Functional and regulatory diversification of circadian rhythm *period* genes during the evolution of vertebrates

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Funding:

The study was supported by The Research Council of Norway (grant no. 325874).

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***Abstract*:**

The Period genes (*Per*) play essential roles in modulating the molecular circadian clock, which regulates the physiological and cellular timing in a broad range of species through the transcription-translation feedback loop in organisms. While the period gene paralogs are widely observed among vertebrates, the evolutionary history and the functional diversification of *Per* gene paralogs across vertebrates are not well known. In this study, we comprehensively investigated the evolution of *Per* genes, their lineage-specific transcriptome landscape and phenotypic effects, and the rapid turnover of transcription binding sites by comparative genomics and transcriptomics. We observed multiple lineage-specific gain and loss events of *Per* genes, though there was no simple association observed between ecological factors and *Per* gene numbers in each species. Most salmonid fish species lost the *per3* gene, while the species that retained the *per3* gene in salmonid did not the signature of relaxed selective constraint, but rather showed the signature of intensified selection, suggesting essential functional roles of the *per3* gene in retained species. We also determined the signature of adaptive diversification of the CRY-binding region in *Per1* and *Per3*, which modulates the circadian rhythm. Collectively, our findings revealed the evolution of *Per* genes and their fine-tuned contribution to the plastic and precise regulation of circadian rhythms in various vertebrate taxa.

Keywords: Circadian rhythm, gene family, molecular evolution, gene expression, development, natural selection

Introduction

The circadian clock regulates the physiological and cellular timing of organisms, adapting to the external environment with the 24-h light/darkness cycle of the solar day in different cells and organs [1–4]. The system is essential for an organism's rhythmic behavior and physiological responses, including sleep/wake cycle and foraging, so that the biological rhythm can efficiently interact with the dynamic and temporal external resources and environments by adjusting its physiological processes [5,6]. The first genetic factor of circadian rhythm regulation, the period gene, was discovered in *Drosophila* mutant strains that showed unusually long or short biological rhythm patterns [7–9]. Mammalian homologs of the period gene were also characterized soon after that [10], and the genetics, molecular mechanism, and behavioral biology of period genes have been extensively studied in model organisms [11–13]. Such studies revealed that in at least fish and mammals, the molecular circadian clock is regulated by transcription-translation feedback loops, core clock genes with changes in external cues, in which Period genes (*Per*) play the key role [14–19]. When there is enough light in the daytime, E-box, the DNA element is bounded by the transcription factors CLOCK-BMAL1, and the level of the PER and Cryptochrome (CRY) proteins are suppressed [20]. When night comes, and once PER and CRY levels have sufficiently dropped in the dark condition, a new cycle of CLOCK–BMAL1 begins to the *Per* and *Cry* transcription [5,21].

Humans and mice have three copies of *PER* genes, *PER1*, *PER2*, and *PER3*, likely due to the two rounds of ancient genome duplications [22], which is one case of the “evolution by genome duplication” theory [23]. In fish species, some known gene absence/presence of *per* genes include a lack of *per3* in sticklebacks (Gasterosteidae) are observed, and multiple additional duplicates and lineage-specific evolution of *per* genes in several fish species [24–26]. *Per* paralogues show some sequence similarity and overlapping functions, and specific functions for each gene. In mice, *Per1* and *Per2* genes are responsible for the circadian rhythm, while *Per3* did not indicate an observable, crucial role in circadian rhythm [27]. On the contrary, a genetic variant in *PER3* in humans is associated with sleep disorders [28]. In non-model species, for example, the evolutionary loss of circadian rhythms in cavefish (*Astyanax* sp.)

is reported, suggesting the impact of ecological factors on circadian gene evolution [16,26]. And a rapid turnover of circadian genes is suggested in amphibians [29]. However, the comprehensive characterization of *Per* genes is yet to be conducted in most non-model vertebrates despite the recent availability of high-quality genome data, and thus their evolutionary history and diversification process are unclear.

At the molecular and physiological levels, it has been thought that the *Per* genes are exclusively expressed in the suprachiasmatic nucleus, a brain region in the hypothalamus, which is responsible for controlling circadian rhythms [30,31]. Contrary to the historical notion, studies revealed that *Per* genes are also expressed in cell lines and non-brain tissues [32], sometimes with circadian rhythmic expression patterns, such as in mouse liver [33] and cell lines [34], and human cell lines [35]. Currently, it is believed that the circadian clock is controlled by the master clock located in the suprachiasmatic nucleus with core neuron gene expressions and is hierarchically conveyed to peripheral clocks in tissues all over the body [5,6]. Interestingly, studies suggested that circadian rhythm starts functioning even before birth in some species [36]. For example, differential regulation of *per2* and *per3* expression during zebrafish embryogenesis is reported [37]. It is reasonable that circadian genes are expressed in oviparous embryos with a transparent eggshell, which is exposed to light in the day and darkness in the night cycles. Studies indicated that circadian rhythm genes are expressed even in mammal embryos that cannot directly sense the day/night cycle by light [38][39], and it is speculated that the mother transmits the fetus information about the day/night cycle so that the fetus can calibrate the circadian rhythms [36,40]. In addition, *Per* genes have non-circadian functions, such as DNA damage response [41] and embryonic neuron development [42]. Although there are numerous reports about the *Per* expression in various conditions in model organisms, *Per* gene paralogs are often not investigated simultaneously, and the comprehensive dynamics of how *Per* gene paralogs share roles and how conserved these roles are across species is not known. Besides, in earlier studies, before genome information was available, *Per* paralogs were sometimes not correctly annotated. Building on that, this paper aims to comprehensively investigate the evolutionary history and the functional diversification of *Per* genes in vertebrates.

Results

Lineage-specific gene gain and loss of period genes in vertebrates

We first curated the *Per* genes in vertebrates (**Fig 1A**) from Ensembl Compara (version 106) and constructed a species-based phylogenetic tree to visualize the evolutionary history of *Per* gene gain and loss. After genome quality filterings (**Materials and Methods**), we included 154 species in total and obtained the sequences of 173 *Per1* genes, 212 *Per2* genes, and *150 Per3* genes. The phylogenetic signal (Pagel's lambda λ) [43] was high in the three *Per* genes reflecting a pattern where closely related species tend to be more similar in *Per* number than the more distantly related species. *Per1* and *Per 3* showed the strongest phylogenetic signal, and *Per3* also showed a high phylogenetic signal: *Per1* λ= 1.00, *Per2* λ= 0.94, and *Per3* λ= 1.00. Most mammalian and amphibian species have three paralogs (*Per1-3*) (**Fig 1A**), one for each family, yet we observed several lineages with marked gene gain/loss. Most reptiles lost the *Per1* gene, while birds lost the *Per3* gene, respectively **(Fig 1B**). The *Per2* number is the most stable among the three genes across taxa, suggesting that *Per2* plays a major, universal role. Elephant sharks (*Callorhinchus milii*) have only one *per2* gene, and in most teleost fish species, two *per2* genes were observed, likely due to the teleost fish-specific whole genome duplication event (Ts3R) [44–46]. In addition, there was a Cyprinidae-specific whole genome duplication (Cs4R) [47] and a salmonid-specific whole genome duplication (Ss4R) [48], which led to the additional duplications of *per* gene in each lineage **(Fig 1C**). Conversely, *per3* gene loss is observed in multiple distant fish lineages, including pike (*Esox lucius*), most salmonid species [49] and cod (*Gadus morhua*), (**Fig 1C**). Although the loss of another circadian gene and retrogression of circadian rhythm is reported in cavefish (*Astyanax mexicanus*) [26], there is no specific trend observed in the *per* gene copy number in cavefish. We observed that *per3* genes are lost in most salmonid species, except for Atlantic salmon *(Salmo salar)*, and brown trout *(Salmo trutta)*, consistent with a previous study [49]. To test if *per3* gene is under relaxed selection in these two species, we used a codon-based statistical test to detect relaxed selection, RELAX

[50], using the *per3* genes in these three species with retained *per3*, compared to the Acanthomorphata group. As a result, we observed a signature for intensified positive selection in the *per3* genes in these species with retained *per3*, compared to the control group (k=1.28, p=0.023) (**Fig S1**). This implies that the *per3* gene maintains indispensable roles in salmonid species which have this gene.

We reconstructed ancestral *Per* states calculating for each gene states transitions based on an equal rate (ER), all-rates-different (AR), and symmetrical (SYM) using the hidden-rate model [51]. The best model of ancestral state transitions for *Per1* was ER (logL = -47.46), ARD for *Per2* (logL = -34.10), and SYM for *Per3* (logL = -45.26). Ancestral state estimations showed a similar pattern for the three *Per* genes in the vertebrate's phylogeny, in which the current observed gene number was derived from a single ancestral copy (**Fig 2**). Dynamic gain/loss pattern changes were more recent clades in *Per1* **(Fig 2A**) and *Per3* (**Fig 2C**) than the duplication pattern noticed in *Per2* (**Fig 2B**). Disparity Through Time (DTT) [52] plots for each *Per* gene revealed differences in the patterns of evolutionary history (**Fig 2D**). *Per1* showed a major increase in the among-clade disparity while *Per2* showed stability, likely driven mainly by the duplication in the whole fish clade, with some slight peaks of within-clade disparity led by the diversification in Cyprinidae, indicating that diversification was high later in the evolutionary history of the group. *Per3* had a pattern of gradual increase in the among-clade disparity.

The evolution of circadian rhythm has been discussed in the context of ecological adaptation and behavioral strategies in both terrestrial and aquatic species, including temperature, diet, habitat, reproduction, and migration; although the effect of these environmental factors on each species is complex, mutually influential, and variable [53–56]. We hypothesized that some environmental factors may have driven the evolution of *Per* gene gain and loss. To investigate the evolutionary association between each *Per* gene number and in each species, we conducted the Phylogenetic Generalized Least Squares test [57] between per gene copy number and environmental/ecological factors of 138 species available. A total of ten environmental/ecological factors we tested included elevation, temperature, precipitation seasonality, solar radiation, windspeed, water vapor pressure, and net primary productivity, and ecological values included migration, body mass, and habitat (**Materials and Methods**). To avoid

noise due to taxa-specific factors, we separated each taxon (mammals, birds and reptiles, amphibians, and fish) in the analysis (**Materials and Methods**). As a result, we did not observe any statistically significant association between *Per* gene numbers and each environmental/ecological variable (Phylogenetic Generalized Least Squares test, *p*-value less than 0.05 was considered to be significant). We assume that ecological adaptation on circadian rhythm is based upon a more complex system, such as spatiotemporal regulation of the enzymatic activity, rather than the simple *Per* gene copy number. We also examined if the copy gain and loss pattern of each *Per* gene family deviated from other gene families available at Ensembl v104 compara gene trees, as previously reported [58]. We calculated coefficient of variation of gene numbers of each gene, and found three gene groups, with (A) low variation, (B) medium variation, and (C) high variation. *Per* genes fell in the medium variation cluster. Genes very low variation included highly conserved essential genes such as Kinesin family-like genes and genes with high variation included dynamically evolving genes, including olfactory receptors (**Fig S2, Table S5**).

Functional diversification of Period genes and their taxonomy-specificity

Previous studies with intensive genetic engineering and behavioral experiments, primarily in mice, suggested that the *Per3* gene has no intrinsic effect on circadian rhythms compared to the *Per1* and *Per2* genes [27], and *Per3* is often excluded from later investigations and discussions probably for this reason [20,59]. However, this makes it difficult to reveal the comprehensive dynamics of how *Per* gene paralogs share roles, and thus how conserved these roles are across species is not known. To investigate if each *Per* gene function is shared or specific and how much they are evolutionarily conserved, we curated and re-analyzed published phenotype datasets. We first inferred that vertebrate *Per* genes is likely to have a single ancestor by constructing a phylogenetic tree based on the amino acid sequences of Per from vertebrate species, with the *Drosophila* Per gene sequence. The *Drosophila* Per sequence was clustered with the Per3 group, suggesting that *Per3* is the most ancestral form of the *Per* gene family (**Fig 3A**). It is speculated that the ancestral *Per3* gene was duplicated twice due to the first and second vertebrate whole genome duplications and further duplicated by the teleost-specific whole genome duplication

corresponding with previous studies [24]. The phylogenetic analysis also supported that lineage-specific whole genome duplications in common carp (*Cyprinus carpio*) and salmon (*Salmo salar*) have also likely contributed to their additional *per* gene duplications.

We next investigated the regulatory and functional differentiation between *Per* paralogs by analyzing public gene-phenotype data sets of multiple species. We examined the genome-wide association study hits of variants in the three *PER* genes in humans that are curated and summarized at GWASATLAS [60]. The gene-level p-value of association with traits was computed by MAGMA [61], a computational method based on a multiple linear principal components regression model to aggregate the effects of variants on traits in a gene. We extracted traits that showed significant association (*p*-value smaller than 10^{-9}) in any of *PER* genes in the human GWAS database (**Fig 3B**). Human *PER1* showed the highest statistical significance with height and medium significance with chronotype and blood-related traits, such as hematocrit, and the percentage of red cells in the blood. *PER2* is also moderately associated with chronotype. *PER3* showed the highest statistical significance with chronotype and also showed some association with height and another blood-related trait, platelet volume. There is a deletion (54 bases) polymorphism reported in the GNOMAD paper [62], which is associated with sleep [63]. This variant is not reported in the 1000 Genomes phase 3 dataset [64] and the updated 1000 genome dataset [65] as it falls in a complex repetitive region, and little is known about the molecular function and evolutionary significance of the repeats.

It is reported that single *Per1* or *Per2* knockout mice have shorter circadian periods with reduced precision and stability, and both *Per1* and *Per2* knockout mice are completely arrhythmic in constant conditions [12,27,66]. Interestingly, *Per3* knockout mice have relatively moderate changes in circadian rhythm, observed light-dependent phenotypes, and reduced body size, growth, and metabolism [63,67]. In our meta-analysis, using data from Mouse Genome Database (MGD), 2021 [68], *Per2* gene has a broad effect on various phenotypes, while *Per1* and *Per3* are only involved in several neurological traits (**Fig 3C**). In zebrafish (*Danio rerio*), based on the Zebrafish Information Network (zfin.org), 2022 [69], it is reported that *per1b* or *per2* deficiency has effects on locomotor, photoperiod, neural development, and

neutrophil migration, a blood-related trait similar to the human case, interestingly. The *per1b* gene has specifically reported phenotypic effects such as memory, and *per2* has specifically reported phenotypic effects such as eye synapse morphology. Meanwhile, there are no marked effects reported for *per1a* and *per3*, except for some expression changes of circadian-related genes (**Fig 3D**). These observations indicate the flexibility of each *Per* gene's roles between species.

Dynamic evolution of Per gene expression pattern between paralogs and species

To understand the regulatory differentiation that may underline such diversity in phenotypic effects of *Per* genes across species, we searched for the *Per* gene expression in various tissues in vertebrates. We compared adult *PER* gene expressions of humans (*Homo sapiens*), from the GTEx portal [70], mouse (*Mus musculus*) expression from the mouse Gene Expression Database (GXD) [71], and African clawed frog (*Xenopus laevis)* Xenbase [72] **(Fig 4A-C).** In humans, *PER1* showed the highest expressions among *PER* genes overall except for Cerebellum. Notably, *PER3* shows a marked expression level at the Cerebellum among other organs, which is strongly influenced by the sleep-wake cycle [73] while it does not show such high expression in other parts of the brain. The *Per2* gene shows mostly low expressions in both the human and the clawed frog, but in the mouse, the *Per2* gene shows a moderate expression level and its expression pattern across species is markedly similar to the *Per3* gene expression. Two *Per3* gene duplicates in the clawed frog, which experienced allotetraploidization around 17–18 Mya [74], showed similar expression patterns each other across tissues **(Fig 4C)**. The orthologous genes, such as *PER2* in humans and *Per2* in mice, did not necessarily show a strong correlation in their expression pattern between species **(**r=-0.07, Spearman's correlation, **Fig 4D)**, suggesting the dynamic evolutionary plasticity of the expression pattern. Rapid transition of tissue-specificity of gene expression has been reported [75], in particular between the ovary and testis, as we also observed in *PER1* in the human and *Per1* in the mouse **(Fig 4AB).**

To further investigate the evolutionary trajectory of this gene expression regulation, we compared the expression pattern of relatively new *Per3* gene duplicates in the African clawed frog, *Xenopus laevis*, which experienced allotetraploidization around 17–18 Mya [74] and the Japanese medaka (*Oryzias*

latipes) which has likely duplicated the *per2* gene because of the teleost genome duplication around 310 Mya [76]. The newly duplicated two *Per3* genes in *Xenopus laevis*, showed a very similar expression pattern both across tissues and across developmental stages Data from Xenbase [72] (**Fig 4F**), such as high expression in the ovary, liver, brain, and lung, and stage 8 and 9, showing their regulatory mechanism is still very similarly conserved. On the contrary, the two Japanese medaka *per2* genes were already differentiated at the regulation level (**Fig 4E**). Collectively, the *Per* gene expression pattern in tissues is not necessarily conserved across species and is dynamically diversifying, probably due to the rapid evolution of gene regulation [77,78]. We further asked about the developmental pattern of *PER* gene in different parts of the human brain using the Brainspan dataset (https://www.brainspan.org/) [79,80] **(Fig S3)**. Overall, *PER1* showed the highest expression across tissues, especially in the 37 post-conception weeks fetus, except for the cerebellar cortex. *PER3* shows high expression in the cerebellar cortex after birth when humans are exposed to the external light and dark cycle. *PER3* also showed high expression in the thalamus, which acts as a sensory hub [81]. Neither the suprachiasmatic nucleus nor the broader region, the hypothalamus, which is responsible for controlling circadian rhythms [30,31], are reported in Brainspan.

In considering the function of period genes, we need to consider their circadian rhythms. Although there have not been many reports on the rhythmicity of all the *Per* genes in one study, West et al. [82] measured the expression of circadian gene paralogues in Atlantic salmon during development in various tissues by using JTK cycle. We extracted the rhythmicity of period gene expression from West et al. [82] (**Fig 4G**). In the Optic tectum, all the expressed *per* genes showed rhythmicity, while in saccus vasculosus, three of seven salmon period genes showed rhythmicity. In gill, only *per1* on chr4 showed moderate rhythmicity.

Evolutionary conservation and diversification at the sequence level.

Per1, *Per2*, and *Per3* do not necessarily show the same phenotype and expression pattern between species, rather, these genes appear to be evolving their expression patterns rapidly. To investigate the genomic basis of this dynamic evolution of the *Per* gene regulation, we investigated how the coding sequences and putative regulatory regions of *Per* genes evolved.

We first explored if there are any sites under diversifying selection on *Per1*, *Per2*, and *Per3* vertebrate genes independently, with MEME (Mixed Effects Model of Evolution) [83] on Datamonkey version 2.0 [84] (**Table S6**). MEME estimates a site-wise synonymous and a two-category mixture of non-synonymous rates and uses a likelihood ratio test to determine the signal from episodic diversification, a combination of strength of selection and the proportion of the lineages affected. As a result, we observed signatures of diversifying selection in the CRY-binding sites in *Per1* (site "2566" in **Table S6** for all species and site "1583" in **Fig 5B** with the selected species) and *Per3* (site "2102" in **Table S6** for all species and site "1393" in **Fig 5B** with the selected species), as shown in **Fig 5AB** and **Fig S4,** contrary to the general notion that protein binding sites in a gene are evolutionarily conserved [85]. As summarized in the introduction section, the PER and Cryptochrome CRY proteins orchestrate the circadian feedback loop with the CLOCK–BMAL1 [21][5]. CRY-binding region of the *Per* gene was characterized in rats (*Rattus norvegicus*) [86]. The site under selection in CRY-binding region is conserved, but after that, the latter binding region shows a dramatic decline in conservation (**Fig 5A and 5B)**, which may indicate sub-functionalization between paralogs, and the molecular basis of functional diversification of *Per* genes between species. *Per2* gene showed a marked signature of diversifying selection around the site 263 in the alignment (**Fig S4, TableS6),** while we did not find known functional annotation there but "Polar residues, Compositional bias" in Uniprot (corresponding with the 46th "N" in NE**N**CS in the human sequence, O15055 · PER2_HUMAN at UNIProt, https://www.uniprot.org/uniprotkb/O15055/entry, last accessed, 3rd. July, 2023,[87].).

We further investigated the conservation and putative regulatory regions of *Per* genes. There have been multiple studies that conducted a comparative analysis of the *Per* gene regulatory region. Nakahata et al. [88] proved that two tandem E-box-like sequences in tandem with a six-base-pair interval are necessary for cell-autonomous oscillation in the mammal and are conserved in various mammalian species. Here, we explored the conservation of E-box elements, expanding their investigations. At the same time, Paquet et al. [89] proposed a model to predict the CLOCK-controlled cis-elements with E-

boxes that regulate *Per* gene expression in various species, from *Drosophila* to mammals; Paquet et al [89]. also indicated that the E-box sequence is highly conserved between fish and mammals.

As general Blast search is not fine-tuned for searching such motif sequences with short query sequences with very rapid turnovers, we used Jbrowse [90,91] -based SalmoBase [92] (https://salmobase.org/, Last accessed, Jan 5th, 2023) to investigate the promoter sequences, allowing ambiguous spacer sequences around the *per* genes in Atlantic salmon. We searched for the three mammalian sequences reported in Nakahata et al. [88], allowing the six-base spacer sequences for against 100K upstream and downstream within each *Per* gene region. Of the combination of three query sequences and seven salmon *per* genes, we only found one *per1-regulatory-like* sequence, CACGTGatctggTGTGTG, near the *per3*, but over 80K apart from the gene, and in a repeat region (**Fig 5C**). In Ensembl Comparative Region Comparison Analysis using the 65 fish EPO-Extended, the sequence (Ssal_v3.1, 22:25993822-25993839) was observed only in the Atlantic salmon genome and Brown trout (*Salmo trutta*) genome. It is thus plausible that the mammalian *Per1*-regularoty-like sequence has arisen independently from mammals in this lineage, and the ancestral sequences underwent rapid turnover and are not detectable. The sequence that Paquet et al. [89] estimated is corresponded to Japanese medaka HdrR (ASM223467v1) 5:28606354-28606380 region, near the *per3* gene, while it to located near the *Per2* sequence in mammalian species [89]. In Ensembl 65 fish EPO-Extended, this region is widely observed in *Percomorpha* (**Fig 5D**) as represented in Paquet et al. [89]. However, the zebrafish (GRCz11, 2: 48,352,711-48,352,738) sequence is located near the *per2* gene, and this region was not detectable in other species. This implies that at least the zebrafish sequence evolved to become similar to the regulatory sequence in its lineage, independent from other lineages. These observations of salmon and zebrafish sequences resemble *per* regulatory regions, suggesting the fast rise and fall of the regulatory sequences concordant with previous observations on the evolution of regulatory elements in general [93,94].

Discussion

One of the major questions in evolutionary genomics is how duplicated genes develop the diverse phenotype in various species by fine-tuning their original functions. In this study, we focused on the highly conserved *Per* gene family in vertebrates, which plays a key role in circadian rhythm. We revealed how the duplicated *Per* gene copies modified their function between species and paralogs. While *Per1*, *Per2*, and *Per3* paralogous genes were duplicated in the common ancestor of vertebrates, there have been multiple lineage-specific gain and loss events. Most birds lost the *Per1* gene, while most reptiles lost the *Per3* gene. The *Per2* gene was observed in most species. Salmonid species lost the *per3* gene except for *Salmo. trutta*, and *S. salar* salmonids. For the three species with the *per3* gene, we detected no signature of relaxed selection but intensified selection compared to the Acanthomorphata group. This is contrary to the current view that *Per3* is the least important, based on earlier knock-out mouse studies, and thus, least studied among the paralogs [27]. By comparative analysis of public gene, phenotype, and transcriptome datasets, we determined that the phenotypic effect of *Per* gene paralogs and their expression patterns are highly variable between species and paralogues, which indicates the rapid evolution of sub-functionalization. We also determined signatures of diversifying selection in the *Per1* and *Per3* genes' CRY-binding region and a rapid turnover of binding site sequences. However, one binding site sequence was widely maintained among taxa (**Fig 3E**), suggesting the lineage-specific conserved role of this region. Collectively, the rapid sequence and regulatory evolution that we observed, despite the relatively stable gene copy number, are likely to contribute to the dynamic effect of the *Per* gene family on the phenotype, both circadian and noncircadian effects in various species.

We postulate that as more transcriptome/gene-phenotype datasets of a variety of species become available, not only genome datasets, the evolution of gene function and regulation will be more comprehensive in the context of how genome evolution contributes to cross-species phenotypic diversity. Although knockout/knock-in studies are a powerful way to understand gene function in a controlled environment, the effects of each *Per* gene are often not investigated within a study. Therefore, when we

encounter a lack of known phenotype report of a gene compared to another, it is difficult to know if this is because that gene was "not investigated" or "was examined, but no observable changes were found/thus not reported", which remains a limitation to investigating the cross-species effects of the paralogs. Our study represents the first step in integrating multiple angles of -omics datasets to understand the functional effect of the *Per* gene duplicates in multiple species. It is important to note that although there are interesting lineage-specific gene loss trends in reptiles and birds at the genomic level, currently, there are not many comprehensive cross-tissue transcriptome data and gene-phenotype data available in these taxa. Also, in most transcriptomic studies, the sampling time is not considered and reported, which may raise limitations for comparative circadian rhythm gene investigation. While large-scale international genome sequencing projects for various non-model organisms are underway, the next step for genomics, especially evolutionary genomics, is to connect insights from genome information to their functional roles on molecular and macroscopic phenotypes and environmental conditions.

Methods

Gene Sequences

We have retrieved all available *Per1*, *Per2*, and *Per3* sequences from Ensembl Compara, version 106, via the Wasabi app (http://wasabiapp.org/). We initially obtained 200 species. To filter out species with potential low-quality genomes, we removed 18 species with sequences containing "N" (script available in Supplementary Material). We also excluded 25 species that have any *Per* genes not starting from methionine code, which suggests patchy gene annotations. We further removed 10 species with inconsistent reports, which had zero *Per1*/*Per2*/*Per3* genes in Ensembl Compara but with *Per1*/*Per2*/*Per3* existing in NCBI orthologs (last accessed, 15. Aug. 2022) and two species with projection-built genomes. We examined the copy gain and loss pattern of *Per* gene in relation to the other gene families, calculating the coefficient of variation for each one using cmstatr package (https://doi.org/10.21105/joss.02265) in R software v4.2.2 (https://www.R-project.org/).

To inspect likely pseudogenes, we removed branches of gene trees that were significantly longer than other genes (likely because they accumulated mutations at a faster rate than others, which is a sign of pseudogenization) by using TreeShrink [95], a software that detects branches that significantly increase the tree diameter. We set the chance of false positives to 10 percent. We thus identified two putative pseudogenes for per3 (ENSAMEG00000030567 from giant panda & ENSCJAG00000000288 from White-tufted-ear marmoset), that were removed from further analysis.

Lastly, we removed six species that are not available at TimeTree5 [96] and four non-vertebrate species, which additionally four do not have geographic range data on Map of Life/IUCN (last accessed, 15. Aug. 2022). A total of 133 vertebrate species were used for subsequent analyses, species lists and *Per* gene numbers are available in **Table S1**. Amino acid alignment was conducted by Mafft version 7 [97].

Evolutionary history of gene gain and loss of period genes in vertebrates

We used the phylogenetic hypothesis from TimeTree5 [96] and was pruned for the species of interest using ape v5.6 package [98] to reconstruct the evolution of each *Per* gene in vertebrates. We reconstruct ancestral *Per* states calculating for each gene states transitions based on an equal rates (ER), all-rates-different (AR), and symmetrical (SYM) using the hidden-rate-model [51] and one category for each level of the trait. The best model was selected according to the Akaike Information Criterion and plotted into the tree, all analyses were performed in phytools v1.5.1 [99]. We estimated the phylogenetic signal for each *Per* gene based on the Pagel's lambda λ parameter and the best rate transition for each gene. To examine how *Per* genes changed through time in vertebrates, we used a disparity-through-time (DTT) approach, this analysis quantifies the disparity for the whole clade and for each node in the phylogeny (subclade). The relative disparity is calculated by dividing each subclade disparity value by the overall disparity of the clade, whereas the mean relative disparity is calculated for each subclade present at each divergence point. Values close to zero suggest that the variation is partitioned among the distinct subclades in the tree (slowdown in trait diversification), while values near one indicate that a major

proportion of the total variation is contained by the subclades in the tree (rapid trait diversification) [52], we performed these analyses in geiger v2.0.10 package [100].

Association between environmental and ecological factors and period gene numbers

Selection of environmental variables

We chose a set of terrestrial and marine world easter layers as likely candidates to predict the evolution of *Per* genes. For terrestrial environments, six climatic layers at 10 arc min (~340 km²) were obtained from Worldclim 2.1 [101]: altitude, annual temperature range, annual precipitation seasonality, annual mean solar radiation, annual mean wind speed, and annual mean water vapor pressure. Additionally, we incorporate the Net Primary Productivity of the MOD17A3 v.55 layers at 0.1 degrees downloaded from NASA for 2000-2016 [102] mean value was calculated for each year, and finally, the mean value for the whole period. For marine environments, we selected bathymetry and annual mean temperature range downloaded from MARSPEC at 10 arc min [103], and annual wind speed downloaded at 5 arc min from Global Marine Environment Datasets 2.0 [104].

All raster layers were scaled to 10 arc min. The geographic range species maps were obtained from the International Union for Conservation of Nature (IUCN. 2022. The IUCN Red List of Threatened Species. Version 2022-2. https://www.iucnredlist.org. Accessed on October 2022) and Map of Life [105] for extracting raster values and mean values calculated for each species using raster v3.6 and exactextractr v0.9.1 [106] in R. The mean latitude was calculated for each species based on the geographic range maps.

Selection of ecological variables.

Ecological traits available for main taxonomic groups were obtained from public databases. For fishes, migration, body mass, vision, and swimming mode were downloaded from [107] through rfishbase v4.0.0 R package [108]. For amphibians, body mass, habitat, and migration were downloaded from AmphiBio

[109]. For birds, body mass, habitat, and migration were downloaded from AVONET [110]. For mammals, activity cycle, body mass, and habitat breadth were downloaded from PanTHERIA [111].

Comparative statistical analyses

To investigate the nature of the evolutionary association between predictors and *Per* gene number we used a Phylogenetic Generalized Least-Squares analysis (PGLS; [57]. Analyses were performed for five taxonomic groups/datasets “Mammals” “Amphibians/Reptiles/birds”, “All fishes”, “Continental fishes”, and “Marine fishes”. A violation of the assumptions about the residuals or model instability can severely affect the conclusions drawn, and hence, it is of crucial importance that these are thoroughly checked and an assessment is made about how much the models can be trusted [112]. Continuous variables were log10 transformed, for altitude/bathymetry predictor with negative values were squared before log-transformed. The distribution of all transformed variables was checked and tested for collinearity between predictors using the Variance Inflation Factors (VIF) with car package v3.1 [113] before running all models. Variables with no normality and a VIF major of 5 were discarded from the final model.

The phylogenetic hypothesis was obtained from TimeTree5 and pruned for species with environmental/ecological available data using ape v5.6 package. PGLS models were computed for each *Per* gene with the final predictors and using a phylogenetic signal calculated using the Pagel’s lambda (λ) estimated by maximum likelihood [43] using caper package v1.0.1 (<https://CRAN.R-project.org/package=caper>). Diagnostic plots of residuals (e.g., outlier analysis, Q-Q plots) were checked to make sure that assumptions were not violated. Species and *Per*/environmental/ecological data analyzed are in **Table S3**.

Gene-phenotype association datasets

Reported phenotypes associated with *PER1*, *PER2,* and *PER3* in humans at GWAS Atlas [60] under PheWAS. Traits with a $-\log_{10}$ p-value of more than nine in at least one of the *PER* genes were considered to be statistically significant and included in the analysis. Traits in the *Per* deficient mouse were retrieved

from Mouse Genome Database (MGD), 2021 [68]. Traits in the *per* deficient zebrafish were retrieved from the Zebrafish Information Network (zfin.org), 2022 [69].

Transcriptome datasets

Human adult transcriptome data set was retrieved from GTEx portal version 8 (last accessed: Jan. 18th, 2023) [70]. Mouse transcriptome dataset was retrieved from The mouse Gene Expression Database (GXD) [71] Mouse embryo dataset: E-ERAD-401, Mouse adult datasets: E-MTAB-2801 and E-MTAB-6798. Frog (*Xenopus laevis*) transcriptome dataset was retrieved from Xenbase [72]. For adult datasets, we selected organs with available gene expression data in all three species for comparison purposes (muscle, ovary, testis, kidney, liver, spleen, heart, lung, and brain or cerreblum). We used Spearman's rank correlation coefficient test to investigate the correlation between gene expression patterns between paralogs.

Molecular evolution analysis

Intensification or relaxation of selection was estimated using [50] implemented in [114]. This software compares a set of “test” branches to a set of “reference” branches and measures intensification ($k > 1$) or relaxation ($k < 1$) of selection in the test group compared to the reference group. The salmonid group (Atlantic salmon, brown trout) *per3* sequences were used as a test group and compared to sequences of the outgroup (Acanthomorphata) as references.

We explored if there are any sites under diversifying selection on *Per1*, *Per2*, and *Per3* vertebrate genes independently, with MEME (Mixed Effects Model of Evolution) [83] on Datamonkey version 2.0 [84]. We conducted the analysis for each *Per* gene independently as *Per1*, *Per2*, and *Per3* paralogs are too diverged to align and thus expected to reduce statistical accuracy if analyzed simultaneously. MEME estimates a site-wise synonymous and a two-category mixture of non-synonymous rates and uses a likelihood ratio test to determine the signal from episodic diversification, a combination of strength of selection and the proportion of the lineages affected.

Transcription binding sites analysis

We explored the conservation of E-box elements, expanding previously reported investigations. As general Blast search is not fine-tuned for searching such motif sequences with short query sequences with very rapid turnovers, we used Jbrowse [90,91] -based SalmoBase [92] (https://salmobase.org/, Last accessed, Jan 5th, 2023) to investigate the promoter sequences, allowing ambiguous spacer sequences around the *per* genes in Atlantic salmon. We searched for the three mammalian sequences reported in Nakahata et al. [88], allowing the six-base spacer sequences for against 100K upstream and downstream within each *Per* gene region.

per1 caggtcCACGTGcgcccgTGTGTGtgacac

per2 cgcggtCACGTTttccacTATGTGacagcg

per3 gaccggCACGCCgcgagcCTCGAGactgcg

We also searched for the predicted CLOCK-controlled cis-elements with E-boxes in figure 5B in Paquet et al [89]:

Medaka cgtTCACGTgga-tccccatGTTACA

Zebratish cggTCACCTgtt-tctccacATGCTG

By using Ensembl Comparative Region Comparison Analysis using the 65 fish EPO-Extended chain.

Acknowledgments

We thank Drs. Shona Wood, Nicola Barson, and Siri Fjellheim for the discussion and comments on the early stage of this study.

Figures and Tables

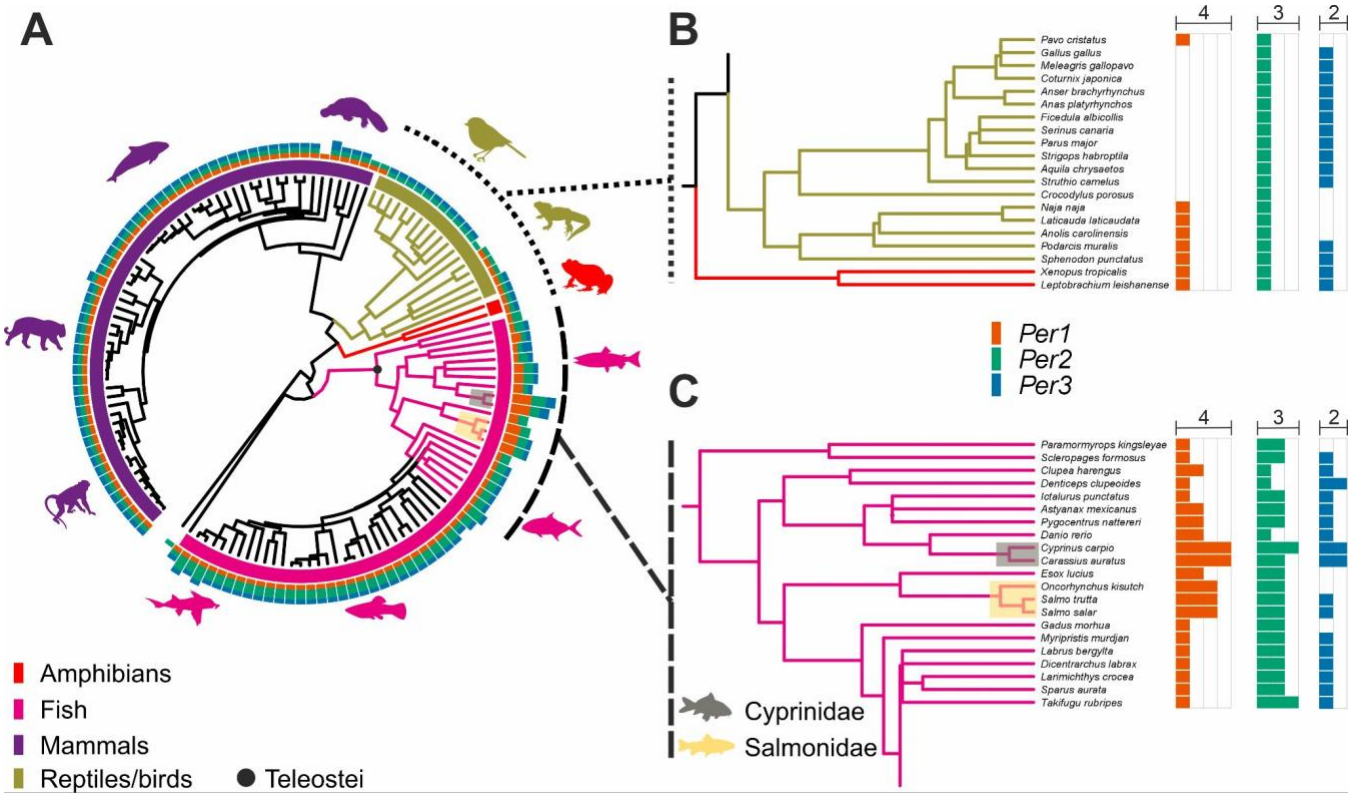

Fig. 1. The evolutionary history of the period gene (*Per*) gain and loss in vertebrates. **A**. Phylogenetic tree and numbers of *Per* paralogs in vertebrates. Bar colors around the tree denote the main vertebrate taxonomic clades; amphibians in red, fishes in deep pink, mammals in purple, and reptiles/birds in olive green. The bar color after the tips indicates the gene number for each species; orange for *Per1*, dark green for *Per2*, and blue for *Per3*. **B**. Zoom in on amphibians, reptiles, and bird clades showing unusual gene loss patterns. **C**. Zoom in on Salmonidae, Cyprinidae, and Chichilidae clades that show unusual gene gain-loss patterns.

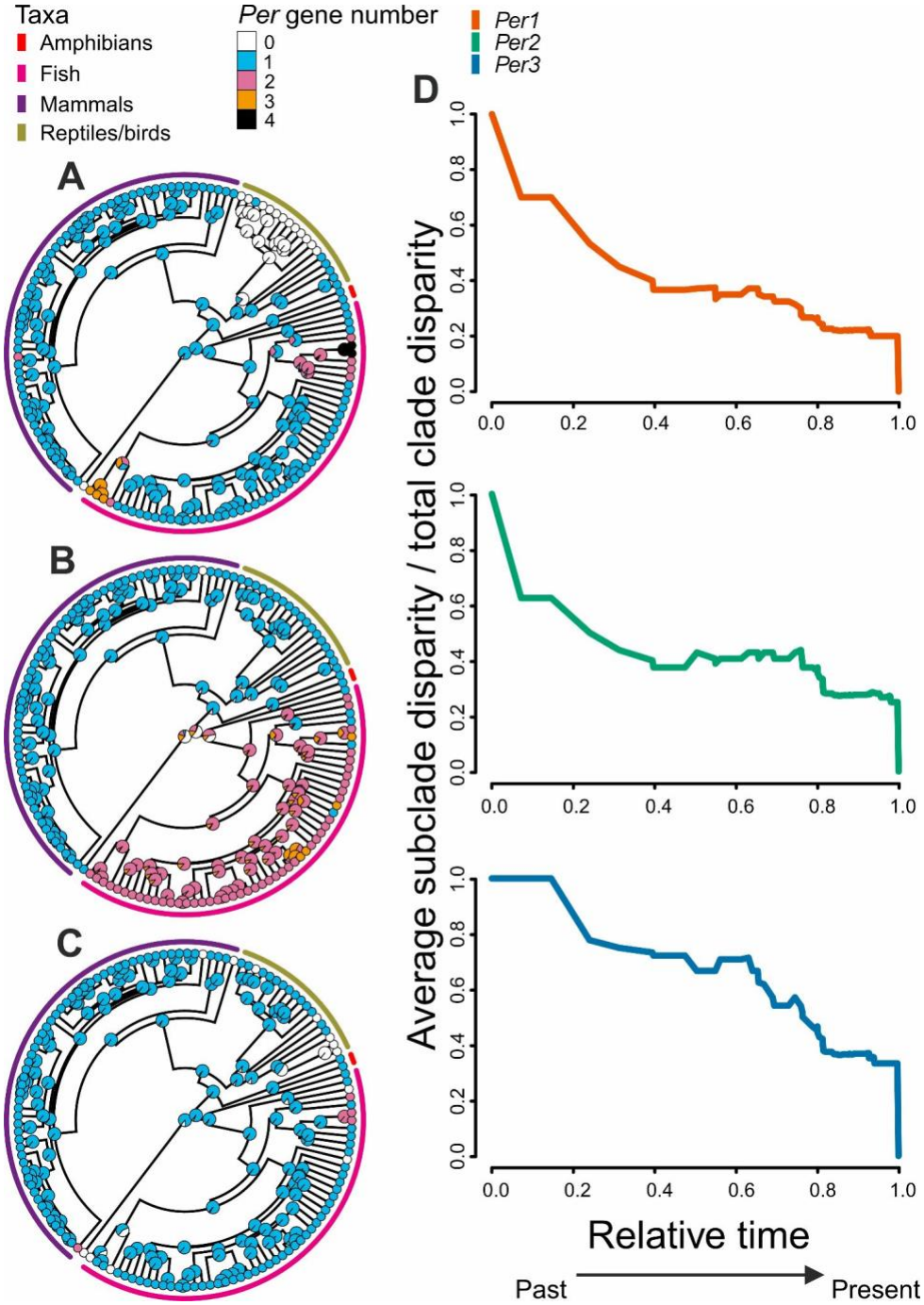

Fig. 2. Ancestral state reconstruction for Period genes (*Per*) gain/loss numbers in vertebrates, **A**. *Per1*, **B**. *Per2*, and **C** *Per3*. **D**. Disparity Through Time (DTT) plots for each *Per* gene. The horizontal axis represents relative time values, 0.0 represents the root, and 1.0 the tips of the tree. The vertical axis represents the average subclade disparity divided by the total clade disparity and is calculated by each internal node of the tree. Higher values of disparity correspond to greater variance values within subclades relative to the disparity of the whole subclade.

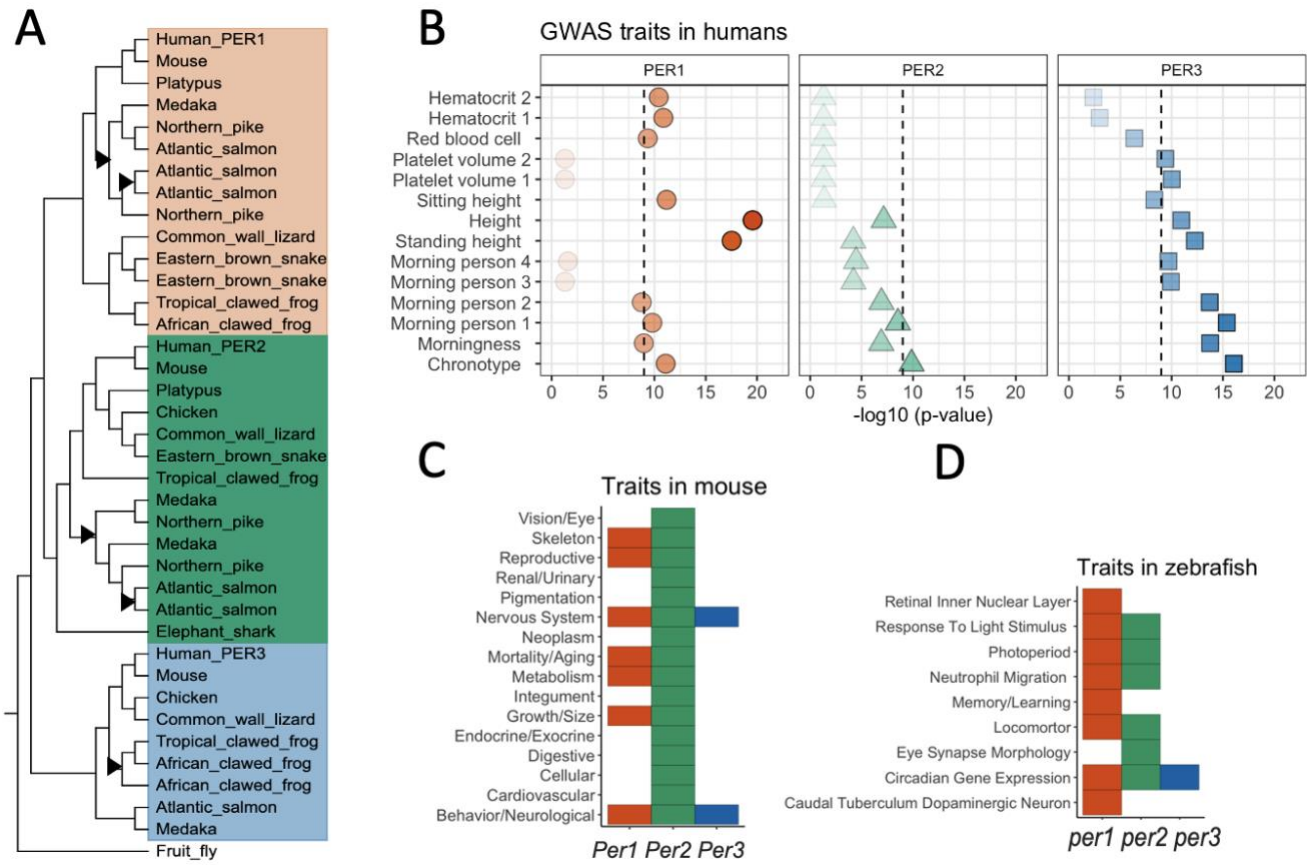

Fig. 3. Phenotypic effect differentiation of period genes (*Per*) in various vertebrate species. **A**. Amino-acid-based phylogenetic tree of vertebrates with the outlier fruit fly (*Drosophila melanogaster*) *per*. Black triangles indicate whole genome duplication events. The background color shows the gene classification (Red: *Per1*, Green: *Per2*, Blue: *Per3*). **B.** Reported phenotypes associated with *PER1*, *PER2*, and *PER3* in humans at GWAS Atlas [60]. The $-\log_{10}$ p-value of the association between genes and phenotypes is plotted on the x-axis. Only phenotypes with $-\log_{10}$ p > 9 in at least one of the *PER* genes are shown. **C**. Reported traits in *Per* deficient mouse (*Mus musculus*) from Mouse Genome Database (MGD), 2021 [68]. **D.** Reported traits in *per* deficient zebrafish (*Danio rerio*), the Zebrafish Information Network (zfin.org), 2022 [69].

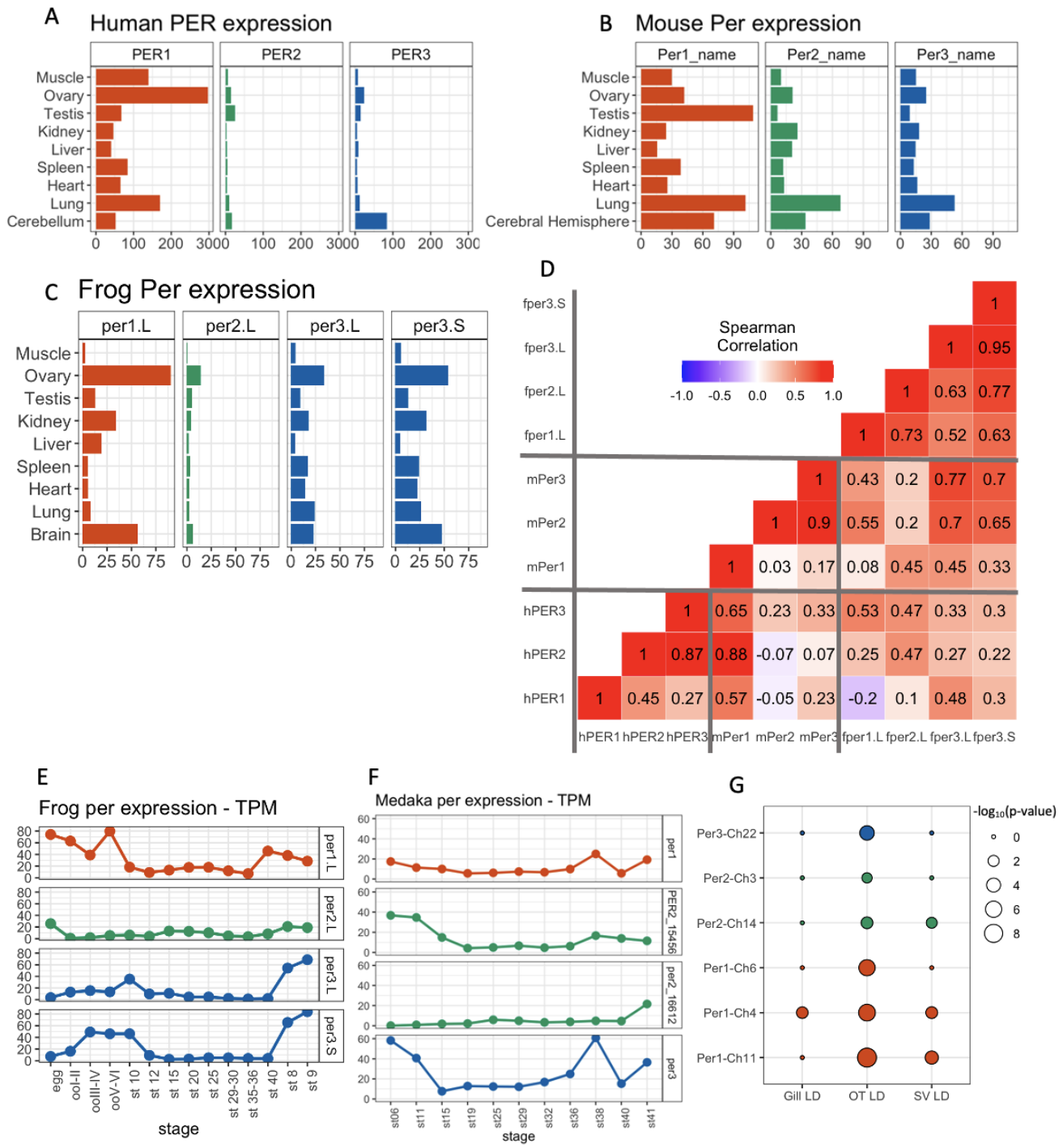

Fig. 4. Transcriptomic differentiation of period genes (*Per*) in **A**. Human, **B**, Mouse (*Mus musculus*), and **C** Frog (*Xenopus laevis*). The expression data was obtained from GTEx, Mouse Genome Database, and the Zebrafish Information Network. **D** Correlation of gene expression patterns between per genes (hPER: human, mPer: mouse, fper: frog). **E**. Medaka development. **F**. Frog development, **G**. Rhythmicity of *per*

genes in tissues in Atlantic salmon (*Salmon salar*), *Optic tectum*, gill, and *saccus vasculosus*. Data is retrieved from supplementary table 8 of [82], one gene with no expression is not displayed.

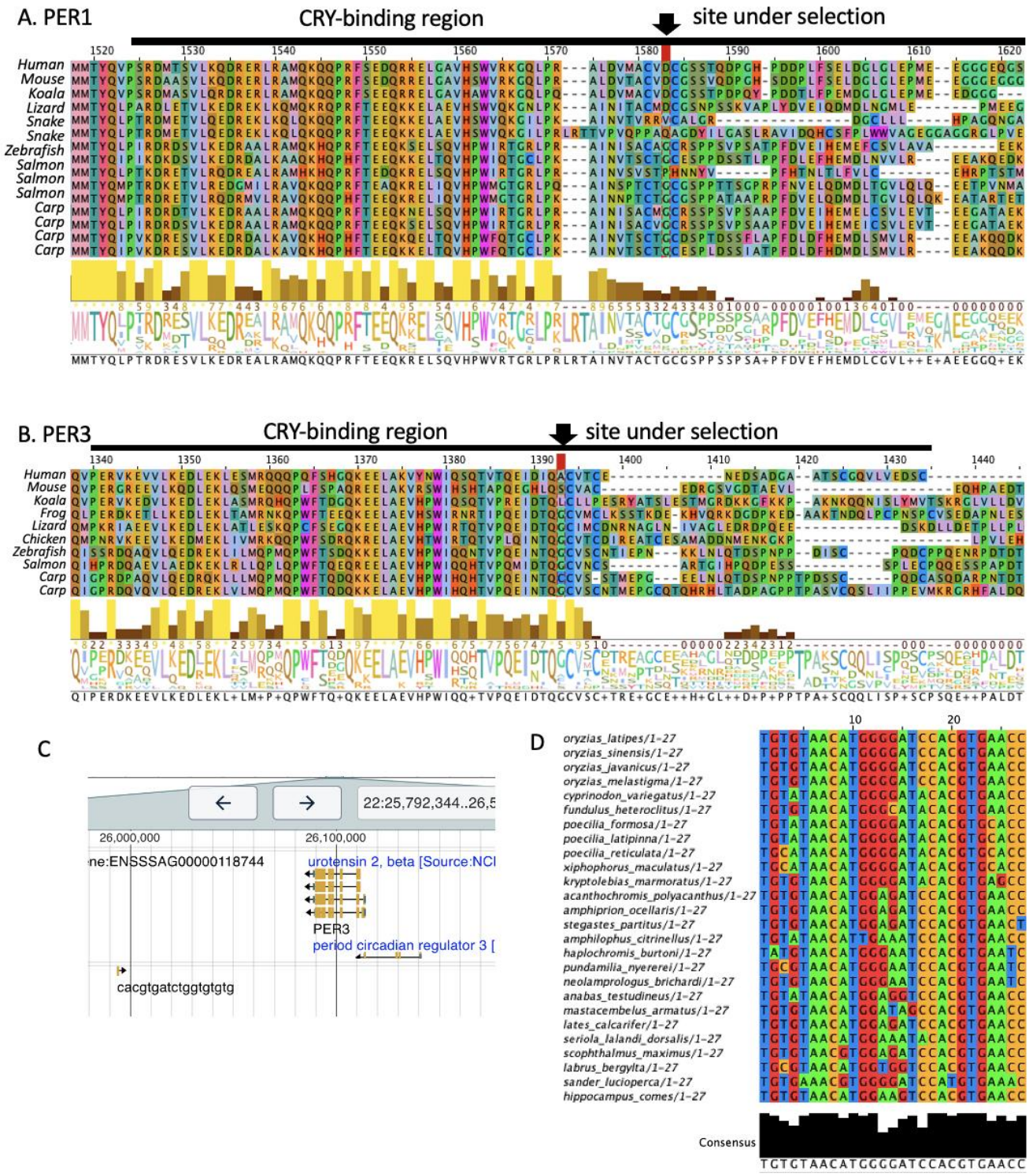

Fig. 5. AB. The site with diversifying selection detected (arrow) by MEME (Mixed Effects Model of Evolution) [83] on Datamonkey version 2.0 [84], which are both located in the CRY_binding sites of *Per1* (**A**) and *Per3* (**B**). As a result, we observed signatures of diversifying selection in the CRY-binding sites in *Per1* (site "1583" in **Fig 5A**) and *Per3* (site "1393" in **Fig 5B** with the selected species). The sites in

Fig 5 and **Table S5** do not have the same numbers because only selected species are aligned and shown in **Fig 5** for a simplified visualization purpose, and **Table S5** is based on the aligned sequences of all the investigated species. **C**. A sequence, which is similar to the mammalian *PER1*-regulatory sequence, CACGTGatctggTGTGTG, near the *per3* gene in Atlantic salmon (*Salmo salar*) on Salmobase, (Ssal_v3.1, 22:25993822-25993839). **D**. Highly conserved *per2* regulatory region of Japanese medaka (*Oryzias latipes*) HdrR (ASM223467v1) 5:28606354-28606380 region in a broad fish species, in *Percomorpha.*

Supplementary Materials

Table S1. Master table of investigated species.

Table S2. Excluded species because of "N" in the period genes.

Table S3. Variables in continental species.

Table S4. Variables in marine species.

Table S5. Coefficient of variation of gene numbers in gene families

Table S6. MEME results for *Per* genes.

Figure S1 RELAX result of salmonid *per3* gene.

Figure S2 The distribution of coefficient of gene copy number variation of each gene family across taxa

Figure S3 Brain *PER* gene expression in humans.

Figure S4 Diversifying selection signature in Period genes in vertebrates.

File S1, S2, S3. Period gene (*Per*) alignment from high-quality species (from Wasabi App).

File S4, R script for sequence curation and visualization.

File S5, A Timetree-based species tree.

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