Mycobiology

Research Article

Bipolaris marantae sp. nov., A Novel Helminthosporoid Species Causing Foliage Blight of the Garden Plant *Maranta leuconeura* in Brazil

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Abstract A severe leaf spot, turning to foliage blight, was observed on leaves of *Maranta leuconeura* growing in a garden in Brazil (state of Rio de Janeiro) in 2015. A dematiaceous hyphomycete bearing a morphology typical of a helminthosporoid fungi was regularly found in association with diseased tissues. The fungus was isolated and pathogenicity was demonstrated through the completion of Koch's postulates. A morphology and molecular analysis led to the conclusion that the fungus belonged to the genus *Bipolaris*, which is characterized by having fusiform conidia, externally thickened and truncate hila and a bipolar pattern of germination. Additionally, homology of internal transcribed spacer and GAPDH sequences with sequences of other *Bipolaris* species, confirmed its generic placement. A phylogenetic study also indicated clearly that the fungus on *M. leuconeura* is phylogenetically distinct from related species of this genus, leading to the proposal of the new species *Bipolaris marantae*.

Keywords Asexual morph, Ornamental, Pathogens, Phylogeny, Pleosporales, Taxonomy

Members of the family Marantaceae are found throughout the tropics [1]. This family includes about 55 genera, 12 of which are native from Brazil, including *Calathea* and *Maranta* [2]. One species within Maranthaceae—*Maranta arundinacea* (arrowroot) is of importance as a crop species [3]. However, plants belonging to this family are also widely used as foliage ornamentals, for their green and variegated leaves, which are flat during the day, and folded up and erect in the evening. One of the most popular ornamentals in the family is *Maranta leuconeura* (popularly known in Brazil as maranta-pena-de-pavão, among other names) which is a perennial, rhizomatous plant having showy elliptical-oval, pale green, leaves with darker green patches [4]. It is broadly used as an indoors ornamental and for covering broad areas in parks and gardens around the world. As for any ornamentals a healthy appearance of the plant stands is a critical issue for its use and any significant biotic or abiotic damage is considered unacceptable.

In May 2015, plant beds of *M. leuconeura* in a private garden in the state of Rio de Janeiro, Brazil (Gávea, Rio de Janeiro) were found to be severely damaged by a disease which started as leaf spots which later grew, coalesced leading to entire leaf blight and, finally, to plant death (Fig. 1A). A dematiaceous hyphomycete, readily recognized as belonging to a helminthosporoid genus complex, was recurrently found in association with the necrotic tissues. Here, the results of the work made towards the clarification of the etiology of the disease on *M. leuconeura* are reported.

Mycobiology 2017 September, **45**(3): 123-128
https://doi.org/10.5941/MYCO.2017.45.3.123
pISSN 1229-8093 • eISSN 2092-9323

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Received January 17, 2017
Revised May 3, 2017
Accepted June 23, 2017

MATERIALS AND METHODS

Collection and isolation. A sample of *M. leuconeura* bearing symptoms of leaf spotting and leaf blight, at various stages, was collected, dried in a plant press and taken to the lab for further examination. Additionally, healthy and diseased individuals were uprooted and kept in sealed plastic bags with water-soaked paper tissue to allow for the maintenance of healthy and diseased plants for later use and cultivation in a greenhouse. The samples were examined for the possible presence of pathogen structures.

A dematiaceous fungus was regularly associated with the necrotic tissues and direct isolation was performed by transfer of individual conidia from fungal colonies to plates containing vegetable broth-agar (VBA)—as described by Pereira *et al.* [5]—with a sterile fine pointed needle. The fungus was incubated at 25°C under a 12-hr photoperiod (light provided by two fluorescent white and one NUV black light lamps located 35 cm above the plates). A representative culture was deposited in the culture collection—Coleção Octávio de Almeida Drumond (COAD) housed at Universidade Federal de Viçosa. A representative herbarium specimen was deposited in the Herbarium of Universidade Federal de Viçosa (VIC).

Morphological study. Fungal structures were either scraped from the leaf surface with a scalpel or removed with an adhesive tape and mounted in lactoglycerol and lactofuchsin. Slides containing the fungal structures were examined and images were produced using an Olympus BX 53 light microscope (Olympus, Tokyo, Japan) equipped with a Motic (Moticam 5) digital camera. Biometric data was based on the observation of a minimum of 30 structures. Additionally, slide cultures were prepared, as described by Waller *et al.* [6], for observation of fine detail of conidiogenesis and conidial germination.

Morphology of colonies and colony pigmentation was described after 7 days of growth on potato dextrose agar (PDA) and VBA incubated at 25°C under a 12-hr light regime (light conditions as above). Colony color terminology followed Rayner [7].

DNA extraction, PCR amplification, and sequencing. A representative monosporic culture was grown on VBA at 25°C under a 12-hr photoperiod for 1 wk. Approximately 50 mg of mycelium was scraped from the colony formed on the plate, placed in a sterile tube containing metal beads, and agitated on mechanical cell disruptor L-Beader-3. DNA was extracted with the Wizard Genomic DNA purification Kit (Promega, Madison, WI, USA) following the manufacturers' protocols. DNA concentration was

Table 1. Strains and NCBI GenBank accession numbers of species used in this study

Species	Voucher/Culture	GenBank accession No.	
		ITS	GAPDH
Bipolaris bamagaensis	BRIP 13577	KX452445	KX452411
	BRIP 10711	KX452444	KX452410
Bipolaris bicolor	CBS 690.96	KJ909762	KM042893
Bipolaris cynodontis	CBS 109894	KJ909767	KM034838
Bipolaris drechsleri	CBS 136207	KF500530	KF500533
	FIP 373	KF500531	KF500534
	MUS 0028	KF500532	KF500535
Bipolaris heveae	CBS 241.92	KJ909763	KM034843
Bipolaris maydis	CBS 136.29	KJ909769	KM034845
	AR 5182	KM230388	KM034848
Bipolaris microlaenae	CBS 280.91	JN601032	JN600974
Bipolaris microstegii	CBS 132549	JX089577	JK089573
	CBS 132550	JX089579	JX089575
Bipolaris panici-miliacei	CBS 199.29	KJ909773	KM042896
	BRIP 12282	KJ415531	KJ415415
Bipolaris oryzae	MAFF 235449	KJ922383	KM042897
	MFLUCC 10-0715	JX256416	JX276430
Bipolaris salviniae	BRIP 16571	KJ415535	KJ415411
Bipolaris setariae	CBS 141.31	EF452444	EF513206
	Bs01JH1a	KT805922	KT982612
Bipolaris sivanesaniana	BRIP 15847	KX452455	KX452421
Bipolaris simmondsii	BRIP 12030	KX452454	KX452420
Bipolaris sorokiniana	CBS 110.14	KJ922381	KM034822
	CBS 480.74	KJ909771	KM034827
Bipolaris urochloae	ATCC 58317	KJ922389	KM230396
Bipolaris victoriae	CBS 327.64	KJ909778	KM034811
	DAOM 147449	KJ909785	KM034812
Bipolaris zeicola	FIP532	KM230398	KM034815
Alternaria alternata (outgroup)	EGS 34.016	AF071346	AF081400
Bipolaris marantae sp. nov.	COAD 2068	KX365749	KX907136

ITS, internal transcribed spacer; GAPDH, glycerol 3-phosphate dehydrogenase.

determined in a spectrophotometer NanoDrop (Thermo Fischer Scientific Inc., Waltham, MA, USA) following the manufacturer's protocols. The primers LR0R and LR5, ITS5, and ITS4 were used to amplify the partial 28S rDNA (LSU) and the internal transcribed spacer regions and intervening 5.8S rDNA gene (internal transcribed spacer [ITS]), respectively [8, 9]. The primers EF-728F and EF-986R were used to amplify the partial translation elongation factor 1-α (*tef1*) [10] and the primers GPD1 and GPD2 [11], were used to amplify the glycerol 3-phosphate dehydrogenase region (GAPDH). Amplicons were analyzed on 0.8% agarose electrophoresis gels stained with GelRed (InstantAgarose) in a 1× TAE buffer and visualized under UV light to check for amplification size and purity. PCR products were purified and sequenced by Macrogen Inc. (http://www.macrogen.com). Sequence electropherograms were analyzed with DNA Dragon 1.4.1 [12]. Resulting sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov).

Phylogenetic analysis. Initially, sequences obtained from the datasets of Tan *et al.* [13], of Manamgoda *et al.* [14], from GenBank, and the novel sequences generated during this study were aligned using MAFFT ver. 7 [15] and whenever necessary, manually improved in MEGA ver. 7.0 [16, 17]. After a preliminary analysis, the dataset was trimmed down to Brazilian isolates and the direct neighbors (Table 1). Appropriate gene models were selected using MrModeltest ver. 2.3 [18] and applied to each gene partition. Based on the results of MrModeltest, a Bayesian phylogenetic analysis was performed in CIPRES web portal [19], using MrBayes ver. 3.2.1 [20], and applying the GTR + I + G substitution model for ITS, and the HKY + I for GAPDH. Posterior probabilities were determined by Markov Chain Monte Carlo sampling in MrBayes ver. 3.2.1. Six simultaneous Markov chains were run for 10,000,000 generations and trees were sampled every 100th generation, until convergence (stopval = 0.01) was reached. Trees were visualized in FigTree [21] and exported to a graphic software. *Alternaria alternata* (isolate EGS 34-016) served as outgroup for the phylogenetic analyses.

Pathogenicity test. The isolate COAD 2068 served as basis for morphological and molecular characterization as well as for confirmation of the fungus pathogenicity. Three healthy adult *M. leuconeura* plants grown in pots containing a sterile commercial compost were inoculated in the test. Inoculum consisted of a conidial suspension (2.2×10^5 conidia/mL) obtained after 7-day-old colonies of COAD 2068 formed on VBA plates at 25°C under a light regime as described above were scraped and suspended in sterile distilled water. Test plants were sprayed with the suspension until runoff, and the plants kept for 2 days in a dew chamber at 25 ± 3°C. Additionally, two plants were sprayed with sterile distilled water and kept under the same conditions to serve as controls. All the plants were inspected periodically, until the appearance of the symptoms.

RESULTS AND DISCUSSION

Phylogenetic study. The ITS, LSU, *tef1*, and GAPDH sequences of the isolate COAD 2068 accession Nos. KX365749, KY198731, KY263645, KX907136, respectively were compared with sequences available in GenBank through a BLASTn search and the following was found: 96% similarity with *Bipolaris panici-miliacei* (KJ909773) for ITS, 96% with *Bipolaris maydis* (AY544645) for LSU, 99% with *Bipolaris oryzae* (KF688977) for *tef1* and 86% with *B. panici-miliacei* (KJ415415) for GAPDH. Although the 28S rDNA and *tef1* sequences were not used for later phylogenetic analyses, they were lodged in GenBank for future studies. The phylogenetic tree inferred from a combined ITS and GAPDH dataset using Bayesian analysis supported that the present isolate is distinct from other *Bipolaris* species for which there are ITS and GAPDH sequences available in GenBank.

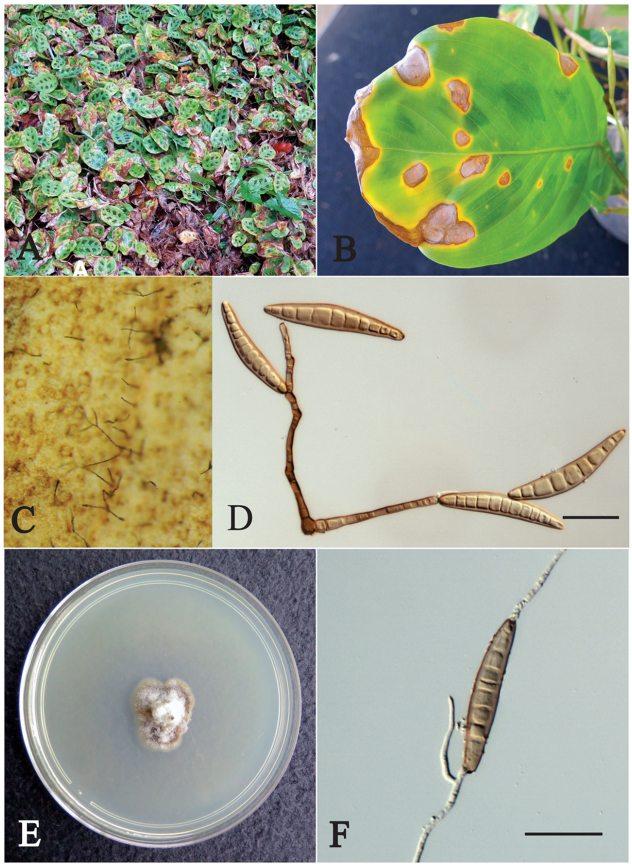

Fig. 1. *Bipolaris marantae* sp. nov. (VIC 44075, holotype) on *Maranta leuconeura*. A, Plant bed in garden (note severely blighted plants); B, Leaf blight on *M. leuconeura* 10 days after inoculation under controlled conditions (Koch's postulates); C, Healthy appearance of a non-inoculated plant kept under the same conditions after 10 days; D, Abundant sporulation on surface of necrotic tissue of *M. leuconeura* (note upright conidiophores and conidia); E, F, Conidia attached to conidiophores (scale bars = 40 μm).

Taxonomy.

Bipolaris marantae J. L. Alves & R. W. Barreto sp. nov. (Fig. 1).

Etymology: referring to the host genus *Maranta*.

Mycobank: MB819048.

Sexual morph not seen. Internal mycelium indistinct. External mycelium absent. Stromata absent. Conidiophores amphigenous, solitary or in loose small groups, cylindrical, proliferating sympodially, 182.5–487.5 × 5–10 µm, 8–17 septate, dark brown. Conidiogenous cell integrated, intercalary and terminal, cylindrical, proliferating sympodially, geniculate, 12.5–40 × 5–10 µm, pale brow to dark brown, smooth. Conidia solitary, straight to somewhat curved, narrowly ellipsoidal, 80–150 × 12.5–22.5 µm, tapering towards the obtuse ends, 7–12 distoseptate (mostly 5–7), first septum median, second septum submedian and third septum supramedian, brown, hilum darkened, thickened, external and truncate, germ tubes bipolar, extending along the main axis of the conidia, smooth (Fig. 1E and 1F). Cultures on PDA and VBA: slow growing (1.5 cm diam after 15 days), colonies flat, aerial mycelium velvety, dark olive, whitish and raised centrally, irregular and pale brown edge, reverse with diurnal zonations alternating dark olive and light grayish rings; covered with short conidiophores and abundant black conidia.

Habitat: Garden plant *Maranta leuconeura* beds with blighted appearance.

Holotype: Brazil, Rio de Janeiro, Gávea on leaves of *Maranta leuconeura*, 10 Nov 2015, Robert W. Barreto (VIC 44075, ex-type culture COAD 2068).

Pathogenicity: Five days after inoculation, symptoms appeared on all inoculated plants. Symptoms were equivalent to those seen on plants in the field. The fungus was found sporulating on necrotic tissues, and reisolated, fulfilling Koch's postulates (Fig. 1B). Non-inoculated plants remained healthy.

Thirty-five fungal taxa have been recorded in association with members of *Maranta* [22] but 12 out of these were identified only at the generic level. The majority of the records were of fungi found on *M. arundinaceae* or *M. leuconeura*. The main list of fungi on plants in Brazil [23] includes nine fungal taxa associated with *M. bicolor* (a rather similar species to *M. leuconeura,* which is also used for the same purpose in gardening) but these all are saprophytes found on decomposed leaves as opposed to the fungus on *M. leuconeura* which was demonstrated to be a severe pathogen.

The taxonomy of the dematiaceous asexual morphs originally placed in *Helminthosporium* has changed considerably along the years. By the end of the 20th century, it was accepted that *Helminthosporium* represented a relatively small assemblage of species mostly found on rotten wood, the plant pathogenic taxa being recognized as separate taxa and transferred to *Bipolaris*, *Curvularia*, *Drechslera*, and *Exserohilum* [24]. Although, *Bipolaris* and *Drechslera* have similarities in their morphology, the sexual morph of *Drechslera* belongs to *Pyrenophora* whereas the sexual morphs of *Bipolaris* and *Curvularia* belong to *Cochliobolus*—an ascomycete genus typified by *C. heterostrophus* which is the sexual morph of a *Bipolaris*, namely *B. maydis* [25-27]. Recently, molecular phylogenetic analysis based on ITS and GAPDH genes corroborated that *Bipolaris* and *Drechslera* are two distinct genera [14, 28, 29]. Nevertheless, the distinction between *Bipolaris* and *Curvularia* has historically been challenging, with discrimination between members of the two genera relying mostly on conidial characters—which were known to present considerable plasticity—and complicated by the fact that *Bipolaris* and *Curvularia* share a common sexual morph. Nevertheless, this complex is being progressively unraveled by recent phylogenetic works [28, 29] which confirmed that the two asexual taxa are distinct, although sharing a morphologically similar sexual morph, and that several species fitting the morphology of *Bipolaris* in fact belong to *Curvularia.*

The morphology of the fungus on *M. leuconeura* fits well within the *Bipolaris-Curvularia* delimitation. This group includes a number of important plant pathogens with worldwide distribution causing leaf spots, leaf blights, and other disease symptoms on many host plants, including several major crops in the family Poaceae [24]. Other monocot crop species such as coconut may also suffer significant losses due to the attack by fungi in this group [30] as well as many ornamentals such as plants belong to *Heliconia* sp., *Calathea* sp., and *Dendrobium* sp. [14].

Currently, only one species of *Bipolaris* has been recorded on members of the Marantaceae, namely *Bipolaris setariae* (= *Drechslera setariae*) found in Florida (USA) on several species of *Calathea* and on *M. leuconeura* bearing leaf spots [31]. This was originally recorded as *Drechslera setariae* based on fungal morphology—the only tool available at the time. It is likely that the fungus studied by Simone [31] belongs to the same taxon of the fungus collected in Brazil, and described herein, as the description of symptoms and fungal morphology matches well with those observed on the newly collected material. Unfortunately, no hebarium specimens nor pure cultures seem to have been deposited which might be used for comparison and clarification. The fungus on *M. leuconeura* is morphologically similar to *B. setariae* and *B. oryzae* but it differs from *B. setariae* by having larger conidia (80–150 × 12.5–22.5 µm in *B. marantae*) than those of *B. setariae* (45–100 × 10–15 µm). It differs from *B. oryzae* for producing longer conidia (63–153 × 14–22 µm in *B. oryzae*) and by conidial pigmentation (pale to mid golden brown in *B. oryzae* and pale to dark brow in *B. marantae*) [24]. Nevertheless, the strongest evidence towards the distinction of *B. marantae* from related taxa was obtained as a result of the phylogenetic analyses of ITS and GAPDH regions. *Bipolaris marantae* clusters far from *B. setariae* and is closely related to *B. panici-miliacei*, having *B. oryzae* as sister clade. The proposal of *B. marantae* as a novel species is hence justified and well supported by this study (Fig. 2).

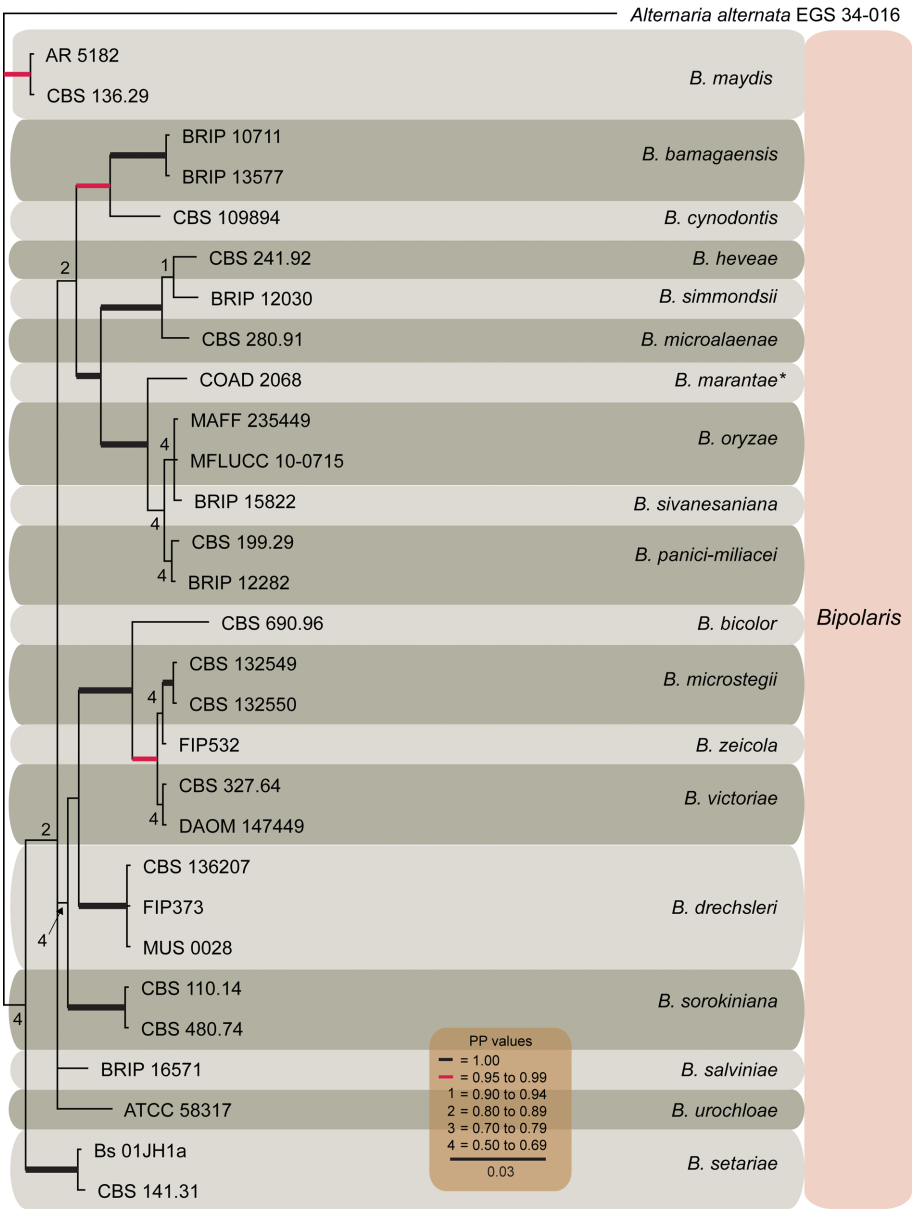

Fig. 2. Consensus phylogram (50% majority rule) of *Bipolaris* species, from a Bayesian analysis of the combined interval transcribed spacer and glyceraldehyde 3-phosphate dehydrogenase alignment. Bayesian posterior probabilities are indicated with colour-coded branches and numbers (see legend) and the scale bar indicates 0.04 expected changes per site. The novel species is indicated with single asterisk. The tree is rooted with *Alternaria alternata* (isolate EGS 34.0160).

ACKNOWLEDGEMENTS

The authors acknowledge financial support by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—CAPES, Conselho Nacional do Desenvolvimento Científico e Tecnológico—CNPq and Fundação de Amparo à Pesquisa do Estado de Minas Gerais – FAPEMIG.

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