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Phytophthora cathayensis sp. nov., a new species pathogenic to Chinese Hickory (*Carya cathayensis*) in southeast China

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Abstract: Crown decline and mortality associated with collar lesions were observed on *Carya cathayensis* (Chinese hickory) trees in a plantation in Zhejiang province, China. Examination of active lesions resulted in the isolation of a homothallic, papillate *Phytophthora* sp. Detailed morphological and physiological studies and phylogenetic analysis, using ITS, beta-tubulin, cytochrome oxidase I, and heat shock protein 90 gene regions, revealed that all isolates belonged to an undescribed species residing in phylogenetic Clade 4, which is described here as *Phytophthora cathayensis sp. nov*. Inoculation trials were conducted under greenhouse conditions on *C. cathayensis* and *C. illinoensis* (pecan) plants to fulfill Koch postulates and hypothesize a possible pathway of the incursion. An existing report of a *Phytophthora* species with the same ITS sequence was reported on *C. illinoensis* from the USA in 2009. The difference in susceptibility of the two inoculated *Carya* species, and the report from the USA, suggest a possible introduction with plant material from the USA to China.

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INTRODUCTION

Carya cathayensis (Chinese hickory) (*Juglandaceae*) is an economically important nut tree in China. Currently, more than 15 000 ha of *C. cathayensis* trees are cultivated in Zhejiang Province. Traditional cultivation methods, monoculture of single varieties, over-fertilization, and excessive application of herbicides, have led to the occurrence of serious phytosanitary problems. Recently, trunk canker caused by *Botryosphaeria dothidea*, has become the most devastating disease of *C. cathayensis* (Zhang & Xu 2012), and nearly 90 % of orchard trees in Zhejiang Province have been affected by this pathogen (Yang *et al.* 2009). On the other hand, *Carya illinoensis* (pecan) is economically the most valuable nut tree native to North America, and is commercially produced in New Mexico, Georgia, Louisiana, and Texas, as well as Mexico. Consumption of pecan nuts in China has boomed since 2008 due to a global walnut shortage and record pecan harvests. However, the supply of Chinese-grown pecan is low and unpredictable, hence, China is the world's largest market for pecan, and imports 50 000 t of US grown pecan annually to satisfy local demand (Wessel 2011, Zhang & Xu 2012). *Carya illinoensis* trees were first introduced to China over 100 years ago. However, productive orchards developed rapidly starting in 2008, when the price of pecan nuts soared, and the nuts were more generally accepted by Chinese people. In 2014, there were about 8 500 ha of commercial pecan orchards in China, mainly distributed in Yunnan, Jiangsu, Zhejiang, and Anhui Provinces. Most of the orchards planted with the recommended cultivars 'Pawnee', 'Wichita', 'Caddo', and 'Jinhua' are starting to bear and showing potential high yields (Zhang *et al.* 2015).

China underwent several intentional introductions of *C. illinoensis* germplasm, seeds, and seedlings from the US since the beginning of 1900, resulting in the establishment of orchards in the same area as those of the native species, *C. cathayensis*. Furthermore, *C. illinoensis*, was also utilized in new plantations as rootstock for *C. cathayensis* scions because of its high resistance to the fungal pathogen *Botryosphaeria dothidea.* It has been observed that *C. cathayensis* grafted on *C. illinoensis* rootstocks are nearly immune to Botryosphaeria canker disease (Yang *et al.* 2009). Repeated introductions of new germplasm greatly increases the risk of host switches of potential threatening microorganisms between the two hosts. Global trade of plants for planting is, however, recognised as the main pathway for unintentional introductions of alien invasive forest and agricultural pests and pathogens worldwide (Brasier 2008, Scott *et al.* 2019).

The number of invasive alien pests and pathogens species impacting ecosystem functioning, human health, and economy has increased dramatically over the last decades (Early *et al.* 2016, Eschen *et al.* 2019). Globalization and international trade have largely facilitated the unintentional long-distance movement of alien plant pests and pathogens into regions outside their native distribution ranges (Seebens *et al.* 2017). In the last decades, the use of sentinel plant systems has been reported as a promising tool to improve the detection of pests

and pathogens before their introduction (Vettraino *et al.* 2017a, Morales-Rodríguez *et al.* 2019a). Among forest pathogens, species from the genus *Phytophthora* showed a high invasion potential specifically because of their dominance in nurseries and nursery stocks and their high aggressiveness (Jung *et al.* 2018, Scott *et al.* 2019).

Phytophthora species are primary pathogens on thousands of trees, shrubs, and crop species worldwide. Depending on whether the lifecycle occurs mainly above- or below-ground, a distinction is made between soilborne *Phytophthora* species causing fine root losses, root and collar rots and bleeding bark cankers, and airborne *Phytophthora* species causing leaf necrosis, shoot blights, fruit rots, and also bleeding bark cankers (Erwin & Ribeiro 1996). The number of described *Phytophthora* species that are associated with woody plants has increased dramatically in the past decade (Hansen *et al.* 2012, Martin *et al.* 2012, Scott *et al.* 2019). New species have been detected either because they were invasive causing severe diseases on new non-coevolved host plants, or because of intensive sampling campaigns, particularly in forest soils and streams (Jung *et al.* 2013). In the case of *Carya* species, *Phytophthora cactorum* is the causal agent of Phytophthora shuck and kernel rot infection of pecan. The disease was first observed in Georgia (USA) in 1988, but the causal agent was only later identified (Reilly *et al.* 1998).

In August 2016, a severe decline and dieback of *C. cathayensis* trees was observed in several orchards in the Zhejiang province, China. Affected trees showed dieback of the crown and cankers at the stem base and along roots, with tongue-shaped, orange-brown lesions of the inner bark (Fig. 1). In 2017, during a survey, isolates of a *Phytophthora* sp. were consistently isolated from the necrotic lesions at the collar of diseased trees (Fig. 1F).

In the present study, a new *Phytophthora* species associated with the decline and mortality of *C. cathayensis* in Zhejiang province is described as *Phytophthora cathayensis sp. nov*. Furthermore, its pathogenicity to *C. cathayensis* and *C. illinoensis* is tested.

MATERIALS AND METHODS

Sampling and *Phytophthora* isolation

Bark samples including cambium and adjacent xylem tissue were taken from active lesions of eight symptomatic trees using a hatchet, a knife, and a scalpel. The samples were taken to the laboratory and rinsed with running cold tap-water overnight and blotted on filter paper (Jung *et al.* 1996). Small tissue pieces were cut from different parts and depths of the phloem and xylem samples and plated onto selective PARPNH amended with 10 µg/mL pimaricin, 200 µg/mL ampicillin, 10 µg/mL rifampicin, 25 µg/mL PCNB, 50 µg/mL nystatin and 50 µg/mL hymexazol (Erwin & Ribeiro 1996). The plates were incubated at 20 °C in the dark and examined daily under the dissecting microscope for phytophthora-like hyphae, which were transferred to V8A (16 g agar, 3 g $CaCO_3$, 100 mL Campbell's V8 juice, 900 mL distilled water) (Erwin & Ribeiro 1996).

At each sampled tree, four soil sub-samples were taken 1–1.5 m apart from the base of a tree in the four cardinal directions and to a soil depth of *ca*. 30 cm after removing the organic layer. Soils were baited in the laboratory as described by Jung *et al.* (1996). A mix of different baits including *Rhododendron* leaf discs, carnation, and rose petals was used. Upon observation of lesions, the baits were plated onto PARPNH selective media. Cultures were stored at 25 °C on V8A for species identification.

Colony morphology, growth rates, and cardinal temperatures

Morphology of hyphae and colony growth patterns were described from 7-d-old cultures grown at 20 °C in the dark on V8A, potato-dextrose-agar (PDA), malt extract agar (MEA), and selective media (PARPHN). Colony morphologies were described according to Erwin & Ribeiro (1996) and Jung & Burgess (2009). For temperature-growth relationships, four replicate V8A plates per isolate were incubated at 10, 15, 20, 25, 27, 30, 32, and 35 °C. All isolates were sub-cultured onto V8A plates and incubated for 24 h at 20 °C to initiate growth. Radial growth rate was recorded after 5–7 d along two lines intersecting the centre of the inoculum at right angles (Hall 1993). When no growth occurred after 5 d, plates were incubated at 25 °C for 5 additional days to determine if the temperature was lethal (Molina *et al.* 2010). The growth test was repeated twice.

Morphology of sporangia and gametangia

Sporangia were obtained by flooding 15 × 15 mm square agar discs taken from growing margins of 3–5-d-old colonies (Simamorra *et al.* 2015) with deionized water and with nonsterile soil extract (Erwin & Ribeiro 1996) in 90 mm Petri dishes and incubating them in the dark at 20–25 °C. After 24–36 h, dimensions and characteristic features of 50 mature sporangia per isolate chosen at random were determined at ×400 magnification (Axioskop microscope and AxioCam ERc5s; Carl Zeiss). For each isolate, dimensions and characteristic features of 50 mature oogonia, oospores, and antheridia chosen at random were measured at ×400 magnification at the surface of 15 × 15 mm square agar plug cut from the centre of 15–20-d-old V8A cultures grown in the dark at 20 °C (Simamorra *et al.* 2015). The oospore wall index was calculated as the ratio between the volume of the oospore wall and the volume of the entire oospore (Dick 1990).

DNA isolation, amplification, and sequencing

The *Phytophthora* isolates were grown on potato dextrose broth at 20 °C for 2 wk and the mycelium was harvested. Genomic DNA was extracted following the protocol recommended by the NucleoSpin Plant II Mini kit (Macherey Nagel, Germany) following the manufacturers' instructions. DNA concentration was assessed by gel electrophoresis, and DNA was diluted 1:10 to perform PCR and finally stored at -20 °C (Morales-Rodríguez *et al.* 2019b). The region spanning the internal transcribed spacer (ITS) region of the ribosomal DNA was amplified using the primers ITS-6 and ITS-4 (White *et al.* 1990, Cooke *et al.* 2000). The PCR amplification mixture, PCR conditions, the clean-up of products, and sequencing were as described by Grünwald *et al*. (2011). The mitochondrial gene *cox1* was amplified with primers Fm84 and Fm83 (Martin & Tooley 2003). The PCR amplification mixture was the same as for the ITS region, but the PCR conditions were as described previously (Martin & Tooley 2003). Moreover, beta-tubulin (*Btub*) and heat shock protein 90 (*HSP90*) were amplified as indicated in Blair *et al*. (2008) using the primers Btub-F1/Btub-R1 and HSP90-F1/HSP90-R2. All PCR products were evaluated for successful amplification

Fig. 1. A–C. Severe dieback and mortality in *Carya cathayensis* orchard in Zhejiang province. **D.** Necrosis descending to the root. **E.** Edge of a collar rot lesion. **F.** Collar rot, tongue-shaped, brown-dark orange necrosis of the inner bark.

using agarose gel electrophoresis. Amplicons were purified with NucleoSpin Gel and PCR Cleanup (Macherey Nagel, Germany). Sequencing reactions were performed by Eurofins Scientific (Luxemburg) and forward and reverse sequences assembled and edited using BioEdit v. 7.0.5.3 (Ibis Bioscience, CA, USA).

Phylogenetic analysis

Sequences of Clade 4 taxa were downloaded from GenBank BLAST hits, IDphy (http://idtools.org/id/phytophthora/index.php), and lists in relevant publications on *Phytophtora* phylogenetic and Clade 4 taxa (Simamora *et al.* 2015, Bose *et al.* 2017). Sequences of *Phytophthora plurivora* (Clade 2) and *P. pseudosyringae* (Clade 3) were used as outgroups. GenBank accession numbers for the sequences generated here and the source and accession numbers for sequences downloaded are listed in Supplementary Material Table S1 and S2. Sequences were aligned using ClustalW, included in MEGA v. 7, under default settings, all the alignments were inspected and adjusted manually if required (Alignments available at TreeBASE: ID 25838). A Bayesian phylogenetic analysis was done using MrBayes v. 3.2.7a (Ronquist *et al.* 2012). As reported by Morales-Rodríguez *et al.* (2019b), evolutionary history was inferred using the maximum-likelihood method based on the general time-reversible model (Nei & Kumar 2000) according to the result obtained using jModelTest v. 2.1.7 (Darriba *et al.* 2012;). Alignments and maximum likelihood analyses were conducted with MEGA v. 7 (Kumar *et al.* 2016).

Under-bark inoculation test

The methodology reported by Ginetti *et al.* (2014) was used for the under-bark inoculation test under greenhouse conditions. One-year-old *C. illinoensis* (stem diam *ca*. 8–10 mm) and 2-yr-old *C. cathayensis* plants (diam *ca*. 15–20 mm) were used for inoculation trials, 10 plants per *Carya* species and per isolate. At 5 cm above the collar, a 0.5 cm disc of bark was removed aseptically, an even-sized V8A disc cut from the margin of freshly growing cultures of *Phytophthora cathayensis* isolates was placed on the wound, covered with the removed bark piece and autoclaved wet gauze, and sealed with Parafilm®. Two isolates were tested. After 10 d, lesion length (mm) and area (mm^2) were measured after removal of the outer bark. Re-isolations were made using PARPNH to fulfill Koch's postulates. The experiment was repeated twice.

Statistical analysis

ANOVA was carried out to determine if morphometric and growth rate differences between isolates were statistically significant. Data normality and equal variances were tested by the Shapiro-Wilk and Bartlett test, respectively. Pathogenicity test data "area of the necrosis" had to be transformed using Ln(x) to get a normal distribution (Sokal & Rohlf 1995). A two-way ANOVA was done with isolate and *Carya* species as factors. Because of the significant interaction between factors the data were analysed with one-way ANOVA; mean separation was accomplished by Tukey's honestly significant difference (HDS) test. Statistical analyses were carried out using GraphPad Prism v. 8 (GraphPad Software, San Diego, CA, USA).

RESULTS

Phytophthora isolation

A unique *Phytophthora* morphotype was isolated from the active lesions on the collar of all *C. cathayensis* symptomatic trees sampled. The same morphotype was never recovered from the baited soil samples. Three isolates were selected for the species description (CP29, CP30, and CP31).

Phylogenetic analysis

All the gene regions sequenced for *P. cathayensis* had a maximum of 96 % similarity with described *Phytophthora* species and, in the case of ITS, a 100 % identity with a non-described *Phytophthtora* isolate from *C. illinoensis* in the USA (isolate P168825, GU997621). GenBank accession numbers for all the gene regions sequenced for *P. cathayensis* are presented on Table S2. According to the result from jModelTest the evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model (Nei & Kumar 2000). The tree with the highest log likelihood (-7025.85) is shown in Fig. 2. A discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+G, parameter = 0.2259)]. The analysis involved 26 nucleotide sequences. There were a total of 3 402 positions in the final dataset. The species most closely related were *P. litchi* and *P. palmivora*.

Taxonomy

Phytophthora cathayensis C. Morales-Rodríguez, Y. Wang & A. Vannini, ***sp. nov.*** MycoBank MB834619. Fig. 3.

Etymology: Name refers to *Carya cathayensis*, the host plant from which all isolates were obtained.

Typus: **China**, Zhejiang, Hangzhou, Lina, Tuankou, isolated from small pieces of cambium and adjacent xylem tissue from *Carya cathayensis* tree with collar canker, 2017, *C. Morales-Rodríguez* CP30 (**holotype** preserved as metabolically inactive culture, China General Microbial Culture Collection, CGMCC No. 19655; ex-type culture, CGMCC No. 19655).

Sporangia (Fig. 3): Papillate persistent sporangia were abundantly produced in distilled water and non-sterile soil extract 8–12 h on simple sporangiophores. Sporangia were rarely observed on solid agar. Semi-papillate sporangia were also occasionally observed. Although predominantly ovoid (90 %, Fig. 3A–C, E), various sporangial shapes were observed including ovoid, elongated ovoid, and limoniform (Fig. 3). Occasionally forming a conspicuous basal plug (Fig. 3C) that protruded into the empty sporangium. Sporangia were typically borne terminally, but some were laterally attached (Fig. 3D–E). Sporangia produced on the tips of radiating hyphae of a hyphal swelling (Fig. 3E) or with short hyphal appendices (Fig. 3B) were common. Sporangia of each isolate released zoospores between 15–20 h after flooding, zoospores were spherical and motile. Sporangia averaged 27.3 ± 4.0 µm in length and 18.6 ± 2.4 µm in breadth (full range), the average length to breadth ratio was 1.5 ± 0.1. The mean papilla dimensions were 5.3 ± 1.1 µm in length and 2.36 ± 0.6 µm in breadth, the average length to breadth ratio

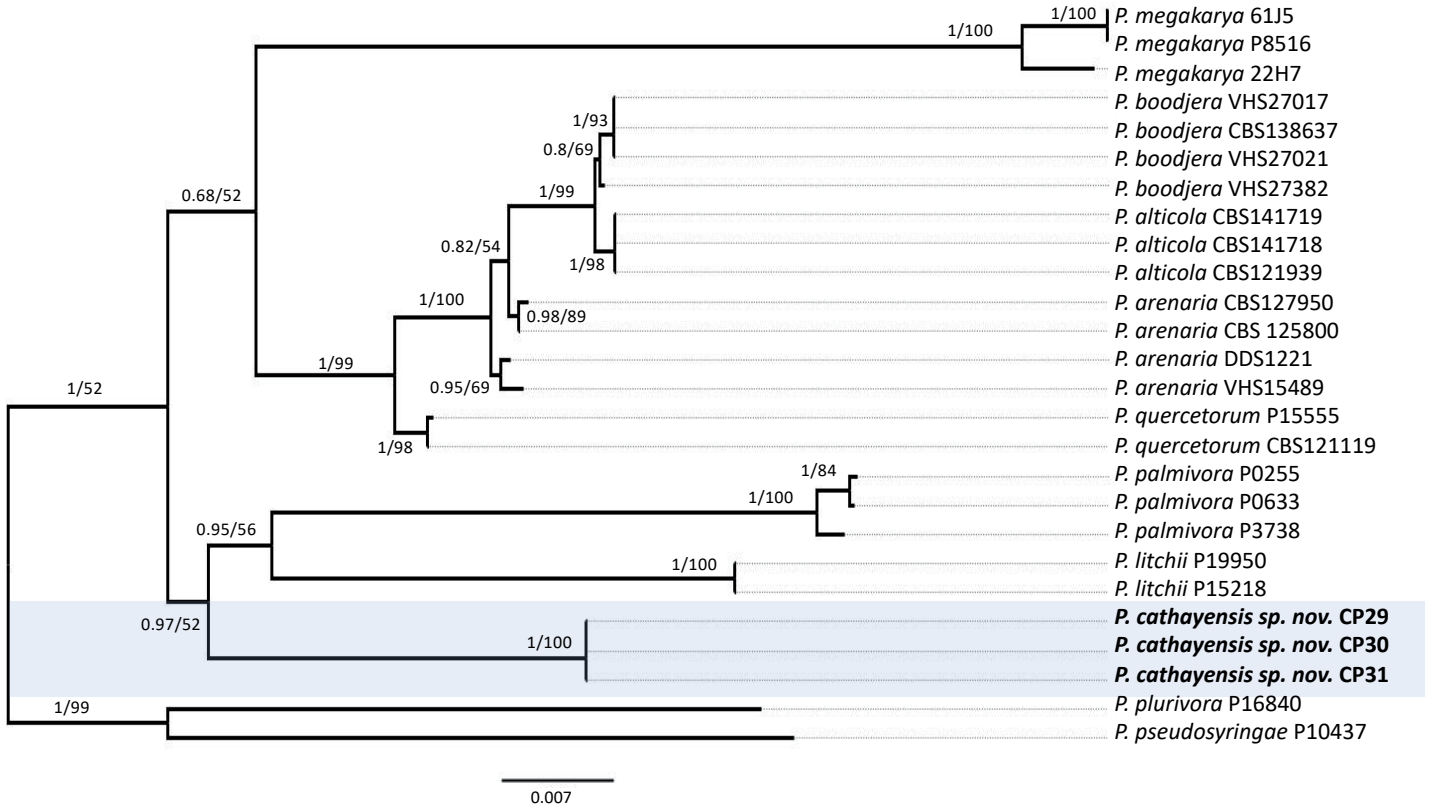

Fig. 2. Bayesian tree for Clade 4 *Phytophthora* species produced from concatenated sequences of the ITS, beta-tubulin, cytochrome oxidase I and heat shock protein 90 gene regions using GTR + G model. Maximum likelihood was conducted on the same dataset with MEGA v. 7 and resulted in the same topology. Numbers above the branches reflect support obtained from the analysis of the same dataset (Bayesian posterior probabilities/ Bootstrap values estimated by MEGA v. 7). *Phytophthora plurivora* (clade 2) and *P. pseudosyringae* (clade 3) were used as outgroup. The scale bar corresponds to substitutions per nucleotide site.

was 2.4 ± 0.6 µm. *Chlamydospores* rarely produced, on average 30.5 ± 3 µm (Fig. 3F). *Oogonia, oospores, and antheridia* (Fig. 3J–O): *Phytophthora cathayensis* is homothallic. Gametangia were readily produced in single culture by all isolates. Oogonia terminal at the main hyphae, globose to slightly subglobose with smooth walls. Mean oogonial diameter on V8A was 24.5 ± 1.6 µm (overall range 20.19–28.99 µm;). Oospores were globose with a mean diameter of 22.2 ± 1.3 µm (overall range 18.35–25.23 µm), an average oospore wall thickness of 1.6 ± 0.2 µm, and a mean oospore wall index of 0.2 ± 0.02 (overall range 0.15–0.26). The mean proportion of plerotic oopores was 80.66 %. The percentage of oogonial or oospore abortion was low (15 %). Antheridia mostly lateral and sessile with a short stalk, one per oogonium, attached near the stalk and rarely displaced, paragynous, cylindrical or club-shaped, averaging 11.4 ± 1.3 × 9.4 ± 1.2 µm. Isolates of *P. cathayensis* formed appressed to submerged colonies with a stellate growth pattern on MEA, stoloniferous felty colonies with submerged margins on PDA and uniform and slightly cottony on PARPNH (Fig. 4). On V8A colony morphology was more variable, ranging from stellate patterns to uniform pattern. Diameters of primary hyphae of *P. cathayensis* averaged 4.5 ± 0.7 µm and varied from 2.7 to 5.8 µm. All isolates tested had identical cardinal temperatures and similar radial growth rates at all temperatures (Fig. 5). The maximum growth temperature for *P. cathayensis* was 30 °C. All isolates were unable to grow at 32 °C and did not resume growth when plates previously incubated for 5 d at 32.5 °C were transferred to 25 °C. The optimum temperature for growth was 25 °C with growth rates of 10.2 ± 0.6 mm/d. At 20 °C *P. cathayensis* showed growth rates of 7.5 ± 0.6 mm/d on V8A, 4.6 ± 0.5 mm/d on PDA, and 5.5 ± 0.2 mm/d on MEA.

Notes: *Phytophthora cathayensis* is phylogenetically related to *P. litchii* and *P. palmivora* (Fig. 2) although, morphologically, it is easily distinguishable from both species as well as from *P. megakarya* by having non-caducous sporangia and a homothallic mating system (Table 1). *Phytophthora cathayensis* produces smaller sporangia with a higher l/b ratio compared to *P. alticola*, *P. arenaria*, *P. boodjera,* and *P. quercetorum*. Terminal chlamydospores can be produced by *P. cathayensis* and *P. quecetorum* but are absent in *P. alticola*, *P. arenaria* and *P. boodjera* (Table 1). The diameter of the oogonium is similar to *P. arenaria* and smaller than in *P. alticola*, *P. boodjera,* and *P. quercetorum* (Table 1).

Under-bark inoculation test

Both isolates of *P. cathayensis* were pathogenic to both *C. illinoensis* and *C. cathayensis* plants with *C. cathayensis* being much more susceptible (Figs 6, 7). The two-way ANOVA showed an interaction between factors (inoculated isolate and species of *Carya*) for both parameters measured, length of necrosis (interaction $F = 9.49$; $P < 0.05$), and area of necrosis ($F = 30.85$; $P < 0.05$). Consequently, a separate one-way ANOVA was performed for the individual data sets. *Carya cathayensis* was significantly more susceptible to *P. cathayensis,* showing longer necroses and larger necrotic areas than *C. illinoensis* ($F = 87.65$; $P < 0.05$ and $F = 101.98$; $P < 0.05$). Because of the low susceptibility of *C.*

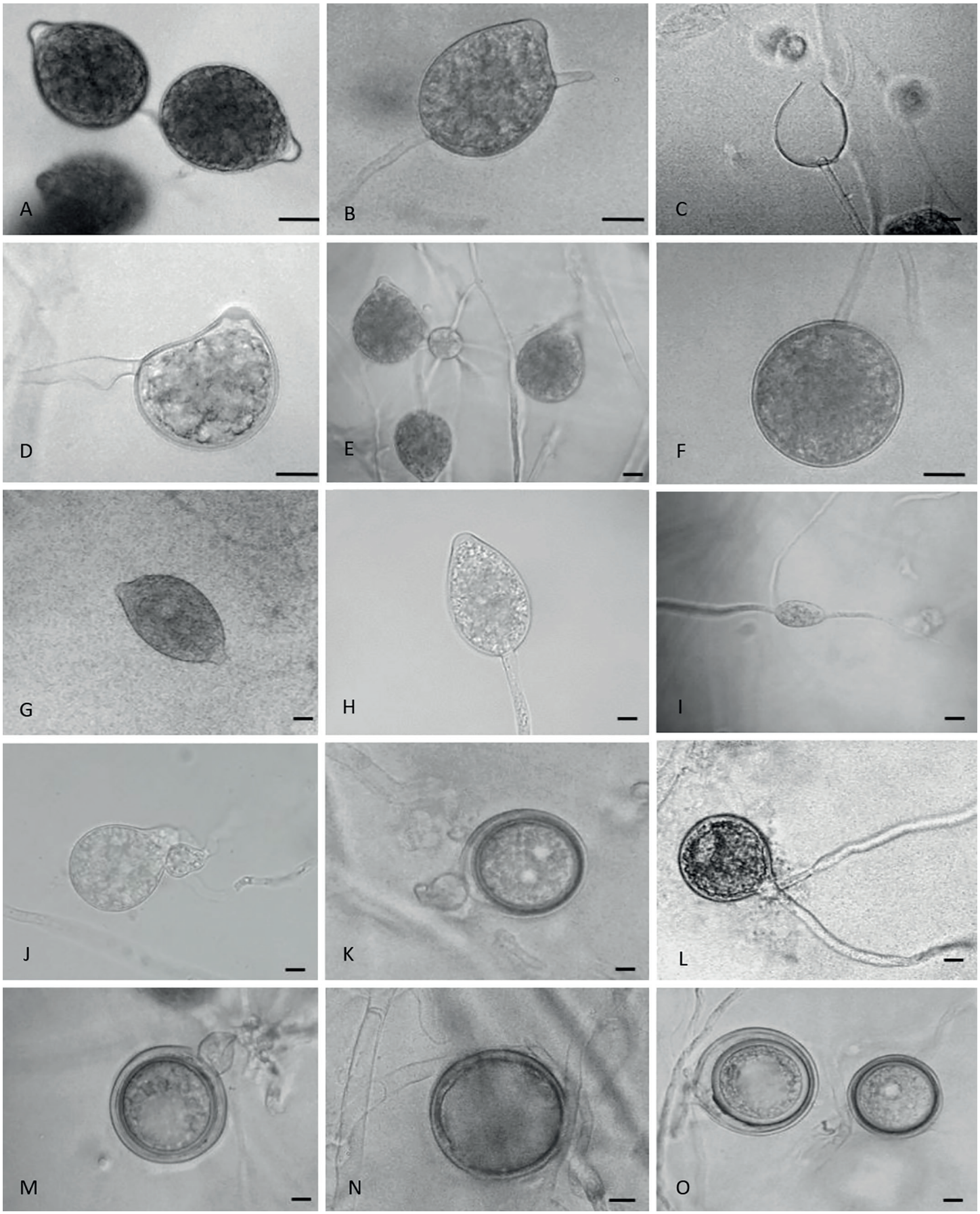

Fig. 3. A. Ovoid papillate, laterally inserted sporangia. **B.** Laterally inserted sporangium with short hyphal appendice. **C.** Conspicuous basal plugs on empty sporangium. **D.** Laterally inserted semipapillate sporangium with markedly curved apex and swelling before sporangial base. **E.** Sporangia produced on the tips of hyphae radiating from a hyphal swelling. **F.** Globose chlamydospore with thin walls. **G.** Limoniform sporangium. **H.** Elongated ovoid semipapillate sporangium. **I.** Hyphal swelling. **J.** Paragynous antheridium on an immature oogonium. **K.** Mature oogonia with thick-walled oospore and two pellucid bodies. **L.** Oospore germination. **M.** Mature aplerotic oogonia with think walled oospore and ooplast. **N.** Aborted oospore. **O.** Aplerotic and plerotic oospores. Scale bars = 5 µm.

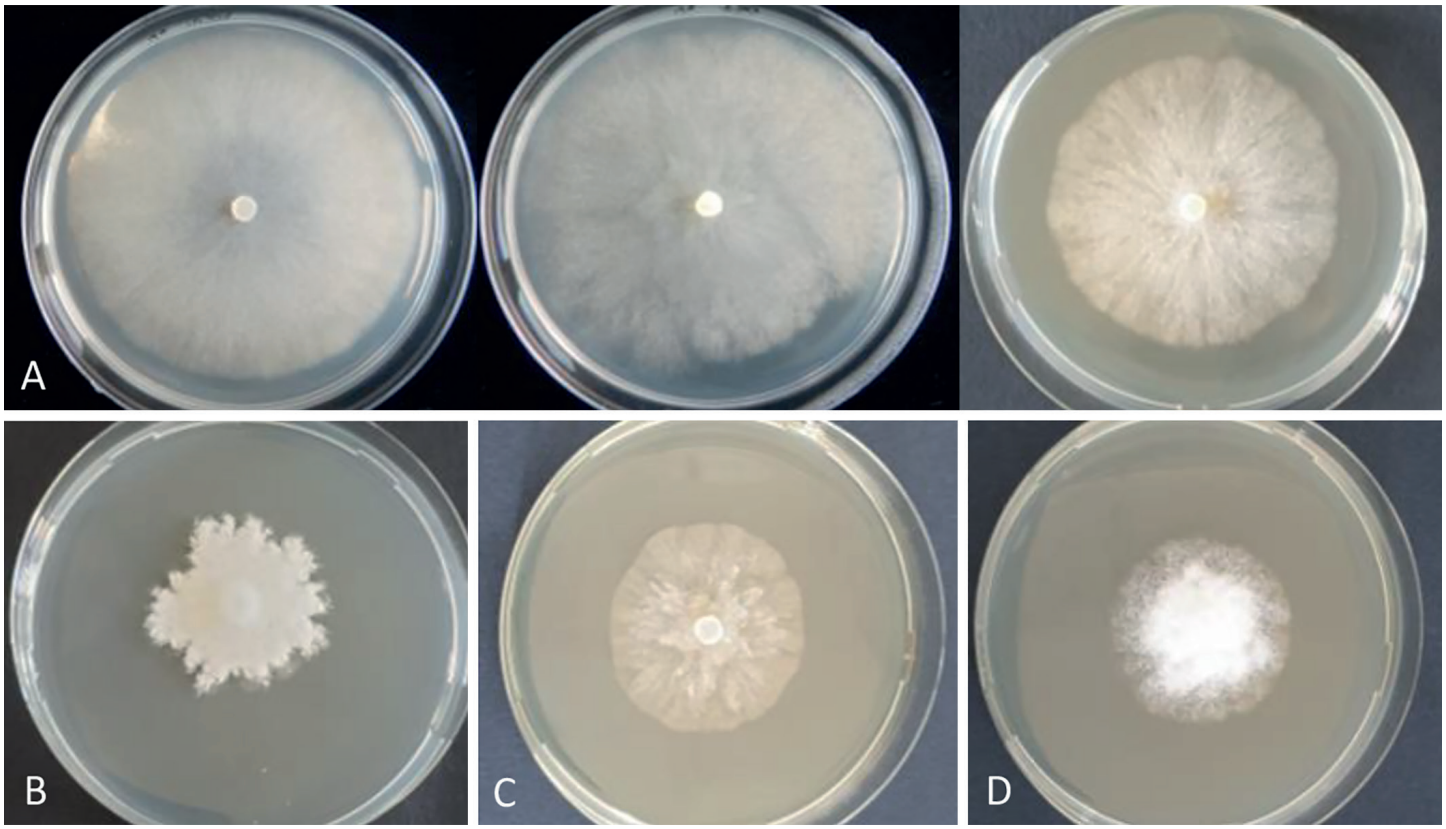

Fig. 4. A–D. Colony morphologies of *Phytophthora cathayensis sp. nov*. Cultures were grown at 20 °C on A (upper line). V8A. **B.** PDA. **C.** MEA. **D.** PARPNH. Photographed 7 d after inoculation.

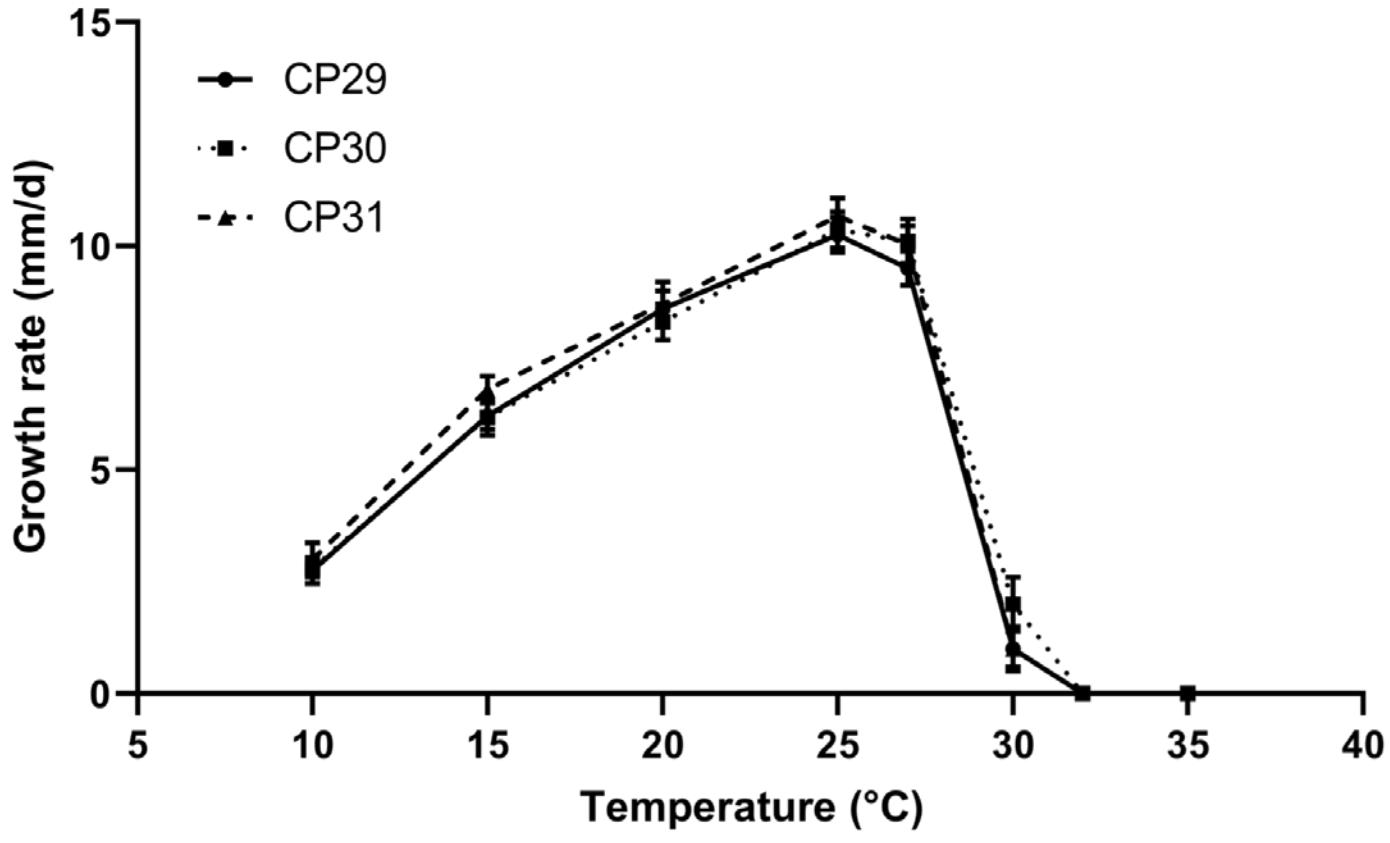

Fig. 5. Radial growth rates (mean ± SE) of three isolates of *Phytophthora cathayensis* on V8 juice agar at different temperatures.

Table 1. Comparison of morphological characters and dimensions, and temperature-growth relations of *Phytophthora cathayensis* and *Phytophthora* species within Clade 4.

Character	***P. cathayensis*** (present study)	***P. alticola*** (Bose *et al.* 2017)	***P. arenaria*** (Rea *et al.* 2011)	***P. boodjera*** (Simamora *et al.* 2015)	***P. litchii*** (idtools.org)	***P. megakarya*** (Erwin & Ribeiro 1996)	***P. palmivora*** (Erwin & Ribeiro 1996)	***P. quercetorum*** (Balci *et al.* 2008)
Sporangia (µm)								
LxB mean	27.3 ± 4.0 × 18.6 ± 2.4	37.6 ± 3.2 × 28.8 ± 4.5	31.8 ± 4.6 × 23.7 ± 3.5	39.2 ± 4.4 × 29.7 ± 3.4	n/a	36 × 26	45.3 × 29.8	40.5 ± 5.7 × 29.7 ± 4
Range of isolates means	17.7–38 × 13.8–26.6	37.9 ± 4.1 × 27.2 ± 4.5	28.9–34.8 × 21.4–28.3	32.6–44.6 × 24.7–33.3	20–33 × 16–22	20–60 × 13–41	40–60 × 25–35	39.1–43.3 × 26.8–32.6
L/B ratio	1.5 ± 0.1	1.28 ± 0.05	1.40 ± 0.17	1.27 ± 0.16	n/a	1.2–1.6	1.5	1.4
Sporangial characteristics	Papillate, rarely semipapillate	Papillate, frequently bipapillate, rarely bilobed.	Papillate, rarely bi/tripapillate or bilobed	Papillate, rarely bipapillate or bilobed	Papillate	Papillate	Prominently papillate	Papillate and occasionally bipapillate
Persistence	Persistent	Persistent	Persistent	Persistent	Caducous with short pedicel	Caducous pedicels of intermediate length (10 to 30 µm)	Caducous with short pedicel (5 µm	Persistent
Sporangiophores	Simple sympodia. Often produced on radiating hyphal swelling. Some case laterally attached	Simple or branched sympodia often with bulbous base, very often laterally attached	Simple or branched sympodia often with bulbous base	Simple or branched sympodia often with bulbous base, very often laterally attached	compound sympodial erected sporangiophores that resemble those produced in downy mildews	Loose sympodium	Sympodial sporangiophores	Unbranched and simple sympodial sporangiophores or intercalary in hyphae
Sporangia shape	Usually ovoid (90 %), also elongated ovoid and limoniform	Ovoid 87 %, obpyriform 9 %, distorted 4 %	Usually ovoid, also obpyriform or distorted	Ovoid 64 %, Limoniform 20 %, peanut-shaped 10 %, distorted 6 %	Globose, ovoid, ellipsoid	Limoniform, obpyriform or elipsoid	Variable in shape, mostly elliptical to ovoid	Ovoid-elongated, globose and peanut-like distorted shapes infrequently
Prolifferation	Absent	Absent	Absent	Absent	n/a	n/a	n/a	n/a
Breeding system	Homothallic	Homothallic	Homothallic	Homothallic	Sterile/homothallic	Heterothallic	Heterothallic	Heterothallic
Chlamydospores	Rare, terminal, on average 30.5 ± 3 µm	Absent	Absent	Absent	Absent	Present (average 30 µm)	Abundant, terminal or intercalary (32–42 µm)	Chlamydospores rarely produced, in average 30 ± 3 mm
Oogonia (µm)								
Mean diameter	24.5 ± 1.6	27.6 ± 1.7	25.3 ± 2.2	29.4 ± 2.3	n/a	26.8	n/a	31.5 ± 3
Diameter range	20–28	22.4–30.3	19.6–34.3	24.3–33.9	25–33 x 22–28	n/a	22.3–34.8	17–40
Oospore (µm)								
Mean diameter	22.2 ± 1.3	24.7 ± 1.9	22.3 ± 1.8	25.5 ± 1.9	n/a	na	22.8 ± 0.1	25 ± 2.5
Diameter range	18–25	19.1–29.2	16.0–28.3	20.92–29.3	18–21	23–28	n/a	14.5–32.5

Table 1. (Continued).

Character	*P. cathayensis* (present study)	*P. alticola* (Bose *et al.* 2017)	*P. arenaria* (Rea *et al.* 2011)	*P. boodjera* (Simamora *et al.* 2015)	*P. litchii* (idtools.org)	*P. megakarya* (Erwin & Ribeiro 1996)	*P. palmivora* (Erwin & Ribeiro 1996)	*P. quercetorum* (Balci *et al.* 2008)
Wall thickness	1.6 ± 0.2	2.48 ± 0.14	2.30 ± 0.34	2.47 ± 0.33	n/a	1.6 to 3.1	n/a	1.9 ± 0.7
Oogonial characteristic	Plerotic and aplerotic oospore. Oogonia terminal, globose to slightly subglobose with smooth walls	Aplerotic oospores, mature oogonia with a slightly wavy surface and golden-brown in colour	Aplerotic oospores, mature oogonia with a slightly wavy surface and golden-brown in colour	Aplerotic oospores, mature oogonia with a slightly wavy surface and golden-brown in colour	Oogonia smooth-walled, globose to ovoid	Plerotic and globose oospore. Oogonium pyriform, tapering at the base to a funnel shape	Aplerotic oospores. Oogonia spherical smooth-walled	Spherical and markedly aplerotic oospores. Oogonia frequently with comma-shaped tapered base
Antheridia (µm)								
Position	Paragynous, attached near the stalk and rarely displaced	Paragynous, often with finger-like projections	Paragynous, often with finger-like projections	Paragynous	Amphigynous	Amphigynous	Amphigynous, sometimes with spine or digitate projections	Antheridia paragynous, cylindrical or club-shaped
lxb mean	11.4 ± 1.3 × 9.4 ± 1.2	10.2 ± 1.2 × 8.2 ± 1.7	11.2 ± 1.7 × 8.4 ± 1.3	10.4 ± 1.9 × 8.3 ± 1.5	n/a	n/a	12–21 × 13–17	11 ± 2.5 × 9 ±1.5
Growth temperatures								
Opt temp (°C)	25	25	25	30	27–28	24–26	27.5–30	22.5
Max temp (°C)	30	30	32.5	35	30	29–30	35	32.5
Min temp (°C)	<10	11–14	11–14	11–14	12	10-11	11	<10
Lethal temp (°C)	32	35	n/a	>37.5	n/a	n/a	n/a	>32.5
Growth rate at optimum (mm/day)	10.2 (V8A)	3.50 (V8A)	5.9–7.4 (V8A)	9.18 (V8A)	n/a	n/a	n/a	7.5 (V8A)

Fig. 6. Necrotic lesions caused by *Phytophthora cathayensis* (isolate CP30) in the under-bark inoculation trial after 10 d 25 °C: on **A.** *Carya illinoensis* and **B.** *Carya cathayensis*. Scale bars = 1 cm.

illinoensis no difference in the pathogenicity between isolates was found in the two parameters, lesion length ($F = 5.70$; $P > 0.05$) and area ($F = 0.59$; $P > 0.05$). In contrast, on *C. cathayensis* isolate CP30 showed greater aggressiveness with significantly higher values in the length of necrosis ($F = 61.30$; $P < 0.05$) and in the area of necrosis ($F = 140.99$; $P < 0.05$).

DISCUSSION

Phytophthora cathayensis is described here based on physiological, morphological, and phylogenetic analyses. All these analyses strongly support the designation of the new species *P. cathayensis* within *Phytophthora* Clade 4.

With the same tree topology, the results presented here are consistent with previous phylogenetic studies obtained for the genus *Phytophthora* (Yang *et al.* 2017), and those specific to clade 4 (Balci *et al.* 2008, Simamora *et al.* 2015, Bose *et al.* 2017). It is possible to differentiate a consistent group formed by *P. quercetorum*, *P. arenaria*, *P. boodjera,* and *P. alticola* from which *P. megakarya* is separated. An additional group includes *P. cathayensis*, *P. litchii,* and *P. palmivora*. This group, although well-defined by the Bayesian posterior probabilities values, presents low bootstrap values in maximum likelihood. According to Russo & Selvatti (2018), the bootstrap test supports the repeatability of the data; that is, the probability of retrieving the same clade using an independent data set (other molecular markers, morphology, *etc*.). Looking at the results obtained from

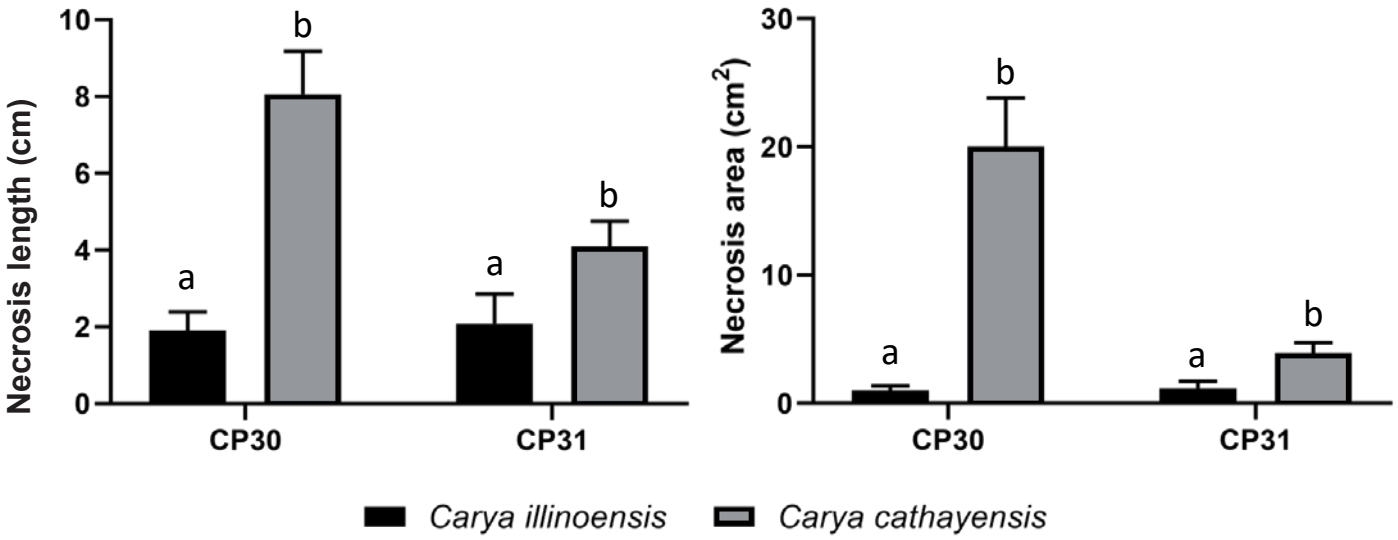

Fig. 7. Mean of length of necrosis (left) and area of the necrosis (right) caused by *Phytophthora cathayensis* isolates on *Carya illinoensis* and *C. cathayensis* 7 d post inoculation. Different letters indicate significant differences at $P < 0.05$, according to Tukey's post-hoc test. Vertical bars indicate standard deviation.

the analysis of the markers separately (Fig. S1), it is evident how the position of this "sub-clade" varies. A more phylogenetically in-depth study including a larger number of isolates is necessary to study the possible existence of different subclades within Clade 4.

Clade 4 represents species of *Phytophthora* with different hosts and diverse origins. *Phytophthora boodjera* has only been found in Western Australia (WA) and has mostly been isolated from dead and dying eucalypt seedlings in plant production nurseries in disturbed urban landscapes. It has been isolated from natural ecosystems on only three occasions (from *Banksia media*, *B. grandis*, and *Corymbia calophylla*) and currently it is considered to be an introduced species (Simamora *et al.* 2015). *Phytophthora arenaria* (Rea *et al.* 2011) has been recovered exclusively from natural Kwongan vegetation on the coastal sand plains of south-west Australia, and it has been suggested to be native to WA. *Phytophthora alticola* has been isolated as a pathogen of cold-tolerant *Eucalyptus* species and from *Acacia mearnsii* plantations, and it is probably native to South Africa (Bose *et al.* 2017). *Phytophthora quercetorum* has been reported from North America where it was isolated from the soil rhizosphere, and is associated with oak (Balci et al. 2008). *Phytophthora megakarya* is an oomycete plant pathogen that causes black pod disease in cocoa trees in west and central Africa (Opoku *et al.* 2000). *Phytophthora palmivora* is a cosmopolitan pathogen with a wide host range, including some very important economic crops such as cacao, papaya, black pepper, rubber, coconut, and citrus. The centre of origin is believed to be southeastern Asia (McHaw & Coffey 1994). *Phytophthora litchi,* formerly *Peronophythora litchi,* has been reported causing blossom blight on *Litchi chinensis* in Taiwan (Ann *et al*. 2012), China (Yu 1998), Vietnam (Vien *et al.* 2001) and Japan (Kobayashi 2007) and on *Euphoria longana* in Taiwan (Ann *et al.* 2012).

The inoculation trials fulfilled Koch's postulates. *Phytophthora cathayensis* was slightly aggressive to *C. illinoensis*, but showed high aggressiveness to *C. cathayensis*. The internal transcribed spacer sequence of *P. cathayensis* shared 100 % identity with an undescribed *Phytophthora* sp. P16825 in the World Phytophthora Genetic Resource Collection (WPC), isolated from *C. illinoensis* in Georgia in 2009. It was isolated specifically from pecan shuck which surrounds the nut (https://chassintranet.ucr.edu/phyto/#/productDetails/5035). *Carya illinoensis* is cultivated for its seed in the southern USA, primarily in Georgia, and in Mexico, which produces nearly half of the world's total production. Georgia is the largest pecan (from *Carya illinoensis*) producing state in the USA, accounting for approximately 30 % of national production (Wells 2014). Nowadays commercial *C. illinoensis* orchards in China are mainly distributed in Yunnan, Jiangsu, Zhejiang, and Anhui Provinces, areas that overlap with the traditional cultivation of *C. cathayensis*. Approximately 90 % of pecan processing in China is done in Lin'an, a city in Zhejiang Province, the origin of *C. cathayensis* (Yang *et al.* 2009) where *P. cathayensis* was isolated.

"Darwinian evolution predicts that being adapted to and co-evolved with their hosts, many of these pathogens are unlikely to do noticeable damage in their native ecosystems, and so are less likely to be detected" (Brasier 2008). Plant and microorganisms in the same natural environment have evolved together in association. These microorganisms often cause little noticeable damage to their host plants, having developed a natural balance through co-evolution. However, when a microorganism is introduced to another region of the world, important problems may arise where native plants have little resistance and the pathogen has eluded its natural enemies (Vettraino *et al.* 2017a). In the Chinese orchards of *C. cathayensis* sampled during this study, it is possible to observe a severe decline and high tree mortality due to *P. cathayensis*. Although *Phytophthora* are important forest pathogens, the present disease has not yet been described or reported in *C. illinoensis* orchards elsewhere in the world, not even in the USA where it seems probable that *P. cathayensis* was isolated for the first time. Furthermore, the pathogenicity analyses performed in this study showed that *C. illinoensis* is much less susceptible to *P. cathayensis* than *C. cathayensis*. Alien pathogens often enter into new countries on either non-hosts or unknown hosts, on infected but asymptomatic hosts,

or associated commodities (Vettraino *et al.* 2017a). According to Darwinian theory, it can be that *C. illinoensis* is a natural host of *P. cathayensis,* and due to their co-evolution, the disease is not that noticeable. According to this assumption, it is likely that *P. cathayensis* was introduced unnoticed with exotic propagation material of *C. illinoensis* from the USA, with a subsequent host shift to *C. cathayensis*. However, more detailed studies are required to clarify the centre of origin of *P. cathayensis* based on genotypic and phenotypic variability between and within the populations at the putative center of origin and area of invasion (Vettraino *et al.* 2017b, Scott *et al.* 2019).

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Supplementary Material: http://fuse-journal.org/

Figure S1. Maximum likelihood phylogenies of individual genes A. ITS; B. heat shock protein 90; C. β-tubulin and D. cytochrome oxidase I for Clade 4 *Phytophthora* species. Numbers above the branches reflect support obtained from the analysis of the same dataset (Bayesian posterior probabilities/Bootstrap values estimated by MEGA v. 7).

Table S1. GenBank accession numbers for sequences used in multi-locus analyses.

Table S2. GenBank accession numbers for all the gene regions sequenced for *Phytophthora cathayensis.*