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Distribution and mating type characterization of chickpea blight (*Didymella rabiei* (Kov.) v. Arx) in Ethiopia

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ABSTRACT

We have assessed the distribution and mating type of chickpea blight (*Didymella rabiei* (Kov.) v. Arx) from a total of 350 and 272 fields in major chickpea (*Cicer arietinum* L.) growing areas of Ethiopia for two consecutive cropping seasons 2017 and 2018, respectively. The prevalence of *D. rabiei* was 20 % for the year 2017 and 7% for 2018. Severity range of 2−9 and incidence of 10–100 % were recorded for both seasons in only 7–20 % of the assed fields. Field prevalence of 2017 and 2018 seasons were significantly different ($df = 31$; $p < 0.001$ and $df = 31$; $p < 0.001$, respectively). Similarly, the disease prevalence of both seasons was significantly different ($df = 31$; $p < 0.001$). The disease prevalence ratio frequency was highly deviated from (1:1) for both seasons ($X^2 = 213.62$ p= <0.001; $X^2 = 144.36$ $p = 0.01$). Genomic DNA of Representative samples were extracted from single spore culture and Mating type 1 (MAT1-1) specific primer SP21, Mating type 2 (MAT1-2) specific primer Tail 5, and a flanking region-specific primer Com1 assay were multiplexed in a single PCR reaction to determine the occurrence of *D. rabiei* mating type in Ethiopia. Out of 156 samples, only 15 samples were positive to MAT1-1 (~10 %) with the ratio of 9:1. MAT 1−2 type was the most dominant and possibly the asexual reproduction of *D. rabiei* is the major type in Ethiopia. The result is important for Ascochyta blight management in breeding strategy.

1. Introduction

Chickpea (*Cicer arietinum* L.) is the third most important grain legume in the world in area coverage, volume of production and trade [1,2]. The crop is primarily grown on the Indian subcontinent, in West Asia, Middle East, Canadian prairies, Mexico, Ethiopia, northern Africa, Australia, southern Europe and northwestern USA [1]. Ethiopia is the largest producer of chickpea in Africa accounting for about 46 % of the continent's production and shares about 4.5 % of the global chickpea market and more than 60 % of Africa's global chickpea market [3–5]. With an increasing trend every year, in 2016, pulses accounted for 6.93 % of Ethiopia's export earnings which represent 3% of world pulse exports, and contributed more than US $ 248 million to the country's hard currency reserves http://www.intracen.org. Chickpea exports were about 25 % of all pulses exported [6]. Chickpea is widely grown in Ethiopia and produced by smallholder farmers either as sole or double cropping with residual moisture on vertisols. It is the third most important pulse crop produced in Ethiopia with 1.63 % (about 208,838 ha) of total area coverage of grain crops [7]. Chickpea covers about 14.5 % and 13.4 % of production volume and area coverage of all pulse crops in Ethiopia respectively [7].

Ascochyta blight caused by *Ascochyta rabiei* (Teleomorph= *Didymella rabiei*) is the most important disease of chickpea production in both small- and large-scale farming of the world [8]. The disease infects all aerial parts of the plant and produces circular necrotic lesions. Under favorable or conducive environmental and genetic conditions, the disease can devastate up to 100 % of the crop yield [9]. Due to unmanaged chickpea blight epidemics, farmers have shifted their common chickpea growing season in Tunisia [10]. The rapidly increasing trend of chickpea production have been limiting by Ascochyta blight outbreak and currently, 95 % of chickpea growing areas are potentially affected [11,12]. In Ethiopia, chickpea blight was reported in 1969 and it is a

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potential threat for chickpea production when favorable conditions prevail [13,14].

Didymella rabiei is heterothallic with a bipolar, biallelic mating system [15]. In heterothallic ascomycete fungi, the presence of isolates of opposite mating types is required to complete their sexual cycle. The two mating types were not reported from all chickpea-growing areas [16]. In all ascomycetes tested to date, sexual reproduction is governed by a single regulatory locus mating-type (MAT) gene and these are designated as MAT1−1 and MAT1−2 [17].

The sexual stage of *D. rabiei* was first reported from Bulgaria in 1936 by Kovachevski [18] and later reported from the USSR [19], Greece [20], Hungary [21], the United States [22], Spain [23], Syria [24], Tunisia, Turkey and Canada [10]. The presence of both mating types (MAT1–1 and MAT1–2) and the teleomorph have been reported from most chickpea-growing regions in the world [17,22,25–31]. The amount of genetic variability of *D. rabiei* is enhanced by the presence of the

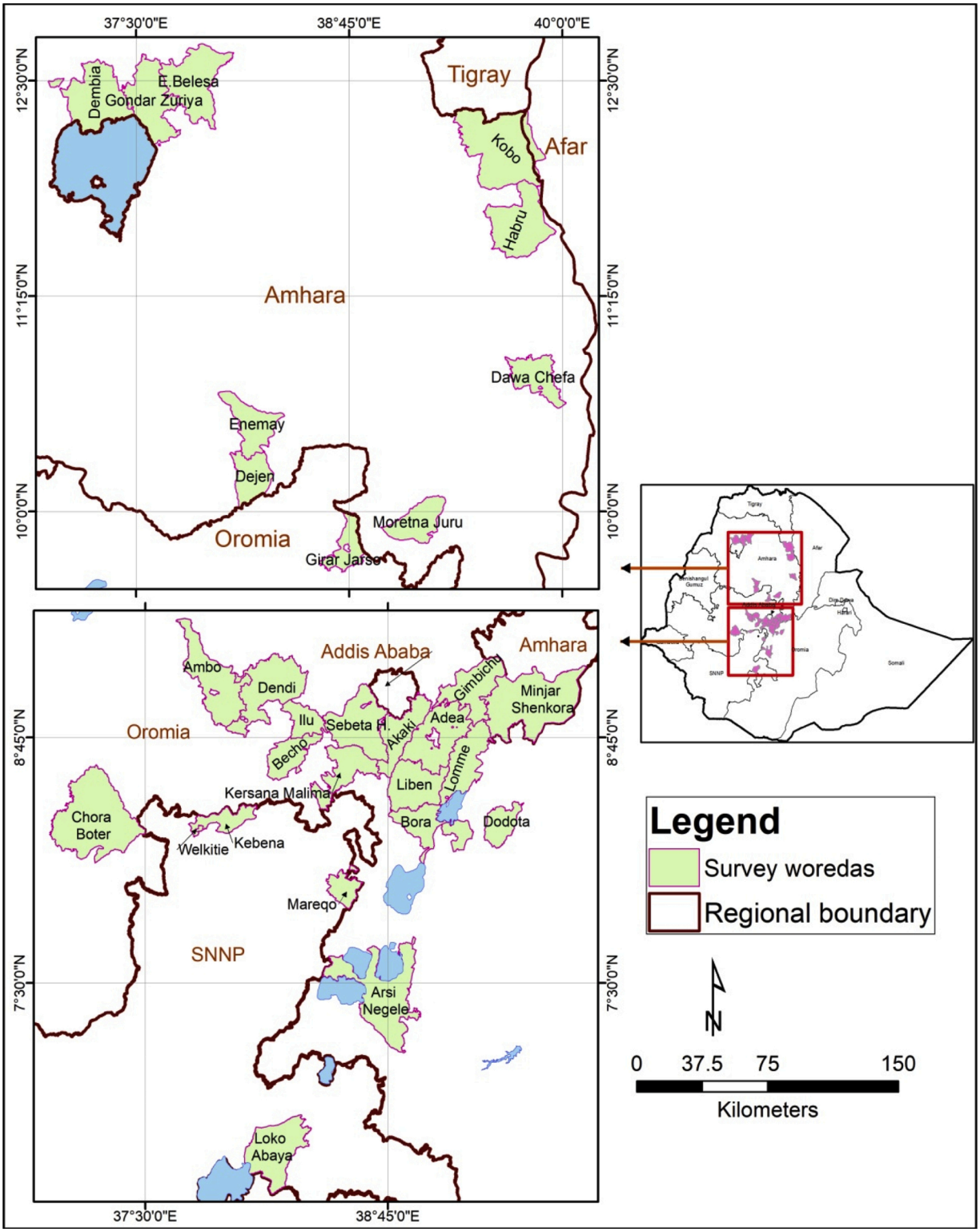

Fig. 1. Map showing Districts of Ethiopia where assessments of Ascochyta blight of chickpea were done and samples collected.

teleomorphic or sexual stage [32]. This may contribute to the emergence of new virulent pathogen strains with resistance to fungicides being used [26,33,34].

Alternate sequences at the mating-type locus are completely dissimilar or idiomorphic and code for different regulatory genes [35]. A DNA binding protein containing domain is coded by the mating-type 1 gene, or MAT1–1, and DNA binding protein that contains a high mobility group domain is coded by MAT1–2 [36,37]. *D. rabiei* has been spread around the world, mainly by human activity through the movement of infected seeds [30]. Although chickpea cultivation and Ascochyta blight have been known for many years in Ethiopia, the sexual stage (the variant of mating-types) of the pathogen has not been studied. This study, report the results of extensive surveillance on the current status of *D. rabiei* and ratio of its mating types across major chickpea growing agro-ecologies using MAT specific primers, and propose its mode of reproduction in Ethiopia.

The major management options in reducing the negative impacts of Ascochyta blight are uses of resistant cultivars and strategic application of fungicides [2,9,45]. In some countries, Ascochyta blight resistant cultivars become susceptible due to increased aggressiveness/virulence of pathogen populations [10,48]. Although Ascochyta blight have been known for many years in Ethiopia, the mating types of the pathogen was not studied. This study, report the results of extensive surveillance on the current status of *D. rabiei* and ratio of its mating types across major chickpea growing agro-ecologies using MAT specific primers, and propose its mode of reproduction in Ethiopia.Therefore, the objective of this suty was to determine the existence of the two mating types and their distribution in chickpea growing areas of Ethiopia.

2. Materials and methods

2.1. Surveys

The prevalence of chickpea blight was assessed for two consecutive seasons (2017 and 2018) in major chickpea growing areas of Ethiopia (Fig. 1). A total of 352 in the first season and 270 chickpea fields in the second season were inspected thoroughly for the presence of chickpea blight (Table 1). The surveillance was done randomly alongside of the main road every 5–10 km distance. Chickpea fields found with infected chickpea plants, counts as positive for chickpea blight. Representative samples (leaves, stem and pods) were collected for further laboratory diagnosis and mating type analyses from each geographic location.

2.2. Pathogen isolation and DNA extraction

Infected samples (leaves, stems and pods) of chickpea were cut into pieces (about 1 cm) and surface-disinfected with 1.5 % concentrated house bleach for 1 min and rinsed with sterile distilled water. Surface-

Table 1
Prevalence of chickpea blight around major chickpea growing areas of Ethiopia in 2017 and 2018.

Region	District	Number of assessed fields with disease prevalence's (per seasons)				% Prevalence comparison of (A) &(B)		Incidence %		Severity (1−9 scale)			
										2017		2018	
		2017 (A)	Prev.	2018 (B)	Prev.	2017 (A)	2018 (B)	2017	2018	Range	Mean	Range	Mean
Amhara	Gonder Zuria	15	0	8	0	0	0	0	0	1	1	1	1
	West Denbia	30	0	10	0	0	0	0	0	1	1	1	1
	West Belesa	15	0	14	0	0	0	0	0	1	1	1	1
	East Dembia	24	0	10	0	0	0	0	0	1	1	1	1
	Kobo	25	16	10	8	64	80	40	30	3−7	3	3−6	3.8
	Minjar	26	12	28	5	46	18	10	15	3−3	3.3	3−3	3.3
	Chefa	1	0	1	1	0	100	0	15	1	1	3	3
	Bichena	3	0	0	0	0	0	0	0	1	1	1	1
	Inewari	2	0	0	0	0	0	0	0	1	1	1	1
	Dejen	3	0	0	0	0	0	0	0	1	1	1	1
	Sirinka	1	0	0	0	0	0	0	0	1	1	1	1
	Becho	10	0	20	0	0	0	0	0	1	1	1	1
	Dendi	20	0	15	0	0	0	0	0	1	1	1	1
	Teji	6	0	5	0	0	0	0	0	1	1	1	1
	Sebeta	12	3	15	0	25	0	30	0	3−4	3.3	1	1
	Lume	15	3	18	0	20	0	20	0	2−3	2.67	1	1
	Adea	35	8	15	1	23	7	35	10	2−7	3.5	2	2
	Dhera	1	1	1	1	100	100	100	100	8	8	8	8
Oromia	Fiche	5	0	0	0	0	0	0	0	1	1	1	1
	Arsi Negele	1	1	0	0	100	0	0	0	5	4	1	1
	Alem Tena	1	1	1	1	100	100	100	100	7	7	7	7
	Tolay	15	6	20	2	40	10	10	5	2−3	2.67	2	2
	Ambo	5	0	3	0	0	0	0	0	1	1	1	1
	Olan komi	5	0	5	0	0	0	0	0	1	1	1	1
	Gimbichu	8	1	0	0	12.5	0	100	0	5	5	1	1
	Akaki	10	3	15	0	30	0	15	0	3	3.67	1	1
	Qersa Malima	15	5	21	0	33	0	25	0	2−9	4.2	1	1
	Gibe Valley	9	6	10	0	67	0	30	0	2	2.83	1	1
	Adulala	12	5	10	0	42	0	20	0	4		1	1
	Walkite	10	0	12	0	0	0	0	0	1	1	1	1
SNNP	Mareko	2	0	3	0	0	0	0	0	1	1	1	1
	Sidama	10	0	0	0	0	0	0	0	1	1	1	1
Total		352	71	270	19	20.2	7						
$\chi^{2=}$			213.62		144.36								
p			P<0.001		P<0.01								
% Prevalence			20.2		7								
Mean t-test variance		11***	2.22	8.44***	0.59	2.22***	0.59						
Pearson correlation		84.84	14.82	62.77	2.76	14.82	2.76						
		0.56		0.29		0.85							

*Range and mean were computed only from *D. rabiei* positive fields; *** = p<0.0001; SNNP = Southern Nation and Nationalities People.

disinfected samples were placed on 2% water agar medium and incubated at room temperature (~25 °C) for 48–72 h. Following the release of conidia, infected plant parts were removed and small cubes of water agar with conidia were transferred to Potato Dextrose Agar (PDA) and incubated at 25 °C from 5 to 7 days. Single spored pure colonies of *D. rabiei* of each isolate were formed, and stored at -20 °C. For DNA extraction, isolates were first cultivated on PDA at 20 °C for 10–15 days. Mycelium was scraped from the surface of the plates and used to initiate cultures in 250 mL flasks containing 50 mL of liquid 2-YEG (Yeast Extract Glucose) medium (2 g per liter yeast extract, 1 g per liter glucose) for 5–6 days on a rotary shaker at 150 rpm and 23 °C. Mycelia harvested from the flasks were lyophilized in 9 cm Petri dishes and stored at −80 °C. Mycelium of each isolate was finely ground using liquid nitrogen and total genomic DNA was extracted at Holetta Agricultural Biotechnology Research Center following using Plant Kit® (Bioline Ltd) with its protocol.

2.3. ITS marker, PCR amplification and sequencing

The ITS1−5.8S-ITS4 regions of the rRNA of *D. rabiei* isolates were amplified using fungal universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGA TATGC-3′) as described by White et al., [38]. The primers were combined in a single PCR reaction with equal concentrations and carried out in a total of 10 μL volumes containing 1 μL genomic DNA, 5 μL of PCR master mix HotStarTaq® (QIAGEN Group), 0.5 μL of each primer and 3 μL nuclease free water. Cycling conditions consisted of an initial denaturation at 95 °C for 15 min followed by 35 cycles of 94 °C for 30 s, 56 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 7 min. Amplified PCR products were separated by 1% agarose gel electrophoresis stained with ethidium bromide (4 ng/mL) and visualized using UV light trans-illumination. The PCR amplicon size was measured using 100 bp DNA size ladder (Invitrogen). Purification of PCR amplicons was done by ExoSAP-IT PCR product Cleanup Reagent (Thermo Fisher Scientific, USA) according to the manufacturer's instructions before sequencing.

2.4. Determination of D. rabiei mating types

The frequency of *D. rabiei* mating types was studied from 156 samples of major chickpea blight hot spot areas across the country. Mating types of *D. rabiei* was determined using three mating-type specific primers and genomic DNA were multiplexed in equal concentrations and amplified in a single reaction. The mating type of each isolate was determined using the MAT-specific primers: MAT1-1-specific SP21 (5′ ACAGTGAGCCTGCACAGTTC 3′), MAT1-2 specific Tail 5 (5′ CGCTATTTTATCCAAGACACACC 3′), and a flanking region-specific Com1 (5′ ACAGTGAGCCTGCACAGTTC 3′) profiles that developed by Barve et al., [17]. PCR reactions were carried out in 10 μL volumes containing 1 μL genomic DNA, 5 μL of PCR HotStar master mix (Quagen ltd), 0.5 μL of each primer and filled to the total volume by nuclease free water. Cycling conditions consisted of an initial denaturation at 95 °C for 15 min followed by 35 cycles of 94 °C for 30 s, 56 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 7 min. Amplified products were separated by 1.5 % agarose gels that stained with ethidium bromides. A 100 bp DNA ladder (Invitrogen) was run in the outer lanes of the gel as a size standard. All mating types and ITS amplicons were sequenced in both directions at Macrogen (Netherlands).

2.5. Data analyses

The data of chickpea blight prevalence, mating type, mating types ratio were collected from diseased fields. Disease prevalence, incidence and severity were taken in 1−9 rating scale (1 = No symptom visible; 2 = minute lesions prominent on the apical stems; 3 = lesions up to 5−10 cm and drooping of the apical stems; 4 = lesions clearly seen and prominent on the apical stems; 5 = lesions on all plant parts and defoliation, breaking of stems started; 6 = lesions as in 5 but defoliation, broken, dry branches common, some plants killed; 7 = lesions as in 5 but defoliation, broken, dry branches common, up to 25 % plants killed; 8 = lesions as in 7, up to 50 % plants killed; 9 = symptoms as in 7, up to 100 % plants killed [45]. The electrophoresis bands of different sizes were analyzed in respect to their size (MAT 1-1 about 700bp and MAT 1-2 about 460bp). Chi-square (χ^2) test with one degree of freedom was computed for both seasons to test mating-type ratios, which are expected to be 1:1 for a randomly mating population [39]. Sequences were aligned, trimmed and Phylogenetic trees of ITS region and mating types sequences were constructed to check further grouping of the sequence with their respective types for confirmation using Geneious version 2019.2 created by Biomatters. Available from https://www.geneious.com.

3. Results

3.1. Prevalence of chickpea blight

Table (1) shows, the majority of assessed fields were free of chickpea blight; but only 20 % and 7 % of the chickpea field were found infected by chickpea blight disease in the first and second seasons, respectively. The chickpea blight disease prevalence fluctuated across the seasons and locations. A wider prevalence of blight was observed in 2017 than in 2018. In 2017, the incidence was unusually observed where it has not been reported before like Gimbichu, Sebeta, and Qersa Malima areas with minor to higher severity of (2−9) with complete loss (on kabuli type 'Shasho' variety). Alem Tena, Dhera, Arsi Negele, Kobo, Tolay and Minjar were areas where higher chickpea blight incidences and severities were recorded in both seasons. The assessed fields of 2017 and 2018 seasons and their respective prevalence were significantly different ($df = 31$; $p < 0.001$ and $df = 31$; $p < 0.001$, respectively) (Table 1). The disease prevalence ratio was highly deviated from (1:1) for both seasons ($\chi^2 = 213.62$ p= <0.001; $\chi^2 = 144.36$ p = 0.01, respectively).

3.2. Inter transcribed spacer (ITS) region for confirmation

About 88 representative isolates from each location were sequenced and confirmed the identity of the organism as *D. rabiei* showing 99–100 % nucleotide sequence similarity on National Center for Biotechnology Information (NCBI) (Only three sequences of them used for tree construction). The tree branches were well supported with a bootstrap score (>50 %), Phenogram of genetic relationship among Ethiopian *D. rabiei* isolates and global collections from GenBank mixed with no geographic area of origin (Fig. 2).

Mating type multiplex PCR and frequencies

The multiplex PCR bands on the gel were differentiated by the two mating types in different sizes (Fig. 3). The upper bands (about 700bp amplicons) belong to MAT1−1 and the lower bands (about 460bp) where belong to MAT1−2. The frequency of occurrence and distribution of MAT1−1 were low (10 %) as compared to MAT1−2 (Table 2; Fig. 4). Both mating types were found only in 5 (35.7 %) areas out of 14 sampling areas (Table 2).

The phylogenetic tree of the gene sequences of the two mating types was clustered clearly in a separate group with high support of bootstrap (Fig. 4).

4. Discussion

Ascochyta blight of chickpea is considered as economically the most important disease of chickpea and cause huge losses to chickpea production around the world [8]. Its epidemics are a repetitive incident in some chickpea growing parts of Ethiopia and it is an important disease in early-planted chickpeas when rainfall extends beyond September [13,14,40,41]. In the current study, chickpea blight prevalence was found lower in major chickpea production areas of the country but there were

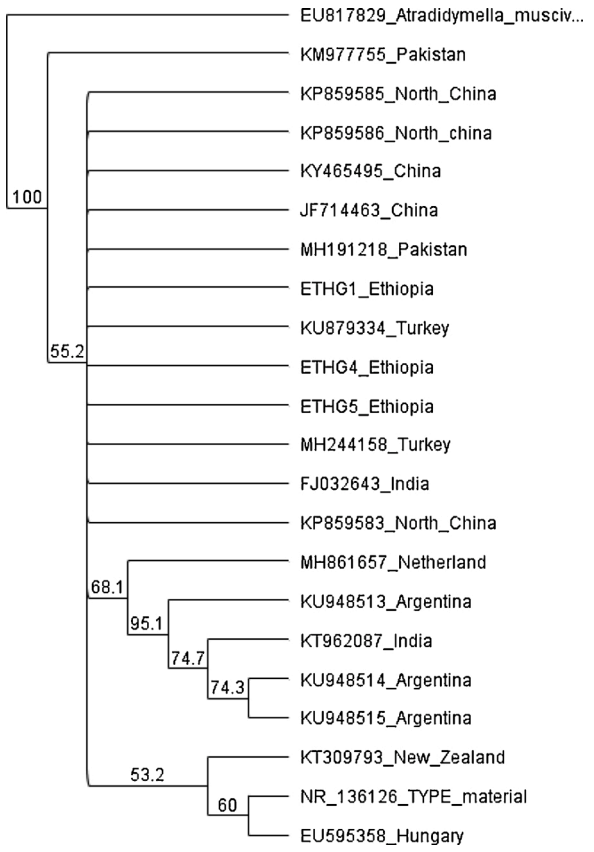

Fig. 2. Neighbor-joining phylogenetic tree generated from the sequences of ITS region of *D. rabiei* isolates along with other country's isolates at bootstrap values (1000 replicates) build by Jukes Canter genetic distance model (*Atradidymella muscivora* used as outgroup). Geneious version 2019.2 was created by Biomatters. Available from https://www.geneious.com.

differences over seasons, which imply its potential occurrence with favorable environmental conditions. The disease was not recorded in both seasons from Gonder and Becho areas (south west Shoa), the major chickpea production areas of the country. This does not mean that the disease will not occur in the future. Earlier, Ascochyta blight of chickpea was not a major disease in Ethiopia and it was reported from few places around Deberzeit, Mekele and Kobo with lower severity about 10 % in 1975/76 cropping season and also lower severity were recorded on the survey done in 2017 [13,14,41]. Chickpea blight is highly dependent on favorable weather conditions, (cool and wet) with an optimum temperature of 16−20 °C and needs a minimum of 6 h of leaf wetness [34,9]. Cloudiness and prolonged wet weather favor rapid development and spread of the pathogen. Disease severity increases with the increase in relative humidity. Chickpea is the dominant pulse crop at Minjar where this disease is prevalent recurrently. For this reason, the resistant kabuli type cultivar "Arerti" was the predominant chickpea type in the area (own data not published). Alem Tena and Dhera are chickpea germplasm blight screening sites of the Ethiopian Institute of Agricultural Research which are hot spot areas of chickpea blight in Ethiopia.

In Ethiopia, the earliest finding of chickpea cultivation was reported as of 1520 B.C. from Lalibela cave and it is considered as a secondary center of diversity and *Cicer cuneatum* is the wild relative of cultivated chickpea found in Tigray region of Ethiopia [42–44]. However, chickpea

Table 2
. Frequency of mating types of *Didymella rabiei* in different locations.

Sample areas	Number of samples	MAT 1−2	MAT 1−1
Debre Zeit	31	31 (100)	0
Chefa Dawa	4	2 (50)	2
Liben	27	27 (100)	0
Dhera	1	1 (100)	0
Dire chukala	9	9 (100)	0
Gimbichu	14	14 (100)	0
Kobo	10	3 (30)	7
Lume	4	4 (100)	0
Meki	2	2 (100)	0
Minjar	15	15 (100)	0
Leman	14	14 (100)	0
Arsi Negele	18	16 (100)	2
Sirinka	1	0 (0)	1
Tolay	6	3 (50)	3
Total	156	141	15
% prevalence		91.1	9.9
Mean	11.14	10.07	1.071
Variance	88.29	97.46	3.92
χ^2		125.8	50.92
P-value		P<0.0001	P<0.0001

*Numbers in parenthesis are the percentage of MAT 1−2 in the total samples per sample areas.

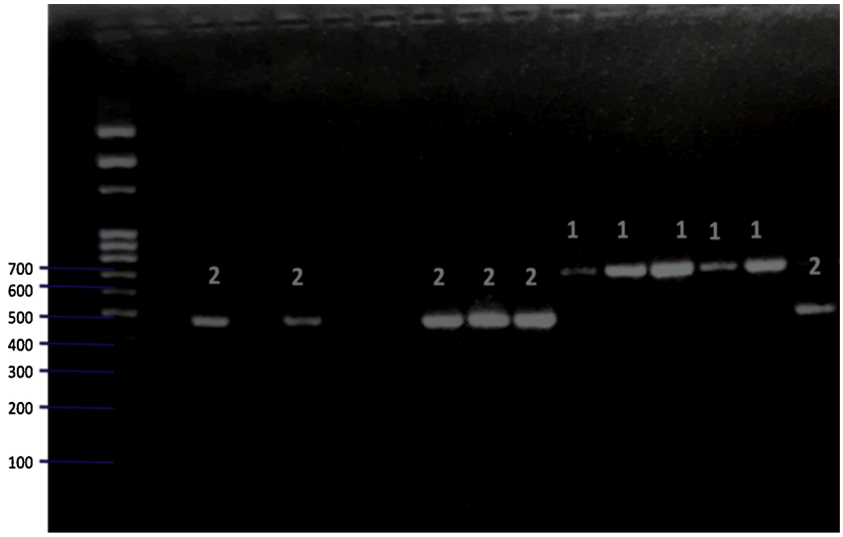

Fig. 3. Multiplex PCR analysis of *D. rabiei* mating types in Ethiopia with primers Com1, SP21 and Tail 5. The ladder (Gene ruler) size marker contains the molecular weight of 100 bp DNA ladder. Lanes with number 1 contain MAT 1-1 isolate of *A. rabiei* (700bp) and lanes with number 2 contain MAT 1-2 isolates of *A. rabiei* (460bp).

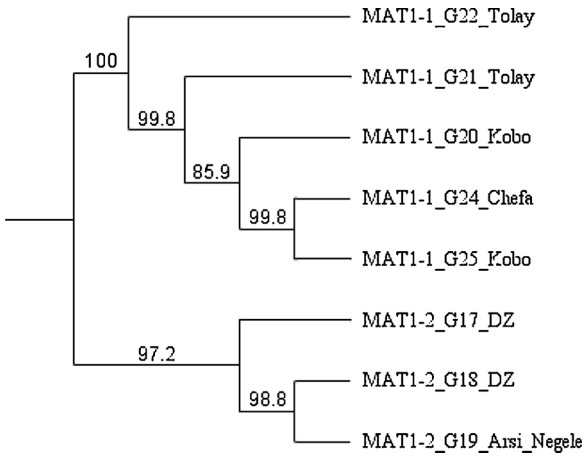

Fig. 4. Phenogram of representative sequences of MAT1-1 and MAT1-2 samples from Tolay, Kobo, Chefa, Debrezeit (DZ) and Arsi Negele.

blight was not well known in Ethiopia until it was first observed around 1969 at Kulumsa- Arsi zone [14] which is now the wheat belt of the country. Chickpea Ascochyta blight has been reported from over 35 countries globally: Asia (Bangladesh, China, India, Iran, Iraq, Israel, Jordan, Lebanon, Pakistan, Syria and Turkey), Africa (Algeria, Cyprus, Egypt, Ethiopia, Kenya, Libya, Morocco, Sudan, Tanzania and Tunisia), Europe (Bulgaria, France, Greece, Hungary, Italy, Portugal, Romania, Spain and Ukraine), North America (Canada and USA), South America (Columbia and Mexico); and Australia [8,45,46]. Both mating types have been reported in at least 15 countries. The presences of both mating types were not yet reported from Ethiopia. Morphological identification using maturation of pseudothecia by crossing isolates of unknown mating type with known tester strains is time-consuming and complicated [15,26]. However, after the development of mating type-specific primers by Barve et al. (2003), which uses multiplex PCR specific primers (Com1, SP21, and Tail 5) for each idiomorph at the MAT locus, became easy and rapid to identify the *D. rabiei* mating types [17]. Com1 was specific to the 3′ flanking regions of the MAT genes, SP21 designed to MAT1-1 and Tail 5 was designed to MAT 1-2 and they amplified an approximate of 700-bp fragment from MAT1-1 isolate, while a 460-bp PCR product was amplified from MAT1-2 isolates [47]. The frequency of occurrence for both mating types of *D. rabiei* in Ethiopia was significantly dominated by MAT1-2. Both mating types (MAT1-1 and MAT1-2) were reported from many chickpea growing countries in the world like Tunisia, Algeria, Morocco, Egypt, Italy, Portugal, Greece, Turkey, Spain, USA and Canada [17,27. 30, 10]. The occurrence and distribution of mating type of *D. rabiei* were not consistent for all production areas of the world. Similar occurrences were reported from India, Iran, Pakistan, Tunisia, and Turkey, where, MAT1-1 dominates over MAT1-2 [10,26]. In some countries like Israel, Australia, Egypt, Jordan, Syria and Lebanon, Cyprus, Greece, Italy, Libya and Morocco, MAT1-2 were more frequent than MAT1-1 [26,31]. But still many occurrences of equal ratio (1:1) were reported from different production areas of the world like the USA and Canada [29,30].

The different forms of alleles on a single locus of mating types are idiomorphic (highly dissimilar) stretches flanked by regions of DNA approximately 1.1 to 5.3 kb in size, which might contain from one to three open reading frames, in isolates of opposite mating type [36]. Resistance breakdown is possibly the greatest challenge in breeding for resistance where most cultivars lack complete resistance [9]. Resistance in breeding lines of chickpea to Ascochyta blight has not been durable because of the high variability in *D. rabiei* populations in several regions where chickpea is grown [49]. Most likely sexual reproduction leads to higher genotypic variability, which in turn increases the chance of evolving virulent pathogen populations. *D. rabiei* survives on infected or contaminated seeds and infected chickpea debris, on which it produces both rain-splashed conidia and windblown ascospores [10,39]. The development of the perfect stage in the natural condition should have to be studied in those areas where both mating types were found. Generally, in the two consecutive seasons, the occurrences of chickpea blight around major chickpea growing areas were very small. MAT1-2 was found the most dominant in Ethiopia than MAT1-1 at the ratio of 9:1, with the later mating-type being recovered only from few places. But, *D. rabiei* genetic diversity and virulence can be acquired through the introduction of the pathogen from its area of origin via planting materials. Relative frequency determination of sexual and asexual reproduction within and between population helps in the strategy of resistant breeding which is best option in Ascochyta blight management [10].

Declaration of Competing Interest

This work is the fulfillment of PhD study which supported by different organization. As per the institutions rule all are acknowledged and the respective authors also mentioned to their contribution. Hence, there is no conflict of interest.

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