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.

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

| Species                  | Voucher/Culture | GenBank accession No. |
|--------------------------|-----------------|-----------------------|
| *Bipolaris bamagaensis*  | BRIP 13577      | KX452445              |
|                          | BRIP 10711      | KX452444              |
| *Bipolaris bicolor*      | CBS 690.96      | KJ909762              |
|                          | CBS 109894      | KJ909767              |
| *Bipolaris cynodontis*   | CBS 136207      | KF500530              |
|                          | FIP 373         | KF500531              |
|                          | MUS 0028        | KF500532              |
| *Bipolaris heveae*       | CBS 241.92      | KJ909763              |
| *Bipolaris maydis*       | CBS 136.29      | KJ909769              |
| *Bipolaris microlaeae*   | AR 5182         | KM230388              |
| *Bipolaris microstegii*  | CBS 132549      | JX089577              |
|                          | CBS 132550      | JX089579              |
| *Bipolaris panici-miliacei* | CBS 199.29 | KJ909773              |
|                          | BRIP 12282      | KJ415531              |
| *Bipolaris oryzae*       | MAFF 235449     | KJ922383              |
| *Bipolaris salviniae*    | BIP 16571       | KJ415535              |
| *Bipolaris setariae*     | CBS 141.31      | EF452444              |
|                          | B001JH1a        | KT805922              |
| *Bipolaris sivanesaniana*| BRIP 15847      | KX452455              |
| *Bipolaris simmondii*    | BRIP 12030      | KX452454              |
| *Bipolaris sorokiniana*  | CBS 110.14      | KJ922381              |
|                          | CBS 480.74      | KJ909771              |
| *Bipolaris urochloae*    | ATCC 58317      | KJ922389              |
| *Bipolaris victoriae*    | CBS 327.64      | KJ909778              |
|                          | DAOM 147449     | KJ909785              |
| *Bipolaris zeicola*      | FIP532          | KM230398              |
| *Alternaria alternata* (outgroup) | EGS 34.016 | AF071346              |
| *Bipolaris marantae* sp. nov. | COAD 2068 | KX365749              |

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

| Species                  | Voucher/Culture | GenBank accession No. |
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| *Alternaria alternata* (outgroup) | EGS 34.016 | AF071346              |
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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 × 10⁵ 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* (KJ145145) 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 brown 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 supramedial, 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 herbarium 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 brown 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. panicili-luicide, 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).


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REFERENCES

1. Christenhusz MJ, Byng JW. The number of known plants species in the world and its annual increase. Phytotaxa 2016; 261:201-17.
2. Vieira S, Souza VC. Four new species of Maranta L. (Marantaceae) from Brazil. Bot J Linn Soc 2008;158:131-9.
3. Simpson BB, Ogorzaly MC. Economic botany: plants in our world. Boston (MA): McGraw Hill; 2001.
4. Lorenzi H. Plantas para jardim no Brasil. Nova Odessa: Instituto Plantarum de Estudos da Flora; 2013.
5. Pereira JM, Barreto RW, Ellison CA, Maffia LA. Corynespora cassiciola f. sp. lantanae: a potential biocontrol agent from Brazil for Lantana camara. Biol Control 2003;26:21-31.
6. Waller JM, Ritchie BJ, Holderness M. Plant clinic handbook. Egham: CABl Publishing; 1997.
7. Rayner RW. A mycological colour chart. Kew: Commonwealth Mycological Institute; 1970.
8. Vilgalys R, Hester M. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several Cryptococcus species. J Bacteriol 1990;172:4238-46.
9. White TJ, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. PCR protocols: a guide to methods and applications. New York: Academic Press; 1990. p. 315-22.
10. Jacobs K, Bergdahl DR, Wingfield MJ, Halik S, Seifert KA, Bright DE, Wingfield BD. Leptographium wingfieldii introduced into North America and found associated with exotic Tomicus piniperda and native bark beetles. Mycol Res 2004;108(Pt 4):411-8.
11. Myllys L, Stenroos S, Thell A. New genes for phylogenetic studies of lichenized fungi: glyceraldehyde-3-phosphate dehydrogenase and beta-tubulin genes. Lichenologist 2002;34:237-46.
12. Hepperle D. DNA Dragon 1.4.1 [Internet]. Klein Raden: SequentiX; 2016 [cited 2016 Oct 22]. Available from: http://www.dna-dragon.com.
13. Tan YP, Crous PW, Shivas RG. Eight novel Bipolaris species identified from John L. Alcorn's collections at the Queensland Plant Pathology Herbarium (BRIP). Mycol Prog 2016;15:1203-14.
14. Manamgoda DS, Cai L, McKenzie EH, Crous PW, Madrid H, Chukeatirote E, Shivag RG, Tan YP, Hyde KD. A phylogenetic and taxonomic re-evaluation of the Bipolaris-Cochliobolus-Curvularia complex. Fungal Divers 2012;56:131-44.
15. Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol 2013;30:772-80.
16. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 2004;32:1792-7.
17. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 2011;28:2731-9.
18. Nylander J. MrModeltest v2. Program distributed by the author. Uppsala: Evolutionary Biology Centre, Uppsala University; 2004.
19. Miller MA, Pfeiffer W, Schwartz T. Creating the CIPRES science gateway for inference of large phylogenetic trees. In: Proceedings of the Gateway Computing Environments Workshop (GCE) 2010; 2010 Nov 14; New Orleans, LA, USA; No. 11705685.
20. Ronquist F, Teslenko M, Van den Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu S, Suchard MA, Huelsenbeck JP. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst Biol 2012;61:399-42.
21. Rambaut A. FigTree 1.2.2. [Internet]. Andrew Rambaut; 2009 [cited 2016 Oct 26]. Available from: http://tree.bio.ed.ac.uk/software/figtree/.
22. Farr DF, Rossman AY, Palm ME, McCray EB. Fungal databases, systematic botany and mycology laboratory, ARS, USDA [Internet]. Beltsville (MD): USDA Agricultural Research Service; 2017 [cited 2017 May 26]. Available from: http://nt.ars-grin.gov/fungaldatabases.
23. Mendes, MAS, Urbén AF. Fungos relatados em plantas no Brasil. Laboratório de Quarentena Vegetal [Internet]. Brasília: Embrapa, Recursos Genéticos e Biotecnologia; 2017 [cited 2017 May 30]. Available from: http://pragawall.cenargen.embrapa.br/aqweb/michtml/michbanco01a.asp.
24. Sivanesan A. Graminicolous species of Bipolaris, Curvularia, Drechslera, Exserohilum and their teleomorphs. Mycol Pap 1987;158:1-261.
25. Drechsler C. Phytopathological and taxonomical aspects of Ophiobolus, Pyrenophora, Helminthosporium and a new genus Cochliobolus. Phytopathology 1934;24:953-83.
26. Alcorn JL. On the genera Cochliobolus and Pseudocochliobolus. Mycotaxon 1983;16:353-79.
27. Alcorn JL. The taxonomy of "Helminthosporium" species. Annu Rev Phytopathol 1988;26:37-56.
28. Manamgoda DS, Rossman AY, Castelbury LA, Crous PW, Madrid H, Chukeatirote E, Hyde KD. The genus Bipolaris. Stud Mycol 2014;79:221-88.
29. Manamgoda DS, Cai L, Bahkali AH, Chukeatirote E, Hyde KD. Cochliobolus: an overview and current status of species. Fungal Divers 2011;51:3-42.
30. Niu XQ, Yu FY, Zhu H, Qin WQ. First report of leaf spot disease in coconut seedling caused by Bipolaris setariae in China. Plant Dis 2014;98:1742.
31. Simone GW. New leaf spot disease of Calathea and Maranta spp. incited by Drechslera setariae. Plant Dis 1983;67:1160-1.