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ORIGINAL ARTICLE

Population structure of *Phytophthora infestans* (Mont.) de Bary in Ethiopia

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Abstract

Late blight disease caused by an Oomycete *Phytophthora infestans* is a major constraint to potato production and is causing significant yield losses in Ethiopia. This study was conducted to characterize the genetic diversity of the pathogen population in the major potato growing regions Awi, East Hararghe, South Gondar, West Arsi, West Gojjam and West Shewa in Ethiopia. In total 138 *P. infestans* isolates were collected using FTA cards in 2017, genotyped using 12-plex SSR markers and characterized for the mitochondrial haplotype. The genotypic patterns were compared to those of reference isolates from the EU2_A1 and US-1 clonal lineages. The population structure analysis using discriminant analysis of principal components (DAPC) and STRUCTURE indicated that most of the Ethiopian isolates were similar to the EU2_A1, while a second cluster of isolates was formed that was clearly different from EU2_A1 as well as the US-1 reference isolates. This new genotype was characterized by private alleles in the SSR D13 locus. We named this new genotype as ET-1 lineage. All isolates had the same mitochondrial haplotype (*Ia*). EU2_A1 was dominant clonal lineage in all locations except West Arsi which was dominated with ET-1 lineage. The old US-1 lineage was not discovered among the Ethiopian samples which suggest that it has been displaced. The West Arsi, West Gojjam and West Shewa populations were found to contain the highest genetic diversity, with the greatest number of multi locus genotypes (MLGs) and a higher diversity index compared to the other locations. The findings of this study establish a baseline of the pathogen population diversity in Ethiopia. Continuous tracking of *P. infestans* population in both potato and tomato is recommended to monitor the changes and migration patterns.

Keywords Clonal lineage · Genetic diversity · Haplotype · Late blight

Introduction

Potato (*Solanum tuberosum* L.) and tomato (*Solanum lycopersicum* L.) production worldwide is constrained by the late blight disease cause by *Phytophthora infestans* (de Vries et al. 2017). Potato is one of the most important food crops and ranks third following wheat and rice in terms food consumption, but the economic loss of production due to late blight is a key threat to food security (Hussain 2017; Hu et al. 2012). Potato was introduced to Ethiopia in 1858 by a German botanist, Wilhelm Schimper (Pankhurst 1964) and the first report on late blight on potato is 80 years later (Laufer and Wilbur 1938). The first reported study on *P. infestans* in Ethiopia found that all isolates were A1 mating type and the *Ia* mt-DNA-haplotype (Schiessendoppler and Molnar 2002). A more recent study found greater genetic variation including not only the *Ia* previously reported lineage but also three new haplotypes *Ib, IIb* and *Ic* (Shimelash et al. 2016). Some tomato isolates from Eastern Ethiopia also had *Ib* and *IIb* haplotypes, which were not found on potato in any location (Shimelash et al. 2016). All the reported lineages belong to the A1 mating type, suggesting that a sexually compatible genotype might not yet exist in the country (Shimelash et al. 2016).

The genomic analysis studies of *P. infestans* done in the last 40 years report alteration in diversity, changes of

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population structure, and in some cases evidence of sexual reproduction and novel destructive strains (Dey et al. 2018). The population changes have had an impact on management of the disease in tomato (Richard and Simko 2005). Reduction in potato and tomato cultivation due to the widespread infection by late blight has been reported in Ethiopia (Tesfahun et al. 1985) and the management of this disease has become challenging (Kassa et al. 2002). *P. infestans* has a high mutation rate and is well known worldwide for its capacity to overcome plant disease resistance mechanisms (Kamoun et al. 2015). Indeed, in Ethiopia some improved varieties that were resistant have succumbed to the disease (Gebremedhin 2013), and the pathogen has also developed resistance to Metalaxyl (Schiessendoppler and Molnar 2002). The knowledge of the phenotypic characteristics of the pathogen strains such as host range, mating type and fungicide sensitivity helps to refine late blight management strategies. Furthermore, *P. infestans* populations can vary histrionically among sites and forecasts regarding their phenotypic behavior need to be based on the precise population structure (Njoroge et al. 2019a). Populations of *P. infestans* have been characterized using a series of genotypic and phenotypic markers. Phenotypically, populations have been characterized through the determination of the mating-type, virulence spectrum and metalaxyl resistance (Fry et al. 1993). The genotypic characterization has included the use of allozyme patterns, mitochondrial DNA (mtDNA) haplotype determination, as well as AFLP and RFLP fingerprints with the probe RG57 (Hussain 2016). In comparison with the previously mentioned markers, simple sequence repeat markers (SSRs, also referred to as microsatellites) seem to offer the greatest potential across a wide range of applications (Cooke and Lees 2004; Imranul Haq et al. 2016). Over the past twenty years, SSR markers have been developed for *P. infestans* (Knapova et al. 2001; Knapova and Gisi 2002; Lees et al. 2006) that formed the basis of the practical method of 12-plex single SSR protocol (Li et al. 2013). This technology has the advantage of the comparison of the SSR marker patterns and has become the principle method to analyze genotypic diversity and tracking the movement of genotypes across continents. For example, a recent study in Eastern Africa using this protocol showed that a new genotype of European origin, EU2_A1, is present in Kenya, Uganda, Tanzania, Burundi, and Rwanda (Njoroge et al. 2019a). Because of this finding it is possible that the same genotype could be also present in Ethiopia. Therefore, our goal was to characterize the genetic diversity of the *P. infestans* population infecting potato in the major potato producing areas in Ethiopia using the same 12-plex assay to be able to compare the genotypes in Ethiopia to those found elsewhere in East Africa.

Materials and methods

Sampling of late blight infected potato leaves

Phytophthora infestans isolates were collected in different major potato producing-areas of the country, i.e. Northwestern Ethiopia (Awi zone, West Gojjam, and South Gondar); Central Ethiopia (West Shewa); Southern Ethiopia (West Arsi), and Eastern Ethiopia (East Hararghe) (Fig. 1). Reference isolates of known clonal lineages were included in the study and were combined with the samples collected in Ethiopia.

Samples of diseased potato leaflets were collected from farmer's fields and field trials on the experimental station of Adet and Holeta Agricultural Research Center. The greatest number of samples came from West Arsi [WA] (57), followed by West Shewa [WS] (43), West Gojjam [WG] (22), East Hararghe [EH] (20), South Gondar [SG] (19), and Awi [AW] (14). A cut of 1–2 cm^2 piece of sporulating single young lesion was placed inside a clean circular sampling area onto the FTA matrix (Whatman FTA Classic Card, catalog number WB120205: GE Healthcare UK Ltd.) sporulating side facing down and was individually crushed in the field; the cards were subsequently air dried before storage and were stored inside the filter paper covered in plastic bags with zip lock until they were moved to the laboratory for SSR analysis. A total of 175 samples were collected in 2017 and world reference DNA samples of the different lineages and variants were included in the analysis.

Genotypic analysis

To wash out the DNAfrom the FTA cards, small discs were cut from the cards using a 2 mm Harris Micro Punch (Whatman, WB100007) and processed according to a modified manufacturer's protocol in FTA purification reagent (Whatman, WB120204) before placing directly into the PCR mix for multiplex SSR analysis. The discs were washed twice with 400 µL of FTA purification reagent and were vortexed and incubated for 4 min at room temperature. Then, the discs were rinsed twice with 400 µL of modified TE^{-1} buffer (10 mM Tris, 0.1 mM EDTA). The discs were transferred to clean microtubes and 80 µL of TE (10 mM Tris, 1 mM EDTA) was added. Following quick spin down the discs were incubated 5 min at 95 °C, and cooled on ice immediately. The FTA discs were left in the final solution and kept at -20 °C until use.

SSR marker analysis

SSR genotypes were identified using the 12-plex microsatellite assay (Table S1; Li et al. 2013). The Type-It Microsatellite PCR

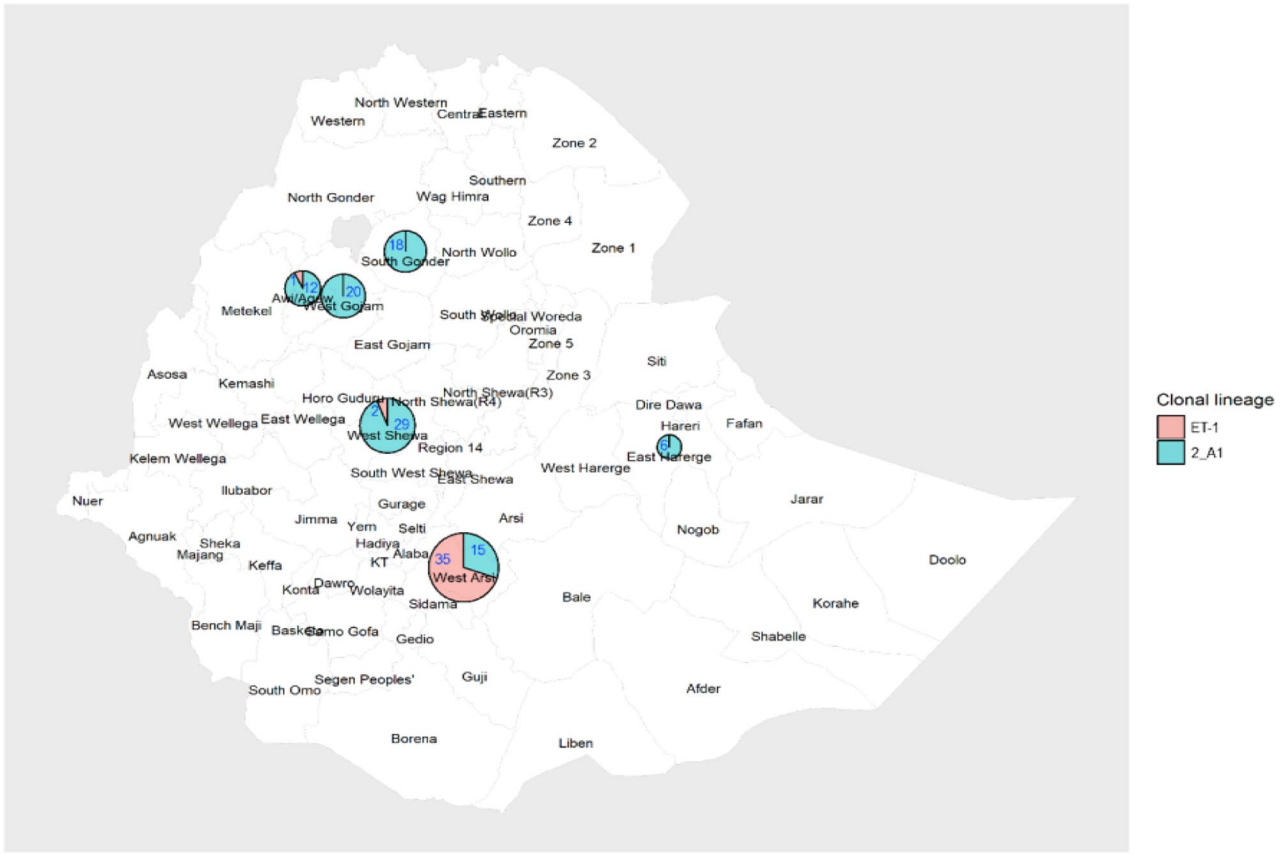

Fig. 1 Geographical distribution of the clonal lineages EU2_A1 and ET-1 among the *Phytophthora infestans* isolates (n = 138) collected in Ethiopia in 2017. The sizes of the pie charts are relative to the sample size

kit (QIAGEN) was used for PCRs following a modified protocol described by Saville et al. (2016). The amplification conditions for the multiplex PCR were as follows: 95 ℃ for 5 min; followed by 33 cycles of 95 ℃ for 30 s, 58 ℃ for 90 s, and 72 ℃ for 30 s; and a final extension at 60 ℃ for 30 min. PCR amplicons were sent to Arizona State University for post-PCR processing in 96-well plates. For fragment analysis, 1–2 µL of PCR product was added to a 10.3 µL reaction mix consisting of 10 µL highly deionized formamide and 0.3 µL LIZ500 size standard (Applied Biosystems). Fragments were then analyzed on an Applied Biosystems 3730xl DNA analyzer at the Genomic Sciences Laboratory at Arizona University. SSR alleles were sized by GENEMARKER v. 1.9 software (SoftGenetics) using the allele sizing parameters defined by Li et al. (2013). Reference isolates from Peru of known SSR genotypes was included on each PCR plate and were used in the analysis (PPU103: US-1). All 12 SSR markers were simultaneously amplified for each sample. Out of 175 samples 37 samples which had high percentage of missing data were discarded. The remaining 138 samples were properly scored. Scores of additional references were added for the analysis (2_A1: EU_2_A1_KE_1(UK), EU_2_A1_KE_2(PG), 2_A1(UK); and US-1: US-1_1(KE), and US-1_2(US) (D. Cooke personal communication and Martin et al. (2019)).

Mitochondrial DNA haplotype

Mitochondrial DNA (mt DNA) haplotypes were determined by comparing the DNA profiles to those of reference isolates having *Ia, Ib, IIa* profiles of the mitochondrial genome (Griffith and Shaw 1998). PE-3 (*Ia*), US-1(*Ib*), and EC-1(*IIa*) reference isolates were used as a standard.

Assignment of clonal lineages

Clonal lineage of *P. infestans* isolates was determined by comparing the marker profiles among the sample isolates from Ethiopia and the reference isolates listed above. The Bayesian model based clustering algorithm STRUCTURE V.2.3.4 (Pritchard et al. 2000) was used to infer population structure. The software clusters individuals into K populations using a Markov Chain Monte Carlo (MCMC) approach. Ten independent runs were performed with 100,000 iterations /MCMC run after a burn-in period of 20,000 for each value of K ranging from 1 to 10. An admixture model was selected as it assumes mixed ancestry of an individual. The optimum value of K was determined using the Evanno et al. (2005) method. CLUMPP V.1.1 was run to aggregate multiple STRUCTURE runs

(Jakobsson and Rosenberg 2007) based on a greedy algorithm, and visualized with the program Distruct v1.1 (Rosenberg 2003).

Data analysis was performed with the statistical software R v. 3.50 (R Core Team 2018) Estimation of genetic distance between individuals was based on the method of Bruvo et al. (2004), which uses a stepwise mutation model to calculate genetic distance of individuals in the population using the R package POLYSAT (Clark and Jasieniuk 2011). Significance was assessed using 1,000 bootstrap replicates. Genetic groupings of individuals within a clonal lineage were viewed using a discriminant analysis of principal components (DAPC) (Jombart et al. 2010) using the R package ADEGENET (Jombart and Ahmed 2011). Minimum-spanning networks to test evolutionary relationships among individuals for each subpopulation were constructed and visualized for different locations, using the R package Poppr (Kamvar et al. 2014). Samples with the same combination of alleles (i.e., similar MLGs) were considered as clones. The number of MLGs in each population and the expected number of MLGs after rarefaction (eMLG) (Hurlbert 1971) were obtained using the R package Poppr (Kamvar et al. 2014). Unweighted paired group method with arithmetic averages (UPGMA) tree was generated based on Bruvo's distance using the clone-corrected data, i.e. each multi locus genotype (MLG) is represented only once.

Genetic diversity indices

Basic indices of genetic variation for each population were calculated for populations defined by lineages. Clonal diversity within the populations was described using the Simpson diversity (Simpson 1949) and the Shannon–Wiener index (Shannon 2001) genetic richness (R), which, in this case, is equivalent to the number of eMLG at the largest shared sample size; and the Hendrick's standardized Index (G`ST), which determines within-population genetic diversity for multiallelic markers (Hedrick 1999, 2005).

Results

P. infestans clonal lineages and population structure

The mitochondrial haplotyping analysis indicated that all 138 *P. infestans* isolates belong to the *Ia* haplotype (Fig. S2). Based on the SSR genotyping the isolates were further separated into different genotypes also known as clonal lineages. The discriminant analysis of principal components clearly separated the isolates into three clusters where most of the Ethiopian isolates clustered with the EU2_A1 reference isolates and the remaining Ethiopian isolates formed a separate cluster distinct from the EU2_A1 or the US-1 reference isolates (Fig. 2). STRUCTURE software also identified three separate clusters as indicated by the ΔK peak at K = 3 (Fig. S1). However, the distinction among the US-1 and EU2_A1 isolates was not as clear as in the discriminant analysis (Fig. 3). The UPGMA dendrogram subdivided the isolates into three groups with a maximum distance of 0.05 (Fig. 4). The EU2_A1 cluster contained 100 isolates from West Shewa (29), West Gojjam (20), South Gondar (18), West Arsi (15), Awi (12), and East Hararghe (6). The ET-1 cluster included 38 isolates from West Arsi (35), West Shewa (2) and Awi (1). The US-1 cluster contained only the reference samples (Fig. 4). Based on these results, the *P. infestans* isolates infecting potato in Ethiopia belong to two separate clonal lineages EU2_A1 and a new clonal lineage, hereafter named as ET-1. The new genotype is characterized by private alleles in the D13 locus, 132/132, in place of 136/136, 144/144, 138/138, 134/134, 140/142, 136/140, 140/140, 134/136, 130/140, 136/170, 138/140 and 136/138 which generally identify the EU2A_1 and US-1 lineages (Table S2).

Population diversity

In total fifty-one MLGs were identified amongst 138 isolates and some of those were shared among locations as indicated by the total number of MLG of 58 (Table 1; Fig. 4; Table S2). The populations from West Arsi, West Gojjam and West Shewa were more diverse with 20, 11 and 11 MLGs identified, respectively (Table 1); 11, 8 and 5 of which were unique MLGs, respectively, to the region. The most frequent MLGs was MLG10 (isolated 39 times), and the other four frequent MLGs were MLG17 (12 times), MLG23 (12 times), MLG19 (7 times) and MLG1 (7 times).

Across the locations West Arsi population had a higher genetic richness at the largest shared sample size (eMLG) than did the other location populations (Table 1). Even though the largest ET-1 lineage subpopulation was found in West Arsi, the lineage was also found in West Shewa and Awi (Fig. 1).

The SSR markers D13, G11, and SSR4 had the highest polymorphism (number of alleles), hence resulting in a higher number of MLGs within the two clonal lineages (Table 2). The *P. infestans* samples had private alleles only on the D13 locus 132/132 (Table S2).

A total of 56 alleles were identified across 12 loci with a mean of 4.667 (Table 2). Among these, the D13, G11 and SSR4 loci had the most alleles, followed by SSR6, SSR3 and SSR2. Loci Pi70, SSR11 and Pi4B were the least diverse with 2 alleles each. Of loci, two or more than two alleles, SSR8, SSR11 and Pi70 were found to be the most evenly distributed alleles (Table 2).

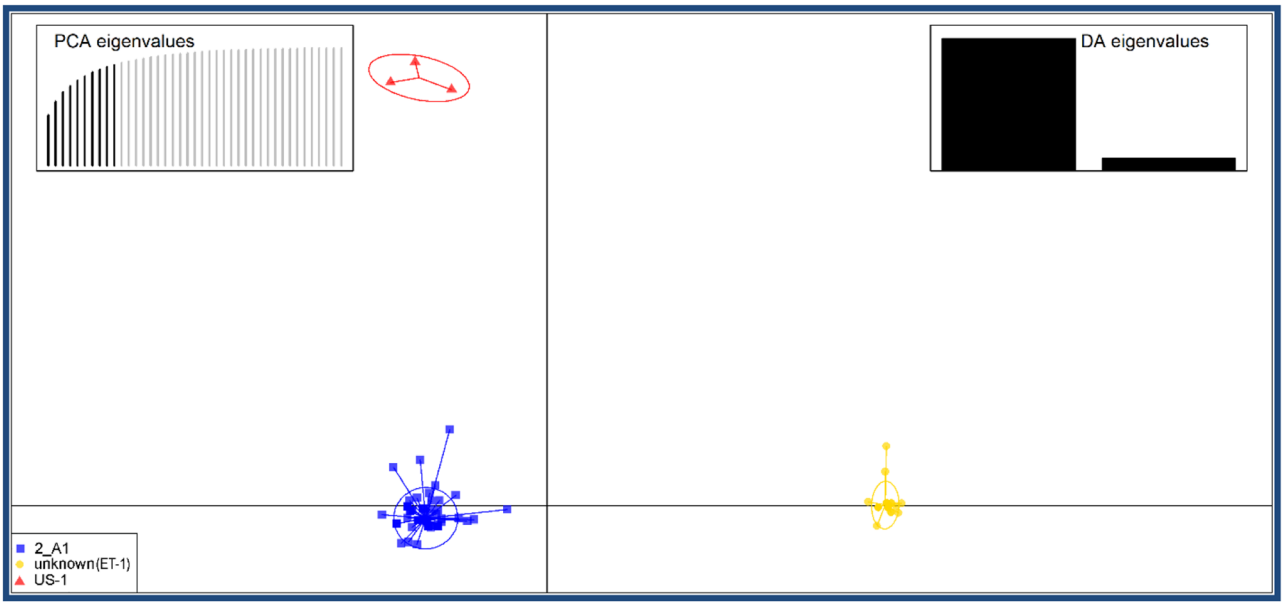

Fig. 2 Discriminant analysis of principal components (DAPC) plot based on the microsatellite marker analysis separating the *Phytophthora infestans* isolates into three clonal lineages EU2_A1, ET-1, and US-1. The axes represent the first two Linear Discriminants (LD). Each circle represents a cluster and each dot represents an individual isolate. Pathogen clonal lineages represented are US_1, 2_A1 and unknown (ET-1)

Discussion

This research investigated the genetic diversity of *P. infestans* population infecting potato crop in the main potato growing-regions of Ethiopia. The STRUCTURE analysis, UPGMA and DAPC analysis of the SSR fingerprints grouped the 138 Ethiopian isolates into two clusters: 100 isolates grouped together with the EU2_A1 control isolates and the remaining 38 formed a separate cluster distinct from the US-1 control isolates and the EU2_A1 cluster. Hence, it appears that the current *P. infestans* population in Ethiopia consists of two clonal lineages: a European EU2_A1 genotype and a new lineage named as ET-1. Both lineages had the same mitochondrial DNA haplotype *Ia*.

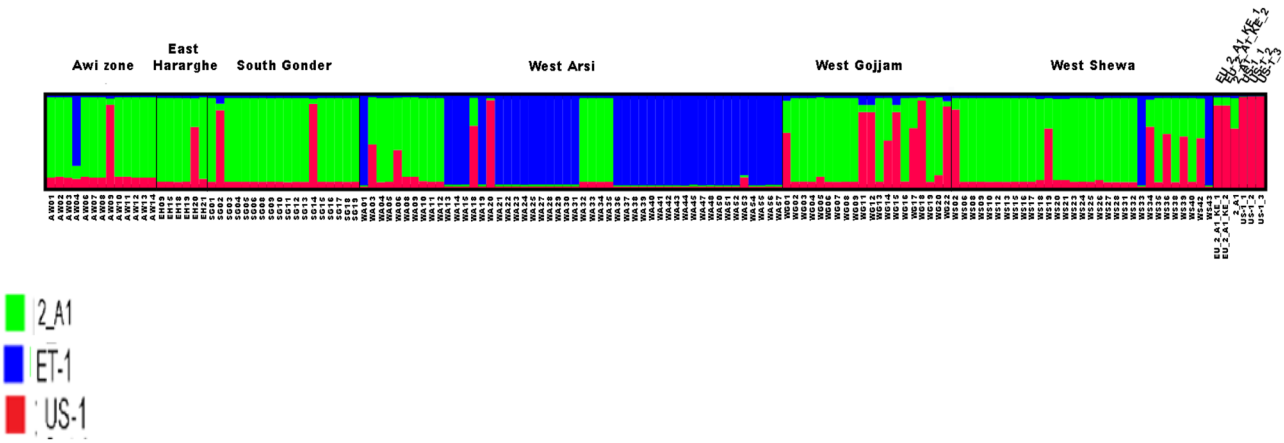

Fig. 3 Bayesian clustering of *P. infestans* genotypes based on the admixture ancestry model using STRUCTURE. The optimum value of K inferred with the Evanno's method was 3. Shown are STRUCUTRE plots for K = 3

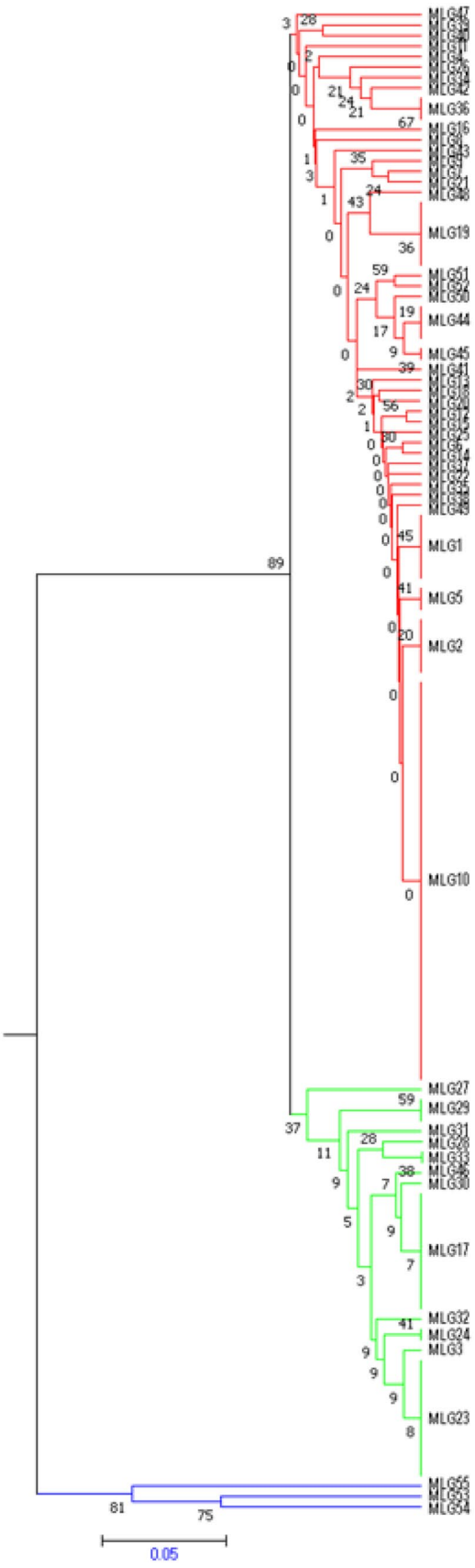

Fig. 4 Unweighted paired group method with arithmetic averages (UPGMA) dendrogram of *Phytophthora infestans* isolates based on Bruvo distance and clone corrected data. The clonal lineages are indicated in different colors: EU2_A1 (red), ET-1 (green) and US-1 (blue)

The old US-1 lineage was not found among the samples suggesting that it is displaced by EU2_A1 and ET-1 lineages. This finding is in line with the global trend of population shifts where old genotypes have been replaced by new ones (Chowdappa et al. 2015; Fry et al. 2015; Saville et al. 2016). It is not known when the EU2_A1 lineage has arrived in Ethiopia and whether it has co-existed with US-1 lineage. In other Eastern African countries Uganda, Rwanda, Burundi and Tanzania the US-1 lineage has been still reported albeit in low frequency, while in Kenya it was displaced by the EU2_A1 lineage (Njoroge et al. 2019a).

It is likely that these new lineages in Ethiopia pose a fitness advantage over the US-1 lineage as has already been shown for the EU2_A1 genotype by Njoroge et al. (2019b). The authors of the study hypothesized that the reason for the reduced aggressiveness of the US-1 lineage could be because of the many mutations accumulated during its relatively long existence (Njoroge et al. 2019a). Indeed, accumulation of deleterious mutations is one of the evolutionary consequences of clonality (Goodwin 1997) and may explain the displacement of HERB-1, the strain of *P. infestans* that was responsible for potato famines of the 1840s in Europe (Yoshida et al. 2013).

The new lineages have been most likely introduced to Ethiopia in imported seed potatoes. Movement of tuber seed, fruits or other plant parts account for most of the global changes in the *P. infestans* populations (Chowdappa et al. 2015). It remains to be studied, what could be the origin of the ET-1 lineage. In any case it is evident that both lineages have already accumulated several mutations as was evidenced by the discovery of several multilocus genotypes among both lineages.

The EU2_A1 genotype was the dominating type in all other regions except for West-Arsi where ET_1 was sampled more frequently. This area in Ethiopia has also previously been found to contain unexpected diversity as a new mt haplotype resembling that of *P. andina* was discovered in potato (Shimelash et al. 2016). The ET_1 isolates are clearly a different genotype as their mt haplotype is *Ia* and the SSR marker pattern is distinct from that of *P. andina* (Forbes et al. 2016). Adaptation of the EU2_A1 genotype to both potato and tomato was discovered in Kenya (Njoroge et al. 2019b). Therefore, it will be important to survey also tomato plants in Ethiopia to investigate the level of host specificity of the two new lineages. This is epidemiologically important because in the absence of host specialization either crop can serve as an inoculum reservoir for the other.

Table 1 Diversity statistics for microsatellite data for the 12 microsatellite loci in populations of *Phytophthora infestans* in Ethiopia by location

Population	N	MLG	eMLG	SE	H	G	lambda	E.5	Hexp	Ia	rbarD
Awi	13	4	3.54	0.57	1.091	2.52	0.604	0.77	0.515	0.49876	0.2862
Eastern Hararghe	6	5	5	0	1.561	4.5	0.778	0.93	0.488	0.2027	0.07383
South Gondar	18	7	4.33	1.02	1.234	2.16	0.537	0.477	0.489	*0.62506*	0.23384
West Arsi	50	20	6.67	1.26	2.452	7.53	0.867	0.616	0.497	0.00259	0.00042
West Gojjam	20	11	6.39	1.11	1.999	4.88	0.795	0.608	0.512	0.40689	0.08243
West Shewa	31	11	5.32	1.14	1.833	4.2	0.762	0.609	0.48	0.1089	0.01586
All	138	58	7.47	1.31	3.164	10.16	0.902	0.405	0.515	1.1487	0.13649

N number of individuals, *MLG* number of multilocus genotypes, *eMLG* expected number of MLGs, *SE* Standard error, *H* Shannon- Weiner Index of MLG diversity, *eHexp* expected heterozygosity, *Ia* Index of Association, *rbarD*. Standardized index of association, *Lambda* Simpson's Index, *E.5* Evenness

A recent analysis of the virulence spectrum of *P. infestans* isolates from potato sampled from Awi, South Gondar and West Gojjam in Ethiopia detected 27 physiological races based on the infection pattern on the late blight differential set (Mihretu et al. 2020). According to the same study almost all isolates were virulent on R1, R2, R3, R4, R6, R7, R10, and R11 differentials, and furthermore, roughly half of the isolates were also virulent on R8, while virulence to R5 and R9 was less frequent (Mihretu et al. 2020). Considering these results, it appears that the resistance based on R9 and R5 might still be functional in Ethiopia. In future studies it will also be important to analyze the virulence spectrum of the isolates from West Shewa and West Arsi, since those populations contain the new ET-1 genotype that may differ in this respect.

Monitoring of the *P. infestans* populations using the 12 plex SSR assay offers the advantage of easy comparison of the marker patterns of already characterized isolates available in databases such as Euroblight. The utility of such a methodology was recently demonstrated by Nnadi et al. (2019) as they were able to identify another European clonal lineage EU_33_A2 for the first time on potato in Nigeria. The appearance of the A2 mating type or other clonal lineages should be carefully monitored, as these may have significant implications on the management strategies of late blight in Ethiopia. To facilitate such comparisons, we have included the allele size information of each SSR locus and isolate characterized in this paper in the Supplementary Table 2.

Table 2 Population statistics of the 12 microsatellite loci in populations of *Phytophthora infestans* in Ethiopia

Locus	Alleles	1-D	Hexp	Evenness
D13	9	0.501	0.503	0.618
SSR8	3	0.666	0.667	0.998
SSR4	8	0.696	0.697	0.864
Pi04	2	0.5	0.502	1
Pi70	3	0.52	0.522	0.917
SSR6	6	0.517	0.519	0.887
Pi63	3	0.666	0.668	1
G11	9	0.562	0.564	0.77
SSR3	5	0.533	0.535	0.857
SSR11	2	0.497	0.499	0.994
SSR2	4	0.488	0.49	0.89
Pi4B	2	0.021	0.021	0.353
Mean	4.667	0.514	0.515	0.846

1-D Simpson index, *eHexp* expected heterozygosity

Conclusions

P. infestans population in Ethiopia is composed of two clonal lineages. Of these one is a genotype of European origin, EU2_A1 that appears to dominate in most of the sampled areas. In addition, a new genotype, called ET-1 was found dominating in in the southern part of the country in West Arsi. The findings of this study establish a baseline of the pathogen population diversity in the country. We recommend a continuous monitoring of the *P. infestans* population in both potato and tomato crops using the 12-plex SSR assay to enable the comparison of the epidemics in time and space and allow for monitoring of the appearance of new lineages. The method can also be utilized to trace the movement of *P. infestans* in tuber seed or other plant parts to aid the phytosanitary measurements.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s42161-021-00820-6.

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Declarations

Conflict of interest No potential conflict of interest.

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