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Research Article

New Insights into Vertebrate Thyroid Hormone Receptor Evolution

Guillaume Holzer[1] and Vincent Laudet[2]

[1]*Institut de Génomique Fonctionnelle de Lyon, Université de Lyon, Université Claude Bernard Lyon 1, UMR CNRS 5242, Ecole Normale Supérieure de Lyon, 46 Allée d'Italie, 69364 Lyon Cedex 07, France*
[2]*Observatoire Océanologique de Banyuls-sur-Mer, UMR CNRS 7232, Université Pierre et Marie Curie Paris, 1 avenue Pierre Fabre, 66650 Banyuls-sur-Mer, France*

Abstract. The lamprey *Petromyzon marinus* belongs to the agnathans, the oldest vertebrate lineage from which jawed vertebrates diverged about 500 million years ago. Therefore, it holds a key phylogenetic position to understand the evolution of vertebrates. As in jawed vertebrates, two thyroid hormone receptors have been described in lamprey. These receptors, referred to as TR1 and TR2, behave as genuine TRs but are considered as an independent duplications when compared to the orthologs characterized in jawed vertebrates, TRα and TRβ. Here, we show that the lamprey genome contains two additional TR sequences. Their assignment to *bona fide* thyroid hormone receptors is supported by sequence alignments and phylogenetic reconstructions. This led us to revisit the phylogeny of thyroid hormone receptors and to detect an acceleration of their evolutionary rates at the basis of vertebrates. Our analysis therefore suggests that major evolutionary shifts occurred at the receptor level just when the modern synthesis of thyroid hormone was established during early vertebrate evolution.

Keywords: Thyroid hormone receptor, nuclear receptor, lamprey, vertebrates, evolution, gene duplication

Corresponding Author
Vincent Laudet
email: vincent.laudet@obs-banyuls.fr

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1. Introduction

Thyroid hormones (THs) are involved in pleiotropic processes in vertebrates [1] such as metabolic control [2], photoperiod signaling [3] or metamorphosis [4]. TH signaling is mediated by thyroid hormone receptors (TRs), ligand-dependent transcription factors that belong to the nuclear receptor superfamily. Like other nuclear receptors, TRs display two major functional domains, the DNA binding domain (DBD) and the ligand binding domain (LBD). In response to the presence of the hormone and in particular the active ligand, triiodothyronine (T3), the receptor exhibits a conformational change and activates a wide variety of target genes, which elicits the biological responses [5, 6]. Two paralogous genes, TRα and TRβ, which originate from a genome duplication predating the gnathostome radiation, have been characterized in jawed vertebrates. Interestingly, TRα and TRβ display different biological properties such as gene regulation or binding properties [6, 7]. However, the precise evolution and timing of duplication of the TRs is difficult to reconcile with the known events of genome duplication that took place earlier on during vertebrate evolution [8–10]. The two sea lamprey TR genes (TR1 and TR2) appear to be the product of an independent duplication [11], but this issue is still debated and complicates our understanding of TR evolution.

The sea lamprey *Petromyzon marinus* belongs to the agnathans, a jawless vertebrate group that branches at the basis of vertebrates. It is believed that jawed vertebrates emerged from an ancestral agnathan *ca.* 450 million years ago. This phylogenetic position makes lamprey a key species to understand the evolution of vertebrates [10]. This is particularly true when gene duplications are concerned since agnathans are located at a key position regarding the two genome-wide duplication events that have shaped the vertebrate genomes [12–14]. However, the timings of these two rounds of vertebrate genome duplication have been difficult to determine precisely, especially regarding the split between agnathans and gnathostomes. Indeed, the question whether these duplications occurred before or after the agnathan/gnathostome split is still debated [9, 15–17]. The most widely accepted model suggests that the two duplications took place before the agnathan/gnathostome split [14, 18]. However, recent comparative data led some authors to propose that, if a single whole-genome duplication effectively occurred at the base of vertebrates, a second event was not supported. These authors proposed instead the occurrence of several evolutionarily-independent duplications in addition to the first whole genome duplication (WGD) [17]. This question of the pattern and tempo of gene/genome duplications that have shaped early vertebrate genomes is particularly important when the TRs are concerned. As mentioned above, the gnathostomes TRα and TRβ are believed to be the product of one of the vertebrate specific genome duplication. If, as it is commonly believed, the 2R model is correct, this means that two of the four duplicates have been secondarily lost. In this context, the existence of two TRs in lamprey that are apparently the products of one independent gene duplication specific to agnathans is of course of interest [19, 20]. However, if the classical 2R model with the two duplications taking place before the agnathan/gnathostome split is true, the observation of an agnathan specific TR duplication implies a large number of independent gene losses.

This question of TR evolution in early vertebrates must be placed in the wider perspective of the evolution of thyroid signaling in vertebrates. Indeed here again, agnathans are a particularly important node. By reconstructing the evolution of thyroglobulin (Tg), the huge protein precursor from which vertebrate THs are synthesized, we have shown that the modern synthesis of these hormones was first put in place in agnathans [21]. In invertebrate chordates such as amphioxus or tunicates, there is nothing reminiscent of a Tg, and although THs are present and active, we do not know how they are produced [22, 23]. In addition, the modern thyroid endocrine gland also originates with lamprey. Indeed, lamprey exhibits a spectacular transformation of an invertebrate chordate-type exocrine endostyle to a modern endocrine thyroid gland during their larval-to-juvenile metamorphosis [24, 25]. Lastly, the metamorphosis of lamprey is, like other known metamorphosis of chordates [26], controlled by THs but with a striking difference: in all known chordates, THs trigger metamorphosis and TH levels surge during metamorphosis, whereas in lamprey TH levels drop during metamorphosis and this process is induced by blocking TH secretion with goitrogens [27, 28]. In other words, the TH control of metamorphosis functions in a reverse orientation when compared to amphioxus or gnathostomes.

The previous studies of TRs in lamprey have resulted in the characterization of two TRs. Like their gnathostome orthologues, lamprey TRs behave as transcription factors activated by the hormone. However, these two TRs do not provide a clear explanation to the striking mode

of control of lamprey metamorphosis by THs. This is why we have scrutinized the genomic data accumulated on agnathans.

Here, we provide a bioinformatic and evolutionary characterization of two new TR genes in the lamprey *Petromyzon marinus*, strongly suggesting that in fact four different loci exist in the genome. We compared their evolution with the evolution of other nuclear receptors, in particular Retinoic Acid Receptors (RARs) and Rev-erbs that are present on the same chromosomal locations. This allowed us to revisit the TR phylogenetic tree and to detect major shifts in the rate of evolution of these genes, strongly suggesting that varying evolutionary pressures were exerted on TR genes during early vertebrate evolution.

2. Material and Method

2.1. Search for TR sequence in *P. marinus* genome

The sequence of the previously identified TR1 and TR2 were used to run a TBLASTN on the lamprey genome assembly available on Ensembl [29]. Coding sequences were predicted from the loci retrieved by TBLASTN, using the Augustus software on the web interface [30]. These predictions were validated by a BLASTP search in Genbank.

2.2. Phylogenetic analysis of TRs

Vertebrate TR, RAR and Rev-erb amino acid sequences were retrieved from Genbank (see Table S1 in Supplementary Material available online at `http://www.agialpress.com/journals/nurr/2017/101287/`) and aligned using MUSCLE 3.8 [31]. For phylogenetic reconstructions, we restricted the analysis to the C-term part of the DBD and the LBD since we were able to retrieve and identify only these domains for all genes and species studied (see Table S2 in Supplementary Material available online at `http://www.agialpress.com/journals/nurr/2017/101287/`). Trees were generated using the Maximum Likelihood (ML) method under a JTT substitution matrix plus an eight category gamma rate correction (α estimated) with the proportion of invariant sites estimated. 1000 bootstrap replicates were performed to evaluate the branch support. Both alignments and tree calculations were performed using the Seaview v4.5 software [32]. A bayesian based phylogenetic tree was also computed using the MrBayes software, using a mixed amino acid model, 5 000 000 steps of Monte Carlo Markov Chain, a sampling every 1000 steps, and a 25% burn-in [33].

3. Results

3.1. Four TR orthologs are present in lamprey

The genome of the sea lamprey *Petromyzon marinus* has been sequenced and assembled, and these data are available in Ensembl [10]. By running a TBLASTN search on the genome using the lamprey TR1 (Genbank DQ320317.1) and TR2 (Genbank DQ320318.1) as queries, we retrieved three loci containing TR-related coding sequences. One of them encodes the previously reported TR2 and the two others contain novel sequences: ENSPMAG00000000524

(referred to as Pm524) on scaffold GL482050, and ENSPMAG00000008742 (referred to as Pm8742) on scaffold GL477881. Interestingly, when we look for the already known TR1 and TR2 in the published genome of *P. marinus*, we are able to retrieve TR2 under the locus ENSPMAG00000005715 on scaffold GL478356, but not TR1. When we Blast TR1, the best hits match on the scaffold GL478356 with 78.3% of identity. Nevertheless, the TR on GL478356 is more similar to TR2 with 96.5% of identity. Thus, we anticipate that TR1 belongs to an unsequenced region of the lamprey genome.

Using the gene prediction software Augustus [30], we retrieved the protein sequence of Pm524 and Pm8742. For Pm524, we were able to retrieve the C-terminal region of the DBD and the complete LBD, which represent 293 amino acids. For Pm8742, we were able to retrieve the complete DBD and the complete LBD, which represent 362 amino acids, but not the A/B region. The alignment of Pm524 and Pm8742 with lamprey TR1 and TR2 and human TRα and TRβ reveals a high level of sequence identity on aligned regions (Figure 1). Pm524 has 72.1% of amino acid identity with lamprey TR1, 67.2% with lamprey TR2, 67.6% with human TRα and 68.9% with human TRβ. Pm8742 has 69.6% of identity with lamprey TR1, 66.0% with TR2, 68.8% with human TRα and 67.1% with human TRβ (see Table S3 in Supplementary Material available online at `http://www.agialpress.com/journals/nurr/2017/101287/`). These scores are even higher when only the DBD (when available) or the LBD are considered (see Table S3 in Supplementary Material available online at `http://www.agialpress.com/journals/nurr/2017/101287/`). This indicates a conservation of these proteins and suggests their assignment as TRs. The level of identity is much lower between Pm524 and Pm8742 and RARs (between 30 and 40% with human RARs, see Table S3 in Supplementary Material available online at `http://www.agialpress.com/journals/nurr/2017/101287/`), which are the nuclear receptors most closely related to TRs in phylogenetic reconstructions [34]. Taken together, these data support the assignment of Pm524 and Pm8742 to TRs. Pm524 and Pm8742 have 62.8% of identity between each other. This is lower than the identity between human TRα and TRβ (76.8%, see Table S3 in Supplementary Material available online at `http://www.agialpress.com/journals/nurr/2017/101287/`), making it unlikely that Pm524 and Pm8742 originated from a recent single locus duplication. Additionally, we also retrieved four TR orthologs in the Japanese lamprey *Lampetra japonica* that align well with human and *P. marinus* TRs, and show high identity scores with human TRα and TRβ (between 60% and 70%, (see Figure S1, Table S3, and Table S4 in Supplementary Material available online at `http://www.agialpress.com/journals/nurr/2017/101287/`).

A detailed sequence analysis highlights an even more striking degree of conservation of some domains. In the DBD of Pm524 and Pm8742, the conservation of the first zinc finger is particularly strong with only 2 out of 21 amino acids that differ in Pm8742 when compared to human TRs. There are more differences in the second zinc finger, which is also observed between human TRα and TRβ. Together, this indicates a strong conservation of the DBD. It also suggests a similar 3D structure between lamprey and human TRs and therefore similar DNA binding properties. In the LBD, the amino acids of the ligand binding pocket and in particular two out of the three amino acids forming direct hydrogen bonds with the ligand in the pocket (R228, S277 and H381, human TRα numeration) are conserved. Importantly, S277 in human TRα is replaced by N331 in human TRβ, and the asparagine residue is conserved in

all lamprey TRs. This indicates that from a pharmacological perspective, lamprey TRs might be more similar to TRβ than TRα or, in other words, that TRα may be a more divergent TR when compared to the ancestral single gene. Together with the overall sequence conservation observed, this residue conservation further supports the assignment of the two newly identified genes as TRs. It also suggests ligand binding specificities similar to the gnathostome TRα and TRβ, and the lamprey TR1 and TR2 paralogues.

3.2. Lamprey TRs are the sister group of gnathostome TRs

To further assess the assignment of Pm524 and Pm8742 to TRs, we constructed a phylogenetic tree of the TR protein sequences by Maximum Likelihood, including RAR and Rev-erbs as outgroups (Figure 2). As Pm524 is partial, we restricted the alignment to the region covered by Pm524. We excluded *Ciona* TRs from this reconstruction because of its high divergence. Similar relative positions of the lamprey genes were obtained using Maximum Likelihood and Bayesian algorithms (Figure 2, see Figure S1 in Supplementary Material available online at `http://www.agialpress.com/journals/nurr/2017/101287/`). In both cases, Pm524 and Pm8742 group with other vertebrate TRs with a strong statistical support (1000 bootstrap support), confirming their identification as TR sequences (Figure 3). The lamprey receptors TR1, TR2 and Pm524 group together with an unsupported node (450 bootstrap), whereas Pm8742 branches at the basis of the whole vertebrate group but with weak support (630 bootstrap). All gnathostome TRs are clustered in strongly supported monophyletic group (990 bootstrap, Figure 2). Interestingly, the branch leading to the vertebrate TRs (Figure 2) is longer than those leading to non-vertebrate TRs with a mean of 1.5 changes per site for this branch. This indicates that vertebrate TRs have a higher evolutionary rate than their non-vertebrate counterparts.

3.3. Synteny suggests Pm8742 and Pm524 as lamprey TRs

In order to confirm the identification of Pm8742 and Pm524 as TRs and to better understand their relationships, we compared the organization of the genomic loci containing these genes to the TRα and TRβ genes characterized in humans (Figure 3). A conserved synteny was previously reported in the lamprey between PmHox2, a RAR gene and a TR gene that clustered over more than 400 kb on scaffold GL4877881 [10] (Figure 3). This organization is reminiscent of the relative position of TRα, RARα and the HoxB cluster on human chromosome 17 (Figure 3). We identify the *P. marinus* TR gene found in the vicinity of PmHox2 as Pm8742, which supports the assignment of Pm8742as a TR. Along the same line, we found another gene coding for a nuclear receptor (ENSPMAG00000000523 or Pm523) next to the Pm524 gene, in the opposite orientation, on scaffold GL482050. Based on Blast identification and phylogeny construction (Figure 2), we identified Pm523 as a Rev-erb gene. Rev-erb family members are also found in the vicinity of TRs, encoded in the opposite direction, on human chromosomes 3 and 17 [35, 36] (Figure 3), which adds support to the identification of Pm524 as a TR. Of note, no Rev-erb ortholog was found in the vicinity of Pm8742. Given the direction of Pm8742 on this scaffold (toward the 3' end) and that Rev-erb genes are found in opposite direction of TRs [35], we would expect to find a Rev-erb gene in the 3' end region of this scaffold. However, we did not find such an associated Rev-erb, certainly because Pm8742 is

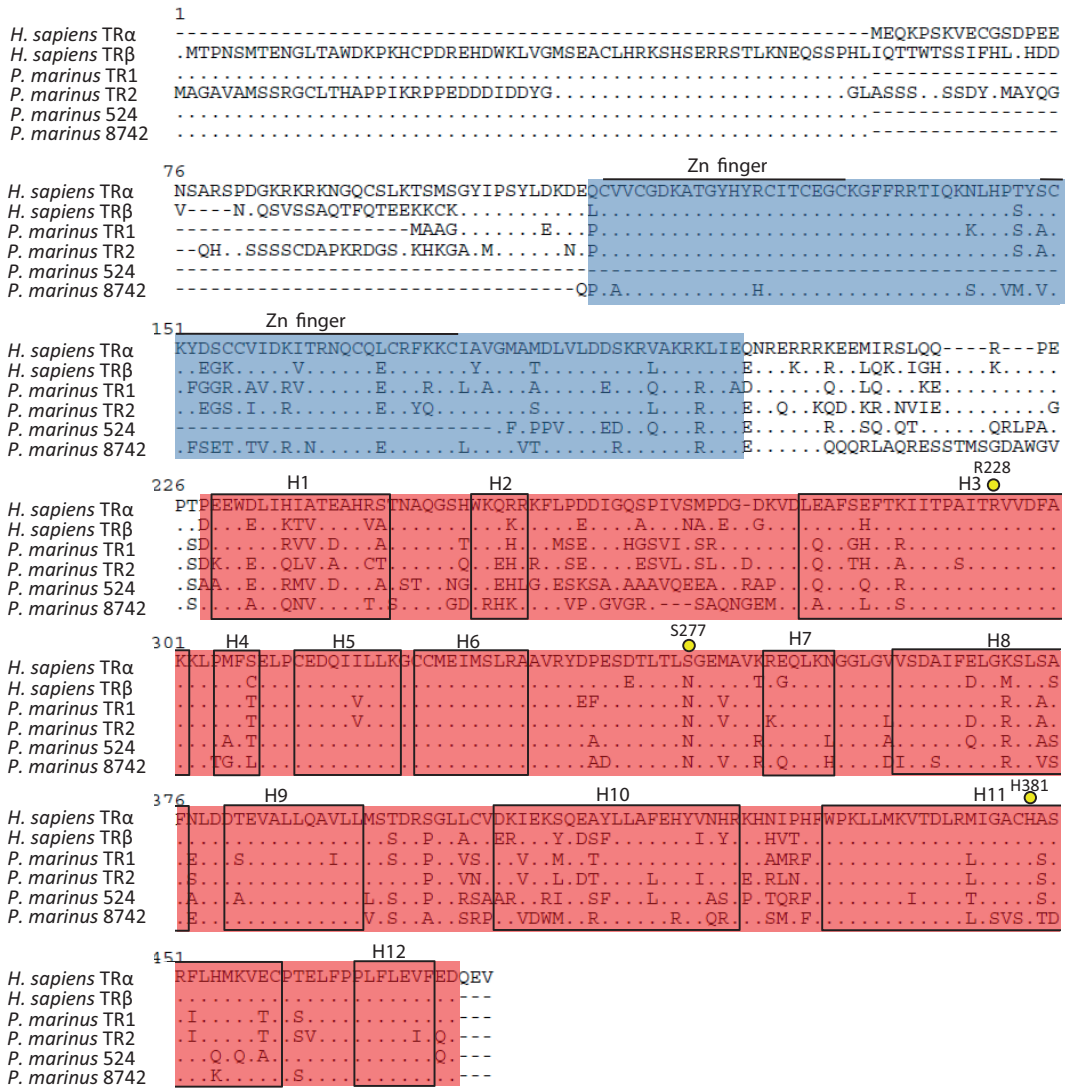

Figure 1: **Alignment of human and lamprey TRs.** Human TRα is used as the reference sequence. Dots indicate that the amino acid in this position is the same as TRα. Dash indicates that there is no amino acid in the sequence at this position. A blue rectangle highlights the DBD and a red rectangle the LBD. The zing fingers of the DBD are indicated as well as the helixes of the LBD. The amino acid forming direct bound with the ligand in the LBD pocket are indicated with a yellow dot.

too close to the 3' end of the scaffold. We expect the Rev-erb associated with Pm8742 to be outside the scaffold GL477881. When we investigated for this Rev-erb ortholog by blasting the gnathostome Rev-erb on the genome, we found the gene ENSPMAG00000008468 (Pm8468) on the scaffold GL74430 but there is no evidence supporting a link between GL4430 and GL477881.

4. Discussion

The identity scores between TR1, TR2, Pm524 and Pm8742 oscillate between 60 and 80%, which is similar to the identity between human TRα and TRβ. Thus, we exclude the possibility that Pm524 and Pm8742 be recent duplications since we would expect higher identity scores.

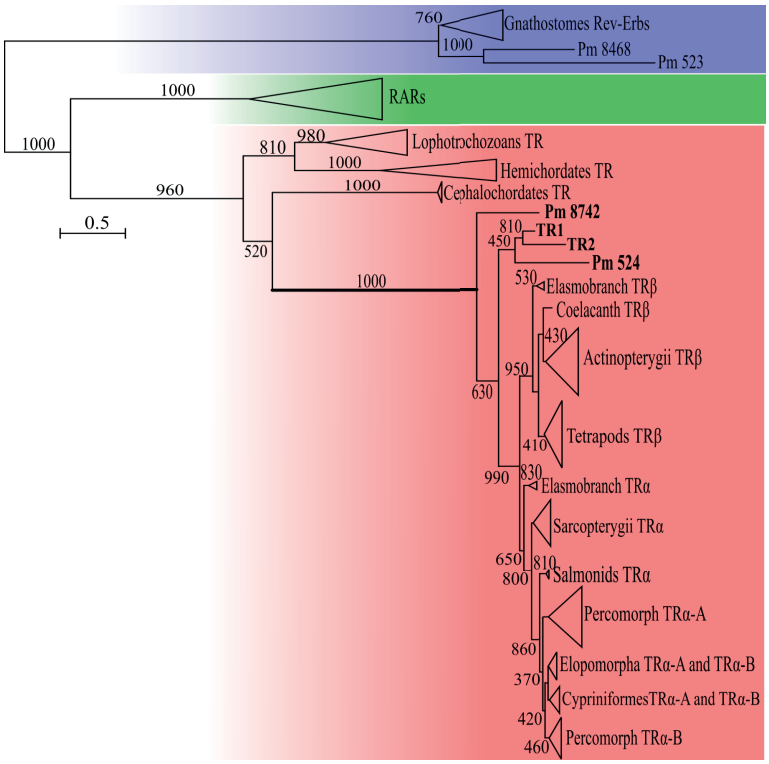

Figure 2: **Phylogeny reconstruction of TRs.** Phylogenetic tree built by maximum likelihood using the DBD and LBD of TR, RAR and Rev-erb amino acid sequence. All bootstrap supports are indicated. TRs are highlighted in red, RARs in green and Rev-erbs in blue. The branch supporting the vertebrate TRs is highlighted with a bold line. The horizontal bar represents the evolution rate in mean substitution per site.

The fact that their amino acid sequences are well conserved, with high conservation of functionally important regions also suggests they are not pseudogenes. The *P. marinus* genome and the cloning of TR1 and TR2 have been performed by different labs with different animal and DNA sources [10, 11]. Thus, it could be argued that the TRs we newly identified are actually polymorphism between individuals. Nevertheless, under this hypothesis we would not expect between 60 of 80% identity betweenTR1/TR2 on the one hand and Pm524/Pm8742 on the other hand, but scores around 90%. Interestingly, we obtain 96.5% of identity when we Blast TR2 on the published genome on Ensembl. We anticipate that this 3.5% difference may therefore give a rough estimate of the degree of polymorphism. In any case, the Blast of TR1 and TR2 in lamprey genome reveal three different scaffolds carrying TRs, indicating that there are at least three TRs in lamprey. Considering that the cloned TR1 is not found in the published lamprey genome, according to our investigation, it brings the total number of lamprey TRs to four. The origin of the cDNA used for the lamprey genome project and the lamprey TR cloning could explain why we did not retrieve TR1 in the genome. *P. marinus* is known to undergo a large-scale programmed elimination of somatic DNA during its development [19, 20]. We suspect that TR1 has been cloned from larval DNA [11], which may have been lost during lamprey development and absent from the adult genome, which is the genome of reference [10]. Therefore, we conclude that there are four TR genes in lamprey: TR1, TR2, Pm524 that we propose to call TR3 and Pm8742 that we propose to call TR4.

The two rounds of WGD that have shaped vertebrate genome likely happened in the common ancestor of all vertebrates, before the agnathan/gnathostome divergence, although it is still

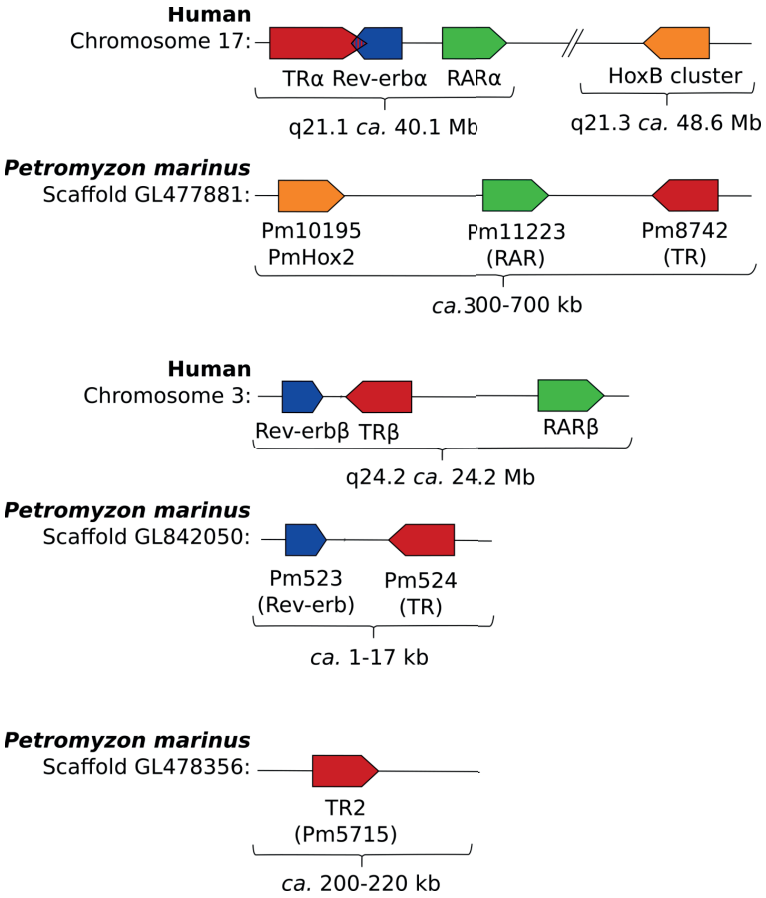

Figure 3: **Two new lamprey TRs in the lamprey genome.** The red boxes indicate the position of the TR orthologs on a scaffold. The blue boxes represent the position of a Rev-erb gene. RAR genes are in green and Hox clusters in orange. The 3' end of each gene is indicated by an arrowhead. Dashed line on the left and right of the bottom panel indicate that the scaffold continues in 5' and 3'. The scaffold number is indicated under each panel.

debated [10]. One of these genome duplications gave rise to the TRα and TRβ paralogs known in gnathostomes [9, 37]. In this context, our discovery of two new TR genes that do not appear to be clear orthologues of either TRα or TRβ is surprising (Figure 2). The genomic data clearly indicate that there are at least three loci encoding TR in lamprey: Pm5715 (that is the already known TR2), Pm524 and Pm 5715 (respectively on the scaffolds GL478356, GL842050 and GL477881). The synteny around the loci Pm524 and Pm8742 are respectively reminiscent of the synteny around the human TRβ and TRα (chromosome 3 and 17 respectively, Figure 3). Indeed, for Pm524, we found a Rev-erb gene in the opposite direction, indicating that Pm524 is a TR. For Pm8742, we found a RAR gene and a Hox cluster in the vicinity, indicating that Pm8742 is a TR. Without more TR sequences from other agnathans, it is difficult to conclude if the topology of the phylogeny we calculated is linked to a methodological artefact (*e.g.* too few information in the tree for a good resolution), or if it is a reflection of the true evolutionary history of these genes. Moreover, the long branch leading to the vertebrates TRs (Figure 2, bold line) suggests an acceleration of amino acid substitutions in TRs. This could explain why the lamprey TRs position is difficult to clearly determine and is not well statistically supported. Nevertheless, if the topology reflects the biological reality, and with the hypothesis that the non robust placement of Pm8742 at the basis of the vertebrate TRs is artefactual, this would require five independent evolutive events in addiction to the two WGD: four losses and one

lamprey-specific duplication in addition to the two early WGD (Figure 4A). However, two other *scenarii* are possible if we consider that, possibly because of an acceleration of evolutionary rates in the lamprey TRs, their position at the base of the Gnathostome tree is artefactual. First, we would have two lamprey orthologues for each of the Gnathostome TRs (Figure 4B). This would therefore require three evolutive events in addition to the two WGDs: one loss of the first whole genome duplicate, and two independent lamprey-specific duplications of the ancestral TRα and TRβ (Figure 4B). Under this hypothesis, the four lamprey TRs would actually correspond to TRα-A, TRα-B, TRβ-A and TRβ-B. There is a third hypothesis that requires two evolutive events in addition to the two WGDs: two independent losses of the TRγ and TRδ copies in the ancestor of the gnathostomes (Figure 4C). Under this hypothesis, the four lamprey TRs would be TRα, TRβ, TRγ and TRδ. Nevertheless, more TR sequences and syntenies from agnathans as well as the clear understanding of the timing of WGD and agnathan/gnathostome split are necessary to definitely solve the phylogeny and propose a clear scenario of TR evolution.

The two new TRs, Pm542 and Pm8742, have not yet been molecularly investigated and could open new perspectives to understand the enigmatic action of TH in lamprey. Indeed, it is well established that TH controls metamorphosis in all chordates [38]. TH treatment induces a precocious metamorphosis and a surge of TH is observed at the climax of metamorphosis in all the investigated species [26, 39, 40]. In lamprey however, it is a decrease of TH, instead of an increase, that triggers metamorphosis [27, 28, 41]. Intriguingly, the already identified lamprey TRs (TR1 and TR2) behave as genuine TRs: they bind T3 at nanomolar concentration and can activate target gene transcription [11]. Therefore, they cannot explain alone how the system can work in a reverse way in lamprey. The two new receptors we identified in this study might provide a part of the explanation of such a reverse system. First, it is possible that Pm542 and Pm8742 behave differently than TR1 and TR2. They might act as constitutive repressors similarly to the vertebrate TRα2 isoform that binds DNA but cannot bind ligand, because of disrupted LBD, which results in an inhibition of gene transcription [42–45]. Nevertheless, this seems unlikely in the case of Pm542 and Pm8742 because the end of the TRα2 LBD is divergent from the TRα1 and TRβ LBDs, which is not observed in Pm542 and Pm8742. Second, Pm542 and Pm8742 could have different pharmacologies from TR1 and TR2. In gnathosthomes, TRα and TRβ have similar binding properties for T3 and T4, but different pharmacologies for other ligands such as Triac, a deaminated T3 derivative [46–48]. This is explained by a different amino acid composition of the ligand pocket between TRα and TRβ [46]. Pm542 and Pm8742 have some differences in their LBD in comparison with TRα and TRβ, and also with TR1 and TR2 (Figure 1). This could account effectively for different pharmacological properties. However, given the overall strong similarity between TR1, TR2, Pm542 and Pm8742, we do not anticipate striking pharmacological differences. Third, some genes have a negative thyroid hormone response element (TRE) in their promoter and are negatively regulated by THs. This means that THs induce their repression instead of their activation. This is the case of genes of the TH pathway such as the thyrotropin-releasing hormone (TRH) gene or the deiodinase 2 [49, 50]. If Pm542 and Pm8742 are somehow specialized in the negative TREs, they may account for the drop of global TH levels observed in lamprey metamorphosis. Nevertheless, the molecular mechanisms of TR negative regulation remain to be fully understood and there is currently no evidence for a variant of TR specialized in negative TRE. Additionally, Pm542 and Pm8742 could

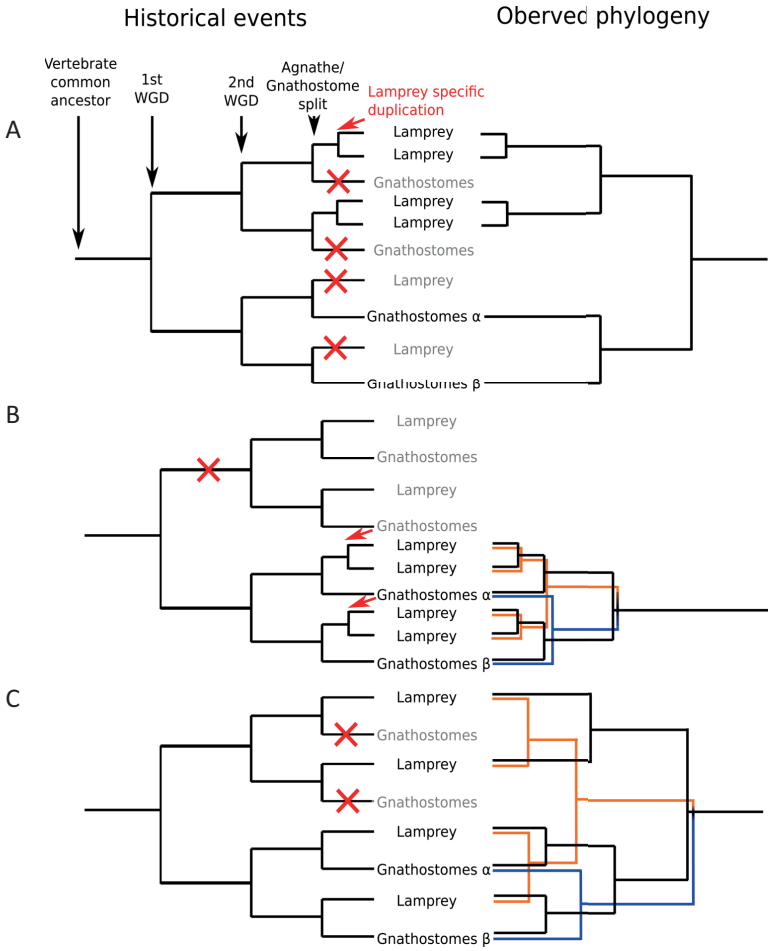

Figure 4: **Hypotheses for TR evolution in vertebrates.** Left panel: historical events such as genome duplication, speciation, gene duplication and gene loss. Right panel: observed phylogeny. A: Representation of the gene losses and duplications required to explain the phylogeny of TRs under the hypothesis that there is no artefact in our phylogeny. B: First alternate hypothesis explaining the phylogeny of TRs under the hypothesis of an artefactual node in the phylogeny. The real relationships are indicated by the black lines and the reconstructed artefactual branches are indicated in orange for lamprey and in blue for gnathostomes. C: Second alternate hypothesis explaining the phylogeny of TRs under the hypothesis of an artefactual node in the phylogeny. The real relationships are indicated by the black lines and the reconstructed artefactual branches are indicated in orange for lamprey and in blue for gnathostomes. The main evolutionary event of genome duplication and speciation are indicated at the top of the figure.

regulate different sets of genes and biological activities than TR1 and TR2, similarly to TRα and TRβ [6, 51, 52]. This does not account for the reverse mechanism of lamprey metamorphosis, but it suggests that gene regulation by THs in lamprey is even more complex than in gnathostomes, because of these two additional TRs. Overall, only the molecular characterization of the DNA and ligand binding properties of these new TRs and the establishment of their expression pattern could shed a new light on the peculiar metamorphosis of lamprey.

The long branch supporting the vertebrate TRs suggests an acceleration of the receptor evolution. Nevertheless, we still do not know enough about TH pathway evolution to completely understand this acceleration. Indeed, the biological role of THs in vertebrates is well investigated [1], with a documented role in development [53], metabolism and thermal acclimation [54], and integration of environmental signals [55]. On the contrary, less is known about the role

of THs outside vertebrates, since the control of metamorphosis has been demonstrated in only a few species (mainly amphioxus) [38]. It is not known if THs have a role in metabolism control since this has not been investigated outside vertebrates. Thus, without the knowledge of the role of THs outside this long branch, it is complicated to fully understand the evolution of TRs. It is interesting to note, however, that major actors of TH pathway are different between vertebrates and invertebrates. For instance, thyroglobulin, the backbone protein necessary for TH synthesis, is a vertebrate innovation [21], as well as the thyroid gland itself [24]. The only deiodinase characterized outside vertebrates has different chemical properties than the vertebrate ones in terms of specificity, as it acts on Triac rather than T3, the classical vertebrate TR ligand [56]. The hypothalamic-pituitary-thyroid endocrine axis was likely set-up at the basis of vertebrates [57, 58]. All these innovations regarding the TH axis arise at the basis of vertebrates and may explain the acceleration of TR evolution in this group.

To conclude, in this paper, we present genomic evidence for the existence of two novel genes encoding TRs in the lamprey *P. marinus*: Pm524 that we propose to call TR3 and Pm8742 that we propose to call TR4. Their identification as such is supported by phylogenetic analyses and synteny arguments, thus unambiguously bringing the total number of TRs to four in the lamprey. This work highlights that the investigation of TH signaling in *P. marinus* is of major importance to fully understand the evolution of this signaling pathway. Moreover, our results call for additional sequencing and characterization of agnathan TRs to fully grasp the peculiar physiology of THs in this taxon.

Competing Interests

The authors declare that they have no competing interests.

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