Endogenous Retrovirus Loci and Induced Changes in Gene Expression in Japanese Indigenous Chickens

Shinya Ishihara[1*], Jun-ichi Shiraishi[1], Saki Shimamoto[2], Daichi Ijiri[2]

[1] Department of Animal Science, Nippon Veterinary and Life Science University, 1-7-1 Kyonancho, Musashino, Tokyo, Japan, 180-8602

[2] Department of Animal Science and Welfare, Joint Faculty of Veterinary Medicine, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan

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Corresponding author:
Shinya Ishihara
Address: Department of Animal Science, Nippon Veterinary and Life Science University, 1-7-1 Kyonancho, Musashino, Tokyo, Japan, 180-8602
E-mail: shinyais@nvlu.ac.jp
Phone: +81-422-31-4151

Summary

When retroviruses infect germ cells and are transmitted to offspring, they become endogenous retroviruses (ERVs), whose insertions influence the expression of nearby genes. This study aimed to identify the genomic loci of ERVs in commercial broiler (Ross308), Tosa-Jidori, and Yakido chickens as well as to elucidate their impact on neighboring gene expression. Whole-genome data were obtained using next-generation sequencing, and candidate ERV loci were identified using the RetroSeq software. The Integrative Genomics Viewer tool was used to confirm target site duplications (TSDs) as evidence of ERV insertions. All reads within 200 bp of these TSDs were extracted to create contigs, confirming the presence of ERV sequences in the contigs using BLASTN. Gene expression levels were estimated by focusing on genes located near the 172 identified ERV loci. Among these, 119 loci were detected in broiler chickens, 80 in Tosa-Jidori chickens, and 86 in Yakido chickens, with 28 loci shared among them. Moreover, of these 172 loci, 75 were located within or near genes. Significant differences in gene expression were observed for N-acetylated alpha-linked acidic dipeptidase 2, glypican 6, and phospholipid scramblase family member 5 depending on the presence of ERV insertions. These results suggest that ERV insertions may influence the expression of certain genes, providing insights into the genetic diversity and evolutionary background of commercial and indigenous chickens. Understanding the effects of ERV insertions on gene expression can inform future genetic research and poultry breeding programs aimed at improving health and productivity.

Author Summary

Recently, endogenous retroviruses (ERVs) have gained significant attention as valuable markers for understanding genetic relationships and evolutionary processes among species.

46 In this study, we investigated the loci and characteristics of ERVs in commercial and
47 traditional Japanese chickens. ERVs are genetic remnants of ancient viral infections that
48 can provide insights into avian evolution. We identified a total of 172 ERV loci in broiler,
49 Tosa-Jidori, and Yakido chickens. Each chicken breed exhibited unique ERV insertion
50 patterns. Notably, we found that ERV insertions near certain genes may influence gene
51 expression. Our research enhances the understanding of how chickens have acquired traits,
52 particularly through genetic mechanisms influenced by ERVs. These insights significantly
53 contribute to our knowledge of biological evolution and the overall biodiversity of birds.
54

55 **Introduction**

56 Endogenous retroviruses (ERVs) are ancient genetic elements comprising virus-derived
57 sequences that have been integrated into the genomes of various organisms. These are
58 formed when viruses insert themselves into a host genome and are subsequently transmitted
59 to the its progeny. Structurally, ERVs possess long terminal repeats (LTRs) at both ends
60 and encode structural proteins (*gag*), reverse transcriptases (*pol*), and envelope proteins
61 (*env*). These sequences accumulate mutations and become fragmented over generations,
62 often leading to a loss of function in many ERVs. Once integrated into the genome, ERVs
63 can influence the transcription and expression of nearby host genes, primarily through
64 regulatory elements present in their LTR sequences, such as promoters and enhancers [1, 2,
65 3]. Recently, ERVs have gained significant attention as valuable markers for understanding
66 genetic relationships and evolutionary processes among species. Because they are inherited
67 vertically across generations, the presence or absence of ERVs in different species may
68 elucidate phylogenetic relationships. While shared ERVs among different species indicate a
69 common ancestral infection, species-specific ERVs suggest independent integration events
70 [4]. Furthermore, as ERVs are genetically stable over long evolutionary timescales, they

71 make reliable markers. Unlike other transposable elements, most ERVs cannot
72 retrotranspose nor spread within the genome, resulting in relatively stable genomic
73 distributions. Consequently, the evolutionary history of a species can be traced by
74 analyzing its ERV integration patterns [5, 6]. In avian species, researchers have focused on
75 the endogenous avian retrovirus (EAV) family and avian leukosis virus subgroup E
76 (ALVE) [7, 8, 9, 10, 11, 12], reporting that the blue eggshells of Araucana chickens are
77 influenced by ERVs. Understanding the role of ERVs in avian species provides significant
78 insight into their evolutionary biology and genetic diversity [13].
79 Japanese indigenous chickens comprise approximately 50 breeds, most of which are
80 classified as fancy or practical breeds based on traits such as unique crowing, ornamental
81 feathers, and fighting characteristics, as well as egg and meat production [14, 15, 16, 17,
82 18]. Examples of these breeds include Tosa-Jidori, the oldest type of Japanese native
83 chicken with a history of over 2000 years, and Yakido, a Malay-type breed with great
84 height and black feathers [19, 20, 21]. A study using 20 microsatellite DNA markers to
85 construct phylogenetic trees showed that these native Japanese breeds possess distinct
86 genetic backgrounds compared to those of foreign breeds such as White Leghorn and
87 Rhode Island Red [22]. These diverse Japanese indigenous chicken breeds, which
88 underwent unique evolutionary processes under different environmental and artificial
89 selection pressures, serve as intriguing models for studying the insertion sites and effects of
90 ERVs. For instance, breeds that are adapted to harsh climates or specific local conditions
91 may exhibit unique ERV profiles.
92 This study aimed to elucidate how ERVs contribute to the genetic diversity and
93 adaptation of Japanese indigenous chickens, which may provide insights for breeding
94 programs and conservation strategies. To achieve this objective, we identified the loci of
95 ERVs in the genomes of commercial broiler (Ross308), Tosa-Jidori, and Yakido chickens

96 and elucidated how these affect gene expression. We employed whole-genome sequencing
97 technologies and bioinformatic analyses to identify ERV insertion sites and assess their
98 regulatory impacts. Our findings suggest that ERV insertions may affect the expression of
99 neighboring genes and demonstrate their contribution to the genetic diversity of these
100 chickens.
101
102 **Results**
103 **Sequencing data quality and identification of the non-reference ERV breakpoint**
104 In this study, 150-bp paired-end reads were aligned using the Burrows-Wheeler Aligner and
105 Mem (BWA-mem) algorithm [23], achieving an overall mean sequence depth of 44.6x
106 (ranging 38.6–57.5) across all chicken samples (Table S1); the detailed mapping results are
107 presented in Table S1. Over 99% of the paired-end reads for each chicken were successfully
108 aligned with the reference Gallus genome, while 1.72–2.77% were classified as improperly
109 mapped reads. Additionally, 0.13–0.16% of the reads were singletons, aligning to a single
110 side. The RetroSeq software [24] identified 167–240 candidate ERV insertion loci per
111 individual (Table S1). In total, 184 ERV loci were identified across the three types of chicken.
112 Of the 184 detected ERV loci, 10 matched the surrounding sequences of the reference
113 genome, and 2 were shorter than 200 bp; consequently, these were excluded from further
114 analysis. The contigs derived from the remaining 172 loci were used in the analysis (Table
115 S2). The distribution of the 172 loci within the genome was as follows: 57 on Chr1, 33 on
116 Chr2, 23 on Chr3, 19 on Chr4, and 12 on ChrZ, representing 81.4% of the total. A strong
117 correlation was observed between chromosome length and the number of detected loci
118 (Pearson's correlation coefficient = 0.96). Broiler, Tosa-Jidori, and Yakido chickens had 117,
119 78, and 82 ERV loci, respectively. The number of loci shared among the breeds is shown in
120 Figure 1, with 28 loci shared among all types of chicken. The ERV loci specific to broiler,

121 Tosa-Jidori, and Yakido chickens were 54, 18, and 23, respectively. Of the 172 loci, 95 were
122 shared with junglefowls, 94 of which were shared with red junglefowls, the ancestor of
123 domestic chickens [25, 26], while the remaining locus was shared with gray junglefowls
124 (Figure S1). The summary statistics for heterozygosity in each breed are presented in Table
125 1. Broilers had higher estimates of observed heterozygosity (Ho=0.28) and expected
126 heterozygosity (He=0.23) than those of Tosa-Jidori (Ho=0.17, He=0.14) and Yakido
127 (Ho=0.19, He=0.15) chickens. In the constructed phylogenetic tree (Figure 1), ERVs were
128 frequently observed at the same loci in the same chicken, although these were not consistently
129 shared at identical loci (Table S2). For example, the number of homozygous ERV loci was 0
130 in broilers, 10 in Tosa-Jidori, and 14 in Yakido. Nonetheless, clusters in the phylogenetic tree
131 were clearly delineated for chickens and junglefowls (Figure 1).
132
133 **Table 1**

Chicken	Observed heterozygosity	Expected heterozygosity
Broiler	0.28	0.23
Tosa-Jidori	0.17	0.14
Yakido	0.19	0.15

134
135 **Analysis of Detected ERV Types and Sequences**
136 The contigs detected from the 172 loci were mapped to ERV sequences in the database (Table
137 S3). The Chr1_159876144 locus was mapped to two locations in EAV-HP (previously
138 described as ev/J, AF125529), while the Chr2_133314053 locus was mapped to two locations
139 in the Murine leukemia virus (MLV) related retroviruse (DQ280312). The numbers of
140 contigs mapped to EAV-HP, EAV-51, EAV-0, and EAV-0/E51 were 79, 9, 33, and 1,
141 respectively (Table 2). Moreover, 8 and 1 contigs were mapped to ALVE and MLV,

142 respectively. The lengths of the obtained contigs varied regardless of ERV type; however,
143 the average length of contigs corresponding to ALVE (573.5 bp, 405–734 bp), was greater
144 than that of other ERVs. Most of these contigs contained LTR sequences, with some
145 including gag, pol, env, or gag/env fusion protein sequences (Figure S2).

146

147 **Table 2**

ERV	Accession No.	Mapped contigs	Contig length						Total
			201-300	301-400	401-500	501-600	601-700	701-800	
EAV-HP	AF125529	24	2	14	4	2	2	0	79
	AJ623291	47	36	6	2	2	1	0	
	NC_005947.1	18	0	9	2	2	5	0	
EAV-51	AM418553	9	6	2	1	0	0	0	9
EAV-0	AM418554	33	1	5	21	5	1	0	33
EAV-0/E51	AM418555	1	0	0	0	0	1	0	1
ALVE	MT263508	8	0	0	1	4	2	1	8
MLV	DQ280312	1	1	0	0	0	0	0	1
LTR	M31063	9	6	3	0	0	0	0	31
	M31065	3	1	1	1	0	0	0	
	M31066	19	13	1	5	0	0	0	
Total		172	66	41	37	15	12	1	172

148

149 **Impact of ERV Insertions on mRNA Expression**

150 The RNA expression levels, classified based on the presence or absence of ERV insertions

151 at each locus, are shown in Figure S3. Of the loci with detected ERVs, 75 were located near
152 or within genes (Table S2). To examine the relationship between the presence of these gene
153 insertions and RNA expression, transcripts per million (TPM) values were calculated
154 (Table S4). Among the 75 genes, 24 were excluded from the analysis for one of three
155 reasons: all individuals had ERV insertions, only one individual had an insertion, or over
156 90% of the individuals showed no gene expression. Figure S3 shows how the presence or
157 absence of ERV insertions for the remaining 51 genes influenced gene expression.
158 Significant differences were observed in the expression of N-acetylated alpha-linked acidic
159 dipeptidase 2 (*NAALAD2*), glypican 6 (*GPC6*), and phospholipid scramblase family
160 member 5 (*PLSCR5*) (adjusted p-values = 0.0022, 0.0119, and 0.0119, respectively). In
161 *NAALAD2* and *GPC6*, ERV insertions were associated with decreased expression, whereas
162 in *PLSCR5*, they were associated with increased expression (Figure 2).
163

164 **Discussion**

165 In this study, we employed next-generation sequencing to identify ERVs that were absent
166 in a reference genome, rigorously trimming the obtained sequence data using stringent
167 criteria. As more than 99% of all read pairs mapped to the Gallus reference genome, the
168 assembled sequence data were considered to be high quality. We subsequently attempted to
169 detect ERVs in broilers and Japanese indigenous chickens using improper pairs and
170 singleton sequence reads that were incorrectly mapped to the reference genome. We
171 identified 172 ERV loci across the three chicken types. Using a similar method, analysis of
172 the genus Gallus—specifically, the red junglefowl—revealed the presence of 116 ERV loci
173 (range: 61–123), which is largely consistent with previous findings [10]. In our study, the
174 numbers of ERV loci detected in broilers, Tosa-Jidori, and Yakido chickens were 119, 80,
175 and 86, respectively, which are comparable to previous findings. Additionally, we visually

176 confirmed the breakpoints detected by RetroSeq using Integrated Genome Viewer (IGV) to
177 identify target site duplications (TSDs) and included the ERV sequences in the contigs
178 assembled from the surrounding sequences. Our results demonstrate the validity of the
179 methods used for detecting ERVs in different chicken breeds and species, confirming their
180 high reliability.

181 The ERV structures at each locus were inferred from the contigs. However, because
182 of the tendency of ERVs to accumulate in repetitive regions [27, 28, 29], short-read
183 sequencing alone may have limitations in accurately detecting complete ERV structures,
184 including the *gag*, *pol*, and *env* regions, or solo-LTRs. To overcome these limitations,
185 single-molecule real-time sequencing or nanopore sequencing may be used to determine
186 complete ERV insertion sequences. This would allow for high-resolution mapping, precise
187 localization of gene insertions, and the identification of mutations. Combining short- and
188 long-read sequencing is expected to improve the differentiation of heterozygosity and
189 homozygosity, leading to more accurate localization of gene insertions and mutations as
190 well as a more refined functional analysis of genetic variation.

191 In this study, we analyzed the ERV insertion sites in the chicken genome to elucidate
192 the genetic diversity and evolutionary background of different chickens. The strong
193 correlation between chromosome length and the number of insertion sites suggests that
194 ERV insertions occur randomly throughout the genome. However, as we did not account
195 for existing ERVs in the reference genome, determining the concentration of ERV
196 insertions in specific genomic regions conclusively was not possible. We found that
197 broilers exhibited higher heterozygosity than that of other chickens, indicating greater ERV
198 diversity. Our phylogenetic analysis based on the presence or absence of ERVs revealed
199 distinct clusters for each chicken, with ERVs frequently observed at the same insertion sites

200 within the same chicken type. This suggests that the observed ERV loci are fixed within
201 specific lineages through selection pressure or genetic drift during breed formation.
202 However, instances of ERV locus loss owing to genetic drift were also observed. For
203 example, while the murine leukemia virus-related ERV (DQ280312) was confirmed in all
204 junglefowls, it was not detected in two broilers. The fixation or loss of ERVs is influenced
205 by selection pressure and genetic drift within the host population [30]; studies on
206 commercial chickens have also reported that certain ERV insertion sites are fixed owing to
207 selection pressure. The findings obtained here suggest that rare indigenous chickens possess
208 unique ERV loci. The high heterozygosity of ERVs in broilers indicates a possible
209 crossbreeding with populations possessing diverse ERV loci during breed formation. Of the
210 172 detected ERV loci, 94 matched those reported in red junglefowl, while the
211 phylogenetic analysis placed commercial and indigenous chicken subspecies in clusters
212 close to that of the red junglefowl. In addition, eight ALVE loci were identified. ALVE has
213 not been found in any junglefowl species other than the red junglefowl, which supports the
214 theory that commercial and indigenous chickens were domesticated from red junglefowl
215 [31]. Although our phylogenetic tree showed separate clusters for gray junglefowl and
216 chickens, these shared 12 ERV loci, one of which was not observed in red junglefowl.
217 Genome analyses of junglefowl suggest that not only red junglefowl but also gray
218 junglefowl contribute to the genetic diversity of poultry; hence, some ERV loci may have
219 been inherited from junglefowl species other than red junglefowl. Although this analysis is
220 based on a limited number of individuals and may not reflect all ERV loci within the
221 population, analyzing more individuals in the future will allow us to accurately understand
222 the ERV gene pool. The results of this study indicate that ERVs may act as powerful
223 markers, similar to microsatellites and single nucleotide polymorphisms, for elucidating
224 evolutionary relationships between breeds or species.

225 In the ERV-type analysis, multiple ERVs, including EAV-HP, EAV-51, and EAV-0,
226 were identified with a particularly high frequency of EAV-HP insertions. On average, the
227 contigs corresponding to ALVE were longer than those of the other ERVs, indicating
228 diverse genomic structures. Many of the obtained contigs contained LTR regions, with
229 some also including gag, pol, and env regions, which demonstrates the structural diversity
230 of ERVs. Analysis of the 51 genes based on ERV insertion and gene expression revealed
231 significant differences in the expression of *NAALAD2*, *GPC6*, and *PLSCR5*. For *NAALAD2*
232 and *PLSCR5*, ERV insertion was associated with decreased expression, whereas *GPC6*
233 showed increased expression. These results suggest that ERV insertions influence the
234 functions of *NAALAD2* in neurotransmitter inactivation, *GPC6* in cancer cell migration and
235 invasion, and *PLSCR5* in phospholipid scrambling of the cell membrane. The impact of
236 ERV insertions may vary depending on the cell type, as specific LTR families are known to
237 promote tissue-specific expression of host genes [32]. LTRs may function as alternative
238 promoters when inserted near genes, with varying effects depending on the insertion site
239 and sequence composition [33, 34]. Known cases of ERV effects in chickens include the
240 blue eggshell color of Araucana, expression of immune- and inflammation-related genes in
241 the liver, and feminization of plumage in Sebright breed males. Although ERVs are not
242 confirmed to function as promoters in these instances, the significant changes in gene
243 expression observed in this study suggest this possibility. Finally, while this study used
244 RNA derived from skeletal muscle, further research is required to determine whether
245 similar effects are observed using other cell types and to elucidate the specific mechanisms
246 by which ERVs influence gene expression.

247 **Conclusion**

248 We successfully identified the genomic loci of ERVs in broiler, Tosa-Jidori, and Yakido
249 chickens. Some of these ERVs were inserted near genes and were correlated with the
250 expression of *NAALAD2*, *GPC6*, and *PLSCR5* genes. Further research is required to
251 determine whether these differences in expression are directly attributable to ERV
252 insertion. Understanding the impact of ERVs on gene expression could reveal new insights
253 into the genetic regulation and evolution of poultry. Future studies should aim to analyze a
254 larger number of individuals and additional chickens to validate these findings and explore
255 the broader implications of ERV insertions in genetic diversity and trait development.
256

257 **Materials and Methods**

258 **Animal samples and genomic DNA and RNA purification**

259 All experimental protocols and procedures were reviewed and approved by the
260 Experimental Animal Committee of the Nippon Veterinary and Life Science University
261 (Tokyo, Japan; Approval number, 2021-S34). The chickens selected for this study include
262 Broilers, representing typical commercial chickens; Yakido, representing Malay-type native
263 Japanese chickens; and Tosa-Jidori, representing traditional native Japanese chickens.
264 Broilers were obtained from a farm in Kagoshima Prefecture. Tosa-Jidori chickens and
265 Yakido chickens were sourced from the Livestock Research Center in Kochi Prefecture and
266 Mie Prefecture, respectively. All chickens were euthanized via decapitation after isoflurane
267 anesthesia at 35 days of age for broilers and 14 days for Tosa-Jidori and Yakido chickens.
268 Blood and breast muscle samples were collected from chickens. Breast muscle was snap
269 frozen in liquid nitrogen and stored at −80 °C until total RNA extraction. Genomic DNA
270 was extracted from the blood samples using the QIAamp DNA Blood and Tissue Kit
271 (Qiagen, Hilden, Germany), while DNA quality was evaluated via gel electrophoresis.
272 Total RNA was extracted using an RNeasy Fibrous Tissue Mini Kit (Qiagen).

273

274 **Whole genome sequence (WGS) data**

275 Genomic DNA from 16 individuals, including 8 broilers, 4 Tosa-Jidori, and 4 Yakido
276 chickens was used to construct each 350-bp sequencing library with a TruSeq DNA PCR-
277 Free Sample Prep Kit (Illumina Inc., San Diego, CA, USA). WGS was performed on 150-
278 bp paired-end reads using an Illumina NovaSeq 6000 System (Illumina Inc.). Nucleotides
279 with low-quality scores were trimmed from these reads, and adapters were removed with
280 Trimmomatic v.0.36 [35] using the ILLUMINACLIP: TruSeq3-PE:2:30:10, LEADING:3,
281 SLIDINGWINDOW:4:20, and MINLEN:30 settings. The reads were mapped to the *Gallus*
282 *gallus* reference genome (GRCg6a, GenBank assembly accession: GCF_000002315.6)
283 using the BWA-mem algorithm [23]. Data were generated with a Binary Alignment Map
284 (BAM) format.

285

286 **Detection of non-reference ERVs**

287 ERVs were detected as previously described [10, 36, 37]. The types of read pairs mapped to
288 the reference genome were defined, and useful sequence reads were extracted. Most paired-
289 end reads were obtained from a reference genome WGS map; however, mismatched read
290 pairs also occurred with unexpected span sizes and orientations. Non-proper pairs were
291 defined as those in which either the 5′ or 3′ end mapped to a contig sequence in the
292 reference genome while the other mapped entirely or partially to an unexpected locus. Ends
293 of a read pair that did not map to the reference genome were denoted as singletons, whereas
294 unmapped read pair was used when both ends of a read pair did not map to the reference
295 genome (Figure S4). Mismatched read pairs can provide insight into LTR-related loci as
296 anchors; these were used with the RetroSeq software to detect non-reference transposon
297 elements [24]. The process flow is illustrated in Figure S4. The ERV sequences used for

298 RetroSeq were obtained from the National Center for Biotechnology Information (NCBI,
299 Bethesda, MD, USA) and are listed in Table S5. The reference genome used was GRCg6a,
300 containing only autosomes and sex chromosomes. In the RetroSeq "call" step, transposable
301 element insertion positions (breakpoints) were estimated using reads detected in the
302 "discover" phase, as previously reported [10, 36, 37]. The call step was set to ≥ 10 to reduce
303 false positives, while the maximum read depth option per call was set to 10,000 to increase
304 BAM coverage. All other RetroSeq options were used at their default values. A minimum
305 of seven filter-level breakpoints was used, with breakpoints detected within 500 bp
306 considered identical and excluded. The IGV [38] was used to detect loci containing TSDs;
307 using the batch script functionality of IGV, a screenshot was obtained at each genomic
308 locus detected using the RetroSeq analysis pipeline and carefully examined. The loci were
309 presumed to be TSDs if they mapped to reads detected during the "discover" phase either
310 from the 5′ or 3′ side, with an overlap ranging 1–10 bp. The 5′ and 3′ reads mapped within
311 150 bp of the TSDs were extracted using SAMtools [39]. The extracted read set was used
312 to generate contig sequences using CAP3 software [40], which were subsequently used for
313 a blastn search [41]. The lowest e-value was used to determine the ERV class. Each 200-bp
314 sequence upstream and downstream of the breakpoint was extracted from the reference
315 genome and subjected to blastn to eliminate the possibility of detecting ERV sequences in
316 the reference genome. Subsequently, the loci that matched the ERVs were excluded from
317 the analysis. Sequences that did not exist in the reference genome were deduced from the
318 contiguous sequence within the region on either side of the TSD sequence or the 6-bp
319 sequence adjacent to the 5′ and 3′ sides of the TSD when its length was insufficient.
320

321 **Analysis of Detected ERV Loci and Sequences**

322 The detected loci in broiler, Tosa-Jidori, and Yakido chickens were compared with

323 previously reported ERV loci in junglefowl [10]. The presence or absence of ERVs at each
324 locus was represented as 1 or 0, respectively, to perform clustering among the species and
325 subspecies. A resulting phylogenetic tree was constructed using the R software *ape* and *ade4*
326 package [42, 43, 44] and "dist.binary" and "hclust" functions. Contigs containing ERV
327 sequences at each locus were mapped to previously reported ERV sequences using the BWA-
328 mem algorithm. Based on the mapping results, each contig was classified according to ERV
329 species and further categorized by length. Homozygosity or heterozygosity was determined
330 using IGV. Contigs were considered homozygous when reads mapped near the TSD were
331 truncated. Conversely, when reads mapped across both the 5' and 3' ends of the TSD, the
332 contig was considered heterozygous. The observed (Ho) and expected heterozygosities (He)
333 for each chicken were estimated using the "popgen" function in the *PopGenKit* package [45]
334 in R [42].
335
336 **Gene expression analysis using RNA-seq and relation**
337 For RNA-seq analysis, the TruSeq cDNA library was constructed using the NEBNext®
338 Ultra™ Directional RNA Library Prep Kit (Illumina, USA) after verifying the quantity and
339 quality of the extracted RNA. Raw RNA-seq data were acquired using the Illumina
340 NovaSeq 6000 platform, trimming was performed using the Trimmomatic software [35],
341 and reads were mapped to the chicken reference genome (GRCg6a) using the STAR
342 program [46]. Expression analysis was conducted using the RSEM program [47] to
343 estimate TPM values. The ERV loci were identified and examined for insertions within the
344 genes using IGV. For loci with ERV insertions within genes, TPM values were compared
345 with those of individuals without ERV insertions at the same loci. A t-test was performed
346 for each locus, with p-values adjusted using the Benjamini-Hochberg method, setting a
347 threshold of statistical significance at $\alpha=0.05$.

348

349 **Data availability**

350 The data used in this study are available from DDBJ (BioProject accession: PRJDB17938).

351

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538 **Figure legend**

539

540 **Figure 1**

541 Number of endogenous retrovirus (ERV) loci detected among broiler, Tosa-Jidori, Yakido
542 and gallus genus as well as phylogenetic trees. (A) Number of ERV loci across broiler,
543 Tosa-Jidori, and Yakido and the overlap between each ERV locus. (B) Number of ERV loci
544 across broiler, Tosa-Jidori, Yakido, and red junglefowl and the overlap between each ERV
545 locus. (C) Phylogenetic tree constructed based on the presence or absence of ERV loci. The
546 scale bar indicates the distance calculated from the presence/absence matrix.

547

548 **Figure 2**

549 Normalized transcripts per million TPM) values for PLSCR5, NAALAD2, and GPC6. Box
550 plots represent the distribution of gene expression levels across three ERV insertion
551 genotypes: no insertion, heterozygosity, and homozygosity. Each box plot shows the
552 median (line within the box), interquartile range (the box), and range of the data (whiskers).
553 Statistical significance was determined using the Tukey honestly significant difference test,
554 indicated by asterisks (adjusted p-value; *$p<0.05$, **$p<0.01$, ***$p<0.001$).

555

556 **Supporting Information**

557

558 **Supplemental Table**

559

560 **Table S1. Sample information, mapping quality and RetroSeq results.**

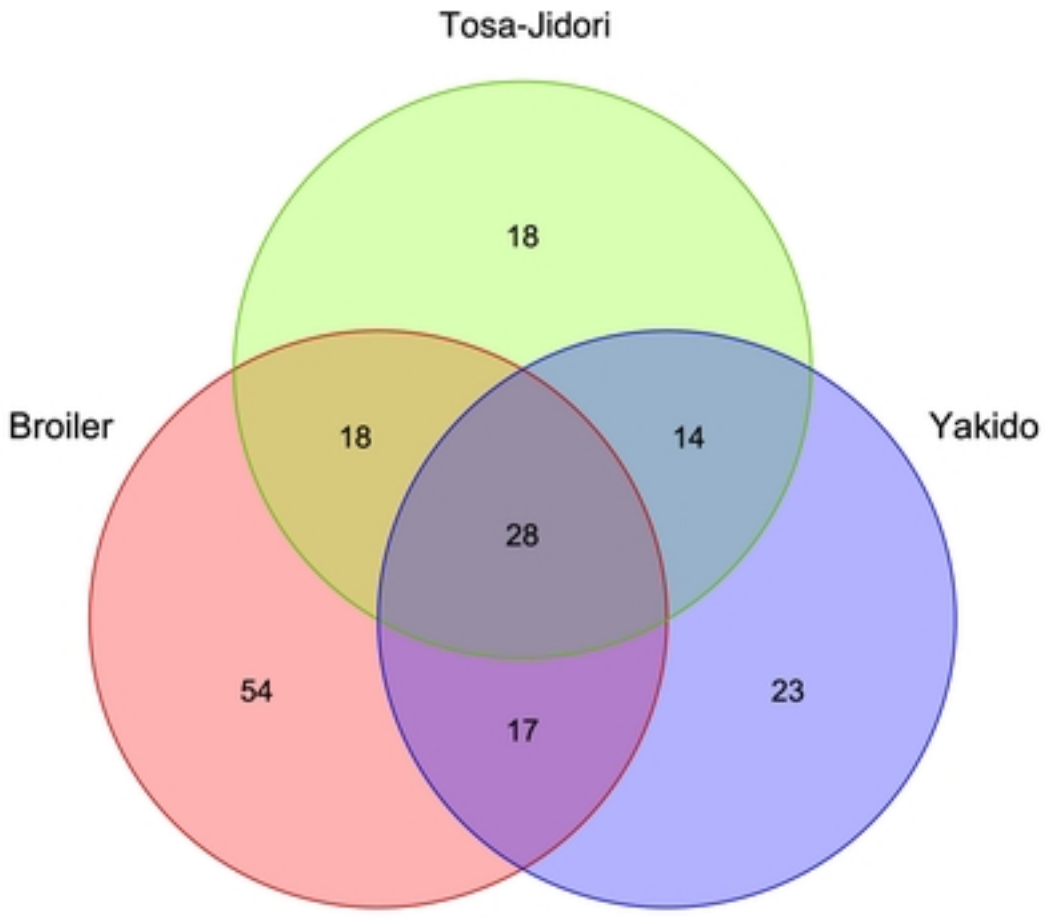
561

562 **Table S2. The loci of endogenous retrovirus detected, and the information about the**
563 **loci.**
564
565 **Supplemental Figure legend**
566
567 **Figure S1.** The Venn diagrams present the intersections of ERV insertion sites among
568 different chicken breeds (Broiler, Tosa-Jidori, Yakido) and wild species (*Gallus Gallus*,
569 *Gallus sonneratii*, *Gallus lafayettii*, and *Gallus varius*). Each circle represents one breed or
570 species, with the numbers within the overlapping regions indicating the count of shared ERV
571 insertions. Non-overlapping regions denote the number of unique insertions specific to each
572 breed or species.
573
574 **Figure S2.** Mapping of contig sequences derived from extracted reads to known
575 endogenous retrovirus (ERV) sequences in chicken genomes using IGV (Integrative
576 Genomics Viewer). The contigs were mapped against the following Accession IDs:
577 Chicken endogenous proviral avian retroviral_LTR (M31063, M31064, M31065, M31066),
578 EAV-0 (AM418554), EAV-51 (AM418553), EAV-0/E51 (AM418555), EAV-HP
579 (NC_005947.1, AJ623291, AF125529), ALV-E (MT263508), MLV-related endogenous
580 retrovirus (DQ280312). M31064 was omitted because there was no mapped contig.
581
582 **Figure S3**. Gene expression levels in different chicken breeds with and without
583 endogenous retrovirus (ERV) insertion. This box plot illustrates the gene expression levels
584 of various genes across the three chickens (broiler, Tosa-Jidori, Yakido) with and without
585 endogenous retrovirus (ERV) insertion. The x-axis labels denote the specific genes and
586 their ERV insertion status (TRUE/FALSE), while the y-axis represents the expression

levels. The boxes represent the interquartile range (IQR), with the black line inside each box indicating the median expression level. The whiskers extend to the smallest and largest values within 1.5 times the IQR from the first and third quartiles, respectively. Individual data points outside this range are considered outliers and are plotted individually. The colors of the data points represent the different chicken breeds: red for broiler, green for Tosa-Jidori, and blue for Yakido.

Figure S4. Pipeline for the detection of non-reference chicken endogenous retroviruses in whole-genome sequencing (WGS) read data. This figure is based on content taken from Ishihara 2023 with modifications. In the right-upper panel, the black line denotes the chicken reference genome sequence. Blue and red boxes connected with lines denote the 5′ and 3′ ends of a paired-end sequencing read. Most paired-end reads were identified as proper mapping, whereas a small percentage of them were improper mapping. Proper pair: both ends of the paired-end sequence mapped accurately (**a**). Discordant read and split read: one end of the paired-end sequence mapped accurately, whereas the other end was only partially identified at the expected locus on the reference genome. The unidentified sequence could be mapped anywhere else on the reference genome (**b**, **c**). Singleton: one end of the paired-end sequence mapped accurately, whereas the other end did not map on the reference genome (**d**). Unmapped read pairs: neither read mapped to the reference genome (**e**). Discordant reads, split reads, and singletons were used for RetroSeq analysis. In the right-middle panel, a representative view of the Integrative Genomics Viewer (IGV) is used to confirm the presence of target site duplications (TSDs) at each locus, detected using RetroSeq, extract support reads from the TSD loci, perform local assembly, and analyze the contigs for the presence of endogenous retroviruses (ERV) - genome junctions from both sides. “A” denotes the individuals that have the TSD, and “B” denotes the individuals that did not have the TSD.

612 The right-lower panel shows a conceptual diagram of local de novo assembly using CAP3.
613 Sequences in red indicate sequences not present on the reference genome, and sequences in
614 purple indicate TSDs. The panels labeled "Homozygosity" and "Heterozygosity" illustrate
615 the differences in read mapping patterns when an ERV insertion is homozygous versus
616 heterozygous. Homozygosity: All reads break at the TSD. Heterozygosity: Some reads break
617 at the TSD, but there are also reads that span across the TSD.
618


Tosa-Jidori
Broiler
Yakido
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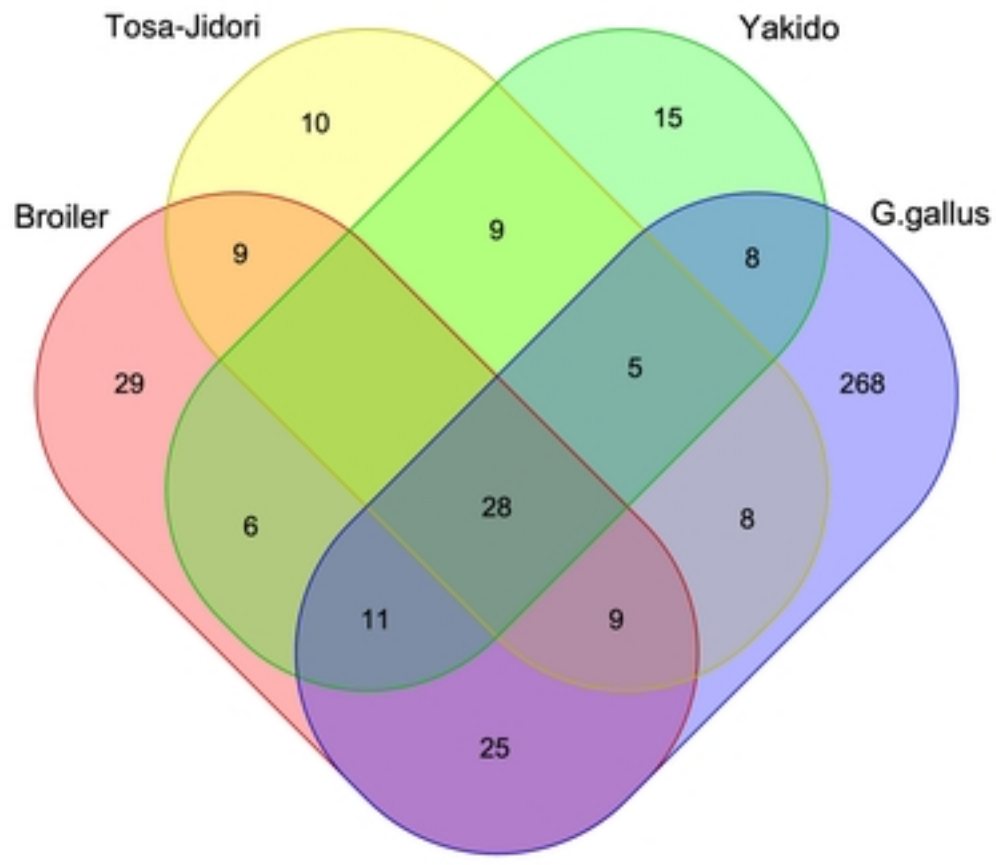
Tosa-Jidori
Yakido
Broiler
G.gallus
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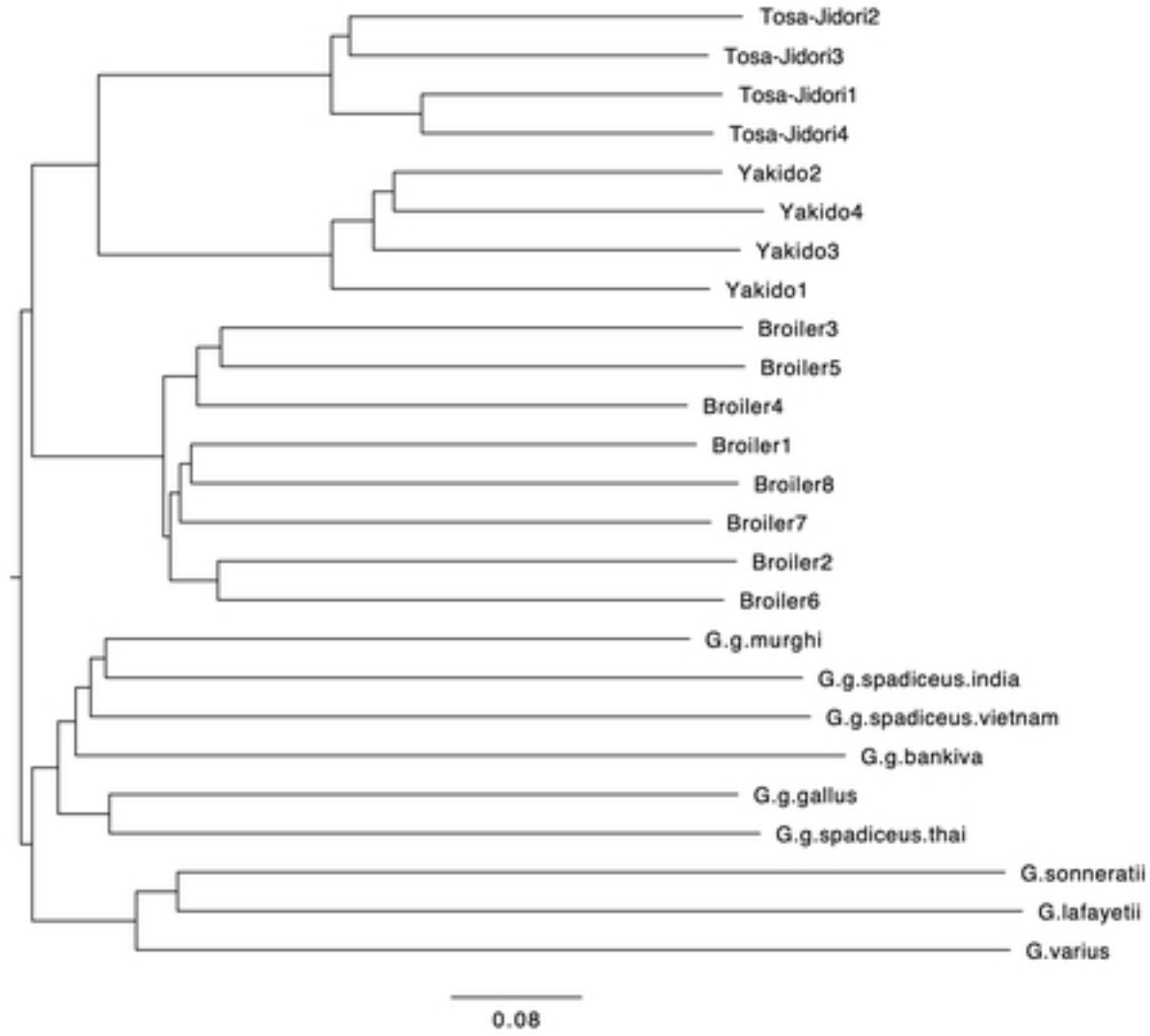
Tosa-Jidori2
Tosa-Jidori3
Tosa-Jidori1
Tosa-Jidori4
Yakido2
Yakido4
Yakido3
Yakido1
Broiler3
Broiler5
Broiler4
Broiler1
Broiler8
Broiler7
Broiler2
Broiler6
G.g.murghi
G.g.spadiceus.india
G.g.spadiceus.vietnam
G.g.bankiva
G.g.gallus
G.g.spadiceus.thai
G.sonneratii
G.lafayetii
G.varius
0.08

Figure1

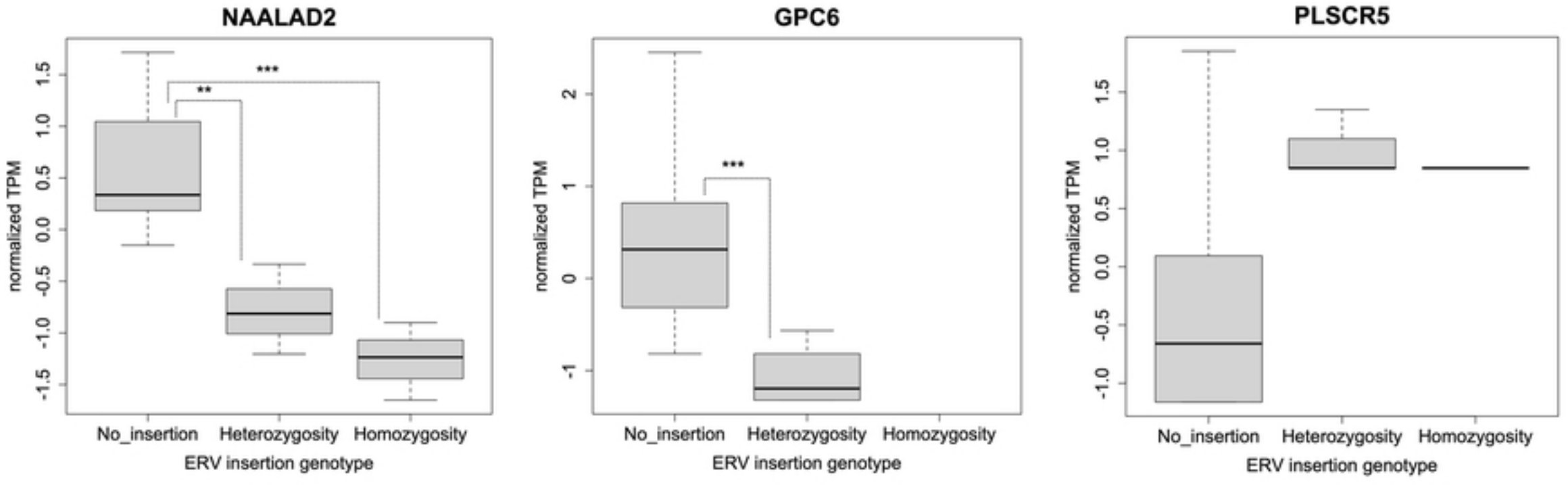
NAALAD2
GPC6
PLSCR5
normalized TPM
***
**
No_insertion
Heterozygosity
Homozygosity
ERV insertion genotype

Figure2