Phylogeny of the Pine Pitch Canker Fungus, *Fusarium circinatum:* An emerging global view

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Introduction

Pitch canker, caused by the ascomycete *Fusarium circinatum*, is one of the most serious diseases of *Pinus spp*. and represents a significant threat to native forests and commercial pine-based forestry. In mature trees the disease results in pitch-soaked wood, crown die-back and stunted growth, whilst in nursery stock, it can cause serious root and root collar disease, resulting in seedling mortality. Globally, pitch canker is known in the USA, Mexico, Chile, Haiti, South Africa, Spain, and Japan, with unconfirmed reports from Italy, Iraq, South Korea and China. Many of these introductions occurred quite recently, indicating a significant need for the development and implementation of appropriate quarantine and disease management practices. To achieve this, the overall objective of our research is to increase our knowledge of the global evolution and population biology of the pitch canker fungus. The aim of this preliminary study was to infer the evolution of the fungus from a subset of pitch canker isolates using the combined sequence information from four unlinked nuclear loci.

Materials and Methods

Thirty fungal isolates from California (isolates 16-20), Florida (isolates 26-30), Mexico (isolates 11-15), Chile (isolates 21-25), South Africa (isolates 06-10) and an undisclosed location (UL; isolates 01-05) were either collected from the field or obtained from the Fusarium Culture Collection (FCC) at FABI. DNA was extracted from these isolates and several housekeeping genes were PCR-amplified and sequenced. These included elongation factor 1-alpha (EF1-α), β-tubulin, the intergenic spacer region (IGS) of the ribosomal RNA operon and the short sequence polymorphic marker, locus FC-9 (Britz *et al*. 2002). These sequences were then aligned using BioEdit v.7.0.5.2 (Hall. 1999) and only those with no missing data were subjected to parsimony analysis using PAUP* v.4.0b10 (Swofford. 2001). The IGS PCR amplicons were also subjected to restriction fragment length polymorphism (RFLP) analyses using the enzymes *Alu*I, *Hha*I and *Hpa*II.

Results and Discussion

RFLP profiles of IGS with *Alu*I, *Hha*I and *Hpa*II varied both between and within populations (Figure 1). The South African isolates had the same RFLP profile as the three USA isolates examined and three of the Mexican isolates. However, the remaining Mexican isolates displayed the same RFLP profile as the Chilean isolates.

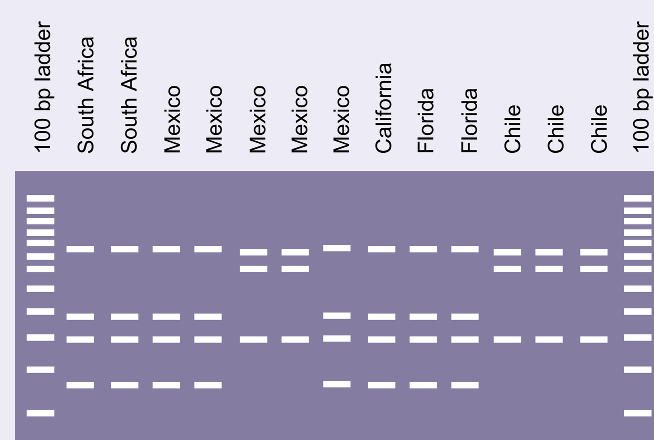

Figure 1. Schematic representation of a 3% agarose gel showing the RFLP profiles of IGS amplicons digested with *Hha*I of various fungal isolates.

Currently, we have complete sequences for the four examined loci from 20 *F. circinatum* isolates. Sequence comparisons revealed the presence of a number of single nucleotide polymorphisms (SNPs) and indels (insertions and/or deletions) among the different populations (Table 1). These are as follows:

EF-1α - 6 SNPs and 1 indel. Three isolates from California, one from Florida and one from South Africa have three SNPs and a 4 bp indel in common. Additionally, one of the SNPs was only present in the three Mexican isolates that share a RFLP profile with the South African and USA isolates (Figure 1); One SNP is present in all the Mexican and Chilean isolates; and two South African isolates share another SNP.

No:	Country	EF-1α				β-tubulin	IGS				FC-9
Indels		328					358				40
SNPs		54,366,557	176	267	532	522		769,809,1342	794	1040	
01	UL					A	158 bp	3 SNPs			
02	UL					A	158 bp	3 SNPs			
03	UL					A	158 bp	3 SNPs			
04	UL					A	158 bp	3 SNPs			
05	UL					A	158 bp	3 SNPs			
06	South Africa	CTTC		T		A					
07	South Africa			T			?	?	?	?	3 bp
08	South Africa								G		3 bp
09	South Africa										6 bp
10	South Africa								G		3 bp
11	Mexico		C		G						
12	Mexico		C		G						
13	Mexico		C		G						
14	Mexico		C				78 bp	?	?	?	
15	Mexico		C				78 bp			G	
16	California							?	?	?	
17	California	CTTC				A					
18	California	CTTC				A		?	?	?	
19	California										
20	California	CTTC				A	?	?	?	?	?
21	Chile		C				78 bp	?	?	?	
22	Chile		C				78 bp			G	
23	Chile		C				78 bp			G	
24	Chile		C				78 bp	?	?	?	
25	Chile		C				78 bp			G	
26	Florida	CTTC						?	?	?	3 bp
27	Florida										
28	Florida					A		?	?	?	3 bp
29	Florida					?	158 bp	3 SNPs			3 bp
30	Florida					A					?

Table 1. Table listing the indels and SNPs found in the four examined loci with their positions in our alignments shown at the top of each column. The 4 bp indel found in EF-1α is marked and the same 5 isolates also have 3 SNPs in common. The indels in IGS and FC-9are indicated by the number of base pairs deleted. ?s denote missing data. The isolates with shaded numbers were not used in the final analysis.

β-tubulin - 1 SNP that grouped all the UL isolates together along with one from South Africa, three from California and three from Florida.

IGS - 5 SNPs towards the 3' end of the gene and 2 indels towards the 5' end of the gene (Figure 2). Three of the SNPs are shared by all the UL isolates and one from Florida. One of the SNPs is common to two South African isolates and one SNP is common to a Mexican and all the Chilean isolates. All the UL isolates and one from Florida share a 158 bp deletion. At the same nucleotide position there is a deletion of 78 bp that is common to all the Chilean isolates and the same two Mexican isolates that shared the same RFLP profile (Figure 2).

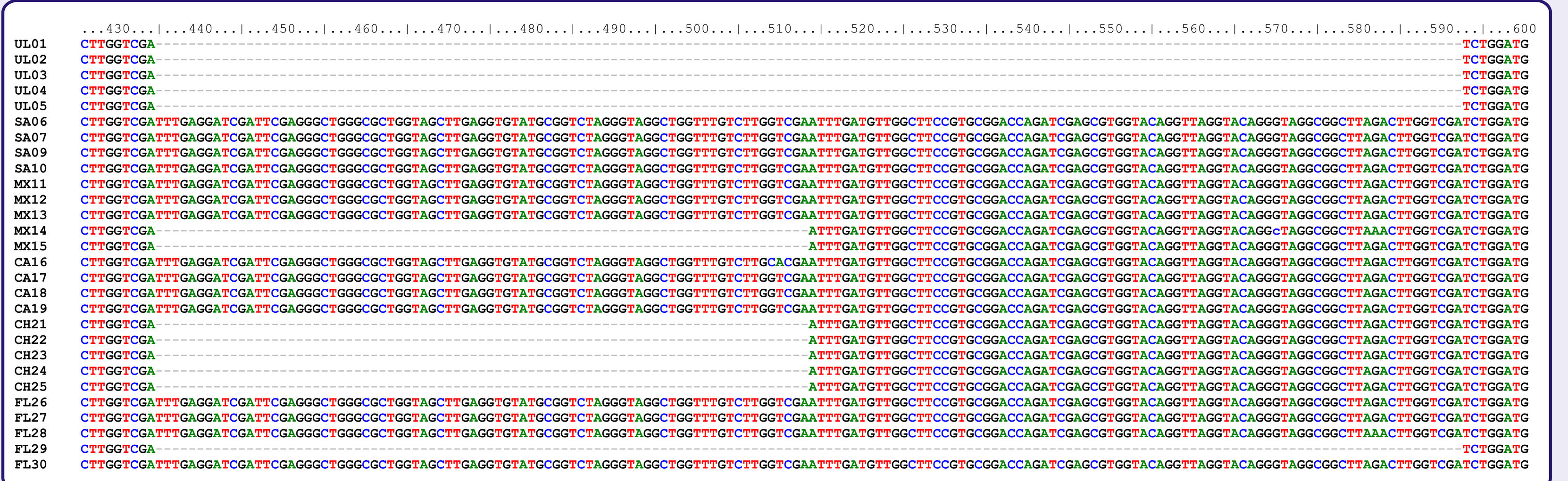

Figure 2. The portion of the IGS alignment showing the indels. The numbers above the sequence indicate their position in the overall alignment. Gaps are indicated by hyphens. UL-undisclosed location; SA-South Africa; MX-Mexico: CA-California; CH-Chile; Fl-Florida.

FC-9 - 2 indels. At this specific postion, most sequences have three trinucleotide repeats (CAA) but three isolates from South Africa and three from Florida have only two repetitions of this trinuceotide repeat and one South African isolate has no repetitions of the trinucleotide.

The combined dataset consisted of only 14 informative characters from a total of 3009 characters. Phylogenetic analyses revealed four distinct groups of isolates (Figure 3). All the Mexican and Chilean isolates grouped together as a distinct South American clade. The five UL isolates also formed a cluster, but this also includes one of the Florida isolates. However, we could not detect any distinct groupings among the USA and South African isolates.

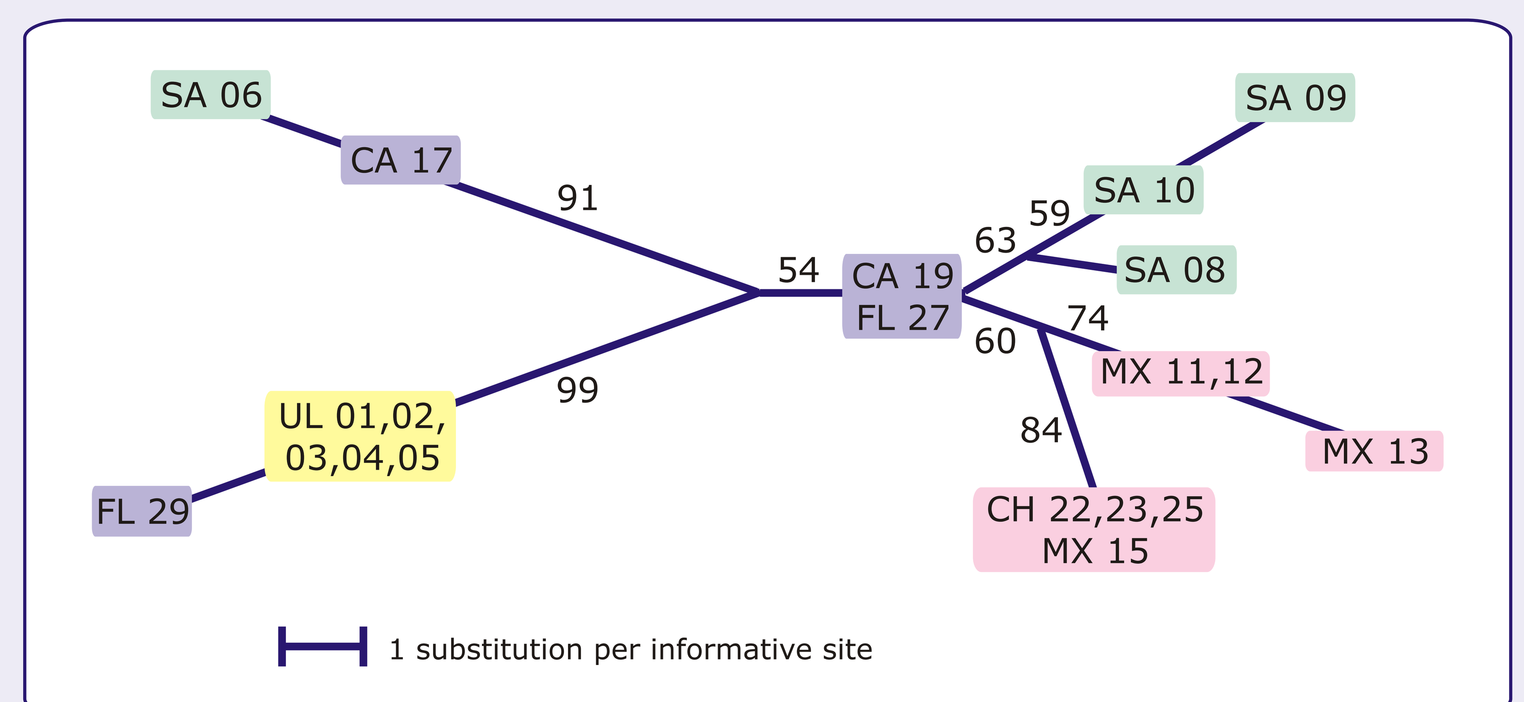

Figure 3. Diagram of an unrooted parsimony tree of concatenated nucleotide sequences of EF-1α, β-tubulin, IGS and FC-9. Bootstrap values from 1000 replicates are given by the branches. SA-South Africa; MX-Mexico: CA-California; CH-Chile; Fl-Florida.

Conclusions

The results of this preliminary study suggest that the Chilean *F. circinatum* isolates of *F. circinatum* are more closely related to the isolates from Mexico than those representing the other populations examined.

As no differences have yet been found among the UL isolates, it is possible that they are a clonal population possibly originating from Florida as indicated by our phylogenetic tree (Figure 3) and the shared 158 bp deletion in IGS (Figure 2).

Although the study included relatively few phylogenetically informative sites, our findings suggest that, by sequencing additional polymorphic regions and increasing the number of informative sites, a well supported and detailed view of the evolution of the pitch canker fungus will emerge. This, together with other data such as vegetative compatibility, microsatellite, and sexual mating analyses will greatly improve our understanding of the biology and possibly the global spread of this important fungus.

Acknowledgements

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