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Identification of *A. arborescens*, *A. grandis*, and *A. protenta* as new members of the European *Alternaria* population on potato

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ABSTRACT

Alternaria species, primarily the small-spored *Alternaria alternata* and the large-spored *Alternaria solani*, are considered a serious threat to potato cultivation. To develop control strategies, it is important to gain insight into the *Alternaria* population. Based on the sequence analyses of the internal transcribed spacer region (ITS) and the *glyceraldehyde-3-phosphate dehydrogenase* gene, the small-spored and large-spored *Alternaria* isolates could be separated from each other. Sequence analyses of the *calmodulin* gene and the RNA polymerase second largest subunit showed that besides *A. solani* also *A. grandis* and *A. protenta* were present in the large-spored *Alternaria* population. Sequence analyses of the *Alternaria* major allergen gene *Alt a 1* and the *elongation factor-α* revealed that both *A. alternata* and species belonging to the *Alternaria arborescens* species complex were present in the small-spored *Alternaria* population. Furthermore, according to the *histone h3* sequence the members of the *A. arborescens* species complex could be subdivided into two groups. Concerning the fitness, it was concluded that the mycelium growth rate of the large-spored isolates was significantly lower compared to the growth rate of the small-spored isolates. In contrast, the spore-germinating capacity and early growth of the large-spored isolates was greater compared to those of the small-spored isolates. Within the groups of small-spored and large-spored isolates there were no significant differences in fitness between the species.

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Introduction

Alternaria species are encountered worldwide in a variety of habitats. Species belonging to this genus can attack various host plants, including crops such as apple, broccoli, cauliflower, carrot, potato, Chinese cabbage, tomato, citrus fruit, different plants used as ornamentals and a number of weeds (Mamgain et al. 2013).

The species within the genus *Alternaria* encompass a considerable morphological diversity. Therefore, in the past, a division into subgeneric groups, based on conidium characteristics has been proposed. According to Neergaard (1945), the genus *Alternaria* can be subdivided into three sections based on catenulation (chain-formation of conidia). Simmons (1992) proposed an organisation of the genus into 14 species-groups, each typified by a representative species, based upon characteristics of conidia and catenulation. However, a major disadvantage of morphological classification is that these characteristics are not only affected by intrinsic factors, but also by environmental conditions, subsequent subculturing, cultivation medium etc (Thomma 2003).

Recently, the genus *Alternaria* has been divided into 25 sections based on multi-gene phylogeny (Lawrence et al. 2013; Woudenberg et al. 2013; Grum-Grzhimaylo et al. 2016). To delineate the large-spored *Alternaria* species in the section Porri, Woudenberg et al. (2014) used a multi-locus sequence analysis. Based on the sequence analysis of the DNA internal transcribed spacer region (ITS) region, the glyceraldehyde-3-phosphate dehydrogenase (*Gpd*) gene, the RNA polymerase second largest subunit (*RPB2*), the translation elongation factor- α (*EF- α*) and the *Alternaria* major allergen gene *Alt a 1*, the section Porri could be divided into 63 morphospecies (e.g. *Alternaria solani*, *Alternaria protenta*, *Alternaria grandis*, etc.).

In order to arrive at a clearer and more stable species classification in the section *Alternaria*, which contains most of the small-spored *Alternaria* species, Woudenberg et al. (2015) performed whole-genome sequencing supplemented with a transcriptome analysis and multi-gene sequencing. The multi-gene phylogeny was based on the sequences of parts of nine genomic regions, namely the ITS regions 1 and 2 and intervening 5.8S nrDNA, the 18S nrDNA (SSU), the 28S nrDNA (LSU), the *Gpd* gene, *RPB2*, *EF- α* , *Alt a 1*, endopolygalacturonase (*endoPG*) and an anonymous gene region (OPA10-2). Based on genome and transcriptome comparisons and molecular phylogenies, these authors concluded that the section *Alternaria* consists of 11 phylogenetic species (e.g. *Alternaria alternata*, *Alternaria longipes*, *Alternaria gossypina* etc.) and one species complex (the *Alternaria arborescens* species complex (AASC)). The morphospecies present in this complex (*Alternaria geophila*, *A. arborescens*, *Alternaria cerealis*, *Alternaria senecionicola*) could not be resolved with the set of nine partial gene sequences used in the study of Woudenberg et al. (2015) and a more detailed study, possibly using whole-genome sequences of additional isolates from this species complex, is needed.

To date, the small-spored *A. alternata* and the large-spored *A. solani* are the predominant pathogenic species reported on potato, causing premature defoliation, which has a significant impact on tuber yield and potato quality. Recently, also the large-spored species *A. grandis* (Rodrigues et al. 2010) and *A.*

protenta (Woudenberg et al. 2014) have been identified as members of the *Alternaria* species complex in potatoes. Moreover, besides *A. alternata*, also the small-spored species: *Alternaria tenuissima*, *Alternaria dumosa*, *Alternaria interrupta*, *A. arborescens*, *Alternaria infectoria*, and *Alternaria arbusti* have been associated with *Alternaria* leaf blight of potato in Iran (Taheri et al. 2009; Ardestani et al. 2010), Russia (Orina et al. 2012), China (Zheng & Wu 2013) and the U.S. (Tymon et al. 2016). Woudenberg et al. (2015) synonymised *A. tenuissima*, *A. dumosa*, and *A. interrupta* under *A. alternata*, although according to Zheng et al. (2015), *A. tenuissima* can be distinguished from *A. alternata* based on the partial coding sequence of the histone *h3* gene. *A. tenuissima* isolates possess a 106 bp insert in the partial coding sequence of the histone 3 gene, which is absent in the *A. alternata* isolates.

The aim of this research was to gain insight into the *Alternaria* population present on naturally infected potato leaves. Therefore, a collection of 83 isolates, obtained during three years (2012–2014) on different potato fields throughout Flanders (Belgium), was used. Microscopically the field isolates were divided into two groups: small-spored and large-spored *Alternaria* isolates. Gaining insight into the population diversity (i.e., which species are present and what is the genetic variability between isolates within one species) is important to improve control measures against the *Alternaria* disease on potato. Therefore, sequences of seven genomic regions, the ITS region, the *Gpd* gene, the *EF- α* , the histone *h3* gene, the *Alt a 1* gene, *RPB2* and the calmodulin gene, were analysed. Additionally, it was verified whether there was a link between spore germination and growth rate on the one hand and genetic profile on the other hand.

Materials and methods

Isolates

Potato leaflets, showing symptoms of early blight, were randomly sampled from naturally infected potato fields throughout Flanders (Belgium) (Fig 1). They were collected from the beginning of July until mid-September at regular intervals during three growing seasons (2012–2014). From each leaf, one lesion was excised, sterilised with 5 % NaOCl and placed on SNA medium ($1 \text{ g L}^{-1} \text{ KH}_2\text{PO}_4$, $1 \text{ g L}^{-1} \text{ KNO}_3$, 0.5 g L^{-1}



Fig 1 – Locations of the potato fields where leaf samples with *Alternaria* symptoms were collected.

MgSO₄·7H₂O, 0.5 g L⁻¹ KCl, 0.2 g L⁻¹ glucose, 0.2 g L⁻¹ sucrose and 15 g L⁻¹ Agar). After one week, outgrowing mycelium was transferred to new Petri dishes. Pure cultures were made by additional transfers. To induce sporulation, plates were placed at 25 °C under near UV light (12 h dark and 12 h light) during two weeks. To obtain single spore colonies, one conidium of each plate was transferred to new SNA medium. Finally, isolated species were microscopically identified by their spore size and shape. Based on the spore size and shape of the single-spored colonies, the isolates could be divided into two categories: large-spored species (150–300 × 15–19 µm) and small-spored species (20–63 × 9–18 µm). For this study a subset of 11 large-spored and 72 small-spored *Alternaria* isolates was used. The fact that we obtained a lot more *Alternaria alternata* isolates compared to *Alternaria solani* isolates is due to the fact that mostly *A. alternata* could be isolated from the leaf samples. An overview of the isolates used in this study is listed in Table A1.

PCR and sequencing

DNA was extracted from 7-day-old mycelium cultured on Potato Dextrose Broth. Then the mycelium was transferred into a 1.5 mL microcentrifuge tube and lyophilized for 24 h. The mycelium was crushed under liquid nitrogen. Genomic DNA extraction was carried out using the Invisorb Spin Plant Mini Kit (Stratec) according to the manufacturer's instructions. The DNA concentration was measured using a Quantus™ Fluorometer (Promega) and adjusted to 5 ng DNA/µL. The primers and the PCR conditions for amplification are given in Table 1. After amplification the PCR product was purified using the E.Z.N.A.® Cycle Pure Kit (Omega Bio-tek). Finally, the purified PCR products were sequenced in both directions by MacroGen Europe Laboratory services (The Netherlands) using the previously described primers (Table 1).

Phylogenetic analysis

To compare the genetic profile of the 83 collected field isolates with each other and with the existing sequences from reference isolates a phylogenetic analysis was performed. The sequences of the genes in study were obtained from GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>). Multiple sequence alignments were generated and manually refined in BioEdit 7.0.9. Further phylogenetic analyses were performed using the R-software (R Core Team 2014). The genetic distance between the different species was evaluated by calculating the pairwise Hamming distance between sequences. The Hamming distance measures the diversity between two aligned sequences with a fixed length by calculating the number of sites at which the corresponding nucleotides differ. Hamming nucleotide distance matrices were generated for each alignment of sequences, and the average fraction of nucleotide sequence identity was calculated. Furthermore, for each set of sequences the number of variable characters and the number of parsimony informative sites was determined. The phylogenetic relationships among the isolates were inferred using maximum likelihood trees based on K2P distances (Kimura 2-parameter distance). Branch support of the groupings obtained in the trees was assessed by bootstrapping with 1000

Table 1 – Primer sequences and references together with the PCR conditions.

Gene	Primer sequence	Reference	PCR conditions
ITS region	TCCTCGGCTTATTGATATGC	White et al. (1990)	5 min 94 °C, 35× (45 s 94 °C, 45 s 52 °C, 90 s 72 °C), 6 min 72 °C.
Gpd gene	GGAACTAAAAGTCGTAAACAAGG	Berbee et al. (1999)	4 min 94 °C, 35× (30 s 94 °C, 45 s 60 °C, 60 s 72 °C), 5 min 72 °C.
	ATGCCACGATATCGAGCAC		
Histone h3	CAAGAGCGGTTGGAGAGAAC	Glass & Donaldson (1995), Rang et al. (2002)	2 min 96 °C, 30× (15 s 96 °C, 30 s 55 °C, 35 s 75 °C), 2 min 72 °C.
	ACTAAGCAGACCCGCCGCGAGG		
Alt a 1	GCGGGGAGCTGGATGCTCTT	Woudenberg et al. (2014)	5 min 94 °C, 40× (30 s 94 °C, 30 s 55 °C, 60 s 72 °C), 7 min 72 °C.
EF-α	ATGCAGTTACCAACCATCGC	Carbone & Kohn (1999) O'Donnell et al. (1998)	5 min 94 °C, 40× (30 s 94 °C, 30 s 52 °C, 45 s 72 °C), 7 min 72 °C.
	ACGAGGGTGAGTAGGGGTC		
Calmodulin	CATCGAGAAGTTCGAGAAGG	Lawrence et al. (2013)	4 min 95 °C, 35× (30 s 95 °C, 30 s 58,5 °C, 1 min 72 °C), 5 min 72 °C.
	GGARGTACCAAGTATCATGTT		
RPB2	AGCAAGTCTCCGAGTTCAAGG	Sung et al. (2007) Liu et al. (1999)	5 min 94 °C, 5× (45 s 94 °C, 45 s 60 °C, 2 min 72 °C), 5× (45 s 94 °C, 45 s 58 °C, 2 min 72 °C), 30× (45 s 94 °C, 45 s 54 °C, 2 min 72 °C), 5 min 72 °C.
	CTTCTGCATCATCACTGGAGG		
	GGGGWGAACAGAAAGGC		
	CCCATRGCTTGTYRCCCAT		

replicates. Bootstrap values $\geq 70\%$ were considered as significant and are indicated in the phylogenetic trees.

Spore germination and growth rate

The growth rate and spore germination of the different *Alternaria* isolates are important factors determining the fitness of the isolates. Therefore, the growth rate on SNA medium was assessed for the isolates under study. Petri-dishes were inoculated with a mycelium plug and incubated in dark at 20 °C. Three, seven, ten, and 14 d after inoculation the mycelium growth was measured and the growth rate was calculated. To investigate the spore germination efficiencies of the isolates, 96-well plates with 180 μL liquid medium (1 g L⁻¹ KH₂PO₄, 1 g L⁻¹ KNO₃, 0.5 g L⁻¹ MgSO₄·7H₂O, 0.5 g L⁻¹ KCl, 0.2 g L⁻¹ glucose, 0.2 g L⁻¹ sucrose) and 20 μL spore solution (10⁴ spores/mL) were incubated for seven days at 20 °C. The spore-germinating capacity and early growth after germination was evaluated by measuring the absorbance of each well at 620 nm using a microplate spectrophotometer (MultiSkan Ascent Plate Reader) directly after inoculation and after seven days to correct for the difference in spore biomass between the large-spored and small-spored isolates. To verify whether there was a correlation between the genetic distance and the difference in fitness, a Mantel test with 1000 permutations was used. This test checks for significant correlations between distance matrices by a permutation procedure. To test if there were significant differences in fitness between species a non-parametric Kruskal–Wallis test was used since the assumptions for parametric tests were not fulfilled.

Results

Multi-locus sequencing

The summary statistics, giving phylogenetic information for the ITS region, the *Gpd* gene, the *EF- α* , the *histone h3* gene, the *Alt a 1* gene, the *calmodulin* gene and *RPB2* are presented in Table A2.

The phylogenetic trees based on the ITS region (Fig A.1A) and *Gpd* gene (Fig A.1B) show that the large-spored *Alternaria* isolates are phylogenetically distinct from the small-spored *Alternaria* isolates; this separate cluster comprising the large-spored isolates is, for each gene, supported by a bootstrap value of 100 %. At first glance, the topology of the *Gpd*-based tree looks rather similar to the ITS tree distinguishing the small-spored and large-spored isolates. Nevertheless, there are some subclusters that are supported by high bootstrap values, but to which none of the reference isolates belong (Fig A.1B).

Since the polymorphisms within the ITS and *Gpd* regions are too low to delineate species within the small-spored or large-spored *Alternaria* isolates, additional sequence analyses of the *calmodulin* gene, *RPB2* (large-spored isolates), *Alt a 1* gene and *EF- α* (small-spored isolates) and the *histone h3* gene (small- and large-spored isolates) were performed.

The sequence analysis of the *calmodulin* gene (Fig A.1C) and *RPB2* (Fig A.1D) together with the resulting trees revealed that within the large-spored *Alternaria* isolates besides *Alternaria*

solani also *Alternaria grandis* and *Alternaria protenta* are present on naturally infected potato leaves in Belgium (Fig 2A).

Concerning the small-spored isolates, similar to Woudenberg et al. (2015), sequence analyses of the *Alt a 1* gene (Fig A.1E) and the *EF- α* (Fig A.1F) were performed. The consensus tree of the *Alt a 1* gene and the *EF- α* (Fig 2B) revealed that the small-spored isolates could be divided into two groups, namely the *Alternaria alternata* isolates and isolates belonging the *Alternaria arborescens* species complex (AASC). Within the AASC group, different subclusters can be distinguished. Most isolates cluster together with the reference isolates. However, some isolates were rather loosely associated with the reference isolates of the AASC.

Additionally, a sequence analysis of the *histone h3* gene was performed (Fig A.1G). In the consensus tree, based on the *histone h3*, *Alt a 1* and *EF- α* sequences (Fig 2C), different subclusters are present. One cluster contains the *A. alternata* isolates. Furthermore, it seemed that the isolates belonging to the AASC according to the *Alt a 1* and *EF- α* sequences, could be further subdivided into two groups according to their *histone h3* sequence. These groups are indicated as the *A. arborescens* group A and the *A. arborescens* group B in Fig 2C. Furthermore, it can be seen that isolates 14.38 and 14.83 are rather loosely associated with the *A. arborescens* group. Additionally, also isolates 14.17 and 12.20.2 form a separated group within the *A. arborescens* group B. In Fig. A.1H the consensus tree for the small-spored species, based on the six different genomic regions under study, is given. Since based on the ITS region and *Gpd* gene no subdivision within the small-spored isolates could be made, this tree shows a similar topology compared to the tree in Fig 2C. However, some subtle differences can be seen.

Microscopic identification

Based on the phylogenetic analyses, it was concluded that there was a subdivision within the small-spored isolates based on the *histone h3*, *Alt a 1* and *EF- α* sequences. Furthermore, the isolates were screened for the presence or absence of arborescent to semi-arborescent conidiophores. Fig 3 parts A and B show the conidia branching of two isolates (14.27 and 14.42) that were identified as *Alternaria alternata*. Parts C and D show the conidia morphology of two isolates (14.79 and 14.259) belonging to the *Alternaria arborescens* group B. In parts E and F of Fig 3 the conidia morphology of two isolates (13.147 and 14.264) of the *A. arborescens* group A can be seen. The isolates that were identified as *A. alternata* (Fig 3A, B) had mostly unbranched chains of conidia, whereas the other isolates showed clusters of branching conidial chains of an arborescent appearance. However, since the differences between isolates were very subtle, we could not classify all isolates according to the results of the sequence analyses.

Fitness screen

To assess the fitness of the different species within the large-spored and small-spored isolates, the mycelium growth rate and spore germination capacity and early growth of a subset of isolates was determined. It can be seen the small-spored *Alternaria* isolates (*Alternaria alternata*, *Alternaria arborescens* group A, and *Alternaria arborescens* group B) grew significantly



Fig 2 – Maximum likelihood trees. The consensus tree based on the calmodulin gene and RNA polymerase second largest subunit (RPB2) for the 11 large-spored species and reference isolates from genbank (A). The consensus tree based on the *Alternaria* major allergen gene (Alt a 1) gene and the elongation factor- α (EF- α) (B). The consensus tree based on the histone h3 gene, the Alt a 1 gene and the EF- α for the 72 small-spored *Alternaria* species together with reference sequences from the *Alternaria* Genome Database (<http://alternaria.vbi.vt.edu/index.html>) (C). Bootstrap values above 70 % are shown in the figures.

faster than the large-spored isolates (*Alternaria solani* and *Alternaria grandis*) (Fig 4A), whereas the spore germination capacity of the large-spored isolates was better than the germination capacity of the small-spored *Alternaria* isolates (Fig 4B). Between *A. alternata*, *A. arborescens* group A or B on the one hand and *A. solani* and *A. grandis* on the other hand, there were never significant differences.

In order to examine the relationship between the fitness on the one hand and genetic distance among the isolates on the other hand, pairwise distance matrices of the genetic distance (measured as the Hamming distance) and the distance based

on fitness were compared using the Mantel test (Table 2). It can be seen that there were no significant associations between the genetic distance and fitness, meaning that isolates clustering together in the phylogenetic tree not necessarily have a similar fitness.

Discussion

To gain insight into the *Alternaria* population causing *Alternaria*-leaf blight on potatoes, the genetic diversity of an

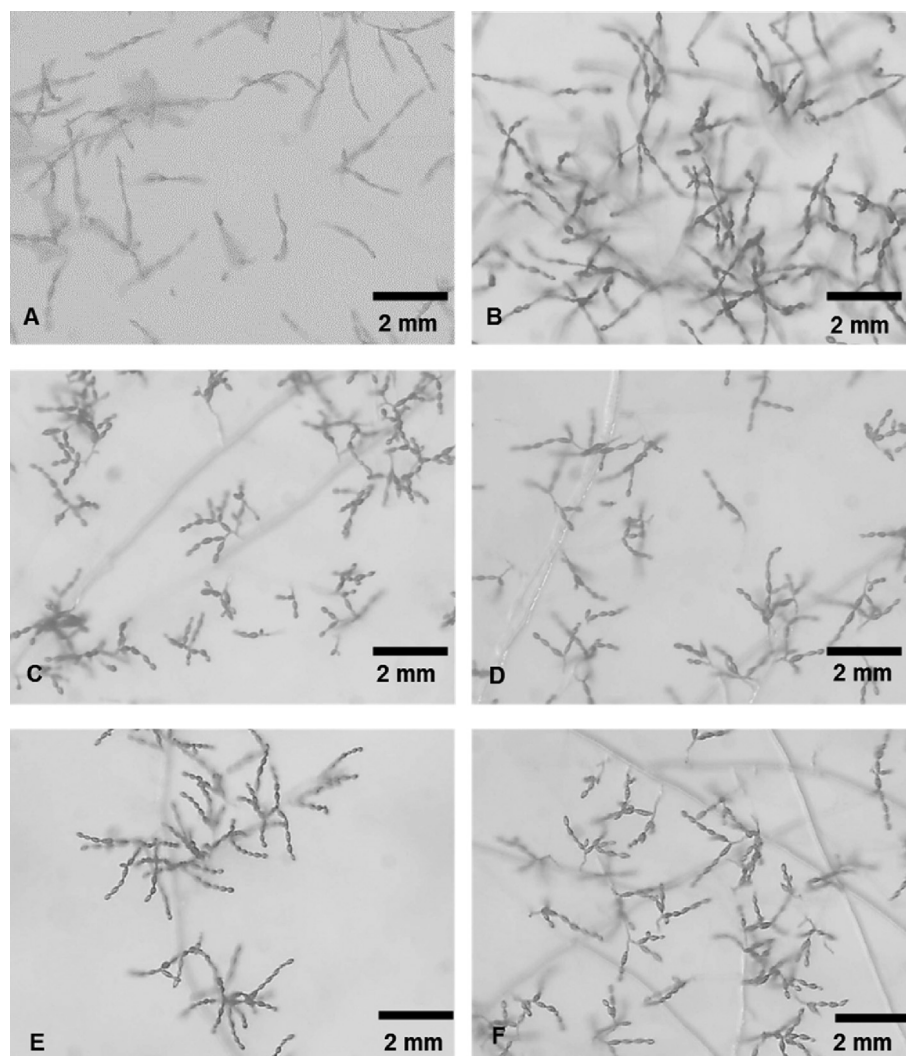


Fig 3 – Conidia of *A. alternata* isolates (14.27 and 14.42) (A, B). Conidia of isolates belonging to the *A. arborescens* group B (14.79 and 14.259) (C, D) and conidia of isolates belonging to the *A. arborescens* group A (13.147 and 14.264) (E, F).

extensive collection of 72 small-spored and 11 large-spored *Alternaria* isolates obtained during 2012–2014 in Flanders (Belgium) was investigated.

A genetic analysis of *Alternaria* species is important to gain further insight into this variability and into the future evolutionary potential of these pathogens. Furthermore, assessing the amount and distribution of the genetic variation in populations gives an idea of disease evolution and adaption of the pathogen to different management methods and environments, which is important to develop adequate control strategies (Morris *et al.* 2000; Aradhya *et al.* 2001; Leiminger *et al.* 2010). Therefore, in this research a sequence analysis of seven different genes of an extended collection of 83 *Alternaria* isolates obtained during three years was performed.

Based on the ITS region and *Gpd* gene the small-spored and large-spored *Alternaria* isolates could be separated. This study confirmed that the discriminative power of the ITS region is too low to gain further insight into the variability within these two groups. The resolution of the sequences of the *Gpd* gene

was clearly higher compared to the ITS region. For this region it was seen that the percentage informative characters within the small-spored isolates is clearly higher than for the large-spored isolates. However, phylogenetic analysis of this region did not result in significant subclusters within the small-spored or large-spored *Alternaria* cluster. Many researchers have indeed reported that differentiation of the *Alternaria* isolates based on the ITS region is difficult due to lack of variation in this genomic region (Elansky *et al.* 2012; Lengi *et al.* 2014; Woudenberg *et al.* 2015; Zheng *et al.* 2015).

To gain further insight into the large-spored *Alternaria* population, sequence analyses of the *calmodulin* gene and *RPB2* were performed. These analyses revealed that *Alternaria solani* was not the only large-spored species on potato leaves, but also *Alternaria grandis* and *Alternaria protenta* were present.

Concerning the small-spored species, Woudenberg *et al.* (2015) reported that the AASC can be distinguished from *Alternaria alternata* by the sequence of the *Alt a 1* gene and a particular region of the *EF- α* sequence. A sequence analysis of both

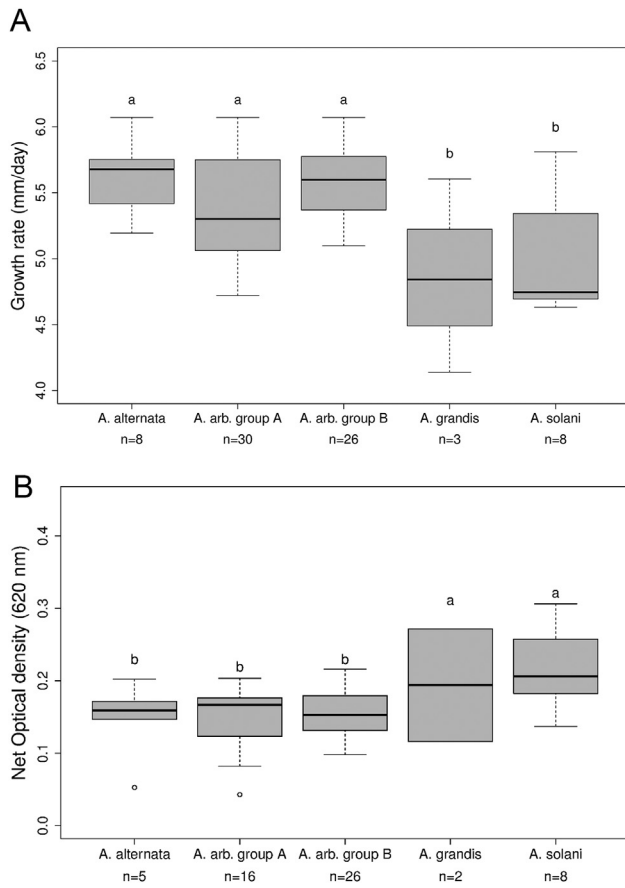


Fig 4 – Distribution of the growth rate (mm day^{-1}) (A) and spore germination capacity and early growth (based on the optical density at 620 nm) (B) for the different small-spored species (*A. alternata*, *A. arborescens* group A and group B) and the large-spored species (*A. solani* and *A. grandis*). *A. protenta* was not taken into account since there was only one *A. protenta* isolate. Different letters above the boxes refer to significant differences between species.

regions revealed that part of our isolates belonged to the AASC and another part was identified as *A. alternata*. An additional sequence analysis of the *histone h3* gene revealed that the isolates belonging to the AASC could be subdivided into two groups. Group A contained isolates with a *histone h3* sequence of 440 bp, whereas in the *histone h3* sequence of the isolates belonging the group B an intron of 106 bp was present, resulting in a *histone h3* sequence of 546 bp. Additionally, some isolates could not be assigned to one of these three groups. These isolates can be the result of genetic recombination. Indeed, the finding of random associations among Simple Sequence Repeat (SSR) markers strongly indicates that most of the *A. alternata* populations sampled from potatoes in China underwent regular recombination (Meng et al. 2015). Based on this available evidence, Meng et al. (2015) concluded that *A. alternata* may adopt an epidemic mode of reproduction by combining many cycles of asexual propagation with fewer cycles of sexual reproduction. However, the presence of Repeat Induced

Table 2 – Mantel correlations (r) between the genetic distance matrices and the distance based on fitness (mycelium growth (mm day^{-1}) rate and spore germination capacity and early growth after germination (OD at 620 nm) and the associated p -value for both the small- and large-spored *Alternaria* species. None of the correlations was significant at $\alpha = 0.05$.

		ITS region		Gpd gene		Histone h3 gene		Alt $\alpha 1$ gene		EF- α		Calmodulin gene		RPB2	
		r	p -value	r	p -value	r	p -value	r	p -value	r	p -value	r	p -value	r	p -value
Growth rate	Small	-0.016	0.547	-0.116	0.973	-0.038	0.895	-0.038	0.885	0.078	0.09	0.041	0.363	0.021	0.423
	Large	-0.109	0.600	0.195	0.233	-0.064	0.560								
Spore germination	Small	0.002	0.417	-0.024	0.610	0.003	0.422	0.084	0.175	0.060	0.199	0.216	0.123	0.042	0.365
	Large	0.034	0.404	0.079	0.384	-0.204	0.806								

Point (RIP) mutation, which is often considered as a hallmark for sexual reproduction, has not yet been studied in *Alternaria* species associated with potato leaf blight (Clutterbuck 2011).

Furthermore, the microscopic analysis revealed that there were no clear differences in conidial branching between the small-spored isolates. Isolates that were identified as *Alternaria arborescens* (group A or B) showed more clusters of branching conidial chains of an arborescent appearance compared to the isolates identified as *A. alternata*. However, the difference was subtle and not consequent for all isolates. It is indeed known that morphological characteristics are fictile and are not only affected by intrinsic factors, but also by environmental conditions, subsequent subculturing, cultivation medium, etc. (Simmons 1992; Thomma 2003) and thus have little value to differentiate between small-spored *Alternaria* isolates.

To effectively control this disease, a thorough characterisation (fitness, fungicide resistance, virulence) of the species involved in the disease complex is inevitable. As previously reported, there exists a considerable variability in the fitness between different *A. alternata* (Lengi et al. 2014) and *A. solani* (Van der Waals et al. 2004) isolates. This was confirmed in this study. It was concluded that the growth rate of the small-spored isolates was significantly faster than the growth rate of the large-spored isolates, whereas the opposite was true for the spore germination capacity. Furthermore, there were no significant differences in growth rate and spore germination capacity between the different small-spored or large-spored *Alternaria* species. Additionally, there was no association between the genetic distance and distance based on fitness. Isolates with a similar fitness did not cluster together in the phylogenetic tree. So, the variability in fitness

parameters cannot be explained by differences in the ITS region, *Gpd* gene, *EF- α* , *histone h3* gene, *calmodulin* gene, *RPB2* gene or *Alt a 1* gene.

In conclusion, this study revealed that the *Alternaria* population present on naturally infected potato leaves in Belgium is more complex than previously expected. At the microscopic and genetic level, the small- and large-spored isolates are easy to distinguish. Based on the *RPB2* and *calmodulin* sequence, it was concluded that besides *A. solani* also *A. grandis* and *A. protenta* are members of the *Alternaria* population of Belgian potato fields. The sequence analyses of the *Alt a 1* gene and the *EF- α* showed that both *A. alternata* isolates and isolates belonging to the AASC were present in the small-spored *Alternaria* population. Based on the *histone h3* gene the isolates belonging to the AASC could be subdivided into two groups.

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Appendix.

Table A1 – List of *Alternaria* isolates examined in this study. The code of the isolate, date of isolation, microscopic identification, the city, coordinates (latitude and longitude) from the locations where the isolates were collected and the identification according to the consensus tree of *histone h3*, *Alt a 1* and *EF- α* (small-spored isolates) and according to the *calmodulin* gene and *RPB2* (large-spored isolates).

Isolate Code	Date of isolation	Spore-type	City	LAT	LON	Species
12.1.1	2012-08-31	Small-spored	Vladslo	51.046	2.916	<i>A. arborescens</i> group A
12.10.2	2012-08-31	Small-spored	Vladslo	51.046	2.916	<i>A. alternata</i>
12.12.1	2012-08-31	Small-spored	Vladslo	51.046	2.916	<i>A. alternata</i>
12.15.1	2012-08-31	Small-spored	Vladslo	51.046	2.916	<i>A. arborescens</i> group B
12.16.1	2012-08-31	Small-spored	Vladslo	51.046	2.916	<i>A. alternata</i>
12.19.1	2012-08-31	Small-spored	Vladslo	51.046	2.916	<i>A. arborescens</i> group A
12.20.2	2012-08-31	Small-spored	Vladslo	51.046	2.916	Undefined
12.26.2	2012-09-05	Small-spored	Kruishoutem	50.928	3.537	<i>A. arborescens</i> group A
12.3.1	2012-08-31	Small-spored	Vladslo	51.046	2.916	<i>A. arborescens</i> group A
12.37.1	2012-08-15	Small-spored	Bottelare	50.967	3.750	<i>A. arborescens</i> group A
12.4.1	2012-08-31	Small-spored	Vladslo	51.046	2.916	<i>A. arborescens</i> group A
12.4.3	2012-08-31	Small-spored	Vladslo	51.046	2.916	<i>A. arborescens</i> group A
12.5.1	2012-08-31	Small-spored	Vladslo	51.046	2.916	<i>A. arborescens</i> group A
12.52.2	2012-08-31	Small-spored	Wetteren	51.003	3.890	<i>A. arborescens</i> group A
12.56.3	2012-08-31	Small-spored	Wetteren	51.003	3.890	<i>A. arborescens</i> group A
12.58.2	2012-08-15	Small-spored	Oosterzele	50.947	3.816	<i>A. arborescens</i> group B
12.8.1	2012-08-31	Small-spored	Vladslo	51.046	2.916	<i>A. arborescens</i> group A
13.10	2013-07-09	Small-spored	Bottelare	50.967	3.750	<i>A. arborescens</i> group A
13.102	2013-08-22	Small-spored	Hoeleden	50.868	4.987	<i>A. arborescens</i> group B
13.107	2013-08-25	Small-spored	Bottelare	50.967	3.750	<i>A. arborescens</i> group A
13.11	2013-08-26	Small-spored	Roeselare	50.950	3.129	<i>A. arborescens</i> group A
13.111	2013-08-27	Small-spored	Zonnebeke	50.873	2.987	<i>A. arborescens</i> group B

(continued on next page)

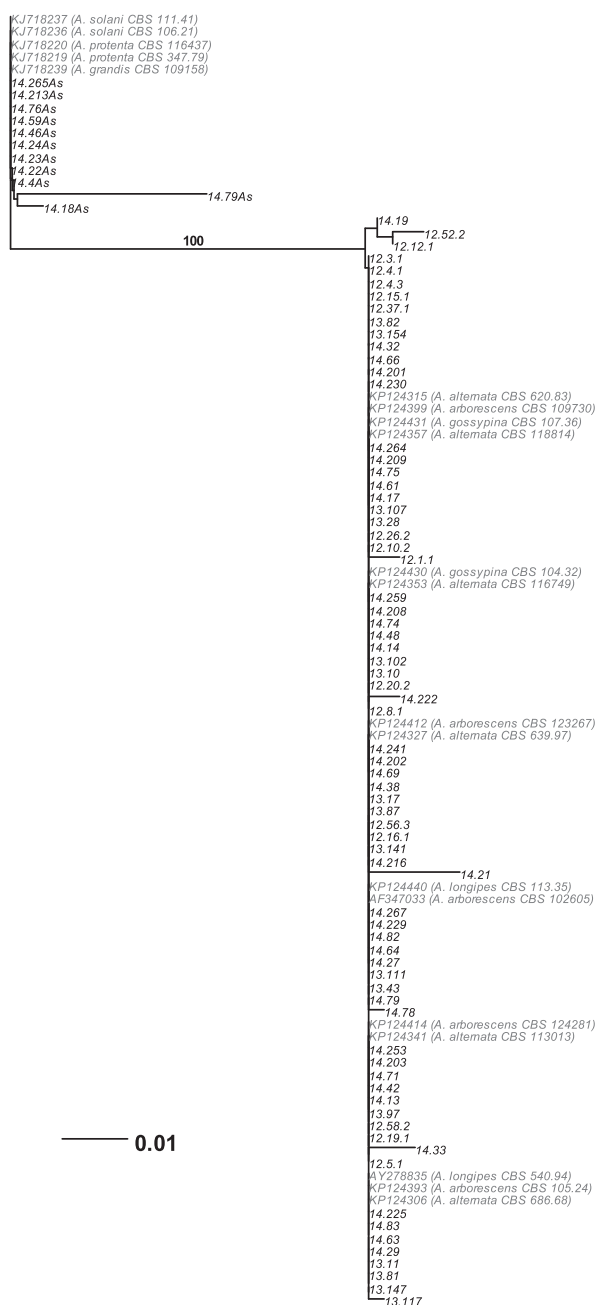
Table A1 – (continued)

Isolate Code	Date of isolation	Spore-type	City	LAT	LON	Species
13.117	2013-08-27	Small-spored	Poperinge	50.845	2.721	<i>A. arborescens</i> group A
13.141	2013-09-02	Small-spored	Leupegem	50.833	3.600	<i>A. arborescens</i> group B
13.147	2013-09-03	Small-spored	Outgaarden	50.765	4.918	<i>A. arborescens</i> group A
13.154	2013-09-05	Small-spored	Kruishoutem	50.928	3.537	<i>A. arborescens</i> group B
13.17	2013-09-23	Small-spored	Kruishoutem	50.928	3.537	<i>A. arborescens</i> group B
13.28	2013-07-24	Small-spored	Deftinge	50.787	3.840	<i>A. arborescens</i> group A
13.43	2013-07-29	Small-spored	Leefdaal	50.850	4.583	<i>A. arborescens</i> group A
13.81	2013-08-19	Small-spored	Horebeke	50.837	3.687	<i>A. arborescens</i> group B
13.82	2013-08-19	Small-spored	Bevere	50.852	3.588	<i>A. arborescens</i> group B
13.87	2013-08-20	Small-spored	Sint-Martens-Bodegem	50.862	4.213	<i>A. arborescens</i> group B
13.97	2013-08-22	Small-spored	Leefdaal	50.850	4.583	<i>A. arborescens</i> group A
14.13	2014-07-31	Small-spored	Outgaarden	50.765	4.918	<i>A. arborescens</i> group A
14.14	2014-08-07	Small-spored	Outgaarden	50.765	4.918	<i>A. arborescens</i> group A
14.17	2014-07-24	Small-spored	Assent	50.952	5.014	Undefined
14.19	2014-08-07	Small-spored	Assent	50.952	5.014	<i>A. arborescens</i> group A
14.201	2014-08-18	Small-spored	Wulpen	51.101	2.699	<i>A. arborescens</i> group A
14.202	2014-08-18	Small-spored	Wulpen	51.101	2.699	<i>A. arborescens</i> group A
14.203	2014-08-18	Small-spored	Veurne	51.073	2.668	<i>A. arborescens</i> group A
14.208	2014-08-06	Small-spored	Roeselare	50.950	3.129	<i>A. arborescens</i> group A
14.209	2014-07-17	Small-spored	Roeselare	50.950	3.129	<i>A. arborescens</i> group A
14.21	2014-08-29	Small-spored	Leefdaal	50.850	4.583	<i>A. arborescens</i> group A
14.216	2014-08-20	Small-spored	Zonnebeke	50.873	2.987	<i>A. arborescens</i> group A
14.222	2014-08-19	Small-spored	Zwevegem	50.813	3.408	<i>A. arborescens</i> group B
14.225	2014-08-19	Small-spored	Helkijn	50.732	3.381	<i>A. arborescens</i> group B
14.229	2014-07-31	Small-spored	Bellegem	50.777	3.278	<i>A. arborescens</i> group B
14.230	2014-07-31	Small-spored	Leefdaal	50.850	4.583	<i>A. arborescens</i> group B
14.241	2014-08-08	Small-spored	Kasterlee	51.241	4.967	<i>A. alternata</i>
14.253	2014-08-22	Small-spored	Mol	51.191	5.117	<i>A. arborescens</i> group A
14.259	2014-08-01	Small-spored	Mol	51.191	5.117	<i>A. arborescens</i> group B
14.264	2014-08-14	Small-spored	Mol	51.191	5.117	<i>A. arborescens</i> group A
14.267	2014-08-08	Small-spored	Weelde	51.400	4.983	<i>A. arborescens</i> group A
14.27	2014-08-07	Small-spored	Watervliet	51.283	3.633	<i>A. alternata</i>
14.29	2014-08-07	Small-spored	Waterland-Oudeman	51.289	3.590	<i>A. arborescens</i> group B
14.32	2014-08-29	Small-spored	Waterland-Oudeman	51.289	3.590	<i>A. arborescens</i> group B
14.33	2014-07-28	Small-spored	Wortegem-Petegem	50.850	3.500	<i>A. arborescens</i> group B
14.38	2014-09-04	Small-spored	Zottegem	50.870	3.811	Undefined
14.42	2014-08-13	Small-spored	Kruishoutem	50.928	3.537	<i>A. alternata</i>
14.48	2014-07-24	Small-spored	Kruishoutem	50.928	3.537	<i>A. alternata</i>
14.61	2014-07-14	Small-spored	Outgaarden	50.765	4.918	<i>A. arborescens</i> group A
14.63	2014-07-31	Small-spored	Outgaarden	50.765	4.918	<i>A. arborescens</i> group B
14.64	2014-08-07	Small-spored	Outgaarden	50.765	4.918	<i>A. arborescens</i> group A
14.66	2014-07-23	Small-spored	Lozer	50.919	3.569	<i>A. arborescens</i> group A
14.69	2014-07-15	Small-spored	Lozer	50.919	3.569	<i>A. alternata</i>
14.71	2014-07-23	Small-spored	Lozer	50.919	3.569	<i>A. arborescens</i> group A
14.74	2014-08-25	Small-spored	Wortegem-Petegem	50.850	3.500	<i>A. arborescens</i> group A
14.75	2014-08-18	Small-spored	Oudenaarde	50.830	3.629	<i>A. arborescens</i> group B
14.78	2014-08-11	Small-spored	Edelare	50.830	3.629	<i>A. arborescens</i> group A
14.79	2014-08-11	Small-spored	Edelare	50.830	3.629	<i>A. arborescens</i> group B
14.82	2014-07-09	Small-spored	Maarkedal	50.799	3.644	<i>A. arborescens</i> group B
14.83	2014-07-24	Small-spored	Goferdinge	50.787	3.840	Undefined
14.18As	2014-07-31	Large-spored	Assent	50.952	5.014	<i>A. solani</i>
14.213As	2014-08-29	Large-spored	Roeselare	50.950	3.129	<i>A. solani</i>
14.22As	2014-09-01	Large-spored	Leefdaal	50.850	4.583	<i>A. grandis</i>
14.23As	2014-09-02	Large-spored	Leefdaal	50.850	4.583	<i>A. solani</i>
14.24As	2014-07-25	Large-spored	Leefdaal	50.850	4.583	<i>A. grandis</i>
14.265As	2014-08-29	Large-spored	Mol	51.191	5.117	<i>A. protenta</i>
14.46As	2014-07-30	Large-spored	Kruishoutem	50.928	3.537	<i>A. solani</i>
14.4As	2014-07-27	Large-spored	Herne	50.723	4.054	<i>A. solani</i>
14.59As	2014-07-31	Large-spored	Leefdaal	50.850	4.583	<i>A. grandis</i>
14.76As	2014-07-29	Large-spored	Oudenaarde	50.830	3.629	<i>A. solani</i>
14.79As	2014-08-07	Large-spored	Edelare	50.830	3.629	<i>A. solani</i>

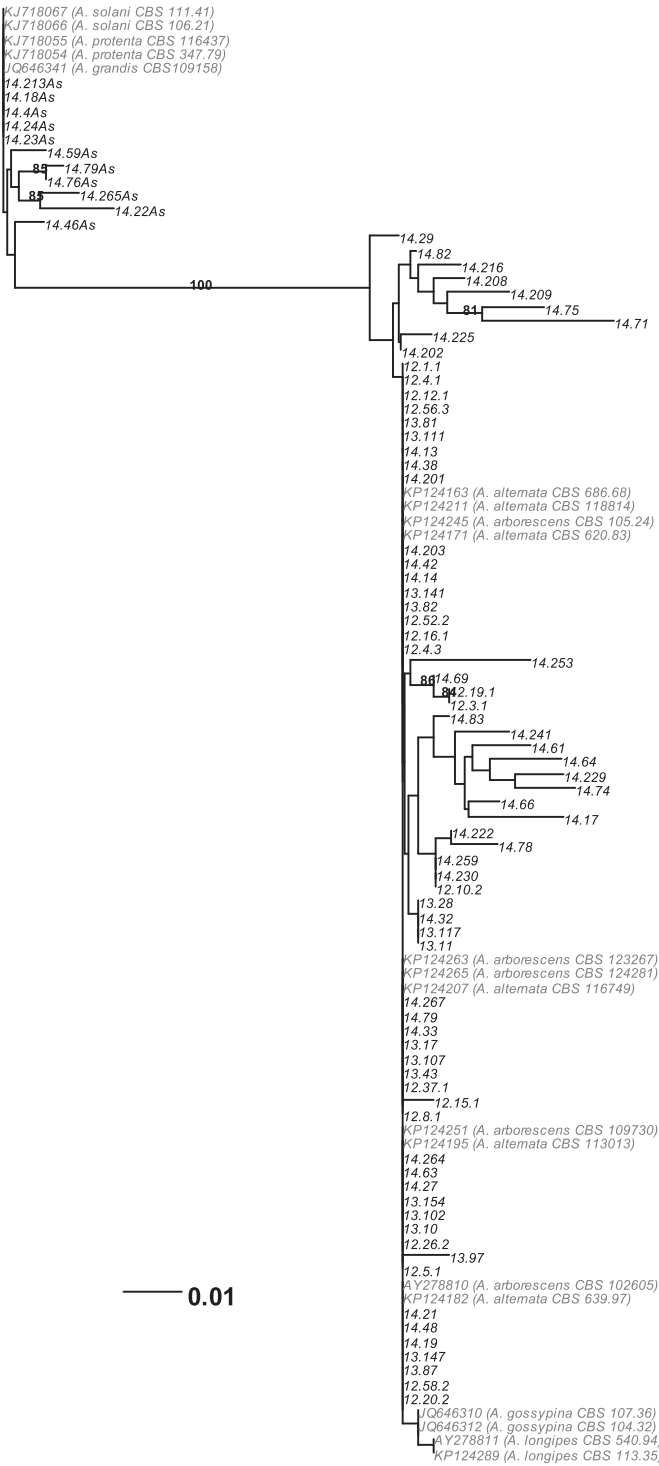
Table A2 – Summary statistics with the phylogenetic information of the seven genomic regions for the small-spored and large-spored *Alternaria* species.

	ITS			GPD			Histone h3			Alt A 1	EF- α	RPB2	Calmodulin
	Small	Large	Both	Small	Large	Both	Small	Large	Both	Small	Small	Large	Large
Number of isolates	72	11	83	72	11	83	72	11	83	72	72	11	11
Number of nucleotides	474	474	474	451	451	451	458	458	458	486	201	419	722
Mean genetic distance (Hamming)	0.09	0.54	1.33	1.034	1.08	0.53	1.98	0.52	2.87	0.70	0.76	0.09	0.48
Standard deviation Hamming distance	0.21	0.98	2.25	1.30	0.70	0.98	1.45	0.42	2.11	0.86	0.81	0.19	0.59
Constant characters	444	458	400	360	416	329	359	452	338	428	195	413	713
Variable characters (polymorphic sites)	30	16	74	104	35	122	99	6	120	58	6	6	9
Percentage variable characters	6.33	3.38	15.61	20.18	7.76	27.05	21.62	1.31	26.20	11.93	2.91	1.43	1.25
Parsimony informative characters	0	0	26	27	3	59	28	6	53	15	4	4	9
Percentage informative characters	0	0	5.49	5.98	0.67	10.91	6.11	1.31	11.57	3.09	1.95	0.95	1.25

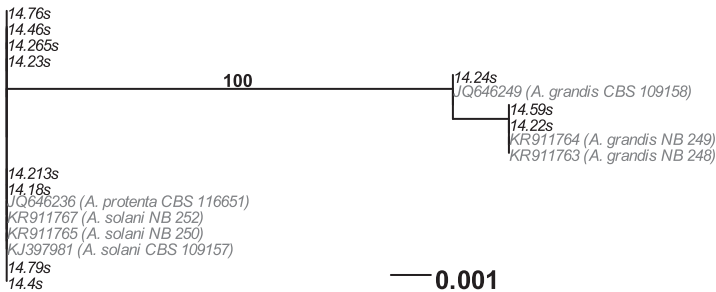
ITS



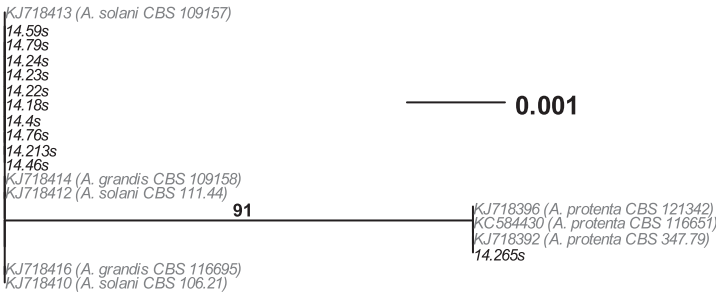
Gpd



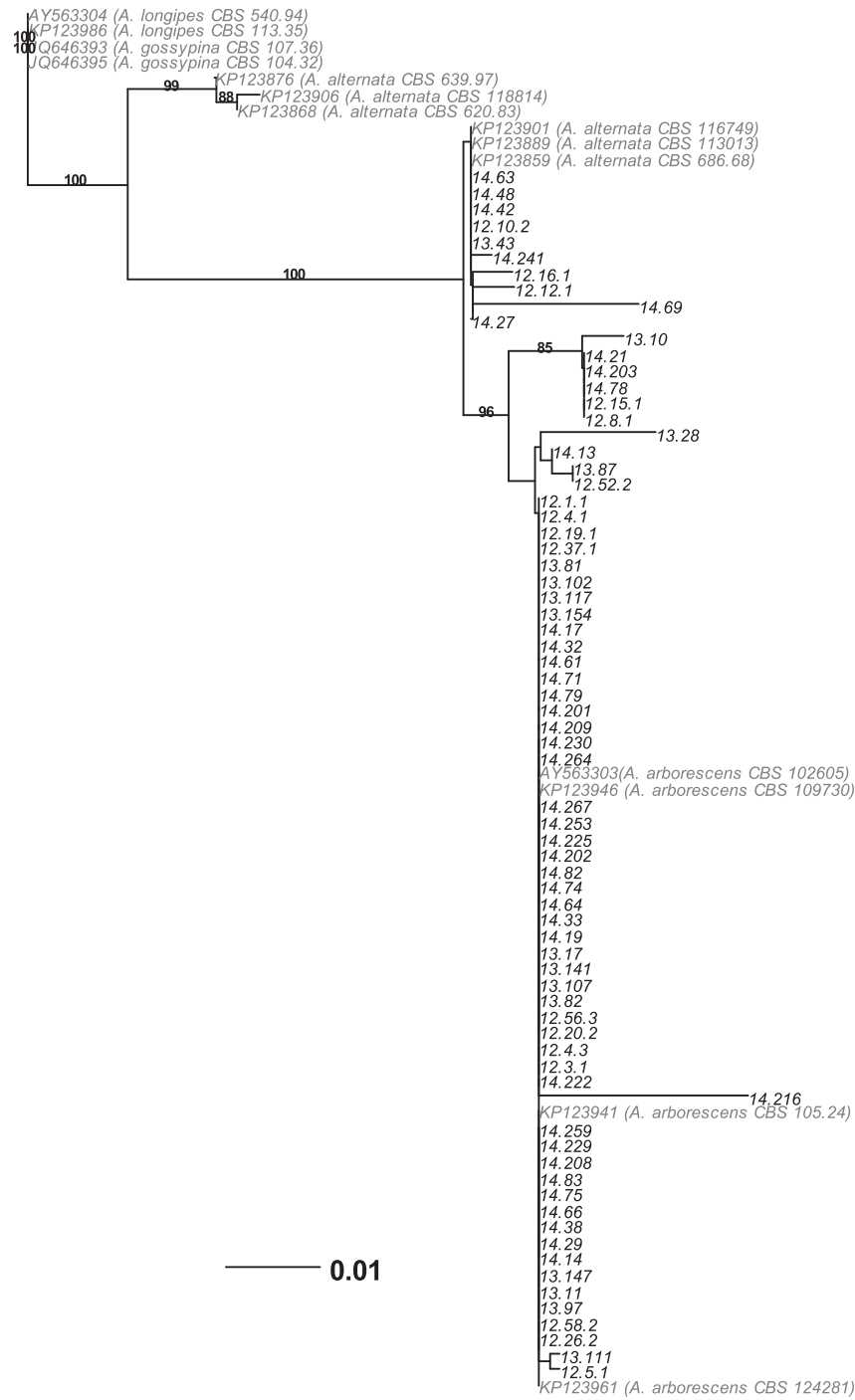
Calmodulin



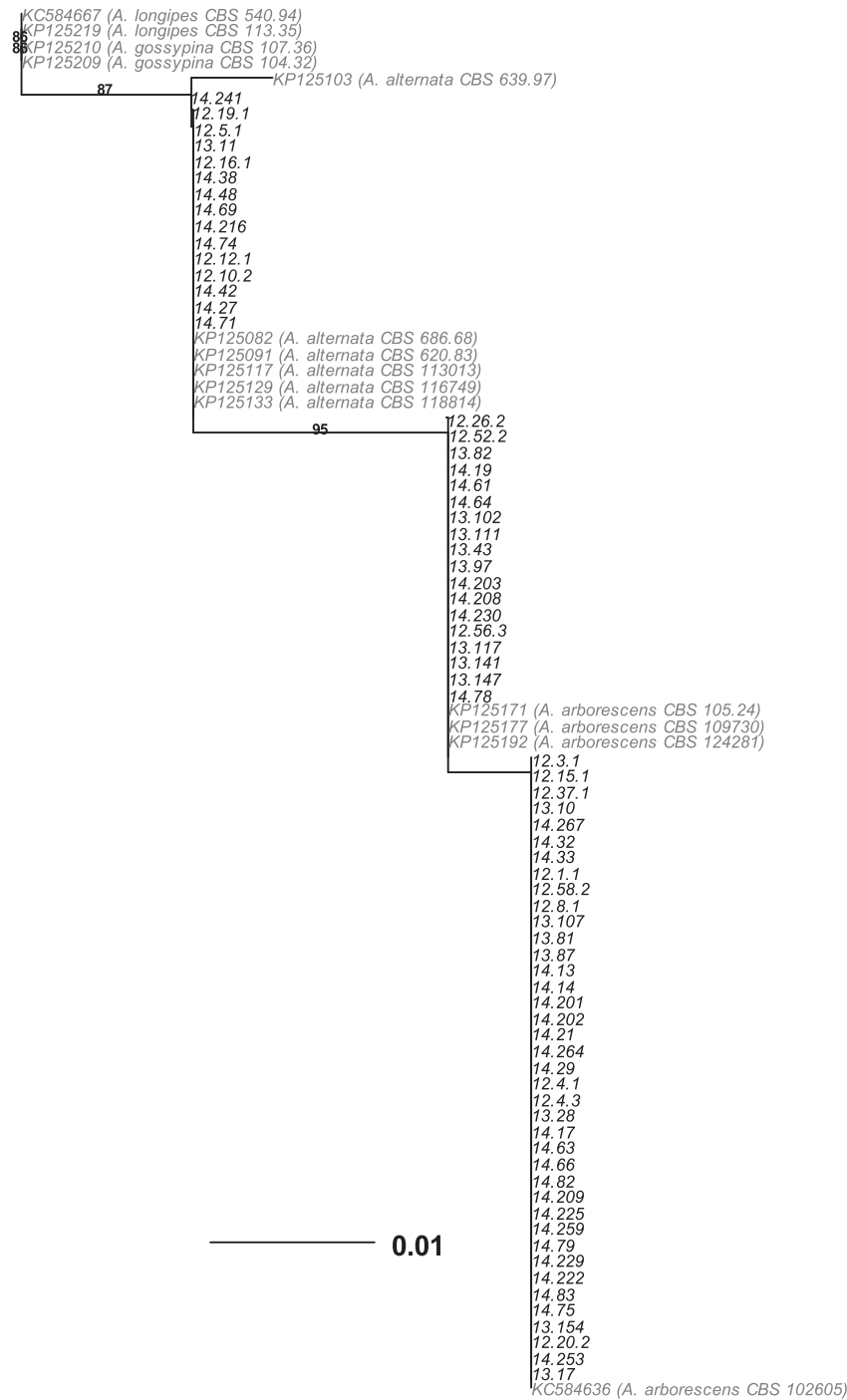
RPB2



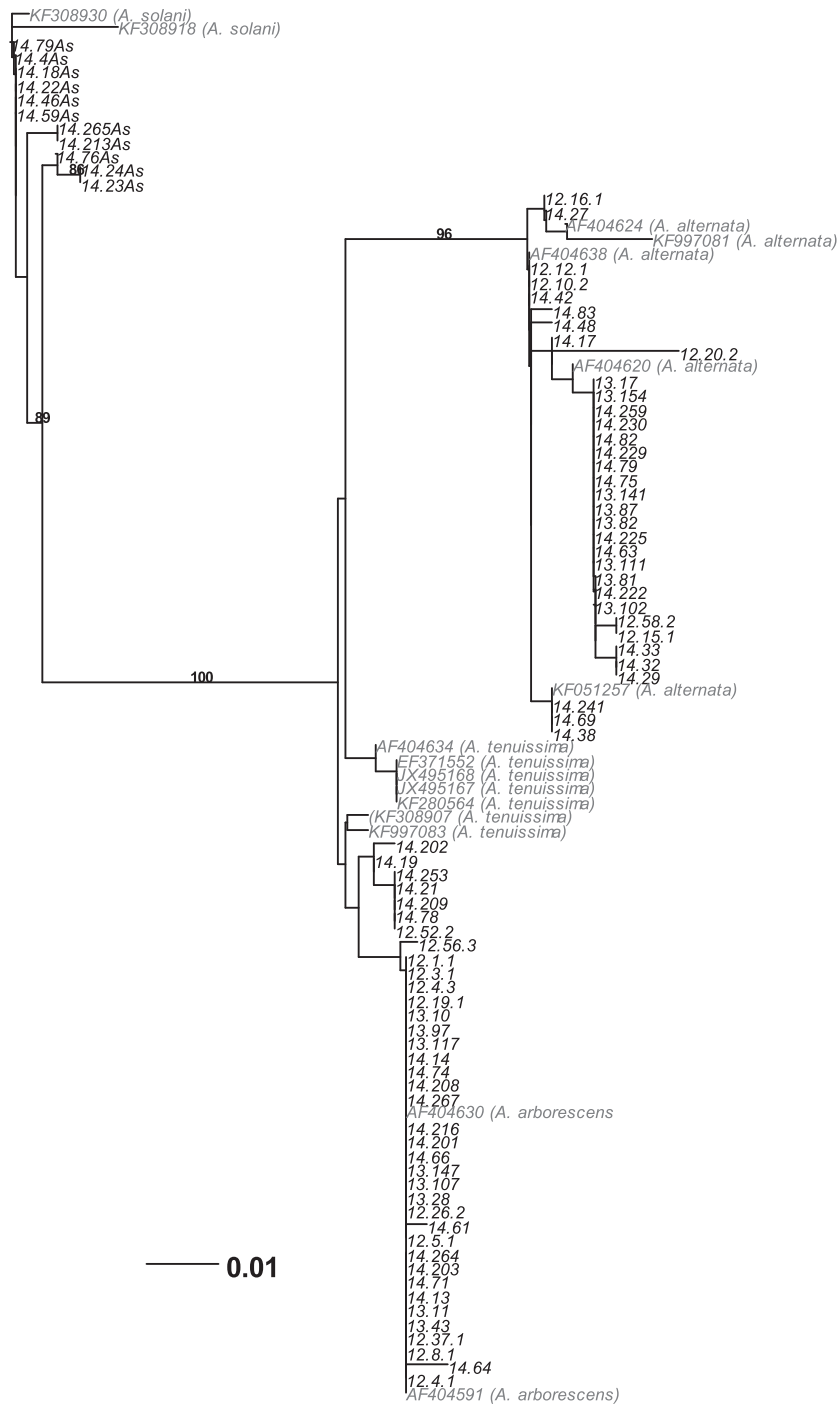
Alt a 1



EF-a



Histone h3



ITS, GPD, EF-a, Histone h3 and Alt a 1



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