Evolutionary History of Chordate PAX Genes: Dynamics of Change in a Complex Gene Family

Vanessa Rodrigues Paixão-Coêrtes, Francisco Mauro Salzano, Maria Cátira Bortolini*

Departamento de Geneática and Programa de Pós-Graduação em Genética e Biologia Molecular, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil

Abstract

Paired box (PAX) genes are transcription factors that play important roles in embryonic development. Although the PAX gene family occurs in animals only, it is widely distributed. Among the vertebrates, its 9 genes appear to be the product of complete duplication of an original set of 4 genes, followed by an additional partial duplication. Although some studies of PAX genes have been conducted, no comprehensive survey of these genes across the entire taxonomic unit has yet been attempted. In this study, we conducted a detailed comparison of PAX sequences from 188 chordates, which revealed restricted variation. The absence of PAX4 and PAX8 among some species of reptiles and birds was notable; however, all 9 genes were present in all 74 mammalian genomes investigated. A search for signatures of selection indicated that all genes are subject to purifying selection, with a possible constraint relaxation in PAX4, PAX7, and PAX8. This result indicates asymmetric evolution of PAX family genes, which can be associated with the emergence of adaptive novelties in the chordate evolutionary trajectory.

Introduction

Paired box (PAX) genes are transcription factors that play key roles in several aspects of embryonic development and organogenesis [1–3]. Although the PAX family is specific to the animal lineage, the evolutionary history of these genes remains uncertain. A unique PAX gene (TriPaxB) has been isolated from Trichoplax adhaerens (Placozoa), the most morphologically simple species of all non-parasitic multicellular metazoan animals. The TriPaxB protein contains the characteristic DNA-binding domain of the PAX family, the paired domain (PD) of 128 amino acids, an octapeptide motif (OC), and a paired-type homeobox DNA-binding domain (HD) [1,4].

Four PAX genes (PAX1/9, PAX2/5/8, PAX3/7, and PAX4/6) have been found in the basal chordates, amphioxus (e.g. Branchiostoma floridae) and tunicates (e.g. Ciona intestinalis) [5–7]. Phylogenetic analyses indicated that a single PAX gene of each subfamily was present in the ancestral chordate and gave rise to the amphioxus PAX. Afterwards, a plus round of whole genome duplications, gave origin to the multiple vertebrate PAX subfamily copies [4–8]. Ohno [9], suggested that the early vertebrate lineage underwent one (1 R) or more (≥2 R) whole genome duplications (WGDs). These processes were considered to provide additional possibilities for diversified evolution and/or speciation. Rapid and widespread evolutionary changes could lead to macroevolutionary emergent properties, since WGD products are able to evolve and reach a greater level of interaction and complexity than would otherwise be possible through cumulative single gene duplications [10–15]. The second round of whole genome duplication most likely occurred after the divergence of invertebrate chordate lineages from the ancestral vertebrate, although there is controversy about the exact branch at which the phenomenon occurred [11,16–18].

After these 2 major duplication events occurred, probably 8 PAX genes emerged. Another partial duplication occurred subsequently, resulting in the 9 PAX genes currently found in mammals (subfamilies: (1) PAX1 and PAX9; (2) PAX2, PAX5, and PAX8; (3) PAX3 and PAX7; (4) PAX4 and PAX6 [1,6]). An alternative scenario would be that more PAX genes would have arisen after 2 WGRD and then lost during the vertebrate evolution history [19,20].

In vertebrates, as well as in other chordates, PAX genes are notably expressed during development. They are also known to play an important role in mature life stages, based on observations of organ/tissue-specific signals (Table S1; [21]). For instance, PAX3 and PAX7 proteins are found in adult cells of the vertebrate muscle tissue [22]. Analogously, in amphioxus, the PAX3/7 gene is most highly expressed in adult muscle [7]. These observations, along with other similar findings, indicate that a PAX-derived gene can maintain similar roles to those present in the putative ancestor. Nonetheless, various novel roles for PAX genes have also emerged during the evolutionary history of vertebrates: they were co-opted for new regulatory networks, diverged, and subsequently gained new functions [5,7,19–27].

Although the presence of PAX genes has been investigated in a variety of organisms [6,19–26], a broad survey of chordate PAX...
genes has yet to be conducted. Therefore, the aim of the present study was to address questions regarding the occurrence and evolution of the \textit{PAX} family in chordates. We used publicly available sequences to evaluate: (1) the presence/absence of \textit{PAX} genes in 188 organisms, and (2) the evolutionary rates and properties of \textit{PAX} genes in chordates. Results of this analysis will contribute to a greater understanding of the mechanisms of change in complex gene families.

**Results and Discussion**

**Search and Identification for Vertebrate \textit{PAX} Genes**

We characterized chordate \textit{PAX} genes using sequences that were available in the Ensembl, UCSC, NCBI, and UniProt databases [28–31]. A total of 188 species were evaluated, including vertebrates (175 jawed and 4 jawless), urochordates (6 tunicates), and cephalochordates (3 amphioxus) [32]; see details in Tables S2 and S3 and Figure 1.

**Basal Chordates**

We retrieved 4 \textit{PAX} genes in cephalochordates (Branchiostoma belcheri, B. floridae, and B. lanceolatum), which is the most basal chordate subphylum. In tunicates, the most basal animals belonging to the Ollactores clade, we recovered 5 \textit{PAX} genes. Three of the genes (\textit{PAX1}/9, \textit{PAX} 3/7, and \textit{PAX4}/6) could be considered equivalent to the ancestral vertebrate \textit{PAX} types, while the others (\textit{PAX2}/5/8a and \textit{PAX2}/5/8b in Branchiostoma lanceolatum) were derived [5–7,33].

**Jawless Vertebrates**

Jawless vertebrates were represented in our study by 1 hagfish species (Eptatretus burgeri) and 3 lamprey species (Lampetra fluviatilis, Lehtenuron cantschaticum, and Petromyzon marinus). Together, they form a sister group of the gnathostome vertebrates, making them a good model to investigate ancestral vertebrate characteristics. The hidden Markov models (HMMER) search recovered 3 \textit{PAX} genes for the hagfish and 4 for all lampreys. In \textit{Petromyzon marinus}, we recovered 2 \textit{PAX} genes (\textit{PAX1}/9 and \textit{PAX1}/9b), and found 2 segments of 158 bp and 144 bp, respectively, showing 88% of identity with the \textit{PAX3} and \textit{PAX7} genes. Interestingly, in vitro studies identified \textit{PAX1}/9 and \textit{PAX1}/9b in this species [33], as well as the \textit{PAX7}, \textit{PAX2} [34], \textit{PAX6} [33–37], \textit{PAX3}/\textit{PAX7} genes [36]. This in vitro information, which was confirmed by our genome data, suggests that \textit{Petromyzon marinus} contains genes corresponding to the 4 ancestral \textit{PAX} genes in addition to a second copy of the \textit{PAX1}/9 and \textit{PAX3}/7 type. This suggests that a duplication of the \textit{PAX1}/9 and \textit{PAX3}/7 genes occurred in the lamprey or jawless lineage, although the possibility of an ancient genome duplication event (before the split between jawless and jawed vertebrates) with subsequent lineage-specific modifications cannot be discarded [12]. The difference between the numbers of \textit{PAX} genes found in the basal chordates, tunicates (4 or 5) and lampreys (6) and the basal jawed vertebrates (9) can be associated with the emergence of adaptive novelties at the tunicate/vertebrate and agnathan/gnathostome transitions.

**Jawed Vertebrates**

We found 6 \textit{PAX} genes in the most basal taxon of this group, the Chondrichthyes (2 skates, 2 sharks, and 1 chimera). The chimera species (Callorhinchus milii; elephant shark), for which the draft genome is already available, contained all 6 genes [1, 9, 8, 3, and 2 copies of 6; Figure 1 and Table S3]. \textit{PAX}X was not retrieved in any search (Genomes, HMMER protein, and BLAST/BLAST; Table S3). However, the absence of \textit{PAX}4 should be interpreted with caution since the elephant shark genome has low coverage (1.4x) and the sequence databases are biased toward the most popular/known genes. The duplicate \textit{PAX6} (named \textit{PAX6}/2) was recently discovered, and based on experimental work and in the conservation of coding and noncoding elements, the authors suggested that although an ancient duplication event occurred in a gnathostome ancestor, the additional copy was independently lost in mammals and birds [30].

All of the expected 9 \textit{PAX} genes were found in 37 species of ray-finned bony fishes. Considering only the 8 ray-finned bony fishes for which complete genomes are available (class Actinopterygii; Table S3), additional duplicate or triplicate copies were found in 7 of the 9 \textit{PAX} genes (exception: \textit{PAX5} and \textit{PAX8}; Figure 1). This situation was probably a consequence of whole genome duplication [39,40], which occurred in the early evolution of teleost fishes approximately 320–530 million years ago [3 RWGD hypothesis; [41]].

Laetaniera chalumnae (coelacanth), a lobe-finned fish, presents all 9 \textit{PAX} genes, suggesting that the ancestor that gave rise to the tetrapod lineage contained all members of the \textit{PAX} family.

The frog species \textit{Xenopus tropicalis} and \textit{Xenopus laevis} also presented 9 \textit{PAX} genes. However, we found duplicated copies of \textit{PAX2} and \textit{PAX6} in \textit{X. laevis} and \textit{X. tropicalis}, respectively. The presence of the additional \textit{PAX2} copy in \textit{X. laevis} could be the result to the fact that this species experienced a recent and specific polyploidization event approximately 40 million years ago. Approximately 32–47% of duplicated genes were observed in its whole genome [15,39,42]. An alternative is that \textit{PAX2} could have been duplicated through local gene duplication. The duplication of \textit{PAX6} in \textit{X. tropicalis} has been reported in a previous study [X6.2 [30]]. For the other 6 species of amphibians, we only retrieved \textit{PAX6} and \textit{PAX7} sequences (6 hits and 1 hit, respectively). For all amphibian species studied here, it was not possible to localize \textit{PAX4}, corroborating a recent paper that proposed that \textit{X. tropicalis} lost \textit{PAX4} [43]. These data suggest that the absence of \textit{PAX4} could be a general characteristic of amphibian taxa.

The analysis of the entire \textit{PAX} family in reptile and bird species (Sauropsida, Table S3 and Figure 1) showed a surprising finding: some branches appear to have lost \textit{PAX}4 and \textit{PAX8}. It was recently suggested [44] that \textit{PAX6} gene was lost after turtles split from other reptiles and birds, which most likely occurred ~240 million years ago [45–47]. We found \textit{PAX8} in 2 of the 3 turtle species studied (Chelysmyx pilita bella and Pelodiscus sinensis; Table S3). We also found 9 \textit{PAX} genes, including a \textit{PAX8} segment, in a snake (\textit{Python molurus}). The unresolved Sauropsida phylogeny [Figure 1; [48]) raises the question as to whether the loss of \textit{PAX4} and \textit{PAX8} is an ancestral event, or whether these losses occurred independently in distinct reptile and bird lineages.

Overall, the searches showed that all 9 \textit{PAX} genes appear to be present in the 74 mammalian species studied (Figure 1). Although some exceptions to this general pattern were found, they are likely a consequence of the low coverage of the genome in question (e.g. \textit{Dipodomys ordii} (kangaroo rat), which had only a 2× coverage), or due to bias toward the most popular genes, rather than a reflection of actual gene loss.

**Shared Synteny and/or Conserved Neighborhood Analysis**

We performed an analysis of shared synteny (genes in the same chromosome) and/or conserved neighborhood (genes side-by-side in the same order) for all 4 \textit{PAX} subfamilies: (1) \textit{PAX1} and \textit{PAX9}; (2) \textit{PAX2}, \textit{PAX5}, and \textit{PAX6}; (3) \textit{PAX3} and \textit{PAX7}; (4) \textit{PAX4} and \textit{PAX6} [7,21]. The most conserved and similar blocks were those in
Figure 1. Phylogenetic tree for the 175 jawed vertebrate species considered in this study. Sequences were obtained from the NCBI Taxonomy Browser and edited with Figtree and hitmap for the presence of PAX genes. The dashed red line indicates the relationship suggested by Crawford et al. [46].

doi:10.1371/journal.pone.0073560.g001
which **PAX1** and **PAX9** were inserted. The others presented distinct levels of neighboring and conserved synteny.

This analysis was also used as additional evidence for the absence of **PAX4** and **PAX8**, as well as for evidence of **PAX6** duplication in some taxa, as described in the previous section.

By using a similar approach, Ravi et al. [38] recently found that **PAX6.2** was located in close proximity to the **RCN3** and **NOSIP** genes in the elephant shark, lizard, zebrafish, and Xenopus. However, no **PAX6**-duplicated ortholog was found in the proximity of **NOSIP-RCN3** or elsewhere in the genomes of birds or mammals. Our analysis confirmed the presence of **PAX6.2** in **RCN3-NOSIP** in Xenopus and in a lizard species (Anolis carolinensis). Additionally, we found the **PAX6.2** gene in this same region in the coelacanth and in the painted turtle (Chrysemys pista bellii; Figure 2A), but failed to find the **PAX6.2-RCN3-NOSIP** block in the genomes of another turtle species (Pilodiscus sinensis) or in mammals and birds. Consequently, our data support the proposal that the duplication that gave rise to the **PAX6.2** gene must have occurred before the split between cartilaginous and bony fish, and that this duplication was followed by multiple independent **PAX6.2** gene losses in distinct vertebrate lineages.

We found a possible fragment of the **PAX4** gene in association with the **ARF5** gene in the budgerigar (Melopsittacus undulatus) genome, which was in the intronic region of the **GCC1** gene. In other vertebrate genomes, the **GCC1-ARF5-FSCN3-PAX4** block formed a conserved neighborhood (Figure 2B). Although the **PAX4** segment appeared to contain a complete paired domain, 3 independent approaches failed to predict a full functional protein.

The syntenic analysis of **PAX6** showed that the block in which it is inserted in mammals and fish is relatively well conserved in birds and reptiles, whose some genomes lack **PAX6**, providing supporting evidence for its loss.

Based on the presence of putatively nonfunctional relics (**PAX4** and **PAX8** segments in *Melopsittacus undulatus* and *Python molurus*, respectively), along with the other findings presented above, we can suggest that the loss of **PAX4** and/or **PAX8** occurred multiple times in tetrapod lineages, which also appears to be the case for **PAX6.2**. This hypothesis is compatible with findings of previous studies [49–56].

![Figure 2. Synteny and neighborhood status for the PAX4 and PAX6 genes.](doi:10.1371/journal.pone.0073560.g002)
Comparative Analysis of PAX Proteins

The PAX protein analysis was performed using the 53 species whose available sequences met our quality criteria (see Material and Methods). Pairwise amino acid distances were calculated between each PAX subfamily member and the PAX protein type of its probable outgroup (the urochordata Ciona intestinalis) (Table S4 and Figure 3). The distances of each protein from that of its probable outgroup were also compared among the 4 subfamilies ((1) PAX1 and PAX9; (2) PAX2, PAX5, and PAX8; (3) PAX3 and PAX7; (4) PAX4 and PAX6). For instance, the distance values of the human PAX1 and PAX9 to the PAX1/9 type from Ciona intestinalis were 0.447 and 0.455, respectively, which was not statistically significant ($p = 0.706$).

Considering all comparisons (53 chordate species and 9 PAX proteins), some specific and general patterns emerged: the distance values between PAX1 and PAX9 paralogs to their putative PAX1/9 protein did not show significant differences ($p = 0.069$). In other words, the distance of PAX1 to the Ciona intestinalis PAX1/9 is the same as the distance to its paralogous gene, PAX9. The same result was found when PAX3 and PAX7 were compared with PAX3/7 ($p = 0.704$).

On the other hand, the PAX4 protein was more dissimilar to its putative outgroup (PAX4/6) than it was to PAX6 ($p = 0.001$). Therefore, this suggests that PAX4 most likely emerged in 2 R together with PAX6. Possibly higher evolutionary rates (inferred by $\omega = dN/dS$) would further confirm this result. A recent study indicated a possible loss of PAX4 expression from the brain in vertebrates, probably after 2 RWGD [43], may have led to relaxed constraints on gene conservation, as suggested by a higher rate of sequence divergence.

When PAX2, PAX5, and PAX8 were compared with their putative PAX 2/5/8 ancestral type, other suggestive patterns appeared. The distance between PAX8 and its ancestor was significantly different from those of the others ($p = 0.001$), whereas the PAX2 and PAX5 distances did not present a significant difference ($p = 0.091$).

These results could indicate that a round of complete vertebrate genome duplication most likely involved PAX2 and PAX5 ancestor, whereas PAX8 emerged through local gene duplication. This would support the idea that PAX8 is the most recent gene to appear by duplication in this family. An alternative scenario to evolution of the PAX2/3/8 subfamily is that after 1 RWGD, two copies of the subfamily genes emerged, one resembling PAX2/5 and the other PAX8. A second duplication event (2 WRGD), resulted in 4 copies of the PAX2/3/8 subfamily, followed by loss of one of the PAX8 duplicates. The result is the presence of PAX2, PAX5, and PAX8 genes in jawed vertebrates [19,20]. The relaxation of selective pressure immediately after this last partial/total duplication would be expected, which could explain the higher variation observed in PAX8 relative to its outgroup (PAX2/5/8). The higher evolutionary rate (inferred by $\omega = dN/dS$; see Material and Methods and next section) could be an alternative explanation; however, the 2 above mentioned possibilities, relative to PAX8, are not mutually exclusive. Redundancy in the expression of these genes likely played a central role in the loss and/or higher divergence rate of PAX8. In mammals, PAX8 is mainly expressed in the kidney, ear, and thyroid gland during development, whereas PAX2 is expressed not only in these organs and tissues, but also in others, such as the eye, pharyngeal arches, and brain [2,3,5,7,19,20,44]. Amphioxus (here considered as an outgroup) contains only PAX2/5/8 and shows pleiotropic expression in most organs and tissues, implying that PAX2, PAX5, and PAX8 have retained most of their ancestral expression patterns [19,20,44].

![Figure 3. Pairwise PAX protein changes observed in different vertebrate taxa compared to that of the sea squirt.](doi:10.1371/journal.pone.0073560.g003)
Selection on PAX Genes

Our molecular evolution analyses (Table 1) revealed ω values <1, indicating that purifying selection has acted on PAX over the majority of the evolutionary history of vertebrates. This result is consistent with the idea that developmental genes are under functional restriction in metazoa [56]. The clade model D [57] performed better in a likelihood ratio test (LTR; p<0.001; Table 1) when compared with the neutral M1a model. This result indicates that ω values obtained for the 4 PAX subfamilies can vary between branches, predicting distinct molecular evolutionary patterns.

Although all estimated ω values were less than 1, which suggested the action of negative selection, a possible relaxation of this selective constraint was revealed when the subfamilies were compared. In 38% of the sites, the PAX4 ω value was 16 times greater than that of the PAX6 ω value. Additionally, in 37% and 31% of the sites, the PAX9 and PAX7 ω values were approximately 2 and 4 times greater than the PAX2-PAX5 and PAX3 ω values, respectively (Table 1). These results suggest that PAX4, PAX6, and PAX7 have experienced relatively more modifications than the other PAX genes.

Gene Phylogeny Analysis

Bayesian Monte Carlo Markov Chain trees were built from 2 PAX subfamily data sets (PAX2, PAX3, and PAX8; PAX4 and PAX6), in which genes were lost in some lineages, and presented greater molecular evolutionary rates wherever they were not lost. Well-defined clusters were observed that separated the 3 and 2 genes of each subfamily, respectively (Figure S1 illustrates the PAX2, PAX5, and PAX8 tree). These results indicate again the conservative nature of purifying selection that has driven molecular evolution of the PAX gene. As expected, the PAX genes found in the tunicate Ciona intestinalis (PAX2/5/8 and PAX4/6) lead to basal branches in Figure S1 in the other subfamily (data not shown). In some cases, recovery of the class phylogeny of the species is apparent as clear mammal, fish, and bird clades can be observed in the PAX2 cluster. Similar topologies and statistical robustness were obtained using the maximum likelihood method (data not shown). The trees, however, do not provide additional evidence about the differences in evolutionary rates of genes observed within each subfamily.

Conclusion

Overall, purifying selection appears to be the main factor responsible for molecular evolution of the PAX family in chordate species. However, there are some indications of potential group-specific changes that are beyond this general pattern. There was a loss of PAX4 and PAX8 in lizards and birds. Accelerated evolutionary rates were suggested for the PAX4, PAX8 and PAX7 genes. The accumulation of variation (at least in some sites), due to an initial relaxation of purifying selection, may indicate the beginning of a process that enabled evolvability of the system.

Results of the present study revealed that some PAX genes experienced striking changes in the course of their evolutionary trajectory, which emphasizes the point that even developmental master genes might not follow universal patterns of molecular evolution. Functional retention and loss, subfunctionalization, as well as neofunctionalization can also be observed in developmental genes.

The asymmetric evolution of the PAX family genes observed here, as evidenced by uneven events of duplications and deletions are compatible with the emergence of adaptive novelties during chordate radiation.

Materials and Methods

Data Collection

Nucleotide and amino acid sequences for all available PAX genes in chordate species were obtained using Biomart (Ensembl v66-70 - http://www.ensembl.org/biomart/martview/; [58,59]). The Protein Domains/Limit filter (InterPro (ID): IPR001522) was used as a parameter to identify the PAX genes or the paired box domains. A second approach was the inspection of one-to-one ortholog gene maps, which were also obtained from Biomart.

BLAST/BLAT searches in Ensembl (http://www.ensembl.org/Multi/blastview), UCSC databases (http://genome.ucsc.edu/), and in the NCBI Genebank (genomic BLAST http://www.ncbi.nlm.nih.gov/sutils/genom_table. cgi?organism=8496&database=8496) were also conducted in order to identify possible unannotated orthologs. The PreEnsembl database (http://pre.ensembl.org/index.html) was used to access the new draft released genomes. Finally, we applied hidden

| Table 1. Branch-site clade model D values for ω (dN/dS ratio) estimated for 2 site classes. |
|-----------------------------------------------|-----------------|-----------------|-----------------|-----------------|
| PAX subfamily | Clade Model D | M1a - Parameter estimates | P* (LRT) |
|----------------|----------------|-----------------|-----------------|-----------------|
| PAX1 and PAX9 | Proportion (p) | Branch type 0 (ω) | Branch type 1 (ω) | M1a X Clade Model |
|----------------|----------------|-----------------|-----------------|-----------------|
| PAX9 | 0.87268 | 0.00186 | 0.00186 | PAX1/PAX9 |
| 0.12732 | 0.08140 | 0.09185 | PAX2/PAX5/PAX8 |
| PAX2/PAX5 and PAX8 | 0.62462 | 0.01192 | 0.01192 | PAX2/PAX5/PAX8 |
| 0.37538 | 0.00027 | 0.00117 | PAX3/PAX7 |
| PAX3 and PAX7 | 0.68577 | 0.01016 | 0.01016 | PAX3/PAX7 |
| 0.31423 | 0.09976 | 0.18923 | PAX4/PAX6 |
| PAX4 and PAX6 | 0.61493 | 0.02335 | 0.02335 | PAX4/PAX6 |
| 0.38507 | 0.30208 | 0.01784 | PAX4/PAX6 |

*Degrees of freedom: 2; LRT: 2 Δl=2l – 1; l=0; doi:10.1371/journal.pone.0073560.t001

PLOS ONE | www.plosone.org 6 September 2013 | Volume 8 | Issue 9 | e73560
Markov models (HMMER) web service searching sequence databases for homologs of PAX amino acid sequences in the NR and Uniprot collections [60].

The genomic sequences of possible unannotated orthologs were verified using three programs that can predict open reading frames (ORF); BESTORF [http://linux1.softberry.com/berry.phhtml?topic=bestorf&group=programs&subgroup=gfind], GeneWise [http://www.ebi.ac.uk/Tools/psa/genewise/] [61], and STAR ORF [http://star.mit.edu/orf/index.html].

The following procedures were also adopted: (a) for genes encoding multiple transcripts, the transcript with the longest genomic transcribed length was selected; (b) a high identity (up to 70%) with the paired domain was accepted as indicating a PAX family member; (c) possible gene losses were accepted only when they were observed in multiple species as well as in high coverage genome assemblies; (d) a subset of 53 species was selected for the evolutionary analyses, since their sequences had the best alignment, and they were optimized for analysis with a higher number of sites.

**PAX Gene Family Synteny and Neighborhood Status**

Mapping adjacent genes into PAX synteny regions was achieved with the Genomicus website v70.01 [http://www.dyogen.ens.fr/genomicus-70.01/cgi-bin/search.pl] [62]. Additionally, we manually searched the Ensembl and UCSC genome browsers for the same purpose.

**Variation in the PAX Family**

The amino acid sequences were aligned using the MUSCLE algorithm [63] included in Mega (version 5.0) [64], which were verified with the GUIDANCE web service using the MAFFT algorithm [65]. Mega (version 5.0) software was employed to evaluate variability in the PAX groups using the pairwise distance of members of each subfamily from the gene of its probable outgroup, the tunicate (*Ciona intestinalis*), SPSS (version 16) software was used to calculate the statistical significance of differences between PAX1/9, PAX2/5/8, PAX3/7, and PAX4/6 paralogous sequences using the paired Student’s t-test.

**Tests for Selection**

Patterns of selection and rates of evolutionary changes in the PAX family were evaluated using standard tests [66–68]. We used the phylogeny-based maximum likelihood analysis of ω (dN/dS) as implemented in the CODEML program of the PAML 4.4 package to statistically test for positive selection and/or relaxation of functional constraints. The heterogeneity of evolutionary rates among paralogous groups was tested using the CODEML program in PAML4.4 clade models [57]. Branches on the phylogeny were divided into 2 clades a priori, and a likelihood ratio test (LRT) was used to evaluate divergences in selective pressures between them, as indicated by different ω ratios. We employed the clade model type D that assumes 2 site classes.

**Supporting Information**

**Figure S1 PAX2, PAX5, and PAX8 gene subfamilies phylogeny based on the Bayesian Monte Markov Chain method.**

**Table S1** Description and functions of PAX genes.

**Table S2** Species considered in this study and font of hits.

**Table S3** Presence of PAX genes in 188 Chordate species.

**Table S4** Pairwise distances between the PAX subfamilies and the outgroup PAX sea squirt.

**Acknowledgments**

We thank the Bioportal at the University of Oslo, where part of the phylogenetic analysis was conducted. We are grateful to reviewers and to Dr. Philippe Vernier and Dr. Marc Robison-Rechavi (Academic Editor) for valuable comments that greatly improved our manuscript.

**Author Contributions**

Conceived and designed the experiments: MCB VRPC. Performed the experiments: VRPC. Analyzed the data: MCB FMS VRPC. Contributed reagents/materials/analysis tools: MCB VRPC. Wrote the paper: MCB FMS VRPC.
and the possible evolutionary precursor of definitive vertebrate neural crest. Evol Dev 1: 133–165.
9. Ohno S (1970) Evolution by gene duplication. New York: Springer Verlag.
10. Dehal P,Boore JL (2005) Two rounds of whole genome duplication in the ancestral vertebrate. PLoS Biol 3: e114.
11. Putnam NH,Butts T, Ferrier DE, Furlong RF, Helentover U, et al. (2008) The amphioxus genome and the evolution of the chordate karyotype. Nature 453: 1064–1072.
12. Kuraku S, Meyer A, Kuratani S (2009) Timing of genome duplications relative to the origin of the vertebrates: did cyclostomes diverge before or after? Mol Biol Evol 26: 47–59.
13. Kuraku S, Meyer A (2009) The evolution and maintenance of Hox gene clusters in vertebrates through the telost-specific genome duplication. Intern J Develop Biol 53: 765–774.
14. Van de Peer Y, Maere S, Meyer A (2009) The evolutionary significance of ancient genome duplications. Nature Rev Genet 10: 725–732.
15. Catestro G (2012) Two rounds of whole-genome duplication: evidence: impact and evidence on the evolution of vertebrate innovations. In Polyphyletic and polypeptide evolution (ed. MS Solis, DE Solins), 309–339. Berlin: Springer.
16. Holland PW, Garcia-Fernandez J (1996) Hox genes and chordate evolution. Dev Biol 173: 392–395.
17. Venken B, Kirschke EF, Loh YH, Halpern AL, Lee AP, Johnson J, et al. (2007) Survey sequencing and comparative analysis of the elephant shark (Callorhinchus milii) genome. PLoS Biol 5: e101.
18. Kuraku S (2013) Impact of asymmetric gene repertoire between cyclostomes and gnathostomes. Semin in Cell Develop Biol 24: 119–127.
19. Bassham S, Catestro C, Poserlehnt WH (2008) Evolution of developmental roles of Pax2/5/8 after independent duplication in urochordate and vertebrate lineages. BMC Biology 6: e35.
20. Goode DK, Elgar G (2009) The PAX2/8 gene subfamily: a comparative perspective. Dev Dynam 238: 2951–2974.
21. Suga H, Tschope P, Graziansu D, Stierwald M, Schmid V, et al. (2010) Flexibly deployed PAX genes in eye development at the early evolution of animals demonstrated by studies on a hydrozoan jellyfish. Proc Natl Acad Sci USA 107: 14263–14268.
22. Buckingham M, Frédéric R (2007) The role of PAX genes in the development of tissues and organs: PAX5 and PAX7 regulate muscle progenitor cell functions. Annu Rev Cell Bio 23: 645–673.
23. Lynch M, Conery JS (2000) The evolutionary fate and consequences of duplicate genes. Science 290: 1151–1155.
24. Lynch VJ, Wagner GP (2008) Resurrecting the role of transcription factor change in developmental evolution. Evolution 62: 2131–2134.
25. Boyle AP, Song L, Lee BK, London D, Keefe D, et al. (2010) High-resolution genome-wide in vivo footprinting of diverse transcription factors in human cells. Genosome 21: 436–464.
26. Balcarek KA, Lai ZC, Kumar S (1997) Evolution of functional diversification of the paired box (PAX) DNA-binding domains. Mol Biol Evol 14: 829–842.
27. Carstenn J, Haycock DC, McIntyre NE, Reeve-Hoyes JS, Mastro R, et al. (1998) PAX-6 origins – implications from the structure of two coral PAX genes. Dev Gen Evol 208: 352–356.
28. Fleck P, Ahmed I, Anode MR, Barrett D, Beal K, et al. (2013) Ensemble 2013: Nuclear Acids Res 41: D43–D55.
29. Meyer LR, Zweig AS, Hinrichs AS, Karolchik D, Kuhn RM, et al. (2013) The UCSC Genome Browser database: extensions and updates. Nuclear Acids Res 41: 66–70.
30. Park Y, Sheehan SL, Ma N, Madden TL, Spouge JL (2012) New finite-size correction for local alignment score distributions. BMC Res Notes 5: e286.
31. The UniProt Consortium (2012) Reorganizing the protein space at the Universal Protein Resource (UniProt). Nuclear Acids Res 40: D142–D147.
32. Delsuc F, Brinkmann H, Chourrout D, Philippe H (2006) Tunicates and not amphioxus are the sister group of ascidians. J Fish Biol 73: 1064–1071.
33. Ogasawara M, Shigetani S, Hirano S, Satoh N, Kuratani S (2000) PAX1/HOMER and PAX3/6 linkage patterns, and the paired box (PAX) DNA-binding domains. Mol Biol Evol 14: 829–842.
34. Yamamoto K, Mirabeau O, Bureau C, Blin M, Michon-Coudouel S, et al. (2012) Evolution of dopamine receptor genes of the D1 class in vertebrates. Mol Biol Evol (published online ahead of press).
35. Freter S, Muta Y, O’Neill P, Vassilev VS, Kuraku S, et al. (2012) More than 1000 ultraconserved elements provide evidence that turtles are the sister group of archosaurs. Biol Lett 8: 783–786.
36. Yang HS, Friedman R (2008) Genome size reduction in the chicken has involved massive loss of ancestral protein-coding genes. Mol Biol Evol 25: 2601–2612.
37. Curtin MJ, Donoghue PCJ (2007) Paleontological evidence to date the true life. Mol Biol Evol 24: 26–33.
38. Ravi V, Bhatia S, Gautier P, Loosli F, Tay BH, et al. (2013) Sequencing of Pax6 genome-wide in vivo footprinting of diverse transcription factors in human cells. Nature 494: 456–460.
39. Zhang G, Cowled C, Shi Z, Huang Z, Bishop-Lilly KA, et al. (2013) Comparative analysis of bat genomes provides insight into the evolution of flight and immunity. Science 339: 456–460.
40. Kuraku S, Kuratani S (2011) Genome-wide detection of gene extinction in early mammalian evolution. Genome Biol Evol 3: 1449–1462.
41. Braasch I, Volff JN, Schartl M (2008) The evolution of teleost pigmentation and the MHC hypothesis: a parrot with a single, highly polymorphic MHC class II B gene. Immunogenetics 60: 219–231.
42. Bates MD, Wells JM, Veukatsh B (2005) Comparative genomics of the Hlx homeobox gene and protein: conservation of structure and expression from fish to mammals. Gene 352: 45–56.
43. Bielawski JP, Yang Z (2004) A maximum likelihood method for functional divergence at individual codon sites, with application to family gene evolution. J Mol Evol 59: 121–132.
44. Freter S, Ballester B, Swope JL, Zehner J, Race P, et al. (2009) BioMart Central Portal – unifired access to biological data. Nuclear Acids Res 37: 23–27.
45. Durinck S, Huber W (2011) The biomart user’s guide. Database 1: 1–22.
46. Finn RD, Clifford J, Eddy SR (2011) HMMER web server: interactive sequence similarity searching. Nuclear Acids Res 39(Web Server Issue): W37–W43.
47. Finzel B, Clamp M, Durbin R (2004) GeneWise and genewise. Genome Res 14: 992–995.
48. Louis A, Muffato M, Crollius HR (2012) Genomicus: five genome browsers for comparative genomics in eukaryota. Nuclear Acids Res 38: 23–28.
49. Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nuclear Acids Res 32: 1792–1797.
50. Nielsen R, Yang Z (1998) Likelihood models for detecting positively selected amino acid sites and applications to the HIV-1 envelope gene. Genetics 148: 929–936.
51. Yaz T, Bielawski J (2000) Statistical methods for detecting molecular evolution. In Polyploidy and genome duplication (ed. JF Duret, M Griffiths), 379–385. Academic Press.
52. Nielsen R, Yang Z (1998) Likelihood models for detecting positively selected amino acid sites and applications to the HIV-1 envelope gene. Genetics 148: 929–936.
53. Nielsen R, Yang Z (1998) Likelihood models for detecting positively selected amino acid sites and applications to the HIV-1 envelope gene. Genetics 148: 929–936.