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A taxonomic revision of *Phytophthora* Clade 5 including two new species, *Phytophthora agathidicida* and *P. cocois*

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Abstract

Phytophthora Clade 5 is a very poorly studied group of species of oomycete chromists, consisting of only two known species *P. castaneae* (≡ *P. katsurae, nom. illegit*.) and *P. heveae* with most isolates from East Asia and the Pacific Islands. However, isolates of two important disease-causing chromists in Clade 5, one of kauri (*Agathis australis*) in New Zealand, the other of coconut (*Cocos nucifera*) in Hawaii, poorly match the current species descriptions. To verify whether these isolates belong to separate species a detailed morphological study and phylogenetic analysis consisting of eight genetic loci was conducted. On the basis of genetic and morphological differences and host specificity, we present the formal description of two new species in Clade 5, *Phytophthora agathidicida sp. nov*. and *Phytophthora cocois sp. nov*. To clarify the typification of the other Clade 5 species, an authentic ex-holotype culture of *Phytophthora castaneae* is designated and *P. heveae* is lectotypified and epitypified.

Key words: nomenclature, oomycete, phylogeny, species description

Introduction

Phytophthora species are important oomycete chromists (Oomycetes, Peronosporales, Pythiaceae) plant pathogens causing significant disease (Kroon *et al*. 2012). Phylogenetic analyses of the genus (Blair *et al*. 2008, Cooke *et al*. 2000, Kroon *et al*. 2004, Martin *et al*. 2014), revealed that *Phytophthora* consists of ten phylogenetically defined clades. Of these, Clade 5 has been poorly studied, with insufficient taxon sampling, unclear species delimitation, and problems with the typification and nomenclature of the extant species. Currently Clade 5 consists of just two species, *Phytophthora castaneae* and *P. heveae*. The first step in resolving the taxonomy of this clade was taken by Pennycook (2013) who reviewed the nomenclature of *P. katsurae*, and found that name to be an illegitimate, superfluous replacement of the original legitimate name, *P. castaneae.*

Kauri (*Agathis australis*), a conifer in the Araucariaceae, is a dominant tree of lowland stands in northern New Zealand. The trees can be very large, with a trunk diameter of over 4.5 meters and with an age exceeding 1,500 years (Ahmed & Ogden 1987). The trees were important to early European settlers in New Zealand as a source of timber and kauri gum; this led to excessive deforestation during the 19th and early 20th centuries, and the species is today virtually restricted to relatively small reserves. The few remaining giant individual trees are accorded special status by New Zealanders, especially the indigenous Māori people. The trees are also major tourist attractions in Waipoua Forest, Northland, the largest remaining kauri stand in the country.

Two species of *Phytophthora* have been reported to cause disease in kauri. *Phytophthora cinnamomi* is found widely in natural kauri stands and occasionally causes disease, especially in regenerating stands on poorly drained sites (Podger & Newhook 1971). A collar-rot of kauri was first reported from a natural stand of unhealthy kauri on Great Barrier Island, a 285 km^2 island, lying in the outer Hauraki Gulf, approximately 100 km north-east of Auckland (Gadgil 1974). Symptoms included yellowing of foliage, canopy thinning and occasional tree death. Additionally, affected trees frequently had bleeding lesions on the lower trunk and main roots. The causative organism was identified as *P. heveae* by J. Stamps of the Commonwealth Mycological Institute (Gadgil 1974).

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In 2006 the *P. heveae* associated disease was reported again from kauri in a forest west of Auckland, both on regenerating and mature trees. This discovery initiated a major joint agency investigation into the identity, etiology, and management of the disease (http://www.kauridieback.co.nz). The original identification of the causative organism as *P. heveae* was questioned, as sequences of the ITS of the newly isolated suspected pathogen were identical to *P. castaneae*. However, the oogonia had only slight wall protuberances, very different from the strongly bullate protuberances of *P. castaneae* and more similar to the smooth oogonia of *P. heveae*. This raised the possibility that the kauri *Phytophthora* was a new species within Clade 5. It was given the interim tag name *Phytophthora* "taxon Agathis" (PTA) (Beever *et al*. 2009).

In the 1970s coconut trees (*Cocos nucifera*) in the Hawaiian island of Kauai were declining and dying with dark fruit rots and premature fruit drop. Over the next decade this disease spread to other Hawaiian islands of Oahu, Hawaii, and Maui. The presumptively same disease was also found on coconuts in the Côte d'Ivoire, and first identified as *Phytophthora heveae* (Quillec & Renard 1984, Quillec *et al*. 1984). However a later study considered that the Hawaiian isolates were more closely related to *P. castaneae*, as the isolates had oogonial protuberances and persistent sporangia (Uchida *et al.* 1992). This identification was considered uncertain as the sporangia were longer those that recorded for *P. castaneae*, and the oogonial ornamentations less prominent and fewer (Uchida *et al.* 1992). Later ITS sequence data grouped the organism in *Phytophthora* Clade 5, distinct from *P. heveae*, *P. castaneae*, and PTA (Beever *et al*. 2009).

This research attempts to elucidate the taxonomic diversity of *Phytophthora* Clade 5, by using a globally diverse set of isolates, and multigene sequencing. The kauri pathogen and the coconut pathogen are both described as new species, and the typification of *P. castaneae* and *P. heveae* is clarified.

Materials and methods

Sampling and Phytophthora *isolation*

An attempt was made to sample the genetic diversity of *Phytophthora* Clade 5 as widely as possible. A BLAST search of GenBank using the ITS sequence of PTA was used to select a genetically diverse set of isolates. Voucher cultures representing this diversity and that of named *P. heveae* and *P. castaneae* species were obtained from international culture collections. Culture collection and fungal herbarium (fungarium) abbreviations used herein are: CBS = Centraalbureau voor Schimmelcultures (Netherlands), ICMP = International Collection of Microorganisms from Plants (New Zealand), DAR = Plant Pathology Herbarium (Australia), NBRC = Biological Resource Center, National Institute of Technology and Evaluation (Japan), PDD = New Zealand Fungal and Plant Disease Fungarium (New Zealand), and WPC = World *Phytophthora* Collection (California, USA).

New Zealand isolates from kauri lesion margins were recovered around the collar (lower trunk) from cork cambium and plated to *Phytophthora* selective media (e.g., V8P_5ARPH; Jeffers 2006). *Phytophthora* was isolated from soil using an extended soil bioassay (using lupin cotyledons (*Lupinus albus*) in flooded soil for 2 days) and cedar needle (*Cedrus deodara*) baits (Dance *et al.* 1971, Erwin & Ribeiro 1994). Cultures were purified by single zoospore isolation.

Isolates studied are listed in Table 1. All cultures are available from ICMP culture collection, where isolates are stored in a metabolically inactive state in liquid nitrogen at -196°C on agar plugs or charcoal filter paper (Stielow *et al*. 2012); additional details on each culture are available on the ICMP website: http://www.landcareresearch.co.nz/resources/collections/icmp

DNA isolation, amplification and sequencing

Mycelium was collected from isolates grown on potato dextrose agar, and manually comminuted with a micropestle in 420 µL of Quiagen DXT tissue digest buffer; 4.2 µL of proteinase K was added and incubated at 55°C for 1 h. After a brief centrifugation 220 µL of the supernatant was placed in a Corbett X-tractorGene automated nucleic acid extraction robot. The resulting 100 µL of pure DNA in TE buffer was stored at -30°C in 1.5 mL tubes until use.

Gene sequences were obtained from five nuclear protein-coding genes: TIGA—transcription unit fusion of triose-phosphate isomerase (TPI) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [1592 bp], YPT1—Ras like GTPase [494 bp], ENL—Enolase [1118 bp], RPL10—60S ribosomal protein L10 gene [463 bp], HSP90—heat shock protein 90 [1595 bp], and two mitochondrial gene regions: COX1—cytochrome oxidase 1 [663 bp], ND1—NADH dehydrogenase subunit 1 [788 bp], and ITS—the internal transcribed spacer gene region [812 bp]. PCR primer sequences used to amplify these genes are shown in Table 2.

TABLE 1. Isolates studied in this research with Genbank accession numbers for each gene. Sequenced isolated indicated, see "specimens examined" for full details including equivalent collection numbers in other collections. Types indicated in bold font.

Isolate	Species	Host	Country	ITS	COX1	YPT1	HSP90	RPL10	ENL	ND1	TIGA
ICMP 17027	***P. agathidicida***	***Agathis australis***	**New Zealand**	**KP295308**	**KP295222**	**KP295429**	**KP295276**	**KP295363**	**KP295255**	**KP295334**	**KP295392**
ICMP 16471	*P. agathidicida*	*Agathis australis*	New Zealand	KP295318	KP295216	KP295439	KP295270	KP295357	KP295263	KP295328	KP295386
ICMP 18244	*P. agathidicida*	*Agathis australis*	New Zealand	KP295309	KP295224	KP295430	KP295278	KP295365	KP295256	KP295336	KP295394
ICMP 18358	*P. agathidicida*	*Agathis australis*	New Zealand	KP295310	KP295225	KP295431	KP295279	KP295366	KP295257	KP295337	KP295395
ICMP 18360	*P. agathidicida*	*Agathis australis*	New Zealand	KP295300	KP295226	KP295432	KP295280	KP295367		KP295338	KP295396
ICMP 18401	*P. agathidicida*	*Agathis australis*	New Zealand	KP295316	KP295227	KP295433	KP295281	KP295368	KP295268	KP295339	KP295397
ICMP 18404	*P. agathidicida*	*Agathis australis*	New Zealand	KP295312	KP295228	KP295434	KP295282	KP295369		KP295340	KP295398
ICMP 18406	*P. agathidicida*	*Agathis australis*	New Zealand	KP295314	KP295229	KP295435	KP295283	KP295370		KP295341	KP295399
ICMP 18407	*P. agathidicida*	*Agathis australis*	New Zealand	KP295317	KP295230	KP295436	KP295284	KP295371	KP295247	KP295342	KP295400
ICMP 18408	*P. agathidicida*	*Agathis australis*	New Zealand	KP295315	KP295231	KP295437	KP295285	KP295372	KP295269	KP295343	KP295401
ICMP 18410	*P. agathidicida*	*Agathis australis*	New Zealand	KP295311	KP295232	KP295438	KP295286	KP295373		KP295344	KP295402
ICMP 19434	***P. castaneae***	***Castanea crenata***	**Japan**	**KP295319**	**KP295234**	**KP295426**	**KP295288**	**KP295375**	**KP295265**	**KP295346**	**KP295404**
ICMP 19435	*P. castaneae*	*Castanea crenata*	Japan	KP295320	KP295235	KP295427	KP295289	KP295376	KP295266	KP295347	KP295405
ICMP 19436	*P. castaneae*	*Castanea crenata*	Japan	KP295321	KP295236	KP295428	KP295290	KP295377	KP295261	KP295348	KP295406
ICMP 19437	*P. castaneae*	*Castanea crenata*	Japan	KP295322	KP295237	KP295442	KP295291	KP295378	KP295267	KP295349	KP295407
ICMP 19635	*P. castaneae*	soil	China	KP295313	KP295243	KP295425	KP295297	KP295384	KP295246	KP295355	KP295413
ICMP 16915	*P. castaneae*	soil	Taiwan	KP295307	KP295219	KP295441	KP295273	KP295360	KP295254	KP295331	KP295389
ICMP 18737	*P. castaneae*	*Castanopsis fargesii*	Taiwan	KP295324	KP295233	KP295440	KP295287	KP295374	KP295264	KP295345	KP295403
ICMP 19450	*P. castaneae*	soil	Taiwan	KP295323	KP295238	KP295443	KP295292	KP295379	KP295245	KP295350	KP295408
ICMP 16948	***P. cocois***	***Cocos nucifera***	**USA, Hawaii**	**KP295304**	**KP295220**	**KP295423**	**KP295274**	**KP295361**	**KP295252**	**KP295332**	**KP295390**
ICMP 16949	*P. cocois*	*Cocos nucifera*	USA, Hawaii	KP295305	KP295221	KP295424	KP295275	KP295362	KP295253	KP295333	KP295391
ICMP 19685	*P. cocois*	*Cocos nucifera*	Ivory Coast	KP295306	KP295244	KP295418	KP295298	KP295385	KP295262	KP295356	KP295414
ICMP 19451	***P. heveae***	***Hevea***	**Malaysia**	**KP295326**	**KP295239**	**KP295419**	**KP295293**	**KP295380**	**KP295250**	**KP295351**	**KP295409**
ICMP 16691	*P. heveae*	soil	Australia	KP295302	KP295217	KP295420	KP295271	KP295358	KP295251	KP295329	KP295387
ICMP 17964	*P. heveae*	soil	China	KP295325	KP295223	KP295422	KP295277	KP295364	KP295259	KP295335	KP295393
ICMP 19452	*P. heveae*	*Persea americana*	Guatemala	KP295327	KP295240	KP295416	KP295294	KP295381	KP295260	KP295352	KP295410
ICMP 16914	*P. heveae*	*Theobroma caco*	Malaysia	KP295303	KP295218	KP295421	KP295272	KP295359	KP295249	KP295330	KP295388
ICMP 19453	*P. heveae*	*Rhododendron*	USA, NC	KP295301	KP295241	KP295417	KP295295	KP295382	KP295258	KP295353	KP295411
ICMP 19454	***P. multivora***	**soil**	**Australia**	**KP295299**	**KP295242**	**KP295415**	**KP295296**	**KP295383**	**KP295248**	**KP295354**	**KP295412**

The PCRs were performed in an Applied Biosystems Veriti Thermal Cycler in a total volume of 25 µL. The PCR mixtures contained 15.8 µL of UV-sterilised ultra-filtered water, 2.5 µL of 10× PCR buffer (with 20 mM $MgCl_2$), 2.5 µL of dNTPs (each 20 µM), 1 µL of each primer (10 µM), 1 µL of BSA, 1 µL of genomic DNA, and 0.2 µL (1 U) of Roche FastStart Taq DNA Polymerase.

The PCR conditions for ITS were 4 min at 94°C, then 38 cycles of 94°C for 30 s, 52°C for 45 s, 72°C for 45 s, and then 7 min at 72°C. The annealing temperatures differed for the other genes, with the optimum for each listed in Table 2. PCR products were purified on a Qiagen MinElute 96 UF PCR Purification Plate.

DNA sequences were obtained in both directions on an Applied Biosystems 3130xl Avant Genetic analyzer using BigDye v. 3.1 chemistry, electropherograms were analysed and assembled in Sequencher v. 4.10.1.

TABLE 2. PCR primers with oligonucleotide sequences and optimal PCR annealing temperatures.

Target region	Annealing temp °C	Name	Sequence
ITS	50	ITS4	TCCTCCGCTTATTGATATGC
		ITS6	GAAGGTGAAGTCGTAACAAGG
COX1	52	LeipF1	ATTCAACCAATCATAAAGATATTGG
		LeipR1	TAAACTTCTGGATGTCCAAAAAATCA
YPT1	58	Yph1F	CGACCATKGGTGTGGACTTT
		Yph2R	ACGTTCTCMCAGGCGTATCT
HSP90	62	HSP90_F1	GCTGGACACGGACAAGAACC
		HSP90_R2	CGTGTCGTACAGCAGCCAGA
RPL10	55	LROR-O	GCTAAGTGTTACCGTTTCCAG
		LR6-O	ACTTCTTGGAGCCCAGCAC
ENL	60	Enl_for	CTTTGACTCGCGTGGCAAC
		Enl_rev	CCTCCTCAATACGMAGAAGC
ND1	52	NADHF1	CTGTGGCTTATTTTACTTTAG
		NADHR1	CAGCAGTATACAAAAACCAAC
TIGA	64	Tig_for	TTCGTGGGCGGYAACTGG
		G3PDH_rev	GCCCCACTCRTTGTCRTACCAC

Phylogenetic analysis

Multiple sequence alignments of each gene were made with Geneious Pro v. 6.1.6 (Drummond *et al.* 2011). Bayesian inference (BI) was used to reconstruct the phylogenies using MrBayes v. 3.2.2 (Ronquist *et al.* 2012). jModelTest v. 2.1.4 (Posada 2008) was used to carry out statistical selection of best-fit models of nucleotide substitution using the corrected Akaike information criteria (AICc). Initial analyses showed that individual genes were broadly congruent, thus nucleotide alignments of all genes were concatenated using Geneious, and separate partitions created for each gene with their own model of nucleotide substitution. Analyses were run twice for 2×10^7 generations. Samples were taken from the posterior every 1000 generations. Convergence of all parameters was checked using the internal diagnostics of the standard deviation of split frequencies and performance scale reduction factors (PSRF), and then externally with Tracer v. 1.5 (Rambaut & Drummond 2007). On this basis the first 25% of generations were discarded as burn-in. An ex-holotype culture of *Phytophthora multivora* (Clade 2) was used as the outgroup.

Sequences derived in this study were lodged in GenBank (Table 1), the concatenated alignment and trees in TreeBASE (www.treebase.org) study number S16065, and taxonomic novelties registered in Index Fungorum.

Morphology of asexual and sexual structures

Sporangia were produced by incubating 10-day-old cultures, grown on 10% clarified V8 juice agar in non-sterile soil extract overnight, at 20°C, under continuous light. Non-sterile soil extract was produced by mixing 200 g of soil with reverse osmosis (RO) water, mixing and then coarsely filtering through paper towels (Jeffers 2006). The following day, the cultures were incubated at 4°C for 15 min prior to fixing with aniline blue and/or acid fuchsin in lacto-glycerol

solution. Microscopic examination included sporangial length × breadth, shape, assessment of caducity and size of exit pore.

Sporangial caducity was assessed using the method described by Hall (1998). This entailed growing 4 mm diameter mycelial plugs cut from 3-day-old cultures on clarified V8 juice agar in sterile RO for 3 days at 24°C. Mycelial plugs were removed from the liquid, agitated gently on a slide, and any dislodged sporangia examined under a compound microscope. Oogonia were produced by growing the 28 isolates on 10% clarified V8-juice agar, for 10 days, in the dark, at 20°C. After this time, samples were fixed with 0.05% trypan blue and/or acid fuchsin in lacto-glycerol solution for microscopic examination of oogonia/oospore diameter and length, gametangial morphology, and wall morphology.

Asexual and sexual structures were examined on a Nikon eclipse 80i microscope and micrographs captured using a Nikon Camera Head DS-Fi1 with images processed through a Nikon "Digital Sight" and NIS Elements.

Colony morphology, growth rates and cardinal temperatures

Isolates were grown at 20°C in darkness on clarified V8-juice agar (V8A), malt extract agar (MEA), corn-meal agar (CMA) and potato-dextrose agar (PDA) (all from BBL, Becton, Dickinson & Co.). Colony morphologies were photographed and described according to Brasier & Griffin (1979), Erwin & Ribeiro (1996) and Jung *et al*. (2003) after 10 days.

To establish the cultures for the growth studies, 6 mm plugs of mycelium were taken from the edge of actively growing of cultures on PDA, and placed in the centre of fresh plates. Radial growth (four measurements per colony, and five replicates per isolate) was recorded after 7 days incubation (Jung *et al*. 1999).

Cardinal temperatures were determined on PDA. Petri dishes were inoculated as above for growth rate studies. Five replicate plates for each isolate were subsequently incubated at temperatures of 3, 6, 10, 15, 17.5, 20, 22.5, 25, 30, and 32.5°C. Radial growth was measured as above after 7 days.

Scanning electron microscopy (SEM)

Oogonial suspensions were pelleted by centrifugation then fixed in 0.25% glutaraldehyde and subjected to a critical point drying procedure where water in the sample was gradually replaced by ethanol which then in turn was replaced with liquid carbon dioxide at high pressure as a transitional fluid. The sample was then coated with an ultrathin layer of gold using a NeoCoater MP-19020NCTR by low volume sputtering. SEM was run at 10 kV and analysed using the NeoScope JCM-5000 machine and software.

Statistical analyses

All morphological character measurements were analysed with the statistical programme "R" 2.14.0 (R Development Core Team 2011). The R package ggplot2 (Wickham 2009) was used for graphical plots. The box plots show the median, upper and lower quartiles, and the 'whisker' extends to the outlying data, or to a maximum of 1.5× the interquartile range, individual outliers outside this rage are shown as dots. Violin plots were smoothed with a bandwidth of 1.4.

Tests of significance were conducted using the Welch two sample t-test, with p values reported. Optimum temperatures were calculated by taking the maximum point on a loess curve ("local regression", Jacoby 2000) fitted to the data.

Results

Phylogenetic analysis

DNA sequences of eight genes were obtained from all 29 isolates included in the study and concatenated to form a supermatrix alignment of 7527 bp. The gene boundaries in the alignment were: RPL10: 1–463, COX1: 464–1126, ENL: 1127–2244, HSP90: 2245–3839, ITS: 3840–4651, ND1: 4652–5439, TIGA: 5440–7031, YPT1: 7032–7525.

A Bayesian inference phylogenetic analysis of this dataset is presented (Fig. 1). This tree is annotated with the species boundaries of the taxa that we accept, ex-type isolates are highlighted in bold and ex-holotype (HT) or ex-epitype (ET) status is indicated.

The posterior probability (PP) support for each of the nodes for the accepted species is 1, the most probable value. Within *Phytophthora castaneae* and *P. heveae* some nodes had lower values reflecting the less certain placement of isolates within these species. As a 50% majority-rule consensus tree, all PP ≤ 0.5 were collapsed to form polytomies.

Individual gene trees were general concordant with the concatenated analysis. The only differences were that not all genes were able to separate each of the four species, due to insufficient sequence variation. For example sequences of ITS were identical for *P. agathidicida* and *P. castaneae*.

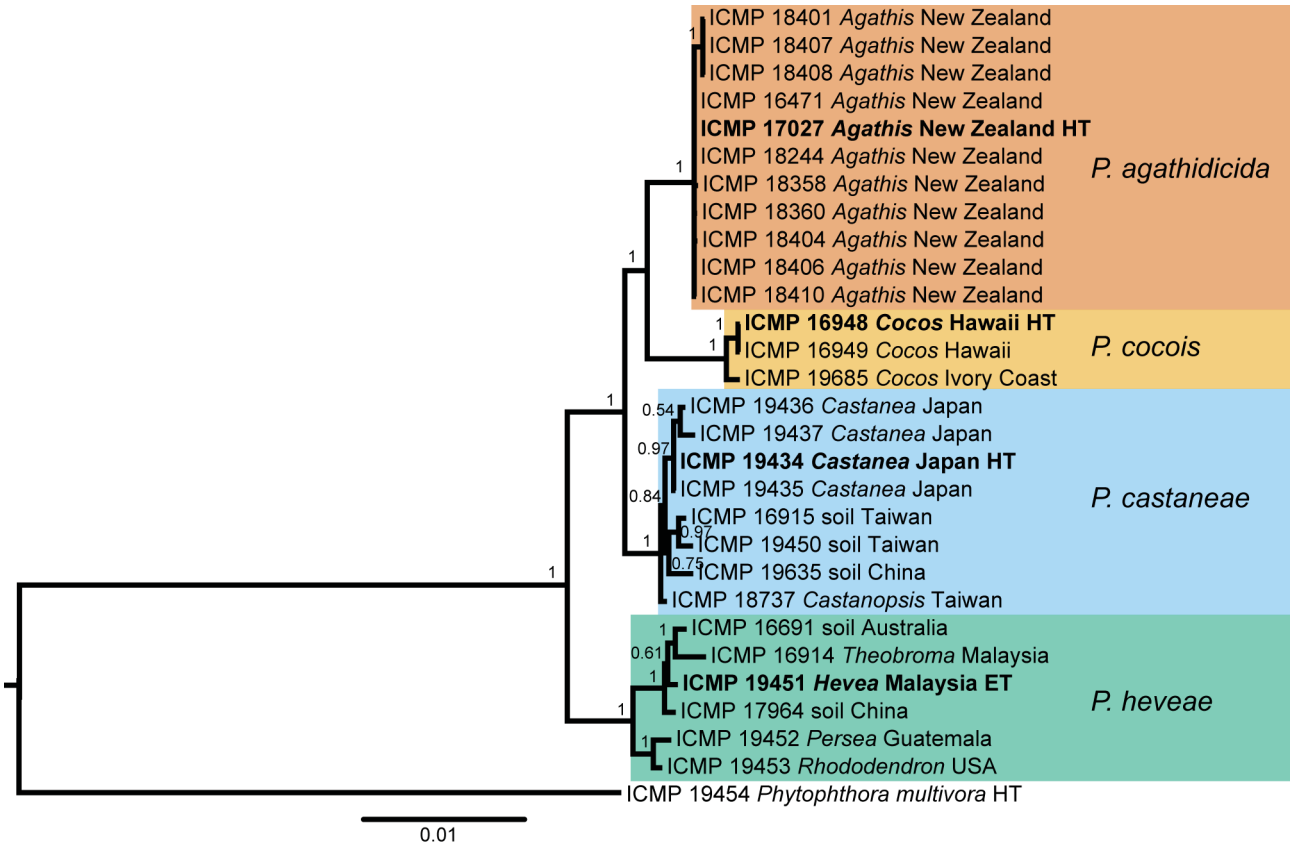

FIGURE 1. A Bayesian inference phylogenetic tree of 28 isolates in the *Phytophthora* Clade 5 group. The tree was built using concatenated sequences of the RPL10, COX1, ENL, HSP90, ITS, ND1, TIGA, and YPT1 genes each with a separate model of DNA evolution. Bayesian posterior probability values ≥0.5 are shown above nodes. Culture accession numbers are listed along with host plant genus and country of origin. Ex-type cultures are emphasised in bold font (HT = ex-holotype; ET= ex-epitype). Species delimitations are indicated with coloured boxes, and *P. multivora* (Clade 2) was used as an outgroup. The scale bar indicates the number of expected changes per site.

Morphology

A full list of morphometric statistics for each of the species in *Phytophthora* Clade 5 is listed in Table 3. Ranges are given in the format (lower extreme–) 25% quartile–median–75% quartile (–upper extreme).

Sexual

Oogonium ornamentation was assessed visually and scored from smooth to strongly bullate, as well as recording oogonium width. However, due to the variable nature of the oogonium ornamentation, the size of the oospore within the oogonium was a more consistent character to assess, and this data is presented visually in Fig. 2 as a violin plot. The mean oospore widths for *P. cocois* and *P. heveae* were not significantly different from each other nor from the Clade 5 mean (p-value = 0.01047), but oospore width of *P. agathidicida* is significantly larger than all other species (p-value $<2.2 \times 10^{-16}$) and *P. castaneae* is significantly smaller than all other species (p-value $<2.2 \times 10^{-16}$). The coloured 'violin' indicates the variability of widths measured, for example in *P. cocois* the mean spore size is 24.2 µm, but the most common spore size measured was 22.5 µm.

TABLE 3. Comparative data for morphological and physiological characters for species within *Phytophthora* Clade 5.

Character	*P. agathidicida*	*P. castaneae*	*P. cocois*	*P. heveae*
No. of isolates	11	6	2	6
Cardinal growth temp (°C)				
minimum	6	6	10	10
maximum	25	30	30	32.5
optimum	21.5	22.3	22	22.5
n	960	240	240	120
Growth rate at 20 °C (7 days)				
CMA mean	20	28.5	19.6	24.9
CMA range	(13–)18–20–22(–34)	(12–)22–31.5–35(–42)	(9–)13.5–21–25(–32)	(12–)21–25–27.3(–42)
MEA mean	28.9	27.6	23.9	21.1
MEA range	(20–)26–28–32(–45)	(12–)15–30–36(–42)	(22–)23–24–25(–25)	(8–)18.8–21–25(–34)
PDA mean	24.3	26.3	24.7	24.4
PDA range	(16–)22–25–27(–32)	(10–)14–31–36(–46)	(22–)23–24.5–26(–27)	(6–)21–27–30(–37)
V8A mean	31.5	31.2	35.4	32.2
V8A range	(20–)28–33–35(–38)	(15–)26–35–37(–43)	(32–)35–35–36(–40)	(11–)31.8–35–37(–42)
n	880	560	160	480
Sporangia				
length mean	39.6	ND	38.4	ND
length range	(14.9–)32.4–37.5–47.5(–75)	ND	(18.6–)31.4–39.6–47.1(–50)	ND
width mean	28.4	ND	25.4	ND
width range	(12.4–)24.8–27.7–32.2(–50)	ND	(12.4–)22.4–27.2–29(–35)	ND
pore mean	5	ND	5.2	ND
pore range	(2.5–)5–5–5(–7.5)	ND	(3.7–)5–5–5(–7.5)	ND
pore type	papillate	papillate	papillate	papillate
sporangial caducity	persistent	persistent	persistent	persistent
n	360		80	
Oogonia				
width mean	31.9	24.2	26.2	28.2
width range	(22.2–)29.7–32.2–32.7(–45)	(14.9–)20–24.8–27.3(–35)	(22.3–)24.8–25–27.3(–35)	(19.8–)25–27.5–29.8(–45)
n	440	280	80	240
Oospore				
width mean	27.7	18.3	24.2	23.4
width range	(19.8–)25–27.5–29.7(–35)	(9.9–)14.9–19.3–20(–29.7)	(19.8–)22.3–24.3–25(–29.7)	(17.3–)21.3–23.8–25(–35)
n	440	280	80	240
Antheridia				
position	amphigynous	amphigynous	reflexed amphigynous	amphigynous
length mean	12	ND	11.5	ND
length range	(7.4–)10.9–12.4–12.5(–17.5)	ND	(7.5–)10–11.3–12.5(–16.3)	ND
width mean	11.1	ND	9.8	ND
width range	(7.4–)10–10.9–12.4(–15)	ND	(7.4–)9.9–9.9–10(–12.5)	ND
n	360		80	
Sexual system	homothallic	homothallic	homothallic	homothallic

Antheridial length and width were collected for the two new species described here. There were no significant differences in length and width, but the *P. cocois* antheridia were often strongly reflexed.

All isolates tested were able to form the sexual oogonial spores in pure culture, and thus have a homothallic mating system.

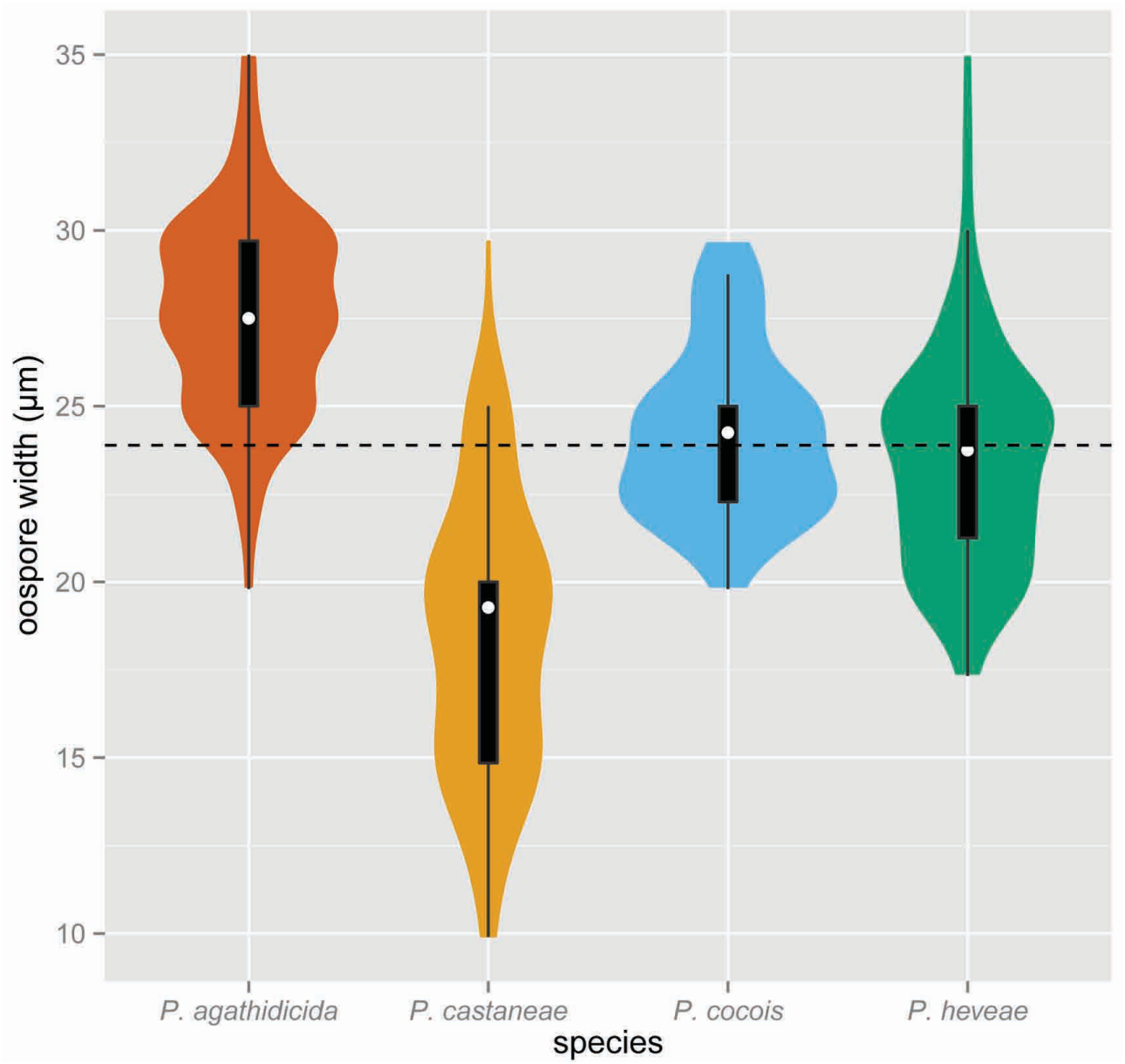

FIGURE 2. Violin plot of oospore with variation in *Phytophthora* Clade 5 species. Inside each violin plot is a box plot summarising ranges and individual means (white dots). The dashed line indicates the mean width over all Clade 5 species (23.89 µm). The mean spore width for *P. cocois* and *P. heveae* cannot be significantly distinguished from each other or the Clade 5 mean. Mean oospore width for *P. agathidicida* is significantly greater and *P. castaneae* significantly less than the Clade 5 mean.

Asexual

Sporangial shape was assessed visually, and data recorded for length, width, and exit pore size. Sporangial morphometrics were collected only for the two new species described; there were no significant differences in sporangial width, length, or pore size. None of the isolates examined had caducous sporangia (*i.e.*, no sporangia were dislodged from their pedicels).

Culture

Colony morphology was assessed on CMA, MEA, PDA, and V8A media after 7 days of growth at 20°C. The

morphology after 7 days was generally uniform within the species tested, consistent differences between species was not noticeable.

There were different growth-rate responses from each of the four species studied (Fig. 3). Overall, *P. castaneae* was the fastest growing on three of the four media tested, but was also the most variable. *P. heveae* culture ICMP 19453 grew much slower than other *P. heveae* cultures on each media resulting in the outliers seen in Fig. 3.

Cardinal growth temperatures were assessed at ten temperatures between 3°C and 32.5°C with five replicates, the full data set was scatter plotted and a trend line to the data fitted as a loess curve (Fig. 4). Although the optimum temperature of all species was similar, the cardinal temperature plot for *P. agathidicida* appeared to be skewed compared to those of the other three species, with *P. agathidicida* growing relatively faster than the other species at 5–22.5°C, but slightly slower at >25°C. Unlike the other three species, *P. agathidicida* did not grow at 30°C.

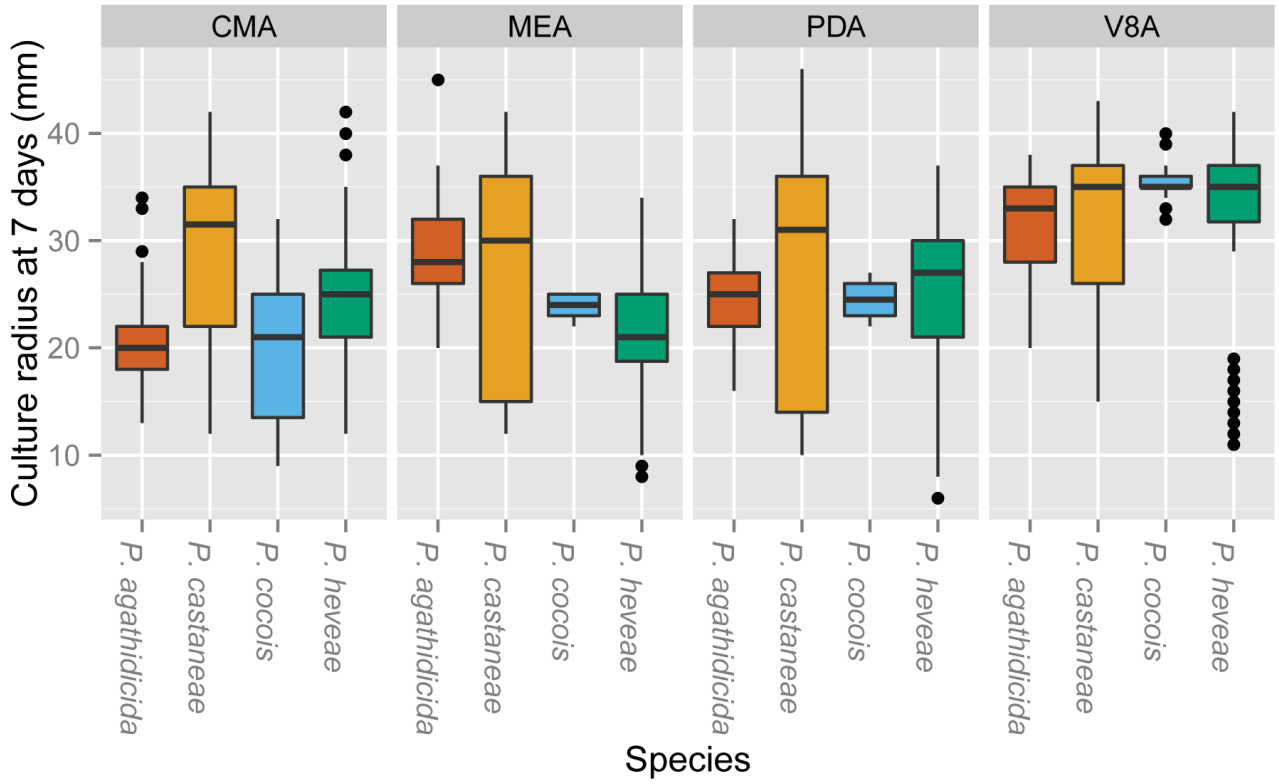

FIGURE 3. Comparative radial growth rates on four selected agar media: (L–R) corn-meal, malt extract, potato-dextrose, V8-juice.

Taxonomy

Phytophthora agathidicida B.S. Weir, Beever, Pennycook & Bellgard, *sp. nov.* (Fig. 5) IF550518

= *Phytophthora* 'taxon Agathis' (PTA) Beever *et al.* (2009), *Phytophthoras in Forests and Natural Ecosystems*, *nom. inval*.

Differs from other *Phytophthora* Clade 5 species in its oogonium ornamentation with occasional and slightly raised protuberances, and its larger mean oospore diameter (31.9 µm). Found in association with *Agathis australis*. Differs from *P. cocois* and *P. heveae* in its DNA barcode sequence of ITS; differs from all other *Phytophthora* species in its DNA sequence of the ND1 gene.

Etymology:—Latin noun: *agathid-* (linguistic stem of *Agathis*) and *-cida* (suffix: one who kills)—the *Agathis*-killer.

Typification:—New Zealand. Coromandel: Great Barrier Island, from bleeding lesion on trunk of *Agathis australis* (D. Don) Lindl. ex Loudon, 23 Mar. 2006, R.E. Beever REB316-14, dried culture specimen, holotype PDD 91595; ex-holotype living culture preserved in a metabolically inactive state as ICMP 17027 = WPC P15175.

Description:—The species is homothallic, with isolates forming oogonia quickly (3–4 days) and abundantly on V8A. Oogonia are globose with a mean width of 31.9 µm, and ranging between (22.2–)29.7–32.2–32.7(–45) µm.

Oogonium wall ornamentation is mildly stipulate. Oospores nearly fill the oogonia with a mean width of 27.7 µm, and ranging between (19.8–)25–27.5–29.7(–35) µm. Antheridia are amphigynous, globose some with knots at the base. Sporangia are globose to ovoid-ellipsoid, papillate, borne terminally from long thin branched sporangiophores and could be formed via internal proliferation. Sporangia are non-caducous (although some isolates have a somewhat defined septum near the base of the sporangium, see Fig. 5H). Sporangia have a mean width of 28.4 µm, and ranging between (12.4–)24.8–27.7–32.2(–50) µm, and a mean length of 39.6 µm, and ranging between (14.9–)32.4–37.5–47.5(–75) µm. Vegetative hyphae are simple, with slight swellings, and lacking chlamydospores in culture. Colony morphology after 7 days was very uniform across the isolates tested on most of the media examined. Colonies are loosely aerial. On 5% clarified V8-juice agar, there is a weakly stellate radial pattern. Minimum growth temperature 6°C; maximum 25°C; optimum 21.5°C. Complete morphometric statistics are presented in Table 3.

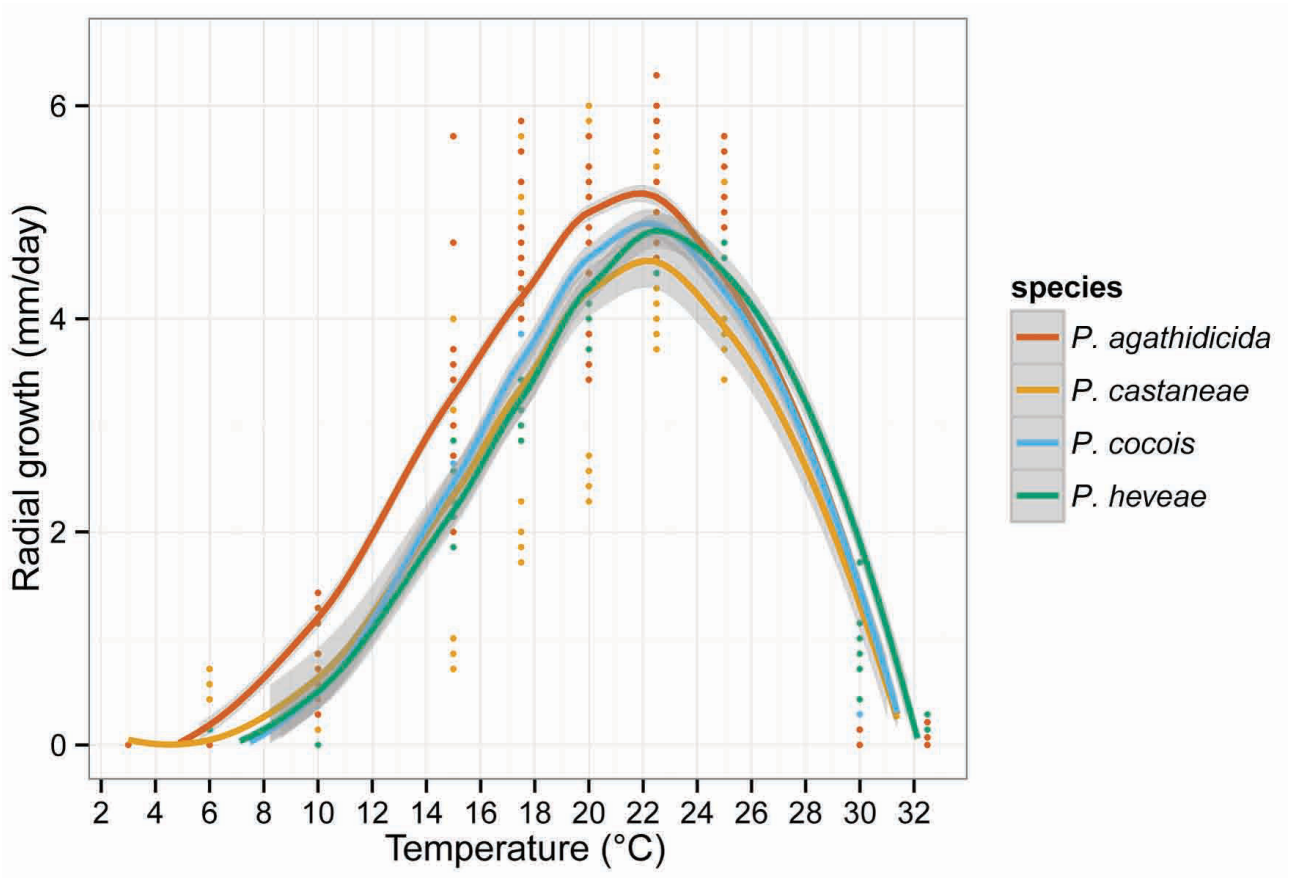

FIGURE 4. Cardinal temperature graph with loess fitted curves. Cardinal temperature plot (on 5% V8-juice agar) of 28 isolates in *Phytophthora* Clade 5. Data variation smoothed using loess curves, with 95% confidence band (grey shading).

Habitat:—Known only from kauri (*Agathis australis*) trees and associated soil in northern (<38°S) New Zealand's mixed podocarp broad-leaf forest.

Other specimens examined:—New Zealand. Northland: Trounson Park, trunk lesion of *Agathis australis*, 23 Mar. 2010, N. Waipara, culture REB 327-41 = ICMP 18404; Northland: Trounson Park, soil under *A. australis*, 23 Mar. 2010, A. Vannini, culture 327-60 = ICMP 18410; Northland: Raetea, trunk lesion of *A. australis*, 23 Mar. 2010, N. Waipara, culture 327-34 = ICMP 18401; Northland: Waipoua Forest, near Tane Mahuta, trunk lesion of *A. australis*, 23 Mar. 2010, N. Waipara, culture 327-47 = ICMP 18407; Northland: Waipoua Forest, trunk lesion of *A. australis*, 23 Mar. 2010, N. Waipara, culture 327-53 = ICMP 18408; Coromandel: Great Barrier Island, from wood of *A. australis*, Mar. 1972, P. Gadgil, culture ATCC 32256 = FRI 135 = ICMP 16471; Coromandel: Great Barrier Island, Kaiarara, from bleeding lesion on trunk of *A. australis*, 21 May 2009, N. Waipara, culture REB 326-154 = ICMP 18360; Auckland: Pakiri, trunk lesion of *A. australis*, 18 Nov. 2008, R.E. Beever, REB326-1 = ICMP 18244; Auckland: Waitakere Ranges, Cascades, from bleeding lesion on trunk of *A. australis*, 7 Oct. 2009, R.E. Beever, culture REB 326-221 = ICMP 18358; Auckland: Huia, soil under *A. australis*, 23 Mar. 2010, A. Vannini, culture 327-46 = ICMP 18406.

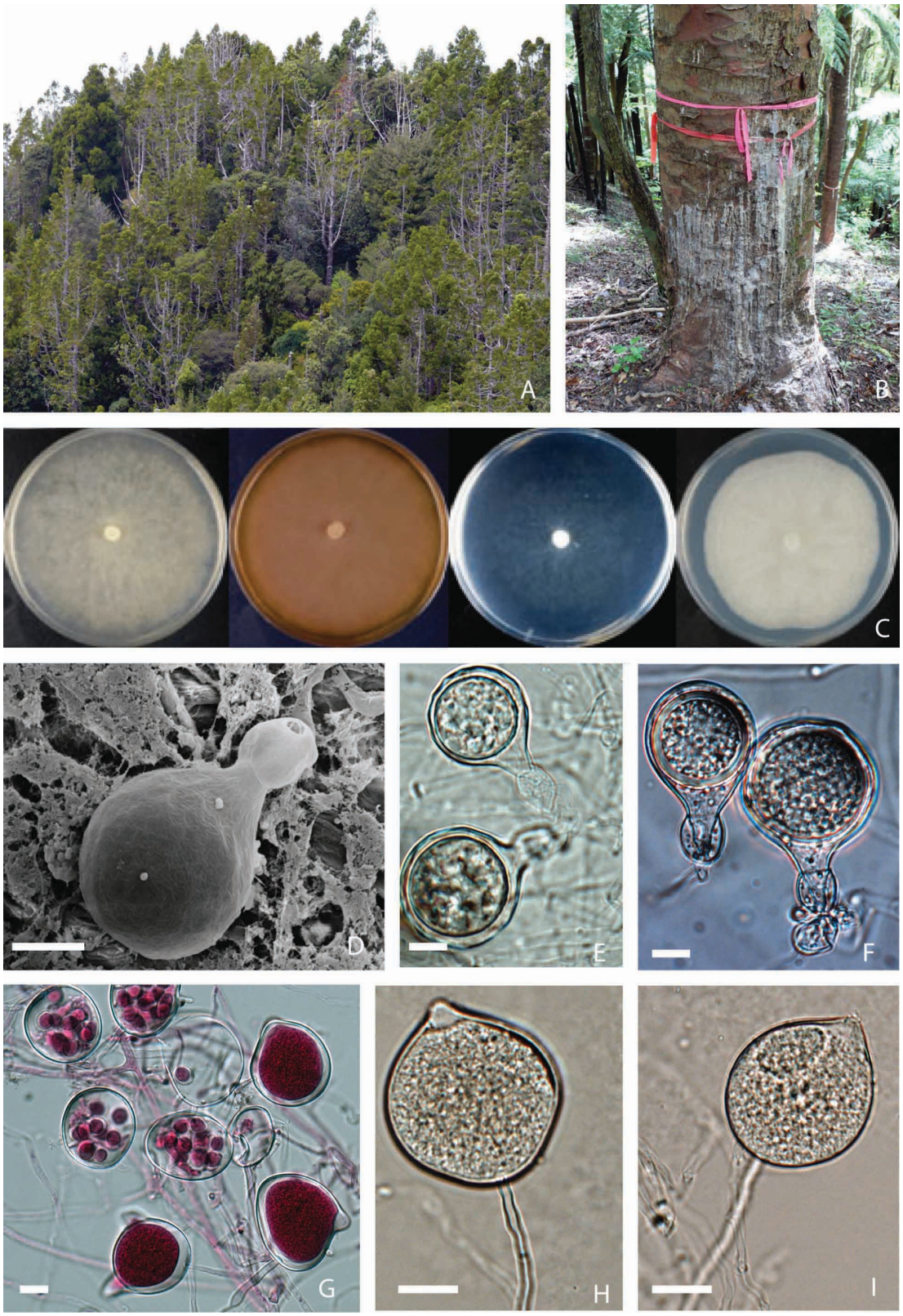

FIGURE 5. *Phytophthora agathidicida*. A. Kauri stand on steep ridge displaying canopy thinning—"little-leaf" syndrome. There is a progressive dieback of the crown in response to parasitism of the cork cambium. B. Bleeding resin ("kauri gum") associated with collar-rot of lower trunk. The advancing lesion will spread laterally, eventually girdling the tree. C. Diffuse non-patterned, colony morphology ex-holotype ICMP 17027 after 10-days incubation at 20°C in the dark. (L–R) Clarified V8 juice agar, MEA, CMA, and PDA. D. Scanning electron micrograph of ex-holotype ICMP 17027 oogonia. E. Ex-holotype ICMP 17027 oogonia with amphigynous, tapering bases. F. ICMP 16471 oogonia with amphigynous, sub-globose antheridia (image courtesy of M.A. Dick, Scion). G. ICMP 18407 Differentiation of the cytoplasm within papillate sporangia into acid fuchsin stained zoospores. H, I. ICMP 17027 papillate, globose sporangia. Scale bars = 10 μm.

Disease and management:—The root and collar rot of kauri (colloquially termed "kauri dieback") is the subject of a long term management response led by the Ministry for Primary Industries, Department of Conservation, regional councils (Auckland, Bay of Plenty, Northland, Waikato), in partnership with the Tangata Whenua Roopu. Delimitation surveys have confirmed impacts upon kauri of all age classes, in forest remnants and plantations, throughout its geographic range. Phytosanitary measures have been put in place in high-access regional parks to control the spread of the pathogen by foot-traffic, and the use of phosphite to control the disease *in planta* has also commenced.

Phytophthora castaneae Katsura & K. Uchida, in Katsura, Trans. Mycol. Soc. Japan 17: 241. 1976. IF283626.

≡ *Phytophthora katsurae* W.H. Ko & H.S. Chang, Mycologia 71: 841. 1979, *nom. illegit*. [superfluous].

Typification:—Japan. Ibaraki: from *Castanea crenata* Siebold & Zucc. trunk rot, 1971, K. Uchida, holotype specimen Herb. 1971-031 (Plant Pathology Herbarium, Kyoto Prefectural University, Japan); ex-holotype culture P8 = NBRC 9753 = ICMP 19434 = WPC P10187.

Ko & Chang (1979) correctly cited the holotype of the species to be the Japanese specimen "No. 1971-031, Plant Pathology Herbarium, Kyoto Prefectural University, Japan", but then described the morphology of an isolate from Taiwan (ATCC 36818); this isolate has subsequently been used as a reference strain for *P. castaneae* (e.g. Q-Bank). However, a culture of 1971-031 isolated by K. Uchida (P8) was deposited in the IFO (now NBRC) culture collection of Japan as NBRC 9753, and is thus an authentic ex-holotype culture.

Description:—Katsura (1976) provided an initial description of this species and Ko & Chang (1979) described Taiwanese specimens. The more recent description of Erwin & Ribeiro (1996) is still useful, although the circumscription encompasses isolates we consider *P. cocois*. The primary morphological characters of isolates considered as *P. castaneae* examined in this work are presented in Table 3.

Habitat:—Known on trunk rot of *Castanea crenata* from Japan and Korea, on *Castanopsis fargesii* Franch. from Taiwan, and soil from China.

Other specimens examined:—China. Hainan Island: forest soil, H. Ho, culture FFM 10-5C = ATCC MYA-3422 = ICMP 19635 = WPC P10661. Taiwan. from soil, F. Panabieres culture F441= ICMP 16915 = SCRP 388; Nan Ton County: Lenhuachih (Lianhuachi), natural forest soil, 13 May 1988, H.S. Chang, Herb. IMI 325914, culture L-2A = ATCC 36818 = ICMP 19450 = CBS 587.85 = IMI 325914 = WPC P15598; Nan Ton County: soil under *Castanopsis fargesii*, 13 Nov 2010, S.E. Bellgard, culture REB326-308 = ICMP 18737. Japan. Ibaraki: from *Castanea crenata* trunk rot, 1971, K. Uchida, culture P1 = NBRC 30433 = ICMP 19435; Ibaraki: from *C. crenata* trunk rot, 1971, K. Uchida, culture P3 = NBRC 30434 = ICMP 19436; Ibaraki: from *C. crenata* shoot, 1971, K. Uchida, culture P12 = NBRC 30435 = ICMP 19437.

Other Remarks:—Pennycook (2013) reviewed the nomenclatural status of *P. katsurae* and found this name to be an illegitimate, superfluous replacement of the legitimate original name, *P. castaneae*.

Phytophthora cocois B.S. Weir, Beever, Pennycook, Bellgard & J.Y. Uchida, *sp. nov.* (Fig. 6). IF550519

Differs from other *Phytophthora* Clade 5 species in oogonium ornamentation with moderately bullate protuberances. Found in association with *Cocos nucifera.* The DNA barcode sequence of ITS distinguish *P. cocois* from all other *Phytophthora* species.

Etymology:—Latin genitive noun: *cocois*—of *Cocos*.

Typification:—USA. Hawaii: Kauai, from diseased fruit (husk) of *Cocos nucifera* L., J.Y. Uchida H1024, May 1990, dried culture specimen, holotype PDD 103199; ex-holotype living culture preserved in a metabolically inactive state as ICMP 16948.

Description:—The species is homothallic, with oogonia formed abundantly on V8A. Oogonia are globose with a mean width of 26.2 µm, and ranging between (22.3–)24.8–25–27.3(–35) µm. Oogonium wall ornamentation is mildly bullate, and often less ornamented isolated in host tissue. Oospores nearly fill the oogonia with a mean width of 24.2 µm, and ranging between (19.8–)22.3–24.3–25(–29.7). Antheridia are amphigynous, globose and often strongly reflexed (bent). Antheridial length in fresh host tissue is often longer than isolates in culture. Sporangia are globose to ovoid, papillate and non-caducous. Sporangia have a mean width of 25.4 µm, and ranging between (12.4–)22.4–27.2–29(–35) µm, and a mean length of 38.4 µm, and ranging between (18.6–)31.4–39.6–47.1(–50) µm. Colony morphology after 7

days was very uniform across the isolates tested on most of the media examined. Colonies are loosely aerial. Minimum growth temperature 10°C; maximum 30°C; optimum 22°C. Complete morphometric statistics are presented (Table 3), and Uchida *et al*. (1992) provide an original description.

FIGURE 6. *Phytophthora cocois*. A. Death of young coconut fronds is the first common symptom of this disease. The young leaves wilt, dry, and are often bent or drooping into the tree canopy. B. Premature loss of fruits is an early sign of disease. In later stages, fruit rots become more common. Infected green fruits have characteristic "green island" black to brown rots that expand irregularly and frequently form green areas surrounded by darkened diseased tissue. C. internal fruit rot. D. Colony morphology of ex-holotype ICMP 16948 after 10-days incubation at 20°C in the dark. (L–R) Clarified V8 juice agar, MEA, CMA, and PDA. E. Scanning electron micrograph of ex-holotype ICMP 16948 oogonia showing reflexed antheridium. F–G. Ex-holotype ICMP 16948 (F) and ICMP 16949 (G) oogonia showing some reflexed antheridia and mildly bullate oogonium ornamentation. H. ICMP 16948 Differentiation of the cytoplasm within papillate sporangia into zoospores. I, J. ICMP 16949 sporangia shape variation. Scale bars = 10 µm.

Habitat:—Known on diseased coconut (*Cocos nucifera*) from Hawaii, and Côte d'Ivoire. (Probably also on cocoa (*Theobroma cacao* L.) from Côte d'Ivoire.)

Other specimens examined:—USA. Hawaii: Kauai, from diseased fruit of *Cocos nucifera*, coll. J.Y. Uchida culture H1026 = ICMP 16949; Côte d'Ivoire. Port Bouet, from nut of *Cocos nucifera*, De Franque-Ville, culture IMI 360596 = ICMP 19685.

Disease and management:—In Hawaii the disease has killed hundreds of coconut trees. Areas with highly conducive environments have lost most of their trees, thus the disease is now less common than in previous decades. Infected trees, which are identified by dead young leaves, cannot be saved by the application of systemic fungicides. Rapid removal and destruction of infected trees and removal of old leaves and petioles is needed to prevent infection of healthy trees (Uchida 2004). No other palm in Hawaii has been found to be affected by *P. cocois* and thus other ornamental palms are planted where coconut fruit is not required.

Phytophthora heveae A.W. Thomps., Malayan Agric. J. 17(3–4): 77, 1929. IF252580.

≡ *Phytophthora palmivora* var. *heveae* (A.W. Thompson) Orellana, Phytopathology 49: 213, 1959.

Typification:—Malaysia. "*P. heveae* Thompson, showing sporangia and oospore formation", Plate 1D (line drawing) in A.W. Thompson, Malayan Agricultural Journal 17(3–4): 53–100 (1929), **lectotype designated here** [IF550520]. Malaysia. Selangor: pod rot of *Hevea brasiliensis* (Willd. ex A. Juss.) Müll. Arg., Dec. 1927, A.W. Thompson, culture preserved in a metabolically inactive state, CBS 296.29 (= IMI 180616 = WPC P3428 = ATCC 58815 = ICMP 19451 = KACC 44943), **epitype designated here** [IF550521].

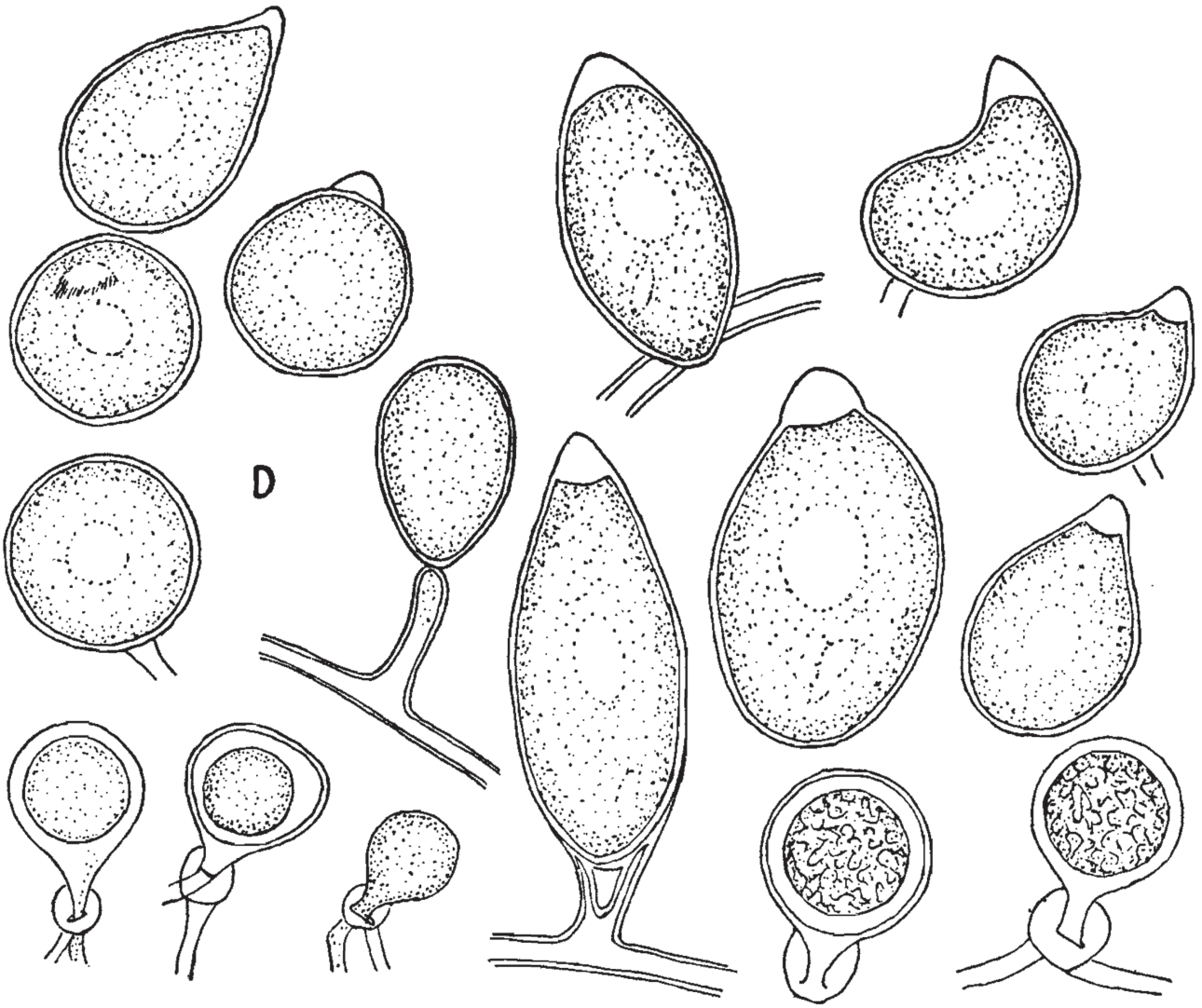

FIGURE 7. Plate ID (lectotype) from Thompson (1929), original caption: "*P. heveae* Thompson, showing sporangia and oospore formation".

Thompson (1929) did not designate a type specimen in his manuscript (not a requirement for valid publication prior to 1958). However, in the same year he deposited a culture of *P. heveae* as CBS 296.29. In 1974 this culture was deposited as IMI 180616, and in 1980 a dried culture of CBS 296.29 was deposited in the CBS herbarium as CBS H-7641. These cultures have the note "isotype" in the collection databases, but we have not been able to find any publication mentioning the typification of *P. heveae*, and the dates of deposition are too far apart for the cultures to be accepted as isotypes. Thus, to clarify the typification of *P. heveae* we designate Thompson's original protologue line drawing of sporangia and oogonia (reproduced here as Fig. 7) as the lectotype, and his authentic culture as the epitype.

Description:—A description of this species is provided by Thompson (1929), and more recently by Erwin & Ribeiro (1996). Descriptions of sporangial caducity are inconsistent in the secondary literature. Caducous sporangia were not noted by Thompson despite extensive description of the sporangia on different media; if free sporangia had been observed he would very likely have described them, as he did for another species (*P. palmivora*) in the same article. The epitype culture CBS 296.29 = ICMP 19451 is described by the fungal database at Q-bank as non-caducous (www.q-bank.org). A *P. heveae* isolated from *Rhododendron* in North Carolina, USA (1400 = ICMP 19453) had persistent sporangia (Benson & Jones 1980). However, Albuquerque *et al.* (1974) reported caducous sporangia on *P. heveae* isolated from leaf blight of Brazil nut (*Bertholletia excelsa* Bonpl.), but also reported some paragynous antheridia, a character not observed in *P. heveae* in our research, nor reported by others. We examined sporangial caducity for an additional two isolates (ICMP 16691 and ICMP 19452) and found only persistent sporangia, even with very vigorous shaking in an attempt to dislodge the sporangium. Therefore we emend the description of *P. heveae* to include persistent (non-caducous) sporangia, the same characteristic as all other *Phytophthora* Clade 5 species (Table 3).

Other specimens examined:—Australia. New South Wales: Wyong, soil under *Eucalyptus pilularis* Sm., 1976, L. Gerrettson-Cornell, culture ATCC 64863 = DAR 27023 = ICMP 16691 = IMI 208224= WPC P3475. China. Hainan Island: forest soil, Hon Ho, culture LMM6 = ICMP 17964 = WPC P10660. Guatemala. Los Aposentos: canker on trunk of *Persea americana* Mill., 1975, G.A. Zentmyer, culture ATCC 38692 = CBS 954.87 = ICMP 19452 = WPC P1000. Malaysia. Trengganu: Jerangau Estate, *Theobroma cacao*, 14 Jan 1968, P.D. Turner, specimen Herb. IMI 131093, culture ICMP 16914 = IMI 131093 = WPC P0578. USA. North Carolina: dieback of *Rhododendron*, 1976, DM Benson, culture 1400 = ATCC 46299 = CBS 958.87 = ICMP 19453 = WPC P1717.

Discussion

Phytophthora Clade 5 has been historically poorly studied, with *P. heveae* described in 1929 and *P. castaneae* described in 1976. In this work, through comprehensive DNA sequencing and analysis, supported by morphological characters, we have described an additional two species, clarified the typification of the extant species, and brought the clade into modern taxonomic context.

The four species within Clade 5 can be discriminated by gene sequencing and analysis. The fungal barcode ITS will distinguish *P. cocois* and *P. heveae*. *Phytophthora agathidicida* and *P. castaneae* have identical ITS sequences, but can be separated by sequences of COX1, ENL, and ND1. A specific diagnostic test has also been developed for *P. agathidicida* (Than *et al*. 2013).

The most reliable morphological character within Clade 5 is gametangial morphology, in particular the degree of oogonium wall ornamentation, which ranges from completely smooth in *P. heveae*, to coarsely bullate in *P. castaneae* (Fig. 8). Antheridial morphology is also important, particularity with the often reflexed antheridia of *P. cocois*.

Following this taxonomic revision it may be necessary to re-identify previous host-association records. For example with the establishment of *P. cocois* as a new species associated with coconut disease it is likely that previous reports of *P. katsurae* or *P. castaneae* on coconut are *P. cocois*. Additionally isolates that were previously identified as *P. castaneae* or *P. katsurae* that are not associated with the hosts *Castanea* or *Castanopsis* in East Asia should be taxonomically re-examined.

Liyanage & Wheeler (1989) reported an isolate IMI 304411 from cocoa in Côte d'Ivoire that was only mildly pathogenic when inoculated into cocoa pods, and was identified as *P. castaneae*. The isolate is no longer viable, but the published photomicrographs of oogonia and antheridia correspond with *P. cocois* rather than *P. castaneae sensu stricto*.

Some previously recorded host/locality data for *P. heveae* (Erwin & Ribeiro 1996) can be excluded, such as those from New Zealand (now *P. agathidicida*), from Côte d'Ivoire coconut (now *P. cocois*), and from Brazilian *Bertholletia* (likely a misidentification, see discussion above about cauducity in *P. heveae*).

This is the first comprehensive study of *Phytophthora* Clade 5, and even after this taxonomic revision there are

only four species, a low number compared to an average of 11 species for the other nine *Phytophthora* clades. It is likely that more species will be discovered and described in this clade, potentially some of them after re-identification of *P. castaneae* and *P. heveae* isolates following the revised modern concepts of those species presented here. One potential new species with the tag name *P.* sp. *"novaeguineae"* was sequenced in the work of Martin *et al.* (2014) and needs further investigation.

The host and geographic associations point to a centre of diversity in the East Asia / Pacific region for *Phytophthora* Clade 5 species, although origins for some of the species are unclear. A comprehensive survey of *Phytophthora* species in undeveloped regions of South East Asia would add valuable knowledge to the origin and diversity of these species.

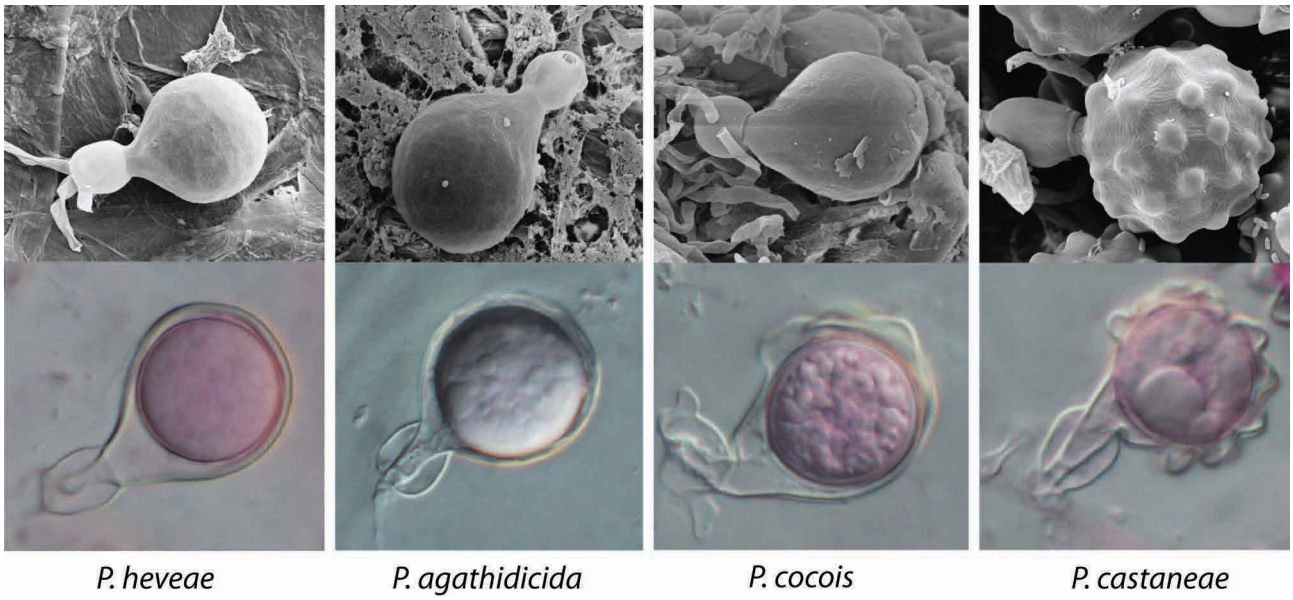

FIGURE 8. Comparative gametangial morphology of *Phytophthora* Clade 5 species, with SEM (top) and light microscopy (bottom). *P. heveae* has smooth walled oogonia with funnel-shaped, amphigynous antheridia. *P. agathidicida* has mildly stipulate oogonia with globose amphigynous antherida. *P.cocois* has mildly bullate oognia with reflexed amphigynous antheridia. *P. castaneae* has coarsely bullate oogonium with rugose protuberances and narrow amphigynous antheridia.

Acknowledgements

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