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Short Notes

# *Curvularia americana* and *Curvularia tropicalis* cause leaf and crown necrosis on Bermuda grass in Italy

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**Summary.** *Curvularia americana* and *C. tropicalis* are described as causes of leaf and crown necroses on Bermuda grass (*Cynodon dactylon* x *Cynodon transvaalensis)* in Veneto, Northern Italy. These pathogens were characterized using morphological characters, and a multilocus molecular phylogenetic analysis based on the nuclear ribosomal internal transcribed spacer (ITS) region, the partial glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) and translation elongation factor-1α (*tef1-α*) genes. Pathogenicity tests and the fulfilment of Koch's postulates confirmed *C. americana* and *C. tropicalis* as foliar pathogens of Bermuda grass. This is the first report of *C. americana* and *C. tropicalis* as pathogens of Bermuda grass, and the first record these two fungi in Europe.

**Keywords.** *Cynodon*, turf disease, multi-locus typing, golf courses.

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## INTRODUCTION

Bermuda grass is a popular turfgrass in temperate, tropical and subtropical regions. Genus crosses and triploid interspecific hybrid Bermuda grass cultivars were developed for various characteristics, including high turf quality, tolerance to low mowing heights, and resistance to pests (Brecht *et al.*, 2007). Hybrid Bermuda cv. Miniverde grass is the product of the interspecific cross between *Cynodon dactylon* and *Cynodon transvaalensis*. In Italy in the last 20 years, use of Bermuda grass is increasing for high maintenance turfgrasses (such as on golf courses and football fields). More than 50 golf courses in Italy have converted the original fairways and tee areas to Bermuda grass, to reduce water consumption and other maintenance inputs. Hybrid Bermuda grass, and other warm season grasses (*Zoysia* spp. and *Paspalum* spp.), are being used under Italian conditions to obtain a high-quality turf,

and to reduce pesticide use in public areas, as requested by the National Action Plan since 2014. Bermuda grass is currently being used to replace bentgrass on golf greens (Magni *et al*., 2018), being less sensitive to water stresses and diseases than other turf species (Turgeon and Kaminski, 2019).

In August 2020, symptoms of a previously unknown disease were observed on *Cynodon dactylon* × *Cynodon transvaalensis* ' Miniverde' in a golf green in the Padova province, Veneto (Northern Italy). The golf course area (approx. 0.5 ha) was covered with Bermuda grass in 2018. The symptoms consisted of leaf spots which were reducing the playability and the smoothness of the green surfaces.

The aims of the present study were: i) to isolate the causal agents associated with the affected host tissues; ii) to test the pathogenicity of isolates by fulfilling Koch's postulates; and iii) to characterize the isolated fungi using morphological, molecular and phylogenetic tools.

## MATERIAL AND METHODS

### *Symptoms and isolation of fungi*

The first symptoms appeared in May 2020 (3 years after the turfgrass was established), as necrotic areas (diam. 2–10 cm) of affected plants. At the end of the growing season smaller spots were completely recovered, but in October 2020 the affected areas of the turf developed involving up to 10–15% of the area (Figure 1a). Diseased plants showed necrotic leaves and crowns with small, dark, sunken spots (Figure 1b). The infected tissues from leaves and crowns (Figure 2) were surface sterilized by immersion in 1% sodium hypochlorite for 1 min, followed by rinsing twice in distilled water, and then drying on sterile absorbent paper. Small fragments (1–2 mm) were cut from the edges of healthy and necrotic tissues and plated on potato dextrose agar (PDA, Oxoid) amended with 25 μg $mL^{-1}$ of streptomycin sulphate (PDA-S, Sigma-Aldrich). The plates were incubated at 25±1°C under a 12 h photoperiod. Hyphae from the margin of each isolate were placed on PDA-S, then, 5 d later, single conidia were transferred into PDA plates to establish pure cultures. Representative isolates with different macroscopic morphological characteristics were selected (5A, 5B, 15A, 15B and 15C) for further molecular characterization. Stock cultures of these isolates are maintained at 4°C and at -20°C in the AGROINNOVA (University of Torino) culture collection, Torino, Italy.

### *Pathogenicity tests*

Isolates 5A, 5B, 15A, 15B and 15C were inoculated on 50-d-old healthy plants of *Cynodon dactylon* x *Cynodon transvaalensis* cultivated in plastic trays (30 × 50 cm, 12 L volume), containing steam disinfected peat plant growth medium. The isolates were grown on PDA amended with streptomycin sulphate (25 mg $L^{-1}$) and kept at 25°C with a 12 h photoperiod for 7 d. The turfgrass sods were each sprayed with a conidium suspension (final concentration of $10^6$ conidia $mL^{-1}$) for each isolate (n. 2 trays per isolate). Non inoculated grass was sprayed with sterile water as experimental controls. Inoculated and non inoculated grass were then kept in a growth chamber at 25°C with a 12 h photoperiod. Fifteen days after inoculation, the symptom severity (SS) associated with each inoculated isolate was evaluated. Small portions (0.3 cm) of symptomatic leaf and crown tissues were placed onto PDA and maintained under the incubation conditions described above. Fungal colonies morphologically similar to those inoculated were con-

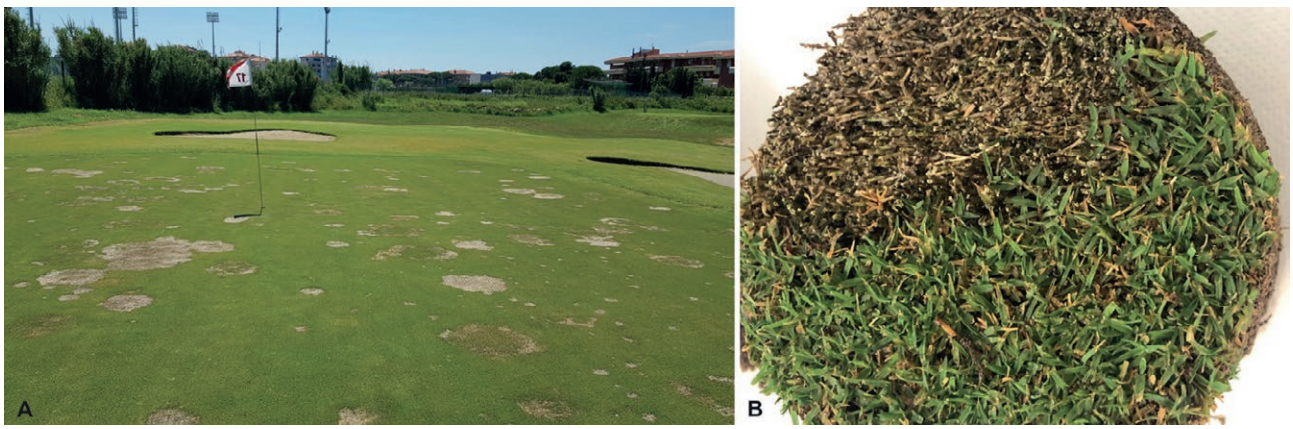


**Figure 1.** Necrotic areas caused by *Curvularia americana* and *C. tropicalis* observed on a golf course green (A), and affected leaves (B).

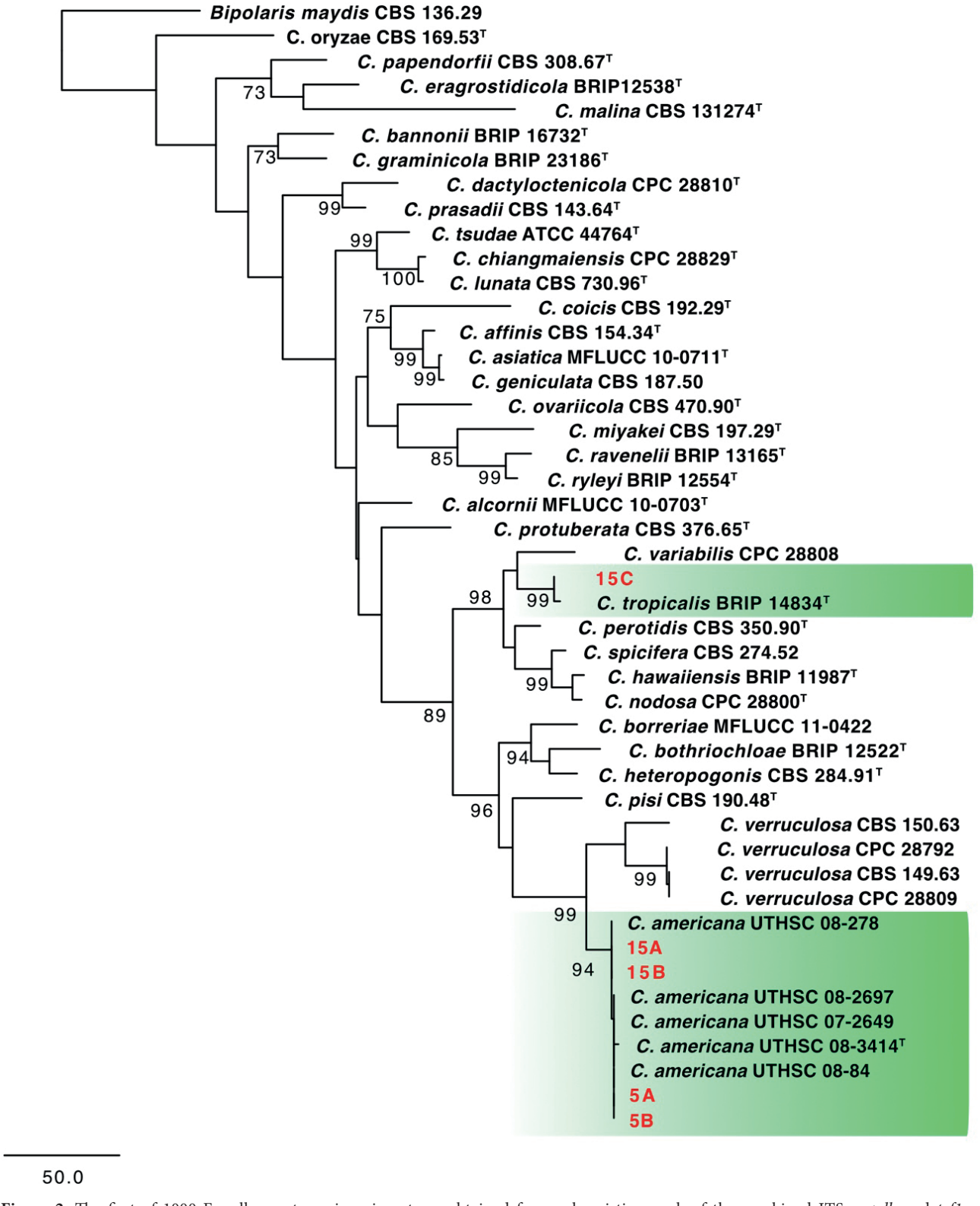


**Figure 2.** The first of 1000 Equally most parsimonious trees obtained from a heuristic search of the combined ITS, *gapdh* and *tef1-α* sequence alignments of *Curvularia* spp. Bootstrap support values are shown at the nodes. The strains isolated in the present study are shown in red font, and the scale bar represents number of changes. The tree was rooted to *Bipolaris maydis* (CBS 136.29). [T] indicates ex-type cultures.

sistently reisolated, and were identified based on their colony characteristics and on nuclear ribosomal internal transcribed spacer (ITS) sequencing.

*Phenotypic characterization*

Agar plugs (5 mm diam.) of representative isolates of each morphology group 5B and 15C were taken from the edge of 10-d-old cultures and transferred to the center of 9 cm diam. Petri dishes containing PDA. These plates were then incubated at 25±1°C under a 12 h photoperiod for 7 d. Colony characters, colour and diameter were observed/measured after 7 d. Cultures were examined over time for development of ascomata, conidiomata and setae. The morphological characteristics were assessed examined by mounting fungal structures in water and examining at 40× magnification (Nikon Eclipse 55i microscope), and 40 measurements were determined for two isolates (5B and 15C).

*DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing*

Total DNA was extracted from 0.1 g of mycelium of each isolate grown on PDA, using the E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-Tek), following the manufacturer's instructions. Species identifications were achieved through DNA amplification and sequencing of a combined dataset of genes: the ITS regions, and the partial glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) and translation elongation factor-1α (*tef1-α*) genes. ITS region of each isolate was amplified using the universal primers ITS1 and ITS4 (White *et al*., 1990). The primer GDF1 and GDR1 were amplified to amplify part of the *gapdh* gene (Guerber *et al*., 2003). The primers EF1-983 and EF1-2218R (Manamgoda *et al*., 2012) were used to amplify part of the *tef1-α* gene. The PCR amplification mixtures and cycling conditions adopted for the three loci were followed as described for the respective cited references (above). An amount of 5 µL of PCR product for each PCR reaction was examined by electrophoresis at 100V on 1% agarose (VWR Life Science AMRESCO® bio chemicals) gels stained with GelRed™. PCR products were sequenced in both directions by Eurofins Genomics Service. The DNA sequences generated were analyzed and consensus sequences were computed using the Genious v. 11.1.5 (Geneious Prime).

*Phylogenetic analyses*

New sequences obtained in this study were blasted against the NCBI's GenBank nucleotide database to determine the closest relatives for develop a taxonomic framework of the studied isolates. Alignments of different gene regions, including sequences obtained from this study and sequences downloaded from GenBank, were initially performed with the MAFFT v. 7 online server (http://mafft.cbrc.jp/alignment/server/index.html) (Katoh and Standley, 2013), and then manually adjusted in MEGA v. 7 (Kumar *et al*., 2016). The program Geneious v. 11.1.5 (Geneious Prime) was used to assemble the generated sequences of DNA and consensus sequences were computed. Alignments of different loci were manually adjusted in MEGA v. 7 (Kumar *et al*., 2016). *Bipolaris maydis* (CBS 136.29) was used as the outgroup. The phylogeny was based on Maximum Parsimony (MP) analysis which was performed through Phylogenetic Analysis Using Parsimony (PAUP) v.4.0b10 (Swofford, 2003). Phylogenetic relationships were estimated through heuristic searches with 100 random addition sequences. Tree bisection reconnection was used, with the 'best trees' as the branch swapping option, with alignment gaps treated as fifth base and all characters weighted equally. Parsimony and the bootstrap analyses (Hillis and Bull, 1993) were based on 1000 replications and on tree length (TL), consistency index (CI), retention index (RI) and rescaled consistence index (RC). The sequences obtained in the present study (Table 1) were deposited in GenBank.

## RESULTS

*Fungal isolations*

Brown to black fungal colonies consistently developed from inoculated leaves (30% frequency) and crowns (46% frequency), after incubation of 48 to 72 h on PDA.

*Pathogenicity tests*

Symptoms described above for leaves and crowns developed on all inoculated plants 15-20 d after inoculation, causing 30 to 40% areas of necroses. The non inoculated plants remained healthy. *Curvularia americana* and *C. tropicalis* were isolated from the symptomatic tissues and their identities were confirmed sequencing the *tef1-α* gene as the most informative, fulfilling the Koch's postulates.

*Phenotypic characterization*

Black to brown colonies were observed for isolate 5B and 15C. Colonies grown on PDA reached 64 mm diam.

**Table 1.** Collection details and GenBank accession numbers of isolates included in this study.

| Species | Isolate No.[1] | Host | Location | GenBank No.[2] | | |
|---|---|---|---|---|---|---|
| | | | | ITS | *gapdh* | *tef1-α* |
| *Bipolaris maydis* | CBS 136.29$^T$ | *Zea mays* | USA | AF071325 | KM034846 | KM093794 |
| *C. affinis* | CBS 154.34$^T$ | Unknown | Indonesia | KJ909780 | KM230401 | KM196566 |
| *C. alcornii* | MFLUCC 10-0703 | *Zea mays* | Thailand | JX256420 | JX276433 | JX266589 |
| *C. americana* | 5A | *Cynodon dactylon* x *C. transvaalensis* | Italy | *OP081046* | *OP114771* | *OP103749* |
| | 5B | *Cynodon dactylon* x *C. transvaalensis* | Italy | *OP081047* | *OP114772* | *OP103750* |
| | 15A | *Cynodon dactylon* x *C. transvaalensis* | Italy | *OP081048* | *OP114773* | *OP103751* |
| | 15B | *Cynodon dactylon* x *C. transvaalensis* | Italy | *OP081049* | *OP114774* | *OP103752* |
| | UTHSC 08-3414$^T$ | Human leg | USA | HE861833 | HF565488 | _ |
| | UTHSC 07-2649 | Human toe tissue | USA | HE861834 | HF565486 | _ |
| | UTHSC 08-84 | Human nasal sinus | USA | HG779015 | HG779115 | _ |
| | UTHSC 08-278 | Human peritoneal dialysis fluid | USA | HE861832 | HF565487 | _ |
| | UTHSC 08-2697 | Human leg | USA | HG779016 | HG779117 | _ |
| *C. asiatica* | MFLUCC 10-0711$^T$ | *Panicum* sp. | Thailand | JX256424 | JX276436 | JX266593 |
| *C. bannonii* | BRIP 16732$^T$ | *Jacquemontia tamnifolia* | USA | KJ415542 | KJ415404 | KJ415450 |
| *C. borreriae* | MFLUCC 11-0422 | Unknown *Poaceae* | Thailand | KP400638 | KP419987 | KM196571 |
| *C. bothriochloae* | BRIP 12522$^T$ | *Bothriochloa bladhii* | Australia | KJ415543 | KJ415403 | KJ415449 |
| *C. chiangmaiensis* | CPC 28829$^T$ | *Zea mays* | Thailand | MF490814 | MF490836 | MF490857 |
| *C. coicis* | CBS 192.29$^T$ | *Coix lacryma-jobi* | Japan | AF081447 | AF081410 | JN601006 |
| *C. dactyloctenicola* | CPC 28810$^T$ | *Dactyloctenium aegyptium* | Thailand | KJ415545 | KJ415401 | KJ415447 |
| *C. eragrostidicola* | BRIP 12538$^T$ | *Eragrostis pilosa* | Australia | MH414899 | MH433643 | MH433661 |
| *C. geniculata* | CBS 187.50 | Unknown seed | Indonesia | KJ909781 | KM083609 | KM230410 |
| *C. graminicola* | BRIP 23186$^T$ | *Aristida ingrata* | Australia | JN192376 | JN600964 | JN601008 |
| *C. hawaiiensis* | BRIP 11987$^T$ | | USA | KJ415547 | KJ415399 | KJ415445 |
| *C. heteropogonis* | CBS 284.91$^T$ | *Heteropogon contortus* | Australia | KJ415549 | JN600969 | JN601013 |
| *C. lunata* | CBS 730.96$^T$ | *Homo sapiens* | USA | JX256429 | JX276441 | JX266596 |
| *C. malina* | CBS 131274$^T$ | *Zoysia matrella* | USA | JF812154 | KP153179 | KR493095 |
| *C. miyakei* | CBS 197.29$^T$ | *Eragrostis pilosa* | Japan | KJ909770 | KM083611 | KM196568 |
| *C. nodosa* | CPC 28800$^T$ | *Digitaria ciliaris* | Thailand | MF490816 | MF490838 | MF490859 |
| *C. oryzae* | CBS 169.53$^T$ | *Oryza sativa* | Vietnam | KP400650 | KP645344 | KM196590 |
| *C. ovariicola* | CBS 470.90$^T$ | *Eragrostis interrupta* | Australia | JN192384 | JN600976 | JN601020 |
| *C. papendorfii* | CBS 308.67$^T$ | *Acacia karroo* | South Africa | KJ909774 | KM083617 | KM196594 |
| *C. perotidis* | CBS 350.90$^T$ | *Perotis rara* | Australia | JN192385 | KJ415394 | JN601021 |
| *C. pisi* | CBS 190.48$^T$ | *Pisum sativum* | Canada | KY905678 | KY905690 | KY905697 |
| *C. prasadii* | CBS 143.64$^T$ | *Jasminum sambac* | India | KJ922373 | KM061785 | KM230408 |
| *C. protuberata* | CBS 376.65 | *Deschampsia flexuosa* | UK | KJ922376 | KM083605 | KM196576 |
| *C. ravenelii* | BRIP 13165$^T$ | *Sporobolus fertilis* | Australia | JN192386 | JN600978 | JN601024 |
| *C. ryleyi* | BRIP 12554 | *Sporobolus creber* | Australia | KJ415556 | KJ415390 | KJ415437 |
| *C. spicifera* | CBS 274.52 | soil | Spain | JN192387 | JN600979 | JN601023 |
| *C. tropicalis* | 15C | *Cynodon dactylon* x *C. transvaalensis* | Italy | *OP081050* | *OP114775* | *OP103753* |
| | BRIP 14834 | *Coffea arabica* | India | KJ415559 | KJ415387 | KJ415434 |
| *C. tsudae* | ATCC 44764$^T$ | *Chloris gayana* | Japan | KC424596 | KC747745 | KC503940 |
| *C. variabilis* | CPC 28808 | *Eleusine indica* | Thailand | MF490819 | MF490841 | MF490862 |
| *C. verruculosa* | CBS 149.63 | *Elaeis guineensis* | Nigeria | HF934909 | HG779110 | _ |
| | CBS 150.63 | *Punica granatum* | India | KP400652 | KP645346 | KP735695 |
| | CPC 28792 | *Cynodon dactylon* | Thailand | MF490825 | MF490847 | MF490868 |
| | CPC 28809 | *Eleusine indica* | Thailand | MF490824 | MF490846 | MF490867 |

[1] ATCC: American Type Culture Collection, Virginia, USA; BRIP: Biosecurity Queensland Plant Pathology Herbarium, Brisbane, Queensland, Australia; CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands; CPC: Culture collection of P.W. Crous, housed at the Westerdijk Institute, Utrecht, the Netherlands; MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand. $^T$ indicates ex-type cultures.

[2] ITS: internal transcribed spacers 1 and 2 together with 5.8S nrDNA; *gapdh:* partial glyceraldehyde-3-phosphate dehydrogenase gene; *tef1-α*: translation elongation factor 1-α gene. Sequences generated in this study indicated in italics.

for isolate 5B and 68 mm diam. for 15C after 7 d at 25°C. Conidia each had three horizontal septa, and were curved at the third cell from the base. This cell was longer and darker than the others. Cells at each end were subhyaline and intermediate cells were medium brown. Conidia of isolate 5B measured 16.9 to 25.6 µm (mean = 21.4 µm) × 6.6 to 11.3 µm (mean = 8.5 µm), resembling the conidia of *C. americana* (Madrid *et al*., 2014). Conidia produced by the isolate 15C measured 20.3 to 30.1 µm (mean = 24.9 µm) × 7.9 to 10.6 µm (mean = 8.9 µm), resembling the morphological characteristics of conidia of *C. tropicalis* (Tan *et al*., 2014).

*Phylogenetic analyses*

The combined locus phylogeny consisted of 46 sequences. A total of 1670 characters (ITS: 1–493, *tef1-α*: 500–1355, *gapdh*: 1362–1670) were included in the phylogenetic analysis. A maximum of 1000 equally most parsimonious trees were saved (Tree length = 1291, CI = 0.510, RI = 0.750, RC = 0.382). Bootstrap support values from the parsimony analysis are plotted on the MP tree presented in Figure 2. In the combined analysis, four of the isolates (5A, 5B, 15A and 15B) obtained from symptomatic *Cynodon* plants clustered with the reference strains of *Curvularia americana*, while one isolate (15C) was grouped with *C. tropicalis* in two separate highly supported clades embedded in *Curvularia*.

## DISCUSSION

*Curvularia* has extensive international distribution, and includes pathogens or saprobes of a wide range of plant hosts (Marin-Felix *et al*., 2020) of the family *Poaceae*. These fungi are important pathogens of grass and staple food crops, including rice, maize, wheat and sorghum (Marin-Felix *et al*., 2017a). *Curvularia* includes than 40 species that are distinguished by differences in the conidium morphology and numbers of septa, and in colony morphology (Zhang *et al*., 2004; Chung and Tsukiboshi, 2005). *Curvularia* includes saprophytes, endophytes and pathogens (Sanchez-Marquez *et al*., 2008), which form a complex with *Cochliobolus* and *Bipolaris* affecting mostly grasses (*Poaceae*) with wide international distributions. The taxonomy of this complex is confusing as frequent nomenclatural changes and refinements have occurred. There is no clear morphological boundary between the asexual genera *Bipolaris* and *Curvularia* (Manamgoda *et al*., 2012), so accurate identifications are based on multilocus sequencing analyses (Manamgoda *et al*., 2012; Tan *et al*., 2014, 2018; Marin-Felix *et al*., 2017a, 2017b), and these are fundamental for achieving specific disease management strategies. For this reason, the species identification in the present study was based on a robust phylogenetic analysis developed through three genomic loci, with high informative level provided to distinguish species within *Curvularia*.

In 2003, the presence of pathogenic *Curvularia* spp. was reported in Wuhan, China, on hybrid Bermuda grass, in a sport turfgrass of *C. dactylon* × *C. transvaalensis* hybrid (Huang *et al*., 2005). A foliar disease of hybrid Bermuda grass, caused by *Curvularia malina*, was observed on several golf courses in China after April 2011 (Zhang *et al*., 2017). A novel species of *Curvularia* was also identified in 2017 as a foliar pathogen of *Cynodon dactylon* in the south of the United States of America (Peterson *et al*., 2017). *Curvularia americana* was reported on *Oryza sativa* in Iran (Heidarian *et al*., 2020) and on *Vitis* sp. in China (Jayawardena *et al*., 2018), and *C. tropicalis* was found associated with leaves of *Coffea arabica* in India (Tan *et al*., 2014).

The present study is the first to report *C. americana* and *C. tropicalis* causing leaf and crown necroses on Bermuda grass in Italy. This study is also the first to record these two species in Europe. Pathogenicity of these fungi was confirmed on Bermuda grass, and symptoms of leaf yellowing and necrosis, and of crown rot were obtained after inoculations under controlled conditions. Despite the use of warm season grasses which are usually less susceptible to the major turf diseases, golf courses and football field environments could induce conditions suitable for disease development, through pathogen sporulation and spread. High temperatures and humidity are likely to be involved with turfgrass diseases caused by *Curvularia* spp. Because of these environments and the susceptibility of turfgrasses to several pathogens, accurate diagnoses of these pathogens are essential for effective disease management. This study highlights the need for accurate diagnostic tools for the identification of pathogens affecting Bermuda grass.

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