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Neopestalotiopsis zimbabwana ISOLATED FROM *Xylaria* STROMATA

Neopestalotiopsis zimbabwana YANG DIISOLASI DARI STROMATA JAMUR *Xylaria*

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ABSTRACT

Neopestalotiopsis is one of pestalotioid fungi. Neopestalotiopsis has versicolourous in the third cell (median cell). This genus is identified as pathogenic and endophytic fungi in the plant. Neopestalotiopsis strain X1 was isolated from Xylaria stromata assumed as endophytic fungi. The surface sterilization was conducted, then isolated into PDA (1/2 receipt of potato dextrose). The morphology was observed in a fresh PDA medium. Genomic DNA was extracted and amplified for the Large Subunit region. A phylogenetic tree was built using RAxML Black Box. The morphology showed that the five cells in a conidium were an appearance with the black color of the septate in the third cell (median cell). The appendage on the basal cell was only a single tubular appendage. The appendage on the apical was two until three tubular appendages. The phylogenetic tree showed that Neopestalotiopsis strain X1 was Neopestalotiopsis zimbabwana. The sequence was deposited into GenBank as MW422813. This study was the first report for Neopestalotiopsis species isolated from micro-mushroom (Xylaria stromata).

Keywords: *median cell, pestalotioid, tubular appendage*

ABSTRAK

Neopestalotiopsis merupakan salah satu kapang pestalotioid. Neopestalotiopsis memiliki warna yang menyolok pada sel ketiga (sel paling tengah). Genus ini teridentifikasi sebagai cendawan patogen dan juga endofit di tanaman. Neopestalotiopsis strain X1 diisolasi dari stromata jamur Xylaria yang diduga sebagai cendawan endofit. Sterilisasi permukaan dilakukan, kemudian bagian stromata diisolasi ke dalam PDA (1/2 penerimaan dekstrosa kentang). Morfologi diamati pada medium PDA segar. DNA genom diekstraksi dan diamplifikasi untuk daerah 28S atau Large Subunit. Pohon filogenetik dibangun menggunakan RAxML Black Box. Morfologi menunjukkan bahwa kelima sel dalam konidium tampak berwarna hitam pada sel ketiga (sel palingtengah) dan bersekat. Pelengkap pada sel bagian dasar hanya sel tambahan berbentuk tubular dan tunggal. Pelengkap pada sel bagian ujung ada dua sampai tiga pelengkap berbentuk tabung. Pohon filogenetik menunjukkan bahwa Neopestalotiopsis strain X1 adalah Neopestalotiopsis zimbabwana. Sekuen DNA disimpan ke GenBank sebagai kode GenBank MW422813. Penelitian ini merupakan laporan pertama spesies Neopestalotiopsis yang diisolasi dari jamur (stromata jamur Xylaria).

Kata kunci: *Pestalotioid, sel paling tengah, tambahan sel berbentuk tabung*

INTRODUCTION

Pestalotioid fungi have unique characters of morphological features on their conidia [1]. The conidia show the septated cells with an appendage on the basal and apical parts. These fungi are common as phytopathogens, saprobes, and endophytes in plants [2]. Pestalotioid fungi are classified into Ascomycota within Pezizomycotina and Xylariomycetidae. These fungi know as asexual fungus. It means the spores are produced through the mitotic process in hyphae [3].

Currently, there are three genera popular in Pestalotioid fungi, i.e., *Neopestalotiopsis*, *Pseudopestalotiopsis*, and *Pestalotiopsis* [2]. Distinguish characters as morphology within their genera are really not sure if only based on the morphological study. The phylogenetic tree analyses are really needed to ensure the genera and the species of Pestalotioid fungi. Maharachchikumbura et al. [2] described the important character of *Neopestalotiopsis*. It is versicolourous median cells. Then, between *Pseudopestalotiopsis* and *Pestalotiopsis* can be distinguished by darker color on the third cell inside the conidia and apical appendages appearance.

According to Index Fungorum, there are 45 name records of *Neopestalotiopsis* [4], 371 name records of *Pestalotiopsis* [5], and 22 name records of *Pseudopestalotiopsis* [6]. Pestalotioid fungi are popular to be studied as taxonomy because of the new species opportunities of these genera [7, 8, 9, 10, 11, 12, 13]. Pestalotioid fungi are well known as phytopathogenic fungi, but some can be

endophytic for any plant. Therefore, endophytic fungal strains can be isolated from the plant. Literally, endophyte means the organisms that live inside the plant, analogous with epiphyte [14]. Organisms, especially microbes that live inside animals or mushrooms, can be usually called endosymbiont. In this study, an isolate of Pestalotioid fungus was isolated from *Xylaria* stromata. In Indonesia, this is the first record that found the Pestalotioid in *Xylaria* stromata. Indonesia was once the place where Pestalotioid species was found, i.e., *Neopestalotiopsis javaensis* and *N. piceana* isolated from *Cocos nucifera* [10].

METHODS

Fungal isolation. The fungus was isolated from *Xylaria* stromata. The *Xylaria* was identified as *Xylaria* sp. assumed as a new species (Hermawan & Khairillah, 2021). The isolation was conducted on 20th July 2019 in the Mycology Laboratory of IPB University. The isolation from *Xylaria* stromata used surface sterilization [15]. The stromata were put into PDA medium (modified for ½ recipe of Potato Dextrose) and incubated in 28 ℃ for four days. Then, the fungal colony that grew in the medium was transferred into the PDA medium (normal composition).

Observation of fungal morphology. The successfully isolated fungi from the *Xylaria* stromata were cultivated into PDA and incubated at 28 ℃ for seven days. The colonies were observed for the hyphae and conidia. The fungus that showed the conidia as *Pestalotiopsis* or *Neopestalotiopsis* was continued for detailed observation, such as size, shape, and color of conidia and its conidiogens.

Fungal molecular. The isolate as X1 was continued for molecular identification. The extraction of genomic DNA used protocol as in [16]. The genomic DNA was amplified using Large Subunit (LSU), i.e., LR0R (5'-GTA CCC GCT GAA CTT AAG C-3') as a forward primer and LR5 (5'-ATC CTG AGG GAA ACT TC-3') as a reverse primer. PCR amplification was done in a 40 µL total reaction. The PCR mixture was 20 µL PCR mix from 2X Kappa Fast 2G,2 µL of 10 pmol of each primer, 4 µL 100 ng template DNA, and 12 µL ddH2O. Amplification used a Thermoline PCR. The PCR was set as follows: initial denaturation at 94 ℃ for 2 minutes, followed by 30 cycles of denaturation at 94 ℃ for 45 seconds, annealing at 55 ℃ for 1 minute, and extension at 72 ℃ for 1 minute. The final extension was set at 72 ℃ for 10 minutes. The amplicon was estimated on 1.5 % agarose gels and visualized by the Gel DocTM XR system. PCR products were sent to the 1st Base Malaysia for sequencing.

Phylogenetic analyses. The sequence was deposited in GenBank. The sequence was conducted Basic Local Alignment Search Tool (BLAST) in GenBank NCBI. The result would show the genera of the sequence. A phylogenetic tree was built containing this sequence, 40 sequences of *Neopestalotiopsis* species, and *Pestalotiopsis diversiseta* (outgroup) (Table 1). Clustal X Ver. 2.1 software was used to align the sequences and then saved as PHYLIP format files [16]. The phylogenetic tree of Randomized Axelerated Maximum Likelihood (RAxML) Black Box was generated on CIPRES [17]. Bootstrap analyses with 1000 replicates assessed the phylogenetic tree. Bootstrap (BS)≥40 was shown on the branch.

Table 1. The taxa used in this study

Species	Strain number	GenBank Acc. No.
		LSU
Neopestalotiopsis acrostichi	MFLUCC 17-1754 TYPE	MK764272
N. brachiata	MFLUCC 17-1555 TYPE	MK764274
N. petila	MFLUCC 17-1738 TYPE	MK764275
N. rhizophorae	MFLUCC 17-1550 TYPE	MK764277
N. sonneratae	MFLUCC 17-1745 TYPE	MK764279
N. thailandica	MFLUCC 17-1730 TYPE	MK764281
N. alpapicalis	MFLUCC 17-2544 TYPE	MK357772
N. aotearoa	CBS 367.54 TYPE	KM199369
N. asiatica	MFLUCC 12-0286 TYPE	JX398983
N. australis	CBS 114159 TYPE	KM199348
N. chrysea	MFLUCC 12-0261 TYPE	JX398985
N. clavispora	MFLUCC 12-0281 TYPE	JX398979
N. cocoes	MFLUCC 15-0152 TYPE	NR_156312
N. coffea-arabicae	HGUP4015 TYPE	KF412647
N. cubana	CBS 600.96 TYPE	KM199347
N. ellipsospora	MFLUCC 12-0283 TYPE	JX398980
N. egyptiaca	CBS 140162 TYPE	KP943747
N. eucalypticola	CBS 264.37 TYPE	KM199376
N. foedans	CGMCC 3.9123 TYPE	JX398987

Species	Strain number	GenBank Acc. No.
		LSU
N. formicarum	CBS 362.72 TYPE	KM199358
N. honoluluana	CBS 114495 TYPE	KM199364
N. iraniensis	CBS 137768 TYPE	KM074048
N. javaensis	CBS 257.31 TYPE	KM199357
N. keteleeria	MFLUCC 13-0915 TYPE	KJ503820
N. magna	MFLUCC 12-0652 TYPE	KF582795
N. mesopotamica	CBS 336.86 TYPE	KM199362
N. musae	MFLUCC 15-0776 TYPE	NR_156311
N. natalensis	CBS 138.41 TYPE	NR_156288
N. pernambucana	GS-2014 RV01 TYPE	KJ792466
N. piceana	CBS 394.48 TYPE	NR_163671
N. protearum	CBS 111506	MH553959
N. rosae	CBS 101057 TYPE	KM199359
N. rosicola	CFCC 51992 TYPE	KY885239
N. samarangensis	MFLUCC 12-0233 TYPE	JQ968609
N. saprophytica	MFLUCC 12-0282 TYPE	KM199345
N. steyaertii	IMI 192475 TYPE	KF582796
N. surinamensis	CBS 450.74 TYPE	KM199351
N. umbrinospora	MFLUCC 12-0285 TYPE	JX398984
N. vitis	MFLUCC 15-1265 TYPE	KU140694
N. zimbabwana	CBS 111495 TYPE	JX556231
N. zimbabwana	X1	MW422813
Pestalotiopsis diversiseta	MFLUCC 12-0287 TYPE	NR_120187

RESULTS AND DISCUSSION

Specimen description. Isolate X1 was identified as an endophytic fungus on the *Xylaria* stromata. The colony was white, cottony, and had circular appearances (Figure 1a). The whitish dot would be appeared when it was more than 6-days old colony. Then, the dot will be blackish when it was more than 10-days old colony. The conidia were produced from hyphae (Figure 1b). Conidia were 20.1–25.0 × 5.2–7.3 µm, fusiform to clavate, straight or erected, 5-cells. The conidia had a basal cell with one long appendage, hyalin, and conical form. The second cell had a young brown color. The third and fourth cells had a more brownish color than the second. The third cell had the form with more rectangular shape than other cells. Then, the fifth cell was hyalin, the same as the basal cell; nevertheless had 2-3 appendage cells. Every septates had a dark brown color; even the septate between the third and fourth cells had more brownish until black color. All cells had a smooth surface. The first and fifth cells had conic to acute form. The first and second cells had thin cell walls. But the other cells had thick cell walls. The first cell had 2.9-3.1 x 2.5-2.7 µm. The second cell had 4.3-4.9 x 4.0-5.2 µm. The third cell had 4.0-4.5 x 4.1-5.4 µm. The fourth had 4.2-4.8 x 3.9-5.1 µm. The fifth cell had 2.6-2.8 x 1.9-2.3 µm. The tubular appendages on the apical cell (fifth cell) were unbranched, flexuous, 21.2-34.4 µm. The basal cell had a single tubular appendage, with unbranched, centric, and 11.3-15.0 µm. Specimen examined: Strain number was X1 and isolated by Rudy Hermawan. GenBank accession number is MW422813.

Pestalotioid fungi have a unique character of the conidia shape. Therefore, the septated cell of conidia makes it easy to identify in their group. There were three groups that commonly be studied as *Pestalotiopsis*, *Pseudopestalotiopsis*, and *Neopestalotiopsis*. *Neopestalotiopsis* can be differed from *Pestalotiopsis* and *Pseudopestalotiopsis* from the versicolourous character of media cells [18]. To ensure the species of isolate X1 needed advanced analyses using molecular study.

The sequencing result was edited using Chromas Pro software. The assembled sequence would be used for the next analyses in BLAST GenBank NCBI. The BLAST result showed that isolate X1 was *Neopestalotiopsis* genus. From 10 top of BLAST results showed that isolate X1 was grouped in *Neopestalotiopsis*. Sometimes, the BLAST result was not really making sure for the species name. Therefore, the analyses should be continued to phylogenetic tree analyses.

Based on Figure 2, *Neopestalotiopsis* isolate X1 was identified as *Neopestalotiopsis zimbabwana* with a 46% BS value. This BS value was not really strong to ensure isolate X1 was *N. zimbabwana*. However, the clade showed that *Neopestalotiopsis* isolate X1 was *N. zimbabwana*. *N. zimbabwana* CBS 111495 as a TYPE isolate and *Neopestalotiopsis* isolate X1 was in the same line in a separated clade with other species. In this study, the phylogenetic tree was reconstructed using LSU (Large Subunit) region. *N. zimbabwana*

had a sister clade with other species such as *N. acrostichi*, *N. surinamensis*, *N. australis*, and *N. vitis*. Maharachchikumbura et al. [2] had also constructed the phylogenetic using the LSU region. The phylogenetic tree made the clade that was similar to Figure 2. Some *Neopestalotiospsis*, such as *N. australis* and *N. surinamensis*, were closed to *N. zimbabwana*.

The identification using molecular study usually needs many genes or regions to ensure the identification. Maharachchikumbura et al. [19] studied the taxonomy of *Pestalotiopsis* using multigene analyses or combining some genes. They used ten genes for molecular identification in *Pestalotiopsis* species. The result showed that the combined genes such as ITS, TUB, and TEF were useful for distinguishing the *Pestalotiopsis* species with a strong BS value in clades. In other genera such as *Neopestalotiopsis*, the multigene phylogenetic tree using ITS, TUB, and TEF was not enough to distinguish *Neopestalotiopsis* species with a strong BS value [2].

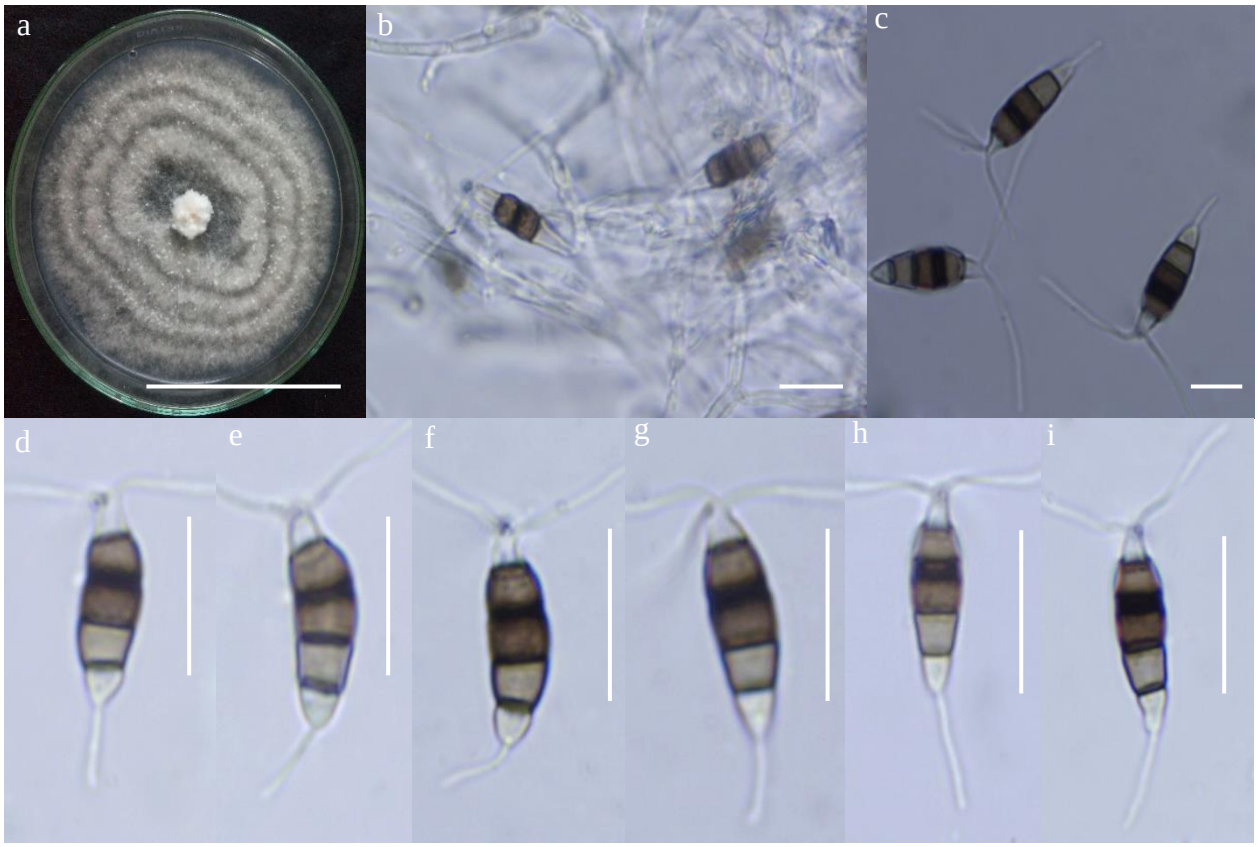

Figure 1. Pestalotioid fungus isolate X1. (a) Colony on PDA 7-days old; (b) Conidium produced in hyphae; (c) Conidia; (d-f) conidia shapes. Scale bars: (a) 3 cm; (b; c) 10 µm; and (d-i) 20 µm

According to Figure 2, the LSU gene was not enough to distinguish the *Neopestalotiopsis* species with a strong BS value. The continued research will do the specific identification using more gene markers. LSU gene was usually used for identification as a powerful identification gene. LSU was one of the genes that were translated and expressed to protein for phenotypic characters. Because *Neopestalotiopsis* showed a specific character with septated cell conidia, the LSU was assumed as a useful identification gene. The optional gene for identification also depended on the genera or group of the specimen. For example, *Trichaleurina* used LSU [16], whereas *Lentinus* used ITS [20]. For general species in the Fungi kingdom, the ITS region or gene was really useful for identification [21].

Neopestalotiopsis species were known as endophytic, parasitic, and saprobic fungi [2, 10]. Currently, some of them were most known as parasitic [22, 9, 18]. As an endophytic and parasitic fungus, *Neopestalotiopsis* had been reported that the host was the plant, not yet was reported from the mushroom. In this study, *Neopestalotiopsis* was isolated from *Xylaria* stromata.

Hermawan & Khairillah [23] found *Xylaria* sp. on the ground in IPB University. The *Xylaria* was tried to be isolated into PDA (1/2 recipe of Potato Dextrose). After the isolate grew, it was transferred into a normal PDA. The colony looked like the Ascomycota group with septate hyphae. Then, the morphological observation was done, and gotten the conidia with pestalotioid character. The isolation was done using surface sterilization as the

protocol in [15]. Andrews [15] evaluated surface sterilization using many doses of disinfectant. It made sure that the phyllosphere or exo-microbes outside stromata were eliminated by the process. Therefore, the microbes that can get inside of stromata would be available isolated into the medium.

This study was the first reported that *Neopestalotiospsis* had been isolated from mushroom or fungi, as *Xylaria* stromata. *Neopestalotiopsis zimbabwana* isolate X1 was endo-microbes as an endosymbiont. But, the trait was not observed yet. This study could only report the *Neopestalotiopsis* live as endosymbiont inside stromata of *Xylaria* sp.

CONCLUSION

Isolate X1 was *Neopestalotiopsis zimbabwana* isolated from *Xylaria* stromata as an endosymbiont. The identification used the Large Subunit region as a single gene analysis. The species had the unique characteristic of Pestalotioid fungi. *N. zimbabwana* isolate X1 had the septated cell of conidia with appendage.

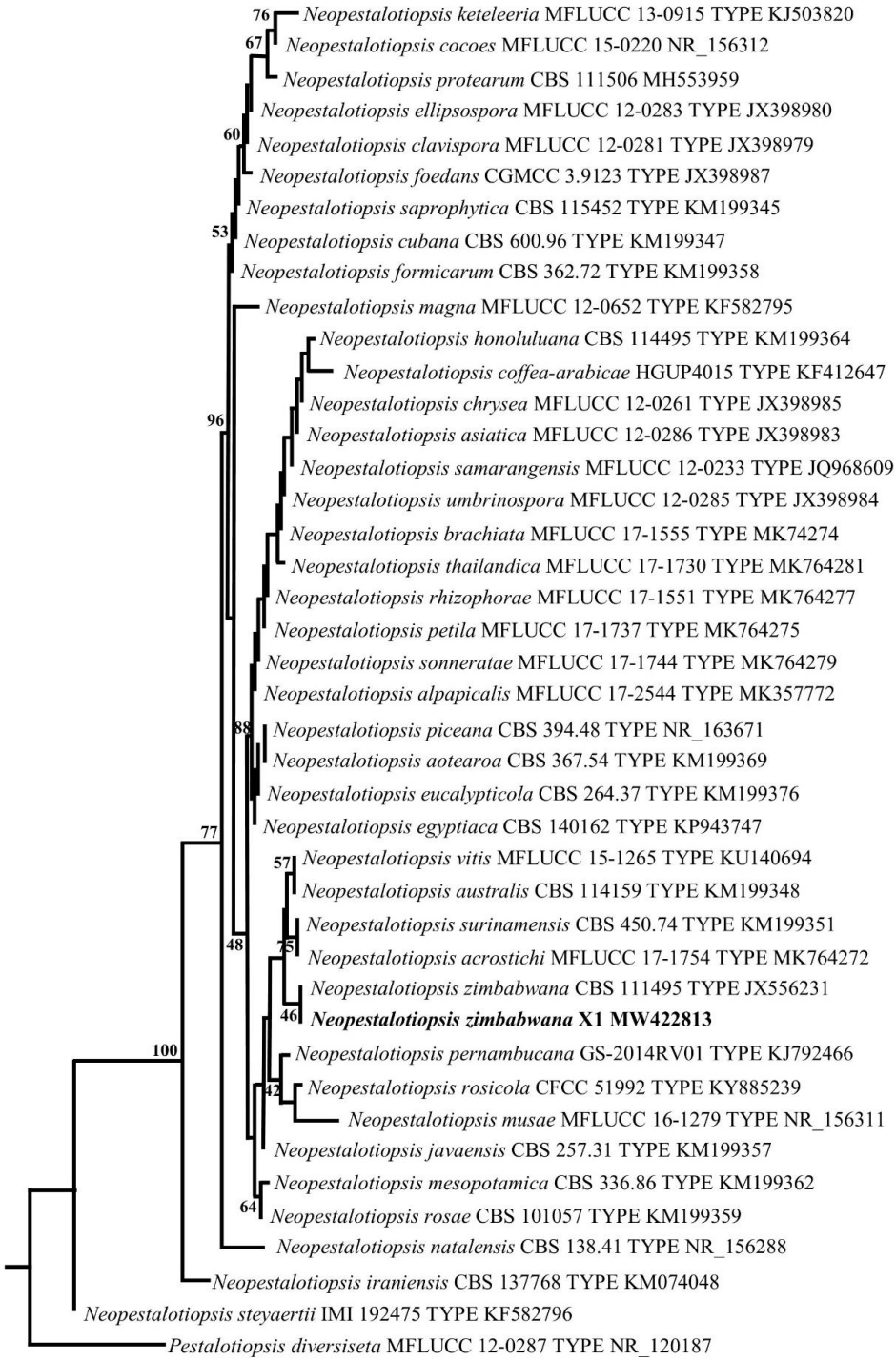

Figure 2. *Neopestalotiopsis* phylogenetic tree based on the ITS4/ITS5 region using RAxML. Bootstrap (BS)≥40 was shown on the branch. The *Neopestalotiopsis* strain X1 must be in bold.

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