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SHORT COMMUNICATION

Postharvest rot and mummification of strawberry fruits caused by *Neofusicoccum parvum* and *N. kwambonambiense* in Brazil

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ABSTRACT

In addition to the rots that are commonly found on strawberries, a new disease was found in 7% of stored fruits during a survey of strawberry diseases at the postharvest stage. Koch's postulates were satisfied, and the fungi were identified as *Neofusicoccum kwambonambiense* and *N. parvum* based on morphology and phylogenetic analysis of the internal transcribed spacers, β-tubulin, RNA polymerase subunit II and transcription elongation factor 1-α regions. This is the first report of *Neofusicoccum kwambonambiense* in Brazil and the first report of *Neofusicoccum* spp. causing mummification and postharvest rot of strawberry.
Key words: *Fragaria* x *ananassa*, Botryosphaeriales, Dothideomycetes, postharvest pathology, stored fruit, tropical fungi.

A major problem in the strawberry production chain is the occurrence of fruit rot caused by fungi, especially at the postharvest stage. In Brazil, the main fungal species that cause postharvest rot in strawberry are *Botrytis cinerea* Pers., *Rhizopus stolonifer* (Ehrenb.) Vuill., *Pilidium concavum* (Desm.) Höhn., *Geotrichum candidum* Link. and *Colletotrichum* spp. Others fungi associated to rotting of strawberry fruit at postharvest stage are *Rhizoctonia solani* Kühn, *Phytophthora* spp., *Sclerotinia sclerotiorum* (Lib.) de Bary, *Pestalotia longisetula* Guba., *Gnomonia comari* Karst. and *Alternaria* spp. (Costa et al., 2003; Tanaka et al., 2005; Lopes, 2011).

The occurrence of diseases in strawberries varies according to the region, climate conditions and crop management (Maas, 1998). Moreover, when the symptoms are similar to those caused by other pathogens, diagnostic errors are common. In such cases, the damage caused by certain pathogens may be ignored or overestimated depending on the situation (Lopes et al., 2010; Lopes, 2011). Therefore, the knowledge regarding the epidemiology and management of the disease depends of its correct etiologic identification such that postharvest damage to strawberry fruits can be reduced.

From March 2009 to February 2010, a survey of diseases in strawberry fruits was performed in the mountainous region of the state of Espírito Santo, Brazil. Fruits from different commercial plantations were collected and stored at 25ºC and 100% relative humidity for seven days. In addition to the rots that are commonly found on strawberries in postharvest, a new fruit rot was verified in 7% of 3,500 stored fruits. Because the symptoms of this postharvest rot have not been previously reported in strawberries, the aim of this research was to determine the etiology of this disease in *Fragaria* x *ananassa* based on morphological and molecular approaches.

The initial symptoms of rot were observed from the first to the fourth day of storage. Initially, the grayish-white mycelium grew, covering the entire fruit, which gradually became gray to black (Figure 1A, B). The fruits became mummified one week after the initial observation. The formation of droplets due to leakage of cellular liquid was commonly observed on the surface of affected fruits (Figure 1C). However, sporulation not was observed on the surface of infected fruits.

The fungi were isolated on potato dextrose agar (PDA) by removing the tip of the hyphae from infected fruits. Three isolates were transferred to 2% water agar medium containing sterilized corn straw to induce sporulation. Single spore isolates were obtained from the samples, and derived cultures were deposited at the "Coleção de Culturas de Fungos Fitopatogênicos Prof. Maria Menezes", Universidade Federal Rural de Pernambuco (UFRPE), Brazil (codes CMM 1842, CMM 1845 and CMM 1846). When the fungi were grown at 25ºC in petri dishes (9 cm in diameter) containing PDA medium, colonies of the CMM1842 covered the medium in the plates in three days whereas it took four days for CMM1845 and CMM 1846 to reach the plate borders. Although CMM 1842 exhibited a higher growth rate, both isolates exhibited maximum growth between 25 and 30ºC. Fungal structures were scraped, mounted in drops of lactophenol on microscope slides and examined under a light microscope. Biometric

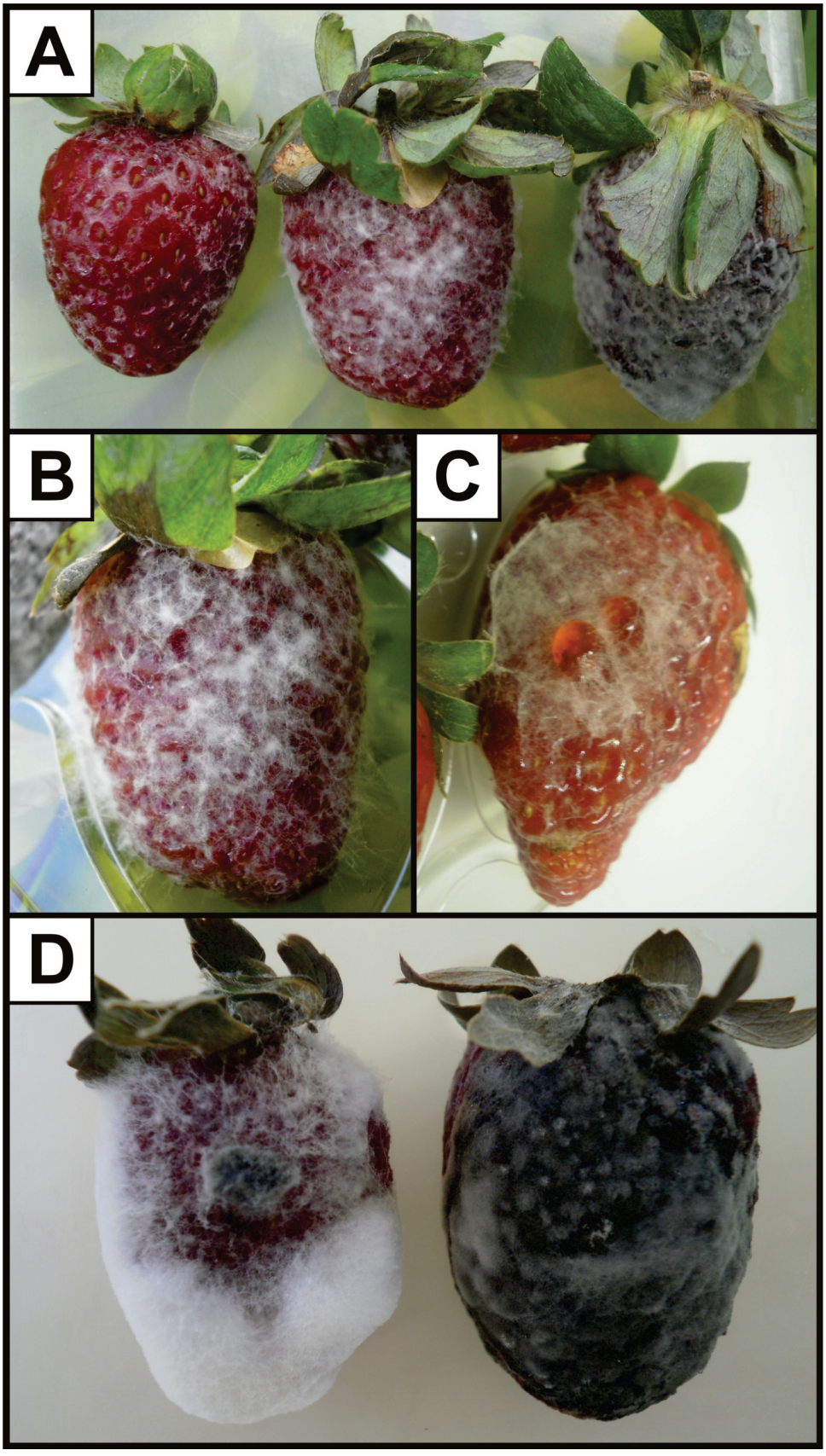

FIGURE 1 - A. Symptoms of rot caused by *Neofusicoccum* spp. at various stages of development; **B**. Mycelium of *Neofusicoccum* spp. on the surface of strawberry fruits; **C**. Extravasation of the cell liquid; **D**. Pathogenicity test showing rot at an early stage of development (left) and mummified fruit (right).

data were based on 30 measurements of various structures. The hyaline conidia were ellipsoidal to fusiform and aseptate with thin, smooth walls, measuring 7.5-20 × 2.5-5 µm. These morphological characteristics indicated that the pathogen belongs to the *Neofusicoccum parvum/N. ribis* complex (Pavlic et al., 2009a, 2009b). However, the species of this complex show conidial dimensions (length and width) that overlap among species, which cannot be distinguished based only on morphological characteristics (Pavlic et al., 2009b). Thus, a molecular characterization of the isolates was performed based on a combined analysis of the following sequences: internal transcribed spacer (ITS) regions 1 and 2 (including the 5.8S rRNA gene and 28S rDNA), β-tubulin, transcription elongation factor 1-α (TEF1-α) and RNA polymerase subunit II (RPB2) (Pavlic et al., 2009b).

To obtain representative fungal DNA, a single spore-derived culture from three selected isolates was grown on PDA at 25ºC for one week. The genomic material was extracted from approximately 40 mg of fungal tissue using the Wizard Genomic DNA Purification Kit (Promega) following the protocol described by Pinho et al. (2012).

The target sequences of the ITS region, 28S rDNA, β-tubulin, TEF1-α and RPB2 were amplified using the primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') for ITS (White et al., 1990), LR0R (5'-ACC CGC TGA ACT TAA GC-3') and LR5 (5'-TCC TGA GGG AAA CTT CG-3') for the partial 28S rDNA (Vilgalys & Hester, 1990), T1 (5'-AAC ATG CGT GAG ATT GTA AGT-3') and Bt2b (5'-ACC CTC AGT GTA GTG ACC CTT GGC-3') for the partial β-tubulin (Glass & Donaldson, 1995; O'Donnell & Cigelnik, 1997), EF1F (5'-TGC GGT GGT ATC GAC AAG CGT-3') and EF2R (5'-AGC ATG TTG TCG CCG TTG AAG-3') for the partial TEF1-α (Jacobs et al., 2004) and 5F2 (5'-GGG GWG AYC AGA AGA AGG C-3)' and 7cR (5'-CCC ATR GCT TGY TTR CCC AT-3') for the partial RPB2 (Sung et al., 2007; Liu et al., 1999) following the protocols of Pinho et al. (2013) and Sung et al. (2007). Amplification of ITS, β-tubulin, TEF1-α and RPB2 produced sequences of approximately 550, 630, 680 and 860 bp, respectively. The nucleotide sequences were edited with BioEdit software (Hall, 2012), and the new sequences were deposited in GenBank. ITS, TEF1-α, β-tubulin and RPB2 sequences from additional species were retrieved from GenBank (Table 1).

Consensus regions were compared with the GenBank database using the MegaBLAST program. The

TABLE 1 - GenBank accession numbers of *Neofusicoccum* spp. DNA sequences used in the phylogenetic analysis.

Species	Isolate	ITS	BT	EF	RPB2
N. parvum	CMM1846	KC507812	KC507806	KC507809	KC507803
N.kwambonambiense	CMM1842	KC507813	KC507807	KC507810	KC507804
N. parvum	CMM1845	KC507814	KC507808	KC507811	KC507805
N. parvum	CMW9071	AY236938	AY236909	AY236880	EU339571
N. parvum	MUCC676	EU339545	EU339482	EU339519	EU339568
N. parvum	CMW9080	AY236942	AY236916	AY236887	EU339572
N. parvum	MUCC211	EU301017	EU339480	EU339517	EU339566
N. parvum	MUCC673	EU339553	EU339483	EU339520	EU339570
N.kwambonambiense	MUCC210	EU301016	EU339478	EU339515	EU339564
N.kwambonambiense	MUCC157	EU339522	EU339479	EU339516	EU339522
N.kwambonambiense	CMW14123	EU821924	EU821864	EU821894	EU821954
N. cordaticola	CMW14124	EU821925	EU821865	EU821895	EU821955
N. cordaticola	CMW14056	EU821903	EU821843	EU821873	EU821933
N. cordaticola	CMW14054	EU821906	EU821846	EU821876	EU821936
N. batangarum	CMW28320	FJ900608	FJ900635	FJ900654	FJ900616
N. batangarum	CMW28637	FJ900609	FJ900636	FJ900655	FJ900617
N. umdonicola	CMW14079	EU821915	EU821855	EU821885	EU821945
N. umdonicola	CMW14127	EU821926	EU821866	EU821896	EU821956
N. umdonicola	CMW14096	EU821913	EU821853	EU821883	EU821943
N. ribis	CMW7772	AY236925	AY26906	AY236877	EU339554
N. ribis	CMW7773	AY236936	AY236907	AY236878	EU339555
N. occulatum	MUCC232	EU301031	EU339473	EU339510	EU339559
N. occulatum	MUCC270	EU339529	EU339471	EU339508	EU339557
N. occulatum	MUCC296	EU301034	EU339475	EU339512	EU339561
N. karanda	MUCC247	EU301028	EU339476	EU339513	EU339562
N. karanda	MUCC125	EU339525	EU339477	EU339514	EU339563
N. australe	CMW6837	AY339262	AY339254	AY339270	EU339573
N. australe	CMW9072	AY339260	AY339252	AY339268	

closest hit sequences were aligned using the multiple sequence alignment program MUSCLE (Edgar, 2004) and built in MEGA v.5 software (Tamura et al., 2011). All of the ambiguously aligned regions within the dataset were excluded from the analyses. Gaps (insertions/deletions) were treated as missing data. The resulting alignment was deposited in TreeBASE (http://www.treebase.org/) under accession number S13850. The manually adjusted alignment contained 28 strains and, of the 1781 characters used in the phylogenetic analysis, 113 were parsimony-informative, 125 were variable and 1655 were conserved. Although the 28S rDNA sequences were not used in the phylogenetic analyses, they were deposited in GenBank (accession nos. KC507815 and KC507816).

Bayesian inference (BI) concatenated analyses employing a Markov Chain Monte Carlo method were performed with all sequences, first with each gene/locus separately and later with the concatenated sequences (ITS, TEF1-α, β-tubulin and RPB2). Before launching the BI, the best nucleotide substitution models were determined for each gene with MrMODELTEST 2.3 (Posada & Buckley, 2004). Once the likelihood scores were calculated, the models were selected according to the Akaike Information Criterion (AIC). The HKY+I model of evolution was used for ITS and TEF1-α, whereas GTR+I was used for RPB2 and β-tubulin. The phylogenetic analysis of the concatenated alignment was performed on the CIPRES web portal (Miller et al., 2010) using MrBayes v.3.1.1 (Ronquist & Huelsenbeck, 2003). The remainder of the phylogenetic analysis was conducted as described by Pinho et al. (2012).

The BI analysis showed that the CMM 1842 isolate belongs to *N. kwambonambiense*, whereas CMM 1845 and CMM 1846 belong to *N. parvum* (Figure 2). *Neofusicoccum parvum* infects a broad range of hosts in a wide range of botanical families (Pavlic et al., 2009a, 2009b; Sakalidis et al., 2011; Farr & Rossman 2013), and in Brazil, it was recently reported as a pathogen in mango (Costa et al., 2010; Marques et al., 2013). *Neofusicoccum kwambonambiense* has been described in *Corymbia*

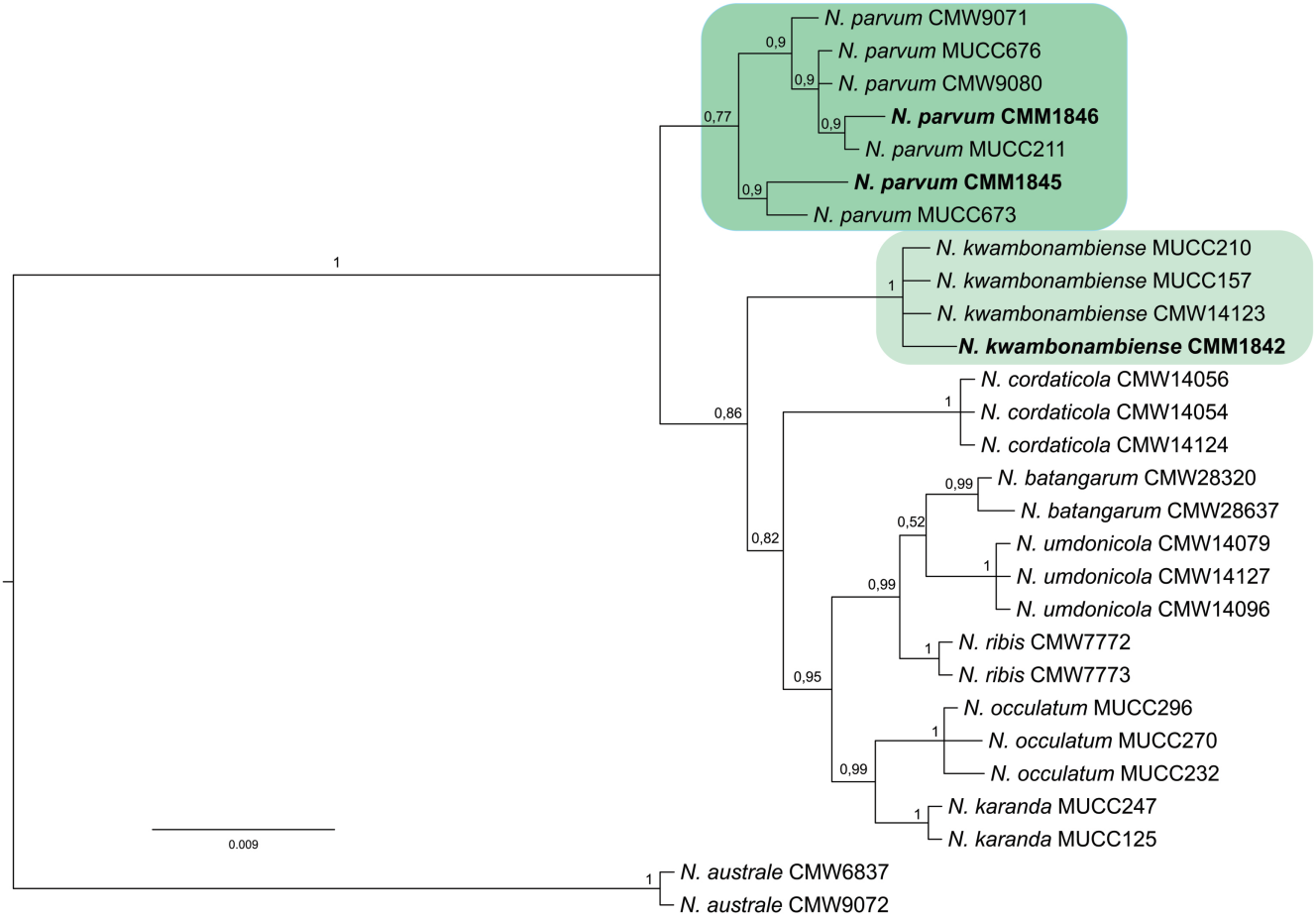

FIGURE 2 - Multilocus phylogenetic tree inferred from Bayesian analysis using the ITS, β-tubulin, EF-1α and RPB2 regions. Bayesian posterior probability values are indicated at the nodes. Strain numbers are indicated after species names, and the specimens used in this study are highlighted in bold. *Neofusicoccum australe* represents the outgroup taxon.

torelliana, *Eucalyptus dunnii* and *Syzygium cordatum* from South Africa (Pavlic et al., 2009a, 2009b; Sakalidis et al., 2011; Farr & Rossman, 2013) but has not yet been reported in Brazil (Mendes & Urben, 2013).

Fungi of the *Neofusicoccum* genus have not been reported to infect strawberry. Only the *Fusicoccum*-like anamorphic fungus *Botryosphaeria obtusa* (Schwein.) Shoemaker (teleomorph: Botryosphaeria-like) is known to cause fruit blotch in strawberry (Gubler & Converse, 1993); however, the fungus found in this research is morphologically distinct from *B. obtusa* (Shoemaker, 1964). In addition, a MegaBLAST search of the nucleotide sequence database (GenBank) using the ITS sequence show that our isolates from strawberries have only 87% identity with *B. obtusa* and belong to *Neofusicoccum*, a genus recently separated from *Botryosphaeria* (Crous et al., 2006).

To verify the pathogenicity of the fungus, 6-mm-diameter plugs of the isolates CMM 1842, CMM 1845 and CMM 1846 (single spore-derived cultures) from three-day-old cultures grown on PDA were inoculated by depositing over the intact surface of sixty fruits (twenty for each isolate) of strawberry fruits of the cultivar "Camarosa", which is widely cultivated in Brazil. An agar plug was deposited in twenty control fruits. The fruits were maintained at 25ºC in plastic trays with individual compartments. Inoculated fruits showed symptoms that were similar to those initially observed, i.e., mycelial growth was observed on the fruit surface at day one, and mummified fruits were observed at seven days after inoculation (Figure 1D) in all inoculated fruits. The control fruits showed no symptoms. *Neofusicoccum* spp. was successfully reisolated from the symptomatic fruits.

This is the first report of *N. kwambonambiense* in Brazil and the first report of *Neofusicoccum* spp. causing postharvest rot and mummification of strawberries. The presence of both species represents new threats to strawberries crop in tropical areas where the climate favors their development. Therefore, control strategies should be investigated, especially for *N. parvum*, which causes serious damage to others important crops (Pavlic et al., 2009a, 2009b; Sakalidis et al., 2011; Farr & Rossman, 2013).

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REFERENCES

Cai L, Udayanga D, Manamgoda DS, Maharachchikumbura SSN, McKenzie EHC, Guo LD, Liu XZ, Bahkali A, Hyde KD (2011) The need to carry out re-inventory of plant pathogenic fungi. Tropical Plant Pathology 36:205-213.

Costa VSO, Michereff SJ, Martins RB, Gava CAT, Mizubuti ESG, Camara MPS (2010) Species of Botryosphaeriaceae associated on mango in Brazil. European Journal Plant Pathology 127:509-519.

Costa H, Zambolim L, Ventura JA (2003) Manejo integrado das doenças do morangueiro. In: Zambolim L (Ed.) Manejo Integrado de Pragas e Doenças: Fruteiras Tropicais. Visconde do Rio Branco, MG. Suprema Gráfica e Editora. pp.131-164

Crous PW, Slippers B, Wingfield MJ, Rheeder J, Marasas WFO, Phillips AJL, Alves A, Burgess T, Barber P, Groenewald JZ (2006) Phylogenetic lineages in the Botryosphaeriaceae. Studies in Mycology 55:235-253.

Edgar RC (2004) MUSCLE: Multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 32:1792-1797.

Farr DF, Rossman AY (2013) Fungal Databases, Systematic Mycology and Microbiology Laboratory, ARS, USDA. Available at: http://nt.ars-grin.gov/fungaldatabases/. Acessed on May 5, 2013.

Glass NL, Donaldson GC (1995) Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. Applied and Environmental Microbiology 61:1323-1330.

Gubler WD, Converse RH (1993) Diseases of strawberry (*Fragaria* × *ananassa* Duch.) Available at: http://www.apsnet.org/publications/commonnames/Pages/Strawberry.aspx Acessed on May 23, 2013.

Hall T (2012) BioEdit v7.0.9: Biological sequence alignment editor for Win95/98/2K/XP/7. Available at: http://www.mbio.ncsu.edu/bioedit/bioedit.html Acessed on July 15, 2012.

Jacobs K, Bergdahl DR, Wingfield MJ, Halik S, Seifert KA, Bright DE, Wingfield BD (2004) *Leptographium wingfieldii* introduced into North America and found associated with exotic *Tomicus piniperda* and native bark beetles. Mycological Research 108:411-418.

Liu YJ, Whelen S, Hall BD (1999) Phylogenetic relationships among ascomycetes: Evidence from an RNA polymerase II subunit. Molecular Biology and Evolution 16:1799-1808.

Lopes UP (2011) Podridões em pós-colheita de morango: Etiologia e efeito de produtos a alternativos. MSc Dissertation, Universidade Federal de Viçosa. Viçosa, MG.

Lopes UP, Zambolim, Lopes UN, Pereira OL, Costa H (2010) First report of *Pilidium concavum* causing tan-brown rot in strawberry fruits in Brazil. Plant Pathology 59:1171-1172.

Maas JL (1998) Compendium of strawberry diseases. St Paul, MN, USA. APS Press.

Marques MW, Lima NB, Morais MA, Michereff SJ, Phillips AJL, Câmara MPS (2013) *Botryosphaeria*, *Neofusicoccum*, *Neoscytalidium* and *Pseudofusicoccum* species associated with mango in Brazil. Fungal Diversity 61:195-208.

Mendes MAS, Urben AF (2013) Fungos relatados em plantas no Brasil Available at: http://pragawall.cenargen.embrapa.br/aiqweb/michtml/fgbanco01.asp Acessed on May 5, 2013.

Miller MA, Pfeiffer W, Schwartz T (2010) Creating the CIPRES science gateway for inference of large phylogenetic trees. In: Gateway Computing Environments Workshop (GCE), Proceedings... New Orleans, LA, USA. pp. 1-8.

O'Donnell K, Cigelnik E (1997) Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are non-orthologous. Molecular Phylogenetics and Evolution 7:103-116.

Pavlic D, Slippers B, Coutinho TA, Wingfield MJ (2009a) Multiple gene genealogies and phenotypic data reveal cryptic species of the Botryosphaeriaceae: A case study on the *Neofusicoccum parvum/N. ribis* complex. Molecular Phylogenetics and Evolution 51:259-268.

Pavlic D, Slippers B, Coutinho TA, Wingfield MJ (2009b) Molecular and phenotypic characterization of three phylogenetic species discovered within the *Neofusicoccum parvum/N. ribis* complex. Mycologia 101:636-647.

Pinho DB, Dutra DC, Pereira OL (2013) Notes on *Ceratocystis paradoxa* causing internal post-harvest rot disease on immature coconut in Brazil. Tropical Plant Pathology 38:152-157.

Pinho DB, Firmino AL, Pereira OL, Ferreira Junior WG (2012) An efficient protocol for DNA extraction from *Meliolales* and the description of *Meliola centellae* sp. nov. Mycotaxon 122:333-345.

Posada D, Buckley TR (2004) Model selection and model averaging in phylogenetics: Advantages of Akaike information criterion and Bayesian approaches over likelihood ratio tests. Systematic Biology 53:793-808.

Ronquist F, Heulsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572-1574.

Sakalidis ML, Hardy GESJ, Burgess T (2011) Use of the Genealogical Sorting Index (GSI) to delineate species boundaries in the *Neofusicoccum parvum-Neofusicoccum ribis* species complex. Molecular Phylogenetics and Evolution 60:333-344.

Shoemaker RA (1964) Conidial states of some *Botryosphaeria* species on *Vitis* and *Quercus*. Canadian Journal of Botany 42:1297-1301.

Sung GH, Sung JM, Hywel Jones NL, Spatafora JW (2007) A multi-gene phylogeny of *Clavicipitaceae* (Ascomycota, Fungi): Identification of localized incongruence using a combinational bootstrap approach. Molecular Phylogenetics and Evolution 44:1204-1223.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular evolutionary genetics analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution 28:2731-2739.

Tanaka MAS, Betti JA, Kimati H (2005) Doenças do morangueiro. In: Kimati H, Amorim L, Rezende JAM, Bergamin Filho A, Camargo LEA (Eds.) Manual de Fitopatologia: Doenças das Plantas Cultivadas. São Paulo, SP. Agronômica Ceres. pp. 489-499.

Vilgalys R, Hester M (1990) Rapid genetic identification and mapping of enzymatically amplifed ribosomal DNA from several *Cryptococcus* species. Journal of Bacteriology 172:4239-4246.

White TJ, Bruns T, Lee S, Taylor J (1990) Amplifcation and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (Eds.) PCR Protocols: A guide to Methods and Applications. San Diego, CA, USA. Academic Press. pp. 315-322.

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