1 *Alternaria* in malting barley: characterization and distribution in relation with climatic
2 conditions and barley cultivars

3 Eliana Castañares[(a)], Lucía da Cruz Cabral[(b,1)], Maria I. Dinolfo[(a)], Birgitte Andersen[(c,2)],
4 Sebastián A. Stenglein[(a)], Andrea Patriarca [(d)*]
5 [(a)] Laboratorio de Biología Funcional y Biotecnología (BIOLAB)-INBIOTEC-CONICET-
6 CICBA, Facultad de Agronomía, UNCPBA. Azul (7300), Buenos Aires, Argentina.
7 [(b)] CONICET - Universidad de Buenos Aires. Instituto de Ecología, Genética y
8 Evolución de Buenos Aires (IEGEBA). Ciudad Autónoma de Buenos Aires, Argentina
9 [(c)] Department of Biotechnology and Biomedicine, Technical University of Denmark,
10 DK-2800 Kgs. Lyngby, Denmark.
11 [(d)] Universidad de Buenos Aires, Facultad de Ciencias Exactas y Naturales,
12 Departamento de Química Orgánica, Laboratorio de Microbiología de Alimentos.
13 CONICET, Instituto de Micología y Botánica (INMIBO), Buenos Aires, Argentina.
14
15 [(1)] Present address: Universidad Tecnológica Nacional, Facultad Regional Chubut
16 (UTN-FRCh), Av. del Trabajo 1536, Puerto Madryn, Chubut, Argentina.
17 [(2)] Present address: Department of the Built Environment, Aalborg University, A.C.
18 Meyers Vænge 15, DK-2450 København SV, Denmark.
19
20 *Corresponding author: andreap@qo.fcen.uba.ar

ABSTRACT

Alternaria is one of the main fungal genera affecting the quality of barley grains. In this study, a polyphasic approach was carried out to characterise the *Alternaria* population infecting different cultivars of barley grains from the major producing regions of Argentina in the 2014 and 2015 seasons. Its relationship with *Fusarium* and correlations between predominant species, barley cultivars, and climatic conditions in the growing regions were evaluated.

Alternaria incidence exceeded that of *Fusarium* in all the barley samples and was higher in the drier season (2015). All the *Alternaria* sp.-grps. identified were present in both growing seasons (2014 and 2015), and their frequency was similar in both years. The dominant *Alternaria* species-group isolated and identified based on morphological characteristics, DNA sequencing, and metabolite profile was *A. tenuissima* (72.9 %), followed by *A. infectoria* (14.6 %). An association between their frequency and field temperature was observed; *A. tenuissima* sp.-grp. was more frequent in northern localities, where higher temperatures were registered, while the opposite was observed for *A. infectoria* sp.-grp. A smaller percentage of *A. arborescens* sp.-grp. (5 %), *A. alternata* sp.-grp. (3.9 %) and *A. vaccinii* (1.4 %) were also identified.

Both secondary metabolite profiles and phylogenetic analysis were useful to distinguish isolates from *Alternaria* section *Alternaria* and section *Infectoriae*. Regarding metabolite profiles, alternariol was the most frequent compound produced by isolates of the section *Alternaria*. Infectopyrones and novae-zelandins were produced by most of the isolates from section *Infectoriae*.

The barley cultivars analysed in this study did not show a particular susceptibility regarding the *Alternaria* population composition, except for Andreia, which presented the highest frequency of contamination with *A. tenuissima* sp.-grp. The rest of the cultivars, when grown in different regions, showed different proportion of the *Alternaria* sp.-grps., suggesting that other factors were determinant in their distribution.

The results obtained in the present study will be a valuable tool for health authorities to assess the need for regulations on *Alternaria* mycotoxins, given the high incidence of *Alternaria* spp. in barley and the diversity of metabolites that might contaminate the grains.

Keywords: barley, *Alternaria*, metabolite profiles, geographic distribution, fungal interactions

1. Introduction

Alternaria is a fungal genus with worldwide distribution due to its diverse role in nature, including saprophytic, endophytic and pathogenic species with the ability to adapt to diverse environmental conditions (Thomma, 2003). Its occurrence has been reported in several fruits such as tomato, apple, nut, and blueberry, among others (Andersen et al., 2015; Ntasiou et al., 2015; Prelle et al., 2013), and is one of the main causes of disease in extensive crops such as wheat, barley, and sorghum (Deshpande, 2002). The production of barley has increased lately in Argentina, mainly due to the rising demand from the beer industry (FAOSTAT, 2021). Barley heads are susceptible to *Alternaria* infection, resulting in yield loss and mycotoxin accumulation in grains, which have negative effects during the malting process, affecting the quality of the final products (Bauer et al., 2016; Beccari et al., 2016; Jedidi et al., 2018; Justé et al., 2011). Besides *Alternaria,* the fungal community colonizing malting barley also includes the genera *Fusarium*, *Claviceps*, *Penicillium* and *Aspergillus* (Beccari et al., 2018; Jedidi et al., 2018; Medina et al., 2006). *Alternaria* and *Fusarium* are considered field fungi, and weather conditions, such as temperature and humidity along different geographical areas, could influence their distribution and affect fungal interactions (Magan et al., 2010; Magan and Lacey, 1984). Several studies have detected a negative correlation between *Fusarium* and *Alternaria* in the field, which can be related to climatic conditions during the growing season (Andersen et al., 1996; González et al., 2008; Schiro et al., 2018). However, the factors determining the prevalence of one or another have not been thoroughly elucidated.

Alternaria contamination in crops is also relevant because of their ability to produce a wide range of mycotoxins, namely alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), altertoxins I, II, III (ATX-I, -II, -III) and tenuazonic acid (TeA), which have genotoxic, mutagenic and carcinogenic properties (Ostry, 2008). Other *Alternaria* secondary metabolites are infectopyrones, phomenins and altertoxin-like metabolites, produced mainly by members of the *A. infectoria* species-group, whose

toxicity has not been fully investigated yet (Andersen et al., 2009, 2015). Although currently there are not worldwide regulations establishing limits for these toxins in food and feed, the European Food Safety Authority (EFSA) has raised concern about *Alternaria* mycotoxins for public health (EFSA, 2011, 2016).

For a long time, the classification of the genus *Alternaria* had been based exclusively on morphological traits following the taxonomic key proposed by Simmons (2007), who introduced the concept of “species-groups” to facilitate identification. With the advances of molecular techniques, the analyses of sequences of conserved regions, such as internal transcribed spacer (ITS), calmodulin, *Alternaria* major allergen (*Alt* a1), glyceraldehydes-3-phospate dehydrogenase (*gpd*), endopolygalacturonase (*endoPG*), plasma membrane ATPase, and an anonymous genomic region OPA, have been used for the identification of *Alternaria*. However, many of them produced incongruent results and did not always correlate with the morphological characterization (da Cruz Cabral et al., 2017; Gherbawy et al., 2018; Lawrence et al., 2014; Siciliano et al., 2018). In an attempt to organize the genus, phylogenetic studies have proposed changes in the taxonomy by elevating 26 clades to the status of section (Lawrence et al., 2016). Thus, the *Alternaria* species commonly infecting cereal grains have been classified in two sections, section *Alternaria*, comprising *A. tenuissima*, *A. arborescens* and *A. alternata* species-groups, among others, and section *Infectoriae*, where the *A. infectoria* species-group was placed.

Given the current complexity of *Alternaria* taxonomy, traditional morphology, sequence analyses and secondary metabolite profiles are combined to achieve more accurate identification (Andersen et al., 2015; Armitage et al., 2015; da Cruz Cabral et al., 2017; Siciliano et al., 2018). The use of metabolite profiles has proved particularly effective to differentiate between isolates from sections *Alternaria* and *Infectoriae* since they have few metabolites in common (Andersen et al., 2002, 2015; Patriarca et al., 2019). Additionally, this type of analysis provides knowledge on the potential metabolomic capacity of a fungal population contaminating crops, which can be useful for monitoring

and establishing risk assessment strategies. Therefore, understanding the distribution of the small-spored *Alternaria* in barley growing regions, is the first step to determine the mycotoxin potential of the crop contaminants and the mycotoxin risk in the final produce. Moreover, factors, such as climatic conditions during the growing season or in different regions, as well as the differential susceptibility of barley cultivars could have a role in selecting the predominant fungal pathogen.

Thus, the aims of this study were 1) to determine the distribution of *Alternaria* spp. in barley grain samples from the main growing areas of Argentina and assessing its relationship with *Fusarium* in the field, 2) to characterise the small-spored *Alternaria* from barley grains by morphological traits, molecular techniques, and secondary metabolite profiles, and 3) to evaluate if a relationship exists between *Alternaria* incidence and climatic conditions or barley cultivars.

2. Materials and Methods

2.1. Sampling and meteorological conditions

The present study was conducted on 33 barley grain samples from six localities of the main producing regions of Argentina (Bordenave, Huanguelén, Miramar, 9 de Julio, Bigand, Paraná) and four barley cultivars (Andreia, Scrabble, Scarlett, Shakira) during two growing seasons, 2014 (15 samples) and 2015 (18 samples) (Table 1). Barley samples were supplied by the National Network of Brewery Barley of the National Institute of Agricultural Technology (INTA, Argentina). Barley grains were conventionally grown under zero-tillage practices, sprayed with foliar fungicide (Orquesta® Ultra; fluxapiroxad + pyraclostrobin + epoxiconazole) at flowering and harvested at physiological maturity (12% humidity). Once received, barley samples (1 Kg) were randomly reduced to 200 g with a grain divider and stored at 4 °C until analysis for a maximum of 15 days.

Meteorological data from the sampled localities (accumulated precipitation and average temperature) were obtained from the Information and Agrometeorological Management

System of INTA and used to characterise the differences between both growing seasons and the influence of climatic conditions on the distribution of the field fungi. Data were collected from flowering to harvest (October to December) and average temperature and accumulated precipitation during the whole period were calculated for each locality in both seasons.

2.2. Mycobiota analysis and morphological identification

Each sample was surfaced disinfected by washing with sodium hypochlorite 5 % and ethanol 70 %, subsequently, during 2 min and rinsed twice with sterilized distilled water. A total of 100 grains per sample were placed on potato dextrose agar (PDA) (Samson et al., 2010) with 0.05 g chloramphenicol/L, at a ratio of 10 grains per plate and incubated at 25 °C for 7 days. The resulting *Fusarium* and *Alternaria* colonies were enumerated and identified at genus level according to reference guides (Samson et al., 2010, Simmons, 2007). The incidence was calculated as the percentage of infected grains for each genus per sample.

Colonies potentially belonging to the *Alternaria* genus were grouped according to similar morphological characteristics. Afterward, a subset of isolates from each group was cultivated on plates containing Potato Carrot Agar (PCA) to obtain single spore cultures. Morphological identification was performed from these cultures by transferring them to PCA plates, inoculating at three equidistant points and incubating at 23 °C under an alternating cycle of 8 h of cool white fluorescent daylight and 16 h darkness. On the fifth day of growth, a rectangular block of agar of about 0.5 x 2.0 cm was removed from each plate to facilitate the observations of sporulation patterns. The colonies continued their growth for further 48 h under the same conditions.

At the 7^{th} day, the three-dimensional sporulation patterns of each isolate were examined directly from the plates on the cut surface using a microscope with 100x magnification. Further examination (branching types, conidial shapes, sizes, colour, and ornamentation, etc.) was done at 400x magnification using slide preparations with transparent adhesive tape on lactic acid. Macroscopic characteristics of the colonies

(colour, diameter, texture) were also recorded from the plates. Four representative strains were used for morphological comparison: *A. tenuissima* (EGS 34.015), *A. infectoria* (EGS 27.198), *A. alternata* (EGS 34.016), *A. arborescens* (EGS 39.128). All *Alternaria* isolates were deposited in the BIOLAB fungal collection.

2.3. DNA extraction and molecular identification

Molecular identification was performed by sequencing two different genomic regions, using specific primers. A subset of 26 isolates from sections *Alternaria* and *Infectoriae* was amplified using the primers ATPDF1/ATPDR1 (Lawrence et al., 2014) for ATPase gene. A second analysis was made by amplifying the anonymous noncoding region OPA10-2 with the primers OPA10-2R/OPA10-2L (Andrew et al., 2009) to differentiate isolates within the *Alternaria* section, since this non-coding region does not generate amplicons in *A. infectoria* isolates (Peever et al., 2004, 2005).

Genomic DNA was extracted from seven-day-old PDA colonies using cetyltrimethylamonium bromide (CTAB) method according to Stenglein and Ballati (2006). DNA quality was examined by electrophoresis in 0.8 % agarose gels with GelRed™ (Biotium, Hayward, USA) at 80 V in 1 X Trisborate-EDTA buffer and visualized under UV light. DNA concentration was calculated by fluorometry (Qubit™ Invitrogen, Argentina). Extracted DNA was stored at -20 °C until analysis.

Both PCR assays were carried out using 10-20 ng of genomic DNA in a total volume of 25 µL containing each of them 10 X reaction buffer, 0.5 mM $MgCl_2$, each primer pair (at 0.5 µM for ATPase gene and at 0.2 µM for OPA10-2 region), 200 µM of each dNTP (Genbiotech S.R.L., Argentina), 1.25 U of Taq DNA polymerase (Inbio-Highway, Argentina). DNA amplifications were performed in a XP Thermal cycler (Bioer Technology Co., China) following cycling conditions described by Lawrence et al. (2014) for ATPase gene and Andrew et al. (2009) for OPA 10-2 region. The effectiveness of the reaction was checked by electrophoresis in 1.5 % (w/v) agarose gels containing GelRed® at 80 V with Trisborate-EDTA buffer and visualized under UV light. PCR products were purified with the PureLink™ PCR Purification kit (Invitrogen,

195 Argentina) and sequenced in a BigDye Terminator v. 3.1 Cycle Sequencing Ready
196 Reaction kit (Applied Biosystems, USA) in a 3130 Hitachi Genetic Analyzer Sequencer
197 (ABI) by CERELA-CONICET Institute, Argentina. Sequences obtained in the present
198 work were deposited in GenBank database (see accession numbers in Table 2).
199 2.4. Secondary metabolite extraction and HPLC-UV analysis
200 A subset of 31 isolates was selected for secondary metabolite profiling. The extraction
201 was made according to Andersen et al. (2015). Briefly, 14 day-old cultures from
202 DRYES at 25 ºC in darkness were extracted by a micro-scale method with 1 mL of
203 ethyl acetate containing 1 % (v/v) formic acid. The extract was transferred to a clean 2
204 mL vial, evaporated to dryness with N_2 at room temperature and re-dissolved in 400 µL
205 methanol. The methanol extract was filtered through a 0.45 µm PTFE filter into a clean
206 2 mL vial and kept at -18 °C prior to analysis.
207 Analyses were performed on an Agilent 1100 HPLC system (Agilent, Waldbronn,
208 Germany) equipped with a diode array detector collecting two ultraviolet–visible (UV–
209 VIS) spectra per sec from 200 to 600 nm. Separations were performed on a 2 × 100
210 mm Luna 3 µm C18 column (Phenomenex, Torrance, CA, USA). The mobile phase
211 consisted of a linear water–acetonitrile gradient at a flow of 0.4 mL/min. The gradient
212 started at 15 % acetonitrile, reached 100 % in 20 min and was held for 5 min. Both
213 eluents contained 50 ppm trifluoroacetic acid. A homologous series of alkylphenones
214 was analysed as external retention time references and used to calculate a bracketed
215 retention index (RI) for each detected peak (Andersen et al., 2008, 2009). Each
216 metabolite was identified by its RI value and its UV–VIS spectrum by comparison with
217 those of standards.
218 2.5. Data treatment
219 Morphological data were analysed by exploratory statistical analysis using the software
220 INFOSTAT version 2012 (Grupo InfoStat, FCA, Universidad Nacional de Córdoba,
221 Argentina). Besides, due to the non-normal distribution of data, the Spearman

nonparametric correlation coefficient was used to evaluate the relationship between the variables of study.

For phylogenetic analysis, DNA sequences were edited using BioEdit v7.0.9.0 (Hall, 1999) and aligned with ClustalW (Thompson et al., 1994). Two different approaches were carried out to infer phylogenetic relationships among the sequences: Bayesian and maximum parsimony analyses. For Bayesian inference, the optimal model of nucleotide substitution was estimated with jModelTest2 (Darriba et al., 2012; Guindon and Gascuel, 2003) through CIPRES Science Gateway v.3.3 (Miller et al., 2010) on the bases of the Bayesian Information Criterion (BIC), which were TrN+G for ATPase gene sequences and K3P+I for OPA region sequences. Bayesian analyses were performed in MrBayes v3.2.3 (Ronquist, et al., 2012) using the Metropolis-coupled Markov chain Monte Carlo (MCMCMC) algorithm. Two independent analyses using four chains, one cold and three incrementally heated, were run using a random starting tree over 1,000,000 generations sampling every 500 generations. The average standard deviation of split frequencies stabilized to a difference of < 1 % and the software Tracer v1.6.0 (Rambaut et al., 2003–2013) were used to assess convergence of the cold chain. The initial 250,000 generations from each run were discarded as “burn-in” when summarizing tree parameters and topology, which was visualized with FigTree v1.4.2 (Rambaut, 2006–2014). Maximum parsimony analysis was performed under a traditional search in TNT v1.1 (Goloboff and Catalano, 2016; Goloboff et al., 2008). Equal weights and no additive characters were used, and gaps were treated as missing data. Before searches, all uninformative characters were deactivated. The analyses were done using Multiple TBR +TBR applied to a series of 1000 random addition sequences retaining 10 cladograms per replicate. Bootstrap values were calculated from 1000 replicates. Sequences used as outgroup for each region and reference strains downloaded from GenBank are depicted in Table 3.

To analyse metabolite data, a dendrogram was constructed with the 31 representative *Alternaria* isolates selected for this study and their production of 20 metabolites. The

250 presence/absence of a particular metabolite was scored as 1/0 in a binary matrix and
251 subjected to cluster analysis with the software NTSYS PC v. 2.0 (Rohlf, 1998) using
252 Jaccard coefficient and UPGMA algorithm.
253
254 3. Results
255 3.1. Meteorological conditions
256 The meteorological conditions in the whole growing region varied between both
257 seasons (Supplementary Table 1). The 2015 growing season was drier and slightly
258 colder; the accumulated precipitation decreased by 25.5% compared to the previous
259 year and the average temperature from flowering to harvest dropped 6.8 % with
260 respect to 2014.
261 The average temperature during the growing months was strongly related to
262 geographic location (Fig. 1). For both seasons, the highest temperatures were
263 recorded in the northern localities (Paraná, Bigand, 9 de Julio), while the southern ones
264 were consistently colder, with similar average temperatures in a range from 17.2 to
265 18.0 ºC. The accumulated precipitation did not follow a geographical pattern. Three of
266 the localities experienced high differences between both years. Bordenave and 9 de
267 Julio suffered droughts reducing the accumulated precipitation in 22 and 35 %,
268 respectively in 2015. Miramar was the locality where the highest decrease (79%) was
269 registered in 2015 when compared with the previous season. In average, the maximum
270 accumulated precipitation was recorded in the centre and southwest region of the
271 sampled area.
272
273 3.2. Mycobiota of barley grains
274 The prevalent fungal genus infecting barley grains in the main growing areas of
275 Argentina was *Alternaria*, which was found in 100 % of the samples analysed (Table 1).
276 Its incidence in malting barley samples (percentage of infected grains per sample)
277 varied between 11 and 64 %. The annual incidence (average incidence of samples

from the same growing season) was significantly higher in 2015 (42 %) than in 2014 (21 %).

The genus *Fusarium* was present in 85 % of the samples and in much lower incidence than *Alternaria* (between 1 and 19 % infected grains per sample). The average annual incidence was slightly higher in 2014 (6 %) than in 2015 (4 %), but no significant differences were observed between both growing seasons.

The relative distribution of both genera in the sampled localities differed in both years. In 2014, a weak negative correlation was observed between them, although it was not statistically significant ($r = 0.48$, $p = 0.07$) (Fig. S1). In 2015, the correlation between both genera followed a positive trend, although it also lacked statistical significance ($r = 0.41$, $p = 0.09$).

3.3. Characterization of *Alternaria* from malting barley

3.3.1. Morphological identification

A total of 280 *Alternaria* isolates were recovered from both healthy and damaged barley grains (either presenting black point or smaller and shrivelled grains). All strains were identified to sp.-grp. level, except for strains corresponding to *A. vaccinii*, whose characteristic three-dimensional pattern allowed identification to species level. Their distribution in each sample is shown in Table 1 and their main microscopic characteristics in Fig. 2.

A. tenuissima was the main sp.-grp. infecting malting barley grains in Argentina, with a total of 204 isolates (72.9 %) out of the 280 samples. The second most common was *A. infectoria* sp.-grp. with a total of 41 isolates (14.6 %). In minor proportion, isolates corresponding to *A. arborescens* sp.-grp. (14 isolates, 5 %), *A. alternata* sp.-grp. (11 isolates, 3.9 %), and *A. vaccinii* (4 isolates, 1.4 %) were identified. Additionally, six isolates (2.1 %) showed intermediate characteristics among the species-groups mentioned above and were referred to as *Alternaria* sp.

3.3.2. Phylogenetic analyses

306 Two genetic regions were used for the phylogenetic analyses of *Alternaria* isolates.
307 Amplicons generated with ATPase region varied in length from 1166 to 1200 bp.
308 Alignment length corresponding to 26 sequences of the *Alternaria* isolates from this
309 study, seven reference strains, and the outgroup was 1292 bp. Meanwhile,
310 amplification of OPA 10-2 region yielded amplicons of 634 bp. In this case, alignment
311 length from nine *Alternaria* isolates from this study, six reference strains, and one
312 outgroup was 562 bp. Both Bayesian and maximum parsimony analyses showed
313 similar topologies for each analysed region (Fig. 3, 4 and Fig. S2 and S3).
314 Phylogenetic trees obtained from ATPase analyses, which included sequences from
315 sections *Alternaria* and *Infectoriae*, yielded two main groups. Group I, contained all the
316 isolates belonging to Section *Infectoriae* (isolates identified as *A. infectoria* sp.-grp., the
317 corresponding reference strains, and the two isolates morphologically identified as *A.*
318 *vaccinii*). Group II included all isolates belonging to section *Alternaria* (*A. tenuissima*, *A.*
319 *alternata* and *A. arborescens* sp.-grps.), section *Embellisioides* (*A. proteae*), section
320 *Japonicae* (*A. japonica*) and the monotypic lineage *A. brassicae*, as well as the
321 reference strains *A. tenuissima*, *A. alternata* and *A. arborescens*. Within Group II, *A.*
322 *arborescens* isolates of this study grouped all together with the correspondent
323 reference strain (Fig. 3 and Fig. S2).
324 The OPA 10-2 region was analysed only for the isolates belonging to section
325 *Alternaria*. This analysis grouped in the same clade (I) all *A. arborescens* sp.-grp.
326 isolates from this study, the reference strains, and one *A. tenuissima* reference strain
327 as well. It was not possible to distinguish *A. tenuissima* from *A. alternata* sp.-grps.
328 isolates which were grouped all together with the reference strains in another cluster
329 (II) (Fig. 4 and Fig. S3).
330 3.3.3. Secondary metabolite profiles
331 A subsample of 31 *Alternaria* isolates from barley grains were selected for this
332 analysis. A total of 20 compounds were detected in the secondary metabolite profiles of
333 the *Alternaria* spp. from barley grains, with a production ranging from 1 to 9 compounds

by strain (Table 4). Twelve of the 20 metabolites were specific to isolates from the *A. tenuissima*, *A. alternata* and *A. arborescens* sp.-grps., all belonging to section *Alternaria* (Gannibal, 2016). Among them, AOH was the most frequently produced (9 isolates), followed by ALT (7 isolates), AME (6 isolates), and altenusin (6 isolates). Only two metabolites, altertoxin-I and alterperylenol, were shared by isolates from sections *Alternaria* and *Infectoriae*. The members of section *Infectoriae*, including the *A. vaccinii* isolates (Gannibal and Lawrence, 2016), showed similar metabolite profiles, producing 8 of the 20 detected compounds. The metabolite most frequently produced by them was infectopyrone (20 isolates), followed by novae-zelandin B (16), novae-zelandin A (14) and alterperylenol (12).

The dendrogram generated by UPGMA using Jaccard coefficient divided the 31 isolates into two main clusters (I and II). Cluster I contained all isolates of *A. infectoria* sp.-grp. and *A. vaccinii*, while the isolates belonging to *A. tenuissima*, *A. arborescens* and *A. alternata* sp.-grp. were grouped all together in cluster II without any particular subdivision (Fig. 5).

3.4. Composition of *Alternaria* population in barley grains

3.4.1. Growing season

All the *Alternaria* sp.-grps. identified were present in both growing seasons (2014 and 2015), and their frequency was similar in both years, except for the *A. vaccinii* isolates, which were only isolated in 2015, and in samples from the same locality (Miramar). In 2014, *A. tenuissima* sp.-grp. was present in a frequency of 83.4 % and *A. infectoria* sp.-grp. in 11.7 %, while in 2015 the values were 81.7 % and 11.3 %, respectively. The Spearman correlation coefficient showed an average negative correlation (r = -0.76, p < 0.05) between both sp.-grps. across all localities in both seasons together (Fig. 6). When seasons were compared, this correlation was higher in 2014 than in 2015 (2014: r = -0.89, p < 0.05; 2015: r = -0.70, p < 0.05).

3.4.2. Barley cultivars

Regarding the susceptibility of the barley cultivars evaluated, all were contaminated with *Alternaria* spp. (Table 1). In general, Andreia showed the highest frequency of *A. tenuissima* sp.-grp. and the lowest of the other sp.-grps., particularly of *A. infectoria* sp.-grp., in both seasons, as can be seen in Fig. 6. In Shakira, Scarlett, and Scrabble, the frequency of *A. tenuissima* sp.-grp. was lower, but the other sp.-grps. occurred in higher proportion than in Andreia (Table 1). Shakira was the cultivar in which *A. infectoria* sp.-grp. was found in higher amount, although its frequency was also affected by the locality.

3.4.3. Localities and climatic conditions

The distribution of the predominant sp.-grps. in the sampled localities showed a higher frequency of *A. tenuissima* in the northern ones, where higher temperatures were registered, while the opposite was observed for *A. infectoria* sp.-grp. (see Fig. 1 for geographic references). Fig. 7 shows the relationship between average temperature in each locality and season and the frequency of both sp.-grps. Likewise, the Spearman correlation coefficient showed a high positive correlation ($r = 0.81$, $p < 0.05$) between *A. tenuissima* sp.-grp. and the average temperature in the locality during the growing season. On the other hand, a moderate to high negative correlation ($r = -0.76$, $p < 0.05$) was found between *A. infectoria* sp.-grp. and temperature, showing higher frequencies in the southern localities, where lower temperatures were registered during both growing seasons (Fig. 7). Regarding the *Alternaria* sp.-grps. isolated in minor proportion, no correlations could be observed between their incidence and the meteorological conditions due to the low number of isolates of each group, and their absence in some of the localities sampled.

4. Discussion

Barley is one of the main grain crops in Argentina and its susceptibility to fungal pathogens is of great concern to both farmers and malt industry. Fusarium head blight (FHB), a disease caused by species of *Fusarium*, has received special attention in the

389 scientific community because of its negative effects on malt quality and the
390 accumulation of mycotoxins in the grain (Pascari et al., 2018; Schawarz, 2017; Wolf-
391 Hall, 2007). *Alternaria* spp. have been associated with the black point disease,
392 characterised by dark brown discoloration closed to the embryo end of the grain,
393 discoloured and shrivelled grains, and with the production of secondary metabolites
394 which can contaminate grains and be transferred to by-products (Tralamazza et al.,
395 2018). However, more information is needed on the *Alternaria* populations infecting
396 barley grains and its secondary metabolites.
397 *Alternaria* incidence exceeded that of *Fusarium* in all the barley samples analysed in
398 the present study, and these results agree with other works carried out in barley and
399 other small grains (Andersen et al., 1996; Beccari et al., 2016; González et al., 2008;
400 Jedidi et al., 2018; Krasauskas, 2017; Masiello et al., 2020; Medina et al., 2006).
401 *Fusarium* incidence was low in both seasons. Several authors suggested a negative
402 correlation between *Alternaria* and *Fusarium* in barley and wheat grains (Andersen et
403 al., 1996; Kosiak et al., 2004). In particular, strong negative associations have been
404 observed when the crop is affected by FHB; González et al., (2008) observed a lower
405 incidence of *A. alternata* during a FHB outbreak. However, the correlations between
406 both genera observed in our study were weak and lacking statistical significance,
407 suggesting that other factors might be involved in fungal competition when climatic
408 conditions are not conducive to the disease.
409 Interestingly, *Alternaria* incidence was higher in the drier season (2015). Similar results
410 were obtained by Schiro et al. (2018) who observed a higher genetic abundance of
411 *Alternaria* on wheat ears during drier microclimate. On the other hand, the opposite
412 was observed by Ramires et al. (2018), who associated the lowest *Alternaria*
413 contamination with the year in which lower levels of rainfall occurred. Climatic
414 conditions during the growing season, such as temperature and humidity are relevant
415 but not the only factors involved in fungal invasion of crops; fungal interactions during

416 infection process, and the different lifestyle of fungi may influence which pathogen
417 prevails in barley grains and determine the consequent mycotoxin contamination.
418 The present study also aimed to characterise the *Alternaria* spp. infecting barley grains.
419 Morphological characteristics allowed the identification of *Alternaria* sp.-grps.
420 (Simmons, 2007), with *A. tenuissima* as the main sp.-grp. isolated, followed by *A.*
421 *infectoria*. To our knowledge, there are few studies about *Alternaria* species
422 composition on barley grains worldwide. Some of them reported *A. alternata* as the
423 main sp.-grp. isolated (Medina et al., 2006; Nguyen et al., 2018), while *A. infectoria*
424 was the dominant species on Danish barley (Andersen et al., 1996). Controversial
425 results were obtained in other crops in Argentina. Patriarca et al. (2019) found *A.*
426 *tenuissima* as the principal sp.-grp. in symptomless wheat grain, followed by *A.*
427 *infectoria*. Nevertheless, *A. infectoria* was the main sp.-grp. associated with black point
428 of Argentinean wheat grains (Perelló et al., 2008). In the present study, damaged
429 grains were not specifically sampled, which might explain the differences with the
430 results reported by the latter.
431 The morphological classification of species in the genus *Alternaria* can be challenging
432 because of overlapping characteristics and a wide variation of the characters among
433 cultures of the same species. Because of this, in the last years, polyphasic approaches
434 combining morphological characteristics, DNA sequencing and metabolite profiles have
435 been extensively used to improve their identification. In our study, and in accordance
436 with others, the sections *Infectoriae* and *Alternaria* were differentiated based on
437 phylogenetic analyses (Masiello et al., 2020; Ramires et al., 2018; Serdani et al., 2002;
438 Somma et al., 2019; Zhu and Xiao, 2015) and their metabolite profiles (Andersen et al.,
439 2002, 2015; Patriarca et al., 2019; Serdani et al., 2002).
440 ATPase gene and the non-coding OPA 10-2 region were reported as useful to
441 discriminate among small-spored *Alternaria* from foods in different studies (Zhu and
442 Xiao, 2015; Siciliano et al., 2018; Somma et al., 2019). However, the *Alternaria* sp.-
443 grps. belonging to section *Alternaria* could be only partially separated by DNA

444 sequencing of ATPase and OPA 10-2 regions. All *A. arborescens* sp.-grp. isolates
445 grouped together in a separate clade with *A. arborescens* reference strains for both
446 genomic regions. Similarly, Siciliano et al. (2018) were able to segregate *A.*
447 *arborescens* from the rest of the isolates of section *Alternaria* in a phylogenetic analysis
448 based on seven regions, including OPA 10-2. Nevertheless, *A. tenuissima* and *A.*
449 *alternata* sp.-grps. could not be discriminated with any of these regions, which agrees
450 with some previous reports (Andrew et al., 2009; da Cruz Cabral et al., 2017; Gannibal,
451 2016; Lawrence et al., 2016; Siciliano et al., 2018). Consequently, it is evident that
452 isolates from section *Alternaria* are closely related.
453 As mentioned earlier, the secondary metabolite profiles were effective to separate
454 isolates from sections *Alternaria* and *Infectoriae* since only two metabolites
455 (alterperylenol and altertoxin-I) were shared by both sections, which is in accordance
456 with results reported by Patriarca et al. (2019). However, segregation between sp.-
457 grps. within sections was not possible through metabolite production, as has already
458 been demonstrated by Andersen et al. (2015), da Cruz Cabral et al. (2017), and
459 Patriarca et al. (2019).
460 All the isolates produced several metabolites *in vitro*, many of which are known as
461 mycotoxins. AOH was the most frequent one produced by isolates of section *Alternaria.*
462 This agrees with an earlier study (Patriarca et al., 2019), where AOH and AME were
463 the most common metabolites produced among this section. A previous study on the
464 natural occurrence of *Alternaria* toxins in the same barley grains, detected AOH as the
465 most frequent toxin contaminating the samples (64 % positive) (Castañares et al.,
466 2020). Thus, toxin contamination in the samples was in accordance with the metabolite
467 profile of the predominant *Alternaria* sp.-grp.
468 Considering that *A. infectoria* sp.-grp. was frequently isolated in the present study, the
469 natural occurrence of metabolites from this section in barley samples deserves to be
470 investigated. Some of them (e.g., altertoxins) have shown genotoxic properties
471 (Schwarz et al., 2012). Infectopyrones and novae-zelandins were produced by most

isolates from this section, but toxicological data on these metabolites are scarce. A possible phytotoxic activity has been suggested, and infectopyrones have been postulated as mycotoxins due to their toxicity to murine cells (Larsen et al., 2003; Patriarca et al., 2019). In a recent study by Drakopoulos et al. (2021), infectopyrone was found in 63 % of the barley grain samples analysed, surpassing the number of positives for AOH (37 %), AME (33 %) and TeA (40 %). For these reasons, more chemical and toxicological research is necessary to determine the risk that the presence of *Alternaria* metabolites in barley grains might represent to animal and human health.

The isolates morphologically identified as *A. vaccinii* grouped together with *A. infectoria* isolates in both the phylogenetic and chemical analyses. Literature description of *A. vaccinii* is limited. In a study of *Alternaria* morphological and secondary metabolite characterization carried out by Andersen et al. (2015) one *A. vaccinii* isolate showed a similar metabolite profile to *A. alternata* sp.-grp., producing AOH, AME, ALT, and tentoxin, amongst others. On the other hand, *A. vaccinii* isolates of our study produced metabolites matching the profile of *A. infectoria* sp.-grp., such as infectopyrone, novae-zeladin A and B, and alterperylenol, among others. These results would agree with the classification proposed by Gannibal and Lawrence (2016), who included *A. vaccinii* in the section *Infectoriae*. Studies on more isolates from this species would be necessary to confirm our results. However, the low incidence of this species in Argentinean grains hinders this purpose.

The insight into a fungal population diversity is important to understand the spread of a pathogen on growing areas and improve control measures. Several factors are involved in determining the distribution of fungal populations in crops. Geographical variations have been observed for different genera, which suggest that adaptation to different environments and climatic conditions are key to the dominance of a species in a specific region (Kosiak et al., 2004; Pitt and Hocking, 2009). Different cultivars or varieties of a crop might have different susceptibility to the colonization from the same

pathogen. The barley cultivars analysed in this study did not show a particular susceptibility regarding the *Alternaria* population composition, except for Andreia, which presented the highest frequency of contamination with *A. tenuissima* sp.-grp. The rest of the cultivars, when grown in different regions, showed different proportion of the *Alternaria* sp.-grps., suggesting that other factors were determinant in their distribution.

A negative correlation was observed between *A. tenuissima* sp.-grp. and *A. infectoria* sp.-grp. frequencies in grain samples, which were associated with the average temperature registered in each growing region. Although *A. tenuissima* sp.-grp. was predominant throughout the growing area, it occurred in higher frequencies in northern localities, where average temperatures were higher than 20 ºC. The opposite trend was observed for *A. infectoria* sp.-grp. which was found in higher proportion in southern localities, characterised by lower average temperatures. This differential incidence associated with temperature may result in different mycotoxin contamination along the geographical growing region, based on the dissimilar metabolite profiles presented by the sections to which these sp.-grps. belong. A study carried out in Australia reported the natural occurrence of TeA, AOH, and AME in weather-damaged wheat in which *A. alternata* was the predominant species, but none of these mycotoxins were detected in grains from regions with primary infection with *A. infectoria* (Webley et al., 1997). Thus, the identification of predominant species inside a fungal population might be a useful tool to predict mycotoxin risk in a certain crop.

5. Conclusions

Alternaria was the dominant fungal genus infecting barley grains from the main growing region in Argentina in the 2014 and 2015 seasons. Its incidence exceeded that of *Fusarium* in all the barley samples and was higher in the drier season (2015).

A. tenuissima sp.-grp. was predominant and associated with the toxic metabolites naturally detected in barley grains in Argentina, like AOH, AME and TeA. The incidence

was higher than that of *A. infectoria* sp.-grp., the second in importance, especially in regions where higher mean temperatures were registered during the growing season. The barley cultivars analysed in this study did not show a particular susceptibility regarding the *Alternaria* population composition, except for Andreia, which presented the highest frequency of contamination with *A. tenuissima* sp.-grp. The rest of the cultivars, when grown in different regions, showed different proportion of the *Alternaria* sp.-grps., suggesting that other factors were determinant in their distribution.

The polyphasic approach used in this study, which included morphological identification, metabolite profile and analysis of DNA sequences, was useful to characterise and distinguish between isolates from sections *Alternaria* and *Infectoriae*. However, *Alternaria* sp.-grps. within section *Alternaria*, i.e. *A. tenuissima*, *A. arborescens* and *A. alternata*, could only be partially separated. Only isolates from *A. arborescens* sp.-grp. clustered together with the correspondent reference strain in the phylogenetic trees obtained from ATPase analyses. The knowledge on fungal populations infecting crops is a valuable tool for monitoring and establishing mycotoxin controls. Since the *Alternaria* isolates more frequently found in grains belonged to sections with two different metabolite profiles (the alternariol profile and the infectopyrone profile), food safety authorities should consider the wide diversity of toxic metabolites that may contaminate barley grains and subsequently the beer, when reviewing risk and safety aspects of *Alternaria* toxins in food and feed.

6. Acknowledgments

This research was supported by Universidad de Buenos Aires [UBACyT 2017, 0020170100094BA], Agencia Nacional de Promoción Científica y Tecnológica-FONCyT [PICT 2055/2017] and UNCPBA.

7. Declarations of interest

None.

556

557 8. References

558 Andersen, B., Thrane, U., Svendsen, A., Rasmussen, I.A., 1996. Associated field
559 mycobiota on malt barley. Can. J. Bot. 74, 854–858. https://doi.org/10.1139/b96-106.

560 Andersen, B., Krøger, E., Roberts, R.G., 2002. Chemical and morphological
561 segregation of *Alternaria arborescens*, *A. infectoria* and *A. tenuissima* species-groups.
562 Mycol. Res. 106, 170–182. https://doi.org/10.1017/S0953756201005263.

563 Andersen, B., Dongo, A., Pryor, B.M., 2008. Secondary metabolite profiling of
564 *Alternaria dauci*, *A. porri*, *A. solani*, and *A. tomatophila*. Mycol. Res. 112, 241–250.
565 https://doi.org/10.1016/j.mycres.2007.09.004.

566 Andersen, B., Sørensen, J.L., Nielsen, K.F., van den Ende, B.G., de Hoog, S., 2009. A
567 polyphasic approach to the taxonomy of the *Alternaria infectoria* species–group. Fungal
568 Genet. Biol. 46, 642–656. https://doi.org/10.1016/j.fgb.2009.05.005.

569 Andersen, B., Nielsen, K.F., Fernández Pinto, V., Patriarca, A., 2015. Characterization
570 of *Alternaria* strains from Argentinean blueberry, tomato, walnut and wheat. Int. J. Food
571 Microbiol. 196, 1–10. https://doi.org/10.1016/j.ijfoodmicro.2014.11.029.

572 Andrew, M., Peever, T.L., Pryor, B.M., 2009. An expanded multilocus phylogeny does
573 not resolve morphological species within the small-spored *Alternaria* species complex.
574 Mycologia 101, 95–109. https://doi.org/10.3852/08-135.

575 Armitage, A.D., Barbara, D.J., Harrison, R.J., Lane, C.R., Sreenivasaprasad, S.,
576 Woodhall, J.W., Clarkson, J.P., 2015. Discrete lineages within *Alternaria alternata*
577 species group: Identification using new highly variable loci and support from
578 morphological characters. Fungal Biol. 119, 994–1006.
579 https://doi.org/10.1016/j.funbio.2015.06.012.

580 Bauer, J.I., Gross, M., Gottschalk, C., Usleber, E., 2016. Investigations on the
581 occurrence of mycotoxins in beer. Food Control 63, 135–139.
582 https://doi.org/10.1016/j.foodcont.2015.11.040.

583 Beccari, G., Caproni, L., Tini, F., Uhlig, S., Covarelli, L., 2016. Presence of *Fusarium*
584 Species and Other Toxigenic Fungi in Malting Barley and Multi-Mycotoxin Analysis by
585 Liquid Chromatography–High-Resolution Mass Spectrometry. J. Agric. Food Chem. 64,
586 4390–4399. https://doi.org/10.1021/acs.jafc.6b00702.
587 Beccari, G., Senatore, M.T., Tini, F., Sulyok, M., Covarelli, L., 2018. Fungal community,
588 Fusarium head blight complex and secondary metabolites associated with malting
589 barley grains harvested in Umbria, central Italy. Int. J. Food Microbiol. 273, 33–42.
590 https://doi.org/10.1016/j.ijfoodmicro.2018.03.005.
591 Broggi, L.E., González, H.H.L., 2007. *Alternaria alternata* prevalence in cereal grains
592 and soybean seeds from Entre Ríos, Argentina. Rev. Iberoam. Micol. 24, 47–51.
593 Castañares, E., Pavicich, M.A., Dinolfo, M.I., Moreyra, F., Stenglein, S.A., Patriarca, A.,
594 2020. Natural occurrence of *Alternaria* mycotoxins in malting barley grains in the main
595 producing region of Argentina. J. Sci. Food Agric. 100, 1004–1011.
596 https://doi.org/10.1002/jsfa.10101.
597 Darriba, D., Taboada, G.L., Doallo, R., Posada, D., 2012. jModelTest 2: more models,
598 new heuristics and parallel computing. Nat. Methods 9, 772.
599 da Cruz Cabral, L., Rodriguero, M., Stenglein, S., Fog Nielsen, K., Patriarca, A., 2017.
600 Characterization of small-spored *Alternaria* from Argentinean crops through a
601 polyphasic approach. Int. J. Food Microbiol. 257, 206–215.
602 https://doi.org/10.1016/j.ijfoodmicro.2017.06.026.
603 Deshpande, S.S., 2002. Handbook of Food Toxicology. New York : Marcel Dekker.
604 Drakopoulos, D., Sulyok, M., Krska, R., Logrieco, A.F., Vogelgsang, S., 2021. Raised
605 concerns about the safety of barley grains and straw: A Swiss survey reveals a high
606 diversity of mycotoxins and other fungal metabolites. Food Control 125,
607 https://doi.org/10.1016/j.foodcont.2021.107919.
608 EFSA Panel on Contaminants in the Food Chain (CONTAM), 2011. Scientific Opinion
609 on the risks for animal and public health related to the presence of *Alternaria* toxins in

610 feed and food: *Alternaria* toxins in feed and food. EFSA J. 9, 2407.
611 https://doi.org/10.2903/j.efsa.2011.2407.
612 EFSA Panel on Contaminants in the Food Chain (CONTAM), 2016. Dietary exposure
613 assessment to *Alternaria* toxins in the European population. EFSA J. 14, 4654.
614 Elfar, K., Zoffoli, J.P., Latorre, B.A., 2018. Identification and Characterization of
615 *Alternaria* Species Associated with Moldy Core of Apple in Chile. Plant Dis. 102, 2158–
616 2169. https://doi.org/10.1094/PDIS-02-18-0282-RE.
617 FAOSTAT, 2021. http://www.fao.org/faostat/en/#data/QC/visualize.
618 Gannibal, P.B., 2016. Distribution of Alternaria species among sections. 2. Section
619 *Alternaria*. Mycotaxon 130, 941–949. https://doi.org/10.5248/130.941.
620 Gannibal, P.B., Lawrence, D.P., 2016. Distribution of *Alternaria* species among
621 sections. 3. Sections *Infectoriae* and *Pseudoalternaria*. Mycotaxon 131, 781–790.
622 https://doi.org/10.5248/131.781.
623 Gherbawy, Y., Hussein, M.A., Runge, F., Spring, O., 2018. Molecular characterization
624 of *Alternaria alternata* population isolated from Upper Egyptian tomato fruits. J.
625 Phytopathol. 166, 709–721. https://doi.org/10.1111/jph.12752.
626 Goloboff, P.A., Catalano, S.A., 2016. TNT version 1.5, including a full implementation
627 of
628 phylogenetic morphometrics. Cladistics 32, 221–238.
629 Goloboff, P.A., Farris, J.S., Nixon, K.C., 2008. TNT, a free program for phylogenetic
630 analysis. Cladistics 24, 774–786.
631 González, H.H.L., Moltó, G.A., Pacin, A., Resnik, S.L., Zelaya, M.J., Masana, M.,
632 Martínez, E.J., 2008. Trichothecenes and Mycoflora in Wheat Harvested in Nine
633 Locations in Buenos Aires Province, Argentina. Mycopathologia 165, 105–114.
634 https://doi.org/10.1007/s11046-007-9084-x.
635 Guindon, S., Gascuel, O. 2003. A simple, fast, and accurate algorithm to estimate large
636 phylogenies by maximum likelihood. Syst Biol. 52:696–704.

637 Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and
638 analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.
639 Jedidi, I., Soldevilla, C., Lahouar, A., Marín, P., González-Jaén, M.T., Said, S., 2018.
640 Mycoflora isolation and molecular characterization of *Aspergillus* and *Fusarium* species
641 in Tunisian cereals. Saudi J. Biol. Sci. 25, 868–874.
642 https://doi.org/10.1016/j.sjbs.2017.11.050.
643 Justé, A., Malfliet, S., Lenaerts, M., De Cooman, L., Aerts, G., Willems, K., Lievens, B.,
644 2011. Microflora during malting of barley: overview and impact on malt quality. Brewing
645 Science 64, 22–31.
646 Kosiak, B., Torp, M., Skjerve, E., Andersen, B., 2004. *Alternaria* and *Fusarium* in
647 Norwegian grains of reduced quality—a matched pair sample study. Int. J. Food
648 Microbiol. 93, 51–62. https://doi.org/10.1016/j.ijfoodmicro.2003.10.006.
649 Krasauskas, A., 2017. Fungi in malting barley grain and malt production. Biologija 63.
650 https://doi.org/10.6001/biologija.v63i3.3583.
651 Larsen, T.O., Perry, N.B., Andersen, B., 2003. Infectopyrone, a potential mycotoxin
652 from *Alternaria infectoria*. Tetrahedron Lett. 44, 4511–4513.
653 Lawrence, D.P., Gannibal, P.B., Dugan, F.M., Pryor, B.M., 2014. Characterization of
654 *Alternaria* isolates from the infectoria species-group and a new taxon from
655 Arrhenatherum, *Pseudoalternaria arrhenatheria* sp. nov. Mycol. Prog. 13, 257–276.
656 https://doi.org/10.1007/s11557-013-0910-x.
657 Lawrence, D.P., Rotondo, F., Gannibal, P.B., 2016. Biodiversity and taxonomy of the
658 pleomorphic genus *Alternaria*. Mycol. Prog. 15. https://doi.org/10.1007/s11557-015-
659 1144-x.
660 Magan, N., Aldred, D., Hope, R., Mitchell, D., 2010. Environmental Factors and
661 Interactions with Mycobiota of Grain and Grapes: Effects on Growth, Deoxynivalenol
662 and Ochratoxin Production by *Fusarium culmorum* and *Aspergillus carbonarius*. Toxins
663 2, 353–366. https://doi.org/10.3390/toxins2030353.

664 Magan, N., Lacey, J., 1984. Effect of water activity, temperature and substrate on
665 interactions between field and storage fungi. Trans. Br. Mycol. Soc. 82, 83–93.
666 https://doi.org/10.1016/S0007-1536(84)80214-4.
667 Masiello, M., Somma, S., Susca, A., Ghionna, V., Logrieco, A.F., Franzoni, M.,
668 Ravaglia, S., Meca, G., Moretti, A., 2020. Molecular Identification and Mycotoxin
669 Production by *Alternaria* Species Occurring on Durum Wheat, Showing Black Point
670 Symptoms. Toxins 12, 275. https://doi.org/10.3390/toxins12040275.
671 Medina, Á., Valle-Algarra, F.M., Mateo, R., Gimeno-Adelantado, J.V., Mateo, F.,
672 Jiménez, M., 2006. Survey of the mycobiota of Spanish malting barley and evaluation
673 of the mycotoxin producing potential of species of *Alternaria*, *Aspergillus* and *Fusarium*.
674 Int. J. Food Microbiol. 108, 196–203. https://doi.org/10.1016/j.ijfoodmicro.2005.12.003.
675 Miller, M.A., Pfeiffer, W., Schwartz, T., 2010. Creating the CIPRES Science Gateway
676 for inference of large phylogenetic trees. Proceedings of the Gateway Computing
677 Environments Workshop (GCE), 14 Nov. 2010, New Orleans, LA pp 1-8.
678 Nguyen, T.T.T., Kim, J., Jeon, S.J., Lee, C.W., Magan, N., Lee, H.B., 2018. Mycotoxin
679 production of *Alternaria* strains isolated from Korean barley grains determined by LC-
680 MS/MS. Int. J. Food Microbiol. 268, 44–52.
681 https://doi.org/10.1016/j.ijfoodmicro.2018.01.003.
682 Ntasiou, P., Myresiotis, C., Konstantinou, S., Papadopoulou-Mourkidou, E.,
683 Karaoglanidis, G.S., 2015. Identification, characterization and mycotoxigenic ability of
684 *Alternaria* spp. causing core rot of apple fruit in Greece. Int. J. Food Microbiol. 197, 22–
685 29. https://doi.org/10.1016/j.ijfoodmicro.2014.12.008.
686 Ostry, V., 2008. *Alternaria* mycotoxins: an overview of chemical characterization,
687 producers, toxicity, analysis and occurrence in foodstuffs. World Mycotoxin J. 1, 175–
688 188. https://doi.org/10.3920/WMJ2008.x013.
689 Pascari, X., Ortiz-Solá, J., Marín, S., Ramos, A.J., Sanchis, V., 2018. Survey of
690 mycotoxins in beer and exposure assessment through the consumption of

commercially available beer in Lleida, Spain. LWT 92, 87–91.
https://doi.org/10.1016/j.lwt.2018.02.021.
Patriarca, A., da Cruz Cabral, L., Pavicich, M.A., Nielsen, K.F., Andersen, B., 2019. Secondary metabolite profiles of small-spored *Alternaria* support the new phylogenetic organization of the genus. Int. J. Food Microbiol. 291, 135–143. https://doi.org/10.1016/j.ijfoodmicro.2018.11.022.
Peever, T.L., Su, G., Carpenter-Boggs, L., Timmer, L.W., 2004. Molecular Systematics of Citrus-Associated *Alternaria* Species. Mycologia 96, 119. https://doi.org/10.2307/3761993.
Peever, T.L., Carpenter-Boggs, L., Timmer, L.W., Carris, L.M., Bhatia, A., 2005. Citrus Black Rot is Caused by Phylogenetically Distinct Lineages of *Alternaria alternata*. Phytopathology 95, 512–518. https://doi.org/10.1094/PHYTO-95-0512.
Perelló, A., Moreno, M., Sisterna, M., 2008. *Alternaria infectoria* species-group associated with black point of wheat in Argentina. Plant Pathol. 57, 379.
Pitt, J.I., Hocking, A.D., 2009. Fungi and Food Spoilage, third ed. Springer, New York.
Prelle, A., Spadaro, D., Garibaldi, A., Gullino, M.L., 2013. A new method for detection of five *Alternaria* toxins in food matrices based on LC–APCI-MS. Food Chem. 140, 161–167. https://doi.org/10.1016/j.foodchem.2012.12.065.
Rambaut, A., Suchard, M.A., Xie, D., Drummond, A.J., 2003–2013. Tracer MCMC Trace Analysis Tool Version v1.6.0. Available from. http://beast.bio.ed.ac.uk/Tracer.
Rambaut, A., 2006–2014. FigTree Tree Figure Drawing Tool Version. pp. v1.4.2. Available from. http://tree.bio.ed.ac.uk/software/figtree/.
Ramires, F., Masiello, M., Somma, S., Villani, A., Susca, A., Logrieco, A., Luz, C., Meca, G., Moretti, A., 2018. Phylogeny and Mycotoxin Characterization of *Alternaria* Species Isolated from Wheat Grown in Tuscany, Italy. Toxins 10, 472. https://doi.org/10.3390/toxins10110472.
Rohlf, F.I., 1998. NTSYSPC. Numerical Taxonomy and Multivariate Analysis System Version 2.0. Applied Biostatistics. New York, NY, USA: Exeter Software.

719 Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Höhna, S.,
720 Larget, B., Liu, L., Suchard, M.A., Huelsenbeck, J.P., 2012. MrBayes 3.2: efficient
721 Bayesian phylogenetic inference and model choice across a large model space. Syst.
722 Biol. 61, 539–542.
723 Samson, R., Houbraken, J., Thrane, J., Frisvad, J.C., Andersen, B., 2010. Food and
724 Indoor Fungi. CBS Laboratory Manual Series 2. CBS-Fungal Biodiversity Centre,
725 Utrecht.
726 Schiro, G., Verch, G., Grimm, V., Müller, M., 2018. *Alternaria* and *Fusarium* Fungi:
727 Differences in Distribution and Spore Deposition in a Topographically Heterogeneous
728 Wheat Field. J. Fungi 4, 63. https://doi.org/10.3390/jof4020063.
729 Schwarz, P.B., 2017. Fusarium head blight and deoxynivalenol in malting and brewing:
730 successes and future challenges. Trop. Plant Pathol. 42, 153–164.
731 https://doi.org/10.1007/s40858-017-0146-4.
732 Serdani, M., Kang, J.-C., Andersen, B., Crous, P.W., 2002. Characterisation of
733 *Alternaria* species-groups associated with core rot of apples in South Africa. Mycol.
734 Res. 106, 561–569. https://doi.org/10.1017/S0953756202005993.
735 Siciliano, I., Franco Ortega, S., Gilardi, G., Bosio, P., Garibaldi, A., Gullino, M.L., 2018.
736 Molecular phylogeny and characterization of secondary metabolite profile of plant
737 pathogenic *Alternaria* species isolated from basil. Food Microbiol. 73, 264–274.
738 https://doi.org/10.1016/j.fm.2018.02.001.
739 Simmons, E.G., 2007. *Alternaria*: An identification manual. CBS Fungal Biodiversity
740 Centre, Utrecht, Netherlands.
741 Somma, S., Amatulli, M.T., Masiello, M., Moretti, A., Logrieco, A.F., 2019. *Alternaria*
742 species associated to wheat black point identified through a multilocus sequence
743 approach. Int. J. Food Microbiol. 293, 34–43.
744 https://doi.org/10.1016/j.ijfoodmicro.2019.01.001.

745 Stenglein, S.A., Ballati, P.A., 2006. Genetic diversity of Phaeoisariopsis griseolain
746 Argentina as revealed by pathogenic and molecular markers. Physiol. Mol. Plant
747 Pathol. 68, 158–167.
748 Thomma, B.P.H.J., 2003. *Alternaria* spp.: from general saprophyte to specific parasite.
749 Mol. Plant Pathol. 4, 225–236. https://doi.org/10.1046/j.1364-3703.2003.00173.x.
750 Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the
751 sensitivity of progressive multiple sequence alignment through sequence weighting,
752 positionspecific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–
753 4680.
754 Tralamazza, S.M., Piacentini, K.C., Iwase, C.H.T., Rocha, L. de O., 2018. Toxigenic
755 *Alternaria* species: impact in cereals worldwide. Curr. Opin. Food Sci. 23, 57–63.
756 https://doi.org/10.1016/j.cofs.2018.05.002.
757 Webley, D.J., Jackson, K.L., Mullins, J.D., Hocking, A.D., Pitt, J.I., 1997. *Alternaria*
758 toxins in weather-damaged wheat and sorghum in the 1995–1996 Australian harvest.
759 Aust. J. Agric. Res. 48, 1249–55. https://doi.org/10.1071/A97005.
760 Wolf-Hall, C.E., 2007. Mold and mycotoxin problems encountered during malting and
761 brewing. Int. J. Food Microbiol. 119, 89–94.
762 https://doi.org/10.1016/j.ijfoodmicro.2007.07.030.
763 Woudenberg, J.H.C., Seidl, M.F., Groenewald, J.Z., de Vries, M., Stielow, J.B.,
764 Thomma, B.P.H.J., Crous, P.W., 2015. *Alternaria* section *Alternaria*: Species, formae
765 speciales or pathotypes? Stud. Mycol. 82, 1-21.
766 http://dx.doi.org/10.1016/j.simyco.2015.07.001.
767 Zhu, X.Q., Xiao, C.L., 2015. Phylogenetic, Morphological, and Pathogenic
768 Characterization of *Alternaria* Species Associated with Fruit Rot of Blueberry in
769 California. Phytopathology 105, 1555–1567. https://doi.org/10.1094/PHYTO-05-15-
770 0122-R.

771 Figure legends

772 Fig. 1. Average temperature (☀) and accumulated precipitation (💧) from flowering to
773 harvest in the sampled localities. Values are the average from 2014 and 2015 data.

774 Fig. 2. Microscopic characteristics of *Alternaria* isolates from barley grains, at 400x in
775 slide preparations (A-E) and in a lateral cut at 100x (F-J) magnification. A-F: *A.*
776 *tenuissima* sp.-grp.; B-G: *A. infectoria* sp.-grp.; C-H: *A. alternata* sp.-grp.; D-I: *A.*
777 *arborescens* sp.-grp.; E-J: *A. vaccinii*.

778 Fig. 3. Bayesian phylogeny estimated from ATPase region sequences of 26 *Alternaria*
779 isolates from this study and seven reference strains. Outgroup: *Pleospora tarda*. Each
780 isolate code is followed by an abbreviation of the corresponding sp.-grp. or species:
781 Ten: *A. tenuissima*; Inf: *A. infectoria*; Arb: *A. arborescens*; Alt: *A. alternata*; Vac: *A.*
782 *vaccinii*. Numbers above branches indicate Bayesian posterior probability values.
783 Branch lengths indicate number of substitutions per site.

784 Fig. 4. Bayesian phylogeny estimated from OPA region sequences of nine *Alternaria*
785 isolates from this study and six reference strains. Outgroup: *A. eichhorniae*. Each
786 *Alternaria* isolate code is followed by an abbreviation of the corresponding *Alternaria*
787 sp.-grp.: Ten: *A. tenuissima*; Arb: *A. arborescens*; Alt: *A. alternata*. Numbers above
788 branches indicate Bayesian posterior probability values. Branch lengths indicate
789 number of substitutions per site.

790 Fig. 5. Dendrogram obtained using Jaccard coefficient and UPGMA algorithm from the
791 analysis of 20 metabolites produced by 31 *Alternaria* isolates. Each isolate code is
792 followed by an abbreviation of the corresponding sp.-grp. or species: Ten: *A.*
793 *tenuissima*; Inf: *A. infectoria*; Arb: *A. arborescens*; Alt: *A. alternata*; Vac: *A. vaccinii*.

794 Fig. 6. Relationship between *A. tenuissima* and *A. infectoria* sp.-grps. frequency in
795 different barley cultivars.

796 Fig. 7. Relationship between the frequency of the main *Alternaria* sp.-grps. and
797 average temperature in the sampled localities. *A. tenuissima* sp.-grp. in localities

798 situated in the South (A) and North (B) of the sampling region; *A. infectoria* sp.-grp. in
799 localities situated in the South (C) and North (D) of the sampling region.
800 Fig. S1. Incidence, expressed as percentage of infected kernels, of *Alternaria* and
801 *Fusarium* in barley grain samples in the 2014 (●) and 2015 (◆) growing season.
802 Fig. S2. Maximum parsimony phylogeny estimated from ATPase sequences of 26
803 *Alternaria* isolates from this study and seven reference strains. Outgroup: *Pleospora*
804 *tarda.* Each *Alternaria* isolate code is followed by an abbreviation of the corresponding
805 *Alternaria* sp.-grp. or species: Ten: *A. tenuissima*; Inf: *A. infectoria*; Arb: *A.*
806 *arborescens*; Alt: *A. alternata*; Vac: *A. vaccinii*. Numbers in branches indicate bootstrap
807 values.
808 Fig. S3. Maximum parsimony phylogeny estimated from OPA sequences of nine
809 *Alternaria* isolates from this study and six reference strains. Outgroup: *A. eichhorniae.*
810 Each *Alternaria* isolate code is followed by an abbreviation of the corresponding
811 *Alternaria* sp.-grp.: Ten: *A. tenuissima*; Arb: *A. arborescens*; Alt: *A. alternata*. Numbers
812 in branches indicate bootstrap values.

Table 1. Incidence (%) of the mycobiota analyzed in malting barley samples grown in Argentina. Data include year of sampling, barley cultivar, locality, incidence of the field fungi evaluated (*Fusarium* and *Alternaria*) and composition of the genus *Alternaria*.

Year	Cultivar	Locality	Incidence (%)		*Alternaria* composition (%)					
			Fusarium	*Alternaria*	Aten	Ainf	Aarb	Aalt	Avac	Alt sp.
2014	Andreia	Huanguelén	6	23	100	0	0	0	0	0
	Scrabble	Huanguelén	10	17	71	23	6	0	0	0
	Scarlett	Huanguelén	4	40	53	45	2	0	0	0
	Andreia	Paraná	9	11	100	0	0	0	0	0
	Scrabble	Paraná	15	18	94	0	0	6	0	0
	Scarlett	Paraná	4	11	82	0	0	0	0	18
	Andreia	9 de Julio	4	14	100	0	0	0	0	0
	Scrabble	9 de Julio	6	22	100	0	0	0	0	0
	Andreia	Bordenave	1	38	79	18	3	0	0	0
	Scrabble	Bordenave	4	30	93	0	3	0	0	3
	Scarlett	Bordenave	0	25	68	28	0	0	0	4
	Andreia	Miramar	19	12	92	0	0	8	0	0
	Scrabble	Miramar	9	21	57	43	0	0	0	0
	Scarlett	Miramar	3	13	77	8	15	0	0	0
	Scarlett	Bigand	0	27	85	11	0	4	0	0
2015	Andreia	Huanguelén	4	45	96	2	0	2	0	0
	Scrabble	Huanguelén	1	41	71	27	2	0	0	0
	Shakira	Huanguelén	0	38	26	66	5	0	0	3
	Andreia	Paraná	12	50	100	0	0	0	0	0
	Scrabble	Paraná	12	42	98	0	2	0	0	0
	Shakira	Paraná	2	17	100	0	0	0	0	0
	Andreia	9 de Julio	4	28	96	0	4	0	0	0
	Scrabble	9 de Julio	5	39	95	5	0	0	0	0
	Shakira	9 de Julio	4	35	89	3	0	8	0	0
	Andreia	Bordenave	0	33	97	0	0	0	0	3
	Scrabble	Bordenave	0	39	38	0	0	44	0	18
	Shakira	Bordenave	1	41	88	12	0	0	0	0
	Andreia	Miramar	7	45	85	13	2	0	0	0
	Scrabble	Miramar	8	64	45	36	6	13	0	0
	Shakira	Miramar	2	33	73	24	0	0	3	0
	Andreia	Bigand	6	51	90	0	10	0	0	0
	Scrabble	Bigand	7	51	100	0	0	0	0	0
	Shakira	Bigand	1	58	83	15	2	0	0	0

Aten: *A. tenuissima* sp.-grp., Ainf: *A. infectoria* sp.-grp., Aalt: *A. alternata* sp.-grp., Aarb: *A. arborescens* sp.-grp., Avac: *A.vaccinii,* Alt sp.: *Alternaria* sp.

Table 2. GenBank accession numbers of sequences of plasma membrane ATPase and anonymous genomic region OPA10-2 of the 26 *Alternaria* isolates selected for phylogenetic analysis.

Strain name	Strain code in figures	Morphological identification	GenBank accession number	
			ATPase	OPA 10-2
M33-1f	A01Inf	*A. infectoria* sp.-grp.	MT977642	--
M34-f4	A04Inf	*A. infectoria* sp.-grp.	MT977643	--
M35-a4	A05Inf	*A. infectoria* sp.-grp.	MT977644	--
M35-g2	A06Inf	*A. infectoria* sp.-grp.	MT977645	--
M36-h3	A08Inf	*A. infectoria* sp.-grp.	MT977646	--
M36-h7	A09Inf	*A. infectoria* sp.-grp.	MT977647	--
M36-h9	A10Inf	*A. infectoria* sp.-grp.	MT977648	--
M36-i8	A12Inf	*A. infectoria* sp.-grp.	MT977649	--
M37-a27	A13Ten	*A. tenuissima* sp.-grp.	MT977650	--
M44-a5	A15Ten	*A. tenuissima* sp.-grp.	MT977651	MT977633
M44-b19	A16Inf	*A. infectoria* sp.-grp.	MT977652	--
M57-1b	A17Vac	*A. vaccinii*	MT977653	--
M58-1	A18Inf	*A. infectoria* sp.-grp.	MT977654	--
M58-2d	A19Inf	*A. infectoria* sp.-grp.	MT977655	--
M58-1c	A20Inf	*A. infectoria* sp.-grp.	MT977656	--
M58-8c	A21Inf	*A. infectoria* sp.-grp.	MT977657	--
M2-3b	A22Inf	*A. infectoria* sp.-grp.	MT977658	--
M4-1	A23Arb	*A. arborescens* sp.-grp.	MT977659	MT977634
M5-1b	A24Ten	*A. tenuissima* sp.-grp.	MT977660	MT977635
M9-9	A25Ten	*A. tenuissima* sp.-grp.	MT977661	MT977636
M25-4	A26Alt	*A. alternata* sp.-grp.	MT977662	MT977637
M32-6	A27Ten	*A. tenuissima* sp.-grp.	MT977663	MT977638
M36-a1	A28Arb	*A. arborescens* sp.-grp.	MT977664	MT977639
M40-10	A29Ten	*A. tenuissima* sp.-grp.	MT977665	MT977640
M60-1d	A30Vac	*A. vaccinii*	MT977666	--
M64-37	A31Arb	*A. arborescens* sp.-grp.	MT977667	MT977641

Table 3. Reference strains and sequences used as outgroup for ATPase and OPA10-2 regions in phylogenetic analyses.

Region	Species name and strain number[a]	Accession number	Use	References
ATPase	*Pleospora tarda* ATCC 42170	JQ671767	Outgroup	Lawrence et al., 2014
ATPase	*A. japonica* EGS 41-158	JQ671840	Reference strain	Lawrence et al., 2014
ATPase	*A. brassicae* EGS 38-032	JQ671847	Reference strain	Lawrence et al., 2014
ATPase	*A. proteae* EGS 39-031	JQ671777	Reference strain	Lawrence et al., 2014
ATPase	*A. infectoria* EGS 27-193	JQ671804	Reference strain	Lawrence et al., 2014
ATPase	*A. tenuissima* EGS 34-015	JQ671875	Reference strain	Lawrence et al., 2014
ATPase	*A. arborescens* EGS 39-128	JQ671880	Reference strain	Lawrence et al., 2014
ATPase	*A. alternata* EGS 34-016	JQ671874	Reference strain	Lawrence et al., 2014
OPA 10-2	*A. eichhorniae* CBS 489.92	KP124740	Outgroup	Woudenberg et al., 2015
OPA 10-2	*A. tenuissima* CBS124278	MF070459	Reference strain	Siliciano et al., 2018
OPA 10-2	*A. tenuissima* CBS124283	MF070453	Reference strain	Siliciano et al., 2018
OPA 10-2	*A. arborescens* CBS124274	MF070456	Reference strain	Siliciano et al., 2018
OPA 10-2	*A. arborescens* CBS 102605	KP124712	Reference strain	Woudenberg et al., 2015
OPA 10-2	*A. alternata* CBS115152	MF070449	Reference strain	Siliciano et al., 2018
OPA 10-2	*A. alternata* CBS 916.96	KP124632	Reference strain	Woudenberg et al., 2015

[a]ATCC: American Type Culture Collection, Manassas, VA 20108; EGS: E.G. Simmons, Mycological Services, Crawfordsville, IN 47933; CBS: Centraalbureau voor Schimmelcultures, Royal Netherlands Academy of Arts and Sciences, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands.

Table 4. Metabolites produced by *Alternaria* isolates from barley grains.

Metabolite	Number of producers					
	A.tenuissima sp.-grp. (n=6)	*A.arborescens* sp.-grp. (n=3)	*A.alternata* sp.-grp. (n=1)	*A.infectoria* sp.-grp. (n=19)	*A.vaccinni* (n=2)	Total (n=31)
Altenuene	4	2	1	0	0	7
Altenusin	3	2	1	0	0	6
Alternariol	5	3	1	0	0	9
Alternariol monomethyl ether	2	3	1	0	0	6
Alterperylenol	1	0	0	10	1	12
Altertoxin analog	2	2	0	0	0	4
Altertoxin-I	2	0	0	2	1	5
Altertoxin-II	3	0	0	0	0	3
Altertoxin-III	1	0	0	0	0	1
Infectopyrone	0	0	0	18	2	20
4Z-Infectopyrone	0	0	0	9	1	10
Novae-zeladin A	0	0	0	12	2	14
Novae-zeladin B	0	0	0	14	2	16
Novae-zeladin derivative	0	0	0	7	2	9
Phomapyrone A	0	0	0	8	1	9
Tenuazonic acid	2	0	1	0	0	3
Tenuazonic acid derivative 1	1	0	1	0	0	2
Tenuazonic acid derivative 2	1	0	0	0	0	1
Tentoxin	1	1	1	0	0	3
Stemphyltoxin-III	2	0	0	0	0	2

Supplementary Table 1. Average temperature and accumulated precipitation from flowering to harvest in the barley growing localities during the 2014 and 2015 harvest seasons.

	2014		2015	
Locality	Average temperature (°C)	Accumulated precipitation (mm)	Average temperature (°C)	Accumulated precipitation (mm)
Miramar	17.80	254.5	16.51	52.5
Huanguelén	17.59	274.3	16.74	267.9
Bordenave	19.20	276.5	16.88	215.5
9 de Julio	21.04	271.2	19.80	175.2
Bigand	22.52	198.2	21.31	183.8
Paraná	22.72	193.3	21.52	180.1

Figure 1

Click here to access/download;Figure;Figure 1.tif

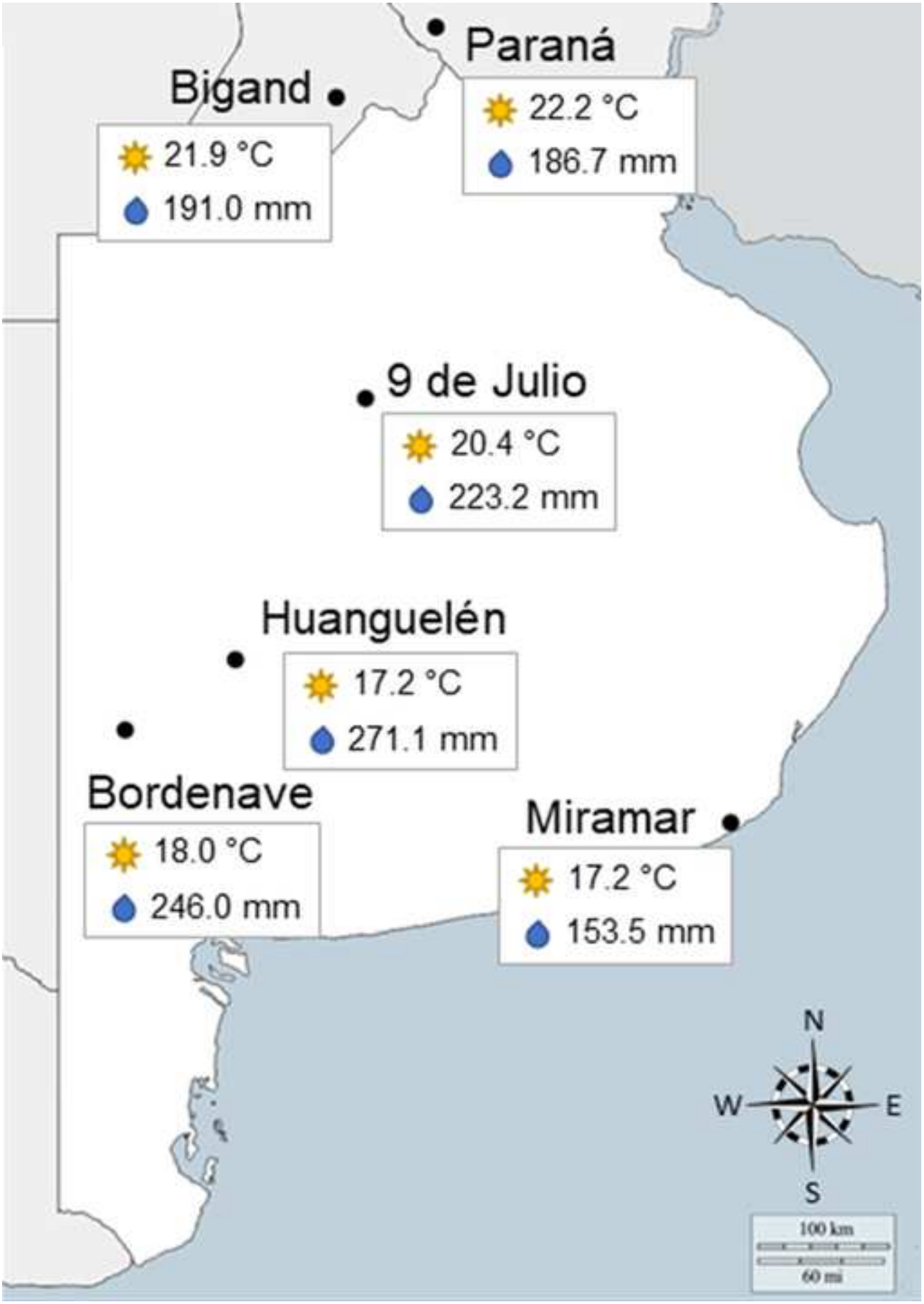


Figure 2

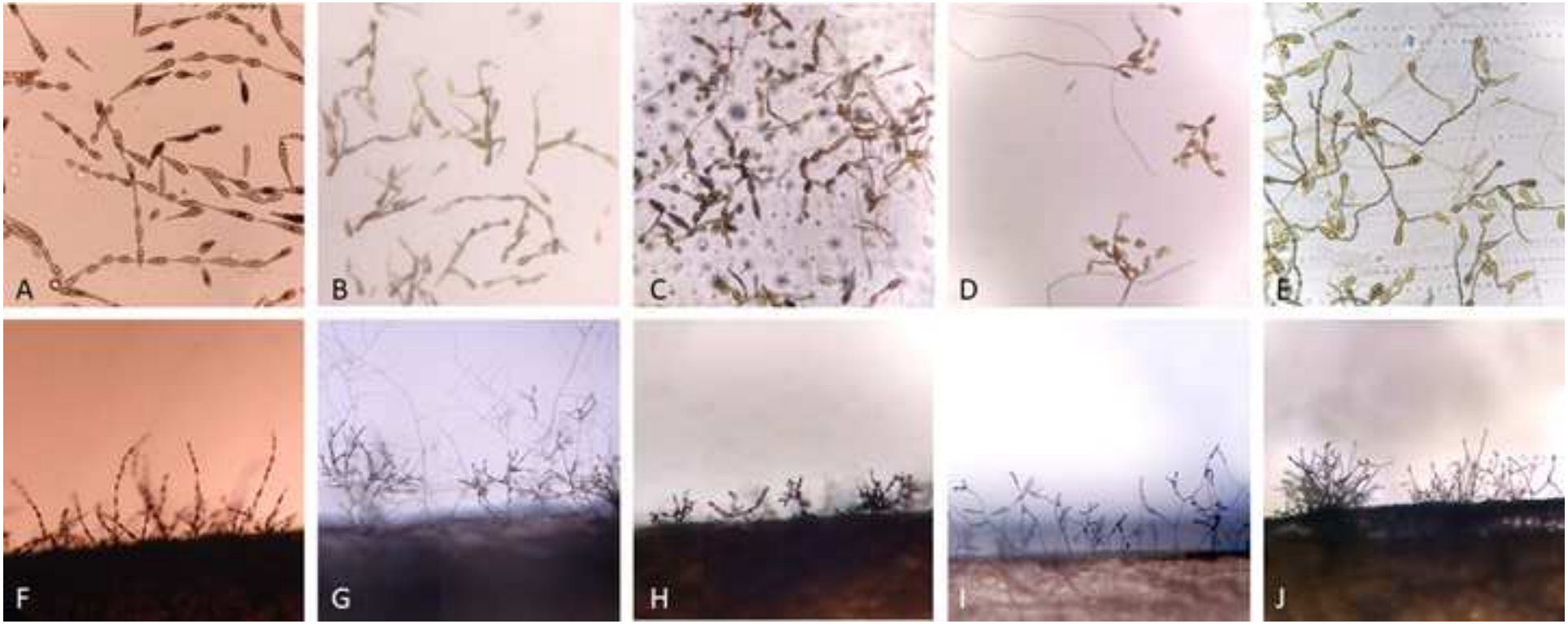


Figure 3

Click here to access/download;Figure;Figure 3.tif

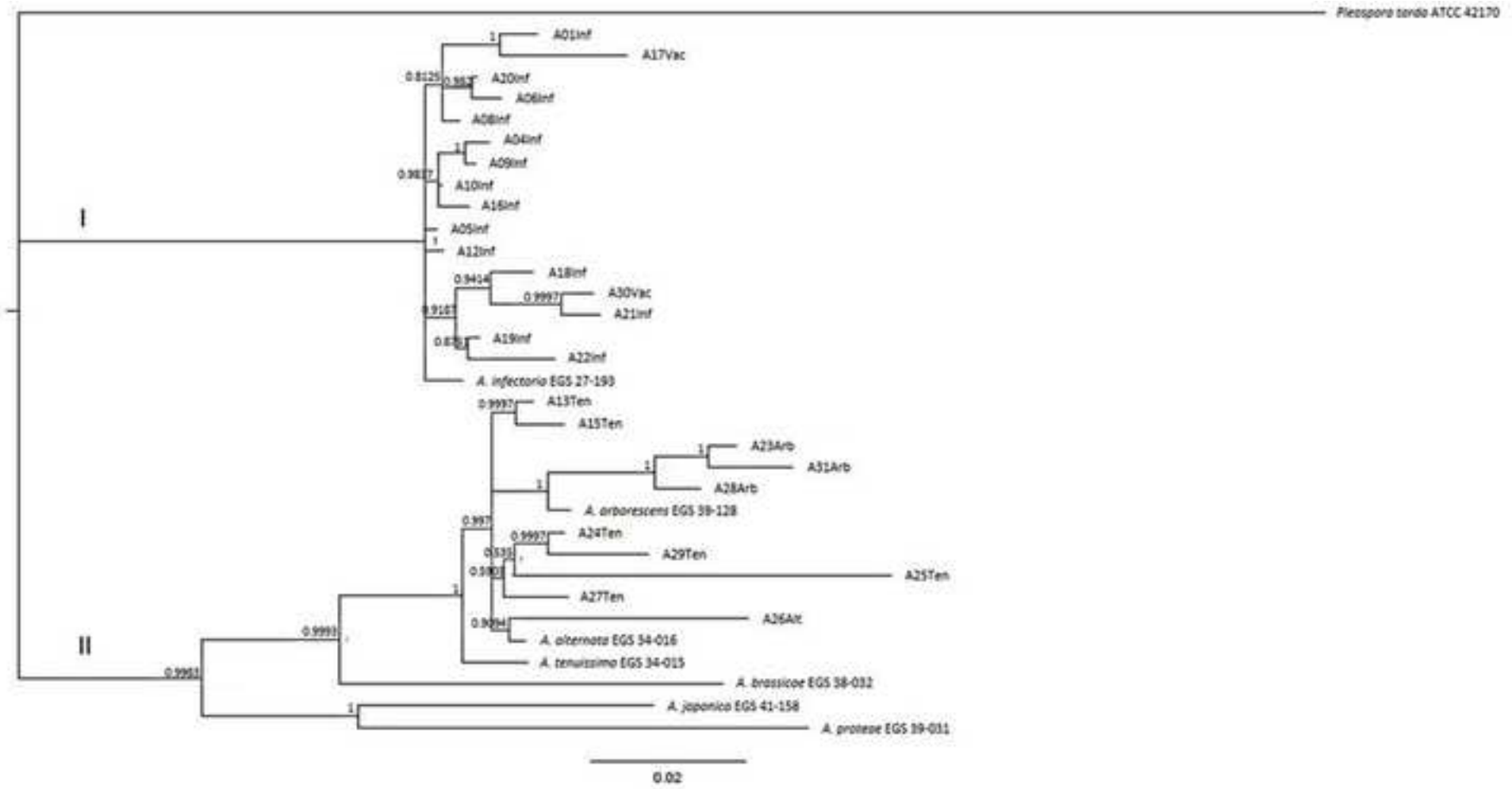


Figure 4

Click here to access/download;Figure;Figure 4.tif

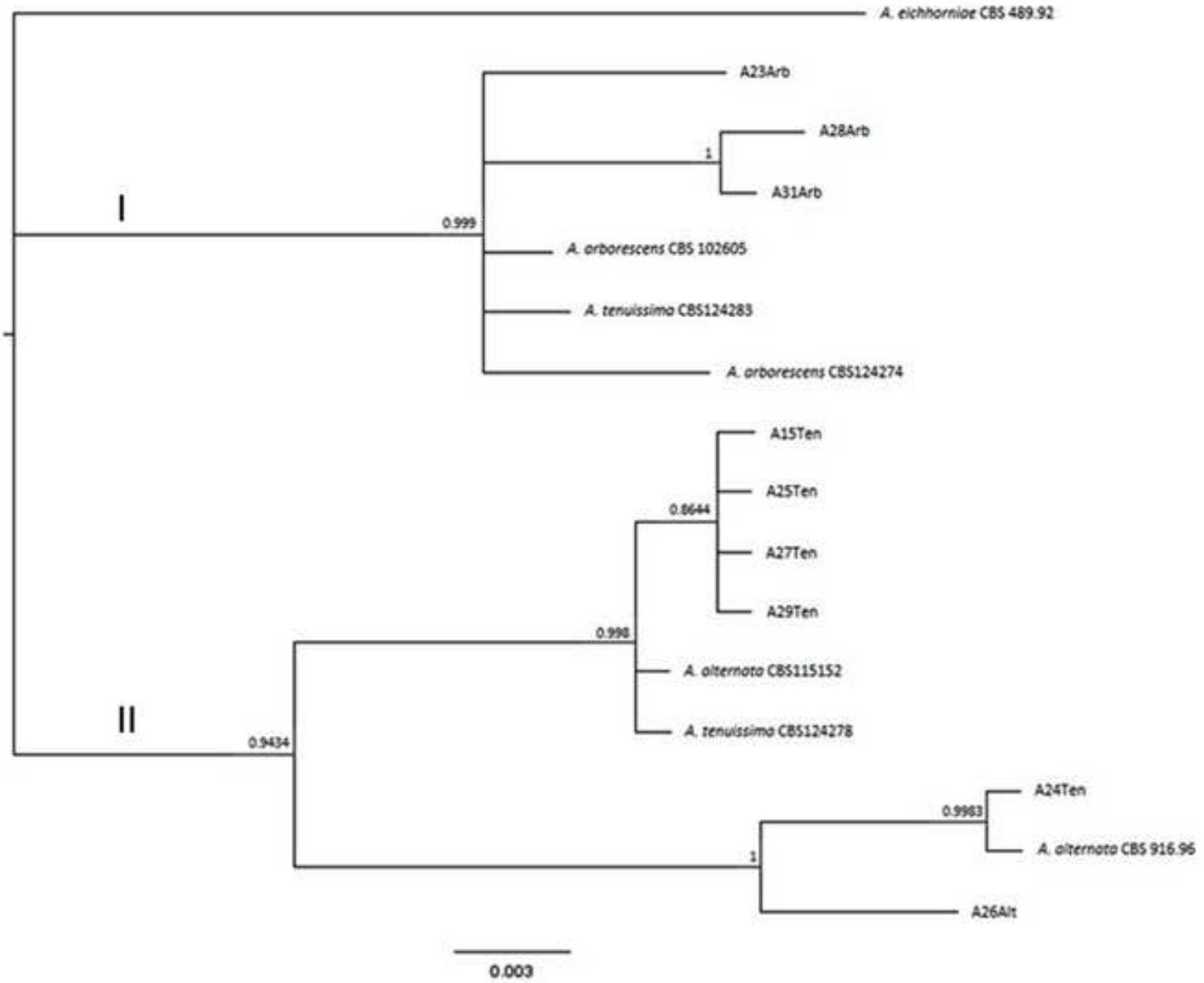


Figure 5

Click here to access/download;Figure;Figure 5.tif

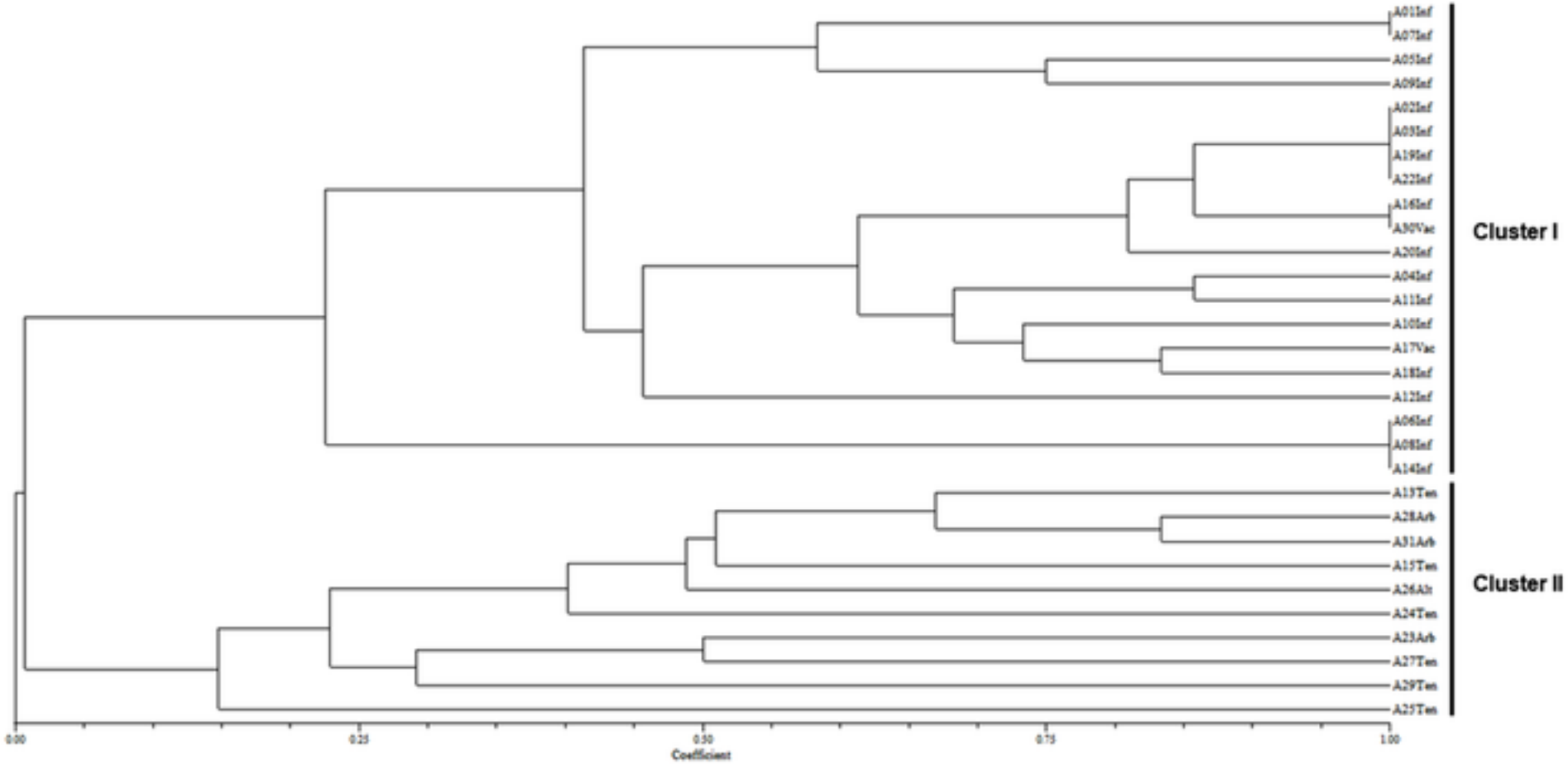


Figure 6

Click here to access/download;Figure;Figure 6.tif

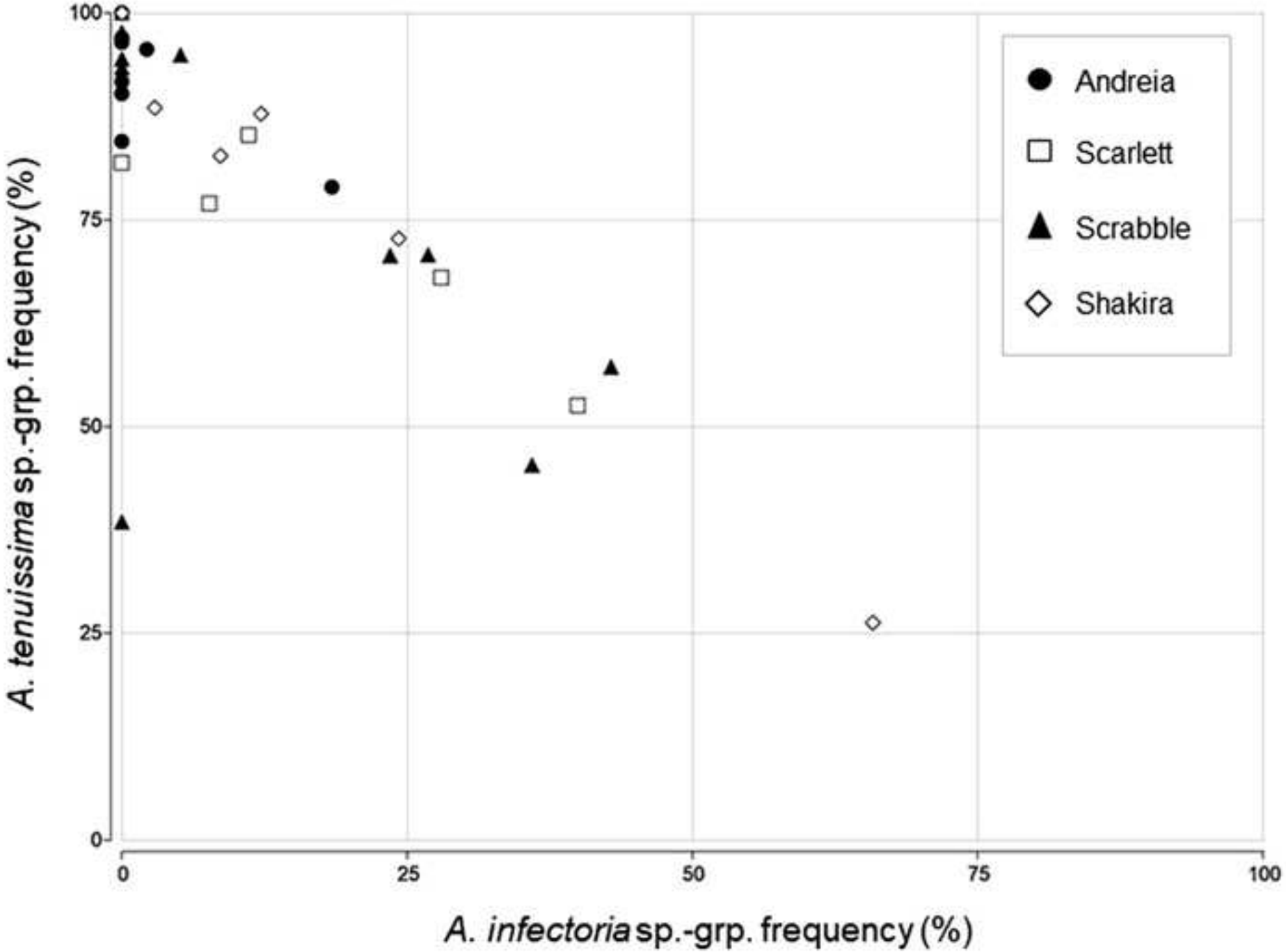
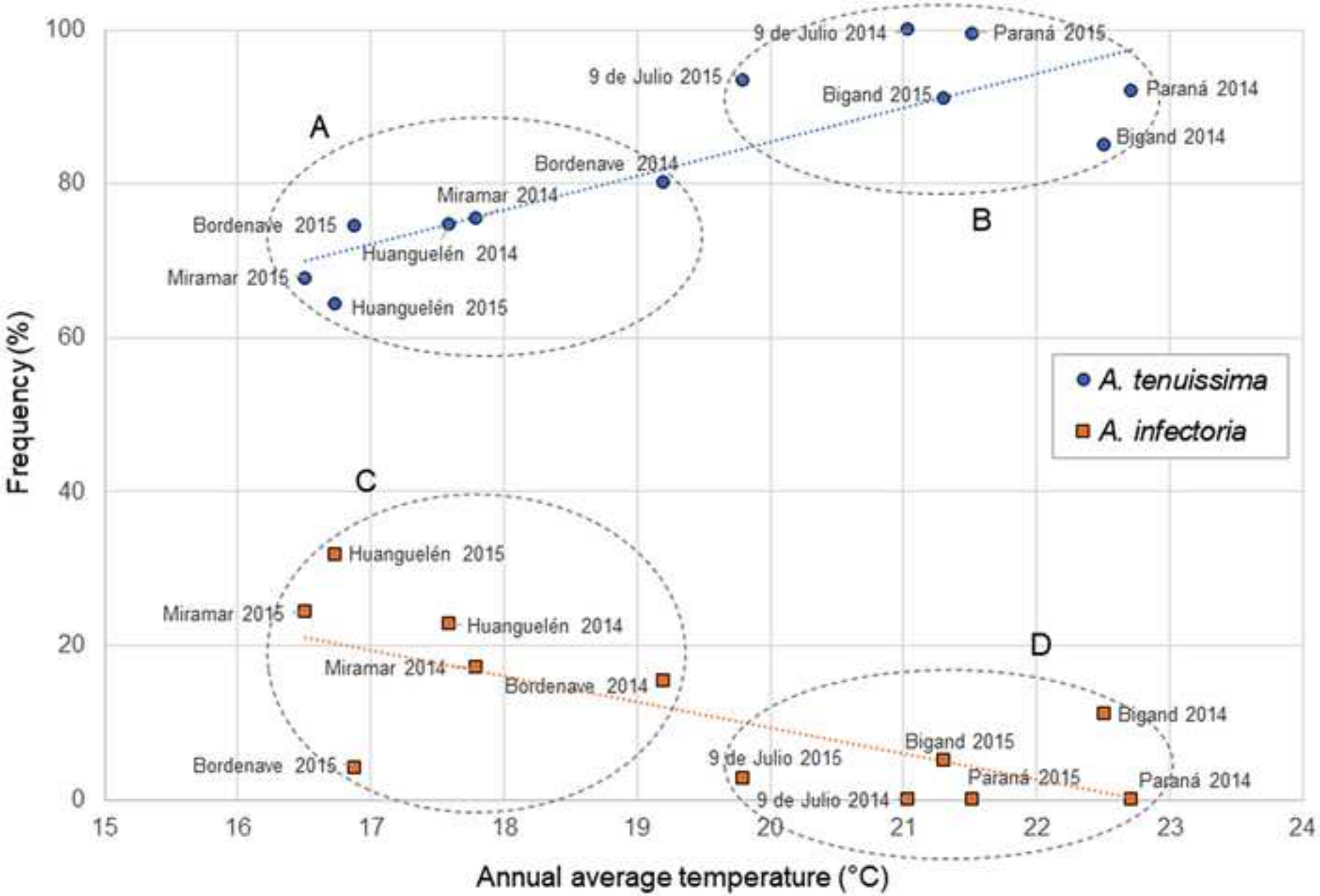
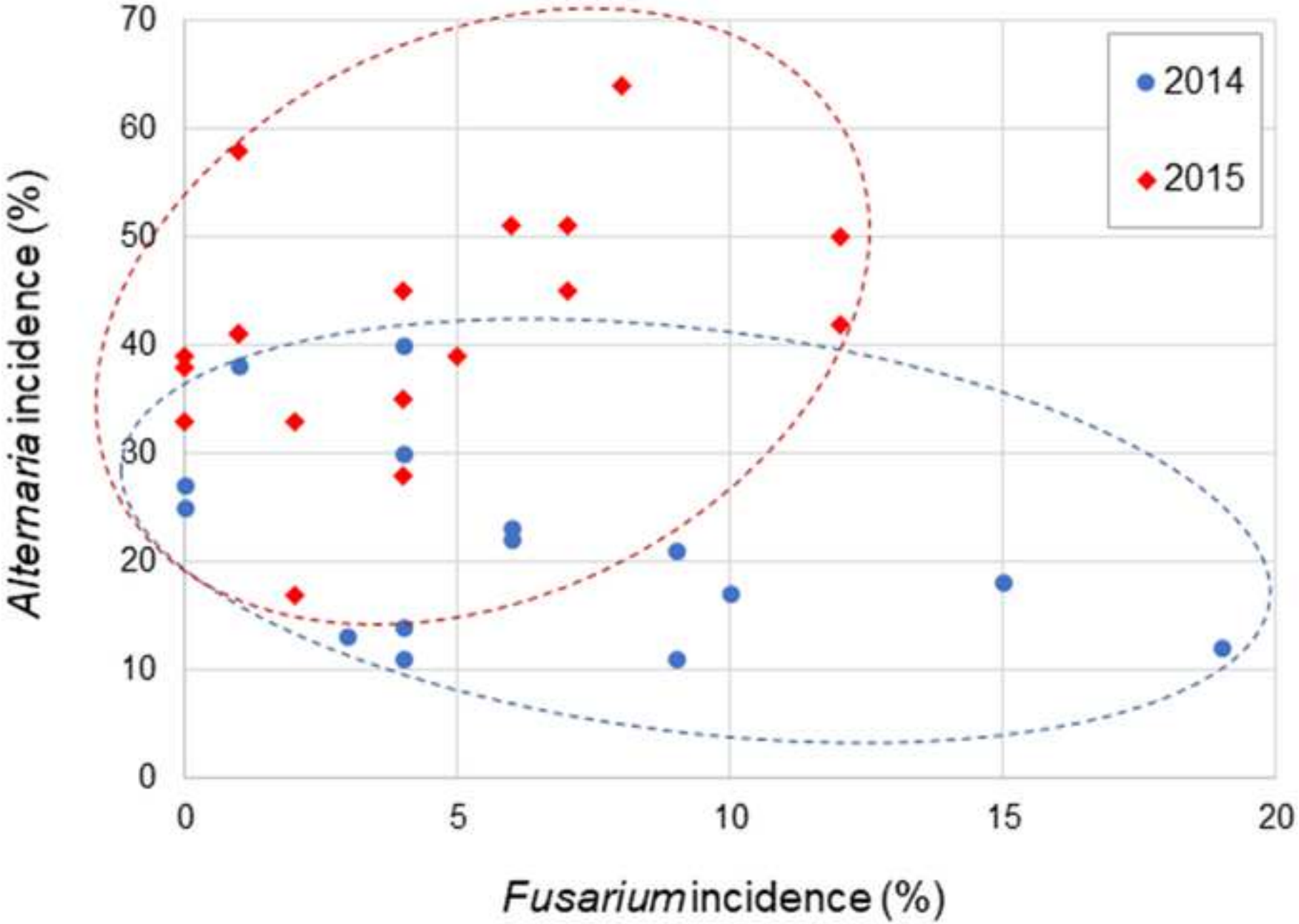


Figure 7



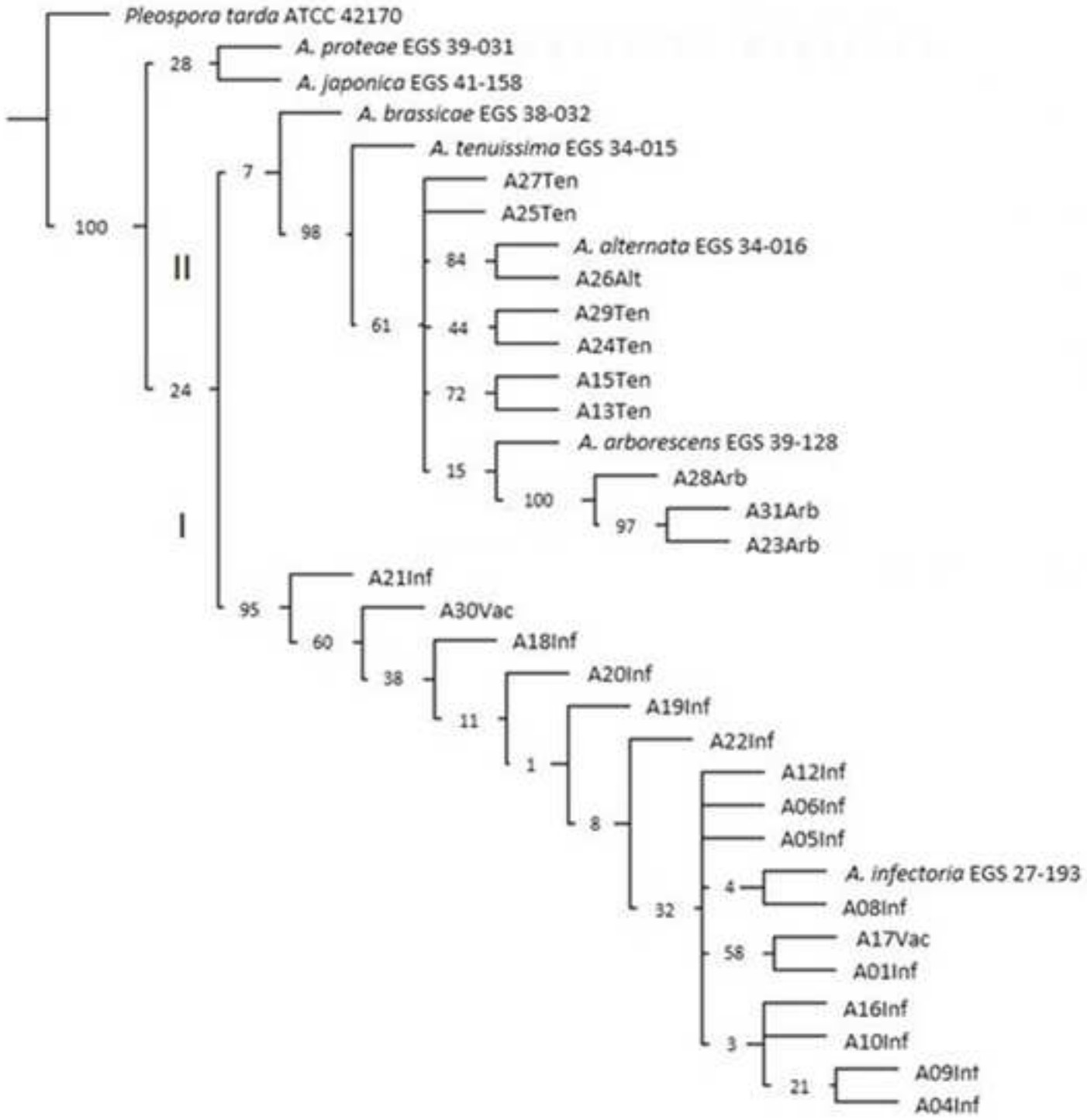
Supplementary Figure 1

Click here to access/download;Figure;Figure S1.tif



Supplementary Figure 2

Click here to access/download;Figure;Figure S2.tif



Supplementary Figure 3

Click here to access/download;Figure;Figure S3.tif