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Review

Incomplete sterols and hopanoids pathways in ciliates: Gene loss and acquisition during evolution as a source of biosynthetic genes

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ABSTRACT

Polycyclic triterpenoids, such as sterols and hopanoids, are essential components of plasmatic membrane in eukaryotic organisms. Although it is generally assumed that ciliates do not synthesize sterols, and many of them are indeed auxotrophic, a large set of annotated genomic sequences and experimental data from recently studied organisms indicate that they can carry putative genes and respond to the presence/absence of precursors in various ways. The pre-squalene pathway, for instance, is largely present in all sequenced ciliates except in *Ichthyophthirius multifiliis*; although *Paramecium tetraurelia* lacks the squalene synthase and *Oxytricha trifallax* the squalene hopene synthase, in addition to the former. On the other hand, the post-squalene pathway, requiring oxygen in several steps, is mostly incomplete in all ciliates analyzed. Nevertheless, a number of predicted genes, with high sequence similarity to C-4 methyl oxidase/s, C-14 demethylase, C-5 and C-7 desaturases and C-24 reductase of sterols are found in *Tetrahymena* and *Paramecium*, and scattered in other Stichotrichia ciliates. Moreover, several of these sequences are present in multiples paralogs, like the C-7 desaturase in *Paramecium*, that carries six versions of the only one present in *Tetrahymena*.

The phylogenetic analyses suggest a mixed origin for the genes involved in the biosynthesis of sterols and surrogates in this phylum; while the genes encoding enzymes of the pre-squalene pathway are most likely of bacterial origin, those involved in the post-squalene pathway, including the processing of sterols obtained from the environment, may have been partially retained or acquired indistinctly from lower eukaryotes or prokaryotes. This particular combination of diverse gene/s acquisition patterns allows for survival in conditions of poor oxygen availability, in which tetrahymanol and other hopanoids may be advantageous, but also conditions of excess oxygen availability and abundant sterols, in which the latter are preferentially phagocyte, and/or transformed. Furthermore, the possibility that some of the genes involved in sterol metabolism may have another biological function in the most studied ciliate *T. thermophila*, was also explored.

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1. Introduction

1.1. Sterols and hopanoids

Sterols and hopanoids are polycyclic triterpenoids that derive from the cyclization of squalene or oxidosqualene by evolutionary related cyclases (Fig. 1). There is a large diversity in the distribution of pentacyclic triterpenoids in nature; as a rule of thumb, sterols are mostly found in eukaryotes, whereas hopanoids are found in prokaryotes (Volkman, 2005).

It is well known that sterols have essential functions in eukaryotic cells: (i) are the major non-polar lipids of cell membranes and, in association with phospholipids, modulate and refine membrane properties forming a liquid-ordered phase (Van Meer et al., 2008), (ii) serve as precursors of bile salts and a number of different steroid hormones implicated in growth, cell cycle and development in mammals, of brassinosteroids in plants (Benveniste, 2004), of antheridiol and oogoniol in aquatic fungi (Brunt and Silver, 1991), of ecdysteroids in arthropods (Gilbert et al., 2002) and of daphacronic acids in nematodes (Motola et al., 2006); (iii) cholesterol is actively involved in the formation of specialized microdomains, termed lipid rafts, which form platforms that function in signaling and trafficking (Hancock, 2006). It is assumed that hopanoids play similar roles in organisms that are not able to synthesize sterols; for instance, they control fluidity in bacterial membranes (Ourisson et al., 1987; Sáenz et al., 2012). However, other distinctly functions were also shown, such as tolerance towards high temperatures and low pH, and a role in the protection of a nitrogenase-complex against oxygen, in nitrogen fixing bacteria (Berry et al., 1993).

Hopanoids and sterols are derived from isopentenyl diphosphate (IPP), a universal precursor which is present and synthesized in the three domains of life (albeit by different pathways) with a clear preference for the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway in bacteria, the classical mevalonate pathway (MVA) in eukaryotes and the alternative MVA pathway in archaea (Fig. 1).

However, exceptions to this rule are also known. For instance some bacteria make use of the MVA pathway, a trait that has been commonly explained by the occurrence of genes likely acquired by

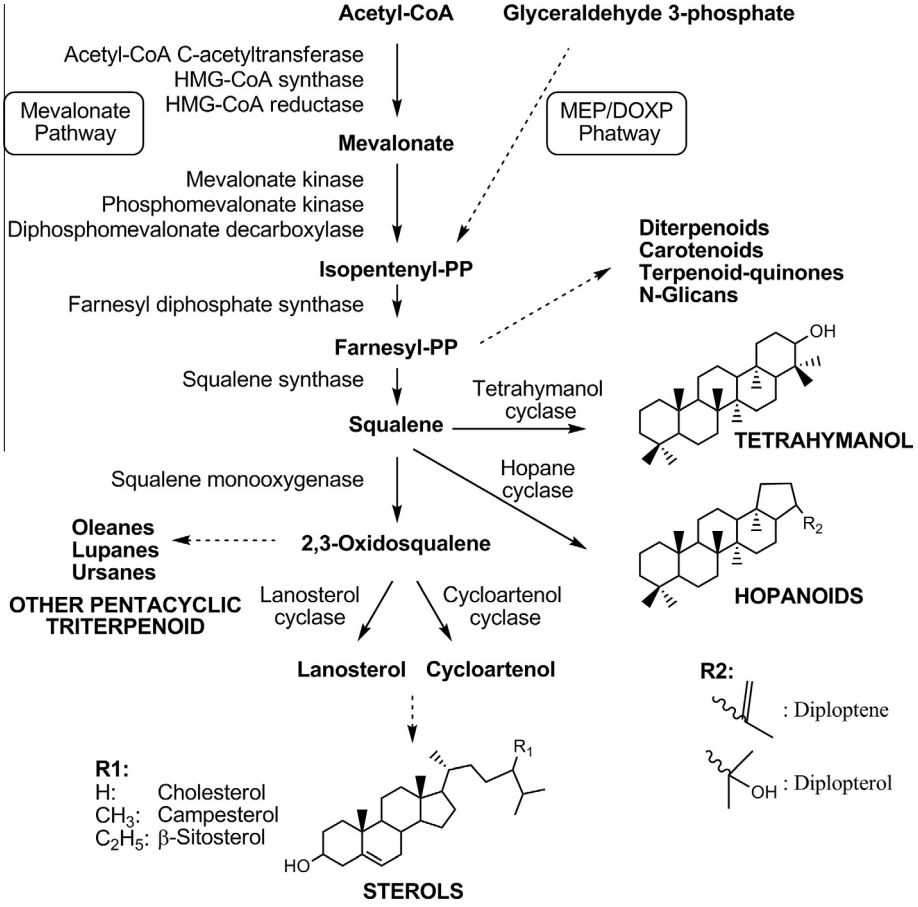

Fig. 1. Canonical polycyclic triterpenoid pathway, leading to hopanoids in bacteria, tetrahymanol in ciliates, sterols in most eukaryotic organism and oleanes, lupanes and ursanes in plants. The biosynthesis of Isopentyl-PP is shown via mevalonate pathway found in eukaryotes and archaea; and via MEP/DOXP pathway found in bacteria and several photosynthetic eukaryotes.

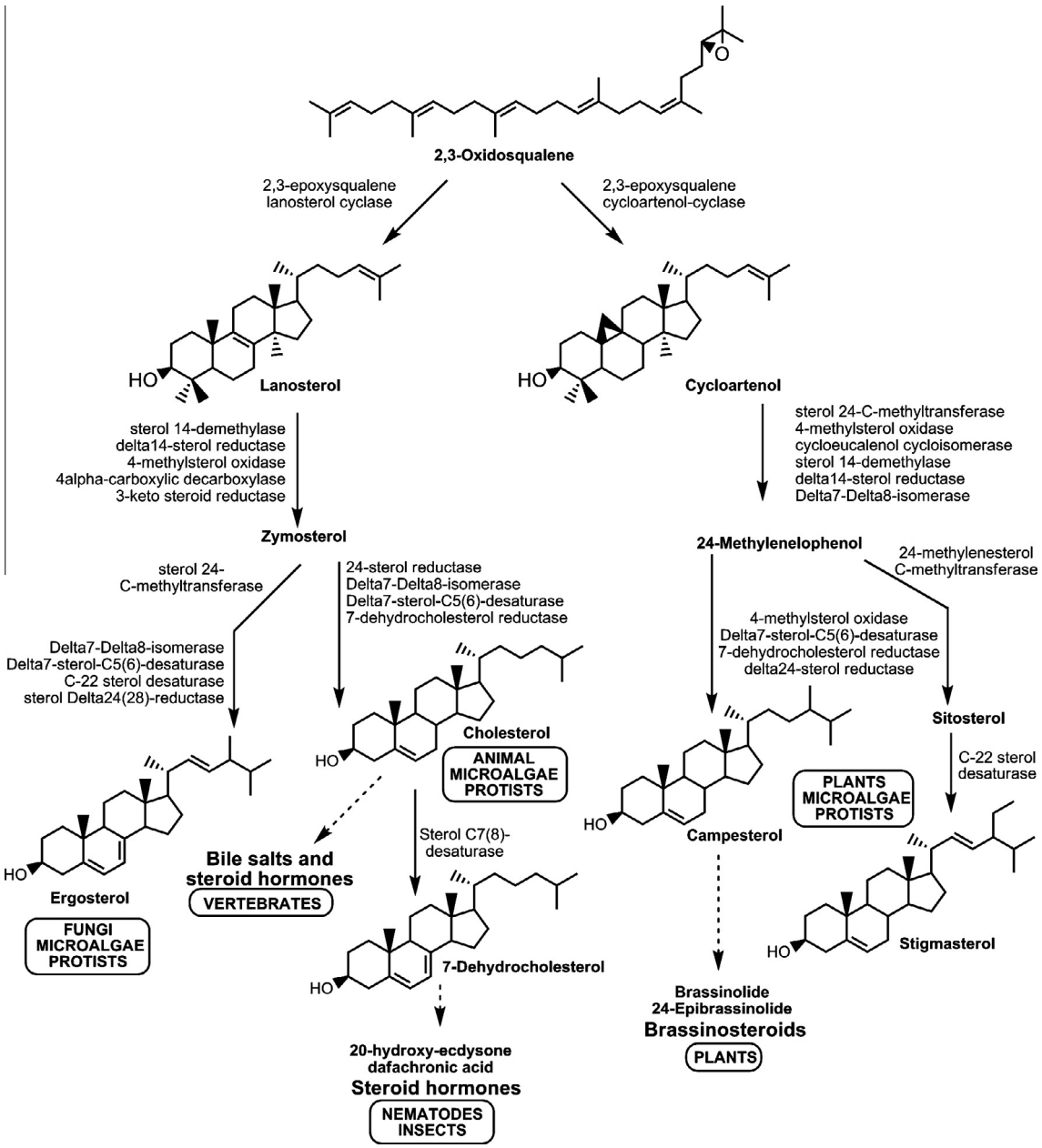

Fig. 2. Post-squalene sterol synthetic pathways in different organisms. Also shown are the steroid hormones in vertebrates, nematodes, insect and plants (brassinosteroids).

horizontal gene transfer (HGT) from archaeal or eukaryotic donors (Boucher and Doolittle, 2000). Alternatively, plants and other photosynthetic eukaryotes, such as microalgae and apicomplexa (a clade of parasitic protists derived from a photosynthetic ancestor) make use of the MEP pathway, in addition to the MVA. All these eukaryotic groups carry plastids suggesting that they may have obtained the MEP pathway through the transfer of genes from the original cyanobacterial endosymbiont that originated the plastids. Finally, most archaeal species lack the last enzymes of the classic (eukaryotic-like) MVA pathway (Desmond and Gribaldo, 2009; Lombard and Moreira, 2011).

Phylogenetic analyses (Lombard and Moreira, 2011) suggest that each of the three routes is ancestral to each domain, but with no enough data to know if these pathways were present in the last common ancestor of all organisms (cenancestor) or not.

The next crucial step in the biosynthesis of hopanoids and sterols derive from the choice to cyclization or oxidation of squalene. In the case of hopanoids, the squalene moiety is modified by a squalene–hopene cyclase (SHC) in a process that does not require oxygen. Hopanoids, particularly the C_{30} diplopterol and diploptene, as well as the C_{35} bacteriohopanes (bacteriohopanetetrol and aminobacteriohopanetriol) are the commonly synthesized sterol surrogates in prokaryotes. They are found in cyanobacteria, methanotrophic and thermoacidophile bacteria (Rohmer, 2010).

In the case of sterols the squalene moiety is first oxidized by a squalene epoxidase, leading to 2,3-oxidosqualene, and then by cyclization in a second step, either to lanosterol or to cycloartenol, by enzymes homologous to SHC (Fig. 1). The subsequent steps in the sterol pathway, including demethylation/s, reduction/s, desaturation/s, isomerization/s and methylation/s (Fig. 2), are highly linked to oxygen availability, suggesting that the last eukaryotic common ancestor (LECA) lived in an oxygenated environment. A commonly accepted hypothesis is thus that the pathway of sterol biosynthesis appeared after the emergence of oxygenic photosynthesis in the Proterozoic eon, where mildly oxic surface waters lay above an oxygen-minimum zone that was persistently anoxic

and commonly sulfidic (Canfield, 1998; Johnston et al., 2009; Parfrey et al., 2011). It has been put forward that the initial role of sterols in eukaryotes may have been that of protection against oxidative stress when oxygen levels rose (Galea and Brown, 2009). Moreover, it has been proposed that the ancestral pathway made cycloartenol as a final product, and that the rising concentrations of oxygen in the atmosphere would have led to an evolution of the pathway beyond cycloartenol toward more stable sterol compounds (Ourisson and Nakatani, 1994).

It is assumed that the LECA synthesized sterols; concomitantly, the origin of some enzymes of the sterol pathway must have been likely recruited from preexisting enzymes of distant bacterial homologs, in parallel to the emergence of the pathway. Other enzymes that do not have any bacterial homologue could presumably have arisen in the eukaryotic lineage itself (Desmond and Gribaldo, 2009).

Eukaryotic organisms able to synthesize sterols are animals, fungi, land plants, and many protists (Volkman, 2005). Animals form cholesterol as the final sterol, fungi synthesize ergosterol and land plants produce a mixture of phytosterols, which include β-sitosterol, stigmasterol and campesterol, as the major representatives (Figs. 1 and 2). Eukaryotic microalgae (microphytes) produce an extraordinary diversity of sterols, depending on the particular class, family, genus and even species. These sterols often carry an ethyl group in position C24 and unusual desaturations at various positions in the sterol moiety (Volkman, 2003) but also ergosterol and other 4-desmethyl sterol are often found (Fig. 2).

Although sterol synthesis seems to be restricted to eukaryotes, some prokaryotes are able to produce them. This is the case in bacteria of the genera Gemmata, Methylosphaera, Methylococcus and few Myxobacteria (Myxococcales), which produce lanosterol derivatives, mono or di-methylated at C-4 (Pearson et al., 2003; Schouten et al., 2000; Bode et al., 2003). In these cases the enzymes involved in this sterol pathway clearly derive from HGT from eukaryotes as showed by phylogenetic analysis (Desmond and Gribaldo, 2009).

In contrast, a number of eukaryotic organisms are not able to synthesize sterols and must take them up from the diet. Among them are the invertebrates such as nematodes and arthropods (Vinci et al., 2008); the alveolate ciliates and apicomplexans, e.g. *Paramecium tetraurelia* (Whitaker and Nelson, 1987) and *Plasmodium falciparum* (Desmond and Gribaldo, 2009) respectively; and flagellate parasites like *Giardia intestinalis* and *Trichomonas vaginalis* (Hernandez and Wasserman, 2006).

Given that metabolic diversity and the release of new sets of sequences related to sterol and hopanoids biosynthesis in ciliates, this investigation focused on assembling current experimental and bioinformatic data available in these organisms, and especially on the advances on sterol metabolism in *Tetrahymena thermophila* and *Paramecium tetraurelia.*

Moreover, new bioinformatic and phylogenomic analyses were performed on enzymes involved in tetrahymanol synthesis and in sterol metabolism in ciliates whose results supported the ongoing hypothesis on the possible biological function of putative genes.

1.2. Ciliates and polycyclic triterpenoids

Ciliates belong to the SAR (stramenopile–alveolate–rhizarian) group which is regarded as one of the major supergroups within the eukaryotes (Parfrey, 2011), (Fig. 3). According to the chromalveolate hypothesis the ciliates are the result of a single secondary endosymbiosis event between a line descending from a bikont and a red alga (Keeling, 2013). Together with apicomplexans, dinoflagellates, oxyrrhis, perkinsids and several flagellates, such as *Colpodella*, ciliates are grouped into the monophyletic lineage Alveolate (Adl et al., 2005) that may have evolved over 1.3 billion years ago (Parfrey, 2011).

Ciliates are a large class of complex-single celled organisms which have colonized many ecological niches and include more than 8000 species divided into two subphyla and 11 classes (Fig. 3). They are characterized by its nuclear dualism and by the arrangement of structures in their surface region (cortex) which includes cilia; they also share conjugation as a sexual process.

It is generally assumed that, in spite of the large metabolic diversity found between alveolates, the ciliates (ciliophora phylum) do not synthesize sterols (Desmond and Gribaldo, 2009). Instead, they either rely on hopanoids or are auxotrophic for sterols, so that the sterols they usually accumulate, or have been found during chemical analyses, are in fact the result of their diet and/or their metabolism.

Several members of the oligohymenophorea class synthesize tetrahymanol as their main pentacyclic triterpenoid, together with minor quantities of diplopterol and other hopanoid analogs (Raederstorff and Rohmer, 1988). Tetrahymanol is a pentacyclic gammaceran-alcohol (Fig. 1) that has stabilizing functions in membranes. Mallory et al. (1963) first isolated tetrahymanol from *Tetrahymena pyriformis*; since then tetrahymanol was reported in many species of the hymenostomatide *Tetrahymena* genus, such as *thermophila*, *setosa*, *vorax*, *patula*, *corlissi*, *paravorax* and *limacis* (Holz and Conner, 1973) as well as in marine scuticociliatia ciliates, including *Pleuronema* sp., *Parauronema acutum*, *Uronema nigricans*, *Anophryoides soldoi* and *Cyclidium* sp. (Ederington et al., 1995; Harvey and Mcmanus, 1991; Harvey et al., 1997). It is interesting to highlight that tetrahymanol is also found in prokaryotes and other non-related eukaryotes. Among prokaryotes it has been reported in the phototrophic alfa proteobacteria *Rhodopseudomonas palustris* (Rashby et al., 2007) and the nitrogen-fixing *Bradyrhizobium* bacteria (Perzl et al., 1997). Reports of tetrahymanol in eukaryotes include the fern *Oleandra wallichii* (Zander et al., 1969), the anaerobic rumen fungus *Piromonas communis* (Kemp et al., 1984) and the anaerobic free-living protist *Andalucia incarcerata* (Takishita et al., 2012).

2. Phylogenomics of tetrahymanol synthesis in ciliates

In ciliates, tetrahymanol is synthesized from isopentenyl diphosphate (IPP) via the mevalonate (MVA) pathway and the final step is catalyzed by squalene–tetrahymanol cyclase (STC). This enzyme catalyzes the cyclization of squalene to tetrahymanol via a non-oxidative proton-initiated reaction (Caspi, 1980), (Fig. 1).

A bioinformatics analysis of the tetrahymanol biosynthetic pathway on complete genomes from four ciliates, *Tetrahymena thermophila*, *Paramecium tetraurelia*, *Ichthyophthirius multifiliis* (oligohymenophorea class) and *Oxytricha trifallax* (spirotrichea class) indicated that (i) *Tetrahymena thermophila* harbors all the coding sequences of the genes necessary in tetrahymanol synthesis, *Paramecium tetraurelia* lacks the squalene and phytoene synthases, *Oxytricha trifallax* lacks the squalene–hopene cyclase in addition to the formers, whereas *Ichthyophthirius* lacks the latter plus the phosphomevalonate kinase and the isopentenyl PP isomerase (Table 1). (ii) Bioinformatic analyses on all *Tetrahymena* sequenced genomes, including *T. malaccensis*, *T. elliotti* and *T. borealis*, show also the complete pathway (data not shown). These data are consistent with reports on the isolation of tetrahymanol in these species, indicating that the capacity of tetrahymanol biosynthesis is a common trait in the genus. In contrast, *Paramecium tetraurelia*, which does harbor a STC ortholog, but not a squalene synthase ortholog, is unable to produce the precursors of hopanoids nor tetrahymanol (Kaneshiro, 1987).

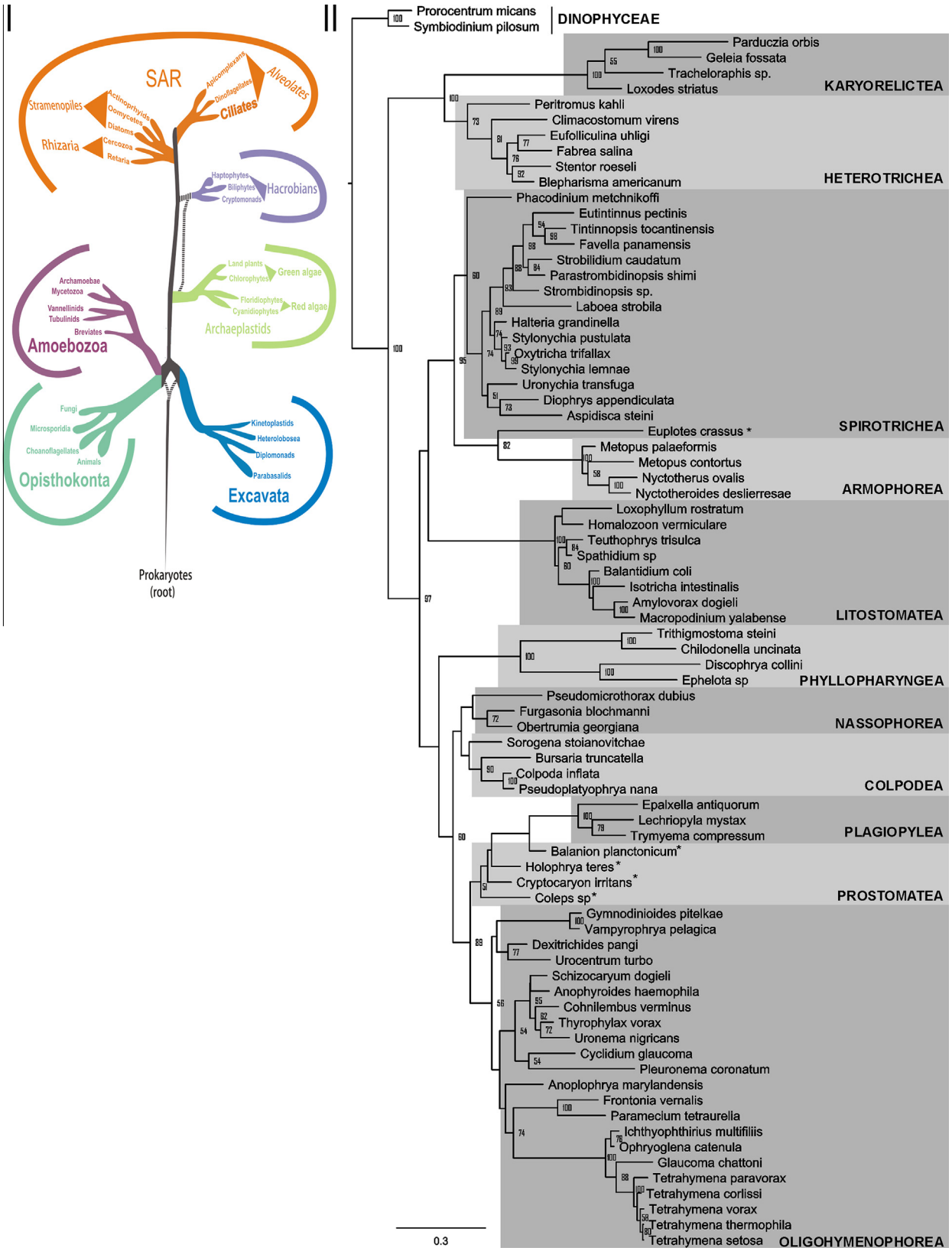

Fig. 3. (I) Schematic representation of the eukaryote tree, showing mayor supergroups and the position of ciliates. SAR: stramenopile–alveolate–rhizarian supergroup. The figure is an adaptation of Keeling, 2013. (II) Phylogenetic analysis of the Phylum Ciliophora. The phylogenetic tree of 88 small subunit rRNA gene sequences (GenBank) was created using maximum-likelihood method with 100 bootstrap replicates using PhyML software (Guindon et al., 2010). The model of nucleotide substitution was calculated with the jModeltest software (Posada, 2008). The parameters proportion of invariant sites and across-site variation (gamma) were estimated and used in the analysis. The sequences were previously aligned using Clustal X 2.1 (Larkin et al., 2007). The tree is based on the molecular phylogeny of ciliates of D. Lynn (Lynn, 2008). The clades show the 11 ciliate classes and the DINOPHYCEAE class from Dinoflagellata Phylum which is used as outgroup. The asterisk shows sequences of ciliates that do not form a monophyletic class group. Only bootstrap values above 50 are shown. The bars indicate percentages of substitution.

Table 1
Orthologs of the enzymes of the tetrahymanol biosynthetic pathway in ciliate genomes.

Class	Oligohymenophorea			Spirotrichea
Order	Hymenostomatia		Peniculia	Stichotrichia
Genus Species	*Tetrahymena thermophila*	*Ichthyophthirius multifiliis*	*Paramecium tetraurelia*	*Oxytricha trifallax*
Acetyl-CoA C-acetyltransferase				
Hmg-CoA synthase				
Hmg-CoA reductase				
Mevalonate kinase				
Phosphomevalonate kinase				
Diphosphomevalonate decarboxylase				
Isopentenyl-PP isomerase				
Farnesyl-PP synthase				
Squalene and phytoene synthases				
squalene-hopene cyclase				

2.1. Bacterial origin for tetrahymanol biosynthetic enzymes

A recent phylogenetic study performed on the triterpene cyclase protein family, which includes the prokaryotic squalene–hopane cyclases (SHCs), the eukaryotic oxidosqualene cyclases (OSCs) and the squalene–tetrahymanol cyclases (STC) from ciliates, fungi and ferns has shown that STCs are grouped in the base of a cluster, together with bacterial squalene cyclases of the SHCs type, in particular from *Stigmatella*, *Methylococcus* and *Gemmata* (Frickey and Kannenberg, 2009). Moreover, another independent study on STC's demonstrated that STC's form a monophyletic group within bacterial SHC's, (Takishita et al., 2012). To test the possible origin of other enzymes of the pre-squalene pathway, a BLAST search was performed on the hydroxymethylglutaryl-CoA reductase (HMG-CoA reductase) predicted sequences present in all the ciliate genomes analyzed, and the squalene synthases (present only in the *Tetrahymena* strains). The search retrieved mostly bacterial sequences in both cases; hence, another observation that arose from the present bioinformatic analysis is that not only the STC gene might be of bacterial origin, as previously reported, but also the HMG-CoA reductase and the squalene synthase show high sequence similarity to bacterial genes (Table 1). To further support this hypothesis a consensus phylogenetic tree generated by Maximum Likelihood and Neighbor-Joining methods with 66 amino acid sequences belonging to HMG-CoA reductases from different kingdoms was analyzed. Fig. 4 clearly shows that the sequences from ciliates are grouped in a different branch within bacterial sequences. The tree also shows the two distinct classes of HMG-CoA reductase enzymes: class I, which comprises eukaryotic and mostly archaeal enzymes, and class II, which comprises eubacteria and other archaea enzymes, where the ciliate sequences are grouped (Friesen and Rodwell, 2004).

Another phylogenetic tree was constructed with 48 amino acid sequences of squalene synthases. The enzyme catalyzes the transformation of farnesyl pyrophosphate into squalene, and is found in the genome of all *Tetrahymena* species, but not in other ciliate genomes analyzed. These sequences are grouped within the bacterial branch and apart from eukaryotic sequences from diverse origin, like opistokonts (animals and fungi), stramenopiles, amoebozoans, archaeplastids (land plants), heteroloboseas and euglenid excavates, among others. This pattern suggests a probable horizontal gene transfer event from bacteria to an ancient ciliate in the early Tetrahymenidae family origin (Fig. 5).

Since most of the bacteria in the branch are in fact cyanobacteria, another possible explanation might be that the squalene synthase found in tetrahymenids had an origin from a lost ancestral plastid. According to the chromalveolate hypothesis, all red secondary plastids are the result of a single endosymbiotic event, and chromalveolates, including chromists (cryptophytes, haptophytes, and stramenopiles) and alveolates (ciliates, apicomplexans, and dinoflagellates) have gained their plastid through secondary endosymbiosis. Later the plastid has been lost, in the case of ciliates and oomycetes, but some genes may have been retained (Cavalier-Smith, 1999; Keeling, 2013). We found only the cyanobacterium version of squalene synthase in tetrahymenids, whereas the eukaryotic version was found in the dinoflagellate *Alexandrium tamarense* and the stramenopiles *Thalassiosira pseudonana*, among others. Assuming the chromalveolate hypothesis, a HGT event from bacteria to an ancient tetrahymenide seems to be a more parsimonius explanation, than multiples losses events, as suggested in other chromalveolates organisms.

A similar branching pattern of likely bacterial origin is observed in Fig. 6 for the SHC's from *Tetrahymena* and *Paramecium*, which are grouped in a cluster together with bacterial squalene cyclases, in agreement with previous reports (Frickey and Kannenberg, 2009; Takishita et al., 2012). The molecular mass analysis of different SHC could also be indicative of a possible bacterial origin in ciliates. The MW of *Tetrahymena thermophila* cyclase is 72 kDa, which is similar to bacterial squalene–hopane cyclases such as the *Bacillus acidocaldarius* (75 kDa) and the alphaproteobacteria *Bradyrhizobium japonicum* (74 kDa) ones, but significantly different from the eukaryotic 2,3-epoxysqualene-lanosterol cyclase from *Saccharomyces cerevisiae* (83 kDa), *Homo sapiens* (83 kDa) and the 2,3-epoxysqualene-cycloartenol cyclase from the plant *Arabidopsis thaliana* or the amoeba *Dictyostelium discoideum* (86 kDa).

2.2. Gene expression analysis

Genome-wide gene expression profiles (publicly available for the ciliates *Tetrahymena thermophila* and *Paramecium tetraurelia* in the *Tetrahymena* Functional Genomics Database (TFGD) and *Paramecium*DB) show the total gene expression profiles at different stages of life cycles, like vegetative growth, starvation and sexual development (conjugation in *Tetrahymena* and autogamy in *Paramecium*) as well as reciliation and regulated exocytosis (only in *Paramecium*) (Arnaiz and Sperling, 2011; Xiong et al., 2011).

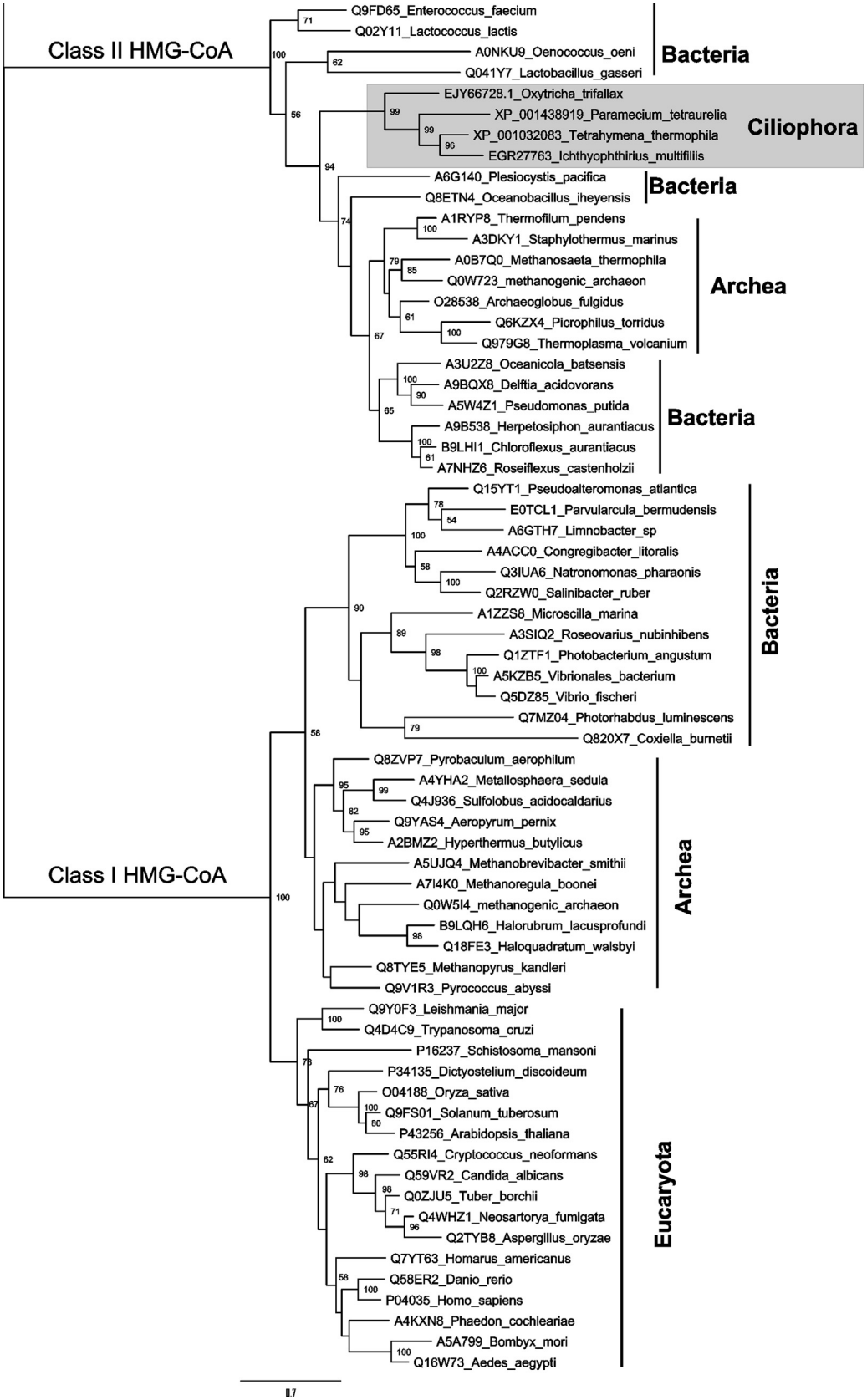

Fig. 4. Phylogenetic analysis of HMG-CoA reductases. The phylogenetic tree of 66 amino acid sequences (UniProtKb) was created using maximum-likelihood method with 100 bootstrap replicates using PhyML software (Guindon et al., 2010). The LG model of amino acid substitution (Le and Gascuel, 2008) was used to analyze amino acid sequence evolution, calculated with the Protest model evolution software (Darriba et al., 2011). The parameters proportion of invariant sites and across-site variation (gamma) were estimated and used in the analysis. Only the parts of the sequences that match with the PF00368 HMG-CoA_red PFam (Punta et al., 2012) were previously aligned using Clustal X 2.1 (Larkin et al., 2007). The ciliate sequences (gray) are grouped within the Class II HMG-CoA reductase, and separated from others eukaryotic sequences grouped within the Class I HMG-CoA reductase. Only bootstrap values above 50 are shown. The bars indicate percentages of substitution.

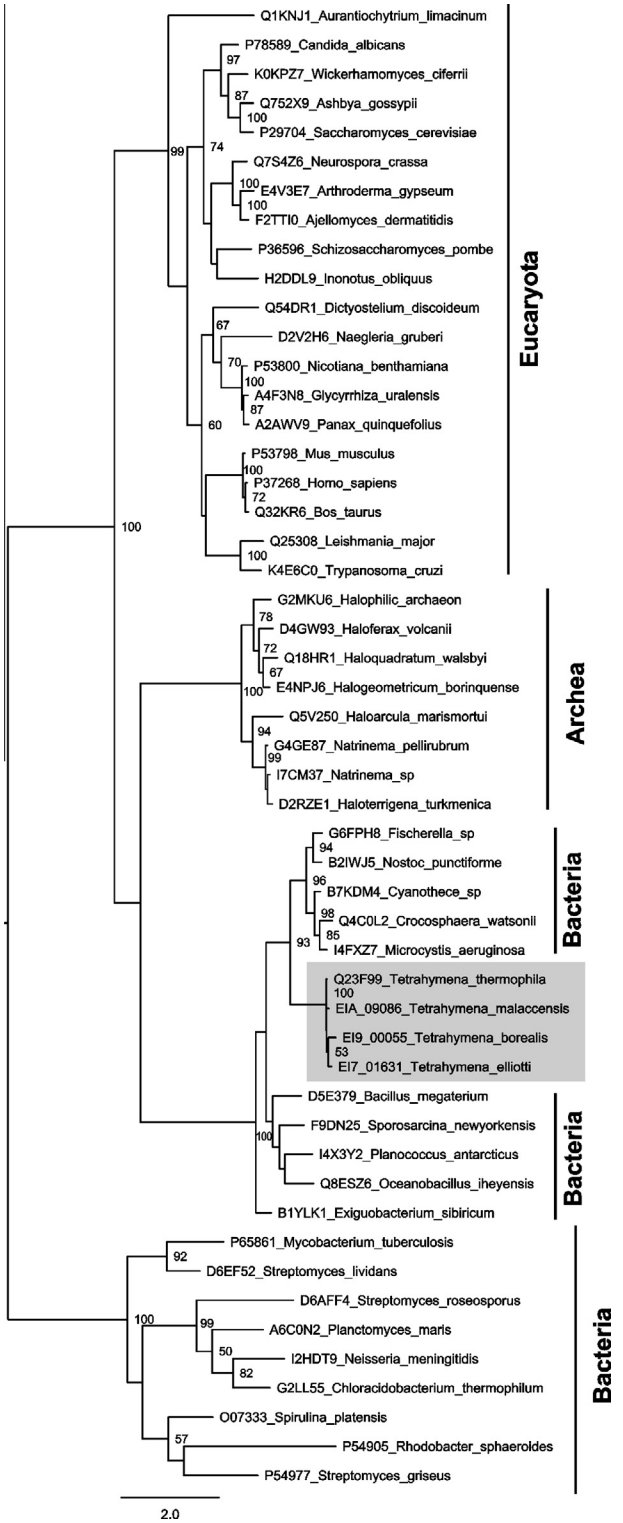

Fig. 5. Phylogenetic analysis of squalene synthases. The phylogenetic tree of 48 amino acid sequences (UniProtKb) was created using maximum-likelihood method with 100 bootstrap replicates using PhyML software (Guindon et al., 2010). The LG model of amino acid substitution (Le and Gascuel, 2008) was used to analyze amino acid sequence evolution, calculated with the Protest model evolution software (Darriba et al., 2011). The parameters proportion of invariant sites and across-site variation (gamma) were estimated and used in the analysis. The sequences were previously aligned using Clustal X 2.1 (Larkin et al., 2007). The tetrahymenids sequences (gray) are grouped within bacterial sequences and separated from others eukaryotic sequences. Only bootstrap values above 50 are shown. The bars indicate percentages of substitution.

The expression profiles of the HMG-CoA reductase and SHC in both ciliates shows only slight (*Tetrahymena*) or not significant changes (*Paramecium*) in all the conditions analyzed. In the case of the squalene synthase, only present in *Tetrahymena*, the gene displays a differential expression pattern among all the stages of its life cycle, suggesting a transcriptional regulatory mechanism for this enzyme that is consistent with previous experimental observations, obtained with labeled substrates, that pointed this enzyme as the key regulatory target in tetrahymanol biosynthesis pathway (Conner et al., 1968; Beedle et al., 1974).

3. Phylogenomics of sterol pathway in ciliates

3.1. The role of sterols in the regulation of tetrahymanol

As indicated previously, ciliates do not synthesize sterols, but they can respond to their presence/absence in the environment in various ways. On one hand, they are able to incorporate them directly from the diet or when added to a culture medium. In the case of oligohymenophorea ciliates, the synthesis of tetrahymanol and hopanoid analogs is immediately suppressed upon sterol uptake, as reported by Conner et al. (1968) and Harvey et al. (1997).

Nevertheless, the oligohymenophorea ciliate *Parauronema acutum* seems to be a controversial case: whereas Sul et al. (2000), have reported the presence of various sterols, such as cholesterol, campesterol, desmosterol and β-sitosterol, in neutral lipids extract, Harvey et al. (1997) reported that no sterols were detected when the ciliate was grown in a bacterized culture. In the latter only tetrahymanol and other hopanoids analogs were detected. Moreover, our in silico analysis of available 7689 EST's of the close related ciliate *Anophryoides haemophila* (same order that *P. acutum*) showed no orthologs of the post-oxidosqualene biosynthetic pathway (unpublished results). Nonetheless, a more detailed analysis of the composition of sterols and their metabolism, as well as whole genome sequencing of *Parauronema actum* would help to resolve the controversy.

3.2. Sterol modifying enzymes

In addition to uptake, some ciliates are able to modify sterols; for example *T. thermophila* and *T. pyriformis* desaturate sterols at positions C-5(6), C-7(8), and C-22(23) and remove the C-24 ethyl group from C_{29} sterols (phytosterols) (Mallory and Conner, 1971). Through the independent activity of the three desaturases (C-5, C-7, and C-22) and the C-24 sterol de-ethylase, the tetrahymenads are able to modify exogenous sterols, and accumulate the unsaturated and de-alkylated products in the membrane. In contrast in *P. tetraurelia* only C-5(6) and C-7(8) sterol desaturase activity have been detected (Conner et al., 1971).

Due to incomplete information available about sterol metabolism in other ciliates, a bioinformatic analysis was performed to search for orthologs of the post-squalene biosynthetic pathway. The analysis included 12 genera of ciliates belonging to 4 different classes (Table 2) and used as query the corresponding enzymes from plant (*Arabidopis thaliana*), metazoan (*Homo sapiens*) and yeast (*Saccharomyces cerevisiae*). It is important to note that not all the ciliates have the complete genome sequenced, thus only available ESTs and GSSs were analyzed in these cases.

The search concluded that none of the ciliates analyzed harbor the complete pathway for sterol biosynthesis. In fact, all the strains lack the squalene epoxidase considered the first enzyme of the group, responsible for the introduction of molecular oxygen into the squalene moiety. This key enzyme was not found in any of the ciliate's databases analyzed, ruling out the possibility of a

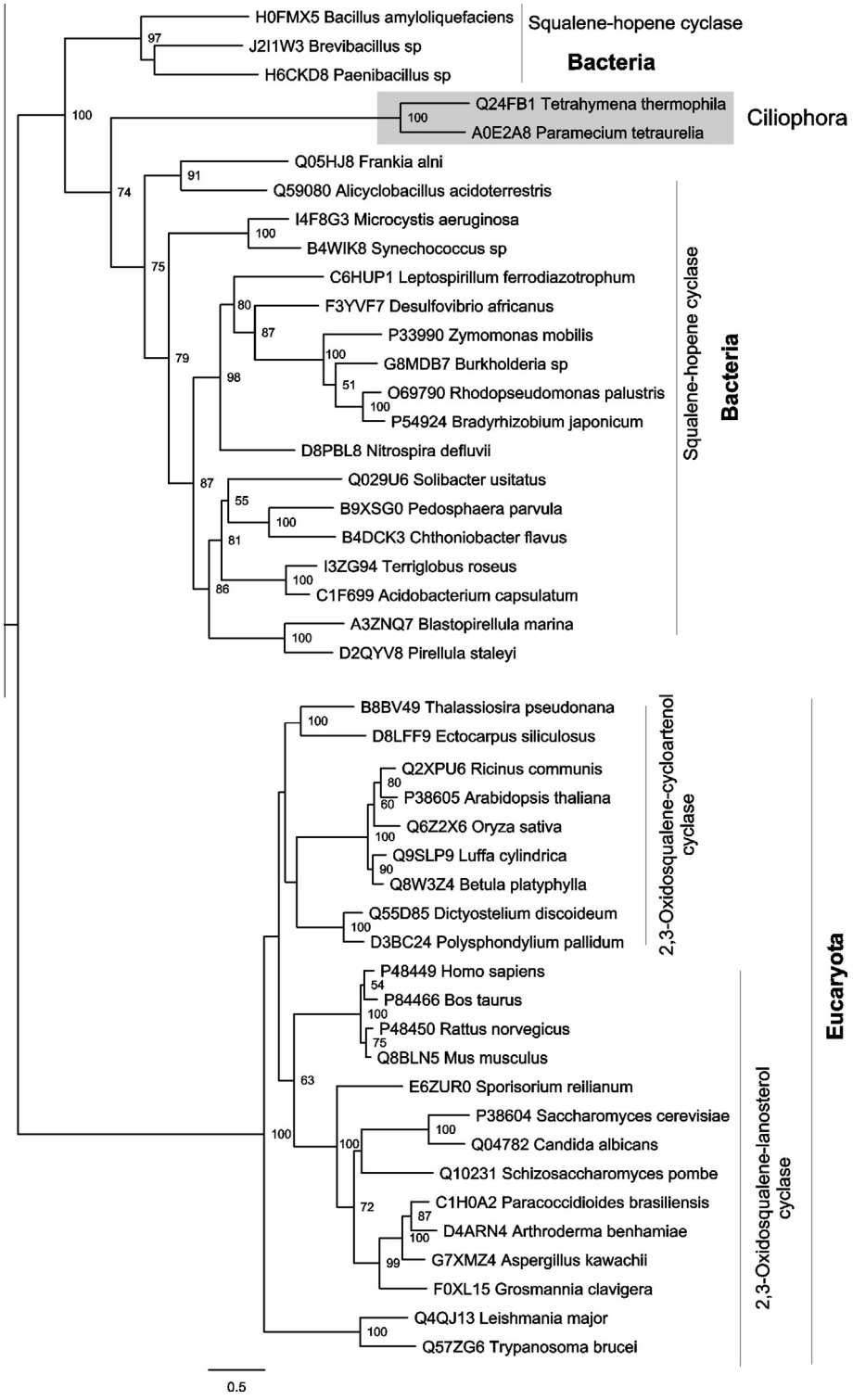

Fig. 6. Phylogenetic analysis of squalene–hopene cyclases. The phylogenetic tree of 46 amino acid sequences (UniProtKb) was created using maximum-likelihood method with 100 bootstrap replicates using PhyML software (Guindon et al., 2010). The LG model of amino acid substitution (Le and Gascuel, 2008) was used to analyze amino acid sequence evolution, calculated with the Protest model evolution software (Darriba et al., 2011). The parameters proportion of invariant sites and across-site variation (gamma) were estimated and used in the analysis. The sequences were pereviusly aligned using Clustal X 2.1 (Larkin et al., 2007). The *Tetrahymena* and *Paramecium* sequences (gray) are grouped within bacterial sequences and separated from others eukaryotic sequences. Only bootstrap values above 50 are shown. The bars indicate percentages of substitution.

metabolic switch between hopane and sterol synthesis in ciliates, as a response to oxygen availability.

For enzymes performing modifications in the sterol moiety, there were identified a few orthologs with an *E*-value <1e−10 in the Oligohymenophorea and Spirotrichea ciliates. The retrieved putative enzymes with a significant *E*-value (<1e−15) belong to two classes of protein families: the C-5 sterol desaturase and C-4 methyl oxidase, both members of the fatty acid hidroxylase family,

Table 2
Orthologs of enzymes of the sterol biosynthetic pathway in ciliates.

Class	OLIGOHYMENOPHOREA				LISTOSTOMATEA		ARMOPHOREA	SPIROTRICHEA		
Subclass	Hymenostomatia		Peniculia	Scuticociliaria	Trichostomatia			Stichotrichia		
Enzyme	*Tetrahymena thermophila* MAC Genome	*Ichthyophthirius multifiliis* MAC Genome	*Paramecium tetraurelia* MAC Genome	*Anophryoides haemophila* 7689 EST	*Vestibuliferida* (Family) 1487 EST	*Entodiniomorphida* (Family) 3009 EST	*Nyctotherus ovalis* 5484 EST + 4240 GSS	*Oxytricha trifallax* MAC Genome	*Stylonychia lemnae* 53,806 Cont seq	*Euplotes crassus* 1820 EST
Squalene epoxidase										
squalene epoxide cyclase										
C-14 demethylase										
C-14 reductase										
C-4 methyloxidase										
C-3 dehydrogenase										
C-3 ketoreductase										
erg 28										
C-8 isomerase (yeast)										
C-8 isomerase (metazoa / plants)										
C5 desaturase										
C-24 methyltransferase										
7DHC reductase										
C-24 reductase (yeast)										
C-24 reductase (metazoa / plants)										
C-22 desaturase										
C-24 sterol desaturase-like (de-ethylation)										
cyclopropilsterol isomeracion										
C-7 sterol desaturase										

and the 7-dehydrocholesterol reductase, C-14 sterol reductase and the C-24(28) sterol reductase, which belong to the ergosterol biosynthesis ERG4/ERG24 family.

3.3. Genes identified in ciliates

The C-5 sterol desaturase from *T. thermophila* (Genbank Accession Number: FJ940725.1) has been identified by analysis of the phenotype of a deletion mutant on the gene TTHERM_01194720 (Nusblat et al., 2009). The deletion mutant, which was fully viable, showed that C-5 sterol desaturase activity was strongly diminished, while C-7(8) and C-22(23) desaturase activities remained unaffected. Moreover, this gene (*DES*5A) was expressed in a yeast mutant, erg3Δ, which has disrupted the gene encoding the *S. cerevisiae* C-5 sterol desaturase, ERG3. The resulting complemented strain accumulated 74% of the wild type level of ergosterol (the final sterol product) and lacks the hypersensitivity to cycloheximide, a phenotype which is associated with the loss of ERG3 function (Poklepovich et al., 2012).

The other putative genes of the fatty acid hydroxylase family identified in ciliates were 4 sequences tentatively characterized as C-4 sterol methyl oxidase (SMO) (see Supplementary table). The corresponding *Tetrahymena* knockouts showed no differences in growth rates or cell shape, when compared with the wild type strain grown in the presence or absence of exogenous sterols, suggesting that none of the four orthologs found in the Tetrahymena Genome Database are essential for the cell. As no demethylation activity was reported in any ciliate, we tested possible C-4 methyl sterols substrates, commonly used by fungi, vertebrates or plants, for their biotransformation in *Tetrahymena* cultures. The wild type strain was therefore grown in the presence of lanosterol, 4,4-dimethylzimosterol, 24-methylen-24-dihydrocycloartenol or 24-ethylidenlophenol, but neither mono nor demethylated products were detected by GC–MS in the lipid extracts, and, nor intermediates of the 4-alpha carboxylic type. Moreover, all the substrates were recovered intact in the cell extracts (unpublished results).

As mentioned, the ciliates *T. thermophila* and *T. pyriformis* are able to perform the dealkylation on exogenous phytosterols of the C_{29} type. Although the reaction has been widely described in organisms such as nematodes, arthropods, and also in sponges, gene/s involved in this activity have not been yet reported (Chitwood et al., 1986; Ikekawa et al., 1993). Recently, the first gene encoding a sterol desaturase-like protein, promoting de-ethylation on C_{29} sterols in *T. thermophila* was identified by a knockout approach (Genbank Accession Number: HM448899.1). Hence, to our knowledge this is the first gene involved in a dealkylation process that was identified in any organism. Sequence analysis of this protein, Des24p, showed that the enzyme is a member of the fatty acid hydroxylase superfamily (FAHS), also present in *T. malaccensis*, *T. elliotti* and *T. borealis* genomes, but not in other ciliates genomes (Table 2). Interestingly, the gene has no clear orthologs in organisms that perform C-24 sterol dealkylation. Previous sequence and phylogenetic analyses have shown that Des24p share similarities with prokaryotic sequences and groups in a branch within bacterial sequences of unknown function, suggesting that the gene could be acquired by lateral transfer (Tomazic et al., 2011). New BLASTp search with Des24p as query, have also retrieved the recently deposited choanoflagellate *Salpingoeca* sp. sequence. This seems to be another event of HGT from bacteria to this closely related metazoan unicellular organism, since no other homologous sequences were found in metazoan descendents. Examination of the choanoflagellate genes indicates that HGT is particularly common in these single-celled organisms with as many as a 1,000 genes appearing to arise from prokaryotic and eukaryotic prey (Tucker, 2013).

Interestingly, the *Tetrahymena DES24* knockout strain was highly sensitive to phytosterols but not to C_{27} or C_{28} sterols, showing, in addition to decrease growth, tetrahymanol regulation and alteration in morphology. These observations suggest that the ethyl group at C-24, may impair the normal growth of the ciliate (Tomazic et al., 2011).

Although *Tetrahymena* can desaturate sterols at position Δ^{22}, no ortholog of the P450-type, typical of all known C-22 sterol desaturases, have been identified in the genome to date (Fu et al., 2009). It is a matter for speculation whether the absence of a clear P450 ortholog, in addition to the observed insensitivity to P450 inhibitors (Nusblat et al., 2005), may be an indication that this enzymatic activity, undoubtedly characterized in *T. thermophila* by numerous reports, represents a new class of C-22 sterol desaturases not yet identified (Morikawa et al., 2006; Skaggs et al., 1996).

The gene encoding a C-7 sterol desaturase in *Tetrahymena thermophila* (*DES7* Gene ID: 7840976) was recently identified by knockout analyses and RNAi approaches (Najle et al., 2013). The enzyme is a Rieske-type monooxygenase, highly conserved in

Table 3
Ka/Ks values for sterol metabolism orthologs in *Tetrahymena*, calculated with *T. thermophila* and *T. malaccensis* species.

Sterol biosyntheticpathway orthologs	Ka	Ks	Ka/Ks
C-4 methyl oxidase	0.024	0.441	0.054
C-5 sterol desaturase	0.025	0.633	0.039
Sterol reductase	0.024	0.525	0.046
C-24 sterol desaturase	0.018	0.449	0.040
C-7 sterol desaturase	0.015	0.361	0.041

protostome animals, involved in the first step of the steroidal hormone biosynthetic pathways in insects. A bioinformatic analysis has revealed the existence of orthologous sequences in the species *T. malaccensis*, *T. elliotti* and *T. borialis*, as well as the presence of multiple parologs (6) in the genome of *Paramecium tetraurelia*. 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). Another ortholog was also identified in the genome of the unicellular Opisthokont choanoflagellate *Capsaspora owczarzaki*, but not in other alveolate organisms, suggesting a probable HGT event to a phagotrophic ancestor ciliate. In *T. thermophila* the knockout strain did not show any significant differences in growth and morphological parameters when compared to the wild type strain, suggesting that *DES7* gene may be nonessential. Although this gene is involved in steroidal hormones biosynthesis in insects, nematodes and Drosophila, it is no clear the role in ciliates, since there are no reports of steroid hormones in these organisms.

3.4. Biological roles of putative genes

As the precise biological role of the putative genes involved in sterols metabolism in ciliates remain largely unknown, we examined the effect of selective pressure on significant orthologs of the sterol biosynthetic pathway in *Tetrahymena*, as a possible indicator of the mode of evolution operating on these sequences. For this purpose, the ratio between the number of nonsynonymous substitutions per nonsynonymous site (Ka) with respect to the number of synonymous substitutions per synonymous site (Ks) were calculated. To obtain the Ka/Ks values for the ciliate sequences we compared the ratios from *T. thermophila* with *T. malaccensis*, in which the activities are not present. The low values obtained in all the genes evaluated are indicative of a purifying selection tendency and suggests a probable biological role in these organisms (Table 3) not identified yet.

4. Conclusions

The analysis of 12 ciliate genomes and other sequences available up to date, belonging to 4 different classes, suggests that these unicellular organisms have lost most of the genes involved in the post-squalene pathway of sterol biosynthesis (present in the LECA), while a few enzymes have been retained, possibly aimed at processing the most frequent sterols that can be obtained from the environment, i.e., cholesterol and phytosterols (Desmond and Gribaldo, 2009).

In the case of ciliates belonging to Oligohymenophorea class, gene losses seem to be combined with the acquisition of bacterial or unicellular Opisthokont genes. HGT from bacteria and archaea has been described previously in rumen ciliates (Listostomatea class). Several of these genes are involved in the catabolism of complex carbohydrates that could have facilitated the colonization of new niches (Ricard et al., 2006). Examples of HGT are also described in *Tetrahymena* and *Paramecium* as an alternative hypothesis of plastid acquisition genes (Reyes-Prieto et al., 2008). The incidence of HGT in ciliates can be explained by their bacteriovoury behavior and also by their nuclear dimorphism, which allows amplification of foreign DNA in the macronucleus (Skovorodkin et al., 2001).

The acquisition of bacterial genes involved in triterpenoid biosynthesis, via HGT, in a phagotrophic ancestor of the Oligohymenophorea class possibly made it feasible the biosynthesis of tetrahymanol in several species of *Tetrahymena* and scuticociliates. This could be the result of an evolutionary process that led to these organisms to adapt to low-oxygen marine environments of the Proterozoic (Dietrich and Michael, 2006) where oxygen-dependent sterol biosynthesis was not possible (same case of rumen ciliates) (Espenshade and Hughes, 2007). The bacterial origin of the squalene–tetrahymanol cyclase, previously reported by other authors (Chen et al., 2007; Desmond and Gribaldo, 2009) plus the occurrence of other bacterial genes, like the HMG-CoA reductase and the squalene synthase, analyzed in this work, reinforces this hypothesis. Additionally, the bioinformatic analysis performed on other oligohymenophorea ciliates, such as *Paramecium tetraurelia* and *Ichthyophthirius multifiliis*, genomes clearly showed the absence of some orthologs involved in triterpenoid synthesis, suggesting that probably the loss of these genes is the reason why these organisms are not able to synthesize neither tetrahymanol nor sterols. These organisms must satisfy their requirements by uptake of sterols from the environment, i.e. stigmasterol in the case of *P. tetraurelia* (Whitaker and Nelson, 1987) and cholesterol, acquired from its fish host, in *I. multifiliis* (Coyne et al., 2011).

Although the biological function of the ciliate genes in environments devoid of sterols is not known, different lines of evidence suggest that they could have a physiological role in these organisms. For example, the KA/KS analysis shows that sterol metabolism orthologs in ciliates have a purifying selection and are conserved during evolution. Furthermore, the presence of differential expression patterns of the gene encoding for squalene synthase suggests the presence of a transcriptional regulatory mechanism in response to different stimuli, not yet discovered. Moreover, the identification of genes that are co-regulated with tetrahymanol cyclase, C-5 and C-7 sterol desaturase and the C-24 sterol desaturase-like further supports the possible biological role of these genes. Surprisingly, it was not possible to identify a C-4 sterol methyl oxidase activity in *T. thermophila*, in spite of the four putative SMOs displayed.

To elucidate the possible biological function of sterol genes is complex. For example, the analysis of the *Tetrahymena DES24* knockout strain previously showed that the enzyme is involved in the C-24 ethyl removal from phytosterols. Further morphological analysis, i.e.SEM, clearly showed that C29 sterols impair the normal growth of the mutant. In contrast, in *Paramecium*, which does not harbor a *DES24* ortholog, phytosterols are necessary for optimal growth. In the case of *DES7* gene, which is linked to steroid hormone biosynthesis in insects, no steroidal compounds were measured in ciliates.

In conclusion, polycyclic triterpenoid metabolism in ciliates seems to be the result of genes losses from the post-squalene pathway combined with acquisition of prokaryotic and/or unicellular eukaryotic genes via HGT, allowing both, the biosynthesis of new compounds and novel mechanisms of triterpenoid metabolism. The physiological role of these genes is an open question that will be surely illuminated with more genomes sequencing and the study of lipid pathways of other ciliates in the near future.

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Appendix A. Supplementary material

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