

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, *tefl*, 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 teleomorfas 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, *tefl*, 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 µm and 425-600 µm; 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 µL 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 on 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

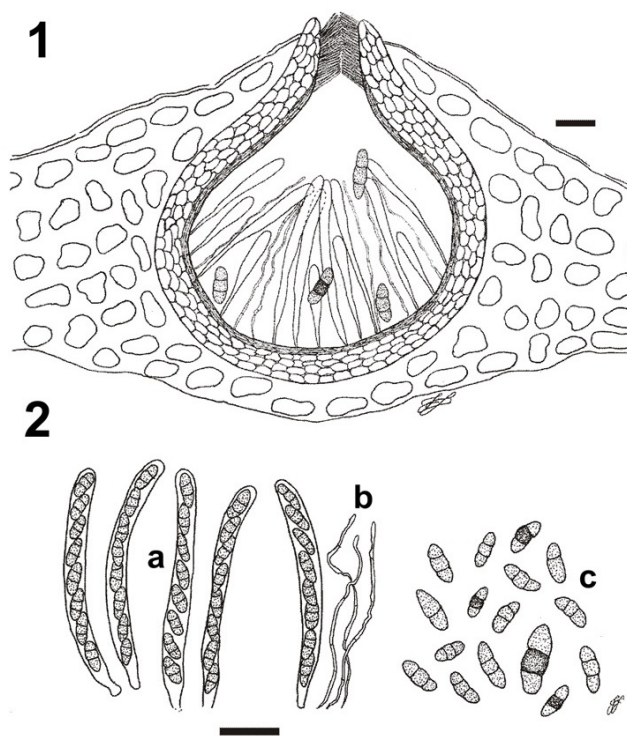
Neopestalotiopsis pernambucana M.L. Silvério, M.A.Q. Cavalcanti et J.L. Bezerra, **sp. nov.**

Figures 1–2, 3

Mycobank MB814915

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, subglobose, $170\text{--}205 \times 182.5\text{--}202.5 \mu\text{m}$, unilocular, glabrous, ostiolate, dark brown, stromata none; peridium $15\text{--}27.5 \mu\text{m}$ 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, $32.5\text{--}45 \times 25\text{--}37.5 \mu\text{m}$. Asci unitunicate, 8-spored, cylindrical to clavate-cylindrical, $52.5\text{--}100 \times 7.5\text{--}10 \mu\text{m}$, short-stipitate, stipe $5\text{--}7.5 \mu\text{m}$ 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 .

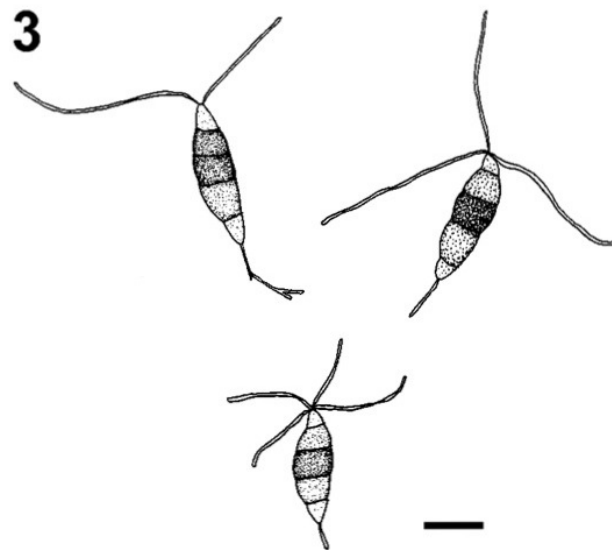


Figure 3 – *Neopestalotiopsis pernambucana*. Conidia with two, three and four apical appendages. Bar = 10 μm .

2.5 µm wide about the same height of the asci or slightly 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, slightly constricted in the septa, hyaline while young, 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
- 1'. Ascospores 1–3 septate..... 8
2. Asci clavate..... 3
- 2'. Asci cylindrical..... 5
3. Ascospores not constricted at the septa..... *P. eugeniae*
- 3'. Ascospores constricted at the septa..... 4
4. Asci 120–134 × 10–15 µm; ascospores verruculose.....
.....*P. austroamericana*
- 4'. Asci 67–90 × 9–15 µm; ascospores smooth..... *P. alpiniae*
5. Asci more than 9 µm wide..... *P. concentrica*
- 5'. Asci less than 9 µm wide..... 6
6. Perithecia solitary, up to 150 µm diameter..... *P. elaeidis*
- 6'. Perithecia aggregated, more than 150 µm diameter..... 7
7. Ascospores pale brown, ellipsoidal; conidiomata acervular.....
.....*P. leucospermi*
- 7'. Ascospores brown, fusoid; conidiomata pycnidial.....
.....*P. maculiformans*
8. Ascospores verruculose..... *P. varia*
- 8'. Ascospores smooth..... 9
9. Ascospores 1–2 septate, slightly constricted at the septa..... 10
- 9'. Ascospores without the combination of characteristics above..... 11
10. Perithecia 210.5–294.7 µm diameter; asci clavate.....
.....*P. jinggangensis*
- 10'. Perithecia 170–205 µm diameter; asci cylindrical to clavate-cylindrical..... *N. pernambucana*
11. Ascospores 2–3 septate..... 12
- 11'. Ascospores may be 1-septate..... *P. accidenta*
12. Asci oblong-cylindrical to oblong-clavate, 10–12.5 µm wide..... *P. gubae*
- 12'. Asci cylindrical, 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).

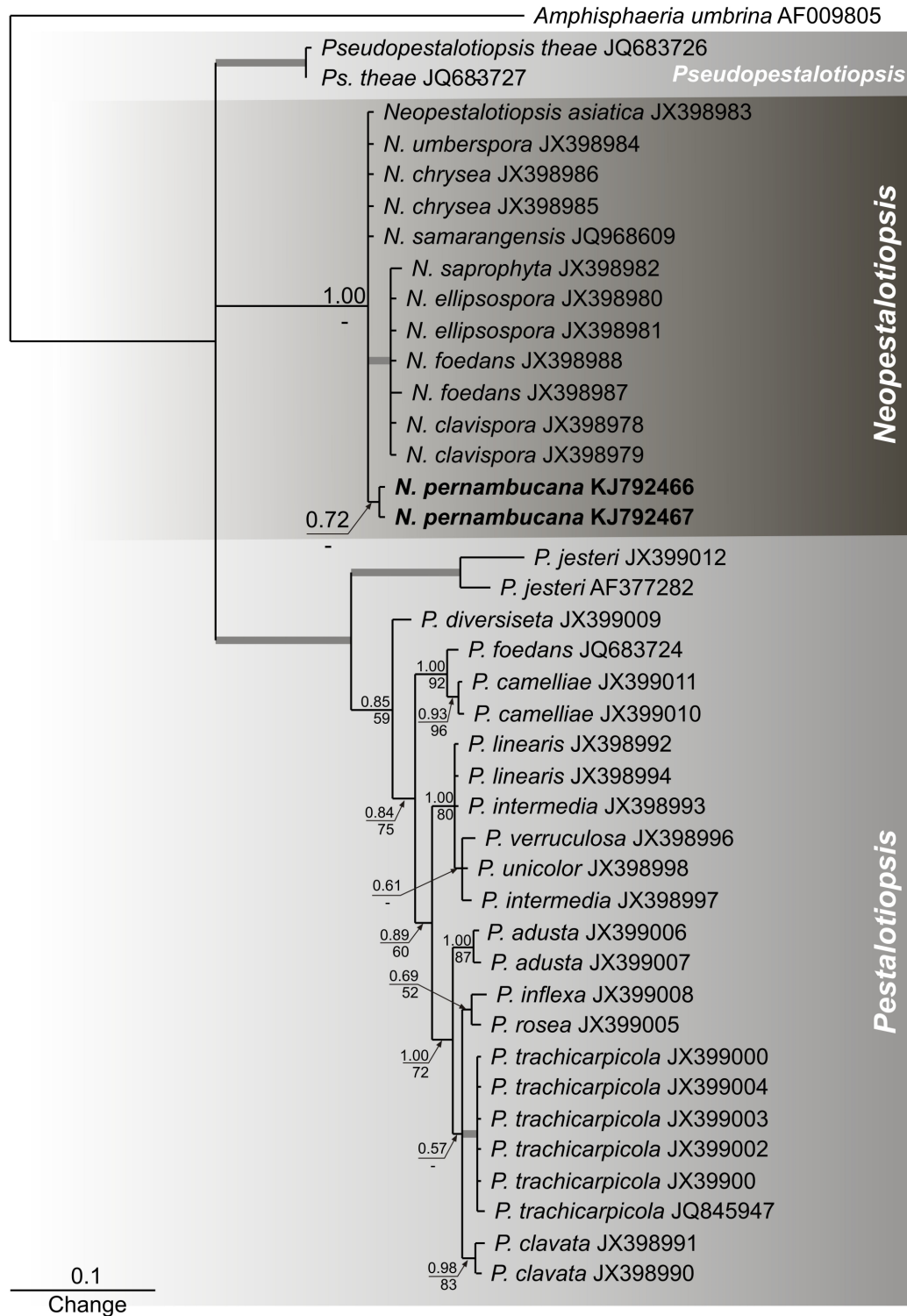


Figure 4 – Phylogenetic tree of the genera *Neopestalotiopsis* obtained by analysis from rDNA sequences (ITS1, 5.8S rDNA and ITS2). Support values are from Bayesian and maximum likelihood (ML) analyses, respectively. Thick branches in grey represent clades with 95% bootstrap support in all analyses. 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.

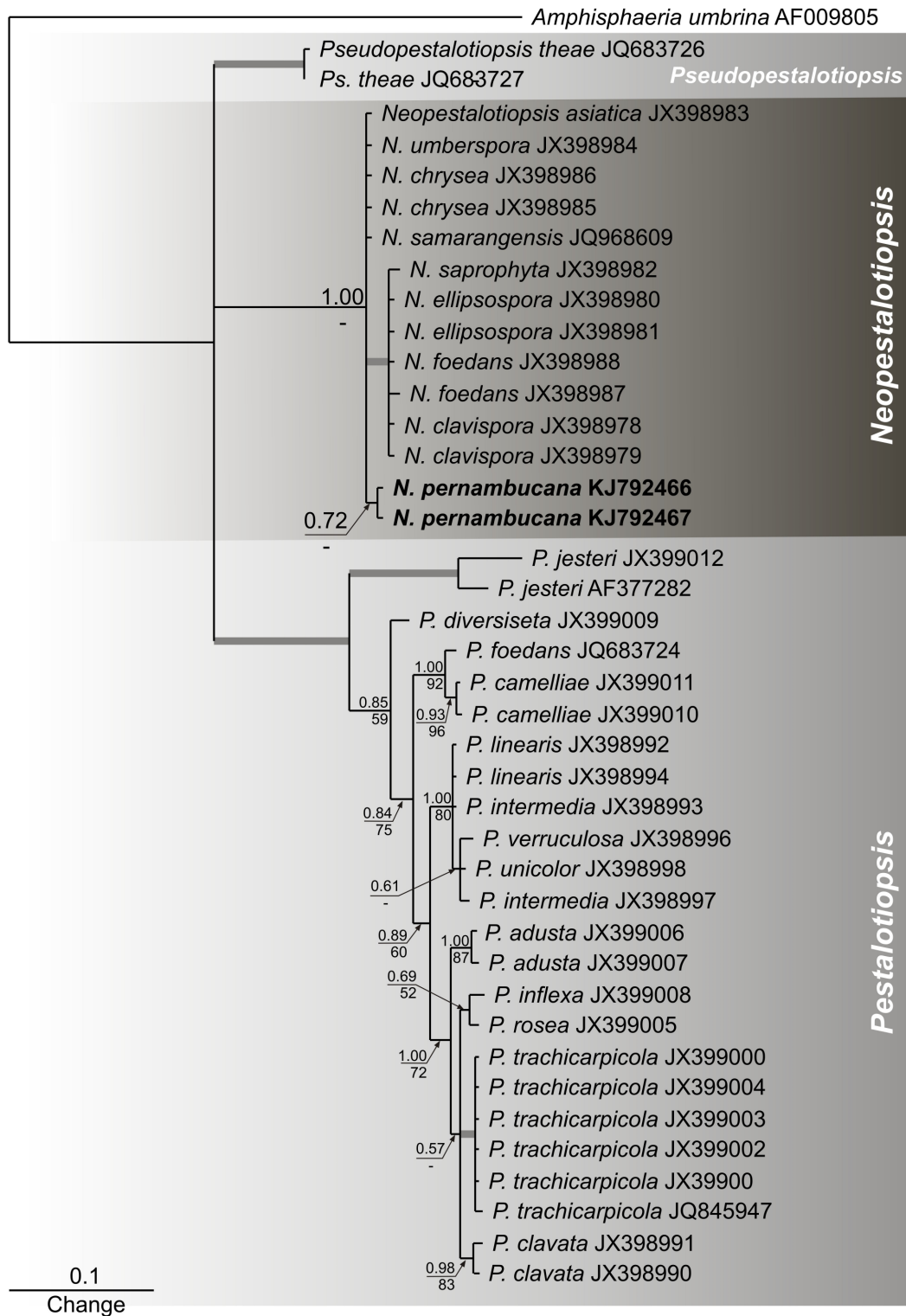


Figure 5 – Phylogenetic tree of the genera *Neopestalotiopsis* obtained by analysis *tef1*. Support values are from Bayesian and maximum likelihood (ML) analyses, respectively. Thick branches in grey represent clades with 95% bootstrap support in all analyses. The tree was rooted by *Seiridium* sp. The 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 *Harknessa americana* in Chile and *P. varia*, on pods of *Acacia koa* in Hawaii. The first record of *P. leucospermi* was done by Samuels; Muller; Petrini (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 *tefl* 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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