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Complete mitochondrial genome of the Amur hedgehog *Erinaceus amurensis* (Erinaceidae) and higher phylogeny of the family Erinaceidae

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ABSTRACT. We sequenced and characterized the complete mitogenome (KX964606) of the Amur hedgehog *Erinaceus amurensis* to provide more data for comparative mitogenomics of the genus *Erinaceus* (Erinaceidae). The mitogenome of *E. amurensis* is a circular molecule 16,941 bp long, consisting of a control region and a conserved set of 37 genes containing 13 protein-coding genes, 22 tRNA genes, and two rRNA genes (12S rRNA and 16S rRNA). The mitogenome of *E. amurensis* is AT-biased, with a nucleotide composition of 33.9% A, 21.1% C, 32.6% T, and 12.4% G. The mitogenomes of *E. amurensis* and the closely related hedgehog species *E. europaeus*, excluding the control region (66.7%), share over 90% sequence similarity. According to the inter-generic relationship based on six mitogenomes described from five genera of Erinaceidae, the subfamilies Erinaceinae and Galericinae are strongly supported as monophyletic groups, with each genus well placed within its own subfamily. Within the subfamily Erinaceinae, *E. amurensis* is a sister species to *E. europaeus*, and the relationship

between *Hemiechinus* and *Erinaceus* is strongly supported. Within the subfamily Galericinae, the clade of *Hylomys* + *Neotetracus* was sister to that of *Echinosorex*, with clades supported by high values. Our findings will help to understand the codon usage pattern and molecular evolution of *E. amurensis*, and provide insight into inter-generic relationships within the family Erinaceidae. In future studies, the inclusion of mitogenomes from other genera would greatly enhance our understanding of higher phylogeny within the Erinaceidae.

Key words: Amur hedgehog; Mitogenome; *Erinaceus amurensis*; Erinaceinae; Galericinae

INTRODUCTION

The family Erinaceidae consists of two subfamilies, which contain the well-known hedgehogs (Erinaceinae) and the gymnures or moonrats (Galericinae). The hedgehogs of the subfamily Erinaceinae are distributed in Eurasia and Africa, whereas gymnures or moonrats of the subfamily Galericinae are found in South-east Asia (Hutterer, 2005; Vaughan et al., 2011). There are 10 described genera and 24 described species of erinaceid (Corbet, 1988; Hutterer, 2005; Vaughan et al., 2011). Hedgehogs of *Erinaceus*, a genus of the subfamily Erinaceinae, include four main species of *E. amurensis* (Amur hedgehog), *E. concolor* (Southern white-breasted hedgehog), *E. europaeus* (European hedgehog), and *E. roumanicus* (Northern white-breasted hedgehog) (Corbet, 1988; Hutterer, 2005). The Amur hedgehog, *E. amurensis,* is similar in appearance and lifestyle to *E. europaeus*, although it is more lightly colored. This species is distributed in the Amur basin and Primorye in Russia, Sichuan and Manchuria in China, and the Korean peninsula (Hutterer, 2005; Tsytsulina, 2008). The Amur hedgehog, *E. amurensis,* usually inhabits the borders between forest and open spaces of valleys and lowlands with mixed coniferous and broadleaf forests (Hutterer, 2005; Tsytsulina, 2008). In Korea, *E. amurensis* lives in wide forests of low altitudes such as cultivated regions, forests, grassland, and scrub. They feed on earthworms and other ground invertebrates as well as the occasional small vertebrate and fruit (Tsytsulina, 2008).

Genome-level analyses, such as the mitogenome, provide useful tools that can be used to infer higher-level phylogeny and to investigate molecular evolution (Boore, 1999). In GenBank, the mitogenomes from only five species of 10 genera within the family Erinaceidae have been completely sequenced and analyzed to date (https://www.ncbi.nlm.nih.gov/genbank/). In this study, we sequenced and characterized the complete mitogenome of the Amur hedgehog *E. amurensis* to provide more data for comparative mitogenomics between various species of hedgehogs. The phylogeny of the family Erinaceidae based on morphological characters is unresolved (He et al., 2012). However, in a recent study, the phylogeny of the family Erinaceidae was well resolved using 3218-bp mitochondrial sequences of 12S rRNA, cytochrome b (*Cyt b*), and NADH dehydrogenase subunit 2 (*Nd2*), including 135 morphological characters (He et al., 2012). In the present study, based on 13 mitochondrial protein-coding genes (PCGs) from the complete mitogenomes of six species of the two subfamilies, we inferred the inter-generic relationship within the family Erinaceidae.

MATERIAL AND METHODS

Sample collection and DNA extraction

During a roadkill survey of wildlife in 2015, an *E. amurensis* individual was collected as roadkill in Gongwon province of South Korea on 26 April 2015. The dead individual was treated according to the standard guideline for road-killed wildlife of the Ministry of Environment, Republic of Korea. A skin tissue was preserved in absolute alcohol and stored at -20°C, and was then used for DNA extraction in May 2016. Total genomic DNA was extracted using a DNeasy® Blood & Tissue Kit (Qiagen, Valencia, CA, USA) following the manufacturer protocol.

Polymerase chain reaction (PCR) and sequencing

For PCR amplification, 15 primer pairs were designed based on sequence alignments of *E. europaeus* (accession No. NC_002080.2) and the *Hemiechinus auritus* (AB099481.1) mitogenomes downloaded from GenBank. The primers were designed within overlapping sequences at both ends of every adjacent fragment with around 0.5-1.5 kb in length (**Table S1**). PCR amplification was performed in a final 25-μL reaction volume containing 10 mM Tris-HCl, pH 8.4, 50 mM KCl, 4 mM $MgCl_2$, 200 mM each dNTP, 50 pmol each primer, 2 U ExTaq polymerase, and 1 μL genomic DNA. The PCR was conducted under the following reaction conditions: an initial denaturation for 5 min at 94°C, followed by 35 cycles of denaturation for 1 min at 94°C, primer annealing for 30 s at 48°-56°C and extension for 1 min at 72°C, and a final extension of 10 min at 72°C. We collected the 15 overlapping PCR products encompassing the whole mitogenome of *E. amurensis*. The PCR products were run on a 1.0% agarose gel by electrophoresis and purified using a DNA Gel Extraction Kit (Qiagen, Valencia. CA, USA). The purified PCR products were sent to Biomedic Co., Ltd. (Bucheon, South Korea) and sequenced using an ABI Prism® Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and a Gene Amp PCR system 9700 (Applied Biosystems). Sequences were determined by automated sequencing on a 3730 DNA Sequencer (Applied Biosystems) on 8% polyacrylamide gels. The 15 DNA fragments were assembled by connecting the adjacent fragments along the overlapping regions of both ends of the DNA fragments.

Genome annotation

The complete mitogenome of *E. amurensis* was aligned with that of *E. europaeus* using the Clustal-W program found in Geneious Pro 5.5.9 (Biomatters, Auckland, New Zealand), and then the *E. amurensis* mitogenome was annotated based on gene organization information with the latter mitogenome as reference. Sequences of 13 mitochondrial PCGs were translated into amino acid sequences using the genetic code of the vertebrate mitochondrial genome. ARWEN web server was used to identify anticodons in each tRNA (Bernt et al., 2013). The formulas used to calculate the skewness of the nucleotide composition were as follows: AT skew = [A - T] / [A + T] and GC skew = [G - C] / [G + C] (Lobry, 1996).

Phylogenetic analysis

Phylogenetic relationships were inferred based on 13 mitochondrial PCGs using maximum-likelihood (ML) and neighbor-joining (NJ) analyses implemented in MEGA

7.0.14 (Kumar et al., 2016). The GTR model with the gamma shape parameter and the estimated fraction of invariant sites (GTR + G + I) was selected by MEGA 7.0.14 as the best model of evolution that fits our data. The confidence of branches in ML trees was assessed using bootstrapping searches of 1000 replicates. A NJ tree was generated using Kimura's 2-parameter model (Kimura, 1980), with bootstrapping searches of 1000 replicates. Three species, including *Didelphis virginiana* (NC_001610.1), *Apodemus peninsulae* (JN546584.1), and *Eothenomys chinensis* (NC_013571.1) from GenBank were used as the outgroup.

RESULTS AND DISCUSSION

Gene organization

The complete mitogenome (KX964606) of *E. amurensis* contains 16,941 bp, which is similar in size to that of *E. europaeus* in the genus *Erinaceus* (Krettek et al., 1995). It consists of a control region and a conserved set of 37 vertebrate mitochondrial genes including 13 PCGs, 22 tRNA genes, and two rRNA genes (*12S rRNA* and *16S rRNA*) (Table 1).

Table 1. Gene organization of the *Erinaceus amurensis* mitogenome.

Gene	Start position	Stop position	Length (bp)	Anticodon	Start codon	Stop codon	Strand
tRNA^{Phe}	1	66	66	GAA			+
12S rRNA	67	1012	946				+
tRNA^{Val}	1013	1080	68	TAC			+
16S rRNA	1081	2647	1567				+
tRNA$^{Leu(UUR)}$	2648	2722	75	TAA			+
Nd1	2724	3677	954		ATA	ATT	+
tRNA^{Ile}	3678	3746	69	GAT			+
tRNA^{Gln}	3744	3816	73	TTG			-
tRNA^{Met}	3816	3884	69	CAT			+
Nd2	3888	4931	1044		ATG	TAA	+
tRNA^{Trp}	4933	4999	67	TCA			+
tRNA^{Ala}	5003	5072	70	TGC			-
tRNA^{Asn}	5073	5145	73	GTT			-
O$_R$	5147	5182	36				+
tRNA^{Cys}	5183	5251	69	GCA			-
tRNA^{Tyr}	5253	5322	70	GTA			-
Cox1	5324	6868	1545		ATG	TAA	+
tRNA$^{Ser(UCN)}$	6866	6934	69	TGA			-
tRNA^{Asp}	6942	7011	70	GTC			+
Cox2	7013	7696	684		ATG	TAA	+
tRNA^{Lys}	7698	7762	65	TTT			+
Atp8	7765	7971	207		ATG	TAG	+
Atp6	7926	8606	681		ATG	TAA	+
Cox3	8606	9389	784		ATG	T--	+
tRNA^{Gly}	9390	9462	73	TCC			+
Nd3	9463	9809	347		ATA	TA-	+
tRNA^{Arg}	9810	9878	69	TCG			+
Nd4L	9879	10175	297		ATG	TAA	+
Nd4	10169	11546	1378		ATG	T--	+
tRNA^{His}	11547	11612	66	GTG			+
tRNA$^{Ser(AGY)}$	11613	11672	60	GCT			+
tRNA$^{Leu(CUN)}$	11672	11742	71	TAG			+
Nd5	11747	13555	1809		ATG	TAA	+
Nd6	13552	14085	534		ATG	AGA	-
tRNA^{Glu}	14086	14152	67	TGT			-
Cyt B	14156	15295	1140	TGG			+
tRNA^{Thr}	15296	15365	70		ATG	TAA	+
tRNA^{Pro}	15367	15432	66	TTC			-
Control region	15433	16941	1509				+

The order and orientation of the *E. amurensis* mitogenome are identical to that of *E. europaeus* (Krettek et al., 1995). *Nd6* and eight *tRNAs* are found on the light strand, while the other 12 PCGs, 14 *tRNAs*, and two *rRNAs* are located on the heavy strand. The control region is located between $tRNA^{Pro}$ and $tRNA^{Phe}$, as seen in the *E. europaeus* mitogenome (Krettek et al., 1995).

Nucleotide composition

The mitogenome of *E. amurensis* is AT-biased, with a nucleotide composition of 33.9% A, 21.1% C, 12.4% G, and 32.6% T. The 13 mitochondrial PCGs consist of 32.8% A, 33.9% T, 22.3% C, and 11.1% G. The 13 PCGs are AT-biased, with a total AT content of 66.7%, ranging from 62.4% in *Cox1* to 71.5% in *Atp8* (Table 2). The AT skew was positive in five PCGs (*Nd2*, *Atp8*, *Nd3*, *Nd5*, and *Nd6*), while the GC skew was negative in all 13 PCGs (Table 2). In the 13 PCGs, the asymmetrical base composition may be due to codon usage bias (Foster et al., 1997; Singer and Hickey, 2000).

Table 2. Nucleotide composition of 13 mitochondrial protein-coding genes of *Erinaceus amurensis*.

Gene	Length (bp)	Proportion of nucleotides (%)					AT skew	GC skew
		A	T	C	G	AT content		
Nd1	954	32.6	33.3	23.4	10.7	65.9	-0.01	-0.37
Nd2	1,044	36.5	33.9	21.4	8.2	70.4	0.04	-0.45
Cox1	1,545	27.7	34.7	21.9	15.7	62.4	-0.11	-0.16
Cox2	684	30.6	33.3	23.0	13.2	63.9	-0.04	-0.27
Atp8	207	39.6	31.9	21.3	7.2	71.5	0.11	-0.49
Atp6	681	31.6	34.1	23.3	11.0	65.7	-0.04	-0.36
Cox3	784	28.1	34.8	23.3	13.8	62.9	-0.11	-0.26
Nd3	347	34.6	34.0	20.7	10.7	68.6	0.01	-0.32
Nd4L	297	32.3	37.4	19.2	11.1	69.7	-0.07	-0.27
Nd4	1,378	34.2	34.8	22.0	9.1	69.0	-0.01	-0.41
Nd5	1,809	34.5	34.4	21.0	10.1	68.9	0.00	-0.35
Nd6	534	45.1	24.0	24.3	6.6	69.1	0.31	-0.57
Cytb	1,140	29.6	35.3	23.6	11.6	64.9	-0.09	-0.34
Total	11,404	32.8	33.9	22.3	11.1	66.7	-0.02	-0.34

Protein-coding genes and codon usage

The 13 mitochondrial PCGs consisted of 11,370 bp, with the exception of stop codons (34 bp), which encode 3790 amino acids (Table 3). Some mammalian mitogenomes contain compact overlapping regions between PCGs (Fernández-Silva et al., 2003). The mitogenome of *E. amurensis* also contains some overlapping regions between PCGs or between PCGs and tRNAs (Table 1). We found a 46-bp overlap between *Atp8* and *Atp6*, a 7-bp overlap between *Nd4L* and *Nd4*, a 4-bp overlap between *Nd5* and *Nd6*, a 1-bp overlap between *Atp6* and *Cox3*, and a 3-bp overlap between *Cox1* and $tRNA^{Ser(UCN)}$. The 13 mitochondrial PCGs of *E. amurensis* use two start codons (ATA for *Nd1* and *Nd3* and ATG for the other 11 PCGs), two incomplete stop codons (TA- for *Nd3* and T-- for *Cox3* and *Nd4*), and four stop codons (ATT for *Nd1*, TAG for *Atp8*, AGA for *Nd6*, and TAA for the other seven PCGs) for translation initiation and termination (Table 1). The most abundant start and stop codons were ATG and TAA, respectively. The frequent pattern of ATG and TAA usage as start and stop codons has also been observed in other mammalian mitogenomes (Kim and Park, 2015; Nam et al., 2015; Yoon and Park, 2015). Incomplete stop codons (T-or T--), which are used in three PCGs (*Nd3*,

Cox3, and *Nd4*), may be completed by poly-adenylation of the 3'-end of the mRNA after transcription (Boore, 1999). Incomplete stop codons or overlaps between PCGs may result from selection pressure to reduce mitogenome size (Rand, 1993).

Table 3. Sequence similarity between the mitogenomes of *Erinaceus amurensis* and *Erinaceus europaeus*.

Gene region	Gene length		Sequence similarity (%)	Indels (bp)
	E. amurensis	*E. europaeus*		
Protein coding genes (PCGs)	11,404	11,405	90.1	3
13 PCGs, except for stop codons	11,370	11,373	89.9	9
Amino acids	3,790	3,791	92.1	3
12S rRNA and 16S rRNA	2,513	2,534	90.3	81
22 tRNAs	1,515	1,517	95.3	6
Control region	1,509	1,988	66.7	3
Total size except for the control region	15,432	15,459	90.6	90
Total	16,941	17,447	86.0	93

Non-coding regions

The two rRNA genes (*12S rRNA* and *16S rRNA*) are located between $tRNA^{Phe}$ and $tRNA^{Leu(UUR)}$, and are separated by $tRNA^{Val}$ (Table 1). The combined size of the two genes is 2513 bp (Table 3). The combined size of 22 tRNA genes is 1515 bp (Table 3), ranging in size from 60 bp [$tRNA^{Ser(AGY)}$] to 75 bp [$tRNA^{Leu(UUR)}$] (Table 1). The tRNA genes include two leucine-tRNA genes [$tRNA^{Leu(UUR)}$ and $tRNA^{Leu(CUN)}$] and two serine-tRNA genes [$tRNA^{Ser(UCN)}$and $tRNA^{Ser(AGY)}$] (Table 1).

Some non-coding regions such as the origin of replication (O_R), intergenic spacers, and the control region are important during replication and maintenance (Fernández-Silva et al., 2003). The non-coding regions were also found in the mitogenome of *E. amurensis* (Table 1). The mitochondrial O_R of *E. amurensis* is located between $tRNA^{Asn}$ and $tRNA^{Cys}$ in the WANCY region, which consists of a cluster of five tRNA genes ($tRNA^{Trp}$, $tRNA^{Ala}$, $tRNA^{Asn}$, $tRNA^{Cys}$, and $tRNA^{Tyr}$), as in other mammals (Kim and Park, 2015; Nam et al., 2015; Yoon and Park, 2015). Intergenic spacers were found in 13 regions of the mitogenome, ranging from seven single-base pair spacers (between $tRNA^{Leu(UUR)}$ and *Nd1*, *Nd2* and $tRNA^{Trp}$, $tRNA^{Cys}$ and $tRNA^{Tyr}$, $tRNA^{Tyr}$ and *Cox1*, $tRNA^{Asp}$ and *Cox2, Cox2* and $tRNA^{Lys}$, and $tRNA^{Tyr}$ and $tRNA^{Pro}$) to a 7-bp spacer (between $tRNA^{Ser(UCN)}$ and $tRNA^{Asp}$) (Table 1). The control region of the *E. amurensis* mitogenome is 1509-bp long and is located between $tRNA^{Pro}$ and $tRNA^{Phe}$ (Table 1).

Sequence comparison between the mitogenomes of *E. amurensis* and *E. europaeus*

The mitogenome (16,941 bp) of *E. amurensis* is 506-bp shorter than that of *E. europaeus* (17,447 bp; NC_002080), which also belongs to the genus *Erinaceus* (Table 3). The difference in length between the two mitogenomes is due to a much shorter control region (479 bp) in *E. amurensis* than in *E. europaeus*. The mitogenome of *E. europaeus* has a similar nucleotide composition to that of *E. amurensis*. The mitogenomes of the two species, with the exclusion of the control region (66.7%), share over 90% sequence similarity, ranging from 90.1% in 13 PCGs to 95.3% in 22 tRNAs.

Phylogenetic relationship

In the family Erinaceidae, the sequences of only five mitogenomes have been deposited

in GenBank to date: *E. europaeus* (NC_002080.2) and *Hemiechinus auritus* (AB099481.1) from the subfamily Erinaceinae (spiny hedgehogs), and *Echinosorex gymnura* (AF348079.1), *Hylomys suillus* (NC_010298.1), and *Neotetracus sinensis* (JX519466.1) from Galericinae (moonrats). The higher phylogenetic relationship of the family Erinaceidae was inferred based on 13 PCGs of the six mitogenomes, with inclusion of the *E. amurensis* mitogenome from this study (Figure 1). The phylogenetic relationships of the family Erinaceidae are consistent with that reported in a previous study (He et al., 2012) based on three mitochondrial genes (*12S rRNA*, *Cyt B*, and *Nd2*) and 135 morphological characters. As shown in the study by He et al. (2012), the two subfamilies are also strongly supported as monophyletic groups, with genera of each subfamily well placed within their own subfamily. Within the subfamily Erinaceinae, *E. amurensis* is a sister to *E. europaeus* and *Hemiechinus* is a sister to the clade of the two *Erinaceus* species. Within the subfamily Galericinae, *Echinosorex* is a sister to the clade *Hylomys* + *Neotetracus* (Figure 1).

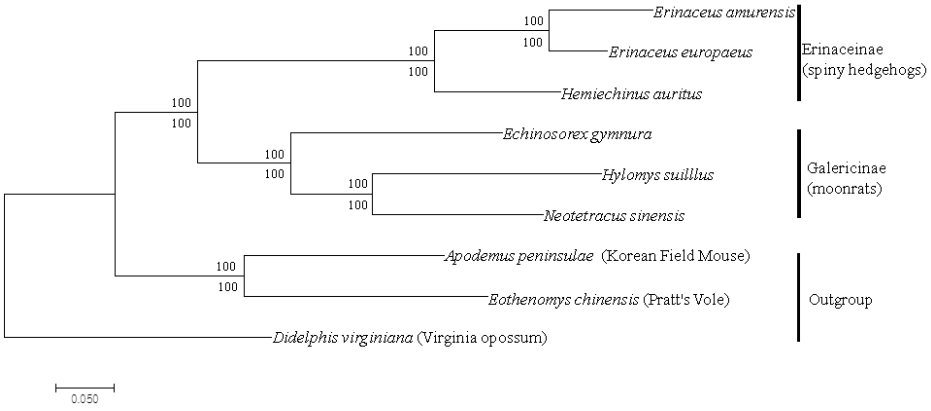

Figure 1. Higher phylogeny of the family Erinaceidae (hedgehogs) inferred from maximum-likelihood (ML) and neighbor-joining (NJ) analyses using 13 mitochondrial protein-coding genes: *Erinaceus europaeus* (accession No. NC_002080.2), *Hemiechinus auritus* (AB099481.1), *Echinosorex gymnura* (AF348079.1), *Hylomys suillus* (NC_010298.1), and *Neotetracus sinensis* (JX519466.1) from GenBank, and *Erinaceus amurensis* (KX964606) from the present study. Three-species of *Didelphis virginiana* (NC_001610.1), *Apodemus peninsulae* (JN546584.1), and *Eothenomys chinensis* (NC_013571.1) from GenBank were used as an outgroup. Only the ML tree is shown here as the tree topology was similar to that of the NJ tree. Numbers on nodes indicate bootstrap values (ML/NJ values).

In conclusion, our study presents characteristics and usage patterns of start and stop codons of the Korean *E. amurensis* mitogenome. The gene order and organization of the *E. amurensis* mitogenome follow the pattern found in other erinaceids. In this study, only six mitogenomes from five genera within the family Erinaceidae, with a total 10 genera, were included to analyze higher phylogeny. To better understand the phylogeny of the family Erinaceidae, mitogenomes of more species should be included in the phylogenetic analyses. The results of the present study will contribute to our understanding of the codon usage pattern and molecular evolution of *E. amurensis*, and provide better insight into inter-generic relationships within the family Erinaceidae. In future studies, the inclusion of mitogenomes from the other five genera of Erinaceidae would greatly enhance our understanding of intergeneric relationships within the Erinaceidae.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary material

Table S1. Primer information used in PCR amplifications of *Erinaceus amurensis*.