Plzeň-Lužany	*Triticum aestivum,* Leguán	2003	1	IV	1
PTR-091	*P. tritici-repentis*	CZE, Znojmo	*Triticum aestivum*	2005	1	IV	1
PTR-092	*P. tritici-repentis*	Argentina	*Triticum aestivum*	2005	1	I	1
PTR-093	*P. tritici-repentis*	Argentina	*Triticum aestivum*	2005	1	IV	1
PTR-094	*P. tritici-repentis*	CZE, Benešov	*Triticum aestivum,* Samanta	2004	1	IV	1
PTR-095	*P. tritici-repentis*	Slovak Republic	*Triticum aestivum*	2004	3	I	0
PTR-096	*P. tritici-repentis*	CZE, Jičín	*Triticum aestivum*, Rheia	2005	3	I	0
PTR-097	*P. tritici-repentis*	CZE, Benešov	*Triticum aestivum,* Samanta	2004	1	I	1
PTR-098	*P. tritici-repentis*	CZE, Pelhřimov	*Triticum aestivum,* Caphorn	2004	1	I	1
PTR-099	*P. tritici-repentis*	Canada (ASC-1)			1	I	1
PTR-100	*P. tritici-repentis*	Canada (86-124)			2	IV	1
PTR-101	*P. tritici-repentis*	USA (DW16)			5	IV	0
PTR-102	*P. tritici-repentis*	CZE, Kladno			1	V	1
PTR-103	*P. tritici-repentis*	CZE, Rakovník	*Hordeum vulgare*		1	IV	1
PTR-104	*P. tritici-repentis*	CZE, Příbram	*Poaceae* spp.		4	III	0
PTR-105	*P. tritici-repentis*	CZE, Příbram	*Poaceae* spp.		4	III	0
PTR-106	*P. tritici-repentis*	CZE, Příbram	*Poaceae* spp.		4	III	0
PTR-107	*P. tritici-repentis*	CZE, Příbram	*Poaceae* spp.		4	III	0
PTR-108	*P. tritici-repentis*	CZE, Příbram	*Poaceae* spp.		4	III	0
PTR-109	*P. tritici-repentis*	CZE, Příbram	*Poaceae* spp.		4	III	0
PTR-110	*P. tritici-repentis*	CZE, Příbram	*Poaceae* spp.		4	III	0
PTR-111	*P. tritici-repentis*	CZE, Příbram	*Poaceae* spp.		4	III	0
PTR-112	*P. tritici-repentis*	CZE, Příbram	*Poaceae* spp.		4	III	0
PTR-113	*P. tritici-repentis*	CZE, Příbram	*Poaceae* spp.		4	III	0
PTR-114	*P. tritici-repentis*	CZE, Příbram	*Poaceae* spp.		4	III	0
PTR-116	*P. tritici-repentis*	CZE, Praha-Ruzyně	*Triticum aestivum*		6	V	0
PTR-117	*P. tritici-repentis*	CZE, Praha-Ruzyně	*Triticum aestivum*		4	V	0
PTR-118	*P. tritici-repentis*	CZE, Praha-Ruzyně	*Triticum aestivum*		4	III	0
PTR-119	*P. tritici-repentis*	CZE, Prostějov	*Triticum aestivum,* Rekord		3	V	0
PTR-120	*P. tritici-repentis*	CZE, Prostějov	*Triticum aestivum,* Semper		1	I	1
PTR-121	*P. tritici-repentis*	CZE, Praha-Kněževes	*Triticum aestivum,* Sulamit		3	V	0
PTR-122	*P. tritici-repentis*	CZE, Praha-Stupice	*Triticum aestivum*		1	V	1
PTR-123	*P. tritici-repentis*	CZE, Praha-Stupice	*Triticum aestivum*		3	V	0
PTR-124	*P. tritici-repentis*	CZE, Praha-Stupice	*Triticum aestivum*		1	V	1
PTR-125	*P. tritici-repentis*	CZE, Praha-Stupice	*Triticum aestivum*		1	V	1
PTR-126	*P. tritici-repentis*	CZE, Praha-Stupice	*Triticum aestivum*		1	V	1
PTR-127	*P. tritici-repentis*	CZE, Praha-Stupice	*Triticum aestivum*		3	V	0

DNA	Isolate	Geographic origin	Host, cultivar	Year of collection	Race	Cluster	Ptr ToxA +
PTR-128	*P. tritici-repentis*	CZE, Praha-Ruzyně	*Triticum aestivum*		3	V	0
PTR-129	*P. tritici-repentis*	CZE, Praha-Ruzyně	*Triticum aestivum*		3	V	0
H603	*P. teres* f. sp. *teres*	CZE, Kroměříž	*Hordeum vulgare*, Monaco	2000		II	1
H614	*P. teres* f . sp. *maculata*	CZE, Lužany	*Hordeum vulgare*	2005		II	1

Ptr ToxA screening. As is a typical initial step for *Ptr ToxA* screening and sequencing analysis, the sequence of the *Ptr ToxA* locus in EMBL database no. AF004369 was used (Ciuffetti et al. 1997). Two pairs of primers (PTR-ToxA1124f TTCTGTACGCGCAATTCCG; PTR-ToxA1124R TCCTCCTTCTCGATCCGACTC and PTR-ToxA1011F TGGGCATCATTGCATGGAC; PTR-ToxA1011R GTGCTTGCTCGCTAATTTTCT) were designed using the Primer Express software –1.5 (Applied Biosystems, Foster City, CA, USA) so that the PCR amplicon covers the entire *Tox A* gene, including a part of the promoter region.

Amplifications were performed in a total volume of 15 µl. Reaction mixtures consisted of 0.33 µM of each primer, 0.25 mM dNTP, 1x PCR reaction buffer with 1.5 mM $MgCl_2$ (Qiagen, Germany), 0.8 mM $MgCl_2$, 1 U Taq polymerase (Qiagen, Germany) and 100 ng DNA template. PCR was performed in a UNO II cycler (Biometra, Germany) under the following conditions: initial denaturing step of 94 °C for 5 min., followed by 35 cycles of 30 s at 94 °C, 30 s at 60 °C, 1 min at 72 °C and 72 °C for 10 min. Products (10 µl of PCR reaction) were analysed using 1.6% agarose gel electrophoresis.

Analysis of retrotransposons. Two methods of studying retrotransposons in PTR were used: SSAP and IRAP. They both detect the variability of DNA amplicons between the LTR or IR parts of transposable elements. The SSAP method is derived from AFLP (Vos et al. 1995). AFLP markers were generated using the Applied Biosystems kit for small genomes (Applied Biosystems, Foster City, CA, USA). DNA digestion was carried out using the restriction enzymes *Eco*RI and *Mse*I. In selective amplification the Pyggy LTR-derived SSAP selective primer was used from the work by Taylor et al. (2004). Selective amplification, using *Mse*I fluorescently marked primers, a selective base, and a Pyggy LTR-derived SSAP primer, was performed as multiplex PCR in a reaction mixture of 10 µl [0.2 mM dNTP, 1 µM *Mse*I primer, 3 × 0.5 µM *Eco*RI primers, 1 U Taq polymerase (Qiagen GmbH, Germany), 1x buffer with 10 mM $MgCl_2$, and 1 µl diluted (1:20) preselective amplification reaction] in the ABI PRISM 7700 cycler (Applied Biosystems, Foster City, CA, USA). Amplification products were separated by capillary electrophoresis in the ABI PRISM 310 sequencer (Applied Biosystems, Foster City, CA, USA) and analysed using GeneScan and Genotyper software (Applied Biosystems, Foster City, CA, USA).

The IRAP segments, between two nearby elements, were amplified using TfoI and Stow-away-MITE primers (Okuda et al. 1998, Smýkal et al. 2006). Amplifications were performed in a total volume of 15 µl. Reaction mixtures consisted of 0.33 µM of each primer, 0.25 mM dNTP, 1 x PCR reaction buffer, 3.3 mM $MgCl_2$, 1 U Tth polymerase (Biotools, Madrid, Spain), and a 100 ng DNA template. PCR was performed in a UNO II cycler (Biometra, Germany) under the following conditions: initial denaturing step of 94 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 50 °C, 1 min at 72 °C, and 72 °C for 10 min. Amplification products were separated electrophoretically in 3% agarose gel and visualised using ethidium bromide in UV light. As the size standard, 100 bp DNA Ladder Plus (Ferments, Vilnius, Lithuania) was used.

Statistical analysis. Cluster analysis was performed to study the relationships between isolates. On the basis of the presence or absence of amplification products, binary data matrices were built. A dissimilarity matrix was computed in the DARwin software using the Jaccard coefficient (Perrier et al. 2003, Perrier and Jacquemoud 2003). A dendrogram was constructed using an unweighted neighbour joining method (NJ). Bootstrap analysis with 1000 replicates was performed to estimate the robustness of the evolutionary tree. Population structure was also studied using STRUCTURE version 2.2 software to support DARwin data. The STRUCTURE program implements the Bayesian clustering method that allows direct estimates of F_{ST} from dominant markers and incorporates uncertainty about the magnitude of within-population inbreeding (Pritchard et al. 2000, Falush et al. 2003).

An exact binomial test for goodness-of-fit was performed to determine whether the presence of the retrotransposon marker correlated with the presence of the *Ptr ToxA* gene (a null hypothesis). Statistical significances were tested by the exact binomial test of goodness-of-fit using an EXCEL spreadsheet calculator (http://udel.edu/~mcdonald/statexactbin.html).

RESULTS

Race assessment

Race assessment of 64 PTR isolates was borrowed from Leisova et al. (2008). From 56 PTR isolates tested within this work, 27 were evaluated de novo and the analysis was repeated with 29 PTR isolates. Race assessment was confirmed by 21 PTR isolates and it was changed by eight isolates. In total, out of 120 PTR isolates tested (Tab. 1), 88 belonged to race 1 (73.3 %). Other races had smaller representations: two isolates were determined to be race 2 (1.7 %), eleven isolates classified as race 3 (9.2 %), 14 isolates as race 4 (11.7 %), two isolates as race 5 (1.7 %), one

Tab. 2. Significant deviation from expected frequency of retrotransposon markers by *Pyrenophora tritici-repentis* isolates containing (*ToxA*+) and not containing (*ToxA*−) the gene for Ptr Toxin A.

	SSAP_C-459		Total	P-value (two-tailed)*
	1	0		
ToxA+	90	1	91	0.000253
ToxA–	16	13	29	8.12E–6
Total	106	14	120	

	SSAP_C-495		Total	P-value (two-tailed)*
	1	0		
ToxA+	31	60	91	0.202
ToxA–	18	11	29	0.023
Total	49	71	120	

	TfoI_2000		Total	P-value (two-tailed)*
	1	0		
ToxA+	70	21	91	0.006432
ToxA–	6	23	29	5.16E–6
Total	76	44	120	

	TfoI_2500		Total	P-value (two-tailed)*
	1	0		
ToxA+	24	67	91	0.116
ToxA–	0	29	29	0.002136
Total	24	96	120	

	TfoI_3000		Total	P-value (two-tailed)*
	1	0		
ToxA+	45	46	91	0.023
ToxA–	1	28	29	0.000024
Total	46	74	120	

	MITE_600		Total	P-value (two-tailed)*
	1	0		
ToxA+	51	40	91	0.026
ToxA–	2	27	29	0.000016
Total	53	67	120	

	MITE_800		Total	P-value (two-tailed)*
	1	0		
ToxA+	89	2	91	0.001918
ToxA–	18	11	29	0.000129
Total	107	13	120	

* – Exact binomial test of goodness-of-fit

isolate as race 6 (0.8 %), and two isolates as race 8 (1.7 %). Race 1 clearly predominates in the Czech Republic. *P. tritici-repentis* isolates belonging to race 4 were collected mostly from wild grasses. One isolate (PTR-103) determined to be race 1 originated from barley.

***Ptr Tox A* screening in the *P. tritici-repentis* population**

The sizes of the amplification products with PTR ToxA1124 and PTR ToxA1011 primer pairs were 1024 and 1011 bp, respectively. PCR product, specific to the *Ptr ToxA* gene, was found in 91 (75.8%) of all studied *P. tritici-repentis* isolates (Tab. 1). Unlike isolates of races 2 and 8, which all demonstrated to be *Ptr ToxA*$^{+}$, three isolates of race 1 were found to be *Ptr ToxA*$^{-}$. Isolates of races 3, 4, 5, and 6 lacked the *Ptr ToxA* gene.

Analysis of retrotransposons

In the SSAP analysis, 171 polymorphic bands were detected using four primer combinations. In the IRAP analysis, two primers were applied (TfoI and Stowaway-MITE), based on the sequence of their terminal repetition region. In total, fifteen polymorphic bands were detected. Neighbour-joining analysis grouped all isolates studied into five clusters (Fig. 1). Cluster II consisted of only two *P. teres* isolates studied, cluster III had fourteen, mainly *P. tritici-repentis*, non-pathogenic, race 4 isolates. Most pathogenic *P. tritici-repentis* isolates (races 1, 2, 3, 5, 6, 7, and 8) were grouped into clusters I and IV (52 and 41 isolates, respectively). Cluster V consisted of thirteen isolates belonging mainly to race 3, but not exclusively. From the *Ptr ToxA* point of view, *Ptr ToxA*$^{+}$ isolates grouped particularly into clusters I and IV, whereas *Ptr ToxA*$^{-}$ isolates grouped into clusters III and V. The Bayesian approach, using the STRUCTURE software, supported the grouping of the studied isolates into five clusters. The analysis of only two isolates (PTR-051 and PTR-120) showed weak evidence of belonging to clusters III and V, respectively, and they should be rather included in cluster I (Fig. 1, Tab. 1).

The average distances (expected heterozygosity) between individuals in clusters were: 0.10 for cluster I, 0.40 for cluster II, 0.18 for cluster III, 0.11 for cluster IV, and 0.13 for cluster V. The mean values of F_{ST} for clusters were: 0.64 for cluster I, 0.19 for cluster II, 0.51 for cluster III, 0.62 for cluster IV, and 0.62 for cluster V. These data indicate that there are highly significant differences between the inferred clusters; all F_{ST} values are significantly different from zero.

An exact binomial test, to measure goodness-of-fit, was performed to determine whether the presence of the retrotransposon marker correlated with the presence of the *Ptr ToxA* gene. Only seven markers (two SSAP markers; three TfoI markers, and two MITE markers) were found to correlate with the presence or absence of the *Ptr ToxA* gene in the *P. tritici-repentis* isolate's genome. Statistical significances tested with the exact binomial test of goodness-of-fit are presented in Tab. 2.

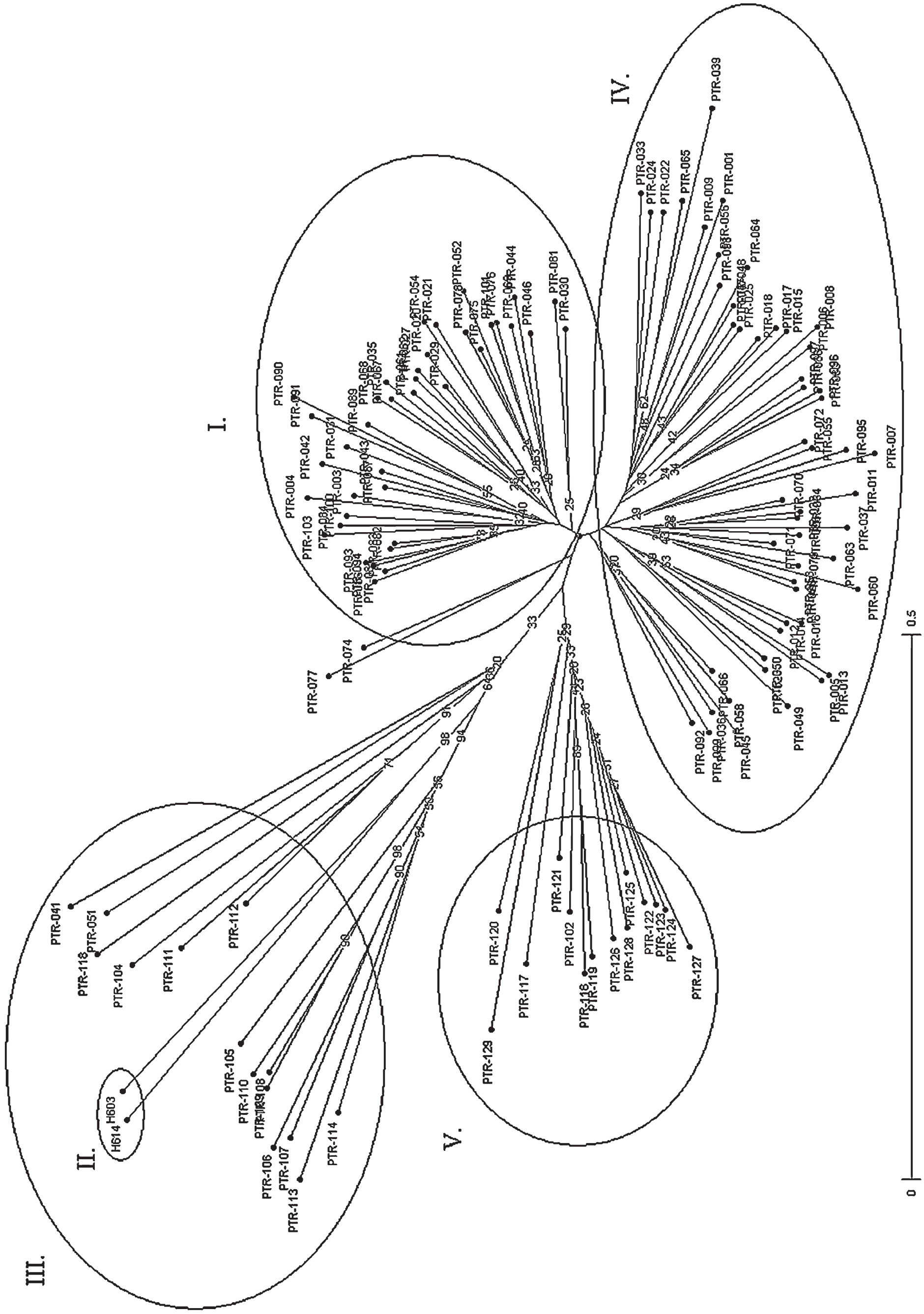

Fig. 1. Phylogenetic tree formed by the unweighed neighbour-joining method with Jaccard dissimilarity coefficients showing the relationship between *Pyrenophora tritici-repentis* and *Pyrenophora teres* isolates based on SSAP and IRAP analyses.

DISCUSSION

Five clusters were distinguished using the NJ method based on retrotransposon analysis (Fig. 1). F_{ST} values indicated that the rate of variability was greater between clusters than within them. With the exception of cluster II, representing *P. teres* isolates, only cluster III covered *P. tritici-repentis* isolates belonging mainly to race 4. The remaining races were included in three other clusters (I, IV, and V). In correspondence with the previous results the genetic similarity among the studied isolates of PTR was independent of race classification, with the exception of race 4 (Friesen et al. 2005, Singh and Hughes 2006, Leisova et al. 2008). Nearly all isolates classified as race 4 were collected from grass – non-wheat – hosts considered to be non-pathogenic on wheat.

The fact that clusters were supported by low values of bootstrap analysis indicates that each cluster represents a reproductively non-isolated population of *P. tritici-repentis*. *P. tritici-repentis* is a homothallic fungus with both types of reproduction. The sexual stage of *P. tritici-repentis* occurs on wheat stubble between crops, whereas the asexual stage occurs during the crop growth. The occurrence of sexual recombination in nature is probably the reason for the high level of genetic variability in isolates of *P. tritici-repentis*. Under favourable conditions conidiospores can travel 10–200 km (De Wolf et al. 1998). PTR is also seed borne (Schilder and Bergstrom 1992), and therefore a fungal inoculum can travel long distances. The occurrence of sexual reproduction and long-distance dispersal of inocula could contribute to the occurrence of genetic variability, independent of race structure or geographic origin.

Although the non-pathogenic isolates of *P. tritici-repentis* are morphologically indistinguishable from the pathogenic isolates, they differ on molecular level (Lichter et al. 2002, Cao et al. 2009). Lichter et al. (2002) found that a part of the chromosome carrying the gene for ToxA, a peptide synthetase, and some repetitive sequence homologues to fungal transposase elements, are missing in the non-pathogenic isolates. The addition of a single gene, encoding the proteinaceous toxin Ptr ToxA, is sufficient for the transformation of a non-pathogenic isolate to a pathogen. Therefore, it was of interest to determine if any differences with regard to this gene could be identified among the various races of *P. tritici-repentis*. The *Ptr ToxA* gene was found in all *P. tritici-repentis* isolates belonging to the races 1, 2, and 8, except for three isolates of race 1 (PTR-050, PTR-058 and PTR-065) that appeared to be *Ptr ToxA*$^-$. This discrepancy may be explained most likely by mutation in primer sites, because only weak signals were detected after amplification. Isolates of races 3, 4, 5, and 6 lacked the *Ptr ToxA* gene.

A significant correlation was found by two SSAP, three TfoI, and two MITE markers between the presence or absence of the marker and the presence or absence of the *Ptr ToxA* gene. The TfoI-transposable element was detected by

Lichter et al. (2002) in the 3.0 Mb-chromosome where the *Ptr ToxA* gene was localised. It is interesting that non-pathogenic isolates contain substantial portions of this chromosome in the size of 2.75 Mb. This fact was supported by Aboukhaddour et al. (2008), who found the *ToxA*-carrying chromosome to be homologous with a related chromosome in the non-ToxA-producing isolates. The repetitive markers homologous to four fungal transposable elements (MAGGY, Grasshopper, TfoI and Fot1/Pot2) revealed major genomic differences between the pathogenic and non-pathogenic strains (Lichter et al. 2002). TfoI and MITE markers with a significant correlation to the *Ptr ToxA* gene could be linked to the *ToxA* gene but this hypothesis needs to be proven by further sequencing analyses.

The analysis of retrotransposons showed to be a useful tool to study the variability of a *P. tritici-repentis* population. It corroborated a noticeable clustering that did not correlate with race assessment. Seven new markers with good correlation with the presence or absence of *Ptr ToxA* gene were found. The genetic background of this relationship is still unknown and it intimates the existence of other differences between pathogenic and non-pathogenic isolates.

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