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Separation of strains of the yeasts *Xanthophyllomyces dendrorhous* and *Phaffia rhodozyma* based on rDNA IGS and ITS sequence analysis

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The type strains of the anamorph *Phaffia rhodozyma* (CBS 5905) and the teleomorph *Xanthophyllomyces dendrorhous* (VKM Y-2786) were analyzed by nucleotide sequence analysis and compared to the sequences found in three additional strains (ATCC 24228, ATCC 24230 and CBS 6938). The results of ribosomal DNA Internal transcribed spacer (ITS) and Intergenic spacer (IGS) region analyses indicate that *P. rhodozyma*, which was isolated from a beech tree, is a distinct species from the other four strains. The latter that were collected from birch trees are considered to be strains of *X. dendrorhous*. These individual strains of *X. dendrorhous*, which have geographically distinct isolation sources, can be distinguished by nucleotide substitutions and deletion/insertion gaps in sub-repeat regions of the Intergenic spacer. The conclusions demonstrate that differences in the IGS region provide molecular markers for denoting strains that may differ in their biochemical and physiological capabilities. The hypothesis is presented that strain differences in the IGS region may be useful to demonstrate geographic and host specificity.

Keywords: yeast; *Phaffia*; molecular markers; strain differentiation

Introduction

The yeast *Phaffia rhodozyma* was originally isolated from slime fluxes of trees in Japan and the west coast of North America [19] with additional collections recorded from Finland and Russia [7,8]. The taxonomic description of *P. rhodozyma* by Miller *et al* [15] defined the species as an anamorphic yeast and designated CBS 5905 as the type strain. A sexual cycle was discovered by Golubev [7] with the description of the teleomorph *Xanthophyllomyces dendrorhous*; strain VKM Y-2786 was designated as the type strain. The *P. rhodozyma* type strain was considered to be con-specific with *X. dendrorhous*, therefore, *P. rhodozyma* was regarded as an anamorph of *X. dendrorhous* [16].

Kucsera *et al* [11] re-examined the sexual cycle and suggested that the anamorph *P. rhodozyma* and the teleomorph *Xanthophyllomyces dendrorhous* represent distinct species. In terms of distributions in nature, isolates were obtained from a variety of species of trees across a wide geographic region; therefore we explored the potential of using a molecular marker to resolve the issue of nomenclature and to distinguish ecologically distinct strains. Strain differentiation could have industrial significance as strains and mutants have been extensively examined for physiological pathways and for biosynthesis of the carotenoid pigment astaxanthin [eg 4,14,23]. Astaxanthin is an important component in aquacultural feeds to provide red coloration for marine fish and crustaceans [10].

The molecular markers that we employed included part of the ribosomal DNA Intergenic spacer region (IGS) and the Internal transcribed spacer (ITS). The IGS region, which is between the 28S-like (LrDNA) and the 18S (SrDNA) genes, includes the 5S gene and a series of sub-repeats. The IGS has been studied for population and strain level differences among a wide range of eukaryotes including *Drosophila*, oats, tomato, rice, nematode and *Daphnia* [2,3,17,20–22]. Of particular interest was the suggested use of the IGS region for typing strains of the yeast *Cryptococcus neoformans* [5]. The ITS region, which is between the LrDNA and the 18S genes, has been extensively studied as a method to discriminate species of ascomycetous yeasts, basidiomycetes and a variety of other eukaryotes [eg 1,6,9].

Methods

Strains, which were obtained from American Type Culture Collection (ATCC), Centraalbureau voor Schimmelcultures (CBS) and WI Golubev (VKM strain), are listed in bold in Table 1. For reference purposes, alternative strain numbers from other collections are also presented. Additional information on the origin of these collections can be found in Phaff *et al* [19] and Golubev [7,8].

Cells from pure cultures were grown for 12–14 h in GYP (2% glucose, 0.5% peptone and 0.1% yeast extract). Cells were centrifuged/washed with distilled water and converted to spheroplasts by incubating them for 2 h at 37°C in 10 mM citrate buffer, pH 5.8, 1 M sorbitol and 10 mg ml^{-1} Lysing Enzymes (containing cellulase, protease and chitinase activities) from *Trichoderma harzianum* (Sigma, St Louis, MO, USA), which was freshly prepared for each extraction procedure. DNA was extracted and purified from the spheroplasts using a QIAamp tissue culture kit (Qiagen,

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Table 1 Strains examined

Strain	Source	GenBank	
		IGS	ITS
ATCC 24228 UCD 68–653C *X. dendrorhous*	*Betula papyrifera*, Rainbow Lake, Alaska	AF139636	AF139631
ATCC 24230 UCD 67–385 *X. dendrorhous*	*Betula tauschii*, Kiso, Japan	AF139635	AF139630
ATCC 96594 **CBS 6938** VKM Y-2793 *X. dendrorhous*	*Betula* sp, Finland	AF139637	AF139632
VKM Y-2786 CBS 7918 *X. dendrorhous* Type	*Betula verrucosa*, Moscow region, Russia	AF139633	AF139628
ATCC 24202 **CBS 5905** UCD 67-210 *P. rhodozyma* Type	*Fagus crenata*, Kyoto, Japan	AF139634	AF139629
CBS 6358 *Cystofilobasidium capitatum*	Antarctic Ocean		AF139627

ATCC = American Type Culture Collection, CBS = Centraalbureau voor Schimmelcultures, UCD = University of California at Davis (HJ Phaff collection), VKM = Golubev strain; numbers in **bold** represent the source of the strain for study; GenBank Accession Number IGS = Intergenic spacer, ITS = Internal transcribed spacer. Type = nomenclatural type strain for the species.

Santa Clarita, CA, USA) following standard protocol. The DNA was amplified with universal fungal primers (see below) using MJ Research Thermal Cyclers (Watertown, MA, USA). The resulting amplicon was purified with the QIAquick PCR purification kit (Qiagen).

The IGS region examined was between the LrDNA and 5S genes. Amplification of this IGS region used two primers, LrDNA primer LR11: 5′ TTA CCA CAG GGA TAA CTG GC and 5S region primer 5SR: 5′ GGA TCG GAC GGG GCA GGG TGC. The 5S primer represents nucleotides 24–44 (reverse and compliment) from the 5′ end in the secondary structure of *Cryptococcus neoformans* [5]. The individual strands were cycle sequenced with 5SR for the reverse strand and LR12 for the forward strand: 5′ CTG AAC GCC TCT AAG TCA GAA, which is closer to the 3′ end of the LrDNA than LR11. Amplification of the ITS region employed universal primers ITS 5: 5′ GGA AGT AAA AGT CGT AAC AAG G and LR6: 5′ CGC CAG TTC TGC TTA CC. ITS cycle sequencing primers included the forward strand primer ITS1, which is near the 3′ end of the SrDNA: 5′ TTC GTA GGT GAA CCT GCG G and the reverse strand primer ITS4 that is located near the 5′ end of the LrDNA: 5′ TCC TCC GCT TAT TGA TAT GC. Nucleotide sequences were obtained using standard Li-Cor protocol with IRD800 conjugate primers and a Li-Cor automated sequencer. All amplification and sequencing procedures were repeated at least twice. Sequences were aligned with Megalign (DNAstar) and visually corrected. Phylogenetic analysis employed PAUP*4.0 using parsimony analysis, random step-wise addition, tree bisection-reconnection, gap handling option as a new state (or 5th base) and as missing data. Complete sequences are available on GenBank (Table 1).

Results

The ITS sequence alignment, which consists of ITS1, 5.8S and ITS2, is depicted in a cladogram (Figure 1). In the ITS2 region, ATCC 24228 and 24230 sequences are identical and differ by one nucleotide from the sequence of strains VKM Y-2786 and CBS 6938. The type strain of *P. rhodozyma* (CBS 5905) differs from the other strains, including the type strain of *X. dendrorhous*, at 43 positions.

The sequence alignment of the IGS region between the LrDNA and 5S genes is presented in Figure 2. The forward primer was approximately 280 nucleotides from the 3′ end of the LrDNA. The sequences for all strains within that region of the LrDNA were identical. Therefore, for presentation purposes, Figure 2 represents the sequences initiating at 241 bases from nucleotide one of the primer. As can be observed by inspection of Figure 2, the IGS, which initiates at ~base 276, is followed by a CT-rich region and a series of short nucleotide repeats and gaps. Parsimony analysis of the 738 nucleotide sequence alignment, with the gap handling option that the deletions represent missing data, is presented in the phylogram in Figure 3. The results indicate that the two Pacific rim strains (ATCC 24228 and ATCC 24230) are identical and that the strains from Europe (CBS 6938 and VKM Y 2786) differ by one nucleotide, at position 665. All four of these strains were isolated from species of birch trees (*Betula*); the two sets of strains differ at seven nucleotide positions which, for visual purposes, are presented in lower case. These differences are in a variety of repeat and non-repeat locations. The difference at position 308 is CTCTc *vs* CTCTT. Positions 394 and 415 represent GT differences in a GT repeat region. Mutations at 468 and 470 are in a region of GAGT repeats: GAGTgTcAGT and GAGTATGAG. Position 618 difference (CAAg *vs* CAAC), which is in a CAAC and CAAG repeat region, may represent a deletion/insertion rather than a transversion. Position 658, which does not appear to be in an area of repeat units, is CTcC *vs* CTGC.

The type strain of *P. rhodozyma*, which was collected from a beech tree (*Fagus*) in Japan, differs from the other strains at more than 30 positions. This number of differences as depicted in Figure 3 is dependent on the alignment, which in this case depicts a minimum number of gaps. Potential alignment differences are evident in the GAGT region; at position 464 the repeat is aAGT, a difference that is repeated seven times and continues to base 552. This region may constitute a single insertion/deletion rather than multiple events as depicted.

PAUP* parsimony analysis with the gap handling option as a new state (Figure 4) confirms visual analysis of the occurrence of insertion/deletions. Specifically, multiple mutation events are strain-specific. *X. dendrorhous* ATCC 24228 and 24230 differ at two locations within the CAAC/CAAG repeat unit: a 17 base gap at 533–550 and 12 bases near position 614. *X. dendrorhous* CBS 6938 and VKM Y2786 differ at two locations (400–452) in the GT

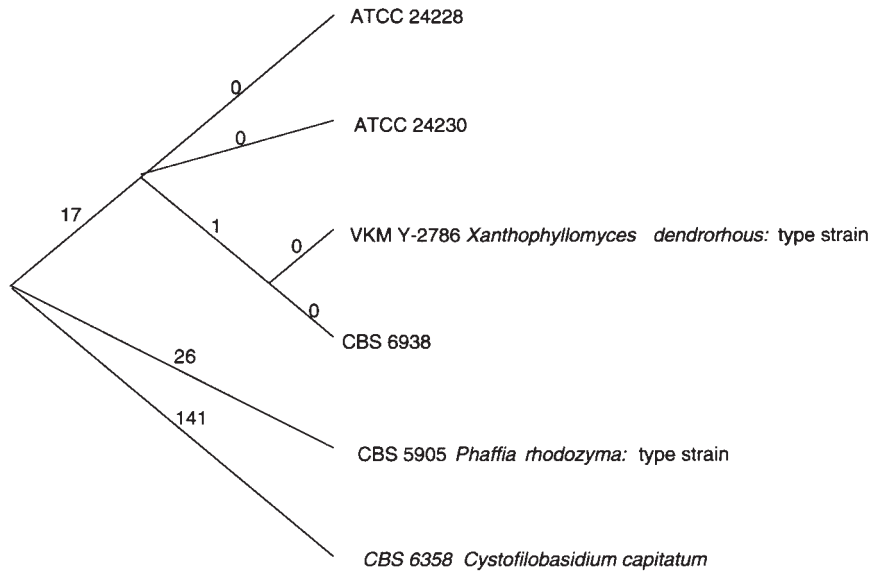

Figure 1 Cladogram of nucleotide sequences of the ITS1-5.8S-ITS2 region based on PAUP*4.0 parsimony analysis, random step-wise addition, tree bisection-reconnection. *Cystofilobasidium capitatum* was the outgroup. Numbers represent branch lengths.

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          241                                                 300                 330                 363
ATCC 24228 TCTGCTGAGGCTAAGCCCTTGTTCTATAGATTTGTTCAACACTTGTTGGACTTTCTTCTCTCTCTCTCTT--CTTCTCTATTCTTTTTT-CTTTCCTTCCTCGCTGCCTTACCCAGGGCCGGA
ATCC 24230 TCTGCTGAGGCTAAGCCCTTGTTCTATAGATTTGTTCAACACTTGTTGGACTTTCTTCTCTCTCTCTCTCTT--CTTCTCTATTCTTTTTT-CTTTCCTTCCTCGCTGCCTTACCCAGGGCCGGA
CBS 6938   TCTGCTGAGGCTAAGCCCTTGTTCTATAGATTTGTTCAACACTTGTTGGACTTTCTTCTCTCTCTCTTTT--CTTCTCTATTCTTTTTT-CTTTCCTTCCTCGCTGCCTTACCCAGGGCCGGA
VKM Y-2786 TCTGCTGAGGCTAAGCCCTTGTTCTATAGATTTGTTCAACACTTGTTGGACTTTCTTCTCTCTCTTTT--CTTCTCTATTCTTTTTT-CTTTCCTTCCTCGCTGCCTTACCCAGGGCCGGA
CBS 5905   TCTGCTGAGGCTAAGCCCTTGTTCTATAGATTTGTTCAACACTTGTTGGACTTTCTTCcCTgTCTtTTTTTCTT------TCTTTTTTCTTTCCTTCCTCGCTGCCTTACCCAGGGCCGGA

          364                                                 400                 440                 487
ATCC 24228 CGTTCCGACTTGTGGTGAGGGGTGTGAAGTGtGTGTGTTTGTATTTGTGGGgGTGAGTGTGTGTG------GGTGTGTGTGGTTGTATTTGT--AAGTGTGAGTgTcAGTGTATTTGTGAGTAT
ATCC 24230 CGTTCCGACTTGTGGTGAGGGGTGTGAAGTGtGTGTGTTTGTATTTGTGGGgGTGAGTGTGTGTG------GGTGTGTGTGGTTGTATTTGT--AAGTGTGAGTgTcAGTGTATTTGTGAGTAT
CBS 6938   CGTTCCGACTTGTGGTGAGGGGTGTGAAGTGGGTGTGTTTGTATTTGTGGGTGTGAGTGTGTGTG----------TTTGT--AAGTGTGAGTATGAGTGTATTTGTGAGTAT
VKM Y-2786 CGTTCCGACTTGTGGTGAGGGGTGTGAAGTGGGT----------GTGAGTGTGTGTGTTTGTGGGTGTGTGTGGTTGTATTTGT--AAGTGTGAGTATGAGTGTATTTGTGAGTAT
CBS 5905   CGTTCCGACTTGTGGTGAGGGGTGTGAAGTGaGa----------GTGGGTGT----------GG-TGTGaG-----TATTTGTATAAGTGTaAGTgTGAGTGT------aAGTAT

          488                                                 550                 580                 614
ATCC 24228 GAGTGT----------CAGTACATGTG-CAATGCTAGTAC-----------------------------------------------AAATACAAGTACACATACAAGTACAAATA
ATCC 24230 GAGTGT----------CAGTACATGTG-CAATGCTAGTACGAGTGCAATGCTAGTAC-------------------------------AAATACAAGTACACATA----------
CBS 6938   GAGTGT----------CAGTACATGTG-CAATGCTAGTACGAGTGCAATGCTAGTAC-------------------------------AAATACAAGTA----------CAAATA
VKM Y-2786 GAGTGTATTTGTGAGTATGAGTGTCAGTACATGTG-CAATGCTAGTACGAGTGCAATGCTAGTACGAGTGCAATGC-TAGTA----------CAAATACAAGTA----------CAAATA
CBS 5905   aAGTGc------aAGTAcaAGTGc----------CAATGCTAGTAC-AGa-CA---CaAGTgCaAGTaCAATGCATAGTATAGTATAGTATAGTG------CAAGTA------CAA-TACAAAcA

          615                 650                 675                 700                 738
ATCC 24228 CAAgTACAAAATACAACTACAAAGCCAGGTCGCACAGATCTCTcCGATGTCGCGGCAGAAAAGCGTCGACTTGTATCCACGGCCATAGGATA-----TCGAAAGCACCCTGCCCCGTCCGATCC
ATCC 24230 CAAgTACAAAATACAACTACAAAGCCAGGTCGCACAGATCTCTcCGATGTCGCGGCAGAAAAGCGTCGACTTGTATCCACGGCCATAGGATA-----TCGAAAGCACCCTGCCCCGTCCGATCC
CBS 6938   CAACTACAAAATACAACTACAAAGCCAGGTCGCACAGATCTCTGCGATGTCGCGGCAGAAAAGCGTCGACTTGTATCCACGGCCATAGGATA-----TCGAAAGCACCCTGCCCCGTCCGATCC
VKM Y-2786 CAACTACAAAATACAACTACAAAGCCAGGTCGCACAGATCTCTGCGATGTtGCGGCAGAAAAGCGTCGACTTGTATCCACGGCCATAGGATA-----TCGAAAGCACCCTGCCCCGTCCGATCC
CBS 5905   CAA-TACA----gCccC-ACAAAGgCAGGTCGaAaAGATCcCgGgGgTGTCGCGGCAGAAAAGtGcCaACTTGTATCCACGGCCATAGGATAGGATATCGAAAGCACCCTGCCCCGTCCGATCC
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Figure 2 Intergenic spacer alignment of strains of *Xanthophyllomyces dendrorhous* and *Phaffia rhodozyma*.

repeat region and at two locations (493–563) in the GAGT region.

Discussion

The results of this study present two major observations: *P. rhodozyma* and *X. dendrohous* may represent separate species, and strains of *X. dendrorhous* can be characterized by deletion/insertion events in the IGS region. Kucsera *et al* [12] suggested, based on an inability of strain CBS 5905 to sporulate, in contrast to other strains studied, that *P. rhodozyma* represents a separate species from *X. dendrorhous*. To corroborate their concept, Kucsera *et al* cited Pfeifer *et al* [18], who analyzed the extrachromosomal genetic elements in six strains of *P. rhodozyma* and demonstrated that, with the exception of the type strain, all strains contained double-stranded DNA. Similarly, Kucsera *et al* [12] noted a report [11] that the type strain was the only strain capable of spontaneous formation of respiratory-deficient petite mutants. Contrasting results [24] based on isoenzyme analysis, RFLP and RAPD patterns led to the conclusion that all strains studied belong to a single species. Also in contradiction to the Kucsera *et al* study, Golubev [7] observed sexual reproduction in the *P. rhodozyma* type strain.

The ITS data (Figure 1), albeit limited to five strains, lend confirmation to the suggestion that *P. rhodozyma* is a distinct taxon that does not represent the anamorphic state of *X. dendrorhous*. The nucleotide differences are spread through different regions, including distinct repeat units,

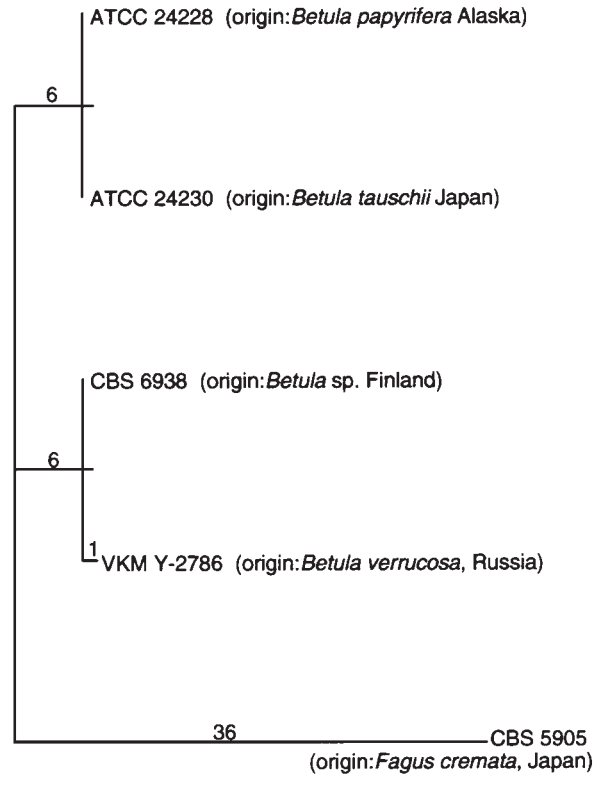

Figure 3 Phylogram of nucleotide sequences of the IGS region between the LrDNA and 5S genes based on PAUP*4.0 parsimony analysis, random step-wise addition, tree bisection-reconnection. Gap handling option = missing data. Organism CBS 5905 was the outgroup; a sequence of a closely related species that could be successfully aligned to Figure 2, was not available. Numbers represent branch lengths.

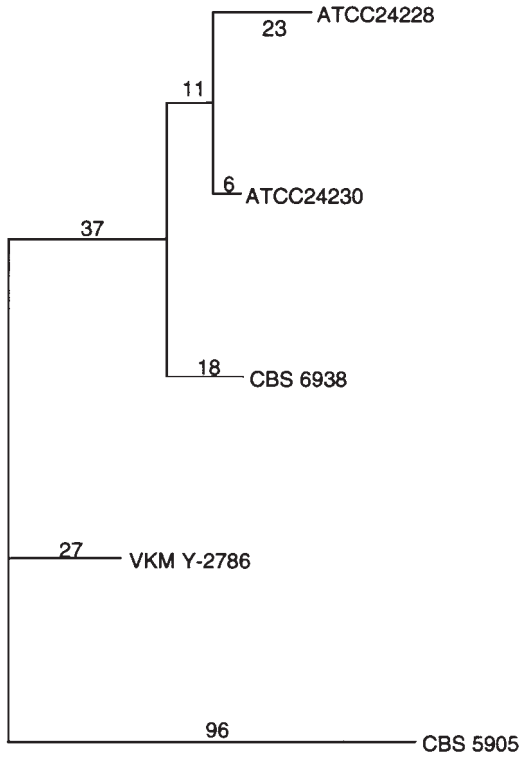

Figure 4 Phylogram of nucleotide sequences of the IGS region between the LrDNA and 5S genes based on PAUP*4.0 parsimony analysis, random step-wise addition, tree bisection-reconnection. Gap handling option = new state (5th base). Organism CBS 5905 was the outgroup; a sequence of a closely related species that could be successfully aligned to Figure 2, was not available. Numbers represent branch lengths.

indicating that the strain differences do not represent a single mutational event. There are no specific guidelines among basidiomycetous yeasts as to the number or percentage of changes in the ITS region that signify separate taxa. However, our unpublished data demonstrate that the closely related taxa *Filobasidiella neoformans* and *F. neoformans* var *bacillispora* differ at four positions in this same region. Also, the closely related, but distinct, species *Filobasidium elegans* and *F. floriforme* differ at 27 positions. Similarly, the IGS data (Figures 2 and 3) indicate that the *Phaffia* type strain is distinct from the other four strains. Our interpretation of the data indicates that all four of these strains belong to the species *X. dendrorhous*, yet there is a phylogenetic difference between the Pacific rim and European strains, possibly at the status of variety. Important confirmatory studies would include a further examination of the sexual cycle of strain CBS 5905 and cross mating experiments of Pacific rim and European strains.

The differences in sequences and occurrences of gaps in the IGS region provide molecular markers for strain differentiation, which could be used for denoting strains that differ on biochemical and physiological capabilities. The insertions or deletions in these repeat areas are generally considered to be the result of replication slippage, although unequal crossover may play a role [13]. The rates of these changes are speculative and may vary with types of organisms and species, although some of the array types in *Daphnia pulex* are considered to be quite old, predating the last, or Wisconsinan, glaciation [3], which was in excess of 70 000 years ago. In ecological terms, the data are not definitive, the suggestion is that the Pacific rim isolates are genetically distinct from the European birch isolates and that each strain can be distinguished based on deletion/insertion patterns in the repeat regions. Also of interest to this study is that the single strain of *P. rhodozyma* was isolated from a beech tree, in contrast to the isolations of *X. dendrorhous* from birch trees.

Due to the paucity of data, ecological conclusions can not be developed, but hypotheses can be advanced that the IGS region may be useful to demonstrate geographic and host specificity and to provide clues as to the rates of genetic change. Molecular analysis of strains obtained from extensive collections, including repeated isolations from individual trees at near and distant geographical regions, will be required to investigate this question.

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