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Haplotype diversity and phylogeny within *Alternaria alternata* and *A. arborescens* species complexes from tomatoes

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ABSTRACT

Tomato (*Solanum lycopersicum* L.) is an economically important vegetable susceptible to various fungal diseases, including leaf spot caused by *Alternaria* spp. from the section *Alternaria*. In our study, a total of 72 tomato-associated *Alternaria* spp. strains from Latvia, Lithuania, Estonia, and Algeria were analysed by integrating morphological data, pathogenicity assay, multi-locus phylogeny, and haplotype assignment. Recovered *Alternaria* spp. strains were characterized by considerable variation in phenotypic diversity, non-pathogenicity to their host of origin and absence of the AAL-toxin biosynthesis gene (*ALT1*). Multi-locus phylogeny of the RNA polymerase II second largest subunit (*rpb2*), putative F-box-domain-containing protein (*ASA-10*), and putative histone-like transcription factor (*ASA-19*) confirmed the occurrence of both *A. alternata* and *A. arborescens* species complexes along with *A. longipes* and *A. postmessia* on symptomatic tomatoes. The discordant tree topology among single-gene phylogenies suggested the occurrence of potential recombination between phylogenetic lineages in the section *Alternaria*, resulting in putative *alternata*-*arborescens* and *alternata*-*longipes* hybrids. DNA polymorphism analysis of the *rpb2*, *ASA-10*, and *ASA-19* loci revealed a high level of genetic diversity in the section *Alternaria*, and the number of single nucleotide polymorphisms (SNPs) and haplotypes varied among loci and lineages studied. A total of 16 and 6 multi-locus haplotypes were assigned in *alternata* and *arborescens* lineages, respectively. Global genetic diversity analysis of *A. alternata* and *A. arborescens* strains at the *rpb2* locus confirmed that major haplotypes described from tomatoes were shared among other hosts of origin.

1. Introduction

Tomatoes (*Solanum lycopersicum* L.) belong to the Solanaceae family and are economically important vegetables worldwide. Diseases caused by fungi, including *Alternaria* spp., are one of the major limiting factors in the production of tomatoes in temperate zones due to defoliation and devastating yield losses (Adhikari et al., 2017; Schmey et al., 2024).

The genus *Alternaria* belongs to the family *Pleosporaceae* and contains more than 360 species divided into 29 sections (Li et al., 2023). *Alternaria* spp. are considered one of the most important mycotoxigenic fungi, which produce more than 70 toxins, thus causing implications for the food safety of fresh and processed tomato products (López et al., 2016; Escrivá et al., 2017; Sanzani et al., 2019; Bertuzzi et al., 2021).

Some of these mycotoxins play an essential role in plant disease development (Meena and Samal, 2019; Wenderoth et al., 2019).

The major pathogens of tomatoes are comprised in the *Alternaria* section *Porri*, which includes 70 species characterized by the production of large conidia with long to filamentous beaks (Li et al., 2023; Zhao et al., 2023). However, a high isolation frequency of several species from the section *Alternaria* has been reported from the family Solanaceae, including both cultivated tomatoes (Bessadat et al., 2017; El Gobashy et al., 2018; Gherbawy et al., 2018; Kokaeva et al., 2018) and their wild relatives (Schmey et al., 2023).

Small-spored *Alternaria* spp. from the section *Alternaria* are mostly considered saprobes, endophytes, or weak plant pathogens that infect a broad host range (DeMers, 2022), and their role in tomato disease

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development has been neglected in comparison with that of large-spored *Alternaria* spp. from the section *Porri* (Adhikari et al., 2020). However, synergistic effects between these species and aggressive large-spored isolates in mixed inoculation on tomatoes have been reported (Bessadat et al., 2017).

The application of morphological data might be helpful for *Alternaria* spp. identification at the section level; however, even for professional mycologists it is very challenging to use morphological characteristics for species delineation as conidial morphology and sporulation vary depending on abiotic factors, such as photoperiod, growing medium, and temperature (Naik et al., 2010; Loganathan et al., 2016; Parvin et al., 2021). Moreover, overlapping of the morphological features to varying degrees among several species in the genus *Alternaria* has been reported, thus highlighting the importance of molecular data implementation for precise delineation of the species (Lawrence et al., 2016).

The taxonomy of *Alternaria* spp. from the section *Alternaria* has undergone several revisions (Lawrence et al., 2013; Woudenberg et al., 2013, 2015; Gannibal, 2016). One of the most comprehensive arrangements in molecular taxonomy of this section was provided by Woudenberg et al. (2015). They applied whole-genome and transcriptome reads in combination with multi-locus phylogeny for species delineation in section *Alternaria*. Based on the analysis of nine genomic regions (*ITS*, *SSU*, *LSU*, *GAPDH*, *RPB2*, *TEF1*, *Alt a 1*, *endoPG*, and *OPA10-2*), 35 morpho-species, including *A. tenuissima*, were synonymized under *A. alternata*, thus reducing the number of phylogenetic species in this section to 11 and one species complex (*A. arborescens*). Since then, the inclusion of morpho-species and the description of new phylogenetic species increased the number of species accommodated in this section to 73 (Li et al., 2023). A subset of the nine genomic regions analysed by Woudenberg et al. (2015) with DNA sequencing of additional less common genes, including histone h3 (Landschoot et al., 2017) and calmodulin, actin, and plasma membrane ATPase (Nasr Esfahani, 2018), has been implemented for improving the resolution of small-spored species in the section *Alternaria*.

Recent phylogenomic studies by Dettman and Eggertson (2021) have indicated the insufficient discriminatory power of common markers for the differentiation of closely related species in the section *Alternaria*. Therefore, several loci have been proposed for species identification in the section *Alternaria* (Dettman and Eggertson, 2021), and new section-specific molecular markers have been developed (Dettman and Eggertson, 2022). Among the candidate loci, the analysis of the putative F-box-domain-containing protein (*ASA-10*) and putative histone-like transcription factor (*ASA-19*) genes in combination with the RNA polymerase II second largest subunit (*rpb2*) provided accurate and reliable assignment of *Alternaria* strains in the main phylogenetic lineages within section *Alternaria* (Dettman et al., 2023). Therefore, these three target genomic regions (*ASA-10*, *ASA-19*, and *rpb2*) were selected for small-spored *Alternaria* spp. identification in our study.

Investigations on the genetic diversity of *A. alternata* and *A. arborescens* are crucial to gain knowledge of pathogen epidemiology and develop effective disease management strategies. Molecular phylogenetic data analysis in the form of haplotype networks has been widely implemented for the evaluation of the population structure of *Alternaria* spp. to provide more insights into interspecies and intraspecies relationships (Adhikari et al., 2020; Blagojević et al., 2020; Xu et al., 2022).

Current studies on disease causal agents of tomatoes in northeastern Europe have implemented a morphology-based approach for the identification of *Alternaria* spp., and molecular data have not been applied for the delineation and characterization of these species (Shidlauskienė and Survilienė, 2002; Dučkēna and Bimsteine, 2023). Therefore, the objectives of our study were to (i) identify *Alternaria* spp. from the section *Alternaria* associated with symptomatic infection of tomatoes by integrating molecular phylogenetic methods, morphological data, and pathogenicity assay; (ii) compare the DNA polymorphism of the *rpb2*, *ASA-10*, and *ASA-19* genes within *A. alternata* and *A. arborescens*

isolates; and (iii) determine the haplotype diversity and distribution and infer phylogenetic relationships with publicly available sequences of *A. alternata* and *A. arborescens*.

2. Materials and methods

2.1. The collection of samples and fungal isolation

The sampling of tomato (*S. lycopersicum* L.) leaves and fruits with typical disease symptoms was conducted in greenhouses and high tunnels in the central part of Latvia, Lithuania, and Estonia. Samples were collected from the beginning of August until the end of September in 2022 and 2023. The leaves and fruits were collected in clean ethylene bags, sealed, and transported to the Plant Pathology Scientific Laboratory of Latvia University of Life Sciences and Technologies. The obtained samples were examined for asexual fungal structures using an Olympus BX43 optical fluorescence microscope with cellSens software (Olympus Cooperation, Tokyo, Japan). For the isolation of fungal strains, small pieces (~1 cm²) from lesions between healthy and symptomatic plant tissue were excised, surface sterilized in 1 % sodium hypochlorite (NaOCl) solution for 1 min, rinsed three times in autoclaved distilled water and then air-dried. Using sterile forceps, plant tissue fragments were transferred to Petri dishes on potato dextrose agar media (PDA) (Scharlau, Barcelona, Spain) supplemented with streptomycin (100 µg/L) and penicillin (100 µg/L). The Petri dishes were incubated at 20 °C under 24 h darkness for seven days, and one representative colony growing from tissues of each sample was chosen as an isolate. Hyphal tips from single hyphae were excised and plated onto new PDA plates for the purification of fungal strains (Leyronas et al., 2012). The obtained pure cultures were analysed at the Institute of Research in Horticulture and Seeds (UMR 1345 IRHS, France) and cryopreserved at –80 °C in 30 % glycerol at the fungal culture collection COMIC (Collection of Microorganisms) of the SFR 4207 QUASAV (42 rue Georges Morel, 49070 Beaucauzé Cedex, France). Fungal strains from Latvia, Lithuania, and Estonia isolated by Lilija Dučkēna are labelled with LD prefixes in this study. *Alternaria* spp. strains from the COMIC collection of Nabahat Bessadat associated with symptomatic tomatoes in Algeria were also included in the study and are marked with NB prefixes (Bessadat et al., 2017, 2019).

2.2. Morphological characterization

For the evaluation of morphological characteristics, 5-day-old colonies of *Alternaria* spp. were transferred onto fresh potato dextrose agar (PDA) plates (Biokar Diagnostics, Pantin Cedex, France). Fungal strains were incubated for 7 day at 23 °C in darkness for the assessment of macroscopic characteristics, including colony texture, margin, elevation, and zonation. The growing media pigmentation and the obverse and reverse colour of the colonies were evaluated according to the Munsell Colour Order System using the Virtual Colour Atlas (Virtual Colour Systems Ltd. 2024) (Smith and Fairchild, 2022). Seven-day-old colonies of *Alternaria* spp. were photographed using a Canon EOS 77D digital single-lens reflex camera with an 18–135 mm Canon EF-S lens (Canon Inc., Tokyo, Japan).

For analysis of microscopic characteristics, fungal strains were transferred to potato carrot agar (PCA) plates (HiMedia Laboratories Pvt. Ltd., Mumbai, India). Conidia formation was observed five to seven days after incubation at 18–22 °C under a natural day–night cycle of 14 h fluorescent light and 10 h darkness and compared with descriptions provided by Simmons (2007). The sporulation patterns of *A. arborescens* and *A. alternata* isolates were characterized using an Optika stereomicroscope at 45 × magnification and Optika ImageView software (Optika Microscopes, Bergamo, Italy).

The conidia and conidiophores were mounted using 2-hydroxypropionic acid without added dye for fluorescent visualisation. Digital image acquisition was performed using a Zeiss Axio Imager Z2

fluorescence microscope and Axiocam 305 colour digital camera (Carl Zeiss, Göttingen, Germany) at 400 × magnification. Images were processed using Zeiss imaging software ZEN 3.3 blue (version 3.3.89.0000) (Carl Zeiss, Göttingen, Germany).

2.3. DNA extraction, PCR amplification and Sanger sequencing

Genomic DNA extraction of mycelia from seven-day-old fungal strains was performed using a microwave mini-prep extraction method (Goodwin and Lee, 1993). The precipitated DNA pellets were rinsed with 80 % ethanol, dried, dissolved in 100 µL TE 10:0.1 (10 mM Tris–HCl, 0.1 mM EDTA, pH 8.0) and stored at –20 °C for polymerase chain reaction (PCR) analyses.

Initial identification of the fungal strains was conducted by PCR amplification of the internal transcribed spacer regions 1 and 2 and intervening 5.8S nrDNA (*ITS*) using the primer pair ITS1/ITS4 (White et al., 1990). Molecular identification of the recovered *Alternaria* spp. strains encompassed the amplification of three additional target regions, namely, the RNA polymerase II second largest subunit (*rpb2*), putative F-box-domain-containing protein (*ASA-10*), and putative histone-like transcription factor (*ASA-19*) genes, which are phylogenetically informative for species resolution in the section *Alternaria* (Dettman and Eggertson, 2022; Dettman et al., 2023). The presence of the AAL-toxin biosynthesis gene (*ALT1*) in small-spored *Alternaria* spp. strains was tested using the primer pair DeH-F/E8T7 (Egusa et al., 2009; Jia et al., 2012).

PCR amplification was carried out in a total volume of 25 µL, containing 1 µL fungal DNA, 1× Green GoTaq® Flexi buffer (Promega, Madison, WI, USA), 1.5 mM MgCl₂ (Promega), 200 µM deoxyribonucleotide triphosphate (dNTP), 1 Unit GoTaq® G2 Hot Start DNA polymerase (Promega), and 0.4 µM of each corresponding oligonucleotide primer. PCR amplification was performed in a Bio-Rad T100™ Thermal Cycler (Bio-Rad Inc). The primers and references of thermocycling parameters for amplification are provided in Table 1.

The amplified PCR products were then subjected to agarose gel electrophoresis using 1.2 % agarose gel in 0.5 × Tris-acetate-EDTA (TAE, pH 8.0) buffer and Midori Green Advance as the staining agent (Nippon Genetics Europe, Germany) and photo documented under ultraviolet (UV) light. The successfully amplified PCR products were purified and sequenced via the TubeSeq Supreme Sanger sequencing service of Eurofins (Eurofins Genomics Europe Sequencing GmbH, Ebersberg, Germany). All the newly generated *ITS*, *rpb2*, *ASA-10*, and *ASA-19* sequences were deposited in the NCBI GenBank (Table 2).

2.4. Phylogenetic analyses

Single gene phylogeny of the internal transcribed spacer regions 1 and 2 and intervening 5.8S nrDNA (*ITS*) region was used for the initial identification of recovered fungal strains by comparing them with selected representatives described by Woudenberg et al. (2013, 2014, 2015, 2017). Phylogenetic reconstruction of *Alternaria* spp. was performed by combining the acquired nucleotide sequences of 72 isolates of

the RNA polymerase II second largest subunit (*rpb2*), putative F-box-domain-containing protein (*ASA-10*), and putative histone-like transcription factor (*ASA-19*) genes. Additional nucleotide sequences of *Alternaria* strains from the section *Alternaria* were obtained from Dettman and Eggertson (2022) and Dettman et al. (2023).

The concatenated DNA sequences of *Alternaria* spp. isolates were aligned using MUSCLE v. 3.8 (Edgar, 2004), and the GBLOCKS tool v. 0.91b was used for alignment curation (Castresana, 2000; Talavera and Castresana, 2007). Phylogeny was rooted with the *A. solani* strain HWC-168-2012p from the section *Porri*. The best fit evolutionary model strategy for the maximum likelihood (ML) phylogenetic tree was identified using IQ-Tree multicore software v. 1.6.12 (Trifinopoulos et al., 2016), the ModelFinder method (Kalyaanamoorthy et al., 2017) and Bayesian information criterion (BIC) analyses. The highest BIC score was found for the TNe + G4 strategy for *rpb2*, K2P for *ASA-10*, K2P + G4 for *ASA-19*, and TNe + R2 for the combined dataset of three genes. The maximum likelihood analyses were run with 1000 ultrafast bootstraps to assess the robustness of the individual nodes, and support values greater than 95 % were considered significant (Minh et al., 2013). Phylogenetic tree construction was performed using the online tool Interactive Tree of Life (ITOL) version 6 (Letunic and Bork, 2024).

2.5. Haplotype assignment and analysis

Haplotypes were defined for *Alternaria* section *Alternaria* strains isolated from tomatoes at three loci: *rpb2*, *ASA-10*, and *ASA-19*. Locus-specific and species-specific haplotypes were assigned based on sequence datasets generated for each locus separately and for concatenated sequences. Sequences were aligned and trimmed using ClustalW implemented in MEGA 11 software (Tamura et al., 2021). To assess the distribution of each mono-locus haplotype identified in the tomato population among a larger set of *Alternaria* spp. strains, the number of sequences with 100 % identity and 100 % query coverage for each haplotype was determined using a standard nucleotide BLAST search in the NCBI GenBank database. PopART (Population Analysis with Reticulate Trees) software version 1.7.3 (Leigh and Bryant, 2015) was used to generate Templeton, Crandall, and Sing's (TCS) networks (Templeton et al., 1992; Clement et al., 2000) for the aligned haplotype sequences, indicate geographic localization of the haplotypes and calculate nucleotide diversity and the numbers of variable and informative sites at each locus.

2.6. Pathogenicity assay on tomatoes (*S. lycopersicum* L.)

The pathogenicity of *Alternaria* spp. isolates was tested on 8-week-old 'Encore F1' tomatoes. Plants were grown in individual pots containing peat substrate (KKS-1, "Laflora", Livberze, Jelgava district, Latvia), a fraction size of 0–7 mm, a pH_{KCl} of 5.2–6, and an EC of 0.3 mS m⁻¹, enriched with a fertilizer PG mixture (NPK 15-10-20, 1 kg m⁻³), dolomite (1.8 kg m⁻³), limestone (6 kg m⁻³), and a wetting agent ("Instant", 0.3 L m⁻³). The tomato plants were grown in a glass greenhouse, and the average day and night temperatures were 25 ± 2 °C and

Table 1

List of primers used for PCR and sequencing in this study.

Target gene	Primer name	Primer sequence (5' to 3')	References
Internal transcribed spacer regions 1 and 2 and intervening 5.8S nrDNA (<i>ITS</i>)	ITS1	TCCGTAGGTGAACCTGCGG	White et al. (1990)
	ITS4	TCTCCGCTTATTGATATGC	
RNA polymerase II second largest subunit (<i>rpb2</i>)	RPB2-5F2	GGGGWGAYCAGAAGAAGGC	Sung et al. (2007)
	rRPB2-7cR	CCCATRGCTTGTYRCCCAT	Liu et al. (1999)
Putative F-box-domain-containing protein gene (<i>ASA-10</i>)	ASA_g821_F15	GCTACGCAAATGGATCTCGC	Dettman and Eggertson (2022)
	ASA_g821_R427	TCGATGACTTGAACCTGGCTGA	
Putative histone-like transcription factor gene (<i>ASA-19</i>)	Asa_g11712_F54	ACCGCCTAGATCAGTACCGA	Egusa et al. (2009); Jia et al. (2012)
	Asa_g11712_R629	ACCCTCCATTGTACACGTAC	
AAL toxin biosynthesis gene (<i>ALT1</i>)	DeH-F	CTCCGCCTGCCAATGTGATTAC	Egusa et al. (2009); Jia et al. (2012)
	E8T7	GCGTACCAAGGCACGTGCTCAA	

Table 2

The list of studied *Alternaria* spp. strains isolated from tomatoes (*Solanum lycopersicum* L.) in Latvia, Lithuania, Estonia, and Algeria, their haplotypes and GenBank accession numbers of analysed loci.

Name of the strain ^a	Species/Species complex	rpb2 haplotype	Multi-locus haplotype (rpb2 + ASA-10 + ASA-19)	Species/Lineage assignment by locus ^b			Country	Site	Year of isolation	Infected plant part	GenBank accession numbers ^c			
				rpb2	ASA-10	ASA-19					ITS	rpb2	ASA-10	ASA-19
LD001	<i>A. alternata</i>	AalH1	AalHm1	alt	alt	alt	Latvia	Berze	2022	Leaf	OQ607989	PP375562	PP375600	PP357358
LD009 ^a	<i>A. arborescens</i>	AarH5	AarHm3	arb	arb	arb	Latvia	Iecava	2022	Fruit	OQ607997	PP375563	PP375601	PP357359
LD011	<i>A. alternata</i>	AalH8	AalHm6	alt	alt	alt	Latvia	Daugmale	2022	Leaf	OQ607990	PP375564	PP375602	PP357360
LD013	<i>A. alternata</i>	AalH6	AalHm11	alt	alt	alt	Latvia	Iecava	2022	Fruit	OQ607955	PP375565	PP375603	PP357361
LD014	<i>A. alternata</i>	AalH9	AalHm7	alt	alt	alt	Latvia	Berze	2022	Leaf	OQ607991	PP375566	PP375604	PP357362
LD016	<i>A. arborescens</i>	AarH2	AarHm2	arb	arb	arb	Latvia	Berze	2022	Leaf	OQ607993	PP375567	PP375605	PP357363
LD017	<i>A. arborescens</i>	AarH2	AarHm4	arb	arb	arb	Latvia	Jaunsvirlauka	2022	Leaf	OQ607970	PP375568	PP375606	PP357364
LD018	<i>A. alternata</i>	AalH9	AalHm7	alt	alt	alt	Latvia	Daugmale	2022	Leaf	OQ607983	PP375569	PP375607	PP357365
LD019 ^a	<i>A. alternata</i>	AalH12	AalHm16	alt	alt	alt	Latvia	Daugmale	2022	Leaf	OQ607998	PP375570	PP375608	PP357366
LD020	<i>A. alternata</i>	AalH8	AalHm6	alt	alt	alt	Latvia	Daugmale	2022	Leaf	OQ607985	PP375571	PP375609	PP357367
LD021	<i>A. alternata</i>	AalH1	AalHm3	alt	alt	alt	Latvia	Daugmale	2022	Leaf	OQ607999	PP375572	PP375610	PP357368
LD022	<i>A. arborescens</i>	AarH4	AarHm1	arb	arb	arb	Latvia	Vircava	2022	Leaf	OQ607994	PP375573	PP375611	PP357369
LD024	<i>A. arborescens</i>	AarH5	AarHm3	arb	arb	arb	Latvia	Iecava	2022	Fruit	OQ608000	PP375574	PP375612	PP357370
LD025 ^a	<i>A. arborescens</i>	AarH5	AarHm3	arb	arb	arb	Latvia	Iecava	2022	Fruit	OQ607987	PP375575	PP375613	PP357371
LD026 ^a	<i>A. alternata</i>	AalH8	AalHm6	alt	alt	alt	Latvia	Jaunsvirlauka	2022	Leaf	OQ607982	PP375576	PP375614	PP357372
LD027	<i>A. arborescens</i>	AarH5	AarHm3	arb	arb	arb	Latvia	Vircava	2022	Leaf	OQ608001	PP375577	PP375615	PP357373
LD029	<i>A. alternata</i>	AalH1	AalHm3	alt	alt	alt	Latvia	Jaunsvirlauka	2022	Leaf	OQ607975	PP375578	PP375616	PP357374
LD031	<i>A. alternata</i>	AalH1	AalHm3	alt	alt	alt	Latvia	Jaunsvirlauka	2022	Leaf	OQ608003	PP375579	PP375617	PP357375
LD032	<i>A. alternata</i>	AalH1	AalHm3	alt	alt	alt	Latvia	Daugmale	2022	Leaf	OQ607965	PP375580	PP375618	PP357376
LD033	<i>A. alternata</i>	AalH2	AalHm4	alt	alt	alt	Latvia	Daugmale	2022	Leaf	OQ607966	PP375581	PP375619	PP357377
LD036	<i>A. alternata</i>	AalH9	AalHm7	alt	alt	alt	Latvia	Jaunsvirlauka	2022	Leaf	OQ607979	PP375582	PP375620	PP357378
LD037	<i>A. alternata</i>	AalH2	AalHm4	alt	alt	alt	Latvia	Vircava	2022	Leaf	OQ607958	PP375583	PP375621	PP357379
LD038	<i>A. alternata</i>	AalH8	AalHm6	alt	alt	alt	Latvia	Daugmale	2022	Leaf	OQ607981	PP375584	PP375622	PP357380
LD039	<i>A. alternata</i>	AalH1	AalHm3	alt	alt	alt	Latvia	Jaunsvirlauka	2022	Leaf	OQ607995	PP375585	PP375623	PP357381
LD040	<i>A. alternata</i>	AalH2	AalHm4	alt	alt	alt	Latvia	Iecava	2022	Leaf	OQ607963	PP375586	PP375624	PP357382
LD042	<i>A. alternata</i>	AalH1	AalHm8	alt	alt	alt	Latvia	Daugmale	2022	Leaf	OQ607967	PP375587	PP375625	PP357383
LD043	<i>A. alternata</i>	AalH9	AalHm7	alt	alt	alt	Latvia	Jaunsvirlauka	2022	Leaf	OQ607977	PP375588	PP375626	PP357384
LD045 ^a	<i>A. alternata</i>	AalH1	AalHm3	alt	alt	alt	Latvia	Berze	2022	Leaf	OQ607980	PP375589	PP375627	PP357385
LD046	<i>A. alternata</i>	AalH8	AalHm6	alt	alt	alt	Latvia	Jaunsvirlauka	2022	Leaf	OQ607978	PP375590	PP375628	PP357386
LD047	<i>A. alternata</i>	AalH2	AalHm4	alt	alt	alt	Latvia	Daugmale	2022	Leaf	OQ607956	PP375591	PP375629	PP357387
LD048	<i>A. alternata</i>	AalH6	AalHm2	alt	alt	alt	Latvia	Daugmale	2022	Leaf	OQ607968	PP375592	PP375630	PP357388
LD049	<i>A. arborescens</i>	AarH5	AarHm3	arb	arb	arb	Latvia	Vircava	2022	Leaf	OQ607984	PP375593	PP375631	PP357389
LD051	<i>A. alternata</i>	AalH8	AalHm6	alt	alt	alt	Latvia	Berze	2022	Leaf	OQ607961	PP375594	PP375632	PP357390
LD053	<i>A. alternata</i>	AalH8	AalHm14	alt	arb?	alt	Latvia	Berze	2022	Leaf	OQ607988	PP375595	PP375633	PP357391
LD054	<i>A. alternata</i>	AalH8	AalHm6	alt	alt	alt	Latvia	Berze	2022	Leaf	OQ607986	PP375596	PP375634	PP357392
LD055	<i>A. alternata</i>	AalH8	AalHm6	alt	alt	alt	Latvia	Berze	2022	Leaf	OQ607957	PP375597	PP375635	PP357393
LD057	<i>A. alternata</i>	AalH2	AalHm4	alt	alt	alt	Latvia	Berze	2022	Leaf	OQ607996	PP375598	PP375636	PP357394
LD058 ^a	<i>A. alternata</i>	AalH6	AalHm5	alt	alt	alt	Latvia	Berze	2022	Leaf	OQ609067	PP375599	PP375637	PP357395
LD090	<i>A. alternata</i>	AalH9	AalHm7	alt	alt	alt	Estonia	Vedu	2023	Leaf	PP631928	PP665560	PP665538	PP665516
LD093 ^a	<i>A. alternata</i>	AalH2	AalHm4	alt	alt	alt	Estonia	Vedu	2023	Leaf	PP631929	PP665561	PP665539	PP665517
LD115 ^a	<i>A. arborescens</i>	AarH5	AarHm3	arb	arb	arb	Estonia	Koimula	2023	Leaf	PP631930	PP665562	PP665540	PP665518
LD116 ^a	<i>A. arborescens</i>	AarH5	AarHm3	arb	arb	arb	Estonia	Koimula	2023	Leaf	PP631931	PP665563	PP665541	PP665519
LD122 ^a	<i>A. alternata</i>	AalH9	AalHm7	alt	alt	alt	Estonia	Jogeva	2023	Leaf	PP631932	PP665564	PP665542	PP665520
LD124 ^a	<i>A. postmessia</i>	AalH8	none	alt	alt	pos	Estonia	Jogeva	2023	Leaf	PP631933	PP665565	PP665543	PP665521
LD131 ^a	<i>A. alternata</i>	AalH2	AalHm4	alt	alt	alt	Estonia	Jogeva	2023	Leaf	PP631934	PP665566	PP665544	PP665522
LD134	<i>A. alternata</i>	AalH1	AalHm4	alt	alt	alt	Estonia	Jogeva	2023	Leaf	PP631935	PP665567	PP665545	PP665523
LD135 ^a	<i>A. alternata</i>	AalH2	AalHm4	alt	alt	alt	Lithuania	Karkiskes	2023	Leaf	PP631936	PP665568	PP665546	PP665524
LD136 ^a	<i>A. alternata</i>	AalH8	AalHm6	alt	alt	alt	Lithuania	Karkiskes	2023	Leaf	PP631937	PP665569	PP665547	PP665525
LD137	<i>A. alternata</i>	AalH2	AalHm4	alt	alt	alt	Lithuania	Karkiskes	2023	Leaf	PP631938	PP665570	PP665548	PP665526
LD138	<i>A. alternata</i>	AalH2	AalHm4	alt	alt	alt	Lithuania	Karkiskes	2023	Leaf	PP631939	PP665571	PP665549	PP665527

(continued on next page)

Table 2 (continued)

Name of the strain ^a	Species/Species complex	<i>rpb2</i> haplotype	Multi-locus haplotype (<i>rpb2</i> + <i>ASA-10</i> + <i>ASA-19</i>)	Species/Lineage assignment by locus ^b			Country	Site	Year of isolation	Infected plant part	GenBank accession numbers ^c			
				<i>rpb2</i>	<i>ASA-10</i>	<i>ASA-19</i>					ITS	<i>rpb2</i>	<i>ASA-10</i>	<i>ASA-19</i>
LD141	<i>A. alternata</i>	AalH2	AalHm4	alt	alt	alt	Lithuania	Virbaliskiai	2023	Leaf	PP631941	PP665573	PP665551	PP665529
LD142	<i>A. alternata</i>	AalH2	AalHm9	alt	alt	alt	Lithuania	Virbaliskiai	2023	Leaf	PP631942	PP665574	PP665552	PP665530
LD143	<i>A. alternata</i>	AalH2	AalHm4	alt	alt	alt	Lithuania	Virbaliskiai	2023	Leaf	PP631943	PP665575	PP665553	PP665531
LD149	<i>A. alternata</i>	AalH9	AalHm7	alt	alt	alt	Lithuania	Ringaudai	2023	Leaf	PP631944	PP665576	PP665554	PP665532
LD153 ^a	<i>A. arborescens</i>	AarH5	AarHm3	arb	arb	arb	Lithuania	Ringaudai	2023	Leaf	PP631945	PP665577	PP665555	PP665533
LD154 ^a	<i>A. arborescens</i>	AarH5	AarHm3	arb	arb	arb	Lithuania	Ringaudai	2023	Leaf	PP631946	PP665578	PP665556	PP665534
LD156	<i>A. alternata</i>	AalH1	nd ^d	alt	alt	alt	Lithuania	Babtai	2023	Leaf	PP631947	PP665579	PP665557	PP665535
LD157	<i>A. alternata</i>	AalH11	AalHm15	alt	alt	alt	Lithuania	Babtai	2023	Leaf	PP631948	PP665580	PP665558	PP665536
LD158	<i>A. alternata</i>	AalH9	AalHm7	alt	alt	alt	Lithuania	Babtai	2023	Leaf	PP631949	PP665581	PP665559	PP665537
NB1	<i>A. alternata</i>	AalH8	AalHm12	alt	alt	alt	Algeria	Mostaganem	2011	Fruit	KT968740	MH232240	PP665582	PP665502
NB38	<i>A. arborescens</i>	AarH4	AarHm5	arb	arb	arb	Algeria	Mostaganem	2011	Fruit	KT968742	MH232242	PP665583	PP665503
NB66	<i>A. alternata</i>	AalH6	AalHm11	alt	alt	alt	Algeria	Mostaganem	2011	Stem	KT968745	MH232244	PP665584	PP665504
NB97	<i>A. alternata</i>	AalH8	AalHm6	alt	alt	alt	Algeria	Oran	2011	Leaf	KT968757	MH232249	PP665585	PP665505
NB107	<i>A. alternata</i>	AalH6	AalHm5	alt	alt	alt	Algeria	Mostaganem	2011	Fruit	KT968760	MH232263	PP665586	PP665506
NB120	<i>A. alternata</i>	AalH6	AalHm13	alt	alt	alt	Algeria	Mostaganem	2011	Fruit	KT968762	MH232255	PP665587	PP665507
NB126	<i>A. alternata</i>	AalH11	AalHm15	alt	alt	alt	Algeria	Mostaganem	2011	Fruit	KT968763	MH232251	PP665588	PP665508
NB164	<i>A. arborescens</i>	AarH4	AarHm5	arb	arb	arb	Algeria	Ain	2011	Leaf	KT968770	MH232254	PP665589	PP665509
NB165	<i>A. alternata</i>	AalH6	AalHm11	alt	alt	alt	Algeria	Témouchent	2011	Leaf	KT968771	MH232258	PP665590	PP665510
NB270	<i>A. longipes</i>	AalH8	none	alt	alt	long	Algeria	Mascara	2013	Leaf	KT968772	MH232256	PP665591	PP665511
NB532	<i>A. alternata</i>	AalH8	AalHm10	alt	alt	alt	Algeria	Mostaganem	2017	Leaf	/	MH232235	PP665595	PP665515
NB553	<i>A. alternata</i>	AalH11	AalHm15	alt	alt	alt	Algeria	Mostaganem	2017	Leaf	/	MH232237	PP665592	PP665512
NB567	<i>A. arborescens</i>	AarH3	AarHm6	arb	arb	arb	Algeria	Mostaganem	2017	Fruit	/	MH232239	PP665593	PP665513

/= no nucleotide sequence available.

^a Fungal strains marked with “a” were selected for pathogenicity assay.

^b Alt = alternata lineage; arb = arborescens lineage; lon = longipes lineage; pos = *A. postmessia*.

^c GenBank accession numbers generated in the study by Bessadat et al. (2017, 2019) are indicated in bold.

^d *ASA-10* nucleotide sequence too short to be considered for haplotype definition.

18 ± 2 °C, respectively. Soluble fertilizer (NPK 3.5–2.3–5.0, Vito, AS Spodribe, Dobele, Latvia) was used to fertilize the tomato plants once a week according to the manufacturer's instructions. A total of 16 *Alternaria* spp. strains from section *Alternaria* were selected for pathogenicity test based on their species or lineage and country of origin, excluding fungal strains from Algeria analysed previously by Bessadat et al. (2017) (Table 2). The large-spored *Alternaria* sp. strain LD030 (section *Porri*) isolated from symptomatic tomatoes in Latvia in 2022 was used as a positive control. The conidia of *Alternaria* spp. were obtained from seven-day-old colonies grown on PCA media at 23 °C under 12 h UV light and 12 h darkness. Conidia were removed by gently scraping the surface of the PCA medium with a Drigalski spatula and placed in a sterile glass jar containing 0.01 % Tween 80 (Chempur, Piekary Śląskie, Poland). The concentration of conidial suspensions was determined with a Bürker counting chamber (Heinz Herenz Medizinalbedarf GmbH, Hamburg, Germany) and adjusted to 1×10^4 conidia/mL. Tomato plants were inoculated with 10 mL of conidial suspensions, and control plants were treated with sterile distilled water. The pathogenicity of each strain was tested in five replicates, and a total of 90 tomato plants were inoculated. After inoculation, tomatoes were covered with perforated high-density polyethylene (HDPE) plastic bags (900 × 480 mm) to maintain relative humidity above 90 % and incubated at 25 ± 2 °C. The perforated HDPE plastic bags were removed after 48 h, and the presence of necrotic areas on the plant tissue was evaluated by visual inspection 7 and 14 days post-inoculation.

3. Results

3.1. Characterization of samples and isolate collection

A total of 588 symptomatic samples were collected in greenhouses and high tunnels during the 2022 and 2023 crop seasons in three countries (viz. Latvia, Lithuania, and Estonia). Accurate identification of disease symptoms caused by *Alternaria* spp. was challenging due to the complex interplay between different pathogens from the family *Pleosporaceae* producing a wide range of symptoms. Based on examinations of conidial morphology, small-spored *Alternaria* spp. were detected in 54 % ($n = 316$) of samples, and they were identified on symptomatic leaves and fruits of tomatoes alone or in complex with large-spored *Alternaria* spp. from the section *Porri* or related pathogens from the genus *Stemphylium*. The isolation of small-spored *Alternaria* spp. from section *Alternaria* was associated with blighted leaves and black, sunken lesions of tomatoes occurring predominantly in the cracks of the peduncular area of fruits (Fig. 1). Molecular phylogeny based on the internal transcribed spacer regions 1 and 2 and intervening 5.8S nrDNA (*ITS*) region (Fig. S1) revealed that recovered fungal strains formed well-established clusters, representing four sections from the genus *Alternaria*, namely *Alternaria*, *Porri*, *Infectoriae*, and *Ulocladioides*; with significant support (>95 % maximum likelihood (ML) bootstrap) for the section *Alternaria*. Furthermore, closely related fungi from the genus *Stemphylium* were also identified on symptomatic tomatoes in our study. The present study focused exclusively on fungal strains from *Alternaria* section *Alternaria* to gain broader understanding of fungal biology in this section and its implications for fungal–plant interactions.

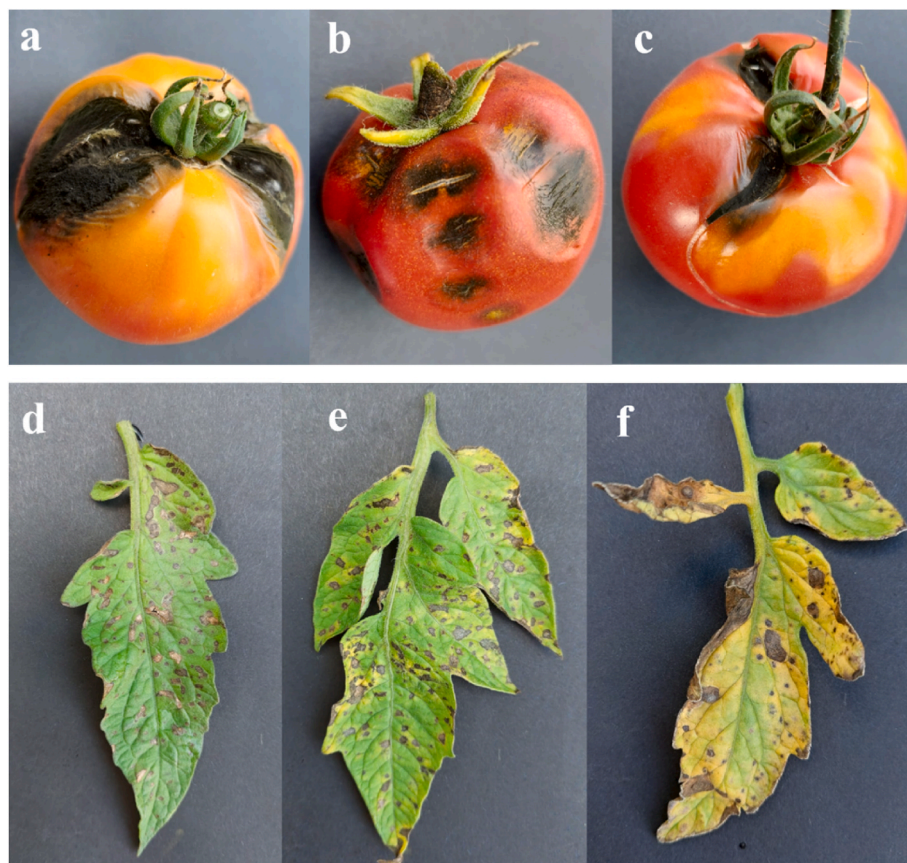


Fig. 1. Symptomatic plant samples selected for small-spored *Alternaria* spp. (section *Alternaria*) isolation in the laboratory. Tomato fruits with black mold, black rot, and sunken lesions with a diffuse edge (a–c); tomato leaves with small, brown spots (d), which become larger, necrotic, and surrounded by diffuse chlorosis (e), leading to coalescent and concentric lesions with blighted appearance (f).

3.2. Morphological characterization of *Alternaria* spp. from section *alternaria*

Considerable diversity of cultural and morphological characteristics was observed for the small-spored *Alternaria* spp. strains from the section *Alternaria* after seven days of incubation on potato dextrose agar (PDA) media at 23 °C in continuous darkness. *Alternaria* spp. strains were cottony or velvety, with or without obvious concentric rings or radial zonation. The elevation of the colonies varied from flat to slightly raised or raised in the centre, and the margins were entire, undulate or irregular. The obverse colour of the *Alternaria* spp. strains varied from light grey (N8, N7) or medium grey (N6, N5) to light greyish brown (5 YR 6/2) or greyish olive green (7.5GY 4/2). The colouration in the centre of the colonies varied from pale green (10GY 7/2) to greyish green (10GY 5/2) and occasionally dark greyish olive green (7.5GY 2/2) with distinctly lighter pigmentation at the edges of the Petri dish. The reverse colour of the *Alternaria* spp. strains varied from dark olive green (7.5GY 2/4) to greyish brown (7.5 YR 3/2), greyish reddish brown (10R 4/2), moderate reddish brown (7.5R 3/4), dark yellowish brown (10 YR 3/4), moderate orange (5 YR 6/8) or dark orange yellow (7.5 YR 6/8) (Fig. 2).

The micro-morphological characteristics confirmed the occurrence of *Alternaria* spp. from the section *Alternaria* on tomatoes, and the production of small (<60 µm in length), obclavate or ellipsoid conidia with a tapering apical beak was observed for the obtained strains (Fig. 3). Some of the *Alternaria* spp. strains produced short, straight, and simple conidiophores with simple or branched chains of 2–14 units, while others were characterized by the presence of short and branched conidiophores producing 2 to 9 catenated conidia in a chain. Based on these sporulation patterns the strains were assigned to *A. alternata* and *A. arborescens* species complexes, respectively.

3.3. Phylogenetic analyses

A multi-gene phylogeny of *rpb2*, *ASA-10*, and *ASA-19* (Fig. 4) was applied for the molecular identification of 72 obtained *Alternaria* spp.

strains at the species and lineage levels by comparing them with 28 selected representatives from each major phylogenetic lineage in the *Alternaria* section *Alternaria* (Dettman and Eggertson, 2022; Dettman et al., 2023).

The combined dataset of *rpb2* (719 nucleotides [nt]), *ASA-10* (253 nt), and *ASA-19* (508 nt) had a total length of 1480 nt and included 120 parsimony-informative sites. The *ASA-19* dataset included the highest percentage of phylogenetically informative sites (11.42 %), followed by the *ASA-10* loci (7.51 %). The smallest amount of informative variation was observed in the *rpb2* dataset, which contained 5.98 % parsimony-informative sites.

The single gene maximum likelihood (ML) phylogenies allocated 70 of 72 recovered small-spored *Alternaria* spp. strains into the main phylogenetic lineages of section *Alternaria* with almost complete agreement between all three separately analysed genomic regions (Figs. S2–S4). Inconclusive results for lineage-level taxonomic assignment were obtained for two fungal strains (LD053 and NB270, respectively). *Alternaria* sp. strain LD053 was assigned to the *alternata* lineage by *rpb2* and *ASA-19* but to the *arborescens* lineage by *ASA-10*. Similarly, strain NB270 was assigned to the *alternata* lineage by *rpb2* and *ASA-10* but to the *longipes* lineage by *ASA-19*, suggesting potential recombination between the lineages in the analysed genomic regions. The topological structure of the lineages of the *ASA-19* locus outperformed that of the two other loci analysed and provided the most similar tree to the combined dataset (Fig. S4).

According to the ML phylogenetic tree of the combined dataset of *rpb2*, *ASA-10*, and *ASA-19*, the small-spored *Alternaria* spp. strains obtained in this study clustered with members of three phylogenetic lineages from section *Alternaria*, including *arborescens*, *alternata*, and *longipes*. High intraspecific genetic divergence was observed within both the *arborescens* and *alternata* lineages, and the formation of eight and four sub-clades with significant bootstrap support (>95 % ML) confirmed the occurrence of *A. alternata* and *A. arborescens* species complexes on tomatoes, respectively. Multi-locus phylogeny revealed that three fungal strains within *alternata* lineage associated with symptomatic tomatoes in Latvia (LD042, LD048, and LD053) formed a

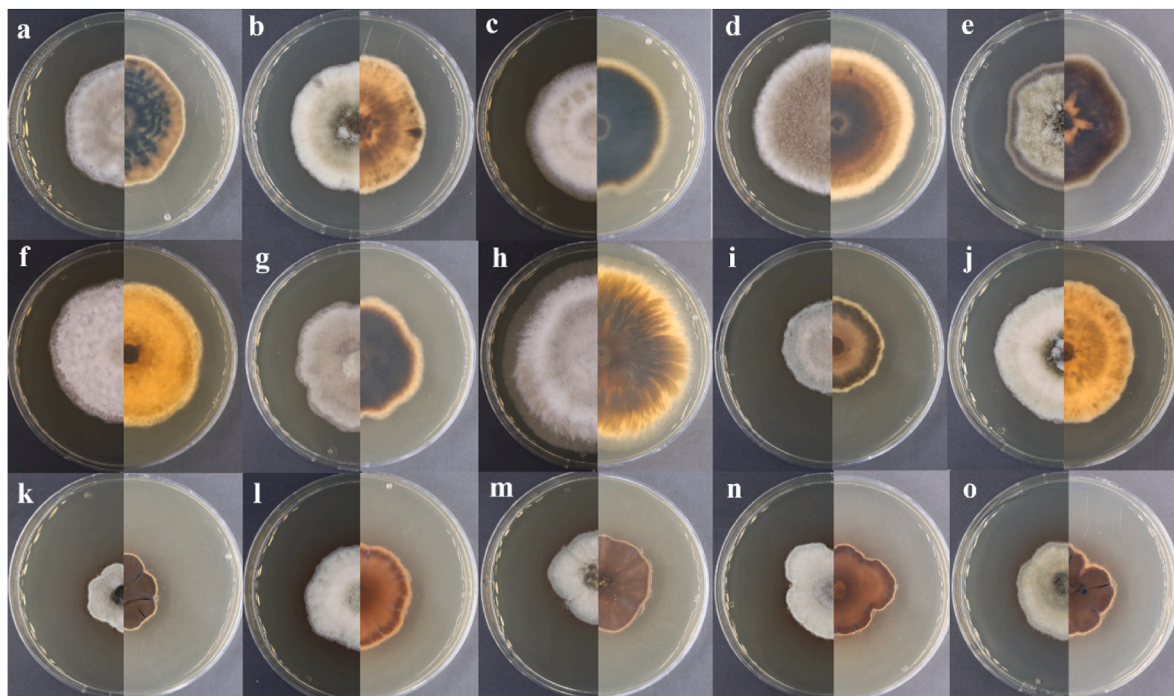


Fig. 2. Phenotypic diversity of small-spored *Alternaria* spp. strains from the section *Alternaria* on PDA medium after 7 days at 23 °C in the dark. The obverse (left) and reverse (right) sides of *A. alternata* strains (LD143, a; LD135, b; LD047, c; LD138, d; LD093, e; LD032, f; LD013, g; LD038, h; LD001, i; LD057, j) and *A. arborescens* strains (LD049, k; LD016, l; LD009, m; LD027, n; LD115, o).

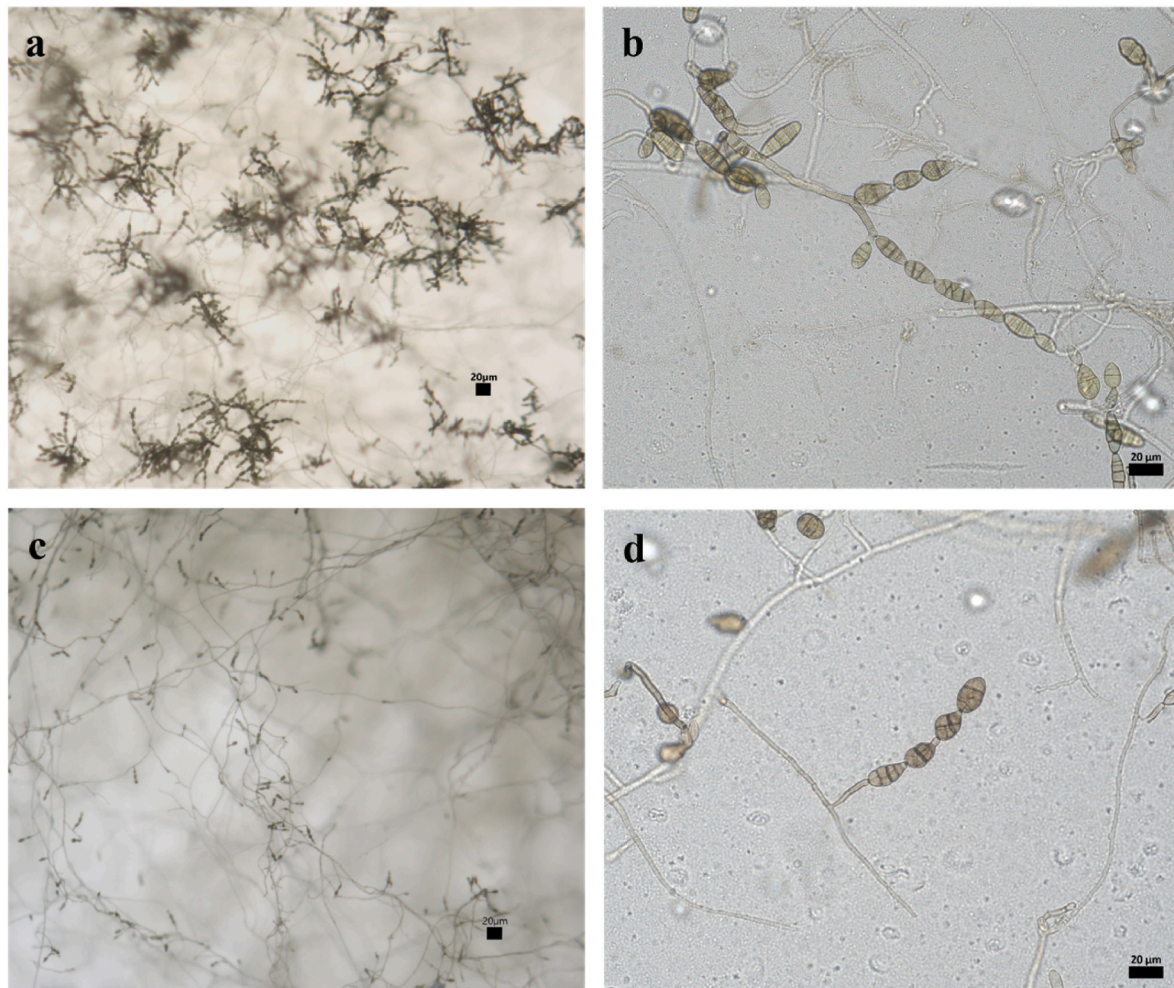


Fig. 3. The catenation and conidial morphology of *A. arborescens* (LD154, a – b) and *A. alternata* (LD093, c – d). Scale bars = 20 µm.

cluster with fungal strains KAS5513 and DET2201 (isolated from cereal crops in Canada and previously described as interlocus recombinants within *alternata* lineage), which was not significantly supported (<95 % ML).

Additionally, we found that strain LD124 clustered with *A. postmessia* (previously synonymized morpho-species of *A. alternata*) and formed a separate branch with significant bootstrap support (100 % ML) based on analyses of combined data from the aforementioned loci (Fig. 4).

3.4. Haplotype diversity of *rpb2* gene at the global level

As a first step to characterize the genetic diversity of *Alternaria* strains from section *Alternaria* associated with tomatoes over a large geographic area, sequences at the *rpb2* locus were selected. The dataset included sequences referenced in different population studies or extracted from currently available assembled genomes. Additional *rpb2* sequence data were obtained by mining the NCBI GenBank Nucleotide database with appropriate keywords till April 2024. Sequences shorter than 580 nucleotides or of poor quality were disregarded. The final dataset included 207 strains from 12 countries isolated from cultivated and wild tomato species (Table 3). An initial phylogenetic analysis of these sequences combined with *rpb2* sequences from representatives of the main lineages within section *Alternaria* was performed to assign each sequence to the *A. alternata* and *A. arborescens* species complexes (Fig. S5). On this basis, the majority of the analysed strains (87 %, $n = 181$) were considered members of the *A. alternata* species complex, whereas 11 % of the strains ($n = 22$) belonged to the *A. arborescens*

species complex. Two strains from South Korea had *rpb2* sequences identical to those of the newly described species from *Solanum* spp. (Gou et al., 2023). As mentioned in Table 2, one strain from Algeria grouped with members of the longipes lineage, and one strain from Estonia clustered with *A. postmessia*. Their geographic repartition is provided in Table 3.

The *rpb2* gene sequence dataset yielded 12 haplotypes (*AalH1* to *AalH12*), of which three were unique, with 17 SNPs for strains from the *alternata* lineage, and seven haplotypes (*AarH1* to *AarH7*), of which two were unique with eight SNPs for strains from the *arborescens* lineage. Strains from the two species complexes shared three polymorphic sites (Table S1). All these SNPs located in the *rpb2* gene corresponded to silent mutations in the translated nucleotide sequence. Fifteen parsimony informative sites were found within the whole population. Haplotypes *AalH8* and *AarH5* accounted for 43 % and 41 % of the total number of strains from tomatoes and were the most frequently encountered within the *alternata* and *arborescens* lineages, respectively (Fig. 5). Regardless of the host origin, the haplotype *AalH8* also represented the majority of the *alternata* lineage (Fig. 5A), whereas *AarH4* was the most prevalent haplotype in the *arborescens* lineage (Fig. 5B). Notably, haplotype *AalH5* which represented 9 % of *A. alternata* strains isolated from tomatoes, was absent in strains from other hosts of origin.

For the estimation of genealogical relationships among the *rpb2* haplotypes in *A. alternata* and *A. arborescens* species complexes, a Templeton, Crandall, and Sing's (TCS) network was constructed considering the allocations to geographic areas of the analysed samples (Fig. 6). As sample size greatly varied among countries, we grouped



Fig. 4. Molecular phylogeny of *Alternaria* section *Alternaria* strains depicted as the maximum likelihood (ML) tree inferred from the multigenic alignment of RNA polymerase II second largest subunit (*rpb2*), putative F-box-domain-containing protein (*ASA-10*), and putative histone-like transcription factor (*ASA-19*) sequence data. The tree was rooted with *Alternaria solani* (section *Porri*) strain HWC-168-2012p. Fungal strains analysed in this study are highlighted in bold. The branches with significant bootstrap support (BS > 95 %) are marked with red stars near nodes. The branch length is indicated as the number of nucleotide substitutions per site.

them into five different geographic areas: North-East Europe (Estonia, Latvia, Lithuania: $n = 56$), North America (USA, Canada: $n = 53$), South America (Chile, Peru: $n = 58$), North Africa (Algeria, Egypt: $n = 27$), and East Asia (Japan, South-Korea: $n = 7$). The network illustrated the relatively good separation of the two lineages. However, equivocal relationships between the two species complexes were observed for three interior haplotypes (*AalH1*, *AarH2*, and *AarH6*, respectively). The two most frequently recovered haplotypes of *A. alternata*, *AalH1* and *AalH8*, were observed in the five geographic areas. In contrast, two haplotypes with considerable sample sizes, *AalH2* and *AalH5*, were restricted to North-East Europe and South America, respectively. Similarly, one haplotype, *AarH5*, that grouped 40 % of the strains assigned to the *A. arborescens* species complex was found only in the North-East of Europe. However, drawing additional conclusions about the arborescens lineage was difficult due to the small sample size.

3.5. Multi-locus haplotype diversity

To further characterize the diversity of *Alternaria* strains from the *A. alternata* and *A. arborescens* species complexes associated with tomatoes multi-locus haplotypes were defined from concatenated sequences of *rpb2*, *ASA-10* and *ASA-19*. As the vast majority of sequences for the two latter loci available in databanks were obtained from strains isolated from Canadian cereal crops, only sequences generated in the present study were considered. The datasets for *A. alternata* and *A. arborescens* grouped 53 and 15 strains, respectively.

The *ASA-10* dataset yielded four haplotypes (*AalHasa10-1* to *AalHasa10-4*) with 3 SNPs for strains from the alternata lineage, and four haplotypes (*AarHasa10-1* to *AarHasa10-4*) with 10 SNPs for strains from the arborescens lineage, which resulted in 3 and 5 amino-acid substitutions in the translated nucleotide sequences, respectively (Fig. S6).

For both lineages, four haplotypes (*AalHasa19-1* to *AalHasa19-4* and *AarHasa19-1* to *AarHasa19-4*) were defined from the *ASA-19* dataset with 11 and 6 polymorphic sites for the alternata and arborescens lineages, respectively. None of these SNPs resulted in amino-acid substitution in the translated nucleotide sequences.

AalHasa10-2 and *AalHasa19-2* were the most abundant haplotypes among strains from tomatoes and other origins, representing up to 98 % of the total number of strains. *AalHasa10-4*, *AalHasa19-1* and *AalHasa19-4* were only retrieved in strains isolated from tomatoes (Fig. S7).

The combined dataset of *rpb2*, *ASA-10*, and *ASA-19* (1452 nt) was resolved into 16 haplotypes for strains from the alternata lineage (*AalHm1-AalHm16*) and 6 haplotypes for strains from the arborescens lineage (*AarHm1-AarHm6*) with 35 SNPs and 20 SNPs, respectively (Table 2).

The TCS network resulting from this dataset and including traits for geographic origins (Fig. 7) revealed that the most abundant haplotypes, *AalHm-4* in the alternata lineage and *AarHm-3* in the arborescens lineage, were found only in European strains. Due to the relatively low number of strains analysed ($n = 68$) and the high variability of the selected loci (Table 4), 60 % of the haplotypes grouped only a single strain. The distribution of *A. alternata* haplotypes according to geographic location revealed that the maximum number of haplotypes was recorded in Latvia (Fig. S8). However, when expressed as a function of the number of strains per geographic location, a higher haplotype diversity was observed in Algeria.

3.6. Pathogenicity assay and toxins

In our study, the pathogenicity of 16 tomato-associated *Alternaria* spp. strains from section *Alternaria* (Table 2) was assessed by inoculating 8-week-old plants of the tomato cultivar 'Encore F1'. Leaf blight symptoms were not observed on tomatoes 7 and 14 days post-inoculation for any of the small-spored *Alternaria* spp. strains tested, including *A. arborescens* ($n = 6$), *A. alternata* ($n = 9$), and *A. postmessia* ($n = 1$), thus indicating their non-pathogenicity to the host of origin. No disease

Table 3
Geographic sources of the *Alternaria* section *Alternaria* strains from tomatoes.

Geographic origin	Number of strains			Host	Reference
	alternata lineage	arborescens lineage	Other lineage/species		
Algeria	9	3	1	<i>S. lycopersicum</i>	Bessadat et al. (2017, 2019)
USA	45	7	0	<i>S. lycopersicum</i>	Adhikari et al. (2020), Dettman and Eggertson (2022) ^b , Woudenberg et al. (2015)
Chile & Peru	58	0	0	Wild tomato spp. ^a	Schmey et al. (2023)
Egypt	15	0	0	<i>S. lycopersicum</i>	El Gobashy et al. (2018)
South Korea	6	0	2	<i>S. lycopersicum</i>	Choi et al., unpublished ^c
Japan	1	0	0	<i>S. lycopersicum</i>	Nishikawa and Nakashima (2020)
Canada	1	0	0	<i>S. lycopersicum</i>	Dettman and Eggertson (2022)
South Africa	2	0	0	<i>S. lycopersicum</i>	Visagie et al., unpublished ^c
Latvia	29	8	0	<i>S. lycopersicum</i>	Present study
Lithuania	10	2	0	<i>S. lycopersicum</i>	Present study
Estonia	5	2	1	<i>S. lycopersicum</i>	Present study
Total	181	22	4		

^a *S. chilense*, *S. habrochaites*, *S. lycopersicoides*, *S. peruvianum*.

^b Extracted from publicly available genome assembly GenBank database.

^c Extracted from GenBank.

symptoms were observed for the negative control treated with sterile distilled water (Fig. 8B). In contrast, typical disease symptoms were observed for tomato seedlings treated with conidial suspensions of the large-spored *Alternaria* sp. strain LD065 (section *Porri*) that served as a positive control. The formation of dark brown leaf spots was observed three days post-inoculation (dpi), indicating successful colonization of the host tissue. Scattered irregularly rounded lesions (3–7 mm in diameter) with concentric lines were visible on leaves 7 dpi, leading to severe damage and discoloration of the plant tissue 14 dpi (Fig. 8A). For the positive control represented by a pathogenic strain LD065, Koch's postulates were fulfilled by reisolation of pathogen from necrotic plant tissue and identification till the genus and section level based on morphological characteristics.

In addition, PCR amplification of the AAL-toxin biosynthesis gene (*ALT1*) confirmed that the small-spored *Alternaria* spp. strains analysed in this study were *ALT1* negative compared with the type strain of *A. arborescens* CBS 102605, which was *ALT1* positive (Fig. S9).

4. Discussion

Our study focused on small-spored *Alternaria* spp. strains from *Alternaria* section *Alternaria* isolated from symptomatic tomatoes in three countries in temperate climate zone (Estonia, Latvia, and Lithuania) and in northwestern Algeria, which has Mediterranean climate conditions. Accurate identification of small-spored *Alternaria* spp. is very challenging due to imperceptible variation in conidial morphology, a high genome identity of up to 98 % (Woudenberg et al., 2015), and the indistinguishable profile of toxic secondary metabolites among several species in the section *Alternaria* (Patriarca et al., 2019). Previous studies on *Alternaria* spp. infecting tomatoes in northeastern Europe have had limitations, especially the application of morphological

data solely for the identification of disease causal agents (Shidlauskienė and Survilienė, 2002; Duckėna and Bimsteine, 2023). Therefore, in this study we identified and characterized 72 tomato-associated *Alternaria* spp. strains based on polyphasic approach, which included morphological data, pathogenicity assay, multi-locus phylogeny, and haplotype analysis.

Considerable variation in phenotypic diversity was observed for small-spored *Alternaria* spp. isolated from symptomatic tomatoes in Latvia, Lithuania, Estonia, and Algeria, and sporulation patterns and conidial morphology did not allow precise identification of all the obtained strains to the species level as described in other studies (Armitage et al., 2015; Woudenberg et al., 2015; Landschoot et al., 2017).

Phylogenetic analysis based on RNA polymerase II second largest subunit (*rpb2*), putative F-box-domain-containing protein (*ASA-10*), and putative histone-like transcription factor (*ASA-19*) sequence data revealed that *Alternaria* spp. strains obtained in this study recovered three phylogenetic clades from the section *Alternaria*, including *alternata* ($n = 55$), *arborescens* ($n = 15$), and *longipes* ($n = 1$), and one of them clustered with *A. postmessia*. High intraspecific genetic variation was observed for both *alternata* and *arborescens* clades, and they were characterized by formation of eight and four sub-clades with significant bootstrap support (>95 % ML), respectively. Based on the revised taxonomy of small-spored *Alternaria* spp. by Woudenberg et al. (2015), *A. arborescens* is referred to as a species complex composed of several morpho-species, including *A. geophila*, *A. arborescens*, *A. cerealis*, and *A. senecionicola*, whereas *A. alternata* represents a phylogenetic species in the section *Alternaria*. However, recent studies have reported that *A. alternata* has a high level of intraspecific genetic divergence and cannot be considered a single species (Aung et al., 2020; Li et al., 2023; Zhu and Xiao, 2015). Moreover, based on the molecular phylogeny of seven loci (*ITS*, *LSU*, *SSU*, *TEF1- α* , *rpb2*, *GAPDH*, and *Alt-a1*), Li et al.

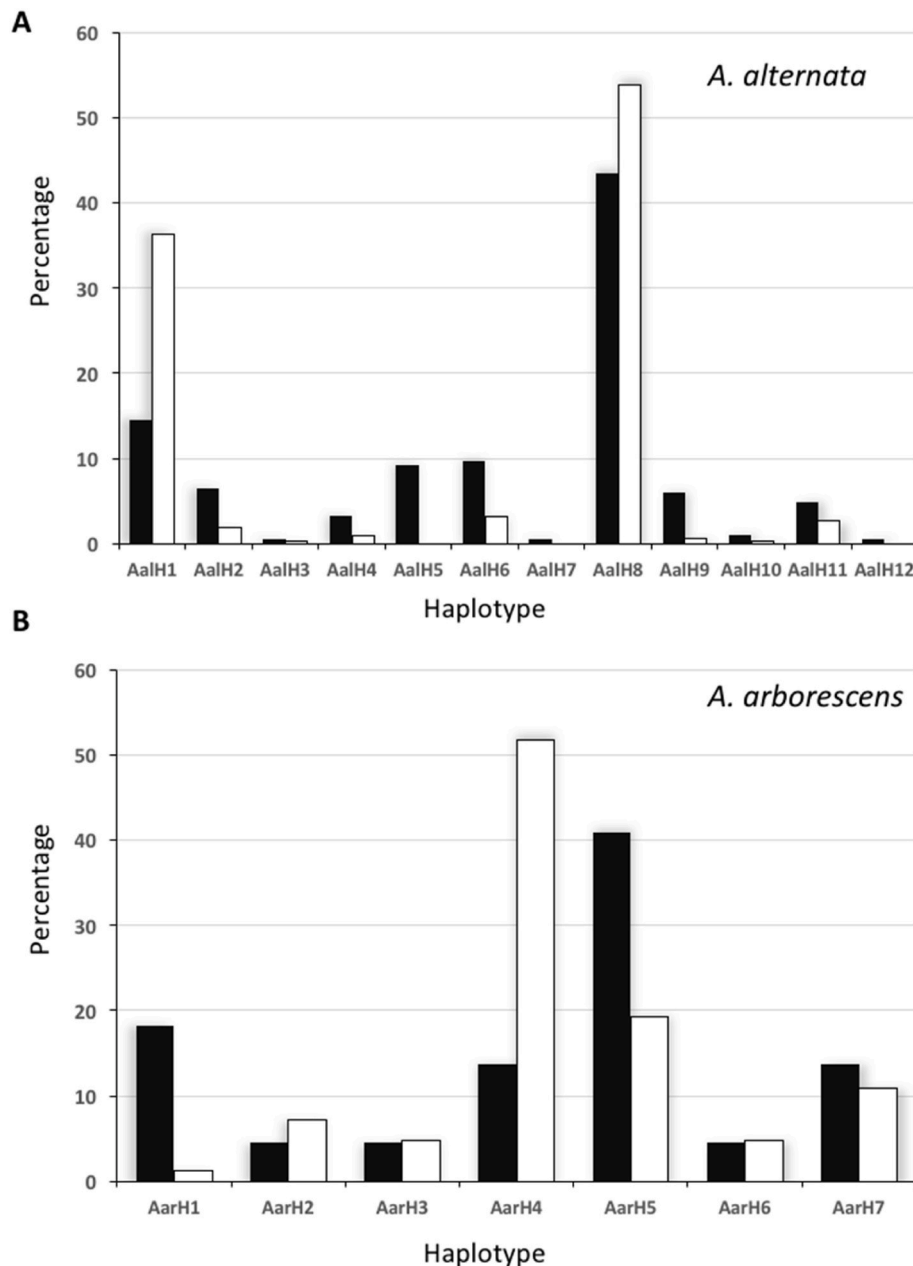


Fig. 5. Distribution of haplotypes of *A. alternata* (A) and *A. arborescens* (B) analysed using the single-copy nuclear gene *rpb2* in isolates from tomatoes (black bars; $n=181$ and 22 for *A. alternata* and *A. arborescens*, respectively) or other hosts (white bars; $n=1523$ and 83 for *A. alternata* and *A. arborescens*, respectively).

(2023) reported the existence of five different clades in *A. alternata* with up to 80 % ML bootstrap support from symptomatic samples collected in China, Italy, and Thailand, thus suggesting that *A. alternata* might constitute five species instead of a single species. Previous studies have also supported the concept of *A. alternata* as a species complex (Armitage et al., 2015, 2020). Based on phylogenetic analyses of five loci (*endo-PG*, *Alt-a1*, *TMA22*, *PGS*, and *REV3*), Armitage et al. (2015) proposed the designation of the *A. alternata* species group into three subspecies, namely *A. alternata* ssp. *arborescens*, *A. alternata* ssp. *tenuissima*, and *A. alternata* ssp. *gaisen*. Our results open the possibility for further studies focused on the molecular taxonomy of both *A. alternata* and *A. arborescens* species complexes.

Interestingly, the fungal strain LD124 isolated from symptomatic tomatoes in Estonia clustered with *A. postmessia* and formed a separate branch with significant bootstrap support (100 % ML), which was closely related to the reference strain of *A. burnsii* (CBS 107.38).

Currently, *A. postmessia* is referred to as a morphospecies of *A. alternata* based on the description of a single citrus-associated strain from the USA (Woudenberg et al., 2015). Recent studies by Dettman et al. (2023) based on molecular analyses of *rpb2*, *ASA-10*, and *ASA-19* loci have also reported *A. postmessia* as a synonym of *A. alternata*, and it was included in *alternata* sub-clade 2 with a unique *ASA-19* allele. Further whole genome sequence analyses are required to clarify the taxonomic position of *A. postmessia*.

Currently the teleomorph has not been found in *Alternaria* section *Alternaria* under natural or artificial conditions (Thomma, 2003; DeMers, 2022); however, we reported a relatively high genetic diversity for asexual fungi. This is demonstrated by the discordant tree topologies observed for the single-gene phylogenetic analyses of *rpb2*, *ASA-10*, and *ASA-19*. We reported two cases of discrepancies at the lineage level for fungal strains isolated from symptomatic tomatoes in Latvia and Algeria, thus suggesting potential recombination between different lineages in

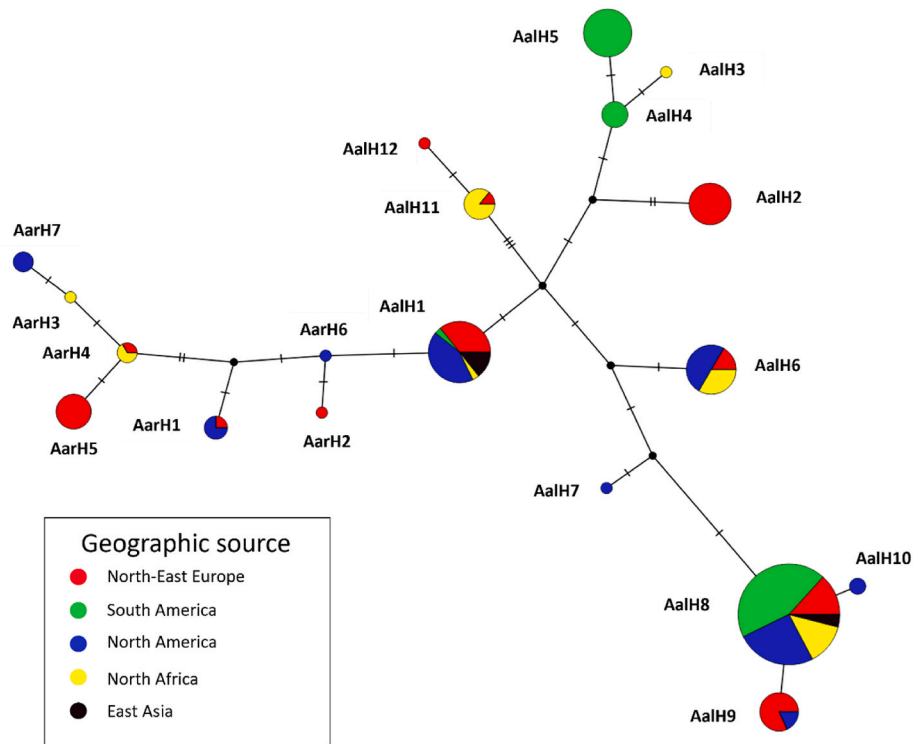


Fig. 6. TCS network for *rpb2*-locus haplotypes of tomato-associated *A. alternata* and *A. arborescens* strains coloured by geographic sources. Mutations are indicated on branches as hatch marks. Circle size is proportional to the number of strains belonging to a particular haplotype.

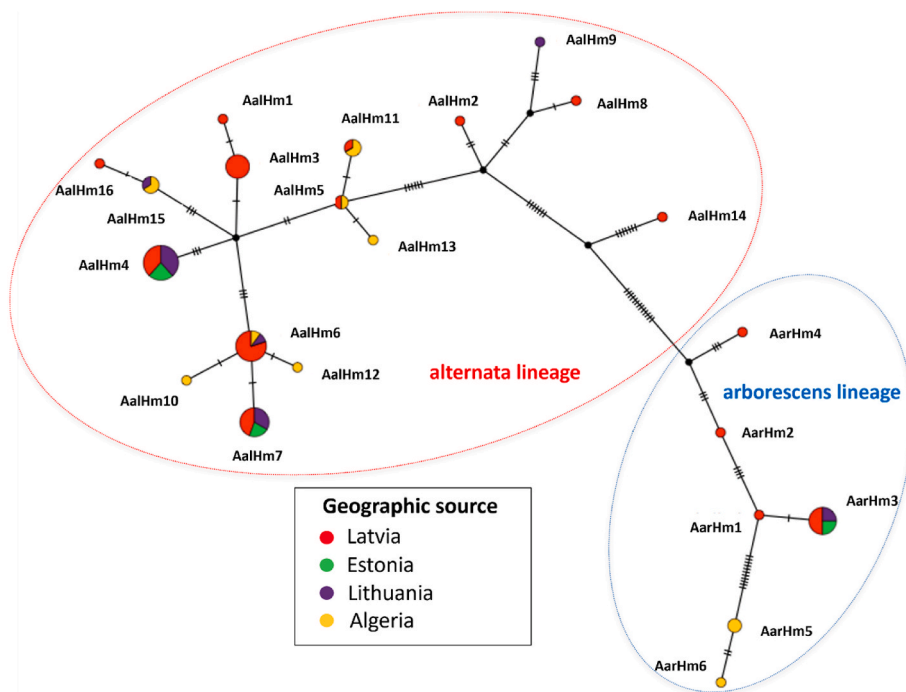


Fig. 7. TCS network for multi-locus haplotypes of tomato-associated *A. alternata* and *A. arborescens* strains coloured by geographic sources. Mutations are indicated on branches as hatch marks. Circle size is proportional to the number of strains belonging to a particular haplotype. Each colour represents a different location.

the section *Alternaria* that results in putative *alternata*-*arborescens* and *alternata*-*longipes* hybrids. Additional analyses will be necessary to confirm the evidence of recombination in these putative hybrid *Alternaria* spp. strains from the section *Alternaria*. Previous studies have also reported the possibility of occurrence of *alternata*-*gaisen* hybrids (Dettman and Eggertson, 2022) and *alternata*-*arborescens* hybrids

(Dettman et al., 2023).

Moreover, we also observed that three fungal strains from Latvia representing *alternata* lineage (LD042, LD048, and LD053) formed a cluster without significant support (<95 % ML) with *A. alternata* strains KAS5513 and DET2201 described by Dettman et al. (2023). These strains were isolated from cereal crops in Canada and had significant

Table 4
Information for single-locus alignments.

Locus	Nucleotide diversity ^a	Alignment length	Number of variable sites ^a	Proportion of variable sites	Number of informative sites ^a	Proportion of informative sites
ASA-10	0.0229	353	21	0.059	9	0.025
ASA-19	0.0204	518	23	0.044	21	0.040
Rpb2	0.0069	581	22	0.037	15	0.025

^a The dataset groups sequences of strains from both alternata and arborescens lineages.

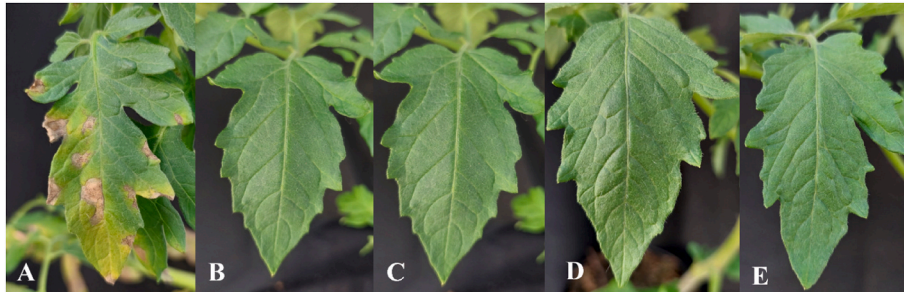


Fig. 8. Results of the pathogenicity test 14 days post-inoculation: disease symptoms caused by large-spored *Alternaria* sp. strain LD065 (section *Porri*) that served as a positive control (A); sterile distilled water treatment that served as a negative control (B); and leaves without visible disease symptoms after inoculation with conidial suspensions of *A. alternata* strain LD019 (C), *A. postmessia* strain LD124 (D), and *A. arborescens* strain LD115 (E).

p-values for recombination based on the MAXChi ($p = 0.0428$) and 3Seq ($p = 1.83E-03$) detection methods, confirming the occurrence of recombination events within alternata lineage (Dettman et al., 2023).

To assess the global genetic variation of alternata and arborescens lineages, the *rpb2* nucleotide sequences obtained in this study were compared with currently available data of this locus in the NCBI GenBank retrieved from tomatoes. In total, 12 and 7 haplotypes were identified in *A. alternata* and *A. arborescens* species complexes, respectively. The major *rpb2* haplotypes found for the tomato population were shared among strains from other hosts of origin; however, the haplotype AalH5 was detected only among isolates associated with symptomatic infection of wild tomatoes in Chile and Peru described by Schmey et al. (2023). Currently, there are no *rpb2* sequences available in the NCBI GenBank originating from commercial tomatoes in South America; therefore, further research and sampling are required to determine whether the haplotype AalH5 is differentiated depending on the host (wild tomato) or geographic origin (South America).

The multi-locus sequence data of *rpb2*, *ASA-10*, and *ASA-19* confirmed the high level of genetic diversity among tomato-associated *Alternaria* spp. strains in our study, and a total of 16 and 6 haplotypes were identified in *A. alternata* and *A. arborescens* species complexes, respectively. The largest number of multi-locus haplotypes was observed in Latvia, representing 53 % ($n = 38$) of the total strains analysed in this study. However, Algeria was host to the most diverse haplotypes when considering the number of strains per geographic location.

Due to the limited number of samples from Algeria ($n = 13$) in the current study, further sampling and genetic diversity analyses of additional strains are necessary to confirm this observation. Previous studies have also revealed a large diversity of *Alternaria* spp. associated with leaf spot symptoms on Solanaceae in this country (Bessadat et al., 2017, 2021). These differences in haplotype diversity might be attributed to several factors, among them sampling areas, which involved only greenhouses and high tunnels in Latvia, contrary to the field-grown tomatoes in Algeria. Additionally, meteorological conditions in Latvia are suitable for one cropping cycle per year in contrast to three cropping cycles in Algeria. Furthermore, Mediterranean areas, such as Algeria, are characterized by extended periods (six to eight months) of the presence of airborne conidia of *Alternaria* spp., with bimodal patterns of high concentrations during spring and autumn. In contrast, northeastern

Europe, including Latvia, is characterized by high concentrations of *Alternaria* spp. conidia for two to three months per year, starting from mid-June to mid-July (Skjøth et al., 2016). Moreover, several cultural practices in Algeria, including the cultivation of tomatoes in fields adjacent to other solanaceous hosts and the absence of crop rotation, also might have an impact on the diversity of *Alternaria* spp. (Ayad et al., 2019).

Other studies have also reported high genetic diversity in small-spored *Alternaria* from section *Alternaria*, indicative of an excess of recent mutations and population expansion (Adhikari et al., 2020; Xu et al., 2022). We observed large number of SNPs for the analysed loci within small sample size representing arborescens lineage; therefore, additional sampling would be required to provide accurate population analysis. The major multi-locus haplotypes in the alternata lineage were shared among strains originating from northwestern Algeria and northeastern Europe. Our results are consistent with current findings across different studies, confirming that *Alternaria* spp. from section *Alternaria* are not geographically differentiated (Andrew et al., 2009; Matić et al., 2020; Adhikari et al., 2021; Achilonu et al., 2023). The distribution of haplotypes in different tomato growing areas might be attributed to the wind dispersal of *Alternaria* spp. conidia (Thomma, 2003). Another important source contributing to the long-distance dispersal of *Alternaria* spp. (section *Alternaria*) is seed contamination with these fungi (Al-Askar et al., 2014).

All selected representatives from section *Alternaria* originating from Baltic countries were non-pathogenic to tomato plants in our study and tested negative for the presence of the AAL-toxin biosynthesis gene (*ALTI*). Bessadat et al. (2017) reported that small-spored *Alternaria* strains (section *Alternaria*) from Algeria not producing host-selective toxins (HST) were weakly aggressive when inoculated alone; however, these strains were shown to develop synergistic interactions with more aggressive species from the section *Porri* in mixed infections of tomatoes. The non-pathogenicity of greenhouse populations of *Alternaria* spp. from Baltic countries in our study might be attributed to the genetic divergence influenced by variations in environmental factors among the analysed samples (Yang et al., 2024).

Studies by Baltzakis et al. (2024) have reported that *Alternaria* spp. (section *Alternaria*) isolated from symptomatic potato (*Solanum tuberosum* L.) lesions did not cause disease symptoms on healthy potato

plants. Additionally, Xu et al. (2024) reported that small-spored *Alternaria* spp. isolated from symptomatic plantain (*Plantago lanceolata* L.) plants did not cause any disease symptoms on their host of origin (whole plants and detached leaves of plantain). These observations suggest that *Alternaria* species from section *Alternaria* do not act as primary plant pathogens, instead they are secondary colonizers or necrosis-dependent opportunists which inhabit the tissue of stressed or damaged plants (Prechsl et al., 2023). Other studies have reported differences in pathogenicity among several species in section *Alternaria*, varying from weakly aggressive (Toome-Heller et al., 2018) to highly aggressive (Ramezani et al., 2019), that is mainly attributed to the production of host-selective toxins (HST) (Hu et al., 2012; Tsuge et al., 2016).

The complexity of the interactions between members of *A. alternata* and *A. arborescens* species complexes is further illustrated by the fact that they might also be represented among the endophytic fungal communities inhabiting asymptomatic plant tissues (Molnár et al., 2023; Wallace, 2023; Mamaghani et al., 2024). Interestingly, endophytic *Alternaria* spp. from the section *Alternaria* produce various bioactive secondary metabolites and can cause the death of other plant pathogens from the kingdom Mycota (He et al., 2024) and Chromista (Musetti et al., 2006). Moreover, recent studies have confirmed that endophytic *A. alternata* strains may transition to necrotrophic pathogens, suggesting that they might represent pathogens of hosts other than their origin or cryptic pathogens (Soltani and Sheikhhamedi, 2023).

5. Conclusions

This study is the first report on implementation of molecular data for the identification of *Alternaria* spp. (section *Alternaria*) on tomatoes in northeastern Europe and their comparison with fungal strains from northwestern Algeria based on a polyphasic approach involving phenotypic assays, multi-locus phylogeny, and haplotype analysis. *Alternaria* spp. strains were non-pathogenic to tomato seedlings and morphological characteristics did not allow their discrimination at the species level; therefore, we applied molecular data of the RNA polymerase II second largest subunit (*rpb2*) along with putative F-box-domain-containing protein (*ASA-10*) and putative histone-like transcription factor (*ASA-19*). Multi-locus phylogeny confirmed the occurrence of both *A. alternata* ($n = 55$) and *A. arborescens* ($n = 15$) species complexes along with *A. longipes* ($n = 1$) and *A. postmessia* ($n = 1$) on symptomatic tomatoes. *Alternaria* spp. strains were characterized by a relatively high genetic diversity, and the discordances observed for single gene phylogenies suggested the occurrence of putative alternata-arborescens and alternata-longipes hybrids among analysed strains in line with previous studies. Further studies are required to confirm the evidence of recombination in these putative hybrid *Alternaria* spp. strains. Based on the DNA polymorphism analysis of the *rpb2*, *ASA-10*, and *ASA-19* loci, a total of 16 and 6 multi-locus haplotypes were assigned to alternata and arborescens lineages, respectively. The major multi-locus haplotypes in *A. alternata* species complex were not geographically differentiated. The estimation of global genetic diversity in *A. alternata* and *A. arborescens* species complexes at the *rpb2* locus revealed that major haplotypes described from symptomatic tomatoes were shared among other hosts of origin.

CRedit authorship contribution statement

Lilija Dučkėna: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation. **Nabihat Bessadat:** Resources, Investigation. **Nelly Bataillé-Simoneau:** Validation, Resources, Investigation, Data curation. **Bruno Hamon:** Resources, Data curation. **Mati Koppel:** Resources, Investigation. **Kaire Loit:** Resources, Investigation. **Neringa Rasiukevičiūtė:** Resources, Investigation. **Gunita Bimšteine:** Supervision, Conceptualization. **Philippe Simoneau:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision,

Methodology, Formal analysis, Data curation, Conceptualization.

Data availability

All newly generated sequences of the internal transcribed spacer regions 1 and 2 and the intervening 5.8S nrDNA (*ITS*), RNA polymerase II second largest subunit (*rpb2*), putative F-box-domain-containing protein (*ASA-10*), and putative histone-like transcription factor (*ASA-19*) genes are deposited in the publicly available National Center for Biotechnology Information (NCBI) GenBank database (<https://www.ncbi.nlm.nih.gov>) as specified in Table 2.

Declaration of competing interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.funbio.2024.10.002>.

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