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Evolutionary biology

Absence of major histocompatibility complex class II mediated immunity in pipefish, *Syngnathus typhle*: evidence from deep transcriptome sequencing

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The major histocompatibility complex (MHC)-mediated adaptive immune system is the hallmark of gnathostome immune defence. Recent work suggests that cod-like fishes (Gadidae) lack important components of the MHC class II mediated immunity. Here, we report a putative independent loss of functionality of this pathway in another species, the pipefish *Syngnathus typhle*, that belongs to a distantly related fish family (Syngnathidae). In a deep transcriptome sequencing approach comprising several independent normalized and non-normalized expressed sequence tag (EST) libraries with approximately 7.5×10^8 reads, sequenced with two next generation platforms (454 and Illumina), we were unable to identify MHC class IIα/β genes as well as genes encoding associated receptors. Along with the recent findings in cod, our results suggest that immune systems of the Euteleosts may be more variable than previously assumed.

1. Introduction

Given the ubiquitous abundance of parasites and pathogens [1], immune systems are of crucial importance for any species. Parasite pressure has resulted in the evolution of highly specific immune defence [2] that discriminates self from potentially dangerous non-self. Innate immune defence components recognize conserved pathogen-associated molecular patterns [3], but their repertoire diversity is limited. Hence, an important evolutionary innovation unique to gnathostomes (jawed vertebrates) is the adaptive immune system along with somatic diversification of receptors of the immunoglobulin family [4,5]. Foreign peptides (epitopes) are recognized upon presentation to specialized lymphocytes that requires the binding of epitopes to specialized receptor molecules, encoded by major histocompatibility complex (MHC) class I and II genes. Whereas MHC class I genes are expressed on all cells and present epitopes from within the cells, class II genes are only expressed on specialized antigen-presenting cells (APC) such as dendritic cells and macrophages. When APCs present motifs of extracellular danger signals from pathogens to specialized T cells ($CD4^+$-type) the MHC II mediated immunity is activated.

Given the core function of the MHC class II mediated pathway in gnathostome immunity, it came as a surprise that members of the cod family lack MHC class IIα and β genes, as well as several important genes of this immune pathway [6]. Here, we report the absence of an MHC class II mediated immune pathway in a member of another, phylogenetically distant fish, the pipefish

Syngnathus typhle (Syngnathidae) based on deep transcriptome sequencing, and compare the repertoire loss to the cod-like fishes.

2. Material and methods

(a) Study species

As a sex-role reversed fish, the pipefish *S. typhle*, is a widely studied model species in evolutionary biology [7,8]. Females lay eggs into the male brood pouch, where they are fertilized and nourished [9]. Pipefish are exposed to bacterial and metazoan pathogens in their coastal habitat [10,11]. Here, we analysed pipefish from the Baltic Sea (Kiel Fjord) and from the Adriatic Sea (Lagoon of Venice). At both sites, fish are continually exposed to commensal and pathogenic *Vibrio* strains [11]. We thus assume that, if present, the MHC class II pathway would be activated. Individuals from Kiel Fjord were fed with live *Vibrio* isolated from their environment in order to stimulate their immune response. Individuals were killed with an overdose of MS222. RNA extracts were prepared immediately from freshly dissected organs.

(b) Molecular methods

Our evidence of absence of the MHC class II pathway is based on three independent methodological approaches: (i) primer-directed approaches using conserved portions of the MHC class IIβ-gene, (ii) preparation and sequencing of a normalized cDNA library and (iii) ultra-high-throughput sequencing of three non-normalized cDNA libraries using an Illumina HiSeq2000 platform.

Using standard PCR-directed cloning and sequencing, we focused on the MHC class IIβ-gene via primer-directed approaches with cDNA and gDNA as a template. Numerous published and self-developed primer pairs were tested in regions that are conserved across six acanthopterygian taxa according to alignments (see the electronic supplementary material, tables S1 and S2). PCR products were characterized using standard TA-cloning approaches and Sanger sequencing.

We performed deep-cDNA sequencing on a Roche 454 Titanium (normalized EST library, pool of individuals from Adriatic Sea/Baltic Sea) and on a Illumina Hi-Seq2000 platform (non-normalized cDNA, electronic supplementary material, tables S3 and S4, fish only from Baltic Sea challenged with *Vibrio*). RNA quality was checked on an Experion RNA analyser (Qiagen). A normalized cDNA library was prepared (pool of five pipefish individuals; pool of gill, liver, head kidney) by GATC (Constance), and sequenced with Roche 454 FLX Titanium chemistry (526 491 reads after quality clipping). On an Illumina HiSeq-2000 platform, we characterized the transcriptome to ultra-high coverage analysing three independent libraries of above organs separately, each derived from *Vibrio*-infected pipefish. In total approximately 750 million paired-end reads were produced (see the electronic supplementary material, table S3; Genbank accession no. SRP018381).

(c) Bioinformatic pipelines and analyses

The raw 454 reads were adaptor and quality-trimmed with SEQCLEAN [12], and reads less than 50 bp were discarded, resulting in 468 126 reads (length 100–951 bp, mean 278 bp). Illumina data were cleaned using SEQPREP and overlapping read-pairs were merged. PRINSEQ [13] was used to maximize read quality by discarding all reads with an undetermined base content of greater than 10 per cent. Random subsamples of merged reads obtained by Illumina-sequencing (length 50–186 bp) were the basis of two hybrid assemblies. They contained all 454 reads and either 10^7 subsampled reads of *Vibrio*-infected pipefish gills, or 5×10^6 reads of each of the three tissues head kidney, gills and liver. The assemblies conducted in MIRA3 [14] produced 96 333 contigs for the 454-gill hybrid data, and 170 726 contigs for all three organ types. To estimate assembly quality, we mapped reads against the contigs resulting from both de novo hybrid assemblies of 454 data and Illumina reads (see the electronic supplementary material, table S4) using the software BOWTIE [15]. For annotation of gene models, the resulting contigs from both de-novo assemblies were blasted (BLASTx) against the non-redundant protein database from NCBI, using an e-value cut-off of 10^{-5}. In the three-tissue assembly these were 106 154 of a total of 170 726 contigs (62.18%).

As a second search strategy, we retrieved a number of cDNA sequences of the most important MHC II pathway genes from GenBank (see the electronic supplementary material, S1) which were queried against all raw reads (approx. 750 Mio) obtained here. We used tBLASTx with very high cut-off values (e-value = 100). For every target gene, we collected the 5000 best hits, which were then annotated using tBLASTx (e-value 1×10^{-5}) against NCBI non-redundant protein database.

To validate our approach (assembly + tBLASTx), we spiked the pipefish read data with short (101 bp) MHC II fragments derived from three-spined sticklebacks (*Gasterosteus aculeatus*) and seahorse (*Hippocampus abdominalis*), and found successful recovery (details in the electronic supplementary material, S2).

3. Results and discussion

The initial purpose of our study was to characterize a putative standard MHC-based immune system of a teleost species with sex-role reversal, the broad-nosed pipefish *S. typhle*. With primer-directed cloning and sequencing approaches, no gene fragment could be identified that even distantly resembled MHC class IIβ. We subsequently performed deep transcriptome sequencing of pathogen-challenged pipefish individuals, where we failed to detect genes crucial for the MHC class II mediated adaptive immune pathways (table 1). Most notable is the absence of genes encoding the MHC class IIα and β chain, as well as the $CD4^+$ receptor. By contrast, all genes important for the MHC class I pathway were present, in particular MHC I, β-2 microglobulin, TAP1 and 2 and the complete complement cascade (table 1). Further, several chemokine genes and associated receptors, interferones and interleukins were identified (table 1).

While the absence of genes directly encoding MHC class II molecules was similar to the situation in cod [6], the receptor encoding gene (CD8β), which is involved in MHC I recognition via the T-cell receptor (TCR) was absent in pipefish but not cod (table 1). Note that CD8β is not mandatory for a MHC I mediated immune response, as CD8α molecules may function as a homodimer [16]. The antigen recognizing TCR γ was also absent. Because the majority of TCRs consist of α/β-heterodimers, functionality of the TCR is still likely [17].

As opposed to cod, where the $CD4^+$-receptor was truncated and non-functional, this gene could not be identified among pipefish transcripts. For the invariant-chain gene, our annotation returned two contigs that aligned almost perfectly to each other, suggesting the same transcript. When translated into the appropriate amino acid sequence, the putative gene model revealed a stop codon approximately 20 amino acid distant from the 3′-end of the gene in other teleosts (see the electronic supplementary material, table S7), suggesting a truncated and non-functional invariant-chain gene. However, the invariant chain also binds to MHC class I molecules [18].

Table 1. Immune gene repertoire of broad-nosed pipefish, *Syngnathus typhle*. The immune gene repertoire is compared with cod [6]. The last column indicates how the transcript was identified, either via contig annotation, or via reciprocal best tBLASTx using teleost queries to the *S. typhle* database (see the electronic supplementary material, S1 and S2; see §2 for details). The results of the tBLASTx search are presented in the electronic supplementary materials, tables S5 and S6.

gene abbreviation	pathway	pipefish, this study	cod	identification
MHC I	MHC I	yes	yes	contig annot
B2-microglobulin	MHC I	yes	yes	contig annot
TAP1	MHC I	yes	yes	contig annot
TAP2	MHC I	yes	yes	contig annot
Tapasin	MHC I	yes	yes	contig annot
PSME1	MHC I	yes	yes	contig annot
PSME2	MHC I	yes	yes	contig annot
PSME3	MHC I	yes	yes	contig annot
PSMB1	MHC I	yes	yes	contig annot
PSMB2	MHC I	yes	yes	contig annot
PSMB3	MHC I	yes	yes	contig annot
PSMB4	MHC I	yes	yes	contig annot
PSMB5	MHC I	yes	yes	contig annot
PSMB6	MHC I	yes	yes	contig annot
PSMB7	MHC I	yes	yes	contig annot
PSMB8	MHC I	yes	yes	tBLASTx
PSMB9	MHC I	yes	yes	tBLASTx
PSMB10	MHC I	yes	yes	tBLASTx
GranzymeB	MHC I	yes	yes	contig annot
Perforin	MHC I	yes	yes	contig annot
FasL	MHC I	yes	yes	contig annot
Fas	MHC I	yes	yes	contig annot
Erap1	MHC I	yes	yes	contig annot
Erap2	MHC I	yes	yes	contig annot
Irap	MHC I	yes	yes	contig annot
UNC93B	MHC I	yes	yes	contig annot
RFXANK	MHC II	yes	yes	contig annot
RFXAP	MHC II	yes	yes	contig annot
RFX5	MHC II	yes	yes	contig annot
RFX7	MHC II	yes	yes	contig annot
CIITA	MHC II	no	yes	n.a.
MHC II alpha	MHC II	no	no	n.a.
MHC II beta	MHC II	no	no	n.a.
invariant chain	MHC II	not functional	no	contig annot
CD3e	T-cell receptors	yes	yes	contig annot
CD8a	T-cell receptors	yes	yes	contig annot
CD8b	T-cell receptors	no	yes	n.a.
CD4	T-cell receptors	no	truncated	n.a.
CD3 zeta	T-cell receptors	yes	yes	contig annot
CD3g/d	T-cell receptors	yes	yes	contig annot
TCR alpha	T-cell receptors	yes	yes	contig annot
TCR beta	T-cell receptors	yes	yes	contig annot
TCR gamma	T-cell receptors	no	yes	n.a.
AIRE	T-cell receptors	yes	yes	tBLASTx

(*Continued*.)

Table 1. (*Continued.*)

gene abbreviation	pathway	pipefish, this study	cod	identification
AICDA	T-cell receptors	yes	yes	contig annot
RAG1	T-cell receptors	yes	yes	contig annot
RAG2	T-cell receptors	yes	yes	tBLASTx
IL1B	interleukins and interferons	yes	yes	contig annot
IL6ST	interleukins and interferons	yes	yes	contig annot
IL8	interleukins and interferons	yes	yes	contig annot
IL10	interleukins and interferons	yes	yes	contig annot
IL12B	interleukins and interferons	yes	yes	tBLASTx
IL15	interleukins and interferons	yes	yes	contig annot
IL17D	interleukins and interferons	yes	yes	contig annot
IL17A F1	interleukins and interferons	yes	yes	tBLASTx
IL22	interleukins and interferons	yes	yes	contig annot
IL2RG	interleukins and interferons	yes	yes	contig annot
IL2RB	Interleukins and interferons	yes	yes	contig annot
IL4RA	interleukins and interferons	yes	yes	contig annot
IL8RB-Like	interleukins and interferons	yes	yes	contig annot
IL12RB2	interleukins and interferons	yes	yes	contig annot
IL17RA	interleukins and interferons	yes	yes	contig annot
IL17RD	interleukins and interferons	yes	yes	contig annot
FOXP3	interleukins and interferons	yes	yes	tBLASTx
TNFa	interleukins and interferons	yes	yes	contig annot
TGFB	interleukins and interferons	yes	yes	contig annot
IFNG	interleukins and interferons	yes	yes	contig annot
IPS1	interleukins and interferons	yes	yes	contig annot
IKKG	interleukins and interferons	yes	yes	contig annot
MYD88	interleukins and interferons	yes	yes	contig annot
C1qT4	complement cascade	yes	yes	tBLASTx
C1qT5	complement cascade	yes	yes	contig annot
C3	complement cascade	yes	yes	contig annot
C4	complement cascade	yes	yes	tBLASTx
C5	complement cascade	yes	yes	contig annot
C6	complement cascade	yes	yes	contig annot
C7	complement cascade	yes	yes	contig annot
C8	complement cascade	yes	yes	contig annot
C9	complement cascade	yes	yes	contig annot
IgM	B cells and APC's	yes	yes	contig annot
Igb	B cells and APC's	yes	yes	contig annot
IgD	B cells and APC's	yes	yes	contig annot
PTPRC	B cells and APC's	yes	yes	contig annot
CD79A	B cells and APC's	yes	yes	contig annot
CD79B	B cells and APC's	yes	yes	contig annot
CD226	B cells and APC's	yes	yes	tBLASTx
CD40L	B cells and APC's	yes	yes	contig annot
CD40	B cells and APC's	yes	yes	contig annot
BLNK	B cells and APC's	yes	yes	contig annot

(*Continued.*)

Table 1. (*Continued.*)

gene abbreviation	pathway	pipefish, this study	cod	identification
IGBP1	B cells and APC's	yes	yes	contig annot
CXCR2	chemokines and receptors	yes	yes	contig annot
CXCR3	chemokines and receptors	yes	yes	contig annot
CXCR4	chemokines and receptors	yes	yes	contig annot
CCR5	chemokines and receptors	yes	yes	contig annot
CCR6	chemokines and receptors	yes	yes	contig annot
CCR7	chemokines and receptors	yes	yes	contig annot
CCR9	chemokines and receptors	yes	yes	contig annot

Thus, even if the identified transcript was functional, this would not provide conclusive evidence for the presence of MHC class II molecules in pipefish.

In our study, we have only captured transcribed genes and not analysed the genome. It is, therefore, possible that too few APCs carrying MHC class II molecules were present in our RNA preparations. This is unlikely, because all individuals were immune challenged with *Vibrio* bacteria, the examined tissues harbour specialized cells involved in the MHC class II pathway, and express MHC class II genes [19]. Second, we identified receptors that are uniquely expressed on specialized immune cells such as *RAG1* and *RAG2* demonstrating that these cell types were present [20].

It was reported earlier that seahorses and pipefish lack spleen and gut associated lymphatic tissue [21] and thus possess no morphologically detectable organ where T cells can reside and proliferate [22]. Syngnathidae are thus supposed to suffer immune deficiency owing to secondary reduction of the adaptive immune system [21]. Nevertheless, among the Syngnathidae, seahorses (*Hippocampus abdominalis*) are reported to possess a single functional MHC class IIβ-gene locus [23] which is, however, divergent in terms of nucleotide sequences from all other Acanthopterygii, in particular in the second exon that codes for the peptide binding region (see the electronic supplementary materials, table S2). Because the *Syngnathus* genus diverged from the general Syngnathidae lineage 34 Ma [24], we postulate that the major immune system modification occurred during the cladogenesis of genus *Syngnathus*.

All cod-like fishes examined thus far seem to lack the MHC class II pathway [6], a trait previously thought to be a key innovation for vertebrate success. The families Syngnathidae and Gadidae are only distantly related and reciprocally monophyletic [25,26], and fish orders that are phylogenetically younger than Syngnathidae possess MHC class II-genes (e.g. many Percomorpha—perch-like fishes). Hence, the absence of the MHC class II pathway in *Syngnathus* can only be explained as a secondary loss independent of (i.e. parallel to) that of cod-like fishes. In the Gadidae, it was speculated that habitat with few pathogens may have facilitated a loss of the MHC class II pathway. Given the ubiquity of macroparasites [10] and microparasites [11] infecting *Syngnathus* species in shallow coastal waters, this explanation is unlikely to apply. This suggests that different selection pressures may result in a functional loss of MHC II mediated adaptive immunity. Moreover, the evolutionary flexibility in the organization of the vertebrate adaptive immune system seems to be higher than previously suggested [27].

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References

1. Windsor DA. 1998 Most of the species on Earth are parasites. *Int. J. Parasitol.* **28**, 1939–1941. (doi:10.1016/S0020-7519(98)00153-2)
2. Schmid-Hempel P, Ebert D. 2003 On the evolutionary ecology of specific immune defence. *Trends Ecol. Evol.* **18**, 27–32. (doi:10.1016/S0169-5347(02)00013-7)
3. Medzhitov R, Janeway CA. 1997 Innate immunity: the virtues of a nonclonal system of recognition. *Cell* **91**, 295–298. (doi:10.1016/S0092-8674(00)80412-2)
4. Cooper MD, Alder MN. 2006 The evolution of adaptive immune systems. *Cell* **124**, 815–822. (doi:10.1016/j.cell.2006.02.001)
5. Flajnik MF, Kasahara M. 2010 Origin and evolution of the adaptive immune system: genetic events and selective pressures. *Nat. Rev. Genet.* **11**, 47–59. (doi:10.1038/nrg2703)
6. Star B *et al*. 2011 The genome sequence of Atlantic cod reveals a unique immune system. *Nature* **477**, 207–210. (doi:10.1038/nature10342)
7. Paczolt KA, Jones AG. 2010 Post-copulatory sexual selection and sexual conflict in the evolution of male pregnancy. *Nature* **464**, 401–404. (doi:10.1038/nature08861)
8. Roth O, Scharsack JP, Keller I, Reusch TBH. 2011 Batemańs principle and immunity in a sex-role reversed pipefish. *J. Evol. Biol.* **24**, 1410–1420. (doi:10.1111/j.1420-9101.2011.02273.x)
9. Kvarnemo C, Mobley KB, Partridge C, Jones AG, Ahnesjö I. 2011 Evidence of paternal nutrient provisioning to embryos in broad-nosed pipefish *Syngnathus typhle*. *J. Fish Biol.* **78**, 1725–1737. (doi:10.1111/j.1095-8649.2011.02989.x)
10. Landis SH, Kalbe M, Reusch TBH, Roth O. 2012 Consistent pattern of local adaptation during an experimental heat wave in a pipefish-trematode host–parasite system. *PLoS ONE* **7**, e30658. (doi:10.1371/journal.pone.0030658)
11. Roth O, Keller I, Landis SH, Salzburger W, Reusch TBH. 2012 Hosts are ahead in a marine host–parasite coevolutionary arms race: innate immune system adaptation in pipefish *Syngnathus typhle* against *Vibrio* phylotypes. *Evolution* **66**, 2528–2539. (doi:10.1111/j.1558-5646.2012.01614.x)

12. Chen YA, Lin CC, Wang CD, Wu HB, Hwang PI. 2007 An optimized procedure greatly improves EST vector contamination removal. *BMC Genomics* **8**, 416. (doi:10.1186/1471-2164-8-416)
13. Schmieder R, Edwards R. 2011 Quality control and preprocessing of metagenomic datasets. *Bioinformatics* **27**, 863–864. (doi:10.1093/bioinformatics/btr026)
14. Chevreux B, Pfisterer T, Drescher B, Driesel AJ, Mueller WEG, Wetter T, Suhai S. 2004 Using the miraEST assembler for reliable and automated mRNA transcript assembly and SNP detection in sequenced ESTs. *Genome Res.* **14**, 1147–1159. (doi:10.1101/gr.1917404)
15. Langmead B, Trapnell C, Pop M, Salzberg SL. 2009 Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* **10**, R25. (doi:10.1186/gb-2009-10-3-r25)
16. Gao GF, Tormo J, Gerth UC, Wyer JR, McMichael AJ, Stuart DI, Jakobsen BK. 1997 Crystal structure of the complex between human CD8$\alpha\alpha$ and HLA-A2. *Nature* **387**, 630–634. (doi:10.1038/42523)
17. Janeway CA, Travers P, Walport M, Shlomchik MJ. 2001 *Immunobiology: the immune system in health and disease*, 5th edn. Garland Publishing.
18. Basha G, Omilusik K, Chavez-Steenbock A, Reinicke AT, Lack N, Choi KB, Jefferies WA. 2012 A CD74-dependent MHC class I endolysosomal cross-presentation pathway. *Nat. Immunol.* **13**, 237–45. (doi:10.1038/ni.2225)
19. Wegner KM, Kalbe M, Kurtz J, Schaschl H, Reusch TBH. 2006 Heritable variation in MHC class II expression and interactions with MHC sequence polymorphism in three-spined sticklebacks. *Mol. Ecol.* **15**, 1153–1164. (doi:10.1111/j.1365-294X.2006.02855.x)
20. Oettinger MA, Schatz DG, Gorka C, Baltimore D. 1990 RAG-1 and RAG-2, Adjacent genes that synergistically activate V(D)J recombination. *Science* **248**, 1517–1523. (doi:10.1126/science.2360047)
21. Matsunaga T, Rahman A. 1998 What brought the adaptive immune system to vertebrates? The jaw hypothesis and the seahorse. *Immunol. Rev.* **166**, 177–186. (doi:10.1111/j.1600-065X.1998.tb01262.x)
22. Rauta PR, Nayak B, Das S. 2012 Immune system and immune responses in fish and their role in comparative immunity study: a model for higher organisms. *Immunol. Lett.* **148**, 23–33. (doi:10.1016/j.imlet.2012.08.003)
23. Bahr A, Wilson AB. 2012 The evolution of MHC diversity: evidence of intralocus gene conversion and recombination in a single-locus system. *Gene* **497**, 52–57. (doi:10.1016/j.gene.2012.01.017)
24. Wilson AB, Orr JW. 2011 The evolutionary origins of Syngnathidae: pipefishes and seahorses. *J. Fish Biol.* **78**, 1603–1623. (doi:10.1111/j.1095-8649.2011.02988.x)
25. Kawahara R, Miya M, Mabuchi K, Lavoue S, Inoue JG, Satoh TP, Kawaguchi A, Nishida M. 2008 Interrelationships of the 11 gasterosteiform families (sticklebacks, pipefishes, and their relatives): a new perspective based on whole mitogenome sequences from 75 higher teleosts. *Mol. Phylogenet. Evol.* **46**, 224–236. (doi:10.1016/j.ympev.2007.07.009)
26. Near TJ, Eytan RI, Dornburg A, Kuhn KL, Moore JA, Davis MP, Wainwright PC, Friedman M, Smith WL. 2012 Resolution of ray-finned fish phylogeny and timing of diversification. *Proc. Natl Acad. Sci. USA* **109**, 13 698–13 703. (doi:10.1073/pnas.1206625109)
27. Star B, Jentoft S. 2012 Why does the immune system of Atlantic cod lack MHC II? *BioEssays* **34**, 648–651. (doi:10.1002/bies.201200005)