Curr Genet
DOI 10.1007/s00294-016-0572-8

ORIGINAL ARTICLE

Mitochondrial DNA assessment of *Phytophthora infestans* isolates from potato and tomato in Ethiopia reveals unexpected diversity

Daniel Shimelash[1] · Temam Hussien[1] · Chemeda Fininsa[1] · Greg Forbes[2] · Jonathan Yuen[3]

Received: 20 April 2015 / Revised: 28 January 2016 / Accepted: 29 January 2016

Abstract Mitochondrial DNA (mtDNA) haplotypes were determined using restriction fragment length polymorphism (RFLP) for *P. infestans* sampled from 513 foliar lesions of late blight found on potato and tomato in different regions of Ethiopia. Among the four reported mitochondrial haplotypes of *Phytophthora infestans*, Ia, Ib and IIb were detected in 93 % of the samples analyzed but the vast majority of these were Ia. The remaining 7 % represented a previously unreported haplotype. DNA sequencing of this new haplotype also confirmed a single base nucleotide substitution that resulted in loss of *EcoRI* restriction site and gain of two additional *MspI* sites in cox1 and atp1 genes, respectively. There were 28 polymorphic sites among all nucleotide sequences including five reference isolates. Sites with alignment gaps were observed in P4 with one nucleotide deletion in 11 Ethiopian isolates. None of the reference sequence produced frame-shifts, with the exception of the 3-nucleotide deletion in the P4 region by *Phytophthora andina*, a feature that can be used to distinguish the new Ethiopian isolates from *P. andina*. While a distinguishing molecular data presented here clearly separated them from *P. infestans*, 7 % of the isolates that share this feature formed an important component of the late blight pathogen causing disease on *Solanum tuberosum* in Ethiopia. Thus, these Ethiopian isolates could represent a novel *Phytophthora* species reported for the first time here.

Keywords *Phytophthora infestans* · Mitochondrial DNA · Diversity · Haplotype

Communicated by M. Kupiec.

✉ Daniel Shimelash
dshamm@gmail.com

[1] School of Plant Sciences, Haramaya University, P.O.Box 138, Dire Dawa, Ethiopia

[2] CIP-China Center for Asia Pacific, Pan Pacific Plaza A12 Zhongguancun Nandajie, Beijing 100081, China

[3] Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences, Box 7026, 750 07 Uppsala, Sweden

Introduction

The diversity of the late blight pathogen linked to *Phythopthora infestans* has not been well studied in Ethiopia. The population structure of *P. infestans* in Ethiopia had been described in only one study where all isolates were described as A1 mating type and Ia mitochondrial DNA (mtDNA) haplotype (Schiessendoppler and Molnar 2002). This study established that while a distinguishing trait of the old US-1 lineage is its Ib mtDNA haplotype, the new lineage formed an important component of the population structure of *P. infestans* detected in Ethiopia. Such displacement of the US-1 lineage by newer lineages is generally associated with increased difficulty of farmers in the management of the disease (Fry et al. 2009). This is consistent with reports of the severity of late blight in Ethiopia (Mesfin et al. 2009). Late blight represents the most important hazard to potato and tomato in Ethiopia.

The mitochondrial genome has been used to study the evolutionary histories of many types of organisms (Chesnick et al. 2000; Galtier et al. 2009; Lang and Forget 1993; Lassiter et al. 2015). This is due to the rapid mutation rate, uniparential inheritance with rare or no recombination, and uniform genetic background due to homoplasmy of the mitochondrial genome (Chen and Hebert 1999; Chesnick et al. 2000). Polymorphisms at various regions of the mitochondrial genome of *P. infestans* have

Published online: 12 February 2016

been successfully employed to study origin, migration and population diversity of this pathogen (Avila-Adame et al. 2006). These polymorphisms have been extensively studied by restriction fragment length polymorphism (RFLP) analysis. Mitochondrial RFLP analysis using a PCR approach and employing different restriction enzyme combinations unambiguously distinguish four haplotypes of *P. infestans,* named Ia, Ib, IIa and IIb (Carter et al. 1990; Griffith and Shaw 1998). A fifth mtDNA haplotype (Ic) was reported in the closely related species, *P. andina* (Ordoñez et al. 2000). Some analyses of the mtDNA genome have questioned the I/II distinction due to convergent evolution at *EcoRI* restriction sites (Martin et al. 2012), and an alternative method for characterizing haplotypes has been proposed using length polymorphisms of PCR amplicons from HVRi and HVRii (Yang et al. 2013). However, neither of these will allow comparison with historical data generated using earlier methods. In addition, comparison of the entire mitochondrial genome of the four haplotypes of *P. infestans* (Avila-Adame et al. 2006) corroborated the difference in size determined previously by restriction enzyme digestion (Carter et al. 1990).

DNA sequencing of genes in the mitochondrial genome has proved to be a very useful tool for comparison of isolates both within and between populations on local and intercontinental scales. There are many examples of this type of study in which researchers are using gene sequence of Avr3a, cox1, Ras, β tubulin for mtDNA haplotype identification and diversity analysis (Blair et al. 2008; Cooke and Lees 2004; Martin 2008). Recently, many researchers have been using simple sequence repeat (SSR) markers because they are highly specific, single locus, codominant, polymorphic, reproducible and a smaller amount of pathogen DNA is needed (Lees et al. 2006). Techniques like multilocus fingerprinting with probe RG57 (Goodwin et al. 1994) and amplified fragment length polymorphisms (AFLPs) (Cooke et al. 2003; Flier et al. 2003) were involved for identification of several organisms including *Phytophthora* species. AFLP finger-printing, for example, discriminates isolates considered identical based on RG57 fingerprint and two SSR markers (Cooke and Lees 2004).

The present study was undertaken to expand the current knowledge of the late blight pathogen population in Ethiopia using mtDNA analysis. According to Berga et al. (1992), potato has been cultivated since its first introduction to Ethiopia in 1858. This suggests that there have been opportunities for migration of *P. infestans* earlier than first reported in 1930s (Laufer 1938). Restriction enzyme digestion of PCR products for RFLP analysis facilitates monitoring of genetic changes among field populations of *P. infestans*. They remain a simple, robust system whereby mtDNA haplotypes could be characterized according to a unified system which was easily comparable between laboratories (Griffith and Shaw 1998). Additionally, DNA sequences in the two regions of the mitochondrial genome also help to find additional polymorphisms making them potentially useful for clarifying relationships among various haplotypes. The objectives of this study were (1) to investigate the mtDNA haplotypes of a collection of Ethiopian isolates of *P. infestans* from both potato and tomato, and (2) to sequence and annotate the mitochondrial genomes of the P2 and P4 regions of some selected isolates and compare them with other populations previously described in different regions worldwide through analysis of mitochondrial diversity.

Materials and methods

Description of the study areas

Late blight surveys on potato and tomato were conducted in the major growing areas in Ethiopia (Fig. 1). In all of these areas, potatoes and tomatoes are planted in early to mid June for the main rainy seasons in 2011 and 2012. Surveys on potato fields included four areas: the central, the eastern, the north-western and the southern areas (Table 1). The central area includes the highlands surrounding the capital, Addis Ababa, within a 100–150 km radius (Fig. 1). About 10 % of Ethiopian potato farmers are located in this area (Hirpa et al. 2010). In central area, a survey was conducted in 16 potato fields of West Shewa and North Shewa. The north-western potato production area, which is situated in the Amhara region, accounted for about 40 % of the country's potato farmers (Hirpa et al. 2010). The survey in

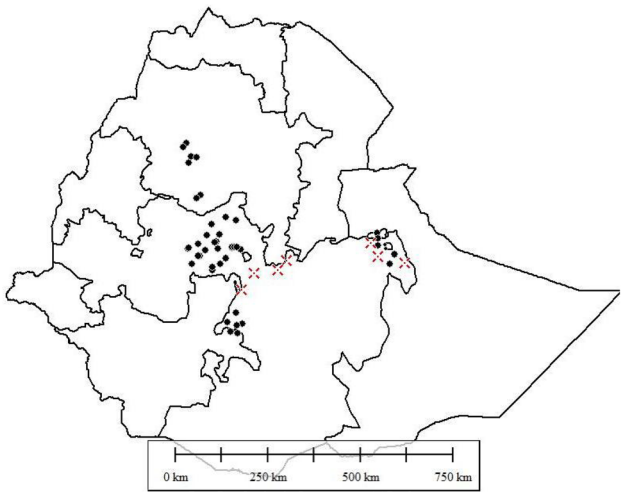

Fig. 1 Late blight survey sites in major potato and tomato growing areas in Ethiopia. Potato fields are indicated by *dot* and tomato by *cross* on the map. Each *dot/cross* represents a field where 15 leaf samples were collected

Table 1 Identity of mitochondrial haplotypes of isolates of *Phytophthora infestans* from late blight infected leaves of potato and tomato collected from different locations in Ethiopia

Year	Region[a]	Zone[b]	Host	Mitochondrial haplotype						
				NF[c]	NL[d]	ia	ib	iia	iib	NA[e]
2011	Central	West Shewa	Potato	9	93	93	0	0	0	0
	Central	North Shewa	Potato	1	9	9	0	0	0	0
	East	East Harerge	Potato	2	18	18	0	0	0	0
	East	East Harerge	Tomato	1	9	6	3	0	0	0
	North Western	Adet	Potato	2	18	18	0	0	0	0
	North Western	East Gojam	Potato	2	24	24	0	0	0	0
	North Western	West Gojam	Potato	1	15	15	0	0	0	0
	Rift valley	East Shewa	Tomato	4	36	36	0	0	0	0
	South	West Arsi	Potato	3	33	33	0	0	0	0
	Total			25	255	252	3	0	0	0
	%					98.8	1.2	0.0	0.0	0.0
2012	Central	West Shewa	Potato	5	45	45	0	0	0	0
	Central	North Shewa	Potato	1	9	9	0	0	0	0
	East	East Harerge	Potato	8	78	78	0	0	0	0
	East	East Harerge	Tomato	1	9	6	2	0	1	0
	North Western	Adet	Potato	1	9	9	0	0	0	0
	North Western	West Gojam	Potato	2	18	18	0	0	0	0
	North Western	East Gojam	Potato	1	15	15	0	0	0	0
	South	West Arsi	Potato	5	75	41	0	0	0	34
	Total			24	258	221	2	0	1	34
	%					85.7	0.8	0	0.4	13.2
Total					513	473	5	0	1	34
%						92.2	1.0	0.0	0.2	6.6

[a] Production regions

[b] Third level administration regions

[c] Number of fields

[d] Number of single lesion leaves

[e] After digestion, the isolates produced a novel band pattern

this area involved East Gojam, West Gojam and Awi. The eastern potato production area mainly covers the eastern highlands of Ethiopia, especially the East Harerge zone. For this part of survey, 10 potato fields were visited. The southern area is mainly located in the Southern Nations', Nationalities', and Peoples' Regional State (SNNPRs) and partly in the Oromiya region. More than 30 % of Ethiopian potato farmers are located in this area (Hirpa et al. 2010). In this area, the survey was conducted in eight potato fields located in the West Arsi zone in Oromiya.

Surveys on tomato fields were carried out in the central rift valley and in eastern Ethiopia (Fig. 1). In the central rift valley area, the survey involved 4 different locations in East Shewa, in the Upper Awash River basin. This area is geographically isolated from potato growing areas found at higher altitudes in the central rift valley system. In all sites, production was for commercial purposes. In Eastern Ethiopia, two tomato fields that are situated in Eastern Harerge zone were sampled.

Sample collection

Late blight infected leaf samples were taken in the fields during occurrence or epidemic of late blight. In all areas, where samples were collected (Fig. 1), fields were at least 20 km apart from each other. The method used in this study followed the standard eucablight protocol for sampling late blight for a disease survey (Cooke and Lees 2006). This protocol describes the collection of 15 lesions per field, consisting of 3 individual lesions from five different plants. In this study, sampling of *P. infestans* was carried out through identification of an individual blight infected plant and collection of three leaves with typical symptom that was repeated for up to three to five plants in an area of infection. During sampling, only leaves with a single sporulating lesion and remaining fresh green tissue were selected. Each leaf sample was put separately in a plastic bag in the field, labelled with place, date of collection, host plant and geographic coordinates.

DNA extraction

Late-blight infected leaves collected from different fields were used to extract DNA. Pieces of the dried leaflets (each with only a single lesion) were cut out and placed in sterile 1.5 ml tubes with glass beads and then homogenized with a Precellys preparation shaker (Bertin Technologies). DNA was extracted according to CETAB (cetyltrimethyl-ammonium bromide) based protocol (Gardes and Bruns 1993). The purified DNA was dissolved in TE (Tris/EDTA) buffer (40 ml). The DNA concentration was measured using a spectrophotometer (ND-1000; NanoDrop) and the DNA samples were diluted to 20 ng μL^{-1} for further analysis.

DNA amplification and restriction of PCR products

Mitochondrial haplotypes were determined using the PCR–RFLP method devised by Griffith and Shaw (1998). Amplification of DNA of each isolate was carried out using primers designed for two specific regions of the mitochondrial genome of *P. infestans* as described by (Griffith and Shaw 1998). The primer pair F2/R2 (F5′ TCCCTTTGTCCTCTACCGAT3′) and (R5′ GCTTATGCTTCAGTTGCTCAT3′) was used to amplify a region of 1070 bp of the mitochondrial genome referred as P2. A 964 bp region of mtDNA (referred to as P4) was amplified using the primer pair F4/R4 (F5′TGGTCATCCAGAGGTTTATGTT3′) and (R5′ CCGATACCGATACCAGCACCAA3′). P2 is the site of the Ia/Ib polymorphism, while P4 is site of polymorphism between haplotype Ia/Ib and haplotype IIa/IIb.

DNA was amplified by thermal cycler in a 25 µL final reactions volume. For each primer set the reactions volume consisted in: 1X Thermo buffer (50 mM KCl, 10 mM Tris–HCl), 1.5 mM $MgCl_2$, 100 µM dNTPs, 0.325 µM each primer, 1.5 units Taq DNA polymerase, and 2 ng DNA. Amplification was conducted with one cycle of 94 °C for 90 s followed by 35 cycles of 92 °C for 30 s, 55 °C for 30 s, and 72 °C for 1.1 min (Griffith and Shaw 1998). All PCR products were evaluated for successful amplification using gel electrophoresis on 1.2 % agarose gels containing 0.5 µg ml^{-1} ethidium bromide in 0.5 X TBE (0.045 M Tris borate, 0.001 M EDTA) running buffer. Products P2 and P4 were digested with *MspI* and *EcoRI*, respectively. Digestion was carried out by thermal cycler in a 30 µL volume restriction digest at 37 °C for 1 h. The digested products (29 µL) were loaded into a slot on a 2 % agarose gel in 1X TBE electrophoresis buffer (containing 0.1 µg of ethidium bromide ml^{-1}). The gel was run at 100 V cm^{-1} for 2.5 h. Restriction patterns were visualized with a UV transilluminator at 254 nm and the images was recorded by a gel documentation system (BIO-RAD, Gel Doc 1000). Carter haplotypes were identified by their restriction patterns as described by Griffith and Shaw (1998).

Mitochondrial DNA sequencing

The portions of mtDNA (P2 and P4) were amplified and sequenced for a subset of 17 *P. infestans* isolates collected in Ethiopia. Eleven of these were chosen since they did not fall into any of the four mtDNA groups used to characterize *P. infestans* haplotypes. The remaining isolates were Ia haplotype and used as standard. Amplification of DNA of each isolate was carried out using the same primers designed for haplotyping *P. infestans* isolates as described earlier (Griffith and Shaw 1998). DNA was amplified by thermal cycler using each primer set (F2/R2; F4/R4) as previously described except that reactions were carried out in a final volume of 50 µL. Amplification was confirmed by gel electrophoresis on 1.2 % agarose gels loaded with 5 µL DNA products as described earlier. The remaining 45 µL PCR products were purified with the Agencourt Ampure PCR purification kit (Agencourt Bioscience Corporation, USA) according to the description provided by the company. Purified PCR products were sent and sequenced in the forward and reverse direction using a Macrogen3730XL system (Macrogen Sequencing System, Korea). PCR products were cycle-sequenced using ABI Prism™ BigDye™ Terminator chemistry with AmpliTaq® DNA Polymerase, and sequenced using a Perkin-Elmer, Applied Biosystems Division, 373 A DNA Sequencer.

Data analysis

The ABI trace files of the forward and reverse sequences data were observed and trimmed for low quality using Sequence Scanner Software 2 (http://www.apliedbiosystems.com). The post trim sequence fragments in both orientations were assembled into contigs using A Contig Assembly Program as implemented in BioEdit version 7.1.9 (Hall 1999). If any regions of sequence ambiguity were observed, the original output files of complementary strands from the ABI 3730 sequencer were compared for correction. The contigs sequence of all isolates were aligned with Clustal-W (Thompson et al. 1997) and manually re-edited for more precise alignment using a software package Mega 5 (Tamura et al. 2011). The blast algorithm (BLAST-NCBI http://www.ncbi.nlm.nih.gov/BLAST/) (Zhang et al. 2000) was performed to compare the P2 and P4 nucleotide sequences generated with those available in GenBank and identify the isolates. Additionally four reference sequences of *P. infestans* isolates and one *P. andina* sequence (since the observed mtDNA pattern was consistent with the Ic reported for this species) were selected from GenBank (www.ncbi.nlm.nih.gov). Accession numbers for the mitochondrial genome sequence data are U17009 (Paquin et al. 1997), AY894835, AY898627 and AY898628 (Avila-Adame et al. 2006) for the Ib, Ia, IIa and IIb

genomes, respectively and HM590419 for *P. andina* (Lassiter et al. 2010).

Annotation of coding regions and prediction of open reading frames (ORFs) was done with BioEdit (Hall 1999) using the universal genetic code. The process of identifying the locations of genes in the coding regions of the mitochondrial genome was facilitated using the previously published sequence of the mitochondrial genome of the Ib mtDNA haplotype of *P. infestans* isolate NC002387 (Lang and Forget 1993; Paquin et al. 1997) available in GenBank (accession number U17009). The annotation data were edited and validated in Sequin, a stand-alone software tool developed by the National Centre for Biotechnology Information (NCBI) for submitting sequences to the GenBank (http://www.ncbi.nlm.nih.gov/Sequin). Accession numbers for sequence data that have been submitted to the GenBank are KM078885 to KM078901 (P2) and KM078902 to KM078918 (P4).

DNA sequences of PCR products (P2 and P4) from 17 Ethiopian isolates were compared with the full-length of the reference sequences generated from GenBank (www.ncbi.nlm.nih.gov). Nucleotide substitutions resulting in gain or loss of restriction sites were ascertained using all nucleotide positions associated with restriction enzyme recognition by the endonuclease enzymes *MspI* and *EcoRI*. The number of polymorphic sites on the DNA sequence data file was analyzed using a software DnaSP v5 (Librado and Rozas 2009).

Results

PCR-RFLP

DNA was extracted from a total of 513 late-blight lesions. Amplification of DNA of each isolate produced a band that corresponded to the two specific regions of the mitochondrial genome of *P. infestans* referred as P2 and P4. After digestion, differences were detected among isolates for the two primer set digested products (Table 1). Among the four possible mitochondrial haplotypes of *P. infestans* that can be detected with this method, Ia, Ib and IIb were detected in 93 % isolates analyzed (Table 1) but the vast majority of these were Ia. The Ib and IIb haplotypes were only observed on tomato samples from East Harerge.

For about 7 % isolates, digestion of product P2 with *MspI* produced a novel band pattern that was not consistent with published results for known haplotypes of *P. infestans*. Although, the largest fragment appears to correspond with one of the sizes of band patterns (720 bp) for haplotypes Ia, IIa and IIb, the remaining two smaller fragments (125 and 100 bp) distinguished the Ethiopian isolates from haplotypes Ia, IIa, and IIb of *P. infestans*. Similarly, amplification with primer set P4 and digestion with *EcoRI* produced a novel two band pattern for these 7 % isolates. One of these bands corresponded with the 209 bp band characteristics of haplotypes Ia and Ib, but the other of approximately 750 bp was not consistent with published results for an *EcoRI* restriction site that characterize all haplotypes of *P. infestans*.

Mitochondrial DNA sequencing and restriction sequence identity

The mtDNA data set comprised 17 contigs sequences assembled using each primer set sequence data in forward and reverse directions for all Ethiopian isolates. In P4 region the contigs length for the sequence alignment in both directions (F4/R4) was a 908 bp. The sequences data in this region predicted to encode two gene products belonged to a portion of the cytochrome c oxidase subunit I (cox1) and ATP synthase F0 subunit 9 (atp9) genes. Additionally, an intronic spacer was identified between the two portions of gens. For P2 region, a 1021 bp contigs sequence was assembled using the complementary strands in both directions (F2/R2). The identified genes encoded by this region include, partial product of the ATP synthase F1 subunit alpha (atp1), the glutamic acid tRNA (trnE) and part of the NADH dehydrogenase subunit 4 (nad4). Sites with alignment gaps were observed with one nucleotide deletion in 11 Ethiopian isolates on the intronic spacer in P4. None of the reference sequence produced frame-shifts, with the exception of the 3-nucleotide deletion in the same P4 region by *P. andina*. Sites with alignment gaps were not observed in P2.

Polymorphism detected in the P4 region

Polymorphisms among isolates were observed in both coding and non-coding regions in P4 (Table 2). Six polymorphic sites were detected in cox1 where nucleotide changes result in synonymous substitutions for 11 out of 17 Ethiopian isolates. Further, a single substitution from G to T detected in cox1 resulted in loss of *EcoRI* restriction site for 11 Ethiopian isolates and *P. andina* (Fig. 2). The remaining thirteen polymorphic sites were identified in non-coding spacer that separates the two coding regions found in the P4 area (Table 2). All Ethiopian isolates and the four *P. infestans* reference isolates had a total of 8 fixed nucleotides differences at this region. The same region was also a source of variation between all Ethiopian isolates and *P. andina*. *EcoRI* links one of these variable sites in the same non-coding spacer to restriction enzyme recognition in all but two sequences representing type II haplotypes (Fig. 2). The absence of this restriction site in all of the Ethiopian specimens from which the target mtDNA

Table 2 Polymorphic nucleotide positions for part of the cytochrome c oxidase subunit 1 gene (coxI) and part of non-coding spacer (intergenic) between cox I and atp9 genes from *Phytophthora infestans* isolates collected in Ethiopia

			coxI						Non-coding region													
									1	1	1	1	1	1	1	1	1	1	1	1	1	1
			9	9	9	9	9	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0
			4	6	7	8	9	9	1	1	1	1	1	1	1	1	1	1	2	2	2	
			2	0	3	9	3	6	2	3	4	8	8	8	8	9	9	9	1	2	5	
Isolate[a]	Accession [b]	Country	7	1	9	8	4	4	1	8	0	4	6	8	9	0	1	3	6	9	1	
ETH-01	KM078902	Ethiopia	T	A	T	C	T	A	-	C	G	G	T	G	T	A	G	A	A	G	A	
ETH-02	KM078903	Ethiopia	T	A	T	C	T	A	-	C	G	G	T	G	T	A	G	A	A	G	A	
ETH-03	KM078904	Ethiopia	T	A	T	C	T	A	-	C	G	G	T	G	T	A	G	A	A	G	A	
ETH-04	KM078905	Ethiopia	T	A	T	C	T	A	-	C	G	G	T	G	T	A	G	A	A	G	A	
ETH-05	KM078906	Ethiopia	T	A	T	C	T	A	-	C	G	G	T	G	T	A	G	A	A	G	A	
ETH-06	KM078907	Ethiopia	T	A	T	C	T	A	-	C	G	G	T	G	T	A	G	A	A	G	A	
ETH-07	KM078908	Ethiopia	T	A	T	C	T	A	-	C	G	G	T	G	T	A	G	A	A	G	A	
ETH-08	KM078909	Ethiopia	T	A	T	C	T	A	-	C	G	G	T	G	T	A	G	A	A	G	A	
ETH-09	KM078910	Ethiopia	T	A	T	C	T	A	-	C	G	G	T	G	T	A	G	A	A	G	A	
ETH-10	KM078911	Ethiopia	T	A	T	C	T	A	-	C	G	G	T	G	T	A	G	A	A	G	A	
ETH-11	KM078912	Ethiopia	T	A	T	C	T	A	-	C	G	G	T	G	T	A	G	A	A	G	A	
ETH-12	KM078913	Ethiopia	C	G	G	T	C	G	A	C	A	T	C	A	C	G	A	C	A	G	A	
ETH-13	KM078914	Ethiopia	C	G	G	T	C	G	A	C	A	T	C	A	C	G	A	C	A	A	A	
ETH-14	KM078915	Ethiopia	C	G	G	T	C	G	A	C	A	T	C	A	C	G	A	C	A	G	A	
ETH-15	KM078916	Ethiopia	C	G	G	T	C	G	A	C	A	T	C	A	C	G	A	C	A	G	A	
ETH-16	KM078917	Ethiopia	C	G	G	T	C	G	A	C	A	T	C	A	C	G	A	C	A	G	A	
ETH-17	KM078918	Ethiopia	C	G	G	T	C	G	A	C	A	T	C	A	C	G	A	C	A	G	A	
	U17009	USA	C	G	G	T	C	G	A	C	A	T	C	A	C	G	A	C	A	G	A	
80029	AY894835	Netherlands	C	G	G	T	C	G	A	C	A	T	C	A	C	G	A	C	A	G	A	
15/99	AY898627	Ireland	C	G	G	T	C	G	A	T	A	T	C	A	C	G	A	C	A	G	A	
94-52	AY898628	USA	C	G	G	T	C	G	A	T	A	T	C	A	C	G	A	C	A	G	A	
EC3425	HM590419	Ecuador	T	A	T	C	T	A	-	C	G	G	T	G	T	A	G	A	-	G	-	

[a] Site numbers (written vertically) refer only to polymorphic sites relative to the complete genome sequence of *P. infestans* mtDNA (GenBank, Accession U17009); Shades indicate identity and dashes gaps

[b] *Phytophthora infestans* reference haplotypes Ib (U17009), Ia (80029), IIa (15/99) and IIb (94-52) and *P. andina* (EC3425)

genes were amplified suggest the Type I haplotypes of *P. infestans* lineages. However, only six Ethiopian isolates had similar restriction sites consistent with Type I haplotypes (Fig. 2). On the other hand, in parts of atp9 gene amplified in the P4 area of mitochondrial DNA, no polymorphism was detected except one missing nucleotide in one sample (Table 2).

Polymorphism detected in the P2 region

Polymorphism detected in the P2 region was associated with two partially amplified coding regions (Table 3). Five nucleotide positions were associated with restriction enzyme recognition by the endonuclease *MspI* (Fig. 3). The atp1 gene accounted for 6 out of the total 9 polymorphic sites detected among all nucleotide sequences including five reference isolates (Table 3). Three nucleotide positions (in the atp1 gene) were associated with restriction enzyme recognition by the *MspI* (Fig. 3). However, the two sites were associated with polymorphism linked to *MspI* restriction site detected among Ethiopian isolates. The eleven isolates had a single nucleotide substitution from A to G that resulted in gain of two additional *MspI* sites that was not consistent with the remaining six isolates and the four

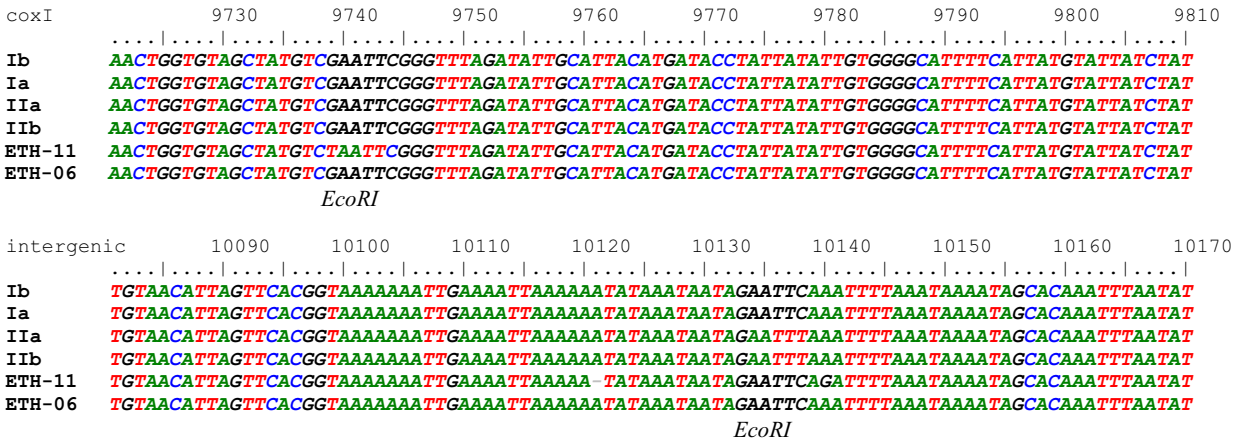

Fig. 2 mtDNA sequences alignments around *EcoRI* restriction site for part of the cytochrome c oxidase subunit 1 gene (coxI) and part of non-coding spacer (intergenic) between cox I and atp9 gene. *Phytophthora infestans* collected in Ethiopia are represented by ETH-11 and ETH-06 for a subset of 11 and 6 isolates sequence data respectively. *Numbers* above alignments correspond to nucleotide positions for Ib haplotypes (U17009)

P. infestans haplotypes lineages (Fig. 3). Throughout the part of nad4 gene, two nucleotide positions were unique for eleven Ethiopian isolates and *P. andina* (Table 3). The only polymorphism linked to *MspI* restriction site detected in this part of the gene was a single nucleotide substitution from C- to- T that resulted in the loss of *MspI* site among all Ethiopian isolates (Fig. 3). No polymorphism was detected in glutamic acid tRNA (trnE) gene and the two non- coding spacers that separate the three coding regions for all analyzed sequences in P2.

Discussion

This study provides new insight into the late blight pathogen, linked to *P. infestans*, in one of the least explored region of the world and demonstrates that *P. infestans* and a novel mtDNA haplotype of *P. infestans* comprise an important component of the population structure attacking potato and tomato. Our current study found greater genetic variation in the population of *P. infestans* in Ethiopia, including not only the Ia previously reported lineage (Schiessendoppler and Molnar 2002) but also two haplotypes reported for the first time in Ethiopia, and one reported for the first time anywhere. As noted, the representativeness of the previous study is not known, and therefore we do not know if the greater diversity we found represents migration of new genotypes or simply a greater sample size. Nonetheless, the level of variations analyzed for mitochondrial diversity in this study would help to expand the current knowledge of *P. infestans* population with reference to important evolutionary mechanisms and implication for disease management.

In this study, haplotype Ia was detected in collections from most areas. It is now clear that this haplotype is always associated with A1 mating type in Ethiopia (Daniel 2015) and seems to be dominant in most of the modern day populations of *P. infestans* studied today (Fry et al. 1993, 2009). Although host adaptation was not examined in current work, it is likely that the population of Ib mtDNA haplotype in Ethiopia may represents an example of a population adapted to tomato. Other studies have also reported persistence of the Ib haplotype on tomato long after it had been displaced on potato by a newer lineage that had the mitochondrial haplotype Ia (Griffith and Shaw 1998; Spielman et al. 1991; Vega-Sánchez et al. 2000). The rare occurrence of the IIb haplotype was surprising since the US-6 presently known to possess the IIb haplotype, had only previously been reported in north-western Mexico, parts of North America and Canada (Gavino and Fry 2002; Goodwin et al. 1994).

Our data incorporate information on novel plant-pathogen and clarify some aspects of their RFLP and molecular characters that enhances the overall knowledge of the late blight pathogen attacking potato. Even though, within the genus *Phytophthora*, only one species named *P. andina* had similar RFLP band pattern designated as Ic mtDNA haplotype (Ordoñez et al. 2000), the Ethiopian had distinguishing molecular characters. A particularly distinguishing feature is the presence two nucleotide deletions in intronic and flanking regions of the P4 region, a feature that can be used to distinguish the new Ethiopian isolates from *P. andina* that have shorter nucleotide length (Lassiter et al. 2010). Molecular data presented elsewhere, also support that genomes' amino acid replacements and the presence indels as distinguishing feature of each member of the Ic

Table 3 Polymorphic nucleotide positions identified for part of the ATP synthase F1 subunit α (atp1) gene and part of the NADH dehydrogenase subunit 4 (nad4) gene from *Phytophthora infestans* isolates collected in Ethiopia

Site[a]			atp1						nad4		
			1	1	1	1	1	1	1	1	1
			3	3	3	3	3	3	4	4	4
			6	7	8	8	8	9	5	5	6
			5	0	0	3	4	3	0	8	0
Isolate	Accession[b]	Country	2	6	5	5	2	5	4	3	4
ETH-01	KM078885	Ethiopia	A	G	T	G	T	C	A	C	T
ETH-02	KM078886	Ethiopia	A	G	T	G	T	C	A	C	T
ETH-03	KM078887	Ethiopia	A	G	T	G	T	C	A	C	T
ETH-04	KM078888	Ethiopia	A	G	T	G	T	C	A	C	T
ETH-05	KM078889	Ethiopia	A	G	T	G	T	C	A	C	T
ETH-06	KM078890	Ethiopia	A	G	T	G	T	C	A	C	T
ETH-07	KM078891	Ethiopia	A	G	T	G	T	C	A	C	T
ETH-08	KM078892	Ethiopia	A	G	T	G	T	C	A	C	T
ETH-09	KM078893	Ethiopia	A	G	T	G	T	C	A	C	T
ETH-010	KM078894	Ethiopia	A	G	T	G	T	C	A	C	T
ETH-011	KM078895	Ethiopia	A	G	T	G	T	C	A	C	T
ETH-012	KM078896	Ethiopia	A	A	T	A	C	T	C	T	T
ETH-013	KM078897	Ethiopia	A	A	T	A	C	T	C	T	T
ETH-014	KM078898	Ethiopia	A	A	T	A	C	T	C	T	T
ETH-015	KM078899	Ethiopia	A	A	T	A	C	T	C	T	T
ETH-016	KM078900	Ethiopia	A	A	T	A	C	T	C	T	T
ETH-017	KM078901	Ethiopia	A	A	T	A	C	T	C	T	T
	U17009	USA	A	A	T	A	C	T	C	T	C
80029	AY894835	Netherlands	A	A	T	A	C	T	C	T	T
15/99	AY898627	Ireland	G	A	C	A	C	T	C	T	T
94-52	AY898628	USA	A	A	T	A	C	T	C	T	T
EC3425	HM590419	Ecuador	A	G	T	G	T	C	A	C	T

[a] Site numbers (written vertically) refer only to polymorphic sites relative to the complete genome sequence of *P. infestans* mtDNA (GenBank, Accession NC_002387); Shades indicate identity and dashes gaps

[b] *Phytophthora infestans* reference haplotypes Ib (U17009), Ia (80029), IIa (15/99) and IIb (94-52) and *P. andina* (EC3425)

clade, including *P. andina* (Blair et al. 2008; Lassiter et al. 2015). Particularly, our data is consistent with Wattier et al. (2003) who ascertained sequence variation in noncoding intergenic mtDNA spacers among species of *Phytophthora* that revealed the potential of these markers in taxonomy and phylogeny.

The novel *Phytophthora* species in Ethiopia, presents the first record of its kind attacking potato. This haplotype is similar, but not identical to the Ic haplotype reported for *P. andina.* The latter fits the morphological description for *P. infestans* (Oliva et al. 2010), but pathogenic analysis indicates host use by *P. infestans* and *P. andina* in Ecuador overlap minimally (Adler et al. 2004; Oliva et al. 2007). Unlike *P. andina*, the Ethiopian isolates appears to be primarily a pathogen of *Solanum tuberosum* indicating a deviation in host status previously reported for Ecuador lineages, which apparently does not infect tomato or potato in the field (Oliva et al. 2010). Further, the Ethiopian isolates tested so far exhibited the A1 compatibility type with *P. infestans* tester isolates (Daniel 2015; Schiessendoppler

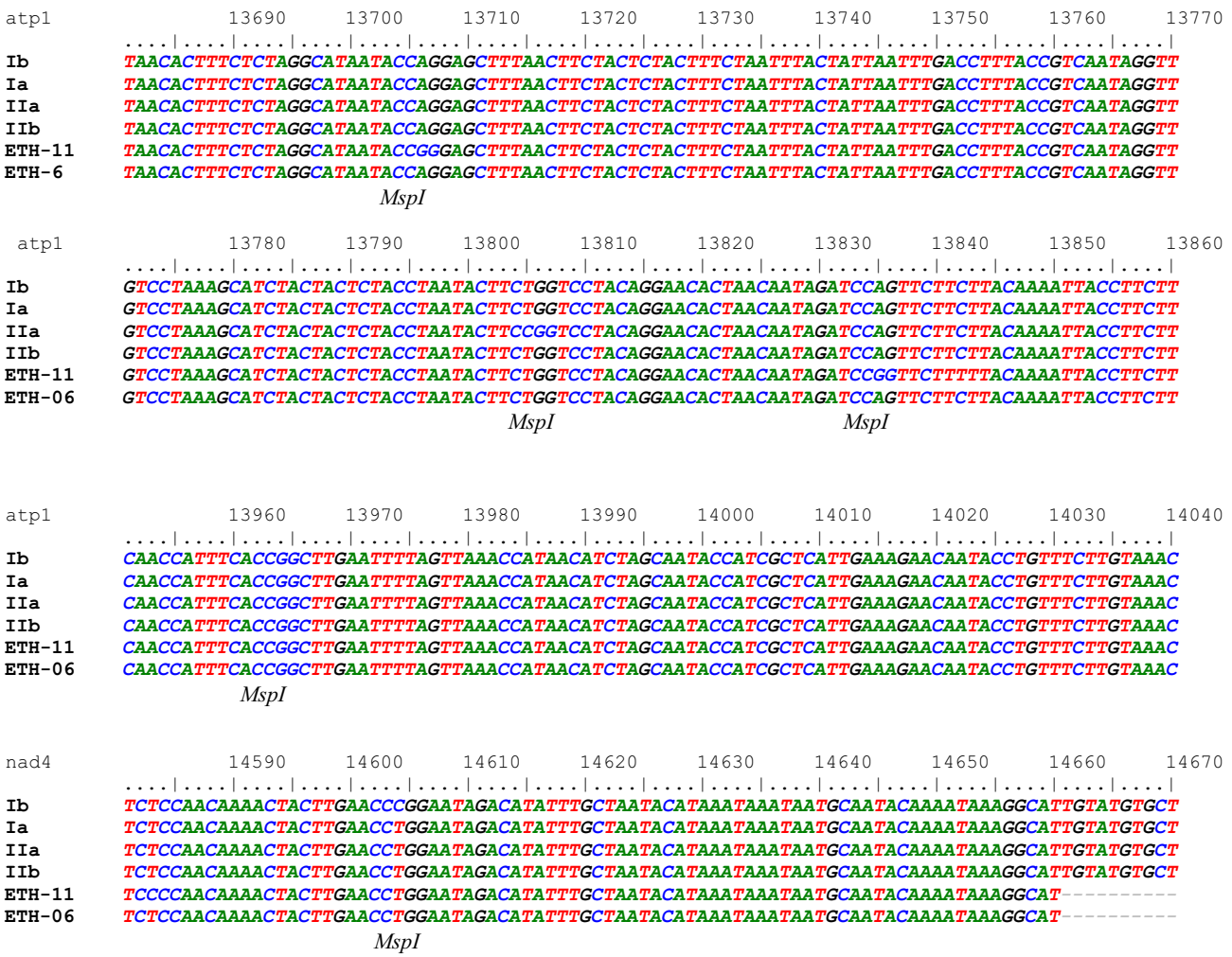

Fig. 3 mtDNA sequences alignments around *MspI* restriction sites for part of the ATP synthase F1 subunit α (atp1) gene part of the NADH dehydrogenase subunit 4 (nad4) gene. *Phytophthora* isolates collected in Ethiopia are represented by ETH-11 and ETH-06 for a subset of 11 and 6 sequence data respectively. *Numbers* above alignments correspond to nucleotide positions for Ib haplotypes (U17009)

and Molnar 2002). In contrast the Ic mtDNA haplotype lineages of *P. andina* was characterized only by the mating type A2 (Oliva et al. 2010). *P. andina* has never been reported outside of South America, but the species is considered to be a hybrid of *P. infestans* and one more unknown species (Goss et al. 2011), indicating that gene flow between these species could be occurring.

Conclusion

The evidence presented here shows that some Ethiopian isolates could represent a novel *Phytophthora* species reported for the first time here. Ethiopia has a recent history of importing potatoes from Peru, the Netherlands, Kenya and Uganda (Gorfu and Woldegiorgis 2013). This might suggest that there could have been opportunities for introduction of *P. andina* from Andean region to Ethiopia. However, a satisfactory answer to this path is yet to be resolved as there was no evidence of natural *P. andina* infection on potato (Adler et al. 2004; Oliva et al. 2010) suggesting the possible source of inoculums via potato seed tuber less likely. While a distinguishing molecular data presented here clearly separated them from *P. infestans*, these Ethiopian isolates formed an important component of the late blight pathogen causing disease on *S. tuberosum* in Ethiopia.

Acknowledgments We thank Dr Bekele Kassa (Plant Pathology Department, Holeta Agricultural Research Centre, Ethiopia) for help with part of sample collection in 2011. We would like to express our thanks to Department of Forest Mycology and Plant Pathology, Swedish University of Agriculture Sciences (SLU) for help with molecular laboratory facility. A Swedish Government Initiative, the SLU Africa Food Security Research Program supports this work.

References

Adler N, Erselius LJ, Chacon MG, Flier WG, Ordonez ME, Kroon LPNM, Forbes GA (2004) Genetic diversity of *Phytophthora infestans* sensu lato in Ecuador provides new insight into the origin of this important plant pathogen. Phytopathology 94:154–162

Avila-Adame C, Gómez-Alpizar L, Zisman V, Jones K, Buell CR, Ristaino JB (2006) Mitochondrial genome sequences and molecular evolution of the Irish potato famine pathogen, *Phytophthora infestans*. Curr Genet 49:39–46

Berga L, Gebre-Medhin W, Teriessa J. Bereketsehai T (1992) Potato agronomy research. In: Herath E, Lemma D (eds) Horticulture research and development in Ethiopia, Proceedings of the 2nd National Horticultural Workshop of Ethiopia, Institute of Agricultural Research, Addis Ababa, 2–5 February, pp. 182–196

Blair JE, Coffey MD, Park SY, Geiser DM, Kang S (2008) A multilocus phylogeny for *Phytophthora* utilizing markers derived from complete genome sequences. Fungal Gen Biol 45:266–277

Carter DA, Archer SA, Buck KW, Shaw DS, Shattock RC (1990) Restriction fragment length polzmorphism of mitocondrial DNA of *Phytophthora infestans*. Mycol Res 94:1123–1128

Chen JZ, Hebert PDN (1999) Intra-individual sequence diversity and hierarchical approach to the study of mitochondrial DNA mutations. Mutat Res 434:205–217

Chesnick JM, Goff M, Graham J, Ocampo C, Lang BF, Seif E, Burger G (2000) The mitochondrial genome of the stramenopile alga *Chrysodidymus synuroideus*. Complete sequence, gene content and genome organization. Nucleic Acid Res 28:2512–2518

Cooke DEL, Lees AK (2004) Markers, old and new, for examining *Phytophthora infestans* diversity. Plant Pathol 53:692–704

Cooke D, Lees A (2006) Eucablight protocol—Sampling of *P. infestans* from foliar epidemics. http://www.eucablight.org/Lib/Eucablight/Protocol/BlightSampling_V1.0.pdf. Accessed 07 March 2011

Cooke DEL, Young V, Birch PRJ, Toth R, Gourlay F, Day JP, Carnegie S, Duncan JM (2003) Phenotypic and genotypic diversity of *Phytophthora infestans* populations in Scotland (1995–97). Plant Pathol 52:181–192

Daniel S (2015) Diversity of *Phytophthora infestans* (Mont.) de Bary on Potato (*Solanum tuberosum* L.) and Tomato (*Solanum lycopersicum* Mill.) in Ethiopia. Dissertation, Haramaya University, Haramay, Ethiopia

Flier WG, Grünwald NJ, Kroon LPNM, Sturbaum AK, van den Bosch TBM, Garay-Serrano E, Lozoya-Saldaña H, Fry WE, Turkensteen LJ (2003) The population structure of *Phytophthora infestans* from the Toluca valley of Central Mexico suggests genetic differentiation between populations from cultivated potato and wild *Solanum* spp. Phytopathology 93:382–390

Fry WE, Goodwin SB, Dyer AT, Ma-tuszak JM, Drenth A, Tooley PW, Sujkowski LS, Koh YJ, Cohen BA, Spielman LJ, Deahl KL, Inglis DA, Sandlan KP (1993) Historical and recent migrations of *Phytophthora infestans*: chronology, pathways, and implications. Plant Dis 77:653–661

Fry WE, Grunwald NJ, Cooke DEL, Mcleod A, Forbes GA, Cao K (2009) Population genetics and population diversity of *Phytophthora infestans*. In: Lamour K, Kamoun S (eds) Oomycete genetics and genomics: diversity, interactions, and research tools, Wiley, pp 139–164

Galtier N, Nabholz B, Glémin S, Hurst GDD (2009) Mitochondrial DNA as a marker of molecular diversity: a reappraisal. Mol Ecol 18:4541–4550

Gardes M, Bruns TD (1993) ITS primers with enhanced specificity of basidiomycetes: application to the identification of mycorrhizae and rusts. Mol Ecol 2:113–118

Gavino PD, Fry W (2002) Diversity in and evidence for selection of the mitochondrial genome of *Phytophthora infestans*. Mycologia 94:781–793

Goodwin SB, Cohen BA, Fry WE (1994) Panglobal distribution of a single clonal lineage of the Irish potato famine fungus. Proc Natl Acad Sci 91:11591–11595

Gorfu D, Woldegiorgis G (2013) Pest risk analysis for potato importation into Ethiopia: a case of four source countries. In: Woldegiorgis G, Schulz S, Berihun B (eds) Seed potato tuber production and dissemination experiences, challenges and prospects. EIAR and ARARI, Bahir Dar, pp 7–20

Goss EM, Cardenas ME, Myers K, Forbes GA, Fry WE et al (2011) The plant pathogen *Phytophthora andina* emerged via hybridization of an unknown *Phytophthora* species and the Irish potato famine pathogen *P. infestans*. PLoS One 6(9):e24543. doi:10.1371/journal.pone.0024543

Griffith GW, Shaw DS (1998) Polymorphism in *Phytophthora infestans*: four mitochondrial haplotypes are detected after PCR amplification of DNA from pure cultures or from host lesions. Appl Envir Microbiol 64(10):4007–4014

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis for Windows 95/98/NT. Nucl Acids Symp Ser 41:95–98

Hirpa A, Meuwissen PMM, Tesfaye A, Lommen JMW, Lansink AO, Tsegaye A, Struik PC (2010) Analysis of seed potato systems in Ethiopia. Am J Potato Res 87:537–552

Lang BF, Forget L (1993) The mitochondrial genome of *Phytophthora infestans*. In: O'Brien SJ (ed) Genetic maps: locus maps of complex genomes. Cold Spring Harbor Laboratory Press, pp 3.133–3.135

Lassiter ES, Russ C, Nusbaum C, Zeng Q, Hu CH, Thorne JL, Ristaino JB (2010) Inferring evolutionary relationships of species in the *Phytophthora* Ic clade using nuclear and mitochondrial genes. Phytopathology, S68

Lassiter ES, Russ C, Nusbaum C, Zeng Q, Saville A, Olarte R, Carbone I, Hu CH, Seguin-Orlando A, Samaniego JA, Thorne JF, Ristaino J (2015) Mitochondrial genome sequences reveal evolutionary relationships of the *Phytophthora* Ic clade species. Curr Genet 61(4):567–577

Laufer B (1938) The American Plant Migration. Part I: The Potato Archeology Series. Field Museum of National History. Vol 28, Number I. Publication 418. Chicago

Lees AK, Wattier R, Shaw DS, Sullivan L, Williams NA, Cooke DEL (2006) Novel microsatellite markers for the analysis of *Phytophthora infestans* populations. Plant Pathol 55:311–319

Librado P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphic data. Bioinformatics 25:1451–1452

Martin FN (2008) Mitochondrial haplotype determination in the oomycete plant pathogen *Phytophthora ramorum*. Curr Genet 54:23–34

Martin FN, Zhang Y, Grünwald N, Coffey MD (2012) Expanded analysis of *P. infestans* mitochondrial haplotypes and correlation with nuclear genotype. Phytopathology 54:76

Mesfin T, Wondirad M, Bekele K (2009) Review of Research on Diseases of Root and Tuber Crops in Ethiopia. In: Abraham T (ed) Increasing crop production through improved plant protection -, vol II. Plant Protection Society of Ethiopia (PPSE) and EIAR, Addis Ababa, pp 169–202

Oliva RF, Chacón MG, Cooke DEL, Lees AK, Forbes GA (2007) Is *Phytophthora infestans* a good taxonomist? Host recognition and co-evolution in the *Phytophthora*/*Solanum* interaction. Acta Horticulturae 745:465–471

Oliva RF, Kroon LPNM, Chacón G, Flier WG, Ristaino JB, Forbes GA (2010) *Phytophthora andina* sp. nov., a newly identified

heterothallic pathogen of solanaceous hosts in the Andean highlands. Plant Pathol 59:613–625

Ordoñez ME, Hohl HR, Velasco JA, Ramon MP, Oyarzun PJ, Smart CD, Fry WE, Forbes GA, Erselius LJ (2000) A novel population of *Phytophthora*, similar to *P. infestans*, attacks wild solanum species in Ecuador. Phytopathology 90(2):197–202

Paquin B, Laforest MJ, Forget L, Roewer I, Wang Z, Longcore J, Lang BF (1997) The fungal mitochondrial genome project: evolution of fungal mitochondrial genomes and their gene expression. Curr Genet 31:380–395

Schiessendoppler E, Molnar O (2002) Characterization of *Phytophthora infestans* populations in sub-Saharan Africa as a basis for simulation modeling and integrated disease management. In: Lizárraga C (ed) Late blight: managing the global threat. Proceedings of the global initiative on late blight conference, Hamburg, Germany, 11–13 July 2002, International Potato Centre, Lima, p 140

Spielman LJ, Drenth A, Davidse LC, Sujkowski LJ, Gu WK, Tooley PW, Fry WE (1991) A second world-wide migration and population displacement of *Phytophthora infestans*? Plant Pathol 40:422–430

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The Clustal X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acid Res 24:4876–4882

Vega-Sánchez ME, Erselius LJ, Rodriguez AM, Bastidas O, Hohl HR, Ojiambo PS, Mukalazi J, Vermeulen T, Fry WE, Forbes GA (2000) Host adaptation to potato and tomato within the US–1 clonal lineage of *Phytophthora infestans* in Uganda and Kenya. Plant Pathol 49:531–539

Wattier RAM, Gathercole LL, Assinder SJ, Gliddon CJ, Deahl KL, Shaw DS, Mills DI (2003) Sequence variation of intergenic mitochondrial DNA spacers (mtDNA-IGS) of *Phytophthora infestans* (Oomycetes) and related species. Mol Ecol Notes 3:136–138

Yang ZH, Qi MX, Qin YX, Zhu JH, Gui XM, Tao B, Xu XH, Zhang FG (2013) Mitochondrial DNA polymorphisms in *Phytophthora infestans*: new haplotypes are identified and re-defined by PCR. J Microbiol Methods 95:117–121

Zhang Z, Schwartz S, Wagner L, Miller W (2000) A greedy algorithm for aligning DNA sequences. J Comput Biol 7:203–214