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Bayesian analyses of five gene regions reveal a new phylogenetic species of *Macrophomina* associated with charcoal rot on oilseed crops in Brazil

Alexandre Reis Machado · Danilo Batista Pinho · Dartanhã José Soares · André Angelo Medeiros Gomes · Olinto Liparini Pereira

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Abstract *Macrophomina* is a genus belonging to Botryosphaeriaceae that comprises well-known necrotrophic pathogens related to hundreds of plant hosts around the world. Historically, *M. phaseolina* is the causal agent of charcoal rot in several crops, mainly in tropical and subtropical areas around the world. However, after a recent genetic diversity study using morphological and molecular approaches, which resulted in the epitypification of *M. phaseolina*, and the description of a new *Macrophomina* species associated with charcoal rot disease, the hypothesis that other cryptic species could be present under the name *M. phaseolina* was raised. Previous studies in Brazil revealed a high genetic diversity and different levels of aggressiveness of *M. phaseolina* isolates associated with charcoal rot in oilseed crops. Thus, the aim of the present study was, through phylogenetic and morphological studies, to determine if isolates of *Macrophomina* obtained from different oilseed crops represent a single species or distinct taxa. Based on the results obtained, it was possible to identify three different *Macrophomina* species: *M. phaseolina*, *M. pseudophaseolina* and a new phylogenetic species, *M. euphorbiicola*. This is first report of *M. pseudophaseolina* in Brazil causing charcoal rot on *Arachis hypogaea*, *Gossypium hirsutum* and *Ricinus communis* and associated with seed decay of *Jatropha curcas*. In addition, a novel species described in the present study, *M. euphorbiicola*, is reported as the etiological agent of the charcoal rot on *R. communis* and *Jatropha gossypifolia*.

Keywords Botryosphaeriales · Cryptic species · Phylogeny · Plant pathology · Soil-borne fungi

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Introduction

The genus *Macrophomina* belongs to the Botryosphaeriaceae and it is characterised by brown, septate mycelium with abundant production of black microsclerotia, pycnidial conidiomata, with aseptate, hyaline conidia with apical mucoid appendages, sometimes becoming dark and septate with age (Sutton 1980; Crous et al. 2006; Phillips et al. 2013). The type species of the genus is the well-known necrotrophic pathogen *M. phaseolina*, which has a wide host range, including plants in many orders and families, distributed throughout

of different climatic zones of the world, but concentred in the tropical and subtropical areas (Dhingra and Sinclair 1978; Sarr et al. 2014). Its occurrence has steadily increased in diverse crop species worldwide (Farr and Rossman 2018). Furthermore, this pathogen can survive for long periods in soil and in crop debris, generally through microsclerotia, and it can also survive as mycelium in asymptomatic seeds and microsclerotia in symptomatic seeds (Dhingra and Sinclair 1978; Short et al. 1980; Singh and Singh 1982; Songa and Hillocks 1998; Gupta et al. 2012).

Several studies, based on different molecular tools, have showed high genetic diversity with distinguishable phylogenetic clades between isolates of *M. phaseolina* (Almeida et al. 2003, 2008; Jana et al. 2003; Baird et al. 2010; Saleh et al. 2010; Gupta et al. 2012). Distinct host preference or pathogenic ability and cultural/biochemical characteristics have frequently been reported among isolates of this pathogen (Pearson et al. 1987; Mihail and Taylor 1995; Mayek-Perez et al. 2001; Rayatpanah et al. 2012), and earlier attempts to establish sub-specific ranks were made (Reichert and Hellinger 1947). However, due to insufficient morphological distinction, and unsuitable host or geographical correlation, the genus was until recently considered to harbour a single species, *M. phaseolina*. It was only following a polyphasic approach using multi-gene DNA data and morphology, that a new species, *M. pseudophaseolina,* was segregated from isolates previously identified as *M. phaseolina* (Sarr et al. 2014).

Shortly after the publication of the work of Sarr et al. (2014), Claudino and Soares (2014), raised the hypothesis that possibly additional cryptic species could occur within *M. phaseolina*, thus potentially explaining the results obtained in their work and throughout the published literature. Thus, the aim of the present study was, through a polyphasic approach, use phylogenetic analysis and morphological characteristics, to verify if the isolates of *Macrophomina* associated to charcoal rot on oilseed crops in Brazil belongs to *M. phaseolina* or represents different species.

Materials and methods

Fungal isolates

Initially 30 isolates of *Macrophomina* obtained from *Arachis hypogaea*, *Glycine max*, *Gossypium hirsutum*, *Helianthus annuus*, *Jatropha gossypifolia*, *Ricinus communis* and *Sesamum indicum* as described in Claudino and Soares (2014) were provided by Embrapa Algodão to the Laboratório de Micologia e Etiologia de Doenças Fúngicas de Plantas of the Universidade Federal de Viçosa for taxonomical and molecular studies. Later, five other isolates were obtained during research on physic nut (*J. curcas*) seeds from Jaíba, in the state of Minas Gerais, and Colatina, in the state of Espírito Santo, Brazil. Details about host and geographical origin of the isolates are listed in Table 1 and also in Claudino and Soares (2014).

Morphological studies

Representative isolates of each species, identified by phylogenetic analysis, were grown in Petri dishes with 2% water agar (WA - Agar Agar, type I, Himedia, Mumbai, India) overlaid with triple-sterilised physic nut seeds, needles or twigs of *Pinus* sp. and incubated at 25 °C with a 12 h light-dark regime for 4 to 8 wk. to induce sporulation (Crous et al. 2006; Sarr et al. 2014). The fruiting bodies were mounted in clear lactophenol. Thirty measurements of all relevant morphological structures (conidia and conidiogenous cells) were made using an OLYMPUS CX31 compound microscope and images were obtained with an OLYMPUS BX 51 compound microscope fitted with a digital camera (OLYMPUS EVOLT330). All isolates of *Macrophomina* investigated in this study were deposited in the culture collection "Coleção de Culturas de Fungos Fitopatogênicos Prof. Maria Menezes" (CMM) at the Universidade Federal Rural de Pernambuco (Recife, Brazil). Additionally, the isolates provided by Embrapa Algodão are maintained in the culture collection "Coleção de Culturas de Microrganismos Fitopatogênicos" (CCMF-CNPA) (Table 1). The holotype of the new species proposed here was deposited in lyophilized form (metabolically inactive culture) in the culture collection "Micoteca URM Profa. Maria Auxiliadora Cavalcanti at the Universidade Federal de Pernambuco (Recife, Brazil).

DNA extraction, sequencing and phylogenetic studies

The isolates were grown on PDA at 25 °C for 1 week., where approximately 40 mg of mycelium was scraped from the agar surface and placed in a sterile 1.5 mL microcentrifuge tube. The extraction was preceded by freezing with liquid nitrogen and grinding into a fine

Table 1 GenBank accession numbers of DNA sequences of Botryosphaeriaceae used in phylogenetic analyses

Species	Isolates	Hosts	Locations	Genbank acession n°				
				ITS	TEF1-α	ACT	βT	CAL
Botryosphaeria dothidea	CMW8000	*Prunus* sp.	Switzerland	AY236949	AY236898	–	–	–
	CBS110302	*Vitis vinifera*	Portugal	AY259092	AY573218	–	–	–
Cophinforma eucalypti	MFLUCC110425	*Eucalyptus* sp.	Thailand	JX646800	JX646865	–	–	–
	MFLUCC110655	*Eucalyptus* sp.	Thailand	JX646801	JX646866	–	–	–
Macrophomina phaseolina	CBS162.25	*Eucalyptus* sp.	–	KF531826	KF531803	–	–	–
	CBS205.47	*Phaseolus vulgaris*	Italy	KF951622	KF951997	KF951804	–	–
	CBS227.33	*Zea mays*	–	KF531825	KF531804	–	–	–
	CMM3615	*Jatropha curcas*	Brazil: Minas Gerais	KF234547	KF226693	–	–	–
	CMM3650	*Jatropha curcas*	Brazil: Espírito Santo	KF234552	KF226710	–	–	–
	CPC21420	*Vigna unguiculata*	Senegal: Saint-Louis	KF951717	KF952088	KF951857	KF952178	KF951940
	CPC21416	*Arachis hypogaea*	Senegal: Louga	KF951715	KF952086	KF951855	KF952176	KF951938
	CPC21419	*Arachis hypogaea*	Senegal: Thiès	KF951716	KF952087	KF951856	KF952177	KF951939
	CMM3875	*Jatropha curcas*	Brazil: Espírito Santo	KF369263	KF553897	–	–	–
	CMM4188 / CCMF-CNPA 617	*Ricinus communis*	Brazil: Paraiba	KU058945	KU058915	–	–	–
	CMM4239 / CCMF-CNPA 281	*Ricinus communis*	Brazil: Bahia	KU058953	KU058923	–	–	–
	CMM4240 / CCMF-CNPA 283	*Ricinus communis*	Brazil: Bahia	KU058954	KU058924	–	–	–
	CMM4575 / CCMF-CNPA 651	*Ricinus communis*	Brazil: Maranhão	KU058955	KU058925	–	–	–
	CMM4238 / CCMF-CNPA 707	*Glycine max*	Brazil: Maranhão	KU058952	KU058922	–	–	–
	CMM4025 / CCMF-CNPA 274	*Sesamum indicum*	Brazil: Paraiba	KU058926	KU058896	–	–	–
	CMM4034 / CCMF-CNPA 277	*Helianthus annuus*	Brazil: Paraiba	KU058927	KU058897	–	–	–
	CMM4129 / CCMF-CNPA 286	*Ricinus communis*	Brazil: Bahia	KU058934	KU058904	–	–	–
	CMM4128 / CCMF-CNPA 285	*Ricinus communis*	Brazil: Bahia	KU058933	KU058903	–	–	–
	CMM4149 / CCMF-CNPA 290	*Ricinus communis*	Brazil: Bahia	KU058938	KU058908	–	–	–
	CMM4159 / CCMF-CNPA 292	*Ricinus communis*	Brazil: Bahia	KU058940	KU058910	–	–	–
	CMM4047 / CCMF-CNPA 279	*Ricinus communis*	Brazil: Bahia	KU058929	KU058899	–	–	–
	CMM4048 / CCMF-CNPA 280	*Ricinus communis*	Brazil: Bahia	KU058930	KU058900	–	–	–
	CMM4054 / CCMF-CNPA 282	*Ricinus communis*	Brazil: Bahia	KU058931	KU058901	–	–	–
	CMM4103 / CCMF-CNPA 284	*Ricinus communis*	Brazil: Bahia	KU058932	KU058902	–	–	–
	CMM4174 / CCMF-CNPA 295	*Ricinus communis*	Brazil: Paraíba	KU058943	KU058913	–	–	–
	CMM4185 / CCMF-CNPA 296	*Ricinus communis*	Brazil: Paraíba	KU058944	KU058914	–	–	–

Table 1 (continued)

Species	Isolates	Hosts	Locations	Genbank acession n°				
				ITS	TEF1-α	ACT	βT	CAL
	CMM4193 / CCMF-CNPA 652	*Ricinus communis*	Brazil: Maranhão	KU058946	KU058916	–	–	–
	CMM4215 / CCMF-CNPA 653	*Ricinus communis*	Brazil: Maranhão	KU058947	KU058917	–	–	–
	CMM4222 / CCMF-CNPA 654	*Ricinus communis*	Brazil: Maranhão	KU058948	KU058918			
M. pseudophaseolina	**CMM3653**	*Jatropha curcas*	Brazil: Minas Gerais	KF369262	KF553906	–	–	–
	CMM4029	*Jatropha curcas*	Brazil: Minas Gerais	KF369270	KF553903	–	–	–
	CMM4030	*Jatropha curcas*	Brazil: Minas Gerais	KF369271	KF553904	–	–	–
	CMM4032	*Jatropha curcas*	Brazil: Minas Gerais	KF369272	KF553905	–	–	–
	CMM4131	*Ricinus communis*	Brazil: Paraíba	KU058935	KU058905	–	–	–
	CMM4155 / CCMF-CNPA 291	*Ricinus communis*	Brazil: Bahia	KU058939	KU058909	–	–	–
	CMM4161 / CCMF-CNPA 293	*Gossypium hirsutum*	Brazil: Rio Grande do Norte	KU058941	KU058911	–	–	–
	CMM4167 / CCMF-CNPA 294	*Gossypium hirsutum*	Brazil: Rio Grande do Norte	KU058942	KU058912	–	–	–
	CMM4230 / CCMF-CNPA 668	*Arachis hypogaea*	Brazil: Rio Grande do Norte	KU058950	KU058920	–	–	–
	CMM4228 / CCMF-CNPA 667	*Arachis hypogaea*	Brazil: Rio Grande do Norte	KU058949	KU058919	–	–	–
	CMM4231 / CCMF-CNPA 669	*Arachis hypogaea*	Brazil: Rio Grande do Norte	KU058951	KU058921	–	–	–
	CPC21400	*Arachis hypogaea*	Senegal: Louga	KF951788	KF952150	–	–	–
	CPC21417	*Arachis hypogaea*	Senegal: Louga	KF951791	KF952153	KF951918	KF952233	KF951986
	CPC21502	*Hibiscus sabdarifa*	Senegal: Saint-Louis	KF951797	KF952159	KF951924	KF952239	KF951991
	CPC21524	*Hibiscus sabdarifa*	Senegal: Saint-Louis	KF951799	KF952161	–	–	–
	CPC21527	*Hibiscus sabdarifa*	Senegal: Saint-Louis	KF951801	KF952163	–	–	–
	CPC21528	*Hibiscus sabdarifa*	Senegal: Saint-Louis	KF951802	KF952164	–	–	–
	CPC21500	*Hibiscus sabdarifa*	Senegal: Saint-Louis	KF951795	KF952157	KF951922	KF952237	KF951989
	CPC21458	*Arachis hypogaea*	Senegal: Louga	KF951793	KF952155	KF951920	KF952235	KF951987
M. euphorbiicola	**CMM4045 / CCMF-CNPA 278**	*Jatropha gossypifolia*	Brazil: Paraíba	KU058928	KU058898	MF457654	MF457657	MF457660
	CMM4134 / CCMF-CNPA 288*	*Ricinus communis*	Brazil: Bahia	KU058936	KU058906	MF457655	MF457658	MF457661
	CMM4145 / CCMF-CNPA 289	*Ricinus communis*	Brazil: Bahia	KU058937	KU058907	MF457656	MF457659	MF457662

The specimens obtained in this study are highlighted in bold (* = ex-type). *CMM* Coleção de Culturas de Fungos Fitopatogênicos Prof. Maria Menezes, *CCMF-CNPA* Coleção de Culturas de Microrganismos Fitopatogênicos da Embrapa Algodão

powder using a microcentrifuge tube pestle. The crushing continued after the addition of 100 μL of Nuclei Lysis Solution from the Wizard® Genomic DNA Purification Kit (Promega Corporation, WI, USA). Subsequently, an additional 500 μL of the previous solution was added. The extraction continued as described by Pinho et al. (2012).

Amplification reactions included the following ingredients for each 25 μL reaction: 12.5 μL of DreamTaq™ PCR Master Mix 2× (MBI Fermentas, Vilnius, Lithuania), 1 μL of 10 μM of each forward and reverse primer synthesised by Invitrogen (Carlsbad, U.S.A), 1 μL of dimethyl sulfoxide (DMSO, Sigma–Aldrich, St. Louis, MO, U.S.A.), 5 μL of 100× (10 mg/mL stock) bovine serum albumin (BSA, Sigma–Aldrich, St. Louis, MO, U.S.A.), 2 μL of genomic DNA (25 ng/μl) and 2.5 μL of nuclease-free water.

The pair of primers ITS1/ITS4 (White et al. 1990), EF1-728F (Carbone and Kohn 1999) EF2R (Jacobs et al. 2004), ACT-512F/ACT-783R (Carbone and Kohn 1999), CAL-228F/CAL-737R (Carbone and Kohn 1999) and T1/Bt2b (O'Donnell and Cigelnik 1997) were used to amplify the target sequences of the Internal Transcribed Spacer regions 1 and 2 including the 5.8S rRNA gene (ITS), Translation Elongation Factor 1-α (TEF1-α), Actin, Calmodulin and β-tubulin, respectively.

The thermal cycle conditions consisted of 95 °C for 5 min, followed by 35 cycles of 94 °C for 1 min (denaturation), 55 °C for 1 min (for TEF1-α and β-tubulin), 52 °C for 1 min (for ITS), 58 °C for 30 s (for actin) or 50 °C for 30 s (for calmodulin) (annealing) and 72 °C for 2 min (elongation), followed by 72 °C for 10 min (final extension). PCR products were analysed with 2% agarose electrophoresis gels stained with GelRed™ (Biotium Inc., Hayward, CA, USA) in a 1× TAE buffer and visualised under UV light to check the amplification size and purity. PCR products were purified and sequenced by Macrogen Inc., South Korea (http://www.macrogen.com). The nucleotide sequences were edited with BioEdit v. 7.2.5 software (Hall 2012). All sequences were checked manually, and nucleotides with ambiguous positions were clarified using both primer direction sequences. New sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov), where sequences of ITS, TEF1-α, Actin, Calmodulin and β-tubulin for additional species were also retrieved (Table 1).

Consensus sequences were compared against NCBI's GenBank nucleotide database using the megaBLAST program. The closest hit sequences were then downloaded in FASTA format and aligned using the multiple sequence alignment program MUSCLE® (Edgar 2004), as implemented in MEGA v. 7 software (Kumar et al. 2015). Alignments were checked, and manual adjustments were made when necessary. All resulting alignments were deposited into TreeBASE (http://www.treebase.org/) under the accession number S18720.

Bayesian inference (BI) analyses employing a Markov Chain Monte Carlo method were performed for all alignments, first with each gene/locus separately and then with the concatenated sequences (ITS and TEF1-α). To confirm the monophyly of the new phylogenetic species clade, unrooted trees were made with the gene regions TEF1-α, β-tubulin, Actin and calmodulin, using the type sequences and few additional representative sequences of each species (Sarr et al. 2014). However, it was not possible to include the type isolate of *M. phaseolina* (CBS205.47) in the analysis of β-tubulin and calmodulin, since no sequences of these gene regions were available for this isolate.. Before launching the BI, the best nucleotide substitution model was determined for each gene with MrMODELTEST 2.3 (Posada and Buckley 2004). Followed by the likelihood scores were calculated, the models were selected according to the Akaike Information Criterion (AIC). Symmetrical model (SYM) of evolution was used for ITS, Hasegawa-Kishino-Yano model with gamma distribution (HKY + G) for TEF1-α and Hasegawa-Kishino-Yano model (HKY) for β-tubulin, Actin and calmodulin. The phylogenetic analyses were performed with the CIPRES web portal (Miller et al. 2010) using MrBayes v.3.1.1 (Ronquist and Heulsenbeck 2003). In concatenated analysis (ITS and TEF1-α), the data were partitioned by locus and the parameters of the nucleotide substitution models for each partition were set as described above. Four chains (MCMC) were run simultaneously, starting from random trees for 10,000,000 generations. Trees were sampled every 1000th generation for a total of 10,000 trees. The first 2500 trees were discarded as the burn-in phase of each analysis. Posterior probabilities (Rannala and Yang 1996) were determined from a majority-rule consensus tree generated with the remaining 7500 trees. Trees were visualized in FigTree v1.3.1 (Rambaut 2009) and exported to graphics programs. The concatenated tree was rooted to *Cophinforma eucalypti* (Botryosphaeriales; Botryosphaeriaceae).

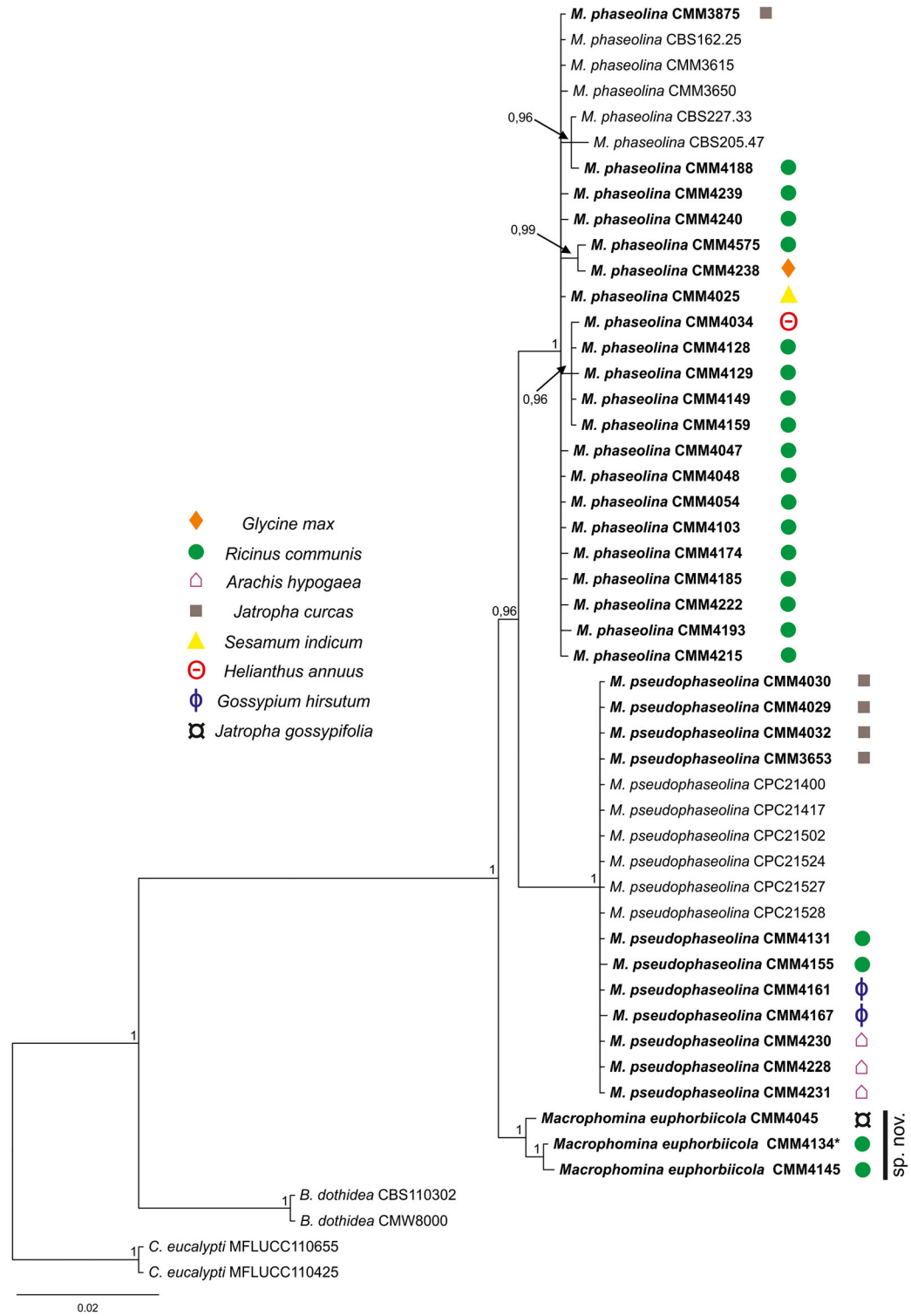

Fig. 1 Phylogenetic tree inferred from Bayesian analysis based on the combined sequences of the ITS and TEF-1α. Bayesian posterior probabilities are indicated above the nodes. The tree was rooted to *Cophinforma eucalypti* MFLUCC110425 and MFLUCC110655. The species obtained in this study are highlighted in bold. *Indicates the ex-type culture. The coloured symbols represent the host of each isolate

Results

Phylogeny

Thirty-five isolates of *Macrophomina*, obtained from seven different hosts in six Brazilian states were analysed (Table 1). PCR reactions were conducted successfully for the regions ITS, TEF1-α for all isolates, and β-tubulin, Actin and Calmodulin for the isolates of new species. The PCR fragments contained approximately 500 bp for ITS, 700 bp for TEF1-α, 600 bp for β-tubulin, 220 bp for Actin and 530 for Calmodulin.

The *Macrophomina* isolates investigated in the present study grouped in three main clades in the concatenated tree (ITS/ TEF1-α), with posterior probabilities values >95% (Fig. 1). The first main clade was represented by isolates that grouped with the ex-epitype (CBS 205.47) of *M. phaseolina* and includes most isolates from *R. communis*, the isolates from *S. indicum*, *H. annus* and *G. max* and one from *J. curcas* seeds. The second main clade grouped with the ex-type (CPC 21417) of *M. pseudophaseolina*, and includes the isolates obtained from *G. hirsutum* and *A. hypogaea,* four remaining isolates from *J. curcas* seeds and two isolates from *R. communis*. The third main clade, which was regarded as a novel species described below, comprises two isolates obtained from *R. communis* and one isolate from *J. gossypifolia*.

The phylogenetic trees obtained with TEF1-α, β-tubulin, Actin and calmodulin separated (Fig. 2) presented a similar topology and confirm the monophyly of the new phylogenetic species proposed here. However,

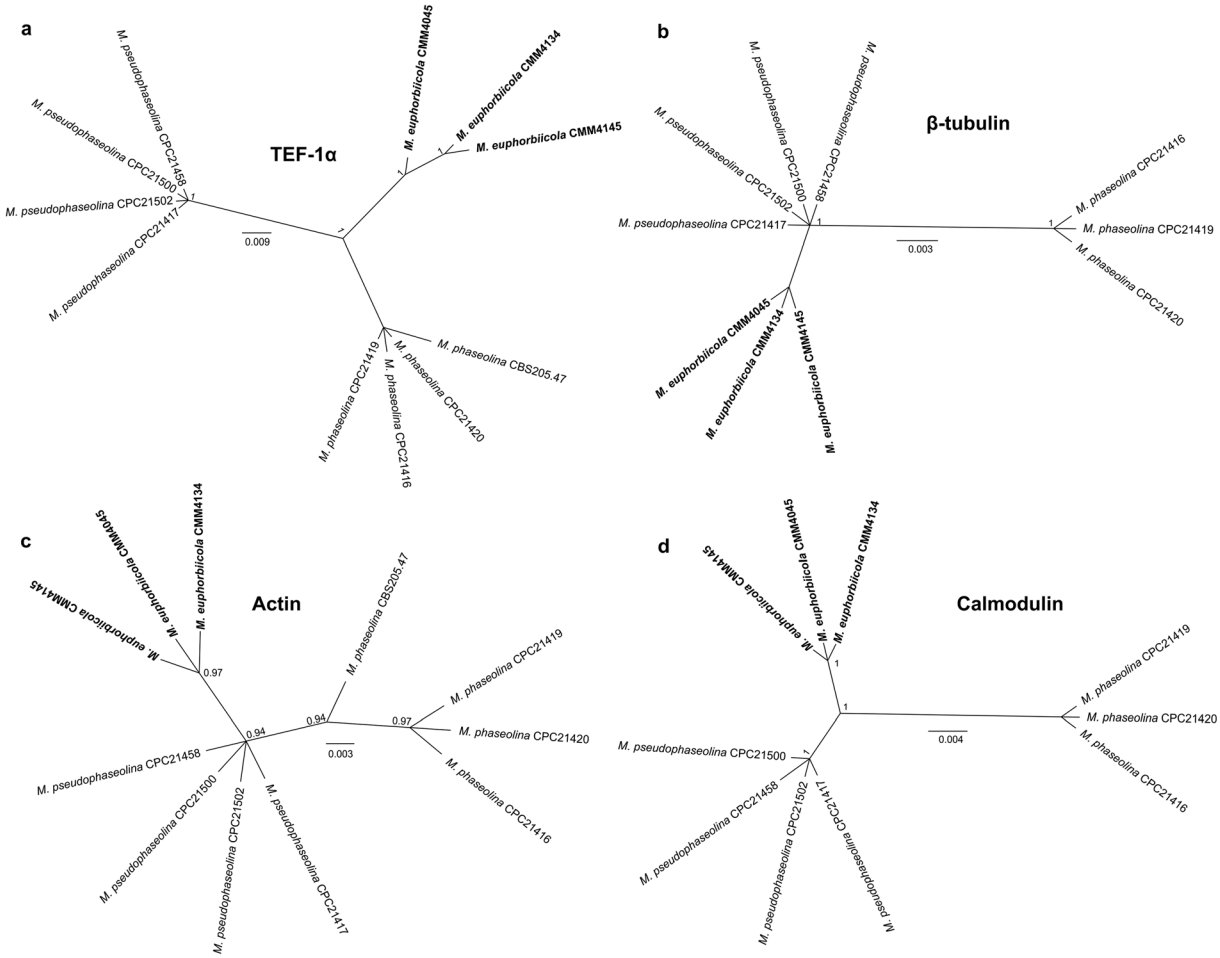

Fig. 2 Unrooted phylogenetic trees inferred from bayesian analysis with representative sequences of the gene regions TEF1-α (**a**), β-tubulin (**b**), Actin (**c**) and Calmodulin (**d**) of the species *M. phaseolina*, *M. pseudophaseolina* and *M. euphorbiicola*. Bayesian posterior probabilities are indicated above the nodes. The species obtained in this study are highlighted in bold

the region ITS alone did not present sufficient variation to separate the three *Macrophomina* species (Data not shown). This region was included in the concatenate analysis only to increase the support of the nodes. The geographic distribution of the three species throughout the sampled areas are shown in Fig. 3.

Taxonomy

Macrophomina euphorbiicola A.R. Machado, D.J. Soares & O.L. Pereira, sp. nov.

MycoBank MB815562.

Etymology: In reference to the host family, *Euphorbiaceae*.

Differs from *M. phaseolina* by nucleotide polymorphisms in five loci (*TEF1-α*, β-tubulin, Actin, Calmodulin and ITS) based on separate alignments: *TEF1-α* positions 3(T), 12 (T), 13(G), 21(A), 22(T), 30(C), 32(C), 55(T), 64(A), 95(A), 133(A), 138(T), 139(T), 140(T), 152(A), 174(C), 182(T), 200(A), 206(−), 207(−), 210(T) and 239(C); β-tubulin positions 38(A), 50(A), 62(C), 71(A), 205(−), 215(C), 267(C), 330(A), 430(A), 411(C), 413(A), 491(T) and 524(T); Actin positions 60(G), 111(A), 133(−) and 220 (C); Calmodulin positions 64(T), 113(G), 123(A), 126(G), 193(T), 194(A), 205(T), 250(C), 258(T), 310(T), 332(A), 428(T) and 497(C); ITS position 31 (T). Differs from *M. pseudophaseolina* on TEF1-α positions 3(T), 12(T), 16(C), 20(C), 22(T), 32(C), 37(G), 74(T), 90(G), 92(C), 95(A), 131(A), 134(T), 138(T), 139(T), 140(T), 174(C), 178(C), 182(T), 189(T), 206(C), 208(T), 209(T), 210(−), 211(−) and 219(T); β-tubulin positions 214(C) and 410(C); Actin position 111(A); Calmodulin

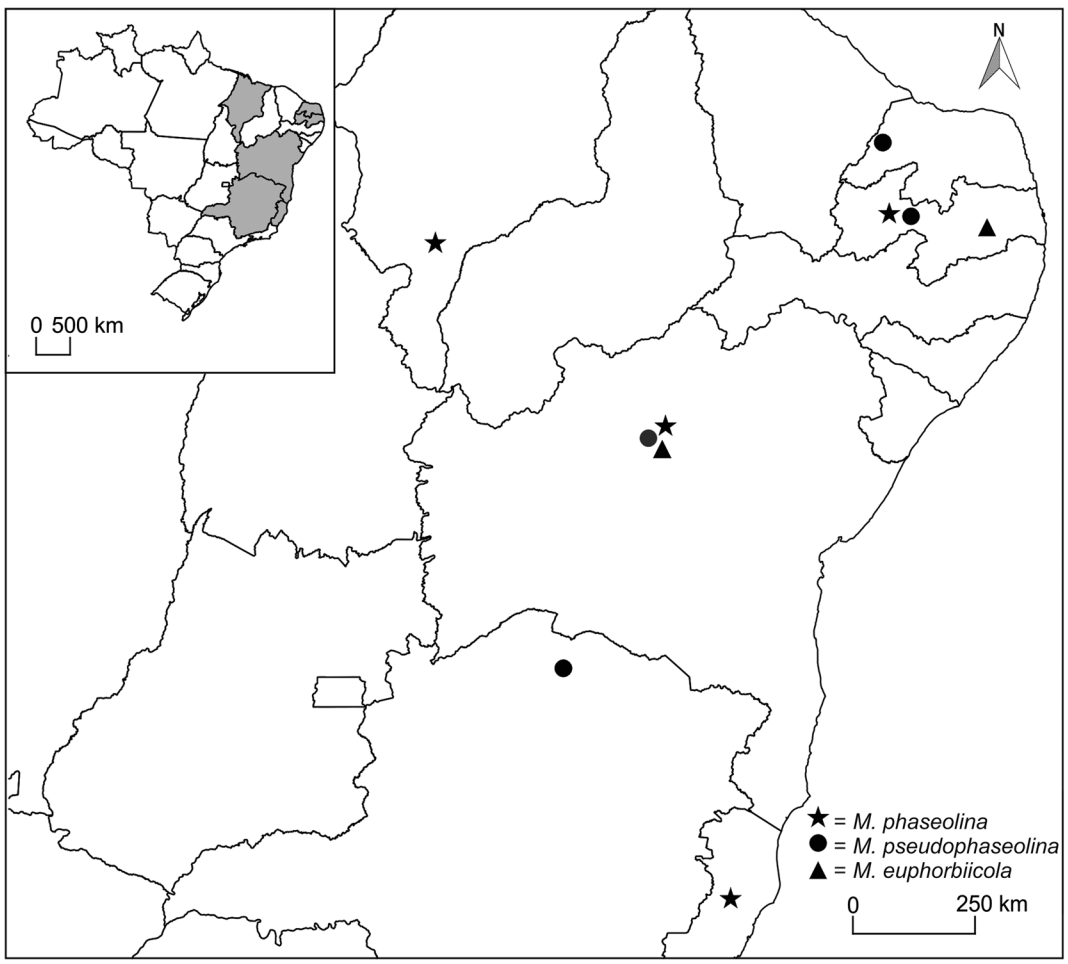

Fig. 3 Map of Brazil showing the distribution of states and different regions from where *Macrophomina* spp. were collected (in grey). Stars indicate *M. phaseolina*, circle indicate *M. pseudophaseolina* and triangle indicate *M. euphorbiicola*

positions 159(A), 250(C), 258(T) and 504(T); No differences in ITS sequences.

Type: **Brazil**: Bahia: Irecê, on *Ricinus communis*, Jul 2010, *D.J. Soares* (URM7464, preserved in a *metabolically inactive* state – holotype; living culture ex-type CMM4134 = CCMF-CNPA 288). Additional cultures examined: Brazil: Paraíba: Lagoa Seca, on *Jatropha gossypifolia*, Jun 2010, *D.J. Soares*

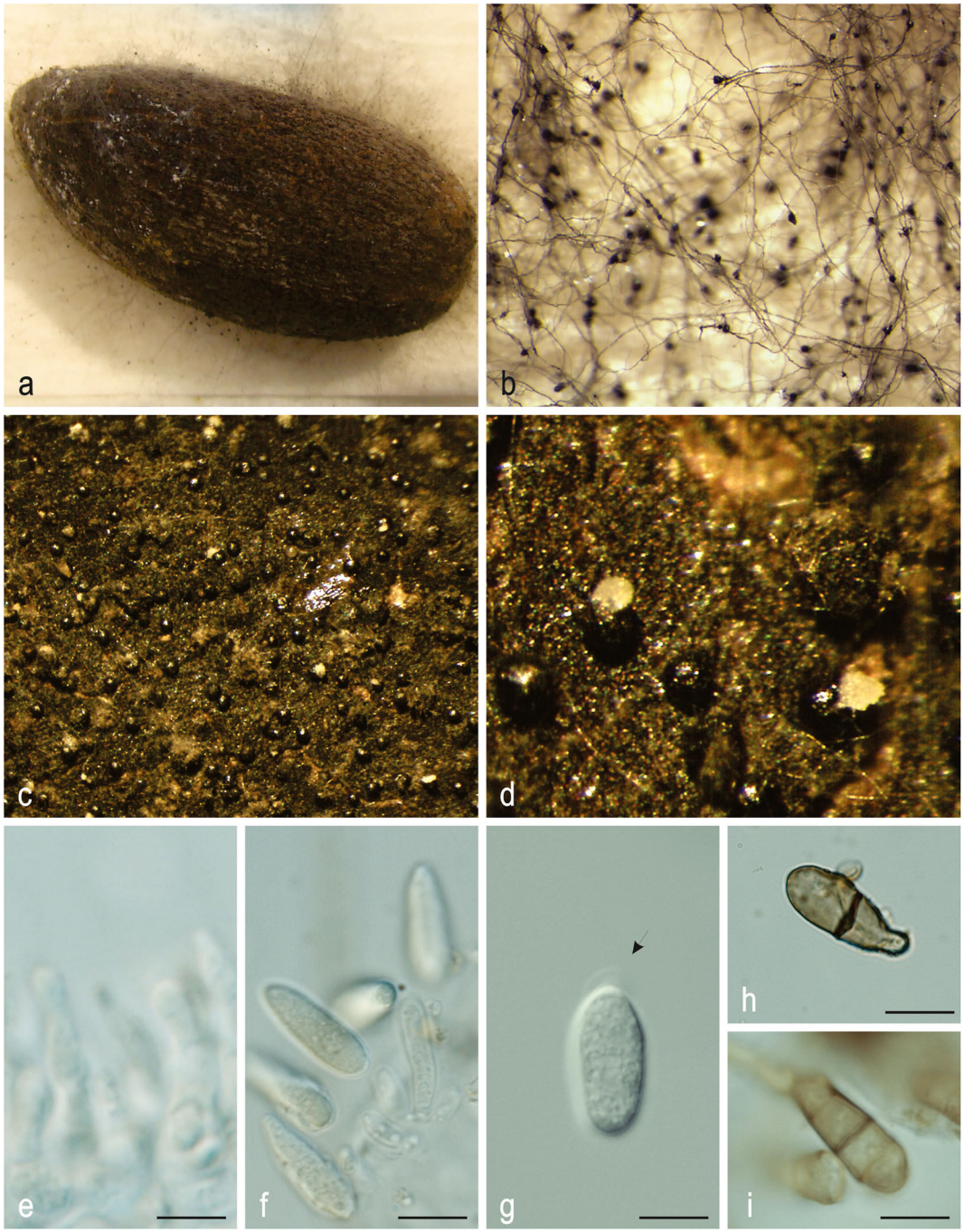

Fig. 4 *Macrophomina pseudophaseolina* (CMM4030). *Jatropha curcas* seed covered by mycelium and sclerotia in blotter test (**a**–**b**). Black sclerotia and conidiomata on seed in culture (**c**–**d**). Conidiogenous cells (**e**). Immature conidia (**f**). Apical mucoid appendages on conidia (**g**). Mature and up to 3-septate conidia (**h**–**i**). Scale bars = 10 μm

(CMM4045 = CCMF-CNPA 278); Brazil: Bahia: Irecê, on *Ricinus communis*, Jul 2010, *D.J. Soares* (CMM4145 = CCMF-CNPA 289).

Notes: *Macrophomina euphorbiicola* is phylogenetically distinct from *M. phaseolina* and *M. pseudophaseolina*, but cultures of the new species failed to sporulate under the conditions described above. It was therefore not possible to carry out a morphological analysis of pycnidial structures. Thus, the new taxon is here introduced based only on molecular data.

Discussion

The present work corroborates to the hypothesis raised by Claudino and Soares (2014) about the presence of cryptic species within *Macrophomina* isolates from oilseed crops in Brazil, previously identified as *M. phaseolina*. However, as concluded by Sarr et al. (2014), no host or geographical correlation was observed. As expected, *M. phaseolina* was the most frequent species found and was present in four of the six Brazilian states (Espírito Santo, Paraíba, Bahia and Maranhão). The distribution range of *M. pseudophaseolina* throughout the sampled areas was surprising, since it was also present in four Brazilian States (Minas Gerais, Paraíba, Rio Grande do Norte and Bahia). Despite the fact that the fungus was initially thought to be restricted to Senegal, Sarr et al. (2014) speculated the possible wider distribution of this species, and our results confirm that hypothesis. Although all five isolates from Rio Grande do Norte, obtained from *G. hirsutum* and *A. hypogaea*, grouped in the *M. pseudophaseolina* clade, these data should be viewed with caution, since all samples were obtained from a single site (same experimental farm, but in different years), and thus do not necessarily represents the pathogen diversity within that State.

Using ITS sequence data alone (data not shown) it was not possible to distinguish the *Macrophomina* species in the present study. However, as already verified for another Botryosphaeriaceae genera, the TEF1-α gene region presented sufficient phylogenetic signal for species discrimination and we believe it can possibly be used as a primary marker for *Macrophomina* species distinction in population screening studies (Hyde et al. 2014). When using a joint analysis approach with ITS and TEF1-α sequences it was possible to discriminate the three *Macrophomina* species with sufficient support (Fig. 1).

Following the approach of the Genealogical Concordance Phylogenetic Species Recognition (Taylor et al. 2000), three additional gene regions were also analysed (Fig. 2), in which corroborated the results obtained with ITS and TEF concatenated, showed concordance between the topologies of different gene regions and confirmed the monophyly of the new species *Macrophomina euphorbiicola*.

The distinction of *Macrophomina* species based only on morphological characters was always a controversial issue, mainly due the wide morphological variation attributed to *M. phaseolina* (Sutton 1980). However, after the epytipification of *M. phaseolina* using a polyphasic approach, which helped to figure out the genetic and morphological application to this name (Sarr et al. 2014), it became possible to segregate new species within this important genus.

Sarr et al. (2014) mentioned that *M. phaseolina* and *M. pseudophaseolina* are very similar, with the exception that conidia of the latter are somewhat shorter. In the present study, conidia of *M. pseudophaseolina* were even smaller (15–22 × 5.5–8 μm), while the conidiogenous cell were slightly longer and larger (10–17 × 4–5 μm) (Fig. 4) than those of *M. pseudophaseolina* provided by Sarr et al. (2014) (Table 2), corroborating the fact that, within the genus *Macrophomina,* classical morphological traits are not informative enough for species differentiation. This fact

Table 2 Main morphological characteristics of *Macrophomina* spp.

Species	Conidial dimensions (μm)	Conidiogenous Cells (μm)	Reference
M. phaseolina	19–30 × 6–9	6–12 × 4–6	Sarr et al. (2014)
M. pseudophaseolina	19–27 × 7.5–9	8–15 × 3–4	Sarr et al. (2014)
M. pseudophaseolina	15–22 × 5.5–8	10–17 × 4–5	This study

highlights the importance of applying the multilocus phylogenetic analysis for an appropriate species discrimination of the genus.

Macrophomina pseudophaseolina is reported for the first time in Brazil causing charcoal rot on *A. hypogaea*, *G. hirsutum* and *R. communis* and associated with *J. curcas* seeds. Until recently, this fungus had been reported only in *Vigna unguiculata*, *Arachis hypogaea*, *Hibiscus sabdarifa*, *Shorghum bicolor* and *Abelmoschus esculentus* in Senegal (Sarr et al. 2014). This species is distributed throughout a wide geographical area within Brazil, and considering the disjunction of the sampled areas, it is probable that it has an even wider distribution within Brazil, and possibly South America.

On the other hand, the newly described species, *M. euphorbiicola*, was recorded causing charcoal rot on *R. communis* and *J. gossypifolia,* and was restricted to two sampled sites. More samples are needed to a better understanding of its ecology, host range and distribution.

A major finding of the present study corroborates to the observations by Sarr et al. (2014), namely that more than one *Macrophomina* species could be present in the same area causing similar symptoms on distinct hosts. Since *Macrophomina phaseolina* sensu *lato* is reported in almost all Brazilian states, from the sub-temperate areas of the Rio Grande do Sul state to the equatorial rain forest in the Amazonian states, broader studies should be conducted to evaluate the diversity of this genus in Brazil, since previous studies demonstrated the existence of a high genetic diversity within *Macrophomina* isolates obtained from different host and Brazilian regions (Almeida et al. 2003, 2008).

Furthermore, until recently charcoal rot diseases were regarded a major problem only in the semiarid Northeast states. However, during the last decades it has been observed as an important pathogen of legumes like soybean, common bean and cowpea, and other crops in the Brazilian Cerrado. Since the present study has demonstrated that more than one *Macrophomina* species can cause similar symptoms on distinct hosts, it is important to perform wider studies to uncover the role of each species in the disease distribution and prevalence throughout the country to help manage this important disease.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest and that the manuscript complies with the ethical standards of the journal.

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