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Allophoma brasiliensis J.L.V.R. Carvalho, J.D.P. Bezerra & Souza-Motta (Didymellaceae, Pleosporales): first occurrence in an agricultural site from Brazilian Atlantic Forest

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Abstract. *Allophoma brasiliensis* J.L.V.R. Carvalho, J.D.P. Bezerra & Souza-Motta is reported for the first time as endophyte from leaves of *Capsicum annuum* L. (Solanaceae) isolated in an agricultural site from Brazilian Atlantic Forest. For species determination, morphological characters were analysed along with a multigene analysis performed using the ITS region of the rDNA, the partial large subunit nuclear ribosomal RNA gene (LSU rRNA), the partial second largest subunit of the RNA polymerase II gene subunit (*rpb2*), and the partial β–tubulin gene (*tub2*). A key to *Allophoma* Qian Chen & L. Cai species is provided. This new record contributes to the global fungal diversity, indicating a new geographical distribution, and a new lifestyle.

Key words. Bell pepper, endophytic fungi, multigene analysis, *Phoma*-like, taxonomy

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INTRODUCTION

The genus *Allophoma* Qian Chen & L. Cai was originally introduced by Chen et al. (2015) in a taxonomic review of *Phoma*-like genera within Didymellaceae to accommodate five species originally described as "*Phoma*": *A. labilis* (Sacc.) Q. Chen & L. Cai (syn. *Phoma labilis*), *A. minor* (Aveskamp, Gruyter & Verkley) Q. Chen & L. Cai (syn. *Phoma minor*), *A. piperis* (Tassi) Q. Chen & L. Cai (syn. *Phoma piperis*), *A. tropica* (R. Schneid. & Boerema) Q. Chen & L. Cai (syn. *Phoma tropica*), *A. zantedeschiae* (Dippen.) Q. Chen & L. Cai (syn. *Phoma zantedeschiae*), and a new species, *A. nicaraguensis* Q. Chen & L. Cai.

Allophoma is a monophyletic lineage in Didymellaceae (Chen et al. 2015) and currently comprises 15 species (Hou et al. 2020a; Wijayawardene et al. 2020). *Allophoma tropica* is the type species of the genus. The asexual morphological features of *Allophoma* are represented by the following characters: one conidiomata pycnidial globose to flask-shaped with ostioles; pycnidial wall (2–5-layered) pseudoparenchymatous; conidiogenous cells phialidic, ampulliform to doliiform; and conidia variable in shape and size and mostly guttulate (Chen et al. 2015).

Allophoma species have been also isolated from coral (*Acropora formosa* Dana) (Hou et al. 2020b), stems (Hou et al. 2020b), twigs (Chen et al. 2015), and leaves (Marin-Felix et al. 2019) of wide host range as saprobes (de Gruyter et al. 1993; Jayasiri et al. 2019), and/or as causal agents of leaf-spot (de Gruyter et al. 1993; Yuan et al. 2021), dieback, and canker diseases (Babaahmadi et al. 2018). Representatives of this genus have also been found in the air (Chen et al. 2017) and in human-eye lesions (Valenzuela-Lopez et al. 2018).

The Atlantic Forest is one of the richest Neotropical hotspots in terms of diversity, encompassing 17 Brazilian states. However, only 12.4% of forest remnants and natural areas of the original vegetation remain (SOS Mata Atlântica 2017). Degraded areas, including by crop plantations, are remarkable within the heterogeneous ecosystems characteristic of the biome (Barbosa et al. 2022; Wilson et al. 2021), and several fungal species from cultivated areas have been described in the last years (Oliveira et al. 2016; Silva et al. 2019).

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Analyzing the diversity of endophytic fungi in leaves of *Capsicum annuum* L. (Solanaceae), which is cultivated under the conventional management system, we identified the isolates URM 8563 and URM 8564 as *Phoma*-like based on their morphological characters. After combined multigene sequence analysis of the ITS, LSU rRNA, *tub2*, and *rpb2*, we identify *Allophoma brasiliensis* J.L.V.R. Carvalho, J.D.P. Bezerra & Souza-Motta (Didymellaceae, Pleosporales), and we highlight the occurrence for the first time as endophyte in leaves of *C. annuum* isolated in an agricultural site from Brazilian Atlantic Forest. In addition, we propose a key to identification the species of the genus.

METHODS

Collecting and isolation. Healthy leaf samples of *Capsicum annuum* were collected in 2021 in an agricultural area of conventional cultivation located in the municipality of Chã Grande (08°15′18″S, 035°29′47″W), Pernambuco, Brazil (Figure 1). The agricultural area is inserted within the Brazilian Atlantic Forest biome at an altitude of 488 m. Endophytic fungi were isolated according to the methodology described by Araújo et al. (2002), modified. The leaves were washed in running water. Afterwards, leaf discs were cut with a sterile metal cork punch (6 mm diameter) and disinfested with 70% ethanol for 30 s, in sodium hypochlorite (3% of active chlorine) for 2 min, and finally washed three times in distilled, sterilized water. After isolation and purification, the colonies were kept in water with 10% glycerol for further analysis.

Representative isolates were deposited in the culture collection of the Micoteca URM Profa. Maria Auxiliadora Cavalcanti WCDM 604 at the Universidade Federal de Pernambuco (UFPE), Recife, Brazil.

Morphological analysis. Isolates of *Allophoma* were cultured on potato dextrose agar (PDA) and malt extract agar (MEA), and incubated up to 15 days at room temperature in a natural (±28 °C) day–night cycle. The isolates sporulated after 15 days, and general culture characteristics were observed (colonies were measured and macroscopic features examined), as well as reproductive structures. Micromorphological characters such as pycnidia (30), conidiogenous cells (three) and conidia (30) were randomly selected and measured. The average of the measurements was used to estimate the size of the structures. The colony colours were described according to Rayner's colour charts (Rayner 1970).

DNA extraction, PCR amplification, and sequencing. Fungal biomass was obtained from colonies grown on PDA plates at room temperature for up to 15 days. Genomic DNA extraction was performed, with the material previously ground, as described by Oliveira et al. (2016). The primers ITS1/ITS4 (White et al. 1990), LR0R/LR5 (Vilgalys and Hester 1990; Vilgalys and Sun 1994), fRPB2-5F2/fRPB2-7cR (Liu et al. 1999; Sung et al. 2007), and Bt2a/Bt2b (Glass and Donaldson 1995) were used to amplify the ITS region of the rRNA (ITS), the partial large subunit nuclear ribosomal RNA gene (LSU rRNA), the partial second largest subunit of the RNA polymerase II gene (*rpb2*) subunit, and the partial β–tubulin gene (*tub2*), respectively. The PCR conditions for ITS and LSU rRNA were an initial denaturation at 95 °C for 3 min, followed by 39 cycles of

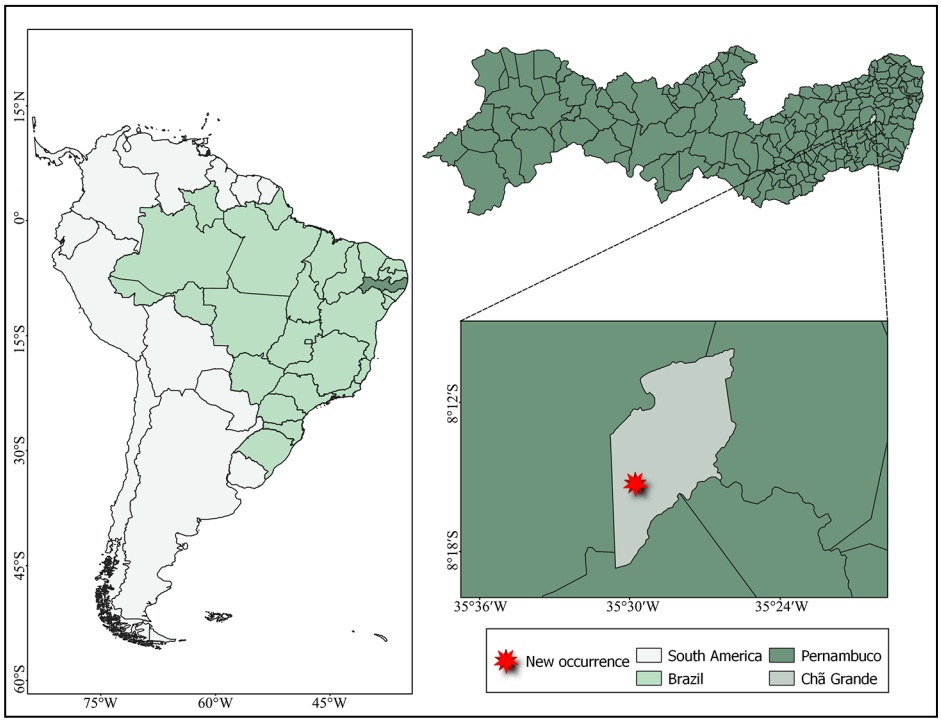

Figure 1. Map of the occurrence of *Allophoma brasiliensis* J.L.V.R. Carvalho, J.D.P. Bezerra & Souza-Motta for the first time as an endophyte in *Capsicum annuum* leaves on an agricultural site in the Brazilian Atlantic Forest.

denaturation at 94 °C for 45 s, annealing at 58 °C for 1 min, and extension at 72 °C for 1 min, with a final extension step at 72 °C for 7 min. The PCR conditions for *rpb2* was an initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at 57 °C for 2 min and extension at 72 °C for 90 s, with a final extension step at 72 °C for 10 min. The PCR conditions for *tub2* was an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s and extension at 72 °C for 1 min, with a final extension step at 72 °C for 7 min. The amplification reactions were carried out in a final volume of 25 µl. All products obtained from the amplification were purified using the enzyme Exonuclease I (Thermo Fisher Scientific) and Shrimp Alkaline Phosphatase (Cellco) and sequenced on the Multiuser Platform for Sequencing and Gene Expression of the UFPE, Centro de Biociências.

Sequence alignment and phylogenetic analysis. For the final molecular characterization of the species of *Allophoma*, four different markers were used: ITS, LSU rRNA, *rpb2*, and *tub2*. The dataset was constructed with the sequences of our isolates together with sequences of type material and representative isolates, totaling 28 sequences of the 15 accepted species of *Allophoma*. *Stagonosporopsis loticola* (Died.) Aveskamp, Gruyter & Verkley (CBS 562.81) was used as the outgroup. The GenBank accession numbers of all sequences used in this study and the newly obtained sequences are shown in Appendix Table A1. Sequences were aligned using the online tool MAFFT v. 7 (Katoh and Standley 2013) and manually edited with the MEGA v. 7 software (Kumar et al. 2016).

The concatenated four-locus datasets were analyzed by maximum likelihood (ML) and bayesian inference (BI) using the RAxML-HPC BlackBox v. 8.2.12 (Stamatakis 2014) and MrBayes on XSEDE v. 3.2.7a (Ronquist et al. 2012), respectively, at the online server of the Cipres Science gateway portal (Miller et al. 2010). ML analyzes were performed with 1000 bootstrap replications using default parameters and the GTR + G + I model test. For BI analyses, the best nucleotide substitution model was estimated by MrModelTest v. 2.3 (Nylander 2004) for each gene region separately (ITS = HKY + I, LSU = GTR + I, *rpb2* = SYM + G, and *tub2* = GTR + G). BI analyzes were performed with two Markov Chain Monte Carlo (MCMC), 1×10^7 generations with a burn value of 25%, and trees were sampled every 1000 generations. In the nodes, BI posterior probability (BPP) and ML bootstrap (ML–BS) values equal to or greater than 0.95 and 70 %, respectively, are presented. The ultimate phylogenetic tree was visualized in FigTree v. 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). The concatenated alignment was deposited in the TreeBASE (study ID 29859).

RESULTS

Allophoma brasiliensis J.L.V.R. Carvalho, J.D.P. Bezerra & Souza-Motta, 2022;
Brazilian Journal of Microbiology, emend.
Figure 2A–J

Materials examined. BRAZIL – Pernambuco • Chã Grande, Rancho Bela Vista; 08°15′18″S, 035°29′47″W; 488 m alt; 29.I.2021; T.G.L. Oliveira leg.; as endophytic fungi in leaves of *Capsicum annuum*; URM 8563 • ibid.; URM 8564.

Distribution. The new record was found as endophyte in leaves of *C. annuum* in an agricultural area inserted in Brazilian Atlantic Forest.

Identification (colony characters, 28 °C, 15 days). PDA: colony regular and white margins, greenish olivaceous, black pycnidia forming a concentric ring, aerial mycelium sparse and whitish; opaque to greenish olivaceous reverse, white near margins. MEA: colony opaque to greenish olivaceous, covered by whitish flaky aerial mycelium, irregular margin, light brown to white near the colony margin; opaque to greenish olivaceous reverse.

Colony diameters, 15 days, in cm. PDA 28 °C 5.8; MEA 28 °C 5.8.

Micromorphology (28 °C, 15 days). PDA: *conidiomata* pycnidial, produced on the agar surface or semi-immersed, mostly isolated, some grouped, globose or subglobose to flask-shaped, pale brown, some dark brown, thin-walled, glabrous, ostiolate, (60–)108–192(–216) × (60–)120–180(–192) µm. *Ostiole* single, slightly papillate. *Conidiogenous cells* phialidic, hyaline, globose to ampulliform, smooth, slightly papillate, 3.5–5(–6.3) × 2.2–3.7(–5.2) µm. *Conidia* oblong with both ends rounded, smooth- and thin-walled, hyaline, aseptate, without guttules, 3.1–4.7(–6.6) × 1.3–2.2 µm. *Conidial matrix* cream. *Chlamydospores* multicellular, mostly intercalary in chain, smooth, dark brown.

Comments. According to phylogenetic analyses, strains URM 8563 and URM 8564 grouped into the same highly supported clade (BPP = 0.95 and ML–BS = 100) together with *A. brasiliensis* ex-type strain (URM 8453) and *A. brasiliensis* URM 8454, isolated from bat flies (*Trichobius* sp. Gervais) in Catimbau National Park, Brazil (Figure 3). Some morphological differences between the endophytes (URM 8563 and URM 8564), isolated from leaves of *C. annuum*, and *A. brasiliensis* ex-type strain (URM 8453) were observed. We

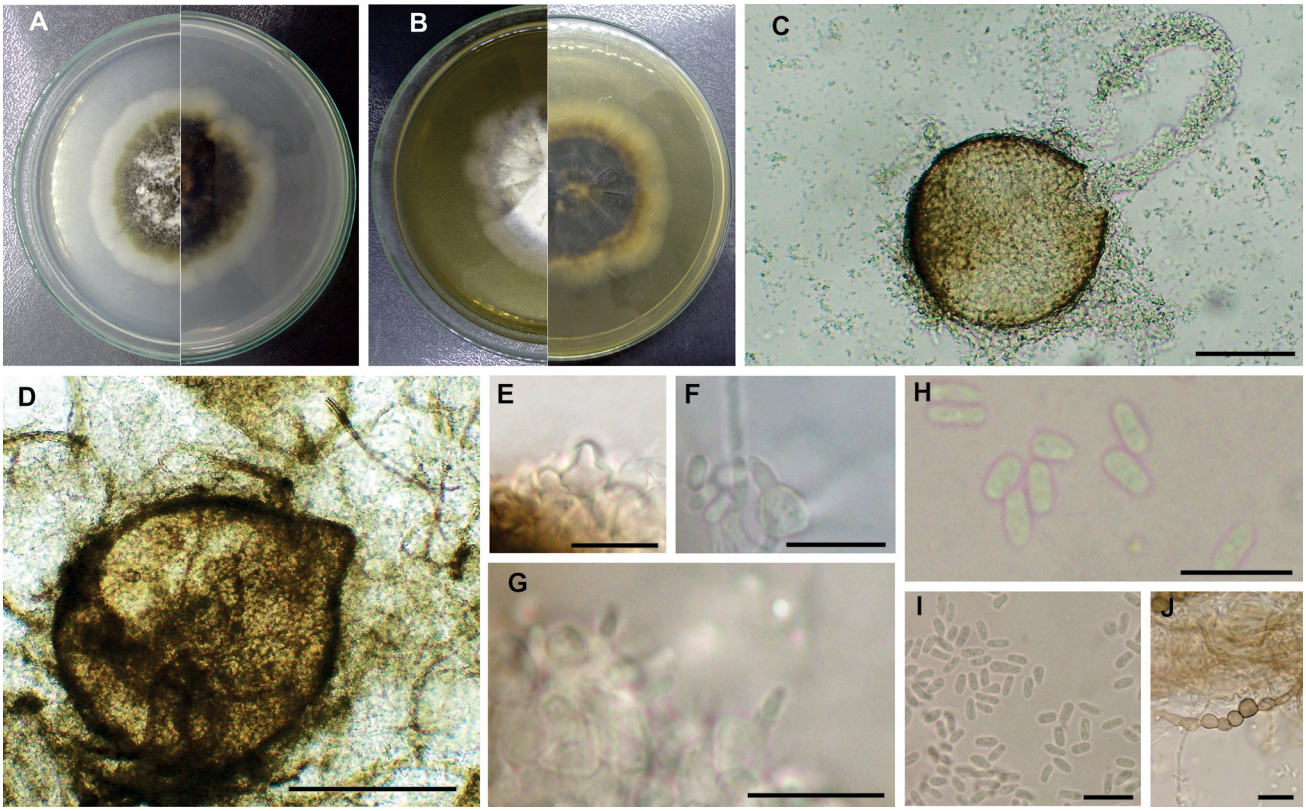

Figure 2. *Allophoma brasiliensis* URM 8563. **A.** Detail of colony on PDA after 15 days. **B.** Detail of colony on MEA after 15 days. **C, D.** Conidiomata pycnidial. **E–G.** Conidiogenous cells and conidia. **H, I.** Conidia. **J.** Chlamydospores. Scale bars: C, D = 100 µm; E–J = 10 µm.

have observed in our specimens pycnidia larger, and conidiogenous cells smaller than ex-type strain (URM 8453). Chlamydospores and conidia without guttules were present in our strains and were not observed in ex-type strain (URM 8453). Furthermore, *A. brasiliensis* (URM 8563 and URM 8564) is reported for the first time as an endophytic fungus.

Key to the *Allophoma* species

1a Conidiomata pycnidial (sub-)globose to elipsoidal or flask-shaped 2
1b Conidiomata pycnidial ovoid 3
2a Conidiomata pycnidial up to 300 µm long 4
2b Conidiomata pycnidial 301–635 µm long 5
3a Conidiomata pycnidial 120–210 × 90–140 µm . . . . . *A. cylindrispora*
3b Conidiomata pycnidial 70–90 × 68–85 µm . . . . . *A. siamensis*
4a Ostiole 1 single, slightly papillate, papillate, or non-papillate 6
4b Ostioles 1–3 slightly papillate, papillate or sometimes non-papillate 7
5a Ostioles 1–3 slightly papillate, papillate, or non-papillate 8
5b Ostioles 1–5 slightly papillate, papillate, or non-papillate 9
6a Conidia oblong to ellipsoidal, hyaline, aseptate, with polar guttules 10
6b Conidia oval, oblong or ellipsoidal, hyaline, aseptate, eguttulate 11
7a Conidia oblong to cylindrical, hyaline, aseptate, with minutes guttules . . . . . *A. thunbergiae*
7b Conidia oblong to ellipsoidal, hyaline to pale brown, 0- or 1-septate, majority 1-septate, guttulate . . . . . *A. hayatii*
8a Conidia oblong, hyaline, aseptate, with 1 or 2 medium-sized polar guttules . . . . . *A. anatiae*
8b Conidia oblong, hyaline, aseptate, with 2 large polar guttules . . . . . *A. alba*
8c Conidia oblong to cylindrical, hyaline, aseptate, with 2 distinct pale-green polar guttules . . . . . *A. oligotrophica*
9a Conidia ellipsoidal to ovoid or slightly allantoid, hyaline, aseptate, (0–)1–3(–4) minute guttules . *A. minor*
9b Conidia ellipsoidal to ovoid or oblong, hyaline, aseptate, 1 or 2 guttules 12
10a Conidial matrix whitish to pale luteous or ochraceous 13
10b Conidial matrix not recorded . . . . . *A. piperis*
11a Conidial matrix greyish and presence of chlamydospores in the mycelium . . . . . *A. zantedeschiae*
11b Conidial matrix cream and presence of chlamydospores in the mycelium . . . . . *A. brasiliensis*
12a Conidial matrix white-yellowish . . . . . *A. tropica*

12b Conidial matrix cream ..... *A. pterospermicola*
13a Negative NaOH spot-test on oatmeal agar ..... *A. nicaraguensis*
13b Positive NaOH spot-test on oatmeal agar ..... *A. labilis*

DISCUSSION

Studying the diversity of endophytic fungi present in bell pepper leaves, we isolated the endophytes URM 8563 and URM 8564, whose morphological characteristics belonged to the *Phoma*-like group. Based on combined multigene sequence analysis of the ITS, LSU rRNA, *rpb2*, and *tub2*, our phylogenetic analyses indicated that our specimens belong to *Allophoma brasiliensis*, which is reported here for the first time as endophyte.

Other species of the genus *Allophoma* have been reported as endophytes elsewhere in the world, including in the stems and wilted leaves of *Ageratina adenophora* (Spreng.) R. M. King & H. Rob. in Yunnan Province, southwestern China (Fang et al. 2019) and in leaves of *Rhizophora apiculata* Blume in Phetchaburi Province, Thailand (Doilom et al. 2017). Members of *Allophoma* are characterized by aseptate conidia that vary both in shape and size, are hyaline and mostly guttulate, and have globose and ostiolate pycnidia (Chen et al. 2015).

The endophytic isolates obtained in this study (URM 8563 and URM 8564) are similar to the description of *Allophoma brasiliensis* culture ex-type URM 8453 proposed by Carvalho et al. (2022), which was isolated from bat flies (*Trichobius* sp.) from Catimbau National Park. This park is within the Caatinga domain and encompasses parts of the municipalities of Buíque, Tupanatinga, and Ibimirim in the state of Pernambuco. Carvalho et al. (2022) used macromorphological characters from colonies in OA, PDA, and MEA cultivated at 27 °C for 7 days in the dark and micromorphological characters such as conidiomata pycnidial, conidiomatal wall, conidiogenous cells, and conidia for identification and confirmation of the species. Multigene analysis (ITS, LSU rRNA, *rpb2*, and *tub2*) demonstrated high identity and phylogenetic relationship with our isolates (Figure 3).

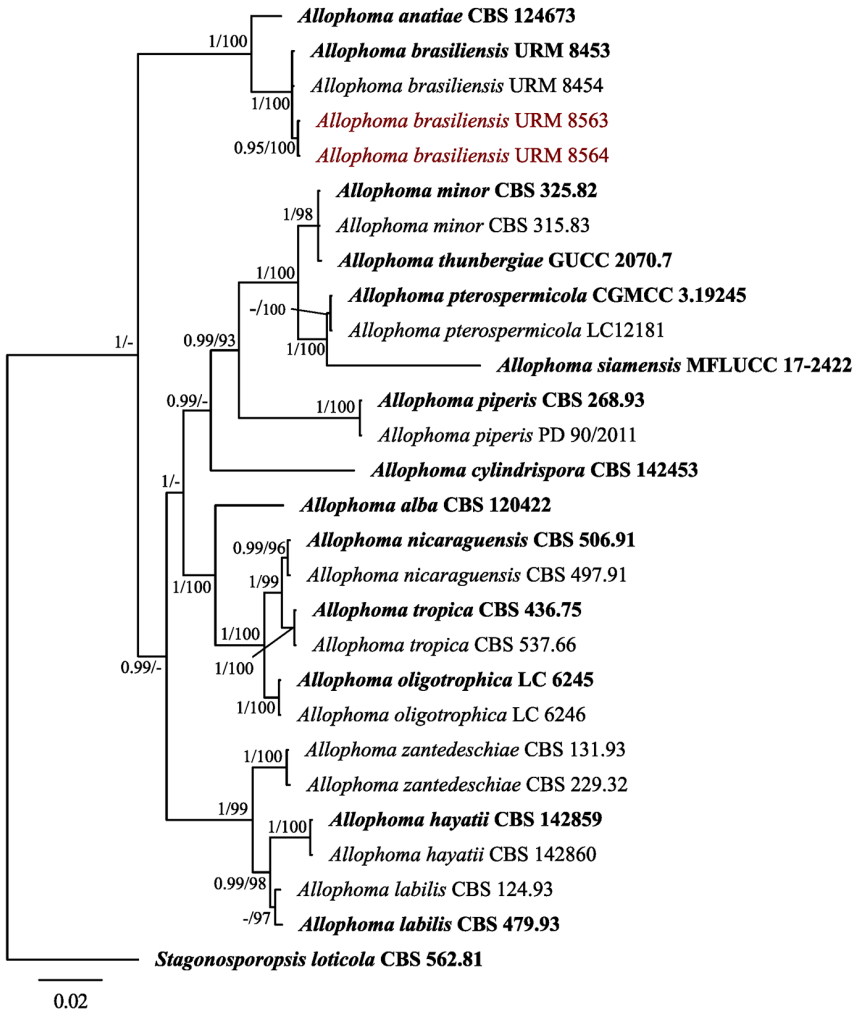

Figure 3. Phylogenetic tree generated from Bayesian inference analysis based on combined *tub2*, *rpb2*, ITS, and LSU rRNA sequences. Sequences from type species and the reference isolates are indicated in bold. The sequence obtained in this study is annotated in red. Bayesian posterior probabilities (above 0.95) and maximum likelihood bootstrap (above 70%) values are shown near nodes. The tree is rooted with *Stagonosporopsis loticola* (CBS 562.81).

The differences in the lifestyle and environment of the isolation for the strains of this fungi possibly are responsible for some variability in morphological characters found. Versatility in the morphological and ecophysiological characteristics of fungi may occur as a form of adaptation to nutrient acquisition, stress tolerance, and interactions with other organisms, in addition to mediating the biogeography of these organisms (Bahram and Netherway 2022). We observed chlamydospores in our specimens, although this was not reported by Carvalho et al. (2022). These authors also have found guttulated conidia, but these structures were not observed by us. The pycnidia found in our isolates were larger than those observed by Carvalho et al. (2022), while the conidiogenous cells from URM 8563 and URM 8564 are smaller than those in the species' original description.

Allophoma brasiliensis (URM 8563 and URM 8564) was found in an agricultural area in the municipality of Chã Grande as endophyte from healthy leaves of Bell Pepper, *Capsicum annuum*, and *A. brasiliensis* species is reported here for the first time in an agricultural site in Brazilian Atlantic Forest. Our study is an important addition to knowledge of the global fungal diversity, as it adds a new geographical occurrence, expands the known geographical distribution, includes a different, previously unreported biome, and reports a new ecological lifestyle for *A. brasiliensis*.

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ADDITIONAL INFORMATION

Conflict of interest

The authors declare that no competing interests exist.

Ethical statement

No ethical statement is reported.

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Author contributions

Conceptualization: TGLO, RMFS, ARM, OMCM, CMSM, AFC, GAS. Data curation: TGLO, RMFS, ARM, OMCM, CMSM, AFC, GAS. Methodology: TGLO, RMFS, GAS. Formal analysis: TGLO, RMFS, ARM, OMCM, AFC, GAS. Visualization: TGLO, RMFS, ARM, OMCM, AFC, GAS. Funding acquisition: GAS. Resources: GAS. Supervision: GAS, ARM, OMCM, TGLO. Project administration: GAS, TGLO. Writing – original draft: TGLO, GAS. Writing – review and editing: TGLO, GAS.

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Data availability

All data that support the findings of this study are available in the main text and the Appendix.

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APPENDIX

Table A1. Isolates used in this study, including the different markers with respective NCBI GenBank accessions numbers, and the culture code. GenBank accessions numbers in bold were generated in this work.

Species	Culture code*	GenBank accession numbers				References
		ITS	tub2	rpb2	LSU	
Allophoma alba	CBS 120422 [T]	MN973469	MT005568	MT018044	MN943671	Hou et al. 2020b
Allophoma anatiae	CBS 124673 [T]	MN973472	MT005571	MT018048	MN943674	Hou et al. 2020b
Allophoma brasiliensis	URM 8453 [T]	OM692214	ON715830	ON715834	ON678611	Carvalho et al. 2022
	URM 8454	OM692215	ON715831	ON715835	ON678612	Carvalho et al. 2022
	URM 8563; PC74	**OP304832**	**OP311925**	**OP311927**	**OP304851**	**This study**
	URM 8564; PC75	**OP304833**	**OP311926**	**OP311928**	**OP304852**	**This study**
Allophoma cylindrispora	CBS 142453 [T]	LT592920	LT592989	LT593058	LN907376	Hou et al. 2020b
Allophoma hayatii	CBS 142859 [T]	KY684812	KY684816	MF095108	KY684814	Hou et al. 2020b
	CBS 142860	KY684813	KY684817	MF095109	KY684815	Hou et al. 2020b
Allophoma labilis	CBS 124.93; PD 87/269	GU237765	GU237619	KT389552	GU238091	Hou et al. 2020b
	CBS 479.93; PD 70/93 [R]	GU237868	GU237620	MN983277	GU238092	Hou et al. 2020b
Allophoma minor	CBS 325.82 [T]	GU237831	GU237632	KT389553	GU238107	Hou et al. 2020b
	CBS 315.83 [R]	GU237826	GU237631	MN983279	GU238106	Hou et al. 2020b
Allophoma nicaraguensis	CBS 506.91; IMI 215229 [T]	GU237876	GU237596	KT389551	GU238058	Hou et al. 2020b
	CBS 497.91; PD 79/209	GU237870	GU237597	—	GU238059	Hou et al. 2020b
Allophoma oligotrophica	CGMCC 3.18114; LC 6245 [T]	KY742040	KY742282	KY742128	KY742194	Hou et al. 2020b
	CGMCC 3.18115; LC 6246	KY742041	KY742283	KY742129	KY742195	Hou et al. 2020b
Allophoma piperis	CBS 268.93; PD 88/720 [T]	GU237816	GU237644	KT389554	GU238129	Hou et al. 2020b
	PD 90/2011	GU237921	GU237645	MT018045	GU238130	Hou et al. 2020b
Allophoma pterospermicola	CGMCC 3.19245 [T]	MK088573	MK088594	MK088587	MK088580	Marin-Feliz et al. 2019
	LC12181	MK088569	MK088590	MK088583	MK088576	Marin-Feliz et al. 2019
Allophoma siamensis	MFLUCC 17-2422 [T]	MK347742	MK412867	MK434912	MK347959	Hou et al. 2020b
Allophoma thunbergiae	GUCC 2070.7 [T]	MW036298	MW116823	MW116819	MW040201	Yuan et al. 2021
Allophoma tropica	CBS 436.75; DSM 63365 [T]	GU237864	GU237663	KT389556	GU238149	Hou et al. 2020b
	CBS 537.66; PD 65/27	MN973468	MT005567	MT018043	MN943670	Hou et al. 2020b
Allophoma zantedeschiae	CBS 131.93; PD 69/140	FJ427084	FJ427188	KT389557	GU238159	Hou et al. 2020b
	CBS 229.32	KT389473	KT389767	KT389558	KT389690	Hou et al. 2020b
Stagonosporopsis loticola	CBS 562.81; PDDCC 6884 [T]	GU237890	GU237697	KT389684	GU238192	Hou et al. 2020b

[R]Reference isolates.
[T]Ex-type strain.
*CBS: Westerdijk Fungal Biodiversity Institute (formerly CBS KNAW), Utrecht, The Netherlands; CGMCC: China General Microbiological Culture Collection, Beijing, China; CPC: Culture collection of Pedro Crous, housed at CBS; DSM: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; GUCC: Culture Collection of the Department of Plant Pathology, Agriculture College, Guizhou University; IMI: International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, UK; LC: LeiCai, corresponding author's personal collection deposited in laboratory, housed at CAS, China; MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; PD: Plant Protection Service, Wageningen, the Netherlands; PDDCC: Plant Diseases Division Culture Collection, Auckland, New Zealand; URM: Culture collection Prof. Maria Auxiliadora Cavalcanti, Recife, Brazil.