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Research article

Molecular detection of *Exserohilum turcicum*, agent of northern corn leaf blight

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Abstract

Importance of the work: *Exserohilum turcicum* has a wide host range and high genetic variability in terms of its morphology, virulence and genetic structure. The recovered isolate varied considerably in morphology, including colony color, growth of pathogen, conidial structures, size and radical mycelial growth.

Objectives: To design a species-specific primer for *E. turcicum* based on inter-simple sequence repeat (ISSR) markers for the detection of the presence of *E. turcicum* on corn leaves.

Materials & Methods: Polymerase chain reaction analysis of 241 isolates of *E. turcicum* using ISSR molecular markers revealed only one primer, $(ACC)_5$, a unique DNA fragment set with 4- consistent polymorphic bands that clearly distinguished all isolates from closely related fungi. A single-specific molecular marker was designed, targeting a portion from a selected fragment region.

Results: The primer set amplified a product of 348 bp from all isolates of *E. turcicum* and did not amplify any DNA of other closely related fungi. In addition, the presence of the SEF/SER-Et41 gene region in the *E. turcicum* isolates was confirmed using dot blot analysis.

Main finding: The specific SEF/SER-Et41 primer set could be used as a reliable diagnostic method and useful tool for epidermiological study for the sensitive and specific detection of *E. turcicum*.

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Introduction

Exserohilum turcicum (Pass.) K.J. Leonard & E.G. Suggs [Synonyms: *Helminthosporium turcicum* teleomorph: *Setosphaeria turcica* (Luttell) K.J. Leonard & E.G. Suggs] is the causal agent of northern corn leaf blight (NCLB), a severe and widespread foliar disease of corn. NCLB causes substantial crop losses in most of the major corn-producing areas throughout the world (Smith and Kinsey, 1993) The most common diagnostic symptom of the disease on corn is cigar-shaped or elliptical, necrotic, gray-green lesions on the leaves that range in length from one to seven inches (Vieira et al., 2014). The lesions appear first on the lower leaves and then spread to upper leaves. Eventually, they turn tan-colored and may contain dark areas of fungal sporulation. The fungus overwinters on infested crop debris from the previous season. The disease causes yield losses of up to 50%, especially when it has heavily established before or at the silking stage and also causes qualitative alterations of seeds, resulting in reduced total sugar content and germinative capacity (Ferguson and Carson, 2004). The pathogen has a wide host range and high genetic variability in terms of virulence and genetic structure. In Thailand, NCLB is distributed in all corn-growing areas, with pseudothecia (sexual state) having been reported on heavily infected corn leaves from natural field production (Bunkoed et al., 2014). Both mating types (A and a) were present in every field population determined. The sexual reproduction of *S. turcica* caused genetic variation in this pathogen. Single conidia isolated from the same single conidial culture differ in color, type of mycelium, rate of growth and sporulation in culture. However, spores produced by *E. turcicum* are insufficiently distinct to visually identify this fungus from the mixed infection or the presence of other fungal pathogens on corn leaves such as *Bipolaris maydis* (Sun et al., 2020). A molecular technique for detecting *E. turcicum* using a specific polymerase chain reaction (PCR) primer producing a single amplicon from genomic DNA from the target pathogen could facilitate accurate, rapid and inexpensive detection. In addition to detection, such tools could be used to study the prevalence, source of inoculum, ecology and epidemiology of the causal organism to enable enhanced disease management. Commonly, the primers designed for such assays are based on the internal transcribed spacer (ITS) region (Bryan et al., 1995); however, such primers may not be specific enough to differentiate among closely related fungi (Chung et al., 2010; Xue et al., 2013). PCR primer pairs based on other sequences that are absent from the genome of other fungi may provide sufficient specificity to detect and identify the target pathogen.

In Thailand, *E. turcicum* is normally detected and identified morphologically by isolation and enumeration on media. Accordingly, identification based on the morphology of this fungus on culture media is time-consuming and requires extensive knowledge of classical taxonomy. Molecular biology techniques, especially PCR, have provided an alternative approach for the detection and identification of plant pathogens and many pathogenic fungi (Vincelli and Tisserat, 2008). Several types of DNA markers are generated by a wide variety of techniques, differing greatly in their reliability, difficulty, expense and the nature of the polymorphism that they detect. ISSR has substantially important advantages compared to other methods, as this technique can generate more polymorphic amplicons than the amplified fragment length polymorphism (AFLP) technique (Meng and Chen, 2001). Furthermore, the ISSR technique requires a smaller amount of genomic DNA of the target pathogen than does the AFLP technique and does not require the process of enzymatic digestion and ligation. ISSR is more specific than the RAPD technique due to the use of longer oligonucleotide sequences, allowing more stringent annealing conditions in PCR amplification (Abadio et al., 2012). It has been reported that ISSR was more reproducible than RAPD and had higher levels of polymorphism (Reddy et al., 2002).

An alternative DNA-based method is desirable to avoid misidentification by the assay, to increase accurate detection of the target species of the pathogen without error in analysis. DNA hybridization is considered to be a reliable and efficient method for routine detection and identification of plant pathogens (Goodwin and Annis, 1991). Therefore, the objectives of the current study were to design a species-specific primer pair for *E. turcicum* based on an ISSR marker and to develop a sensitive and specific PCR-based method for the detection of the presence of *E. turcicum* in corn leaves. A method for extracting fungal DNA directly from plant tissues was optimized and a dot blot hybridization assay target DNA probe was developed from the same specific primer set for more reliable detection of *E. turcicum*.

Materials and Methods

Fungal isolates and culture conditions

Infected corn leaves showing NCLB symptoms were collected from different agroclimatic zones in Thailand

during 2013–2015. Leaf material was surface sterilized and incubated in a moist chamber for 3 d. A single conidium of *E. turcicum* from an infected lesion was transferred to water agar plates (agar 20 g, water 1 L) and incubated at room temperature (25–30°C) for 72 hr. A hyphal tip from the single conidium was transferred to potato dextrose agar (PDA) plates as pure culture. The pure cultures were maintained on PDA slants and kept in a refrigerator at 5°C for further study. Other fungal pathogens of corn (*E. rostratum*, *Bipolaris maydis*, *H. carbonum*, *Peronosclerospora sorghi*, *Fusarium verticillioides* and *Macrophomina phaseoli*) were also isolated from symptomatic leaves of corn, as described above. Each isolate was identified based on standard morphological criteria, comparison with type isolates, pathogenicity testing and published descriptions.

Isolation of total DNA

Total DNA from the mycelia of *E. turcicum* and other fungal pathogens were extracted using a modified hexadecyltrimethylammonium bromide (CTAB) extraction method according to Moller et al. (1993). Samples (each 30 mg) of fungal mycelia were scraped from culture aged 10 d on PDA (three replications were conducted), manually ground in separate 1.5 mL microfuge tubes using a micro pestle in 700 µL of pre-warmed CTAB buffer (60°C) and incubated at 60°C for 60 min. DNA was extracted by adding an equal volume of chloroform-to-octanol (24:1) and centrifuging at 13,000 rpm for 10 min. The DNA was precipitated by adding 700 µL of cold isopropanol and centrifuging at 13,000 rpm for 10 min. The DNA was washed twice with 70% ethanol, then suspended in 200 µL of TE (Tris EDTA) buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA (Ethylene diamine tetra-acetic acid). The quantity and quality of DNA was determined using a spectrophotometer by measuring the absorbance at 260 and 280 nm. On the basis of the spectrophotometer readings, 50 ng/µL solutions were prepared and stored at -20°C for further PCR amplification. The specific primers of *S. turcica*, based on Chung et al. (2010) were designed based on the internal transcribed spacer 1 (ITS1) region of *S. turcica* and used to provide molecular evidence to prove the fungal species. PCR amplification resulted in a specific fragment of 170 base pairs.

Inter-simple sequence repeat primer screening and amplification

E. turcicum genotypes were screened for polymorphism using 19 ISSR primers which were purchased from BioDesign (Thailand). PCR amplifications were carried out according to the method described by Williams et al. (1990). The final reaction volume was 10 µL and contained 1 µL of 1 mM of each primer (Bio Design; Thailand), 5 µL of Dream Taq Green PCR Master Mix (2X) (Thermo Scientific; Singapore), 2 µL of RNase-free water and 2 µL (approximately 10 ng) of template DNA. Distilled water served as a negative control to verify the absence of contamination. DNA amplification was conducted on a programmable thermal cycler (Hybaid; USA).

Analysis of the amplification products

Each of the DNA bands of electrophoretic pattern was a molecular marker. All the DNA fragments amplified by one primer were arranged in molecular size. The banding profiles generated by each primer were recorded as binary data ("1" or "0"). The dendrograms were generated using the unweighted pair group average method based on the average similarity indices between isolates according to the SAHN program of the NTSYS-pc package 2.0 (Exeter Software, USA) (Rohlf, 1993).

Cloning and sequencing of inter-simple sequence repeat fragments

The ISSR marker screening revealed 11 out of 19 primers produced the best PCR amplicons for a set of 14 isolates of *E. turcicum*. Among them, the $(ACC)_5$ primer clearly discriminated between target and non-target species based on their allele patterns at the loci that amplified a 4-typical fragment set only from isolates of *E. turcicum.* The four amplicons of *E. turcicum* amplified by the ISSR primer $(ACC)_5$ were further developed for a single species-specific molecular marker. The amplicons were purified from the gel using a QIAquick Gel Extraction Kit (QIAGEN; Germany), ligated into the pGEM-T easy vector (Promega Corporation; USA) and incubated overnight at 4°C. Ligated DNA was transformed using *Escherichia coli* DH5*α* cells. The recombinant plasmids were identified as white colonies on Luria Bertani (LB) plates supplemented with X-gal and IPTG (Isopropyl-β-D thiogalactopyranoside). The white colonies of *E. coli* DH5*α* containing specific PCR product in pGEM-T plasmid were grown in 2 mL LB medium containing 100 µg/mL ampicillin. Plasmid DNA was isolated using a Wizard Plus plasmid DNA purification kit (Promega Corporation; USA) according to the manufacturer's recommendations. The clones were sequenced in both forward and reverse directions using $(ACC)_5$. Sequencing was done at Macrogen Inc., Korea.

Primer design, polymerase chain reaction optimization and specificity and sensitivity of primer set

Oligonucleotide primers were designed based on the obtained sequence data of cloned ISSR fragments using the Primer 3 software (S. Rozen and H. J. Skaletsky; Whitehead Institute for Biomedical Research; USA) and tested for optimal PCR conditions. The PCR containing species-specific primers was carried out in a 20 µL reaction mixture, including 2 µL of 1 mM of each primer (Bio Design; Thailand), 10 µL of Dream Taq Green PCR Master Mix (2X) (Thermo Scientific; USA), 2 µL of RNase-free water and 4 µL (approximately 10 ng) of template DNA. The master mix prepared and modified by adding bovine serum albumin (BSA) at a final concentration of 0.1 mg/mL DNA template was obtained from pure culture and infected plant samples. The amplification reaction was performed with a program consisting of pre-denaturing at 94°C for 3 min, 40 cycles (94°C for 1 min, 62.5°C for 1 min and 72°C for 2 min) and a final extension at 72°C for 5 min. The PCR products were electrophoresed on 1.6% agarose gels and stained with ethidium bromide before visualizing under UV light.

The specificity of the PCR primer pairs was determined individually by using genomic DNA of 241 *E. turcicum* isolates from corn and other fungal pathogens closely related with *E. turcicum*, including *E. rostratum*, *B. maydis*, *H. carbonum*, *P. sorghi*, *Fusarium* sp. and *M. phaseoli.*

To test the sensitivity of the primers designed on the limit of detection of the assay, 10-fold serial dilutions of purified *E. turcicum* DNA were prepared in TE buffer concentration in the range 1–100 ng. The ability of the primer set to direct the amplification of the target DNA fragment from decreasing concentrations of DNA was considered as the reliable assay for the sensitivity of the PCR-designed primers.

Detection of target pathogen from infected plant based on inter-simple sequence repeat-derived specific primer product

Random collection was made of infected corn samples displaying NCLB lesions obtained from artificial inoculation in the greenhouse and natural infection in the fields. Plant materials without NCLB symptoms (healthy leaves) served as the control. Infected leaves (at 4 d, 7 d and 10 d after inoculation) were removed, kept in plastic bags and stored at 4°C for DNA isolation. The DNA of *E. turcicum* was directly extracted from the infected leaf samples using a Soil DNA Isolation Mini Kit (Favorgen®; Biotech Corp.; Taiwan) according to the manufacturer's protocol. The specificity and detection of each of the designed primer sets for *E. turcicum* was tested individually using genomic DNA extracted from infected leaves and PCR assays, as previously described. The sensitivity of specific primers in the leaf assay was determined using a piece of 1x1 cm leaf lesion for each sample (Park et al., 2004) and the target sequence of *E. turcicum* was detected from undiluted DNA extracts of this lesion size.

Dot blot hybridization assay

The DNA dot-blot confirmed the DNA region of *E. turcicum* rather than protein due to the fact that genomic DNA was used for hybridization in this experiment. Oligonucleotide probes were designed from the same ISSR specific primer using the aforementioned methods. The PCR products (100 ng) corresponding to specific markers were labeled as a probe with digoxigenin using a DIG-High Prime DNA Labeling Kit (Roche; Germany), following the manufacturer's instructions. A sample (100 ng) of DNA isolated from each of the 50 analyzed *E. turcicum* isolates and other fungal pathogens of corn (*E. rostratum*, *B. maydis*, *H. carbonum*, *P. soghi*, *Fusarium* sp. and *M. phaseoli*) were spotted on a nylon membrane, along with standard DNA (positive controls). These were fix to DNA on the membrane to bind the labeled probe DNA. Hybridization with the labeled probes was carried out for less than 1 hr at 65°C, with the washing and detection steps according to the DIG system recommendations (Roche, Germany).

Results

Fungal isolates

The morphological and cultural characteristics of 241 isolates of *E. turcicum* were observed at 12 d after incubation on PDA medium at 25°C. Based on the colony color and growth on PDA, the results showed that almost all of the *E. turcicum* isolates had good-excellent growth, with colony diameters in the range 64–86 mm. The colony color varied being whitish, grayish, yellowish brown, olivaceous brown, grayish green, slightly light brown, or dark brown (Fig. 1).

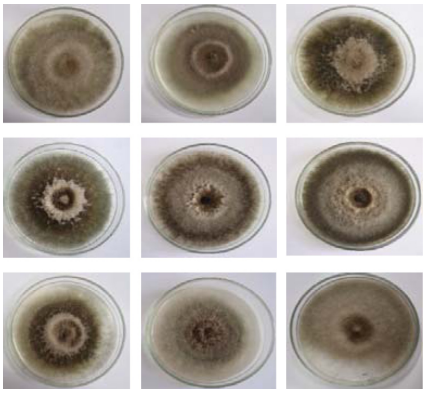

Fig. 1 Colony color variation of *Exserohilum turcicum* on potato dextrose agar medium after incubation for 12 d

Inter-simple sequence repeat fingerprints

There were 11 primers that produced the best PCR amplifications for 14 isolates of *E. turcicum*. The number of bands varied in the range 2–10, with sizes in the range 200–1,000 bp. They were separated into three main clusters by 93%, 88% and 84% similarity, respectively. The distribution of the three clusters showed a lesser degree of aggressive correlation, which was relative to neither geographic origin nor mating type. None of the 11 primers used provided clear genotypic characterization of the 241 *E. turcicum* isolates in this study (data not shown). Among them, one primer, the $(ACC)_5$- PCR fragments, from 29 isolates representing *E. turcicum* in the range 400–700 bp that exhibited 11 polymorphic bands, was clearly discriminated against the other related species (Fig. 2).

All *E. turcicum* isolates were similar with specific $(ACC)_5$-type bands in four- different combinations. This $(ACC)_5$ pattern for *E. turcicum* became an excellent phylogenetic marker for resolving interspecies relationships within the closely related species (Fig. 3). The results obtained led to the construction of a single specific primer for species identification and detection of the pathogen causing NCLB disease.

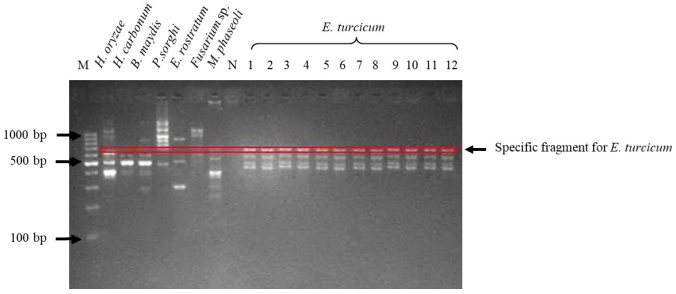

Fig. 3 Agarose gel electrophoresis of polymerase chain reaction-amplified products of *Exserohilum turcicum* using inter-simple sequence repeat $(ACC)_5$ primer, where lanes 1–12 = *E. turcicum* isolates representing four combination bands, lanes M and N = 100 bp DNA ladder and negative control (nuclease-free sterile water) respectively, with six lanes between M and N being fungal-related species

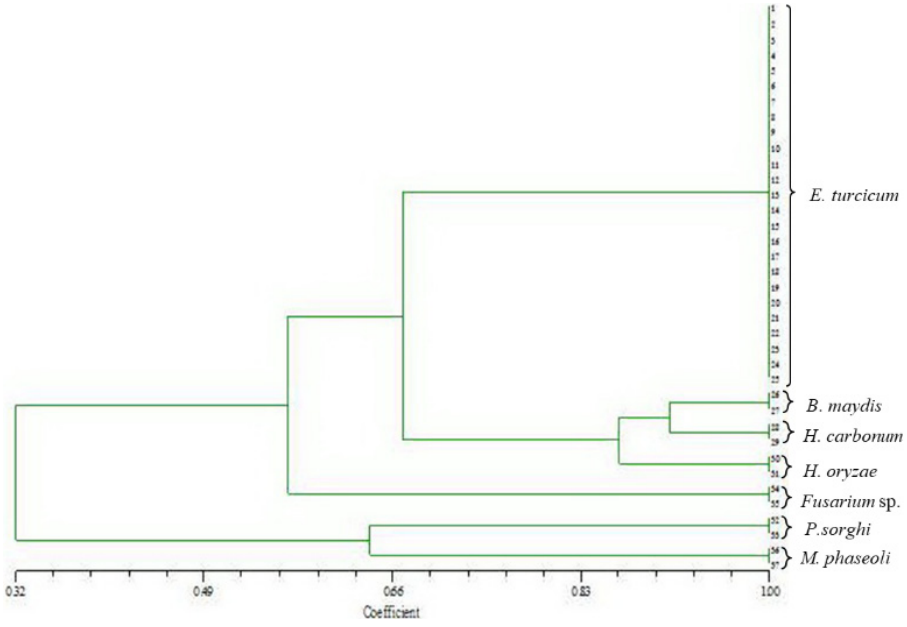

Fig. 2 Unweighted pair-group method with arithmetic average dendrogram of relationships of 24 isolates of *Exserohilum turcicum* and related species based on $(ACC)_5$ molecular marker from inter-simple sequence repeat-polymerase chain reaction analysis.

Cloning, sequencing of inter-simple sequence repeat fragments and primer design

Two of the four fragments considered to be specific for *E. turcicum* (Fig. 2) were cut out from the gel and re-amplified using the ISSR primer $(ACC)_5$. One specific fragment (680 bp) was chosen and cloned to pGEM-T Easy Vector (Promega; Germany). The cloned ISSR-specific fragments were sequenced using the $(ACC)_5$ primer. The sequencing results showed that the cloned ISSR fragments had the correct size (630 bp). This fragment showed 100% similarity with partial mRNA sequences for *Setosphaeria turcica* hypothetical protein (Et28A strain; CP054645.1). Based on the sequence information derived from the cloned ISSR bands, 18-mer oligonucleotide primer pairs were designed as SEF 5'-CCACGCACCATCCCAAGA-3' and SER 5'-ACCGCTAT CAGGTGTACA-3' (Fig. 4). Then, these primers were named as SEF/SER-Et41 and synthesized for PCR specificity assay.

PCR optimization and testing for specificity

To check the specificity of the designed primers, a large collection of other fungal isolates occurring on corn and rice was tested in PCR assay with the designed primers SEF and SER-Et41. These primers amplified the PCR product of 348 bp with DNA from the 241 *E. turcicum* isolates tested. The PCR condition that was specific for these primers was performed at 94°C for 3 min, 40 cycles (94°C for 1 min, 62.5°C for 1 min and 72°C for 2 min) and a final extension at 72°C for 5 min. No amplification occurred when the specific primers were tested with other fungal-related pathogens (*E. rostratum*, *B. maydis*, *H. carbonum*, *P. soghi*, *Fusarium* sp. and *M. phaseoli*), as shown in Fig. 5. These results indicated that the SEF/SER-Et41 primers designed were specific to *E. turcicum*.

Detection of $(ACC)_5$ derived specific primer product from infected plants

The detection of *E. turcicum* in artificially inoculated and naturally infected corn leaves was evaluated to test the specificity of the SEF/SER-Et41 primers. When DNA extracted directly from infected corn leaves was tested using the conventional PCR set of specific SEF/SER-Et41 primers, a 348-bp PCR amplification product specific to *E. turcicum* was detected on the infected corn leaf DNA for every NCLB randomly sampled from the field and greenhouse. No amplification occurred when the specific primers were tested with uninfected corn leaves (Fig. 6).

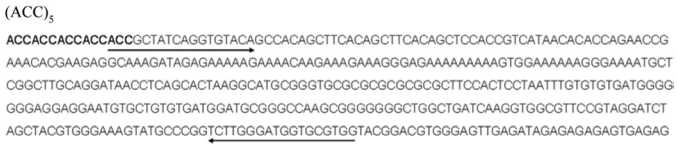

Fig. 4 Sequence of 630 bp obtained from specific fragment amplified by inter-simple sequence repeat $(ACC)_5$ primer, where sequence was used in development of specific marker linked to *Exserohilum turcicum* and $(ACC)_5$ primer sequence indicated in bold and positions of specific primers (SEF/SER-Et41) are underlined

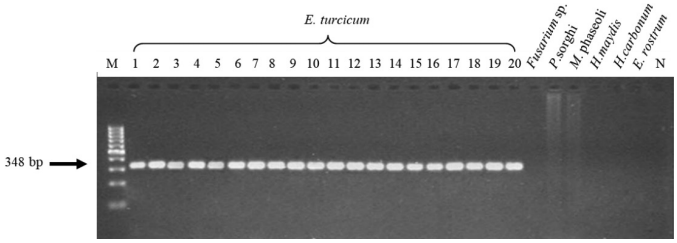

Fig. 5 Agarose gel electrophoresis of polymerase chain reaction-amplified products using *Exserohilum turcicum*-specific primers SEF/SER-Et41, where lanes 1–20 = *E. turcicum* isolates, lanes 21–26 = other fungal pathogens isolated from corn and rice, lane N = negative control and lane M = 100 bp DNA ladder

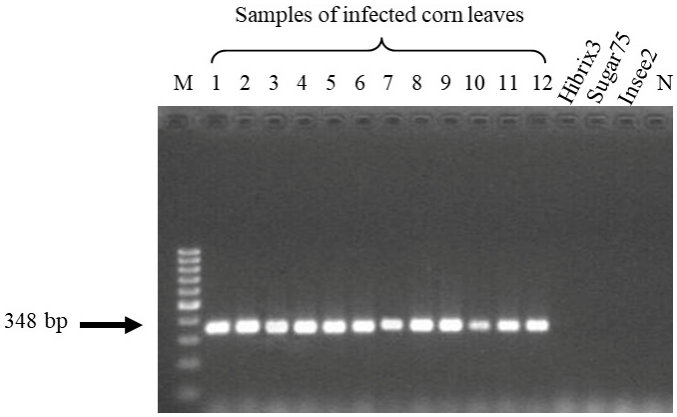

Fig. 6 Agarose gel electrophoresis of polymerase chain reaction-amplified products using *Exserohilum turcicum*-specific primers SEF/SER-Et41, where lanes 1–12 = infected leaves with different isolate of *E. turcicum*, lanes 13–15 = leaf samples of three sweet corn cultivars with no northern corn leaf blight symptoms, lane N = negative control and lane M = 100 bp DNA ladder

In the sensitivity testing based on serial dilutions of *E. turcicum* total genomic DNA, the lowest concentration of total genomic DNA from which the target fragment of 348 bp was amplified for a specific primer set (the limit of detection) was 2 ng in lane b (Fig. 7). In addition, gels in lanes 7 and 8 exhibited faint bands of fungal DNA at 1 ng to 100 pg/mL, respectively.

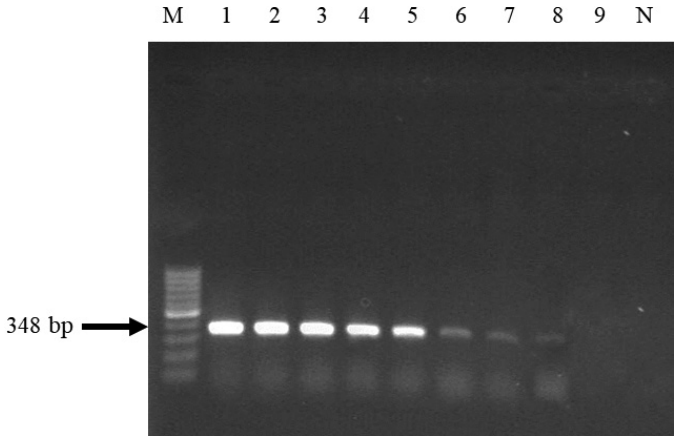

Fig. 7 Sensitivity of polymerase chain reaction (PCR) with primers SEF/SER-Et41 using different concentrations of *Exserohilum turcicum* DNA, where lanes 1–9 = DNA of *E. turcicum* isolate 42 at concentrations of 50 ng/mL, 30 ng/mL, 20 ng/mL, 10 ng/mL, 5 ng/mL, 2 ng/mL and 1 ng/mL and at 100 pg/mL and 10 pg/mL, respectively, in 20 µL PCR reaction, lane N = negative control and lane M = 100 bp DNA ladder

Specificity of dot blot hybridization

Analysis of the DNA-dot blot was used to confirm the specificity of the SEF/SER-Et41 gene region present in *E. turcicum* isolate No.41. The SEF/SER-Et41 probe hybridized to only *E. turcicum* isolates, with a high hybridized signal but did not hybridize to other non-targeting fungi of the 6 species tested (Fig. 8). This demonstrated that the genomic DNA of the NCLB pathogen harbored an SEF/SER-Et41 region of the specific gene that did not exist or share homology with any other fungi that were tested.

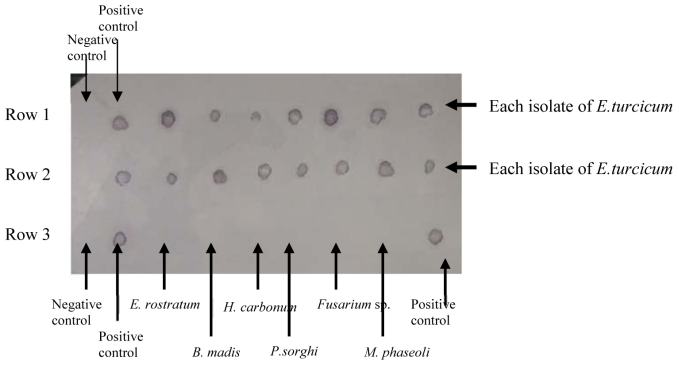

Fig. 8 Dot blot of SEF/SER-Et41 probe with DNA sample from *Exserohilum turcicum* starting from left in column 3 of row 1 and row 2, where in all 3 rows, columns 1 and 2 present negative and positive controls, respectively, DNA samples from *E. rostratum*, *B. maydis*, *H. carbonum*, *P. soghi*, *Fusarium* sp. and *M. phaseoli* are against positive control, respectively, starting in column 3 of row 3

Discussion

The greatest problem in identification was the limited identifying morphology of *E. turcicum*. However, *E. turcicum* is described, illustrated and compared with other *Exserohilum* species for similar conidial morphology. The conidia of *E. turcicum* were similar to other graminicolous species (*Bipolaris*, *Curvularia*, *Drechslera*, *Helminthosporium* and another *Exserohilum* spp.). Molecular biological techniques have been developed for some time to improve the sensitivity and specificity detection of pathogenic fungi. Increasingly, molecular methods have been used to explore genetic variability in fungi (Caligiorne et al., 1999). PCR is the most sensitive and specific technique for detecting a specific DNA sequence, with species-specific PCR-diagnosis methods having been applied to plant pathogens. The ITS regions have been used successfully to generate specific primers capable of differentiating closely related fungal species (Bryan et al., 1995). The ITS region remains an important locus for molecular identification of fungi. However, more sequence data are being collected from a wider range of fungal isolates. A design strategy of DNA barcodes specific for fungal detection generally uses genomic data in GenBank and public databases to identify a particular sequence common to multiple isolates or races of the target fungi but that is absent from other sequences of related species (Xue et al., 2013). PCR primer pairs based on those sequences have been designed and tested empirically using numerous target and non- target fungal species. The spectrum of variability in the phylogenetic relationships among related species should provide optimal rDNA sequences to develop and validate a PCR method for the specific detection of the target fungus.

The PCR-based tools to identify *E. turcicum* isolates have been developed. For example, Haasbroek et al. (2014) used the mating type primers (MAT) designed from this fungal genome sequences to flank microsatellite regions (SSR marker, SSR27), that was specific to *E. turcicum* and could differentiate the target species from *E. rostratum*. The ITS1 regions designed from the *E. turcicum* genome have been reported for detection of this particular fungus (Chung et al., 2010). Such primers of the ITS1 portion yielded the target fragment of 170 bp and demonstrated that the 241 *E. turcicum* Thai isolates collected around the main growing areas of the country resided in the same clade within other fungi on corn that were not clearly separated from those of 6 related species (*E. rostratum*, *B. maydis*, *H. carbonum*, *P. soghi*, *Fusarium* sp. and *M. phaseoli*).

As such, species-specific primers for detecting and identifying these Thai isolates need to be developed to provide insight into the population biology of NCLB pathogen that might lead to improvements in disease management.

ISSR molecular markers were used in the current study to search for suitable band fragments that could provide a better understanding of the genetic structure of all the Thai isolates tested. The ISSR markers were selected because they were species- or gene-specific, highly polymorphic, easy to amplify using PCR and worked well with a small amount of DNA. Furthermore, the ISSR technique does not require the process of enzymatic digestion and ligation. Hence, the developed ISSR procedure has a number of advantages over other molecular techniques such as RAPD or AFLP (Abadio et al., 2012). Phylogenetic analysis using 11 out of the 19 screened ISSR primers separated all *E. turcicum* isolates into three main groups that revealed genetic diversity with poor characterization of morphological typing (data not shown). All 241 isolates of *E. turcicum* in the three distinct groups demonstrated less correlation with aggressive phenotypes and did not correlate with mating type or geographic area from where they had been collected. However, one primer $(ACC)_5$ out of the 11 ISSR markers generated non-polymorphic bands among *E. turcicum* isolates that provided a unique region set for the target fungus compared to other pathogenic species. PCR amplification with the template $(ACC)_5$ primer resulted in the production of a set of four- single-specific sequence repeating markers from all 241 *E. turcicum* isolates in the range 400–700 bp. Primer $(ACC)_5$ of the universal-ISSR molecular markers showed the same fingerprint pattern in the target but different sized fragments in non-target fungi that produced a degree of interspecific similarity differentiation among the species. These four size of DNA fragments provided good phylogenetic discrimination; hence, they were suitable candidates for a single-designed primer set specific for the detection of *E. turcicum* in the fungal species complex associated with the corn plant. These 4-type bands found in the NCLB pathogen may have been due to either polymorphism within the rDNA repetitive unit or the presence in the same isolate of more than one nucleus bearing different rDNA sequences (Sanders et al., 1996). These four fragments were developed to amplify gene specific for *E. turcicum*. The sequencing of the four-cloned fragments confirmed that the profile generated was from *E. turcicum*. Among them, one of obvious target sequences of 700 bp provided the greatest genetic discrimination that could be developed as a species-specific primer for the target fungus. A PCR product of 348 bp from the polymorphism band of 700 bp was produced in reaction with the *E. turcicum* (SEF/SER Et41) primer set without yielding this DNA-sized product from other fungi. A sensitive and rapid PCR assay using the SEF/SER-Et41 primer set was successfully achieved for species-specific detection of *E. turcicum* in pure culture complexes of different species and in corn plants. The SEF/SER Et41 primer developed in the current study showed 100% identity with a part region of the gene encoding a hypothetical protein (*BcHP*) in *Setosphaeria turcica* Et 28A strain (NCBI Blast). It was induced in a multiple gene phylogeny that provided good genetic discrimination for *E. turcicum* in the fungal species complex. It is believed that none of these gene regions have been examined in a large number of *E. turcicum*. The *BcHP* gene was identified to be associated with virulence of *Botrytis cinerea*, causing gray mold disease of several plant species, including tomato (DafaAlla et al., 2017). However, there has been investigation to date on whether the aggressiveness of the *E. turcicum* Thai isolates is associated with the *BcHP* gene that may link to the phenotype of either lesion number or lesion size of NCLB disease. Genes involved in virulence represent a small fraction of the genes in the pathogen genome and may be subjected to strong selection by the host (Pfennig, 2001).

Use of the species-specific primer SEF/SER-Et41 and PCR protocols developed in this article is sufficient for sensitive and accurate detection of *E. turcicum*. The detection system presented for the target fungus consists of a combination of techniques in PCR-and DNA-based assays to optimize the extraction and amplification protocols and allow for reliable detection of this fungus from infected corn leaves. The PCR amplification of the target *E. turcicum* DNA from the total DNA extracted from corn leaves was consistent in the current study because the recommended BSA (an additive for enhancing the PCR-amplified yield and reducing PCR inhibitions released from corn leaves) was used. This is a common problem, particularly when fungal DNA is directly extracted from plant materials (Farell and Alexandre, 2012). It is necessary to optimize the elimination assay of those PCR inhibitors for quantifying and qualifying the target DNA during the extraction process. Other assays designed for the detection of fungi in plant tissues or in complex biological samples have also successfully used BSA to reduce PCR inhibitors (Farell and Alexandre, 2012).

The specificity of the SEF/SER-Et41 primers was high under the conditions of our tests that produced single amplicons of 348 bp using genomic DNA from the target fungus. They provided rapid and direct detection which could amplify only

E. turcicum and did not yield any DNA from a wide range of related fungi. The PCR-based assay using the SEF/SER-Et41 primer set required a relatively short period (4 hr) to detect the target fungus from corn leaves compared with 2 wk for the direct plating method. Rapid detection was achieved at an early stage of infection by a causal fungus at 4 d after inoculation using the new primer set. The PCR protocols allowed the accurate and rapid detection directly from corn leaves that had been artificially inoculated or naturally infected by *E. turcicum*, demonstrating that the specific primers were sufficient and could be applied to infected plant tissues, which eliminated time-consuming morphological comparisons.

Other DNA-based methods for at least two PCR tests for pathogen detection would be essential for ordinal levels to avoid misdiagnosis and confirm positive results. The current study incorporated dot blot assay with the PCR-based method targeting the same designed primers (SEF/SER-Et41) as a hybridized probe developed for *E. turcicum* detection and identification. The DNA hybridization is considered to be convenient and can be effectively applied for reliable and sensitive detection and identification of plant pathogens (Tsui et al., 2011). However, dot blot assay may not be convenient enough when a large number of samples of related fungal species must be considered (Lévesque et al., 1998). The representative *E. turcicum* isolates all showed a high hybridized signal of 348 bp with no cross reaction with other related fungi. The dot blot hybridization using the DNA probe developed from the same specific primer pairs was an effective supplement for the detection of *E. turcicum*, with 100% agreement between the primers (SEF/SER-Et41) and the probe dot blot (SEF/SER-E41). Utilizing these two assays with the same portion of the *BcHP* gene achieved excellent specificity for *E. turcicum* that generated positive results with the cloned *BcHP* gene regions from all 14 *E. turcicum* isolates tested

The current study demonstrated that the SEF/SER-Et41-PCR and dot blot were a suitable combination for detecting *E. turcicum* and providing accurate, specific and rapid steps for the diagnosis of the target fungus. This study was the first to use the SEF/SER-Et41 marker from the *BcHP* gene regions to assess the detection and identification of 241 *E. turcicum* Thai isolates from mixed culture and corn plants.

The method allows for the early detection of *E. turcicum* and can effectively distinguish gray speck disease, round spot and leaf blight on corn. The ITS region is most important aspect in designing a primer. Sometimes, ITS primers bind to a non-target sequence and amplify it, which results in variable results. Therefore, it is important to select more than one primer pair for detection. This primer was highly specific in only binding to *E. turcicum* species and did not show affinity with other fungal pathogen on corn. A detection system developed for *E. turcicum* based on the techniques developed in the current article may be applicable in the detection of other fungal pathogens in infested plant tissues once the right primers have been developed.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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