RESEARCH ARTICLE

Phytophthora betacei, a new species within *Phytophthora* clade 1c causing late blight on *Solanum betaceum* in Colombia

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Abstract   Over the past few years, symptoms akin to late blight disease have been reported on a variety of crop plants in South America. Despite the economic importance of these crops, the causal agents of the diseases belonging to the genus *Phytophthora* have not been completely characterized. In this study, a new *Phytophthora* species was described in Colombia from tree tomato (*Solanum betaceum*), a semi-domesticated fruit grown in northern South America. Comprehensive phylogenetic, morphological, population genetic analyses, and infection assays to characterize this new species, were conducted. All data support the description of the new species, *Phytophthora betacei* sp. nov. Phylogenetic analyses suggest that this new species belongs to clade 1c of the genus *Phytophthora* and is a close relative of the potato late blight pathogen, *P. infestans*. Furthermore, it appeared as the sister group of the *P. andina* strains collected from wild *Solanaceae* (clonal lineage EC-2). Analyses of morphological and physiological characters as well as host specificity showed high support for the differentiation of these species. Based on these results, a complete description of the new species is provided and the species boundaries within *Phytophthora* clade 1c in northern South America are discussed.

INTRODUCTION

Oomycetes represent a diverse group of fungus-like eukaryotic microorganisms widely distributed in nature. Their ecological characteristics have been extensively studied to reveal a wide diversity of ecological niches (e.g., Soanes et al. 2007). Particular emphasis has been placed on the study of plant pathogens for which ecological speciation seems a common process due to specialization to particular host species (Harrington et al. 2002, Tellier et al. 2010). Furthermore, oomycetes show an unprecedented plasticity in terms of genome size and ploidy (Haas et al. 2009, Yoshida et al. 2013), which could influence rates of speciation and extinction (Santini et al. 2009, Wood et al. 2009, Muir & Hahn 2015, Puttick et al. 2015). Recently, new hybrid species were described showing that interspecific hybridisation also plays a major role in adaptation and speciation of *Phytophthora* species (Brasier et al. 2004, Jung et al. 2017).

Molecular taxonomy can use DNA sequences to identify and delimitate species that are not amenable to genetic crosses (Roe et al. 2010). In combination with classical taxonomy, mo-

lecular studies are useful in describing allopolyploid interspecific *Phytophthora* hybrids as distinct species (Brasier et al. 2004, Man in 't Veld et al. 2012, Bertier et al. 2013, Husson et al. 2015, Jung et al. 2017). The premise of these approaches is to identify discrete genetic groups that have ceased genetic exchanges with other groups. The number of studies defining species in this way has increased recently, mainly due to the ease of obtaining information on population-level DNA variation (Roe et al. 2010, Singh et al. 2015). However, this approach has inherent limitations. Gene genealogies tend to overestimate the number of species as the population structure within a species may be mistaken for species boundaries (Dettman et al. 2003). Furthermore, multi-locus species delimitation, relying on reciprocal monophyly and strict genealogical congruence, may fail to differentiate among recently diverged lineages (Hickerson et al. 2006, Knowles et al. 2007, Shaffer & Thomson 2007).

The most notable genus within oomycetes, *Phytophthora*, includes pathogens that infect a broad range of hosts in both agricultural and natural environments, causing adverse economic consequences (Erwin & Ribeiro 1996, Duncan 1999, Fry 2008, Forbes et al. 2013). To date, the genus *Phytophthora* comprises more than 150 recognised species, classified into 10 phylogenetic clades (Blair et al. 2008, Kroon et al. 2012, Martin et al. 2014). Over the past few decades, the numbers of recognised species within most divisions of the genus *Phytophthora* have nearly tripled within 20 years since Erwin and Ribeiro in 1996 listed 52 species, and greater species diversity has been considerably reported in all *Phytophthora* clades (Cooke et al. 2000, Kroon et al. 2012, Martin et al. 2012, Forbes et al. 2013). However, defining clear and objective species boundaries, as is the case for most oomycetes, remain a challenge in all *Phytophthora* clades.

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Within the genus, *P. infestans* has become a 'model system' because of its undoubted economic impact. This pathogen affects important crops, such as potatoes (*Solanum tuberosum*) and tomatoes (*Solanum lycopersicum*) (Haverkort et al. 2008, 2009), making it one of the most threatening plant disease agents in the world. Although *P. infestans* was once considered a single species (henceforth referred to as *P. infestans* s.lat.), it has been shown to be a species complex (Forbes et al. 2013). Four other species related to *P. infestans* s.str. have been identified over the last 35 years. *Phytophthora mirabilis* has been found in Central America (Galindo & Hohl 1985) infecting only *Mirabilis jalapa*, an ornamental and medicinal plant in the region. *Phytophthora ipomoeae* (Flier et al. 2002) infects two morning glory species endemic to the highlands of central Mexico, *Ipomoea longipedunculata* and *I. purpurea* (Flier et al. 2002, Badillo-Ponce et al. 2004). *Phytophthora phaseoli*, initially classified as *P. infestans* (Thaxter 1889), is distributed globally but infects only lima beans (*Phaseolus lunatus*). Due to host-preference studies, *P. phaseoli* was described as a different species and for over 60 years thought to be the closest relative of *P. infestans*. Genetic comparisons have also revealed the existence of a separate group composed of strains from Ecuador and Peru that are collectively called *P. andina* (Oliva et al. 2010). To date, host range and distribution of *P. andina* is restricted to the Andean region of Ecuador and Peru where it has been reported in tree tomato crops (these isolates belong to the EC-3 clonal lineage) and wild solanaceous species (isolates belonging to the EC-2 clonal lineage) (Oliva et al. 2010). These observations, along with previous reports based on phylogenetic analyses using both nuclear and mitochondrial markers, reveal this species as polyphyletic, suggesting it might be a species complex comprising at least two genetically distinct lineages (Adler et al. 2002, Kroon et al. 2004, Chacón et al. 2006, Oliva et al. 2010, Cárdenas et al. 2012, Forbes et al. 2013, Goss et al. 2014, Lassiter et al. 2015, Forbes et al. 2016).

As in Ecuador and Peru, the southern region of Colombia has suffered a recent increase of late blight, leading to devastating declines in and disease outbreaks on tree tomato (*Solanum betaceum*) crops caused by *Phytophthora*. However, these have not been formally described. In this study, an integrative approach that leveraged phylogenetic study, morphological assays, population genetics and host specificity was used to characterise them. Here, a new species of *Phytophthora* infecting the tree tomato was found and defined. Morphological, physiological and molecular characteristics were used to provide a complete description of this species. Finally, this new species was formally described as *Phytophthora betacei* sp. nov. Furthermore, as this new species was described, the boundaries of other species within clade 1c were re-defined. For convenience, this new species will be referred by the proposed name throughout this article.

MATERIALS AND METHODS

Sampling and Phytophthora isolation

All samples were collected in southern Colombia between 2008 and 2009. Three to four leaves from 10 randomly selected tree tomato (*Solanum betaceum*) plants per plantation that showed symptoms resembling late blight were collected from 34 locations from two Colombian states, Nariño and Putumayo. The initial collections comprised over 970 putatively infected leaves. One to three lesions per leaf were excised (~ 0.5 to 1 cm^2) from the margin between necrotic and healthy tissues. Excised leaf pieces were surface-sterilised by submerging them in 70 % ethanol for 20 to 30 s and then washing them with sterile distilled water to remove the excess ethanol (~ 10 s). The leaf pieces were dried on a sterile paper towel and subsequently

transferred to a tree tomato agar (TTA) medium. The medium was prepared with tree tomato fruit (0.25 g of $CaCO_3$, 0.5 g of yeast extract, 25 g of sucrose, 15 g of agar and 100 mL of tree tomato extract, composed of 550 g of tree tomato fruit per litre of water). Subsequently, single zoospores were isolated from sporangia washed from the tree tomato medium with sterile distilled water. In total, 128 *P. betacei* strains were successfully isolated. All single zoospore isolates were cultured in a tree tomato medium for seven to 15 d at 18 ºC and then stored in the *Phytophthora* collection of the Museum of Natural History at Universidad de los Andes. Isolate P8084 was selected as the type culture for further taxonomic description. All *P. betacei* isolates collected in this study are deposited in the Museum of Natural History under accession numbers Andes-F 1081 to Andes-F 1207.

Phylogenetic analyses and population genetics

DNA extraction and sequencing

The mycelia of each *Phytophthora* strain were grown in a liquid Plich medium (Van der Lee et al. 1997) for 15 d at 20 °C. Subsequently, mycelia were washed with sterile distilled water and macerated thoroughly in liquid nitrogen using a cooled mortar and pestle. The macerated mycelia (0.1 g) were immediately transferred into a micro-centrifuge tube (1.5 mL) and DNA was extracted using the DNA kit OmniPrep (G-Biosciences) while following the manufacturer's instructions. The DNA was suspended in Tris-EDTA buffer (pH 8.0) and was treated with RNAse. The quality and quantity of the DNA were measured, using NanoDrop ND-1000.

Whole genome sequencing and mitochondrial genome assembly

Two *P. betacei* isolates (P8084 and N9022) were sequenced using Illumina sequencing. A standard shotgun library (1 × 200 bp) was constructed and sequenced by Beijing Genomic Institute (BGI, Hong Kong, China) using an Illumina Hiseq2000 platform with paired-ends chemistry and 100 cycles. In total 40 Gb of 96–100 bp paired-end reads from two libraries with insert lengths of 200 bp was generated. Furthermore, 22 Gb of Illumina mate-pair libraries (6 kb insert size) for each of the *P. betacei* isolates was also generated. Read mapping was done with BWA-MEM 0.7.12 (Li 2013) with the parameter k = 10 using *P. infestans* as a reference (Haas et al. 2009). Variants were called with GATK 3.2-2 (McKenna et al. 2010) using default parameters.

Phylogenetic analysis using mitochondrial genomes

Two newly assembled mitochondrial genomes of *P. betacei* (data taken from the whole genome sequences) were compared to data from Martin et al. (2016) in order to infer their phylogenetic position within clade 1c. MITObim (Hahn et al. 2013) was used to assemble the mitochondrial genome of each isolate. The process included 30 iterations using the quick approach with T30-4 haplotype Ia as the reference genome. Individual gene regions were aligned using MAFFT v. 7.187 (Katoh & Toh 2010). Then, maximum likelihood (ML) analyses were performed using RAxML v. 7.6.3 (Stamatakis 2006), as implemented on the CIPRES portal (Miller et al. 2010). The sequence alignment was partitioned into five subsets (rRNA genes, tRNA genes, first and second codon positions, third codon positions and intergenic regions) according to Martin et al. (2016). The GTRGAMMA (GTR + G) model for nucleotide substitution was used but allowed for the estimation of different shapes, GTR rates, and base frequencies for each partition. The majority rule criterion implemented in RAxML (-autoMRE) was used to assess clade support.

Phylogenetic analyses of SNP data using genotyping-by-sequencing

Genomic DNA was isolated with the DNeasy® Plant Mini Kit (QIAGEN, Germany). Genotyping-by-sequencing (GBS) was performed (Elshire et al. 2011) at the Institute of Genomic Diversity (Cornell University) for a total of 70 *Phytophthora* strains in total. These included 12 isolates (10 from Colombia and two reference strains from the United States, US-8 and US-12). For *P. betacei*, 35 isolates were included (clonal lineage EC-3), one *P. andina* (clonal lineage EC-3) and five *P. andina* (clonal lineage EC-2). Finally, three *P. andina* isolates of unknown clonal lineage, as well as five *P. mirabilis*, eight *P. ipomoeae*, and one *P. phaseoli* isolates were also included (Table 1). Briefly, genome complexity was reduced by digesting the total genomic DNA from individual samples with the type II restriction endonuclease *Ape*KI, which recognises a degenerate 5-bp sequence (GCWGC, where W is A or T) and creates a 5' overhang (3 bp). The digested products were then ligated to adapter pairs with enzyme-compatible overhangs; one adapter contained the barcode sequence and the other the binding site for the Illumina sequencing primer. The GBS library fragment-size distributions were checked on a BioAnalyzer (Agilent Technologies, Inc., USA). The PCR products were quantified and diluted for sequencing on an Illumina HiSeq 2500 sequencer (Illumina Inc., USA). A 96-well plate, comprising 70 samples and one blank, was multiplexed on a single Illumina flow cell lane.

To sort each of the GBS barcode samples into separate fastq files, *Phytophthora* samples were demultiplexed using sabre (https://github.com/najoshi/sabre), allowing no mismatches within the barcode. In total, 1 992 701 tags were analysed and mapped against the *P. infestans* T30-4 reference genome (Haas et al. 2009), using Bowtie v. 2.2.3 (Langmead 2010). Of this total number of reads, 917 890 (46.1 %) were aligned to unique positions, 573 880 (28.8 %) were aligned to multiple positions and 500 931 (25.1 %) could not be aligned to the reference genome. For SNP calling, each SAM sample alignment file was converted into a BAM file, followed by sorting and indexing using SAMtools. SNPs and indels were called simultaneously using the variant caller GATK v. 4.3.10 (McKenna et al. 2010). The final dataset consisted of 70 samples with 23 480 SNPs obtained from GATK. To discard the presence of sequencing errors in the data, all samples that did not fulfil the following criteria were filtered out: mapping QUAL > 30, an overall coverage between 8 and 32X (cutoff values between 5 % and 95 % coverage) and a minor allele frequency (MAF) > 0.05. To infer the phylogenetic relationships of species from *Phytophthora* clade 1c (Table 1), a matrix was created with all high-quality SNP loci obtained from the GBS analyses concatenated into a single alignment. An ML phylogenetic tree was generated using RAxML (Stamatakis 2006) under the general reversible nucleotide substitution model (GTR) with 1 000 bootstrap replicates to quantify branch support. The software jModelTest v. 2.1.7 was used to select the best-fit substitution model, while *P. phaseoli* was used as an outgroup. The phylogenetic tree was visualised using Figtree v. 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/) (Rambaut 2009).

Restriction fragment length polymorphism analysis using mitochondrial haplotyping and probe RG57

Phytophthora lineages have been characterised by using mitochondrial haplotyping (Carter et al. 1990, Griffith & Shaw 1998) and a restriction fragment length polymorphism (RFLP) analysis with the highly polymorphic probe RG57 (Goodwin et al. 1992). The same approach to compare *P. betacei* with previously reported lineages within the genus *Phytophthora* was used in this study. The mitochondrial haplotype was determined using the PCR-RFLP method with reference strains US-1 and US-8 included as controls (Griffith & Shaw 1998, Ordoñez et al. 2000, Adler et al. 2002, Gavino & Fry 2002).

Population genetic analyses using microsatellite data

The *Phytophthora* isolates used in this study were genotyped and compared with the closely related species *P. infestans* and *P. andina* using simple sequence repeats (SSRs). All SSRs were sequenced and analysed using the protocols developed previously by Lees et al. (2006) and described in the Eucablight Network's Protocol section dated March 2008 (www.eucablight.org). In total 116 *P. betacei* isolates obtained in this study, 117 *P. infestans* isolates and 17 *P. andina* isolates reported in Goss et al. (2014) were included in the analysis. Among the 117 *P. infestans* isolates, there were 17 distinct clonal lineages, as well as genotypically diverse isolates from Mexico and Northern Europe. A principal component analysis (PCA) was conducted for the combined *P. betacei*, *P. infestans* and *P. andina* microsatellite data implemented in the adegenet package (Jombart et al. 2008). The allele frequencies at bi-allelic sites for the triploid *P. infestans* isolates (1/3 or 2/3) were unknown. To account for this uncertainty, the alleles at each locus for each isolate were subsampled. Because adegenet treats ploidy as a global parameter, resampled datasets for the strains from all species were generated, assuming all the individuals across species had the same ploidy. To account for both the uncertainty in allele frequencies at bi-allelic sites in triploid *P. infestans* isolates, as well as for the fact that adegenet would require all samples to have the same ploidy, each 100 independent diploid and 100 independent triploid resampled datasets for the PCA were generated (i.e., within each subsampled dataset, all individuals were diploids or triploids), and adegenet was run independently on each of them.

To estimate the number of populations that would best explain the genetic variance in the group of isolates studied, the Bayesian model-based clustering program STRUCTURE v. 2.3 (Pritchard et al. 2000) was used. To account for allele frequency uncertainty at the bi-allelic triploid *P. infestans* loci and because ploidy is a global parameter in STRUCTURE, the same 200 resampled datasets for the PCA were used. STRUCTURE was run in total 32 000 times: (2 ploidies) × (100 resampled datasets) × (8 populations, K = 1 to 8) × (20 repetitions for K selection). Each run involved 1 000 000 MCMC steps with a burn-in of 100 000 and the following parameters were used: NOADMIX = 0, LINKAGE = 0, INFERALPHA = 1, ALPHA = 1.0, UNIFPRIORALPHA = 1, ALPHAMAX = 10.0 and FREQSCORR = 0. The ΔK method (Evanno et al. 2005) was used to infer the most likely number of clusters by evaluating the rate of change in the log probability of data between successive K values for each resampled dataset.

Population genetics analyses using genotyping-by-sequencing data

To corroborate the results of the population structure analyses obtained by using 11 microsatellite loci, a PCA was conducted based on the 23 480 high-quality SNP markers obtained from the GBS analyses. High quality was defined as SNPs with MAF > 0.05 and with less than 20 % missing data, that is, SNPs that were present in at least 80 % of the strains assessed. Genetic structure was also estimated using the Bayesian assignment test implemented in the program STRUCTURE v. 2.3 (Pritchard et al. 2000) for high-quality SNP markers. These are defined as SNPs with MAF > 0.05 and with less than 10 % missing data. In total, 48 samples were used: 12 for *P. infestans*, 29 for *P. betacei* and 7 for *P. andina* (EC-2). The run parameters were as follows: 24 runs with four repetitions with 100 000 MCMC steps and a burn-in period of 10 000 for six populations (K = 1 to 6), under the NOADMIX ancestry model and allele frequencies correlated. The ΔK of Evanno (Evanno

Table 1 Description of isolates of *P. infestans*, *P. andina* and *P. betacei* used for the morphological, physiological, phylogenetic and host preference assays.

Species	Sample ID	Locality (Country/State/Locality)	Original host	Year	Locus lineage	Mitochondrial haplotype	Mating type	Assay[1]	Source
P. infestans T-30-4 strain[2]	1826	Scotland (SCRI)	NA	NA	NA	NA	NA	C	Grünwald lab culture collection
P. infestans T-30-4 strain[3]	4392	Scotland	NA	2007	NA	NA	NA	C	Grünwald lab culture collection
P. infestans	C0008S	Colombia/Putumayo/Sibundoy	*S. tuberosum*	2013	NA	NA	NA	C	LAMFU[4]
	C003210	Colombia/Nariño	*S. tuberosum*	2013	NA	NA	NA	C	LAMFU[4]
	C003B21	Colombia/Nariño	*S. tuberosum*	2013	NA	NA	NA	C	LAMFU[4]
	PUA10096	Colombia/Cundinamarca/Guasca	*S. phureja*	2013	NA	NA	A1	C	LAMFU[4]
	RB003	Colombia/Nariño	*S. tuberosum*	2013	NA	NA	NA	C	LAMFU[4]
	RB005	Colombia/Nariño	*S. tuberosum*	2013	NA	NA	NA	C, D	LAMFU[4]
	RC1-6	Colombia/Cundinamarca/Rosal	*S. tuberosum*	2015	EC-1	IIa	A1	A, B	LAMFU[4]
	SP03562	Colombia/Putumayo/Sibundoy	*S. tuberosum*	2013	NA	NA	NA	C	LAMFU[4]
	STG100	Colombia/Nariño/Guachucal	*S. tuberosum*	2013	EC-1	IIa	A1	A, B	LAMFU[4]
	STT161	Colombia/Nariño/Túquerres	*S. tuberosum*	2013	EC-1	IIa	A1	A, B	LAMFU[4]
	US040009	USA/New York	*S. tuberosum*	NA	US-8	NA	A2	A, B	Fry lab culture collection
	US940480	NA	NA	NA	US-8	Ia	A2	C	Fry lab culture collection
	US940494	USA	*S. lycopersicum*	NA	US-12	NA	A1	C	Fry lab culture collection
	US970001	USA/Florida	*S. lycopersicum*	1997	US-17	NA	A1	A, B	Fry lab culture collection
	VPC7-10	Colombia/Cundinamarca/Villa Pinzon	*S. tuberosum*	2015	EC-1	IIa	A1	A, B	LAMFU[4]
	Z3-2	Colombia/Cundinamarca/Zipacón	*S. phureja*	2007	NA	NA	A1	A, B, C, D	Vargas et al. (2009)
P. andina[5]	EC 3163	Ecuador	Anarrhichomenum	NA	EC-2	Ic	A1	C	Oliva et al. (2010), Goss et al. (2011)
	EC 3189	Ecuador	Anarrhichomenum	NA	EC-2	Ic	A2	C	Oliva et al. (2010), Goss et al. (2011)
	EC 3399	Ecuador	Anarrhichomenum	NA	EC-2	Ia	A2	A, B, D	Oliva et al. (2010), Goss et al. (2011)
	EC 3399	Ecuador	Anarrhichomenum	NA	EC-2	Ia	A1	C	Oliva et al. (2010), Goss et al. (2011)
	EC 3510	Ecuador	*S. betaceum*	NA	EC-3	Ia	A1	A, B, D	Goss et al. (2011)
	EC 3510	Ecuador	*S. betaceum*	NA	EC-3	Ia	A1	C	Goss et al. (2011)
	EC 3563	Ecuador	*S. quitoense*	NA	NA	Ia	A1	C	Goss et al. (2011)
	EC 3678	Ecuador	Anarrhichomenum	NA	EC-2	Ic	A1	C	Goss et al. (2011)
	EC 3780	Ecuador	*S. hispidum*	NA	NA	Ic	NA	C	Goss et al. (2011)
	EC 3818	Ecuador	Anarrhichomenum	NA	EC-2	Ia	A2	A, B, D	Oliva et al. (2010), Goss et al. (2011)
	EC 3818	Ecuador	Anarrhichomenum	NA	EC-2	Ia	A2	C	Oliva et al. (2010), Goss et al. (2011)
	EC 3821	Ecuador	Anarrhichomenum	NA	NA	Ia	NA	C	Goss et al. (2011)
	EC 3836	Ecuador	*S. betaceum*	2008	EC-3	Ia	A1	D	Goss et al. (2011)
P. betacei	A01492	Colombia/Antioquia	*S. betaceum*	2012	EC-3	Ia	ND	C	This study
	CO1298	Colombia/Putumayo/Sibundoy	*S. betaceum*	2012	EC-3	Ia	ND	C	This study
	MFM-N9012	Colombia/Nariño/Buesaco	*S. betaceum*	2009	EC-3	Ia	ND	C	This study
	MFM-N9022	Colombia/Nariño/Buesaco	*S. betaceum*	2009	EC-3	Ia	ND	A, B, C, D	This study
	MFM-N9025	Colombia/Nariño/Buesaco	*S. betaceum*	2009	EC-3	Ia	ND	C	This study; Forbes et al. (2016)
	MFM-N9039	Colombia/Nariño/Buesaco	*S. betaceum*	2009	EC-3	Ia	ND	A, B, C	This study
	MFM-N9041	Colombia/Nariño/Consaca	*S. betaceum*	2009	EC-3	Ia	ND	C	This study
	MFM-N9046	Colombia/Nariño/Pasto	*S. betaceum*	2009	EC-3	Ia	ND	C	This study
	MFM-N9056	Colombia/Nariño/Consaca	*S. betaceum*	2009	EC-3	Ia	ND	C	This study
	MFM-N9057	Colombia/Nariño/Consaca	*S. betaceum*	2009	EC-3	Ia	ND	C	This study
	MFM-N9065	Colombia/Nariño/Iles	*S. betaceum*	2009	EC-3	Ia	ND	C	This study
	MFM-N9071	Colombia/Nariño/Iles	*S. betaceum*	2009	EC-3	Ia	ND	A, B, C	This study

Table 1 (cont.)

Species	Sample ID	Locality (Country/State/Locality)	Original host	Year	Locus lineage	Mitochondrial haplotype	Mating type	Assay[1]	Source
P. betacei (cont.)	MFM-P8012	Colombia/Putumayo/Colon	*S. betaceum*	2008	EC-3	Ia	ND	C	This study
	MFM-P8029	Colombia/Nariño/Buesaco	*S. betaceum*	2008	EC-3	Ia	ND	C	This study
	MFM-P8050	Colombia/Putumayo/Colon	*S. betaceum*	2008	EC-3	Ia	ND	C	This study
	MFM-P8064	Colombia/Putumayo/Santiago	*S. betaceum*	2008	EC-3	Ia	ND	C	This study
	MFM-P8071	Colombia/Putumayo/Colon	*S. betaceum*	2008	EC-3	Ia	ND	C	This study
	MFM-P8075	Colombia/Putumayo/Colon	*S. betaceum*	2008	EC-3	Ia	ND	C	This study
	MFM-P8077	Colombia/Putumayo/Colon	*S. betaceum*	2008	EC-3	Ia	ND	A, B, C	This study
	MFM-P8084	Colombia/Putumayo/Colon	*S. betaceum*	2008	EC-3	Ia	ND	A, B, C, D	This study
	MFM-P8093	Colombia/Putumayo	*S. betaceum*	2008	EC-3	Ia	ND	C	This study
	MFM-P8096	Colombia/Putumayo/San Francisco	*S. betaceum*	2008	EC-3	Ia	ND	C	This study
	MFM-P8099	Colombia/Putumayo/San Francisco	*S. betaceum*	2008	EC-3	Ia	ND	C	This study
	MFM-P9105	Colombia/Putumayo/San Francisco	*S. betaceum*	2009	EC-3	Ia	ND	C	This study
	MFM-P9127	Colombia/Putumayo/San Francisco	*S. betaceum*	2009	EC-3	Ia	ND	C	This study
	MFM-P9128	Colombia/Putumayo/Sibundoy	*S. betaceum*	2009	EC-3	Ia	ND	C	This study
	MFM-P9129	Colombia/Putumayo/Sibundoy	*S. betaceum*	2009	EC-3	Ia	ND	C	This study
	MFM-P9146	Colombia/Putumayo/Sibundoy	*S. betaceum*	2009	EC-3	Ia	ND	C	This study
	MFM-P9147	Colombia/Putumayo/Sibundoy	*S. betaceum*	2009	EC-3	Ia	ND	C	This study
	MFM-P9151	Colombia/Putumayo/Sibundoy	*S. betaceum*	2009	EC-3	Ia	ND	C	This study
	MFM-P9153	Colombia/Putumayo/Sibundoy	*S. betaceum*	2009	EC-3	Ia	ND	C	This study
	S00321	Colombia/Putumayo/Sibundoy	*S. betaceum*	2012	EC-3	Ia	ND	C	This study
	S06298	Colombia/Putumayo/Santiago	*S. betaceum*	2012	EC-3	Ia	ND	C	This study
	S07198	Colombia/Putumayo/Sibundoy	*S. betaceum*	2012	EC-3	Ia	ND	C	This study
	S07398	Colombia/Putumayo/Sibundoy	*S. betaceum*	2012	EC-3	Ia	ND	C	This study
P. mirabilis	1260	Mexico/Texcoco[6]	NA	NA	NA	NA	NA	C	Grünwald lab culture collection
	1276	Mexico	*M. jalapa*	1998	NA	NA	NA	C	Grünwald lab culture collection
	1991	Mexico/Coyoacán	*M. jalapa*	2000	NA	NA	NA	C	Grünwald lab culture collection
	1992	Mexico/Coyoacán	*M. jalapa*	2000	NA	NA	NA	C	Grünwald lab culture collection
	1994	Mexico/Coyoacán	*M. jalapa*	2007	NA	NA	NA	C	Grünwald lab culture collection
P. ipomoeae	1270	Mexico	*I. longipedunculata*	1999	NA	NA	NA	C	Grünwald lab culture collection
	1271	Mexico	*I. longipedunculata*	1999	NA	NA	NA	C	Grünwald lab culture collection
	1989	Mexico	*I. longipedunculata*	2000	NA	NA	NA	C	Grünwald lab culture collection
	1990	Mexico/Michoacan	*Ipomoea* spp.	1999	NA	NA	NA	C	Grünwald lab culture collection
	4666	Mexico	*I. longipedunculata*	1999	NA	NA	NA	C	Grünwald lab culture collection
	4667	Mexico	*I. longipedunculata*	1999	NA	NA	NA	C	Grünwald lab culture collection
	4669	Mexico	*I. longipedunculata*	1999	NA	NA	NA	C	Grünwald lab culture collection
	4670	Mexico	*I. longipedunculata*	1999	NA	NA	NA	C	Grünwald lab culture collection
P. phaseoli	5134	USA	*Phaseolus lunatus*	2003	NA	NA	NA	C	Grünwald lab culture collection

NA data not available.
ND Not determined; Oospores formed showed abnormal appearance in mating crosses between of *P. betacei* (isolate Andes-F 1172) and *P. infestans* strain of the A2 mating type (US040009).
[1] Isolates used for A = morphological, B = Physiological, C = Phylogenetic analysis with GBS data, D = Host preference assay.
[2] F1 of a cross between two aggressive strains of *P. infestans* originally isolated from potato in The Netherlands. Isolate used for genome sequence.
[3] Duplicate of #1826.
[4] LAMFU: The Mycology and Plant Pathology Laboratory at Universidad de los Andes, Bogotá, Colombia.
[5] Isolates classified as *P. andina* according to Oliva et al. (2010).
[6] From CBS in The Netherlands, ATCC 64130.

et al. 2005) was calculated using the application Structure Harvester v. 0.6.94 (Earl & vonHoldt 2012) to infer the most likely number of clusters.

Nuclear genome size determination

The nuclear genome size of *P. betacei* was assessed using flow cytometry. Nuclear DNA was isolated from one strain of each species (*P. infestans*: T30-4, *P. betacei*: P8084 and *P. andina*: EC3510). Sample preparation and laser flow cytometry conditions were set according to Catal et al. (2010). Approximately 200 mg of fresh mycelium was grown in a liquid Plich medium for 10–15 d at 20 °C. The grown mycelia were washed by centrifugation, and sliced for 3–4 min in 1 mL of PBS containing 0.1 % Triton X-100 at pH 7.4. The processed samples were subsequently maintained on ice for 4 h. The solution was then filtered through a nitrocellulose membrane (0.45 µm; diam 25 mm; Bio-Rad). The filtrate was then pelleted by centrifugation at $5\,000 \times g$ for 10 min at 4 °C and it was washed with sterile PBS. Afterwards, nuclei isolated from samples were stained with propidium iodide (PI) using the BD cycle test plus DNA Reagent kit (BD Biosciences, Heidelberg, Germany) according to the manufacturer's instructions. Briefly, 1 mL of buffer solution was added to the pellet obtained from previous centrifugation, and cells were subsequently resuspended at a low speed. The experiment was run twice. A volume of 250 µL of Solution A (Trypsin) was added and mixed by tapping and then incubated for 10 min at room temperature. Next, 200 µL of solution B (RNase A and trypsin inhibitor) was added and mixed by tapping and was again incubated for 10 min at room temperature. Finally, 200 µL of solution C (PI) at 4 °C was added to each tube, mixed by tapping, and then incubated for 10 min in the dark on ice. The sample was filtered and run on a FACS Canto II cytometer. Results were visualised using the FACSDiva software (BD Bioscience) and data were recorded using the FlowJo v. 7.5.5 software (Tree Star, Inc. Ashland, OR, USA). Diploid cells from avian erythrocytes were used as standards for instrument calibration. The measurements of relative fluorescence intensity were performed on $5\,000$ to $20\,000$ nuclei for each sample (Galbraith et al. 1983). Three independent samples per isolate were prepared and run separately. The estimation of DNA content was calculated as done by Dolezel et al. (2007). Heterogeneity across runs was minimised by calculating the genome size of each of the *Phytophthora* isolates using the internal reference standard (chicken; 2C = 2.4 pg; Nakamura et al. 1990). Genome size differences were compared across species by fitting a one-way ANOVA where species was the only factor and genome size was the response. Pairwise comparisons were done using Tukey's honest significant difference (HSD) test with the R library 'multcomp' (function 'glht').

Morphological characterization of P. betacei isolates

Morphological traits, including sporangial morphology, the presence of hyphal swelling or chlamydospores, oogonia, oospores and antheridia were examined to determine whether isolates of *P. betacei* presented morphological differences with isolates of the close species, *P. andina* and *P. infestans* (Table 1). Sporangia morphology was evaluated on four culture media V8 juice agar (V8, 1.0 g $CaCO_3$/100 ml V8 Juice, 15 g bacteriological agar), Potato Dextrose Agar (PDA, Oxoid Ltd, UK), Corn Meal Agar (CMA, Oxoid Ltd, UK) and TTA (described above), and quantitative measurements of sporangial sizes were scored on each isolate-medium combination. Sporangia were collected from a Petri dish incubated at 20 °C in the dark for 2 wk (optimum growth temperature for all isolates; see results), and the length (µm), breadth (µm) and length : breadth ratio (µm) of sporangia were measured. Furthermore, the three-dimensional volume of each sporangia was calculated according to Seidl Johnson et al. (2014). Briefly, the volume of each sporangia was calculated based on a standard equation: Volume = $(4/3) \times \pi \times A \times B^2$, where A is half of the major axis and B is half of the minor axis (assuming that each sporangium has a spherical shape).

To harvest sporangia, each Petri plate was flooded with sterile distilled water, and colonies were gently scraped with a sterile glass rod to dislodge sporangia. The resulting sporangia suspension was placed in sterile plastic 2-mL microcentrifuge tubes and it was examined using the 40X oil objective in a fully motorized Olympus IX81 microscope (40X/1.3 N.A objective). Measurements of 30 randomly selected sporangia were taken for each isolate-medium combination described above. The resulting transmitted light images were processed and were merged using the Helicon Focus software (http://www.helicon-soft.com/). Pictures of sporangia were further analysed and processed using the ImageJ software. Two biological replicates were conducted for each isolate-medium combination.

The shape, type of apex, papillation, caducity and special qualitative features of sporangia were also recorded. The presence of hyphal swelling or aggregations were evaluated using mycelia from 15-d-old actively growing colony margins collected using a scalpel and immediately suspended in a drop (~ 50 µL) of sterile distilled water. Furthermore, differences in microscopic hyphal characteristics were detected among *P. betacei*, *P. infestans* and *P. andina*. The width of the primary hyphae was also included as a character for identification (Fichtnera et al. 2011). Briefly, the width of the primary hyphae was determined by growing each isolate in the four-culture medium described above. The primary hyphae for each isolate-medium combination were evaluated using mycelia from 15-d-old actively growing colony margins collected using a scalpel and immediately suspended in a drop (~ 50 µL) of sterile distilled water. Pictures of the primary hyphae were processed as described above and were further analysed using the ImageJ software. In total, 20 measurements of hyphal width were taken of each colony, and the average width of the primary hyphae per colony was recorded.

The production of gametangia structures (oogonia and antheridia) was evaluated for all isolates of *P. betacei* (Table 1). Oogonia were produced by growing each *P. betacei* isolate with a known isolate of *P. infestans*, either the A1 mating type (US970001 US-17 genotype) or A2 mating type (US040009, US-8 genotype), on Petri plates containing V8-Rye Agar (50 % clarified V8 (10 %), 50 % clarified rye (10 %), 1.5 % agar, and 1 mL of β-sitosterol per litre, adjusted to pH 8.5) (Mayton et al. 2000). Polycarbonate membrane test was used to separate physically *P. betacei* isolates from the *P. infestans* A2 mating type (Ko 1978, Gallegly & Hong 2008). Petri plates were kept at 18 °C for 15–30 d in the dark. To evaluate the number of oospores produced, 1 cm² of agar in the hyphal interface of the two colonies was excised using a scalpel. Samples were fixed for microscopic examination of the diameter and length, gametangial morphology, and wall morphology of oogonia/oospore.

Colony morphology, radial growth and cardinal temperature

To assess the effect of temperature and culture media on the colony morphology and on radial growth rate of *P. betacei* isolates (Table 1), four distinct media and eight different incubation temperatures were used. The four culture media tested were: V8, PDA (Oxoid Ltd, UK), CMA and TTA. Nine representative isolates were selected: three isolates of *P. betacei* (P8084, N9022, P8077 (EC-3)), three isolates of *P. andina* (EC3510 (EC-3, Ia), EC3399 (EC-2, Ia), EC3818 (EC-2, Ia)) and three isolates of *P. infestans* (Z3-2 (EC-1), US040009 (US-8), US970001 (US-17)) (Table 1). One circular inoculum plug (~ 5 mm diam) of

an actively growing culture of a 15-d-old culture on V8 medium was placed in the centre of each Petri dish (90 mm diam). The colony morphology and radial growth rate for each isolate-media combination were evaluated after 15 d of incubation at different temperatures in a dark chamber with constant humidity. Colony morphology was described according to Erwin & Ribeiro (1996) and Gallegly & Hong (2008). The mean characteristics, including texture, density and growth were identified by visual observations while the radial growth rate was measured by taking pictures using a Canon Digital EOS Rebel T3i / 600D camera (Tokyo, Japan) and importing the images into the ImageJ software (rsb.info.nih.gov/ij/).

To evaluate the optimum temperature for mycelial growth, all isolate-medium combinations previously described were incubated at 4, 10, 15, 20, 25, 30, and 35 °C. Before evaluating each temperature, all isolates (Table 1) were sub-cultured onto V8 agar and incubated at 18 °C in a dark chamber with constant humidity for growth stimulation (Jung et al. 2002, 2017). All combinations of isolates and media were tested in two independent blocks (biological replicates) with two technical replicates per combination. For the temperatures 10, 15, 20, 25, 30 and 35 °C, identical incubators were used. The refrigeration equipment was used to keep the isolates at 4 °C. The radial growth rate was measured at 3, 7, 11 and 15 d post inoculation (dpi) using the same procedure described above.

Statistical analyses to determine the radial growth rate were only conducted for isolates incubated at the optimum temperature (see results). The normality of the residuals of the linear models for each trait measured was assessed. In all cases, they were not normally distributed (Shapiro-Wilk test; P < 0.05) and thus, differences in the radial growth rate of the three species at different temperatures and different media were assessed.

The observations of PDA were excluded because *P. betacei* did not grow on this medium. The observations obtained were pooled from all other media and a fitted linear model was created where the radial mycelial growth rate was the response, and the interaction between temperature and species was the only effect of the model. Pairwise comparisons were done using Tukey's HSD (honest significant difference) test with the R library 'multcomp' (function 'glht').

Quantitative morphological variation among *Phytophthora* species

Quantitative variation in terms of morphological traits among species was evaluated using a linear model approach, where the measurements were the response variables and the species was the only fixed effect. The normality of the residuals of each linear model was assessed using the Shapiro-Wilk test (function 'shapiro.test', package 'stats'; R Core team 2013). Based on the Shapiro-Wilk test, residuals were not normally distributed in any of the linear models (P < 0.05). Thus, the non-parametric Kruskal-Wallis test was used. To identify which groups of isolates differed from the others, multiple comparisons using non-parametric Nemenyi post hoc tests were performed. All Kruskal-Wallis tests were conducted using the R package 'stats' (R Core team 2013) and the Nemenyi and Tukey post hoc tests were conducted using the Pairwise Multiple Comparison of Mean Ranks Package ('pmcmr') implemented in R (Pohlert 2014).

Next, whether the morphology of *P. betacei* and other *Phytophthora* species differed was established by visualising all the morphological traits in a bi-dimensional plane using a discriminant function analysis (DA) based on the linear combination of morphological variables. To this end, a matrix with six traits (radial mycelial growth rate, primary hyphal width, sporangia length, sporangia breadth, sporangia volume and the sporangia

length-to-breadth ratio) and a total of 15 isolates (five isolates for *P. betacei*, seven isolates for *P. infestans*, one isolate for *P. andina* clonal lineage EC-3 and two isolates for *P. andina* clonal lineage EC-2) was generated. Analyses were conducted using the 'lda' function from the package 'mass' in R (Venables & Ripley 2002).

Host pathogenicity assays and evaluation of host preference

A group of six isolates was used to estimate the effect of host specialization among *P. infestans*, *P. andina* and *P. betacei*. Two isolates of each species were used for all infection assays: strains P8084 and N9022 for *P. betacei*, strains Z3-2 and RB005 for *P. infestans* and strains EC3510 (EC-3, Ia) and EC3836 (EC-3, Ia) for *P. andina*, all isolated from tree tomatoes (Table 1). The *P. andina* strains EC3399 (EC-2, Ia) and EC3818 (EC-2, Ia), isolated from hosts in the *Anarrichomenum* complex (Table 1) were also included. Each *Phytophthora* isolate was inoculated onto three different *Solanum* host species: *S. tuberosum* group *phureja* (yellow potato), *S. lycopersicum* (tomato) and the *S. betaceum* variety Común (tree tomato). For isolates EC3399 and EC3818, no symptoms of infection were detected in inoculations done on *S. tuberosum*, *S. betaceum* and *S. lycopersicum*. Thus, these isolates were excluded from the final analysis.

Plants were grown in a greenhouse (17–19 °C) and leaves or leaflets were harvested after 8–10 wk. Detached leaves were placed abaxial side up, on the base of 90-mm Petri plates containing moist paper towels. Three leaves were used per isolate as technical replicates. Each leaf was inoculated at four points with two 20 µL droplets of a sporangial suspension (3.5×10^4 sporangia/mL) on each side of the main vein. The Petri dishes were sealed with Parafilm and incubated at 15 °C with a 16 h light period. Each experiment consisted of four hosts, three genotypes, two isolates per genotype, three leaflets per isolate and four inoculation points per leaflet. The whole experiment was repeated three times.

The latent period, total lesion area and number of sporangia produced were documented by taking daily pictures of the inoculated leaves from day 1 to day 9. The latent period was scored as the number of days it took from inoculations until sporangia were observed. The lesion area was scored as the necrotic area around the inoculation site at 9 d post-inoculation (dpi), and it was measured using ImageJ (rsb.info.nih.gov/ij/). The number of sporangia produced on each leaf nine dpi was assessed by pooling individual lesions into 15 mL disposable polypropylene culture tubes with 3 mL of sterile distilled water. After vortexing for 10 s sporangial numbers were counted at least twice using a haemocytometer. The total number of sporangia was calculated by averaging the sporangia counts per aliquot, and then multiplying the result by the dilution factor.

The total number of sporangia per day was calculated by dividing the total number of sporangia produced after 9 d by the number of days when sporangia were visible (9 d – latent period). The number of sporangia produced was calculated by subtracting the total number of sporangia produced (9 dpi) from the sporangial concentration in the original inoculum suspension (2 800 sporangia per leaflet).

A fitness parameter for each replicate was calculated as the reproductive rate of each genotype on each host as follows:

$$Fitness = \frac{\ln(Sporangia_{day\,9} - 2{,}800)}{9 - LP} \quad (1)$$

To quantify the variation in fitness, a full factorial linear mixed model was fitted with the R package 'nlme' (function 'lme'; Pinheiro et al. 2013). In the linear model, fitness was the response variable, both genotype (*P. infestans*, *P. betacei* or *P. andina*)

and host (capiro potato, yellow potato, tomato or tree tomato) were fixed effects and strain (two independent isolates per genotype) was a random effect nested within a genotype. The significance of all interactions was assessed with Crawley's (1993, 2002) ML approach, in which the full model containing all factors and interactions was fitted and then simplified by a series of stepwise deletions, starting with the fixed-effect interaction and progressing to the interaction terms. The critical probabilities for retaining factors and determining whether effects or interactions were significant were 5 % for main effects and 1 % for two-way interactions. The linear model followed the formula:

$$Fitness \sim genotype_i + host_j + genotype(strain)_k + (genotype \times host)_{ij} + E_{ijk}. (2)$$

Because the residuals of this linear model were not normally distributed (Shapiro-Wilk normality test, W = 0.8342; P < 1 × 10^{-15}) they were analysed in a nonparametric framework. Fitness calculations of each host were compared among genotypes, using a Kruskal-Wallis test with the R package 'stats' (R Core team 2013), followed by pairwise comparisons with a Nemenyi test with a Tukey-Dist approximation for independent samples, using the R package 'pmcmr' (Pohlert 2014).

Molecular diagnosis of P. betacei based on SNP data

To distinguish among *P. betacei*, *P. andina* (EC-2) and *P. infestans*, SNPs obtained from GBS data were analysed for 55 *Phytophthora* isolates in total (12 *P. infestans*, 35 *P. betacei* and eight *P. andina* (EC-2); Table 1). Potentially diagnostic SNPs were selected by calculating the allele frequencies and allele
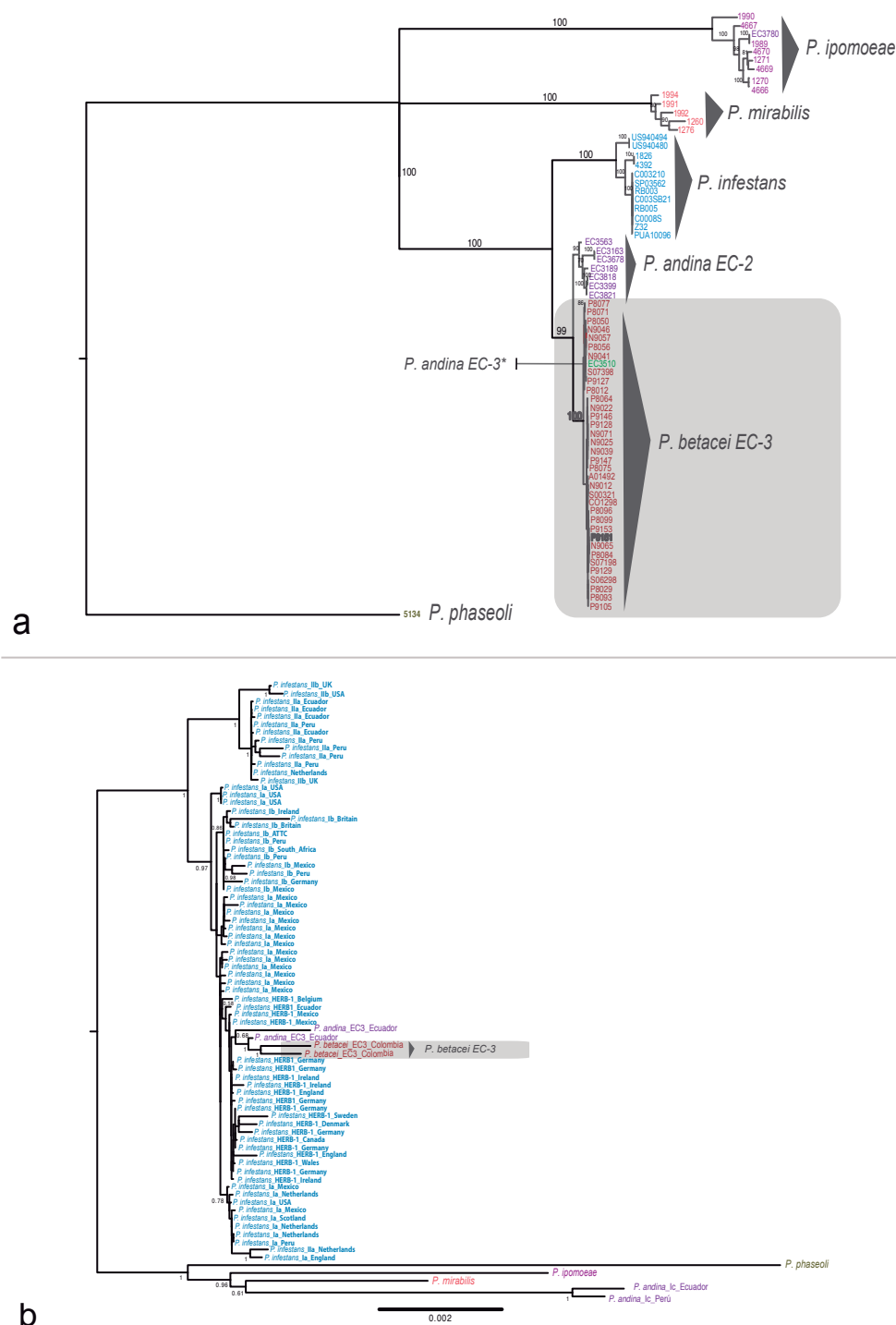
Fig. 1 Phylogenetic relationships of the *Phytophthora betacei* isolates and the closely related species, based on genotyping by sequencing (GBS) data and phylogeny of complete mitochondrial genomes. a. The tree was inferred using maximum likelihood (ML) with *P. phaseoli* as an outgroup. Support values associated with branches correspond to ML bootstrap support values (BS); b. ML phylogeny of complete mitochondrial genomes showing the phylogenetic position of *P. betacei* sp. nov. Only bootstrap support values above 50 % are shown.

counts of each SNP for the entire dataset (55 samples and 22 788 SNPs). Samples belonging to each species were separated into three different files (*P. betacei*, *P. andina* (EC-2) and *P. infestans*) and allele counts were calculated for each dataset. SNPs with changes in the major allele in *P. betacei* were selected as candidates of differentiations relative to *P. infestans* and *P. andina* (EC-2) isolates.

RESULTS

Phylogenetic relationships of the Phytophthora clade 1c species using nuclear and mitochondrial genomes

A phylogenetic reconstruction using 23 480 nuclear SNPs showed *P. betacei*, *P. andina* and *P. infestans* as more closely related to each another than to *P. ipomoeae* or *P. mirabilis* (Fig. 1a). The former three species formed a monophyletic group. All *P. infestans* clonal lineages (EC-1, US-8 and US-12) formed a monophyletic group. *Phytophthora betacei* appeared as the sister group of the *P. andina* strains collected from other wild plants of the *Solanaceae* family. This *P. andina* group comprised the EC-2 clonal lineage with mitochondrial haplotypes Ia and Ic and some isolates of unknown clonal lineage. The two clades, *P. betacei* and *P. andina* (EC-2 clonal lineage), were reciprocally monophyletic, providing evidence of the divergence of the two species. The only isolate formerly identified as *P. andina* clonal lineage EC-3 that was included in the analysis grouped together with *P. betacei*. This *P. andina* strain was also isolated from *S. betaceum* (Fig. 1a). A phylogenetic analysis of the mitochondrial genome sequences did not differentiate among the three species of the *P. infestans* s.lat. complex, with one notable exception: the *P. andina* clonal lineage EC-2 with the Ic mtDNA type appeared as the sister species of *P. mirabilis* (Fig. 1b).

Nuclear genome size determination

Genome sizes differed significantly among the three species ($F_{2,103}$ = 6.09, P < 0.01). Post-hoc comparisons revealed that *P. betacei* has the largest genome among the three species (mean size genome *P. betacei* = 1.13pg). The *P. infestans'* genome was almost half the size of that of *P. betacei* (mean size genome *P. infestans* = 0.67pg). Differences in genome size between *P. betacei* and *P. infestans* were significant (Tukey HSD test comparing *P. betacei* and *P. infestans*: t-value = 3.45, P < 0.01). The mean genome size of *P. andina* seems to intermediate between *P. betacei* and *P. infestans* (mean size genome *P. andina* = 0.97). Differences in genome size between *P. andina* and *P. betacei*, or between *P. andina* and *P. infestans* were not significant (Tukey HSD test comparing *P. andina* and *P. infestans*: t-value = 0.08213; Tukey HSD test comparing *P. andina* and *P. betacei*: 0.48; P > 0.05 in both comparisons) (data not shown).

Population structure analyses

Microsatellite and whole-genome SNP data differed among the populations of *P. infestans*, *P. betacei* and *P. andina* (Fig. 2a–e). The results obtained from the PCA, using microsatellite data, suggested three genetic groups (Fig. 2a). The PC1 separated *P. infestans* and *P. betacei* (mean variance
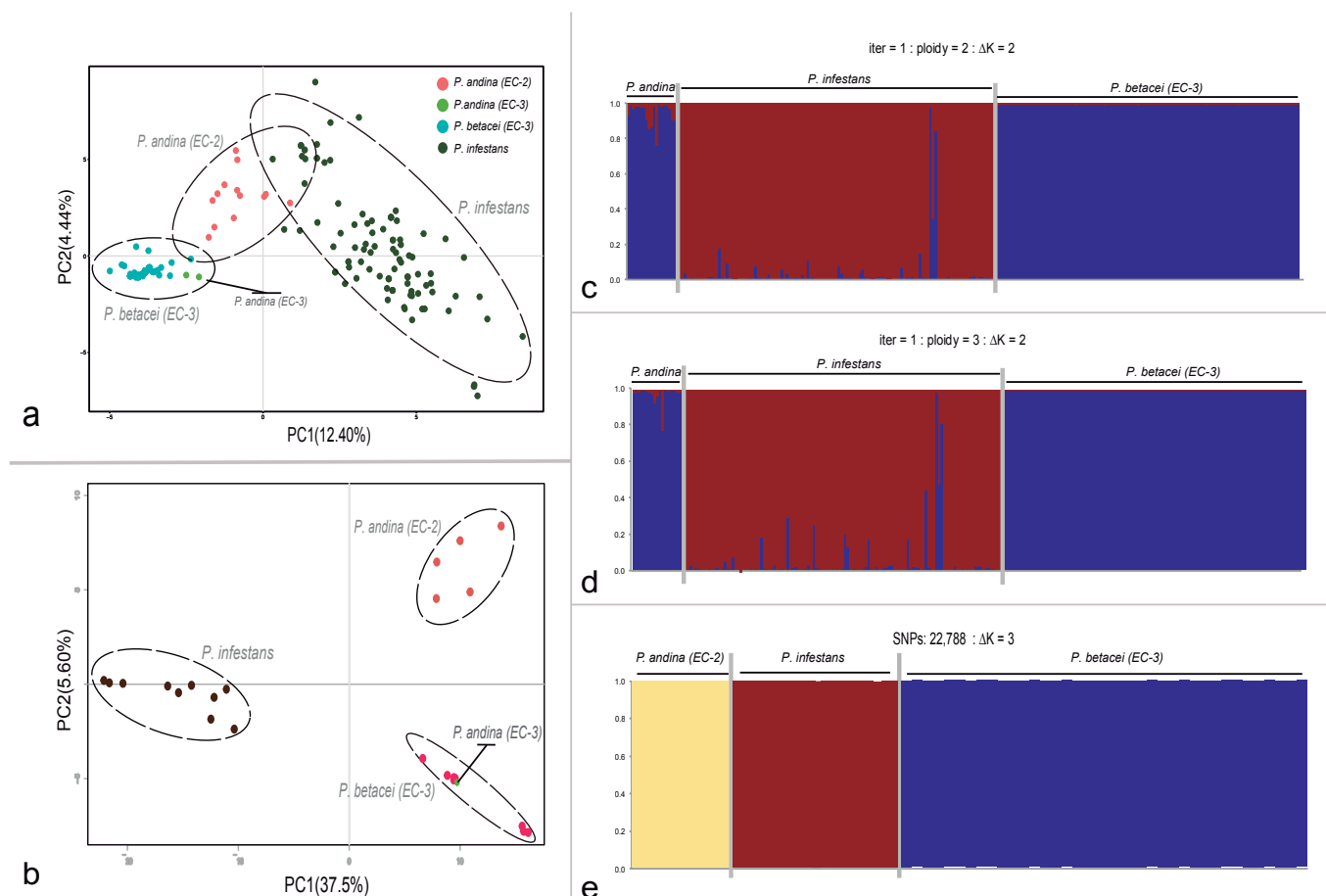

Fig. 2 Principal component analysis (PCA) and STRUCTURE results for microsatellite and Genotyping-by-sequencing data showing the genetic structure for *P. betacei*, *P. andina* and *P. infestans*. a–b. Results for PCA analysis for SSR and GBS data, respectively. Principal components (PC) 1 and 2 are shown and the percentage of variance explained by each eigenvalue is shown within parentheses on each axis. Individuals of *P. infestans* are shown in blue, *P. andina* in orange and *P. betacei* in green; c–d. STRUCTURE results for SSR data for resampled diploid and triploid datasets, respectively (ΔK = 2); e. STRUCTURE results for Genotyping-by-sequencing data. data (ΔK = 3). Classification of *Phytophthora* samples was established according to the optimal population number (Evanno's method). The distribution of the individuals in different populations is indicated by the different colours on each plot.

explained = 12.40 %), indicating that a large proportion of the genetic variation is explained by the genetic difference between isolates belonging to these two species. The PC2 (mean variance explained = 4.44 %) showed an intraspecific variation within *P. infestans* that was larger than the variations within *P. betacei* or *P. andina*. The two *P. andina* strains of the EC-3 clonal lineage were grouped closely with *P. betacei*. For this analysis, PCs 3, 4 and 5 primarily represent intraspecific variation within *P. infestans* (data not shown). Similar results were obtained for the GBS data supporting the clustering of the SSR analysis (Fig. 2b). The PCA on the GBS data including

23 480 SNPs showed strong genetic difference among strains of *P. infestans*, *P. betacei* and *P. andina* (Fig. 2b). The PC1 accounted for 37.5 % of the total variation and separated *P. infestans* from the *P. andina/P. betacei* clades. The PC2 identified 5.8 % of the variation between the strains and separated *P. betacei* and *P. andina* (Fig. 2b).

The Bayesian assignment test conducted in STRUCTURE for the SSR and GBS data showed similar results (Fig. 2c–e). The STRUCTURE analysis showed two genetic clusters or populations that could explain the genetic variance for the SSR data (ΔK = 2; Fig. 2c–d). The two genetic clusters matched
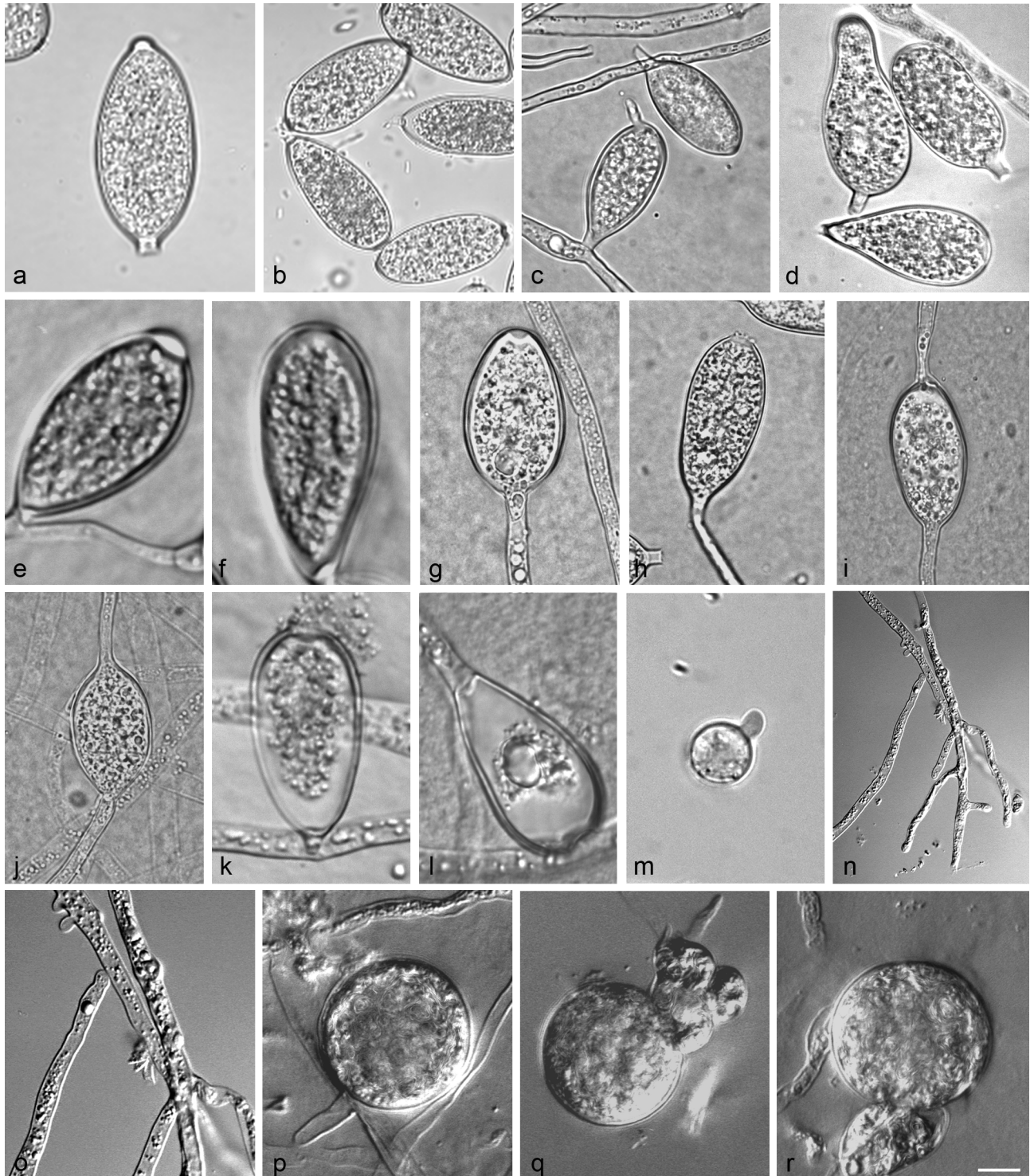
Fig. 3 *Phytophthora betacei* morphology. a–c. Typical *P. betacei* sporangia. a. Limoniform; b–c. elongated ellipsoid; d. distorted or irregular shape; e. sporangia with papillate; f. semi-papillate or non-papillate sporangia; g–j. sporangia proliferation; k–m. zoospores cysts diameter; n–o. absence of hyphal swelling or chlamydospores and characteristics of primary hyphae; p–r. *Phytophthora betacei* gametangia. Abnormal oospores detected for *P. betacei* isolates (MFM-N9022, MFM-P8084, MFM-P8077). — Scale bar = 10 μm.

P. infestans s.str. and *P. betacei*. The third species, *P. andina* (both lineages EC-2 and EC-3) could not be assigned and seemed to cluster with *P. betacei* but not with *P. infestans* (Fig. 2c–d). Population assignment was robust to changes in ploidy; the most likely clustering was with two populations across diploid and triploid subsample datasets (∆K = 2; Fig. 2c–d). The STRUCTURE analysis supports genetic differentiation between *P. infestans* and *P. betacei*, with uncertain clustering for *P. andina*. For the GBS data, the STRUCTURE analysis showed that the most likely clustering was three populations (∆K = 3; Fig. 2e). The first genetic clusters matched with *P. infestans* s.str. and the second matched with *P. betacei*. The third cluster was assigned to *P. andina* samples (lineage EC-2) supporting the genetic differentiation among *P. infestans*, *P. andina* and *P. betacei* (Fig. 2e).

TAXONOMY

Phytophthora betacei Mideros, L.E. Lagos & S. Restrepo, *sp. nov*. — MycoBank MB815748; Fig. 3

Etymology. Name refers to *Solanum betaceum*, the host plant from which the isolates were obtained.

Typus. Colombia, Putumayo, Colon, San Pedro locality, isolated from small pieces of infected leaves from *S. betaceum* plants, 2008, *M.F. Mideros* (Andes-F 1172 holotype, culture on TTA, MFM-P8084 ex-type culture).

Sporangia (Fig. 3a–d) — *Phytophthora betacei* produce sporangia typically borne terminally with limoniform (Fig. 3a), elongated ellipsoid (Fig. 3b–c) and distorted or irregular shape (Fig. 3d). No relationship between sporangia shape and culture medium was found. Sporangia were caducous with papillate (Fig. 3e; 75 % on average) and semi-papillate or non-papillate sporangia (Fig. 3f; 25 % on average) with short pedicels (2.6 ± 0.5 µm length). Typically, sporangia proliferated terminally in a simple sympodium with external proliferation close to the sporangial base and occasionally intercalary (Fig. 3g–j). Zoospores of *P. betacei* were discharged directly through a small exit pore 4.2–4.6 µm wide (overall average 4.4 ± 0.2 µm) (Fig. 3k–l). They were spherical and motile. The average diameter of the cysts was 7.74 ± 0.9 µm (Fig. 3m). Sporangia from five isolates of *P. betacei* on V8 agar averaged 36.3 ± 6.0 µm in length × 17.3 ± 2.9 µm in breadth with a range of isolate means of 24.27–40.45 × 11.8–23.1 µm. The average length-to-breadth ratio on V8 was 2.1 ± 0.4. Sporangia from the same isolates on CMA averaged 37.6 ± 5.4 µm of length × 16.9 ± 2.3 µm of breadth with a range of isolate means of 27.6–47.8 × 12.25–22.25 µm. The average length-to-breadth ratio on CMA was 2.2 ± 0.2. Finally, on TTA sporangia of all isolates averaged 39.3 ± 4.8 µm in length × 15.8 ± 5.7 µm in breadth with a range of isolate means of 31.6–49.2 × 12.13–19.23 µm. The average length-to-breadth ratio on TTA was 2.6 ± 0.2. Chlamydospores absent. Catenulate, ellipsoid or globose hyphal swellings were not observed (Fig. 3n–o). The primary hyphae of *P. betacei* varied from 2.6–3.5 µm in width with a range of isolate means of 2.8 ± 0.7 µm.

Oogonia, oospores and antheridia (Fig. 3p–r) — *Phytophthora betacei* is heterotallic with a low oospore production (15.4 ± 10.9 oospores per mm²) and it has an abnormal appearance when crossed with a *P. infestans* strain of the A2 mating type (US040009). Some isolates never formed oospores and the abortion rate was very high (60–80 % on average; Fig. 3q–r). In the polycarbonate membrane test, oogonia from four isolates were borne terminally or laterally and not ornamented. The average oogonial diameter among the isolates was 22.2–26.1 µm (overall average 25.0 ± 0.9 µm). Oogonia were usually globose to subglobose with a smooth wall (Fig. 3p). Oospores were produced within 25 to 30 days after pairing with the

P. infestans isolate A2 mating type (US040009) on the rye agar medium. Oogonia were often turned golden-brown on maturity. Antheridia were amphigynous with mean dimensions of 12.0 ± 0.3 × 9.0 ± 0.5 µm (Fig. 3q–r).

Colony morphology, growth rate and cardinal temperatures (Fig. 4a–l) — All *P. betacei* isolates tested formed colonies with different growth pattern and irregular margins on V8, CMA, TTA and PDA (Fig. 4a–l). *Phytophthora betacei* isolates grew on V8 juice agar (V8) producing fluffy colonies with non-defined margin. Aerial mycelium is sparse and velvety with no discernible pattern (Fig. 4a). The optimum temperature for the growth of *P. betacei* on V8 was between 10 and 20 °C with an average radial growth rate of 1.19 ± 0.6 mm/d. On CMA, *P. betacei* isolates produced colonies that were fluffy and smooth, similar to V8 medium with irregular margins (Fig. 4b). The optimum temperature for the growth of *P. betacei* on CMA was 20 °C with an average radial growth rate of 0.65 ± 0.3 mm/d (Fig. 5). The growth of *P. betacei* isolates on PDA was limited (Fig. 4d). The radial growth rate was on average 0.02 ± 0.01 mm/d (Fig. 5). No sporulation was detected on PDA. Finally, on TTA, *P. betacei* produced white colonies with sparse and with dense, fluffy aerial mycelium. The radial growth rate and sporulation of *P. betacei* isolates were more abundant on the TTA medium than on any of the other three media tested (Fig. 4d). The average radial growth rate at the optimum temperature of 20 °C was 2.38 ± 0.19 mm/d. No growth was recorded below 10 °C or above 25 °C, as these temperatures were lethal to most isolates, thus, the maximum temperature for growth was 20 °C (Fig. 5).

Additional specimens examined. Table 1.

Notes — Disease caused by *P. betacei* can lead to the complete loss of the *S. betaceum* crops (tree tomato) 5–10 d after the first symptoms are detected. In the field, the pathogen is able to defoliate the tree completely in approximately 1 wk. The symptoms of *P. betacei* on tree tomatoes differed from those generated by *P. infestans* on potatoes in forming concentric blighted areas that produce sporangia, and covered large areas of the leaves and petioles. In the field, no symptoms were observed on fruits, and the disease was rarely found on stems. From the GBS data, we identified 22 788 SNPs in total, 150 of which were able to discriminate *P. betacei* from *P. andina* (EC-2) and *P. infestans*. Although all 150 SNPs were classified as potentially diagnostic SNPs, this set of markers should then be validated using a larger *P. betacei* collection to confirm their robustness to diagnose this species. *Phytophthora betacei* can be morphologically distinguished from the *P. andina* or *P. betacei* species by sporangia size, with *P. betacei* isolates showing a greater length-to-breadth ratio of sporangia (ranges from 2.13–2.56) than *P. infestans* and *P. andina* isolates. *Phytophthora betacei* also showed an elongated ellipsoid or limoniform sporangia shape in contrast to *P. infestans* or *P. andina*, which showed ovoid sporangia on all isolates. In addition, the new species can be distinguished from *P. andina* (EC-2) and *P. infestans* isolates using a combination of morphological and physiological traits. A discriminant analysis showed that length-to-breadth ratio of sporangia and the width of the primary hyphae are the morphological traits that explain the 99 % of the variance of the data (Fig. 6). The first function (LD1) characterized the groups based mostly on the length-to-breadth ratio of sporangia. For the second function (LD2), the primary hyphal width and the length-to-breadth ratio of sporangia helped discriminate among these groups of isolates. Sporangial measurements (length (µm), breadth (µm), length-to-breadth ratio) differed significantly among all species (Kruskal-Wallis rank sum test, $P < 0.01$), confirming that this morphological character can be used to separate *P. betacei* from the other two related species. However, some differences were more striking between species depending
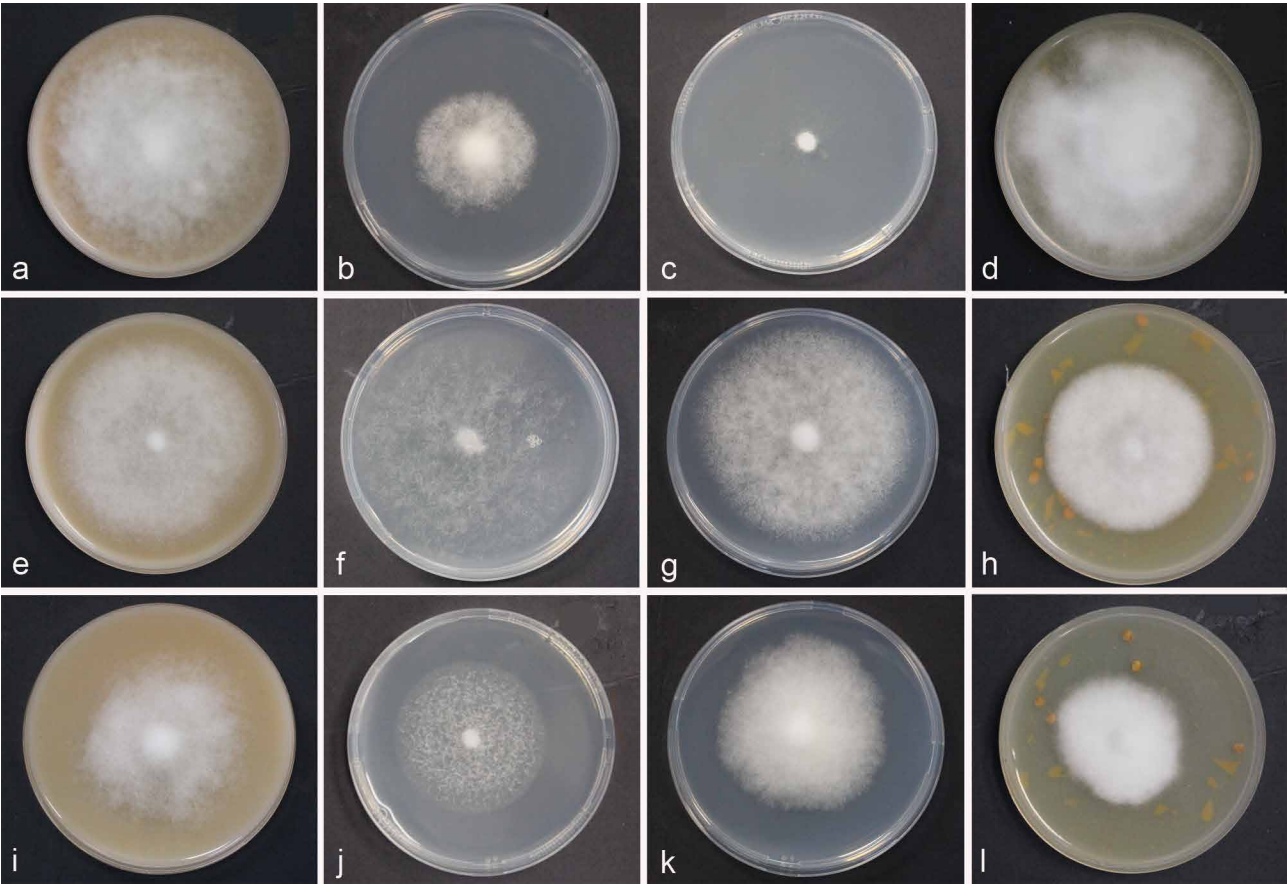
Fig. 4 Colony morphology, of *Phytophthora betacei*, *P. infestans* and *P. andina*. a–l. Colony morphology and radial growth for *P. betacei* (ex-type culture Andes-F 1172), *P. infestans* (isolate VPC7-10) and *P. andina* (isolate EC 3818) (from top to bottom) after 15 d incubation at 18 °C on V8 juice agar (V8), corn meal agar (CMA), potato dextrose agar (PDA), and tree tomato agar (TTA) (from left to right).
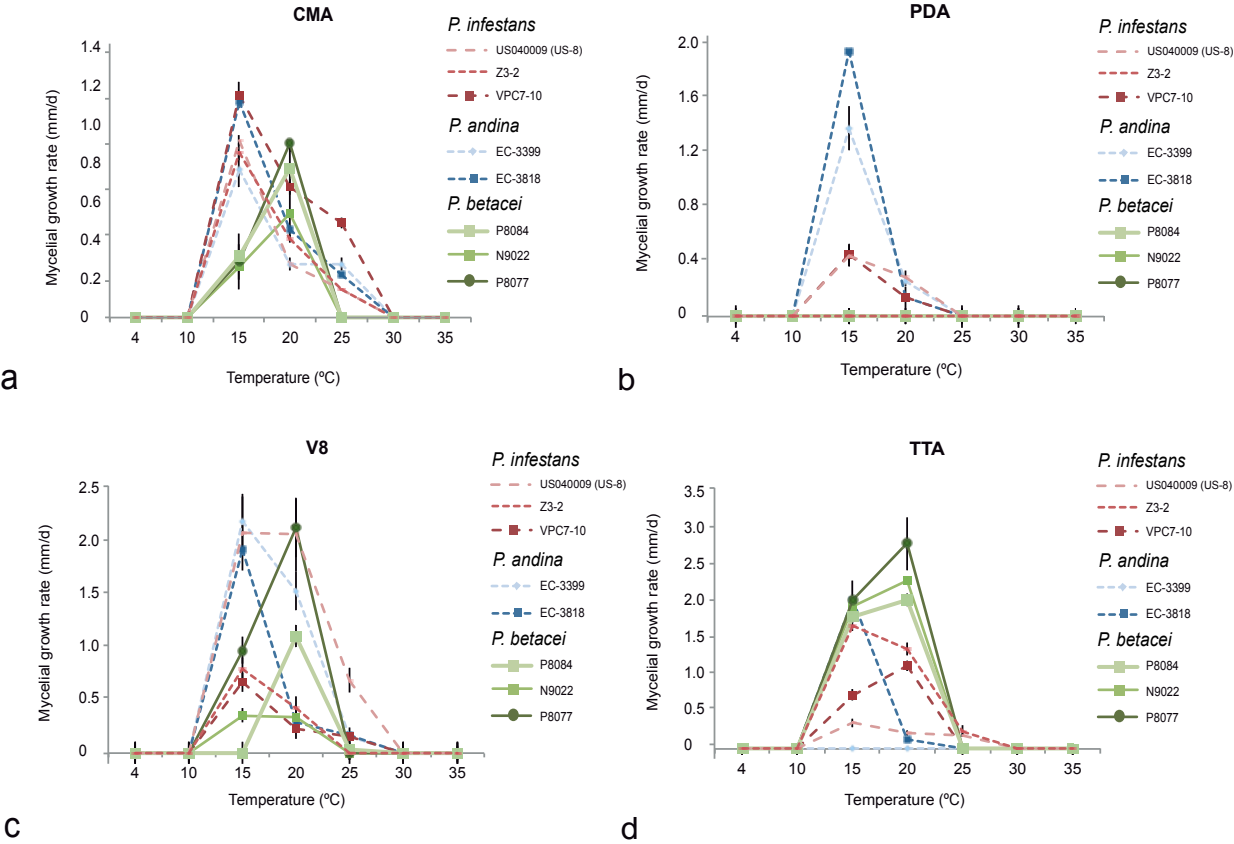

Fig. 5 Radial growth rate of *Phytophthora betacei* on four culture media at different temperatures. Means and standard errors were calculated from three isolates: green lines = *P. betacei* isolates (ex-type Andes-F 1172, isolate P8077 and N9039), blue lines = *P. andina* (isolates EC 3818 and EC 3399), and red lines = *P. infestans* (isolates VPC7-10, Z-32 and US040009), on: a. Corn meal agar (CMA); b. potato dextrose agar (PDA); c. V8 juice agar (V8); d. tree tomato agar (TTA).
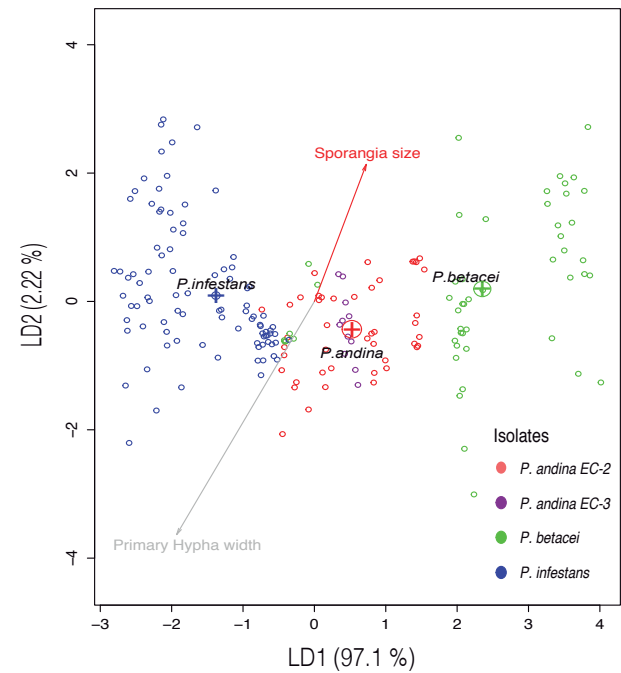
Fig. 6 Plot of the morphological traits of *Phytophthora* species using a discriminant analysis (DA) showing the first and second discriminant components. *Phytophthora betacei* strains are shown in blue, *P. infestans* in green and *P. andina* in red.

on the medium used (comparisons of *P. betacei* with either *P. infestans* or *P. andina*). The optimal and lethal temperatures for *P. betacei*, *P. infestans* and *P. andina* (EC-2) were similar on all media tested. However, at the optimal temperature (20 °C), *P. betacei* grew more rapidly than the other two species (linear coefficients: *P. betacei* vs *P. infestans*: 12.36; *P. betacei* vs *P. andina*: 9.37, both P < 0.001). Isolates of *P. infestans* and *P. andina* were also able to grow at 25 °C in at least one of the four media tested. In contrast, none of the *P. betacei* isolates was able to grow at 25 °C (Fig 5). The type of culture media (V8, PDA, TTA and CMA) affected the radial growth rate of the different species at 15 °C. No significant differences were found when comparing isolates of *P. betacei* with those of *P. infestans* and *P. andina* (EC-2) growing on the CMA culture medium. However, a notable exception for *P. betacei* isolates was detected on the PDA. *Phytophthora betacei* showed low rates of growth on the PDA and a complete absence of sporulation. Significant differences were also detected among all isolates of *P. betacei*, *P. andina* and *P. infestans* growing on the V8 and TTA culture media (Kruskal-Wallis χ² = 80.703, df = 3, P < 0.001). The original descriptions of the *P. infestans* and *P. andina* isolates were congruent with the data obtained in the present study. The host range of *P. betacei* is only restricted to *S. betaceum*, showing a strong host-plant specialisation in reciprocal inoculation experiments. *Phytophthora betacei* could not infect either tomatoes or potatoes but showed the highest fitness on *S. betaceum* (Fig. 7).

Host pathogenicity assays and evaluation of host preference

Because the *P. infestans*, *P. betacei* and *P. andina* (EC-2) strains were isolated from different hosts, the hypothesis that the three species were host specialised or had a reduced fitness on their alternate host was tested. Isolates of *P. andina* of the EC-2 and EC-3 clonal lineages were included to make all possible pairwise comparisons. Isolates of *P. andina* of the EC-2 clonal lineage did not produce any symptoms on any of the hosts used. *Phytophthora infestans* had a higher fitness on tomatoes and yellow potatoes compared to *P. betacei* and *P. andina* (Table 2),

Table 2 Pairwise comparisons of overall fitness on four different hosts. Pairwise comparisons were made using a Kruskal Wallis rank sum test followed by a Nemenyi test with multiple comparisons. Statistically significant P-values (P < 0.05) are shown in **bold**.

| Host	Mean fitness (Standard Deviation)			Kruskal Wallis rank sum test		Nemenyi pairwise comparisons		
	P. infestans	*P. andina* (EC-3)	*P. betacei*	χ², df = 2	P-value	*P. infestans* vs *P. andina* (EC-3)	*P. infestans* vs *P. betacei*	*P. betacei* vs *P. andina* (EC-3)
Tomato (*S. lycopersicum*)	0.0481 (± 0.0589)	0.0421 (± 0.1055)	0 (± 0)	18.0462	1.206 × 10⁻⁴	0.5063	**0.0097**	0.2034
Potato (*S. tuberosum* var. *capiro*)	0.0146 (± 0.0341)	0.0267 (± 0.0486)	0 (± 0)	4.7732	0.09194	0.89	0.76	0.45
Yellow potato (*S. phureja*)	0.0604 (± 0.0450)	0.0545 (± 0.0574)	0 (± 0)	14.6133	6.711 × 10⁻⁴	0.999	**0.019**	**0.021**
Tree tomato (*S. betaceum*)	0.0659 (± 0.0622)	0.0298 (± 0.0462)	0.1025 (± 0.0583)	28.9611	5.143 × 10⁻⁷	**0.036**	**0.033**	**5.7 × 10⁻⁷**
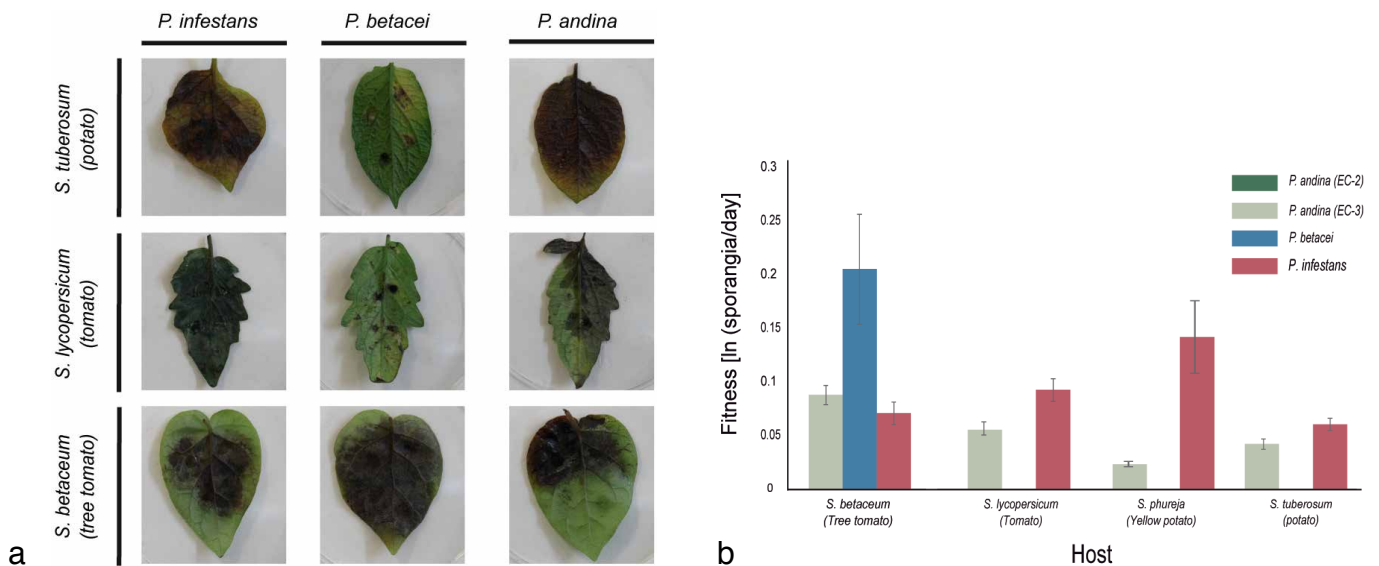
Fig. 7 Host specialisation results in strong premating reproductive isolation of *P. infestans*, *P. betacei* and *P. andina* isolates. a. Fitness obtained from reciprocal infection assays. Results of infection for *P. infestans*, *P. betacei* and *P. andina* (EC-3 clonal lineage) on the main hosts evaluated: potatoes (*S. tuberosum*), tomatoes (*S. lycopersicum*) and tree tomatoes (*S. betaceum*) after 9 d post inoculation (dpi). The isolates of *P. andina* of the EC-2 clonal lineage did not produce any symptoms on any of the hosts; b. values of fitness (sporangia per day) for *P. infestans*, *P. betacei* and *P. andina* (EC-2 and EC-3 clonal lineages). On tree tomato, isolates of *P. betacei* showed significantly higher fitness than *P. infestans* and *P. andina* (EC-3 clonal lineage). Conversely, isolates of *P. betacei* are unable to infect other hosts where *P. infestans* thrives. Additionally, *P. andina* (EC-3 clonal lineage) shows significantly lower fitness on all hosts tested.

while *P. betacei* could not infect neither tomatoes nor potatoes but showed the highest fitness on tree tomatoes (Table 2). The formerly *P. andina* (EC-3) strains assayed here were able to infect all hosts but showed a lower fitness than *P. infestans* on the three hosts (tomatoes, yellow potatoes and tree tomatoes). They also displayed a lower fitness on tree tomatoes compared to *P. betacei*. All pairwise comparisons indicated that strains of *P. infestans* and *P. betacei* displayed different fitness properties on every host assessed (Fig. 7a–b).

DISCUSSION

Here, a new species of *P. betacei* that is closely related to *P. infestans* and *P. andina* is described. This finding provides novel insights into the evolutionary history of the Irish famine pathogen *P. infestans* and its close relatives. Furthermore, the species boundaries within the complex of *P. andina*, originally described as a polyphyletic taxon, are refined. These two sets of results are discussed in the following two subsections.

Phytophthora betacei is a new species

In this study, the new taxon, *P. betacei* was described based on physiological, morphological, population genetic and phylogenetic analyses, as well as differences in host specificity. All these analyses strongly support the designation of the new species *P. betacei* within *Phytophthora* clade 1c.

The first line of evidence for the distinction between *P. betacei* and the other species of *Phytophthora* clade 1c is the high genetic differentiation among the genetic groups. Nuclear phylogenies indicate that the triad *P. infestans*, *P. betacei* and *P. andina* form a monophyletic clade whose closest known relatives are other members of the *Phytophthora* clade 1c (i.e., *P. ipomoeae*, *P. phaseoli* and *P. mirabilis*). The three species, *P. betacei*, *P. infestans* and *P. andina* (from the clonal lineage EC-2; see below) are reciprocally monophyletic, suggesting they lack recent gene flow and can be considered different species. Interestingly, the mitochondrial markers do not separate the three species. This result is consistent with a scenario of speciation with secondary contact and mitochondrial introgression, a phenomenon common across the tree of life (Funk & Omland 2003).

A second line of evidence for the existence of *P. betacei* as a separate species from *P. infestans* s.str. involves differences in allele frequencies in each of these genetic groups. All analyses using both SSR loci and SNP markers suggest the existence of two discrete genetic clusters that correspond to *P. infestans* s.str. and *P. betacei*. *Phytophthora andina* has a less clear origin and this is discussed below. The data further suggests that *P. infestans* and *P. betacei* are isolated genetic groups with little detectable nuclear gene flow between them.

In addition to genetic variation, morphological differences among *P. betacei*, *P. andina* and *P. infestans* were determined. Four morphological characteristics (primary hyphal width and sporangial length, breadth, and length-to-breadth ratio) and mycelial radial growth on four different media traits were measured. The discriminant analysis clearly separated *P. betacei* from *P. infestans* in all media tested. The most striking morphological differences between *P. betacei* and *P. infestans* are the length-to-breadth ratio of sporangia, the primary hyphal width (μm) and the radial mycelial growth rate. Differences between *P. andina* and *P. infestans* or *P. betacei* are not as clear as the statistical differences between *P. andina* and *P. infestans* and between *P. andina* and *P. betacei*, and they are dependent on the medium tested. Combining all the morphological variables, it is evident that the *P. betacei* strains collected in Colombia comprise a well-differentiated group of strains (Fig. 6).

The final line of evidence comes from infection assays on the native host range of the three species and from observations in nature. The host pathogenicity assays indicate that *P. betacei* is a tree tomato specialist unable to colonise potatoes and tomatoes (Fig. 7). Conversely, *P. infestans* has a low fitness on tree tomatoes, the only known host of *P. betacei*. These reciprocal differences in host pathogenicity represent a strong reproductive isolating mechanism between the two species (Restrepo et al. 2014). Host specificity is considered one of the most important isolating mechanisms between species of plant pathogens (reviewed in Harrington & Rizzo 1999, Coyne & Orr 2004). In asexual populations, host specialisation could be associated with a strong niche partition, which is common in species with asexual reproduction and strong local adaptation to the host (Poulin 2005, Halkett et al. 2006). Plant pathogens are commonly restricted to their hosts; thus, host specialisation

can result in a strong premating barrier (Stukenbrock 2013, Vialle et al. 2013, Restrepo et al. 2014).

Notably, all isolates of *P. betacei* belonging to the EC-3 clonal lineage are closely related to the isolates previously described as *P. andina* EC-3 (Oliva et al. 2010, Goss et al. 2011, 2014, Lassiter et al. 2015, Martin et al. 2016), which in turn should be considered *P. betacei*. All EC-3 isolates form a monophyletic group differentiated from *P. infestans* and *P. andina* EC-2. The results support a finer definition of the species boundary of *P. andina*.

Phytophthora andina previously characterized as a polyphyletic group, includes only EC-2

In the literature, *P. andina* has been reported to be polyphyletic and include the following three clonal lineages: the *P. andina* EC-2 mitochondrial haplotype Ia, the *P. andina* EC-2 mitochondrial haplotype Ic, and *P. andina* EC-3 (Adler et al. 2004, Gómez-Alpizar et al. 2008). This species has been controversial since its erection, as species are expected to be monophyletic with the expectation of descent from one common ancestor. *Phytophthora andina* was shown to be a hybrid based on cloning nuclear haplotypes from several loci showing that one ancestor is *P. infestans* while the other ancestor remains to be described (Goss et al. 2011). Later, it has been hypothesized to have arisen from hybridization based on the conflicting phylogenetic information of mitochondrial and nuclear genealogies (Martin et al. 2016). Based on the observed hybrid nature and the polyphyletic mitochondrial phylogenies in *P. andina*, *P. andina* was not appropriately described as a new species (Cárdenas et al. 2012). The identification of *P. betacei* as a new species, distinct from *P. infestans*, sheds some light on the origin of *P. andina*.

The results support a monophyletic grouping of the EC-2 *P. andina* clonal lineages of mitochondrial haplotypes Ia and Ic that are closely related and that form a monophyletic group distinct from *P. betacei* and *P. infestans*. Supported by phylogenetic and population genetic analyses, *P. andina* EC-3 should now be considered *P. betacei*. These results indicate that the initial definition of *P. andina* included isolates that were either *P. betacei* or were closely related to *P. betacei*, namely, the EC-3 clonal lineage. However, further work is needed to determine whether all isolates of *P. andina* of the EC-3 clonal lineage collected from tree tomato in Ecuador (mitochondrial haplotype Ia, reported in Oliva et al. 2010, Goss et al. 2011, 2014, Lassiter et al. 2015, Martin et al. 2016) indeed cluster with isolates of *P. betacei*. Generally, the results confirmed previous observations that *P. andina*, as currently described, is a polyphyletic group that requires redefinition (Gómez-Alpizar et al. 2008, Cárdenas et al. 2012, Forbes et al. 2012). Redefining *P. andina* including lineages of clonal lineage EC-2 makes this group monophyletic and provides a biologically rigorous species definition. Thus, *P. andina* s.lat. is proposed to be the proper description of *P. andina* EC-2.

Whether there is reciprocal host specificity between *P. betacei* and *P. andina* (EC-2) remains an open question, yet some predictions can be formulated. Because all known EC-2 *P. andina* isolates have been collected in *Anarrichomenum* and other wild species, it is possible the species will be specialised on these plants. This study showed that strains of *P. andina* of the EC-2 clonal lineage could not infect *S. betaceum*, the only known host of *P. betacei*. Several authors have documented host specificity between the *P. andina* EC-2 and EC-3 clonal lineages in nature (Adler et al. 2004, Gómez-Alpizar et al. 2007, Oliva et al. 2010). Isolates of the EC-3 clonal lineage have always been collected from *S. betaceum* plants, again suggesting a strong isolating mechanism between *P. betacei* and *P. andina* (EC-2).

However, it is important to mention that a group of strains referred to as *P. andina* has recently been reported as infecting *S. betaceum* in Peru (lineage PE-8) (Forbes et al. 2016). Thus, further analyses and a greater number of isolates are needed to determine the host range and fitness of isolates belonging to the PE-8 clonal lineage.

Conclusions

This study provided several lines of evidence supporting the claim that *P. betacei* is a distinct, previously undescribed species within *Phytophthora* clade 1c. The findings also resolve the polyphyletic nature of *P. andina*. Together, the evidence shows that the dyad *P. andina* / *P. betacei* is a sister clade of the potato blight pathogen, *P. infestans*. The strong host specialisation of *P. infestans* and *P. betacei* may act as a premating barrier that restricts gene flow between these two species in nature. It remains unclear whether host specialisation facilitated or initiated the speciation process in the *P. infestans* s.lat. complex. However, in this report, it was shown that ecological differences are important in the persistence of *P. infestans* and *P. betacei* as genetically isolated units across an overlapping area in the northern Andes. More studies are needed to further characterise the evolution of the closely related species and to understand the process of divergence in this group. In general, the results obtained have implications for the understanding of how new plant pathogen species originate and persist. The findings also highlight the importance of sampling plant pathogens of semi-domesticated or undomesticated hosts.

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