Diversification and coevolution of the ghrelin/growth hormone secretagogue receptor system in vertebrates

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Abstract

The gut hormone ghrelin is involved in numerous metabolic functions, such as the stimulation of growth hormone secretion, gastric motility, and food intake. Ghrelin is modified by ghrelin O-acyltransferase (GOAT) or membrane-bound O-acyltransferase domain-containing 4 (MBOAT4) enabling action through the growth hormone secretagogue receptors (GHS-R). During the course of evolution, initially strong ligand/receptor specificities can be disrupted by genomic changes, potentially modifying physiological roles of the ligand/receptor system. Here, we investigated the coevolution of ghrelin, GOAT, and GHS-R in vertebrates. We combined similarity search, conserved synteny analyses, phylogenetic reconstructions, and protein structure comparisons to reconstruct the evolutionary history of the ghrelin system. Ghrelin remained a single-gene locus in all vertebrate species, and accordingly, a single GHS-R isoform was identified in all tetrapods. Similar patterns of the nonsynonymous (dN) and synonymous (dS) ratio (dN/dS) in the vertebrate lineage strongly suggest coevolution of the ghrelin and GHS-R genes, supporting specific functional interactions and common physiological pathways. The selection profiles do not allow confirmation as to whether ghrelin binds specifically to GOAT, but the ghrelin dN/dS patterns are more similar to those of GOAT compared to MBOAT1 and MBOAT2 isoforms. Four GHS-R isoforms were identified in teleost genomes. This diversification of GHS-R resulted from successive rounds of duplications, some of which remained specific to the teleost lineage. Coevolution signals are lost in teleosts, presumably due to the diversification of GHS-R but not the ghrelin gene. The identification of the GHS-R diversity in teleosts provides a molecular basis for comparative studies on ghrelin’s physiological roles and regulation, while the comparative sequence and structure analyses will assist translational medicine to determine structure–function relationships of the ghrelin/GHS-R system.
2014), or alternative splicing to eventually become a gene family. Whole-genome duplication (WGD) has been considered as the most common mechanism resulting in the diversification of ligand and receptor gene families (Taylor et al. 2003). Especially in teleosts, the whole genome has undergone a teleost-specific WGD (Meyer and Scharl 1999; Meyer and Van de Peer 2005). Many ligand and binding-receptor duplicates have been preserved in the genome after duplication through subfunctionalization or neo-functionalization either maintaining the original function or evolving new functions (Zhang and Cohn 2008). Theories on gene duplication stipulate that new duplicates are usually redundant and one of the two paralogous duplicates is free of selective constraints, accumulating deleterious mutations to become nonfunctional pseudogene (Kellogg 2003; Zhang and Gaut 2003) and eventually being deleted from the genome (Wagner 1998; Zhang and Gaut 2003). In rare cases, both paralogous duplicates are maintained active because they differ in their functional aspects (Nowak et al. 1997; Zhang and Gaut 2003; Hughes 2005). After the duplication event, endogenous ligand duplicates have to maintain their properties to selectively recognize and bind to their specific receptors, and any alteration in their sequences may compromise this recognition (Tillier and Charlebois 2009b). This implies that the evolution of the protein ligand structure should be correlated with the evolution of their binding proteins, which can be studied as ligand–receptor coevolution (Kaiya et al., 2013c). The coevolution of orthologs and/or paralogs can be investigated using phylogenetic reconstruction and evolutionary distance approaches (Tillier and Charlebois 2009b; Chan et al. 2013). The phylogenetic approach is based on the fact that orthologs with similar phyletic patterns may have coevolved during the course of evolution, while the group of orthologs with different phylogenetic profiles may have evolved differently (Koszul et al. 2004; Chen et al. 2009; Cohen et al. 2012). The evolutionary distance measured by average dn/ds differences between ortholog pairs is used to identify genes that may have coevolved (Tillier and Charlebois 2009a). This approach relies on the principle that the evolutionary rate of protein coding genes is strongly related to their functions (Chan et al. 2013). While there are initially strong ligand/receptor specificities, such interactions can be disrupted during the course of evolution through evolutionary changes including duplication, divergence, rearrangement, and recombination (Laisney et al. 2010). The interaction specificities can persist if both ligands and receptors have undergone similar evolutionary constraints and if they have coevolved after these evolutionary events have occurred (Moyle et al. 1994; van Kesteren et al. 1996). Many structural features of proteins such as the position of the transmembrane domain (TMD), α-helix or β-strand in the secondary or tertiary structure, and relative solvent accessibility (RSA) are known to influence their evolution (Bustamante et al. 2000; Ramsey et al. 2011). The RSA, which measures the extend amino acid residue exposure, has been particularly used to investigate the biophysical and evolutionary properties of proteins (Shaytan et al. 2009; Tien et al. 2013). It is well established that the RSA correlates with the evolutionary rates of proteins (Goldman et al. 1998; Bloom et al. 2006; Franzosa and Xia 2009; Tien et al. 2013), especially for G protein-coupled receptors, which mediate the actions of various protein ligands (Spelman and Wilke 2013).

Ghrelin is a gastrointestinal peptide belonging to the same family as the motilin genes. Secreted from the stomach, ghrelin is involved in various physiological processes including appetite regulation, food uptake, and glucose metabolism (Müller et al. 2015). Besides neuroendocrine and cardiovascular functions, ghrelin action has also been implicated in cell differentiation and proliferation (Asakawa et al. 2005; Chen et al. 2009; Kaiya et al. 2013b). The mature ghrelin peptide is modified specifically at the amino acid residue serine-3 by the ghrelin O-acyltransferase (GOAT) enzyme (Kitazawa et al. 2015). Ghrelin acylation is required to accomplish all of its physiological activities. The actions of ghrelin are mediated by growth hormone secretagogue receptor (GHS-R), a G-coupling protein receptor with seven TMDs (Howard et al. 1996; Kaiya et al. 2013c). Ghrelin binds to the TMD, and ghrelin/GHS-R signaling increases intracellular Ca²⁺ concentration (Howard et al. 1996).

While only one ghrelin isoform has been so far described in all vertebrates (Kaiya et al. 2011b), several GHS-R variants were identified in many species (Kaiya et al. 2003, 2010, 2014a, 2014b). Two GHS-R isoforms were characterized in mammals based on their amino acid sequence composition and length. GHS-Ra is derived from regular splicing and is functionally active, whereas GHS-Rb, whose function is not well understood, results from alternative splicing (Howard et al. 1996). Nonmammalian vertebrates other than fishes possess GHS-Ra which is similar to the GHS-Ra of mammals and is activated by the ghrelin gene (Kaiya et al. 2011a, 2013a, 2013b). Previous studies support that the GHS-Ra was duplicated in some fish species and is subdivided into two paralogs, GHS-R1a and GHS-R2a. While GHS-R1a is more similar to mammalian GHS-Ra, GHS-R2a is more different but nevertheless possesses the functional domain which is activated by the ghrelin peptide (Kaiya et al. 2013a, 2014a). Another isoform named GHS-R1a-like (GHS-R1a-LR) has been so far found only in fishes (Kaiya et al. 2013a, 2014a).

The ghrelin/GHS-R system offers an excellent opportunity to investigate the coevolution of ligands and their binding receptors. Why do teleosts only have a single
ghrelin isoform, but three or more GHS-R isoforms? Are all these GHS-R isoforms functional and activated by the same ghrelin isoform? If the diversification of GHS-R has resulted from teleost-specific WGD, was the ghrelin gene affected by the same evolutionary events? Were certain ghrelin duplicates subsequently lost after duplications and did this happen due to functional redundancy? Were GHS-R duplicates maintained by evolving new functions? All these questions can only be addressed by first investigating whether the ghrelin gene and its receptors have coevolved in all vertebrates. The functional triangle consisting ghrelin, its activating enzyme (GOAT) and the binding receptors (GHS-R) support the hypothesis that these genes may have coevolved. The main objective of this study aimed to gain insights into the evolutionary dynamics of the ghrelin/GHS-R system and to determine whether the ligand ghrelin coevolved with its binding receptors and activating enzyme GOAT during the course of vertebrate evolution. To this end, we first searched and identified all orthologous and paralogous isoforms of ghrelin, GOAT (also known as MBOAT4) and the GHS-R in a broad range of species covering all vertebrate lineages. We compared the selection patterns of ghrelin/GHS-R and ghrelin/GOAT, as well as the selection patterns between ghrelin and other MBOAT isoforms (MBOAT1/2) to determine whether these patterns are different from the MBOAT4 selection profiles. We then performed whole-genome comparative analyses including similarity search, conserved synteny analyses, phylogenetic reconstructions, and protein secondary structure comparisons. These analyses predict scenarios of coevolution and the interaction within the ghrelin/GHS-R system. Our analysis uncovers the functional diversification of GHS-R and may assist in the understanding of the physiological roles in diverse vertebrate lineages, in particular the teleost fishes.

**Materials and Methods**

**Identification of ghrelin ligand and GHS-R**

Protein sequences of GHS-R of the zebrafish, *Danio rerio*, and of humans, *Homo sapiens*, were blasted against the NCBI nonredundant database to identify orthologs and paralogs in vertebrate species. The sequence information from NCBI was complemented by BLAST similarity search in species whose complete genome is available in the ENSEMBL Genome Browser. Two GHS-R loci were considered as paralogs or orthologs when the two corresponding protein sequences matched on aligned blocks with an average length of at least 80% with ≥70% identity. Synteny-based analyses were then performed to confirm whether the genes identified are real GHS-R paralogs or orthologs. These synteny analyses consisted of performing a comprehensive comparative analysis of the genomic region harboring GHS-R genes to identify their upstream and downstream flanking genes. When a GHS-R gene was not identified in a given species by similarity search using the protein sequence of its ortholog, the sequences of flanking genes were used for its identification by means of BLAST search. When the flanking genes were identified and no GHS-R was predicted between them, the genomic region potentially harboring these flanking genes was extracted and re-annotated using de novo and/or similarity-based annotation approaches. A gene annotated by de novo or similarity-based approach was considered to be a GHS-R locus when it matched the well-characterized GHS-R protein sequences on aligned blocks with an average length of at least 80% with ≥70% identity. The protein sequences of predicted genes from the de novo annotation were then confirmed as GHS-R by BLAST against the well-characterized GHS-R genes using the above criteria. The same similarity and synteny search criteria (aligned blocks of protein sequences that match with an average length of at least 80% with ≥70% identity, re-annotation of genomic regions potentially harboring orthologs) were applied to identify ghrelin orthologs in NCBI databases and in all vertebrate species whose genome is present in the ENSEMBL Genome Browser. Likewise, the same similarity search approach was used to identify MBOAT4 (GOAT) and MBOAT1/2 orthologs and paralogs in a wide range of vertebrate species. The sequences of MBOAT isoforms were used in this study only for the evolutionary investigation, that is, the comparison of dN/dS patterns.

**Phylogenetic analyses**

The ghrelin and GHS-R phylogenetic trees of both mammalian and nonmammalian vertebrates were reconstructed using protein sequences of species belonging to these respective lineages. The protein sequences of GHS-R isoforms from a broad range of mammalian species were aligned using MAFFT version 7 (Katoh and Standley 2013). The protein sequences of GHS-R of nonmammalian vertebrates, and the ghrelin protein sequences of all vertebrates analyzed in this study, were also separately aligned using MAFFT. The Gblocks Server was used to improve these alignments (Castrésana 2000; Talavera and Castresana 2007). The well-aligned blocks identified by Gblock software were then used to reconstruct a phylogenetic tree using MEGA software version 6 (Tamura et al. 2013). The maximum-likelihood method with the Jones–Taylor–Thornton (JTT) substitution model was used to constructed the phylogenetic trees, which was rooted with the reptile GHS-R protein sequence (*Chelonia mydas* and *Pelodiscus sinensis*) for mammal tree and lamprey
(Petromyzon marinus) GHS-R protein sequence for non-mammal tree. The vertebrate ghrelin tree was rooted using the midpoint rooting approach. The GHS-R actually belongs to the same family as motilin (MLN-R). These two receptors share some homologies and might be mixed up when considering only similarity search by BLAST. We therefore conducted phylogenetic reconstruction of a tree including MLN-R genes from teleost and tetrapods.

**Evolutionary analyses**

The \( dN/dS \) by site was used to measure the selective pressure exerted on ghrelin, GOAT, MBOAT1/2, and GHS-R genes. The \( dN/dS \) ratio is a common method used to measure the evolutionary selective pressure exerted on genes. It is commonly accepted that the theoretical limit between positive and negative selection is a \( dN/dS \) ratio of one. A \( dN/dS \) ratio less than one is indicative of negative selection whereas a ratio greater than one is a sign of positive selection. Pairwise comparisons of \( dN/dS \) ratios were conducted between vertebrate ghrelin orthologs using nucleotide sequences, and also between vertebrate GHS-R orthologs and paralogs. Similar pairwise comparisons of the average \( dN/dS \) of GOAT and MBOAT1/2 orthologs were also conducted. The \( dN/dS \) ratios between GHS-R paralogs and orthologs were calculated using both naïve empirical Bayes and Bayes empirical Bayes model implemented in JCoDA (Steinway et al. 2010). There are several methods incorporated in the JCoDA software (Department of Biology, The College of New Jersey, Ewing, USA) for the estimation of \( dN/dS \) ratios, which include NG (Nei and Gojobori 1986), YN (Yang and Nielsen 2000), and LPB (Li 1993; Pamilo and Bianchi 1993). All these methods were applied, and the results did not differ significantly. Finally, the NG method was applied and a Fisher’s exact test was used to test the significance of differences in \( dN/dS < 1 \) and \( dN/dS > 1 \). The multiple-comparisons Turkey’s test was used to assess the significance of differences in the average \( dN/dS \) ratios of ghrelin orthologs between lineages. The same test was used to evaluate the significance of the average \( dN/dS \) ratios between GHS-R and MBOAT clusters and between lineages. Sliding window \( dN/dS \) was used to identify codons of the GHS-R and ghrelin proteins that are under positive selection. Codons that are under selective constraints were graphically visualized using the graph sliding \( dN/dS \) window option as implemented in JCoDA software. The size of the window was set at 200 bp, with a jump of 25 bp between windows.

**Protein structure prediction**

The SABLE server (Adamczak et al. 2004) was used for the protein structure prediction, which included finding the number of TMDs, predicting the secondary structure, quantifying the RSA of amino acid residues along the protein sequences, and identifying physical–chemical property profiles. The RSA measures the solvent surface accessible to amino acid residues in a protein. The RSA represents the solvent-accessible surface areas normalized by the surface area of the residue in the unfolded state. A RSA value of 0 means completely buried whereas a value of 9 is indicative of a fully exposed surface area. The predicted structures were visualized using the POLYVIEW-2D viewer (Porollo et al. 2004). Correlations between RSA and other structural features of the protein, including \( \alpha \)-helix, \( \beta \)-strand, and coil structures, were investigated; as were correlations between RSA and \( dN/dS \) ratios.

**Results**

**Mammalian ghrelin and its orthologs in other vertebrates**

There are currently about 100 mammalian, 50 avian, and 50 fish genomes deposited at NCBI. This study that aimed on investigating the coevolution of the ghrelin/GHS-R system in the main vertebrate lineages. The species selected for represent a fraction for which ghrelin/GHSR annotations were found in NCBI protein database using similarity search (Fig. 1). The selection was also based on the availability of completely sequenced genomes that allowed performing conserved synteny analyses in ENSEMBL. The similarity search revealed a single ghrelin gene encoding for a single isoform in mammals and nonmammalian vertebrates such as birds, reptiles, amphibians, teleosts, euteleosts, and the coelacanth. The selection of species can be retrieved from the phylogenetic tree (Figs. 2–4). The conserved synteny analyses revealed that the upstream and downstream flanking genes of ghrelin are the orthologs of SEC13 homolog (SEC13) and TatD DNase domain-containing 2 (TATDN2) in mammals and the coelacanth. The ghrelin of teleost fishes including D. rerio and the cave fish, A. mexicanus, is flanked downstream by TATDN2 but upstream is flanked by a different gene annotated as coiled coil domain-containing 174 (CCDC174). While birds (G. gallus) possess SEC13 upstream similar to mammals, the downstream flanking gene is interleukin-1 receptor-associated kinase-like 2 (IRAK2).

The structural organization analyses of the ghrelin gene varied in the exon and intron counts between vertebrate lineages (Appendix S1). The analysis of protein structure revealed that the entire protein is constituted by a soluble part, and no TMD was predicted in the protein sequence of any analyzed species (Appendix S2). The number of
α-helices, β-strands, and coils of the ghrelin gene in mammals, birds, amphibians, reptiles, teleosts, and coelacanth is summarized in Appendix S1. The analysis of RSA revealed values ranging from 2 to 8, with most of them being equal or greater than 4 (Appendix S3). There were few amino acid residues with a RSA value in the first α-helix of the protein sequence. The main characteristic of the amino acid residues of the first helix is hydrophobicity, as compared to the other helices (consisting polar or charged residues).

**GHS-R in tetrapods**

Two GHS-Ra isoforms were previously reported in tetrapods. One isoform results from regular pre-mRNA splicing, while the other derives from alternative splicing of the same gene (Kaiya et al. 2013a, 2014a). As the two mammalian GHS-R isoforms are encoded by the same locus, only one variant was considered in this study. A list of mammalian species possessing GHS-Ra is provided in Table 1. The orthologous isoform of this receptor was found in a total number of 22 mammalian species. The synteny analyses in available genomes of human, chimpanzee, cow, pig, cat, mouse, and rat revealed that the GHS-Ra isoform is flanked upstream by the tumor necrosis factor (ligand) superfamily, member 10 gene (TNFSF10) and downstream, by fibronectin type III domain-containing 3B (FNDC3BB). The orthologs of the mammalian GHS-Ra gene were located in amphibian species (X. laevis, X. Silurana tropicalis, Hyla japonica, R. catesbeiana) and in reptiles (C. mydas, P. sinensis, A. sinensis) as well as in birds (G. gallus, M. gallopavo) (Table 2). Similar to the mammalian GHS-Ra, the flanking genes are orthologous to FNDC3BB and TNFSF10. The amino acid residues D99, C116, E124, M213, S217, and H280 that have been shown to play key roles in the function of GHS-R are present in all tetrapod groups.

**GHS-R1a/2a in fishes and the coelacanth are the mammalian orthologs**

Two orthologs (GHS-R1a and GHS-R2a) of the mammalian GHS-Ra were identified in *D. rerio*, with the GHS-R1a isoform comprising of 360 amino acids (aa), consisting 2 exons and 1 intron. GHS-R2a is 365 aa in size with identical exon–intron arrangement. Both of these isoforms are flanked upstream by FNDC3BB as found for all other vertebrates but downstream by a different gene, phospholipase D1a (PLD1A). The *D. rerio* GHS-R1a isoform is more similar to the orthologs of other vertebrates.
GHS-R1a is found in a limited number of other fish species (Carassius auratus, Lepisosteus oculatus, Clupea harengus and Astyanax mexicanus) where it is flanked by the same genes as the mammalian isoform. The GHS-R2a ortholog was also found only in a limited number of teleost species such as Carassius auratus and Carassius carpio with the same flanking genes as GHS-R1a.

Identification of teleost-specific isoform GHS-R1a-LR

Another ortholog flanked by the same genes (FNDC3BB and PLD1A) as D. rerio GHS-R1a and GHS-2a was identified in a large number of teleost species including the pufferfishes (Takifugu rubripes and Tetraodon nigroviridis), tilapias (Oreochromis niloticus and Oreochromis mossambicus), Gasterosteus aculeatus, Oryzias latipes, Gasterosteus aculeatus, Xiphophorus maculates, Poecilia formosa, Fundulus heteroclitus, Dicentrarchus labrax, and Larimichthys crocea. This isoform is more identical to D. rerio GHS-R1a and is named as GHS-R1a-like receptor (GHS-R1a-LR) in this study.

The order of GHS-R and surrounding genes relative to each other on the forward strand of the genomic fragment is PLD1A-FNDC3BB-GHSR-TNFSF10 in H. sapiens and L. oculatus, TNFSF10-GHSR-FNDC3BB-PLD1A in A. carolinensis and G. gallus, and PLD1A-GHSR-FNDC3BB-(3 other genes)-TNFSF10 in D. rerio. Considering the
gene direction, it is evident that the order of the genes relative to each other is identical in *H. sapiens*, *G. gallus*, *A. carolinensis*, and *L. oculatus*. The coordinates of the whole chromosomes in *G. gallus* and *A. carolinensis* are probably inverted, possibly at a higher level inversion. This explains the different order compared to *H. sapiens* and *L. oculatus*. In *D. rerio*, a lower level inversion of (*TNFSF10*+, *GHS-R*+, *FNDC3BB*–) *PLD1A*+, followed by a translocation of *TNFSF10*, resulted in: (*FNDC3BB*+, *GHS-R*–, translocated) *PLD1A*+. In conclusion, the difference in the downstream flanking gene between fish *GHS-R1a*, *GHS-R2a* *GHS-R1a-LR*, and tetrapod *GHS-Ra* is the consequence of inversion and translocation of *TNFS10* in fishes. This arrangement appears to be common but specific to teleost species, as in the euteleost *L. oculatus*, *GHS-R1a* is flanked downstream by *TNFS10*. Together, synteny and similarity suggest that *GHS-R1a-LR* is probably the same isoform as to *GHS-R1a*, which in turn is paralogous to *GHS-Ra*.

**Identification of teleost-specific isoforms**

*GHS-Ra-LR2a/b*

A new isoform was named *GHS-Ra-like receptor 2a* (*GHS-Ra-LR2a*) in this study as it displays high similarity to *GHS-R1a-LR*. *GHS-Ra-LR2a* was found in a significant number of teleost species such as *L. oculatus*, *D. rerio*, *T. rubripes*, *O. latipes*, *F. heteroclitus*, *P. reticulate*, *O. niloticus*, and *Neolamprologus brichardi*. Table 2 summarizes the number of amino acid residues for each species and the exon and intron counts. The *GHS-Ra-LR2a* isoform is flanked upstream by enoyl-CoA delta isomerase 1 (*ECI1*) and downstream by tachykinin 4 (hemokinin) gene (*TAC4*). In *D. labrax*, the downstream flanking gene
Figure 4. ML phylogenetic tree of GHS-R in nonmammal vertebrates constructed with protein sequences and using the Jones–Taylor–Thornton (JTT) substitution model. The tree was rooted with the lamprey (Petromyzon marinus) GHS-R protein sequence. WGD1: whole-genome duplication 1; WGD2: whole-genome duplication 2.
Table 1. GHS-Ra isoform in mammal vertebrates and its flanking genes.

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<th>Protein ID</th>
<th>Length (aa)</th>
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<th>Intron</th>
<th>Genomic location</th>
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</table>
is pyridoxal-dependent decarboxylase domain-containing protein 1 (PDXDC1I). The flanking genes of GHS-Ra-LR2a are also different in D. rerio and A. mexicanus where the upstream flanking gene is protein phosphatase 1 regulatory subunit 35 (PPP1R35) for both species, whereas the downstream gene different and are bromo adjacent homology domain-containing 1 (BAHD1) and calmodulin-binding transcription activator 2 (CAMTA2), respectively.

Phylogenetic analysis grouped another isoform of the fish GHS-R to GHS-Ra-LR2a (Fig. 4). Thus, this gene was named GHS-Ra-like receptor 2b (GHS-Ra-LR2b) and identified in a limited number of fish species including D. rerio, A. mexicanus, O. latipes, F. heteroclitus, P. formosa, X. maculates, and D. labrax. The length and exon/intron counts of GHS-Ra-LR2b are summarized in Table 2. The upstream flanking gene of this isoform is diablo, IAP-binding mitochondrial protein (DIABLO) in D. rerio, P. reticulate, and O. nilotica, but its downstream flanking gene is prolyl 4-hydroxylase, alpha polypeptide III (P4HA3), and uncharacterized proteins (Table 2). In D. labrax and O. latipes, the upstream flanking gene is FNDC3BB whereas the downstream flanking genes are RCSD1 and an uncharacterized protein, respectively.

**Secondary structure characteristics of GHS-R isoforms in vertebrates**

Key roles in the function of GHS-R are mediated by the amino acid residues D99, C116, E124, M213, S217, and H280 (Miki et al. 1992; Howard et al. 1996; Kaiya et al. 2008, 2013a, 2014a), which are present in tetrapods. While these functional amino acid residues were also found in all fish GHS-R isoforms, their relative positions...
differed in some species either due to insertion or deletion of amino acid residues. All GHS-Ra orthologs and GHS-R1a/2a isoforms analyzed in this study possess seven TMD (Appendices 4 and 5). The GHS-R1a-LR has seven TMDs in all analyzed species including G. aculeatus, G. morhua, L. crocea, N. burchardi, O. mossambicus, and D. labrax, except in X. maculatus where this isoform has exceptionally nine TMDs. The GHS-Ra-LR2a/b isoforms both comprised seven TMDs except in O. latipes and L. oculatus where GHS-Ra-LR2b has only six TMDs. The comparative results of the protein secondary structure, including the number of \( \alpha \)-helices, \( \beta \)-strands, and coils predicted for each GHS-Ra in each species, are indicated in Appendix S5. The number of these structural units of the GHS-R protein varies between species but also between isoforms.

These different isoforms differ in the length of the second extracellular loop (ECL2) connecting the TMD4 and TMD5 which is comprised of 24-26 amino acid (aa) residues for GHS-R1a/2a and GHS-Ra isoforms. The ECL2 of GHS-Ra-LR1 (Appendix S4) is much larger (~36 aa residues) than that of GHS-R1a/2a and GHS-Ra isoforms. The GHS-Ra-LR2a and GHS-Ra-LR2b display ECL2 lengths similar to that of GHS-R1a/2a (Appendix S4) in most of the species. The GHS-Ra-LR2a is mainly characterized by a large ICL3, with a length ranging from 90 to up 237 aa residues. This ICL3 is exceptionally large in F. heteroclitus (275 aa residues). The GHS-Ra-LR2b is characterized by an ICL3 that is shorter (5–16 aa residues) than that of the other GHS-R isoforms (Appendix S4) where it is comprised of 19–26 aa residues, respectively.

The highest RSA values were observed in P. sinensis, which also has the highest number of residues with higher RSA values (RSA \( \geq 7 \)). The lowest number of residues with RSA was recorded for birds (F. albicolis and M. gallopavo). H. sapiens and G. gorilla have the same number of RSA residues (181) and higher RSA values (RSA \( \geq 7 \)). The GHS-R1a has a higher number of residues with RSA values (276 vs. 177 aa) compared to other fish isoforms. The number of residues with higher RSA values was lower in GHS-R1a-LR compared to GHS-Ra-LR2a/b, which display similar number of residues with high RSA values. For both ghrelin and GHS-R genes, null or lower RSA values were observed in the TMD. For most of the isoforms, RSA were higher in the \( \alpha \)-helix and \( \beta \)-strands structures compared to coils. The only exception was observed for GHS-R1a where the higher RSA values were observed in coil structures.

**Phylogenetic analyses**

The phylogenetic reconstructions grouped vertebrate ghrelin genes into different clades, with the main groups being supported by high bootstrap values. All teleost ghrelin genes were grouped in the same clade (Fig. 2), which is the sister group of amphibian ghrelin genes. Reptilian ghrelin genes are clustered together and form the sister group of avian ghrelin. All mammalian ghrelin genes were grouped into a same clade (Fig. 2), which is distinct from all other vertebrate lineages. The phylogenetic analysis of mammalian GHS-Ra revealed minor divergence and close relation between species (Fig. 3). This is also evident from the low bootstrap values (Fig. 3). The comprehensive phylogenetic reconstruction also allowed regrouping non-mammalian GHS-R into different clades which were supported by higher bootstrap values (Fig. 4). Bird and reptile GHS-Ra are grouped into two different clades. Amphibian and coelacanth GHS-Ra are grouped into the same clade (Fig. 4). These groups are closely related to both fish GHS-Ra, which are subdivided into subclades GHS-R1a and GHS-R2a. Another subclade (GHS-R1a-LR) that belongs to the same superclade than GHS-R1a was identified in teleosts. Teleost GHS-R1a and GHS-R1a-LR were grouped into the same clade, which is a sister group of GHS-R2a found in a limited number of teleost species (Fig. 4). Clade GHS-R1a-LR comprised isoforms that were exclusively found in actinopterygian fishes. Among the particularities of the isoforms that constitute this clade, the ECL2 is longer as compared to its counterpart of the group GHS-R2a. Two other clades (GHS-Ra-LR2a and GHS-Ra-LR2b) are also identified in fishes from the phylogenetic reconstruction (Fig. 4). The clades of GHS-Ra-LR2a and GHS-Ra-LR2b diverged from the other fish receptors (Fig. 4). Clade GHS-Ra-LR2a is comprised of genes exclusively identified in teleost fishes, and no orthologous isoforms were found in other vertebrates. The particularity of this clade is represented by genes with ICL3 larger than their analogous loop of the other isoforms. Clade GHS-Ra-LR2b also consists of isoforms found only in teleosts, without any orthologs in other vertebrate species. The characteristic of this clade compared to its sister group is resembled by the size of the ICL3, which is much shorter than in the other isoforms. The phylogenetic reconstruction including MLN-R showed that fish and tetrapod MLN-R are grouped in the same clade, which is a sister group of the GHS-Ra-LR2a/GHS-Ra-LR2b cluster (Appendix S6). The phylogenetic tree indicated that MLN-R derived from clade B prior to GHS-Ra-LR2a/b duplication whereas tetrapod GHS-Ra derived from clade A whose duplication in teleosts has resulted in GHS-R1a/GHS-R1a-LR/GHS-R2a (Appendix S6).

**Natural selection**

The average \( dN/dS \) ratio, which is used to measure the selective pressure exerted on ghrelin, was significantly
(P ≤ 0.05) higher in birds and lower in amphibians compared to reptiles, mammals and fishes Table 3. Sliding window analysis applied to all ghrelin orthologs pairwise revealed regions of the protein sequences that are under positive selection. These regions are located between 110 and 250 bp (Fig. 5A), more precisely between 110 and 130 bp and around sites 146, 160, 230, and 240 bp of the nucleotide sequence. The residues under positive selection are located in regions where α-helix structures are predicted, which also correspond to regions where the amino acid residues have the highest RSA values.

The average dN/dS ratio of GHS-Ra was lower in amphibians compared to birds, reptiles, and mammals. In teleosts, the average dN/dS was lower for GHS-R1a/2a cluster compared to GHS-R1a-RL, GHS-R2a-RL2a, and GHS-R-RL2b clusters (Table 3). The average dN/dS ratio was significantly lower for amphibian GHS-Ra and fish GHS-R1a/GHS-R2a cluster compared to mammals, birds, reptiles, and teleost GHS-R1a-LR, GHS-Ra-LR2a, and GHS-Ra-LR2b clusters. The sliding window dN/dS analyses of pairwise comparisons conducted along protein sequences allowed the detection of amino acid residues that are under selective constraints (Fig. 5B). The comparison of all pairwise GHS-R sequences between different lineages showed similar profiles. The same regions of the proteins that are under positive selection in fishes were also found to be under positive selection in all tetrapods. Likewise, the pairwise comparisons of the sliding window between all clusters identified by the phylogenetic tree revealed similar profiles between clades, with the same codons being under positive selection at the same position of the protein sequences. The exact position of codons with dN/dS indicative of positive selection is highlighted in Fig. 5B. They are all located in the coding region between 110 and 250 bp, around the positions 120, 146, 160, 210, 230, and 240 codons, in regions of the proteins where the α-helix and TMD are predicted except for those around codon 240 which, for some isoforms, is located in ICL3. The residues under positive selection are also located in regions of the proteins with low RSA values (RSA ≤ 1).

The average dN/dS ratio of the MBOAT4 (GOAT) cluster was significantly lower in amphibians compared to other lineages where it did not show differences (Table 3). Average dN/dS of MBOAT4 and MBOAT1 clusters in teleost were not significantly different. The average dN/dS of MBOAT1 and MBOAT2 were not significantly different either, except in teleost, where the average dN/dS of MBOAT1 was significantly higher than that of MBOAT2 (Table 3). The comparison with ghrelin revealed similar dN/dS patterns between MBOAT4 and ghrelin in all lineages except birds. In birds, the average dN/dS ratio of ghrelin was higher than in the other lineages. However, bird MBOAT4 dN/dS is equivalent to that of reptiles, mammals, and fishes. The graph dN/dS by sites indicates that the same codons are under positive selection, and these codon positions are the same not only for all clusters or lineages, but also for all isoforms (Fig. 6A–C). The sites under positive selection (Fig. 6A–C) are located between codons 110–130, around codons 148, 160, 210, 230, and 240.

**Discussion**

The ghrelin/ghrelin receptor hormone system is not only an important therapeutic target in translational medicine as several important physiological functions are systemically controlled in mice and men; it also represents an interesting biological system to investigate the evolutionary relationships between the unique ligand and receptor (Kaiya et al. 2011b). Uncovering the divergence and spectrum of this hormone system in the animal kingdom may assist in unraveling specific and unspecific, as well as direct and indirect functions of ghrelin. In order to shed light onto the evolution of the ghrelin/ghrelin receptor system, we found that the interaction of ghrelin and ghrelin receptor is based on a single gene in most vertebrates except teleost fishes. In the teleosts, we found several ghrelin receptor isoforms that we used to reconstruct the evolution of the ghrelin system and that we further analyzed to highlight conversed and diverse secondary structures.

It can be assumed that the novel orthologs named GHS-Ra-RL2a and GHS-Ra-RL2b in this study are also motilin receptor paralogs and/or orthologs. Interestingly, all GHS-R variants found in this study were either annotated as GHS-R or GHS-R-like. Moreover, the syntenic analysis revealed that in most of the teleosts, the novel orthologs are flanked upstream and downstream by ECL1 and TAC4, respectively. The only exceptions are D. labrax and O. latipes, where the upstream gene is FNDC3BB, while RCD51 and an unknown gene are located downstream. The vertebrate MLN-R, tetrapod GHS-R-, and teleost GHS-R1a, GHS-R2a, and GHS-R1a-LR have the same upstream flanking gene (FNDC3BB). From these syntenic results, it appears that MLN-R is more similar to GHS-Ra, GHS-R1a, GHS-R2a, and GHS-R1a-LR than GHS-Ra-RL2a/b. As MLN-R and the ancient (previously characterized) GHS-R isoforms (GHS-R, GHS-R1a, GHS-R2a, and GHS-R1a-LR) have the same upstream flanking gene in both tetrapods and teleosts, which is different from that of novel orthologs (GHS-Ra-RL2/b), it cannot be concluded from the syntenic results that these novel orthologs are similar to GHS-R and MLN-R only in teleosts. Moreover, the syntenic results indicate that GHS-R1a-LR and GHS-R1a have the same upstream and downstream flank-
ing genes (FNDC3BB and PLD1A, respectively), suggesting that GHS-R1a-LR is probably the same isoform as GHS-R1a, which in turn is paralogous to tetrapod GHS-Ra and teleost GHS-R2a. Thus, if GHS-Ra-LR1 equals GHS-Ra, then GHS-Ra-LR2a and GHS-Ra-LR2b are paralogs from a fish-specific WGD after their ancestral gene has derived from motilin receptor. The novel orthologs identified in this study are not derived from alternative splicing. Therefore, none of them is referred to as GHS-Rb. Overall, the nomenclature given to the ancient GHS-R orthologs in this study is in accordance with that of previous studies (Kaiya et al. 2013a, 2014a). Also, the nomenclature given to the novel isoforms reflects their similarity with the ancient GHS-R isoforms and MLN-R. Motilin receptor gene was probably duplicated as for GHS-R, but the duplicates were subsequently lost. This may explain why only one MLN-R isoform exists in both tetrapods and teleosts.

Figure 5. Graph sliding window dN/dS ratio of ghrelin genes (A) and GHS-R all isoforms combined (B). The NG method was applied for the estimation of dN/dS ratios. The size of the window was set at 200 bp, with a jump of 25 bp between windows. Codons under positive selection with empirical Bayes (NEB) model are indicated in blue and in green with Bayes empirical Bayes (BEB) model. Nearly neutral codons are indicated in red.

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Figure 6. Graph $dN/dS$ by site for (A) MBOAT4, (B) MBOAT1, and (C) MBOAT2 estimated using the $\nuG$ method. The size of the window was set at 200 bp, with a jump of 25 bp between windows. Codons under positive selection with Empirical Bayes (NEB) model are indicated in blue and in green with Bayes empirical Bayes (BEB) model. Nearly neutral codons are indicated in red.
The phylogenetic reconstructions of this study suggest that the ancestral gene of GHS-R has undergone successive rounds of duplications that have resulted in different isoforms. While duplicated isoforms are usually lost during the course of evolution due to redundancy, the maintenance of several GHS-R genes suggests some diversification of the ghrelin/ghrelin receptor system in teleosts. The evolutionary scenario that has led to the different GHS-R isoforms is illustrated in Figure 7. The ancestral gene of GHS-R was duplicated during early vertebrate evolution into two main clades: Clade A comprises GHS-Ra (GHS-R2a/GHS-R1a-LR/GHS-R1a) orthologs in teleosts; and Clade B comprises GHS-Ra-LR2a/GHS-Ra-LR2b, both subgroups clustering in distinct clades. A teleost-specific whole-genome duplication of clade A has resulted to GHS-R1a-LR/GHS-R1a and GHS-R2a, with GHS-R1a-LR being the same than GHS-R1a. GHS-R2a has been subsequently lost in all teleosts except in some cypriniforme species. Thus, teleost GHS-Ra/GHS-R1a-LR is a sister group of mammalian GHS-Ra which in turn is orthologous to fish GHS-R2a and tetrapod GHS-Ra. The synteny results uncovered that the neighboring genes of mammal, bird, reptile, and amphibian GHS-Ra are FNDC3B and TNFSF10. In teleosts, GHS-R1a, GHS-R1a-LR, and GHS-R2a are all flanked by FNDC3B and PLD1A. However, there is a TNFSF10 gene next to PLD1A (at the fourth position of genes located downstream of PLD1A) in humans, suggesting that the TNFSF10 gene has been translocated in teleosts. This indicates that GHS-R1a-LR is the same isoform as GHS-R1a, which is the paralog of GHS-Ra. This interpretation of the origin of GHS-R1a/2a differs from that of previous studies, which suggested that these two isoforms resulted from a duplication event that specifically occurred in some cypriniforme species (Kaiya et al. 2013a, 2014a). We suggest that there is confusion on the origin of GHS-Ra2 from a recent WGD (tetraploidization) in fishes. From completely sequenced genomes, GHS-R2a is only present in carp (Cyprinus) but not in grass carp, suggesting that GHS-R2a is highly specific to the genus Cyprinus and possibly some other cypriniformes. While the similarity search indicated that GHS-R1a and GHS-R1a-LR are more similar compared to other isoforms, the structural protein results showed that the ECL2 length differs between these two isoforms. This may be seen as a contradiction of the above interpretation (GHS-R1a same variant as GHS-R1a-LR), but the larger ECL2 of GHS-R1a-LR may have resulted from structural changes that specifically have affected this isoform (Kaiya et al. 2013a). The presence of only one GHS-Ra isoform in the elephant shark, Callorhinchus milii, genome supports the idea that the duplication specifically occurred in teleost fishes. The GHS-R1a and GHS-R2a isoforms were identified on corresponding chromosomes 2 and 24 in zebrafish, which provides evidence that they originated from WGD (Meyer and Schartl 1999; Jaillon et al. 2004; Kaiya et al. 2013a, 2014a). This interpretation is also in agreement with the synteny between homologous genomic regions harboring the two isoforms from this duplication event, which are still conserved. The same WGD has affected clade B and has resulted in GHS-Ra-LR2a and GHS-Ra-LR2b. We believe that the same WGD that has resulted to GHS-R1a/GHS-R1a-LR and GHS-R2a has also resulted to GHS-Ra-LR2a and GHS-Ra-LR2b, which constitute the clade B. Although the GHS-Ra-LR2a and GHS-Ra-LR2b isoforms still remain in the genome, the micro-synteny of genomic regions harboring them is no longer conserved, probably because these regions have undergone genomic re-arrangements including insertions, deletions, and translocations that may have altered sequence homology signals. This may explain why GHS-Ra-LR2a and GHS-Ra-LR2b isoforms were not detected in corresponding genomic regions in any of the fish species. GHS-R1a, GHS-R2a, and GHS-Ra-LR2a have an ECL2 with similar length, which is also equivalent to the

Figure 7. Evolutionary scenario explaining duplication events that gave rise to the different GHS-R isoforms in vertebrates. Duplication in early vertebrate evolution (WGD1); fish-specific whole-genome duplication (WGD2); GHS-R1a-LR same than GHS-R1a; GHS-R2a lost in fish species other than Cypriniformes; (CA = Common ancestor).
length of MLN-R ECL2, suggesting that ECL2 length is a characteristics that may have been inherited from the common ancestral gene of GHS-R and MLN-R. The large ECL2 length of GHS-R1a-LR has probably resulted from structural changes that have specifically affected these two GHS-R isoforms after duplication. Similarly, the exception of larger ICL3 of GHS-Ra-LR2a was probably acquired through structural changes that have specifically occurred in this GHS-R isoform after duplication. By contrast, the shorter ICE3 of GHS-Ra-LR2b seems to have common characteristics with MLN-R, which also have one ICE3 with a similar length. If MLN-R and GHS-R have both been considered as belonging to the motilin–ghrelin protein gene family, it has never been demonstrated that MLN-R shares more similarity with GHS-Ra-LR2a/b isoforms that were not clearly characterized. The phylogenetic reconstruction showed that fish MLN-R and GHS-R1a-LR2/GHS-Ra-LR are sister groups and certainly resulted from duplication of the same common ancestral gene.

The biological activities of ghrelin are mediated by GHS-R. However, ghrelin acylation by ghrelin O-acyltransferase (GOAT) enzyme is required for the accomplishment of all its physiological activities. The ghrelin amino acid residues Gly-Ser-Ser(n-octanoyl)-Phe-NH2 are required for the activation of ghrelin (Kojima and Kan-gawa 2005) and are conserved among most species analyzed in this study, except in few species including the amphibian R. catesbeiana, where Ser3 was replaced by Thr3. While the conservation of these essential amino acid residues in all lineages is indicative of common functions in vertebrates, the replacement of Ser by Thr may have altered the properties of ghrelin gene as experimental replacement of Ser3 by Leu led to a complete inhibition of ghrelin gene activity (Matsumoto et al. 2001; Delporte 2013). Ser3 > Thr is not amphibian-specific, and R. catesbeiana may be an exception.

The functionality of GHS-R isoforms is not experimentally confirmed here, but the structural properties shared by all variants suggest that they may all be responsive to the ghrelin gene. The key residue D99 is located in the TMD2 of GHS-Ra, GHS-R1a, GHS-R2a, and GHS-Ra-LR2b isoforms whereas C116 and E124 on one hand and M213 and S217 on the other hand are located in TMD3 and TMD5 of the same isoforms, respectively. The H280 residue is located in TMD6 of the above-mentioned isoforms, and in TMD7 of the GHS-Ra-LR2a isoform. The location of these key residues on the same structural components, despite slight differences in positions, suggests that they have not altered the properties of GHS-R. Taken together, these results indicate that all GHS-R isoforms have preserved the basic functions of the ancestral ghrelin receptor gene.

Given the conservation of amino acids required for ghrelin acylation, all GHS-R isoforms possess key residues that play crucial roles in receptor binding, strongly supporting the coevolution of the ligand/receptor pair. Despite sequence divergence, the functional key residues are preserved by all isoforms and positional shades, which probably have resulted from inversions or deletions, particularly in the GHS-R isoforms. Ghrelin, GOAT, and GHS-R are physiologically and biochemically interlinked (Gutierrez et al. 2008; Yang et al. 2008a, 2008b). There is a functional triangle between these three genes that might be reflected in their evolutionary profiles (Yang et al. 2008b). Coevolution between genes can result either from their proximities in the genome or reflect functional relationships (Tillier and Charlebois 2009b; Chan et al. 2013). Neighboring genes can coevolve due to the action exerted by local evolutionary factors, such as the existence of genomic regions with differential recombination activities. The density of chromatin in the regions where these genes are located (Chan et al. 2013). It has been demonstrated that evolutionary events such as retrotransformation are regulated by the chromatin status, which influences the mediating L1 element activity (Seleme et al. 2006). The identification of coevolution signals between distant loci could reflect an ancestral genomic rearrangement that may have resulted in initial neighboring genes being distantly located in the genome after rearrangements (Zhang and Gaut 2003; Koszul et al. 2004). Coevolution interactions also include gene gain or loss that can be inferred from phylogenetic reconstruction profiles and conserved synten analyses (Chan et al. 2013; Borges et al. 2015). Our synteny results did not show signals of ancestral genomic rearrangements that may have altered the proximity of GHS-R. Likewise, it is unlikely that GHS-R isoforms were initially proximately located in the genome because they did not originate from tandem duplications that result in paralogs being adjacent in genome. The comparisons of substitution ratios (all isoforms combined) showed similar profiles of sliding window dN/ dS between lineages, with the same codon positions being under positive selection. Similar results were observed from GHS-R clade comparisons of sliding window dN/dS, which showed same the profiles between clusters, which is also similar to the patterns observed between lineages. These codons under positive selection would not affect the receptor functionality or promote functional differences because they all fall within TMDs and are neither lineage- nor isoform-specific. They are found in all GHS- R clusters, which argues against the existence of specific functional constraints that may have altered the original function or led to novel functions exclusive to any of these isoforms. Although recent studies have demonstrated the existence of correlation between RSA and the
evolutionary rate of proteins (Shaytan et al. 2009; Tien et al. 2013), we did not identify a linear relationship between RSA and dN/dS ratio. The comparison of dN/dS patterns between MBOAT isoforms and ghrelin indicated that MBOAT4 selection patterns are more similar to ghrelin selection profiles. Although not strong, there is signal of coevolution between ghrelin and MBOAT4 compared to MBOAT1/2. However, the graph of dN/dS by sites shows the same amino acid residues under positive selection in all MBOAT isoforms. Therefore, the comparison of the selection patterns does not allow to draw definitive conclusions concerning why ghrelin specifically binds MBOAT4, but not MBOAT1/2 isoforms. This suggests that there must exist other factors allowing MBOAT4/ghrelin specificity.

Conclusion

The combination of similarity-based BLAST search, phylogenetic reconstructions, conserved synteny, and protein structure analyses appeared to be the best approach for an exhaustive clarification of the evolutionary history of gene families. In addition to confirming the presence of a single ghrelin locus in vertebrates, one GHS-R isoform in all tetrapods and three isoforms previously characterized in fishes, we identified two new GHS-R variants in teleosts. The combined conserved synteny analyses and phylogenetic reconstructions showed that two of fish GHS-Rs, namely GHS-R1a and GHS-R1a-LR previously described as different isoforms, are the same variant. GHS-R1a and GHS-R1a-LR differed only from the larger ECL2 length of GHS-R1a-LR that has probably resulted from structural changes that have specifically occurred in species having this variant. The phylogenetic reconstruction, together with conserved synteny analyses, showed that all GHS-R isoforms have resulted from different WGD, some of which were specific to teleosts. The retention of GHS-R isoforms in teleosts after duplications and their functional diversification offer excellent opportunities for investigating neo- and/or subfunctionalization following gene duplications, processes that are responsible for the functional divergence and diversification of protein gene families. The most commonly characterized GHS-R isoforms (GHS-Ra, GHS-R1a, and GHS-R2a) share common functions with MLN-R. From the phylogenetic reconstructions, it can be expected that MLN-R shares more functionalities with GHS-Ra-RL2a and GHS-Ra-RL2b isoforms, to which it is more closely related. The identification of new GHS-R isoforms and the clarification of the evolution history of this receptor group provide further insights for studies on structure–function relationships and may assist in determining the physiological role of the ghrelin/GHS-R system.

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Conflict of Interest

None declared.

References


Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Ghrelin genes in vertebrates: Accession number, exon/intron structure, and secondary sericulture

Appendix S2. Secondary structure of ghrelin gene in vertebrate lineages

Appendix S3. Relative solvent accessibility (RSA) of secondary ghrelin gene in vertebrate lineages

Appendix S4. Secondary structure of GHS-R isoforms

Appendix S5. Composition of GHS-R secondary structure

Appendix S6. GHS-R phylogenetic tree including MLN-R.