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Review

Molecular Characterization of *Alternaria* spp. and Presence of Toxin in Isolated Genes: Review

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Abstract: The precision and efficiency of phytosanitary regulatory pursuits are conditional upon correct recognition of solitary confinement material, which in turn is reliant upon an exact taxonomic structure. Traditional disease diagnosis methods include the study of indicators on the host, isolation of fungi in suitable culture media, and ascertaining sexual and asexual edifices' attributes along with spores used for the taxonomic classification. Attainment of components allowing pathogenicity on hosts has been indicated to have led the conversion in *Alternaria alternata* from a saprophytic to a plant pathogenic lifestyle. These elements are described as being "host-specific" or "host-selective" because they provide specific pathogenicity to a particular host or range of hosts. Barcoding uses a set of primers with broad specificity to magnify genetic regions typically 500-800 bp in length. Cytochrome oxidase subunit 1 is the animal barcoding locus and has been commonly used in fungi. Phylogenetic examinations have endeavored to define whether *A. alternata* pathotypes containing specific toxin genes are different phylogenetic taxa, and three descriptions can be deemed for the allotment of pathotypes all through the *Alternaria* phylogeny. Based on the above, this review aimed to present the molecular characterization of *Alternaria* spp. and toxin in isolated genes.

Keywords: molecular characterization; mycotoxins; *Alternaria*; gene isolation.

1. Introduction

The accuracy and effectiveness of phytosanitary regulatory activities depend upon correct identification of quarantine material, which is dependent upon an accurate taxonomic framework [1]. Conventional methods of disease diagnosis involve the study of symptoms on the host, isolation of fungi in suitable culture media, and determining the characteristics of sexual and asexual structures and spores used for the taxonomic identification [2]. Isolates need to be assigned a taxonomic name to register and monitor the occurrence of disease [3]. In the *Alternaria* this has traditionally relied upon observations of sporulation structures under standard conditions [4]. Identification based upon molecular methods, rather than morphological examination, is becoming more prevalent in modern diagnostic laboratories [5]. Molecular techniques offer generic tests, meaning that less expertise needs to be maintained, allowing a cost-effective, sustainable service. Molecular methods can also provide

advantages in speed, automation can be performed at a high-throughput and are less ambiguous in their results [6].

Genetic markers showing resolution between quarantine and nonquarantine pathogens are therefore required [7]. Recent progress has been made in resolving the *Alternaria* genus and related organisms' taxonomy, but the *A. alternata* species group is still unresolved. The quarantine pests *Alternaria mali* and *Alternaria gaisen* belong to this unresolved group [8]. Current barcoding loci need to be assessed for their suitability to differentiate quarantine from nonquarantine species within the *A. alternata* species group [9]. Commonly used barcoding loci do not determine taxa, then more specific barcoding loci must be developed for particular groups [10]. Variable phylogenetic loci that show species and population-level variation in other systems need to be assessed for their potential as secondary molecular markers across *Alternaria* taxa [11]. If previously used loci do not show resolution, then novel loci may be developed, taking advantage of the increased access to genomic sequence data. DNA barcoding is an approach to identify species using short, standard genetic markers rapidly [12]. Often combinations of species recognition criteria are employed. Distinct groups of genetically isolated individuals identified by GSR and supported by MSR may be the closest that species can satisfy the definition of the ESC. Where species are truly asexual, then a combination of the MSR and PSR may be considered the most relative to fulfill an ESC [13].

2. Host-Selective Production by *Alternaria* spp. Toxins

An acquisition of factors allowing pathogenicity on hosts has been suggested to have led the transition in *A. alternata* from a saprophytic to a plant pathogenic lifestyle [14]. These factors are referred to as being "host-specific" or "host-selective" because they provide specific pathogenicity to a particular host or range of hosts. The term "host-selective toxin" (HST) is typically used as these toxins may confer pathogenicity to a range of hosts [15]. Host-selective pathogenicity in *A. alternata* is determined by the production of host selective toxins (HSTs). Tsuge et al. [14] summarise previous definitions of an HST as "a compound that possesses the following characteristics: (1) host-selective toxicity, (2) selective toxicity matching the specificity of the HST-producing pathogen, (3) plants insensitive to the HST must be resistant to the pathogen producing the compound, (4) the compound can reproduce the initial physiological changes in host cells caused by the HST-producing pathogen and (5) the initial physiological changes caused by HST in host cells leads to penetration or initial colonization by the HST producing pathogen." These criteria have been met for then taxa within the *Alternaria* genus (Table 1). Purified samples of three of the *Alternaria* HSTs have been shown to lose toxicity when heated or by the addition of proteinase K, indicating that they are proteinacious. Structures of five HSTs have been identified and show structural similarity to polyketides. This is supported through the rough lemon toxin being demonstrated to require a specific polyketide synthase gene for its production.

Table 1. Host-selective toxins produced by *Alternaria* spp. pathogens [14].

Pathogen	Toxin	Structure
Alternaria mali	AMT	Polyketide
Alternaria gaisen	AKT	
Alternaria citri	ACRT	
Alternaria tangelonis	ACTT	
Alternaria arborescens	AALT	
Alternaria brassicicola	ABT	
Alternaria brassicae	Destruxin B	Protein
Alternaria panax	APT	
Alternaria longipes	ATT	Unknown
Alternaria tenuissima	ATCT	

3. Barcoding Loci and Phylogenetic Species Concept of *Alternaria* spp.

Barcoding uses a set of primers with broad specificity to amplify genetic regions typically 500-800 bp in length [16]. Cytochrome oxidase subunit 1 (CO1) is the animal barcoding locus [17] and, as such, has been commonly used in fungi [18]. The cytochrome oxidase locus has been concluded to be less appropriate for the barcoding of fungi than the internal transcribed spacer (ITS) region [19]. The ITS locus is a region of ribosomal DNA (rDNA) cistron covering two noncoding parts between the 18S and 28S rDNA and including the 5.8S rDNA (Figure 1).

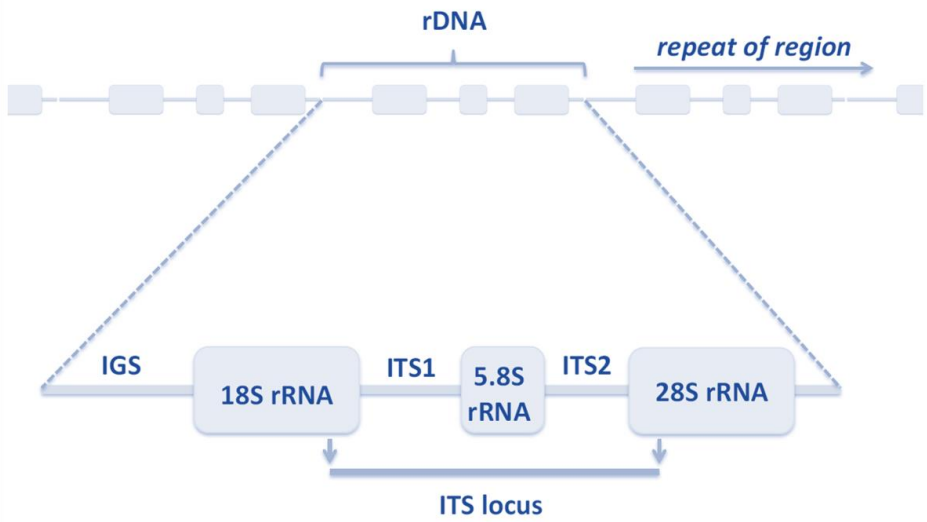

Figure 1. Structure of fungal nuclear ribosomal DNA: Showing regions coding for 18S rRNA, 5.8S rRNA, and 28s rRNA. Noncoding sequences at the intergenic spacer (IGS) and internal transcribed spacer regions (ITS1 and ITS2) is presented. The position of the "ITS locus," an area commonly used for barcoding, is marked [20].

The locus's structure is conserved enough across the fungal kingdom to allow for high amplification success using standard primers. It shows high levels of interspecific variation and intra-specific variation. It is widely considered as the fungal barcoding region [20]. This is reflected in the prevalence of sequencing of this locus in routine diagnostics, as of February 2012, there were over 172,000 full-length fungal ITS sequences deposited on Genbank, representing 15,000 species [21]. The ITS region has been reported as showing no inter-specific variability within some species-rich fungal genera such as *Penicillium* and *Fusarium* which has led to criticism in its use as a barcoding gene [22]. It has shown some resolution within the *Alternaria* genus to identify major morphological species groups and differentiate the *A. alternata* species group from other small-spored species such as *Alternaria infectoria* [11]. The locus has shown little or no resolution when studies have attempted to resolve morphologically described species within the *A. alternata* species group [8,23]. This led to the suggestion that morphological species described within the *A. alternata* species group can be considered intraspecific variants of *A. alternata* [24].

Other commonly used barcoding loci have been used to investigate phylogenetics within the *Alternaria* genus [25]. The 18S nuclear ribosomal subunit rDNA (SSU) and 28S large ribosomal subunit are commonly used loci in phylogenetics of fungi and have been evaluated as potential fungal barcoding loci [26]. Throughout the fungal kingdom, the SSU has shown greater intraspecific variation than the ITS region. The LSU region is recognized as showing a higher resolution than the ITS region in the Ascomycete yeasts but is inferior to ITS in other fungal kingdom regions. Both loci have been used to resolve the *Alternaria* genus but have previously reported low variability within the *alternata* species group [26]. Where standard barcoding loci do not show resolution within taxa,

other loci showing greater variability are required. These are often based upon single copy genes rather than on rDNA regions [27].

Phylogenetic species concepts (PSC) consider distinct clades in genetic phylogenies to represent species [28]. Phylogenetic species recognition uses framework, where "the smallest diagnosable cluster of individual organisms within which there is a pattern of ancestry and descent" delimits a species. Like the morphological species concept, this definition struggles to acknowledge the possibility of subspecies or population-level variation. As such, PSR often uses other species recognition concepts to support it [29]. The development of the PSC was the incorporation of principles from the BSC [30]. This led to genealogical concordance species recognition (GSR). This denotes species boundaries by identifying limits of gene flow between populations as indicated by the dichotomy between multiple genetic phylogenies [30]. Where tree topologies are identical, there is no gene flow between populations as polymorphic sites have become genetically fixed [31]. If gene flow can occur between populations, populations do not have their own "historical fate" and do not have distinct "evolutionary tendencies [32]." If gene flow does not occur, we can consider these groups as distinct species (Figure 2).

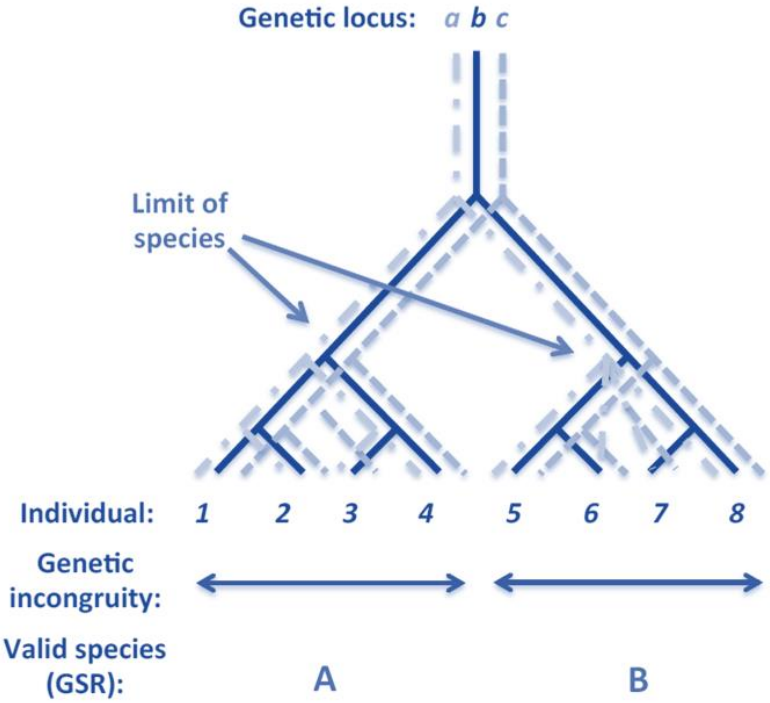

Figure 2. Explanation of genealogical concordance species recognition: Gene flow is present in the incongruity between tree topologies (as shown in genetic loci a, b and c). In this example, evidence of gene flow is shown within two groups of individuals but not between them [33].

Like the BSR, GSR cannot be clearly applied to asexual fungi [34]. This stems from their basis on the ESC, which identifies a species based on strains being a single lineage, having their evolutionary tendencies and historical fate. Following these criteria, each progeny from an asexual species could be considered a new species [35]. To deal with this problem, GSR denotes the species boundary to be the point at which sexuality was lost, and recombination cannot be detected. Application of this form of GSR would be expected to lump whole genera of asexual species into single species [36]. This approach has not been widely tested, but evidence of cryptic sexuality and parasexual recombination shows greater gene flow in putatively asexual species than previously thought [36].

4. Phylogenetic distribution of toxin-synthesis genes

Phylogenetic studies have attempted to determine whether *A. alternata* pathotypes containing specific toxin genes are distinct phylogenetic taxa [37]. The ITS sequence is identical for all pathotypes

leading to these isolates all being designated as *A. alternata*. With the identification of highly variable molecular markers, studies can be performed investigating the distribution of isolates carrying toxin genes within *A. alternata* species group phylogenies [38]. Results of phylogenetics of the *A. alternata* pathotypes could be used to inform diagnostics. If genes on essential chromosomes can be identified, that can phylogenetically resolve the different *A. alternata* pathotypes, then these could act as molecular markers. QBOL, a recent initiative to barcode and develop molecular diagnostics for quarantine pathogens in the EU, is based upon this approach. Establishing whether identification can be performed based on sequencing of phylogenetic loci or toxin-gene specific primers, is of particular importance for the *A. alternata* apple pathotype and the *A. alternata* pear pathotype due the phytosanitary risk posed by their potential establishment in the Europe [39].

Studies that have sampled *Alternaria* from the field often report many of these strains as non-pathogenic on the host they were tested [40]. This was the case in Simmons and Roberts [41], where it was estimated that only 10% of about 400 isolates isolated from pear orchards in East Asia were pathogenic on a susceptible host. This was also the case in a survey of airborne *Alternaria* spores in Japanese orchards of susceptible pear where 2.5% of 515 isolates produced the pear toxin (AKT) and only one in 575 isolates from a resistant orchard produced AKT [42]. Higher percentages were reported in isolations from apple orchards in Italy, but only 35% of 44 single spored isolates could induce necrosis on unwounded leaves of *cv.* Golden delicious in detached leaf assays. Plant pathogenic interactions are often complicated, with suites of effectors and resistance genes typically determining whether a strain of a fungal pathogen will be pathogenic on a particular cultivar of a plant host [43]. Resistance to AMT is reported as being determined by apple cultivars being homozygous or heterozygous for a single dominant allele. Production of HSTs may be a major factor in pathogenicity in *A. alternata*, but not the only factor [43].

The research suggested that due to *A. alternata* being prevalent in air samples, saprophytic airborne spores may take advantage of pre-existing necrotic leaf tissue and cause infection [44]. Synergistic effects have been shown between *Alternaria* spp. and other pests [45]. European red mites feed on the upper and lower surfaces of apple leaves [46]. They have been observed in orchards affected by *Alternaria* leaf blotch that regularly suffer over 60% defoliation through leaf abscission. *Alternaria* leaf blotch could be worse in apple orchards in years that European red mites were at high density and that these two pests had synergistic effects on inducing defoliation in orchards [47,48]. Interactions between fungal pathogens and other factors such as mechanical damage by insect pests are not necessarily recorded when collecting field specimens [48]. *A. alternata* have been shown to produce over 30 non-host specific toxins [49]. *A. alternata* apple pathotype isolates have also been shown to be able to make tentoxin, a nonspecific plant toxin, in addition to AMT in pure culture [14]. All *A. alternata* are considered able to form appressoria to penetrate host cells and are opportunist necrotrophs, as shown by their ability to cause infections in human systems [49]. Some research described the function of HSTs in *A. alternata* as being to affect the regulation of metabolism, permeability, and other vital processes and suppress the induction of defense responses [14].

5. Origin of Conditionally Dispensable Chromosomes

Three explanations can be considered for the distribution of pathotypes throughout the *Alternaria* phylogeny [50]: (a) *A. alternata* CDCs were present in the last common ancestor of the *A. alternata* species group, before undergoing divergence or loss during vertical transmission; (b) CDCs were acquired in multiple independent events; (c) CDCs originated from the core genome through gene duplication and divergence. Similar explanations were tested for the origin of CDCs [51] ("lineage specific regions") within the *Fusarium oxysporum* f. sp. *lycopersici* genome [52]. Analysis of genes on lineage specific regions revealed that only 50% of genes had orthologs in essential chromosomes. This and differences in codon usage indicated that a horizontal gene transfer event had occurred, possibly from related *Fusarium* species. A difference in codon usage was detected between genes on *A. alternata* tomato pathotype essential chromosomes and the CDC. This study showed that only three of 40 genes involved in HST synthesis had orthologs in *A. alternata* genomes

not carrying CDCs [52]. This supports the first two explanations for the origin of CDCs, and future work should focus on testing these hypotheses for the origin of *A. alternata* CDCs (Figure 3).

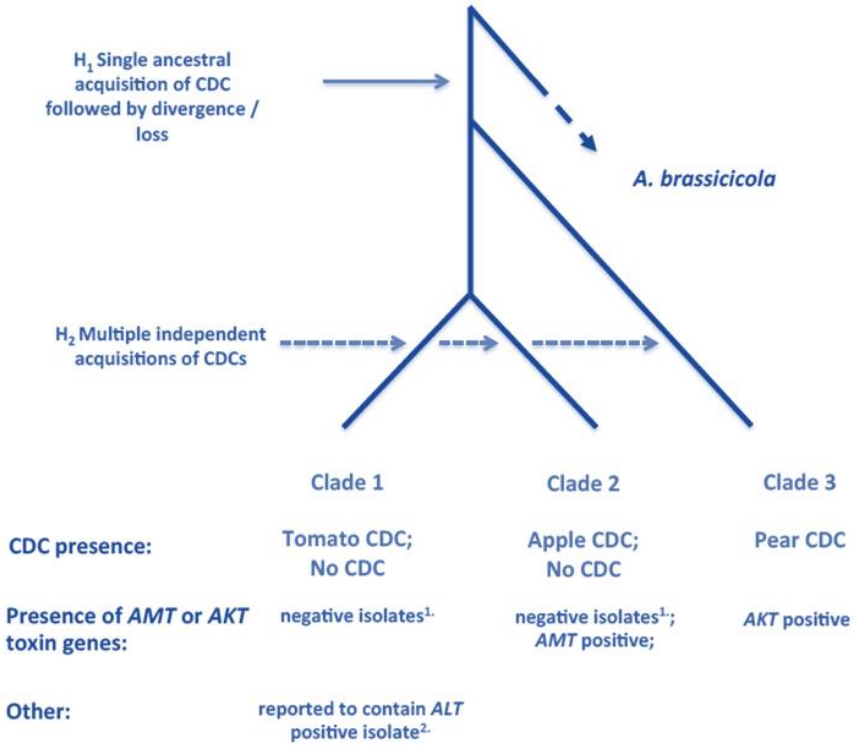

Figure 3. Hypotheses for the acquisition of conditionally dispensable chromosomes in *Alternaria alternata*: Two hypotheses (H1 or H2) to acquire CDCs by horizontal gene transfer shown on an idealized phylogeny representing phylogenetic clades. The presence of toxin genes on the CDCs of apple (AMT) and pear (AKT) is determined by genome sequencing and suggested by PCR screens. [1.] PCR assays indicated that *AMT* or *AKT* genes might be present in some isolates. [2.] The *Alternaria arborescens* reference isolate (*EGS 39.128*) carries the tomato pathotype CDC [53].

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Conflicts of Interest: The authors declare no conflict of interest.

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