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Evolution of the Rax family of developmental transcription factors in vertebrates

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ABSTRACT

Rax proteins comprise a small family of paired-type, homeodomain-containing transcription factors with essential functions in eye and forebrain development. While invertebrates possess only one *Rax* gene, vertebrates can have several *Rax* paralogue genes, but the evolutionary history of the members of the family has not been studied in detail. Here, we present a thorough analysis of the evolutionary relationships between vertebrate *Rax* genes and proteins available in diverse genomic databases. Phylogenetic and synteny analyses indicate that *Rax* genes went through a duplication in an ancestor of all jawed vertebrates (Gnathostomata), giving rise to the ancestral vertebrate *Rax1* and *Rax2* genes. This duplication event is likely related to the proposed polyploidisations that occurred during early vertebrate evolution. Subsequent genome-wide duplications in the lineage of ray-finned fish (Actinopterygii) originated new *Rax2* paralogues in the genomes of teleosts. In the lobe-finned fish lineage (Sarcopterygii), the N-terminal octapeptide domain of Rax2 was lost in a common ancestor of tetrapods, giving rise to a shorter version of Rax2 in this lineage. Within placental mammals, the *Rax2* gene was lost altogether in an ancestor of rodents and lagomorphs (Glires). Finally, we discuss the scientific literature in the light of *Rax* gene evolution and propose new avenues of research on the function of this important family of transcriptional regulators.
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1. Introduction

The *Rax* - also called *Rx* - gene was first described in three independent reports that appeared in 1997. Furukawa et al. (1997) identified Rax as a paired-type homeodomain protein that was expressed in the anterior neural plate and later in the eyes and hypothalamus of mouse embryos, while Casarosa et al. (1997) described a *Rax* homologue, *Xrx1*, from the frog *Xenopus laevis*. Mathers et al. (1997) described the expression of *Rax* homologues in the mouse, zebrafish and *Xenopus laevis*, showing that expression pattern of the gene was conserved in vertebrates. They also found that engineered mice that lacked *Rax* expression were eyeless and had forebrain abnormalities, establishing *Rax* as a gene with crucial functions in early mammalian development (Mathers et al. 1997). Indeed, a hypomorph *Rax* allele is found in the *eyeless* (*ey1*) mouse, a mutant strain that lacks eyes and optic cups (Tucker et al. 2001). Early surveys identified two or three *Rax* genes in the genomes of some vertebrate model organims like teleost fishes, *X. laevis* and chicken (Mathers et al. 1997; Ohuchi et al. 1999), and a second *Rax*-like gene (called *RAX2* or *QRX*) was later identified in humans (Wang et al. 2004). Interestingly, this second gene is absent from the mouse genome, which harbours only one *Rax* gene (Wang et al. 2004; Zhong and Holland 2011). Both human *Rax* paralogues are important for eye development, since mutations in *RAX* (Voronina et al. 2004; Gonzalez-Rodriguez et al., 2010; Lequeux et al. 2008; Abouzeid et al. 2012) and *RAX2* (Wang et al. 2004; Yang et al. 2015) are associated with absence of eyes or eye defects in human patients.

Apart from studies in mouse mutants and human patients, the function of *Rax* genes has been studied in several vertebrate models, including teleosts like medaka and zebrafish (Winkler et al. 2000; Loosli et al. 2003; Rojas-Muñoz et al. 2005), the frogs *X. laevis* and *X. tropicalis* (Andreazzoli et al. 1999, 2003; Pan et al. 2006; Wu et al. 2009; Giudetti et al. 2014; Fish et al. 2014) and the chicken (Ohuchi et al. 1999; Chen and Cepko 2002). *Rax* genes have also been studied in the context of eye evolution, as in African cychlids (Schulte et al. 2014) and the cavefish, *Astyanax mexicanus* (McGaugh et al. 2014). The interpretation and comparison between all these studies is made difficult by the inconsistent gene names and the lack of a thorough evolutionary analysis and classification of vertebrate *Rax* genes, even when the phylogeny of a few members of the Rax family has been described (Wang et al. 2004; Wu et al. 2009; Schulte et al. 2014). Here, we analyse *Rax* genes and proteins from several model organisms and sequenced genomes and present a thorough picture of the evolutionary history of this important group of transcription factors.

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2. Results

2.1. Analysis of Rax protein sequences

Invertebrates, including cnidarians, protostomes and chordates like amphioxus and sea squirts, possess only one *Rax* gene (Mazza et al. 2010), but vertebrates can have more than one *Rax* paralogue. To obtain a clearer picture of the evolution of the *Rax* gene family, we scoured sequenced vertebrate genomes for *Rax* homologues, paying special attention to species in key phylogenetic positions. In particular, we obtained predicted Rax peptide sequences from species like the Japanese lamprey (*Lethenteron japonicum*), a jawless vertebrate; the elephant shark (*Callorhinchus milii*), a cartilaginous fish; the spotted gar (*Lepisosteus oculatus*), a ray-finned fish representing a sister group to teleost fishes; and the West Indian Ocean coelacanth (*Latimeria chalumnae*) a lobe-finned fish that represents a sister lineage to tetrapods (amphibians, reptiles, birds and mammals). In addition, we also retrieved Rax sequences from teleost fishes and several tetrapod genomes, in particular from the mammalian phylogenetic tree. Fig. 1 shows a schematic tree of vertebrate phylogeny with the main events of *Rax* evolution that are discussed below.

An alignment of vertebrate Rax proteins distinguishes two groups of these proteins, namely a Rax1 and a Rax2 subgroup (Supplementary Fig. S1), which are also evidenced in phylogenetic and genomic analyses (see below). As observed previously (Furukawa et al. 1997; Wu et al. 2009; Mazza et al. 2010), Rax proteins possess three conserved regions of special interest: i) a N-terminal octapeptide motif that is also found in transcription factors of the Pax family, among others; ii) the central homeodomain (HD) region that binds to DNA, and iii) the C-terminal OAR domain, so called due to its presence in the transcription factors Otp, Aristaless and Rax, among others (Furukawa et al. 1997). All Rax proteins in our alignment possess the HD and the OAR domains, but Rax2 proteins of all tetrapods are shorter and lack the octapeptide motif. Since both Rax1 and Rax2 of non-tetrapods have the octapeptide (Suppl. Fig. 1), this indicates that the loss of this segment happened in a lobe-finned fish (Fig. 1).

2.2. Rax phylogeny

To understand the evolutionary history of vertebrate *Rax* genes, we estimated the phylogeny of Rax proteins using the maximum likelihood method as implemented in PhyML 3.0 (Guindon et al. 2010). Statistical support for the branches was estimated using the bootstrap (Felsenstein 1985). Since many Rax proteins lack the octapeptide domain, the alignment used for phylogeny estimation was mostly restricted to the homeodomain and C-terminal regions (including the OAR motif) of the proteins.

The resulting phylogenetic tree (Fig. 2) shows that Rax proteins can be divided into two large groups, which we named the Rax1 and Rax2 subgroups. The Japanese lamprey Rax protein falls within the Rax2 subgroup. No other *Rax* gene could be retrieved from the Japanese lamprey draft genome, and no *Rax* gene was found in the draft genome of the sea lamprey, *Petromyzon marinus* (Smith et al. 2013). Due to the incomplete nature of the genome assemblies of agnathans, however, it cannot be discarded that another *Rax* gene might be identified in the future in this clade. The sequence of the Rax protein of the Japanese lamprey is quite divergent in relation to the other Rax proteins, as indicated by the long branch length of the lamprey Rax in the tree of Fig. 2. It is important to note that the Rax1 and Rax2 subgroups are retrieved even when the lamprey sequence is excluded from the phylogenetic analysis (data not shown), indicating that this divergent sequence is not altering the phylogenetic reconstruction and the resulting tree topology.

As for jawed vertebrates, all species analysed possess Rax1 and Rax2 representatives, except for some mammals (see below). The most basal phylogenetic group is represented by the elephant shark, a cartilaginous fish. The elephant shark possesses two *Rax* genes, one in each subgroup. The basal ray-finned fish, the spotted gar, as well as the basal lobe-finned fish, the coelacanth, also possess one *Rax1* and one *Rax2* representative each. This indicates that the duplication of the *Rax* genes occurred very early in vertebrate evolution, and that the genomes of the ancestors of both teleosts and tetrapods carried two *Rax* genes (Fig. 1). However, teleosts have more than two *Rax* genes; two that belong to the Rax2 subgroup, namely *rx1* and *rx2*, and one member of the Rax1 subgroup, *rx3*. This is true for the zebrafish (*Danio rerio*), medaka (*Oryzias latipes*) and pufferfish (*Tetraodon nigroviridis*), which are shown in the tree in Fig. 2, as well as for other species like the Mexican blind cavefish (*Astyanax mexicanus*), the Nile tilapia (*Oreochromis niloticus*) and other teleosts (data not shown). This observation, together with synteny analyses (see below) suggests that the three *Rax* genes present today in teleosts originated in the whole-genome duplication (WGD) that happened in a teleost ancestor after the divergence of the spotted gar lineage (Fig. 1; Glasauer and Neuhauss 2014). *rx1* and *rx2* were retained after WGD, while one *rx3* paralogue was subsequently lost from the teleost ancestor.

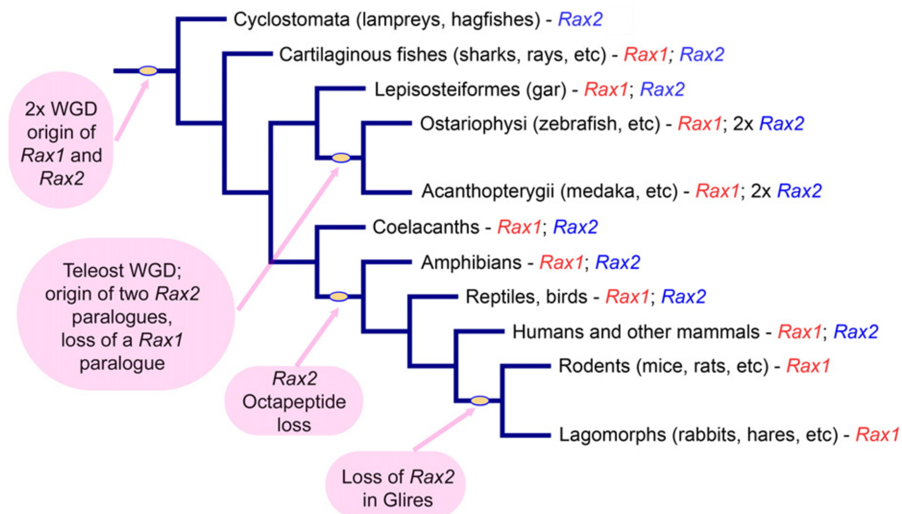

Fig. 1. Rax evolution in vertebrates. The tree schematically represents the phylogenetic relationships of vertebrate clades relevant for the present study. The relative timing of important events in the evolution of *Rax* genes are indicated. Note that the timing of the two rounds of whole genome duplications (2× WGD) in a vertebrate ancestor is still controversial; it is represented here as happening in a common ancestor of cyclostomes and jawed vertebrates (see Smith et al. 2013; Mehta et al. 2013; Smith and Keinath 2015). The presence and number of *Rax1* (red) and *Rax2* (blue) paralogues in each clade is indicated.

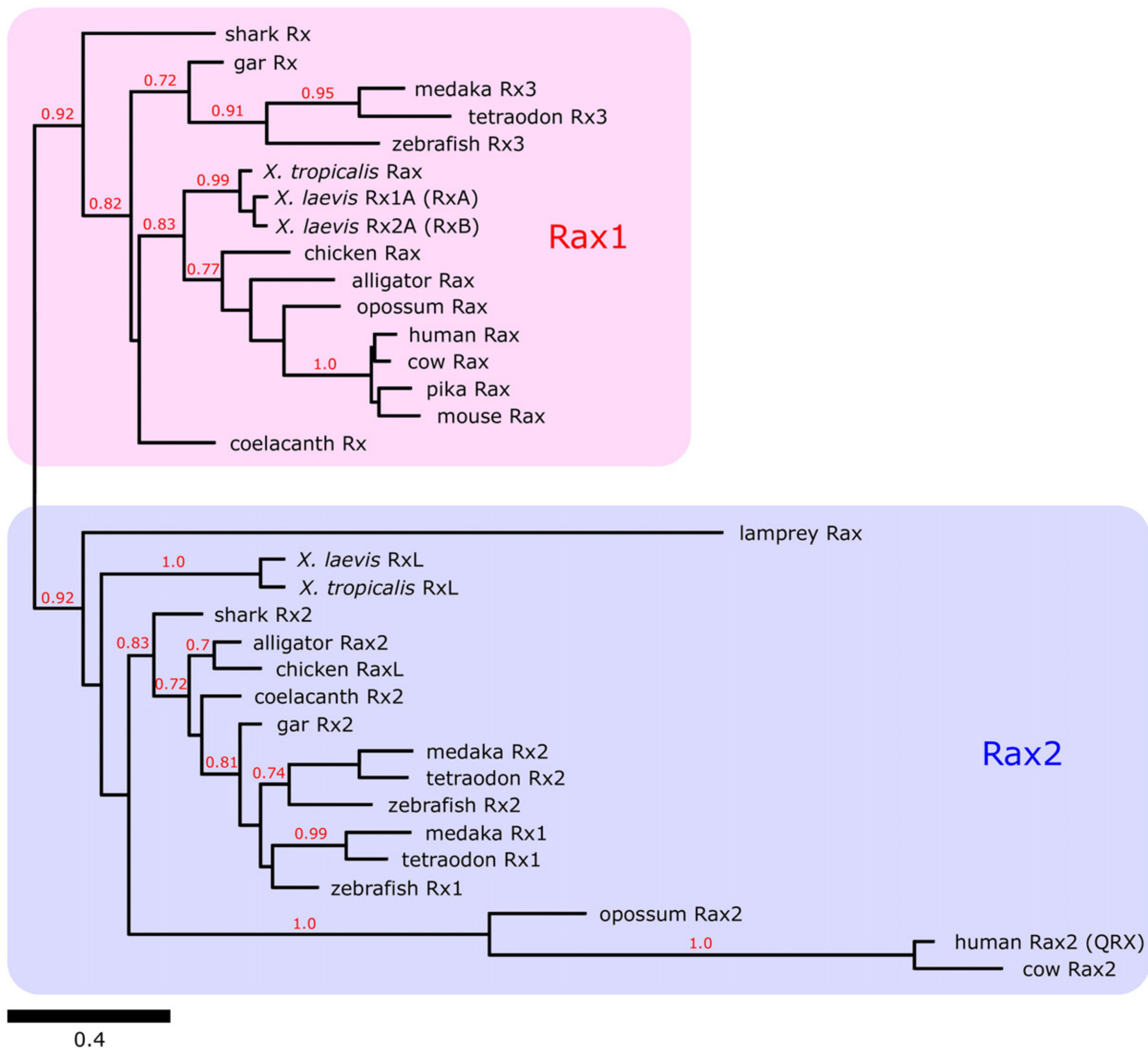

Fig. 2. Phylogenetic analysis of Rax proteins. Amino acid sequences of Rax proteins were aligned and a maximum likelihood reconstruction of the phylogenetic relationships was performed using PhyML 3.0 (Guindon et al. 2010). Sequences were trimmed to remove ambiguously aligned regions, including the octapeptide domain. Nodes with over 70% (0.7) bootstrap support are indicated. The scale bar represents amino acid residue substitutions per site. Note that Rax proteins are subdivided into two branches, named Rax1 and Rax2 (highlighted). The Rax protein from the Japanese lamprey falls within the Rax2 subgroup.

Among frogs of the *Xenopus* genus, *X. laevis* also possesses more than one *Rax* gene (Fig. 2; Wu et al. 2009). Our analysis indicates that Rx1A (RaxA) and Rx2A (RaxB) belong to the Rax1 subgroup, while Rx-like (RxL) belongs to the Rax2 subgroup. Rax1A and Rax2A are very closely related, and are likely the result of the tetraploidisation event that has happened in a *X. laevis* ancestor (Pollet and Mazabraud 2006). *X. tropicalis*, which is diploid, has only one protein belonging to the Rax1 subgroup (Rax) and one that belongs to the Rax2 subgroup (RxL), as in the ancestral situation.

Birds and reptiles, represented here by the chicken (*Gallus gallus*) and the American alligator (*Alligator mississipiensis*), have one Rax1 and one Rax2 member each (Fig. 2).

Most mammals, including the marsupial gray short-tailed opossum (*Monodelphis domestica*) also have two Rax proteins, one in each subgroup (Fig. 2). Mammalian Rax2 sequences are more divergent from those of other vertebrates, as indicated by their long branch lengths in the phylogenetic tree. As noticed by others (Wang et al. 2004; Zhong and Holland 2011), the mouse genome possesses only one *Rax* gene, which encodes a member of the Rax1 subgroup (Fig. 2). We have searched the genomes of other rodents and identified *Rax1* genes in all of them, while *Rax2* could not be identified in the genomes of the rat (*Rattus norvegicus*), the naked mole-rat (*Heterocephalus glaber*), the squirrel (*Ictidomys tridecemlineatus*), the Guinea pig (*Cavia porcellus*), the Chinese hamster (*Cricetulus griseus*), and the kangaroo rat (*Dipodomys ordii*; data not shown). The closest sister group of rodents, the lagomorphs, also seems to lack *Rax2* genes, since only one gene related to *Rax1* could be retrieved from the genomes of the common rabbit (*Oryctolagus cuniculus*) and the pika (*Ochotona princeps*; Fig. 2). Thus, mammals originally possessed two *Rax* genes, but *Rax2* was secondarily lost from the genome of a common ancestor of rodents and lagomorphs (clade Glires; Fig. 1).

2.3. Rax synteny analyses

In the phylogenetic tree presented in Fig. 2, the separation of Rax proteins into Rax1 and Rax2 gene subfamilies is supported by a branch with high statistical support. Nevertheless, the branching order within each subfamily often does not coincide with the vertebrate phylogenetic tree (see Fig. 1). Incongruencies between protein and species phylogenetic trees are a common phenomenon that can have many causes (Rokas et al. 2003; Philippe et al. 2011). In the case of Rax proteins, the relatively short alignable sequence (around 212 amino acid residues) as well as the fast pace of Rax2 evolution may interfere with phylogenetic reconstruction. To reassure that the Rax1 and Rax2 subgroups retrieved in the phylogenetic tree are true paralogues, we analysed *Rax* loci of various representative species to check the degree of

conservation of synteny surrounding the genes. The genomic neighbourhood of the Japanese lamprey *Rax* locus could not be evaluated as it is the only gene in its scaffold (data not shown). No synteny conservation was detected between *Rax* loci of vertebrates and basal chordates like sea squirts and amphioxus (data not shown).

In general, we observed that the gene neighbourhoods of *Rax1* and *Rax2* are partially conserved in all vertebrates analysed (Fig. 3 and data not shown). When inspecting the neighbourhoods of *Rax* paralogues of the elephant shark and gar genomes, it is evidenced that *Rax1* and *Rax2* loci have a few genes in common, namely *malt1a/b-rax1/2-cplx4a/c*, which were present in this order in an ancestral vertebrate (Fig. 3). This indicates that the ancient duplication that originated the *Rax* paralogues in a jawed vertebrate ancestor was not a single-gene duplication, but the result of a higher order event. In the human genome, *Rax1* is located on chromosome (Chr.) 18, while *Rax2* is located on Chr. 19p13 (Fig. 3). When synteny conservation of human *Rax* loci is analysed using the Synteny Database (Catchen et al. 2009; see Materials & Methods) it is observed that Chr. 18 and Chr. 19p13 share nine different paralogue genes, indicating that synteny conservation extends beyond *Rax* and its close neighbours. Moreover, an analysis that compared linkage groups of the amphioxus genome with the human genome suggests that human Chr. 18 and Chr. 19p13 originated from an ancient duplication of a chordate chromosome, being part of an ancient conserved cuadruple synteny block (see Table 1 in Putnam et al. 2008). All this points to *Rax1* and *Rax2* having originated in the two rounds of whole genome duplications (WGD) that ocurred in a vertebrate ancestor (Fig. 1; Cañestro et al. 2013). Since these duplications presumably gave rise to four *Rax* genes, it seems that two of these paralogues were lost very early, before the separation of cartilaginous and bony fish lineages.

Within each *Rax* subgroup, synteny is conserved between zebrafish and human (Fig. 3). For *Rax1*, a gene neighbourhood comprising *ccbe1-lman1-cplx4-rax1-grp-oacylp-zfn532* is conserved between the zebrafish *rx3* locus and human *RAX1*; while for *Rax2*, the genes *atcay-zfr2-matk-rax2* are also conserved between zebrafish *rx1/2* and human *RAX2* loci. As noted earlier, the *Rax2* subgroup in the zebrafish genome is represented by two different loci, *rx1* and *rx2*, located in chromosomes 2 and 22, respectively (Fig. 3). Analyses of the zebrafish genome indicate that chromosomes 2 and 22 share many paralogues and likely descend from a common ancestral chromosome (see Fig. 3b in Howe et al. 2013). Thus, *rx1* and *rx2* are likely derived from the teleost-specific WGD, while a second *rx3* paralogue was lost from the teleost genome after the WGD event (Fig. 1).

An analysis of the synteny of *X. tropicalis* and chicken *Rax* genes also support their assignments to the *Rax1* and *Rax2* groups as indicated in the phylogenetic tree of Fig. 2 (data not shown). In conclusion, synteny

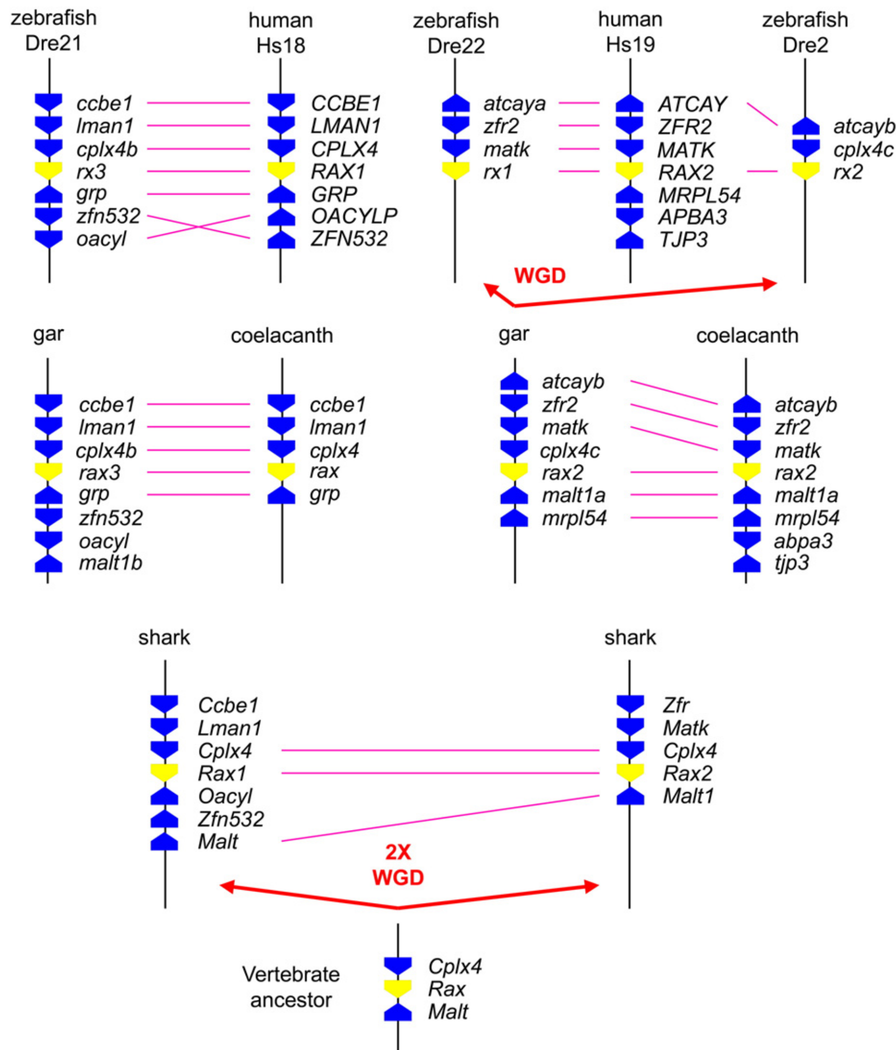

Fig. 3. Synteny analysis of vertebrate *Rax* loci. Schematic representations of the gene composition surrounding *Rax1* and *Rax2* loci in the elephant shark, spotted gar, coelacanth, zebrafish and human genomes. Orthologues are connected by lines. The hypothetical locus at the bottom represents the genes surrounding *Rax* in a vertebrate ancestor. Whole genome duplications (WGD) in early vertebrate and teleost lineages are indicated. Chromosomes are not drawn to scale, and some intervening genes were omitted for clarity.

analyses confirm the phylogenetic relationships of Rax proteins of Fig. 2 and support the existence of *Rax1* and *Rax2* gene subfamilies in vertebrates.

3. Discussion

In this work, we used database searches, phylogenetic tree reconstructions and synteny analyses to trace the evolutionary history of *Rax* genes in vertebrates. Although the number of *Rax* genes can vary between species, we found that all vertebrate Rax proteins can be classified as belonging to either of two subgroups, namely Rax1 and Rax2. These subgroups have a very ancient history, as they originated at least before the split between cartilaginous and bony fishes lineages, which happened around 525 million years ago (Blair and Hedges 2005). Moreover, synteny analyses suggest that the genes appeared as a result of the polyploidisation events that happened in a vertebrate ancestor, possibly before the split between jawless and jawed vertebrates (Panopoulou and Poutska, 2005; Cañestro et al. 2013; Smith et al. 2013).

The evolutionary framework of *Rax* genes presented here may facilitate the interpretation of the many functional studies of these genes in various vertebrate species. During early embryogenesis, all vertebrate *Rax1* and *Rax2* genes studied to date are expressed in the anterior neural territory that gives rise to the eyes and part of the forebrain and/or in the developing eyes (Mathers et al. 1997; Chuang et al. 1999; Ohuchi et al. 1999; Pan et al. 2006; Bailey et al. 2004; Muranishi et al. 2012). Loss of function models of *Rax1* orthologues in mice (Mathers et al. 1997; Zhang et al. 2000; Tucker et al. 2001; Swindell et al. 2008), medaka (Loosli et al., 2001), zebrafish (Loosli et al. 2003; Rojas-Muñoz et al. 2005) and *Xenopus* (Andreazzoli et al. 2003; Fish et al. 2014) show that *Rax1* genes are crucial for eye development in vertebrates, as evidenced by the absence or reduction of optic vesicle evagination, coupled to absence or strong reduction in retina and lenses formation in these *Rax1*-deficient models. As for *Rax2* orthologues, loss-of-function studies also point to an important role for this gene in eye development. But, in contrast to what is observed in *Rax1* mutants, reducing *Rax2* expression does not lead to a near absence of eyes, but causes phenotypes that affect proliferation and differentiation of retinal cell types in the zebrafish (Nelson et al., 2009; Reinhardt et al. 2015), *Xenopus* (Pan et al. 2006; Wu et al. 2009) and chicken (Chen and Cepko 2002; Sakagami et al. 2003). Importantly, hypomorphic mutations in human *Rax1* are associated with microphthalmia and anophthalmia (Voronina et al. 2004; Lequeux et al. 2008; London et al. 2009; Gonzalez-Rodriguez et al., 2010; Abouzeid et al. 2012), while heterozygous mutations in human *Rax2* are associated with retinal degeneracy and dystrophy (Wang et al. 2004; Yang et al. 2015). Thus, the available evidence indicates that the functions of *Rax1* and *Rax2* in eye development are not identical, with *Rax1* having a particularly important role in the general specification of the eye field, optic cup evagination and retinal differentiation and *Rax2* being necessary at later developmental stages in cell proliferation and differentiation within the retina.

Another major site of expression of *Rax* genes after the closure of the neural folds is the hypothalamus, but in this region there are major differences between *Rax1* and *Rax2* expression. In the zebrafish and medaka, the *Rax1* orthologue *rx3* is expressed in the eyes and hypothalamus, while the *Rax2* orthologues, *rx1* and *rx2*, are only expressed in the eye fields (Mathers et al. 1997; Deschet et al. 1999; Chuang et al. 1999; Chuang and Raymond 2001; Winkler et al. 2000). Something similar occurs in *X. laevis*, where the *Rax1* orthologue *Rx1A* (*RxA*) is expressed in the eye field and hypothalamus, whereas the *Rax2* orthologue, *RxL*, is only expressed in the eyes (Casarosa et al. 1997; Mathers et al. 1997; Pan et al. 2006). In the chick embryo, both *Rax1* (*Rax*) and *Rax2* (*RaxL*) genes are initially expressed in the anterior neural fold and in the eyes but, as development progresses, *Rax1* expression is maintained in the hypothalamus while *Rax2* expression is downregulated in this region (Chen and Cepko 2002). In the mouse, *Rax1* is expressed in the hypothalamus, and mutations in the gene cause profound abnormalities in the development of the hypothalamus and pituitary gland (Mathers et al. 1997; Zhang et al. 2000; Medina-Martinez et al., 2009; Lu et al. 2013; Orquera et al. 2016). The function of *Rax1*-group genes in hypothalamic development is conserved in fishes, since *rx3* deficiency also causes profound abnormalities in the patterning of the hypothalamus and pituitary in the zebrafish (Stigloher et al. 2006; Dickmeis et al. 2007; Muthu et al. 2016).

What could have been the primitive functions of *Rax* in a vertebrate ancestor, before its divergence into *Rax1* and *Rax2* clades? *Rax* is an ancient gene present in a single copy in the genomes of cnidarians and bilaterians (Mazza et al. 2010). In marine larvae of cnidarians and several bilaterians, *Rax* (*rx*) is part of the conserved molecular signature of the apical plate region, indicating an ancestral role in the patterning of the larval ectoderm (see Marlow et al. 2014 and references therein). Interestingly, *Rax* is later expressed in the anterior-most portion of the developing nervous system of distant protostomes like the polychaete annelid *Platynereis dumerilii* and the centipede *Strigamia maritima* (Tessmar-Raible et al. 2007; Marlow et al. 2014; Hunnekuhl and Akam 2014). In these organisms, *Rax*-expressing territory in the anterior nervous system gives rise to a neurosecretory centre that expresses several genes also found in the vertebrate hypothalamus, suggesting a deep conserved function of *Rax* in bilaterian nervous system development (Tessmar-Raible et al. 2007; Hunnekuhl and Akam 2014). In *Drosophila*, *Rax* is not expressed in the neurosecretory centre (*pars intercerebralis*), but it is nevertheless necessary for the formation of the ellipsoid and mushroom bodies, also located in the anterior portion of the fly brain (Eggert et al. 1998; Davis et al. 2003; Kraft et al. 2016). As for the eyes, the picture differs between protostomes and deuterostomes. *Rax* is neither expressed nor necessary for the development of eyes of protostomes like *Drosophila* and the annelid *P. dumerilii* (Eggert et al. 1998; Davis et al. 2003; Arendt et al. 2004). Among chordates, however, *Rax* is associated with eye structures. Thus, in the amphioxus *Branchiostoma floridae*, *Rax* is expressed in the neural territory that will give rise to the frontal eye (Vopalensky et al. 2012) and, in the urochordate *Ciona intestinalis*, *Rax* is expressed and is necessary for the development of the light-sensing ocellus (D'Aniello et al. 2006; Yoshida and Saiga 2011). Thus, it would seem that the primitive function of *Rax* in bilaterians was related to the development of the anterior neuroectodermal portion of larvae and embryos and the subsequent formation of the neurosecretory centre of the brain. In the chordate lineage, as complex eyes with ciliary-type opsins and photoreceptors evolved (Arendt 2003; Lamb 2013), *Rax* function became crucial for eye development.

Considering the evolutionary framework depicted above, it seems that vertebrate *Rax* genes maintain two primitive functions inherited from a chordate ancestor; namely 1) a role in anterior forebrain and hypothalamic patterning and development and 2) a role in eye and photoreceptor development. In addition, it can be envisaged that soon after the vertebrate ancestor acquired two copies of the primitive *Rax* gene over 525 million years ago, the newly arisen *Rax1* and *Rax2* paralogues assumed different subfunctions of the ancestral *Rax* gene, a process called subfunction partitioning or subfunctionalisation (Force et al. 1999; de Souza et al. 2005; Cañestro et al. 2013). While *Rax1* became the main gene involved in the early development of eye structures and in the development of the hypothalamus, *Rax2* function became more specialised in controlling proliferation and differentiation within the retina. Future studies on the expression of *Rax* genes in agnathans may shed some light on the early functional evolution of these genes in vertebrates. In teleosts, the two *Rax2* paralogues that appeared after the teleost-specific WGD also appear to have underwent subfunction partitioning, as the functions of *rx1* and *rx2* in retinal development overlap only partially (Nelson et al. 2009).

Although the phenotypic effects of the loss of function of *Rax1* seem more drastic than the effects of losing *Rax2*, there can be no doubt of the importance of *Rax2* in eye development, particularly considering that human patients with retinal degeneration have been found to carry

mutations in this gene (Wang et al. 2004; Yang et al. 2015), which is consistent with the recent finding that a *Rax2* homologue is needed for retinal stem cell maintenance in adult medaka fish (Reinhardt et al. 2015). The study of *Rax2* expression and function in a mammalian model would be needed to better understand its role in the human eye, including the degree of its functional overlap with *Rax1*, but unfortunately neither rodents nor lagomorph models can be used. Although some expression studies have been done in the bovine retina (Wang et al. 2004), a full characterisation of the developmental roles of *Rax2* may need to be performed in another mammalian model or in in vitro organogenesis models (Sasai et al. 2012). Studies on the degree of overlap of *Rax1* and *Rax2* function in mammalian eye development might also shed light on how rodents and lagomorphs have compensated for the loss of *Rax2* from their genome.

Rax proteins have a N-terminal octapeptide domain, a homeodomain and a C-terminal OAR domain. The homeodomains of Rax1 and Rax2 proteins are almost identical and, although little is known about the direct targets regulated by these proteins, both seem to recognise similar DNA motifs, to have similar transactivating capabilities and even to interact with some of the same protein partners (Chen and Cepko 2002; Wang et al. 2004; Pan et al., 2006). One of the intriguing aspects of Rax protein evolution is the loss of the octapeptide domain from Rax2 proteins in a common ancestor of all tetrapods. Although the octapeptide domain is found in many homeodomain transcription factors of the Pax family, its function in protein activity is still unknown. More studies on the functions of Rax protein domains, their partners and the target genes regulated by these proteins are needed before we can attempt to understand the evolutionary significance of the loss of the octapeptide domain from tetrapod Rax2 proteins and the loss of *Rax2* genes from the genomes of Glires.

In summary, we presented an evolutionary framework of *Rax* genes in vertebrates that, we hope, will help inform future studies on the roles of this family of transcription factors on vertebrate development.

4. Materials & methods

4.1. . Sequences and databases

Rax genes and proteins were retrieved from GenBank (Clark et al. 2016) and from genome assemblies in the Ensembl (Flicek et al. 2014) and UCSC (Speir et al. 2016) databases. A lamprey Rax sequence was retrieved from the Japanese Lamprey Genome Project (http://jlampreygenome.imcb.a-star.edu.sg/). Database accession numbers for the sequences used in phylogenetic analysis are shown in Supplementary Table 1.

4.2. Phylogenetic analysis

Rax protein sequences were edited to remove the N-terminal region, including the octapeptide domain, and were aligned with Clustal Omega (Sievers and Higgins 2014). The resulting multiple alignment was trimmed using AliView (Larsson 2014) to eliminate ambiguously aligned sequences, and used to construct bootstrapped maximum likelihood phylogenetic trees with PhyML 3.0 (Guindon et al. 2010) using the LG protein substitution model (Le and Gascuel 2008). Gamma correction was used to account for heterogeneity in evolutionary rates with four discrete classes of sites, an estimated alpha parameter and an estimated proportion of invariable sites as implemented in PhyML 3.0. A total of 100 bootstrap replicates were used to assess robustness. The tree was visualised and edited with TreeDyn (Chevenet et al. 2006).

4.3. Synteny analysis

Assembled genomes in the Ensembl and UCSC genome databases were searched to determine the gene composition and order surrounding *Rax* loci of several species. The location of *Rax* genes shown in Fig. 3 are the following: in the elephant shark genome (version calMil1 in the UCSC browser), *Rax* is located in scaffold KI635957 (coordinates 1,679,783–1,690,348) and *Rax2* in scaffold KI636053 (650,599–656,685); in the spotted gar genome (LepOcu1 in the Ensembl browser), *Rax1* (*rx3*) is located in linkage group LG2 (49,446,278–49,452,812) and *Rax2* (*rx2*) is located in LG19 (5,547,300–5,558,799); in the coelacanth genome (LatCha1 in the Ensembl browser), *Rax1* (*Rax*) is located in scaffold JH127807.1 (464,934–468,459) and *Rax2* (*rx2*) in scaffold JH127062.1 (864,940–875,022). The well-annotated zebrafish genes *rx1*, *rx2* and *rx3* are located in chromosomes 22, 2 and 21, respectively, and human *RAX* and *RAX2* are located in chromosomes 18 and 19, respectively.

Paralogous genes surrounding *Rax* loci in human chromosomes Hs18 and Hs19 were identified with the Synteny Database version Ens70 (Catchen et al. 2009; http://syntenydb.uoregon.edu/synteny_db/) using the *Ciona instestinalis* genome as outgroup. Retrieved cluster #21467 shows nine conserved paralogue genes surrounding *Rax* genes in Hs18 and Hs19.

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