Algal Research 35 (2018) 125–141

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Proposal of a new thraustochytrid genus *Hondaea* gen. nov. and comparison of its lipid dynamics with the closely related pseudo-cryptic genus *Aurantiochytrium*

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ARTICLE INFO

Keywords:
Biotechnological potential
Phylogeny
Sterol–thraustochytrid–triacylglycerol
ω3 fatty acid

ABSTRACT

Thraustochytrids are marine protists highly ecologically relevant in mangrove environments. The family Thraustochytriaceae underwent profound taxonomical rearrangements in the last decade, with the description and emendation of several genera. Here, we identified two new thraustochytrid strains (CCAP 4062/1 and CCAP 4062/3) collected from the same mangrove environment in Mayotte Island (Indian Ocean) and representative of two sister clades in the phylogenetic *Aurantiochytrium* super clade. Phylogenomic (on 2389 genes) and phylogenetic analyses on 18S rDNA sequences led us to propose the description of a new genus, *Hondaea* gen. nov. (CCAP 4062/3), closely related and pseudo-cryptic to *Aurantiochytrium* (CCAP 4062/1). Compared to *Aurantiochytrium*, *Hondaea* did not produce amoeboid cells and its zoospores were smaller. Chemotaxonomical traits, such as fatty acid, sterol, and carotenoid profiles measured along the growth curves, validated the new genus description. Genome sequencing and manual annotation of lipid metabolism genes revealed similar pathways in both strains. However, such pathways showed different dynamics during the growth phases. *Aurantiochytrium* accumulated carotenoids (canthaxanthin) and large amounts of triacylglycerols enriched in ω3-docosahexaenoic acid in the stationary phase, while squalene and free cholesterol increased during the early exponential phase. In contrast, *Hondaea* accumulated low amounts of triacylglycerols enriched in odd and saturated fatty acids during the early exponential phase, whereas free-sterol and carotenoid contents were little affected. These results suggest that these genera evolved independently, although phylogenetically and

Abbreviations: ACAA, 3-ketoacyl-CoA thiolase; ACAT, acetyl-CoA acetyltransferase; ACP, acyl carrier protein; CACT, carnitine-acylcarnitine translocase; CCT, CTP:phosphocholine cytidylyltransferase; CDP-DAG, cytidyldiphosphate-diacylglycerol; CDP-EtA, cytidyldiphosphate-ethanolamine; CDS, CDP-DAG synthase; Chol, choline; CK, choline kinase; CLS, cardiolipin synthase; CPT1, carnitine palmitoyltransferase; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferases; DGDG, digalactosyldiacylglycerol; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; DPG, diphosphatidylglycerol; DGK, diacylglycerol kinase; DMDS, dimethyl disulfide; DW, dry weight; ECH, enoyl-CoA hydratase; ECT/CTP, phosphoethanolamine cytidylyltransferase; EPA, eicosapentaenoic acid; EtA, ethanolamine; EK, ethanolamine kinase; ESI, electrospray ionization; FA, fatty acid; FAD, fatty acid desaturase; FAE, fatty acid elongase; FAME, fatty acid methyl ester; FAS, fatty acid synthase; FDR, false discovery rate; FID, flame ionization detector; G3P, glycerol-3-phosphate; Glyco-PE, glycosylated-phosphatidylethanolamine; GOEA, gene ontology enrichment analysis; GPAT, glycerol-3-phosphate acyl transferases; HADH, hydroxyacyl-CoA dehydrogenase; HMG, 3-hydroxy-3-methyl-glutaryl; LPAAT, lysoPA acyl transferases; LPCAT, lyso-phosphatidylcholine acyltransferase; LPEAT, lyso-phosphatidylethanolamine acyltransferase; LPGAT, lyso-phosphatidylglycerol acyltransferase; LPIAT, lyso-phosphatidylinositol acyltransferase; MAG, monoacylglycerol; MAT, malonyl-CoA ACP transacylase; MGDG, mono-galactosyldiacylglycerol; PA, phosphatidic acid; PAP, phosphatidic acid phosphatase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEAMT, phosphoethanolamine *N*-methyl transferase; PEMT, phosphatidylethanolamine methyltransferase; PG, phosphatidylglycerol; PGP, phosphatidylglycerol phosphate; PGPP, phosphatidylglycerol phosphatase; PGPS, phosphatidyl glycerol phosphate synthase; PI, phosphatidylinositol; Pi-Chol, phosphocholine; Pi-EtA, phosphoethanolamine; PIS, phosphatidylinositol synthase; PP, pyrophosphate; PKS-like, polyketide synthase like complex; PLMT, phospholipid *N*-methyl transferase; PPT, phosphopantetheine transferase; PS, phosphatidylserine; PSD, phosphatidylserine decarboxylase; PSS, phosphatidylserine synthase; SQDG, sulfoquinovosyl diacylglycerol; TAG, triacylglycerol; VLCPUFA, very long chain polyunsaturated fatty acid; WGD, whole genome duplications

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https://doi.org/10.1016/j.algal.2018.08.018
Received 30 March 2018; Received in revised form 20 June 2018; Accepted 16 August 2018

ecologically closely related. This comparative study also showed that the biotechnological potential of thraustochytrids cannot be deduced solely from phylogenetic and genomic analyses.

1. Introduction

Thraustochytrids, formerly known as 'lower fungi', are marine Stramenopiles [1] (order Thraustochytrida, family Thraustochytriaceae [2]), characterised by obligatory heterotrophy. Their inability to photosynthesise results from the evolutionary loss of their chloroplast [3]. Thraustochytrids have colonised a wide range of habitats, including the deep sea [4], but they are particularly abundant in turbid waters [5–7]. In subtropical mangrove habitats thraustochytrids are the major leaf litterfall colonisers [8].

Thraustochytrid life cycle and morphology are rather complex and several attempts have been made to rearrange taxonomy and systematics of this order [9–13]. Genus and species descriptions often include behavioural (e.g. presence or absence of binary divisions, [5,13]) or chemotaxonomic traits (e.g. production of specific compounds like fatty acids or pigments [14]). Unfortunately, these features may vary with culture conditions, prompting the need to specify the culture medium used for life or cell cycle observations [15]. The difficulty to taxonomically homogenise and systematically define taxa in this order has led to a plethora of criteria to identify and describe taxa. The traits used for taxonomy can be shared by a set of taxa but may be absent in their common ancestor, i.e. some traits may have appeared multiple times during evolution (homoplasy), as already hypothesised [5]. Such homoplasic features do not contain phylogenetic information. Similarly to what reported in other systems like e.g. diatoms [16], a multiphasic approach including molecular taxonomy has been implemented in thraustochytrids as well to describe new taxa. Indeed, the use of sequences from the gene encoding for the small sububit of the ribosome (SSU) for molecular taxonomy spread in different systems because it is present in all living organisms and it accomplishes the same function [17].

Besides their ecological relevance as eukaryotic biomineralisers, thraustochytrids have attracted biotechnological interest because they naturally accumulate high levels of triacylglycerols (TAGs), as seen with many other microalgae [18], but they also display a high content of very long chain polyunsaturated fatty acid (VLCPUFA), primarily ω3-docosahexaenoic acid (DHA, 22:6) [19–23]. DHA and other n − 3 (ω3) PUFAs, such as eicosapentaenoic acid (EPA, 20:5), are poorly synthesised in animals. DHA and EPA are considered as 'essential FAs' to be obtained from the diet, offering multiple benefits to human health [24]. Thraustochytrids may accumulate up to 30–40% of their dry weight as lipids, with DHA frequently representing 30% or more of the total FA content [20,23,25]. Thraustochytrid growth rates and biomass accumulation are often well adapted to biotechnological exploitation, although the yield of lipid and DHA production can vary considerably from one strain to another [19,26] and with the growth conditions [21,27–29]. Noteworthy, some thraustochytrids have also the ability to produce relatively high amounts of squalene [30–32], a key precursor of sterols and carotenoids. The former are crucial components of the lipid rafts embedded in the membranes and play a central role in membrane fluidity and structural organisation [33]. The latter are effective antioxidants [32] and widely used in cosmetics [34].

Here we compare two thraustochytrid strains isolated in Mayotte Island (Indian Ocean) and describe a new genus based on the results obtained from morphological, phylogenetic, genomic, and lipidomic analyses. Phylogenetic and phylogenomic analyses indicate that strain CCAP 4062/1 belongs to the *Aurantiochytrium limacinum* species; morphological and chemotaxonomic observations corroborate this conclusion. Strain CCAP 4062/3 belongs to a different taxon, phylogenetically closely related to *A. limacinum* but morphologically, genomically and metabolically different. This led us to the formal description of a new genus, *Hondaea* gen. nov. Using optimal growth conditions we determined for both strains three phases of growth, the early exponential, the exponential and the stationary phases. At each of these phases, we analysed total fatty acids (FA) and glycerolipid contents, illustrating the interplay between the membrane (phospholipids) and the storage (TAGs) lipids. We also determined the amount of squalene, and the sterol and carotenoid compositions associated with the different stages of growth. The results indicate strong differences between the two strains, CCAP 4062/1 (*Aurantiochytrium*) accumulating on a dry cell weight basis four to five times more glycerolipids and squalene than CCAP 4062/3 (*Hondaea)*.

2. Materials and methods

2.1. Strains and culture conditions

CCAP 4062/1 and CCAP 4062/3 strains were collected in Mayotte Island (Indian Ocean, 12°48′51.8″S, 45°14′21.7″E) and grown in liquid R medium (Supplemental Table S1). In this medium, glucose represents the main source of organic carbon (> 90%) whereas the yeast extract provides little organic carbon (< 10%) and almost all the nitrogen and phosphorus (> 98%). Growth curves were performed in triplicate, in Infors incubators at 20 ± 1 °C 16 h:8 h light:dark photoperiod to mimic natural light conditions, light intensity of 50 μmol photons·m^{-2}·s^{-1}, and constant shaking at 100 rpm. Nomenclature follows the International Code of Nomenclature for algae, fungi, and plants [35].

2.2. DNA extraction and phylogenetic analyses

The genomic DNA from CCAP4062/1 strain was extracted, and 18S rDNA was amplified and sequenced as described in the Supplementary methods. Bacterial clones sequenced showed no differences. CCAP 4062/3 18S rDNA sequence was obtained from whole genome sequencing and assembly [36]. CCAP 4062/1 (MG946762) and CCAP 4062/3 (MF766427) 18S rDNA sequences were analysed together with a total of 555 18S rDNA sequences from various thraustochytrids (Supplemental Table S2). A final subset of 415 sequences was used for Maximum Likelihood phylogenetic reconstruction using MEGA7 [37] software. Five thousand bootstrap replications were performed (see Supplementary methods).

2.3. Phylogenomic analysis

A phylogenomic analysis was carried out to determine the relationship between strain CCAP 4062/3 and other members of the Thraustochytriaceae. In order to obtain the maximum possible number of protein coding genes, only the species with a full genome assembly were considered, i.e. strain CCAP 4062/3 [36], *Aurantiochytrium limacinum* ATCC MYA-1381 (=SR21) (http://genome.jgi.doe.gov/Aurli1/Aurli1.home.html), *Schizochytrium* sp. CCTCC M209059 (GCA_000818945.1), *Aurantiochytrium* sp. T66 (GCA_001462505.1), *Thraustochytrium* sp. ATCC 26185 (GCA_002154235.1) and *Phytophthora infestans* (used as outgroup, GCA_000142945.1). Since no genome annotation was available for *Schizochytrium*, *Aurantiochytrium* sp. T66 and *Thraustochytrium*, an ab initio gene prediction was performed with Augustus using the prediction model previously generated for strain CCAP 4062/3 [36]. The predicted proteomes of the six species were then analysed with OrthoMCL pipeline (release 5, http://orthomcl.org/orthomcl/) to identify single copy genes that were