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IDENTIFICATION OF *Tilletia* SPECIES USING REP-PCR FINGERPRINTING TECHNIQUE

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Analyzing 167 non-processed seed samples of wheat, it was found that 145 samples (86.8 %) were contaminated with *Tilletia* species, while 22 (13.2 %) samples were not contaminated. By using rep-PCR fingerprinting technique, it was found that DNA isolates of *T. tritici* originated from Serbian wheat samples had 80 % similarity with positive control for *T. tritici*. One isolate shared similarity of 60% with *T. tritici, T. controversa* and *T. laevis.* It was supposed that this isolate belongs to *T. bromi*. Isolate of *T. laevis* shared a similarity of 70 % with isolates of *T.*

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tritici and *T. controversa,* while *T. walkeri* was more than 10 % similar with *T. tritici, T. controversa* and *T. laevis.* Although *T. controversa* and *T. tritici* had high percent of genetic similarity, they were clustered separately. Our results suggest that rep-PCR fingerprinting could be a useful tool for monitoring presence of morphologically similar *Tilletia* species in wheat production areas.

Key words: identification, rep-PCR fingerprinting, *Tilletia tritici, Tilletia controversa, Tilletia laevis, Tilletia bromi, Tilletia walkeri*, wheat

INTRODUCTION

Bunt of wheat is a worldwide distributed disease caused by *Tilletia* species. Except wheat, hosts of *Tilletia* species are rye, barley, triticale and many other species of family *Poaceae*. *Tilletia tritici* (Bjerk.) G. Wint. and *T. laevis* J.G. Kühn, which incite common bunt, are the most reported *Tilleta* species in Serbia (JEVTIĆ *et al.*, 1997). KOSTIĆ and SMILJKOVIĆ (1968) reported *T. triticoides* Savalescu and *T. intermedia* Grassner in northeastern part of the country. Both *T. controversa* J.G. Kühn which incites dwarf bunt and *T. indica* Mitra which causes Karnal bunt of wheat are quarantine species for Serbia. *T. indica* is also listed as an A1 quarantine pest for the EPPO region (European and Mediterranean Plant Protection Organization).

No researches on diversity and distribution of *Tilletia* species in Serbia has been undertaken for the last 10 years. Bearing in mind that characteristics of pathogens can be changed, as well as their virulence, it is important to monitor the presence of *Tilletia* species, varieties and races, in wheat production areas (JEVTIĆ, 1998).

Identification of *Tilletia* species on the basis of teliospores morphological characteristics is very unreliable. Teliospores isolated from individual sort, different races, as well as from geographical area can vary in the depth of reticulation. According to GOATES *et al.* (1996), there is an overlap of about 10% in the morphology of *Tilletia* species with reticulate episporium. This makes identification of some individual teliospores of quarantine *T. controversa* and non-quarantine *T. tritici,* difficult or impossible. There is also overlap in teliospore morphology between *T. controversa* and certain members of *T. fusca* Ellis & Everh complex which causes bunt of grass (MATHRE, 1996). Members of *T. fusca*. complex are *T. fusca* var. *bromi-tectorum*, *T. fusca* var. *guyotiana* and *T. fusca* var. *fusca*. The hosts of *T. fusca* var. *bromi-tectorum* and *T. fusca* var. *guyotiana* are *Bromu*s sp, so some authors consider these two varieties as *T. bromi* (Brockm.) Brockm. (BOYD and CARRIS, 1997) The hosts of *T. fusca* var. *fusca* are *Vulpia* sp. Given that *Bromus* and *Vulpia* sp. are found in and around wheat fields, the harvested grain can be contaminated with teliospores of *T. fusca*, so it could make quarantine decisions difficult (MATHRE, 1996).

Biochemical techniques played a significant role in identification of different plant pathogens, thus improving morphological identification (LEVIĆ and PENČIĆ, 1993; IVANOVIĆ *et al.,* 1995; STOJKOV *et al.,* 1996; STOJKOV and

IGNJATOVIC, 1998; MATHRE, 1996). However, the most accurate results can be achieved by the use of molecular techniques. These techniques are unavoidable in identification of different plant (IGNJATOVIĆ-MICIĆ *et al*., 2007, 2009; PEJIĆ *et al*., 1998; VERMA *et al*., 1999; MIAN *et al*., 2002) and pathogen (MCDONALD *et al*., 2000; FERNANDEZ – ORTUNO *et al*., 2011; LADHALAKSHMI *et al.,* 2009) genotypes.

Initially, PCR (*polymerase chain reaction*) based genomic fingerprinting techniques with markers such are RAPD (*random amplified polymorphic DNA*), AFLP (*amplified fragment length polymorphism*) and PCR-RFLP (*restriction fragment length polymorphism*) of ITS rDNA region were applied, in order to detect polymorphism between *T. controversa*, *T. tritici* and grass bunt fungi (BOYD *et al.,* 1998, PIMENTEL *et al*., 1998; SHI *et al*., 1996). In general, these methods have not been precise in distinguishing *T. controversa* from *T. trtici*, but they could make clear difference between wheat and grass bunt fungi. Although these techniques did not make clear difference between *T. controversa* and *T. tritici*, significant divisions between members of *T. fusca* comlex, based upon host specificity, were obtained (MCDONALD *et al*., 2000). One such division was obtained in *T. fusca* var. *fusca,* where RAPD profiles of isolates from *Vulpia octoflora* (Walt.) Rydb. shared only 20 to 35% similarity with isolates from *V. microstachys* (Nutt.) Benth. (BOYD *et al*., 1998, BOYD and CARRIS, 1998). Using RAPD markers it was also obtained that *T. fusca* var. *bromi-tectorum* and *T. fusca* var. *guyotiana* are similar of about 55% (BOYD and CARRIS, 1998).

Repetitive-sequence-based polymerase chain reaction (rep-PCR*)* fingerprinting technique was shown to be a molecular technique which is successful in distinguishing *T. controversa* from *T. tritici* (MCDONALD *et al*., 2000). This technique is based on PCR-mediated amplification of DNA sequences located between repeated elements which are termed BOX, REP (repetitive extragenic palindromic), and ERIC (enterobacterial repetitive intergenic consensus) elements. These repeated elements were first detected in prokaryotic genome, but they were also found very useful for genome variability characterization of several fungal genera, such are *Verticillium* (ARORA *et al*., 1996), *Fusarium* (EDEL *et al*., 1995), *Stagonospora* (CZEMBOR *et al*., 1999), *Septoria* (CZEMBOR *et al*., 1999), and *Leptosphaeria* (JEDRYCZKA, *et al*., 1999). Rep-PCR products separated on agarose gels yield DNA fingerprints that can be either visually compared or subjected to computer assisted pattern analysis (RADEMAKER *et al*., 1997). The main advantages of rep-PCR fingerprinting over RAPD technique are simplicity, tolerance to wider range of DNA concentrations and generation of reproducible results (MCDONALD *et al*., 2000).

QING *et al.* (2009) and LIU *et al.* (2009) found highly selective primers for distinguishing *T. controversa* from *T. tritici*, using a random amplified polymorphic DNA (RAPD) primer-mediated asymmetric-PCR (RM-PCR) technique and AFLP techniques, respectively. Even though highly selective primers for economically important *Tilletia* species were found, their use is not suitable for distinguishing varieties within *Tilletia* species. Therefore, at present rep-PCR fingerprinting technique is valuable not only for studying taxonomic diversity of pathogens, but

also for monitoring the presence of *Tilletia* species and their varieties in wheat production areas.

The aim of this study was to identify *Tilletia* species found in seed samples collected in Serbia using rep-PCR fingerprinting techniques. Considering the fact that the resistance breakdown is the constant threat, precise identification of *Tilletia* species would be of great importance, in order to make plant protection more efficient and reliable. MCDONALD *et al.* (2000) found this technique very useful for distinguishing *T. indica* from *T. walkeri* Castlebury & Carris, as well as *T. controversa* from *T. tritici*, and they suggested that rep-PCR fingerprinting techniques could be used for diagnostic purposes.

MATERIALS AND METHODS

Collection of wheat seed samples

One hundred and sixty seven non-processed seed samples of wheat used in this study were collected in cooperation with regional phytosanitary laboratory Agroinstitut-Sombor during the 2007-2008 harvest season. Wheat grain was collected from 39 municipalities in 7 districts of Vojvodina and 6 municipalities in 5 districts of Central Serbia. For teliospore extraction, 50 g of subsamples were used (OEPP/ EPPO, 2007)

Teliospore extraction

Teliospores were extracted from 50 g subsamples of each grain sample using the size-selective sieving wash method (PETERSON *et al.*, 2000) and OEPP/EPPO diagnostic protocol for *T. indica* (2007). Except that a 10-µm mesh nylon sieve was used instead of 20-µm nylon sieve. Extracted teliospores were mounted in 15% glycerol and examined by light microscopy at 250x magnification.

Method for isolation and germination of teliospores

The protocol for isolation of teliospores from both the microscope slide and the cover slip was a modification of that described in OEPP/EPPO diagnostic protocol for *T. indica* (2007). Except that a 10 µm mesh nylon sieve was used instead of 20 µm mesh nylon sieve and teliospores were surface-sterilized with 5% commercial bleach solution (approximately 0.2% active NaOCl) instead of 10% commercial bleach solution (approximately 0.4% active NaOCl). One part of teliospore suspension was plated on 2% water agar with antibiotics and incubated at 16°C from 10 to 15 days, whereas the other part of suspension, which was also plated on 2% antibiotic water agar, was incubated at 5°C in the presence of light from 3 to 6 weeks. These are environmental conditions required for teliospore germination and production of primary sporidia and small colonies with secondary sporidia of *T. tritici*, *T. laevis* and *T. controversa* (GOATES, 1996) Mycelial mat for DNA isolation was produced according the OEPP/EPPO diagnostic protocol for *T. indica* (2007), but the cuts of small blocks of agar bearing germinated teliospores or colonies were incubated at 16-20°C in the dark instead of at 21°C in the light.

Isolation of DNA and rep-PCR amplification

DNA was isolated from mycelial mats using the procedure of MÖLLER *et al.* (1992). Rep-PCR reactions were performed using 19 DNA isolates of *Tilletia* species. Fifteen DNA isolates were obtained from nine samples of wheat seed, while four DNA isolates were positive controls for *T. tritici, T. controversa, T. laevis* and *T. walkeri* (Table 1). The collection of *T. tritici,* used as positive control, was obtained from the Institute for Field and Vegetable Crops, Novi Sad DNA isolates used as positive controls for *T. controversa, T. laevis* and *T. walkeri* were obtained from strains that originate from the CBS collection (Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands).

The rep-PCR protocol was carried out using a combination of two procedures. One was described by MCDONALD *et al.* (2000) and the other was OEPP/EPPO diagnostic protocol for *T. indica* (2007). Rep-PCR fingerprints were obtained with the use of the following primers: BOX 1A1R (5'-CTACGGCAA GGC GACGCTGACG 3'); ERIC 1R (5'ATGTAAGCTCCTGGGGATTCAC3') and 2I (5'-AAGTAAGTGACTGGGGTGAGCG-3'); and REP 1R (5'-IIIICGICGICATCI GGC-3') and 2I (5'-ICGICTTATCIGGCCTAC-3'). Briefly, 100ng of purified DNA was used as template in 50 µl reaction mixture containing 0.5 µM of each primer, 0.2 mM of each deoxynucleotide triphosphates, 6.25 units of *Taq* DNA polymerase, 1x PCR buffer and 1.5 mM $MgCl_2$. PCR cycling parameters were: an initial denaturation at 95°C for 7 min; 35 cycles consisting of 94°C for 3 s, 92°C for 30 s, then either 40°C (REP primers) or 50°C (ERIC/BOX primers) for 1 min; extension at 65°C for 8 min; and a single final extension at 65°C for 8 min, followed by cooling at 4°C. Amplified PCR products (9 µl per sample) were separated on 1.5% agarose gels in 1x TAE (0.04 M Tris-acetate and 1 mM EDTA, pH 8.0) buffer for 18 to 19 h at 2 V/cm, at 4°C. DNA bands were stained with ethidium bromide, visualized under UV transilluminator and photographed with digital camera. Gels were normalized using 1-kb DNA ladder (Fermentas) loaded on both sides and in the center of 20-lane gels. Positions of DNA fragments on agarose gel were scored visually. Using a band-based method, a matrix of binary variables (band present - 1 and band absent - 0) was generated for each genotype. Genetic similarities were estimated using the similarity coefficient defined by JACCARD (1908). Cluster analysis was carried out on the matrix of genetic similarities using the unweighted pair group method with arithmetic averages (UPGMA). The computing of binary data including coefficients of similarities and UPGMA clustering were performed using NTSYS-pc software (ROHLF, 2000).

Table 1 DNA isolates designated by isolate number and supplier

sample number	Sources	Supplier
1	positive control for *Tilletia walkeri*	CBS
2	positive control for *Tilletia laevis*	CBS
3	positiv control for *Tilletia controversa*	CBS
4	mercantile wheat 1	Agroinstitut, Sombor
5	mercantile wheat 1	Agroinstitut, Sombor
6	mecantile wheat 1	Agroinstitut, Sombor
7	mercantile wheat 4	Agroinstitut, Sombor
8	mercantile wheat 4	Agroinstitut, Sombor
9	mercantile wheat 2	Agroinstitut, Sombor
10	mercantile wheat 2	Agroinstitut, Sombor
11	mercantile wheat 11	Agroinstitut, Sombor
12	mercantile wheat 11	Agroinstitut, Sombor
13	cultivar Ljiljana	Agroinstitut, Sombor
14	cultivar Ljiljana	Agroinstitut, Sombor
15	mercantile wheat 6	Agroinstitut, Sombor
16	mercantile wheat 7	Agroinstitut, Sombor
17	positive control for *Tilletia tritici*	Institute for Field and Vegetable Crops, Novi Sad
18	artificialy contaminated wheat seed with *Tilletia tritici*	Institute for Field and Vegetable Crops, Novi Sad
19	mercantile wheat 14	Agroinstitut, Sombor

RESULTS

Trees with early growing season onset were predominant in the studied walnut Examination of 167 seed samples collected from Vojvodina and Central Serbia, showed that 145 (86.83%) samples were contaminated with teliospores of *Tilletia* species, while 22 (13.17%) samples were not contaminated.

Identification of Tilletia species by rep-PCR genomic fingerprinting

Rep-PCR fingerprint patterns were obtained from 19 DNA isolates of *Tilletia* species. Each primer pair generated ~25 to 30 bands visible on agarose gel. The size of the amplification products ranged from 500 bp to 3500 bp (Figure 1). Four DNA isolates numbered as 4, 10, 11 and 13 were not analyzed because bands were not clearly visible when REP primers were used. Dendrogram obtained from similarity matrix is presented in Figure 2.Positive control for *T. tritici* and ten out of eleven DNA isolates obtained from Serbian wheat seed samples were clustered together with more than 80% similarity, and thus were confirmed as *T. tritici* (Figure 2). Isolate 14 and isolates of *T. tritici,T. controversa* and *T. laevis* shared a similarity of 60%, although isolate 14 was obtained from the seed sample where only

teliospores of *T. tritici* were found using microscopy method. Positive controls for *T. controversa* and *T. laevis* were not clustered together with isolates of *T. tritici*. Isolate of *T. controversa* shared a similarity of 75% with isolates of *T. tritici,* while isolate of *T. laevis* shared a similarity of 70% with isolates of *T. tritici* and *T. controversa* (Figure 2). Similarity between *T. walkeri* and *T. tritici, T. controversa* and *T. laevis* was approximately 10% (Figure 2).

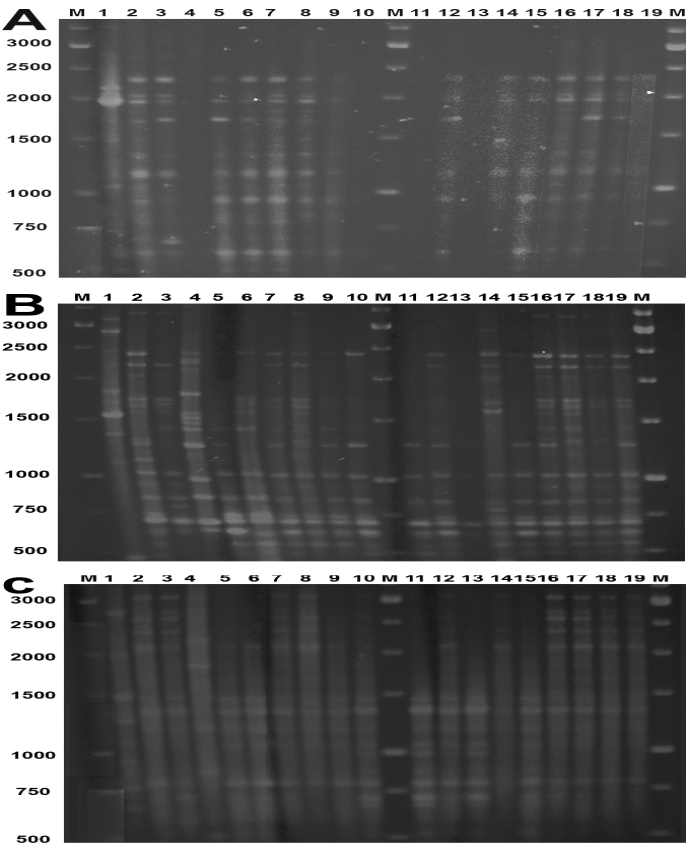

Figure 1 *DNA fingerprints of 19 Tilletia genotypes obtained by rep-PCR technique.*

(A) REP-PCR, **(B)** ERIC-PCR, **(C)** BOX-PCR. Lane 1=positive control for *Tilletia walkeri;* Lane 2=positive control for *Tilletia laevis*; Lane 3=positive control for *Tilletia controversa*; Lane 4=mercantile wheat 1.1; Lane 5= mercantile wheat 1.2; Lane 6=mercantile wheat 1.3; Lane 7=mercantile wheat 4.1; Lane 8=mercantile wheat 4.2; Lane 9= mercantile 2.1; Lane 10=mercantile wheat 2.2; Lane 11=mercantile wheat 11.1; Lane 12=mercantile wheat 11.2; Lane 13=cultivar Ljiljana 1; Lane 14=cultivar Ljiljana 2; Lane 15=mercantile wheat 6; Lane 16=mercantile wheat 7;Lane 17 = positive control for *Tilletia tritici*; Lane 18= artificialy contaminated wheat seed with *Tilletia tritici;* Lane 19=mercantile wheat 14; Lane M= DNA molecular size markers (1 kbp ladder); sizes in base pairs.

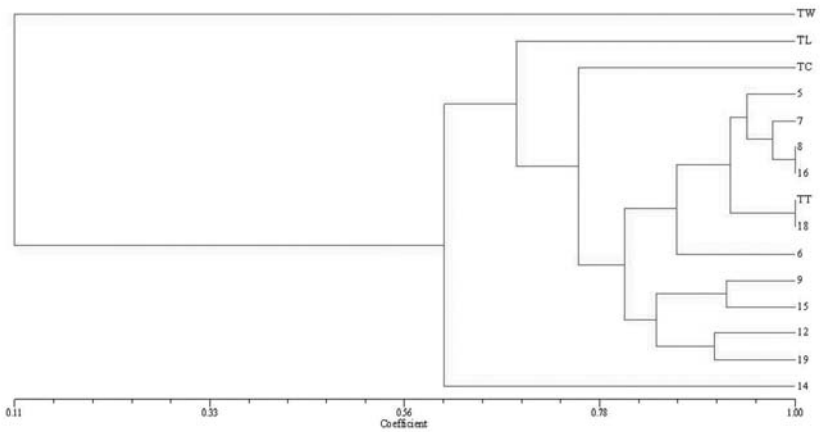

Figure 2 Dendrogram of 19 Tilletia genotypes derived from UPGMA analysis.

TW=positive control for *Tilletia walkeri;* TL=positive control for *Tilletia laevis*; TC=positive control for *Tilletia controversa*; TT = positive control for *Tilletia tritici;* 5= mercantile wheat 1.1; 6=mercantile wheat 1.2; 7=mercantile wheat 4.1; 8=mercantile wheat 4.2; 9= mercantile wheat 2.1; 12=mercantile wheat 11.2; 14=cultivar Ljiljana 2; 15=mercantile wheat 6; 16=mercantile wheat 7; 18= artificialy contaminated wheat seed with *Tilletia tritici;* 19=mercantile wheat 14

DISCUSSION

Examination of 167 seed samples of wheat from Vojvodina and Central Serbia, showed that 145 (86.83%) samples were contaminated with teliospores of *Tilletia* species. These results were in line with results obtained by STOJANOVIĆ *et al*. (1993) and JEVTIĆ *et al*. (1997). STOJANOVIĆ *et al.* (1993) examined 179 seed samples of wheat from 43 locations in Serbia, and found that 73.7% samples were contaminated with *T. tritici*. JEVTIĆ *et al*. (1997) examined 120 seed samples collected from Srem District, south part of Vojvodina, and revealed that 85.3% samples were contaminated with *T. tritici*.

Identification of *Tilletia* species, based on morphological characteristics of teliospores, is very difficult or in some cases impossible. Deep reticulations and prominent sheath are the best characteristics for distinguishing *T. controversa* from *T. tritici*, but there is an overlap of about 10% in the morphology of *Tilletia* species with reticulate exosporium, so individual teliospores of *T. controversa* with short reticulations and sheath thickness of 1.5 µm can be wrongly identified as *T. tritici* (GOATES, 1996). LIANG *et al.* (1982) tried to determine *T. controversa* and *T. tritici* based on depth of reticulations, thickness of gelatinous sheath, and germination test, with a success rate of 70%.

The similarity in morphology between *T. controversa* and certain members of *T. fusca (T. bromi)* complex is another problem for distinguishing *T. controversa* in a survey of the health of cereal seed. PETERSON *et al.* (2009) developed risk assessment model for importation of United States milling wheat containing *T. controversa* to People's Republic of China. They analyzed presence of *T. controversa* in samples of wheat export shipments. *T. controversa* was distinguished from *T. tritici* based on reticulation depth. If reticulation depth was $\geq$ 0.95 μm, teliospores were identified as *T. controversa*. This method does not differentiate *T. controversa* from *T. bromi*, so there is the risk of possible misidentifying grass smut which leads to risk for possible commodity rejection. Grass smuts are commonly present in wheat export shipments but in the low number. Although they are not pathogens of wheat, their hosts, *Bromus* and *Festuca* sp, can be found in and around wheat fields.

The aim of this study was to identify *Tilletia* species which were found in seed samples of wheat, using rep-PCR fingerprinting technique. Analysis of eleven DNA isolates of *Tilletia* species, obtained from nine seed samples, revealed that ten out of eleven isolates belong to *T. tritici*. These isolates and the positive control for *T. tritici* had more than 80% similarity, which was consistent with result obtained by MCDONALD *et al*. (2000). In our study only one isolate of *T. laevis* and one isolate of *T. controversa* were used and they shared similarity of about 70% with isolates of *T. tritici*. MCDONALD *et al*. (2000) analyzed large number of *T. controversa*, *T. tritci* and *T. laevis* isolates and they also shared 70% similarity. Isolate 14 obtained from the seed sample contaminated with teliospores of *T. tritici* had 60% similarity with isolates of *T. tritici, T. laevis* and *T. controversa*. Positive control for *T. bromi* was not used in this study, but on the basis of similarity of our results and these obtained by MCDONALD *et al*. (2000), it might be assumed that isolate 14 belongs to *T. bromi*. This assumption is supported by the fact that teliospores of *T. tritici* and *T. bromi* can be morphologically similar, thus they could have been misidentified. According the MCDONALD *et al.* (2000) *T. bromi* shared >55% similarity with *T. tritici, T. laevis* and *T. controversa*. Similarity between *T. walkeri* and *T. tritici, T. controversa* and *T. laevis* was approximately 10%, which was in line with result obtained by MCDONALD (2000).

The results of our study indicate the high sensitivity of size selective sieving method as well as the study of PETERSON *et al*. (2000). By using this method it was possible to detect $\leq$5 teliospores in 50g seed samples. However there is still problem of producing mycelial mats for DNA isolation if the seed samples are contaminated with one or several teliospores. This is the reason why we have successfully isolated DNA from only nine out of 167 examined seed samples. It is important to say that these nine samples were highly contaminated with teliospores of *Tilletia* species, unlike the rest of seed samples which were contaminated with about 5 teliospores of *Tilletia* species.

Regardless of the difficulties in obtaining mycelial mat, MCDONALD *et al*. (2000) suggested that rep-PCR has potential as a diagnostic method when it's important to distinguish quarantined *T. indica* from *T. indica*-like teliospores.

Furthermore, *T. tritici*, *T. controversa* and *T. laevis* were clustered separately despite the high genetic similarity of 70%. *T. bromi* which has morphologically similar teliospores to those of *T. controversa* also clustered separately, providing differentiation of these two species. *T. bromi* can be distinguished from *T. controversa* using RAPD markers (SHI *et al*., 1996; BOYD *et al*., 1998), but according the MCDONALD *et al.* (2000) rep-PCR fingerprinting technique has advantages due to its simplicity, the universality of PCR primers and tolerance of a wider range of DNA concentrations in generating reproducible results. QING *et al.* (2009) and LIU *et al*. (2009) developed high selective primers for identification of *T. controversa*, and for the first time it was possible to distinguish *T. controversa* from *T. tritici* on the basis of the presence of one unique DNA fragment. This is a very important discovery that could provide accurate identification of *T. controversa,* avoiding confusion during identification of this quarantine species. Nevertheless, rep-PCR fingerprinting is a useful technique for studying, diversity, phylogenetic relationships and taxonomic status of wheat bunt species, considering the fact that different isolates of the same variety, such as *T. fusca* var. *fusca* isolates on *V. microstachys* and *V. octoflora,* could share only 20 to 35% of similarity. (BOYD *et al.,* 1998; MCDONALD *et al*., 2000). These findings could be of a great importance not only for studying diversity of *Tilletia* species, but also for understanding relationships between a pathogen and its hosts.

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REFERENCES

ARORA, D. K., P. R. HIRSCH, and B. R. KERRY (1996): PCR-based molecular discrimination of *Verticillium chlamydosporum* isolates. Mycol. Res. *100*:801-809.

BOYD, M. L., and L. M. CARRIS (1997): Molecular relationships among varieties of the *Tilletia fusca* (*T. bromi*) complex and related species. Mycol. Res. *101*:269-277.

BOYD, M. L., and L. M. CARRIS (1998): Evidence supporting the separation of *Vulpia*- and *Bromus* - infectiong isolates in the *Tilletia fusca* (*T. bromi*) complex. Mycologia *90*: 1031-1039.

BOYD, M. L., L. M. CARRIS, and P. M. GRAY (1998): Characterization of *Tilletia goloskokovii* and allied species. Mycologia *90*:310-322.

CZEMBOR, P. C., and E. ARSENIUK (1999): Study of genetic variability among monopycnidial and monopycnidiospore isolates derived from single pycnidia of *Stagonospora* spp. and *Septoria tritici* with the use of RAPD-PCR, MP-PCR and rep-PCR techniques. J. Phytopathol. *147*:539-546.

EDEL, V., C. STEINBERG, I. AVELANGE, G. LAGUERRE, and C. ALABOUVETTE (1995): Comparison of three molecular methods for the characterization of *Fusarium oxysporum* strains. Phytopathology *85*:579-585.

FERNANDEZ-ORTUÑO D., S. L. ATKINS, B. A., FRAAIJE (2011): The use of *CYP51C* gene based PCR-RFLP assay for simultaneous detection and identification of *Fusarium avenaceum* and *F. tricinctum* in wheat. International Journal of Food Microbiology *145*(1): 370-374.

GOATES, B. J. (1996): Common Bunt and Dwarf Bunt. (In R. D. Wilcoxson, E. E. Saari, G. P. Hettel & A. McNab (Eds), *Bunt and Smut Diseases of Wheat- Concepts and Methods of Disease Management*, (pp:12-25), Mexico: CIMMYT)

IGNJATOVIĆ-MICIĆ, D., S. MLADENOVIC DRINIĆ, A. NIKOLIĆ, V. LAZIĆ-JANČIĆ (2007): Comparison of AFLP and SSR markers for genetic diversity studies in maize populations. Maydica *52*: 399-406.

IGNJATOVIĆ-MICIĆ, D. K. MARKOVIĆ, D. RISTIĆ, S. MLADENOVIĆ DRINIĆ, S. STANKOVIĆ, V.LAZIĆ-JANČIĆ, M. DENIĆ (2009): Variability analysis of normal and *opaque2* maize inbred lines. Genetika *41*(1): 81 -93.

IVANOVIĆ, D., R. OSLER, N. KATIS, M. S. IVANOVIĆ and D. IGNJATOVIĆ (1995): Principal maize viruses in Mediterranean countries. Agronomie *15*: 443-446.

JACCARD, P. (1908): New researches on plant distribution. Nouvelles rescherches sur la distribution florale. Bull. Soc. Vaud. Sci. Nat. *44*: 223-270

JEDRYCZKA, M., T. ROUXEL, and M-H. BALESDENT (1999): Rep-PCR based genomic fingerprinting of isolates of Leptosphaeria maculans from Poland. Eur. J. Plant Pathol. *105*:813-823.

JEVTIĆ, R., S. STOJANOVIĆ, M. DOPUĐA, D. MATIJEVIĆ, and M. MILOŠEVIĆ (1997): Occurrence of bunt and smuth in Serbia. XXXI Agronomy Conference. Book of abstracts *29*: 217-223. (original text in Serbian)

JEVTIĆ, R. (1998): Races, gene identification and resistance sources; Resistance of varieties and cropping practice defense. Chapters in book: *Wheat blunt*, Milošević, M., Stojanović, S., Jevtić, R., Matijević, D., Rajković, Snežana. Institute of Field and Vegetable Crops, Novi Sad; Institute of Agricultural Research SERBIA, Belgrade; Institute for Plant Protection and Environment, Belgrade. Feljton, Novi Sad, pp.: 47-78. (original text in Serbian)

KOSTIĆ, B., and H. SMILJAKOVIĆ (1968): Wheat diseases under intensive production and their suppression. Agrohemija *7-8*: 331-342. (original text in Serbian)

LADHALAKSHMI D., A. VIJAYASAMUNDEESWARI, V. PARANIDHARAN, R. SAMIYAPPAN, R. VELAZHALAN (2009): Molecular identification of isolates of Peronosclerospora sorghi from maize using PCR-based SCAR marker. World Journal of Microbiology and Biotechnology *25*(12): 2129-2135.

LEVIĆ, J., V. PENČIĆ (1993): Morphology of a new pathotype of *Bipolaris zeicola* (Stout) Shoemaker. J. Phytopathology 139: 339-346.

LIANG, Z., Y. GUO, Y. ZHU, and Y. SONG (1982): Statistic analysis based on the teliospores morphological characters in differentiating dwarf smut from stinking smut. Acta Phytophylacica Sin. *9*: 243-250.

LIU, J.H., L. GAO, T.G. LIU, and W.Q. CHEN (2009): Development of a sequence-characterized amplified region marker for diagnosis of dwarf bunt of wheat and detection of *Tilletia controversa* Kühn. Letters in Applied Microbiology *49*: 235-240.

MATHRE, D. E. (1996): Dwarf Bunt: Politics, Identification, and Biology. Annu. Rev. Phytopathol, *34*: 67-85

MCDONALD, J.G., E. WONG, and G. P. WHITE (2000): Differentiation of Tilletia Species by rep-PCR Genomic Fingerprinting. Plant Dis. *84* (10): 1121-1125.

MIAN ROUF M.A., A.A. HOPKINS, J.C. ZWONITZER (2002): Determination of genetic diversity in tall fescue with AFLP markers. Crop Sci. *42***:** 944-950.

MÖLLER E.M., G. BAHNWEG, H. SANDERMANN, and H. H. GEIGER (1992): A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected tissues. Nucleic Acids Research. *20* (22) 6115-6116.

OEPP/EPPO (2007): Diagnostic Protocols for Regulated Pests. EPPO Standards PM 7/29 (2) Tilletia indica. Bulletin OEPP/EPPO Bulletin 37, 503-520.OEPP/EPPO (Paris).

PEJIĆ, I., P. AJMONE-MARSAN, M. MORGANTE, V. KOZUMPLICK, P. CASTIGLIONI, G. TARAMINO, M. MOTTO (1998): Comparative analysis of genetic similarity among maize inbred lines detected by RFLPs, RAPDs, SSRs, and AFLPs. Theor. Appl. Genet. *97***:**1248-1255.

PETERSON, G. L., M. R. BONDE, and J. G. PHILLIPS (2000): Size-Selective Sieving for Detecting Teliospores of *Tilletia indica* in Wheat Seed Samples. Plant Dis. *84*: 999-1007

PETROSON, G. L., T. B. WHITAKER, R. J. STEFANSKI, E. V. PODLECKIS, J. G. PHILLIPS, J. S. WU, and W. H. MARTINEZ (2009): A Risk Assessment Model for Importation of United States Milling Wheat Containing *Tilletia contraversa*. Plant Dis. *93* (6): 560-573.

PIMENTEL, G., L. M. CARRIS, and T. L. PEEVER (1998): Characterization of interspecific hybrids between *Tilletia controversa* and *Tilletia bromi*. Mycologia *92* (3): 411-420.

RADEMAKER, J. L. W., and F. J. DE BRUIJN (1997): Characterization and classification of microbes by rep-PCR geno mic fingerprinting and computer-assisted pattern analysis (In: G. Caetano-Anollés and P. M. Gresshoff (Eds), *DNA Markers: Protocols, Applications and Overviews* (pp:151-171) John Wiley & Sons, New York).

ROHLF, F.J. (2000): NTSYS-pc. Numerical taxonomy and multivariate analysis system. Version 2.0 Exeter Software, Setaket, N.Y

STOJKOV, S., J. LEVIĆ, , D. IGNJATOVIĆ, D. IVANOVIĆ, V. PENČIĆ (1996): *Periconia* pathogenes on maize root and stalk.. Zaštita bilja, vol. 47(3), 217: 229-240. (original text in Serbian).

STOJKOV, S., D. IGNJATOVIĆ (1998): Variability of fungus *Microdochium bolleyi* isolate - maize root and stalk pathogen. 7 th International Congress of Plant Pathology. 9-16. Avgust, Edinburgh.

VERMA, K.S., V. KHANNA, N. SINGH (1999): Random Amplified Polymorphic DNA Analysis of Indian Scented Basmati Rice (*Oryza sativa* L.) Germplasm for Identification of Variability and Duplicate Accessions, if any. Electrophoresis, *20*: 1786–1789.

QING, Y., N. SIJI, Y. YOUPING, L.MINHUI, C. JUN, and W. ZHONGKANG (2009): Development of a PCR-based diagnostic tool specific to wheat dwarf bunt, caused by *Tilletia controversa*. Eur. J. Plant Pathol. *124*: 585-594.

SHI, Y. L., P.LOOMIS, D. CHRISTIAN, L. M. CARRIS, and H. LEUNG (1996): Analysis of the genetic relationships among the wheat bunt fungi using RAPD and ribosomal DNA markers. Phytopathology *86*: 311-318.

STOJANOVIĆ, S., M. DOPUĐA, J. STOJANOVIĆ, and S. GUDŽIĆ (1993): Bunt as a frequent wheat disease in Serbia. I Yugoslav Conference on Plant Protection. Vrnjačka Banja. Book of Abstracts, p. 18 (original text in Serbian)

IDENTIFIKACIJA VRSTA RODA *Tilletia* rep-PCR *FINGERPRINTING* TEHNIKOM

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I z v o d

Istraživanjem kontaminiranosti uzoraka semena pšenice teleutosporama *Tilletia* vrsta, utvrđeno je da je od 167 uzoraka nedorađenog semena pšenice, bilo kontaminirano 145 (86,8%), dok su 22 uzorka (13,2%) smatrana nekontaminiranim. Identifikacija *Tilletia* vrsta izvršena je rep-PCR fingerprinting tehnikom. Izolati prikupljeni na teritoriji Republike Srbije identifikovani su kao *T. tritici,* s obzirom da su sa pozitivnom kontrolom imali genetičku sličnost veću od 80%. Jedan od izolata koji je vodio poreklo iz opštine Apatin bio je oko 60% genetički sličan sa izolatima *T. tritici, T. controversa* i *T. laevis*. Pretpostavljeno je da pripada vrsti *T. bromi*. Genetička sličnost izolata *T. walkeri* i vrsta: *T. tritici, T. laevis* i *T. controversa*, iznosila je nešto više od 10%. Genetička sličnost *T. tritici, T. controversa* i *T. laevis* bila je oko 70 %. I pored visokog procenta genetičke sličnosti između *T. controversa* i *T. tritici*, napravljena je razlika među njima, što rep-PCR fingerprinting tehniku čini veoma podesnom za praćenje prisustva morfološki sličnih *Tilleria* vrsta prilikom kontrole kvaliteta semena pšenice.

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