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RESEARCH ARTICLE

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# Morphological, molecular and pathogenic characterization of *Phytophthora palmivora* isolates causing black pod rot of cacao in Colombia

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## Abstract

*Aim of study:* To characterize isolates of *Phytophthora sp.* causing black pod rot (BPR) of cacao (*Theobroma cacao* L.).

*Area of study:* Eight cocoa-growing regions in Colombia.

*Material and methods:* Sixty isolates of *Phytophthora sp.* were obtained from tissues of cacao pods showing symptoms of BPR. Isolates were characterized using the morphology of sporangia and chlamydospores, molecular sequencing of regions of nuclear DNA (rDNA-ITS) and mitochondrial (COX) and virulence in different genotypes of cocoa pods.

*Main results:* A high phenotypic variability between the isolates was determined, being the pedicel length and the length/width ratio (L/W) the most stable characters for species identification. Short pedicels with an average of 3.13 µm ± 0.28 and a length/width ratio of sporangia (L/W) with an average of 1.55 µm ± 0.11 were established as the most consistent morphological characteristics within *palmivora* species.

*Research highlights: Phytophthora pamivora* was the only species associated to BPR, identified using morphology together with sequence analyses.

**Additional keywords:** *Theobroma cacao* L.; *Phytophthora* sp.; sporangia; pedicel; chlamydospores.

**Abbreviations used:** ADL (average diameter of the lesion); AV8 (agar-V8 juice); BPR (cacao black pod rot disease); ITS (internal transcribed spacer); L/W (length to width ratio)

**Authors' contributions:** ERP: conceived the research project, conceived and designed the experiments, wrote the article. JGM: performed genetic and statistical analyses, critical revision of the manuscript for important intellectual content. MMA: molecular sequence analysis of ITS and Cox regions. MLCG: morphology of the colonies and biometric characteristics of sporangia and chlamydospores and BPR severity of selected *Phytophthora* isolates. JDSA: morphology of the colonies and biometric characteristics of sporangia and chlamydospores BPR severity of selected *Phytophthora* isolates. All authors read and approved the final manuscript.

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## Introduction

It is widely accepted that cacao tree (*Theobroma cacao* L.) is native to tropical forests of northern South America and was later introduced by man in Central America (Motamayor *et al.,* 2002). Most cultivated varieties are generally susceptible to pests and diseases that cause large losses and limit cacao sustainability (Acebo *et al.,* 2012; Gutiérrez *et al.,* 2016; Marelli *et al.,* 2019). Cacao black pod rot disease (BPR), caused by various species of

*Phytophthora,* occurs in most cacao growing areas around the world (Ploetz, 2016). It is estimated that about 30% of cacao production is lost by BPR disease (Drenth & Guest, 2004; Ndoumbe *et al.,* 2004), corresponding to US$ 1.6 billion in 2017 (Bymolt *et al.,* 2018).

Of the various species of the microbial oomycete *Phytophthora* that cause BPR, only *P. palmivora* Butler, shows a global distribution. *P. megakarya* Brasier and Griffin, which is confined to the African continent, is considered the most destructive pathogen of BPR. *P. capsici* Leonian and *P. citrophthora* Leonian are present in Central and South America, and *P. capsici* has been identified also in Camerún, Africa (Zentmyer *et al.,* 1981; Kellam & Zentmyer, 1986); *P. hevea* Thompson has been reported in Malaysia (Turner, 1961) and México (Lozano & Romero, 1974); *P. megasperma* Drechsler in Venezuela and Cuba (Reyes & Capriles, 2000); and *P. arecae* Coleman, in India and Sri Lanka (Stamps *et al.,* 1990).

BPR may cause 100% of crop losses in the tropics during environmental conditions favourable to disease development or poor management practices (Guest, 2007). Cacao is susceptible to BPR during all stages of plant development. Main symptoms include necrotic spots of grizzly color in seedlings, stem canker and foliar spots in mature trees, brown necrotic spots in the pod Surface and rot of beans reducing production and affecting cacao quality (Guest, 2007).

*Phytophthora* identification at the species level is usually made by morphological characteristics and the length to width ration of sporangia, pedicel length and other structures of the microorganism and corresponding colonies (Wilson, 1914; Al-Hedaithy & Tsao, 1979; Brasier & Griffin, 1979; Appiah *et al.,* 2003; Erwin & Ribeiro, 2005). In cacao, colony pattern and growth rate have been used as an approach for identification of *Phytophthora* species causing BPR (Appiah, 2001; Appiah *et al.,* 2003). However, accurate identification based only in morphology is sometimes difficult because of the large variability observed in the populations of different species associated to cacao trees (Brasier *et al.,* 1981; Erwin & Ribeiro, 1996).

In recent decades the number of species classified in the genus *Phytophthora* have increased at an accelerated rate, mainly because more researchers are interested in this important genus that affects not only crops but plants in natural ecosystems and natural evolution of new species (Ersek & Ribeiro, 2010). A number of biochemical and molecular techniques have been used as support for morphological identification of *Phytophthora* species such as electrophoretic patterns of proteins (Bielenin *et al.,* 1988), isozyme analysis (Oudemans & Coffey, 1991), restriction fragment length polymorphisms (RFLP) and other molecular approaches (Förster *et al.,* 1990). Recently, sequence analysis of mitochondrial and genomic genes and regions or whole genome has become widely used, mainly due to the availability of high throughput techniques and lower costs. The internal transcribed spacer (ITS) region of rRNA, the elongation factor (EF1a), β-tubulin, CoxI, Cox II and NADH subunit I genes and regions have shown to be useful for identification of different species of *Phytophthora* (Coulibaly *et al.,* 2018; Maizatul-Suriza *et al.,* 2019).

Genetic resistance is the most cost-effective method for disease control; therefore, reliable quantification of BPR progress rate is very important for cacao breeding. BPR development may be measured using inoculation of pods in the laboratory or under field conditions. Since a positive correlation has been observed when comparing results between both methods, it is more appropriate to perform evaluations in the laboratory to avoid disease dissemination and contamination of other trees in cacao orchards (Nyassé, 1997; Pokoua *et al.,* 2008).

Recently, *P. megakarya* has dispersed and displaced *P. palmivora* in Africa, where 55% of cacao is produced in the world, posing a threat to the industry not only in Africa but in other places of cacao production such as Asia and America (Drenth & Sendal, 2004). For this reason, prompt and accurate disease diagnosis and management is key for cacao crop sustainability. In addition, more research on basic biology of causal agents addressing their origin, diversity, biological fitness, epidemiology, ecological relationships and adaptive abilities, is needed to prevent and manage BPR disease appropriately.

In Colombia, *Phytophthora* spp. associated to BPR have not yet been accurately identified and virulence and diversity of pathogen populations is unknown. This knowledge is crucial for cacao breeding for resistance to BPR and disease management. In the present research, morphological characteristics together with molecular sequencing of the ITS-rRNA and Cox DNA regions of Colombian isolates were used to identify the species of *Phytophthora* causing BPR disease in cacao and to determine phenotypic and genotypic diversity. In addition, virulence of selected isolates of *Phytophthora* sp. on different genotypes of cacao, was measured.

# Material and methods

## Isolates

Sixty isolates of *Phytophthora* spp. were collected from eight departments of Colombia where cacao is grown (Table 1 and Table S1 [suppl.]). Tissues of cacao pods showing symptoms of BPR, were incubated in P5ARPH (cornmeal agar amended with 10 mg/L pimaricin, 250 mg/L, ampicillin, 5 mg/L rifampicin, 100 mg/L PCNB and 50mg/L hymexazol) culture media for microorganism isolation and purification following procedures described in Jeffers & Martin (1986).

**Table 1.** Characteristics of colonies, sporangia and chlamydospores of *Phytophthora isolates*.

| Cluster | Isolate code | Colony characteristics | | | | Sporangia characterisitcs | | | | Pedicel lenght (µm) [i] | Chlamydospore diameter (µm) [j] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Growth pattern [a] | Texture [b] | Edge [c] | Aerial development of mycelia [d] | Shape [e] | Lenght (µm) [f] | Width (µm) [g] | Ratio l/w (µm) [h] | | |
| 2 | 11-016 | SCR | SAF | R | E | ELP | 53.538 | 35.787 | 1.496 | 2.929 | 37.121 |
| 2 | 11-018 | SCR | SAF | R | E | OV, ELP | 54.701 | 35.86 | 1.525 | 2.829 | 38.379 |
| 2 | 11-019 | CR | SAF | R | E | LM,OV | 60.203 | 37.067 | 1.624 | 3.063 | 36.917 |
| 2 | 11-021 | ET | SAF | R | E | GL | 56.012 | 37.519 | 1.493 | 2.7 | 39.985 |
| 2 | 11-022 | ET | AF | R | E | ELP | 51.61 | 36.109 | 1.429 | 2.846 | 37.973 |
| 2 | 11-027 | ET | AF | R | E | GL | 51.462 | 34.402 | 1.496 | 2.817 | 40.301 |
| 3 | ANMA259 | ET | AF | R | E | GL | 45.387 | 30.125 | 1.507 | 3.123 | 30.726 |
| 3 | ANMA265 | ES | AF | R | E | LM, OV | 42.689 | 24.091 | 1.772 | 3.18 | 34.604 |
| 3 | ANMT281 | ET | AF | R | M | OV, ELP | 48.685 | 27.702 | 1.757 | 3.508 | 31.043 |
| 2 | ANRE248 | ET | AF | R | E | OV, ELP | 53.172 | 29.666 | 1.792 | 3.415 | 33.51 |
| 2 | ANSR271 | ET | AF | R | E | OBV | 49.833 | 37.379 | 1.333 | 3.366 | 34.244 |
| 3 | ANVE249 | SN | AF | I | E | OV, ELP | 38.253 | 23.745 | 1.611 | 3.434 | 33.267 |
| 3 | ANVE250 | ET | AF | R | E | OBV, ELP | 47.329 | 28.664 | 1.651 | 3.428 | 31.173 |
| 3 | ANYA228 | ET | SAF | R | E | GL, OV | 43.51 | 28.176 | 1.544 | 3.665 | 33.474 |
| 3 | ANYA230 | ET | AF | R | E | OV, ELP | 38.185 | 30.137 | 1.267 | 3.452 | 27.127 |
| 2 | ANYA247 | P | AF | R | E | GL | 46.404 | 35.055 | 1.324 | 3.099 | 42.804 |
| 3 | ARAR153 | ET | AF | R | E | GL | 41.563 | 27.86 | 1.492 | 3.443 | 33.933 |
| 3 | ARAR155 | ET | AF | R | E | OV, ELP | 41.98 | 27.071 | 1.551 | 3.17 | 29.418 |
| 2 | ARSR116 | ET | SAF | R | E | OV, ELP | 47.387 | 33.248 | 1.425 | 2.923 | 38.943 |
| | ARSR121 | | | | | | | | | | |
| 3 | ARSR128 | CR | SAF | R | A | OV, ELP | 41.677 | 23.118 | 1.803 | 3.061 | 31.808 |
| 3 | ARSR133 | SCR | SAF | R | M | LM,OV | 41.964 | 23.65 | 1.774 | 2.835 | 33.098 |
| 1 | ARTA161 | ET | AF | R | E | ELP | 33.765 | 19.032 | 1.774 | 3.01 | 25.226 |
| | ARTA164 | | | | | | | | | | |
| | ARTA173 | | | | | | | | | | |
| 2 | CAMAR318 | SCR | AF | R | E | LM,OV | 59.492 | 37.698 | 1.578 | 3.215 | 35.832 |
| 2 | CAMAR320 | SCR | SAF | R | E | ELP | 54.304 | 35.814 | 1.516 | 3.183 | 36.951 |
| 2 | CAVI300 | SCR | SAF | R | E | GL | 51.669 | 34.183 | 1.512 | 2.914 | 35.863 |
| 2 | CAVI303 | SET | SAF | R | E | OV, ELP | 55.648 | 37.316 | 1.491 | 2.779 | 35.531 |
| 2 | CAVI307 | CR | AF | R | M | LM,OV | 50.152 | 32.947 | 1.522 | 3.285 | 37.992 |
| 2 | CAVI308 | ET | AF | R | E | GL, OV | 55.396 | 35.67 | 1.553 | 3.123 | 31.773 |
| 2 | F1-001 | P | AF | R | E | GL, OV | 56.977 | 35.405 | 1.609 | 3.45 | 32.439 |
| 2 | F2-003 | SET | AF | R | E | OV, | 49.978 | 31.748 | 1.574 | 3.627 | 31.426 |
| 3 | F5-002 | SET | AF | R | E | GL, OV | 45.61 | 28.43 | 1.604 | 3.602 | 31.734 |
| 2 | F5-003 | P | AF | R | E | OV, ELP | 50.405 | 34.709 | 1.452 | 3.386 | 31.624 |
| 1 | HUAL07 | RS | AG | O | A | GL | 23.987 | 16.424 | 1.46 | 3.018 | 21.31 |
| 2 | HUAL12 | ET | SAF | R | E | LM,OV | 52.578 | 36.722 | 1.432 | 3.081 | 38.177 |
| | HUAL18 | | | | | | | | | | |
| 1 | HUGAR60 | SET | AF | R | E | LM,OV | 29.564 | 19.123 | 1.546 | 2.763 | 22.413 |
| | HUGAR63 | | | | | | | | | | |
| 1 | HUGAR70 | ET | AF | O | A | LM,OV | 30.292 | 21.842 | 1.387 | 2.847 | 21.399 |
| | HUGAR71 | | | | | | | | | | |
| 1 | HURV19 | CR | AF | O | M | GL, OV | 27.656 | 17.732 | 1.56 | 3.429 | 20.566 |
| | HUTE39 | | | | | | | | | | |
| 2 | SACAR216 | ET | SAF | R | E | LM,OV | 52.78 | 38.225 | 1.381 | 2.753 | 36.242 |
| 3 | SACAR222 | ET | SAF | R | E | ELP | 43.829 | 32.139 | 1.364 | 2.552 | 33.352 |
| | SAPLY290 | | | | | | | | | | |
| 3 | SARIO189 | SN | SAF | A | E | GL | 43.726 | 27.395 | 1.596 | 3.744 | 32.774 |
| 3 | SARIO197 | ET | SAF | R | E | ELP | 41.771 | 26.369 | 1.584 | 3.283 | 32.672 |
| 3 | SASCH204 | ET | AF | R | E | ELP | 43.188 | 26.744 | 1.615 | 2.745 | 38.736 |
| 3 | SASCH207 | CR | SAF | R | M | ELP, OBV | 41.512 | 23.701 | 1.751 | 3.024 | 23.948 |
| 2 | TOAT113 | ET | SAF | R | M | ELP | 50.147 | 30.397 | 1.65 | 2.957 | 40.418 |
| 2 | TOAT115 | SN | AG | R | M | OV, ELP | 49.845 | 31.229 | 1.596 | 2.851 | 39.465 |
| 2 | TOCHA74 | SET | AF | R | E | OV, ELP | 54.172 | 38.231 | 1.417 | 3.117 | 39.32 |
| 1 | TOCHA78 | ET | AF | O | A | OV, ELP | 27.608 | 18.321 | 1.507 | 3.253 | 21.35 |
| | TORB103 | | | | | | | | | | |
| 1 | TORB104 | ET | SAF | R | E | ELP | 28.093 | 18.106 | 1.552 | 3.258 | 21.658 |
| 2 | TORB90 | P | AF | R | E | OV, ELP | 58.871 | 37.895 | 1.554 | 2.724 | 38.234 |
| | TOVR03 | | | | | | | | | | |
| 3 | TOVR01 | ET | AF | R | M | OV, ELP | 38.931 | 23.423 | 1.662 | 3.261 | 25.269 |

[a] Growth pattern of each colony in AV8 media after 4 days of incubation. ET starry; CR chrysanthemum; SN without defined pattern; P petaloid; SET semi-starry; RS pinkish; SCR semi-pinkish; ES stoloniferous. [b] Colony texture. AF plush; AG cottony; SAF semi-plush. [c] Colony edge. I irregular, O wavy; R regular. [d] Aerial development of mycelia. A abundant; E scarce; M medium. [e] Shape of sporangia. ELP ellipsoid, GL bulbous, LM lemon-shaped, OBV obovoid, OV ovoid. [f] Length of sporangia. Mean calculated from 30 independent measurements. [g] Width of sporangia. Mean calculated from 30 independent measurements. [h] Length to width ratio of sporangia. Mean calculated from 30 independent measurements. [i] Length of pedicel. Mean calculated from 30 independent measurements. [j] Diameter of chlamydospore. Mean calculated from 30 independent measurements. [a,b,c,d]: Mean calculated from data obtained from 4 petri dishes per each isolate after 4 days of incubation of inocula in fresh media. [e,f,g,h,I,j]: Mean calculated from 30 independent measurements.

## Morphology of the colonies and biometric characteristics of sporangia and chlamydospores

Fifty isolates were grown in agar-V8 juice (AV8) in petri dishes (20% v/v V8 vegetable juice, Campbell; 0.3% w/v $CaCO_3$; 1.8 w/v agar, pH adjusted to 6.0 with NaOH 10% w/v). Morphology of the colonies was determined according to the growth pattern and the format of the edge of each colony in AV8, after 4 days of incubation in darkness at 28 ± 2 °C (Brasier & Griffin, 1979; Erwin & Ribeiro, 1996). In addition, other variables of each colony were described such as texture, aerial development of mycelia and color. Evaluation for morphology determination was performed four times for each colony.

For microorganism morphometrics, microorganisms were grown placing a disc of fresh AV8 media in the center of a sterile microscope slide (~ 13 mm), that is placed on a bended glass road inside a Petri dish with a humid filter paper. Growing mycelia were collected with an inoculation loop and were spread around the disc of AV8 media in the microscope slide. Another sterile microscope slide was put on top of the disc of media AV8 and the Petri dish was covered with the top and was sealed with parafilm and incubated at 25 °C under continuous white light. After 5 days of incubation, the microscope slide was withdrawn and a drop of stain (lactophenol / cotton blue / lactofucsin) was placed on growing mycelia and was covered with a glass cover slip. Sporangia obtained by this method in 4 or 5 days were clean and mature enough for further use in morphometric measurements (Appiah *et al.,* 2003). Thirty measurements of each structure were performed per isolate. Length, width and length to width ratio (L/W) of sporangia were measured, diameter of chlamydospores, length of the pedicel and presence or absence of papilla and chlamydospores were determined. Sporangia dehiscence was determined following the test reported by Cerqueira *et al.* (1999). All measurements were performed using a light microscope with an objective of 40X (Carl Zeiss Primo star, coupled with camera Axio CamERc5s Zeiss and software Zem 2011).

## Molecular sequence analysis of ITS and Cox regions

DNA sequence analysis of the genomic ITS and the mitochondrial Cox regions was used as support for identification of isolates at the species level (Martin & Tooley, 2003; Kroon *et al.,* 2004) in 60 isolates. DNA was purified using the Plant/Fungi DNA isolation kit following the manufacturer instructions (Norgen Biotek, Corp.). Quality and quantity were determined by spectrophotometry in a Nanodrop equipment (NanoDrop Thermo Scientific) and agarose (0.8% w/v) gel electrophoresis (5 v/cm) in TBE buffer (89 mM Tris-borate and 2 mM EDTA, pH 8.3) stained with EZVision (Amresco) following the manufacturer guidelines and visualized under UV in a transilluminator (BioRad). Purified DNA was stored at -20 °C for further use. Best amplification conditions for PCR reaction were determined for each pair of primers of a combination of six primers for ITS and three primers for the Cox regions (Table 2). Once the best primer pair/PCR condition was determined, it was used for amplification of ITS and Cox regions of each isolate evaluated.

**Table 2.** Primers used for sequencing the ITS and Cox regions (based on Martin & Tooley, 2003; Kroon *et al.,* 2004)

| Primer | Sequence 5'→3' |
|---|---|
| ITS1 | TCC GTA GGT GAA CCT GCG G |
| ITS1F | CTT GGT CAT TTA GAG GAA GTA A |
| ITS4 | TCC TCC GCT TAT TGA TAT GC |
| ITS4B | CAG GAG ACT TGT ACA CGG TCC AG |
| ITS5 | GGA AGT AAA AGT CGT AAC AAG G |
| ITS6 | GAA GGT GAA GTC GTA ACA AGG |
| FMPh-8 | dAAG GTG TTT TTT ATG GAC AAT GTA |
| FMPhy-8b | AAA AGA GAA GGT GTT TTT TAT GGA |
| FMPhy-10b | GC AAA AGC ACT AAA AAT TAA ATA TAA |

## Internal Transcribed Spacer (ITS) genomic region

A reaction mix was prepared containing the following components at final concentrations: buffer 1X (Thermo Scientific), 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.4 µM of each forward and reverse primers, 1.5 U *Taq* Polymerase enzyme (Fermentas), 2 ng/µL of DNA template, and molecular biology grade water to a final volume of 25 µL. Amplification was carried out in a thermalcycler (LabNet) with the following program: initial denaturation 95°C for 3 min; 40 cycles of denaturation at 95°C for 30 s, primer annealing at 55°C for 1 min, extension at 72°C for 1 min; after the 40 cycles finished, a final extension at 72°C for 10 min was performed.

## Cox mitochondrial region

A reaction mix was prepared containing the following components at final concentrations: buffer 1X (Thermo Scientific), 3 mM $MgCl_2$, 100 µM dNTPs, 1 µM of each forward and reverse primers, 2 U Taq Polymerase enzyme (Fermentas), 2 ng/µL of DNA template, and molecular biology grade water to a final volume of 25 µL. Amplification was carried out in a thermocycler (LabNet) with the following program: initial denaturation 95°C for 3 min; 40 cycles of denaturation at 95°C for 30 s, primer annealing at 60 °C for 1 min, extension at 72°C for 1 min;

**Table 3.** Scale of resistance / susceptibility to BPR disease based on mean diameter of lesion (cm) caused by *Phytophthora* sp. isolates in cacao pods (Phillips-Mora & Galindo, 1989).

| Level of resistance/susceptibility of cacao genotypes | Mean diameter of the lesion (cm)[a] | |
|---|---|---|
| | 6 days | 10 days |
| Resistant (R) | 0 - 2 | 0 - 3 |
| Moderately resistant (MR) | 2.1 - 4 | 3.1 - 6 |
| Moderately susceptible (MS) | 4.1 - 6 | 6.1 - 9 |
| Susceptible (S) | >6 | >12 |

[a] Mean diameter of cacao pod lesion calculated from length and width measurements.

after the 40 cycles finished a final extension at 72°C for 10 min was performed.

Amplified fragments of ITS and Cox regions were analyzed in agarose (2% w/v) gel electrophoresis as described before. PCR products from 3-4 reactions showing a single and clear band were purified using the GeneJET PCR purification kit (Thermo Scientific) following the manufacturer instructions. Purified products were quantified by spectrophotometry using a Nanodrop equipment and verified by agarose gel electrophoresis (2% w/v) as described. Purified fragments were sent for sequencing in both forward and reverse sense following the company guidelines (Macrogen, Republic of Korea). Obtained sequences were manually assembled, cleaned and edited using BioEdit software. Sequences were aligned using the Clustal W algorithm implemented in BioEdit software (Larkin *et al.,* 2007). Homologies were identified by comparison with databases using the algorithm BLASTn (http://blast.ncbi.nlm.nih.gov/blast.cgi).

### Genetic analyses

Alignment was used to identify the best substitution model and to perform the dendrogram. The model showing the lowest value of Bayesian information criteria (BIC) was selected as the best substitution pattern of each gene. Phylogenetic reconstructions were performed with the Maximum likelihood method with a Bootstrap of 1000 iterations. All calculations were performed with the computational package MEGA X (Kumar *et al.,* 2018).

### BPR severity of selected *Phytophthora* isolates

Incidence and severity of BPR caused by isolates ANYA 228, SARIO 189, ARAR 153, TOVR 01 and HURV 19 (Table 1), on cacao clones CCN51, ICS 95, EET 8, TSH 565, IMC 67 and PA 46, were measured. Isolates were grown in AV8 media incubated at 28 ± 2 °C for 10 days, 4 days in darkness and 6 days under a photoperiod of 12 h white light and 12 h in darkness. For zoospore release, sterile distilled water at 10 °C was added to Petri dishes and were incubated for 25 min at 5 °C and then incubated for 30 min at 25 °C. A disc of filter paper of 0.5 cm of diameter impregnated with a suspension of zoospores at a concentration of 1,5 x $10^5$ zoospores/mL, was placed in the equatorial zone of each of 10 cacao pods of 4.5 months old per each clone tested. As control, discs impregnated with sterile distilled water were placed in pods of each clone evaluated (Rodríguez & Vera, 2015). Pods were incubated in a humid chamber and incidence and the average diameter of the lesion (ADL) were measured at 6 and 10 days after inoculation. The diameter of the lesion was measured in two perpendicular directions and with values obtained, the average was calculated and used for rating the disease development according to the scale proposed by Phillips-Mora & Galindo (1989) (Table 3). A completely randomized design with ten replicates per treatment was used and experiments were performed twice through time.

### Statistical analyses

Isolates were grouped by homogeneous characteristics by multivariate analysis of conglomerates using the squared Euclidian distance and the minimum variance grouping method of Ward. Comparison of groups with quantitative variables (length, width, sporangia length to width ratio) was performed by analysis of variance (ANOVA) ($p \leq 0.05$) followed by the multiple mean comparison test of Tukey ($p \leq 0.05$). Contingency tables and Chi-squared test were applied for analysis of qualitative variables and the generated groups. ADL data were analyzed by ANOVA followed by the Tukey test ($p \leq 0.05$) for identification of differences between the mean of treatments. ADL data from the two experiments performed through time were combined after variance homogeneity was verified by the Cochran test (Gomez & Gomez, 1983). All statistical analyses were performed using the software SAS 9.3.

## Results

### Biometric characteristics of sporangia and chlamydospores

Three groups of isolates were formed; 14% of isolates in the first group (I), 50% in the second (II) and 36% in the

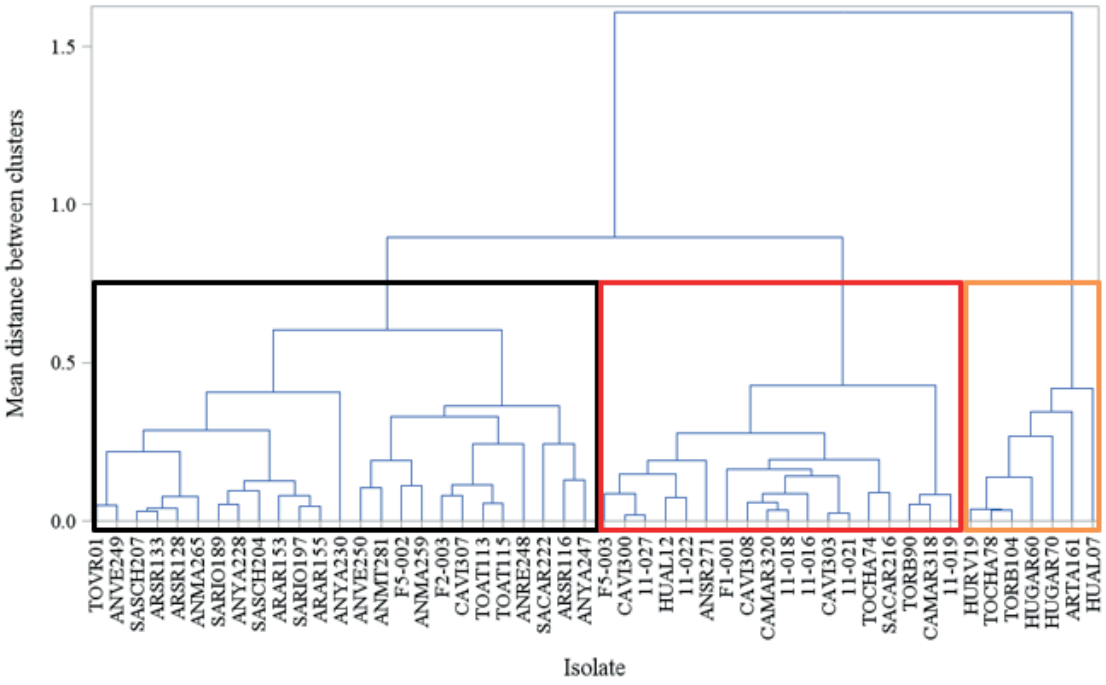


**Figure 1.** Groups of isolates obtained by multivariate analysis of conglomerates using the squared Euclidian distance and the minimum variance grouping method of Ward. Isolates characteristics used in the analysis: length, width and L/W ratio of sporangia, and pedicel length. Fifty isolates were used for the analysis. Yellow, cluster I; black, cluster II; and red, cluster III.

third (III) (Fig. 1). In group I, seven isolates from Arauca (ARTA 161), four from Huila (HUAL07, HUGAR60, HUGAR70, HURV19) and two from Tolima (TORB104, TOCHA78) grouped together. Less diversity was found in I than in II and III, and similarly, I showed the lowest values of length and width of sporangia and chlamydospore diameter ($p \leq 0.05$) (Fig. 2). No significant differences were found between groups for L/W ratio of sporangia and pedicel length (Fig. 3). Cluster II grouped 25 isolates and was the most diverse. This cluster had isolates from all regions studied with a higher percentage from Caldas and Bolivar. In cluster II, the highest values for all groups ($p \leq 0.05$) of length (53.069 ± 0.650 µm) and width of sporangia (35.211 ± 0.493 µm) and diameter of chlamydospore (36.859 ± 0.611 µm), were observed (Fig. 2). In cluster III, 18 isolates were grouped with seven isolates from Antioquia, five from Santander and four from Arauca. From Tolima and Nariño only one isolate from each department were found in this cluster (Fig. 1). Isolates in this group showed significant intermediate values ($p \leq 0.05$) for length of sporangia (42.766 ± 0.765 µm), width of sporangia (26.808 ± 0.581 µm) and diameter of chlamydospore (31.564 ± 0.720 µm) (Fig. 2).

## Morphology of the colony

Significant differences ($p \leq 0.001$) were observed between groups for edge and growth pattern, texture ($p \leq 0.0243$) and aerial development of mycelia ($p \leq 0.0033$) (Table 4). In cluster I, 47.62% of isolates showed regular edge and 42.86% wavy edge; 52.38% exhibited starry growth pattern, 71.43% plush texture, 76.19% scarce aerial development of mycelia and 100% of isolates showed two concentric rings. In cluster II 98.67% of isolates showed regular edge; 37.33% exhibited starry growth pattern, 53.33% plush texture, 76.19% scarce aerial development of mycelia and 100% of isolates

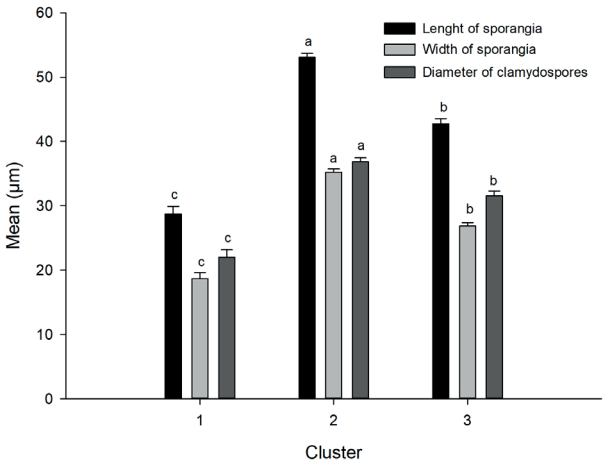


**Figure 2.** Mean biometric characteristics: length and width of sporangia and diameter of chlamydospores, in µm. Mean of each characteristic was calculated from 30 measurements. Different letters represent significant differences between each biometric characterization of different cluster identified by Tukey test ($p \leq 0.05$)

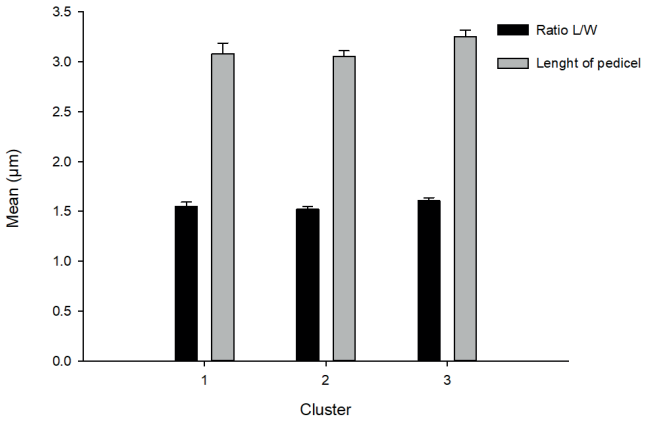


**Figure 3.** Mean biometric characteristics. Length to width ratio of sporangia (L/W) and length of pedicel in µm. Means were calculated from 30 independent measurements for each structure.

showed two concentric rings. In cluster III, 81.48% of isolates showed regular edge; 53.70% exhibited starry growth pattern, 62.96% plush texture, 70.37% scarce aerial development of mycelia and 69.23% of isolates showed two concentric rings (Table 4). Only the aerial development of mycelia was present in the three clusters identified. Growth pattern and number of concentric rings showed a higher variation between groups.

## DNA sequence analyses

All sequences analyzed showed high homology with sequences from *P. palmivora* available in GenBank, using the Blast algorithm (Table 1). For ITS, the best substitution model was Hasegawa-Kishino-Yano + Gamma distribution with five categories and for Cox sequences best model was Tamura 3-parameter + Gamma distribution with five categories. Non-uniformity of evolutionary rates among sites were modeled by using a discrete Gamma distribution (+G) with five rate categories (Nei & Kumar, 2000; Kumar *et al.,* 2018). In Fig. 4 (ITS sequences) and Fig. 5 (Cox sequences), phylogenetic relationships between *Phytophthora* sp. isolates may be observed. Phylogenetic analyses for both Cox and ITS regions showed that sequences of *P. palmivora* grouped together confirming the identification of all isolates. Sequences of *P. arecae* from the palm trees *Areca catechu* and *Cocos nucifera* were found in the same group with *P. palmivora* for both Cox and ITS regions. In

**Table 4.** Comparison of morphological characteristics of colonies in the three groups identified.

| Characteristics of the colonies[a] | Categories [b] | Groups (in %) | | | *p*-value $\chi^2$ |
|---|---|---|---|---|---|
| | | I (n=7) | II (n=25) | III (n=18) | |
| Edge | Aracnoid | 9.52 | 0.00 | 5.56 | <0.0001**[d] |
| | Irregular | 0.00 | 1.33 | 12.96 | |
| | Wavy | **42.86** [e] | 0.00 | 0.00 | |
| | Regular | **47.62** | **98.67** | **81.48** | |
| Growth pattern [c] | CR | 14.29 | 10.67 | 12.96 | <0.0001** |
| | ES | 0.00 | 0.00 | 1.85 | |
| | ET | **52.38** | **37.33** | **53.70** | |
| | P | 0.00 | 16.00 | 0.00 | |
| | RS | 14.29 | 0.00 | 0.00 | |
| | SRM | 0.00 | **20.00** | 5.56 | |
| | SET | 14.29 | 9.33 | 9.26 | |
| | SN | 4.76 | 6.67 | 16.67 | |
| Texture | Plush | **71.43** | **53.33** | **62.96** | 0.0243* |
| | Cottony | 14.29 | 5.33 | 0.00 | |
| | Semi-plush | 14.29 | 41.33 | 37.04 | |
| Aerial development of mycelia | Abundant | 19.05 | 0.00 | 7.41 | 0.0033** |
| | Scarce | **76.19** | **85.33** | **70.37** | |
| | Medium | 4.76 | 14.67 | 22.22 | |
| Number of concentric rings | 1 | 0.00 | 11.11 | 7.69 | 0.3253ns |
| | 2 | **100.00** | **55.56** | 23.08 | |
| | 3 | 0.00 | 27.78 | **69.23** | |
| | 4 | 0.00 | 5.56 | 0.00 | |

[a] Colonies of *P. palmivora* grown in agar V8 media 4 days after incubation. [b] CR: chrysanthemum; ES: stoloniferous; ET: starry; P: petaloid; RS: pinkish; SRM: semi-pinkish; SET: semi-starry; SN: without defined pattern. [c] Growth pattern of the colony in agar V8 media after 4 days of growth. [d] * significant, ** highly significant, ns not significant by *p*-value $\chi^2$. [e] Bold numbers indicate the highest percentage within each category

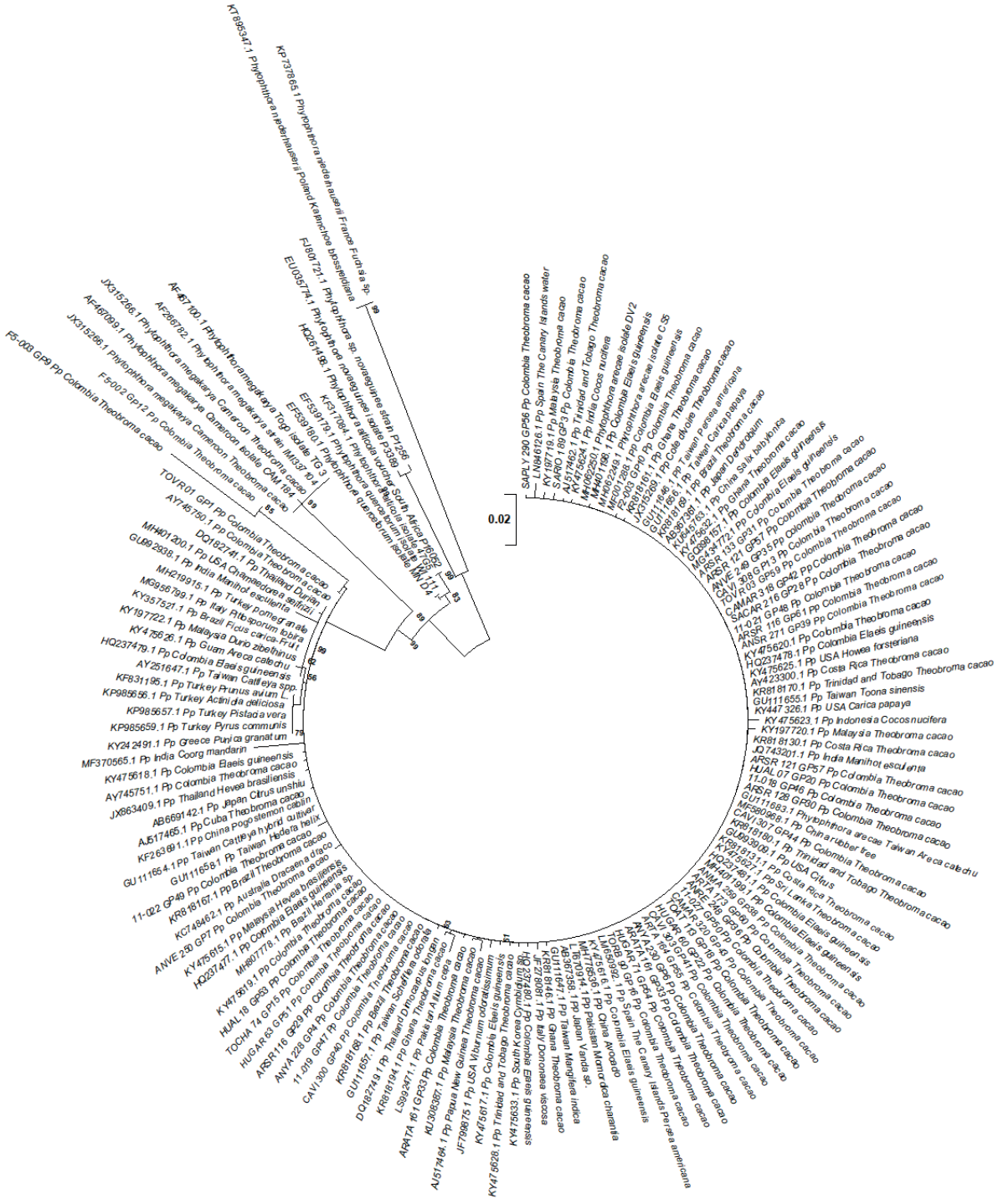


**Figure 4.** Molecular phylogenetic analysis of rADN-ITS sequences from *P. palmivora* (Pp) isolates. The evolutionary history was inferred by using the Maximum Likelihood method and Hasegawa-Kishino-Yano model (Hasegawa *et al.,* 1985). The tree with the highest log likelihood (-2998.64) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G,* parameter = 0.7314)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 145 nucleotide sequences. There were a total of 941 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al.,* 2018).

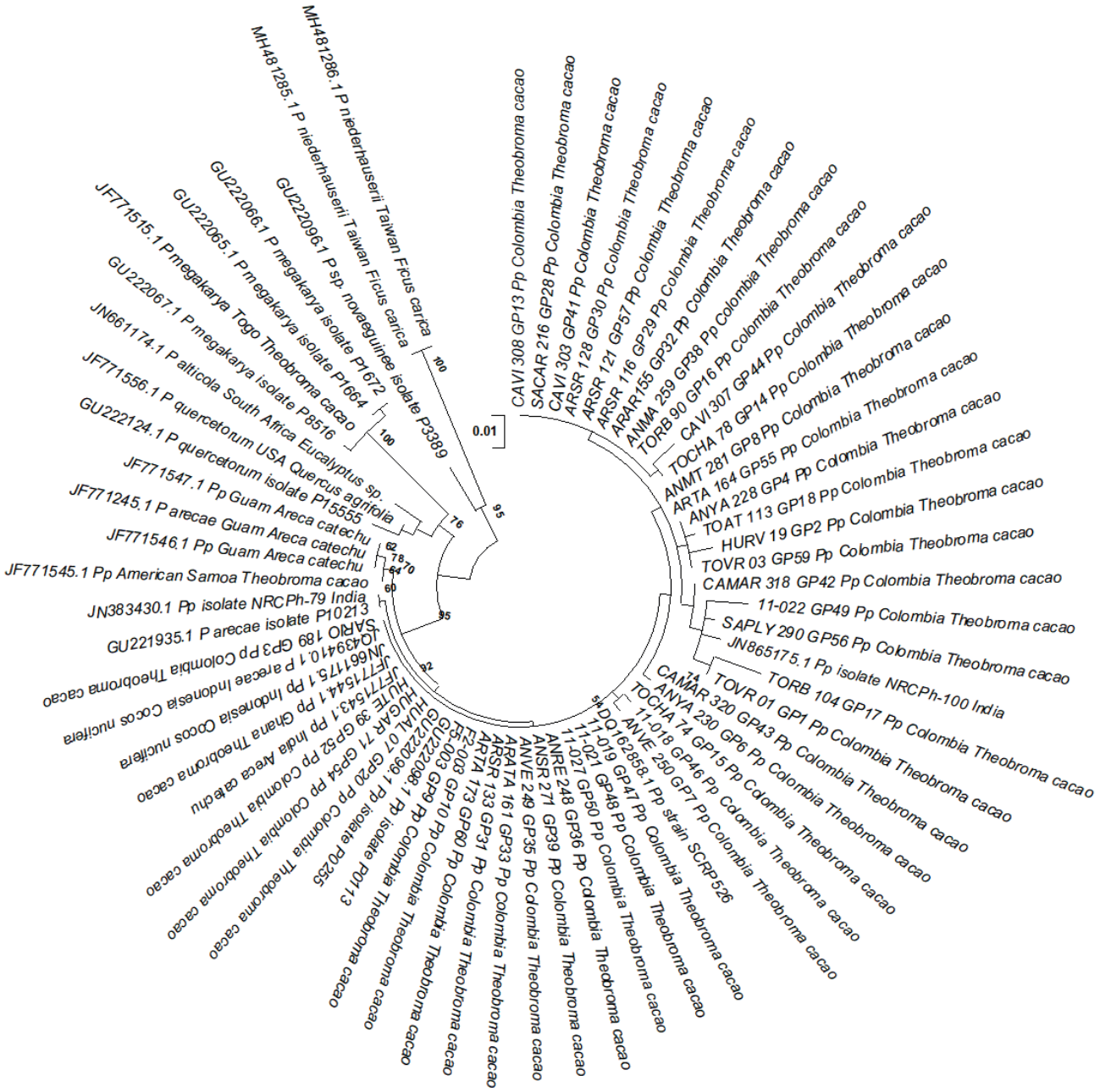


**Figure 5.** Molecular phylogenetic analysis of COX sequences from isolates of *P. palmivora* (Pp). The evolutionary history was inferred by using the Maximum Likelihood method and Tamura 3-parameter model (Tamura, 1992). The tree with the highest log likelihood (-12449.39) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G,* parameter = 0.2104)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 66 nucleotide sequences. There were a total of 473 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al.,* 2018).

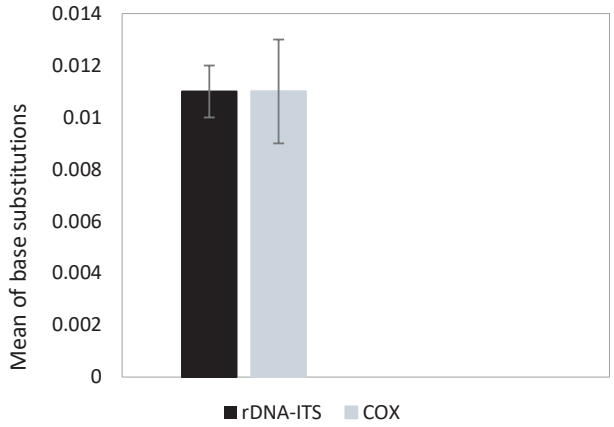


**Figure 6.** Mean evolutionary divergence from all sequence pairs (rDNA-ITS and COX). Bars represent the number of base substitutions per site from averaging over all sequence pairs. Variation rate between sites was modeled using a Gamma distribution. Error bars represent the standard error obtained by bootstrap using 1000 iterations.

addition, sequences from other *Phytophthora*-related species such as *P. niederhauserii, P. novaeguinee, P. megakarya*, *P. quercetorum* and *P. alticola,* formed independent groups for both Cox and ITS regions.

Mean general distance observed for ITS sequences was of 0.011 (SE = 0.001) and for Cox sequences was of 0.011 (SE = 0.002) (Fig. 6). As expected, low genetic variability was observed between isolates because sequences used were conserved and confirmed taxonomy of isolates.

### Response of cacao genotypes to inoculation with *P. palmivora*

Incidence was 100% for all genotypes inoculated. ANOVA results indicated significant differences in lesion development in cacao pods between genotypes tested at 6 days after inoculation ($p \leq 0.05$) (Table 5). Genotype CCN51 showed the highest value for lesion size with 46.08% and 46.39% higher compared to genotypes IMC 67 and PA 46, respectively. Genotype CCN51 was classified as susceptible together with ICS 95, EET8 and TSH 565; and PA 46 and IMC 67 were classified as moderately susceptible (Table 5). Ten days after inoculation, genotypes CCN51, ICS 95 and EET8 exhibited the highest values of lesion size in the same group of significance (21.86, 19.29 and 19.25 cm, respectively) ($p \leq 0.05$) (Table 5). Lowest scores of diseases were registered for genotypes TSH 565, IMC 67 and PA 46 (15.30, 13.95 and 13.25 cm, respectively).

### Virulence of isolates of *P. palmivora*

Significant differences ($p \leq 0.05$) were identified between five isolates of *P. palmivora* tested for virulence on pods of cacao genotypes (Table 6). More virulent isolate was HURV19 (in Cluster I) with a lesion value of 13.79 cm and 45.98% higher than value found for ANYA 228 (in Cluster II), which exhibited the lowest virulence of all isolates tested. Isolates SARIO 189 (Cluster II) and ARAR 153 (Cluster II) showed intermediate values of lesion size with 10.84 and 12.11 cm, respectively (Table 6).

## Discussion

In the present work, morphological and DNA variability and virulence of *P. palmivora* isolates collected from different cacao growing regions in Colombia, were studied. Morphology of *P. palmivora* has been extensively studied since was identified as a plant pathogen. As early as in 1924, Gadd described that isolates obtained from rubber, orchids and bread fruit, exhibited smaller sporangia and chlamydospores than those isolated from cacao plants. Morphometric analyses were also used for identification of *P. palmivora* subgroups according to geographical re-

**Table 5.** Level of resistance / susceptibility of cacao pods of different genotypes to *P. palmivora* (isolates ANYA 228, SARIO 189, ARAR 153, TOVRO1, HURV19).

| Clone | 6 days after inoculation | | | 10 days after inoculation | | |
|---|---|---|---|---|---|---|
| | Mean diameter of lesion (cm) [a] | Reaction of resistance/ susceptibility[b] | Incidence (%) | Mean diameter of lesion (cm) [a] | Reaction of resistance/ susceptibility | Incidence (%) |
| CCN 51 | 9.83 A | S | 100 | 21.86 A | S | 100 |
| ICS 95 | 8.18 AB | S | 100 | 19.29 A | S | 100 |
| EET 8 | 7.36 BC | S | 100 | 19.25 A | S | 100 |
| TSH 565 | 6.67 BC | S | 100 | 15.30 B | S | 100 |
| IMC 67 | 5.30 C | MS | 100 | 13.95 B | S | 100 |
| PA 46 | 5.27 C | MS | 100 | 13.25 B | S | 100 |

[a] Different letters in the same column represent significant differences identified by the Tukey test ($p \leq 0.05$). [b ] S: susceptible, MS: moderately susceptible

**Table 6.** Virulence of selected isolates of *P. palmivora* on five genotypes of cacao pods (CCN51, ICS95, EET8, TSH 565, IMC 67, PA 46).

| Code of isolate | Department of collection | Mean diameter of lesion (cm) [a] |
|---|---|---|
| ANYA 228 | Antioquia | 7.45 A |
| SARIO 189 | Santander | 10.84 B |
| ARAR 153 | Arauca | 12.11 B |
| TOVRO1 | Tolima | 12.6 C |
| HURV19 | Huila | 13.79 C |

[a] Different letters in the same column represent significant differences identified by the Tukey test ($p \leq 0.05$).

gion of origin (Chee, 1971; Mchau & Coffey, 1994) and host (Brasier & Griffin, 1979; Chee, 1969). Our results indicate that morphology of various microbial structures was significantly variable between isolates in agreement of what has been reported before (Erwin & Ribeiro, 1996; Kroon *et al.,* 2012; Martin *et al.,* 2012). Morphometry may be influenced not only by natural variation but by environmental conditions as well, thereupon species identification based solely in morphological characters had caused confusion when characterizing within and between *Phytophthora* sp. populations, generating the need to find stable characters (Erwin & Ribeiro, 1996). Morphological analyses of sporangia, pedicel and chlamydospores grouped isolates in three main clusters. In these three clusters, only the pedicel length and length to width ratio characters showed stability, pointing to pedicel length as a consistent characteristic within this species. Similar results were reported by Al-Hedaithy & Tsao (1979), who identified pedicel length as both intra and interspecific stable diagnostic character in the genus *Phytophthora*. This character has been useful for initial identification of two *Phytophthora* sp. pathogenic on cacao (*P. palmivora* and *P. capsici*) (Griffin, 1977; Cerqueira *et al.,* 1999). In *P. palmivora* a short pedicel is usually observed (2.0–4.0 µm), in clear contrast to *P. capsici* where a deciduous long pedicel is registered (18.3–40.4 µm) (Cerqueira *et al.,* 1999). Other studies of *P. palmivora* on cacao populations have shown similar findings with a continuous range of variation of morphological characteristics (Torres, 2016; Maora *et al.,* 2017).

Length to width ratio did not show significant differences between groups suggesting that, despite the large variation observed in sporangia measurements, the proportion is consistent in *P. palmivora*. Erwin & Ribeiro (1996), pointed that the L/W ratio may be useful as a species characteristic to circumvent difficulties associated to subjective descriptions such as ovoid, obvoid and others. A high variability was found for colony characteristics and shape of sporangia. Similar findings have been reported widely, so many authors do not recommend using these characteristics for species identification using it as supplementary information only (Erwin & Ribeiro, 1996). Identification of isolates as *P. palmivora* sensu Buttler, using morphology was confirmed by molecular sequencing of nuclear rDNA-ITS and mitochondrial Cox regions (Griffin, 1977; Brasier & Griffin, 1979). Sequence results have been used to define clear limits between species of *Phytophthora* (Robideau *et al.,* 2011). Rahman *et al.* (2014) found that a phylogenetic tree obtained using the Cox region only resulted in a similar tree constructed with combined sequences from five genes (ITS, LSU, COX I, β-tubulin and EF1-a), confirming its use for species identification in oomycetes.

In our work, *P. arecae* grouped together with *P. palmivora* for both regions sequenced (*i.e.,* ITS and Cox). However, *P. arecae* is similar to *P. palmivora* in morphology, isozyme analysis and DNA markers (SSCP), and now is considered a synonymous species of *P. palmivora* (Oudemans & Coffey, 1991; Mitchell & Kannwischer-Mitchell, 1992; Cooke *et al.,* 2000). Therefore, it can be concluded that all sequences obtained in the present research from Cox and ITS regions corresponded to *P. palmivora* (Gallegly & Hong, 2008). No clear grouping was identified within *P. palmivora* sequences according to country of origin or host. *P. palmivora* is a redoubtable, ubiquitous and pantropical plant pathogen with over a thousand host species reported and thrives in a range of different environmental conditions causing severe diseases not only on cacao but on oil palm, mango, black pepper, Hevea sp., ornamentals, pineapple, papaya, citrus, coconut and many others (Widmer, 2014). Consequently, it is understandable that *P. palmivora* sequences of isolates from diverse countries and host plants grouped together using conserved molecular markers that has proven useful for taxonomical identification at the species level (Cooke *et al.,* 2000; Robideau *et al.,* 2011). Further analysis about molecular identification of pathogenicity factors such as effector proteins or host preferences is crucial for a better understanding of *P. palmivora* populations.

Genetic analyses in Papua New Guinea showed that *P. palmivora* strains isolated from cacao lesions belonged to one clonal lineage with limited variability; however, isolates from soil in the same regions showed higher genetic diversity, suggesting continuous selection for pathogenicity from a genetic pool of *P. palmivora* (Maora *et al.,* 2017). It is not known whether or not there are in Colombia genetic differences between plant or soil populations of *P. palmivora.* Therefore, further research is required to answer this question. In a similar finding, *P. palmivora* populations obtained from oil palm crops in Colombia and Malaysia were not separated by phylogenetic analysis, supporting previous results and highlighting the importance to continue research expanding host species, number of isolates from different geographical regions and environmental conditions, looking for a better understanding of *P.*

*palmivora* populations as a key tool for disease management programs (Maizatul-Suriza *et al.,* 2019).

Virulence varied between five isolates tested in cacao genotypes with 45.98% of difference between the most (HURV 19, cluster I) and the less aggressive (ANYA 228, cluster III) isolate in the mean diameter of the lesion. It is considered that 90% of known cacao genotypes are susceptible to BPR disease (Iwaro *et al.,* 1997). In our research, cacao genotype PA 46 showed the smallest disease score. High levels of resistance to cacao BPR have been usually associated to the named 'Forastero' of Amazonian cacao genotypes such as SCA 6, PA 150, P7 and P 46 (Tahi *et al.,* 1999; Bartley, 2005). 'Trinitario' genotypes such as CCN 51, ICS 95, EET 8 and TSH 565, have been reported more frequently as susceptible (Paulin *et al.,* 2008). No clear relationship was established between morphological, virulence and genetic groups, identified in the present research. Contrasting results have been reported around the world on aggressiveness of *P. palmivora* isolates from different hosts and countries (Surujdeo-Maharaj *et al.,* 2001; Thevenin *et al.,* 2012; Torres, 2016; Fuzitani *et al.,* 2018). Usually, plant breeding programs use a narrow sample of the genetic variability of pathogens for disease resistance selection. Pathogen populations exhibit complex dynamics in constant evolution, new genotypes are constantly emerging posing a great challenge for sustainable agriculture, as evidenced for a morphotype identification in Central America (Johnson *et al.,* 2007; Cooke *et al.,* 2012). In the present research, high variation was identified, therefore indicating that accurate and prompt identification of *Phytophthora* sp. attacking cacao is of crucial importance in an effective and efficient integrated crop management program. As most pathogen populations may be complex, wide studies and higher representation of pathogen diversity should be used in breeding programs to increase the possibility of selection of cacao genotypes, which may adapt to a wider range of pathogen variability in different environments.

Our knowledge about the origin and evolution of *P. palmivora* populations in Colombia with the whole host range is poor, more basic research is needed to understand the complex ecological relationships, sources of variation, mating type, gene flow, and many other aspects of pathogen biology to design better or new management tools for this important microorganism.

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