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Diverse *Fusarium solani* isolates colonise agricultural environments in Ethiopia

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Abstract *Fusarium solani* is a fungal pathogen that infects many different genera of plants. It represents one of the two *Fusarium* spp. commonly isolated from agricultural soils and plant tissues in Ethiopia. To determine the diversity of *F. solani* in Ethiopia, we studied 43 isolates using Amplified Fragment Length Polymorphism (AFLP) and nucleotide sequences of the Translation Elongation Factor 1α (TEF-1α) and β-tubulin genes. TEF-1α sequences from GenBank, representing previously described species and clades of the *F. solani-Haematonectria haematococca* complex, were also included for comparative purposes. Phylogenetic analyses of the TEF-1α data separated the isolates into three groups corresponding with the three previously described clades (Clades 1–3) for this fungus. The Ethiopian isolates aggregated into one group corresponding to Clade 3. TEF-1α, β-tubulin and AFLPs further separated the Ethiopian isolates into a number of clusters and apparently novel phylogenetic lineages. Although the biological and ecological significance of these lineages and clusters is unclear, our data show that the Ethiopian agricultural environment is rich in species and lineages of the *F. solani-H. haematococca* complex.

Keywords AFLP · β-tubulin · Ethiopia · *Fusarium solani* · TEF-1α

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Introduction

Fusarium solani (teleomorph *Haematonectria haematococca*; Rossman et al. 1999; syn. *Nectria haematococca*) is a widely distributed soil-borne fungus pathogenic to at least 111 plant species spanning 87 genera (Kolattukudy and Gamble 1995). It causes wilt and rot diseases on a wide variety of crops including *Cucurbita* spp. (Hawthorne et al. 1992), *Pisum sativum* (Van Etten 1978) and *Phaseolus vulgaris* (Li et al. 1995).

Fusarium solani is distinguished from other *Fusarium* spp. based on a number of morphological features (Leslie and Summerell 2006). The most important of

these are the conditions under which it grows in culture (Matuo and Snyder 1973), the appearance of its chlamydospores, as well as long monophialides and macroconidia, although the macroconidia have been shown to vary in size with geographical origin (Burgess et al. 1994). Within this species, isolates are typically grouped based on pathogenicity, sexual compatibility and DNA-sequence comparisons. Based on pathogenicity to specific plant hosts, *F. solani* has been divided into ten *formae speciales* (Matuo and Snyder 1973), of which *forma specialis cucurbitae* includes two distinct races (Toussoun and Snyder 1961). These two races and five of the known *formae speciales* have been shown to represent distinct biological species designated as mating populations (MPs) I–VII, with *H. haematococca* as the teleomorph (Matuo and Snyder 1973; Van Etten and Kistler 1988). However, application of pathogenicity and sexual compatibility tests for diagnostic purposes is impractical, time-consuming and often inconclusive. This is because these assays are affected by factors such as the environment and genetic make-up of the host species, in the case of pathogenicity test, and fertility of the interacting individuals, in the case of sexual compatibility tests (Leslie and Summerell 2006). Furthermore, only positive mating tests provide guaranteed identifications (Matuo and Snyder 1973), and loss of virulence can result in lack of repeatability of experiments (Windels 1991).

Various DNA-based methods have been employed to identify or characterise isolates of *F. solani* (*e.g.*, O'Donnell 2000; Godoy et al. 2004; Zhang et al. 2006). Of these, phylogenetic analysis of nucleotide sequences for multiple loci appears to be the most informative, and has been used to show that *F. solani* represents a species complex (O'Donnell 2000) consisting of over 26 discrete phylogenetic species, seven of which correspond to MPs I–VII (O'Donnell 2000; Aoki et al. 2003, 2005). Another very informative DNA-based method for resolving inter- and intra-specific relationships is Amplified Fragment Length Polymorphism (AFLP, Vos et al. 1995). Although this technique has not been used previously for *F. solani*, we successfully employed it to study the phylogeny of *Fusarium oxysporum* isolates (Bogale et al. 2006). A major advantage offered by the AFLP technique is that it allows genome-wide sampling rather than considering specific regions as is the case with nucleotide sequences (Majer et al. 1996).

The objective of this study was to assess the phylogenetic relationships of *F. solani* isolates obtained from agricultural soils and plant tissues in Ethiopia using AFLP and DNA sequence analyses. Furthermore, we compared the Ethiopian collection with isolates representing previously characterised species and/or groups in the *F. solani-H. haematococca* species complex.

Materials and methods

Sample collection and isolation of *F. solani* strains

Soil samples were collected in September 2000 from 23 farms previously planted to different cereals and pulses (Table 1). Four composite samples were collected from an area of 4–6 m^2 at a depth of 3–15 cm along the diagonals of each farm visited. Wheat and barley samples were collected in August 2001 from three different farms in the Shoa region (Table 1). From each farm, samples were collected at five points along the diagonals of the field, where 10–15 plants close to harvesting were sampled from an area of ~4 m^2 at each point. Stem samples were collected from plants cut close to the ground. Banana root samples were also collected in August 2001 from 100 Cavendish plants grown on a commercial farm at Arbaminch (Table 1). Sampled banana plants were at least 10 m apart from each other. Five to eight root pieces, each ~5 cm in length, were taken from the selected banana plants at 5–10 cm depth from the surface of the ground.

Fusarium strains were isolated from air-dried soil samples following the dilution plate method (Nelson et al. 1983). Plant tissues (wheat stems, barley stems and banana roots) were surface-sterilised using NaOCl (1.5% *v*/*v*) before fungal isolation. Strains were first isolated from these samples on a *Fusarium*-selective pentachloronitrobenzene (PCNB) medium (Nash and Snyder 1962). Individual colonies were then transferred to MEA medium (2% *w*/*v* malt extract, 1.5% *w*/*v* agar), and incubated for 10–15 days at 25°C in the dark. Spores were gently washed from the surface of the cultures, diluted with sterile distilled water, and spread on a sterile water agar medium (1.5% *w*/*v*). After incubation at 25°C for 16–24 h under fluorescent light, single germinating spores were identified using a stereomicroscope, aseptically transferred to MEA plates and incubated

Table 1 Strain numbers, source and geographic origin of the *F. solani* isolates used in this study

Strain number	Source (host/substrate)[a]	Location[b]	
		Region	Lat./Long.
FCC3631, FCC3681, FCC3734, FCC3782, FCC3789, FCC3794, FCC3809, FCC4359, FCC4632	Banana root	Arbaminch	06.04/37.55
FCC3749	Barley stem	North Shoa	09.85/39.76
FCC2926, FCC3723, FCC4636	Soil (Barley)	Bale	07.86/39.63
FCC2934	Soil (Barley)	West Shoa	09.07/38.50
FCC3735	Soil (Barley)	Gondar	12.12/37.78
FCC3810	Soil (Barley)	Sidamo	05.88/38.98
FCC3695	Soil (Maize)	East Shoa	08.60/39.12
FCC3814	Soil (Maize)	Wollo	11.08/39.74
FCC3820, FCC4633	Soil (Maize)	Zeway	07.93/38.71
FCC3612	Soil (Pulses)	Gondar	12.12/37.78
FCC3639	Soil (Pulses)	Bale	07.08/38.70
FCC3689	Soil (Pulses)	Gondar	11.73/38.47
FCC3649, FCC3650	Soil (Sorghum)	Wollo	12.50/39.53
FCC4634	Soil (Sorghum)	Gondar	11.86/38.01
FCC2930	Soil (*tef*)	Gondar	11.60/37.40
FCC3613	Soil (*tef*)	Sidamo	05.63/38.23
FCC3632, FCC3686	Soil (*tef*)	Gojjam	10.69/37.26
FCC3661	Soil (*tef*)	Gondar	11.92/37.70
FCC3736	Soil (*tef*)	Wollo	10.72/39.87
FCC3776	Soil (*tef*)	Shoa	08.66/38.21
FCC3815, FCC3816, FCC3623	Soil (*tef*)	East Shoa	08.55/39.27
FCC4631	Soil (*tef*)	Wollega	09.09/36.53
FCC3625	Soil (Wheat)	Bale	06.97/39.18
FCC3670	Soil (wheat)	North Shoa	09.80/38.73
FCC4635	Soil (Wheat)	Wollega	09.50/35.50
FCC3727, FCC3748	Wheat stem	North Shoa	08.66/38.21
FCC4448	Wheat stem	West Shoa	08.98/37.85

[a] The crops in parentheses reflect those planted on the respective farms during the cropping season before the soil samples were collected in the subsequent off-season. The pulses were predominantly broad bean and peas, [b] Geographical positions (latitude/longitude) and regions where the farms were located

in the dark at 25°C for 10–15 days. Spore suspensions were prepared from these single-spore cultures and stored at −80°C as glycerol (15% *v*/*v*, aqueous solution) stocks for later use. Forty-three of these isolates were morphologically identified as *F. solani* (Table 1) as described by Nelson et al. (1983).

A *F. oxysporum* f.sp. *dianthi* (CBS No. 491.97; Table 2) strain obtained from the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands, was included in the study as an outgroup taxon for phylogenetic analyses. All of the strains used in this study are maintained in the *Fusarium* Culture Collection (FCC) at the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

DNA extraction and sequencing

DNA was extracted from isolates using CTAB (N-cetyl-N,N,N-trimethyl-ammonium bromide) (Murray and Thompson 1980). Fragments of approximately 650 base pair (bp) and 290 bp of the genes encoding Translation Elongation Factor 1α (TEF-1α) and β-tubulin, respectively, were then amplified using PCR. For this purpose primer sets EF1/EF2 (O'Donnell et al. 1998) and 2A/2B (Glass and Donaldson 1995), and their respective reaction and cycling conditions were used. PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Germany), and sequenced in both directions using the same primers as for the respective PCRs. The BigDye terminator

Table 2 Strain information for the various TEF-1α sequences obtained from GenBank and included in this study to represent known species and clades of the *F. solani-H. haematococca* complex

Strain number	Species	MP	Origin	GenBank Acc. No.
NRRL20438	*F. ambrosium*	-	India	AF178332
NRRL31779[a]	*F. brasiliense*	-	Brazil	AY320150
NRRL31949[a]	*F. cuneirostrum*	-	Brazil	AY320161
NRRL22090[b]	*F. illudens*	-	New Zealand	AF178326
CBS No. 491.97	*F. oxysporum* f. sp. *dianthi*	-	The Netherlands	DQ220144
NRRL31156[c]	*F. phaseoli*	-	USA	AY220187
NRRL22402[b]	*F. solani* f.sp. *batatas*	II	USA	AF178344
NRRL22142[b]	*F. solani* f.sp. *cucurbitae*	V	USA	AF178347
NRRL22153[b]	*F. solani* f.sp. *cucurbitae*	I	-	AF178346
NRRL22157[b]	*F. solani* f.sp. *mori*	III	Japan	AF178359
NRRL22570[b]	*F. solani* f.sp. *piperis*	-	Brazil	AF178360
NRRL22820[b]	*F. solani* f.sp. *pisi*	VI	USA	AF178355
NRRL22586[b]	*F. solani* f.sp. *robiniae*	VII	USA	AF178353
NRRL22277[b]	*F. solani* f.sp. *xanthoxyli*	IV	Japan	AF178336
NRRL22098	*F. solani* f.sp. *cucurbitae*	I	-	AF178327
NRRL22101	*F. striatum* (homothallic)	-	Panama	AF178333
NRRL31950[a]	*F. tucumaniae*	-	Brazil	AY320157
NRRL22823[c]	*F. virguliforme*	-	USA	AF395647
NRRL22354	*Fusarium* sp.	-	French Guiana	AF178338
NRRL22632 [b]	*Nectria plagianthi*	-	New Zealand	AF178354
NRRL22389	*Nectria* sp. (homothallic)	-	USA	AF178340
NRRL22436[d]	*Neocosmospora africana*	-	South Africa	-
NRRL22468[d]	*Neocosmospora ornamentata*	-	Guinea	-
NRRL22166	*Neocosmospora vasinfecta*	-	USA	AF178350

[a] (Aoki et al. 2005), [b] (O'Donnell 2000), [c] (Aoki et al. 2003), [d] Unpublished sequence kindly supplied by K. O'Donnell

sequencing kit (Version 3.1, Applied Biosystems, USA) and an ABI PRISM™ 3100 DNA sequencer (Applied Biosystems) were used to obtain sequence data. All amplification and sequencing reactions were performed on a GeneAmp PCR System 9700 (Applied Biosystems).

Phylogenetic analyses

TEF-1α sequences representing previously described species and clades (Clades 1–3) of the *F. solani-H. haematococca* complex (O'Donnell 2000; Aoki et al. 2003, 2005) were obtained from GenBank for inclusion in our analyses (Table 2). DNA sequences generated in this study have been deposited in GenBank under accession numbers DQ220207-DQ220247 (β-tubulin) and DQ220248-DQ220288 (TEF-1α). Nucleotide sequences were assembled using Sequence Navigator (Version 1.0.1, Applied Biosystems), and aligned using Multiple sequence Alignment based on Fast Fourier Transform (MAFFT, Version 6, Katoh et al. 2002). Phylogenetic Analysis Using Parsimony (PAUP, Version 4.0b 10, Swofford 2002) was used to estimate parsimony-based phylogenetic relationships. For heuristic searches, 1,000 random additions of sequences and tree bisection-reconnection were used with branch swapping only on best trees. All characters were weighted equally and alignment gaps were treated as missing data. Bootstrap Proportions (BP; Hillis and Bull 1993) were estimated using 1,000 replications.

AFLP analyses

AFLP analysis was performed as described previously (Bogale et al. 2006), using *Eco*RI (E) and *Mse*I (M) adaptor-specific primer sets E-AC/M-AG and E-TC/M-AG (Inqaba Biotechnologies, RSA), each of which contained two selective nucleotides at their 3'-ends. Bands with similar migration patterns were considered identical, and the presence or absence of alleles at a particular locus was scored as one or 0, respectively.

Only bands that were between 100 and 500 bp were considered; and rare alleles with a frequency of <5%, and ambiguous bands that were very intense or very faint, were excluded. Pair-wise distances among the isolates were calculated from the binary matrix using the simple mismatch coefficient (Sneath and Sokal 1973) recommended for dominant markers in haploid organisms (Kosman and Leonard 2005). The resulting distance matrices were used to cluster the isolates by the Unweighted Pair-Group Method using Arithmetic means (UPGMA) implemented in the Molecular Evolutionary Genetics Analysis (Version 2.1, Kumar et al. 2001). The goodness of fit of dendrograms and the respective distance matrices were determined using the cophenetic correlation analysis (Sneath and Sokal 1973).

Results

Phylogenetic analyses

Phylogenetic analysis was done with and without the 23 ingroup GenBank sequences (Table 2). The aligned TEF-1α dataset in the presence of these 23 GenBank sequences contained 773 characters, of which 631 were parsimony uninformative, and thus excluded. Phylogenetic analysis using the remaining 142 informative characters separated the Ethiopian isolates and the taxa included from GenBank into three groups (Fig. 1). These groups corresponded with the three clades (Clades 1–3) previously characterised for the *F. solani-H. haematococca* species complex (O'Donnell 2000; Aoki et al. 2003, 2005). All of the Ethiopian isolates resided in Clade 3 (BP=98%). Within this clade, the Ethiopian isolates resolved into six well-supported lineages (Lineages 1–6) with FCC3815 as the only Ethiopian isolate residing in Lineage 6. The aligned sequence data excluding the 23 GenBank sequences had 33 informative sites. Phylogenetic analysis based on these 23 informative sites for the 43 Ethiopian isolates and the outgroup taxon also recovered the six lineages although the bootstrap support for Lineage 6 was <70% (Fig. 2a).

The aligned β-tubulin dataset contained 292 characters. Of these, only 12 were parsimony–informative, and did not allow full resolution of the taxa included (tree not shown). This dataset only resolved Lineage 5 of the Ethiopian *F. solani* isolates as distinct. However, inspection of the aligned sequences revealed the presence of three nucleotide sites that were fixed differentially (data not shown), separating the Ethiopian *F. solani* isolates into two distinct groups corresponding to the major AFLP Clusters A and B (see below, Fig. 2b). These polymorphisms were all situated at third codon positions within exons and resulted in synonymous substitutions.

AFLP analyses

A total of 65 distinctly polymorphic bands were scored for the 43 Ethiopian isolates included in this study. This dataset separated the Ethiopian *F. solani* isolates into two major clusters (Cluster A, 85% BP; and Cluster B, 74% BP) at a similarity of about 75% (~25% dissimilarity, Fig. 2b). The isolates in these clusters further resolved into six AFLP-based lineages although only Lineage 4 was well supported (BP=71%). These AFLP-based lineages corresponded to the six lineages that emerged from the TEF-1α sequence data (Figs. 1 and 2). Cluster A contained Lineages 1 and 2, while Cluster B contained Lineages 3–6.

Discussion

In this study, we considered the phylogenetic diversity of *F. solani* isolates associated with agricultural soils and plant tissues from Ethiopia. The occurrence of *Fusarium* spp. in general and *F. solani* in particular, has not been well documented in Ethiopia or any other African country, except for South Africa (Fandohan et al. 2003). The only detailed account of *Fusarium* spp. in Ethiopia was made by Bekele and Karr (1997), where more than 19 different species were reported to be associated with head blight and other diseases of wheat. Bogale et al. (2006) reported that diverse members of the *F. oxysporum* species complex were associated with Ethiopian soils and plant tissues. We have shown here that both of these ecological niches in Ethiopia are also occupied by diverse *F. solani* isolates, the majority of which represent novel phylogenetic lineages in the *F. solani-H. haematococca* species complex. Our findings, together with those from the limited number of previous studies, therefore, suggest that agricultural environments in Ethiopia and other such under-explored African regions represent significant reservoirs of novel

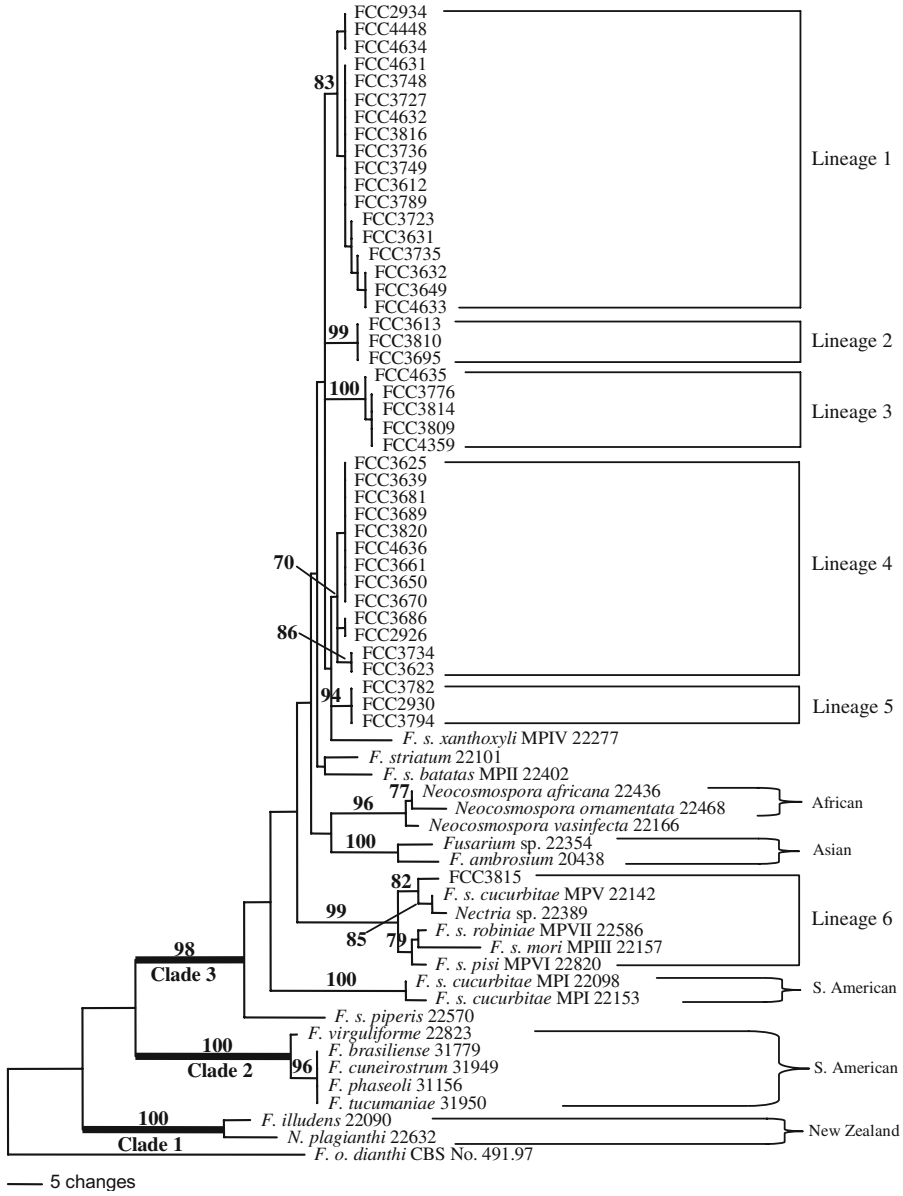

Fig. 1 One of 268 most parsimonious trees inferred from the TEF-1α sequences of the Ethiopian isolates, as well as 23 GenBank sequences representing previously described species and clades (Clades 1–3; O'Donnell 2000) of the *F. solani-H. haematococca* species complex. These clades are indicated with thick lines. *Fusarium oxysporum* f.sp. *dianthi* (CBS No. 491.97) was used as an outgroup. Bootstrap values of 70% and higher are indicated above internodes. The tree scores were: length, 340; consistency index, 0.612; retention index, 0.818; rescaled consistency index, 0.500. Numbers following species names are NRRL numbers of strains, the TEF-1α sequences of which we downloaded from GenBank (Table 2)

species and lineages of the genus *Fusarium*. A number of other fungal species have been shown to have higher diversity in native than in cultivated soils and plants (*e.g.*, Edel et al. 1997; Gordon et al. 1992). This is mainly due to the higher diversity of the plant species in native soils, which supports more diverse species and strains of fungi. The diversity of *F. solani* in native soils and plants in Ethiopia may similarly be higher than that reported here for the sampled cultivated soils and plants.

The Ethiopian isolates of *F. solani* examined here appear to share a common ancestor with those from a variety of different plant hosts (Fig. 1). In the phylogenetic study of O'Donnell (2000), three major *F. solani-H. haematococca* clades (Clades 1–3) were identified. The hypothesis presented in that study was that vicariance associated with the fragmentation of Gondwanaland played a central part in shaping the evolutionary history of the fungi in this complex. Following this model, the complex arose near the New Zealand component of Gondwanaland because isolates collected in that region make up the most basal clade, Clade 1 (Fig. 1). Clade 2, consisting mainly of isolates collected in South America, and

Clade 3, including isolates from Asia and Africa, are most likely to have emerged later because of the reproductive barrier imposed by fragmentation of the ancient southern hemisphere super continent. O'Donnell (2000) attributed the presence of non-gondwanan isolates (*e.g.*, isolates collected in Africa and Asia, Fig. 1) in Clade 3 to distributions associated with agriculture. Consistent with this hypothesis, all of our Ethiopian *F. solani* isolates formed part of Clade 3. The isolates examined here, despite being associated with agricultural environments are, therefore, probably native to this region.

Analyses of TEF-1α and AFLP data in this study separated the 43 Ethiopian *F. solani* isolates into six well-supported and concordant lineages (Figs. 1 and 2). AFLPs further grouped these lineages into two clusters (Fig. 2b). Cluster A included Lineages 1 and 2, and Cluster B included Lineages 3–6. The separation of these clusters was also supported by the presence of β-tubulin nucleotide sites that were fixed differently for the two clusters. The fact that AFLP analysis samples the entire genome, as opposed to specific nuclear or mitochondrial regions in the case of gene genealogies (Vos et al. 1995; Majer et al. 1996), suggests that AFLP analyses provide independent measures of the evolutionary history of the organism. Consequently, phylogenies supported by the two techniques are likely to reliably reflect the evolutionary history of the *F. solani* isolates included in this study.

The biological significance of the distinct clusters and multiple phylogenetic lineages in Clade 3 is unclear (Figs. 1 and 2). None of the lineages or

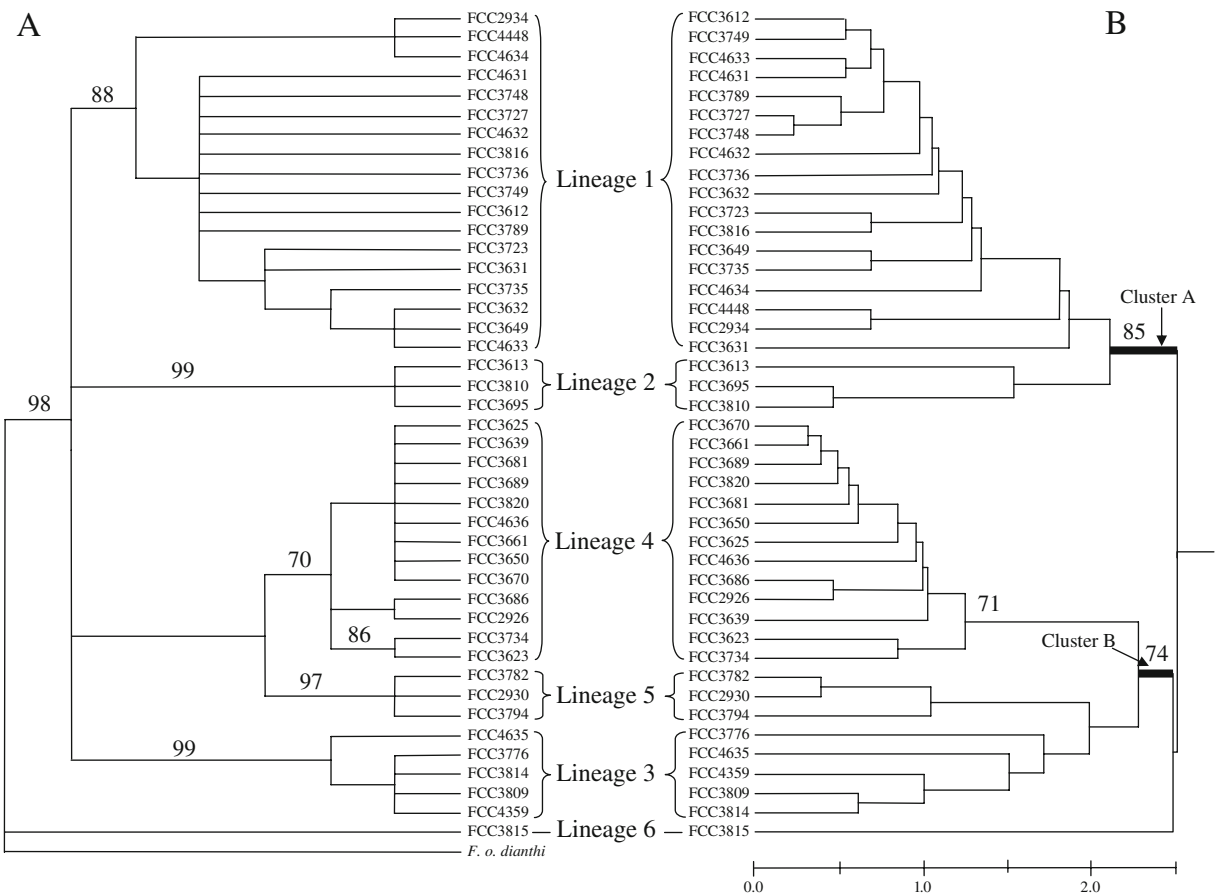

Fig. 2 TEF-1α- and AFLP-based phylogeny of the 43 Ethiopian isolates. **a** A cladogram representing the 45 most parsimonious trees inferred from the TEF-1α sequences using *F. oxysporum dianthi* as an outgroup. The tree scores were: length, 45; consistency index, 0.956; retention index, 0.986; and rescaled consistency index, 0.942. Bootstrap values of 70% and higher are indicated above internodes. **b** UPGMA dendrogram generated from the AFLP data. The scale bar shows the percentage dissimilarity. Bootstrap values of 70% and higher are indicated above internodes. Thick lines lead to clusters

clusters correlated to geographic origin or source (host or substrate) of isolates (Table 1; Figs. 1 and 2). The isolates also did not appear to form part of known lineages or species residing in this complex. The only exception was Lineage 6 (represented by FCC3815) that grouped with a homothallic isolate of *Haematonectria* sp. (NRRL22389) and an isolate of *H. haematococca* MP V (NRRL22142). Nevertheless, the fact that such lineages in species complexes, especially of *Fusarium*, potentially represent discrete species is increasingly recognised (*e.g.*, O'Donnell et al. 1998, 2004). As a result, the morphology, pathology and ecology of a number of *F. solani* isolates have been re-examined and described as distinct taxa in the *F. solani-H. haematococca* complex (Aoki et al. 2003, 2005). Also, analyses of the reproductive mode of such novel lineages may reveal the presence of additional biological species in the complex. For example, Covert et al. (2007) recently showed that *F. tucumaniae* (previously also recognised as *F. solani* representing a distinct lineage in the complex) is heterothallic and capable of producing viable ascospores upon interaction between compatible individuals. Insight into the significance of the different lineages of *F. solani* reported here will, therefore, require a comprehensive sampling of native and agricultural soils, as well as extensive mating compatibility, pathogenicity and morphological studies.

Sequence information from the β-tubulin gene region was less informative than that from TEF-1α for inference of phylogenetic relationships among isolates of the *F. solani-H. haematococca* complex. Contrary to the findings of O'Donnell (2000), we did not detect any divergent paralogues of this gene. Although our isolates probably also harbour such multiple copies of the β-tubulin gene, the PCR primers used in this study did not allow their detection. The primer set (May et al. 1987) used by O'Donnell (2000) and the primer set (Glass and Donaldson 1995) employed here, target different regions of the gene. The fact that we did not detect extra copies of the gene may, therefore, be because the region sequenced is highly conserved, thus limiting our ability to detect divergent nucleotide positions. It is also possible that the primer set used in this study amplified only some of the copies of the gene. Even so, our β-tubulin sequences harboured nucleotide sites diagnostic of the two AFLP clusters of Ethiopian isolates of the *F. solani-H. haematococca* complex.

The findings presented in this study contribute to the growing body of knowledge on the evolution and biology of fungi in the *F. solani-H. haematococca* complex. In terms of the evolution of this group of fungi, we support the view that *F. solani* isolates in Ethiopia and possibly the rest of Africa are potentially of gondwanan origin. In terms of biology and ecology, we have clearly highlighted some of the *F. solani* diversity associated with agricultural soils, stem samples of wheat and barley plants, and roots of banana plants collected from different locations in Ethiopia. We have further demonstrated that these *F. solani* isolates share complex phylogenetic relationships that are not easily interpretable in terms of geographic origin, host or substrate. Although their pathogenicity is unknown, studies on other *Fusarium* spp. have shown that pathogenic strains can arise from presumably non-pathogenic strains, and *vice-versa*, through mutations involving only a few genes (Baayen et al. 2000). To fully understand the ecological significance of the *F. solani* isolates associated with the examined hosts and substrates, future studies should, therefore, also consider factors such as pathogenicity, distribution, and reproduction.

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