Eur J Plant Pathol (2009) 124:577–583
DOI 10.1007/s10658-009-9443-1

Cercospora zeina is the causal agent of grey leaf spot disease of maize in southern Africa

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Received: 23 June 2008 / Accepted: 2 February 2009 / Published online: 17 February 2009

Abstract The aim of our study was to identify the causal agent of grey leaf spot disease of maize in southern Africa. Single-conidial cultures were recovered from maize leaves with typical disease symptoms sampled from several fields in South Africa, Zambia and Zimbabwe. Morphology, cultural characteristics, and a PCR-based test using *Cercospora zeae-maydis* and *C. zeina*-specific primer sets identified all single-conidial cultures as *C. zeina*. In addition, sequence alignment of DNA fragments of the internal transcribed spacer region (ITS1, ITS2, and the 5.8S gene) and elongation factor 1-α grouped all cultures in the same clade as the *C. zeina* ex-type culture CBS 118820. To by-pass cultivation of the slow-growing fungus, a rapid method to isolate DNA directly from lesions was successfully applied for PCR identification of *C. zeina* with species-specific ITS and histone primers. Koch's postulates were fulfilled for *C. zeina* by artificially inoculating maize plants in a greenhouse, re-isolating conidia emerging from lesions and verifying pathogen identity with molecular techniques. These results provide evidence that confirms the presence of *C. zeina* and absence of *C. zeae-maydis* in commercial maize plantations in southern Africa.

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Keywords Diagnostic species-specific PCR assay · Grey leaf spot · Maize · Koch's postulates · *Cercospora zeina* · *Cercospora zeae-maydis*

Abbreviations

CI	consistency index
EF	elongation factor 1-α
GLS	grey leaf spot
ITS	internal transcribed spacer
RI	retention index

Introduction

Grey leaf spot (GLS) is a yield-limiting foliar disease of maize (*Zea mays*) of great economic importance in many countries (Latterell and Rossi 1983; Ward et al. 1999). GLS was first reported in Illinois (USA) by Tehon and Daniels (1925). Reduced-tillage practices and continuous monoculture of maize have recently led to a dramatic increase in disease prevalence in warm and humid climates (Ward et al. 1999). Occurrence of GLS in Africa was initially observed in KwaZulu-Natal (South Africa) in 1988 (Ward 1996). The disease then spread rapidly north into the continent (Ward 1996; Nowell 1997; Ward et al. 1997).

More recently, molecular techniques have allowed a detailed analysis of the genetic variability of the pathogen population and the existence of two very distinct groups was revealed. Isolates of Group I were found throughout various areas of the USA and in Mexico, whereas Group II isolates were found in the eastern third of the USA, Brazil, Kenya, Rwanda, Uganda, South Africa, Zambia and Zimbabwe (Wang et al. 1998; Dunkle and Levy 2000; Goodwin et al. 2001; Okori et al. 2003; Shim and Dunkle 2005). Crous et al. (2006) reclassified Group II as a distinct species now called *C. zeina*. Because of the higher genetic variability of *C. zeina* in Africa compared to the USA, it was postulated that the GLS pathogen from Africa was introduced to the USA (Dunkle and Levy 2000).

The aim of our study was to further test whether *C. zeae-maydis* is the primary cause of GLS in southern Africa. For this purpose, we collected infected leaf samples with typical GLS symptoms from several fields in South Africa, Zambia and Zimbabwe. Characteristic single conidia were recovered from these diseased leaves and identified as *C. zeina* based on morphology, cultural characteristics, and a PCR-based test using *C. zeae-maydis* and *C. zeina*-specific primer sets designed to a fragment of the histone H3 gene (Crous et al. 2006). Sequences from the internal transcribed spacer region (ITS1, ITS2, and the 5.8S gene) and the elongation factor 1-α (EF) gene were obtained for 71 fungal cultures and compared. To avoid the time-consuming cultivation of single conidia of the slowly-growing pathogen, DNA was isolated directly from typical GLS lesions and used in a diagnostic PCR. In addition, we fulfilled Koch's postulates for *C. zeina* by inoculating maize plants in a greenhouse with *C. zeina* conidia from sporulating cultures, re-isolating single spores from lesions, and confirming identity of the pathogen with molecular techniques.

Materials and methods

Sample collection and isolation procedures

Leaves from five to ten maize plants per field with representative GLS lesions were sampled in the South African province KwaZulu-Natal from two fields in Cedara, two fields in Greytown, three fields in Karkloof and one field in Winterton. In addition, two fields in Zambia and one in Zimbabwe were sampled (Table 1). The dimensions of at least 50 conidia and 25 conidiophores each from lesions on five to ten leaves per field were measured with an ocular micrometer, and characteristics of the conidia and conidiophores were recorded.

Single conidia were isolated, transferred to V8 medium (800 ml of distilled water, 200 ml of V8 juice, 15 g of agar, 2 g of $CaCO_3$) and cultured for up to 3 months in the dark at 25°C. The *C. zeae-maydis* ex-type culture CBS 117757 was obtained from the culture collection of the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. Cultures used

Table 1 Fungal isolates collected from grey leaf spot disease lesions of maize and used for this sequence study

Isolate no.[a]	Field	Area	Country	Total number of isolates analysed	GenBank acc. no. (EF, ITS)
CMW 25465	C3 8A	Cedara	South Africa	5	EU569209, EU569220
CMW 25462	Vimy Ridge	Cedara	South Africa	5	EU569210, EU569224
CMW 25463	Chipperfield Farm	Greytown	South Africa	9	EU569211, EU569221
CMW 25448	Hildesheim Farm	Greytown	South Africa	6	EU569208, EU569219
CMW 25454	Preston	Karkloof	South Africa	10	EU569212, EU569222
CMW 25452	Gartmore	Karkloof	South Africa	7	EU569213, EU569229
CMW 25466	Near Yarrow	Karkloof	South Africa	5	EU569214, EU569223
CMW 25459	Brandkraal	Winterton	South Africa	5	EU569215, EU569226
CMW 25467	Mkushi	Mkushi	Zambia	6	EU569218, EU569227
CMW 25445	Gart Farm	Lusaka	Zambia	8	EU569217, EU569225
CMW 25442	Art Farm	Harare	Zimbabwe	5	EU569216, EU569228

[a] CMW = culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa

in this study are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa, and representative cultures have been deposited in the Mycothèque de l'Université catholique de Louvain (MUCL), Belgium.

Artificial inoculation of maize plants

Maize plants of the *C. zeina*-susceptible hybrid PAN 6724B were planted in a greenhouse at 28±4°C under a 16-h day length. Sporulation of *C. zeina* culture CMW 25463 grown on V8 medium was enhanced by incubating the plates under diurnal light (12 h of fluorescent light, 12 h of dark). Conidia were dislodged with a brush and rinsed with 0.01% Tween 20. The inoculum was diluted to 3×10^4 conidia ml^{-1} and applied onto all three leaves (V3 stage) of the maize plants with a small brush. Control plants were treated with 0.01% Tween 20. Plants were covered with plastic bags for 5 days.

DNA isolation and PCR analyses

Mycelium was scraped off V8 plates, ground to a fine powder in liquid nitrogen and fungal DNA isolated according to Möller et al. (1992). DNA from lesions was isolated by the protocol of Griffith and Shaw (1998) using a modified extraction buffer which contained 100 mM Tris–HCl [pH 8.0], 1.4 M NaCl, 2% CTAB, and 20 mM EDTA [pH 8.0]. Integrity of the DNA was checked on a 0.8% agarose gel and concentration was determined spectrophotometrically.

Approximately 30 ng of DNA served as template in PCRs (25 µl) containing 18 µl of distilled water, 1.7 µM each of the primers, 1.0 mM of each dNTP (Fermentas, St. Leon-Roth, Germany), 2.7 mM $MgCl_2$, 2.5 µl of 10× NH_4 reaction buffer and 2.5 U of BIOTAQ™ DNA Polymerase (Bioline, London, UK). A partial sequence of histone H3 was amplified from DNA isolated from fungal mycelium with the general histone primer pair CYLH3F/CYLH3R and the *Cercospora* species-specific primer pairs CzeaeHIST/CylH3R and CzeinaHIST/CylH3R (Crous et al. 2006). Part of the nuclear rRNA operon spanning the 3′ end of the 18S rRNA gene, the first ITS region, the 5.8S rRNA gene, the second ITS region and the 5′ end of the 28S rRNA gene was amplified from DNA isolated from fungal mycelium with the primer pair ITS1 and ITS4 (White et al. 1990). A *C. zeae-maydis*/*C. zeina*-specific ITS region was amplified from DNA isolated from typical lesions with the primers ITSC2fwd (5′-gtcggagtttaag taaattaaacaa-3′) and ITS4 in the presence of 1% 2-pyrrolidone (Chakrabarti and Schutt 2001).

The primers EF1-728F and EF1-986R were used to amplify a fragment of the elongation factor 1-α (EF) gene from DNA isolated from fungal mycelium (Carbone and Kohn 1999). Cycling conditions were 2 min at 94°C followed by 35× (20 s at 94°C, 30 s at 52°C for the general histone PCR, 53°C for both ITS PCRs and EF PCR and 58°C for the species-specific histone PCR, 40 s at 72°C). A final elongation step was performed at 72°C for 5 min. Quality of all PCR products was verified by gel electrophoresis. PCR products were purified with the Invisorb® Spin PCRapid Kit (Invitek Gesellschaft für Biotechnik & Biodesign mbH, Berlin, Germany).

DNA sequencing and data analysis

Purified PCR products were used as template for sequencing reactions on an ABI PRISM™ 3100 automated DNA sequencer (Perkin Elmer, Norwalk, CT). The ABI Prism Big Dye Terminator Cycle sequencing reaction kit v1.1 (Perkin Elmer Applied Biosystems, Foster City, CA) was used for the sequencing reactions conducted with the same forward primers used for the PCR. Sequences were analysed by Sequence Navigator 1.0.1 (Perkin-Elmer Applied Biosystems) and blastN (Altschul et al. 1990). Sequences were aligned using the Clustal function of Sequence Navigator and gaps were inserted manually where necessary.

Parsimony analyses were conducted on the combined EF and ITS1/ITS4 data matrices obtained using PAUP 4.0b10 (Swofford 2002). Following a partition homogeneity test, data from both loci could be combined. Trees were generated using a heuristic search algorithm with random addition of taxa and tree bisection reconnection branch swapping. Bootstrap analysis of 1,000 replicates on the aligned sequences was done to gain confidence at the nodes. Settings were the same as within the parsimony analysis but with sequences added sequentially. Measures to evaluate the quality of the cladogram included a consistency index (CI) and retention index (RI). The cladogram was rooted to two strains of *Mycosphaerella thailandica* (CPC10548 and CPC10549).

Results

Identification of isolates

No differences in the results of the morphological and molecular tests applied could be found among the five to ten isolates obtained from plants with GLS disease symptoms at each location (Fig. 1a–c). Therefore, we only present data obtained from one representative isolate per field (11 isolates among the 71 mono-conidial isolates sampled in total).

Morphological characteristics of conidiophores and conidia obtained from all sampled lesions and from single-conidial cultures were examined. Conidiophores ranged from 42.8 to 125.8 μm long and 4.4 to 8.3 μm wide with mean dimensions of 78.7× 5.9 μm (Fig. 1d). Conidia were 39.7 to 71.3 μm long and 4.8 to 9.1 μm wide with mean dimensions of 56.2×6.6 μm (Fig. 1e). Fungal cultures were olivaceous-grey with white patches and grew about 3 mm in diameter per week (Fig. 1f).

Fungal DNA of all single-conidial cultures listed in Table 1 and the *C. zeae-maydis* culture CBS 117757 was proven to be intact by amplification of a 389-bp histone fragment using the general histone primers (CYLH3F/CYLH3R; Fig. 2a). The *C. zeae-maydis*-specific primers (CzeaeHIST/CylH3R) amplified a 284-bp histone gene fragment with DNA from the *C. zeae-maydis* culture CBS 117757 only (Fig. 2b), while the *C. zeina*-specific primer set (CzeinaHIST/CylH3R) produced a 284-bp histone gene fragment in all single-conidial cultures obtained from Africa (Fig. 2c). Formation of additional amplicons was observed when these histone PCRs were multiplexed as suggested by Crous et al. (2006). Therefore, the primer sets were run in separate reactions to obtain single PCR products.

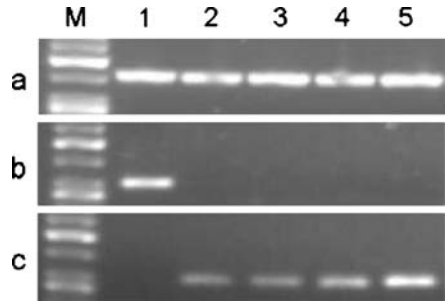

Fig. 2 PCR to distinguish *Cercospora* species. Amplicons produced with general histone primers CYLH3F/CYLH3R that served as a positive control (**a**), *C. zeae-maydis*-specific primers CzeaeHIST/CylH3R (**b**) and *C. zeina*-specific primers CzeinaHIST/CylH3R (**c**) are shown. *Lane M* GeneRuler™ 50-bp DNA ladder (Fermentas), *1 C. zeae-maydis* isolate CBS 117757, *2* CMW 25465, *3* CMW 25462, *4* CMW 25463, *5* CMW 25448

Sequencing of the ITS1/ITS4 region of the rDNA operon and the EF gene fragment resulted in nucleotide information of approximately 470 and 270 bp, respectively, for all isolates listed in Table 1. Sequence alignment of DNA fragments of the ITS1/ITS4 and EF regions revealed no differences between the single-conidial cultures. All 71 isolates collected

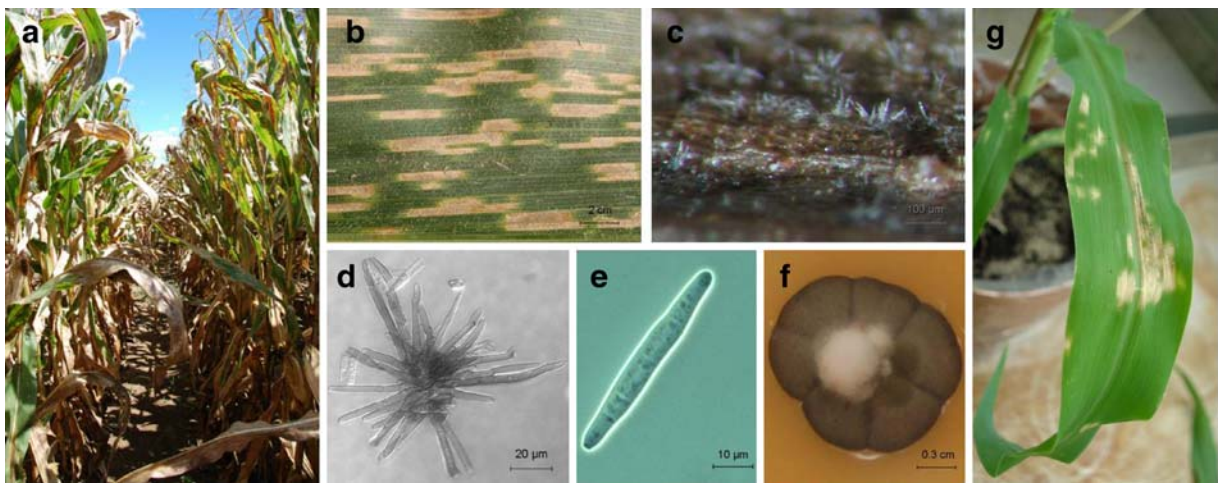

Fig. 1 Grey leaf spot on maize caused by *Cercospora zeina*. **(a)** Disease symptoms in the field, **(b)** close-up of GLS lesions on maize leaf, **(c)** conidiophores on leaf surface, **(d)** conidiophores, **(e)** conidia, **(f)** *C. zeina* in culture, and **(g)** artificially inoculated leaf

in southern Africa in this study could be classified as *C. zeina* (Fig. 3) which is consistent with the observation of Dunkle and Levy (2000), Okori et al. (2003) and Crous et al. (2006).

A partition homogeneity test showed that both loci could be combined (P=0.53). Among the 692 characters analysed, 179 were parsimony-informative. Heuristic tree searches based on parsimony produced one cladogram with a length of 203 steps, with CI=0.947 and RI=0.960. All isolates representing *C. zeina* formed in a monophyletic group supported by 100% bootstrap support (Fig. 3).

DNA isolation from host lesions

DNA from typical GLS lesions (Fig. 1b) was directly isolated successfully and used in PCR to amplify and sequence an ITS rDNA fragment. PCR with a primer (ITSC2fwd) specific to *C. zeae-maydis* and *C. zeina* and the commonly used ITS4 primer produced an amplicon of approximately 400 bp from DNA isolated from fungal cultures of both *C. zeae-maydis* and *C. zeina* and host lesions caused by *C. zeina* (Fig. 4). Amplification of the ITS fragment was exclusive for fungi since the DNA from plant tissue did not yield a PCR product. The nucleotide sequence from the host lesion had 100% identity to an ITS fragment from *C. zeina* (ex-type culture CBS 118820).

Amplification of a 389-bp histone fragment with the general histone primers (CYLH3F/CYLH3R) from DNA isolated from GLS lesions was successful (Fig. 5). Primers (CzeaeHIST/CylH3R) designed to the *C. zeae-maydis* histone sequence did not produce a fragment, while the *C. zeina*-specific primer set (CzeinaHIST/CylH3R) generated a 284-bp histone gene fragment (Fig. 5).

Koch's postulates

Koch's postulates were fulfilled for *C. zeina*. The CMW 25463 culture was obtained from a single conidium isolated from a symptomatic maize leaf

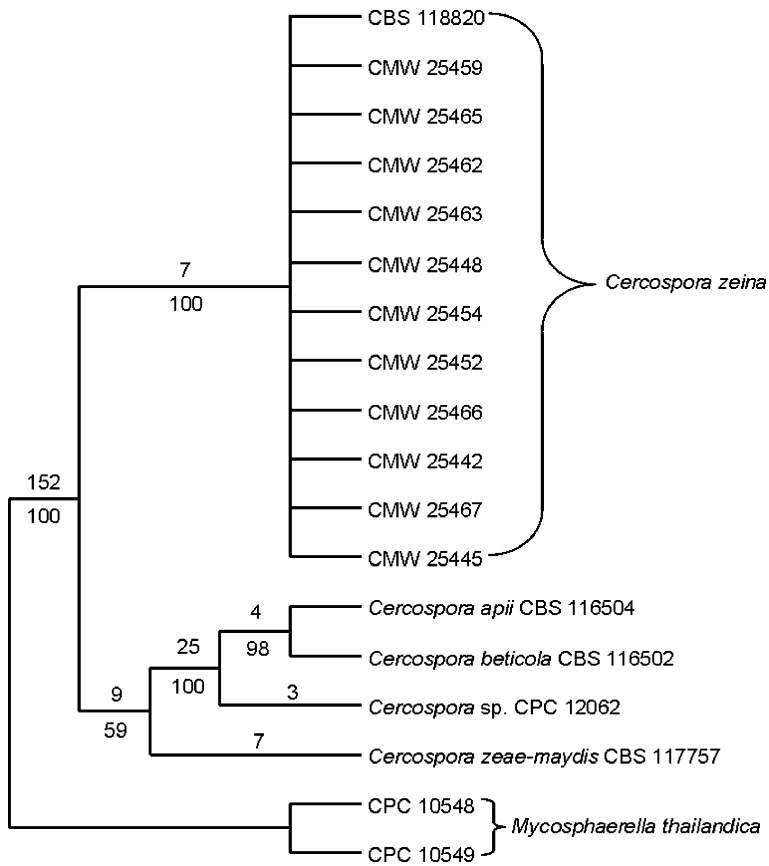

Fig. 3 Cladogram to classify *Cercospora* species based on elongation factor 1-α gene and internal transcribed spacer region ITS1 and ITS2 DNA sequences. The tree was rooted to two *Mycosphaerella thailandica* strains. Branch lengths are given *above the tree branches* and percentage bootstrap (1,000 replicates) values are shown *below the tree branches*

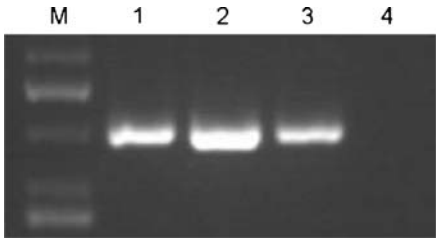

Fig. 4 PCR to amplify an ITS fragment from lesions caused by *Cercospora zeina* with the ITSC2fwd/ITS4 primers. *Lane M* GeneRuler^{TM} 50-bp DNA Ladder (Fermentas), *1* CBS 117757 (*C. zeae-maydis*), *2* CMW 25465 (*C. zeina*), *3* DNA from *C. zeina*-inoculated plant, *4* DNA from non-inoculated control plant

collected at the Chipperfield Farm in Greytown, South Africa. Morphological and molecular analyses proved that the isolate was *C. zeina*. Greenhouse-grown plants of the maize hybrid PAN 6724B were inoculated with conidia from sporulating cultures of *C. zeina* isolate CMW 25463. Typical symptoms of GLS were observed after 19 days (Fig. 1g). Mock-treated plants remained free of symptoms. Conidia were re-isolated from lesions and transferred to V8 medium and the fungal culture obtained was verified as *C. zeina* by sequencing part of the ITS1/ITS4 region (data not shown).

Discussion

The taxonomy of the causal agent of GLS was clarified with the re-classification of *C. zeae-maydis* Group II isolates as *C. zeina* (Crous et al. 2006). In previous studies conducted on GLS from African maize samples, Dunkle and Levy (2000) analysed 15 isolates from Zimbabwe, 15 isolates from Uganda and a total of six isolates from Zambia and South Africa. Okori et al. (2003) worked on 15 isolates from Kenya, three from Zimbabwe, eight from Rwanda and 55 from Uganda. Crous et al. (2006) worked with two isolates from South Africa. None of these studies produced any evidence of *C. zeae-maydis* (former Group I) on the continent. In our study we wanted to identify which *Cercospora* sp. was causing GLS in maize fields in southern Africa (Fig. 1a). We analysed 52 samples from KwaZulu-Natal, the province in South Africa where GLS symptoms were found initially (Ward 1996) and where the disease currently occurs the most frequently. In addition, we analysed 19 leaf samples from Zimbabwe and Zambia, two countries to the north that the African GLS pathogen must have passed through if it spread through the continent from an initial focus in South Africa (Table 1).

Measurements obtained for conidiophores and conidia as well as characteristics of the fungal cultures were typical for *C. zeina* and in agreement with the findings of Dunkle and Levy (2000) and Crous et al. (2006). Molecular techniques allowed analysis of fragments of the histone H3 (Fig. 2) and EF gene and ITS region. Thus, all eleven single-conidial cultures in our study, which grouped in the same clade as the *C. zeina* ex-type CBS 118820, are *C. zeina* (Fig. 3). This provides evidence that *C. zeae-maydis* is rare or not present in Africa based on the results obtained from 190 samples analysed in our and previous studies.

Because of the slow growth of the fungus, we adapted a rapid method to extract DNA directly from lesions (Griffith and Shaw 1998). Successful amplification of *C. zeina*-specific regions of the histone gene and ITS region proved that isolation of the fungus is not essential for identification (Figs. 4 and 5).

Artificial inoculation of maize plants with *C. zeina* in a greenhouse is challenging due to the high humidity required for germination of conidia and establishment of infection. Fully developed lesions typically appear 19 days after inoculation, as observed. Numerous cycles of sub-culturing lead to formation of a white sterile mycelium but incubation of fresh cultures under diurnal light increases sporulation, as observed previously by Beckman and Payne (1983). Artificial inoculation has been reported for *C. zeae-maydis* only (Beckman and Payne 1982, 1983; Bair and Ayers 1986; Asea et al. 2005; Paul and Munkvold 2005). By

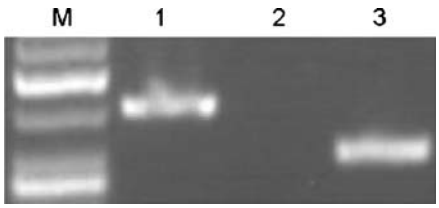

Fig. 5 PCR to amplify histone gene fragments from lesions caused by *Cercospora zeina*. *Lane M* GeneRuler^{TM} 50-bp DNA ladder (Fermentas), *1* amplicon generated with general histone primers (CYLH3F/CYLH3R), *2 C. zeae-maydis*-specific primers CzeaeHIST/CylH3R, *3 C. zeina*-specific primers CzeinaHIST/CylH3R

fulfilling Koch's postulates we verified that *C. zeina* is the causal agent of GLS in Africa.

Acknowledgements We thank Martin Coetzee for assistance with the phylogenetic analysis. The funding of this work was provided by the Department of Science and Technology through the PlantBio Trust, South Africa and a Post-doctoral Fellowship from the University of Pretoria to BM.

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