The Sterol-C7 Desaturase from the Ciliate *Tetrahymena thermophila* Is a Rieske Oxygenase, Which Is Highly Conserved in Animals

Sebastián R. Najle,[1] Alejandro D. Nusblat,[2] Clara B. Nudel,[2] and Antonio D. Uttaro*,[1]

[1]Instituto de Biología Molecular y Celular de Rosario, CONICET, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina

[2]Cátedra de Microbiología Industrial y Biotecnología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina

***Corresponding author:** E-mail: toniuttaro@yahoo.com.ar.

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Abstract

The ciliate *Tetrahymena thermophila* incorporates sterols from its environment that desaturates at positions C5(6), C7(8), and C22(23). Phytosterols are additionally modified by removal of the ethyl group at carbon 24 (C24). The enzymes involved are oxygen-, NAD(P)H-, and cytochrome b_5 dependent, reason why they were classified as members of the hydroxylases/desaturases superfamily. The ciliate's genome revealed the presence of seven putative sterol desaturases belonging to this family, two of which we have previously characterized as the C24-de-ethylase and C5(6)-desaturase. A Rieske oxygenase was also identified; this type of enzyme, with sterol C7(8)-desaturase activity, was observed only in animals, called Neverland in insects and DAF-36 in nematodes. They perform the conversion of cholesterol into 7-dehydrocholesterol, first step in the synthesis of the essential hormones ecdysteroids and dafachronic acids. By adapting an RNA interference-by-feeding protocol, we easily screened six of the eight genes described earlier, allowing the characterization of the Rieske-like oxygenase as the ciliate's C7(8)-desaturase (Des7p). This characterization was confirmed by obtaining the corresponding knockout mutant, making Des7p the first nonanimal Rieske-sterol desaturase described. To our knowledge, this is the first time that the feeding-RNAi technique was successfully applied in *T. thermophila*, enabling to consider such methodology for future reverse genetics high-throughput screenings in this ciliate. Bioinformatics analyses revealed the presence of Des7p orthologs in other Oligohymenophorean ciliates and in nonanimal Opisthokonts, like the protists *Salpingoeca rosetta* and *Capsaspora owczarzaki*. A horizontal gene transfer event from a unicellular Opisthokont to an ancient phagotrophic Oligohymenophorean could explain the acquisition of the Rieske oxygenase by *Tetrahymena*.

Key words: **Rieske oxygenase, ciliates, sterol desaturase, phylogeny, RNA interference.**

Article

Introduction

Sterols are a diverse group of amphipathic lipids indispensable for a multitude of physiological processes in most eukaryotic organisms. They have a structural role in modulating membrane fluidity and permeability barrier properties (Chang et al. 2006). In addition, sterols are essential for signaling processes, as they also serve as precursors of oxysterols, including steroid hormones and vitamins D in mammals (Chang et al. 2006), brassinosteroids in plants and fungi (Benveniste 2002), ecdysteroids in arthropods (Gilbert et al. 2002), and dafachronic acids in nematodes (Motola et al. 2006).

In those eukaryotes that are unable to synthesize sterols de novo, there is a preservation of genes involved in sterol metabolism (Vinci et al. 2008), which allows these organisms perform limited structural modifications of the sterol moiety, such as dehydration, reduction, oxidation, dealkylation, and desaturation (Tomazic et al. 2011). For instance, the nematode *Caenorhabditis elegans* and the insects *Drosophila melanogaster* and *Bombyx mori* take up sterols from the diet and convert phytosterols, such as campesterol, sitosterol, and stigmasterol, into cholesterol (Ikekawa et al. 1993), through modifications involving sterol ring desaturations and the removal of the alkyl group at carbon 24 (C24).

The peculiar metabolism of sterols in the ciliate *Tetrahymena thermophila* was first described by Conner et al. (1968, 1969). In their absence, *Tetrahymena* synthesizes triterpenoid alcohols, mainly tetrahymanol (a sterol surrogate similar to the hopanoids found in prokaryotes) (fig. 1). In contrast, when *Tetrahymena* cells are grown in the presence of sterols, tetrahymanol biosynthesis is suppressed, and sterols are preferentially incorporated into the cells, with or without modification, depending on the sterol supplied. Four modifications have been confirmed in *Tetrahymena*: desaturations at positions C5(6), C7(8), and C22(23) and the removal of the ethyl group at position C24 from 29-carbon (29C) sterols, leading to the accumulation of provitamin D analogs (fig. 1*A*) (Conner et al. 1969). The transformation of cholesterol into the C7 unsaturated derivative (provitamin D_3 or cholesta-5,7-dien-3β-ol) has attracted particular attention, because of the pharmaceutical and food-related applications,

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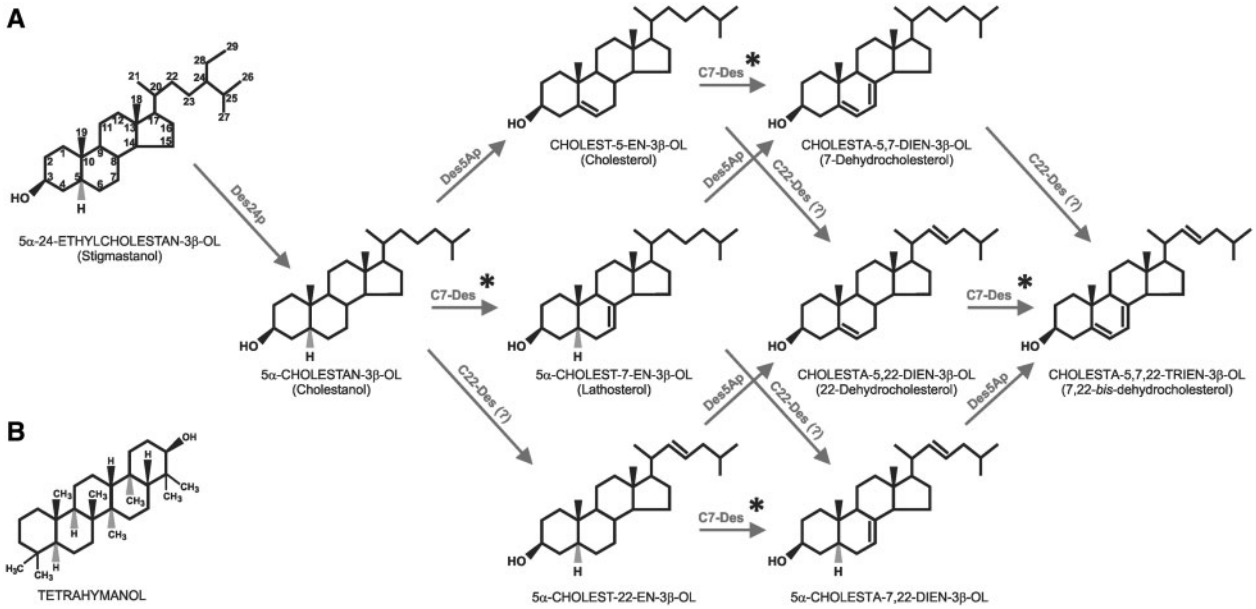

Fig. 1. Pathway of sterol metabolism in *Tetrahymena thermophila*. (*A*) Intermediates and final product formed in cultures supplemented with stigmastanol. The end product 7,22-*bis*-dehydrocholesterol is formed through the removal of the ethyl group in C24 of the stigmastanol moiety by Des24p (Tomazic et al. 2011), as well as a series of desaturations at positions C5(6) by Des5Ap (Nusblat et al. 2009), C7(8) by the enzyme described in this work (asterisk), and C22(23) by an as yet unknown C22-desaturase. (*B*) Tetrahymanol is synthesized in the absence of added sterols.

this enzyme may have to decrease the cholesterol content in foodstuffs and the coupled production of provitamin D_3 in a single step (Valcarce et al. 2000, 2001).

Previous biochemical characterizations of sterol desaturase activities in *T. thermophila* have revealed several typical features, such as their oxygen, cytochrome b_5, and NAD(P)H dependence (Nusblat et al. 2005), tentatively grouping these enzymes within the superfamily of fatty acid hydroxylases/desaturases. A bioinformatic analysis of the *T. thermophila* genome shows the presence of seven sterol desaturase-like genes that contain the canonical motif of a desaturase (Tomazic et al. 2011). We have characterized the product of two of these genes, TTHERM_01194720 and TTHERM_00438800, respectively, as sterol C5-desaturase (Des5Ap) and sterol C24-de-ethylase (Des24p), the latter being involved in the removal of the ethyl group from position C24 of 29C phytosterols (Nusblat et al. 2009; Tomazic et al. 2011).

The identity of the enzyme/s responsible for the conversion of sterols into C7-unsaturated derivatives in *T. thermophila* still remains unknown. Recently, it was reported by two independent groups that Rieske [2Fe-2S]-containing oxygenases are the sterol C7(8)-desaturases in insects and nematodes (Wollam et al. 2011; Yoshiyama-Yanagawa et al. 2011). This catabolic step is of utmost relevance for reproduction and development in those animals. In insects, cholesterol is metabolized to the ecdysteroids, 20-hydroxyecdysone (20E), and ecdysone (E), hormones involved in both molting and metamorphosis (Gilbert et al. 2002). In contrast, the nematode *C. elegans* metabolizes cholesterol into the 4-methyl sterols (dafachronic acids) required for its development and longevity (Entchev and Kurzchalia 2005; Motola et al. 2006). The first step in the biosynthesis of ecdysteroids and dafachronic acids is the conversion of cholesterol acquired from diet into 7-dehydrocholesterol (Rottiers et al. 2006; Yoshiyama et al. 2006). The enzymes catalyzing this conversion were named "Neverland" (Nvd) in *B. mori* and *D. melanogaster* (Yoshiyama et al. 2006) and DAF-36 in *C. elegans* (Rottiers et al. 2006).

This work reports the identification of a Rieske [2Fe-2S] oxygenase, belonging to the Nvd/DAF-36 family of proteins, as the sterol C7(8)-desaturase of *T. thermophila*. Furthermore, we provide strong phylogenetic evidence showing that this family of proteins is evolutionarily conserved not only in animals but also in ciliates of the clade Oligohymenophorea.

Results

Identification of *T. thermophila DES7* by Testing Candidate Genes Using RNA Interference by Feeding

To identify the enzyme responsible for the sterol C7(8)-desaturase activity in *T. thermophila*, we designed a simple and rapid screening for six candidate genes based on RNA interference by feeding. The feeding-RNAi technology was designed for the large-scale analysis of interferents in the nematode *C. elegans* (Timmons and Fire 1998), showing systemic effects produced by the interference in each of its cells (Grishok 2005). The protocol was subsequently applied to organisms for which there are no classic genetic tools, such as parasitic nematodes (Kalinna and Brindley 2007; Félix 2008), planarians (Newmark et al. 2003), and *Hydra* sp. (Chera et al. 2006) among others. This powerful technology proved applicable in the genetic analysis of ciliated protists as well. It was first utilized in *Paramecium tetraurelia* (Galvani and Sperling 2002), and further adjusted to be used in three species of spirotrichous ciliates (*Euplotes aediculatus*,

Oxytricha nova, and *Stylonichia lemnae*), to interfere with the expression of genes belonging to different families (Paschka et al. 2003). The technique could potentially be applied to any phagotrophic organism able to feed on bacteria (Galvani and Sperling 2002) and possessing the molecular machinery for RNA interference, which is the case for *T. thermophila*. A different method for *T. thermophila* RNAi was described by Howard-Till and Yao (2006), based on overexpression of RNA hairpins encoded in a plasmid vector in the ciliate cell, which led to specific gene silencing through degradation of their complementary mRNAs, throughout the complete ciliate's cell cycle. These results showed the feasibility of applying RNAi technology in *Tetrahymena*. The feeding-RNAi protocol is, from a methodological point of view, more straightforward than the one proposed by Howard-Till and Yao and potentially useful for high-throughput screenings. As far as we know, the former approach has not been used with *Tetrahymena* before.

The protocol followed in this work was adapted from that used with *Paramecium* (Beisson et al. 2010) and basically consists in feeding the ciliates with bacteria that produce and accumulate double-stranded RNA (dsRNA) homologous to a portion of the target gene. The targeting sequence is cloned in a plasmid vector between two convergent T7 promoters, which then is used to transform the *Escherichia coli* strain HT115, devoid of RNAse III activity, and expressing the T7 polymerase gene under the control of an IPTG-inducible promoter.

DNA fragments (500–800 bp length) homologous to the sequences TTHERM_00446080 and TTHERM_00758950, assigned as C5 sterol desaturases, and TTHERM_00077800, TTHERM_00876970, and TTHERM_00348230, assigned as C4 sterol methyl oxidases on the basis of previous phylogenetic analyses (Nusblat et al. 2009; Tomazic et al. 2011) as well as a putative ortholog of Nvd/DAF-36, TTHERM_00310640, were obtained by polymerase chain reaction (PCR) and used to construct the recombinant *E. coli* lines. Production of the corresponding dsRNAs was induced by adding IPTG to the bacterial cultures, which were used subsequently to feed *T. thermophila*.

All sterol modifications known to occur in *T. thermophila* can be detected by analysis of the conversion products formed when the organism is cultured in media supplemented with a specific substrate (fig. 1). For example, when the cells are grown in the presence of cholesterol (cholest-5-en-3β-ol), this sterol is converted into the diunsaturated derivative 7,22-*bis*-dehydrocholesterol (cholesta-5,7,22-trien-3β-ol), although formation of minor quantities of the precursors, 22-dehydrocholesterol (cholesta-5,22-dien-3β-ol), and 7-dehydrocholesterol (cholesta-5,7-dien-3β-ol) can be observed as well (Conner et al. 1969; Mallory and Conner 1971; Mulheirn et al. 1971).

After 4 days of interference by feeding, cultures were supplemented with 10 μg/ml cholesterol and maintained for another 2 days. As a control, cells were fed with equally induced bacteria containing the vector without insert. On the 6th day, cultures were harvested and their sterol profiles analyzed by gas chromatography–mass spectrometry (GC/MS) as described in Materials and Methods. We did not detect any significant modifications of the sterol profiles after interference of the first five putative genes tested (hydroxylases/desaturases/methyl-oxidases family members), when compared with the control strain (data not shown). Interestingly, interference of gene TTHERM_00310640, by using two different cloned fragments, $rnai_{560}$ (from base number 362 to 921 of the gene) and $rnai_{797}$ (from 125 to 921) (fig. 2*A*), produced, respectively, an average of 56% and 40% reduction of C7-desaturated derivatives (fig. 2*C*). The shorter fragment ($rnai_{560}$) appeared to be more effective in the interference process, probably due to its increased production of dsRNA when compared with $rnai_{797}$, as seen in figure 2*B*.

These results suggested that TTHERM_00310640 coded for a protein with sterol C7(8)-desaturase activity; and therefore we named it *DES7*, with its corresponding protein product, Des7p. Moreover, these results also illustrate the feasibility of feeding-RNAi as a simplified technique for the screening of multiple genes in *Tetrahymena*.

Construction of a *DES7* Somatic Knockout Strain

To confirm the function of the protein encoded by the TTHERM_00310640 sequence, we targeted this gene with the transformation sequence C*DES7*, which can insertionally inactivate *DES7* and provide paromomycin resistance encoded in the *neo4* cassette (Mochizuki 2008). The construct was introduced into the macronucleus by somatic transformation, using a biolistic bombardment protocol (see fig. 3*A* for a schematic representation of the construct) (Cassidy-Hanley et al. 1997). Transformants were selected upon growing in 150 mg/ml paromomycin and were shown to carry the selection marker correctly integrated into the proper locus (*DES7*). Allele-specific PCR amplification of genomic DNA from the wild type (WT) and the knockout mutant *DES7* showed the correct construction of the mutant strain (fig. 3*B*). The WT amplification band observed in the mutant corresponds to the copies of the gene present in the transcriptionally silent micronucleus. An expression analysis by reverse transcription (RT)-PCR further confirmed this result, showing that the gene is expressed only in the WT and not in the knockout cell line, neither with or without cholesterol added to the growth medium (fig. 3*C*).

As expected from *T. thermophila*'s sterol prototrophy, *DES7* appears not to be essential for the cells, as there were no differences in growth rates nor cell shape between WT *T. thermophila* and its KO mutant strains, neither in the presence nor in the absence of added sterols (not shown).

Sterol C7(8)-Desaturation Is Completely Abolished in the KO*DES7* Mutant

Comparative analysis of the conversion products formed by the WT and the KO*DES7* mutant, grown in SPP medium supplemented with 10 μg/ml cholesterol, shows a clear difference in the final products formed by each strain after 48 h of incubation (fig. 3*D*). Analysis of peak areas from the chromatogram (fig. 3*F*) shows that the sterols recovered from

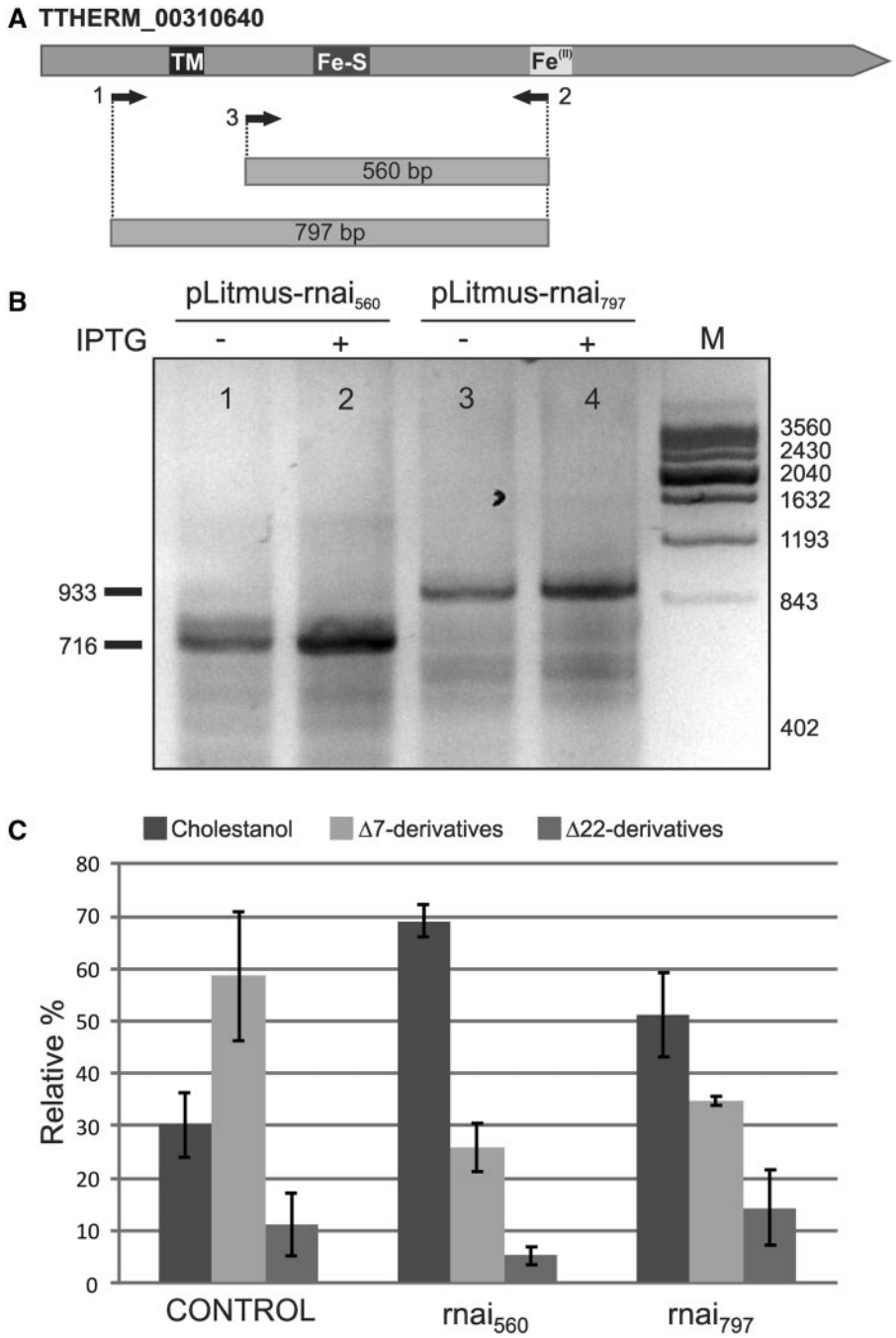

Fig. 2. Inhibition of sterol C7(8)-desaturase activity by RNA interference by feeding. (*A*) Schematic representation of the TTHERM_00310640 gene indicating the PCR-amplified regions used to construct the feeding vectors. TM, transmembrane; Fe-S, Rieske [2Fe-2S] motif; $Fe^{(II)}$, nonheme iron (II) motif. (*B*) Denaturing agarose gel electrophoresis showing the production of specific targeting dsRNAs. DNAse I-treated total RNA samples were loaded on a 1.5% agarose gel: *E. coli* HT115 containing the pLitmus-rnai$_{560}$ or pLitmus-rnai$_{797}$ plasmids, with or without 0.5 mM IPTG. The size of the bands observed is according to the distance between T7 promoters of each plasmid. M, marker for fragment length. (*C*) Quantification of peak areas of GC/MS chromatograms obtained from the sterol fraction of cultures supplemented with 10 μg/ml cholestanol (5α-cholestan-3β-ol), after 6 days of interference (see Materials and Methods). CONTROL: ciliates fed with HT115 bacteria transformed with the empty vector.

cultures where, in the case of the WT strain, 83.44% 7,22-*bis*-dehydrocholesterol (cholesta-5,7,22-trien-3β-ol; peak 2) as the major derivative, besides cholesterol itself (peak 3), 0.55% 7-dehydrocholesterol (cholesta-5,7-dien-3β-ol; peak 4), and 3.84% 22-dehydrocholesterol (cholesta-5,22-dien-3β-ol; peak 1). In contrast, in the KO*DES7* cell line, the only conversion product recovered was 22-dehydrocholesterol (92.76%). The Δ7-desaturated products in this strain (7-dehydrocholesterol and 7,22-*bis*-dehydrocholesterol) if any were present only in trace amounts (<0.1%). This result confirmed unequivocally that the gene TTHERM_00310640 encodes a protein with sterol C7(8)-desaturase activity.

To evaluate whether other sterol modifying activities described in *Tetrahymena* (i.e., C5- and C22-desaturases and

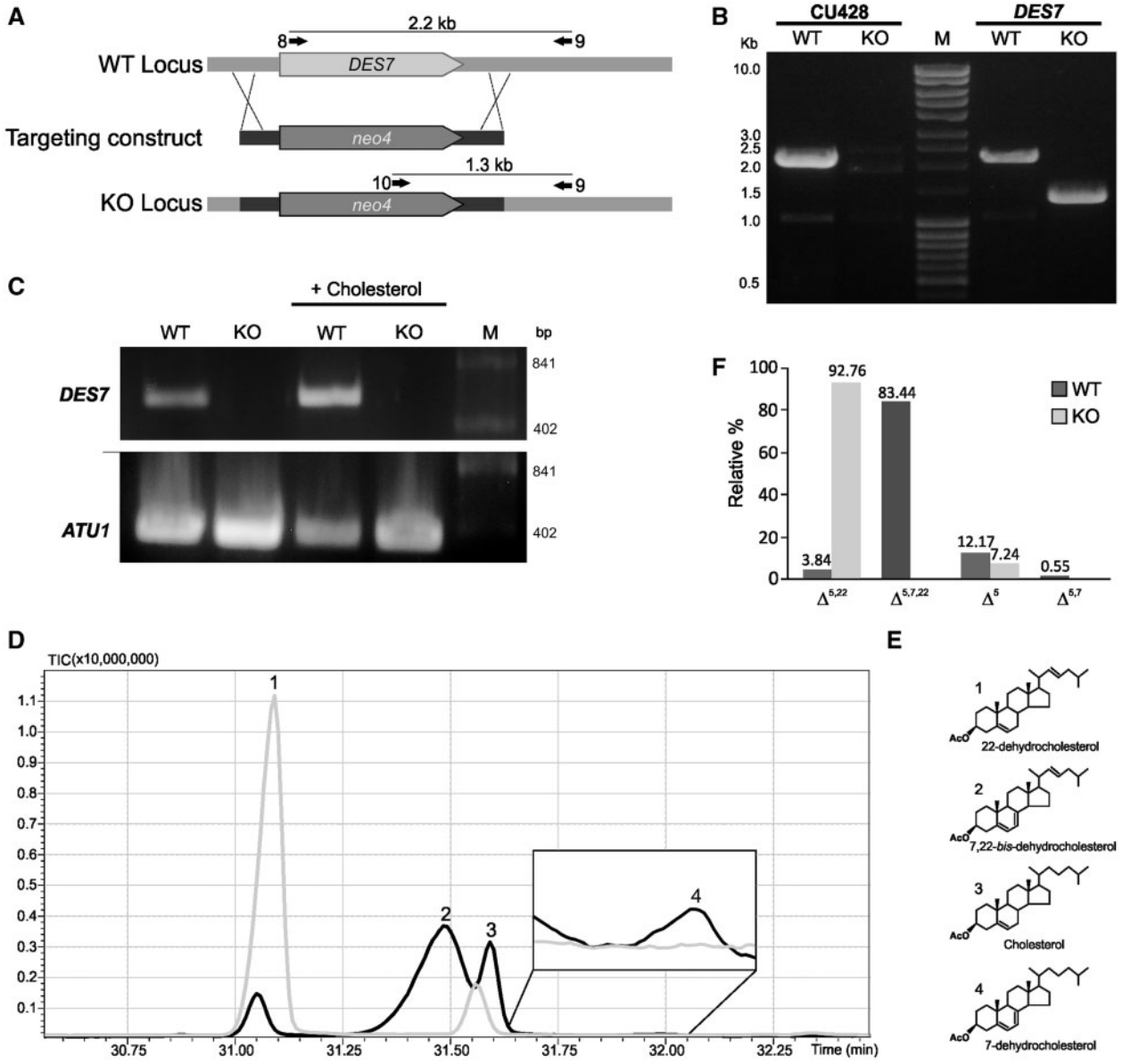

FIG. 3. Generation and characterization of the *DES7* knockout mutant. (*A*) Schematic representation of the gene replacement strategy used to knockout the gene TTHERM_00310640. The target construction is inserted by homologous recombination generating the knockout locus (see Materials and Methods for details on the strain construction and phenotypic assortment). The numbered arrows indicate primers used for PCR amplifications to check proper integration of the construct, and the sizes of the corresponding fragments are indicated above them. (*B*) DNA amplification of the WT and KO loci from both the WT and *DES7* cells, respectively, using the same reverse primer (primer 9) and two locus-specific forward primers (primers 8 and 10), as shown in (*A*). The 2.2 kb DNA fragment from the WT locus observed in the KO strain (lane 4) correspond to the WT copies of the gene present in the micronucleus. The 1.3-kb DNA fragment from the KO locus is present only in the *DES7* strain (lanes 2 and 5). Lane *M*: marker for fragment length. (*C*) RNA transcripts from the TTHERM_00310640 ORF measured in the WT and *DES7* mutant by RT-PCR. The fragment was amplified with primers 1 and 2. The *ATU1* gene (α-Tubulin) was used as control, amplified with primers 15 and 16. See supplementary table S1, Supplementary Material online, for primer descriptions. (*D*) GC/MS chromatogram of acetylated sterols obtained from WT (black line) and KO*DES7* (gray line) strains cultured for 48 h in SPP medium supplemented with 10 μg/ml cholesterol. Peak identity was determined by comparison of the corresponding mass spectra with the NIST08 Library, and the chemical structure of the compounds is indicated in (*E*). (*F*) Quantification of integrated peak areas from the chromatogram in (*D*) showing the relative percentage of each sterol found. (Δ^5: cholesterol; $\Delta^{5,7}$: 7-dehydrocholesterol; $\Delta^{5,22}$: 22-dehydrocholesterol; $\Delta^{5,7,22}$: 7,22-*bis*-dehydrocholesterol).

C24-de-ethylase) were affected in the *DES7* mutant, cultures of WT and KO*DES7* strains were grown in media supplemented with different sterol substrates. All the compounds recovered with their relative abundance are presented in table 1. In the WT strain, the conversion of all substrates tested led to the accumulation of the final product 7,22-*bis*-dehydrocholesterol, whereas the sterol profiles obtained from cultures of the *DES7* mutant all showed the accumulation of 22-dehydrocholesterol as the end product, independently of the original substrate. For example, in the case of stigmastanol (24-ethyl-5α-cholestan-3β-ol), a 29C fully saturated phytosterol, the products recovered included the

Table 1. Sterols Recovered from WT and KO*DES7* Mutant Cultured with Different Substrates.

Substrate	Products Obtained from Each Strain (Relative %)	
	CU428 (WT)	KO*DES7*
Cholestanol (5α-cholestan-3β-o l)	Cholestanol (4.23) Cholest-22-en-3β-ol (1.72) Cholesta-5,7-dien-3β-ol (1.08) Cholesta-5,7,22-trien-3β-ol (92.97)	Cholestanol (11.7) Cholest-22-en-3β-ol (85.94) Cholesta-5,22-dien-3β-ol (2.36)
Stigmastanol (5α-24-Ethylcholestan-3β-ol)	Stigmastanol (22.75) 5α-24-Ethylcholest-7-en-3β-ol (32.37) 24-Ethylcholest-5-en-3β-ol (0.18) 24-Ethylcholesta-5,7-dien-3β-ol (19.67) 24-Ethylcholesta-5,22-dien-3β-ol (0.74) 24-Ethylcholesta-5,7,22-trien-3β-ol (3.34) Cholest-22-en-3β-ol (1.07) Cholest-7-en-3β-ol (4.52) Cholesta-5,7-dien-3β-ol (1.63) Cholesta-7,22-dien-3β-ol (7.51) Cholesta-5,7,22-trien-3β-ol (6.22)	Stigmastanol (38.22) 24-Ethylcholest-5-en-3β-ol (1.47) 24-Ethylcholest-22-en-3β-ol (25.93) 24-Ethylcholesta-5,22-dien-3β-ol (0.81) Cholestanol (0.7) Cholest-22-en-3β-ol (32.36) Cholesta-5,22-dien-3β-ol (0.51)
Stigmasterol (24-Ethylcholesta-5,22-dien-3β-ol)	Stigmasterol (8.38) 24-Ethylcholesta-5,7,22-trien-3β-ol (37.15) Cholesta-5,22-dien-3β-ol (4.23) Cholesta-5,7,22-trien-3β-ol (50.24)	Stigmasterol (58.72) Cholesta-5,22-dien-3β-ol (41.28)

Δ5-, Δ22-, and Δ5,22-unsaturated derivatives both from the "cholestan" (27C) and "stigmastan" (29C) series, as well as cholestanol, the 27C saturated sterol produced by the removal of the ethyl group from C24 by Des24p (Tomazic et al. 2011). Taken together, these results indicate that the KO*DES7* strain is impaired specifically in its sterol C7(8)-desaturase activity, without any evident effect on the remaining spectrum of sterol modifications known for this ciliate.

Sequence Analysis of the *T. thermophila* Ortholog of Neverland/DAF-36

Rieske-type oxygenases belonging to the Nvd/DAF36 family were initially described in insects and nematodes (Rottiers et al. 2006; Yoshiyama et al. 2006) and found to be highly conserved in both protostome and deuterostome animals, except in mammals (Yoshiyama-Yanagawa et al. 2011). These enzymes constitute the terminal oxygenase of two- or three-component systems in which NAD(P)H-derived electrons are transferred to the Rieske's active site via an electron transport chain. The reductive power is thus used to activate molecular oxygen for the oxygenase component that performs the catalysis step (Ferraro et al. 2005; Capyk and Eltis 2011).

Amino acid sequence alignments show that members of this family, including *T. thermophila* Des7p, share the following structural architecture (fig. 4*A*): 1) presence of at least one N-terminal hydrophobic segment; 2) the Rieske domain, harboring its namesake [2Fe-2S] cluster coordinated by two His and two Cys (C-X-H-X_{16-17}-C-X_2-H) residues that works as electron acceptor; 3) a C-terminal catalytic domain harboring the active-site nonheme Fe(II) motif, coordinated by a His-His-carboxylate facial triad (Capyk and Eltis 2011) (fig. 4*A*, bottom right). In addition, Des7p shares, respectively, 29% and 28% amino acid identity with *D. melanogaster* Nvd (NP_001097670) and *C. elegans* DAF-36 (NP_505629). The ciliate protein has, as a distinctive feature, a 25-amino acid insertion between the Rieske and the nonheme iron motifs, compared with Opisthokont sequences (fig. 4*A*).

According to the *T. thermophila* genome database (Eisen et al. 2006; Coyne et al. 2008), the TTHERM_00310640 gene model was predicted to be composed of three exons of 45, 77, and 1,421 bp, respectively. However, after the extensive reannotation of the *Tetrahymena* genome, and the recently published data from deep RNA sequencing transcriptomic analysis (Xiong et al. 2012), the *Tetrahymena* Functional Genomics Database (http://tfgd.ihb.ac.cn/) indicated that the model for the TTHERM_00310640 gene was mispredicted and that, in its corrected version, the complete ORF is encoded in one single exon (fig. 4*B*). Accordingly, its mRNA would have a 107-bp 5′-UTR and a 145-bp 3′-UTR, flanking the 1,362 bp ORF encoding a protein composed of 453 amino acids.

A Des7p:eGFP Fusion Protein Localizes in Membrane Compartments

Previous studies by our group have shown that the sterol C7(8)-desaturase activity in *Tetrahymena* is localized in the microsomal fraction of cell-free extracts (Valcarce et al. 2000; Nusblat et al. 2005). With the aim of establishing if the subcellular location of Des7p is in accordance with those results, a *T. thermophila* strain was constructed expressing Des7p fused to enhanced green fluorescent protein (eGFP) (see Materials and Methods for details on the construction). The construct was prepared, so that the eGFP coding sequence was fused in frame to the 3′-end of the *DES7* coding region. In this way, the expression of the fusion protein is under the control of the endogenous *DES7* promoter. Microscopic analysis (fig. 5) showed Des7p:eGFP localized in defined compartments compatible with microsomes, as well as in membranes surrounding both micro and macronucleus (fig. 5*B*). Although

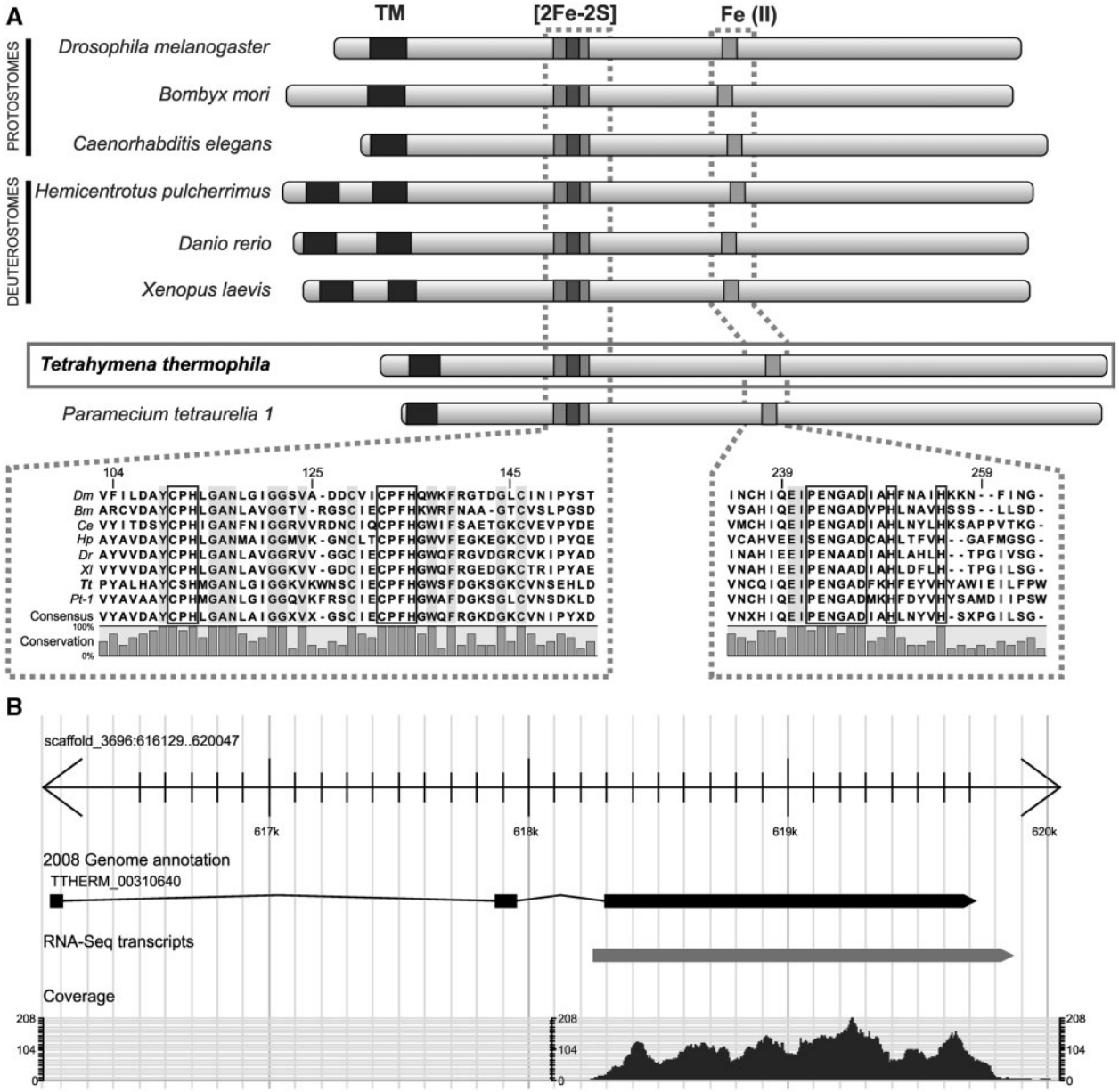

Fig. 4. Domain architecture of Nvd/DAF-36 proteins and structure of the TTHERM_00310640 gene. (*A*) Schematic interpretation of the predicted Nvd/DAF-36 polypeptides from different animals and from the ciliates *Tetrahymena thermophila* (Des7p, boxed) and *Paramecium tetraurelia*. Only one of the six paralogs from *P. tetraurelia* is shown (fig. 6). Proteins are aligned by their Rieske [2Fe-2S] motifs. The transmembrane (TM) domains were predicted by TMHMM 2.0. Partial sequence alignments of the Rieske [2Fe-2S] motif ($CXHX_{16}CX_2H$) and the nonheme iron binding motif ($(D/E)X_3DX_2HX_4H$) are illustrated at the bottom. Boxed are consensus residues important for catalysis. (*B*) GBrowse snapshot showing the mispredicted 2008 version of the TTHERM_00310640 gene, and the current gene model according to RNA-seq data (modified from http://tfgd.ihb.ac.cn/).

Des7p:eGFP was inactive as sterol desaturase (data not shown), these results are in agreement with previous reports on the localization of sterol C7(8)-desaturase activity in this species (Valcarce et al. 2000; Nusblat et al. 2005). A similar microsomal localization pattern was reported for the *B. mori* ortholog, Bm-Nvd:GFP, overexpressed in *Drosophila* S2 cells (Yoshiyama-Yanagawa et al. 2011) as well as for the *T. thermophila*'s sterol C5(6)-desaturase, Des5Ap (Poklepovich et al. 2012).

Interestingly, although there seems to be no difference in Des7p:eGFP localization when the cells are grown with or without added sterols, fluorescence is clearly induced by the presence of cholesterol in the growth media (fig. 5). Taking into consideration that the strain was constructed by placing the *DES7:eGFP* fusion gene under the control of the *DES7* endogenous promoter, this result suggests the existence of specific sterol sensing and transcriptional control mechanism/s, which would require further investigation.

Evolution of Rieske-Type Oxygenases in Ciliates

Previous reports on *nvd/daf-36* sterol desaturases emphasized the high degree of conservation of this family of genes across the animal phyla, including insects and nematodes, and

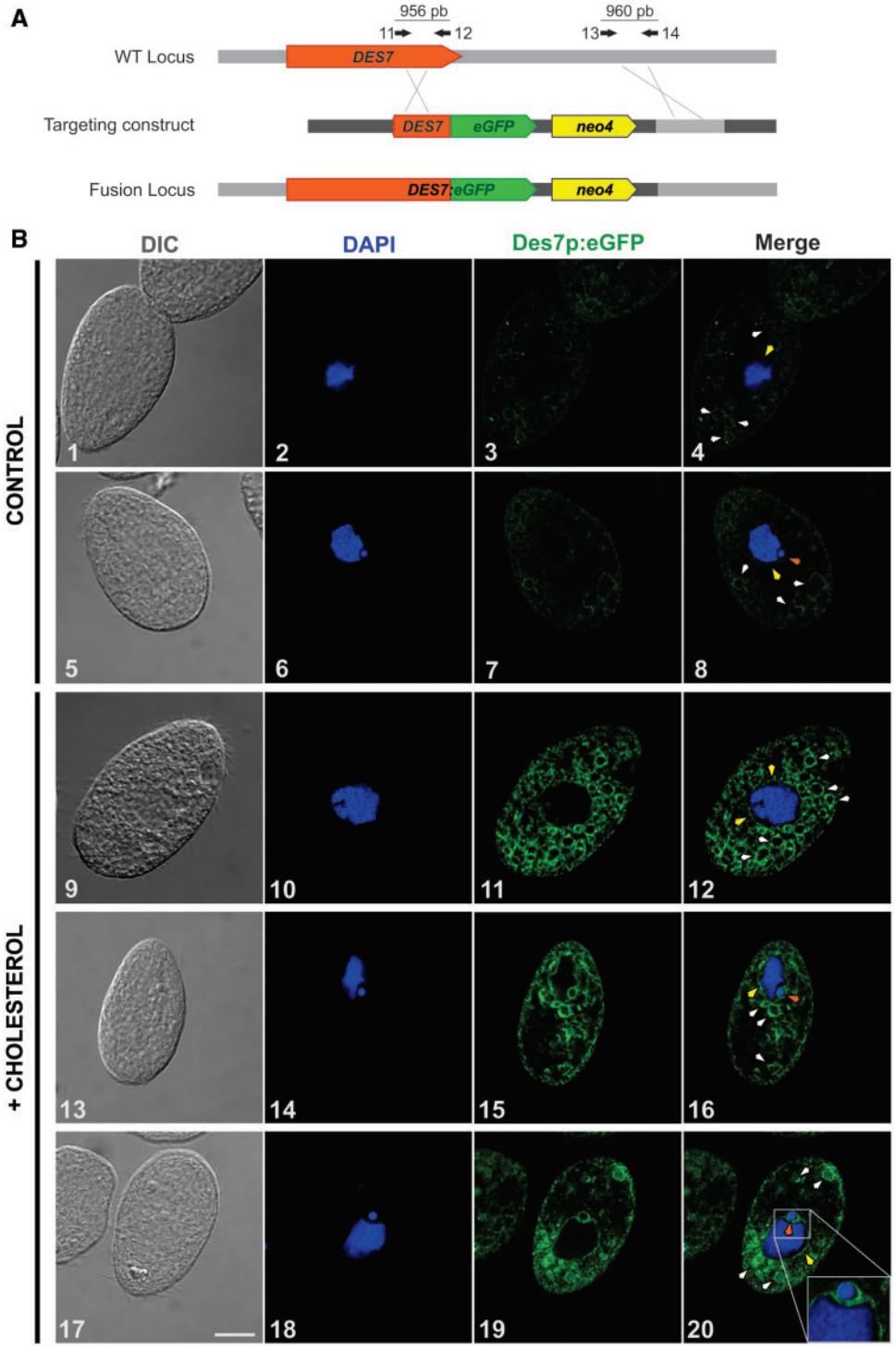

Fig. 5. Subcellular localization of Des7p. (*A*) Schematic representation of the strategy used to construct the Des7p:eGFP strain. The fusion protein is under the control of the *DES7* endogenous promoter. (*B*) Confocal laser scanning micrographs of cells grown in SPP medium (1–8) and in SPP medium supplemented with 10 μg/ml cholesterol (9–20). The fusion protein (green) is localized in membranes surrounding both the macro- and micronucleus (yellow and orange arrowheads, respectively—see inset in 20) as well as in widely distributed punctate microsomes (white arrowheads). Scale bar: 10 μm.

several deuterostome species, with the exception of mammals (Yoshiyama et al. 2006; Yoshiyama-Yanagawa et al. 2011). A BLASTp search using the available sequence information in different genome databases showed that, besides the already reported animal sequences, clear orthologs are present in the genomes of ciliates and other protists species. A maximum likelihood phylogenetic reconstruction was conducted with 62 amino acid sequences, as described in Materials and Methods. As shown in the phylogenetic tree (fig. 6), one Nvd/DAF-36 ortholog was found in the genome of each *Tetrahymena* species hitherto sequenced (*T. thermophila*, *T. malaccensis*, *T. eliotti*, and *T. borealis*)

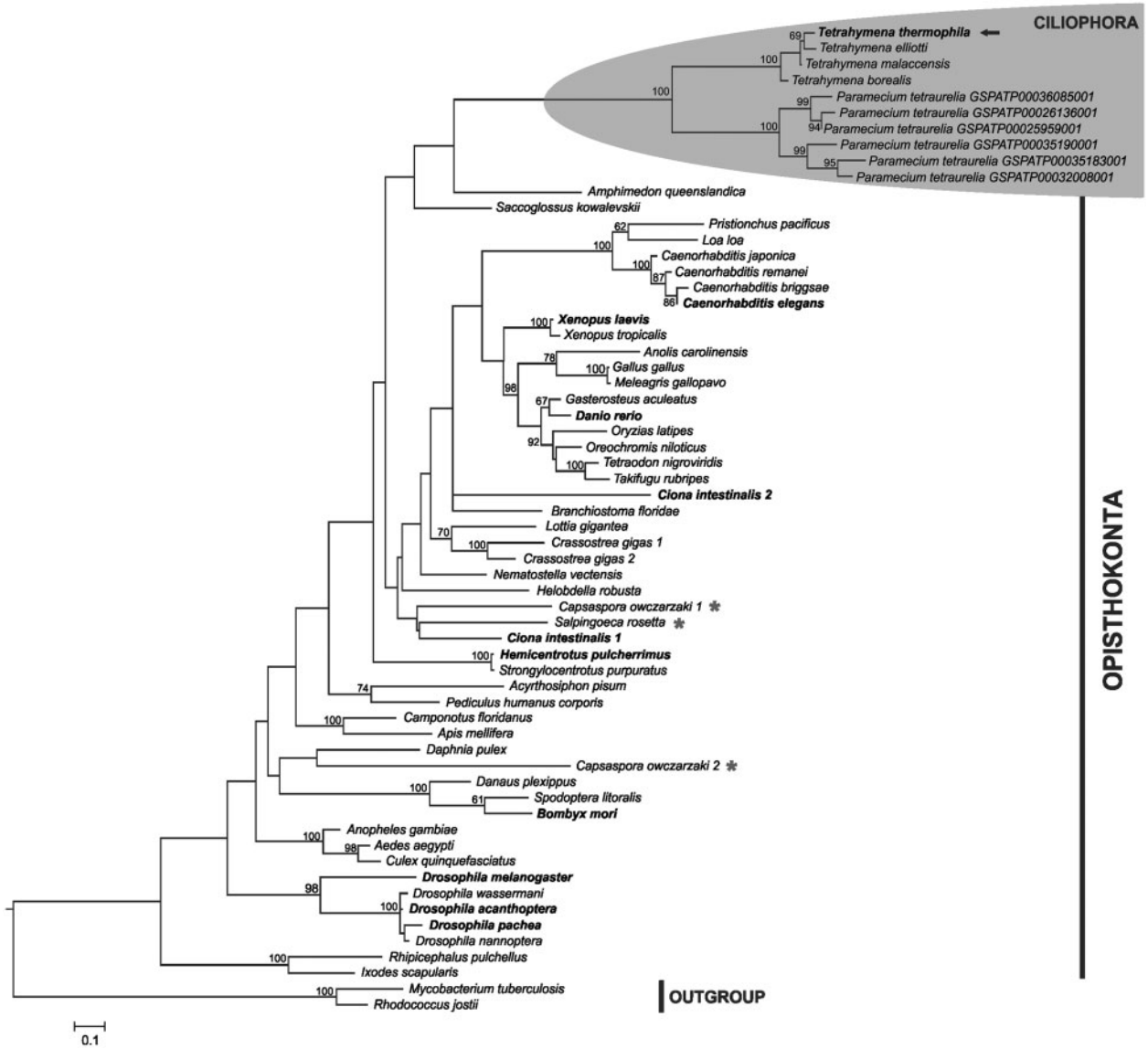

Fig. 6. Phylogenetic analysis of Nvd/DAF-36 Rieske-type oxygenases. A maximum likelihood phylogenetic reconstruction was conducted with 62 amino acid sequences (accession numbers listed in supplementary table S2, Supplementary Material online). Orthologs whose sterol C7-desaturase activity was experimentally demonstrated are indicated in bold. The sequence from *Tetrahymena thermophila* characterized in this work is indicated by an arrow. Sequences from unicellular Opisthokonts are indicated by an asterisk. Bootstrap values greater than 50% are shown. It should be noted that some branches do not reflect the normally assumed phylogenetic relationships between taxa.

(http://www.broadinstitute.org/annotation/genome/Tetrahymena/MultiHome.html), as well as six paralogs (44–46% amino acid identity with Des7p), with a high degree of conservation, in the genome database of the related ciliate *P. tetraurelia* (Arnaiz and Sperling 2011). No ortholog was found in the genome database of the *Tetrahymena*'s relative parasitic species *Ichthyophthirius multifiliis*, but this organism seems to have lost the set of genes required for sterol modifications and tetrahymanol biosynthesis; in fact, it is only capable of producing cholesterol esters from cholesterol acquired from its fish host (Coyne et al. 2012). We were also unable to find orthologs in the genome database of the spirotrichous ciliate *O. trifallax* (OxyDB, http://oxy.ciliate.org) nor in the genome databases of Apicomplexa (http://EupathDB.org). Interestingly, besides the ciliate sequences, other sequences of protist origin were also identified in the genomes of the unicellular Holozoans *Salpingoeca rosetta* (Choanomonada) and *Capsaspora owczarzaki* (Filasterea), suggesting a horizontal gene transfer event, probably from a unicellular Opisthokont to a phagotrophic ancestor of Oligohymenophorean ciliates. In support of this hypothesis, Holozoan sequences have greater identity (31–34%) than the metazoan ones (28–29%) when compared with Des7p. According to the current evidence, these results can be explained assuming that a *nvd/daf-36* ortholog was horizontally transferred, most probably from an unicellular Opisthokont to a common ancestor of *Tetrahymena* and *Paramecium* within the ciliate lineage before these genera diverge, an event that is estimated to have occurred about 550 Ma (Parfrey et al. 2011). More genomic data (taxon sampling) are

needed to further understand the evolution of this protein family in ciliates.

Our phylogenetic analysis includes orthologs from metazoan orders that were not taken into account in an earlier report (see fig. S2 in Yoshiyama-Yanagawa et al. [2011]), implying that this protein family is more widely distributed within the Opisthokonta than previously proposed.

Discussion

Conversion of cholesterol into 7-dehydrocholesterol is a crucial step in steroid hormone biosynthesis in insects and nematodes. This reaction is catalyzed by Rieske-type oxygenases belonging to the Nvd/DAF-36 family of proteins. These enzymes are highly conserved in animals, and functional orthologs were described for both protostomes and deuterostomes, including several *Drosophila* species, *B. mori*, *C. elegans*, the ascidian *Ciona intestinalis*, the sea urchin *Hemicentrotus pulcherrimus*, the frog *Xenopus laevis*, and the zebrafish *Danio rerio* (Wollam et al. 2011; Yoshiyama-Yanagawa et al. 2011; Lang et al. 2012). We report here the first example of a nonanimal member of this family of proteins with sterol C7(8)-desaturase activity in *T. thermophila*. We also found Nvd/DAF-36 orthologs in the genome database of the ciliates *T. malaccensis*, *T. eliotti*, *T. borealis*, and *P. tetraurelia* (fig. 6). In the last case, six paralogs were identified. The presence of more than one paralog of a particular gene in the genome of *P. tetraurelia* is not surprising given its particular evolutionary history involving at least three whole genome duplications (Aury et al. 2006). *Paramecium*, itself a sterol auxotroph, requires exogenous 29C phytosterols, such as β-sitosterol or stigmasterol, which the ciliate converts into the end product 7-dehydrostigmasterol (Whitaker and Nelson 1987).

Our phylogenetic analysis showed the presence of orthologs in the genomes of the protists *S. rosetta* and *C. owczarzaki*, suggesting that Nvd/DAF-36 could be an ancient character within the Opisthokonta. Nevertheless, the probable enzymatic activity of these putative proteins is merely speculative given the lack of experimental data regarding sterol metabolism in these species.

The gene coding for the sterol C7(8)-desaturase in *T. thermophila*, TTHERM_00310640, was identified by RNA interference. The feeding-RNAi procedure resulted in 40–56% decrease in Δ7-unsaturated derivatives, obtained from the interfered cultures when compared with control (fig. 2). To our knowledge, this is the first time that a feeding-RNAi protocol is applied with success for the screening of several genes in *Tetrahymena*. The strategy can be applied for high-throughput analysis, as it permits avoiding the methodological difficulties related to constructing knock-out strains in this species.

Once the gene TTHERM_00310640 was identified as coding for a sterol C7(8)-desaturase, it was named *DES7*, and its identity was further unequivocally confirmed by constructing a somatic knock-out strain, which was completely impaired in C7(8)-desaturase activity (fig. 3). Nevertheless, the remaining sterol modifying activities, named C5(6) and C22(23)-desaturation and the removal of the ethyl group from C24 of 29C phytosterols, were unaffected in cultures of KO*DES7* grown in the presence of diverse sterol substrates (table 1). These results imply that Des7p catalyzes the specific introduction of a double bond at the C7(8) position of a wide range of sterol substrates, independently of the presence of other modifications in the sterol moiety. Orthologs from *B. mori* and different *Drosophila* species are known to have more than one activity, allowing sterol desaturases to introduce double bonds at positions C5(6) and C7(8), depending on the sterol utilized as substrate (Lang et al. 2012). These insect species lack cytochrome b_5-dependent C5(6) desaturases, relying on the bifunctional Nvd proteins to desaturate sterols at that position, whereas in *T. thermophila*, *DES5A* appears to encode the sole ciliate's enzyme with cytochrome b_5-C5(6)-desaturase activity. It was functionally characterized in our laboratory. Although we originally reported a remaining 5% of C5(6) desaturase activity in the knocked out cells (Nusblat et al. 2009), now we know that this was due to an incomplete assortment of the somatic mutant strain. After selective pressure with higher concentrations of paromomycin, the KO*DES5A* strain was completely impaired in such desaturase activity.

A strain expressing a Des7p:eGFP fusion allowed us to subcellularly localize this protein in the membranes of discrete microsomal compartments, similar to what was reported for *B. mori* Nvd expressed in insect S2 cells (Yoshiyama-Yanagawa et al. 2011), as well as surrounding macro- and micronucleus. It is compatible with putative endoplasmic reticulum localization, perhaps forming a multienzymatic complex with C5(6) and C22(23) desaturases and C24-de-ethylase, probably sharing similar components or domains in electron chain/s and cofactor requirements. Nevertheless, it is striking that the two up to date identified enzymes, Des5p and Des7p, performing the same type of chemical modification on the same substrate, belong to different superfamilies and, most probably, make use of different electron donor chains (cyt b_5/cyt b_5 reductase and Rieske/ferredoxin reductase, respectively), also suggesting a different evolutionary origin. A horizontal gene transfer event from an opisthokont protist to an ancestral phagotrophic ciliate is a plausible scenario in the acquisition of the Rieske oxygenase. In addition, by growing this strain in the presence of cholesterol, we notice a significant upregulation of Des7p:eGFP, in comparison with the cells grown without cholesterol (fig. 5*B*). The expression profile of *DES7* (formally TTHERM_00310640) obtained by Xiong et al. (2012), publically available at the *Tetrahymena* Functional Genomics Database (http://tfgd.ihb.ac.cn/search/trans/tname/gene_000020936), shows this gene is almost unvaryingly expressed during the vegetative growth phase and displays a differential expression pattern in starvation conditions and during the conjugation process (supplementary fig. S1, Supplementary Material online). It would therefore not be surprising that an underlying mechanism regulates transcription of *DES7* in response to different stimuli.

The fact that this enzyme is directly involved in the biosynthesis of steroid hormones in many organisms (Rottiers et al. 2006; Yoshiyama et al. 2006) suggests that it could play a

similar role in *T. thermophila*. Interestingly, it has been stated that *Tetrahymena* respond to hormones of different nature, including steroids (Csaba 2012; Kohidai et al. 2003). However, there is no evidence for further modification of the triunsaturated sterols produced by this ciliate. The conservation of the molecular machinery used by this free living protist to modify sterols, while it is absolutely capable of growing without added sterols by synthesizing tetrahymanol as surrogate, still remains a mystery.

Materials and Methods

Tetrahymena Strain and Growth Conditions

Tetrahymena thermophila strain CU428, *mpr1-1/mpr1-1* (mp-s, VII), designated the WT in this work, was a gift from Martin A. Gorovsky (University of Rochester, NY). Cells were grown in 250 ml flasks containing 100 ml SPP medium of the following composition (wt/vol): 1% beef peptone, 0.1% yeast extract, 0.2% glucose, and 0.003% iron citrate. In sterol desaturase activity assays, medium was supplemented with cholestanol (5α-cholestan-3β-ol), cholesterol (cholest-5-en-3β-ol), stigmastanol (5α-24-ethylcholestan-3β-ol), or stigmasterol (24-ethylcholesta-5,22-dien-3β-ol) (all from Sigma-Aldrich) at a final concentration of 10 μg/ml, which were added from 5 mg/ml stock solutions in ethanol. When indicated, paromomycin (Sigma-Aldrich) was added from a 300 mg/ml stock solution in water, together with 1 μg/ml of $CdCl_2$, which was prepared as a 0.5 mg/ml stock solution in water.

Media were inoculated daily with a 1:10 dilution of a 24-h culture. Cultivation was carried out in a rotary shaker (180 rpm) at 30 °C.

Standard DNA and RNA Manipulation Procedures

Genomic DNA from *T. thermophila* CU428 was prepared with DNAzol reagent (Invitrogen, Carlsbad, CA). The isolation of plasmid DNA from bacteria was performed with a Wizard Plus SV miniprep DNA purification system kit (Promega, Madison, WI). Total RNA was prepared from *T. thermophila* cultures grown for 24 h using TRIzol reagent (Invitrogen, Carlsbad, CA). Nucleic acid fragments were amplified by PCR using *Taq* DNA polymerase. For high-fidelity DNA amplification, *Pfx50* enzyme (Invitrogen, Carlsbad, CA) was the choice. The PCR products were separated on 1–2% agarose gels, isolated, and recovered using the illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare, UK). DNA sequencing was performed by the University of Maine DNA Sequencing Facility (Orono, ME). For RNA analysis, RT reactions were carried out using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) according to the manufacturer's instructions. All RNA samples were treated with DNAse I before amplification. cDNA synthesis was monitored by PCR with α-tubulin primers. The primers used for all amplification reactions are listed in supplementary table S1, Supplementary Material online.

Construction of Vectors for dsRNA Production and RNA Interference by Feeding Protocol

DNA fragments homologous to the gene to be targeted were PCR amplified and cloned into the PCR-Blunt-TOPO II vector (Invitrogen, Carlsbad, CA). Once checked by sequencing, inserts were subcloned into the *Eco*RI site of plasmid pLitmus28i (New England BioLabs, Ipswich, MA). These vectors were used to transform the RNAse-III-deficient *E. coli* strain HT115 (F^-, *mcrA*, *mcrB*, IN [*rrnD-rrnE*]1, lambda$^-$, *rnc4*::*Tn10* [DE3 lysogen: *lacUV5* promotor-T7 polymerase]), used to feed the ciliates (Takiff et al. 1989; Timmons and Fire 1998). The HT115 strain was a generous gift from Dr Martin C. Simon, University of Kaiserslautern, Germany.

The feeding bacteria were prepared in 5 ml of Luria-Bertani broth, supplemented with 100 μg/ml ampicillin: 0.5 ml of an overnight culture was inoculated into fresh medium and incubated at 37 °C in a rotary shaker set at 250 rpm for 1 h, to reach an OD_{600} between 0.4 and 0.6. Production of dsRNA was induced by adding IPTG to a final concentration of 0.5 mM. After 4 h of induction, bacteria were collected by centrifugation and resuspended in glucose-depleted modified SPP medium (1% beef peptone, 0.1% yeast extract, and 0.003% iron citrate, wt/vol) to be used to feed the ciliates.

Ciliates were fed daily with induced bacteria in glucose-depleted modified SPP medium supplemented with 100 μg/ml ampicillin and 0.5 mM IPTG to ensure the continued induction of dsRNA production by bacteria. Because of the fast growth of *Tetrahymena* cells, the cultures were diluted daily with fresh medium at a concentration of 10,000 cells/ml and incubated in a rotary shaker set at 30 °C and 180 rpm. After 4 successive days of feeding with the *E. coli* cells, *Tetrahymena* cultures were supplemented with 10 μg/ml cholesterol and maintained for another 2 days, without further addition of bacteria. As a control, cells were fed with induced bacteria containing the vector without insert. On the 6th day, cultures were harvested and their sterol profiles were analyzed by GC/MS.

Construction of the Transformation Sequence *CDES7* and the KO*DES7* Mutant

For *DES7* gene disruption in *T. thermophila*, we constructed the transformation sequence *CDES7* to perform the somatic knockout. Briefly, the flanking regions of the TTHERM_00310640 putative sequence were PCR amplified, as a 0.90 kb fragment upstream (UP) and 0.88 kb fragment downstream (DW) sequences using primers 4–5 (UP) and 6–7 (DW) (supplementary table S1, Supplementary Material online). The two fragments were cloned into *Hind*III and *Pst*I sites, respectively, of pEGFP-*neo4* (Kataoka et al. 2010) using directional In-Fusion Cloning techniques (Clontech) as described (Bright et al. 2010). The *CDES7* construct was then released with *Pst*I and *Xho*I restriction enzymes from the vector and was introduced into the macronucleus of *T. thermophila* CU428 cells by biolistic transformation (Cassidy-Hanley et al. 1997). The knockout mutant was generated by replacement of the WT gene for the KO construct by homologous recombination. The somatic

transformants were selected with 120 μg/ml of paromomycin in the presence of 1.0 μg/ml $CdCl_2$, which induces the *MTT1* promoter of the *neo4* expression cassette (Mochizuki 2008), and were transferred daily in increasing concentrations of paromomycin to allow phenotypic assortment. Single cells were then isolated and the clones expanded with daily transfers for 2 weeks at increasing concentrations of paromomycin until 150 mg/ml, where only mutant alleles remained, thus producing complete macronuclear-knockout strains. Proper integration of the construct was checked by PCR, and the level of gene segregation was assayed by RT-PCR. In the first case, the elimination of the *DES7* gene from the macronucleus was confirmed by allele-specific PCR by using primer pairs 8–9 and 10–9 (supplementary table S1, Supplementary Material online) for WT or KO locus, respectively. For transcript-level assays, WT and KO*DES7* mutant strains were grown in SPP medium. After RNA extraction and purification, cDNAs were obtained and analyzed by PCR amplification with primers 1 and 2 (supplementary table S1, Supplementary Material online), designed on part of the single exon of *DES7*. The products were separated on 1% agarose gels. α-Tubulin (*ATU1*) transcript detection, amplified with primers 15 and 16, was used as a control (supplementary table S1, Supplementary Material online).

Construction of a *T. thermophila* Strain Expressing the Des7p:eGFP Fusion Protein

A 956-bp C-terminal fragment of the TTHERM_00310640 gene, lacking the stop codon (primers 11–12), and a downstream flanking region of 960 bp (primers 13–14) were amplified by PCR and cloned into the *Bam*HI and *Xho*I sites, respectively, of pEGFP-*neo4* (Kataoka et al. 2010) using directional In-Fusion Cloning techniques (Clontech). The *DES7-eGFP* construct was then released with *Nhe*I and *Kpn*I restriction enzymes from the vector and was introduced into the endogenous *DES7* locus by homologous integration as diagrammed in figure 5*A*. Somatic transformants were obtained as described earlier.

Microscopic Analysis

The *T. thermophila DES7:eGFP* strain was grown in SPP medium (5 ml) with and without 10 μg/ml cholesterol for 24 or 48 h. Cells were collected by centrifugation, washed twice with PHEM buffer (60 mM PIPES [piperazine *N,N'*-bis{2-ethane-sulfonic acid}], 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, pH 6.9), and fixed for 30 min with 3% paraformaldehyde in PHEM buffer. Fixed cells were rinsed twice with phosphate-buffered saline (PBS) and stained with 1 mM DAPI (4',6-diamidino-2-phenylindole, diluted in PBS) for 1 h at room temperature. After being rinsed with PBS, cells were mounted onto poly-L-lysine-coated coverslips, allowed to settle for 20 min, and placed onto slides with a drop of Vectashield mounting medium (Vector Laboratories). Cells were examined by Confocal Laser Scanning and Nomarski Differential-Contrast Microscopy with a Nikon Eclipse TE-2000-E2 microscope. Images were analyzed with EZ-C1 FreeViewer Gold version 3.90 (Nikon Corporation) and edited with Adobe Photoshop CS3 version 10.0 (Adobe Systems). Photoshop edition consisted only in cropping the images to fit them to the final figure design, and no other modifications were performed.

Identification of Sterols by GC/MS

Cells from cultures with added sterol were collected by centrifugation at 3,000 × g for 5 min at 4 °C and washed twice with 20 ml of distilled water, and the lipids were extracted according to Bligh and Dyer (1959). The organic phase was evaporated to dryness under a N_2 stream, and the lipids were saponified. After 2-fold extraction with 2 ml hexane, the organic solvent was evaporated under N_2 stream, and the residue was resuspended in 50 μl of distilled pyridine. One hundred microliters of acetic anhydride was added, and the mixture was incubated for 40 min at 80 °C. The composition of the steryl acetate ester derivatives was analyzed by running samples through an SPB-1 column (30 m × 0.25 mm × 0.25 μm; Supelco) in a Shimadzu GC-2010 Plus gas chromatograph. The column was temperature programmed at 5 °C/min from 160 to 320 °C and subsequently held for 10 min at 320 °C. MS was carried out using a GCMS-QP2010 Plus mass detector, operated at an ionization voltage of 70 eV with a scan range of 20–600 atomic mass units. The retention times and mass spectra of all new peaks obtained were compared with those of standards (Sigma-Aldrich) and those available in the National Institute of Standards and Technology mass spectral library.

Phylogenetic and Sequence Analyses

The data set for the phylogenetic analysis was prepared with sequences obtained from Genbank, JGI, Ensembl, UniProtKB, and Broad Institute (62 sequences, accession numbers in supplementary table S2, Supplementary Material online) using Basic Local Alignment Search Tool against the nonredundant protein database. Amino acid sequences in the final data sets were aligned using the MAFFT algorithm (Katoh et al. 2002) implemented in the online resource at CBRC, Japan (http://mafft.cbrc.jp/alignment/server/). Confidence of the alignment was assessed with GUIDANCE (Penn et al. 2010). Unreliable columns below a confidence score of 0.93 were removed. The phylogenetic reconstruction was performed by the maximum likelihood method using the RaxML algorithm version 2.2.3 (Stamatakis 2006) implemented in the TOPALi 2.5 software (Milne et al. 2004). The WAG empirical model (Whelan and Goldman 2001) was used to analyze amino acid sequence evolution. The parameters proportion of invariant sites and across-site variation (gamma) were estimated for each data set and used in the analysis. Branch support was evaluated by 300 bootstrap pseudoreplicates.

Supplementary Material

Supplementary tables S1 and S2 and figure S1 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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