

Differentiation of the closely related species, *Alternaria solani* and *A. tomatophila*, by molecular and morphological features and aggressiveness

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Abstract The main causal agent of early blight, the noxious disease of solanaceous crops, is generally considered to be *Alternaria solani* Sorauer (in a broad sense). However, heterogeneity in many morphological features of this pathogen has been noted suggesting that the disease may not be caused by a single species. Recent research has revealed that several large-spored *Alternaria* species may cause disease of potato and tomato including *A. solani sensu stricto* and *A. tomatophila*. The goal of our research was to compare Russian large-spored *Alternaria* isolates from tomato and potato to test the hypothesis that early blight of tomato and potato are caused by different species. Cluster analysis of genetic distances estimated from 12 polymorphic molecular markers (universally primed-polymerase chain reaction and randomly amplified polymorphic DNA) revealed two groups of isolates accepted here as *A. solani* and *A. tomatophila* that were supported by morphology and host plant association. Differentiation of species was supported by phylogeny derived from the DNA sequences of a portion of the *Alt a1*, *gpd* and calmodulin genes. Species-specific primers based on the *Alt a1* and calmodulin gene sequences for both species were designed. Under laboratory conditions, *A. solani* isolates were equally aggressive on both tomato and potato, whereas *A. tomatophila* was highly aggressive to tomato but only weakly aggressive to

potato. In the field, *A. solani* was isolated from potato, tomato and from several wild potato species including *S. schickii*, *S. papita* and *S. kurtzianum*. The majority (90 %) of *A. solani* isolates carried the mating type locus 1 (*MAT1*) idiomorph *MAT1-1* while the majority (88 %) of *A. tomatophila* isolates carried the *MAT1-2* idiomorph.

Keywords Aggressiveness · Early blight · Molecular phylogeny · Potato · Specialization · Species-specific PCR · Tomato

Introduction

Early blight is an economically important disease of worldwide importance affecting solanaceous crops and their wild relatives (Neergaard 1945; Sherf and MacNab 1986; Rotem 1994; van der Waals et al. 2004; Chaerani and Voorrips 2006; Kumar et al. 2007). This disease can cause yield losses that occasionally reach 20 % for potato and 78 % for tomato (Rotem 1994). Early blight occurs in all regions of Russia (Dmitrieva 1988; Kozlovsky and Filippov 2007; Orina et al. 2010) and neighbouring countries (Ivanyuk et al. 2003) where potato and tomato are cultivated. The main causal agent of early blight is generally considered to be *Alternaria solani* Sorauer. It is a ubiquitous fungus which was previously presumed to infect eggplant and nightshade in addition to tomato and potato (Neergaard 1945) as well as non-solanaceous plants (Akhtar et al. 2011).

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Genetic and phenetic polymorphisms within *A. solani* have been of interest for many research teams. Sufficient heterogeneity in pathogenic characters (aggressiveness and substrate preferences) as well as cultural characters (colony growth rate and sporulation intensity) have been observed in *A. solani* populations (Remneva and Ivanjuk 1968; Dmitrieva 1988; Weir et al. 1998; Martinez et al. 2004; Weber and Halterman 2012). Differences in pathogenic properties gave a basis to describe races within *A. solani*. In such a way seven physiological races have been described from Germany (Henning and Alexander 1952), 12 in Cuba (Izquierdo 1981), and five in Russia (Dmitrieva 1988). In spite of the large body of work the cultural, morphological and physiological subdivision was not always well-defined and the race concept in *A. solani* was not widely accepted. Pathogenic and cultural characters have been studied more thoroughly for Byelorussian isolates (Ivanyuk 1983; Ivanuk and Palilova 1996; Ivanyuk et al. 2003). Those isolates have been distinguished by substrate preferences, different levels of specialization and aggressiveness. The authors have described isolates infecting predominantly potato leaves, tomato leaves, stems or fruits. Some types of isolates (“races”) were specialized but the others have been found on both crops. Besides pathogenic characters the isolates had cultural markers. All isolates have been conditionally divided into three morphotypes: ‘mycelial’ (sterile), ‘sporulating’ and ‘intermediate’. Each type had some additional individual characters such as colony colour, colony growth rate, and conidia size.

Lately, a detailed morphology-based taxonomic revision of *Alternaria* specimens from Solanaceae has been performed. Several new species have been recognized among *A. solani*-like isolates obtained from different plants (Simmons 2000). The dominant species from potato preserved the name *A. solani*, but the similar fungus that is common on tomato has been described as a new species, *A. tomatophila* E. G. Simmons (Simmons 2000). At present it is important to estimate the phylogenetic and genomic distances between morphospecies and to analyze the predictive value of morphology-based taxonomy for plant pathology. Morphological similarity between species and variability of morphological traits have made it necessary to develop some easy and effective molecular methods of species identification that can be used by non-taxonomists, including practical plant pathologists.

Simmons’ morphological species conception has been supported by evidence from RAPD and AFLP data that revealed two types of the distinction: (i) differences between geographic populations of *A. solani sensu lato* isolated from a single host plant, and (ii) sufficient genetic distance between populations of the fungus occurring on potato and tomato (Weir et al. 1998; Martinez et al. 2004). In other studies, application of RFLP data to *A. solani sensu lato* has not produced clear results. No correlation has been found between molecular marker patterns and host-plant, geography, and resistance to fungicides (Adachi et al. 1996; Morris et al. 2000; Aradhya et al. 2001). Additional evidence supporting the supposition of the existence of two species has come from biochemical data. Metabolite profiles of *A. solani* and *A. tomatophila* obtained using HPLC were rather different (Andersen et al. 2008). Lourenço et al. (2009) have identified five “tomato” and two “potato” haplotypes by analyzing nucleotide sequences of three genes of *A. solani sensu lato* isolates from Brazil. This fact has clearly indicated some degree of divergence between pathogens that originated from two host plants. However, the presence of several species was not concluded. At the same time, the same team (Rodrigues et al. 2010) analyzed Brazilian *A. solani* and *A. tomatophila* by morphological and molecular markers and proposed to consider them as different species.

The existence of polymorphisms for molecular, morphological and physiological characters in the large-spored *Alternaria* isolates from solanaceous crops is obvious. At least two *Alternaria* species are common on tomato and potato. However *A. solani sensu stricto* and *A. tomatophila* have never been described in Russia as two dissimilar species. It is not clear how large is the range of variability of morphological and physiological features between these two species. The goal of our research was to compare large-spored *Alternaria* isolates sampled from tomato and potato in Russia by molecular and morphological characters and aggressiveness to test the hypothesis that early blights of potato and tomato are caused by different species.

Materials and methods

Fungal cultures Sixty-three single-conidium large-spored *Alternaria* isolates (*A. solani sensu lato*) were sampled for this research. They were sampled from different hosts (29 from potato, 30 from tomato, four

from three wild potato species) and regions of Russia (Fig. 1). This sample was supplemented with three representative cultures of *A. solani* and *A. tomatophila* obtained from the collection of Dr. E.G. Simmons (USA). A representative strain of *A. dauci* was used as an outgroup for the molecular analyses as described in Fig. 2.

DNA extraction Isolates were grown in Petri dishes containing Czapek agar in darkness at 23 °C for 7–10 days. Mycelium was harvested by a sterile scalpel, collected into 1.5-ml tubes and kept at –80 °C until extraction. DNA was isolated using the chloroform extraction method with buffer containing 2 % CTAB (Ausubel et al. 2002). DNA was precipitated with isopropyl alcohol and dissolved in 25 µl of TE buffer.

Amplification To get molecular fingerprints, PCR with universal primers (UP-PCR) was used as a main method. Randomly amplified polymorphic DNA (RAPD) data were used to increase the number of polymorphic bands. Amplification was performed in a thermal cycler C1000 (BioRad, USA). UP-PCR and RAPD-PCR with each primer carried out twice using DNA probes with primary concentration and diluted ten times.

PCR with universal primers (UP-PCR) was carried out in 20 µl of PCR-mixture containing approx. 1 ng of DNA; 0.8 U/µl of Taq DNA polymerase (Helicon,

Russia); 1× Taq DNA polymerase buffer with no Mg^{2+} (Helicon, Russia); 2 mM $MgCl_2$; 0.4 mM of each dNTP; 0.8 µM primer. The amplification program was: 94 °C for 2.5 min.; 30 cycles: 92° for 50 s, 50° for 90 s, 72° for 60 s; and final elongation 72° for 3 min. Four universal primers were used: L45, 15/19, AS4, and AS15inv (Bulat et al. 1995, 2000; Lübeck et al. 1999).

PCR with random primers (RAPD-PCR) was used in addition to UP-PCR to assess a broader range of the genome and to get a few more polymorphic markers. RAPD-PCR procedure was performed as described by Williams et al. (1990). Amplification was carried out in 20 µl of PCR-mixture containing approx. 1 ng of DNA; 0.8 U/µl of Taq DNA polymerase (Helicon, Russia); 1× Taq DNA polymerase buffer with no Mg^{2+} (Helicon, Russia); 2.5 mM $MgCl_2$; 0.4 mM of each dNTP; 0.5 µM primer. The amplification programme was the following: 94 °C for 2.5 min.; 30 cycles: 92° for 50 s, 36° for 60 s, 72° for 60 s; final elongation 72° for 3 min. The number of polymorphic amplified fragments and the clarity of the electrophoretic profiles were preliminarily assessed using five random primers (I09, I10, A08, A09, and A10). One primer, A10 (5'-GTG ATC GCA G), was chosen.

Amplification products were resolved on 1.5 % agarose gels stained with ethidium bromide and photographed with a ChemiDoc XRS (Bio-Rad, USA) imaging system. Bands produced by UP-PCR and

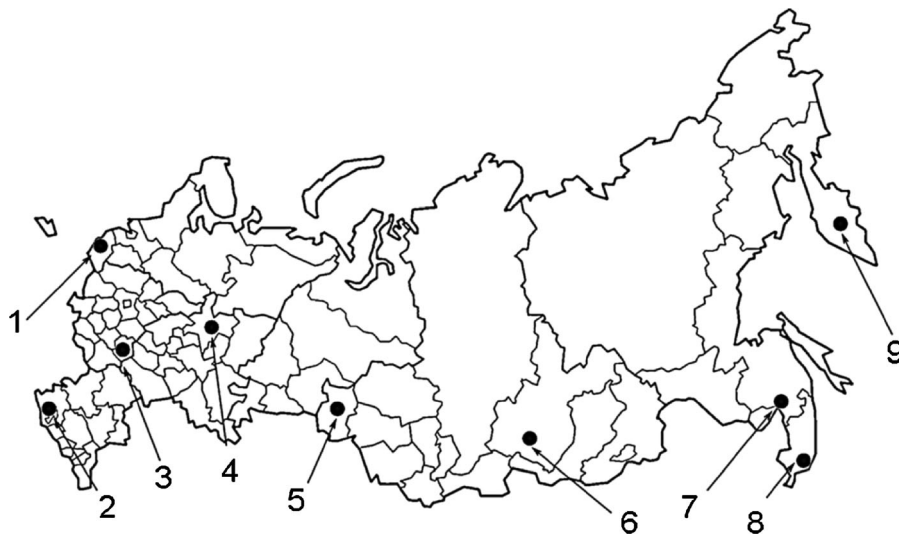


Fig. 1 Map of Russia showing the locations of potato and tomato areas sampled for *Alternaria* isolates used in this survey. Region codes: 1 – Pskovskaya oblast (sampling year – 2010); 2 – Republic of Adygeya (2008); 3 – Tambovskaya oblast (2008); 4 –

Kirovskaya oblast (2008); 5 – Omskaya oblast (2008); 6 – Irkutskaya oblast (2008); 7 – Khabarovskiy kray (2006); 8 – Primorskiy kray (2006); 9 – Kamchatskiy kray (2010)

RAPD were detected manually and recorded as a 0/1 (absent/present) matrix. Only reproducible, bright, unambiguous bands were scored.

Two PCRs were carried out to amplify idiomorphs (*MAT1-1* or *MAT1-2*) at the mating-type locus. *MAT1-1* was amplified using forward primer CHO13 (Berbee et al. 2003) and reverse primer Amat1r: 5'-GAC CAG GCT TTC GYC ATC (Gannibal and Kazartsev 2013). *MAT1-2* was amplified with primers MCHMG1/MCHMG2 (Berbee et al. 2003). Amplification was performed in 25 µl of PCR-mixture containing 1 U/µl Taq DNA polymerase (Helicon, Russia); 1× Taq DNA polymerase buffer with 2.5 mM MgCl₂ (Helicon, Russia); 0.2 mM each dNTP; 0.5 µM of each primer. The amplification programme was the following: 94 °C for 2.5 min.; 30 cycles: 92° for 50 s, 55° (for reaction with primers CHO13/Amat1r) or 60° (MCHMG1/MCHMG2) for 40 s, 72° for 50 s; final elongation 72° for 3 min. Amplification products were resolved on agarose gels as described above. *MAT1-1* isolates were identified by the amplification of an 800-bp band and *MAT1-2* isolates by the presence of a 280-bp band.

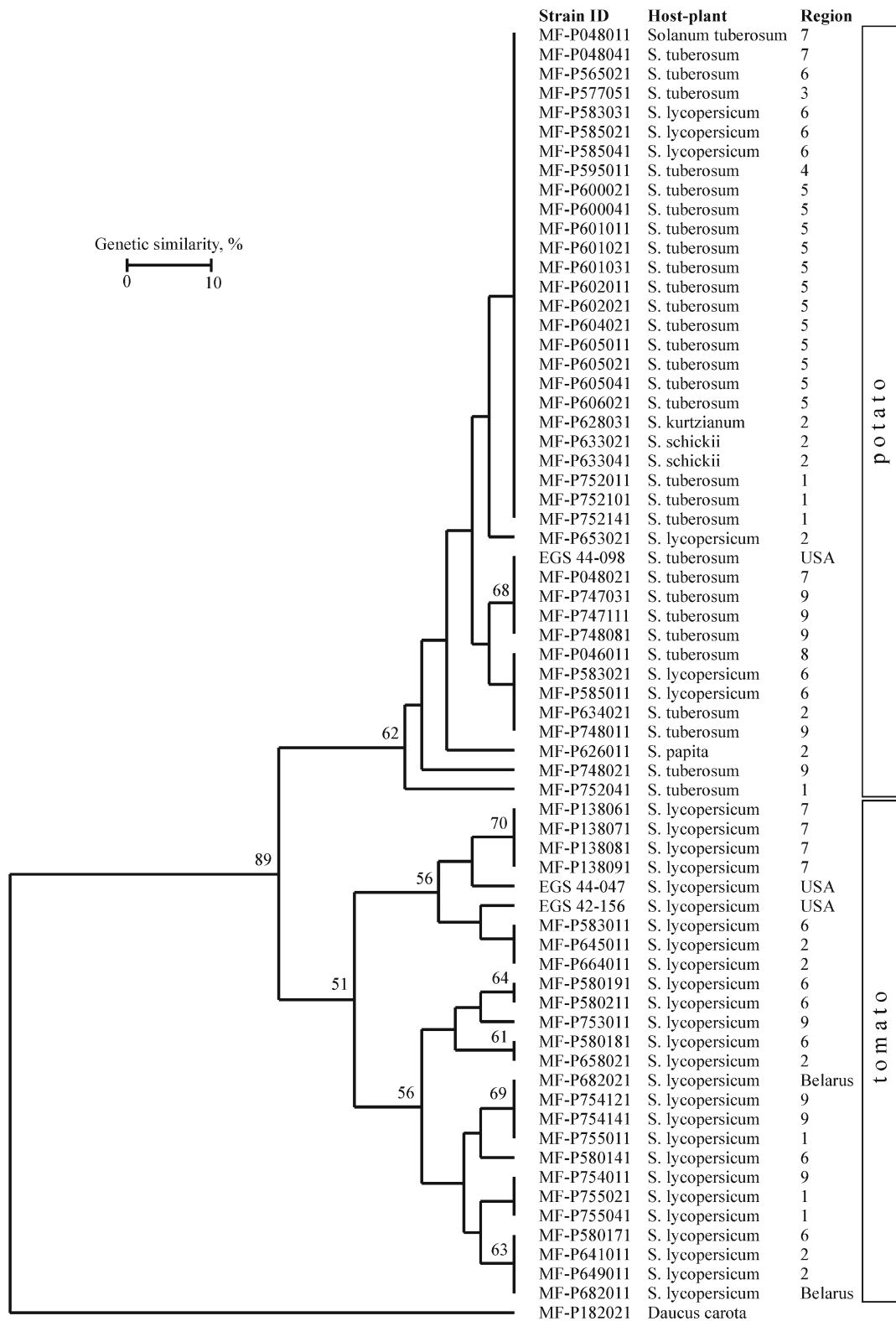
Species-specific primers for *A. solani* and *A. tomatophila* were designed on the basis of a comparison of the *Alternaria* major allergen *Alt a1* and calmodulin genes, respectively. To design primers, appropriate sequences of representative *Alternaria* strains were downloaded from Genbank. Primers were designed using online software Primer3Plus (Untergasser et al. 2007). Each primer set had three marker nucleotide positions differentiating *A. solani* from *A. tomatophila*. *A. solani*-specific primers OAsF7 (5'-CGACGAGTAA GTTGCCCTCA) and OAsR6 (5'-TGTAGGCGTCAG AGACACCATT) were expected to amplify a product of 164 bp in length. Another primer pair, OAtF4 (5'-TGCG GCTTGCTGGCTAAGGT) and OAtR2 (5'-CAGTCG ATGCGGCCGTC), theoretically could amplify a 483-bp fragment in *A. tomatophila* and some other large-spored *Alternaria* species excluding *A. solani*. Primer specificity was tested on DNA samples from the 63 isolates of *A. solani sensu lato* mentioned above, representative cultures of potato-pathogenic *A. solani*, *A. grandis*, tomato-pathogenic *A. tomatophila*, *A. subcylindrica* and 23 authors' strains of 12 *Alternaria* species including large-spored *A. acalyphae*, *A. cirsinoxia*, *A. dauci*, *A. helianthiinficiens*, *A. macrospora*, *A. porri*, *A. silybi*, *A. zinniae* and non-large-spored *A. alternata*, *A. arborescens*, *A. brassicicola* and *A. tenuissima*. Amplification was

performed in 25 µl of PCR-mixture similar to those used for mating-type locus amplification. The amplification programme for reaction with primers OAsF7/OAsR6 was the following: 94 °C for 2.5 min; 30 cycles: 92° for 20 s, 68° for 30 s, 72° for 30 s; final elongation 72° for 3 min. The programme for reaction with OAtF4/OAtR2 differed by combining the primer annealing and DNA elongation steps into one (72° for 40 s).

DNA sequencing and phylogenetic analysis Sequences of fragments of three protein-coding genes, *Alternaria* major allergen (*Alt a1*), glyceraldehyde 3-phosphate dehydrogenase (*gpd*) and calmodulin, were used for phylogenetic study. Sequences of these genes of all available ex-type or representative strains of Solanaceae-associated large-spored *Alternaria* species were obtained from the GenBank database (<http://www.ncbi.nlm.nih.gov>). Fourteen *Alt a1*, 14 *gpd* and seven calmodulin gene sequences were collected for *A. crassa* (AY563293, AY278804), *A. capsici* (AY562408, AY563298, JQ646256), *A. cretica* (JQ646426, JQ646342, JQ646250), *A. cyphomandrae* (JQ646444, JQ646360), *A. grandis* (JQ646425, GQ180077, JQ646249), *A. nitritinali* (JQ646442, JQ646358), *A. solani* (AY563299, AY278807, JQ646232), *A. solani* (GQ180096, GQ180080), *A. solani* (GQ180099, GQ180083), *A. solani* (GQ180097, GQ180081), *A. solani* (GQ180098, GQ180082), *A. solani-nigri* (JQ646417, JQ646333, JQ646233), *A. subcylindrica* (JQ646429, JQ646345, JQ646254), and *A. tomatophila* (GQ180101, GQ180085, JQ646257).

The same three genes, *Alt a1*, *gpd* and calmodulin, were sequenced for two Russian *A. solani* isolates (MF-P048011 from potato and MF-P585011 from tomato) and two *A. tomatophila* isolates (MF-P138061 and MF-P580171 from tomato). The calmodulin gene sequence

Fig. 2 Dendrogram of distances among *Alternaria* isolates of different substrate and geographic origin. Distances were estimated from universal-primed PCR and random amplified polymorphic DNA profiles using the unweighted pair group method with arithmetic means (UPGMA) algorithm. Numbers at branches indicate percent occurrence of the cluster to the right of the branch in 1,000 bootstrapped dendrograms. Only branches occurring in 50 % or more of the bootstrapped dendrograms are mentioned. Region codes: 1 – Pskovskaya oblast (sampling year – 2010); 2 – Republic of Adygeya (2008); 3 – Tambovskaya oblast (2008); 4 – Kirovskaya oblast (2008); 5 – Omskaya oblast (2008); 6 – Irkutskaya oblast (2008); 7 – Khabarovskiy kray (2006); 8 – Primorskiy kray (2006); 9 – Kamchatskiy kray (2010)



of the representative *A. solani* strain EGS 44–098 was obtained as well. Amplification of genes *Alt a1*, *gpd* and calmodulin utilized primer pairs Alt-for/Alt-rev, *gpd1/gpd2* and CaldF1/CaldR1 (Hong et al. 2005; Berbee et al. 1999; Lawrence et al. 2013, respectively). The nucleotide sequence of both forward and reverse DNA strands from PCR products was determined with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and ABI automated DNA sequencer ABI PRIZM 3500 (ABI-Hitachi, Japan). The sequences were proofread and edited in ContigExpress of Vector NTI Suite 8.0. The sequences were deposited in the GenBank (KJ397975–KJ397987).

The alignments of the sequences were performed using ClustalX. The RAxML (randomised accelerated maximum likelihood) software (v. 7.2.8, Stamatakis 2006) was used for the Maximum Likelihood (ML) analysis. The robustness of trees in the ML analyses was evaluated by 100 bootstrap replications. The phylograms were rooted with *A. calendulae*.

Morphological examination For analysis of cultural characters, 20 *A. solani* isolates and 10 *A. tomatophila* isolates were grown in Petri dishes with Czapek agar and YES (Samson et al. 2000). For examination of conidial morphology isolates were grown on PCA (potato carrot agar; Simmons 2007) and V-4 (Mikhailova et al. 2002) that is an analogue of the V-8 medium used for description of large-spored and other *Alternaria* species (Simmons 2007). Isolates were incubated for 7 days at 23 ± 1 °C without exposure to light (for cultural study) or under an alternating light/dark cycle consisting of 12 h of cool-white fluorescent daylight (to study conidia morphology). For size measurement, conidia were collected from the middle part of the colony radius. Length and width of conidial body and the beak length of 30 conidia per isolate were measured for 30 isolates. Presence and degree of beak branching were also estimated.

Plant cultivation and preparation Potato cultivar ‘Nevskiy’ and tomato cultivar ‘Baltiyskiy’ were planted in plastic vessels under laboratory conditions for 4–6 weeks post emergence. 10-mm disks were cut out from leaves of the middle tier. Leaf disks were incubated in plastic boxes on filter paper wetted with sterile tap water (for tomato) or 4 μ M 2,4-dichlorophenoxyacetic

acid (2,4-D) (for potato; Bussey and Stevenson 1991). Boxes were covered with transparent lids.

Inoculum preparation Sixty-six isolates were subjected to the pathogenicity test. Isolates were cultivated on V-4 medium in glass Petri dishes under continuous near-UV light (tubes LE-30, Russia; emission at 280–380 nm, maximum emission at 310–320 nm) at 22–24 °C. After 7 days aerial mycelium was removed with a sterile scalpel and Petri dishes were incubated in a refrigerator for 1–2 days to produce sporulation. Conidia were collected by washing with 5 ml of sterile water with 0.01 % Tween-60. Conidia were counted in a Goriaev chamber.

Aggressiveness evaluation Each leaf disk (10 mm diam.) was inoculated with a 10- μ l drop of a conidial suspension (5×10^3 conidia/ml) in the centre of each disk. Wet chambers with leaf disks were incubated under an alternating day/night cycle consisting of 16 h of cool-white fluorescent daylight at 24 °C and 8 h in the dark at 18 °C. Lesion sizes were estimated visually after 5 dpi as percent area of each disk affected by necrosis. All inoculation experiments used three replicates of ten leaf disks each.

Data analysis UP-PCR and RAPD data matrices were combined. Dendrograms were obtained by cluster analysis of all isolates using the unweighted pair group method with arithmetic means (UPGMA) based on the simple matching similarity coefficient. The Treecon 3.1b program was employed for this analysis (Van de Peer and De Wachter 1994). Confidence in specific clusters of the resulting topology was estimated by bootstrap analysis with 1,000 replicates. The degree of subdivision of isolate groups between regions and host plants was measured by Wright’s fixation index (F_{ST}) (Wright 1943), using the AMOVA (analysis of molecular variance) algorithm of the software Arlequin version 2.000 (Schneider et al. 2000). For the processing of inoculation results one-way ANOVA (analysis of variance) from the software package Statistica 6.0 was applied.

Results

Molecular fingerprinting and mating-type locus analysis Amplification of large-spored *Alternaria* isolate DNA by UP- and RAPD-PCR produced 18

polymorphic bands, six of which were found only in the profile of the outgroup species *A. dauci*. Thirteen and 5 markers were obtained from UP-PCR and RAPD amplifications, respectively. Analysis of the combined data matrix resulted in construction of a dendrogram (Fig. 2). Dendrograms based on data from amplification with solitary primers in general were similar.

Molecular markers allowed resolution of 18 haplotypes among 66 isolates. All isolates excluding the outgroup strain of *A. dauci* were divided into two major clusters consisting of 40 and 26 isolates, respectively. Genetic difference between those groups was about 30 %.

Each cluster consisted of isolates of different geographic origin. Isolates from the Republic of Adygeya, Irkutskaya oblast, Pskovskaya oblast, Kamchatskiy kray and Khabarovskiy kray were present in both clusters. Within each cluster isolates of the same origin were grouped mostly together. For instance all 12 isolates from potato from Omskaya oblast belonged to the same haplotype and probably to the same clone. Complete identity was found for PCR profiles of four isolates from tomato from Khabarovskiy kray. At the same time six isolates collected from tomato in Irkutskaya oblast had different haplotypes, but were distributed within one major cluster. Thus, the molecular markers did not reveal a strong dependence of PCR pattern similarity on the geographic origin of isolates.

Clusters in the dendrogram were separated according to host plants from which they were obtained. The first major cluster mainly consisted of isolates from cultivated and wild potato (30 isolates from *Solanum tuberosum*, two from *S. schickii*, one from *S. kurtzianum* and one from *S. papita*), but also included six isolates from tomato (*S. lycopersicum*). Interestingly, those isolates from tomato and collection samples of wild potato found in the first cluster were obtained from plants grown in fields in the neighbourhood with cultivated potato fields in Irkutskaya oblast and the Republic of Adygeya.

The first major cluster contained isolates of seven haplotypes, two of which were common for isolates from potato and tomato, and one haplotype was represented by a single isolate from tomato. The most abundant haplotype was represented by 26 isolates, obtained from four of the five studied *Solanum* species from seven regions situated in the European part of the country, as well as in Siberia and the Russian Far East.

The second cluster consisted of 26 isolates (11 haplotypes) obtained solely from tomato. This cluster could be divided into two subclusters (nine and 17 isolates) with satisfactory bootstrap support. No correlation was detected between isolate distribution among the subclusters and geographic distribution was not detected.

Clustering of representative strains was correlated with host plant as it was for the other isolates. A representative *A. solani* strain was placed in the first “potato” cluster while two *A. tomatophila* strains appeared in the second “tomato” cluster. Therefore we consider the two clusters as two different *Alternaria* species.

Calculation of Wright's F_{ST} indicated sufficient genetic difference between the two clusters ($F_{ST}=0.832$). The index F_{ST} was slightly lower (0.764) when it was calculated for isolates collected only in three regions (Adygeya, Pskovskaya oblast and Irkutskaya oblast) which were better represented in both clusters. When isolates of each cluster from those three regions were compared pairwise the Wright's index was not higher than 0.11, but it was always no lower than 0.65 when pairwise comparison of “potato” and “tomato” cluster isolates from each of those regions was performed.

Surprisingly, a majority of isolates (36 isolates, 90 %) of the first “potato” cluster had mating-type locus idiomorph *MAT1-1* (Table 1). The alternative idiomorph, *MAT1-2*, was found only in three isolates of this group from potato from Kamchatskiy kray and in one isolate from tomato from Irkutskaya oblast. In contrast, 23 (88 %) of 26 “tomato” cluster isolates had *MAT1-2* with the exception of three isolates from tomato obtained in Adygeya and Khabarovskiy kray.

Phylogenetic analysis Alignment of the *Alt a1 + gpd* sequences with those of 14 *Alternaria* species resulted in a 1,030-character data set of which 71 characters (6.9 %) were phylogenetically informative. Aligned *Alt a1 + gpd* + gene for calmodulin data set for seven species included totally 1,793 characters and 75 phylogenetically informative characters (4.2 %). Figures 3 and 4 show that three species from tomato (*A. tomatophila*, *A. subcylindrica* and *A. cretica*), including a strain from tomato annotated as *A. solani*, are accommodated in a well-supported, monophyletic clade and that strains of *A. solani* and *A. grandis* from potato form another independent, well-supported clade. Both clades do not include *Alternaria* species from other solanaceous plants of which sequences were available in the GenBank.

Table 1 List of studied large-spored *Alternaria* isolates from cultivated potato, three wild potato species, and tomato

Strain ID ^a	Origin	Year	Matyng type
<i>Solanum tuberosum</i>			
EGS 44-098	USA, Washington	1996	1
MF-P046011	Primorskiy kray	2006	1
MF-P048011, MF-P048021, MF-P048041	Khabarovskiy kray	2006	1
MF-P565021	Irkutskaya oblast	2008	1
MF-P577051	Tambovskaya oblast	2008	1
MF-P595011	Kirovskaya oblast	2008	1
MF-P600021, MF-P600041, MF-P601011, MF-P601021, MF-P601031, MF-P602011, MF-P602021, MF-P604021, MF-P605011, MF-P605021, MF-P605041, MF-P606021	Omskaya oblast	2008	1
MF-P634021	Republic of Adygeya	2008	1
MF-P747031	Kamchatskiy kray	2010	1
MF-P747111, MF-P748011, MF-P748021, MF-P748081			2
MF-P752011, MF-P752041, MF-P752101, MF-P752141	Pskovskaya oblast	2010	1
<i>S. kurtzianum</i>			
MF-P628031	Republic of Adygeya	2008	1
<i>S. papita</i>			
MF-P626011	Republic of Adygeya	2008	1
<i>S. schickii</i>			
MF-P633021, MF-P633041	Republic of Adygeya	2008	1
<i>S. lycopersicum</i>			
EGS 42-156	USA, Indiana	1995	2
EGS 44-047		?	2
MF-P138061, MF-P138071, MF-P138091	Khabarovskiy kray	2006	2
MF-P138081			1
MF-P580141, MF-P580171, MF-P580181, MF-P580191, MF-P580211, MF-P583011	Irkutskaya oblast	2008	2
MF-P583021, MF-P583031, MF-P585011			1
MF-P585021			2
MF-P585041			1
MF-P641011, MF-P645011, MF-P649011	Republic of Adygeya	2008	2
MF-P653021, MF-P658021, MF-P664011			1
MF-P682011 MF-P682021	Belarus	2008	2
MF-P753011, MF-P754011, MF-P754121, MF-P754141	Kamchatskiy kray	2010	2
MF-P755011, MF-P755021, MF-P755041	Pskovskaya oblast	2010	2

Acronym EGS means collection of E. G. Simmons, Crawfordsville, IN, USA

^a Isolate numbers with acronym MF means collection of the Laboratory of Mycology and Phytopathology, All-Russian Institute of Plant Protection, St. Petersburg, Russia

Sequences of four Russian *A. solani* and *A. tomatophila* isolates were identical to those of the respective representative strains. This result supports identification made based on molecular fingerprinting and morphological study. The “potato” clade contained

both Russian *A. solani* isolates obtained from potato as well as from tomato.

Species-specific primers Analysis of *Alt a1*, *gpd* and calmodulin gene sequences revealed six, four, and four

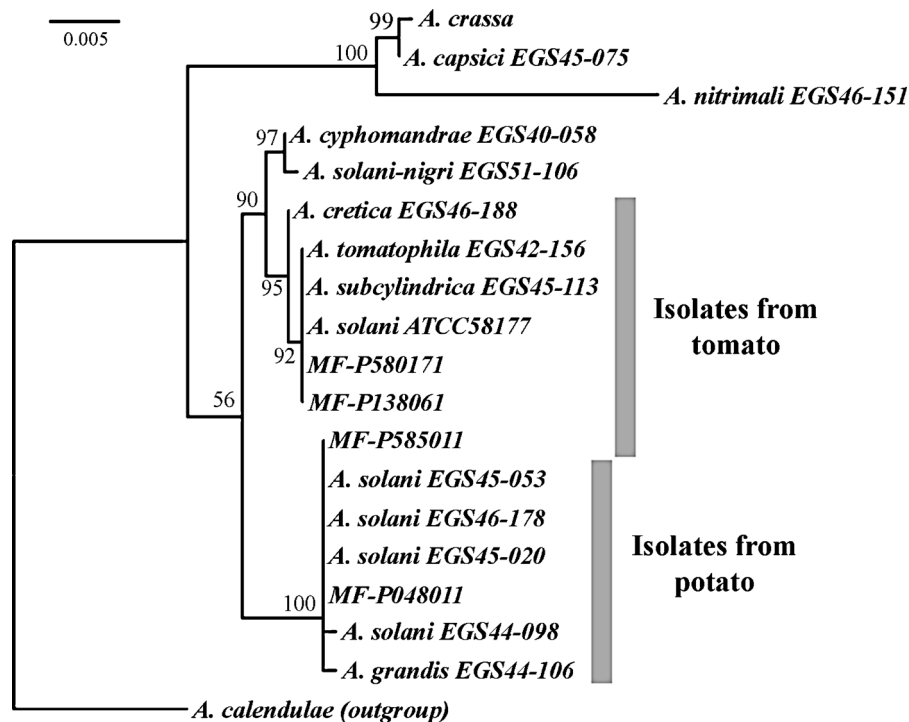


Fig. 3 Best-scoring Maximum Likelihood tree calculated using RAXML, based on partial *Alt a1* and *gpd* sequences showing the relationships among large-spored *Alternaria* species found on plants of the family Solanaceae. The acronym EGS indicates ex-type or representative strains; the acronym MF indicates authors'

isolates. The bootstrap support percentages of the maximum likelihood (ML) analysis are presented at the nodes. The bar indicates the number of substitutions per site. The tree is rooted with *Alternaria calendulae*

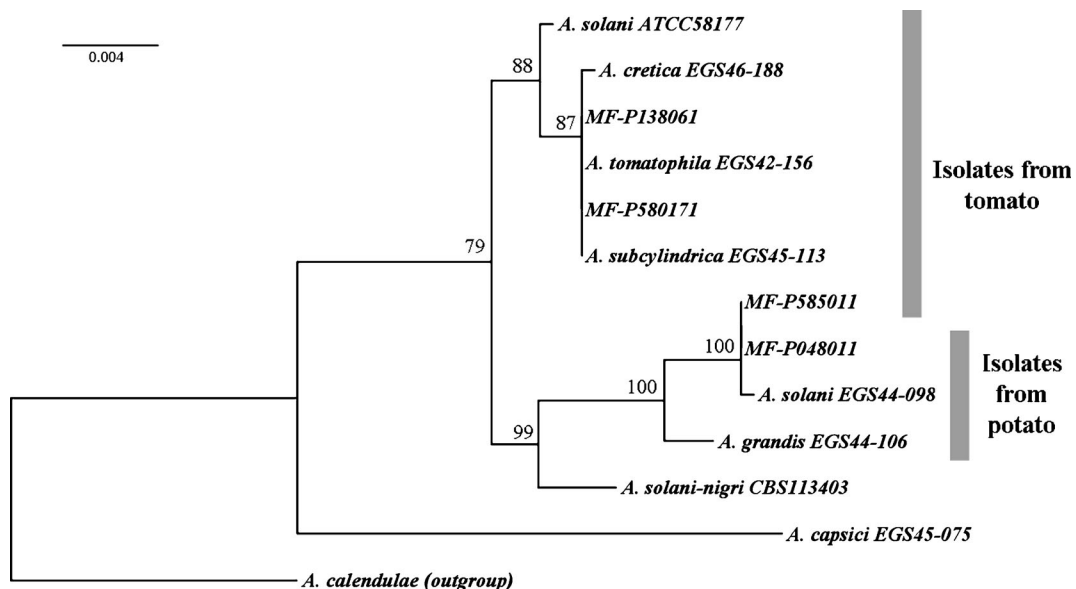


Fig. 4 Best-scoring Maximum Likelihood tree calculated using RAXML, based on partial *Alt a1*, *gpd*, and calmodulin gene sequences showing the relationships among large-spored *Alternaria* species found on plants of the family Solanaceae. The acronym EGS indicates ex-type or representative strains; the

acronym MF indicates authors' isolates. The bootstrap support percentages of the maximum likelihood (ML) analysis are presented at the nodes. The bar indicates the number of substitutions per site. The tree is rooted with *Alternaria calendulae*

single-nucleotide polymorphisms, respectively, differentiating *A. solani* and *A. tomatophila* which make it possible to design species-specific primers. Results of PCR with novel specific primers completely agreed with the cluster analysis of DNA fingerprints. All strains of the “potato” cluster gave an amplicon of the expected size with *A. solani*-specific primers and gave no product when they were amplified with *A. tomatophila*-specific primers. The opposite results were obtained with isolates of the “tomato” cluster. All PCR assays with DNA of the “tomato” cluster strains as well as the sole *A. subcylindrica* strain gave positive results. PCR with DNA of all other *Alternaria* species tested was negative.

Morphology Isolates of the first and second groups had morphological characters very similar to those described by Simmons (2000, 2007) for sporulation of *A. solani* and *A. tomatophila*. Comparison of our isolates with representative strains of both species supported the high level of similarity. No isolates similar to other large-spored potato and tomato-associated species (*A. cretica*, *A. elegans*, *A. grandis*, *A. subcylindrica*, and *A. subtropica*) were found. Therefore, these two groups (“potato” and “tomato” clusters) are accepted below as *A. solani* and *A. tomatophila*.

Average and individual size of conidia of *A. solani* and *A. tomatophila* isolates varied within a broad range (Table 2). Ranges of variation of all studied morphological characters of both species overlapped. But in general, conidia of *A. tomatophila* differed from those of *A. solani* by a shorter and thinner body and

by a longer beak (Fig. 5). On PCA both species produced conidia on average with a longer body and beak than those on V-4.

On V-4, which is more similar to the V-8 medium used by Simmons (2007) to describe many large-spored *Alternaria* species, the most important differentiating feature was beak length. This character can be used to draw an approximate boundary line between the two species (Fig. 6). For smaller conidia with short bodies (60–70 µm) the boundary value was 130 µm, but for conidia with larger bodies (95–105 µm) it was near 150 µm. However, ranges of variation of *A. solani* and *A. tomatophila* morphological parameters were slightly overlapping. This boundary line was operable for cultures grown on V-4 and V-8 under light/dark cycle only.

On V-4 the proportion of conidia with several beaks or one branched beak varied between 0 and 50 % in *A. solani* isolates and between 6 and 44 % in *A. tomatophila* isolates. On PCA the proportion of such conidia in *A. solani* was 0–60 % and in *A. tomatophila* 6–44 %. On V-4 *A. solani* isolates had solitary or double beaks while *A. tomatophila* isolates produced beaks with 1–2(–3) branches. On PCA branching of beaks occurred more often and was more intensive. The maximal number of branches was three in *A. solani* isolates and four in *A. tomatophila* isolates.

After 7 days of incubation on the V-4 medium *A. solani* isolates produced colonies of 47–55 mm in diam. Diameter of colonies of *A. tomatophila* isolates was 50–58 mm. On the Czapek agar diameters were 27–33 and 27–37; on YES diameters were 22–33 and 20–

Table 2 Size of conidia of *Alternaria solani* and *A. tomatophila* cultures, obtained on three different media

Substratum	Species	Size of conidia, µm						
		Body length		Beak length		Total length		Body width
		Mean	Range	Mean	Range	Mean	Range	
V-4 ^a	<i>A. solani</i>	84±1 ^b	54–143	115±2	40–194	198±2	122–302	21±1
	<i>A. tomatophila</i>	75±2	43–128	170±4	61–306	247±3	144–396	18±1
PCA ^a	<i>A. solani</i>	78±1	40–115	135±2	36–216	214±3	90–313	22±1
	<i>A. tomatophila</i>	73±1	43–112	204±4	97–288	284±4	140–385	23±1
V-8 ^c	<i>A. solani</i>	109–115		80–118		–		18–26
	<i>A. tomatophila</i>	83–114		99–217		–		16–23

^a Original data, obtained from study of 20 *A. solani* and 10 *A. tomatophila* isolates (30 conidia per isolate)

^b Digits after ± indicate 95 % confidence interval

^c Simmons 2007; size of longest conidia with unbranched solitary beak

25 mm, respectively. Ranges of variation were sufficiently overlapping in all cases. Statistically significant differences ($p \leq 0.05$) between averages were not discovered. Reliable and reproducible characters differentiating the two species by colony morphology were not found.

Aggressiveness All 66 isolates caused disease symptoms on potato and tomato leaf disks. The ANOVA test of significance used to compare aggressiveness of *A. solani* and *A. tomatophila* isolates on potato and tomato revealed four significant factors for inoculations under laboratory conditions. The P value for the factors strain, year of sampling and treated plant species was < 0.0001 . The factor fungal species was estimated using fresh isolates (2008 and 2010, $n=54$) and was sufficient whether the inoculated plant was potato ($P < 0.0001$) or tomato ($P=0.0084$).

The necrotic area at 5 dpi varied among isolates from 2 to 41 % of a total leaf disk area that indicated different aggressiveness of the isolates (Table 3). Variation in aggressiveness was higher when potato was inoculated with *A. solani* and tomato was inoculated with *A. tomatophila*, i.e., higher when inoculum was placed on the fungus's main host plant than when it was placed on the non-principal host plant. Fresh isolates on average were more aggressive. *A. solani* isolates on average were equally aggressive on both tomato and potato ($P=$

0.5567). *A. tomatophila* isolates on average were highly aggressive to tomato but only weakly aggressive to potato ($P < 0.0001$). Sufficiently larger lesions on potato leaf disks were observed after inoculation with *A. solani* than *A. tomatophila*. Tomato was more strongly affected by *A. tomatophila*.

Discussion

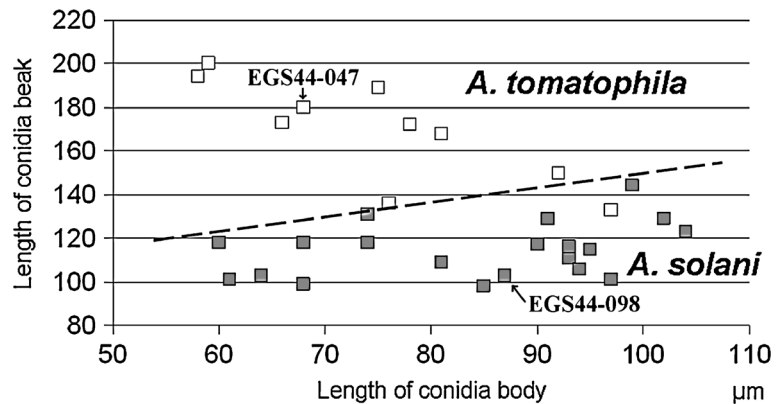
Taxonomy and species recognition of large-spored *Alternaria* species are based on morphology. Apart from *A. solani* and *A. tomatophila*, nine other large-spored *Alternaria* species have been described from *Solanaceae*: *A. cretica*, *A. elegans*, *A. subtropica*, and *A. tomato* on tomato, *A. grandis* on cultivated potato and *A. africana*, *A. beringelae*, *A. nitrimali*, and *A. solani-nigri* on other species of the genus *Solanum* (Simmons 2007). At this moment those species are characterized by morphology only, primarily by conidia shape and size. We have no information concerning research on aggressiveness and host specialization of these fungi.

The results derived from molecular, morphological and physiological experiments were congruent. All tested Russian isolates were divided into only two groups according to differentiation by molecular markers (UP-



Fig. 5 Conidia of *Alternaria solani* (left) and *A. tomatophila* (right) on PCA medium. Scale bar=50 μ m

Fig. 6 Length of conidia body and beak of *Alternaria solani* (shaded squares) and *A. tomatophila* (blank squares) isolates obtained on V-4 medium under alternating light/dark cycle consisting of 12 h of cool-white fluorescent daylight. Representative strains of both species are marked with arrows. The dotted line denotes the conditional boundary line between the two species



PCR, RAPD, PCR with specific primers, and partly by sequencing). Morphological study in general supported this division. Comparison with representative strains enabled us to ascertain that those groups represent the two species *A. solani* and *A. tomatophila*. They have different levels of aggressiveness regarding potato and tomato. Russian *A. solani* and *A. tomatophila* isolates had morphological characters on V-4 very similar to those described by Simmons (2007) on V-8 using representative cultures. Isolates from our collection had identical or slightly shorter and thinner conidial body and a longer beak. The most important characters for identification which should be examined carefully are: length of beak and its branching. Size of body has less diagnostic value. Summarizing our original data and data obtained from the literature it is possible to define a diagnosis of *A. solani* on V-4 or V-8 under dark/light cycle: body of mature conidia 50–120(–143)×18–23(–26) μm; apical beak mainly 80–130 μm long, but no longer than 200 μm; beak sometimes double or rarely triple. Conidia body of *A. tomatophila* 50–120(–128)×16–20(–23) μm; beak 100–220(–306) μm long and consists of 2–3 or rarely 4–5 parts (branches).

V-4 and PCA both allow differentiating the two species, however it is essential to take into account that conidia size and shape depend on substratum including composition of agar medium. In comparison with V-4, on PCA conidia of both species are somewhat larger and have beaks with bigger average numbers of branches. The same has been described for other large-spored species, e.g., *A. calendulae* (Simmons 2007).

Cultural characters have been used in some work to differentiate races in *A. solani* populations (Ivanuk and Palilova 1996; Ivanyuk et al. 2003). Unfortunately it

was not possible to obtain representative strains of the races described by Ivanuk and Palilova (1996). We failed to find obvious and reproducible difference in colony morphology between species using 30 isolates. It has been mentioned previously (Rotem 1994) that cultural features of *A. solani* are unstable. It is notorious that many fungi can change their cultural features after several consecutive subculturings. Altogether those facts suggest that use of cultural characters for identification of large-spored species and moreover for differentiation of their races or pathotypes is not fruitful. Identification of a pattern of micromycete biodiversity on the level of species or lower should be based on molecular techniques which are broadly available at the present.

Several groups of morphologically similar large-spored *Alternaria* species have been described from the same host plant or similar plants of one family. For instance, five species are known on spurge (*Euphorbia* spp., *Euphorbiaceae*) and three species on sunflower

Table 3 Aggressiveness of *Alternaria solani* and *A. tomatophila* isolates when inoculated to potato and tomato

Fungal species	Necrotic area on leaf disk ^a , %			
	Potato		Tomato	
	Mean	Range	Mean	Range
<i>A. solani</i>	11.0±0.3 ^b	1.7–21.7	11.2±0.4	1.5–20.0
<i>A. tomatophila</i>	6.9±0.2	1.9–15.8	14.1±0.5	2.8–41.0

^a Lesions were estimated visually after 5 dpi as percent area of each disk affected by necrosis

^b Digits after ± indicate 95 % confidence interval

(*Helianthus annuus*, *Asteraceae*). *Alternaria* species pathogenic for nightshades (*Solanum sensu lato*) are the most complicated group for taxonomic and related studies because it includes 11 large-spored *Alternaria* species. As it was noticed above this group is polyphyletic. Probably large-spored species possess ecologically effective and simultaneously genetically flexible physiological and biochemical traits that ensure the capability of rapid change of substratum. The process of host shift by pathogenic microfungi is a very interesting biological issue that has great practical value. *Alternaria* species can serve in the future as a good model for such study.

No special large-scale molecular study has been done on correspondence between morphospecies, their specialization and phylogenetic pattern. However some results can be obtained by analyzing phylogenetic trees. Different fungal species associated with related host plants can be close relatives or not. For instance, two species from *Euphorbiaceae*, *A. pseudorostrata* and *A. euphorbiicola*, have some differences in sequences of the *Alt a 1* and *gpd* genes, but have a common nearest ancestor (Hong et al. 2005). Two pathogens of solanaceous plants (datura and pepper), *A. crassa* and *A. capsici*, have identical sequences. But the last pair of species and *A. solani* have less genomic similarity and are descended from different nearest ancestors in spite of host preference. In another work (Rodrigues et al. 2010), it was found that sequences of the same genes in *A. solani* and *A. tomatophila* have slight, but phylogenetically informative differences. This can indicate that adaptation of *Alternaria* for parasitism on solanaceous crops appeared at least twice and resulted in the formation of at least two groups of related species.

An interesting chemotaxonomic study of four large-spored species pathogenic for plants from three families has been carried out (Andersen et al. 2008). Cluster analysis of metabolite profiles revealed that *A. solani*, *A. tomatophila* and *A. dauci* (pathogen of carrot, *Apiaceae*) have unique combinations of metabolites. Each species had three compounds which were not found in the other species. However the results were ambiguous for attempts to describe the chemical individuality of *A. porri* (pathogen of onions, *Alliaceae*).

In our research, the genetic distance between *A. tomatophila* and *A. solani* calculated on the basis of DNA fingerprinting data was relatively low (30 %), which is lower than the distance between those species

and another large-spored taxon, *A. dauci*. At the same time the Wright's index calculated for comparison of *A. tomatophila* and *A. solani* was rather high ($F_{ST}=0.764$). For instance the index estimated for very distant populations (6 000 km apart) of the single species *A. tenuissima* was much lower ($F_{ST}=0.283$) (Gannibal et al. 2007). It verifies the difference between *A. tomatophila* and *A. solani* at the species level.

Some isolates from tomato were clustered in the “potato” branch and represent *A. solani*. It is interesting, that disease of tomato and wild potato caused by *A. solani* was the result of proximity of potato and tomato fields. Despite the more frequent appearance of this fungus on potato it can infect under field conditions at least five *Solanum* species, in concordance with laboratory tests. Under laboratory conditions *A. solani* infected both potato and tomato to an equal degree.

A. tomatophila probably has stronger specialization and is associated with tomato only. This was revealed by field observations and laboratory tests. This species is able to infect potato leaf fragments, but with sufficiently lesser aggressiveness than for tomato. It is not unique for facultative saprotrophs to express moderate or poor pathogenicity in relation to some non-host plants which are weakened by conditions of laboratory experiments. Thus *A. solani* is aggressive for both tomato and potato to an equally moderate degree, but *A. tomatophila* is highly aggressive for tomato and weakly aggressive for potato. This result does not contradict the data of previous studies (Ivanyuk 1983) where it was reported that the “potato” subpopulation of *A. solani sensu lato* contained mainly medium-aggressive isolates when the “tomato” subpopulation was represented primarily by high-aggressive isolates.

Genes of mating-type loci in sexually reproducing fungi regulate mating preceding the formation of meiospores and probably other functions related to reproduction. These loci have been found in all (or at least in almost all) presumably asexual ascomycetes and should not perform their main functions. Naturally the ratio of two idiomorphs (*MAT1-1* and *MAT1-2*) in a clonal population can sufficiently deviate from 1:1 due to stochastic processes like genetic drift. The *MAT1* locus has been studied in several *Alternaria* species with no known sexual stage. Both *MAT* idiomorphs have been found in isolates of *A. tenuissima*, *A. brassicicola* and *A. brassicae* (Berbee et al. 2003). Isolates of opposite mating type but from the same species had

identical sequences of the ribosomal ITS regions. Berbee and her colleagues suggested that isolates of opposite mating type were exchanging substitutions through cryptic sexual or parasexual reproduction. If any sexuality was lost, it was lost after the period of evolutionary history represented by the shared substitutions. So far no signature of recombination has been found in the genetic structure of *A. solani* populations (Lourenço et al. 2009). Bias in occurrence of the different mating types in *A. solani* and *A. tomatophila* suggests the dominance of clonal reproduction.

Van der Waals and colleagues (2004) have used randomly amplified microsatellites (RAMS) and groups of vegetative compatibility to analyze the South African populations of *A. solani sensu lato*. They found that genetic diversity was relatively high for asexual species. But as mentioned above, recombination has not been found in this species at least in South America (Lourenço et al. 2009). At the same time in a number of studies (Weir et al. 1998; van der Waals et al. 2004; Lourenço et al. 2009) as well as in our research no significant correlation between genetic markers and geographic origin of isolates has been defined. This suggests intensive gene flow between distant populations in combination with mutagenesis. Mutagenesis, the ability to produce several generations per vegetation season, and intensive migration provide *A. solani* and *A. tomatophila* with significant evolutionary potential which is limited only by the absence of frequent recombination events. Both of these species have population genetic resources for moderately fast overcoming of host plant resistance as well as for development of tolerance to fungicides.

Summarizing all of the data we can suggest that *A. solani* and *A. tomatophila* were formed from a common ancestor after the moment when this ancestor acquired the ability to infect solanaceous plants with low host specificity. Later a number of climatic, breeding, agrotechnological and other factors led to emergence of several isolated species with different levels of specificity and aggressiveness.

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