A NEW EPIFOLIAR SPECIES OF Neopestalotiopsis FROM BRAZIL

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Neopestalotiopsis pernambucana, a new Ascomycota species found on leaves of Vismia guianensis in Atlantic Rain Forest of Pernambuco State, Brazil, is described and illustrated. The species is characterized by having morphological and DNA data different from the other species of the genus. A key for identification of Neopestalotiopsis and Pestalotiopsis with sexual morph (previously known as Pestalosphaeria) species is also provided. The phylogenetic relationship between N. pernambucana and other related species is discussed.

Key words: Amphisphaeriaceae, ITS, taxonomy, tef1, tropical fungi.

Uma nova espécie epifoliar de Neopestalotiopsis do Brasil. Neopestalotiopsis pernambucana, uma nova espécie de Ascomycota encontrada sobre folhas de Vismia guianensis na Mata Atlântica de Pernambuco, Brasil é descrita e ilustrada. A espécie é caracterizada por ter dados morfológicos e de DNA diferentes das outras espécies do gênero. Uma chave para identificação das espécies teleomórficas de Neopestalotiopsis e Pestalotiopsis (previamente alocadas em Pestalosphaeria) é apresentada. As relações filogenéticas entre N. pernambucana e outras espécies são discutidas.

Palavras-chave: Amphisphaeriaceae, ITS, taxonomia, tef1, fungos tropicais.
Introduction

Vismia guianensis (Aubl.) Pers. is a small tree of the Angiosperms group (Angiospermae), Hypericaceae family (Reichardt, 1878) (previously Guttiferae or Clusiaceae). It presents cosmopolitan and neotropical distribution, occurring in Amazonia, Cerrado and Atlantic Forest biomes and almost all Brazilian territory (CRIA, 2007). Viégas (1961) published a catalogue of fungi associated with higher plants from Brazil and South America which lists 10 ascomycetes on Vismia species. Later, Mendes et al. (1998) listed the foliicolous fungi studied in Brazil, mentioning only Hypocrella camerunensis (Aubl.) Pers., Micropeltella vismiae Bat., Peres & Holanda and Sphaerulina vismiae Bat. & J.L. Bezerra on Vismia spp. However, Farr and Rossman (2016) recorded Pestalotiopsis spp. on V. guianensis, V. obtusa Spruce ex Reichardt, V. baccifera (L.) Planch. & Triana and V. baccifera subsp. ferruginea (Kunth) Ewan in Ecuador and Venezuela.

Pestalotiopsis Steyaert is a cosmopolitan genus frequently reported in many states of Brazil comprising saprobic, pathogenic and endophytic species. The 211 species of Pestalotiopsis recorded in Brazil were cataloged in association with 53 host plants (Kruschewsky; Luz; Bezerra 2014). According to Maharachchikumbura et al. (2011), most species of Pestalotiopsis lack sexual morphs and only 13 species have been recorded to reproduce sexually, and they were previously treated as belonging to the genus Pestalosphaeria. The sexual morph of Pestalotiopsis has not been reported in Brazil. The genus Neopestalotiopsis was segregated from Pestalotiopsis by Maharachchikumbura et al. (2014) based on phylogenetic analysis and morphological differences, such as versicolorous median cells and conidiophores indistinct, often reduced to conidiogenous cells, and according to Index Fungorum currently has 25 recognized species (CABI, 2016).

A new species of Neopestalotiopsis, found on Vismia guianensis from the Atlantic Rain Forest of Pernambuco State, Brazil, is here described and illustrated.

Materials and Methods

Morphological study - collection, isolation and characterization

During a mycological expedition to the ‘Reserva Ecológica de Dois Irmãos’ (08°00’36.9”S and 34°56’57.2”W), an important remnant of the Atlantic Rain Forest of Pernambuco State, Northeast of Brazil, attached and fallen spotted leaves of Vismia guianensis were collected. Some of the leaves were incubated for about 30 days in moist chambers consisting of Petri dishes lined with wet filter paper. Handmade transversal sections of leaves with the fungus colonies, using a razor blade were mounted between slides and cover slides with PVLG plus cotton blue, Melzer’s reagent or water and examined under a light microscope Leica DM500 equipped with a drawing tube and a digital camera. Ascospores obtained ‘in nature’ were transferred to Potato Dextrose Agar (PDA) culture medium and incubated at temperature about 28°C and 12 hours light/dark regime. The fungal morphological features ‘in nature’ and artificial medium were described, measured and illustrated. The exsiccates and cultures were deposited in the Herbarium URM and URM Culture Collection of the Universidade Federal de Pernambuco (UFPE), respectively.

Molecular analyses

The fungal biomass was obtained from cultures grown on malt agar contained in test tubes and kept at 28°C for up to six days. All mycelium was removed from the test tube with the aid of a platinum loop, the material was transferred to 2 mL micro-tubes with screw caps, being added in each tube 0.5 g of glass beads with two different diameters in the 1:1 ratio (acid-washed, 150-212 im and 425-600 im; Sigma, U.S. sieve). The material was crushed by stirring at high speed in a FastPrep.

The genomic DNA extraction procedures followed Góes-Neto; Loguercio-Leite; Guerreiro (2005). The mycelium was washed with chloroform:isoamyl alcohol (24:1), followed by a homogenization in CTAB buffer at 2%, isopropanol precipitation, wash in 70% ethanol, and re-suspension in 50 mL of ultrapure water.

For ITS rDNA amplifications the primers ITS5/ITS4 (White et al., 1990) were used. The polymerase chain reactions were carried as described by Oliveira et al. (2014). For tef1 amplifications the primers EF1-526F/EF1-1567R (Rehner, 2001) were used. The polymerase chain reactions were carried as Maharachchikumbura et al. (2012).
The final amplicons were purified with the PureLink PCR Purification Kit (Invitrogen). Sequencing was performed by the Human Genome Research Center (São Paulo, Brazil). Sequence data were compared with similar sequences available on EMBL and GenBank databases through BLASTn. The obtained sequences were deposited in the NCBI database under the accession numbers KJ792466 and KJ792467 (ITS rDNA), KU306739 and KU306740 (tef1).

**Phylogenetic analyses**

The phylogeny was reconstructed by analyses from sequences of the ITS1, 5.8s and ITS2 of the rDNA and tef1 gene. The fungal sequences were aligned in ClustalX (Larkin et al., 2007) and edited with the BioEdit program (Hall, 1999). Prior to phylogenetic analysis, the model of nucleotide substitution was estimated using Topali 2.5 (Milne et al., 2004). Bayesian (two runs over 1 × 10^6 generations with a burnin value of 2500) and maximum likelihood (1,000 bootstrap) analyses were performed, respectively, in MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) and PhyML (Guindon & Gascuel, 2003), launched from Topali 2.5.

**Results**

**Taxonomy**

*Neopestalotiopsis pernambucana* M.L. Silvério, M.A.Q. Cavalcanti et J.L. Bezerra, sp. nov.

**Figures 1–2, 3**

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**Etymology** – name reflects the original place of the species, Pernambuco State, Brazil.

**Foliicola. Sexual morph:** Ascomata perithecial, epiphyllous, abundant, isolated, immersed in the host tissue, with neck slightly erumpent, subglobe, 170–205 × 182.5–202.5 µm, unilocular, glabrous, ostiolate, dark brown, stromata none; peridium 15–27.5 µm thick, inner stratum hyaline to subhyaline, composed of elongated, thin-walled, compressed cells; outer stratum more developed, dark brown, with bigger and thicker-walled cells; ostiolar canal periphysate, 32.5–45 × 25–37.5 µm. Asci unitunicate, 8-spored, cylindrical to clavate-cylindrical, 52.5–100 × 7.5–10 µm, short-stipitate, stipe 5–7.5 µm high, apical ring amyloid, flattened; paraphyses flexuous, vacuolated, simple, thin, intertwined, smooth, septate, hyaline, semi-evanescent.

**Figures. 1–2 – Neopestalotiopsis pernambucana on Vismia guianensis.** 1. Vertical section of ascomata. 2. a) Asci; b) Paraphyses; c) Ascospores. Bars = 20 µm.

**Figure 3 – Neopestalotiopsis pernambucana.** Conidia with two, three and four apical appendages. Bar = 10 µm.

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becoming olivaceous to brown when mature, slightly constricted in the septa, hyaline while young, 2-septate, occasionally with 1 supramedian septum, slightly curved, 8–17 (–22) × 4–7.5 (–10) µm, usually smaller. Ascospores uniseriate, smooth, usually ellipsoidal, sometimes irregularly oblong, straight to slightly curved, 8–17 (–22) × 4–7.5 (–10) µm, usually 2-septate, occasionally with 1 supramedian septum, becoming olivaceous to brown when mature, concoloured or the middle cell slightly darker.

Asexual morph: Conidiomata acervular, epiphyllous, black, abundant, isolated, sub-epidermal, irregularly distributed on the leaf surface, erumpent at maturity. Conidiophores (conidiogenous cells) hyaline, short. Conidia fusiform to subclavate, smooth, straight, 18–32 × 6–10 µm, 4-septate; three median cells versicoloured, the middle cell or the two upper cells darker; apical cell hyaline to subhyaline, 2–3 (–4) appendages, 7–33 µm long, filiform, simple or branched; basal cell hyaline, 1–2 appendages, 3–15 µm long, simple or occasionally branched.

Colonies on PDA fast-growing, 6.5 cm diam. after five days at about 28°C, white, cottony, odorless, without exudate, with black dots in the center corresponding to the conidiomata (acervuli); reverse smooth, pale cream. Mycelium hyaline, septate, smooth hyphae, 13–22 × 2–6 µm; acervuli isolated or aggregated. Conidia 5-celled (4-septate), fusiform to subclavate, versicoloured, usually with the two upper median cells dark brown, 14–24 × 5–7 µm; apical cell hyaline, with 2–3 appendages, filiform, simple or branched, 8–32 µm long; median cells 11–20 µm long; basal cell hyaline, with 1 (–2) appendage, filiform, simple or scarcely branched, 3–11 µm long.

Material examined – Brazil, Pernambuco, Recife, Reserva Ecológica de Dois Irmãos, 08°00’36.9”S, 34°56’57.2”W, elev. 30 m, on living and fallen leaves of Vismia guianensis (Aubl.) Pers. (Clusiaceae), 24 Apr 2009, M.L. Silvério (holotype, UFPE-Herbarium URM, 80210; UFPE-URM Culture Collection, 7148).

Notes - The new proposed species, N. pernambucana, differs from other congeneric species by size, septation and form of its ascospores, as shown in the key of the species with sexual morph below. In this study, both conidiomata and conidia developed on PDA presented characters similar to those found in nature. According to Misaghi et al. (1978), conidia obtained from nature are usually more uniform in size and morphology than those from artificial media.

Key to current species of Pestalotiopsis and Neopestalotiopsis with sexual morphs

1. Ascospores strictly 2-septate..................................................... 2
2. Asci clavate........................................................................... 3
3. Ascospores not constricted at the septa............................... P. eugeniae
4. Asci 120–134 × 10–15 µm; ascospores verruculose.................. P. austroamericana
5. Asci 67–90 × 9–15 µm; ascospores smooth.................. P. alpinae
6. Perithecia solitary, up to 150 µm diameter......................... P. elaeidis
7. Ascosporas pale brown, ellipsoidal; conidiomata acervular...... P. maculiformans
8. Ascospores verruculose.......................................................... P. varia
9. Ascospores 1–2 septate, slightly constricted at the septa...... 10
10. Perithecia 210.5–294.7 µm diameter; asci clavate........... P. jinggangensis
11. Ascospores 2–3 septate......................................................... 12
12. Ascospores may be 1-septate........................................... P. accidenta
13. Asci oblong-cylindrical to oblong-clavate, 10–12.5 µm wide.................. P. gubae
14. Ascospores 7.7–9 µm wide.............................. P. hansenii

Phylogenetic analyses

Phylogenetic analyses from sequences of the ITS gene showed that Neopestalotiopsis pernambucana forms a distinct clade (Figure 4) and phylogenetic analyses from sequences of the tef1 gene separated N. pernambucana from other species used in this study, with high bootstrap support (Figure 5).
A new species of *Neopestalotiopsis* from Brazil

**Figure 4** – Phylogenetic tree of the genera *Neopestalotiopsis* obtained by analysis from rDNA sequences (ITS1, 5.8S rDNA and ITS2). Support values are from Bayesian and maximum likelihood (ML) analyses, respectively. Thick branches in grey represent clades with 95% bootstrap support in all analyses. The tree was rooted by *Amphisphaeria umbrina*. The new species is shown in boldface. The database accession number are labeled with the name of the fungal species.
Figure 5 – Phylogenetic tree of the genera *Neopestalotiopsis* obtained by analysis *tef1*. Support values are from Bayesian and maximum likelihood (ML) analyses, respectively. Thick branches in grey represent clades with 95% bootstrap support in all analyses. The tree was rooted by *Seiridium* sp. The new species is shown in boldface. The database accession number are labeled with the name of the fungal species.
Discussion

In the review of the genus *Pestalotiopsis*, Maharachchikumbura et al. (2014) made a phylogenetic reconstruction of the Amphisphaeriaceae based on analysis of LSU of the rRNA sequence data. Furthermore, two novel genera were segregated from *Pestalotiopsis*, namely *Neopestalotiopsis* and *Pseudopestalotiopsis* based on combined morphological and DNA data.

The genus *Pestalosphaeria* (Amphisphaeriaceae, Xylariales) was established in 1975 by Margaret E. Barr, as the sexual morph of *Pestalotiopsis*, to allocate the pathogenic species *P. concentrica*, found on living leaves of *Rhododendron maximum* L., ornamental plant normally cultivated in North-American gardens. The features of the type species included perithecia globose, immerse in the host tissues and with erumpent ostiole, asci cylindrical, 70-95 × 9-12 µm, unitunicate, with short to elongated stipe and apical ring amyloid and ascospores ovoid-elliptical, 13.5-20 × 7-10 µm, brown when mature and 2-septate.

Later, Van der Aa (1976) proposed the transfer of *Leptosphaeria elaeidis* C. Booth & J.S. Robertson for the genus *Pestalosphaeria*, after comparing structural features of the asci. This new combination, *P. elaeidis*, was redescribed and illustrated by Hyde (1996).

In 1982, Shoemaker & Simpson described *Pestalosphaeria hansenii* on *Pinus caribaea* var. *hondurensis*, while Nag Raj (1979; 1985) found the species *P. austroamericana*, on dead leaves of *Harknessia americana* in Chile and *P. varia*, on pods of *Acacia koa* in Hawaii. The first record of *P. leucospermi* was done by Samuels; Muller; Petrinii (1987) in New Zealand and *P. gubae* was discovered in Japan by Kobayashi; Ishihara; Ono (2001). *Pestalosphaeria accidenta, P. jinggangensis, P. alpiniae* and *P. eugeniae* were reported in China (Zhu et al., 1991; Chi, 1994) and *P. maculiformans* was found in South Africa, on dead leaves of several hosts (Marincowitz et al., 2008).

According to the Amsterdam Declaration on Fungal Nomenclature, only one name can be applied to any fungal species (Hawksworth et al., 2011). Maharachchikumbura et al. (2011) suggested that *Pestalotiopsis* should be adopted instead of *Pestalosphaeria* because it is an older and more common name. The genus *Neopestalotiopsis* was introduced by Maharachchikumbura et al. (2014) as sexual morph not observed. The new species presently described is referred to as *Neopestalotiopsis pernambucana* and it is the first species of the genus encountered with the sexual morph. Phylogenetic analysis of the sequences of ITS and tef1 genes showed that *Neopestalotiopsis pernambucana* forms a distinct clade (Figures 4 and 5) with high bootstrap support. The versicolorous morphology of the conidia, a feature of the genus *Neopestalotiopsis*, corroborate this molecular result.

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