

Geographical variation and positive diversifying selection in the host-specific toxin *SnToxA*

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SUMMARY

The host-specific toxin ToxA produced by the wheat pathogens *Pyrenophora tritici-repentis* and *Phaeosphaeria nodorum* interacts with the product of the dominant plant gene *Tsn1* to induce necrosis. The *ToxA* gene is thought to have been acquired by *Py. tritici-repentis* from *Ph. nodorum* through a recent horizontal gene transfer event. PCR and sequence analysis indicate that the level of *ToxA* variation, including gene deletion, in *Ph. nodorum* (*SnToxA*) is significantly higher than in *Py. tritici-repentis* (*PtrToxA*). We PCR-screened 788 isolates of *Ph. nodorum* originating from eight geographical regions to infer the pattern of *SnToxA* deletions. The frequency of deletions differed significantly among populations, ranging from 0% (Australia) to 98% (China). Sequence analysis of the *SnToxA* gene in 123 *Ph. nodorum* isolates revealed 13 distinct haplotypes. The distribution and diversity of haplotypes varied significantly among populations. The majority of *SnToxA* mutations were non-synonymous resulting in changes at the protein level. We applied different models of selection to infer the mode of evolution operating at the *ToxA* locus. Evidence for positive diversifying selection supports the hypothesis that evolution of the *ToxA* locus is driven by selection imposed by the host. The distribution of *SnToxA* alleles and deletions may reflect the distribution of different *Tsn1* alleles in the corresponding host populations.

INTRODUCTION

Gene-for-gene models provide a useful framework for understanding coevolution between plants and their pathogens. The concept of gene-for-gene interactions was first described by Flor (1955) in a study of flax rust. He found that for every gene determining resistance in the host there was a corresponding and complementary gene for avirulence in the pathogen. According

to this model the incompatible interaction that leads to resistance in the plant depends on the recognition of a pathogen elicitor (encoded by the avirulence gene) by a receptor in the plant (encoded by the resistance gene) (Gabriel and Rolfe, 1990). Resistance in the plant is generally dominant to susceptibility, and in the pathogen avirulence is dominant to virulence. If a host lacks a specific resistance gene, the corresponding avirulence gene cannot be detected and a resistance response is not initiated. The changes between incompatible and compatible interactions are driven by the continuous replacement of new avirulence and new resistance genes, leading to an arms race between host and pathogen (Stahl and Bishop, 2000).

A similar coevolutionary process can occur for host-specific toxins encoded by pathogens. Host-specific toxins (HSTs) have been characterized in a number of plant pathogens (for reviews see Markham and Hille, 2001; Walton, 1996; Wolpert *et al.*, 2002). These toxins are determinants of host specificity and in most cases are required for pathogenicity such that disease does not occur in the absence of toxin production. Molecular studies have demonstrated that a single gene often conditions host sensitivity to a given HST and disease does not occur in the absence of the dominant toxin sensitivity allele. Only the compatible interaction between the toxin and the host sensitivity gene induces host cell death in the plant tissue (Walton, 1996). In contrast to the elicitor–receptor interaction, toxin production and host sensitivity are generally dominant characters, thereby producing a mirror-image of Flor's classic gene-for-gene system (Flor, 1956; Wolpert *et al.*, 2002).

Several examples consistent with the arms race model have demonstrated how selective maintenance of genetic variability drives the evolution of genes involved in gene-for-gene interactions in plant pathosystems (Bishop *et al.*, 2000; Ludere *et al.*, 2002; Meyers *et al.*, 1998; Noel *et al.*, 1999; Parniske *et al.*, 1997; Rose *et al.*, 2004). Diversifying selection, also referred to as positive diversifying selection, was demonstrated in fungal avirulence genes as an excess of non-synonymous mutations (Kang *et al.*, 2001; Liu *et al.*, 2005; Schürch *et al.*, 2004). Diversifying selection and other non-neutral changes such as loss-of-function mutations or complete gene deletions can potentially

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lead to virulence of a pathogen, thereby also selecting for new resistance alleles in the host. HSTs and their corresponding sensitivity genes are likely to coevolve in a similar way as avirulence and resistance genes. Although most studies have focused on the function of HSTs, little is known about the evolution of these 'agents of compatibility' (Walton, 1996).

One of the best-studied host-specific toxins is *ToxA* produced by the wheat infecting pathogen *Pyrenophora tritici-repentis* (Balance *et al.*, 1989; Ciuffetti *et al.*, 1997). The *Py. tritici-repentis* species consists of eight different races characterized by the presence or absence of at least three different toxins produced during infection of the host (Lamari *et al.*, 2003; Strelkov *et al.*, 2002). *ToxA* is responsible for the development of necrosis in susceptible host cultivars carrying the dominant toxin sensitivity gene *Tsn1* (Faris *et al.*, 1996; Lamari and Bernier, 1989). A compatible interaction between *PtrToxA* and *Tsn1* is possible only when both the toxin and the corresponding plant gene are expressed. Thus far, the *Tsn1* gene has not been cloned and the mechanism of interaction between *Tsn1* and *ToxA* is not known.

A homologue to the *Py. tritici-repentis ToxA* gene was identified recently in another fungal wheat pathogen, *Phaeosphaeria nodorum* (Friesen *et al.*, 2006). This finding was surprising as the gene does not exist in any closely related species of either pathogen and because HSTs are usually regarded as being unique to only one pathogen species (Markham and Hille, 2001). The *ToxA* gene in *Ph. nodorum* was shown to interact with the *Tsn1* gene in an indistinguishable manner as in *Py. tritici-repentis* (Liu *et al.*, 2007). Friesen *et al.* (2006) analysed a large collection of isolates of both pathogen species. While the level of variation in the *ToxA* gene was surprisingly high in *Ph. nodorum*, only one allele was identified in *Py. tritici-repentis*. Friesen *et al.* (2006) presented evidence that the high homology between the *ToxA* genes was due to a very recent horizontal transfer of the gene from *Ph. nodorum* into *Py. tritici-repentis*. To distinguish specifically the *ToxA* gene in *Ph. nodorum* from the gene in *Py. tritici-repentis* we here define the genes as *SnToxA* in *Ph. nodorum* and *PtrToxA* in *Py. tritici-repentis* and *ToxA* as a general term for the locus in both pathogens.

Genetic variability in populations of *Ph. nodorum* and *Py. tritici-repentis* has so far only been described using neutral genetic markers (Friesen *et al.*, 2005; Keller *et al.*, 1997a,b; Stukenbrock *et al.*, 2006). In spite of the different levels of *ToxA* variability among the two pathogen species, they have very similar life histories and population structures, with both pathogens exhibiting high levels of genetic diversity mediated through high levels of gene flow and frequent recombination (Friesen *et al.*, 2005; Stukenbrock *et al.*, 2006).

Because *ToxA* affects pathogenicity and fitness, it is likely to be involved in local adaptation and may experience different types of selection in different environments. The finding of high nucle-

otide diversity in the *SnToxA* gene could be a result of positive diversifying selection driven by a gene-for-gene interaction between *SnToxA* and *Tsn1* or a different plant gene. The observation that deletions occur at a high frequency (Friesen *et al.*, 2006) suggests that the deletion is selectively advantageous to the pathogen in some environments.

In this study we explore the pattern of gene deletions and nucleotide diversity in *SnToxA* in eight geographical populations of *Ph. nodorum*. The finding that diversity is considerably higher in the exons of the *SnToxA* gene suggests that selection is operating on this gene. This pattern resembles the evolution of avirulence genes also involved in gene-for-gene interactions with the host plant. We here apply different statistical tests and models of evolution to a large number of *ToxA* sequences obtained from *Ph. nodorum* and *Py. tritici-repentis* in order to understand processes affecting selection and evolution of the genes encoding host-specific toxins.

RESULTS

Deletion frequency

We screened 788 isolates from eight regional populations of *Ph. nodorum* for the presence of the *SnToxA* gene (Table 1). PCR analysis resulted in 199 positive *SnToxA* amplifications, corresponding to an average deletion frequency of 60% (Table 2). The *SnToxA* deletion frequency varied considerably between geographical populations of *Ph. nodorum*. In East Asia only two isolates carried the *SnToxA* gene in a sample of 93 isolates (deletion frequency = 98%). Europe and North America similarly exhibited high frequencies of gene deletions (90 and 75%, respectively). By contrast, no deletions of the *SnToxA* gene were observed in the Australian population. Intermediate frequencies of gene deletions were encountered in *Ph. nodorum* populations originating from Central America (58%), Central Asia (46%), the Middle East (63%) and South Africa (51%).

Haplotype distribution

In total, 123 *SnToxA* amplicons were sequenced. The *SnToxA* gene (excluding the promoter region) includes two exons encompassing 537 bp and one intron of 50 bp. Most of the polymorphic sites (21/22) in the *SnToxA* gene were located in the two exon regions and only one occurred in the intron. Figure 1 shows a haplotype alignment of the *SnToxA* exon regions. The 21 polymorphic sites were distributed among 13 different haplotypes of *Ph. nodorum*. Seven of these sites were parsimoniously informative. When the three *PtrToxA* sequences were included in the alignment, the total number of polymorphic sites was 25, ten of which were parsimoniously informative. Haplotype sequences were deposited in the NCBI database under accession numbers

Table 1 Continental populations of *Ph. nodorum* used to study distribution of *SnToxA*.

Population	Location	Host plant	Year	Collector	Total no. of isolates
Australia	Narogin	<i>T. aestivum</i>	2001	B.A. McDonald & R. Loughman	58
Central America	Michoacan, Mexico	<i>T. aestivum</i>	1993	L. Gilchrist	34
Central Asia	Kazakhstan	<i>T. aestivum</i>	2003/2004	H. Marite & E. Duveiller	32
	Kyrgyzstan	<i>T. aestivum</i>	2004	E. Duveiller	1
	Russia	<i>T. durum/T. aestivum</i>	2003	H. Marite & E. Duveiller	9
	Tadjikistan	<i>T. aestivum</i>	2004	E. Duveiller	7
	Uzbekistan	<i>T. aestivum</i>	2004	E. Duveiller	1
East Asia	Fujian Province, China	<i>T. aestivum</i>	2001	R. Wu	91
Europe	Denmark	<i>T. aestivum</i>	1994	M. Rasmussen	4
	England	<i>T. aestivum</i>	1992	M. Shaw	12
	Sweden*	<i>T. aestivum</i>	2005	E. Blixt	56
	Switzerland	<i>T. aestivum</i>	1999	B.A. McDonald & V. Michel	93
Middle East*	Ali Abad, Iran	<i>T. aestivum</i>	2005	M. Razavi	54
North America	Ohio	<i>T. aestivum</i>	2003	T. Friesen	18
	Minnesota	<i>T. aestivum</i>	2003	T. Friesen	3
	North Dakota	<i>T. aestivum</i>	1992/2002/2003	Leonard Francl & T. Friesen	54
	Oregon	<i>T. aestivum</i>	1993	M. Smith	96
	Texas	<i>T. aestivum</i>	1992	B.A. McDonald & L. Nelson	92
South Africa	South-Western Cape	<i>T. aestivum</i>	1995	P. Crous	73
Total					788

*Not included in the study by Friesen *et al.* (2006).

Table 2 *SnToxA* gene diversity in the geographical populations.

Continental region	% deletion of <i>SnToxA</i>	Sequenced isolates	No. of <i>SnToxA</i> haplotypes	Gene diversity (SD)
Australia	0	13	3	0.603 (0.088)
Central America	58.2	4	2	—*
Central Asia	46	27	4	0.39 (0.112)
East Asia	97.8	2	1	—
Europe	89.9	19	2	0.409 (0.10)
Middle East	63	18	2	0.209 (0.116)
North America	74.5	20	1	0.0 (0)
South Africa	50.7	20	7	0.835 (0.07)
All populations	60.1	123	13	0.77 (0.07)

*Gene diversity was not calculated for Central America and East Asia due to the low sample size.

EF108451–EF108463. In the previous study by Friesen *et al.* (2006) 11 *SnToxA* haplotypes were identified among 97 sequences. The two new haplotypes identified in this study both originated from Central Asia.

Translation of the *SnToxA* nucleotide sequences revealed six synonymous and 15 non-synonymous mutations. Six South African isolates represented the H3 haplotype that differed by nine mutations from the closest haplotype, H10 (Fig. 1). All nine mutations were G→A transitions and unique to this haplotype. Translation of the nucleotide sequence showed that two of the

base pair substitutions encoded stop codons, suggesting that the H3 allele is non-functional. Stop codons were not found in other *SnToxA* alleles. If the non-functional *SnToxA* allele in H3 is excluded, there were five synonymous and seven non-synonymous mutations in the haplotype alignment.

The frequency and distribution of haplotypes varied among the eight *Ph. nodorum* populations (Fig. 2). The lowest haplotype diversity was found in North America where only one *SnToxA* haplotype was present (Table 2). In East Asia only two positive haplotypes were present (Table 2). Two haplotypes were found in each of the population samples from Central America, Europe and the Middle East. In the Australian population, where we found the highest frequency of the gene, three haplotypes were present. In the Central Asian population sample we found four unique haplotypes. The highest diversity of haplotypes was in South Africa where seven haplotypes of the *SnToxA* gene were found; five of these were unique to South Africa.

The relationship between haplotypes and the distribution of synonymous and non-synonymous mutations among the 13 *SnToxA* haplotypes are illustrated in a haplotype network (Fig. 3). The position of a haplotype in a network shows how many mutations separate it from other haplotypes. More ancestral haplotypes can be identified by their internal position in the network and by their high frequency. The tip clades that are connected to the remainder of the network by only one connecting branch are considered more recently derived haplotypes. There were few mutational

Position	35	73	98	102	115	146	160	168	179	238	268	297	300	310	340	361	362	363	390	401	412	419	487	499	528	
Site number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	
Consensus	C	G	A	T	G	G	C	C	G	G	G	A	G	A	A	A	C	T	G	C	G	A	T	A		
Non-syn. ^a	*	*	*		*	*	*		*	*	*	*		*	*					*	*	*	*	*		
Site type ^b	v	t	t	t	t	t	v	t	t	t	t	v	t	t	t	-	-	v	t	t	v	t	v	v	v	
Character type ^c	-	-	-	i	-	-	-	-	-	-	-	i	-	i	i	i	i	i	-	-	i	-	i	i	-	
H1 (27)
H2 (1)	A	G	
H3 (6)	.	A	.	A	A	.	.	A	A	A	.	A	G	A	.	A	
H4 (3)	.	G	C	T	
H5 (44)	.	.	C	T	
H6 (3)	.	.	C	G	T	A	
H7 (2)	.	.	C	T	C	
H8 (21)	.	.	C	T	T	.	
H9 (1)	G	G	
H10 (12)	G	
H11 (1)	G	T	
H12 (1)	G	T	A	
H13 (2)	T	.	
H14 (59)	G	C	G	A	
H15 (1)	.	.	C	G	C	G	A	.	G	.	.	A	.	.	.	
H16 (1)	.	.	C	.	.	T	G	C	G	A	.	G	.	.	A	.	.	.	

Fig. 1 Haplotype alignment showing the distribution of haplotypes and synonymous and non-synonymous mutations among *SnToxA* sequences (exon regions) from 123 isolates of *Phaeosphaeria nodorum* (haplotypes 1–13) and three *PtrToxA* sequences of *Pyrenophora tritici-repentis* (haplotypes 14–16). Nucleotide positions are numbered relative to the start codon of the *ToxA* sequence. Numbers in parentheses show the number of sequences representing each haplotype.

^a Non-synonymous mutations marked with an asterisks.
^b t: transitions; v: transversions
^c i: parsimoniously informative site; -: uninformative site

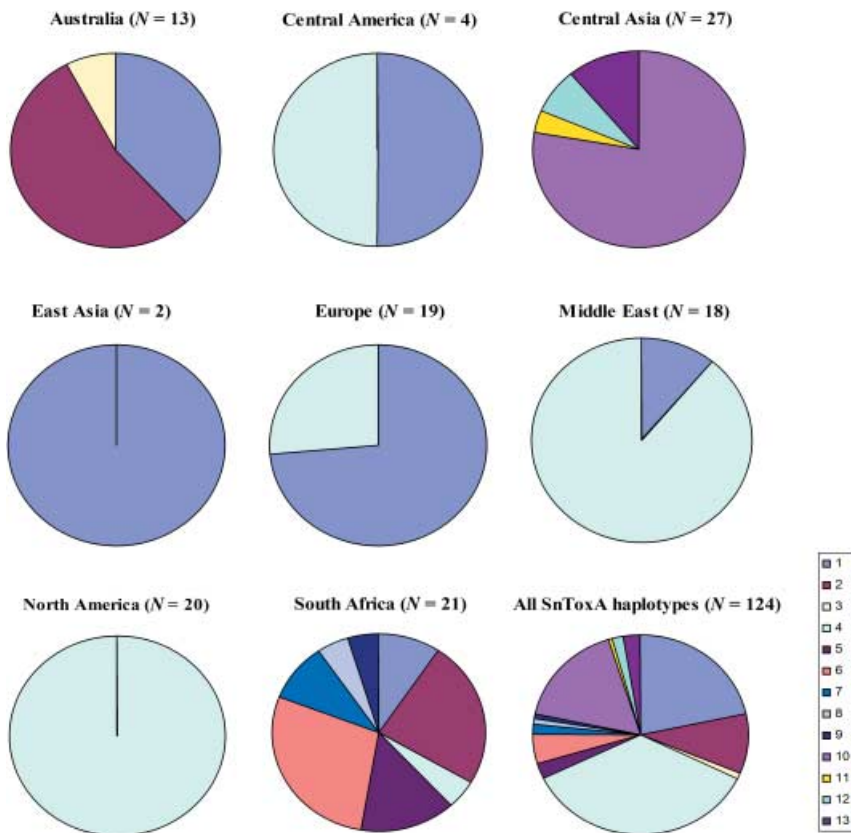


Fig. 2 Distribution of the 13 *SnToxA* haplotypes among and within *Phaeosphaeria nodorum* populations originating from six geographical regions.

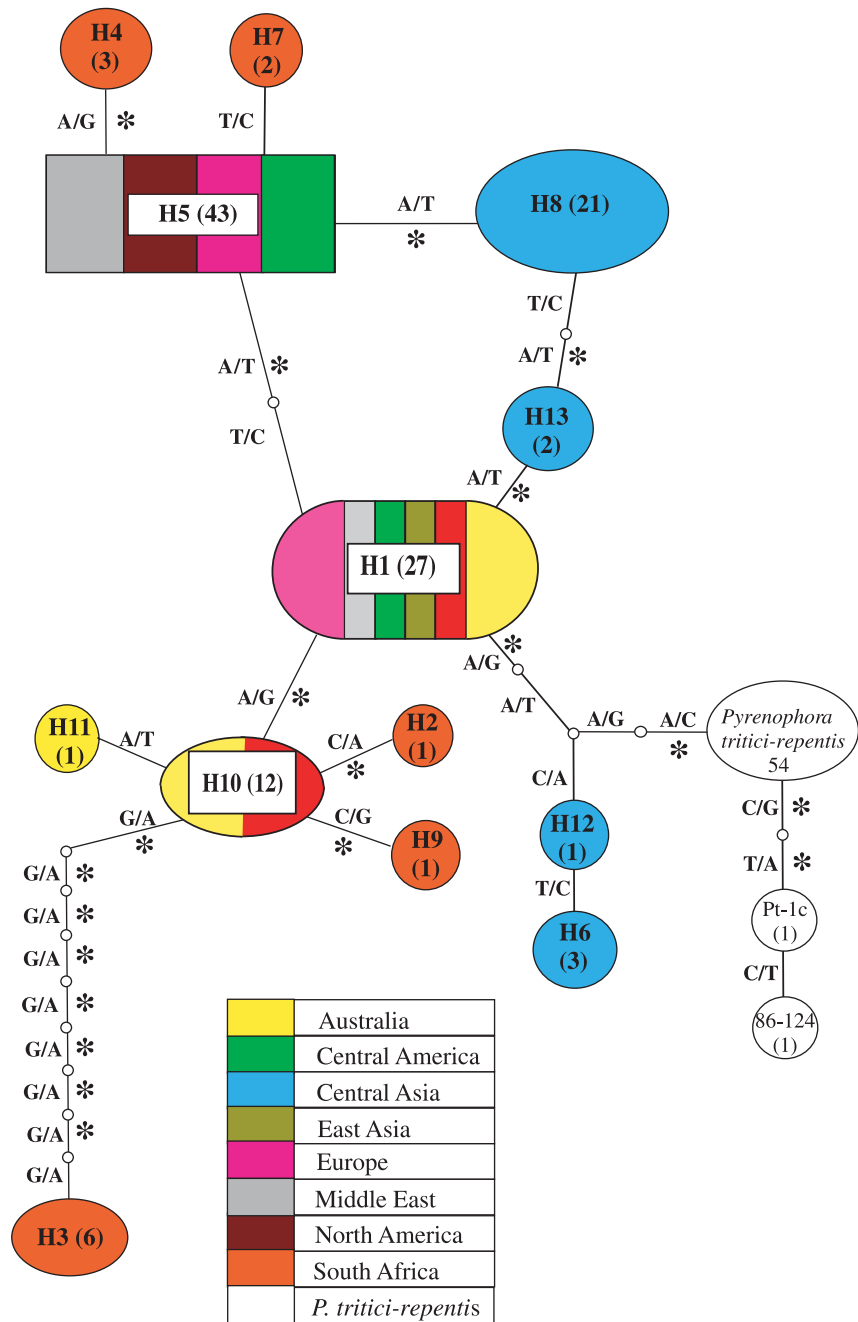


Fig. 3 Parsimonious haplotype network for the *SnToxA* gene amplified from 123 *Phaeosphaeria nodorum* isolates. The most common haplotype is shown in a rectangle. Each haplotype is identified with a unique number and numbers in parentheses refer to the number of isolates represented by this haplotype. If there is no number, the haplotype is represented by one isolate. Origins of haplotypes are indicated by different colours. The three haplotypes of *Pyrenophora tritici-repentis* are also included in the network. Dots are hypothetical missing intermediate haplotypes. Asterisks show non-synonymous mutations. Because of homoplasy in the network (shown by a loop between haplotype H5, H8, H13 and H1), two non-synonymous mutations are shown twice.

steps missing between the haplotypes, suggesting that a large fraction of the existing *SnToxA* diversity was present in the *Ph. nodorum* isolates included in the analysis. The lone exception was the South African haplotype H3 containing two stop codon mutations. No pattern of geographical grouping was revealed in the haplotype network. The unique South African haplotypes were found at the tips of the haplotype network while the two most frequent haplotypes were located at the centre of the network. The most frequent haplotype representing 43 isolates was found in Central America, Europe, the Middle East and North

America. We included the three *PtrToxA* sequences known in *Py. tritici-repentis* to illustrate their location among the *SnToxA* haplotypes. The *PtrToxA* haplotype described in Friesen *et al.* (2006) was found in 54 isolates originating from Europe, North and South America. The two additional *PtrToxA* haplotypes were previously published by Balance *et al.* (1996) and Ciuffetti *et al.* (1997). The haplotype network demonstrates a close phylogenetic relationship between the *SnToxA* haplotypes and the *PtrToxA* haplotypes. The *PtrToxA* haplotype representing 54 geographical samples differed by only three mutations from the *SnToxA*

Table 3 Parameter estimates and likelihood scores under models of variable ω ratios among sites for the *ToxA* gene.

Nested model pairs	d_N/d_S (Pn)	Parameter estimates (Pn)	l (Pn)	$2\Delta l$ (Pn)	P (Pn)	d_N/d_S (Pn + Ptr)	Parameter estimates (Pn + Ptr)	l (Pn + Ptr)	$2\Delta l$ (Pn + Ptr)	P (Pn + Ptr)
M0: one ratio	0.61	$\omega = 0.61$	-828.21	—	—	0.67	$\omega = 0.67$	-879.35	—	—
M3: discrete	0.77	$\rho_0 = 0.009,$ $\rho_1 = 0.96,$ $\rho_2 = 0.03,$ $\omega_0 = 0.0,$ $\omega_1 = 0.25,$ $\omega_2 = 16.04$	-823.88	—	—	0.88	$\rho_0 = 0.00,$ $\rho_1 = 0.99,$ $\rho_2 = 0.012,$ $\omega_0 = 0.0,$ $\omega_1 = 0.44,$ $\omega_2 = 36.00$	-868.89	—	—
M0/M3				8.66	0.07				20.92	< 0.001
M1a: neutral	0.35	$\rho_0 = 0.65,$ $\rho_1 = 0.35,$ $\omega_0 = 0.0,$ $\omega_1 = 1.0$	-826.65	—	—	0.34	$\rho_0 = 0.66,$ $\rho_1 = 0.34,$ $\omega_0 = 0.0,$ $\omega_1 = 1.0$	-876.44	—	—
M2a: selection	0.77	$\rho_0 = 0.97,$ $\rho_1 = 0.0,$ $\rho_2 = 0.03,$ $\omega_0 = 0.24,$ $\omega_1 = 1.0,$ $\omega_2 = 15.74$	-823.88	—	—	0.87	$\rho_0 = 0.52,$ $\rho_1 = 0.47,$ $\rho_2 = 0.01,$ $\omega_0 = 0.0,$ $\omega_1 = 1.0,$ $\omega_2 = 39.43$	-868.86	—	—
M1a/M2a				5.54	0.06				15.52	< 0.001
M7: beta	0.4	$\rho = 0.006,$ $q = 0.008$	-823.89	—	—	0.4	$\rho = 0.005,$ $q = 0.007$	-876.52	—	—
M8: beta & ω	0.75	$\rho_0 = 0.92$ ($\rho_1 = 0.083$), $\rho = 0.05,$ $q = 4.93,$ $\omega = 9.06$	-826.68	—	—	0.87	$\rho_0 = 0.99$ ($\rho_1 = 0.01$), $\rho = 0.02,$ $q = 0.03,$ $\omega = 39.09$	-868.86	—	—
M7/M8				5.58	0.06				15.32	< 0.001

Likelihood analyses performed for the 12 functional *Phaeosphaeria nodorum* *SnToxA* haplotypes (Pn).

Likelihood analysis performed for both the *Ph. nodorum* haplotypes and the three *Pyrenophora tritici-repentis* haplotypes (Pn + Ptr).

haplotype H12 from Central Asia. Among the three *PtrToxA* haplotypes we found three mutations of which two were non-synonymous.

Selection

The higher number of non-synonymous mutations suggests that the *SnToxA* gene may be under positive diversifying selection. We applied different approaches to test for selection in the gene. For all analyses of selection the non-functional allele of haplotype H3 was excluded.

We calculated Tajima's D and Fu and Li's F^* and D^* statistics to test the hypothesis of neutral selection in the *ToxA* gene (Fu and Li, 1993; Tajima, 1989). Analyses were initially performed for the entire sample. The three tests failed to reject the hypothesis of neutral evolution (Tajima's $D = -0.853$, $P > 0.10$; Fu and Li's $D = 0.418$, $P > 0.10$; Fu and Li's $F = -0.084$, $P > 0.10$). We also performed the test for each regional population but no departures from neutrality were found in any of the populations.

The d_N/d_S ratios (ω) across the entire coding sequence were calculated using nucleotide sequences of the 12 functional *SnToxA* alleles. We found that d_N exceeded d_S in 34 of 66 possible pair-wise comparisons of the 12 sequences. In 29 comparisons d_S exceeded d_N while d_S was 0 in three comparisons. These results suggest that the average value of ω is greater than one and that positive diversifying selection may contribute to the evolution of the *SnToxA* gene.

A maximum-likelihood (ML) method (Nielsen and Yang, 1998; Yang *et al.*, 2000) was used to estimate ω ratios and to test different evolutionary models against the data set (Table 3). The ML analyses were applied for two data sets. One data set included only the 12 functional *Ph. nodorum* *SnToxA* alleles. The second data set also included the *PtrToxA* alleles.

For the *Ph. nodorum* data set we obtained weak ($P = 0.06$ – 0.07) evidence of positive diversifying selection (Table 3). A neutral model M1 that allows ω to be either 0 (purifying selection) or 1 (neutral selection) fitted the data less well than the selection model M2 that includes an additional category of sites with ω

Table 4 Likelihood ratio tests for positive selection of amino acid sites.

ToxA	Model 1	Model 2	LRT statistics†	P value	Positively selected codons	Posterior probability ($\omega > 1$)	ω value
<i>Ph. nodorum</i>	M1	M2	5.54	0.06	121 N 163 I	0.996** 0.982*	15.993 15.77
	M0	M3	5.54	0.06	121 N 163 I	0.996** 0.982*	15.993 15.772
	M7	M8	5.64	0.06	121 N 163 I	0.996** 0.982*	15.986 15.761
	M1	M2	15.16	< 0.001	121 N 163 I	1.000** 0.537	39.428 21.653
<i>Ph. nodorum</i> and <i>Py. tritici-repentis</i>	M0	M3	15.16	< 0.001	121 N 163 I	1.000** 0.783	35.99 28.291
	M7	M8	16	< 0.001	121 N 163 I	1.000** 0.555	39.154 22.173

**Positively selected sites with posterior probability (P) values of > 0.99 ; *positively selected sites with posterior probability values of > 0.95 (Anisimova *et al.*, 2000). †LRT statistics follow a χ^2 distribution with degrees of freedom equaling 2.

estimated from the data ($P = 0.06$). The model M8 (beta & ω) allowing the value of ω to exceed 1 fit the data better than the model M7 (beta) where ω is only allowed to range from 0 to 1 ($P = 0.06$). Finally we tested how the discrete model M3, which allows the ω ratio to vary among branches, fit the genealogy. This model was compared with the model M0 where ω is held constant among branches. The model M3 fit the genealogy of the *Ph. nodorum* haplotypes better than the M0 model, suggesting that ω varies among the different haplotypes in the *SnToxA* genealogy ($P = 0.07$).

For the data set including the three *PtrToxA* haplotypes we found strong evidence of positive diversifying selection. The statistical significance was high ($P < 0.001$) for all likelihood ratio tests (LRTs) (Table 3). We found that the two site models M2 and M8 had the highest likelihood estimates. For the branch models the selection model M3 fit the *ToxA* genealogy better than the M0 model.

To test further whether specific sites were evolving under positive selection we also compared likelihood estimates of the three tests. The ω ratio is an average of all sites in the gene. For instance using the selection model M3 for the *ToxA* gene, the average ω ratio is 0.77 (only *SnToxA*) and 0.88 (*PtrToxA* included in the data set). The majority of sites are under purifying selection; however, the analysis identified two sites under positive diversifying selection with ω values > 1 . These two sites were amino acids 121 and 163 encoded by the base pairs 361–363 and 485–487, respectively. The ω values of these two codon sites were estimated as 15.99 and 15.77 (only *SnToxA*) and 39.99 and 28.29 (*PtrToxA* included in the data set). The likelihood estimates showed strongest support for the selection models in the data set that included the *PtrToxA* sequences. Table 4 summarizes the likelihood ratio tests for specific codon sites.

Recombination

In the coding region of the *SnToxA* gene, five pairs of sites ([102,361], [102,362], [102,363], [102,487] and [297, 487]) were found to be in conflict. Conflict between sites in a sequence can be caused by either recombination or homoplasy, where the same mutation occurs in different lineages of a phylogeny. Interestingly, the four sites introducing conflict in the data set (361, 362, 363 and 487) were all mutations unique to the Central Asian population. The amino acids changed by the mutations affecting base pairs 361–363 and 487 (amino acids 121 and 163) were both suggested to be under positive diversifying selection in the ML analysis (Table 4) as described above. No conflicting sites were present in the alignment if the Central Asian haplotypes were excluded from the analysis.

When we performed the same analysis including the three *PtrToxA* sequences we found only four pairs of conflicting sites ([102,340], [102,361], [102,487] and [297,487]). The conflicting sites [102,340] represented a mutation (site 102) in the highly frequent *SnToxA* haplotype H5 and a mutation (site 340) common for the three *PtrToxA* alleles. The remaining three pairs of conflicting sites were due to mutations unique to the Central Asian population.

DISCUSSION

Non-neutral modifications of the *Ph. nodorum SnToxA* locus occur through either non-synonymous mutations, stop codon mutations rendering the gene non-functional, or complete gene loss via deletion. The observed high level of non-neutral modifications suggests that the evolution of the *SnToxA* locus is driven by selection for different alleles or for genotypes in which the gene is not expressed at all.

We found significant differences in the frequencies of *SnToxA* deletions in eight geographical populations of *Ph. nodorum*. The high deletion frequencies found in China, Europe and North America suggest that there is a trade-off associated with the presence of the gene. It is possible that *SnToxA* was never present in the entire *Ph. nodorum* population, but rather originated as a horizontal gene transfer into the genome of one or a few *Ph. nodorum* strains. The finding of only one non-functional pseudogene supports this possibility. Interestingly, the *SnToxA* gene was fixed in the Australian population and lower frequencies of gene deletions were encountered in other populations such as Central America and South Africa. A global population survey of *Ph. nodorum* suggested that Australian and South African populations were more recently founded populations of the pathogen (Stukenbrock *et al.*, 2006). However, the different deletion frequencies may be due to different local selection pressures operating on the *SnToxA* locus in the different geographical regions whereby the absence of a compatible *Tsn1* allele selects for complete absence of the *SnToxA* locus. A number of other toxins produced by *Ph. nodorum* have been identified (Jørgensen and Smedegaard-Petersen, 1999) including two other host-specific toxins (Liu *et al.*, 2004; T.L. Friesen *et al.* unpublished data), and thus it is possible that a range of other toxins could compensate for the loss of *ToxA*.

The distribution of *SnToxA* haplotypes also varied among populations. The number of haplotypes per population ranged from one (China and North America) to seven (South Africa). A population genetic study using neutral microsatellite markers and a global collection of *Ph. nodorum* isolates showed low global population subdivision consistent with high amounts of gene flow (Stukenbrock *et al.*, 2006). It was suggested that the successful global spread and establishment of the pathogen could be explained by the highly homogeneous agricultural systems and host crops found world-wide coupled with the global trade in wheat grain and seed. However, in spite of the relatively homogeneous agricultural systems world-wide, we here find evidence that local conditions can select strongly for specific alleles or complete deletion of a pathogenicity-related gene. We hypothesize that the distribution and diversity of *SnToxA* haplotypes and the frequency of gene deletions reflect the composition and frequencies of different *Tsn1* alleles present in local host populations.

This is the first population study to demonstrate how non-neutral selection drives the evolution of a host-specific toxin in a fungal pathogen. Using both an approximate method (Nei and Gojobori, 1986) and an ML method (Nielsen and Yang, 1998) we obtained weak evidence for positive diversifying selection in the *SnToxA* gene. Among the three *PtrToxA* haplotypes two out of three mutations were non-synonymous. Although this is not conclusive evidence of positive diversifying selection in the gene, it demonstrates that non-neutral changes also occur in this species.

The *SnToxA–Tsn1* interaction presents a mirror image of the selection scenario presented by the classical gene-for-gene system. We hypothesize that genetic variability is likely to be maintained in the plant susceptibility gene (e.g. *Tsn1* in this case) in order to avoid a compatible interaction with *SnToxA*. Under this scenario, the emergence of new, resistant *Tsn1* alleles would be followed by selection for new, toxigenic *SnToxA* alleles to regain compatibility with the host gene. *Tsn1* is located in a recombination hot spot in the chromosome 1AS in wheat (Haen *et al.*, 2004), suggesting that frequent recombination could be a mechanism to create new alleles of the toxin sensitivity gene just as recombination is known to be a mechanism creating new alleles of host resistance genes (Ellis *et al.*, 2000). The future cloning and characterization of *Tsn1* will allow population samples to be compared to test the hypothesis of positive diversifying selection in the host gene.

We also considered whether recombination could play a role in the evolution of *SnToxA*. Recombination has previously been shown to occur at higher rates in genes that are under positive diversifying selection (Ellis *et al.*, 2000; McDowell *et al.*, 1998; Polley and Conway, 2001). We found five incompatible sites among the *Ph. nodorum* haplotypes. When the *PtrToxA* sequences were included in the analysis, the number of conflicting sites was reduced to four. The four mutations introducing conflict into the alignment between *Ph. nodorum* haplotypes were all unique to the Central Asian population. Three of these mutations (base pairs 361–363) were also found in the *Py. tritici-repentis* haplotypes and conferred a change in the protein sequence. Frequent sexual recombination has been demonstrated in populations of *Ph. nodorum* (Keller *et al.*, 1997a; Sommerhalder *et al.*, 2006; Stukenbrock *et al.*, 2006) and if recombination occurred regularly in the *SnToxA* locus, we would expect to find incompatible sites in other populations. As the gene is under positive diversifying selection, we consider it more likely that the conflicts, especially those involving sites 361–363, are a result of convergent evolution in the gene due to selection for specific mutations or combinations of mutations. The ML analysis indicated that amino acids 121 and 163, encoded by nucleotides at positions 361–363 and 487, respectively, are indeed under positive selection, offering support for this hypothesis.

Another possibility is that a host factor other than *Tsn1* could also impose selection on *SnToxA*. Under this scenario, the toxin gene could play a secondary role as an elicitor for plant resistance in resistant cultivars as observed for the *NIP1* gene in the pathogen *Rhynchosporium secalis* (Schürch *et al.*, 2004). The elicitor encoded by *NIP1* is recognized as an elicitor for plant resistance in barley cultivars carrying the resistance gene *Rrs1* (Rohe *et al.*, 1995). The *NIP1–Rrs1* interaction is an example of a classic gene-for-gene interaction where positive diversifying selection on the elicitor gene is driven by an antagonistic coevolution with the host resistance gene. However, in non-resistant cultivars the *NIP1* protein also functions as a necrosis-inducing peptide providing a

benefit to the pathogen upon infection. As found for *SnToxA* in *Ph. nodorum*, highly variable *NIP1* deletion frequencies were observed in *R. secalis*, ranging from 0 to 100% among different geographical populations and probably reflecting the frequency of the host gene *Rrs1* (Schürch *et al.*, 2004). Could the *SnToxA* gene product have a similar dual function? Friesen *et al.* (2003) showed that *PtrToxA* plays a role as a virulence factor in *Py. tritici-repentis* by influencing the degree of disease in susceptible host cultivars. Although other plant genes may be affected by the toxin, *Tsn1* was identified as the major determinant for susceptibility to both *Py. tritici-repentis* and *Ph. nodorum* (Liu *et al.*, 2007). If another host-encoded protein operates as a standard resistance gene that can detect *ToxA* and activate host defences, this also could impose positive diversifying selection on *ToxA*. One prediction under this hypothesis is that some host plants carrying *Tsn1* would show less susceptibility to *ToxA*-producing pathogen strains.

Different hypotheses may be considered to explain the geographical distribution of *SnToxA* haplotypes and deletion frequencies. First, it is possible that the origin of either the *ToxA* gene or *Ph. nodorum* itself is in South Africa. The high *SnToxA* haplotype diversity in South Africa suggests that the gene may have existed in this population for a long time, while the lower level of gene diversity found using neutral microsatellite markers indicates that South Africa is a derivative population and not the centre of origin of *Ph. nodorum*. This pattern is consistent with the possibility that the *ToxA* gene originated in South Africa. Wheat was introduced into South Africa in 1652 with Dutch settlers. It is likely that associated seed-borne pathogens, including *Ph. nodorum*, were introduced with the crop plant. *Ph. nodorum* could have acquired the *ToxA* gene in South Africa through a horizontal gene transfer, subsequently evolving a number of different *ToxA* haplotypes. The lack of variation in the *ToxA* intron supports either a recent selective sweep or an introduction into *Ph. nodorum* within the last 350 years. The *ToxA* gene is located close to a transposase-like sequence (Friesen *et al.*, 2006) and could have been inserted into the *Ph. nodorum* genome from, for example, a bacteria or another fungus also associated with wheat. Estimates of migration showed that the South African *Ph. nodorum* population is mainly a sink population of immigrants (Stukenbrock *et al.*, 2006). The paucity of wheat exports from South Africa to other populations could explain why only few *SnToxA* alleles are present elsewhere.

An alternative hypothesis is that both the South African and the Central Asian populations were isolated from the global trade in wheat grain and germplasm due to apartheid and the Cold War, respectively. Transport of infected plant material and seeds is believed to be a major contributor to the spread of *Ph. nodorum*, and the isolation of South Africa and Central Asia may have created more differentiated populations of the pathogen in these places. Different wheat cultivars grown more

often in South Africa and Central Asia and used less often in other parts of the world could have strongly selected for a higher diversity of *SnToxA* alleles.

A third hypothesis is that *SnToxA* used to be present at high frequencies in all populations and that the haplotype diversity was similarly higher in the past in populations including Europe, East Asia and North America. Haplotype diversity may have been reduced as the development of new wheat varieties selected for a few specifically adapted *SnToxA* alleles such as the dominant haplotypes H1 and H5 or for the complete deletion of the gene. Haplotype diversity could have been maintained in the more isolated populations such as South Africa and Central Asia. As a general component of the different hypotheses, our results suggest that strong selection imposed by the local host population shaped the distribution of *SnToxA* alleles and gene deletions in the different populations.

Evolution is likely to occur more quickly for genes that are under strong selection, such as those involved in host-parasite gene-for-gene interactions (Bergelson *et al.*, 2001; Brodie and Brodie, 1999; Zheng *et al.*, 2004). Wheat has been cultivated by humans for approximately 12 000 years (Salamini *et al.*, 2002). Within this time frame adaptation and speciation of the wheat pathogen *Ph. nodorum* has occurred. Although this is a relatively short time in evolution, rapid co-adaptation and co-speciation of pathogens to new agricultural crops have previously been demonstrated (Couch *et al.*, 2005; Stukenbrock *et al.*, 2007). We hypothesize that a considerable number of adaptive mutations have accumulated in the *SnToxA* gene within a relatively short time frame to counter-adapt to new alleles of the *Tsn1* locus or in response to a second plant gene interacting with the toxin. Similar analyses conducted with genes encoding other HSTs will be needed to differentiate among the different hypotheses regarding the evolutionary processes affecting HSTs. Additional knowledge of the emergence and speciation of *Ph. nodorum* will be needed to understand better the molecular coevolution of the pathogenicity-related gene *SnToxA*.

EXPERIMENTAL PROCEDURES

Isolate collection

A total of 788 *Ph. nodorum* isolates were screened for the presence of *SnToxA*. The isolates originated from 19 field populations representing eight geographical regions: Australia (58 isolates), Central America (34), Central Asia (50), East Asia (91), Europe (165), Middle East (54), North America (263) and South Africa (73) (Table 1). Because sampling strategies differed among the 19 populations, the geographical populations represented different spatial scales within each region. The populations from Australia, Central America, East Asia, Europe except Sweden, North America and South Africa were characterized in earlier studies (Keller

et al., 1997a,b; Sommerhalder *et al.*, 2006; Stukenbrock *et al.*, 2006). The population from Sweden was kindly provided by E. Blixt and consisted of isolates obtained from a hierarchical sampling within one field. The population from the Middle East consisted of isolates originating from eight plots within one wheat field. The collection from Central Asia was kindly provided by E. Duveiller and H. Marite and consisted of randomly sampled isolates from five different regions: Kazakhstan, Kyrgyzstan, Russia, Tajikistan and Uzbekistan. All isolations were made from bread wheat except the Russian collection in which three isolations were made from durum wheat. Isolation procedures and DNA extractions were as described previously (McDonald *et al.*, 1994). We also included 54 *PtrToxA* sequences used in the study of Friesen *et al.* (2006) and two *PtrToxA* sequences published by Balance *et al.* (1996) and Ciuffetti *et al.* (1997) (accession numbers U79662 and AF004369, respectively).

Data collection

In the previous study by Friesen *et al.* (2006) 97 *SnToxA* sequences were obtained. In addition to these 97 sequences, we sequenced the *SnToxA* gene from 18 isolates originating in the Middle East and eight isolates from Central Asia not included in the previous study. PCR of *SnToxA* was performed as described in Friesen *et al.* (2006) using forward primer ACCGTCGGCTACCTAGCAA and reverse primer CGTTCGGTTGTGCTCTCCT. The annealing temperature was 58 °C. The deletion frequency was assessed by determining the number of isolates in which the *SnToxA* amplification failed. For 400 randomly chosen isolates the *SnToxA* amplification was repeated as a control of PCR error. The same PCR results were obtained in the second PCR. In a previous study Southern blot analyses were performed to confirm the deletion of the gene when PCRs were negative (Friesen *et al.*, 2006). Sequencing of *SnToxA* was performed as described in Banke *et al.* (2004). Samples were analysed on an ABI 3100 automated sequencer. Sequences for each isolate were obtained with forward and reverse primers and were aligned and edited manually using the program Sequencher 4.1 (Gene Codes Corp., Ann Arbor, MI). Further processing of sequence alignments was performed using the free software Bioedit Sequence Alignment Editor (www.mbio.ncsu.edu/BioEdit/bioedit.html/bioedit.com).

Data analyses

Gene diversity and haplotype networks

The number of haplotypes and gene diversity of the *SnToxA* sequences were calculated for each geographical population using the program Dnasp version 3.53 (Nei, 1987; Rozas and Rozas, 1999).

Alignments were initially processed in the SNAP workbench Java program package, which implements and coordinates

several programs to analyse gene genealogies and population parameters (Price and Carbone, 2005). Using the program SNAP Map (Aylor *et al.*, 2006) we collapsed sequences into unique haplotypes categorizing base substitutions as phylogenetically informative or uninformative, transitions or transversions, and non-synonymous or synonymous mutations. We analysed the haplotype alignments in the program TCS 1.3 (Clement *et al.*, 2000). This program applies a statistical parsimony method to infer unrooted cladograms based on Templeton's 95% parsimony connection limit (Templeton *et al.*, 1992). A haplotype alignment showing the distribution of haplotypes was generated by the program. The mutational steps in the haplotype network were identified manually and defined as either synonymous or non-synonymous mutations.

Tests of selection

We used different approaches to test for neutral selection. For all tests only the 12 functional *SnToxA* alleles were included. Tajima's *D* and Fu and Li's *D** and *F** test statistics based on estimates of nucleotide polymorphism were calculated for all *Ph. nodorum* sequences using Dnasp (Fu and Li, 1993; Rozas and Rozas, 1999; Tajima, 1989).

In the next analysis we compared synonymous and non-synonymous substitution rates. The ratio between non-synonymous and synonymous nucleotide substitutions (ω) indicates which type of selection is acting on the gene under consideration. If purifying selection removes non-synonymous substitutions ω will be < 1 . If non-synonymous mutations are neutral they will be fixed at the same rate as synonymous mutations and ω will be $= 1$. If non-synonymous substitutions are beneficial they will be retained by positive diversifying selection and ω will be > 1 . The rates of non-synonymous nucleotide substitutions per synonymous site across all amino acid sites were estimated using the approximate method of Nei and Gojobori (1986) using the YN00 program implemented in the PAML software package (Yang, 1997).

As an additional test for positive diversifying selection we applied an ML method developed by Yang and colleagues (Nielsen and Yang, 1998; Yang, 1998). Six codon substitution models were used to investigate whether positive selection was acting on the *ToxA* gene. These models viewed the codons as the fundamental unit of evolutionary change and took into account the genealogical history when estimating likelihood scores. The likelihood scores evaluated the quality of the fit of the input data to the conditions of the model. Analyses were conducted in the program CODEML also implemented in the PAML software package (Yang, 1997). Both a maximum-parsimony and a neighbour-joining genealogy of the 12 functional *SnToxA* alleles were initially constructed in the program PAUP using a heuristic search strategy (Swofford, 1998). ML analyses were performed in CODEML using both the maximum-parsimony tree and the neighbour-joining tree as in-files.

First, we applied four different site models to the genealogy in which ω is allowed to vary across codons while being held constant along branches (Nielsen and Yang, 1998; Yang *et al.*, 2000). We compared the neutral model M1a in which mutations are either neutral or deleterious to the selection model M2 which includes a third category of advantageous mutations. Next, we compared likelihood estimates of the model M7 where the distribution of ω was allowed to range from 0 to 1 across all codon sites (beta distribution) with the alternative model M8 where ω was allowed to exceed 1 (beta & ω). Finally, we examined how models of evolution that allowed ω to vary among branches (branch models, Yang, 1998) fit the *SnToxA* genealogies. Likelihoods were estimated where ω was held constant along all branches (M0) and where ω was allowed to vary freely among branches (M3). Likelihood estimates of the evolutionary models were compared using an LRT.

Recombination

SnToxA haplotypes were also examined for evidence of past recombination events. We used the four-gamete test and Hudson and Kaplan recombination *R* statistics (Hudson and Kaplan, 1985). The four-gamete test was executed on the complete *SnToxA* alignment to identify incompatible pairs of sites. Conflicting sites were also recognized in the SNAP workbench by generating compatibility matrices using the program SNAP Matrix (Markwordt *et al.*, 2003; Price and Carbone, 2005).

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