Twelve Group I Introns in the Same Pre-rRNA Transcript of the Myxomycete Fuligo septica: RNA Processing and Evolution

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The ribosomal DNA region of the myxomycete Fuligo septica was investigated and found to contain 12 group I introns (four in the small subunit and eight in the large subunit ribosomal RNAs). We have performed molecular and phylogenetic analyses to provide insight into intron structure and function, intron-host biology, and intron origin and evolution. The introns vary in size from 398 to 943 nt, all lacking detectable open reading frames. Secondary structure models revealed considerable structural diversity, but all, except one (subclass IB), represent the common group IC1 intron subclass. In vitro splicing analysis revealed that 10 of the 12 introns were able to self-splice as naked RNA, but all 12 introns were able to splice out from the precursor rRNA in vivo as evaluated by reverse transcription PCR analysis on total F. septica RNA. Furthermore, RNA processing analyses in vitro and in vivo showed that 10 of 12 introns perform hydrolytic cleavage at the 3′ splice site, as well as intron circularization. Full-length intron RNA circles were detected in vivo. The order of splicing was analyzed by a reverse transcription PCR approach on cellular RNA, but no strict order of intron excision could be detected. Phylogenetic analysis indicated that most Fuligo introns were distantly related to each other and were independently gained in ribosomal DNA during evolution.

Introduction

All group I intron sequences known in the nucleus interrupt the ribosomal RNA genes of protists and fungi, and more than 1,300 examples at 50 different integration sites are known (Cannone et al. 2002; Jackson et al. 2002). Group I introns are characterized by conserved secondary RNA structures consisting of at least 10 paired segments (P1 to P10) common to most group I ribozymes, and usually several optional segments (P11 to P17) present in subsets of introns (Lehnert et al. 1996; Einvik et al. 1998). The highly conserved catalytic core responsible for the self-splicing reaction consists of P4 to P6 and P3 to P9 (Michel and Westhof 1990; Golden et al. 1998). Group I intron RNAs have been divided into 12 subclasses within five main groups (IA1-3, IB1-4, IC1-3, ID, and IE), based on conserved secondary structure, tertiary interactions, and phylogenetic analyses (see Michel and Westhof [1990] and Suh, Jones, and Blackwell [1999]). Group I intron RNAs are ribozymes catalyzing their own splicing reactions, resulting in perfectly ligated exons. The self-splicing reaction is initiated by a nucleophilic attack of an exogenous guanosine at the 5′ reaction is initiated by a nucleophilic attack of an endogenous hydroxyl group of the last intron residue (Cech and Herschlag 1996). A parallel, competing, intron RNA–processing reaction, known as the intron circularization pathway, is initiated by hydrolytic cleavage at the 3′ splice site (Cech and Herschlag 1996). A parallel, competing, intron RNA–processing reaction, known as the intron circularization pathway, is initiated by hydrolytic cleavage at the 3′ splice site (Nielsen et al. 2003). Here, the free 3′ hydroxyl group of the last intron residue (οG) generated by hydrolysis attacks the 5′ exon-intron junction and results in the formation of full-length circular (FLC) intron RNAs, as well as nonligated exons. Thus, the circularization pathway generates nonfunctional RNAs and appears to challenge the viability of the host. It is generally considered that nuclear group I introns benefit from a ribosomal DNA localization because they are replicated as part of the host chromosomes and cotranscribed by RNA polymerase I as an integrated part of the pre-rRNA (Lin and Vogt 1998; Jackson et al. 2002). Once transcribed, the group I introns have to be precisely spliced out to generate functional pre-rRNAs essential to host cell viability. The excised group I intron RNAs are either rapidly degraded (Brehm and Cech 1983), reversed spliced into cognate or related RNA sites (Roman and Woodson 1998), or, in the case of some mobile introns, further processed into homing endonuclease messenger RNAs (Lin and Vogt 1998; Vander, Nielsen, and Johansen 1999; Decatur, Johansen, and Vogt 2000). The widespread distribution and patterns of relatedness suggest that group I introns are selfish genetic elements able to spread both vertically and horizontally between evolutionarily distinct lineages and are thought to be the product of multiple insertions and selective losses (see Goddard and Burt [1999]). Despite the large number of nuclear group I introns known, very few have been subjected to experimentally tests for RNA processing activities in vitro as naked RNA and in vivo from endogenous pre-rRNAs. Among these are the Tetrahymena introns, their mobile cognate intron from Physarum, and the complex twin-ribozyme introns in Didymium and Naegleria. We recently reported that all these introns generate FLC intron RNAs when incubated in vitro (Haugen, De Jonckheere, and Johansen 2002; Nielsen et al. 2003). Whereas the Tetrahymena introns do not generate detectable FLCs in vivo, the Didymium intron forms a significant amount of FLCs in the nucleus, thus challenging the viability of the cell. In this report we have characterized the small subunit (SSU) and large subunit (LSU) ribosomal DNA (rRNA) from Fuligo septica. The rDNA was found to contain 12 group I introns, which is the highest group I intron content reported for a single precursor RNA species. Thus, F. septica serves as an attractive model system in the characterization of biological roles and molecular evolution of group I introns. Here, we...
present the results from molecular and phylogenetic analyses of the \textit{Fuligo} introns to provide new insight into fundamental questions such as intron structure and function, intron-host biology, and intron origin and evolution.

Materials and Methods

\textit{Fuligo septica} Culturing, DNA Isolation, and Sequencing

The myxomycete \textit{F. septica} (NY-1 [Haugen et al. 2003]) was grown on DS/2 media (Johansen et al. 1997) at 26°C feeding on \textit{Escherichia coli}. Before DNA isolation, cells were harvested and washed twice in DS/2 media by centrifugation at 3,000 rpm to separate amoeboid cells from bacteria. Total DNA was isolated as previously described (Haugen et al. 2003), and \textit{F. septica} rDNA was amplified by PCR using various primers designed from known myxomycete DNA sequences. Automatic sequencing was performed by the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (PerkinElmer) running on an ABI Prism 377 system (PerkinElmer), and manual sequencing was performed by the Thermo Sequenase Radiolabeled Terminator Cycle Sequencing kit (USB Corp.) according to the manufacturer’s instructions.

Plasmid Construction

All introns from \textit{F. septica} rDNA were PCR amplified with a forward T7 promoter-containing primer located approximately 30 nt upstream of the 5' splice site, as well as a reverse primer located approximately 100 nt downstream the 3' splice site. For introns closely spaced, neighbor intron sequences were included to obtain flanking regions of appropriate length. The amplified products were inserted into pUC18 using the SureClone ligation kit (Amersham Pharmacia Biotech) and transformed into \textit{E. coli} JM105 as described by Sambrook and Russel (2001). Recombinant plasmids were isolated from \textit{E. coli} using the DNA purification Wizard SV plus kit (Promega) and subsequently confirmed by sequencing. Site-specific mutagenesis was performed by the PCR-based Quick Change site-directed mutagenesis kit (Stratagene).

Reverse Transcription PCR Determination of Intron Splicing Order, Intron Circle, and Ligated Exon Junctions

Total RNA was isolated from \textit{F. septica} amoeboid cells by the TRIzol reagent (Invitrogen). The DNA was removed by incubating the RNA preparation with RQ1 DNase (Promega) and subsequent RNA Cleanup by the RNeasy kit (Qiagen). Reverse transcription (RT) PCR reactions were performed by using the First-Strand cDNA Synthesis kit (Amersham Bioscience) or Superscript II RNaseH-Reverse Transcriptase (Invitrogen) according to the manufacturer’s instructions. Splicing order of introns in rRNA was determined by RT-PCR on total-cellular RNAs with nested reverse primers. PCR amplifications using the same primer sets were also performed directly on total-cellular RNA as a control. Intron circle and ligated exon junctions were determined by RT-PCR on purified cellular RNA, in vitro spliced RNA, and RNA purified from polyacrylamide gels, as previously described (Haugen, DeJonckheere, and Johansen 2002). All primer sequences used in this study are available on our Web site (see Acknowledgments).

In vitro Transcription and Group I Intron Splicing

RNA was transcribed by T7 RNA polymerase off restriction enzyme–linearized intron plasmid using [35S] CTP aS (10 mCi/ml [Amersham Pharmacia Biotech]) for labeling, purified by phenol extraction and ethanol precipitation, and dissolved in DEPC-treated water. RNA was subjected to self-splicing conditions essentially as described by Johansen and Vogt (1994) at 50°C for 0 to 60 min in low-salt buffer (40 mM Tris-HCl pH 7.5, 200 mM KCl, 2 mM spermidine, 5 mM DTT, 10 mM MgCl2, 0.2 mM GTP) or high-salt buffer (40 mM Tris-HCl pH 7.5, 1 M KCl, 2 mM spermidine, 5 mM DTT, 25 mM MgCl2, 0.2 mM GTP). Splicing intermediates and products were analyzed by denaturing polyacrylamide gel electrophoresis (5% polyacrylamid, 7 M urea) and visualized by autoradiogra.

Nucleic Acid Sequence and Phylogenetic Analyses

Computer analyses of DNA sequences were performed in general by the DNASTAR version 5 software package (DNASTAR Inc.). For the phylogenetic analyses, a nucleotide sequence alignment based on approximately 100 conserved positions within the catalytic core of the group I introns was constructed according to the secondary structure of the introns. ClustalX version 1.81 (Thompson et al. 1997) alignments gave similar tree topologies compared with those generated by manual alignment according to secondary structure. The ClustalX alignment was used to calculate distance matrices, p-distance method (Nei and Kumar 2000), from which phylogenetic trees were constructed. Phylogenetic analyses performed with the neighbor-joining (NJ) and maximum-parsimony (MP) methods gave similar tree topologies. A bootstrap test with 1,000 resamplings was performed. The phylogenetic trees were generated by MEGA version 2.1 (Kumar et al. 2001).

Results

Structural Features of 12 Group I Introns in \textit{Fuligo septica} rDNA

Sequencing analysis of \textit{F. septica} rDNA revealed 12 group I introns at conserved regions in the SSU and LSU rRNA genes (fig. 1). Six of the rDNA sites (S516, S956, L1090, L1949, L2449, and L2500) were previously known to contain group I introns in either nuclear or organellar genomes in some eukaryotic microorganisms (see Cannone et al. [2002]), but others appear novel (S911, S1065, S569, L1898, L1911, and L2584). The \textit{Fuligo} introns, which are named according to Johansen and Haugen (2001), range in size from 398 nt to 943 nt, and lack recognizable open
reading frames. All introns, except Fse.L1949 and Fse.L2449 (see below), represent typical group IC introns with structural features resembling the well studied Tetrahymena rDNA intron (see Lehnert et al. [1996] and Golden et al. [1998]). Whereas Fse.S956 and Fse.S1065 contain all base-paired segments proposed for the Tetrahymena intron, others (Fse.S516, Fse.S911, Fse.L569, Fse.L1090, and Fse.L2584) have gained an additional segment in the P4 to P6 domain (P5d). Here, Fse.L569 is unusual because of a 16-nt direct-repeat motif (5′-CTAAGGGTTCACGGAT-3′) present in 17 identical copies at P5d. One intron (Fse.S911) has a small one-segment P9 region, and three introns (Fse.S516, Fse.S1065, and Fse.L2584) contain the P13 tertiary base pairing between L2.1 and L9.1.

Two Fuligo introns possess unusual secondary structural features. Fse.L1949 and Fse.L2449 resemble cognate introns of the related myxomycetes Physarum polycephalum and P. flavicomum (Johansen, Johansen, and Haugli 1992; Vader et al. 1994). Fse.L1949 and Fse.L2449 contain large extension sequences in L1, which includes three copies of a nearly perfect 45-nt direct-repeat motif in Fse.L1949. Furthermore, Fse.L1949 contains a U:A pair in place of the conserved U:G pair at the 5′ splice site. Fse.L2449 contains large additional extensions at peripheral loops, as well as the unusual P5 hairpin P5.1 (fig. 2). Fse.L1949 folds into a group IC–like structure lacking a recognizable P8 segment, and Fse.L2449 folds into a group IC–like secondary structure (fig. 2).

Ten of 12 Fuligo Introns Self-Splice As Naked RNA In vitro

Catalytic activities of the Fuligo rDNA introns were analyzed from in vitro transcribed and purified RNAs, subsequently incubated at splicing conditions. All the introns, except Fse.L1949 and Fse.L2449, were found to self-splice but at slightly different optimal conditions. Representative time-course experiments show that a significant fraction of precursor RNAs was spliced after 60 min of incubation, generating free intron RNAs and ligated exons (fig. 3A). RT-PCR and sequencing analyses of processed RNAs confirmed that all the self-splicing Fuligo introns perform perfect exon ligation in vitro (data not shown). Lack of self-splicing of Fse.L1949 and Fse.L2449 may be assigned to their unusual sequence features (see above). These observations corroborate results of the cognate introns in the myxomycetes Didymium and Physarum, where no in vitro splicing products were detected (Johansen, Johansen, and Haugli 1992; Vader et al. 1994). All 12 Fuligo introns splice accurately in vivo, a conclusion based on an RT-PCR and sequencing approach using exon-specific oligo primers (figure 4). Thus, the lack of in vitro self-splicing of Fse.L1949 and Fse.L2449 is probably because of the dependence on essential splicing factors (maturase-like activities) in vivo.

No Essential Order of Intron Excision and Splicing In vivo

In vitro self-splicing analysis of two cotranscribed intron pairs (Fse.L1898/Fse.L1911 and Fse.L2500/Fse.L2584) demonstrated that one of the intron partners (Fse.L1898 and Fse.L2584, respectively) performed the splicing reaction faster than the other when incubated at same reaction conditions (data not shown). These observations suggested a preferred order of group I intron excision in Fuligo pre-rRNA similar to what was previously reported for three P. polycephalum rDNA introns (Rocheleau and Woodson 1995). However, the observed in vitro splicing order for the Fuligo intron pairs appeared not to be essential. Inactivation of the most efficient splicing partner (Fse.L1898 or Fse.L2584) by mutating the P7 guanosine-binding site (G:C to C:G), with subsequent cotranscription and in vitro splicing, showed that both Fse.L1911 and Fse.L2500 were able to self-splice before the mutated Fse.L1898 and Fse.L2584 (data not shown).

To investigate the order of splicing in vivo, endogenous Fuligo RNA was isolated and subjected to an RT-PCR approach (see Rocheleau and Woodson [1995]). Here, splicing intermediates of isolated pre-rRNA were detected by the use of primer pairs complementary to sequences within introns, as well as the flanking exons (fig. 4). By this approach, the presence or absence of a given intron can be investigated in a pre-RNA intermediate that contains a second intron. Because of the large number of introns in Fuligo rDNA, the analysis was restricted to neighboring intron pairs. All amplified products were confirmed by DNA sequencing.

The majority of endogenous rRNA sequences appeared completely spliced because only intron-lacking segments could be amplified from exon-specific primer pairs (figure 4, primer combination F1/R2). No essential
Fig. 2.—Secondary structure models of the *F. septica* rDNA group I introns S516, L569, L1949, and L2449. The paired segments P1 to P10 and P13 are indicated according to Cech, Damberger, and Gutell (1994) and Lehnert et al. (1996). The intron sequences are shown in upper case letters, and exon sequences are shown in lowercase letters. Arrows indicate the 5' splice site (5' SS) and the 3' splice site (3' SS). The conserved core position nucleotides used in phylogenetic analysis are indicated as bold letters in Fse.S516. Key features of introns are presented in table 1, and structural features of the additional *Fuligo* introns are shown as Supplementary Material online at the MBE Web site.
order of intron excision and exon splicing was detected from the intron pair analysis of Fse.L1898/Fse.L1911 and Fse.L2500/Fse.L2584. These results support the in vitro mutation analysis (see above) but do not exclude the possibility of different rates of splicing of particular introns in vivo. Similar results were seen for all intron combinations investigated because all the experiments detected processed RNAs lacking both introns and lacking one of the two introns (schematically presented in figure 4). Based on these observations, we conclude that there is no essential order of intron excision and splicing in Fuligo pre-rRNA.

Full-Length Intron RNA Circles Are Generated Both In vitro and In vivo

Gel analysis of in vitro self-splicing intron RNAs identified slow-migrating circular RNA species in all samples (fig. 3A). An RT-PCR and sequencing approach of gel-purified circular RNAs (see Einvik et al. [1997]) confirmed that the majority of Fuligo rDNA introns generate full-length circle (FLC) RNAs during incubation (table 1). FLCs are generated from the circularization pathway initiated by hydrolytic cleavage at the 5' splice site, a reaction distinct from the intron-splicing pathway (figure 3B and Nielsen et al. [2003]). A representative example from the analysis of ligated exons and FLCs is shown for the Fse.L1090 intron (fig. 3C). Similar results were obtained when the RT-PCR sequencing approach was applied on endogenous Fuligo pre-rRNA (table 1). All the self-splicing Fuligo introns were found to generate FLCs either in vitro or in vivo. Furthermore, additional intron RNA circles were observed for six introns in vitro and two introns in vivo (table 1). These circles are truncated at the internal guide sequence (IGS) and thus lack nucleotide residues corresponding to the 5' end of the introns.

Multiple Origin of Fuligo Group I Intron in Nuclear Ribosomal DNA

A comparison of the Fuligo introns with other representative nuclear group I introns (table 2) was based on conserved sequence positions within the ribozyme core. All intron cores were folded according to established group I intron RNA secondary structure models (Cech, Damberger, and Gutell 1994; Lehner et al. 1996) before alignment analysis. The p-distance method (Nei and Kumar 2000) was used to generate the distance matrix because the number of introns included in the analysis was relatively high, and few positions of each intron could be unambiguously aligned. The tree topology was found to be similar for both NJ (fig. 5) and MP analyses.

The overall pattern seen from the NJ tree (fig. 5) corroborates previous reports on group I intron evolution (Bhattacharya et al. 1994; Shinohara, LoBuglio, and Rogers 1996; Nishida, Tajiri, and Sugiyama 1998; Suh, Jones, and Blackwell 1999; Jackson et al. 2002; Haugen et al. 2003). Here, group IE introns are found to be distantly related to the group IC1 introns, and introns at the same rDNA site from distantly related hosts tend to be more similar than introns at different sites in closely related hosts. An illustrating example is given in figure 5 by the highly similar introns present at position 1925 in LSU rDNA (L1925) of the ciliate Tetrahymena and the distantly related myxomycete Physarum. Features relevant to the origin and evolution of Fuligo introns are seen from the NJ tree in figure 5. First, most Fuligo introns are interspersed among nuclear group I introns present in distantly related organisms. Second, introns located within the same gene (SSU or LSU rRNA gene) appear not more closely related than introns between the genes. Third, some of the intron sites (S516, S956, L1949, and L2449) are known to contain group I introns in other myxomycetes that are more closely related to those in Fuligo (Johansen, Johansen, and Haugli 1992; Vader et al. 1994; Haugen et al. 2003). Finally, three of the 12 Fuligo introns (Fse.L1898, Fse.L1911, and Fse.L2500) seem more related to each other than to any other group I intron in the database (Cannone et al. 2002) and may have originated by intron transfer between sites in Fuligo rRNA (intron transposition). We conclude that the Fuligo group I introns represent a complex evolutionary history and that most introns have been independently acquired in the Fuligo rDNA. Thus, both horizontal and vertical intron transfers have to be considered to explain the multiple origins and high number of group I introns in Fuligo rDNA.

Discussion

Posttranscriptional processing of pre-rRNA in eukaryotes consists of a complex pathway of RNA cleavages, digestions, and covalent modifications performed by a large number of trans-acting protein or ribonucleoprotein factors (e.g., Venema and Tolleryve [1995]). The existence of 12 group I introns in F. septica rRNA, which have to be perfectly removed from the pre-rRNA either cotranscriptionally or posttranscriptionally to render the rRNAs functional, is an additional challenge to the host cells. Thus, it is surprising that the multiple Fuligo rDNA introns are not removed in a strict order. The relaxed splicing order implies that exon sequence contexts are not critical for the removal of individual introns, a situation similar to what found in the multiple group I intron–containing gene psba of the Chlamydomonas chloroplast (Deshpande, Bao, and Herrin 1997). However, ordered splicing of nuclear group I introns has previously been reported in Physarum polycephalum pre-rRNA, where a mobile, self-splicing intron was removed before two obligate conserved myxomycete introns (Rocheleau and Woodson 1995). A very different case of ordered splicing in pre-rRNA was observed in Didymium iridis. Here, the spliceosomal intron found inserted into a group I self-splicing intron was removed subsequently to the group I intron (Vader, Nielsen, and Johansen 1999). When the group I intron RNA is inactivated by endonucleolytic cleavage, the spliceosomal intron remains unspliced in vivo (Vader, Johansen, and Nielsen 2002). Finally, a highly preferred, but not obligate order of splicing was reported in a multi-intron mammalian pre-mRNA transcript (Kessler, Jiang, and Chasin 1993). Ordered spliceosomal intron splicing is the result of individual differences in the splicing kinetics between introns of the same transcripts (Audibert, Weil, and Dautry 2002).
FIG. 3.—Analyses of the in vitro RNA products from the 10 self-splicing F. septica introns. (A) Gel analysis of time-course self-splicing experiments. Introns were in vitro transcribed, PAGE purified, and incubated (0, 2, 5, 15, 30, and 60 min) at self-splicing conditions (low-salt and high-salt buffer) at 50°C. RNA was analyzed on a denaturing polyacrylamid gel, and visualized by autoradiography. The observed processed RNAs are full-length intron circles (♀), precursor (○), 5’ exon-intron (●), excised intron (○), ligated exons (▲), and free 3’ exon (▲). The small 5’ exons were run off the gel M, RNA size marker. (B) Schematic presentation of RNA processing pathways of F. septica group I introns. The splicing pathway (left) results
Pre-rRNA

RT-PCR

Processed RNA detected

FIG. 4.—Analysis of splicing orders of *F. septica* rDNA group I introns. A possible strict order of excision among neighboring introns was assessed by an RT-PCR approach designed to detect the presence or absence of introns in endogenous RNA transcripts. A schematic presentation of neighboring introns (A and B) in precursor rRNA (pre-rRNA) and detected processed RNA are shown. Total RNA isolated from growing *F. septica* cells was subjected to RT-PCR using the primer combinations F1/R2, F2/R2, and F1/R1, and corresponding products were gel purified and DNA sequenced. Intron pairs analyzed were S516/S911, S911/S956, S956/S1065, L569/L1090, L1090/L1898, L1898/L1911, L1911/L1949, L1949/L2449, L2449/L2500, and L2500/L2584. The three variants of processed RNAs were detected for all intron pairs. F1 and F2 are forward primers. R1 and R2 are reverse primers. The primer sequences used in this experiment are listed as Supplementary Material online at the MBE Web site.

What are the costs and benefits for host cells of having group I introns in the essential and heavily transcribed rRNA genes? Early studies in *Tetrahymena pigmmentosa* concluded that the presence of a group I intron in rDNA appears neutral with no observed phenotypic feature compared with corresponding cells lacking an intron (Nielsen and Engberg 1985; Nielsen, Simon, and Engberg 1992). This observation may be explained by the fact that group I introns, such as the *Tetrahymena* intron, spliced out very efficiently and at the same time are self-sufficient by encoding the ribozyme-based splicing activity (Brehm and Cech 1983). However, recent studies have unveiled several group I intron activities that may challenge the host. First, the excised linear intron could reverse splice into cellular ribosomal RNA sequences, or perhaps other endogenous RNAs, and, thus, interfere with cellular gene expression (Roman, Rubin, and Woodson 1999). Second, internal intron RNA processing could render the host RNA nonfunctional. During starvation-induced encystment in *Didymium*, internal processing of a mobile rDNA group I intron results in a partial processed pre-rRNA transcript incompatible with the formation of functional rRNAs (Vader, Johansen, and Nielsen 2002). Finally, we have recently shown that the formation of full-length circular (FLC) intron RNAs from nuclear group I introns is common in vivo (Haugen, De Jonckheere, and Johansen 2002; Nielsen et al. 2003). FLC-formation is dependent on a processing pathway different from splicing, and initiated by hydrolytic cleavage at the 3′ splice site of the intron (figure 3B and Nielsen et al. [2003]). Similar to the internal processing event described above, FLC formation results in nonfunctional rRNAs from the same transcript. The observation that 10 of the 12 *Fuligo* introns are able to form FLCs, and that FLCs were detected from eight introns in vivo, is consistent with the idea of an ongoing competition between FLC formation and splicing in *Fuligo* nuclei.

In contrast, there are few examples of group I introns with reported advantageous effects to the host. Splicing of...
FIG. 5.—Neighbor-joining (NJ) phylogenetic tree of the *F. septica* rDNA introns and representative nuclear group I introns based on distance matrix generated with the $p$-distance method. The intron phylogenetic analysis was based on about 100 sequence positions within the catalytic core aligned according to the secondary structure. Bootstrap values (1,000 replications) higher than 50% are shown at the branches. The *Fuligo* rDNA introns are shown in bold. The three main subclasses of nuclear group I introns (B, C1, and E) are indicated. Abbreviations and accession numbers of introns are found in table 2.
introns in the psbA gene of *Clambydomonas* chloroplasts is light dependent and regulates the expression of the psbA encoded D1 protein (Deshpande, Bao, and Herrin 1997). A different case of an advantageous effect for the host is that the presence of introns makes the DNA resistant to endonucleases that cleave in the vicinity of the intron insertion site. Double-strand breaks generated by such endonucleases (homing endonucleases) are fatal to the host unless rapidly repaired. Homing endonucleases are known to be present in a variety of microorganisms and with specificities that overlap several of the *Fuligo* rDNA intron insertion sites (Johansen et al. 1997; Elde, Willassen, and Johansen 2000; Chevalier and Stodard 2001).

The intron phylogeny supports an independent and multiple gain of group I introns by *Fuligo* rDNA. A similar conclusion was obtained by Cummings, Michel, and McNally (1989), who studied 16 introns (14 group I introns) present in the CO I gene of the *Podospora* mitochondrial genome. The fact that introns with identical insertion sites tend to cluster together regardless of the host species indicates lateral intron transfer during evolution. The actual mechanism for lateral transfer is not known, but both RNA-based and DNA-based pathways have been proposed (Lambowitz and Belfort 1993). The observation that many fungi and protists harbor viruses (Schuster and Dunnebacke 1986; Ghabrial 1998; Van Etten and Meints 1999) and that group I introns have been detected in some of these (Nishida et al. 1998) makes viruses interesting as possible intron vectors. A common feature among organisms harboring related group I introns is that they share natural niches. Intron transfers may be facilitated through predation, endosymbiosis, endoparasitism, or interspecies hybridization (Nishida and Sugiyama 1995; Adams, Clements, and Vaughn (1998). Nikoh and Fujiwara (2001) noted that organisms possessing multiple group I introns seem to be highly specialized for symbiotic or parasitic life styles. They proposed that the slow-growing endosymbiont/parasites are particularly well suited for multiple introns. The negative fitness effect of multiple insertions in rDNA would be much less for these organisms than for free-living organisms where optimal fitness through growth rate and nutrient utilization is critical for survival and reproduction. Indeed, lichen-forming fungi are known to contain multiple group I introns in nuclear rDNA (Gargas, De Priest, and Taylor 1995; Bhattacharya, Friedl, and Helms 2000). In contrast, *F. septica* is neither endosymbiotic nor slow growing, and, unlike most other known myxomycetes, it feeds on almost all kinds of microorganisms at the forest floor. Perhaps the large number of introns accumulated in *Fuligo* rDNA reflects its natural behavior and promiscuous feeding habits and, thus, is a case in point of the phrase “you become what you eat.”

### Supplementary Material

Secondary structural features of the eight additional *Fuligo* introns are presented as supplementary material to figure 2, and primer sequences and specificities are included as supplementary materials to figure 4.

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