ΔΙΙΔΡΥΜΑΤΙΚΟ ΠΡΟΓΡΑΜΜΑ ΜΕΤΑΠΤΥΧΙΑΚΩΝ ΣΠΟΥΔΩΝ

Περιβαλλοντική Βιολογία ENVIRONMENTAL BIOLOGY

Building the genome reference of *Caligus minimus*, an ectoparasite with a growing negative impact in the European sea bass aquaculture

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Abstract

In Greece, *Caligus minimus* infestations in European sea bass (*Dicentrarchus labrax*) have increased, emphasizing the need for targeted research. This study aimed to develop the reference genome for *C. minimus*. Specimens were collected from European sea bass, farmed in Leros with Genomic DNA and RNA extracted at HCMR. Oxford Nanopore MinION technology was used for genomic sequencing at HCMR, whereas the transcriptome short-read sequencing was outsourced (Illumina NovaSeq X Plus Series).

After removing contaminants, the refined reference genome consisted of 314 Mb, with an N50 equal to 1.9 Mb, and L50 to 47 contigs (BUSCO score 94.4%). The predicted genes were 31,343 while 30,490 proteins functionally annotated. *Caligus minimus* was phylogenetically studied and systematically confirmed, and proteins were aligned with related species' proteins involved in antioxidant response, antigenicity and virulence. In the long run, these findings can contribute to effective control strategies against parasitic infestations in aquaculture.

Introduction

Infestation of *Caligus minimus* at the European sea bass

- To date, crustacean parasites threaten the Mediterranean aquaculture leading to severe pathological effects and financial loss. Among them, the ectoparasite *Caligus minimus* (Fig. 1) is one of the major problems in European the sea bass aquaculture (6).
- The presence of *Caligus minimus* in the European sea bass can be detected in the skin and fins, as well as in the buccal and branchial cavities (Fig. 2) (6).
- The infected fishes exhibit distress, sluggish swimming in the surface, extreme mucus production, opercula swelling, gill hemorrhages and necrotic lesions in skin and kidney, increasing susceptibility to bacterial infections and causing osmoregulatory imbalance (14,6).
- Another critical issue is the transmission of these parasites in wild populations as evidenced by infections in brown wrasse (*Labrus merula*) fishes during their stay close to the sea cages for feeding (13).

Preventative methods and treatments of salmon lice

- The management of these parasites includes delousing treatments and preventative methods. Despite all efforts, the control of sea lice disease remains a grave challenge since the lack of methods to effectively combat this concern (7).
- Nowadays, Next Generation Sequencing (NGS) and the potential of *in silico* analysis of genomes facilitate vaccine production being fundamental for sustainable and cost-efficient fish farming. The commercial injectable vaccine for *Caligus rogercresseyi* infestations paves the way for similar efforts to *C. minimus* (1).

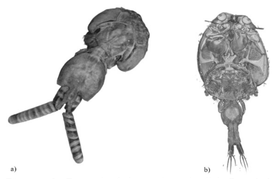

Figure 1. Female (a), and male (b) *C. minimus.* The picture is not scaled. The images originate from the micro-CT of HCMR (kindly offered by P. Katharios and N. Keklikoglou).

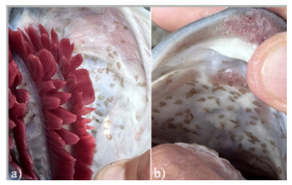

Figure 2. Presence of *Caligus minimus* specimens in the branchial (a) and buccal (b) cavity of sea bass (kindly offered by P. Katharios)

Materials and methods

Laboratory analysis

- Total genomic DNA was extracted using the Monarch HMW DNA Extraction Kit. The quantity and purity were assessed by a Nanodrop ND-1000 spectrophotometer, a Qubit spectrophotometer, and agarose gel electrophoresis.
- RNA was extracted using NucleoZOL and NucleoSpin RNA Set for NucleoZOL. The integrity of the total RNA was evaluated through denaturing agarose gel electrophoresis.

Bioinformatic analysis

- The *de novo* genome was assembled using Flye and evaluated through BUSCO. Contaminants of the reference genome were identified using BlobToolKit.
- The quality of mRNA raw reads was assessed (FastQC) and low-quality sequences were removed (trimmomatic). BRAKER3 was used for genome annotation, combining RNA raw reads and the final assembly.
- The phylogenetic analysis of *C. minimus* was performed using Mega11, while proteins from related species were aligned with proteins of *C. minimus* via BLASTp analysis.

Results

Assembly statistics and contamination

- The 370 Mb *C. minimus* assembly, includes ~12,000 contigs with the longest at 7.6 Mb. The N50 was 1.6 Mb, indicating that half of the reference genome consisted of contigs larger than this size. BUSCO analysis showed 97% integration of arthropod lineage ortholog genes into the assembly.
- Six non-Arthropod taxonomic groups were identified within the genome of *C. minimus* (70 Mb) (fig.3). Among them the phylum Cnidaria, although the contigs initially identified as Cnidaria were misclassified and were retained in the genome after verification (fig.4).

Assembly statistics of the final genome and genome annotation

- The final reference genome is 314 Mb in size consisting of 1,631 contigs, with an N50 of 1.9 Mb and an L50 of 47 contigs, meaning that 50% of the genome is contained within these 47 contigs. GC content was 33% and the BUSCO score 94.4%.
- The analysis revealed 31,343 genes and 35,083 alternative protein-coding transcripts. Functional annotation, based on similarity searches against UniProt, resulted in 30,490 annotated proteins.

Phylogenetic analysis and alignment of C. *minimus* proteins with related species

- The construction of a phylogenetic tree using COI sequences confirmed *C. minimus* identity, with 100% bootstrap support. A second tree with 18S rRNA sequences reinforced its close relation to *C. centrodonti*, *C. lacustris* and *C. curtus*.
- In Table 1, alignments with 16 antioxidants, antigens, or putative virulence factors of *L. salmonis,* and *C. rogercresseyi are depicted.* Fourteen showed >80% coverage being highly conserved, and 10 out of the 16 alignments have more than 70% percentage of identity.

Table 1. Three out of sixteen candidate proteins of *C. minimus* for antioxidants, antigens, and putative virulence factors. Alignment metrics of reverse blast (query coverage, e-value, and percentage of identity) are represented.

Contigs	Candidate proteins	Query coverage	E-value	Percentage of identity	accession number	Proteins	Proteins' description
contig_1321: 648029- 648547	g4770.t1	89%	5e-135	70.57%	ACO10845.1	Glutathione S-transferase kappa 1 (*C. rogercresseyi)*	Antioxidant response to H2O2 and Deltamethrin (3,4)
contig_13390: 298167 -297727	g5335.t1	95%	0.0	87.42%	ACO10357.1	Cathepsin L precursor (*C. rogercresseyi)*	Potential antigen (2)
contig_688: 555182 -56131	g27104.t1	94%	3e-108	62.20%	XP_040563761.1	Trypsin-1-like (L. salmonis)	Putative virulence factor (10)

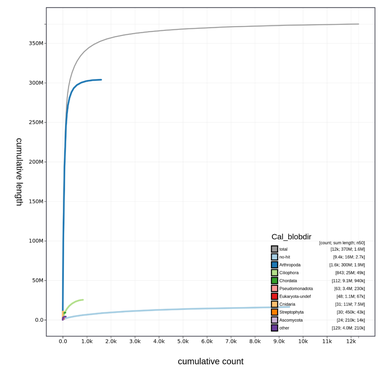

Figure 3. Blobtoolkit cumulative contig plot for the assembly which shows the span of the assembly that is covered by different phyla in comparison with the number of contigs.

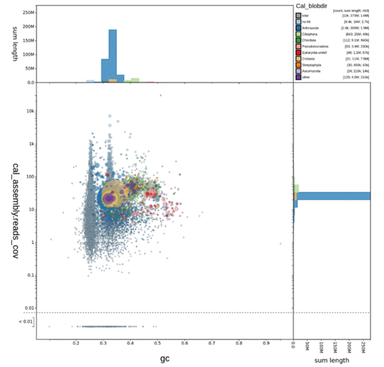

Figure 4. Blobtoolkit blob plot of base coverage in *Caligus minimus* against GC proportion for contigs in the assembly. Every circle represents a contig, and they are colored by phylum.

Discussion

Insights into the sea lice genomes and explaining the presence of contaminants

- The genome size of C. minimus was significantly smaller than that of *C. rogercresseyi (*478.2 Mb), and *L. salmonis (*647 Mb); data are retrieved from NCBI.
- The GC content estimation of *Caligus minimus (33%)* slightly differed from *C. rogercresseyi (33.5%) and L. salmonis (31%)* following these reported in copepods around 30% (9).
- The genomes of *L. salmonis* and *C. rogercresseyi* encompass 14,014 and 19,128 protein-coding genes, respectively (data retrieved from NCBI). Even though the annotated genes of *C. minimus* were 31,343, they were split while transposable elements have not yet been masked.

Explaining the presence of contaminants

- The genome sequences from six different phyla found in *C. minimus* genome can possibly be explained by the parasite's microbiome (Pseudomonadota) (5,8), symbionts of Crustacea (Cilliophora) (11), parasites of copepods (Ascomycota) (12), part of the external enviroment (Streptophyta), genome of host (Chordata) and missclassified contigs (Cnidaria).

Practical implications of aligned proteins with those of related species

- The quantitative PCR (qPCR) technique could provide insights into the expression profile of enzymes of *C. minimus* responsible for drug resistance.
- Development of vaccines through the identification of potential antigens.

Conclusions

The infestation of *Caligus minimus* on European sea bass poses significant challenges for the aquaculture sector in Greece, highlighting the urgent need for effective control strategies. The present study is the first attempt to assemble and annotate the reference genome of this sea louse. Findings can be largely instrumental in developing efficient methods for combating *Caligus minimus*. However, further analyses are required to remove any remaining contamination, increasing purity of the genome while masking transposable elements is essential, as it can impact the annotation accuracy. Additionally, further DNA sequencing is also necessary to improve the contiguity of the genome and avoid gene fragmentation. The current research can pave the way for addressing the sea lice infestation crisis on European sea bass and support the sustainable thriving of aquaculture.

Αναφορές

1. Barrett, L. T., Oppedal, F., Robinson, N., & Dempster, T. (2020). Prevention not cure: a review of methods to avoid sea lice infestations in salmon aquaculture. *Reviews in Aquaculture*, *12*(4), 2527-2543.
2. Casuso, A., Valenzuela-Muñoz, V., Benavente, B. P., Valenzuela-Miranda, D., & Gallardo-Escárate, C. (2022). Exploring sea lice vaccines against early stages of infestation in Atlantic salmon (*Salmo salar*). *Vaccines*, *10*(7), 1063.
3. Chávez-Mardones, J., Asencio, G., Latuz, S., & Gallardo-Escárate, C. (2017). Hydrogen peroxide modulates antioxidant system transcription, evidencing sex-dependent responses in Caligus rogercresseyi. *Aquaculture Research*, *48*(3), 969-978.
4. Chavez-Mardones, J., & Gallardo-Escárate, C. (2014). Deltamethrin (AlphaMax™) reveals modulation of genes related to oxidative stress in the ectoparasite Caligus rogercresseyi: Implications on delousing drug effectiveness. *Aquaculture*, *433*, 421-429.
5. Dalvin, S., Skaftnesmo, K. O., Malde, K., Glover, K. A., Leong, J. S., Koop, B. F., ... & Sandlund, N. (2020). Microbial communities associated with the parasitic copepod Lepeophtheirus salmonis. *Marine Genomics*, *49*, 100688.
6. El-Deen, A. N., Mahmoud, A., & Hassan, A. (2013). Field studies of caligus parasitic infections among cultured seabass (Dicentrarchus labrax) and mullet (Mugil cephalus) in marine fish farms with emphasis on treatment trials. *Glob. Vet*, *11*, 511-520.
7. Guragain, P., Tkachov, M., Båtnes, A. S., Olsen, Y., Winge, P., & Bones, A. M. (2021). Principles and methods of counteracting harmful salmon–arthropod interactions in salmon farming: Addressing possibilities, limitations, and future options. *Frontiers in Marine Science*, *8*, 701793.
8. Gonçalves, A. T., Collipal-Matamal, R., Valenzuela-Muñoz, V., Nuñez-Acuña, G., Valenzuela-Miranda, D., & Gallardo-Escárate, C. (2020). Nanopore sequencing of microbial communities reveals the potential role of sea lice as a reservoir for fish pathogens. *Scientific reports*, *10*(1), 2895.
9. Jørgensen, T. S., Petersen, B., Petersen, H. C. B., Browne, P. D., Prost, S., Stillman, J. H., ... & Hansen, B. W. (2019). The genome and mRNA transcriptome of the cosmopolitan calanoid copepod Acartia tonsa Dana improve the understanding of copepod genome size evolution. *Genome biology and evolution*, *11*(5), 1440-1450.
10. Lewis, D. L., Barker, D. E., & McKinley, R. S. (2014). Modulation of cellular innate immunity by Lepeophtheirus salmonis secretory products. *Fish & shellfish immunology*, *38*(1), 175-183.
11. Savage, R. L., Maud, J. L., Kellogg, C. T., Hunt, B. P., & Tai, V. (2023). Symbiont diversity in the eukaryotic microbiomes of marine crustacean zooplankton. *Journal of Plankton Research*, *45*(2), 338-359.
12. Spatafora, J. W., Aime, M. C., Grigoriev, I. V., Martin, F., Stajich, J. E., & Blackwell, M. (2017). The fungal tree of life: from molecular systematics to genome-scale phylogenies. *The fungal kingdom*, 1-34.
13. Tanrikul, T. T., & Percin, F. (2012). Ectoparasitic Sea lice, Caligus minimus (Otto 1821, copepoda: Caligidae) on brawn wrasse, Labrus merula l., in Izmir bay, Aegean Sea. *Italian Journal of Animal Science*, *11*(2), e38.
14. Tavares-Dias, M., & Oliveira, M. S. (2023). Global distribution patterns of Caligus Müller, 1785 (Copepoda: Caligidae) associated to teleost fishes, with physiological and histopathological data and description of treatment strategies. *Anais da Academia Brasileira de Ciências*, *95*(1), e20220281.

ΤΜΗΜΑ ΒΙΟΛΟΓΙΑΣ
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