The role of transposable elements in functional evolution of amphioxus genome: the case of opsin gene family

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Transposable elements (TEs) are able to jump to new locations (transposition) in the genome, usually after replication. They constitute the so-called selfish or junk DNA and take over large proportions of some genomes. Due to their ability to move around they can change the DNA landscape of genomes and are therefore a rich source of innovation in genes and gene regulation. Surge of sequence data in the past years has significantly facilitated large scale comparative studies. Cephalochordates have been regarded as a useful proxy to ancestral chordate condition partially due to the comparatively slow evolutionary rate at morphological and genomic level. In this study, we used opsin gene family from three Branchiostoma species as a window into cephalochordate genome evolution. We compared opsin complements in terms of family size, gene structure and sequence allowing us to identify gene duplication and gene loss events. Furthermore, analysis of the opsin containing genomic loci showed that they are populated by TEs. In summary, we provide evidence of the way transposable elements may have contributed to the evolution of opsin gene family and to the shaping of cephalochordate genomes in general.

Transposable elements (TEs) are complicated biological entities able to replicate and jump to new locations (transposition) in the genome. Rather simple models have been defined to study their dynamics, while their classification is also problematic. The first TE-classification system distinguishes two classes of TEs, based on the transposition intermediate: RNA (class I or retrotransposons) and DNA (class II or DNA transposons), which follow a “copy-and-paste” and “cut-and-paste” mechanism, respectively. This system was later modified in order to include bacterial, non-autonomous TEs (such as the Miniature Inverted Repeat Transposable Elements - MITEs) and other types of TEs that couldn't fall in any of these two categories. Curcio and Derbyshire categorized transposons according to the way they move, determined by their transposase proteins. A hierarchical classification system for eukaryotic TEs has been proposed by Wicker, et al., which takes into account not only the replication strategy but also the structure of the encoded proteins and of the non-coding domains, the presence and size of the target site duplication (TSD) and even some phylogenetic data.

It was long ago speculated that TEs can “control the time and type of activity of individual genes”, or in other words they play key role in a variety of gene regulatory networks and lately there is accumulating information in favor of this theory (revised by Chuong et al. and Bourque). This can be achieved either by the insertion of TEs in the proximity of genes and consequently the generation of new regulatory elements or the emergence of new regulatory proteins. In fact, TEs occupy a large proportion of the regulatory control regions (revised by Feschotte). On one hand, TEs alter gene expression (activate or inactivate genes); on the other hand, they promote inversions and deletions of chromosomal DNA, they can create new genes (or exons), or serve as illegitimate recombination hotspots. Consequently, they contribute to the shaping of the genome's architecture, its evolution and the emergence of genetic innovations. TE-associated chromosomal rearrangements can be driven by two mechanisms, in particular via homologous recombination or by an alternative transposition process.

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Transposable Elements Analysis. Genomic scaffolds containing opsins and those expected to contain opsin genes based on synteny analyses were screened for repetitive elements using Censor\cite{48} in the RepBase database\cite{48}. NCBI Accession numbers for *B. floridae* scaffolds used are NW_003101565 (Bf_scaffold6), NW_003101418 (Bf_scaffold_187), NW_003101537 (Bf_scaffold_36), NW_003101507 (Bf_scaffold_98) and NW_003101409 (Bf_scaffold_196). The genomic regions used were: Bf_scaffold_6: 305,868–729,662 or 305,868–729,662 (CNR); Bf_scaffold_36: 4,567,754–4,488,902, Bf_scaffold_98: 4,135,366–4,213,900, Bf_scaffold_187: 4,135,366–4,628,754 or 4,135,366–4,378,895 (CNR); Bl_Sc0000011: 2,118,981–2,146,160; Bb_Sc0000116: 547,140 (Comparison of Narrow Regions, CNR); Bf_scaffold_196: 2,792,247–2,817,466, Bl_Sc0000011: 2,118,981–2,146,160; Bb_Sc0000116: 763,100–794,099.

Animal Collection. *B. floridae* adults were collected in Old Tampa Bay (Florida, USA, no permission required for amphioxus collection). Housing of animals and *in vivo* experiments in the present study were performed in accordance with guidelines established by the Institute of Molecular Genetics and in compliance with national guidelines (ID#12135/2010–17210). All animal works were also conducted according to the National
in simple repeated sequences of varying size (2–65 bp) have been detected within and in the proximity of the duplicated in the region where cated in the latter, with more striking example that of Bf73045 (Fig. 1B). Duplication of other genomic fragments Bf_op6 cated genes and genomic fragments in Bl_Sc0000005 (see Supplementary Fig. 4A for names of TEs). A similar vicinity (Supplementary Fig. 4B). No other conservation at genomic level is observed between B. floridae genes and in their even more appealing is the number and type of transposable elements within and and transposon Harbinger-N11 (data not shown).

Frozen in RNAlater® Stabilization Solution (ThermoFisher Scientific), under light conditions.

Cloning and Sequencing of Opsin Gene Fragments/Transcripts. For validation of the in silico predicted gene models, cloning and sequencing of opsin gene fragments and complete transcripts from B. floridai was performed, according to Pantzartzi et al.40. Primers used are included in Supplementary Table 2.

qRT-PCR. Primers used are provided in Supplementary Table 2. Experiments and analysis of results were performed according to Pantzartzi et al.40. TBP was used as the housekeeping gene.

Identification, classification and genome organization of opsin genes in the Branchiostoma genus. We initially performed a thorough comparative analysis of the opsin gene repertoires of three cephalochordate species. We used the recently reported genes from B. lanceolatum41 together with previously reported genes from B. floridai and B. belcheri42–44 many of which had to be re-predicted and some were de novo identified in the current study (Supplementary Table 1). Final transcripts and encoded proteins for newly characterized and modified opsins from B. floridai and B. belcheri as well as details on gene organization and genomic location are provided in Supplementary File 1. Orthology of identical genes was validated by synteny and phylogenetic analysis (Supplementary Fig. 1). The alignments of orthologs for each opsin gene from the three Branchiostoma species are provided in Supplementary File 1. Orthologs have the same number of exons; the sole exceptions are op7 and op20. Orthologous exons have almost identical size, however, pronounced changes are observed in the size of the last exon. Furthermore, there is a great similarity among orthologs in terms of sequence, with the C-terminus being the most variable. Evidently, opsin genes are spread over 16 genomic regions (scaffolds) in B. floridai and 14 in B. belcheri (Supplementary Fig. 2). Phylogenetic analysis (Supplementary Fig. 1) in combination with the arrangement of opsin genes in the genomes of the three species (Supplementary Fig. 2) supports the fact that the majority of opsin genes are represented by an ortholog in all three species (Table 1). This is not the case for op6, op12b, op13b, and op17b, which seem to be the result of a gene duplication.

We further analyzed the opsin expression pattern across different developmental stages (Supplementary Fig. 3) of B. floridai. Onset of several opsin gene expression starts at L1 stage, in which frontal eye and lamellar body (ciliary photoreceptive organs) start to develop. In agreement with B. lanceolatum41, the majority of the B. floridai opsin genes show most predominant expression in L2/L3 stages, where all of the known amphioxus photoreceptor organs are differentiated. Nevertheless, differences are observed between the two species in regard to the onset of expression of op13a. Interestingly, op6, a gene detected only in B. floridai, follows a distinct pattern in regard to the other two neuropsins (i.e. op7 and op8), for which expression patterns are the same for both B. floridai and B. lanceolatum.

Transposable elements and opsin genes in the Branchiostoma genus. Differences have been noted among the three Branchiostoma species in regard both to the structure and the number of opsin genes (Table 1 and Supplementary File 1). Since transposable elements (TEs) have been vastly implicated in gene structure alteration as well as gene duplications and losses, we scanned scaffolds containing altered genes against RepBase to locate TEs populating these regions; for opsin orthologs that are absent from one or two Branchiostoma species (Table 1), we found the syntenic scaffolds and also scanned them against RepBase.

The beginning of forth exon of Bl_op2 is occupied by small repeated sequences, a fact that leads to elongation of the third cytoplasmic loop (Supplementary File 1). Noticeably, the fifth intron of Bl_op8 highly resembles a satellite locus from Salmo salar (SAT-11_Ssa in RepBase). In fact, the beginning of the last exon is one of the repeat units. It is also worth mentioning that the last exon of Bl_op16 is longer in size than the respective exons from the B. floridai and B. belcheri orthologs due to palindromic repeats at its end (Supplementary File 1). Bl_op16 is flanked by a truncated and a complete copy of the DNA transposon Ginger2-1 and the non-autonomous DNA transposon Harbinger-N11 (data not shown).

Comparison of the syntenic scaffolds related to op6 is portrayed in Fig. 1. High similarity is observed among the genomic regions containing op7 in B. floridai, B. lanceolatum and B. belcheri (Fig. 1A). Similarity is also observed between the genomic regions flanking op6 in B. floridai and B. lanceolatum Sc0000005 and B. belcheri scaffold484, however, there are no traces of op6 in the other two species. Some of the immediately flanking genes of Bf_op6 have their orthologs in B. lanceolatum (only one seems to be eliminated, namely B210534), but are duplicated in the latter, with more striking example that of Bf73045 (Fig. 1B). Duplication of other genomic fragments in the region where Bf_op6 was supposed to be is also evident. Numerous families of transposable elements and simple repeated sequences of varying size (2–65 bp) have been detected within and in the proximity of the duplicated genes and genomic fragments in Bl_Sc0000005 (see Supplementary Fig. 4A for names of TEs). A similar case of duplicated genomic fragments populated by transposable elements is also observed in B. belcheri. What is even more appealing is the number and type of transposable elements within Bf_op6 and Bf_op7 genes and in their vicinity (Supplementary Fig. 4B). No other conservation at genomic level is observed between B. floridai scaffolds 6 and 187, apart from the opsin genes and various transposable elements, as shown in Supplementary Fig. 4B.
Differences are observed among the three species in regard to op12 and op13 copies (Table 1, Fig. 2 and Supplementary Fig. 2). In general, these genes exhibit high sequence similarity and contain the same number of exons. Size of the exons is almost identical, with a strikingly smaller last exon in Bb_op12b (Supplementary File 1). Comparison of scaffolds bearing op12a, op12b and op13a from the three species (Fig. 2A) shows that there is high conservation in opsin genes as well as in their flanking regions. However, no significant similarity exists in the intergenic regions of op12a and op13a. Interestingly, opsin genes in B. belcheri are flanked by complete copies of DNA transposons (Supplementary Fig. 4C). The absence of op13b ortholog from B. floridae and B. belcheri is evident from the comparison of syntenic scaffolds (Fig. 2B). On the other hand, scaffolds containing the B. lanceolatum op13a and op13b paralogs (Fig. 2C, Supplementary Fig. 4C) show a high degree of similarity only in the genic regions and their immediate neighborhood which does not extend further in the region of Bl_op12a. The region of similarity is bordered by simple repeats as well as complete or partial copies of TEs.

Another example of putative gene duplication and loss event is that of op17a and op17b (Fig. 3). Using the neighboring genes of Bf_op17a we detected the syntenic scaffold in B. belcheri. Comparison of the three scaffolds shows conservation in the flanking regions but no traces of a Bb_op17a gene. Instead, in the region where Bb_op17a is expected to be, there are copies of retrotransposons52 (Supplementary Fig. 4D). Bl_op17a and Bl_op17b genes are as well flanked by autonomous and non-autonomous transposons.

To summarize our previous findings, we could say that independent events of gene duplications and losses occurred during the evolution of Branchiostoma opsins (Fig. 4A). Taking into account the higher similarity between B. lanceolatum and B. belcheri regions, the almost identical structure of Bf_op6 and Bf_op7 and the presence of common transposable elements within and outside these two genes, we could conclude that op6 is the result of a duplication event in B. floridai, after its split from B. lanceolatum. However, we cannot rule out the possibility that op6 existed in the common ancestor of the Branchiostoma species and it was eliminated in the lineages of B. lanceolatum and B. belcheri. We could also conclude that Bb_op12a and Bl_op13a were independently duplicated in B. belcheri and B. lanceolatum. Finally, we assume that op17a was lost in B. belcheri and op17b is the result of a gene duplication only in B. lanceolatum (Fig. 4A). Figure 4B outlines what the ancestral state could have been for each of the duplicated/lost genes and the putative mechanisms through which gene gains and losses took place. Complete and partial copies of TEs identified in the vicinity of opsin genes probably served as illegitimate spots for recombination, leading to misalignment, unequal crossover and hence duplication of an opsin gene, as in the case of op12 and op13, or caused crossing over of the same chromosome, leading to the deletion of op17 in B. belcheri.

Discussion

Cephalochordates are often used as a proxy to the ancestral chordates. This is in large part due to the presumed slow evolutionary rate of their genomes. In this study we used the Branchiostoma opsin gene family as an example
Figure 1. Comparison of genomic loci containing or lacking op6 and op7. (A) Comparison of the op6 containing Bf_scaffold_6 and the op7 containing Bf_scaffold_187 with the BL_Sc0000005 and the Bb_scaffold48 that apparently contain only op7 and lack op6. (B) Comparison of more narrow regions of Bf_scaffold_6 (delimited by arrows in (A)), with BL_Sc0000005 (left) and Bb_scaffold48 (right). A high degree of duplicated regions was observed for B. lanceolatum, with the most striking example that of Bf73045 (left). Duplicated regions were also observed for B. belcheri (right). Red and black (complete and partial copies based on the RepBase database) symbols mark the position of simple tandem repeats and various families of Transposable Elements (TEs) (see key legend for explanation and Supplementary Fig. 4A for TE names). For the sake of clarity, predicted B. floridae gene models are listed only in the internal part of the Bf_scaffold_6 in (B). Ribbons connecting syntenic scaffolds under comparison denote similarity at genomic level.
Figure 2. Comparison of genomic loci containing or lacking op12a, op12b, op13a and op13b opsins. (A) Comparison of Bl_Sc0000154 with Bf_scaffold_36 and Bb_scaffold_23 (B) Comparison of B. lanceolatum scaffold containing the op13b gene with the syntenic scaffolds from B. floridae (Bf_scaffold_98) and B. belcheri (Bb_Sc0000263). (C) Comparison of B. lanceolatum scaffolds bearing opsins op13a (Sc00000154) and op13b (Sc0000040). Red and black (complete and partial copies based on the RepBase database) symbols mark the position of simple tandem repeats and various families of transposable elements (TEs) (see key legend for explanation and Supplementary Fig. 4B and C for TE names). Predicted B. floridae gene models are listed in the internal part of the scaffolds.
of how TEs can shape cephalochordate genomes, by deleting or creating new genes, by altering the number and size of exons or influencing their expression patterns. We further reconstructed the evolutionary history of opsin family in the Branchiostoma genus, via comparison of primary sequence, structure and expression patterns of opsin genes from three cephalochordate species.

The species-specific duplicates Bl_op13a and Bl_op13b differ in their spatial (tissue-specific) but overlap in their temporal expression patterns and are already detected at an earlier stage than B. floridae (Pantzartzi et al. and Supplementary Fig. 3). The first one is indicative of subfunctionalization, where the two genes seem to have optimized for specific tasks in tissues with different type of photoreceptor cells (ciliary and rhabdomeric), while the latter implies that Bl_op13a underwent neofunctionalization, due to which expression is triggered at an earlier stage. The relatively large size of the Go group and the retention in the genome of the duplicated opsins (Bb_op12b and Bl_op13b) could be an indication of fine tuning between these opsins in order for specific photoreception-related tasks to be fulfilled. Similarly, retention of Bf_op6 and Bf_op7 in the genome of B. floridae could be attributed to subfunctionalization, since changes are noted in their temporal expression pattern (Supplementary Fig. 3B).

The role of transposable elements (TEs) in shaping the genome and promoting evolution has been the focus of many studies, and what was formerly characterized as “junk” or “selfish DNA” is gaining more and more value and functional importance. TEs may act in the same or completely different way, depending on selection forces. This is nicely exemplified by the ParaHox loci in Ciona, amphioxus and vertebrates. ParaHox cluster in Ciona has lost the tight organization present in chordates and this degeneration could be attributed to the invasion of TEs in the locus, specifically of MITEs. On the other hand, even though the amphioxus ParaHox cluster was found to be a hotspot for TE insertion, selection constraints probably inhibit this disruptive elements from influencing the ParaHox locus. Another example of how TEs may influence the gene structure is that of PRHOXB gene, for which the gain of an intron was reported, in which the miniature inverted-repeat transposable element (MITE) LanceletTn-2 was detected.
An increase in the number of opsin gene has been previously reported for various species, owing either to local gene duplications or whole genome duplications. In some cases, the number or structure of opsin genes seems to be shaped under the influence of TEs. The presence of an incomplete Alu element upstream the human middle wavelength sensitive (MW) opsin gene may imply that Alu elements have been involved in the initial gene duplication responsible for the MW and long-wavelength sensitive (LW) genes in the Old World primates and the high frequency of gene loss and gene duplication within the opsin gene array. It is suggested that unequal crossover is the mechanism through which this duplication occurred. In the swordtail fish, Xiphophorus helleri, one of the four LW copies was found to be the result of a retrotransposition event. On the other hand, the loss of function of the Takifugu rubripes RH2-2 gene is reported to follow a transposon-induced deletion that truncated the N-terminal of the protein.

We have provided information about how TEs might have led to gene duplications and losses in the Branchiostoma opsin family, or alterations in the number and size of exons. In fact, the Branchiostoma opsin
family could serve as an example of how TEs can play an important role in the shaping of a gene family and of the genome per se, through gene gain and loss events due to unequal cross-over or moving of genes between different loci in the genome (Fig. 5). Moreover, TEs may also lead to neofunctionalization of duplicate genes, which typically occurs by the acquisition of new regulatory elements. Overrepresentation of transcription factor binding sites is evident for TEs residing in promoter regions of not only human genes, but of amphioxus as well. Retention of Branchiostoma gene copies in the genome and differences in their spatiotemporal expression pattern, together with the presence of different types of TEs, could also imply that TEs were not implicated only in the birth or death of opsin genes but in their control as well.

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Author Contributions
C.N.P., J.P. and Z.K. conceived and designed the experiments; C.N.P. performed in silico analysis, J.P. performed expression analysis experiments; C.N.P., J.P. and Z.K. wrote the manuscript.

Additional Information
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