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# Oomycete species associated with *Theobroma cacao* crops in Colombia

Julie Ramírez Martínez[1] | Martha Cárdenas Toquica[1] | Marcela Guevara-Suarez[1,2] | Diannefair Duarte[1,3] | Laura Daniela Victorino Jimenez[1] | Bibian Katherine Argüello Bernal[1] | Edwin Gutiérrez Rodríguez[1,4] | Silvia Restrepo Restrepo[1]

[1]Laboratory of Mycology and Phytopathology - Laboratorio de Micología y Fitopatología (LAMFU), Department of Chemical and Food Engineering, Universidad de los Andes, Bogotá, Colombia

[2]Applied Genomics Research Group, Vicerrectoria de Investigación y Creación, Universidad de los Andes, Bogotá, Colombia

[3]Federación Nacional de Cacaoteros (FEDECACAO) – Fondo Nacional del Cacao (FNC), San Vicente de Chucurí – Santander, Colombia

[4]Federación Nacional de Cacaoteros (FEDECACAO) – Fondo Nacional del Cacao (FNC), Bogotá, Colombia

**Correspondence**
Silvia Restrepo Restrepo, Laboratory of Mycology and Phytopathology - Laboratorio de Micología y Fitopatología (LAMFU), Department of Chemical and Food Engineering, Universidad de los Andes, Cr 1 # 18 a 12 Bogotá, Colombia.
Email: srestrep@uniandes.edu.co

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## Abstract

The study of oomycetes associated with crops is highly important due to the economic losses they might cause. In cacao, the genus *Phytophthora* has been extensively studied, but little is known about other genera and species of oomycetes associated with this plant. This study aimed to determine the oomycetes' diversity present in Colombian cacao crops. A total of 146 isolates were obtained from diseased plants and soil in 11 departments. Analysis of internal transcribed spacer (ITS) and cytochrome oxidase subunit I (*coxI*) sequences was performed along with the assessment of morphological characteristics. Nine species were identified, distributed in four genera: *Phytophthora* (*P. palmivora*, 54%; *P. nicotianae*, 1%), *Phytopythium* (*Phy. chamaehyphon*, 15%; *Phy. cucurbitacearum*, 9%; *Phy. vexans*, 7%; *Phy. helicoides*, 1%), *Globisporangium* (*G. splendens*, 3%), and *Pythium* (*Py. delicense*, 1%; *Py. inflatum*,1%). Additionally, an unidentified and possibly new species of *Phytophthora* (5%) and three unidentified species of *Phytopythium* (3%) were found. This is the first report of *Globisporangium*, *Phytopythium*, and *Pythium* in cacao crops of Colombia and the first report of the species *Phy. chamaehyphon* in the country. Interestingly, some isolates of *Phytopythium* spp. were isolated from necrotic leaves and vascular section of stems, which may suggest a role in cacao diseases traditionally associated with *Phytophthora*. Also, it is proposed that the new species of *Phytophthora* may be contributing significantly to black pod disease in Colombian cacao crops, and we highlight that the study of *P. palmivora* is urgent because of its distribution all over the country.

**KEYWORDS**
black pod, *Globisporangium*, *Phytophthora*, *Phytopythium*, *Pythium*, stem canker

## 1 | INTRODUCTION

Oomycetes are a large group of microorganisms classified in the kingdom Protista and related to heterokont, biflagellate, golden-brown algae. The study of oomycetes is important for agriculture because many of these organisms are highly specialized pathogens of crops or can act as pathogens if the opportunity is given (Marano et al., 2016); consequently, they can cause large economic losses (Kamoun, 2003).

In *Theobroma cacao* (cacao) crops, the most important and studied oomycete pathogen is *Phytophthora* (Adeniyi, 2019). The main reason for interest in this pathogen is that some of the most important diseases in cacao are linked to it, such as black pod disease, stem canker, and leaf blight. Black pod disease is the most

  

significant, causing up to 60%–100% of yield losses depending on the environmental conditions (Adeniyi, 2019). However, other oomycetes remain under-studied, even though different genera have been frequently found in other crops causing damping-off, root damage, and other diseases (Choudhary et al., 2016). Thus, it is very possible that they are also present in cacao crops, possibly affecting them.

For a long time, it was assumed that the only causal agent of black pod on *T. cacao* was the species *Phytophthora palmivora* (Brasier & Griffin, 1979). However, in the last decades, some studies based on morphological, physiological, and chromosome differences made possible the differentiation of three species within an original species complex: *Phytophthora capsici*, *Phytophthora megakarya* and *P. palmivora* sensu stricto (Brasier & Griffin, 1979). Later on, with the use of molecular markers such as the sequences of the internal transcribed spacer (ITS) genomic region and the cytochrome oxidase (*cox*) gene and its subunits (*coxI* and *coxII*; Bailey et al., 2002), it was found that this division also had supporting genetic evidence.

The use of molecular tools as well as morphological characteristics has made possible the identification of several species associated with cacao; the most commonly reported species are *P. palmivora*, followed by *P. citrophthora*, *P. capsici*, and *P. megasperma* in America, while in Africa the most frequently reported species after *P. palmivora* is *P. megakarya* (Akrofi, 2015; Faleiro et al., 2003; Molina et al., 2016).

In Colombia, cacao is cultivated in several regions and its production is an important source of income for many farmers (Ramírez, 2016). Also, it is expected that cultivation in the country will increase in the coming years due to the international recognition that the Colombian cacao has been gaining for its fine flavour and aroma, and to the current national policy of encouraging the replacement of illicit crops of coca (*Erythroxylum* sp.) with cacao crops (Barrera-Ramírez et al., 2019). Therefore, it is necessary to study the factors that may affect its production, such as the associated oomycetes.

Ramírez (2016) was the first to record oomycete species associated with cacao crops in Colombia, with the use of morphological identification. In that study, *P. palmivora* was found to be associated with black pod, along with another species of the same genus that could not be identified. Since then, only two other studies in Colombia have been made, both identifying *P. palmivora* with black pod disease (Rodríguez-Polanco et al., 2020; Villamizar-Gallardo et al., 2019).

Therefore, the aim of this study was to characterize the oomycete species associated with diseased *T. cacao* trees in different regions of Colombia using molecular and morphological characters. We describe several species reported as the causal agents of several diseases in tropical areas, including *Phytophthora* species and species belonging to the complex *Phytopythium vexans*. Further characterization of these species, and their pathogenicity to cacao, will be the focus of future studies.

## 2 | MATERIALS AND METHODS

### 2.1 | Sampling and isolation

Sampling was performed during the second half of 2018 in regions of Colombia where cacao is grown commercially. A total of 82 farms, distributed in 42 municipalities of 11 departments of Colombia, were visited and samples collected. One farm belongs to the company Casa Luker, and the remainder are affiliated to the Federación Nacional de Cacaoteros de Colombia (FEDECACAO) (Figure 1). Five types of samples were taken: pods, leaves and stems with symptoms (vascular section), roots from diseased plants, and bulk soil from plants with symptoms. These samples were taken from different cacao trees on each cultivated area, and a maximum of five samples per type were taken at each locality of the FEDECACAO-associated farms, depending on the availability of diseased plants. In the Casa Luker farm a total of 29 samples were taken (15 leaves, 12 pods, and two stems).

For isolation of oomycetes, different methodologies were employed for each sample type following the methods described by Sendall and Drenth (2001) with modifications (Table 1). In all cases, the same culture medium was used: modified PARPNH/V8-juice agar (pimaricin 10 mg/L, ampicillin 250 mg/L, rifampicin 10 mg/L, pentachloronitrobenzene 25 mg/L, nystatin 50 mg/L; no hymexazol was added). The inoculated medium was incubated at 20 °C in darkness until colonies with morphological characteristics of oomycetes were obtained.

Monosporic cultures were obtained by successive dilutions of sporangia in peptone water (0.1%) until a concentration of 100–200 sporangia/ml was obtained. Then 0.1 ml of this suspension was placed on potato dextrose agar (PDA; Difco) plates and spread. The plates were incubated in darkness at 20 °C and checked daily under a dissecting microscope until germinated sporangia were found. Then, the germinated sporangia were individually transferred to V8 agar plates (V8 juice 100 ml/L, $CaCO_3$ 1 g/L), and one of the colonies obtained per isolate was chosen to carry on with the study.

### 2.2 | Molecular identification

For molecular identification, DNA extraction was performed according to Griffith and Shaw (1998) and the DNA was stored at −30 °C until used. PCR amplification of two markers was performed: ITS region of the ribosomal RNA gene cluster using the primers ITS6 and ITS4 (Cooke & Duncan, 1997; White et al., 1990) and the *coxI* gene using the primers OomCoxILevup and Fm85mod (Robideau et al., 2011). The annealing temperatures for the ITS and *coxI* primers were 58 and 51 °C, respectively. The PCR was performed with an initial denaturation step at 95 °C for 10 min; followed by 31 cycles for ITS and 35 cycles for *coxI* at 95 °C for 1min, respective annealing temperature for 1 min and 72 °C for 1 min; and then a final elongation step at 72 °C for 10 min.

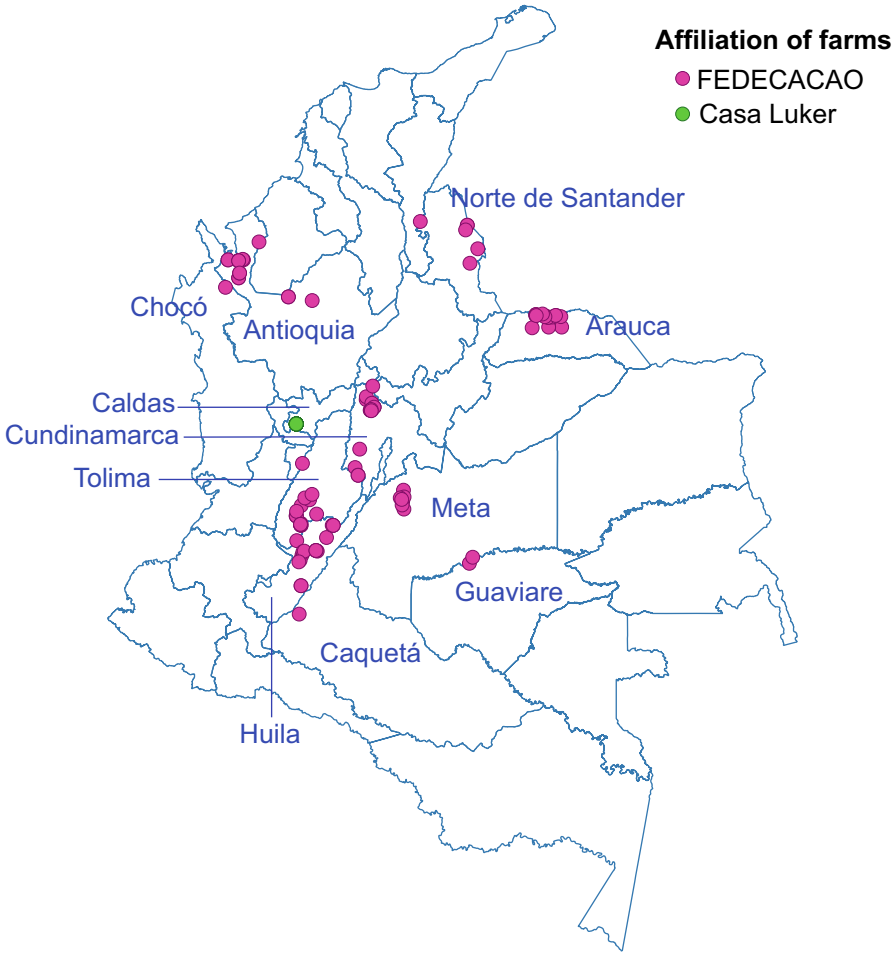


**FIGURE 1** Geographic distribution of sampling sites in Colombia. The administrative divisions (Departments) of the country are displayed, and the names of departments where samples were collected are shown. Dots represent sampling sites, and colours correspond to the affiliation of the farms

**TABLE 1** Oomycete isolation methods according to the different sample types based on the methodology proposed by Sendall and Drenth (2001)

| Sample | Methodology |
|---|---|
| Pod | Surface-sterilized in 70% ethanol 30 s, washed in sterile distilled water, dried with sterilized paper. 1 $cm^2$ pieces of tissue were placed on selective medium |
| Leaf | Surface-sterilized in 70% ethanol 30 s, washed in sterile distilled water, dried with sterilized paper. 1 $cm^2$ pieces of tissue were placed on selective medium |
| Stem | Surface-sterilized in 70% ethanol 60 s, pieces of 1 $cm^2$ were flamed and placed on selective medium |
| Root | Rinsed under water stream, surface-sterilized in 70% ethanol 30 s, pieces of roots were placed in a plastic jar, covered with water, chopped roots were placed on selective medium after a week |
| Soil | 5 g was placed in a plastic jar, covered with water and disinfected pieces of rose petals were put on the surface as baits. After a week, pieces of petals were placed on medium. |

The PCR products were purified and then sequenced at the GenCore facility of the Universidad de Los Andes (Bogotá, Colombia) with an ABI 3500 sequencer (Applied Biosystems). The sequences were assembled and manually edited using GeneiousPrime v. 2019.2.1 (https://www.geneious.com).

Preliminary identification of the isolates was performed with a similarity search for the ITS sequences using the BLASTn algorithm (parameters by default) against the National Center for Biotechnology Information nr database (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Westerdijk Fungal Biodiversity Institute (http://www.westerdijkinstitute.nl) database.

To establish the identity of the isolates to the species level, phylogenetic analyses were conducted, first individually for each locus and then as multilocus sequence analyses using ITS and *coxI* data sets. Sequences for each locus were aligned independently using MAFFT on the webserver of the European

Bioinformatics Institute (EMBL-EBI) (http://www.ebi.ac.uk/Tools/msa/mafft/). Alignments were checked and manually edited if necessary, using MEGA v. 7 (https://www.megasoftware.net/). Sequences of ex-type and reference strains of species in the genera involved (Blair et al., 2008; de Cock et al., 2015) were retrieved from GenBank (Table S1) and included in the phylogenetic analyses. Phylogenetic reconstructions by maximum likelihood (ML) and Bayesian inference (BI) were carried out using MEGA v. 7.0 and MrBayes v. 3.1.2, respectively. For ML analyses, the trees were inferred using nearest-neighbour-interchange as a heuristic method and gaps were treated as partial deletion with a 95% site coverage cut-off. Phylogeny support for internal branches was assessed by 1,000 ML bootstrapped pseudoreplicates and bootstrap support (bs) ≥70 was considered significant. BI analyses included two parallel runs of 5,000,000 generations, with the stop rule option and a sampling frequency set to every 1,000 generations. The 50% majority rule consensus trees and posterior probability (PP) values were calculated after discarding the first 25% of the samples as burn-in. The resulting trees were plotted using FigTree v. 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree) and edited in Adobe Illustrator CS3. Before being concatenated, individual gene data sets were assessed for incongruence by checking their individual phylogenies for conflicts between clades with significant ML and BI support (Mason-Gamer & Kellogg, 1996). Once topologies were found to be congruent, we proceeded with the concatenation. Evolutionary models were calculated using MrModelTest v. 2.3 selecting the best-fit model for each data partition according to the Akaike criterion.

### 2.3 | Morphological characterization

Plugs of oomycete monosporic cultures were transferred to V8 medium and potato-carrot medium (PCM) (Crous et al., 2009) and incubated at 27 ℃ in the dark.

After 7 and 14 days' incubation, the colony appearance, colour (Ridgway, 1912), and growth pattern were described. After 14 days, microscopic characteristics were assessed. Colour and presence of septa in the mycelium were evaluated; 30 sporangia and 30 chlamydospores were measured (when present), and these results were compared with previous morphological descriptions of oomycetes (de Cock et al., 2015; Erwin & Ribeiro, 1996; Fuller & Jaworski, 1986).

Some isolates did not show sporulation during the 14 day period in the previously described media and so several methods for sporulation induction were assayed. Nonsporulated isolates were transferred to modified soil extract agar (boiled solution of soil 100 ml/L, agar 15 g/L), water agar (agar 14 g/L), water agar with carnation leaves, and water agar with grass blades, a method that has been proved to be effective to induce sporulation in oomycetes (Mendoza & Prendas, 1988). Also, plugs of isolates were submerged in distilled water amended with hemp seeds or grass blades on the surface. Isolates were incubated at 27 ℃ for solid media and 19 ± 2 ℃ for water. Microscopic features were checked every five days for 60 days.

## 3 | RESULTS

A total of 146 isolates were obtained from the different types of samples (Table 2). Preliminary identification based on morphological

**TABLE 2** Number of isolates of oomycetes associated with cacao crops, classified according to taxonomic identification and type of tissue or sample from which they were obtained

| | | Number of isolates | | | | | |
|---|---|---|---|---|---|---|---|
| **Genus** | **Species** | **Pod** | **Leaf** | **Stem** | **Root** | **Soil** | **Total** |
| *Phytophthora* | *P. palmivora* | 47 | 19 | 12 | — | 1 | 79 |
| | *Phytophthora* sp. | 5 | 2 | — | — | — | 7 |
| | *P. nicotianae* | 1 | — | — | — | — | 1 |
| | Total | 53 | 21 | 12 | — | 1 | 87 |
| *Phytopythium* | *Phy. chamaehyphon* | — | — | 3 | 5 | 14 | 22 |
| | *Phy. cucurbitacearum* | — | — | — | 2 | 11 | 13 |
| | *Phy. vexans* | — | 1 | 1 | 4 | 4 | 10 |
| | *Phy. helicoides* | — | — | — | — | 1 | 1 |
| | *Phytopythium* sp. 1 | — | — | 1 | 1 | — | 2 |
| | *Phytopythium* sp. 2 | — | — | — | — | 2 | 2 |
| | *Phytopythium* sp. 3 | — | — | 1 | — | — | 1 |
| | Total | — | 1 | 6 | 12 | 32 | 51 |
| *Globisporangium* | *G. splendens* | — | — | — | — | 5 | 5 |
| *Pythium* | *P. deliense* | — | — | — | — | 2 | 2 |
| | *P. inflatum* | — | — | — | — | 1 | 1 |
| | Total | — | — | — | — | 3 | 3 |
| | Total per sample type | 53 | 22 | 18 | 12 | 41 | 146 |

—, no isolates obtained.

description and the ITS BLASTn search showed that the isolates were distributed in four oomycete genera: *Phytophthora* (60%), *Phytopythium* (35%), *Globisporangium* (3%), and *Pythium* (2%), as shown in Table 2. Phylogenetic analyses for each genus were done in order to identify the corresponding species; we found 13 species, from which we were able to identify nine (Table 2) as explained below. All sequences are available in GenBank (Table S2). The length, number of phylogenetic informative and variable sites, and substitution models (for ML) for each data set are summarized in Table 3.

All the isolates were deposited in the fungal collection of the Natural History Museum of the Universidad de Los Andes, Bogotá, Colombia (catalog numbers in Table S2) and are included in "Contrato de Acceso a Recursos Genéticos y sus productos derivados" no. 305, 05/02/ 2021.

### 3.1 | Species identification of *Phytophthora*

For isolates representing the genus *Phytophthora* (*n* = 87), the topology of the phylogenetic tree showed well-delimited clades inside the genus that correspond to the clades proposed by Blair et al. (2008). The *Phytophthora* isolates were distributed into the three clades 1, 2, and 4 (Figure 2). Most of the isolates were placed in clade 4 along with *P. palmivora* (*n* = 79, 92%) with a bootstrap support value/Bayesian posterior probability score (BS/BPPS) of 100/1. One isolate was placed in clade 1 and was identified as *P. nicotianae* (1%) with BS/BPPS of 100/1. The other isolates belonged to a species that could not be identified (*n* = 7, 8%) but were clustered within clade 2 of the genus *Phytophthora* and formed a delimited group with a BS/BPPS of 85/—. *Phytophthora* isolates were obtained from all sample types except from roots (Table 2).

Overall, *P. palmivora* isolates exhibited the same morphology; the colonies on V8 agar were white (colour 19, YO-Y according to Ridgway, 1912) with a cottony appearance and a stellate pattern of growth (Figure 3a–g). Compared to the morphology obtained on PCM, the colony was less fluffy, but overall, the morphology was very similar.

The microscopic characteristics of *P. palmivora* isolates were generally similar among the used media. Sporangia were prominently papillated and ellipsoid to ovoid in both media (Figure 3). On V8 agar, the average length × width was 38.6 (*SD* 8.1) × 23.8 (*SD* 5.6) µm, with a length:width (l:w) ratio of 1.68 (*SD* 0.42), and on PCM the width was similar (average 27.2, *SD* 4.6) but the length was noticeably longer: average 44.1 (*SD* 9.6) µm (Figures S1 and S2) although the l:w ratio was similar to that obtained on V8, 1.65 (*SD* 0.39). In both cases, the sporangia were caduceus with short pedicels, 2.6 µm (*SD* 0.9) on V8 and 3.0 µm (*SD* 0.7) on PCM, and were produced in simple sympodial sporangiophores. Abundant chlamydospores were found in intercalary and terminal positions with an average diameter of 26.9 (*SD* 5.5) on V8 and 30.1 (*SD* 6.8) µm on PCM (Figure S3). Hyphae were slightly septate and swellings were not observed overall. All these characteristics mostly agreed with morphological descriptions of the species, but curiously, the sporangia on V8 were smaller than previously reported in common guides (Erwin & Ribeiro, 1996), although inside the range reported for Colombian cacao crops (Rodríguez-Polanco et al., 2020).

The *P. nicotianae* isolate had a white colony with cottony appearance and a radiate semistellate pattern of growth (Figure 3h–k). This morphology did not change on the different media tested (Figure S4). This isolate had papillated, ovoid-pyriform sporangia and sometimes the papilla was not in apical position (Figure 3i). The sporangia were produced on branched sporangiophores and were mainly noncaduceus. They had an average length × width of 33.7 (*SD* 4.2) × 27.2 (*SD* 3.3) µm with an average l:w ratio of 1.25 on V8; on PCM, they measured 33.2 (*SD* 3.6) × 27.1 (*SD* 3.7) with an average ratio of 1.24 (*SD* 0.2). Abundant chlamydospores were observed, with an average diameter of 30.1 (*SD* 3.9) µm on V8 and 31.5 (*SD* 4.1) µm on PCM. Hyphae were slightly septate, and swelling was rarely observed. All these characteristics agreed with the corresponding morphological description of the species (Erwin & Ribeiro, 1996). The isolate was obtained from a pod sample.

**TABLE 3** Overview of models and details for the phylogenetic analyses of *Phytophthora*, *Phytopythium*, *Pythium*, and *Globisporangium*

| Data set | | *Phytophthora* | *Phytopythium* | *Pythium* | *Globisporangium* |
|---|---|---|---|---|---|
| ITS | Length (bp) | 961 | 979 | 925 | 1,244 |
| | Pvar | 573 | 574 | 428 | 620 |
| | Pi | 433 | 387 | 227 | 457 |
| | Model[a] | HKY + G | HKY + G | HKY + G | TN93 – G |
| *coxI* | Length (bp) | 665 | 682 | 680 | 680 |
| | Pvar | 185 | 202 | 165 | 145 |
| | Pi | 148 | 147 | 108 | 84 |
| | Model[a] | GTR + G + I | GTR + G | GTR + G + I | GTR + G + I |
| Concatenated | Length (bp) | 1,628 | 1,661 | 1,605 | 1,924 |
| | Pvar | 758 | 534 | 593 | 765 |
| | Pi | 581 | 335 | 335 | 541 |
| | Model[a] | GTR + G + I | GTR + G + I | GTR + G | GTR + G |

ITS, internal transcribed spacer; *coxI*, cytochrome oxidase subunit I; Pvar, variable sites; Pi, phylogenetic informative sites.
[a]Maximum-likelihood substitution models.

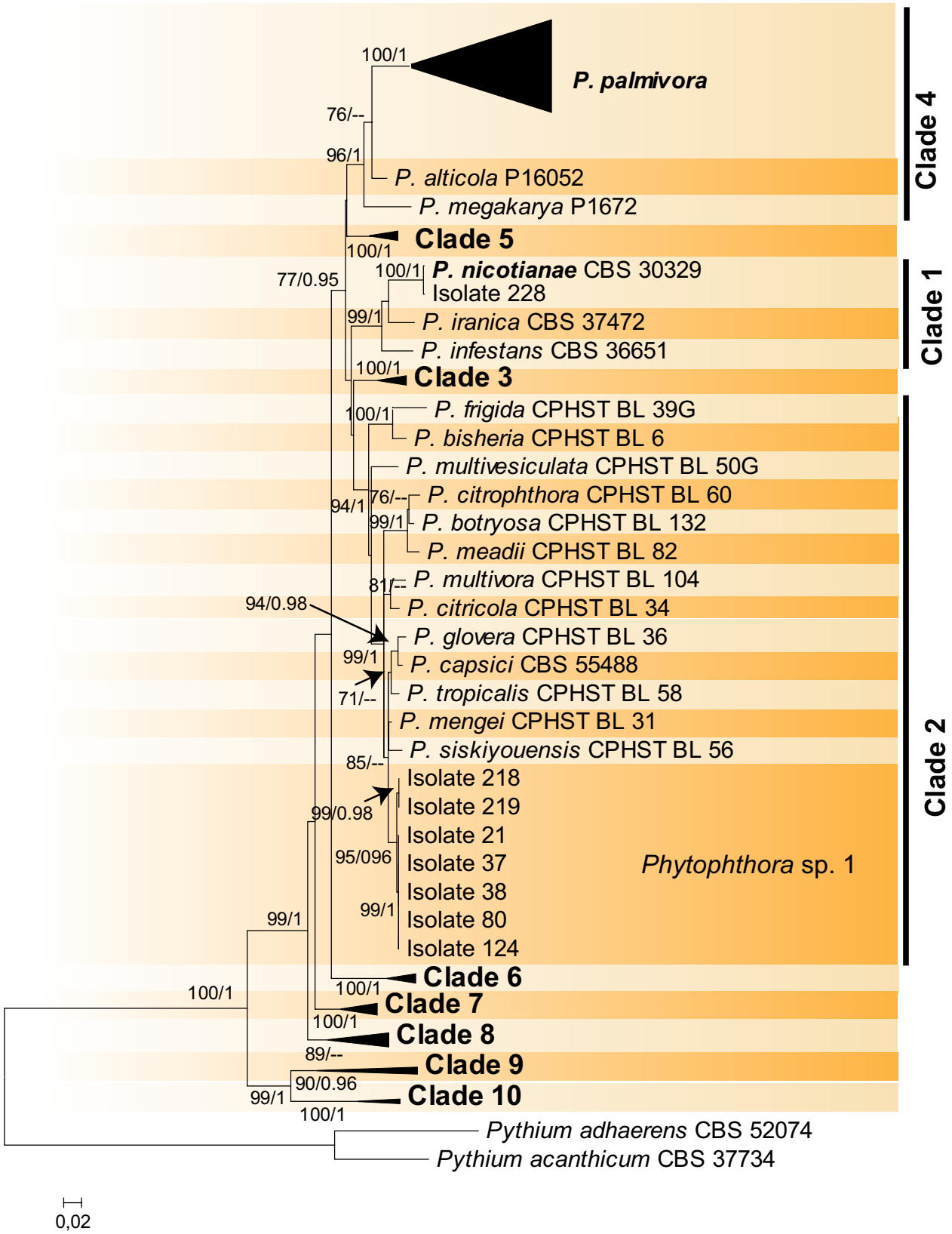


**FIGURE 2** Maximum-likelihood tree showing the distribution of *Phytophthora* isolates from cacoa relative to reference species, constructed using concatenated sequences from cytochrome oxidase subunit I (*coxI*) and the internal transcribed spacer (ITS). The *Phytophthora palmivora* branch is collapsed and contains reference strain CBS 29829 and 79 isolates. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores above 70%/0.95 are indicated on the nodes. The tree is rooted to *Pythium acanthicum* CBS 37734 and *Pythium adhaerens* CBS 52074

*Phytophthora* isolates that could not be identified were isolated from pods and leaves, and formed a defined group in the phylogenetic analysis, sharing the same morphological features. The colonies of these isolates were white, cottony with a rosette semistellate pattern of growth in both media used (Figure 3l–o, Figure S5). Sporangia were noncaduceus, elongated, became prominently papillated with age, and seemed catenulate or were at the end of catenulate hyphal swellings. They had a length × width of 43.8 (*SD* 10) × 23.9 (*SD* 5.6) µm and their average l:w ratio was 1.9 (*SD* 0.5) when grown on V8 medium. On PCM, they measured 49.5 (*SD* 7.1) × 26.9 (*SD* 5.9) µm with an average l:w ratio of 1.9 (*SD* 0.5). Chlamydospores were present, with an average diameter of 38.7 (*SD* 18.6) µm on V8, and 24.7 (*SD* 8.5) µm on PCM. Hyphae were slightly septate and hyphal swelling was abundant in both media. Neither of these isolates matched any published DNA sequence in GenBank or any published morphological description.

### 3.2 | Species identification in the *Phytopythium*, *Globisporangium*, and *Pythium* genera

For the *Phytopythium* isolates (*n* = 51), a phylogenetic tree with all the reference strains of the species reported for the genus was made (data not shown); however, not all species were phylogenetically closely related to our isolates and consequently those were eliminated. A final phylogenetic analysis (Figure 4) showed that most of the isolates of *Phytopythium* were distributed in four species (see also Table 2): *Phy. chamaehyphon* was the most frequent (*n* = 22, 43%), followed by *Phy. cucurbitacearum* (*n* = 13, 25%), *Phy. vexans* complex (*n* = 10, 20%), and *Phy. helicoides* (*n* = 1, 2%). Five isolates (42, 55, 70, 94, and 116) could not be identified at the species level (*n* = 5, 10%). The isolates 70 and 42, isolated from root and stem, respectively, formed an independent, well-supported branch near the clade of *Phy. helicoides*; therefore, they were not considered to belong to that species, but to be related to it. In contrast, isolate 90, which formed a single branch with the reference strain *Phy. helicoides* CBS 286.31, was considered to belong to that species. We performed an additional phylogenetic analysis (data not shown) to evaluate the intraspecific variability of this species, but the only additional strain in the GenBank database was CBS 16768 and the same results were obtained. The isolates 55 and 94, both isolated from soil, and 116, isolated from stem, were related to the *Phy. vexans* complex, but 55 and 94 clustered in a different, well-supported branch from the reference sequence, and 116 was separated from all sequences including the reference sequence. For all these reasons,

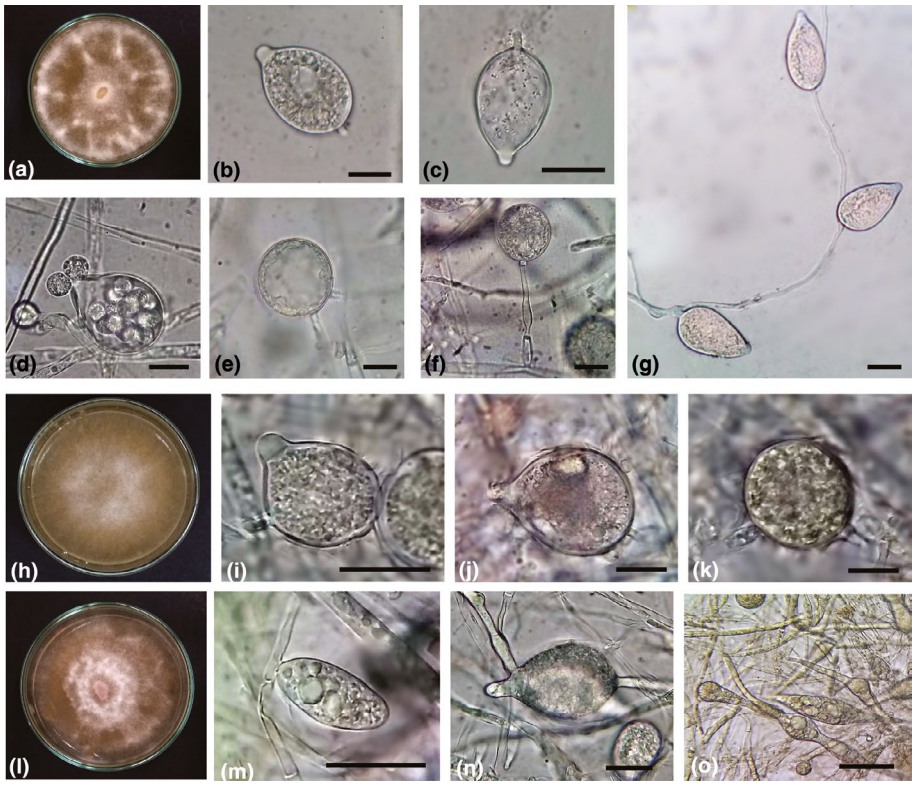


**FIGURE 3** Morphological features of *Phytophthora* isolates from cacao on V8 agar. (a–g) *Phytophthora palmivora* isolates. (b), (c), (e), and (f) correspond to isolate 5, (d) isolate 107, (g) isolate 87. (a) Colony on V8 agar, cottony appearance and stellate pattern; (b,c) sporangia with pedicel; (d) sporangium and zoospores; (e) intercalary chlamydospore; (f) terminal chlamydospore; (g) sympodial sporangiophore. (h–k) *Phytophthora nicotianae* isolate. (h) Colony on V8 agar, cottony appearance and radiate semistellate pattern; (i,j) papillated sporangia; (i) excentric papillated sporangium; (k) intercalary chlamydospore. (l–o) *Phytophthora* sp. isolate 21. (l) Colony on V8 agar, cottony appearance and semistellate, rosette pattern; (m) young papillated sporangium; (n) noncaduceous papillated sporangium; (o) chlamydospores and catenulate hyphal swellings. Scale bars =20 µm

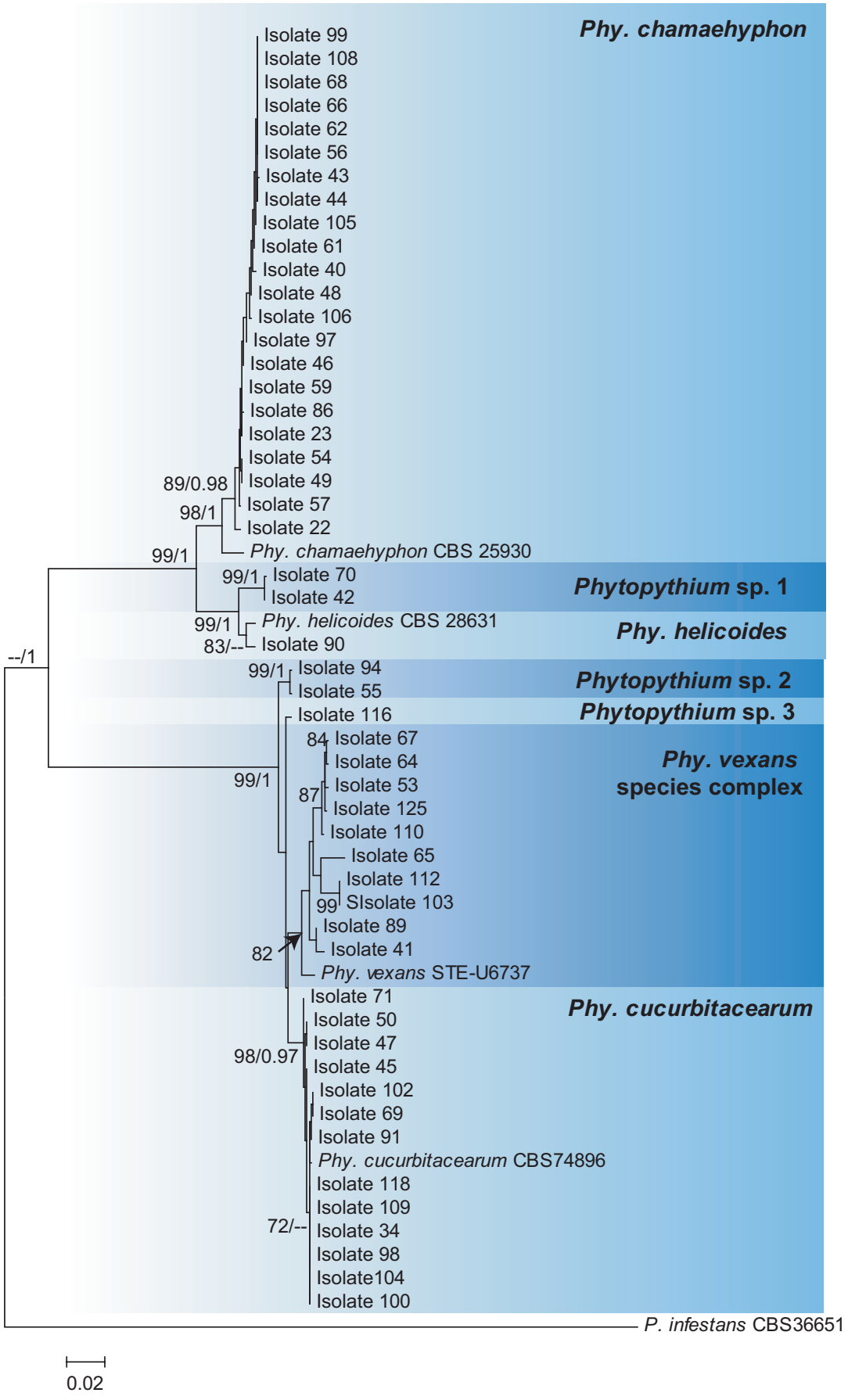
Phy. chamaehyphon
Isolate 99
Isolate 108
Isolate 68
Isolate 66
Isolate 62
Isolate 56
Isolate 43
Isolate 44
Isolate 105
Isolate 61
Isolate 40
Isolate 48
Isolate 106
Isolate 97
Isolate 46
Isolate 59
Isolate 86
Isolate 23
Isolate 54
89/0.98
Isolate 49
98/1
Isolate 57
Isolate 22
99/1
Phy. chamaehyphon CBS 25930
99/1
Isolate 70
Isolate 42
Phytopythium sp. 1
99/1
Phy. helicoides CBS 28631
Phy. helicoides
83/--
Isolate 90
--/1
99/1
Isolate 94
Isolate 55
Phytopythium sp. 2
Isolate 116
Phytopythium sp. 3
84
Isolate 67
Isolate 64
Phy. vexans
species complex
99/1
87
Isolate 53
Isolate 125
Isolate 110
Isolate 65
Isolate 112
99
SIsolate 103
Isolate 89
82
Isolate 41
Phy. vexans STE-U6737
Isolate 71
Phy. cucurbitacearum
Isolate 50
Isolate 47
98/0.97
Isolate 45
Isolate 102
Isolate 69
Isolate 91
Phy. cucurbitacearum CBS74896
Isolate 118
Isolate 109
72/--
Isolate 34
Isolate 98
Isolate104
Isolate 100
P. infestans CBS36651
0.02

**FIGURE 4** Maximum-likelihood tree showing the distribution of *Phytopythium* spp. isolates from cacoa relative to reference species, constructed using concatenated sequences of the internal transcribed spacer (ITS) and cytochrome oxidase subunit I (*coxI*). Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores above 70%/0.95 are indicated on the nodes. The tree is rooted to *Phytophthora infestans* CBS 36651

these isolates were considered to be a different species. In general, the isolates belonging to this genus were isolated mostly from roots and soil samples, some were also obtained from stems and leaves, but the genus was not found in pod samples.

Regarding the morphological identification, isolates of *Phytopythium* could not be easily identified because they did not sporulate well on V8, PCM, or any of the media used. It was only possible to observe some microscopic features. The colonies had similar morphology: all the isolates had white (colour 19, YO-Y according to Ridgway, 1912) cottony colonies, and a radial growth pattern (Figure 5).

For the *Phy. chamaehyphon* isolates, it was possible to observe some sporangia. These were papillated and were observed in terminal and intercalary positions; abundant terminal and intercalary spherical chlamydospores were also observed, with a 15–25 µm diameter (Figure 5a–d). The formation of sufficient sporangia could not be induced to give a reliable estimation of their length or width. Hyphae were slightly septate. It was not possible to observe a germinal tube production or zoospore release. For the other isolates of this genus, a few features were observed, such as intercalary papillate sporangia and sexual structures (Figure 5e–h). It was not possible to observe germinal tube production or zoospore release in any isolate, even after 60 days' incubation.

The isolates belonging to the genus *Globisporangium*, defined by Uzuhashi et al. (2010) from former E–I clades of the *Pythium* genus (de Cock et al., 2015; Lévesque & de Cock, 2004), corresponded to one species, *Globisporangium splendens* (*n* = 5, 100%). For these isolates a tree was constructed with the corresponding reference sequences listed in Table S1 (Figure 6); this tree showed that the identity of all five isolates was *G. splendens*. All the isolates from this species came from soil samples. Colonies were white (colour 19, YO-Y according to Ridgway, 1912) and cottony, and hyphae were slightly septate. Sporangia or sexual structures were not observed in any of the tested media even after 60 days' incubation.

For the isolates belonging to the *Pythium* (*n* = 3) genus, a tree was constructed with the corresponding reference sequences included in Table S1 (Figure 7). Two species could be identified: *Py. deliense* (*n* = 2, 67%) and *Py. inflatum* (*n* = 1, 33%) belonging to the clades A and B, respectively. These clades were proposed by Lévesque and de Cock (2004) and modified by de Cock et al. (2015). All the isolates were obtained from soil samples. Colonies were white (colour 19, YO-Y according to Ridgway, 1912) and cottony, and hyphae were slightly septate. Nonetheless, sporangia or sexual structures were not observed in any of the tested media, even after 60 days' incubation.

## 4 | DISCUSSION

This is the first study describing the genera and species of oomycetes associated with cacao crops in Colombia. Results show that not only *P. palmivora*, but also several oomycete species are associated with symptomatic plants of cacao and they are distributed into four different genera.

The genus *Phytophthora* was not only the most abundant among the isolates, but also at least three species were associated with Colombian cacao crops, contrary to the results obtained by Rodríguez-Polanco et al. (2020). Neither *P. nicotianae* nor a species similar to the unidentified species found in this work have been previously reported in cacao crops of the American continent. It is interesting that not a single isolate obtained in this study belonged to the other species reported in America, such as *P. megasperma*, *P. capsici*, *P. citrophthora*, *P. tropicalis*, or *P. hevea* (Faleiro et al., 2003; Molina et al., 2016). This gives insights into the distribution of the *Phytophthora* spp. associated with cacao in the American continent. Nonetheless, the presence of these other species cannot be completely disregarded in Colombian cacao crops as they might be present in very low frequency and therefore could not be isolated in the present study.

The most abundant species isolated was *P. palmivora*, which was isolated from all sample types except roots. The fact that this species was isolated most abundantly from pods and leaves reflects the importance of aerial spread of this pathogen. Therefore, diseased aerial parts must be removed and destroyed to avoid secondary infections in the field or neighbouring fields. One isolate came from soil, indicating soil may be an important reservoir for this pathogen. As this species has been found so abundantly associated with diseased crops, the characterization of its populations and the design of control strategies are essential.

Although *P. nicotianae* has been previously reported infecting cacao in Asia (Suwastika et al., 2019), no reliable report had been made in cacao from America. Overall, this species has not been considered to be of economic importance for cacao production and it is more common to find it in other crops, such as those from the genera *Citrus* and *Nicotiana* (Biasi et al., 2016). However, it was not very surprising to find this species infecting cacao, because *P. nicotianae* is one of the most aggressive oomycete plant pathogens, with a wide host range that includes more than 255 species over five continents and a wide diversity of climates (Panabières et al., 2016). More work is needed to determine the pathogenicity of this species on cacao in Colombia and to understand the extent to which it contributes to disease symptoms.

One potentially new species belonging to the clade 2 of *Phytophthora* was isolated. The phylogenetic analysis performed showed a distinguishable group that did not resemble any of the other *Phytophthora* spp. found. The group was closely related to *P. siskiyouensis* and other species of the clade, but differed from these species in their ITS and *coxI* sequences as well as their morphological features. The morphological characteristics of these unidentified

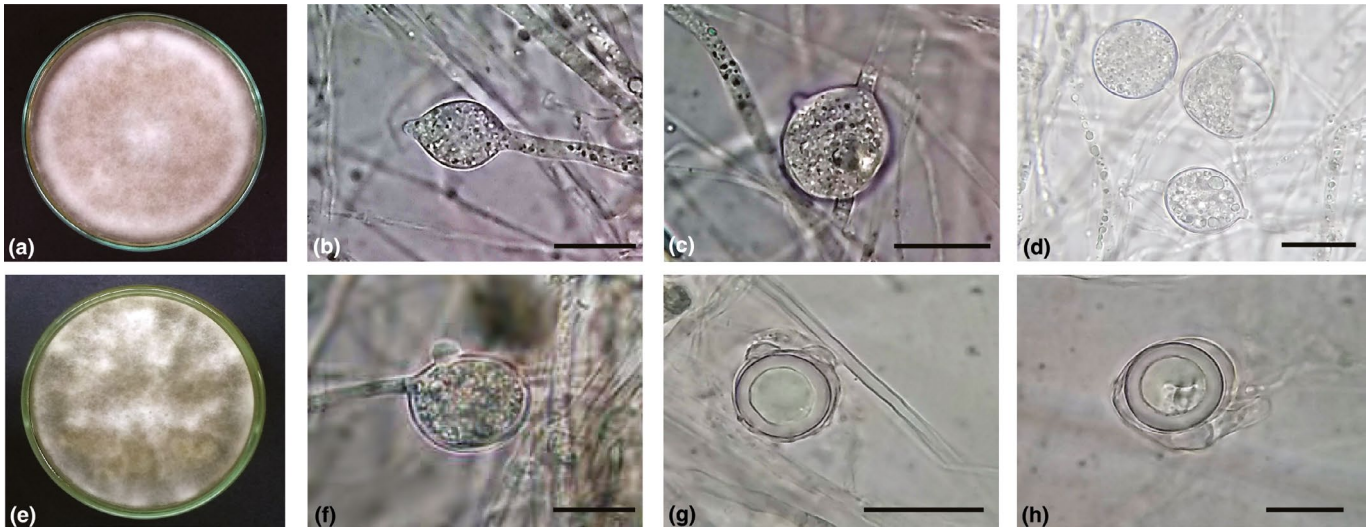


FIGURE 5 Morphological features *Phytopythium* isolates from cacao. (a–d) *Phytopythium chamaehyphon* isolate 99. (a) Colony on V8 agar, cottony appearance radiate pattern; (b) papillate terminal sporangium in water with grass blades; (c) intercalary papillate sporangium in water with grass blades; (d) chlamydospores on V8 agar. (e–h) Morphological features of some unidentified *Phytopythium* species. (e–g) Isolate 94; (e) colony on V8 agar, cottony appearance radiate pattern; (f) intercalary papillate sporangium in water with grass blades; (g) oospore and bilobate antheridium; (h) isolate 116, elongated antheridium. Scale bars = 20 µm

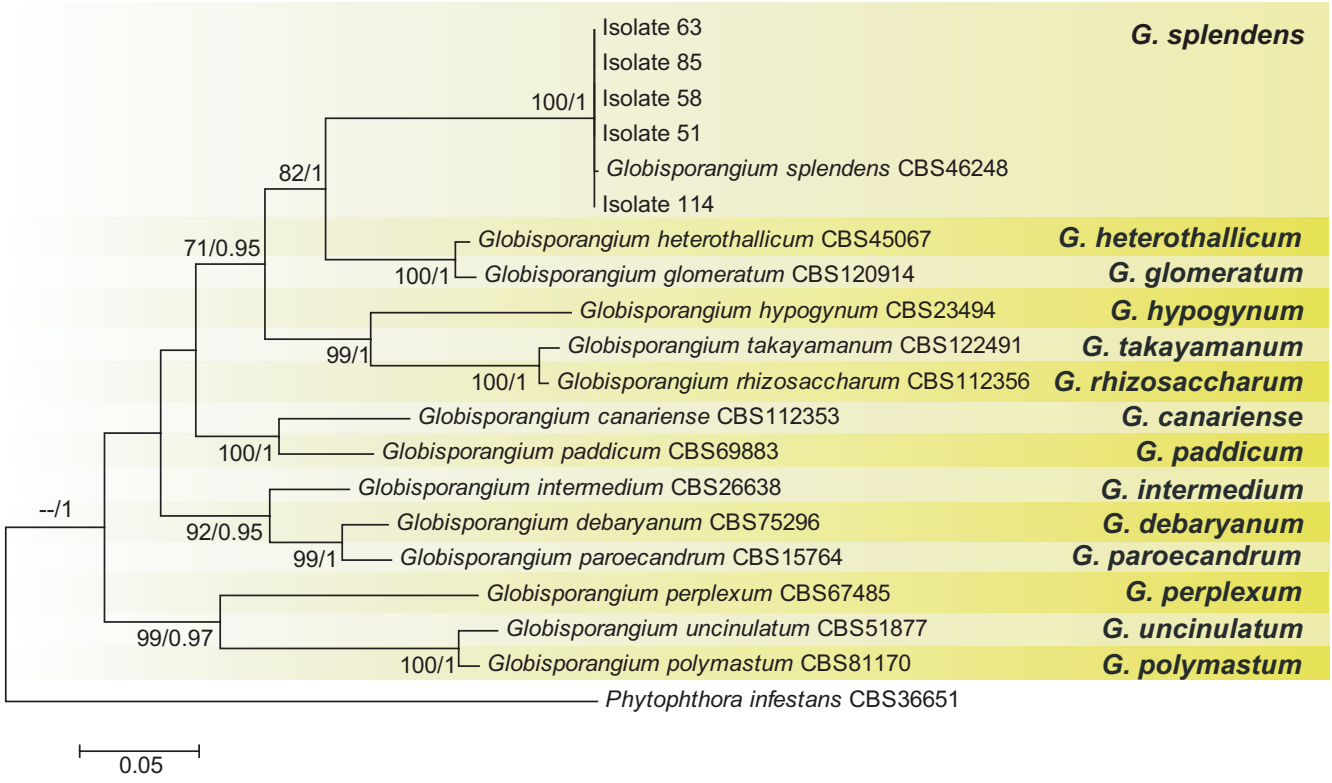


FIGURE 6 Maximum-likelihood tree showing the distribution of isolates from cacao of the *Globisporangium* genus, relative to reference species, constructed using internal transcribed spacer (ITS) and cytochrome oxidase (*coxI*) concatenated sequences. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores above 70%/0.95 are indicated on the nodes. The tree is rooted to *Phytophthora infestans* CBS 36651.

isolates were more similar to those of other relatively newly described species, such as *Phytophthora* taxon *castitis* from clade 8 (Bertier et al., 2013) and *P. litoralis* from clade 6 (Jung et al., 2011), because they produced sporangia at the end of catenulate hyphal swellings or had catenulate sporangia. However, the rest of their features, such as macroscopic appearance or sporangia size, were not similar.

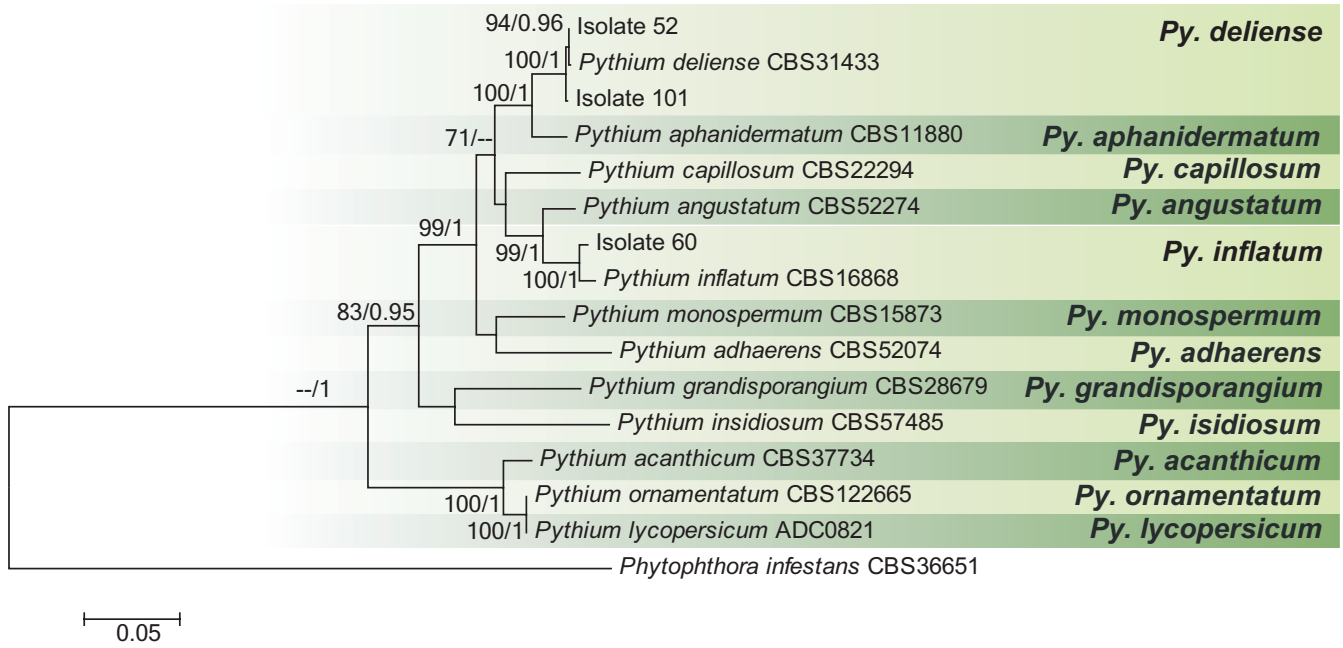


**FIGURE 7** Maximum-likelihood tree showing the distribution of isolates from cacao of the *Pythium* genus, relative to reference species, constructed using internal transcribed spacer (ITS) and cytochrome oxidase (*coxI*) concatenated sequences. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores above 70%/0.95 are indicated on the nodes. The tree is rooted to *Phytophthora infestans* CBS 36651

It is interesting that this species was not isolated from one single location but from five different farms distributed in three geographically distant departments (minimum 250 km, maximum 500 km), and was found with a relatively high frequency, considering the low number of samples per type that were obtained from each farm. Also, it is worth noting that this species was isolated mostly from pods, which may indicate that this is an important species associated with Colombian cacao crops that may be playing a significant role in black pod disease, requiring further investigation.

As shown in the results, none of the isolates was identified as *P. megakarya*. This is an important result because this pathogen, originally from Cameroon/Nigeria in Africa, has devastated cacao crops in other regions of Africa and its spread to other locations needs to be prevented (Akrofi, 2015).

The genus *Phytopythium* was present not only in soil and roots, where it is typically found (Choudhary et al., 2016), but was also isolated from samples of necrotic leaves and vascular section of stems with canker. This is an interesting result because it indicates this species has a role in diseases commonly associated with *Phytophthora* spp. There is a precedent of a similar phenomenon in rubber trees studied by Zeng et al. (2005). They addressed the controversy of the identity of the causal agent of rubber stem canker and concluded that, even though *Phytophthora* spp. are the most acknowledged aetiological cause, species of *Phytopythium* such as *Phy. vexans* play a role in the progression of the disease initiated by *Phytophthora* and can also act as a primary causal agent of disease. There may be a similar scenario in cacao; thus, the question of whether *Phytopythium* is contributing to diseases in cacao that show symptoms of *Phytophthora* spp. infections needs further investigation.

Some of the isolates of *Phytopythium* were particularly difficult to place in a taxonomic group and were assigned to three unidentified species. However, two of them were related to the species *Phy. vexans* for which the taxonomic and phylogenetic status is not very clear, according to de Cock et al. (2015). Those authors suggested that *Phy. vexans* is an unresolved complex that has not been completely described using morphological characters or molecular markers such as ITS, *coxI*, small subunit (SSU), or large subunit ribosomal ribonucleic acid (LSU) sequences. This may be one reason why two of the *Phytopythium* species could not be identified in the present study. The other reason, which also applies for the third unidentified species, is the absence of sporulation of the isolates that made it impossible to describe their morphology fully.

Many isolates formed a well-supported clade with the *Phy. cucurbitacearum* reference sequences. This species is not a valid taxon because the ex-type is no longer viable and it lacks a complete Latin diagnosis (de Cock et al., 2015). Despite this, its ITS and *coxI* sequences are available in the GenBank database, making it possible to include it in this study. Although de Cock et al. (2015) considered this species as part of the *Phy. vexans* complex, our results support the hypothesis that this taxon is different from *Phy. vexans*. It is necessary to carry out a lectotyping process along with complete Latin diagnosis in the future to validate the taxon. Another noteworthy result regarding the *Phytopythium* genus is that this is the first report of *Phy. chamaehyphon* in the country and the isolate was collected from the aerial part of the plant.

Further study of this oomycete and its role in the Colombian cacao agrosystems might be very valuable.

For the *Pythium* and *Globisporangium* isolates, the molecular identification was quite conclusive, but, as for *Phytopythium* isolates, other methods must be implemented to induce sporulation in the future to supplement genetic data with morphological data.

Besides the possibility that *Phytopythium* might play a role in pod canker and leaf blight of cacao, this study has shown that all *Phytopythium*, *Pythium*, and *Globisporangium* isolates were found abundantly below the soil surface, in rhizosphere soil and roots; thus, there is a possibility that they may be relevant for this crop as soilborne pathogens causing damping-off of seedlings, as reported for other crops (Rai et al., 2020; Uzuhashi et al., 2010).

In general, this study achieved the identification of nine different species of oomycetes associated with Colombian cacao crops, most of which have not been previously reported in cacao crops of Colombia or any other country.

New findings presented here provide a better understanding of cacao Colombian agrosystems and should lead to further studies. Specifically, pathogenicity tests for the species found need to be performed, particularly for those that have not previously been reported in cacao crops. In addition, we strongly recommend further study of *P. palmivora* populations due to the abundance of this species, its wide distribution all over the country, and its association with cacao diseases. The presence of this pathogen in aerial parts of the plant, pods, and leaves, highlights the importance of continuous inspections of the crops and elimination of all diseased tissues to avoid secondary infections.

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## DATA AVAILABILITY STATEMENT

The ITS and *coxI* sequences of the isolates that support the findings of this study are openly available in NCBI at GenBank (https://www.ncbi.nlm.nih.gov) under the accession numbers provided in Table S2.

## ORCID

*Julie Ramírez Martínez* https://orcid.org/0000-0001-7589-5900
*Martha Cárdenas Toquica* https://orcid.org/0000-0002-2009-4126

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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