Diversity of *Neopestalotiopsis* and *Pestalotiopsis* spp., Causal Agents of Guava Scab in Colombia

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Abstract

Common guava (*Psidium guajava* L.) is a fruit tree of global economic importance. It is grown in Asia, South and Central America, and Hawaii for its exquisite aroma and flavor, and nutritional and medical properties. However, guava production is limited by guava scab, caused by fungi in the *Pestalotiopsis* genus. Characteristic symptoms of guava scab are corky, ovoid or round lesions on fruit surfaces. These lesions may thicken, affecting the flesh below and reducing fruit quality and commercial value. We characterized 81 isolates isolated from guava scab lesions on guava leaves and fruit in different regions of Colombia, and identified them as *Pestalotiopsis*

and *Neopestalotiopsis* spp. We analyzed the morphology, pathogenicity, and genetic diversity of the isolates based on the sequences of the internal transcribed spacer, β -tubulin, and elongation factor genes. Isolates were morphologically, pathogenically, and genetically diverse but the diversity did not correlate with geographical origin, or guava cultivar or tissue from which the isolates were recovered. Selected monosporic isolates included in the multiple-gene analysis were identified as belonging to two genera: *Neopestalotiopsis* (65 isolates with versicolorous conidia) and *Pestalotiopsis* (4 isolates with concolorous conidia).

Guava (*Psidium guajava* L.) is a small tree that belongs to the genus *Psidium* (Gutiérrez et al. 2008). The center of origin for guava extends from Mexico to Peru but it is cultivated in most tropical and subtropical countries (Gutiérrez et al. 2008). Guava grows in soils of differing textures, drainage, and pH (between 4.5 and 9.4). It can grow in regions with high mean annual rainfall or in areas subject to drought (Keith et al. 2006).

In Colombia, guava is usually consumed fresh or processed into a firm, sweet pulp known as *bocadillo* that is used for juices and jams (Melgarejo et al. 2010). The fruit is a good source of calcium, iron, and phosphorus (Pachanawan et al. 2008). It also provides vitamin A, ascorbic acid (vitamin C), three essential amino acids (tryptophan, lysine, and methionine), and three vitamin B compounds (thiamine or B1, riboflavin or B2, and niacin or B3) (Gutiérrez et al. 2008). In Colombia, guava production averaged 1.3 million tons per year over the past 5 years (Romero-Frías et al. 2015).

Guava production is socioeconomically important in Colombia, providing more than 4,800 jobs in places such as La Hoya del Río Suárez, Colombia (Rodríguez-Borray and Rangel-Moreno 2005). Guava is an important commodity for domestic markets but also has great export potential. However, guava production is subject to various production constraints, including pests and diseases. Guava scab, also known as pestalotiopsis or nailhead rust, is the most important disease constraint to guava cultivation in Colombia, affecting fruit, leaves, and shoots (Buriticá-Céspedes 1999; Keith et al. 2006). It is caused by a group of fungi in the genus *Pestalotiopsis*. The first visible symptoms of guava scab are small brown (coffee-bean colored) spots that develop into corky scabs on fruit surfaces. The scabs develop “heads” that resemble oxidized nails. The lesions coalesce to form large lesions that cover part of the fruit and sink into the flesh and, eventually, deform the whole fruit. Leaf symptoms appear as brown lesions that usually show up first on leaf edges and apices. As they expand, lesions become brittle and gray with mycelium growth.

Farfán et al. (2006) reported that *Pestalotia* spp. are widely distributed throughout La Hoya del Río Suárez, Colombia. They also found

that, in the municipality of Vélez, guava fruit are most susceptible to infection in the transition periods between dry and rainy seasons in March (90.6%) and between rainy and dry in July (93.1%), (Farfán et al. 2006). Guava scab has also been reported in Mexico (Morera and Blanco 2009), India, Australia (Jeewon et al. 2003), and the United States (Hawaii) (Keith et al. 2006).

The causal agents of guava scab in Colombia are *Pestalotia* spp. (Morera and Blanco 2009) and *Pestalotiopsis* spp. (Keith et al. 2006). However, there have been no efforts to characterize and identify the *Pestalotiopsis* spp. attacking the guava crop in Colombia. Additionally, the pathogen’s distribution in Colombia’s principal production areas has not been determined. In general, the guava scab pathogens have been characterized by their morphological features and not by molecular techniques such as multiple-gene analysis. De Notaris (1839) initially described the genus *Pestalotia*, characterizing it as having a fusiform conidium composed of six cells, and appendages in the apical and basal extremes. Over a century later, Steyaert (1955) divided the genus into three genera according to the number of cells comprising the conidia; specifically, conidia of *Pestalotia*, *Pestalotiopsis*, and *Truncatella* genera possessed six, five, and four cells, respectively. This last classification is still in current use after more than 60 years (Maharachchikumbura et al. 2011). However, the separation of *Pestalotiopsis* and *Pestalotia* into distinct genera remained controversial until 1980, when Sutton (1980) used electron microscopy to examine the development of the cell wall in two species of *Pestalotiopsis* and in *Pestalotia pezizoides*. Sutton’s findings supported Steyaert’s classification (Griffiths and Swart 1974a,b).

Until the 1990s, the taxonomy of *Pestalotiopsis* and related genera was based on describing conidial characteristics because of the stability of their traits. Specifically, the pigmentation of the three medium cells of the conidia are concolorous in *Pestalotiopsis* and versicolorous in *Neopestalotiopsis* (Maharachchikumbura et al. 2011, 2012). However, the sole use of conidial characteristics for species identification was controversial because of the wide variability of other morphological characteristics such as colony color, texture, and shape observed for *Pestalotiopsis* and related genera when grown on culture media (Egger 1995; Hu et al. 2007).

Molecular techniques have become the norm for species differentiation and identification. Maharachchikumbura et al. (2012) evaluated 10 groups of primers, seeking the best regions or genes on which to perform phylogenetic analyses of multiple, replicable, and reliable genes. The authors succeeded in establishing the internal transcribed spacer (ITS) region and the β -tubulin and translation elongation 1 (*tef1*) genes as the best regions because of greater amplification

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Fig. 1. Sampling areas from which tissues of guava (*Psidium guajava* L.), infested with scab were obtained.

Table 1. Details of *Pestalotiopsis* and *Neopestalotiopsis* isolates obtained from guava fruit and leaves collected from Boyacá, Santander, and Valle del Cauca in Colombia included in this study

Species	Isolate code	Origin	Source	Genotype	Growth rate (mm) ^z
<i>Neopestalotiopsis</i>	SSbu1	San Benito, Santander	Fruit	Roja	16.9
<i>Neopestalotiopsis</i>	SVsnp8	Vélez, Santander	Leaf	Roja	16.7
<i>Neopestalotiopsis</i>	VTman1	Toro, Valle	Fruit	Pera	16.7
<i>Neopestalotiopsis</i>	VUve3	La Unión, Valle	Fruit	Pera	16.5
<i>Neopestalotiopsis</i>	SVsnp3	Vélez, Santander	Leaf	Roja	15.6
<i>Neopestalotiopsis</i>	SVpa1	Vélez, Santander	Leaf	Roja	15.6
<i>Neopestalotiopsis</i>	BVayr1	Pauna, Boyacá	Fruit	Roja	15.4
<i>Neopestalotiopsis</i>	VTman2	Toro, Valle	Fruit	Pera	15.3
<i>Neopestalotiopsis</i>	VTman3	Toro, Valle	Leaf	Pera	15.3
<i>Neopestalotiopsis</i>	SBma1	Barbosa, Santander	Fruit	Roja	15.1
<i>Neopestalotiopsis</i>	SPugra2	P. Nacional, Santander	Fruit	Roja	15.1
<i>Neopestalotiopsis</i>	SVpo3	Vélez, Santander	Fruit	Roja	15
<i>Neopestalotiopsis</i>	SVsnp11	Vélez, Santander	Fruit	Roja	15
<i>Neopestalotiopsis</i>	VUve2	La Unión, Valle	Fruit	Coronilla	14.9
<i>Neopestalotiopsis</i>	SBac2	Barbosa, Santander	Leaf	Roja	14.9
<i>Neopestalotiopsis</i>	VRes2	Roldanillo, Valle	Fruit	Manzana	14.9
<i>Neopestalotiopsis</i>	SPugra1	P. Nacional, Santander	Fruit	Roja	14.8
<i>Neopestalotiopsis</i>	SVpa4	Vélez, Santander	Fruit	Roja	14.7
<i>Neopestalotiopsis</i>	SVpo7	Vélez, Santander	Leaf	Roja	14.7
<i>Neopestalotiopsis</i>	SPugra5	P. Nacional, Santander	Fruit	Roja	14.6
<i>Neopestalotiopsis</i>	BPpa2	Pauna, Boyacá	Fruit	Roja	14.5
<i>Neopestalotiopsis</i>	SVpo5	Vélez, Santander	Leaf	Roja	14.4
<i>Pestalotiopsis</i>	VTsin3	Toro, Valle	Leaf	Pera	14.4
<i>Neopestalotiopsis</i>	VRte1	Roldanillo, Valle	Leaf	Pera	14.4
<i>Neopestalotiopsis</i>	SSbu2	San Benito, Santander	Fruit	Roja	14.1
<i>Neopestalotiopsis</i>	SSre5	San Benito, Santander	Leaf	Roja	14.0
<i>Neopestalotiopsis</i>	SVsnp12	Vélez, Santander	Fruit	Roja	13.8
<i>Neopestalotiopsis</i>	SVpa3	Vélez, Santander	Fruit	Roja	13.7
<i>Neopestalotiopsis</i>	SVsnp2	Vélez, Santander	Fruit	Roja	13.7
<i>Neopestalotiopsis</i>	SVsnp7	Vélez, Santander	Fruit	Roja	13.7
<i>Neopestalotiopsis</i>	BPva1	Pauna, Boyacá	Fruit	Roja	13.6
<i>Neopestalotiopsis</i>	SVsnp1	Vélez, Santander	Leaf	Roja	13.6
<i>Neopestalotiopsis</i>	VRes4	Roldanillo, Valle	Fruit	Manzana	13.5
<i>Pestalotiopsis</i>	VTman5	Toro, Valle	Fruit	Pera	13.5
<i>Neopestalotiopsis</i>	BTca2	Tununguá, Boyacá	Fruit	Roja	13.3
<i>Neopestalotiopsis</i>	SVpo2	Vélez, Santander	Fruit	Roja	13.3
<i>Neopestalotiopsis</i>	VUgy	La Unión, Valle	Fruit	Pera	13.2
<i>Neopestalotiopsis</i>	SVsnp9	Vélez, Santander	Fruit	Roja	13.1
<i>Pestalotiopsis</i>	VTsin2	Toro, Valle	Leaf	Pera	13.1
<i>Neopestalotiopsis</i>	SVpa8	Vélez, Santander	Fruit	Roja	12.9

(Continued on next page)

^z Rate of growth on potato dextrose agar: high = 14.4 to 16.9, medium = 12.1 to 14.1, and low 5.8 to 11.7 mm.

through polymerase chain reaction (PCR) and sharper definition of species limits.

Recently, Maharachchikumbura et al. (2014) reclassified *Pestalotiopsis* into three genera. The sequence analyses of several species based on the large subunit gene, together with the results of the 2012 multiple-gene analyses, clearly show three highly diverse lineages. Therefore, two new genera—*Neopestalotiopsis* and *Pseudopestalotiopsis*—were introduced. The objective of this study was to characterize and assess the genetic diversity of the isolates causing guava scab in Colombia.

Materials and Methods

Sampling, isolation, and storage. Samples of infected guava tissues (leaves and fruit) were collected from commercial crops in the Colombian regions of Boyacá, Santander, and Valle del Cauca (Fig. 1). These regions produce different guava varieties with different crop management and edaphoclimatic conditions.

We obtained infected guava leaf and fruit samples ($n = 200$) from 45 farmers' fields. Samples had symptoms that included spots that were blackish gray, necrotic, amorphous, and brittle, and were usually located on leaf apices; or round to oval, brown, corky lesions that were located on the fruit epidermis. The symptomatic guava varieties that were sampled included Palmira ICA-1, Pera, Coronilla, Regional Roja, and Manzana.

Samples (5 mm²) were washed first in deionized water for 10 min, followed by 1 min in 1% sodium hypochlorite and 1 min in 70% alcohol. Subsequently, samples were washed twice with sterilized distilled water for 1 min. Samples (5 mm²) were then dried on sterilized paper towels, and plated onto potato dextrose agar (PDA; Difco Laboratories

Inc.) modified with 25% lactic acid at 1 ml/liter (Merck & Co. Inc.) (five samples per plate). Plates were incubated at 22°C ± 1 under 12 h of light and 12 h of darkness for 5 days.

To obtain single-spore cultures, we started with 8- to 10-day-old colonies. One acervulus from each colony was picked up using a fine needle and washed into a Petri dish containing water agar with one drop of sterilized distilled water. Drops of water carrying acervuli were then spread over the entire surface of the medium, using a sterilized glass spreader. A stereomicroscope was used to identify one conidium physically separated from the others. A fragment of the culture medium carrying this conidium was transferred to a plate containing fresh PDA medium supplemented with 25% lactic acid at 1 ml/liter (Merck & Co., Inc.). The colonies were incubated at 22°C ± 1 under 12 h of light and 12 h of darkness for 1 to 2 days.

Morphological characterization. We characterized 81 monoconidial isolates from different acervuli of *Pestalotiopsis* spp. based on morphological features. From the edges of 5-day-old colonies, discs (5 mm in diameter) were transferred to PDA plates and incubated at 22°C under 12 h of light and 12 h of darkness for 7 days. The morphological characteristics of these cultures were examined 7 days later. Specifically, cultures were examined for colony color, growth rate, presence or absence of acervuli, conidia length and width, apical and basal appendage length, and other characteristics, as described by Keith et al. (2006) and Maharachchikumbura et al. (2011). To determine the growth rate, isolates were cultured in 90-mm Petri dishes containing PDA in a randomized complete block design with three replications. The experiment was repeated twice. Disks (5 mm in diameter) were cut from the edge of a 5-day-old colony and placed in the center of Petri dishes with

Table 1. (Continued from previous page)

Species	Isolate code	Origin	Source	Genotype	Growth rate (mm) ²
<i>Neopestalotiopsis</i>	VUve1	La Unión, Valle	Fruit	Pera	12.8
<i>Neopestalotiopsis</i>	BPca2	Pauna, Boyacá	Fruit	Roja	12.7
<i>Neopestalotiopsis</i>	SSre3	San Benito, Santander	Leaf	Roja	12.7
<i>Neopestalotiopsis</i>	SSbu3	San Benito, Santander	Fruit	Roja	12.7
<i>Neopestalotiopsis</i>	SSre2	San Benito, Santander	Leaf	Roja	12.7
<i>Neopestalotiopsis</i>	VUgu1	La Unión, Valle	Fruit	Pera	12.6
<i>Neopestalotiopsis</i>	SVpo1	Vélez, Santander	Fruit	Roja	12.5
<i>Neopestalotiopsis</i>	SVsnp13	Vélez, Santander	Fruit	Roja	12.5
<i>Neopestalotiopsis</i>	VTman6	Toro, Valle	Leaf	Pera	12.5
<i>Neopestalotiopsis</i>	SVpo6	Vélez, Santander	Fruit	Roja	12.3
<i>Neopestalotiopsis</i>	SVsnp6	Vélez, Santander	Fruit	Roja	12.3
<i>Neopestalotiopsis</i>	VTsin1	Toro, Valle	Leaf	Pera	12.2
<i>Neopestalotiopsis</i>	SVpa7	Vélez, Santander	Fruit	Roja	12.2
<i>Neopestalotiopsis</i>	SVsnp5	Vélez, Santander	Leaf	Roja	12.1
<i>Neopestalotiopsis</i>	SVpo2-2	Vélez, Santander	Fruit	Roja	12.1
<i>Neopestalotiopsis</i>	BPca1	Pauna, Boyacá	Fruit	Roja	11.7
<i>Neopestalotiopsis</i>	SVpa6	Vélez, Santander	Fruit	Roja	11.7
<i>Neopestalotiopsis</i>	SBac1	Barbosa, Santander	Leaf	Roja	11.7
<i>Neopestalotiopsis</i>	SBma3	Barbosa, Santander	Fruit	Roja	11.6
<i>Pestalotiopsis</i>	SSpla	San Benito, Santander	Fruit	Roja	11.6
<i>Neopestalotiopsis</i>	VRes1	Roldanillo, Valle	Fruit	Manzana	11.5
<i>Neopestalotiopsis</i>	VUve4	La Unión, Valle	Fruit	Pera	11.5
<i>Neopestalotiopsis</i>	SVpa5	Vélez, Santander	Fruit	Roja	11.5
<i>Neopestalotiopsis</i>	SPugra4	P. Nacional, Santander	Fruit	Roja	11.3
<i>Neopestalotiopsis</i>	VUgu2	La Unión, Valle	Fruit	Pera	11.1
<i>Neopestalotiopsis</i>	SPugra3	P. Nacional, Santander	Fruit	Roja	11
<i>Neopestalotiopsis</i>	SVpo4	Vélez, Santander	Fruit	Roja	11
<i>Neopestalotiopsis</i>	BTca1	Tununguá, Boyacá	Fruit	Roja	11
<i>Neopestalotiopsis</i>	VRes3	Roldanillo, Valle	Fruit	Manzana	10.8
<i>Neopestalotiopsis</i>	VRlep	Roldanillo, Valle	Fruit	Pera	10.8
<i>Pestalotiopsis</i>	SVsnp4	Vélez, Santander	Fruit	Roja	10.5
<i>Neopestalotiopsis</i>	BPpa1	Pauna, Boyacá	Fruit	Roja	9.7
<i>Neopestalotiopsis</i>	SVsnp10	Vélez, Santander	Leaf	Roja	8.9
<i>Neopestalotiopsis</i>	VTman4	Toro, Valle	Leaf	Pera	8.5
<i>Neopestalotiopsis</i>	SBma2	Barbosa, Santander	Fruit	Roja	8.4
<i>Neopestalotiopsis</i>	BVayr2	Pauna, Boyacá	Fruit	Roja	8.3
<i>Neopestalotiopsis</i>	VTpo	Roldanillo, Valle	Fruit	Pera	8.1
<i>Neopestalotiopsis</i>	SVpa2	Vélez, Santander	Fruit	Roja	8.1
<i>Neopestalotiopsis</i>	SSre4	San Benito, Santander	Fruit	Roja	7.9
<i>Neopestalotiopsis</i>	VUgu3	La Unión, Valle	Fruit	Pera	7.2
<i>Neopestalotiopsis</i>	SSre1	San Benito, Santander	Leaf	Roja	5.8

Fig. 2. Symptoms of guava scab on A, leaf, B, immature fruit, and C, mature fruit.

Table 2. Details of isolates representing species in the phylogenetic clades of *Pestalotiopsis* and *Neopestalotiopsis* used in this study^y

Species	GenBank accession number ^z	Host, substrate	Location
<i>Neopestalotiopsis aotearoa</i>	CBS 367.54; ATCC 11763; QM 381	Canvas	New Zealand
<i>N. asiatica</i>	MFLUCC 12-0286; NN0476380	Unidentified tree	China
<i>N. australis</i>	CBS 114159; STE-U 3017	<i>Telopea</i> sp.	Australia
<i>N. chrysea</i>	MFLUCC 12-0261; NN042855	Dead leaves	China
<i>N. chrysea</i>	MFLUCC 12-0262; NN047037	Dead plant	China
<i>N. clavispora</i>	CBS 447.73	Decaying wood	Sri Lanka
<i>N. clavispora</i>	MFLUCC 12-0280; NN043011	<i>Magnolia</i> sp.	China
<i>N. clavispora</i>	MFLUCC 12-0281; NN043133	<i>Magnolia</i> sp.	China
<i>N. cubana</i>	CBS 600.96; INIFAT C96/44-4	Leaf litter	Cuba
<i>N. ellipsospora</i>	CBS 115113; HKUCC 9136	<i>Ardisia crenata</i>	Hong Kong
<i>N. ellipsospora</i>	MFLUCC 12-0283	Dead plant materials	China
<i>N. eucalypticola</i>	CBS 264.37; BBA 5300	<i>Eucalyptus globulus</i>	...
<i>N. egyptiaca</i>	CBS 140162	<i>Mangifera indica</i>	Egypt
<i>N. foedans</i>	CGMCC 3.9123	Mangrove plant	China
<i>N. foedans</i>	CGMCC 3.9178	<i>Neodopsis decaryi</i>	China
<i>N. foedans</i>	CGMCC 3.9202	<i>Calliandra hematocephala</i>	China
<i>N. formicarum</i>	CBS 115.83	Plant debris	Cuba
<i>N. formicarum</i>	CBS 362.72	Dead <i>Formicidae</i> (ant)	Ghana
<i>N. honoluluana</i>	CBS 111535; STE-U 2078	<i>Telopea</i> sp.	United States: Hawaii
<i>N. honoluluana</i>	CBS 114495; STE-U 2076	<i>Telopea</i> sp.	United States: Hawaii
<i>N. iraniensis</i>	CBS 137768	<i>Fragaria</i> × <i>ananassa</i>	Iran
<i>N. javaensis</i>	CBS 257.31	<i>Cocos nucifera</i>	Indonesia: Java
<i>N. magna</i>	MFLUCC 12-652; ICMP 20011	<i>Pteridium</i> sp.	France
<i>N. mesopotamica</i>	CBS 299.74	<i>Eucalyptus</i> sp.	Turkey
<i>N. mesopotamica</i>	CBS 336.86	<i>Pinus brutia</i>	Iraq
<i>N. natalensis</i>	CBS 138.41	<i>Acacia mollissima</i>	South Africa
<i>N. piceana</i>	CBS 225.30	<i>Mangifera indica</i>	...
<i>N. piceana</i>	CBS 254.32	<i>Cocos nucifera</i>	Indonesia: Sulawesi
<i>N. piceana</i>	CBS 394.48	<i>Picea</i> sp.	United Kingdom
<i>N. protearum</i>	CBS 114178; STE-U 1765*	<i>Leucospermum cuneiforme</i>	Zimbabwe
<i>N. rosae</i>	CBS 101057	<i>Rosa</i> sp.	New Zealand
<i>N. rosae</i>	CBS 124745	<i>Paeonia suffruticosa</i>	United States
<i>N. samarangensis</i>	CBS 115451; HKUCC 9095	Unidentified tree	Hong Kong
<i>N. saprophytica</i>	CBS 115452; HKUCC 8684	<i>Litsea rotundifolia</i>	Hong Kong
<i>Neopestalotiopsis</i> sp.	CBS 233.79	<i>Crotalaria juncea</i>	India
<i>Neopestalotiopsis</i> sp.	CBS 110.20	...	...
<i>Neopestalotiopsis</i> sp.	CBS 177.25	<i>Dalbergia</i> sp.	...
<i>Neopestalotiopsis</i> sp.	CBS 274.29	<i>Cocos nucifera</i>	Indonesia: Java
<i>Neopestalotiopsis</i> sp.	CBS 322.76	<i>Camellia</i> sp.	France
<i>Neopestalotiopsis</i> sp.	CBS 664.94	<i>Cocos nucifera</i>	Netherlands
<i>Neopestalotiopsis</i> sp.	CBS 164.42	Dune sand	France
<i>Neopestalotiopsis</i> sp.	CBS 360.61	<i>Cinchona</i> sp.	Guinea
<i>Neopestalotiopsis</i> sp.	CBS 119.75	<i>Achras sapota</i>	India
<i>Neopestalotiopsis</i> sp.	CBS 266.80	<i>Vitis vinifera</i>	India
<i>Neopestalotiopsis</i> sp.	CBS 266.37; BBA 5087; IMI 083708	<i>Erica</i> sp.	Germany
<i>Neopestalotiopsis</i> sp.	CBS 323.76	<i>Erica gracilis</i>	France

(Continued on next page)

^y Phylogenetic clades of genus *Pestalotiopsis* and *Neopestalotiopsis* as described by Maharachchikumbura et al. (2014), Ariyawansa et al. (2015), Liu et al. (2015), and Jayawardena et al. (2016).

^z CBS = culture collection of the Centraalbureau voor Schimmelmicrocultures, Fungal Biodiversity Centre, Utrecht, The Netherlands; CGMCC = China General Microbiological Culture Collection Center, Institute of Microbiology, Chinese Academy of Sciences, Beijing; ICMP = International Collection of Microorganisms from Plants, Auckland, New Zealand; IFRDCC = International Fungal Research & Development Centre Culture Collection, China; MFLUCC = Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; and IMI = International Mycological Institute, England.

mycelium in direct contact with PDA medium. The isolates were incubated at 22°C under 12 h of light and 12 h of darkness for 7 days and radial growth was measured daily. Analysis of variance (ANOVA) of characteristics described above was carried out with the Statistical Analysis System (SAS software, v. 9.0) ($P < 0.01$), and the Ryan-Einot-Gabriel-Welsch (REGW) multiple-range test was used to separate means ($\alpha = 0.05$).

To determine the average length, width, and number of appendages of conidia, 20 mature conidia were obtained from each 10-day-old isolate. Conidia were mounted on slides and stained with cotton blue for contrast. Photographs were taken with a Leica microscope at $\times 400$ magnification and measurements were made using the ImageJ software (Image Processing and Analysis in Java). The 20 measurements

were subjected to an ANOVA with 20 replications using SAS v. 9.0 software and the REGW multiple-range test to separate means ($\alpha = 0.05$).

Pathogenicity tests. The aggressiveness of the 81 isolates was evaluated by inoculating them onto detached, physiologically mature guava fruit (variety Palmira ICA-1). The fruit were disinfected by immersion into 2% sodium hypochlorite for 2 min, then 70% alcohol for 1 min, and finally rinsing twice in sterilized distilled water for 1 min each time. Fruit were air dried on sterilized paper towels in a laminar flow chamber for 30 min.

The fruit were inoculated as follows: a 5-mm disc of PDA medium with mycelia from a 5-day-old culture was placed on a 1.5-mm wound made by removing a piece of fruit epidermis. The disc was

Table 2. (Continued from previous page)

Species	GenBank accession number ^z	Host, substrate	Location
<i>Neopestalotiopsis</i> sp.	CBS 361.61	<i>Cissus</i> sp.	Netherlands
<i>N. steyaertii</i>	IMI 192475	<i>Eucalyptus viminalis</i>	Australia
<i>N. surinamensis</i>	CBS 111494; STE-U 1779	<i>Protea eximia</i>	Zimbabwe
<i>N. umbrinospora</i>	MFLUCC 12-0285; NN042986	Unidentified plant	China
<i>N. vitis</i>	MFLUCC 15-1265	<i>Vitis vinifera</i>	China
<i>N. zimbabwana</i>	CBS 111495; STE-U 1777	<i>Leucospermum cuneiforme</i>	Zimbabwe
<i>Pestalotiopsis adusta</i>	ICMP 6088	On refrigerator door PVC gasket	Fiji
<i>P. anacardiacearum</i>	IFRDCC 2397	<i>Mangifera indica</i>	China
<i>P. arceuthobii</i>	CBS 434.65	<i>Arceuthobium campylopodum</i>	United States
<i>P. arengae</i>	CBS 331.92	<i>Arenga undulatifolia</i>	Singapore
<i>P. australasiae</i>	CBS 114126; STE-U 2896	<i>Knightia</i> sp.	New Zealand
<i>P. australis</i>	CBS 111503; STE-U 1770	<i>Protea neriifolia</i> $\times$ <i>susannae</i>	South Africa
<i>P. biciliata</i>	CBS 124463	<i>Platanus</i> $\times$ <i>hispanica</i>	Slovakia
<i>P. biciliata</i>	CBS 236.38	<i>Paonia</i> sp.	Proteaceae
<i>P. biciliata</i>	CBS 790.68	<i>Taxus baccata</i>	Taxaceae
<i>P. brassicae</i>	CBS 170.26	<i>Brassica napus</i>	New Zealand
<i>P. camelliae</i>	CBS 443.62	<i>Camellia sinensis</i>	Turkey
<i>P. chamaeropsis</i>	CBS 113604; STE-U 3078	...	...
<i>P. clavata</i>	MFLUCC 12-0268; NN0471340	<i>Buxus</i> sp.	China
<i>P. colombiensis</i>	CBS 118553; CPC 10969	<i>Eucalyptus eurograndis</i>	Colombia
<i>P. diploclisiae</i>	CBS 115449; HKUCC 9103	<i>Psychotria tutcheri</i>	Hong Kong
<i>P. diversiseta</i>	MFLUCC 12-0287; NN0472610	<i>Rhododendron</i> sp.	China
<i>P. ericacearum</i>	IFRDCC 2439	<i>Rhododendron delavayi</i>	China
<i>P. furcata</i>	MFLUCC 12-0054; CPC 20280	<i>Camellia sinensis</i>	Thailand
<i>P. gaultheria</i>	IFRD 411-014	<i>Gaultheria forrestii</i>	China
<i>P. grevilleae</i>	CBS 114127; STE-U 2919	<i>Grevillea</i> sp.	Australia
<i>P. hawaiiensis</i>	CBS 114491; STE-U 2215	<i>Leucospermum</i> sp.	United States: Hawaii
<i>P. hollandica</i>	CBS 265.33	<i>Sciadopitys verticillata</i>	Netherlands
<i>P. humus</i>	CBS 115450; HKUCC 9100	<i>Ilex cinerea</i>	Hong Kong
<i>P. inflexa</i>	MFLUCC 12-0270; NN0470980	Unidentified tree	China
<i>P. intermedia</i>	MFLUCC 12-0259; NN0476420	Unidentified tree	China
<i>P. jesteri</i>	CBS 109350 = MONT 6M-B-3	<i>Fragaria bodenii</i>	Papua New Guinea
<i>P. kenya</i>	CBS 442.67	<i>Coffea</i> sp.	Kenya
<i>P. knightiae</i>	CBS 111963; STE-U 2905	<i>Knightia</i> sp.	New Zealand
<i>P. linearis</i>	MFLUCC 12-0271; NN0471900	<i>Trachelospermum</i> sp.	China
<i>P. malayana</i>	CBS 102220	<i>Macaranga triloba</i>	Malaysia
<i>P. monochaeta</i>	CBS 144.97	<i>Quercus robur</i>	Netherlands
<i>P. montelica</i>	MFLUCC12-0279	...	Thailand
<i>P. novae-hollandiae</i>	CBS 130973	<i>Banksia grandis</i>	Australia
<i>P. oryzae</i>	CBS 111522; STE-U 2083	<i>Telopea</i> sp.	United States: Hawaii
<i>P. papuana</i>	CBS 331.96	Coastal soil	Papua New Guinea
<i>P. parva</i>	CBS 265.37; BBA 2820	<i>Delonix regia</i>	...
<i>P. portugalia</i>	CBS 393.48	...	Portugal
<i>P. rhododendri</i>	IFRDCC 2399	<i>Rhododendron sinogrande</i>	China
<i>P. rosea</i>	MFLUCC 12-0258; NN0471350	<i>Pinus</i> sp.	China
<i>P. shorea</i>	MFLUCC12-0314	<i>Shorea obtusa</i>	Thailand
<i>P. scoparia</i>	CBS 176.25	<i>Chamaecyparis</i> sp.	...
<i>P. spatulata</i>	CBS 356.86	<i>Gevuina avellana</i>	Chile
<i>P. telopeae</i>	CBS 113606; STE-U 3082	<i>Telopea</i> sp.	Australia
<i>P. trachicarpicola</i>	IFRDCC 2403	<i>Podocarpus macrophyllus</i>	China
<i>P. unicolor</i>	MFLUCC 12-0275; NN0473080	Unidentified tree	China
<i>P. verruculosa</i>	MFLUCC 12-0274; NN0473090	<i>Rhododendron</i> sp.	China

then covered with the piece of epidermis and sealed with parafilm. Fruit were placed on a plastic grate inside translucent polypropylene boxes. Water was added to a level below the plastic grate to maintain 100% relative humidity. The boxes were then hermetically sealed and incubated at 22°C under 12 h of light and 12 h of darkness for 12 days.

Aggressiveness was evaluated as “lesion diameter” (mean diameter of three lesions per day) at 4, 8, and 12 days after inoculation. Each isolate was replicated three times and the experiment was conducted twice. Fruit inoculated in a similar manner with PDA discs with no mycelia served as negative controls. Data were analyzed by ANOVA ($P < 0.01$), and mean separation tests were conducted using the REGW multiple-range test ($\alpha = 0.05$), using SAS v. 9.0 software.

Phylogenetic analysis of multiple genes. *DNA extraction.* To extract DNA, we used the methodology reported by Gramaje et al. (2009). Fragments of 5-day-old fungal mycelium grown on PDA were transferred to 1.5-ml Eppendorf tubes, each containing 200 μ l of cetyltrimethylammonium bromide (CTAB) extraction buffer (0.2 M Tris, 1.4 M NaCl, 20 mM EDTA, and of CTAB at 0.2 g/liter). The mycelium was macerated in 600 μ l of buffer using a sterilized micropestle (Sigma-Aldrich), until pulverized. The tubes were then incubated at 65°C for 15 min, after which 400 μ l of chloroform-isoamyl alcohol (24:1) was added to each tube. Tubes were mixed by inverting and immediately centrifuged at 15,800 $\times g$ for 5 min.

The supernatant (approximately 500 μ l) was transferred to a fresh tube and 600 μ l of cold isopropanol and 166 μ l of cold 7.5 M ammonium acetate were added to obtain a final concentration of 2.5 M. The tubes were then incubated at room temperature for 15 min and centrifuged at 15,800 $\times g$ for 5 min. The supernatant was discarded, saving only the pellets. Cold 70% ethanol (1 ml) was added to each tube and centrifuged at 15,800 $\times g$ for 5 min. The supernatant was discarded and the pellet was dried in a SpeedVac dryer at 37°C for 50 min. After drying, the pellets were resuspended in Tris-EDTA buffer and 1.5 μ l of RNase was added to each tube. Tubes were incubated at 37°C for 30 min.

*Amplification of the ITS, β -tubulin, and *tefl* genes.* The PCR assay was used to amplify the ITS region and the β -tubulin and *tefl* genes. The three regions were amplified using primers and amplification conditions that were previously described (Glass and Donaldson 1995; Maharachchikumbura et al. 2012; Rehner 2001; White et al. 1990). For the PCR amplifications, a 25- μ l reaction volume was prepared, consisting of 10 ng of DNA, a final 1 $\times$ concentration of Promega GoTaq Green Master Mix 2X, and 0.5 mM each primer. The reactions were carried out in a PTC-100 thermal cycler (MJ Research Inc.).

The PCR products were first cleaned by adding 25 μ l of polyethylene glycol (20%) and NaCl (2.5 M) solution to each sample. The mixture was homogenized using a vortexer (Scientific Industries) and incubated at room temperature for 15 min. The solution was centrifuged at 13,000 rpm for 15 min, and the supernatant was discarded. Then, 70% ethanol (100 μ l) was added to the tube and the solution was centrifuged at 13,000 rpm for 3 min. The ethanol was carefully removed and each pellet was dried by incubation at 38°C for 30 min. Finally, each DNA pellet was resuspended in 20 μ l of ultrapure distilled water (Invitrogen). The amplicons were sent to the DNA Facility of the Iowa State University, Ames, for sequencing. The sequences were aligned to obtain a consensus sequence of the amplified regions for all isolates. This sequence was analyzed using ChromasPro (version 1.49 beta) and MEGA 5.2 (Kumar et al. 2012). Homologies were sought using the BLAST tool in GenBank. The reference sequences were downloaded from GenBank species type (holotype, epitype, and ex-epitype) and others previously referred to by Maharachchikumbura et al. (2014), Ariyawansa et al. (2015), Liu et al. (2015), and Jayawardena et al. (2016) (Table 1). These sequences were aligned with the sequences obtained from the isolates collected from guava using MEGA 5.2. The aligned sequences were analyzed using Bayesian Inference of Phylogeny, variant Metropolis coupled Markov Chain Monte Carlo, and MrBayes software 3.2.1 (<http://mrbayes.sourceforge.net/download.php>) (Ronquist et al. 2012). The best model for substituting nucleotides for the sequences of each

primer was determined separately, using the application jModelTest 2.1.4 (<http://darwin.uvigo.es/our-software/>) (Posada 2008). For the multilocus analysis of the combined ITS region, β -tubulin, and *tefl* genetic loci, 30 million generations were run, sampling every 1,000 generations and omitting the first 25% of trees generated. From the remaining trees sampled, the consensus tree was estimated at a domain of more than 50%. The resulting trees were printed with Fig Tree, version 1.4.0 (<http://tree.bio.ed.ac.uk/software/figtree/>), and the layout was made with Adobe Illustrator Cs (V.6).

Results

Isolating the fungi. We collected 81 isolates from the three regions sampled as follows. We recovered 76 *Neopestalotiopsis* isolates, with 9 coming from Boyacá, 44 from Santander, and 23 from Valle del Cauca. We also recovered five *Pestalotiopsis* isolates, with three from Santander and two from Valle del Cauca. We isolated *Neopestalotiopsis* spp. from fruit in all phenological stages of development and from mature leaves, whereas *Pestalotiopsis* spp. were isolated only from fruit (Fig. 2). *Neopestalotiopsis* isolates came from tissues of productive trees of the four most common varieties (Pera, Manzana, Coronilla, and Regional Roja) found in the sampling zones. In contrast, *Pestalotiopsis* isolates were found only on Regional Roja and Pera.

Morphological characterization—Macroscopic. No morphological differences were found between *Pestalotiopsis* and *Neopestalotiopsis* isolates. The 81 isolates grouped into morphotypes 1 through 9 according to colony characteristics (Table 1). The principal descriptors were surface color, texture, mycelium production (high, medium, or low), presence or absence of surface acervuli, and edge type. However, surface color, mycelium production, and texture were given the most weight because the other characteristics were highly variable. The 81 isolates were distributed throughout the 9 morphotypes as follows: 1 = 33 (40.7%); 2 = 16 (19.7%); 7 = 6 (7.4%); 3, 4, 5, and 9 = 5 (6.17%) each; 8 = 4 (4.9%); and 6 = 1 (1.2%).

In all, 24 (30%) isolates had a high growth rate on artificial culture media, while 31 (38%) grew at medium rates. The remaining 26 (32%) isolates grew slowly (Table 2). Moreover, no correlation was found between growth rate on artificial culture medium and colony morphology.

Morphological characterization—Microscopic. Microscopic characterization was carried out on 48 isolates that produced fruiting bodies with conidia. Sporulation was low in the remaining 33 isolates. On subculturing, these isolates lost morphological characteristics, including the ability to produce fruiting bodies.

The minimum and maximum values for conidium length were 19.05 and 29.25 μ m, respectively, while the minimum and maximum values for conidium width were 4.40 and 10.40 μ m, respectively. The averages ranged from 21.38 to 25.74 μ m for length and 5.13 to 8.34 μ m for width.

The minimum and maximum values for length of apical appendages were 11.54 and 34.8 μ m, respectively, and the minimum and maximum values for length of basal appendages were 3.00 and 6.75 μ m, respectively. For most isolates, appendage number per conidium was constant, with most isolates presenting conidia with three apical and one basal appendages (Table 3).

Of the 48 isolates, 46 were *Neopestalotiopsis* spp. Analysis of their median cells showed that they had versicolored conidia, with two upper median cells being darker than the lowest median cell. Of these isolates, 45 had dark and pale coffee-colored conidia and 1 (Vr1eP) presented blackish-gray conidia. The two remaining isolates (VTman5 and SSpla) were *Pestalotiopsis* spp. and had concolorous conidia (Fig. 3).

The posthoc test used in ANOVA on conidial length and width data showed significant differences ($P = 0.05$) only for isolates VTman5 and SSpla, which formed one group, and for isolate Vr1eP, which formed another group by itself. The characteristics shared by all other isolates were not significantly different. Isolates VTman5 and SSpla also stood out for their long and thin conidia, which were 5.42 and 5.13 μ m wide, respectively. Their length-to-width ratio was also the highest and, although Vr1eP had a similar length-to-width ratio, it had a higher average width (5.94 μ m).

Pathogenicity tests. Isolates that did not form lesions were considered nonpathogenic, and did not differ significantly from the control. Of the 81 isolates, 4 were not pathogenic. Different degrees of aggressiveness were observed among the 77 pathogenic isolates by means of two variables: diameter of the lesion (DL) and area under the disease-progress curve. SAS cluster analysis (Ward's Minimum Variance Cluster Analysis) separated the isolates into three groups according to their level of aggressiveness ($P = 0.05$): low, intermediate, and high.

The ANOVA ($P < 0.01$) for variable DL showed significant differences for aggressiveness, according to the REGW multiple-range test ($\alpha = 0.05$). The high aggressiveness group was composed of *Neopestalotiopsis* isolates, mainly from Santander and Valle del Cauca and one (BVayr1) from Boyacá. The most aggressive isolate was SVpo5 from Vélez (Santander), standing out from all the others, including the highly aggressive VRes2, SVpa1, BVayr1, SVsnp8, and VRte1.

Fruit attacked by mildly aggressive isolates developed small lesions with few visible acervuli on their surfaces. Intermediately aggressive isolates usually produced a larger quantity of surface acervuli but small quantities of mycelia. Highly aggressive isolates produced large quantities of mycelia and surface acervuli by 12 days after inoculation.

Molecular characterization. *Phylogenetic analysis.* The phylogenetic analysis included reference sequences from 52 *Pestalotiopsis*

and 27 *Neopestalotiopsis* spp., all 79 epitypified, and 13 *Neopestalotiopsis* spp. published by Maharachchikumbura et al. (2014), Ariyawansa et al. (2015), Liu et al. (2015), and Jayawardena et al. (2016). The sequences from this study are available in GenBank as accession numbers KR493595 to KR493664.

Sequence analysis enabled the separation of isolates into two genera: *Pestalotiopsis* (Fig. 4) and *Neopestalotiopsis* (Fig. 5). Moreover, this analysis enabled us to establish that, under our study's conditions, the *Neopestalotiopsis* isolates were more numerous ($n = 63$) than the *Pestalotiopsis* isolates ($n = 4$). Therefore, the phylogenetic analysis presents them in separate cladograms.

The four *Pestalotiopsis* are grouped into two clades (Fig. 4); the first two (VTsin2 and SSpla) formed one clade, *Pestalotiopsis* sp. 1, and the other two isolates (SVsnp4 and VTman5) formed a separate clade with *Pestalotiopsis australasiae*.

For the *Neopestalotiopsis* group (Fig. 5), the 63 isolates in this study were distributed across 11 well-defined clades. Of the 63 isolates, 40 were grouped into two clades, *Neopestalotiopsis* sp. 1 and *Neopestalotiopsis* sp. 3, with no reference sequences being present. Clade *Neopestalotiopsis* sp. 1 was more homogeneous.

Some sequences of *Neopestalotiopsis* spp. were closely related to reference species. For example, BVayr1 and SPugra5 formed a clade with *Neopestalotiopsis surinamensis*. Likewise, VR1ep formed a

Table 3. Morphological, cultural, and pathogenic characteristics of 65 *Neopestalotiopsis* and 4 *Pestalotiopsis* isolates obtained from guava and grouped according to genetic diversity clusters, as identified through multilocus analysis

Clade ^u	Tissue ^v	Type ^w	Genus, species	Origin	Conidium size (μm) ^t		L/W ^x	AUDPC ^y	Growth ^z
					L	W			
1 p (2)	Fruit	1	<i>Pestalotiopsis</i>	Santander, Valle del Cauca	24.7	5.4	4.5 a	27.7 (36.6–18.8) ab	11.9
2 p (2)	Fruit, leaf	1, 5	<i>Pestalotiopsis australasiae</i>	Santander, Valle del Cauca	22.9	5.1	4.4 a	28.6 (36.5–20.7) ab	12.3
1 (19)	Fruit, leaf	1, 2, 3, 4, 5, 7, 8, 9	<i>Neopestalotiopsis</i>	Santander, Valle del Cauca	23.5	6.6	3.5 cd	27.5 (7.5–75.7) abc	12.7
2 (3)	Fruit	1, 3	<i>Neopestalotiopsis</i>	Boyacá	23.1	6.2	3.7 cd	31.6 (24.9–39.4) b	15.3
3 (21)	Fruit, leaf	1, 2, 3, 4, 5, 6, 7, 8, 9	<i>Neopestalotiopsis</i>	Boyacá, Santander, Valle del Cauca	23.4	7.1	3.2 ef	38.1 (0–99.2) abc	13.5
4 (2)	Fruit	1, 2	<i>Neopestalotiopsis surinamensis</i>	Boyacá, Santander	22.7	6.6	3.4 de	62.6 (58.8–66.4) a	15.0
5 (8)	Fruit	1, 2, 3, 9	<i>Neopestalotiopsis</i>	Santander, Valle del Cauca	22.8	6.3	3.5 cd	24.8 (3.5–54) abc	12.3
6 (3)	Fruit, leaf	1	<i>Neopestalotiopsis</i>	Santander, Valle del Cauca	22.5	6.5	3.4 de	4.6 (0–7) c	13.4
7 (1)	Fruit	2	<i>N. egyptiaca</i>	Valle del Cauca	-	-	-	11.8 c	10.8
8 (4)	Fruit, leaf	2, 3, 4	<i>Neopestalotiopsis</i>	Santander, Valle del Cauca	23.5	6.1	3.8 cd	48.0 (70.4–15.9) ac	12.5
9 (1)	Fruit	1	<i>N. foedans</i>	Valle del Cauca	24.5	5.9	4.1 b	11.8 c	10.8
10 (1)	Fruit	8	<i>Neopestalotiopsis</i>	Valle del Cauca	21.9	6.8	3.2 ef	11.9 c	11.1
11 (2)	Leaf	1, 7	<i>Neopestalotiopsis</i>	Santander, Valle del Cauca	24.3	8.0	3.0 f	34.0 (28.7–39.3) ab	14.5

^t Average length (L) and width (W).

^u Multilocus clades. Values in parentheses show the number of isolates in each clade.

^v Infected tissue.

^w Nine colony morphotypes, where 1 = bright-white colony, cottony texture, intermediate mycelium density, acervuli in the middle, regular edges; 2 = bone-white colony, velvety texture, high mycelium density, no acervuli, regular edges; 3 = bright-white colony, spiky cottony texture, low mycelium density, scattered acervuli, irregular edges; 4 = white colony, powdery spiky texture, intermediate mycelium density, acervuli in the middle, irregular edges; 5 = white to bright-yellow colony, powdery texture, low mycelium density, scattered acervuli, regular edges; 6 = white colony, powdery texture, low mycelium density, scattered acervuli, regular edges; 7 = white colony, powdery texture, intermediate mycelium density, abundant acervuli, regular edges; 8 = white colony, powdery texture, low mycelium density, scattered acervuli, irregular edges; and 9 = black colony, oily texture, low mycelium density, scattered acervuli, irregular edges.

^x Length-to-width ratio (L/W). Mean difference between each group is significant at the 0.05 level; values with the same letters in a column do not differ significantly, according to the Ryan-Einot-Gabriel-Welsch (REGW) multiple-range test.

^y Area under the disease-progress curve (AUDPC) average (maximum to minimum). Mean difference between each group is significant at the 0.05 level; values with the same letters in a column do not differ significantly, according to the REGW multiple-range test.

^z Average rate of growth (mm/day) on potato dextrose agar, with no significant differences between clades.

Fig. 3. Typical conidia of **A**, *Pestalotiopsis* spp., showing concolorous median cells and **B**, *Neopestalotiopsis* spp., showing versicolored median cells. (Photos taken at $\times 100$.)

clade with two sequences of *N. foedans*; VUgu2 with two sequences that have not yet been determined; and SVsnp10, SVsnp12, SPugra3, and SVsnp8 formed a clade with *N. egyptiaca* and VTman2, showing high sequence similarity (98.6%) (Fig. 5).

Although no relationship was observed among the isolates based on geographical origin, most of the clades were formed by isolates from Santander and Valle de Cauca. Nevertheless, some clades and subclades consisted of grouped isolates from specific regions. For

example, clade *Neopestalotiopsis* sp. 1 has a subclade of three isolates (SSre1, SSre2, and SSre4) that came from the same region in Santander; clade *Neopestalotiopsis* sp. 2 is formed by three isolates from Boyacá; and clade *Neopestalotiopsis* sp. 8 is formed by two isolates from Santander.

Beyond these similarities, we could not determine the species of most of the isolates because there are only a few nucleotides of difference between the sequences of the species in the genetic loci we

Fig. 4. Phylogenetic consensus tree based on Bayesian inference, illustrating the relationships within the *Pestalotiopsis* epitypified species and of isolates obtained from guava. The tree was built using concatenated sequences of the genes internal transcribed spacer, β -tubulin, and translation elongation 1, and run for 1×10^7 generations, each with a separate model of DNA evolution. *Neopestalotiopsis saprophyta* (NN047136) was used as the outgroup.

Fig. 5. Phylogenetic consensus tree based on Bayesian inference, illustrating the relationships within the *Neopestalotiopsis* epitypified species and of isolates obtained from guava. The tree was built using concatenated sequences of the genes internal transcribed spacer, β -tubulin, and translation elongation 1, and run for 1×10^7 generations, each with a separate model of DNA evolution. *Pseudopestalotiopsis theae* (NN047136) was used as the outgroup.

studied. However, both *Pestalotiopsis* and *Neopestalotiopsis* genera are clearly associated with the disease in Colombia, and the isolates showed substantial diversity.

Discussion

This is the first study documenting the genetic relationships between isolates of *Neopestalotiopsis* and *Pestalotiopsis* causing guava scab. Molecular and morphological characterization along with virulence assays demonstrated that guava scab was caused by species of more than one genus (*Neopestalotiopsis* and *Pestalotiopsis*). Selection due to geographic location was not an important factor determining genetic structure of clades. There was no correlation among origin, morphology, pathogenicity, and molecular clades based on multiple gene analysis of the isolates in Colombia (Table 1).

In this study, 77 of 81 isolates recovered from guava scab lesions were pathogenic to detached guava fruit. Virulence assays on fruit of guava cultivar Pera1 also revealed that isolates SVpo5, VRes2, SVpa1, and BVayr1 were more aggressive than isolates SVsnp5, SVsnp11, and SVpa8. In addition, isolates VUgu3, VTman4, SVpa2, and BVayr2 were not pathogenic. The aggressive strains produced symptoms of guava scab on fruit tissues.

We observed the same variability in colony color (white, pale buff, pale saffron, or pale olive) and production of acervuli as Keith et al. (2006). Conidium length also was similar to that reported by Keith et al. (2006). In other studies, conidium length was reported as being as short as 15 μm to as long as 35 μm (Hu et al. 2007; Maharachchikumbura et al. 2013, 2014).

Most of the isolates recovered from guava scab lesions exhibited variability in the morphological characteristics evaluated. When subcultured, the isolates changed one or more colony characteristics. This was previously reported by Hu et al. (2007), who indicated that *Pestalotiopsis* spp. colony morphology is not stable under subculturing (e.g., for color, growth rate, and texture). Indeed, a single isolate was sometimes seen as having two different morphologies. Jeewon et al. (2003), Tejesvi et al. (2007), and Maharachchikumbura et al. (2011) suggest that colony morphology characteristics are plastic and variable. In contrast, conidial characteristics are more stable, especially the length, width, and color of median cells. These traits were more useful for differentiating isolates, enabling statistical analyses to separate the four *Pestalotiopsis* spp. evaluated (VTsin2, SSpla, SVsnp4, and VTman5) from those of the *Neopestalotiopsis* spp. However, it was impossible to separate or group isolates, especially those with unreliable traits. This situation made it necessary to use phylogenetic analysis to better understand the other traits and variables analyzed in our study. Maharachchikumbura et al. (2012) came to the same conclusion.

Phylogenetic analyses revealed genetic variability among the guava scab isolates, particularly those of the *Neopestalotiopsis* spp. The possibility of finding more than one species causing guava scab was already reported by Keith et al. (2006), who found five *Pestalotiopsis* spp. (*P. microspora*, *P. clavispora*, *P. disseminata*, *P. neglecta*, and *Pestalotiopsis* sp. GJ-1) among 23 isolates collected from different guava varieties in Hawaii. Unfortunately, none of these species is epitypified and, therefore, could not be included in our study.

Phylogenetic analysis also revealed close relationships between some reference sequences and isolates in our study. For *Pestalotiopsis*, isolates SVsnp4 and VTman5 were related to *P. australasiae*; and, for *Neopestalotiopsis*, BVayr1 and SPugra5 were related to *N. surinamensis*. Additionally, VTman2 was closely related to *N. egyptiaca*, Vrlep was closely related to *N. foedans*, and VUgu2 was related to *Neopestalotiopsis* sp. CBS 360.61 and *Neopestalotiopsis* sp. CBS 162.42. Despite this, the expertise of a taxonomist specializing in these genera should be consulted because sequence similarity is not sufficient to identify the species. The foregoing is evident in the studies by Maharachchikumbura et al. (2012, 2014), where two species were differentiated by only one pair of nucleotides. For example, in the 2014 study, *N. honolulua* and *N. zimbabweana* differed by only six nucleotides but were defined as two species. In contrast, in the 2012 study, one of the *N. foedans* isolates differed by six nucleotides but was considered to be the same species as the other two *N. foedans* isolates evaluated.

This situation shows the importance of combining morphological traits with phylogenetic analysis when determining a new species. In our study, we could not reach this level of depth, because our objective was phytopathological rather than taxonomic. Nevertheless, our data showed that the guava scab in Colombia is caused by a complex of species of the genera *Pestalotiopsis* and *Neopestalotiopsis*. These results agree with those of Keith et al. (2006), taking into account that, in their study, the *Pestalotiopsis* and *Neopestalotiopsis* genera were classified as one genus.

The isolates belonging to 6 of the 11 clades (*Neopestalotiopsis* spp. 1, 2, 3, 5, 6, and 11) could be defined as new species (S. S. N. Maharachchikumbura, personal communication). It is even possible that some clades may include more than one species; for example, clades *Neopestalotiopsis* spp. 1, 3, and 5 (Fig. 5). Our findings represent an important contribution to the etiology and epidemiology of guava scab in Colombia. We provide evidence for diversity in both the *Neopestalotiopsis* and *Pestalotiopsis* spp. associated with the disease in guava. Our study facilitates the development of future epidemiological research in guava and other crops. It offers information relevant to the design of management strategies, such as appropriate chemistries and crop rotation, and to the evaluation of resistance in *Psidium* germplasm to the different *Neopestalotiopsis* and *Pestalotiopsis* isolates.

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