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Phylogenetic relationships of *Phytophthora andina*, a new species from the highlands of Ecuador that is closely related to the Irish potato famine pathogen *Phytophthora infestans*

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Abstract: Phylogenetic relationships of *Phytophthora infestans sensu lato* in the Andean highlands of South America were examined. Three clonal lineages (US-1, EC-1, EC-3) and one heterogeneous lineage (EC-2) were found in association with different host species in genus *Solanum*. The EC-2 lineage includes two mitochondrial (mtDNA) haplotypes, Ia and Ic. Isolates of *P. infestans sensu lato* EC-2 fit the morphological description of *P. infestans* but are different from any genotypes of *P. infestans* described to date. All isolates of *P. infestans sensu lato* from Ecuador were amplified by a *P. infestans* specific primer (PINF), and restriction fragment length patterns were identical in isolates amplified with ITS primers 4 and 5. The EC-1 clonal lineage of *P. infestans sensu lato* from *S. andreanum*, *S. columbianum*, *S. paucijugum*, *S. phureja*, *S. regularifolium*, *S. tuberosum* and *S. tuquerense* was confirmed to be *P. infestans* based on sequences of the cytochrome oxidase I (*cox* I) gene and intron 1 of *ras* gene. The EC-2 isolates with the Ic haplotype formed a distinct branch in the same clade with *P. infestans* and *P. mirabilis*, *P. phaseoli* and *P. ipomoeae* for both *cox* I and *ras* intron 1 phylogenies and were identified as the newly described species *P. andina*. *Ras* intron 1 sequence data suggests that *P. andina* might have arisen via hybridization between *P. infestans* and *P. mirabilis*.

Key words: Oomycetes, *Phytophthora*, potato, Stramenopiles

INTRODUCTION

Genus *Phytophthora* is classified within the diploid, algae-like Oomycetes in the Stramenopiles (Adl et al 2005, Cavalier-Smith 1986, Gunderson et al 1987, Yoon et al 2002) and contains more than 60 species that occupy a variety of terrestrial and aquatic habitats and are responsible for economically important diseases of a wide range of agronomic and ornamental crops (Erwin and Ribeiro 1996). *Phytophthora infestans* (Mont.) de Bary, the causal agent of late blight disease, is one of the most important *Phytophthora* species and infects potato and tomato worldwide and caused the Irish potato famine in the 1840s (Gómez-Alpizar et al 2007). *Phytophthora infestans* is included in Waterhouse's taxonomic Group IV, which contains both homothallic and heterothallic species with amphigynous antheridia and semipapillate sporangia (Newhook et al 1976, Stamps et al 1990, Waterhouse 1963). Other members of this group are *P. colocasiae* Racib. 1900, *P. hibernalis* Carne 1925, *P. ilicis* Buddenhagen & R.A. Young 1957, *P. mirabilis* Galindo & H.R. Hohl 1986 and *P. phaseoli* Thaxt. 1889. Cooke et al (2000) reported a phylogenetic analysis of 50 *Phytophthora* species, based on sequence analysis of the internal transcribed spacer regions (ITS1 and ITS2) of the rRNA genes, and this phylogeny is considered the most comprehensive to date. The monophyletic genus comprises eight main lineages designated clades 1–8. Clade 1 was subdivided further into clades 1a, 1b and 1c. *Phytophthora infestans* clustered in Cooke's clade 1c with *P. mirabilis* and *P. phaseoli*, indicating that these species share a most recent common ancestor. The phylogenetic relationship among species in clade 1c has been confirmed further based on sequence analysis of mitochondrially encoded cytochrome oxidase I and II genes (*cox* I, *cox* II) (Martin and Tooley 2003).

New *Phytophthora* species have been described and their taxonomic status has been clarified on the basis of morphology, physiology and molecular relatedness (Brasier et al 2003, Cooke et al 1999, Galindo and

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TABLE I. Diversity of *P. infestans sensu lato* on *Solanaceous* plants in Ecuador

Lineage	mtDNA haplotype[a]	Mating type	Allozyme genotype[b]		Hosts
			Gpi	*Pep*	
US-1	Ib	A1	86/100	92/100	Tomato (*Solanum lycopersion*), *S. ochrantum*, *S. carpinense*, pear melon (*S. muricatum*), *S. andreanum*[c]
EC-1	IIa	A1	90/100	96/100	Potato (*S. tuberosum*, *S. phureja*) and wild potato (*Solanum* spp in the section *Petota*, tuber-bearing species)
EC-2	Ic	A2	100/100	76/100	*Anarrhichomenum*-complex[d], pear melon (*S. muricatum*)
EC-2[e]	Ia	A1	100/100	76/100	*Anarrhichomenum*-complex[d]
EC-3	Ia	A1	86/100	76/100	Tree tomato (*S. betaceum*)

[a] Mitochondrial DNA haplotype determined by methods of Griffith and Shaw (1998).
[b] *Gpi* = Glucose-6-phosphate isomerase, *Pep* = Peptidase.
[c] S. *andreanum* is a tuber-bearing species in section *Petota*. It is also attacked by isolates of the EC-1.
[d] *Anarrhichomenum-complex* includes *S. brevifolium*, *S. tetrapetalum* and other species not clearly identified.
[e] Have the same RFLP fingerprint as EC-2 but have different mating types and mtDNA haplotypes (Adler et al 2004).

Hohl 1985, Jung et al 2003). In addition to morphological approaches other methods have been used to identify *Phytophthora* species, including protein patterns, isozymes, serology, restriction fragment length polymorphism (RFLP) analysis of nuclear and mitochondrial genes, single-strand-conformation polymorphism (SSCP) and DNA sequence analyses of the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) (Benson 1991, Ersek et al 1994, Forster et al 1990, Kong et al 2003, Martin and Tooley 2004, Mills et al 1991, Ristaino et al 1998).

Flier et al (2002) described *P. ipomoeae* Flier & Grünwald 2002 as a new species and the causal agent of leaf blight disease on *Ipomoea longipedunculata*, a morning glory native to the highlands of central Mexico (Toluca Valley). Phylogenetic analyses based on ITS and other nuclear and mitochondrial genes showed that *P. ipomoeae* is also a member of the 1c clade (Flier et al 2002, Kroon et al 2004). Kroon et al (2004) used multigene-based phylogenies that included other nuclear and mitochondrial loci and added one isolate of *P. andina* nom. prov. 2006 and *P. ipomoeae* into their analysis. The Toluca Valley has been proposed as the center of origin of *P. infestans*, *P. mirabilis*, *P. phaseoli* and *P. ipomoeae* (Brasier and Hansen 1992, Flier et al 2002, Goodwin et al 1992, Niederhauser 1991). Flier et al (2002) suggested that the central highlands are the center of origin of all Waterhouse's group IV *Phytophthora* species.

However other evidence indicates that the Andean region of South America is more likely to be the center of origin of *P. infestans* and that other lineages morphologically related to *P. infestans* are associated with both tuber-bearing and non-tuber-bearing wild *Solanum* species in South America (Abad and Abad 1997, Adler et al 2004, Garry et al 2005, Gómez-Alpizar et al 2007, May and Ristaino 2004, Ordoñez et al 2000, Ristaino et al 2001). Three clonal lineages (US-1, EC-1, EC-3) and one heterogeneous lineage (originally designated EC-2) were found in association with different *Solanum* species (TABLE I) in the Andean highlands of Ecuador. Due to taxonomic uncertainties of the EC-2 and EC-3 lineages, the population of *P. infestans* in Ecuador was referred to as *P. infestans sensu lato* (Adler et al 2004). The EC-2 and EC-3 lineages fit the morphological description of *P. infestans* but are different from any genotypes of *P. infestans* described to date and are considered indigenous to the Andean highlands of South America (Adler et al 2004). Genetic diversity of isolates of *P. infestans sensu lato* from the non-tuber-bearing hosts is high, occurring principally among and not within host-specific groups.

The EC-2 lineage originally was described as clonal (Ordoñez et al 2000) but subsequently found to comprise at least two lineages (Adler et al 2004) (TABLE I). One is characterized by the A2 mating type, the Ic mitochondrial haplotype (mtDNA) and allozyme genotypes for glucose phosphate isomerase (*Gpi*) and peptidase (*Pep*), 100/100 and 76/100 respectively and will be referred to as EC-2 (Ic haplotype). The other lineage is characterized by the A1 mating type, Ia mtDNA haplotype, Gpi 100/100 and Pep 76/100 and will be referred to as EC-2 (Ia haplotype). Most isolates from EC-2 originally were isolated from species in section *Anarrhichomenum* of genus *Solanum* and for that reason were jointly referred to by Adler et al (2004) as the *Anarrhichomenum* complex. A number of EC-2 (Ic haplotype) isolates also were collected on two occasions from the Andean pear melon (*S. muricatum*) (Adler et al 2002). Isolates in the EC-3 lineage

attack cultivated tree tomato (*S. betaceum*), and this clonal lineage is characterized by A1 mating type, Gpi (86/100) and Pep (76/100) allozyme genotypes and the Ia mtDNA haplotype (TABLE I).

The taxonomic status of isolates assigned to EC-2 (Ia and Ic haplotypes) and EC-3 lineages has not been clearly resolved (Adler et al 2004). Molecular phylogenetic analyses can help to clarify and elucidate the relatedness of these lineages to *Phytophthora infestans* and other closely related species. Some evidence already has been presented that suggests members of the EC-2 lineage might belong to a new *Phytophthora* species. Wattier et al (2003) sequenced five intergenic mitochondrial DNA spacers from two isolates of EC-2 (Ic haplotype) lineage from the *Anarrhichomenum* species *S. brevifolium*. These two isolates from *S. brevifolium* were different from the isolates of *P. infestans* from potato. The EC-2 (Ic haplotype) lineage had identical nucleotide sequences but was polymorphic for 45 nucleotides within the five intergenic spacers when compared to the reference sequence from the US-1 (Ib mtDNA haplotype) (Lang and Forget 1993). Both isolates from *S. brevifolium* were also highly polymorphic when compared to *P. ipomoeae*, *P. mirabilis* and *P. phaseoli*.

Kroon et al (2004) included one isolate of EC-2 (Ic haplotype) from Ecuador in a phylogenetic study of 48 *Phytophthora* species and used nuclear (translation elongation factor 1α and β-tubulin) and mitochondrial (*cox* I and *nadh*) genes and proposed the name *P. andina* for the Ecuadorian isolate. *Phytophthora andina* formed a new branch on both nuclear and mitochondrial phylogenies and clustered together with clade 1c species that include *P. infestans*, *P. ipomoeae*, *P. mirabilis* and *P. phaseoli*. Kroon et al (2004) suggested that *P. andina* could be of hybrid origin and speculated that *P. infestans* might be one of the parental species.

Five isolates of EC-2 (Ic haplotype) from *Anarrhichomenum* species were included in our study of the Andean origin of *P. infestans* (Gómez-Alpizar et al 2007). These isolates were highly polymorphic across the nuclear (*ras* and β-tubulin genes) and mitochondrial (*rpl14*, *rpl5*, tRNA and *cox* I) loci analyzed. They formed an independent lineage that shared a common Andean ancestor with *P. infestans* and were different from the lineages associated with potato and tomato in both mitochondrial and nuclear genealogies.

The objective of this research was to elucidate the phylogenetic relationships of isolates of EC-2 with other isolates of *P. infestans sensu lato* and other *Phytophthora* species, particularly those within clade 1c.

MATERIALS AND METHODS

Isolates.—Ecuadorian isolates of *P. infestans sensu lato* from different hosts and clonal lineages were kindly provided by Drs Nicole Adler, Peter Bonants, Wilber Flier and Francine Govers (TABLE II). Twelve isolates from the EC-1 clonal lineage from *Solanum tuberosum* and six other *Solanum* species with the IIa mtDNA haplotype were studied (TABLE II, Forbes et al 1997). One isolate of the EC-2 (Ia haplotype) lineage and nine isolates of the EC-2 (Ic haplotype) lineage (TABLE II) from *Anarrhichomenum* species were studied (Ordoñez et al 2000). An additional isolate (EC3421) from the EC-2 (Ic haplotype) lineage from *S. muricatum* (pear melon), which was named *P. andina* by Kroon et al (2004), also was characterized (TABLE II). Eight isolates from the EC-3 lineage that are the Ia mitochondrial DNA haplotype and were isolated from *S. betaceum* (tree tomato) also were studied.

Other *Phytophthora* species used for the phylogenetic analysis, their origins and collection number are provided (TABLE III). Cultures of *P. infestans sensu lato* were grown and maintained on Rye-V8 medium at 18 C, while other *Phytophthora* species were grown on lima bean agar at 25 C.

Preparation of DNA.—Isolates of *P. infestans sensu lato* were grown in Eppendorf tubes (2 mL) containing pea broth (120 g frozen garden peas boiled 10 min in 1 L distilled water, filtered and autoclaved) 1–2 wk at 18 C. Other *Phytophthora* spp were grown at 25 C. The mycelium was harvested by centrifugation at 13 000 rpm, rinsed in sterile distilled water, dried and either stored at −20 C or used immediately for DNA extraction. Total DNA was extracted from the mycelium by using a cetyltrimethylammonium bromide (CTAB, Sigma Chemical Co., St Louis, Missouri) protocol (Ristaino et al 2001). DNA was diluted 1:10 or 1:100 and stored at −20 C until use.

Identification of Ecuadorian isolates.—Two polymerase chain reaction (PCR) assays were used to determine the relatedness of the isolates of *P. infestans sensu lato* from Ecuador with other isolates of *P. infestans* and the closely related species *P. mirabilis*. The *P. infestans*-specific primer, PINF, (Trout et al 1997) was used in combination with the ITS5 primer (White et al 1990) and yields an approximately 600 bp product specific to *P. infestans* (Trout et al 1997). The second PCR assay consisted of a PCR and restriction fragment length polymorphism (RFLP) analysis of ribosomal DNA (rDNA) amplified with primers ITS4 and ITS5 (Ristaino et al 1998). Both protocols were conducted according to the methods described by Trout et al (1997) and Ristaino et al (1998). PCR was conducted in a 50 μL reaction volume with thin-walled 0.2 mL tubes. One microliter of DNA template (1:10 or 1:100 dilution) was added to a 49 μL master reaction mixture containing 5 μL 10× PCR buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, and 500 mM KCl, pH 8.3), 36.6 μL sterile distilled H_2O, 1 μL 10 mM $MgCl_2$, 2 μL 2 mM dNTPs, 2 μL 10 μM of each forward and reverse primers (PINF and ITS5 or ITS4 and ITS5 respectively) and 0.4 μL *Taq* polymerase (5 U/μL; Invitrogen, Carlsbad, California). All reactions were overlaid with sterile mineral oil before thermal cycling. Thermal

TABLE II. Ecuadorian isolates of *P. infestans sensu lato* in this study

Isolate number	Host species	Mating type	mtDNA haplotype[b]	RFLP genotype	GenBank accession numbers	
					cox1	Intron 1 of *ras*
EC3090	*Solanum phureja*	A1	IIa	EC-1	EF011126	DQ864587
EC3094	*Solanum phureja*	A1	IIa	EC-1	EF011127	DQ864588
EC3099[a]	*Solanum columbianum*	A1	IIa	EC-1	EF011141	DQ864589
EC3154	*Solanum andreanum*	A1	IIa	EC-1	EF011131	DQ864590
EC3199	*Solanum tuquerense*	A1	IIa	EC-1	EF011128	DQ864591
EC3253	*Solanum columbianum*	A1	IIa	EC-1	EF011129	DQ864592
EC3300	*Solanum paucijugum*	A1	IIa	EC-1	EF011130	DQ864593
EC3330	*Solanum paucijugum*	A1	IIa	EC-1	EF011147	DQ864585
EC3331	*Solanum paucijugum*	A1	IIa	EC-1	EF011143	DQ864594
EC3335	*Solanum tuberosum*	A1	IIa	EC-1	EF011144	DQ864595
EC3336	*Solanum tuberosum*	A1	IIa	EC-1	EF011145	DQ864596
EC3449[a]	*Solanum regularifolium*	A1	IIa	EC-1	EF011142	DQ864597
EC3414[a]	*Anarrhichomenum complex*	A1	Ia	EC-2	EF011148	DQ864576
EC3163	*Anarrhichomenum complex*	A2	Ic	EC-2	EF011132	DQ864567
EC3164	*Anarrhichomenum complex*	A2	Ic	EC-2	EF011133	DQ864568
EC3165	*Anarrhichomenum complex*	A2	Ic	EC-2	EF011134	DQ864569
EC3166	*Anarrhichomenum complex*	A2	Ic	EC-2	EF011135	DQ864570
EC3167	*Anarrhichomenum complex*	A2	Ic	EC-2	EF011136	DQ864571
EC3189	*Anarrhichomenum complex*	A2	Ic	EC-2	EF011149	DQ864572
EC3190	*Anarrhichomenum complex*	A2	Ic	EC-2	EF011150	DQ864573
EC3417[a]	*Anarrhichomenum complex*	A2	Ic	EC-2	EF011151	DQ864574
EC3421[a]	*Solanum muricatum*	A2	Ic	EC-2	AY564160	DQ864575
EC3425[a]	*Anarrhichomenum complex*	A2	Ic	EC-2	EF011152	DQ864584
EC3237	*Solanum betaceum*	A1	Ia	EC-3	EF011154	DQ864577
EC3238	*Solanum betaceum*	A1	Ia	EC-3	EF011155	DQ864578
EC3240	*Solanum betaceum*	A1	Ia	EC-3	EF011156	DQ864579
EC3308	*Solanum betaceum*	A1	Ia	EC-3	EF011157	DQ864580
EC3309	*Solanum betaceum*	A1	Ia	EC-3	EF011158	DQ864581
EC3310	*Solanum betaceum*	A1	Ia	EC-3	EF011159	DQ864582
EC3364[a]	*Solanum betaceum*	A1	Ia	EC-3	EF011146	DQ864598
EC3394[a]	*Solanum betaceum*	A1	Ia	EC-3	EF011153	DQ864583

[a] All isolates were collected in Ecuador and obtained from CIP- Ecuador except isolates designated with [a] that were obtained from W. Flier, formerly of Plant Research International and published in Adler et al (2004) or Olivia et al (2002).

[b] Mitochondrial haplotypes determined by the methods of Griffith and Shaw (1998).

cycling parameters were initial denaturation at 96 C for 2 min, followed by 35 cycles consisting of denaturation at 96 C for 1 min, annealing at 55 C for 1 min, and extension at 72 C for 2 min. A final extension at 72 C for 10 min followed. Electrophoresis of amplified products was conducted on 1.6% agarose gels containing ethidium bromide at 0.5 µg/mL with 1× Tris-Borate EDTA (TBE) running buffer. A 100 bp DNA ladder was included in each gel as a molecular size standard. Gels were viewed under UV light. The 946 bp PCR product was digested with the restriction enzymes *AluI*, *MspI* and *RsaI*. Ten microliters of the PCR product was digested with 1 unit of the restriction enzyme for 4 h at 37 C and then at 65 C for 10 min. Individual digested products were resolved by electrophoresis in 1.6% agarose gels containing ethidium bromide as described above.

Mitochondrial DNA (mtDNA) haplotypes of the isolates of *P. infestans sensu lato* lineages EC-1, EC-2 and EC-3 were determined according to the methods of Griffith and Shaw (1998). PCR products were digested and resolved by electrophoresis in 1.6% agarose gel containing ethidium bromide in TBE buffer.

DNA amplification and sequencing.—Primer pair F4/R4 (Griffith and Shaw 1998) was used to amplify and sequence the P4 region of the mitochondrial genome that contains a portion of the *cox* I gene from the Ecuadorian isolates. The portion of the *ras* gene amplified and sequenced consisted of intron 1 located in the 5′ untranslated region of the gene (Chen and Roxby 1996). The *ras* intron 1 primer pair sequences were IntronRas F 5′ TTGCAGCACAACCCAA GACG 3′ and IntronRas R 5′ TGCACGTACTATTCGG GGTTC 3′. Primers F4/R4 and IntronRas F/R produce PCR fragments of 964 bp and 223 bp respectively. For each primer set two 50 µL reactions were carried out per individual. Each reaction contained 1× PCR buffer (Invitrogen, Carlsbad, California), 2.75 mM $MgCl_2$, 1 unit *Taq* polymerase (Invitrogen), 200 µM each dNTP, 0.4 µM each

TABLE III. Isolates of *Phytophthora* spp. in this study and GenBank accession numbers for *cox1* and *ras* intron 1 sequences

Species name	Group[a]	Isolate number[b]	GenBank accession numbers	
			Cox1[c]	*ras* Intron 1[d]
Phytophthora andina	IV	EC3421[K]	AY564160	DQ864575
Phytophthora botryosa	II	CBS533.92[K], P44[G]	AY564166	DQ864613
Phytophthora cactorum	I	NY568[MT], P6183[K], P7[G], P59[G]	AY129174, AY564167	DQ864605, DQ864606
Phytophthora capsici	II	302[MT], P8[G], P30[G]	AY129166	DQ864618, DQ864619
Phytophthora citricola	III	P1817[K], P33[G], P53[G]	AY564170	DQ864621, DQ864622
Phytophthora citrophthora	II	CBS274.33[K], P96[G], P132[G]	AY564171	DQ864611, DQ864612
Phytophthora colocasiae	IV	P1696[MT]	AY129173	ND
Phytophthora gonapodyides	IV	NY353[MT]	AY129175	ND
Phytophthora hibernalis	IV	P3822[MT]	AY129170	ND
Phytophthora ilicis	IV	P3939[MT], 384[F]	AY129172	DQ864607
Phytophthora infestans	IV	94-1[R], US6[Fr],US7 [Fr],US8 [R], US11 [Fr],US15 [Fr],US17 [Fr]	EF011125	DQ864586
Phytophthora inflata	III	IMI342898[K], P122[G]	AY564187	DQ864623
Phytophthora ipomoeae	IV	Pic99165[K], Pic99139[F]	AY564158	DQ864602
Phytophthora meadii	II	CBS219.88[K], P74[G], P75[G]	AY564192	DQ864614, DQ864615
Phytophthora megakarya	II	IMI337098[K], P42[G]	AY564193	DQ864610
Phytophthora mirabilis	IV	P3007[MT], Pic99129[K], G4-4[F], G9-5[F]	AY129171, AY564153 EF011139, EF011140	DQ864601, DQ864599, DQ864600
Phytophthora nicotianae	II	332[MT], P582[K], P21[G], P70[G]	AY129169, AY564196	DQ864603, DQ864604
Phytophthora palmivora	II	CBS236.30[K], P26[G], P65[G]	AY564197	DQ864608, DQ864609
Phytophthora phaseoli	IV	330[MT], CBS556.88[K], PhyP22E[E]	AY129168, AY564159	EF012279
Phytophthora pseudotsugae	I	H270[MT]	AY129167	ND
Phytophthora syringae	III	IMI045169[K], P35[G]	AY564203	DQ864620
Phytophthora tropicalis	—	PD97/11132[K], P27[G], P118[G]	AY564161	DQ864616, DQ864617
Outgroup species				
Pythium aphanidermatum			AY564163	ND

[a] Waterhouse morphological group (Waterhouse 1963).

[b] Isolate number according to Tom Evans, Univ. DL (E), P. Bonants and W. Flier, PRI, NL (F), W. Fry (Fr), Cornell Univ., M. Gallegly (G) WV Univ., Kroon et al 2004, (K), Martin and Tooley 2003, (MT), or Jean Ristaino (R).

[c] Sequences for the *Cox* 1 region deposited by Kroon et al (2004), Martin and Tooley (2003) or Gómez-Alpizar et al (2007).

[d] The ras intron 1 region was sequenced for isolates provided by M. Gallegly. ND = not determined.

forward and reverse primer, 160 μg/μL BSA (20 mg/mL, Roche, Mannheim, Germany) and 5–10 ng genomic DNA. Cycling conditions for the P4 region were: 94 C (1.5 min), then 40 cycles of 94 C (40 s), 55 C (1 min), 72 C (1.5 min), a final extension of 72 C (10 min); and for the *ras* Intron 1 region, 96 C (1 min); then 35 cycles of 96 C (1 min), 55 C (1 min), 72 C (2 min) and a final extension of 72 C (10 min). PCR products were pooled and purified with QIAquick PCR Purification Kits (QIAGEN, Valencia, California). Purified fragments were sequenced directly on both strands with the same primers as those used in the initial amplification. Sequencing reactions were prepared with the ABI PRISM® BigDye Terminator Cycle Sequencing Ready Reaction Kit and analyzed on an ABI PRISM® 377 automated sequencer (Applied Biosystem, Foster City, California).

Sequence data for the P4 region (including part of *cox* I gene) and *ras* intron 1 were obtained for all the Ecuadorian isolates (TABLE II). Sequence data for *ras* intron 1 also was obtained for a subset of the *Phytophthora* species (TABLE - III). Sequences from the *cox* I gene from various *Phytophthora* species and *Pythium aphanidermatum* (outgroup) were obtained from GenBank (Kroon et al 2004, Martin and Tooley 2003).

Phylogenetic analysis.—All sequences were aligned with Clustal X (Thompson et al 1997) with subsequent visual adjustment and editing in BioEdit (Hall 1999). All

polymorphisms were rechecked from the chromatograms. Sites showing the presence of two coincident peaks in the forward and reverse sequence chromatograms were observed for Intron Ras, indicating heterozygous sites and were labeled according to the IUPAC coding system. Phylogenetic and molecular evolutionary analyses were conducted separately on each gene region with maximum likelihood (ML), maximum parsimony (MP), neighbor joining (NJ) and Bayesian inference (BI). Both maximum parsimony (MP) and neighbor joining (NJ) analyses were conducted with MEGA software version 4 (Tamura et al 2007). A bootstrap test with 1000 replicates was used for both methods (Felsenstein 1985). The close neighbor interchange (CNI) with search level 1 was selected for MP analysis and the Kimura 2-K parameter method was used for the NJ tree (Kimura 1980). The ML analyses used RAxML-VI-HPC (randomized axelerated maximum likelihood for high performance computing) (Stamatakis 2006). Computations were performed on the computer cluster of the CyberInfrastructure for Phylogenetic RESearch project (CIPRES, www.phylo.org) at the San Diego Supercomputing Center. Best scoring trees were determined after the bootstraps and the other parameters were maintained at default settings. Bayesian inference was performed with Mr Bayes version 3.1.2 running on Windows XP (Huelsenbeck and Ronquist 2001). For the Intron-Ras dataset the HKY substitution model was used and 2 000 000 generations run to achieve a convergence of < 0.01. With the Cox1 dataset the general time reversible substitution model was used and 3 000 000 generations run to achieve a convergence of < 0.01. An invariant gamma distribution (default settings) was used for both analyses. Determination of the substitution models was done with a Web implementation of ModelTest 3.7 (http://hcv.lanl.gov/content/hcv-db/findmodel/findmodel.html) (Posada and Crandall 1998).

RESULTS

A 600 bp product was obtained with Ecuadorian isolates of *P. infestans sensu lato*, *P. infestans* and *P. mirabilis* when amplified with PINF/ITS5 primers (data not shown). Restriction digests of ITS + 5.8S rDNA from the EC-1 and EC-2 lineages yielded identical PCR-RFLP patterns with restriction enzymes *RsaI*, *MspI* and *AluI* and were identical to those found among isolates of *P. infestans* and *P. mirabilis*. Restriction digests with the same set of enzymes of amplified ITS and 5.8S rDNA from isolates from the EC-3 lineage (TABLE II) yielded identical patterns. ITS sequences of all isolates were identical (data not shown).

All isolates of EC-1 of *P. infestans sensu lato* from *Solanum* species in Ecuador were identical and clustered with isolates of *P. infestans* for the *cox* I phylogeny using ML analysis with RAxML, indicating that they are true isolates of *P. infestans* (FIG. 1). Isolates of *P. mirabilis*, *P. ipomoeae* and *P. phaseoli* formed distinct branches from *P. infestans* and the EC-1 clonal lineage of *P. infestans* for the *cox* I mitochondrial locus. Isolates of EC-2 (Ic haplotype) from the *Anarrhichomenum* complex formed a distinct group within clade 1c and clustered with the isolate EC3421 named *P. andina* by Kroon et al (2004) (FIG. 1). The NJ, MP and Bayesian trees documented similar relationships to the RaxML tree among the species (data not shown).

The *ras* intron 1 phylogeny also supports the designation of the Ecuadorian isolates from the EC-2 (Ic mtDNA haplotype) as distinct from true isolates of *P. infestans* (FIG. 2). The EC-2 (Ic haplotype) isolates clustered with Ecuadorian isolate EC3421 from *S. muricatum* that Kroon et al (2004) designated *P. andina* (FIG. 2).

The single isolate of EC-2 Ia (EC 3414, Ia mtDNA, A1) clustered with isolates of *P. infestans* in the *cox* I phylogeny (FIG. 1) and *P. andina* in the *ras* intron 1 phylogeny (FIG. 2). In addition one isolate of EC-1 from *S. paucjugum*, clustered with isolates of *P. infestans* in the *cox* I phylogeny but with isolates of *P. andina* in the *ras* intron 1 phylogeny.

The mitochondrial (*cox* I) and nuclear (*ras* intron 1) sequences of all of the EC-2 (Ic haplotype) isolates from Ecuador were identical and clearly different from those of isolates of *P. infestans* from *S. tuberosum* and the other EC-1 isolates of *P. infestans sensu lato* from the tuber-bearing species *S. phureja*, *S. columbianum*, *S. andreanum*, *S. paucijugum*, *S. regularifolium* and *S. tuquerense* from Ecuador. Six heterozygous sites were found in the *ras* intron 1 sequence of EC-2 (Ic haplotype) isolates (TABLE IV). No heterozygous sites were observed for Ecuadorian isolates of *P. infestans sensu lato* from other hosts or from the isolates of *P. infestans* from *S. tuberosum* or *P. mirabilis*. The heterozygous sites found in the *ras* intron 1 sequence of EC-2 (Ic haplotype) isolates could have originated from hybridization between *P. infestans* and *P. mirabilis* (TABLE IV). In a study of the migration history of *P. infestans* six heterozygous sites were observed in the *ras* intron 1 sequence of isolates of *P. infestans* from Brazil, Bolivia, Peru, USA and Ireland and only one of them (site 603 in GenBank accession number U30474) was found in isolates of EC-2 (Ic haplotype) from Ecuador (Gómez-Alpizar et al 2007).

Phylogenetic relationships among the other *Phytophthora* species based on *ras* intron 1 sequences (FIG. 2) reflect the groupings reported by Cooke et al (2000) based on ITS sequences and showed only minor differences with the phylogeny based on the combined analysis of nuclear encoded β-tubulin and elongation factor-1 genes (Kroon et al 2004).

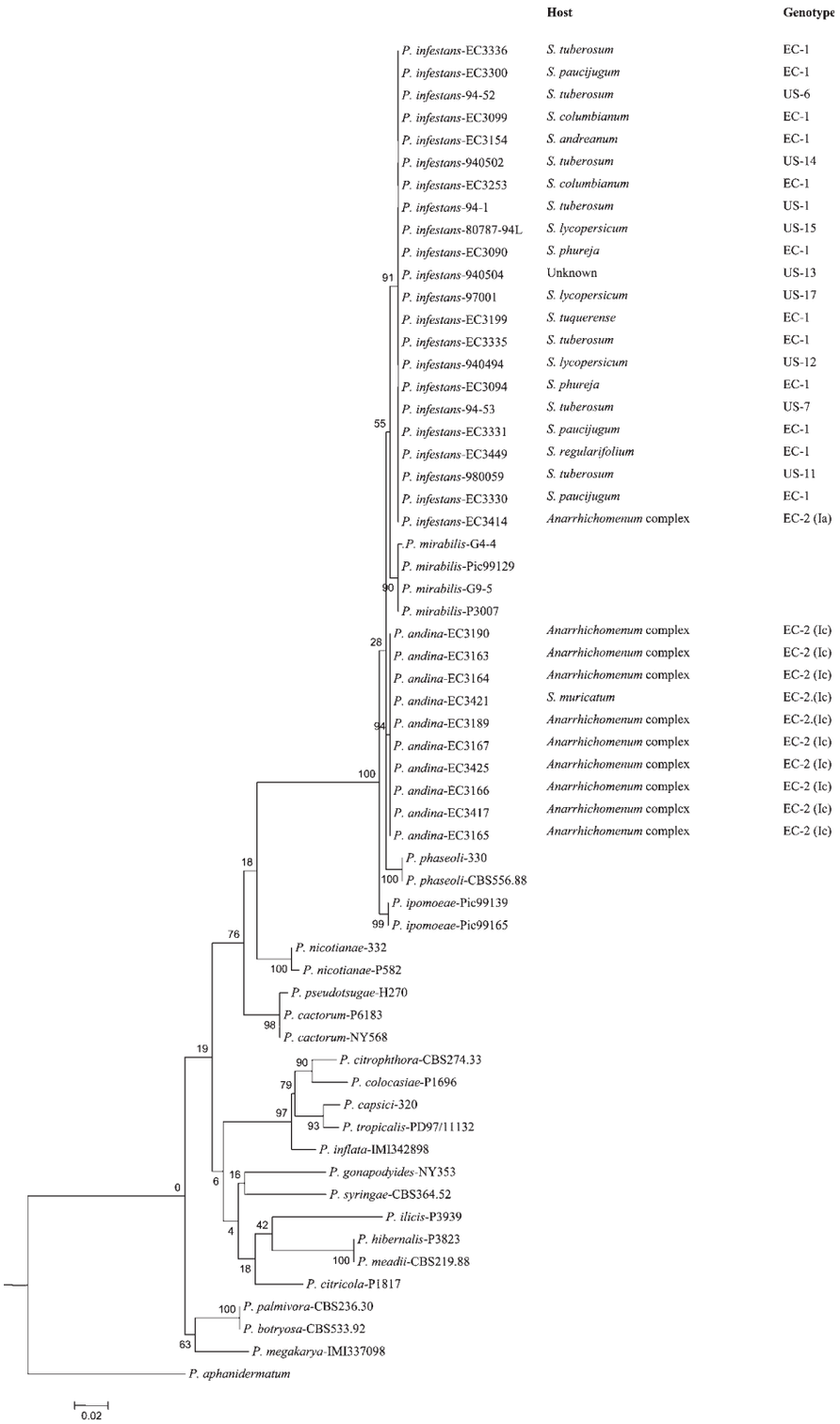

FIG. 1. Maximum likelihood (ML) tree illustrating phylogenetic relationship among *Phytophthora* species, including isolates of lineages EC-2 (Ic haplotype) and EC-2 (Ia haplotype) of *P. infestans sensu lato* from section *Anarrhichomenum* of genus *Solanum*, based on the sequence of the *cox* I gene from the mitochondrial genome. The analysis was done with RAxML-VI-HPC (randomized axelerated maximum likelihood for high performance computing).

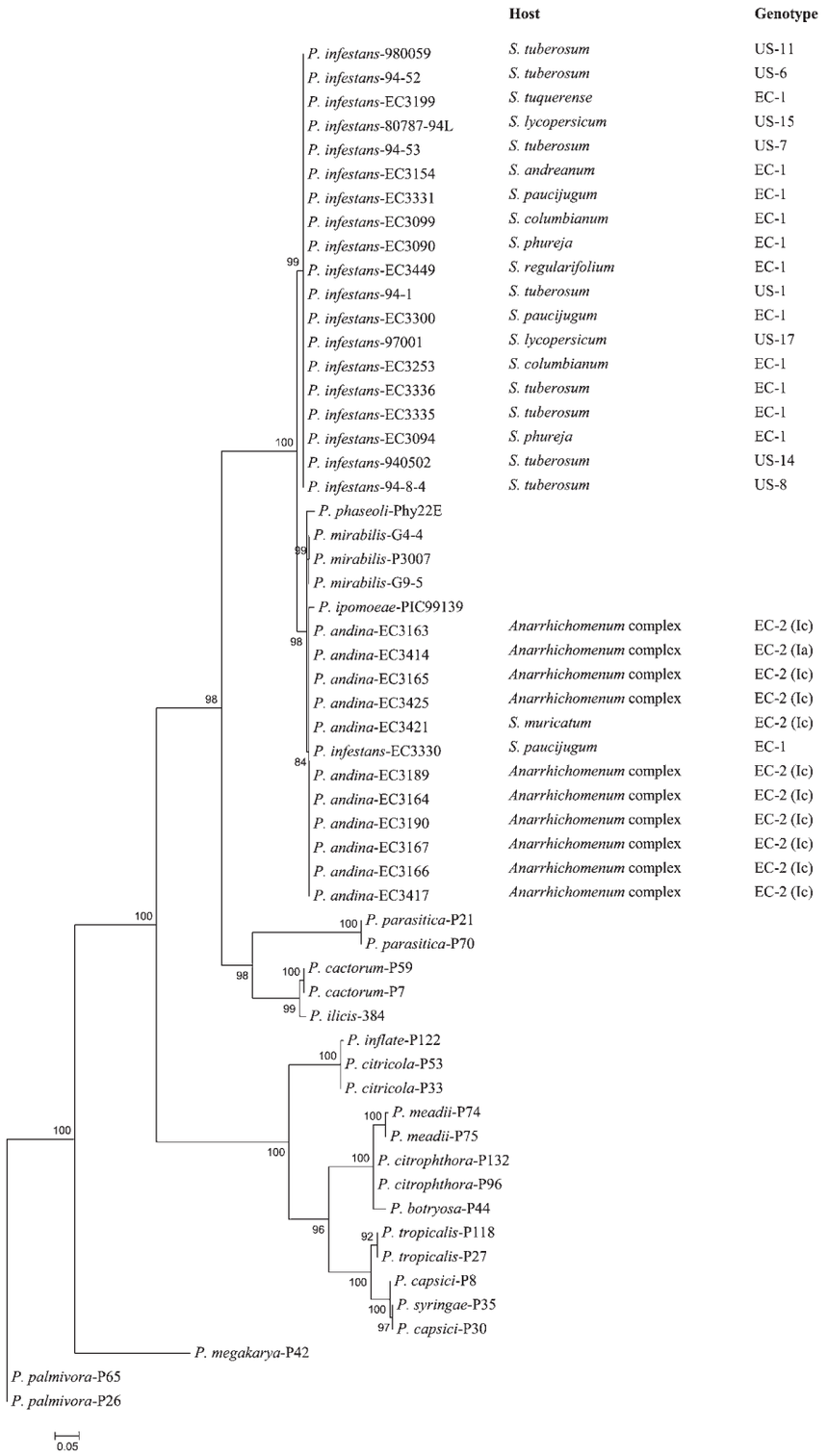

FIG. 2. Maximum likelihood (ML) tree illustrating phylogenetic relationship among *Phytophthora* species, including isolates of lineages EC-2 (Ic haplotype) and EC-2 (Ia haplotype) of *P. infestans sensu lato* from section *Anarrhichomenum* of genus *Solanum*, based on the sequence of the intron 1 of the single-copy *ras* gene from the nuclear genome. The analysis was done with RAxML-VI-HPC (randomized axelerated maximum likelihood for high performance computing).

TABLE IV. Heterozygous (two bases same site) and homozygous (one base same site) sites within the 223 bp of Ras Intron 1 region of isolates of *P. infestans*, *P. infestans sensu lato* from the EC-2 (Ic haplotype) lineage attacking hosts in the *Anarrhichomenum* complex in Ecuador and *P. mirabilis*

Species	Site[a]								
	519	551	589	593	598	603	610	688	706
P. infestans	G	C	C	A	A	A	A	C	G
P. andina (EC-2 Ic haplotype[b])	G/A	T	A/C	A/G	A/C	A/C	G	T	G/A
P. mirabilis	G	T	A	G	C	C	G	C	G

[a] Numbering is that of GenBank accession No. U30474 (Chen and Roxby 1996).

[b] Three extra nucleotides (YYG) between site 680 and 681 were found in all EC-2 Ic (haplotype) isolates and at the same site three nucleotides (TCG) were found in *P. mirabilis*.

DISCUSSION

We found in previous research that isolates of *P. infestans sensu lato* in the EC-2 (Ic haplotype) lineage from *Anarrichomenum* hosts formed a divergent lineage from *P. infestans* and share a common evolutionary ancestor and were distinct from true isolates of *P. infestans* based on both mitochondrial and nuclear DNA sequences (Gómez-Alpizar et al 2007). The lineage formed by the EC-2 (Ic haplotype) isolates fit the morphological description of *P. infestans*, raising questions about their taxonomic status (Adler et al 2004, Gómez-Alpizar et al 2007).

Restriction digest of the internal transcribed spacer (ITS + 5.8S rDNA) region of the ribosomal DNA (rDNA) did not separate isolates of *P. infestans sensu lato* from Ecuador from *P. infestans* or *P. mirabilis*, indicating high ITS-sequence similarity. ITS-sequence similarity among *P. infestans* and closely related species *P. mirabilis*, *P. phaseoli*, *P. ipomoeae* and *P. andina* also has been reported to be 99.9% (Kroon et al 2004).

PCR amplification with the PINF/ITS5 primer combination also did not differentiate any of the Ecuadorian *P. infestans sensu lato* isolates from *Solanum* species from potato isolates of *P. infestans* or *P. mirabilis*. The PINF primer is considered a *P. infestans*-specific primer on potato (Trout et al 1997). In addition to *P. infestans* only isolates of *P. mirabilis* and *P. cactorum* were amplified by the PINF/ITS5 primer combination (Trout et al 1997). *Phytophthora mirabilis* was described as a new species in 1985 by Galindo and Hohl 1985. Isozyme and RFLP analysis demonstrated that *P. mirabilis* and *P. infestans* were unique and distinguishable species (Goodwin et al 1999, Martin and Tooley 2003).

Mitochondrial and nuclear phylogenies (FIGS. 1 and 2) support the designation of Ecuadorian EC-2 (Ic haplotype) isolates as a new species called *P. andina*. *P. andina* is closely related to *P. infestans* and is a member of clade 1c (Cooke et al 2000). In contrast isolates of *P. infestans sensu lato* from *S. andreanum*, *S. columbianum*, *S. phureja*, *S. regularifolium*, *S. tuberosum* and *S. tuquerense* from Ecuador of the EC-1 clonal lineage (IIa haplotype) (TABLE I) were in the same clade and most closely related to isolates of *P. infestans* (US genotypes) from *S. tuberosum*, indicating that they are true isolates of *P. infestans*. Mitochondrial genome sequences of isolates of *P. andina* were highly polymorphic, and gene genealogies indicated that they shared a common ancestor with *P. infestans* in the Andes (Gómez-Alpizar et al 2007).

EC-2 (Ic haplotype) isolates clustered with the EC3421 isolate from *S. muricatum* that Kroon et al (2004) designated as *P. andina*, with a bootstrap support values of 94% in the *cox* I phylogeny (FIG. 1). It therefore is likely that the EC-2 (Ic haplotype) isolates we analyzed are identical to or closely related to the isolate analyzed by Kroon et al (2004) and therefore all can be referred to as *P. andina*. Wattier et al (2003) sequenced an intergenic region found in the P4 region (*cox* I gene) from Ecuadorian isolates that also came from the *Anarrhichomenum* complex of hosts. Their sequence data also matched ours (100%) for that region. Therefore EC-2 (Ic haplotype) lineage appears to be primarily a pathogen of several species in section *Anarrhichomenum* of genus *Solanum* and occasionally of *S. muricatum*.

The taxonomic resolution of the other lineage from the EC-2 lineage, EC-2 (Ia mtDNA, A1; and *Gpi* [100/100] and *Pep* [76/100]), which also has been associated with the *Anarrhichomenum* complex (Adler et al 2004), is less clear and is under investigation. In our study the single EC2 isolate with the Ia haplotype clustered with isolates of *P. infestans* in the *cox* I phylogeny but *P. andina* in the *ras* intron 1 phylogeny. However, until more isolates from this lineage are evaluated phylogenetically, we will refer to them as *P. infestans sensu lato*. The EC-2 lineage is heterogeneous, and data from single sequence repeats suggest that *P. andina* and the EC-2 (Ia haplotype) lineage are closely related (Olivia et al

unpubl). EC-2 (Ia haplotype) also shares common allozyme alleles at both the *Pep* and *Gpi* loci with the EC-2 (Ic haplotype) lineage (TABLE I).

Isolates of *P. infestans sensu lato* from the EC-3 lineage from *S. betaceum* were not clearly resolved. Mitochondrial *cox* I phylogenies show a close relationship between these isolates and *P. infestans*, but the nuclear gene phylogeny supported a closer relationship between these isolates and *P. andina* (data not shown). Further collections from *S. betaceum* and phylogenetic studies of the pathogen also are under investigation to resolve the taxonomic delineation of EC-3 isolates.

Flier et al (2002) hypothesized that the central highlands of Mexico, which include the Toluca Valley, were the center of origin of the Waterhouse group IV *Phytophthora* species (analogous to clade 1c, Cooke et al 2000) and a speciation hot spot. The highlands have been presumed to be the center of origin of *P. infestans*, *P. mirabilis*, *P. phaseoli* and the newly described species *P. ipomoeae* (Brasier and Hansen 1992, Flier et al 2003, Goodwin et al 1992, Niederhauser 1991). These species show host specificity in the highlands with *P. infestans* associated with Solanaceous hosts (potato and wild *Solanum*) and *P. mirabilis*, *P. phaseoli* and *P. ipomoeae* associated with *Mirabilis jalapa* (four o'clocks), *Phaseolus* and *Ipomoea longipedunculata* respectively. It was suggested that these species might have undergone sympatric speciation in the Toluca Valley (Flier et al 2003, Goodwin et al 1999).

The EC-2 (Ic haplotype) lineage of *P. andina* appears to be indigenous to the Andean highlands (Adler et al 2004). This raises questions about the center of origin and the historical migration route throughout South and Central America of all species in clade 1c of *Phytophthora*. The Andean region is either a center of origin or center of diversity for all hosts in clade 1c (Gepts 1998, Rajapakse et al 2004). Common host and plant origins would be consistent with an Andean origin of clade 1c; however *P. mirabilus* and *P. ipomoeae* never have been reported from South America, even though the wild hosts exist. It should be noted however that we are unaware of any serious attempts to search for *P. mirabilus* or *P. ipomoeae* in South America and that *P. ipomoeae* was described only recently from Mexico (Flier et al 2002). Efforts to confirm the presence of these *Phytophthora* species in South America or of *P. andina* on *Anarrhichomenum* species that occur in Mexico (Correll 1962) could help clarify the evolutionary history of clade 1c.

One of the arguments for central highlands of Mexico as the center of origin of *P. infestans* is the high degree of diversity of the pathogen found in that region relative to other locations (Flier et al 2002). However a study on the *P. infestans* population attacking *S. ochranthum* in Ecuador revealed undetected levels of diversity, including an unreported RFLP fingerprint that shared some bands with isolates for the EC-2 (Ic haplotype) lineage (Chacón et al 2006). Another reason for considering the central highlands of Mexico as the center of origin of the pathogen is that the population is sexual, with both A1 and A2 mating types found in similar frequencies (Grünwald et al 2005). To date only the A1 mating type has been found for *P. infestans* in the Andes. Data from a recent work suggest that the Andean highlands are the center of origin of *P. infestans* (Gómez-Alpizar et al 2007) and support the Andean theory, first proposed by Berkeley (1846). Flier et al (2003) found that the mitochondrial haplotype associated with *P. ipomoeae* was the Ic mtDNA described by Ordoñez et al (2000) for the isolates of *P. infestans sensu lato*. This same mitochondrial haplotype was found in isolates of EC-2 (Ic haplotype) on the *Anarrhichomenum* group hosts in Ecuador. This suggests a common mitochondrial origin of all the *Phytophthora* species from these hosts.

Kroon et al (2004) postulated a hybrid origin of *P. andina* (EC-2 Ic haplotype) because the presence of several heterozygous sites in the sequences of nuclear genes and suggested that *P. infestans* was one of the parental species. Our data agree with this suggestion. The origin of new *Phytophthora* species through interspecific hybridization has been documented for other species (Brasier et al 1999). The *ras* intron 1 sequence (223 bp) of the EC-2 (Ic haplotype) isolates (*P. andina*) had six heterozygous sites (TABLE IV). Other Ecuadorian isolates of *P. infestans* from different hosts were homozygous for all sites, suggesting *P. infestans* as a putative parent. Of note, *P. mirabilis* isolates were also homozygous for all sites but polymorphic in the same sites compared to *P. infestans*. Hybridization between *P. infestans* and *P. mirabilis* might have resulted in the formation of the heterozygous sites found in the isolates of *P. andina* (TABLE IV). Goodwin and Fry (1994) demonstrated that interspecific hybridizations between *P. infestans* and *P. mirabilis* are possible, although F1 progeny were unable to infect any of the hosts infected by either parent. However they did not consider other non-tuber-bearing *Solanum* hosts such as (*Solanum muricatum*) as possible species for post-mating host specialization.

Phylogenetic relationships among *Phytophthora* species are based mainly on sequence data of the rDNA internal transcribed spacers (ITS) (Cooke et al 2000); however some *Phytophthora* species cannot be

resolved based on ITS data, particularly closely related ones. For example *P. infestans*, *P. mirabilis* and *P. phaseoli* are poorly resolved with ITS data. Mitochondrial genes (*cox* II and *cox* I) (Martin and Tooley 2003) and multigene based phylogenies (Kroon et al 2004) have been published, and these and other groups were clearly differentiated. Both the phylogenies presented here were largely congruent with the ITS phylogeny described by Cooke et al (2000). We used sequence data from the intron 1 (223 bases) found in the *ras* gene, a single-copy gene (Chen and Roxby 1996). Although we sequenced only a limited subset of other *Phytophthora* species from our collection, the groupings recovered are congruent with those found with other nuclear and mitochondrial sequences (Cooke et al 2000, Kroon et al 2004, Martin and Tooley 2003). Our data indicate that the *ras* intron 1 region might have sufficient variation to be useful as another tool for reconstructing the evolutionary history in genus *Phytophthora* and deserves further testing on more *Phytophthora* species. Exons and introns in genes are subjected to different evolutionary selection pressures. Mutations in introns are less likely to affect gene function than mutations in exons. Therefore introns accumulate more mutations, while the mutation rates of the coding exon sequences are constrained (Rajapakse et al 2004). Most *Phytophthora* genes lack introns; however the *ras* gene (Chen and Roxby 1996) and *niaA* gene, a gene encoding nitrate reductase (Pieterse et al 1995), are notable exceptions (Ospina-Giraldo and Jones 2003).

Species recognition traditionally is based on morphology (morphological species recognition or MSR), mating or reproductive behavior (biological species recognition or BSR) and DNA sequence divergence (phylogenetic species recognition or PSR) (Taylor et al 2000). Every approach has its limitations and advantages; therefore congruence among them gives strong support to the creation of a new species. Ecuadorian isolates of the EC-2 lineage (Ic haplotype) fit the morphological description of *P. infestans*. However Oliva et al (2002) and Adler et al (2004) demonstrated the existence of pre- and postmating isolating mechanisms among EC-2 isolates and other *P. infestans* isolates. Phylogenetic analyses using multiple methods in this study and in others (Kroon et al 2004), based on different nuclear and mitochondrial genes, show that the isolates of *P. andina* form an independent lineage closely related to *P. infestans* and other species of clade 1c (Cooke et al 2002). Therefore both biological and phylogenetic species recognition strongly support this group as a new species, *Phytophthora andina*, a name given by Kroon et al (2004) for a related isolate from *S. muricatum* from Ecuador.

Isolates of EC-2 have been collected rarely from hosts that are also attacked by *P. infestans*. One exception is EC-2 (Ic haplotype) lineage of *P. andina*, which is an A2 mating type and has been isolated several times from *S. muricatum* (Adler et al 2002), a host that is attacked generally by the US-1 lineage of *P. infestans*, which is an A1 mating type. Thus pear melon could be a bridge species and a potential host for novel species evolution in the Andes. Postmating mechanisms of reproductive isolation have been documented in subpopulations of *P. infestans sensu lato* in Ecuador (Oliva et al 2002). Host specialization might be the driving force for the evolution of several other *Phytophthora* species as well (Cooke et al 2000).

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