Shotgun Label-Free Proteomic Analyses of the Oyster Parasite *Perkinsus Marinus*

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Abstract

Perkinsus marinus is an intracellular parasitic protozoan that is responsible for serious disease epizootics in marine bivalve molluscs worldwide. Despite all available information on *P. marinus* genomics, more baseline data is required at the proteomic level. Our aim was to study the proteome profile of in vitro cultured *P. marinus* isolated from oysters *Crassostrea* spp. using a label-free shotgun UDMS^E approach. A total of 4073 non redundant proteins were identified across three biological replicates with stringent identification. Proteins specifically related to adaptive survival, cell recognition, antioxidants, regulation of apoptosis and others were detected. Important virulence factors of *P. marinus* were identified including serine protease and iron-dependent superoxide dismutase. Other proteins with involvement in several pathogens invasion strategies were rhoptries, serine-threonine kinases and protein phosphatases. Interestingly, peptides corresponding to retroviruses polyproteins were identified in all replicates. The interactomic analysis of *P. marinus* proteins demonstrated important cluster networks related to biological processes. In conclusion, we provide the first comprehensive proteomic profile of *P. marinus* that can be useful for further investigations on *Perkinsus* biology and virulence mechanisms.

Introduction

Marine bivalve molluscs production worldwide is being affected by disease epizootics caused by intracellular protozoan parasites of the genus *Perkinsus*. Numerous species have been described but only two species namely *Perkinsus marinus* and *Perkinsus olseni* require notification to the World Organization for Animal Health [1]. *P. marinus* has been associated with significant mortalities of the eastern oyster *Crassostrea virginica* but other species such as the pacific oyster *Crassostrea gigas* and the mangrove oyster *Crassostrea rhizophorae* are also susceptible [2].

The parasite belongs to a new independent phylum *Perkinsozoa* that is positioned between the phyla Apicomplexa and Dinoflagellata [3]. Species of *Perkinsus* are believed to be transmitted directly from host to host during the feeding process [4]. Different developmental stages are observed that are all believed to be infective [2]. No effective therapies have been developed to date.

A considerable amount of genomic information is available for *P. marinus*. In contrast, few studies available on comparisons of proteomic profiles of different *Perkinsus* species and on proteome variability of *P. olseni* resulted in low throughputs: 28 and 19 annotated proteins, respectively [5, 6]. More proteomic baseline data is required for a better understanding of *P. marinus* biology, including virulence mechanisms. Proteome techniques nowadays allow us to observe whole cellular events by directly visualizing the proteins being expressed. Neither genome nor transcriptome investigations would allow the analysis of such complex parasitic responses [7].

In the present study, we analyzed the proteomic profile of in vitro cultures of *P. marinus* by a high throughput label-free shotgun UDMS^E approach using nano ultra-performance liquid chromatography mass spectrometry (nanoUPLC-MS).

Material and Methods

P. marinus cultures were established from infected gills and mantle tissues of native oysters of the genus *Crassostrea* obtained from São Francisco do Sul, State of Santa Catarina, Brazil, following methods developed for *Perkinsus* spp. isolation [8]. Clonal cultures of trophozoites were established through isolation of individual cells by a standard limiting dilution method until it reached exponential phases of growth in DMEM/F-3 medium [9]. Parasite densities were estimated by counting with a hemocytometer.

Proteomic analysis was conducted using *P. marinus* ($1,7 \times 10^8$ cells) with three biological replicates. Briefly, *P. marinus* cells were centrifuged (1000 g for 10 min at 10 °C) and proteins were extracted by suspending pelleted cells in lysis buffer (42 % urea, 15 % thiourea, 4 % CHAPS, 12.5 mM Tris-HCl pH 7.5 and 1.5 % DTT). A protease inhibitor mix at 1 % (GE Healthcare, UK) was also added. Samples were then centrifuged (22000 g) at 4 °C for 40 min and washed four times in 50 mM NH_4HCO_3. Proteins were quantified using Qubit protein assay kit (Thermo Fisher, UK).

Samples were submitted to tryptic digestion using 50mM NH_4HCO_3 and 0.2% RapiGest SF (Waters) at 80 °C for 15 min, following treatment with 100 mM DTT at 60 °C for 30 min and then carboxamidomethylated in 300 mM of Iodoacetamine at room temperature for 30 min. Next, trypsin (Promega, USA) were added and incubated at 37 °C for 16 hours. Finally, 5% TFA was added at 37 °C for 90 min and samples were then centrifuged (22000 g) at 6 °C for 30 min. Supernatants were then transferred/ to the Total Recovery Vials (Waters, USA) and subjected to nanoUPLC-MS analysis using a Synapt G2Si mass spectrometer.

Qualitative bidimensional nanoUPLC (multiplexed DIA - data-independent acquisition) analysis were conducted using both a 1-h reverse-phase gradient from

7 % to 40 % (v/v) acetonitrile (0.1 % v/v formic acid) and a 450 nL.min-1 nanoACQUITY UPLC 2D Technology system. MS analysis of tryptic peptides was performed using a mass spectrometer equipped with a T-Wave-IMS device in MSE and UDMS^E modes [10]. MSE and UDMS^E raw data generated for each replicate were submitted to ProteinLynx Global Server software (PLGS) version 3.0.2 (Waters), with the following settings: maximum of 1 missed cleavage by trypsin, a fixed modification: carbamidomethyl (cysteine) and variable modification: oxidation (M). Mass spectra were searched against *P. marinus* protein database UniProt (Proteome ID UP000007800; 23,114 proteins). Protein identification was considered valid only when the following criteria were met: auto curate value Green (representing 99% of spectrum confident), default maximum false discovery rate (FDR) of 4 % and proteins with at least two distinct peptides. FDR were determined by searching against the reversed-sequence decoy database version of *P. marinus* protein sequences. For quantitative analysis, the dynamic range of protein abundance was calculated using the average of "MatchedProductIntenSum" column of PLGS data of all replicates. The data was decreasingly ordered and plotted using R software [11] (Fig. 3). A protein-protein interaction network was built using the algorithm STRING [12]. STRING data setting adjusted parameters were active interaction sources to

experiments, gene fusion, databases, co-ocurrence and co-expression; and the minimum required interaction score to custom value of 0.980. Proteins present in all replicates with at most a unique peptide were included as query in STRING web server (n = 881).

Results

A total of 4073 non redundant proteins were identified across the replicates and a combined total of 2810 proteins were present in at least two of the biological replicates (Figure 1). The calculated FDR was ~1 % when detection was set at agreement of all replicates. The complete list of identified proteins can be found in the Supplementary Table S1. The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [13] partner repository with the dataset identifier PXD003727.

The identified proteins were divided into 14 categories, according to Gene Ontology (GO) classification. Most of the identified proteins (82.09 %) were involved in metabolic, cellular and single-organisms processes. Other identified proteins were involved in response to stimulus, localization, biological regulations, cellular component organization or biogenesis, signaling, detoxification, developmental process, biological adhesion and less than only 1 % were involved in multicellular, organismal process and locomotion (Figure 2). The predicted proteins were blasted against Uniprot/
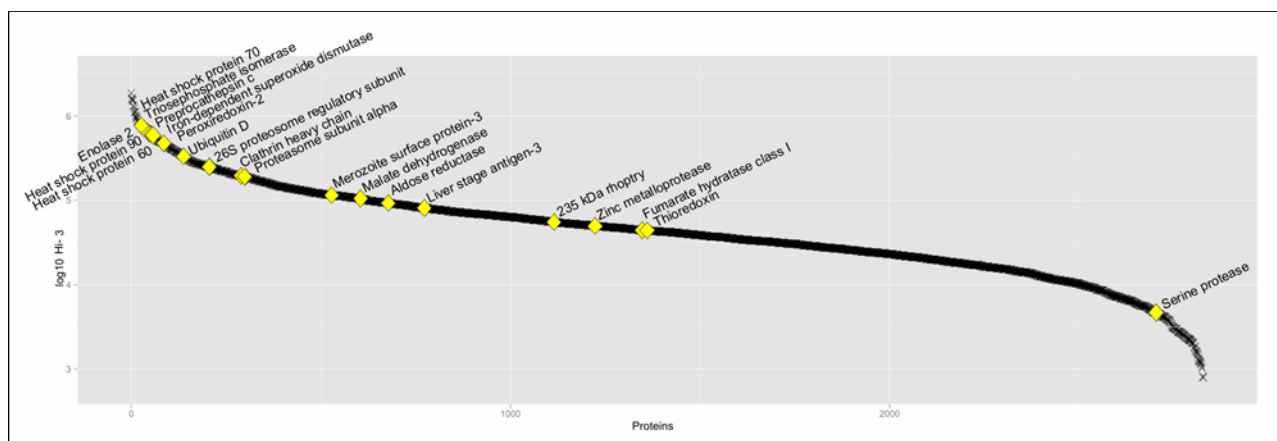

Figure 3. Dynamic range of the method based on protein abundance estimates, data points derived from LC-UDMS^E analysis. Y-axis shows $\log_{10}$ of Hi-3 matched peptides abundance for each protein. Yellow square shows the proteins cited along this work.
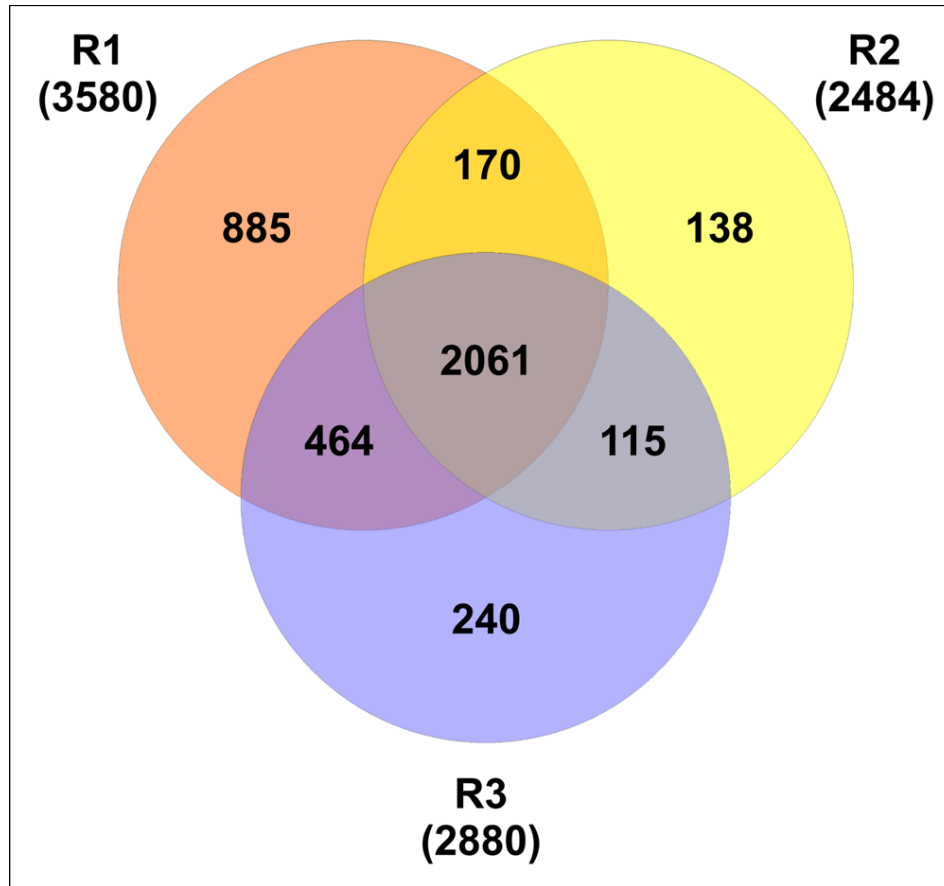
Figure 1. Venn diagram showing the numbers of unique and overlapping proteins identified between all biological replicates (R1, R2 and R3).
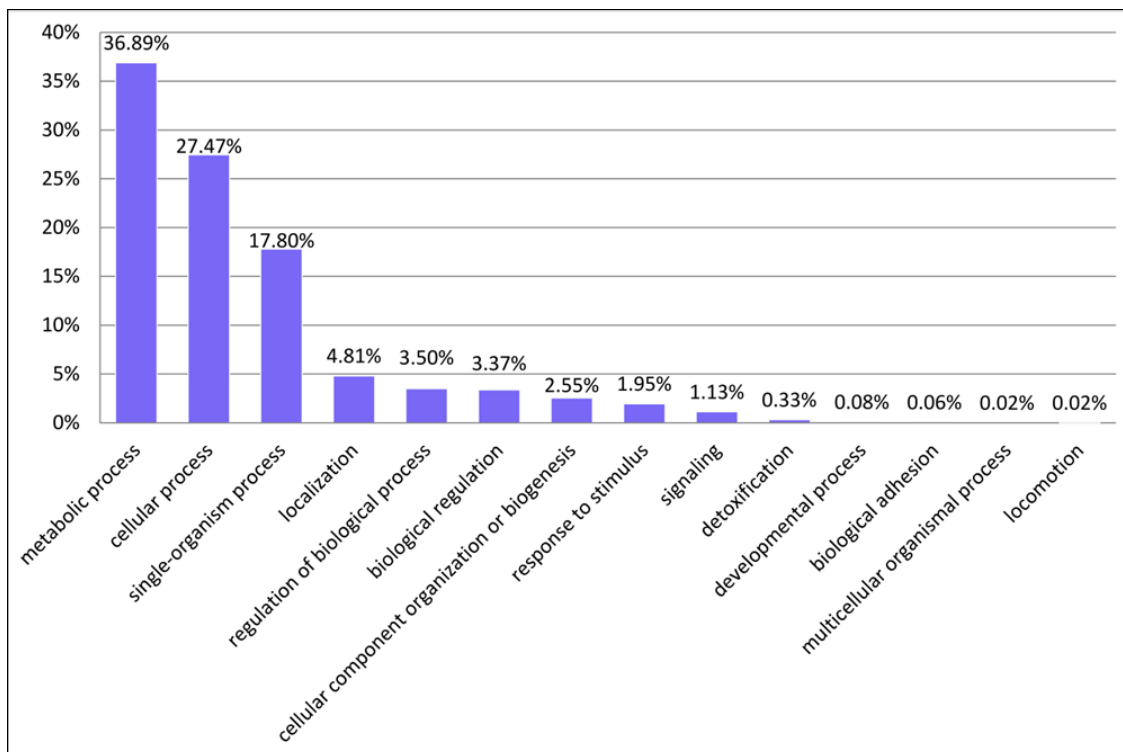

Figure 2. Classification of all detected protein**s** of in vitro cultures of *P. marinus*, using GO annotation. The distribution was made based on biological processes.

TrEmbl local database with e-value of 1e-06, percent of identity of 90, and maximum number alignments of 20. The GO function was mapped using a local database. Briefly, 907 (~26 %) proteins had unknown functions, whereas 4860 GO terms were assigned on Level 2 for Biological Process to the other 3166 proteins. The dynamic range (~3 logs) of quantified proteins of *P. marinus* was analyzed aiming to generate a global view of their relative abundance distribution. In our study, it was observed a high estimated abundance of proteins that were mostly related to biological processes and virulence in *P. marinus* (Figure 3).

Results from the interactomic analyses revealed that the greatest number of interactions were verified in the proteins related to ubiquitin-proteasome complex (cluster with 41 proteins), ribosomal proteins (41 proteins), iron-sulfur fumarate hydratase (20 proteins), TCP-1 (15 proteins), ATP synthase (17 proteins), pyruvate ferredoxin (7 proteins), triose phosphate isomerase/phosphoglucose isomerase/enolase (14 proteins) and clathrin interactome (15 proteins) (Fig 4).

Discussion

The proteome of in vitro cultured *P. marinus* was analyzed. In this study, we provided the first broad-based proteomic view into the basic biology and cellular metabolism of *P. marinus*.

Differences were observed in the number of identified proteins between biological replicates. Species of *Perkinsus* under culture conditions exhibits morphologically distinct life stages [8] making it quite difficult to produce homogeneous cultures of *P. marinus*. These differences would probably have an effect on the nature of the protein being expressed by each biological replicate.

For proteome dataset analyses, particular attention was given to proteins that are known to represent virulence factors in *P. marinus* and in other pathogens, including closely related apicomplexans. Several heat shock proteins were detected in all replicates mostly from the 70 kDa family that are the most common expressed heat shock proteins in

response to stress [14, 15]. Heat shock proteins are necessary for *P. marinus* adaptive survival repertoire [16, 17]. Antioxidant proteins including peroxiredoxin-2 and thioredoxin were detected in all triplicates, as well as proteins that are known to have immunosuppressive action, such as cyclophilins [17]. Proteins that are involved in the regulation of apoptosis including TP53-regulated inhibitor of apoptosis, liver stage antigen 3, pterin-4a-carbinolamine dehydratase, ubiquitin, polyubiquitin and adenylate kinase B were identified in at least two replicates [15, 17].

Serine proteases and iron-containing superoxide dismutase that are suggested to be virulence determinants of *P. marinus* were detected in at least two replicates [18, 19]. Proteases are known to play important roles in *P. marinus* disease pathogenesis causing cellular and tissue damage [17, 18]. Other identified proteases of great importance in virulence included: serine/threonine kinases, cathepsin b, c, L and z, preprocathepsin c precursor, Thiolproteinases, aspartyl aminopeptidase, 26S proteases regulatory subunit, proteasomes, m1 zinc metalloprotease, intracellular alkaline proteases and cysteine protease [15, 17].

Additional proteins that are important for the pathogenesis of *P. marinus* and a variety of bacterial and parasitic organisms were detected in at least two replicates, including: aldose reductase [20], betaine aldehyde dehydrogenase [21], helicases [15, 22], malate dehydrogenase [6], and aspartyl aminopeptidase [23].

Rhoptries are club-shaped secretory organelles that discharge their contents during infection. Rhoptry proteins were detected in all replicates. *P. marinus* is well known to infect oyster hemocytes residing inside phagosome-like vesicles where they remain viable and multiply. Rhoptries are considered to be key mediators of virulence by enabling many parasites, including apicomplexans to invade hosts erythrocytes [24].
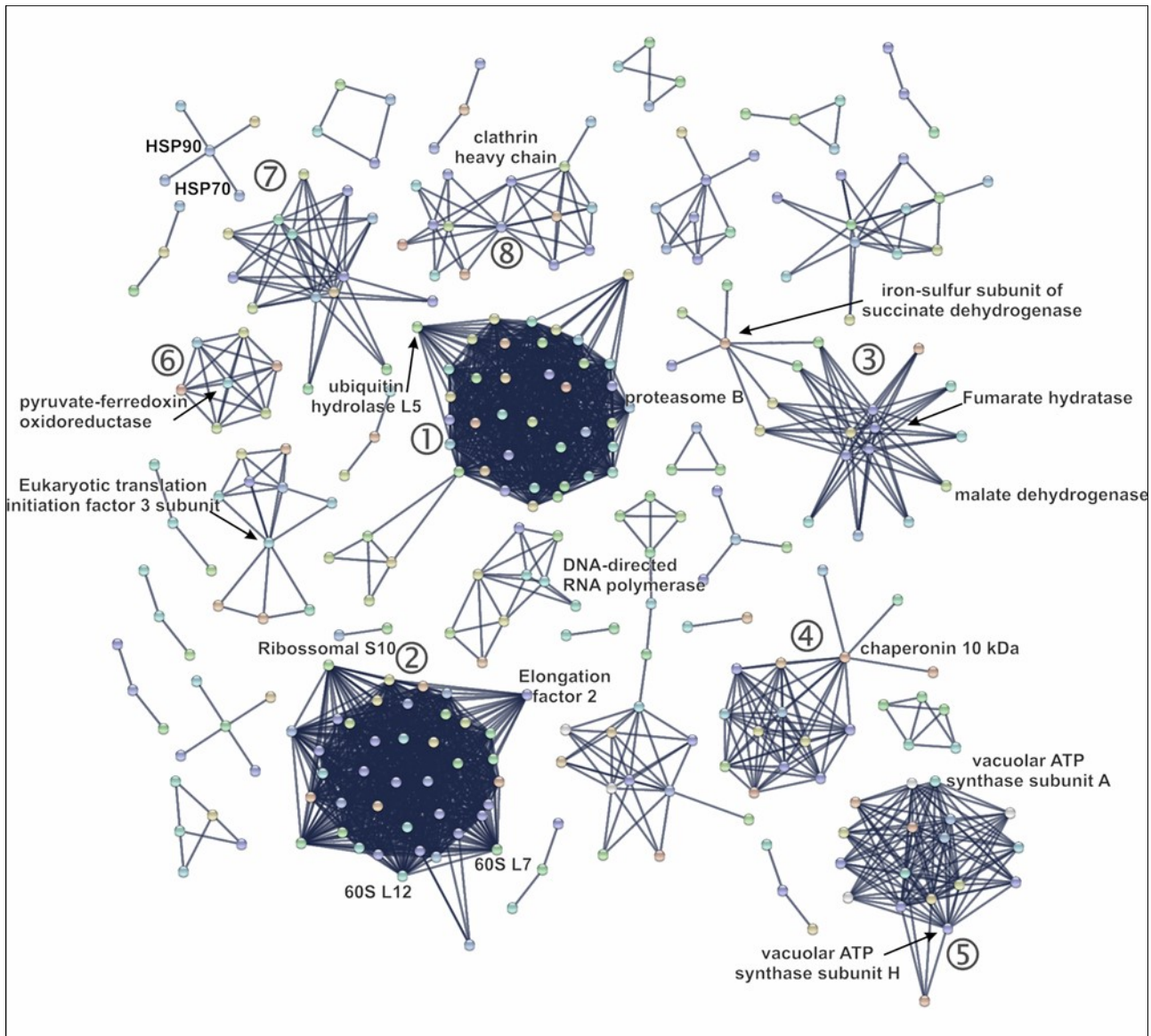
Figure 4. Protein-protein interaction networks of *Perkinsus marinus* proteome. STRING algorithm was used to build a interaction map among the proteins identified in all three biological replicates. Thicker lines denote interactions with score ≥ 0.980. (1) Ubiquitin-proteasome complex. (2) Ribosomal protein cluster. (3) Iron-sulfur/fumarate hydratase cluster. (4) T-complex protein-1 (chaperonin) cluster. (5) ATP synthase network. (6) Pyruvate ferredoxin. (7) Triosephosphate isomerase/phosphoglucose isomerase/enolase cluster and (8) Clathrin interactome.

Rhoptries contains a number of novel proteins (ROP) including serine-threonine kinases and protein phosphatases that were detected in all replicates and are also believed to be parasitic virulence determinants [24]. Furthermore, the merozoite surface protein 3 was detected in all replicates. This protein is reported to be important parasitic surface antigens as well as virulence factors [25].

One remarkable finding was that peptides corresponding to retroviruses polyproteins were detected. An analysis of the *P. marinus* genome reveals that there are three putative endogenous retroviruses present and our findings shows that these sequences are being transcribed and translated. The presence of a virus and/or retrotransposon elements in the *P. marinus* genome based on genomic annotation together with EST data was previously reported [15]. The implications of the presence of these viruses are still unknown; however, they might have an impact in *Perkinsus* survival and possibly even its pathogenicity.

A protein-protein interaction network was analyzed to better understand the biological outcome of the detected proteins of *P. marinus* and a great number of interactions were observed. A prominent feature of the life cycle of *Perkinsus* spp. is the numerous morphological changes they undergo during development in the oysters hosts, which must involve extensive and carefully controlled proteolytic activity. There are several reports that have confirmed the role of proteasomes in parasite differentiation and proliferation processes, which are key steps in pathogen colonization [26]. A great variety of proteasome inhibitors have been studied as molecular targets for treatment development of many parasitic diseases by selectively killing or disrupting parasite multiplication [26, 27]. It would be interesting to examine *P. marinus* proteasome as potential drug targets for mollusc diseases. Abundant interactions for ribosomal proteins might be suggestive of the rapid and extensive protein translation that accompanies parasite differentiation and multiplication following host-cell invasion [15]. The iron-sulfur cluster fumarase activity has been shown to be essential for protozoan parasites such as *Trypanossoma cruzi* since its mitochondrial isoform is part of the tricarboxylic acid cycle and as such being central to aerobic respiration [28]. The TCP1 complex belongs to the HSP60 family protein that was detected in *P. marinus* from our study. This protein is involved in folding and assembly of wide range of cytosolic proteins after stress related denaturation being critical for maintaining the integrity of cellular proteins [15]. In most organisms, ATP synthase taps the energy stored in the proton gradient generated by the respiratory complexes to synthesize ATP from ADP and phosphate (P_i). However, it has been suggested that in blood-stage trypanosomes that has no mitochondrial respiratory enzymes, this enzyme might operate in reverse, as an ATPase hydrolyzing ATP into ADP and P_i and pumping protons into the intermembrane space. This reverse reaction would be important to maintain the membrane potential during anoxia [29]. Since *P. marinus* primarily infects host hemocytes, it is interesting to further examine *Perkinsus* ATP synthase mechanisms. It has been reported that *P. marinus* possesses genes for a plant-type ferredoxin system that possibly encodes plastid-targeting signals [30]. In addition to ferredoxins, triose phosphate proteins that are also predicted to target plastids were detected in our study, however its implications are unknown. Clathrin-mediated trafficking is known to be responsible for endocytosis and post-Golgi transport in trypanosomes. It also represents an important interface with the host, in addition to play multiple roles in immune evasion and host cell invasion that are vital for effective infection and persistence [31].

In conclusion, this is the first comprehensive identification of *P. marinus* proteins by a label-free shotgun proteomic approach. These results might serve as a valuable resource for future investigations involving

comparative proteomics, potential drug targets, mechanisms of adaptation under stress-related conditions, as well as host-parasite interactions.

Supplementary table

Supplementary table 1

References

1. OIE World Organization for Animal Health (2017). Manual of diagnostic test for aquatic animals. Available at: http://www.oie.int/international-standard-setting/aquatic-manual Accessed in: April, 26 of 2017.

2. Villalba, A., Reece, K.S., Ordás, M.C., Casas, S.M., and Figueras, A. (2004). Perkinsosis in molluscs: A review. *Aquat. Living Resour*. 17, 411-432.

3. Mangot, J.F., Debroas, D., and Domaizon, I. (2011). Perkinsozoa, a well-known marine protozoan flagellate parasite group, newly identified in lacustrine systems: A review. *Hydrobiologia* 659, 37-48.

4. Allam, B., Carden, W. E., Ward, J. E., Ralph, G., and Winnickia, S. (2013). Early host-pathogen interactions in marine bivalves: Evidence that the alveolate parasite *Perkinsus marinus* infects through the oyster mantle during rejection of pseudofeces. *J. Invertebr. Pathol*. 113, 26-34.

5. Fernández-Boo, S., Chicano-Galvez, E., Alhama, J., Barea, J. L., Villalba, A., et. al. (2014). Comparison of protein expression profiles between three *Perkinsus* spp., protozoan parasites of molluscs, through 2D electrophoresis and mass spectrometry. *J. Invertebr. Pathol*. 118, 47-58.

6. Fernández-Boo, S., Villalba, A., and Cao, A. (2015). Cell proteome variability of protistan mollusc parasite *Perkinsus olseni* among regions of the Spanish coast. *Dis Aquat. Organ*. 113, 245-256.

7. Huang, K-Y., Huang, P-J., Ku, F-M., Lin, R., Alderete, J.F., et. al. (2012). Comparative transcriptomic and proteomic analyses of *Trichomonas vaginalis* following adherence to fibronectin. *Infect. Immun*. 80, 3900-3911.

8. Dungan, C.F., and Reece, K.S. (2006). In vitro propagation of two *Perkinsus* spp. parasites from Japanese Manila clams *Venerupis philippinarum* and description of *Perkinsus honshuensis* n. sp. *J. Eukaryot. Microbiol*. 53, 1-11.

9. Gauthier, J.D., and Vasta, G.R. (1995). In vitro culture of the eastern oyster parasite *Perkinsus marinus*: optimization of the methodology. *J. Invertebr. Pathol*. 66, 156-168.

10. Distler, U., Kuharev, J., Navarro, P., Levin, Y., Schild, H., et. al. (2014). Drift time-specific collision energies enable deep-coverage data-independent acquisition proteomics. *Nat. Methods* 11, 167-170.

11. R Core Team, (2013) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL:http://www.R-project.org/.

12. Szklarczyk, D., Franceschini, A., Wyder, S., Forslund, K., Heller, D., et al. (2015). STRING v10: protein–protein interaction networks, integrated over the tree of life. *Nucleic Acids Res*. 43(D1), D447-D452.

13. Vizcaíno, J.A., Csordas, A., Del-Toro, N., Dianes, J. A., Griss, J., et. al. (2016). Update of the PRIDE database and related tools. *Nucleic Acids Res*. 44, D447-D456.

14. Lindquist, S. (1992). Heat-shock proteins and stress

tolerance in microorganisms. *Curr. Opin. Genet. Dev.* 2, 748-755.

15. Joseph, S.J., Fernandez-Robledo, J.A., Gardner, M.J., El-Sayed, N.M., Kuo, C-H. , et. al. (2010). The alveolate *Perkinsus marinus*: biological insights from EST gene discovery. *BMC Genomics* 11, 228.

16. Tirard, C.T., Grosspeld, R.M., Volety, A.K., and Chu, F.L.E. (1995). Heat shock proteins of the oyster parasite *Perkinsus marinus*. *Dis. Aquat. Org.* 22, 147 -151.

17. Espinosa, E.P., Corre, E., and Allam, B. (2014). Pallial mucus of the oyster *Crassostrea virginica* regulates the expression of putative virulence genes of its pathogen *Perkinsus marinus*. *Int. J. Parasitol*. 44, 305-317.

18. La Peyre, J.F., Yarnall, H.A., and Faisal, M. (1996). Contribution of *Perkinsus marinus* extracellular products in the infection of eastern oysters (*Crassostrea virginica*). *J. Invertebr. Pathol.* 68, 312-313.

19. Wright, A.C., Ahmed, H., Gauthier, J.D., Silva, A.M., and Vasta, G.R. (2002). cDNA cloning and characterization of two iron superoxide dismutases from the oyster parasite *Perkinsus marinus*. *Mol. Biochem. Parasitol*.123, 73-77.

20. Adebayo, A.S., and Anumudu, C.I. (2013). Annotation of virulence factors in schistosomes for the development of a SchistoVir database. *J. Comput. Biol. and Bioinform*. Res. 5, 6-14.

21. Lee, J.J., Kim, J.H., Kim, D.J., Kim, D.H., Simborio, H.L., et. al. (2014). Characterization of betaine aldehyde dehydrogenase (BetB) as an essential virulence factor of *Brucella abortus*. *Vet. Microbiol*. 168, 131-40.

22. Netterling, S., Bäreclev, C., Vaitkevicius, K., and Johansson, J. (2015). RNA Helicase important for *Listeria monocytogenes* hemolytic activity and

virulence factor expression. *Infect Immun*. 84, 67-76.

23. Naglik, J.R., Challacombe, S.J., and Hube, B. (2003). *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. *Microbiol. Mol. Biol. Rev*. 67, 400-428.

24. Bradley, P.J., and Sibley, L.D. (2007). Rhoptries: an arsenal of secreted virulence factors. *Curr. Opin. Microbiol*. 10, 582-587.

25. Fankhauser, N., Nguyen-Ha, T.M., Adler, J. and Mäser, P. (2007). Surface antigens and potential virulence factors from parasites detected by comparative genomics of perfect amino acid repeats. *Proteome Sci*. 5, 20.

26. Cardoso, J., Lima, C.D.P., Leal, T., Gradia, D.F., Fragoso, S.P., et al. (2011). Analysis of proteasomal proteolysis during the in vitro metacyclogenesis of *Trypanosoma cruzi*. *PLoS ONE* 6, e21027.

27. Muñoz, C., Francisco, J.S., Gutiérrez, B., González, J. (2015). Role of the ubiquitin-proteasome systems in the biology and virulence of protozoan parasites. *Biomed Res. Int*. 5, 13.

28. De Padua, R.A.P., Kia, A.M., Filho, A.J.C., Wilkinson, S.R., and Nonato, M.C. (2017) Characterization of the fumarate hydratase repertoire in *Trypanosoma cruzi*. *Int. J. Biol. Macromol.* 102, 42-51.

29. Nina, P.B., Morrisey, J.M., Ganesan, S.M., Ke, H., Pershing, A.M., et.al. (2011). ATP Synthase Complex of *Plasmodium falciparum*: Dimeric assembly in mitochondrial membranes and resistance to genetic disruption. *J. Biol. Chem*. 286, 41312-41322.

30. Stelter, K., El-Sayed, N.M., and Seeber, F. (2007). The expression of a plant-type ferredoxin redox system provides molecular evidence for a plastid in the early dinoflagellate *Perkinsus marinus*. *Protist*. 158,119-130.

31. Kalb, L.C., Frederico, Y.C.A., Boehm,C., Moreira, C.M.N., Soares, M.J. et. al. (2016). Conservation and divergence within the clathrin interactome of *Trypanosoma cruzi*. *Sci. Rep.* 6, 31212.