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Proteomic analysis of differentially expressed proteins in the two developmental stages of *Ichthyophthirius multifiliis*

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Abstract Ichthyophthirius is a severe disease of farmed freshwater fish caused by the parasitic ciliate *Ichthyophthirius multifiliis* (Ich). This disease can lead to considerable economic loss, but the protein profiles in different developmental stages of the parasite remain unknown. In the present study, proteins from trophonts and theronts of Ich were identified by isobaric tags for relative and absolute quantitation (iTRAQ). A total of 2300 proteins were identified in the two developmental stages, of which 1520 proteins were differentially expressed. Among them, 84 proteins were uniquely expressed in the theronts stage, while 656 proteins were expressed only in trophonts. The differentially expressed proteins were catalogued (assorted) to various functions of Ich life cycle, including biological process, cellular component, and molecular function that occur at distinct stages. Using a 1.5-fold change in expression as a physiologically significant benchmark, a lot of differentially expressed proteins were reliably quantified by iTRAQ analysis. Two hundred forty upregulated and 57 downregulated proteins in the trophonts stage were identified as compared with theronts. The identified proteins were involved in various functions of the *I. multifiliis* life cycle, including binding, catalytic activity, structural molecule activity, and transporter activity. Further investigation of the transcriptional levels of periplasmic immunogenic protein, transketolase, zinc finger, isocitrate dehydrogenase, etc., from the different protein profiles using quantitative RT-PCR showed identical results to the iTRAQ analysis. This work provides an effective resource to further our understanding of Ich biology, and lays the groundwork for the identification of potential drug targets and vaccines candidates for the control of this devastating fish pathogen.

Keywords Proteomic analysis · *Ichthyophthirius multifiliis* · iTRAQ · Differentially expressed proteins

Introduction

Ichthyophthirius multifiliis (Ich), the etiologic agent of "white spot" disease, is a highly pathogenic ciliate that infects virtually all species of freshwater fish. While *Ichthyophthirius* poses a major threat to commercial aquaculture worldwide, there are currently no effective vaccines for disease prevention, and acceptable strategies for the treatment of large-scale outbreaks are not yet available (Traxler et al. 1998; Matthews 2005).

The life cycle of *I. multifiliis* comprises several morphologically distinct stages, each fulfilling discrete function in the life history of *I. multifiliis* (MacLennan 1935; Nigrelli et al. 1976; Ewing and Kocan 1992). The trophont is the feeding stage which resides on the basal layers of the skin and gill epithelia of fish. Following a growth period, it leaves the fish as tomonts, which then encyst and subsequently undergo multiple divisions, and finally release numerous free-swimming infective theronts. The theront attaches to fish skin and penetrates into the epidermis where it settles as a trophont and

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completes the life cycle. Killing the infective theront or the detached trophont with various antiprotozoal drugs can stop the reproductive cycle and prevent spread of the disease to other fish (Tucker and Robinson 1990; Schäperclaus 1991; Wise et al. 2004).

Although the expression of stage-specific genes in the *I. multifiliis* has been detected with various transcriptome, cDNA cloning, and RNA sequencing approaches (Cassidy-Hanley et al. 2011; Coyne et al. 2011), *I. multifiliis* biology at each developmental stage, including developmental regulation, nutrition metabolism, and cell cycle, remains unclear. Clearly, a more thorough understanding of the proteins and metabolic pathways associated with the different stages of the parasite life cycle is critical to the development of effective control strategies.

Proteomic approaches are available to identify proteins involved in complex biological progress and allow for large-scale screening of potential vaccine candidate, drug target proteins from parasites. Isobaric tag for relative and absolute quantization (iTRAQ) is a common technique used in proteomics (Cha et al. 2012; Marancik et al. 2013; Lu et al. 2014). iTRAQ, a stable isotope method for protein measurement by using mass spectrometry (Ross et al. 2004), can be used for the comparison of four or eight different samples at the same time (Ross et al. 2004; Pierce et al. 2008), With these advantages, the iTRAQ method has now attracted more attentions which are applied in various studies (Jeswin et al. 2016).

To obtain a more complete picture of the *I. multifiliis* proteome, in this study, we performed differential proteomics to identify differentially expressed proteins in the infective theront and the detached trophont developmental stages of *I. multifiliis*, which provided valuable clues and insights to guide future studies on parasite development. This study laid the foundation for the discovery of therapeutic targets and the development of vaccine against *I. multifiliis*.

Materials and methods

Parasites

The sources of *I. multifiliis*, its propagation on grass carp, and collection of the cysts have been described by Yao et al. (2014). Several heavily infected grass carp (*Ctenopharyngodon idella*) obtained from the aquatic fry farm of Zhejiang Institute Freshwater Fisheries in China were placed into filtered aquarium water for 30–60 min. Mature trophonts were allowed to dislodge from the host by body movements of the fish while in close proximity. Isolated trophonts were sieved to remove containing debri, and then harvested by low speed centrifugation at 252 g for 5 min. Harvested trophonts were randomly divided into two batches, one was used to proteomic assay, and the other placed in a

plastic beaker with double aerated distilled water and incubated for 24 h at 23°C. The next day, cultures were examined by microscopic examination (×100 magnifications) to insure that development was complete. Theronts were then sieved and collected by centrifugation at 1500×*g* for 3 min.

Protein preparation

Prepared theront and trophont samples were ground into powder in liquid nitrogen, then 300 μg of the powder protein was diluted in STD buffer (4% SDS, 150 mM Tris-HCl, 100 mM DTT, pH 8.0), heated at 100°C for 5 min, and then sonicated. After centrifugation, the supernatants were collected and protein content was measured by using a BCA protein assay reagent (Beyotime Institute of Biotechnology, China). Protein digestion was conducted according to the FASP procedure (Wisniewski 2009). Briefly, each sample was placed on an ultrafiltration filter (30 kDa cutoff, Sartorius, Germany) containing 200 μL of UA buffer (8 M urea, 150 mM Tris-HCl, pH 8.0) followed by centrifugation at 14,000×*g* for 30 min and an additional washing step with 200 μL of UA buffer; the process was stopped by adding 100 μL UA buffer (contain 50 mM iodoacetamide), then incubated for 30 min at room temperature in the dark, 14,000×*g* for 30 min after washing twice by 100 μL of UA buffer and dissolution buffer (Applied Biosystems, Foster City, CA, USA); the protein suspensions were then digested with 40 μL of trypsin (Promega, Madison, WI, USA) buffer (2 μg trypsin in 40 μL dissolution buffer) at 37°C for 16–18 h and then centrifuged at 14,000×*g* for 30 min. The resulted peptides were collected and the peptide concentration was analyzed at OD280.

iTRAQ labeling and high-pH reversed-phase (HpH) fractionation

iTRAQ labeling was performed according to the manufacturer's instructions (Applied Biosystems). Briefly, the peptide mixtures were reconstituted with 30 μL of iTRAQ dissolution buffer. Each sample (100 μg) was labeled according to iTRAQ Reagent-8plex Multiplex Kit (AB SCIEX). The iTRAQ-labeled peptides were subjected to high-pH reversed-phase fractionation in 1100 Series HPLC Value System (Agilent) with the following chromatographic conditions: buffer A (10 mM Ammonium acetate, pH 10.0) and buffer B (10 mM ammonium acetate, 90% *v/v* ACN, pH 10.0) were eluted at a flow rate of 1 mL/min according to gradient elution procedure. The elution process was monitored by measuring absorbances at 214 nm, and fractions were collected every 1 min. The collected fractions (approximately 30) were finally combined into ten pools. Each fraction was concentrated via vacuum centrifugation and reconstituted in 40 μL of 0.1% *v/v* trifluoroacetic acid. All samples were stored at −80°C until LC-MS/MS analysis.

LC–ESI-MS/MS analysis

The iTRAQ-labeled samples were then subjected to Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). A total of 1 μg of each sample was loaded onto Thermo Scientific EASY column (two columns) using an autosampler at a flow rate of 150 nL/min. The mass spectrometer was operated in positive ion mode, and MS spectra were acquired over a range of 300–2000 m/z. The resolving powers of the MS scan and MS/MS scan at 200 m/z for the Orbitrap Elite were set as 60,000 and 15,000, respectively. The top ten most intense signals in the acquired MS spectra were selected for further MS/MS analysis. The isolation window was 1 m/z, and ions were fragmented through higher energy collisional dissociation with normalized collision energies of 35 eV. The maximum ion injection times were set at 50 ms for the survey scan and 150 ms for the MS/MS scans, and the automatic gain control target values for full scan modes was set to 1.0×10^{-6} and for MS/MS was 5×10^{4}. The dynamic exclusion duration was 30 s.

Protein data analysis

The raw MS data files were converted using Proteome Discoverer 1.4 software (Thermo Fisher Scientific). Protein identification was performed using the Mascot software Matrix 2.3.02 (Science, London, UK) against the *I. multifiliis* database downloading from UniProt of EMBL. The differentially expressed proteins were defined as those with 1.5-fold change relative to one another, with $p < 0.05$. The gene name, functional annotations, protein orthologous classification, molecular interaction, and reaction networks were analyzed by Blast, Gene Ontology, and KEGG Pathway, respectively.

Quantitative real-time RT-PCR verification

Total RNA was extracted from theronts and trophonts by TRIzol Reagent (Simgen). cDNA was then synthesized using the Reverse Transcriptase M-MLV Kit (TaKaRa) following the instructions. The real-time quantitative PCR was performed using THUNDERBIRD SYBR qPCR Mix Kit (Toyobo), and carried out in astratagene MxProSystem (Stratagene mx3005p, USA) in 96-well reaction plates. All PCRs were performed at least three times. Additional dissociation-curve analysis was performed and showed a single melting curve in all cases. Data were analyzed by the Stratagene MxPro software (Stratagene mx3005p, USA).

Results

Identification of differentially expressed proteins

The spectra generated from the iTRAQ experiment matched 2300 proteins through BLAST in GenBank and NCBI. The differentially expressed proteins (DEPs) in the two developmental stages (theronts and trophonts) were analyzed using comparison of their identified spectra. A total of 1520 proteins were differentially expressed in the two arbitrary stages. Gene ontology (GO) analysis of total proteins was based on cellular component, biological process, and molecular function. Differentially expressed proteins were also identified using GO for gene function classification. The molecular function ontology analysis was as follows: binding (trophonts 45.76%; theronts 42.69%,), catalytic activity (39.09%; 38.95%), structural molecule activity (5.99%; 7.36%), transporter activity (4.36%; 5.66%), electron carrier activity (1.25%; 1.60%), and molecular function regulator (1.81%; 1.71%). The percentages of proteins classified into bonding and catalytic activities were the highest among molecular functions, which were up to 84.85 and 81.64% for theronts and trophonts, respectively. The GO analysis of the identified proteins was shown in Fig. 1.

Clusters of Orthologous Groups of proteins (COG) classification in the two developmental stages revealed that the proteins were primarily involved in signal transduction mechanisms, translation, ribosomal structure and biogenesis, post-translational modification, protein turnover, chaperones, general function prediction, energy production and conversion, lipid transport and metabolism, amino acid transport and metabolism, carbohydrate transport and metabolism, nucleotide transport and metabolism, and replication, recombination, and repair (Fig. 2). The results indicated that proteins from the two developmental stages were involved in every aspect of parasite growth and metabolism.

Protein profiling of the stage-specific differentially expressed proteins

Among the differentially expressed proteins, 84 proteins were unique expressed in theronts stage (Table 1), including Ymf75 (mitochondrion, accession no. 345894133), flagellar microtugule protofilament ribbon protein (accession no. 471219372), intraflagellar transport protein (accession no. 471221951), oxoglutarate dehydrogenase (accession no. 471228421), radial spoke head protein, 471232003, kinesin family member 9 (accession no. 471234104), phosphoglycerate kinase (471221776), and inorganic pyrophosphatase (accession no. 471223495) papain family cysteine protease (accession no. 471233695). As for trophonts (Table 2), there were 656 specific proteins only expressed in this development such as carbon-nitrogen family protein (accession no.
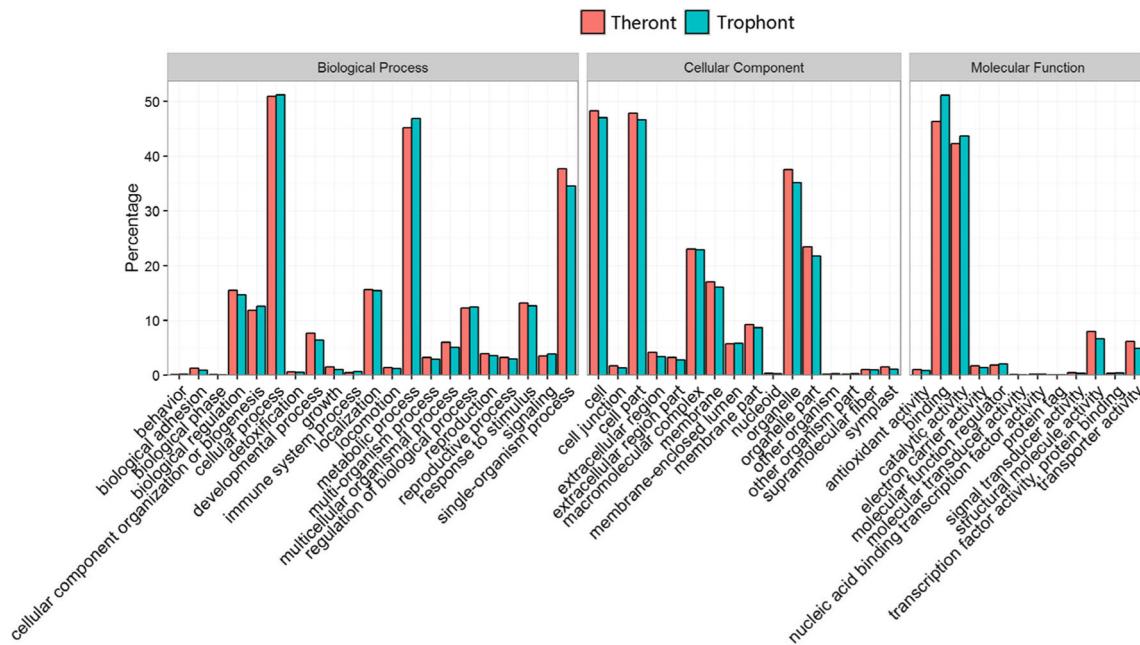
Fig. 1 Comparative analysis of GO categories associated with theront and trophont. The proteins were categorized according to the annotation of GO, and the number of each category is displayed based on biological process, cellular components, and molecular functions

471226451), tryptophanyl-tRNA synthetase (accession no .471226600), Ran binding protein (accession no. 471226744) DNA-directed polymerase II polypeptide J (accession no. 471227247), zinc-binding dehydrogenase family protein (accession no. 471228852), copper/zinc superoxide dismutase family protein (accession no. 471230086), and parkin co-regulated gene protein (accession no. 471235274). Stage-specific differentially expressed proteins were also identified using GO for gene function classification, and the

cellular component ontology analysis was listed in Figs. 3 and 4.

iTRAQ quantification

Using a 1.5-fold change in expression as a physiologically significant benchmark, a lot of differentially expressed proteins were reliably quantified by iTRAQ analysis. Two hundred forty upregulated and 57 downregulated proteins in the
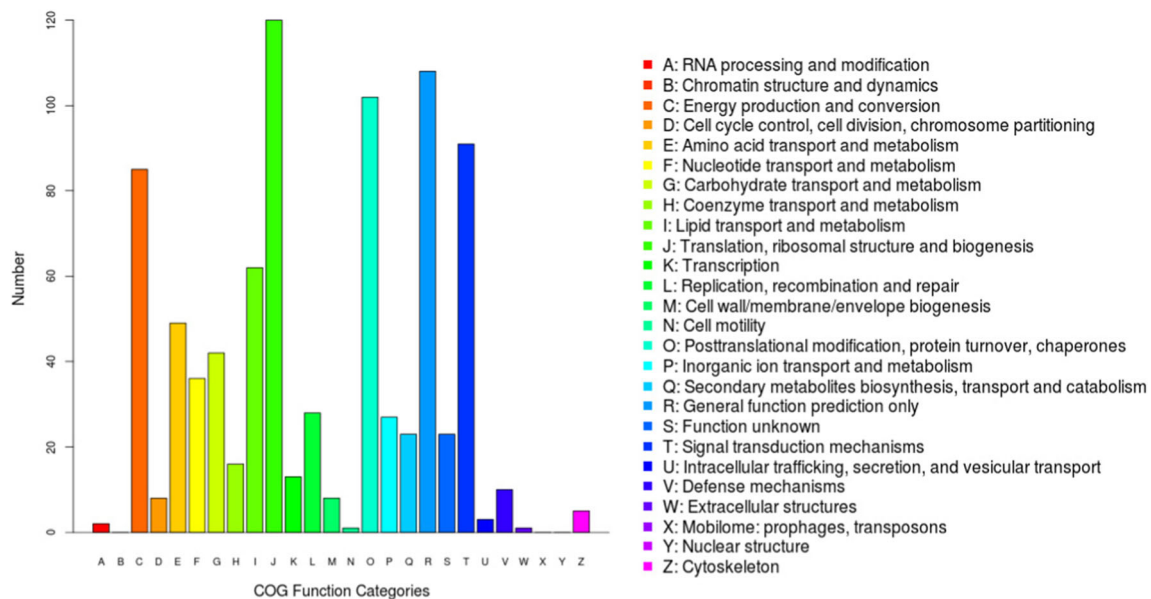
Fig. 2 COG coverage of the protein sequence. A total of 24 groups of differentially expressed proteins were clustered by orthologous groups

Table 1 Details of downregulated proteins (trophonts compared with theronts)

Accession no.	Description	Fold (P/T)	MW (Da)	Coverage (%)
471221496	Secretory granule protein t4-b, putative (*Ichthyophthirius multifiliis*)	0.262778653	41363.54	45.6
471226797	K antiporter P-type alpha subunit family protein, putative (*Ichthyophthirius multifiliis*)	0.128693533	135107.79	14.2
471219876	WD repeat protein (*Ichthyophthirius multifiliis*)	0.135913306	72275.35	15.9
471233910	Pyrimidine-nucleosidephosphorylase, putative (*Ichthyophthirius multifiliis*)	0.288436704	54931.29	20.3
471236849	Isocitrate dehydrogenase, putative (*Ichthyophthirius multifiliis*)	0.250034254	39223.27	20.5
471228460	Radial spoke head protein, putative (*Ichthyophthirius multifiliis*)	0.313904587	59686.08	12.9
471220932	Radial spoke head protein, putative (*Ichthyophthirius multifiliis*)	0.263383082	33113.34	24.6
471225240	Ornithine aminotransferase, putative (*Ichthyophthirius multifiliis*)	0.191287055	46633.52	19.3
471222719	Radial spoke head protein, putative (*Ichthyophthirius multifiliis*)	0.214904054	26219.74	21.6
471226098	spfh domain band 7 family protein (*Ichthyophthirius mulrifiliis*)	0.274790479	25814.63	11.5
471227705	Protein kinase domain protein (*Ichthyophthirius mulrifiliis*)	0.336157351	43416.25	4.3
471235683	IQ calmodulin-binding motif family protein, putative (*Ichthyophthirius multifiliis*)	0.179855413	31725.03	7.5
471234844	Splicing factor subunit 3, putative (*Ichthyophthirius multifiliis*)	0.260652442	109371.3	15.1
471228977	Zinc finger lsd1 subclass family protein, putative (*Ichthyophthirius multifiliis*)	0.281505927	61926.17	8.3
471219697	Normocyte-binding protein, putative (*Ichthyophthirius multifiliis*)	0.112094549	23943.04	25.4

We here only listed more than threefold expressed proteins

P trophonts, *T* theronts

trophonts stage were identified as compared with theronts. The proteins expressed at a high level in trophonts were mainly propionates—ligase, kap beta 3 protein, ribosomal protein, zinc-binding dehydrogenase family protein, phenylalanyl-tRNA alpha subunit (Table 1). The proteins that were more abundant in theront than trophonts were K antiporter P-type alpha subunit family protein, ornithine aminotransferase, IQ calmodulin-binding motif family protein, normocyte-binding protein, etc. (Table 2).

Quantitative real-time RT-PCR analysis validation of DEPs

Six genes from DEPs designated redoxin domain protein (471224523), protein kinase domain protein (471227705), spfh domain band 7 family protein (471226098), normocyte-binding protein (471219697), immobilization antigen isoform (471231290), and leishmanolysin family protein (471230932) were selected for RT-PCR analysis to quantify their transcriptional levels (Fig. 5). The results revealed that the genes encoding normocyte-binding protein, protein kinase domain protein, and spfh domain band 7 family protein were significantly higher expressed in theronts than in trophonts stages. The redoxin domain protein was upregulated

in trophonts than in theronts. Leishmanolysin family protein (471230932) only expressed in trophonts, but in contrast to iTRAQ analysis, immobilization antigen isoform (471231290) were expressed in both development stages. The PCR results were mainly consistent with the iTRAQ analysis, which suggested that the proteomics analysis tool used in this study is reliable.

Discussions

In recent years, an increasing number of ichthyophthiriosis outbreaks have led to substantial economic loss in fisheries and aquaculture (Yao et al. 2010, 2011). Numerous strategies including immunoprophylaxis and chemotherapeutics had been used to control the parasite; however, no methods resulted in great success. Therefore, it is necessary to screen new drug targets and vaccine candidates of *I. multifiliis*, and the understanding of the life-stage biology, host pathogen interactions, signal transduction, and regulation, the key enzyme invasion, and metabolic pathway of the parasite can facilitate further studies. Numerous proteins are involved in differential regulation of distinct life cycle stages of *I. multifiliis*. The differentially expressed genes at each developmental stage

Table 2 Details of the upregulated protein (trophonts compared with theronts)

Accession no.	Description	Fold (P/T)	MW	Coverage (%)
471229171	Leucine rich repeat protein (*Ichthyophthirius multifiliis*)	8.716408104	99,959.99	9.8
471230261	pci domain protein, partial (*Ichthyophthirius multifiliis*)	8.590308024	50,110.68	21.5
471229005	uv excision repair protein, putative, partial (*Ichthyophthirius multifiliis*)	10.35108937	44,934.18	14.9
471225341	Ribosomal protein, putative (*Ichthyophthirius multifiliis*)	5.246205794	44,301.5	15.2
471228359	Ribosomal protein, putative (*Ichthyophthirius multifiliis*)	6.721355554	17,891.83	13.4
471228769	Stress-induced protein sti1 family protein, putative, partial (*Ichthyophthirius multifiliis*)	7.128942378	67,742.98	35.9
471231913	Polyadenylate-binding protein 2, putative (*Ichthyophthirius multifiliis*)	5.063186939	75,002.06	28.5
471236448	Valyl-tRNA synthetase, putative (*Ichthyophthirius multifiliis*)	9.643968963	116,915.18	17.5
471228157	Protein kinase domain protein (*Ichthyophthirius multifiliis*)	5.959365252	65,611.05	27
471222021	Nucleolar protein 5a, putative (*Ichthyophthirius multifiliis*)	8.800074257	55,523.94	38
471220389	kap beta 3 protein, putative (*Ichthyophthirius multifiliis*)	31.04764785	58,077.85	39
471222257	Propionate-ligase, putative (*Ichthyophthirius multifiliis*)	39.40266525	43,226.85	35.3
471231310	Triosephosphate isomerase, putative (*Ichthyophthirius multifiliis*)	7.025236888	28,541.21	54.7
471228693	Threonyl-tRNA synthetase, putative, partial (*Ichthyophthirius multifiliis*)	9.635104647	89,813.29	15.5
471235990	Nucleosome assembly protein, putative (*Ichthyophthirius multifiliis*)	11.86241807	38,806.53	27
471223303	Phenylalanyl-tRNA alpha subunit, putative (*Ichthyophthirius multifiliis*)	12.98600981	57,913.4	20.2
471235144	Profilin family protein, putative (*Ichthyophthirius multifiliis*)	5.108759077	16,149.31	50
471226670	Zinc-binding dehydrogenase family protein, putative (*Ichthyophthirius multifiliis*)	13.1983258	100,604.4	8.2
471219060	Ribosomal protein, putative (*Ichthyophthirius multifiliis*)	10.19816376	16,638.66	20.8
471223314	Transketolase, putative, partial (*Ichthyophthirius multifiliis*)	8.850479253	55,177.36	7.5
471236150	Periplasmic immunogenic protein, putative, partial (*Ichthyophthirius multifiliis*)	12.39325288	23,853.64	32.5
471221014	ran binding protein 1, putative (*Ichthyophthirius multifiliis*)	5.166319345	22,007.05	41.1
471219197	Ribosomal protein, putative (*Ichthyophthirius multifiliis*)	17.70066207	24,036.03	27.5
471224523	Redoxin domain protein (*Ichthyophthirius multifiliis*)	8.32419123	18,701.62	30.8
471220696	ras oncogene family protein, putative (*Ichthyophthirius multifiliis*)	5.983516971	23,477.09	43.6
471221054	Proteasome subunit, putative (*Ichthyophthirius multifiliis*)	5.327648024	27,903.73	21.4
471224817	Poly binding protein 6 pab6 RNA binding translation initiation factor, putative, partial (*Ichthyophthirius multifiliis*)	5.119082038	59,912.81	8.7

We here only listed more than threefold expressed proteins

P trophonts, *T* theronts

have been identified in previous studies (Abernathy et al. 2011). However, the protein profiles in their developmental stages remain largely unknown. Herein, we applied a quantitative proteomics approach with the iTRAQ technique to fully
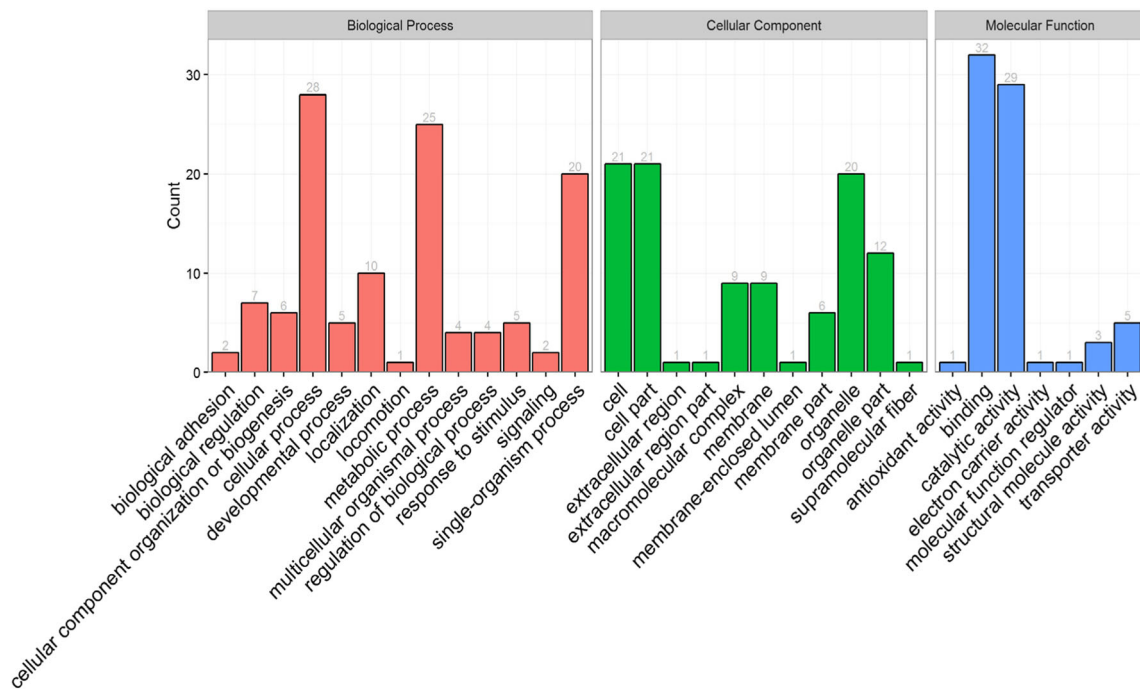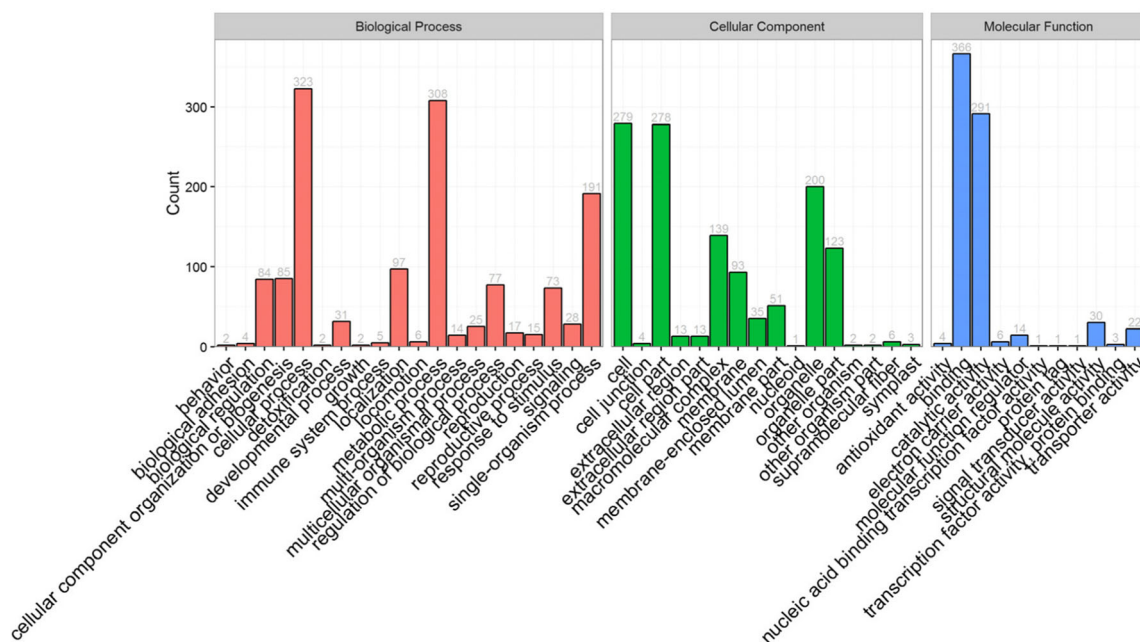
Fig. 3 Classification of uniquely expressed protein (theronts) functions by Gene Ontology (GO). The proteins were categorized according to the annotation of GO, and the number of each category is displayed based on biological process, cellular components, and molecular functions

reveal the protein expression profiles of the two developmental stages (theronts and trophonts). A total of 1520 proteins were identified involved in binding, catalytic activity, structural molecule activity, transporter activity, electron carrier activity, and molecular function regulator.

Among the differentially expressed proteins in the two development stages of *I. multifiliis*, 84 of them were uniquely expressed in the theronts stage. In line with the previous study, flagellar microtugule protofilament ribbon protein, intraflagellar transport protein, kinesin family member 9 were highly expressed in the theronts stage (Table 2), which almost certainly reflects the critical energy needs of these cells for rapid and sustained motility associated with host finding (Hennessey et al. 2002; Wood et al. 2007; Cassidy-Hanley

Fig. 4 Classification of uniquely expressed protein (trophonts) functions by Gene Ontology (GO). The proteins were categorized according to the annotation of GO, and the number of each category is displayed based on biological process, cellular components, and molecular functions

Fig. 5 Confirmation of differentially expressed genes of *Ichthyophthirius multifiliis* by RT-PCR. Note: RDP24523: redoxin domain protein, PDP27705: protein kinase domain protein, SBS26098: spfh domain band 7 family protein, NBP19697: normocyte-binding protein, IAI31290: immobilization antigen isoform, LFP30932: leishmanolysin family protein
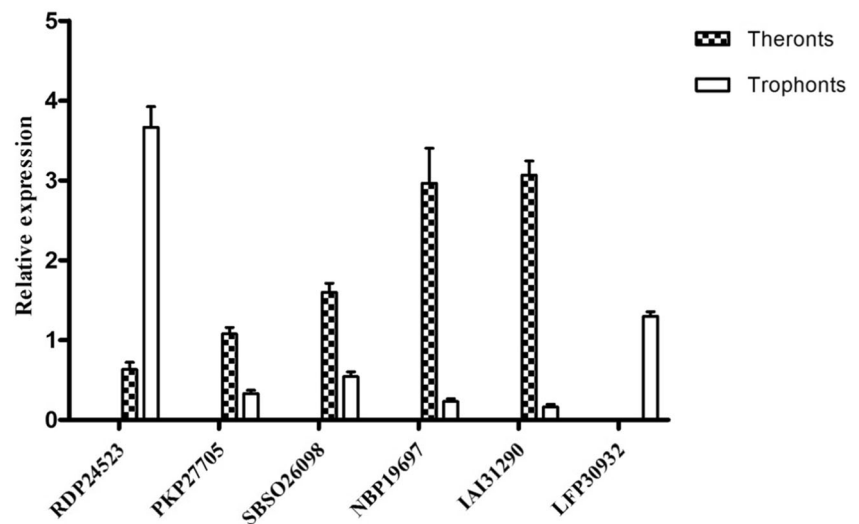
et al. 2011). Another specific expressed protein in theronts, phosphoglycerate kinase (PGK), has traditionally been studied as the sixth enzyme of the glycolytic pathway in which it equilibrates phosphate transfer between position 1 of 1,3-bisphosphoglycerate and the g-phosphate of $MgATP^{2-}$. PGK has also been shown to influence DNA replication and repair in mammalian cell nuclei (Vishwanatha et al. 1992; Popanda et al. 1998) and stimulate viral messenger RNA synthesis in the cytosol (Ogino et al. 1999). Interestingly, the leishmanolysin family protein (accession no. 471230932) that contribute to invasion, migration, and adhesion of Leishmania species within the extracellular matrix of host tissues (McGwire et al. 2003; d'Avila-Levy et al. 2008) were also highly expressed in the theronts stage. As for trophonts, we found 656 specific expressed proteins mainly associated with the cellular process and binding and catalytic activity. ATP synthase alpha subunit precursor (47122655), the purine salvage, and metabolism enzymes were highly expressed only in the trophonts stage; as we know, enzymes involved in purine salvage are of particular interest since a variety of parasitic protozoa are incapable of synthesizing purines de novo, so it may be an attractive target for pharmacological intervention in parasite control (Abernathy et al. 2007; Sullivan et al. 2005). These unique proteins may offer additional targets for vaccine and therapeutic drug development.

Proteases in parasitic protozoa have long been considered potential drug targets due to their crucial roles in parasite development and infection, and the feasibility of designing specific inhibitors (Atkinson et al. 2009; Blackman 2008; Kuang et al. 2009; Wu et al. 2003). Onara et al. (2008) reported that carp infected with Ich could increase the expression levels of a2M3, a protease inhibitor of endogenous and exogenous proteases which indicated that anti-proteases could be viable anti-infectives. In the present study, we identified 105 protease homologs from the two development stages of Ich. The inorganic pyrophosphatase, oxoglutarate dehydrogenase,

phosphoglycerate kinase, isocitrate dehydrogenase, and transketolase and lactate dehydrogenase were significantly downregulated in the theront as compared to the trophont stage, which indicated maximal activation of gluconeogenesis in theront which reflects the more energy needs of the parasite during invasion and migration (Janke and Kneussel 2010). Though our study identified only half of the protease homologs revealed by comparative genomic analysis (Coyne et al. 2011), it significantly expands the range of protease targets. More functions of the proteins should be further investigated.

For proteins of known function, their differential expression often reflects the biological activities associated with each stage of the parasite life cycle. In the present study, the normocyte-binding protein (71219697) was about 10 times more prevalent in theronts than in trophonts. Normocyte-binding proteins are members of reticulocyte-binding proteins which are characterized in malaria parasite species including *Plasmodium cynomolgi* (Okenu 2005) and *Plasmodium yoelii* (Ogun 2011). It was identified as a key mediator of *Plasmodium knowlesi* human infection and may be a target for vaccine development against this emerging pathogen (Ahmed et al. 2016). Karyopherin (kap) beta 3 protein (471220389), an import receptor for cargo interacts in the cytoplasm and nuclear, is transported through the nuclear pore complex, and is released inside the nucleus (Moroianu 1999; Nakielny and Dreyfuss 1999; Wente 2000). Coyne et al. (2008) reported that there are 88% as many mitochondrial carriers in Ich as in Tetrahymena. They mention that such a high representation of these carriers suggests a strong dependence of Ich's energy generation on mitochondrial aerobic respiration. It may become an attractive drug target because Ich mitochondrial ATP synthase is highly divergent from its vertebrate form. As mentioned in Table 1, calmodulin-binding motif family protein (471235683) was highly prevalent in theronts than in trophont. As described by Coyne et al. (2008), Ich and other ciliates contain a large number of

calcium- and calmodulin-regulated protein kinases, which is absent in vertebrates. Calcium-regulated pathways have come under study as promising therapeutic targets against apicomplexan parasites (Nagamune et al. 2008; Billker et al. 2009) and also could be considered as targets against Ich.

In conclusion, a total of 2300 proteins were identified by iTRAQ technique in the two developmental stages, and 1520 proteins were differentially expressed in the trophonts stage compared with theronts. GO analysis revealed that some proteins are involved in important functions related to biological process, cellular component, and molecular function that occur at distinct stages. This work provides an effective resource to further our understanding of Ich biology, and lays the groundwork for the identification of potential drug targets and vaccines candidates for the control of this devastating fish pathogen.

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