Characterization of *Fusarium oxysporum* isolates from Ethiopia using AFLP, SSR and DNA sequence analyses

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Fusarium oxysporum is one of the most important fungal pathogens of agricultural crops. Yet the significance of this fungus in Ethiopian agriculture has not been well investigated. We employed Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeat (SSR) markers and DNA sequence analyses to study 32 strains of *F. oxysporum* from Ethiopia. For comparative purposes, we also included 18 representatives of *F. oxysporum formae speciales* as well as sequences from GenBank representing the three phylogenetic clades that were designated previously. All the three methods used in this study separated the strains into three lineages, which corresponded with the three clades of *F. oxysporum*. Inspection of the sequence data revealed five fixed nucleotide sites in the Translation Elongation Factor-1α (TEF-1α) partition, which uniquely distinguished the three lineages. Thirty of the Ethiopian isolates grouped in Lineage 2, whereas the remaining two isolates grouped in Lineages 1 and 3. The 18 *formae speciales* included in this study did not separate according to host with any of the three DNA-based techniques used. This confirmed that pathogenicity of isolates does not necessarily correlate with phylogenetic grouping. The genetic diversity observed among the Ethiopian isolates was low. This most probably reflects the nature of the Ethiopian agricultural system that heavily relies on local crop varieties, thereby restricting the introduction of new genotypes of the fungus via infected seeds.

Key words: AFLP, β-tubulin, Ethiopia, *Fusarium oxysporum*, mtSSU, SSR, TEF-1α

Introduction

Fusarium oxysporum Schlecht. (emend. Snyd. & Hans.) is a widely distributed soil inhabiting fungus, which is known to be phylogenetically diverse. Most strains assigned to this species are saprotrophic. Some non-pathogenic strains have been used as biological control agents (eg., Postma and

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Rattink, 1992), while others have veterinary and/or medical significance (Pereiro *et al*., 2001). However, *F. oxysporum* is best known for the plant pathogenic strains, which cause wilt, root rot and crown rot diseases on a wide variety of crops, often limiting crop production (Nelson *et al*., 1981).

The significance of *F. oxysporum* in Ethiopian agriculture has not been carefully investigated. The only reports of this fungus are those of Bekele (1985) and Bekele and Karr (1997). These studies represented crop disease inventories (Bekele, 1985) and a diagnostic survey for causal agents of *Fusarium* head blight of wheat (Bekele and Karr, 1997).

Most studies of *F. oxysporum* have focused on the plant pathogenic isolates (eg., Mohammadi *et al*., 2004; Pasquali *et al*., 2004). However, the non-pathogenic members make up a significant proportion of the isolates encountered, and harbour most of the genetic diversity within this species complex (Bao *et al*., 2002). There is a great deal of genetic relatedness between pathogenic and non-pathogenic *F. oxysporum* isolates (Baayen *et al*., 2000a; Skovgaard *et al*., 2002), which has led some researchers to conclude that particular pathogenic isolates might have evolved from non-pathogenic strains by mutations involving a few loci (Skovgaard *et al*., 2002). Some non-pathogenic isolates have also been considered to have evolved from pathogenic strains through loss of virulence (Skovgaard *et al*., 2002). Consequently, assessment of genetic diversity within the non-pathogenic populations is important.

DNA-based techniques have increasingly become the tool of choice for understanding the genetic diversity and phylogeny of *Fusarium* species. For example, DNA sequence analysis has been used to reveal a large number of phylogenetic species in the *F. solani-Nectria haematococca* (O'Donnell, 2000), *F. graminearum* (O'Donnell *et al*., 2000), and the *F. oxysporum* (O'Donnell *et al*., 1998) species complexes. Amplified Fragment Length Polymorphisms (AFLPs) have been used to distinguish species and phylogenetic groups in a number of *Fusarium* species (Baayen *et al*., 2000a; Zeller *et al*., 2003; Belabid *et al*., 2004; Leslie *et al*., 2004) including *F. oxysporum* (Baayen *et al*., 2000a; Belabid *et al*., 2004). Simple Sequence Repeats (SSRs, also known as microsatellites) have also been used in other fungal species because of the high resolution that they provide (eg., Jarne *et al*., 1996; Barnes *et al*., 2005; Enjalbert *et al*., 2005).

The objective of this study was to examine the diversity of *F. oxysporum* strains isolated from agricultural soil and plant samples in Ethiopia. We also considered how these isolates compare with those representing known phylogenetic groups previously characterized in this species complex. This was achieved using AFLP, SSR and DNA sequence comparisons.

Materials and methods

Isolates

The *F. oxysporum* isolates (Fig. 1) that formed the basis of this study were obtained from agricultural soils and plant tissues collected from Ethiopia. These isolates have also previously been used in the development of SSR markers in *F. oxysporum* (Bogale *et al*., 2005). Eighteen isolates representing different *formae speciales* of *F. oxysporum* were obtained from the Centraalbureau voor Schimmelcultures (CBS), the Netherlands, and used for comparative purposes (Fig. 1). Sequences representing the three clades of *F. oxysporum* described by O'Donnell *et al*. (1998) were obtained from GenBank and included in our analyses (Fig. 1). Also, relevant sequences of a *Fusarium* sp. (NRRL25184) were obtained from GenBank and used for outgroup purposes (Fig. 1) since this isolate was used for the same purpose in the study (O'Donnell *et al*., 1998) where groupings in *F. oxysporum* were first described.

DNA extraction and sequence analyses

DNA was extracted from isolates using the CTAB (N-cetyl-N,N,N-trimethyl-ammonium bromide) method (Murray and Thompson, 1980). Fragments of the genes encoding Translation Elongation Factor 1α (TEF-1α), mitochondrial Small Subunit ribosomal RNA (mtSSU rRNA) and β-tubulin were then amplified by PCR using primer pairs EF1/EF2, MS1/MS2 and 2A/2B and the conditions described by O'Donnell *et al*. (1998), White *et al*. (1990) and Glass and Donaldson (1995), respectively. PCR products were purified using the QIAquick PCR purification kit (QIAGEN, GmbH, Germany), and sequenced in both directions using the respective PCR primers. For this purpose, the BigDye terminator sequencing kit (Version 3.1, Applied Biosystems) and an ABI PRISM^{TM} 3100 DNA sequencer (Applied Biosystems) were used. All PCRs and sequencing reactions were performed on a GeneAmp PCR System 9700 (Applied Biosystems).

Gene sequences were assembled using Sequence Navigator (Version 1.0.1, Applied Biosystems), and aligned using ClustalX (Version 1.8, Thompson *et al*., 1997), after which the alignments were manually corrected where needed. Sequence navigator was also used to predict amino acid sequences from nucleotide sequences. The predicted amino acid sequences were then compared with the corresponding amino acid sequences in GenBank to determine the possible positions of introns. PAUP (Phylogenetic Analysis Using Parsimony, Version 4.0b 10, Swofford, 2002) was used to estimate phylogenetic relationships and to test combinability of datasets. For this

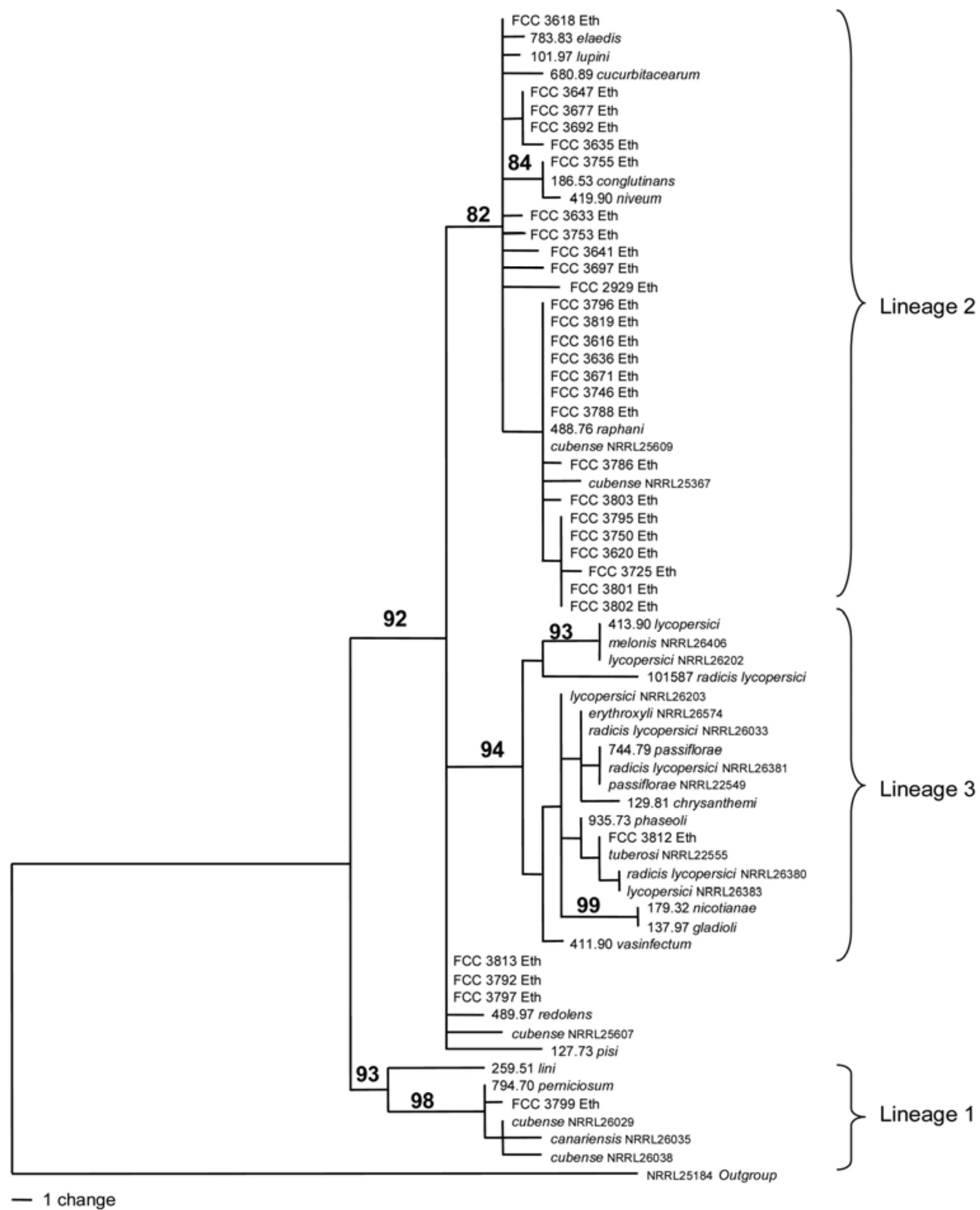

Fig. 1. One of 120 most parsimonious trees inferred from the combined TEF-1α, β-tubulin and mtSSU data set using the Ethiopian isolates, *formae speciales*, and *F. oxysporum* sequences from GenBank. Ethiopian isolates are indicated by FCC numbers followed by 'Eth', whereas CBS isolates are indicated by CBS numbers followed by names of *formae speciales*. Names of *formae speciales* followed by NRRL numbers indicate strains for which sequences were downloaded from GenBank. *Fusarium* sp. NRRL125184 was used as outgroup. Bootstrap support values of 70% and higher are shown above nodes.

purpose, heuristic searches based on 1,000 random addition sequences and tree bisection-reconnection were used with the branch swapping option set on ‘best trees’ only. To test the combinability of datasets, a partition homogeneity test with heuristic searches was employed using 10,000 replications. All characters were weighted equally and alignment gaps were treated as missing data. Bootstrap analysis (Hillis and Bull, 1993) was based on 1,000 replications.

AFLP and SSR analyses

AFLP analysis was performed as described by Vos *et al*. (1995). For this purpose, three 5’-end Cy5.5-labelled (Inqaba Biotechnologies, RSA) *Eco*RI (E) and unlabelled *Mse*I (M) primer pairs, each primer containing two selective nucleotides (E-CC/M-TA, E-TC/M-AA and E-TC/M-AG), were used. Amplification and resolution of SSR loci was performed with the labelled SSR primers and conditions described by Bogale *et al*. (2005). For both the AFLP and SSR analyses, the presence/absence of an allele at a particular locus was scored as 1/0, and the pair-wise distance among the isolates was calculated from the binary matrix using the simple mismatch coefficient (Sneath and Sokal, 1973) that is recommended for haploid fungi (Kosman and Leonard, 2005). The resulting distance matrices were used to cluster the isolates by the UPGMA (Unweighted Pair-Group Method using Arithmetic means) method implemented in the Molecular Evolutionary Genetics Analysis (MEGA, 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).

Genotypic diversity (H) among isolates was estimated from allelic frequencies using the equation

$$H = 1 - \Sigma x_i^2,$$

where, x_i is the frequency of the ith allele (Nei, 1973).

Genetic identity (I) was computed as

$$I_{xy} / \sqrt{I_x I_y}$$

where, I_{xy}, I_x, and I_y are the averages over all loci of $\Sigma x_i y_i$, Σx_i^2, and Σy_i^2; and x_i and y_i are the frequencies of allele i for populations x and y, respectively (Nei, 1973).

Population differentiation was calculated using the equation

$$\delta_T = (n/n\text{-}1)(1\text{-}\Sigma p_i^2),$$

where, p_i is the frequency of the ith allele, and n is the number of individuals sampled (Gregorious, 1987).

The coefficient of population subdivision (G_{ST}) was computed as

$$(H_t - H_s)/H_t,$$

where, H_t is the total genetic diversity, and H_s is the average gene diversity over all subgroups (Nei, 1973).

Results

Sequence analyses

One mitochondrial (mtSSU rRNA) and two nuclear (TEF-1α and β-tubulin) genes were sequenced for each isolate. These sequences have been deposited in GenBank under accession numbers DQ220021, DQ220022, DQ220024-28, DQ220030-43, DQ220045, DQ220047-53, DQ220055-59, DQ220062, DQ220064-71, DQ220073-75, DQ220077, DQ220079 and DQ220080, for β-tubulin; DQ220083, DQ220084, DQ220086-90, DQ220092-105, DQ220107, DQ220109-115, DQ220117-121, DQ220124, DQ220126-133, DQ220135-137, DQ220139, DQ220141 and DQ220142, for TEF-1α; and DQ220145, DQ220146, DQ220148-152, DQ220154-167, DQ220169, DQ220171-177, DQ220179-183, DQ220186, DQ220188-195, DQ220197-199, DQ220201, DQ220203 and DQ220204, for mtSSU. Although we sequenced a 656 base pair (bp) mtSSU fragment from the Ethiopian isolates and the *formae speciales*, only a 213 bp sequence at the 3'-end of these sequences was used in the analyses. This was to match the size of these sequences to those obtained from GenBank. Inclusion of the remaining 5'-end portion of the mtSSU sequence in the analyses of our isolates and some GenBank sequences, for which we had the complete sequence information, resulted in trees with the same topology as those based on the smaller fragment, but with slightly higher bootstrap support values (results not shown). The TEF-1α, β-tubulin and mtSSU sequences were analysed individually and in all possible combinations since they represented homogeneous partitions ($p = 0.033$). Sequence alignment characteristics and scores of the various phylogenetic trees generated are summarized in Table 1; but only the tree inferred from the combined TEF-1α, β-tubulin and mtSSU sequence data is presented here (Fig. 1).

The combined TEF-1α, β-tubulin and mtSSU sequence data separated most of the taxa analysed into three well-supported lineages (Lineages 1 – 3, Fig. 1). These lineages corresponded with the three previously recognised phylogenetic clades of *F. oxysporum* (O'Donnell *et al*., 1998). Three Ethiopian isolates (FCC3813, FCC3792, and FCC3797), *F. o. redolens*, *F. o. cubense*, and one of the taxa (NRRL25607) representing Clade 2 of O'Donnell *et al.* (1998) did not resolve with respect to the three lineages. These unresolved taxa, however, grouped closer to Lineages 2 and 3 than to Lineage 1. Most of

the Ethiopian isolates grouped within Lineage 2. The isolates representing different *formae speciales* of *F. oxysporum* were spread out across the three lineages showing no obvious pattern of grouping (Fig. 1). In trees inferred from the individual TEF-1α dataset, the bootstrap support for Lineage 3 was weak, and some isolates from this lineage formed a separate group (results not

Table 1. Sequence alignment characteristics and phylogenetic tree scores inferred from the individual TEF-1α, mtSSU and β-tubulin partitions and the combined datasets.

Dataset	**Aligned Sites**[a]	**Constant Sites**[b]	**Pars. Info. Sites**[c]	**MPTs**[d]	**Tree scores**[e]	**Nodes > 50% BS**[f]
TEF-1α	676	590 87.3%	38 5.62%	98	L = 104, CI = 0.865, RI = 0.953, RC = 0.825	21
β-tubulin	278	263 94.6%	2 0.72%	12	L = 54, CI = 0.296, RI = 0.050, RC = 0.015	1
mtSSU	213	189 88.7%	11 5.16%	26	L = 27, CI = 0.926, RI = 0.984, RC = 0.911	5
TEF-1α+ β-tubulin	954	848 88.9%	44 4.61%	105	L = 141, CI = 0.794 RI = 0.924, RC = 0.734,	22
TEF-1α + mtSSU	889	773 87.0%	49 5.51%	116	L = 122, CI = 0.943, RI = 0.984, RC = 0.927	25
β-tubulin + mtSSU	491	451 91.9%	13 2.65	48	L = 61, CI = 0.721, RI = 0.903, RC = 0.651	5
TEF-1α + β-tubulin + mtSSU	1167	1036 88.8%	56 4.80%	120	L = 172, CI = 0.824, RI = 0.943, RC = 0.778	27

[a] Number of aligned sites including gaps.
[b] Number of constant sites.
[c] Number of parsimony informative sites.
[d] Number of most parsimonious trees.
[e] Tree scores (L = tree length, CI = consistency index, RI = retention index, RC = rescaled consistency index).
[f] Number of nodes with bootstrap values of 50% or more.

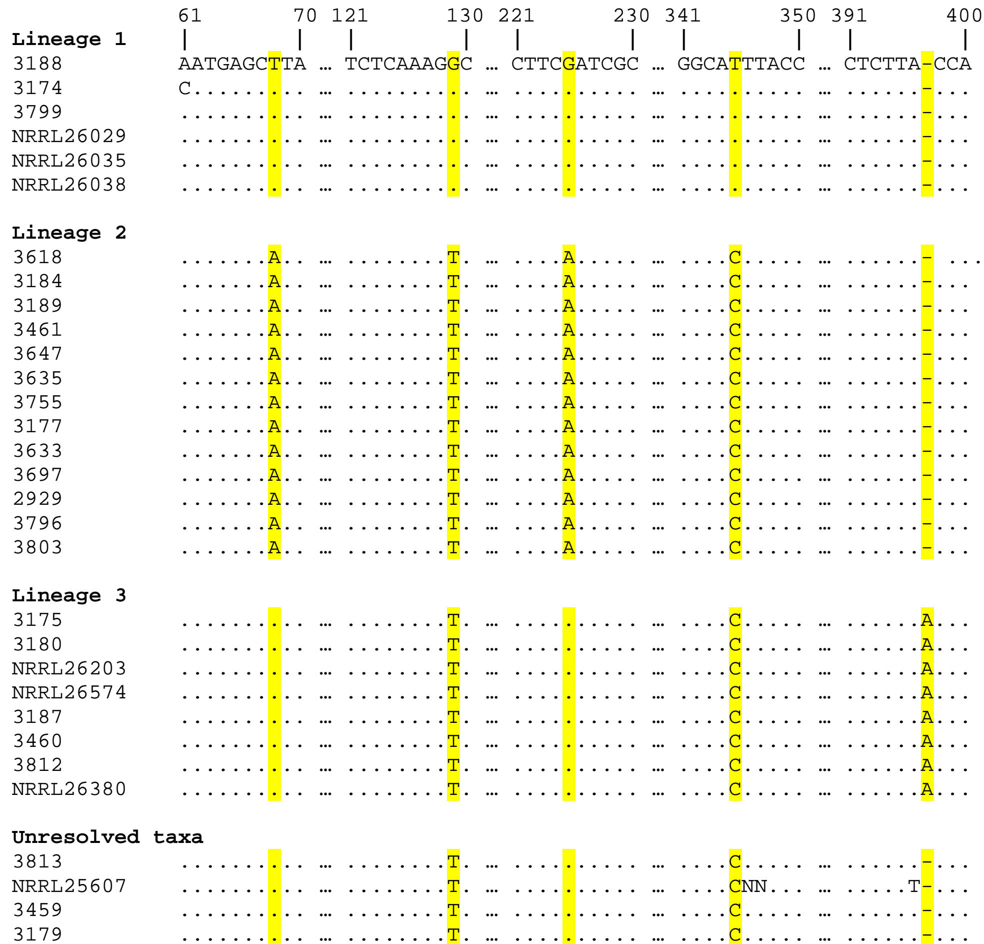

Fig. 2. The partial TEF-1α sequences (5'→3', sites 61 – 400) of TEF-1α haplotypes representing the three lineages and the unresolved taxa (isolates that did not resolve with respect to Lineage 2 and Lineage 3 of our multigene tree, Fig. 1). The shaded sites show nucleotides that uniquely distinguish particular lineages. The dots indicate nucleotides that are identical to the ones in the first sequence at the respective sites. The numbers at the top of sequences indicate nucleotide sites.

shown). There was only a weak bootstrap support for Lineage 2 in trees inferred from the mtSSU partition, whereas only Lineage 2 had a strong bootstrap support on trees inferred from the β-tubulin partition (results not shown).

Inspection of the sequence partitions revealed the presence of TEF-1α sites (Fig. 2) that uniquely differentiated each lineage from the other two as well as from the unresolved taxa. Positions 129 and 345 distinguished Lineage 1; positions 68 and 225 distinguished Lineage 2; and position 397

distinguished Lineage 3. None of these characters uniquely distinguished the unresolved taxa (FCC3813, FCC3459, FCC3179, *F. o. redolens, F. o. cubense* and *F. o. pisi*, shown in figure 4). These taxa shared characters 129 and 345 with Lineage 2 and Lineage 3, and character 397 with Lineage 1 and Lineage 2. Comparison of predicted amino acid sequences for our TEF-1α nucleotide sequences with relevant amino acid sequences in the GenBank showed that all these unique nucleotide sites were located in non-coding intron positions. Such distinguishing nucleotide sites were absent from the mtSSU and β-tubulin partitions.

AFLP and SSR analyses

The AFLP data (Fig. 3) separated the Ethiopian isolates and the *formae speciales* of *F. oxysporum* into two major groups (91% bootstrap values each). One of these major AFLP-based groups corresponded to Lineage 1 that emerged from the sequence data. The second major group included four unresolved taxa, Lineage 2 and Lineage 3. Only isolate FCC3812 grouped in a different lineage from that indicated by DNA sequence comparisons. This isolate grouped in Lineage 2 in the AFLP-based dendrogram (Fig. 3), whereas it had a position in Lineage 3 in the phylogram based on sequence data (Fig. 1).

The SSR analysis (Fig. 4) resolved the Ethiopian isolates and the *formae speciales* into three groups, which broadly corresponded with the three lineages that emerged from the sequence and AFLP analyses. However, the taxa that did not resolve with respect to Lineage 2 and Lineage 3 in the sequence and AFLP analyses, grouped in Lineage 2 in the SSR analyses. A higher similarity was observed among the isolates using SSR (Fig. 4, ~90% similarity; $I = 0.892$) than AFLP (Fig. 3, ~75% similarity; $I = 0.749$). The genetic diversity among the Ethiopian isolates was low. However, this estimate was higher using SSR ($H = 0.550$) than AFLP ($H = 0.268$). The genetic diversity among isolates in Lineage 2 was higher than that among isolates in Linage 3 (Table 2). However, the genetic differentiation between the two lineages was low, which was also reflected in the high genetic identity among all the isolates of the two lineages (Table 2).

Discussion

All three of the techniques used in this study separated the Ethiopian *F. oxysporum* isolates and the *formae speciales* into three lineages. The lineages that emerged from the SSR, AFLP and DNA sequence analyses were also concordant. Most of the Ethiopian isolates resided in a single lineage, whereas

the isolates that represented different *formae speciales* and included in this study for comparative purposes were widely spread across the three lineages. Therefore, the three lineages did not correspond with host or geographical origin of the isolates studied. Sequence analyses also showed that the three lineages emerging from this study were the same as the three clades previously described for *F. oxysporum* by O'Donnell *et al*. (1998).

None of the three techniques applied in this study provided host-related resolution for the *formae speciales*. The sequence analyses, which included a larger number of different *formae speciales*, also indicated the lack of resolution among the different *formae speciales*. This suggests that phylogenetic groups have little connection with virulence groups. Other studies reported similar findings (Baayen *et al*., 2000a; Bao *et al*., 2002), which is perhaps not surprising as *formae speciales* are based on a phenotypic character (pathogenicity to specific plants). The latter is influenced by a range of factors and not necessarily linked to phylogenetic placement.

The results of our phylogenetic analyses correspond largely to those of O'Donnell *et al*. (1998) and Baayen *et al*. (2000a), since we also recovered three strongly-supported lineages (Lineages 1 – 3), which corresponded with the three clades (Clades 1 - 3) reported by O'Donnell *et al*. (1998). However, within the larger Lineage 2/Lineage 3 assemblage, a number of isolates remained unresolved. These results suggest that the *F. oxysporum* species complex might include more than the three main phylogenetic clades designated by O'Donnell *et al*. (1998). To address this issue many more *F. oxysporum* isolates from diverse sources will need to be evaluated.

Three Ethiopian isolates (FCC3813, FCC3792 and FCC3797), and two isolates representing *F. o. pisi* and *F. o. redolens* did not resolve with respect to Lineage 2 and Lineage 3, based on the sequence and the combined AFLP analyses. However, the SSR analysis placed these isolates within Lineage 2. An isolate of *F. o. cubense* (NRRL25607) was also amongst the unresolved taxa. This strain represented clone 1 of *F. o. cubense* and was reportedly from *F. oxysporum* Clade 2 (O'Donnell *et al*., 1998) based on TEF-1α and mtSSU rDNA sequence information. This suggests that the unresolved taxa may belong to Lineage 2, which corresponds to Clade 2 of O'Donnell *et al*. (1998).

Table 2. Genetic diversity and population differentiation statistics between Lineage 2 and Lineage 3 as determined from the AFLP and SSR analyses.

	Using AFLP					**Using SSR**			
	H	**δ_T**	**G_{ST}**	**I**		**H**	**δ_T**	**G_{ST}**	**I**
Lineage 2	0.608	0.684	0.008	0.799	Lineage 2	0.956	0.981	0.036	0.910
Lineage 3	0.606	0.622			Lineage 3	0.851	0.957		

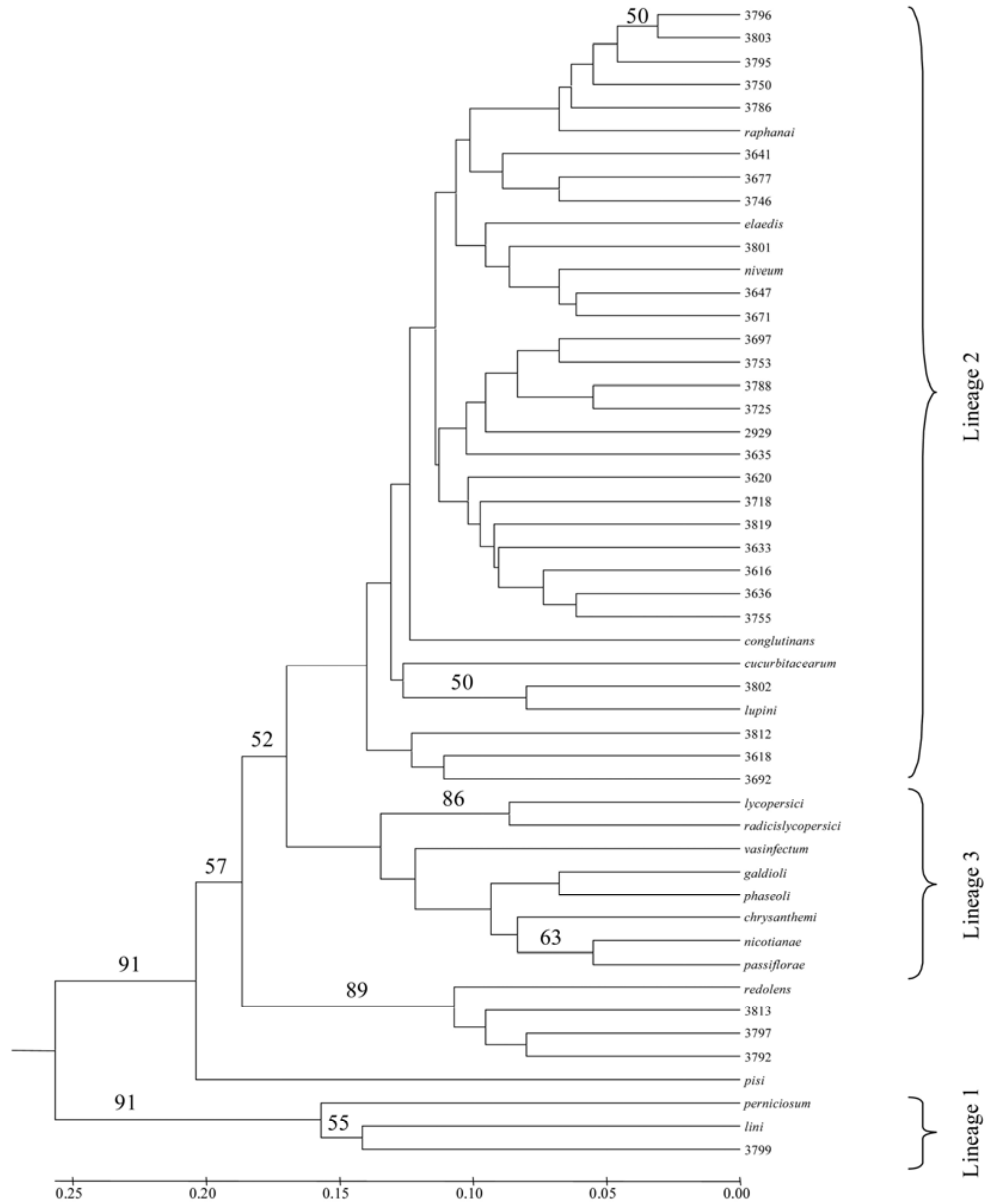

Fig. 3. Dendrogram generated using the AFLP dataset for the *F. oxysporum* isolates obtained from Ethiopia (indicated by strain numbers) and *formae speciales* obtained from the CBS (names of *formae speciales* indicated). Bootstrap values of 50% and more are indicated above nodes. The scale bar indicates the dissimilarity index.

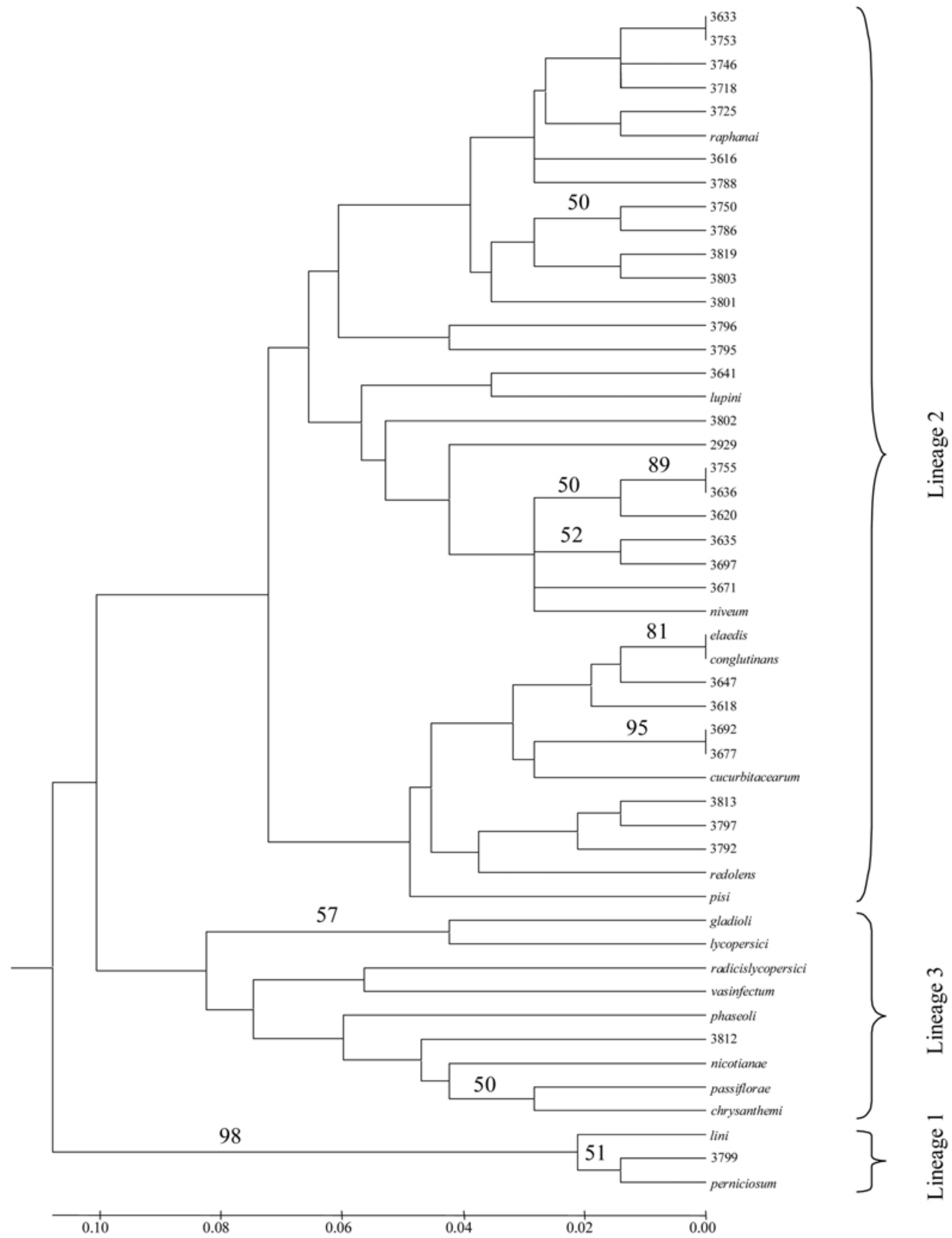

Fig. 4. Dendrogram generated using the SSR dataset for the *F. oxysporum* isolates obtained from Ethiopia (indicated by strain numbers) and *formae speciales* obtained from the CBS (names of *formae speciales* indicated). Bootstrap values of 50% and higher are indicated above nodes. The scale bar indicates the index of dissimilarity.

There were some discrepancies among the results of the three techniques used to resolve the taxonomic position of the Ethiopian *F. oxysporum* isolates. One of these was the relative position of isolate FCC3812 from Ethiopia. Sequence and SSR analyses placed this isolate in Lineage 3, whereas AFLP analyses placed it in Lineage 2. This difference in the position of isolate FCC3812 may be due to the homoplasy associated with AFLP. Homoplasy usually arises in AFLP because alleles are not easily recognized, allelic fragments are scored as independent where they are not so, and changes in fragment size rather than changes in site are scored (Majer *et al*., 1996).

The 32 Ethiopian isolates studied were obtained from various sources and different locations in the country. Consequently, these isolates most probably represent a large proportion of the genetic diversity of the fungus that exists in Ethiopia. The fact that 30 of these isolates grouped in a single lineage reflects the low genetic diversity among the Ethiopian *F. oxysporum* isolates. The traditional subsistence farming in Ethiopia accounts for most of the crop production in the country, which almost entirely relies on local crop and seed varieties. The low genetic diversity observed among the Ethiopian isolates is, therefore, not surprising since introduction of new genotypes of the fungus via infected seeds from outside is expected to be limited. The strains included here have not been tested for pathogenicity, and thus cannot be considered in terms of this characteristic.

There was a low genetic diversity among the isolates in Linage 2 and Lineage 3, although the estimate was higher using SSR than AFLP. Only a small amount of the total variation among the isolates was partitioned between the two lineages. This was evident from the low coefficient of genetic differentiation, and the high genetic similarity among all the isolates as well as the small number of fixed TEF-1α sites. Furthermore, the higher genetic variation observed within each of the two lineages using SSR than AFLP may reflect the higher resolution power of SSR at the sub-species level.

The three lineages revealed in this study shared ~74% AFLP-based and close to 90% SSR-based similarity. Considering the minimum AFLP-based similarity of 70% observed within isolates of particular *Fusarium* species (Abdel-Satar *et al*., 2003; Zeller *et al*., 2003; Leslie *et al*., 2004), the three lineages revealed in this study can be considered to constitute the same species. This cut off point for AFLP-based species recognition in *Fusarium*, however, seems to be rather arbitrary. Baayen *et al*. (2000b), for example, have reported AFLP-based similarity of only 40% among some isolates of *F. proliferatum*. Other studies have also revealed that isolates within particular *Fusarium* species may share only 50-55% isozyme-based (Yli-Mattila *et al*., 1996; Baayen *et al*., 1997) and RFLP-based (Baayen *et al*., 1997) similarities. The

three lineages revealed in this study, however, represent distinct phylogenetic entities. The significance of these lineages and the three clades (O'Donnell *et al*., 1998) to which they correspond, remains to be determined in terms of the overall biology of the fungus and its significance in agriculture.

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