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Discrete lineages within *Alternaria alternata* species group: Identification using new highly variable loci and support from morphological characters

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ABSTRACT

The *Alternaria alternata* species group is ubiquitous in the environment acting as saprotrophs, human allergens, and plant pathogens. Many morphological species have been described within the group and it is unclear whether these represent re-descriptions of the same species or discrete evolutionary taxa. Sequencing of five loci identified three major lineages within the *A. alternata* species group. These loci included three new phylogenetic loci (TMA22, PGS1, and REV3) identified as highly variable based on publically available genome sequence data for Dothideomycete species. Lineages were identified as *A. alternata* ssp. *arborescens*, *A. alternata* ssp. *tenuissima*, and *A. alternata* ssp. *gaisen* in accordance with the placement of reference isolates. The phylogenetic results were supported by morphological analysis, which differentiated strains in *A. alternata* ssp. *arborescens* and *A. alternata* ssp. *tenuissima* and also aligned with previous morphological species descriptions for *A. arborescens* and *A. tenuissima*. However, phylogenetic analysis placed the morphologically described species *A. alternata* and *A. mali* within the *A. alternata* ssp. *tenuissima* and did not support them as discrete taxa. As *A. alternata* are of phytosanitary importance, the molecular loci used in this study offer new opportunities for molecular identification of isolates by national plant protection organizations.

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Introduction

An accurate taxonomic framework is required by plant and human health organizations to identify and record *Alternaria*

spp. involved in disease. Over 100 plant species are affected by *Alternaria* spp., which cause stem and leaf spot diseases and post-harvest rots. In addition, *Alternaria* spp. are important allergens and opportunist human pathogens in

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immuno-compromised patients. The *Alternaria alternata* group is a species complex containing saprophytes, generalist pathogens, and host-selective pathotypes. However, despite being found ubiquitously in the environment, the taxonomy of this closely related, but diverse species group is largely unresolved. Identification of strains following isolation is difficult due to morphological plasticity and genetic similarity. Overlapping spore characters between morphologically described species complicates identification and has led to the recommendation that morphological characters are too variable to be of practical use for identification (Smith et al. 1992). All strains possess an identical ITS haplotype (Kusaba & Tsuge 1995; Pryor & Michailides 2002), and previous studies using highly variable phylogenetic loci have found a lack of support between phylogenetic clades and morphologically described species (Andrew et al. 2009). As a result, uncertainty remains over whether morphological variation represents discrete species (Simmons 1999; Roberts 2001; Simmons 2007), or whether this variation is continuous within the species group, which may therefore be a single species *A. alternata* (Nishimura 1980; Nishimura & Kohmoto 1983; Kusaba & Tsuge 1994; Kusaba & Tsuge 1995).

Recent progress using molecular approaches has begun to resolve the taxonomy of the genus *Alternaria*. Lawrence et al. (2013) performed a phylogenetic analysis of 176 species representing *Alternaria* and the related genera *Stemphylium*, *Embellisia*, *Nimbya*, and *Ulocladium* using five phylogenetic loci and identified eight phylogenetic lineages within the *Alternaria* genus, assigning them the taxonomic rank of section (Lawrence et al. 2013). One of these represented the *A. alternata* species group and was named *Alternaria* sect. *alternata*. A similar study by Woudenberg et al. (2013) supported the designation of the *A. alternata* species group's taxonomic designation as a section, though naming it *Alternaria* sect. *alternata*. Taxonomic hierarchy within the species group has been attempted but has largely focused on specific pathosystems, such as apple (Kang et al. 2002; Rotondo et al. 2012), citrus (Timmer et al. 2003; Peever et al. 2004; Peever et al. 2005; Stewart et al. 2014), and pistachio (Pryor & Michailides 2002). These studies have made use of genetic loci with greater variability than traditional barcoding genes to perform phylogenetic analyses. The *Alternaria* major allergen 1 (Alt a1) gene showed variability between sections within the *Alternaria* genus, whereas endopolygalacturonase (*endo*-PG) showed variability within *A. sect. alternata* spp. in investigations using citrus pathogens (Timmer et al. 2003; Peever et al. 2004; Peever et al. 2005; Stewart et al. 2014). These phylogenetic loci therefore offer potential to determine evolutionary relationships within the *A. sect. alternata*.

The wealth of genomic data available from Dothideomycete genome sequencing projects has allowed opportunities to develop novel loci for phylogenetics and identification. Within the fungal kingdom, 246 genes have been identified as present in a single copy in 31 fungal genomes (Marthey et al. 2008). Nucleotide and amino acid sequence is available for these genes on the Fungal Phylogenomic Database (FUNYBASE; <http://genome.jouy.inra.fr/funybase>) as they appear in the *Saccharomyces cerevisiae* genome (Marthey et al. 2008). Homologs to these protein sequences can be identified in Dothideomycete genomes and used to determine genetic regions

that are highly variable between species, including the *A. alternata* species group.

The *A. alternata* species group is of phytosanitary importance and is subject to quarantine regulations in Europe (Smith et al. 1997). A clear taxonomic hierarchy is required to enable regulation by National Plant Protection Organizations (NPPOs) to discriminate harmful regulated taxa from common saprotrophic strains that are associated with decay in storage. Commonly encountered morphologically described species within the *A. alternata* species group include *Alternaria tenuissima*, *Alternaria arborescens*, and *A. alternata* (Simmons & Roberts 1993; Andrew et al. 2009; Rotondo et al. 2012). However there are many morphological species descriptions from strains isolated from specific hosts including *Alternaria mali* (ex. apple) and *Alternaria gaisen* (ex. pear). Work in the citrus pathosystem has indicated that a number of morphologically described species cannot be supported under a phylogenetic species concept (Peever et al. 2004). This leads to further questions as to whether the species group represents a single taxon, a small number of commonly encountered taxa or a large number of host specific taxa. The aim of this study was to investigate whether there is support for *A. gaisen*, *A. alternata*, *A. tenuissima*, *A. mali*, and *A. arborescens* as discrete evolutionary lineages within the *A. alternata* species group. Previously published and newly identified variable phylogenetic loci were used to resolve taxa within the species group and was supported by additional morphological data using a range of different strains. New genetic loci that resolved taxa within the species group offer potential for the molecular identification of important quarantine pathogens within the *A. alternata* species group by NPPOs.

Methods

Collection and maintenance of strains

90 *Alternaria* strains representing a diverse range of hosts and geographic origins (Table 1) were obtained from culture collections at the UK Food and Environmental Research Agency, the United States Department of Agriculture, the All Russia Institute of Plant Protection and from the private collection E.G. Simmons. These included the reference strains for *Alternaria alternata*, *Alternaria arborescens*, *Alternaria gaisen*, *Alternaria mali*, and *Alternaria tenuissima* which are the basis for the morphological species descriptions as presented in Simmons (2007).

Strains were grown on 1 % potato dextrose agar (1 % PDA) and potato carrot agar. 1 % PDA plates were made by autoclaving 15 g agar powder (Agar-agar technical, Merck) and 0.23 g potato dextrose broth (Fluka, Sigma Aldrich) dissolved in 1 L of distilled water at 121 °C for 15 min. Potato carrot agar was made as described in Simmons (2007) where 20 g of potato tuber and 20 g of carrot root tissue were diced and autoclaved in a small amount of distilled water. This slurry was then forced through a fine sieve and water was added to make the solution to 1 L. 20 g of agar powder (Agar-agar technical, Merck) was added and the solution was autoclaved a second time. Agar plates of both media were poured into 90 mm Petri dishes after cooling to 65 °C.

Table 1 – Details of 90 *Alternaria alternata* species group strains sequenced across five phylogenetic loci and their use in morphological analysis including whether they sporulated in all three repeats of the morphological experiment.

Isolate	Host	Source ^a	Morph. analysis	Sporulation	Isolate	Host	Source	Morph. analysis	Sporulation
FERA 326	<i>Pyrus pyrifolia</i>	FERA	✓		FERA 1551	<i>Malus domestica</i>	FERA	✓	✓
FERA 348	<i>Malus domestica</i>	FERA	✓	✓	FERA 2038	<i>Solanum lycopersicum</i>	FERA	✓	
FERA 469	<i>Citrus reticulata</i>	FERA	✓	✓	FERA 2040	<i>Malus domestica</i>	FERA	✓	
FERA 478	<i>Fragaria</i> sp.	FERA	✓		FERA 2041	<i>Malus domestica</i>	FERA	✓	
FERA 479	<i>Fragaria</i> sp.	FERA	✓	✓	FERA 2043	<i>Malus domestica</i>	FERA	✓	✓
FERA 482	<i>Fragaria</i> sp.	FERA	✓	✓	FERA 2044	<i>Malus domestica</i>	FERA	✓	✓
FERA 484		FERA	✓	✓	FERA 2103	<i>Citrus</i> sp.	FERA	✓	
FERA 488	<i>Prunus persica</i>	FERA	✓	✓	FERA 2277	<i>Impatiens walleriana</i>	FERA	✓	
FERA 538	<i>Pyrus pyrifolia</i>	FERA	✓		FERA 2280	<i>Impatiens walleriana</i>	FERA	✓	
FERA 540	<i>Pyrus</i> sp.	FERA	✓	✓	FERA 2712	<i>Citrus</i> sp.	FERA	✓	✓
FERA 631	<i>Pyrus communis</i>	FERA	✓	✓	FERA 3250		FERA	✓	✓
FERA 632	<i>Pyrus</i> sp.	FERA	✓		FERA 9285	<i>Impatiens walleriana</i>	FERA	✓	✓
FERA 634	<i>Malus domestica</i>	FERA	✓		FERA 15968	<i>Solanum tuberosum</i>	FERA	✓	✓
FERA 635	<i>Malus domestica</i>	FERA	✓	✓	FERA 21677	<i>Pyrus</i> sp.	FERA	✓	✓
FERA 647	<i>Pyrus</i> sp.	FERA	✓		FERA 23766	<i>Pyrus pyrifolia</i>	FERA	✓	✓
FERA 648	<i>Pyrus</i> sp.	FERA	✓	✓	FERA 24350	<i>Pyrus pyrifolia</i>	FERA	✓	✓
FERA 650	<i>Pyrus</i> sp.	FERA	✓	✓	CBS 603.78		FERA	✓	✓
FERA 675	<i>Pyrus pyrifolia</i>	FERA	✓	✓	EGS 34.015	<i>Dianthus</i> sp.	EGS	✓	✓
FERA 678	<i>Malus domestica</i>	FERA	✓	✓	EGS 34.016	<i>Arachis</i> sp.	EGS	✓	✓
FERA 679	<i>Malus domestica</i>	FERA	✓		EGS 38.029	<i>Malus domestica</i>	EGS	✓	✓
FERA 681	<i>Pyrus</i> sp.	FERA	✓	✓	EGS 39.128	<i>Solanum lycopersicum</i>	ARIPP		
FERA 704	<i>Pyrus pyrifolia</i>	FERA	✓		EGS 90.0512	<i>Pyrus pyrifolia</i>	EGS	✓	
FERA 743	<i>Malus domestica</i>	FERA	✓		RGR 97.0007	<i>Malus domestica</i>	USDA	✓	✓
FERA 833	<i>Malus domestica</i>	FERA	✓	✓	RGR 97.0008	<i>Malus domestica</i>	USDA	✓	✓
FERA 1082	<i>Malus domestica</i>	FERA	✓	✓	RGR 97.0009	<i>Malus domestica</i>	USDA	✓	✓
FERA 1105		FERA	✓	✓	RGR 97.0010	<i>Malus domestica</i>	USDA	✓	✓
FERA 1164	<i>Malus domestica</i>	FERA	✓	✓	RGR 97.0011	<i>Malus domestica</i>	USDA	✓	✓
FERA 1165	<i>Malus domestica</i>	FERA	✓	✓	RGR 97.0012	<i>Malus domestica</i>	USDA	✓	✓
FERA 1166	<i>Malus domestica</i>	FERA	✓	✓	RGR 97.0013	<i>Malus domestica</i>	USDA	✓	✓
FERA 1169	<i>Malus domestica</i>	FERA	✓		RGR 97.0014	<i>Malus domestica</i>	USDA	✓	✓
FERA 1170	<i>Malus domestica</i>	FERA	✓		RGR 97.0015	<i>Malus domestica</i>	USDA	✓	✓
FERA 1171	<i>Malus domestica</i>	FERA	✓		RGR 97.0016	<i>Malus domestica</i>	USDA	✓	✓
FERA 1172	<i>Malus domestica</i>	FERA	✓		RGR 97.0017	<i>Malus domestica</i>	USDA	✓	✓
FERA 1177	<i>Malus domestica</i>	FERA	✓		RGR 97.0018	<i>Malus domestica</i>	USDA	✓	✓
FERA 1310	<i>Malus domestica</i>	FERA	✓	✓	RGR 97.0019	<i>Malus domestica</i>	USDA	✓	✓
FERA 1490	<i>Fragaria</i> sp.	FERA	✓	✓	RGR 97.0020	<i>Malus domestica</i>	USDA	✓	✓
FERA 1491	<i>Fragaria</i> sp.	FERA	✓		RGR 97.0021	<i>Malus domestica</i>	USDA	✓	✓
FERA 1512	<i>Dendrothema</i> sp.	FERA	✓	✓	RGR 97.0022	<i>Malus domestica</i>	USDA	✓	✓
FERA 1518	<i>Solanum lycopersicum</i>	FERA	✓	✓	RGR 97.0024	<i>Malus domestica</i>	USDA	✓	✓
FERA 1519	<i>Gossypium</i> sp.	FERA	✓	✓	RGR 97.0025	<i>Malus domestica</i>	USDA	✓	✓
FERA 1520	<i>Lactuca</i> sp.	FERA	✓	✓	RGR 97.0026	<i>Malus domestica</i>	USDA	✓	✓
FERA 1521	<i>Prunus armeniaca</i>	FERA	✓	✓	RGR 97.0027	<i>Malus domestica</i>	USDA	✓	✓
FERA 1522	<i>Actinidia</i> sp.	FERA	✓	✓	RGR 97.0067	<i>Malus domestica</i>	USDA	✓	✓
FERA 1523	<i>Citrullus lanatus</i>	FERA	✓	✓	HA-1	<i>Malus domestica</i>	ARIPP		
FERA 1539	<i>Juglans regia</i>	FERA	✓		O-159	<i>Malus domestica</i>	ARIPP		

a Isolates were received from the UK Food and Environmental Research Agency (FERA), the US Department of Agriculture, the All Russia Institute of Plant Protection (ARIPP) and the private collection of Prof. E. G. Simmons (EGS).

Routine culturing of *Alternaria* strains was performed on 1 % PDA, with cultures grown for 14–28 d at 23 °C in 12 h day/night cycles in an incubator (MLR-350/T, Sanyo), under a gradually drying atmosphere (no humidity control and unsealed plates). New plates were inoculated from growing cultures using the end of a sterilized wooden cocktail stick to touch a young, representative sporulating region of the culture and then stab-inoculate the new agar plate.

Long-term storage of cultures was performed by freezing colonized filter paper. Ten 0.5 cm discs of sterile filter paper

were placed on un-colonized 1 % PDA agar plates. Plates were then inoculated and cultures grown as described above. Once mycelium/spores could be seen emerging from the upper side of the filter paper they were removed and desiccated for 48–72 h in a freeze drier (Modulyo, Edwards). They were then stored until required at –20 °C. Cultures were revived by growing on potato carrot agar plates for seven days under the conditions described above.

Alternaria strains were cultured for DNA extraction by inoculating 2 ml eppendorf tubes containing 1.5 ml of sterile

potato dextrose broth (23 g L⁻¹) (Fluka, Sigma Aldrich) with spores from actively growing cultures. Eppendorf tubes were then covered with sterile cotton wool and aluminium foil. These were grown for 14–21 d at 23 °C (12 h day/night cycle). Mycelium was prepared for DNA extraction by removing it from the tubes, placing on filter paper and washing with sterile water in a Buchner funnel before it was desiccated for 24 h in a freeze drier (Modulyo, Edwards). DNA extraction was then performed using a DNeasy Plant Mini Kit (Qiagen) following the manufacturers protocol.

Molecular phylogeny

Novel loci for phylogenetics were developed using the FUNYBASE database. Orthologs of 26 genes listed on FUNYBASE were identified in six Dothideomycete fungi with publicly available genome sequence (*Alternaria brassicicola*, *Phaeosphaeria nodorum*, *Pyrenophora tritici-repentis*, *Cochliobolus heterostrophus*, *Mycosphaerella graminicola*, and *Mycosphaerella fijiensis*) using tblastn BLAST searches. ClustalW alignments were made for genomic sequences of these 26 genes in MEGA 5.1 (Tamura et al. 2011). From these alignments, a subset were selected for further analysis and potential primer sites identified within alignments of *A. brassicicola*, *P. tritici-repentis*, *P. nodorum*, and *C. heterostrophus*. The positions of variable and conserved regions were noted. Primers were designed in conserved regions either side of regions that showed variability between the Dothideomycete taxa and were less than 650 bp in length, to allow sequencing using a standard ABI 3100 sequencer (Applied Biosystems).

Sequence data was generated for five genetic loci (*endo*-PG, *Alt a1* and three novel FUNYBASE loci) for all 90 *Alternaria alternata* species group strains (Table 1). PCR reaction mixtures consisted of 10 µl REDTaq ReadyMix (Sigma–Aldrich), 2 µl DNA, 1 µl of forward and reverse primer, and 6 µl purified water (Sigma–Aldrich). PCR reactions were performed in Genius Thermo Cyclers (Techne) using the following conditions:

initial denaturation at 94 °C for 1 min followed by 35 cycles of denaturation at 94 °C for 30 s, with a 30 s annealing step (set at an appropriate temperature for the primers (Table 2)), and an extension step at 72 °C for 1 min. A final extension was performed at 72 °C for 7 min.

PCR purification was performed using a QIAquick PCR Purification Kit (Qiagen, UK) and the purified PCR product was submitted to the LIGHTRun service (GATC, Germany) for sequencing of amplicons in both directions. Sequencing data was aligned, edited, and saved as consensus sequences using SeqMan (Burland 2000). Sequence alignment was then performed using the ClustalW algorithm in Geneious (Kearse et al. 2012).

A multi-locus phylogeny was determined from concatenated sequence alignments using the MrBayes plugin in Geneious (Kearse et al. 2012). The custom command block was used to specify partitioning of the dataset by locus and to assign the appropriate evolutionary model to each locus. The run was performed using four heated chains, set at a heat of 0.1 and sampled every 1000 generations. Following 20 million generations, average standard deviations were confirmed to be below 0.01 for the run. The first 25 % of trees were discarded as burn-in, after which log likelihood values were observed to be stationary. Geneious was used to calculate 50 % consensus phylogenies from each MrBayes run. Images were exported from Geneious displaying strain name, host or reference strains these images were merged using the image manipulation program GIMP 2.8.14. Single locus sequence data was submitted to Genbank (accessions KP275690–KP276139). Sequence alignments and molecular phylogenies were uploaded to Treebase (submission 16842).

Morphological dendrogram

Morphological analysis was performed for all 90 *Alternaria* spp. strains except EGS 39.128, HA1, and O-159, which were acquired after the experiment. Identifying information was

Table 2 – Details of the primer pairs used for PCR and sequencing of five loci in this study.

Locus ^a	Primer	Primer sequence (5'-3')	Primer source	Annealing temperature (°C)	Amplicon length (approx. no. bp)
<i>endo</i> -PG	PG3	TACCATGGTCTCTTCCGA	Andrew et al., (2009)	50	485
	PG2b	GAGAATTCRCARTCRCTCYTGRTT	Andrew et al., (2009)		
<i>Alt-a1</i>	Alt-for	ATGCAGTTCACCACCATCGC	Hong et al., (2005)	57	507
	Alt-rev	ACGAGGGTGAYGTAGGCGTC	Hong et al., (2005)		
<i>Tma22</i>	MS432-A-F	CAAGCTCTCTCTTTCGCGTC	This study	62	355
	MS432-A-R	GAGGGAGGCCATGTTCTGCTG	This study		
<i>Pgs1</i>	MS550-A-F	CAGACGCCTGCCGAGTTTAT	This study	60.5	537
	MS550-A-R	CCTTCGTTGATGCGTTTAGG	This study		
<i>Rev3</i>	MS578-A-F	CTGTAGAGAGTATTGAAGATC	This study	54.8	407
	MS578-A-R	AGGTGCTCTGGGGCCTTCTTG	This study		

a Endopolygalacturonase (*endo*-PG), *Alternaria* major allergen 1 (*Alt-a1*), Translation Machinery Associated protein (*TMA22*, FUNYBASE gene MS432), CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase (*PGS1*, FUNYBASE gene MS550), Catalytic subunit of DNA polymerase zeta (*REV3*, FUNYBASE gene MS578).

removed from all cultures in long-term storage and replaced with a randomized number, so that the experiment was performed under blind conditions. The experiment was performed three times, with one-week intervals between the start of each repeat. Revived *Alternaria* cultures were used to inoculate a new potato-carrot agar plate by removing an agar plug with a sterile 5 mm cork borer from a sporulating region of the culture approximately 5 mm within the leading edge of mycelium. Plugs were placed in the centre of agar plates and incubated using the same conditions as described above. Morphological data was recorded seven days after inoculation. Colony diameter was measured and spore characteristics assessed by microscopy as described by Simmons (2007). Briefly, transparent adhesive tape was used to collect spores from the colony surface, placed on a glass microscope slide and a few drops of lactic acid added before a cover slip was placed on top. The slide was then gently heated to fix the spores. Images were taken at three locations for each slide at 500 times magnification using a camera (C5050 Zoom, Olympus) attached to a microscope (SZX7, Olympus). The three locations were approximately 25 %, 50 %, and 75 % across the length of the slide. The sporulation images were then analysed by recording the following morphological characters: 1) the number of conidia present in the image; 2) the number of a) conidiophore b) intercalary c) basal branches present in the image (Fig 1A); 3) the number of conidia comprising the longest chain of conidia (Fig 1B). 4) a representative selection of ten spores chosen from the image and the following characters measured using Image J (Abramoff et al. 2004): a) conidial length b) conidial width c) number of transverse septae d) number of latitudinal septae. An image of haemocytometer gridlines at 500 times magnification was taken at the beginning of each microscopy session (Improved Neubauer, Webb England). This allowed all measurements made in Image J to be calibrated to a known number of pixels μm^{-1} . Mean values for each morphological character were calculated. A principal components analysis was then performed to explain variation within the dataset using Genstat 13 (VSN international, UK). Data from the ten morphological

variables was used to form a correlation matrix. Ten principal components were calculated and a scree plot made to show the relative contribution of each of these to summarising the data. From this, the most informative components were selected and investigated for biological meaning.

Association of strains with similar morphological characters was investigated through construction of a dendrogram using Genstat 13. A Euclidean correlation matrix was calculated from scores from principal components 2–6 and the dendrogram determined using the furthest neighbour algorithm. Clades that were present at a 95 % similarity level were considered representative of major groupings within the dendrogram and were used for further analysis. A constant data range was used to form the Euclidean matrix, allowing the relative weightings of principal components to be maintained within the matrix. This was equal to the largest range between a maximum and minimum principal component score observed within one of the principal components in the dataset. Principal component scores for strains were plotted against each other and clades that were positively or negatively associated with each principal component identified. Genstat 13 produced dendrogram images in eight parts, which were merged into a single image using GIMP 2.8.14.

Results

Molecular phylogeny

Of the 26 genes downloaded from FUNYBASE, three were selected for phylogenetic analysis; Translation Machinery Associated protein (TMA22, FUNYBASE gene MS432), CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase (PGS1, FUNYBASE gene MS550) and the catalytic subunit of DNA polymerase zeta (REV3, FUNYBASE gene MS578) as characterized in *Saccharomyces cerevisiae* (Marthey et al. 2008). These genes showed equivalent or greater levels of variability than *Alt a1* or *endo-PG* in alignments between the 90 *Alternaria alternata* species group strains that were sequenced (Table 3). Alignments for the five loci were concatenated, resulting in an alignment of 2578 bp, containing 237 parsimoniously informative sites for the *A. alternata* species group strains. The Bayesian phylogeny determined from



Fig 1 – Characters used in morphological analysis. Spores demonstrating apical branching (A), intercalary branching (B) and basal branching (C). longest conidial chain. The morphological character longest conidial chain (D) was defined as the number of spores between the base and the apex. Scale bars represent 30 μm .

Table 3 – Length of alignments and the number of single nucleotide polymorphisms (SNPs) presents in alignments of 90 *A. alternata* species group strains.

Locus	Length of alignment	No. SNPs in alignment ^{a,b}
<i>endo-PG</i>	448	24
<i>Alt-a1</i>	468	37
<i>Tma22</i>	429	26
<i>Pgs1</i>	597	99
<i>Rev3</i>	620	42

^a Excluding SNPs that only showed variability between *A. alternata* and the out-group *A. brassicicola*.

^b Phylogenetic loci developed in this study *Tma22*, *Pgs1* and *Rev3* showed similar or greater variability to previously described phylogenetic loci *endo-PG* and *Alt-a1*.

Table 4 – Mean values and standard error of morphological characters measured across strains in morphological clades.

Morphological clade		Colony diameter (mm)	Number of conidia ^a	Longest chain ^a	Conidial length (μm) ^b	Number of transepta ^a	Conidial width (μm) ^b	Number of longisepta ^b	Number of basal branches ^a	Number of intercalary branches ^a	Number of conidiophore branches ^a
A	(13 strains)	64.06 ± 0.94	32.47 ± 3.14	3.43 ± 0.17	23.92 ± 0.35	3.36 ± 0.05	7.39 ± 0.08	0.13 ± 0.01	0.21 ± 0.06	0.39 ± 0.10	0.30 ± 0.09
B	(21 strains)	66.70 ± 0.81	46.96 ± 4.02	3.21 ± 0.12	19.49 ± 0.31	2.69 ± 0.05	7.26 ± 0.07	0.11 ± 0.01	0.32 ± 0.06	0.48 ± 0.07	2.50 ± 0.21
C	(6 strains)	63.16 ± 1.32	41.94 ± 8.43	2.85 ± 0.25	20.10 ± 0.67	2.68 ± 0.08	7.19 ± 0.21	0.08 ± 0.02	0.83 ± 0.28	1.06 ± 0.32	0.94 ± 0.26
D	(2 strains)	57.25 ± 3.82	30.72 ± 12.12	3.66 ± 0.67	19.32 ± 1.67	2.98 ± 0.26	6.36 ± 0.29	0.11 ± 0.04	0.72 ± 0.26	0.88 ± 0.51	0.33 ± 0.21
E	(14 strains)	66.91 ± 0.74	50.14 ± 5.58	3.87 ± 0.21	20.92 ± 0.30	2.30 ± 0.05	6.50 ± 0.09	0.06 ± 0.01	0.43 ± 0.10	1.09 ± 0.20	0.81 ± 0.16
F	(2 strains)	68.92 ± 2.46	49.72 ± 8.23	3.67 ± 0.15	19.49 ± 0.57	2.66 ± 0.06	8.20 ± 0.49	0.36 ± 0.05	0.22 ± 0.14	1.06 ± 0.25	2.11 ± 0.41
G	(4 strains)	68.21 ± 1.52	54.60 ± 16.20	3.00 ± 0.18	17.49 ± 0.47	2.10 ± 0.09	7.49 ± 0.10	0.05 ± 0.01	0.33 ± 0.09	0.44 ± 0.17	1.88 ± 0.40
H	(1 strain)	65.33 ± 3.94	92.33 ± 34.09	3.11 ± 0.22	20.54 ± 0.47	2.90 ± 0.07	7.15 ± 0.11	0.09 ± 0.05	0.33 ± 0.19	0 ± 0.00	6.22 ± 1.78
I	(1 strain)	40.17 ± 0.73	39.11 ± 6.94	3.67 ± 0.51	22.56 ± 2.87	3.08 ± 0.68	7.90 ± 0.39	0.28 ± 0.01	0.67 ± 0.38	0.44 ± 0.11	0.67 ± 0.51

a Count data from image of microscopy slide at 500× magnification.

b Measurements made from ten representative spores in image of microscopy slide at 500 times magnification.

Table 5 – Weightings of morphological characters within principal components (PC1-6) explaining morphological variation in the *A. alternata* species group.

Principal component	Variation explained (%) ^a	Colony diameter (mm)	Number of conidia	Longest chain	Conidial length (μm)	Number of transepta	Conidial width (μm)	Number of longisepta	Number of basal branches	Number of intercalary branches	Number of conidiophore branches
PC1	30.61	0.018	0.454	0.367	0.194	0.126	0.269	0.236	0.479	0.448	0.216
PC2	22.64	0.305	0.225	−0.060	−0.552	−0.580	−0.009	−0.184	0.038	0.072	0.415
PC3	14.77	0.208	0.038	0.396	0.005	0.166	−0.639	−0.527	0.105	0.211	−0.172
PC4	9.2	0.772	0.064	0.081	0.304	0.246	0.164	0.008	−0.290	−0.269	0.238
PC5	7.29	0.444	−0.271	0.117	−0.236	−0.181	0.034	0.455	0.045	0.285	−0.580
PC6	6.16	0.206	0.076	−0.608	0.249	−0.055	0.272	−0.465	0.287	0.239	−0.294

a Variation within the dataset explained by each principal component.

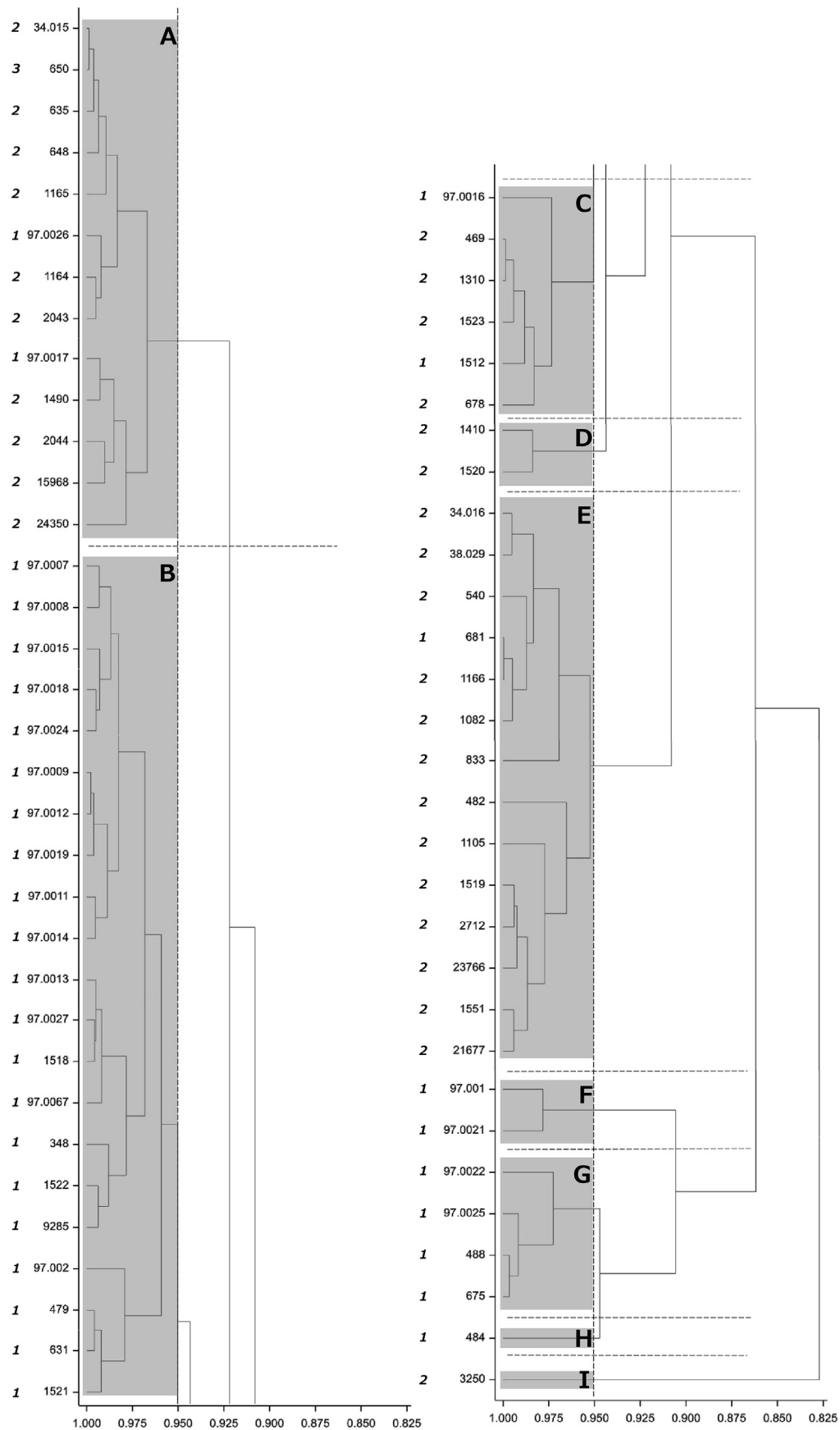


Fig 3 – Dendrogram determined from principal components 2–10 explaining variation in *Alternaria* spp. morphological characters. Major clades (A–I) are identified at a 95 % similarity level and strains are marked by the phylogenetic lineage they represent (1–3).

this dataset identified three evolutionary lineages whose separation was concordant across all single locus phylogenies (Fig 2). None of the clades within these three lineages were distinct or concordant in all five single locus phylogenies (Supplementary material).

The placement of strains from different hosts in phylogenetic clades and the placement of reference strains were noted. Phylogenetic lineage 3 contained the *Alternaria gaisen* reference strain (EGS 90.0512) and two other strains ex. Pear (EMR 632 and EMR 650). Phylogenetic lineage 2 contained the reference strains for *Alternaria mali*, *Alternaria tenuissima*, and *A. alternata* (EGS 38.029, EGS 34.015, and EGS 34.016) as well as strains isolated from a diverse range of hosts, including pear, apple, strawberry, carnation, peanut, citrus, lettuce, potato, and cotton. Phylogenetic lineage 1 contained the *Alternaria arborescens* representative strain (EGS 39.128) and strains from a diversity of hosts, including apple, pear, tomato, strawberry and impatiens.

Morphological analysis

Values for morphological characters were concordant with characters for *Alternaria* species previously described (Table 4), as presented by Simmons (2007). However, the number of spores on the longest chain was an exception to this, with average values between two and five conidia long whereas species descriptions of morphological species in the *Alternaria alternata* species group would expect chain sizes of approximately 12–15 conidia long for *Alternaria tenuissima* and *Alternaria mali*. Statistical analysis was based upon 68 of the 90 strains that sporulated in all three repeats of the experiment while data collected for remaining 22 strains that did not sporulate in one or more repeats of the experiment were discarded.

Principal components analysis (PCA) revealed that 85 % of the variation in the dataset could be explained in five principal components (PC 1–5) and that each additional PC explained less than 5 % of the remaining variation. The contribution of each morphological character to the score of a PC (Table 5), was used to understand how PCs explained biological variation.

All coefficients in PC1 contributed positively to the score for an individual (Table 5). This PC contrasted strains that had large values for morphological characters against individuals with low values for morphological characters and was considered to separate strains on the basis of how well they sporulated. Strong weightings were put on individuals that had large values for numbers of conidia, maximum chain length and presence of branching. Therefore a morphological dendrogram was determined from the PC scores for each strain from PC 2–10 (Fig 3) resulting in the identification of nine morphological clades (A–I) at a 95 % similarity cutoff. The PCs responsible for the separation of each of morphological clades were identified through plots of strain's PC scores against one another (Fig 4).

PC2 separated strains with a greater occurrence of branching from conidiophores and shorter, more rounded spores with few transverse septa. This contrasted against strains with longer spores, more transverse and longitudinal septae and a low occurrence of conidiophore branching. Strains positively associated with PC2 were considered to possess a more

Alternaria arborescens-like morphology, whereas strains negatively associated with PC2 were considered to possess a more *A. alternata* or *A. tenuissima*-like morphology. Morphological clades B and G were positively associated with PC2 whereas morphological clades A and I were negatively associated with PC2.

Strains that were positively associated with PC3 had longer chains of spores. Their conidia had more transeptae, were thinner, had a lower occurrence of longisepta, were rarely branched from conidiophores but had more branching from within conidia. These characters were considered to be representative of *A. tenuissima*-like and *A. mali*-like morphologies. Strains negatively associated with PC3 had shorter chains with fewer transeptae, were wider with more longitudinal septation and had greater degrees of conidiophore branching but with less branching from within conidia. These characters represented a more *A. arborescens*-like morphology. Morphological clade E was positively associated with the PC3 and morphological clade F was negatively associated with the PC3.

PC4 put a strong positive weight on colony size and apical branched spores whereas basally and mid-branched spores were negatively weighted. Patterns of morphological association with PC4 were considered to reflect an *A. alternata*-like morphology. Morphological clades C and D were negatively associated with PC4 but no morphological clades were positively associated PC4.

Alternaria strains that were positively associated with PC5 had larger colonies, fewer spores, smaller spores, more longisepta and more intercalary branching. Strains that were negatively correlated with PC5 had smaller colonies, more conidia, larger spores with fewer longisepta and less branching from within spores but had more conidiophore branching. These morphological characters were not considered to represent any morphological species descriptions. Morphological clade F was positively correlated with PC5 and morphological clade H was negatively associated with PC5.

Each morphological clade that was identified (A–I) largely contained strains of a single phylogenetic clade. Morphological clades B, F, G, and H contained strains exclusively from phylogenetic lineage 1 (Fig 3) and had characters associated with *A. arborescens* as well as the *A. arborescens* reference strain (Fig 2). Phylogenetic lineage 2 consisted of 28 strains from morphological clades A, E, and I (*A. tenuissima*/*A. mali*-like morphology) and eight strains from morphological clades C and D (*A. alternata*-like morphology) (Fig 2). Reference strains for *A. tenuissima*, *A. mali*, and *A. alternata* were all present in this phylogenetic lineage (Fig 2). The single strain from phylogenetic lineage 3 that showed sufficient sporulation in all three time points to be included in the morphological analysis was present in morphological clade A. However, there was insufficient data to determine whether the morphology of the strain in phylogenetic lineage 3 was distinct from other taxa.

Discussion

This study demonstrates for the first time that strains from the *Alternaria alternata* species group from a broad range of

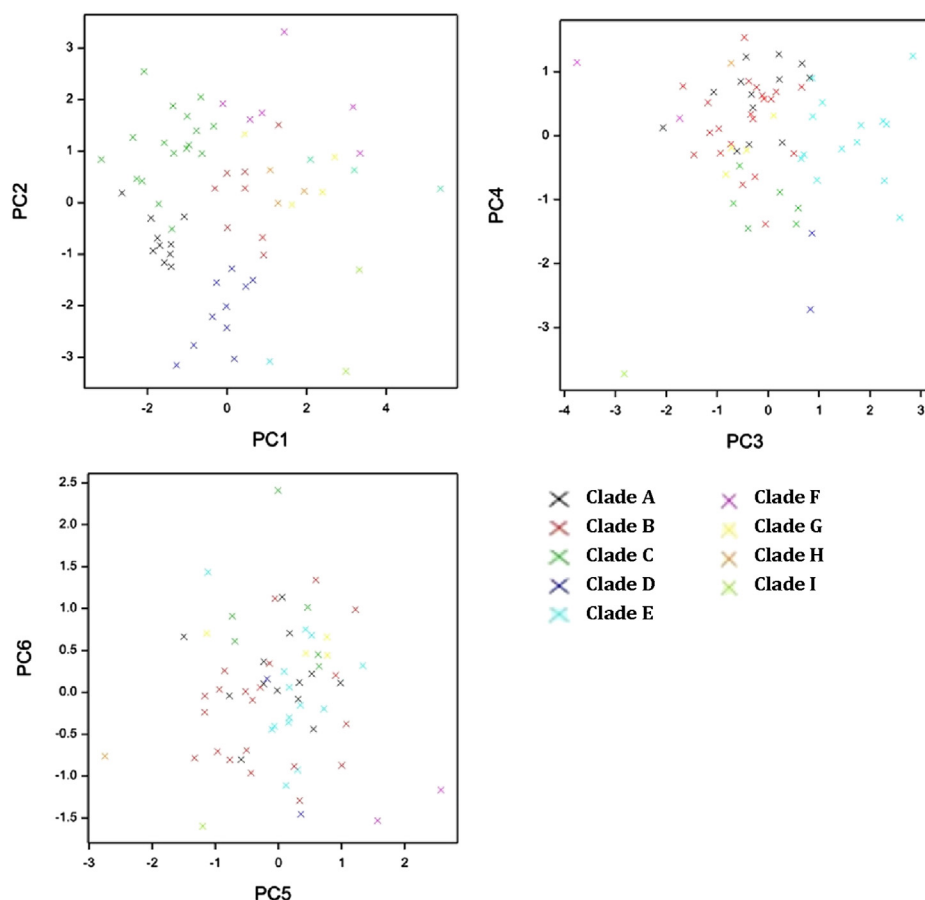


Fig 4 – Plot of strain’s principal component (PC) scores for the first six principal components. Strains are coloured by their morphological clade as determined in Fig 4.6. Morphological clades show separation by principal components.

hosts and locations can be separated into three evolutionary lineages. Resolution of these three lineages was congruent between *endo*-PG, *Alt a1*, TMA22, PG51, and REV3 loci, meaning that any one of these five loci could be used for molecular characterization of unknown strains.

The results of this work have implications for the taxonomy of the *A. alternata* species group. All strains in the *A. alternata* species group possess identical sequences for the fungal barcoding locus ITS (Kusaba & Tsuge 1995; Pryor & Michailides 2002), leading to the suggestion that all morphological species could be collapsed into a single taxon *A. alternata* (Nishimura 1980; Nishimura & Kohmoto 1983; Kusaba & Tsuge 1994; Kusaba & Tsuge 1995; Andrew et al. 2009). Currently the binomial name *A. alternata* is used in plant pathology, medical research, and environmental studies to describe this taxonomically complex group of organisms. Having identified discrete evolutionary lineages within the *A. alternata* species group, we have demonstrated that there are discrete taxa within this group. We propose that lineages identified in this study represent diverging subspecies *A. alternata* ssp. *arborescens* (phylogenetic lineage 1), *A. alternata* ssp. *tenuissima* (phylogenetic lineage 2), and *A. alternata* ssp. *gaisen* (phylogenetic lineage 3). This taxonomic framework is congruent with the current designation of *A. alternata* species group strains that

produce host-selective toxins as pathotypes of *A. alternata* (Tsuge et al. 2013).

This study did not support the taxonomic framework of Simmons (2007) where *A. alternata*, *Alternaria tenuissima*, and *Alternaria mali* are considered distinct taxa. This supports findings in the citrus pathosystem, where two evolutionary lineages contained ten morphologically described species (Stewart et al. 2014). The morphologically described *A. tenuissima* and *A. mali* species in Simmons (2007) possess very similar morphological characters including overlapping ranges of spore characters and produce similar chains of spores. These results indicate that *A. mali* is a re-description of the morphological species *A. tenuissima*. Isolates possessing an *A. alternata*-like morphology have been described as rare in the environment (Simmons & Roberts 1993; Simmons 1999) and it has been suggested that most identifications of *A. alternata* are in fact misidentifications of *A. tenuissima* Simmons (2007). It may be the case that *A. alternata* does not represent a distinct taxon within the *A. alternata* species group but instead represents a combination of morphological characters possible within those possible in a single *A. tenuissima* lineage.

Identification of an evolutionary lineage containing *Alternaria arborescens*-like characters distinct from a lineage containing *A. tenuissima*-like characters within the *A. alternata*

species group is supported by results from a study investigating *A. alternata* species group isolates ex. apple leaves (Rotondo et al. 2012). Use of multi-locus sequence data, AFLP, and inter simple spacer repeats indicated the presence of two to three distinct phylogenetic clades and morphological analysis indicated one clade as having an *A. arborescens* morphology and one or two, closely related clades containing mixed *A. alternata*/*A. tenuissima* morphologies.

Although the *A. alternata* ssp. *gaisen* lineage was represented by only three strains in this study, it was highly divergent from the other two lineages and contained the reference strain for *Alternaria gaisen* (EGS 90.0512). This strain has previously been used as an out-group when performing phylogenetics on *A. alternata* species group strains causing disease in citrus (Akimitsu et al. 2003; Peever et al. 2004). This study confirms that this taxon is indeed a distinct lineage within the *A. alternata* species group.

The merit of using morphology in taxonomy of the *A. alternata* species group has been unclear (Andrew et al. 2009). However, by identifying the morphological variation associated with a strain's ability to sporulate using PCA and removing that variation from further analysis, morphological support was found for all the phylogenetic lineages that we identified in the species group. Similar techniques have previously been used in the *A. alternata* species group. Clustering of metabolite profiles and subsequent cladistics has been used to support differentiation of *Alternaria infectoria* from *Alternaria malorum*, *Alternaria cetera*, and *Embellisia abundans* (Andersen et al. 2009) and multivariate morphological data was used to aid characterization of *A. alternata* species group spore morphologies associated with apple leaf spot in Italy (Rotondo et al. 2012), but this is first time multivariate data has been used to characterize the *A. alternata* species group as a whole.

The importance of consistent sporulating cultures is stressed in *Alternaria* identification manuals. However, as morphological species descriptions include overlapping characters between taxa, good culturing practice can only minimise natural variation. This study showed that morphological characters measured from a single culture are too variable to be of practical use for identification in the *A. alternata* species group; instead reference strains must be grown and replicate cultures used. Practically, isolate identification using molecular methods means that less expertise needs to be maintained, allowing delivery of a cost-effective and sustainable identification service (Boonham et al. 2008). Furthermore, molecular methods can also offer advantages in speed (as a culturing step is not always required), automation, can be performed at a high throughput and are generally less ambiguous in their results. Molecular identification should be performed as a two-step process, by first sequencing the ITS region to confirm that a strain belongs to the *A. alternata* species group. Following this the TMA22 locus should be sequenced to identify subspecific taxa as it shows clear resolution between the *A. alternata* ssp. *arborescens*, *A. alternata* ssp. *tenuissima*, and *A. alternata* ssp. *gaisen* lineages.

This study also demonstrated that FUNYBASE was a useful tool to identify novel phylogenetic loci and highlights the utility of publicly available genome sequence data. As TMA22, PGS1, and REV3 primers were generated from conserved sites between *Alternaria brassicicola* and other Dothideomycete

genomes, they should be appropriate for phylogenetics in other Dothideomycete systems. This would be particularly applicable to closely related genera that still have unresolved taxonomies such as *Embellisia*.

This study demonstrated that isolate morphology is associated with evolution of the core genome. However, host selective pathogenicity is associated with presence of an additional conditionally dispensable chromosome (CDC), which is thought to have been acquired through horizontal gene transfer (HGT) (Tsuge et al. 2013). Further work is now required to establish the evolution of pathogenicity in the *A. alternata* species group lineages, particularly the adaptation to hosts within these lineages. Of particular interest is the distribution of host-selective pathotypes between the three *A. sect. alternaria* lineages. Each of the three strains present in lineage 3 were ex. Asian pear (*Pyrus pyrifolia*) including the representative strain for *A. gaisen* (EGS 90.0512). This isolate is known to possess the conditionally dispensable chromosome (CDC) responsible for the production of the pear host selective toxin (Roberts et al. 2012). Similarly HA1 and 0–159 are known to possess CDCs for the production of apple toxins and EGS 39.128 is known to possess the CDC for production tomato toxins (Johnson et al. 2000; Harimoto et al. 2007; Hu et al. 2012). Evolutionary relationships determined in this study were based on loci within the essential genome of *A. alternata*. As such, this phylogeny provides a background upon which the distribution of CDCs can be identified. This may provide insight into the gain or loss of CDCs and past HGT events.

Conclusions

We identified three major phylogenetic lineages within the *Alternaria alternata* species group using a diverse collection of 90 strains. Position of neotype isolates within the phylogeny allowed the validity of morphologically described species to be assessed. Representative isolates for the morphologically described species *Alternaria arborescens* and *Alternaria gaisen* were present in two distinct lineages whereas *Alternaria tenuissima*, *A. alternata*, and *Alternaria mali* were not distinct from one another and present in a third lineage. Morphological data collected under blind conditions supported the separation of two phylogenetic lineages, one showing more *A. arborescens*-like morphological characters and the second showing more *A. tenuissima*-like morphological characters. Despite clear resolution using highly variable phylogenetic loci, previous studies have shown that common barcoding loci fail to resolve taxa at this level. As such it is proposed that these lineages represent subspecific-taxa within a single species *A. alternata*. This new framework has further benefit to taxonomy within this previously unresolved species group as it allows incorporation of *A. alternata* pathotype designations (based upon presence of an additional chromosome) into *A. alternata* taxonomy.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.funbio.2015.06.012>.

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