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Phylogenetic analysis of the protein homologs of hemoxygenases HO-1 and HO-2 inferred from the transcriptomes of dinoflagellates

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Summary

The potentially toxic marine dinoflagellates *Prorocentrum cordatum* (Ostenfeld) Dodge, 1975 are responsible for harmful algal blooms in many coastal ecosystems and have recently colonized the brackish-water Baltic Sea. Their ability to adapt to changing environmental conditions is partly mediated by cytoprotective proteins that provide the effective physiological stress response. One such protein, heme oxygenase (HO/HSP32), not only catalyzes the degradation of heme but also protects cells from oxidative stress caused by a number of environmental factors. In this study, we phylogenetically characterized the HO-like protein sequences of dinoflagellates found in the unannotated transcriptomes represented in the Marine Microbial Eukaryote Transcriptome Sequencing Project database. The homologues sequences identified in the database shared amino acid identity with HO family proteins of other taxa and contained typical conserved motifs. Phylogenetic analysis showed that HO-like homologs are widely represented in the dinoflagellate transcriptomes. Overall, sequences of dinoflagellates can be classified into two distinct groups. The first group is closely related to other unicellular protists and cyanobacteria. The second group clusters separately from all other taxa. We made a comparative analysis of the HO-1-like and HO-2-like protein trees to evaluate topological and branch length differences between the trees. We found that both trees possessed different topologies, thus indicating that these two proteins evolved at different rates.

Key words: dinoflagellates, heme oxygenase, HO-1, HO-2, phylogeny, *Prorocentrum cordatum*, transcriptome

Introduction

Dinoflagellates are eukaryotic microalgae that are widely spread in the marine and freshwater environments and are among the most important primary producers in aquatic ecosystems. Some dinoflagellate species are responsible for harmful algal blooms (HABs) that cause economic loss and are potentially dangerous to human health due to the toxins they produce (Hallegraef, 2003; Berdalet et al., 2015). Some dinoflagellates are known as successful, highly adaptive invasive species. For

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example, the potentially toxic dinoflagellates *Prorocentrum cordatum* (Ostenfeld) Dodge, 1975 (synonym: *Prorocentrum minimum* (Pavillard) Schiller, 1933) invaded oligohaline waters of the Baltic Sea in the early 1980th, where they have successfully naturalized and eventually outcompeted the native congener *P. balticum* (Telesh et al., 2016), demonstrating remarkable adaptation strategies (Knyazev et al., 2018; Khanaychenko et al., 2019; Pechkovskaya et al., 2017; Skarlato et al., 2018a, 2018b; Telesh et al., 2021). However, molecular mechanisms of stress response and adaptation to environmental changes (in particular, the critical salinity conditions) of dinoflagellates are insufficiently studied. Meanwhile, molecular data are crucial for understanding the regulation mechanisms and physiological characteristics of the organism. Thus, biomarkers of stress can be useful tools for understanding the complex interactions that regulate organisms' responses to ecological stressors at molecular level (Pechkovskaya et al., 2020, 2021).

Among the various molecular biomarkers, stress proteins are of special interest due to their ability to increase synthesis rates in response to unfavorable environmental conditions (Kültz, 2005). The changing levels of stress proteins indicate the activation of protection and reparation processes in the cell in response to harmful effects. A number of researchers have investigated the role of some dinoflagellate HSP-family proteins in cells' stress response (Rosic et al., 2011; Guo et al., 2015; Deng et al., 2019), but molecular studies of the vast majority of other cytoprotective proteins are scarce so far. One of these proteins, heme oxygenase (HO), plays a significant role not only in heme catalysis, but also in protecting cells against oxidative stress caused by a number of environmental factors (Jansen et al., 2010; Gozzelino et al., 2010).

HO, also indicated as HSP32, is an evolutionary conserved protein, homologs of which were found in most taxa across different domains and kingdoms (Li and Stocker, 2009). Bacterial and human heme oxygenases share up to 70% homology within the heme-degrading motif (Wilks, 2002). There are two HO isoenzymes, namely HO-1 (enzyme classification EC1.14.99.3) and HO-2 (EC1.14.99.39) encoded by different genes. HO-1 is rapidly induced by diverse stress stimuli and is regarded as the heat shock protein, whereas HO-2 is constitutive and not inducible (Maines and Gibbs, 2005). HO-1 is inducible by a large number of physical and chemical effects, including light intensity, temperature shifts, carbon source, etc. (Rhie and Beale, 1994; Leong et al., 2012; Strasky et al., 2013). The HO system oxidatively cleaves heme to produce free iron, carbon monoxide (CO) and biliverdin, which is further reduced to bilirubin, a potent endogenous antioxidant (McDonagh, 2010) (Fig. 1). Unconjugated bilirubin is capable of scavenging singlet oxygen with high efficiency, reacting with superoxide anions and peroxyl radicals and serving as a reducing substrate for peroxidases in the presence of hydrogen peroxide or organic hydroperoxides (Stocker and Ames, 1987; Stocker et al., 1987). HO-1 and HO-2 both share cytoprotective mechanisms and play an important role in maintaining cellular homeostasis, enhancing cell survival and suppressing the apoptotic pathways (Shibahara, 2003; Maines and Gibbs, 2005).

Information about HO-1 activity in microalgae is very scarce. The study of red algae revealed the essential role of HO-1 in protecting the cells from oxidative stress induced by heavy metal treatments (Richaud and Zabulon, 1997; Elbaz et al., 2010). HO-1 family homologous sequences were found in metatranscriptome of symbiotic dinoflagellates *Symbiodinium* sp. and the coral holobiont (Hongo et al., 2017). Our previous study demonstrated changes in HO-1 protein synthesis rates in dinoflagellates *P. cordatum* in response to salinity shifts (Pechkovskaya et al., 2021). Still, there is no data concerning HO-2 activity in eukaryotic microalgae. We believe that these proteins can be considered as biomarkers of stress, especially oxidative stress induced by salinity alterations and other fluctuating environmental factors.

The structure and evolutionary history of dinoflagellate genes is poorly studied. Molecular data is still rather limited for dinoflagellates due to the technical difficulties of sequencing their enormous genomes (up to 250 Gb) and other molecular peculiarities that distinguish them from other eukaryotes (Lin, 2011). Transcriptome databases are providing an alternative tool for studying the nucleotide and protein sequences of dinoflagellates.

In this article, we analyzed the amino acid sequences of HO homologous proteins, the potent biomarkers of stress, which can be a useful tool for studying the complex interactions that regulate organisms' responses to external stressors. For this purpose, we identified HO-like protein sequences in the unannotated transcriptomes of a number of dinoflagellate species and performed a phylogenetic analysis to evaluate their evolutionary relationship to

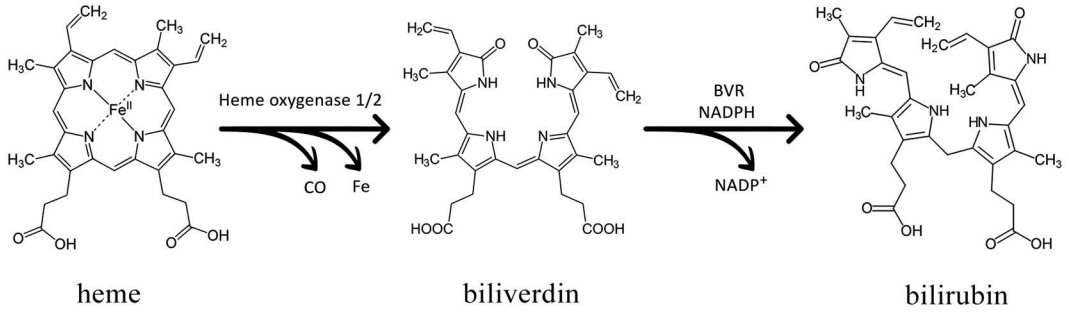

Fig. 1. Heme degradation catalyzed by heme oxygenase (orig.). Heme oxygenases 1 and 2 degrade heme to CO, Fe^{2+} and biliverdin, which is then converted to bilirubin by biliverdin reductase (BVR). All end products of heme catabolism (CO, Fe, biliverdin and bilirubin) are cytoprotective.

other taxa. Additionally, we performed a comparative analysis of the HO-1-like and HO-2-like protein trees of dinoflagellates to evaluate topological and branch length differences between the trees and estimate their evolutionary relationships.

Material and methods

1. HO family homologs identification in unannotated transcriptomes

We identified amino acid homologous sequences of the HO family proteins in the unannotated dinoflagellate transcriptomes available in the database of the Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP, Keeling et al., 2014). The red algae *Porphyra purpurea* heme oxygenase 1 amino acid sequence (accession number AAC08157) from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/protein) was used as query for a local BLAST search performed by means of the BioEdit 7.2.5 software (Hall, 1999) with BLOSUM62 matrix (E-value < 10^{-50}).

2. Sequence analysis

Protein conserved domains were characterized using Conserved Domains Search service (Marchler-Bauer et al., 2017). The signature motifs and conserved residues for HO family proteins were described by Wilks (2002). We analyzed unannotated sequences to find predicted targeting tags for determining the subcellular localization of these proteins by SignalP 4.1 (Petersen et al., 2011), TargetP 1.1 (Emanuelsson et al., 2007), WoLF PSORT (Horton et al., 2007), Mitoprot (Claros et al., 1996), Localizer (Sperschneider et al., 2017) and DeepLoc-1.0 (Almagro Armenteros et al., 2017). Amino acid sequences were aligned using the MAFFT online service (Katoh et al., 2019). The alignments were manually inspected and edited.

3. HO phylogeny analysis

The phylogenetic analysis of the HO-like amino acid sequences of the dinoflagellate species and the other taxa' HOs available in GenBank database and sufficient for phylogenetic reconstructions was performed. Conserved blocks were selected manually with the SeaView software (Gouy et al., 2010). The resulting dataset of HO homologs contained 60 amino acid sequences and had 325 positions. The resulting dataset of HO-1 homologs contained 19 amino acid sequences and had 228 positions. The resulting dataset of HO-2 homologs contained 25 amino acid sequences and had 362 positions. Phylogenetic analysis was performed by means of the RAxML 8.2.1 (Stamatakis, 2014), available online on CIPRES Science Gateway (Miller et al., 2010). For the analysis, we generated 100 RaxML tree searches to obtain the best ML tree as the starting tree. ML analysis was performed using LG (Le and Gascuel, 2008) substitution matrix. The most suitable substitution matrix was determined by the means of MEGA software (Kumar et al., 2018) and IQ-Tree web server (Trifinopoulos et al., 2016) in the CIPRES Science Gateway. Topological robustness was statistically tested by non-parametric bootstrap analysis from 1000 bootstrap replications. The tree was visualized using SeaView software. The tanglegram was done in RStudio (RStudio Team, 2020) using the packages DECIPHER (Wright, 2016) and dendextend (Galili, 2015).

Table 1. Heme oxygenase subcellular localization prediction in *Prorocentrum cordatum*.

Protein in the transcriptome CCMP1329	SignalP4	TargetP1.1	WoLF PSORT	Mitoprot	DeepLoc-1.0	Localizer 1.0	Result
HO-1	noTM	Chloroplast	nucl: 6 chlo: 4 cyto: 3 mito: 1	0,05	Plastid, Membrane	Chloroplast	Chloroplast
HO-2	noTM	—	cyto: 6.5, cyto_E.R.: 4.33333 chlo: 2 nucl: 2 E.R._vacu: 1.33333 plas: 1 pero: 1	0,005	Mitochondrion, Membrane	—	—

Results and discussion

1. HO family homologs characteristics

HO homologous protein sequences were identified in the unannotated transcriptomes of 19 dinoflagellate species presented in the MMETSP database. Three of them possess 1 paralogous HO sequences in their transcriptomes, 11 have 2, 4 have 3 and 1 of them has 4. The analysis revealed some sequences to be the short copies of the same transcript, so they were not included in the analysis. Amino acid sequences encoding proteins with estimated molecular mass of about 32 kDa were considered to be HO-1 homologs. Amino acid sequences of putative HO-2 homologs encoded proteins with the average mass of 53.7 kDa and heme-binding conserved Cysteine-Proline-Phenylalanine (CPF) residues specific to HO-2 sequences (Maines and Gibbs, 2005). Conservative domains of the HemeO-like superfamily were identified for all found HO sequences in dinoflagellates using the CD-Search algorithm in NCBI.

In both available *P. cordatum* transcriptomes, we identified 2 paralogs of HO homologous proteins. In strain CCMP1329, HO-1 homologous sequence is 302 aa long encoding the putative 32.9 kDa protein with theoretical isoelectric point (pI) 5.4. Hypothetical HO-2 homolog is 426 aa long and encode the putative 45.6 kDa protein with predicted pI 9.0. In strain CCMP2233, hypothetical HO-1 homologous sequence is 319 aa long and encodes the putative 34.5 kDa protein with theoretical pI 5.6. Predicted HO-2 homolog was 485 aa long encoding the 51.6 kDa protein with pI 6.8.

P. cordatum HO-1 and HO-2 sequences only share 35% identity. HO-1 is showing the strongest identity with HO-1 from *Azadinium spinosum* 3D9.186885 (71%), yet HO-2-like sequence is more similar to procaryotic HO-1 from *Cyanobacterium aponinum* AFZ530 with 44% identity. In general, all putative HO-2 sequences were remarkably longer than HO-1-like sequences with an average length of 510 aa (Fig. 2, A). Cnidarian sequences possess similar feature, although no similarity was found between the dinoflagellate and cnidarian sequences outside of the conservative region. In silico analysis of subcellular localization showed that HO-1-like amino acid sequences were predicted as chloroplast-localized protein (Table 1). However, analysis of the HO-2-like protein sequence did not reveal a definite specific location of the protein, which may be due to the molecular features of dinoflagellate protein sequences.

The analysis of sequences revealed that there is evolutionary conservation between dinoflagellates HO-like protein homologs and HO family proteins from different other taxa studied. For all these sequences, a conserved HO signature sequence (GDLSG) and essential histidine residue His-25 involved in heme-iron binding, which are typical for animal, bacterial and protist sequences (Richaud and Zabulon, 1997; Wilks, 2002) was found (Fig. 2, B).

2. HO family homologs phylogeny

To examine the relationship between the dinoflagellates' HO-like homologs and other taxa' HO family proteins and compare them, a maximum likelihood tree was constructed using RAxML. HO-1 and HO-2 animal amino acid sequences were included in the analysis to provide the depth to the tree. Plant HO homologs were not included

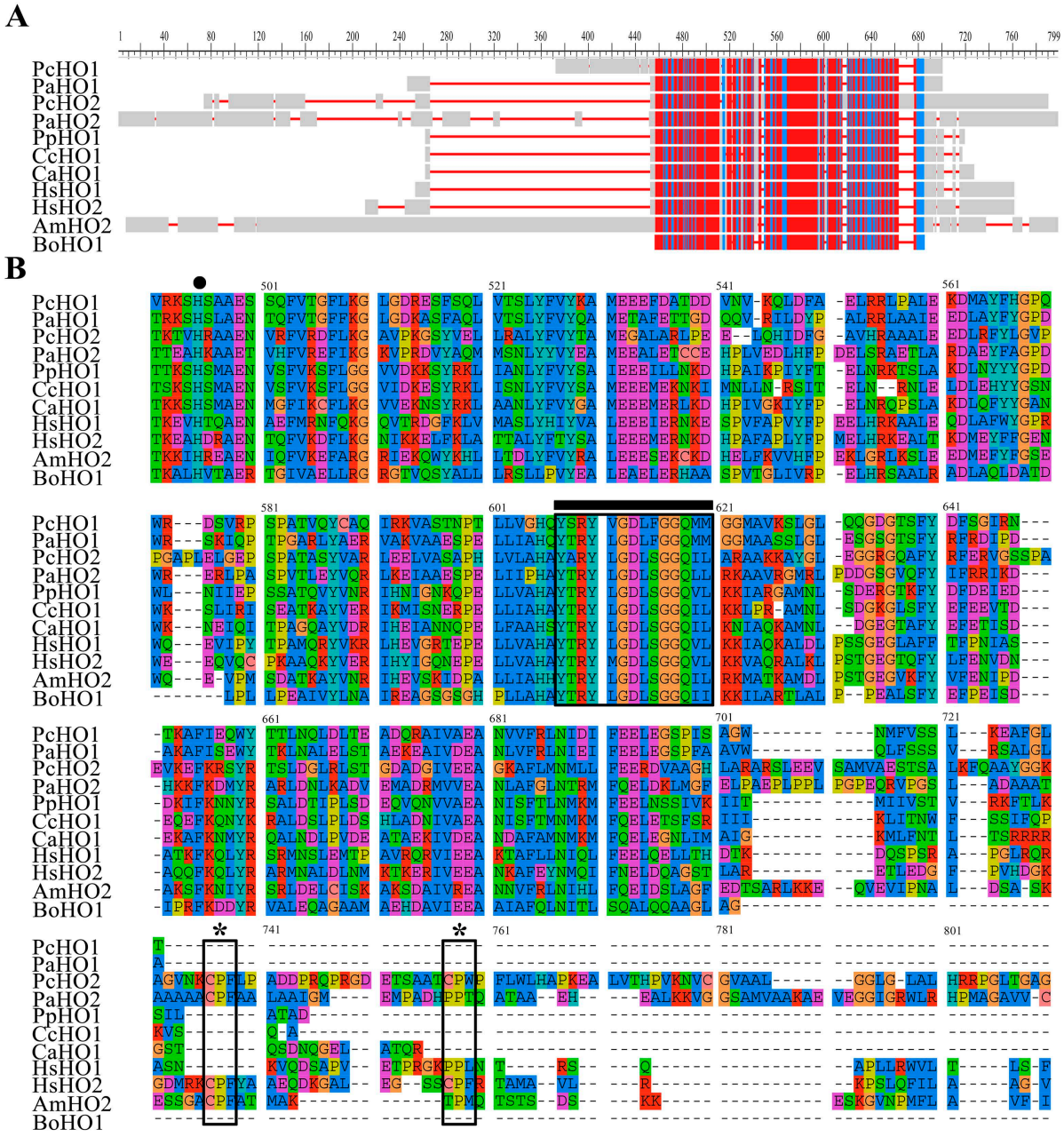

Fig. 2. Amino acid sequence alignment of HO family homologs of the representatives of the major taxonomic groups. A. Schematic structures of the full protein sequences. Conserved regions of the sequences are colored with red. B. The black dot highlights the conserved His-25 residue. The black bar marks the highly conserved region within heme oxygenase family. The asterisks mark the sites specific to HO-2. *Abbreviations* for species and accession number in MMETSP or GenBank: PcHO1 – *P. minimum* CCMP1329 39462; PcHO2 – *P. minimum* CCMP1329 6127; PaHO1 – *Peridinium aciculiferum* PAER2 31704; PaHO2 – *P. aciculiferum* PAER2 31704; PpHO1 – *Porphyra purpurea* AAC08157; Cc – *Cryptomonas curvata* A0A222AHE8; Ca – *Cyanobacterium aponium* AFZ53007; HsHO1 – *Homo sapiens* NP002124; HsHO2 – *H. sapiens* BAA04789; AmHO2 – *Acropora millepora* XP029200410; BoHO1 - *Bradyrhizobium oligotrophicum* KIZ47043.

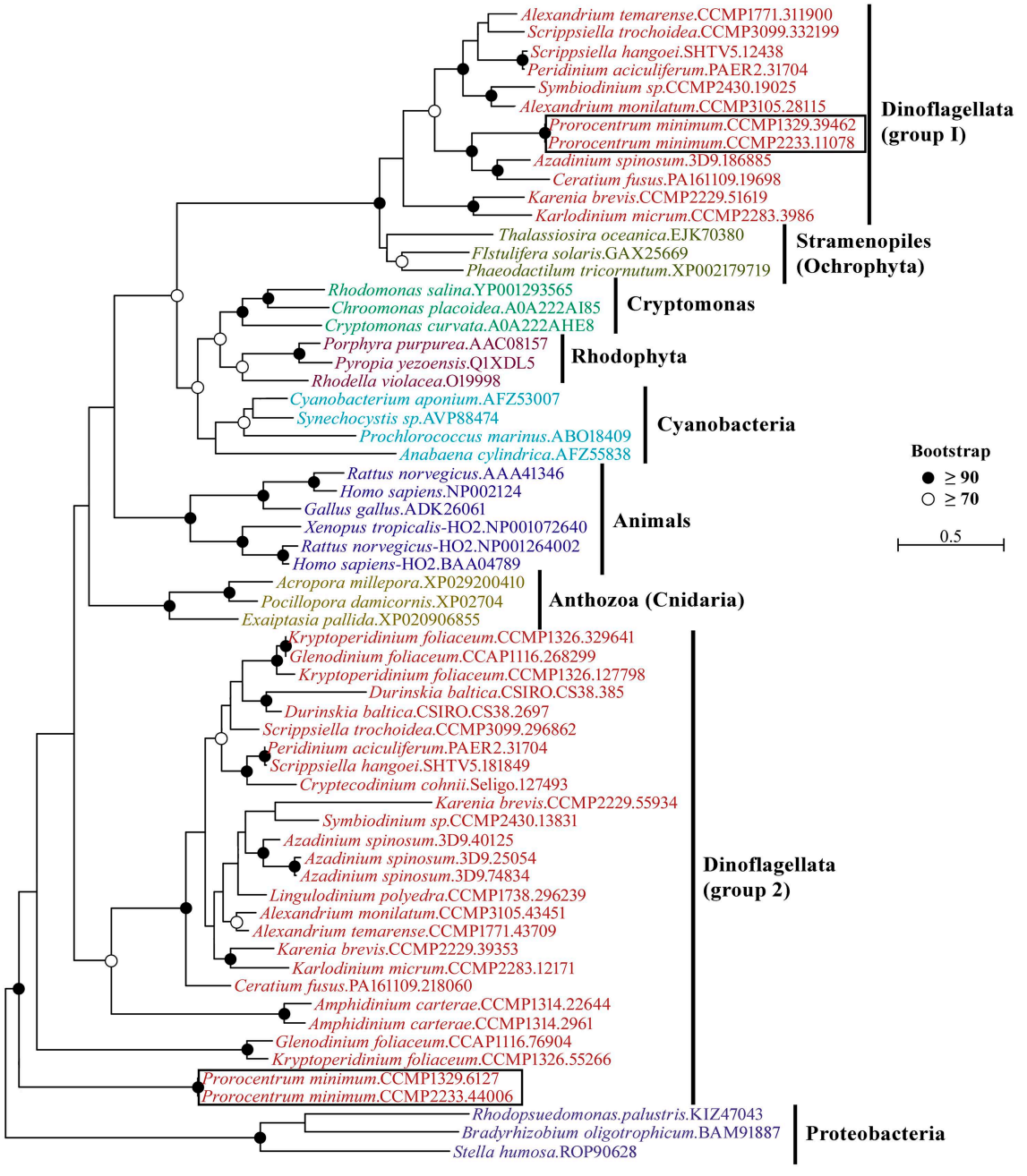

Fig. 3. Protein maximum likelihood phylogeny of HO homologous protein sequences using LG substitution model. Given scale represents the relative divergent time, bootstrap numbers (>70) are indicated. Sequences are colored according to taxonomic groups. All species names are given in accordance with database. *P. cordatum* sequences are highlighted with frame. The number corresponding to the number of sequences in the unannotated transcriptomes or the accession number in the database (GenBank) is indicated. Major eukaryotic lineages are bracketed to the right. Proteobacteria were used as an outgroup.

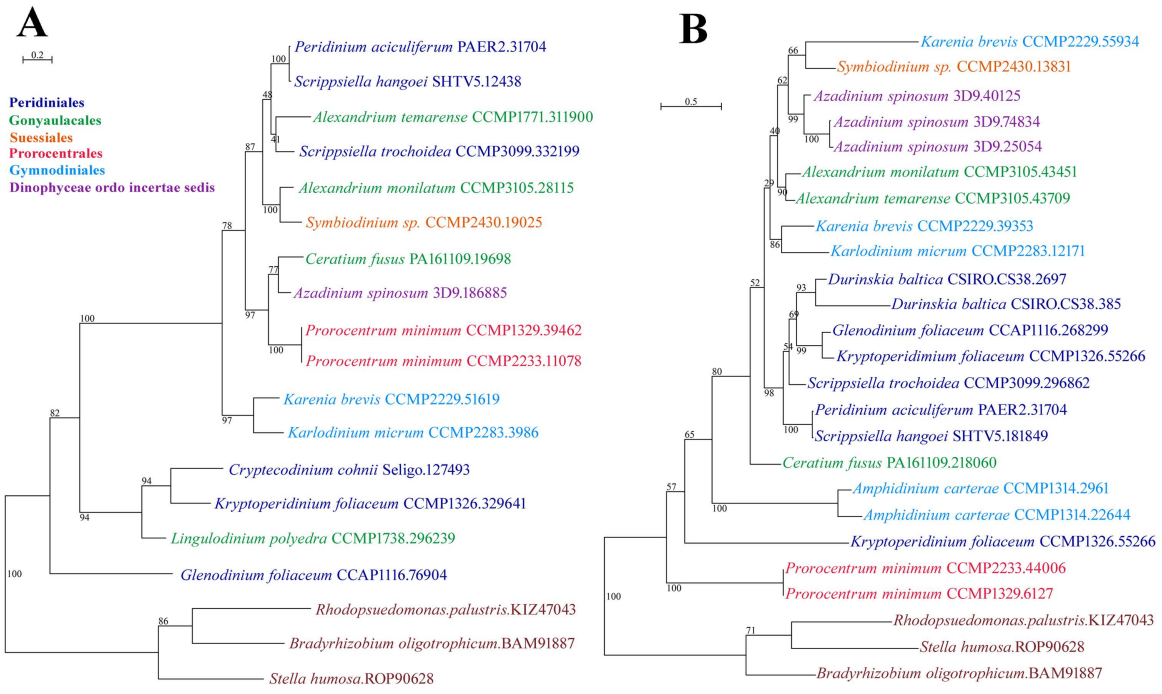

Fig. 4. Protein maximum likelihood phylogeny of HO-1-like and HO-2-like dinoflagellate protein sequences using LG substitution model. A – The ML tree of dinoflagellate HO-1 homologs; B – the ML tree of dinoflagellate HO-2 homologs. Given scale represents the relative divergent time. The family groups are coded by the color. All species names are given in accordance with the database.

in the analysis due to their significant evolutionary diversity. The tree was rooted with proteobacterial sequences.

Phylogenetic analysis of HO sequences revealed that the dinoflagellate HOs clustered into two major groups (Fig. 3). The group 1 consists of HO-1-like dinoflagellate homologs, clustered together with Ochrophyta species, suggesting they share a common evolutionary origin. Red algae, cryptophyta and cyanobacteria formed the distinct group, demonstrating sister relationship with HO-1-like sequences of dinoflagellates with high bootstrap support. Remarkably, there is a residue substitution of serine to phenylalanine in a conservative region (GDLSGG to GDLFGG), which is typical for all the sequences of the group 1. The group 2 of dinoflagellate HO homologs include all the sequences with average length of 510 encoding proteins with average mass 50.8 kDa with conservative heme-binding CPF residues and also 4 sequences encoding putative 32 kDa proteins (*C. cohnii* 127493, *K. foliaceum* 329641, *L. polyedra* 296239 and *G. foliaceum* 76904). These sequences possess the molecular features of HO-1-like sequences with the lack of the CPF residues. In Fig. 4, A, HO-1-like sequences are shown; they form separate group with a high statistical support.

Our phylogeny analysis of HO homologs is consistent with another HO phylogeny performed for a much larger number of taxa in Sharaf (2019) and provides a better insight into the genetic diversity of the HO-like proteins in dinofagellates. In general, HO homologs are ubiquitous in photosynthetic dinoflagellates and possess high similarity with HO of red algae, cyanobacteria and ochrophytas (Fig. 2). The amino acid sequences reported for higher plants' HO family homologs differ greatly from those in animals and unicellular algae. Moreover, anthozoan HO sequences are not clustered with the metazoan sequences presumably due to the evolutionary diversifcation of protein and its possible functional alterations.

3. HO-1 and HO-2 phylogeny comparison

In several transcriptomes of the MMETSP database, such as *Kryptoperidinium foliaceum* CCMP1326, *Glenodinium foliaceum* CCAP1116,

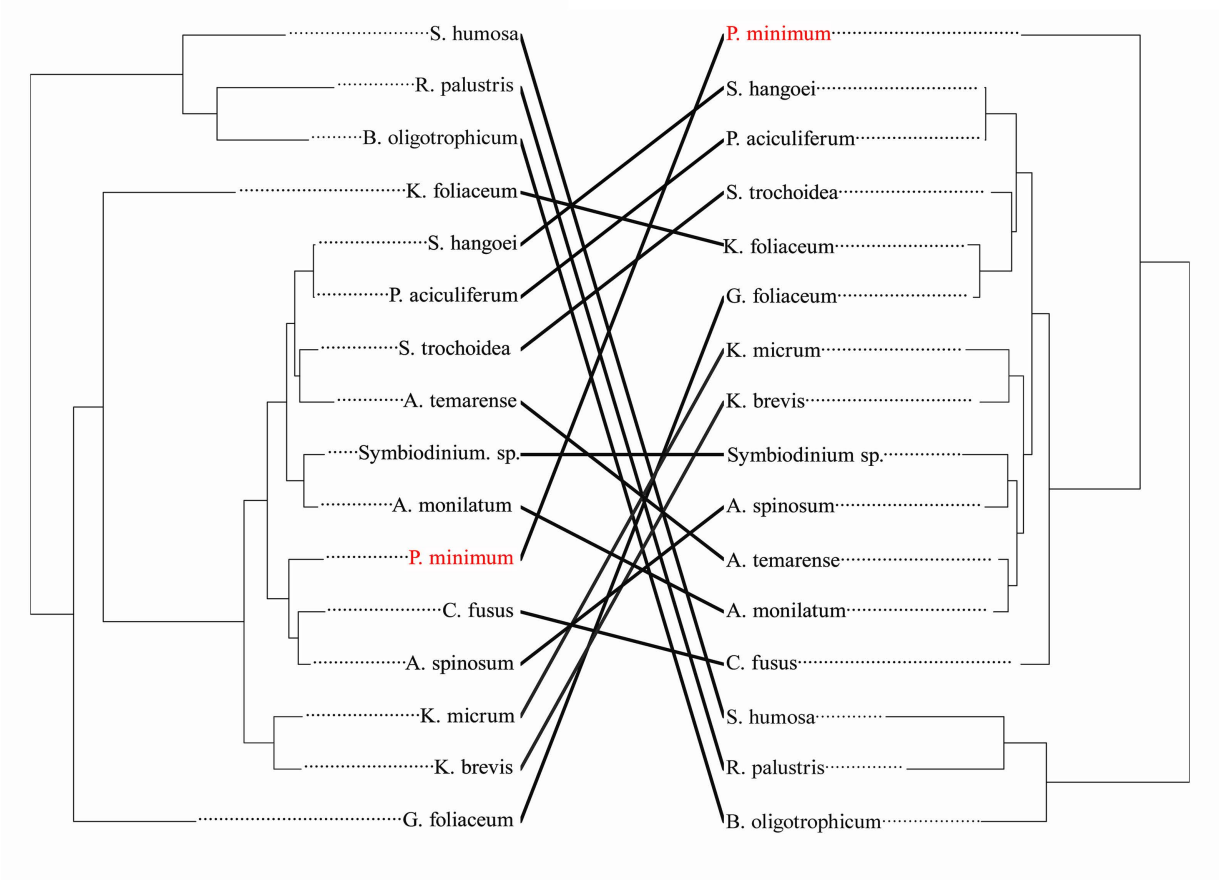

Fig. 5. Comparison of protein tree topologies HO-1 (left side) and HO-2 (right side) performed using R package for dinoflagellates. *P. cordatum* sequences (named *P. minimum*) are marked by the color. Proteobacteria were used as outgroup. The connecting lines are tangled due to the abundance of topology difference for each tree.

Karenia brevis, and *Azadinium spinosum*, we found the single HO-1-like transcript and several copies of HO-2-like sequences that might be a result of gene duplication. Presumably, divergence of two isoforms of HO occurred before the speciation of dinoflagellates and may be related to the protein acquisition from symbiosis during multiple plastid losses and replacements.

In order to compare the topology of trees and evolutionary rates of HO-1-like and HO-2-like dinoflagellates sequences, we analyzed these two proteins separately. The analysis was carried out with the same sequences; the trees were constructed according to the molecular features of HO-1 and HO-2 amino acid sequences and the obtained results (Fig. 3).

In the case of HO-1, dinoflagellate species from different families were distributed across the tree (Fig. 4, A). *P. cordatum* sequences grouped together and formed sister relationship with *A. spinosum* and *C. fusus*. Phylogenetic analysis of the HO-2-like proteins showed, that *P. cordatum* sequences were placed at the base of the tree, separated from the other dinoflagellates (Fig. 4, B). All the Peridiniales grouped together, except for the *K. foliaceum* paralog, with high bootstrap support. Other dinoflagellate orders like Gymnodiniales and Gonyaluacales were placed at the different parts of the tree.

To evaluate topological differences between the groups, we performed a comparative analysis of HO-1 and HO-2 protein trees. The results demonstrate that due to the very different topology, many branches are not comparable (Fig. 5). The different tree topologies and branch lengths suggest that these two proteins were evolving at different rates.

In conclusion, a phylogenetic analysis made in this study of the HO family proteins of the dinoflagellates revealed their relationship to other taxa, which allows for better understanding of their evolutionary history. HO-1-like and HO-2-like

homologs formed two distinct groups characterized by the substitution in the conserved region of the amino acid sequence. A comparative analysis of the HO-1-like and HO-2-like protein showed that these two proteins were likely evolving at different rates.

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