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Alternaria alternata, the causal agent of leaf blight of sunflower in South Africa

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Abstract Sunflower (*Helianthus annuus* L.) is an important oilseed crop in South Africa, and is grown in rotation with maize in some parts of North West, Limpopo, Free State, Mpumalanga and Gauteng provinces. Alternaria leaf blight is currently one of the major potential disease threats of sunflower and is capable of causing yield losses in all production regions. *Alternaria helianthi* was reported as the main cause of Alternaria leaf blight of sunflower in South Africa; however small-spored *Alternaria* species have been consistently isolated from leaf blight symptoms during recent surveys. The aim of this study was to use morphological and molecular techniques to identify the causal agent(s) of Alternaria blight isolated from South African sunflower production areas. *Alternaria helianthi* was not recovered from any of the sunflower lesions or seeds, with only *Alternaria alternata* retrieved from the symptomatic tissue. Molecular identification based on a combined phylogenetic dataset using the partial internal transcribed spacer regions, RNA polymerase second largest subunit, glyceraldehyde-3-phosphate dehydrogenase, translation elongation factor and *Alternaria* allergen gene regions was done to support the morphological identification based on the three-dimensional sporulation patterns of *Alternaria*. Furthermore, this study aimed at evaluating the pathogenicity of the recovered *Alternaria* isolates and their potential as causal agents of Alternaria leaf blight of sunflower. Pathogenicity tests showed that all the *Alternaria alternata* isolates tested were capable of causing Alternaria leaf blight of sunflower as seen in the field. This is the first report of *A. alternata* causing leaf blight of sunflower in South Africa.

Keywords *Helianthus annuus* · Alternaria leaf blight · Oilseed

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Introduction

Alternaria species are ubiquitous and are able to develop under a wide range of temperatures, utilizing locally available sources of moisture (Lourenco Jr. et al. 2009). The genus *Alternaria* includes nearly 300 species that occur worldwide (Rotem 1994; Pryor and Gilbertson 2000; Simmons 2007) as either plant pathogenic or saprotrophic microorganisms (Konstantinova et al. 2002). *Alternaria* leaf blight of sunflower (*Helianthus*

annuus L.) is an important disease that has been reported to cause a reduction of up to 80% and 33% seed and oil yield, respectively (Calvet et al. 2005). *Alternaria helianthi* (Hansf.) Tubaki and Nishihara is regarded as the main cause of Alternaria leaf spot of sunflowers (van der Westhuizen and Holtzhausen 1980; Allen et al. 1983). Nine other *Alternaria* species have been reported on sunflower, including *A. alternata* (Fries) Kiessler, *A. helianthicola* Rao and Rajagopalan, *A. helianthinficiens* Simmons, *A. leucanthemi* Nelen (syn. *A. chrysanthemi* Simmons and Grosier), *A. longissima* Deighton and MacGarvey, *A. protenta* Simmons, *A. tenuissima* (Fries) Wiltshire and *A. zinniae* Ellis (Lapagodi and Thanassoulopoulos 1998). Based on Woudenberg et al. (2015a), *A. tenuissima* is now synonymised with *A. alternata*.

Alternaria genus is a complex phylogenetic grouping whose taxonomy has always been controversial with an ongoing debate to synonymize certain species (Peever et al. 2004; Logrieco et al. 2009). The *Alternaria* genus has an abundant number of recognized species, therefore the identification becomes more difficult when the genus as a whole is considered (Pryor and Michailides 2002; Woudenberg et al. 2013). Firstly, some of the species have a spore dimension range and genetic make-up that overlaps other small-spored *Alternaria* species. Secondly, catenation and conidial morphology of *Alternaria* species is affected by the conditions of growth such as substrate, light and humidity and thus may be unreliable for morphological characterisation of the genus (Andersen et al. 2002; Pryor and Michailides 2002). Finally, the genus *Alternaria* was previously also often characterised based on host association (Rotem 1994), but it is known however to be able to infect more than 4000 host plants (Lawrence et al. 2013) and this has resulted in new species descriptions from around the world (Nishikawa and Nakashima 2013).

Small-spored *Alternaria* species are a group of fungi with few morphological or molecular characteristics that allow distinctive discrimination among the taxa (Andrew et al. 2009). Previously, Kusaba and Tsuge (1995) and Peever et al. (2005), suggested that small-spored *Alternaria* should not be separated at species level but should be classified as *Alternaria alternata* with differentiation at species level as *formae specialis*. Single-gene phylogenies often yield poorly supported trees due to a limited number of informative sites that leads to inaccurate phylogenetic hypotheses. Multi-gene phylogenetic analysis combined with morphological species concepts will result in a species description that is strong enough to withstand most challenges (Peever et al. 2004; Andrew et al. 2009). Recent studies by Woudenberg et al. (2013) and Lawrence et al. (2013) have shown that molecular methods using multi-gene phylogenetics can classify or segregate *Alternaria* species. In this study, the extent of cultural, phylogenetic diversity, geographical association and pathogenic variability were studied among 27 isolates of *A. alternata* collected from different sunflower production localities of South Africa.

Materials and methods

Alternaria leaf blight sampling

Alternaria species were recovered from sunflower seeds and symptomatic leaves. The leaves were sampled from the major sunflower production areas of South Africa; North West, Limpopo, Free State, Mpumalanga and Gauteng provinces during surveys in 2013 and 2014 (Table 1). Samples of diseased sunflower leaves were placed in individual paper bags to prevent rapid desiccation, and transported to the laboratory in an ice box. For each locality in this study, four to eight leaves showing leaf blight symptoms were sampled. Furthermore, *Alternaria* was recovered from 19 sunflower seed-lots (seed and grain) received from South African seed companies.

Fungal isolation

Approximately four 5 mm^2 segments of the leaf per lesion (10–15 lesions per leaf sample) were cut with a disinfected scalpel blade. Each leaf piece included both healthy and infected tissue. The leaf pieces and seed ($n = 400$ seeds per seed-lot) samples were disinfected by immersion in a 1.5% sodium hypochlorite solution for 3 min and rinsed in sterile distilled water for a minute. The excised leaf pieces and seeds were placed on potato dextrose agar (PDA) (Merck, Modderfontein) amended with chloramphenicol (0.01 g l^{-1}) (Biologica Pharmaceuticals, Pretoria). The Petri dishes were incubated at 25 °C under 12 h alternating cycles of near ultra-violet (NUV)-light (360 nm wavelength) and darkness for 3 days. All the *Alternaria* cultures were purified from the master plate and plated onto PDA media containing the same antibiotics as above. Morphological

Table 1 Origin of *Alternaria* isolates recovered from sunflower grown at major production localities of South Africa and the pathogenicity of these isolates toward artificially inoculated PAN 7351sunflower leaves

Isolate	Locality	Plant tissue isolated from	*Pathogenicity test disease rating
PPRI 11433	Greytown, Kwa-Zulu Natal	Leaves	3.2 b
PPRI 13496	Lichtenburg, North West	Leaves	2.4 d
PPRI 13462	Arlington, Free State	Seeds	3.4 ab
PPRI 13500	Bloemhof, Free State	Leaves	1.8 e
PPRI 13468	Dwaalboom, Limpopo	Leaves	2.1 d
PPRI 13476	Delmas, Mpumalanga	Leaves	2.3 d
PPRI 13505	Viljoenskroon, Free State	Seeds	3.0 bc
PPRI 13488	Bapsfontein, Gauteng	Seeds	3.0 bc
PPRI 13517	Viljoenskroon, Free State	Seeds	3.2 b
PPRI 13525	Viljoenskroon, Free State	Seeds	2.6 c
PPRI 13528	Bloemfontein, Free State	Seeds	3.8 a
PPRI 13538	Vierfontein, Free State	Seeds	2.0 de
CBS 916.96	India	*Arachis hypogaea*	2.8 c
PPRI 13464	Settlers, Limpopo	Leaves	2.2 d
PPRI 13467	Bloemhof, Free State	Leaves	2.6 cd
PPRI 13471	Kroonstad, Free State	Leaves	2.0 de
PPRI 13473	Sannieshof, North West	Leaves	3.0 bc
PPRI 13478	Bela Bela, Limpopo	Leaves	3.4 b
PPRI 13504	Nylstroom, Limpopo	Leaves	3.0 bc
PPRI 13484	Potchefstroom, North West	Leaves	2.0 d
PPRI 13491	Bapsfontein, Gauteng	Seeds	2.4 d
PPRI 13513	Bapsfontein, Gauteng	Seeds	2.8 c
PPRI 13522	Bloemfontein, Free State	Seeds	2.4 d
PPRI 13531	Ventersdorp, North West	Seeds	3.4 b
PPRI 13535	Bultfontein, Free State	Seeds	2.0 de
PPRI 13541	Vredefort, Free State	Seeds	2.8 c
PPRI 18986	Wesselsbron, Free State	Seeds	2.6 cd
Control			0.7 f
Grand mean			2.63
Fisher's L.S.D.			0.502
CV%			20.7

*Means followed by the same letter in a column indicate no significant difference with $P \leq 0.05$

identification was based on colony characteristics, conidial sizes, conidial catenulation and three–dimensional (3-D) sporulation pattern (Simmons 2007).

Morphological identification

Twenty-seven purified representative *Alternaria* isolates were selected from cultures recovered from sunflower seeds and symptomatic leaves, based on the sampling locality and morphology and were used further in this study. The isolates were cultured on half strength PDA for 7 days at 25 °C under 12 h alternating cycles of NUV-light and darkness. The morphological characteristics of the isolates was observed and examined under a Zeiss Stereomicroscope. The *Alternaria* spp. isolated from the infected leaves and seed-lots were compared to the three-dimensional sporulation pattern of the *Alternaria* spp. described by Simmons (2007). All the representative cultures were deposited in the National Collection of Fungi at the Agricultural Research Council–Plant Protection Research (ARC-PPR), Pretoria, South Africa.

DNA extraction, PCR amplification, sequencing and phylogenetic analyses

The genomic DNA of the selected *Alternaria* isolates was extracted using the cetyltrimethylammonium bromide (CTAB)-method (Ausubel et al. 1998). The ITS, *rbp*2, *gpd*, *tef*1 and *alt* a1 gene regions were amplified using primer sets described by Woudenberg et al. (2013) and Lawrence et al. (2013). The PCRs were performed using a 25 μl reaction volume containing a reaction mixture of 18.25 μl of sterile double-deionised water, 5 μl MyTaq buffer (Bioline, Celtic Molecular Diagnostics, Cape Town), 0.25 μl MyTaq DNA polymerase (Bioline, Celtic Molecular Diagnostics, Cape Town), 0.25 μl of the respective primer sets (200 nM) and 1 μl template DNA (15 ng μl^{-1}). PCR amplifications were performed in an MJ Mini: Personal Thermal Recycler (Bio-Rad, Johannesburg). The conditions for PCR amplification for ITS consisted of initial denaturation step of 5 min at 94 °C, followed by 35 cycles of 94 °C for 30 s, 48 °C for 30 s, and 72 °C for 90 s, and a final elongation step of 7 min at 72 °C. Conditions for *tef*1 deviated from the ITS by using 40 cycles and 52 °C for annealing, whereas for *gpd* 55 °C for annealing was used. A touchdown PCR protocol was used for the *rbp*2 region consisting of five cycles of 94 °C for 45 s, 60 °C for 45 s and 72 °C for 2 min, followed by five cycles with a 58 °C annealing temperature and 30 cycles with a 54 °C annealing temperature. For Alt al, PCR conditions were an initial denaturation step at 95 °C for 5 min followed by 35 cycles of 95 °C for 40 s, 57 °C for 40 s, and 72 °C for 1 min followed by a 10 min final extension at 72 °C. PCR products were analysed in 1.5% agarose gels, stained with gel red and visualized under UV light. An hpII ladder (Bioline, Celtic Molecular Diagnostics, Cape Town) was used as a molecular weight marker.

PCR products were purified with the GeneJet™ PCR purification kit (InqabaBiotec, Pretoria) following the manufacturer's instruction. The purified PCR products were sequenced in both directions at the University of Pretoria DNA Sequencing Facility using an ABI Prism DNA Automated Sequencer (Perkin Elmer, California). Consensus sequences of the *Alternaria* isolates were created using the BioEdit Sequence Alignment v 7.0.0 (Hall 1999), and manually adjusted when required. The sequences were compared with the species in the section *Alternaria* based on Woudenberg et al. (2015a; Table 2), imported from GenBank (http://www.ncbi.nlm.nih.gov/genbank/), and then aligned using MAFFT version 7 (http://mafft.cbrc.jp/alignment/server/).

Phylogenetic analyses were performed based on maximum parsimony (MP) using PAUP 4.0* (Phylogenetic Analysis Using Parsimony* and Other Methods version 4) (Swofford 2002). Gaps were treated as missing data. Analyses were conducted by heuristic searches consisting of 100 random-addition of sequences with branch swapping by tree-bisection-reconnection algorithm. Branch stability for individual dataset and concatenated dataset were evaluated by 1000 bootstrap replications to produce a majority rule consensus tree with nodal support values. Congruency among datasets ($P \geq 0.05$) was evaluated with the partition homogeneity test (PHT) implemented in PAUP v4.0. *Alternaria alternantherae* was used as an out-group in all the analyses based on results of Woudenberg et al. (2015a).

Pathogenicity test and Koch's postulates

Sunflower seeds (cultivar PAN 7351) received from Pannar (PTY, LTD), Bapsfontein, South Africa were used for the pathogenicity test due to their low *Alternaria* infection rate (29%). The seeds were surface sterilised with 1.5% sodium hypochlorite solution for 3 min and rinsed in sterile water for a minute. Seeds were sown in 15 cm diameter (1.5-l) plastic pots, one seed per pot, containing pasteurised soil (light red, pH (H_2O): 6.6, pH (KCl): 6, sand: 85%, clay: 11%, silt: 4%, texture: loamy sand). The plants were maintained in a greenhouse at temperatures of 25 to 30 °C and a relative humidity of 50–80% with a photoperiod of 16 h and watered daily. The plants were left to grow until 6-weeks-old before use in the subsequent experiments. Sunflower plants were then inoculated with a 4×10^5 spores ml^{-1} conidial spore suspension prepared from 2-week-old *Alternaria* isolates. The spore suspensions were prepared by adding 5 ml of sterile distilled water to each Petri dish, and dislodging the conidia with a sterile hockey stick. Each suspension was poured in a beaker and amended with 0.05 μl Tween 20 (Merck, Modderfontein). The solution was passed through a cheesecloth to remove mycelial fragments from the spore suspension. The spore concentration was determined using a haemocytometer. The conidial suspension was stirred to prevent clumping of conidia and inoculated immediately after preparation by spraying the leaves until run-off with an automatic aerosol

Table 2 *Alternaria* isolates used for phylogenetic analysis in this study and their GenBank accession number

Species name	Locality	Isolate number	ITS	GPD	TEF	RBP2	Alt a1
Alternaria arborescens SC	South Africa	PPRI 11433	MF381794	MF381768	MF381820	MF381846	MF381742
Alternaria alternata	South Africa	PPRI 13462	MF381795	MF381769	MF381821	MF381847	MF381743
Alternaria alternata	South Africa	PPRI 13464	MF381796	MF381770	MF381822	MF381848	MF381744
Alternaria alternata	South Africa	PPRI 13467	MF381797	MF381771	MF381823	MF381849	MF381745
Alternaria alternata	South Africa	PPRI 13468	MF381798	MF381772	MF381824	MF381850	MF381746
Alternaria alternata	South Africa	PPRI 13471	MF381799	MF381773	MF381825	MF381851	MF381747
Alternaria alternata	South Africa	PPRI 13473	MF381800	MF381774	MF381826	MF381852	MF381748
Alternaria alternata	South Africa	PPRI 13476	MF381801	MF381775	MF381827	MF381853	MF381749
Alternaria alternata	South Africa	PPRI 13478	MF381802	MF381776	MF381828	MF381854	MF381750
Alternaria alternata	South Africa	PPRI 13484	MF381803	MF381777	MF381829	MF381855	MF381751
Alternaria alternata	South Africa	PPRI 13488	MF381804	MF381778	MF381830	MF381856	MF381752
Alternaria alternata	South Africa	PPRI 13491	MF381805	MF381779	MF381831	MF381857	MF381753
Alternaria alternata	South Africa	PPRI 13496	MF381806	MF381780	MF381832	MF381858	MF381754
Alternaria alternata	South Africa	PPRI 13500	MF381807	MF381781	MF381833	MF381859	MF381755
Alternaria alternata	South Africa	PPRI 13504	MF381808	MF381782	MF381834	MF381860	MF381756
Alternaria alternata	South Africa	PPRI 13505	MF381809	MF381783	MF381835	MF381861	MF381757
Alternaria alternata	South Africa	PPRI 13513	MF381810	MF381784	MF381836	MF381862	MF381758
Alternaria alternata	South Africa	PPRI 13517	MF381811	MF381785	MF381837	MF381863	MF381759
Alternaria alternata	South Africa	PPRI 13522	MF381812	MF381786	MF381838	MF381864	MF381760
Alternaria alternata	South Africa	PPRI 13525	MF381813	MF381787	MF381839	MF381865	MF381761
Alternaria alternata	South Africa	PPRI 13528	MF381814	MF381788	MF381840	MF381866	MF381762
Alternaria alternata	South Africa	PPRI 13531	MF381815	MF381789	MF381841	MF381867	MF381763
Alternaria alternata	South Africa	PPRI 13535	MF381816	MF381790	MF381842	MF381868	MF381764
Alternaria alternata	South Africa	PPRI 13538	MF381817	MF381791	MF381843	MF381869	MF381765
Alternaria alternata	South Africa	PPRI 13541	MF381818	MF381792	MF381844	MF381870	MF381766
Alternaria alternata	South Africa	PPRI 18986	MF381819	MF381793	MF381845	MF381871	MF381767
Alternaria alstroemeriae	USA	CBS 118808	KP124296	KP124153	KP125071	KP124764	KP123845
Alternaria alstroemeriae	Australia	CBS 118809 T	KP124297	KP124154	KP125072	KP124765	–
Alternaria alternantherae	China	CBS 124392	KC584179	KC584096	KC584633	KC584374	KP123846
Alternaria alternata	USA	CBS 106.24	KP124298	KP124155	KP125073	KP124766	KP123847
Alternaria alternata	Egypt	CBS 103.33	KP124302	KP124159	KP125077	KP124770	KP123852
Alternaria alternata	Denmark	CBS 117.44	KP124303	KP124160	KP125079	KP124772	KP123854
Alternaria alternata	USA	CBS 194.86	KP124316	KP124172	KP125092	KP124784	KP123869
Alternaria alternata	Canada	CBS 195.86	KP124317	KP124173	KP125093	KP124785	JQ646398
Alternaria alternata	India	CBS 916.96 T	AF347031	AY278808	KC584634	KC584375	AY563301
Alternaria alternata	UK	CBS 918.96	AF347032	AY278809	KC584693	KC584435	AY563302
Alternaria alternata	USA	CBS 102598	KP124329	KP124184	KP125105	KP124797	KP123878
Alternaria alternata	Turkey	CBS 102599	KP124330	KP124185	KP125106	KP124798	KP123879
Alternaria alternata	Israel	CBS 102604	KP124334	AY562410	KP125110	KP124802	AY563305
Alternaria alternata	South Africa	CBS 113014	KP124342	KP124196	KP125118	KP124810	KP123890
Alternaria alternata	USA	CBS 118812	KC584193	KC584112	KC584652	KC584393	KP123905
Alternaria alternata	USA	CBS 118814	KP124357	KP124211	KP125133	KP124825	KP123906
Alternaria alternata	USA	CBS 121454	AY278836	AY278812	KP125145	KP124837	JQ646402
Alternaria arborescens SC	Switzerland	CBS 101.13	KP124392	KP124244	KP125170	KP124862	KP123940

Table 2 (continued)

Species name	Locality	Isolate number	ITS	GPD	TEF	RBP2	Alt a1
Alternaria arborescens SC	USA	CBS 102605	AF347033	AY278810	KC584636	KC584377	AY563303
Alternaria arborescens SC	South Africa	CBS 112749	KP124401	KP124253	KP125179	KP124871	KP123948
Alternaria arborescens SC	New Zealand	CBS 119544	KP124408	JQ646321	KP125186	KP124878	KP123955
Alternaria arborescens SC	New Zealand	CBS 119545	KP124409	KP124260	KP125187	KP124879	KP123956
Alternaria betae-kenyensis	Kenya	CBS 118810	KP124419	KP124270	KP125197	KP124888	KP123966
Alternaria burnsii	India	CBS 107.38 T	KP124420	JQ646305	KP125198	KP124889	KP123967
Alternaria burnsii	Mozambique	CBS 110.50	KP124421	KP124271	KP125199	KP124890	KP123968
Alternaria burnsii	India	CBS 118817	KP124424	KP124274	KP125202	KP124893	KP123971
Alternaria eichhorniae	India	CBS 489.92 T	KC146356	KP124276	KP125204	KP124895	KP123973
Alternaria eichhorniae	Indonesia	CBS 119778	KP124426	KP124277	KP125205	KP124896	–
Alternaria gaisen	Japan	CBS 632.93	KC584197	KC584116	KC584658	KC584399	KP123974
Alternaria gaisen	Japan	CBS 118488	KP124427	KP124278	KP125206	KP124897	KP123975
Alternaria gaisen	Portugal	CPC 25268	KP124428	KP124279	KP125207	KP124898	KP123976
Alternaria gossypina	Zimbabwe	CBS 104.32 T	KP124430	JQ646312	KP125209	KP124900	JQ646395
Alternaria gossypina	USA	CBS 102597	KP124432	KP124281	KP125211	KP124902	KP123978
Alternaria gossypina	Colombia	CBS 102601	KP124433	KP124282	KP125212	KP124903	KP123979
Alternaria iridiaustralis	New Zealand	CBS 118404	KP124434	KP124283	KP125213	KP124904	KP123980
Alternaria iridiaustralis	Australia	CBS 118486 T	KP124435	KP124284	KP125214	KP124905	KP123981
Alternaria iridiaustralis	Australia	CBS 118487	KP124436	KP124285	KP125215	KP124906	KP123982
Alternaria jacinthicola	Mauritius	CBS 878.95	KP124437	KP124286	KP125216	KP124907	KP123983
Alternaria jacinthicola	Mali	CBS 133751 T	KP124438	KP124287	KP125217	KP124908	KP123984
Alternaria longipes	USA	CBS 540.94	AY278835	AY278811	KC584667	KC584409	AY563304
Alternaria longipes	USA	CBS 121332	KP124443	KP124292	KP125222	KP124913	KP123989
Alternaria longipes	USA	CBS 121333	KP124444	KP124293	KP125223	KP124914	KP123990
Alternaria tomato	Unknown	CBS 103.30	KP12445	KP124294	KP125224	KP124915	KP123991
Alternaria tomato	Unknown	CBS 114.35	KP12446	KP124295	KP125225	KP124916	KP123992

CBS = Culture collection of the Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht; CPC = Personal collection of P.W. Crous, Utrecht, The Netherlands; PPRI = Agricultural Research Council–Plant Protection Research, Pretoria; SC = species complex; USA = United States of America

sprayer. Control plants were sprayed with sterile distilled water. The plants were then covered with polyethylene bags to maintain a high relative humidity (>95%) within the surrounding area of the plants and incubated in a random block design in the greenhouse at 25 °C. All treatments consisted of five replicates, with one pot per replicate. The evaluation of the *Alternaria* infection was done after 7 days. The resulting lesions were scored on a 5-point rating system modified from Pryor and Michailides (2002): 0 = No lesion, 1 = Lesion surrounded by a yellow halo, 1 to 25% leaf infection, 2 = Lesions enlarge in diameter and become necrotic, 26 to 50% leaf infection, 3 = Multiple lesions that coalesce and enlarge further in diameter, 51 to 75% leaf infection, 4 = Defoliation of the leaf, leaf severely damaged, 76 to 100% leaf infection (Fig. 1). Separation of means was done using the least significant difference (LSD) Fishers's LSD test ($P \leq 0.05$). The disease severity was analysed using the standard analysis of variance (ANOVA) using Statistical Analysis System (SAS v9.4) software package (SAS Institute Inc 2013).

Results

Fungal isolations and morphological identification

Leaves showing the Alternaria blight symptoms consistently yielded *Alternaria* isolates on the PDA plates.

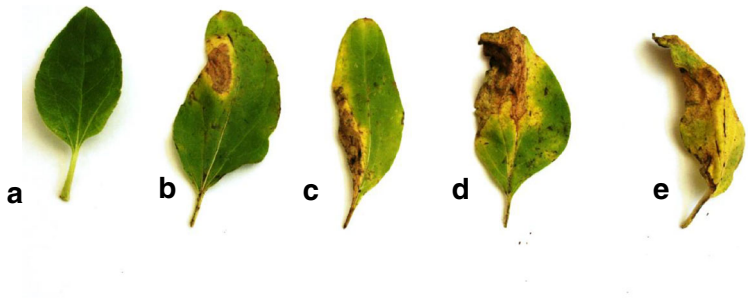

Fig. 1 Disease rating used in the pathogenicity tests of *Alternaria alternata* recovered from sunflower. The rating scale for lesion development on sunflower was as follows in leaves from left to right: (**a**) Rating 0 = no lesion development, (**b**) Rating 1 = 1 to 25% leaf infection (**c**) Rating 2 = 26 to 50% leaf infection (**d**) Rating 3 = 51 to 75% leaf infection, (**e**) Rating 4 = 76 to 100% leaf infection

Twenty seven representative isolates (14 seed and 13 leaf samples) were selected. Isolates had relatively small conidia in concatenate chains indicating that they all belonged to *Alternaria* sect. *Alternaria*. *Alternaria* isolates had typical *Alternaria* colony growth patterns of alternating circles on half strength PDA (Fig. 2a). The colony texture of the *Alternaria* isolates was generally woolly. Conidiophores were 12 to 50 μm long and rose singly or in branches, conidial width ranged from 8 to 15 μm and the length ranged from 12 to 34 μm. The conidial colours were pale to mid olivaceous green to pale green. There were three types of sporulation patterns noted in the study of which two fitted the description of *Alternaria alternata* (Fries) Kiessler and *A. arborescens* (Simmons). Group 1 (Fig. 2b) was characterised by short primary conidiophores and conidial chains that were seldom branched. Conidiophores rose singly and straight, and lead to conidia which had slender beaks. This group included isolates PPRI 13462 and PPRI 13468. This group fitted in the description of *A. tenuissima* which is now synonymised to *A. alternata* (Woudenberg et al. 2015a).

Group 2 (Fig. 2c) was characterised by short primary conidiophores and conidial chains that were branched out. Conidiophores arose either singly or in branches. Some isolates had less secondary conidiophore branching and longer conidial chains which included isolates PPRI 13500, PPRI 13541 and PPRI 18986. Other isolates had conidiophores were more branched out with secondary conidiophores as seen in Fig. 2d. This group included isolates PPRI 13478, PPRI 13496, PPRI 13488 and PPRI 12522. This group fitted in the description of *A. alternata.*

Group 3 (Fig. 2e) was characterised by long secondary conidiophores that developed from the conidia. The secondary conidiophores were branched and difficult to distinguish from mycelium resulting in complex branching that was observed as bushy. The conidia were mid-golden brown in colour. The conidia appeared to be oval and often inversely club-shaped. This group fitted the description of *A. arborescens* and included only one isolate, PPRI 11433.

Phylogenetic analyses

Phylogenetic analyses were based on a consensus parsimony analyses of five loci (*alt* a1, *rbp*2, *gpd*, *tef*1 and ITS) using 69 in-group taxa from the section *Alternata* and *Alternaria alternantherae* as an outgroup taxa (Fig. 3). Alignment of the combined dataset resulted in 2611 total characters (2242 constant, 182 parsimony uninformative, and 187 parsimony informative). Maximum parsimony analyses of the concatenated dataset produced a phylogenetic tree with clades that were consistent with that of Woudenberg et al. (2013) as seen in Fig. 3. Isolate PPRI 11433was the only isolate of *A. arborescens* recovered from sunflower fields supported by a bootstrap value of 74% within the *A. arborescens* species complex. The other recovered *Alternaria* isolates all grouped with *A. alternata*. However, there was sub-clustering within the *A. alternata* clade. In this study, there was no observed association between phylogenetic clade and geography or disease symptom expression. Most clades contained isolates from more than one geographic association.

Pathogenicity tests

Following morphological and molecular identification, the pathogenicity of the isolates was tested in the

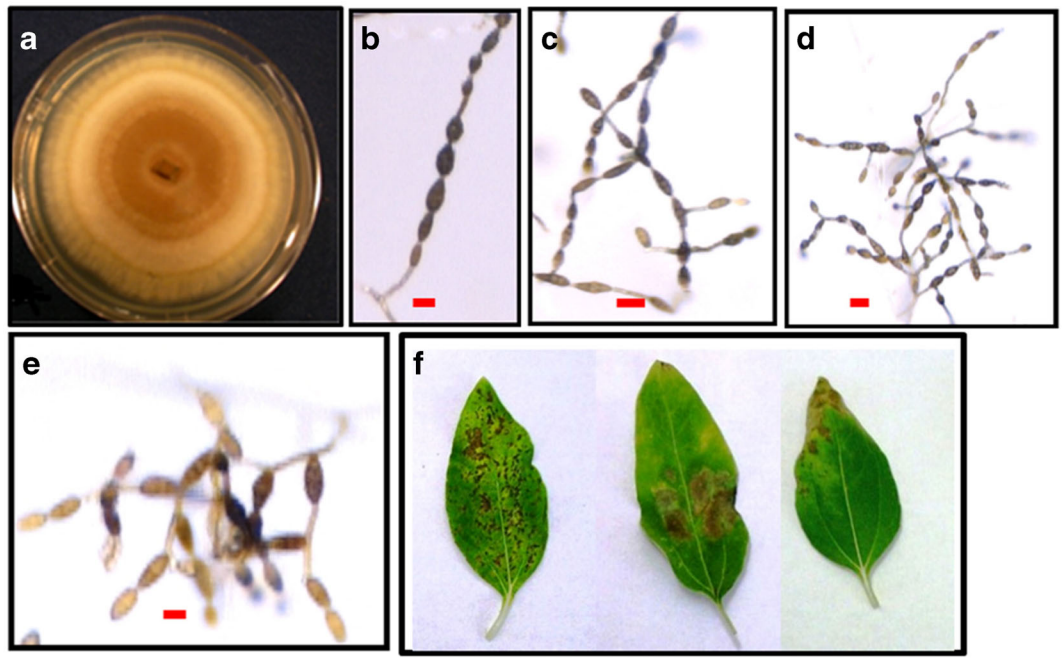

Fig. 2 **a** Culture and sporulation structures of *Alternaria* species on half-strength potato dextrose agar (½ PDA) isolated from lesions of sunflower leaves and infected seeds. **b** Sporulation pattern of *A. tenuissima* (on ½ PDA after four days). (**c** and **d**) Sporulation pattern of *A. alternata* (on ½ PDA after four days). **e** Sporulation pattern of *A. arborescens* (on ½ PDA after four days). **f** Pathogenicity tests results depicting different Alternaria leaf blight lesions on sunflower. Each bar represents 10 μm

greenhouse. A disease severity index was compiled to determine the disease severity of the *Alternaria* isolates during pathogenicity tests (Fig. 1). All the isolates tested caused Alternaria leaf blight on the plants; though there was a significant difference in pathogenicity amongst the *Alternaria* isolates (Table 1). Two types of disease symptoms were expressed. The first type of symptom was seen as a small to large lesion surrounded by a chlorotic halo. The lesion was either found on the leaf tip or centre of the leaf. The second type of symptom was seen as tiny lesions with chlorotic halos that were closely clustered together (Fig. 1f).

Discussion

Small-spored *Alternaria* species are increasingly becoming of great economic importance in the agricultural industry due to their cosmopolitan nature and ability to cause disease on a large number of crops (Rotem 1994; Pryor and Michailides 2002; Lawrence et al. 2013). The aim of this study was therefore to determine the diversity of *Alternaria* species causing Alternaria blight of sunflower.

Morphological identification was mainly based on the three–dimensional (3-D) sporulation pattern under stringent conditions of 25 °C, alternating NUV and darkness cycles and grown on PCA or half strength PDA as described by Simmons (2007). Colony pigmentation, conidial sizes and shapes were not the key identification factors as *Alternaria* morphology may vary under different culture conditions and several species have overlapping spore sizes (Pryor and Michailides 2002). Andrew et al. (2009) reported that morphological characters used to delineate species in the *Alternaria* section *Alternaria* are phenotypically plastic and do not allow the reproducible differentiation of several morpho-species. These characteristics may therefore be misleading in the description and comparison of small-spored catenulate *Alternaria* isolates in this study (Pryor and Michailides 2002; Park et al. 2008).

Five gene regions were used in this study as the use of single-gene phylogenies often yields poorly supported trees due to limited number of informative sites that leads to inaccurate phylogenetic hypotheses. Lawrence et al. (2013) stated that *tef*1 and *rbp*2 are slow evolving and would therefore be good to resolve early divergence, whereas *alt* a1 is fast evolving and would

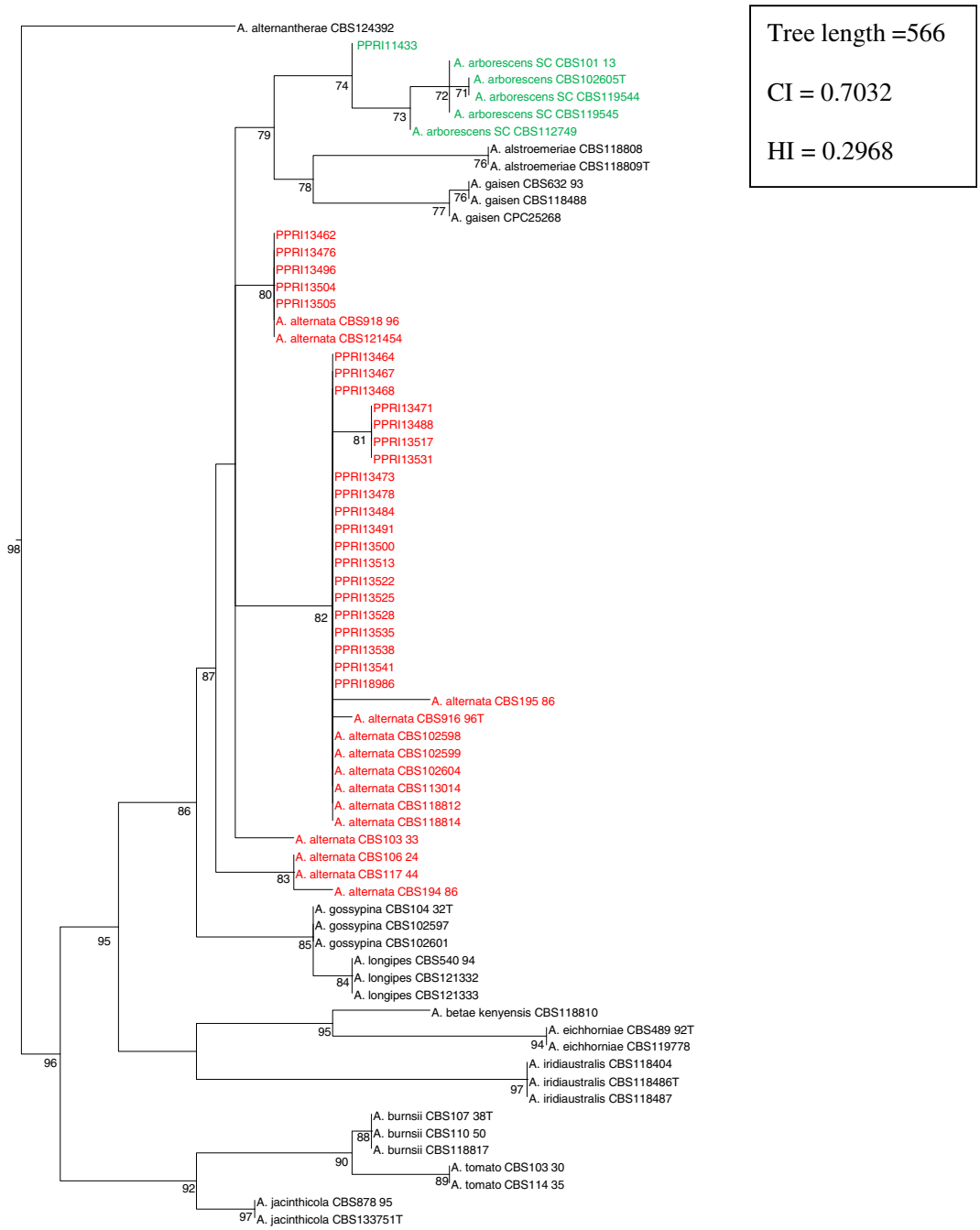

Fig. 3 One of the most parsimonious trees of *Alternaria* section *Alternaria* isolates generated from maximum parsimony analysis of five-gene combined data set (*alt* a1, *rbp*2, *gpd*, *tef*1 and ITS gene sequences). The tree was outrooted to *Alternaria alternantherae* (CBS 124392). The bootstrap values are given at the nodes. The *A. alternata* and *A. arborescens* species complex are highlighted in red and green, respectively. There was no geographical and symptom expression grouping amongst the *Alternaria alternata* isolates

therefore be useful in resolving recently diverged *Alternaria* species. A combined analysis of the five genes was done to minimise any discrepancies.

Kusaba and Tsuge (1995) and Andrew et al. (2009) have previously suggested that small-spored *Alternaria* species should all be classified as *A. alternata,* as there is

no adequate evidence to phylogenetically differentiate these species. Furthermore, Woudenberg et al. (2015a) showed that whole-genome alignments of *Alternaria* species such as *A. tenuissima* Wiltshire (CBS 918.96), *A. mali* Roberts (CBS 106.24), *A. malvae* Roum. and Letell, *A. lini* Dey, *A. citri* (Penz.) Mussat, *A. angustiovoide* Simmons, etc., revealed 96.7–98.2% genome identity with reference to the genome of *A. alternata* (CBS 916.96), and are now referred to as *A. alternata*.

There was some genetic diversity within the *A. alternata* population as three subclades were formed. The diversity between the *A. alternata* isolates may be caused by mutation, migration and cryptic recombination (Aradhya et al. 2001; Meng et al. 2015; Woudenberg et al. 2015b). Meng et al. (2015) found that *A. alternata* reproduces by combining many cycles of asexual propagation with fewer cycles of cryptic sexual reproduction thus facilitating its adaption to the ever changing environment.

Pathogenicity tests indicated that all the isolates including isolate CBS 916.96 recovered from *Arachis hypogaea* in India caused Alternaria leaf blight disease although at different disease severity indices, which concurs with the findings of Zhu and Xiao (2015) from the studies done on *Alternaria* on blueberry where all the tested *Alternaria* isolates caused necrosis around the inoculation point. Phylogenetic analysis by means of multiple genes displayed no geographical or symptoms expression grouping amongst the isolates (Aradhya et al. 2001; Peever et al. 2004; Andrew et al. 2009). This implies that *Alternaria* species may be capable of causing disease on agricultural crops due to their non-host specific toxins (Pryor and Michailides 2002).

The large-spored *Alternaria helianthi* was reported as the main causal agent of Alternaria leaf blight of sunflower in South Africa in 1980 based on morphological characteristics and symptom expression (van der Westhuizen and Holtzhausen 1980) but no *A. helianthi* isolates were available from the PPRI collection to include in the present study for comparison. *Alternaria helianthi* was not recovered from any of the surveyed areas in this study. The dominance of small-spored *A. alternata* from a disease previously reported to be caused by large-spored *Alternaria* species, is similar to early blight of potato caused by large-spored *Alternaria solani* Sorauer and small-spored *A. alternata*. *Alternaria solani* was initially regarded as the main causal agent of early blight of potato, while *A. alternata* was only noted to cause secondary infections (Kumar et al. 2008; Stammler et al. 2014). However, recent studies have reported *A. alternata* as the most prevalent and more aggressive causal agent of early blight of potato (Kapsa and Osowski, 2012; Fairchild et al. 2013). This implies that there may be a shift in pathogen populations. Possible reasons may be that *A. alternata* has a high reproductive rate and widespread dispersal of spores (Aradhya et al. 2001); is more resistant to fungicide treatment (Fairchild et al. 2013) and *A. alternata* proliferates at higher temperatures than *A. solani* and *A. helianthi* (Stammler et al. 2014) and can therefore be more prevalent in the field.

In conclusion, the *Alternaria* species recovered from the sunflower seeds and leaves were predominantly distinguished as *A. alternata* based on the morphological and phylogenetic analyses. There was however one distinct isolate which grouped with the *A. arborescens*-species complex. The *A. arborescens*-species complex needs to be studied further for better resolution. The most important finding of this study was that all *Alternaria* isolates tested were able to cause leaf blight of sunflower. Thomma (2003) regarded *Alternaria* species as being mainly saprotrophic fungi commonly found in soil or on decaying plant tissues, but as most have acquired pathogenic capacities collectively causing disease over a broad host range, this notion can be altered in that *Alternaria* species should all be referred to as plant pathogens capable of being saprotrophs and living on dead decaying matter as an overwintering or survival strategy. This is the first study where *A. alternata* and *A. arborescens* have been recovered from sunflower leaves and seeds in South Africa. Further studies should be considered to determine potential yield loss caused by these species, as well as control strategies to limit the spread of this pathogen.

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Compliance with ethical standards The research presented in this research does not implicate nor Human Participants and/or Animals.

Conflict of interest The authors declare that they have no conflict of interest.

References

Allen, S. J., Brown, J. F., & Kockman, J. K. (1983). Effect of leaf age, host growth stage, leaf injury and pollen on the infection of sunflower by *Alternaria helianthi*. *Phytopathology, 73*, 896–898.

Andersen, B., Kroger, E., & Roberts, G. (2002). Chemical and morphological segregation of *Alternaria arborescens*, *A. infectoria* and *A. tenuissima* species-groups. *Mycological Research, 106*(2), 170–182.

Andrew, M., Peever, T. L., & Pryor, B. M. (2009). An extended multilocus phylogeny does not resolve morphological species with the small-spored *Alternaria* species complex. *Mycologia, 101*(1), 95–109.

Aradhya, M. K., Chan, H. M., & Parfitt, D. E. (2001). Genetic variability in the pistachio late blight fungus, *Alternaria alternata*. *Mycological Research, 5*(3), 300–306.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., & Struhl, K. (1998). *Short protocols in molecular biology*. New Jersey: Wiley.

Calvet, N. P., Ungaro, M. R. G., & Oliveira, R. F. (2005). Virtual lesion of Alternaria blight on sunflower. *Helia, 28*(42), 89–100.

Fairchild, K. L., Miles, T. D., & Wharton, P. S. (2013). Assessing the fungicide resistance in populations of Alternaria in Idaho potato fields. *Crop Protection, 49*, 31–39.

Hall, T.A. (1999). BioEdit: A user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. Nucleic Acids Symposium Series, 41, 95–98.

Kapsa, J.S. & Osowski, J. (2012). Host – pathogen interaction between *Alternaria* species and *S. tuberosum* under different conditions. Thirteenth Euroblight workshop: St. Petersburg, Russia. Special Report, 15, 107–112.

Konstantinova, P., Bonants, P. J. M., Gent-Pelzer, M. P. E., Zouwen, P., & Bulk, R. (2002). Development of specific primers for detection and identification of *Alternaria* spp. in carrot material by PCR comparison with blotter and plating assays. *Mycological Research, 106*(1), 23–32.

Kumar, V., Haldar, S., Pandey, K. K., Singh, R. P., Singh, A. K., & Singh, P. C. (2008). Cultural, morphological, pathogenic and molecular variability amongst tomato isolate of *Alternaria solani* in India. *World Journal of Microbiology and Biotechnology, 24*, 1003–1009.

Kusaba, M., & Tsuge, T. (1995). Phylogeny of *Alternaria* fungi known to produce host-specific toxins on the basis on the variation in the internal transcribed spaces of the ribosomal DNA. *Current Genetics, 28*, 491–498.

Lapagodi, A. L., & Thanassoulopoulos, C. C. (1998). Effect of a leaf spot disease caused by *Alternaria alternata* on sunflower yield in Greece. *Plant Disease, 82*, 41–44.

Lawrence, D. P., Gannibal, P. B., Peever, T. L., & Pryor, B. M. (2013). The sections of *Alternaria*: formalising species-groups concepts. *Mycologia, 105*(3), 530–546.

Logrieco, A., Moretti, A., & Solfrizzo, M. (2009). *Alternaria* toxins and plant diseases: an overview of origin, occurrence and risks. *World Mycotoxin Journal, 2*(2), 129–140.

Lourenco Jr., V., Moya, A., Gonzalez-Candelas, F., Carbone, I., Maffia, L. A., & Mizubuti, E. S. G. (2009). Molecular diversity and evolutionary processes of *Alternaria solani* in Brazil inferred using genealogical and coalescent approaches. *Phytopathology, 99*, 765–774.

Meng, J. W., Zhu, W., He, M. H., Wu, E. J., Duan, G. H., Xie, Y. K., Jin, Y. J., Yang, L. N., Shang, L. P., & Zhan, J. (2015). Population genetic analysis reveals cryptic sex in the phytopathogenic fungus *Alternata alternata*. *Scientific Reports, 5*, 18250.

Nishikawa, J., & Nakashima, C. (2013). Taxonomic characterization and experimental host ranges of four newly recorded species of *Alternaria* from Japan. *Journal of Phytopathology, 161*, 604–616.

Park, M. S., Romanoski, C. E., & Pryor, B. M. (2008). A re-examination of the phylogenetic relationship between the causal agents of carrot black rot, *Alternaria radicina* and *A. carotiincultae*. *Mycologia, 100*(3), 511–527.

Peever, T. L., Su, G., Carpenter-Boggs, L., & Timmer, L. W. (2004). Molecular systematic of citrus-associated *Alternaria* species. *Mycologia, 96*(1), 119–134.

Peever, T. L., Carpenter-Boggs, L., Timmer, L. W., Carris, L. M., & Bhatia, A. (2005). Citrus black rot is caused by phylogenetically distinct lineages of *Alternaria alternata*. *Phytopathology, 95*, 512–518.

Pryor, B. M., & Gilbertson, R. L. (2000). Molecular phylogenetic relationships amongst *Alternaria* species and related fungi based upon analysis of nuclear ITS and mt SSU rDNA sequences. *Mycological Research, 104*(11), 1312–1321.

Pryor, B. M., & Michailides, T. J. (2002). Morphological, pathogenic, and molecular characterization of *Alternaria* isolates associated with Alternaria late blight of pistachio. *Phytopathology, 92*, 406–416.

Rotem, J. (1994). The genus Alternaria: Biology, epidemiology and pathogenicity. APS Press: St Paul.

SAS Institute Inc. (2013). *Base SAS® 9.4 procedures guide: Statistical procedures*. Cary: SAS Institute Inc..

Simmons, E.G. (2007). Alternaria: An identification manual. CBS Biodiversity 6, CBS Fungal Biodiversity Centre, Utrecht, The Netherlands.

Stammler, G., Bohme, F., Philippi, J., Miessner, S., & Tegge, V. (2014). Pathogenicity of *Alternaria* species on potatoes and tomatoes. *Fourteenth EuroBlight Workshop: Limassol, Cyprus.* Special Report, 16, 85–96.

Swofford, D. L. (2002). PAUP*. *Phylogenetic analysis using parsimony (*and other methods)*. Version 4. Sinauer Associates. Sunderland, Massachusetts.

Thomma, B. P. H. J. (2003). *Alternaria* spp.: From general saprophytic to specific parasite. *Molecular Plant Pathology, 4*(4), 225–236.

van der Westhuizen, G. C. A., & Holtzhausen, M. A. (1980). *Alternaria helianthi* on sunflower in South Africa. *Phytopathologica, 12*, 49–52.

Woudenberg, J. H. C., Groenewald, J. Z., & Crous, P. W. (2013). *Alternaria* redefined. *Studies in Mycology, 75*(1), 171–212.

Woudenberg, J. H. C., Seidi, M. F., Groenewald, J. Z., de Vries, M., Stielow, J. B., Thomma, B. P. H. J., & Crous, P. W. (2015a). *Alternaria* section *Alternaria*: Species, *formae speciales* or pathotypes? *Studies in Mycology, 82*, 1–21.

Woudenberg, J. H. C., van der Merwe, N. A., Jurjevic, Z., Groenewald, J. Z., & Crous, P. W. (2015b). Diversity and movement of indoor *Alternaria alternata* across the mainland USA. *Fungal Genetics and Biology, 81*, 62–72.

Zhu, X. Q., & Xiao, C. L. (2015). Phylogenetic, morphological and pathogenic characterization of *Alternaria* species associated with fruit rot of blueberry in California. *Phytopathology, 105*, 1555–1567.