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Basic

Short Communication

Lymphocyte pathway analysis using naturally lymphocyte-deficient fish

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Comparative phylogenetic analyses are of potential value to establish the essential components of genetic networks underlying physiological traits. For species that naturally lack particular lymphocyte lineages, we show here that this strategy readily distinguishes trait-specific actors from pleiotropic components of the genetic network governing lymphocyte differentiation. Previously, three of the four members of the DNA polymerase X family have been implicated in the junctional diversification process during the somatic assembly of antigen receptors. Our phylogenetic analysis indicates that the presence of terminal deoxynucleotidyl transferase is strictly associated with the facility of V(D)J recombination, whereas PolL and PolM genes are retained even in species lacking Rag-mediated somatic diversification of antigen receptor genes.

Keywords: DNA polymerase X · Lymphocyte-deficient fish · V(D)J recombination

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Despite considerable technical improvements, genetic screens in vertebrates [1, 2] typically address only a single or at best a few physiological traits and follow-up studies often require massively parallel efforts to reconstruct the components of lineage-specific genetic networks. However, provided that species differ in a particular trait, inter-specific genome comparisons [3] may offer an alternative in silico strategy to catalog gene content in pathways of interest.

The presence and dichotomy of lymphocyte lineages is a hallmark of vertebrate adaptive immune systems [4–7]. Yet not all species conform to this canonical structure. Among the Ceratioidei suborder of anglerfishes, T^+B^+, T^-B^+, and T^-B^- species were identified [8] (Fig. 1A). Because about 100 My have elapsed between the emergence of Ceratioidei and the present day [9], genes originally active specifically in the now defunct adaptive lymphocyte lineage(s) can be expected to be either lost or pseudogenized [8]. Thus, species-specific differences among Ceratoidei provide an unprecedented opportunity to interrogate the physiological pathways of lymphopoiesis. The blunt-snouted clingfish (*Gouania willdenowi*) has lost immunoglobulin genes and other B lymphocyte-lineage-related genes [10] and can thus be considered a T^+B^- variety. Inclusion of this species complements the lymphocyte pathway analysis through phylogeny (LYPAP) strategy proposed here.

Results and discussion

Outline of the LYPAP strategy

Among the Ceratoidei, we focused on three species for the present comparative analysis. *Chaunax abei*, belonging to the closely

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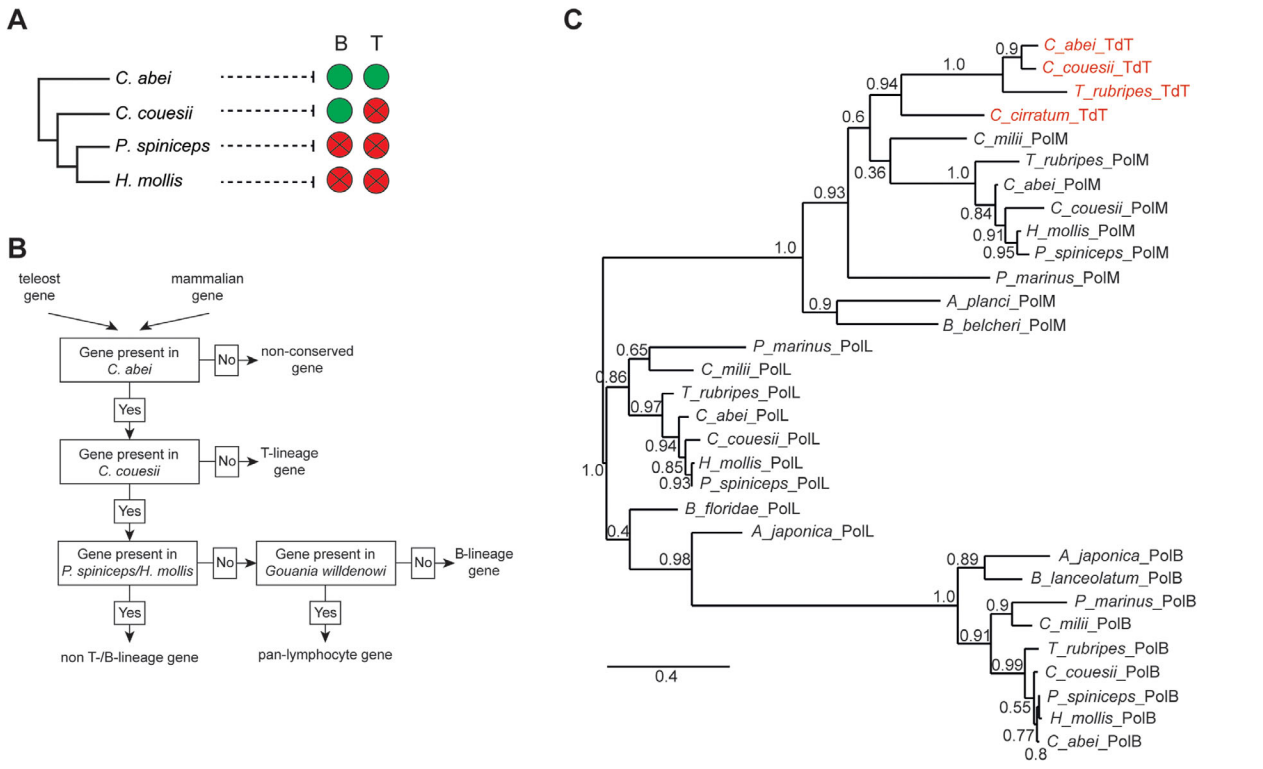

Figure 1. Lymphocyte pathway analysis through phylogeny. (A) Schematic cladogram of the four anglerfish species used in this study. (B) Decision tree to evaluate the lineage specificity of individual genes, based on the genome analysis of *C. abei*, *C. couesii*; *P. spiniceps/H.mollis*, and *G. willdenowi*. (C) Cladogram of PolX gene family members generated by the PhyML 3.1/3.0 aLRT algorithm; the branch support values are indicated. For PolM, PolL, and TdT, only the polymerase domain sequences were used for alignment; for sources of protein sequences, see Materials and methods. TdT sequences are highlighted in red font.

related family of Lophiiformes, serves as a benchmark for the comparative analysis since it possesses the canonical set of genes essential for adaptive immunity. *Cryptopsaras couesii* lacks the T cell lineage, but retains the key components of the canonical B cell lineage; *Photocorynus spiniceps* and *Haplophryne mollis* both lack T cells and B cells [8]. From this constellation, we expect that a gene strictly associated with the T cell lineage (and thus without any other non-redundant function in the developing or adult organism) should be present in *C. abei*, but be either non-functional or missing in the genomes of *C. couesii*, *P. spiniceps*, and *H. mollis*. Likewise, a gene that functions in the B lymphocyte lineage should be present in the genomes of *C. abei* and *C. couesii*, but be either non-functional or missing in the genomes of *P. spiniceps* and *H. mollis*; genes that have a function in both T and B cells (such as the *rag1* and *rag2* genes) should also be absent in *P. spiniceps* and *H. mollis*. As no anglerfish species was found to definitively lack B cells, but retain T cells, analysis of the *G. willdenowi* genome was included to resolve ambiguity in identifying pan-lymphocyte and B lymphocyte-lineage genes (Fig. 1B).

Validation of the LYPAP strategy

To explore the usefulness of the LYPAP strategy, we examined the status of two potentially lineage-specific genes. The interleukin 7 receptor (il7r) is a key component of homeostatic cytokine signaling in T cells [11–13] of all species so far investigated; because its requirement for B cell development varies among species [14–16], we asked whether it is required for teleost B cell development. We find that the *il7r* gene is present in the genome of *C. abei*, but is absent in the genomes of the three other species (Table 1; Supporting Information S1). Hence, in teleosts, the function of *il7r* is not essential for B cell development; expression studies in zebrafish support this view (Supporting information Fig. S1A and B). Thus, LYPAP correctly identified a T cell lineage-specific function of *il7r* in Ceratoidei.

Next, we examined the role of the *slp65* gene, encoding an adaptor protein (also known as BLNK) orchestrating the formation of a signaling complex that transmits engagement of the B cell receptor with antigen to the nucleus; in mice, *Slp65* deficiency impairs B cell development, whereas T cell development is unaffected [17]. Genome analyses indicate that *slp65* is present in *C. abei* and *C. couesii*, but absent in intact form in *P. spiniceps* and *H. mollis*, where it is found in pseudogenized form (Table 1; Supporting information Fig. S1C; Supporting information S1). Of note, whereas the expression pattern (Supporting information Fig. S1A and D) would suggest a pleiotropic function of *slp65* in the hematopoietic system, the analysis of gene models via LYPAP reveals that *slp65* is essential only for lymphocyte development. Indeed,

Table 1. Cell type and gene content of anglerfish genomes.

Cell type/gene	Species *C. abei*	*C. couesii*	*H. mollis*	*P. spiniceps*
T cells	+	−	−	−
B cells	+	+	−	−
il7r	+	−	−	−
slp65	+	+	−	−
fli1a	+	+	+	+
fli1b	+	+	+	+
atad5a	+	+	+	+
atad5b	+	+	(+)	(+)

Note: The presence of an intact gene is denoted by "+"; absence or pseudogenization is indicated by "–"; (+) indicates a gene with alterations of uncertain functional consequence. The accession numbers for nucleotide sequences are listed in Supporting information Table S1; alignments of conceptually translated atad5b protein sequences are shown in Supporting information Fig. S3.

its presence in the *G. willdenowi* genome (Genbank accession number XM_028468772) assigns a pan-lymphocyte function to *slp65*.

In a second application, we turned to the validation of results from forward genetic screens, during which we identified many components of the genetic network underlying larval T cell development in zebrafish [13]. We used LYPAP to ask whether the identified candidate genes (and/or their paralogs) have T lineage-specific or pleiotropic activity. By way of example, we addressed two paralogous gene pairs, *fli1a/fli1b*, and *atad5a/atad5b*, for which *fli1a* and *atad5a* were identified in the screens [13] (Supporting information Fig. S2A and B). As summarized in Table 1, *fli1a* and *fli1b* (Supporting information S1) can be found even in those genomes that lack canonical adaptive lymphocyte lineages, suggesting that they are required in lineages other than lymphocytes, in line with scRNA-seq expression analysis of zebrafish whole kidney marrow cells (Supporting information Fig. S2C–E). With respect to *atad5a* and *atad5b* genes, we found that both *atad5a* and *atad5b* are present in all four species. However, the *atad5b* genes of *H. mollis* and *P. spiniceps* lack exon 2, which encodes two short disordered regions (Supporting information Fig. S3); since no abnormality was noted in the T cell centered-screen at 5 dpf (Supporting information Fig. S2B), this gene may not be required for larval lymphopoiesis (c.f., Supporting information Fig. S2C and G).

Investigation of the *PolX* gene family

Finally, we applied the LYPAP paradigm to examine a long-standing question about the roles of the members of the DNA polymerase X (PolX) family during somatic antigen receptor diversification. The PolX family has four members, PolB, POLM, POLL, and terminal deoxynucleotidyl transferase (TdT; encoded by *dntt*); PolM, PolL, and TdT share a characteristic BRCT domain that is lacking in PolB [18]. TdT is a major contributor to both immunoglobulin (Ig) and T cell receptor gene diversification during V(D)J recombination [19, 20]; in addition, PolL and PolM have been shown to augment the junctional diversification of immunoglobulin heavy and light genes, respectively in mice [21], raising the question of whether the latter activities are a species-specific phenomenon or represent a constitutive part of the somatic diversification process of antigen receptor genes. Phylogenetic analysis (Fig. 1C) supports the view that TdT is characteristic of jawed vertebrates [22]. Interestingly, *P. spiniceps* and *H. mollis*, which lack intact *rag1*, *rag2, ig* and *tcr* genes [8], possess *polB*, *polL* and *polM* genes, but lack *dntt* (Table 2; Supporting information S1). The LYPAP analysis thus not only defines TdT as a uniquely lymphocyte-specific polymerase[23] but also indicates that the functions of PolL and PolM are not essential for V(D)J recombination (as recognized in mice).

Conclusion

Here, we have introduced the concept of LYPAP, suggesting that comparative analyses of genomes of species that lack one or both of the canonical adaptive lymphoid lineages are an opportunity to uncover the genetic underpinnings of adaptive immunity. The trait-specific phylogenetic analysis as exemplified here should be generalizable to other aspects of immune-related phenotypes; with deeper phenotyping of vertebrate species and analysis of their associated genome resources[24] this approach will become even more useful.

Materials and methods

Animals

The zebrafish (*Danio rerio*) strains Ekkwill (EKK), Tüpfel long fin (TL), wild-type-in-Kalkutta (WIK), AB, Assam (ASS), and Tubingen (TU) were maintained in the animal facility of the Max Planck Institute of Immunobiology and Epigenetics. All animal experiments were approved by the institute's review committee and conducted under licenses from the local governments (Regierungspräsidium Freiburg [AZ 35–9185.81/G-

Table 2. Comparative analysis of lymphoid cell types and *PolX* genes.

Cell type/gene	Species *Tunicate*	*Lamprey*	*Shark*	*C. abei*	*C. couesii*	*H. mollis*	*P. spiniceps*
T cells	n/a	+	+	+	−	−	−
B cells	n/a	+	+	+	+	−	−
polb	+	+	+	+	+	+	+
poll	+	+	+	+	+	+	+
polm	+	+	+	+	+	+	+
dntt	−	−	+	+	+	−	−

19/69; AZ 35–9185.81/G-14/41; AZ 35–9185.81/G-17/ 79; AZ 35–9185.81/G-13/70]; Regierungspräsidium Tübingen [AZ AP1/02]).

Zebrafish morphants

Morpholino antisense oligonucleotides (morpholinos) targeting the sequences of initiation codons (to block translation of both maternal and zygotic mRNAs) of target gene mRNAs were designed by and sourced from GeneTools, LLC. Lyophilised morpholinos were resuspended in nuclease-free water at a concentration of 1mM and stored at 4°C. Morpholinos diluted in 1× Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM CaCl2, 5 mM HEPES, pH 7.6) were titrated and injected in a volume of 1–2 nL into wild-type embryos at the 1-cell stage as described previously [13]. The phenotypes of morphants were determined by RNA in situ hybridization, comparing the *rag1/gh* ratio (thymopoietic index; see below) of injected versus un-injected fish at 5 dpf. The sequences of anti-sense morpholinos are as follows. *fli1a*: 5-cgcctccttaatagttccgtccatt; *fli1b*: 5′-agtacagtccattgcagatttctgt; *atad5a*: 5′-ggcaatgcaacaaccccagccatct; *atad5b*: 5′-acgaatccattgttgccataaatat.

Thymopoietic index

Thymic *rag1* gene expression is a marker of ongoing assembly of T cell receptor genes. Hence, the intensity of the RNA in situ signal correlates with the number of differentiating T cells, which we consider to be a measure of T cell development. In order to provide an internal control (technical, with respect to the hybridization process as such; and, biological, with respect to the tissue specificity of the observed genetic effects), we employed a probe specific for the growth hormone (*gh*) gene, which marks a subset of cells in the hypophysis. Determination of *rag1/gh* ratios was carried out as follows: After RNA in situ hybridization with *rag1* and *gh* probes, ventral images of 4–5 dpf zebrafish larvae were taken on an MZFLIII (Leica) microscope using a digital camera DFC300FX (Leica), essentially generating a two-dimensional projection of the 3D structure. The areas of *rag1* and *gh* signals were measured using ImageJ (ImageJ 1.52a; available at http://imagej.nih.gov/ij), and the ratio of average of the *rag1*-positive area vs. *gh*-positive area was calculated as a measure of thymopoietic activity.

Genomic resources

Whole genome sequences of Ceratioidei were described previously[8].

Contig assembly from customized BLAST databases

Genomic sequence collections were searched for gene-specific sequences using the BLASTn algorithm on the SequenceServer BLAST server (version 1.0.9) [25], installed in-house. BLAST parameters were set to an expectation cutoff of 1E−5, allowing a maximum number of 1000 returned sequences. From the resulting hits, contigs were assembled with SeqMan Pro (version 13.0.0, DNASTAR) using a match size of 25 nucleotides and a minimum match percentage of 98% with otherwise default parameters. Contigs were manually curated and used as queries against the non-redundant National Center for Biotechnology Information protein database using the BLASTn and BLASTx algorithms to identify gene-specific regions. The DNA and protein alignments were performed using the MUSCLE alignment algorithm in DNASTAR's MegAlign Pro applications [26] and other sequence analysis tools [27]. Gene models of anglerfish genes were determined using *T. rubripes* (or orthologous teleost) protein sequences as templates for tBLASTN searches and assembled into virtual cDNA sequences (Genbank accession numbers are listed in Supporting information Table S1).

Phylogenetic analysis

For phylogenetic analysis, the PhyML 3.1/3.0 aLRT algorithm implemented at the pyhlogeny.fr platform was used with default parameters [28]. Apart from sequences tabulated in Supplementary Data 1 (conceptually translated from the virtual cDNA [Supporting information Table S1]), the following sequences were retrieved from Genbank. PolB sequences (Anneissia japonica: XP_033124559; Branchiostoma lanceolatum: CAH124683; Callorhinchus milii: XP_042200499; Petromyzon marinus:

XP_032817546; Takifugu rubripes: XP_003974975). PolL sequences (Anneissia japonica: XP_033125579; Branchiostoma: XP_035671919; Callorhinchus milii: XP_007909582; Petromyzon marinus: XP_032808362; Takifugu rubripes: XP_003972614). PolM sequences (Acanthaster planci: XP_022091844; Branchiostoma belcheri: XP_019635777; Callorhinchus milii: XP_042200446; Petromyzon marinus: XP_032807934; Takifugu rubripes: XP_003965471). TdT sequences (Ginglymostoma cirratum: AAG53984; Takifugu rubripes: NP_001027915).

Single–cell RNA sequencing data analysis

Clustering analysis and visualization of all datasets [29] were performed by the VarID algorithm [30]. The Uniform manifold approximation and projection for dimension reduction representation was used for cell cluster visualization [31]. Information on how to generate the Uniform manifold approximation and projection for dimension reductions for the genes shown in Supporting information Figs. S1 and S2 can be retrieved from GitHub (https://github.com/ElliotBoehm/LYPAP).

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Data availability statement: Whole genome sequences of Ceratioidei were described previously [8]; Genbank accession numbers of relevant sequences are listed in Supporting information Table S1.

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Abbreviations: **Il7r**: interleukin 7 receptor · **LYPAP**: lymphocyte pathway analysis through phylogeny

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