Phylogenetic position of the freshwater ciliate *Euplotes daidaleos* within the family of Euplotidae, obtained from small subunit rDNA gene sequence*

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Abstract: The Culture Collection of Algae and Protozoa (CCAP) (http://www.ccap.ac.uk) is the largest and most genotypically diverse microbial Biological Resource Centre (BRC) in Europe. The collection includes a wide variety of ciliates including ten *Euplotes* cultures, four of which are in the public collection. This study focuses on the freshwater ciliate *E. daidaleos* Diller & Kounaris, 1966, the only species of *Euplotes* that lives in endosymbiosis with '*Chlorella*' like green algae. We sequenced the small subunit of the nuclear ribosomal operon (SSU rDNA) and compared this sequence with others of 40 taxa of different *Euplotes* species. In our phylogenetic analyses we determined six, well-supported clades and two single taxa (*E. rariseta* and *E. euryhalinus*). *Euplotes daidaleos* belongs to a clade containing dominantly freshwater species of *Euplotes* of which *E. octocarinatus* is its closest relative.

Key words: BRC (Biological Resource Centre), importance of collections, endosymbiosis, phylogeny.

Introduction

Many aspects of microbiologically orientated science depend on having defined, stable cultures, which can be repeatedly used. In the case of protists these uses can include such diverse applications as: being reference strains in taxonomy (authentic strains, "type cultures"), starter cultures in aquaculture, ecotoxicity testing and, of course, pure/ applied research. In all the above examples, including research, cultures become **de facto** biological standards. It is at this point that the role of Biological Resource Centres (BRCs), or culture collections, become of real value to the scientific community. All collections, and for that matter many researchers, designate a unique identifier (non taxonomic reference) to individual strains, e.g. the ecotoxicity test strain *Chlorella vulgaris* CCAP 211/11B that is used in internationally agreed algal growth inhibition tests (OECD 1984). By having a unique identifier (strain number) and having the strain available in a public/service BRC, research is facilitated; furthermore, it ensures that where work needs to be comparative, or involves more than one lab, that all workers are employing the same strain and that published data in the scientific literature can be directly comparable.

CCAP's core service and research activities are funded by the UK Natural Environment Research Council (NERC), of which it is a National Facility. More generally, it is recognized as an important resource for UK and European science, both academic and commercial. The Collection comprises more than 2700 strains in the public domain, of which 1050 are marine algae, 1300 freshwater algae, and 350 protozoa (http://www.ccap.ac.uk). The primary mission of CCAP is to maintain and distribute defined cultures and their associated information to its customers. It also has a support and advisory function on all aspects of protistan science. In addition, it is involved in the training of students and researchers in algal identification and culture techniques.

There have been many attempts to unravel the taxonomy of the class Spirotrichea and subclass Hypotrichia within the order Euplotida. Initially, the taxonomy of this order was based purely on morphological characters and mating patterns; however, the use of morphological features etc. has its limitations, not least because of its dependence on an ever reducing number of experts. More recently molecular techniques have been applied to this order and Chen et al. (2000), Bernhard et al. (2001), Petroni et al. (2002), and Li & Song (2006) have all demonstrated that it is possible to establish the phylogenetic position of ciliates by using the small subunit rDNA gene sequences. In addition, this approach helps to separate species from each

* The authors would like to dedicate this paper to Prof. Wilhelm Foissner on the occasion of his 60th birthday in recognition of his contribution to protozoology, protistan taxonomy and teaching.

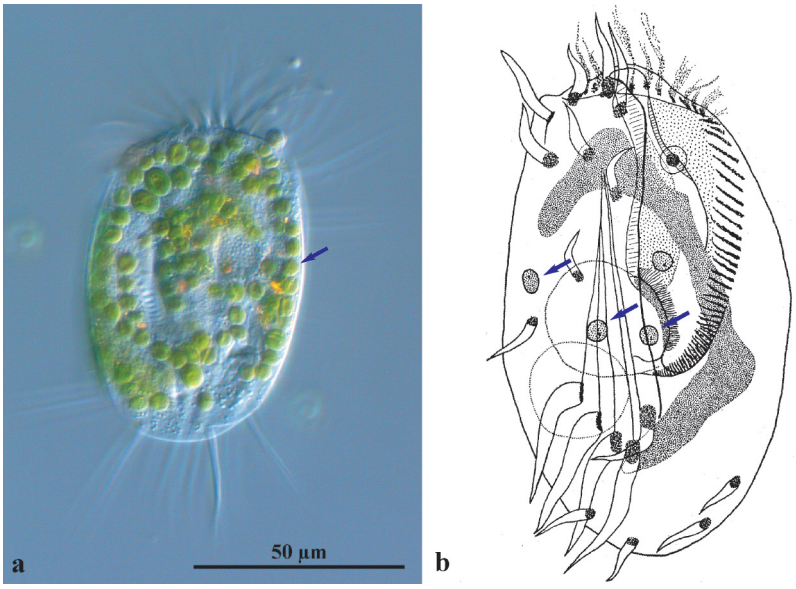

Fig. 1: Photomicrograph (**a;** as seen from dorsal) and schematic drawing (**b**) of *Euplotes daidaleos.* Drawing based on DILLER & KOUNARIS (1966). Blue arrows indicate green algal endosymbionts.

other and aids the reconstruction of phylogenetic relationships (SCHLEGEL et al. 1988, 1991; ORIAS et al. 1991; KUSCH & HECKMANN 1996; JEROME & LYNN 1996, LYNN et al. 1999).

This study investigates the phylogenetic position of one CCAP strain *Euplotes daidaleos* DILLER & KOUNARIS CCAP 1624/15, within the genus *Euplotes*. This hypotrich ciliate (class Spirotrichea, order Euplotida) was first described by DILLER & KOUNARIS (1966) and differs from other *Euplotes* species by having green algal endosymbionts. This project was undertaken as a pilot study to enhance the genetic and other data available on CCAP strains and to show the complications that can occur when many strains are not deposited at a BRC and therefore not available for researchers wishing to verify or demonstrate their phylogenetic position.

Material and methods

Culture and maintenance: The culture of *Euplotes daidaleos* CCAP 1624/15 has been maintained at CCAP since 1988 and was isolated from Priest Pot a small eutrophic lake in the Lake District, Cumbria, UK. It is one of the ciliate strains that grow well in artificial culturing conditions. This culture is grown in bi-phasic soil/water tubes at 20 ºC and under a 12:12 h light-dark regime (PRINGSHEIM 1946; modified; for recipe see http://www.ccap.ac.uk).

Microscopy: *E. daidaleos* was observed using a Polyvar microscope employing phase contrast and the image acquired using a Leica DFC320 with the Leica IM50 Version 5 software.

DNA extraction, PCR, cloning procedure and sequencing: An aliquot (10 ml) of a late stationary phase *E. daidaleos* culture grown in glass tubes was centrifuged for 3 min at 3000 rpm. The supernatant was removed from the tube and the remaining ciliate pellet of around 1 ml was transferred to a safe-lock Eppendorf tube. The Eppendorf tube was then inserted into liquid N_2 for about 1 min and the pellet was then manually destroyed using a 'destroy-stick'. DNA was extracted from cell cultures using the DNeasy Plant Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The amplification of the DNA was performed by using the QIAGEN Taq PCR Master Mix using EAF3 and ITS055R (PRÖSCHOLD et al. 2001) as PCR primers. The DNA was cloned using a QIAGEN PCR Cloning^{plus} kit, employing Qiagen EZ Competent Cells, with all procedures following the methods described in the QIAGEN manual. The sequencing was performed on an ABI-system.

Phylogenetic analyses: In order to establish where *E. daidaleos* phylogenetically belongs within the Subclass Hypotrichia we retrieved additional Euplotida sequence data from NCBI GenBank. These sequences were then manually aligned according to their secondary structure using the sequence editor MacVector 8.1 (Accelrys Inc.). The secondary structure of the SSU rRNA of *E. daidaleos* (CCAP 1624/15) was determined by comparison of the structure presented for *Tetrahymena canadensis* (M26359: SOGIN et al. 1986; http://bioinformatics.psb.ugent.be/webtools/rRNA).
The data set used for the phylogenetic analyses contained 41 taxa with 1732 unambiguous positions. The phylogenetic tree presented in Figure 2 was calculated using PAUP 4.0b10 (SWOFFORD 2002). To determine the evolutionary model that best fitted our data set the program Modeltest 3.7 (POSADA & CRANDALL 1998) was used, which employs two statistics: the likelihood ratio test (LRT) and the Akaike information criterion (AIC; AKAIKE 1974). Based on the results of these tests, the best models were selected by the hierarchical LRT for this data set. For our data set, the best model was the Tamura-Nei model (TrN: TAMURA & NEI 1993), with the proportion of invariable sites (I), the gamma shape parameter (G), and equal base frequencies (TrN+I+G). The tree topology (Fig. 2) represents a maximum likelihood (ML) tree using the best evolutionary model estimated by Modeltest (TrN+I+G). The confidence of branching for the data set was assessed using 1000 bootstrap replicates for neighbour-joining (NJ; using TrN+*I*+G) and unweighted maximum parsimony (MP) and 100 replicates for ML analysis (using TrN+*I*+G; FELSENSTEIN 1985). Strain designations and GenBank accession numbers are given also in Figure 2.

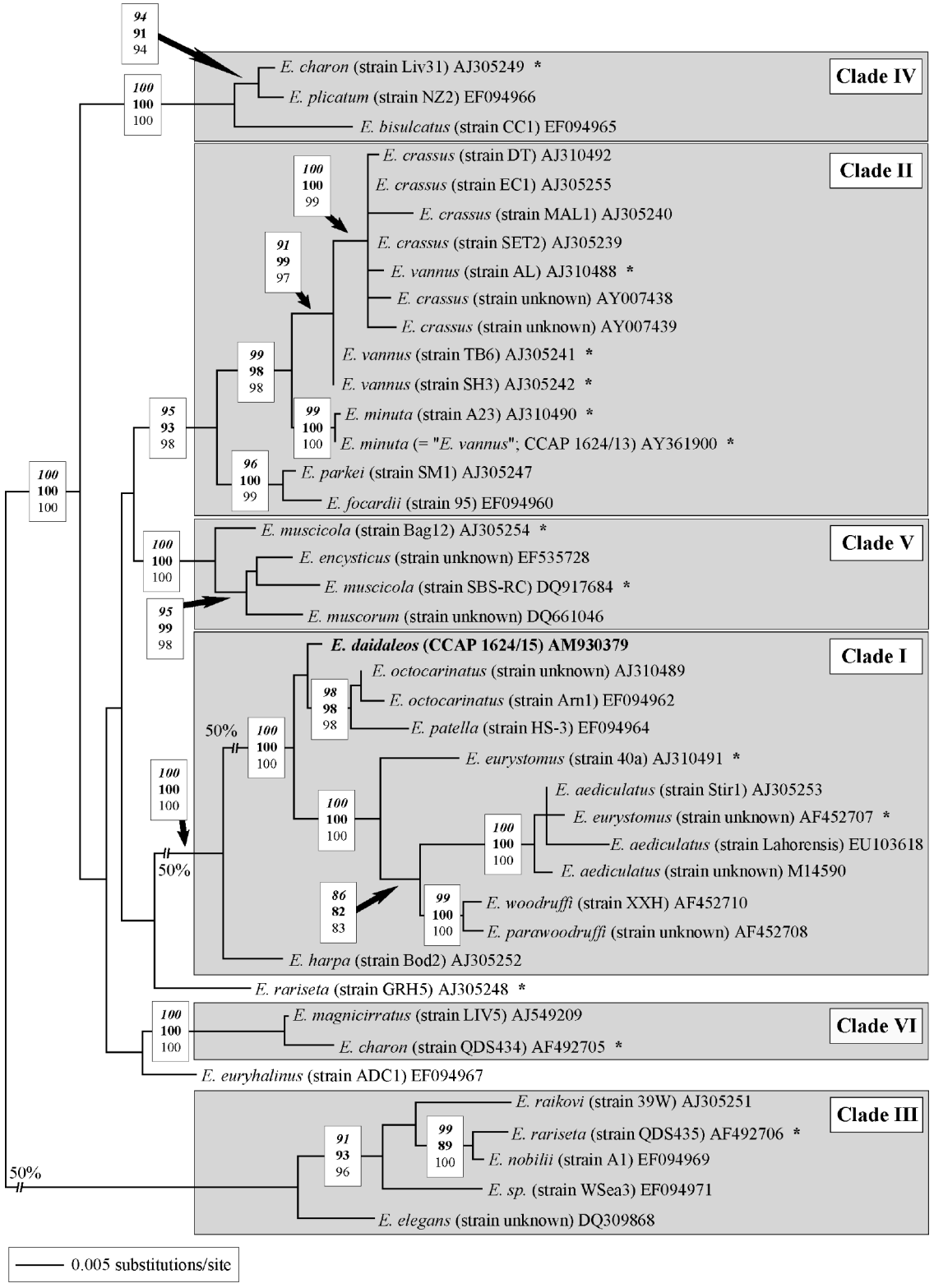

Fig. 2: Molecular phylogeny of the genus *Euplotes* inferred by SSU rDNA sequence comparison using 1732 aligned positions. The rooted tree shown (using Clade III as outgroup) resulted from a maximum likelihood analysis [using the model of Tamura & Nei (1993) with estimated gamma shape (G = 0.76) and proportions of invariable sites (I = 0.54), TrN+I+G, calculated as the best model by Modeltest 3.7 (Posada & Crandell 1998)] of 41 taxa; bootstrap percentage values (>80%) were determined for maximum likelihood (using TrN+I+G; 100 replicates; bold italic), neighbour-joining (using TrN+I+G; 1000 replicates; bold) and unweighted maximum parsimony (1000 replicates; not bold) methods. The new sequence of *E. daidaleos* is highlighted in bold. The strain designations and GenBank accession numbers are given. Anomalies of species designations are marked with an asterisk.

Table 1: Assemblages proposed in the literature for *Euplotes* strains.

Species	Subgroups		
	BORROR & HILL (1995)	PETRONI et al. (2002)	This study
Euplotes crassus	Genus *Moneuplotes*: single argyrome; 10 frontoventral cirri	Clade II	Clade II
Euplotes vannus			
Euplotes minuta			
Euplotes aediculatus	Genus *Euplotoides*: double argyrome; 9 frontoventral cirri, lack of cirrus V/3; Polynucleobacter like symbionts; freshwater with one exception		Clade I
Euplotes daidaleos		Not included in the study	
Euplotes octocarinatus			
Euplotes patella			
Euplotes eurystomus			
Euplotes woodruffi			
Euplotes parawoodruffi			
Euplotes harpa	Genus *Euplotes*: double or complex argyrome; 10 frontoventral cirri; marine with one exception	Clade II	
Euplotes magnicirratus		Unresolved	Clade VI
Euplotes charon			Clade IV or VI
Euplotes rariseta			Unresolved or Clade III
Euplotes euryhalinus		Not included in the study	Unresolved
Euplotes focardii			Clade II
Euplotes nobilii			Clade III
Euplotes raikovi	Genus *Euplotopsis*: double or multiple argyrome; 7–9 frontoventral cirri, lack of cirrus V/2; marine, freshwater or soil	Clade I	
Euplotes elegans		Not included in the study	
Euplotes encysticus			Clade V
Euplotes parkei		Clade III	Clade II
Euplotes muscicola		Unresolved	Clade V

Results and discussion

Over the past 20–30 years data from ultrastructural studies, and more recently from DNA and protein sequencing have helped clarify protistan taxonomy, and today it is considered that there are in the order of 50–70 unicellular and simple, multicellular lineages (WILLIAMSON & DAY 2007). A full review of all known species' origins and relationships (phylogeny) is now underway through a $29m NSF program, Assembling the Tree of Life (2005–10); for information on eukaryotic aspects, see www.eutree.org. A considerable cross-section of protistan biodiversity is held in protistan BRCs, in fact over 16,000 strains are held in >90 European protistan collections alone (DAY & SAXON 2005). Although the majority of conserved taxa are algal and worldwide, only a couple of major BRCs hold significant sized (>250 strains) collections of free-living, non-pathogenic protozoa.

The isolate of *E. daidaleos* investigated in this study (Fig. 1) was very similar to the original description by DILLER & KOUNARIS (1966). *Euplotes daidaleos* CCAP 1624/15 cells measures about 80 × 50 µm, which is within the previously reported range of 77–119 µm in body length and 43–80 µm in body width (DILLER & KOUNARIS 1966). All cells contain large numbers of symbiotic '*Chlorella*-like' green algae.

There have been a number of attempts to resolve the interrelationships of *Euplotes* strains, these have involved structural/ultrastructural analyses (CURDS 1975, GATES & CURDS 1979), with more recent studies using both phenotypic and molecular characters (PETRONI et al. 2002; SCHWARZ et al. 2007). In this paper we analyse the currently available molecular data and attempt to link these with previously suggested *Euplotes* assemblages (Tab. 1). Phylogenetic analysis of SSU rDNA sequence data for the 72 *Euplotes* species, listed on the NCBI web-database, employing a maximum likelihood calculation revealed six stable, statistically well-supported clades (Clades I–VI in Fig. 2). However, for simplicity and clarity we have omitted double or multiple entries of *Euplotes* species from Figure 2, where the sequences, obtained from different strains of the same species, were very similar or identical.

The SSU rDNA of *E. daidaleos* CCAP 1624/15 consists of 1876 bp, which confirmed that the SSU rDNA sequences of different *Euplotes* species are longer than of other ciliates as described by BERNARD et al. (2001) and MIAO et al. (2007). It was noted that this endosymbiont containing organism (*E. daidaleos*) "harmonised" within the largely freshwater and euryhaline species in Clade I (bootstrap values of 100 in all three analyses; Fig. 2). The SSU rDNA data obtained suggests that there is a closer phylogenetic relationship among *E. daidaleos*, *E. octocarinatus* and *E. patella*; furthermore, there is a more distant relationship to *E. eurystomus*, *E. aediculatus*, *E. woodruffi*, *E. parawoodruffi* and *E. harpa* strains (Fig. 2). It was observed that one marine *Euplotes* species (*E. harpa*) was grouped within Clade I, which suggests that both have a common ancestor. This is highly supported in all bootstrap analyses (Fig. 2). All the other taxa listed in Clade I are either freshwater or euryhyaline species including *E. woodruffi* and *E. parawoodruffi*. Utilizing the genetic information available from Genbank, our analyses agree with Clades I, II and III sensu PETRONI et al. (2002) and SCHWARZ et al. (2007); how-

ever, with the additional data of other *Euplotes* strains available we suggest three additional clades (designated as Clades IV–VI in Fig. 2; see also Tab. 1). In contrast, our analyses (Fig. 2) did not support to split the genus *Euplotes* into different genera as suggested by BORROW & HILL (1995); *Moneuplotes*, *Euplotoides*, *Euplotes* and *Euplotopsis* (see Tab. 1). For simplicity we retained the clade numbering described in SCHWARZ et al. (2007), but have added additional *Euplotes* strains to them and to the newly proposed clades (Fig. 2, Tab. 1). These new clades are supported by high bootstrap values in our phylogenetic analyses (Fig. 2). We do not attempt to, or suggest at this stage that *Euplotes* species should be, split into separate genera, as suggested by BORROR & HILL (1995), although phylogenetically Clade III could justifiably be considered to be a separate genus from the other more closely related clades. Clade III is effectively an out-group and shows a split between *E. raikovi* (AJ305251), *E. rariseta* (AF492706), *E. nobilii* (EF094969), *Euplotes* sp. (EF094971) and on a separate discrete branch *E. elegans* (DQ309868) (Fig. 2). This clade is the strongest candidate to be re-designated as a genus separate from *Euplotes*, because the type-species of the subgenus *Euplotes* (*Neteuplotes*) JANKOWSKI (*Euplotes elegans*; for details see JANKOWSKI 1979) belongs to this clade. Therefore, this group could be designated as *Neteuplotes*. However, JANKOWSKI (1979) also includes the species *E. muscorum* and *E. muscicola* in *Neteuplotes* which clearly belong to other clades according to our phylogenetic results (Fig. 2, Tab. 1; see also BORROR & HILL 1995).

Within our phylogenetic tree (Fig. 2) two *Euplotes* strains appeared to be separate from all other congeneric species (*E. rariseta* and *E. euryhalinus*). They do not "fit" in any of the proposed clades on the basis of their available sequences and so their taxonomic and phylogenetic position remains unresolved. It is possible that they represent two additional clades, or it is certainly possible that they might have been misidentified and without more data (phenotypic and molecular) it will not be possible to resolve these anomalous taxa. It is also worth noting that there are some additional problems associated with our suggested phylogeny (marked with an asterisk in Fig. 2). There are two strains of *Euplotes charon* (AJ305249 and AF492705), which belong to two different clades (Clades IV and VI in Fig. 2). For taxonomy, this problem needs to be clarified, because *E. charon* is the type species of *Euplotes*. Another example of the same problem was where two strains of *E. rariseta* (AJ305248 and AF492706) were separated into two different clades, with one of them falling into our defined out-group (Fig. 2). In addition, on the basis of the molecular data, *E. vannus* (AJ310488) could possibly be a *E. crassus*? The same issue was noted for *E. eurystomus*, which appears in two different subclades within Clade I, and another anomaly in Clade V where two strains of *E. muscicola* (AJ305254 and DQ917684) are separated from each other. Such anomalies, often associated with misidentification, are common in the molecular data hosted online. For example BRIDGE et al. (2003) revealed that of 206 named sequences of the ribosomal RNA gene cluster in fungi up to 20% of their identifications were considered "unreliable", this further highlights the scientific need to have access to live material as well as curated molecular data.

In our analyses, freshwater and euryhaline taxa are limited to two clades (Clades I and V). In both cases these clades also incorporate taxa, including *E. encysticus* (EF535728) and *E. muscorum* (DQ661046) in Clade V, where we do not have access to either the culture, or data, that would confirm whether these are freshwater, euryhaline or marine isolates.

In all the anomalies and/or unresolved questions of the above examples, we cannot confirm phenotypic characters of the *Euplotes* species listed in our analyses, because the strains concerned are not available in any BRC. In fact, the only strains available for further research in Figure 2 are the two CCAP strains listed (CCAP 1624/13 and CCAP 1624/15). This problem ('the lack of availability of live strains') is equally true for other suggested taxonomies (Tab. 1), where without access to live material phenotypic and genotypic characters cannot be confirmed.

This initial study is a component of a much larger program of research and curatorial effort at CCAP where live material is linked with curated molecular data that will be available via the CCAP website www.ccap.ac.uk. The objective is that this website will act as a comprehensive knowledgebase for the protistan strains held in CCAP and it will also act as a portal to live links with molecular data on CCAP isolates hosted at GenBank and elsewhere. Our ultimate objective is that this website becomes a model for protistan BRCs and others working on protists and cyanobacteria, thus helping the scientific community to resolve many pressing phylogenetic, taxonomic, ecological and other queries.

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