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Molecular Evidence of Fungal Signatures in the Marine Protist *Corallochytrium limacisporum* and its Implications in the Evolution of Animals and Fungi

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Fungi, animals, and single-celled organisms belonging to the choanozoans together constitute the supergroup Opisthokonta. The latter are considered crucial in understanding the evolutionary origin of animals and fungi. The choanozoan *Corallochytrium limacisporum* is an enigmatic marine protist of considerable interest in opisthokontan evolution. Several isolates of the organism were obtained from a coral reef lagoon in the Lakshadweep group of islands of the Arabian Sea. The capability of these cultures to grow on media containing inorganic nitrogen sources prompted us to examine the possible presence of fungal signatures, namely the enzyme α-aminoadipate reductase (α-AAR) involved in the α-aminoadipate (AAA) pathway for synthesizing lysine and ergosterol, in one of the isolates. These features, as well as the sterol C-14 reductase gene involved in the sterol pathway of animals and fungi, were detected in the organism. Phylogenetic trees based on the α-AAR gene suggested that *Corallochytrium limacisporum* is a sister clade to fungi, while those based on the C-14 reductase gene did not adequately resolve whether the organism was more closely related to fungi or animals. While many studies indicate that *Corallochytrium* is a sister clade to animals, we suggest that further studies are required to examine whether this protist is in fact more closely related to fungi rather than to animals.

Key words: α-aminoadipate reductase; *Corallochytrium limacisporum*; marine protist; sterol C-14 reductase.

Introduction

The common origin of animals and fungi, the recognition of a group of organisms belonging to choanozoans as 'animal—fungal allies', and the establishment of the supergroup Opisthokonta comprising these three are some of the major developments to emerge in eukaryote evolutionary biology in recent times (Adl et al. 2005; Cavalier-Smith 1998; Keeling et al. 2005; Mendoza

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et al. 2002; Steenkamp et al. 2006). The phylum Choanozoa sensu Cavalier-Smith is comprised of several enigmatic single-celled organisms, including a group of parasitic and saprotrophic protists belonging to the Ichthyosporea or the Mesomycetozoea (the DRIP clade), the choanoflagellates, the corallochytreans (*Corallochytrium limacisporum*), the ministeriids, and the nucleariids (Herr et al. 1999; Mendoza et al. 2002; Ragan et al. 1996). These organisms have received much attention in recent years in order to unravel the origins of animals and fungi. While several choanozoans have generally been considered to constitute a sister clade to animals (Cavalier-Smith 1998; Mendoza et al. 2002; Stechmann and Cavalier-Smith 2004), a few of them, such as nucleariids and ichthyosporeans, have recently been found to be more closely related to fungi (Ragan et al. 2003; Steenkamp et al. 2006).

Corallochytrium limacisporum Raghukumar is one of the organisms in the complex group of the choanozoans and has frequently been studied in connection with opisthokontan evolution (Cavalier-Smith and Chao 2003). This protist was originally described as a thraustochytrid (Raghukumar 1987). Subsequently, the organism was found to be phylogenetically closer to the choanoflagellates based on its 18S rRNA gene sequence (Cavalier-Smith and Allsopp 1996). Other studies also indicate such a close relationship (Mendoza et al. 2002). According to Cavalier-Smith (1998), *C. limacisporum* is the only member of the Class Corallochytrea belonging to the subphylum Choanozoa, phylum Neomonada of the kingdom Protozoa. Adl et al. (2005) place it under the supergroup Opisthokonta, first rank Mesomycetozoa.

We recently re-isolated this marine protist from the coral reef lagoon of the Kavaratti Island of the Lakshwadeep atoll in the Arabian Sea. The organism grew luxuriantly both in a medium containing either peptone or ammonium nitrate as the organic or inorganic nitrogen source respectively. Growth of *C. limacisporum* in synthetic medium suggested its capability to synthesize amino acids from inorganic nitrogen. This prompted the question whether *C. limacisporum* was capable of synthesizing its amino acid lysine through the AAA pathway that is characteristic of fungi (Vogel 1964) or the α, ε-diaminopimelic acid (DAP) pathway that characterizes bacteria, chromists, and vascular plants (Brown and Cooksey 1984). Although the former has recently been found to occur in some prokaryotes, the α-AAR is a key enzyme evolutionarily conserved in the fungal lysine synthesis pathway (Nishida and Nishiyama 2000). Yet another signature of fungi is ergosterol, which is an integral membrane lipid of fungi and has been used as a biomarker for fungi (Lindblom et al. 2004). It is also a common target for many anti-fungal drugs like polyenes (Jia et al. 2002). Therefore, we studied the relationship of this marine protist to fungi by analyzing the presence of α-AAR and its gene, as well as ergosterol and the sterol C-14 reductase gene involved in its biosynthesis.

Results

The identity of *C. limacisporum* NIOCC#27 was first verified by its colony and microscopic morphology. All isolates produced rough, pink, raised colonies on BK medium. The morphology corresponded exactly with that described for the species. Vegetative cells were unicellular, underwent repeated binary divisions and released limax-shaped amoeboid spores (Fig. 1). The 18S rRNA sequence of the organism was subjected to a BLAST analysis. The sequence showed 99% identity and 0% gap with the 18S rRNA gene sequence of *C. limacisporum* (L42528).

Corallochytrium limacisporum NIOCC #27 showed no growth in distilled water and grew poorly at 10 ppt seawater. Good growth was observed at salinities of 20—35 ppt (mg dry wt biomass of 250 ± 50), demonstrating its obligate marine nature. All isolates grew luxuriantly both in BK medium, as well as in VG medium. Growth in the latter was about 50% less of that obtained in the medium with peptone.

α-AAR activity was detected in the crude homogenate of the culture grown in both the organic, as well as in the synthetic media (Table 2). The enzyme activity in *C. limacisporum* was 150% higher when compared with *Neurospora crassa* (strain 74-OR23-1 *mat* A; FGSC 987) for both media.

The absorbance spectrum of sterols extracted from *C. limacisporum* NIOCC #27 was similar to that of the spectrum of control cultures of *N. crassa* with absorbance maxima at 272, 282, and 293 nm (Fig. 2). It differed from that of *N. crassa* erg-3 mutant (erg-3 *mat a* [FGSC 2725]) that showed an absorbance maximum at 250 nm.

The BLAST results showed that the amino acid sequence of the partial (1485 bp) α-AAR gene sequenced from *C. limacisporum* NIOCC#27

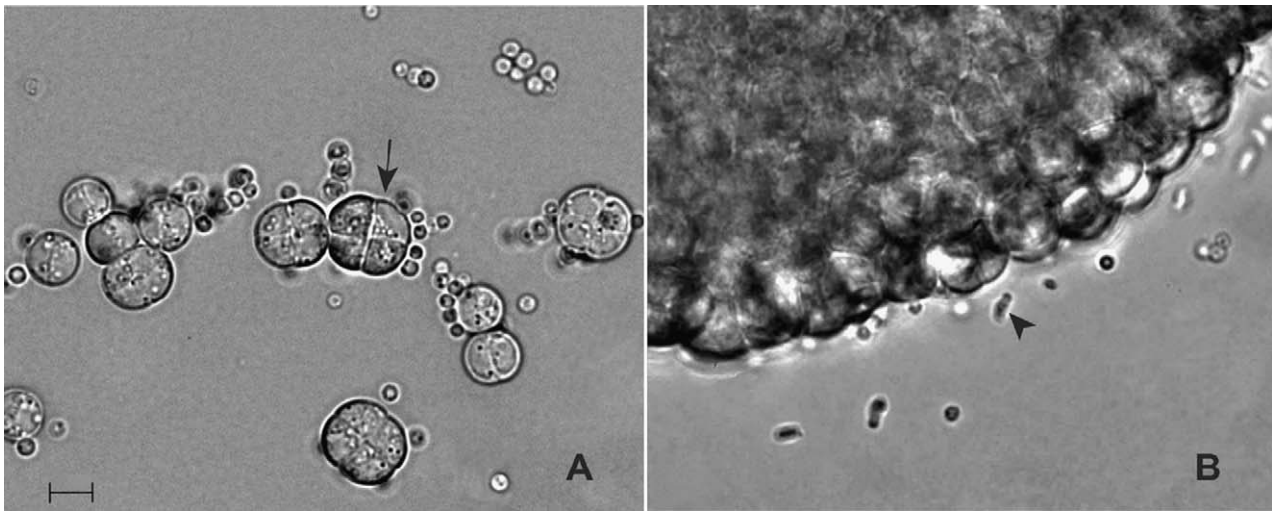

Figure 1. Photomicrograph of *Corallochytrium limacisporum* cells. (**A**) The organism is characterized by single cells, diads, and tetrads (arrow). (**B**) Reproduction takes place by the production of limax-shaped amoeboid spores (arrowhead). Bar in (**A**) represents 5 μm and is common to (**B**).

(DQ223866) had similarity with 30 fungal α-AAR gene sequences and 2 peptide synthetase genes of cyanobacteria and 3 of bacteria. The partial (894 bp) sterol C-14 reductase gene sequence of *C. limacisporum* NIOCC#27 (Accession No. DQ223865) showed maximum similarity to that of the fungus *Schizosaccharomyces pombe* with 45% identity. BLAST results showed a similarity with 15 fungal, 8 metazoan, and 1 euglenozoan sterol C-14 reductase gene sequences as well as 8 metazoan lamin B receptor sequences.

Phylogenetic analysis of the partial α-AAR gene sequence using 2 codons based on both the NJ and ML methods placed *C. limacisporum* in a separate clade, distinct from the rest of the fungi with branch lengths of 0.249 and 0.360 respectively (Figs 3, 4). The NJ tree constructed using 3 codons also indicated a similar relationship (tree not shown). However, the ML tree using 3 codons placed this protist within fungi, along with *Acremonium, Penicillium, Aspergillus nidulans*, and *N. crassa* with a bootstrap value of 35% and branch length 0.405 (Fig. 5).

The constructed NJ tree based on the partial sterol C-14 reductase gene using 2 codons (Fig. 6), as well as the NJ and ML trees using 3 codons of the same gene (trees not shown) placed *C. limacisporum* as a sister clade to animals. However, the ML tree constructed using 2 codons placed *C. limacisporum* as a sister group to fungi and not to animals (Fig. 7). All the trees showed a poor bootstrap support.

Discussion

The group of mesomycetozoan and choanozoan organisms, previously classified as Protozoa, and comprising choanoflagellates, corallochytreans, nucleariids, ministeriids, and ichthyosporeans is considered to be critical in understanding the early evolution of animals and fungi (Cavalier-Smith 1998; Lang et al. 2002; Steenkamp et al. 2006). Cavalier-Smith (1998) suggested that early stem choanozoan-like uniflagellate organisms were the common ancestors of animals and fungi. It is obvious that an understanding of the extant crown choanozoans will throw light on animal—fungal origins. Among such organisms, those that can be easily cultured will provide an ideal tool to explore molecular aspects of animal and fungal evolution (Mendoza et al. 2002). Unfortunately, only a few members of this group, such as *Sphaerosoma arcticum* and *Ichthyophonus hoferi,* have been cultured so far, while most other parasitic forms have not been cultivated. *Corallochytrium limacisporum*, by virtue of its capability to grow well in organic as well as defined media, may provide a convenient tool for the purpose.

This study has examined the relationships of *C. limacisporum* to fungi by studying the gene α-AAR, which encodes a key enzyme that is involved in the AAA pathway for lysine synthesis in fungi. Synthesis of the amino acid lysine is known to take place through two distinct enzymatic pathways: (1) the DAP pathway that involves

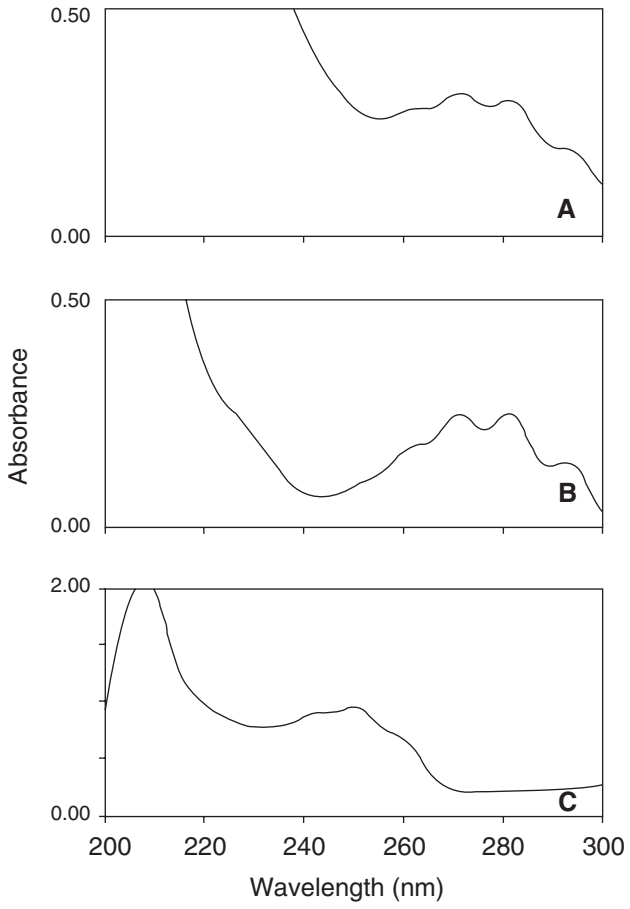

Figure 2. The UV absorption spectra of the sterols. Profile of the sterols extracted from (**A**) *Corallochytrium limacisporum* (NIOCC # 27) (**B**) *Neurospora crassa* 74-OR23-1 *mat* A (FGSC 987), and (**C**) *Neurospora crassa* mutant erg−3 *mat a* (FGSC 2725).

α,ε-diaminopimelic acid as an intermediate or (2) the AAA pathway that involves α-aminoadipic acid. Prokaryotes use the DAP pathway; while the AAA pathway is characteristic of fungi (Vogel 1964). It has been hypothesized that lysine biosynthesis in cyanobacteria, green algae, and vascular plants has evolved from bacterial DAP pathway (Brown and Cooksey 1984). Protists and metazoans have lost the ability to synthesize lysine, which is an essential amino acid for these organisms. However, fungi and some euglenids seem to have undergone adaptive mutation through the course of evolution to develop an elaborate lysine biosynthetic pathway through the AAA pathway (LeJohn 1971). In this pathway, lysine is synthesized from 2-oxoglutarate and acetyl-CoA through α-aminoadipic acid. The AAA pathway was considered to be common and unique to fungi; however, recent studies have shown that the thermophilic bacteria *Thermus thermophilus* and the archaean *Pyrococcus horikoshii* also synthesize lysine through a modified form of the AAA pathway (Nishida et al. 1999). However, the pathway is not exactly similar as in fungi and α-AAR is still believed to be a key enzyme in the evolution of fungal lysine synthesis (Nishida and Nishiyama 2000). The biosynthetic step catalyzed by α-AAR that leads to the formation of α-aminoadipic acid 6-semialdehyde from α-aminoadipic acid is identified to be a key enzyme in the evolution of fungal lysine synthesis and fungal evolution in general (An et al. 2002; Nishida et al. 1999). Defining the starting point when this pathway began to evolve might prove crucial in understanding the divergence of animal/fungal evolution. *Corallochytrium limacisporum* possesses the α-AAR gene, with a homology of between 45% and 55% to 30 fungal α-AAR sequences. Further, the α-AAR gene is apparently functional since *C. limacisporum* was capable of producing the α-AAR enzyme.

Likewise, ergosterol is the principal sterol of fungi (Palermo et al. 1997), although it has been reported in a few organisms, such as sponges (Gunatilaka et al. 1981). This signature molecule of fungi has been used as a biochemical marker for estimating fungal biomass. It is a functional analog of cholesterol in animal cells, and plays an essential role in the modulation of membrane fluidity and as a signal for cell division. Besides possessing α-AAR, *C. limacisporum* NIOCC #27 shows similarity to fungi also by the possession of ergosterol (Fig. 2). We studied the sterol C-14 reductase gene involved in the reduction of the C-14−15 double-bond in the sterol intermediate 4,4-dimethylcholesta 8,14,24-trienol, which leads to the formation of 4,4-dimethylzymosterol. Sterol C-14 reductase is an intermediary enzyme in cholesterol biosynthesis and is present in many eukaryotes (Holmer et al. 1998; Roberti et al. 2002). Our phylogenetic analyses based on the amino acid sequence of this gene from *C. limacisporum* shows a similarity of 35−45% to 18 sterol C-14 reductase sequences of fungi. Further, ergosterol was detected in the organism (Fig. 2) based on the UV absorbance spectrum.

Is *C. limacisporum* by possessing the fungal signatures of the α-AAR gene and ergosterol, as well as the fungal/animal character of the C-14 reductase gene more closely related to fungi or animals? This question needs to be discussed in light of other studies, which have examined the

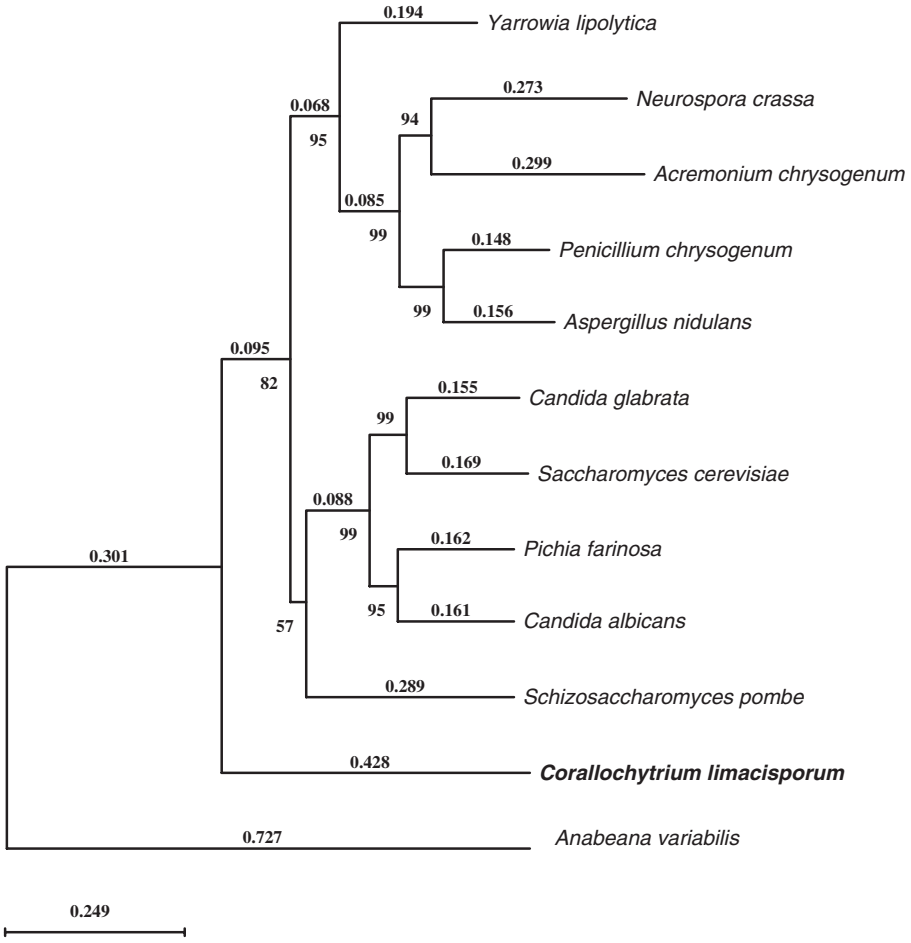

Figure 3. NJ tree based on the α-AAR gene. Neighbor-joining tree constructed using the Kimura 2-parameter model for the α-aminoadipate reductase (α-AAR) sequence, based on the first two codon positions. The numbers at each internal branching are bootstrap values computed from 500 bootstrap replicates. The numbers on the branches are the branch lengths. The bar represents 0.249 mutations per site.

phylogenetic relationship of *C. limacisporum* and other choanozoans to animals and fungi.

Several studies have suggested that *Corallochytrium* and other choanozoans are a sister clade to animals. Using HsP90 sequences, Stechmann and Cavalier-Smith (2004) have shown that *C. limacisporum* and other choanozoans form a sister clade to animals. Likewise, Lang et al. (2002) found that choanoflagellates are a sister group to animals, based on mitochondrial gene sequences. Phylogenetic trees based on 18S ribosomal RNA genes showed that *C. limacisporum* forms a sister clade to choanoflagellates, in turn a sister clade to the Mesomycetozoea, the entire group being sister to animals (Mendoza et al. 2002). Similar results were obtained by King and Carroll (2001) based on the elongation factor 2, α-tubulin, β-tubulin, and actin that demonstrated that choanoflagellates and metazoans are more closely related to each other than to fungi. Likewise, Steenkamp et al. (2006) placed *Corallochytrium* as a sister clade to animals. Thus, most of the studies report that the choanozoans form a sister clade to animals and not to fungi.

In contrast to the above, some studies have indicated doubts regarding the affinities of a few choanozoans to animals rather than fungi. Thus, nucleariids, generally placed under the choanozoans have recently been shown to be a sister clade to fungi based on EF-1α, actin, HSP70, and β-tublin (EAH β) protein sequences (Steenkamp et al. 2006). Ragan et al. (2003), based on EF1-α sequences, showed that *Ichthyophonus irregularis* belongs to the Ophisthokonta, but further resolution of its position along with fungi or animals could not be resolved unambiguously in

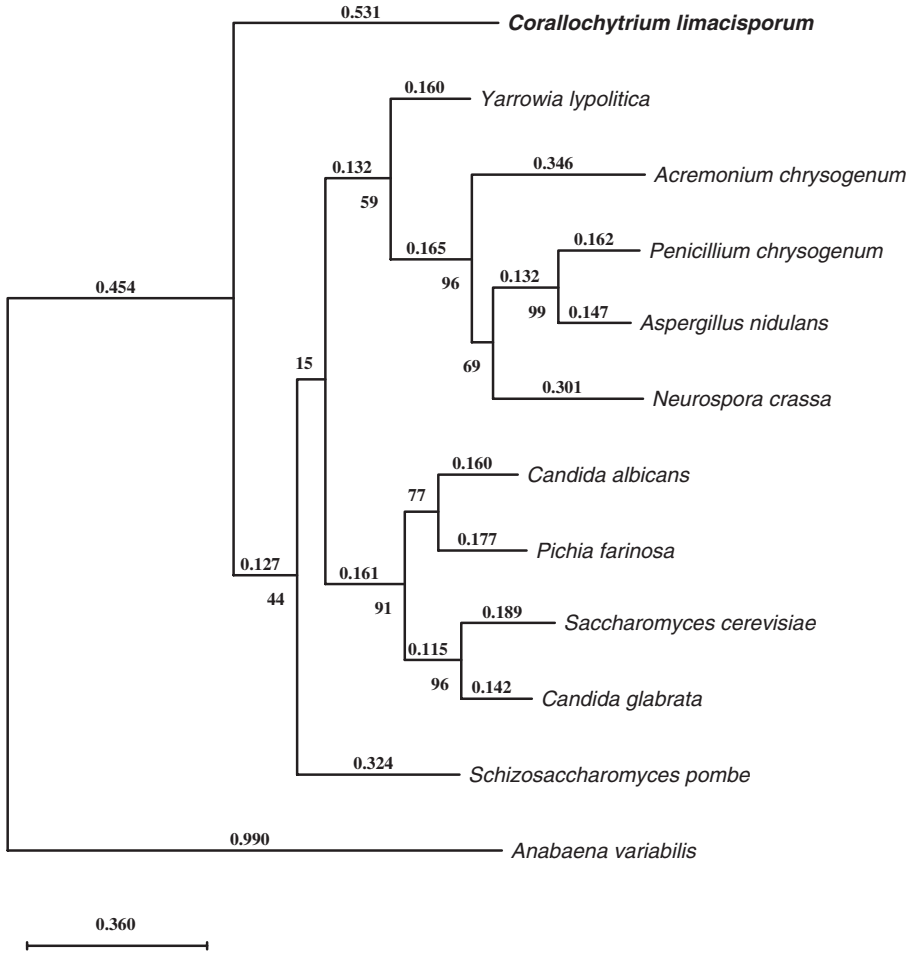

Figure 4. ML tree based on α-AAR. Maximum-likelihood tree constructed using the Kimura 2-parameter model for the α-aminoadipate reductase (α-AAR) sequence based on the first two codon positions. The numbers at each internal branching are bootstrap values computed from 500 bootstrap replicates. The numbers on the branches are the branch lengths. The bar represents 0.360 mutations per site.

their study. These authors remarked that this may be achieved by analyzing concatenated gene phylogenies. Tanabe et al. (2005) have shown that the ichthyosporeans are characterized by a deletion of a sequence encoding 2 amino acids in their EF-1 α gene, a characteristic of fungi.

All phylogenetic trees constructed using the α-AAR gene, except the ML tree using 2 codon positions, indicated that *C. limacisporum* is a sister clade to fungi (Figs 3, 4). The AAA pathway is considered to be specific to fungi and no animal sequences of this gene were available in the NCBI database for comparison. Our phylogenetic trees using the C-14 reductase gene, which is common to the ergosterol pathway of fungi as well as the lanosterol pathway of animals, were not conclusive regarding the placement of *C. limacisporum* as a sister clade to either animals or fungi. (Figs 6, 7).

There are two likely explanations for the presence of the fungal signatures namely the presence of ergosterol and the α-AAR gene in *C. limacisporum* (Fig. 8). If this protist is a sister clade to animals, the ability of *C. limacisporum* to synthesize lysine through the AAA pathway could be a plesiomorphic character, having been present in the common fungi-animal ancestor. These fungal characters apparently persisted in the common choanozoan-animal clade and extended to *C. limacisporum,* the sister clade to other choanozoans. This lysine-synthesizing gene in animals possibly became redundant due to their phagotrophic mode of nutrition (Fig. 8A). If *C. limacisporum* is a sister clade to fungi rather

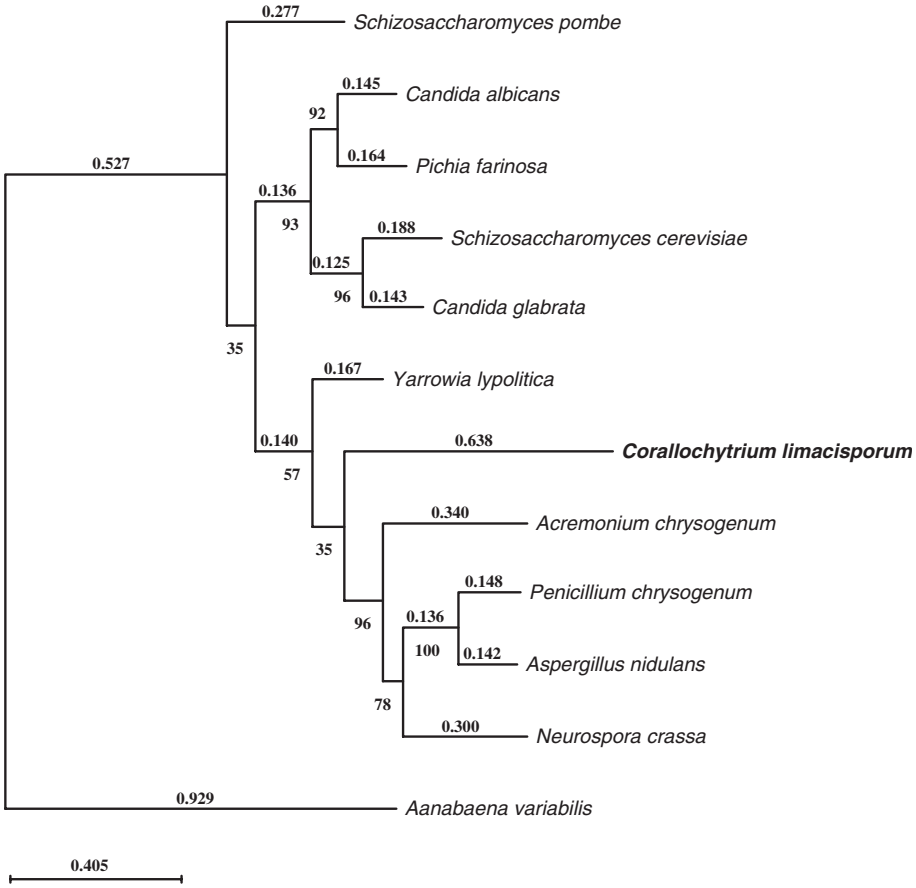

Figure 5. Maximum-likelihood tree constructed using the Kimura 2-parameter model for the α-aminoadipate reductase (α-AAR) sequence based on all the three codon positions. The numbers at each internal branching are bootstrap values computed from 500 bootstrap replicates. The numbers on the branches are the branch lengths. The bar represents 0.405 mutations per site.

than animals, one might expect to find the α-AAR gene only in the clade leading to fungi and their sister clades, but not in the clade leading to animals and those choanozoans forming a sister clade to animals (Fig. 8B).

Cavalier-Smith and Chao (2003) opined that the position of *Corallochytrium* could be better understood by studying the wall chemistry and protein sequences in *C. limacisporum.* Although Steenkamp et al. (2006) placed *Corallochytrium* and ichthyosporeans as a sister clade to animals, the latter are now thought to be close to fungi (Tanabe et al. 2005). While discussing the above two organisms, Cavalier-Smith and Chao (2003) remarked that bootstrap support of 18S rRNA sequences was too low to exclude paraphyly of choanozoans and that the placement of nucleariids, Ichthyosporea, and *Corallochytrium* as a sister clade to fungi rather than animals could not be ruled out. The presence of the fungal signatures ergosterol and the α-AAR genes in *C. limacisporum* leads us to believe that it will be worthwhile examining whether this protist is in fact a sister clade to fungi as suggested in Figure 8 B and not to animals. Future studies on fungal signatures in other organisms of the choanozoan complex, as carried out in this study, will help to refine our understanding of animal/fungal evolution.

Yet another approach to understand fungal origins is to discover and study novel fungi-like organisms from the sea. Estimates on the date of origin of fungi vary with different authors; many suggesting them to be more than 1 billion years old (Berbee and Taylor 2001; Heckman et al. 2001). Molecular clocks of well-represented genes among animals, plants, and fungi showed that the divergence times among animal phyla, must have taken place 1200-670 million years ago (Ma). Plants, animals, and fungi probably diverged 1576

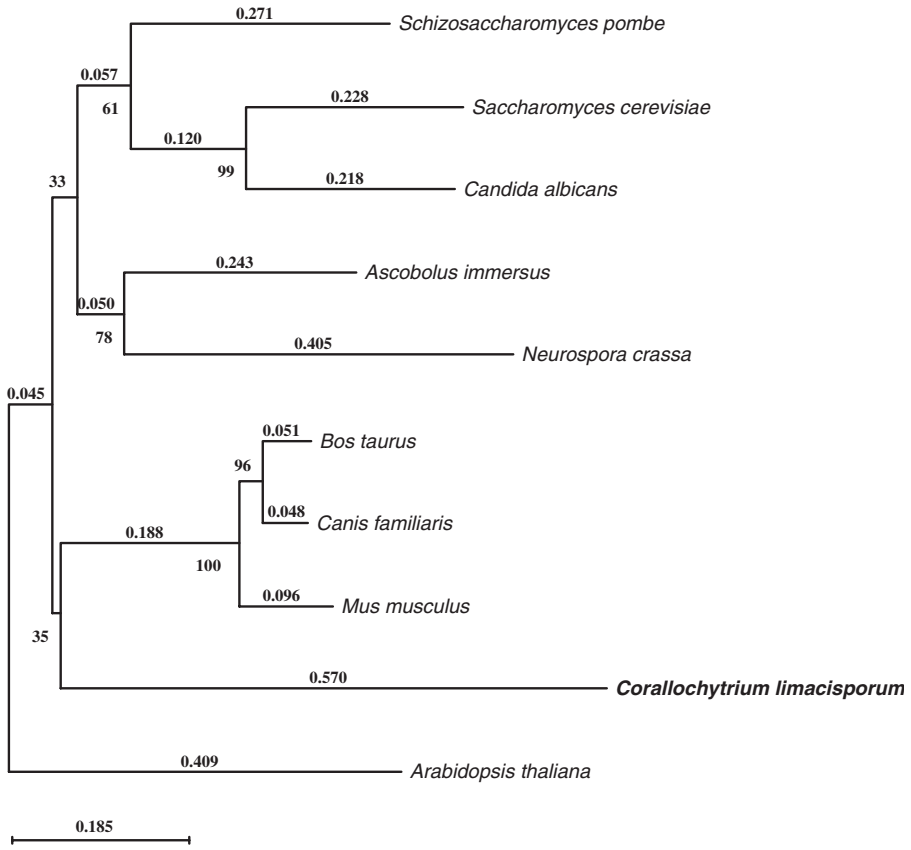

Figure 6. NJ tree based on sterol C-14 reductase. Neighbor-joining tree constructed using the Kimura 2-parameter model for sterol C-14 reductase gene sequence based on the first two codon positions. The numbers at each internal branching are bootstrap values computed from 500 bootstrap replicates. The numbers on the branches are the branch lengths. The bar represents 0.185 mutations per site.

+/− 88 Ma (Wang et al. 1999). This divergence probably took place in the sea, which is the likely source of ancient fungi, and the fungi may have conquered land as symbionts in association with plants (Heckman et al. 2001). Hence, we believe that studies on the yet unknown marine eukaryotic diversity may be a key to understanding the origins of fungi. Refinements in culture techniques, such as those used for rearing uncultured bacteria in the laboratory (Connon and Giovannoni 2002), might bring to light more organisms such as *C. limacisporum* that are situated at the base of the choanozoans and of animals and fungi. Early fungi in the sea were probably unicellular and had an amoeboid stage in their life cycle, this characteristic may probably also being occasionally reflected in zoospores belonging to the chytridiaceous fungi (Cavalier-Smith 1998). In this context, our studies on *Corallochytrium*, a marine organism far removed in morphology from fungi, yet displaying certain fungal characteristics, may be significant. A study of *C. limacisporum* using molecular clocks might provide interesting information.

Methods

Isolation and maintenance of culture: *Corallochytrium limacisporum* was isolated from degrading sea grass blades of *Thalassia hemprichii* (Ehrenb.) Aschers and bleached corals of *Porites lutea* were collected from the coral reef lagoon of Kavaratti Island, of the Lakshwadeep atoll in the Arabian Sea located between 10°30′—11.0° N and 72.0—73.0°E. Samples were plated on Boyd and Kohlmeyer (BK) medium (Kohlmeyer and Kohlmeyer 1979) fortified with 0.0075 g of streptomycin and 0.025 U of penicillin per 100 ml of medium to prevent bacterial growth. Colonies of *Corallochytrium limacisporum* could be easily detected on the plates by virtue of their pinkish color and

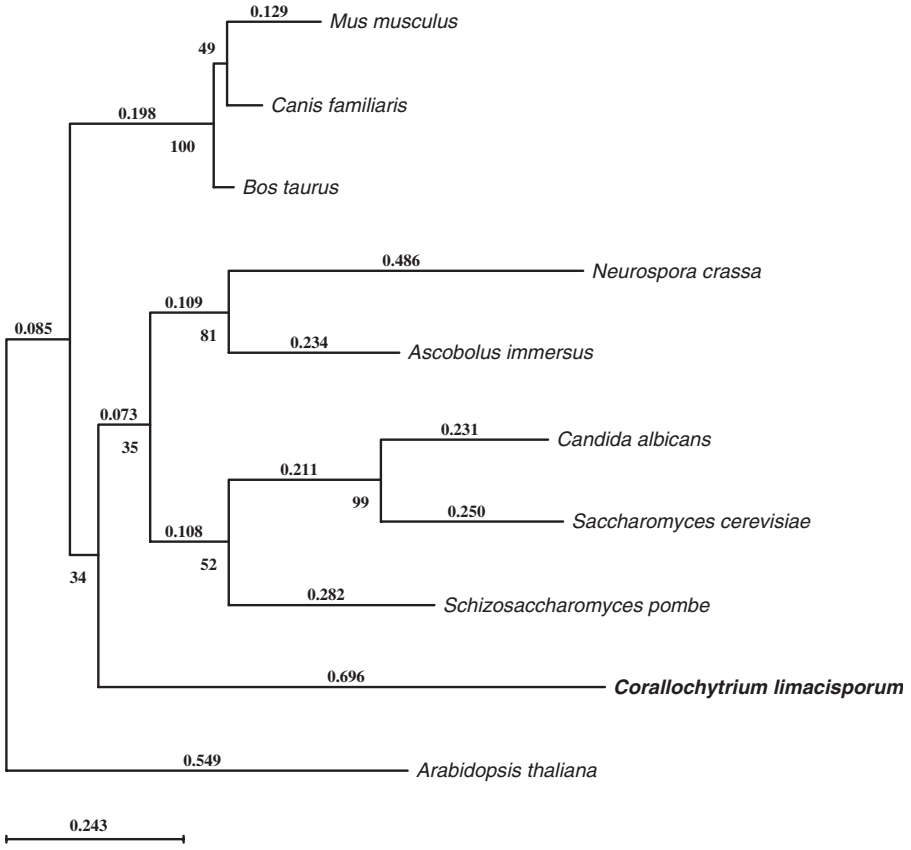

Figure 7. ML tree based on sterol C-14 reductase. Maximum-likelihood tree constructed using the Kimura 2-parameter model for sterol C-14 reductase gene sequence based on the first two codon positions. The numbers at each internal branching are bootstrap values computed from 500 bootstrap replicates. The numbers on the branches are the branch lengths. Bar represents 0.243 mutations per site.

convex, raised nature. The cultures were transferred to fresh media and identified based on their typical morphological characteristics (Raghukumar 1987). A total of 27 isolates were obtained. The identity of one of these isolates, designated NIOCC #27, was further confirmed by 18S rDNA sequence analysis and compared with the existing sequence in the GeneBank (L42528). This culture was maintained under axenic conditions in BK agar tubes with 0.6% agar prepared in seawater and was used as inocula. The isolate NIOCC #27 is deposited at the NIO, Goa, and is available upon request.

Inocula for experiments using *Corallochytrium limacisporum* NIOCC#27 were raised by transferring a small piece of agar-grown culture of the organism into 20 ml of BK broth in 100 ml Erlenmeyer flask and incubated on a shaker at 150 rpm speed and 28 °C $\pm$ 2 °C. After 4 days, the cells were harvested by centrifugation and re-suspended in 0.5 ml of seawater. A 0.1 ml ($\sim$15—20 mg wet wt of cells) aliquot was used as inoculum for all growth experiments. Wild-type *Neurospora crassa*, culture '74-OR23-1 *mat* A (FGSC 987)', and a sterol C-14 reductase mutant, designated 'erg—3 *mat a* (FGSC 2725)' were used for some of the experiments.

Growth studies: Growth at different salinities was estimated by growing the culture in 100 ml of BK liquid medium prepared in seawater of salinity 35, 30, 20, 10, and 0 ppt. The different dilutions were obtained by diluting seawater with appropriate amounts of distilled water. Growth after 5 days was estimated as dry weight of lyophilized biomass. All experiments were carried out in triplicates.

Growth using organic and inorganic nitrogen sources was estimated by growing the culture in BK broth (organic nitrogen source of 0.2% peptone) and in Vogel's minimal medium (Davis and De Serres 1970) supplemented with

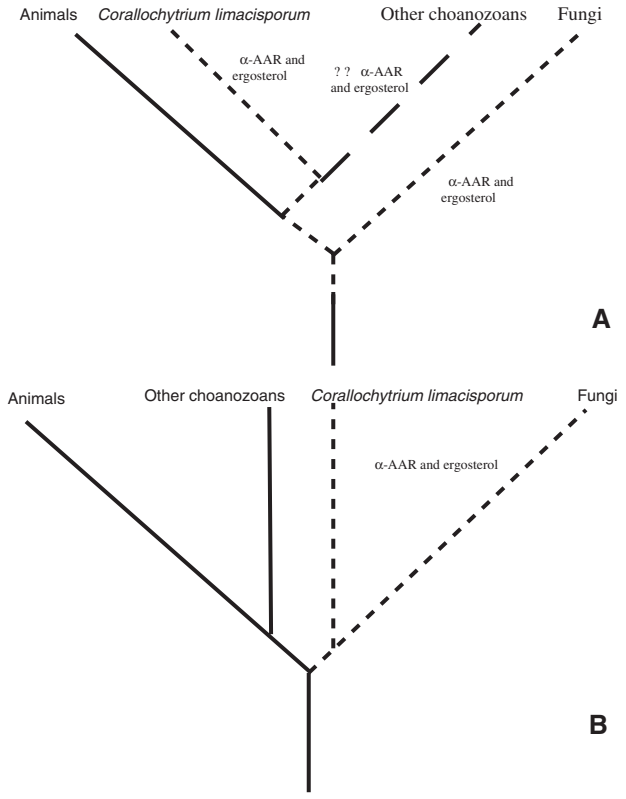

Figure 8. Possible relationships of *Corallochytrium limacisporum* to animals, choanozoans, and fungi. (**A**) represents *Corallochytrium limacisporum* as a sister clade to choanozoans and both groups as a sister clade to animals. (**B**) represents *Corallochytrium limacisporum* as a sister clade to fungi. The dotted line indicates the presence of α-aminoadipate reductase (α-AAR) and ergosterol; the unbroken line indicates the absence of the two fungal signatures; and the broken line indicates lack of information on their presence.

1.5% glucose (VG) (inorganic nitrogen source of 0.2% ammonium nitrate), the media were made up in seawater. Growth was estimated in triplicates as dry weight. The cells were harvested by centrifugation and lyophilized for estimation of the growth as dry weight and the same was used for estimating the α-AAR enzyme as described below.

Biochemical studies: Estimation of α-AAR enzyme activity and of sterols. The methodology described by Bhattacharjee and Bhattacharjee (1999) was followed for estimating α-AAR enzyme activity. An amount of 200 mg of lyophilized culture was suspended in 2 ml of 10 mM Tris—HCl (pH 8.0) on ice for 30 min and ultrasonicated 5 times at 50 W (30 sec each with an interval of 30 sec). The supernatant was collected by centrifugation for 30 min at 3000*g* at 4 °C and was used for total protein and enzyme assays. Protein was estimated by the Bradford method (Bradford 1976).

The reaction mixture for the enzyme assay contained 12.5 mM α-amino adipate (α-AA) (Sigma, St. Louis, Mo.), 15 mM ATP, 10 mM $MgCl_2$, 1mM glutathione reductase (Sigma, St. Louis), 0.625 mM NADPH, 250 mM Tris—HCl (pH 8.0), and an appropriate amount of enzyme, usually 500—600 µl (containing 600—800 mg protein) solution in a total volume of 1 ml. The crude cell homogenate was used as an enzyme source. Reaction mixture with no α-AAR was used as a negative control. The reaction was carried out at 30 °C for 1 h and terminated by the addition of 1 ml of 2% *p*-methylaminobenzaldehyde (DAB) in 2-methoxyethanol and heated at 100 °C for 15 min. The coagulated protein was removed by centrifugation and the enzyme activity was determined by quantifying α-AA-δ semialdehyde which forms a yellow complex with DAB. One unit of α-AAR is defined as the activity that produces an increase of absorbance of 0.01 per min at 460 nm. All analyses were carried out in triplicate.

Sterols were extracted from 50 mg of lyophilized culture as described by Prakash et al. (1999) and the UV absorption spectrum (200—300 nm) was recorded on a Hitachi spectrophotometer. The absorption maxima at 272, 282, and 293 nm was compared with the sterols extracted from wild-type *Neurospora crassa* 74-OR23-1 *mat* A (FGSC 987) and *Neurospora crassa* mutant erg—3 *mat a* (FGSC 2725).

Molecular studies: General protocols for DNA extraction, PCR amplification, radiolabeling, restriction digestion, ligation, gel purification, and sequencing were carried out as described by Sambrook et al. (1989) or according to the manufacturer's instructions.

Primers for the α-AAR gene were designed as follows. Multiple alignment of α-AAR amino acid sequences for 10 fungi deposited in the NCBI (National Center for Biotechnology Information, USA) was carried out using the MultAlin software (Corpet 1988). The sequences used were *Acremonium chrysogenum* (CAC22111), *Candida albicans* (AAC02241), *Saccharomyces cerevisiae* (AAA34747), *Yarrowia lipolytica* (CAG79208), *Penicillium chrysogenum* (CAA74300), *Neurospora crassa* (CAB97293), *Bullera alba* (BAD01573), *Rhodotorula minuta* (BAD01575), *Mixia osmundae* (BAD01574), and *Leptosphaeria maculans* (AAO49452) (the aligned sequences can be made

Table 1. Oligonucleotide sequences used in this study.

	Oligonucleotide sequences (5′—3′)	Purpose
AAR-F AAR-R	GCBTTCTTYGTBGGBGAC CCNARYTCRATDCKRAANCC	Degenerate oligos used for α-aminoadipate reductase gene
AA-inF AA-inR	CGGATGGTTCTGTGGAGTG CAACACCTGTCAGCATCTTG	Inverse PCR oligos for α-aminoadipate reductase gene
SCF[a] SCR[a]	ATIGGIMGIGARYTIAAYCCIMG CCARTCICCIARRTARTTIATRTG	Degenerate oligos used for sterol C-14 reductase gene
SC-inF SC-inR	TGTGCTCTCGACGATGGACATC GGCCTGAGTTCGCAAACGTAC	Inverse PCR oligos for sterol C-14 reductase gene

[a]Aparna K, Sandrock RW, Kasbekar DP (1998) Cloning of the sterol C-14 reductase gene of the tomato pathogenic fungus *Septoria lycopersici* and its complementation of the erg-3 mutation of *Neurospora crassa*. J Genet 77: 71—75

Table 2. Growth and α-AAR activity in *Corallochytrium limacisporum* and *Neurospora crassa.*

Parameters	Culture Medium	
	VG[a]	BK[b]
Corallochytrium limacisporum NIOCC#27		
Biomass (mg dry wt)	50 ± 00	101 ± 30
α-AAR activity (U mg^{-1} protein)	14.5 ± 2.1	14.8 ± 2.4
Neurospora crassa		
74-OR23-1 mat A (FGSC 987)		
Biomass (mg dry wt.)	509 ± 20	554 ± 70
A-AAR activity (U mg^{-1} protein)	9.6 ± 3.2	9.8 ± 3.7

[a]contains ammonium nitrate as N source.
[b]contains peptone as N source.

available on request). Based on the alignment, conserved regions were identified and degenerate primers AAR-F and AAR-R were designed (Table 1). A partial fragment (500 bp) of the α-AAR gene was amplified using these primers. For the sterol C-14 reductase gene, degenerate primers (Aparna et al. 1998) were used. The PCR conditions were an initial denaturation step at 94°C for 5 min, followed by 34 cycles each of 1 min, denaturation at 94°C, annealing at 50°C, and extension at 72°C followed by a final extension at 72°C for 5 min.

Inverse PCR (iPCR) is a procedure that extends the utility of PCR for geometric amplification of unknown DNA sequence that flanks a core of known sequence (Howard et al. 1990). This approach was followed to obtain flanking regions of the PCR products obtained by using degenerate primers as above. Based on the sequence information of the PCR product, specific primers for iPCR (AA-inF, AA-inR, SC-inF, and SC-inR) were designed (Table 1). Genomic DNA, self-ligated after digestion with either *Hind*III or *Nco*I restriction enzyme, was used as template for iPCR to obtain extra sequences for the α-AAR and sterol C-14 reductase genes respectively. The enzymes were identified by carrying out a restriction digestion with different enzymes, *Hind*III, *EcoR*I, *EcoR*V, *Nde*I, *Not*I, *Pvu*II, and *Nco*I followed by a Southern blot analysis using an α-P^{32}-labeled PCR product as probe. The enzymes *Hind*III and *Nco*I were selected because they did not have restriction sites in the desired gene and digested the genomic DNA in a manner that the gene was in a less than 3 kb fragment. The iPCR conditions were the same as for normal PCR given above, except that the annealing temperature was increased to 5°C.

The PCR and iPCR products were gel purified using the QIAgen QIAquick gel extraction kit and direct sequencing was done on an automated ABI PRISM 377TM DNA sequencer using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). The sequence

identity was obtained by doing a homology BLAST search in the GenBank database (National Center for Biotechnology Information, USA: NCBI http://www.ncbi.nlm.nih.gov).

Sequences for phylogenetic analysis were obtained by carrying out a BLAST search in the GenBank database to identify sequences related to the sequence of the two genes α-AAR and sterol C-14 reductase of *Corallochytrium limacisporum* NIOCC#27. Sequences retrieved from the NCBI GeneBank database were aligned and this was used for phylogenetic analysis. The nucleotide sequences were aligned using the programs Clustal-X (http://www.igbmc.u-strasbg.fr/BioInfo/) (Higgins and Sharp 1989) and GenDoc (http://www.psc.edu/biomed/genedoc) (Nicholas and Nicholas 1997) and also checked manually for large gaps. The aligned sequences were flushed at the ends to avoid missing information for any compared reference entries. Phylogenetic analysis was carried out using all three codon positions and also by using only two codon positions to avoid divergence based on codon bias. Phylogenetic analysis of the α-AAR gene was carried out using 12 sequences and the sequence of *Anabena* was used to root the tree. For the sterol C-14 reductase gene, 10 sequences were used for tree construction and *Arabidopsis* sequence was used as outgroup. The aligned sequences were then used to derive corrected Kimura two-parameter distance (Kimura 1980) estimates and infer phylogenetic relationships using both distance-based neighbor-joining (NJ) (Saitou and Nei 1987) and maximum likelihood (ML) (Strimmer and Haeseler 1997) methods, with analytical routines available in the software package PHYLOwin (Galtier et al. 1996).

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