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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

et al. 1995; Talhinhas et al. 2002), although its usefulness in *Alternaria* systematics has not been assessed.

The objectives of this work were to examine diversity among small-spored catenulate species of *Alternaria* associated with fruit necrosis of walnut and hazelnut and compare molecular-based groupings based upon AFLP, ISSR, and H4 sequence analysis with those based upon morphology. In addition, the robustness of these analyses was evaluated both as single data sets and in concatenation, to establish methods that should be given priority in subsequent studies of relationships among small-spore, catenulate *Alternaria* taxa.

Materials and methods

Fungal isolates and nucleic acid extraction

Thirty single-conidial isolates of small-spored catenulate *Alternaria* taxa were isolated from symptomatic hazelnut and English walnut fruit (Table 1). All isolates used in this study were pre-screened for pathogenicity, and only isolates that were pathogenic both on leaves and on fruit of the respective host were included (Belisario et al. 2004). All isolates were cultured on weak potato dextrose agar (WPDA) (Pryor & Michailides 2002) and incubated in a fully programmable growth chamber (Conviron ATC 10-3, Conviron Controlled Environments, Pembina, ND) using the following environmental parameters: temperature, constant at 22 °C; humidity, constant at 25 % RH; lighting, 60 μmoles/m^2/s using Sylvania FB031/841/XP 31-watt fluorescent lamps (Osram Sylvania, Danvers, MA) at a 10:14 light:dark cycle. Morphological characterization and grouping were based upon criteria established in a previous study (Pryor & Michailides 2002). Type or representative cultures of *A. alternata*, *A. tenuissima*, *A. arborescens*, and *A. infectoria* (EGS 34-016, EGS 34-015, EGS 39-128, and EGS 27-193, respectively) were included for comparative purposes and as reference for each morphological group.

For DNA extraction, isolates were grown on potatoes dextrose agar (PDA; Difco, Detroit, MI) for one week at 22 °C in the dark. Total genomic DNA was extracted from 120 mg of fresh mycelium powdered in 1.5 ml Eppendorf tubes with a sterile pestle in liquid nitrogen. The remainder of the procedure for DNA extraction followed the protocol provided by Puregene for Genomic DNA Purification Kit (Gentra Systems, Minneapolis, MN). Total DNA was resuspended in 50 ml of sterile TE buffer and stored at −20 °C. DNA concentration was estimated visually in 1 % agarose gels by comparing band intensity with known quantities of λ DNA markers

Table 1 – Isolates used in this study and their sources

Species or morphogroup	Code	Host	Origin/Source	Date
Alternaria infectoria	EGS 27-193 (Ex-type)	*Triticum* sp.	*E. G. Simmons*	
A. alternata	EGS 34-016 (Representative)	*Arachis hypogaea*	*E. G. Simmons*	
A. tenuissima	EGS 34-015 (Representative)	*Dianthus* sp.	*E. G. Simmons*	
A. arborescens	EGS 39-128 (Ex-type)	*Lycopersicon esculentum*	*E. G. Simmons*	
A. alternata group	ISPaVe 1769	*Juglans regia*	Treviso, Italy	2001
A. alternata group	ISPaVe 1779	*J. regia*	Labretonie, France	2001
A. alternata group	ISPaVe 1781	*J. regia*	Treviso, Italy	2001
A. alternata group	ISPaVe 1782	*J. regia*	St Livrade sur Lot, France	2001
A. alternata group	ISPaVe 1796	*Corylus avellana*	Viterbo, Italy	2002
A. arborescens group	ISPaVe 1722	*J. regia*	Ferrara, Italy	1998
A. arborescens group	ISPaVe 1768	*J. regia*	Treviso, Italy	2001
A. arborescens group	ISPaVe 1771	*J. regia*	Treviso, Italy	2001
A. arborescens group	ISPaVe 1772	*J. regia*	Rovigo, Italy	2001
A. arborescens group	ISPaVe 1774	*J. regia*	Rovigo, Italy	2001
A. arborescens group	ISPaVe 1775	*J. regia*	Treviso, Italy	2001
A. arborescens group	ISPaVe 1776	*J. regia*	Rovigo, Italy	2001
A. arborescens group	ISPaVe 1778	*J. regia*	Treviso, Italy	2001
A. arborescens group	ISPaVe 1780	*J. regia*	Jusix, France	2001
A. arborescens group	ISPaVe 1784	*J. regia*	Agen, France	2001
A. arborescens group	ISPaVe 1787	*C. avellana*	Viterbo, Italy	2001
A. arborescens group	ISPaVe 1791	*C. avellana*	Viterbo, Italy	2001
A. arborescens group	ISPaVe 1793	*C. avellana*	Viterbo, Italy	2001
A. arborescens group	ISPaVe 1794	*C. avellana*	Viterbo, Italy	2001
A. arborescens group	ISPaVe 1795	*C. avellana*	Viterbo, Italy	2002
A. arborescens group	ISPaVe 1797	*C. avellana*	Viterbo, Italy	2002
A. tenuissima group	ISPaVe 1770	*J. regia*	Rovigo, Italy	2001
A. tenuissima group	ISPaVe 1773	*J. regia*	Treviso, Italy	2001
A. tenuissima group	ISPaVe 1783	*J. regia*	Labretonie, France	2001
A. tenuissima group	ISPaVe 1786	*C. avellana*	Viterbo, Italy	2001
A. tenuissima group	ISPaVe 1788	*C. avellana*	Viterbo, Italy	2001
A. tenuissima group	ISPaVe 1789	*C. avellana*	Viterbo, Italy	2001
A. tenuissima group	ISPaVe 1790	*C. avellana*	Viterbo, Italy	2001
A. tenuissima group	ISPaVe 1792	*C. avellana*	Viterbo, Italy	2001
A. tenuissima group	ISPaVe 1798	*C. avellana*	Viterbo, Italy	2002

(Amersham Biosciences, Piscataway, NJ). The stock DNA was diluted to10 ng/ml and stored in TE buffer at 4 °C to standardize concentrations for working DNA solutions.

AFLP analysis

AFLP DNA fingerprinting analysis was performed as described by Vos *et al.* (1995) except that a fluorescence-labelled primer was used instead of a radio-labelled primer. Template DNA was prepared by digestion with the restriction enzymes, *Eco*RI and *Mse*I, and ligation with *Eco*RI-adapter and *Mse*I-adapter. Pre-selective PCR was performed using a thermal cycler (PTC-100, Bio-Rad, Waltham, MA) with two primers for each adapter, E-0 (GACTGCGTACCAATTC) and M-0 (GATGAGTCCT GAGTAA). Selective PCR was performed using two set of primers, E-AG/M-GA and E-AG/M-GT, where the code following E or M refers to additional selective nucleotides at the 3′ end of the two non-selective primers, E-0 and M-0. E-AG primer was labelled with 6-carboxyfluoescein (Sigma-Aldrich, St. Louis, MO) at the 5′ end. PCR products were analysed by a Perkin-Elmer 3100 sequencing machine (Applied Biosystems, Foster City, CA) with the size marker, GeneScan-500 (ROX; Applied Biosystems).

Peak profiles were read with GenescanView software (available at http://bmr.cribi.unipd.it/). Filtering and alignment of peaks were conducted in three steps. Initially, peaks smaller than 5 % of highest peak were removed. Retained peaks were aligned based upon DNA fragment size. Peaks smaller than 10 % of the highest peak were removed unless their peak heights were over 75 % of the corresponding peaks from related isolates that were accepted. This three-step filtering and alignment method was used to reduce possible errors resulting from peak height variation among AFLP PCR reactions. Peaks between 50 and 450 bp were scored and a binary matrix was constructed. Cluster analysis of the data matrix was performed by the UPGMA with Jaccard's similarity coefficient (Sneath & Sokal 1973) and the goodness of fit was measured by cophenetic correlation (r) analysis using the software NTSYSpc ver. 2.1 (Exeter Software, Setauket, NY).

ISSR analysis

Among the eight primers tested, two minisatellite primers (M13 and T3B, 5′- GAGGGTGGCGGTTCT-3′ and 5′-AGG TCGCGGGTTCGAATCC-3′, respectively) and two microsatellite primers [$(GACA)_4$ and $(CAA)_5$] were selected based upon the production of distinct and reproducible banding patterns. Amplification reactions were performed using a thermal cycler (Gene Amp® System 9700 Applied Biosystems, Foster City, CA) in volumes of 25 μl containing 2 μl of working solution of template DNA (approximately 20 ng), 0.5 mM primer, 0.2 mM each dNTP, 2.5 mM $MgCl_2$, and 1 unit of Ampli*Taq* DNA polymerase in 1× Ampli*Taq* PCR buffer II (Applied Biosystems). The PCR was carried out in the following conditions: 94 °C for 1 min, 50 °C for 1.5 min, 72 °C for 2 min for 40 cycles. Ten microlitres of PCR products were resolved in 1 % agarose gels in 0.5 % Tris–borate–EDTA (TBE) buffer and visualized by UV illumination after staining in ethidium bromide. Gels were photographed on a UV-transilluminator using a digital imaging system (Gel DOC, Bio-Rad, Hercules, CA) and bands were scored as present or absent (1/0). ISSR analyses were performed three times to confirm reproducibility of amplification products. The same clustering procedures and goodness of fit measurements were conducted as for AFLP fingerprint analysis for PCR products between 200 and 2000 bp.

H4 PCR and sequencing

Amplification reactions were performed using the H4-1a/H4-1b primer pair (Glass & Donaldson 1995). PCR was performed using a thermal cycler (Gene Amp® System 9700 Applied Biosystems, Foster City, CA), and each 50-μl amplification reaction consisted of the following: 2 μl of working solution of template DNA (approximately 20 ng), 0.3 mM primer, 0.2 mM each dNTP, 2.5 mM $MgCl_2$, and 0.5 unit of Ampli*Taq* DNA polymerase in 1× Ampli*Taq* PCR buffer II (Applied Biosystems, Foster City, CA). The PCR was carried out in the following conditions: 94 °C for 1 min, 63 °C for 1.5 min, 72 °C for 2 min for 35 cycles. PCR products were visualized on 1 % agarose gels after staining in ethidium bromide, following the procedure above described. PCR products were purified using a Microcon PCR purification kit (Amicon, Millipore, Bedford, MA) according to the manufacturer instructions. Cycle-sequencing reactions were carried out using dye terminator procedure with fluorescent dideoxynucleotides of the BigDye Terminator V 1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). The PCR products were sequenced in both directions using the same primers used in the amplification reactions for confirmation.

Test of hypothesis

Parsimony criterion was used to test congruency of data with hypotheses for groupings based upon results from morphology, AFLP, ISSR, and sequence data. Parsimony trees were reconstructed under the constraints of each hypothesis by heuristic search using tree bisection–reconnection (TBR) branch swapping on starting trees generated by random sequence addition (100 reps) using PAUP 4.0 10b (Swofford 2002). Ten randomly selected trees from the equally parsimonious trees from each tree reconstruction condition were compared with the best trees reconstructed without any constraints by Kishino–Hasegawa (KH) (Kishino & Hasegawa 1989), Templeton (Templeton 1983b), and Winning-sites tests (Templeton 1983a) implemented in PAUP 4.0.

Results

Morphological grouping

Isolates of *Alternaria* recovered from walnut and hazelnut fruit were grouped into three morphological groups, the *Alternaria alternata* group, the *A. tenuissima* group, and the *A. arborescens* group (Table 1). Isolates included in the *A. alternata* group ($n = 5$) developed primary conidium chains 6–12 conidia in length, and secondary conidiophores originating from terminal, median, or basal conidium cells that resulted in abundant secondary and tertiary branches 2–8 conidia in length.

Branching also originated as a result of elongation of conidiophores (primary and secondary) below existing conidia (sub-conidium conidiophore elongation) resulting in geniculated sporulating structures. Isolates included in the *A. tenuissima* group ($n = 9$) developed primary conidium chains 6–16 conidia in length, and developed secondary conidiophores infrequently from terminal, median, or basal conidium cells that resulted in short secondary branches 1–4 conidia in length. Sub-conidium conidiophore elongation was minimal. Isolates included in the *A. arborescens* group ($n = 16$) developed primary conidium chains 4–8 conidia in length, and secondary conidiophores almost exclusively from distal terminal conidium cells. Branching of sporulating structure was almost exclusively as a result of sub-conidium conidiophore elongation resulting in a highly geniculated sporulating structure with secondary, tertiary, and quaternary branches 2–6 conidia in length.

AFLP fingerprint analysis

After filtering procedures, 16–27 peaks were retained from each isolate when E-AG and M-GA primers were used for amplification of restriction fragments, and 15–24 peaks were retained when E-AG and M-GT primers were used. When peaks from walnut and hazelnut isolates and standard isolates of *Alternaria alternata*, *A. arborescens*, and *A. tenuissima* were aligned based upon the DNA fragment size, amplified fragments using E-AG/M-GA primers produced 79 aligned characters and amplified fragments using E-AG/M-GT primers produced 52 characters. *Alternaria infectoria*, which was used as an outgroup, did not share any peaks with walnut and hazelnut isolates, nor with other type or representative isolates. The UPGMA tree, based upon Jaccard similarity coefficients calculated from the combined dataset of AFLP fingerprinting using E-AG/M-GA and E-AG/M-GT selective primer sets for all isolates, is presented in Fig 1 ($r = 0.97534$). The isolates of the *A. alternata* and *A. tenuissima* groups formed a single clade with representative isolates of *A. alternata* and *A. tenuissima*. The *A. alternata* and *A. tenuissima* groups were not discriminated from each other. The isolates of the *A. arborescens* group formed a single clade with the type isolate of *A. arborescens*. The UPGMA tree of each AFLP dataset using E-AG/M-GA or E-AG/M-GT primer sets produced similar groupings with only minor variation in each group (data not shown).

ISSR analysis

Combined dataset of ISSR fingerprints using four primers had 22 to 34 bands for each isolate. Alignment of bands resulted in a 60-character data set. The UPGMA tree, based upon the Jaccard similarity coefficients calculated from combined data sets, is presented in Fig 2 ($r = 0.90770$). Two distinct groups were recognized as in AFLP analysis. However, grouping of isolates was not identical with that from AFLP data. Three isolates of the *Alternaria alternata* group, ISPaVe 1779, 1781, and 1782, were included in the clade containing isolates of the *A. arborescens* group.

H4 gene sequence analysis

PCR amplification of partial H4 genes using the H4-1a/H4-1b primer set produced 245 bp DNA fragments. Alignment of sequences of the H4 gene resulted in five polymorphic sites,

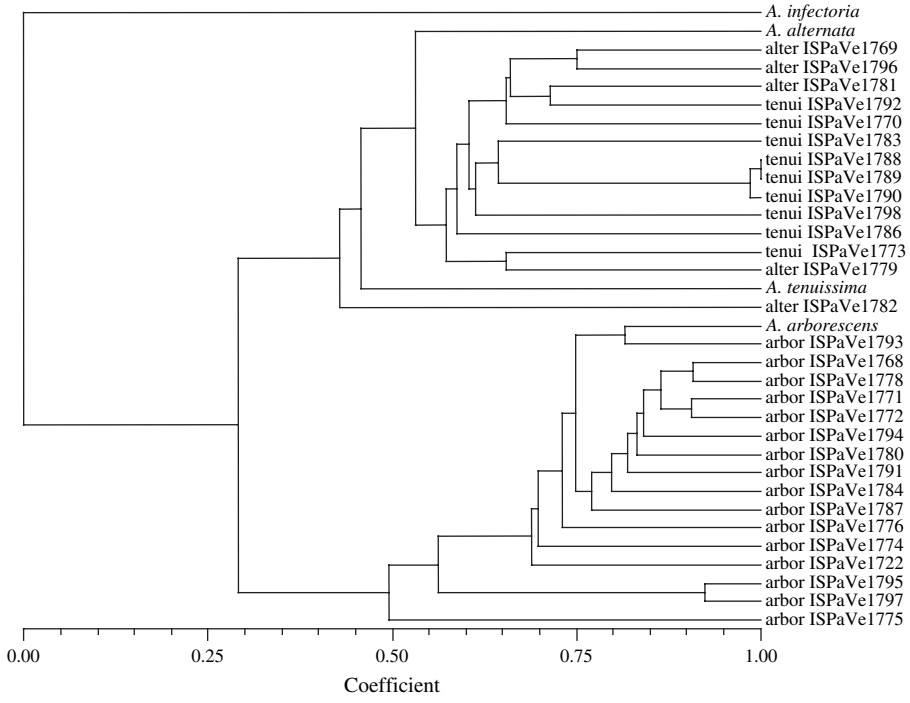

Fig 1 – UPGMA dendrogram of combined dataset of AFLP fingerprint data from *Alternaria* recovered from walnut and hazelnut. Data from type or representative isolates of *A. infectoria*, *A. arborescens*, *A. alternata*, and *A. tenuissima* were included for comparison.

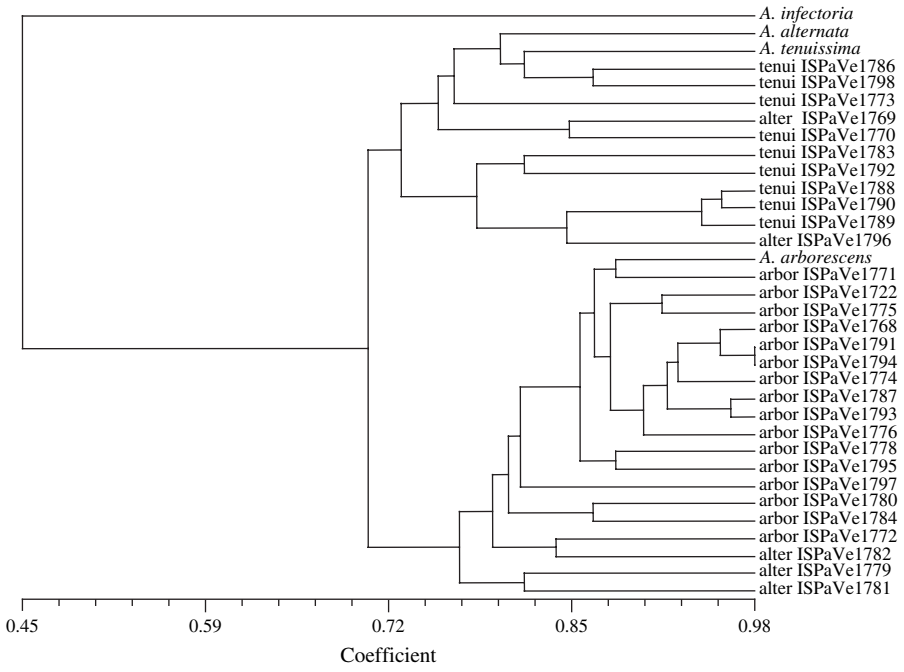

Fig 2 – UPGMA dendrogram of combined dataset of ISSR fingerprint data from *Alternaria* recovered from walnut and hazelnut. Data from type or representative isolates of A. infectoria, *A. arborescens*, *A. alternata*, and *A. tenuissima* were included for comparison.

three in the intron region and two in the exon region (Table 2). The polymorphism found at nucleotide position 73 was not discriminative in delineating three species-groups because only two isolates of the *Alternaria arborescens* group, ISPaVe 1795 and ISPaVe 1797, had a different base from the other isolates. These two isolates were also closely related base upon AFLP data (Fig 1). The only variation at nucleotide position 146 was found in the representative isolate of *A. alternata*. Two polymorphic sites, 77 and 143, had discriminative information in delineating the *A. arborescens* group from the *A. alternata* and *A. tenuissima* groups. This result was consistent with groupings by morphological characteristics and AFLP fingerprint data, but slightly different from ISSR data. Base G at nucleotide position 82 was shared by 11 isolates of the *A. arborescens* group including the type isolate. These isolates did not form a monophyletic lineage in either the AFLP or the ISSR tree. No polymorphic site was found that discriminated the *A. alternata* group from the *A. tenuissima* group.

Test of hypothesis

Three hypotheses of grouping were tested with parsimony criterion (Fig 3). The first hypothesis (Fig 3A) was drawn from the grouping based upon the AFLP fingerprint data. In this hypothesis, isolates of the *Alternaria alternata* and the *A. tenuissima* groups formed a monophyletic lineage, and isolates of the *A. arborescens* group formed the other monophyletic lineage. The second hypothesis (Fig 3B) was drawn from the combined view of AFLP and morphological study. In this hypothesis, the monophyletic lineage of the *A. alternata* and *A. tenuissima* groups in the first hypothesis was further resolved for each group to form monophyletic lineage. The third hypothesis was drawn from the grouping based upon the ISSR data. In this hypothesis, overall grouping of isolates was same with the first grouping, but three isolates of the *A. alternata* group, ISPave 1779, ISPave 1981, and ISPave 1782, were grouped with isolates of the *A. arborescens* group.

Heuristic search of AFLP data without any constrains produced 89 equally parsimonious trees of 299 steps. Heuristic search under constraints of Fig 3B and C produced 174 equally parsimonious trees of 314 steps and 518 equally parsimonious trees of 327 steps, respectively. The null hypothesis of no difference between the best tree and constraint trees were rejected by KH, Templeton, and WS tests implying that the two hypotheses are not consistent with AFLP data (Table 3).

Heuristic search with ISSR data without any constraints produced 229 trees of 193 steps. Heuristic search under constraints of Fig 3A and B produced 350 equally parsimonious trees of 200 steps and 1192 equally parsimonious tress of 209 steps, respectively. The null hypothesis of no difference between the best trees and trees reconstructed under the constraints Fig 3A was not rejected, implying that the hypothesis A is consistent with ISSR data (Table 3). Test of hypothesis B was not congruent among three tree topology tests. Only one of the ten tested tree topologies was inconsistent by KH tests, but six and ten among ten tested tree topologies were inconsistent with ISSR data by Templeton and winning-sites tests, respectively (Table 3).

Discussion

This study provides molecular support for the morphology-based groupings of species and taxa within the phylogenetically-supported alternata species-group using AFLP, ISSR,

Table 2 – Polymorphic sites of the H4 gene

Morphogroup	Species/strain	*Nucleotide position				
		73	77	82	143	146
Alternaria alternata	EGS 34-016	T	T	C	C	A
A. alternata	ISPaVe 1769	T	T	C	C	G
A. alternata	ISPaVe 1779	T	T	C	C	G
A. alternata	ISPaVe 1781	T	T	C	C	G
A. alternata	ISPaVe 1782	T	T	C	C	G
A. alternata	ISPaVe 1796	T	T	C	C	G
A. tenuissima	EGS 34-015	T	T	C	C	G
A. tenuissima	ISPaVe 1770	T	T	C	C	G
A. tenuissima	ISPaVe 1773	T	T	C	C	G
A. tenuissima	ISPaVe 1783	T	T	C	C	G
A. tenuissima	ISPaVe 1786	T	T	C	C	G
A. tenuissima	ISPaVe 1788	T	T	C	C	G
A. tenuissima	ISPaVe 1789	T	T	C	C	G
A. tenuissima	ISPaVe 1790	T	T	C	C	G
A. tenuissima	ISPaVe 1792	T	T	C	C	G
A. tenuissima	ISPaVe 1798	T	T	C	C	G
A. arborescens	EGS 39-128	T	C	G	T	G
A. arborescens	ISPaVe 1722	T	C	C	T	G
A. arborescens	ISPaVe 1768	T	C	C	T	G
A. arborescens	ISPaVe 1771	T	C	G	T	G
A. arborescens	ISPaVe 1772	T	C	G	T	G
A. arborescens	ISPaVe 1774	T	C	G	T	G
A. arborescens	ISPaVe 1775	T	C	G	T	G
A. arborescens	ISPaVe 1776	T	C	G	T	G
A. arborescens	ISPaVe 1778	T	C	C	T	G
A. arborescens	ISPaVe 1780	T	C	G	T	G
A. arborescens	ISPaVe 1784	T	C	G	T	G
A. arborescens	ISPaVe 1787	T	C	C	T	G
A. arborescens	ISPaVe 1791	T	C	G	T	G
A. arborescens	ISPaVe 1793	T	C	G	T	G
A. arborescens	ISPaVe 1794	T	C	G	T	G
A. arborescens	ISPaVe 1795	C	C	C	T	G
A. arborescens	ISPaVe 1797	C	C	C	T	G

*Numbering starts at the 5′ end of the primer H4-1a. Exon 1 ranges from nucleotide positions 1–49. Intron 1 and exon 2 ranges nucleotide positions 50–102 and 103–245, respectively.

and sequence data from the H4 gene for resolving relationships. These types of data have not been used to date in studies involving these specific fungi, and should prove to be a valuable compliment to the other DNA fingerprinting and sequence data that are available.

Different molecular approaches have been undertaken to support the differentiation of small-spored *Alternaria* spp. into distinctive morphological and sporulation patterns. However, confidence must first be established regarding the morphological groups defined. Simmons and others have frequently documented the pleomorphic capacity of this group of *Alternaria* that is dependent upon and impacted by changes in culture conditions (Simmons 1992; Simmons & Roberts 1993; Pryor & Michailides 2002). Although this impact has never been fully quantified, it is well established that culture parameters of media, temperature, lighting, and humidity must be held constant and be reproducible for meaningful comparative studies to proceed. Moreover, such conditions must be invariant between facilities for robust comparisons of results among different laboratories. In many previous studies, descriptions of culture conditions have been either lacking or restricted to statements of temperature and the type of lights used (e.g. Sylvania Cool-white fluorescent) as well as the light:dark cycles maintained during incubation. However, these descriptions generally referred to average conditions encountered on the laboratory bench, which are often not strictly controlled. In this study, all isolates were subjected to perhaps the most completely described culture conditions ever reported for characterization of *Alternaria* into morphological groups. The fact that these conditions were maintained in a programmable incubator reveals that they were also strictly controlled. Although it is recognized that such conditions might not be readily attainable in other laboratories, this study should establish benchmark criteria for future morphological studies. Nevertheless, even with carefully standardized culture conditions to mitigate morphological plasticity, exact delimitation of groups based upon morphological characters may still encounter difficulties due to the presence of isolates with intermediate characteristics.

This study recognized three generalized morphological groupings within the alternata species-group among isolates recovered from walnut and hazelnut fruit, the *A. alternata* group, the *A. tenuissima* group, and *A. arborescens* group. These groups have been recognized by other studies involving isolates recovered from a number of different hosts as well (Simmons 1990; Simmons & Roberts 1993; Roberts *et al.* 2000; Teviotdale *et al.* 2001; Pryor & Michailides 2002; Serdani *et al.* 2002; Andersen *et al.* 2002). Although culture conditions encountered in other studies were not exactly the conditions described in this study, it is interesting to note that the same general groupings were recovered. This suggests that these groupings are reproducible entities, providing there is adherence to minimum standards of culture conditions. However, considering the morphological plasticity of individual isolates and the differences in culture conditions reported, the reproducibility of exact membership within each group can not be evaluated without more controlled comparative studies.

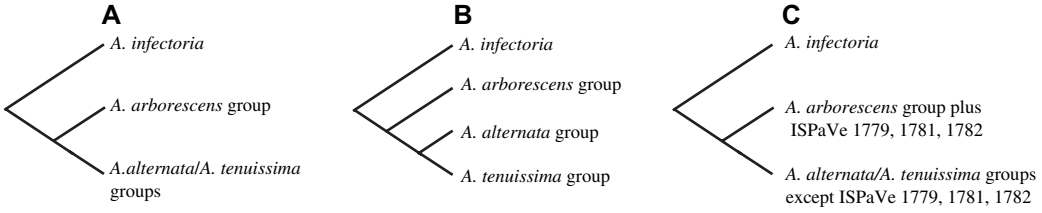

Fig 3 – Three hypotheses for phylogentic relationships among morphology-based groups of small-spored catenulate *Alternaria*: (A) monophyly of *A. arborescens* group and *A. alternata*/*A. tenuissima* groups; (B) monophyly of *A. alternata*, *A. tenuissima*, and *A. arborescens* groups; and (C) grouping produced by ISSR data.

Table 3 – Topology tests under varying constraint

Data set	Constraints[a]	Steps (best tree)	P (KH)	P (Templeton's)	P (Winning-sites)
AFLP	B	314 (299)	0.005 - 0.0186	0.0054 - 0.0207	0.0118 - 0.0266
AFLP	C	327 (299)	<0.0001	<0.001 - 0.002	<0.001 - 0.001
ISSR	A	200 (193)	0.2260 - 0.3495	0.1379 - 0.2416	0.1338 - 0.2478
ISSR	B	209 (193)	0.0480 - 0.1029	0.0316 - 0.0574	0.0106 - 0.0433

AFLP, amplified fragment length polymorphism; ISSR, inter simple sequence repeat.
a Constraint trees are presented in Fig 3

Sequences of ITS, gpd, mtSSU, mtLSU, alt a l, endoPG, and anonymous sequences have been analysed in previous studies in an efforts to recognize distinct *Alternaria* species and/or morphological groups within the alternata species-group using robust phylogenetic criteria (Kusaba & Tsuge 1995; Pryor & Gilbertson 2000; Pryor & Michailides 2002; de Hoog & Horre 2002; Peever *et al.* 2002, 2004, 2005; Pryor & Bigelow 2003; Hong *et al.* 2005a). For each of these loci, groupings of defined species and undefined taxa were resolved at varying degrees, although frequently with minimal statistical support. However, no study has revealed loci suitable for delimitation of all morphologically defined, small-spored catenulate *Alternaria* spp. into monophyletic lineages. Results from analysis of the H4 locus in this study provided no additional resolution. This failure to resolve morphologically distinct taxa into genetically distinct lineages is particularly significant because most of the loci used in these studies have been successfully used in related studies to resolve members of other *Alternaria* species-groups and/or other fungal genera to the species level (Baayen *et al.* 2001; Berbee *et al.* 1999; Hong *et al.* 2005a; Pryor & Bigelow 2003; Talhinhas *et al.* 2002). That these loci have not provide comprehensive resolution of many taxa in the alternata species-group reveals a very close genetic relatedness among members and suggests that many of these taxa lie at the species–population interface where only more highly variable loci would reveal monophyletic lineages, if, in fact, they exist. Whether or not these lineages represent distinct species or infraspecific populations would be dependent upon a necessary clarification of species concepts within *Alternaria*.

Among the three groups morphologically differentiated, only the *A. arborescens* group (which included *A. arborescens*) formed a monophyletic lineage distinct from the *A. alternata* and the *A. tenuissima* species-groups. Monophyly was revealed in AFLP analysis, as well as by shared polymorphic bases in H4 sequence analysis. ISSR data produced a slightly different grouping from AFLP data as three *A. alternata* group isolates, ISPaVe 1779, 1781, 1782, were marginally included in the *A. arborescens* group cluster. However, this did not result in a contradiction of AFLP grouping as evidenced by the tree topology test. Distinction of the *A. arborescens* group from the *A. alternata* and *A. tenuissima* groups has been supported in most other studies previously mentioned, as well as by analysis of metabolite profiles (Andersen *et al.* 2002) and restriction mapping of IGS region (Hong *et al.* 2005b). On the basis of the results of our work together with those from previous investigations, it is clear that the *A. arborescens* group represents a unique genetic lineage with diagnostic morphology that is distinct from the *A. alternata* and *A. tenuissima* groups.

Monophyletic lineages were not revealed for the *A. alternata* and *A. tenuissima* groups with ISSR, AFLP, and H4 datasets. ISSR data were inconsistent with the hypothesis of monophyletic grouping of each morphological group based upon Winning-sites topology tests, whereas the KH and Templeton tests results were inconclusive. However, AFLP data were inconsistent with the hypothesis of monophyletic grouping of each morphological group based on all three topology tests (KH, Templeton's, Winning-sites). Moreover, the H4 sequence analysis failed to reveal any synapomorphic characters that would resolve the *A. alternata* group and the *A. tenuissima* group as independent lineages. The failure to resolve these two groups, which have distinct sporulation patterns, has already been reported in previous studies based upon PCR-RFLP and RAPD fingerprint analyses, and ITS sequence analysis (Pryor & Michailides 2002), and restriction mapping of the IGS region (Hong *et al.* 2005b). Similarly, this study also failed to resolve these groups based upon two methods of DNA fingerprint analysis and sequence analysis of a different genetic locus.

Although certain members of each morphological group have been recognized as distinct species in previous studies, e.g. *A. alternata*, *A. dumosa*, and *A. hesperidearum* in the *A. alternata* group (Simmons 1990, 1999a), for many isolates of small-spore catenulate *Alternaria*, particularly when large numbers are recovered in environmental studies, the assignment of exact names has been withheld in favour of the temporary use of less precisely defined morphological 'groups'. The difficulties presented in developing more exact systematic structure within the alternata species-group is evidenced by the fact that even experts on these fungi have often chosen to place taxa into 'groups' rather than attempt creation of potentially erroneous nomenclature. The finding that these three morphological groups of small-spored catenulate *Alternaria* are associated with fruit disease in walnut and hazelnut, as well as in citrus, pear, pistachio, almond, and apple, as demonstrated in other works (Belisario *et al.* 2004; Peever *et al.* 2004; Kang *et al.* 2002; Pryor & Michailides 2002; Teviotdale, *et al.* 2001; Simmons 1990; Simmons & Roberts 1993), reveals that these diverse morphological groups are widely distributed and occupy similar ecological niches such as leaf tissue, fruit tissue, or both. However, the inability to precisely link pathogenicity, symptom development, or even host range to specific morphological groupings impedes the development of diagnostic criteria and exact nomenclature for these pathogenic *Alternaria* taxa, and a robust understanding of their ecology.

Insight into how such a morphologically diverse assemblage of taxa can be associated with disease development on a single host or on multiple hosts, i.e. occupy a similar

ecological niche across a range of plant species, may be gained through an understanding of the role of fungal toxins in pathogenesis. *Alternaria* species, particularly those in the alternata species-group, are well known for producing numerous toxic secondary metabolites. These toxins, which collectively are chemically quite varied, often function in plant pathogenesis, and in some cases, are the single determinants of pathogenicity or virulence in several plant–pathogen interactions (Scheffer 1992; Montemurro & Visconti 1992). For many *Alternaria* toxins, such as tenuazonic acid, tentoxin, and zinniol, the mode of action is not host specific (Stoessl 1981). However, for certain metabolites toxigenicity is exhibited only on susceptible hosts or in some cases, specific cultivars of that host. For example, all isolates of *Alternaria* that produce AK toxin cause black spot disease of pear only on specific cultivars of *Pyrus pyrifolia*, particularly cv. Nijisseiki (Nishimura & Nakatsuka 1983). Similar relationships between toxigenic *Alternaria* strains and hosts are found with isolates that produce AM toxin and Alternaria blotch of apple, isolates that produce AT toxin and brown spot of tobacco, isolates that produce AF toxin and black spot of strawberry, isolates that produce AAL toxin and stem canker of tomato, and isolates that produce ACT toxin and ACR toxin cause brown spot of tangerine and Alternaria leaf spot of rough lemon, respectively. However, in at least three of these toxin-mediated diseases, black spot of pear, Alternaria blotch of apple, and brown spot of tangerine, toxigenicity, and consequently pathogenicity, is not limited to a single morphologically-defined species (Simmons & Roberts 1993; Simmons 1999b). This demonstrates that, at least in some cases, the production of specific toxins is not restricted to specific taxa or species of *Alternaria* and that morphology alone cannot be used as a predictor of pathogenicity and host range.

Alternaria disease of citrus caused by members of the alternata species-group is interesting in that two distinct toxins are involved, ACT and ACR toxin. In a sampling of small-spored *Alternaria* from citrus in Florida, three distinct genetic lineages were recovered; two that included isolates that produce ACT toxin and one that included isolates that produce ACR toxin (Peever *et al.* 1999). Interesting, within each lineage, numerous non-pathogenic isolates were recovered, suggesting that the genetic background itself was not sufficient to predict toxin production, and consequently, host range. Perhaps more interesting, all three lineages could be recovered together from rough lemon even though many of the isolates (the ACT toxin producers and the non-toxin producers) were incapable of causing disease on the source plant. Furthermore, a single isolate was recovered from the rough lemon collections that produced both citrus toxins and the quantity of each toxin produced and the concomitant pathogenicity was similar to that of each pathotype independently (Masunaka et al 2005). The ability to produce two separate host-specific toxins did not appear to be a result of a species hybridization event as the ACR-ACT isolate had the genetic background of a normal ACR-producing strain. Rather, it was hypothesized that ACT toxin production appeared to be due to the acquisition of small conditionally dispensable chromosomes (CDC) containing the genes for ACT toxin. CDCs in filamentous fungi are generally not required for growth but often confer an advantage in colonizing certain ecological niches (Covert 1998). They have been shown to harbour genes for toxin production and are required for pathogenicity in several small-spored *Alternaria* pathosystems (Hatta *et al.* 2002; Akamatsu *et al.* 1999). Although the acquisition or transfer of CDCs has not been conclusively documented in *Alternaria*, there is molecular evidence for this type of transfer in other asexual fungi such as *Colletotrichum gloeosporioides*, even when the interacting strains are vegetatively incompatible (Masel *et al.* 1996). The exact mechanism and frequency for transfer of CDCs between asexual fungi has not been elucidated. However, if even a rare event, this type of transfer could explain how a complex of closely related *Alternaria* found sympatric on so many different hosts, including walnut and hazelnut, could collectively possess the ability to cause disease on their associated host despite segregation into different morphological groups and/or species that transects host species boundaries.

Similar ecology and close genetic relatedness among morphologically-defined groups of small-spored catenulate *Alternaria*, even in the presence of differential pathogenicity, suggests that many of these taxa lie at the species–population interface. Until such a time as the morphological plasticity of this group can be reasonably reconciled along with the numerous taxa of intermediate characters, the utility of designated morphological groups within this species-group will be maintained. However, it is increasingly apparent that molecular data from highly variable loci will be necessary for further resolution of *Alternaria* taxonomy. This resolution will provide critical insight into the genetic basis of morphological variation and plasticity, which will further the development of definitive diagnostic criteria for taxon identification and the establishment of robust systematic structure.

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