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Development of a SCAR Marker by Inter-Simple Sequence Repeat for Diagnosis of Dwarf Bunt of Wheat and Detection of *Tilletia controversa* KÜHN

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ABSTRACT. Dwarf bunt of wheat, caused by *Tilletia controversa* KÜHN, is a destructive disease on wheat as well as an important internationally quarantined disease in many countries. The primer ISSR818 generated a polymorphic pattern displaying a 867-bp DNA fragment specific for *T. controversa*. The marker was converted into a sequence characterized amplified region (SCAR), and specific primers (TCKSF3/TCKSR3) designed for use in PCR detection assays; they amplified a unique DNA fragment in all isolates of *T. controversa* but not in the related pathogens. The detection limit with the primer set (TCKSF3/TCKSR3) was 5 ng of DNA which could be obtained from 5.5 μg of teliospores in a 25-μL PCR reaction mixture.

Abbreviations

AFLP	amplified fragment length polymorphism	RAPD	random amplification of polymorphic DNA
CAPS	cleavage amplified polymorphic sequence	RFLP	restriction fragment length polymorphism
ISSR	inter-simple sequence repeat	SCAR	sequence characterized amplified region
ITS	internal transcribed spacer	SSR	simple sequence repeat
PCR	polymerase chain reaction		

Tilletia controversa KÜHN is the causal organism of wheat dwarf bunt (Duran and Fischer 1961). Mature sori (bunt balls) consist almost entirely of teliospores and are covered by a thin, modified ovary wall; wheat yield reductions due to bunt are nearly equal to the percentage of infected spikes, and can reach 20–50 % and even more than 75 % during severe disease epidemics (Goates 1996).

Many research projects have been developed and numerous papers on the identification of *T. controversa* have been published (Mathre 1996). However, in the smut fungi, few features are available for use as taxonomic criteria (spore size, shape, morphology, germination type, host range, hyphal separation and zones of host-range interactions) (Vánky 1991; Bauer *et al.* 1997). The use of DNA-based molecular techniques for examining relationships within and among different fungi at the species, subspecies, *formae speciales*, and race levels has also been applied to smut fungi in recent years. These techniques have several potential advantages, such as improved accuracy and reliability (Bakkeren and Kronstad 1994; Gang and Weber 1995; Shi *et al.* 1996). Therefore, a molecular assay for rapid identification and accurate detection of *T. controversa* would be useful. Recently, the application of molecular markers for the identification of smut fungi has been used on *T. controversa*, *T. indica*, *T. walkeri*, *T. caries*, *T. foetida* and related fungi (Gang and Weber 1995; Frederick *et al*. 1998; Pimental 2000; Josefsen and Christiansen 2002; Eibel *et al.* 2005; Liang *et al*. 2006). However, none of these investigations readily and clearly distinguished *T. controversa* from similar pathogens. Many investigations of *T. controversa* indicated that conserved genes lacked sufficient variation and could not successfully identify this species (Josefsen and Christiansen 2002; Liang *et al*. 2006).

Screening random regions of the fungal genome may reveal species specificity (Liu *et al*. 2009). RAPDs (Williams *et al.* 1990), SSRs or microsatellites (Tautz 1989), and AFLPs (Vos *et al.* 1995) were three widely-used PCR-based markers. Each technique has its own advantages and disadvantages. RAPD markers are very quick and easy to develop but lack reproducibility (Karp *et al.* 1997; Hansen *et al.* 1998; Virk *et al.* 2000). AFLP has medium reproducibility but is labor intensive and has high operational and development costs (Karp *et al.* 1997). Microsatellites are specific and highly polymorphous (Karp *et al.* 1997), but they require knowledge of the genomic sequence to design specific primers and, thus, are limited primarily to economically important species.

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The choice of a molecular marker technique depends on its reproducibility and simplicity (Bornet and Branchard 2001). Since 1994, a new molecular marker technique called ISSR has been available, which amplifies inter-microsatellite sequences at multiple loci throughout the genome by a single primer 16–18 bp long composed of a repeated sequence anchored at the 3′ or 5′ end by 2–4 arbitrary nucleotides (Zietkiewicz *et al.* 1994). Such amplification does not require genome sequence information and leads to multilocus and highly polymorphous patterns (Zietkiewicz *et al.* 1994; Nagaoka and Ogihara 1997). ISSR amplification is also a PCR-based method that can differentiate closely related individuals, which is quick and easy to handle like RAPD. This technique was shown by Hantula *et al.* (1996) to be able to generate DNA markers in a variety of fungi. A hypothesis was formulated that there would be a greater likelihood of finding polymorphisms with ISSRs than with most other techniques, including RAPDs, because the evolutionary rate within ISSRs is considerably higher than in most other types of DNA (Charlesworth *et al.* 1994). ISSR technique also combines most of the benefits of AFLP and SSR markers with the universality of RAPD (Pradeep Redy *et al.* 2002), which are dominant inheritance markers and can generate large numbers of highly informative and reproducible alleles.

Hantula *et al.* (1996) found that ISSRs are common in *Ustilago* genomes by conducting a computer search on all available *Ustilago* sequences. In case of *T. controversa*, such techniques have mainly been used to delineate the species and to elucidate their genetic relationship to other members of the genus *Tilletia* (*e.g.*, Shi *et al.* 1996; McDonald *et al.* 2000; Levy *et al.* 2001; Menzies *et al.* 2003). The use of specific SCAR markers (Weber *et al.* 2002) is also a powerful tool to unambiguously identify a particular strain; these markers are designed based on certain known genome sequences and are used as co-dominant markers (Bautista *et al.* 2003). Notably, SCAR analysis is relatively inexpensive and straightforward. Thus, for further specific and reliable discrimination of *T. controversa*, development of SCAR markers is very essential.

In the present study, ISSR technique has been successfully applied to development a SCAR marker for diagnosis of dwarf bunt of wheat and detection of *T. controversa*.

MATERIALS AND METHODS

Isolates and preparation of DNA templates. Eight isolates of *T. controversa* were collected in the United States by Prof. B. Goates (*National Small Grains Germplasm Research Facility*, USDA-ARS). Other related species were obtained from Dr. Zhongkang Wang (*Chongqing University*, China), Dr. Pinshan Wu (*Institute of Animal and Plant Quarantine, Chinese Academy of Inspection and Quarantine*) and our laboratory stock. The collection included *T. caries*, *T. foetida*, *Ustilago tritici*, *U. maydis*, *U. scitaminea*, *Sorisporium crueuta*, *Puccnia striiformis*, *P. triticina*, *P. graminis*, *Erysiphe graminis* and *Fusarium graminearum*. Genomic DNA was extracted and the final solution of genomic DNA was treated according to Liu *et al.* (2009).

ISSR procedure. Sixty ISSR primers (UBC primer set no. 9; *University of British Columbia*, Canada; **http://www.michaelsmith.ubc.ca/services/NAPS/Primer_Sets/**) were used.

PCR reaction mixture (25 μL) contained: 2.5 μL of 10× PCR buffer (Mg^{2+} free), 2.0 μL of Mg^{2+} (25 mmol), 0.25 μL of dNTP (10 mmol), 3 μL ISSR primer (10 μmol) (ISSR818: 5′-TGT GTG TGT GTG TGT GRC-3′), 0.4 μL of *TaKaRa Taq* (2.5 U/μL), 1.5 μL of DNA (20 ng/μL) and sterile water.

Amplification was carried out in a programmable peltier thermocycler PTC-200 (*MJ Research*, USA) with the following cycling conditions: an initial denaturing cycle (1 min at 94 °C) followed by 40 cycles of 1 min at 94 °C, 1 min at the annealing temperature (32–75 °C depending on the primer), 2 min elongation at 72 °C and final extension (5 min at 72 °C). The products were stored at 4 °C.

Amplification products were subjected to electrophoresis on an agarose gel (1.5 %) at 140 V for 2 h in 1× TAE buffer (40 mmol/L Tris–acetic acid, 1 mmol/L EDTA, pH 8.0) at room temperature. Gels were stained with ethidium bromide, visualized under UV light and photographed using a gel documentation system (*Bio-Rad*, USA). A molecular size marker D2000 was used.

SCAR marker development. For the development of SCAR markers, the species-specific 867-bp amplicon generated by the primer ISSR818, was excised from the gel and purified with the QIAquick Gel DNA Extraction Kit (*Qiagen*, Germany) according to the manufacturer's instructions. The amplicon was cloned into the *pEASY*-T1 vector using cloning kit (*TransGen Biotech*, China). Ligated plasmids were used to transform *Escherichia coli* competent cells according to the manufacturer's protocol. Plasmids were extracted from positive clones according to Sambrook *et al.* (1989). The cloned fragment was sequenced by *Sangon Biological Engineering Technology and Services, Ltd.* (Shanghai, China) using M13 vector specific primers.

The sequence of the 867-bp fragment was analyzed with Chromas and subjected to BLASTn/BLASTx and PROSITE to check for potential homologies with sequences or domains. Based on full-length sequences of the 867-bp amplicon, a SCAR was designed with the DNAMAN 5.0 computer software to amplify a diagnostic product of 419 bp. The primers were synthesized by *Sangon Biological Engineering Technology and Services*.

Universality, specificity, and sensitivity of the SCAR marker. The universality and specificity of the SCAR marker was tested by PCR with purified genomic DNA of *T. controversa* (race 7 from Dr. Zhongkang Wang) and *T. caries*.

SCAR amplification was performed in 25 μL of reaction mixture containing 20 ng of genomic DNA, 2.5 μL of 10× PCR buffer (Mg^{2+} free), 2.0 μL of Mg^{2+} (25 mmol) (means a volume of 2 μL of Mg^{2+} solution was added to the mixture, the total volume of 25 μL was kept constant) and the final concentration of Mg^{2+} was thus 1 μmol/μL), 0.3 μL of dNTP (10 mmol), 1 μL of SCAR primer (10 mmol) TCKSF3 and TCKSR3, respectively, 0.3 μL *TaKaRa Taq* (2.5 U/μL), and ddH_2O. PCR amplification was carried out with an initial denaturation (5 min at 94 °C) followed by 30 cycles (30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C) with a final extension (10 min at 72 °C). The amplified products were separated by electrophoresis on a 1.5 % (*W*/*V*) agarose gel and run at 100 V for 1 h in 1×TAE buffer, and then stained with ethidium bromide and visualized on a UV transilluminator.

The sensitivity of the SCAR markers was tested with purified genomic DNA of *T. controversa*, which was diluted to give the following DNA templates in 25-μL PCR reaction mixture – in ng: 100, 90, 80, 70, 60, 50, 40, 30, 20, 10, 5, 1, and 100 pg, 10 pg, 1 pg; UV spectrophotometer (*Hitachi* U-2000) was used for the detection. The reaction mixture, amplification conditions and gel conditions were the same as *above*.

RESULTS

The specific band screening. Most primers tested in this study amplified multiple polymorphic DNA fragments from all of the organisms tested. Among them, ISSR818 generated a polymorphic profile that distinguished all isolates of *T. controversa* from *T. caries* and *T. foetida* (Fig. 1).

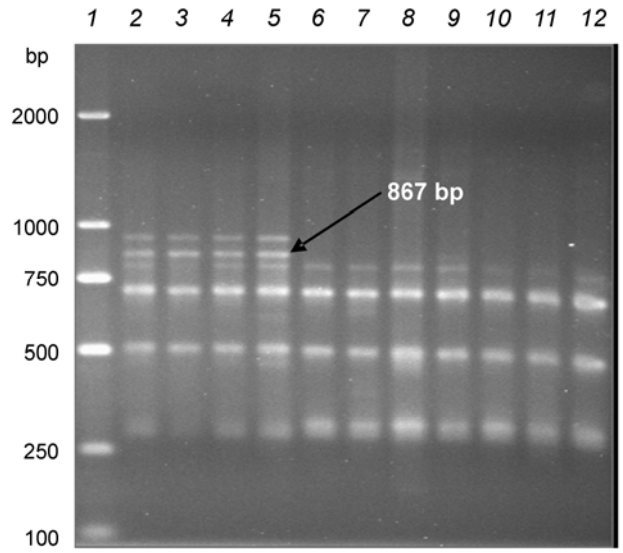

Fig. 1. ISSR patterns generated by ISSR818 primer in four races of *T. controversa*; *1* – D2000 DNA ladder, *2–5* – *T. controversa*, *6–9* – *T. caries*, *10–12* – *T. foetida.*

SCAR marker development. Based on the sequence of the unique, *T. controversa*-specific fragment (Fig. 1), we designed SCAR primers by DNAMAN 5.0 of TCKSF3/TCKSR3: 5′-CAC ACA CAC ACA GGA AGC A-3′ and 5′-CGA GGA AGC AGA CAA GGC AT-3′ (Fig. 2).

Universality, specificity, and sensitivity of the new SCAR marker. The SCAR primers amplified a 419-bp DNA fragment from *T. controversa* but no products from 38 related smut fungi (Fig. 3). The sensitivity of the SCAR markers was tested using a serial dilution of genomic DNA extracted from *T. controversa* (race 7). The electrophoresis pattern indicated that stronger amplification could be obtained with higher template concentrations and the detection limit of the primer set TCKSF3/TCKSR3 was 5 ng of DNA which could be obtained from by 5.5 μg of teliospores in the 25-μL PCR reaction mixture (Fig. 4).

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1     CACACACACA CACACAGGAA GCAAGGCGTG GGGCCAGCTC CGGGCAAAAC TAGAATCGGC
61    TCGGGGCAAA ACTTTTTGCT AGGGACAAAA CTCCAAAGCG CCGAGGTGGT GTGGAAGATG
121   GGAAGGTGGT GGTGAAAGAG TTGGACGAGC AGAACACGTC GAGCTCTTTT GAGCAGCACA
181   GGAAGGCAGC ACATATGAGA AAAGGATACT GGATAATGCA GAGATTCATG TCATGAGAAA
241   GAAAGGTAAT GCATACATAT GAGAGTTGAG ACCGAAGACA AGCCGCAGCG CTCATATGTC
301   ATCATAAAAG ACATGAGTTG ACCTTGTGTT CGACGGACTT GCATCGGCCG CAAGGCTGTG
361   GACAGCGGCT GCAATTATGC CCGGCTTGCA TGCAGTTGCT ACAATGCCTT GTCTGCTTCC
421   TCGCTTTAGC CTTTGCCAAT TCTGCCGCCG TGAGAAGACG GCCGGTTGCC TTGGTGGCGG
481   CAGCGACCTT GCGCTGCTCC GGGTTTAGAG GGTCCAAAAG ACGAGGGAAA CGGACATCCT
541   GGAGGCCCTC ATCCGTGCGC CACTGAGGAT GTATGCTGGA GAGCGGAATC GGCTGACTGC
601   TCGCGCGTAG TTGACAGAGG GTGTGCGAGC AAGGAAGACC CATGGTGACC GTGATAATGC
661   AGGTGCATGT AATCACCCCG GTCTCATTTG ATCTCCGTGC CGCTTCCACA GTCTGCCGTC
721   CCATTTCGTC CTGCGCCTGG ATGAGCCGAA GCGCATATCT GGAGACGGTA TTGGCGACCT
781   CGACGTAAAA TCGATCCCCT AGAATATTGA TGGGGACTTT GTTGTGATCG TTTGCAATGT
841   CGCTTTCAAT CTGTGTGTGT GTGTGTG
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Fig. 2. Sequence of a specific DNA fragment of dwarf bunt of wheat; underlined are SCAR primers.

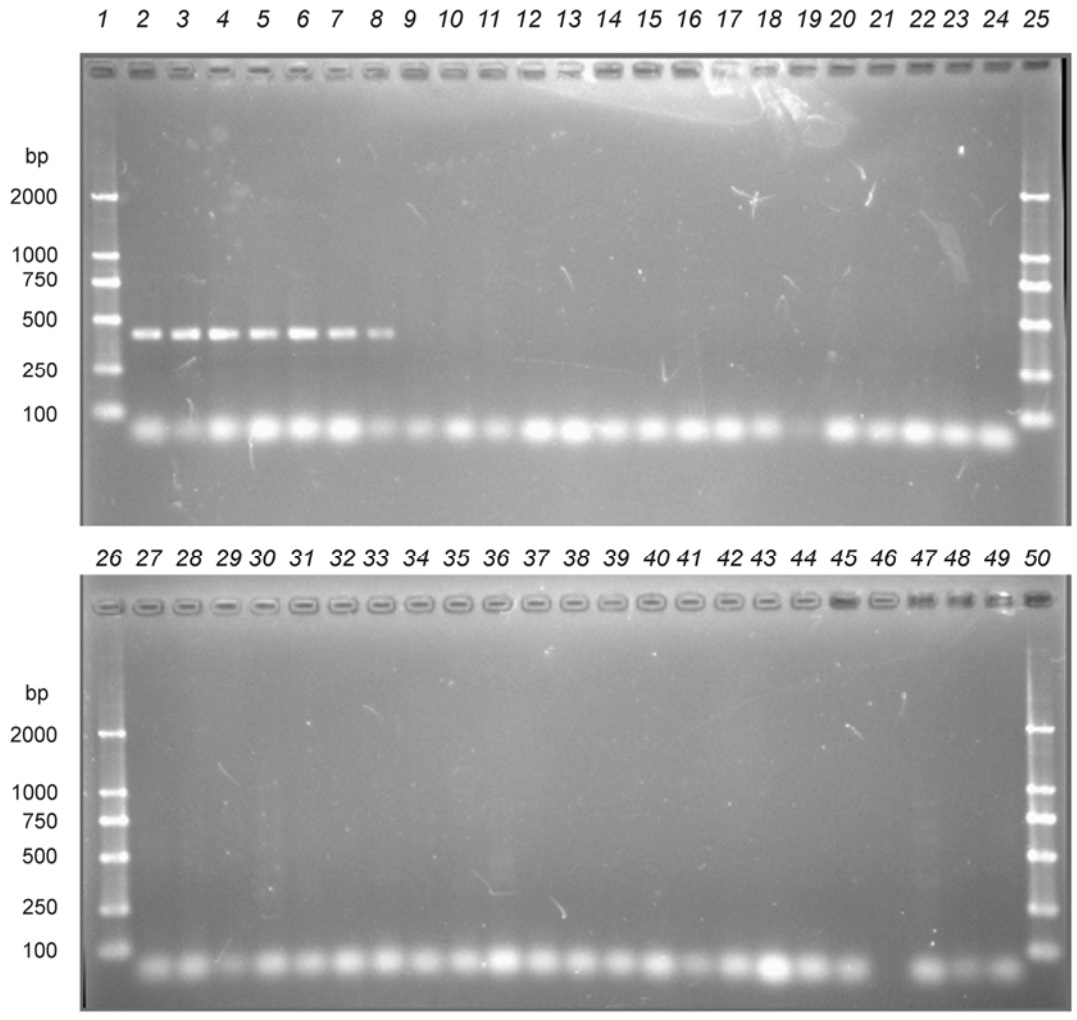

Fig. 3. The universality and specificity of the SCAR marker. *1*, *25*, *26*, *50* – D2000 DNA ladder, *2*–*8* – *T. controversa*, *9*–*16* – *T. caries*, *17*–*22* – *T. foetida*, *23*–*24* and *27*–*28* – *U. tritici*, *29*–*33* – *U. scitaminea*, *34*–*36* – *S. crueuta*, *37*–*40* – *U. maydis*, *41*–*42* – *P. striiformis*, *43*–*44* – *P. triticina*, *45*–*46* – *P. graminis*, *47* – *E. graminis*, *48* – *F. graminearum*, *49* – control (ddH_2O).

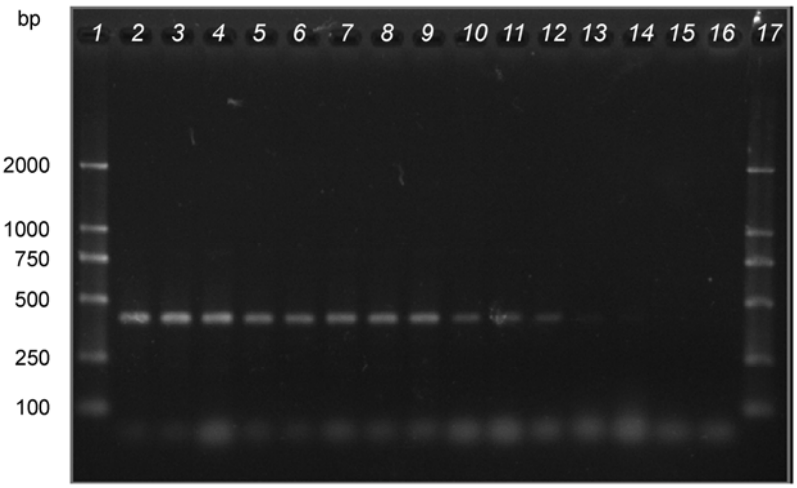

Fig. 4. The sensitivity test of the SCAR marker with different amounts of DNA template in a 25-μL PCR reaction mixture. *1*, *17* – D2000 DNA ladder; in ng: *2* – 100, *3* – 90, *4* – 80, *5* – 70, *6* – 60, *7* – 50, *8* – 40, *9* – 30, *10* – 20, *11* – 10, *12* – 5, *13* – 1; in pg: *14* – 100, *15* – 10, *16* – 1.

DISCUSSION

The ITS regions of the ribosomal subunit have been used as a target region for species-specific primers (O'Gorman *et al.* 1994; Salaza Julian and Rubio 2000; Errampalli *et al.* 2001; Pažoutová 2009). However, in the case of common bunt, it was not possible to design species-specific primers in the sequenced ITS region, as there is no variation between the ITS2 region of *T. tritici*, *T. laevis*, *T. controversa*, and *T. bromi* (Josefsen and Christiansen 2002). A similar conclusion was drawn by Mulholland and McEwan (2000) who designed *Tilletia*-specific primers in the large subunit of the ribosomal RNA genes to be used in a PCR-based healthy seed assay. *T. laevis* and *T. tritici* are closely related to *T. controversa* (Duran and Fischer 1961). Genomic fingerprinting and sequence data on *Tilletia* isolates (Shi *et al.* 1996; McDonald *et al.* 2000; Levy *et al.* 2001) do not provide support for a screening process for bunt resistance in wheat; no further search was made for primers that were more specific.

DNA molecular marker technology, which are based on sequence variation of specific genomic regions, provide powerful tools for identification with the advantages of time-saving, less labor-consumption and more efficiency (Mongkolporn *et al.* 2004; Dongre and Parkhi 2005; Garg *et al.* 2006). Compared with several types of DNA markers, *i.e.* RFLP, AFLP, RAPD, CAPS, SSR, and SCAR markers, ISSR is a novel molecular marker technique with some advantages (Agarwal *et al.* 2008). Firstly, ISSR is a PCR-based method that is similar to RAPD technique except that the ISSR primer sequences are longer and are designed from microsatellite regions. Therefore the annealing temperatures used are higher than those used for RAPD markers which could lead to higher consistency of the PCR products. Secondly, the effective multilocus markers used for diversity analysis, fingerprinting and genome mapping (Nghia *et al.* 2008) are easy to employ and are highly reproducible compared with other technique, such as RAPD, and no prior sequence knowledge is required (Goldwin *et al.* 1997). The ISSR have been successfully applied to study the genetic diversity of pathogenic fungi, *e.g.*, *Fusarium graminearum* (Mishra *et al.* 2004), *Sphaeropsis sapinea* (Burgess *et al.* 2001), *Phialophora gregata* (Meng and Chen 2001), *Cryphonectria cubensis* (Van *et al.* 2003), *Trichaptum abietinum* (Kauserud and Schumacher 2003), *Ustilago* spp. (Menzies *et al.* 2003), *Serpula lacrymans* (Kauserud 2003) and *Beauveria bassiana* (Elena Estrada *et al.* 2007). ISSRs have been used to characterize genetic variation within fungi but, to date, not with the smut fungi and *T. controversa*.

ISSR analytic system was set up and successfully used in the identification of *T. controversa* firstly in this study with limited samples, which revealed the possibility to identify *T. controversa* by this method. The use of SCAR produces genetic markers that are highly specific, which is a powerful tool to unambiguously identify a particular strain (Weber *et al.* 2002). The SCAR primers specific for the target sequence could be used to amplify the characterized genomic regions under PCR conditions, which makes the markers more specific than ISSR markers. In our SCAR analyses, primers were designed based on the *T. controversa*-specific band, which made possible to amplify specific DNA fragments of *T. controversa* that differ from all other tested related genera and species, including *T. caries*, *T. foetida*, *U. tritici*, *U. maydis, U. scita-*

minea, *S. crueuta*, *Puccinia striiformis*, *P. triticina*, *P. graminis*, *Erysiphe graminis* and *F. graminearum*. Now, it is thus completely possible to distinguish *T. controversa* using only SCAR markers.

We have previously also reported a SCAR for detecting *T. controversa* by AFLP method (Liu *et al.* 2009). Compared with ISSR, AFLP is more laborious and has higher operation and development costs. The SCAR obtained by AFLP have a sensitivity of 10 ng per 25-μL PCR reaction mixture with race 1 while, in this report, the SCAR obtained by ISSR could also detect 5 ng per 25-μL PCR reaction mixture with race 7. Because of limited DNA sample, we have not compared the sensitivity with the same race. Whatever method we have been using for development of SCAR, both studies confirmed that we could detect *T. controversa* from similar smut fungi quickly, easily and correctly. Both can lead to the development of a throughput kit for the detection of *T. controversa*, which could be a supplement to each other SCAR in the future.

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