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# Diversity and movement of indoor *Alternaria alternata* across the mainland USA

J.H.C. Woudenberg [a,b,*], N.A. van der Merwe [c], Ž. Jurjević [d], J.Z. Groenewald [a], P.W. Crous [a,b,c]

[a] CBS-KNAW Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands
[b] WUR, Laboratory of Phytopathology, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands
[c] Department of Genetics, Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria 0002, South Africa
[d] EMSL Analytical, Inc., 200 Route 130 North, Cinnaminson, NJ, USA

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ABSTRACT

*Alternaria* spp. from sect. *Alternaria* are frequently associated with hypersensitivity pneumonitis, asthma and allergic fungal rhinitis and sinusitis. Since *Alternaria* is omnipresent in the outdoor environment, it is thought that the indoor spore concentration is mainly influenced by the outdoor spore concentration. However, few studies have investigated indoor *Alternaria* isolates, or attempted a phylogeographic or population genetic approach to investigate their movement. Therefore, the aim of the current study was to investigate the molecular diversity of indoor *Alternaria* isolates in the USA, and to test for recombination, using these approaches. *Alternaria* isolates collected throughout the USA were identified using ITS, *gapdh* and *endoPG* gene sequencing. This was followed by genotyping and population genetic inference of isolates belonging to *Alternaria* sect. *Alternaria* together with 37 reference isolates, using five microsatellite markers. Phylogenetic analyses revealed that species of *Alternaria* sect. *Alternaria* represented 98% (153 isolates) of the indoor isolates collected throughout the USA, of which 137 isolates could be assigned to *A. alternata*, 15 to the *A. arborescens* species complex and a single isolate to *A. burnsii*. The remaining 2% (3 isolates) represented sect. *Infectoriae* (single isolate) and sect. *Pseudoulocladium* (2 isolates). Population assignment analyses of the 137 *A. alternata* isolates suggested that subpopulations did not exist within the sample. The *A. alternata* isolates were thus divided into four artificial subpopulations to represent four quadrants of the USA. Forty-four isolates representing the south-western quadrant displayed the highest level of uniqueness based on private alleles, while the highest level of gene flow was detected between the south-eastern (32 isolates) and south-western quadrants. Genotypic diversity was high for all quadrants, and a test for linkage disequilibrium suggested that *A. alternata* has a cryptic sexual cycle. These statistics could be correlated with environmental factors, suggesting that indoor *A. alternata* isolates, although extremely diverse, have a continental distribution and high levels of gene flow over the continent.
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## 1. Introduction

Although environmental *Alternaria* spp. are not considered as pathogens, their omnipresence is frequently associated with hypersensitivity pneumonitis, asthma and allergic fungal rhinitis and sinusitis in humans (Pastor and Guarro, 2008). Allergic rhinitis is the most common form of noninfectious rhinitis (Randriamanantany et al., 2010), while allergic (extrinsic) asthma is the most common form of asthma, affecting over 50% of 20 million asthma sufferers (Salo et al., 2006).

The primary dispersal method of species of *Alternaria* is by the release of conidia (asexual spores) into the air. It has been suggested that changes in temperature and relative air humidity can trigger spore release from plant material (Timmer et al., 1998). The concentration of allergenic airborne spores can thus be linked to the release of spores from infected plants during dry/wet cycles. Additionally, it is possible that the environment contributes to the genetic diversity of populations of airborne *Alternaria*. For example, areas with large fluctuations in humidity and temperature, and where agricultural activities are prevalent, may be conducive to the generation of diversity that can counteract the selective pressures imposed by the environment. Since *Alternaria* is omnipresent in the outdoor environment, it is thought that the indoor spore concentration is mainly influenced by the outdoor spore

* Corresponding author at: CBS-KNAW Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands.
*E-mail address:* j.woudenberg@cbs.knaw.nl (J.H.C. Woudenberg).

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concentration. However, the indoor level of fungal spores in the air is influenced by the activity in the room, fluctuations in temperature and relative humidity, and the ventilation rate (Samson et al., 2010).

*Alternaria alternata* (belongs to *Alternaria* sect. *Alternaria*) (cf. Lawrence et al., 2013; Woudenberg et al., 2013) is thought to be the main airborne allergen of the genus *Alternaria* (Horner et al., 1995; Kuna et al., 2011; Pulimood et al., 2007). *Alternaria* sect. *Alternaria* consists of more than 50 pathogenic and non-pathogenic morpho-species (Woudenberg et al., 2013). These morpho-species display very low levels of DNA sequence variation, and are therefore difficult to distinguish at the sequence level (Andrew et al., 2009; Peever et al., 2004). A recent study based on whole-genome sequencing supplemented with transcriptome profiling and multi-gene sequencing only recognized 11 phylogenetic species and one species complex in sect. *Alternaria* (Woudenberg et al., unpublished results). As a result, 35 morpho-species were placed in synonymy with *A. alternata*. *A. alternata* is also associated with diseases of citrus, and like other airborne fungi, it displays a worldwide distribution (Stewart et al., 2014). Nonetheless, several studies (e.g., Peever et al., 2004, 2005; Stewart et al., 2014) were able to delineate geographically or host-restricted lineages of *Alternaria*, indicating the potential for phylogeographic studies. In contrast to plant pathogenic fungi, or fungi that have restricted geographic and host ranges, airborne fungi have been neglected as subjects for phylogeographic and population genetic studies (Slippers et al., 2005). It is generally believed that such fungi would display a lack of population subdivision due to their ease of spread, and that diversity levels would be extremely high due to high migration rates. Thus, the lack of data on the population genetics of non-pathogenic airborne fungi can be ascribed to these untested assumptions.

Few studies have investigated indoor *Alternaria* isolates specifically, although multiple studies mention the detection of *Alternaria* in the indoor environment (de Ana et al., 2006; Li and Kendrick, 1995; Solomon, 1975). One large study of dust-borne *A. alternata* allergens in USA homes assessed the concentration of *Alternaria* allergens in dust with a polyclonal anti-alternaria antibody assay (Salo et al., 2005). That study revealed that exposure to *A. alternata* allergens is common, and that residential characteristics such as smoking, mold and moisture problems, and cleaning frequencies influence the indoor antigen levels in house dust. Nonetheless, no reports exist on the genotypic or allelic composition of indoor *Alternaria* isolates from the USA. In addition to the few studies on indoor *Alternaria* species, more studies were performed on *Aspergillus* and *Penicillium* species. These two genera are poorly represented in outdoor air, but they are frequently isolated indoors (Araujo et al., 2010; Henk et al., 2011; Scott et al., 2004, 2007). A study on the genotypic variation in *ca.* 200 *Penicillium chrysogenum* strains from Canadian homes showed no evidence of recombination, indicating a strictly clonal population (Scott et al., 2004). Additionally, a study on the genotypic variation of the *Penicillium brevicompactum* group in house dust in Canada revealed that the two predominant taxa, *P. brevicompactum* and *P. bialowiezense*, also showed a predominantly clonal mode of reproduction (Scott et al., 2007).

Sexual reproduction in filamentous fungi is controlled by the mating-type (or *MAT*) locus (Coppin et al., 1997; Turgeon, 1998). These mating-type loci have been identified from several asexual fungi based on PCR and whole genome sequencing (e.g. Goodwin et al., 2003; Groenewald et al., 2006; Paoletti et al., 2005; Pöggeler, 2002; Sharon et al., 1996; Woo et al., 2006). The discovery of cryptic sexual cycles is important in understanding the evolution of fungal diversity. *Alternaria* is considered to be an asexual fungal genus; however, the connection to a sexual morph, formerly called *Lewia*, is known for some species (Simmons, 1986, 2007). With the recent division of the genus into sections, these sexual connections seem to be restricted to specific sections (Lawrence et al., 2013; Woudenberg et al., 2013). However, the mating-type loci have also been identified from several *Alternaria* spp. which are supposedly asexual (Arie et al., 2000; Berbee et al., 2003; Linde et al., 2010; Stewart et al., 2011).

The first aim of the current study was to identify which *Alternaria* species are present in the indoor environment in the USA, by sequencing two protein-coding genes and one non-translated locus. Secondly, we wanted to investigate the molecular diversity of indoor *Alternaria* isolates in the USA, by genotyping and population genetic inference of the sect. *Alternaria* isolates, using five microsatellite markers (Tran-Dinh and Hocking, 2006). A third aim was to investigate whether alleles at these five microsatellite loci are randomly associated, *i.e.* to test for recombination.

## 2. Materials and methods

### 2.1. Isolates and DNA extraction

Isolates were collected throughout the USA over a period of 6 months from December 2011 to May 2012 (Table 1). Most of the samples (137/156) were collected as malt extract agar (MEA) settle plates by homeowners from their own homes. The MEA plates were purchased by homeowners from hardware stores and sent to EMSL Analytical, Inc. for identification after exposure to indoor air. Ten air samples were collected with a single stage bio-aerosol impaction sampler (EMSL VP-400 Microbial Sampler), three were swab samples and four were dust samples (Table 1). The media used for fungal isolation was MEA. No further information is available on the individual homes. For the microsatellite typing experiment, 37 reference isolates were included (Table 1). For DNA isolation, the isolates were grown on potato–carrot agar (Crous et al., 2009) for 7 d at ambient temperature (~22 °C). Total genomic DNA was extracted using the UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions.

### 2.2. PCR, sequencing and sequence analyses

The internal transcribed spacers (ITS) of the ribosomal DNA operon, including the 5.8S rDNA gene, and a section of the glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) gene region were amplified from genomic DNA as described by Woudenberg et al. (2013) with the primers V9G (De Hoog and Gerrits van den Ende, 1998) and ITS4 (White et al., 1990) for the ITS region, and gpd1 and gpd2 (Berbee et al., 1999) for the *gapdh* region. A section of the endopolygalacturonase (*endoPG*) gene was amplified with the primers PG3 and PG2b (Andrew et al., 2009). The PCR mixture consisted of 1 µl genomic DNA (*ca.* 50 ng), 1 × PCR reaction buffer (Bioline, Luckenwalde, Germany), 2 mM $MgCl_2$, 20 µM of each dNTP, 0.2 µM of each primer, and 0.5 U *Taq* DNA polymerase (Bioline). The PCR program consisted of an initial denaturation step of 5 min at 94 °C followed by 40 cycles of 30 s at 94 °C, 30 s at 50 °C and 30 s at 72 °C and a final elongation step of 7 min at 72 °C. The PCR reactions were performed in a 2720 Thermal Cycler (Applied Biosystems, Foster City, California), in a total volume of 12.5 µl. PCR amplicons were sequenced in both directions using the PCR primers and the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions, and analysed using an ABI Prism 3730xl DNA Analyzer (Applied Biosystems). Consensus sequences were assembled from forward and reverse sequences using the

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**Table 1**
Isolates used in this study with the substrate, locality and date they were collected, and their sequence type (ST) and eBURST group based on microsatellite data.

| Isolate#[a] | Substrate | Locality | Date | Name | ST[c] | eBURST group[c] |
|---|---|---|---|---|---|---|
| CPC 22417 | Air[b], bedroom 2nd floor | USA, NJ | Dec. 2012 | *A. arborescens* SC | 12 | Singleton |
| CPC 22418 | Dust, carpet | USA, PA | Dec. 2012 | *A. alternata* | 39 | Singleton |
| CPC 22419 | Air, bedroom | USA, CA | Jan. 2013 | *A. alternata* | 79 | Singleton |
| CPC 22420 | Air, bedroom | USA, MD | Jan. 2013 | *A. alternata* | 44 | 1 |
| CPC 22421 | Air, bathroom | USA, TX | Jan. 2013 | *A. alternata* | 18 | 1 |
| CPC 22422 | Air, recreational vehicle | USA, CA | Jan. 2013 | sect. *Infectoriae* | na | na |
| CPC 22423 | Air, bedroom | USA, NJ | Dec. 2012 | *A. arborescens* SC | 106 | Singleton |
| CPC 22424 | Air, living room | USA, KS | Dec. 2012 | *A. alternata* | 167 | Singleton |
| CPC 22425 | Air, office | USA, NJ | Dec. 2012 | *A. alternata* | 118 | 19 |
| CPC 22426 | Air, office | USA, MI | Dec. 2012 | *A. alternata* | 44 | 1 |
| CPC 22427 | Air, kitchen | USA, MD | Dec. 2012 | *A. alternata* | 158 | Singleton |
| CPC 22428 | Air, bedroom | USA, WI | Dec. 2012 | *A. alternata* | 86 | 3 |
| CPC 22429 | Air, living room | USA, NJ | Jan. 2013 | *A. alternata* | 134 | 2 |
| CPC 22430 | Air, class room | USA, TX | Jan. 2013 | *A. alternata* | 105 | 1 |
| CPC 22431 | Air, office | USA, CO | Dec. 2012 | *A. alternata* | 91 | 1 |
| CPC 22432 | Air, bedroom | USA, IL | Dec. 2012 | *A. alternata* | 59 | 1 |
| CPC 22433 | Air, basement | USA, NJ | Dec. 2012 | *A. alternata* | 52 | 1 |
| CPC 22434 | Air, 2nd floor | USA, NJ | Jan. 2013 | *A. alternata* | 133 | 2 |
| CPC 22435 | Air[b], office | USA, NY | Jan. 2013 | *A. alternata* | 61 | 1 |
| CPC 22436 | Air, bathroom | USA, MD | Jan. 2013 | *A. alternata* | 47 | 1 |
| CPC 22437 | Swab, store | USA, NY | Jan. 2013 | *A. alternata* | 157 | Singleton |
| CPC 22438 | Air, basement | USA, CT | Jan. 2013 | *A. alternata* | 145 | Singleton |
| CPC 22439 | Air, living room | USA, NJ | Jan. 2013 | *A. alternata* | 111 | 1 |
| CPC 22440 | Air, bedroom | USA, WA | Jan. 2013 | sect. *Pseudoulocladium* | na | na |
| CPC 22441 | Dust, rug | USA, PA | Jan. 2013 | *A. alternata* | 82 | Singleton |
| CPC 22970 | Dust, rug | USA, PA | Jan. 2013 | *A. alternata* | 67 | Singleton |
| CPC 22971 | Air, class room | USA, NY | Jan. 2013 | *A. alternata* | 68 | 1 |
| CPC 22972 | Air, hallway | USA, CA | Jan. 2013 | *A. alternata* | 71 | 1 |
| CPC 22973 | Air, hallway | USA, CA | Jan. 2013 | *A. alternata* | 23 | 10 |
| CPC 22974 | Air, kitchen | USA, GA | Jan. 2013 | *A. alternata* | 141 | 15 |
| CPC 22975 | Air, kitchen | USA, SD | Jan. 2013 | *A. alternata* | 32 | Singleton |
| CPC 22976 | Air, basement | USA, OH | Jan. 2013 | *A. alternata* | 49 | Singleton |
| CPC 22977 | Air, living room | USA, PA | Jan. 2013 | *A. alternata* | 46 | 1 |
| CPC 22978 | Air, living room | USA, MD | Jan. 2013 | *A. arborescens* SC | 98 | Singleton |
| CPC 22979 | Air, bedroom | USA, OK | Jan. 2013 | *A. alternata* | 174 | 1 |
| CPC 22980 | Air, living room | USA, CA | Jan. 2013 | *A. alternata* | 74 | Singleton |
| CPC 22981 | Air, living room | USA, OH | Jan. 2013 | *A. alternata* | 159 | Singleton |
| CPC 22982 | Air, living room | USA, CA | Jan. 2013 | *A. alternata* | 62 | 1 |
| CPC 22983 | Air, bedroom | USA, IL | Jan. 2013 | *A. alternata* | 62 | 1 |
| CPC 22984 | Air, basement | USA, NY | Jan. 2013 | *A. alternata* | 80 | Singleton |
| CPC 22985 | Air, office | USA, AZ | Jan. 2013 | *A. alternata* | 125 | 1 |
| CPC 22986 | Swab, bedroom | USA, TX | Jan. 2013 | *A. alternata* | 166 | 1 |
| CPC 22987 | Air[b], outside | USA, DE | Feb. 2013 | *A. alternata* | 89 | 1 |
| CPC 22988 | Air[b], office | USA, DE | Feb. 2013 | *A. alternata* | 85 | 3 |
| CPC 22989 | Air[b], warehouse | USA, DE | Feb. 2013 | *A. alternata* | 41 | 9 |
| CPC 22990 | Air[b], office | USA, AZ | Feb. 2013 | *A. alternata* | 127 | Singleton |
| CPC 22991 | Air[b], elevator | USA, MO | Feb. 2013 | *A. alternata* | 171 | Singleton |
| CPC 22992 | Air, living room | USA, IL | Feb. 2013 | *A. alternata* | 64 | 7 |
| CPC 22993 | Dust, carpet | USA, MD | Feb. 2013 | *A. arborescens* SC | 146 | 14 |
| CPC 22994 | Air, office | USA, GA | Feb. 2013 | *A. alternata* | 128 | Singleton |
| CPC 22995 | Air, office | USA, TX | Feb. 2013 | *A. alternata* | 125 | 1 |
| CPC 22996 | Air, garage | USA, TX | Feb. 2013 | *A. alternata* | 153 | Singleton |
| CPC 22997 | Air, bedroom | USA, CO | Feb. 2013 | *A. alternata* | 115 | Singleton |
| CPC 22998 | Air, bedroom | USA, TX | Feb. 2013 | *A. arborescens* SC | 172 | 12 |
| CPC 22999 | Air, bedroom | USA, FL | Feb. 2013 | *A. alternata* | 47 | 1 |
| CPC 23000 | Air, living room | USA, WA | Feb. 2013 | *A. arborescens* SC | 104 | Singleton |
| CPC 23001 | Air, bedroom | USA, OR | Feb. 2013 | *A. alternata* | 19 | 1 |
| CPC 23002 | Air, bedroom | USA, IL | Feb. 2013 | *A. alternata* | 90 | 1 |
| CPC 23003 | Air, outside | USA, OK | Feb. 2013 | *A. alternata* | 101 | Singleton |
| CPC 23004 | Air, living room | USA, TX | Feb. 2013 | *A. alternata* | 138 | 1 |
| CPC 23005 | Air, bedroom | USA, IL | Feb. 2013 | *A. alternata* | 148 | Singleton |
| CPC 23006 | Air, bedroom | USA, PA | Feb. 2013 | *A. alternata* | 122 | 17 |
| CPC 23007 | Air, bedroom | USA, CA | Feb. 2013 | *A. alternata* | 100 | Singleton |
| CPC 23008 | Air, bathroom | USA, TX | Feb. 2013 | *A. alternata* | 161 | Singleton |
| CPC 23009 | Air, storage room | USA, MD | Feb. 2013 | *A. alternata* | 16 | Singleton |
| CPC 23010 | Air, kitchen | USA, TX | Feb. 2013 | *A. arborescens* SC | 151 | 14 |
| CPC 23011 | Air, bedroom | USA, MO | Feb. 2013 | *A. alternata* | 50 | 1 |
| CPC 23012 | Air, bedroom | USA, FL | Feb. 2013 | *A. alternata* | 58 | 1 |
| CPC 23013 | Air, bedroom | USA, FL | Feb. 2013 | *A. alternata* | 47 | 1 |
| CPC 23014 | Air, living room | USA, GA | Feb. 2013 | *A. alternata* | 96 | 1 |
| CPC 23015 | Air, dining room | USA, NJ | Feb. 2013 | *A. arborescens* SC | 112 | Singleton |
| CPC 23016 | Air, bedroom | USA, GA | Feb. 2013 | *A. alternata* | 117 | 19 |
| CPC 23017 | Air, office | USA, MS | Feb. 2013 | *A. alternata* | 123 | 17 |
| CPC 23018 | Air, bathroom | USA, TX | Feb. 2013 | *A. alternata* | 124 | 1 |

(*continued on next page*)

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**Table 1** (*continued*)

| Isolate#[a] | Substrate | Locality | Date | Name | ST[c] | eBURST group[c] |
|---|---|---|---|---|---|---|
| CPC 23019 | Air, living room | USA, ME | Feb. 2013 | *A. alternata* | 71 | 1 |
| CPC 23020 | Air, bedroom | USA, IL | Feb. 2013 | *A. alternata* | 69 | 1 |
| CPC 23021 | Air, bathroom | USA, PA | Feb. 2013 | *A. alternata* | 135 | Singleton |
| CPC 23022 | Air, office | USA, CA | Feb. 2013 | *A. alternata* | 26 | Singleton |
| CPC 23023 | Air, bedroom | USA, TX | Mar. 2013 | *A. alternata* | 27 | 1 |
| CPC 23024 | Air, bathroom | USA, CA | Mar. 2013 | *A. arborescens* SC | 15 | 12 |
| CPC 23025 | Air, bathroom | USA, MA | Mar. 2013 | *A. alternata* | 59 | 1 |
| CPC 23026 | Air, bedroom | USA, CO | Mar. 2013 | *A. arborescens* SC | 8 | 16 |
| CPC 23027 | Air, hallway | USA, MI | Mar. 2013 | *A. alternata* | 126 | 1 |
| CPC 23028 | Air, living room | USA, TX | Mar. 2013 | *A. alternata* | 136 | Singleton |
| CPC 23029 | Air, bedroom | USA, IN | Mar. 2013 | *A. alternata* | 114 | Singleton |
| CPC 23030 | Air, bedroom | USA, LA | Mar. 2013 | *A. alternata* | 162 | 8 |
| CPC 23031 | Air, bedroom | USA, NJ | Mar. 2013 | *A. alternata* | 170 | 13 |
| CPC 23032 | Air, break room | USA, TX | Mar. 2013 | *A. alternata* | 88 | 1 |
| CPC 23033 | Air, family room | USA, GA | Mar. 2013 | *A. alternata* | 20 | 1 |
| CPC 23034 | Air, bedroom | USA, CA | Mar. 2013 | *A. alternata* | 65 | 7 |
| CPC 23035 | Air, bathroom | USA, TX | Mar. 2013 | *A. alternata* | 129 | 1 |
| CPC 23036 | Air, bedroom | USA, PA | Mar. 2013 | *A. alternata* | 140 | 1 |
| CPC 23037 | Air[b], office | USA, AZ | Mar. 2013 | *A. alternata* | 40 | 9 |
| CPC 23038 | Air, garage | USA, NJ | Mar. 2013 | *A. alternata* | 48 | 1 |
| CPC 23039 | Air, kitchen | USA, TX | Mar. 2013 | *A. alternata* | 95 | Singleton |
| CPC 23040 | Air, outside | USA, CA | Mar. 2013 | *A. arborescens* SC | 11 | 18 |
| CPC 23041 | Air, bathroom | USA, TX | Mar. 2013 | *A. alternata* | 150 | 2 |
| CPC 23042 | Air, living room | USA, PA | Mar. 2013 | *A. alternata* | 56 | 1 |
| CPC 23043 | Air, living room | USA, CA | Mar. 2013 | *A. arborescens* SC | 9 | Singleton |
| CPC 23044 | Air, office | USA, NE | Mar. 2013 | *A. alternata* | 45 | 1 |
| CPC 23045 | Air, living room | USA, GA | Mar. 2013 | *A. alternata* | 142 | 15 |
| CPC 23046 | Air, office | USA, IL | Mar. 2013 | *A. alternata* | 139 | 1 |
| CPC 23047 | Air, bedroom | USA, KY | Mar. 2013 | *A. alternata* | 144 | Singleton |
| CPC 23048 | Air, bathroom | USA, SC | Mar. 2013 | *A. alternata* | 87 | 3 |
| CPC 23049 | Air, dining room | USA, NM | Mar. 2013 | *A. alternata* | 77 | Singleton |
| CPC 23050 | Air, class room | USA, MO | Mar. 2013 | *A. alternata* | 78 | 1 |
| CPC 23051 | Air, bedroom | USA, TX | Mar. 2013 | *A. alternata* | 107 | 1 |
| CPC 23052 | Air, office | USA, FL | Mar. 2013 | *A. alternata* | 149 | 2 |
| CPC 23053 | Air[b], bathroom | USA, NJ | Mar. 2013 | sect. *Pseudoulocladium* | na | na |
| CPC 23054 | Air, outside | USA, MD | Mar. 2013 | *A. alternata* | 160 | Singleton |
| CPC 23055 | Air, bedroom | USA, TN | Apr. 2013 | *A. alternata* | 113 | 13 |
| CPC 23056 | Air, living room | USA, CT | Apr. 2013 | *A. alternata* | 70 | 1 |
| CPC 23057 | Air, outside | USA, CA | Apr. 2013 | *A. arborescens* SC | 7 | 16 |
| CPC 23058 | Air, bedroom | USA, AL | Apr. 2013 | *A. alternata* | 53 | 1 |
| CPC 23059 | Air, outside | USA, GA | Apr. 2013 | *A. alternata* | 132 | 2 |
| CPC 23060 | Air, bathroom closet | USA, NJ | Apr. 2013 | *A. alternata* | 34 | 4 |
| CPC 23061 | Air, bedroom | USA, TX | Apr. 2013 | *A. alternata* | 169 | Singleton |
| CPC 23062 | Air, bedroom | USA, TX | Apr. 2013 | *A. alternata* | 147 | Singleton |
| CPC 23063 | Air, bedroom | USA, FL | Apr. 2013 | *A. burnsii* | 5 | 5 |
| CPC 23064 | Air, office | USA, FL | Apr. 2013 | *A. alternata* | 60 | 1 |
| CPC 23065 | Air, bedroom | USA, NY | Apr. 2013 | *A. alternata* | 109 | Singleton |
| CPC 23066 | Air, bedroom | USA, CA | Apr. 2013 | *A. alternata* | 81 | Singleton |
| CPC 23067 | Air, class room | USA, NC | Apr. 2013 | *A. alternata* | 31 | Singleton |
| CPC 23068 | Air, living room | USA, NJ | Apr. 2013 | *A. alternata* | 37 | 4 |
| CPC 23069 | Air, family room | USA, CA | Apr. 2013 | *A. alternata* | 75 | 11 |
| CPC 23070 | Air, utility room | USA, MS | Apr. 2013 | *A. alternata* | 55 | 1 |
| CPC 23071 | Air, living room | USA, CA | Apr. 2013 | *A. alternata* | 102 | 6 |
| CPC 23072 | Air, basement | USA, PA | Apr. 2013 | *A. alternata* | 49 | Singleton |
| CPC 23073 | Air, living room | USA, MO | Apr. 2013 | *A. alternata* | 72 | 1 |
| CPC 23074 | Air, bedroom | USA, MO | Apr. 2013 | *A. alternata* | 168 | 1 |
| CPC 23075 | Air, dining room | USA, TX | Apr. 2013 | *A. alternata* | 102 | 6 |
| CPC 23076 | Air, bedroom | USA, FL | Apr. 2013 | *A. alternata* | 84 | 3 |
| CPC 23077 | Air, kitchen | USA, IL | Apr. 2013 | *A. alternata* | 116 | 1 |
| CPC 23078 | Air, living room | USA, NY | Apr. 2013 | *A. alternata* | 137 | Singleton |
| CPC 23079 | Air, bathroom | USA, FL | Apr. 2013 | *A. alternata* | 43 | 1 |
| CPC 23080 | Air, bedroom | USA, MI | Apr. 2013 | *A. alternata* | 173 | Singleton |
| CPC 23081 | Air, bedroom | USA, AZ | Apr. 2013 | *A. alternata* | 76 | 11 |
| CPC 23082 | Swab, wine barrel | USA, PA | Apr. 2013 | *A. alternata* | 22 | 1 |
| CPC 23083 | Air, living room | USA, GA | May 2013 | *A. alternata* | 30 | 2 |
| CPC 23084 | Air, bathroom | USA, IL | May 2013 | *A. alternata* | 163 | Singleton |
| CPC 23085 | Air[b], warehouse | USA, DE | May 2013 | *A. alternata* | 41 | 9 |
| CPC 23086 | Air, bathroom | USA, CA | May 2013 | *A. alternata* | 93 | Singleton |
| CPC 23087 | Air, basement | USA, PA | May 2013 | *A. alternata* | 36 | 4 |
| CPC 23088 | Air, bedroom | USA, FL | May 2013 | *A. alternata* | 143 | Singleton |
| CPC 23089 | Air, bedroom | USA, CA | May 2013 | *A. arborescens* SC | 10 | 18 |
| CPC 23090 | Air, kitchen | USA, AZ | May 2013 | *A. alternata* | 165 | Singleton |
| CPC 23091 | Air, bedroom | USA, IA | May 2013 | *A. alternata* | 35 | 4 |
| CPC 23092 | Air, bedroom | USA, RI | May 2013 | *A. alternata* | 164 | Singleton |
| CPC 23093 | Air, living room | USA, GA | May 2013 | *A. alternata* | 73 | Singleton |

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**Table 1** (*continued*)

| Isolate#[a] | Substrate | Locality | Date | Name | ST[c] | eBURST group[c] |
|---|---|---|---|---|---|---|
| CPC 23094 | Air, bathroom | USA, CA | May 2013 | *A. alternata* | 25 | 10 |
| CPC 23095 | Air, bathroom | USA, VA | May 2013 | *A. arborescens* SC | 152 | Singleton |
| CPC 23096 | Air, office | USA, TX | May 2013 | *A. alternata* | 130 | 1 |
| CPC 23097 | Air, office | USA, OK | May 2013 | *A. alternata* | 155 | Singleton |
| CPC 23098 | Air, basement | USA, MA | May 2013 | *A. alternata* | 131 | 2 |
| CPC 23099 | Leaf, green house | USA, NC | May 2013 | *A. alternata* | 102 | 6 |
| CPC 23100 | Leaf, green house | USA, NC | May 2013 | *A. alternata* | 110 | 6 |
| CBS 101.13 | Unknown | Unknown | <Jan. 1913 | *A. arborescens* SC | 8 | 16 |
| CBS 103.33 | Soil | Egypt | <Jan. 1933 | *A. alternata* | 24 | 10 |
| CBS 107.38 | *Cuminum cyminum* | Unknown | <Dec. 1938 | *A. burnsii* | 3 | 5 |
| CBS 117.44 | *Godetia* sp. | Denmark | Jul. 1942 | *A. alternata* | 28 | Singleton |
| CBS 194.86 | *Quercus* sp. | USA | 1953 | *A. alternata* | 33 | Singleton |
| CBS 195.86 | *Euphorbia esula* | Canada | 1982 | *A. alternata* | 103 | 6 |
| CBS 479.90 | *Citrus unshiu* | Japan | 1968 | *A. alternata* | 42 | 9 |
| CBS 632.93 | *Pyrus pyrifolia* | Japan | Jul. 1990 | *A. gaisen* | 14 | 8 |
| CBS 540.94 | *Nicotiana tabacum* | USA | <Nov. 1971 | *A. longipes* | 97 | Singleton |
| CBS 916.96 | *Arachis hypogaea* | India | Dec. 1980 | *A. alternata* | 99 | Singleton |
| CBS 918.96 | *Dianthus chinensis* | UK | Feb. 1981 | *A. alternata* | 21 | 1 |
| CBS 102595 | *Citrus jambhiri* | USA | <Jul. 1997 | *A. alternata* | 54 | 1 |
| CBS 102596 | *Citrus jambhiri* | USA | <Jul. 1997 | *A. alternata* | 92 | 1 |
| CBS 102597 | *Minneola tangelo* | USA | <Aug. 1997 | *A. gossypina* | 17 | Singleton |
| CBS 102598 | *Minneola tangelo* | USA | <Feb. 1998 | *A. alternata* | 175 | 1 |
| CBS 102599 | *Minneola tangelo* | Turkey | May 1996 | *A. alternata* | 51 | 1 |
| CBS 102600 | *Citrus reticulata* | USA | Jun. 1975 | *A. alternata* | 57 | 1 |
| CBS 102601 | *Minneola tangelo* | Colombia | <Nov. 1996 | *A. gossypina* | 17 | Singleton |
| CBS 102602 | *Minneola tangelo* | Turkey | May 1996 | *A. alternata* | 51 | 1 |
| CBS 102604 | *Minneola tangelo* | Israel | <Nov. 1996 | *A. alternata* | 66 | Singleton |
| CBS 102605 | *Solanum lycopersicum* | USA | Apr. 1990 | *A. arborescens* SC | 8 | 16 |
| CBS 118404 | *Iris* sp. | New Zealand | Jan. 2001 | *A. iridiaustralis* | 6 | Singleton |
| CBS 118488 | *Pyrus pyrifolia* | Japan | Jul. 1990 | *A. gaisen* | 13 | 8 |
| CBS 118809 | *Alstroemeria* sp. | Australia | Jul. 2005 | *A. alstroemeriae* | 119 | Singleton |
| CBS 118810 | *Beta vulgaris* var. *cicla* | Kenya | 2001 | *A. betae-kenyensis* | 1 | Singleton |
| CBS 118811 | *Brassica oleracea* | USA | Apr. 1982 | *A. alternata* | 94 | Singleton |
| CBS 118812 | *Daucus carota* | USA | Jan. 1984 | *A. alternata* | 83 | 3 |
| CBS 118814 | *Solanum lycopersicum* | USA | Jun. 1996 | *A. alternata* | 156 | 3 |
| CBS 118816 | *Rhizophora mucronata* | India | Oct. 1995 | *A. burnsii* | 2 | 5 |
| CBS 118817 | *Tinospora cordifolia* | India | Sep. 1987 | *A. burnsii* | 4 | 5 |
| CBS 118818 | *Vaccinium sp.* | USA | Oct. 1973 | *A. alternata* | 108 | 1 |
| CBS 119399 | *Minneola tangelo* | USA | Dec. 1980 | *A. alternata* | 121 | Singleton |
| CBS 119408 | *Euphorbia esula* | USA | Nov. 1992 | *A. alternata* | 38 | 4 |
| CBS 119543 | *Citrus paradisi* | USA | Jun. 1947 | *A. alternata* | 63 | 7 |
| CBS 121454 | *Cuscuta* sp. | USA | Aug. 1997 | *A. alternata* | 154 | Singleton |
| CBS 121455 | *Broussonetia papyrifera* | China | Sep. 1996 | *A. alternata* | 29 | 2 |
| CBS 124392 | *Solanum melongena* | China | Unknown | *A. alternantherae* | 120 | Singleton |

[a] CBS: Culture collection of the Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, The Netherlands; CPC: Personal collection of P.W. Crous, Utrecht, The Netherlands.
[b] Collected with a single stage bio-aerosol impaction sampler.
[c] na: not analysed.

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BioNumerics version 4.61 software package (Applied Maths, St-Martens-Latem, Belgium).

Sequence alignments were generated with MAFFT version 7 (Katoh and Standley, 2013), and manually adjusted where necessary. A Bayesian inference analysis was conducted with MrBayes version 3.2.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) on the individual datasets. The K80 model with gamma distribution was used for the ITS region, and the GTR-model with gamma distribution for the *gapdh* and *endoPG* regions, as suggested by the on-line tool FindModel (http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html). The two Markov Chain Monte Carlo (MCMC) analyses used four chains and started from a random tree topology. The analysis ran with the sample frequency set at 1000 and the temperature value of the heated chain at 0.05 and stopped when the average standard deviation of split frequencies fell below 0.01. Burn-in was set to 25%, after which the likelihood values were stationary. The convergence of chains was verified with Tracer version 1.5.0 (Rambaut and Drummond, 2009), and TreeView version 1.6.6 (Page, 1996) was used to visualise the phylogenetic tree. Both the sequence alignments and phylogenetic trees were deposited in TreeBASE (http://www.treebase.org).

### 2.3. Microsatellite typing

Five primer pairs previously designed for *A. alternata* (Tran-Dinh and Hocking, 2006; Table 2) were used to characterize the indoor *Alternaria* sect. *Alternaria* population from the USA, together with 37 reference isolates (Table 1). By performing a genomic search of the primer sequences against a draft *A. alternata* genome (Woudenberg et al., unpublished results), the relative positions of the microsatellites on the genome were located. From each primer pair, one primer was labelled with the Fluorobrite oligo FAM (loci AEM3 and AEM5), SOL (locus AEM6) or ZEL (loci AEM9 and AEM13) (Biolegio BV, Nijmegen, the Netherlands; Table 2). Loci AEM3/AEM5 and AEM9/AEM13 were amplified in a multiplex PCR. The PCR mixture consisted of 1 µl DNA (*ca.* 50 ng), 1 × PCR buffer (Bioline), 40 µM of each dNTP, 1.6 mM $MgCl_2$, 0.2 µM of each primer, and 0.25 U *Taq* polymerase (Bioline) in a total volume of 12.5 µl. The amplification was performed on a 2720 Thermal Cycler (Applied Biosystems) and consisted of a 5 min initial denaturation step (94 °C) followed by 35 cycles of 30 s at 94 °C, 55 °C and 72 °C, and a final 7 min elongation step (72 °C). For fragment analysis, the PCR products were diluted 1:1000 and combined per biological sample, which resulted in one

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**Table 2**
PCR primer sequences, repeat motifs, number of alleles and allele distribution observed for microsatellite markers in *Alternaria* sect. *Alternaria* based on 153 isolates.

| Locus | Primer sequence (5′-3′)[a] | Repeat motif | No. of alleles | Allele distribution |
|---|---|---|---|---|
| AEM3 | F: TGA TCC CAC GTC ACA GAA AG<br>R: [F]GGT TGT CCA AGT ACC CCA TAG A | $(AAG)_9$ | 39 | Uneven |
| AEM5 | F: [F]TAC AGA CGG AGG GAG GAC AC<br>R: CAC AGC TCG TCA TCC GAG TA | $(GAA)_{10}$ | 13 | Even |
| AEM6 | F: TGA CGA GCT GTG AGG AGT GT<br>R: [S]CGT GTG TAG GGT CTT CGT CTC | $(CA)_5(CT)_5$ | 15 | Uneven |
| AEM9 | F: GAA GCC CAT TCC ACT CAC A<br>R: [Z]GCT CCA TCT CCC ACA GTA ACA | $(CAA)_{12}$ | 11 | Uneven |
| AEM13 | F: TGC GAA ACC GTG GAT ACT G<br>R: [Z]TCG GAA ATG GCT GCA ATA GT | $(GAC)_7(GAA)_{38}$ | 47 | Even |

[a] [F], [S] or [Z] indicates the use of respectively FAM, SOL or ZEL as fluorescent label.

well per isolate for the fragment analysis. MCLAB's Orange Size Standard (Nimagen, Nijmegen, the Netherlands) was used as internal marker. Samples were electrophoresed using an ABI Prism 3730xl DNA Analyzer (Applied Biosystems), and analysed with the freeware Peak Scanner version 1.0 (Applied Biosystems). Individual alleles at each locus were assigned using fragment lengths (Table S1).

### 2.4. Population genetic analyses

The online program eBURST version 3 (http://eburst.mlst.net/v3; Feil et al., 2004) was used to identify clusters of closely related genotypes. The allelic profiles were assigned to sequence types (STs; Table S1), and eBURST identified groups of STs that only differed at one locus (known as single locus variants).

For subsequent population genetic analyses, the *A. alternata* isolates were divided into four artificial subpopulations representing four quadrants of the USA. Isolates from NE-USA were excluded due to small sample size. The program MultiLocus version 1.3 (Agapow and Burt, 2001) was used to simulate genotypic diversity against the number of loci (1000 randomizations per locus combination), in order to test whether sampling was sufficient for population genetic analyses. The same software was used to calculate the genotypic diversity and linkage disequilibrium in sub-populations of isolates (10,000 data randomizations). For this analysis, locus AEM6 was excluded due to the fact that it was physically linked to locus AEM5 and was also less polymorphic than that locus. The Index of Association ($I_A$) between alleles at different loci was normalized as $\bar{r}_d$ as an indication of random association between loci. The null hypothesis for this test was that alleles are randomly associated, and deviation from random association is measured as a confidence interval. The $\theta$-values of population differentiation between pairwise combinations of subpopulations, *i.e.* SW-USA, NE-USA, and SE-USA (Fig. 1), were used to estimate the pairwise number of migrants per generation (Slatkin, 1995), which equates to gene flow ($\widehat{M}$).

The Stoddart and Taylor (1988) genotypic diversity was manually calculated for isolates from each included quadrant of the USA, and the genotypic diversity of each subpopulation was normalized with sample size to yield $\widehat{G}$ (the percentage of maximum genotypic diversity), which can be used to make inter-sample comparisons. The significance of differences between $\widehat{G}$ values was assessed using a two-tailed *t*-test at a significance level of 99% ($P = 0.01$) with $N_1 + N_2 - 2$ degrees of freedom, where $N$ is the sample size.

In order to assess diversity that is independent of genotypes, the allelic (gene) diversity (Nei, 1973) was calculated. This statistic provides an indication of heterozygosity, or the probability of obtaining two different alleles at a locus when two individuals are randomly sampled from a haploid population. $H \rightarrow 1$ for diverse populations, while $H \rightarrow 0$ for populations that display allelic homogeneity. Additionally, the level of uniqueness ($\varphi$) (Van der Merwe et al., 2012) for each subpopulation was calculated. This statistic estimates the probability of sampling a unique (private) allele belonging to a subpopulation, when a random individual is drawn from the total population. In other words, $\varphi$ is an indication of allelic segregation in a subpopulation.

## 3. Results

### 3.1. Phylogeny

From the 193 included isolates we were not able to amplify the *endoPG* sequences from five isolates (CPC 22422, CPC 22440, CPC 23053, CPC 23063 and *A. alternantherae* CBS 124392). The sequences of the ITS (554 characters), *gapdh* (580 characters) and *endoPG* (448 characters) gene regions consisted of respectively 58, 88 and 47 unique site patterns. After discarding the burn-in, the Bayesian analysis resulted in respectively 4308, 5200 and 4218 trees from both runs. Based on their ITS, *gapdh* and *endoPG* sequences, 153 of the 156 isolates (i.e., 98%) belonged to sect. *Alternaria*, while two isolates belonged to sect. *Pseudoulocladium* (CPC 22440, CPC 23053), and one belonged to sect. *Infectoriae* (CPC 22422) (Table 1). From the 153 isolates that belonged to sect. *Alternaria*, CPC 23063 could be assigned to *A. burnsii* and 15 other isolates could be assigned to the *A. arborescens* species complex (AASC). The remaining 137 isolates were identified as *A. alternata*. Both the *gapdh* and ITS phylogeny could distinguish the sect. *Alternantherae*, sect. *Pseudoulocladium* and sect. *Infectoriae* isolates from the sect. *Alternaria* isolates. The *endoPG* locus from the isolates outside sect. *Alternaria* could not be amplified. Within sect. *Alternaria* the ITS phylogeny could only distinguish *A. betae-kenyensis*, *A. burnsii*, *A. iridiaustralis* and *A. longipes*. The other five included *Alternaria* species, *A. alstroemeriae*, *A. alternata*, *A. gaisen*, *A. gossypina*, and the AASC, all clustered together based on their ITS sequences. The *gapdh* and *endoPG* phylogenies separated all included species in sect. *Alternaria* except AASC/*A. alternata* and *A. gossypina*/*A. longipes*, respectively. The clustering of the *A. alternata* isolates with respect to the other recognized species in sect. *Alternaria* was not consistent throughout the three sequenced genes, as inconsistent sub-clusters were formed.

### 3.2. Microsatellite typing

Comparisons of microsatellite loci to a draft genome sequence revealed that three of the loci, namely AEM3, AEM9 and AEM13 each resided on a different genomic scaffold. Loci AEM5 and AEM6 resided on a single scaffold, and the AEM5-R and AEM6-F primers overlapped with 12 nt. We found 142 allelic profiles (or sequence types, ST) from the 153 collected isolates (Table 1). When the 37 reference isolates were included, 175 allelic profiles

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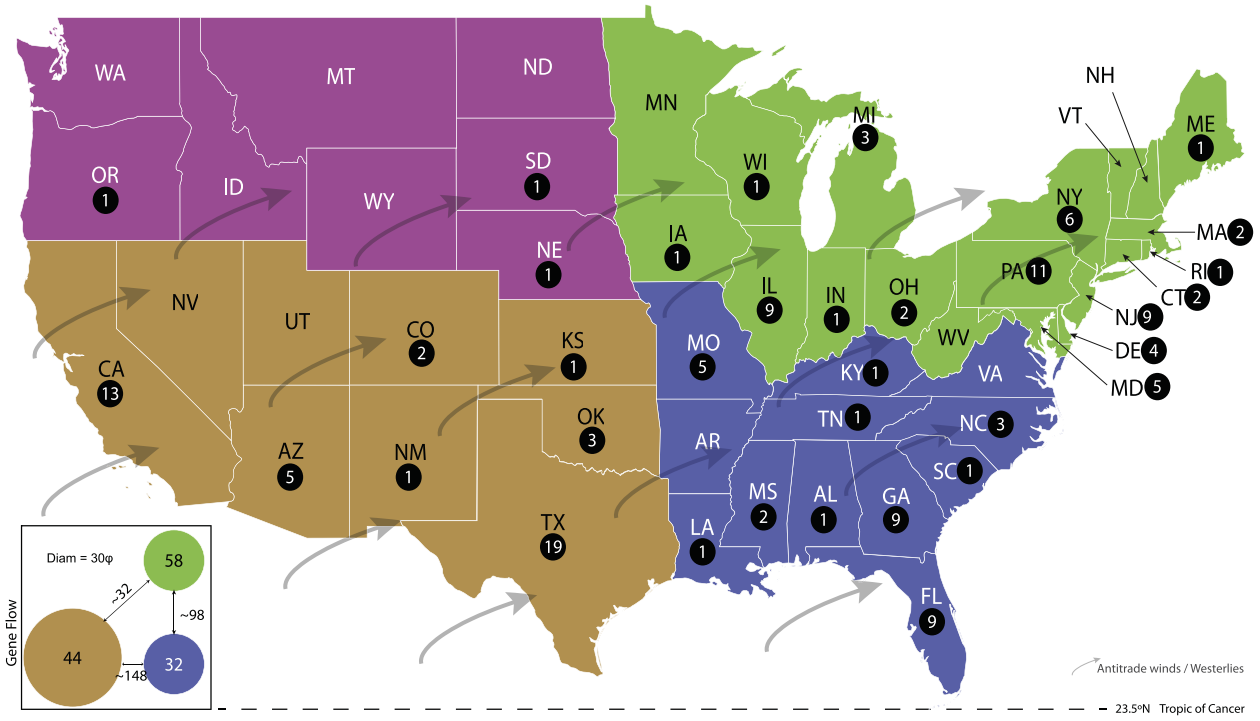


**Fig. 1.** Map of the mainland USA (Mercator projection) indicating the four artificially defined quadrants using different colors, and the general north-easterly direction of the antitrade winds over the subcontinent (grey arrows). Numbers in black filled circles are the numbers of isolates from each state. The boxed insert depicts gene flow estimations between the south-west, south-east and north-east quadrants. Diameters of the circles are proportionate to the level of uniqueness ($\varphi$) of each of the subpopulations. The north-west quadrant was excluded from these analyses due to lack of a sufficient number of isolates.

were observed. Loci AEM3 and AEM13 showed the largest number of alleles (Table 2). However, within AEM3 there was an uneven distribution of the different alleles, with allele 257 being observed in 58 of 153 isolates (~35%). For loci AEM6 and AEM9 the distribution across the different alleles was more unbalanced. At these loci, alleles 161 and 278 were observed in ~70% of the isolates. For loci AEM5 and AEM13 there was an even distribution among the different alleles. The locus AEM13, which displayed the highest number of alleles and an even distribution of these alleles, contributed most to the genotypic variation, followed by AEM3 with a high number of alleles but with an uneven distribution. Loci AEM6 and AEM9 were the least informative loci, with a low number of alleles and an uneven distribution.

### 3.3. Population genetic analyses

An eBURST analysis of 190 isolates (153 sect. *Alternaria* isolates and 37 reference isolates), representing 175 STs, resulted in 19 groups and 65 singletons (Fig. 2, Table 1). Group 1 was the largest, and included 62 isolates representing 54 STs (including eight reference isolates forming seven STs). Group 2 contained eight isolates representing eight STs (including 1 reference isolate) while group 3 contained six isolates and six STs (including 2 reference isolates). The remaining groups, namely 4–19, included five or less isolates. The isolates assigned to the *A. arborescens* complex based on their *endoPG* sequence formed groups 12, 14, 16, and 18, while six isolates were singletons. The assignment of CPC 23063 to *A. burnsii* based on the *gapdh* sequence is supported by the microsatellite data, since all *A. burnsii* isolates clustered in eBURST group 5. No correlation was found between the location and place of isolation and the eBURST groups assigned to the isolates based on their allelic profiles. Almost all eBURST groups contained isolates from different states in the USA and different places of isolation, e.g. bathroom, bedroom, kitchen, etc. The only exceptions were group 13, which consists of two bedroom isolates, but isolated in two different states, and groups 15 and 18, which both consisted of two isolates from the same state, respectively Georgia and California, but from different places of isolation.

When the microsatellite alleles for the *A. alternata* (137) isolates were combined into multilocus genotypes (haplotypes), 126 distinct genotypes could be recovered. While most of these genotypes were observed only once, the most frequent genotype was observed three times. Modelling of the genotypic diversity vs. the number of loci revealed that both microsatellite loci and genotypes were adequately sampled to continue with population genetic analyses (Fig. 3). Index of Association values for three quadrants of the USA, namely the south-west, north-east, and south-east quadrants (SW-USA, NE-USA, SE-USA) indicated that alleles were randomly associated for all three subpopulations, as well as for the metapopulation (Fig. 4, Table 3). Additionally, alleles of the two physically linked loci, namely AEM5 and AEM6, were in linkage disequilibrium ($P < 0.0001$), while all other loci were in pair-wise equilibrium with each other and with AEM5. Population differentiation ($\theta$; Table 4) was very low when pair-wise combinations of these three subpopulations were analysed. Subsequently, the estimated numbers of migrants per generation ($\widehat{M}$) were high between all three pair-wise combinations of subpopulations (Table 4). However, the migration rate between SE-USA and SW-USA ($\widehat{M} = 147.5$) was much higher than the other two combinations, and the migration rate between SW-USA and NE-USA ($\widehat{M} \cong 32$) was the smallest.

The maximum likelihood estimator of genotypic diversity ($\widehat{G}$) revealed that all subpopulations consisted of an extremely large diversity of genotypes (Table 3). No significant differences between the estimated $\widehat{G}$-values could be detected using a two-tailed *t*-test. Gene diversity ($\overline{H}$) values were 0.952, 0.923, and 0.916 for SW-USA, SE-USA, and NE-USA, respectively. Thus, alleles were most unevenly distributed in the SW-USA subpopulation ($N = 58$). An estimation of the level of uniqueness ($\varphi$) of each subpopulation

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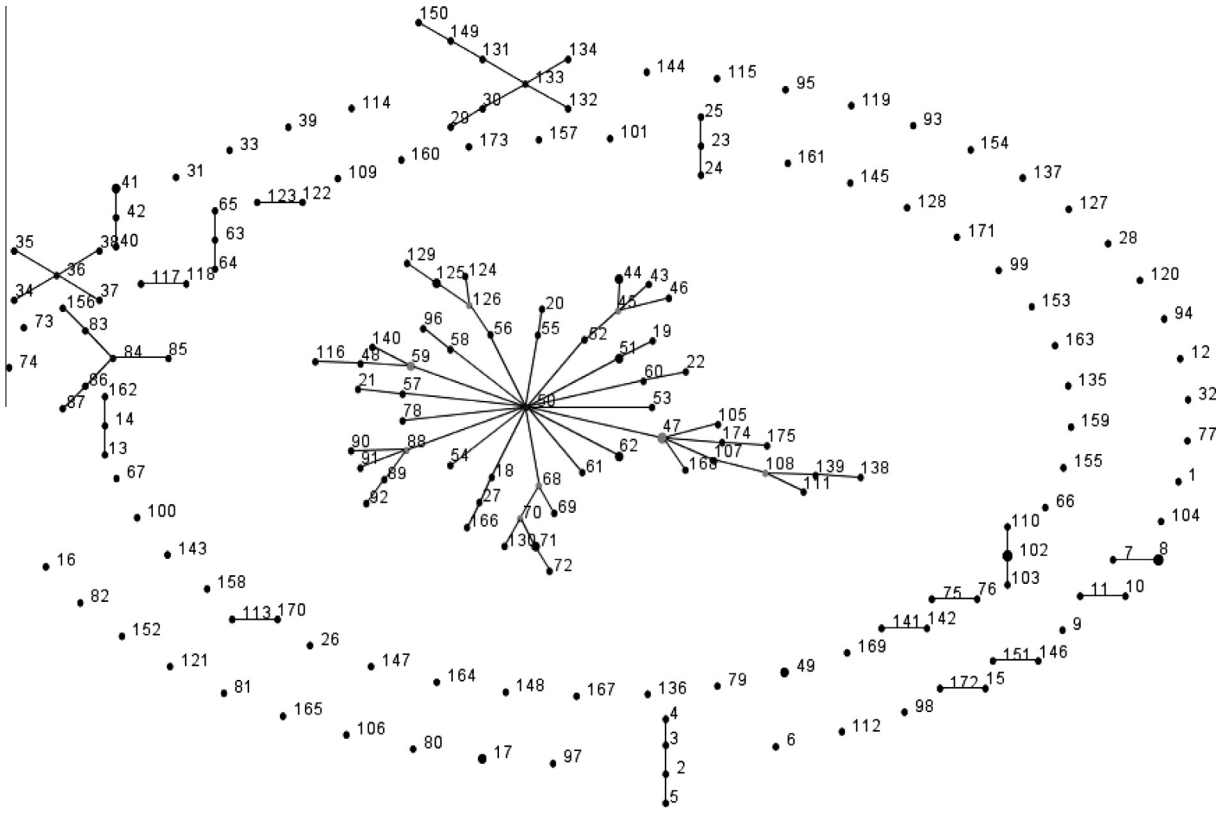

**Fig. 2.** eBURST diagram of 190 *Alternaria* isolates. The numbers correspond to sequence type numbers, the size of the dot correlates to the number of isolates.

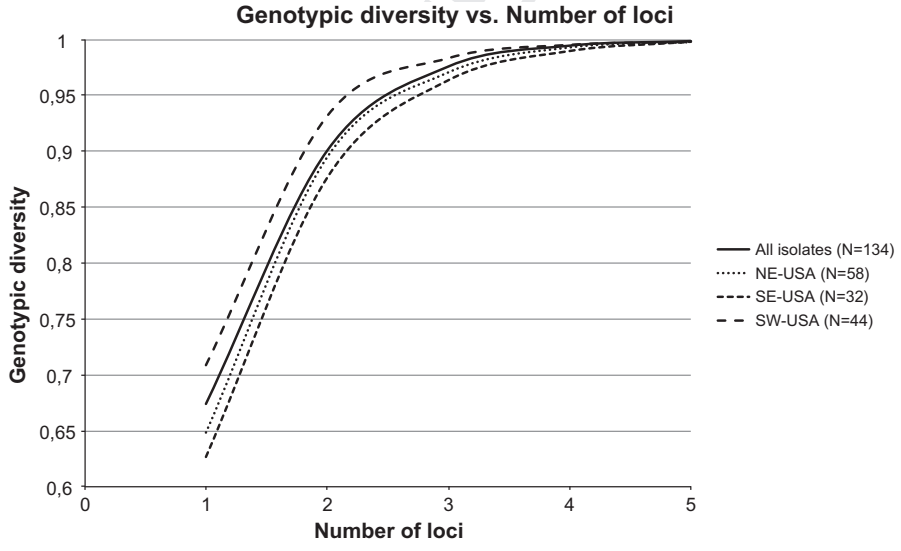


**Fig. 3.** Results from modeling genotypic diversity against the number of loci. Each locus-combination was repeated 1000 times, resulting in a mean genotypic diversity for that combination. The graph reaches a plateau at four microsatellite loci, indicating that both the number of isolates and the number of loci were sufficient for population genetic analyses.

indicated that the SW-USA subpopulation was most unique ($\varphi = 0.915$), while the SE-USA and NE-USA subpopulations were equally unique ($\varphi = 0.564$ and $\varphi = 0.568$, respectively).

## 4. Discussion

*Alternaria* species from sect. *Infectoriae*, the *A. arborescens* group and *A. tenuissima* (both sect. *Alternaria*) are described as common species from food and the indoor environment (Samson et al., 2010). Three species from the former genus *Ulocladium*, recently synonymized under *Alternaria* (Woudenberg et al., 2013), are also common in food and the indoor environment (Samson et al., 2010); *A. cucurbitae*, *A. atra* (both sect. *Ulocladioides*) and *A. alternariae* (sect. *Ulocladium*). Our results largely support these observations for the indoor samples, although the species from sect. *Alternaria* were by far the most prevalent in the US homes included in this study. We only found one isolate from sect. *Infectoriae* and two isolates from sect. *Pseudoulocladium*, that resembles sect. *Ulocladioides* and *Ulocladium* based on morphology.

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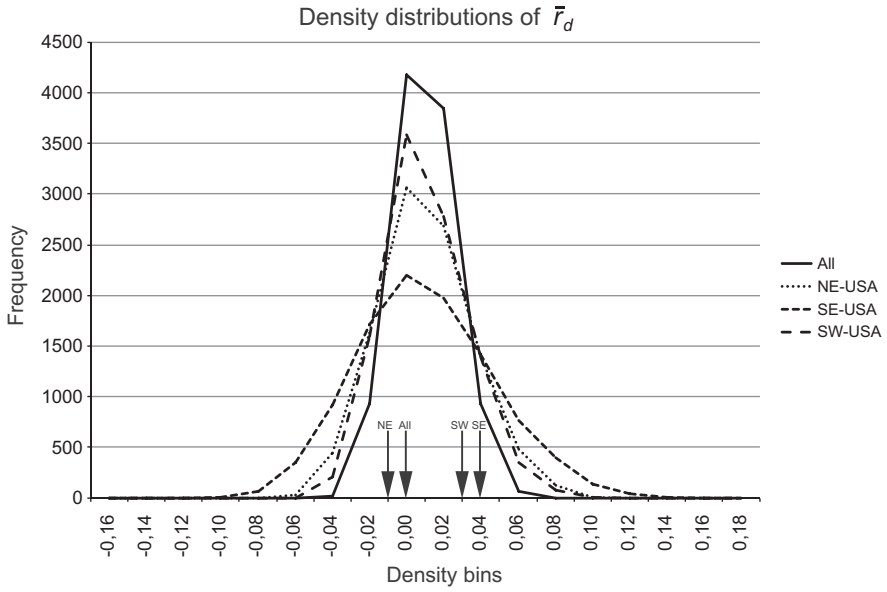


**Fig. 4.** Graph of linkage disequilibrium estimation densities ($\bar{r}_d$ values, which are normalized Index of Association values) resulting from 10,000 randomizations of each of the artificially-defined subpopulations, as well as for these three subpopulations combined. The observed linkage disequilibrium values are indicated using arrows, and these are inside the 95% confidence intervals of the distributions. Thus, the null hypothesis of random mating in these populations cannot be rejected.

**Table 3**
Summary statistics for indoor *Alternaria alternata* isolates from the USA.

| Statistic | All isolates[a] | South-West USA | North-East USA | South-East USA |
|---|---|---|---|---|
| Number of isolates, $N$ | 134 | 44 | 58 | 32 |
| Number of genotypes | 122 | 42 | 54 | 31 |
| Genotypic diversity[b], $\widehat{G}$ | 81.71% | 91.67% | 87.88% | 94.12% |
| Number of alleles (all loci) | 104 | 72 | 66 | 52 |
| Gene diversity, $\overline{H}$ | 0.968 | 0.952 | 0.916 | 0.923 |
| Private alleles (all loci) | – | 27 | 15 | 14 |
| Uniqueness, $\varphi$ | – | 0.915[c] | 0.568 | 0.564 |
| Gametic equilibrium[d] | Yes ($P = 0.448$) | Yes ($P = 0.086$) | Yes ($P = 0.695$) | Yes ($P = 0.135$) |

[a] Excludes three *A. alternata* isolates from the NW quadrant of the USA.
[b] None of the maximum likelihood estimators of genotypic diversity were significantly different in any of the pair-wise combinations.
[c] A uniqueness of 0.915 implies that there is a 91.5% chance that an isolate containing a unique allele, relative to the meta-population, can be drawn from this sub-population.
[d] $P$-values indicate the probabilities of rejecting the null hypothesis of random association of alleles. A $P$-value of less than 0.05 is regarded as significant.

**Table 4**
Population differentiation ($\theta$) and estimated number of allelic migrants per generation ($\widehat{M}$) between the three artificial sub-populations of *Alternaria alternata* from the south-west, south-east, and north-east quadrants of the USA.

| Comparison | $\theta$ | $\widehat{M}$ |
|---|---|---|
| NE-USA vs. SE-USA | 0.00506 | 98.33 |
| NE-USA vs. SW-USA | 0.01537 | 32.04 |
| SE-USA vs. SW-USA | 0.00338 | 147.50 |

No correlation was found between the location and place of isolation and the eBURST groups assigned to the isolates based on their allelic profiles. Since most groups contained indoor isolates as well as reference isolates, there did not seem to be a specific indoor cluster. However, there was subjective correlation between the eBURST groups and phylogeny; *Alternaria gaisen*, *A. gossypina* and *A. burnsii* isolates clustered together in both analyses. The other species that could be distinguished based on phylogeny, namely *A. alstroemeriae*, *A. alternantherae*, *A. betae-kenyensis*, *A. iridiaustralis* and *A. longipes*, were also separated using eBURST. The *A. arborescens* isolates did not form a single group based on their allelic profiles, but the isolates did cluster together in several eBURST groups (12, 14, 16 and 18) or remained as singletons (6). Furthermore, 15 out of the 17 isolates from the *A. arborescens* species-complex had allele 125 at locus AEM5 and allele 281 at locus AEM9. The two remaining isolates had one of the mentioned alleles but differed at the other locus. Although the *A. iridiaustralis* isolate also had these alleles, these loci have some potential as markers for species in the *A. arborescens* complex.

Surprisingly, analyses to test for random association of alleles in isolates of *A. alternata* showed that the allele associations between microsatellite loci were not significantly different from what can be expected in a randomly mating population. Nonetheless, alleles of AEM5 and AEM6, which were on the same locus, were in linkage disequilibrium. The last mentioned observation can be explained by the improbability of cross-over events between the two adjacent stretches of DNA. For these reasons, the less polymorphic of these two loci, *i.e.* AEM6, was excluded when disequilibrium was tested between loci.

Two possible explanations can be proposed for gametic equilibrium and, thus, outcrossing. The first is that cryptic sexual recombination could account for the lack of allelic associations. Evidence

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is accumulating for the occurrence of cryptic sex in filamentous fungi that are thought to be asexual (Kück and Pöggeler, 2009). For example, another study of an *A. alternata* population causing citrus brown spot in Florida revealed three subpopulations of which two were clonal and one showed the ability to recombine through a cryptic sexual cycle or parasexual cycle, based on six fast evolving loci and the presence of both mating-types (Stewart et al., 2013). A second explanation for random association of alleles in *A. alternata* can be arrived at when we consider the nature of microsatellites. These loci change via birth-and-death evolution (Buschiazzo and Gemmell, 2006) such that they are highly polymorphic. It is possible that over long periods of asexual reproduction a microsatellite locus can become hyper-mutated in very large populations such as *A. alternata*. If this process acts equally on all microsatellites, such a situation could account for the random association of independently evolving alleles that were detected in this study. Thus, this explanation accounts for two possibilities: either the lack of allele association was due to experimental error (the inability of the available microsatellites to discriminate between randomly and non-randomly associated alleles), or *A. alternata* has been asexual for so long that the loci are hyper-mutated. A simulation of the observed data showed that sampling was adequate in both dimensions (i.e., number of isolates and number of loci). Additionally, due to size limitations on microsatellite loci (e.g., Buschiazzo and Gemmell, 2006) there is a very high probability of size homoplasy, confounding the detection of hyper-mutation. Therefore, recombination is the most parsimonious explanation for the data.

High levels of diversity can be caused only by a limited set of evolutionary processes. The most important of these are mutation, recombination, and migration (Ayala, 1982; Halliburton, 2004; Hartl and Clark, 2007; Hedrick, 2000; Nielsen and Slatkin, 2013). Our data indicated that recombination is a contributor, but that hyper-mutation is not a viable explanation for the diversity of *A. alternata*. Although no sub-populations could be statistically identified, the levels of uniqueness provided important information regarding the movement of the fungus across the mainland USA. Since the SW-USA sub-population was most unique, we can hypothesize that either this sub-population results directly from sexual reproduction, or the alleles have an alternate origin but are concentrated in this region.

The SW-USA and SE-USA sub-populations appear to exchange a very high number of inter-population allelic migrants, and this pattern correlates with the anti-trade winds. *Alternaria* spores are known as dry air spores that are dispersed by wind (Andersen et al., 2012). Long-distance dispersal in the air can only occur if there is a susceptible host in the target area (Brown and Hovmøller, 2002). Since *A. alternata* has been described from more than 100 host plants (Rotem, 1994), it is possible that these genotypes move through the air in a west-to-east direction across the southern USA. This is then possibly followed by south-to-north movement out of the SE-USA sub-population towards to NE-USA. This long-distance movement of fungal spores from the southern USA to the northern USA has already been reported for the air-borne plant pathogens *Puccinia graminis* and *Phakopsora pachyrhizi* (Andersen et al., 2012).

The high genotypic diversity within the *A. alternata* isolates was also visible with our gene sequencing, as inconsistent sub-clusters existed within the three single-gene phylogenies. In a more extensive phylogenetic study on sect. *Alternaria*, where eleven individual gene regions were sequenced, the incongruent clustering within the *A. alternata* isolates was demonstrated even more clearly (Woudenberg et al., unpublished results). We hypothesize that this high genotypic diversity derives from Mexico/Central America, where many agricultural crops have evolved. From here the fungi moved through the USA via the antitrade winds.

## 5. Conclusions

This study showed that the most prevalent species in the indoor environment in USA homes is *A. alternata*, with a high genotypic diversity. The SW-USA subpopulation displayed the highest level of uniqueness and the highest amount of gene flow, between SW-USA and SE-USA, coincided with prevailing winds over the subcontinent. Lastly, *A. alternata* in the continental USA displays random mating. This is the first report of such an observation in indoor samples of this fungus from homes in the USA.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2015.05.003.

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