Evolution of the *ToxB* gene in *Pyrenophora tritici-repentis* and related species.

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Abstract

Pyrenophora tritici-repentis (tan spot) is a destructive foliar pathogen of wheat with global impact. This ascomycete fungus possesses a highly plastic open pangenome shaped by the gain and loss of effector genes. This study investigated the allelic variations in the chlorosis-encoding gene, *ToxB*, across 422 isolates representing all identified pathotypes and worldwide origins. To gain better insights into *ToxB* evolution, we examined its presence and variability in other *Pyrenophora* spp. A *ToxB* haplotype network was constructed, revealing the evolutionary relationships of this gene (20 haplotypes) across four *Pyrenophora* species. Notably, *toxb*, the homolog of *ToxB*, was detected for the first time in the barley pathogen *Pyrenophora teres*. The *ToxB*/*toxb* genes display evidence of selection that is characterized by loss of function, duplication, and diverse mutations. Among *ToxB*/*toxb* open reading frame, 72 mutations were identified, including 14 synonymous, 55 nonsynonymous, and 3 indel mutations. Remarkably, a ~5.6 Kb Copia-like retrotransposon, named Copia-1_Ptr, was found inserted in the *toxb* gene of a race 3 isolate. This insert disrupted the *ToxB* gene's function, a first case of effector gene disruption by a transposable element in *P. tritici-repentis*. Additionally, a microsatellite with 25-nucleotide repeats (0 to 10) in the upstream region of *ToxB* suggested a potential mechanism influencing *ToxB* expression and regulation. Exploring ToxB-like protein distribution in other Ascomycetes revealed their presence in 19 additional species, including the Leotiomycetes class for the first time. The presence/absence pattern of ToxB-like proteins defied species relatedness compared to a phylogenetic tree, suggesting a past horizontal gene transfer event during the evolution of *ToxB* gene.

Introduction

The fungus *P. tritici-repentis*, which causes tan spot, a destructive foliar disease of wheat worldwide, has often been used as a model system to study new disease emergence and necrotrophic effector (NE) evolution. *P. tritici-repentis* secretes several NEs, and its ability to infect a given wheat genotype is directly linked to the production of these effectors, which interact in a specific manner with matching sensitivity genes in the host. So far, three NEs have been identified in *P. tritici-repentis*; ToxA is the necrosis inducing effector while ToxB and ToxC are the chlorosis-inducing effectors. Eight different 'races' of *P. tritici-repentis* have been identified based on their ability to produce different combinations of these three effectors, and were designated as race 1 to 8 (Lamari et al. 2003). Races 2, 3, and 5 produce one effector each (ToxA, ToxC, and ToxB, respectively). Races 1, 6, and 7 produce combinations of two effectors each (ToxA and ToxC; ToxB and ToxC; ToxA and ToxB, respectively). Race 8 produces all three effectors, while race 4 is incapable of producing any of these effectors (Lamari et al. 2003). Additional effectors have been indicated by several researchers and are awaiting further identification (reviewed in Aboukhaddour et al. 2021; Gourlie et al. 2022).

Although the exact molecular identity and coding gene for ToxC has yet to be determined, ToxA and ToxB are confirmed as two unrelated, small, secreted apoplastic proteins (Dagvadorj et al. 2022; Kim et al. 2010; Strelkov et al. 1998) and each can trigger programmed cell death in susceptible hosts (reviewed in Friesen and Faris 2021). The coding genes for both ToxA and ToxB have been known for over 20 years (reviewed in Ciuffetti et al. 2010), and the allelic variation in these coding genes have been studied by various research groups since their

identification (Friesen et al. 2006; Ghaderi et al. 2020; Hafez et al. 2020, Hafez et al. 2022; McDonald et al. 2013; Strelkov et al. 2005; Stukenbrock and McDonald 2007).

ToxA has been the subject of intensive research over the past 30 years since its identification (reviewed in Aboukhaddour et al. 2023), and in part this is due to the predominance of ToxA-producing isolates in North America and Australia where the majority of the research output on tan spot has been generated. Whereas, research on ToxB was of significant value but has been so far sporadic. Unlike the *ToxA* gene, which has a global distribution, the *ToxB* gene is currently restricted to certain geographical regions in North Africa and the Fertile Crescent and encompassing regions where wheat originated and was domesticated 10,000 years ago (Kamel et al. 2019). The *ToxB* gene appears to be absent in Australia and is extremely rare in the Americas, although its inactive homolog, *toxb*, is present in race 3 and 4 isolates in the Americas and in other *Pyrenophora*-related species (Aboukhaddour et al. 2021; Guo et al. 2020; Wei et al. 2020). ToxB is a 6.5 kDa protein encoded by the multi-copy *ToxB* gene, which has an ORF consisting of 261 nucleotides that translate into an 87 amino acid pre-protein with a 23 amino acid signal peptide (Martinez et al. 2001; Strelkov and Lamari 2003). Not much is known about the various *ToxB* haplotypes present in *P. tritici-repentis* or other species, as access to these isolates is limited and there is a lack of molecular tools to detect full *ToxB*/*toxb* coding sequences with their flanking regions. Contrary to the assumption that *ToxB* was acquired via vertical inheritance, we recently reported on the multi-copy tandem repeat of *ToxB*/*toxb* in a cluster that was embedded in a large putative Starship transposon called "Icarus" carried on a chromosome of essential nature in *P. tritici-repentis* (Gourlie et al. 2022). This

suggests an alternative or combination of mechanisms of *ToxB/toxb* acquisition including potential horizontal gene transfer (Gourlie et al. 2022).

In this study, we further explored the *ToxB*/*toxb* sequence polymorphisms from a large collection of *P. tritici-repentis* isolates and in other related species. In addition, we showed the role of transposons in disrupting the function of *ToxB* and studied the prevalence of ToxB-like proteins in Ascomycetes. Overall, we have provided novel insights into the *ToxB* gene evolution in *P. tritici-repentis* and many other related species.

Results

Distribution of *ToxB* and its homolog *toxb* in a worldwide collection of isolates

A worldwide collection of *P. tritici-repentis* isolates (422) were included in this study, and the species identity was confirmed by single spore isolation followed by sequence verification using the ITS region and *β-tub* gene (Table 1; Supplementary Table S1). The presence or absence of *ToxB* and its homolog *toxb* was confirmed using specific primers Bb-F and Bb-R (Table 1; Supplementary Table S2; Supplementary Fig. S1). Out of the 422 tested isolates, *ToxB* and *toxb* were detected in 88 (20.9%) and 25 (5.9%) isolates, respectively (Supplementary Table S3). The functional *ToxB* encoding gene was only detected in Algeria [in 7 isolates out of 14], Azerbaijan [in 7 isolates out of 8], Syria [in 1 isolate out of 3], and Tunisia [in 73 isolates out of 96]. The *toxb* gene was only present in isolates of races 3 and 4, and these races were reported in Canada [in 23 isolates out of 79], and Syria [in 2 isolates out of 3] (Supplementary Table S3). The homolog *toxb* gene was never detected in the same isolate with

ToxB gene, and was found only in race 3 (ToxC-producing) and race 4 (no known effector) isolates (Supplementary Table S2).

ToxB/toxb sequence polymorphism and haplotype network

A total of 157 *ToxB*/*toxb* sequences were analyzed for haplotype identification, including 116 obtained in this study (113 from *P. tritici-repentis* and 3 from *Pyrenophora seminiperda*) and 41 previously released sequences from *P. tritici-repentis* (Gourlie et al. 2022; Guo et al. 2020; Martinez et al. 2001; Mironenko et al. 2021; Moolhuijzen et al. 2022; Strelkov et al. 2005; Strelkov and Lamari 2003) and other *Pyrenophora* species*: Pyrenophora bromi* (Andrie et al. 2008), *Pyrenophora teres* (Wyatt and Friesen 2021), and *P. seminiperda* (Soliai et al. 2014). Four copies of *ToxB* were identified in *P. tritici-repentis* isolate I-73-1 (Supplementary Table S4). Sequence analysis of these four copies and its flanking sequences (+500 bp up and downstream) indicated that the ORF (*ToxB1*) and the flanking regions in all these copies were identical (Supplementary Fig. S2). In total, 20 *ToxB* haplotypes were identified in four Ascomycetes fungi: *P. tritici-repentis* (nine haplotypes), *P. bromi* (six haplotypes), *P. seminiperda* (three haplotypes), and *P. teres* (two haplotypes). Haplotypes that were identified in *P. seminiperda* (*toxb16-toxb18*) and *P. teres* (*toxb19* and *toxb20*), were found to be highly divergent (percentage of homology compared to the *ToxB1* ORF in Alg3-24: 72.3%, 73.8%, 68.5%, 67.8%, and 64.4%, respectively).

A total of 72 polymorphic sites were found among the different *ToxB* haplotypes in *P. tritici-repentis* (Supplementary Table S5). Transition substitutions were more common than transversion substitutions among these polymorphic sites (Supplementary Fig. S3; Supplementary Table S5; Supplementary Table S6). Translation to amino acid sequences

revealed 14 synonymous and 58 nonsynonymous mutations, including a 3-nucleotide indel mutation at positions 212-214, relative to *ToxB* start codon (Supplementary Fig. S3; Supplementary Table S5; Supplementary Table S6).

A *ToxB* haplotype network was constructed here and for the first time, to show the genetic relationships among the 20 *ToxB*/*toxb* haplotypes in four *Pyrenophora* species (Fig. 1). Nine haplotypes were identified in *P. tritici-repentis* (*ToxB1* to *ToxB5* and *toxb12* to *toxb15*), with *ToxB1* being the most prevalent (Supplementary Table S6). Six haplotypes were identified in *P. bromi* (*ToxB6* to *ToxB11*), with *toxb10* represented by three sequences and all other haplotypes represented by a single sequence. Three haplotypes, *toxb16* (one isolate), *toxb17* (one isolate) and *toxb18* (two isolates) were identified in *P. seminiperda* (Supplementary Table S6). Haplotype *toxb16* was identified using BLASTn, and haplotypes *toxb17* and *toxb18* were identified in *P. seminiperda* isolates (Supplementary Table S7) by PCR using Bb-F and Bb-R degenerate *ToxB*-specific primers. Two haplotypes, three sequences each, were identified in *P. teres* (*toxb19* and *toxb20*) that differ by a single nonsynonymous mutation. Haplotype *toxb19* was found in three *P. teres* f. *maculata*, while haplotype *toxb20* was found in two *P. teres* f. *maculata* and one *P. teres* f. *teres* sequence (Supplementary Table S6 for accession numbers). The two *P. teres* haplotypes were identified using BLASTn searches. PCR screening for the reference *P. teres* isolates (Supplementary Table S7) produced negative results.

The *ToxB*-network identified three different types of mutations: synonymous, nonsynonymous, and indel. No nonsense mutations were observed. Out of 72 polymorphic site, the prevalence of nonsynonymous mutations (76.4%) was higher than that of synonymous

mutations (19.4%). Indel mutations were found at three different positions in the network (Fig. 1; Supplementary Fig. S3). The impact of indel mutations at positions 212-214 on ToxB protein structure was investigated. Each of the insertions split the 5'-ACT-3' codon (encoding for threonine "T" at position 71) into two amino acid residues encoding for asparagine "N" and proline "P' at positions 71 and 72, respectively (Supplementary Fig. S4). The ToxB protein has a secondary structure consisting of six antiparallel beta sheets (β1-β6), and the location of these two amino acid residues was found to be in the loop between β5 and β6 sheets (Supplementary Fig. S4).

ToxB flanking sequence characterization

The BUS-F/BDS-R primer pair (Table 1) amplified a fragment ranging from 734 to 879 bp, representing *ToxB/toxb* ORFs as well as the upstream and downstream flanking regions (Supplementary Fig. S1). When specific primers for isolates encoding haplotype *toxb3* (3US-F) and haplotype *toxb12* (12US-F) were paired with BDS-R primer, PCR amplicons of 794 and 756 bp were produced, respectively. This primer set enabled the detection and the amplification of all the *ToxB*/*toxb* haplotypes that were identified in *P. tritici-repentis* to-date.

We investigated the *ToxB* flanking 5′ UTR and 3' UTR in two of the *Pyrenophora* species, *P. tritici-repentis* and *P. bromi*. The 5' UTR region in all *ToxB* haplotypes started with a conserved core promotor sequence (TATA-box: 5'-TATAAA-3'), except haplotype *toxb12* in race 3 isolates I-72-1 and I-72-7 collected from Syria (Fig. 2). The region between the TATA-box and intron contains a short microsatellite consisting of a highly conserved 25 bp. These 25 nucleotides were repeated a variable number of times (0 to 10) among different haplotypes (Fig.

2; Supplementary Table S8). An intron (52 nucleotides) was located just upstream of the *ToxB* start codon in all haplotypes, except for haplotype *ToxB5* in isolate Alg215 (sequence is truncated). The intron was identified based on comparative sequence analysis to *ToxB* cDNA from Alg3-24 (Strelkov et al. 2005) and DW7 (Martinez et al. 2004), in addition to the sequence features for nuclear/spliceosomal introns (5'-GT and AG-3'). The *ToxB*-ORF start codon was identical in all haplotypes (5'-ATG-3'). The 3' UTR region was found to be homologous in all *ToxB* haplotypes, except for haplotype *toxb13* (*P. tritici-repentis* isolate SC29-1, a Canadian race 3) and some isolates within haplotype *ToxB1* (*P. tritici-repentis* isolates I-34-5 and T181-1, races 5 and 7, respectively). The stop codon in all *ToxB*-ORFs were identical (5'-TAG-3'), except for haplotype *toxb13* (represented by one isolate, SC29-1). In this isolate, the *ToxB* ORF ended with a 5'-TGA-3' stop codon.

A Copia-like element disrupted the *ToxB* ORF

Copies of *ToxB* from the long-read assembled genomes of *P. tritici-repentis* isolates I-73-1 and D308 (Gourlie et al. 2022) were investigated further here. A comparative sequence analysis of the four *ToxB* ORF copies from I-73-1 showed that these copies were identical to *ToxB1*. Comparison to DW5 (PRJNA315205) indicated that these copies were on the anti-sense strands in the I-73-1 isolate. Alignment of a single copy from I-73-1 to the only *toxb* copy found in race 3 isolate, D308, revealed a large insertion (5,661 bp) within the ORF of D308 inactive *toxb*. Within the insertion there were three primary open reading frames. These ORFs shared sequence similarity to: DoeR (deoxyribonucleoside) transcriptional regulator, GAG (capsid protein), INT (integrase), Pol (retrovirus-related polyprotein), and RT (reverse transcriptase) domains (Fig. 3). The edges of this insertion were repetitive producing 192 bp long terminal

repeats (LTRs), and a four bp target site duplication (5'-CCTA-3') was identified in I-73-1. A search of RepBase resulted in some homology to a number of different Copia and Copia-like elements. These features point to the insertion of a Copia-like retrotransposon disrupting the coding sequence of *ToxB*. Although many Copia and Copia-like elements have been identified in *P. tritici-repentis* (Manning et al. 2013; Gourlie et al. 2022), but to our knowledge none have been identified in the ORF of an effector gene in this pathogen. Following the convention of RepBase, we are naming this element Copia-1_Ptr (accession number OR553420). A search of GenBank revealed hits (>60% identity over <27% query) in *P. teres* f. *teres* (GCA_900232045.3) and *P. teres* f. *maculata* (GCA_014334815.1; GCA_14334795.1), as well as an LTR retrotransposon in *P. nodorum* (AJ277966.1) which was not present in our version of RepBase.

ToxB chlorosis bioassay

A representative single spore isolate of *P. tritici-repentis* from each *ToxB* haplotype was selected to test its ability to induce chlorosis symptoms on the wheat genotype 6B662, which is known to be sensitive to ToxB but insensitive to ToxA and ToxC. The *ToxB1* encoding isolate, Alg3-24, produced small dark penetration sites surrounded by chlorosis. Lesions coalesced and most of the infected area turned chlorotic 5 to 7 days after inoculation, isolates that produced similar symptoms were given a score of 5 (extensive chlorosis). Both 92-171-R5 (*ToxB4*) and Alg215 (*ToxB5*) isolates produced small dark sites surrounded by a chlorotic region and Alg215 was the only isolate tested for infection/symptoms in Australia. For these two isolates (92-171-R5 and Alg215) the lesions have not expand or coalesce, and were given a score of 3 (weak

chlorosis). Isolates coding for the *toxb* haplotypes produced no visible chlorosis and were given a score of 1 (non-chlorotic) (Fig. 4).

Identification of ToxB-like proteins in the Ascomycete classes

Sequence similarity searches of fungal genomes identified ToxB protein homologs in three classes of Ascomycetes: Dothideomycetes, Sordariomycetes, and Leotiomycetes (Supplementary Table S9). Previously ToxB-like proteins were identified in Dothideomycetes and Sordariomycetes only (Andrie et al. 2008; Nyarko et al. 2014). Species reported for the first time to carry ToxB-like proteins in this study include: *Bipolaris oryzae*; *Colletotrichum* spp. (C. *karsti, C. nymphaeae , C. orbiculare, C. salicis, C. scovillei, C. scovillei, C. sidae, C. simmondsii, C. spinosum, C. trifolii*); *Diplodia corticola*; *Diaporthe* spp. (*D. batatas, D. citri*); *Lasiodiplodia theobromae*; *Leptosphaeria biglobosa*; *Pyricularia pennisetigena*; and *P. seminiperda* (Supplementary Table S9). In addition, a ToxB-like protein is reported here in the Leotiomycetes species, *Hyaloscypha bicolor*, for the first time. The homology to full length ToxB1 protein (Alg3-24) ranges between 100% (*P. tritici-repentis*) to 31.4% (*C. spinosum*).

Analysis using ConSurf, a tool for estimating the evolutionary conservation of amino acid positions in proteins, showed sequence and structural similarities among all ToxB-like proteins. The position of four cysteine residues (which form disulfide bonds) are highly conserved among all sequences (Fig. 5A). Residues in regions corresponding to β1, β2, and β5 are more highly conserved than in the other β-strands (Fig. 5B). Conservation is highest at the end of β1 (3 identical residues), and the end of β2 (2 identical residues), and highly variable at β3-β4 and β5-β6 loops (Fig. 5). Amino acid residues are more homologous in the β1-β6 regions,

with all ToxB-like proteins showed ≥50% sequence similarity to ToxB1 (except for *L. biglobosa*, 46.8%). Percentage identity for all investigated ToxB-like proteins (full length and β1-β6 regions) is indicated in Supplementary Table S9. A mirror-tree for ToxB-like proteins and ITS sequences for all ascomycete species identified in this study was generated to study their co-evolution (Supplementary Fig. S5; Supplementary Table S10). The tree showed that, ToxB-like sequences are not well correlated with the species evolutionary relationships. This inconsistent grouping pattern indicates a possible horizontal movement for *ToxB*/*toxb* between different Ascomycete species.

Discussion

This study provides one of the most comprehensive insights into the variability of the *ToxB* gene and its homolog *toxb* from a worldwide isolate collection spanning North and South America, Europe, North Africa, the Fertile Crescent, Japan, and Australia. In contrast to *ToxA*, that has been investigated in depth by many research groups, not much was known about genetic variability of the *ToxB* coding sequence and its flanking regions in *P. tritici-repentis* or other species. This is because access to *ToxB*/*toxb* encoding isolates is limited and there is lack of suitable molecular tools. Our results indicated the presence of one predominant active haplotype in *P. tritici-repentis*, *ToxB1*, but also showed the extensive polymorphism in the *toxb* haplotypes and showed the presence of inactive *toxb* in the barley pathogen *P. teres* for the first time.

Moreover, comparative analysis of the *ToxB* gene in fully sequenced genomes revealed the presence of a Copia-like retrotransposon element inserted in the *toxb* ORF in D308, a race 3 isolate of *P. tritici-repentis*. This confirms the role of transposons in *ToxB* evolution in this

species as this insertion interrupts the coding sequence of *ToxB*, and caused the absence of ToxB chlorosis related symptoms when inoculating with D308, as confirmed in the bioassay in this study and in previous work (Strelkov et al. 2005; Aboukhaddour et al. 2013) Additionally, comparative genomics of the *ToxB*/*toxb* sequences revealed the presence of microsatellite upstream to the coding sequence and also showed variability in the promoter region which likely play a role in the *ToxB* regulation and expression. The study also expanded on the evolution of the ToxB effector, and ToxB-like proteins in Ascomycetes, and reported the presence of these homolog proteins in an additional 19 species in the Dothidiomycetes and Sordariomycetes classes and its identification in the Leotiomycetes class for the first time. The presence of these ToxB-like proteins across several ascomycete species independent of these species evolutionary relatedness, suggests a possible role for horizontal movement of *ToxB*/*toxb*.

ToxB sequence polymorphism and its predicted evolution

ToxB, the second necrotrophic effector identified in *P. tritici-repentis*, has been largely restricted to regions of North Africa, Fertile Crescent and the Caucasus. It is rarely found in North America and has never been reported in Australia. The inactive homolog (*toxb*) is present in races 3 and 4 isolates in North America and other places (Ciuffetti, et al. 2010; Kim and Strelkov 2007; Strelkov et al. 2005). There is a lack of information about *P. tritici-repentis* effectors from Europe, as tan spot is not known for its damage there. Most research on *ToxB* has been concentrated in a few research groups, mainly in Canada and in the United States and on a limited number of isolates. In this study, we utilized a unique collection of isolates that have *ToxB* and its homolog *toxb*, offering a more in-depth investigation on the diversity in *ToxB/toxb* in *P. tritici-repentis* and in other species. We reported the presence of 20 different *ToxB*/*toxb*

haplotypes among four different *Pyrenophora* species, with the majority lacking chlorosis activity on wheat (denoted small *toxb*). In *P. tritici-repentis*, only one haplotype (*ToxB1*) was associated with a strong chlorosis activity, and two haplotypes (*ToxB4* and *ToxB5*) were associated with a weak chlorosis. *ToxB1* is the predominant haplotype and was detected in 76.6% of tested *ToxB*-coding *P. tritici-repentis* isolates. It appears that *ToxB/toxb* has undergone selection that is characterized by loss of function, duplication, and different types of mutation (synonymous, nonsynonymous, and indel mutations). While *ToxB5* is one point mutation away from *ToxB1*, the puzzling situation of *ToxB4* remains to be explored, as it is distantly positioned away from *ToxB1* and *ToxB5* and is more related to the *toxb* haplotypes found in other grass species in the network. *ToxB1* and *ToxB5* are both encoded by isolates collected from Algeria, whereas, *ToxB4* is encoded by 92-171-R5, the only race 5 isolate found in Canada. *ToxB1*, may be under positive selection pressure in chlorosis inducing *P. tritici-repentis* which may explain its predominance in chlorosis inducing isolates from various regions.

Comparison of the long-read contigs containing *ToxB* and *toxb* in I-73-1 and D308 isolates respectively, revealed the insertion of a Copia-like element in the D308 ORF of this virulence gene. Previous work with theses isolates had showed Class I LTR retrotransposons comprise ~11% of the *P. tritici-repentis* genome, with Copia elements specifically representing ~5.5% of the genome (Gourlie et al. 2022). Previously, in an effort to explain the inactive nature of *toxb* in D308, Strelkov et al. (2005) hypothesized an alternative start codon which results in a 318 bp ORF. Based on our ORF prediction, the insertion of Copia-1_Ptr produces a new start codon creating an ORF precisely matching this previous prediction. Others have also noted the remnants of Copia and Gypsy elements around *ToxB* (Manning et al. 2013). It is now well

established that transposons play a role in virulence evolution, but may often be a 'double-edged sword'. For example, gains of virulence have been noted in at least two pathogens *Fusarium oxysporum* f. *lycopersici* and *Magnaporthe oryzae* via disruption of avirulence genes and the subsequent inability of the hosts to recognize these pathogens (Inami et al. 2012; Wu et al. 2015). Additionally, *P. tritici-repentis* has acquired *ToxA* through the horizontal movement of a hAT transposon (Friesen et al. 2006; McDonaled et al. 2018; McDonaled et al. 2019) and recently we reported the movement of *ToxA* and its hAT transpson via a large Starship (Gourlie et al., 2022). Here we have provided a concrete example of how transposons may negatively impact a pathogen through the disruption of another important virulence gene, the *ToxB* gene.

Variability and lack of chlorosis activity

Previous studies have shown that the variation in *ToxB*-copy number among various *P. tritici-repentis* isolates may underline changes in virulence (Aboukhaddour et al. 2012; Amaike et al. 2008; Strelkov et al. 2005). The silencing of *ToxB* in virulent race 5 isolate, Alg3-24, have resulted in a reduction in the ability of the mutants to induce chlorosis, this reduction was proportional to the reduced quantity of ToxB protein expressed (Aboukhaddour et al. 2012). This reduction was also correlated with a reduction in the pathogen's ability to form appressoria, suggesting that *ToxB* or its homologs may play a role in the pathogen's fitness in addition to its virulence dosage (Aboukhaddour et al. 2012). The future release of full genomes with long read sequences for *ToxB*-coding isolates will hopefully highlight important aspects of *ToxB* evolution as a multi-copy virulence gene. On the other hand, the lack of chlorosis activity in *toxb*-coding isolates can be explained by enough mutations in the coding gene to significantly alter the translated protein, such the case of *P. tritici-repentis* race 4 isolate 90-2. Comparison of the

ToxB1 haplotype sequence from Alg3-24 and *toxb2* from 90-2 showed the presence of 15 amino acid substitutions and one insertion in the predicted protein product from 90-2 (Strelkov and Lamari 2003). When the *toxb* gene from 90-2 was heterologously overexpressed, it induced only trace amounts of chlorosis (Kim and Strelkov 2007).

In another situation, the lack of chlorosis activity was explained by mutations in the ORF's first few nucleotides and the upstream flanking sequence that affects the secretory pathway, this was the case of *toxb3* in D308 (a race 3 isolate). In this isolate, the insertion of a retrotransposon has altered the putative start codon and resulted in a longer secretory signal peptide (Strelkov et al. 2005). Preliminary results show evidence of similar transposon activity in other *toxb3*-encoding isolates (SW21-5 and 331-2) (Aboukhaddour and Gourlie, personal communication). In haplotype *toxb13*, the 3' end appears to be highly mutated. However, we hypothesize that the large variation may be due to another transposon insertion, possibly also Copia-1_Ptr but inserted further downstream. The short-read assembly of SC29-1 is insufficient to expand on this question, long-read sequencing would be required to explore this further.

The TATA-box is a component of the eukaryotic core promoter and is recognized in a sequence-specific manner by TATA box-binding protein (TBP) to initiate transcription by RNA polymerase II in eukaryotes (Wang and Stumph 1995). The consensus TATA-box sequence in eukaryotes is 5'-TATA(A/T)A(A/T)-3' (Watson 2014). All *ToxB* sequences investigated here were found to have 5'-TATAAA-3' sequence upstream of the start codon except the two isolates with *toxb12* haplotype (Fig. 2). Interestingly, *toxb12*, a haplotype present in race 3 isolates collected from Syria, I-72-1 and I-72-7, was only one nucleotide different from the ORF in

ToxB1, but despite this high sequence similarity (>99%), these two isolates did not induce chlorosis symptoms on a susceptible genotype. The lack of chlorosis activity in *toxb12*-coding isolates may be associated with mutations affecting *ToxB* expression regulatory sequences rather than the single mutation in the ORF. In *toxb12*, the TATA-box promotor sequence is absent and this may have caused gene transcription failure, although experimental confirmation is needed. Moreover, the TATA-box sequence in the core promoter has been found to contribute to the determination of light-dependent gene expression in plants (Kiran et al. 2006). It is possible that the TATA-box sequences also plays a role in controlling the expression of light-dependent genes in fungi, such as *ToxB* (Strelkov et al. 1998). This finding emphasizes the importance of analyzing *ToxB* (and other effector) flanking sequences and confirming genotypic characterization for effector-encoding genes through phenotypic experiments using susceptible lines to confirm the activity of the effector in the host.

Identification of conserved microsatellite upstream of *ToxB*/*toxb* ORFs

In this study, we identified a microsatellite sequence in the 5′ UTR of *ToxB/toxb* haplotypes consisting of 25 nucleotides repeated several times, from 0 to 10, in these haplotypes. In eukaryotes, the 5′ UTR is a regulatory region of DNA located at the 5′ end of all protein-coding genes that is transcribed into mRNA but not translated into a protein (Bradnam and Korf 2008), and likely this region contains various regulatory elements that play a major role in the control of translation initiation and expression regulation (reviewed in: Barrett et al. 2012; Kochetov et al. 1998; Pickering and Willis 2005). The microsatellites sequence we identified here is identical in 20 positions and highly conserved in the remaining five positions (Fig. 2). These microsatellites could be involved in splicing of the 52-nucleotides intron upstream of

ToxB ORF, or may affect the regulation of *ToxB* expression (up- or down-regulation) by forming RNA secondary structures that impact ribosome binding. We showed that the folding of the 5′ UTRs in *ToxB* produced many stem-loop structures (Supplementary Fig. S6), and this may or may not affect translation rate, or the post transcriptional regulation upon interacting with RNA-binding proteins and either up- or down-regulate gene expression (Dmitriev et al. 2009; Ringnér and Krogh 2005). More experimental work is needed to validate the role of these microsatellites in expression regulation of *ToxB*.

The distribution and evolution of ToxB-like proteins in Ascomycetes

It has been suggested that *ToxB*, unlike *ToxA*, was acquired vertically from a common Ascomycete ancestor (Andrie et al. 2008; Ciuffetti et al. 2014) however, recent research has revealed that *ToxB* and its homologs in *P. tritici-repentis* were associated with a putative, possibly inactive large transposon inserted into an essential chromosome, suggesting that *ToxB* may have been capable of horizontal transfer via transposons at some point in the past (Gourlie et al. 2022). *ToxB* homologs have been identified in various genera of the Dothidiomycetes class, including *Pyrenophora*, *Cochliobolus* and *Alternaria*, as well as in the distantly-related Sordariomycete *Magnaporthe grisea*, the rice blast pathogen (Andrie et al. 2008). In this study, ToxB-like protein were also identified in other members of the Dothidiomycetes and Sordariomycetes classes, as well as in the Leotiomycetes endophyte species *Hyaloscypha bicolor* (Supplementary Table S9). The irregular distribution of ToxB and its homolog proteins in certain Ascomycete species, as well as the total absence of the *ToxB* gene or its homologs in certain races of *P. tritici-repentis*, could be explained by gene gains and losses in the evolutionary

history of *ToxB/toxb*. Further research is needed to fully understand the evolution, distribution and possible gain or loss of *ToxB* and its homologs across ascomycetes.

In all the ToxB-like proteins identified in these three Ascomycete classes, the positions of the four cysteine residues are highly conserved (Fig. 5) and form the two disulfide bonds which stabilize the ToxB structure against heat (Kim and Strelkov 2007) and organic compounds (Orolaza et al. 1995; Strelkov et al. 1999). The stability provided by these disulfide bonds is necessary for ToxB to function effectively in the harsh conditions of the plant apoplast (Figueroa et al. 2015). The β1-loop-β2 sequence, which is thought to be the site of the interaction with the putative receptor (Nyarko et al. 2014) is also highly conserved in ToxB-like sequences from the three Ascomycete classes. Previous work has shown that substitutions in β1-loop-β2 region can reduce ToxB activity and renders the protein partially inactive (Betts et al. 2011).

Investigating the allelic variation of effector genes in a pathogenic species can provide a comprehensive understanding of the co-evolutionary patterns at the population level, and how selection has shaped the diversity and distribution of a particular effector within and among distant populations. In the last decade, there has been an increasing trend in studying the diversity of effector genes among different pathogenic species in order to infer the mechanisms driving their evolution. Our study, which utilized a large collection of isolates, representative of all known pathotypes from worldwide origins, is one of the most comprehensive insights into the diversity of *ToxB*/*toxb* in *P. tritici-repentis* and related species. The added details into *ToxB*, its homologs, and its evolution via duplication or loss of function and the variation in its upstream

sequences in various isolates or species also adds significant value to the effector research community.

Materials and methods

Fungal isolates and DNA extraction

A total of 422 *P. tritici-repentis* isolates were used in this study and are described in Supplementary Table S2. These isolates were collected from various geographic regions including North America (Canada and USA), South America (Argentina), Australia, Japan, Europe (Denmark, Germany and United Kingdom), the Fertile Crescent and encompassing regions in North Africa (Algeria, Azerbaijan, Iran, Syria, and Tunisia). The race designation of the selected isolates were confirmed in this study (see below), and in previous studies (Aboukhaddour et al. 2009; Aboukhaddour et al. 2011; Aboukhaddour et al. 2013; Hafez et al. 2022; Kamel et al. 2019; Moolhuijzen et al. 2022; Wei et al. 2020). For each isolate, single conidia were used to inoculate 50 mL of ¼-strength potato dextrose broth (PDB) medium, which was incubated at room temperature for seven days without shaking. Fungal mats were collected, washed with dH_2O, freeze-dried, and stored at -20 °C until ground into powder using liquid nitrogen for DNA extraction. Genomic DNA (gDNA) was extracted using the DNeasy Plant Mini Kit (Qiagen, Mississauga, ON, Canada) following the manufacturer's instructions. Reference isolates of *P. teres* (two isolates) and *P. seminiperda* (three isolates) from the Canadian Collection of Fungal Cultures (DAOMC) were included to screen for the presence of *ToxB*/*toxb* by PCR (Supplementary Table S7).

PCR amplification

The internal transcribed spacer (ITS) region was amplified from the fungal isolates using the primers ITS-1 and ITS-4 (White et al. 1990), and the beta tubulin gene (*β-tub*) was amplified using the primer pair T1/T22 (O'Donnell and Cigelnik 1997). PCR conditions was conducted as described in Hafez et al. 2020. Degenerate primers Bb-F and Bb-R (Table 1; Supplementary Fig. S1) were developed for this study to amplify the full ORF of the *ToxB* gene (264 bp, including the stop codon) and *toxb* homologs (267 bp, including the stop codon). These primers are a mix of oligonucleotide sequences that contain a number of possible nitrogenous bases at certain positions to cover all possible nucleotide combinations for *ToxB* and *toxb* homologs identified to-date in *P. tritici-repentis* (See Table 1 for details on all the primers used in this study) .The specificity of the newly designed primers, Bb-F and Bb-R, was tested *in silico* using BLASTn (Altschul et al. 1990) searches and *in vitro* using a set of 20 *P. tritici-repentis* isolates known to contain both *ToxB* or *toxb* homologs. The PCR conditions were: initial denaturation at 94 ºC for 3 minutes, followed by 35 cycles of 94 ºC for 1 minute, 51 ºC for 30 seconds, and 72 ºC for 30 seconds, and a final extension step at 72 ºC for 5 minutes. Additional set of degenerate primers was developed to amplify *ToxB*/*toxb* with upstream and downstream flanking regions. Previous *ToxB*/*toxb* sequences (Gourlie et al. 2022; Strelkov et al. 2005) were analyzed to design *ToxB*/*toxb* universal primers, BUS-F and BDS-R (Supplementary Fig. S1), which amplify a fragment of 734-879 bp (variable length between different isolates) from isolates belonging to all races except some race 3 isolates. Two isolate-specific forward primers were designed to amplify *toxb* ORF plus flanking sequences from some race 3 isolates: 3US-F (specific to *toxb3*-encoding isolates: D308, SW21-5, 331-2) and 12US-F (specific to *toxb12*-encoding isolates: I-72-1, I-72-7). When paired with the reverse BDS-R primer (*ToxB*/*toxb* universal primer), 3US-F and 12US-

F produce fragments of 794 and 756 bp, respectively. PCR reactions were performed using the DreamTaq Green PCR Master Mix with the following conditions: initial denaturation at 94 ºC for 3 minutes, followed by 35 cycles of 94 ºC for 1 minute, 51 ºC for 30 seconds, and 72 ºC for 1 minute, and a final extension step at 72 ºC for 5 minutes.

Gel electrophoresis was used to analyze PCR amplicons. Agarose gel of 1-2% in 1X TBE buffer (89 mM Tris-borate, 10 mM EDTA, pH 8.0) was used. The sizes of the PCR amplicons were estimated using the GeneRuler 1 kb plus DNA ladder (Thermo Fisher Scientific, Canada) and were visualized under UV light after staining with RedSafe™ (iNtRON Biotechnology Inc., South Korea). Purified PCR amplicons for the ITS region, *β-tub*, *ToxB* and *toxb* genes were sequenced in two directions by Psomagen Inc. (Rockville, MD, U.S.A.). Initial nucleotide sequence alignments were done with Clustal-X v2 (Thompson et al. 1997) and the resulting alignments were refined with GeneDoc v2.5.010 (Nicholas 1997). BLAST searches (Altschul et al. 1990) on GenBank were conducted to confirm the species identity of the fungal isolates.

ToxB haplotype networks

Two primers, Bb-F and Bb-R, were used to amplify *ToxB* and its homolog *toxb* from 113 *P. tritici-repentis* and 3 *P. seminiperda* isolates. In addition to these isolates, 41 *ToxB*/*toxb* sequences were retrieved from GenBank resulting in a total of 157 sequences analyzed (Supplementary Table S6). The ORF finder program (https://www.ncbi.nlm.nih.gov/orffinder/) was used to identify coding sequence (ORF) of *ToxB*. The method from Templeton et al. (1992) was used in PopART v. 1.7 (Leigh and Bryant 2015; http://popart.otago.ac.nz) to build a TCS (Templeton, Crandall, and Sing) haplotype network. The generated TCS network was then edited and visualized by CorelDraw x4 Graphic for better readability. Mutations in *ToxB* coding

sequence were identified using GeneDoc (Nicholas 1997). In order to determine the *ToxB* haplotype(s) in *P. tritici-repentis* isolates, *ToxB* ORFs generated in this study were aligned with previously published *ToxB* sequences from *P. tritici-repentis*. Additionally, BLASTn searches (Altschul et al. 1990) on GenBank for all previously published *ToxB* sequences was conducted. GenBank accession numbers for all sequences used to generate *ToxB* haplotype network are listed in Supplementary Table S6. BLASTp (search protein databases using ToxB protein 'AAN39056' query) and BLASTx (search protein data bases using a translated *ToxB* nucleotide 'AF483831.1' query) were conducted to identify putative ToxB-like protein homologs in fungal genomes through NCBI Entrez molecular sequence database system (>70% query coverage and >30% ID to *ToxB1* haplotype). The amino acid sequences of all ToxB and ToxB-like proteins retrieved from GenBank are listed in Supplementary Table S9, along with information about the protein ID, organism, host, and associated disease.

Bioassay and symptoms rating

The 6B662 wheat genotype, which is susceptible to ToxB producing isolates, was used in a bioassay to study its reaction to different ToxB isoforms. The experiment was conducted as described in Aboukhaddour et al. (2013). Briefly, 6B662 seeds were planted in 10 cm diameter pots at a rate of eight seeds per pot with three replicates. The plants were grown in a growth chamber under a 16 h photoperiod (20 °C day/ 18 °C night) at a light intensity of a 180 mmol m^{-2} s^{-1}. At the two-leaf stage (14 days old), the seedlings were sprayed with a conidial suspension (3000 conidia ml^{-1}) and transferred to a humidity chamber for 24 hours. Seedlings were then transferred back to the growth chamber. Symptoms development, chlorosis, were assessed on the

mid section of the 2nd leaves five to seven days after inoculation on a scale of 1 to 5. The bioassay was repeated twice for each isolate.

ToxB structural modeling

The secondary structure of the ToxB protein was predicted using AlphaFold (Jumper et al. 2021). This was done to identify and compare the positions of nonsynonymous substitutions between different ToxB isoforms. ConSurf (Ashkenazy et al. 2016: https://consurf.tau.ac.il/) was used to analyze the amino acid sequences of all ToxB and ToxB-like proteins (Supplementary Table S9), in order to estimate evolutionary conservation of each amino acid position in the ToxB protein. A maximum-likelihood (ML) phylogenetic analysis was conducted using PhyML v3.1, which is included in Phylogeny.fr (http://www.phylogeny.fr/index.cgi). The analysis used 1000 bootstrap replicates and the WAG substitution model, which was selected by PhyML as the best-fitting model for this analysis. The ML tree was visualized and edited using iTOL v3 (Letunic and Bork 2016). ToxB protein sequences identified in ascomycete groups were used to construct ML tree (as explained above) to be compared with species tree of the same group based on ITS sequences to investigate the possibility of ToxB horizontal transfer. All ToxB and ITS sequences used in the analysis are indicated in Supplementary Table S10.

The *ToxB* flanking sequences for *P. tritici-repentis* were obtained from NCBI BioProject PRJNA803191 (Gourlie et al. 2022), and from GenBank (accession numbers: AY242114, AY242115, AY243033, AY243460 and AY243461) (Strelkov et al. 2005). *ToxB* flanking sequences for *P. bromi* were also obtained from GenBank (accession numbers EF452435-EF452442) (Andrie et al. 2008). A DNA logo for short sequence repeats (microsatellites: 25

nucleotides) in the *ToxB* 5' untranslated region (5' UTR) was generated using the online program WebLogo v2.8.2 (Crooks et al. 2004; http://weblogo.berkeley.edu/logo.cgi). The DNA logo was generated from 40 microsatellites in the *ToxB* 5' UTR region to visually display the sequence similarity of this region between different *ToxB* haplotypes (see Supplementary Table S8 for DNA accession numbers).

The copy number of the *ToxB* gene was determined in two *P. tritici-repentis* isolates that had been fully sequenced using PacBio RS II long-reads (Gourlie et al. 2022) (Supplementary Table S4). BLAST searches were conducted using the Alg3-24 *ToxB* ORF sequence as a query. The physical distance between *ToxB* copies in the same contig were measured and the orientation of *ToxB* copies were confirmed by comparison to isolate DW5 (Moolhuijzen et al. 2020). Alignment of contigs containing *ToxB* and *toxb* from I-73-1 and D308, respectively, were performed with MAFFT (v1.5.0; Katoh and Standley 2013). ORFs were predicted with Geneious Prime v 2022.1 (Biomatters Ltd), ORF domains identified with UnitProt (UniProt Consortium), and homology to known transposons were checked in RepBase (Jurka et al. 2005) and GenBank.

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Authors' contributions:

MH performed most of the work including experimental design, genotyping, primer design, sequence analysis, haplotype network, identify ToxB-like proteins in Ascomycete classes and figures creation. RA conceived the project, drafted the introduction and the discussion, helped in single spore isolation and rating of symptoms. MH with RA prepared the first and the revised draft. MT helped in DNA extraction, primer validation, amplification and sequencing *ToxB/toxb* genes. RG helped analyze *ToxB/toxb* and flanking sequences, and performed transposon identification and created Figure 3. PTS performed DNA extraction and sequence analysis. All authors contributed to editing and improving the manuscript and approved the final version.

References

Aboukhaddour, R., Cloutier, S., Lamari, L., and Strelkov, S. 2011. Simple sequence repeats and diversity of globally distributed populations of *Pyrenophora tritici-repentis*. Can. J. Plant Pathol. 33:389-399.

Aboukhaddour, R., Cloutier, S., Ballance, G. M. and Lamari, L. 2009. Genome characterization of *Pyrenophora tritici-repentis* isolates reveals high plasticity and independent chromosomal location of *ToxA* and *ToxB*. Mol. Plant Pathol. 10:201-212.

Aboukhaddour, R., Kim, Y. M. and Strelkov, S.E. 2012. RNA-mediated gene silencing of *ToxB* in *Pyrenophora tritici-repentis*. Mol. Plant Pathol. 13:318-326.

Aboukhaddour, R., Turkington, T. K., and Strelkov, S. E. 2013. Race structure of *Pyrenophora triciti-repentis* (tan spot of wheat) in Alberta, Canada. Can. J. Plant Pathol. 35:256-268.

Aboukhaddour, R., Hafez, M., McDonald, M., Moffat, C., Navathe, S., Friesen, T. L., Strelkov, S.E., Oliver, R. P., Tan, K. C., Liu, Z., Moolhuijzen, P. M., Phan, H., See, P. T., and Solomon, P. S. 2023a. A revised nomenclature for *ToxA* haplotypes across multiple fungal species. Phytopathology 113:1180-1184.

Aboukhaddour, R., Hafez, M., McDonald, M., Moffat, C., Navathe, S., Friesen, T. L., Strelkov, S.E., Oliver, R. P., Tan, K. C., Liu, Z., Moolhuijzen, P. M., Phan, H., See, P. T., and Solomon, P. S. 2023b. *ToxA* haplotypes alleles in the wheat leaf spot complex. Github. https://github.com/AAFC-Cereal-Pathology/ToxA-haplotypes-alleles-in-the-wheat-leaf-spot-complex

Aboukhaddour, R., Hafez, M., Strelkov, S. E. and Fernandez, M. R., 2021. Tan spot disease under the lenses of plant pathologists. In Achieving durable disease resistance in cereals (pp. 589-621). Burleigh Dodds Science Publishing.

Altschul, S. F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403-410.

Amaike, S., Ozga, J. A., Basu, U., and Strelkov, S. E. 2008. Quantification of *ToxB* gene expression and formation of appressoria by isolates of *Pyrenophora tritici-repentis* differing in pathogenicity. Plant Pathol. 57: 623-633.

Andrie, R. M., and Ciuffetti, L. M., 2011. *Pyrenophora bromi*, causal agent of brownspot of bromegrass, expresses a gene encoding a protein with homology and similar activity to Ptr ToxB, a host-selective toxin of wheat. MPMI 24: 359-367.

Andrie, R. M., Schoch, C. L., Hedges, R., Spatafora, J. W., and Ciuffetti, L. M., 2008. Homologs of *ToxB*, a host-selective toxin gene from *Pyrenophora tritici-repentis*, are present in the genome of sister-species *Pyrenophora bromi* and other members of the Ascomycota. Fungal Genet. Biol. 45: 363-377.

Ashkenazy, H., Abadi, S., Martz, E., Chay, O., Mayrose, I., Pupko, T., and Ben-Tal, N. 2016. ConSurf 2016: an improved methodology to estimate and visualize evolutionary conservation in macromolecules. Nucleic Acids Res. 44:W344-W350.

Barrett, L. W., Fletcher, S., and Wilton, S. D. 2012. Regulation of eukaryotic gene expression by the untranslated gene regions and other non-coding elements. Cell. Mol. Life Sci. 69: 3613-3634.

Betts, M. F., Manning, V. A., Cardwell, K. B., Pandelova, I., and Ciuffetti, L. M. 2011. The importance of the N-terminus for activity of Ptr ToxB, a chlorosis-inducing host-selective toxin produced by *Pyrenophora tritici-repentis*. Physiol. Mol. Plant Pathol. 75: 138-145.

Bradnam, K. R., and Korf, I. 2008. Longer first introns are a general property of eukaryotic gene structure. PloS one 3:p.e3093.

Ciuffetti, L. M., Manning, V. A., Pandelova, I., Betts, M. F., and Martinez, J. P. 2010. Host-selective toxins, Ptr ToxA and Ptr ToxB, as necrotrophic effectors in the *Pyrenophora tritici-repentis*–wheat interaction. New Phytol. 187: 911-919.

Ciuffetti, L. M., Manning, V. A., Pandelova, I., Faris, J. D., Friesen, T. L., Strelkov, S. E., Weber, G. L., Goodwin, S. B., Wolpert, T. J., and Figueroa, M. 2014. *Pyrenophora tritici-repentis*: a plant pathogenic fungus with global impact. In Genomics of plant-associated fungi: monocot pathogens (pp. 1-39). Springer, Berlin, Heidelberg.

Crooks, G. E., Hon, G., Chandonia, J. M. and Brenner, S. E. 2004. WebLogo: a sequence logo generator. Genome Res. 14:1188-1190.

Dagvadorj, B., Outram, M. A., Williams, S. J. and Solomon, P. S. 2022. The necrotrophic effector ToxA from *Parastagonospora nodorum* interacts with wheat NHL proteins to facilitate Tsn1-mediated necrosis. Plant J. 110: 407-418.

Dmitriev, S. E., Andreev, D. E., Ad'ianova, Z. V., Terenin, I. M., and Shatskiĭ, I. N. 2009. Efficient cap-dependent in vitro and in vivo translation of mammalian mRNAs with long and highly structured 5'-untranslated regions. Mol. Biol. 43: 119-125.

Figueroa, M., Manning, V. A., Pandelova, I., and Ciuffetti, L. M. 2015. Persistence of the host-selective toxin Ptr ToxB in the apoplast. MPMI 28:1082-1090.

Friesen, T. L. and Faris, J. D. 2021. Characterization of effector–target interactions in necrotrophic pathosystems reveals trends and variation in host manipulation. Annu. Rev. Phytopathol. 59:77-98.

Friesen, T. L., Stukenbrock, E. H., Liu, Z., Meinhardt, S., Ling, H., Faris, J. D., Rasmussen, J. B., Solomon, P. S., McDonald, B. A., and Oliver, R. P. 2006. Emergence of a new disease as a result of interspecific virulence gene transfer. Nat. Genet. 38:953-956.

Ghaderi, F., Sharifnabi, B., Javan-Nikkhah, M., Brunner, P. C. and McDonald, B. A. 2020. *SnTox*A, *SnTox1*, and *SnTox3* originated in *Parastagonospora nodorum* in the Fertile Crescent. Plant Pathol. 69:1482-1491.

Gourlie, R., McDonald, M., Hafez, M., Ortega-Polo, R., Low, K. E., Abbott, D. W., Strelkov, S. E., Daayf, F., and Aboukhaddour, R. 2022. The pangenome of the wheat pathogen *Pyrenophora tritici-repentis* reveals novel transposons associated with necrotrophic effectors *ToxA* and *ToxB*. BMC biol. 20:1-21.

Guo, J., Shi, G., Kalil, A., Friskop, A., Elias, E., Xu, S. S., Faris, J. D., and Liu, Z. 2020. *Pyrenophora tritici-repentis* race 4 isolates cause disease on tetraploid wheat. Phytopathology 110:1781-1790.

Hafez, M., Despins, T., Nakajima, K., and Aboukhaddour, R. 2022. Identification of a novel *ToxA* haplotype of *Pyrenophora tritici-repentis* from Japan. Phytopathology 112:1597-1602.

Hafez, M., Gourlie, R., Despins, T., Turkington, T. K., Friesen, T. L., and Aboukhaddour, R. 2020. *Parastagonospora nodorum* and related species in Western Canada: Genetic variability and effector genes. Phytopathology 110:1946-1958.

Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O., Tunyasuvunakool, K., Bates, R., Žídek, A., Potapenko, A. and Bridgland, A. 2021. Highly accurate protein structure prediction with AlphaFold. Nature 596: 583-589.

Kamoun, S. 2006. A catalogue of the effector secretome of plant pathogenic oomycetes. Annu. Rev. Phytopathol. 44: 41-60.

Inami, K., Yoshioka-Akiyama, C., Morita, Y., Yamasaki, M., Teraoka, T., and Arie, T. 2012. A genetic mechanism for emergence of races in *Fusarium oxysporum* f. sp. l*ycopersici*: inactivation of avirulence gene AVR1 by transposon insertion. PloS One 7: e44101.

Jurka, J., Kapitonov, V.V., Pavlicek, A., Klonowski, P., Kohany, O. and Walichiewicz, J. 2005. Repbase Update, a database of eukaryotic repetitive elements. Cytogenet. Genome Res. 110: 462-467.

Kamel, S., Cherif, M., Hafez, M., Despins, T., and Aboukhaddour R. 2019. *Pyrenophora tritici-repentis* in Tunisia: race structure and effector genes. Front. Plant Sci. 10:1562.

Katoh, K., and Standley, D. M. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30: 772-780.

Kim, Y. M., and Strelkov, S. E., 2007. Heterologous expression and activity of Ptr ToxB from virulent and avirulent isolates of *Pyrenophora tritici-repentis*. Can. J. Plant Pathol. 29:232-242.

Kim, Y. M., Bouras, N., Kav, N. N., and Strelkov, S. E. 2010. Inhibition of photosynthesis and modification of the wheat leaf proteome by Ptr ToxB: a host-specific toxin from the fungal pathogen *Pyrenophora tritici-repentis*. Proteomics 10:2911-2926.

Kiran, K., Ansari, S. A., Srivastava, R., Lodhi, N., Chaturvedi, C. P., Sawant, S. V., and Tuli, R. 2006. The TATA-box sequence in the basal promoter contributes to determining light-dependent gene expression in plants. Plant physiol. 142: 364-376.

Kochetov, A. V., Ischenko, I. V., Vorobiev, D. G., Kel, A. E., Babenko, V. N., Kisselev, L. L., and Kolchanov, N. A. 1998. Eukaryotic mRNAs encoding abundant and scarce proteins are statistically dissimilar in many structural features. FEBS lett. 440: 351-355.

Lamari, L., Strelkov, S. E., Yahyaoui, A., Orabi, J., and Smith, R. B., 2003. The identification of two new races of *Pyrenophora tritici-repentis* from the host center of diversity confirms a one-to-one relationship in tan spot of wheat. Phytopathology 93:391-396.

Leigh, J. W., and Bryant, D. 2015. POPART: full-feature software for haplotype network construction. Methods Ecol. Evol. 6:1110-1116.

Letunic, I., and Bork, P. 2016. Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. Nucleic Acids Res. 44:W242-W245.

Manning, V. A., Pandelova, I., Dhillon, B., Wilhelm, L. J., Goodwin, S. B., Berlin, A. M., Figueroa, M., Freitag, M., Hane, J. K., Henrissat, B., and Holman, W. H. 2013. Comparative genomics of a plant-pathogenic fungus, *Pyrenophora tritici-repentis*, reveals transduplication and the impact of repeat elements on pathogenicity and population divergence. G3: Genes, Genomes, Genet. 3: 41-63.

Martinez, J. P., Oesch, N. W., and Ciuffetti, L. M. 2004. Characterization of the multiple-copy host-selective toxin gene, *ToxB*, in pathogenic and nonpathogenic isolates of *Pyrenophora tritici-repentis*. MPMI 17: 467-474.

Martinez, J. P., Ottum, S. A., Ali, S., Francl, L. J., and Ciuffetti, L. M. 2001. Characterization of the *ToxB* gene from *Pyrenophora tritici-repentis*. MPMI 14:675-677.

McDonald, M. C., Ahren, D., Simpfendorfer, S., Milgate, A., and Solomon, P. S. 2018. The discovery of the virulence gene *ToxA* in the wheat and barley pathogen *Bipolaris sorokiniana*. Mol. Plant Pathol. 19: 432-439.

McDonald, M. C., Oliver, R. P., Friesen, T. L., Brunner, P. C., and McDonald, B. A., 2013. Global diversity and distribution of three necrotrophic effectors in *Phaeosphaeria nodorum* and related species. New Phytol. 199:241-251.

McDonald, M. C., Taranto, A. P., Hill, E., Schwessinger, B., Liu, Z., Simpfendorfer, S., Milgate, A., and Solomon, P.S. 2019. Transposon-mediated horizontal transfer of the host-specific virulence protein ToxA between three fungal wheat pathogens. MBio 10: e01515-19.

Mironenko, N. V., Orina, A. S., and Kovalenko, N. M., 2021. Genetic polymorphism of *ToxB*+ *Pyrenophora tritici-repentis* strains. Ecol. Genet. 19:121-129.

Moolhuijzen, P., See, P. T., and Moffat, C. S., 2020. PacBio genome sequencing reveals new insights into the genomic organisation of the multi-copy *ToxB* gene of the wheat fungal pathogen *Pyrenophora tritici-repentis*. BMC Genom. 21:1-12.

Moolhuijzen, P., See, P. T., Shi, G., Powell, H.R., Cockram, J., Jørgensen, L. N., Benslimane, H., Strelkov, S. E., Turner, J., Liu, Z., and Moffat, C. S. 2022. A global pangenome for the wheat fungal pathogen *Pyrenophora tritici-repentis* and prediction of effector protein structural homology. BioRxiv, doi: https://doi.org/10.1101/2022.03.07.482416.

Nicholas, K. B. 1997. GeneDoc: analysis and visualization of genetic variation. Embnew. news 4:14.

Nyarko, A., Singarapu, K. K., Figueroa, M., Manning, V. A., Pandelova, I., Wolpert, T. J., Ciuffetti, L. M., and Barbar, E. 2014. Solution NMR structures of *Pyrenophora tritici-repentis* ToxB and its inactive homolog reveal potential determinants of toxin activity. J. Biol. Chem. 289:25946-25956.

O'Donnell, K., and Cigelnik, E. 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. Mol. Phylogenetics Evol. 7:103-116.

Orolaza, N. P., Lamari, L., and Ballance, G. M. 1995. Evidence of a host-specific chlorosis toxin from *Pyrenophora tritici-repentis*, the causal agent of tan spot of wheat. Phytopathology 85:1282-1287.

Pickering, B. M., and Willis, A. E. 2005. The implications of structured 5′ untranslated regions on translation and disease. In Seminars in cell & developmental biology (Vol. 16, No. 1, pp. 39-47).

Ringnér, M., and Krogh, M. 2005. Folding free energies of 5′-UTRs impact post-transcriptional regulation on a genomic scale in yeast. PLoS Comput. Biol. 1:e72.

Sánchez-Vallet, A., Fouché, S., Fudal, I., Hartmann, F. E., Soyer, J. L., Tellier, A., and Croll, D., 2018. The genome biology of effector gene evolution in filamentous plant pathogens. Annu. Rev. Phytopathol. 56:21-40.

Sautua, F. J., and Carmona, M. A. 2021. Detection and characterization of QoI resistance in *Pyrenophora tritici-repentis* populations causing tan spot of wheat in Argentina. Plant Pathol. 70:2125-2136.

See, P. T., Marathamuthu, K. A., Iagallo, E. M., Oliver, R. P., and Moffat, C. S. 2018. Evaluating the importance of the tan spot *ToxA–Tsn1* interaction in Australian wheat varieties. Plant Pathol. 67:1066-1075.

Shi, G., Kariyawasam, G., Liu, S., Leng, Y., Zhong, S., Ali, S., Moolhuijzen, P., Moffat, C. S., Rasmussen, J. B., Friesen, T. L., and Faris, J. D. 2022. A conserved hypothetical gene is required but not sufficient for Ptr ToxC production in *Pyrenophora tritici-repentis*. MPMI 35:336-348.

Soliai, M. M., Meyer, S. E., Udall, J. A., Elzinga, D. E., Hermansen, R. A., Bodily, P. M., Hart, A. A., and Coleman, C. E., 2014. De novo genome assembly of the fungal plant pathogen *Pyrenophora semeniperda*. PLoS One 9:e87045.

Strelkov, S. E., and Lamari, L. 2003. Host–parasite interactions in tan spot [*Pyrenophora tritici-repentis*] of wheat. Can. J. Plant Pathol. 25: 339-349.

Strelkov, S. E., Kowatsch, R. F., Ballance, G. M., and Lamari, L. 2005. Characterization of the *ToxB* gene from North African and Canadian isolates of *Pyrenophora tritici-repentis*. Physiol. Mol. Plant Pathol. 67:164-170.

Strelkov, S. E., Lamari, L., and Ballance, G. M., 1998. Induced chlorophyll degradation by a chlorosis toxin from *Pyrenophora tritici-repentis*. Can. J. Plant Pathol. 20:428-435.

Strelkov, S. E., Lamari, L., and Ballance, G. M. 1999. Characterization of a host-specific protein toxin (Ptr ToxB) from *Pyrenophora tritici-repentis*. MPMI 12:728-732.

Stukenbrock, E. H., and McDonald, B. A. 2007. Geographical variation and positive diversifying selection in the host-specific toxin SnToxA. Mol. Plant Pathol. 8:321-332.

Templeton, A. R., Crandall, K. A., and Sing, C. F., 1992. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. Genetics 132:619-633.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25:4876-4882.

Wang, Y., and Stumph, W. E. 1995. RNA polymerase II/III transcription specificity determined by TATA box orientation. Proc. Natl. Acad. Sci. 92: 8606-8610.

Watson, J. D. 2014. Molecular biology of the gene. Watson, James D., 1928- (Seventh ed.). Boston.

Wyatt, N. A., and Friesen, T. L. 2021. Four reference quality genome assemblies of *Pyrenophora teres f. maculata*: a resource for studying the barley spot form net blotch interaction. MPMI 34:35-139.

Wei, B., Moscou M. J., Sato, K., Gourlie, R., Strelkov, S., Aboukhaddour, R. 2020. Identification of a locus conferring dominant susceptibility to *Pyrenophora tritici-repentis* in barley. Front. Plant Sci. 11:158.

White, T. J., Bruns, T., Lee, S. J. W. T., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications 18:315-322.

Wu, J., Kou, Y., Bao, J., Li, Y., Tang, M., Zhu, X., Ponaya, A., Xiao, G., Li, J., Li, C., and Song, M.Y. 2015. Comparative genomics identifies the *Magnaporthe oryzae* avirulence effector AvrPi9 that triggers Pi9-mediated blast resistance in rice. New Phytol. 206: 1463-1475.

Table 1: List of PCR primers used during the current study. The reference of each primer is indicated. Bb-F/Bb-R degenerate primers designed to amplify the complete ORF (including the stop codon) from both *ToxB* and its homolog *toxb*. BUS-F and BDS-R primers used to amplify *ToxB/toxb* ORF plus flanking sequences.

Primer	Sequence (5' → 3')	Targeted gene/region	Band length (bp)	Reference
ITS-1	TCCGTAGGTGAACCTGCGG	ITS	~700	White et al. 1990
ITS-4	TCCTCCGCTTATTGATATGC			
T1	AACATGCGTGAGATTGTAAGT	*β-tub*	~1300	O'Donnell and Cigelnik 1997
T22	TCTGGATGTTGTTGGGAATCC			
Bb-F	**M**TG**R**C**R**CCTATATT**YV**AGACT**R**C*	*ToxB/b* ORF	264-267	This study
Bb-R	CTAACAACGTCCTCCACTT**KS**C*			
BUS-F	GCTTAGGTCTAGAA**YY**ATTT**R**CTCG*	*ToxB/toxb* flanking seq.	734-879	
BDS-R	GTTA**R**CTTTT**R**CGTTAGCAAGC*			
3US-F (a)	GATAGTACAGACATCTTGTCGATGAG	*toxb*	794	
12US-F (b)	TGTTTCAGCCAATCCGAGCG	*toxb*	756	

* **K**=G or T; **M**= A or C; **R**= A or G; **S**= C or G; **V**= A or C or G; **Y**= C or T.

(a) This primer produce a 794 bp band when paired with BDS-R primer for isolates belonging to *toxb3* haplotype (D308, SW21-5 and 331-2).

(b) This primer produce a 756 bp band when paired with BDS-R primer for isolates belonging to *toxb12* haplotype (I-72-1 and I-72-7).

Supplementary Tables (see excel file).

Supplementary Table S1: GenBank accession numbers for the ITS region and *β-tub* gene in isolates investigated in the current study (See note below Table).

Supplementary Table S2: List of *Pyrenophora tritici-repentis* isolates used in this study with information about: race, country, collection year, host cultivar and presence/absence of *ToxB* and *toxb* genes. Missing information is represented by NA (Not Available).

Supplementary Table S3: Genotype frequencies and number of isolates harboring *ToxB/toxb* genes.

Supplementary Table S4: *ToxB* copy numbers in some *Pyrenophora tritici-repentis* isolates.

Supplementary Table S5: Number and types of polymorphisms observed in *Pyrenophora tritici-repentis ToxB*/*toxb* ORF.

Supplementary Table S6: *ToxB/toxb* sequence accession numbers used to generate the haplotype network. All *ToxB/toxb* sequences generated in this study and previously published from *Pyrenophora tritici-repentis* and other *Pyrenophora* spp. are included to indicate the genetic diversity in *ToxB* open reading frame (ORF). Activity of ToxB protein encoded by each haplotype is indicated (*ToxB*=haplotypes produced active protein isoform; *toxb*=haplotypes produced inactive protein isoform).

Supplementary Table S7: List of *Pyrenophora teres* and *Pyrenophora seminiperda* isolates screened for the presence of *ToxB*/*toxb* gene by PCR using the newly designed primers Bb-F and Bb-R. Missing information is represented by NA (Not Available).

Supplementary Table S8: *ToxB/toxb* sequence accession numbers used to generate the DNA logo for a short microsatellites at the 5' untranslated region (5' UTR).

Supplementary Table S9: ToxB and ToxB-like protein accession numbers in different Ascomycete classes, orders and families. (*) ToxB protein homologs reported for the first time.

Supplementary Table S10: GenBank accession number for ToxB protein and ITS sequences used to investigate the possibility of ToxB horizontal transfer.

Figure legends:

Fig. 1: A global *ToxB* haplotype network in *Pyrenophora tritici-repentis* and other *Pyrenophora* spp. (*Pyrenophora bromi, Pyrenophora teres* and *Pyrenophora seminiperda*). Each circle represents a unique *ToxB* haplotype and the size of the circle indicates the relative frequency belonging to a particular haplotype (smallest circle, 1 sequence; largest circle, 105 sequences). The hatch marks along the network branches indicates the number of mutations. Red and black hatch marks represent synonymous and nonsynonymous, respectively. Indel mutations are also indicated by green (insertion) or pink (deletion) hatch marks. *toxb19* and *toxb20* are differ from *ToxB1* by 10, 73 and 3 synonymous, nonsynonymous and indel mutations, respectively. *toxb16* - *toxb18* are differ from *ToxB4* by 18 and 20 synonymous and nonsynonymous mutations, respectively. The ORF in isolates with *toxb3* haplotype is interrupted by Copia-1_Ptr (5661 bp) transposon. Dashed circles represent *in silico* identified haplotypes in *P. teres* and *P. seminiperda*. *ToxB*/*toxb* haplotypes named according to Aboukhaddour et al. 2023a.

Fig. 2: Sequence variation among *ToxB* haplotypes in *Pyrenophora tritici-repentis* and *P. bromi*. Reference isolate for each *ToxB/toxb* haplotype is indicated; number in parenthesis represent the total number of isolates/sequences identified in each haplotype. The open reading frame (ORF) in *ToxB1*, *ToxB4,* and *ToxB5* produces active ToxB protein (orange color), while *toxb* homologs in other *P. tritici-repentis* haplotype ORFs (green color) do not produce active ToxB protein. *ToxB1* and *toxb3* ORFs are identical, with the latter being interrupted by Copia-like transposon (Copia-1_Ptr: 5661 bp) to produce inactive ToxB (*toxb3*). *toxb* homologs were found in *Pyrenophora bromi*, sister species of *P. tritici-repentis*. Heterologously expressed proteins from these haplotypes (Andrie and Ciuffetti 2011) can induce chlorosis on ToxB-sensitive wheat cultivars (faint blue color), except haplotype *toxb10* (yellow color). Sequence analysis of the upstream flanking untranslated regions (5' UTR) reveals the presence of core promotor sequence (TATA-box; 5'-TATAAA-3') in all haplotypes except *toxb12*. In haplotype *ToxB5*, upstream sequence is truncated. Region between TATA-box and the intron contains microsatellites

consisting of 25 nucleotides (purple squares), that is repeated several times (varied in number between different haplotypes from 0 to 10). A DNA logo (created by WebLogo version 2.8.2; Crooks et al.2004) for a single microsatellite from 40 *ToxB* sequences (32 *P. tritici-repentis* and eight *P. bromi*) is shown in the bottom and indicate higher level of conservation among all sequences. The 3' UTR region looks homologous (black color) in all *ToxB* haplotypes except *toxb13* (grey color) and some isolates within *ToxB1* (brown color).

Fig. 3: Schematic of Copia-1_Ptr interrupting D308 *toxb* ORF; I-73-1 *ToxB* shows the target site duplication (5'-CCTA-3'). Copia-1_Ptr is inserted in the opposite direction to *ToxB*/*toxb*. Within Copia-1_Ptr we identified ORFs containing DoeR (transcriptional regulator), GAG (capsid protein), INT (integrase), Pol (retrovirus-related polyprotein), and RT (reverse transcriptase) domains.

Fig. 4: Reaction of 6B662 wheat genotype (ToxB sensitive) to inoculation with *Pyrenophora tritici-repentis* isolates belonging to different *ToxB* haplotypes. *ToxB* haplotypes, *P. tritici-repentis* isolates and chlorosis score (1-5) are indicated. ToxB activity (i.e chlorosis symptoms) was only observed in *ToxB1* (Alg3-24), *ToxB4* (92-171-R5), and *ToxB5* (Alg215), with extent of chlorosis symptom development associated with *ToxB4* and *ToxB5* is much less (score 3) than for *ToxB1* (score 5). Bioassay picture for Alg215 isolate (*ToxB5*) adapted from Moolhuijzen et al. 2022.

Fig. 5: Distribution of ToxB and other ToxB-like amino acid sequences in members of Ascomycetes. **[A]** ToxB-like sequences obtained from NCBI using BLASTp (protein-protein BLAST), and aligned with GeneDoc. ToxB-like sequences were found in *Pyrenophora tritici-repentis* and other members of Ascomyvcetes in Dothidiomyceyes (Pleosporales and Botryosphaeriales), Leotiomycetes (Helotiales) and Sordariomycetes (Diaporthales, Glomerellales and Magnaporthales). ToxB sequence alignment used to generate a Maximum Likelihood (ML) tree with bootstrap values from lower to higher represented by gradient colour (black to red to green, respectively) on the tree branches. The four stars (*) indicate the conserved position of cysteine (C) residues, highlighted in green, that form disulfide bonds (S-S: green lines). Amino acid residue designation: White on black for identical; white on grey for conservative; black on grey for semiconservative and black on white for unique or shared between ≤50% of sequences. Relative position of the six β-strands (β1-β6) is indicated at the top. **[B]** ToxB protein sequences from the tree were analyzed with ConSurf (https://consurf.tau.ac.il/) to estimate the evolutionary conservation of each amino acid position in ToxB protein. β1-β6, N-terminus (N) and C-terminus (C) are indicated. A color-blind friendly scale is used to indicate conservation degree for each position from 1 (variable) to 9 (conserved).

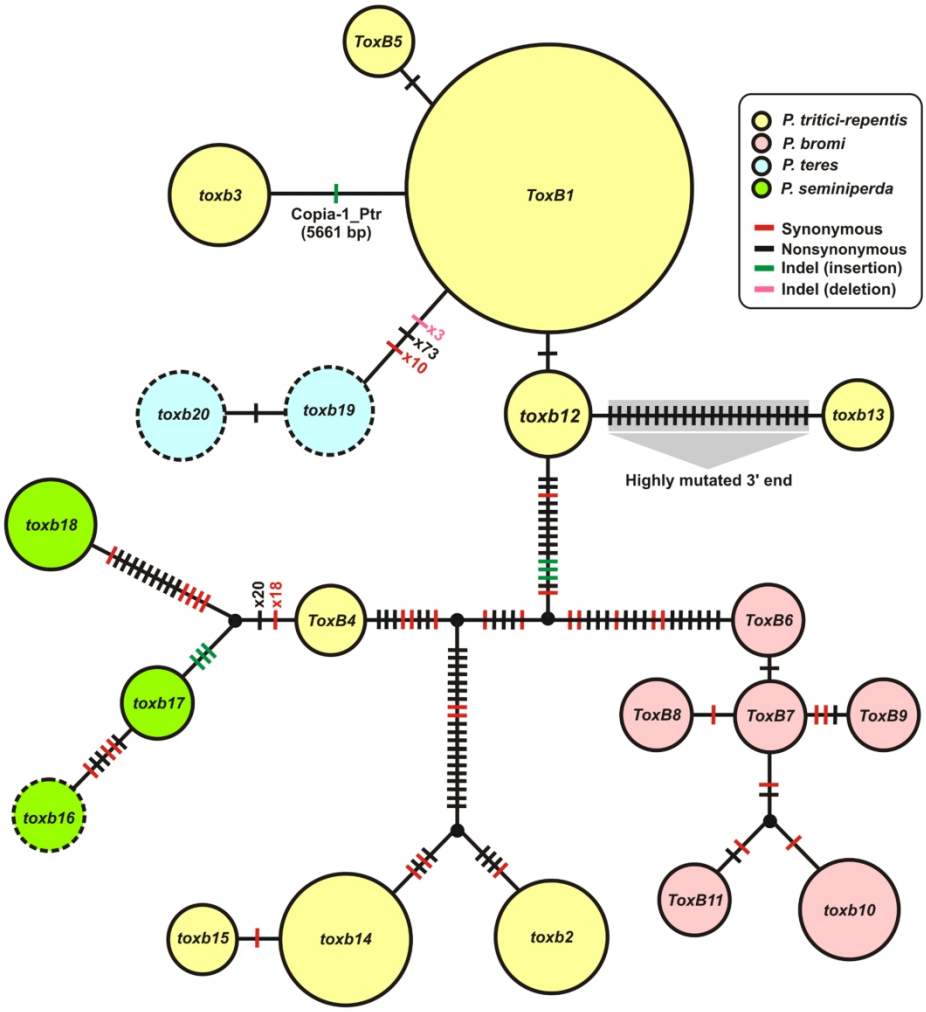

Fig. 1

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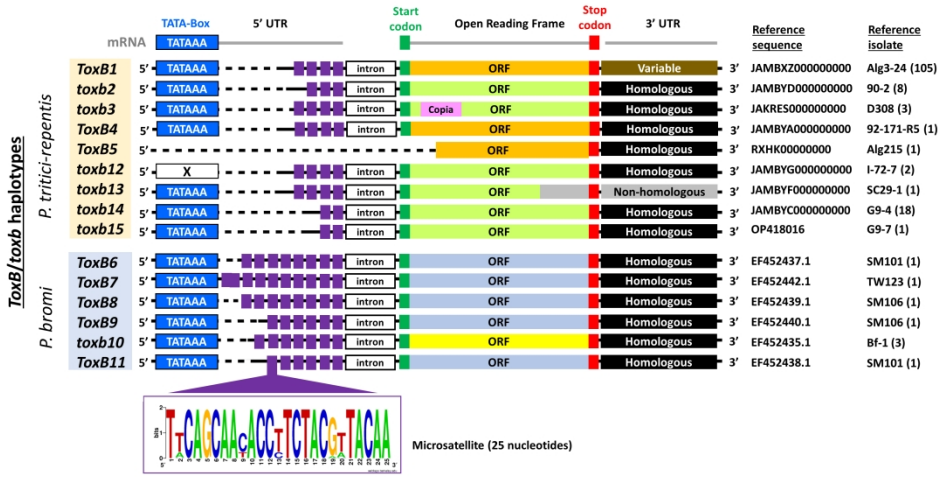

Fig. 2

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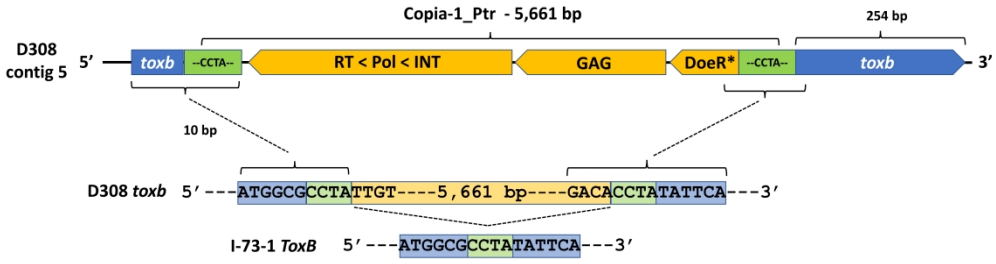

Fig. 3

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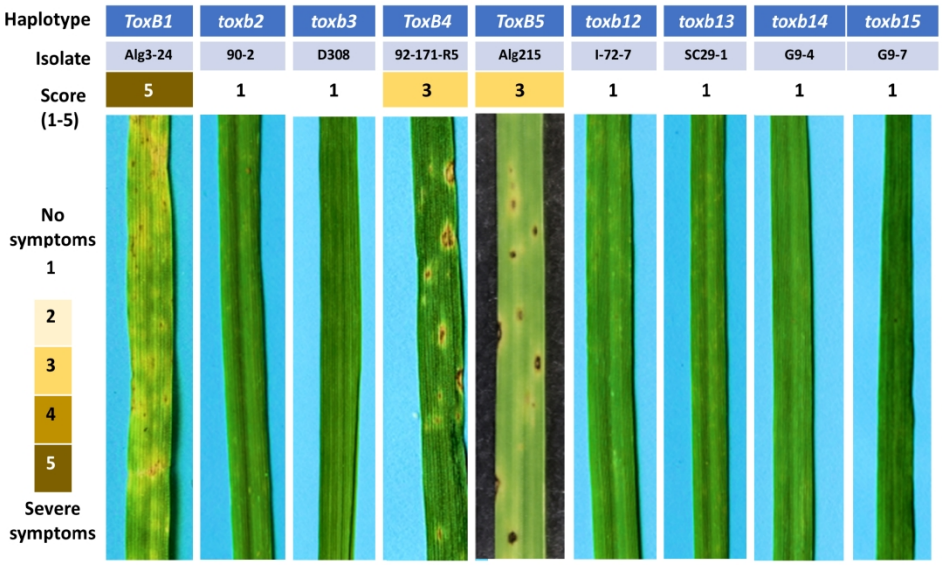

Fig. 4

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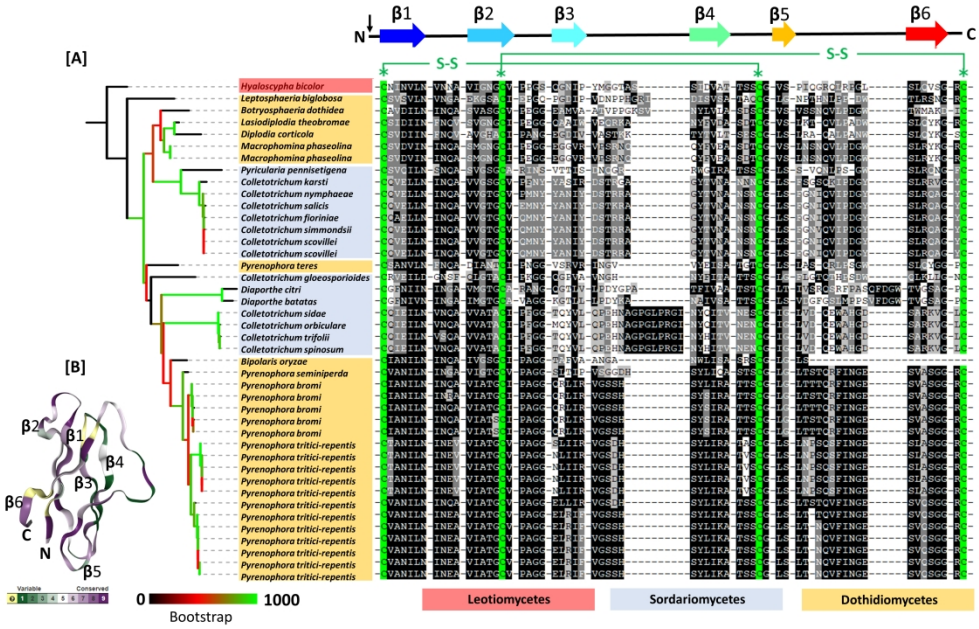

Fig. 5

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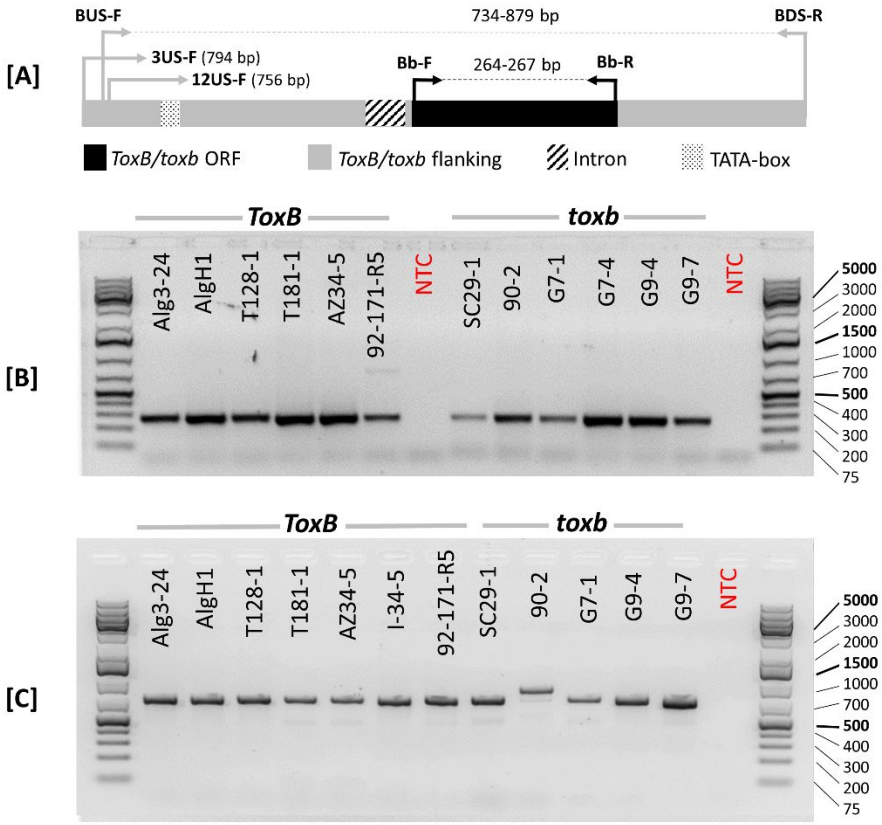
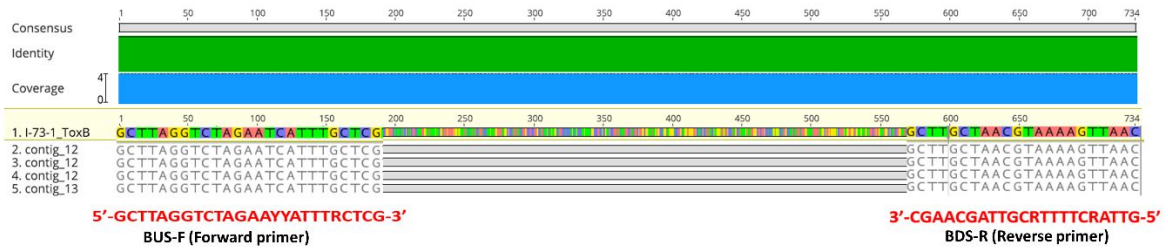
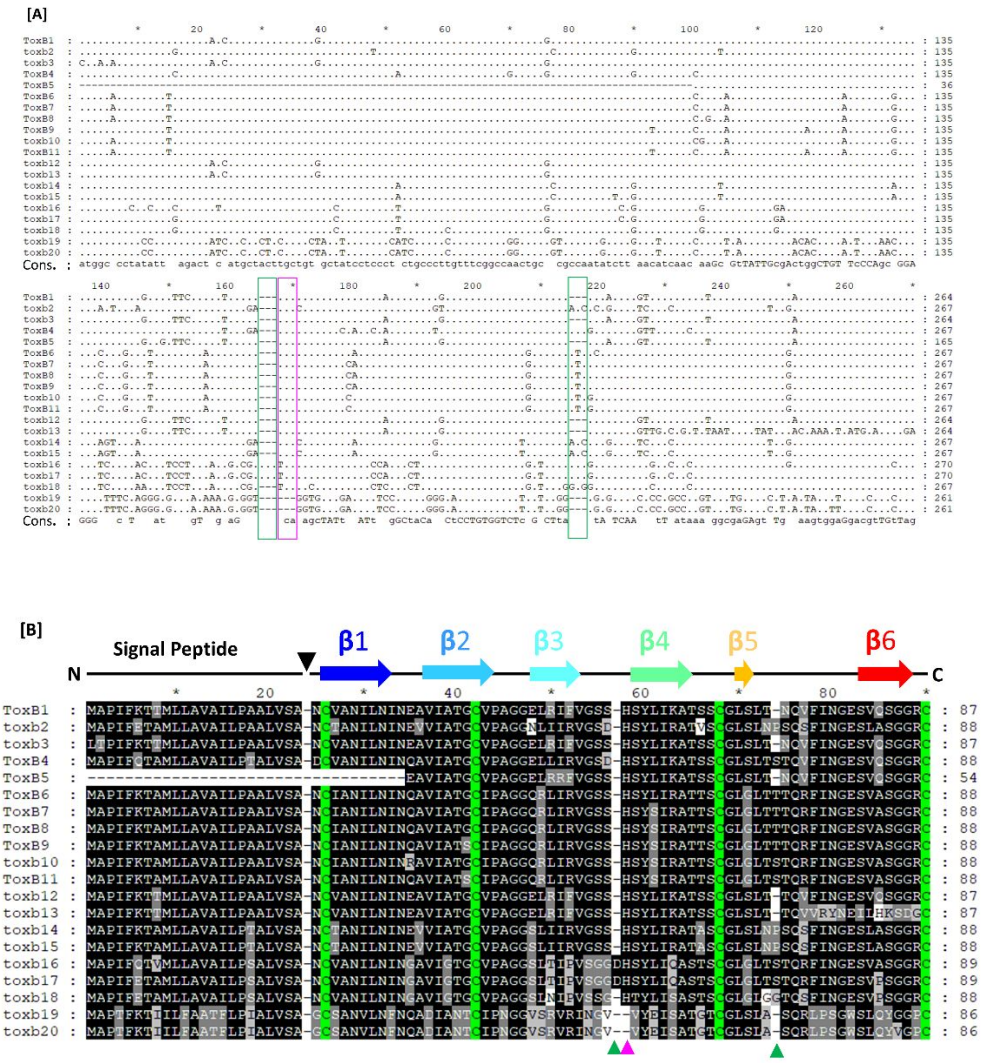


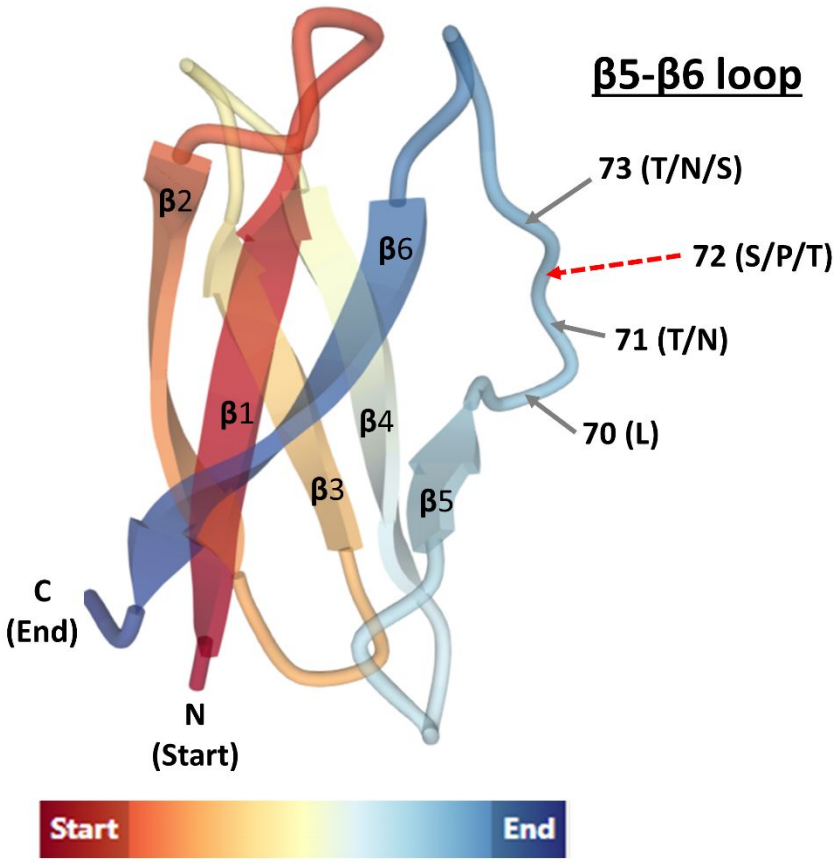
Supplementary Fig. S1: [A] *ToxB* gene map showing ORF and flanking regions. Bb-F/Bb-R universal primers used to amplify *ToxB* and *toxb* ORFs including stop codon (264 and 267 bp, respectively). BUS-F/BDS-R primer pair used to amplify *ToxB/toxb* ORFs in addition to upstream and downstream flanking regions (734-879 bp) are indicated. Specific forward primers for isolates belonging to haplotype *toxb3* (3US-F) and haplotype *toxb12* (12US-F) when paired with the universal reverse BDS-R primer produce 794 and 756 bp PCR amplicons, respectively. **[B]** PCR amplification of *ToxB* ORF (264 bp) and its homolog (*toxb* – 267 bp) from selected *Pyrenophora tritici-repentis* isolates using Bb-F/Bb-R primer pair. **[C]** PCR amplification of *ToxB/toxb* ORF with flanking regions using BUS-F/BDS-R primer pair. Band size varied among different isolates, with most isolates produce 734 bp band and 90-2 isolate produce a 879 bp band. NTC lane indicate the negative control and ladder lane contain GeneRuler 1 kb Plus DNA Ladder (Thermo Fisher Scientific).



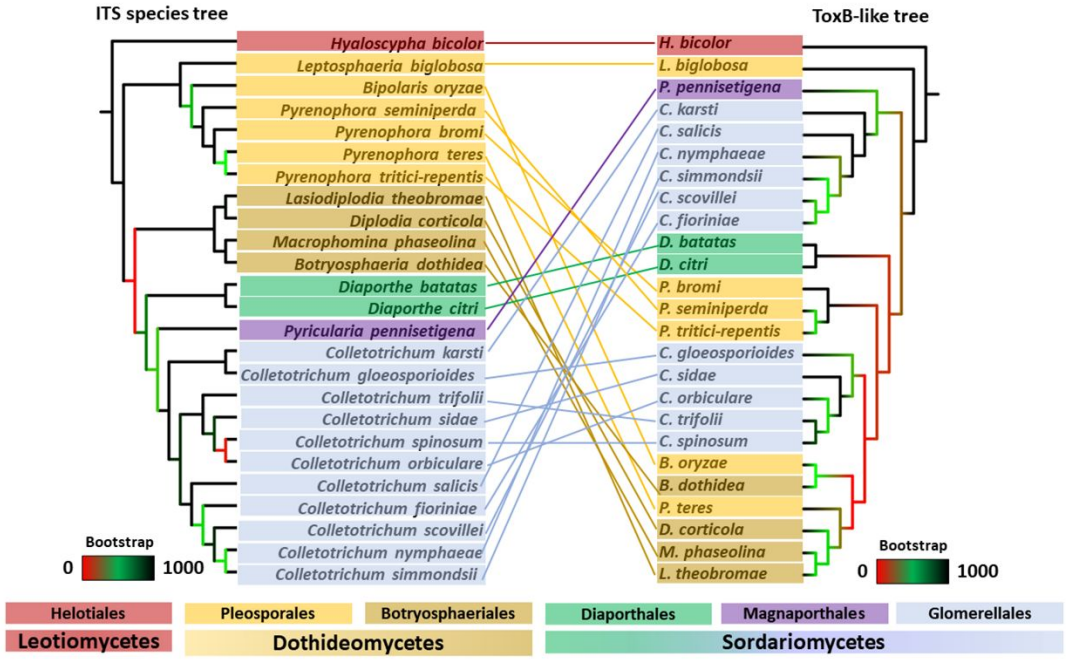
Supplementary Fig. S2: Sequence alignment of *ToxB* coding plus flanking regions in *Pyrenophora tritici-repentis* I-73-1. Four *ToxB* copies were identified: three copies in contig number 12 (accession: JAKRER010000006.1) and one copy in contig number 13 (accession: JAKRER010000008.1). The four *ToxB* copies were identical and belongs to *ToxB1* haplotype, flanking regions were also identical. Primers to amplify *ToxB* ORF plus flanking sequences (BUS-F and BDS-R) and DNA binding sites for these two primers are indicated.



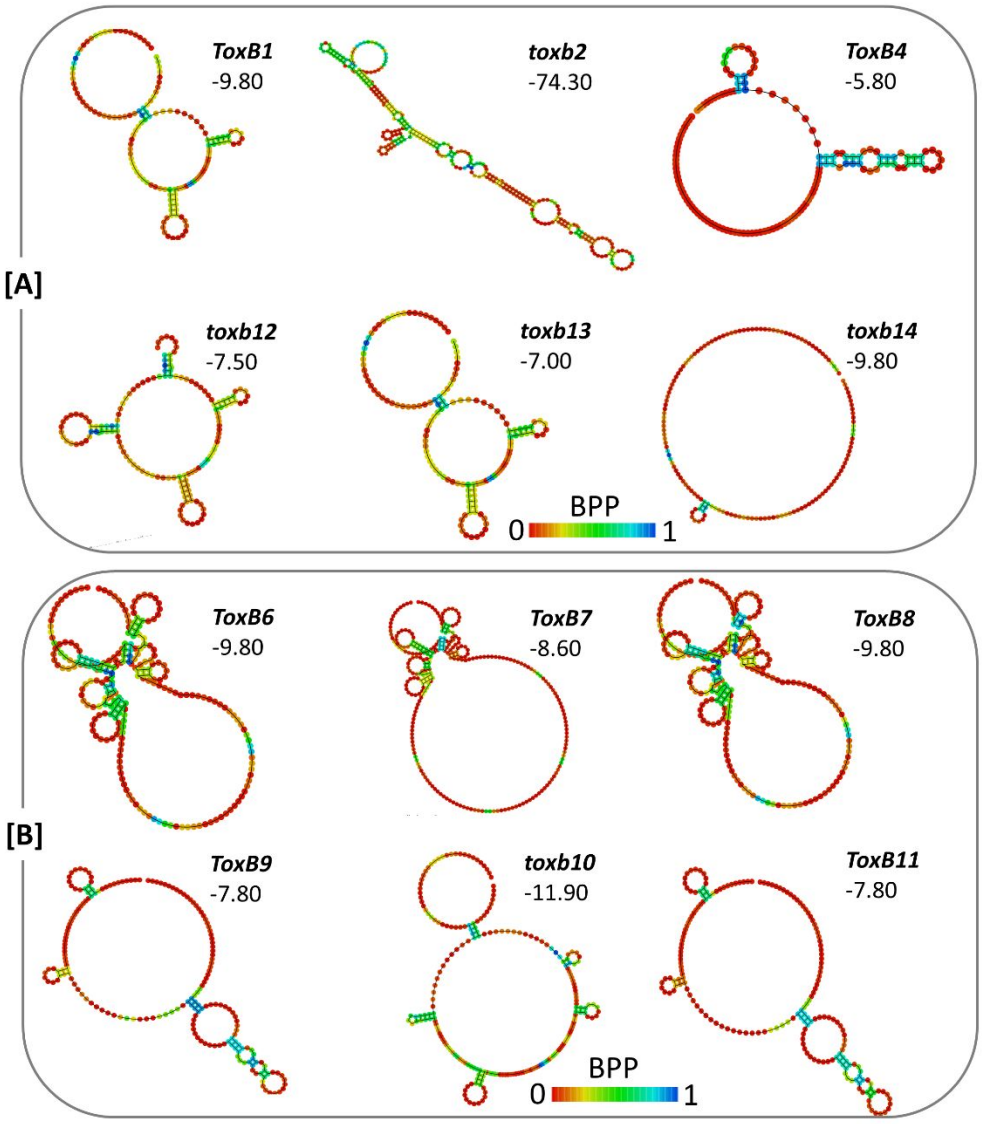
Supplementary Fig. S3: [A] *ToxB/toxb* gene haplotypes sequence alignment in *Pyrenophora tritici-repentis* (*ToxB1-ToxB5* & *toxb12-toxb15*), *Pyrenophora bromi* (*ToxB6-ToxB11*), *Pyrenophora seminiperda* (*toxb16-toxb18*) and *Pyrenophora teres* (*toxb19* and *toxb20*). *ToxB/toxb* gene consisting of intronless 264-267 nucleotides (including stop codon), and all *ToxB/toxb* sequences could be collapsed into 20 unique haplotypes. In *P. tritici-repentis*, a total of 72 polymorphic sites were found among the different nine *ToxB/toxb* haplotypes. Consensus sequence (Cons.) is indicated at the bottom line. Insertion and deletion positions are indicated by green and pink lines, respectively. **[B]** *ToxB/toxb* encoding for 86-89 amino acid proteins. The black triangle denotes cleavage site of the signal peptide following a conserved alanine (A) residue. Conserved cysteine (C) residues highlighted in green. The mature ToxB/toxb protein consisting of six antiparallel beta sheets (β1-β6). Table S6 provide information about sequences used to generate this figure and GenBank accession numbers, and Table S5 contains information about polymorphic sites within *Pyrenophora tritici-repentis ToxB/toxb* ORF. Positions of amino acids insertion and deletion are indicated by green and pink triangles, respectively.



Supplementary Fig. S4: The indel mutation (three nucleotides insertion at positions 212-214) in some *Pyrenophora tritici-repentis* and *Pyrenophora bromi toxb* haplotypes splitting the 5'-ACT-3' codon (212-214) encoding for threonine "T" at position 71 into two amino acid residues at positions 71 and 72. ToxB protein secondary structure model consisting of six antiparallel beta sheets (β1-β6) show the location of 70-73 amino acid residues in β5-β6 loop is indicated. Model predicted by AlphaFold Protein Structure Database (https://alphafold.ebi.ac.uk/). The AlphaFold per-residue confidence score (pLDDT) for the predicted model is very high: pLDDT > 90.



Supplementary Fig. S5: Phylogenetic relationships between ToxB protein evolution (based on ToxB-like amino acid sequences) and their corresponding species evolution (based on ITS sequences) in Dothidiomycetes (Pleosporales and Botryosphaeriales), Leotiomycetes (Helotiales) and Sordariomycetes (Diaporthales, Glomerellales and Magnaporthales). The tree topology is based Maximum Likelihood (ML) with bootstrap values from lower to higher represented by gradient color (red to green to black, respectively) on the tree branches. The trees showed that, ToxB-like sequences are not well correlated with the species evolutionary relationships. This inconsistent grouping pattern might indicate a possibility of horizontal transfer for *ToxB* between different ascomycete groups.



Supplementary Fig. S6: RNA secondary structure models for the 5' untranslated region (5' UTR) of different *ToxB/toxb* haplotypes in **[A]** *Pyrenophora tritici-repentis* and **[B]** *Pyrenophora bromi.* The RNAfold web server (http://rna.tbi.univie.ac.at//cgi-bin/RNAWebSuite/RNAfold.cgi) used to predict the secondary structure model for the 5' UTR between TATA-box and ToxB start codon after removing the intron sequence. The optimal secondary structure with a minimum free energy (MFE) are given for each haplotype. MFE (kcal/mol) and base-pair probability (BPP: 0-1) are indicated for each model.