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Secondary metabolite profiling of *Alternaria dauci*, *A. porri*, *A. solani*, and *A. tomatophila*

Birgitte ANDERSEN[a,*], *Anita DONGO*[b,1], *Barry M. PRYOR*[c]

[a]*The Mycology Group, Center for Microbial Biotechnology, BioCentrum-DTU, Building 221, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark*
[b]*Plant Protection Institute, University of Veszprém, Deák Ferenc u. 57. H-8360 Keszthely, Hungary*
[c]*Department of Plant Pathology, University of Arizona, Tucson, AZ 85721, USA*

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ABSTRACT

Chemotaxonomy (secondary metabolite profiling) has been shown to be of great value in the classification and differentiation in *Ascomycota*. However, few studies have investigated the use of metabolite production for classification and identification purposes of plant pathogenic *Alternaria* species. The purpose of the present study was to describe the methodology behind metabolite profiling in chemotaxonomy using *A. dauci*, *A. porri*, *A. solani*, and *A. tomatophila* strains as examples of the group. The results confirmed that *A. dauci*, *A. solani*, and *A. tomatophila* are three distinct species each with their own specific metabolite profiles, and that *A. solani* and *A. tomatophila* both produce altersolanol A, altertoxin I, and macrosporin. By using automated chemical image analysis and other multivariate statistic analyses, three sets of species-specific metabolites could be selected, one each for *A. dauci*, *A. solani*, and *A. tomatophila*.

Introduction

Foliar blights of carrots, onions, potatoes, and tomatoes caused by *Alternaria*, especially *A. dauci*, *A. porri*, and *A. solani*, are diseases with worldwide distribution and result indirectly in severe crop loss due to defoliation (Rotem 1994). Neergaard (1945) considered these three foliar pathogens to belong to the same species, because of similarities in morphology. Phylogenetically, *A. dauci*, *A. porri*, and *A. solani* cluster in the same clade as *A. crassa* and *A. macrospora*, but all as separate species (Pryor & Bigelow 2003). Simmons re-examined *A. dauci* in 1995 and various *Alternaria* species on *Solanaceae* in 2000. He treated *A. dauci*, *A. porri*, and *A. solani* as separate species and extracted several new species from the '*A. solani*-complex', including *A. tomatophila*, the common and widely distributed incitant of early blight of tomato (Simmons 2000).

Chemotaxonomy (secondary metabolite profiling) has been shown to be of great value in the classification and differentiation of species in many genera in *Ascomycota* (Frisvad *et al.* 2008), and even though a large number of secondary metabolites are known from plant pathogenic *Alternaria* spp. (Montemurro & Visconti 1992; Rotem 1994), few studies have investigated metabolite production for classification and identification purposes. According to the literature, zinniol is the only metabolite that *A. dauci*, *A. porri*, and *A. solani* have in common (Montemurro & Visconti 1992; Horiuchi *et al.* 2003). Alterporriols, altersolanols, macrosporin, and tentoxin have been reported from various cultures of *A. porri* and *A. solani*

* Corresponding author.
E-mail address: ba@biocentrum.dtu.dk
[1] Current address: Plant Protection Institute, Hungarian Academy of Sciences, PO Box 102, H-1525 Budapest, Hungary.
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(Suemitsu et al. 1990a, b, 1992; Montemurro & Visconti 1992). Alternariol monomethyl ether has been reported from *A. dauci*, erythroglaucin and other anthraquinones from *A. porri*, and alternaric acid, alternariol, solanapyrones and zinnolide from *A. solani* (Montemurro & Visconti 1992).

The main purpose of this study was to describe the methodology behind metabolite profiling in chemotaxonomy using *A. dauci*, *A. porri*, *A. solani*, and *A. tomatophila* strains as examples. One objective was to examine the chemical diversity of the four aforementioned *Alternaria* species and compare metabolite profiles with the currently accepted classification. A second objective was to suggest species-specific metabolites that could be adopted as chemotaxonomic markers in taxon identification.

Materials and methods

Fungal strains, growth media, and conditions

Fifty-six fungal strains belonging to the genus *Alternaria* were used. The strains were first inoculated on potato carrot agar (PCA) (Simmons 1992) and grown under standardized conditions (Andersen et al. 2005). In brief, the unsealed 9 cm plates were incubated in one layer for 7 d at 23 °C under an alternating light/dark cycle consisting of 8 h of cool-white fluorescent daylight and 16 h darkness. Each strain was then inoculated onto dichloran–Rose Bengal–yeast extract–sucrose agar (DRYES) (Frisvad 1983) as three-point inoculations and onto another PCA for confirmation of identity. After inoculation, the DRYES plates were packed in perforated plastic bags and incubated for 14 d in the dark at 25 °C. The PCA cultures were incubated as mentioned above and examined morphologically after 7 d according to Simmons (1995, 2000). If sporulation was unsatisfactory, the plates were scarified and incubated longer. The original and confirmed identity, identification number, host plant, and geographic origin of all strains are presented in Table 1. All strains are available from the authors' private collections and the IBT collection at BioCentrum-DTU.

Metabolite extraction procedure

Metabolite extractions were performed on the 14-d-old DRYES cultures. The extraction protocol used was a micro-scale extraction method modified for *Alternaria* metabolites (Andersen et al. 2005). In brief, three 6 mm agar plugs were cut from the centre of the three colonies and the nine plugs were placed in a 2 ml screw-top vial. The plugs were extracted with 1 ml ethyl acetate containing 1 % formic acid (v/v) in ultrasound for 60 min in a Branson 3210 water-bath (Ultrasonics Corporation, Danbury, CT). The extract was transferred to a clean 2 ml vial, evaporated to dryness in a rotary vacuum concentrator (Christ, Gefriertrocknungsanlagen, Osterode am Harz), re-dissolved ultrasonically in 400 µl methanol, and filtered through a 0.45 µm filter (National Scientific Company, Rockwood, TN) into a clean 2 ml vial prior to hplc analysis. The experiment was repeated once with all strains, and for strains with low metabolite production, a third time with twice the number of plugs.

hplc UV–visible analysis

The hplc analyses were performed on a HP-1100 high-performance liquid chromatograph (Agilent, Waldbronn) equipped with an auto-sampler injecting 3 µl sample^{-1} and a diode array detector collecting 2 UV–visible (UV–vis) spectra s^{-1} from 200–600 nm. Separations were done on a 100 × 2 mm Luna 3 µ C18(2) 100 Å column (Phenomenex, Torrance, CA) with a 4 × 2 mm C18(2) guard column (Phenomenex). The column temperature was 40 °C. The mobile phase consisted of a linear gradient starting at 85 % water and 15 % acetonitrile reaching 100 % acetonitrile in 20 min. One hundred percent acetonitrile was maintained for 5 min. Thereafter, the gradient was returned to 15 % acetonitrile in 3 min and allowed to equilibrate for 5 min before the next analysis. Both eluents contained 50 ppm trifluoroacetic acid. The flow rate was 0.4 ml min^{-1}. A homologous series of alkylphenones was analysed as external retention time references and used to calculate a bracketed RI for each detected peak (Frisvad & Thrane 1987). Origin and preparation of fungal standards, such as altenuene, alternarials, altersolanol A, altertoxin I, erythroglaucin, tentoxin, and tenuazonic acid, are given in Nielsen & Smedsgaard (2003). All solvents were hplc grade, chemicals were analytical grade, and the water was double distilled.

Data treatment

A flow sheet of the various steps from automated and manual analyses of the hplc data to the suggestion of species-specific metabolites is shown in Fig 1. The raw hplc data files were first subjected to an automated chemical image analysis (CIA) (Hansen 2003) and the resulting dendrogram evaluated in respect to original fungal identification and chemical clustering. In parallel: (1) the identity of the strains was checked by microscopic methods and (2) a full manual metabolite matrix was made containing fungal identities and all recognizable peaks/UV–vis spectra from all hplc chromatograms. Strains whose identity could not be confirmed and strains that did not cluster with the main body of strains with the same ID in the CIA dendrogram were excluded before the matrix was subjected to partial least squares regression PLS-R. Based on the resulting loadings plot, the reduced manual metabolite matrix was constructed and the excluded strains were reintroduced. The new matrix was then subjected to a cluster analysis and after evaluation of the dendrogram, species-specific metabolites were manually selected.

Chemical Image Analysis (CIA)

The raw hplc data files, which are quantitative two-dimensional matrices (*x*-axis: time, *y*-axis: wavelength, value in matrix: UV–vis absorbance), were transferred from the hplc to a standard PC and analysed by an in-house written CIA program (Hansen 2003). No manipulations or peak selections were made before processing. Each hplc file was processed first by a $\log_{10}$ scaling (to account for concentration differences among extracts), then a baseline correction and finally an alignment (to account for drift in baseline and retention time among identical metabolites in different runs) (Hansen 2003). Each hplc file was then compared with the other 55

Table 1 – *Alternaria* strains used in the study

Collection no.	Original identification[a]	Identification confirmed[b]	Host plant	Substratum and origin	No. in other collections
AD 113	*Alternaria dauci*	+	*Daucus carota*	Carrot, -, NZ	
AD 180	*A. dauci*	+	*D. carota*	Carrot, leaf, Germany	
AD 181	*A. dauci*	+	*D. carota*	Carrot, leaf, Germany	
AD 183	*A. dauci*	+	*D. carota*	Carrot, seed, NL	CBS 101592
AD 185	*A. dauci*	+	*D. carota*	Carrot, seed, -	CBS 106.48
AD 188	*A. dauci*	+	*D. carota*	Carrot, leaf, Hungary	
AD 189	*A. dauci*	+	*D. carota*	Carrot, leaf, Hungary	
BA 1489	*A. dauci* R	+	*D. carota*	Carrot, seed, CA, USA	EGS-46-006
BA 1491	*A. dauci* R	+	*D. carota*	Carrot, leaf, NZ	EGS-46-152
BA 1492	*A. dauci* R	+	*D. carota*	Carrot, seed, CA, USA	EGS-47-131
BMP 155	*A. dauci*	+	*D. carota*	Carrot, seed, -	
BMP 156	*A. dauci*	+	*D. carota*	Carrot, leaf, CA, USA	
BMP 157	*A. dauci*	+	*D. carota*	Carrot, leaf, CA, USA	
BMP 158	*A. dauci*	+	*D. carota*	Carrot, seed, -	
BMP 159	*A. dauci*	+	*D. carota*	Carrot, seed, -	
BMP 160	*A. dauci*	+	*D. carota*	Carrot, seed, -	
BMP 161	*A. dauci*	+	*D. carota*	Carrot, seed, -	
BMP 163	*A. dauci*	+	*D. carota*	Carrot, seed, -	
BMP 164	*A. dauci*	+	*D. carota*	Carrot, seed, -	
BMP 165	*A. dauci*	+	*D. carota*	Carrot, leaf, CA, USA	
BMP 167	*A. dauci*	+	*D. carota*	Carrot, leaf, FL, USA	ATCC 36613
BMP 168	*A. dauci*	+	*D. carota*	Carrot, seed, -	
BMP 171	*A. dauci*	+	*D. carota*	Carrot, -, CA, USA	
AD 186	*A. porri*	NS	*Allium cepa*	Onion, seed, DK	CBS 114.38
BA 1451	*A. porri* R	+	*A. cepa*	Onion, leaf, NY, USA	EGS-48-147
BA 1545	*A. porri*	+	*A. cepa*	Onion, -, NY, USA	EGS-48-152
BA 1548	*A.* cfr *porri*	*A.* cfr *porri*	*A. ascalonicum*	Shallot, -, NZ	EGS-46-052
BA 1553	*A. porri*	+	*A. cepa*	Onion, - , Mexico	EGS-49-065
BA 1557	*A. porri*	+	*A. cepa*	Onion, -, NY, USA	EGS-48-153
BMP 178	*A. porri*	*A.* cfr *porri*	*A. ampeloprasum*	Leek, leaf, Australia	
AD 155	*A. solani*	*A.* cfr *solani*	*Solanum tuberosum*	Potato, leaf, Hungary	
AD 187	*A. solani*	NS	*S. aviculare*	New Zealand nightshade, leaf, DK	CBS 110.41
BA 1444	*A. solani* R	+	*S. tuberosum*	Potato, leaf, WA, USA	EGS-44-098
BA 1546	*A. solani*	+	*S. tuberosum*	Potato, -, NY, USA	EGS-46-125
BA 1552	*A. solani*	+	*S. tuberosum*	Potato, -, CA, USA	EGS-45-020
BA 1555	*A. solani*	+	*S. tuberosum*	Potato, -, NZ	EGS-45-053
BA 1558	*A. solani*	+	*S. tuberosum*	Potato, -, NY, USA	EGS-46-133
BMP 181	*A. solani*	+	*Lycopersicon esculentum*	Tomato, fruit, CA, USA	
BMP 182	*A. solani*	+	-	-, -, CA, USA	
BMP 183	*A. solani*	+	-	-, -, CA, USA	
BMP 185	*A. solani*	*A.* cfr *solani*	*S. tuberosum*	Potato, tuber, CA, USA	
BMP 186	*A. solani*	+	*S. nigrum*	Black nightshade, leaf, CA, USA	
BMP 187	*A. solani*	NS	*L. esculentum*	Tomato, -, Mexico	ATCC 58177
BA 1443	*A. tomatophila* R	+	*L. esculentum*	Tomato, leaf, IN, USA	EGS-44-074
BA 1523	*A. tomatophila*	+	*L. esculentum*	Tomato, leaf, Australia	EGS-44-024
BA 1524	*A. tomatophila*	+	*L. esculentum*	Tomato, leaf, Australia	EGS-44-036
BA 1525	*A. tomatophila*	+	*L. esculentum*	Tomato, stem, FL, USA	EGS-44-046
BA 1526	*A. tomatophila*	+	*L. esculentum*	Tomato, leaf, NZ	EGS-46-161
BA 1527	*A. tomatophila*	+	*L. esculentum*	Tomato, leaf, Venezuela	EGS-48-026
BA 1528	*A. tomatophila*	+	*L. esculentum*	Tomato, -, PA, USA	EGS-50-065
BA 1541	*A.* cfr *tomatophila*	+	*L. esculentum*	Tomato, leaf, IN, USA	EGS-44-073
BA 1542	*A.* cfr *tomatophila*	+	*L. esculentum*	Tomato, leaf, IN, USA	EGS-44-169
BA 1544	*A. tomatophila* T	+	*L. esculentum*	Tomato, leaf, IN, USA	EGS-42-156
BA 1549	*A.* cfr *tomatophila*	+	*L. esculentum*	Tomato, leaf, IN, USA	EGS-44-170
BA 1550	*A.* cfr *tomatophila*	+	*L. esculentum*	Tomato, leaf, IN, USA	EGS-44-072
BA 1554	*A.* cfr *tomatophila*	+	*L. esculentum*	Tomato, fruit, IN, USA	EGS-46-012

a Original identity given by the authors (A.D. & B.M.P.). B.A. strains received from Emory G. Simmons. R, representative strain for the species; T, ex-type strain for the species.

b Confirmed at BioCentrum-DTU. +, identification confirmed compared to representative and ex-type strains; *cfr*, deviated morphologically compared to the representative strains and was reintroduced in the final analysis; NS, no sporulation and was removed from final analyses.

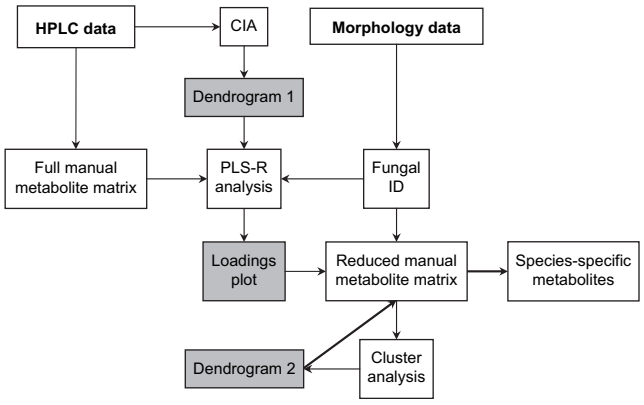

Fig 1 – Flowchart of the procedures, decisions, and analyses leading to the selection of species-specific metabolites.

hplc files, pair-wise, using an algorithm described by Hansen (2003) giving a similarity value for each pair, which was entered into a new matrix. The resulting 56×56 similarity matrix was then used to calculate a dendrogram using WARD clustering method.

Partial Least Squares Regression (PLS-R)

The binary matrix was made manually by scoring each metabolite as present or absent from the printed chromatograms and subjected to multivariate statistics using The Unscrambler version 9.2 (CAMO ASA, Oslo). This full manual metabolite matrix consisted of 64 *x*-variables (known and unknown metabolites) and four *y*-variables (four species names) for the 43 objects (fungal strains with confirmed ID and clustering according to ID). The matrix was analysed using PLS-R, which relates the variations in response variables (*y*-matrix) to the variations of several predictors (*x*-matrix) (Wold *et al.* 2001). By plotting the PLS components one can view main associations between *x*-variables (metabolites) and *y*-variables (strain ID), and also interrelationships within *x*- and *y*-data.

Cluster analyses

The full manual metabolite matrix was reduced according to the PLS-R result and contained consistently produced metabolites with both known and unknown chemical structures. The binary, reduced metabolite matrix, consisting of 53 strains and 34 species-specific metabolites, was subjected to cluster analysis using NTSYS-pc version 2.11N (Exeter Software, Setauket, NY) without standardization using Yule (Y) as correlation coefficient and UPGMA as clustering method. The matrix was also analysed by simple matching (SM) and Jaccard (J) similarity coefficients in NTSYS.

Results

Chemical Image Analysis (CIA)

The result of the automated and unbiased CIA of 56 *Alternaria* extracts is shown in Fig 2. The analysis is based on unedited chromatograms (hplc files) of raw fungal extracts that contained compounds from the growth media (e.g. chloramphenicol and dichloran), impurities from extraction solvents, fungal-specific (e.g. ergosterol), species-specific, and strain-specific metabolites. The dendrogram shows two main clusters, A and B. Cluster A contained *A. solani* and *A. tomatophila* strains and cluster B contained *A. dauci* and *A. porri* strains. Cluster A consisted of three minor clusters: one *A. solani* cluster (A1), which included one *A. porri* strain (BA 1553), but not the representative strain of *A. solani* (BA 1444); one miscellaneous cluster (A2) with *A. dauci* (BMP 163), two *A. solani* strains (AD 155 and AD 187), two *A. porri* strains (BA 1548 and AD 186) strains and the representative strain of *A. solani* (BA 1444); and one *A. tomatophila* cluster (A3), including one *A. solani* strain (BMP 187). Cluster B also contained three clusters: one *A. dauci* cluster (B1) and one *A. porri* cluster (B2) with a cluster (B3) of three *A. dauci* strains (AD 185, AD 113, and BMP 165). Construction of the full manual metabolite matrix showed that the extracts of strains in the miscellaneous cluster (A2) and the small *A. dauci* cluster (B3) had between 60 and 85 % fewer metabolites compared with extracts in the nearest sister clusters. Repeated experiments (growth, extraction, and hplc analysis) with these strains resulted in the same low metabolite production.

Partial Least Squares Regression (PLS-R)

Before a PLS-R could be performed, a consensus between chemical and morphological data was necessary. The identity of all strains was confirmed by comparison with the type or representative strains of each species (see Table 1). Morphological re-examination lead to the permanent exclusion of *Alternaria porri* (AD 186) and *A. solani* (AD 187 and BMP 187) from any further analyses, because it was not possible to induce sporulation in these strains again and thereby confirm their identity. *A. porri* (BA 1548 and BMP 178) and *A. solani* (AD 155 and BMP 185) were temporarily omitted, because of morphological discrepancies when compared with representative strains. Examination of chemical data lead to the temporary omission of *A. dauci* (AD 185, AD 113, BMP 163, and BMP 165), *A. porri* (BA 1553), and *A. solani* (BA 1444), because they either clustered in the miscellaneous cluster (A2 in Fig 2) or clustered away from the representative strain of the species. Five strains that arrived as putative *A. tomatophila* were confirmed, morphologically and chemically, as *A. tomatophila* and remained in the analyses. The resulting consensus reduced the number of *Alternaria* strains for PLS-R to 43 where both morphology and chemistry were in agreement.

The full manual metabolite matrix was constructed from the 43 hplc chromatograms as a binary (presence or absence) matrix and gave 64 metabolites that were produced in high concentration and that had distinct UV–vis spectra. These 64 metabolites constituted the 'master metabolite profile' for the four *Alternaria* species on which subsequent matrices would be based. The PLS-R was made to select consistently produced species-specific metabolites and deselect common and inconsistently produced metabolites. The plot in Fig 3 shows the calculated model, which explains 78 % of the fungal identity (*y*-data) and 58 % of the chemical diversity (*x*-data) in the first two axes out of the four axes that constituted the

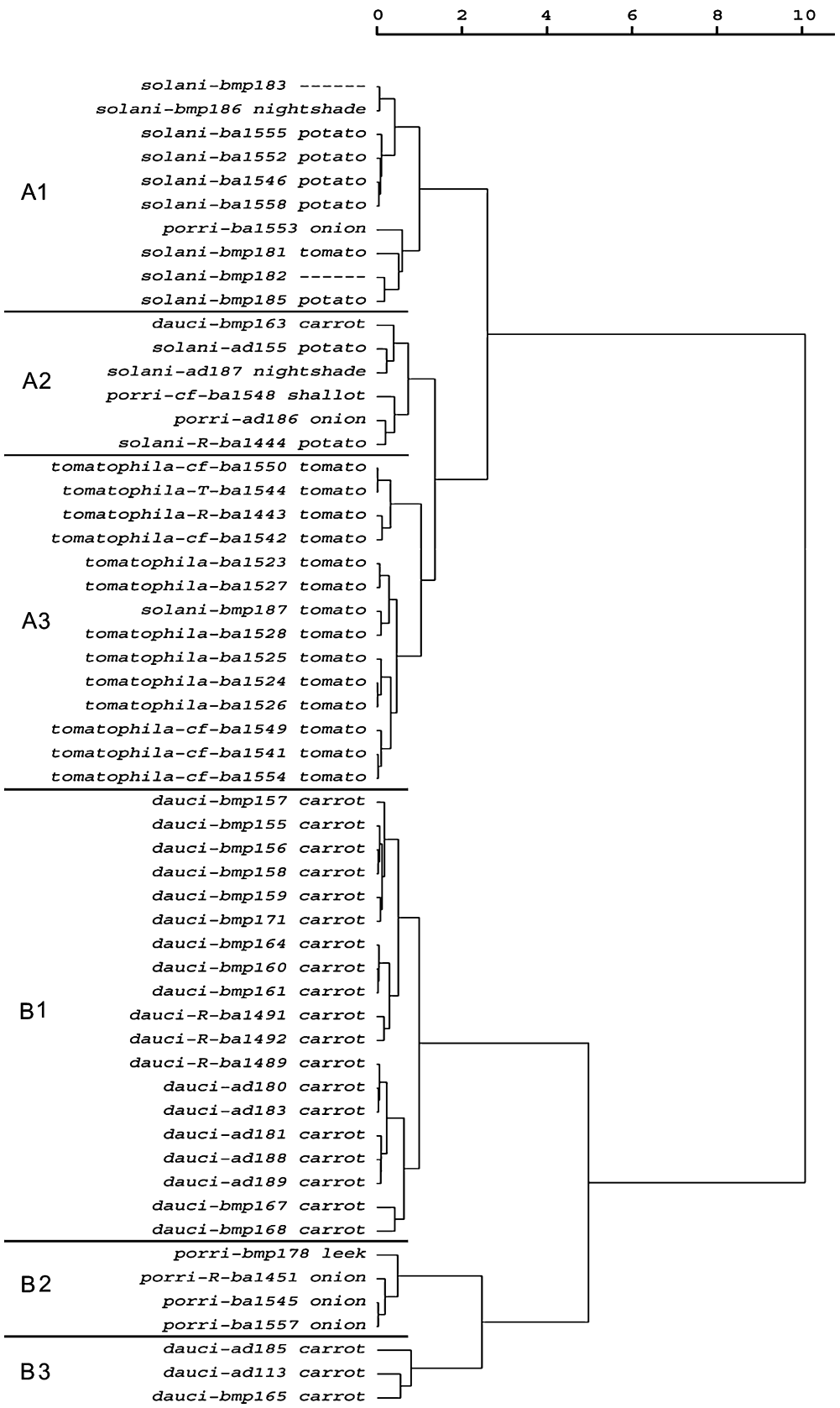

Fig 2 – Dendrogram based on an automated CIA of all metabolites of 56 *Alternaria* strains using the Ward clustering method. Strain labels read as follows 'species ID-ID number-substratum'. Ex-type and representative strains are marked with T and R, respectively. Arbitrary scale.

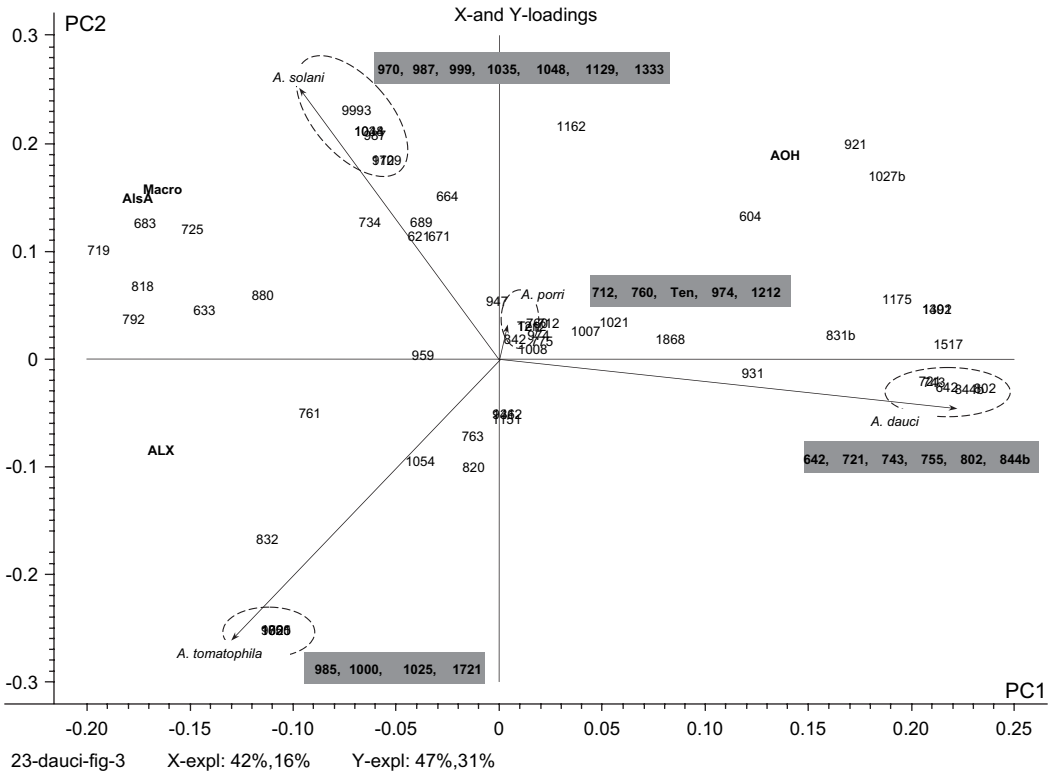

Fig 3 – Loadings plot based on a PLS-R of the full manual metabolite matrix of 64 metabolites of 43 *Alternaria* strains. Each metabolite is given by its RI value. Species-specific metabolites are found along the species arrows. The closer a metabolite is to the arrowhead the more significant it is for the given species. The most important species-specific metabolites are encircled and written out in grey. Arbitrary axes.

model. The correlation coefficient between the 'predicted' fungal identity and the 'calculated' identity was 0.989 with full cross-validation. The first and second axes (arbitrary scales) separated *A. dauci*, *A. solani*, and *A. tomatophila* and the third, orthogonal axis segregated *A. porri*. The 64 metabolites are represented in the plot by their RI values. The model in Fig 3 gives the species-specific metabolites by arranging them along a line (arrow) from the centre of the coordinate system to the given species. The closer a metabolite is to the arrowhead and the species name, the more specific it is and the larger a percentage of the strains in the given species produce it. For example, 100 % of the *A. dauci* strains produce the metabolite with RI value 802. Metabolites that are located between two species-arrows are common to both species, whereas metabolites located diametrically opposite a species-arrow are not produced by the given species. For example, metabolite 1517 is common to both *A. dauci* and *A. porri*, but not found in *A. solani* or *A. tomatophila*. Likewise, metabolite 719 is not produced by any of the 19 *A. dauci* strains, but by 21 out of the remaining 24 strains. Metabolites common in all strains would be located in the centre of the coordinate system.

Cluster analysis

After reviewing the result of the PLS-R (Fig 3), the full manual metabolite matrix could be reduced to 34 species or group-specific metabolites. The ten strains that were temporarily omitted in the PLS-R analysis were reintroduced. The result of the cluster analysis (Y/UPGMA) of the reduced manual metabolite matrix of 53 *Alternaria* strains and 34 metabolites is shown in Fig 5. The cophenetic correlation (r) for Y/UPGMA was 0.85, whereas r was 0.90 and 0.95 for SM/UPGMA and J/UPGMA, respectively. All three analyses gave the same clustering, but only the Y/UPGMA is shown. The dendrogram shows the same overall clustering as the CIA dendrogram (Fig 2), but now with all *A. dauci* in the same cluster. The four confirmed *A. porri* strains from onion formed one cluster with *A.* cfr *porri* (BMP 178) from leek as an outlier. All the *A. tomatophila* strains also formed one cluster with *A. tomatophila* (BA 1528) as an outlier. The cluster with all the *A. solani* strains split up into two with the strains from cluster A2 (Fig 2) in one sub-cluster and with *A.* cfr *porri* (BA 1548) from shallot as an outlier.

Species-specific metabolites

Combining the results in Figs 3 and 5, species-specific metabolites were selected from the reduced manual metabolite matrix and selected ones are given in Table 2 together with the production of metabolites with known structure. None of the species-specific metabolites could be identified with any known chemical structure, based on their UV–vis spectra and compared to the metabolite standards in the collection at BioCentrum-DTU (Nielsen & Smedsgaard 2003), except for tentoxin (Ten). Table 2 gives the species-specific metabolites

Table 2 – Production of known *Alternaria* metabolites and selected, unknown species-specific metabolites for each species and for the strains that were omitted in the selection procedure

Metabolites from the full matrix	RI[a]	*A. dauci*[b] (19)	*A. porri*[b] (3)	*A. solani*[b] (8)	*A. tomatophila*[b] (13)	AD 185[c]	BMP 163	BA 1548	BA 1553	BMP 178	AD 155	BA 1444	BMP 185
Alternariol	860	18	–	8	1	–	–	–	–	–	–	–	–
Altersolanol A	652	–	3	8	8	–	–	1	–	1	1	1	1
Altertoxin	844a	–	–	6	12	–	–	–	–	1	–	–	–
Dauci-specific	642	17	–	–	–	1	1	–	–	–	–	–	–
Dauci-specific	755	19	–	–	–	1	1	–	–	–	–	–	–
Dauci-specific	802[d]	19	–	–	–	1	1	–	–	–	–	–	–
Dauci-specific	844b	18	–	–	–	1	–	–	–	–	–	–	–
Macrosporin	1062	–	3	8	7	–	–	1	–	1	1	1	1
Porri-specific	760	–	3	–	–	–	–	–	1	–	–	–	–
Porri-specific	1212	–	3	–	–	–	–	–	–	1	–	–	–
Solani-specific	999	–	–	8	–	–	–	–	–	–	–	–	1
Solani-specific	1035[d]	–	–	8	–	–	–	–	–	–	–	–	–
Solani-specific	1048	–	–	8	–	–	–	–	–	–	–	–	–
Solani-specific	1333	–	–	8	–	–	–	–	–	–	–	–	1
Tentoxin	868[d]	–	3	–	–	–	–	–	–	–	–	–	–
Tomatophila-specific	985	–	–	–	12	–	–	–	–	–	–	–	–
Tomatophila-specific	1000	–	–	–	12	–	–	–	–	–	–	–	–
Tomatophila-specific	1025[d]	–	–	–	12	–	–	–	–	–	–	–	–
Tomatophila-specific	1721	–	–	–	12	–	–	–	–	–	–	–	–
Dauci/porri specific	1517[d]	18	3	–	–	–	–	–	1	1	–	–	–

a RI: retention index value for each metabolite.
b *Alternaria* species and corresponding number of strains used in detecting species specific metabolites.
c The *A. dauci* strain AD 185 shows the same pattern as AD 113 and BMP 165.
d The UV–vis spectra of selected species-specific metabolites with unknown chemical structures and of tentoxin are shown in Fig 5.

for each *Alternaria* species and for ten strains that were omitted in the PLS-R.

As can be seen from Table 2, the production of known metabolites, such as alternariol, altersolanol A, altertoxin I, and macrosporin shown in Fig 4, were not as consistently produced as the selected unknown metabolites and production was found in more than one species. Alternariol was detected in *A. dauci* and *A. solani*, whereas altersolanol A and macrosporin were detected in *A. porri* and *A. solani*. Tentoxin was detected in *A. porri*, and altertoxin in *A. solani*. In *A. tomatophila*, formerly *A. solani* from *Lycopersicon esculentum*, altersolanol A, altertoxin, and macrosporin were detected in most strains, whereas alternariol was detected in one strain only (BA 1528). Alternariol was not detected in any of the *A. porri* strains, and altersolanol A, altertoxin, macrosporin, and tentoxin were not detected in any of the 23 *A. dauci* strains. Altenuene, erythroglaucin, and tenuazonic acid were not detected in any of the 56 extracts.

The specific metabolites in each species consisted of four to ten metabolites that had similar UV–vis spectra (i.e. belong to same chromophore family). Each series (dau, sol and tom in Table 2) is represented by one characteristic UV–vis spectrum as shown in Fig 5. The porri-specific metabolites (RIs 760 and 1212 in Table 2) had the same uv–vis chromophore as the dau/por metabolite 1517 (shown in Fig 5), which is common to both *A. dauci* and *A. porri*, but with a different retention time.

Discussion

The chemotaxonomic approach presented in this study shows that profiles of secondary metabolites can be powerful discriminators in fungal classification provided that they are based on extracts derived from correctly identified fungal cultures grown under standardized conditions. Unfortunately, the CIA does not reveal the individual metabolites that are responsible for a particular clustering and manual construction

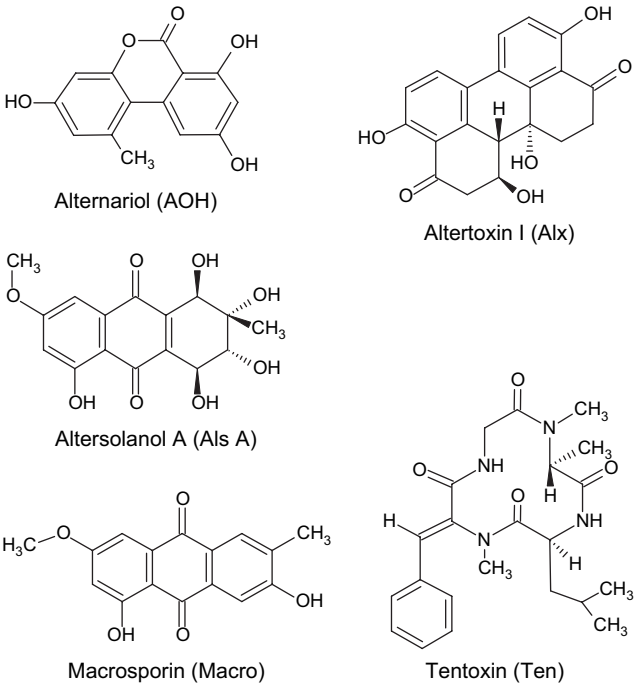

Fig 4 – Chemical structures of the known secondary metabolites inconsistently produced by the four *Alternaria* spp.

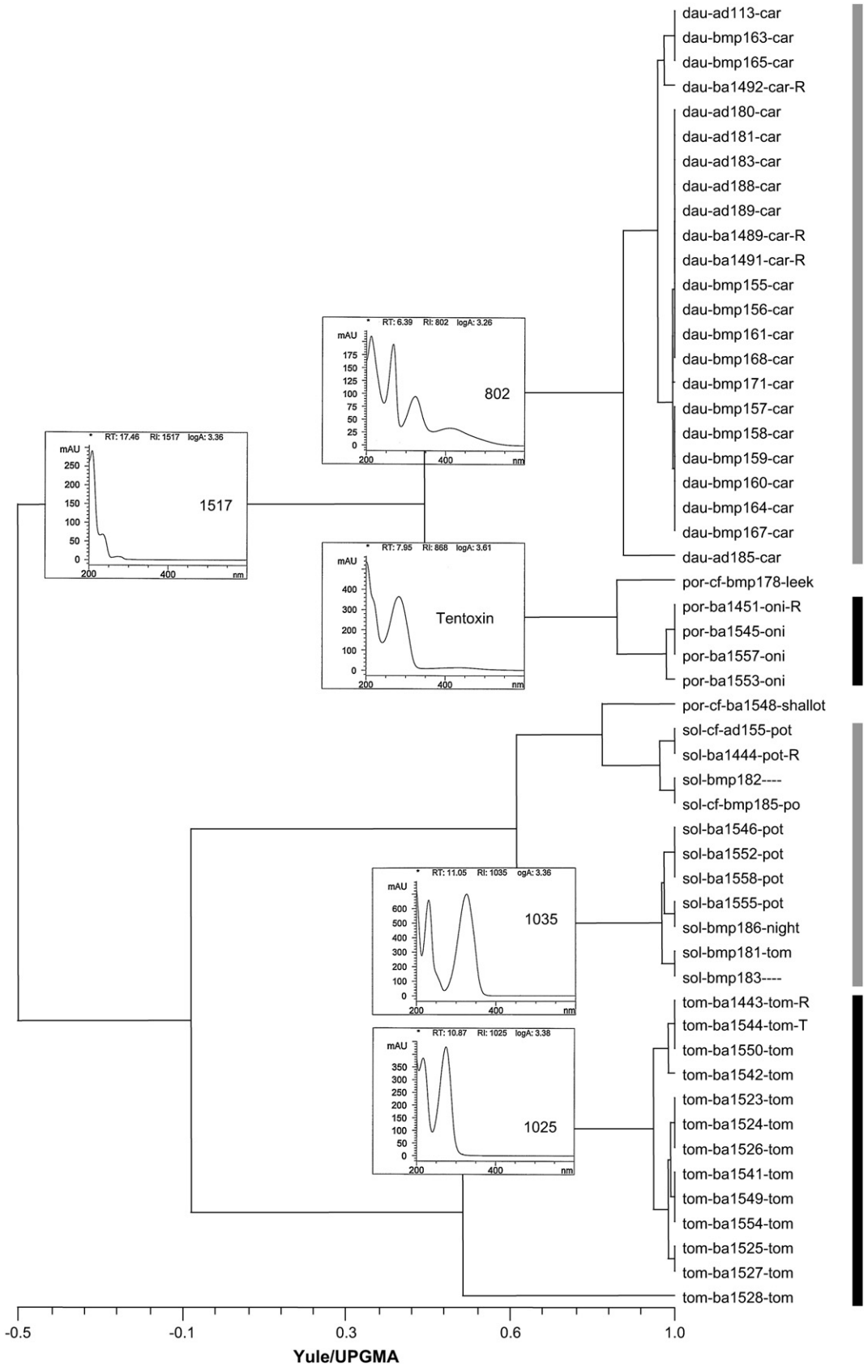

Fig 5 – Dendrogram based on a cluster analysis of 35 species-specific metabolites of 53 *Alternaria* strains using Yule (Y) as the correlation coefficient and UPGMA as the clustering method. Strain labels read as follows 'species ID-ID number-substratum'. Ex-type and representative strains are marked with T and R, respectively. A representative UV–vis spectrum of each of the four species or group-specific metabolites with unknown chemical structures and of tentoxin is located on each branch. Arbitrary scale.

of the actual metabolite profiles is still necessary. However, it can be done with much greater confidence when the overall clustering is known. The approach also shows that individual metabolites can function as chemotaxonomic markers in fungal identification, when they are selected from a large, uniform group of sound strains from each taxon. This study confirmed *Alternaria dauci*, *A. solani*, and *A. tomatophila* as three distinct species, each with their own profile of species-specific metabolites.

When using multivariate statistics to analyse correlations between species ID and metabolite profiles, metabolites with high segregation power at species level can be selected from the profiles. Metabolites, such as ergosterol (common in all fungal mycelia) and altersolanol A (inconsistently produced by more than two species), do not contribute to any segregation or classification. Likewise, metabolites found in only one strain out of many in a taxon do not contribute either. These metabolites are still a part of the strain's total metabolite profile, but can only rarely be used for classification or identification purposes. Species-specific metabolites can only be identified with the highest level of confidence if the analysis contains a large enough number of reliable strains of the same taxon. Chemotaxonomy should not be done on only one strain per taxon if additional strains exist, and it is suggested that five or more strains per expected taxon are used. The number of reliable *A. porri* strains in this study (three strains) is too few to ensure a fully reliable chemotaxonomy for this species. The two *Alternaria* cfr *porri* strains (BA 1548 from shallot, New Zealand and BMP 178 leek, Australia), have morphologies and metabolites that are unique to each and may represent new species, but additional isolates of the same taxa are needed for a robust chemical classification.

Only strains that can be unequivocally identified morphologically [being in a 'normkultur' state (Appel & Wollenweber 1910)] can be used in the selection process of species-specific metabolites or chemotaxonomic markers. *A. porri* AD 186 (syn. CBS 114.38) and *A. solani* AD 187 (syn. CBS 110.41) located in cluster A2 in Fig 2 are regarded as 'abkultur', because they had stopped sporulating and only produced a few, non-specific metabolites, which made it impossible to confirm their identity morphologically or chemically. The reason for this deficiency in metabolite production is not known. However, it is likely that metabolic processes degenerate in culture over time, as do morphology and pathogenicity (Burkholder 1925; Neergaard 1945; Otani & Kohmoto 1992; Simmons 1992; Rotem 1994). The *A. solani* strain (BMP 187, syn. ATCC 58177) located in cluster A3 (Fig 2) is also regarded as 'abkultur', but to a lesser extent than AD 186 and AD 187. BMP 187 had also stopped sporulating, but still produced a complete profile of tomatophila-specific metabolites. Had it been possible to make this strain sporulate again, morphological examination would most certainly place it with *A. tomatophila*.

When chemotaxonomic markers have been selected, a subset of metabolites from the profile of any new strain can be entered into the reduced manual matrix and subjected to a new cluster analysis. In this way the same procedures can be used as a confirmation or identification system. This is illustrated with the four *A. dauci* strains ('normkultur' regarding morphology, but 'abkultur' regarding the metabolite production), which were originally located in clusters A2 and B3 (Fig 2) and omitted during the selection of species-specific metabolites. The four strains, in spite of their low metabolite production, came together in the dauci-cluster in the final cluster analysis (Fig 5) when they were reintroduced.

The detection of alternariol, altersolanol A, and macrosporin in this study corresponds well with the reports in the literature (Suemitsu *et al.* 1990a, b; Montemurro & Visconti 1992), except that none of the *A. solani* strains produced tentoxin, as reported by Suemitsu *et al.* (1992). Furthermore, this is the first report of production of altersolanol A, altertoxin I, and macrosporin by *A. tomatophila* (former *A. solani* from *Lycopersicon esculentum*). It is also the first report on the production of altertoxin I by *A. solani*.

The lack of standards for many known *Alternaria* metabolites makes it difficult to identify many peaks in the chromatograms using only hplc-dad. The species-specific metabolites selected in this study are currently of unknown chemical structures, but ongoing purification and structure elucidation will determine whether all are new compounds or whether they can be identified as some of the many metabolites known for *A. porri* and *A. solani*. Judging by the chemical diversity of the extracts and the presence of several metabolites in chromophore families, each species seems to have its own unique biosynthetic pathways, which might be responsible for production of unique host-specific metabolites.

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