Variation in digit number has occurred multiple times in the history of archosaur evolution. The five digits of dinosaur limbs were reduced to three in bird forelimbs, and were further reduced in the vestigial forelimbs of the emu. Regulation of digit number has been investigated previously by examining genes involved in anterior-posterior patterning in forelimb buds among emu (*Dromaius novaehollandiae*), chicken (*Gallus gallus*) and zebra finch (*Taeniopygia guttata*). It was described that the expression of posterior genes is conserved among these three birds, whereas expression of anterior genes *Gli3* and *Alx4* varied significantly. Here we re-examined the expression pattern of *Gli3* and *Alx4* in the forelimb of emu, chicken and zebra finch. We found that *Gli3* is expressed in the anterior region, although its range varied among species, and that the expression pattern of *Alx4* in forelimb buds is broadly conserved in a stage-specific manner. We also found that the dynamic expression pattern of the BMP antagonist *Gremlin1* (*Grem1*) in limb buds, which is critical for autopodial expansion, was consistent with the digital pattern of emu, chicken and zebra finch. Furthermore, in emu, variation among individuals was observed in the width of *Grem1* expression in forelimb buds, as well as in the adult skeletal pattern. Our results support the view that the signalling system that regulates the dynamic expression of *Grem1* in the limb bud contributes substantially to variations in avian digital patterns.
the other two species investigated, and thus the expression patterns of emu Gli3 and Alx4 differ from those described previously. In particular, the expression of Alx4 in forelimb buds is broadly conserved across species in a stage-sensitive manner. We also found that the dynamic expression pattern of Grem1 in early limb buds is consistent with the avian digital patterns. These results support the view that the signalling system regulating dynamic expression of Grem1 in the limb bud contributes substantially to variations in the digital patterns among avian species.

Results and Discussion
First, we re-examined the expression patterns of Gli3 and Alx4 in limb buds of emu, chicken and zebra finch embryos (Figs 1, S1, S2). To ensure an accurate staging of all embryos, the hindlimb shape was used as morphological criteria for identifying the Hamburger-Hamilton stages in chicken14, which was adapted for staging zebra finch19 and emu20 embryos. Specifically, stage 25 is defined by a faint demarcation of one digit in the hindlimb plate, and stage 26 by three digit indentations clearly visible in the hindlimbs.

Gli3 expression was extensively expressed in the emu forelimb buds at stage 25, although it was more intense in the anterior region (Fig. 1a). In the chicken forelimb bud, Gli3 expression was detected in the anterior region (Fig. 1d), while it was extended posteriorly in the forelimb bud of zebra finch (Fig. 1g). Although the stage of the emu embryos was different from that in the previous study4, our data also suggest that the extent of Gli3 expression in limb buds vary among emu, chicken and zebra finch.

In contrast, the expression pattern of Alx4 in the forelimb buds was broadly conserved among emu, chicken and zebra finch. We detected the transcripts of Alx4 in the anterior one-third of the emu forelimb buds at stage 25 (Fig. 1b) as well as in chicken and zebra finch forelimb buds (Fig. 1e,b). The posterior expression of Alx4 reported in the emu forelimb bud33 was also seen in both chicken and zebra finch at stage 26 (Fig. 1c,f,j), as well as in their hindlimb buds (Fig. 1i–o). Similar posterior expression of Alx4 was previously shown in the chicken forelimb bud at late stage 2532. Although it was reported that Alx4 expression extended posteriorly (similar to Gli3 expression) in the zebra finch3, we detected Alx4 transcripts in the anterior one-third of wing buds (Fig. 1h). Therefore, the anterior Alx4 expression in forelimb buds was broadly conserved among emu, chicken and zebra finch in a stage-specific manner.

We then aimed to understand the contribution of the SHH/GREM1/AER-FGF feedback loop9,10,11 to the variation in digital pattern among birds, focusing on the BMP antagonist Gremlin1, a key node of this system10–12. For this purpose, we isolated Grem1 of each species (Fig. S3) and examined its expression pattern. The width of the Grem1 expression domain in the limb bud was consistent with the resulting skeletal pattern (Fig. 2), supporting the view that the level of BMP activity is critical for creating variation in the digital pattern14,17. As Gli3, a key component of SHH signalling, directly controls the expression of Grem1 in limb buds22, differences in the expression of Gli3 in forelimb buds among emu, chicken and zebra finch observed here (Fig. 1a,d,g) are likely to contribute to the resulting distal Grem1 expression patterns and digit number. Interestingly, in emu forelimb buds, the width of distal Grem1 along the anterior-posterior axis varied among individuals (Fig. 2a,b). Distal Grem1 expression area relative to total forelimb area showed a greater variation in emu (0.778 ± 0.264, mean ± s.d., n = 7) than in chicken (0.762 ± 0.114, mean ± s.d., n = 10) (Fig. S4). The skeletal elements of adult emu wings demonstrated a great range of individual variation as well (Fig. 3a–c). Among the 24 wings that we examined, 10 had a small rudiment of digit 2 fused to digit 3 in the proximal region, and 15 had a partial digit 4 at the posterior margin (Fig. 3a–c, digits are referred to by their embryological origin, not by their osteological identity). A high degree of individual variation was also recognised in the cartilage pattern of developing forelimbs (Fig. 3d–f), suggesting that this range of individual variation might already be present in early limb bud stages as indicated by Grem1 expression. Our results suggest that the expression pattern of Grem1 in the early limb bud reflects both intraspecific and interspecific variation in digital patterns in avian species.

In conclusion, our results support the hypothesis that variation in digit number arise from regulation of the feedback loops that promote limb outgrowth and patterning, the SHH/GREM1/AER-FGF system. The extent of the expression of Gli3, a key component of the SHH pathway, varied between emu, chicken and zebra finch (Fig. 1a,d,g). We agree with the interpretation that the spatiotemporal expression pattern of the anterior gene Gli3 is critical for creating the variation in the resulting digital pattern, as it directly controls Grem1 expression and digit number10,17.

Unlike previously reported8, the expression pattern of another anterior gene, Alx4, was broadly conserved across species8. A posterior expression domain of Alx4 appears at stage 26 in the forelimb bud of all three birds, suggesting that it is not unique to emu. In addition, the emu forelimb bud has been proposed to develop heterochronically based on delayed SHH expression during the initiation of limb outgrowth31. The conserved expression pattern of Alx4 observed in this study is inconsistent with this model at least at stages 25 and 26. Still, we do not exclude the possibility that slight differences in Alx4 expression levels could affect the skeletal pattern. In fact, Grem1 expression is upregulated in the anterior part of Alx4 mutant limb buds32. It is also important to point out that anterior propagation of Grem1 expression depends on the level of HoxA and HoxD expression25, and anterior skeletal elements can be influenced by subtle changes in the duration or level of posterior Shh expression37, even though de Bakker et al. suggested that Hoxd11, Hoxd12 and Shh had similar expression patterns in emu, chicken and zebra finch8. Thus, differences in the expression of both anterior and posterior genes, which affects the expression of Grem1, can lead to the variation in the final digital pattern.

In this study, we showed that the spatiotemporal expression pattern of Grem1 was highly consistent with the final digital pattern of birds. Furthermore, Grem1 expression in the emu forelimb bud was consistent with their variation of the adult skeletal pattern. Experimental manipulations of Grem1 expression alter the skeletal pattern in several models. The formation of additional phalanges can be induced by infection of chicken forelimbs with Grem1-expressing virus14. Furthermore, in mouse embryos, inhibiting BMP signalling throughout limb buds by overexpressing Grem1 between E10.5 and E11.5 leads to the elongation of digits as well as the formation of
both pre- and post-axial polydactyls. In the limb buds, proliferation of mesenchymal cells terminates after the downregulation of Grem1, and subsequently these cells undergo chondrogenic differentiation. It is more likely that the avian digital pattern is regulated by the well-documented SHH/Gremlin1/AER-FGF feedback loops, in which Gremlin1 is the critical node linking each signalling module, and both intraspecific and interspecific variation in the digital pattern can be recognized as the expression pattern of Grem1 in early limb buds.

Finally, a recent study showed that the co-option of Nkx2.5 in the emu forelimb bud leads to the reduction of forelimb growth and digit loss; however, it remains unknown how Nkx2.5 inhibits the expansion of limb bud. Future studies should determine whether Nkx2.5 expression in emu forelimb bud leads to their extreme digit reduction via regulation of the SHH/GREM1/AER-FGF feedback loops, or via another pathway.
Methods

Data reporting. No statistical methods were used to predetermine sample size. The experiments were not randomised and the investigators were not blinded to allocation during experiments and outcome assessment. The sex of the embryos is unknown.

Animals. Chicken (Gallus gallus) eggs were incubated at 38 °C and staged\(^\text{18}\). Fertilised emu (Dromaius novachollandiae) eggs were purchased from Kakegawa Kachoen and Okhotsk Emu Pasture, incubated at 36.5 °C and staged as described\(^\text{20}\). Zebra finch (Taeniopygia guttata) eggs were collected, incubated at 38 °C and staged as described\(^\text{19}\). For in situ hybridization, embryos were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline, dehydrated in a graded methanol series and stored in 100% methanol at −20 °C. All animal work was performed in accordance with guidelines for animal experiments of the Tokyo Institute of

Figure 2. Expression patterns of Grem1 in limb buds is consistent with the resulting skeletal pattern. (a–g) Distal Grem1 expression in forelimb and hindlimb buds of stage 25 emu, chicken and zebra finch embryos is correlated with the digital skeletal patterns shown on the left (a, b, e n = 9; c, f, n = 10; d, g, n = 3). a, b Left limb buds flipped horizontally. Note that size of the distal Grem1-positive area (bracket) varies among emu embryos at the same stage.
Technology, RIKEN, and The Jikei University School of Medicine, and the experimental protocols were approved by the committees of Tokyo Institute of Technology, RIKEN, and the Jikei University of Medicine.

Gene isolation and phylogenetic analysis. Total RNA was extracted from stage 20 chick, stage 25 emu and stage 17–25 zebra finch embryos using RNasea kit (Qiagen). cDNA was synthesised by reverse transcription and used as a template for PCR. To isolate emu, chick and zebra finch genes, we used the following avian universal primers: avian Alx4, 5′-CTACTACAACGCAGCCTCCC-3′ and 5′-CTTYGCTTTCATCCTCAGGGC-3′; avian Gli3, 5′-ATATCGCACCTTCCCGAACC-3′ and 5′-GATGAGTGGAGGGCTGTGTC-3′; avian Grem1, 5′-TCCTGTCAAGGATCAGCCCA-3′ and 5′-GATGAGTGGAGGGCTGTGTC-3′; avian Grem1, 5′-TCCTGTCAAGGATCAGCCCA-3′ and 5′-ACACCGGCACTCCTTAACTC-3′. The gene fragments were cloned into pGEM T-easy vector (Promega). The partial coding sequences for D. novaehollandiae Grem1, D. novaehollandiae Gli3, D. novaehollandiae Alx4, T. guttata Grem1, T. guttata Gli3, and T. guttata Alx4 were submitted to GenBank under accession numbers MH352496–MH352501, respectively. Amino acid sequences were aligned using ClustalW version 2.1.13.

Probe synthesis and in situ hybridisation. D. novaehollandiae, G. gallus and T. guttata Alx4, Gli3 and Grem1, all of which were in pGEM T-easy vector, were used as templates for riboprobe synthesis. Whole-mount in situ hybridisation was carried out as described.32 We used both G. gallus and T. guttata Grem1 probes for expression analysis of Grem1 in T. guttata embryos as they produced the same results.

Measurement of Grem1 expression ratio. Measurements were made using ImageJ (https://imagej.net/Downloads). The expression area and limb area were delimited manually. Then, the ratio between the distal
Grem1 expression area and whole limb area was calculated. To normalize any experimental variation during in situ hybridization staining, we divided the forelimb Grem1 expression ratio by the hindlimb Grem1 expression ratio of the same embryo. The Grem1 expression ratio was defined as mean ± s.d.

Alcian blue staining. Embryos were fixed in 4% PFA, stained in 0.1% Alcian blue in 1% HCl/70% ethanol, dehydrated in ethanol and cleared in methyl salicylate.

Computed Tomography imaging. Computed Tomography (CT) Imaging was performed by Micro-CT system (Latheta LCT-200, Hitachi Aloka Medical Ltd., Tokyo, Japan) for adult emu forelimb skeletons. Acquired slice data were rendered as three-dimensional images using the VGStudio MAX2.0 software (Volume Graphics GmbH, Heidelberg, Germany).

Data Availability The authors declare that all data supporting the findings of this study are available within the article and its Supplementary Information Files or from the corresponding author upon reasonable request.

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**Acknowledgements**
We thank Dr. Q. Zhou for providing wing samples of emu embryos, Tokyo Nodai Bioindustry Corporation for providing wing samples of adult emu, Kakegawa Kacho-en and Okhotsk Emu Pasture for providing emu eggs, and the Biotechnology Center of Tokyo Institute of Technology for sequencing services. This work was supported in part by a Grant-in-Aid for Scientific Research (C)(17KT0106), Mitsubishi Foundation and Yamada Science Foundation to M.T.

**Author Contributions**
K.K., I.R.C. and M.T. designed the project and wrote the manuscript. K.K. isolated cDNA fragments and constructed plasmids. K.K., I.R.C., S.U., C.N., and Y.M. performed *in situ* hybridisation. I.R.C. performed statistical analysis. K.K., S.U., C.N., and R.Y. performed cartilage staining. G.S. provided zebra finch and emu embryos and related materials. M.K. took CT images, M.O. assisted with CT scanning.

**Additional Information**
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-019-44913-w.

**Competing Interests:** The authors declare no competing interests.

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