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ORIGINAL ARTICLE

Bacterial endophytes from wild and ancient maize are able to suppress the fungal pathogen *Sclerotinia homoeocarpa*

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Abstract

Aims: The aim of this study was to determine if endophytes from wild and ancient *Zea* plants (corn family) have anti-fungal activities, specifically against the most important fungal pathogen (*Sclerotinia homoeocarpa*) of creeping bentgrass, a relative of *Zea*, used here as a model grass.
Methods and Results: A library of 190 bacterial endophytes from wild, ancient and modern *Zea* plants were tested for their ability to suppress *S. homoeocarpa in vitro*, followed by *in planta* testing of candidates using greenhouse trials. Three endophytes could suppress *S. homoeocarpa*, originating from wild maize and an ancient Mexican landrace, consistent with our hypothesis. 16S phylogenetic analysis and BOX-PCR DNA fingerprinting suggest that the anti-fungal endophytes are distinct strains of *Burkholderia gladioli*. One strain (3A12) was confirmed to colonize creeping bentgrass using green fluorescent protein (GFP) tagging. Evans blue vitality staining demonstrated that the bacterial endophytes exhibited fungicidal activities against the pathogen. The endophytes inhibited a wide spectrum of plant-associated fungi including diverse crop pathogens.
Conclusions: The results support the hypothesis that wild and ancient *Zea* genotypes host bacterial endophytes that can control fungal pathogen(s).
Significance and Impact of the Study: These results suggest that wild and ancient crops may be an unexplored reservoir of anti-fungal bacterial endophytes.

Introduction

Microbes that inhabit host plant living tissue without showing disease symptoms are referred to as endophytes (Hallmann *et al.* 1997; Strobel 2003). Some endophytes seem to establish this nonharmful relationship with plants to gain access to their protected habitats (Saikkonen *et al.* 2004). In return, endophytes help their host plants to acquire nutrients or fight bacterial and fungal pathogens (Rosenblueth and Martínez-Romero 2006; Ryan *et al.* 2008; Johnston-Monje and Raizada 2011).

Wild plants grow and resist pathogens without the use of fungicides, which has primarily been attributed to plant genome-mediated immunity (Rosenthal and Dirzo 1997; Dávila-Flores *et al.* 2013; de Lange *et al.* 2014). In barley, however, endophytes isolated from the roots of a wild relative were shown to have potent anti-pathogen activities (Murphy *et al.* 2015). Our group has previously cultured ~200 bacterial endophytes from the agriculturally important genus *Zea* which includes maize (corn). These endophytes were isolated from modern maize (*Zea mays* ssp. *mays*) as well as its wild and ancient relatives (Johnston-Monje and Raizada 2011; Johnston-Monje *et al.* 2014). These relatives included the primary ancestor of modern maize, known as Parviglumis (*Zea mays* ssp. parviglumis) which is an annual teosinte grass that continues to grow today in southwestern Mexico (Piperno *et al.* 2009), as well as *Zea diploperennis*, a unique perennial teosinte grass from Mexico (Iltis and Doebley 1980). Following its domestication ~9000 years ago, maize was migrated by indigenous farmers throughout the Americas and bred into diverse landraces (Matsuoka *et al.* 2002), including the

ancient Mexican landrace Chapalote (*Zea mays* ssp. *mays* landrace Chapalote). In the 20th century, the landraces were further bred by professional breeders into modern inbreds and hybrids. We hypothesized that the wild and ancient relatives of modern maize may host endophytes that assist plants to control fungal pathogens.

Creeping bentgrass (*Agrostis stolonifera*) is a relative of *Zea* within the grass family (Poacea) (Rotter *et al.* 2007). Similar to maize, it is a domesticated plant (Rotter *et al.* 2007) and is the most widely used turfgrass species on golf greens (Casler 2006). The most economically important disease that affects creeping bentgrass is dollar spot disease, caused by the fungus *Sclerotinia homoeocarpa* (soon to be reclassified to the *Rutstroemiaceae* family) (Beirn *et al.* 2013; Rioux *et al.* 2014). *Sclerotinia homoeocarpa* enters the plant through wounds, natural openings on the leaves (stomata) or by appressorium formation (Orshinsky *et al.* 2012). The disease appears as straw coloured lesions with reddish brown borders (Walsh *et al.* 1999). Clusters of lesions may reach the $1 US dollar coin, hence the name dollar spot. There are increasing demands to find bio-based methods to control dollar spot disease, to overcome the problems associated with the use of synthetic fungicides (Compant *et al.* 2005), and other cost/labour intensive cultural control methods (Ellram *et al.* 2007; Giordano *et al.* 2012). *Sclerotinia homoeocarpa* is widespread among the grass relatives of *Zea* plants, affecting at least 11 genera (Walsh *et al.* 1999).

The objective of this study was to test the hypothesis that wild and ancient *Zea* plants host endophytes that control the fungal pathogens of modern crops, using creeping bentgrass as a model grass system. Here, we screened 190 bacterial endophytes derived from seeds, roots and shoots of *Zea* for their ability to suppress *S. homoeocarpa*. The results show that the wild and ancient relatives of modern maize possess endophytes that can control this pathogen.

Materials and methods

Source of endophytes

The bacterial endophytes used in this study were isolated from seeds, roots and shoots of 14 different genotypes belonging to *Zea* (Fig. 1 and Table S1), as previously reported (Johnston-Monje and Raizada 2011; Johnston-Monje *et al.* 2014).

In vitro screen for endophytes with activity against *Sclerotinia homoeocarpa*

Sclerotinia homoeocarpa was cultured in YPD media at 25°C at 80 rev min^{-1} for 3 days. Presterilized potato dextrose agar (PDA) was melted, cooled to 50°C, inoculated with an *S. homoeocarpa* culture at a ratio of 1 : 25 (fungal culture: media, v/v), and then the inoculated PDA was poured into Petri dishes (150 mm × 15 mm) and permitted to solidify. Wells were created in the agar using sterilized Pasteur pipettes. Maize endophytes were cultured using LB media and permitted to grow overnight at 37°C with shaking at 250 rev min^{-1}. The endophyte cultures were adjusted to OD_{595} of 0·4–0·6. Thirty microliters from each endophyte culture were added to triplicate agar plates containing *S. homoeocarpa*. The plates were subsequently incubated at 25°C for 3–5 days,

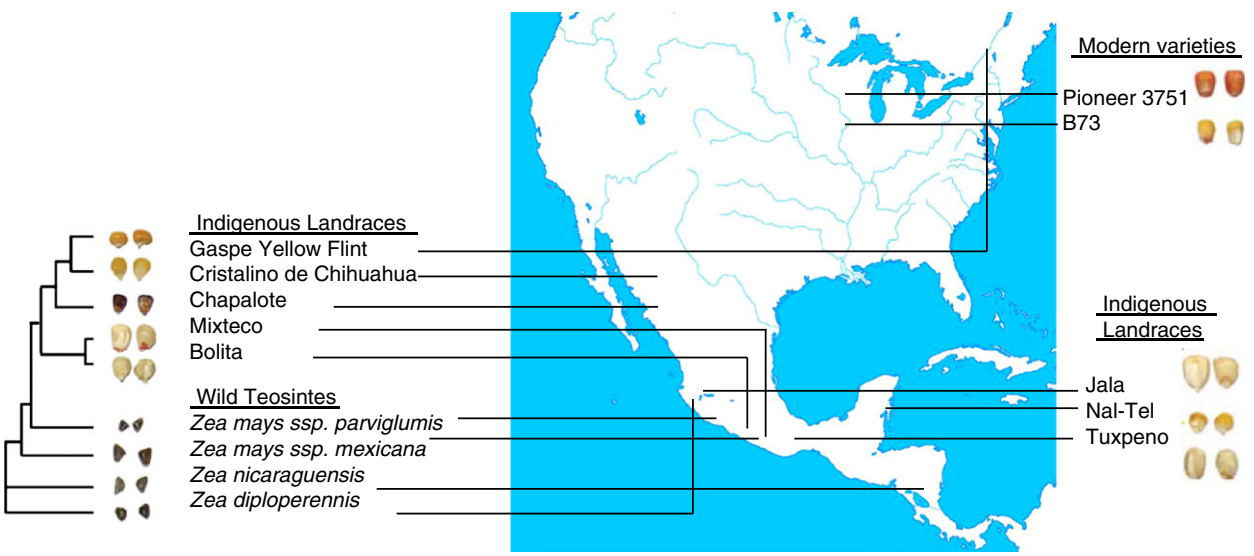


Figure 1 A map showing the different *Zea* genotypes previously used to collect the endophytes used (Johnston-Monje and Raizada 2011; Johnston-Monje *et al.* 2014).

and zones of inhibition of *S. homoeocarpa* hyphal growth were measured. Endophytes associated with a fungal zone of inhibition were selected for further experiments. Nystatin fungicide (N581; PhytoTechnology Laboratories, Overland Park, KS) was used as a positive control at a concentration of 303 units well^{-1} (0·05 mg well^{-1}).

Taxonomic identification of candidate endophytes

Endophytic strain 3A12 was previously identified using its full genome sequence (Ettinger *et al.* 2015). For all other endophytes, the full length 16S rRNA sequence was used for taxonomic identification. DNA was extracted using a Bacterial Genomic Miniprep Kit (NA2110; Sigma, St. Louis, MO). A NanoDrop ND-1000 machine (Thermo Scientific, Waltham, MA) was used to quantify DNA, then per endophyte, 100 ng of DNA was used as template in a PCR reaction with universal 16S rDNA primers (Frank *et al.* 2008; Ghyselinck *et al.* 2013) in a total volume of 40 μl. The reaction mixture contained: 20 μl of GoTaq® Green Master Mix (M712C; Promega, Madison, WI), 1 μl of 10 μmol l^{-1} 27f primer with sequence AGAGTTTGATCMTGGCTCAG, 1 μl of 10 μmol l^{-1} 1492r primer with sequence GGTTACCTTGTTACGACTT, and double distilled water up to 40 μl. For degenerate primers, M = A, C. The following amplification conditions were used: 94°C for 5 min, 35 amplification cycles (94°C for 45 s, 50°C for 1 min, 72°C for 2 min), and a final extension at 72°C for 7 min. PCR amplicons were gel purified (Illustra GFX; GE Healthcare, Little Chalfont, UK), submitted for sequencing using both forward and reverse primers (Laboratory Services, University of Guelph) and identified using BLAST searches. To confirm bacterial identity at the species level, reference 16S sequences were obtained from GenBank. These sequences were then used to construct phylogenetic trees using Phylogeny.lirmm.fr using default parameters (Castresana 2000; Edgar 2004; Dereeper *et al.* 2008).

Genomic fingerprinting of candidate endophytes

The BOX-PCR reaction consisted of 20 μl of GoTaq® Green Master Mix (M712C; Promega, Madison, WI), 50 ng of DNA, 2 μmol l^{-1} BOX-A1R primer with sequence CTACGGCAAGGCGACGCTGACG and double distilled water up to 40 μl. The amplification conditions were: 95°C for 7 min, 30 amplification cycles (95°C for 1 min, 53°C for 1 min, 65°C for 8 min), and a final extension at 65°C for 15 min (Cottyn *et al.* 2001). From each PCR product, 20 μl were loaded onto 1% agarose gel in TAE and run at 75 V for 11 h beside O'GeneRuler DNA Ladder Mix (SM1173, Thermo Fisher Scientific, Waltham, MA).

Confirming the endophytic ability of strain 3A12

Please see supplemental methods (Appendix S1).

Greenhouse testing of antifungal endophyte candidates using creeping bentgrass

Experimental design

Field cores (cup cuts, 12 cm diameter, 9 cm deep) were harvested using a golf cup cutter from a creeping bentgrass putting field (cultivar Mackenzie, grown on 80:20 sand:peat, according to USGA specification) taken from the Guelph Turfgrass Institute (Guelph, ON Canada) and placed into pots (12 cm diameter). Pots were incubated in a greenhouse at 23°C day/18°C night, 16-h daylight, supplemented when natural light fell below 500 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) (at pot level) with a mixture of 400–600 W high pressure sodium lights to provide an additional 70–80 μmol m^{-2} s^{-1} PPFD (at pot level). Light was measured using a quantum meter (model QMSS; Apogee Instruments Inc, Logan, UT). Treatments included: buffer only control; individual endophyte treatments (3A12, 3C11, 4H12, 5C9) as well as 3A1 (as a negative control); pyramided endophyte treatment (mixture of equal volumes of 3A12, 3C11 and 5C9); and a fungicide (positive control, see below). For each endophyte treatment, there were four replicate field cores inoculated with *S. homoeocarpa* and four controls that were not inoculated with *S. homoeocarpa*. Controls that were treated with an endophyte but not inoculated with the fungal pathogen were used to measure for any pathogenicity caused by the endophytes. The fungicide treatment consisted of the maximum label application rate for Banner MAXX (51 ml 100 m^{-2}, Propiconazole 14·3%, 60207-90-1; Syngenta Crop Protection, Guelph, Ontario, Canada). Pots were randomized, and the entire greenhouse trial was independently replicated.

Endophyte inoculant preparations

To prepare the endophyte inoculants, each bacterium was cultured for ~20 h in 200 ml of LB in 500 ml flasks with shaking at 250 rev min^{-1} at 30°C. Cells were collected by centrifugation, washed in 10 mmol l^{-1} Tris HCl pH 7, resuspended in 10 mmol l^{-1} Tris HCl pH 7 to OD_{595} = 0·5.

Pathogen inoculum preparation

The pathogen inoculums were prepared by autoclaving 200 g of Kentucky bluegrass seeds, adding 100 ml of sterile water, which were then mixed, allowed to stand overnight, and mixed with ½ plate of *S. homoeocarpa* grown on PDA plates (100 mm × 15 mm, cut into small pieces). The inoculated seed carriers were then covered

and incubated for 2–3 weeks, spread out on newspaper to air dry, passed through a 2 mm sieve to break clumps, placed into plastic bags and refrigerated.

Endophyte and pathogen applications
After field cores had been transplanted into the greenhouse, each pot received 5 ml of endophyte suspension in the form of a canopy spray. Control pots were sprayed with 5 ml of buffer (10 mmol l^{-1} Tris HCl, pH 7). One week later, the grass was clipped to ~3 cm and re-sprayed with endophytic bacteria. Pots were irrigated daily with a top spray of water and fertilizers (20-8-20). One week later, 0·2 g of *S. homoeocarpa* (coated onto Kentucky bluegrass seed as carrier) was sprinkled into each pot. Following inoculation of the fungal pathogen, turf pots were then covered with plastic bags to help maintain high humidity to favour disease development. No irrigation or clipping was performed after pathogen inoculation. Pots were observed daily for disease symptoms.

Disease scoring and statistical analysis
Disease scoring was performed quantitatively using ASSESS software (43696M5; American Phytopathological Society, Saint Paul, MN) from picture scans. The software colour threshold was set to 105 for the upper threshold and 31 for the lower threshold to differentiate healthy areas from diseased areas. For statistical analysis, one-way ANOVA was used (PRISM; Graphpad Software, La Jolla, CA).

Characterization of *in vitro* interactions between pathogen and endophytes

The *in vitro* interactions between endophytes and *S. homoeocarpa* were visualized on microscope slides. *Sclerotinia homoeocarpa* was cultured in YPD media for 2–3 days at 25°C at 80 rev min^{-1}. The endophytes were cultured in liquid LB overnight at 37°C with shaking at 250 rev min^{-1}. One ml of PDA was spread on sterilized glass slides placed in Petri dishes and allowed to solidify. A fragment of *S. homoeocarpa* was applied to the centre of each slide, and then 20 μl of each endophyte culture was applied to one side of *S. homoeocarpa*, and on the other side 20 μl of LB media was applied. Slides were incubated at 25°C overnight. Controls consisting of 1% Banner MAXX (60207-90-1; Syngenta Crop Protection, Guelph, Ontario, Canada) in water and 1% Nystatin (N581, PhytoTechnology Laboratories, Overland Park, KS) in DMSO were included. Slides were stained using Evans blue (#206334; Sigma, St. Louis, MO), then examined using light microscopy (B1372, Axiophot; Zeiss, Oberkochen, Germany) and NORTHERN ECLIPSE software.

Anti-fungal target spectrum of strain 3A12, 3C11 and 5C9

Using the dual culture method, the anti-fungal target spectra of endophytes 3A12, 3C11 and 5C9 were tested against a library of diverse plant-associated fungi including crop pathogens obtained from the Agriculture and Agrifood Fungal Type Collection (AAFC, Guelph, Canada). The library was comprised of 14 species; the species name along with the major crops affected (in brackets) are: *Alternaria alternate* (strawberry and pear) (Ito *et al.* 2004). *Aspergillus niger* (maize, grapes and peanut) (Palencia *et al.* 2010), *Davidiella tassiana* (pear, grape, cherry and date palm) (Barbosa *et al.* 2001), *Diplodia pinea* (pine) (Waterman 1943), *Fusarium avenaceum* (maize, wheat) (Shaner 2003; Kang *et al.* 2005), *Fusarium lateritium* (sweet potato, groundnut and olive) (Vitale *et al.* 2011), *Fusarium sporotrichioides* (maize) (Logrieco *et al.* 2002), *Gibberella avenacea* (maize) (Shaner 2003), *Nigrospora oryzae* (rice) (Sempere and Santamarina, 2008), *Nigrospora sphaerica* (blueberry and date palm) (Wright *et al.* 2007; Abass *et al.* 2013), *Paraconiothyrium brasiliense* (peach, nectarine and plum) (Damm *et al.* 2008), *Penicillium commune* (pear and apple) (Sanderson and Spotts 1995), *Penicillium expansum* (apple) (Lai *et al.* 2014) and *Trichoderma longibrachiatum* (biocontrol agent and opportunistic human pathogen) (Kuhls *et al.* 1999). The different fungal strains were cultured in YPD media for 3 days at 25°C at 80 rev min^{-1}. Previously sterilized PDA was melted, allowed to cool to 50°C, mixed with each fungal culture, poured into plates and allowed to completely solidify. Holes were created into the agar using a sterile Wessermann tube, and the resulting agar plugs were removed using a sterilized wire loop. The day before, strains 3A12, 3C11 and 5C9 were cultured in LB media and allowed to grow overnight at 37°C with shaking at 250 rev min^{-1}. Thirty microlitres of each culture (OD_{595} = 0·8) were applied in each hole for each fungus, on triplicate agar plates. Plates were incubated at 25°C for 3–5 days, and inhibition zones of fungal growth were measured and recorded.

Results

In vitro screen for endophyte activity against *Sclerotinia homoeocarpa*

Using dual culture assays (Fig. 2a), 190 endophytes were screened for *in vitro* antifungal activity against *S. homoeocarpa*. Five endophytes showed zones of inhibition of fungal growth (Fig. 2b–c, Table S1).

Figure 2 *In vitro* testing of maize endophytes for antifungal activity. (a) Example of a Petri dish dual culture screen showing zones of inhibition of *Sclerotinia homoeocarpa* by endophytes 3A12 and 3C11. (b) Graph showing the mean zone of inhibition diameter (cm) associated with each candidate endophyte and controls. Asterisks indicate significant difference from the negative control. (c) Summary of candidate endophytes with anti-*Sclerotinia* activity, their predicted taxonomy, and host plant source. (d–f). 16S phylogenetic trees to assist with taxonomic identification of: (d) endophyte 3A12, 3C11, 5C9, (e) endophyte 3H8, and (f) endophyte 4H12. (g–h) Genome fingerprinting of strains 3A12, 3C11 and 5C9. (g) fingerprinting pattern after gel electrophoresis where lanes from left to right show DNA ladder, strain 3A12, strain 3C11, strain 5C9 and a negative control, and (h) the corresponding lane profiles for strains 3A12, 3C11 and 5C9. (i–j) Confirmation of the endophytic ability of strain 3A12 through visualization of GFP tagged strain 3A12 in creeping bentgrass shoots using confocal microscopy. The scale bar is 10 μm.

Taxonomic identification of candidate endophytes

One endophytic candidate (3A12) was classified after whole genome sequencing as most closely matching *Burkholderia gladioli* (Ettinger *et al.* 2015). The other four candidate endophytes were identified based on 16S rRNA sequencing combined with phylogenetic tree analysis. Two endophytes (3C11 and 5C9) most closely resembled *B. gladioli* (100%) (Accession numbers KP455296 and KP455294) (Fig. 2d); the three predicted *Burkholderia* endophytes (3A12, 3C11 and 5C9) showed 100% DNA sequence identity to one another in their 16S rRNA genes. Genomic fingerprinting using BOX-PCR showed that the *B. gladioli* isolates represent three distinct strains (Fig. 2g–h). Strain 3H8 most closely resembled *Bacillus subtilis* (100%) (accession number KP455298) and 4H12 resembled *Paenibacillus* polymyxa (99%) (accession number KP455297) (Fig. 2e–f). Respectively, 3A12, 3C11 and 3H8 originated from seeds of: an ancient Mexican maize landrace (*Zea mays* ssp *mays* landrace Chapalote); a wild Central American perennial maize (*Zea diploperennis*); and a modern commercial hybrid (*Zea mays* ssp *mays*, Pioneer 3751). Strain 4H12 originated from roots of the Pioneer 3751 hybrid, while 5C9 originated from roots of the extant wild ancestor of modern maize (*Zea mays* ssp *parviglumis*) (Fig. 2c, Table S1).

Confirming the endophytic ability of strain 3A12

Confocal microscopic examination of creeping bentgrass plants that had been seed coated with GFP tagged strain 3A12 showed that the microbe can colonize shoots of creeping bentgrass, suggesting that it behaves as an endophyte in creeping bentgrass (Fig. 2i–j).

Greenhouse testing of antifungal endophyte candidates

The candidate antifungal endophytes were tested for their ability to suppress dollar spot disease in replicated greenhouse trials. To determine the reliability of ASSESS software for disease scoring, positive and negative controls were first evaluated (Fig. S1).

After validating the disease scoring methodology, the endophytes were applied as sprays on creeping bentgrass field cores prior to inoculation with *S. homoeocarpa* (Fig. 3; Figs S2–S5). One endophyte (3H8) was excluded from greenhouse testing, as it failed to suppress the disease in a pretrial involving test tubes (data not shown). Endophytes 3A12, 3C11, 5C9 as well as a mixture of all three endophytes were found to reduce mean dollar spot disease symptoms in two independent trials (Fig. 3a–f, j–o, s, t; Figs S2 and S4, Table S2). The endophyte mixture did not show better antifungal activity when compared to individual endophytes. Endophyte 4H12 did not show disease suppression in either trial (Fig. 3g,p,s,t; Figs S2 and S4). Endophyte 3A1 (used here as a negative control) did not reduce disease symptoms (Fig. 3h,q,s,t; Figs S2 and S4) while the fungicide Banner MAXX was able to reduce disease symptoms (Fig. 3i,r,s,t; Figs S2 and S4). Endophytes 3A12, 3C11, 5C9 and the endophyte mixture did not cause pathogenicity on creeping bentgrass (Figs S3, S5 and S6 and Table S2).

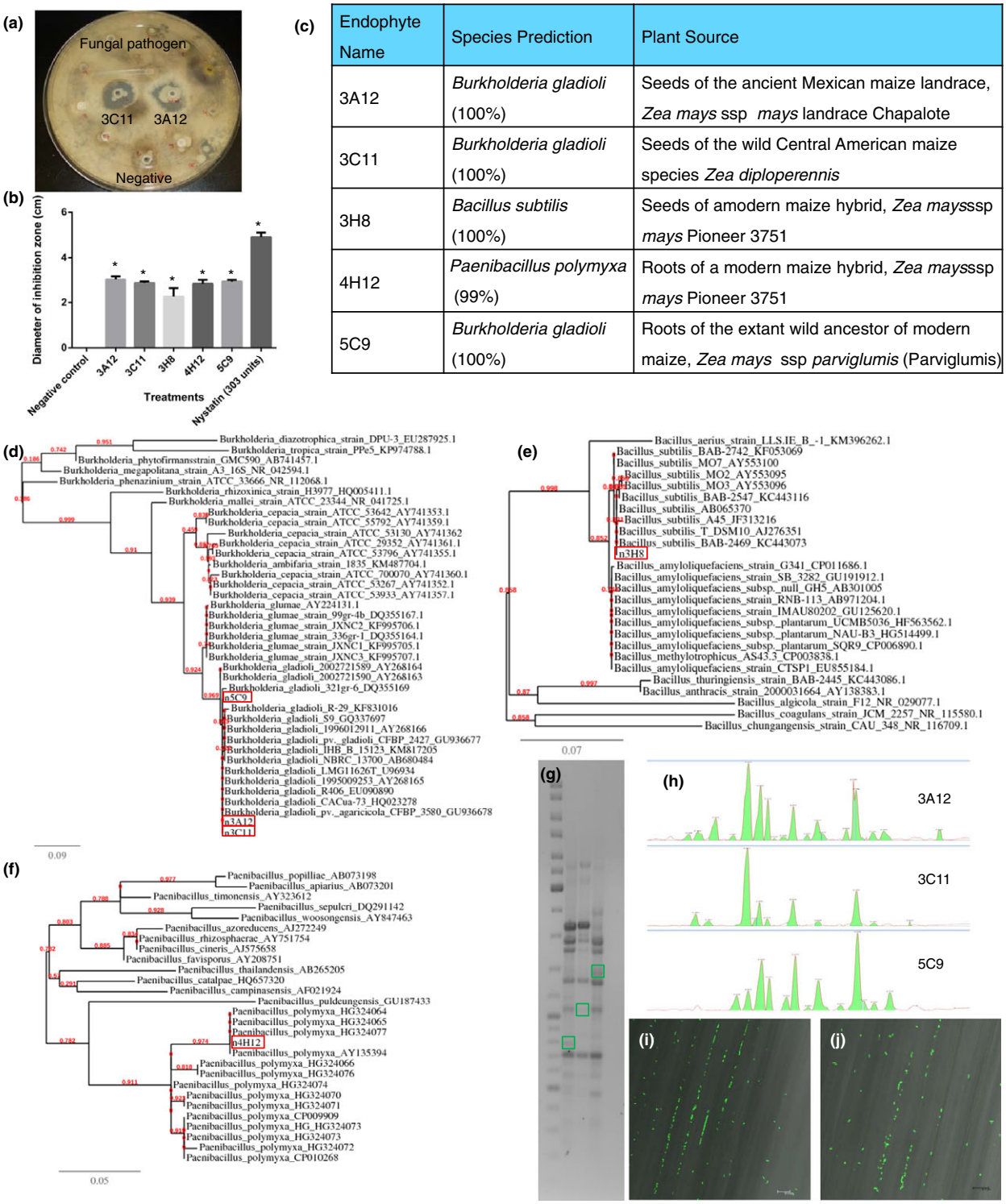
Characterization of *in vitro* interactions between the pathogen and endophytes

To help understand the anti-fungal mode of action of the candidate endophytes, they were grown side by side with *S. homoeocarpa* on microscope slides then stained with Evans blue, which stains mycelia blue when dead (Fig. 4a).

None of the fungal mycelia stained blue on the side exposed only to LB media (control) (Fig. 4b,d,h,j,l,n) but they stained blue when in contact with endophytes 3A12, 3C11, 4H12 and 5C9 indicating mycelial death (Fig. 4c,e, i,k); endophyte 3H8 was the exception (Fig. 4f,g). The fungal mycelia also stained blue when in contact with the fungicides, Banner MAXX (Fig. 4m) or Nystatin (Fig. 4o). We conclude that endophytes 3A12, 3C11, 4H12 and 5C9 exhibit fungicidal activity against *S. homoeocarpa in vitro*, whereas 3H8 may be fungistatic.

Anti-fungal target spectrum of candidate antifungal endophytes

We then tested whether the candidate endophyte strains could inhibit other plant-associated fungi including well known crop pathogens. Using dual culture assays to test for the antifungal activity of strain 3A12, 3C11 and 5C9



Endophyte Name	Species Prediction	Plant Source
3A12	*Burkholderia gladioli* (100%)	Seeds of the ancient Mexican maize landrace, *Zea mays* ssp *mays* landrace Chapalote
3C11	*Burkholderia gladioli* (100%)	Seeds of the wild Central American maize species *Zea diploperennis*
3H8	*Bacillus subtilis* (100%)	Seeds of amodern maize hybrid, *Zea mays*ssp *mays* Pioneer 3751
4H12	*Paenibacillus polymyxa* (99%)	Roots of a modern maize hybrid, *Zea mays*ssp *mays* Pioneer 3751
5C9	*Burkholderia gladioli* (100%)	Roots of the extant wild ancestor of modern maize, *Zea mays* ssp *parviglumis* (Parviglumis)

against diverse plant fungi revealed that endophytes 3A12, 3C11 and 5C9 were active against 14, 14 and 13 out of 14 tested fungi respectively (Fig. 5). These results suggest that the bacterial endophytes with potential antifungal activity against *S. homoeocarpa* have a broad target spectrum of antifungal activity.

Discussion

Based on the results obtained from the *in vitro* screen and the greenhouse trials, three candidate endophytes from *Zea* were discovered that have potential to suppress the grass pathogen, *S. homoeocarpa* (endophytes 3C11, 5C9 and 3A12). The full draft genome sequence of one of the potent anti-fungal microbes, endophyte 3A12, showed that the closest taxonomic match was to *B. gladioli* (Ettinger *et al.* 2015), while molecular analyses predict that the other two anti-fungal strains are also *B. gladioli.* Genomic DNA fingerprinting demonstrated that the three *B. gladioli* isolates are distinct strains. All bacterial endophytes with potential antifungal activity originated from wild maize (Diploperennis, Parviglumis) or ancient maize (Chapalote) (Fig. 6), providing support for our hypothesis that wild and ancient relatives of crops may be a reservoir of bacterial endophytes that can suppress fungal pathogens.

Ancient selection for endophytes?

Wild Diploperennis plants originate from the Sierra de Manantlan region, Cuatitlan, Jalisco, Mexico. This area experiences rainfall (about 1800 mm) for up to 6 months, and the temperature of this region varies from cool to very warm (Iltis and Doebley 1980). By contrast, wild Parviglumis plants originate from the tropical forest in the Central Balsas of southwestern Mexico (Piperno *et al.* 2009). Hence, both Parviglumis and Diploperennis derive from wet conditions, which are known to favour fungal pathogens. It may be that these plants selected for endophytes with antifungal activity. By contrast, the landrace Chapalote originates from the dry lowlands of Sonora and Sinaloa, Northern Mexico (Wellhausen 1952; Carpentier 2004). One possible hypothesis is that the antifungal endophyte(s) co-evolved with *Zea* under wet conditions but were retained in early domesticated maize, including Chapalote, as it was migrated by indigenous farmers to the dry lowlands of Mexico.

Diploperennis is a wild perennial plant (Iltis *et al.* 1979). Parviglumis is a wild annual teosinte that was domesticated 9000 years ago into modern maize (Matsuoka *et al.* 2002). Chapalote is considered the missing link between wild and modern maize. It is an ancient indigenous landrace, that emerged in the archaeological records >3000 years ago in Mexico (Wellhausen 1952). Hence, the three predicted anti-fungal *B. gladioli* endophyte strains from this study were isolated from a range of host genotypes that span the stages of crop evolution, from perennialism to annualism in the wild, to domestication by humans (from wild annual plants to farmer landraces). The bacilli strains may represent more recent genetic selection from traditional landraces to modern cultivars by professional breeders (Rosenthal and Dirzo 1997).

Though *B. gladioli* is a well-recognized pathogen of humans and plants (Kanj *et al.* 1997; Ura *et al.* 2006; Nandakumar *et al.* 2009; Dursun *et al.* 2012; Zhou *et al.* 2015), recent evidence suggests that *B. gladioli* may also be isolated from healthy plant tissues or soils and have beneficial activities (Gupta *et al.* 2012; Ko *et al.* 2012; Pereira *et al.* 2012; Suárez-Moreno *et al.* 2012; Rombola *et al.* 2014; Jha *et al.* 2015). A preliminary RAST-server based search of the draft sequence of strain 3A12 did not show this strain to possess genes encoding toxins, superantigens or virulence/disease factors (Ettinger *et al.* 2015). The search did reveal the presence of genes that encode the plant hormone auxin, suggesting that it may stimulate plant growth (e.g. roots).

Anti-fungal mechanisms of action

With respect to the anti-fungal mechanism(s) of action of the *Burkholderia* endophytes, the genome sequence of strain 3A12 predicts that it can produce antifungal compounds including chitinase and phenazine (Ettinger *et al.* 2015). In a previous report, *B. gladioli* was found to have antifungal activity against the pathogens, *Botrytis cinerea, Aspergillus flavus, Aspergillus niger, Penicillium digitatum, Penicillium expansum, Sclerotinia sclerotiorum and Phytophthora cactorum* (Elshafie *et al.* 2012). The potential mechanism of action was the production of a volatile organic compound (cyclic terpene) (Elshafie *et al.* 2012). In another study, involving *B. gladioli* strain CHB101, the antifungal activity was associated with chitinase production (Kong *et al.* 2001). Other members of the genus *Burkholderia,* comprising >40 species (Vial *et al.* 2007), are well known to produce antimicrobials including phenazine, chitinase, lipopeptides, quinolinones, altericidins, pyrrolnitrin, cepacidines, siderophores and volatile compounds (Vial *et al.* 2007; Schmidt *et al.* 2009). For example, *Burkholderia cepacia* BC11 was found to control *Rhizoctonia solani* in cotton through production of the lipopeptide AFC-BC11 (Kang *et al.* 1998), while another report showed that *B. cepacia* strain 5·5B inhibited *R. solani* by producing a phenazine derivative (Cartwright *et al.* 1995). *Burkholderia cepacia* PC II was reported to inhibit growth of *Phytophthora capsici* in red pepper probably through production of 4-quinolinone metabolites (Moon *et al.* 1996). *Burkholderia cepacia* AF2001 was shown to synthesize cepacidine A which was found to have biocontrol activity against *Pythium ultimum* on cotton and cucumbers (Lee *et al.* 2000). *Burkholderia cepacia* B37w was shown to produce pyrrolnitrin which inhibited

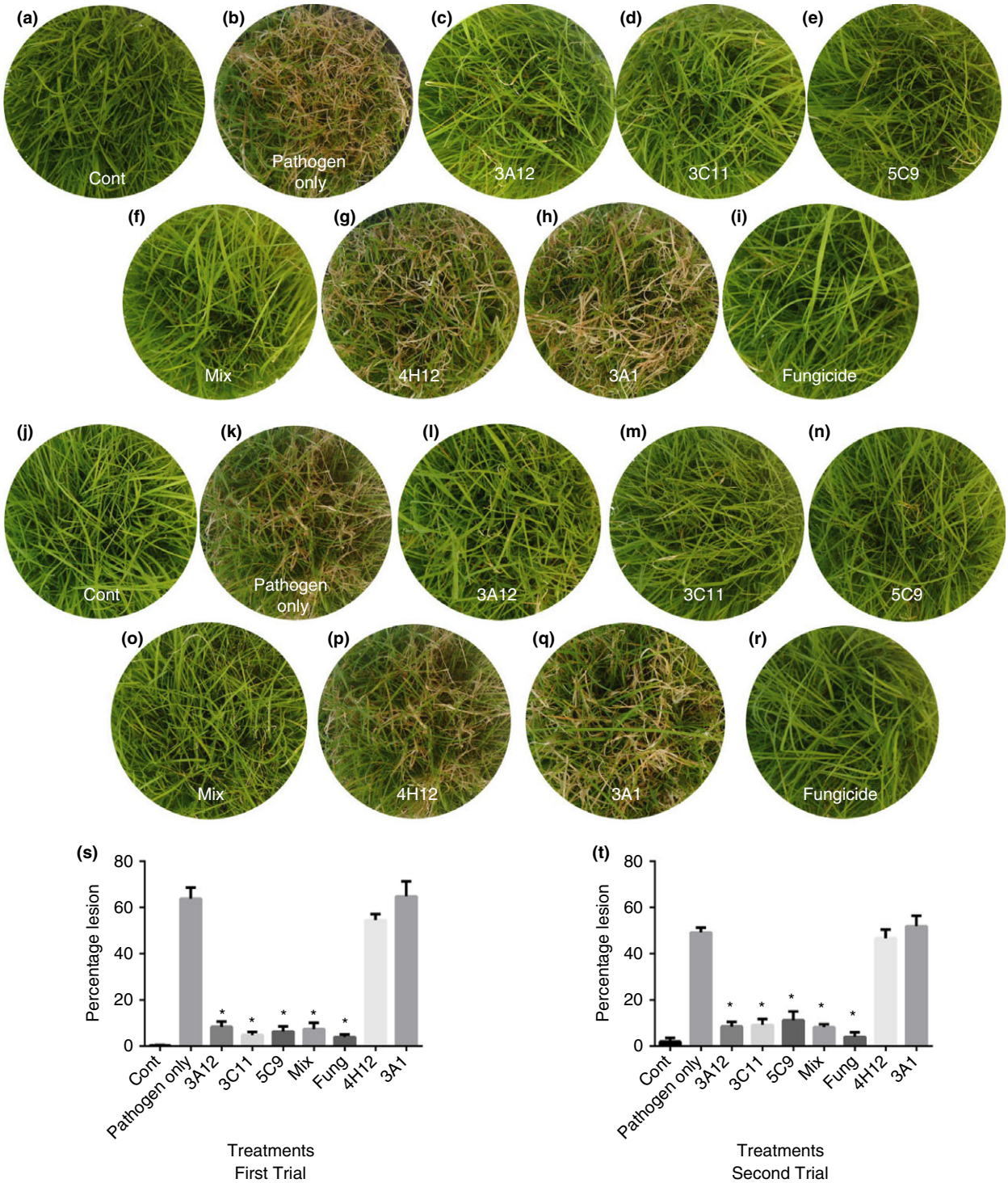


Figure 3 Testing antifungal endophyte candidates on field cores of creeping bentgrass, after inoculation with *Sclerotinia homoeocarpa*. (a–i) Trial 1 and (j–r) Trial 2. (a and j) no endophyte, no pathogen treatment (control); (b and k) no endophyte, pathogen-only treatment (control); (c and l) pathogen with endophyte 3A12; (d and m) pathogen with endophyte 3C11. (e and n) pathogen with endophyte 5C9; (f and o) pathogen with mixture of endophytes 3A12, 3C11 and 5C9; (g and p) pathogen with endophyte 4H12; (h and q) pathogen with endophyte 3A1 (negative control endophyte); and (i and r) pathogen with fungicide treatment (Banner MAXX); (s and t) Graphs showing the mean percentage lesion after each treatment (n = 4) as measured by ASSESS software from Trial 1 and Trial 2 respectively. The histograms represent the mean values, and the error bars represent the standard error of the mean (SEM). Asterisks indicate significant difference at 0·05 compared to the respective pathogen-only control.

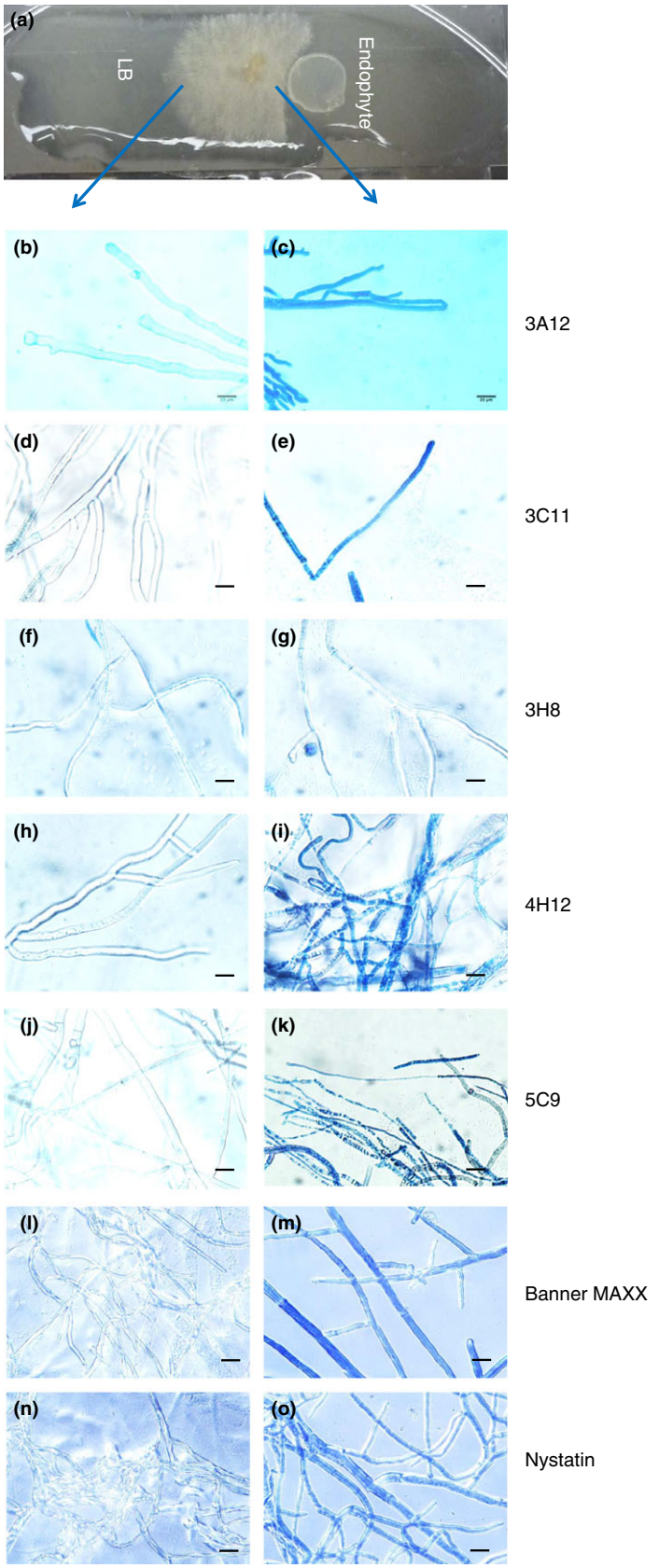


Figure 4 The interactions of candidate bacterial endophytes with *Sclerotinia homoeocarpa* after staining with the Evans blue stain. (a) Methodology used: potato dextrose agar coated microscope slide with *S. homoeocarpa* in the centre, flanked by a candidate endophyte or control antifungal (right) or LB buffer control (left). (b, d, f, h, j, l, n) Mycelia of *S. homoeocarpa* from the no-endophyte or antifungal side (negative controls). (c, e, g, i, k, m, o) Corresponding mycelia of *S. homoeocarpa* from the side exposed to: (c) endophyte 3A12, (e) endophyte 3C11, (g) endophyte 3H8, (i) endophyte 4H12, (k) endophyte 5C9, (m) Banner MAXX, and (o) Nystatin. The scale bar in all images is 20 μm.

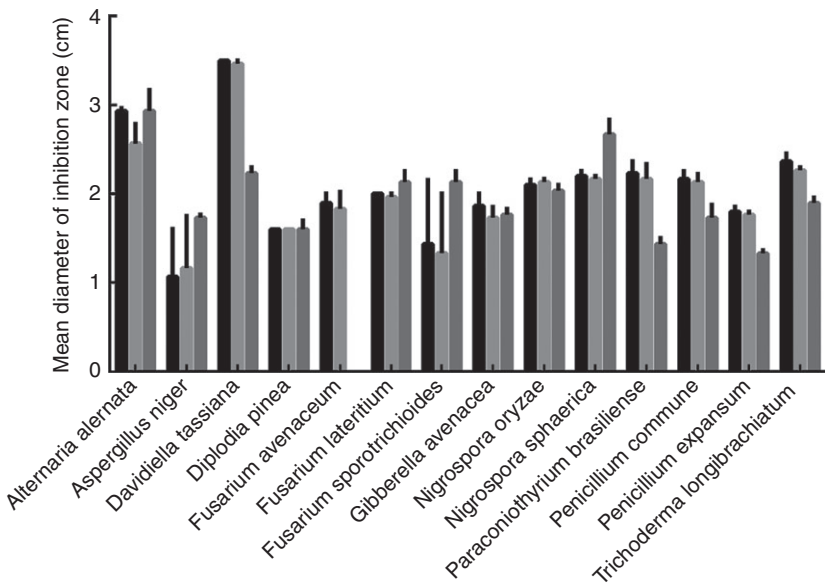


Figure 5 Graph showing the target spectrum of antifungal activity of endophyte strains 3A12, 3C11 and 5C9 against 14 diverse plant-associated fungi including well known crop pathogens. Error bars represent the standard error of the mean. (■) 3A12, (■) 3C11 and (■) 5C9.

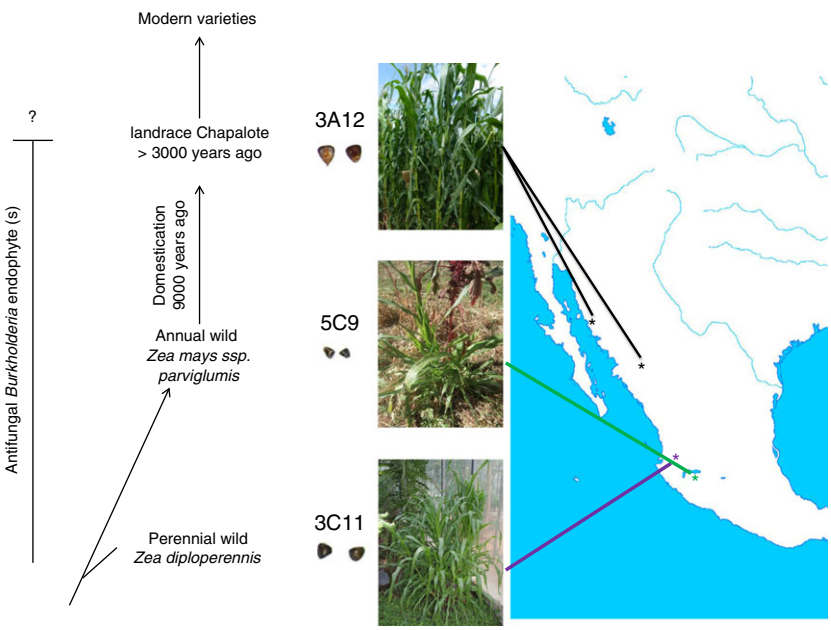


Figure 6 Co-evolutionary model of anti-fungal *Burkholderia* endophyte(s) across maize evolution, domestication, breeding and migration in Mexico.

Fusarium sambucinum, the causative agent of potato dry rot (Burkhead *et al.* 1994).

Some of the above *Burkholderia* compounds may have broad-spectrum anti-fungal activities: for example, pyrrolnitrin from *Burkholderia pyrrocinia* was used as an antifungal agent to treat opportunistic fungal infections in humans (Vial *et al.* 2007). The wide spectrum, diverse antimicrobial arsenal encoded by the *Burkholderia* genome may have made it an ideal endophyte for selection and retention by *Zea*.

Future applications

Dollar spot is an important disease in creeping bentgrass and there is no current and efficient control against it except for the use of synthetic fungicides which are facing increased regulation or use restrictions (Wisniewski and Wilson 1992; Geiger *et al.* 2010). There is concern that extensive use of fungicides is causing the development of resistant strains of *S. homoeocarpa* and causing environmental toxicity due to its slow degradation (Jo

et al. 2008; Ok *et al.* 2010). Other culture-based control strategies (e.g. nitrogen application, dew removal) are cost or labour intensive (Ellram *et al.* 2007; Giordano *et al.* 2012). Development of biological agents to control this disease is thus of great importance. Field trials are now needed to determine if the bacterial endophytes with potential antifungal activity identified in this study can partially control dollar spot disease under real world conditions. Endophyte spray formulations will need to be optimized to improve their efficacy. For example, preliminary data using qPCR suggests that all three endophyte strains (3A12, 3C11, 5C9) colonize creeping bentgrass shoots while only endophyte 5C9 appears to colonize roots effectively (data not shown); this methodology can be used in the future to improve endophyte colonization and persistence. Furthermore, our data showing that the endophytes can target a wide spectrum of modern crop pathogens *in vitro* suggest that these ancient endophytes may be useful in the fight against diverse fungal diseases of modern crops. Nevertheless, the practical application of these endophytes to biological control may be limited, depending on the results of future human safety testing – but our results suggest the endophytes may hold benefits to their native host plants.

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Authors' contributions

HRS helped to design the study, carried out all experiments, performed the analyses, and wrote the manuscript. KSJ and EML helped to design the study and generated materials. MNR helped to design the study and edited the manuscript. All authors read and approved the final manuscript.

Conflict of Interest

No conflict of interest declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Validation of the disease assessments used in this study, using positive and negative controls as follows: (a–d) First trial. (e–h) Second trial. (i–l) Third trial.

Figure S2 Testing of antifungal endophyte candidates (Trial 1) as foliar sprays on field cores of creeping bentgrass, after 10 days of inoculation with *S. homoeocarpa* fungal pathogen.

Figure S3 Testing pathogenicity of antifungal endophyte candidates (Trial 1) on field cores of creeping bentgrass, after 17 days of inoculation with foliar spray of endophytes.

Figure S4 Testing of antifungal endophyte candidates (Trial 2) as foliar sprays on field cores of creeping bentgrass, after 10 days of inoculation with *S. homoeocarpa* fungal pathogen.

Figure S5 Testing pathogenicity of antifungal endophyte candidates (Trial 2) on field cores of creeping bentgrass, after 17 days of inoculation with foliar spray of endophytes.

Figure S6 Testing pathogenicity of antifungal endophyte candidates on field cores of creeping bentgrass, after 17 days of inoculation with foliar spray of endophytes.

Table S1 List of *Zea* endophytes used in this study and their corresponding zones of inhibition of *S. homoeocarpa* growth.

Table S2 Percentage of lesions in turfgrass field cores from greenhouse trials as measured by ASSESS software.

Appendix S1 Confirming the endophytic ability of strain 3A12 (Materials and methods).