Automated and Unbiased Image Analyses as Tools in Phenotypic Classification of Small-Spored *Alternaria* spp.

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ABSTRACT

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For more than 25 years, controversy has surrounded the characterization and differentiation of small-spored *Alternaria* spp. And, therefore, the application of names of several species that are involved in the pathology of diseases related to host-specific toxin production. The name *A. alternata* often has been broadly applied to various morphologically and chemically distinct groups of isolates from different hosts. The purpose of this study was to develop and evaluate automated and

unbiased image analysis systems that will analyze different phenotypic characters and facilitate testing and application of the morphological species concept in *Alternaria* taxonomy. Host-specific toxin-producing *Alternaria* isolates assigned to five morpho-species were compared with representative isolates of morphologically distinct *A. alternata*. Combined results of growth rates at different temperatures, colony morphology, and metabolite profiles were found to be useful in characterization and differentiation of small-spored *Alternaria* spp. when standardized conditions are applied and representative isolates employed for comparison.

Additional keywords: chemotaxonomy, metabolite profiling, phylogeny.

Much of the controversy over the identification and naming of small-spored *Alternaria* pathogens originated through a misinterpretation by Nishimura et al. (20) of a discussion of the original specimen of *A. alternata* (Fr.:Fr.) Keissl. (30). Nishimura et al. (20), using a statistical treatment of conidial size from a potato dextrose agar culture, concluded that three small-spored *Alternaria* pathogens, *A. citri*, *A. longipes*, and *A. mali*, belonged to what they called a “collective species” of *A. alternata* if it were accepted that spore size was the primary taxonomic criterion classifying small-spored *Alternaria* spp. Since then, various authors (13,16,39,40) have accepted the statements of Nishimura et al. (20) without testing the underlying hypothesis that conidium size is a unique characteristic useful in synonymizing otherwise distinguishable species. Under this collective species hypothesis, the number of “pathotypes” of *A. alternata* has risen to six: *A. citri* (citrus pathotype), *A. longipes* (tobacco pathotype), *A. mali* (apple pathotype), *A. gaisen* (Japanese pear pathotype, syn. = *A. kikuchiana*), *A. arborescens* (tomato pathotype, syn. = *A. alternata* f. sp. *lycopersici*), and an unnamed strawberry pathotype (19). Followers of this classification approach continue to use the name *A. alternata* for increasing numbers of morphologically, pathologically, and physiologically diverse *Alternaria* spp. The scientifically adverse result of this practice is the submersion of masses of discriminating data in the literature under a single nondiscriminating name (1,13,39,40), even though standardized morphological methods, reference isolates, and more extensive species characterization on most of the host-specific toxin-pro-

ducing *Alternaria* spp. have been published (31,33–35). If the same hypothesis (spore size as the primary taxonomic criterion) were used in the genus *Penicillium*, the result would be no more than a handful of species, which would be against all existing knowledge, be it morphological, molecular, or chemical.

The controversy surrounding small-spored *Alternaria* spp. did not subside with the arrival of molecular cladistics and phylogeny; quite the contrary. Cladistic analyses of commonly used internal transcribed spacer (ITS) and β -tubulin sequences supported the original hypothesis of Nishimura et al. (20), but not the existing morphological classification based on a combination of characteristics (sporulation pattern) within the small-spored species of *Alternaria* (16,24,26,27). Although Nishimura et al. (18,20) originally omitted morphology from their classification of small-spored *Alternaria* pathogens, they did, inadvertently, adopt a chemotaxonomic approach to differentiating the pathogens by their production of host-specific toxins. The qualitative analysis of secondary metabolites, of which host-specific toxins are a part, has shown high value in the classification and differentiation of *Penicillium* and *Stachybotrys* spp. (5,17,37). Classification based on several different phenotypic characteristics, such as metabolite profiling and colony appearance on standardized media, also has been shown to be congruent with classification of morphologically defined small-spored *Alternaria* spp. as distinct species (3) and species-groups (2,4,6,8). However, manual scoring of metabolite profiles and description of colony color and texture for classification and identification is laborious and time-consuming and has been criticized for being inconsistent and biased due to human error.

Information under the single nondiscriminating name of *A. alternata* has continued to enter the broader literature of phytopathology, because the value of phenotypic data (e.g., chemotaxonomy) on morphologically distinguishable species (3) has not been generally recognized. The purpose of this study, therefore, was to develop and evaluate automated and unbiased (without human interpretation) image analysis systems based on the phenotype for

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use in *Alternaria* spp. classification. In this study, six small-spored *Alternaria* spp. which are at the center of the controversy were used to calibrate the Digital Image Analysis and Chemical Image Analysis systems: *A. gaisen* and *A. longipes*, which have been labeled as Japanese pear and tobacco pathotypes, respectively, of *A. alternata* by Nishimura et al. (19,20); *A. limoniasperae*, *A. tangelonis*, and *A. turkisafrica*, which have been segregated as new species (33) from the citrus pathogen complex, but regarded as “pathotypes” of *A. alternata* by Peever et al. (24); and *A. alternata* (33,34).

MATERIALS AND METHODS

Fungal cultures, growth media, and conditions. In all, 28 fungal isolates belonging to the genus *Alternaria* Nees were used. To check identity, ensure purity, and generate inoculum, the 28 isolates were inoculated on potato carrot agar (PCA) (31) and grown under standardized conditions. The unsealed plates (9 cm in diameter, plastic) were incubated in one layer for 7 days at 23°C under an alternating light-and-dark cycle consisting of 8 h of cool-white fluorescent daylight and 16 h of darkness. The lamps (TLD, 36W/950, Philips) were placed 40 cm from the plates. The identity of the isolates was checked against the species descriptions (33,34) after 7 days of growth. All but two isolates produced abundant amounts of conidia. Two *A. gaisen* isolates, EGS 39-1590 and EGS 90-0391, produced much aerial mycelium and very few conidia, respectively. The identity, sporulation pattern group, geographic origin, and substratum of all isolates are shown in Table 1. The isolates are held at the IBT culture collection at BioCentrum-DTU. After identity verification, each isolate then was inoculated onto five plates of dichloran rose bengal yeast extract sucrose agar (DRYES) (9) as three-point inoculations (three colonies on one plate). After inoculation, the unsealed DRYES plates were placed in stacks of seven, packed in perforated plastic bags, and incubated for 21 days in the dark at 15, 20, 25, 30, and 37°C.

Diameter measurements. Colony diameters were measured every seventh day until they reached the edge of plate. Colonies were measured in millimeters along the broadest diameters from the reverse side of the plate as shown by Pitt and Hocking (25). The mean of the three colonies on each plate was calculated at each temperature, and nine diameter means per isolate (three at 15°C and two at 20, 25, and 30°C, respectively) were stored as a rectangular matrix in an Excel workbook.

Digital image acquisition and feature extraction. Digital pictures were taken of the entire DRYES plate of every isolate at all temperatures every seventh day with a VideometerLab system (Videometer A/S, Hørsholm, Denmark). The cultures were digitized using a highly accurate digital camera system consisting of an integrating sphere combined with a photometrically calibrated camera system based on the 3CCD (charged coupled device) color camera. In the digitization procedure, colors were mapped into discrete arrays of pixel values representing a digital image. The result was a floating-point red-green-blue (RGB) image with a spatial resolution of 768 by 576 pixels for each DRYES plate. Each image was calibrated with respect to color and texture prior to analysis, thereby gaining a set of directly comparable images.

The obtained images contained a large amount of information (5 MB in HIPS [heritage image processing system] format) of both colonies and bare medium surface. To reduce the computational complexity, the three colonies and inoculation points were localized on each plate using an in-house written script and segmented (10,11,38). After segmentation, each colony-containing segment was divided into six separate concentric regions and features were extracted. The first features (e.g., mean value for red, green, and blue and standard deviation of red, green, and blue) were based on calibrated color measurements (histogram) and features from the same regions in the three colonies were pooled to account for natural variations on each plate. The subsequent features were based on the colony texture (e.g., degrees of folding or roughness) in the same six regions. After

TABLE 1. Identity, isolate number, substratum, origin, and sporulation pattern group of *Alternaria* isolates studied

<i>Alternaria</i> spp.	EGS number ^a	Substratum	Origin	Group ^b
<i>A. alternata</i>	34-016 ^c	<i>Arachis hypogaea</i>	India	4
<i>A. alternata</i>	34-039 ^c	<i>Datura metel</i>	India	4
<i>A. alternata</i>	35-193 ^c	<i>Musa sapientum</i>	India	4
<i>A. gaisen</i>	37-1321 ^c	<i>Pyrus pyrifolia</i>	Japan	2
<i>A. gaisen</i>	37-1332	<i>P. pyrifolia</i>	Japan	2
<i>A. gaisen</i>	39-1590	<i>P. pyrifolia</i>	Japan	2
<i>A. gaisen</i>	90-0391	<i>P. pyrifolia</i>	Japan	2
<i>A. gaisen</i>	90-0512 ^c	<i>P. pyrifolia</i>	Japan	2
<i>A. limoniasperae</i>	39-185 ^c	<i>Citrus jambhiri</i> , leaf lesion	United States	5
<i>A. limoniasperae</i>	39-187	<i>C. jambhiri</i> , leaf lesion	United States	5
<i>A. limoniasperae</i>	45-080 ^c	<i>C. jambhiri</i> , leaf lesion	United States	5
<i>A. limoniasperae</i>	45-100	<i>C. jambhiri</i> , leaf lesion	United States	5
<i>A. limoniasperae</i>	46-159	<i>C. jambhiri</i> , leaf lesion	United States	5
<i>A. longipes</i>	30-033 ^c	<i>Nicotiana tabacum</i>	United States	5
<i>A. longipes</i>	30-034	<i>N. tabacum</i>	United States	5
<i>A. longipes</i>	30-048	<i>N. tabacum</i>	United States	5
<i>A. longipes</i>	30-051	<i>N. tabacum</i>	United States	5
<i>A. longipes</i>	30-080	<i>N. tabacum</i>	United States	5
<i>A. tangelonis</i>	41-175	<i>C. paradisi</i> × <i>C. reticulata</i> , stem	Colombia	5
<i>A. tangelonis</i>	45-014 ^d	<i>C. paradisi</i> × <i>C. reticulata</i> , fruit lesion	Colombia	5
<i>A. tangelonis</i>	45-016 ^d	<i>C. paradisi</i> × <i>C. reticulata</i> , fruit lesion	Colombia	5
<i>A. tangelonis</i>	45-114 ^c	<i>C. paradisi</i> × <i>C. reticulata</i>	United States	5
<i>A. tangelonis</i>	45-121	<i>C. paradisi</i> × <i>C. reticulata</i>	United States	5
<i>A. turkisafrica</i>	44-159 ^{c,d}	<i>C. paradisi</i> × <i>C. reticulata</i>	Turkey	5
<i>A. turkisafrica</i>	44-166 ^d	<i>C. paradisi</i> × <i>C. reticulata</i>	Turkey	5
<i>A. turkisafrica</i>	45-056 ^d	<i>C. paradisi</i> × <i>C. reticulata</i> , fruit lesion	South Africa	5
<i>A. turkisafrica</i>	45-057 ^d	<i>C. paradisi</i> × <i>C. reticulata</i> , fruit lesion	South Africa	5
<i>A. turkisafrica</i>	45-058 ^d	<i>C. paradisi</i> × <i>C. reticulata</i> , fruit lesion	South Africa	5

^a Isolates from the culture collection of E. G. Simmons (Crawfordsville, IN).

^b Sporulation pattern groupings are according to Simmons and Roberts (28).

^c Isolates used by Peever et al. (24).

^d Isolates used by Peever et al. (23).

processing, the extracted features from each image were stored as a vector in an ASCII file which could be read into an Excel workbook. The method described by Hansen et al. (10,11) was used as descriptors.

Seven images (three at 15°C, two at 20°C, and one each at 25 and 30°C) of each isolate were used in the analysis. Irregular growth or production of sectors did not qualify for removal. Only one picture was removed from the analyses (*A. limoniasperae* 46-159, grown for 21 days at 15°C) due to contamination.

Combined diameter, color, and texture. The diameter data and color and texture data were pooled by pasting the non-standardized diameter matrix into the standardized matrix with colors and texture features. No other manipulations were done on the combined data matrix.

Metabolite extraction procedure. Metabolite profiling was done on the 14-day-old DRYES cultures grown at 25°C. The extraction protocol used was adapted from a micro-scale extraction method designed by Smedsgaard (36) modified for *Alternaria* metabolites. Three agar plugs (6 mm in diameter) were cut from the middle, inoculation point, and edge of each of the three colonies and placed in a 2-ml screw-top vial. The nine plugs were extracted with 1.0 ml of ethyl acetate containing 1% formic acid by sonication for 60 min. The extract was transferred to a clean 2-ml vial, evaporated to dryness in a rotary vacuum concentrator (Christ, Gefriertrocknungsanlagen GmbH, Germany), redissolved ultrasonically in 500 µl of methanol, and filtered through a 0.45-µm filter into a clean 2-ml vial prior to high-performance liquid chromatography (HPLC) and electrospray ionization mass spectrometry (ESI-MS) analyses.

HPLC-UV analysis. The HPLC analyses were performed on a HP-1100 high-performance liquid chromatograph equipped with a diode array detector using a 6-mm flow cell collecting approximately two UV spectra per second from 190 to 600 nm (bandwidth of 4 nm) (Agilent, Germany). Separations were done on a 125-by-2-mm (interior diameter [i.d.]) HP Hypersil BDS-C₁₈ (3-µm particle size) cartridge column (Agilent) with a 10-by-2-mm (i.d.) Superspher 100 RP-18 guard column (Agilent). The mobile phase consisted of a linear gradient starting at 90% water and 10% acetonitrile, and reaching 50% acetonitrile in 30 min and 100% acetonitrile in 40 min; 100% acetonitrile was maintained for 5 min. Thereafter, the gradient was returned to 10% acetonitrile in 8 min and allowed to equilibrate for 6 min before the next analysis. Both eluents contained trifluoroacetic acid at 50 µl/liter. The flow rate was 0.3 ml min⁻¹, the column temperature was 40°C, and the injection volume per sample was 3.0 µl. All solvents were HPLC grade, chemicals were analytical grade, and the water was double distilled. A standard of six commercially available *Alternaria* metabolites (altenuene, alternariol, alternariol monomethyl ether, altertoxin I, tentoxin, and tenuazonic acid; Sigma-Aldrich, St. Louis) was used as first and last sample to ensure that the gradient had been stable during the run.

Direct infusion ESI-MS analysis. Analysis by direct infusion ESI-MS was adapted from Smedsgaard and Frisvad (37). A 1-µl aliquot of extract was infused into the ESI source at a rate of 15 µl/min using 100% methanol as carrier from an Agilent 1100 microflow pump. Just prior to the ESI, source water with 2% formic acid was added at a rate of 5 µl/min from a Harvard syringe pump, giving a combined flow of 75% methanol with 0.5% formic acid at a rate of 20 µl/min. The quadrupole time-of-flight mass spectrometer (Q-tof) (Micromass, UK) was tuned to a resolution better than 8500 FWHM and calibrated on a polyethylene glycol (PEG) solution (mix of PEG 200 at 0.5 µg/ml, PEG 400 at 1.0 µg/ml, and PEG 600 at 1.5 µg/ml in 2.0 mM ammonium acetate in water:acetonitrile [1:1]) to a residual error less than 2 mDa. One scan per second was collected from *m/z* 150 to 1,000 as continuum spectra.

Chemical image analysis of HPLC data. The HPLC data were analyzed by an in-house written chemical image analysis

(CIA) program, which calculated the dissimilarity matrix directly from the raw data files. No manipulation or peak selection was made before processing of the HPLC chromatograms. However, each data file was checked to ensure a peak height of 300 milli-absorption units (mAU) or higher, that there were 20 peaks or more with a UV spectrum other than an end-absorption, and that no UV spectrum was distorted due to too high a concentration. The full three-dimensional chromatographic data files (time, wavelength, and absorbance) were processed first by a log₁₀ scaling (to account for concentration differences among extracts), then by a baseline correction, and, finally, by an alignment (to account for drift in baseline and retention time among identical metabolites in different runs) as described by Hansen (10). Each data file then was compared to the other 27 data files in the set using an algorithm described by Hansen (10), resulting in a 28-by-28 dissimilarity matrix.

CIA of MS data. Mass profiles generated from the direct infusion MS were used directly to calculate the dissimilarity matrix using the dot-product coefficient/algorithm (10). The cluster analysis was done using the Complete linkage clustering method and produced a single dendrogram. The full two-dimensional data files (*m/z* and intensity) from the MS analyses were processed first by a centroid conversion (to account for concentration differences among extracts), then by a mass correction, and, finally, by an alignment (to account for drift in *m/z* baseline among identical metabolites in different runs) as described by Hansen (10). Each data file then was compared with the other 27 data files in the set using the dot-product distance (10) resulting in a 28-by-28 dissimilarity matrix.

Cluster analyses. The two rectangular matrices with diameters and colony characteristics were analyzed separately and together using NTSYS-pc (version 2.11N; Exeter Software, NY). The diameter matrix (28 isolates by nine diameters) was subjected to analysis without standardization using the Manhattan coefficient, yielding a 28-by-28 dissimilarity matrix. The color/texture matrix (28 isolates by [seven pictures by nine features]) was standardized (MAX = divided by the maximum value of each variable) and subjected to analysis also using the Manhattan coefficient. These two matrices were pooled into one large colony appearance matrix (diameter data were kept unstandardized and color data standardized) and analyzed as above (Manhattan). These three dissimilarity matrices and the two with chemical (HPLC and MS) data were clustered in NTSYS using the Complete linkage method. All matrices also were analyzed using Euclid as dissimilarity coefficient or unweighted pair group method with arithmetic means (UPGMA) as clustering method. The Manhattan or Complete results were similar or identical to Euclid or UPGMA results and, therefore, are not shown.

HPLC-MS analysis of known metabolites. The production of individual metabolites in culture was analyzed by high-resolution HPLC-MS: 3.0 µl of extract was injected into an Agilent 1100 system coupled to an LCT time of flight mass spectrometer by an electrospray ion source with a LockMass probe (Micromass, UK). Separation was done on a 50-by-2-mm (i.d.) Luna C₁₈ column (Phenomenex) at a flow rate of 0.4 ml/min. The mobile phase consisted of a linear gradient starting at 90% water and 10% acetonitrile and reaching 100% acetonitrile in 20 min; 100% acetonitrile was maintained for 5 min. Thereafter, the gradient was returned to 10% acetonitrile in 8 min and allowed to equilibrate for 6 min before the next analysis. Both eluents contained 20 mM formic acid. The instrument was tuned to a resolution better than 6,000 FWHM and calibrated on a PEG solution (as for ESI-MS). A solution of leucineenkephaline (1 µg/ml in 50% acetonitrile with 0.1% formic acid) was used as lockmass at a flow rate of 5 µl/min. Two centroid mass measured scans were collected per second from *m/z* 150 to 900. A standard of six *Alternaria* metabolites (see HPLC-UV analysis) was used for confirmation of identity.

RESULTS

Diameter measurements. For each of the 28 *Alternaria* isolates, colony diameters on DRYES were measured every seventh day until the colonies reached the edge of the petri dish, which happened after 14 days at 20, 25, and 30°C and 21 days at 15°C.

Fig. 1. Dendrogram produced by Manhattan coefficient and Complete linkage based on nine diameter measurements from colonies on dichloran rose bengal yeast extract sucrose agar at five different temperatures.

No growth was seen for any of the 28 *Alternaria* isolates after 3 weeks at 37°C, and no growth was seen for *A. longipes* and *A. limoniasperae* after 3 weeks at 30°C.

Nine diameter values per isolate were used in the cluster analysis, which was based on raw data without any prior evaluation or standardizing. The cluster analysis (Manhattan/Complete) yielded one single dendrogram (Fig. 1). The analysis segregated five of the six *Alternaria* spp. and gave three major clusters. The first major cluster contained two distinct species clusters, one with *A. alternata* (alt) and one with *A. turkisafrica* (tur). The second major cluster also showed two distinct species clusters, one with *A. tangelonis* (tan) and one with only three *A. gaisen* isolates. The last major cluster contained *A. longipes* (lon) and *A. limoniasperae* (lim) in two separate species clusters. The analysis failed to cluster the two last *A. gaisen* isolates: gai-90-0391 was linked to the *A. turkisafrica* cluster and gai-37-1321 was linked to the *A. tangelonis* cluster.

The diameter ranges also were calculated for each species as a whole using the means from the cluster analysis. Mean diameters $\pm$ twice the standard deviation as measured after 7 days of growth at five different temperatures are shown in Table 2. It shows that *A. alternata* and *A. turkisafrica* had highest growth rates and that colony diameters of *A. gaisen* and *A. alternata* had the largest standard deviations compared with the other species.

Color and texture measurements. Images of petri dishes with the 28 *Alternaria* isolates were captured every time the colonies were measured. Nine features, numerical values representing the red, green, and blue colors and the roughness and folding of the colony, were automatically extracted from predefined areas on the colonies of each of the 196 images. No other evaluation or sorting of the raw data, except standardization, was done prior to cluster analyses.

The dendrogram resulting from the cluster analysis is shown in Figure 2. Only a single dendrogram was found. It segregated three of the six *Alternaria* spp. and gave two major clusters. In the top cluster, *A. tangelonis* and *A. turkisafrica* segregated into species cluster, whereas the *A. alternata* and *A. gaisen* isolates were split into several scattered clusters. Two *A. alternata* isolates clustered together outside the above-mentioned species clusters, while the last, alt-35-193, was linked to the *A. turkisafrica* species cluster. Four of the *A. gaisen* isolates clustered together in two neighbor clusters next to the *A. tangelonis* species cluster, while the last, gai-37-1321, was an outlier on a branch linked to this top cluster. In the bottom cluster, *A. longipes* was in a species cluster with three *A. limoniasperae* isolates. Two *A. limoniasperae* isolates formed their own cluster.

Combined diameter, color, and texture. The only dendrogram from the combined analysis is shown in Figure 3. It segregated four of the six *Alternaria* spp. and gave three distinct clusters, each containing two species. The top cluster contained the *A. tangelonis* species cluster with gai-37-1321 tied on and the four remaining *A. gaisen* isolates in their own cluster. The middle cluster contained an *A. longipes* species cluster with a cluster of two *A. limoniasperae* isolates tied on and three *A. limoniasperae* isolates in a separate cluster. The bottom cluster contained *A. alternata* and *A. turkisafrica* in two separate species clusters.

TABLE 2. Colony diameters at different temperatures after 7 days of growth on dichloran rose bengal yeast extract sucrose agar (DRYES)

Species (no. of isolates)	Mean diameter (mm) on DRYES $\pm$ 2 SD at given temperatures (°C) ^a				
	15	20	25	30	37
<i>Alternaria alternata</i> (3)	16 $\pm$ 2	30 $\pm$ 4	45 $\pm$ 7	51 $\pm$ 7	0
<i>A. gaisen</i> (5)	13 $\pm$ 4	23 $\pm$ 2	36 $\pm$ 6	38 $\pm$ 8	0
<i>A. limoniasperae</i> (5)	13 $\pm$ 1	21 $\pm$ 3	31 $\pm$ 6	0	0
<i>A. longipes</i> (5)	11 $\pm$ 2	18 $\pm$ 2	27 $\pm$ 2	0	0
<i>A. tangelonis</i> (5)	13 $\pm$ 1	20 $\pm$ 2	30 $\pm$ 3	38 $\pm$ 2	0
<i>A. turkisafrica</i> (5)	16 $\pm$ 1	26 $\pm$ 2	42 $\pm$ 3	49 $\pm$ 2	0

^a Diameter $\pm$ two times standard deviation (SD).

Metabolite profiling using HPLC-UV. The result of the cluster analysis of the chromatographic profiles based on the three-dimensional HPLC data files from the CIA program is shown in Figure 4. Each HPLC profile contained between 30 and 70 recognizable peaks representing metabolites of both known and unknown chemical structure. It produced a single dendrogram and each species was segregated into its own species cluster, except for *A. gaisen*. The analysis showed one major cluster and one minor cluster, the latter with only *A. alternata* isolates. The major cluster contained the four species clusters of *A. tangelonis*, *A. longipes*, *A. turkisafrica*, and *A. limoniasperae*. The five *A. gaisen* isolates had split into two clusters, one with two isolates linked to the *A. longipes* species cluster and another with two isolates tied to the *A. turkisafrica* species cluster. The last, gai-39-1590, was on its own branch linked to the *A. turkisafrica* species cluster.

Fig. 2. Dendrogram produced by Manhattan coefficient and Complete linkage based on 63 features of color and texture from colonies on dichloran rose bengal yeast extract sucrose agar.

Metabolite profiling using direct infusion MS. The cluster analysis of mass profiles, each containing between 100 and 400 ions, gave a single dendrogram (Fig. 5) and shows that four of the six *Alternaria* spp. were segregated. The dendrogram shows three major clusters. The top cluster contained the *A. alternata* species cluster and the two stray isolates, *lim*-46-159 and *lon*-30-048. The middle cluster contained four isolates each of the *A. longipes* and the *A. tangelonis*, while the bottom one held the *A. turkisafrica*, the *A. limoniasperae*, and the *A. gaisen* species clusters.

Species specific production of known metabolites. The production of known metabolites detected manually in the HPLC-UV or HPLC-MS chromatograms according to species is shown in Table 3. *A. alternata* was the only species which produced altenuene and *A. gaisen* was the only species in which AK toxin was detected, although it was only detected in three of five *A. gaisen* isolates (39-1590, 90-0391, and 90-0512). Tenuazonic acid

Fig. 3. Dendrogram produced by Manhattan coefficient and Complete linkage based on the combined data sets of diameter measurements and colony color and texture.

DISCUSSION

The results of color and texture analysis (Fig. 2) show that the colony does contain some features that can be used in taxonomy, but the method needs to be further developed. *A. longipes*, *A. tangelonis*, and *A. turkisafría* had consistent colony color and texture and, therefore, formed species clusters, whereas isolates within *A. alternata*, *A. gaisen*, and *A. limoniasperae* were less homogeneous. This suggests that the extraction of color and texture features from the digital photos may be too strict and that the parameters of the analysis need to be reevaluated. Growth rates, on the other hand, expressed as colony diameters at different temperatures and times (Fig. 1; Table 2) proved to have high taxonomic value. In particular, lack of growth at 30°C differentiated *A. limoniasperae* and *A. longipes* from the four other species. When the color or texture were analyzed together with diameter data (Fig. 3), species characteristics became more prominent and individual isolate traits were leveled out. Image analyses of the metabolite profiles also had high utility as a taxonomic tool, especially the direct infusion MS analysis. When using HPLC-UV (Fig. 4), all isolates, except the *A. gaisen* isolates, formed their own distinct and well-separated clusters. When direct infusion MS was used (Fig. 5), all isolates, including the *A. gaisen* isolates, formed species-specific clusters, with the exception of one *A. longipes* isolate and one *A. limoniasperae* isolate. This shows that the two different detection methods complimented each other, because some metabolites, such as the AK toxin (21), are better detected using MS. In an earlier study (3) using only HPLC-UV, tentoxin was not detected in *A. alternata* or *A. gaisen*. In this study, tentoxin was detected using HPLC-MS in *A. alternata*, *A. gaisen*, *A. tangelonis*, and *A. turkisafría* (Table 3). Furthermore, the metabolite labeled "unknown C (RI: 1425)" in Andersen et al. (3) has been identified as altersetin (12) using both HPLC-UV and HPLC-MS and has been detected in *A. gaisen*, *A. tangelonis*, and *A. turkisafría*. This study is also the first report of the production of tenuazonic acid, altertoxin I, alternariol, and alternariol monomethyl ether by *A. limoniasperae* and the production of tenuazonic acid, tentoxin, altertoxin I, alternariol, alternariol monomethyl ether, and altersetin by *A. tangelonis* and *A. turkisafría* (Table 3).

Fig. 5. Dendrogram produced by Ward clustering based on metabolite profiles from direct mass spectrometry.

TABLE 3. Qualitative production of known metabolites in pure culture detected by high-performance liquid chromatography (HPLC)-UV, HPLC-mass spectrometry, or both

Species (no. of isolates)	Metabolites on dichloran rose bengal yeast extract sucrose agar ^a							
	Alt	TeA	Ten	Alx	AOH	AME	AK	Als
<i>Alternaria alternata</i> (3)	+	–	(+) ^b	(–) ^c	+	+	–	–
<i>A. gaisen</i> (5)	–	+	(+) ^d	+	+	+	(+) ^e	(+) ^d
<i>A. limoniasperae</i> (5)	–	+	–	(+) ^f	+	+	–	(–) ^g
<i>A. longipes</i> (5)	–	+	–	+	(–) ^h	(–) ^h	–	–
<i>A. tangelonis</i> (5)	–	+	+	(+) ⁱ	+	+	–	+
<i>A. turkisafria</i> (5)	–	+	(+) ^j	+	+	+	–	(+) ^j

^a Alt = altenuene, TeA = tenuazonic acid, Ten = tentoxin, Alx = altertoxins I, AOH = alternariol, AME = alternariol monomethyl ether, AK = AK toxin, and Als = altersetin.

^b Not detected in alt-34-039.

^c Detected in alt-35-193.

^d Not detected in gai-37-1321.

^e Not detected in gai-37-1321 and gai-37-1332.

^f Not detected in lim-46-159.

^g Trace amount found in lim-45-100.

^h Trace amount found in lon-30-048 and lon-30-051.

ⁱ Not detected in tan-45-014.

^j Not detected in tur-45-057.

The dispute over morphological classification of the small-spored *Alternaria* spp. has been ongoing for at least 25 years. Several authors (1,23,24) advocate using the single nondiscriminating name of *A. alternata* for all their isolates, because they saw no resolution in their molecular phylogeny that corresponded to unique phenotypic characters (i.e., morphology) among their isolates. However, the lack of resolution or segregation in any kind of analysis does not necessarily mean that the isolates are all the same species. Rather, it indicates that the isolates are identical with respect to the particular sequence under study. Sequencing of “housekeeping genes”, such as ITS, mitochondrial small-subunit (SSU), and mitochondrial large-subunit (mtLSU), which have been used with success in other genera, have not yielded any segregation among the small-spored *Alternaria* pathogens from sporulation group 5 listed in Table 1 (15,16,24,26,27). Some sequences from functional genes, such as β -tubulin, translation elongation factor α , calmodulin, actin, chitin synthetase, and 1,3,8-trihydroxynaphthalene reductase also failed to differentiate the small-spored *Alternaria* pathogens whereas others, such as the partially sequenced endopolygalacturonase gene and two anonymous genomic regions, did provide some resolution (24). Recent research suggests that there can be incongruence among alternative phylogenies generated from different single-gene data sets. Rokas et al. (29) showed that the number of genes sufficient to support all branches in the species phylogram of genus *Saccharomyces* ranged from 8 to 20. The small-spored, host-specific toxin-producing *Alternaria* spp. have the production of some metabolites in common (e.g., alternariols) and probably are very closely related. Therefore, they must have several functional genes that control these pathways in common as well. A combination of these functional genes for phylogenetic analysis might provide a better resolution of species relationships among the small-spored *Alternaria* pathogens.

Since the publication of standardized methods (31,35), evidence in the literature on morphologically distinguishable small-spored *Alternaria* spp. (33,34) has accumulated and should not be ignored. Correct names based on scientific data communicate and predict, and referring to each of these taxa as *A. alternata* buries the important biological differences between them. The metabolite profile of *A. alternata* is different from metabolite profiles of the five pathogenic *Alternaria* spp. examined (Table 3; Figs. 4 and 5). *A. alternata* is the only species in this study to produce alternuene and, unlike in the other species, tenuazonic acid was not detected in *A. alternata*. All isolates used in this study had basically the same range in conidial size which, therefore, cannot be used as the primary taxonomic criterion for distinguishing species. The three-dimensional sporulation pattern (length of primary conidiophores, branching types and origin of branching, conidial shapes, colors, and ornamentations), however, could discriminate among the six species (33,34). Likewise, all isolates produced alternariols and had the basically the same growth rate at 15°C; therefore, these characteristics cannot be used to distinguish species. On the other hand, presence or absence of alternuene and tentoxin, and growth or no growth at 30°C, discriminate between species such as *A. alternata* and *A. longipes*. Application of combined phenotypical characters will compliment the morphological classification as long as growth conditions, extraction procedures, and chemical analyses are standardized and reference isolates are used.

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