

1 **Molecular diversity and allergenic profiles of *Alternaria* spp. from desert**
2 **environments in Arizona.**

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4 Francesca Rotondo^{1,4}

5 Soon Gyu Hong^{2,4}

6 Tobin Peever³

7 Barry M. Pryor^{4*}

8 *¹ Department of Plant Pathology, The Ohio State University - OARDC, Wooster, OH*

9 *44691*

10 *² Korea Polar Research Institute, Korea Polar Research Institute, Incheon 406-840,*

11 *Republic of Korea*

12 *³ Department of Plant Pathology, Washington State University, Pullman, WA 99164*

13 *⁴ School of Plant Sciences, College of Agriculture, University of Arizona, Tucson, Arizona*

14 *85721, USA*

15

16 *Corresponding author

17 Name: Barry M. Pryor

18 Address: Division of Plant Pathology and Microbiology, Department of Plant Sciences,

19 College of Agriculture, Forbes 204, P.O. Box 210036, Tucson, AZ 85721, USA

20 Telephone: (520) 626-5312

21 Fax: (520) 621-9290

22 E-mail: bmpryor@ag.arizona.edu

23

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25 **desert environments in Arizona.**

26

27 **Abstract**

28 This study examined the genetic diversity of small-spored *Alternaria* species in the
29 southwest desert of the USA by sampling 552 isolates from different habitats (soil and
30 plant debris) in different locations (urban and an undisturbed desert). To estimate the
31 genetic diversity, Amplified Fragment Length Polymorphism (AFLP) fingerprinting
32 analysis was performed for all isolates. Strains representative of the sampled genotypic
33 diversity (n=125) were further characterized according their sporulation pattern and the
34 capability to produce allergens. Morphological characterization assigned the majority of
35 the strains to the *A. alternata* and *A. tenuissima* morpho-groups with only two isolates
36 assigned to the *A. arborescens* morpho-group. AFLP fingerprinting differentiated the *A.*
37 *arborescens* morpho-groups, but could not distinguish between the *A. alternata* and *A.*
38 *tenuissima* morpho-groups. Western blot analysis showed that a large number of
39 allergenic proteins were produced by strains. These proteins were not specific for any
40 morpho-group nor source of isolation. A hierarchical analysis of molecular variance was
41 performed on the AFLP data to quantify molecular variation and partition this variation
42 among sampled locations and habitat. No statistically significant differentiation among
43 locations and habitat was detected indicating a lack of population structure across
44 environments.

45 **Key words:** *Alternaria*, *allergens*, *AFLP*, *desert*

46 **Introduction**

47 Over the last twenty years, the percentage of the population affected by inhalant allergies
48 has increased around the world, particularly noted in industrialized countries (Kobayashi
49 *et al.* 2009; Wang *et al.* 2015; Vianello *et al.* 2016). World Allergy Organization (WAO)
50 White Book on Allergy (Pawankar *et al.* 2013), confirms that the incidence of asthma and
51 rhinitis is increasing across the globe, and that this increase is in both industrialized and
52 developing countries (Pawankar *et al.* 2013). According to the World Health
53 Organization, the number of persons suffering from asthma is projected to reach 400
54 million by 2025 (Pawankar 2014).

55 A wide variety of fungi such as *Alternaria*, *Aspergillus*, *Cladosporium*, and
56 *Penicillium* have demonstrated the ability to induce allergic symptoms and to sensitize
57 patients (Esch *et al.* 1993; Bush & Prochnau 2004). Most significantly, sensitization to
58 *Alternaria alternata*, a cosmopolitan mold, is a significant risk factor for asthma
59 development in both adults and children, especially those raised in a desert environment
60 (Halonen *et al.* 1997; Salo *et al.* 2006). In a nationwide epidemiological study, Gergen
61 and collaborators (Gergen *et al.* 1997) demonstrated that 3.6% of the general population
62 of the United States was sensitized to *A. alternata*. Sensitivity and exposure to airborne
63 *Alternaria* spores have been associated with respiratory arrest and severe episodes of
64 potentially fatal asthma (O'Hollaren *et al.* 1991; Gergen *et al.* 1997; Neukirch *et al.*
65 1999; Black *et al.* 2000; Sanchez & Bush 2004; Denning *et al.* 2006). While generally
66 considered as outdoor allergens (Perzanowski *et al.* 1998; Corden & Millington 2001;
67 Bush & Prochnau 2004), sensitized populations are at risk even while occupying indoor
68 environments (Licorish *et al.* 1985; Li & Kendrick 1995; Flannigan 2001, Bush &
69 Prochnau 2004; de Ana *et al.* 2006).

70 Multiple allergens are produced by *Alternaria* species. Burge *et al.* (1989)
71 demonstrated a high diversity of allergenic proteins among strains of *A. alternata* through
72 the use of immunodetection methods. Comparing strains from diverse geographic areas,
73 Burge *et al.* (1989) highlighted that variability in allergen production was influenced both
74 by strain and culture conditions. The glycoprotein fraction Alt-1 produced by *A.*
75 *alternata* (strain ATCC 6663) was the first allergen compound to be characterized and its
76 unique molecular structure was elucidated using crystallographic analysis (Yunginger *et*
77 *al.* 1980; Chruszcz *et al.* 2012). Subsequent studies were able to isolate the major
78 allergenic component Alt a 1 (Portnoy *et al.* 1990; Deards & Montague 1991; Paris *et al.*
79 1992; Mathiensen *et al.* 1992), as well as other minor allergenic components (Bush &
80 Sanchez 1993; Achtaz *et al.* 1995; De Vogue *et al.* 1996). It was shown that all species
81 of *Alternaria* tested, as well as many species in related genera, carried sequences
82 homologous to the gene encoding the major allergen, *Alt a1*, suggesting it has primary
83 metabolic function (Cramer & Lawrence 2003; Hong *et al.* 2005). However, the
84 biological role of *Alt a1* in the life history of *Alternaria* spp. is unknown, as is the
85 function of the majority of these fungal allergenic products. Recent works suggest that
86 Alt a 1 plays an important role in phytopathogenesis (Gomez-Casado *et al.* 2014;
87 Garrido-Arandia *et al.* 2014)

88 Characterization of *Alternaria* species has historically been based on
89 morphological features of the conidia and sporulation apparatus. Simmons (2007)
90 published an identification manual, intended to cover the entire genus with detailed
91 descriptions, drawings, culture, and isolation media. These characters have been used to
92 delineate morphologically-defined species-groups typified by representative *Alternaria*

93 species (Pryor & Michailides 2002; Andrew *et al.* 2009; Rotondo *et al.* 2012; Lawrence
94 *et al.* 2013). Subsequent phylogenetic analyses of molecular data have shown how these
95 morphological groupings encompass distinct phylogenetic lineages or sections (Peever *et*
96 *al.* 2005; Hong *et al.* 2006; Andrew *et al.* 2009; Rotondo *et al.* 2012; Lawrence *et al.*
97 2013). However, little is known about genetic variation below the species level within
98 these lineages, partly due to difficulty in obtaining unambiguous identification of closely
99 related species and strains by classical criteria (Breitenbach & Simon-Nobbe 2002).

100 Population genetics allows researchers to infer the evolutionary process and the
101 mechanisms that determine the genetic diversity within and among natural populations
102 (Hedrick 1985; Hartl & Clark 1997; Milgroom & Peever 2003). In the last two decades,
103 molecular techniques have allowed a broader characterization of the population structure
104 of many plant pathogens, including *Alternaria*, and allowing discrimination of closely
105 related species and an examination of diversity at the sub-species level (Bock *et al.* 2002;
106 Peever *et al.* 2002). Within the *Alternaria* citrus pathosystems, analyses of population
107 structure suggested that there are several independently evolving *Alternaria* lineages
108 infecting different citrus cultivars (Peever *et al.* 1999; Peever *et al.* 2000). These results
109 have subsequently been confirmed with phylogenetic and network analyses (Peever *et al.*
110 2002; Stewart *et al.* 2013). Likewise, phylogenetic analyses of *A. solani* sampled from
111 potato and tomato in Brazil similarly identified several independently evolving
112 phylogenetic lineages of the pathogen that caused similar disease symptoms (Lourenco *et*
113 *al.* 2009). However, there is still considerable ambiguity concerning the species
114 boundaries among some closely related species (Stewart *et al.* 2014). This taxonomic
115 deficiency has a direct impact on the ability of medical personnel to accurately assess

116 extrinsic factors contributing to asthmatic conditions and provide therapies toward
117 disease management.

118 The objective of this study was to characterize genetic and allergenic diversity of
119 *Alternaria* spp. sampled from two different habitats, soil and plant debris, and different
120 location, urban and desert areas, of the desert of the southwest USA. Isolates were also
121 classified morphologically and correlated with genetic diversity revealed by neutral
122 AFLP markers and immunological studies. Diversity was compared between samples
123 from undisturbed desert areas and human inhabited urban desert areas. Further
124 comparison was made among samples from soil and plant debris in each area. Another
125 fundamental aspect of this work was to compare the characteristics of the sampled *A.*
126 *alternata* with those of typical *A. alternata* isolates from which commercial allergen
127 preparations are made for clinical sensitization testing.

128

129 **Material and methods**

130 **Sampling protocol.**

131 Soil and plant debris were sampled from 6 locations in native Sonoran desert
132 ecosystems and 23 urban locations within and surrounding the city of Tucson, AZ. For
133 each native desert site, sample plot delimitation was conducted according to native
134 habitat sampling protocol described in Stock *et al.* (1999). Briefly, at each site, three
135 sample plots (20 m²) were established 100 m apart and samples were collected from two
136 different habitats (desert soil and decaying vegetation). The same protocol was applied to
137 sample the urban landscape sites, with the exception of the distance between plots as
138 some sites did not permit a distance of 100 m.

139 Isolation of *Alternaria* spp.

140 Plant debris and soil samples were processed following the method described in
141 Pryor & Michailides (2002) with a few modifications. Briefly, 4 g of each sample were
142 added to 40 mL of 0.1% agar solution (1/10 dilution w/v) and mixed on an orbital shaker
143 for 1 hr at 180 rpm. Serial dilutions (1/10 and 1/100) were obtained and 200 µl of each
144 dilution were inoculated on acidified potato dextrose agar (APDA) supplemented with
145 0.25 mL of Mertect 340-F (Syngenta, Basel, Switzerland) (Hong & Pryor, 2004). Plates
146 were incubated for 7 days at 25°C under fluorescent light. Fungal colonies were
147 examined using a stereo-microscope at 40x magnification (Olympus, SZX12) and
148 *Alternaria* spp. were transferred to acidified weak potato dextrose agar (AWPDA, Pryor
149 & Michailides 2002) supplemented with 0.2 g/L the fungicide Bayleton WP (Miles, Inc,
150 Kansas City, MO). When contaminating fungi were encountered, *Alternaria* colonies
151 were further subcultured to AWPDA supplemented with 5 g/L of the fungicide Botran
152 75WP (Gowan Company, Yuma, AZ). The modified APDA and AWPDA efficiently
153 promoted the selective growth *Alternaria* species from soil and plant debris samples
154 while suppressing the growth of bacteria and other contaminant fungi. Once pure
155 cultures were obtained from the positive *Alternaria* isolates, single-spored cultures were
156 obtained and stored on sterilized silica gel at 4°C.

157 DNA extraction and AFLP analysis

158 For DNA extraction and genetic analysis, *Alternaria* isolates were grown on
159 potato dextrose agar (Difco Laboratories, Detroit, MI), covered with sterile cellophane
160 film, at 23°C for one week. A subset of isolates, representative of all haplotypes
161 recovered in the Tucson area, was selected for further analysis. Also included in the

162 analysis were the three strains of *Alternaria* commonly used in comparative studies and
163 representing three distinct sporulation morphologies; the *alternata* group (EGS 34-016,
164 ex-epitype of *A. alternata*), the *tenuissima* group (EGS 35-015, commonly used as a
165 surrogate for the type of *A. tenuissima*), and the *arborescens* group (EGS 39-128, ex-type
166 of *A. arborescens*). (Pryor & Michailides 2002; Rotondo *et al.* 2012; Simmons 2007;
167 Woudenberg *et al.* 2015). Also included was an isolate commonly used in the
168 preparation of allergen extracts used in sensitization testing, ATCC 1168 (American Type
169 Culture Collection, Manassas, VA).

170 Mycelia were collected in 1.5 mL microfuge tubes by gently scraping the plate
171 with a sterile spatula. DNA extraction was performed following the method described in
172 Hong & Jung (2004). Amplified Fragment Length Polymorphism (AFLP) was
173 performed using modifications of the method of Vos *et al.* (1995) using a fluorescence-
174 labelled primer as described in Hong *et al.* (2006). Selective PCR was carried out
175 utilizing the single primer combination Eco-AG/Mse-GC. All PCR were conducted
176 using a PTC-100 thermal cycler (Bio-Rad, Waltham, MA). Two independent AFLP
177 reactions were performed for each isolate to assess repeatability of fingerprint patterns.
178 PCR products were analyzed by a Perkin-Elmer 3100 sequencing machine (Applied
179 Biosystems, Foster City, CA) with the size marker, GeneScan-500 (ROX; Applied
180 Biosystems). AFLP profiles were read using automatic peak filtering and alignment
181 performed with GelCompareII software (Applied Math, Kortrijk, Belgium). AFLP
182 profiles were used to estimate a distance tree using an Unweighted Pair Group Method
183 with Arithmetic Mean (UPGMA) algorithm based upon curve-based Pearson correlation
184 coefficient. Manual peak filtering and alignment were conducted in three steps as

185 described by Hong *et al.* (2006). Only those peaks that showed consistent and
186 reproducible amplification between replicate AFLP reactions were scored. The profiles
187 of 125 haplotypes, representative of the genetic diversity observed, were interpreted with
188 a binary score, where 1 indicates the presence of the band and 0 the absence. Cluster
189 analysis was performed with an UPGMA algorithm based on the Dice's coefficient using
190 the software PAST v. 1.63. Bootstrap support for phenogram nodes was estimated using
191 1000 pseudoreplicated datasets (Hammer *et al.* 2001).

192 Population structure among sampled isolates was estimated by partitioning AFLP
193 variation by geographical location (undisturbed desert vs. urban area) or habitat (plant
194 debris vs. soil). Hierarchical analysis of molecular variance (AMOVA) nested within the
195 locations or within the habitats, implemented in GenAlEx v. 6.5 (Peakall & Smouse
196 2006), were performed to estimate genetic differentiation among location or habitat
197 (PhiRT), among populations with the same habitat or location (PhiPR), and within
198 populations (PhiPT). The significance of each variance partition was determined by a
199 random permutation test (Peakall & Smouse 2006).

200 **Morphological characterization**

201 Three dimensional sporulation patterns of the 125 haplotypes, representative of the
202 genetic variability assessed with the AFLP fingerprinting, were analyzed for following
203 the guidelines of previous studies (Pryor & Michailides 2002; Hong *et al.* 2006; Rotondo
204 *et al.* 2012). Fungi were cultured on weak potato dextrose agar [WPDA (Pryor &
205 Michailides 2002)] under strictly defined incubation conditions (Hong *et al.* 2006) and
206 the three dimensional sporulation apparatus was observed using a stereo microscope at
207 40x magnification. Characters scored included the number of spores in a chain,

208 frequency of branching, and secondary conidiophore proliferation from apical cell or
209 median cell of conidia (Rotondo *et al.* 2012). Included in this study were the three strains
210 of *Alternaria* commonly used in comparative studies and representing three distinct
211 sporulation morphologies; the *alternata* group with EGS 34-016, the *tenuissima* group
212 with EGS 35-015, and the *arborescens* group with EGS 39-128 (Pryor & Michailides
213 2002; Rotondo *et al.* 2012; Simmons 2007; Woudenberg *et al.* 2015). All isolates were
214 assigned into one of the three morphological groups according to their similarity with the
215 reference strains used. If isolates exhibited sporulation characteristics of both *A.*
216 *alternata* and *A. tenuissima* (mostly long unbranched chains >6 conidia in length but with
217 increasing numbers of secondary and tertiary chains forming) they were assigned as
218 intermediates, Aa/At intermediate if they resembled more *A. alternata* (secondary and
219 tertiary chain proliferation common), or as At/Aa intermediates if they resembled more *A.*
220 *tenuissima* (secondary chain proliferation infrequent, tertiary proliferation rare).

221 **Protein extraction, IEF gels, and Western blotting**

222 Profiles of allergenic proteins were examined for the subset of Tucson isolates
223 and four reference strains. *Alternaria* isolates were grown on V8 juice agar (Simmons
224 1992) at 25°C for 10 days with a photoperiod of 10/14 hours light/dark. Fungal mycelia
225 were harvested by gently scraping the agar surface with a spatula and then ground in
226 liquid nitrogen. The powder obtained was transferred to 5 ml Coca's solution (0.3%
227 NaHCO₃, 0.9% NaCl, and 0.5% phenol) and shaken at 200 rpm for 24 hrs. Cell debris
228 was spun down by centrifugation at 16,000 g for 15 min. Supernatants were dialyzed
229 against 20 mM ammonium bicarbonate buffer for 48 hr and concentrated by dialyzing
230 against 10% polyethylene glycol (PEG) 20,000 solution buffered with 20 mM ammonium

231 bicarbonate for 24 hr using dialysis tubing (Spectra/Por MWCO: 3,500). Proteins were
232 resuspended in 200 μ L of protein dissolving buffer [25 mM Tris-HCl (pH 7.5) and 0.1%
233 (w/v) CHAPS] and stored at -80°C until needed. Protein concentration was determined
234 by Bradford's method using the Coomassie Protein Assay Kit (Pierce, Rockford, IL).

235 Proteins were resolved by isoelectrofocusing (IEF) gel electrophoresis using
236 Model III Mini IEF Cell (Bio-Rad, Hercules, CA). Polyacrylamide IEF gels [acrylamide
237 monomer (5% T, 0.6% C), 5% glycerol, and 2% ampholyte (pH 3-10)] were cast on the
238 surface of glass plates. Two microliters of protein samples (5 g/ μ L) were loaded on the
239 gel using a loading template. Gels were run for 20 min at 100 V, 20 min at 200 V, and 1
240 hr at 450 V. Proteins were transferred to Hybond-P membrane (GE Healthcare,
241 Waukesha, WI) using a trans-blot SD semi-dry electrophoretic transfer cell (Bio-Rad,
242 Hercules, CA) and 0.7% acetic acid as the transfer buffer. Allergens were detected by
243 ECL plus Western Blotting Detection Reagents (GE Healthcare, Waukesha, WI), 1:20
244 diluted composite human serum from 50 patients with reported asthma by age 5 yr, and
245 1:10,000 diluted HRP-labeled goat anti-human IgE antibodies (Antibodies Inc., Davis,
246 CA or KPL, Gaithersburg, MD) as secondary antibodies following manufacturer's
247 directions. The resulting allergen profiles were scored as a presence/absence binary
248 matrix and data were analyzed according an UPGMA algorithm based on Dice's
249 coefficient using the software PAST v. 1.63.

250

251 **Results**

252 **Isolation of *Alternaria* spp**

253 A total of 552 *Alternaria* spp. isolates were sampled. Of these, 126 were obtained from
254 plant debris collected in desert areas, 50 from soil samples collected in desert areas, 243
255 from plant debris collected in urban areas, and 133 from soil samples collected in urban
256 areas. Single-conidial isolates were established from each isolate to minimize the
257 potential for isolation of multiple genotypes and prevent contamination by genetically
258 heterogeneous *Alternaria* species or other fungal species. Isolates were preserved in
259 sterilized silica gel for long term storage.

260 **AFLP analysis**

261 Based upon AFLP fingerprinting, a subset of 125 strains, representative of the genetic
262 diversity sampled, was selected. For this subset of strains, the final filtering and
263 alignment of peaks resulted in 11 to 31 peaks per isolate. Of a total of 81 peaks, 26 were
264 unique to single strains, and 1 was shared by all strains. Twenty peaks were shared by
265 more than 50 strains and 8 peaks were shared by more than 100 strains. The cluster
266 analysis based on the Dice's coefficient clearly discriminated the *A. arborescens*
267 morphological group as a well-supported cluster with a bootstrap support of 100 (Fig.1).
268 No other significant clusters, on the basis of the geographical location (desert vs. urban)
269 or source of sampling (plant debris vs. soil), were inferred.

270 AMOVA revealed that the majority of genetic variation (94%) was highly
271 localized within populations defined by location and habitat of isolation (Table 2). Low
272 genetic differentiation was found among locations and this differentiation was significant.
273 No significant genetic differentiation was detected among habitats.

274 **Morphological characterization**

275 Characterization of sporulation pattern showed that the majority of the 125 strains
276 belonged to the *A. alternata* and *A. tenuissima* morpho-groups. Only two strains
277 (BMP1089 and BMP1616 isolated from desert plant debris and urban soil, respectively)
278 were characterized as members of the *A. arborescens* morpho-group. On the basis of the
279 comparison with the *A. alternata* and *A. tenuissima* reference strains EGS34-016 and
280 EGS 34-015, respectively, only a few isolates exhibited the typical sporulation pattern of
281 each species described by Simmons (1992) and by Pryor & Michailides (2002). Of the
282 125 isolates analyzed, twenty-seven isolates were assigned to the *alternata* morpho-group
283 and seventeen were assigned to the *tenuissima* morpho-group. The remaining isolates had
284 an intermediate branching pattern and were classified as Aa/At intermediates. No
285 obvious association was found between morpho-group assignment and geographical
286 location (desert vs. urban) or habitat (plant debris vs. soil).

287

288 **Immunological variation**

289 Western blot analysis revealed the presence of a total of twenty-four unique
290 bands, nine of which were common to approximately 70% of the strains. The number of
291 bands ranged from 4 to 18 per isolate, but most of the isolates were characterized by at
292 least 8 bands. While *A. alternata* isolate ATCC 11680 clustered closely with other
293 sampled isolates, it had a distinct profile, (Fig. 2). The cluster analysis based on allergen
294 bands did not separate the *A. arborescens* isolates as a defined group and no other
295 clusters were observed on the basis of the location and or habitat of isolation (Fig. 3).

296

297 **Discussion**

298 Previous studies of allergies associated with *Alternaria* spp. have focused on the
299 characterization of the major allergens produced by *Alternaria alternata* isolates from
300 different locations (Deards & Montague 1991; Achatz *et al.* 1995; Kurup & Banargee
301 2000; Bush & Prochnau 2004). This study is the first to attempt to characterize a large
302 number of *Alternaria* isolates collected from different locations and from different
303 habitats, based on their ability to produce allergenic proteins and their underlying genetic
304 diversity. Interestingly, our morphological characterization revealed only two isolates
305 displaying typical traits of the *A. arborescens* morphological group. This was concordant
306 with the output of the cluster analysis of the AFLP profiles that resolved these two
307 isolates as a genetically distinct cluster. In the case of plant pathosystems, previous
308 research has highlighted how both species *A. alternata* and *A. arborescens* can be
309 predominantly associated with a specific disease (Serdani *et al.* 2002; Peever *et al.* 2005;
310 Harteveld *et al.* 2014; Rotondo *et al.* 2012). The rarity *A. arborescens* suggests that this
311 species is uncommon in the desert habitats and is likely to be less significant in the
312 production of allergic inhalant disease for populations in this area. However, the allergen
313 profiles were similar to those produced by the *alternata* and *tenuissima* morpho-groups.

314 Population genetic studies of *Alternaria* species associated with plant disease
315 have shown that distinct lineages are associated with each host, resulting in host-
316 pathogen specificity (Akimitsu *et al.* 2003; Lourenco *et al.* 2009). In this study, even if
317 significant, genetic differentiation related to sampling location was low with high genetic
318 variability within both location and habitat. Thus, the hypothesis that different

319 environments support distinct *Alternaria* populations has to be rejected in favor of a more
320 global regional population or populations not captured in this study. The heterogeneity
321 found is likely related to the fact that the genus *Alternaria* encompasses different species
322 of saprophytes that are commonly found in soil or on decaying plant tissues (Thomma
323 2003).

324 Although morphological traits may be used to identify distinctive morphological
325 groups, the high variability observed in the 3-dimensional sporulation pattern made the
326 morphological traits unsuitable to group the isolates in well-defined clusters within the *A.*
327 *alternata* morpho-group and *A. tenuissima* morpho-group. Previous systematic studies
328 encountered analogous difficulties in defining the boundaries of species within this group
329 of fungi, suggesting that all stable characters, both molecular and morphological, should
330 be utilized to obtain a consistent identification (Pryor & Gilbertson 2002; Pryor &
331 Bigelow 2003; Hong *et al.* 2006; Andrew *et al.* 2009; Rotondo *et al.* 2012; Lawrence *et*
332 *al.* 2013).

333 The western blot analysis revealed that all isolates were able to produce a large
334 number of allergenic compounds with some shared among isolates and others unique to
335 particular isolates. In contrast to the AFLP genotyping data and the morphological
336 characterization, cluster analysis of allergen profiles did not differentiate *A. arborescens*
337 isolates from *A. alternata/A. tenuissima* isolates. This suggests that the production of
338 allergenic proteins is not a taxon-specific character in contrast with previous reports of
339 secondary metabolites tracking phylogenetic relationships among *Alternaria* species
340 (Andersen *et al.* 2008).

341 The high variability detected from the allergen profile of *Alternaria* strains
342 suggests that the production of allergenic compounds is related to the characteristics of
343 individual spores and mycelial fragments, not fungal species. In fact, studies on
344 aerosolized fungal fragments showed how the high concentration of this particle is tightly
345 associated with allergenic reaction and manifestation of severe asthmatic episodes (Li &
346 Kendrick 1995; Li & Kendrick 1996). The association of allergens with fungal cell walls
347 is supported by Mitakakis *et al.* (2002), who speculate that the Alt a 1 protein is involved
348 in spore germination. Further support is given by the Twaroch and colleagues' findings
349 (2012), which show the Alt a 1 protein is localized exclusively in the cell wall of
350 *Alternaria* species spores, which mediate the allergic reactions once inhaled.
351 Interestingly, Lizaso *et al.* (2006) found that the culture filtrate from *Alternaria* cultures
352 to be the most adequate source of allergens for establishing an in-house reference (IHR)
353 preparation for *A. alternata* allergen extract manufacturing, suggesting the protein is
354 secreted in some way. Subsequent studies have led to the isolation and characterization
355 of additional allergenic components and similarly their functions are under investigation
356 (Budd *et al.* 1983; Portnoy *et al.* 1990; Paris *et al.* 1991 Deards & Montague 1991; Bush
357 & Sanchez 1993; Breintenbach *et al.* 1995).

358 Immunotherapy for IgE mediated allergic disease has been proven successful in
359 case of asthma and rhinitis (Higgins & Looney 2004). Fungal sensitivity is well
360 recognized, but the wide range of allergens produced by different strains can make the
361 recognition of sensitized patients less accurate, especially considering the practice of
362 using only one strain to prepare allergenic extract for skin tests. Despite the significant
363 progress in allergen standardization, it still remains difficult to assess the effective dose

364 and composition for a successful immunotherapy (Nelson 2009). In the present study, we
365 show how *Alternaria* isolates investigated share some allergen proteins with the strains
366 ATCC 11680, but they also were characterized by unique allergen proteins. Clarifying
367 the role of specific allergens is a fundamental step to determine the exact relationship of
368 allergenic response and development of asthma. This study highlights the high genetic
369 and allergenic variability within *Alternaria* isolates even within a defined region and
370 suggests the potential for commercial development of region-specific *Alternaria* allergen
371 preparations for use in standardized tests that determine allergen sensitization in children.

372

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380

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664 **Figure captions**

665 **Fig. 1.** Distance phenogram estimated among 125 haplotypes, based on UPGMA
666 clustering of genetic distances generated from the AFLP data. Bootstrap values greater
667 than 70% are indicated at each node. Location and habitat of isolation are indicated as
668 follow: DPD: desert plant debris, DS: desert soil, UPD: urban plant debris, US: urban
669 soil.

670

671 **Fig. 2.** Detection of allergenic proteins by Western analysis with human serum pools
672 from 50 patients with reported asthma since 5 years age. Proteins were resolved by
673 isoelectrofocusing (IEF) gel electrophoresis. 1-13) *A. alternata* ATCC 11680; *A.*
674 *alternata* EGS 34-016; *A. arborescens* EGS 39-128; *A. tenuissima* EGS 34-015, BMP
675 1073; BMP 1117; BMP 1118; BMP 1119; BMP 1125; BMP 1176; BMP 1179; BMP
676 1200; BMP 1229.

677

678 **Fig. 3.** Distance phenogram estimated among 125 haplotypes, based on UPGMA
679 clustering of genetic distances generated from the allergen data. Location and habitat of
680 isolation are indicated as follow: DPD: desert plant debris, DS: desert soil, UPD: urban
681 plant debris, US: urban soil.
682