Comparative Biochemistry and Physiology, Part D 6 (2011) 382–392

Contents lists available at SciVerse ScienceDirect

Comparative Biochemistry and Physiology, Part D

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

Gene expression profiling of a fish parasite *Ichthyophthirius multifiliis*: Insights into development and senescence-associated avirulence

Jason Abernathy [a], De-Hai Xu [b], Eric Peatman [a], Huseyin Kucuktas [a], Phillip Klesius [b], Zhanjiang Liu [a,*]

[a] The Fish Molecular Genetics and Biotechnology Laboratory, Department of Fisheries and Allied Aquacultures, Program of Cell and Molecular Biosciences, Aquatic Genomics Unit, 203 Swingle Hall, Auburn University, Auburn, AL 36849, USA
[b] Aquatic Animal Health Research Unit, Agricultural Research Service, United States Department of Agriculture, 990 Wire Road, Auburn, AL 36832, USA

ARTICLE INFO

Article history:
Received 1 June 2011
Received in revised form 9 August 2011
Accepted 10 August 2011
Available online 16 August 2011

Keywords:
Catfish
Ciliate
Genome
EST
Gene expression
Microarray
Parasite
Protozoa

ABSTRACT

The ciliate parasite *Ichthyophthirius multifiliis* (Ich) infects many freshwater fish, causing white spot disease that leads to heavy economic losses to aquaculture and ornamental industries. Despite its economic importance, molecular studies examining fundamental processes such as life stage regulation and infectivity have been scarce. In this study, we developed an oligo microarray platform using all available *I. multifiliis* expressed sequence tag (EST) information as well as probes designed through comparative genomics to other protozoa. Gene expression profiling for developmental and virulence factors was conducted using this platform. For the developmental study, the microarray was used to examine gene expression profiles between the three major life stages of Ich: infective theront, parasitic trophont, and reproductive tomont. A total of 135 putative *I. multifiliis* genes were found to be differentially expressed among all three life-stages. Examples of differentially expressed transcripts among life stages include immobilization antigens and epiplasmin, as well as various other transcripts involved in developmental regulation and host-parasite interactions. *I. multifiliis* has been shown to lose infectivity at later cell divisions potentially due to cellular senescence. Therefore, the microarray was also used to explore expression of senescence-associated genes as related to the passage number of the parasite. In this regard, comparison between tomont early and late passages yielded 493 differently expressed genes; 1478 differentially expressed genes were identified between trophont early and late passages. The EST-derived oligo microarray represents a first generation array of this ciliate and provided reproducible expression data as validated by quantitative RT-PCR.

1. Introduction

The protozoan *Ichthyophthirius multifiliis* is one of the most widespread ciliate parasites of freshwater fish worldwide (Matthews, 2005), causing ichthyophthiriosis, or white spot disease. The disease is characterized by white spot cysts covering the host skin and gills, and cause high mortalities and severe economic losses to both aquaculture and ornamental fish industries. *I. multifiliis* has a life-cycle with three developmental stages: a reproductive tomont, an infective theront, and a parasitic trophont (Matthews, 2005). The trophont lives within the host, where it feeds and grows; once matured, the trophont emerges from the host, drops to a substrate, and forms a cyst wall. The tomont rapidly divides producing daughter cells within the cyst; these cells develop into theronts. When fully developed, the cyst bursts, releasing the free-swimming theronts that actively locate a host, where the parasite invades the fish epithelium (Xu et al., 2000; Matthews, 2005).

The trophont stage is an obligate parasitic stage and must be propagated on a host fish. A difficulty in long-term maintenance of any *I. multifiliis* isolate is the reduction in infectivity after a number of life-cycles, presumably related to senescence as the isolate undergoes serial passages from fish to fish in the laboratory (Houghton and Matthews, 1986; Burkart et al., 1990; Xu and Klesius, 2004). Currently, *in vitro* methods used to culture *I. multifiliis* remain elusive as no media or cryopreservation technique has been developed, which is needed for long-term maintenance. Xu and Klesius (2004) described the effect of senescence on an isolate maintained *in vivo* on channel catfish over a two-year period involving a total of 105 serial passages. The authors noted that infectivity of *I. multifiliis* declined as the number of serial passages increased, causing lower and lower fish mortalities. Furthermore, the time period required for trophont emergence from the infected fish was found to be significantly increased at the later passages (91–105 cycles) as compared to that at earlier passages (Xu and Klesius, 2004).

The mechanisms of senescence in the serial passages of laboratory *I. multifiliis* isolates remained unclear. Genomic characteristics of *I. multifiliis* may play a role in senescence. *I. multifiliis*, typical of the phylum Ciliophora, has nuclear dimorphism; the genome consists of a

* Corresponding author. Tel.: +1 334 844 8727; fax: +1 334 844 4694.
E-mail address: zliu@acesag.auburn.edu (Z. Liu).

1744-117X/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbd.2011.08.003

macronucleus and a micronucleus (Matthews, 2005). The characteristics of ciliate nuclear dimorphism include both germ line and somatic cell divisions, where the micronucleus contains the germ line that can undergo recombination through meiotic conjugation, and the macronucleus that is transcriptionally active for cellular functions. As such, ciliates can reproduce both sexually and asexually. Asexual cell division through binary fission has been well studied. While the mechanisms of *I. multifiliis* sexual reproduction are unresolved, some evidence exists to support sexual conjugation (Matthews, 2005). It has been suggested that senescence of laboratory isolates may be related to sexual reproduction (Scholz, 1999; Matthews, 2005), or lack thereof, due to a lack of recombination of the germ line.

Several biological processes are known to be capable of inducing senescence, including changes in gene expression, activation of oncogenes, epigenetic effects, and DNA damage and replicative damage in the form of telomeric shortening (Ben-Porath and Weinberg, 2004, 2005; Campisi, 2005; Herbig et al., 2006; Herbig and Sedivy, 2006; Peters, 2008). Therefore, undertaking a study of senescence-associated gene expression would be an important step in the identification of factors of senescence in the *I. multifiliis* macronuclear genome.

Despite these intriguing observations, follow up studies have been difficult due to the lack of necessary genomic tools. The objectives of this study were to develop a unique clustered set of sequences using all existing ESTs, to construct an oligo microarray using the unigenes, and to utilize the microarray for capture of expression profiles of *I. multifiliis* related to its developmental stages and passages.

2. Materials and methods

2.1. Biological samples

I. multifiliis was isolated from an outbreak of ichthyophthiriosis at a local pet shop. It was cultured by serial infections of channel catfish (*Ictalurus punctatus*) as previously described (Xu et al., 2000, 2005). The trophont, tomont, and theront samples were collected into separate tubes. Trophonts were collected from surface of fish skin when the white spots were visible; tomonts were collected from culture dishes after attachment of the parasite to culture dish and rounds of division; theronts were collected after the burst of the cysts. Theronts were first passed through a fine mesh and then collected by centrifugation. Because of small biological samples that can be collected from each fish, tomont, trophont, and theront samples were pooled from multiple fish, and distinct pools were collected to serve as biological replicates for the microarray analysis. Samples of each pool were collected from approximately 8 infected fish: 2 for trophonts, 3 for theronts and 3 for tomonts. Trophonts were placed in Petri dishes and allowed to attach and develop into tomonts and theronts.

Each life stage was also collected over approximately a two-year period of serial passages, based on the observation that *I. multifiliis* loses infectivity over the course of serial passages (Xu and Klesius, 2004). The first passage after wild isolation (passage 1) and passage 100 samples were collected as described above. All samples were suspended in phosphate buffered saline (PBS, pH 7.4) and flash-frozen in liquid nitrogen. Samples were then stored at −80 °C until RNA extraction.

2.2. Genetic information

The *I. multifiliis* microarray was constructed using EST sequence information. ESTs available in the dbEST at the GenBank were analyzed to determine a set of unique genes. The EST sequences were first downloaded to a local database, and then contiguous sequences (contigs) were assembled. A total of 33,515 ESTs were used for sequence assembly including 8432 ESTs from a previous sequencing effort (Abernathy et al., 2007) and an additional 25,083 ESTs in the public domain. Before sequence assembly, all ESTs were trimmed of poly A/T sequences at both the 5′ and 3′ ends using the default settings of the Vector NTI Advance version 10 software (Invitrogen, Carlsbad, CA). Next, the trimEST feature at EMBOSS (http://emboss.sourceforge.net/) was employed using the settings of minimum 4 poly A/T and mismatch setting at 2 bases. The ESTs were then clustered to create contigs using the ContigExpress program of the Vector NTI software (Invitrogen) with the settings of 90% identity and 40 bp overlap. All other cluster settings used were the default. This produced a set of 9129 total unique *I. multifiliis* expressed sequences, comprising 2824 contigs and 6305 singletons. *I. multifiliis* contig sequences are provided in Supplemental File 1. Unique *I. multifiliis* sequences were used in BLAST analyses and gene ontology comparisons, and for the construction of the microarray. BLAST searches were performed using information at the National Center for Biotechnology Information (NCBI) and, where indicated, the J. Craig Venter Institute. Preliminary sequence data for the *I. multifiliis* scaffolds was obtained from the J. Craig Venter Institute.

2.3. Microarray construction

High-density *in situ* 385 K oligonucleotide microarrays were constructed by Nimblegen (Madison, WI, USA). All unique EST sequences were submitted for probe design. Since the unique ESTs comprised 9129 sequences, extra space was available on the 385 K microarrays to place additional features. At the time of microarray construction, no whole genome sequence of *I. multifiliis* was publically available. Therefore, to facilitate comparative genomic analysis and to potentially increase gene content through cross-hybridization, gene coding sequences of *Tetrahymena thermophila* and *Plasmodium falciparum* were also added to the microarrays. *T. thermophila* and *P. falciparum* sequences were selected since both organisms are unicellular protists, *P. falciparum* is parasitic and *T. thermophila* is phylogenetically close to *I. multifiliis* (Clark et al., 1992; Wright and Lynn, 1995; Van Den Bussche et al., 2000; Abernathy et al., 2007), and both have a whole-genome sequence available. A total of 26,273 *T. thermophila* and 5184 *P. falciparum* gene coding sequences were included in the array for probe design. The probe design strategy was to create 60 base pair (60mer) oligonucleotide probes, including 12 60mer oligos per unique sequence, and 10 60mer oligos for both *T. thermophila* and *P. falciparum* sequences. The array for both developmental and passage study can be accessed at the NCBI Gene Expression Omnibus (GEO) database under the accession numbers **GSE18556** and **GSE22466**.

2.4. RNA extraction and synthesis of cDNA

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Replicates from each life-stage were processed separately to avoid cross-contamination. Quantity and quality of total RNA was assessed on a spectrophotometer and by denaturing agarose gel electrophoresis containing formaldehyde.

Total RNA was used for the synthesis of first-strand cDNA, using an oligo$(dT)_{12-18}$ primer (Invitrogen) and components from the SuperScript™ Double-Stranded cDNA Synthesis kit (Invitrogen) according to protocols from Nimblegen Systems, Inc. (Roche Nimblegen, Madison, WI, USA). For first-strand cDNA synthesis, 10 μg of each total RNA was combined with 100 pmol of oligo(dT) primer in total reaction volumes of 11 μL. Samples were heated to 70 °C for 10 min to denature and immediately placed on ice. Then, 4 μL of 5× First Strand Buffer, 2 μL of 0.1 M DTT, and 1 μL of 10 mM dNTP mix were added to each reaction. Reactions were mixed, incubated for 2 min at 42 °C, and then 2 μL of RT was added (200 U/μL SuperScript II, Invitrogen). Incubations were continued at 42 °C for 1 h and placed on ice.

Next, second-stranded cDNA was synthesized, also with kit components from the SuperScript™ Double-Stranded cDNA Synthesis kit (Invitrogen). A master mix was made for each first-strand reaction sample in a total volume of 150 μL as follows: 20 μL of first-strand reaction was combined with 91 μL of nanopure water, 30 μL of 5×

Second Strand Buffer, 3 μL of 10 mM dNTP mix, 1 μL of 10 U/μL DNA ligase, 4 μL of 10 U/μL DNA polymerase I, and 1 μL of 2 U/μL RNase H. Samples were gently mixed and incubated at 16 °C for 2 h. Then, 2 μL of 5 U/μL T4 DNA polymerase was added and the incubation was continued for an additional 5 min. Samples were then placed on ice and 10 μL of 0.5 M EDTA added to each reaction to complete the synthesis of double-stranded cDNA. One-microliter of 10 mg/mL RNase A Solution was added to each reaction and incubated at 37 °C for 10 min. The samples were extracted with TE-saturated 25:24:1 phenol:chloroform:isoamyl alcohol (Sigma-Aldrich, St. Louis, MO, USA) and the aqueous phase saved for cDNA precipitation. Second strand cDNA was precipitated by adding 16 μL of 7.5 M ammonium acetate to each sample. Samples were mixed by repeated inversion and 7 μL of 5 mg/mL glycogen was added and mixed. Cold (−20 °C) absolute ethanol (326 μL) was added and mixed by inversion. Samples were centrifuged at 14,000 ×*g* for 20 min and the supernatants removed and discarded. To wash the samples, 500 μL of 80% ethanol (−20 °C) was added to each tube and mixed by inversion. Samples were centrifuged at 14,000 ×*g* for 5 min and the supernatants removed. Washing with ice-cold 80% ethanol was repeated. Pellets were air-dried under a laminar flow hood for 10 min and reconstituted with 20 μL of nanopure water. Concentration of each cDNA sample was determined by spectrophotometry, and ≥2 μg cDNA from each sample was provided to Nimblegen (Madison, WI, USA) for labeling, hybridization, and image acquisition.

2.5. Microarray hybridization and analysis

The microarray was used to assess gene expression profiles on each of the three life-stages of *I. multifiliis*, and to assess gene expression in early to late life-cycles based on serial passages. For a comparison between life-cycle stages, a total of nine hybridization experiments were performed utilizing the microarray design, including three biological replicates from each of the three life-stages (tomont, trophont, and theront life-stages). For the infectivity study, a total of 18 hybridization experiments were performed to include three biological replicates at passage 1 and passage 100 of each of the three life-stages. Second-stranded cDNA, created as described above in Section 2.4, from each was hybridized to microarrays and used to generate gene calls. Gene call data for each array was provided by Nimblegen. NimbleScan software was used to normalize the raw images by quantile normalization (Bolstad et al., 2003). Gene calls of the normalized data were generated using the Robust Multichip Average (RMA) algorithm (Irizarry et al., 2003). Normalized RMA call expression values and standard errors were loaded into the DNA-chip analyzer (dChip) software (Li and Wong, 2001). This software was used to perform data comparisons and generate fold-change of expression values. All default comparison criterion statistics were selected in dChip, along with a cut-off value of 2-fold expression difference minimum among all three-life stages. A 2-fold cut-off difference was chosen to increase the likelihood of gene discovery. Additional dChip analyses were performed comparing one-to-one life-stage expression changes with a 5-fold difference cut-off. This included an analysis comparing trophont to tomont life-stage, tomont to theront life-stage, and theront to trophont life-stage, mimicking the continual nature of the parasite development. A 5-fold difference cut-off was chosen to increase the confidence in hybridization results between the life-stages and also between the passage data. A global false discovery rate of 10% was assessed using 500 permutations for all analyses.

2.6. Quantitative RT-PCR

A selected subset of genes was chosen for microarray validation using quantitative PCR. For quantitative real-time RT-PCR (qRT-PCR), total RNA of *I. multifiliis* was extracted using the RNeasy Plus Mini kit (Qiagen, Valencia, CA, USA). Briefly, aliquots of *I. multifiliis* cells from each life-stage were homogenized in lysis buffer (6 μL of 14.3 M β-mercaptoethanol; 600 μL Buffer RLT Plus) using a 20-gauge needle attached to a sterile syringe according to the protocol from the RNeasy Plus Mini kit (Qiagen). Each homogenized lysate was centrifuged to remove cellular debris, and the supernatants were filtered through a gDNA eliminator spin column to eliminate the genomic DNA. The total RNA was further extracted using the protocol supplied from the manufacturer. Total RNA from each life-stage replicate was isolated for qRT-PCR. Each of the total RNA samples was adjusted to 100 ng/μL to use as template. One-step qRT-PCR was performed on a LightCycler 1.0 real-time instrument (Roche Applied Science, Indianapolis, IN, USA). The qRT-PCR reactions were made using the LightCycler RNA Master SYBR Green I kit (Roche Applied Science), with modifications. A 10 μL total reaction volume was used (9 μL reaction mix + 1 μL RNA temple) for each sample. Each reaction mix contained 2.65 μL of PCR grade water, 0.65 μL of 50 mM Mn $[OAc]_2$, 1 μL of 5 μM forward primer, 1 μL of 5 μM reverse primer, and 3.7 μL of SYBR Green I. The thermal cycling profile used was as follows: (i) Reverse transcription, 61 °C for 20 min; (ii) Denaturation, 95 °C for 2 min; (iii) Amplification, 50 cycles of 95 °C for 5 s, 56 °C for 10 s, and 72 °C for 20 s. Melting curve analysis was performed on each run and confirmed single product amplification. Cycle threshold (Ct) values were generated for each triplicate sample, both for the genes of interest and 18S rRNA gene. The 18S rRNA primers were those as reported by Jousson et al. (2005); the forward primer reads 5′-AGTGACAAGAAATAGCAAGCCAGGAG-3′ and the reverse primer reads 5′-ACCCAGCTAAATAGGCAGAAGTTCAA-3′. These primers amplified a segment of 193 bp.

Relative expression levels were determined using Ct values and the Relative Expression Software Tool (REST) version 2009 (Pfaffl, 2001; Pfaffl et al., 2002), assuming 100% PCR efficiencies and statistics based on 1000 randomizations. The 18S rRNA gene was used as the normalizer. Negative control PCR reactions (PCR using the total RNA templates, minus RT) were also performed to test for amplification of genomic DNA.

3. Results and discussion

3.1. EST analysis

A total of approximately 33,000 *I. multifiliis* ESTs were available for analysis. Cluster analysis of these ESTs indicated the presence of 9129 unique sequences. These ESTs were further subjected to BLASTX analysis to obtain their putative identities. As summarized in Table 1, of the 9129 unique sequences, 4730 (52%) had significant BLASTX hits to the non-redundant (*nr*) database at the NCBI. A complete list of BLASTX search results with taxonomic identities are provided in Supplemental Table 2. A close examination of putative identities suggested the presence of three main types of transcripts: those with highest similarities to protozoa sequences; those with highest similarities to teleost sequences; and those with highest similarities to bacterial sequences (Table 1), suggesting that the *I. multifiliis* EST database may actually contain ESTs with a mixture of origins.

The vast majority, 3717 (78.6%), of the *I. multifiliis* ESTs with significant (E-value<1E-5) BLASTX hits had the highest similarities to protozoan sequences, as expected (Table 1 and Supplemental Table 2). However, a fraction of 387 (8.2%) ESTs with significant BLASTX hits had the highest similarities to teleost sequences. Similarly, a fraction of 469 (9.9%) ESTs with significant BLASTX hits had the highest similarities to bacterial sequences. To draw conclusions based on this informatic analysis, it is likely that the *I. multifiliis* EST database contained sequences of various origins: those of *I. multifiliis* as intended, those from fish hosts, and those from bacteria. It is reasonable to assume that those ESTs with the highest similarities to protozoan sequences were likely of *I. multifiliis* origin, representing truly *I. multifiliis* ESTs, while those with the highest similarities to teleost sequences may have been derived from a collection of host fish tissues along with parasitic

Table 1
BLASTX summary of all the unique *I. multifiliis* ESTs.

Description	Value	Taxonomy	% of significant hits
Total unique *I. multifiliis* ESTs	9,129		
BLASTX significant (<1e−5) hits	4,730		
I. Number of top hits to eukaryotes	4,259		90
Hit distribution	3,519	*Tetrahymena* sp.	74
	152	*Paramecium tetraurelia*	3
	25	*Ichthyophthirius multifiliis*	<1
	2	*Plasmodium* sp.	<1
	174	Others	4
i. Number of top hits to teleosts	387		8
Hit distribution	236	*Danio rerio*	5
	36	*Tetraodon nigroviridis*	<1
	33	*Salmo salar*	<1
	25	*Ictalurus punctatus*	<1
	1	*Ictalurus furcatus*	<1
	56	Others	1
II. Number of top hits to prokaryotes	469		10
Hit distribution	191	*Rickettsia* sp.	4
	95	*Chryseobacterium gleum*	2
	13	*Flavobacterium* sp.	<1
	6	*Wolbachia* endosymbiont	<1
	4	Candidatus bacteria	<1
	160	Others	3
III. Other			
Hit distribution	2	Synthetic constructs	<1

trophont samples for cDNA library creation. Those ESTs with the highest similarities to bacterial sequences may have been derived from bacterial origin. Indeed, symbiotic bacteria have been reported for this parasite (Sun et al., 2009). Since *I. multifiliis* is an obligate parasite, complete removal of contaminating fish RNA remains difficult with the best of efforts, especially when the parasitic trophont stages is used for study. Removal of endosymbiotic signals is improbable, particularly since it is currently unclear if *I. multifiliis* can survive without the symbionts, and the trophont and tomont life-stages both contain symbiotic bacteria within their cytoplasm (Sun et al., 2009). Nonetheless, based on our computational analysis, caution must be exercised in dealing with ESTs from obligate parasitic organisms such as *I. multifiliis*.

To that end, all *I. multifiliis* contigs and singletons were searched against the *I. multifiliis* genome sequence, available in preliminary form at the J. Craig Venter Institute. BLAST analysis was performed using the *I. multifiliis* scaffolds and annotated amino acid sequence translations that are currently available (version: ich_jcvi_0407), with a cut off Expect value<1E^{-}5. Results of the BLAST analysis are available in Supplemental Table 3. There were 5361 significant hits (59%) to the genome scaffolds out of the possible 9129 sequences used in the search. There were 4651 transcripts with significant identity based on BLASTX searches of the translated amino acid sequence of the genome. The largest percentage (58%) of significant BLASTX hits, a total of 2683 sequences, was assigned to hypothetical proteins. Taken together with the *nr* database search, in which approximately 18% of sequences had highest BLAST identity to either fish or bacteria, approximately 23% of *I. multifiliis* sequences remain uncharacterized. This could be due to: (1) the preliminary translation and unfinished nature of the current *I. multifiliis* genome sequence, (2) clustering issues from *de novo* assembly, particularly since several repeat domains (e.g. WD–MORN–leucine–repeats) and gene families (e.g. ubiquitin, zinc finger) were identified in the ESTs, (3) the discovery of unique transcripts or (4) likely some combination.

Whole genome information of two well-studied protists, *T. thermophila* and *P. falciparum*, has been a valuable comparative genomic resource for previous studies e.g. (Pomel et al., 2006; Abernathy et al., 2007; Abernathy et al., 2009; Damaj et al., 2009) as well as the current study. Now, the preliminary *I. multifiliis* genome sequence is a useful tool going forward to align expressed sequences to genomic DNA for gene discovery. The genome will be of particular importance in screening of ESTs for contaminating sequences, not only because this organism contains symbiotic bacteria, but also because *I. multifiliis* cannot be propagated outside of a fish host. The bioinformatic analyses described in this section includes a combination of comparative genomics, *nr* BLAST at the NCBI, and whole-genome screening. This data will be utilized to provide confidence in the results and conclusions of the microarray and quantitative PCR through the exclusion of sequences of putative bacterial or teleost origin herein.

Subsequent to the above analyses, all *I. multifiliis* ESTs with a significant BLAST hit were filtered to remove potential teleost and bacterial homologues, and the remaining sequences were analyzed using gene ontology (GO) searches (Fig. 1). A total of 1816 sequences were assigned GO terms within the biological processes, with 31% and 39% being involved in metabolic and cellular processes, respectively; 1284 sequences were assigned functions under the cellular component category; and 1948 sequences were assigned under the molecular functions category, with 42% for binding functions, and 44% for catalytic activities. The overall functional diversity of the sequences remains high based on analysis using 2nd level GO terms. All sequences assigned to GO categories did not exceed 44% assigned to any one category (Fig. 1).

3.2. Microarray design and construction

A microarray was constructed using the unique 9129 *I. multifiliis* EST sequences. Because of excess capacity on the microarray for additional features, gene coding sequences from *Tetrahymena thermophila* and *Plasmodium falciparum* were also included on the microarray to potentially provide more information via cross-hybridization to the related protozoa: one highly-related and non-pathogenic ciliate (*T. thermophila*) and one highly pathogenic apicomplexan (*P. falciparum*). The resultant microarray included a total of 38,728 features, with 7271 features designed from the *I. multifiliis* sequences, and 31,457 designed from the related organisms. Some of the 9129 unique *I. multifiliis* EST sequences (1858) were excluded from probe design after quality control.

3.3. Differentially expressed genes among different life stages

The cDNA samples derived from theront, trophont, and tomont stages were used as probes to explore gene expression profiles at the three different life-stages of *I. multifiliis*. Comparison of hybridization data indicated that a total of 173 transcripts were differentially expressed among all three life-cycle stages. The 173 transcripts represented 115 singletons and 57 contigs of the *I. multifiliis* ESTs, and one feature representing a hypothetical protein sequence of *T. thermophila*. Of the 173 differentially expressed transcripts, 95 were assigned putative identities by BLASTX at E-value of E<1e^{-}5.

After filtering the transcripts for putative contaminating sequences of bony fishes and bacteria, 135 transcripts were identified as differentially expressed, representing 85 singletons and 49 contigs from *I. multifiliis* along with the single feature representing a hypothetical protein sequence of *T. thermophila*. Of the 135 filtered differentially expressed transcripts, 51 were assigned putative identities. The complete listing of results is provided in Supplemental Table 4. The top 5 most up-regulated and down-regulated transcripts are shown (Table 2). Several of the transcripts have a reciprocal relationship: the most down-regulated transcript in one life-stage was one of the most up-regulated in another stage (Table 2 and Supplemental Table 4).

Examination of the 135 transcripts reveals some prominent genes involved in protozoan cellular regulation including immobilization

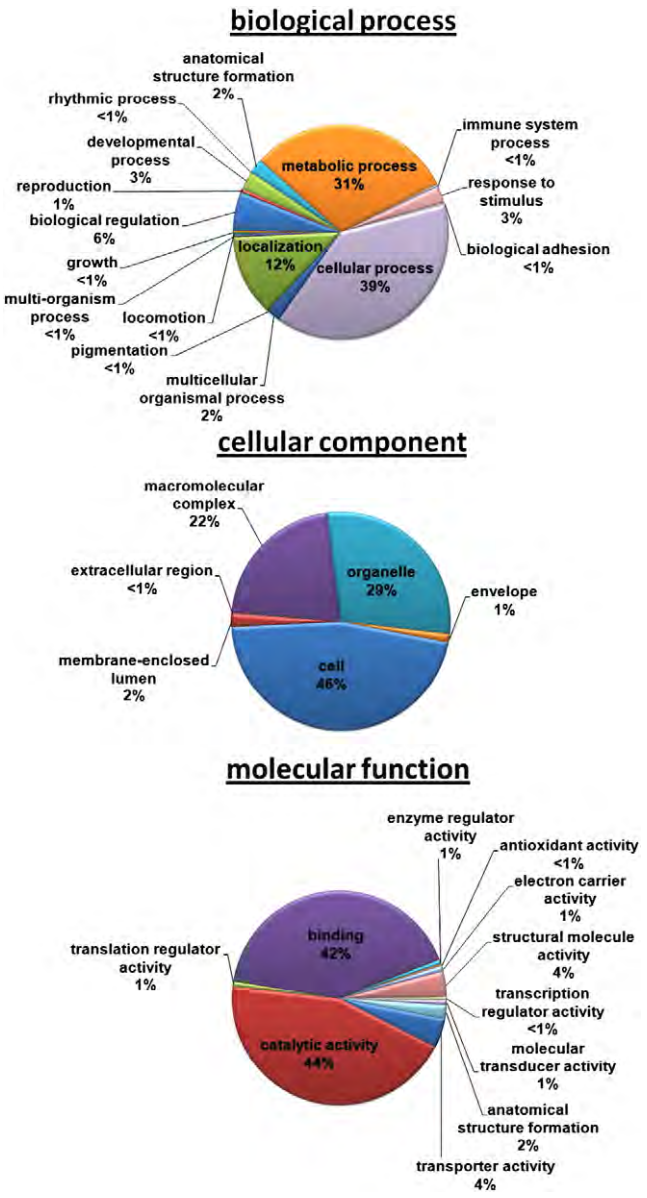

Fig. 1. Gene ontology. Level 2 gene ontology (GO) of *I. multifiliis* sequences. BLASTX analysis was performed on all unique ESTs, and then results filtered to remove teleost and bacterial homologues. Filtered results were entered into the Blast2GO software (Conesa et al., 2005) where sequences were assigned to GO categories. Level 2 GO categories include biological process (top panel), cellular component (middle panel), and/or molecular function (lower panel).

antigens (I-antigens), epiplasmin, and cysteine proteases (Supplemental Table 4). *I. multifiliis* I-antigens are surface proteins associated with cilia and cell cortex that strongly invoke the host immune response. The presence of host antibodies to these antigens renders the parasite immobile (Dickerson et al., 1989) and provides the host teleost some protective immunity against subsequent exposure (Swennes et al., 2007; Xu et al., 2008). As such, I-antigens have long been studied for their potential as an immunogen source (reviewed in (Matthews, 2005)). However, differential expression of I-antigens across all three life stages has not been previously examined. The results of the microarray experiments, as also confirmed with qRT-PCR (Section 3.8), suggested that I-antigen was expressed at low level in the parasitic trophont, at a slightly increased level in the reproductive tomont, and at a highly elevated level in the infective theront (Fig. 2). These results confirmed a previous report indicating differential regulation of this protein (Clark et al., 1992), and further specify relative amounts. The results here and elsewhere (Clark et al., 1992) indicate that, while *I. multifiliis* is most closely related to free-living ciliates such as *Tetrahymena*, there is clearly some developmental control of I-antigens that has evolved to coincide with its parasitic nature.

A similar expression pattern was observed with epiplasmin 1, a gene representing the major component of the ciliate membrane skeleton and member of a multigenic family of high-interest in evolutionary and genomic studies (Pomel et al., 2006; Damaj et al., 2009). Epiplasmins were demonstrated to be of critical importance for normal cell development and morphogenesis in the ciliate *Paramecium tetraurelia* (Damaj et al., 2009), and it may play a similar role in *I. multifiliis*.

Cathepsin L cysteine protease was also found to be differentially expressed among all life stages. This gene was previously found to be differentially regulated between *I. multifiliis* life-stages using quantitative RT-PCR (Jousson et al., 2007). Cathepsin L appears to play important roles in host–pathogen interactions. Proteases from parasitic protozoa have been demonstrated to function in host cell invasion and emergence, encystment and excystment, cytoadherence, stimulation and evasion of host immune responses, and catabolism of host proteins for a nutrient source (Klemba and Goldberg, 2002; Sajid and McKerrow, 2002). In ciliate fish parasites, proteases have been implicated in host invasion strategies and degradation of host cells from both *I. multifiliis* (Jousson et al., 2007) and *Philasterides dicentrarchi* (Parama et al., 2004). It has been speculated that fish parasites release proteolytic enzymes to degrade collagen and other structural molecules to assist in disruption of external epithelia as an invasion strategy (Parama et al., 2004). Indeed, we observed the largest up-regulation of cathepsin L cysteine protease in the infective theront (Table 2), as seen elsewhere (Jousson et al., 2007). Further, up-regulation was also observed in the tomont, where these proteases could be essential for encystment functions in this free-living stage (Villalobo et al., 2003; Parama et al., 2004).

A total of 31 of the 135 genes identified as differentially expressed among all three life-cycle stages had top BLASTX hit and highest sequence identity to the closely related *Tetrahymena* species (Supplemental Table 4). Many of these genes identified are involved in cell structure and cell regulation. They include genes involved in protein assembly, folding, and translocation such as with HSP70, HSP90, DnaJ, DnaK, prefoldin, Ras family and TCP1 gamma family proteins. A few core structural proteins were identified including ribosomal protein S25, TPR domains, and histones. Several other transcripts involved in cell-cycle functions were identified including proteins involved in fatty acid oxidation and the citric acid cycle, amino acid biosynthesis, ion binding and transport, phosphorylation, nucleic acid modification and translation termination. Three transcripts also had homology to hypothetical proteins. One final transcript was identified as a proteasome A-type and B-type family protein (Supplemental Table 4). Proteasomes represent a ubiquitous central component in eukaryotic cells involved in protein turnover. These proteins have been extensively characterized in protozoan parasites including *Giardia*, *Entamoeba*, *Leishmania*, *Trypanosoma*, *Plasmodium* and *Toxoplasma* species (Paugam et al., 2003). The proteasome has been demonstrated to be critical for cell differentiation and replication in protozoa; as such, proteasomes have been studied for their use as a therapeutic target to help the control of such parasites (Paugam et al., 2003). The current study shows that the transcript for the *I. multifiliis* proteasome is differentially regulated in all three life-cycle stages and potentially critical to cell regulation; therefore, this gene could also be a potential target for a therapeutic agent in the biocontrol of the parasite.

3.4. Differentially expressed genes between life stages

Microarray hybridization results were also examined between each life-stage: tomont versus theront, theront versus trophont, and trophont versus tomont. These data represent a significant portion of

Table 2
I. multifiliis developmental array microarray. List of the 5 most down-regulated and most up-regulated transcripts with a BLASTX identity among all life-cycles based on the microarray analysis. Fold-change values are listed based on comparisons between tomont and theront (Tom→Thr), theront and trophont (Thr→Tro), and trophont and tomont (Tro→Tom) life-stages. Bold values indicate the Expression parameter by which the data was sorted.

Expression	Accession number/contig ID	BLASTX description	E-value	Tom→Thr	Thr→Tro	Tro→Tom
Down – theront	Ich_Contig_1769	Histone H3	2.66E−68	**−44.99**	11.44	3.93
Down – theront	AAP49009	Transposase	2.76E−12	**−39.5**	114.69	−2.9
Down – theront	CAA82360	Putative transposase	9.63E−14	**−36.5**	105.93	−2.9
Down – theront	XP_002337912	Predicted protein	2.05E−13	**−35.15**	107.38	−3.05
Down – theront	AAW24907	SJCHGC06398 protein	1.61E−16	**−27.51**	99.76	−3.63
Down – trophont	Ich_Contig_2514	Immobilization antigen isoform	3.44E−15	24.96	**−109.83**	4.4
Down – trophont	EG958024	Hypothetical protein	8.93E−23	7.62	**−29.31**	3.85
Down – trophont	Ich_Contig_256	Protein with unknown function	2.6E−26	4.78	**−28.53**	5.97
Down – trophont	EL922591	Cathepsin L cysteine protease	5.9E−112	4.65	**−28.17**	6.06
Down – trophont	Ich_Contig_260	CHORD family protein	1.17E−46	4.26	**−24.39**	5.73
Down – tomont	Ich_Contig_1476	Predicted protein	9.97E−26	−8.62	57.05	**−6.62**
Down – tomont	EL928230	Small subunit ribosomal protein 14	9.55E−09	−18.27	89.68	**−4.91**
Down – tomont	EG962656	Transposase	5.91E−11	−14.28	61.33	**−4.29**
Down – tomont	Ich_Contig_2262	Unnamed protein product	7E−13	−6.97	27.43	**−3.94**
Down – tomont	EG965699	actin	1E−6	−7.7	30.3	**−3.93**
Up – theront	Ich_Contig_2514	Immobilization antigen isoform	3.44E−15	**24.96**	−109.83	4.4
Up – theront	Ich_Contig_40	Protein kinase domain containing protein	0	**18.8**	−6.59	−2.85
Up – theront	EG958024	Hypothetical	8.93E−23	**7.62**	−29.31	3.85
Up – theront	Ich_Contig_481	Fumarylacetoacetase family protein	7.39E−59	**5.6**	−19.82	3.54
Up – theront	Ich_Contig_256	Protein with unknown function	2.6E−26	**4.78**	−28.53	5.97
Up – trophont	EG960199	Transposase	2.76E−12	−39.5	**114.69**	−2.9
Up – trophont	EG958591	Predicted protein	2.05E−13	−35.15	**107.38**	−3.05
Up – trophont	Ich_Contig_2584	Putative transposase	9.63E−14	−36.5	**105.93**	−2.9
Up – trophont	EG963124	SJCHGC06398 protein	1.61E−16	−27.51	**99.76**	−3.63
Up – trophont	EL928230	Small subunit ribosomal protein 14	9.55E−09	−18.27	**89.68**	−4.91
Up – tomont	Ich_Contig_1536	Calcium-translocating P-type ATPase	3.77E−20	2.63	−23.07	**8.76**
Up – tomont	Ich_Contig_2788	Cystathionine beta-lyase	8.91E−45	3.06	−21.7	**7.08**
Up – tomont	EL922591	Cathepsin L cysteine protease ICP2	5.9E−112	4.65	−28.17	**6.06**
Up – tomont	Ich_Contig_256	Protein with unknown function	2.6E−26	4.78	−28.53	**5.97**
Up – tomont	Ich_Contig_260	CHORD family protein	1.17E−46	4.26	−24.39	**5.73**

the steady-state transcriptome between the life-cycle stages of *I. multifiliis* and further provide a material basis for future developmental candidate gene expression studies. Raw comparisons between tomont and theront yielded 576 putative differently expressed genes, between theront and trophont 787 putative differently expressed genes, and between trophont and tomont 162 putative differently expressed genes.

After filtering of the transcripts to remove bony fish and bacterial sequences, 515 differently expressed genes were found between tomont and theront, 666 differently expressed genes were found between theront and trophont, and 109 differently expressed genes were found between trophont and tomont (Supplemental Table 5). A total of 32, 335, and 310 transcripts identified as differentially expressed and with highest BLASTX similarities to *Tetrahymena* species were identified between trophont versus tomont, theront versus trophont, and tomont versus theront, respectively. In the trophont–tomont dataset, the two

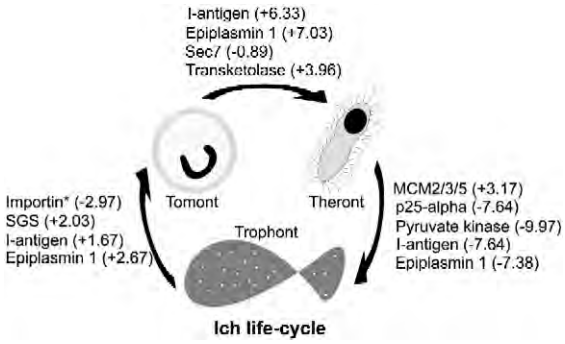

Fig. 2. *I. multifiliis* developmental stages quantitative PCR. Quantitative real-time RT-PCR of selected transcripts from *I. multifiliis*. Values indicated are $\log_2$ transformed expression data generated from the REST 2009 software (Pfaffl, 2001; Pfaffl et al., 2002) as described in Section 2.6. All data are significant at $p<0.05$. Asterisk indicates a hypothetical protein with putative similarity. Full gene information is listed in Table 3.

most abundantly identified transcripts were hypothetical proteins and dynein heavy chain proteins; the remaining transcripts included cell structural and regulatory proteins such as granule lattice, tubulin, and histone, and dehydrogenase, lyase, and intraflagellar transport proteins. In the theront–trophont dataset, the three most abundantly identified transcripts were hypothetical proteins, protein kinases, and leishmanolysins. Various other family proteins were identified including zinc-finger domains family, EF hand family, and ATPase family proteins. In the tomont–theront dataset, the three most abundantly identified transcripts were identical to the theront to trophont dataset: hypothetical proteins, protein kinases, and leishmanolysins.

The identity of the transcripts with BLASTX similarity to *Tetrahymena* hypothetical proteins remains unclear; interestingly however, protein kinases and leishmanolysins are the most abundant differentially expressed genes in two of the three datasets. Protein kinases are proteins that modify other proteins through kinase activity, or phosphorylation. They are one of the largest families of proteins in the cells of most eukaryotes; they comprise at least 2% of human genes (Doerig et al., 2002; Manning et al., 2002) and upwards of 3.9% of *T. thermophila* genes (Doerig et al., 2002; Eisen et al., 2006). The importance of overwhelming identification of protein kinases could be useful from *I. multifiliis*, since these proteins have been studied as potential therapeutic targets for control of other protozoan parasites (Doerig et al., 2002; Doerig, 2004). Leishmanolysins are proteases originally identified from *Leishmania major* and are believed to be involved in processing surface proteins (Eisen et al., 2006). These proteins have since been identified from *T. thermophila* (Eisen et al., 2006) and *Cryptocaryon irritans* (Lokanathan et al., 2010) both ciliates highly related to *I. multifiliis*. As previously shown in *I. multifiliis*, cysteine proteases can be utilized for detection of the pathogen (Jousson et al., 2007). Parasitic proteases are highly immunogenic, and may be useful as biomarkers, vaccine candidates, and/or therapeutic targets (Sajid and McKerrow, 2002). Based on these findings, further data is warranted on the characterization of leishmanolysins in species other than *L. major* to

determine their potential roles as critical virulence factors in pathogenic ciliates.

3.5. Differentially expressed genes identified through cross-hybridization

Given additional capacity on the array, features designed from genes of *T. thermophila* and *P. falciparum* were included. The microarray hybridization data suggested that a total of 35 transcripts were identified as differentially expressed genes through cross-hybridization with *T. thermophila* or *P. falciparum* sequences. This included 16 transcripts by tomont–theront comparison, 16 transcripts by theront–trophont comparison, and 3 transcripts by trophont–tomont comparison. Transcript identities include a translation initiation factor and an erythrocyte membrane protein from *P. falciparum*, and an immobilization antigen and IBR domain containing protein from *T. thermophila*. There were also multiple identities to hypothetical proteins identified from *T. thermophila* (Supplemental Table 5).

As expected due to the evolutionary relationships of the three protozoa (Wright and Lynn, 1995; Abernathy et al., 2007), the largest degree of cross-hybridization was observed with *T. thermophila* sequences as compared to *P. falciparum* sequences with the *I. multifiliis* probes. Additional comparative genomic data was obtained when the stringency was reduced from a 5-fold cut-off to a 2-fold cut-off; a total of 118 additional differentially expressed transcripts were identified, including 23 from *P. falciparum* and 95 from *T. thermophila* (data not shown). Results from comparative analyses should facilitate additional gene discovery and annotation in *I. multifiliis*, particularly since relatively little expression data is known and whole genome sequence is currently preliminary from *I. multifiliis*, whereas the whole genome sequences are available and well-annotated for both *T. Thermophila* and *P. falciparum*.

3.6. Differentially expressed genes from low and high passages of I. multifiliis

The ciliate parasite *I. multifiliis* was found to lose its infectivity and thereby its virulence upon a high number of passages (Xu and Klesius, 2004). *I. multifiliis* infectivity decreased significantly after 26–105 passages in the lab on fish hosts (Xu and Klesius, 2004). In this study, the microarray was used to assess gene expression patterns in low and high passages. Probes from tomont and trophont at passage 1 (P1) and passage 100 (P100) were used to study the differentially expressed genes. After filtering, a total of 195 transcripts were found to be differentially expressed among both tomont and trophont between passage 1 to passage 100 (Supplemental Table 6). Of the 195 transcripts differentially expressed, 150 were assigned putative identities by BLASTX at E-value of $E<1e^{-5}$. Of the 150 transcripts assigned putative identities, 130 of these were identified as having highest similarity to *Tetrahymena* species by BLASTX analysis. One transcript was also identified through cross-hybridization of *T. thermophila* probes and was identified as a hypothetical protein. Interestingly, all but two of the 195 shared transcripts were differentially expressed in concert: trophont genes down-regulated in P100 were also down-regulated in P100 of tomont, and likewise trophont genes up-regulated in P100 were also up-regulated in P100 of tomont. Since the parasite is replicating asexually, this suggests that the contribution of gene/transcript copy number from each life stage could ultimately play a role in the senescence of the parasite, an observation that certainly warrants further study based on this microarray data.

The four most commonly found transcripts identified were hypothetical proteins, leishmanolysins, protein kinases, and major facilitator superfamily proteins. All protein kinases were found to be up-regulated in the later passage (P100) of both trophont and tomont life-cycle stages. Similarly, all leishmanolysin family proteins were identified as up-regulated in P100 when compared to P1. All major facilitator superfamily proteins likewise were up-regulated in the later passages. Major facilitator superfamily proteins comprise one of the largest families of membrane transport proteins known. These proteins transport a wide array of substances including sugars, nucleosides, ions, metabolites, amino acids, and drugs across the cell membranes (Pao et al., 1998). This family has long been studied as a means of multidrug resistance in many pathogens from bacteria to eukaryotes, as these proteins are involved in transmembrane transport and are actively removing cytotoxic compounds out of the cell (Sa-Correia and Tenreiro, 2002). As with other protozoa, these proteins may have the potential for the biocontrol of *I. multifiliis*, especially as it harbors endosymbiotic bacteria (Sun et al., 2009) that could possibly be used as a target for a therapeutic agent.

Microarray hybridization results were also examined between each passage separately: tomont P1 versus tomont P100, and also trophont P1 versus trophont P100. After filtering, comparison between tomont P1 to tomont P100 yielded 493 putative differently expressed genes and 1478 putative differentially expressed genes between trophont P1 to trophont P100 were identified (Supplemental Table 7). This data provides a large repertoire of genes putatively involved in *I. multifiliis* infectivity or the loss-of-virulence in tomont and trophont life-cycle stages. In the tomont passage analysis (P1 to P100), 226 transcripts were down-regulated while 267 transcripts were up-regulated. In the trophont passage analysis (P1 to P100), 91 transcripts were down-regulated while 1387 transcripts were up-regulated. A total of 339 transcripts were assigned putative identities by BLASTX when comparing tomont P1 to P100, and 1233 transcripts were assigned putative identities by BLASTX when comparing trophont P1 to P100. Unfortunately, a sufficient quantity of RNA was not achieved in the theront life-cycle stage to obtain microarray results for theront passages P1 and P100.

Eleven transcripts in this analysis were identified as differentially regulated by cross-hybridization of *I. multifiliis* cDNA to *T. thermophila* sequences on the microarray. This included 5 transcripts by tomont P1 to P100 analysis and 6 by trophont P1 to P100 analysis. All were identified as hypothetical proteins except one in the trophont analysis. That sequence was identified as a NAC domain containing protein (Supplemental Table 7).

I-antigens and other surface proteins were found to be differentially expressed between passages (Supplemental Table 7). Other frequently observed transcripts included leishmanolysin, zinc-finger domains, insect antifreeze proteins and neurohypophysial hormones. Interestingly, these types of similar proteins are characterized as meckelin transmembrane family proteins in mammals. These proteins are believed to be associated with ciliatory diseases (Smith et al., 2006). It would be highly interesting to understand the function of these similar sequences in *I. multifiliis* and if, in fact, they are related to cilia damage or dysfunction in the later passages and if this correlates with a loss of infectivity.

The complete molecular mechanisms of senescence remain poorly understood. Several biological processes have been suggested as capable of inducing senescence, including changes in gene expression, activation of oncogenes, epigenetic effects, and DNA damage and replicative damage in the form of telomeric shortening (Takahashi et al., 2007; Peters, 2008). Telomerase has long been identified from *T. thermophila* (Collins and Gorovsky, 2005), and later from other protozoa such as *Leishmania* (Giardini et al., 2006). As such, telomerase activity may be active in *I. multifiliis*. We were not able to identify a telomerase homolog from *I. multifiliis* through the analysis of EST data and therefore telomerase gene activity could not be assessed by cDNA microarray analysis. However, known senescence proteins were found to be differentially regulated in *I. multifiliis*. The silent information regulator 2 (*sir2*) gene was found to be highly (>33-fold) up-regulated in the later passage, P100, of trophonts (Supplemental Table 7). Sir2 is a histone deacetylase that functions in yeast in silencing of transcription at repetitive DNA including mating-type loci, telomeres, ribosomal DNA (rDNA), and also represses recombination in rDNA (Imai et al., 2000;

Blander and Guarente, 2004; Kennedy et al., 2005). Overexpression of Sir2 in yeast has been shown to extend the replicative lifespan of this organism (Kaeberlein et al., 1999), and similar observations were later found in fruit fly and worms (Blander and Guarente, 2004; Kennedy et al., 2005). The current findings in *I. multifiliis* suggest that selection of trophont cells that survive to later ages may have resulted, at least in part, through overexpression of Sir2. Future cytogenetic studies would be useful to determine if telomeric shortening and/or alterations of cilia are occurring in the laboratory and if these processes lead to a loss of infectivity in *I. multifiliis*.

Recently in *P. falciparum*, the protozoan responsible for malaria in humans, two Sir2 paralogs were characterized, with one Sir2 gene shown to affect telomere length (Tonkin et al., 2009). Further, the two Sir2 paralogs also play a role in antigenic gene variation by regulation of *var*, the gene family that encodes the major *P. falciparum* antigen: erythrocyte membrane protein 1. Approximately 60 subtelomeric *var* genes encode different variants of the protein; with one type being expressed at a time. Sir2 homologs were found to be major effectors on silencing the *var* genes (Tonkin et al., 2009). Gene switches in *var* expression cause variation of the protein enabling the parasite to evade the host immune system (Borst and Genest, 2006; Tonkin et al., 2009). Other pathogenic protozoa, including *Cryptosporidium* and *Trypanosoma brucei*, have surface protein gene families associated with subtelomeric regions and possess some gene switching activity (Templeton, 2007). As such, there remains great interest in elucidating the complete mechanism of Sir2 as related to *I. multifiliis* senescence and/or loss of infectivity. Similar to *P. falciparum*, a single serotype of *I. multifiliis* surface antigen (I-antigen) appears to be expressed at any given time (Clark et al., 2001); however, there is no current evidence of antigen switching in clonal isolates of *I. multifiliis* (Swennes et al., 2007). Also, it has yet to be demonstrated whether the I-antigen gene family is located adjacent to telomeres.

The question therefore arises as to which of the mechanism(s) are responsible for *I. multifiliis* senescence and loss of infectivity in the lab passages. Two mechanisms are described here relating to changes in gene expression: a replicative senescence effect as the cells age presumably due to an evolutionarily similar mechanism of telomere shortening, or partial loss of function in the mechanisms that regulate antigenic variation of the immunogens. Current knowledge of *I. multifiliis* biology indicates that antigenic switching may not occur; rather, that senescence could be an artefact of cloning (Scholz, 1999; Matthews, 2005). A particular *I. multifiliis* serotype is passaged clonally in the laboratory setting and there is no opportunity for the isolate to regenerate its cell or recombine its DNA through sexual conjugation, as occurs in related free-living ciliates such as *Tetrahymena* (Matthews, 2005). Since *I. multifiliis* bridges the gap between free-living ciliates and parasitic protozoa, the mechanisms that affect gene regulation as related to senescence and virulence may have characteristics of both, somewhat revealed through analysis of *I. multifiliis* genes (Lokanathan et al., 2010; Abernathy et al., 2007; 2009; Wright and Lynn, 1995; this study). Overall, the complete mechanisms related to *I. multifiliis* senescence and loss of infectivity evaluated by gene expression warrant further study; the current findings based on gene expression analysis provide the material basis for such future studies.

3.7. Potential role of endosymbiotic bacteria in senescence

Endosymbiotic bacteria may be another factor to consider in relation to *I. multifiliis* senescence. Endosymbiotic bacteria, particularly *Rickettsia* species and sphingobacteria, have been discovered in *I. multifiliis* (Sun et al., 2009). Could changes in gene expression of the endosymbionts be a contributing factor in the decline of infectivity? To address this question, the microarray data was further examined for bacterial sequences. Since a portion of the ESTs on the microarray likely represent the endosymbiotic bacteria based on sequence homology, we can putatively assess their gene expression. All but three genes representative of *Rickettsia* species by BLAST analyses were dramatically ($-5.7 \geq$ fold-change ≤ -68.9) down-regulated in Passage 100, including surface antigens, surface proteins, outer membrane proteins, ribosomal protein S15, and GRoeL (Supplemental Table 8). A DnaK transcript with sequence similarity (E-value $= 4e^{-35}$) to *Acinetobacter* species was also sharply down-regulated (-19.9 fold-change) in the tomont P100 (Supplemental Table 8). Both DnaK and GRoeL are chaperones that have been indicated in bacterial senescence (Ballesteros et al., 2001; Nystrom, 2002). Similarly to the *I. multifiliis* gene expression patterns observed, a potential role in senescence could be due to the replicative loss of gene/transcript copy number in the later passages of the parasite. Further, the role of bacterial surface antigens from symbionts could modulate the host immune response, potentially explaining a previous report of longer periods of trophont emergence in high passage of *I. multifiliis* (Xu and Klesius, 2004). The interplay of the *I. multifiliis* and symbiotic genomes may also affect fish immunity. While interesting, further study is needed to elucidate the contribution of the host-symbiont relationship to senescence.

3.8. Validation using quantitative RT-PCR

In order to validate the microarray and the results from the microarray study, 21 differentially expressed genes were analyzed using quantitative real time reverse transcription PCR (qRT-PCR). For the developmental microarray study, based on the microarray data and BLAST analysis, a subset of sequences with putative identities and BLASTX hit to other protozoa was selected for qRT-PCR. This included two genes (I-antigen and epiplasmin 1) that were differentially regulated among all three life-cycle stages. Validation by qRT-PCR was also performed for various other developmental genes represented in the microarray when comparing one-to-one life-stage expression changes (Table 3). At least two genes differentially expressed between each of the three life-cycle stages were also chosen for validation, to represent at least one unique up-regulated and one unique down-regulated transcript. For the passage microarray study, at least one common down-regulated and one common up-regulated transcript in both the tomont and trophont passages were selected for validation (Table 4 and Supplemental Table 6). An additional subset of genes was also chosen for validation by qRT-PCR, to represent at least two unique up-regulated transcripts and two unique down-regulated transcripts between the tomont and trophont passages (Table 4 and Supplemental Table 7).

Transcripts tested by qRT-PCR for the developmental study concurred with the microarray hybridization results. For comparisons between theront and trophont life-stages, p25-alpha and pyruvate kinase were determined to be down-regulated and MCM2/3/5 was determined to be up-regulated in trophont. For comparisons between tomont and theront life-stages, Sec7 was found to be down-regulated and transketolase was found to be up-regulated in theront. For comparisons between trophont and tomont life-stages, importin was determined to be down-regulated and SGS was found to be up-regulated in tomont (Fig. 2). The results from quantitative RT-PCR (Fig. 2) confirmed those from the microarray hybridization experiments, whether comparing expression levels from all three life-stages simultaneously (Supplemental Table 4) or with comparisons between each life-stage (Supplemental Table 5), validating the design and reproducibility of the microarray between developmental life stages.

Transcripts tested by qRT-PCR for the passages study also concurred with the microarray hybridization results (Fig. 3). Two transcripts were found to be up-regulated in the later (P100) passages of *I. multifiliis* tomonts including a high mobility group protein and casein kinase II. Two transcripts were found to be down-regulated in the later (P100) passages of tomonts including a TPR domain containing protein and a prenyltransferase/squalene oxidase repeat family protein. In the later passage of trophont, three transcripts were validated to be down-regulated including a Sec61beta family protein,

Table 3
I. multifiliis developmental array quantitative RT-PCR. List of genes selected for qRT-PCR analysis. Information includes putative gene identities based on BLAST analysis, GenBank accession numbers, life-stages examined, and primer sequences. The fold-change data are $\log_2$ transformed values from the microarray and from qRT-PCR, using one life-stage as baseline data (B) and another life-stage as experimental data (E) for comparisons. Probe names (ID) are also listed when the primer was constructed from a single EST within a contig.

Gene name	Accession/ID	Comparison	Fold-change (μarray)	Fold-change (qRT-PCR)	Primer sequence (5′–3′), F: forward; R: reverse
18S rRNA gene	U17354	All			F: GTGACAAGAAATAGCAAGCC R: CCCAGCTAAATAGGCAGAAG
Epiplasmin I	EL906016/Ich_ Contig_ 670	Theront B) → Trophont (E)	−3.84	−7.38	F:TGTTCACTAACCTGTACAAGAAGC
		Trophont (B) → Tomont (E)	1.60	2.67	R:AAACAAGTGGTGCTCCTACAGTC
		Tomont (B) → Theront (E)	2.24	7.03	
I-antigen	EL905772	Theront (B) → Trophont (E)	−2.81	−7.64	F: TTTGCGAAAGTGGAACTGG
		Trophont (B) → Tomont (E)	1.56	1.67	R: AACATCTGGTTTTGCAGCAG
		Tomont (B) → Theront (E)	1.24	6.33	
p25-alpha family protein	EL922674/Ich_contig_479	Theront (B) → Trophont (E)	−5.61	−7.64	F: CTCTGGAAAAGCTGAAATGGA R: TTAAGGACCTCCAGCTGAAGTAA
Pyruvate kinase family protein	EL925641	Theront (B) → Trophont (E)	−5.48	−9.97	F: TTGCAGACGGTACTTTAGTATGTATTG R: TCTTCTTTTTCGGTTACTGTTGG
MCM2/3/5 family protein	EL912787	Theront (B) → Trophont (E)	4.25	3.17	F: TCCTTCGCCTTCTGATGATACAGGAAT R: TGGCAGGCAGAAGAAACAATCGTG
Sec7 domain containing protein	EL907900	Tomont (B) → Theront (E)	−4.27	−0.89	F: GGAGAATTATTTGGGTCTGATAATG R: AAGCACCAGTAGCCGATTTG
Transketolase	EL916987/Ich_contig_1786	Tomont (B) → Theront (E)	4.88	3.96	F: TGAAGGGTCAGTTTGGGAAG R: GCCATCCAAAAGCTTCAAATC
Similar to Importin-3/5	EL909221	Trophont (B) → Tomont (E)	−2.11	−2.97	F: TCAGAAGCTCTTGAAAGTTTCCTT R: TTCATTGCATTCTGGTTTGC
SGS domain containing protein	EG962593/JAIch_018B_H09	Trophont (B) → Tomont (E)	3.72	2.03	F: GAATATGGTGAAGATCCTATGAATG R: GGAGGTTCAGGTCTATCTTTTCC

UDP-sugar pyrophosphorylase, and heat shock protein 90. In the later passage of trophont, three transcripts were validated to be up-regulated including an Acyl-CoA oxidase family protein, a WD repeat domain, and cathepsin z. A common down-regulated gene (ankyrin 2,3/unc44) and a common up-regulated gene (phosphoglucomutase/parafusin) were also validated (Fig. 3). To test the utility of the microarray for the theront life-stage, real time RT-PCR was also performed on the two common differentially expressed genes. The transcript for parafusin was also up-regulated in the theront stage (data not shown).

In all cases, transcripts tested by qRT-PCR concurred with the microarray hybridization results, thus demonstrating the feasibility, reproducibility, and usefulness of the microarrays in the study of *I. multifiliis* gene regulation.

Table 4
I. multifiliis passages array quantitative PCR. List of genes selected for quantitative PCR analysis using RNA of *I. multifiliis* passage 1 (P1) and passage 100 (P100). Detailed description of data is given in Table 3.

Gene name	Accession/ID	Comparison	Fold-change (μarray)	Fold-change (qRT-PCR)	Primer sequence (5′–3′), F: forward; R: reverse
18S rRNA gene	U17354	All			F: GTGACAAGAAATAGCAAGCC R: CCCAGCTAAATAGGCAGAAG
High mobility group protein	EG963277/Ich_ Contig_ 20	Tomont P1(B) → Tomont P100 (E)	−4.74	−9.97	F: GGCTAAACCCTCTGCATCAA R: GCAACTTTCCATTAATCAGCAAT
Casein kinase II	EL923519	Tomont P1(B) → Tomont P100 (E)	−4.39	−5.16	F: TGTCCTGATATTGATGGATGCT R: CATCTTCAGCAAATCTTGATCCT
TPR domain containing protein	EL912984	Tomont P1(B) → Tomont P100 (E)	3.49	2.35	F: GCAGTATTTGACGGCTTGGT R: TACACCCGACCCTTGGTTTA
Prenyltransferase and squalene oxidase repeat family protein	EL907482	Tomont P1(B) → Tomont P100 (E)	3.31	5.89	F: TGGGGAGAAGTGGATACTCG R: ATACGCCCCATGACTTTCTG
Sec61beta family protein	EL914941/Ich_Contig_1766	Trophont P1(B) → Trophont P100 (E)	−2.87	−6.27	F: TGGTGGATCAAAAAGTTCTGG R: TGCAGCAGATGAAGTTCCAC
UDP-sugar pyrophosphorylase	EL908359	Trophont P1(B) → Trophont P100 (E)	−2.65	−3.61	F: TGCCATTCACAATTACGAACA R: AAATGTGCTGAATGCAATATTTGT
Heat shock protein 90	EG961165/ JAIch_014C_D08	Trophont P1(B) → Trophont P100 (E)	−2.48	−5.21	F: TCTTGGGTGTGAACCTGGAT R: CGAAAGGAGATTAAGCTACAGAGG
Acyl-CoA oxidase family protein	EL910216	Trophont P1(B) → Trophont P100 (E)	6.39	5.72	F: TTGGAACCCTTCCCACTGTA R: AACATTGGAACCATGGCCTA
Similar to WD repeat domain	EG958609/ JAIch_006D_C03	Trophont P1(B) → Trophont P100 (E)	6.35	4.97	F: AGACGCGGTTTCATAGGTTG R: TTTAGAATCTCCCCCTGCTG
Cathepsin z	EL923418/Ich_Contig_487	Trophont P1(B) → Trophont P100 (E)	6.10	4.32	F: CAATGCAGGAGGATCATGTG R: TTACAGCCCAGCATCTACCA
Ankyrin 2,3/unc44	EG965438/JAIch_026A_E02	Tomont P1(B) → Tomont P100 (E)	−2.97	-0.96	F: TATATACGGCGTGCCAGGA R: CACCCGCTGTGAGTAACGTA
		Trophont P1(B) → Trophont P100 (E)	−3.55	−8.97	
Phosphoglucomutase/parafusin	EL912504/Ich_Contig_910	Tomont P1(B) → Tomont P100 (E)	2.58	4.00	F: GGGATTGCAAGAAGGCAATA R: AGTCGCTCCTTCAGATCCAG
		Trophont P1(B) → Trophont P100 (E)	4.55	5.05	

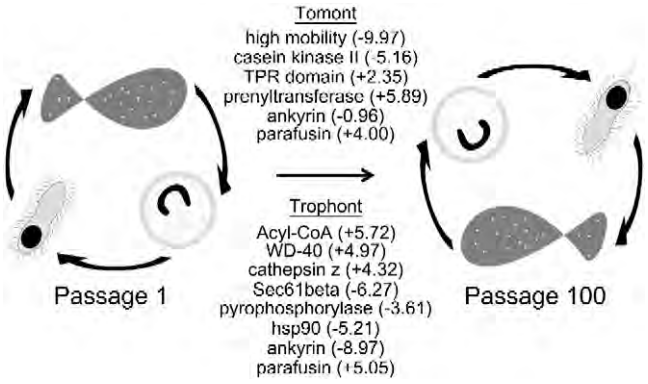

Fig. 3. *I. multifiliis* passages quantitative PCR. Quantitative real-time RT-PCR of selected transcripts from *I. multifiliis*. Values indicated are $\log_2$ transformed expression data generated from the REST 2009 software (Pfaffl, 2001; Pfaffl et al., 2002) as described in Section 2.6. All data were significant at $p<0.05$. Full gene information is listed in Table 4.

4. Conclusions

We conducted EST analysis of *I. multifiliis* and constructed an oligo cDNA microarray using the EST information. The microarrays were used to assess the gene expression profiles on each of the three life-stages. Microarray hybridization results were confirmed by results from real time quantitative RT-PCR using a selected set of genes, providing assessment for the reproducibility of the microarray design. This work therefore provides a technological platform for future gene expression studies of *I. multifiliis* at the genome scale. Currently, genome resources are limited in *I. multifiliis*, and genome sequence data is preliminary and un-validated; however, the use of cDNA microarrays based on ESTs in species with limited genomic information has been highly effective (Stewart et al., 2003; Campisi, 2005). The application of microarrays to address parasitological questions involving developmental and life-stage biology, host–pathogen interactions, virulence factors, and comparative genomics has been well-established (Boothroyd et al., 2003). The current work provides a first-generation framework and platform which can facilitate further transcriptional studies while the gene expression data provides a structure for functional host–pathogen study in this important fish parasite.

Supplementary materials related to this article can be found online at doi:10.1016/j.cbd.2011.08.003.

Acknowledgements

This project was supported by a grant from the USDA NRI Animal Genome Basic Genome Reagents and Tools Program (USDA/NRICGP award# 2009-35205-05101), and partially by a Specific Cooperative Agreement with the USDA ARS Aquatic Animal Health Laboratory under the contract number 58-6420-5-030. Preliminary sequence data for the *I. multifiliis* scaffolds was obtained from the J. Craig Venter Institute.

References

Abernathy, J.W., Xu, P., Li, P., Xu, D.H., Kucuktas, H., Klesius, P., Arias, C., Liu, Z., 2007. Generation and analysis of expressed sequence tags from the ciliate protozoan parasite *Ichthyophthirius multifiliis*. BMC Genomics 8, 176.

Abernathy, J.W., Xu, D.-H., Li, P., Klesius, P., Kucuktas, H., Liu, Z., 2009. Transcriptomic profiling of *Ichthyophthirius multifiliis* reveals polyadenylation of the large subunit ribosomal RNA. Comp. Biochem. Physiol. Part D Genomics Proteomics 4, 179–186.

Ballesteros, M., Fredriksson, A., Henriksson, J., Nystrom, T., 2001. Bacterial senescence: protein oxidation in non-proliferating cells is dictated by the accuracy of the ribosomes. EMBO J. 20, 5280–5289.

Ben-Porath, I., Weinberg, R.A., 2004. When cells get stressed: an integrative view of cellular senescence. J. Clin. Invest. 113, 8–13.

Ben-Porath, I., Weinberg, R.A., 2005. The signals and pathways activating cellular senescence. Int. J. Biochem. Cell Biol. 37, 961–976.

Blander, G., Guarente, L., 2004. The *Sir2* family of protein deacetylases. Annu. Rev. Biochem. 73, 417–435.

Bolstad, B.M., Irizarry, R.A., Astrand, M., Speed, T.P., 2003. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 19, 185–193.

Boothroyd, J.C., Blader, I., Cleary, M., Singh, U., 2003. DNA microarrays in parasitology: strengths and limitations. Trends Parasitol. 19, 470–476.

Borst, P., Genest, P.A., 2006. Parasitology: switching like for like. Nature 439, 926–927.

Burkart, M., Clark, T., Dickerson, H., 1990. Immunization of channel catfish, *Ictalurus punctatus* Rafinesque, against *Ichthyophthirius multifiliis* (Fouquet): killed versus live vaccines. J. Fish Biol. 13, 401–410.

Campisi, J., 2005. Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. Cell 120, 513–522.

Clark, T.G., McGraw, R.A., Dickerson, H.W., 1992. Developmental expression of surface antigen genes in the parasitic ciliate *Ichthyophthirius multifiliis*. Proc. Natl. Acad. Sci. U.S.A. 89, 6363–6367.

Clark, T.G., Gao, Y., Gaertig, J., Wang, X., Cheng, G., 2001. The I-antigens of *Ichthyophthirius multifiliis* are GPI-anchored proteins. J. Eukaryot. Microbiol. 48, 332–337.

Collins, K., Gorovsky, M.A., 2005. *Tetrahymena thermophila*. Curr. Biol. 15, R317–R318.

Conesa, A., Gotz, S., Garcia-Gomez, J.M., Terol, J., Talon, M., Robles, M., 2005. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21, 3674–3676.

Damaj, R., Pomel, S., Bricheux, G., Coffe, G., Vigues, B., Ravet, V., Bouchard, P., 2009. Cross-study analysis of genomic data defines the ciliate multigenic epiplasmin family: strategies for functional analysis in *Paramecium tetraurelia*. BMC Evol. Biol. 9, 125.

Dickerson, H.W., Clark, T.G., Findly, R.C., 1989. *Icthyophthirius multifiliis* has membrane-associated immobilization antigens. J. Protozool. 36, 159–164.

Doerig, C., 2004. Protein kinases as targets for anti-parasitic chemotherapy. Biochim. Biophys. Acta 1697, 155–168.

Doerig, C., Meijer, L., Mottram, J.C., 2002. Protein kinases as drug targets in parasitic protozoa. Trends Parasitol. 18, 366–371.

Eisen, J.A., Coyne, R.S., Wu, M., Wu, D., Thiagarajan, M., Wortman, J.R., Badger, J.H., Ren, Q., Amedeo, P., Jones, K.M., Tallon, L.J., Delcher, A.L., Salzberg, S.L., Silva, J.C., Haas, B.J., Majoros, W.H., Farzad, M., Carlton, J.M., Smith Jr., R.K., Garg, J., Pearlman, R.E., Karrer, K.M., Sun, L., Manning, G., Elde, N.C., Turkewitz, A.P., Asai, D.J., Wilkes, D.E., Wang, Y., Cai, H., Collins, K., Stewart, B.A., Lee, S.R., Wilamowska, K., Weinberg, Z., Ruzzo, W.L., Wloga, D., Gaertig, J., Frankel, J., Tsao, C.C., Gorovsky, M.A., Keeling, P.J., Waller, R.F., Patron, N.J., Cherry, J.M., Stover, N.A., Krieger, C.J., del Toro, C., Ryder, H.F., Williamson, S.C., Barbeau, R.A., Hamilton, E.P., Orias, E., 2006. Macronuclear genome sequence of the ciliate *Tetrahymena thermophila*, a model eukaryote. PLoS Biol. 4, e286.

Giardini, M.A., Lira, C.B., Conte, F.F., Camillo, L.R., de Siqueira Neto, J.L., Ramos, C.H., Cano, M.I., 2006. The putative telomerase reverse transcriptase component of *Leishmania amazonensis*: gene cloning and characterization. Parasitol. Res. 98, 447–454.

Herbig, U., Sedivy, J.M., 2006. Regulation of growth arrest in senescence: telomere damage is not the end of the story. Mech. Ageing Dev. 127, 16–24.

Herbig, U., Ferreira, M., Condel, L., Carey, D., Sedivy, J.M., 2006. Cellular senescence in aging primates. Science 311, 1257.

Houghton, G., Matthews, R.A., 1986. Immunosuppression of carp (*Cyprinus carpio* L.) to ichthyophthiriasis using the corticosteroid triamcinolone acetonide. Vet. Immunol. Immunopathol. 12, 413–419.

Imai, S., Armstrong, C.M., Kaeberlein, M., Guarente, L., 2000. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature 403, 795–800.

Irizarry, R.A., Hobbs, B., Collin, F., Beazer-Barclay, Y.D., Antonellis, K.J., Scherf, U., Speed, T.P., 2003. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 4, 249–264.

Jousson, O., Pretti, C., Di Bello, D., Cognetti-Varriale, A.M., 2005. Non-invasive detection and quantification of the parasitic ciliate *Ichthyophthirius multifiliis* by real-time PCR. Dis. Aquat. Organ. 65, 251–255.

Jousson, O., Di Bello, D., Donadio, E., Felicioli, A., Pretti, C., 2007. Differential expression of cysteine proteases in developmental stages of the parasitic ciliate *Ichthyophthirius multifiliis*. FEMS Microbiol. Lett. 269, 77–84.

Kaeberlein, M., McVey, M., Guarente, L., 1999. The SIR2/3/4 complex and *SIR2* alone promote longevity in *Saccharomyces cerevisiae* by two different mechanisms. Genes Dev. 13, 2570–2580.

Kennedy, B.K., Smith, E.D., Kaeberlein, M., 2005. The enigmatic role of *Sir2* in aging. Cell 123, 548–550.

Klemba, M., Goldberg, D.E., 2002. Biological roles of proteases in parasitic protozoa. Annu. Rev. Biochem. 71, 275–305.

Li, C., Wong, W.H., 2001. Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. Proc. Natl. Acad. Sci. U.S.A. 98, 31–36.

Lokanathan, Y., Mohd-Adnan, A., Wan, K.L., Nathan, S., 2010. Transcriptome analysis of the *Cryptocaryon irritans* tomont stage identifies potential genes for the detection and control of cryptocaryonosis. BMC Genomics 11, 76.

Manning, G., Whyte, D.B., Martinez, R., Hunter, T., Sudarsanam, S., 2002. The protein kinase complement of the human genome. Science 298, 1912–1934.

Matthews, R.A., 2005. *Ichthyophthirius multifiliis* Fouquet and Ichthyophthiriosis in Freshwater Teleosts. Adv. Parasitol. 59, 159–241.

Nystrom, T., 2002. Translational fidelity, protein oxidation, and senescence: lessons from bacteria. Ageing Res. Rev. 1, 693–703.

Pao, S.S., Paulsen, I.T., Saier Jr., M.H., 1998. Major facilitator superfamily. Microbiol. Mol. Biol. Rev. 62, 1–34.
Parama, A., Iglesias, R., Alvarez, M.F., Leiro, J., Ubeira, F.M., Sanmartin, M.L., 2004. Cysteine proteinase activities in the fish pathogen *Philasterides dicentrarchi* (Ciliophora: Scuticociliatida). Parasitology 128, 541–548.
Paugam, A., Bulteau, A.L., Dupouy-Camet, J., Creuzet, C., Friguet, B., 2003. Characterization and role of protozoan parasite proteasomes. Trends Parasitol. 19, 55–59.
Peters, G., 2008. An INKlination for epigenetic control of senescence. Nat. Struct. Mol. Biol. 15, 1133–1134.
Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45.
Pfaffl, M.W., Horgan, G.W., Dempfle, L., 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 30, e36.
Pomel, S., Diogon, M., Bouchard, P., Pradel, L., Ravet, V., Coffe, G., Vigues, B., 2006. The membrane skeleton in *Paramecium*: molecular characterization of a novel epiplasmin family and preliminary GFP expression results. Protist 157, 61–75.
Sa-Correia, I., Tenreiro, S., 2002. The multidrug resistance transporters of the major facilitator superfamily, 6 years after disclosure of *Saccharomyces cerevisiae* genome sequence. J. Biotechnol. 98, 215–226.
Sajid, M., McKerrow, J.H., 2002. Cysteine proteases of parasitic organisms. Mol. Biochem. Parasitol. 120, 1–21.
Scholz, T., 1999. Parasites in cultured and feral fish. Vet. Parasitol. 84, 317–335.
Smith, U.M., Consugar, M., Tee, L.J., McKee, B.M., Maina, E.N., Whelan, S., Morgan, N.V., Goranson, E., Gissen, P., Lilliquist, S., Aligianis, I.A., Ward, C.J., Pasha, S., Punyashthiti, R., Malik Sharif, S., Batman, P.A., Bennett, C.P., Woods, C.G., McKeown, C., Bucourt, M., Miller, C.A., Cox, P., Algazali, L., Trembath, R.C., Torres, V.E., Attie-Bitach, T., Kelly, D.A., Maher, E.R., Gattone 2nd, V.H., Harris, P.C., Johnson, C.A., 2006. The transmembrane protein meckelin (MKS3) is mutated in Meckel–Gruber syndrome and the wpk rat. Nat. Genet. 38, 191–196.
Stewart, S.A., Ben-Porath, I., Carey, V.J., O'Connor, B.F., Hahn, W.C., Weinberg, R.A., 2003. Erosion of the telomeric single-strand overhang at replicative senescence. Nat. Genet. 33, 492–496.
Sun, H.Y., Noe, J., Barber, J., Coyne, R.S., Cassidy-Hanley, D., Clark, T.G., Findly, R.C., Dickerson, H.W., 2009. Endosymbiotic bacteria in the parasitic ciliate *Ichthyophthirius multifiliis*. Appl. Environ. Microbiol. 75, 7445–7452.
Swennes, A.G., Findly, R.C., Dickerson, H.W., 2007. Cross-immunity and antibody responses to different immobilisation serotypes of *Ichthyophthirius multifiliis*. Fish Shellfish Immunol. 22, 589–597.
Takahashi, A., Ohtani, N., Hara, E., 2007. Irreversibility of cellular senescence: dual roles of p16INK4a/Rb-pathway in cell cycle control. Cell Div. 2, 10.
Templeton, T.J., 2007. Whole-genome natural histories of apicomplexan surface proteins. Trends Parasitol. 23, 205–212.
Tonkin, C.J., Carret, C.K., Duraisingh, M.T., Voss, T.S., Ralph, S.A., Hommel, M., Duffy, M.F., Silva, L.M., Scherf, A., Ivens, A., Speed, T.P., Beeson, J.G., Cowman, A.F., 2009. Sir2 paralogues cooperate to regulate virulence genes and antigenic variation in *Plasmodium falciparum*. PLoS Biol. 7, e84.
Van Den Bussche, R.A., Hoofer, S.R., Drew, C.P., Ewing, M.S., 2000. Characterization of histone H3/H4 gene region and phylogenetic affinity of *Ichthyophthirius multifiliis* based on H4 DNA sequence variation. Mol. Phylogenet. Evol. 14, 461–468.
Villalobo, E., Moch, C., Fryd-Versavel, G., Fleury-Aubusson, A., Morin, L., 2003. Cysteine proteases and cell differentiation: excystment of the ciliated protist *Sterkiella histriomuscorum*. Eukaryot. Cell 2, 1234–1245.
Wright, A.D., Lynn, D.H., 1995. Phylogeny of the fish parasite *Ichthyophthirius* and its relatives *Ophryoglena* and *Tetrahymena* (Ciliophora, Hymenostomatia) inferred from 18S ribosomal RNA sequences. Mol. Biol. Evol. 12, 285–290.
Xu, D.H., Klesius, P.H., 2004. Two year study on the infectivity of *Ichthyophthirius multifiliis* in channel catfish *Ictalurus punctatus*. Dis. Aquat. Organ. 59, 131–134.
Xu, D., Klesius, P.H., Shoemaker, C.A., Evans, J.J., 2000. The early development of Ich*thyophthirius multifiliis* in channel catfish in vitro. J. Aquat. Anim. Health 12, 290–296.
Xu, D.H., Klesius, P.H., Shoemaker, C.A., 2005. Cutaneous antibodies from channel catfish, *Ictalurus punctatus* (Rafinesque), immune to *Ichthyophthirius multifiliis* (Ich) may induce apoptosis of Ich theronts. J. Fish Dis. 28, 213–220.
Xu, D.H., Klesius, P.H., Shoemaker, C.A., 2008. Protective immunity of Nile tilapia against *Ichthyophthirius multifiliis* post-immunization with live theronts and sonicated trophonts. Fish Shellfish Immunol. 25, 124–127.