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Evolutionary relationship and species separation of four morphologically similar stichotrichous ciliates (Protozoa, Ciliophora)

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Abstract

Phylogenetic and taxonomic studies on ciliate protists using molecular approaches have been demonstrated to be very reliable to form strong conclusions and results. In the present work, species separation of some morphologically similar stichotrichous ciliates, two species of *Pseudokeronopsis* and two species of *Apokeronopsis*, was reexamined using amplified ribosomal DNA restriction analysis (PCR-RFLP). Five of 10 restriction enzymes revealed species-specific polymorphic patterns, of which four similar stichotrichs could be significantly separated and identified. Among them, *Eco*R I offered almost no significantly different restriction fragment patterns, but the four species could be separated from one another and identified with Hae III. Distinctly different restriction digestion haplotypes and similarity indices separated the species, and were used to construct a phylogeny. Phylogenies based on ITS2 nucleotide sequences and ITS2 secondary structures supported the separation of *Pseudokeronopsis* and *Apokeronopsis* using RFLP analysis, although three *Pseudokeronopsis carnea* populations did not cluster together. In addition, phylogenetic analyses using multiple algorithms confirmed that these two genera formed two distinct groups within the urostylids.

Keywords: Ciliophora; *Pseudokeronopsis*; *Apokeronopsis*; PCR-RFLP; ITS2 secondary structures

1. Introduction

Species of ciliated protists in the subclass Stichotrichia are widespread and common in mostly benthic habitats. Their taxonomy is extremely confused compared to that of most other ciliates because their distinguishing morphological features often overlap between species and genera [1–3]. The genus *Pseudokeronopsis* containing many species and, as more and more were described, was considered to be a polyphyletic assemblage by some workers based on the morphological evidence provided by the unusual species *Pseudokeronopsis qingdaoensis* [4,5]. Berger [6] designated *P. qingdaoensis* as a synonym of *Thigmokeronopsis crassa* (Claparéde & Lachmann, 1858), and Shao et al. [7] erected the new genus *Apokeronopsis* for *T. crassa* based on the pattern of ciliature and features revealed in its morphogenesis. However, more data obtained by different methods are needed to validate the separation of *Apokeronopsis* from *Pseudokeronopsis* because they share some common morphological characteristics [4,5,7].

Criteria for recognizing species of ciliates and the study of variation within and among populations have been the concerns of ciliate systematists for about 70 years [8]. The analysis of restriction fragment length polymorphisms (RFLPs) obtained from amplified and digested ribosomal

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DNA is a promising method for the detection of variation and the identification of species closely related with a high degree of conservation and species-species specificity of ribosomal RNA components [9,10].

The sequence of the ITS2 region has been found to evolve comparatively rapidly, giving it a wide application for phylogenetic analyses at the level of species and genus [11]. Conversely, it has been characterized as a double-edged tool for the evolutionary analyses of eukaryotes because of a conservation of secondary structure [12]; thus the knowledge of RNA secondary structure is becoming increasingly important for phylogenetic analysis.

In the present investigation, the separation of morphologically similar species of *Pseudokeronopsis* and *Apokeronopsis* was studied using PCR-RFLP. Furthermore, the relationships of four species and their constituent populations were analyzed using a combination of PCR-RFLP fingerprinting, and the analyses of SSrRNA gene sequences, ITS2 sequences, and ITS2 secondary structures.

2. Materials and methods

2.1. Collection and identification of ciliates

Samples of six strains were all collected from the coast of the Yellow Sea in the vicinity of Qingdao (36°08′N; 120°43′E), using microscope slides immersed for one to several weeks as artificial substrates. Identifications and morphological studies were done according to the methods of Hu and Song [4,13].

2.2. Extraction of genomic DNA, amplification of rRNA and restriction digests

Extraction of genomic DNA and PCR amplification were carried out according to the methods described by Chen et al. [14]. PCR products were purified with a TIANgel Midi Purification Kit (TIANGEN Bio. Co., China), and the ribosomal DNA regions were digested with different restriction enzymes for 3 h under conditions suggested by the manufacturer (Takara Bio. Co., Japan).

Restriction fragments from the digest were separated by electrophoresis on a 1.2% agarose gel along with two DNA molecular weight markers. Gels were stained with ethidium bromide and were photographed under UV light. Identification scheme of DNA bands and analyses of the data were performed as described in Ref. [10]. The genetic similarities (S) for each sample were expressed by the formula $S = 2N_{AB}/(N_A + N_B)$ [15], where N_A and N_B are the number of bands scored in ciliate A and B, respectively, and N_{AB} is the number shared by both [16]. PHYLIP 3.67 [17] was used for the calculation of genetic similarity.

2.3. Secondary structure prediction and sequence alignment

Sequences of the ITS2 region were obtained from the GenBank database: *Pseudokeronopsis carnea* population I–III (DQ503580–DQ503582), *Pseudokeronopsis flava* (DQ503579), *Apokeronopsis bergeri* (DQ777741), *Apokeronopsis crassa* (DQ537483). The default settings of the mfold website (http://frontend.bioinfo.rpi.edu/applications/mfold) [18] were used to produce the secondary structure and sequence in dot-bracket structural format of ITS2 RNA transcripts. The structures were edited for aesthetic purposes with RnaViz 2.0 [19].

The sequences of six strains were aligned using Clustal X 1.81 [20] with default parameters. The ITS2 sequences with the secondary structure format were aligned using the MARNA web server (http://biwww2.informatik.uni-freiburg.de/Software/MARNA/index.html) [21], based on both the primary and secondary structures.

2.4. Phylogenetic analyses

All SSrRNA gene sequences used in the present investigation are available from GenBank.

The final alignments of SSrRNA gene and ITS2 region sequences were used to construct phylogenetic trees by the following methods: Maximum parsimony (MP) using PAUP* 4.0b10 [22], and Bayesian inference (BI) using MrBayes 3.1.2 [23]. Binary model was selected for the restriction sites. GTR+G and GTR+G+I models of nucleotide substitution generated by likelihood ratio tests and AIC criteria in MrModeltest v.2 [24] were used in the BI analysis of SSrRNA gene and ITS2 region sequences, respectively. The third analysis was conducted for the secondary structure alignment by the complex model, which included the doublet model with 16 states of nucleotide doublets for the stem region and the standard model of DNA substitution with four nucleotide states for loops and bulges.

3. Results

3.1. RFLP patterns and identification scheme

The length of amplified rDNA was approximately equal (ca 3000 bp) for the six populations. Of the 10 restriction enzymes used in the present study, five (*Eco*R V, Hae III, Pst I, *Hin*d III, *Hin*f I) yielded different RFLP patterns among the six strains studied, while *Eco*R I offered almost no significantly different RFLP patterns. Four stichotrichs could be separated and identified with Hae III, and *Hin*d III produced different patterns for three *P. carnea* populations (Fig. 1). These four species could be clearly defined with a combination of different RFLP patterns revealed by five restriction enzymes therefore. The other four enzymes (*Msp* I, Taq I, Xba I, *Bam*H I) gave no restriction bands. In all, 28 bands were scorable in our study, and 24 of those were polymorphic bands.

Genetic similarities (S-values) between species investigated in the present study are shown in Table 1. The S-value of the two species of *Apokeronopsis* was 0.4615. Three strains of *P. carnea* shared a high degree of genetic similarity, with S-values of 0.8571–0.9444. This also was

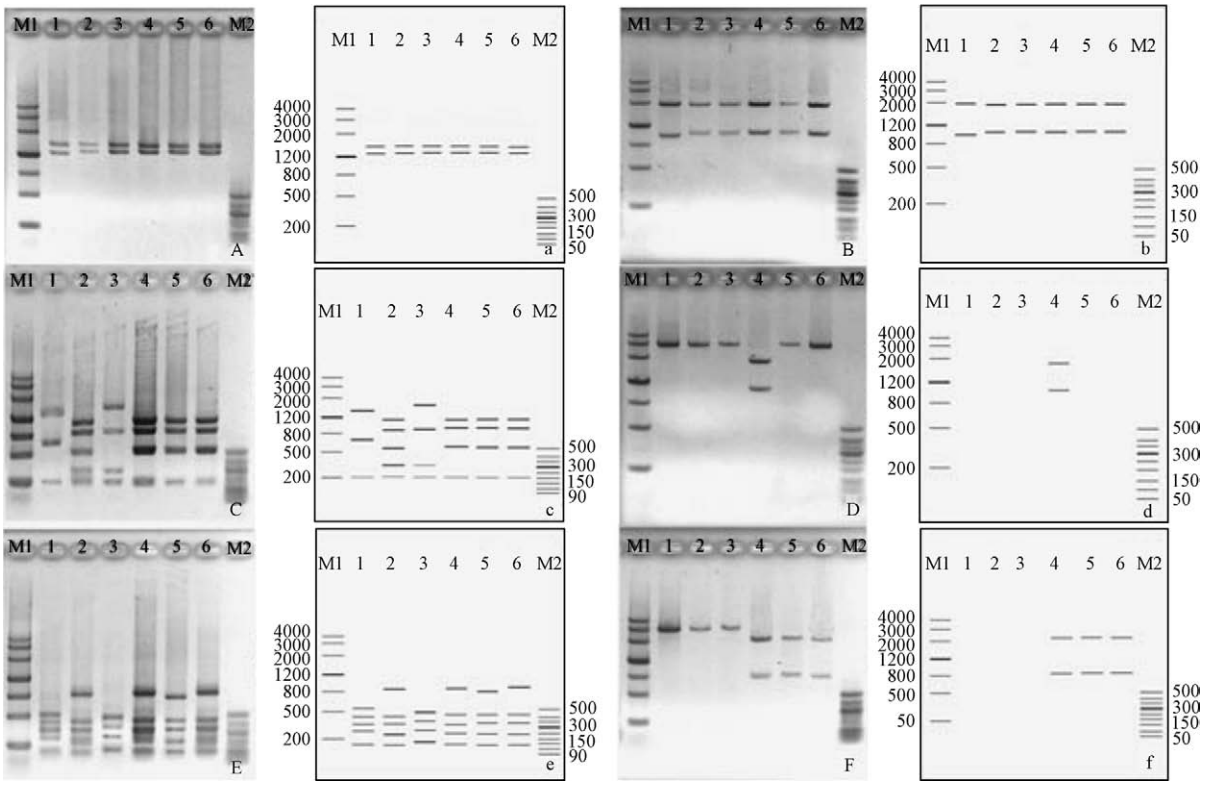

Fig. 1. Riboprinting patterns (A–F) and schematic representations (a–f) of SS-LS rRNA fragments for four populations of *Pseudokeronopsis* and two species of *Apokeronopsis*. Enzyme: A, *Eco*R I; B, *Eco*R V; C, *Hae* III; D, *Hin*d III; E, *Hin*f I; F, *Pst* I.

Table 1
Genetic similarity (*S*-values) calculated.

	A. crassa	*A. bergeri*	*P. flava*	*P. carnea* popI	*P. carnea* popII
A. bergeri	0.4615				
P. flava	0.5518	0.5517			
P. carnea popI	0.5000	0.4375	0.8571		
P. carnea popII	0.5517	0.4828	0.8125	0.8571	
P. carnea popIII	0.5333	0.4667	0.9091	0.9444	0.9091

true for the two species of *Pseudokeronopsis*, which had *S*-values of 0.8125–0.9091. Similarity values between the two genera ranged from 0.4375 to 0.5333.

Relationships of four morphospecies inferred from RFLP patterns are shown in Fig. 2a. *Pseudokeronopsis flava* formed a well-supported clade with three populations of *P. carnea* (0.83 BI, 95% MP). The three populations of *P. carnea* clustered together into a moderately well-supported clade (0.66 BI, 66% MP). *A. bergeri* and *A. crassa* constituted an unresolved pair of taxa that were separated from the *Pseudokeronopsis* clade and did not associate with each other.

3.2. Prediction of ITS2 secondary structure and analyses of relationships based on sequences of the ITS2 region and ITS2 secondary structures

As shown in Fig. 3, the motif in gray denotes the differences among *Apokeronopsis bergeri* and other three species,

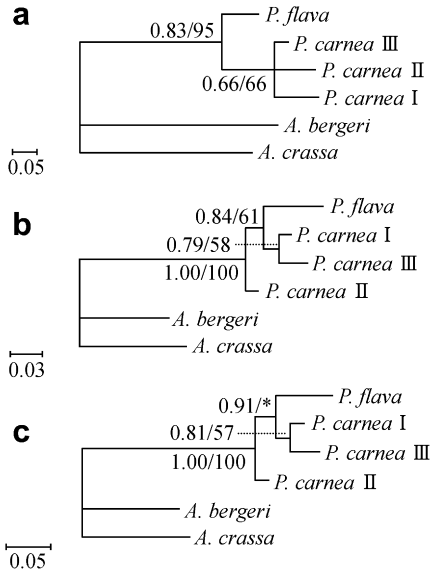

Fig. 2. Bayesian trees showing the relationships of four populations of *Pseudokeronopsis* and two species of *Apokeronopsis*. Numbers near branches are the posterior probability values and bootstrap values, respectively. *Represents nodes that differ in the MP and BI phylogenies. The scale bar corresponds to 3/5 substitutions per 100 nucleotide positions. (a) Phylogenetic trees inferred from the restriction fragment patterns. (b and c) Phylogenetic trees inferred from ITS2 nucleotide sequences and ITS2 secondary structures, respectively.

while region in ellipse shows the difference of loop size among *P. flava* and other three species. Two minor differ-

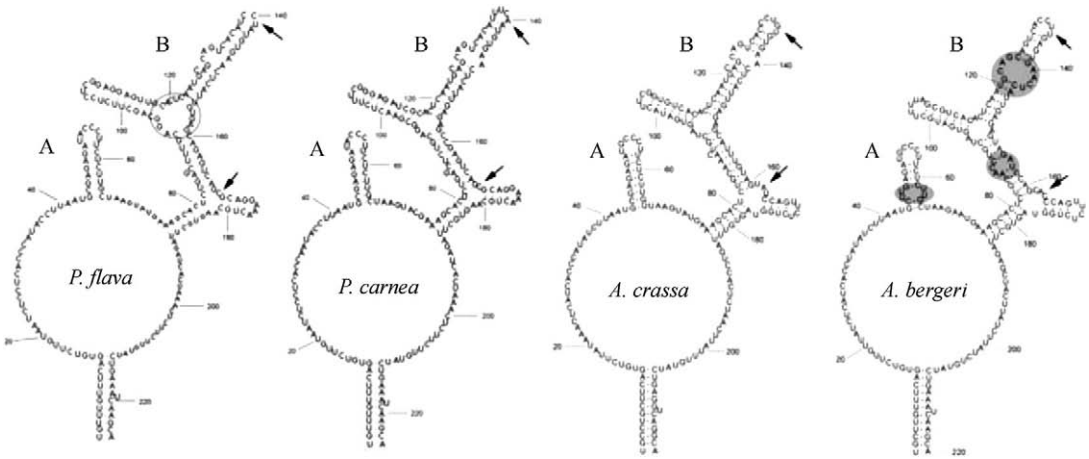

Fig. 3. Morphology and secondary structures of the internal transcribed spacer 2 (ITS2) RNA transcripts of two *Pseudokeronopsis* spp. and two *Apokeronopsis* spp. The diagrams illustrate the two helices, labeled A and B, respectively, present in the class Spirotrichea [25]. Arrows indicate two minor differences between two genera. The distinct regions of *A. bergeri* are highlighted in gray, and that of *P. flava* is enclosed in ellipse.

ences among the secondary structures of two genera are marked with arrows: *Apokeronopsis* has two bigger loops in these two denoted regions (Fig. 3).

Phylogenetic trees inferred from ITS2 nucleotide sequences (Fig. 2b) and ITS2 secondary structures (Fig. 2c) showed essentially the same results as those obtained from the analysis of RFLP patterns. In both trees, *Pseudokeronopsis* spp. formed a monophyletic clade, separated from *Apokeronopsis* spp. However, the association of the three populations of *P. carnea* was not seen in either tree (Fig. 2b and c).

3.3. Phylogenetic analyses of Pseudokeronopsis and Apokeronopsis based on SSrRNA gene sequences

Phylogenetic trees produced with different methods generated nearly congruent relationships, in which each class appears as monophyly in the phylum Ciliophora based on SSrRNA sequences (Fig. 4). In all the analyses, *Pseudokeronopsis* and *Apokeronopsis* consistently fell into the order Urostylida. Three *Pseudokeronopsis* species always formed a distinct cluster, sister to *Pseudourostyla*, whereas the clade containing two *Apokeronopsis* spp. consistently

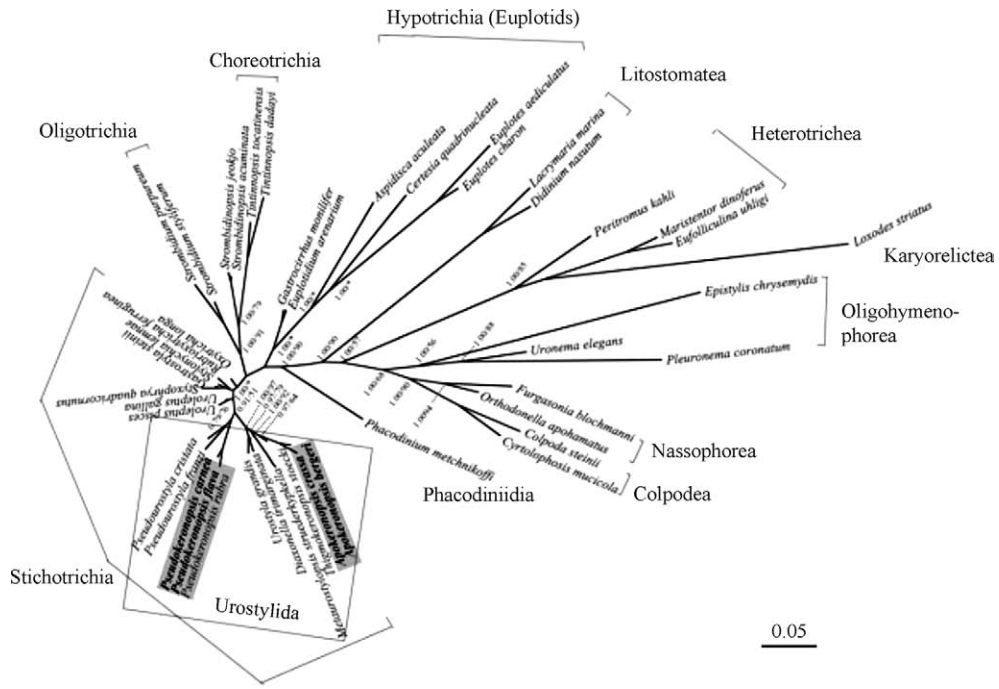

Fig. 4. Bayesian trees inferred from SSrRNA gene sequences showing the relationships of Ciliophora. *Pseudokeronopsis* and *Apokeronopsis* are highlighted in gray. Numbers near branches are the posterior probability value and bootstrap value, respectively. *Represents nodes that differ in the MP and BI phylogenies. The scale bar corresponds to 5 substitutions per 100 nucleotide positions.

clustered with *Thigmokeronopsis* with a high posterior probability (0.97 BI) and a low bootstrap value (64% MP).

4. Discussion

4.1. Species delimitation based PCR-RFLP analyses

The four stichotrichous ciliates that we studied could be clearly identified by their RFLP patterns. This held even for the morphologically similar species *Pseudokeronopsis carnea* and *P. flava*. These were consistent with the previous studies that suggested that PCR-RFLP might be regarded as a promising and reliable tool for species delimitation for some morphologically similar species [9,10].

Most previous researches indicated that there was no intraspecific variation among PCR-RFLP patterns of populations [9,10,26]. However, Chen and Song [27] confirmed that there were some differences between three strains of *Diophrys oligothrix* in regard to riboprint patterns obtained with Msp I. In their opinion, the possible reason for this was a small number of transversions in the nucleotide sequences of rRNA among three strains of *D. oligothrix* owing to high biological diversity. Similarly, our investigation found that there were some differences among PCR-RFLP patterns of three *P. carnea* populations obtained with *Hin*d III. This resulted from the inclusion of somewhat variable regions (ITS1 and ITS2) along with conserved regions (SSrRNA, 5.8S rRNA and the 5′ end of the LS rRNA) in the universal amplified region.

PCR-RFLP is proved to be useful for separating the four morphospecies we investigated, using even a single restriction enzyme. And the PCR-RFLP patterns are helpful for these four stichotrichs identification in the following investigations. However, the use of more conserved regions (such as SSrRNA) and more restriction enzymes is suggested for identifying and distinguishing sibling species.

4.2. Comparisons of ITS2 secondary structures

The secondary structures presented here (Fig. 3) resembled those found in the members of the class Spirotrichea by Coleman [25]. As Coleman suggested, the helix that we labeled as "B" contained the most conserved stretch of primary nucleotide sequence, and the helix labeled "A" showed a pyrimidine–pyrimidine bulge near the base in almost all the cases. The typical eukaryote association of the 3′ region of the 5.8S rRNA gene with the 5′ region of the ribosomal LS rRNA gene [25] was also seen in Fig. 3.

Three populations of *Pseudokeronopsis carnea* shared the same secondary structure because all the variations of nucleotides in paired regions of the secondary structure preserved the pairing potential with a compensatory base change (CBC) or hemi-CBCs (compensatory change on only one side of a helix pairing) [12]. In contrast, the four species of morphologically similar stichotrichs could be separated using ITS2 secondary structures, although the separation of *Pseudokeronopsis* from *Apokeronopsis* was not obvious (Fig. 3).

4.3. Relationships of the six stichotrichous populations

Phylogenetic analyses based on SSrRNA gene sequences (Fig. 5) showed that both *Pseudokeronopsis* and *Apokeronopsis* were monophyletic, and they were rather distant from each other, though both of them were urostylids undoubtedly. This result confirmed that *Pseudokeronopsis* and *Apokeronopsis* are two well-defined genera.

The phylogenetic tree based on PCR-RFLP fingerprinting (Fig. 2a) had a topology similar to those based on ITS2 nucleotide sequences (Fig. 2b) and ITS2 secondary structures (Fig. 2c). All phylogenetic analyses were in accordance with the morphological data, which showed that *Pseudokeronopsis* and *Apokeronopsis* were rather distant genera, though *Apokeronopsis* might not be monophyletic. The separation of these two genera was also hinted in the comparison of ITS2 region sequences (Fig. 5). However, the monophyly of three populations of *P. carnea* was not supported by the ITS2 nucleotide and ITS2 secondary structure trees (Fig. 2b and c). The different PCR-RFLP patterns of these three populations obtained with *Hin*d III (Fig. 1D and d) led to a similar conclusion. This same

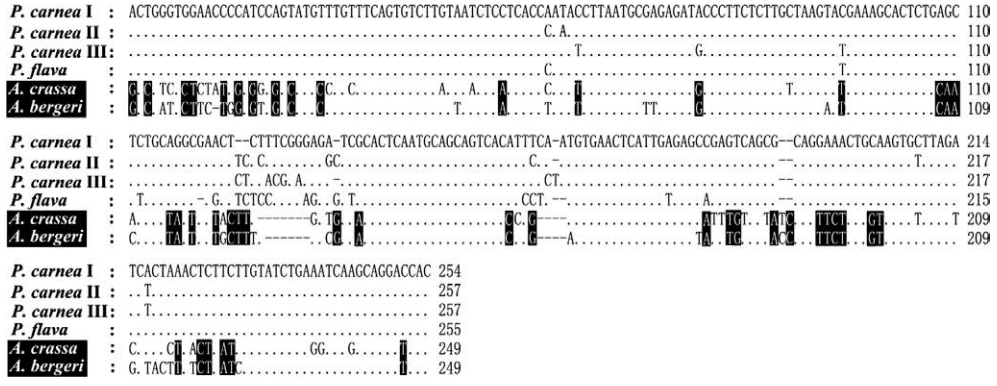

Fig. 5. Alignment of ITS2 sequences of six stichotrichous strains from which the tree shown in Fig. 2b was derived. Homologous groups of nucleotides for the genus *Apokeronopsis* are indicated in black boxes.

result was also obtained in a previous investigation that used multiple comparisons of 13 morphological characters [28]. The variability among populations of *P. carnea* seen in ITS2 nucleotide sequences and secondary structures was consistent with the morphological data. For example, some aspects of the infraciliature show a great deal of variability among the populations of the same species [2,28]. However, the morphogenetic processes of *P. flava* and *P. carnea* shared similar characteristics, which might cause the grouping of *P. flava* and two populations of *P. carnea*.

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