Mycotoxicology Society of Nigeria

Mycotoxicology, 2016, 3: 1-14

Molecular investigations of food-borne *Cladosporium* and *Fusarium* species from Nigeria

[a]Moore G. G. and [b*]Fapohunda S. O.

[a] Southern Regional Research Center, Agricultural Research Service, United States Department of Agriculture, New Orleans, Louisiana, USA; 1100 Robert E. Lee Blvd, New Orleans, LA 70124
Phone: 504-286-4361; Fax: 504-286-4533; geromy.moore@ars.usda.gov
[b]Department of Biosciences and Biotechnology, Babcock University, Ilishan-Remo, Nigeria
*Corresponding author: oystak@yahoo.co.uk

ABSTRACT

A sampling of contaminated foodstuffs throughout southwest Nigeria yielded three fungal isolates belonging to the genus *Fusarium* and two belonging to the genus *Cladosporium*. These 2 are known to produce mycotoxins and volatile organic compounds (VOCs) respectively. In this study we subjected these isolates to various molecular investigations. The morphological species identifications were confirmed or refined with BLAST queries for sequences from two genomic regions (translation elongation factor-1 α and ITS). BLAST results uncovered species identification inconsistencies for one *Fusarium* isolate, SRRC1606, and for both *Cladosporium* isolates based on the examined loci. Using additional species sequences obtained from GenBank, the phylogenetic associations for each genomic region were explored and observed species haplotype associations for the *Fusarium* sequences; however, this was not the case for the *Cladosporium* sequences. There was evidence of recombination in both loci for the *Fusarium* species, but only in the translation elongation factor locus for the *Cladosporium* sample population. *Fusarium* coalescent analyses for both loci inferred two lineages, one containing only *F. oxysporum* sequences and the other containing the remaining species examined. These same analyses for the Cladosporia inferred ancient segregation of one lineage, containing only the outgroup taxa, from a second lineage that exhibited recent divergence events among the other taxa examined. Mycological population dynamics and analyses can achieve a better understanding of interventions to protect consumers from contaminated foods.

Keywords: fungal contaminants, genotyping, phylogenetics, recombination, speciation

1.0 INTRODUCTION

The genus *Fusarium* includes species noted for the production of secondary metabolites and cause of diseases in both plants and animals [Kovalsky-Paris *et al.,* 2014, Tamura *et al.,* 2015], while *ladosporium* species are most often associated with cutaneous and pulmonary infections [De Hoog *et al.,* 2000]. The 2 isolates occupy very diverse habitats such as plumbing drains [Short *et al.,* 2011], tomato plants [Rollan *et al.,* 2013], cosmetics [Pinto *et al.,* 2012], and hospitals [Migheli *et al.,* 2010, Oeschsler *et al.,* 2010]. Previous research has emphasized the value of molecular techniques, beyond morphological investigation, as critical components for reliable fungal classification. For example, utilization of a molecular technique on *Fusarium*, known as PCR-ITS-RFLP, was able to further distinguish between strains within the *Fusarium solani* species complex (FSSC) [Chehri *et al.,* 2011]. A newly developed species-specific primer, for the conserved regions of 28s-rDNA and the intergenic spacer, to genotype *F. oxysporum* f. sp. *psidii* was reported by Mishra and co-workers [Mishra *et al.,* 2013]. New genotypes and races of *F. oxysporum* f. sp. *vasinfectum* were discovered, through comparison with previously described races, using sequences from translation elongation factor, phosphate permease, and beta-tubulin genes [Holmes *et al.,* 2009]. Similar attention has been focused on the use of molecular techniques to differentiate *Cladosporium* species [Braun 2001; Braun *et al.,* 2003;Braun *et al.,* 2007;Bensch *et al.,* 2011]. The identities of two races of *C. fulvum* were recently confirmed by amplifying and sequencing 580bp of their respective ITS regions [Rollan *et al.,* 2013]. Molecular association of metabolite production with fungal geneshas also been investigated such as in the *FUM-1* (fumonisin) gene,and both *TRI-13* and *TRI-7*trichothecene genes in *F. proliferatum* and *F. verticillioides*, respectively [Sampietro *et al.,* 2010]. Also, molecular techniques were used to elucidate the genotypes of non-mycotoxigenic strains of *F. proliferatum*, *F.verticillioides*, and the *F. graminearum* species complex strains [Sampietro *et al.,* 2012]. The aim of this preliminary study, not disregarding the sample size, was to analyze genomic loci in previously described *Fusarium* and *Cladosporium* isolates, sampled from

contaminated foodstuffs, to observe species diversity and to assure the integrity of their taxonomic placements. The molecular analysis was also designed to enhance in -depth fungal knowledge, which is a critical and strategic input in fungal control and management

2.0 MATERIALS AND METHODS

2.1 *Fungal identification*

Fungal strains selected for this study, originally sampled from contaminated foods in Nigeria, were identified based on morphological characters as in Fapohunda *et al.,* [2012] and all isolates are currently housed in the Southern Regional Research Center (SRRC) fungal collection in New Orleans, Louisiana, USA. Three *Fusarium* isolates (SRRC1606, SRRC1630 and SRRC1633) were morphologically identified from among the collected samples. Also, two *Cladosporium* isolates were identified based on morphology (SRRC1616 and SRRC1634). In addition to morphology, BLAST queries of sequences from two genomic regions were performed to verify species identification for each isolate: the internal transcribed spacer (ITS) region and the translation elongation factor-1 α (*tef1α*). The *tef1α* locus is considered a good delimiter of species for fungal genera such as *Fusarium* and *Cladosporium* [Castano *et al.,* 2014; Schoch *et al.,* 2006]. All isolates were PCR-amplified for ITS as in Fapohunda *et al*. [2012]. Amplification of the *tef1α* locus involved primers reported in Carbone and Kohn [Carbone *et al.,* 1999]. Amplicons for each isolate were purified and sequenced, and the sequences were trimmed, cleaned, and aligned using Sequencher version 5.3 (Gene Codes Corporation). Individual sequences for each isolate/locus were BLAST queried to verify species associations previously determined by morphological observations. In this study we did not test any isolates for the presence of mycotoxins.

2.2 ***Comparative analyses***

Fungal sampling was done on less than four isolates per genus, and searches for each locus were performed using the NCBI database and additional species sequences from each genus were included in the respective sequence alignments. Whenever possible, the same accessioned isolate (or at least species) was used in alignments for the genomic loci examined. In addition to the various sequences acquired to build sample populations for each genus and locus, outgroup taxa were selected for each genus. *Fusarium chlamydosporum* [Darvishnia, 2013] and *Cladosporium salinae* [Zalar *et al.,* 2007] have been reported as appropriate phylogenetic outgroup taxa for their respective genera, and; therefore, were chosen for this study. All sequence alignments were exported in nexus format for analyses in SNAP Workbench [Price and Carbone, 2005]. The first analysis component involved collapsing the sequences into haplotypes using SNAP: Map [Aylor *et al.,* 2006]. Resulting outfiles were examined for haplotype associations and evidence of the ancestral sequence. The ancestral sequence is evident when its nucleotide composition is identical to the consensus sequence. Next to be performed was the phylogenetic inference for each locus; additionally, bootstrap values and heuristics (e.g., consistency indices and numbers of most-parsimonious trees) were determined using PAUP* software [Swofford, 2003]. Comparative analyses of recombination and coalescence were also performed for each locus and sample population with the recombination events for each locus/species group was performed using RecMin [Myers and Griffiths, 2003]. If evidence of recombination was observed, an ancestral recombination graph (ARG) was inferred using the beagle algorithm with one million simulations [Lyngso *et al.,* 2005]. Finally, to explore divergence patterns among the species for each locus, without the interference of recombination, coalescent analyses were performed which only consider mutational differences among isolates [Griffiths and Tavare, 1994]. The GenBank accession numbers for the *Fusarium* isolates are KP771785-KP771787 (ITS) and KT950249-KT950251 (*tef1α*). The accession numbers for the *Cladosporium* isolates are KP771788-KP771789 (ITS) and KT950252-KT950253 (*tef1α*).

RESULTS

3.1 *Molecular confirmation of species*

Table 1 lists the sampled Nigerian isolates, the foodstuffs from which they were sampled and their BLAST identification results .

3.2 *Haplotype associations*

Sequence comparisons, for the *Fusarium* species, revealed enough polymorphisms within their respective ITS regions to segregate the sequences into six haplotypes, and evidence of the ancestral ITS sequence affiliated with haplotype H5, comprised of isolate SRRC1633 and the two other *F. incarnatum* sequences.

Table 1. Nigerian isolates with sampling sources and species identifications based on two genomic loci

SRRC ID	Sample Source	ITS	*tef1*
SRRC1606	Raw yam	*Fusarium equiseti*	*Fusarium incarnatum*
SRRC1630	Cooked rice	*F. oxysporum*	*F. oxysporum*
SRRC1633	Bread	*F. incarnatum*	*F. incarnatum*
SRRC1616	Cooked rice	*Cladosporium cladosporioides*	*Cladosporium tenuissimum*
SRRC1634	Raw groundnut	*C. cladosporioides*	*C. tenuissimum*

3.0

Table 2. *Fusarium* species and haplotype designations for phylogenies, ARGs and genetrees

Species	GenBank Accession	Haplotypes	
		Phylogeny and ARG[a]	Genetree[b]
ITS			
F. chlamydosporum[c]	EU715622[e]	H1	H1
F. equiseti (SRRC1606)[d]	KP771785	H3	H4
F. equiseti	EU326202[e]	H4	H3
F. equiseti	KC254029[e]	H6	H3
F. incarnatum (SRRC1633)[d]	KP771786	H5	H1
F. incarnatum	KP453980[e]	H5	H1
F. incarnatum	KJ572780[e]	H5	H1
F. oxysporum (SRRC1630)[d]	KP771787	H2	H2
F. oxysporum	EU326216[e]	H2	H2
F. oxysporum	HQ651161[e]	H2	H2
tef1α			
F. chlamydosporum[c]	HM134858[e]	H1	H1
F. equiseti	KR071777[e]	H6	H5
F. equiseti	KP450714[e]	H6	H5
F. equiseti	JQ429377[e]	H6	H5
F. incarnatum (SRRC1606)[d]	KT950249	H5	H6
F. incarnatum (SRRC1633)[d]	KT950250	H5	H6
F. incarnatum	KF255493[e]	H7	H7
F. oxysporum (SRRC1630)[d]	KT950251	H2	H2
F. oxysporum	FJ904872[e]	H4	H4
F. oxysporum	KF728241[e]	H3	H3

[a] Haplotypes based on collapsing sequences with recoded indels and excluded infinite sites violations. Loci with fewer than four haplotypes will not have an inferred phylogeny or ARG.

[b] Haplotypes based on collapsing sequences with excluded indels and excluded infinite sites violations. Loci with fewer than four haplotypes will not have an inferred phylogeny or ARG.

[c] Out-group species used for each alignment.

[d] Sequences obtained from Nigerian isolates and accessioned in GenBank.

[e] Sequences borrowed from previously accessioned data.

Table 3. *Cladosporium* species and haplotype designations for phylogenies, ARGs and genetrees

Species	GenBank Accession	Haplotypes	
		Phylogeny and ARG[a]	Genetree[b]
ITS			
C. salinae[c]	NR_119606[e]	H1	H1
C. cladosporioides (SRRC1616)[d]	KP771788	H2	H2
C. cladosporioides (SRRC1634)[d]	KP771789	H2	H2
C. cladosporioides	KJ558398[e]	H2	H2
C. cladosporioides	KF444172[e]	H2	H2
C. oxysporum	HM148119[e]	H2	H2
C. tenuissimum	KP131832[e]	H2	H2
C. tenuissimum	AY545639[e]	H2	H2
C. tenuissimum	JN033474[e]	H2	H2
C. tenuissimum	JQ246357[e]	H2	H2
tef1α (with *C. salinae*)			
C. salinae[c]	JN906993[e]	H1[b]	H1
C. cladosporioides	JF499872[e]	H3[b]	H3
C. cladosporioides	HM148290[e]	H7[b]	H7
C. cladosporioides	HM148248[e]	H9[b]	H9
C. oxysporum	HM148363[e]	H8[b]	H8
C. tenuissimum (SRRC1616)[d]	KT950252	H2[b]	H2
C. tenuissimum (SRRC1634)[d]	KT950253	H4[b]	H4
C. tenuissimum	HM148466[e]	H10[b]	H10
C. tenuissimum	HM148461[e]	H6[b]	H6
C. tenuissimum	HM148459[e]	H5[b]	H5
tef1α (without *C. salinae*)			
C. cladosporioides[c]	JF499872[e]	H1	H1
C. cladosporioides	HM148290[e]	H7	H7
C. cladosporioides	HM148248[e]	H8	H8
C. oxysporum	HM148363[e]	H6	H6
C. tenuissimum (SRRC1616)[d]	KT950252	H2	H2
C. tenuissimum (SRRC1634)[d]	KT950253	H3	H3
C. tenuissimum	HM148466[e]	H9	H9
C. tenuissimum	HM148461[e]	H5	H5
C. tenuissimum	HM148459[e]	H4	H4

[a] Haplotype based on collapsing sequences with recoded indels and excluded infinite sites violations. Loci with fewer than four haplotypes will not have an inferred phylogeny or ARG.

[b] Haplotypes based on collapsing sequences with excluded indels and excluded infinite sites violations. Loci with fewer than four haplotypes will not have an inferred phylogeny or ARG.

Haplotype designations for the examined *Fusarium* loci are listed in Table 2. Isolate SRRC1630 shared a haplotype (H2) with the other two *F. oxysporum* sequences examined. There was enough diversity to segregate the three *F. equiseti* sequences, including that of SRRC1606, into individual haplotypes (H3, H4 and H6). The *tef1α* locus segregated the *Fusarium* species into seven haplotypes, and no evidence of the ancestral sequence could be observed for this group of fungi. SRRC1606 and SRRC1633 had identical *tef1α* sequences (H5) and could not be segregated as individuals. This locus resulted in different haplotype segregations compared to ITS. For example, SRRC1630 and the other *F. oxysporum* sequences segregated into individual haplotypes (H2, H3 and H4) while the *F. equiseti* sequences shared a single haplotype (H6). For the *Cladosporium*sequence dataset, a majority of the ITS sequences were identical, resulting in only two haplotypes (Table 3). Haplotype H1 included only the *C. salinae* sequence, and the second haplotype included all the remaining sequences examined. No evidence of an ancestral sequence was observed for the ITS.The *tef1α* locusfor the *Cladosporium*sequences were distinct enough to segregate each of the sequences into its own haplotype for both inference types (Table 3).An evidence was observed of the ancestral sequence within a *C. tenuissimum* sequence (H6) downloaded from GenBank when *C. salinae* was used as the outgroup taxa, but without this sequence the inference of the ancestral sequence was lost. A total of five *C. tenuissimum* sequences, including SRRC1616 (H2) and SRRC1634 (H4), examined for this locus were considered as separate individuals.

3.3 *Phylogenetic inference*

Using a population framework, phylogenetic inference for the Fusaria at the *tef1α* locus (Figure 1; Table 2)

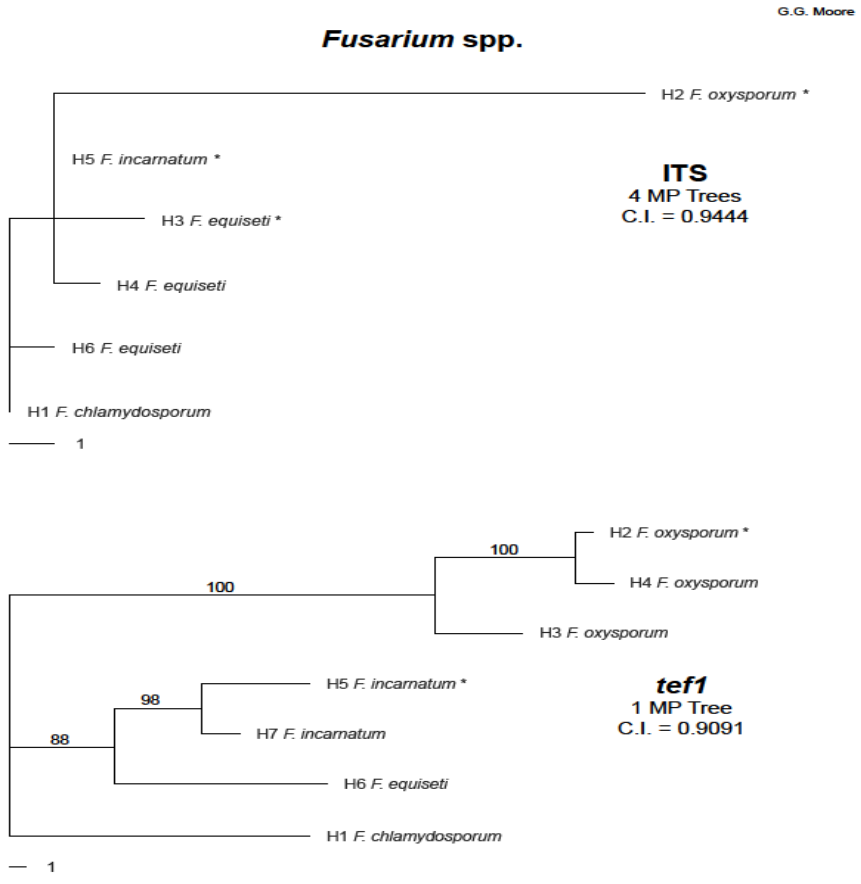

Figure. 1 Phylogenies for two genomic regions in multiple *Fusarium* species. The outgroup species (H1) used for the inferred trees was *F. chlamydosporum*. Mutational distance separating each haplotype can be determined using the scale bar beneath each tree. Bootstrap values above 70 are displayed at the nodes. Also displayed beneath each locus name is the consistency index (C.I.) value and number of most parsimonious (MP) trees obtained. The haplotype designations and GenBank accessions for each locus are shown in Table 2.

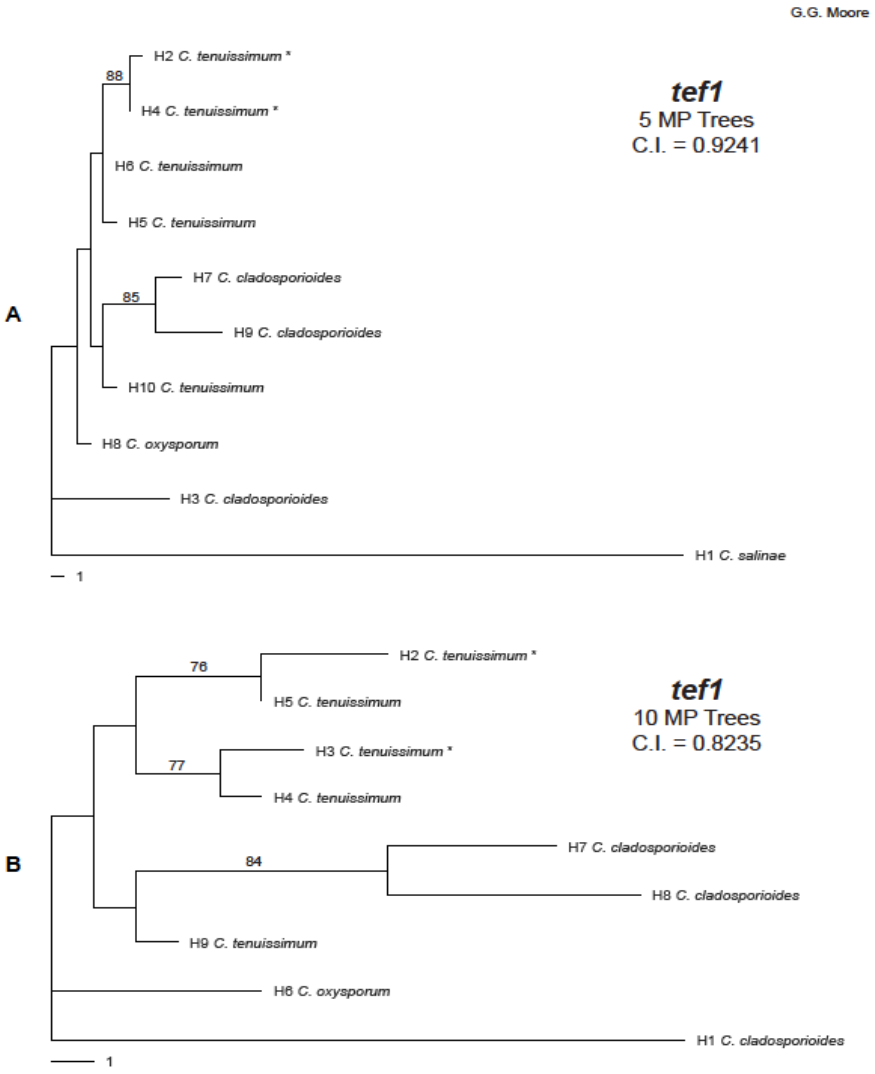

Figure. 2 Phylogenies for the *tef1α* locus in multiple *Cladosporium* species. The respective outgroup species (H1) used for inferred tree A was *C. salinae* and for inferred tree B was *C. cladosporioides*. No ITS phylogeny could be inferred because too few haplotypes resulted when the sequences were collapsed. Mutational distance separating each haplotype can be determined using the scale bar beneath each tree. Bootstrap values above 70 are displayed at the nodes. Also displayed beneath each locus name is the consistency index (C.I.) value and number of most parsimonious (MP) trees obtained. The haplotype designations and GenBank accessions for each tree are shown in Table 3.

resulted in the formation of three lineages:*F. oxysporum*, which included SRRC1630,exhibited greater phylogenetic distance from the other species examined *F. incarnatum*, which included SRRC1606 and SRRC1633,shared a clade with the *F. equiseti* sequences and the outgroup taxa *F. chlamydosporum*. Strong bootstrap support was observed for each clade. Similar associations were inferred for the ITS phylogeny (Figure 1; Table 2) although clade groupings were not as clear and no bootstrap support could be determined. The *tef1α* locus for the *Cladosporium* sequences failed to infer a phylogeny with recoded indels, so two different PHYLIP alignments were created that allowed for phylogenetic inference. For the first, we excluded indels and infinite sites violations in the alignment when collapsing sequences in SNAP:Map, which allowed a phylogeny to be inferred using PAUP*. For the second, we removed *C. salinae* as the outgroup taxa and instead used a *C. cladosporioides* sequence downloaded from GenBank as the root; we were then able to recode indels during our collapse of the sequences, and subsequently use PAUP* to infer a phylogeny. Both phylogenies are displayed in Figure 2, and their haplotype designations are shown in Table 3.Two Nigerian *C. tenuissimum* isolates (SRRC1616 and SRRC1634) shared strong cladal associations (H2 and H4, respectively) and bootstrap support, for the *tef1α* locus (Figure 2A), within a larger clade that included two other *C. tenuissimum* sequences (H5 and H6) obtained from

GenBank. However, a fifth *C. tenuissimum* sequence (H10) shared greater cladal association with two *C. cladosporioides* sequences (H7 and H9) and marginal association with the *C. oxysporum* sequence (H8). One *C. cladosporioides* sequence (H3) and the outgroup taxa (*C. salinae*) were observed as distinct lineages from the remaining sequences. In Figure 2B we observed similar haplotype associations. For example, the *C. cladosporioides* sequence representing H3 in Figure 2A remained a distinct lineage as H1 in Figure 2B, and the same *C. tenuissimum* sequence (H10) that shared a cladal association with the other two *C. cladosporioides* sequences (H7 and H9) in Figure 2A again exhibited close phylogenetic associations in Figure 2B (H7-H9). The differences we observed related to *C. oxysporum* as a distinct lineage (H6) instead of sharing a clade with other species. The two Nigerian isolates (*C. tenuissimum*) exhibited different haplotype groupings(Figure 2A). The *Cladosporium*ITS sequences were too similar and resulted in only two haplotypes for the ten sequences (representing four species); therefore, phylogenetic inference could not be obtained for this region.

3.4 *Comparative analyses*

RecMin analyses among the *Fusarium* species indicated a minimum of 11 recombination events for the *tef1α* locus, and one for the ITS region; therefore, an ARG was inferred for each (Figure 3; Table 2).

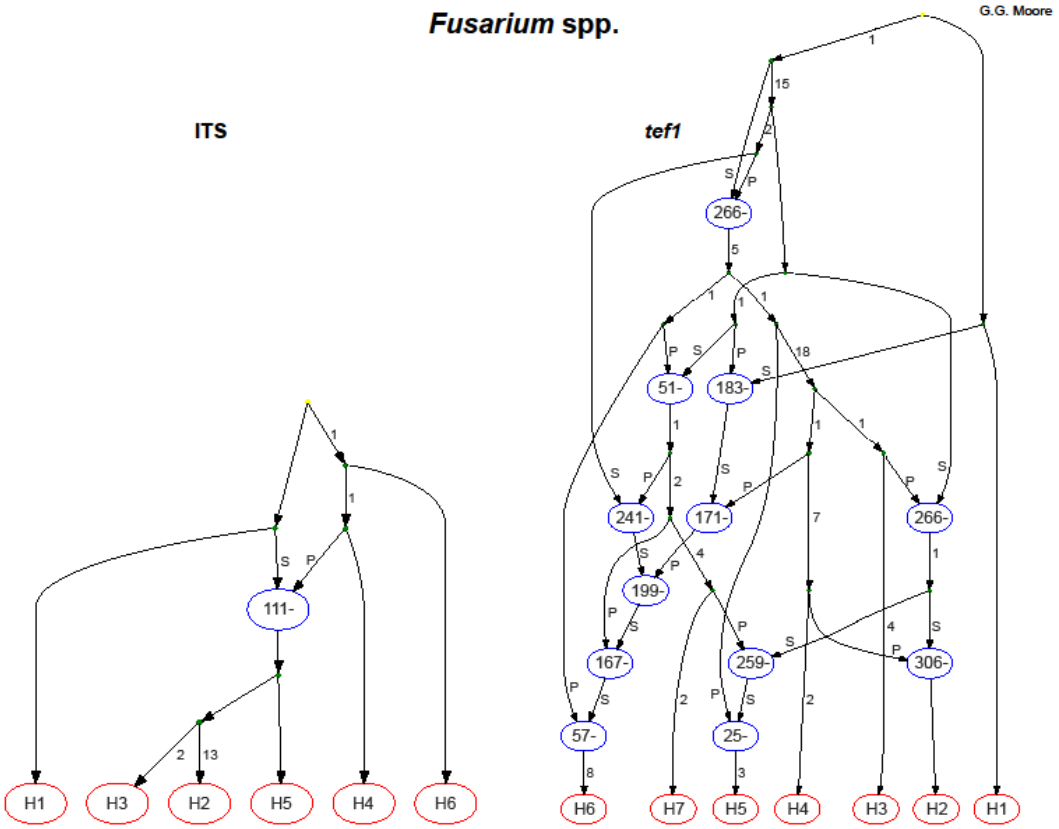

Figure. 3 Ancestral recombination graphs (ARGs) for two genomic regions in multiple *Fusarium* species. Each ARG was inferred using the Beagle heuristic method for all polymorphisms within the analyzed sequences. Each ARG shows the possible mutation and recombination paths that result in the sampled haplotypes (red ovals). The top of the ARG represents the past and the bottom represents the present. The paths leading to the recombination nodes (blue ovals) are labelled with a P (prefix) or S (suffix), indicating the 5' to 3' segments of the recombinant sequence, respectively; the number in each oval indicates the variable position immediately to the left of the recombination breakpoint. Numbers along the path lines indicate polymorphisms. The haplotype designations for each locus are shown in Table 2.

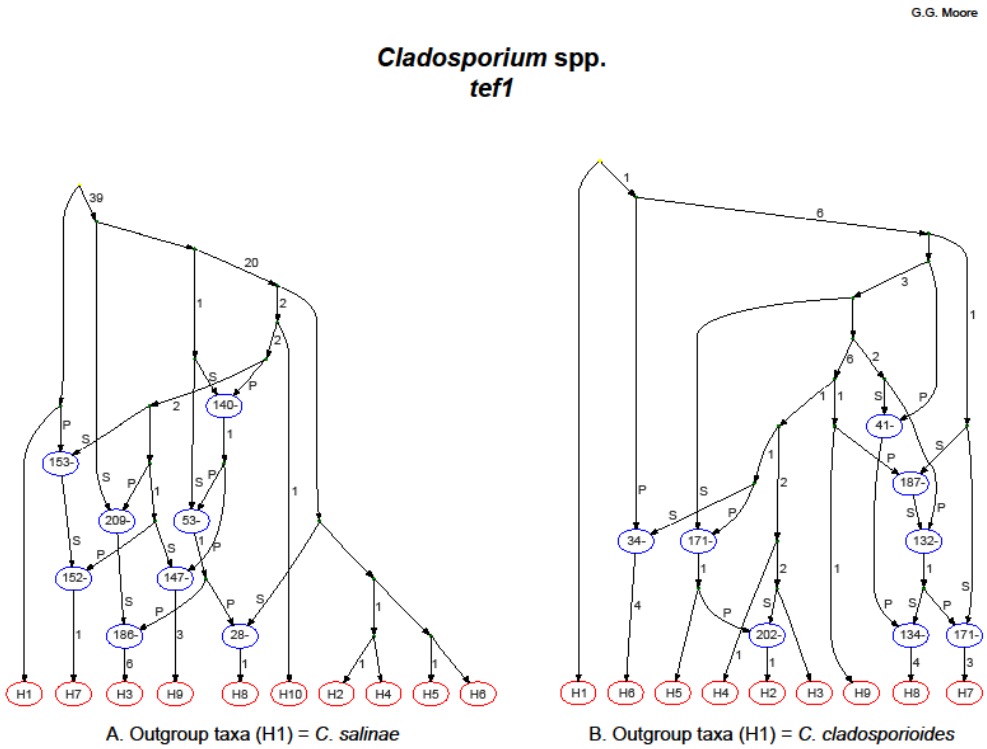

Figure. 4 Ancestral recombination graphs for the*tef1α* locus in multiple *Cladosporium* species. The respective outgroup species (H1) used for inferred ARG A was *C. salinae* and for inferred ARG B was *C. cladosporioides*. No ITS ARGs could be inferred because too few haplotypes resulted when the sequences were collapsed. The ARGs were inferred using the Beagle heuristic method for all polymorphisms within the sequences analyzed. Each ARG shows the possible mutation and recombination paths that result in the sampled haplotypes (red ovals). The top of the ARG represents the past and the bottom represents the present. The paths leading to the recombination nodes (blue ovals) are labeled with a P (prefix) or S (suffix), indicating the 5' to 3' segments of the recombinant sequence, respectively; the number in each oval indicates the variable position immediately to the left of the recombination breakpoint. Numbers along the path lines indicate polymorphisms. The haplotype designations for each ARG are shown in Table 3.

According to the ARG for the *tef1α* locus, 12 recombination events existed throughout the histories of all species/haplotypes examined, with the exception of the outgroup taxa (*F. chlamydosporum*, H1). However, evidence existed to suggest the shuffling of the *tef1α* sequence from *F. chlamydosporum* into other species/haplotypes. Haplotype H6 (*F. equiseti*) appeared to result from successive recombination events; and this haplotype, as well as H5 (*F. incarnatum*), appeared to result from recombination events that occurred on a recent time scale. Haplotype H5 included Nigerian isolates SRRC1606 and SRRC1633. Two *F. oxysporum* sequences (H3 and H4) experienced a single, ancient recombination event in their respective histories, but the sequence obtained from isolate SRRC1630 resulted from two recombination events. The ARG inferred for the ITS region suggested that haplotypes H1, H4 and H6 (*F. chlamydosporum* and two *F. equiseti* GenBanksequences, respectively) had no histories of recombination; however, an inferred recombination between H1 and H4 appeared to result in the formation of haplotypes H2 (*F. oxysporum* which included SRRC1630), H3(SRRC1606/*F. equiseti*) and H5 (*F. incarnatum* which included SRRC1633). RecMin inferred a minimum of eight recombination events for the *Cladosporium tef1α* locus that included *C. salinae*, and its ARG (Figure 4A; Table 3) showed eight recombination events in the histories of haplotypes H3, H7 and H9 (*C. cladosporioides*), as well as H8 (*C. oxysporum*), with the most recent recombination events resulting in haplotypes H3 and H8. The remaining haplotypes represented the *C. salinae* and *C. tenuissimum* (including SRRC1616 and SRRC1634) species sequences which were not inferred to result from previous recombination events, but they had contributed genetic material to the recombinant sequences mentioned above.

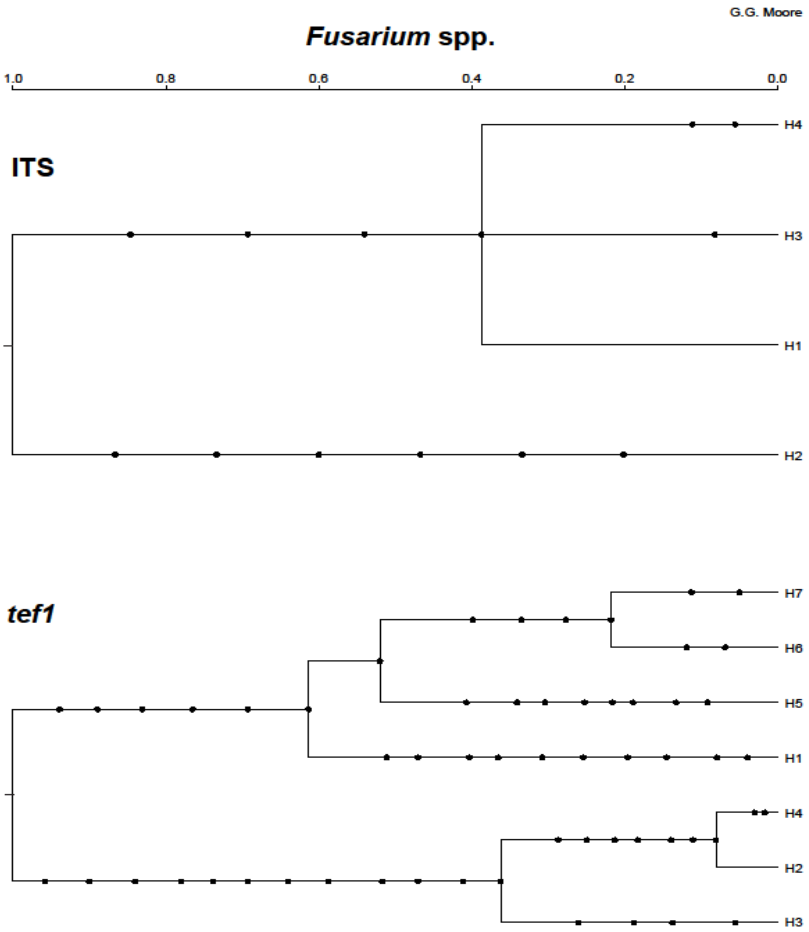

Figure. 5. Gene genealogies for two genomic regions in multiple *Fusarium* species. These coalescent-based trees show mutations as dots along branches and time is scaled to the most recent common ancestor (TMRCA) of 1.0 for each locus. The haplotype designations for each locus are shown in Table 2.

RecMin inferred a minimum of six recombination events for the alignment without*C. salinae* as the outgroup taxa, and the ARG revealed eight possible recombination events (Figure 4B), but the inferences change in regard to most of the haplotypes. Four haplotypes were inferred to have histories of recombination in Figure 4A, but in Figure 4B that number changes to five. The *C. cladosporioides* isolate represented as H3 in Figure 4A was inferred to have derived from a recent recombination event, but this isolate was considered the root for the inferences in Figure 4B. In these types of analyses it is assumed that the root is not a recombinant. Nigerian isolate SRRC1616 was not inferred to have a history of recombination for Figure 4A, but was inferred to result from a recent recombination in Figure 4B. The only two haplotypes that were consistently inferred to have no histories of recombination were H4 and H10 (Figure 4A) and H3 and H9 (Figure 4B). These haplotypes, for both ARGs, contained the same representative sequences (Table 3). Since the *Cladosporium*ITS region resulted in too few haplotypes, no recombination analyses could be performed. In the coalescent analyses and haplotype designations for the *Fusarium* species as shown in Figure 5 and Table 2, two *Fusarium* lineages for the *tef1α* region were observed , with *F. oxysporum* being the sole species represented in one of them, while the second lineage experienced a series of speciation events that resulted in the other Fusaria examined. The *F. oxysporum* lineage had experienced bifurcations that segregated into three haplotypes (H2-H4). The other lineage showed the evolutionary time point when *F. chlamydosporum* (H1) diverged from *F. equiseti* (H5) as well as the time point when *F. equiseti* diverged from what is now *F. incarnatum*. Even *F. incarnatum* exhibited a bifurcation into two distinct branches represented by haplotypes H6 (Nigerian isolates) and H7.

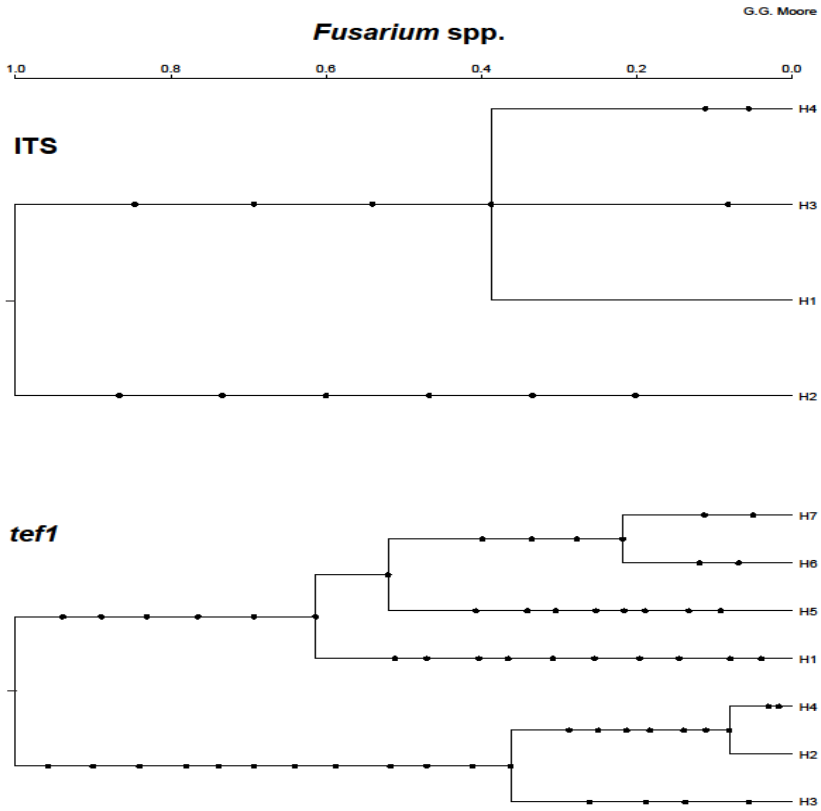

Figure. 6. Gene genealogies for the*tef1α* locusin multiple *Cladosporium* species. The respective outgroup species (H1) used for inferred genetree A was *C. salinae* and for inferred genetree B was *C. cladosporioides*. No ITS ARGs could be inferred because too few haplotypes resulted when the sequences were collapsed. These coalescent-based trees show mutations as dots along branches and time is scaled to the most recent common ancestor (TMRCA) of 1.0 for each locus. The haplotype designations for each locus are shown in Table 3.

The ITS locus also inferred two ancient lineages whereby *F. oxysporum* (H2), as a species/complex, had persisted as its own lineage. The second lineage offered little resolution for the remaining species examined, because a single bifurcation, occurring millions of years ago, gave rise to three branches undergoing a clonal amplification. Two of those branches are represented by *F. equiseti* (H3 and H4/SRRC1606) and the third branch showed sequence identity for *F. chlamydosporum* and *F. incarnatum*(H1). The *Cladosporium tef1α* gene tree for which *C. salinae* was the outgroup taxa(Figure 6A; Table 3) inferred *C. salinae* as an ancient species and sole representative of its own lineage, while the other species shared a second lineage. A fairly recent bifurcation in the second lineage appeared to segregate *C. tenuissimum* into multiple haplotype branches. One of those branches showed the speciation events associated with *C. cladosporioides* and *C. oxysporum*. The most recent bifurcation for this group resulted in the segregation of the Nigerian isolates (H2 and H4).The genetree without *C. salinae* (Figure 6B) offered more resolution for the group of fungi sharing the second lineage in Figure 6A. We observed bifurcations resulting in haplotypes H1-H6. Haplotypes H7-H9 were not resolved and appeared to undergo clonal amplification since their divergence from H2-H5. No genetree could be inferred for the ITS region due to having only two haplotypes.

4.0 DISCUSSION

It has been shown, through these investigations, that two of the Nigerian *Fusarium* isolates were consistently identified using different genomic loci. This would indicate that ITS and *tef1α* are adequate species delimiters for this particular genus. The exception for this group of isolates was SRRC1606, identified as *F. equiseti* based on ITS sequence, but also as *F. incarnatum* based on its *tef1α* sequence. Both species are part of the *Fusarium* incarnatum-equiseti species complex (FIESC)[O'Donnell *et al.*, 2009] and share a most recent common ancestor. In SRRC1606 we see evidence of recombination whereby the ancestry of the ITS region originated with an *F. equiseti* strain and the *tef1α* locus originated with an *F. incarnatum* strain. Reports of *F. equiseti* and *F. incarnatum* as specifically pathogenic to yams are lacking although both species

have been reported as pathogens of potatoes [Goswani *et al.,* 2008, Thrane, 2014]. Some of the mycotoxins associated with *F. equiseti* are nivalenol, A and B trichothecenes and zearalenone [Goswami *et al.,* 2008, Kristensen *et al.,* 2005], while *F. incarnatum* has been reported to produce beauvericin and zearalenone [Thrane, 2014]. Isolate SRRC1633 wassampled from bread and identified as *F. incarnatum* based on ITS and *tef1α* regions. Although reports of *F. incarnatum* as a cereal grain pathogen are rare, they do exist [Mackinaite *et al.,* 2006]. Isolate SRRC1616 was consistently identified as *F. oxysporum* based on both genomic regions examined. This isolate/species was sampled from cooked rice which correlated with its reported pathogenicity of cereal grains, and beauvericin and moniliformin are among the mycotoxins that may be produced by *F. oxysporum* [Thrane, 2014, Hsuan *et al.,* 2010]. ITS and *tef1α* did not offer consistency for species delimitation for sampled *Cladosporium* species from Nigeria. We determined the respective identifications of isolatesSRRC1616 and SRRC1634 as *C. cladosporioides*(ITS) and *C. tenuissimum*(*tef1α*). Taxonomically, *C. Cladosporioides* shares no synonymy with *C. tenuissimum*, which supported that these are distinct species. However, these two species are part of the same (*C. cladosporioides*) complex [Sandoval-Denis *et al.,* 2015] and share a most recent common ancestor. Interestingly, no evidence of recombination was observed in the histories of these isolates. This likely relates to the necessity of removing indels and infinite sites violations in order to proceed with our haplotype associations and comparative inferences. SRRC1616 was sampled from cooked rice while SRRC1634 was sampled from groundnut (peanut). Our findings supported previous reports of a host-pathogen relationship between these two fungal species and cereal grains such as rice, but only *C. cladosporioides* has been reported on groundnut [Pitt and Hocking 1997). There have been no reported mycotoxins produced by *Cladosporium* species[Milgroom, 1996]. The exact reason for two conserved loci offering different taxonomy is uncertain, but may be the result of ancient interspecific recombination, or horizontal gene transfer, of the loci examined from one species to another that has maintained in isolates like SRRC1606, SRRC1616 and SRRC1634.

From a phylogenetic perspective, the Fusaria exhibited an expected segregation for the loci examined. Cladal associations could be observed, and overall the species/complex distinctions were maintained. For example, *F. chlamydosporum* represents its own species complex (FCSC) but is considered a closely-related lineage to the FIESC complex that includes the *F. incarnatum* and *F. equiseti* species [O'Donnell *et al.,* 2009]. The most divergent lineage of species examined was *F. oxysporum* which represents its own species complex (FOSC). Inferring phylogenetic association for the Cladosporia;we were unable to infer a phylogeny for the ITS region because 99% of the sequences obtained were identical. The examined species are encompassed within the *C. cladosporioides* species complex. The observation that not enough haplotype diversity existed for the*Cladosporium* ITS region suggests that these are indeed closely-related species whose diversity will likely exist in other loci. For example, we observed greater sequence diversity for the *tef1α* locus. As expected for closely-related species, two *C. cladosporioides*sequences were observed sharing a clade with *C. tenuissimum* while a third *C. cladosporioides* sequence was inferred as its own lineage.The high degree of diversity among *C. tenuissimum* sequences could relate to geography or host specificity, but could also relate to misidentifications which can occur for closely-related species. Future studies should involve an increase in the numbers of sampled isolates for more refined inferences of intra-species population dynamics.

Contemporary recombination is an occurrence that is more likely within panmictic populations, but with conserved loci it is still possible to infer ancient recombination events, despite geographic isolation, among co-evolved species [Milgroom 1996, Carbone and Kohn 2004]. We were able to infer recombination within the histories of multiple *Fusarium* species based on two different genomic regions that are considered "conserved" [Bradshaw *et al.,* 2006]. This finding would support that (1) historical recombination likely contributed to species diversity among these fungi [Kerenyi *et al.,* 2004, Stergiopolous *et al.,* 2007], and (2) true species delimitation for these fungi would require genome-scale comparisons and a more holistic approach to identification [Samson and Varga, 2009]. Recombination among *Cladosporium* species was only observed for the *tef1α* locus, but the richness of species diversity may require examining other loci and additional species. Based on our inferences of recombination, the different species within each genus, are either exhibiting ancient genetic configurations that have been maintained in certain species, or are exchanging genetic material on a recent time scale as part of their co-evolution. This is because they share a common ancestor,

Coalescent analysis removes the influence of recombination from species evolution by simply

counting mutations and applying them to a time scale based on the assumption of a neutral mutation rate [Griffiths and Tavare, 1994]. Basically, the greater the number of mutations along a sequence, the longer that sequence has been in existence. The finding that ITS sequences from three *F. oxysporum* isolates segregated as a distinct lineage, with no divergence, suggests it is an ancient organism that long ago diverged from the common ancestor of the other examined *Fusarium* species. Despite being species from closely-relatedcomplexes, *F. chlamydosporum*, *F. equiseti* and *F. incarnatum* exhibited recent divergence of their respective ITS regions with no further resolution. More evident patterns of divergence/speciation were observed for the *tef1α* locus. For example, in the *F. oxysporum* lineage isolate SRRC1630 was derived from a recent divergence event. The divergence of FCSC and FIESC occurred millions of years ago, and not too long after their divergence a speciation event segregated *F. equiseti* and *F. incarnatum*. The *tef1α* locus for the Cladosporia also suggested an ancient divergence for which the *C. salinae* lineage is shown as much older than the other species examined.Resolution of the divergence of the remaining *Cladosporium* species is moderate, but what can be inferred are recent divergence/speciation events for species that share a complex. As well, the Nigerian isolates appeared to be the most recently derived.

In conclusion, the sampling of *Fusarium* species on foodstuffs in Nigeria is important when one considers the potential for the identified species to produce mycotoxins. We did not perform any mycotoxin assays for our sampled isolates in this study. The identification of a fungus on a particular host for which it is not considered a pathogen may indicate the presence of new species that happen to share genomic sequence with a known species/pathogen. Our findings support the need for a more holistic approach to species identification; particularly, when those fungi are contaminating foods and feeds and even cosmetics(Pinto *et al.,* 2012).Of recent, there was a hint that *Cladosprorium* species produced mycotoxins(Alwatban *et al.,* 2014).This further underscores the need for in depth molecular analysis of this genus as an emerging toxin producer.

ACKNOWLEDGEMENTS

The authors would like to thank Mrs. Shannon B. Beltz for her assistance in the morphological identification of the Nigerian isolates, and all reviewers who helped to improve this body of work.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest. This article does not contain any studies with human participants The authors did not also use animal subjects during the experiments.

REFERENCES

Alwatban, M.A, Hadi, S and Moslem, M.A. (2014.) Mycotoxin production in *Cladosporium* species influenced by temperature regimes *Journal of Pure Applied. Microbiology*, 8(5):4061-4069 ·

Aylor D.L, Price E.W and Carbone I. (2006). SNAP: Combine and Map modules for multilocus population genetic analysis. *Bioinformatics* 22: 1399-1401

Bensch K, Groenewald J.Z, Dijksterhuis J, Starink-Willemse M, Andersen B, Summerell BA, Shin H.D, Dugan F.M, Schroers H.J, and Braun U (2010). Species and ecological diversity within the *Cladosporium cladosporioides* complex (Davidiellaceae, Capnodiales). *Studies in Mycology*; 67:1-94

Bradshaw R.E, Foster S.J, and Monahan B.J. (2006). Molecular diagnostic tools for detection of plant pathogenic fungi. In Rao JR, Fleming CC, Moore JE, editors. Molecular Diagnostics: Current Technology and Applications. Norfolk, United Kingdom: Horizon Bioscience;.pp. 47-70.

Braun U. (2001). *Cladosporium exoasci, C. exobasidii* and some allied species. *Schlechtendalia*; 7: 53–58.

Braun U, Crous P.W, Dugan F.M, Groenewald J.Z, and de Hoog GS (2003). Phylogeny and taxonomy of cladosporium-like hyphomycetes, including *Davidiella* gen. nov., the teleomorph of *Cladosporium s.str. Mycological Progress*; 2: 3-18.

Braun U, and Schubert K (2007). Taxonomic revision of the genus *Cladosporium* s. lat. 7. Descriptions of new species, a new combination and further new data. *Schlechtendalia* 16: 61-76.

Carbone I, and Kohn L. (2004). Inferring process from pattern in fungal population genetics. *Applied Mycology and .Biotechnology*; 4: 1-30.

Castaño R, Scherm B, and Avilés M. (2014). Genetic Diversity of *Fusarium oxysporum* f. sp. *dianthi* in Southern Spain. *Journal of Mycology*; Article ID 582672, 14 pages. doi:10.1155/2014/582672.

Chehri K, Salleh B, Yli-Mattila T, Reddy K.R.N and Abbasi S. (2011). Molecular characterization of pathogenic *Fusarium* species in cucurbit plants from Kermanshah province, Iran. *Saudi Journal of Biological Sciences;* 18: 341-351.

Carbone I. and Kohn L.M. (1999). A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* 91: 553-556.

Darvishnia M. (2013). Morphological and Phylogenetic Studies of *Fusarium* species in Iran. *Journal.of Novel Applied Science* 2: 1134-1142

De Hoog G.S, Guarro J, Gené J, and Figueras M.J. (2000). Atlas of Clinical Fungi.2nd ed. Utrecht, The Netherlands: *Centraalbureau voor Schimmelcultures*;

Fapohunda SO, Moore GG, Ganiyu OT, and Beltz S.B. (2012). Toxigenic *Aspergillus flavus* and other fungi of public health concern in food and organic matter in southwest Nigeria. *Mycology: An International Journal of. Fungal Biology* 3: 210-219.

Goswami, R.S., Dong Y. and Punja Z.K. (2008) Host range and mycotoxin production by *Fusarium equisetti* isolates originating from ginseng fields. *Canadian Journal of Plant Pathology* 30: 255-260

Griffiths RC and Tavaré S. (1994) Ancestral inference in population genetics. *Statistical Science* 9: 307-319.

Holmes EA, Bennett RS, Spurgeon DW, Colyer PD, and Davis R.M (.2009) New genotypes of *Fusarium oxysporum* f. sp. *vasinfectum* from the southeastern United States. *Plant Disease* 93: 1298-1304.

Hsuan HM, Zakaria L, and Salleh B. (2010). Characterization of Fusarium isolates from rice, sugarcane and maize using RFLP-IGS. *Journal of Plant Protection Research* 50: 409-415.

Kerényi Z, Moretti A, Waalwijk C, Oláh B, and Hornok L. (2004). Mating type sequences in asexually reproducing *Fusarium* species. *Applied Environmental Microbiology* 70: 4419-4423

Kovalsky-Paris MP, Schweiger W, Hametner C, Stückler R, Muehlbauer GJ, Varga E, Krska R, Berthiller F, and Adam G. (2014) Zearalenone-16-O-glucoside: a new masked mycotoxin. *Journal of Agricultural Food Chemistry* 62:1181-1189.

Kristensen R, Torp M, Kosiak B, and Holst-Jensen A. (2005) Phylogeny and toxigenic potential is correlated in *Fusarium* species as revealed by partial translation elongation factor 1 alpha gene sequences. *Mycological Research* 109: 173-186.

Lyngsø RB, Song YS, and Hein J. (2005). Minimum recombination histories by branch and bound. *Proceedings of the 5th International Workshop on Algorithms in Bioinformatics (Lecture Notes in Bioinformatics 3692)* pp. 239-250.

Mačkinaitė R, Kačergius A, Lugauskas A, and Repečkienė J. (2006) Contamination of cereal grain by *Fusarium* micromycetes and their mycotoxins under Lithuanian climatic conditions. *Ekologija*; 3: 71-79.

Migheli Q, Balmas V, Harak H, Sanna S, Scherm B, Aoki T, and O'Donnell K (2010). Molecular phylogenetic diversity of dermatologic and other human pathogenic fusarial isolates from hospitals in northern and central Italy. *Journal of Clinical Microbiology* 48: 1076-1084

Milgroom M.G. (1996) Recombination and multilocus structure of fungal populations. *Annual Review of Phytopathology* 34: 457-477.

Mishra RK, Pandey BK, Singh V, Mathew AJ, Pathak N,Zeeshan M. (2013). Molecular detection and genotyping of *Fusarium oxysporum f. sp. psidii* isolates from different agro-ecological regions of India.. *Journal of Microbiology* 1: 405-412.

Myers SR, and Griffiths RC. (2003) Bounds on the minimum number of recombination events in a sample history. *Genetics* 163: 375-94.

O'Donnell K, Sutton DA, Rinaldi MG, Gueidan C, Crous PW, and Geiser DM. (2009). Novel multilocus sequence typing scheme reveals high genetic diversity of human pathogenic members of the *Fusarium incarnatum-F.equiseti* and *F. chlamydosporum* species complexes within the United States. *Journal of Clinical Microbiology* 47: 3851-3861

Oechsler RA, Feilmeier MR, Ledee DR, Miller D, Diaz MR, Fini ME, Fell JW,and Alfonso EC (2009). Utility of molecular sequence analysis of the ITS rRNA region for identification of *Fusarium* spp. from ocular sources. *Investigative Ophthalmology & Visual Science* 50: **2230-2236.**

Pinto FCJ, Braga de Lima D, Agustini BC, Dallagassa CB, Shimabukuro MF, Chimelli M, Brand D, Telles Fadel-Picheth CM, and Bordin Bonfim TM. (2012). Morphological and molecular identification of filamentous fungi isolated from cosmetic powders. *Brazilian Archives of Biology and Technology* 55: 897-901.

Pitt JI, and Hocking AD. (1997). Fungi and food spoilage. 2nd ed. Sydney: Springer Press;

Price EW, and Carbone I. (2005)SNAP: workbench management tool for evolutionary population genetic analysis. *Bioinformatics* 21: 402-404.

RollanC, Protto V, MedinaR, Lopez S, Vera-Bahima J, Ronco L, Saparrat M, and Balatti P. (2013). Identification of races 0 and 2 of *Cladosporium fulvum* (syn *Passalora fulva*) on tomato in the Cinturón Hortícola de La Plata, Argentina.*Plant Disease*; 97:992.

Sampietro DA, Marín P, Iglesias J, Presello DA, Vattuone MA, Catalán CA, and Gonzalez-Jaen MT. (2010). A molecular based strategy for rapid diagnosis of toxigenic *Fusarium* species associated to cereal grains from Argentina. *Fungal Biology* 114: 74-81.

Sampietro DA, Ficoseco ME, Jimenez CM, Vattuone MA, and Catalán CA. (2012) Trichothecene genotypes and chemotypes in Fusarium

graminearum complex strains isolated from maize fields of northwest Argentina. *International Journal of Food Microbiology*; 153: 229-233.

Samson RA, and Varga J. (2009). What is a species in *Aspergillus*? *Medical Mycology*; Suppl 1: S13-S20

Sandoval-Denis M, Sutton DA, Martin-Vicente A, Cano-Lira JF, Wiederhold N, Guarro J, and Gené J. (2015). *Cladosporium* species recovered from clinical samples in the United States. *Journal of Clinical Microbiology*; 53: 2990-3000

Schoch C.L, Shoemaker R.A, Seifert KA, Hambleton S, Spatafora JW, and Crous PW. (2006). A multigene phylogeny of the Dothideomycetes using four nuclear loci. *Mycologia*; 98: 1041-1052.

Short D.P, O'Donnell K, Zhang N, Juba JH, and Geiser DM. (2011). Widespread occurrence of diverse human pathogenic types of the fungus *Fusarium* detected in plumbing drains. *Journal of Clinical Microbiology;* 49: 4264-4272.

Stergiopoulos I, De Kock M.J, Lindhout P, and De Wit PJ. (2007). Allelic variation in the effector genes of the tomato pathogen *Cladosporium fulvum* reveals different modes of adaptive evolution. *Molecular Plant Microbe Interaction*; 20: 1271-1283.

Swofford D.L. (2003). *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4.0*. Sinauer Associates, Sunderland, Massachusett

Tamura M, Naoki M, Nagatomi Y, Harayama K, Akira T, and Hayakawa K. (2015). A method for simultaneous determination of 20 *Fusarium*toxins in cereals by High-Resolution Liquid Chromatography-Orbitrap Mass Spectrometry with a pentafluorophenyl column. *Toxins*; 7: 1664-1682.

Thrane U (2014). *Fusarium*. In: Batt C.A, Tortorello ML, editors. Encyclopedia of Food Microbiology. New York: Academic Press; p. 76-81.

Zalar P, de Hoog G.S, Schroers H.J, Crous P.W, Groenwald J.Z, and Gunde-Cimerman M. (2007). Phylogeny and ecology of the ubiquitous saprobe *Cladosporium sphaerospermum*, with descriptions of seven new species from hypersaline environments. *Studies in Mycology*; 58: 157-183.