




Noninsect-Based Diet Leads to Structural and Functional Changes of Acidic Chitinase in Carnivora

Eri Tabata ^{1,2}, Akihiro Itoigawa ³, Takumi Koinuma,¹ Hiroshi Tayama,¹ Akinori Kashimura,¹ Masayoshi Sakaguchi,¹ Vaclav Matoska,⁴ Peter O. Bauer,^{4,5} and Fumitaka Oyama ^{*,1}

¹Department of Chemistry and Life Science, Kogakuin University, Tokyo, Japan

²Research Fellow of Japan Society for the Promotion of Science (PD), Tokyo, Japan

³Department of Cellular and Molecular Biology, Primate Research Institute, Kyoto University, Aichi, Japan

⁴Laboratory of Molecular Diagnostics, Department of Clinical Biochemistry, Hematology and Immunology, Homolka Hospital, Prague, Czech Republic

⁵Bioinova JSC, Prague, Czech Republic

*Corresponding author: E-mail: f-oyama@cc.kogakuin.ac.jp.

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Abstract

Acidic chitinase (Chia) digests the chitin of insects in the omnivorous stomach and the chitinase activity in carnivorous Chia is significantly lower than that of the omnivorous enzyme. However, mechanistic and evolutionary insights into the functional changes in Chia remain unclear. Here we show that a noninsect-based diet has caused structural and functional changes in Chia during the course of evolution in Carnivora. By creating mouse-dog chimeric Chia proteins and modifying the amino acid sequences, we revealed that F214L and A216G substitutions led to the dog enzyme activation. In 31 Carnivora, *Chia* was present as a pseudogene with stop codons in the open reading frame (ORF) region. Importantly, the Chia proteins of skunk, meerkat, mongoose, and hyena, which are insect-eating species, showed high chitinolytic activity. The cat *Chia* pseudogene product was still inactive even after ORF restoration. However, the enzyme was activated by matching the number and position of Cys residues to an active form and by introducing five meerkat Chia residues. Mutations affecting the Chia conformation and activity after pseudogenization have accumulated in the common ancestor of Felidae due to functional constraints. Evolutionary analysis indicates that *Chia* genes are under relaxed selective constraint in species with noninsect-based diets except for Canidae. These results suggest that there are two types of inactivating processes in Carnivora and that dietary changes affect the structure and activity of Chia.

Key words: digestive enzyme, insectivores, carnivores, gene loss, acidic chitinase, Chia.

Introduction

The ancestor of all placental mammals was a tiny insect-eating creature that evolved soon after the mass extinction of the dinosaurs (O'Leary et al. 2013). This event opened a niche for species with diets that led to the emergence of a wide variety of mammals, from carnivores to herbivores and omnivores. Such dietary changes have been a significant adaptive force in shaping mammalian variation, sometimes causing loss of protein-coding genes (Zhao et al. 2010; Hecker et al. 2019). In addition to the genetic information, the function of the translated product is also an evolutionarily important aspect. However, knowledge on how and why they lost their function is still limited.

Acidic chitinase (Chia; also referred to acidic mammalian chitinase, "AMCase"; Boot et al. 2001, 2005) hydrolyzes the β -1, 4 glycoside bonds of chitin, a major structural polysaccharide in insects (Bueter et al. 2013; Wysokowski et al. 2015; Van Dyken and Locksley 2018). Chia is expressed in the stomachs

of insectivorous and omnivorous animals including bat (Strobel et al. 2013), mouse (Ohno et al. 2016), pangolin (Ma et al. 2018), crab-eating monkey (Uehara et al. 2018, 2021), and common marmoset (Tabata, Kashimura, et al. 2019). In addition, omnivorous livestock animals, such as chicken and pig, express Chia at high levels in their stomachs and are able to degrade chitin and chitin-containing materials such as shrimp shells and mealworm larvae shells under normal gastrointestinal conditions (Tabata, Kashimura, Wakita, Ohno, Sakaguchi, Sugahara, Imamura, et al. 2017; Tabata, Kashimura, Wakita, Ohno, Sakaguchi, Sugahara, Kino, et al. 2017; Tabata, Kashimura, Wakita, et al. 2018; Tabata, Wakita, et al. 2019). On the other hand, certain animals, such as dog and bovine, express low levels of Chia mRNA and proteins accompanied by a marked decrease in chitinolytic activity (Tabata, Kashimura, Kikuchi, et al. 2018). Based on these results, we hypothesized that in the absence of dietary chitin, functional constraints on the *Chia* gene might have been

relaxed, allowing for the accumulation of mutations and resulting in a decrease of the chitinolytic activity.

Recent genomic research has shown that the insectivorous placental mammalian ancestor possessed five *Chia* genes and that repeated losses of these genes occurred in mammals with a limited amount of invertebrates in their diet (Emerling et al. 2018). Additionally, the number of paralogous *Chia* genes in nonhuman primates is related to insect consumption and body size (Janiak et al. 2018). Thus, the *Chia* gene modifications leading to functional loss provide a molecular record for a gene that has evolved from an insectivorous ancestor (Emerling et al. 2018).

Here, we report that the structure and activity of carnivorous *Chia* have changed in species with a noninsect-based diet. Our results indicate that two amino acid substitutions reduced the activity of *Chia* in Canidae. Simultaneously, *Chia* was pseudogenized in these species (except for Canidae) and Cys mutations and amino acid substitutions at functional sites occurred due to relaxation of functional constraints. In contrast, Carnivora species that feed on insects retain highly active *Chia*. The data presented here may help to better understand the changes in gene evolution associated with noninsect-based diets.

Results

F214L and A216G in Dog *Chia* Are Crucial for High Chitinolytic Activity

Recently, we reported that chitin is well digested in omnivores due to the expression of sufficient levels of *Chia* mRNAs and active proteins as compared with carnivores and herbivores, with low levels and activities of these enzymes (Tabata, Kashimura, Kikuchi, et al. 2018). The activity of dog *Chia* is several times lower than that of mouse despite their sequence homology being ~80%.

To identify the regions responsible for the reduced chitinolytic activity in dog *Chia*, we expressed chimeric mouse-dog enzymes in *Escherichia coli* (fig. 1A; supplementary figs. S1–S3, Supplementary Material online) and analyzed the chitinolytic activity of each chimera using a synthetic fluorogenic substrate 4-methyl umbelliferyl β -D-N, N'-diacetyl chitobioside [4-MU-(GlcNAc)₂].

Chimeras C1 (coded by mouse exons 3–10/dog exon 11) and C2 (coded by mouse exons 3–7 and dog exons 8–11) exhibited strong chitinolytic activity comparable to that of wild-type (WT) mouse *Chia* (fig. 1B). However, the activity dropped with the presence of dog exons 6–11 (Chimera C3; fig. 1B). The chitinolytic activities were very low in chimeras C4 and C5 as compared with that of mouse *Chia*. In contrast, chimera C6 showed strong activity and the level was similar to that of the mouse enzyme (fig. 1B). These results indicate that exons 6 and 7 are essential in chitinolytic activity determination in *Chia*, with mouse sequence activating and dog sequence deactivating the enzyme.

When only the region coded by exons 6 and 7 was replaced between the two enzymes, the mouse enzyme was inactivated by exons 6 and 7 from the dog (Chimera C7). Conversely, the dog enzyme with mouse exons 6 and 7 was

activated (Chimera C8) (supplementary fig. S4, Supplementary Material online). These results confirm that exons 6 and 7 control dog and mouse *Chia* activity.

Further analysis of the chimeric and mutant proteins (Chimeras C9–C12, dog FLG, and dog TE mutants) revealed that three amino acids at the N-terminus of the exon 7 region are strongly involved in the inactivation of dog *Chia* (supplementary fig. S4, Supplementary Material online). To narrow down the amino acids involved in this activation, we introduced F214L and A216G to dog *Chia* (dog LG mutant; fig. 1C; supplementary figs. S2 and S3, Supplementary Material online). These two amino acid substitutions achieved a 9-fold increase in the activation of the enzyme (fig. 1D). Conversely, we introduced L214F and G216A into mouse *Chia* (mouse FA mutant; fig. 1C; supplementary figs. S2 and S3, Supplementary Material online). Strikingly, the mouse FA mutant had significantly lower chitinase activity than the WT mouse *Chia* (fig. 1D). Interestingly, substitutions of single amino acids had no significant effect on the enzymatic activity (dog 214L or 216G mutants; supplementary fig. S4, Supplementary Material online), confirming that both amino acids are required for dog *Chia* activation.

We created a homology model of the mouse and dog *Chia* catalytic domain (CatD) based on the X-ray crystal structure of the same region in a human CHIA CatD (Olland et al. 2009). The L214 and G216 residues (mouse type) align near Y212 and D213, which are essential for catalytic reactions (Olland et al. 2009; fig. 1E, left). In dog *Chia*, these residues have been changed to F214 and A216, the former having bulky aromatic side group that seems to be an obstacle for sugar-substrate conformation (fig. 1E, right).

Chia ORFs Are Maintained Only in Canidae and Insectivorous Species

The activity of dog *Chia* was restored by F214L and A216G substitutions. As described above, mice are omnivorous and feed on chitin-containing insects. In contrast, dogs are carnivorous and their diet largely lacks chitin-containing organisms. To further clarify the relationships between the noninsect-based diet and the two amino acid substitutions, we analyzed the diets and *Chia* open reading frame (ORF) integrities of 40 carnivorans concerning two inactivating amino acid substitutions identified in dog *Chia*. We obtained *Chia* nucleotide sequences of these carnivorans from the NCBI Genome database (supplementary table S1 and data file 1, Supplementary Material online). Since phylogenetic trees generated from an alignment of the coding sequences did not reflect the established phylogenetic relationships (supplementary fig. S5, Supplementary Material online), we used a published phylogeny of Carnivora to examine the relationship between dietary habits and the protein-coding regions of *Chia* (fig. 2A–D).

The amino acids F214 and A216 were conserved only in Canidae, including *Lycan pictus*, *Vulpes vulpes*, and *Vulpes lagopus*, while the sequences being otherwise close to that of mouse *Chia*. This observation indicates that the *Chia* inactivation event observed in dogs occurred only in Canidae (fig. 2A).

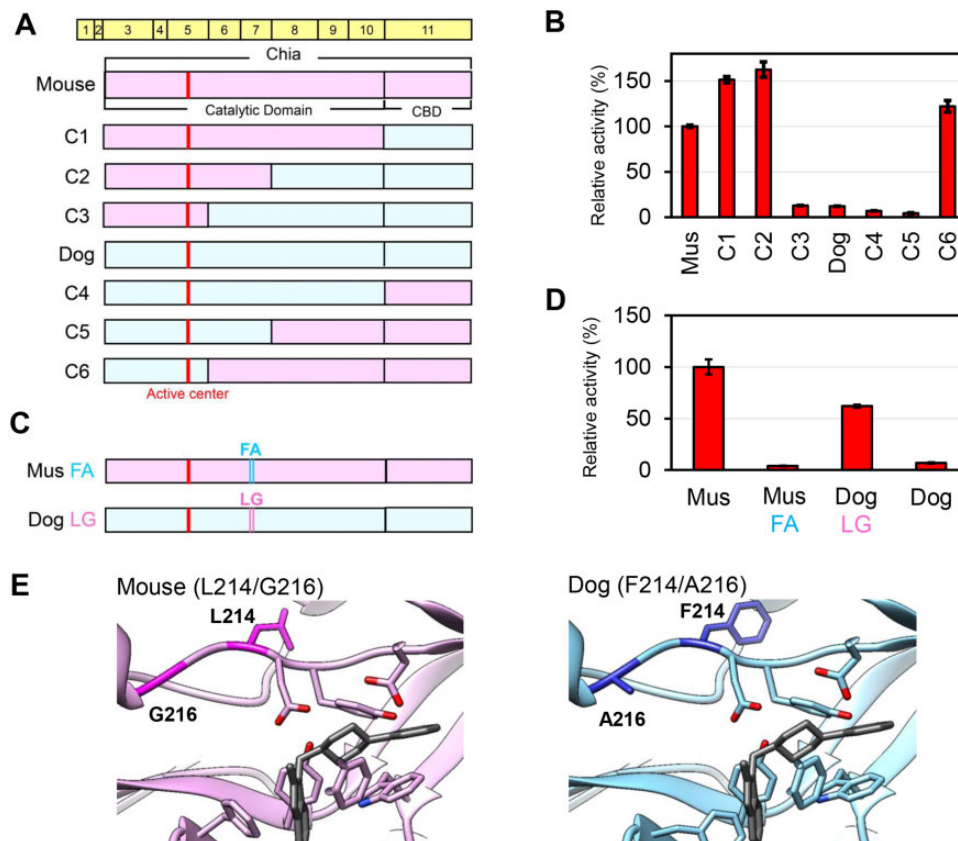


Fig. 1. Two amino acid substitutions can activate dog Chia. (A) Schematic representation of *E. coli*-expressed Chia chimeric proteins. The amino acid sequences are color-coded as follows: pink, mouse sequence; blue, dog sequence. (B) Comparison of the chitinolytic activities of Chia proteins outlined in (A). (C) Schematic representation of *E. coli*-expressed Chia mutant proteins. (D) Comparison of the chitinolytic activities of mutant proteins outlined in panel C with mouse and dog Chia. (E) A homology model of the CatD shows that the identified amino acid substitutions (pink in mouse, left; blue in dog, right) are close to the residues interacting with the carbohydrate chain (gray).

Species with noninsect-based diets (meat, fish, fruits, or leaves) contain premature stop codons in the coding regions (fig. 2A–D) caused by deletions, insertions, and substitutions. Felidae shares a deletion in exon 3 that causes a frameshift leading to a premature stop codon (fig. 2C; supplementary figs. S6 and S7, Supplementary Material online). A premature stop codon due to an amino acid substitution was also found in exon 10 in Mustelidae. Phocoidea family shares 2-bp substitutions and 11–13-bp insertions in exon 11. Chia of Ursidae has five premature stop codons due to a 1–2-bp deletion in exon 1, a 1-bp deletion and substitution in exon 7, a 2-bp substitution in exon 10, and a 1-bp substitution in exon 11. These mutations are not conserved in *Cryptoprocta ferox*, *Crocota crocuta*, *Paradoxurus hermaphroditus*, or *Ailurus fulgens*. Since exon 1 of *Chia* could not be found in the genomes of the Mustelidae and Ailuridae, the common ancestor of the two groups may have pseudogenized due to the lack of a partial sequence containing the start codon. Thus, the pseudogenization of *Chia* seems to have evolved at least seven times independently in Carnivora (fig. 2A).

In contrast to Canidae, Mephitidae, Hyaenidae, and Herpestidae, containing skunk, hyena, mongoose, and meerkat, which often feed on insects, preserved the full-length *Chia* ORF with 214L and 216G residues (fig. 2A–D). These

observations strongly suggest that species with noninsect-based diets have lost parts of the *Chia* ORF, whereas insect-eating species maintain the full-length ORF, which leads to a gene product with high chitinolytic activity (fig. 2A–D).

Chia Exhibits Strong Chitinase Activity in Insect-Eating Species

Above, we have shown the relationship between *Chia* gene conservation and feeding behavior in carnivorans (fig. 2). The *Chia* sequences without protein-coding regions had insertions, deletions, and mutations in active sites or Cys residues (supplementary fig. S7, Supplementary Material online).

Insectivorous species have a conserved catalytic motif (DXXDXDXE) and 12 conserved Cys residues in the Chia proteins. In addition, these enzymes have L and G residues at positions 214 and 216, respectively (mouse type) (fig. 3A; supplementary figs. S2 and S3, Supplementary Material online). Based on our previous findings (Tabata, Kashimura, Kikuchi, et al. 2018) and the gene structure of *Chia*, we anticipated that insectivorous Chia proteins would have high chitinolytic activity. To test this assumption, we synthesized five insectivorous *Chia* cDNAs (banded mongoose, meerkat, common dwarf mongoose, striped hyena, and skunk) and expressed them as described above. All enzymes displayed

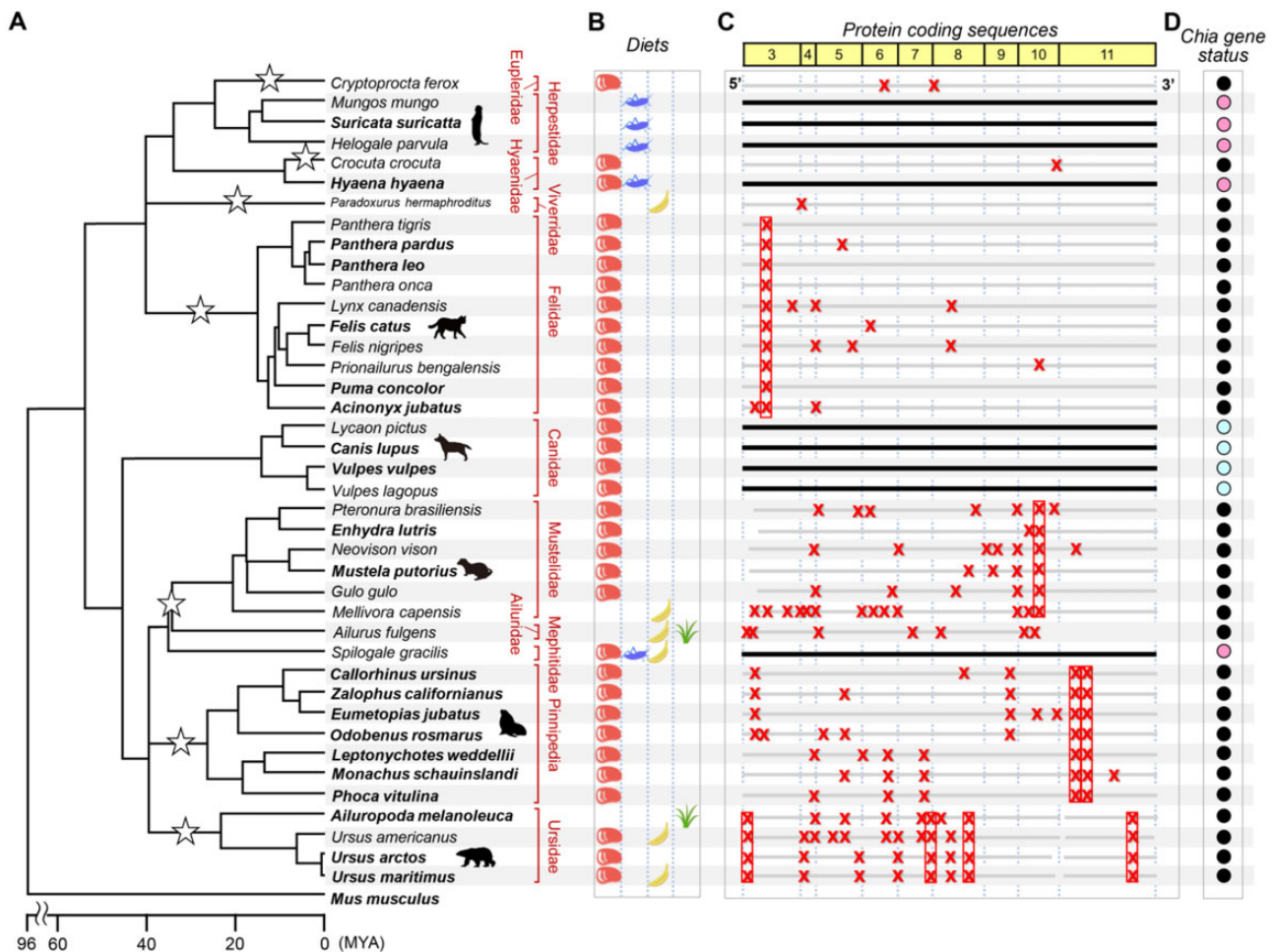


Fig. 2. *Chia* gene loss in Carnivora. (A) Phylogenetic tree of 40 carnivorans. Phylogenetic relationships and divergence times were obtained from TimeTree version 3.0 (<http://www.timetree.org/>). A scientific name in bold means that *Chia* sequences were annotated genes and otherwise assembled manually from the genome by BLAST using the dog *Chia* cDNA sequence. Stars indicate a pseudogenization event, but the timing is unknown. (B) The main diet in each species is shown in the illustration; pink, meats; purple, insects or crustaceans; yellow, fruits or nectar; green, plants. (C) The protein-coding region of *Chia*. X, the positions of loss-of-function mutations and the mutations shared in each family are indicated by boxes. (D) Whether it is a protein-encoding gene is indicated by the closed circle; pink, the protein-encoding gene with 214L and 216G (active type); blue, protein-encoding gene with 214F and 216A (inactive type); black, lack of full-length protein-coding region (pseudogene). The illustrations in (A) and (B) (copyright AC Works Co., Ltd.) are used with permission.

the highest activity at pH 2.0, 4–9 times higher than that of dog *Chia* (fig. 3B). These results further suggest that the chitinolytic activity of *Chia* is strongly related to feeding behaviors in the Carnivora group.

Estimating the Time of Replacement of L214F and A216G in Canidae

To estimate when the inactivation substitutions occurred in dog *Chia*, a total of ten *Chia* sequences from Canidae species were analyzed (fig. 4A). These sequences were identified from the NCBI Gene database or were estimated from the NCBI Genome database or the NCBI Sequence Read Archive (SRA) database using dog *Chia* as a reference gene (supplementary table S2 and data file 1, Supplementary Material online).

We found that inactivating substitutions (F214 and A216) were conserved in almost all Canidae except for gray fox (*Urocyon cinereoargenteus*). The gray fox conserved Gly at position 216 (active type), but a single substitution

significantly reduced the enzyme activity (supplementary fig. S4, Supplementary Material online), indicating that *Chia* is inactive in extant Canidae species (fig. 4B). The genus *Urocyon* is considered the most basal of living canids (Lindblad-Toh et al. 2005). Thus, *Chia* was inactivated in a common ancestor of Canidae, which may have occurred at least 13 Ma.

We also found a substitution in lycaon *Chia* within for 12 conserved Cys in *Chia* described above. The first Cys was replaced by Ser, affecting the enzyme activity (fig. 4C). To test this possibility, lycaon *Chia* (WT-lycaon) and the enzyme with a mutation to Cys (Cys-lycaon) were expressed in *E. coli*, and the enzyme activity was measured as described above (fig. 4C, supplementary figs. S1 and S2, Supplementary Material online). While WT-lycaon *Chia* showed no chitinase activity, the Cys-lycaon enzyme showed activity equal to that of WT-dog *Chia* (fig. 4D, supplementary fig. S3, Supplementary Material online). This result suggests that

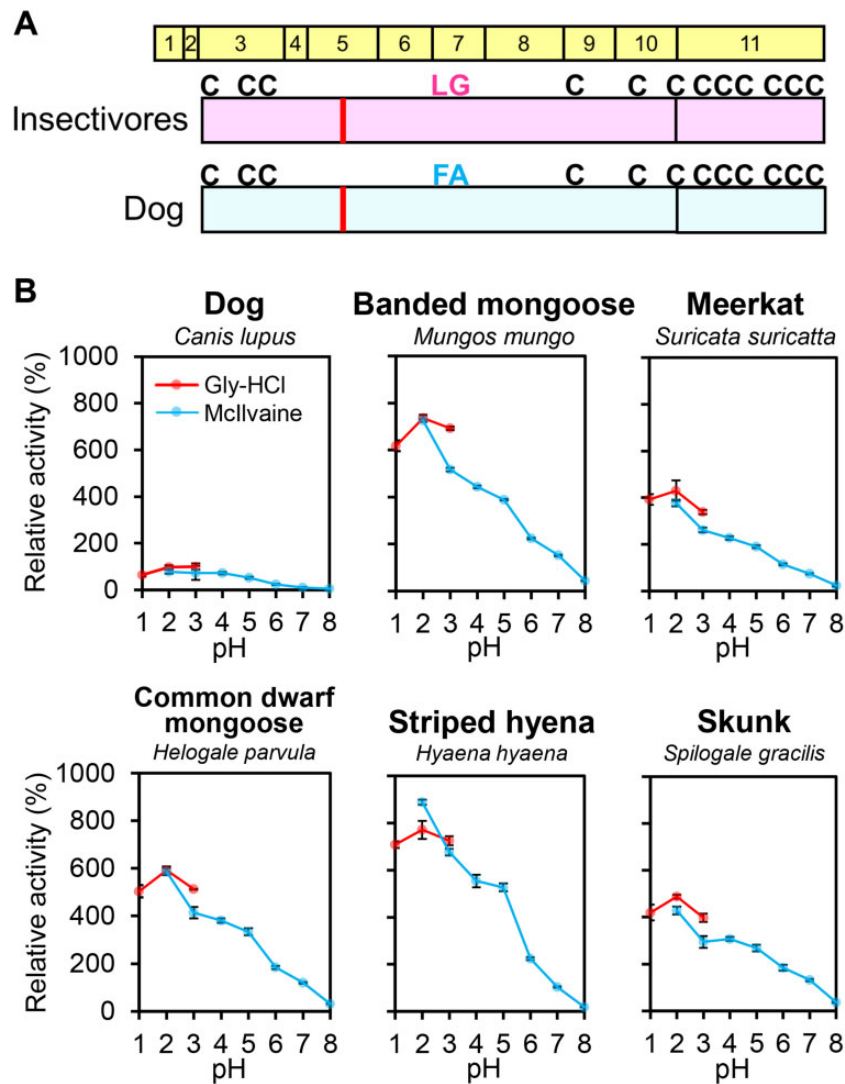


FIG. 3. Insect-eating species encode full-length chitinase and exhibit high chitinase activity. (A) Schematic representation of *E. coli*-expressed dog and insectivorous Chia. C, Cys residues conserved in active Chia proteins; FA or LG, the amino acids that controlled the activity of Chia are color-coded as follows: pink, mouse sequence; blue, dog sequence. (B) Comparison of the chitinolytic activities of dog, banded mongoose, meerkat, common dwarf mongoose, striped hyena, and skunk Chia proteins using 4-MU-(GlcNAc)₂. Error bars represent the mean \pm SD from a single experiment conducted in triplicate.

lycaon Chia has undergone a mutation to Cys after a previous two-amino acid substitution, reducing its activity.

Activation of the Cat Chia Pseudogene Revealed the Structure Required for Chitinase Activity

To clarify how *Chia* was pseudogenized, we attempted to activate cat Chia. The ORFs of the corresponding *Chia* pseudogenes were restored by engineering the molecule with “incorrect” bases (we named it created-cat or Cre-Cat Chia) (fig. 5A; supplementary figs. S2 and S3, Supplementary Material online). Cre-Cat Chia was expressed as a soluble protein but showed no activity (fig. 5B, left).

Next, we focused on the conservation of Cys residues in the Chia molecule. Chia enzymes from omnivorous animals such as mouse, chicken, pig, and marmoset show high chitinolytic activity. They have six Cys residues in

the CatD and in the chitin-binding domain. Thus, we postulated that these 12 Cys residues conserved in Chia are essential for restoring chitinolytic activity. Four mutations to Cys were corrected and expressed as Cys-Cat Chia, but the enzyme remained inactive (fig. 5B, middle). This result indicates the relaxation of the functional constraint not only in Cys residues comprising disulfide bonds but also some unknown amino acid positions involved in protein structure and function.

Then we created meerkat-cat chimeric enzymes (fig. 5C; supplementary figs. S2 and S3, Supplementary Material online) and analyzed the chitinolytic activity of each chimera to identify the regions responsible for the loss of chitinolytic activity in cat Chia, in the same manner as described above. Chimeras C1 and C2 coded by meerkat exons 3–7 and cat exons 8–11 exhibited strong chitinolytic activity comparable to that of WT meerkat Chia (fig. 5D). However, the activity

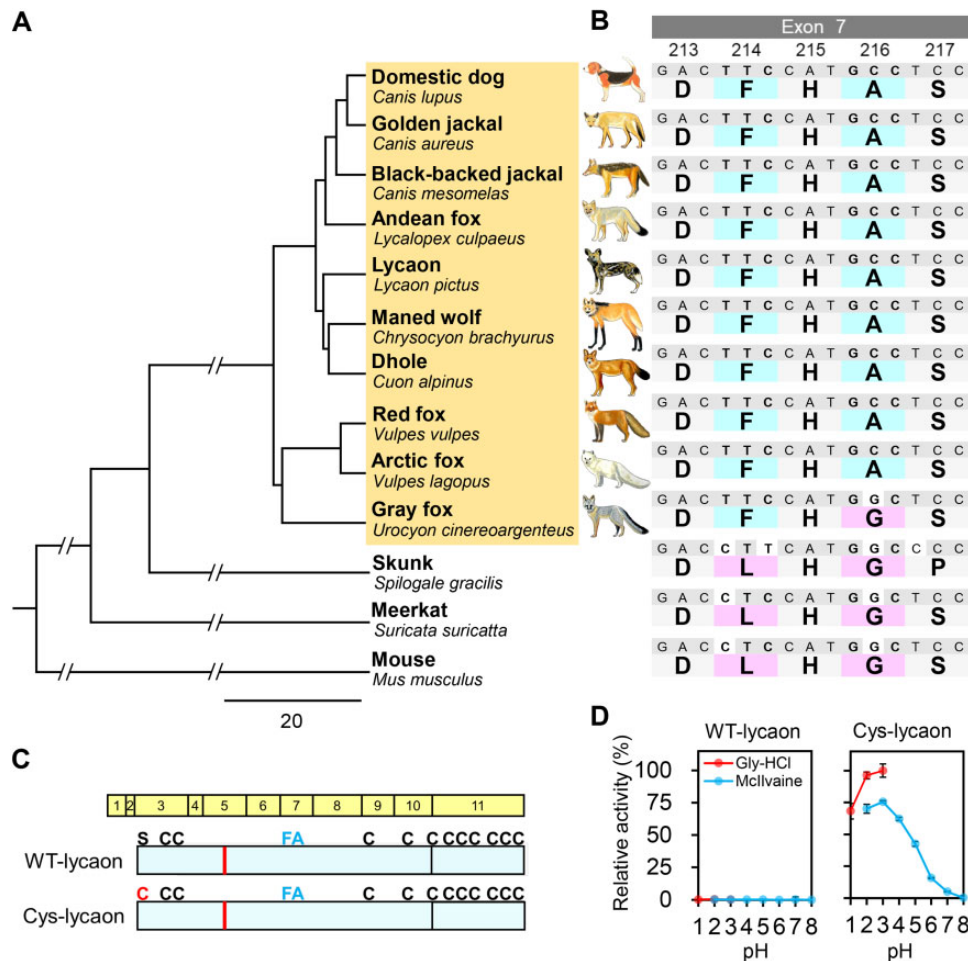


FIG. 4. The timing of Chia's inactivating substitution events in Canidae. (A) Phylogenetic tree of ten canids, including outgroup species. The scale bar indicates the divergence times (20 Mya) obtained from TimeTree version 3.0 (<http://www.timetree.org/>). (B) Amino acid sequences of exon 7 regions of canids and outgroup *Chia*. The amino acids at positions 214 and 216 that control the activity of Chia are color-coded as follows: pink, mouse sequence; blue, dog sequence. (C) Schematic representation of WT lycaon Chia (WT-lycaon) and Chia mutant protein named Cys-lycaon. (D) Comparison of the chitinolytic activities of Chia proteins outlined in (C). The relative activity when the WT lycaon Chia activity level at pH 2.0 was set to 100% is shown. Error bars represent the mean \pm SD from a single experiment conducted in triplicate. The illustrations were drawn by Eri Tabata.

dropped with cat exons 6–11 (Chimera C3; [fig. 5D](#)). In contrast, chitinolytic activities were not detected in chimeras C4, C5, and C6 ([fig. 5D](#)). These results indicate that the N-terminus region encoding exons 3–7 is essential for gaining chitinolytic activity in cat Chia.

To reveal the contribution of each exon to activity regulation, we generated further meerkat-cat chimeras C7–C10 ([supplementary figs. S2, S3, and S8, Supplementary Material online](#)) and showed that the meerkat exons 5 and 7 regions are responsible for activating the cat enzyme ([supplementary fig. S8, Supplementary Material online](#)). Next, we constructed mutants to narrow down the amino acids involved in this activation ([supplementary figs. S2 and S3, Supplementary Material online](#)). Five amino acid substitutions named Cys-Cat-5 Mut (N118T, D143G, and P158L in exon 5; P215H and G223A in exon 7) ([fig. 5E, supplementary fig. S7, Supplementary Material online](#)), showed the highest activity at pH 2.0 and achieved 50% activity of the meerkat enzyme ([fig. 5B, right](#)). This result provided an experimental evidence

of the relaxation of functional constraints on amino acid positions involved in protein structure and function after the ORF disruption ([fig. 5F](#)).

The Selective Constraints Were Relaxed in *Chia* Pseudogenes

Based on the enzymatic experiments shown above, we can divide Carnivora into three groups: 1) species feeding on insects and maintaining *Chia* (skunk, meerkat, mongoose, and hyena); 2) species not feeding on insects but maintaining full-length *Chia* (dog); and 3) species not feeding on insects and that have lost *Chia* (ferret, walrus, bear, cat, etc.).

To test whether the selective constraints on the *Chia* genes differed among the above three groups, we performed analysis using the CODEML program in the PAML package ([Yang 2007](#)), estimating the dN/dS ratios (ω) for different branches. We evaluated the fit of the following branch models to the data: 1) the null one-ratio model (M0), which assumes the same ω value for all branches; 2) a two-ratio model (M2), in

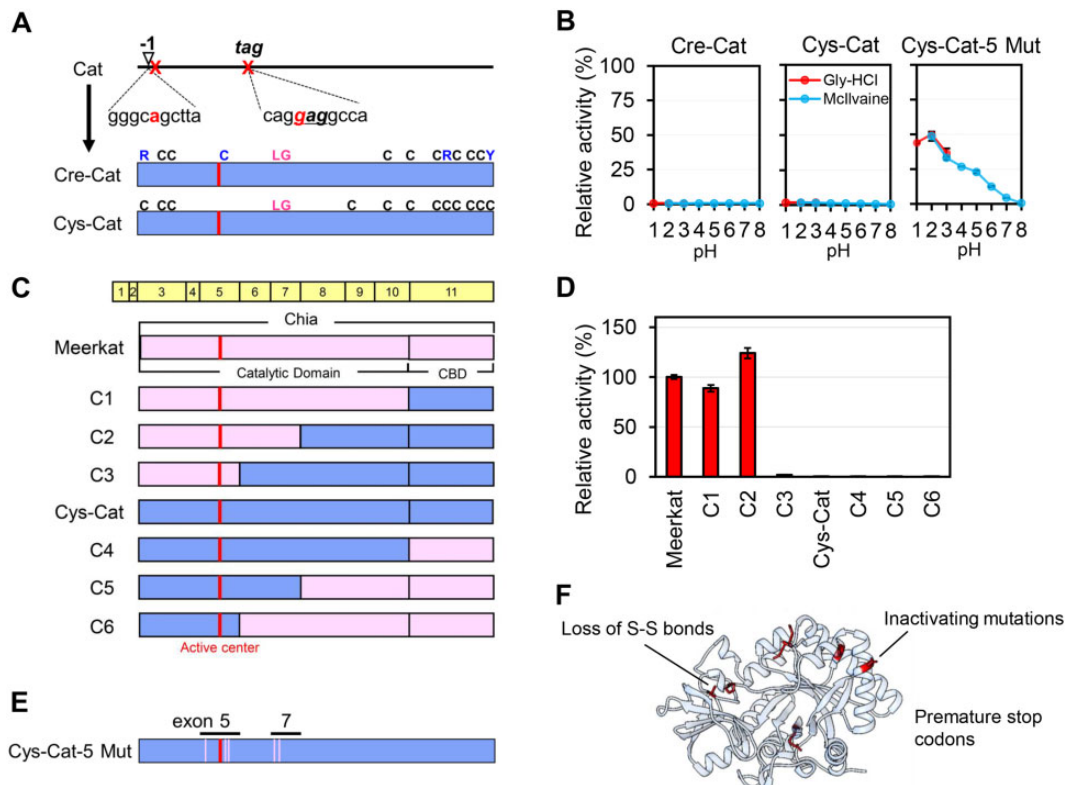


Fig. 5. The evolutionary process of the cat *Chia* pseudogene from ancestrally active proteins. (A) Graphical display of the coding region of cat *Chia* and the positions of the inactivating mutations. ATG, start codon; indel, with the number above indicating the number of bases deleted; X, stop codon. Cre-cat, creation of functional *Chia* proteins that restored their ORFs from their genes. C (black), a Cys position conserved in mammalian chitinase, and C (blue), a Cys distinct from the conserved position among mammalian chitinases. FA, the amino acid that controls the activity of *Chia* and is the same as the mouse sequence. Cys-cat, creation of functional *Chia* proteins that restored their positions and number of Cys. (B) Comparison of the chitinolytic activities of *Chia* proteins outlined in (A) and (E). We showed relative activity when the meerkat *Chia* activity level at pH 2.0 was set to 100%. Error bars represent the mean \pm SD from a single experiment conducted in triplicate. (C) Schematic representation of *Chia* chimeric proteins narrowing down the responsible region. The amino acid sequences are color-coded as follows: pink, meerkat sequence; blue, Cys-cat sequence. (D) Comparison of the chitinolytic activity of *Chia* proteins outlined (C) and their comparison with a meerkat and Cys-cat *Chia*. (E) Schematic representation of the *Chia* mutant protein named Cys-cat-5 Mut. (F) A homology model of the cat *Chia* mutant proteins shows *Chia* pseudogene evolution from an ancestrally active enzyme. The identified amino acid substitutions were color-coded red.

which two separate ω values were estimated for species with pseudogenes ($\omega_{\text{foreground}}$) and functional genes ($\omega_{\text{background}}$); and 3) a three-ratio model (M3), where pseudogenes (ω_1), Canidae (ω_2), and insectivorous ($\omega_{\text{background}}$) branches were allowed to have different ω . We then compared the fit of models using likelihood ratio tests (LRTs). M2 provided a significantly better fit than M0 ($\chi^2 = 94.7$, $P < 0.001$). Pseudogenes had almost three times higher ω ($\omega_{\text{foreground}} = 0.711$) than the background ω of species with functional genes ($\omega_{\text{background}} = 0.236$), indicating relaxation of purifying selection (table 1). However, M3 did not show better fitness than M2 ($\chi^2 = 0.329$, $P = 0.566$), indicating that there was no significant difference in the strength of functional constraints between Canidae and insectivorous species (table 1).

We then used another approach implemented in RELAX, which computes the values and distribution of three ω using a branch-site model (Wertheim et al. 2015). RELAX tests whether the test branch has relaxed or enhanced selection compared with the reference branch during evolution. The magnitude of convergence depends on a parameter, k , which

tends to zero as selection tends to complete relaxation. In contrast to expectations, this test did not support the hypothesis of relaxed selection in species where the gene underwent pseudogenization ($k = 1.02$, $P = 0.811$, LR = 0.06, table 2, supplementary fig. S9, Supplementary Material online). The second test comparing Canidae to insectivores also showed no significant changes in selection constraints acting on Canidae branches ($k = 1.18$, $P = 0.265$, LR = 1.24, table 2, supplementary fig. S9, Supplementary Material online). These results suggest that there are no significant differences in the selection intensity on the *Chia* gene between Canidae and insectivores.

Chia Molecules Evolved from Insectivorous Ancestors in Carnivora

Previous reports have shown that ancestral mammals were insectivores. After the Cretaceous–Paleogene boundary, which marked the end of the age of the dinosaurs ~ 65 Ma, these mammals were subjected to noninsect-based diets (O’Leary et al. 2013; Emerling et al. 2018). Based on our results, we speculated on how the structure and function of the *Chia*

Table 1. Estimated parameters of relaxed selection tests using branch models in CODEML.

Model	$\omega_{\text{background}}$	$\omega_{\text{foreground}}$	κ	TL	np	log L	LR	P
M0 (null):one-ratio model	0.507	0.507	2.96	4.06	94	-11,481	—	—
M2: two-branch classes	0.236	0.711	2.97	4.08	95	-11,434	94.7	0.000
M3: three-branch classes	0.221	ω_1 :0.711	2.97	4.05	96	-11,434	0.329	0.566
	—	ω_2 :0.260	—	—	—	—	—	—

ω , ratio of nonsynonymous to synonymous substitutions, ω_1 , foreground for pseudogene branches; ω_2 , foreground for Canidae branches; κ , transition/transversion rate; TL, tree length; np, number of parameters; log L, log likelihood; P, P-value of the LRT of M0 versus M2 or M2 versus M3 models.

Table 2. Estimated Parameters of Relaxed Selection Using RELAX.

Test Branches	Reference Branches	Model	log L	np	k	AIC _c	LR	P
Pseudogenes	Functional genes	Null	-11,250	115	1	—	—	—
		Alternative	-11,250	116	1.02	—	0.06	0.811
Canidae	Insectivores	Null	-11,242	115	1	—	—	—
		Alternative	-11,242	116	1.18	—	1.24	0.265

k, selection intensity; AIC_c, sample size-corrected Akaike Information Criterion; P, P value of likelihood ratio of alternative relative to null for each test.

gene changed from insectivorous ancestral mammals to carnivorans (fig. 6). Species with insect-based diets such as skunk and meerkat still retain Chia in an active form that can digest insect-derived chitin (fig. 6, upper). Species that feed on insects have a strong functional constraint on the gene (Active-type Chia). In contrast, Chia was inactivated in species with noninsect-based diets, dividing them into two groups. Even in the lineages with intact *Chia*, carnivorous lineages such as Canidae have inactive-type Chia, suggesting the strong association of Chia function with feeding habits in Carnivora (Inactive-type Chia) (fig. 6, middle). In most lineages with a noninsect-based diet, the functional constraints on the *Chia* gene were relaxed, leading to its pseudogenization (Functional loss-type Chia; fig. 6, lower).

Discussion

In this study, we created chimeric and mutant Chia proteins comprising mouse and dog sequences. We found two crucial amino acid substitutions that caused dog Chia inactivation. In 31 carnivoran species, *Chia* underwent pseudogenization by the accumulation of mutations throughout the whole coding region. In addition, insect-eating species such as skunk, meerkat, mongoose, and hyena with highly active enzymes show preserved complete ORFs with conserved Cys residues.

Dogs are among the first domesticated animals, occurring from at least 15,000 years ago (Pang et al. 2009; Skoglund et al. 2011). Although dietary changes leading to high levels of starch consumption had a significant effect on the dog's genomic signature (Axelsson et al. 2013) and the composition of their gut microbiota (Lyu et al. 2018), the protein sequence of dog Chia, which has lost its activity, still shows 98% and 100% identity to those of fox and wolf, respectively. This is probably because the domestication of dogs did not increase the consumption of insects and changed their diet from carnivorous to omnivorous. However, if they do not depend on insect digestion, why does the *Chia* gene have a preserved sequence? The evolutionary analysis based on the dN/dS ratio showed no differences in selection intensity on the *Chia* gene between Canidae ($\omega_2 = 0.260$) and insectivorous species

($\omega_{\text{background}} = 0.221$). From these results, we considered two possibilities: the first is the scenario where the loss of *Chia* is disadvantageous for survival. Unlike other carnivorans, Canidae completely lost another active chitinase gene (chitotriosidase, *Chit1*), and maintaining Chia activity was essential for purposes other than digestion. Since chitin is also present in pathogens such as mites and molds, Chia may be involved in biological defenses (Van Dyken et al. 2017). Another scenario is that the evolution of *Chia* has occurred relatively recently in this lineage and no ORF deterioration has occurred yet. Even though the functional constraints on Canidae *Chia* have not been relaxed, there was a mutation in the functional site of the gene. In particular, *Lycaon (L. pictus)* have a Cys-to-Ser-mutation that caused loss of enzyme activity. These results may indicate the relaxation of the functional constraints on *Chia* in this lineage.

Early in its evolutionary history, Carnivora evolved carnassial teeth that allowed them to be omnivorous. Later, the order split into two groups, Caniformia (dog-like carnivores; e.g., skunk) and Feliformia (cat-like carnivores; e.g., meerkat; Van Valkenburgh and Wayne 2010). Both skunk and meerkat are insectivorous and possess active Chia, suggesting that Chia maintained its active form in early carnivorans. The species that subsequently expanded their dietary niche to insects has experienced intense selective pressure for retaining the active form of Chia into the present.

Many reports on gene losses related to specific diets or environmental changes have been published (Koike et al. 2007; SharMa et al. 2018; Hecker et al. 2019). However, few studies have attempted to restore their function to understand how these genes lost their function. We have previously identified the inactivating factor in human CHIA by restoring its function (Okawa et al. 2016). Another study showed that functionally restored human-specific bitter taste receptor pseudogenes differed from chimpanzee functional orthologues (Risso et al. 2017). Additionally, the activation of the *Petunia secreta* transcription factor AN2 pseudogene revealed its role in color evolution in *Petunia* (Esfeld et al. 2018). Here, we restored the cat *Chia* pseudogene and found that, in

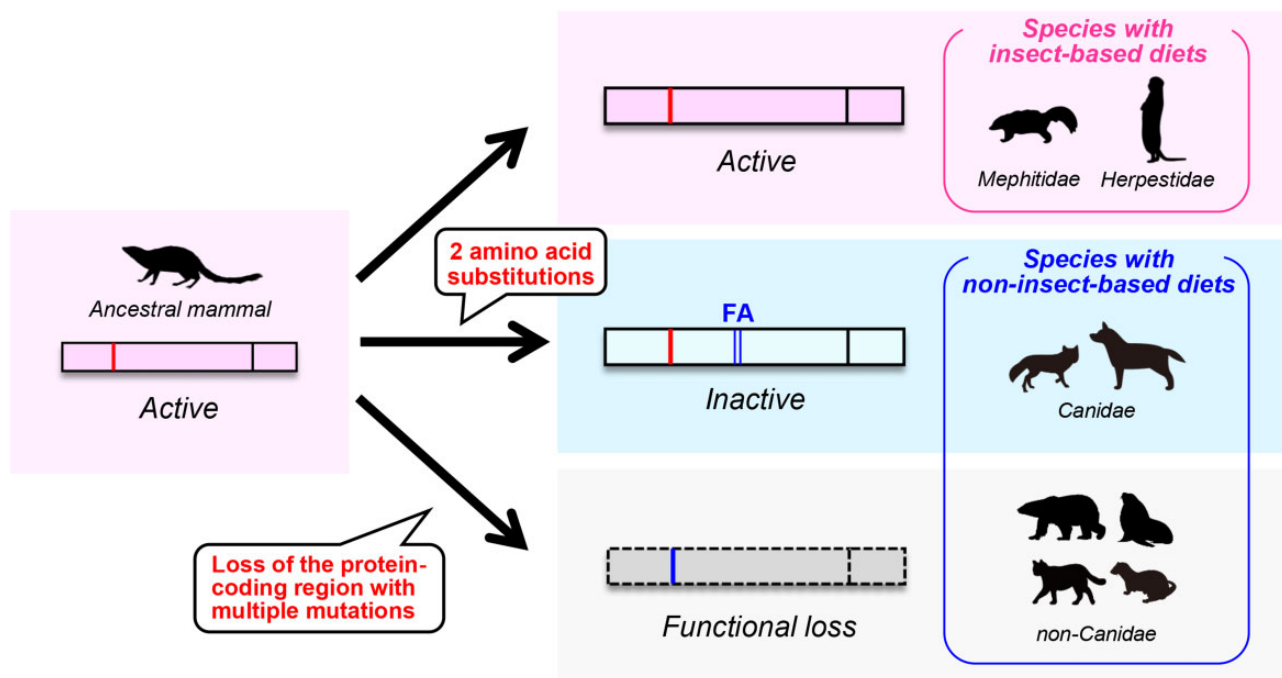


FIG. 6. Model explaining the evolution of the *Chia* gene in Carnivora. Schematic illustration of *Chia* gene evolution from an insectivorous ancestor with dietary changes. Species that feed on insects retain *Chia* with high chitinolytic activity (Active-type *Chia*). In species that do not feed on insects, the structure and activity of the gene have changed. The activity of *Chia* in Canidae was reduced by two amino acid substitutions (Inactive-type *Chia*). Except for Canidae, *Chia* has evolved into a nonfunctional gene (Functional loss-type *Chia*) due to the relaxation of functional constraints and the accumulation of mutations that cause stop codons and/or loss of activity.

addition to ORF disruption, the enzyme lost its activity due to Cys-mutations and 5 amino acid substitutions. Because some of these mutations were shared in Felidae, this lineage may first have experienced the loss of the ORF or Cys residues, followed by accumulation of further mutations involving residues affecting *Chia* activity due to the relation of functional constraints. The presented approach could provide a new perspective of gene loss as a pervasive source of genetic change with great potential in promoting evolutionary diversity.

Chia expression and/or activity levels are markedly altered in various diseases such as asthma and allergic inflammation (Zhu et al. 2004; Reese et al. 2007; Seibold et al. 2009; Okawa et al. 2016). *Chia*-deficient mice spontaneously accumulate environmentally derived chitin in their airways and develop age-related lung fibrosis, which can be ameliorated by the restoration of chitinase activity (Van Dyken et al. 2017; Van Dyken and Locksley 2018). In contrast, humans have significantly lower *Chia* levels and activity in their lungs (Ohno et al. 2013; Okawa et al. 2016). Specific polymorphisms and haplotypes of *Chia* are associated with bronchial asthma in humans (Bierbaum et al. 2005; Seibold et al. 2009; Okawa et al. 2016). From this point of view, the creation of active *Chia* is essential for preventing chitin accumulation or reducing lung inflammation. Recently, hyperactive mouse *Chia* has been generated using error-prone PCR for supplementation of lung chitinase activity (Barad et al. 2020). We succeeded in activating dog *Chia* by replacing two amino acid residues conserved in the mouse. This approach to gene activation could potentially be applied to some medical fields.

Analysis of Carnivora *Chia* revealed that insectivorous species retain functional *Chia*, while in species with diets devoid of insects, the enzymes devolved into inactive molecules. Additionally, we clarified that this inactivation process has two modes: first, amino acid replacements inactivate *Chia* (Canidae type). Second, the functional constraints on *Chia* shift to relaxation and more dramatic genetic alterations disrupt the protein-coding region (non-Canidae type). Our concept outlined in this study provides essential information for a better understanding of the evolutionary history of mammals.

Materials and Methods

Preparation of Expression Vector, Chimeric, and Mutant Protein cDNAs

We expressed *Chia* as fusion proteins with Protein A-*Chia*-V5-His using pEZZ18, which is driven by the *Staphylococcus aureus* Protein A promoter in *E. coli* BL21 (DE3) (Merck Millipore, Tokyo, Japan). In this report, we changed the promoter system from pEZZ18 to pET22b using the T7 promoter system and expressed mouse and dog *Chia* proteins and chimeric and mutant proteins.

We expressed dog *Chia* as a recombinant fusion protein with pre-Protein A (PA) and V5-His (pEZZ18/PA-dog *Chia*-V5-His; Tabata, Kashimura, Kikuchi, et al. 2018). In this report, PA-dog *Chia*-V5-His and their derivative chimeric or mutant proteins were expressed by pET22b using the T7 promoter system (designated pET22b/PA-*Chia*-V5-His). We constructed pET22b/PA-*Chia*-V5-His as follows: The region encoding Protein A-dog *Chia*-V5-His was amplified from

pEZZ18/PA-Chia-V5-His by PCR employing KOD Plus DNA polymerase (Toyobo, Kyoto, Japan) and primers (Eurofins Genomics, Tokyo, Japan) anchored with the restriction sites for NdeI and Sall. The forward primer (5'-**GGAGATATACATATGAAAAAGAAAAACATTTATTC**AAT-3') contains an EcoRI recognition sequence (underlined) and 24 bases long Protein A region with their signal sequence for extracellular secretion. The reverse primer (5'-**CATGCCTGCAGGTCGACTAGAAAGTCGAGGCTGATCA**-3') contained a Sall restriction site (underlined) and is complementary to nucleotides 1,080–1,106 of the pcDNA3.1/V5-His C vector (http://tools.invitrogen.com/content/sfs/vectors/pcdna3_1v5hisc_seq.txt, last accessed November 24, 2021). Both primers contain the 9 or 11 bases long extra nucleotides (boldfaced) for efficient cleavage close to the end of the amplicons. The PCR product was purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and digested with NdeI and Sall. The cleaved DNA fragments were purified from a 1.5% agarose gel and subcloned into the NdeI and XhoI sites of the pET22b expression vector. The entire nucleotide sequence of the resulting pET22b/PA-dog Chia-V5-His was confirmed by sequencing (Eurofins Genomics).

The pEZZ18/PA-mouse Chia-V5-His was digested with EcoRI and XhoI. The cleaved DNA fragments were purified and subcloned into similarly digested pET22b/PA-dog Chia-V5-His to produce pET22b/PA-mouse Chia-V5-His. Other Protein A-mouse Chia-V5-His DNAs were cloned in the same way, as described below.

Construction of Chimeric Proteins

We constructed mouse/dog chimeric proteins. Since both molecules have similar exon structures at the nucleotide level, we fused two units at the junctions among exons 3–5, exons 6–7, exons 8–10, and exon 11 ([supplementary fig. S1 and tables S3 and S4, Supplementary Material](#) online).

Construction of Chimeras C1, C2, and C3 was carried out as follows: the 5'-parts of C1, C2, and C3 were amplified from pEZZ18/Protein A-mouse Chia-V5-His (mouse version) using Protein A_Fw_2533 and Mu_Dog_C1_Rv (C1), Mu_Dog_C2_Rv (C2), or Mu_Dog_C3_Rv (C3). The 3'-parts were from pEZZ18/Protein A-dog Chia-V5-His (dog version) by Mu_Dog_C1_Fw (C1), Mu_Dog_C2_Fw (C2) or Mu_Dog_C3_Fw (C3), and Sal_BGH_Rv. The resulting fragments were purified, mixed, and amplified by PCR using Protein A_Fw_2533 and Sal_BGH_Rv. The amplified cDNA was digested with EcoRI and XhoI and cloned into the pET22b-Protein A-V5-His vector to create the pET22b/PA-Chimera-V5-His.

Other chimeras were also produced by the combination of template and primers ([supplementary tables S3 and S4, Supplementary Material](#) online), essentially as described above.

Preparation of Chia Mutant Proteins

Chia mutant proteins were prepared by PCR using primers as previously described ([Okawa et al. 2016](#)). We first amplified two fragments, which consisted of 5'- and 3'-products of the

final product using the forward and reverse primers with the base substitution ([supplementary tables S3 and S4, Supplementary Material](#) online). The fragments were gel purified, mixed, and amplified by PCR using Protein A_Fw_2533 and Sal_BGH_Rv. The amplified cDNA was digested with EcoRI and XhoI and cloned into the pET22b/PA-V5-His vector, as described above.

Using the plasmid DNAs (the pET22b/PA-Chia-V5-His), *E. coli* BL21 (DE3) was transformed to express PA-Chia-V5-His proteins. Transformed *E. coli* were grown in 250 mL of LB medium containing 100 µg/mL ampicillin at 37 °C for 18 h. After induction with 0.1 mM isopropyl β-D-thiogalactopyranoside, the bacteria were further cultured for 2 h in LB medium. Cells were harvested by centrifugation at 6,500 × g for 20 min at 4 °C. The recombinant protein was prepared from *E. coli* and purified by IgG Sepharose (GE Healthcare, Piscataway, NJ, USA) chromatography as described previously ([Kashimura et al. 2013](#)). The protein-containing fractions were desalted using PD MidiTrap G-25 (GE Healthcare) equilibrated with TS buffer (20 mM Tris-HCl (pH 7.6), 150 mM NaCl and a protease inhibitor [Complete, Roche, Basel, Switzerland]). The recombinant products were detected by Western blot using an anti-V5-HRP monoclonal antibody (Thermo Fisher Scientific, Waltham, MA, USA).

Chitinase Enzymatic Assays

The chitinolytic activity was determined using a synthetic fluorogenic substrate, 4-MU-(GlcNAc)₂ (Sigma-Aldrich, St. Louis, MO, USA), as described previously ([Kashimura et al. 2013](#)). The fluorescence of liberated 4-methyl umbelliferon was measured using a GloMax Discover Multimode Microplate Reader (Promega) with excitation at 365 nm and emission at 445 nm.

For determination of the optimal pH, the chitinase activity was evaluated by incubating the enzyme with the 4-MU-(GlcNAc)₂ substrate in 0.1 M Gly-HCl buffer (pH 1.0–3.0) or McIlvaine's buffer (0.1 M citric acid and 0.2 M Na₂HPO₄; pH 2.0–8.0) at 37 °C for 30 min.

Sequence Analysis

We conducted BLAST searches against whole-genome assemblies of 36 carnivoran genomes from the NCBI Genome Database and 6 Canidae sequencing data from the SRA database using the dog *Chia* gene sequence (XM_038667732.1) as a query. In addition to these sequences, we also used annotated gene sequences of *V. vulpes*, *Suricata suricatta*, and *Hyaena hyaena* available in GenBank. GenBank accession numbers and deduced *Chia* nucleotide sequences are described in [supplementary tables S1 and S2](#) and data file 1, [Supplementary Material](#) online. We obtained additional gene models from non-Carnivora mammals (*Mus musculus*, *Callithrix jacchus*, *Sus scrofa*, and *Bos taurus*) as outgroups, imported all of the sequences into MEGA X ([Kumar et al. 2018](#)), and aligned them using the MUSCLE algorithm ([Edgar 2004](#)). The evolutionary relationships of the *Chia* genes in Carnivora were estimated by the maximum-likelihood method, but this tree was incongruent with established trees ([supplementary fig. S5, Supplementary Material](#) online).

Molecular Evolution Analysis

We performed selective intensity analyses of genes based on a variation in the ratios of nonsynonymous to synonymous nucleotide substitutions (dN/dS or ω) using the CODEML program of PAML (Yang 2007) and RELAX implemented in HyPhy version 2.22 (Wertheim et al. 2015). For these analyses, premature stop codons were removed from pseudogene sequences by excising the insertions, deletions, or substitutions (supplementary data file 2, Supplementary Material online). The phylogenetic relationships of 46 carnivorans and mouse (outgroup) were inferred using TimeTree (<http://www.timetree.org/>, last accessed November 24, 2021; Kumar et al. 2017).

For the CODEML analysis, we used branch models, where ω is assumed to be different between foreground and background branches. We labeled the tested branches as follows: 1) a null one-ratio model (M_0), which assumes the same ω value for all branches; 2) a two-ratio model (M_2), where two separate ω values were estimated for species with pseudogenes (foreground branches) and species with functional genes (background branches); and 3) a three-ratio model (M_3), where species with pseudogenes (foreground branches_1), species with functional genes in Canidae (foreground branches_2) and species with functional genes in insectivores (background branches) were allowed to have different ω . Its goodness-of-fit was analyzed using LRTs.

We also used RELAX to detect relaxed selection on the *Chia* gene in a codon-based phylogenetic framework. RELAX tests selection intensity in one set compared with the other. In this analysis, we set the two categories of branches as follows: 1) pseudogene branches versus all other branches and 2) Canidae versus insectivorous branches. RELAX estimates the distribution of ω for each set using a random effects branch-site model and then fits a selection parameter k . Intensified selection is indicated by $k > 1$, whereas relaxed selection is indicated by $k < 1$. The goodness-of-fit for two given models was analyzed using the LRT by comparison with each null model whose k parameter was constrained to 1.

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

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Authors' Contributions

Project design, coordination: E.T., P.O.B., and F.O.; Performed research: E.T., T.K., H.T., A.K., and F.O. Analyzed data: E.T., A.I., and M.S. Wrote the paper: E.T., V.M., P.O.B., and F.O. Contributed to the critical appraisal of the paper and approved the final version: E.T., A.I., T.K., H.T., A.K. M.S., V.M., P.O.B., and F.O.

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