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Polyphasic classification of *Alternaria* isolated from hazelnut and walnut fruit in Europe

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ABSTRACT

Brown apical necrosis of English walnut and grey necrosis of hazelnut are destructive fruit diseases caused by a complex of opportunistic fungi including several small-spored catenulate *Alternaria* taxa. Thirty *Alternaria* isolates recovered from walnut and hazelnut fruit that were pathogenic on their respective host were compared along with type or representative isolates of *A. alternata*, *A. tenuissima*, *A. arborescens*, and *A. infectoria* using morphological and molecular criteria. Morphological examination using standardized procedures separated the walnut and hazelnut isolates into three morphological groups: the *A. alternata* group, the *A. tenuissima* group, and the *A. arborescens* group based upon common characteristics of the conidium and the sporulation apparatus. To evaluate genetic relationships among these groups, AFLP markers, inter simple sequence repeat (ISSR) markers, and histone gene sequence data were compared. Based upon AFLP data, the *A. alternata* and *A. tenuissima* groups comprised a single lineage, and the *A. arborescens* group comprised a separate lineage. ISSR data supported the grouping by AFLP data except for three isolates of the *A. alternata* group that clustered with the *A. arborescens* group. Base substitution of the H4 gene supported the discrimination of the *A. arborescens* group from the *A. alternata* and *A. tenuissima* groups. Tests of hypotheses based upon groupings derived from the various data sets supported the discrimination of the *A. arborescens* group but did not support the discrimination of the *A. alternata* group from the *A. tenuissima* group.

Introduction

Brown apical necrosis (BAN) on English walnut (*Juglans regia*) and grey necrosis (GN) on hazelnut (*Corylus avellana*) are recently described diseases reported in Italy (BAN and GN) and France (BAN) (Belisario *et al.* 2002, 2003, 2004). Both diseases cause severe fruit drop resulting in yield loss often exceeding 30 % (Belisario *et al.* 2004). Previous works revealed the causal agents of both diseases to include a number of opportunistic fungi such as *Colletotrichum* sp., *Fusarium* spp., and *Phomopsis* sp. (Belisario *et al.* 2002, 2003), as well as a complex of morphologically diverse small-spored catenulate *Alternaria* taxa (Belisario *et al.* 2004). *Alternaria* isolates recovered from diseased tissue and tested for pathogenicity on fruit of their respective host could be separated into three distinct morphological groups, each typified by a representative *Alternaria* species: the *A. alternata* group, the *A. tenuissima* group, and the *A. arborescens* group, which shared collective characteristics of

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the conidium and three-dimensional sporulation apparatus (Belisario *et al*. 2004). Interestingly, this morphological diversity appeared to be fully represented across hosts, with no significant differences in pathogenicity or symptom development among isolates within each host-defined group (Belisario *et al*. 2004).

The finding of a morphologically diverse complex of small-spored catenulate *Alternaria* taxa recoverable from a single host is not unusual. Similar complexes have been recovered from a number of additional hosts including citrus (Simmons 1990), pear (Simmons & Roberts 1993), cherry (Roberts *et al*. 2000), almond (Teviotdale *et al*. 2001), pistachio (Pryor & Michailides 2002), apple (Serdani *et al*. 2002), and barley (Andersen *et al*. 2002). Perhaps the most comprehensively documented case was the work of Simmons & Roberts, which involved taxa recovered from necrotic lesions on pear and were considered putative causal agents of black spot of pear (Simmons & Roberts 1993). In this study, six distinct morphological groups were recovered, notable groupings being the *A. gaisen* group (most of which represented *A. gaisen*), the *A. alternata* group, the *A. infectoria* group, and the arborescent group [later typified by *A. arborescens* (Simmons 1999b)]. Further work revealed that pathogenicity on pear could be attributed to members of three of the six groups with no differences in pathogenicity or symptom development noted among toxigenic isolates (Simmons & Roberts 1993). Results from studies that included isolates from citrus, cherry, almond, pistachio, apple, and barley were similar to those of the pear study in that two to four distinct morphological groupings of taxa were revealed per host, most typically including the *A. alternata* group, the *A. tenuissima* group, the *A. arborescens* group, and/or the *A. infectoria* group. Importantly, pathogenicity was always attributed to more than one morphological group.

Although it appears well documented that there exists a number of morphologically distinct groups of small-spored catenulate *Alternaria* recoverable as a complex from a number of different hosts, the phylogenetic relationship among these groups is less clear. Studies based upon sequence analysis have revealed that small-spored catenulate *Alternaria* cluster into three distinct monophyletic clades termed species-groups: the brassicicola species-group (uncommonly encountered), the infectoria species-group, and the alternata species-group (Pryor & Gilbertson 2000; Pryor & Bigelow 2003). Note that the term 'species-group' has been adapted from previous usage by Simmons (1992), and when preceded by the non-italicized specific epithet of a representative taxon has been adopted as the nomenclatural format for phylogenetically-based infrageneric groupings of *Alternaria* in general (Pryor & Gilbertson 2000). This usage is to be differentiated from the more specific application of the term 'group' (preceded by the Latin name of a representative taxon) often used in discussions of morphologically similar small-spored catenulate taxa as initiated by Simmons (1990). Members of the *A. infectoria* (morphological) group all belong to the infectoria species-group, which is genetically distinct and phylogenetically distant from other species-groups. However, the other morphological groups discussed, the *A. alternata*, *A. tenuissima*, *A. gaisen*, and *A. arborescens* groups, all are encompassed within the alternata species-group, which reveals very close phylogenetic relatedness among these groups (Pryor & Gilbertson 2000; Pryor & Bigelow 2003). Moreover, sequence variation in loci most commonly used for phylogenetic studies (e.g. ITS, mtSSU) has not been sufficient for robust discrimination among morphological groups within the alternata species-group and has presented significant challenges for systematic, diagnostic, and population studies (Kusaba & Tsuge 1995; Chou & Wu 2002; de Hoog & Horre 2002; Pryor & Michailides 2002; Pryor & Bigelow 2003; Serdani *et al*. 2002; Kang *et al*. 2002; Konstantinova *et al*. 2002).

A number of studies have employed DNA fingerprinting for analysis of relationships among morphologically distinct taxa or groups within the alternata species-group. The most common techniques used for this purpose have been RAPD-PCR, RFLP, and PCR-RFLP analysis (Kusaba & Tsuge 1994; Weir *et al*. 1998; Roberts *et al*. 2000; Pryor & Michailides 2002; Peever *et al*. 2002). However, results from these studies have not been in agreement in regard to which morphological groups represent distinct phylogenetic lineages. RAPD analysis of isolates recovered from pear and cherry, primarily, supported segregation of the *A. alternata*, *A. tenuissima*, *A. arborescens*, *A. gaisen*, and *A. infectoria* groups based upon morphology (Roberts *et al*. 2000). However, studies using RAPD and PCR-RFLP data from groups recovered from pistachio only supported segregation of the *A. arborescens* and *A. infectoria* groups, but isolates in the *A. alternata* and *A. tenuissima* groups resolved as a single clade with no segregation of morphological types (Pryor & Michailides 2002).

Other fingerprinting methods commonly used in studies of populations or closely related taxa include AFLP and inter simple sequence repeat (ISSR) analyses. Since its development (Vos *et al*. 1995), AFLP analysis has been applied to population studies of diverse organisms including bacteria (Janssen *et al*. 1996, 1997), fungi (Mueller *et al*. 1996; Arenal *et al*. 1999; de Barros Lopes *et al*. 1999), plants (Travis *et al*. 1996), and animals (Folkertsma *et al*. 1996). Along with the widespread application across all phyla, AFLP analysis provides advantages over other methods in its reproducibility (Janssen *et al*. 1996). ISSR analysis involves selective amplification of regions lying between tandemly repeating arrays of short oligonucleotide sequences, described as minisatellite or microsatellite sequences depending on their size (Hamada *et al*. 1982; Jeffreys *et al*. 1985). ISSR analysis has been shown informative when comparing closely related species or elements of the same species, which has made it useful in a variety of genetic studies in plant, animal, and fungal species (Liu & Wendel 2001; McCall *et al*. 2004; Zhou *et al*. 2001). However, its use in resolving relationships among morphological groups within *Alternaria* has not yet been assessed.

For loci previously mentioned, sequence analysis has not been a suitable method for reconstructing relationships within the alternata species-group. However, several less-commonly used loci appear to have potential. The endo-PG gene has been shown to be informative for discrimination among closely related *Alternaria*, and has been used successfully, along with sequences from anonymous regions, for reconstructing relationships among small-spored taxa recovered from citrus (Peever *et al*. 2004, 2005). The histone 4 (H4) gene region has also been shown to provide high resolution in discriminating closely related species and sub-specific groups among *Fusarium* and *Colletotrichum* spp. (Donaldson