Genomic Insights into Processes Driving the Infection of *Alexandrium tamarense* by the Parasitoid *Amoebophrya* sp.

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The regulatory circuits during infection of dinoflagellates by their parasites are largely unknown on the molecular level. Here we provide molecular insights into these infection dynamics. *Alexandrium tamarense* is one of the most prominent harmful algal bloom dinoflagellates. Its pathogen, the dinoflagellate parasitoid *Amoebophrya* sp., has been observed to infect and control the blooms of this species. We generated a data set of transcripts from three time points (0, 6, and 96 h) during the infection of this parasite-host system. Assembly of all transcript data from the parasitoid (> 900,000 reads/313 Mbp with 454/Roche next-generation sequencing [NGS]) yielded 14,455 contigs, to which we mapped the raw transcript reads of each time point of the infection cycle. We show that particular surface lectins are expressed at the beginning of the infection cycle which likely mediate the attachment to the host cell. In a later phase, signal transduction-related genes together with transmembrane transport and cytoskeleton proteins point to a high integration of processes involved in host recognition, adhesion, and invasion. At the final maturation stage, cell division- and proliferation-related genes were highly expressed, reflecting the fast cell growth and nuclear division of the parasitoid. Our molecular insights into dinoflagellate parasitoid interactions point to general mechanisms also known from other eukaryotic parasites, especially from the Alveolata. These similarities indicate the presence of fundamental processes of parasitoid infection that have remained stable throughout evolution within different phyla.

Dinoflagellates, ciliates, and apicomplexa belong to the Alveolata superphylum (1–3). The dinoflagellates are among the most important primary producers in the marine ecosystem. Some of these species can form harmful algal blooms (HABs) that can be noxious to animals and aquatic ecosystems and thus profoundly impact marine environments (4, 5). One of the most prominent microalgae recorded to form such HABs is the toxicogenic dinoflagellate *Alexandrium* sp., associated with paralytic shellfish poisoning (PSP) (5, 6). *Alexandrium* life cycle transitions include sexual and clonal reproduction, and both play important roles in the dynamics and recurrence of blooms (7, 8). Sexual reproduction results in the formation of resting cysts that can remain viable in sediments for years, whereas clonal reproduction is responsible for proliferation that may lead to HABs (7–9). The formation of HABs and their ability to sustain themselves over time are strongly dependent on favorable abiotic conditions, such as solar radiation, nutrient concentration, salinity, and water mass stability (10, 11), but also on biotic parameters, for instance, the avoidance of competition and grazing (12). Recent studies demonstrated the active participation of parasitic pathogens in the control of toxic bloom formation and development in both field observations (13–15) and model predictions (16).

The parasitoid *Amoebophrya* sp. has been observed to infect populations of the bloom-forming dinoflagellate *Alexandrium minutum* (16, 17), with a prevalence up to 40% infected cells observed in the Penzé estuary (Brittany, France) (13). Specific characteristics make the effects of parasitoid on HABs different from those of predators or parasites. First, a parasitoid infects only one host during its lifetime, whereas a predator kills many prey (18). Second, infection by a parasitoid suppresses further host division and the host inevitably is killed to complete the parasitoid’s life cycle, whereas a parasite has effects on the host fitness, influencing its viability only indirectly (18, 19). The *Amoebophryidae* belong to Syndiniales (Alveolata), and only one genus, *Amoebophrya*, is known, but this genus has a high genetic diversity (20). Corresponding environmental sequences belonging to this group were clustered into the widespread eukaryotic group marine alveolate group II (MALV II) (21).

The life cycle of *Amoebophrya* elucidated by Cachon in 1964 (22) was recently confirmed using electron microscopy (23, 24). The life cycle begins with small infective biflagellate cells termed dinospores (22, 25, 26) (see Fig. S1 in the supplemental material). After finding and recognizing the host and adhering to the host’s surface, the dinospores exhibit electron-dense bodies within a microtubular basket. *Amoebophrya* seems to use this structure to enter the host cytoplasm, resembling the rhoptries employed by apicomplexa parasites (27, 28). The dinospores lose their flagella and penetrate first into the host cytoplasm protected by a parasitophorous membrane. In some cases, the parasitoid crosses the nuclear envelope into the host nucleus, losing this protecting membrane (23). The parasitoid maturation, after being initiated inside the nucleus, takes 2 to 3 days. During this time, the trophont of the parasitoid increases in size, followed by consequent cellular divisions during sporogenesis to ultimately form a typical intracellular- and multicellular stage called the beehive structure. A motile vermiform stage of brief duration, composed of several rows of...
biflagellate cells with concerted swimming behavior, is then released from the host after the intracellular maturation of the parasitoid. Soon after this release, this structure dissociates into hundreds of free-living infective dinospores (26, 29).

Amoebophrya infection has been studied on morphological and physiological levels, and yet the molecular processes of infection are poorly understood. Gene expression data are scarce. At present, only Bachvaroff et al. (30) have performed a survey of a host-parasite system at the gene expression level, publishing 898 expressed sequence tags (ESTs) from the host Karlodinium veneficum and the parasitoid Amoebophrya sp. However, the transcriptional changes during the host-parasitoid interaction still remain enigmatic.

The objective of this study was to obtain transcriptomic insights into the life cycle of the parasitoid Amoebophrya sp. During infection of the toxic dinoflagellate host Alexandrium, we profiled the transcriptome of Amoebophrya sp. at three different life stages: the pure dinospore stage (0 h), the initial infection/penetration stage (6 h), and the maturation stage (96 h). By analyzing the ESTs obtained from different life stages, we could identify processes and genes that may be relevant to these three different life stages. These data foster our understanding of complex host-parasitoid interactions and deliver a mechanistic understanding of the genetic basis enabling Amoebophrya sp. to dominate over toxic Alexandrium blooms.

MATERIALS AND METHODS

Cultures. The Alexandrium tamarense strain (Alex5) used in this study was isolated from the North Sea coast of Scotland (31). The strain was grown in K-medium (32) prepared from water from the North Sea filtered through a 0.2-μm-pore-size sterile filter (VacuCap; Pall Life Sciences, Dreieich, Germany).

The Amoebophrya sp. strain (AT5.2) used was isolated from host Alexandrium cells sampled from the Gulf of Maine in the United States (33). Amoebophrya sp. infecting Alexandrium was maintained on the above-described Alexandrium strain from the North Sea (3 to 4 days per generation). All cultures were grown at 15°C, with cool-white fluorescent lamps providing 150 μmol photons m⁻² s⁻¹ on a light:dark cycle of 14 h:10 h.

Fixation and counting methods. Host samples (10 ml) were fixed with Lugol’s solution (10 g potassium iodide, 5 g iodine, 100 ml distilled water) with a final concentration of 2% (31), and three 1-ml aliquots were counted after sedimentation in chambers under an inverted microscope (Zeiss Axiosvert 200M). The total number of cells counted was always >400 per sample. The growth rate (μ) of Alexandrium was calculated with the following formula (31, 34):

\[ \mu = \frac{1}{t_2 - t_1} \ln \frac{N_2}{N_1} \]

where μ is the growth rate (day⁻¹), N₁ and N₂ are the abundances of Alexandrium at t₁ and t₂, respectively, and t is the sampling day.

The persistence of green autofluorescence indicated the survival of the dinospores. Samples (10 ml) were fixed with formaldehyde (10% CaCO₃-buffered formaldehyde; 2% final concentration), and three 1-ml aliquots were counted in duplicate after sedimentation in chambers using a microscope (Zeiss Axiosvert 200M) (26). Parasitoid prevalence (i.e., the percentage of infected host cells) was assessed by detecting well-maturing parasitoid stages using the natural autofluorescence of the parasitoid (29). More than 400 cells from each aliquot were screened using an epifluorescence microscope (Carl Zeiss AG, Göttingen, Germany).

Infection experiments. The infection experiments were set up to cover one complete life cycle of the parasitoid and included three harvesting time points (0 h, 6 h, and 96 h; see Fig. 1 in the supplemental material). Infection of the host culture was established following the methods of Coats and Park (26). Infective parasitoid dinospores for the experiment were harvested from an infected host culture by gravity filtration through a 10-μm-pore-size mesh. Three 1-ml harvested cultures were checked under an inverted microscope (Zeiss Axiosvert 200M) to make sure that no host cells remained. One part of this dinospore culture (15 ml) was used for RNA extraction (time point 0 h). The remaining dinospore culture was immediately used to inoculate the triplicate exponential-phase cultures of Alexandrium with a parasitoid:host ratio of 10:1. Triplicate cultures of 400 ml Alexandrium with a concentration of approximately 3,000 cells ml⁻¹ were initially prepared in three flasks (500-ml Erlenmeyer flasks) for the treatment. Three vials for each triplicate culture with hosts only (~400 ml Alexandrium at a concentration of 3,000 cells ml⁻¹) served as controls. Two incubation times were chosen after adding the dinospores: 6 and 96 h. At each time point, samples were taken from the same batch of each triplicate parasitoid-treated culture for (a) fixation and counting, (b) parasitoid prevalence assessment, (c) RNA extraction and sequencing, and (d) PSP toxicity analysis.

RNA extraction and sequencing. Samples (100 ml) were taken for RNA extraction. Cells for RNA extraction were filtered through a 10-μm-pore-size-size in order to remove the living free dinospores, suspended from the filter using fresh K-medium, and harvested by centrifugation at 4°C for 10 min. The supernatant was decanted, and the resulting pellet was immediately resuspended in 1 ml of 60°C (hot) Tri Reagent (Sigma-Aldrich, Steinheim, Germany) and transferred to a 2-ml cryovial containing acid-washed glass beads. Cells were lysed using a Bio101 FastPrep instrument (Thermo Savant ILLkirch, France) at maximum speed for 45 s. Afterwards, 200 ml of pure chloroform was added and the sample was subjected to vortex mixing for 15 s. The mixture was incubated for 10 min at room temperature and then centrifuged at 4°C for 15 min at 13,000 × g. The upper aqueous phase was transferred to a new vial, which was filled with an equal volume of 100% isopropanol, subjected to vortex mixing, and incubated for 2 h at −20°C to precipitate the RNA. The RNA pellet was collected by 20 min of centrifugation at 4°C at 13,000 × g. The pellet was washed twice, first with 70% ethyl alcohol (EtOH) followed by 10 min of centrifugation at 4°C at 13,000 × g and then with 96% EtOH followed by 5 min of centrifugation at maximum speed, air dried, and dissolved with 30 μl RNase-free water (Qiagen, Hilden, Germany). The RNA sample was further cleaned by the use of an RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol for RNA cleanup, including on-column DNA digestion. An RNA quality check was performed using a NanoDrop ND-100 spectrometer (PeqLab, Erlangen, Germany) and a RNA Nano Chip assay by the use of a 2100 Bioanalyzer (Agilent Technologies, Böblingen, Germany).

Subsamples of the triplicate RNAs from each treatment were pooled and were used for cDNA library preparation. The construction of the cDNA library was done by Vertis Biotechnology AG (Freising-Weihenstephan, Germany). In brief, poly(A)⁺-positive [poly(A)⁺] RNA was prepared from the total RNA and first-strand cDNA synthesis was primed with random hexamers. The 454 sequencing adaptors were ligated to the 5′ and 3′ ends of the cDNA. The cDNA was amplified with 19 PCR cycles using a proofreading polymerase. The amplified cDNA was normalized by 1 cycle of denaturation and reannealing. The cDNA was passed over a hydroxyapatite column to separate the reassociated cDNA from the single-stranded cDNA (ss-cDNA). The ss-cDNA obtained was then amplified with 9 PCR cycles. cDNAs with a size range of 450 to 650 bp were cut off and eluted from an agarose gel and converted to a 454 Roche titanium sequencing library according to the manufacturer’s protocol for RNA cleanup, including on-column DNA digestion. An RNA quality check was performed using a NanoDrop ND-100 spectrometer (PeqLab, Erlangen, Germany) and a RNA Nano Chip assay by the use of a 2100 Bioanalyzer (Agilent Technologies, Böblingen, Germany).

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Sequence reads were quality trimmed and assembled using a CLC Genomics Workbench (CLC Bio, Aarhus, Denmark) with default settings. In order to identify the cDNA sequences derived from the parasitoid genome, we mapped all assembled contigs to parasitoid Amoebophrya draft genome sequence data of the same strain. This draft genome cur-
rently consists of a bona fide Amoebophrya raw sequence bin (\%/H11022/20\%/H11003/cov-\%/H11022/20\%/H11022/20\%/H11003/erage) comprised of approximately 100 Mb, from which various contaminating sequences of bacterial origin were removed (U. John, unpublished data). A further test of contamination of the transcript contigs to be analyzed was done using all available host Alexandrium ESTs from the NCBI nucleotide database. The read mapper from the CLC Genomics Workbench was used to align all single reads from each of the three time points to the reference Amoebophrya contigs. The numbers of hits were extracted as read counts and were used to classify the occurrence of Amoebophrya contigs at each time point.

Functional annotation. Amoebophrya cDNA contigs (larger than 200 bp) were annotated by BLAST search (blastx) (\%/H35/6) against the nonredundant protein sequence database of the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov) and the Universal Protein Resource (Uniprot; http://www.uniprot.org). Searches were conducted with Blast2GO (BioBam Bioinformatics S.L., Valencia, Spain) with an e-value cutoff of 1e-6. For further functional annotation, the Amoebophrya genes were translated into amino acid sequences with the Virtual Ribosome tool (http://www.cbs.dtu.dk/services/VirtualRibosome), and the batch web CD-search tool (http://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) was used to assign eukaryotic orthologous groups (KOGs) with an e-value cutoff of 1e-7. The translated sequences were additionally screened for the occurrence of Pfam domains and families (http://pfam.sanger.ac.uk). Homology searches and potential coding sequence searches were performed following the Trinotate annotation suite guidelines (36, 37). Briefly, putative orthologs were predicted from reciprocal best BLAST hits, peptide sequences were predicted with use of the Trinity transdecoder, and protein families, signal peptides, and transmembrane domains were determined using Pfam (38), signalP (39), and tmHMM (40), respectively. Comparisons to currently curated annotation databases were derived from the eggNOG/GO (41), Gene Ontology (42), and KEGG (43) pathway databases. Significant enrichments of the contigs were tested by calculating the P value from a hypergeometric distribution at the background level of all KOGs and Pfam families (44). KOGs and Pfam families were considered significantly enriched for a given experimental time point when test statistics gave a P value of \%/H11022/0.05/\%/H11003/.

RESULTS

Time course study of infected cultures. To assess the host-parasitoid interaction, host abundances and infection percentages were measured over a 96-h period (Fig. 1). As a control, we used the noninfected culture of Alexandrium. The growth rate of the infected culture ranged from \%/H9262/0.32/\%/H9262/0.17 at 24 h to 72 h and was significantly lower than that of the control (\%/H9262/0.46/\%/H9262/0.38 at 72 h) (one-way analysis of variance [ANOVA], \%/H19.96/\%/H0.05) (Fig. 1A; see also Table S1 in the supplemental material).

The percentage of parasitoid infection increased slightly during the first 48 h. Afterward, a drastic increase appeared after 72 h, when the infection level ascended rapidly from 2% to 36%. The
infection coverage remained high and increased during the following 24 h, finally reaching 39% after 96 h (Fig. 1B).

**Sequencing and assembly statistics.** Sequencing the three different libraries over the infection cycle yielded the following results: (i) parasitoid dinospores (time point 0), 445,296 reads/106 Mbp; (ii) the initial infection/penetration stage mixed with host and parasitoid (6 h), 309,199 reads/106 Mbp; and (iii) the maturation stage mixed with host and parasitoid (96 h), 301,377 reads/106 Mbp. Assemblies of the reads yielded 17,780 contigs for 0 h and 16,495 contigs for 96 h (pure dinospores) and that 4,291 and 11,911 contigs were present at the 6-h initial penetration stage and the 96-h maturation stage, respectively, with 3,587 contigs in common to all time points (Table 1 and Fig. 3). To investigate the potential gene products and pathways regulated during infection, we assessed the transcriptional changes between the time points. At the background of all 14,336 contigs, the significantly enriched contigs were sorted by calculating the P value from the hypergeometric distribution (44) (see Data set S1 in the supplemental material). Complete lists of the Pfam domains and families of over-represented genes (P value = < 0.05) and KOG enrichments (P value = < 0.05) are provided in Table S2 and Table S3, respectively. In the following, we describe our main findings.

**Comparison of three time points (0 h, 6 h, and 96 h) of the infection cycle.** All single reads from each of the three time points were mapped back to the *Amoebophrya* contigs (14,336 contigs). The read mapping indicated that 12,227 contigs were present at 0 h (pure dinospores) and that 4,291 and 11,911 contigs were present at the 6-h initial penetration stage and the 96-h maturation stage, respectively, with 3,587 contigs in common to all time points (Table 1 and Fig. 3).

| Contig category or database | No. of contigs | e-value or significance | % contigs identified |
|----------------------------|----------------|-------------------------|----------------------|
| Long (>200 bp)             | 14,336         |                         |                      |
| Spliced leader (SL)        | 51             |                         |                      |
| Poly(A) tail               | 91             |                         |                      |
| Database                   |                |                         |                      |
| All annotated              | 6,662          | e^-6                    | 46.47                |
| NCBL Nr                    | 2,938          | e^-12                   | 20.49                |
| Uniprot                    | 2,003          | e^-8                    | 13.97                |
| Pfam                       | 4,944          | e^-11                   | 34.49                |
| KOG                        | 1,174          | e^-7                    | 8.19                 |
| Gene Ontology              | 2,771          | e^-11                   | 19.33                |
| KEGG enzyme                | 1,325          | e^-11                   | 9.24                 |

**Table 2 Gene content and annotation summaries**

*Amoebophrya*-specific EST contigs. In the combined EST set, we annotated all *Amoebophrya* contigs (14,336 contigs) and were able to assign a putative function to 6,662 (46.47%) contigs based on BLAST searches and Trinotate results obtained using the NCBI Nr, the Uniprot, Pfam, KOG, Gene Ontology, and KEGG databases (Table 2). Annotated sequences were classified into functional categories according to KOGs (Fig. 2), whereby 33% of the genes were assigned to metabolism, 33% to cellular processes and signaling, 21% to information storage and processing, and 13% to the category “poorly characterized.” Within these categories, more than half of the functional annotated sequences fell into six subcategories: subcategory O, posttranslational modification, protein turnover, and chaperones (16%); subcategory J, translation, ribosomal structure, and biogenesis (12%); subcategory C, energy production and conversion (10%); subcategory T, signal transduction mechanisms (7%); subcategory E, amino acid transport and metabolism (6%); and subcategory R, general function prediction only (11%). A full list of the genes with annotations can be retrieved from the supplemental material (see Data set S1 in the supplemental material).

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trate cycle [TCA cycle], electron transport chain, ATP synthase complex) were downregulated. Table 3 summarizes the presence of genes for mitochondrial pathways in Amoebophrya.

(iii) Maturation stage (96 h). At the time point of 96 h, several oxidative pathway components, including peroxiredoxin, thioredoxin, and glutaredoxin, were expressed during infection. The genes related to cell division, reconstruction, and proliferation (e.g., chromosome segregation ATPases, meiosis-specific nuclear
FIG 3 Venn diagram of all 14,336 contigs (assembled from all time points) categorized with reads mapped from three time points: T0, pure dinospores; T6, initial penetration stage; T96, maturation stage. The number of raw reads mapping to each contig can be found in Data set S1 in the supplemental material.

structural) were observed in this maturation stage. From 6 to 96 h, the absolute number of expressed stress response genes (including those encoding heat shock protein 90, DnaJ, Cpn10, and cold shock protein) increased.

There was an abundant range of proteases (in total, 30 different Pfam domains and families) expressed by Amoebophrya, including ATP-dependent protease, cathepsin cysteine protease, subtilisin-like serine protease, cysteine protease, serine protease, and ubiquitin-specific proteases. Table S4 in the supplemental material lists the proteases identified in the Amoebophrya data set, which are discussed below.

### DISCUSSION

Transcriptional activity of genes can be used to assess functional changes within organisms. Dinoflagellates use trans-splicing to generate mature mRNA species (45). This mechanism may influence the posttranscriptional fate of transcripts and thus might impair the correlation of the amount of transcripts to the amount of proteins. Despite this potential decoupling of functions from transcription, transcriptional changes indicate a reaction of an organism to environmental stimuli. Our report demonstrates that the host-parasitoid interaction causes significant changes in parasitoid gene expression over the time course of the infection. Our major findings are summarized in Fig. 4, where we depict the occurrence of genes related to cell adhesion, glycan-related enzymes, and lectins, genes involved in energy metabolism and signal transduction, and genes encoding cytoskeleton proteins, heat shock proteins, and proteases.

The importance of proteases for a parasitic lifestyle has been shown for Perkinsus marinus, a protozoan parasite of the oyster. The Perkinsozoa (P. marinus) are the earliest-diverging group of the dinoflagellates, with the phylogenetic position at the base of the dinoflagellate branch (46, 47). Because of their close relationship, the Perkinsozoa are considered a key taxon to investigate parasitism within the Alveolata (48). In P. marinus, extracellular serine proteases are produced to degrade oyster protein in order to gain nutrients to support the parasite basic cell function (49) and to affect oyster host defenses (50). Parasites with increased expression of low-molecular-mass protease (LMP; 30 to 45 kDa) appeared to be more infective of highly susceptible oysters (Crassostrea virginica) in host-supplemented medium (51, 52). Some of these serine proteases were isolated and characterized and were found to be encoded by a subtilisin-like gene(s) from the P. marinus genome (53). In our data set, we also found high expression of serine protease and subtilisin-like serine protease together with cysteine protease, cathepsin cysteine protease, and ubiquitin-specific proteases by Amoebophrya which might be involved in host-parasitoid interactions of Amoebophrya in a manner similar to that seen with Perkinsus (see Data set S1 in the supplemental material; see also Table S4). Surprisingly, diverse proteases in Amoebophrya showed expression during all time points analyzed. Thus, their expression appears not to be triggered by contact or entry of the parasitoid. Rather, the proteases had been already produced and stored to enable a rapid provision of these degrading enzymes after entering the host.

**Dinospores (0 h).** This report represents the first broad-scale gene expression study of pure dinospores. In general, a large number of genes were expressed during this stage, indicating that the living free and actively swimming dinospores, although not dividing, make use of an extensive and specific gene set. In particular, glycan-related enzymes and carbohydrate-binding proteins (lectins) serve as an important cellular surface recognition mechanism in host-parasite interactions (54–56). Lectins of free-living, nonparasitic, heterotrophic dinoflagellates are involved in prey recognition and discrimination prior to phagocytosis (57). Our study revealed that two types of lectins were present in Amoebophrya dinospores: ricin-type beta-trefoil lectin and C-type lectin. Pfam analysis detected similarities to two types of plant lectins: legume-like lectin and jacalin-like lectin. The jacalin-like lectins (JRLs), the common name of B-prism-I lectins, were first identified from the seeds of jackfruit (Artocarpus heterophyllus) (58). Lectins of this family have been found to exhibit a repertoire of functions due to their high sensitivity in recognizing cell surface carbohydrates (59). The expression of these surface lectins therefore most likely contributes to the attachment of the parasite to the host cell. Hence, parasitic dinoflagellates may use lectins in the same way as their apicomplexan counterpart, whereas the lectin function probably has also evolved toward prey recognition in heterotrophic dinoflagellate lineages (57).

**Similarities between 0 and 6 h of infection.** During this stage of the parasitoid life cycle, the dinospores have a demand of energy due to active swimming for the purpose of finding and penetrating their host and maybe already for the initiation of development of the trophont. To date, not much is known about the energy metabolism of Amoebophrya. A comparison with their sister lineage, apicomplexa (diverging ~800 to 900 million years ago), may help efforts to understand the nutritional mode of these parasites (2, 60, 61). The apicomplexa include many human and animal pathogens such as Plasmodium (causative agent of malaria) (62), Toxoplasma (parasitic toxoplasmosis disease) (63), and Cryptosporidium (diarrhea in mammals). In the sexual stage, Plasmodium lives in the mosquito host and shows greater activity in electron transport and oxidative phosphorylation (64–66). While in the asexual stage, the parasite lives in a glucose-rich environment (human blood) and gets sufficient ATP through the glycolysis pathway alone (67, 68). We detected genes for mitochondrial pathways in Amoebophrya at all three time stages investigated, albeit at different expression levels (Table 3). At 6 h, levels of expression of
TABLE 3 Genes related to mitochondrial metabolic pathways

| Functional category          | Definition                               | Name              | E-value | T0 Read count | T6 Read count | T96 Read count |
|------------------------------|------------------------------------------|-------------------|---------|---------------|---------------|---------------|
| TCA cycle                    | Aconitase                                | Contig_2081       | 2E-125  | 30            | 7             | 70            |
|                             | Citrate synthase                         | Contig_2141       | 1.4E-50 | 19            | 0             | 17            |
|                             | Citrate synthase                         | Contig_2724       | 1.5E-61 | 10            | 0             | 20            |
|                             | Malate/lactate dehydrogenases            | Contig_1233       | 8.1E-26 | 25            | 0             | 12            |
|                             | Malate/lactate dehydrogenases            | Contig_7089       | 3.1E-45 | 5             | 0             | 12            |
|                             | NAD/NADP transhydrogenase alpha subunit  | Contig_528        | 1E-12   | 123           | 0             | 105           |
|                             | NAD/NADP transhydrogenase beta subunit   | Contig_4871       | 3.6E-75 | 28            | 0             | 11            |
|                             | Succinate dehydrogenase/fumarate reductase, Fe-S protein subunit | Contig_7660 | 2.3E-55 | 33            | 48            | 72            |
|                             | Succinate dehydrogenase/fumarate reductase, flavoprotein subunit | Contig_5497 | 3.3E-23 | 26            | 1             | 21            |
|                             | Succinate dehydrogenase/fumarate reductase, flavoprotein subunit | Contig_6096 | 5.5E-12 | 13            | 0             | 14            |
|                             | Succinate dehydrogenase/fumarate reductase, flavoprotein subunit | Contig_4542 | 4.5E-17 | 8             | 0             | 0             |
|                             | Succinyl-CoA synthetase, beta subunit    | Contig_4953       | 5.8E-95 | 19            | 0             | 17            |
|                             | Succinyl-CoA synthetase, beta subunit    | Contig_11475      | 3.5E-23 | 6             | 0             | 0             |
| Electron transport chain     | Cytochrome b                             | Contig_5154       | 3.6E-08 | 9             | 0             | 17            |
|                             | Cytochrome b                             | Contig_2214       | 4.5E-18 | 6             | 0             | 24            |
|                             | Cytochrome b                             | Contig_11545      | 5.8E-18 | 4             | 4             | 6             |
|                             | Cytochrome c₁                            | Contig_14469      | 2.6E-30 | 9             | 3             | 0             |
|                             | Cytochrome c₂                            | Contig_7748       | 6.1E-44 | 28            | 0             | 47            |
|                             | Electron transfer flavoprotein, alpha subunit | Contig_17848 | 9.2E-58 | 8             | 5             | 2             |
|                             | Electron transfer flavoprotein, beta subunit | Contig_41980 | 6.8E-35 | 4             | 3             | 7             |
|                             | NADH dehydrogenase, FAD-containing subunit | Contig_410 | 1.1E-24 | 73            | 0             | 38            |
| Additional dehydrogenases   | Glycerol-3-phosphate dehydrogenase       | Contig_10030      | 1.8E-16 | 3             | 0             | 22            |
| ATP synthase complex         | F0F1-type ATP synthase, alpha subunit    | Contig_3133       | 1E-175  | 5             | 0             | 0             |
|                             | F0F1-type ATP synthase, alpha subunit    | Contig_21601      | 1.3E-74 | 6             | 0             | 0             |
|                             | F0F1-type ATP synthase, beta subunit     | Contig_7952       | 1.8E-61 | 23            | 0             | 1             |
|                             | F0F1-type ATP synthase, beta subunit     | Contig_7654       | 1E-137  | 17            | 0             | 4             |
|                             | F0F1-type ATP synthase, delta subunit    | Contig_3817       | 8.8E-15 | 8             | 2             | 0             |
|                             | F0F1-type ATP synthase, delta subunit    | Contig_41749      | 1E-15   | 0             | 0             | 12            |
|                             | F0F1-type ATP synthase, gamma subunit    | Contig_41538      | 2.8E-13 | 0             | 0             | 6             |
|                             | F0F1-type ATP synthase, subunit c/Archaeal/vacuolar-type H+ -ATPase, subunit K  | Contig_13688 | 1.3E-08 | 3             | 1             | 44            |
|                             | F0F1-type ATP synthase, subunit c/Archaeal/vacuolar-type H+ -ATPase, subunit K  | Contig_13690 | 2.1E-11 | 1             | 0             | 24            |
| ATPase                       | Archaeal/vacuolar-type H+ -ATPase subunit A | Contig_20433 | 3.5E-82 | 11            | 4             | 1             |
|                             | Archaeal/vacuolar-type H+ -ATPase subunit A | Contig_43169 | 2.5E-52 | 0             | 0             | 5             |
|                             | Archaeal/vacuolar-type H+ -ATPase subunit A | Contig_44048 | 2.3E-90 | 2             | 3             | 2             |
|                             | Archaeal/vacuolar-type H+ -ATPase subunit C | Contig_8043 | 1.1E-09 | 7             | 0             | 0             |
|                             | Archaeal/vacuolar-type H+ -ATPase subunit D | Contig_6053 | 5.9E-43 | 2             | 4             | 4             |
|                             | Archaeal/vacuolar-type H+ -ATPase subunit F | Contig_1984 | 2E-11   | 8             | 0             | 0             |
|                             | Archaeal/vacuolar-type H+ -ATPase subunit I | Contig_1670 | 1.5E-10 | 2             | 0             | 6             |
|                             | Archaeal/vacuolar-type H+ -ATPase subunit I | Contig_1671 | 2.9E-10 | 0             | 0             | 3             |

*T0, pure dinospores; T6, initial penetration stage; T96, maturation stage; FAD, flavin adenine dinucleotide.

glucokinase were comparably high and many genes related to mitochondrial metabolic pathways were downregulated. This may suggest the importance of glycolysis and thus of anaerobic energy production in the initial penetration stage of *Amoebophrya*. At 0 and 96 h, the high expression of genes for the TCA cycle, electron transport chain, and ATP synthase complex indicated that oxidative phosphorylation may play a key role in generating ATP in dinospores and mature trophonts of parasitoid *Amoebophrya*. Unlike apicomplexa, the parasitoid *Amoebophrya* may need ATP synthesis from complete carbohydrate oxidation in mitochondria, congruent with the observation that mitochondria of the parasitoid *Amoebophrya* infecting *Akashiwo sanguinea* ranged in size from relatively large in dinospores to small in initial trophonts (at 12 and 36 h) and elongated in mature trophonts (at 48 h) (23). We found the majority of sequences encoding key components of mitochondrial pathways in *Amoebophrya* (Table 3), but the sequence encoding mitochondrial pyruvate dehydrogenase (PDH) was missing. In general, the PDH complex links glycolysis to the TCA cycle by converting pyruvate to acetyl coenzyme A (acetyl-CoA), which is thought to be a key regulator in the carbon flux in mitochondria (69). There is evidence that the apicoplast and some dinoflagellate lost their mitochondrial PDH, stopping the conversion of pyruvate into acetyl-CoA (66–68, 70). Therefore, we expect that the source of acetyl-CoA in the mitochondria of *Amoebophrya* is not pyruvate but might be found in degradation of branched-chain amino acids or β-oxidation of fatty acids. This shedding of metabolic functions of *Amoebophrya* may reflect its efficient parasitoid energy production, similar to that...
revealed in well-supported examples of apicomplexa and other parasitic protists. For instance, Helicosporidium, the obligate parasitic green alga, has lost nearly all genes associated with light harvesting and photosystems but contains an almost complete pathway for carbon fixation (71).

Initial penetration stage (6 h). The majority of the genes overrepresented at the time point of 6 h were related to cytoskeletal organization and signal transduction, pointing to processes that enable recognition, adhesion, and penetration of the host. The high expression of cytoskeleton-related genes implies its strong role during infection. This is supported by the ultrastructure research by Miller et al. (23), who observed a motility system based on microfilament polymerization that was used to enter the host cytoplasm.

The expression of heat shock protein 70 (HSP70) increased at 6 h of the infection (see Table S3 in the supplemental material). This observation is congruent with further literature data where it has been shown that the HSP70 gene is highly expressed in the parasitic apicomplexa Toxoplasma gondii (72, 73), in P. marinus, and in the parasitic ciliate Cryptocaryon irritans (74) to overcome the stress from the host environment (55). In addition, further heat shock proteins (HSP90, DnaJ, Cpn10) and a cold shock protein (cold shock domain [CSD]) were expressed in Amoebophrya both at the initial penetration stage at 6 h and at the late maturation stage at 96 h and also in dinospores (see Data set S1 in the supplemental material). Heat shock proteins are highly conserved proteins which play an essential role in the response to stress (75–77), and many heat shock proteins, such as the small HSP (78) and HSP90, are also chaperones essential for activating signaling proteins and protein folding in the eukaryotic cell (79). Cold shock proteins are associated with posttranscriptional regulation in eukaryotes (80) and have been found to play an important role in regulating translation in the dinoflagellate Lingulodinium (81). Taken together, our data indicate that the heat shock gene products in Amoebophrya may be needed to overcome the host defense response.

Late infection and maturation stage (96 h). Genes expressed after 96 h reflect the fast cell growth and nuclear division of the parasitoid during this life stage. In total, 7,388 genes, including a wealth of genes related to cell division, reconstruction, and proliferation, are expressed at the 96-h stage (see Data set S1 in the supplemental material). During this life stage, the parasitoid trophont undergoes karyokinesis, and each cell forms its own flagellum (26).

Analogous to P. marinus, Amoebophrya sp. might experience an oxidative burst reaction of the host cell that is counteracted through the expression of antioxidant genes (55). P. marinus uses superoxide dismutases (SOD) to protect itself from reactive oxygen intermediates (ROIs) generated by the host’s oxidative enzymes. SODs were found at the 96-h maturation stage and at the dinospore stage in Amoebophrya. With the rapid growth of the parasitoid in the host cell, the major host defense against pathogens may thus be production of reactive oxygen species (ROS) with a toxic effect on the pathogen by damaging DNA, proteins, and lipids (82, 83).

Conclusion. Our analysis shows that parasitism is at least partly driven by common mechanisms in different eukaryote groups. Differentially expressed genes are associated with different metabolic
pathways at each time point such as those corresponding to proteases, the parasitoid-host cellular surface recognition mechanism, antioxidant defense, energy production such as glycolysis, TCA cycle, and other mitochondrial proteins. Further comparative analyses of the dinoflagellate parasitoid with the closely related Apicomplexa will show whether these similarities have a common evolutionary basis within the Alveolata.

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