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Note

**Characterization and expression of DNA sequences encoding the growth hormone gene in African
Pygmy Mouse (*Mus minutoides*)**

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Running head: GROWTH HORMONE IN MUS MINUTOIDES

Abstract

We determined the nucleotide sequence of the *growth hormone (Gh)* gene in *Mus minutoides*, one of the smallest mammals, where was predicted to be distinct in the functional regions between *M. minutoides* and *Mus musculus*. To investigate the evolutionary characteristics of *Gh* in *M. minutoides*, we constructed a phylogenetic tree based on the putative amino acid sequences of *Gh*, suggesting that the *Gh* of *M. minutoides* diverged earlier than *M. musculus*. Furthermore, the *Gh* gene expressed higher in *M. minutoides* than in *M. musculus*. Our results suggest that the specific feature of the *Gh* in *M. minutoides* is in rather the regulatory mechanism than the sequence.

Keywords: body size, evolution, growth hormone, mouse, *Mus minutoides*

Mammals have various body sizes, ranging from large whales to small rodents. Each species also has an inherent size, but it is not clear how this is determined and maintained. *Mus minutoides* (African pygmy mouse) is one of the smallest mammals in the world, measuring about 30 mm in body length and about 3 g in body weight. *M. minutoides* originally inhabit the south of Saharan in Africa and is now bred as a pet animal in many countries [5, 7] . The sexual maturity of *M. minutoides* is about 8 weeks old, the gestation period is about 20 days, and the lifespan is about 2 years [15], which is similar to that of the common laboratory mice, but the chromosomes of *M. minutoides* have unique characteristics that differ from those of *Mus musculus* (House mouse). There are individuals that differ with different numbers of chromosomes in *M. minutoides* [7]. Moreover, *M. minutodes* has a special sex-determination pattern [12]. However *M. minutoides* is a quite small mammal, and despite its obvious characteristics, few studies have focused on its size.

Many factors seem to be involved in determination of unique body size and growth rates in different animal species, and they are thought to interact in complex manner. One of the most representative factors involved in growth is growth hormone (Gh). Many studies have been conducted to identify the structure, functional and binding sites of Gh and Gh receptors, and to determine the regulatory mechanisms of growth and homeostasis [1, 4, 8, 10, 16]. In animals, Gh has been also well-studied, and the sequences of the *Gh* gene have been identified not only in rodents but also in dogs [2], cats [6], and pandas [11], and so on.

In general, dwarfism is also due to impaired function of growth hormone regulators in the anterior pituitary gland and neuroendocrine and tissue transcription factors in the anterior pituitary in mice [3, 14]. In addition, understanding the regulatory mechanisms of Gh and other pituitary hormones is particularly

important in body size determination mechanisms because many spontaneous dwarf mice may be deficient in pituitary hormones.

The purpose of this study is to elucidate the basic mechanisms that determine the size of *M. minutoides* by analyzing firstly the nucleotide sequences of *Gh*, which is involved in animal body size.

All animal experiments were approved by the Experimental Animal Care and Use Committee of Yamaguchi University (protocol number: 291). *M. minutoides* used in this study were prepared for experiments immediately after purchase from different pet stores (Increase, Inc., Himeji, Japan and Yanohashi pet Trading, Yamaguchi). The species was determined from appearance and body weight at 25 weeks old (Supplemental fig. 1). *M. musculus* (C57BL/6J) were purchased from Japan SLC (Hamamatsu, Japan) and housed in groups with *ad libitum* access to water and food.

Primers were designed based on the nucleotide sequence of the Ensembl database (Mouse (GRCm38.p6)) of the *Gh* gene, and the genomic DNA extracted from the tail (n=3) was used for sequencing. The primers used for nucleotide sequencing were designed to divide the entire gene length into three regions based on the sequence of *M. musculus*. The primer sequences are listed in Table 1.

The *Gh* gene was amplified by PCR using the genome extracted from *M. minutoides* and compared with *M. musculus*. The *Gh* gene of *M. musculus* consists of a 1595 bp sequence and contains five exons. Alignment of the nucleotide sequence of *M. musculus* showed substitutions, deletions, and insertions in all regions including exons in *M. minutoides* (Fig. 1A). The DNA sequence of the *Gh* gene of *M. minutoides* was registered to DNA Data Bank of Japan (accession number: LC5671320).

Furthermore, the sequence of *M. minutoides* of the *Gh* gene was translated into a putative amino acid sequence and compared with the amino acid sequence of *M. musculus* using BLAST. The length of the putative amino acid sequence was 216 amino acids in *M. minutoides*, and identical to that of *M. musculus* (Fig. 1B). At the DNA sequence level, the *Gh* gene in *M. minutoides* differed from those in *M. musculus*, but the homology at the putative amino acid level was very high except for two amino acids, suggesting that *M. minutoides* also may have a functional Gh. The results showed that only the 25th and 37th amino acid sequences of *M. musculus* are serine, respectively, while two sites are different in *M. minutoides*: glycine and alanine. These mutations were found in the N-terminal region responsible for signal transduction and in the region that promotes Gh protein maturation, respectively [9]. The 25th amino acid in *M. musculus* is serine, while the 25th amino acid in *M. minutoides* is glycine, suggesting that the binding ability of Gh protein to receptors may be altered (<https://www.uniprot.org/uniprot/P06880/protvista>). Serine, the 37th amino acid in *M. musculus*, is part of the site responsible for the maturation of the Gh protein, suggesting that the maturation process or post-translational modification of the Gh protein in *M. minutoides* may be different from that in *M. musculus*. *M. minutoides* purchased from the pet store was maintained in closed colonies. Since the sequences were identical in *M. minutoides* purchased from different pet stores, the sequences we found in this study are considered to be unique to *M. minutoides*.

Next, to elucidate the evolutionary characteristics of the amino acid sequence of Gh in *M. minutoides*, we constructed a phylogenetic tree of Gh in other *Mus* genus and mammals (Fig. 2) by the neighbor-joining method using the Poisson model by MEGAX with 1,000 bootstraps (The Biodesign Institute, Tampe, AZ, USA), suggesting that the Gh of *M. minutoides* diverged earlier than that of *M. musculus* but evolved within the *Mus* genus. Therefore, we considered that not only the sequence of the *Gh* but also its regulatory mechanism may be different between *M. minutoides* and *M. musculus*.

103
104 Finally, we analyzed the *Gh* gene expression using qRT-PCR in the pituitary gland of adult male *M.*
105 *minutoides* (n=3). Table 2 lists the primers used to detect *Gapdh* and *Gh*. We found that the expression of
106 the *Gh* gene was higher in *M. minutoides* than in *M. musculus* (Fig. 3). It is unlikely that the sequence of
107 the *Gh* gene is directly related to the secretion of Gh protein, but the relatively high expression of the *Gh*
108 gene in *M. minutoides* may be related to body size, such as in association with other growth-related
109 factors. In dogs, growth promotion has been correlated with the level of Igf1, a downstream factor of Gh,
110 rather than with the Gh secretory [9]. In our preliminary experiments, we have found that the expression
111 level of both the *Igf1* gene and *Growth hormone receptor (Ghr)* gene in *M. minutoides* is much lower than
112 that in *M. musculus*. Igf1 is mainly secreted from the liver, which is triggered by Gh signaling [13].
113 Although further studies on the regulation of *Gh* gene expression in *M. minutoides* are needed, it is
114 possible that *Gh* gene expression may be promoted by a decrease in *Ghr* gene expression and subsequent
115 decrease in *Igf1* gene expression and blood levels.

116
117 The present study suggests that Gh of *M. minutoides* evolved within the *Mus* genus and may have
118 different functions and signaling from *M. musculus* at the presumed amino acid sequence level.
119 Furthermore, the expression analysis of the *Gh* indicated that the regulatory mechanism of the *Gh* in
120 *M. minutoides* may differ from that in *M. musculus*. In order to further elucidate the characteristic
121 growth-related characteristics of *M. minutoides*, particularly Gh, it will be necessary to analyze
122 related factors such as growth hormone receptors and Igf1.

124 **POTENTIAL CONFLICTS OF INTEREST**

125 The authors have nothing to disclose.

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Figure legends

Fig. 1 Alignment of a nucleic acid sequence of the genomic region and a putative amino acid sequence of the *Gh* gene. (A) Alignment of a nucleic acid sequence of the genomic region of the *Gh* gene between *Mus musculus* and *Mus minutoides*. Arrows indicate primers for the nucleotide sequence and qRT-PCR. The upper row is the sequence of *M. musculus* and the lower row is that of *M. minutoides*. The region between right-angled arrows is the genomic sequence of the *Gh* gene in *M. musculus*. * indicates matching nucleic acids between *M. musculus* and *M. minutoides*. Deletions and insertions across the entire region, including exons, were observed in the genomic DNA of *M. minutoides*. The shaded indicates five exons of *M. musculus*. (B) Alignment of a putative amino acid sequence of *Gh* between *M. musculus* and *M. minutoides*. The 25th and 37th amino acid sequences of *M. musculus* are serine, respectively, while two sites are different in *M. minutoides*: glycine and alanine.

Fig. 2 Phylogenetic tree of the vertebrate *Gh*. The tree was constructed with the neighbor-joining method using MEGAX from *Mus minutoides* and 13 other vertebrate *Gh* amino acid sequences. *Gh* amino acid sequences were obtained from the Ensembl database. Numbers on nodes represent the frequency with which the node is recovered per 100 bootstrap replications in a total of 1,000.

Fig. 3 Transcript levels of *Gh* in the pituitary gland of *Mus minutoides* and *Mus musculus*. Left: The relative expression level for *Gh* was normalized against the *Gapdh* expression level. The expression of the *Gh* gene in *M. minutoides* was higher than those in *M. musculus*. Data represent the mean $\pm$ standard deviation. Right: RT-PCR analysis was performed for confirmation of the specificity of the primer set.

Supplemental fig. 1 Appearance of *Mus minutoides* at 25 weeks old. The body weight is 4.81 g. Bar: 1 cm.

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Figure 1 Matsuya *et al.*

A

	F1
Mus_musculus	TGTCAGTGGCCCCAGCCATGAATAAATGTATAGGGGAAAGGCAGGAGCCTTGGGGTTCGAGGAAACAGGTAGGGTATAAAAGGGCACGAAGGGACCAAGTCAGCATCCTAGAGTCC
Mus_minutoides	TGTCAGTGGCCCCAGCCATGAATAAATGTATAGGGGAAAGGCAGGAGCCTTGGGGTTCGAGGAAACAGGTAGGGTATAAAAGGGCACACGAAGGACCAAGTCAGCATCCTAGAGTCC

Mus_musculus	AGATTCCAAACTGCTCAGAGTCTGTGGACAGATCACTGCTTGGCAATGGCTACAGGTAAGCATGCGAAATCCCCTGGGTGTGGTTTGGGACCCAGGGCCCCGAAGATGGATCTGAG
Mus_minutoides	AGATTCCAAACTGCTCAGGGTCTGTGGACAGATCGCTGCGTGGCAATGGCTACAGGTAAGCATGG-----GTGTGGTTTGGGACCAAGGGGCCCTGAAGATGGAGCTGAG

Mus_musculus	GCTTCTAATGTGAGTGCCTTCCAACCTTCTGCCATGTTGGGAATACTCTGGGTCCCTATGGGATTGGGAGAGATCGGCCATTGCTCCAGGTTTCTCTGCCCTCCTG-----TCTCTCTC
Mus_minutoides	GCTTCTAATGTGAGTGTGTCCCAACTTCCACCATGTCCGGAACACTCTGGGTCCCAAGGGGATTGGGAGAGATTGGTCATTGCTCCCTGGTTTCTCTGCCCTCCTGTCTCTCTCTC

Mus_musculus	TAGACTCTCGGACCTCCTGGCTCTGACCGTCAGCTGCTCTGCTGCTCTGGCTCAGGAGGCTAGTGCTTTTCCGCCATGCCCTTGTCCAGTCTGTTTCTAATGCTGTCTCCGAG
Mus_minutoides	TAGACTCTCGGACCTCCTGGCTCTGACCGTCAGCTGCTCTGCTGCTCTGGCTCAGGAGGCTGGTGCTTTCCCGCCATGCCCTTGTCCAGTCTGTTTGGCAATGCTGTCTCCGAG

	F2
Mus_musculus	CCCAGCACCTGCACCAAGCTGGCTGTGACACCTACAAGAGTTTCGTAAGTTCCCCAG-AGATGGGTGCCCGTTTGGGAAGCAGGAAGGGGACAGGTCTACCCCATACTCTGCCCCAG
Mus_minutoides	CCCAGCACCTGCACCAAGCTGGCTGTGACACCTACAAGAGTTTGTAAAGTTCCCCAGAGATGGGTGCCCGTCTGGGAAGCAGGAAGGGGACAGGTCCACCCCATACTCTGCCCCAA

	R1
Mus_musculus	GAAGTCATTGAGGAAACTATGGGGTAGGGGAATCTTAGCCAATGCTGTACCATAGTAATGATGGTGACGAGACACAAGCTGGTCCCTCAGTGACCACCTTCTTCCAGGAGCGTGCCTA
Mus_minutoides	GAAGTCAAGAGGGAAACTATGGGGTAGGGGAATCTTAGCCAATGCTGTACCATAGTAATGATGGTGAGGAGCACAAGCTGGTACCTCAGTGACCACCTCTCTCCAGGAGCGTGCCTA

	F (for qRT-PCR)
Mus_musculus	CATTCCCGAGGGACAGCGCTATTCCATTCAAGATGCCAGGCTGCTTCTGCTTCTCAGAGACCATCCCGGCCCCACAGGCAAGGAGGAGGCCAGCAGAGAACCCTGAGTAGTCCAG
Mus_minutoides	CATTCCCGAGGGACAGCGCTATTCCATTCAAGATGCCAGGCTGCTTCTGCTTCTCAGAGACCATCCAGGCCCCACAGGCAAGGAGGAGGCCAGCAGAGAACCCTGAGTAGTCCAG

Mus_musculus	GCCTTGTCTGCACAAATCTCGTTTCCCTCCATGCAGCCCTAACTGCACCTCAGGCCAGGGACCAAGCTCCTCCTGAAAGCTGGGGTAACCTGGGAGTCCAGGCAGAGGTCACTAGGCAA
Mus_minutoides	GCCTTGTCTGCACAAATCTCTTTTCCCTCCACGACAGCCCTAACTGCACCTCAGGCCAGGGACCAAGCTCCTCCTGAAAGCTGGGGTAACCTGGGAGTCCAGGCAGAGGTAACTAGGCAA

	F3
Mus_musculus	TACACTAACCCAGCCCTTTTTCCTCCCTCAGGACATGGAATTGCTTCGCTTCTCGTGTCTCATCCAGTCATGGCTGGGGCCCGTCAGTTCTCAGCAGGATTTTACCAACAG
Mus_minutoides	TACACTGCACCCAGCCCTTTT-CCCCCTCAGGACATGGAATTGCTTCGCTTCTCTGCTGTCTATCCAGTCGTGGCTGGGGCCCGTCAGTTTCTCAGCAGGATTTTACCAACAG

	R2
Mus_musculus	CCTGATGTTTCGGACCTCGGACCGTGTCTATGAGAACTGAAGGACCTGGAAGAGGGCATCCAGGCTCTGATGCAGGTGAGGATGGACTAG-----CCTGGGGTTATGCTCGGAGC
Mus_minutoides	CCTGATGTTTGGCACCTCAGACCGTGTCTATGAGAACTGAAGGACCTGGAAGAGGGCATCCAGGCTCTGATGCAGGTGAGGATGGAGTGGACCCGCGGTCCCTAACTGGGGTTATACCTGGAGC

Mus_musculus	CTAGGTGGGG-CTCACTGCTCTGTGT--TTACCGGTGAGCCCTTAGACCTTGAGAAGGCTTCTTCTTCTCATTTTCTTTATGAAGCTCCAGGCTTTTCTTGGTCTGGGGTGG
Mus_minutoides	CTAGGTGGATGCTTACTGCTCTTCTGTTTATCGTCAAGCCCTTAGACCTTGAGAAG-----GCTTCTTCTTCTCCTTATGAAGCTCCAGGCTTTTCTTGGTCTGGGGTGG

	R (for qRT-PCR)
Mus_musculus	AGGGAGGCACAGCTCCCGAGTCTCTGCCCTTCTTTCCACGACAGAGCTGGAAGATGGCAGCCCCCGTGTGGGCAGATCCTCAAGCAAACCTATGACAAGTTTGACGCCAACATGCG
Mus_minutoides	AGGGAGGCACAGCTCCCGAGTCTCCAGCCCTTCTTTCCATGACAGGAGCTGGAAGACGGCAGCCCCCGTGTGGGCAGATCCTCAAGCAAACCTACGACAAGTTTGATGCCAACATGCG

Mus_musculus	CAGCGACGACGCGTCTCAAAACTATGGGCTGCTCTCTGCTTCAAGAAGGACCTGCACAAAGCGGAGACCTACCTGCGGGTCATGAAGTGTCCGCGCTTTGTGGAAGCAGCTGTGC
Mus_minutoides	CAGCGACGACGCGTCTCAAAACTATGGGCTGCTCTCTGCTTCAAGAAGGACCTGCACAAAGCAGAGACCTATCTGCGGGTCATGAAGTGTCCGCGCTTGTGGAAGCAGCTGTGC

	R3
Mus_musculus	TCTTTCTGTTATGGGATGGAGCAGTTGTGTCA
Mus_minutoides	-----

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B

Mus_musculus	MATDSRTSWLLTVSLLCLLWPQEASAFPAMPLSSLFSNAVLRAQHLHQLAADTYKEFERA
Mus_minutoides	MATDSRTSWLLTVSLLCLLWPQEAGAFPAMPLSSLFANAVLRAQHLHQLAADTYKEFERA

Mus_musculus	YIPEGQRYSIQNAQA AFCFSETIPAPTGKEEAQQRDMELLRFSLLLIQSWLGPVQFLSR
Mus_minutoides	YIPEGQRYSIQNAQA AFCFSETIPAPTGKEEAQQRDMELLRFSLLLIQSWLGPVQFLSR

Mus_musculus	IFTNSLMFGTSDRVYEKLDLEEGIQALMQELEDGSPRVGQILKQTYDKFDANMRSDDAL
Mus_minutoides	IFTNSLMFGTSDRVYEKLDLEEGIQALMQELEDGSPRVGQILKQTYDKFDANMRSDDAL

Mus_musculus	LKNYGLLS CFKKDLHKAETYLRVMKCRRFVESSCAF
Mus_minutoides	LKNYGLLS CFKKDLHKAETYLRVMKCRRFVESSCAF

Fig. 1. Alignment of a nucleic acid sequence of the genomic region and a putative amino acid sequence of the *Gh* gene. (A) Alignment of a nucleic acid sequence of the genomic region of the *Gh* gene between *Mus musculus* and *Mus minutoides*. Arrows indicate primers for the nucleotide sequence and qRT-PCR. The upper row is the sequence of *M. musculus* and the lower row is that of *M. minutoides*. The region between right-angled arrows is the genomic sequence of the *Gh* gene in *M. musculus*. * indicates matching nucleic acids between *M. musculus* and *M. minutoides*. Deletions and insertions across the entire region, including exons, were observed in the genomic DNA of *M. minutoides*. The shaded indicates five exons of *M. musculus*. (B) Alignment of a putative amino acid sequence of *Gh* between *M. musculus* and *M. minutoides*. The 25th and 37th amino acid sequences of *M. musculus* are serine, respectively, while two sites are different in *M. minutoides*: glycine and alanine.

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Fig. 2. Phylogenetic tree of the vertebrate Gh. The tree was constructed with the neighbor-joining method using MEGAX from *Mus minutoides* and 13 other vertebrate Gh amino acid sequences. Gh amino acid sequences were obtained from the Ensembl database. Numbers on nodes represent the frequency with which the node is recovered per 100 bootstrap replications in a total of 1,000.

Figure 3 Matsuya *et al.*

Fig. 3. Transcript levels of *Gh* in the pituitary gland of *Mus minutoides* and *Mus musculus*. Left: The relative expression level for *Gh* was normalized against the *GAPDH* expression level. The expression of the *Gh* gene in *M. minutoides* was higher than those in *M. musculus*. Data represent the mean + standard deviation. Right: RT-PCR analysis was performed for confirmation of the specificity of the primer set.

Supplemental figure 1 Matsuya *et al.*

Supplemental fig. 1. Appearance of *Mus minutoides* at 25 weeks old. The body weight is 4.81 g. Bar: 1 cm.

Table S1 Primer sequences for the genome sequence of Gh

	Primer sequences (5'→3')
F1	TGTCAGTGGCCCCAGCCATG
R1	CCAGCTTGTGTCTCGTCACCATC
F2	GAGTTCGTAAGTTCCCCAGAGATGG
R2	CTGGATGCCCTCTTCCAGGTCC
F3	GCTCATCCAGTCATGGCTGGG
R3	TGACACAACCTGCTCCATCCCATAAC

Table S2 Primer sequences for RT-PCR

genes		Primer sequences (5'→3')
<i>Gh</i>	F	CGAGGGACAGCGCTATTCCATTC
	R	GCTTGAGGATCTGCCCAACACG
<i>Gapdh</i>	F	GTGCTGAGTATGTCGTGGAGTC
	R	CATACTTGGCAGGTTTCTCCAG