The Ghrelin Receptor Gene in Animal Production

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For Tsuneyuki Tsuda, who gave me the fun of ruminant physiology

CONTENTS

PREFACE

It is my pleasure and honour to welcome the reader of this book dedicated to "The Ghrelin Receptor Gene in Animal Production: A light on short tandem repeats in the genome". The ghrelin receptor, growth hormone secretagogue receptor (GHSR), is involved in many important functions, including growth hormone (GH) release, appetite regulation, and energy metabolisms in animals. It is well known that ghrelin is the result of reverse pharmacology, which began more than 30 years ago with these historical milestones: the development of synthetic growth hormone-releasing peptide (GHRP) in 1984, the discovery of non-peptidyl GH secretagogues (GHSs) in 1993, the discovery of the GHS receptors (GHSR1a and GHSR1b) in 1996, and the discovery of a natural GHS, ghrelin, in 1999.

However, my research group had started a linkage analysis between microsatellite DNA markers on the genetic map and growth traits in Japanese Black cattle (JBK) in 1998, because microsatellite DNA markers were the primary tools for identifying candidate genes on quantitative traits in animal production in the late-1990's through the mid-2000's. We identified the *GHSR1a* gene as one of the candidate genes for birth weight and chest width in JBK in 2002. It was extremely interesting for us that, in cattle, the bi-nucleotide microsatellite $(TG)_n$ exists within the 5'UTR intron region of *GHSR1a* gene. The *GHSR1a.5'UTR_*$(TG)_n$ is transcribed in the non-spliced type *GHSR1b* transcript but not in the spliced type *GHSR1a* transcript. Further, the *GHSR1a.5'UTR_*$(TG)_n$ does not exist in the human, mouse, rat, and other non-ruminant animal genomes. At first, we did not predict that the *GHSR1a.5'UTR_*$(TG)_n$ was a quantitative trait nucleotide (QTN) for growth traits in cattle. However, based on our subsequent studies over more than 15 years, we believe that the *GHSR1a.5'UTR_*$(TG)_n$ is a QTN for carcass and fatty acid composition traits because the $(TG)_n$ may affect the secondary structure of *GHSR1b* mRNA and thus the translational efficiency of *GHSR1b* transcript and additive effect of each $(TG)_n$ allele on carcass weights. Further, GHSR plays a central role in growth and fatty acid metabolism in a sex-dependent fashion due to gene-gene interactions between *GH*, *GH receptor*, and *GHSR1a* genes. After the mid-2000s, it was reported that hetero-dimerization of GHSR1a with GHSR1b can occur and reduces the

signalling capacity of GHSR1a. Because GHSR1a can pair with other GPCRs, these heterodimerisations affect ligand selectivity, G-protein coupling, and the downstream signalling of each receptor. Moreover, GHSR1b can form heterodimers with other GPCRs. Furthermore, my research group identified the arginine (R) repeat polymorphism [4 arginine residues (*4R*) or 3 arginine residues (*3R*, *DelR242*)] in the third intracellular loop of GHSR1a, with a high frequency in Japanese Shorthorn bulls. Further investigation indicated that the *4R/3R* heterozygotes had a selective advantage in weaner bulls because of their higher average daily gain and feed efficiency than those of homozygotes. Finally, my research group revealed age/lactation-related changes and comprehensive tissue distributions of the *GHSR1a/1b* and *GH releasing hormone receptor* (*GHRHR*) mRNA expressions in cattle and found several interesting bovine-specific mRNA expression patterns of these genes.

The *5'UTR_(TG)*$_n$ and *DelR242* [*4R/3R*, *4R*:$(AGG)_2$(AGA)(CGC), *3R*:(AGG)(AGA)(CGC)] loci of the *GHSR1a* gene are bi-nucleotide and tri-nucleotide (arginine) tandem repeats, respectively. Repetitive DNA sequences constitute a large fraction of the genomes of animal species. Tandem repeats (TRs) are a major class of repetitive DNA, and TR polymorphisms (TRPs) provide a unique source of genomic variability. TRs can change length during meiosis and mitosis, providing a dynamic source of genetic variation. The mutation rate of TRPs is higher and the extent of polymorphism is far more diverse than that of single nucleotide polymorphisms (SNPs). Whereas SNPs are invariably binary in nature, TRPs generally exhibit extended digital (multi-allelic) distributions, which provide a much richer range of polymorphic variants and thus a wider possible genetic contribution to quantitative traits in animals.

In 2015, Nessa Carey published an extremely exciting book, "Junk DNA: A Journey Through the Dark Matter of the Genome." The "bi-nucleotide (TG)" tandem repeats [*GHSR1a.5'UTR_*$(TG)_n$] in cattle are considered "junk DNA". Specifically, I believe that the "TG repeat" is an example of "junk DNA" and a light in dark matter of the genome in cattle. I hope that many "lights" will be found in domestic animal genomes in the near future.

It is my hope that this book will provide readers with an exhaustive picture of present knowledge in the ghrelin receptor field in endocrinology and animal production. Given the rapid pace of new information and discoveries, it is clear that this book will soon be obsolete; nevertheless, it will provide a good foundation for better understanding the novelties awaiting us in the near future.

I am indebted to all the co-authors who contributed to chapters of this book and to our five manuscripts of the bovine *GHSR1a* gene already published. I also would like to thank several people for their assistance with data and DNA sample collection, genotype determination, and data and statistical analyses. This research was supported by the National Agriculture and Food Research Organization (NARO) grant (No.212-j-01-001), the National Institute of Agrobiological Sciences (NIAS) Gene Bank project, the collaborative research projects between the National Agricultural Research Centre for Western Region (WeNARC) and the Shirakawa Institute of Animal Genetics - Japan Livestock Technology Association (SIAG- JLTA), and between the National Institute of Livestock and Grassland Science (NILGS) and the Maebashi Institute of Animal Science - Livestock Improvement Association of Japan (MIAS-LIAJ), and the collaborative research projects involving NILGS and the five Prefectural Livestock Research Centres in Japan. Support was also provided by the Platform for Drug Discovery, Informatics, and Structural Life Science from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

Masanori Komatsu

CHAPTER ONE

FUNCTION, NUCLEOTIDE POLYMORPHISMS, AND PHYLOGENY OF THE GHRELIN RECEPTOR (*GHSR1A*) GENE IN ANIMALS[1]

MASANORI KOMATSU, YUKI FUJIMORI, AND YOICHI SATO

Abstract

The ghrelin receptor, or growth hormone secretagogue receptor (GHSR), is implicated in many important functions including growth hormone (GH) release, food intake, and energy homeostasis. We have summarized (1) history of the hypothalamo-GH axis research and the hypothalamic neuronal network of GH release and feeding regulation, (2) ghrelin: interspecific variation, tissue distribution, functions, and genetic associations with quantitative trait loci (QTL) in domestic animals, (3) ghrelin receptor (GHSR): amino acid sequence and gene structure, GHSR dimerisation, molecular phylogeny, arginine repeats in the third intracellular loop domain, and genomic structure and transcriptional regulation, (4) bovine ghrelin receptor (*GHSR1a*) gene: structure, genetic variation, and genetic associations with QTL in domestic animals and humans. We focus on (1) two different kinds of mRNA transcripts: [i] spliced type [without a microsatellite $(TG)_n$ within the 5'UTR (*GHSR1a*)], and [ii] non-spliced type {with a $(TG)_n$ [*GHSR1b*]}, and (2) the arginine repeats in the third intracellular loop of GHSR1a. Molecular phylogeny of the *GHSR1a* gene suggests that the 5'UTR_(*TG*) repeats and arginine

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repeats of *GHSR1a* gene are coordinated with the *ghrelin* gene and may contribute to animal evolution.

1-1 Introduction

According to Kostyo (1999) [1], the control of growth is one of the primary importance of the endocrine system. The concepts and tools of cellular and molecular biology became available to endocrinologists, who have used them to discover how hormones act on target cells and influence their functions and expression of their genes [1]. Moreover, the tools of molecular biology have been used to advantage to clarify the nature of receptors for hormones and to identify the intracellular molecules with which these receptors communicate [1]. It is clearly defined that body growth was regulated not only by hormones such as pituitary GH, thyroid hormone, and insulin, but also by their receptors that may act globally in an endocrine manner or locally in a paracrine or autocrine manner to promote growth [1]. The chemical nature of the hypothalamic hormones that regulate GH secretion provided the basis for producing synthetic forms of these hormones and made possible a large number of researches leading to detailed understanding of how GH secretion is controlled by these and other hormones and receptors [1]. Knowledge of the physiological control of GH secretion elucidated how the growth process can be modulated in health, disease, and animal production [1].

The ghrelin receptor, growth hormone secretagogue receptor (GHSR), is involved in many important functions, including GH release, food intake, and energy homeostasis in animals [4, 48]. It is well known that ghrelin is the result of reverse pharmacology, which began more than 30 years ago with these historical milestones: the development of synthetic, growth hormone-releasing peptide (GHRP) by Bowers *et al.* in 1984 [2], the discovery of non-peptidyl GH Secretagogues (GHSs) by Smith *et al.* [3] in 1993, the discovery of the GHS receptors (GHSR1a and GHSR1b) by Howard *et al.* in 1996 [4], and the discovery of a natural GHS, ghrelin, by Kojima *et al.* in 1999 [5].

In this chapter, we summarize knowledge of the physiological control of GH secretion and feeding regulation, ghrelin and its functions, ghrelin receptor (GHSR) and its functions, nucleotide polymorphisms, and phylogeny of the *GHSR1a* gene in animals.

1-2 History of the hypothalamo-GH axis research, and hypothalamic neuronal network of GH release and feeding regulation

1-2 History of the hypothalamo-GH axis research, and hypothalamic neuronal network of GH release and feeding regulation

History of the hypothalamo-GH axis research

According to Murray *et al*. (2015) [6], as shown in Table 1-1, the growth-promoting function of the pituitary gland was discovered in 1921 [7], and the pituitary was recognized to produce a growth factor in 1944 [8]. Since then, our understanding of the neuroendocrinology of the hypothalamo-GH axis has included identification of the central components of the hypothalamo-GH axis, somatostatin (SST) and GH-releasing hormone (GHRH), in the late-1960s to early-1970s [9, 10] and 1980s [2, 11–14], and their receptors in 1980s to the mid-1990s [15–19], non-peptidyl GHSs in 1993 [3], GHS receptors in the mid-1990s [4, 20], ghrelin in 1999 [5], and ghrelin *O*-acyl transferase (GOAT) in 2008 [21, 22]. Characterization of the physiological control of the axis was significantly advanced by frequent blood sampling studies in the mid-1970s through the 1990s [23–25]; the pulsatile pattern of GH secretion and the factors that influenced the frequency and amplitude of the pulses have been elucidated [23–25]. Furthermore, it was firmly established that ghrelin had physiological roles in controlling GH release and appetite in the 2000s [26–30]. After the mid-2000s, it was found that heterodimerisation with GHSR1b blocks the GHSR1a signalling [31–33]. Moreover, in the 2010s, heterodimeric receptors consisting of GHSR1a and GHSR1b in combination with another G-protein-coupled receptor (GPCR) were identified [33–39]. Our essential history and current understanding of the hypothalamo-GH axis are summarised in Table 1-1 and Fig.1-1.

Table 1-1 History of the hypothalamo-GH axis research

Year	Research	Reference
1921	Discovery of the growth-promoting principle of the pituitary gland (rats were treated with extracts from bovine anterior pituitary glands and showed increased growth)	Evans & Long [7]
1944	Isolation of bovine growth hormone (GH)	Li & Evans [8]
1954	Discovery of X/A-like cell (*secretion of ghrelin in stomach)	Davis [40]
1959	Recognition of the species specificity of GH in the primate	Knobil & Greep [41]
1960	The concept that GH is regulated by the central nervous system	Reichlin [42]
1968	Discovery of a substance from hypothalamic extract that inhibits GH release	Krulich *et al.* [9]
1973	Isolation and characterization of hypothalamic GH-inhibitory substance (somatostatin [SST]-14)	Brazeau *et al.* [10]
1976	Evidence for an endogenous ultradian rhythm governing GH secretion	Tannenbaum & Martin [23]
1980	First growth hormone secretagogue (GHS)	Bowers *et al.* [43]
1982 1982 1983 1984	Discovery of growth hormone releasing hormone (GHRH)	Rivier *et al.* [11] Guillemin *et al.* [12] Spiess *et al.* [13] Ling *et al.* [14]
1984	Development of synthetic, non-natural growth hormone releasing peptide (GHRP)	Bowers *et al.* [2]
1984	Evidence for two SST-14 receptor (SSTR) types	Reubi [15]
1987	Cloning of GH receptor (GHR)	Leung *et al.* [16]
1992	Cloning of SST receptor (SSTR)	Yamada *et al.* [17]
1992 1993	Cloning of GHRH receptor (GHRHR)	Mayo [18] Gaylinn *et al.* [19]
1992 1998	SST acts to reduce GH secretion by inhibiting the response of the pituitary to GHRH	Spoudeas *et al.* [24] Wagner *et al.* [25]

1993	First nonpeptidyl GHS	Smith *et al.* [3]
1996 1997	Cloning of GHS receptor (GHSR) (*ghrelin receptor) (GHSR1a, functional receptor; GHSR1b, truncated nonfunctional receptor)	Howard *et al.* [4] McKee *et al.* [20]
1998 2001 2002	Cloning/characterization of the 5'-flanking region of the human GHSR gene (*splicing of intron in the 5'-untranslated region occurs only for GHSR1a)	Kaji *et al.* [44] Petersenn *et al.* [45] Petersenn [46]
1999	Discovery of ghrelin in stomach	Kojima *et al.* [5]
2001	Orexigenic activity of ghrelin	Nakazato *et al.* [26] Shintani *et al.* [27]
2003	Hypothalamic circuit of ghrelin cell	Cowly *et al.* [28]
2004 2005	Function of ghrelin was firmly established (*control of GH release and appetite)	van der Lely *et al*. [29] Kojima & Kangawa [30]
2004 2007 2013	Heterodimerisation with GHSR1b blocks GHSR1a signalling	Chan & Cheng [31] Leung *et al.* [32] Mary *et al.* [33]
2008	Discovery of ghrelin *O*-acyl transferase (GOAT)	Gutierrez *et al.* [21] Yang *et al*. [22]
2006 2006 2011 2012 2013	Several functional receptors consist of GHSR1a or GHSR1b in combination with other GPCRs	Jiang *et al*. [34] Takahashi *et al.* [35] Rediger *et al.* [36] Kern *et al.* [37] Park *et al.* [38] Schellekens *et al.* [39] Mary *et al.* [33]
2014	Molecular evolution of ghrelin/ghrelin receptors based on the amino acid sequences of the exons	Kaiya *et al*. [94]
2018	LEAP2** is an endogenous antagonist of the ghrelin receptor	Ge *et al*. [137]

*annotation by authors, **LEAP2, liver-expressed antimicrobial peptide 2.

Hypothalamic neuronal network of GH release and feeding regulation

The secretion of GH from the pituitary gland is regulated by a neuroendocrine system that involves both neural and feedback regulatory components (Fig.1-1). Central nervous system (CNS) control of GH release

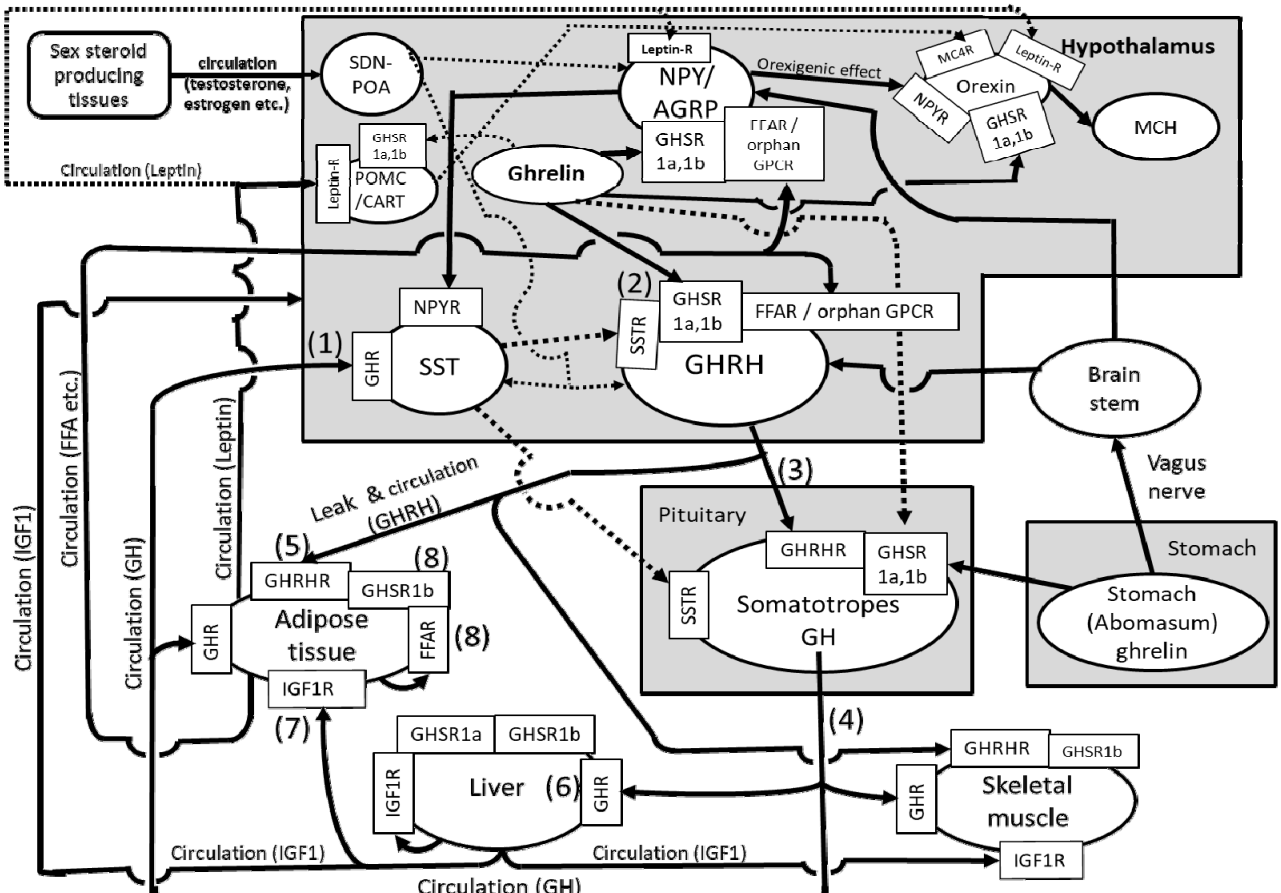

Sex steroid producing tissues
circulation (testosterone, estrogen etc.)
SDN-POA
Leptin-R
NPY/ AGRP
Orexigenic effect
MC4R
Leptin-R
Hypothalamus
Orexin
NPYR
GHSR 1a,1b
MCH
GHSR 1a,1b
POMC /CART
Leptin-R
Circulation (Leptin)
Ghrelin
FFAR / orphan GPCR
(2)
NPYR
GHSR 1a,1b
FFAR / orphan GPCR
SSTR
(1)
GHR
SST
GHRH
Brain stem
Vagus nerve
Circulation (FFA etc.)
Circulation (Leptin)
Leak & circulation (GHRH)
(3)
Pituitary
(5)
(8)
GHRHR
GHSR1b
GHSR 1a,1b
SSTR
Somatotropes GH
Stomach
Stomach (Abomasum) ghrelin
GHR
Adipose tissue
FFAR
(8)
Circulation (IGF1)
Circulation (GH)
IGF1R
(7)
(4)
GHSR1a
GHSR1b
GHRHR
GHSR1b
IGF1R
Liver
(6)
GHR
GHR
Skeletal muscle
IGF1R
Circulation (IGF1)
Circulation (IGF1)
Circulation (GH)

Fig. 1-1 (see previous page): A hypothetical model of hypothalamic neuronal network of GH release and feeding regulation in cattle by Komatsu *et al*. (2018) [49] using the interactions between ghrelin/GHSR signalling pathways and the GH neuroendocrine axis (Wagner *et al*. 2009) [50]. Stomach (ruminants: abomasum)-derived ghrelin conveys a signal via the vagus nerve to the hypothalamus (GHRH and NPY release) and via the circulation to the pituitary (GH release action). Ghrelin in the hypothalamus stimulates GHRH and NPY neurons via GHSR1a/1b receptors and also influences the somatostatin (SST) block at the pituitary (increased GH release). NPY/AGRP-containing neurons, which mediate the orexigenic action, are activated by ghrelin.

For Chapter Five: A hypothesis explains epistatic effects of fatty acid (FA) composition traits in steers as follows {Komatsu *et al*. (2018) [49]}. (1) The release of SST is activated by a GH feedback signal through the GHRs on SST neurons. (2) An interaction between the SSTR and GHSR1a attenuates the GHRH release from the GHRH neurons in the hypothalamus. (3) Lower GHRH leak into circulation is brought about by the lower GHRH release from the hypothalamus to the pituitary gland. (4) GH secretion from the pituitary gland to circulation is low. (5) In the adipose tissue, GHRH signalling (i.e. cell proliferation) is brought about by GHRH leak from the hypothalamus. (6) The variant 1A of *GHR* (*GHR1A*) mRNA is expressed in the liver. Hepatic GHR (GHR1A) expression is highly correlated with *IGF-1* mRNA expression in the liver and blood IGF1 level. (7) The IGF1 from the liver interacts with IGF1 receptor (IGF1R) in the adipose tissue. (8) The GHSR1b receptor interacts with FFARs or GHRHR by heterodimerisation on the adipose tissue [49].

Abbreviations: SDN-POA, the sexually dimorphic nuclei of the pre-optic area; NPY, neuropeptide Y; NPYR, NPY receptor; Leptin-R, leptin receptor; AGRP, agouti-related peptide; GHSR, growth hormone secretagogue receptor; GH, growth hormone; GHR, GH receptor; GHRH, GH–releasing hormone; GHRHR, GHRH receptor; POMC, proopiomelanocortin; CART, cocaine- and amphetamine-regulated transcript; MC4R, melanocortin 4 receptor; MCH, melanin-concentrating hormone; IGF1, insulin like growth factor 1; IGF1R, IGF1 receptor; FFA, free fatty acids; FFAR, FFA receptor; GPCR, G-protein-coupled receptor; SST, somatostatin; SSTR, SST receptor. Stimulating pathways are represented by full lines whereas dotted lines show inhibitory routes.

is achieved by an exquisite interplay between at least two hypothalamic hormones a stimulatory GHRH , and an inhibitory SST [50]. Their interaction generates a striking pulsatile pattern of GH release ([23, 47]). GHRH is secreted by arcuate neurons into the hypothalamic portal vessels and stimulates GH release by activating GHRH receptor on portal vessels and stimulates GH release by activating GHRH receptor on pituitary somatotrophs [50]. SST inhibits the activation of GHRH neurons through its receptor and hyperpolarizes somatotroph membranes to inhibit GH release [50]. Furthermore, a third independent pathway regulating GH release has been identified from studies of growth hormone secretagogues

(GHSs) [51–55]. GHSs are synthetic compounds that are potent stimulators of GH release, working through a GPCR, the GHS receptor (GHSR) [4, 56, 57]. Because GHSs are a group of artificial compounds, it was postulated that there must exist an endogenous ligand that binds to GHSR and performs similar function to GHSs [58–60]. Kojima *et al.* (1999) [5, 61] succeeded in the purification and identification of the endogenous ligand for the GHSR from the stomach and named it "ghrelin". Ghrelin is a GH-releasing and appetite-stimulating peptide [5, 61].

1-3 Ghrelin: interspecific variation, tissue distribution, functions, and genetic associations with QTL

According to Kojima and Kangawa (2005) [30], and Hosoda *et al*. (2006) [62], ghrelin is predominantly produced by the stomach (abomasum in ruminants), but substantially lower amounts are also derived from the bowel, pancreas, kidney, pituitary, and placenta. Ghrelin, the ligand of GHSR1a, acts to initiate and increase GH pulse amplitude by increasing GHRH level and antagonizing SST action mediated by GHSR1a and SST receptor (SSTR) interactions at the hypothalamus and the pituitary [50, 63]. The GHSR1a is the receptor engaged in ghrelin modulation of GH pulse amplitude and feeding [50, 63].

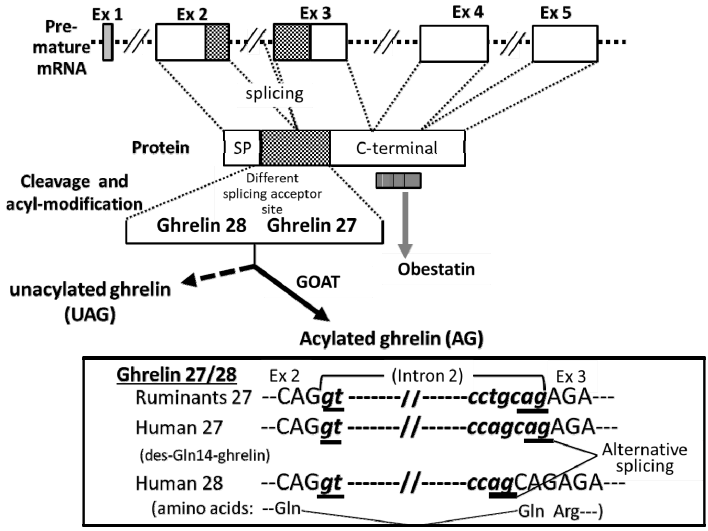
Interspecific variation

As shown in Table 1-2, human, pig, mouse, rat, dog, horse, and camel have the 28-amino acid peptide form (ghrelin 28), whereas many ruminants such as cattle, sheep, and goats have the 27-amino acid peptide form that, such as des-Gln14 ghrelin, lacks the Gln14 residue (ghrelin 27 or des-Gln14-ghrelin) [29, 30, 62]. Chicken has the 26-amino acid peptide in which the two amino acids (proline and arginine) of 3' end of ghrelin 28/27 are deleted [29, 30, 62]. Dickin *et al*. (2004) [64] have revealed that the ruminant type ghrelin 27 has a deletion within the first acceptor site within intron 2 by a transition event (adenine to thymine). Interestingly, opossums and killer whales also have ghrelin 27 (Table 1-2). The ghrelin amino acid sequences of chickens and turkeys are characteristically different from those of mammals (Table 1-2). In the "ghrelin 28" animals such as humans, pigs, and rats, two types of active ghrelin peptide are produced in the stomach, the ghrelin 28 and des-Gln14-ghrelin by alternative splicing at the end of intron 2 (Table 1-2) [30]. Des-Gln14-ghrelin is only present in low amounts in the stomach, indicating

Table 1-2 Interspecific variation of ghrelin amino acid sequence and overview of ghrelin gene structures and functionally relevant ghrelin gene derivative variants.

O=C-$(CH_2)_6$-CH_3
O n-Octanoyl (C8:0)

Animal species	★ 10	Amino-acid sequence 20	No. of amino acids* 28	Accession No.
Human	GSSFLSPEHQ	RVQQRKESKK PPAKLQPR	28*	NP_001289750.1
Mouse	----------	KA-Q---S--P AK-Q--	28	NP_067463.2
Rat	----------	KA-Q---S--P AK-Q--	28	NP_067701.1
Dog	----------	KL-Q---S--P AK-Q--	28	NP_001003052.1
Camel	--------R-	RA-Q---S--S AK-Q--	28	XP_006173390.2
Pig	----------	KV-Q---S--A AK-K--	28	NP_001009853.1
Cat	----------	KV-•---S--P AK-Q--	27	NP_998972.1
Cattle	----------	KL-•---A--S GR-K--	27	NP_776492.1
Yak	----------	KL-•---A--S GR-K--	27	AMM02735.1
Buffalo	----------	KL-•---P--S GR-K--	27	XP_006077547.1
Sheep	----------	KL-•---P--S GR-K--	27	NM_001009721.3
Goats	----------	KL-•---P--S GR-K--	27	XP_017893717.1
Killer whale	----------	KV-•---S--S AKPK--	27	XP_004274877
Horse	---------H	KV-H---S--P AK-K--	28	XP_023475741.1
Opossum	---------P	KT-•---T--S VK-Q--	27	XM_016424492.1
Chicken	--------TYK	NI-Q Q-DTR--T AR-H••	26	NP_001001131.1
Turkey	--------AYK	NI-Q Q-DTR--T AR-HPR	28	XP_003210257.1



The 27-amino acid form of the mature ghrelin peptide (ghrelin 27 or des-Gln14-ghrelin) is produced by different or alternative splicing at the end of Intron 2,

respectively [29, 30]. Identical amino acids in species are indicated as bar (-). ★, acyl-modified third amino acid (S). ●, deleted amino acids. SP, signal peptide. The black under line indicates donor-accepter sites (*gt-ag*) for splicing. Intronic sequence is shown in bold-italic lower case.

that ghrelin 28 is the major active form [30]. In ghrelin's amino acids, the serine-3 (Ser3) is *n*-octanoylated (acylated ghrelin), and this modification is essential for ghrelin's activity [29, 30]. Ghrelin is the first known case of a peptide-hormone modified by a fatty acid [29, 30]. Ghrelin octanoylation is mediated by the acyltransferase (GOAT, ghrelin *O*-acyl transferase) [21, 22]. Ghrelin 27 is formed from the deletion of Gln14 that has the same potency of activities as that of ghrelin, even retaining the *n*-octanoic acid modification [29, 30]. In addition, the highest mRNA level of the ghrelin gene is in the proventriculus in chickens [64]. There is no structural homology between ghrelin and peptide GHSs such as GHRP-6 or hexarelin [3].

Tissue distribution

According to Kojima and Kangawa (2005) [30], and Hosoda *et al*. (2006) [62], in all vertebrate species, ghrelin is mainly produced in the stomach (abomasum in ruminants), duodenum, jejunum, ileum, colon, pancreas, and many other tissues. The main molecular forms of stomach and intestinal ghrelin are acylated ghrelin (AG) and nonacylated ghrelin (des-acyl ghrelin) (UAG) [30, 62]. Ghrelin is also found in the hypothalamic arcuate nucleus (ARC), an important region for controlling feeding, and hypothalamic neurons adjacent to the third ventricle between the dorsal, ventral, paraventricular, and arcuate hypothalamic nuclei [30, 62]. The ghrelin-containing neurons send efferent fibres to neurons that contain neuropeptide Y (NPY) and agouti-related protein (AgRP) and stimulate the release of these orexigenic peptides [30, 62] (Fig.1-1). Furthermore, the pancreatic ghrelin profile changes dramatically during foetal development; the onset of pancreatic ghrelin expression precedes that of gastric ghrelin [30, 62]. Pancreatic ghrelin expression is highest in the prenatal and neonatal periods [30, 62]. In contrast, gastric ghrelin levels are low during the prenatal period and increase after birth [30, 62]. Moreover, pancreatic ghrelin levels are not affected by fasting [30, 62].

Function (1): ghrelin and GH secretion

According to Kojima and Kangawa (2005) [30], ghrelin is a multifunctional peptide hormone which stimulates GH release directly or indirectly. In addition to stimulating GH secretion, ghrelin also stimulates prolactin and ACTH secretion and exhibits orexigenic and adipogenic

effects when injected centrally or peripherally [29, 30]. According to van der Lely *et al*. (2004) [29], numerous other effects of ghrelin have been documented, including those on gut motility, insulin secretion, sleep, response to stress, learning, memory, cardiovascular performance, cell proliferation, cell differentiation, cell survival, and immunological response [29]. Secretion of ghrelin is decreased by food intake and increased by food deprivation, hypoglycaemia, and leptin administration [66]. In addition, the co-administration of ghrelin and GHRH synergistically affect GH secretion [67, 68]. Ghrelin increases GH secretion via a direct effect on pituitary somatotrophs by depolarising the cell membrane and increasing GH secretion per cell and by stimulatory actions on GHRH release at the hypothalamus with a weaker element of SST inhibition [69]. According to Hosoda *et al*. (2006) [62], the stimulatory effects of ghrelin for GH release needs the vagus nerve. Peripheral administration of ghrelin induces *c-Fos* expression in GHRH neurons in the ARC of the hypothalamus [62]. Ghrelin evokes GH secretion from the pituitary by modulation of hypothalamic GHRH via the afferent vagus nerve system [62] (Fig.1-1). Furthermore, according to Hosoda *et al*. (2006) [62], peripherally-injected peptides do not pass through the blood-brain diffusion barrier, and it is suggested that ghrelin signals from the stomach are transmitted to the brain via the vagus nerve by the detection of ghrelin receptors on vagal afferent neurons in the rat nodose ganglion [70–72]. Therefore, according to Hosoda *et al*. (2006) [62], the vagus afferent nerve is the major pathway conveying peripheral signals of ghrelin for starvation and GH secretion to the brain [62]

Function (2) Ghrelin and feeding

According to Hosoda *et al*. (2006) [62], ghrelin is the first identified circulating hormone that promotes food intake (Fig.1-1). Furthermore, according to Hosoda *et al*. (2006) [62], the ghrelin-containing neurons in the ARC send efferent fibres onto NPY- and AgRP-expressing neurons to stimulate the release of these orexigenic peptides [orexins, melanin-concentrating hormone (MCH)] and onto pro-opiomelanocortin (POMC) neurons to suppress the release of this anorexigenic peptide. In addition, according to Willesen *et al.* (1999) [73], *GHSR* mRNA is expressed in 94% of the neurons in the ARC that express NPY, in 8% of cells that express POMC, in 30% of those that express somatostatin, and in 20-25% of those that express *GHRH* mRNA in rats [73].

According to Schwartz *et al*. (2000) [74], ghrelin affects food intake by stimulating NPY/AGRP neurons in the ARC to facilitate the production and secretion of NPY and AgRP peptides. The ARC is an important target

site of leptin, an anorexia-mediating molecule produced from adipose tissue [74]. Most NPY/AgRP-containing neurons and POMC/CART-containing neurons also express leptin receptors, and both types of neurons are regulated by leptin in an opposing manner [74]. Furthermore, leptin inhibits ghrelin-induced food intake, and ghrelin considerably reverses the anorexic effect of leptin, exhibiting that ghrelin antagonize leptin function by controlling the NPY-AgRP system [74] (Fig.1-1).

According to Sakurai [2003] [75], orexin, an orexigenic hypothalamic neuropeptide, is engaged in the regulation of feeding and arousal [75]. Furthermore, isolated orexin neurons are stimulated by ghrelin and are inhibited by glucose and leptin [76]. Thus, feeding behaviour is regulated in part by cooperative activity between ghrelin and orexin [30] (Fig. 1-1**)**. In addition, Zhang *et al*. (2005) [77] reported the role of multiple alternative peptides encoded by the ghrelin gene (e.g. obestatin) (Table 1-2). According to Murray *et al*. (2015) [6], ghrelin is now recognised as a pleiotropic hormone, like the multifunctional SST.

Genetic associations with QTL in domestic animals

Association studies between the nucleotide polymorphisms of ghrelin (*GHRL*) gene and QTL have been reported in cattle, pigs, chickens, and ducks.

Sherman *et al*. (2008) [78] found that one SNP in intron 3 of the *GHRL* gene showed a minor association with the feed efficiency traits effects ($P < 0.10$) but not with the body weight of the 381 steers (Continental × British hybrid beef). Zhang *et al*. (2012) [79] found that some SNPs within the promoter region of the *GHRL* gene were associated with ischium width in Nanyang cattle aged 18 months ($P < 0.05$) but not with other growth traits, such as body weight (n = 66). Furthermore, Braz *et al*. (2015) [80] found that three SNPs in intron 3, intron 4, and exon 5 (3'UTR) of the *GHRL* gene were associated with growth traits, feed efficiency, and carcass traits ($P = 0.05–0.01$) using 231 Nellore cattle. However, Sun *et al*. (2011) [81] detected 11 SNPs in the *GHRL* gene but did not find any significant association between any variant sites and body weight, average daily gain, and body sizes for different growth periods using Nanyang cattle (n = 390), as well as for the milk yield at 305 days, milk protein rate, and milk fat percentage using Chinese Holstein cattle (n = 350).

In pigs, Kim *et al.* (2004) [82] found that the RFLP *BsrI* GHRL polymorphism of the *GHRL* gene was associated with marbling, back fat, and bone-in loin and bone-in ham muscle weights, but not with boneless muscle weight in the commercial Berkshire population (n = 49–74) ($P <$

0.06–0.10). Furthermore, Wojtysiak and Kaczor (2011) [83] also demonstrated associations of the RFLP-*BsrI* polymorphisms with carcass and meat quality traits in 168 barrows of the Polish Landrace breed.

In chickens, Fang *et al*. (2007) [84] observed that an 8 bp (CTAACCTG) indel in exon 1 of the *GHRL* gene is associated with body weight and body composition traits ($P < 0.05$ or $P < 0.01$) using 450 birds of the F2-designed resource population constructed by reciprocal crossing between White Recessive Rock (WRR) and Xinghua (X) chickens. Nei *et al*. (2009) [85] also found that three SNPs of the *GHRL* gene were significantly associated with abdominal fat weight ($P = 0.01$) or crude protein content of leg muscle ($P = 0.02$ or 0.0009) using 411 birds (219 males and 193 females). Furthermore, Jin *et al.* (2014) [86] showed that an SNP of the *GHRL* gene was significantly associated with body weight at 70 days of age, body weight gain from 49 to 70 days of age, and feed conversion ratio in the interval ($P < 0.05$) using 724 birds of two yellow meat-type populations. In ducks, Nei *et al*. (2009) [85] observed that an SNP of the *GHRL* gene was significantly associated with subcutaneous fat thickness ($P = 0.04$) using 139 birds from 9 duck populations.

Taken together, the *GHRL* gene may be a more important candidate for the identification of genetic variations that influence traits of economic importance in chickens than of those in cattle and pigs.

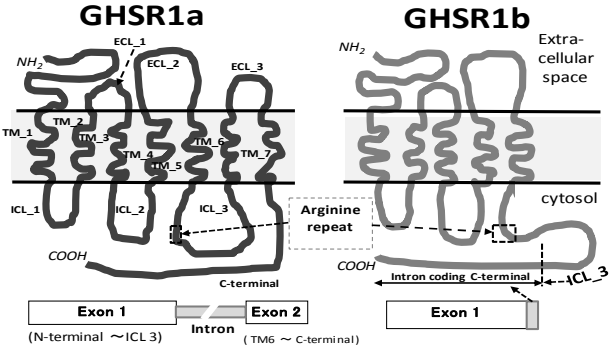
1-4 Ghrelin receptor (GHSR): amino acid sequence and gene structure, GHSR dimerisation, molecular phylogeny, arginine repeats in the third intracellular loop domain, and genomic structure and transcriptional regulation

Amino acid sequence and gene structure

According to Howard *et al*. (1996) [4], McKee *et al*. (1997) [20], and Petersenn (2002) [46], ghrelin receptor (GHSR) is a typical GPCR with seven transmembrane domains (7-TM). Two distinct GHSR cDNAs have been isolated [4, 20, 46]. The first, GHSR1a type, encodes a 7-TM GPCR with binding and functional properties consistent with its role as ghrelin's receptor [4, 20, 46]. The GHSR1a has features characteristic of a typical GPCR, including conserved cysteine residues in the first two extracellular loops (ECL_1 and ECL_2), several potential sites for posttranslational modifications (N-linked glycosylation and phosphorylation), and an aromatic triplet sequence (E/DRY) located immediately after TM-3 in the second intracellular loop (ICL_2) [4, 20, 46]. Another type, GHSR1b, is produced by a non-mRNA splicing mechanism [46]. GHSR1b is derived from only the first exon and encodes only five of the seven TM domains [4,

Table 1-3 Amino acid numbers of the GHSR1a and GHSR1b, and the arginine repeat type in various animals.

Species	Number of amino acids					Arginine repeat type
	GHSR1a	GHSR1b (predicted)	GHSR1a/1b N-terminal	GHSR1a C-terminal	Intron coding-GHSR1b (predicted)	
Cattle	366/365[1,2)]	292/291[1)]	40/40	40/40	27	4R/3R
Yak	366	292	40	40	27	4R
Baffalo	366	292	40	40	44	4R
Goats	366	292	40	40	27	4R
Sheep	366	307	40	40	42	4R
Horse	366	289	40	40	24	4R
Whale	366	289	40	40	24	4R
Pig	366[3)]	289[3)]	40	40	24	RRKR
Human	366[3)]	289[3)]	40	40	24	4R
Chimpanzy	366	289	40	40	24	4R
Mouse	364	266	39	40	3	3R
Rat	364[4)]	336[4)]	39	40	73	3R
Rabbit	366	383	40	40	118	RRKR
Cat	366	394	40	40	129	RRKR
Dog	349	330	23	40	82	RRGR
Chicken	347/331[5)]	267/267[6)]	21/21	40/40	21	RRKR
Turkey	347	277	21	40	31	RRKR
Opossum	362	265	36	40	4	RRKR
Black seabream	385[7)]	295[7)]	39	47	14	QRHR



ICL, intracellular loop; ECL, extracellular loop. [1-6)]cDNA cloning: [1)]AM931584 [105]. [2)]AB236903 [98]. [3)][4]. [4)][20]. [5)]AB095996 [109] and [6)]AB095997. [7)]AY151040 and AY151041 [65].

20, 46]. The GHSR1b receptor is thus a COOH-terminal truncated form of the GHSR1a and is pharmacologically inactive [4, 20, 46] (Table 1-3).

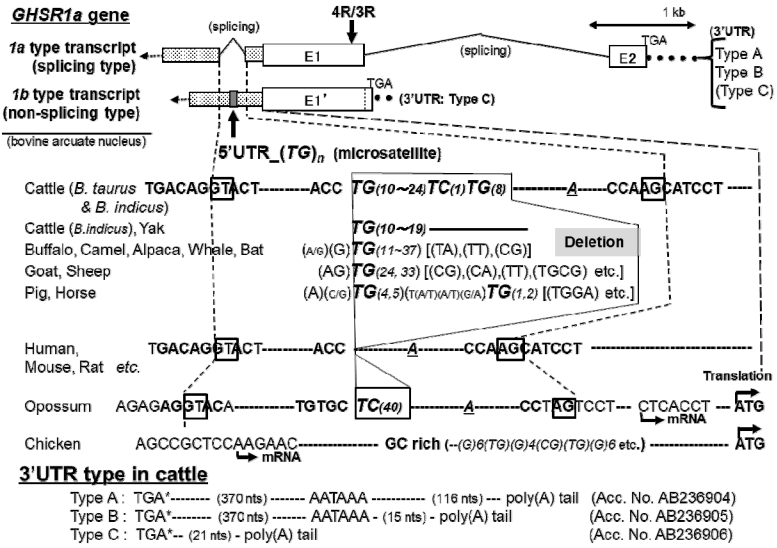


Fig. 1-2: Structure of the 5'UTR region of *GHSR1a* gene in various animals and 3'UTR type in cattle (tissue: arcuate nucleus).

The *GHSR1a* gene is composed of two exons separated by one intron [4, 20, 46]. Exon 1 is composed of a 5'-untranslated region and encodes the first 265 amino acids (human, cattle, sheep, goat, pig, etc.; chicken: 246 amino acids) from the N-terminal segment to ICL region 3 (ICL_3), including the transmembrane region (TM-1~TM-5) [4, 20, 46]. Exon 2 encodes 101 amino acids from the TM-6 to C-terminal segment and includes the 3'-untranslated region [4, 20, 46] (Table 1-3). The *GHSR1a* gene encodes two types of *GHSR* mRNA, *1a* and *1b*, by mRNA splicing or non-splicing [46] (Fig. 1-2). The *GHSR1a* mRNA is encoded by exon 1 and 2 and the intronic sequence (intron 1: ~2142 nucleotides [nts] in cattle; ~2181 nts in pigs; ~2152 nts in humans; ~2471 nts in chickens) is removed from the pre-RNA by splicing [46]. As a result, the GHSR1a is a protein of 366/365 amino acids with seven TM domains in cattle (Table 1-3). In contrast, the *GHSR1b* mRNA is encoded by the exon 1 and a part of intron 1 by non-mRNA splicing, which produces polypeptides of 292/291 amino

acids (in cattle) with only five of seven TM domains plus 27 differing amino acids (in cattle) at the C-terminal segment (Table 1-3).

The GHSR1a is well conserved across mammalian species (see review Kaiya *et al*. (2014) [94]). This strict conservation suggests that GHSR1a serve important biological functions. Therefore, as shown in Table 1-3, a high sequence identity is found in the amino-acid sequences of GHSR1a across the different mammalian species. It is interesting that the number of amino-acid sequences of N-terminal segments of GHSR1a/1b in dogs, chickens, and turkeys is smaller than those of other animals whereas the length of C-terminal segments is the same number as those of other animals (40 amino-acid sequences) (Table 1-3). In addition, the numbers of intron-coding amino acids of GHSR1b (partially predicted) in these animals are substantially different from that in other animals (Table 1-3).

Only GHSR1a is activated by ghrelin and the GHSs [46] because only the GHSR1a induces intracellular Ca^{2+} signalling that mediates the activation of a G-protein subtype, Gαq/11, by agonist treatment [4]. The GHSR1b does not induce Ca^{2+} signalling due to the lack of TM 6 and 7 [4]. The N-terminal segment of Ser3 acyl-ghrelin binds to the transmembrane cores of GHSR1a, determining a particular orientation of the molecule where the C-terminal segment of acylated-ghrelin interacts with the exoloops and/or with the N-terminal segment of the GHSR1a [89].

The *GHSR1a* mRNA is markedly expressed in the ARC and in multiple hypothalamic nuclei as well as in the pituitary [62]. Comprehensive analysis with real-time PCR of the expression of *GHSR1a* and *GHSR1b* mRNA in tissues has been accomplished in humans [90], and *GHSR1a* mRNA was mainly expressed in the pituitary gland and at much lower levels in the thyroid gland, pancreas, spleen, myocardium, and adrenal gland, whereas *GHSR1b* mRNA was found in all tissues explored [90]. In the next chapter (chapter two), we describe age-related changes and comprehensive tissue distributions of the *GHSR1a/1b* and *GHRHR* mRNA expressions in cattle.

GHSR dimerisation

Normally, GHSR1a is considered to form a functional homodimer [91]. However, a heterodimerisation with GHSR1b has been shown to occur and reduces the signalling capacity of GHSR1a [31–33], suggesting a predominantly negative role for GHSR1b in GHSR1a signalling [36]. Moreover, according to Schellekens *et al.* (2013) [39], the GHSR1a can pair with other GPCRs, e.g., melanocortin 3 receptor (MC3R), dopamine receptors (DRD1 and DRD2), serotonin 2C receptor (HTR2C), and somatostatin receptor-5 (SSTR5), or with members of the prostanoid

receptor family such as prostacyclin receptor, prostaglandin E2 receptor subtype EP3-I, and the thromboxane A2 receptor [39]. These heterodimerisations affect ligand selectivity, G-protein coupling, and the downstream signalling of each receptor [39]. Furthermore, the GHSR1b can form heterodimers with other GPCRs, e.g., the neurotensin receptor 1 [35] (see review [92]).

Molecular phylogeny of the *GHSR1a* gene

Molecular phylogenetic trees of the GHSR1a based on the amino acid sequences of the exons in mammalian and non-mammalian vertebrates have been constructed using the neighbour-joining (NJ) method with MEGA4 [93] by Kaiya *et al*., (2014) [94]. In this chapter, we have constructed a molecular phylogenetic tree of the GHSR1a based on nucleotide sequences, rather than the amino acid sequences, of the exons in 35 animal species, including domesticated ruminants (cattle, sheep, and goats, for example) and chickens using the NJ method with MEGA6 (http//www.megasoftware.net/) (Fig. 1-3; Komatsu, unpublished data). The result showed three major groups: (1) chicken, turkey, turtle, coelacanth, and frog, (2) Tasmanian devil and opossum, and (3) mammals. Furthermore, the mammalian group was subdivided into fifteen groups as follows: (1) camel/alpaca, (2) pig, (3) cattle/yak, (4) water buffalo, (5) sheep/goats, (6) whale, (7) human/R. monkey, (8) bat, (9) cat, (10) rabbit/manatee, (11) dog, (12) horse, (13) D. m. rat, (14) squirrel/jerboa, and (15) mouse/rat/G. hamster (Fig. 1-3). The result is different from those of the mammals by Kaiya *et al.* (2014) [94]. The reason for this difference may be the difference in molecular basis (amino acid sequences vs. nucleotide sequences).

It is of great interest that a polymorphic microsatellite $(TG)_n$ is located within the 5'UTR region in cattle but not in humans, mice, or rats (Table 1-4). We summarize interspecific differences in the structure of the 5'UTR microsatellite region of the *GHSR1a* gene in Table 1-4 and Fig. 1-2. The animals have been divided into four groups: {1} non-(TG or TC) repeat group (chicken, turkey, mallard, turtle, coelacanth, frog); {2} (TC) repeat group (opossum, Tasmanian devil); {3} (TG) repeat group (camel, alpaca, pig, cattle, yak, water buffalo, sheep, goat, whale); and {4} non-(TG) repeat group (human, chimpanzee, rabbit, mouse, rat, etc.) (Fig. 1-3, Table 1-4). In group {4}, the (TG) repeat region may have been deleted in the process of evolution (Fig. 1-3) (Komatsu, unpublished data). On the basis of molecular phylogenetic tree of nucleotide sequence of the exons of the *GHSR1a* gene (Fig.1-3), we have estimated a route of molecular evolution as follows: {1} → {2} → {3} → {4} (Fig. 1-3).

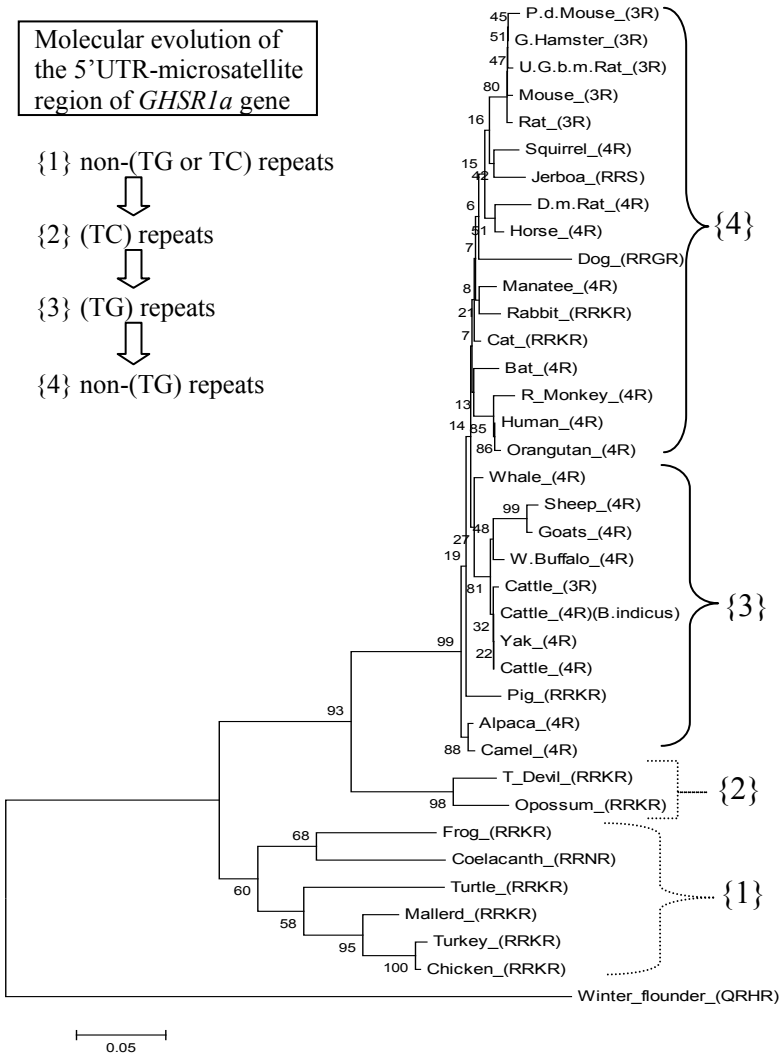


Fig. 1-3: Molecular phylogenetic tree of the *GHSR1a* gene in 35 animal species (Komatsu, unpublished data). The phylogenetic tree of nucleotide sequences of the exons was constructed using the neighbour-joining (NJ) method with MEGA6 (http//www.megasoftware.net/). 3R, 4R, RRKR, the arginine repeat type (amino acid symbols); {}, 5'UTR_microsatellite type: P.d. mouse, Prairie deer mouse; D.m. rat, Damara mole-rat (NW_011045175.1); U.G.b.m Rat, Upper Galilee mountains blind mole rat (NW_008344095.1).