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Phylogeny of *g6pc1* Genes and Their Functional Divergence Among Sarcopterygian Vertebrates: Implications for Thermoregulatory Strategies

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Glucose-6-phosphatase catalytic subunit 1 (G6PC1) catalyzes the final rate-limiting step in endogenous glucose production and is critically important for glucose homeostasis. Although a single *g6pc1* gene is present in mammals, other vertebrates possess two to five paralogs. Functional divergence between paralogs has been reported in actinopterygians and has been implicated in the acquisition of adaptive characteristics. Such reports make sarcopterygian *g6pc1* an interesting research topic because unlike the aquatic habitat of actinopterygians, sarcopterygians have successfully adapted to terrestrial environments. However, little is known about the evolution of sarcopterygian *g6pc1*. In the present study, the evolutionary history of sarcopterygian *g6pc1* was investigated using molecular phylogeny, synteny analyses, and comparison of the genomic environment. Functional divergence between paralogs was also investigated in a reptilian species, the Japanese gecko, with a focus on gene expression in the liver. Evolutionary analyses suggested that amphibians and amniotes acquired duplicated genes independently. Among the amniotes, gene duplication occurred at the root of the reptilian-avian lineage, giving rise to *g6pc1-1* and *g6pc1-2* classes. While the avian lineage subsequently lost the *g6pc1-1*, the reptiles retained both classes. This co-occurrence of gene loss and endothermy acquisition, together with the observation that mammals possess only a single gene, suggests that the duplicated *g6pc1* is dispensable for endotherms. Quantitative RT-PCR analyses revealed that the two gecko genes respond differently to E2 administration, as the expression of *g6pc1-1* was downregulated by E2, whereas *g6pc1-2* showed no significant response. Such paralog-specific responses suggest functional divergence between paralogs, which is possibly related to reproduction.

Key words: *g6pc*, glucose metabolism, molecular evolution, gene duplication, functional divergence

INTRODUCTION

Glucose homeostasis is critically important for animal survival, because the central nervous system is largely reliant on this substrate (Dienel, 2019). In addition to carbohydrates obtained from diet, animals rely on endogenous glucose production as a source of glucose. Glucose can be produced by the breakdown of glycogen (glycogenolysis) or the conversion of non-carbohydrate sources (gluconeogenesis) such as amino acids, glycerol, lactate, and pyruvate (Berg et al., 2002). Both processes yield glucose-6-phosphate (G6P), which is metabolized in the cells. However, G6P cannot be transported across the cell membrane via glucose transporters (Rui, 2014). Therefore, G6P must be converted to glucose before being released into circulation. The conversion of G6P into glucose is catalyzed by the glucose-6-phosphatase catalytic subunit (G6PC) (Chou et al., 2010).

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Three isoforms of *g6pc*, namely, *g6pc1* (often called *g6pcα* or simply *g6pc*), *g6pc2*, and *g6pc3*, have been identified in vertebrates. Among these isoforms, *g6pc1* is exclusively expressed in gluconeogenic organs such as the liver, kidney, and small intestine. This enzyme maintains systemic glucose homeostasis by facilitating glucose production in these organs (Hutton and O'Brien, 2009). Consequently, defects in the *g6pc1* gene result in glycogen storage disease type Iα (GSD-Iα), the symptoms of which are characterized by hypoglycemia, hyperlipidemia, and hyperuricemia (Bali et al., 2006; Chou and Mansfield, 2008).

Vertebrates possess two to five *g6pc1* paralogs. Marandel et al. (2017) reported that the ancestral *g6pc1* gene duplicated as a result of 2nd round whole genome duplication (2R-WGD), giving rise to two paralogs, which they designated *g6pca* and *g6pcb*. The authors also suggested that, while actinopterygians retained both paralogs, sarcopterygians lost *g6pca* after divergence from the actinopterygian lineage. As a result, only *g6pcb* was present among the sarcopterygians. However, several sarcopteryg-

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ian species possess more than one *g6pc1* paralog, possibly as a result of lineage-specific duplication. For example, the green anole and western clawed frog have two *g6pc1* genes (Marandel et al., 2017).

Functional divergence between the *g6pc1* paralogs has been implicated in the acquisition of adaptive novelty among actinopterygian species (Marandel et al., 2017). In this regard, the resurgence of duplicated *g6pc1* among sarcopterygian lineages is an interesting topic. In contrast to actinopterygians, sarcopterygians have successfully invaded and conquered terrestrial environments. This process, often called "conquest of land," certainly required physiological changes. Therefore, the evolutionary aspect of sarcopterygian *g6pc1* may shed light on vertebrate adaptations to land from a physiological perspective. Unfortunately, little has been investigated regarding the evolutionary history of the sarcopterygian *g6pc1*. In addition, the physiological roles of duplicated paralogs in non-mammalian species have yet to be examined.

This study aimed to provide basic knowledge regarding the evolution and function of *g6pc1* in sarcopterygians. We investigated the evolutionary process of sarcopterygian *g6pc1* by reconstructing molecular phylogeny and syntenic analyses and comparing the genomic environment upstream of paralogs. To infer functional divergence, gene expression of *g6pc1* paralogs was investigated in the liver of the Japanese gecko (*Gekko japonicus*), a reptilian species whose genome data are available (Liu et al., 2015). The results suggest that multiple duplication and loss events, rather than sporadic species-specific duplication, gave rise to multiple paralogs among sarcopterygians. In addition, each gecko paralog responded differently to the other when 17β-estradiol (E2) was administered, implying functional divergence between paralogs.

MATERIALS AND METHODS

Comparison of the amino acid sequences and syntenic analyses of sarcopterygian *g6pc1*

The CLUSTAL W program embedded in MEGA (version 7.0.21) (Kumar et al., 2016) was used with default settings to align the nucleotide and deduced amino acid sequences of the sarcopterygian *g6pc1*. Syntenic analyses of sarcopterygian *g6pc1* were performed using the genome data deposited in GenBank and Ensembl (see Supplementary Table S1).

Construction of molecular phylogenetic tree of vertebrate g6pc1

Molecular phylogenetic trees of vertebrate *g6pc1* were constructed together with the *g6pc2* and *g6pc3* genes. In all trees, *g6pc3* genes were used to root the trees. This is because the vertebrate *g6pc* family first bifurcates between *g6pc1*/*g6pc2* and *g6pc3* (Al-Daghri et al., 2017). Codon alignments of the CDS nucleotide sequences were constructed using the CLUSTAL W program embedded in MEGA (version 7.0.21) (Kumar et al., 2016) and were subsequently used for tree construction.

Three trees were selected for the study. First, a neighbor-joining (NJ) tree was drawn based on amino acid sequences to determine whether the results of Marandel et al. (2017) could be reproduced. A nucleotide-based tree was then constructed using the maximum likelihood (ML) method to re-examine the evolutionary history of vertebrate *g6pc1*. These trees contain both actinopterygian and sarcopterygian genes. To infer the evolution of sarcopterygian *g6pc1* genes, another ML tree was drawn based exclusively on the nucleotide sequences of sarcopterygian genes. The amino acid-based NJ tree was drawn by selecting the "partial deletion" option for gap treatment with 95% of site coverage cutoff. Nucleotide-based trees were drawn by selecting the "partial deletion" option for gap treatment with 95% of site coverage cutoff. A total of 340 positions were included in the actinopterygian-sarcopterygian trees, whereas 342 positions were included in the sarcopterygian ML tree. Before constructing ML trees, "Find Best DNA/Protein Models" was run for the selection of "Substitution Model" and "Rates and Patterns". The best setting for the actinopterygian + sarcopterygian nucleotide-based tree was the General Time Reversible model for "Substitution Model" and Gamma Distributed with Invariant sites (G + I) for "Rates and Patterns" (GTR + G + I). The best setting for the sarcopterygian nucleotide-based tree was the General Time Reversible model for the "Substitution Model" and Gamma Distributed (G) for "Rates and Patterns" (GTR + G). The number of bootstrap replicates for the estimation of the robustness of the internal nodes was set to 1000 for NJ and 100 for ML trees. The full species names and GenBank accession numbers are summarized in Supplementary Table S1.

Comparison of the nucleotide sequences upstream of *g6pc1*

Human and mouse *g6pc1* genes have multiple response elements in the region within –1000 base pairs (bp) from the transcription start site (Schmoll et al., 1996; Streeper et al., 1997, 1998; Qiu et al., 2017). We predicted that the genomic environment corresponding to this region would have a significant effect on transcriptional regulation. In this study, nucleotide sequences –1000 to –1 bp upstream of the transcription start site were aligned using the LAGAN program (Brudno et al., 2003) in the Vista package (http://genome.lbl.gov/vista/mvista/submit.shtml) (Frazer et al., 2004).

Animals

Collection of Japanese geckos

The Japanese geckos used in this study were collected from a field in the Tokyo metropolitan area of Japan. The dates of collection were as follows: fasting experiment – 19 April 2016, 17β-estradiol administration – 25 April 2017; comparison of free-living individuals – 25 April and 26 September 2017. Males larger than 45 mm and females larger than 50 mm in snout-to-vent length (SVL) were used for subsequent experiments, because males larger than 44.9 mm and females larger than 50 mm were regarded as sexually mature in previous studies (Ikeuchi, 2004; Jono and Inui, 2012). All animals used in this study were treated according to the guidelines of the Bioscience Committee of the University of Tokyo (permission number 16-4).

Animal care

The captured animals were reared in individual cages under a 12-h light and 12-h dark condition. Room temperature was set to maintain around 25°C. Animals were fed ad libitum with *Tenebrio molitor* larvae until they were used for the experiments. All animals were euthanized by rapid decapitation and exsanguination. Liver samples were collected, frozen in nitrogen liquid, and stored at –80°C until further use for RNA extraction.

Fasting experiments

Fourteen male Japanese geckos were divided into two groups of seven individuals each. After the acclimation period, one group was fasted for 23 days, whereas the other group was fed ad libitum.

Comparison of seasonal differences in free-living population

Male and female Japanese geckos were captured on 25 April 2017 (n = 5 for each sex) and on 26 September 2017 (n = 6 for each sex). All of the geckos were sacrificed on the same day that they were captured.

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Administration of 17β-estradiol (E2)

Gecko species can store sperm in the oviduct for months after copulation (Murphy-Walker and Haley, 1996; Yamamoto and Ota, 2006). Therefore, the inadvertent onset of vitellogenesis and concomitant increase in blood estrogen levels are possible. In this experiment, such an effect was avoided by using males. Sixteen male Japanese geckos were divided into two groups of eight individuals each. After the acclimation period, the E2 treatment group was intraperitoneally injected with 17β-estradiol in sesame oil at a dose of 1 μg/g body weight every alternate day for 2 weeks. The control group was intraperitoneally injected with sesame oil alone. Both groups were provided with food and water ad libitum. Two individuals in the E2 treatment group died at 2 and 13 days after the first administration, respectively. Therefore, subsequent analyses were performed on six specimens from the E2 treatment group and eight specimens from the control group.

RNA extraction and cDNA synthesis

Total RNA was extracted from the liver samples using ISOGEN (Nippon Gene, Tokyo, Japan). For the construction of the DNA complementary to RNA (cDNA) templates, 3 μg of total RNA was used. The cDNA templates for quantitative real-time PCR were constructed as follows: denatured total RNA was reverse-transcribed using an oligo (oligodeoxyribonucleotide) (dT) primer and M-MLV reverse transcriptase (Promega, Madison, WI). The reaction volume contained oligo (dT) primers at 5 pM, 2 mM of each deoxyribonucleoside triphosphate (dNTP), 200 units of M-MLV reverse transcriptase, and 1 × M-MLV buffer (Promega). The volume was brought to 20 μL with nuclease-free water (Qiagen, Tokyo, Japan). The reaction was performed at 42°C for 90 min, and then 70°C for 15 min.

Quantitative real-time PCR

Five microliters of each 25- or 100-fold-diluted cDNA from liver samples was amplified in a 20 μl reaction mixture containing each primer (0.3 μM) and the KAPA SYBR Fast qPCR Kit (KAPA Biosystems, Century City, South Africa) using a LightCycler 480 Instrument II (Roche Diagnostic). The primers were designed to span at least one intron of more than 400 bp based on the genome sequences of the Japanese gecko in the NCBI database. The primer sequences used are listed in Table 1. The PCR reaction conditions were as follows: 95°C for 5 min, 45 cycles of 95°C for 10 s, 56°C for 10 s, and 72°C for 10 s, followed by melting curve analysis by which the PCR product was confirmed. The PCR product was also confirmed using RNA samples without RT reaction as a template for PCR. The specificity of the primers for each *g6pc1* gene was confirmed by sequencing PCR products. The reaction efficiency was greater than 90% for all the primer sets. The amplicons from each gene were designed as follows: 115 bp for *g6pc1-1*; 142 bp for *g6pc1-2*; 112 bp for *pck2*; 88 bp for *36b4*; 121 bp for *rpl8*.

Table 1. Primers used for quantitative RT-PCR analyses.

Gene	Sense/Antisense	Primer sequence
36b4	Sense	AACACCATGATGCGCAAGGC
36b4	Antisense	CCACATTGCCACGGATATGAGG
g6pc1-1	Sense	CCATCTGTTTCCACCTGAAG
g6pc1-1	Antisense	CAGTAGGGTCTCTGTCCAAAG
g6pc1-2	Sense	TCGATCCAGCAGTTCCCTGTG
g6pc1-2	Antisense	GTTTCCAGGACGGTTGTTTGTTC
pck2	Sense	ATGCTTTGCTCTCCGTATCG
pck2	Antisense	CGCCACGTACTTCTTCTTCC
rpl8	Sense	ATGCCACTGTTATCTCCCAC
rpl8	Antisense	CCACCACCAGCAACAATACC

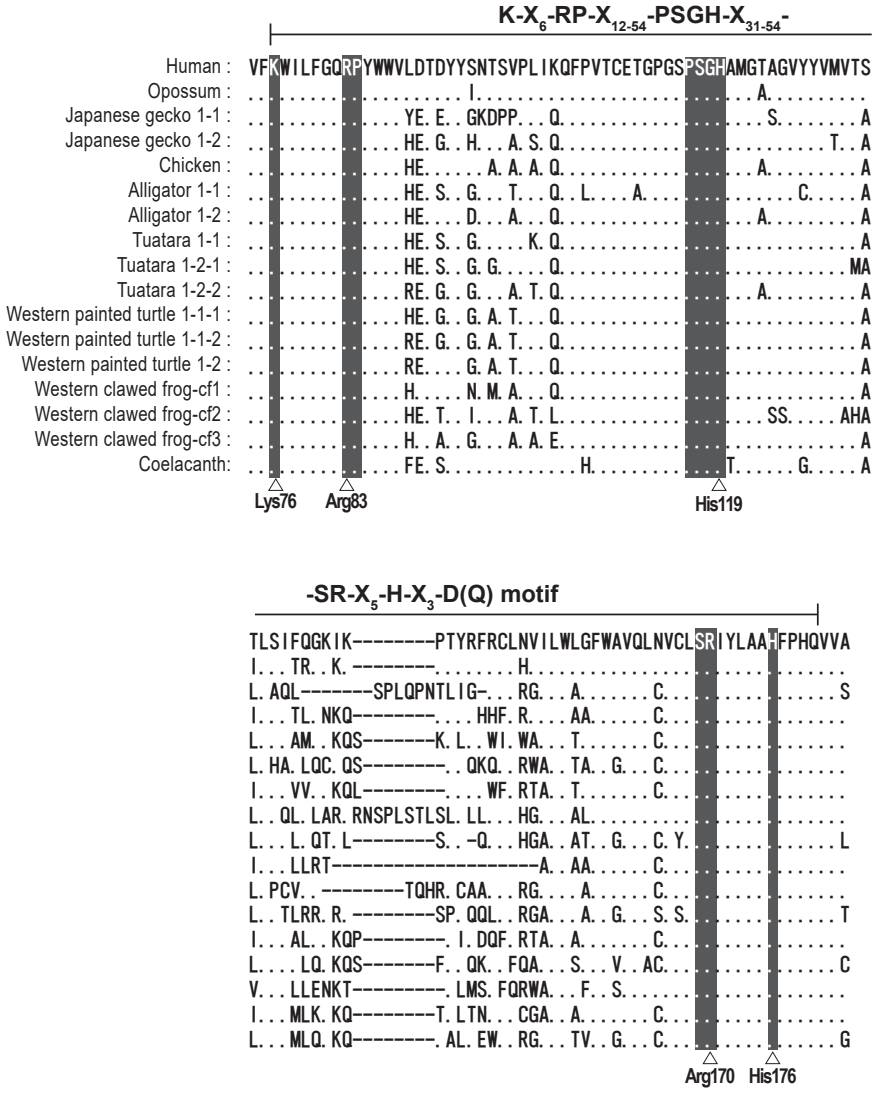

Fig. 1. Amino acid sequences deduced from sarcopterygian *g6pc1* genes. The amino acid sequences of G6PC1, deduced from sarcopterygian genes, were aligned. It should be noted that only functionally important regions are presented. Species were selected from the major extant sarcopterygian lineages (Eutherian mammal, Marsupial, Squamata, Aves, Tuatara, Crocodilia, Chelonia, Amphibia, and coelacanth). Amino acids corresponding to active centers for enzymatic activities (Lys76, Arg83, His119, Arg170, and His176 in human G6PC1) are denoted by open triangles. All species and paralogs conserved both the phosphatase motif and the active centers.

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Reference genes for normalization were selected using the NormFinder (Version 0.953) algorithm (Andersen et al., 2004), which is available as an Excel add-in at https://moma.dk/normfinder-software. *36b4*, *ef1a1*, and *actb* levels were examined in the fasting experiment. Among these genes, *36b4* was selected as the most stable gene, with a stable value of 0.013. To compare the free-living individuals and for the E2 administration experiment, *36b4*, *ef1a1*, *actb*, *sdha*, and *rpl8* were examined. In both experiments, the geometric means of *36b4* and *rpl8* were selected as the most stable reference, with stable values of 0.006 (free-living population) and 0.001 (E2 administration).

Search for hormone responsive elements upstream of Japanese gecko *g6pc1*s

Two types of hormone responsive elements, estrogen responsive element (ERE) and progesterone responsive element (PRE), were searched in the genomic regions upstream of the Japanese gecko *g6pc1* genes. Genomic sequence information corresponding to 1 kilobase pair (kbp) upstream of the transcription start site was obtained from the GenBank database, and ERE and PRE half sites (AGGTCA and TGACCT for the former, AGAACA and TGTTCT for the latter) were manually searched with the aid of Windows Notepad.

Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM). The differences of the expression levels of the genes between the groups were analyzed using the Mann–Whitney *U* test. All analyses were conducted using KyPlot 5.0 software (KyensLab, Tokyo, Japan).

RESULTS

Comparison of deduced amino acid sequences and syntenic analyses of *g6pc1* genes

The amino acid sequences of *g6pc1* deduced from the genome data revealed that all sarcopterygian paralogs possessed a phosphatase motif. They also conserved five active center sites corresponding to Lys76, Arg83, His119, Arg170, and His176 of human G6PC1 (Chou and Mansfield, 2008) (Fig. 1).

Syntenic analysis of *g6pc1* revealed that the coelacanth had a single *g6pc1* gene (Fig. 2). According to GenBank annotation, the western clawed frog had two *g6pc1* and one *g6pc2* genes arranged in tandem on chromosome 10. However, this "*g6pc2*" gene was not included in the *g6pc2* clade in the phylogenetic trees constructed in this study (Figs. 3, 4). In addition, no other sarcopterygian species had the *g6pc1* and *g6pc2* genes on the same chromosome (Fig. 2). Therefore, we designated this "*g6pc2*" as a *g6pc1* gene. Among amniotes, mammals and aves had one *g6pc1* gene, whereas reptiles had two or three genes. Among reptiles, squamates, crocodilians, and the softshell turtle had two genes. In contrast, the tuatara, green sea turtle, and western painted turtle had three. In all species investigated, the regions containing *g6pc1* genes were flanked by the *aoc* and *aarsd1* genes (Fig. 2).

Molecular phylogenetic analyses

To infer the phylogenetic relationships among vertebrate *g6pc1* paralogs, three molecular phylogenetic trees were constructed. First, an NJ tree was constructed based on the amino acid sequences of the vertebrate *g6pc* family genes. This tree includes both actinopterygian and sarcopterygian species. In this NJ tree, the gnathostome *g6pc1* paralogs formed a monophyletic clade. The gnathostome *g6pc1* clade was divided into two subclades, each representing the *g6pca* and *g6pcb* class genes (see Supplementary Figure S1).

Next, an ML tree was constructed based on the nucleotide sequences. In this ML tree, actinopterygian *g6pca* and *g6pcb* formed a clade distinct from chondrichthyan and sarcopterygian *g6pc1* genes (Fig. 3).

Phylogenetic relationships among amniote *g6pc1* genes were inferred from an ML tree constructed based on the nucleotide sequences of the sarcopterygian genes (Fig. 4).

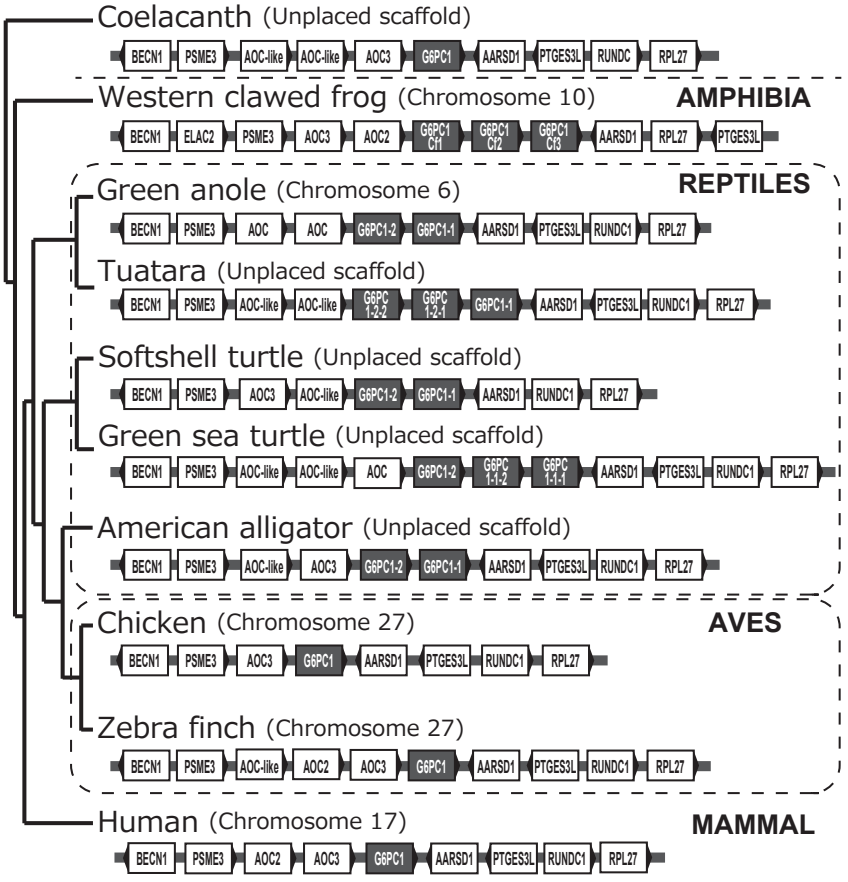

Fig. 2. Comparative syntenic analyses of *g6pc1* genes among sarcopterygian lineages. The synteny around *g6pc1* in the sarcopterygian species was compared. While the coelacanth, human, and avian species have only one *g6pc1*, the western clawed frog and reptiles have two or three paralogs. Regardless of the number of paralogs, *g6pc1* genes were flanked by *aoc* and *aarsd1* genes in all species examined.

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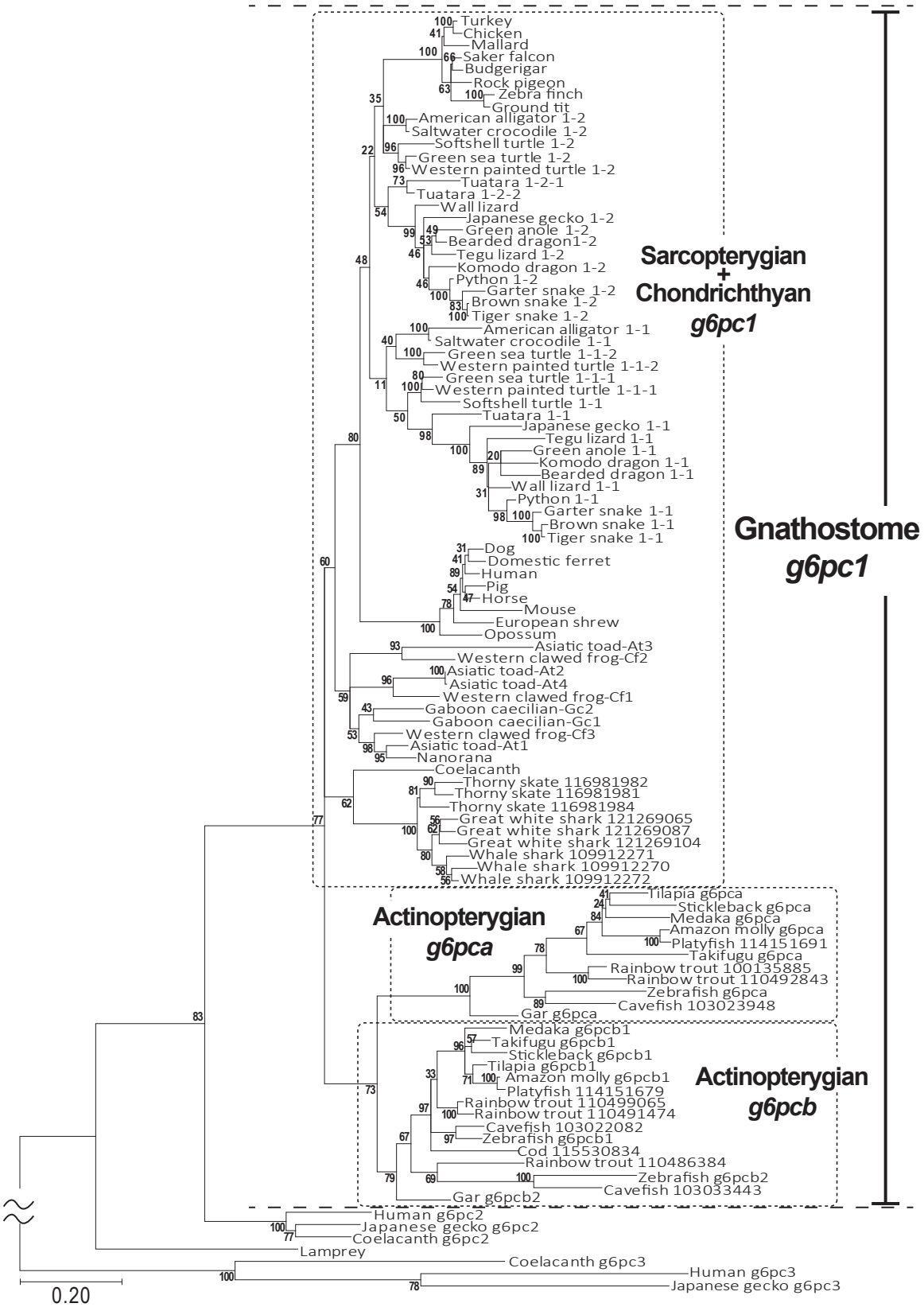

Fig. 3. Maximum likelihood phylogenetic tree based on nucleotide sequences of vertebrate *g6pc1*, *g6pc2*, and *g6pc3*. A molecular phylogenetic tree of vertebrate *g6pc* family genes was constructed using MEGA software (version 7.0.21). The tree was rooted in *g6pc3* genes. The scale bar beneath the tree indicates the estimated evolutionary distance units (these settings also apply to Fig. 4). Actinopterygian *g6pca* and *g6pcb* formed a clade distinct from chondrichthyan and sarcopterygian *g6pc1* genes. This implies that the *g6pca* and *g6pcb* classes emerged only in the actinopterygian lineage.

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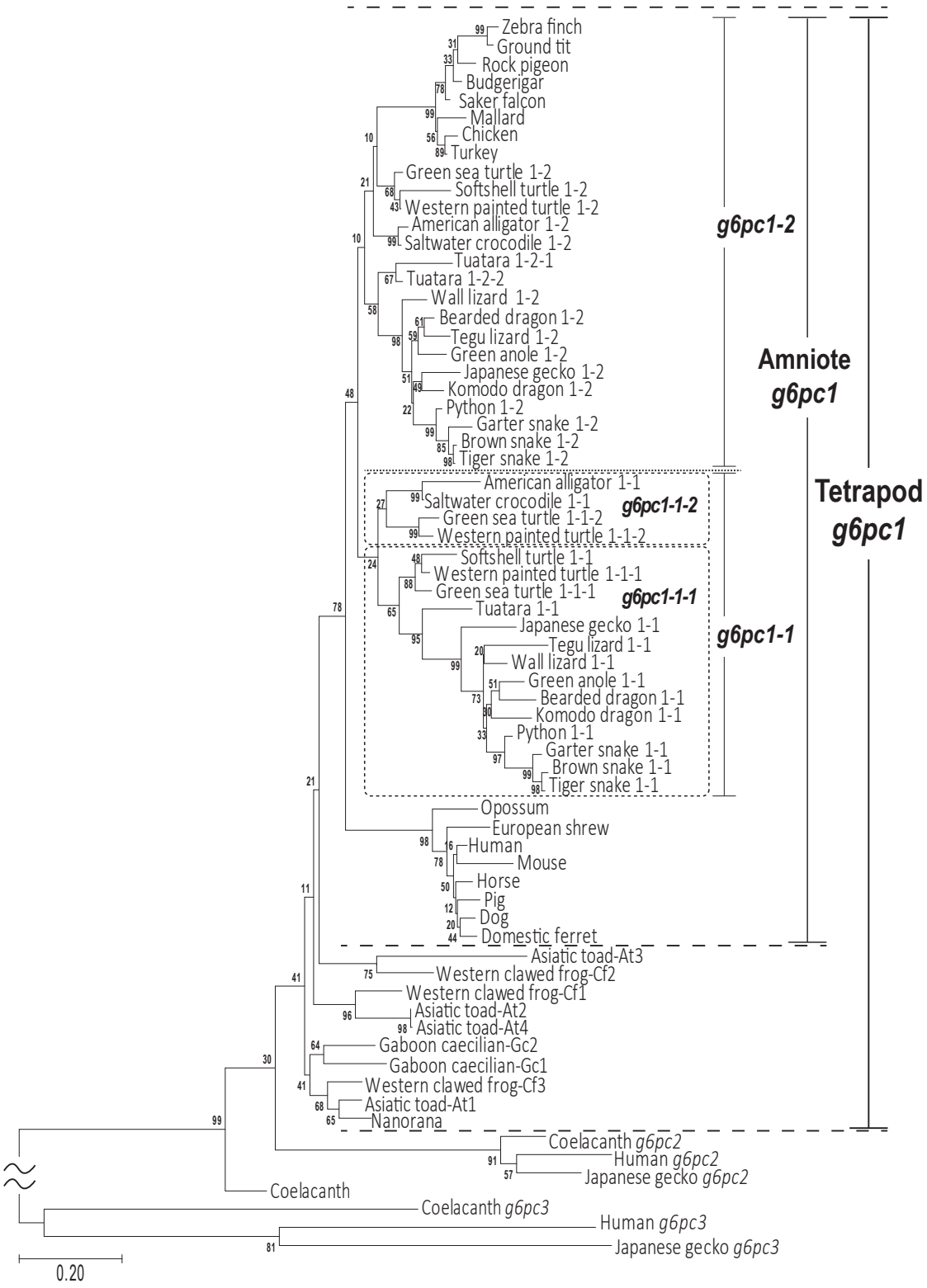

Fig. 4. Maximum likelihood phylogenetic tree based on nucleotide sequences of sarcopterygian *g6pc1*, *g6pc2*, and *g6pc3*. The nucleotide-based sarcopterygian tree suggested the existence of two *g6pc1* classes among amniotes. These were designated *g6pc1-1* and *g6pc1-2*. The former was further divided into two subclasses: *g6pc1-1-1* and *g6pc1-1-2*.

In this sarcopterygian ML tree, the amniote *g6pc1* genes formed a single clade, suggesting a monophyletic origin of *g6pc1* within this group. The *g6pc1* clade of amniotes was divided into two subclades. One contained all the mammalian and avian genes, together with at least one gene from each reptilian species. The other subclade consisted exclu-

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sively of reptilian genes. We designated the former subclade as "*g6pc1-2* class" whereas the latter as "*g6pc1-1* class".

The *g6pc1-1* class was further divided into two subclasses, designated as *g6pc1-1-1* and *g6pc1-1-2*. The former contained tuatara, squamates, and one paralog from each of the chelonian species. The latter contained crocodilian paralogs and one paralog each from the green sea turtle and the western painted turtle.

Comparison of genomic environment upstream of *g6pc1*

Genomic regions upstream of the transcription start site were compared to infer phylogenetic relationships among the reptilian genes (Fig. 5).

When the painted turtle *g6pc1-1-1* was used as a query, a similar region was detected in sea turtle *g6pc1-1-1* and softshell turtle *g6pc1-1* (Fig. 5A). On the other hand, regions similar to painted turtle *g6pc1-1-2* were detected in sea turtle *g6pc1-1-2* and crocodilian *g6pc1-1* (Fig. 5B). In both comparisons, no similarity was detected between the *g6pc1-1-1* and *g6pc1-1-2* subclasses.

Hepatic gene expression of *g6pc1* genes and *pck2* in the Japanese gecko

Fasting had no significant effect on the expression of *g6pc1* genes. In contrast, *pck2* expression was significantly downregulated (Fig. 6A). Comparison of gene expression in free-living female geckos between seasons revealed significantly lower expression of *g6pc1* and *pck2* in April than in September. In contrast, males showed no significant change between seasons (Fig. 6B). Male geckos administered E2 intraperitoneally showed significantly lower expression levels of *g6pc1-1* and *pck2* compared to those administered sesame oil alone. In contrast, the expression of *g6pc1-2* showed no significant change between the E2 treatment and control groups (Fig. 6C).

Search for hormone responsive elements upstream of *g6pc1* genes

A single ERE (AGGTCA) was present –928bp upstream of the *g6pc1-1* transcription start site. In contrast, no ERE or its half site was present at least 1kbp upstream of *g6pc1-2*. No PRE was present upstream of either of the *g6pc1* genes.

DISCUSSION

Conservation of functional sites among sarcopterygian *g6pc1* paralogs

As mentioned in the Introduction, the primary function of *g6pc1* is the dephosphorylation of G6P. In this study, we investigated whether functionally important active center sites and a phosphatase motif (Chou and Mansfield, 2008) are conserved among sarcopterygian *g6pc1* genes. For this purpose, the amino acid sequences of *g6pc1* from species representing each sarcopterygian lineage were aligned. Alignment revealed that all sarcopterygian genes conserved

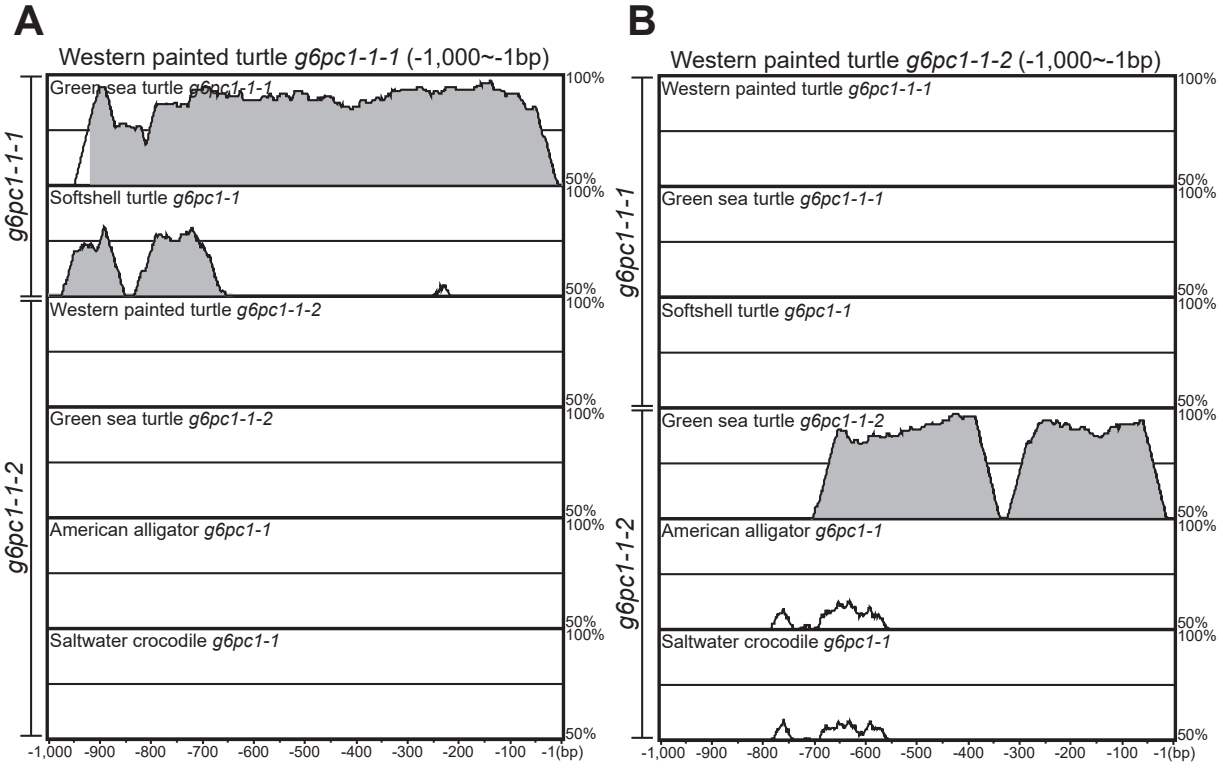

Fig. 5. Comparison of genomic sequences upstream of *g6pc1*. Nucleotide sequences upstream of the *g6pc1* paralogs (–1 kbp from the transcription start site) were aligned and compared using the Vista package. Sequence similarities of over 50% are represented as peaks. Conserved non-coding sequences (CNSs) detected by the program are highlighted. **(A)** When the painted turtle *g6pc1-1-1* was used as a query, similar regions were detected in sea turtle *g6pc1-1-1* and softshell turtle *g6pc1-1*. **(B)** When the painted turtle *g6pc1-1-2* was used as a query, similarity was detected in sea turtle *g6pc1-1-2* and crocodilian *g6pc1-1*. No similarity was detected between *g6pc1-1-1* and *g6pc1-1-2* subclasses in any of the comparisons described above.

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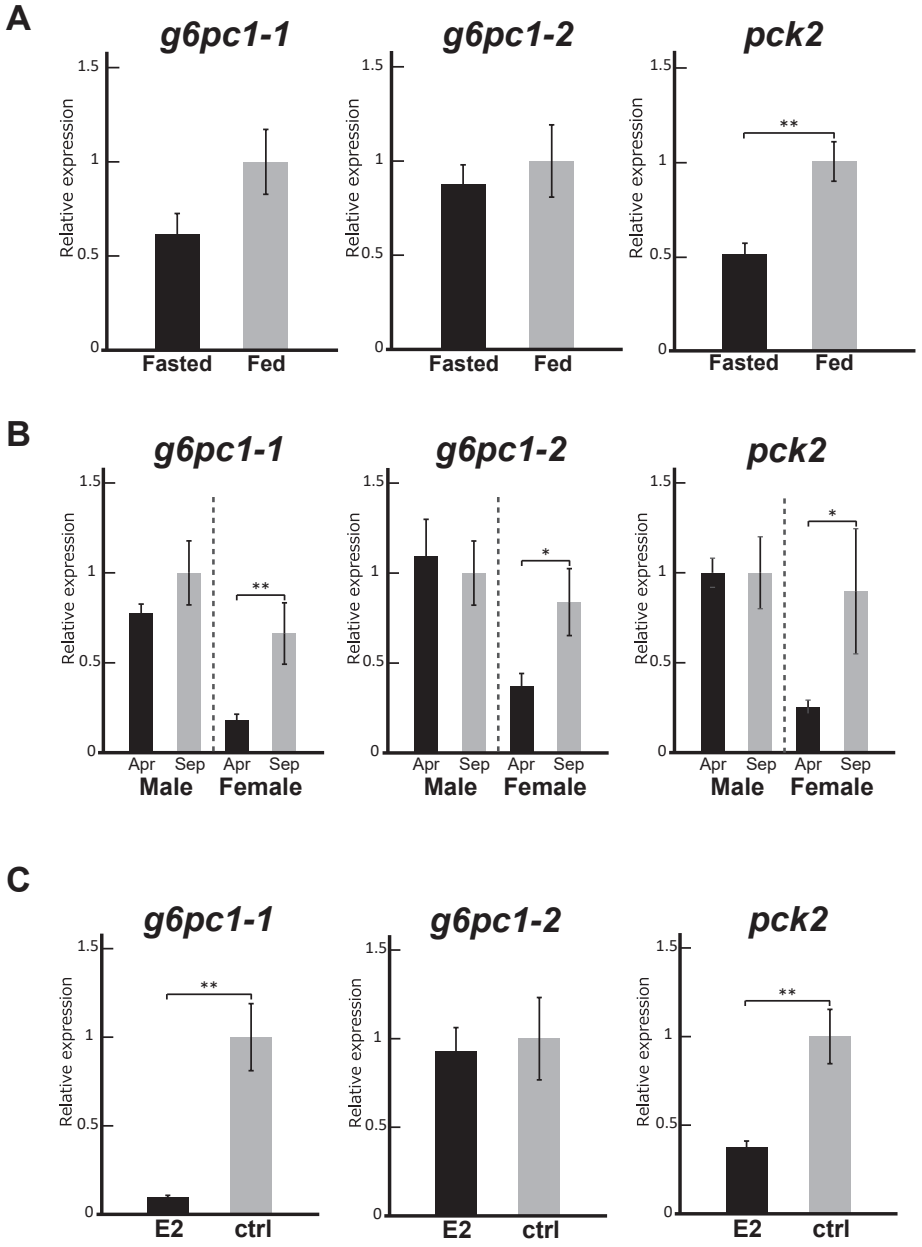

Fig. 6. Quantitative RT-PCR analyses of Japanese gecko *g6pc1* and *pck2* in the liver. The results are shown as the mean ± SEM, and the expression levels of the control group (fasting and E2 administration) and males captured in April (seasonal variation) are expressed as 1. * $P < 0.05$, ** $P < 0.01$ (Mann–Whitney U test). **(A)** Gene expression in response to fasting for 3 weeks. The expression of *g6pc1* genes showed no significant difference between groups. In contrast, *pck2* expression was significantly downregulated by fasting. **(B)** Seasonal changes in gene expression were examined in males and females. Although no significant seasonal change was observed in males, the expression of both *g6pc1* genes and *pck2* in females was significantly lower in April than in September. **(C)** Gene expression in response to E2 treatment. The expression of *g6pc1-1* and *pck2* was significantly lower in the E2 treatment group, whereas that of *g6pc1-2* was not significantly different between groups.

five active center sites and a phosphatase motif (Fig. 1). Such conservation of sites suggests that all genes retain the catalytic activity of G6P dephosphorylation.

Evolutionary process of duplicated *g6pc1* genes

The evolutionary process of *g6pc1* was investigated by drawing phylogenetic trees, syntenic analyses, and comparing the upstream sequences. Three molecular phylogenetic trees were constructed in this study, of which two are presented. Although the bootstrap robustness of internal nodes is not always good, insightful information can be obtained from the other analyses.

Gene duplication around actinopterygian-sarcopterygian split

In the amino acid-based NJ tree, the *g6pc1* clade was divided into the *g6pca* and *g6pcb* subclades (see Supplementary Figure S1). This tree topology was in accordance with a previous report (Marandel et al., 2017), which suggested that 2R-WGD in the ancestral gnathostome gave rise to subclades. However, the nucleotide-based ML tree constructed in this study did not support a sister relationship between *g6pca* and *g6pcb*. Instead, the actinopterygian *g6pca* and *g6pcb* genes formed a distinct clade from the chondrichthyan and sarcopterygian *g6pc1* genes (Fig. 3). This tree topology suggested that *g6pca* and *g6pcb* emerged in the actinopterygian lineage after the actinopterygian-sarcopterygian split. Therefore, the evolutionary origins of *g6pca* and *g6pcb* may need to be reconsidered.

Basal sarcopterygian and amphibian paralogs

In the sarcopterygian ML tree, all *g6pc1* genes except that of coelacanth formed a monophyletic clade, which was designated as “tetrapod *g6pc1* clade” (Fig. 4). Syntenic analyses revealed only a single *g6pc1* gene in the coelacanth, whereas multiple genes were present among the tetrapod species (Fig. 2). This observation suggests that the sarcopterygian *g6pc1* genes have emanated from a single ancestral gene.

Among the amphibian species, *Nanorana parkeri* was found to possess only a single *g6pc1* gene. In contrast, other species, including anurans and caecilians, were found to possess two to four genes (Fig. 4), suggesting lineage-specific gene duplication.

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Gene duplication in the ancestral amniotes

Our syntenic analyses revealed that mammals possess only a single *g6pc1* gene, whereas reptiles possess two to three genes (Fig. 2). This distribution of genes within amniotes suggests that gene duplication occurred in the ancestral reptilian lineage after the mammalian-reptilian split. Molecular phylogeny supports this hypothesis. In both ML trees drawn in this study, the amniote *g6pc1* clade bifurcated between the mammalian clade and reptilian-avian clade (Figs. 3, 4).

Gene duplication and loss among reptilian lineages

In the sarcopterygian ML tree, the reptilian-avian clade was divided into two subclades, which were designated as *g6pc1-1* and *g6pc1-2* classes (Fig. 4). Squamates, crocodilians, and softshell turtles were found to possess two genes, one from each subclass. The tuatara had one more *g6pc1-2* class gene, which was likely produced by lineage-specific duplication after divergence from squamates (Fig. 4).

The sea turtle and painted turtle were found to possess three *g6pc1* genes, two from the *g6pc1-1* class and one from the *g6pc1-2* class. The two *g6pc1-1* genes were designated *g6pc1-1-1* and *g6pc1-1-2* (Fig. 4). According to Crawford et al. (2015), these two species and the softshell turtle belong to two sister superfamilies: the former two to Durocryptodira, the latter one to Trionychia. Considering the sister-relationship between these superfamilies, the simplest explanation for the origin of the *g6pc1-1-1* and *g6pc1-1-2* subclasses is the duplication of the ancestral *g6pc1-1* gene in the durocryptodiran lineage (Fig. 7A). However, this hypothesis is not supported by molecular phylogeny. In the sarcopterygian ML tree, durocryptodiran *g6pc1-1-1* and *g6pc1-1-2* were not immediate siblings. Instead, the former was sister to the softshell turtle *g6pc1-1-1*, whereas the latter was sister to the crocodilian *g6pc1-1* (Fig. 4). An alternative hypothesis that can explain such a tree topology is as follows: First, *g6pc1-1* duplicated in the common ancestor of chelonians and crocodilians, giving rise to *g6pc1-1-1* and *g6pc1-1-2* subclasses. While durocryptodirans retained both subclasses, the softshell turtle and crocodilians lost one of the two subclasses (Fig. 7B). Support for this alternative hypothesis was obtained by comparing the genomic environment. Regions similar to durocryptodiran *g6pc1-1-1* were detected in softshell turtle *g6pc1-1* but not in durocryptodiran *g6pc1-1-2* (Fig. 5A). In contrast, regions similar to *g6pc1-1-2* were detected in crocodilian *g6pc1-1*, but not in durocryptodiran *g6pc1-1-1* (Fig. 5B). These results suggest a close relationship between *g6pc1-1-1* and softshell turtle *g6pc1-1* and between *g6pc1-1-2* and crocodilian *g6pc1-1*.

Loss of *g6pc1-1* class from avian lineage

Syntenic analyses revealed only a single *g6pc1* gene in the avian species (Fig. 2). This gene belonged to the *g6pc1-2* class in the sarcopterygian ML tree (Fig. 4). Although the zebra finch was previously reported to possess two *g6pc1* genes (Marandel et al., 2017), only a single gene was present in the current version of the genome assembly (bTaeGut2.pat.W.v2). The presence of two *g6pc1* classes in crocodilians suggests the loss of *g6pc1-1* from the avian lineage after crocodilian-avian split. Interestingly, mammals possess only one *g6pc1* gene. Since mammals and aves are two amniote lineages that have acquired endothermy independently from each other, acquisition and loss of the duplicated *g6pc1* gene could be related to thermoregulatory strategies (Fig. 8).

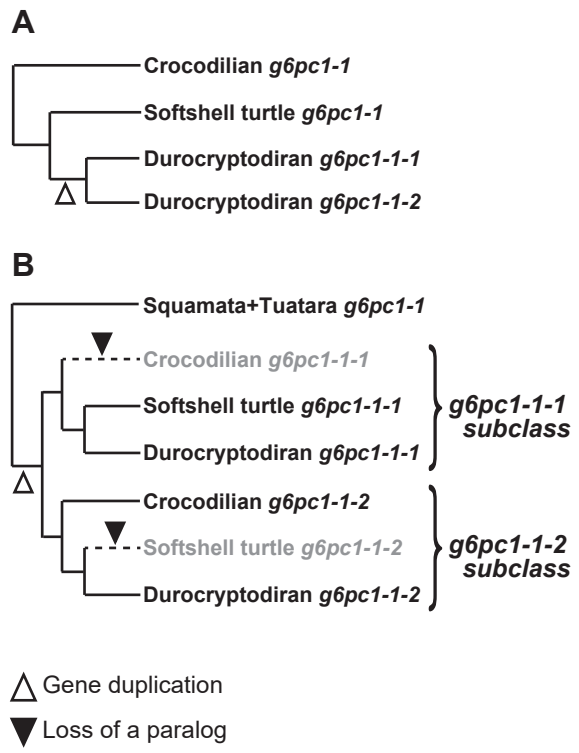

Fig. 7. Hypotheses regarding the evolution of reptilian *g6pc1*. **(A)** Presence of two *g6pc1-1* subclasses (*g6pc1-1-1* and *g6pc1-1-2*) only in the durocryptodiran turtles suggests duplication within this lineage. **(B)** In contrast, the sarcopterygian ML tree and similarity of genomic environment suggest duplication in the common ancestor of crocodilians and chelonians, followed by lineage-specific loss of subclasses.

Transcriptional regulation of gecko *g6pc1* genes and implications for physiological significance

In this study, the functional divergence between *g6pc1* genes was investigated, focusing on transcriptional regulation in the liver. This is because the transcription of *g6pc1* is regulated by multiple hormonal and nutritional stimuli, which in turn results in altered glucose production from gluconeogenic organs (Caseras et al., 2002; Salgado et al., 2004; Hutton and O'Brien, 2009; Yabaluri and Bashyam, 2010; Rui, 2014). In addition, paralog-specific responses to nutritional status have been reported in rainbow trout (Marandel et al., 2015). We also investigated the gene expression of phosphoenolpyruvate carboxykinase 2 (*pck2*) because *pck2* catalyzes the first irreversible step of gluconeogenesis and thus is expected to reflect hepatic gluconeogenic activities.

In the current study, neither of the *g6pc1* genes showed significant changes in expression after 3 weeks of fasting. In contrast, *pck2* expression was significantly downregulated after fasting (Fig. 6A). Downregulation of *pck* under prolonged fasting has also been reported in other vertebrates,

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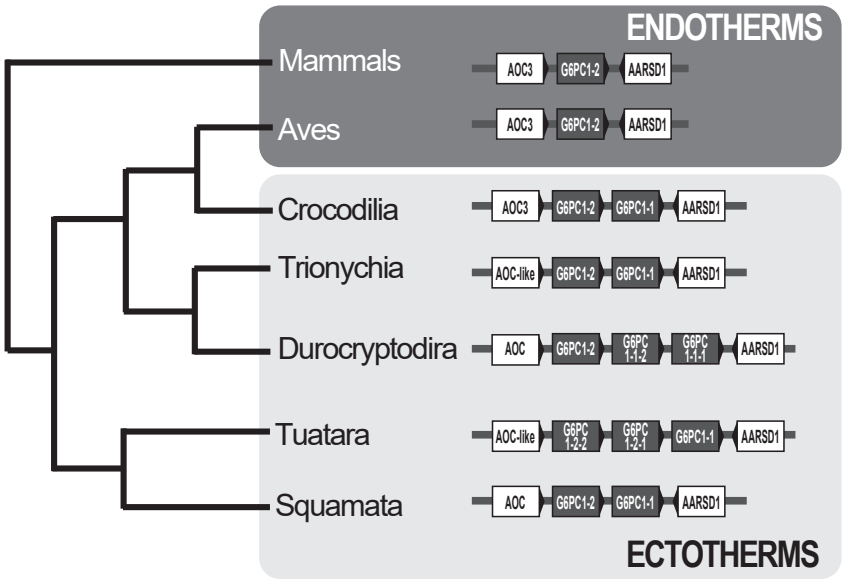

Fig. 8. Distribution of *g6pc1* paralogs and thermoregulatory strategies among amniotes. Among amniotes, mammals and aves acquired endothermy independently of each other. These endothermic lineages possess only a single *g6pc1-2* class paralog. In contrast, all ectothermic reptiles retained both the *g6pc1-1* and *g6pc1-2* class paralogs. Such a distribution of genes suggests that the retainment of more than one *g6pc1* is beneficial only to ectotherms.

such as the large yellow croaker (*Larimichthys crocea*) (Qian et al., 2016). In mammals and birds, gluconeogenesis is repressed within a day or two because excessive breakdown of proteins is harmful (Berg et al., 2002; Wang et al., 2006). Such protein sparing could also be the reason for the downregulation of the gecko *pck2* gene.

Stable expression of gecko *g6pc1* genes under prolonged fasting might reflect reliance on glycogen for energy storage, because *g6pc1* catalyzes the most downstream reaction shared by gluconeogenesis and glycogenolysis. Although glycogen has been considered to serve only as a short-term energy storage in mammals (Wang et al., 2006), it serves as a long-term energy source in other vertebrates, such as side-blotched lizard (*Uta stansburiana*) (Zani et al., 2012) and common carp (*Cyprinus carpio*) (Wang et al., 2006).

Seasonal gene expression changes were significant only in females, which showed lower expression of both *g6pc1* and *pck2* in April than in September (Fig. 6B). This implies the involvement of sex-related factors. One possible factor is estrogen. Rise of blood E2 levels during vitellogenesis is reported from two close relatives of the Japanese gecko: the leopard gecko (Rhen et al., 2000) and Madagascar ground gecko (Weiser et al., 2012). Since female Japanese geckos undergo vitellogenesis from April to July on Honshu Island (Ikeuchi, 2004), an effect of estrogen was expected. E2 administration resulted in the downregulation of *g6pc1-1* and *pck2* expression, but not *g6pc1-2* expression (Fig. 6C). To infer the molecular basis behind such paralog-specific responses of *g6pc1*, the ERE was searched within genomic regions up to 1 kbp upstream of the *g6pc1* transcription start site. A single ERE half-site was present –928 bp upstream of *g6pc1-1*, whereas no ERE was present upstream of *g6pc1-2*. These results suggest that the paralog-specific E2 response is determined by the distribution of the ERE upstream of each gene.

As *g6pc1-2* expression in female geckos was lower in April than in September, seasonal and sex-specific factors other than estrogen were expected to regulate this gene. One possible candidate for this was progesterone. Blood progesterone levels have been reported to increase during the reproductive period in other gecko species, including the leopard gecko and Madagascar ground gecko (Rhen et al., 2000; Weiser et al., 2012). However, a PRE was not present within at least 1 kbp upstream of either of the *g6pc1* genes. This result implies that progesterone is not involved in the regulation of *g6pc1* genes. Further investigation is required to fully understand the seasonal expression of *g6pc1-2*.

The advantage of such paralog-specific responses to E2 could be related to the maintenance of fertility. The downregulation of *g6pc1-1* and *pck2* expression by E2 suggests that this hormone represses gluconeogenesis. Similar observations have been reported for both mammalian and non-mammalian vertebrates (Petersen et al., 1983; Qiu et al., 2017). Such downregulation has been proposed to be beneficial for the maintenance of glucose homeostasis under normal conditions (Qiu et al., 2017). However, this can adversely affect female reproduction. Using medaka (*Oryzias latipes*) as a model, Hasebe et al. (2017) revealed that the firing activity of female GnRH1, which is essential for reproduction, is induced only when blood glucose levels are sufficiently high. That report implies that maintenance of blood glucose levels is essential for reproduction. However, an increase in blood estrogen levels during vitellogenesis can downregulate gluconeogenesis, resulting in lower blood glucose levels and impaired fertility. Under such conditions, estrogen-insensitive *g6pc1-2* can maintain glucose production and safeguard female fertility. One important feature of *g6pc1* is that it facilitates not only gluconeogenesis but also glycogenolysis. While other gluconeogenic enzymes, such as *pck2*, only regulate reactions in the gluconeogenesis pathway, *g6pc1* catalyzes the common and last reaction shared by gluconeogenesis and glycogenolysis. This glycogenolytic function enables *g6pc1* to safeguard glucose production even when gluconeogenesis is downregulated.

The "safeguarding" role of duplicated *g6pc1* genes could also explain why non-avian reptiles retained more than one *g6pc1* gene (Fig. 8). According to Wieser (1985), ectotherms invest a larger portion of metabolizable energy into reproduction than endotherms do, and are susceptible to mortality from excess investment in reproduction. As a result, ectotherms need to take great care when partitioning energy between their own survivorship and reproduction. Duplicated *g6pc1* genes may aid in energy partitioning by fine-tuning glucose production. As described above, *g6pc1*

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catalyzes the last reaction shared by gluconeogenesis and glycogenolysis. Thus, this gene is in a position to single-handedly affect the net glucose production. By retaining more than one *g6pc1* gene and putting them under independent control, ectotherms, in theory, can enjoy greater flexibility in regulating glucose production than endotherms, which only possess a single *g6pc1*.

CONCLUSION

The present study re-examined the evolutionary history of the vertebrate *g6pc1* gene, with a particular focus on sarcopterygians. Molecular phylogenetic analyses suggest that *g6pca* and *g6pcb* emerged in the actinopterygian lineage after the actinopterygian-sarcopterygian split. This contrasts with a previous report (Marandel et al., 2017) that assumed the origin of *g6pca* and *g6pcb* before the actinopterygian-sarcopterygian split. Comprehensive analyses of molecular evolution among sarcopterygians revealed the duplication of *g6pc1* in the common ancestor of reptiles and aves, giving rise to the *g6pc1-1* and *g6pc1-2* classes. While the aves lost all but one gene, the ectothermic reptiles retained at least two genes. Quantitative RT-PCR analyses in the liver of Japanese geckos revealed that *g6pc1-1* and *g6pc1-2* respond to E2 differently. This observation implies that functional divergence between paralogs is beneficial for the reproduction of ectothermic reptiles.

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COMPETING INTERESTS

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

GY designed the study, carried out all experiments, drafted and prepared the manuscript. MKP designed the study and helped with the preparation of the manuscript. SM participated in the design of the study and helped prepare the manuscript. All authors gave final approval for publication.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available online. (URL: https://doi.org/10.2108/zs210113)

Supplementary Table S1. List of *g6pc* family genes used for phylogenetic analyses.

Supplementary Figure S1. Neighbor-joining phylogenetic tree based on amino acid sequences of vertebrate *g6pc1*, *g6pc2*, and *g6pc3*.

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