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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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Abstract

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.

Introduction

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

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

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

Materials & Methods

Isolate collection

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

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

Phylogenetic analysis

The EndoPG, Alta-1 and OPA 10-2 sequence datasets generated in our study were supplemented with data from previous studies. The sequence datasets that enabled taxonomic identification of isolates in these previous studies (Armitage et al. 2015; Gur et al. 2017; Harteveld et al. 2013b; Rotondo et al. 2012; Woudenberg et al. 2015a) were used as a reference in our phylogenetic analysis. As we performed molecular identification using an MLST approach, we decided to use the taxonomy of the *Alternaria* section *Alternaria* proposed by Woudenberg et al. (2015a), which consists of 11 phylogenetic species and one species complex. In other words, we used the *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

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

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

Results

Molecular identification of strains

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

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

Identification of *Alternaria* isolates causing ALB in France

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

Screening for the *Alternaria* apple pathotype

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

several unexpected bands appeared after gel electrophoresis of the PCR product (data not shown) and these primers were discarded for subsequent molecular tests. The results obtained with the Italian strains are more difficult to explain because the initial study of Rotondo et al. (2012) performed several confirmation tests (including sequencing of the products). One hypothesis that may explain the difficulty in amplifying these loci is the occurrence of partial or total chromosomal loss in isolates. This phenomenon has previously been reported in the apple pathotype by Johnson et al. (2001) and is due to chromosomal instability in culture. To avoid this problem, in the analysis of French isolates, our tests targeting *AMT1*, *AMT2* and *AMT14* were performed right after isolation, avoiding several subculturing cycles. However, in all the cases where the presence of *AMT1*, *AMT2* and *AMT14* was assessed (in the five reference isolates; Supplementary material S1), all three gene-specific PCR assays gave positive results. Based on the results of our study, we suggest that the apple pathotype should be detected from pure cultures by using at least two or more of the existing molecular tests to target *AMT1* and *AMT2*, which is a good option if the objective is to specifically detect the apple pathotype. By optimising and validating current tools or developing new molecular tests, it might be possible to detect diseases *in planta* from symptomatic leaves, which could avoid isolate subculturing cycles while minimising the risk of chromosomal loss.

Co-existence of *Alternaria alternata* and the *Alternaria arborescens* species complex in French orchards

The second objective of this study was to identify *Alternaria* species or groups associated with *Alternaria* leaf blotch (ALB) and *Alternaria* fruit spot (AFS) in French orchards. The phylogenetic trees generated after using MLST clearly showed that these diseases are caused by

two phylogenetic clades: *A. alternata* and *A. arborescens* SC*,* regardless of the apple cultivar. Our results also showed that both taxa may co-exist in the same orchard. These results confirm that these two *Alternaria* taxa are the major cause of ALB and AFS in regions of the world where these diseases have been studied so far (Gur et al. 2017; Harteveld et al. 2013b; Rotondo et al. 2012; Toome-Heller et al. 2018; Wenneker et al. 2018). In addition, our results suggest that sequencing two loci, i.e. Alta-1 and EndoPG, is enough to be able to distinguish *Alternaria* isolates involved in these diseases. Firstly, these two loci enable the two major phylogenetic clades — *A. alternata* and *A. arborescens* SC — to be distinguished. Secondly, the loci also clearly distinguish these two clades from other *Alternaria* taxa within the *Alternaria* section. Including the OPA 10-2 locus did not substantially improve molecular identification of the strains.

Alternaria alternata and the _Alternaria arborescens_ SC are responsible for defoliation in French apple orchards

We showed that all the strains isolated from necrotic leaves were able to produce symptoms on detached apple leaves of varieties Gala and Golden Delicious. The latter variety was more susceptible under our conditions, as shown by measurements of the diseased leaf area after 7 and 10 dpi, a quantitative trait generally used to measure pathogen aggressiveness. Our results also showed that the entire subset of *Alternaria* isolated from apple leaves or fruit fulfilled Koch's postulates. The pathogenicity tests showed that there are no significant differences between isolates of *A. alternata* and *A. arborescens* SC as assessed by the number of lesions per leaf on 4 dpi or the proportion of the diseased leaf area on 4 and 7 dpi. Both results are in agreement with previous studies that suggest that pathogenicity may be isolate-dependent rather than species-

dependent (Harteveld et al. 2014; Rotondo et al. 2012). One of the limits of our study is that we could not assess the pathogenicity of any of the reference apple pathotype strains because too few spores could be obtained during cultivation. It is important to highlight, however, that previous studies comparing the pathogenicity of apple pathotype isolates with other *Alternaria* isolates in apples have shown discrepant results: while Armitage et al. (2020) showed that apple pathotype strains were significantly more pathogenic than other isolates that do not carry CDCs, Rotondo et al. (2012) did not observe any difference in levels of pathogenicity between apple pathotype isolates and other *Alternaria* isolated from apple leaves or fruit. Unexpectedly, we observed symptoms on apple leaves inoculated by *A. brassicicola,* which has never been reported as pathogenic on apple to our knowledge. These results suggest that *Alternaria* isolates from other *Alternaria* sections that do not carry CDCs involved in the production of HTS may also cause ALB symptoms under controlled conditions. This is probably associated with the production of nonspecific Alternaria toxins that can affect many plants regardless of whether they are or are not a host of the pathogen (Tsuge et al. 2013). However, as shown here and elsewhere, under natural conditions only small-spore Alternaria (*Alternaria* section *Alternaria*) have so far been described as apple pathogens causing ALB and AFS. Recent genomic resources, including the genome of *A. brassicicola* (Belmas et al. 2018) and isolates of *Alternaria* involved in AFS and ALB (Armitage et al. 2020) will allow comparative genomics analysis that may clarify these pathogenicity mechanisms.

Finally, this study identified the *Alternaria* taxa involved in ALB and AFS in France, but did not determine the cause of the increased severity in these diseases over recent years (e.g. introduction of the apple pathotype). However, alternative explanations may be suggested based 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

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

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References

Armitage AD, Barbara DJ, Harrison RJ, Lane CR, Sreenivasaprasad S, Woodhall JW, and Clarkson JP. 2015. Discrete lineages within *Alternaria alternata* species group: Identification using new highly variable loci and support from morphological characters. *Fungal Biology* 119:994-1006. http://dx.doi.org/10.1016/j.funbio.2015.06.012

Armitage AD, Cockerton HM, Sreenivasaprasad S, Woodhall J, Lane CR, Harrison RJ, and Clarkson JP. 2020. Genomics Evolutionary History and Diagnostics of the *Alternaria alternata* Species Group Including Apple and Asian Pear Pathotypes. *Frontiers in Microbiology* 10. 10.3389/fmicb.2019.03124

499 Belmas E, Briand M, Kwasiborski A, Colou J, N'Guyen G, Iacomi B, Grappin P, Campion C,
500 Simoneau P, Barret M, and Guillemette T. 2018. Genome sequence of the necrotrophic
501 plant pathogen *Alternaria brassicicola* Abra43. *Genome Announc* 6.
502 Bhat K, Peerzada S, and Anwar A. 2015. *Alternaria* epidemic of apple in Kashmir. *African*
503 *Journal of Microbiology Research* 9:831-837.
504 Brooks ME, Kristensen K, van Benthem KJ, Magnusson A, Berg CW, Nielsen A, Skaug HJ,
505 Machler M, and Bolker BM. 2017. glmmTMB balances speed and flexibility among
506 packages for zero-inflated generalized linear mixed modeling. *The R journal* 9:378-400.
507 Carvalho DD, Alves E, Batista TR, Camargos RB, and Lopes EA. 2008. Comparison of
508 methodologies for conidia production by *Alternaria alternata* from citrus. *Brazilian*
509 *Journal of Microbiology* 39:792-798.
510 Dickens JSW, and Cook RTA. 1995. Japanese pear black spot and apple *Alternaria* blotch.
511 *EPPO Bulletin* 25:651-659. 10.1111/j.1365-2338.1995.tb01117.x
512 Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high
513 throughput. *Nucleic Acids Research* 32:1792-1797. 10.1093/nar/gkh340
514 Fernández-Rodríguez S, Sadyś M, Smith M, Tormo-Molina R, Skjøth CA, Maya-Manzano JM,
515 Silva-Palacios I, and Gonzalo-Garijo Á. 2015. Potential sources of airborne *Alternaria*
516 spp. spores in south-west Spain. *Science of The Total Environment* 533:165-176.
517 Filajdić N, and Sutton T. 1991. Identification and distribution of *Alternaria mali* on apples in
518 North Carolina and susceptibility of different varieties of apples to *Alternaria* blotch. *Plant*
519 *Disease* 75:1045-1048.
520 Filajdić N, and Sutton T. 1992. Influence of temperature and wetness duration on infection of
521 apple leaves and virulence of different isolates of *Alternaria mali*. *Phytopathology*
522 82:1279-1283.
523 Gouy M, Guindon S, and Gascuel O. 2010. SeaView version 4: a multiplatform graphical user
524 interface for sequence alignment and phylogenetic tree building. *Mol Biol Evol* 27:221-
525 224.
526 Gur L, Reuveni M, and Cohen Y. 2017. Occurrence and etiology of *Alternaria* leaf blotch and
527 fruit spot of apple caused by *Alternaria alternata* f. sp. *mali* on cv. Pink lady in Israel.
528 *European Journal of Plant Pathology* 147:695-708. 10.1007/s10658-016-1037-0
529 Harimoto Y, Hatta R, Kodama M, Yamamoto M, Otani H, and Tsuge T. 2007. Expression
530 Profiles of Genes Encoded by the Supernumerary Chromosome Controlling AM-Toxin
531 Biosynthesis and Pathogenicity in the Apple Pathotype of *Alternaria alternata*. *Molecular*
532 *Plant-Microbe Interactions* 20:1463-1476. 10.1094/MPMI-20-12-1463
533 Harimoto Y, Tanaka T, Kodama M, Yamamoto M, Otani H, and Tsuge T. 2008. Multiple copies
534 of AMT2 are prerequisite for the apple pathotype of *Alternaria alternata* to produce
535 enough AM-toxin for expressing pathogenicity. *Journal of General Plant Pathology*
536 74:222-229. 10.1007/s10327-008-0089-1
537 Harteveld D, Akinsanmi OA, Chandra K, and Drenth A. 2013a. Timing of Infection and
538 Development of *Alternaria* Diseases in the Canopy of Apple Trees. *Plant Disease*
539 98:401-408. 10.1094/PDIS-06-13-0676-RE
540 Harteveld D, Akinsanmi OA, and Drenth A. 2013b. Multiple *Alternaria* species groups are
541 associated with leaf blotch and fruit spot diseases of apple in Australia. *Plant Pathology*
542 62:289-297. 10.1111/j.1365-3059.2012.02637.x
543 Harteveld D, Akinsanmi OA, and Drenth A. 2014. Pathogenic variation of *Alternaria* species
544 associated with leaf blotch and fruit spot of apple in Australia. *European Journal of Plant*
545 *Pathology* 139:789-799. 10.1007/s10658-014-0433-6
546 Hartig F. 2017. DHARMa: residual diagnostics for hierarchical (multi-level/mixed) regression
547 models. *R package version 01* 5.

548 Hatta R, Ito K, Hosaki Y, Tanaka T, Tanaka A, Yamamoto M, Akimitsu K, and Tsuge T. 2002. A
549 conditionally dispensable chromosome controls host-specific pathogenicity in the fungal
550 plant pathogen *Alternaria alternata*. *Genetics* 161:59-70.
551 Horlock CM. 2006. *Management of Alternaria leaf and fruit spot in apples*: Horticulture Australia.
552 Hothorn T, Bretz F, and Westfall P. 2008. Simultaneous inference in general parametric models.
553 *Biometrical Journal* 50:346-363.
554 Huson DH, and Scornavacca C. 2012. Dendroscope 3: an interactive tool for rooted
555 phylogenetic trees and networks. *Systematic biology* 61:1061-1067.
556 Johnson L, Johnson RD, Akamatsu H, Salamiah A, Otani H, Kohmoto K, and Kodama M. 2001.
557 Spontaneous loss of a conditionally dispensable chromosome from the *Alternaria*
558 *alternata* apple pathotype leads to loss of toxin production and pathogenicity. *Current*
559 *Genetics* 40:65-72.
560 Johnson R, Johnson L, Kohmoto K, Otani H, Lane CR, and Kodama M. 2000. A Polymerase
561 Chain Reaction-Based Method to Specifically Detect *Alternaria alternata* Apple
562 Pathotype (*A. mali*), the Causal Agent of *Alternaria* Blotch of Apple. *Phytopathology*
563 90:973-976. 10.1094/PHYTO.2000.90.9.973
564 Kim C-H, Cho W-D, and Kim S-C. 1986. An empirical model for forecasting *Alternaria* leaf spot
565 in apple. *Korean journal of applied entomology* 25:221-228.
566 Lawrence DP, Gannibal PB, Peever TL, and Pryor BM. 2013. The sections of *Alternaria*:
567 formalizing species-group concepts. *Mycologia* 105:530-546. 10.3852/12-249
568 Letunic I, and Bork P. 2016. Interactive tree of life (iTOL) v3: an online tool for the display and
569 annotation of phylogenetic and other trees. *Nucleic Acids Research* 44:W242-W245.
570 Li Y, Hu X-L, Trigiano R, Aldwinckle H, and Cheng Z-M. 2019. Evaluation of 110 Apple Cultivars
571 for Resistance to *Alternaria* Blotch Caused by *Alternaria alternata* Apple Pathotype.
572 *HortScience* 54:1268-1274. 10.21273/HORTSCI13841-18
573 Meena M, Gupta SK, Swapnil P, Zehra A, Dubey MK, and Upadhyay RS. 2017. *Alternaria*
574 Toxins: Potential Virulence Factors and Genes Related to Pathogenesis. *Frontiers in*
575 *Microbiology* 8:1451.
576 Ozgonen H, and Karaca G. 2006. First report of *Alternaria mali* causing necrotic leaf spot of
577 apples in Turkey. *Plant Pathology* 55:578-578.
578 Ronquist F, Huelsenbeck J, and Teslenko M. 2012a. MrBayes version 3.2 manual: tutorials and
579 model summaries. *Available with the software distribution at*
580 *mrbayessourceforgenet/mb32_manualpdf*.
581 Ronquist F, Teslenko M, Van Der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L,
582 Suchard MA, and Huelsenbeck JP. 2012b. MrBayes 3.2: efficient Bayesian phylogenetic
583 inference and model choice across a large model space. *Systematic biology* 61:539-542.
584 Rotondo F, Collina M, Brunelli A, and Pryor BM. 2012. Comparison of *Alternaria* spp. collected
585 in Italy from apple with *A. mali* and other AM-toxin producing strains. *Phytopathology*
586 102:1130-1142. 10.1094/PHYTO-04-12-0076-R
587 Talavera G, and Castresana J. 2007. Improvement of phylogenies after removing divergent and
588 ambiguously aligned blocks from protein sequence alignments. *Systematic biology*
589 56:564-577.
590 Thomma BPHJ. 2003. *Alternaria* spp.: from general saprophyte to specific parasite. *Molecular*
591 *Plant Pathology* 4:225-236. 10.1046/j.1364-3703.2003.00173.x
592 Toome-Heller M, Baskarathevan J, Burnip G, and Alexander B. 2018. First report of apple leaf
593 blotch caused by *Alternaria arborescens* complex in New Zealand. *New Zealand Journal*
594 *of Crop and Horticultural Science*:1-6.
595 Tsuge T, Harimoto Y, Akimitsu K, Ohtani K, Kodama M, Akagi Y, Egusa M, Yamamoto M, and
596 Otani H. 2013. Host-selective toxins produced by the plant pathogenic fungus *Alternaria*
597 *alternata*. *FEMS Microbiology Reviews* 37:44-66. 10.1111/j.1574-6976.2012.00350.x

598 Wang M, Fu H, Shen X-X, Ruan R, Rokas A, and Li H. 2019. Genomic features and evolution of
599 the conditionally dispensable chromosome in the tangerine pathotype of *Alternaria*
600 *alternata*. *Molecular Plant Pathology* 20:1425-1438. https://doi.org/10.1111/mpp.12848
601 Wenneker M, Pham K, Woudenberg J, and Thomma BP. 2018. First report of *Alternaria*
602 *arborescens* species complex causing leaf blotch and associated premature leaf drop of
603 'Golden delicious' apple trees in the Netherlands. *Plant Disease* 102:1654.
604 Woudenberg J, Seidl M, Groenewald J, de Vries M, Stielow J, Thomma B, and Crous P. 2015a.
605 *Alternaria* section *Alternaria*: Species, formae speciales or pathotypes? *Studies in*
606 *Mycology* 82:1-21.
607 Woudenberg J, Van Der Merwe N, Jurjević Ž, Groenewald J, and Crous P. 2015b. Diversity and
608 movement of indoor *Alternaria alternata* across the mainland USA. *Fungal Genetics and*
609 *Biology* 81:62-72.
610 Woudenberg JHC, Groenewald JZ, Binder M, and Crous PW. 2013. *Alternaria* redefined.
611 *Studies in Mycology* 75:171-212. http://dx.doi.org/10.3114/sim0015
612 .

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.

Table 1. 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.

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**

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.

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

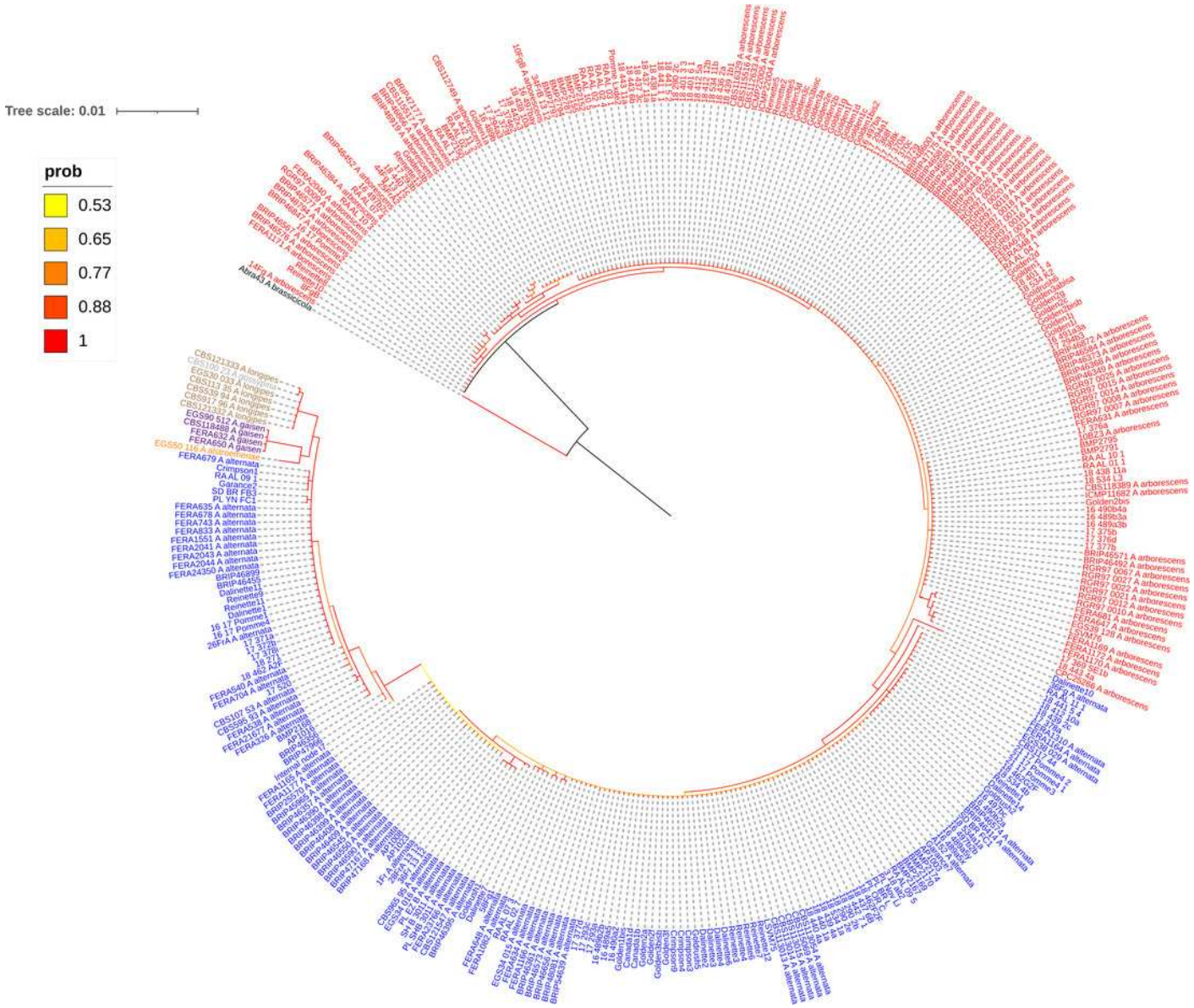
Tree scale: 0.01
prob
0.53
0.65
0.77
0.88
1

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.

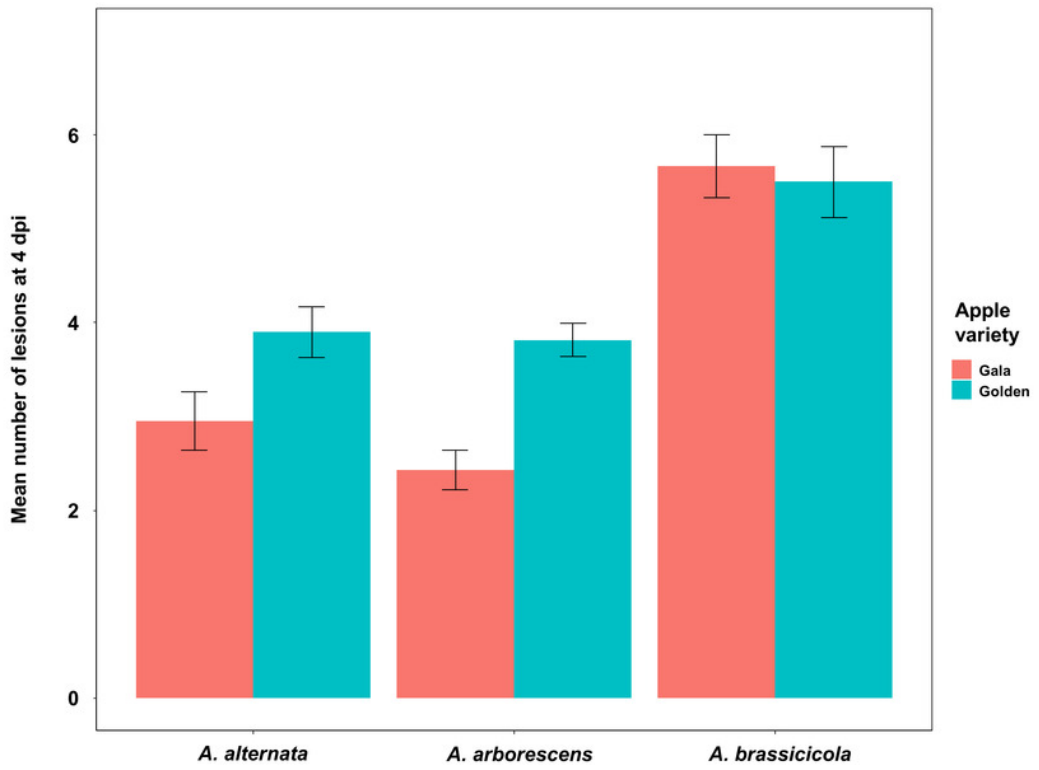

Figure 3

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; χ^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; χ^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; χ^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; χ^2=91.82; p<0.001) was also observed.