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Abstract: This review will outline what is known about the origins and evolution of type 2 cytokines and their receptors in vertebrates. It takes advantage of the recent advances made in gene identification from the many vertebrate genomes that have now been sequenced. It will also describe what functional studies have been performed to date, giving clues to the role of these molecules and signalling pathways in non-mammalian vertebrates.

15th March 2015

Editor, Cytokine.

Dear Editor,

Please find enclosed our invited review article on "The evolution of IL-4 and IL-13 and their receptor subunits", which we submit for publication in the special issue of Cytokine on "IL-4 and IL-13". We hope you like the article and find it suitable for publication in the special issue.

Yours sincerely,

Chris Secombes

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The evolution of IL-4 and IL-13 and their receptor subunits

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Abstract

This review will outline what is known about the origins and evolution of type 2 cytokines and their receptors in vertebrates. It takes advantage of the recent advances made in gene identification from the many vertebrate genomes that have now been sequenced. It will also describe what functional studies have been performed to date, giving clues to the role of these molecules and signalling pathways in non-mammalian vertebrates.

1. Introduction

Interleukin (IL)-4 and IL-13 are canonical type 2 cytokines, that play overlapping but distinct roles in mammalian immune responses to extracellular parasites, via production of high affinity IgE, the generation of alternatively activated macrophages and the differentiation of Th2 cells [1]. Whilst their amino acid (aa) identity is low (~23%) they can bind to a common receptor composed of the IL-4Rα and IL-13Rα1 subunits (type II receptor), although they can individually bind to the type I receptor composed of the IL-4Rα and γC (CD132) subunits (IL-4), or to the IL-13Rα2 receptor (IL-13). IL-4 and IL-13 are found side by side in the mammalian genome, suggesting they arose from a tandem duplication event at some point in vertebrate evolution. In this review we will examine the evidence for the presence of these cytokines and receptors in other vertebrate groups, with a focus on the Gnathostomes (jawed vertebrates) in which RAG-mediated adaptive immunity arose.

2. IL-4, IL-13 and IL-4/13 molecules

With the relatively low homology seen between IL-4 and IL-13 within mammals (eg 42% - 57% aa identity for IL-4 and 53% - 64% aa identity for IL-13), it is not surprising that it has been difficult to find these genes or their homologues in other vertebrate groups. In essence it became possible to search the IL-4 locus in detail once genomes became available, taking advantage of the presence of genes such as KIF3A and RAD50, that are on either side of IL-4 and IL-13 in mammals, that are relatively well conserved between different vertebrate groups

(92% - 95% aa identity for human KIF3A and 71% - 77% aa identity for human RAD50 vs chicken, frog and fish equivalents). In particular a considerable number of fish genomes have been sequenced in the last decade and this has been very informative. The first non-mammalian genes were identified in chicken *Gallus gallus*, where sequencing of a bacterial artificial chromosome (BAC) clone allowed the identification of IL-4 and IL-13, together with IL-3, IL-5, GM-CSF and a novel cytokine-like transcript termed KK34 located between GM-CSF and P4HA2 [2]. The locus was subsequently confirmed to be on chicken chromosome 13 [3]. The five known genes are present in the same order relative to neighbouring genes, and have the same transcriptional direction as seen in mammals (Fig. 1), with aa identity to equivalent mammalian genes between 16% - 30%. However, one of the genes, IL-5, could not be cloned from cDNA and appeared to lack a recognisable promoter, prompting the suggestion it could be a pseudogene. KK34 has 20.5% aa identity to the predicted chicken IL-5, and was suggested to be IL-5-like, perhaps functioning in a similar way to IL-5. Although there is no evidence of transcription from its genomic DNA in transfected cells, the chicken IL-5 gene can be transcribed and introns spliced out when it is driven by the CMV promoter. Furthermore, recombinant IL-5 from the CMV driven mRNA can bind to the chicken IL-5Rα, confirming its identity as a chicken IL-5 gene. However, recombinant KK34 does not bind to IL-5Rα, and thus its function remains obscure [4]. Chicken IL-4, IL-5 and IL-13 (amongst others) can be highly up-regulated during disease states, as seen with infectious bursal disease virus (IBDV) infection [5].

IL-4 and IL-13 have since been found in other bird species, such as turkey *Meleagris gallopavo* [6,7] and grey partridge *Perdix perdix* [8], and some aspects of their bioactivities have been studied. For example, chicken rIL-4 and rIL-13 can induce the proliferation of chicken B cells when co-stimulated with CD154 (CD40 ligand) [2]. In macrophages (HD11 cells - an avian macrophage cell line) chicken rIL-4 activates NO synthesis but rIL-4 pre-treatment suppresses the NO response to subsequent stimulation with microbial PAMPs [9]. However, rIL-4 pre-treatment enhances the oxidative burst (ROS) response when HD11 cells are exposed to *Salmonella enteriditis*. rIL-4 has also been shown to up-regulate CCR9 expression (~400-fold) in splenocyte/thymocyte cultures but not in either population alone [10]. When such treated cells, labelled with CFSE, were injected into 5 week old chicks of the same MHC haplotype, they were found to preferentially migrate to the caecal tonsils (a gut associated lymphoid tissue). Chicken rIL-4 has also been shown to drive dendritic cell maturation in combination with GM-CSF when added to chicken bone-marrow cells [11]. A

few studies have also used cloned IL-4 in an expression plasmid to examine IL-4 function. Chicks from eggs injected with IL-4 containing plasmids were found to have enhanced anti-coccidia immune responses [12] and had larger caecal tonsils, increased numbers of CD8+ cells in the caecal tonsils and a higher CCR9 mRNA level in caecal tonsils compared to control chicks [10]. Lastly, co-administration of IL-4 to chickens in a plasmid vector (pVIVO2) also containing two genes of Newcastle disease virus as a DNA vaccine (injected intramuscularly on three occasions), led to higher levels of IgY and higher protection (40% vs 10%) compared to the DNA vaccine without IL-4 [13], suggesting it may have value as a molecular adjuvant.

In 2007 a gene with relatedness to IL-4 and IL-13 was discovered in the pufferfish (*Tetraodon nigroviridis*) genome by Li et al. [14]. This gene was next to RAD50 and was initially reported to be an IL-4 like gene, but the very low homology (11-16% aa identity) made it difficult to be sure of its true identity, although clearly a gene related to type 2 cytokines. A second IL-4 like gene was also discovered in zebrafish *Danio rerio* at a different locus [15], next to KIF3A. Subsequent analysis of these two loci has revealed they likely arose as a consequence of the third round (3R - 1R and 2R occurred at the base of the vertebrate lineage) whole genome duplication (WGD) event that happened in the teleost fish ancestor [16], and it was proposed to call these two genes IL-4/13A and IL-4/13B [17]. Within individual 3R teleost species the number of IL-4/13 genes can be increased by local duplication events, as seen with medaka *Oryzias latipes* IL-4/13A [17] and in seabass *Dicentrarchus labrax* (unpublished). In addition, due to a fourth (4R) WGD in some teleost lineages there can be further duplication of the loci, giving additional copies as seen in salmonids that have two copies of IL-4/13B (unpublished). More recently the genomes of several 2R bony fish have been sequenced, and in species such as the spotted gar *Lepisosteus oculatus* it is apparent that a single IL-4/13 gene exists between KIF3A and RAD50 (Fig. 1). Most recently the elephant shark *Callorhinchus milii* genome has been sequenced and is the first cartilaginous fish genome to be analysed [18]. Whilst initial analysis of the type 2 cytokine locus could not find any apparent homologs, subsequent analysis revealed the presence of at least two IL-4/13 genes between KIF3A and RAD50 [19,20]. Two further genes in the *C. milii* genome described as IL-5 like (IL-5A and IL-5B) were also reported by Dijkstra [19] immediately upstream of the IL-4/13 locus. However, this has been contested [21] and the jury is still out as to their origin and function. Analysis of an amphibian genome (the frog *Xenopus tropicalis*, genomic scaffold_3) revealed a well conserved synteny at the

KIF3A/RAD50 locus between frog and spotted gar with an IL-4/13 gene linked to KIF3A and potentially another IL-4/13 gene linked to RAD50, although this needs to be confirmed by cloning and functional studies (Fig. 1). Whilst there was a flip-over of both KIF3A and RAD50 related genes on the chromosome, the transcriptional directions of the frog IL-4/13 genes relative to KIF3A or RAD50 are the same as seen in other vertebrates. Taken overall, it seems likely that a single IL-4/IL-13 gene existed in ancestral Gnathostomes, which has been duplicated in different lineages by WGD and/or tandem duplication events.

Multiple alignment of the aa sequences of selected IL-4, IL-13 and IL-4/13 proteins reveals that in general four cysteine residues are present in each protein but the patterns of the cysteine residues are lineage-specific, ie mammalian IL-4 and IL-13, teleost fish IL-4/13, and other vertebrate IL-4/13 (Fig. 2). Two cysteine residues (C3 and C9, Fig. 2) distinguish the bony fish (including the 2R spotted gar) IL-4/13 molecules from IL-4 and IL-13 (Fig. 2), as reported previously [17], and the IL-4/13 molecules from other vertebrates presented here.

Few studies have looked at the function of these fish IL-4/13 molecules. In zebrafish injection of fish with rIL-4/13A results in higher numbers of peripheral blood leucocytes (PBL) that express the IgZ-2 isoform [22] after two days, or DC-SIGN after 5 days [23]. rIL-4 administration into zebrafish for three days also increased IgM^+ B cell number in PBL (from ~11% in PBS injected fish to ~35% in fish given 1 μg rIL-4/13A) and the transcript expression of mIgM, MHC class II and CD80 in these cells [24]. In addition, three injections of rIL-4, spaced 12 h apart, prior to immunisation with keyhole limpet hemocyanin (KLH) gave higher serum antibody titres (at day 28) compared to fish given KLH alone, with higher titres seen using 1 μg vs 0.1 or 0.01 μg rIL-4 [24]. High constitutive expression of IL-4/13A has been found in trout mucosal tissues (gill, skin), suggesting an important role at these sites [25]. Expression was mainly found in IgM^- cells when lymphoid cells from the gill were isolated by FACS using a monoclonal antibody (mAb**)** to trout IgM. In experiments carried out *in vitro* with unfractionated head kidney primary cell cultures, it was shown that IL-4/13A was up-regulated by PHA 24 h post-stimulation. These cultures contain lymphoid cells, macrophages and granulocytes. In addition, IL-4/13A has been found to be up-regulated in rainbow trout epidermis 9 days post-infection with *Ichthyobodo necator* compared with uninfected fish, and this was associated with up-regulation of GATA3 but not T-bet or FOXP3 [26]. Interestingly, a cell line (KoThL5) that expresses IL-4/13B has been

established from carp *Cyprinus carpio* [27]. These cells also express TcR chains, and CD4-1, the four Ig domain containing CD4-like molecule present in fish [28], and thus have a phenotype similar to Th2 cells.

3. IL-4 and IL-13 receptors

As outlined above, there are four receptor chains, namely IL-4Rα, IL-13Rα1, IL-13Rα2 and γC, that form three types of receptors for IL-4 and IL-13 in mammals. The γC is also a subunit of the receptors for IL-2, IL-7, IL-9, IL-15 and IL-21 [29]. As with the ligands, it has become clear that these receptor chains exist throughout the jawed vertebrates (Fig. 3).

γC was one of the first cytokine receptor chains to be found outwith mammals, in both fish (trout) and birds (chickens). In chickens the gene organisation was very similar to that in mammals, with an 8 exon/ 7 intron structure, although intron 1 was relatively large in the chicken gene and the other introns relatively small [30]. It was also discovered that an alternatively spliced variant existed, where inton 5 was retained, called chicken γC-b (chγC-b). This means two transcripts of ~1.4 kb (chγC-a) and ~1.5 kp (chγC-b) can be detected by Northern blot analysis. Both forms were highly expressed in lymphoid tissues such as spleen, thymus, bursa and caecal tonsils, and in both IgM^+ and IgM^- cell populations from spleen. However, when splenic lymphocytes were stimulation with ConA *in vitro* there was a switch to just chγC-a at 24 h - 48 h post-stimulation. Mapping of the chγC ectodomains has identified two conserved fibronectin type III domains and a residue (Q^{96}) critical for IL-2 binding [31]. mAb to chγC have shown that ~3% of splenic mononuclear cells express γC, and that this is increased to 18% after ConA stimulation. Curiously, FACS analysis of different γC expressing cell populations during IBDV infection has shown that $\gamma C^+/CD8^+$ cells are decreased in the bursa whereas $\gamma C^+/CD25^+$ cells are increased and $\gamma C^+/CD4^+$ cells show no change [32]. Thus the balance between the different γC expressing lymphoid populations may determine the outcome of infection. γC has also been cloned in other bird species more recently, including duck *Anas platyrhynchos* and Japanese quail *Coturnix japonica* [33,34], and it appears that alternative splicing to retain intron 5 is a common feature in birds. However, in ducks the insertion of intron 5 led to a frameshift that altered the hydrophobic profile of the downstream transmembrane domain (encoded in exon 6), with the

potential to make a soluble form of γC in this species. Whilst the transcripts were again most apparent in lymphoid tissues of duck and quail, γC-a was the major transcript in both species.

In fish γC was first identified in rainbow trout [35], where it was shown to be highly expressed in tissues such as blood, spleen, gill and kidney. In trout macrophage cultures (primary cells and/or RTS-11 cells) it was shown that γC expression could be up-regulated by stimulation with trout rIL-1β and LPS. Subsequent analysis of the zebrafish genome has revealed that two genes for γC are present [36], and this appears to be a common phenomenon in fish (Fig. 3) with two γC genes now known to be also present in trout, tilapia, gar and elephant shark [18,37]. Whilst the two γCs in elephant shark, spotted gar and tilapia are linked at the same chromosome, zebrafish γCs are on different chromosomes (ch 10 and 14). In trout the two genes (γC1 and γC2) have 89% aa identity, whilst in zebrafish they have 28% aa identity, and this hints that the mechanisms giving rise to the increased gene number are likely different in different species/fish groups. Comparative expression analysis of trout γC1 and γC2 showed that in general γC1 had a higher expression level, and that both genes were most highly expressed in thymus and spleen. γC was also relatively highly expressed in RTS-11 cells (compared to other trout cell lines) and a small increase in γC1 expression was seen in these cells after LPS or poly I:C stimulation [37].

The IL-4Rα has also been identified in birds and fish [36,38,39]. Analysis of the chicken and zebrafinch *Taeniopygia guttata* genomes for elevated allelic diversity revealed that the IL-4Rα gene had an enhanced rate of nonsynonymous substitutions [40]. After sequencing of the gene in 6 related bird species, 70 global village chickens and 20 commercial broilers, it was concluded that there are a number of sites which are under positive selection. Since aa substitutions in the human IL-4Rα can affect disease susceptibility [41], this phenomenon in chickens may reflect selection of specific variants in response to pathogen challenge. In trout, as with γC, two paralogues of IL-4Rα (IL-4Rα1 and IL-4Rα2) are found that share ~85% aa identity. Whilst the trout aa sequence generally shows conservation of the known IL-4Rα domain structure, the WSXWS motif in domain 2 is missing and the intracellular domain is relatively short, lacking the box 2 motif and two (Y3 and Y5) of five conserved tyrosine residues in the mammalian sequences [39]. In addition, an ITIM motif in mammalian IL-4Rα molecules is only present in trout IL-4Rα2. In general IL-4Rα2 was more highly expressed than IL-4Rα1, in a wide range of tissues, and in RTS-11 cells. Both genes could be up-regulated in RTS-11 cells by poly I:C, LPS and trout rIFNγ, with IL-4Rα2 being more

responsive. In head kidney primary leucocyte cultures rIFN-γ could also induce expression of IL-4Rα2 but not IL-4Rα1, whilst PHA could induce both genes. However, these genes were refractory to stimulation by LPS or poly I:C in the primary cultures.

In zebrafish IL-4Rα exists as two isoforms located ~ 36 kb apart on chromosome 3 [24]. They differ in that one of the isoforms (DrIL-4Rα-iso) has an early stop codon in the last exon (exon 11) that prevents the translation of the last 203 aa of the intracellular tail. In addition, with both genes an alternatively spliced variant can be produced by retaining "pseudo"exon 7 that is normally spliced out, and this results in a premature stop of translation at the end of exon 6. These variants contain the extracellular domain but lack the transmembrane domain and intracellular region, and thus may represent soluble forms of the two receptor isoforms. Whilst the membrane bound forms were found to be expressed in a wide range of tissues, the putative secreted forms were only found constitutively expressed in liver, brain and muscle. A pull down assay confirmed that zebrafish rIL-4/13A could bind with rIL-4Rα and administration of the soluble form of rIL-4Rα or an anti-IL-4Rα Ab (for receptor blockade) was found to neutralise the biological effects of rIL-4/13A administration in vivo (described above). Lastly, dual labelling of blood leucocytes showed the presence of IgM^+/IL-4Rα^+ B cells.

IL-13Rα1 and IL-13Rα2 are less well studied but also present throughout the jawed vertebrates [18,36,39] (Fig. 3). In chickens IL-13Rα2 has 37% - 39% aa identity to mammalian genes, and is highly expressed in liver, gonads and brain [42]. Transcript expression in a monocytic chicken leukemia cell line (IN24) could be induced by LPS stimulation. The extracellular domain contains three potential glycosylation sites, and that the molecule can be glycosylated was confirmed by incubation of LPS stimulated IN24 cells with/without tunicamycin to block N-linked glycosylation. In the presence of tunicamycin a protein of ~41 kDa was detected with an anti-chicken IL-13Rα2 Ab, whilst in the absence of tunicamycin a protein of ~45kDa was detected by Western blot analysis. That IL-13Rα1 can be up-regulated by infection has also been demonstrated, in chicken cell cultures [43,44].

In fish the IL-13Rα2 gene was first discovered in trout [45], with 31% aa identity to human IL-13Rα2, and high constitutive expression was seen in gill, spleen and head kidney. Subsequent studies revealed that two genes of both IL-13Rα1 and IL-13Rα2 are present in this species [39]. Both paralogues of IL-13Rα2 are similar (79% aa identity) but IL-13Rα1b

lacks the N-terminal S-type Ig domain (D1) and so the identity is lower (34%) to IL-13Rα1a. Since the S-type Ig domain is critical for binding of IL-13 but not IL-4 in mammals, its absence may allow discrimination between the fish IL-4/13A and IL-4/13B molecules. The highest constitutive expression levels IL-13Rα1a were seen in scales, gills and skin, whilst the highest expression of IL-13Rα1b was seen in ovary. The two genes showed differential responses to stimulation in different cells lines. For example, poly I:C induced IL-13Rα1a expression in all cell lines studied, with highest induction seen at 24 h post-stimulation. In contrast trout rIFN-γ induced IL-13Rα1b and maximal responses to poly I:C were often seen earlier. In the case of the IL-13Rα2 paralogues, highest constitutive expression of IL-13Rα2a was seen in spleen, head kidney and mucosal tissues, whilst IL-13Rα2b was highest in ovary, kidney and liver. However, no expression of either gene was detectable in RTS-11 cells and IL-13Rα2b expression was undetectable in all cell lines studied.

4. Conclusions

It is clear that the known IL-4/IL-13 ligands/receptors exist throughout the jawed vertebrates, with multiple copies present for some genes in some species. The receptor genes likely arose from an expansion of ancestral dome and gp130-like receptors present in invertebrates, correlated with the appearance of adaptive immunity [36]. The origin of the ligands is less clear but at least one ancestral type 2 cytokine was present in early jawed vertebrates, that subsequently expanded in different lineages by tandem duplication and WGD events, to give rise ultimately to the complex Th2 cytokine locus present in birds and mammals. This predicts that Th2 type cells will exist throughout the vertebrates, to control Ig production and the activation of macrophages via the alternative route. Whilst these responses are present in fish, their regulation is still to be discovered and whether alternative mechanisms/ cell populations exist for their control via the type 2 cytokines that exist will be fascinating to determine.

Figure legends

Figure 1. Gene synteny at the Th2 loci across vertebrates. The information for frog and elephant shark is extracted from NCBI genomic sequence NW_004668234 (frog *Xenopus (Silurana) tropicalis*) and NW_006890145 (shark *Callorhinchus milii*). The KIF3A/IL-4/IL-13/RAD50 loci in other vertebrates were analysed using the Genomicus program (http://www.genomicus.biologie.ens.fr/genomicus, database release-78.01). The spotted gar locus was used as a reference with genes on the left of KIF3A/IL-4/IL-13/RAD50 highlighted with a yellow background and genes on the right highlighted with a blue background. KIF3A and RAD50 are highlighted in red. The IL-4, IL-13 and IL-4/13 genes are highlighted in green, with further IL-4/13 related genes and other homeotherm cytokine genes (IL-3, IL-5, CSF2, KK34) highlighted in black.

Figure 2. Multiple alignment of mammalian IL-4 and IL-13, and IL-4/13 related molecules from bony fish and other vertebrates (A) and the patterns of cysteine residues in different vertebrate groups (B). The multiple alignment was produced using ClustalW, and conserved amino acid residues were shaded using BOXSHADE. The spotted gar IL-4/13 gene was predicted on chromosome LG6 using FGENESH program. The IL-4/13A and IL-4/13B molecules of tetraodon, fugu and stickleback were reported by Ohtani et al. [17]. The accession numbers for other sequences used for this alignment are B3IWZ9 (zebrafish IL-4/13A), D1YSM1 (zebrafish IL-4/13B), I0IV50 (carp IL-4/13A), H1AFL4 (carp IL-4/13B), XP_007900235 (shark IL-4/13A), XP_007900233 (shark IL-4/13B), XP_006023629 (alligator IL-4/13), A9JPI4 (xenopus IL-4/13), C4PAF0 (chicken IL-4), C4PA62 (chicken IL-13), P05112 (human IL-4), P07750 (mouse IL-4), P30367 (cow IL-4), P35225 (human IL-13), P20109 (mouse IL-13), and Q9XSV9 (cow IL-13).

Figure 3. Neighbour joining (NJ) phylogenetic tree of the subunits of IL-4/IL-13 receptors (IL-4Rα, IL-13Rα1, IL-13Rα2, γC). The tree was constructed using an amino acid multiple alignment and the NJ method within the MEGA6 program [46]. The evolutionary history was inferred by using the method based on the JTT matrix-based model using pair-wise deletion option. The percentage of trees in which the associated taxa clustered together is shown next to the branches based on 10,000 bootstrap replications. The accession number for each sequence is given after the common species name and molecular type.

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Figure1

Fig. 1 Wang & Secombes

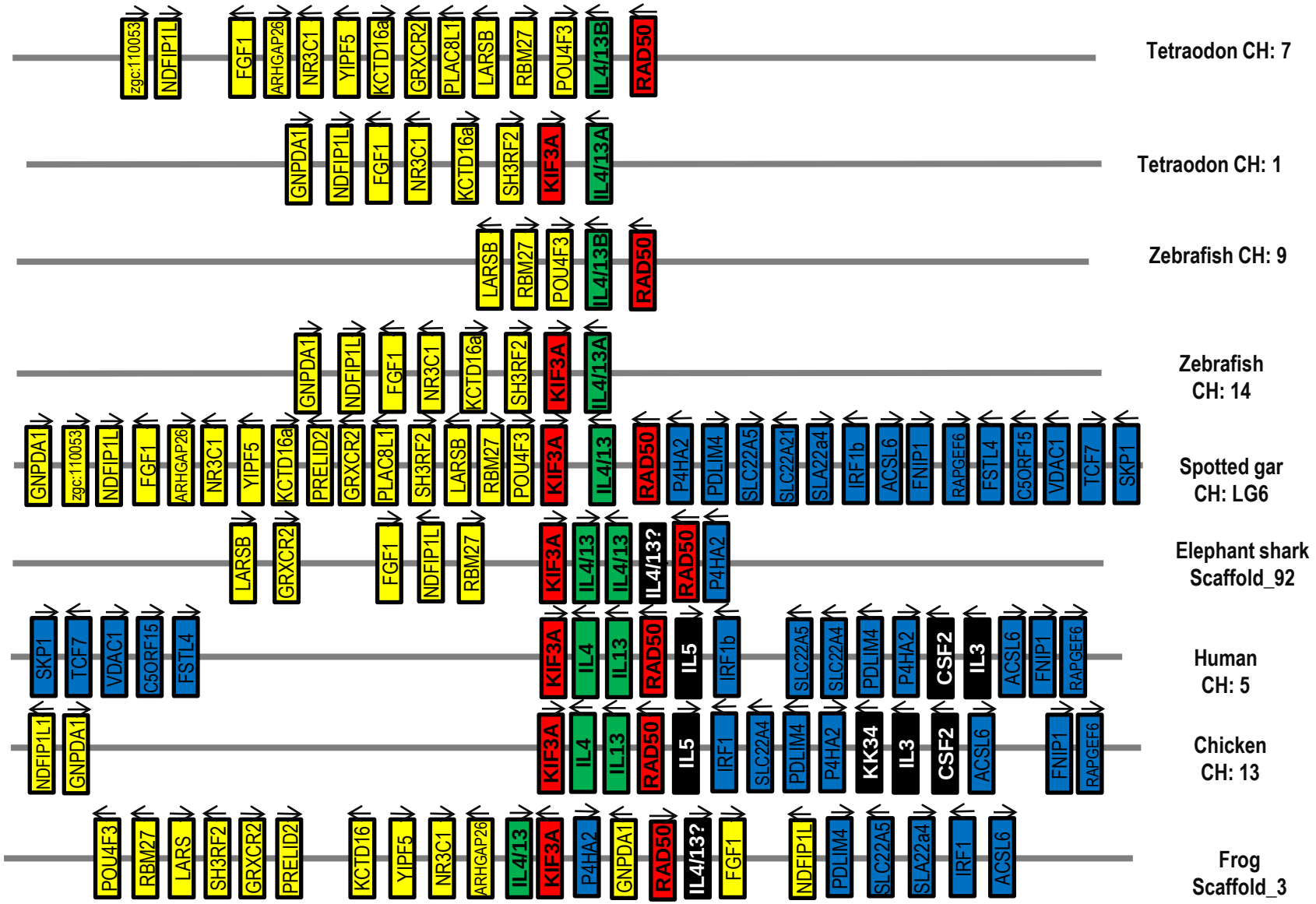
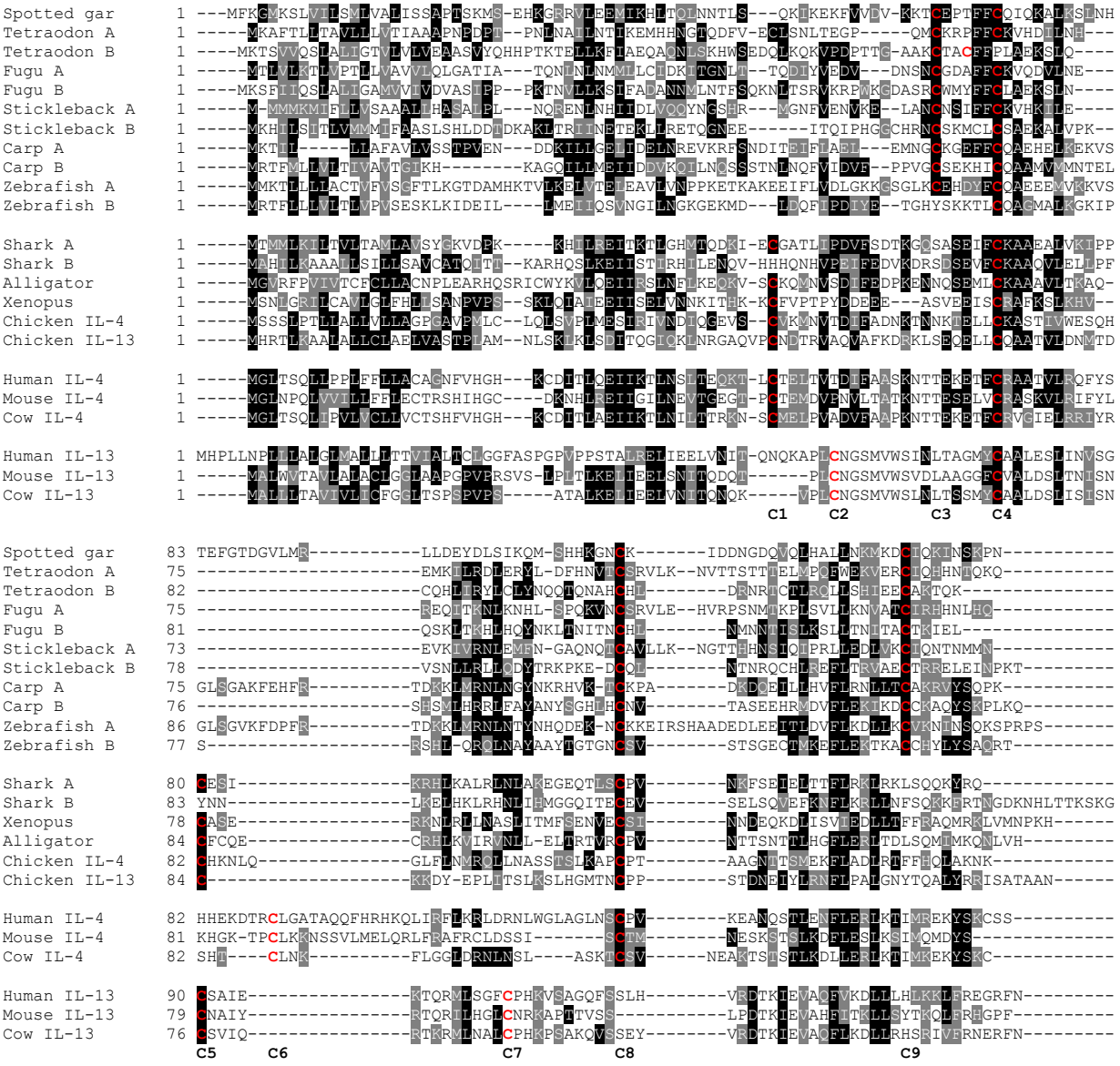


Figure 2

Fig. 2 Wang & Secombes

A.



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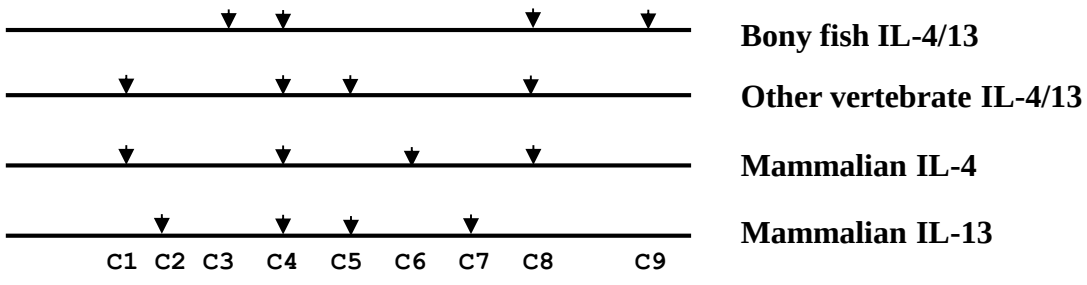

Figure 3
Fig. 3