Identification and pathogenicity of groups of *Alternaria* species associated with a recent surge in leaf blotch disease and defoliation in French apple orchards

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Leaf blotch caused by *Alternaria* is a common disease in apple-producing regions. The disease is usually associated with one phylogenetic species and one species complex, *Alternaria alternata* and *Alternaria arborescens* species complex (*A. arborescens* SC) respectively. Both taxa may include the *Alternaria* apple pathotype, a quarantine or regulated pathogen in several countries. The apple pathotype is characterised by the production of host-selective toxins (HSTs) which are involved in pathogenicity towards the apple. A cluster of genes located on conditionally dispensable chromosomes (CDCs) are involved in the production of these HSTs (namely *AMT* in the case of the apple pathotype). Since 2016, leaf blotch and tree defoliation attributed to *Alternaria* spp. have been observed in apple-producing regions of central and south-eastern France. Our study aimed to identify the *Alternaria* species involved in apple tree defoliation and assess the presence of the apple pathotype in French orchards. From 2016-2018, 166 isolates were collected and identified by multi-locus sequence typing (MLST). This analysis revealed that all these French isolates belonged to either the *A. arborescens* SC or *A. alternata*. Specific PCR detection targeting three genes located on the CDC did not indicate the presence of the apple pathotype in France. Pathogenicity was assessed under laboratory conditions on detached leaves of Golden Delicious and Gala apple varieties for a representative subset of 28 *Alternaria* isolates. All the tested isolates were pathogenic on detached leaves of Golden Delicious and Gala, but no differences were observed between the pathogenicity levels of *A. arborescens* SC and *A. alternata*. However, the results of our pathogenicity test suggest that Golden Delicious is more susceptible than Gala to *Alternaria* leaf blotch.

Implications in the detection of the *Alternaria* apple pathotype and the taxonomic assignment of *Alternaria* isolates involved in *Alternaria* leaf blotch are discussed.

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27 ## Abstract
28

29 Leaf blotch caused by *Alternaria* is a common disease in apple-producing regions. The disease is
30 usually associated with one phylogenetic species and one species complex, *Alternaria alternata*
31 and *Alternaria arborescens* species complex (*A. arborescens* SC) respectively. Both taxa may
32 include the *Alternaria* apple pathotype, a quarantine or regulated pathogen in several countries.
33 The apple pathotype is characterised by the production of host-selective toxins (HSTs) which are
34 involved in pathogenicity towards the apple. A cluster of genes located on conditionally
35 dispensable chromosomes (CDCs) are involved in the production of these HSTs (namely *AMT* in
36 the case of the apple pathotype). Since 2016, leaf blotch and tree defoliation attributed to
37 *Alternaria* spp. have been observed in apple-producing regions of central and south-eastern
38 France. Our study aimed to identify the *Alternaria* species involved in apple tree defoliation and
39 assess the presence of the apple pathotype in French orchards. From 2016-2018, 166 isolates
40 were collected and identified by multi-locus sequence typing (MLST). This analysis revealed
41 that all these French isolates belonged to either the *A. arborescens* SC or *A. alternata*. Specific
42 PCR detection targeting three genes located on the CDC did not indicate the presence of the
43 apple pathotype in France. Pathogenicity was assessed under laboratory conditions on detached
44 leaves of Golden Delicious and Gala apple varieties for a representative subset of 28 *Alternaria*
45 isolates. All the tested isolates were pathogenic on detached leaves of Golden Delicious and
46 Gala, but no differences were observed between the pathogenicity levels of *A. arborescens* SC
47 and *A. alternata*. However, the results of our pathogenicity test suggest that Golden Delicious is
48 more susceptible than Gala to *Alternaria* leaf blotch. Implications in the detection of the
49 *Alternaria* apple pathotype and the taxonomic assignment of *Alternaria* isolates involved in
50 *Alternaria* leaf blotch are discussed.

51 ## Introduction
52
53 *Alternaria* spp. are ubiquitous fungi comprising more than 300 different species. They have
54 different lifestyles and can be isolated from a large number of substrates (Thomma 2003;
55 Woudenberg et al. 2013). *Alternaria* spp. are major pathogenic fungi in agriculture and the food
56 industry, leading to serious diseases in many economically important crops (Meena et al. 2017;
57 Thomma 2003). Several taxa of *Alternaria* have been associated with *Alternaria* leaf blotch
58 (ALB) and *Alternaria* fruit spot (AFS) diseases (Armitage et al. 2015; Gur et al. 2017; Harteveld
59 et al. 2013b). ALB is characterised by the development of round or irregular brown spots on
60 leaves, bordered by dark brown to purple margins (Rotondo et al. 2012). These symptoms
61 generally start in late spring or early summer, developing to yellowing leaves that can lead to
62 early tree defoliation and a premature fruit drop associated with a reduction in tree vigour and
63 fruit quality over the following years (Harteveld et al. 2013b; Rotondo et al. 2012). ALB may
64 cause up to 80% of defoliation in some susceptible apple cultivars (Filajdić & Sutton 1991) and
65 consequently may drastically decrease fruit yields (Harteveld et al. 2013b; Horlock 2006). Less
66 frequent, AFS is characterised by necrotic spots on the skin of the fruit surrounded by a red halo
67 centred on the lenticels (Harteveld et al. 2013b; Horlock 2006; Rotondo et al. 2012) and in some
68 cases can result in calyx cracking and fruit rot (Gur et al. 2017). AFS may consequently
69 downgrade the fruit's value, resulting in a significant financial burden to apple growers (Gur et
70 al. 2017; Harteveld et al. 2014). Both ALB and AFS have been reported in nearly all apple-
71 producing regions of the world (Dickens & Cook 1995; Filajdić & Sutton 1991; Gur et al. 2017;
72 Harteveld et al. 2013b; Kim et al. 1986; Ozgonen & Karaca 2006; Rotondo et al. 2012;
73 Wenneker et al. 2018). Taxa causing ALB and AFS are part of the *Alternaria* section *Alternaria*
74 that comprises the small-spored *Alternaria* species. As for the whole genus *Alternaria,*

75 identification of isolates within the section *Alternaria* is challenging due to morphological
76 plasticity and genetic similarity (Armitage et al. 2015; Lawrence et al. 2013; Woudenberg et al.
77 2013). However, recent advances in multi-gene phylogeny, comparative genomics and
78 transcriptomics have allowed the different *Alternaria* sections to be redefined and delineated,
79 with accurate molecular differentiation and identification of isolates (Armitage et al. 2020;
80 Woudenberg et al. 2015a). Woudenberg et al. (2015a), for example, have shown that the
81 *Alternaria* section *Alternaria* consists of 11 phylogenetic species and one species complex. The
82 taxonomic implications of this study are major because 35 morphospecies, which could not be
83 distinguished through multi-gene phylogeny, were synonymised under *Alternaria alternata*
84 (including the morphospecies and important plant pathogens *A. alternata*, *A. tenuissima* and *A.*
85 *citri*). ALB and AFS have been commonly associated with the phylogenetic species *A. alternata*
86 and the *Alternaria arborescens* species complex (*A. arborescens* SC) (Gur et al. 2017; Harteveld
87 et al. 2013b; Rotondo et al. 2012; Toome-Heller et al. 2018; Wenneker et al. 2018), with both
88 taxa also known as saprophytic and generalist opportunistic pathogens affecting a variety of
89 important crops (Armitage et al. 2015; Thomma 2003). Both *A. alternata* and *A. arborescens* SC
90 may include the apple pathotype, which has been recently shown to be polyphyletic (Armitage et
91 al. 2020). The apple pathotype, formerly known as *Alternaria mali,* causes significant problems
92 in apple orchards in south-eastern Asia (Li et al. 2019), was responsible for ALB in south-eastern
93 USA in the early nineties (Filajdić & Sutton 1991) and has been associated with severe AFS in
94 Israel (Gur et al. 2017). It is also listed either as a quarantine or a regulated pathogen in several
95 countries throughout the world (https://gd.eppo.int/). In *Alternaria*, pathotypes are characterised
96 by the production of polyketide host-selective toxins (HSTs), which are linked to pathogenicity
97 affecting specific hosts (Tsuge et al. 2013). To date, at least seven pathotypes have been

98 described, each producing a unique HST essential to pathogenicity in apples (*AMT*), Japanese
99 pears *(AKT*), strawberries (*AFT*), tangerines (*ACT*), tomatoes (*AAL*) rough lemons (*ACR*) and
100 tobacco (*AT*) (Tsuge et al. 2013; Wang et al. 2019). The production of these HSTs involves a
101 cluster of genes located on conditionally (or accessory) dispensable chromosomes (CDCs), so
102 named because they are not essential for saprophytic growth and reproduction of pathogens
103 (Hatta et al. 2002; Wang et al. 2019). In the case of the apple pathotype, at least 17 genes could
104 be involved in synthesis of *AMT* apple toxin (Harimoto et al. 2007) but so far only four, i.e.
105 *AMT1*, *AMT2*, *AMT3* and *AMT4*, have been demonstrated to be involved in this process. To date,
106 molecular detection of this pathotype is only possible by PCR targeting one of the genes
107 involved in the production of the *AMT* apple toxin (Armitage et al. 2020; Harimoto et al. 2007;
108 Johnson et al. 2000) or by identifying these genes in the genome of *Alternaria* isolates using
109 bioinformatics (Armitage et al. 2020). However, molecular taxonomic assignment to either
110 *Alternaria alternata* or the *A. arborescens* SC in routine diagnostics requires the construction of
111 multi-gene phylogenies (Armitage et al. 2015; Harteveld et al. 2013b; Rotondo et al. 2012;
112 Woudenberg et al. 2015a). Indeed, multi-locus sequence typing (MLST) with relevant
113 phylogenetic markers for the *Alternaria* genus has been used to identify *A. alternata* and the *A.*
114 *arborescens* SC. It has also enabled researchers to understand their association with ALB and
115 AFS in several countries (Armitage et al. 2015; Gur et al. 2017; Harteveld et al. 2013b; Rotondo
116 et al. 2012; Toome-Heller et al. 2018; Wenneker et al. 2018).
117 ALB has been observed for years in French orchards without causing serious damage. However,
118 since 2016, significant defoliation in trees infected by *Alternaria* has been reported in four
119 regions of central and south-eastern France. The reported presence of the apple pathotype in
120 northern Italy (Rotondo et al. 2012) has raised serious concerns for both the French plant health

121 authorities and apple growers. The first objective of this study was to assess whether the upsurge
122 in ALB symptoms observed in French orchards was due to the emergence of the apple pathotype.
123 To identify the pathogens responsible for these unusual cases of defoliation, we conducted
124 MLST analyses to determine the phylogenetic position of the French *Alternaria* isolates. We also
125 assessed the presence of the apple pathotype by PCR tests targeting two genes involved in the
126 production of the *AMT* apple toxin, and a gene found in the apple pathotype CDC that has
127 homologue genes in the pear and strawberry pathotypes (Armitage et al. 2020). Pathogenicity
128 tests were then carried out and Koch's postulates were assessed on the Gala and Golden
129 Delicious apple cultivars using a representative panel of isolates. Finally, both the phylogenetic
130 position and pathogenicity of the French *Alternaria* isolates were compared with *Alternaria*
131 isolates from different countries and/or isolated from crops other than apple.

132 ## Materials & Methods

133 **Isolate collection**

134

135 French *Alternaria* spp. isolates (166) were obtained from symptomatic field samples of leaves
136 (156) and fruit (10) from ten different apple cultivars or cultivar groups collected in apple
137 orchards in four major apple-producing regions in central and south-eastern France: Auvergne-
138 Rhône-Alpes (ARA), Provence-Alpes-Côtes d'Azur (PACA), Occitanie (OCC) and Nouvelle-
139 Aquitaine (NA). The samples were collected over a 3-year period (seasons 2016-17, 2017-18 and
140 2018-19, Table 1, Supplementary material S1). Leaves and fruit surfaces were first disinfected
141 with 70 % ethanol and necrotic spots were excised using a sterile scalpel blade then plated onto
142 Petri dishes containing malt medium supplemented with chloramphenicol (0.2 g/L) (MALTCH
143 medium). The cultures were incubated for four to seven days at 22 °C with a 12 h alternating
144 dark and light cycling period. A plug of actively growing cultures was then transferred to malt

145 extract medium and incubated under the same conditions as described above. The isolate
146 collection was supplemented with 43 *Alternaria* isolates either associated with ALB or AFS
147 from Australia (16), Israel (8), Italy (14) and New Zealand (5). Furthermore, the Food and
148 Environmental Research Agency (FERA) contributed 12 isolates obtained from fruit
149 importations showing AFS and intercepted in the UK (unknown origin). Nine additional ALB or
150 AFS isolates were obtained from the Westerdijk Institute's collection (https://www.wi.knaw.nl).
151 Other *Alternaria* isolates associated with post-harvest apple rot problems in Argentina (4) and
152 South Africa (8) were also included. Additionally, the collection was completed with *Alternaria*
153 isolates from other hosts belonging to different botanical families (19 isolates). Five isolates in
154 the collection were identified as apple pathotype isolates. All the isolates were single-spored
155 prior to analysis. Details of the isolate collection studied in this work are presented in
156 Supplementary material S1.
157

158 **Genomic DNA extraction and loci sequencing**

159 DNA extractions were performed with approximately 0.5 g of *Alternaria* mycelium, scraped
160 from a fresh culture on malt extract, using the NucleoSpin® Plant II kit (Macherey Nagel).
161 Mycelium was ground by placing two sterilised steel beads (3 mm in diameter) in an Eppendorf
162 tube containing mycelium with 400 µL of lysis buffer and 10 µL of RNAse (both provided with
163 the DNA extraction kit). Samples were subsequently ground twice for 60 s at 30 Hz in a MM
164 400 mixer mill (Retch®). DNA was extracted according to the manufacturer's instructions. The
165 concentration of the DNA extracts (100 µL final volume) was estimated with a NanoDrop TM
166 2000 Spectrophotometer (ThermoFisher). All the extracted DNA was stored at -30°C until use.
167 The endopolygalacturonase (EndoPG) and the *Alternaria* major allergen (Alta-1) genes, two loci

168 commonly used in *Alternaria* identification and phylogenetics (Table 2, Armitage et al. 2015;
169 Harteveld et al. 2013b; Lawrence et al. 2013), were sequenced for all the isolates. In addition, the
170 anonymous region OPA 10-2 (Table 2, Rotondo et al. 2011, Woudenberg et al. 2015) was
171 sequenced for a subset of 100 isolates and used to assess putative differences in taxonomic
172 identification by comparison with EndoPG and Alta-1. For PCR amplification of the three loci,
173 the reaction mixtures contained 1X PCR reaction buffer (HGS Diamond Taq, Eurogentec), 2.5
174 mM $MgCl_2$ (4.0 mM for EndoPG), 4 x 0.25 mM dNTPs, 0.2 µM of forward and reverse primers
175 (Table 2), 1 U of HGS diamond Taq (Eurogentec), 2 µL of DNA extract and molecular grade
176 water to complete up to 25 µL. PCR conditions consisted of an initial denaturation step at 95°C
177 for 10 min, followed by 40 cycles at 94°C for 45 s, annealing temperatures of 57°C for Alta-1,
178 56°C for EndoPG and 62°C for OPA 10-2 for 30 s (Table 2), 72°C for 1 min and a final
179 extension step at 72°C for 7 min. The GENEWIZ sequencing platform (Leipzig, Germany) was
180 used for bidirectional Sanger sequencing of the amplicons. Consensus sequences were obtained
181 after manual correction using the Geneious R11 programme.

182 **Phylogenetic analysis**

183

184 The EndoPG, Alta-1 and OPA 10-2 sequence datasets generated in our study were supplemented
185 with data from previous studies. The sequence datasets that enabled taxonomic identification of
186 isolates in these previous studies (Armitage et al. 2015; Gur et al. 2017; Harteveld et al. 2013b;
187 Rotondo et al. 2012; Woudenberg et al. 2015a) were used as a reference in our phylogenetic
188 analysis. As we performed molecular identification using an MLST approach, we decided to use
189 the taxonomy of the *Alternaria* section *Alternaria* proposed by Woudenberg et al. (2015a),
190 which consists of 11 phylogenetic species and one species complex. In other words, we used the
191 *Alternaria alternata* phylogenetic species without including any results of morphospecies (e.g. *A.*

192 *tenuissima* was taxonomically assigned to the phylogenetic species *A. alternata*), an approach
193 used in other studies that described isolates morphologically (Armitage et al. 2015; Rotondo et
194 al. 2012). DNA sequences were first analysed with SeaView version 4 (Gouy et al. 2010). These
195 analyses included sequence alignments using MUSCLE (Edgar 2004) and elimination of poorly
196 aligned positions with Gblocks (Talavera & Castresana 2007). MrBayes version 3.2 (Ronquist et
197 al. 2012b) was used for multi-locus phylogeny analysis on concatenated sequences for EndoPG
198 and Alta-1 (two-locus MSLT phylogenetic tree) and EndoPG, Alta-1 and OPA 10-2 (three-locus
199 MLST phylogenetic tree) separately. Runs were performed under the Bayesian MCMC model
200 jumping approach, which provides a convenient alternative to model selection prior to analysis
201 (command lset applyto= (all) nst=mixed). In model jumping, the Markov Chain Monte Carlo
202 (MCMC) sampler explores di□erent models and weights the results according to the posterior
203 probability of each model (Ronquist et al. 2012a). Four MCMC chains were run using the default
204 heating with tree sampling performed every 5000 generations. Runs were performed for at least
205 20 million generations, and stopped when the standard deviation of split frequencies was below
206 0.01 (Ronquist et al. 2012a). Homologous sequences of Alta-1 and Endo-PG for *A. brassicicola*
207 (isolate Abra43) were used as an outgroup in all the generated trees. The consensus tree was
208 obtained by using the command sumt. The resulting phylogenetic trees were visualised and
209 annotated with the interactive tree of life (iTOL) online tool (Letunic & Bork 2016). The
210 taxonomic identification of 100 isolates using the concatenated trees EndoPg/Alta-1 and
211 EndoPg/Alta-1/OPA 10-2 was compared with the function tanglegram implemented in
212 DENDROSCOPE 3.2.10 (Huson & Scornavacca 2012). A subset of single-locus sequence data
213 for the corresponding loci was submitted to Genbank (accession nos. MN975269-MN975340,
214 Supplementary material S1).

215 **PCR detection of the Alternaria apple pathotype**

216

217 We searched for the *Alternaria* apple pathotype among all the French isolates by PCR targeting
218 two genes involved in AMT apple toxin biosynthesis — namely *AMT1* and *AMT2* — using
219 primers developed by Johnson et al. (2000) and Harimoto et al. (2007) respectively (Table 2).
220 Additionally, 44 out of these French isolates were also tested by PCR targeting *AMT14*, a gene
221 found in the apple pathotype toxin gene cluster and for which homologous genes also exist in
222 pear and strawberry pathotypes (Armitage et al. 2020). Other non-French isolates were tested by
223 PCR targeting either *AMT1* / *AMT2* or *AMT1* / *AMT2* / *AMT14* genes (27 and 47 isolates
224 respectively, Supplementary material S1). PCRs were performed in 25-µL reaction mixtures
225 containing 1X PCR reaction buffer (HGS Diamond Taq, Eurogentec), 2.5 mM $MgCl_2$, 4 x 0.25
226 mM dNTPs, 0.2 µM of forward and reverse primers (Table 2), 1 U of HGS diamond Taq
227 (Eurogentec), 2 µL of DNA extract and molecular grade water to complete up to 25 µL. PCR
228 conditions comprised an initial 10 min denaturation step at 95°C followed by 40 cycles of a
229 denaturation step at 94°C for 30 s, an annealing step at 65°C for *AMT1*, 57°C for *AMT2*, 66°C
230 for *AMT14* (Table 2) and an extension step at 72°C for 60 s. These cycles were followed by a
231 final extension at 72°C for 7 min. All PCRs were performed in duplicate. Controls were included
232 in all reactions. Positive controls included either gDNA (*Alternaria* apple pathotype isolate
233 LSVM 75) for *AMT14* testing or a plasmid solution of *AMT1* and *AMT2* genes inserted in a
234 vector using the pCR4-TOPO cloning kit (Invitrogen) following the manufacturer's instructions.
235 Negative controls consisted of sterile distilled water (SDW).

236

237

238 **Pathogenicity assays and Koch's postulates**

239

240 Twenty-eight isolates were selected for pathogenicity assays: ten *A. alternata,* 17 *A. arborescens*

241 SC and one *A. brassicicola* (Supplementary material S1). No apple pathotype isolate could be

242 tested, because no strain in our collection could produce a sufficient amount of conidia in

243 culture, despite several attempts. The *A. brassicicola* isolate Abra43 was included as a non-

244 pathogenic control. Assays were performed on detached apple leaves from the cultivars Golden

245 Delicious clone X972 and Gala clone X4712. For simplicity, both clones will be referred

246 hereafter to as Golden Delicious and Gala. Spore suspensions were obtained from isolates grown

247 at 22°C for 21 days on MALTCH medium under specific light condition cycles (Carvalho et al.

248 2008): an incubation period of 7 days under a 12 h alternating dark then light cycling period

249 followed by 2 days under an 8 h-UV/16 h dark conditions (UV light induced by a black

250 fluorescent near UV lamp (Philips/15W, T8-BLB) and a final cycle of 12 days in full darkness.

251 For each isolate, the inoculum was obtained by flooding the culture with 2 mL of SDW before

252 dislodging spores by scraping the plate with an L-shaped spreader. After a filtration step with a

253 sterile gauze, the spore suspensions were counted and adjusted to a concentration of $1x10^5$

254 conidia/mL with a haemocytometer. Leaves from the third or fourth node were detached from

255 fresh branches of apple saplings grown in a glasshouse. Eight leaves cleaned with 70% ethanol

256 were placed in plastic boxes containing two white absorbent paper towels humidified with SDW

257 and conserved at ambient temperature overnight prior to leaf inoculations. An experimental

258 replicate consisted of one strain inoculated on five different leaves (placed in five different

259 plastic boxes) per cultivar. Unwounded abaxial leaf surfaces were inoculated at six points with

260 10 µL of conidial suspension. Each plastic box contained a negative control that consisted of one

261 leaf inoculated with SDW. Inoculated leaves were incubated at 20°C for 10 days under an

262 alternating 12 h dark then light cycling period. Each isolate was tested twice in independent
263 experiments. The results from each experiment were analysed separately at the three data
264 collection times: 4, 7 and 10 days post-inoculation (dpi). Two types of analysis were performed.
265 For data from 4 dpi, a zero-inflated Poisson general linear mixed model (GLMM) was used to
266 assess the number of lesions per leaf. The model included the following explanatory variables:
267 taxon of the tested isolate (*A. alternata* or *A. arborescens* SC), the apple leaf variety (Gala or
268 Golden Delicious), and an experiment repeat variable (each isolate was tested twice in
269 independent experiments). An isolate effect was taken into account as a random variable. For
270 data from 7 and 10 dpi, zero-inflated beta GLMMs were performed. On both 7 and 10 dpi the
271 response variable was the proportion of the diseased leaf area on detached leaves that
272 corresponds to the lesion size. The diseased leaf area proportion (necrosis) was assessed by
273 visual inspection and coded between 0 and 1. The model included the same explanatory and
274 random effect variables used for the 4 dpi data model: taxon, variety, repeat and isolate (random
275 effect). All GLMM analyses (on 4, 7 and 10 dpi) took into account only isolates of *A. alternata*
276 and *A. arborescens* SC, as only one *A. brassicicola* isolate was used, which is not enough to be
277 included in the isolate random effect. All the models were run in the R environment (version
278 4.0.3) using the glmmTMB package (Brooks et al. 2017). Excess zeros were in all cases tested
279 with the function testZeroInflation of the DHARMa R package (Hartig 2017), which compares
280 the distribution of expected zeros in the data with the observed zeros. Model residual diagnostics
281 of all models were performed with the DHARMa package. Analysis of variance type II
282 (ANOVA type II) analyses were performed to assess the effect of each explanatory variable.
283 Koch's postulates were assessed by the re-isolation on MALTCH medium of 23 randomly

284 chosen tested isolates (Supplementary material S1) and the re-sequencing of Alta-1 and EndoPG
285 loci.

286 Results

287 Molecular identification of strains

288 Concatenated Alta-1 and Endo-PG sequences resulted in a length of 900 bp; these sequences were
289 used for phylogenetic analyses. Depending on the isolate, the number of bases/residues that
290 differed between isolates of *A. alternata* and isolates of other taxa of the *Alternaria* section
291 *Alternaria* included in the analysis (*A. arborescens* SC, *A. gaisen*, *A. longipes*, *A. gossypina* and
292 *A. alstroermeriae*) ranged from 6 to 37. The two-marker phylogenetic tree distinguished two major
293 clades: *A. alternata* and *A. arborescens* SC (Figure 1). The *A. alternata* phylogenetic clade
294 encompassed four subclades, while the *A. arborescens* SC encompassed two. The analysis could
295 also distinguish these two clades from other taxa in the *Alternaria* section *Alternaria*: *A. gaisen*,
296 *A. longipes*, *A. gossypina* and *A. alstroemeriae* (Figure 1). The three concatenated genes (Alta-
297 1/Endo-PG/OPA10-2) resulted in a 1534-bp sequence length which included 18–65
298 differences/residues between *A. alternata* and other taxa of the *Alternaria* section *Alternaria*
299 included in the analysis. As for the two-marker concatenated tree, the phylogenetic analysis using
300 three markers distinguished two major clades, *A. alternata* and *A. arborescens* SC (Supplementary
301 material S2). It could also distinguish these clades from other taxa in the *Alternaria* section
302 *Alternaria* (*A. gaisen*, *A. longipes*, *A. gossypina* and *A. alstroemeriae*). The phylogenetic tree
303 pattern was similar to that determined with only two loci (i.e. Alta-1 and EndoPG). The *A.*
304 *alternata* clade was divided into four subclades, whereas the *A. arborescens* SC was separated into
305 two subclades. Adding a third locus to the analysis (OPA 10-2) did not improve the resolution
306 within *A. alternata* and *A. arborescens* SC (Supplementary material S2). The two phylogenetic

307 analyses did, however, refine identification: two Australian strains were consequently assigned to
308 *A. alternata* whereas they had previously been assigned to *A. longipes* (BRIP46356 and
309 BRIP46455) by Harteveld et al. (2013b) (Figure 1, Supplementary materiel S1).

310 **Identification of *Alternaria* isolates causing ALB in France**
311
312 Isolates from France were identified as either *A. arborescens* SC (91 isolates, 55%) or *A. alternata*
313 (75 isolates, 45%) based on the taxonomic identification with the Alta-1 and EndoPG markers. No
314 changes in the taxonomic identification were observed for the subset of isolates with sequences of
315 Alta-1, EndoPG and OPA 10-2 markers (Supplementary material S1). The distribution of isolates
316 differed according to the cultivar (Table 1). It was observed that *A. arborescens* SC isolates were
317 more frequent on Gala and Golden Delicious varieties, whereas *A. alternata* isolates were more
318 frequent on Reinette grise du Canada, Dalinette and Crimson. On Braeburn and Pink lady, there
319 was a similar number of isolates of *A. arborescens* SC and *A. alternata*. On Garance and GoldRush
320 (Coop38cov) varieties, too few isolates were recovered to make any comparison (Table 1).

321 **Screening for the *Alternaria* apple pathotype**
322
323 None of the 166 French isolates were identified as the apple pathotype by PCR tests targeting the
324 *AMT* apple toxin (*AMT1*, *AMT2*) or cross-pathotype (*AMT14*) loci (Supplementary material S1).
325 All five apple pathotype reference isolates behaved as expected and yielded positive results for
326 *AMT1* and *AMT2* PCR tests and also for the tests targeting *AMT14,* which is common to apple,
327 pear and strawberry pathotypes (Supplementary material S1). However, four isolates formerly
328 identified as apple pathotype in earlier studies in Italy (Rotondo et al. 2012) and Israel (Gur et al.
329 2017) gave negative PCR results for the three loci in our conditions, thus overturning their
330 identification (Supplementary material S1).

331

332 **Pathogenicity assays and Koch's postulates**

333 On 4 dpi, 27 isolates — including the negative control *A. brassicicola* — were able to induce at
334 least one necrotic spot on detached leaves of apple varieties Golden Delicious and Gala. The only
335 exception was an *A. arborescens* SC isolate (16_489b3a) that did not induce necrotic spots on
336 detached Gala leaves. The zero-inflated Poisson GLMM used to assess the number of lesions per
337 leaf on 4 dpi, indicated a significant effect of the experiment repetition (Type II Wald; χ^2=17.75;
338 df=2; p=0.00014) but not of the taxa (Type II Wald; χ^2=2.44; df=1; p=0.118) or the apple variety
339 (Type II Wald; χ^2=0.06; df=1; p=0.810) (Figure 2). All 28 tested isolates were able to induce leaf
340 blotch after 7 dpi on Golden Delicious and Gala. On 7 dpi, the zero-inflated beta GLMM used to
341 assess the proportion of the diseased leaf area indicated a significant effect of the experiment
342 repetition (Type II Wald; χ^2=84.013; df=2; p<0.001) and the apple variety (Type II Wald;
343 χ^2=17.296; df=1; p=3.199e^{-5}), but not of the taxa (Type II Wald; χ^2=0.001; df=1; p=0.97) (Figure
344 3a). On 10 dpi the zero-inflated beta GLMM indicated a significant effect of the experiment
345 repetition (Type II Wald; χ^2=91.82; df=2; p<2.2e^{-16}) and the apple variety (Type II Wald; χ^2=11.80;
346 df=1; p=0.0006), but not of the taxa (Type II Wald; χ^2=0.06; df=1; p=0.801) (Figure 3b). On 7 and
347 10 dpi, leaves of the Golden Delicious variety were more susceptible than those of Gala, as
348 measured by the proportion of the diseased leaf area (Figure 3). Raw measurements at 4, 7 and 10
349 dpi are presented in the supplementary material S4 section. Finally, the identity of 23 of these
350 strains was confirmed by re-isolating and sequencing (Alta-1 and EndoPG loci), fulfilling Koch's
351 postulates (Supplementary material S1). No disease symptoms were observed on leaves inoculated
352 with water. Some examples of the carried pathogenicity tests are shown in the supplementary
353 material S5 section.

354

355 Discussion

356 Severe symptoms of apple leaf blotch (ALB) reported in French orchards since 2016 raised
357 questions about the identity of the *Alternaria* spp. responsible for unprecedented early
358 defoliation in orchards. Our principal objectives were to verify whether the *Alternaria* apple
359 pathotype could have emerged in France, to accurately identify the *Alternaria* taxa involved in
360 such defoliation, and to assess the pathogenicity of tested isolates.
361

362 **The *Alternaria* apple pathotype was not found in French orchards**

363 We firstly checked whether we were witnessing the emergence of the apple pathotype in French
364 orchards. We did this through PCR assays targeting three genes located in the conditionally
365 dispensable chromosome (CDC) — *AMT1* (Johnson et al. 2000), *AMT2* (Harimoto et al. 2008)
366 and *AMT14* (Armitage et al. 2020) — which characterises isolates of the apple pathotype. Our
367 results showed that this pathogen was not present in France within the sampled regions and
368 years. To date, molecular detection of the apple pathotype has only been possible by PCR tests
369 that target genes present in the CDC. These targets are associated with secondary metabolite
370 clusters involved in the production of the apple pathotype host specific toxin *AMT* (Armitage et
371 al. 2020; Harimoto et al. 2007; Johnson et al. 2000). Although all our apple pathotype reference
372 strains gave positive results using the three markers, the Italian and Israeli strains previously
373 identified as apple pathotype by PCR based on the amplification of a modified PCR test targeting
374 *AMT1* (Rotondo et al. 2012) and *AMT3* (Gur et al. 2017) gave negative results in our study with
375 tests targeting loci *AMT1*, *AMT2* and *AMT14*. In the case of the Israeli strains, the primers
376 targeting *AMT3* (Harimoto et al. 2007), showed unsatisfactory results in our preliminary tests as

377 several unexpected bands appeared after gel electrophoresis of the PCR product (data not shown)
378 and these primers were discarded for subsequent molecular tests. The results obtained with the
379 Italian strains are more difficult to explain because the initial study of Rotondo et al. (2012)
380 performed several confirmation tests (including sequencing of the products). One hypothesis that
381 may explain the difficulty in amplifying these loci is the occurrence of partial or total
382 chromosomal loss in isolates. This phenomenon has previously been reported in the apple
383 pathotype by Johnson et al. (2001) and is due to chromosomal instability in culture. To avoid this
384 problem, in the analysis of French isolates, our tests targeting *AMT1*, *AMT2* and *AMT14* were
385 performed right after isolation, avoiding several subculturing cycles. However, in all the cases
386 where the presence of *AMT1*, *AMT2* and *AMT14* was assessed (in the five reference isolates;
387 Supplementary material S1), all three gene-specific PCR assays gave positive results. Based on
388 the results of our study, we suggest that the apple pathotype should be detected from pure
389 cultures by using at least two or more of the existing molecular tests to target *AMT1* and *AMT2*,
390 which is a good option if the objective is to specifically detect the apple pathotype. By
391 optimising and validating current tools or developing new molecular tests, it might be possible to
392 detect diseases *in planta* from symptomatic leaves, which could avoid isolate subculturing cycles
393 while minimising the risk of chromosomal loss.
394
395 **Co-existence of *Alternaria alternata* and the *Alternaria arborescens* species complex in**
396 **French orchards**
397 The second objective of this study was to identify *Alternaria* species or groups associated with
398 *Alternaria* leaf blotch (ALB) and *Alternaria* fruit spot (AFS) in French orchards. The
399 phylogenetic trees generated after using MLST clearly showed that these diseases are caused by

400 two phylogenetic clades: *A. alternata* and *A. arborescens* SC*,* regardless of the apple cultivar.
401 Our results also showed that both taxa may co-exist in the same orchard. These results confirm
402 that these two *Alternaria* taxa are the major cause of ALB and AFS in regions of the world
403 where these diseases have been studied so far (Gur et al. 2017; Harteveld et al. 2013b; Rotondo
404 et al. 2012; Toome-Heller et al. 2018; Wenneker et al. 2018). In addition, our results suggest that
405 sequencing two loci, i.e. Alta-1 and EndoPG, is enough to be able to distinguish *Alternaria*
406 isolates involved in these diseases. Firstly, these two loci enable the two major phylogenetic
407 clades — *A. alternata* and *A. arborescens* SC — to be distinguished*.* Secondly, the loci also
408 clearly distinguish these two clades from other *Alternaria* taxa within the *Alternaria* section.
409 Including the OPA 10-2 locus did not substantially improve molecular identification of the
410 strains.
411
412 ***Alternaria alternata* and the *Alternaria arborescens* SC are responsible for defoliation in**
413 **French apple orchards**
414 We showed that all the strains isolated from necrotic leaves were able to produce symptoms on
415 detached apple leaves of varieties Gala and Golden Delicious. The latter variety was more
416 susceptible under our conditions, as shown by measurements of the diseased leaf area after 7 and
417 10 dpi, a quantitative trait generally used to measure pathogen aggressiveness. Our results also
418 showed that the entire subset of *Alternaria* isolated from apple leaves or fruit fulfilled Koch's
419 postulates. The pathogenicity tests showed that there are no significant differences between
420 isolates of *A. alternata* and *A. arborescens* SC as assessed by the number of lesions per leaf on 4
421 dpi or the proportion of the diseased leaf area on 4 and 7 dpi. Both results are in agreement with
422 previous studies that suggest that pathogenicity may be isolate-dependent rather than species-

423 dependent (Harteveld et al. 2014; Rotondo et al. 2012). One of the limits of our study is that we
424 could not assess the pathogenicity of any of the reference apple pathotype strains because too
425 few spores could be obtained during cultivation. It is important to highlight, however, that
426 previous studies comparing the pathogenicity of apple pathotype isolates with other *Alternaria*
427 isolates in apples have shown discrepant results: while Armitage et al. (2020) showed that apple
428 pathotype strains were significantly more pathogenic than other isolates that do not carry CDCs,
429 Rotondo et al. (2012) did not observe any difference in levels of pathogenicity between apple
430 pathotype isolates and other *Alternaria* isolated from apple leaves or fruit. Unexpectedly, we
431 observed symptoms on apple leaves inoculated by *A. brassicicola,* which has never been
432 reported as pathogenic on apple to our knowledge. These results suggest that *Alternaria* isolates
433 from other *Alternaria* sections that do not carry CDCs involved in the production of HTS may
434 also cause ALB symptoms under controlled conditions. This is probably associated with the
435 production of nonspecific Alternaria toxins that can affect many plants regardless of whether
436 they are or are not a host of the pathogen (Tsuge et al. 2013). However, as shown here and
437 elsewhere, under natural conditions only small-spore Alternaria (*Alternaria* section *Alternaria*)
438 have so far been described as apple pathogens causing ALB and AFS. Recent genomic resources,
439 including the genome of *A. brassicicola* (Belmas et al. 2018) and isolates of *Alternaria* involved
440 in AFS and ALB (Armitage et al. 2020) will allow comparative genomics analysis that may
441 clarify these pathogenicity mechanisms.
442 Finally, this study identified the *Alternaria* taxa involved in ALB and AFS in France, but did not
443 determine the cause of the increased severity in these diseases over recent years (e.g.
444 introduction of the apple pathotype). However, alternative explanations may be suggested based
445 on previous epidemiological studies. Firstly, it seems that the disease develops better in

446 relatively hot (between > 20°C and 30°C) and rainy weather (Bhat et al. 2015; Filajdić & Sutton
447 1992; Harteveld et al. 2013a; Kim et al. 1986). Potential changes in these two parameters, or
448 other climatic factors, should be studied in greater depth in the French regions concerned in order
449 to draw conclusions. Another hypothesis is the introduction of more virulent strains. This could
450 occur by the long-distance movement of spores carried by wind currents that may have
451 transported *Alternaria* air inoculum into apple orchards from sources in other apple-producing
452 regions (Fernández-Rodríguez et al. 2015; Woudenberg et al. 2015b). Finally, the emergence of
453 fungicide resistance among strains should not be ruled out, considering that apple orchards are
454 treated intensely with fungicides, mainly used to control apple scab caused by *Venturia*
455 *inaequalis*, which also contributes to the control of ALB and AFS (Horlock 2006).
456

457 Conclusions

458 Since 2016, apple leaf blotch and fruit tree defoliation attributed to *Alternaria* spp. have been
459 observed in apple-producing regions in central and south-eastern France. The emergence of the
460 *Alternaria* apple pathotype was suspected following its observation in northern Italy. The
461 presence of the apple pathotype in French orchards was therefore assessed by a specific PCR
462 targeting three genes located on conditionally dispensable chromosomes across a large collection
463 of 166 *Alternaria* isolates from different varieties and production areas during the 2016-2018
464 period. Our results showed that the *Alternaria* apple pathotype was not present. Taxonomic
465 identification of these isolates, assessed by multi-locus sequence typing and construction of
466 phylogenetic trees, indicates that apple leaf blotch and fruit tree defoliation in France are
467 associated with isolates of *A. alternaria* and *A. arborescens* SC. Pathogenicity tests of a
468 subsample of isolates demonstrated that they were all able to induce necrotic symptoms on
469 detached apple leaves of the varieties Gala and Golden Delicious. Our results also showed that

470 there are no significant differences in levels of pathogenicity between isolates of *A. alternata* and
471 *A. arborescens* SC. Our controlled pathogenicity tests do suggest, however, that Golden
472 Delicious is more susceptible to *Alternaria* leaf blotch. In the future, genetic and epidemiological
473 approaches are required to clarify why *Alternaria* leaf blotch events have increased in frequency
474 and severity in some regions of France.

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476

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Table 1 (on next page)

Distribution of *Alternaria* isolates obtained from French orchards during the 2016-2019 period.

The table shows the apple variety, the number of samples, the taxa (*Alternaria arborescens* SC or *Alternaria alternata*) and the co-occurrence of the isolates in the same orchard. The samples were identified by sequencing EndoPg and Alta-1. a: varieties Galastar and Royal Gala included, b: variety Reinette grise du Canada (Canada), c: variety Crimson Crisp included and d: variety Rosy Glow included.

1 **Table 1.** Distribution of *Alternaria* isolates obtained from French orchards during the 2016-2019
2 period. The table shows the apple variety, the number of samples, the taxa (*Alternaria arborescens*
3 SC or *Alternaria alternata*) and the co-occurrence of the isolates in the same orchard. The samples
4 were identified by sequencing EndoPg and Alta-1. a: varieties Galastar and Royal Gala included,
5 b: variety Reinette grise du Canada (Canada), c: variety Crimson Crisp included and d: variety
6 Rosy Glow included.

Variety	No. of samples (including fruit samples)	*A. arborescens* species complex	*A. alternata*	Co-occurrence/sample (including fruit samples)
Braeburn	2	4	4	1
Gala[a]	13 (2)	16	8	3
Golden Delicious	10	39	10	5
Canada[b]	9	14	17	3
Dalinette	4	5	12	4
Crimson[c]	4	2	8	1
Belchard	1	1	1	1
Pink Lady[d]	7 (3)	9	10	4 (2)
Garance	1	0	2	0
GoldRush	1	1	3	1
Total	**52 (5)**	**91**	**75**	**23**

7

8

Table 2(on next page)

Characteristics of primer pairs used in this study for multi-locus sequence typing (MSLT) identification of isolates and specific PCR.

1 **Table 2.** Characteristics of primer pairs used in this study for multi-locus sequence typing (MSLT) identification of isolates and specific
2 PCR.

Locus / function	Primer	Primer sequence (5'-3')	Reference	Annealing temperature (°C)	Amplicon length (pb)
Alta-1 / Alternaria major allergen 1	Alt-for	ATGCAGTTCACCACCATCGC	(Hong et al. 2005)	57	472
	Alt-rev	ACGAGGGTGAYGTAGGCGTC			
EndoPG / Endopolygalacturonase	PG3	TACCATGGTTCTTTCCGA	(Andrew et al. 2009)	56	464
	PG2b	GAGAATTCRCARTCRTCYTGRTT			
OPA 10-2 / Anonymous noncoding region	OPA10-2L	TCGCAGTAAGACACATTCTACG	(Andrew et al. 2009)	62	634
	OPA10-2R	GATTCGCAGCAGGGAAACTA			
AMT-1 / Non-ribosomal peptide synthethase	LinF1	TATCGCCTGGCCACCTACGC	Johnson et al. (2000)	65	496
	LinR	TGGCCACGACAACCCACATA			
AMT-2 / Aldo-keto reductase	AMT2-f2	GTTGCAGAATCGCAAACTCA	(Roberts et al. 2012)	57	653
	AMT2-r2	GGCTCTTGGTCTCAAATCCA			
AMT-14 / Unknown function	AMT14-EMR-F	TTTCTGCAACGGCGKCGCTT	Armitage et al. (2015)		
	AMT14-EMR-R	TGAGGAGTYAGACCRGRCGC		66	436

3

Figure 1

Bayesian phylogenetic tree of Alta-1 and EndoPg markers. The tree was constructed with sequences of 352 *Alternaria* isolates (261 were generated in this study).

The colour legend refers to the Bayesian posterior probabilities of the tree nodes. *Alternaria alternata* isolates are shown in blue. The *Alternaria arborescens* SC is shown in red. Isolates from other taxonomic groups of the *Alternaria* section *Alternaria* are represented in orange (*Alternaria alstroemeriae*), purple (*Alternaria gaisen*), brown (*Alternaria longipes*) and grey (*Alternaria gossypina*).

Tree scale: 0.01

Figure 2

Mean number of leaf lesions per variety (Gala in red and Golden Delicious in blue) 4 days post-inoculation (4 dpi).

Results are reported for isolates of *Alternaria alternata*, *Alternaria arborescens* SC and *Alternaria brassicicola*, which were identified by multi-locus sequence typing (MLST). Pathogenicity tests were performed on unwounded abaxial leaf surfaces with six separate point inoculations of 10 µl of *Alternaria* conidial suspensions (concentration of $1x10^5$ conidia/µL). Statistical tests were only performed on *Alternaria alternata* and *Alternaria arborescens* SC. No differences were observed between isolates of *Alternaria alternata* and *Alternaria arborescens* SC or the apple variety (Golden and Gala). A significant effect of the experiment repetition (Type II Wald; $\chi^2=17.75$; $p<0.001$) was observed.

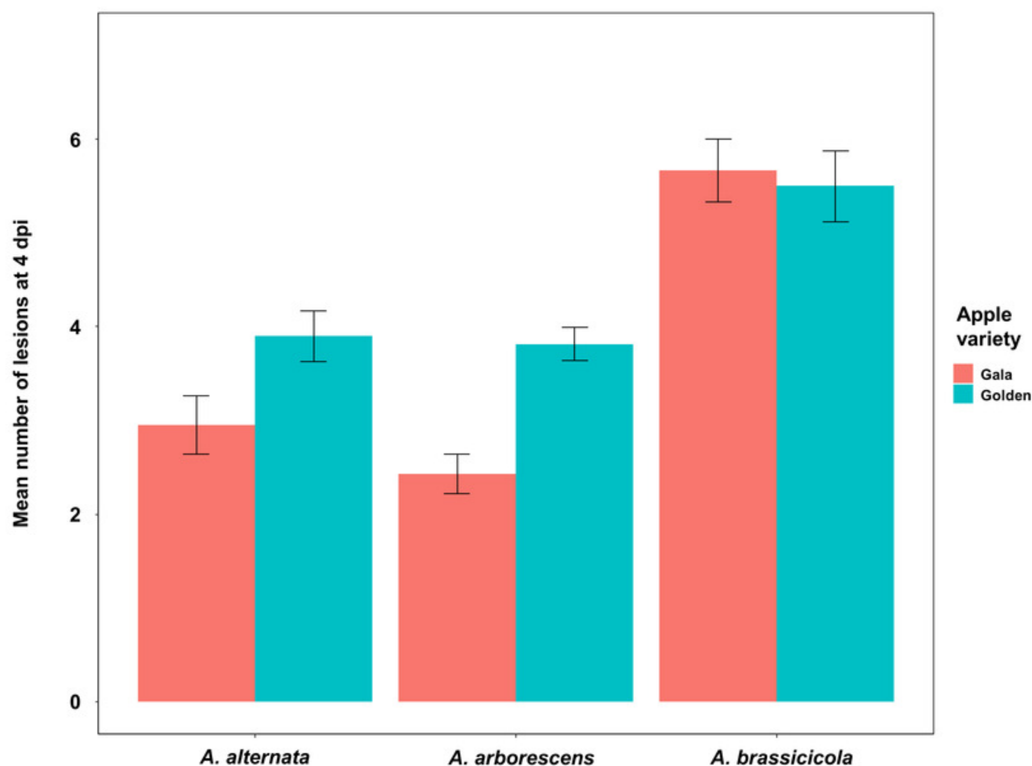

Figure 3

Mean proportion of the diseased leaf area per variety 7 and 10 days post-inoculation (7, 10 dpi) reported for *Alternaria alternata*, *Alternaria arborescens* SC and *Alternaria brassicicola.*

a. Mean proportion of the diseased leaf area per variety (Gala in red and Golden Delicious in blue) 7 days post-inoculation (7 dpi) reported for isolates of *Alternaria alternata*, *Alternaria arborescens* SC and *Alternaria brassicicola*, identified by multi-locus sequence typing (MLST). Statistical tests were only performed on *Alternaria alternata* and *Alternaria arborescens* SC. The results showed that on 7 dpi, leaves of the Golden Delicious variety were more susceptible than leaves of the Gala variety (Type II Wald; $\chi^2=17.296$; $p<0.001$). No differences were observed between isolates of *Alternaria alternata* and *Alternaria arborescens* SC. A significant effect of the experiment repetition (Type II Wald; $\chi^2=84.013$; $p<0.001$) was also observed.

b. Mean proportion of the diseased leaf area per variety (Gala in red and Golden Delicious in blue) 10 days after inoculation (10 dpi) reported for isolates of *Alternaria alternata*, *Alternaria arborescens* and *Alternaria brassicicola*, identified by multi-locus sequence typing (MLST). Statistical tests were only performed on *Alternaria alternata* and *Alternaria arborescens* SC. The results showed that on 10 dpi, leaves of the Golden Delicious variety were more susceptible than leaves of the Gala variety (Type II Wald; $\chi^2=11.80$; $p<0.001$). No differences were observed between isolates of Alternaria alternata and *Alternaria arborescens* SC. A significant effect of the experiment repetition (Type II Wald; $\chi^2=91.82$; $p<0.001$) was also observed.