Genetic variation of *Alternaria alternata,* an endophytic fungus isolated from *Pinus tabulaeformis* as determined by random amplified microsatellites (RAMS)

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Genetic variation of 112 isolates of *Alternaria alternata,* an endophytic fungus isolated from *Pinus tabulaeformis,* was carried out using two RAMS primers (CCA and CGA). All 1273 bands produced were clear and reproducible and ranged from 200 to 2000 base pairs. Analysis of the two primers revealed a high level of genetic diversity (*H'* = 2.79) among the 112 isolates tested. A total of 20 markers were scored. Two markers were present in all isolates, while the other 18 (90%) markers occurred in combinations of 105 (93.8%) different genotypes among the 112 isolates. Genetic similarity coefficients between pairwise isolates varied from 0.3 to 1 based on an unweighted paired group method of arithmetic average (UPGMA) cluster analysis. There was no relationship between fungal genotypes and host tissue ages, and the endophytic fungus *A. alternata* appears to have the potential to evolve relatively quickly and maintain significant genetic variation.

Key words: endophyte, genotype, pine, RAMS.

Introduction

   Alternaria alternata (Fr.) Keissler is common saprobe found on many plants and other substrata worldwide, including pine needles (Lu *et al*., 2000; Grunden *et al*., 2001; Tokumasu and Aoiki, 2002). This species is also an opportunistic pathogen affecting many cultivated plants in the field and during post harvest storage of fruit and vegetables. The taxon is the principal causative agent of black mould of ripe tomatoes (Pearson and Hall, 1975; Davis *et al*., 1997), brown spot on citrus (Kohmoto *et al*., 1979), brown necrotic lesions on foliage and black pit disease of potatoes (Droby *et al*., 1984), and late blight on California pistachios (Aradhya *et al*., 2001).

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Alternaria alternata has often been isolated as an endophyte in previous endophyte studies (Petrini and Fisher, 1988; Sieber, 1989; Collado *et al.*, 1999; Taylor *et al.*, 1999; Kumaresan and Suryanarayanan, 2001, 2002; Romero *et al.*, 2001). In this survey of endophytic fungi from *Pinus tabulaeformis* in the Dongling Mountain range, Beijing Forest Ecosystem Research Station of the Chinese Academy of Sciences, *A. alternata* was isolated as the most dominant species. The genetic variation of saprobic and pathogenic *A. alternata* isolates has previously been assessed based on the analyses of RAPD, RFLPs, DNA hybridization, AFLP, and DNA sequences (Tanabe *et al.*, 1990; Adachi *et al.*, 1993; Morris *et al.*, 2000; Aradhya *et al.*, 2001; Peever *et al.*, 2002). Endophytic isolates of *A. alternata*, however, have not been investigated using random amplified microsatellite (RAMS) markers.

The RAMS technique, which was originally used to measure genetic diversity of plants and animals (Zietkiewicz *et al.*, 1994), has also been applied in studies of fungi (Hantula *et al.*, 1996, 1998; Hantula and Müller, 1997; Zhou *et al.*, 1999, 2001), and particularly endophytic fungi. For examples, Burgess *et al.* (2001) clearly distinguished between morphotypes of *Sphaeropsis sapinea*, a fungal endophyte of *Pinus* spp., using RAMS markers. Grünig *et al.* (2001) have also differentiated between the dark septate endophytic fungi *Phialocephala fortinii* and type I of a non-sporulating mycelium, which had the same allozyme phenotype, based on RAMS analysis.

The aim of the present study is to investigate the usefulness of RAMS for assessing intraspecific genetic variation and to address the questions of whether or not there are significant genetic differences within the endophyte *Alternaria alternata* population isolated from *Pinus tabulaeformis* in one location, and there is relationship between fungal genotypes and host tissue ages. This information will be used as the basis for further study of population genetic structure.

Materials and methods

Site and sampling procedure

Ten individual mature *Pinus tabulaeformis* pines were randomly chosen for this study at Dongling Mountain mixed woodland, Beijing Forest Ecosystem Research Station of the Chinese Academy of Sciences, 117 km west of Beijing (39˚58'N, 115˚26'E). The pines were about 30 years old and located at an average altitude of 1211 metres above sea level. The mean annual temperature is 2-7 C and the mean annual precipitation is about 500 mm. Three branches, each containing three age-classes (1-, 2-, and 3-year-old branches),

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were randomly collected from each plant in February 2001. These branches were immediately placed in plastic bags, labelled, and taken to the laboratory. Samples were stored at 4 C and processed within 3 days of collection.

Isolation and identification of endophytic fungi

Each selected branch was divided into three parts based on age class, i.e. 1-, 2-, and 3-year-old branches with needles. Needles were removed from each age-class branches, and the branches were cut into segments (5 mm long). Three branch segments and three needles were randomly selected from each age class. The needles were also cut into segments (5 mm long), and the branch segments were separated into bark and xylem. Three needle, three bark, and three xylem segments were randomly selected from each age class. Surface sterilization was performed using the method of Guo *et al*. (2000). After surface sterilization, sets of four segments were then evenly placed in each 90 mm Petri-dish containing malt extract agar (MEA, 2%) supplemented with Rose Bengal (30 mg l^{-1}) to slow fungal growth. Streptomycin sulphate (50 mg l^{-1}, North China Medicine Inc., Shijiazhuang, China) was added to suppress bacterial growth. Petri dishes were sealed, incubated for 2 months at 25 C, and examined periodically. When colonies developed, they were transferred to new Petri-dishes.

Subcultures were then incubated on potato carrot agar (PCA, 2%) and examined periodically. *Alternaria alternata* were identified based on morphological characters according to descriptions of Simmons (1967, 1990, 1995, 1999) and Ellis (1971). Concurrently, three isolates of *A. alternata* were randomly selected, and the ITS (ITS1, 5.8S, ITS2) region was sequenced in the present study. The three ITS sequences were identical and one sequence was used as query sequence to search for similar sequences from GenBank using BLAST program. The result indicated that this sequence had highly similarity with reference ITS sequences of *A. alternata* in GenBank, particularly identical with some reference ITS sequences, e.g. accession nos. AF455537, AF455441, AF397233, AF397242, and AY160211. A total of 112 *A. alternata* isolates were obtained from three age-class branches of ten plants investigated in this study (Table 1).

DNA extraction

Single-spore cultures of *A. alternata* were incubated on MEA in the dark at 25 C for 3-7 days. Genomic DNA was extracted using the modified CTAB protocol (Guo *et al*., 2000). About 50mg fresh fungal mycelia scraped

from

Table 1. *Alternaria alternata* strains isolated from *Pinus tabulaeformis* used in this study.

Host number	1-year old	2-year old	3-year old
1	1, 2, 3, 4	5, 6, 7, 8	9, 10, 11
2	12, 13, 14, 15	16, 17, 18, 19	20, 21, 22
3	23, 24, 25, 26	27, 28, 29, 30	31, 32, 33
4	34, 35, 36, 37	38, 39, 40, 41	42, 43, 44
5	45, 46, 47, 48	49, 50, 51, 52	53, 54, 55
6	56, 57, 58, 59	60, 61, 62, 63, 64	65, 66, 67
7	68, 69, 70, 71	72, 73, 74, 75,	76, 77, 78
8	79, 80, 81, 82	83, 84, 85, 86,	87, 88, 89
9	90, 91, 92, 93	94, 95, 96, 97, 98	99, 100, 101
10	102, 103, 104, 105, 106	107, 108, 109, 110	111, 112

the surface of the agar plate was transferred into a 1.5ml Eppendorf microcentrifuge tube with 700μl of preheated (60 C) 2 × CTAB extraction buffer (2% (w/v) CTAB, 100mM Tris-HCl, 1.4M NaCl, 20mM EDTA, pH 8.0) and *ca.* 0.2g sterilized sand (white quartz, Sigma). Fungal mycelia were ground using a glass pestle for 5-10 minutes and then incubated in a 60 C water bath for 30 minutes with occasional gentle swirling. Seven hundred microlitres of phenol:chloroform:isoamyl alcohol (25:24:1) was added into each tube and mixed thoroughly to form an emulsion. The mixture was spun at 13,000 g for 15 minutes at room temperature in a microcentrifuge, and the upper aqueous phase was removed into a new 1.5 ml tube. The aqueous phase containing DNA was re-extracted with chloroform:isoamyl alcohol (24:1) until no interface was visible. Fifty microlitres of 5M KOAc was added into the aqueous phase followed by 400 μl of isopropanol and inverted gently to mix. The genomic DNA was precipitated at 9,000 g for 2 minutes in a microcentrifuge. The DNA pellet was washed with 70% ethanol twice and air-dried. The DNA pellet was then resuspended in 100 μl TE buffer (10mM Tris-HCl, 1mM EDTA). The final concentration of total DNA was *ca.* 150 ng μl^{-1}.

RAMS assay

Two 5'-anchored RAMS-primers were used as described by Hantula *et al*. (1996) (Table 2). The DNA fragments were amplified in an automated thermal cycler (PTC-100™, MJ Research, Inc., Watertown, MA, USA). Amplification was performed in a 25 μl reaction volume which contained PCR buffer (10 mM KCl, 10mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, pH 8.8, 0.1% Triton X-100), 1.5 mM $MgCl_2$, 200 μM of each deoxyribonucleotide triphosphate, 2 μM of primer, *ca.* 30 ng template DNA, and 1.25 units of *Taq* DNA

Table 2. Primer sequence and annealing temperature used in this study.

Primer	Sequence*	Annealing temperature (C)
CCA	$DDB(CCA)_5$	64
CGA	$DHB(CGA)_5$	61

*The following designations are used for degenerate sites: H (A, T, or C), B (G, T, or C), and D (G, A, or T)

polymerase (Promega, Madison, WI, USA). The thermal cycling program was as follows: 3 minutes initial denaturation at 95 C, followed by 35 cycles of 30 seconds denaturation at 94 C, 50 seconds annealing at a temperature dependent on the primer (Table 2), 2 minutes extension at 72 C, and a final 10 minutes extension at 72 C. A negative control using water instead of template DNA was included in the amplification process. Four microliters of PCR products from each PCR reaction were examined by electrophoresis in a 1.8% (w/v) agarose gel in $1 \times$ TAE buffer (40mM Tris, 1mM EDTA, pH 8.0) and visualized under UV light after staining with ethidium bromide (0.5µg ml^{-1}). The lengths of the amplification products were estimated by comparison with a 200 bp DNA ladder.

Data analysis

Amplified fragments were scored 1 for presence and 0 for absence. All fragments generated by the two primers were recorded and combined into one data matrix. Statistical analysis of the data was performed using the NTSYS-pc version 1.70 (Rohlf, 1992). The degree of genetic relatedness or similarity was estimated using simple matching coefficients (Sneath and Sokal, 1973): $S=(a+d)/(a+b+c+d)$, where a is the number of (1, 1) matches between pairs, b and c equal the number of (0, 1) and (1, 0) mismatches respectively, and d equals the number of (0, 0) matches. Based on simple matching coefficients, a dendrogram was constructed using SAHN clustering in the unweighted pair group method with arithmetic means (UPGMA) in NTSYS-pc.

Genetic diversity within the population of *Alternaria alternata* was estimated using the Shannon-Weaver diversity index (H'), which was calculated by the formula $H' = -\sum_{i=1}^{k} pi \times \ln pi$, where k is the total number of amplification fragments, and pi is the frequency of a given band (Chalmers *et al.*, 1992).

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Results

Occurrence of RAMS markers

Amplification of genomic DNA of 112 isolates of endophytic *Alternaria alternata* was performed by the two RAMS primers (CCA and CGA), which produced 1273 bands. All the bands were clear and reproducible, and the size ranged from 200-2000 base pairs. No visible fragment was produced below 200 bp or above 2000 bp. Due to the high number of amplification products, complex band patterns were obtained (Fig. 1). The relationship between loci and alleles was not determined and the variation observed was considered as on/off type polymorphism. Thus, the variation was analyzed according to the presence or absence of the markers.

A total of 20 markers were scored from all the amplification products using two primer combinations, and each marker was denoted by the primer which was followed by the approximate length (in base pairs) of the band. Two markers, CCA 200 and CGA 470, were present in all the isolates, and another two markers, CGA 850 and CGA 670, were observed in 107 (95.5%) out of 112 isolates, whereas marker CGA 370 was unique to one isolate.
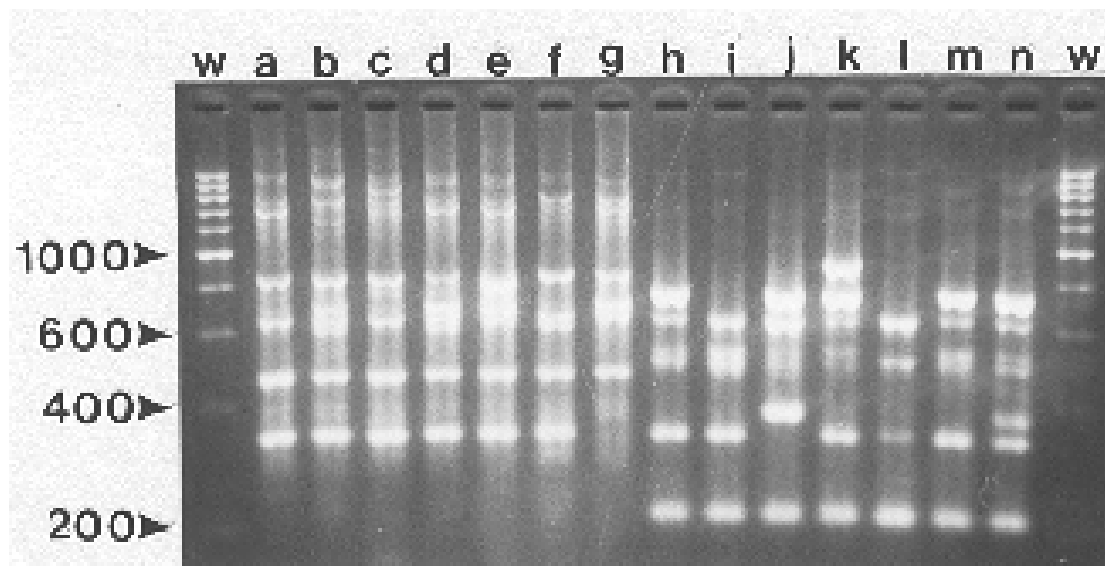

Fig. 1. An example of random amplified microsatellite (RAMS) marker patterns of *Alternaria alternata*. Lane w, the molecular weight marker of a 200 bp ladder and numbers on the left margin indicated length of marker fragments in base pairs; lane a-g, PCR products with primer CCA; lane h-n, PCR products with primer CGA.

Variation in RAMS patterns

Analysis of the two primers revealed a high level of genetic diversity among the 112 isolates tested (Shannon-Weaver index $H' = 2.79$). The polymorphisms in the 20 markers analyzed resulted in 105 (93.8%) different banding patterns among the 112 isolates, and these patterns are referred to as genotypes, thus most isolates were identified as separate genotypes and allowed to be distinguished easily within the *Alternaria alternata* population. The UPGMA cluster analysis based on pairwise genetic similarity coefficients revealed that these 112 isolates had a 66.3% level of similarity. Seven pairs of isolates, i.e. (i) 58 and 82, (ii) 38 and 43, (iii) 11 and 101, (iv) 24 and 80, (v) 29 and 37, (vi) 14 and 75, and (vii) 96 and 109, had identical genotypes (Fig. 2). Genetic similarity coefficients between pairwise isolates varied from 0.3 to 1.

In addition, some isolates had the same genotype, but they were isolated from different tissue ages, e.g. isolates 38 and 43, 29 and 37, and 14 and 75. On contrary, the cultures isolated from the same tissue ages had the different genotypes (Fig. 2). Therefore, there was no relationship between genotypes of *A. alternata* and host tissue ages

Discussion

The genetic variation of an endophytic fungus *Alternaria alternata* population isolated from *Pinus tabulaeformis* in China was investigated using RAMS markers. The high number of RAMS markers (20 markers) observed among *A. alternata* isolates in the present study was comparable with the other studies using the same technique, e.g. in *Gremmeniella abietina* (Hantula and Müller, 1997), *Peridermium pini* (Hantula *et al*., 1998), *Phlebiopsis gigantea* (Vainio *et al*., 1998), *Phialocephala fortinii* (Grünig *et al*., 2001), and *Sphaeropsis sapinea* (Burgess *et al*., 2001). This indicates that the frequencies of microsatellites with CGA- and CCA-motif are high in these fungi and RAMS technique is powerful in evaluating the population genetic variation.

Although the teleomorph *Lewia* have been identified for some *Alternaria* species, the sexual stage of *A. alternata* is unknown (Simmons, 1978, 2002). Therefore, *A. alternata* is likely to be haploid, existing in a vegetative phase, reproducing asexually, and this would be expected to display a low genotype variation. However, our observation did not support this hypothesis. We have found that there was a high genetic variation (105 genotypes) in 112 isolates, and RAMS fingerprinting identified variation between isolates because 90% markers were polymorphic. A high genetic variation in *A. alternata* populations has been reported at the molecular level in

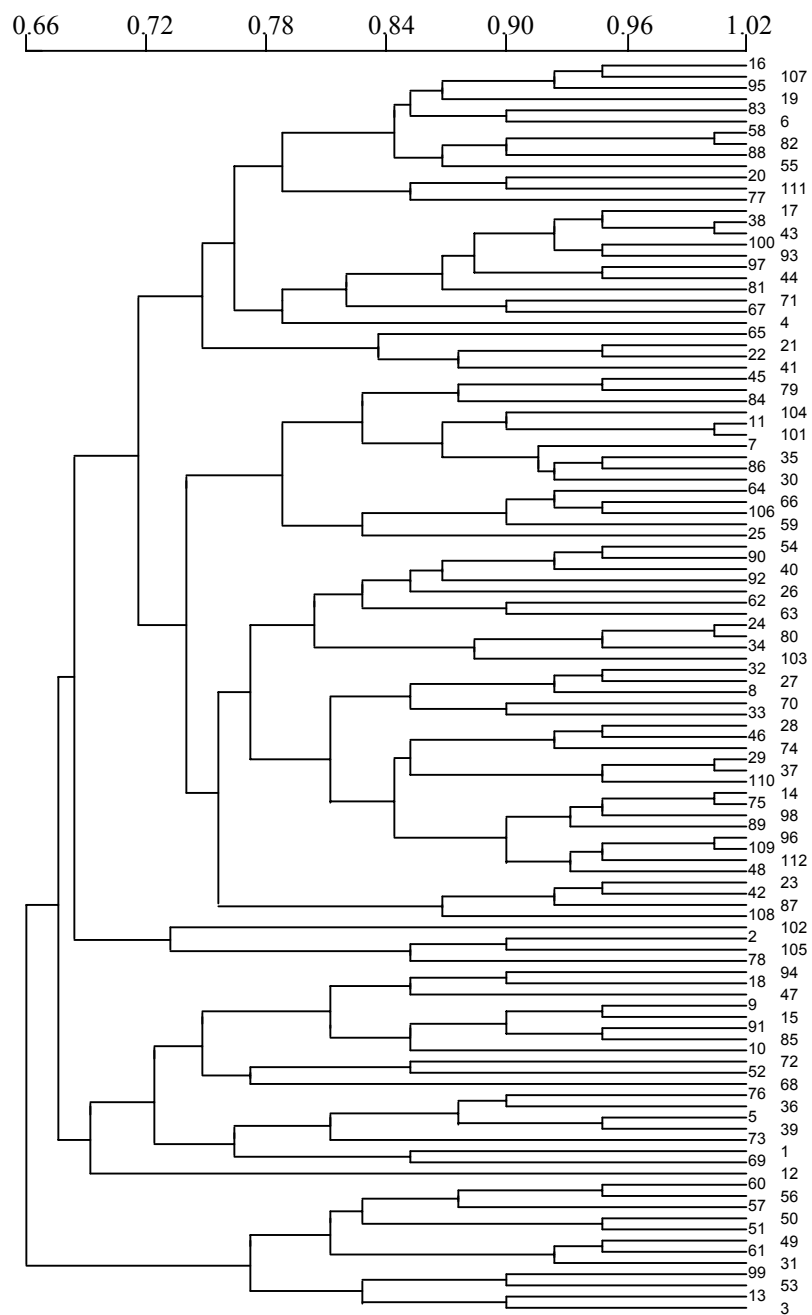previous   studies   (Weir

Fig. 2. Dendrogram generated by the unweighted pair group method with arithmetic means (UPGMA) of simple matching coefficients based on the data amplified from the 112 isolates of *Alternaria alternata* using two random amplified microsatellite (RAMS) primers.

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et al., 1998; Roberts *et al.*, 2000; Bock *et al.*, 2002). Morris *et al.* (2000) distinguished 69 isolates of *A. alternata* isolated from tomato fruit with characteristic sunken black lesions in California using RAPD markers, and Aradhya *et al.* (2001) identified 34 different haplotypes among 56 *A. alternata* isolates collected from four central valley locations in California by means of RFLP analysis. Twenty-six genotypes were found in 65 *A. alternata* isolates sampled from citrus brown spot lesions of six countries using RAPD markers (Peever *et al.*, 2002).

A low genetic variation of an *A. alternata* population, i.e. eight rDNA variants in 271 isolates, however, was reported from central and western regions of Japan, based on RFLP analysis (Adachi *et al.*, 1993). It is likely that RFLP markers are no more sensitive and do not offer any higher resolution than RAMS markers.

The result of the present study indicated that there was no relationship between genotypes of *A. alternata* isolates and host tissue ages. It is highly possible that *A. alternata* isolates with various genotypes can penetrate the *P. tabulaeformis*, regardless of tissue ages. However, Häemmerli *et al.* (1992) analyzed 30 endophyte strains of *Discula umbrinella* isolated from beech, chestnut and oak using RAPD markers, and pointed out that the fungal genotypes were related to host origin. The possible explanation is that the *D. umbrinella* isolates with some genotypes can only penetrate specific hosts. The real relationships between fungal genotypes and host and tissue origins need further study.

The source of genetic variation, e.g. mutation, somatic hybridization and heterokaryosis, in many apparently asexual fungi is unknown (Burdon and Silk, 1997; Bock *et al.*, 2002), although the level of recombination can be typical of the sexual system (Burt *et al.*, 1996; Geisler *et al.*, 1998; McDonald *et al.*, 1999). In this study, however, the high genetic variation was distributed within a small geographic area indicated that the endophytic *A. alternata* population has the potential to evolve relatively quickly and to maintain significant genetic variation.

There are arguments against using RAMS techniques, as compared to AFLP and RAPD, in population genetic studies. Although microsatellite alleles are considered to be codominant markers, differences in alleles are measured based solely on size. There is therefore the possibility of single point mutations within the flanking sequence that do not result in a change in the fragment length. Furthermore fragments from different genomic regions can co-migrate because they are the same size. It is possible that different indels could result in fragments of the same size that have different sequences. In addition, markers may not be independent, due to genetic linkage or being alternative alleles at

the same locus. In an asexual fungus, however, meiotic segregation of markers cannot occur, and although co-migration may occur, this does not negate the usefulness of this approach. Thus a dominant marker system is suitable for assessing haploid, asexual populations without overestimating variation due to co-segregation (Bock *et al*., 2002).

The RAMS technique is comparatively cheap, fast, and easy to perform. It is similar to RAPD analysis but longer primers with 18 nucleotides are used and the conditions (e.g. annealing temperature) during amplification are more stringent. Furthermore, genomic regions containing microsatellites are evolving and mutating more rapidly than other areas of genome. This is due to slipped-strand mispairing during replication, with the slippage rate dependent on the length of the repeat (Levinson and Gutman, 1987; Burgess *et al*., 2001). Therefore, the use of higher annealing temperatures and longer nucleotide primers results in highly reproducible RAMS markers that are much more robust than the RAPD markers used previously (Weir *et al*., 1998; Roberts *et al*., 2000; Peever *et al*., 2002). The RAMS markers are also more powerful than the RFLP profiles generated from rDNA (Adachi *et al*., 1993; Aradhya *et al*., 2001) and sequence analysis of ITS regions (Kusaba and Tsuge, 1995) in revealing genetic variation among a set of closely related isolates. Thus, RAMS technique combines most of the benefits of RAPD and microsatellite analyses, and is ideal for studies of genetic variation.

Acknowledgements

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