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Bioinformatics and functional characterization of taxonomically-restricted genes in Myxozoa

Ph.D. thesis

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Annotation

Evolutionary dynamics, generating novel phenotypic traits, embody an essential route to either lineage-specific adaptations or the creation of endless forms in organisms. Understanding the evolution of such ultimate novelties requires the study of the origin and the role of the functional units in generating unique traits, which represent the taxon-specific genes (TRGs). Myxozoa, the endoparasitic cnidarians, have become a suitable model for the study of evolutionary innovations, with their example of single-celled novelty: polar capsules. This uniquely evolved structure within the spore stage represents a lineage-specific analogy of cnidarian stinging cells (nematocyst). In Cnidaria, nematocysts contain several uniquely emerged genes, rendering the assembly of this complex structure. The prominent fraction represents minicollagens, extremely shortened structural peptides forming the wall and tubule during the nematocyst development. Unlike in Cnidaria, the role of TRGs such as minicollagens in myxozoan development is unknown. The thesis describes the evolution and functional characterization of minicollagens in Myxozoa. Using the bioinformatics and molecular approaches, we explored the diversity and genomic organization and comprehensively characterized minicollagen gene expressions during myxospore development and localization of minicollagens in the polar capsule.

Declaration [in Czech]

Prohlašuji, že jsem autorem této disertační práce a že jsem ji vypracoval pouze s použitím pramenů a literatury uvedených v seznamu použitých zdrojů.

České Budějovice, Date: 29.4.2022

Mgr. Jiří Kyslík

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List of publications and author's contribution

Paper I.: Kyslík, J., Kosakyan, A., Nenarokov, S., Holzer, A.S., Fiala, I. (2021) The myxozoan minicollagen gene repertoire was not simplified by the parasitic lifestyle: computational identification of a novel myxozoan minicollagen gene. BMC Genomics 22: doi.org/10.1186/s12864-021-07515-3. [IF= 3.9]

JK executed the bioinformatics pipelines, performed

data analyses, and drafted the manuscript. (80%)

Paper II.: Kyslík, J., Vancová, M., Bartošová-Sojková, P., Lövy, A., Holzer, A.S., Fiala, I. Expressional profiling and cellular localization of myxozoan minicollagens demonstrate their involvement in polar capsule formation during sporogenesis. Manuscript submitted to International Journal for Parasitology.

JK carried out all experiments, analysed gene expression

and immunostainings, and drafted the manuscript. (80%)

Paper III.: Wiśniewska, M., Alama-Bermejo G., **Kyslík J**., Kolísko, M., Holzer A.S., Kosakyan A. Game of genes: Exploring the major parasitic strategies in myxozoans, case study: *Sphaerospora molnari*. Manuscript in advanced preparation.

JK contributed to taxonomically-restricted genes identification, visualization,

and edited the manuscript. (30%)

Co-authors agreement

The senior and corresponding authors of the manuscripts included in this thesis, hereby confirm that Jiří Kyslík contributed significantly to these publications, according to the statement above:

RNDr. Ivan Fiala, Ph.D.

Anush Kosakyan, Ph.D.

Table of content

1. Introduction to Myxozoa

1.1. Overview of Myxozoa and main characteristics

Myxozoa Grassé, 1970, a group of endoparasites found almost exclusively in the aquatic (freshwater and marine) environment, represents a lineage of considerably diversified cnidarians (Cnidaria Hatschek, 1888). The ultimate parasitic life strategy, complex life cycles, and body plan reduction of diploblastic Myxozoa underscore how the seemingly simple nature of an organism can be complex. With the growing number of described species, currently counting >2400, Myxozoa represent around 20% of cnidarian biodiversity (Zhang 2011). Myxozoans alter between vertebrate and invertebrate hosts in their life cycle. Despite fish being their predominant hosts, the incidence of myxozoan infections has also been reported in other lineages across the vertebrates (amphibians, reptiles, birds, and mammals) (Eiras, 2005; Jirků et al. 2006; Prunescu et al. 2007; Dyková et al. 2007; Bartholomew et al. 2008; Dyková et al. 2011). Nevertheless, myxozoans also exhibit an evolutionary transition to hyperparasitism, exploiting ecto-and endo-parasitic fauna (Freeman & Shinn, 2011; Overstreet, 1976; Siau et al. 1981). Bryozoans and the freshwater and marine annelids are the definitive hosts during their complex life cycle (Annelida; Lamarck, 1809).

This subphylum of obligatory endoparasitic cnidarians is known to cause severe diseases in the aquaculture industry with global economic impact. The global losses to parasitism in the hatchery, including myxozoan infections, have been tentatively estimated at $1–5 billion (Shinn et al. 2015). Notably, they can cause several well-known fish diseases affecting fish populations in the aquatic industry (Álvarez-Pellitero et al. 1993; Kent et al. 1994; Nehring & Walker, 1996; Moran et al. 1999; Pote et al. 2000; Palenzuela et al. 2002; MacKenzie et al. 2005). However, the pathogenicity of myxozoans is not a common attribute of all described species (Holzer et al. 2021).

The intriguing aspects, including extreme body reduction, adaptation to parasitic life strategy, and complicated taxonomic classification history, were already the subject of interest from the beginning of the 19th century. The mutual affinity of the currently considered cnidarian group Myxozoa to their free-living relatives has gone through many counterintuitive explanations (Gurley, 1890; Weill, 1934; Shulman, 1966; Levine, 1969; Grasse´ & Lavette, 1978; Kent et al. 1994). Due to considerable loss of metazoan apomorphies and extreme reduction

of the body plan, the classification of Myxozoa, with respect to the morphological features, underwent difficulties until the application of advent molecular techniques. There are two formally accepted clades, Myxosporea and Malacosporea, in Myxozoa. The former is the group that underwent radiation and represents many of myxozoan described species. Malacosporea, the previously unrecognized myxozoans (Canning et al. 2000), parasitizing freshwater bryozoans joined Myxozoa after the unanticipated rediscovery of worm shape life form of *Buddenbrockia plumatellae* (Monteiro et al. 2002; Jimenéz-Guri et al. 2007). Their concealed diversity and basal evolutionary placement within Myxozoa have been further ascertained (Bartošová-Sojková et al. 2014; Hartikainen et al. 2014; Naldoni et al. 2019).

The incongruences between traditional spore morphotype-based and molecular classification of Myxozoa (Myxosporea and Malacosporea classes) resulted in the convergence regarding morphological plasticity of myxozoan myxospores (Fiala & Bartošová 2010; Fiala et al. 2015). Currently, the phylogeny of Myxozoa comprises four lineages, according to multiple evolutionary routes, including host environment, tissue tropism, definitive hosts, etc. (reviewed in Fiala et al. 2015). Contemporary with recent advances in genomics and transcriptomics, new venues for studying the Myxozoa were opened and revealed countless surprising findings of this mysterious group of multicellular parasites, and there are not many lefts.

1.2. History, evolution, and origin of Myxozoa

Since their discovery more than 190 years ago (Jurine 1825), Myxozoa have gone through quite a long history of research. Early classification of Myxozoa was associated with various protistan taxa. Throughout this prolonged period of protistan reassignment, Myxozoa were classified together with Microspora, currently called Microsporidia Balbiani, 1882, into a common subclass Cnidospora of the group Sporozoa Leuckart, 1879. Later, the profound biological differences between these two parasitic groups elevated them into their own phyla (Levine, 1969). However, an inability of linking them to any other protozoan lineage, the protozoan origin of Myxozoa was questioned. Grassé & Lavette (1978) recognized specific ultrastructural features (terminal intrasomatic differentiation and desmosome-like structures) within myxozoan spores, suggesting their possible metazoan origin. This fact also

supported the previous remarks (Štolc, 1899; Emery 1909; Ikeda 1912). However, these observations were commonly opposed in the context of the similar ameboid appearance of plasmodia to Amobeozoa (Shulman, 1966) or uncertain reason for metazoan relation of Myxozoa upon considerable lack of multicellular features (Cavalier-Smith 1993). Therefore, other records (Lom and de Puytorac, 1965; Lom, 1969; Grassé and Lavette, 1978) advised the hypothesis of the cnidarian origin of Myxozoa. Later, this observation led to the inner reclassification culminated in the demise declaration of the protistan origin of Myxozoa (Kent et al. 1994).

Emerging modern molecular technologies have driven the uncertain and controversial classification of Myxozoa, and the demise of the phylum Myxozoa followed onwards. When the first molecular confirmation of the relationship of Myxozoa to metazoans was demonstrated, an artificial bilaterian relationship was found (Smothers et al. 1994). The enrichment of the analyses by morphological and ontogenetic data proposed the placement of Myxozoa to class Narcomedusae; Haeckel, 1879 (Siddal et al. 1995). This long-study puzzle in molecular classification of Myxozoa has been discussed in terms of divergent fast-evolving genes and their incorrect inference caused by the issue of long-branch attraction artifact (Hanelt et al. 1996; Pawlowski et al. 1996; Schlegel et al. 1996; Winnipenninckx et al. 1998; Kim et al. 1999; Zrzavý & Hypša, 2003). Hence the recent phylogenomic studies partially resolved the negative compositional bias and heterogeneity hampering the phylogeny of Myxozoa and supported previous morphological suggestions of cnidarian affinity of Myxozoa (Okamura et al. 2002; Jimenéz-Guri et al. 2007; Nesnidal et al. 2013). The growing compelling molecular evidence using omics strategies by genome and transcriptome sequencing consequently supported the cnidarian origin of Myxozoa (Nesnidal et al. 2013; Feng et al. 2015; Chang et al. 2015; Yahalomi et al. 2020).

The examination of the history of Myxozoa has also been demonstrated by understanding their common evolutionary history with the hosts. Prior to acquiring a complex life cycle, myxozoans congruently evolved with origin of invertebrates (Holzer et al. 2018). The origin of complex life cycle has been then hypothesized by “feed-integration” of invertebrates (Lisnerová et al. 2020). Further radiation of Myxozoa in the fish groups has been accompanied by diversification of fish hosts (Holzer et al. 2018; Lisnerová et al. 2020). The complex life histories with the hosts represent the driving force of successful myxozoan diversification and

vast biodiversity (Holzer et al. 2018). Moreover, the cryogenic origin of Myxozoa (~651 Ma) premised on molecular dating has been determined (Holzer et al. 2018). Despite the sister relationship between Myxozoa and *Polypodium hydriforme*, Endocnidozoa (Zrzavý & Hypša 2003), the origin and acquisition of fish host of *P. hydriforme* represents rather an independent lineage that transited to endoparasitism (Holzer et al. 2018).

1.3. Myxozoan life cycle and cellular development

The discovery of the myxozoan heteroxenous life cycle (Fig. 1) alternating between two hosts revolutionized and reconsidered the myxozoan taxonomy (Wolf & Markiw, 1984). Myxospore represents the life stage that forms within the vertebrate host, mainly in fish. The prominent appearance of myxospores comprises the spore valves enclosing the infectious agent, sporoplasm, and apically positioned omnipresent organelles with eversible tubule, polar capsules. The numbering of shell valves, polar capsules, and the structural features of the surface of myxospore represent a key taxonomic feature for genus/species description (Lom & Dyková, 2006).

The initial invasion steps of myxosporean infection start with the waterborne actinospore stages, released from the invertebrate hosts, attaching to the vertebrate hosts via polar capsule discharge strategy. Once the infectious agent of the spore, multicellular amoeboid sporoplasm, is expelled, it penetrates the host tissue (El-Matbouli et al. 1999). Sporoplasm represents a primary stage that lacks secondary cells, containing structures with unknown function – so called sporoplasmosomes (Lom & Dyková, 1997; Hedrick et al. 2004). The process of cell proliferation in further development of proliferative stages is accompanied by endogenous budding via engulfment of one cell by the other (Lom & Dyková, 2006). The sporoplasm enters intra/intercellularly the host tissue and transforms into vegetative trophic stage spore-producing pseudoplasmodium or plasmodium (Bjork & Bartholomew 2010; Ohnishi et al. 2013). These stages are mono/di/poly-sporic and produce either one, two or many spores (Kent et al. 2000, Feist et al. 2015).

Prior to sporogony, when myxospores are being produced, the myxozoan stages undergo the presporogony development comprising the schizogony of cells from the sporoplasm and subsequent development of plasmodial stages (El-Matbouli & Hoffman, 1998; Morris &

Freeman, 2010). However, only for a few species, a detailed description of presporogonic development is available (Yokoyama et al. 1990; El-Matbouli et al. 1995; El-Matbouli & Hoffman, 1998; Canning et al. 2002; Morris & Adams 2007). During the sporogony, multiple cellular divisions occur terminated by pansporoblast formation.

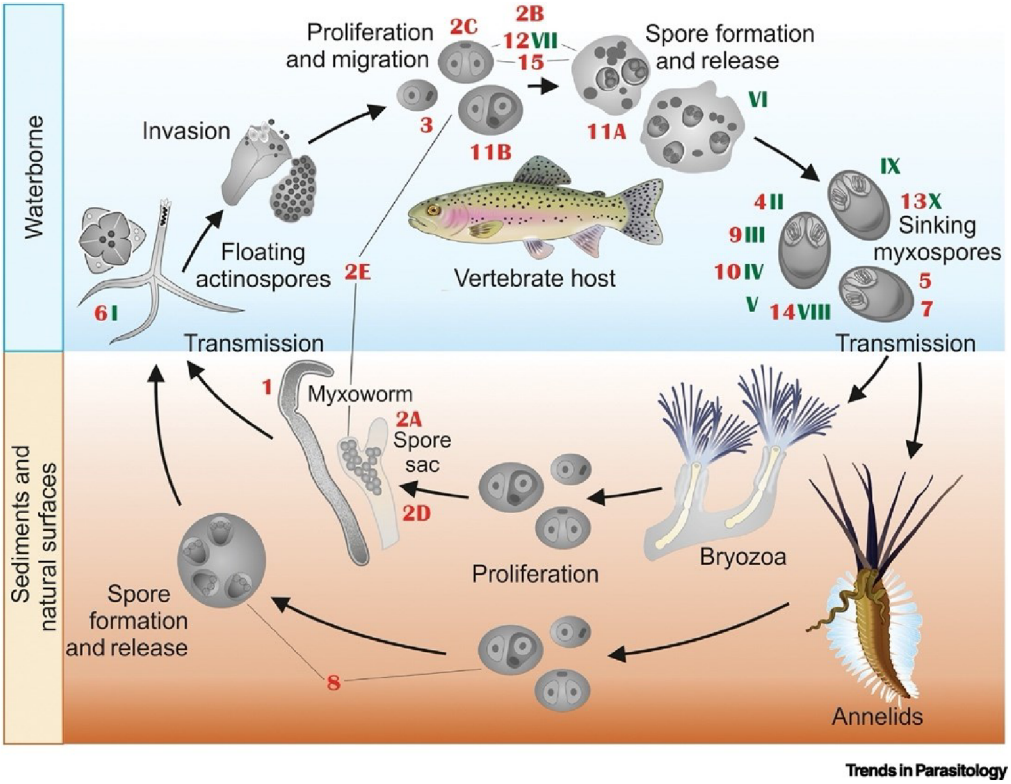

Figure 1: Schematic drawing of myxozoan life cycle. Letters refer to different datasets of the same species. Lines to different parasite stages indicate comparative studies. Transcriptomic and genomic datasets of the same study are listed together (e.g., 4 II, 9 III, etc.). 2B is unspecified with regard to parasite stages but the sample was taken 140 days postexposure of the fish host. Reprinted with permission of the authors (Alama-Bermejo & Holzer, 2021).

The manner of sporogony differs by the occurrence of the pansporoblast, typifying the plasmodia, whereas this structure is absent in pseudoplasmodia (Feist et al. 2015). In general, plasmodia contain single or multiple nuclei of generative cells. Although in some species, single spores are produced in one plasmodium (pseudoplasmodium), whereas other myxozoan species generate numerous spores from a single polysporic pseudoplasmodium (Lom & Dyková 2006).

Once plasmodium enters the sporogony phase of development, the endogeny of generative cells appears. This process gives rise to either pansporoblast (pericyte with sporogonic cells) or sporoblast (solely sporogonic cells). Within the (pan)sporoblast, the cellular precursors generating the individual myxospore compartments are created. Among these, capsulogenic (polar capsule), valvogenic (spore valves), and sporoplasmogenic cells (sporoplasm) are contemporaneously developed via intensive cell division and endogeny processes (Feist et al. 2015). The cellular processes associated with sporogonic development, including the presence of pansporoblast or maturation of sporoplasm, alter in terms of the phase of the life cycle or taxon (Feist et al. 2015; Hulbert et al. 1977; Desser et al. 1983; Feist, 1995; Casal et al. 2002). In capsulogenic cells, a capsular primordium of the capsule body and the polar filament are formed. These cells are typical of a substantial amount of endoplasmic reticulum surrounding the primordium structure (Feist et al. 2015). Within developing pan/sporoblast, another specific cell type, valvogenic cells, continuously lose the cellular compartments, are present. The valvogenic cells create the spore envelope (valve), which shape represents a characteristic morphological feature for the classification of myxospore species. The last prominent part, sporoplasmogenic cell, which typifies the precursor producing transmissive myxospore compartment, sporoplasm, consists of structures with unknown content, sporoplasmosomes (Lom et al. 1989).

Since the myxospore is formed and released from the vertebrate host, the infection heads on the invertebrate host. Actinospores typically possess a wide range of morphotypes distinct from myxospores (Fig. 2). The typical morphotype of actinospore is triradiate symmetry, numerous sporoplasms, and three polar capsules (Fig. 2) (Lom & Dyková 2006). Besides the similar fashion of formation, the development of the actinospore possesses some differences contrary to myxospore. Although detailed ultrastructural studies in myxospore formation have been published (Hulbert et al. 1977; Desser et al. 1983; Feist, 1995; Casal et al. 2002), the development of actinospore, utilized within definitive invertebrate hosts by meiosis, is limited to a few species (Hallet & Lester, 1999; Morris et al. 2010, 2012; Morris & Freeman, 2010; Rangel et al. 2012). The main discrepancy in the actinosporean phase of the life cycle compared to myxosporean phase is the presence of meiotically created haploid germ cells surrounded by other cells from which the sporoblasts are formed. In contrast to myxospore, actinosporean sporogony occurs in pansporocysts containing sporoblasts, where via fusion

and open mitosis, sporoplasms and valvocapsulogenic cells develop. Towards the end of actinospore maturation, valvocapsulogenic cells divide, and likewise, myxospore valvogenic and capsulogenic cells create the valves and polar capsules.

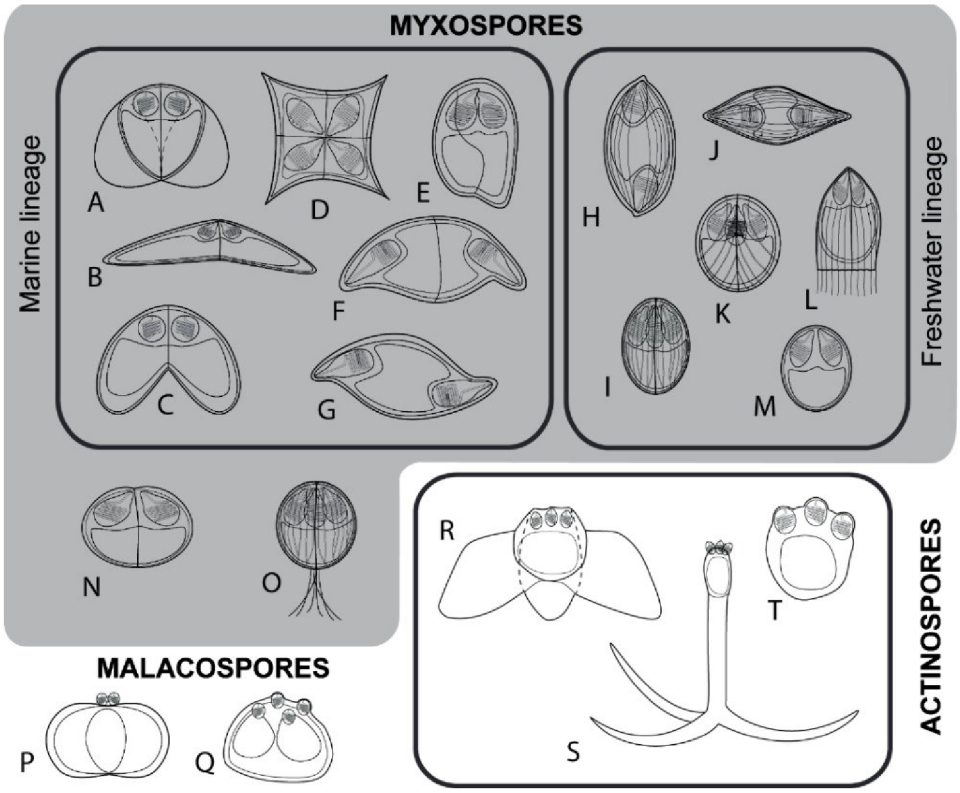

Figure 2: Morphotypes of myxozoan spores. (A) *Bipteria*, (B) *Ceratomyxa*, (C) *Ceratonova*, (D) *Kudoa*, (E) *Parvicapsula*, (F) *Enteromyxum*, (G) marine *Myxidium*, (H) freshwater *Myxidium*, (I) freshwater *Chloromyxum*, (J) *Myxidium lieberkuehni*, (K) *Chloromyxum careni*, (L) *Hoferellus*, (M) *Myxobolus*, (N) *Sphaerospora*, (O) marine *Chloromyxum*. (P-Q) Malacosporean morphotypes, (P) fishmalacospore of *Tetracapsuloides*, (Q) malacospore of *Tetracapsuloides* from bryozoan host (R-T) Common actinospore morphotypes, (R) aurantiactinomyxon, (S) triactinomyxon, (T) tetractinomyxon. Reprinted with the persmission of the author (Kodádková, 2014).

Although spore formation in Malacosporea undergoes a similar fashion, there are some cellular differences of development in either bryozoan or fish hosts. The life cycle of some malacosporean myxozoans comprises some specific life stages created within bryozoan host like the worm-like stage with muscle cells (myocytes) and sac stage in *Buddenbrockia plumatellae* (Canning et al. 2002). Similarly, another species, *Tetracapsuloides bryosalmonae*, also possesses sac stages (Hedrick et al. 2004; McGurk et al. 2005; Morris & Adams 2008). Fish

malacorospores are formed inside pseudoplasmodium, which undergoes a similar process of sporogony as for myxospores (Kent & Hedrick 1986; Clifton-Hadley & Feist 1989; Morris & Adams 2008). However, the exact cellular process of cell development remains unexplored.

1.4. Myxozoan polar capsules

The mysterious relatedness of 'micro-jellyfish' parasites, Myxozoa, to Cnidaria has been accompanied historically by a resemblance of their prominent structures to nematocyst with coiled filament, a polar capsule. Over the frequently noted parallels and discharge properties between polar capsules and cnidarian stinging cells (nematocysts) (Weill, 1934; Lom & de Puytorac, 1965; Lom, 1969), such descriptions were therefore consistently dismissed (Shulman 1966; Levine 1969; Cavalier-Smith, 1993). Remarkably, the final compelling evidence, refuting previous doubts about the cnidarian origin of Myxozoa, was the discovery of lineage-specific genes restricted to Cnidaria (Holland et al. 2011). However, a recent proposal amended the terminology of the polar capsule as nematocyst regarding their commonalities (Americus et al. 2020).

Given the conspicuousness, facilitating the diagnostics and taxonomy of myxozoans, polar capsules possess accordant structures with the bilayered wall enclosing the coiled, membrane-bound tubule, polar filament like cnidarian nematocysts. Unlike their free-living relatives, nematocysts, polar capsules lack some nematocyst features, including spines (Okamura et al. 2015). Despite the typical weaponry-like fashion of discharge of polar capsules and nematocysts, the principle of myxozoan polar capsules is to attach to the host. Myxozoan species possess a various number of polar capsules within their spores (Lom & Dyková, 2006). While the tremendous morphological plasticity and complexity of nematocysts in Cnidaria, up to 30 types (Weill, 1934; Mariscal, 1974; Fautin, 2009), one morphotype of the polar capsule is present in Myxozoa (Cannon & Wagner, 2003). Either simplification or adaptation might reflect this fact during the evolution of their parasitic life strategy. Nevertheless, some hidden morphology is questionable (Okamura et al. 2015).

Cell type diversification represents one of the key mechanisms required to emerge novel phenotypic traits and cell function (Erwin et al. 2009). The cell fate of cnidarian nematocysts begins at multipotent interstitial cells that also arise neuronal cells, gland cells, etc. (Tardent,

1995). Recently the mechanism by which neuronal phenotype is suppressed and nematocysts are developed has been revealed (Babonis et al. 2021). Yet, in Myxozoa, none of these regulatory mechanisms is known. In contrast to free-living cnidarians, Myxozoa probably possesses retention of the nervous system that seems primitive (Guo et al. 2022a).

The astonishing uniqueness of polar capsules and nematocysts represent their kinetics of discharge mechanism, one of the fastest across the animal kingdom (Holstein & Tardent, 1984; Nuchter et al. 2006; Karabulut et al. 2021). Nematocysts and myxozoan polar capsules possess a similar discharge mechanism. Nevertheless, the dynamics of polar capsules revealed contraction of the tubule while everting, which is absent in their free-living relatives (Ben-David et al. 2016). Nematocysts of free-living cnidarians perforate the tissue of their prey and inject the toxins only by using the tip of the tubule (Lotan et al. 1995; Tardent, 1995). In contrast, the injection apparatus, releasing the content via side lateral apertures, seems unique in Myxozoa. These specific characteristics may suggest the adaptation strategy of myxozoans during the immanent evolution from their free-living counterparts (Ben-David et al. 2016).

Even though both organelles possess similar morphological hallmarks, their molecular content is distinct. Throughout the extensive study of the proteomic content of nematocysts in Cnidaria (Balasubramanian et al. 2012; Brinkman et al. 2012; Moran et al. 2013; Weston et al. 2013; Li et al. 2014; 2016; Ponce et al. 2016), comparison of the protein profiles between representatives of the main cnidarian lineages revealed a dynamically evolving cocktail of proteins (Rachamim et al. 2015). Contrary to nematocysts, myxozoan polar capsules reflect their evolutionary modification during adaptation to distinct function with loss and partial retention of the molecular content (Piriatinskiy et al. 2017). Intriguing insight revealed the recent observation of evolutionary pattern bearing the driving force of adaptation evolution of nematocysts (Guo et al. 2022b). Notably, mutations in nematocyst proteins, as well as an increase in the evolutionary rate of adaptation of these phenotypic novelties, are crucial to encouraging rapid diversification and successful adaptation of nematocysts and polar capsules (Guo et al. 2022b).

2. Next-generation omics in Myxozoa

2.1. History and advances in myxozoan omics

The era of next-generation technologies made splendid strides in the last sixteen years. Currently, the attainment of genetic information is a widespread method used for broad research directions. Encouraged by this fact, the field of research on the parasitic group of Myxozoa has joined this lately. Initially, data on myxozoans were obtained in two pilot studies based on expressed sequence tags (ESTs) of cloned cDNA libraries (Jimenéz-Guri et al. 2007; Holland et al. 2011). Later on, the partial genome of the myxozoan stalwart *Myxobolus cerebralis* was gained (Nesnidal et al. 2013). Therefore, the quantitative and qualitative ambitiousness of obtained next-gen data prompted the need for other good quality omics myxozoan data.

The first genome of *Thelohanellus kitauei* was obtained, endeavoring to describe the general biology and metabolic processes of the Myxozoa with their connection to the fish host (Yang et al. 2014). Soon after, a comprehensive study of three annotated myxozoan genomes (*Kudoa iwatai* and *M. cerebralis*) and obligate parasitic cnidarian *Polypodium hydriforme* enhanced the understanding of myxozoan transition to parasitism by revealing the genome reduction and gene depletion manifestations (Chang et al. 2015). The recent groundbreaking discovery provided sequencing of *Henneguya salminicola* and *Myxoxobolus squamalis* genomes, most notably by the surprising absence of mitochondrial genome in the former (Yahalomi et al. 2020). Interestingly, research of epigenetic mechanisms in the genomes of distantly related myxozoans *Ceratonova shasta* and *H. salminicola* showed the complete disappearance of DNA methylation (Kyger et al. 2020). Recent insight into the sophistication of the myxozoan genome uncovered sequencing of *Myxobolus honghuensis* (Guo et al. 2022a) with emphasis on the evolution of myxozoan genomes by the combination of expansion and streamlining theory of beneficial aspect of the smaller size of the genome for reproduction also known for bacterial genomes (Giovannoni et al. 2014).

Sequencing of the mitochondrial genomes showed another remarkable perspective in Myxozoa. The pioneering study of sequencing of three *Kudoa* species (*Kudoa septempunctata*, *K. hexapunctata*, and *K. iwatai*) pointed to poorly encoded mitochondrial genomes, including the fastest evolutionary rate of the genes contained (Takeuchi et al. 2015). A similar pattern

possessed *Enteromyxum leei* with eight circular chromosomes, the largest known across the animal kingdom, and reassessment of *K*. *iwatai* genomes showed some plasticity and partially revealed the evolutionary placement of Myxozoa within Cnidaria (Yahalomi et al. 2017). Surprisingly, the complete loss of mitochondrial genome revealed genome sequencing of *H. salminicola*, indicating the absence of essential components for aerobic respiration towards adaptation of parasitic Myxozoa (Yahalomi et al. 2020). However, the evolution of such genomes within Myxozoa remains uncharted.

In parallel with the genome sequencing of Myxozoa, there was the prevailing expansion of transcriptomic datasets generation. While some RNA-sequencing (RNA-seq) data represent an auxiliary source within genome sequencing (see above), many intriguing facts were retrieved solely based on assembled transcriptomes. Importantly, the identification of candidate protease-encoding proteins for vaccine development (Hartigan et al. 2020; Faber et al. 2021) or differences between gene expressions of the parasite in both in/vertebrate hosts (Faber et al. 2021) highlights some outcomes revealed by transcriptomes of myxozoans. Yet, 22 species of a wide spectrum of myxozoans have been sequenced, whereas 16 represent available assemblies. Beyond the first generation of transcriptomes, evolutionary background, and diversity of taxon-specific genes, unifying Cnidaria (see Chapter 3.2.), were assessed (Shpirer et al. 2014; Foox et al. 2015). A majority of conducted RNA-seq of Myxozoa has prompted the exciting description of either host-parasite interactions or immunological response to the fish host, different levels of virulence in comparison with the host-genotype, etc. (Robledo et al. 2014; Ronza et al. 2016; Zhao et al. 2020; Picard-Sánchez et al. 2020; Hartigan et al. 2020; Alama-Bermejo et al. 2020; Kumar et al. 2020, 2022; Faber et al. 2021).

The upscaling technology of myxozoan research represents eDNA metabarcoding used for tracking biodiversity in ecosystems (reviewed in Ruppert et al. 2019). This highly applicable method, implemented mainly for studying microbial communities, has also recently expanded to multicellular organisms (Port et al. 2016; Deiner et al. 2017; Djurhuus et al. 2018, 2020). So far, such technology has been used only in a few studies regarding aspects of methodological assessment or species distribution of Myxozoa (Hartikainen et al. 2016; Richey et al. 2020).

2.2. Pitfalls of Myxozoa sequencing

Starting with the application of High-Throughput Sequencing (HTS) strategies, notable limitations in myxozoan datasets creation were observed. The challenging issue of host contamination presents the major obstacle in sequencing outputs of myxozoans in relation to their life cycle and host-parasite interactions. The filtering strategies of primary assemblies of myxozoan data for host subtraction with GenBank datasets of their fish hosts were applied. Notably, the first in silico pipeline underscoring transcripts assignment and host decoupling enabled transcriptome generation and detected <30% of contamination (Foox et al. 2015). Aggravation of problems in myxozoan HTS assemblies was caused by chimerical reads of host and parasite disabling the correct parasite assembly and host continuous presence in these data (Yang et al. 2014). In fact, the reduction of such bias was eliminated by the application of pre-assembly filtering methods (Alama-Bermejo et al. 2020; Hartigan et al. 2020; Yahalomi et al. 2020).

Despite the general absence of in vitro cultures and cell lines of myxozoans, a novel approach has been assessed to clean up the parasite of the host presence and thus alleviate the quality of further sequenced HTS data (Born-Torrijos et al. 2022). Regarding the high genome reduction and fast-evolving genes of Myxozoa (Chang et al. 2015), the gene predictions mostly generate inaccuracies of the peptide sequences falling under *ab initio* or empirical prediction models. Yet, no genome to date possesses a chromosome-level assembly regarding the questioned diploidy during the life cycle in Myxozoa (Okamura et al. 2015).

2.3. Characteristics of myxozoan HTS-data

Continuous sequencing of myxozoan genomes elucidated parallel reduction toward general body plan simplification in evolutionarily modified myxozoans like other parasitic organisms (Chang et al. 2015; Jackson et al. 2015). Despite genome size variation in Myxozoa (22,5 to 260 Mb), myxozoans possess the smallest genome in the whole animal kingdom (Chang et al. 2015). The smallest myxozoan genome known to date (22.5 Mb) was estimated for *Kudoa iwatai*, which is almost 25-fold smaller than the size of the closest myxozoan relative *Polypodium hydriforme* with its 561 Mb genome displaying a higher number of genes than those in Myxozoa (Chang et al. 2015). In contrast to free-living cnidarian species, such a

genome represents only 10% of the size (Alama-Bermejo & Holzer, 2021). Intriguingly, a supposedly largest myxozoan genome of *Myxobolus wulli* (260 Mb) compared to *M. honghuensis* has been recently published (Guo et al. 2022b). In addition to the evolutionary constraints of myxozoans in transitioning to a parasitic life strategy, their genomes show apparent evolvability in reducing the size and GC content. However, mosaicism in evolution illustrates the recently obtained genome of *Myxobolus honghuensis* (Guo et al. 2022a). Thus, myxozoans might possess hidden complexity and genome plasticity for their successful evolution as parasites. Along with genome reduction, the biology of Myxozoa supports the general standpoint, describing Myxozoa as an example of highly degenerate organisms. Bioinformatic analyses, comparing gene ontology and the number of expressed genes to the cnidarian model species, revealed either depletion or simplification of evolutionarily conserved biological pathways (Chang et al. 2015). In the context of body plan formation, the pivotal signaling pathways (Hox-like, Runx, Wnt pathway, Hedgehog pathway) appears to be absent from the myxozoan genomes (Chang et al. 2015). However, some ancestrally speciated orthologs of both Wnt and Hox-like pathways were identified in malacosporean *Tetracapsuloides bryosalmonae* (Faber et al. 2021). Henceforward, these observations point to the uniqueness of these parasites and continuously open new venues in myxozoan research.

3. Taxon-specific genes in Myxozoa

3.1. Evolution and characterization of TRG genes

By looking at the tree of life, the biology systems evolved in tremendous diversity and uniqueness, making organisms to carry distinctive phenotypes, but what makes this nature of specific traits? The generation of phenotypic novelties in organisms requires deep consideration of evolutionary aspects on the genome level with respect to genes. There are two concepts, including regulatory elements and gene duplication, that are generally considered to underlie the emergence of diverse traits (Ohno, 1970; Prud'homme, 2007; Shubin et al. 2009). However, another essential mechanism shaping the phylogenetic uniqueness and species-specific properties of organisms are genes lacking homology in other organisms – so-called taxonomically-restricted genes (TRGs).

Since the first genomes were sequenced, the presence of such ''orphan'' genes was conceptually discussed (Casari et al. 1996; Fisher & Eisenberg, 1999). Essentially, any extant genome possesses up to 20 % TRGs (Khalturin et al. 2009). Besides the abundance of these lineage-specific genes, the molecular processes triggering the evolution of TRGs are poorly understood.

A plausible scenario of control and acquisition of evolutionary novelty is determined by the evolution of genes and their roles in promiscuity in the genome (Canici, 2010). The emergence of new genes is associated with many events, mostly either gene duplication or transposition mechanisms (Singh & Wurtele, 2020). In a manner akin to this, arising TRGs would undergo a gene duplication followed by gene diversification, or *de novo* origination from non-coding genomic regions can be considered (reviewed in Tautz & Domazet-Lošo, 2011 and Schlötterer, 2015). Nevertheless, the nature of limitations, including these mechanisms, makes understanding the evolution of TRGs more complicated. In the process of gene duplication-diversification, a single gene undergoes from duplication event mediated either by recombination or transposition to diversification under fast adaptive evolution, resulting in high dissimilarity toward duplicated ancestor (Lynch & Kajtu, 2004; Conant et al. 2008). In the context of TRGs, the process of sequestration of these genes to be duplicated and preserving their ancestral copy is so far unexplored. Moreover, a substantial number of lineage-specific genes encode functional domains, hardly accumulating the mutations (Albà & Castresana, 2007). However, a modified model of the duplication-diversification process, including the involvement of the insertion of transposable elements (e.g., retro/transposons), and a process of gene rearrangement through recombination that ultimately supports adaptive evolution, has been discussed (Long et al. 2003; Zhou et al. 2008; Kaessmann, 2010).

Another possible explanation for the emergence of TRGs is given by *de novo* origination from non-coding, ''junk DNA'' which were considered unlikely in classical genetics in the first place. This enigmatic birth of genes consists of the formation of the functional open reading frame from random RNA regulatory sites with the further creation of RNA transcript (Cai et al. 2008; Heinen et al. 2009). Therefore, this approach is currently being accepted and applied by recent reports of *de novo* emergence using the example of antifreeze proteins in fish during evolution (Baalsrud et al. 2018; Levy, 2019).

Overall, the plasticity and evolutionary dynamics of orphan genes have been studied in various organisms, including in/vertebrates and prokaryotes (Long et al. 2003; Levine et al. 2006; Wilson et al. 2007; Zhou et al. 2008; Toll-Riera et al. 2009; Ekman et al. 2010; Wissler et al. 2013). Intriguingly, the function of TRGs stems from the findings regarding the origin of TRGs upon specific adaptation of the organism to the environment (Colbourne et al. 2011; Donoghue et al. 2011; Voolstra et al. 2011) or new morphological traits (Khalturin et al. 2008; Milde et al. 2009).

With advances in comparative genomics (Werner et al. 2018; Zhang et al. 2019) and deep transcriptomics (Blevins et al. 2021), more comprehensive results have been identified regarding the epigenetic signature and evolutionary mechanism of positive selection of TRGs.

Nonetheless, neither the pathways for determining the conserved nor the lineage-specific features are known in the level of importance – hence ultimately, the significance of either conserved genes or TRGs in triggering the phenotypic innovations is questioned (Johnson, 2018).

3.2. Diversity and evolution of TRGs in Cnidaria

Among the evolutionary basal lineages, the ancient phylum Cnidaria including hydroids, jellyfish, sea anemones, and corals, became a valuable model for empirical research of TRGs in evolutionary innovations. Cnidaria possess an example of a common unique structure, nematocyst, utilized for defense, prey capture, and locomotion (David et al. 2008). Unlike the contentious origin of nematocysts, Cnidaria underwent extreme radiation in both body plan and the morphology of stinging cells, resulting in ~30 types (David et al. 2008). In addition, the structural and genetic setup of the nematocyst's sophisticated structure was subjected to detailed understanding. The essential model represents *Hydra* with a relatively simple body plan and numerous nematocyst types (David et al. 2008).

Using the expression profiles of control and interstitial cells (i-cell)-free *Hydra's*, several nematocyte-specific genes that lack the homology in other taxa were identified (Hwang et al. 2007). Similarly, a transcriptomic comparison of three *Hydra* species revealed the contribution of TRGs to tentacle formation (Khalturin et al. 2008). Using subtracted cDNA libraries of control and mutant i-cell-free *Hydra's* showed a considerable repertoire of TRGs restricted to

developing nematocytes. Moreover, the transgenic line of *Hydra* under the control of the selected TRG gene locus indicated a spatiotemporal pattern of expression within the different tissues. (Milde et al. 2009). Henceforward, studies on taxon-specific genes continuously supported the role of TRGs in adaptation to the environment (Fraune et al. 2010; Franzenburg et al. 2013; Baumgarten et al. 2015) and developmental processes in Cnidaria (Engel et al. 2001, 2002; Hellstern et al. 2006; Adamczyk et al. 2008; Denker et al. 2008; Forêt et al. 2010; Franzenburg et al. 2013; Hwang et al. 2010 Babonis et al. 2016).

Recent studies of cnidarian genomes provided an overview of phylum-specific traits in Cnidaria. Genome sequencing of jellyfish *Aurelia* life stages did not identify the role of TRGs in the origin of novel life forms, e.g., *medusa* stage, but rather the redeployment of conserved genes (Gold et al. 2019). Therefore, a remarkable function of TRGs with spatiotemporal expression has been supported (Sunagar et al. 2018; Khalturin et al. 2019). Hence, Cnidaria bring a step forward in knowledge of taxon-specific genes in early basal multicellular lineages.

A detailed outline of the genetic toolbox generating the taxon-specific novelty in Cnidaria is exemplified by nematocyst-specific genes. The molecular nature of cnidarian nematocysts revealed several unique structural proteins involved in the assembly of subcellular structures of stinging cells. Besides the minicollagens (see Chapter 3.3.), the glycoprotein (NOWA) was found to be involved in nematocyst capsule outer wall formation (Engel et al. 2002) as well as other proteins found exclusively in tubule spines (Koch et al. 1998; Hellstern et al. 2006) or nematogalectins in nematocyst tubule (Hwang et al. 2010; Adamczyk et al. 2010).

3.3. Minicollagens, orphans of Cnidaria

Despite the vast diversity of morphotypes and dynamic protein content of cnidarian nematocysts (David et al. 2008; Rachamim et al. 2015), a group of essential phylum-specific genes unifying Cnidaria was identified. Among these, a family of proteins with triple collagen helices, minicollagens, represents the most prominent part. Since the first discovery of these unusually short collagen proteins in nematocysts (Lenhoff et al. 1957; Blanquet & Lenhoff, 1966; Kurz et al. 1991), their significant role in the diversity and evolution of nematocysts has been later elucidated.

The notable feature of minicollagens is the domain architecture (Fig. 3). In the vicinity of the center collagen domain surrounded by poly-proline stretches, a set of repeating cysteines, cysteine-rich domains (CRDs), with disulfide cross-links on each terminal part of minicollagen proteins (N-CRD/C-CRD), is presented (Kurz et al. 1991 Holstein et al. 1995).

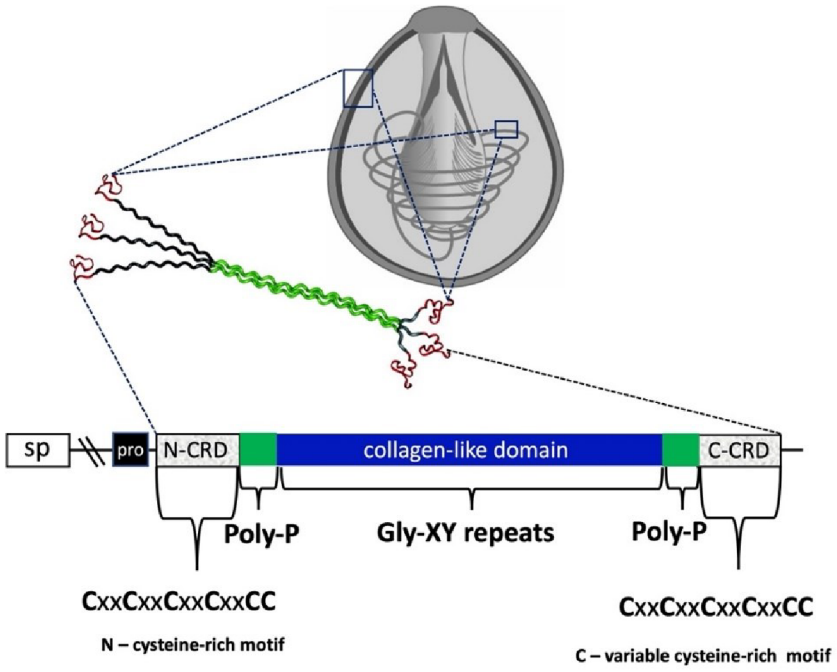

Figure. 3: Schematic drawing of minicollagen in the nematocyst. Signal peptide (SP), propeptide (pro), N-terminal cysteine-rich domain (N-CRD), PolyProline streatch (Poly-P), C-terminal cysteine-rich domain (C-CRD).

While a limited number of nematocyst morphotypes in Anthozoa (David et al. 2008), the Medusozoa have evolved a great variety of these excretory organelles and have become true predators. This morphological expansion of nematocysts was associated with the parallel manifestation of CRDs, resulting in the evolutionary radiation of stinging cells in Cnidaria (David et al. 2008). The biological role and structural features of CRDs revealed important intermolecular disulfide bonds of cysteines and reshuffling, supported by ensuing studies of minicollagen biochemical properties (Engel et al. 2002; Özbek et al. 2002; Meier et al. 2004). The recently described functional kinetics of CRDs outlined the gain of function domain of CRD for the origin of the high-pressure resistant nematocysts in Medusozoa (Tursch et al. 2016). The description of minicollagen as a major component of the nematocyst wall represents an

initial insight into the structural role in nematocyst biogenesis (Engel et al. 2001). The cell fate of nematocysts is determined by i-cells, which form the precursor of nematocysts, nematocyte, but also nerve, gland, and germ-line cells (Tardent, 1995). The cellular cascade of nematocyst morphogenesis includes the production of the giant cytoplasmic post-Golgi vacuole within the nematocyte, where a nematocyst is being assembled via continuous vesicular protein transport (Engel et al. 2001, 2002; Hwang et al. 2010; Özbek 2011), including minicollagens (Engel et al. 2001). During the maturation of the stinging cells, minicollagens assemble into polymers at the inner side of the double-layered nematocyst wall to form highly compacted molecular structures (Özbek et al. 2002). Later, compelling evidence showed that other minicollagen of *Hydra* (minicollagen 15) are restricted to the tubule structure of the nematocyst (Adamczyk et al. 2008). A study of minicollagens in the anthozoan *Nematostella* showed a possible ancestral state of minicollagens related to the simple molecular architecture of a particular nematocyst morphotype that lacks the conserved cnidarian minicollagen, Ncol-1 (Zenkert et al. 2011).

During the morphogenesis of stinging cells in Cnidaria, minicollagens were expressed exclusively in developing nematocytes (Engel et al. 2001; Adamczyk et al. 2008; Zenkert et al. 2011). Consistent with minicollagen polymerization during the wall assembly of nematocysts, the loss of detection was observed in fully developed nematocysts (Engel et al. 2001; Adamczyk et al. 2008; Tursch et al. 2016). Accordingly, the dynamic landscape of expression during the nematogenesis (nematocyst biogenesis) of *N. vectensis*, including nematocyst-specific transcription factors and structural proteins, also displayed the spatiotemporal expression with a dramatic reduction in mature nematocyst capsules (Sunagar et al. 2018). Intriguingly, comparative genomics of cnidarian lineages illuminated the organization of minicollagens into phylotypic clusters with a collinear expression resembling the Hox gene clusters (Khalturin et al. 2019), which underscores the important strategy for the evolution of taxonomic novelties.

3.4. Minicollagens in parasitic cnidarians, Myxozoa

From the first records of striking morphological similarities between polar capsules and nematocysts, a hypothesis of myxozoan relation to Cnidaria was proposed but withal fully

proven (see Chapter 1.4.). Yet, sequencing the first myxozoan minicollagen gene has provided compelling evidence of the affinity of obscure parasitic group Myxozoa to free-living Cnidaria (Holland et al. 2011).

Parallel increase of sequenced genomes and transcriptomes of myxozoan species revealed a retainment of some cnidarian nematocyst-specific genes like minicollagens (Shpirer et al. 2014; Foox et al. 2015). Interestingly, compared to Cnidaria, a low number of minicollagens was observed in Myxozoa. Thus, a depleted gene repertoire of minicollagens has been linked to the overall reduction of the body plan in Myxozoa with a simple polar capsule morphology (Shpirer et al. 2014). In contrast, the identification of an additional minicollagen ortholog suggested a more complex molecular content in myxozoan polar capsules (Foox et al. 2015). The recent proteomic profile of the polar capsule has supported the similar content of polar capsules to cnidarian nematocyst (Piriatiskyi et al. 2017). However, while the core proteins were present in both extrusive organelles, several losses of proteins such as toxins suggested the consequence of adaptation in transition to the parasitic strategy of Myxozoa (Piriatiskyi et al. 2017). Recent insights into the evolution of cnidarian adaptation, including the role of nematocysts/polar capsules, revealed species-specific adaptation of nematocyst proteins (Guo et al. 2022). Intriguingly, the conspicuous miss of minicollagens in polar capsule proteomic contents was found. This surprising absence of such abundant constituents like minicollagens was linked to lineage-specific characteristics (Guo et al. 2022). However, the evolutionary origin or the role of minicollagens and the general characterization of taxonomically-restricted genes in Myxozoa, including polar capsules, remained unexplored.

4. Research objectives

The thesis aims to ascertain the following objectives:

- Identification of taxonomically-restricted genes (TRGs) in available next generation myxozoan sequencing data with a focus on genes responsible for the formation of the polar capsule.
- Diversity and evolution of TRGs in Myxozoa.
- Expressional profiling of selected TRGs using transcriptomic analysis of sporogonic stages of myxozoan *Myxidium lieberkuehni* and extrasporogonic stage of *Nephrocystidium pickii*.
- Localization of selected taxonomically-restricted genes during myxospore development of Myxozoa.
- Stage-specific expression profile of myxospore development of *Sphaerospora molnari.*

5. Results and research publications

5.1. Paper I.

Kyslík, J., Kosakyan, A., Nenarokov, S., Holzer, A.S., Fiala, I.

The myxozoan minicollagen gene repertoire was not simplified by the parasitic lifestyle: computational identification of a novel myxozoan minicollagen gene.

BMC Genomics (2021) 22:198

doi.org/10.1186/s12864-021-07515-3

This part is comprised of 14 pages published data, which is present in the original thesis deposited at the Faculty of Science, University of South Bohemia

Background

Lineage-specific gene expansions represent one of the driving forces in the evolutionary dynamics of unique phylum traits. Myxozoa, a cnidarian subphylum of obligate parasites, are evolutionarily altered and highly reduced organisms with a simple body plan including cnidarian-specific organelles and polar capsules (a type of nematocyst). Minicollagens, a group of structural proteins, are prominent constituents of nematocysts linking Myxozoa and Cnidaria. Despite recent advances in the identification of minicollagens in Myxozoa, the evolutionary history and diversity of minicollagens in Myxozoa and Cnidaria remain elusive.

Results

We generated new transcriptomes of two myxozoan species using a novel pipeline for filtering of closely related contaminant species in RNA-seq data. Mining of our transcriptomes and published omics data confirmed the existence of myxozoan Ncol-4, reported only once previously, and revealed a novel noncanonical minicollagen, Ncol-5, which is exclusive to Myxozoa. Phylogenetic analyses support a close relationship between myxozoan Ncol-1–3 with minicollagens of *Polypodium hydriforme*, but suggest independent evolution in the case of the myxozoan minicollagens Ncol-4 and Ncol-5. Additional genome- and transcriptome-wide searches of cnidarian minicollagens expanded the dataset to better clarify the evolutionary trajectories of minicollagen.

Conclusions

The development of a new approach for the handling of next-generation data contaminated by closely related species represents a useful tool for future applications beyond the field of myxozoan research. This data processing pipeline allowed us to expand the dataset and study the evolution and diversity of minicollagen genes in Myxozoa and Cnidaria. We identified a novel type of minicollagen in Myxozoa (Ncol-5). We suggest that the large number of minicollagen paralogs in some cnidarians is a result of several recent large gene multiplication events. We revealed close juxtaposition of minicollagens Ncol-1 and Ncol-4 in myxozoan genomes, suggesting their common evolutionary history. The unique gene structure of myxozoan Ncol-5 suggests a specific function in the myxozoan polar capsule or tubule. Despite the fact that myxozoans possess only one type of nematocyst, their gene repertoire is similar to those of other cnidarians.

5.2. Paper II.

Kyslík, J., Vancová, M., Bartošová-Sojková, P., Lövy, A., Holzer, A.S., Fiala, I.

Expressional profiling and cellular localization of myxozoan minicollagens demonstrate their involvement in polar capsule formation during sporogenesis

(Manuscript submitted to the International Journal for Parasitology)

This part is comprised of 28 pages unpublished data, which is present in the original thesis deposited at the Faculty of Science, University of South Bohemia

Abstract

Minicollagens are major structural components in the biogenesis of nematocysts in Cnidaria. Recent sequence mining and proteomic analysis of myxozoan polar capsules, homologous structures of cnidarian nematocysts, have confirmed the presence of minicollagens in this evolutionarily ancient cnidarian endoparasitic group. Nonetheless, the presence and abundance of nematocyst-associated genes/proteins in polar capsule morphogenesis has never been studied in Myxozoa. Here, we report gene expression profile of three myxozoan minicollagens, *ncol-1*, *ncol-3*, and the recently identified noncanonical *ncol-5*, during the intrapiscine development of *Myxidium lieberkuehni*, the myxozoan parasite of Northern pike *Esox lucius*. Moreover, we performed the localisation of myxozoan-specific minicollagen Ncol-5 in the developing myxosporean stages by western blotting and by immunofluorescence and immunogold electron microscopy. We found that expression of minicollagens was spatiotemporally restricted to developing polar capsules within the myxospores during sporogenesis. Intriguingly, Ncol-5 was localised predominantly in the wall of polar capsule and with a lower abundancy in the capsule tubule. Overall, we demonstrate that though being significantly reduced in their morphology, myxozoans have retained similar structural components associated with the polar capsule development as reported for the nematocysts of free-living cnidarians. Furthermore, our findings have practical implications as minicollagens are useful targets for a more accurate identification of the developmental phase of myxozoan parasites.

5.3. Paper III.

Wiśniewska, M., Alama-Bermejo, G., **Kyslík, J.,** Kolísko, M., Holzer, A.S., Kosakyan, A.

Game of genes: Exploring the major parasitic strategies in myxozoans, case study: *Sphaerospora molnari*.

(Manuscript in advanced preparation)

This part is comprised of 31 pages unpublished data, which is present in the original thesis deposited at the Faculty of Science, University of South Bohemia

Abstract

Myxozoans are a unique group of metazoan parasites that diverged from their free-living cnidarian ancestors to endoparasites. These parasites have received considerable attention since many of them cause severe diseases in farmed and wild fish populations. *Sphaerospora molnari* is a myxozoan parasite infecting common carp in the central Europe. It develops mature spores in the gills of fish causing respiratory distress. Before reaching the gills of the host, *S. molnari* undergoes presporogonic development in the blood and liver of the host. We used *S. molnari* as a model to understand genetic mechanisms that parasite uses to successfully invade and proliferate within the host. While the most common functional gene groups in the gill stages were related to cellular differentiation and cytoskeletal rearrangement, blood and liver stages gene groups were related to parasite feeding and immune evasion strategies. We identified homologs of these genes in other parasitic organisms (e.g., *Plasmodium*, *Giardia*, *Trypanosoma*), that are essential for their successful survival in their hosts, and proposed a list of "pathogenicity-related" gene families. We have uncovered genes that are critical for each developmental stage of *S. molnari*, suggesting potential candidates for disease control in myxozoans. Moreover, *S. molnari* species-specific and cnidarian taxonomically-restricted genes were identified. Additionally, we compared all the advantages and flows of different methods of transcriptome analysis (i.e. genome-based, genome-guided, and *de novo*) and discussed the optimal RNA-seq pipeline for this group of organisms.

6. Summary of results and discussion

The decisive outcomes of the thesis are present in the following subheadings according to the attached publications and described as follows:

Paper I: Diversity and evolution of myxozoan minicollagen gene repertoire

- Diversity and evolution of minicollagens in Myxozoa and Cnidaria
- Identification of novel minicollagen in Myxozoa (Ncol-5)
- *De novo* transcriptome assemblies of two myxosporean species (*M. lieberkuehni*, *N. pickii*)
- Gene clustering of minicollagens in myxozoan genomes

Paper II. Expressional profiling of minicollagens and localization of novel Ncol-5 in Myxozoa

- Screening of myxospore development of *Myxidium lieberkuehni*
- Expressional profiling of minicollagens during myxospore development
- Localization of novel minicollagen Ncol-5 in myxozoan polar capsule

Paper III. Stage-specific expression study of *Sphaerospora molnari* myxospore development

- Stage-specific expression of taxonomically-restricted genes
- Identification of unique genes for *S. molnari* and their gene expression profile

6.1. Diversity and evolution of myxozoan minicollagen gene repertoire

Since the discovery of minicollagen homologue (Ncol-1) in Myxozoa (Holland et al. 2010), the diversity of taxonomically-restricted genes has been expanded with the identification of two

additional minicollagen orthologs (Ncol-2 and Ncol-3) in myxozoans using high throughput sequencing data (Shpirer et al. 2014). Due to the low number of minicollagen genes in Myxozoa, the hypothesis of reduction of gene repertoire under simplification of either body plan or myxozoan nematocyst homolog, polar capsule, has been suggested (Shpirer et al. 2014). This postulate was weakened by the identification of another minicollagen (Ncol-4), proposing a more complex polar capsule content (Foox et al. 2015). However, the evolution of minicollagens in both Myxozoa and Cnidaria was poorly understood.

To address this point, we conducted an extensive search for minicollagens in Myxozoa and Cnidaria and attempted to determine their evolutionary history (Paper I). Despite the complex evolutionary trajectories of minicollagens, our results supported the previous scenario of more complex content of myxozoan polar capsule repertoire presented by Foox et al. (2015) and challenged the hypothesis of the simplicity of the myxozoan minicollagen gene repertoire. Essentially, the evolutionary analyses of minicollagens revealed a similar basic repertoire of these genes in Myxozoa and Cnidaria, as our phylogenetic analysis suggested a recent gene multiplication of several minicollagens in free-living Cnidaria resulting in a higher number of minicollagens typical of *Hydra*, for example. The repetitive character of the minicollagen sequences reported in David et al. (2008) scrutinized the phylogenetic analyses resulting in an unstable topology and polytomic character, which do not allow us to infer the phylogenetic relationships of the minicollagen orthologs. Hence, this problem also underscores the lack of ancestral orthologs of minicollagens (Guo et al. 2022b), including the yet unknown evolutionary origin of minicollagens. In our study, we have improved the dataset of cnidarian minicollagen genes that can better clarify the minicollagen evolution and the origin of nematocyst, including polar capsules.

Importantly, we have identified a novel myxozoan minicollagen, Ncol-5, that is unique to Myxozoa. The description of the noncanonical gene architecture of Ncol-5 revealed the substitution of typical minicollagen PolyProline stretches and variable C-terminal cysteine-rich motif by glycine/serine repeats. These remarkable changes in the structural domains of Ncol-5 were also detected in Ncol-4, suggesting a specific function of these minicollagens that might evolve as the result of the adaptation of polar capsules, which corresponds with proteomic analysis of myxozoan polar capsules (Piriatinskiy et al. 2017; Guo et al. 2022b).

In addition, two transcriptomes of the model *Myxidium lieberkuehni* and extrasporogonic *Nephrocystidium picki* (Sokolov et al. 2019), selected for this thesis, were generated. Accordingly, we have also created and applied a novel bioinformatics pipeline for filtering cross-contamination of closely-related species within the attached study (Paper I). Although various tools have been developed to remove such contaminations (Cibulskis et al. 2011; Lafond-Lapalme et al. 2017), certain limitations of filtering closely related species were reported (Simion et al. 2018). With high accuracy of filtering, the pipeline can be applied for cross-contamination of species with homology above 90% and thus can improve the future acquisition of omics data from the species limited by coinfections.

The study also describes the genomic clustering of myxozoan minicollagens showing a juxtaposition of two minicollagens, whereas other minicollagen genes were not placed on a single genomic region. Besides phylotypic clustering of minicollagens in genomes of free-living Cnidaria (Khalturin et al. 2019), myxozoan genomes displayed a distinct transcriptional direction. However, the low genome coverage and low assembly level of myxozoan genomes complicate the proper inference of gene clustering. The study of phylotypic clusters of minicollagens in Myxozoa can elucidate the transcriptional regulation in the development of polar capsules.

6.2. Expressional profiling of minicollagens and localization of novel Ncol-5 in Myxozoa

The common evolutionary history of cnidarian nematocysts and myxozoan polar capsules has been widely supported by numerous studies (Shpirer et al. 2014; Piriatiskyi et al. 2017; Americus et al. 2021; Guo et al. 2022). The recently obtained proteomes of the polar capsules (Piriatinskiy et al. 2017; Guo et al. 2022) represent an essential glimpse into the proteomic content of the nematocyst homolog. However, the detailed characterization of the core structural components of polar capsules, including nematocyst-related proteins, remains elusive in Myxozoa. Despite extensive research on nematocyst's minicollagens in free-living Cnidaria (Engel et al. 2001; Adamczyk et al. 2008; Zenkert et al. 2011; Tursch et al. 2016; Beckmann & Özbek, 2012), no study to date described the kinetics of expression nor cellular localization of these lineage-specific genes in Myxozoa. This particular point addresses the

second part of the present thesis (Paper II) following the identification of the newly identified minicollagen, Ncol-5 (Paper I). We performed the gene expression study of three myxozoan minicollagens (Ncol-1, Ncol-3, Ncol-5) and the localization of Ncol-5 during the development of the myxospore. To ascertain gene expression, the development myxospores was examined in the myxozoan model *M. lieberkuehni*. Minicollagens were found to exhibit the expression restricted to the sporogonic phase of myxospore morphogenesis, including polar capsule biogenesis. This restricted pattern of minicollagen expression was also reported in cnidarian nematocysts (Engel et al. 2001; Adamczyk et al. 2008; Tursch et al. 2016). Similar to the mesoglea collagen molecules reported by Turch et al. (2016), some myxozoan minicollagens can be expressed outside the sporogony process. Therefore, our obtained results represent a deep insight into the molecular background of selected taxonomically-restricted genes in phylum-specific traits, like polar capsules in Myxozoa. Moreover, we performed immunostaining analyses to reveal the localization of the uniquely-evolved Ncol-5 minicollagen protein (Paper II). Unlike the noncanonical gene architecture, including domain substitutions (described in a previous study, Paper I), the subcellular localization of Ncol-5 was restricted to the polar capsule wall, as reported for the canonical minicollagen 1 of free-living cnidarian nematocysts (Engel et al. 2001). We thus proved that the identification of Ncol-5 as a fifth known myxozoan minicollagen was correct and that myxozoans possess the unique noncanonical character of minicollagens.

Functional research on developmental processes in Myxozoa is limited by the general absence of in vitro culture (Alama-Bermejo & Holzer, 2021). Therefore, the myxozoan expression profiling is problematic and needs live parasitic stages freshly isolated from the host tissues. Thus, this thesis represents original research in Myxozoa.

6.3. Stage-specific expression study of *Sphaerospora molnari* myxospore development

Despite extensive RNA-sequencing studies on Myxozoa (Faber et al. 2021; Hartigan et al. 2020; Alama-Bermejo et al. 2020; Barrett & Bartholomew, 2021), the transcriptomic profiling of stage-specific development of Myxozoa is missing. We performed comparative transcriptomics of tissue-related developmental stages of *Sphaerospora molnari* to understand the genetic mechanisms underlying development and successful parasitic

strategies in Myxozoa (Paper III). In addition to the various candidate proteins for host invasion and disease control of myxozoans identified in the study, the stage-specific expressional profile of taxonomically-restricted genes, including newly emerged species-specific genes, has been uncharted.

Using datasets of myxozoan nematocyst-related genes (Shpirer et al. 2014, 2018; Paper I), we detected orthologs in transcriptomic assemblies of *S. molnari* (Paper III). Obtained gene expression values for individual developmental stages (blood stages, liver stages, spore-forming stages) of *S. molnari* revealed a spatiotemporal pattern of expression restricted to spore-forming stages. Overall, we detected upregulation of cnidarian-related markers, such as minicollagens, nematogalectins, and additional nematocyst-specific genes in spore-forming stages. Accordingly, a stage-specific expression of these genes was also observed during the development of *M. lieberkuehni* myxospore (Paper II) and transcriptomic data of *Tetracapsuloides bryosalmonae* from the bryozoan host (Faber et al. 2021). Furthermore, exclusive expression of minicollagen transcripts in spore-forming stages consistently supports the previous reports of spatiotemporal expression of minicollagens during nematocyst biogenesis of Cnidaria (Engel et al. 2001; Adamczyk et al. 2008; Tursch et al. 2016). Interestingly, a duplicated paralog of minicollagen of *S. molnari* (Ncol-1a), described in the first study (Paper I), also possesses exclusive expression in spore-forming stages. In the present study (Paper III), this gene paralog is present in a set of unique genes for *S. molnari* that exemplifies the taxon-specific origin of the gene on the species level. Therefore, the function of Ncol-1a in *S. molnari* remains unexplored. In addition, the described pattern of expression of nematocyst-specific markers demonstrates the robustness of the accurately obtained transcriptomic data, which confirms the proposed translational relevance of these markers for either comparative or functional studies in myxozoan research (Paper II). Recent exploration of the proteomic content of polar capsules supported the role of these lineage-specific organelles for successful myxozoan adaptation in evolution (Piritiskyi et al. 2017; Guo et al. 2022b). However, similar to Microsporidia (Haag et al. 2014), the evolution of morphological novelty, like a polar capsule in Myxozoa, might represent a consequence of adaptation after genome compaction.

Among a large number of genes, a considerable proportion are species-specific genes -so-called unique for *S. molnari*. Although many of these taxonomically-restricted genes lack

either gene ontology enrichment or homology to both Cnidaria and other Metazoa (Paper III), we determined the expression profile of these genes in transcriptomes of *S. molnari*. In contrast to *S. molnari*-specific genes that showed intersected expression in all developmental stages of *S. molnari* examined, a group of genes was described whose expression was restricted to individual stages (Paper III). Due to the apparent lack of homology in other species of Myxozoa, these genes can be classified as orphans, as reported for other organisms, including Cnidaria (Khalturin et al. 2008, 2009; Gold et al. 2019). Nevertheless, the existence of these singletons in the genome represents a common feature of organisms (Marsden et al. 2006). Interestingly, the number of these unassigned genes compared to repetitively evolving gene families could be greater than the number of regular genes in each lineage during evolution (Lee et al. 2005). One can suggest that acquisition of such species-specific "orphan" genes in myxozoans may represent a key to understanding the emergence of novelty in the context of adaptation to new ecological niches (Wilson et al. 2005), host environments, or myxozoan parasitic strategies.

7. Conclusions and future perspectives

In summary, this thesis makes an important contribution to the study of taxonomically-restricted genes in myxozoan research. In the present thesis, the diversity of minicollagens in parasitic Myxozoa and free-living Cnidaria has been deciphered. Importantly, a new minicollagen Ncol-5 has been identified in Myxozoa, including a description of the expression and localization of this structural protein specific to myxozoans. Moreover, this study illustrated different transcriptional orientation and clustering of minicollagens in myxozoan genomes. Furthermore, we developed a bioinformatics pipeline for a filtering strategy in genomic and transcriptomic data. Also, we conducted a gene expression study to understand the transcriptional level of minicollagens during the development of polar capsules within the myxospore development. Finally, the stage-specific profiling of *Sphaerospora molnari* development provided supportive evidence of restricted expression of cnidarian-related genes, including minicollagens. Additionally, a set of unique genes were identified in *S. molnari* that revealed spatiotemporal dynamic of expression. Given the outcomes of the present work, future studies of taxonomically-restricted genes could elucidate other intriguing aspects

regarding the fascinating nature of Myxozoa. Unlike currently described minicollagen diversity, additional searches for minicollagen orthologs can enrich the evolutionary trajectories that could have a great payoff for deciphering the evolution of nematocysts and polar capsules. At present, the genomes of myxozoan species are limited in the size of assembled contigs. Hence, the establishment of a reference genome for Myxozoa with high coverage and assembly could enable the expansion of knowledge of phylotypic clustering of taxonomically-restricted genes in Myxozoa and thus uncover the complete genetic toolbox that controls myxozoan polar capsule assembly. Applied more generally, gene expression profiles of minicollagens can help to more accurately determinate of developmental stage in either functional or descriptive studies in myxozoan research.

8. References

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9. Appendix

Curriculum vitae

Jiří Kyslík (24.2.1992, Český Krumlov, Czech Republic)

Present position

since 1.10. 2016: Ph.D. student at Laboratory of Fish Protistology, Institute of Parasitology, Biology Center, Czech Academy of Sciences, České Budějovice, Czech Republic.

Education

2016 – up to now Faculty of Science, University of South Bohemia in České Budějovice - Ph.D. Study program: Parasitology: Ph.D. thesis: Bioinformatics and functional characterization of taxonomically-restricted genes in Myxozoa.

2014-2016 Faculty of Science, University of South Bohemia in České Budějovice – MSc. study program: Parasitology: MSc. thesis: Phylogeny of Myxozoa based on cnidarian specific genes.

2011-2014 Faculty of Science, University of South Bohemia in České Budějovice – BSc. study program: Biology: BSc. thesis: New phylogenetic markers for phylogenetic reconstruction of Myxozoa.

Teaching experience

Teaching involvement at University of South Bohemia, Faculty of Science, Czech Republic with the following topics:

teaching assistant of the practical course Biology of parasitic protists

teaching assistant of the practical course Field parasitology

Pedagogical activities

University of South Bohemia Faculty of Science open days, Institute of Parasitology AS CR open days, The week of the Czech Academy of Sciences

Awards

2019: EMBO Short-term fellowship (Scientific Exchange Grant), University of Haifa, Israel – 3 months.

Expertise relevant to the proposed project

Microscopy: extensive experience in light and electron microscopy, confocal microscopy, imaging.

Cell culturing: ample experience in parasite culturing methods.

Molecular biology methods: extensive experience in DNA/RNA isolation, PCR, RT-qPCR, cloning, RNAi, sequencing, cDNA library preparation.

Bioinformatics: protein structure modelling, assembly and annotation of transcriptomic data. Very experienced in phylogenetic reconstruction, skills in the various phylogenetic programs, e.g.: Geneious Prime, MAFFT, RAxML, PAUP*, MrBAYES, PhyloBayes, PHYLIP, BLAST.

Skills

Troubleshooting skills, working independence, teamwork skills, critical thinking skills, communication, creativity.

Languages

Czech (native), English (advanced), Spanish (Elementary), German (Elementary)

List of publications

Kyslík J., Kosakyan A., Nenarokov S., Holzer A., Fiala I. (2021) The myxozoan minicollagen gene repertoire was not simplified by the parasitic lifestyle: computational identification of a novel myxozoan minicollagen gene BMC Genomics 22: 198. DOI: 10.1186/s12864-021-07515-3

Bartošová-Sojková P., **Kyslík J.**, Alama Bermejo G., Hartigan A., Atkinson S.D., Bartholomew J., Palenzuela O., Picard-Sánchez M., Faber M.N., Holland J.W., Holzer A. (2021) Evolutionary Analysis of Cystatins of Early-Emerging Metazoans Reveals a Novel Subtype in Parasitic Cnidarians 10: 110. DOI: 10.3390/biology10020110

Conferences

Lövy, A., **Kyslík, J.**, Gahurová, L., Krejčí, A., Holzer, A.S. Is anybody out there? Attempt to peek behind the door of Notch signalling in myxozoans (Cnidaria). 20th International Conference on Diseases of Fish and Shellfish (EAFP), September 2021 – online.

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Bartošová-Sojková, P., **Kyslík, J.**, Holzer, A.S. Early evolution and functional diversification of cysteine protease inhibitors drived by parasite life strategy. 11th General Meeting of the International Proteolysis Society, October 2019, Marianské Lazně, Czech Republic.

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Kyslík J., Hartigan A., Holzer A.S., Fiala I. New Markers for Resolving Myxozoan Phylogenetic Relationships. 45th Jíroveć s Protozoologial Days, May 2015, Dubovice, Czech Republic.