

## Restriction mapping of the IGS region in *Alternaria* spp. reveals variable and conserved domains

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Accurate identification of *Alternaria* spp. is dependent upon the production of diagnostic morphological characters under defined cultural conditions and the proper assessment of character variation. This process is often compromised by variation in laboratory facilities and technical expertise. To assist taxon identification and phylogenetic studies, restriction site information from the intergenic spacer (IGS) region of nuclear rDNA was evaluated. Restriction maps were constructed from 15 species of *Alternaria* and *Stemphylium botryosum* (telemorph *Pleospora herbarum*) for 11 restriction enzymes using a new method for restriction mapping based on differential priming of IGS amplicons. IGS fragment size varied among species from 2.2–3.9 kb. Based upon restriction site homology among closely-related and more distantly related species, the IGS region could be divided into conserved and variable domains. The conserved domain was approximately 0.75 kb in size and was located at the 3' end of the IGS region. Restriction site homology within this region was very high, especially among closely related taxa. The remainder of the region comprised the variable domain, which encompassed considerable differences in size and restriction sites among taxa. The presence or absence of restriction sites among taxa was analyzed using methods of neighbor-joining. Phylogenetic relationships based on this method were concordant with those previously resolved based upon other methods and other genomic regions.

### INTRODUCTION

The genus *Alternaria* includes a diverse assemblage of species that occur worldwide in a variety of habitats (Rotem 1994). Estimates of the number of species range from one to several hundreds, although, specific data are difficult to assess due to the proliferation of nomenclatural synonyms of dubious taxonomic validity (Simmons 1992). Many species are plant pathogens that cause a range of diseases on a large variety of important agronomic host plants including cereals, ornamentals, oil-crops, vegetables, and fruits (Thomma 2003). In addition, several species are important post-harvest pathogens that contribute to considerable spoilage of agricultural output and contamination of food and feed through the production of mycotoxins and other biologically active compounds (Montemuro & Visconti 1992, Rotem 1994, Wilson & Wisniewski 1994). *Alternaria* species are also gaining prominence as emerging human pathogens, particularly in immunocompromised patients (Anaissie, Bodey & Rinaldi

1989, Rossmann, Cernoch & Davis 1996). Moreover, *Alternaria* spores are one of the most common and potent airborne allergens (Wilken-Jensen & Gravesen 1984, Karlsson-Borga, Jonsson & Rolfsen 1989). Because of their high impact on humans and human activities, information on the extent of intra- and interspecific variation leading to accurate and rapid identification systems would be of considerable value.

To date, classification and identification of *Alternaria* species have been based primarily on morphological characteristics of conidia and conidiation patterns. Using these criteria, species have been described and many have been placed into one of several species-groups based upon common sporulation characteristics (Simmons 1992). However, there have been debates on whether these morphological characters should be used as the sole criteria for delimiting species, especially regarding small-spored catenulate taxa (Otani & Kohmoto 1992, Rotem 1994, Kusaba & Tsuge 1995, Andersen, Krøger & Roberts 2001, 2002, Peever, Su & Carpenter-Boggs 2004). These debates are based, in part, on the presence of considerable variability in conidium size, shape, and septation within each species

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as well as within a single *Alternaria* colony (Simmons 1992). Moreover, conidium characteristics often overlap between closely related species, hindering the establishment of distinct and unambiguous species boundaries.

Recently, molecular approaches using RAPD, RFLP, karyotype, and sequence analysis have been applied in systematic studies of *Alternaria* species. RAPD fingerprinting methods have been applied to several groups of closely related species and revealed applicability for discrimination among closely related species (Weir *et al.* 1998, Morris, Connolly & St Clair 2000, Roberts, Reymond & Andersen 2000, Pryor & Michailides 2002). Although this technique has been used extensively because of its relative ease and discriminatory power, it has been criticized as poorly reproducible and containing artifactual information (Ellsworth, Rittenhouse & Honeycutt 1993, Perez, Albornoz & Dominguez 1998, Bagley, Anderson & May 2001, Herzberg, Fischer & Titze 2002). RFLP analysis of rDNA has been applied in population studies of plant pathogens by Southern hybridization or restriction digestion of amplified DNA (Adachi *et al.* 1993, Aradhya, Chan & Parfitt 2001, Pryor & Michailides 2002). However, without information on the position of restriction sites, the potential for character homoplasy must be considered when using this type of data for phylogenetic reconstruction (Upholt 1977, Slade *et al.* 1993). Karyotype analysis of *Alternaria* species revealed considerable variation in the number and size information of chromosomes even among closely related isolates, which precluded its use in robust phylogenetic studies (Akamatsu *et al.* 1999). Thus, direct sequence determination provides the most robust data from which phylogenetic reconstruction can proceed with confidence. The most contemporary phylogenetic studies have been based on ITS, mt SSU, glyceraldehyde 3-phosphate dehydrogenase (*gpd*), mt LSU,  $\beta$ -tubulin, *endo*-polygalacturonase (*endo*-PG), and anonymous genomic regions (OPA1-3 and OPA2-1) sequence analysis, and have supported the organization of *Alternaria* species into distinct species-groups (Pryor & Gilbertson 2000, de Hoog & Horre 2002, Pryor & Bigelow 2003, Peever *et al.* 2004). Composition of species-groups was relatively well supported, but relationships among species within the same species-group were often not well resolved because of insufficient genetic information contained within the loci chosen for genetic analysis.

Sequence data or restriction mapping data from IGS regions of nuclear rDNA has been used to discriminate closely related species or populations in several fungal species including *Fusarium oxysporum*, *Amylostereum areolatum*, *Metarhizium* spp., and *Pyrenophora graminea* (Appel & Gordon 1996, Pecchia, Mercatelli & Vannacci 1998, Slippers *et al.* 2002, Pantou, Mavridou & Typas 2003). Data from these studies revealed that the IGS region is highly variable. In studies of *Alternaria*, RFLP patterns of the IGS region were

examined for *A. alternata*, *A. arborescens*, *A. infectoria*, and *A. tenuissima*. Differences among isolates were generally concordant with RAPD fingerprinting data and were supportive of data derived from sequence analysis of various genetic loci (Pryor & Michailides 2002). These data suggest that the IGS region may be useful for developing informative restriction maps for *Alternaria* spp. as well, and may be useful in more robust phylogenetic analyses of relationships among closely related, taxonomically problematic groups.

The objectives of this study were to construct restriction maps of the IGS region from representative species of several *Alternaria* species-groups, and to compare restriction site profile among species. Based upon the presence and position of restriction sites, phylogenetic analysis of restriction data was performed and compared to sequence-based phylogenetic analysis in order to evaluate the potential use of such data as new genetic markers for systematic studies of *Alternaria*.

## MATERIALS AND METHODS

### *Fungal strains, DNA extraction, and IGS amplification*

We used 15 isolates representing species from four species-groups of *Alternaria*, and a species of *Stemphylium*, for restriction mapping of the IGS region (Table 1). Species identity and species-group affiliation for each isolate were based upon previous studies (Andersen *et al.* 2001, Pryor & Bigelow 2003). Culture methods, DNA extraction, and purification were conducted according to previously established protocol (Pryor & Gilbertson 2000).

The IGS region from each isolate was amplified via PCR using three combinations of four IGS-specific primers resulting in PCR products A, B, and C (Table 2, Fig. 1). PCR reaction mixture contained 5  $\mu$ l of 10X *Taq* reaction buffer (100 mM Tris-HCl (pH 8.3) and 500 mM KCl), 5  $\mu$ l of 25 mM MgCl<sub>2</sub>, 10 ng template DNA, 5 pmole of each primer, 4  $\mu$ l of 2.5 mM dNTPs, and 2 units of AmpliTaq polymerase (Applied Biosystems, Foster City, CA). Total volume was adjusted to 50  $\mu$ l with deionized water. PCR amplification was conducted using the following conditions: an initial denaturation at 94 °C for 1 min, 35 amplification cycles of 94 ° for 1 min, 57 ° for 1 min 30 s, and 72 ° for 2 min, and a final extension phase at 72 ° for 10 min. PCR products were visualized by 1.0% agarose gel electrophoresis, EtBr staining, and uv-illumination.

### *Restriction digestion and mapping*

In order to facilitate the mapping process, a preliminary screening of 22 restriction enzymes (4, 5, and 6-base cutters) was performed using PCR product A from *Alternaria alternata*, *A. radicina*, and *A. crassa*. Restriction enzymes were obtained from New England Biolab (Beverly, MA) or MBI Fermentas (Hanover, MD) and restriction digestions were conducted

**Table 1.** Isolates used in restriction mapping of the IGS region.

Species name	†Strain number	‡Species-group	*Size of IGS-PCR product (kb)
<i>A. alternata</i> (Fr.) Keissler	EGS 34-016	alternata	2.8
<i>A. tenuissima</i> (Fr.) Wiltshire	EGS 34-015	alternata	2.8
<i>A. arborescens</i> Simmons	EGS 39-128	alternata	2.9
<i>A. gaisen</i> Nagano	RGR 91.0091	alternata	2.6
<i>A. longipes</i> (Ell. & Ev.) Mason	EGS 30-033	alternata	2.9
<i>A. brassicicola</i> (Schw.) Wiltshire	EEB 2232	brassicicola	3.0
<i>A. mimicola</i> Simmons	EGS 01-056	brassicicola	2.9
<i>A. japonica</i> Yoshii	ATCC 13618	brassicicola	3.1
<i>A. carotiincultae</i> Simmons	EGS 26-010	radicina	2.8
<i>A. radicina</i> Meier, Drechsler & Eddy	ATCC 96831	radicina	3.9
<i>A. petroselini</i> (Neergaard) Simmons	EGS 09-159	radicina	2.2
<i>A. selini</i> Simmons	EGS 35-178	radicina	3.0
<i>A. crassa</i> (Sacc.) Rands	DDG acr1	porri	3.0
<i>A. taetica</i> Shome & Mustafae	EGS 44-044	porri	3.0
<i>A. dauci</i> (Kuhn) Groves & Skolko	ATCC 36613	porri	2.6
<i>Stemphyllium botryosum</i> Wallr.	ATCC 42170		2.7

† Abbreviations for strain number: ATCC, American Type Culture Collection, Manassas, VA 20108; DGG, D. G. Gilchrist, Department of Plant Pathology, University of California, Davis, CA 95616; EEB, E. E. Butler, Department of Plant Pathology, University of California, Davis, CA 95616; EGS, E. G. Simmons, Mycological Services, Crawfordsville, IN 47933; RGR, R. G. Roberts, USDA-ARS Fruit Tree Laboratories, Wenatchee, WA 98801.

‡ Species-group designations were based on the previous studies (Andersen *et al.* 2001, Pryor & Bigelow 2003).

\* PCR product by primer set 26S3111F and IGS27 (product A).

**Table 2.** Primers used in amplification of the IGS region of rDNA.

Primer	Sequence	Direction	<i>S. cerevisiae</i> numbering	Reference
26S3111F	AGGGAACGTGAGCTGGGTTTAG	Forward	LSU 2932–2953	This study
GVA#30	CTGAACGCCTCTAAGTCAGAA	Forward	LSU 3106–3126	van der Auwera <i>et al.</i> (1994)
IGS27	AATGAGCGATTCGCACTTC	Reverse	SSU 83–102	Subbarao <i>et al.</i> (1995)
CNS1	GAGACAAGCATATGACTACTG	Reverse	SSU 18–38	Appel & Gordon (1996)

according to manufacturers' recommendations. Only those enzymes that revealed 1–3 restriction sites within the IGS region were selected for further study. From the initial screening, 11 restriction enzymes were selected: *Cla*I, *Dra*I, *Eco*RI, *Hinc*II, *Hind*III, *Mbo*I, *Nhe*I, *Pvu*II, *Sma*I, *Xba*I, and *Xho*I. PCR products A, B, and C were digested with the selected enzymes and the sizes of restriction fragments were compared in 1.0% agarose gel. The sizes of restriction fragments were estimated with the aid of Labworks 4.0 software (UVP Bioimaging System, Cambridge, UK). To locate the position of restriction sites neighboring 5' or 3' ends, restriction digestion profiles were compared among products A, B, and C. If a restriction fragment obtained from digestion of PCR product B was shorter than a fragment obtained from digestion of product A or C by approximately 65 bp, the restriction fragment was determined to be positioned at the 3' end. If a restriction fragment obtained from digestion of PCR product C was shorter than the fragments obtained from digestion of product A or B by approximately 170 bp, the restriction fragment was considered to be positioned at 5' end. If there were three restriction sites in an IGS PCR product, agarose gel analysis of DNA fragments produced by double-digestion of PCR products A were used to locate the third restriction site.

### Phylogenetic analysis

Considering positive location (distance from 5' end), negative location (distance from 3' end) and neighboring restriction sites, restriction sites were aligned among different species. Based upon this alignment, the presence or absence for each restriction site was coded as 1 or 0, respectively, and a distance matrix was generated based upon the distance model for restriction site variation (Nei & Li 1979). Phylogenetic analyses of the data matrix were performed using the neighbour-joining algorithm (Saitou & Nei 1987) in PAUP phylogenetic software (Swofford 2002). For these analyses, *Stemphyllium botryosum* was used as an outgroup to root the tree. Bootstrap confidence values were calculated from 1000 randomly re-sampled data sets using the same algorithm.

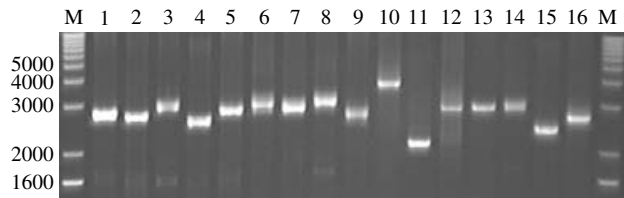
## RESULTS

### PCR amplification

The size of PCR products amplified with 26S3111F and IGS27 primer set (products A) ranged from 2.2–3.9 kb (Fig. 2, Table 1). Species of the *alternata* species-group, *brassicicola* species-group, and *porri* species-group showed relatively low size variation ranging from



**Fig. 1.** Schematic diagram of the rDNA repeat unit showing three PCR amplification schemes with four different primers to amplify the intergenic spacer (IGS) region.

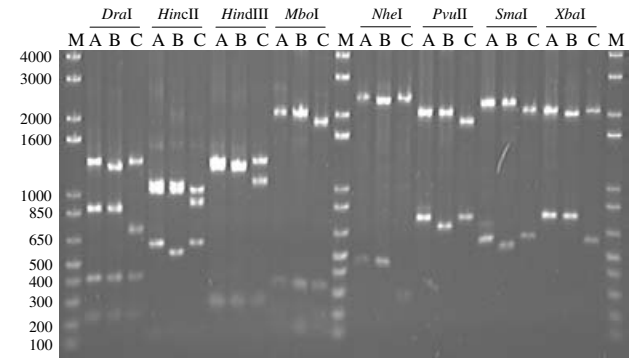


**Fig. 2.** IGS-PCR amplification products from 16 species of *Alternaria* and *Stemphylium* using primers 26S3111F and IGS27. Lane M, DNA size marker, 1-kb (+) DNA ladder (fragment size indicated on the left); lane 1, *A. alternata*; lane 2, *A. tenuissima*; lane 3, *A. arborescens*; lane 4, *A. gaisen*; lane 5, *A. longipes*; lane 6, *A. brassicicola*; lane 7, *A. mimicula*; lane 8, *A. japonica*; lane 9, *A. carotinicultae*; lane 10, *A. radicina*; lane 11, *A. petroselini*; lane 12, *A. selini*; lane 13, *A. crassa*; lane 14, *A. tagetica*; lane 15, *A. dauci*; lane 16, *S. botryosum*.

2.6–2.9 kb, 2.9–3.1 kb, and 2.6–3.0 kb, respectively. However, among species of the radicina species-group there was high level of size variation ranging from 2.2–3.9 kb. In most cases, only a single PCR product was evident. For *A. brassicicola*, a minor secondary band was also produced. Minor secondary bands were also noted for this species in PCR products B and C (data not shown).

### Restriction digestion and mapping

Restriction digestions resulted in unambiguous fragment patterns from all enzyme-PCR product combinations. Restriction maps were developed by comparing restriction fragments from the three PCR products as described above. For example, digestion of IGS PCR product from *A. alternata* with *DraI* produced four restriction fragments (Fig. 3). The largest fragment was estimated to be 1.3 kb in PCR product A and C, but slightly smaller in PCR product B. Therefore, this fragment was located at the 3' end. The next fragment was estimated to be 0.9 kb in PCR product A and B, but smaller by about 170 bp in PCR product C. Therefore, this fragment was located at the 5' end. The remaining two fragments were 0.4 kb and 0.2 kb, which reveals that there was one more *DraI* site between two specified *DraI* sites as above mentioned. To locate the site, size differences of restriction fragments among *HincII* single digestion, *HincII* and *DraI* double digestion, *DraI* single digestion, *DraI* and

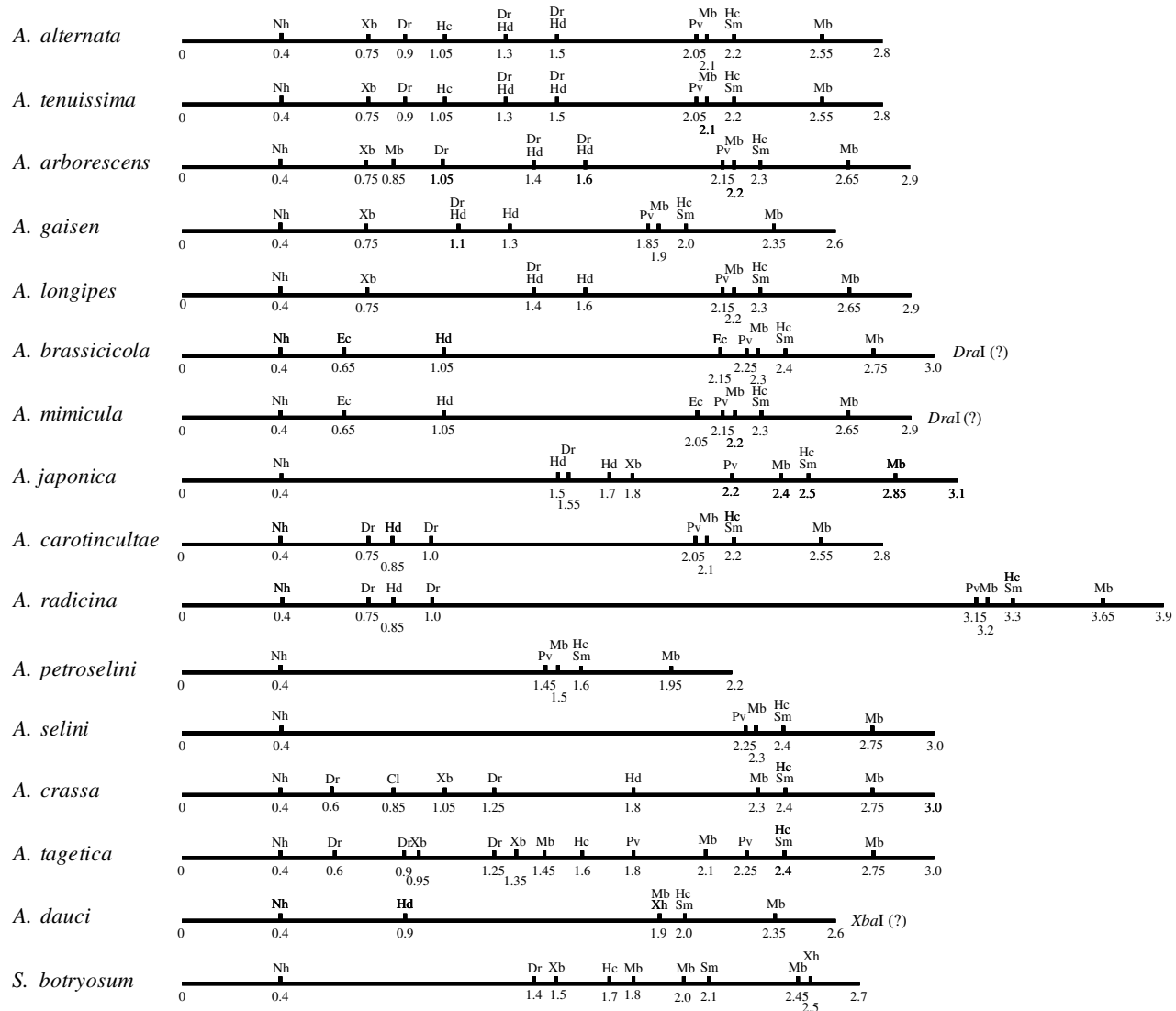


**Fig. 3.** Restriction fragments of IGS-PCR amplification products from *Alternaria alternata* using three different primer sets (lane A, 26S3111F and IGS27; lane B, 26S3111F and CNS1; lane C, GVA#30 and IGS27). Lane M, DNA size marker, 1-kb (+) DNA ladder (fragment size indicated on the left).

*HindIII* double digestion, and *HindIII* single digestion were compared (data not shown). *DraI* sites of *A. brassicicola* and *A. mimicula* and *XbaI* sites of *A. dauci* were not mapped because those enzymes produced many small DNA fragments.

Comparing restriction maps among species revealed that the IGS region is composed of a conserved domain and a highly variable domain. *SmaI* (–0.6) and *MboI* (–0.25) sites were located within the IGS region and were conserved in all the species examined (Fig. 4). *HincII* (–0.6), *MboI* (–0.7) and *PvuII* (–0.75) sites were also located within the IGS region and were conserved in most species. Therefore, the 750 bp 3' domain that includes these enzyme sites could be regarded as a relatively conserved domain. The remainder of the IGS fragment was highly variable in size and restriction sites except among very closely related species. The *NheI* (0.4) site near the 5' end of the amplified fragment was conserved in all the species examined (Fig. 4). However, based upon the position of forward primers, this site was located near the 3' end of 26S rDNA, not within the IGS region. By these results, the IGS region could be divided into two domains depending on restriction site variability: a conserved 3' domain (0.75 kb) and a variable 5' domain (1.05–2.75 kb depending on species).

Five species of the *alternata* species-group shared additional restriction sites, *XbaI* (0.75), *DraI* (–1.5 and –1.3), and *HindIII* (–1.5 and –1.3), in the variable



**Fig. 4.** Restriction maps of the IGS region from 15 *Alternaria* species and *Stemphylium botryosum* for eleven restriction enzymes. Abbr: Cl, *Cl*I; Dr, *Dra*I; Ec, *Eco*RI; Hc, *Hinc*II; Hd, *Hind*III; Mb, *Mbo*I; Nh, *Nhe*I; Pv, *Pvu*II; Sm, *Sma*I; Xb, *Xba*I; Xh, *Xho*I.

domain (Fig. 4). Size differences among whole IGS PCR products originated from the size differences between *Xba*I (0.75) and *Dra*I/*Hind*III (−1.5) sites. In addition to the size variation, restriction site variation was also observed in this region. *A. alternata* and *A. tenuissima* had identical restriction maps. *A. gaisen* and *A. longipes* had very similar restriction maps. The only difference was the size variation between *Xba*I (0.6) and *Dra*I/*Hind*III (−1.5) sites. *A. brassicicola* and *A. mimicula* of the brassicicola species-group shared the same restriction maps except for minor size difference between *Hind*III (1.05) and *Eco*RI (−0.85). However, *A. japonica*, placed in the same species-group in previous works, did not share restriction enzyme sites in the variable domain nor the *Pvu*II (−0.75) site in the conserved domain. Four species of the radicina species-group showed peculiar restriction patterns. They shared all of the restriction sites in the conserved domain, but restriction pattern in the variable domain was

completely different between two sub-groups, the *A. carotiincultae*/*A. radicina* subgroup and the *A. petroselini*/*A. selini* subgroup. *A. carotiincultae* and *A. radicina* had the same restriction sites except 1.1 kb size difference between *Dra*I (1.0) and *Pvu*II (−0.75) sites. The same phenomenon was observed between *A. selini* and *A. petroselini*. They also shared the same restriction sites except the 0.8 kb size difference between *Nhe*I (0.4) and *Pvu*II (−0.75) sites. Three species of porri species-group showed high variation of restriction sites in the variable domain including the *Mbo*I (−0.7) and *Pvu*II (−0.75) sites, which were conserved within other species-groups.

#### Phylogenetic analysis

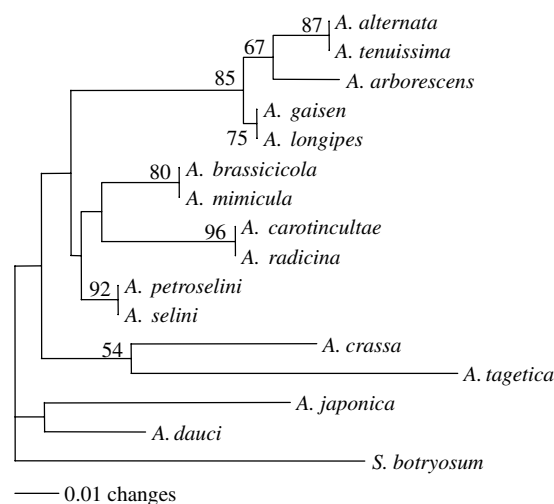
Since restriction sites near the 3' end of the conserved domain were shared by most of the species and there was high size variation within the variable domain,

restriction sites that were mapped at the same distance from either the 5' or 3' ends were considered homologous. For example, *Sma*I sites of *A. alternata* (2.2 or -0.6) and *A. petroselini* (1.6 or -0.6) were regarded as homologous because they were mapped at the same distance from the 3' end. Most of the shared sites in the variable domain were among species of the same species-group. Three restriction sites, *Dra*I (0.90), *Dra*I (-1.3), and *Mbo*I (-0.90), were shared between species of different species-groups. These sites were thought to be homoplasious because neighbouring restriction sites were not shared between them. However, they were treated as homologous sites because there was no direct sequence evidence to confirm homoplasy. After aligning restriction sites using these principles, presence or absence was coded as 1 or 0 (Table 3).

In a phylogenetic tree reconstructed by the neighbour-joining method (Fig. 5), five species of the *alternata* species-group comprised a monophyletic group supported by high bootstrap value. Among them, *A. alternata*, *A. tenuissima*, and *A. arborescens* comprised a monophyletic group leaving the other two species, *A. gaisen* and *A. longipes*, as sister taxa. *A. brassicicola* and *A. mimicula* comprised a monophyletic group. *A. carotiincultae* and *A. radicina* comprised another monophyletic group. Although these two monophyletic groups were grouped as sister clades, bootstrap support for this relationship was very low. Two other species that shared all restriction sites, *A. petroselini* and *A. selini*, comprised another monophyletic group. Relationships among other species except those mentioned above were not clear. Although *A. crassa* and *A. tagetica* of the porri species-group clustered together, and *A. japonica* of the brassicicola species-group and *A. dauci* of the porri species-group clustered together, bootstrap support for these groupings were very low.

## DISCUSSION

This study examined restriction site distribution within the IGS region for a number of *Alternaria* species and *Stemphylium botryosum* (teleomorph *Pleospora herbarum*). These data were then used to evaluate the utility of restriction maps in assessing relationships among taxa by comparison with relationships established from previous sequence analysis. From restriction maps of the IGS region of *Alternaria* species, it was found that sizes of the IGS region are highly variable even between closely related species of the same species group. For example, the IGS regions of *A. radicina* and *A. carotiincultae* had the same restriction maps yet showed 1.1 kb size difference. Previous studies have shown that *A. radicina* and *A. carotiincultae* are very closely related based upon ITS, mt SSU, and *gpd* sequences, and had one base difference in ITS sequences (de Hoog & Horre 2002, Pryor & Bigelow 2003). Additional studies have proposed that these two species are conspecific (Pryor & Gilbertson 2002). Size differences between species



**Fig. 5.** Phylogenetic tree reconstructed by the neighbour-joining method based on the distance values calculated by the distance model of Nei & Li (1979). Bootstrap confidence measures greater than 50% from 1000 bootstrap replicates are indicated.

with the same restriction maps were also found with *A. selini* and *A. petroselini*, *A. brassicicola* and *A. mimicula*, and *A. gaisen* and *A. longipes*. Regarding *A. selini* and *A. petroselini*, and *A. brassicicola* and *A. mimicula*, analyses have also shown that these pairs have identical ITS sequences and are very closely related (Pryor & Bigelow 2003, unpubl.). Intraspecific length variation of the IGS region has been reported in many fungi including *Cochliobolus heterostrophus* (Garber *et al.* 1988), *Coprinus cinereus* (Wu, Cassidy & Pukkila 1983), *Fusarium oxysporum* (Appel & Gordon 1996), and *Pyrenophora graminea* (Pecchia *et al.* 1998), where the length variation was attributed to insertions or deletions in the array of sub-repeats within the IGS region. Thus, it is proposed that the size differences of the IGS region among closely related *Alternaria* species do not contradict close relationships inferred from shared IGS restriction sites or sequence analyses of rDNA and single-copy protein gene. The minor band observed in *A. brassicicola*, which was not present in *A. mimicula*, was observed from all primer sets used in this study. At this point, the origin of this band is uncertain and further work will be needed to clarify the relationship of this band with the major band.

When insertion or deletion occurs frequently, relationships among species can be misinterpreted by RFLP analysis. For example, *A. gaisen* and *A. longipes*, which have identical restriction maps except 0.3 kb size variation between *Xba*I (0.75) and *Dra*I/*Hind*III (-1.5), shared only 20 among 36 total restriction fragments. Deviation between the RFLP results and restriction site map can occur whenever there is size variation caused by an insertion or deletion events. As revealed in this study, the IGS regions of *Alternaria* species are highly variable in size and it is recommended to use restriction site map data instead of RFLP data when resolving phylogenetically informative characters. In most cases,

**Table 3.** Coding of restriction sites.

Enzyme Location	<i>NheI</i> 0.40	<i>DraI</i> 0.60	<i>EcoRI</i> 0.65	<i>DraI</i> 0.75	<i>XbaI</i> 0.75	<i>Clal</i> 0.85	<i>HindIII</i> 0.85	<i>MboI</i> 0.85	<i>DraI</i> 0.90	<i>HindIII</i> 0.90	<i>XbaI</i> 0.95	<i>DraI</i> 1.00	<i>DraI</i> 1.05	<i>HincII</i> 1.05	<i>HindIII</i> 1.05	<i>XbaI</i> 1.05	<i>DraI</i> 1.25	<i>XbaI</i> -1.65	<i>HindIII</i> -1.60	<i>DraI</i> -1.55	<i>MboI</i> -1.55	
<i>A. alternata</i>	1	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0
<i>A. tenuissima</i>	1	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0
<i>A. arborescens</i>	1	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0
<i>A. gaisen</i>	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>A. longipes</i>	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>A. brassicicola</i>	1	ND	1	ND	0	0	0	0	ND	0	0	ND	ND	0	1	0	ND	0	0	ND	0	0
<i>A. mimicula</i>	1	ND	1	ND	0	0	0	0	ND	0	0	ND	ND	0	1	0	ND	0	0	ND	0	0
<i>A. japonica</i>	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
<i>A. carotincultae</i>	1	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
<i>A. radicina</i>	1	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
<i>A. petroselini</i>	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>A. selini</i>	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>A. crassa</i>	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0
<i>A. tagetica</i>	1	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	1	0	0	0	1
<i>A. dauci</i>	1	0	0	0	ND	0	0	0	0	1	ND	0	0	0	0	0	ND	0	ND	0	0	0
<i>S. botryosum</i>	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Enzyme Location	<i>DraI</i> -1.50	<i>HindIII</i> -1.50	<i>HincII</i> -1.40	<i>HindIII</i> -1.40	<i>DraI</i> -1.30	<i>HindIII</i> -1.30	<i>XbaI</i> -1.30	<i>HindIII</i> -1.20	<i>PvuII</i> -1.20	<i>XbaI</i> -1.20	<i>HincII</i> -1.00	<i>MboI</i> -0.90	<i>PvuII</i> -0.90	<i>EcoRI</i> -0.85	<i>PvuII</i> -0.75	<i>MboI</i> -0.70	<i>XhoI</i> -0.70	<i>HincII</i> -0.60	<i>SmaI</i> -0.60	<i>MboI</i> -0.25	<i>XhoI</i> -0.20	
<i>A. alternata</i>	1	1	0	0	1	1	0	0	0	0	0	0	0	0	1	1	0	1	1	1	0	0
<i>A. tenuissima</i>	1	1	0	0	1	1	0	0	0	0	0	0	0	0	1	1	0	1	1	1	1	0
<i>A. arborescens</i>	1	1	0	0	1	1	0	0	0	0	0	0	0	0	1	1	0	1	1	1	1	0
<i>A. gaisen</i>	1	1	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	1	1	1	1	0
<i>A. longipes</i>	1	1	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	1	1	1	1	0
<i>A. brassicicola</i>	ND	0	0	0	ND	0	0	0	0	0	0	0	0	1	1	1	0	1	1	1	1	0
<i>A. mimicula</i>	ND	0	0	0	ND	0	0	0	0	0	0	0	0	1	1	1	0	1	1	1	1	0
<i>A. japonica</i>	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	1	0	1	1	1	1	0
<i>A. carotincultae</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	1	1	0
<i>A. radicina</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	1	1	0
<i>A. petroselini</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	1	1	0
<i>A. selini</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	1	1	0
<i>A. crassa</i>	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1	1	1	1	0
<i>A. tagetica</i>	0	0	1	0	0	0	0	0	1	0	0	1	0	0	1	0	0	1	1	1	1	0
<i>A. dauci</i>	0	0	0	0	0	0	ND	0	0	ND	0	0	0	0	0	1	1	1	1	1	1	0
<i>S. botryosum</i>	0	0	0	0	1	0	0	0	0	1	1	1	0	0	0	1	0	0	1	1	1	1

it takes considerable time and effort to obtain restriction site maps by the standard double-digestion method. However, by using the new method employed in this study, where three different PCR products were digested by the same restriction enzymes and compared in the same gels, restriction mapping is much more straightforward. In addition, the direction of the restriction map will be determined together with the location of restriction sites.

Restriction sites in the variable domain were unique to closely related species except for three sites, *DraI* (0.90), *DraI* (−1.3), and *MboI* (−0.90). However, homology among these sites is doubtful because neighbouring restriction sites were not conserved. Two species of the brassicicola species-group, *A. brassicicola* and *A. japonica*, which have shown moderate nucleotide difference (98 % similarity) in ITS sequences (Pryor & Gilbertson 2000), did not share restriction sites in the variable domain. Three species of the porri species-group, *A. crassa*, *A. dauci*, and *A. tagetica*, also did not share restriction sites in the variable domain. Similarity values of the ITS region among these three species varies from 98.4–99.1% (Pryor & Gilbertson 2000, Chou & Wu 2002). *A. selini* and *A. radicina*, representatives of two subgroups of the radicina species-group, also did not share restriction sites in the variable domain, and ITS sequence similarity between these species was 99.1% (Pryor & Gilbertson 2000). In contrast, restriction sites in the variable domain were shared only among species for which corresponding ITS sequences were identical or varied by only one base pair. This suggests that sequence data from the variable domain, which would likely expose considerably more variable sites than revealed by restriction mapping, may provide critical information for discriminating among very closely related species. This may be particularly useful for resolving relationships among members of the alternata species-group including *A. alternata*, *A. arborescens*, *A. gaisen*, *A. longipes*, and *A. tenuissima*, which vary by only one nucleotide difference in the ITS region (Wang & Zhang 2001, Pryor & Bigelow 2003).

In contrast to the variable domain, the 3' end was well conserved among all of the species tested, with minor variations in *PvuII* (−0.75), *MboI* (−0.70), *XhoI* (−0.70), *HincII* (−0.60), and *XhoI* (−0.20). From the level of restriction site variation, it is expected that sequence data from the IGS conserved domain will provide informative molecular markers for higher-order phylogenetic study of *Alternaria* species and related taxa.

This study revealed that the IGS region of *Alternaria* is highly variable in size and restriction sites among species, and should provide useful information for discriminating among species having varying degrees of relatedness. The restriction site mapping method introduced here provides specific restriction site information that can be easily obtained in a straightforward manner. These data are superior to the more commonly

generated restriction fragment polymorphisms, which would clearly be problematic in cases of size-variable regions such as the IGS. Based on the high variability of restriction sites in the 5' region and relatively low variability in the 3' region, it is expected that sequence data of the 5' region and the 3' region will provide useful information to discriminate closely related species and for phylogenetic studies of more distantly related *Alternaria* species, respectively. Additional studies are needed to determine the degree of intra-specific variation in IGS size and/or restriction sites within *Alternaria* species, which will further elucidate the utility of these data in phylogenetic analyses and may provide additional information useful for studies at the population level.

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## REFERENCES

- Adachi, Y., Watanabe, H., Tanabe, K., Doke, N., Nishimura, S. & Tsuge, T. (1993) Nuclear ribosomal DNA as a probe for genetic variability in the Japanese pear pathotype of *Alternaria alternata*. *Applied and Environmental Microbiology* **59**: 3197–3205.
- Akamatsu, H., Taga, M., Kodama, M., Johnson, R., Otani, H. & Kohmoto, K. (1999) Molecular karyotypes for *Alternaria* plant pathogens known to produce host-specific toxins. *Current Genetics* **35**: 647–656.
- Anaissie, E. J., Bodey, G. P. & Rinaldi, M. G. (1989) Emerging fungal pathogen. *European Journal of Clinical Microbiology and Infectious Diseases* **8**: 323–330.
- Andersen, B., Krøger, E. & Roberts, G. (2001) Chemical and morphological segregation of *Alternaria alternata*, *A. gaisen* and *A. longipes*. *Mycological Research* **105**: 291–299.
- Andersen, B., Krøger, E. & Roberts, G. (2002) Chemical and morphological segregation of *Alternaria arborescens*, *A. infectoria* and *A. tenuissima* species-groups. *Mycological Research* **106**: 170–182.
- Appel, D. J. & Gordon, T. R. (1996) Relationships among pathogenic and nonpathogenic isolates of *Fusarium oxysporum* based on the partial sequence of the intergenic spacer region of the ribosomal DNA. *Molecular Plant-Microbe Interactions* **9**: 125–138.
- Aradhya, M. K., Chan, H. M. & Parfitt, D. E. (2001) Genetic variability in the pistachio late blight fungus, *Alternaria alternata*. *Mycological Research* **105**: 300–306.
- Bagley, M. J., Anderson, S. L. & May, B. (2001) Choice of methodology for assessing genetic impacts of environmental stressors: polymorphism and reproducibility of RAPD and AFLP fingerprints. *Ecotoxicology* **10**: 239–244.
- Chou, H. H. & Wu, W. S. (2002) Phylogenetic analysis of internal transcribed spacer regions of the genus *Alternaria*, and the significance of filament-beaked conidia. *Mycological Research* **106**: 164–169.
- de Hoog, G. S. & Horre, R. (2002) Molecular taxonomy of the *Alternaria* and *Ulocladium* species from humans and their identification in the routine laboratory. *Mycoses* **45**: 259–276.
- Ellsworth, D. L., Rittenhouse, K. D. & Honeycutt, R. L. (1993) Artfactual variation in randomly amplified polymorphic DNA banding patterns. *Biotechniques* **2**: 214–217.



- Garber, R. C., Turgeon, B. G., Selker, E. U. & Yoder, O. C. (1988) Organization of ribosomal RNA genes in the fungus *Cochliobolus heterostrophus*. *Current Genetics* **14**: 573–582.
- Herzberg, M., Fischer, R. & Titze, A. (2002) Conflicting results obtained by RAPD-PCR and large-subunit rDNA sequences in determining and comparing yeast strains isolated from flowers: a comparison of two methods. *International Journal of Systematic and Evolutionary Microbiology* **52**: 1423–1433.
- Karlsson-Borga, A., Jonsson, P. & Rolfsen, W. (1989) Specific IgE antibodies to 16 widespread mold genera in patients with suspected mold allergy. *Annals of Allergy, Asthma & Immunology* **63**: 521–526.
- Kusaba, M. & Tsuge, T. (1995) Phylogeny of *Alternaria* fungi known to produce host-specific toxins on the basis of variation in internal transcribed spacers of ribosomal DNA. *Current Genetics* **28**: 491–498.
- Montemurro, N. & Visconti, A. (1992) *Alternaria* metabolites: chemical and biological data. In *Alternaria: biology, plant diseases and metabolites* (J. Chelkowski & A. Visconti, eds): 449–557. Elsevier Science Publishers, Amsterdam.
- Morris, P. F., Connolly, M. S. & St Clair, A. (2000) Genetic diversity of *Alternaria alternata* isolated from tomato in California using RAPDs. *Mycological Research* **104**: 286–292.
- Nei, M. & Li, W. H. (1979) Mathematical model for studying genetic variation in terms of restriction endonuclease. *Proceedings of the National Academy of Sciences, USA* **76**: 5269–5273.
- Otani, H. & Kohmoto, K. (1992) Host-specific toxins of *Alternaria* species. In *Alternaria: biology, plant diseases and metabolites* (J. Chelkowski & A. Visconti, eds): 123–156. Elsevier Science Publishers, Amsterdam.
- Pantou, M. P., Mavridou, A. & Typas, M. A. (2003) IGS sequence variation, group-I introns and the complete nuclear ribosomal DNA of the entomopathogenic fungus *Metarhizium*: excellent tools for isolate detection and phylogenetic analysis. *Fungal Genetics and Biology* **38**: 159–174.
- Pecchia, S., Mercatelli, E. & Vannacci, G. (1998) PCR amplification and characterization of the intergenic spacer region of the ribosomal DNA in *Pyrenophora graminea*. *FEMS Microbiology Letters* **166**: 21–27.
- Peever, T. L., Su, G. & Carpenter-Boggs, L. (2004) Molecular systematics of citrus-associated *Alternaria* species. *Mycologia* **96**: 119–134.
- Perez, T., Albornoz, J. & Dominguez, A. (1998) An evaluation of RAPD fragment reproducibility and nature. *Molecular Ecology* **7**: 1347–1357.
- Pryor, B. M. & Bigelow, D. M. (2003) Molecular characterization of *Embellisia* and *Nimbya* species and their relationship to *Alternaria*, *Ulocladium*, and *Stemphylium*. *Mycologia* **95**: 1139–1152.
- Pryor, B. M. & Gilbertson, R. L. (2000) Molecular phylogenetic relationships amongst *Alternaria* species and related fungi based upon analysis of nuclear ITS and mt SSU rDNA sequences. *Mycological Research* **104**: 1312–1321.
- Pryor, B. M. & Gilbertson, R. L. (2002) Relationships and taxonomic status of *Alternaria radicina*, *A. carotiinculatae*, and *A. petroselini* based upon morphological, biochemical, and molecular characteristics. *Mycologia* **94**: 49–61.
- Pryor, B. M. & Michailides, T. J. (2002) Morphological, pathogenic, and molecular characterization of *Alternaria* isolates associated with *Alternaria* late blight of pistachio. *Phytopathology* **92**: 406–416.
- Roberts, R. G., Reymond, S. T. & Andersen, B. (2000) RAPD fragment pattern analysis and morphological segregation of small-spored *Alternaria* species and species groups. *Mycological Research* **104**: 151–160.
- Rossmann, S. N., Cernoch, P. L. & Davis, J. R. (1996) Dematiaceous fungi are an increasing cause of human disease. *Clinical Infectious Diseases* **22**: 73–80.
- Rotem, J. (1994) *The Genus Alternaria*. American Phytopathological Society Press, St Paul, MN.
- Saitou, N. & Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**: 406–425.
- Simmons, E. G. (1992) *Alternaria* taxonomy: current status, viewpoint, challenge. In *Alternaria: biology, plant diseases and metabolites* (J. Chelkowski & A. Visconti, eds): 1–35. Elsevier Science Publishers, Amsterdam.
- Slade, R. W., Moritz, C., Heideman, A. & Hale, P. T. (1993) Rapid assessment of single-copy nuclear DNA variation in diverse species. *Molecular Ecology* **2**: 359–373.
- Slippers, B., Wingfield, D., Coutinho, T. A. & Wingfield, M. J. (2002) DNA sequence and RFLP data reflect geographical spread and relationships of *Amylostereum areolatum* and its insect vectors. *Molecular Ecology* **11**: 1845–1854.
- Subbarao, K. V., Chassot, A., Gordon, T. R., Hubbard, J. C., Bonello, P., Mullin, R., Okamoto, D., Davis, R. M. & Koike, S. T. (1995) Genetic relationships and cross pathogenicities of *Verticillium dahliae* isolates from cauliflower and other crops. *Phytopathology* **85**: 1105–1112.
- Swofford, D. L. (2002) *PAUP\*: phylogenetic analysis using parsimony (\*and other methods)*. Version 4. Sinauer Associates, Sunderland, MA.
- Thomma, B. P. H. J. (2003) *Alternaria* spp.: from general saprophyte to specific parasite. *Molecular Plant Pathology* **4**: 225–236.
- Upholt, W. B. (1977) Estimation of DNA sequence divergence from comparison of restriction endonuclease digests. *Nucleic Acids Research* **4**: 1257–1265.
- van der Auwera, G., Chapelle, S. & De Wachter, R. (1994) Structure of the large ribosomal subunit RNA of *Phytophthora megasperma*, and phylogeny of the oomycetes. *FEBS Letters* **338**: 133–136.
- Wang, H. K. & Zhang, T. Y. (2001) Application of sequencing of 5.8S rDNA, ITS1 and ITS2 on identification and classification of *Alternaria* at species level. *Junwu Xitong* **20**: 168–173.
- Weir, T. L., Huff, D. R., Christ, B. J. & Romaine, C. P. (1998) RAPD-PCR analysis of genetic variation among isolates of *Alternaria solani* and *Alternaria alternata* from potato and tomato. *Mycologia* **90**: 813–821.
- Wilken-Jensen, K. & Gravesen, S. (1984) *Atlas of Moulds in Europe Causing Respiratory Allergy*. Foundation for Allergy Research in Europe, ASK Publishing, Copenhagen, Denmark.
- Wilson, C. L. & Wisniewski, M. E. (1994) *Biological Control of Post-harvest Diseases: theory and practice*. CRC Press, Boca Raton, FL.
- Wu, M. M., Cassidy, J. C. & Pukkila, P. J. (1983) Polymorphisms in DNA of *Coprinus cinereus*. *Current Genetics* **7**: 385–392.