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Ancient DNA integrates fossil and modern giant salamander taxonomy

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The genus *Andrias* includes the largest extant salamanders, and is comprised of one Japanese species *A. japonicus* and four Chinese species. The fossil record of the giant salamander is incomplete and modern giant salamanders are not differentiated osteologically among species, making it difficult to identify bones at the species level. In this study, we re-examined a fossil series of giant salamander discovered from a cave on Shikoku Island, Japan. We obtained ancient DNA from the fossil and confirmed that the partial sequence of mitochondrial DNA was identical to that of extant *A. japonicus*. These remains were dated to the Late Pleistocene, however, the result of carbon-14 dating in this study estimated the age as more recent, approximately 3,500–4,100 years ago. Currently, there is a small population of *A. japonicus* in Shikoku, but it is far removed from the fossil discovery area. Our findings suggest that wild *A. japonicus* in western Shikoku may have been extirpated very recently.

Cryptobranchidae is a family of giant salamanders that includes the largest extant amphibians which has a total body length of over 1.8 m. Two extant genera are recognized: *Andrias* in East Asia and *Cryptobranchus* in North America. *Andrias* consists of four species from China and one species, *A. japonicus*, from Japan. According to the latest molecular phylogenetic analyses, the Cryptobranchidae appeared around 61.3 Ma, and *Andrias japonicus* and Chinese *Andrias* spp. are estimated to have diverged around 15.8 Ma[1]. Furthermore, molecular and morphological studies strongly suggest an Asian origin for cryptobranchids with subsequent expansions into Europe and North America[2]. However, the fossil record in Asia after the Miocene is not well documented. Thus, the time of the origin of these species is poorly understood.

The genus *Andrias* was first proposed for the fossil species *A. scheuchzeri* but now includes both fossil and extant species. This is because the morphological characters of fossil species from Europe and Asia are highly conserved. The extant species *A. davidianus* was once misclassified and synonymized with the fossil species *A. scheuchzeri*[3]. On the other hand, the extant Chinese giant salamander *A. davidianus* has recently been separated into four species: *A. davidianus* sensu stricto, *A. jiangxiensis*, *A. sligoi*, and *A. cheni*[4–6], this revision is largely based on molecular data but also includes some minor differences in external morphology. However, molecular data was unavailable from fossil species, and comparative osteological studies on extant *Andrias* species are scant. Due to this situation, the taxonomy of *Andrias* has proven very confusing and it has been extremely difficult to taxonomically integrate fossil and extant species.

In this context, giant salamander remains were discovered in the Shikimizu bed and exposed at a quarry in Ozu City, Ehime Prefecture, Shikoku Island, Japan (Fig. 1B). The fissure deposits at the quarry of the Shikimizu bed are mainly composed of brown brecciated clay 14 m thick underlain by layers of black sand, yellow clay, and gravel, and overlain by red clay and black humus[7]. A total of nearly 1,900 fossils have been collected from this deposit, of which about 1,800 are vertebrates including fishes, amphibians, reptiles, birds, and mammals[8]. The exact age of the deposit is unknown, but it has been inferred to date from the Late Pleistocene or Early Holocene based on the mammalian species composition[9].

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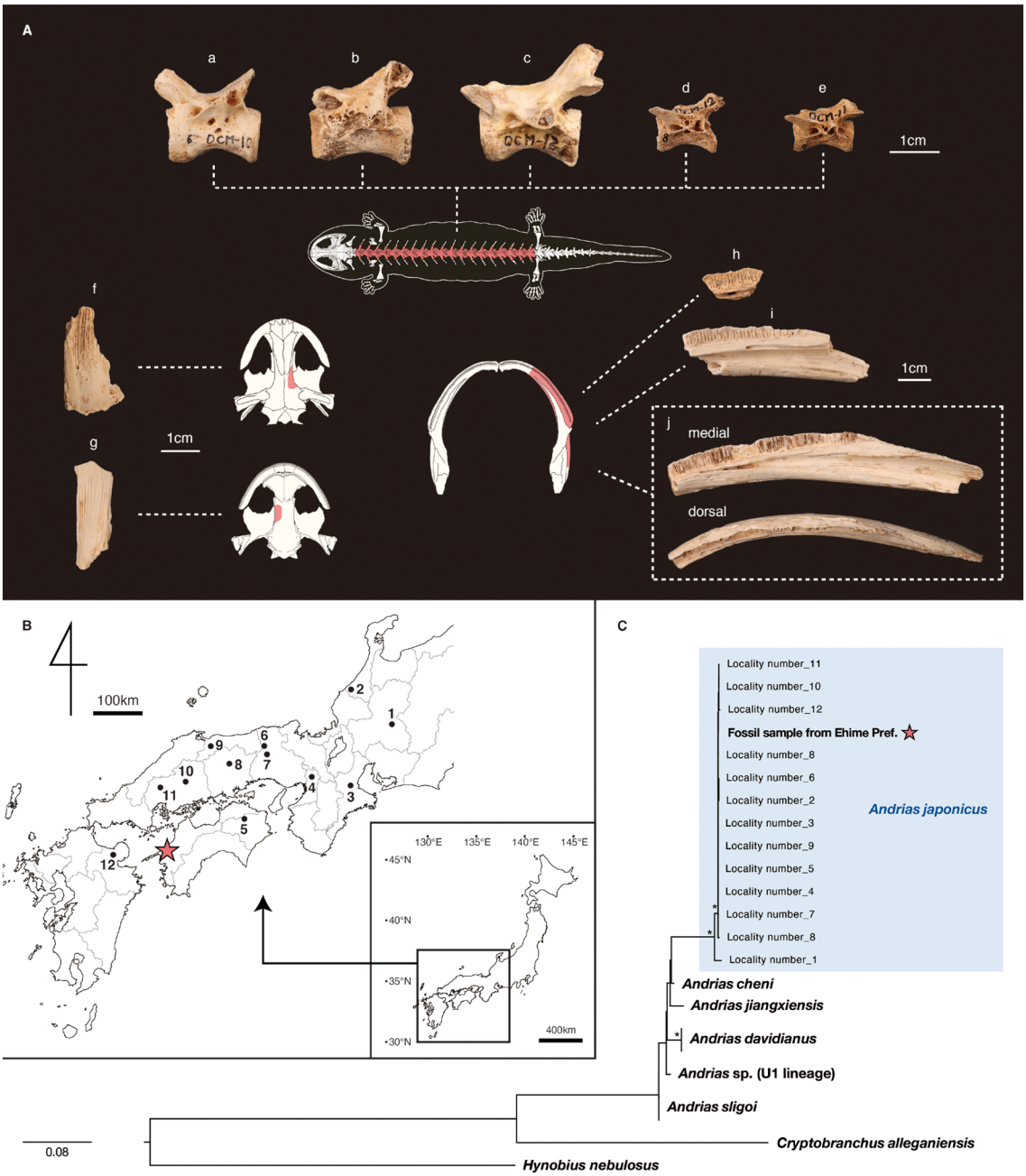

Fig. 1. (**A**) The fossil remains of *Andrias japonicus* from Ehime Prefecture. a–e (OCM-10, OCM-14, OCM-13, OCM-12, OCM-11): left lateral view of trunk vertebrae; f (OCM-8): dorsal view of right parietal; g (OCM-9): ventral view of right parasphenoid; h–j (OCM-7, OCM-6, OCM-5); medial view of right dentaries. (**B**) Map of central and southwestern Japan showing localities of samples used in the study. The red star indicates the locality of the fossil sample of *A. japonicus*. For locality numbers, refer to Table 1. (**C**) Maximum likelihood phylogenetic reconstruction of partial cyt b gene sequences from giant salamanders. Asterisks on nodes indicate significant supports (Bootstrap value ≥70%). Locality numbers are concordant to Fig. (B). This figure was created using Adobe Photoshop 26.1 and Adobe Illustrator 29.1.

The giant salamander remains from Ehime contain the right parietal, a chunk of parasphenoid close to its right border, three right dentaries, and three trunk vertebrae (Fig. 1A). All specimens are incomplete and have yellowish-white color. In a previous study, these remains were identified as the extant species *A. japonicus* based on morphological comparisons only with *A. japonicus* skeletons, and at least four individuals can be identified based on size differences and duplicated elements; at least one individual is small-sized (approximately 500 mm long) and at least three are large-sized (approximately 900 mm long)[7].

However, as mentioned above, given the taxonomic issues within *Andrias*, it is impossible to identify the fragmentary remains from Ehime Prefecture as *A. japonicus* without considering comparisons with other

species. Moreover, unidentified Pliocene Cryptobranchid fossils were reported from Oita, Japan, which may represent a new species of *Andrias* or even a different genus[10]. So, it is also not appropriate to identify the remains from Ehime as *A. japonicus* solely because it was discovered in Japan. Identifying the giant salamander remains from Ehime has been challenging, but we believe that recent advances in ancient DNA methodology may overcome this problem. The aim of this study is to reexamine the taxonomic assignment of a rare series of Asian giant salamander remains through an integrated approach combining morphological comparisons, ancient DNA analysis, and radiocarbon dating.

Identification based on morphological characteristics of bones

The giant salamander from Ehime can be assigned to Cryptobranchoidea and Cryptobranchidae because of its large body size, absence of the spinal nerve foramina in trunk vertebrae, and unicapitate transverse processes in trunk vertebrae[11]. They are also assigned to Cryptobranchinae because the vertebrae are rectangular in lateral view, and the prezygapophyses usually project above the bases of the neural spine[12]. Furthermore, these remains can be assigned to the genus *Andrias* based on a larger angle (about 40 degrees) between the neural spine and the axis of the vertebral centrum (19–37 degrees in *Andrias* vs. 15–20 degrees in *Cryptobranchus*)[3,13]. The giant salamander from Ehime differs from the Miocene to Pliocene *A. scheuchzeri*[3,14,15] by possessing a more slender and slightly curved dentary. Therefore, the morphological character of the Ehime giant salamander fossil suggests that it may be assignable to a recent extant species.

However, the diagnostic features that differentiate *A. japonicus* and *A. davidianus* sensu lato (maxilla and anterior part of dentary) were not preserved. We can provisionally assign the fossil only to the generic level until reliable data on the comparative osteology of the modern species of *Andrias* or new material from giant salamander remains appear from Ehime.

Radiocarbon dating and phylogenetic analysis

We obtained collagen and ancient DNA from one of the trunk vertebrae. This sample was dated to 4,140–3,587 cal BP by carbon-14 dating. The mitochondrial cytb sequence extracted from the remain of *Andrias* sp. from Ehime Prefecture, Shikoku Island, Japan is identical with *A. japonicus* samples collected from Ishikawa, Mie, Nara, Shiga, Kyoto, Osaka, Tokushima, Hyogo, Okayama, Tottori, Hiroshima, Shimane, Oita and Kumamoto prefectures in Japan. The phylogenetic analysis recovered the fossil as part of the Western Japan clade of the species (Fig. 1C), and hence we identify the fossil sample as the extant species, *A. japonicus*.

Most ancient DNA (aDNA) studies were conducted on mammals including humans, with very little focus on amphibians. This is because amphibian habitats are mainly wetlands, ponds and other watery places, where genetic information is often destroyed by DNase. Among the extremely few examples of amphibian aDNA, successful extraction has been achieved from frog remains in Australia[16] and small salamander remains in the United States[17], both discovered in caves. The remains we analyzed in this study were also found in caves. This suggests that the cave environment may be relatively suitable for preserving DNA.

Currently, there are small populations of *A. japonicus* in Shikoku[18], but they are not in the area where these fossils were discovered. This suggests that wild *A. japonicus* in Ehime Prefecture likely became extinct very recently. Giant salamander bones were also found, along with fish and mammal bones, in a cave site inhabited by humans in Hiroshima[19]. In the Ehime caves, several individuals were also found together with fishes and mammals. Therefore, it is highly likely that humans consumed giant salamanders as a food or a ritual. Although direct evidence like a cut mark was not found on the present bones, human activities might have contributed to the local extirpation of *A. japonicus* in this area.

Materials and methods

Samples

The fossil of *Andrias japonicus* is designated as the cultural property of Ozu City and is deposited at the Ozu City Museum. These specimens include three right dentary fragments (OCM-5, OCM-6, OCM-7), a right parietal fragment (OCM-8), a right parasphenoid fragment (OCM-9), and five trunk vertebrae (OCM-10, OCM-11, OCM-12, OCM-13, OCM-14).

Radiocarbon dating

We extracted collagen fraction with >30k Da from a small fragment (~ 100 mg) of each bone for C dating following the procedures[20,21], and the ^{14}C ages were measured using the HVEE Tandetron accelerator mass spectrometer at Nagoya University[22]. We used CALIB[23] v8.2 for ^{14}C age calibration.

DNA extraction

DNA extractions were conducted in a purpose-built positive-pressure ancient DNA (aDNA) laboratory located within the Museum of Natural and Environmental History, Shizuoka. This laboratory is physically isolated from other molecular laboratories with independent air intake, and there has been strict compliance with the 'one-way rule'[24] upon using these laboratories. We followed procedures employed[20] for aDNA extraction. A negative extraction was included in each aDNA extraction procedure.

Mitochondrial DNA sequencing and phylogenetic analysis

We amplified the partial mitochondrial gene cytochrome b using the newly designed primers andrias_cytb4 F (ACAGGGTCAAGCAATCCAAC) and andrias_cytb4R (TGGGAGTAGCAGTGAAATCAAT). The expected amplification length of the targeted gene fragment is 93 bp (excluding primers). The total volume of the PCR reaction was 25 µl containing 0.48 µM of each primer, 0.16 mM of each dNTP, 4 mM MgCl2, 1 M betaine, 1 U of AmpliTaq Gold 360 DNA Polymerase (Applied Biosystems), 1× PCR buffer, and 2 µL of extracted DNA.

Species	Specimen Number	Locality Number	Locality	GenBank	Reference
Andrias japonicus	no voucher	1	Gujyo-shi, Gifu, Japan	AB445781	18
Andrias japonicus	no voucher	2	Kaga-shi, Ishikawa, Japan	AB445776	18
Andrias japonicus	no voucher	3	Iga-shi, Mie, Japan	AB445780	18
Andrias japonicus	no voucher	4	Takatsuki-shi, Osaka, Japan	AB445779	18
Andrias japonicus	no voucher	5	Mima-shi, Tokushima, Japan	AB445778	18
Andrias japonicus	KUHE 26,613	6	Yabu-shi, Hyogo, Japan	AB445777	18
Andrias japonicus	no voucher	7	Asago-shi, Hyogo, Japan	AB445775	18
Andrias japonicus	no voucher	8	Kagamino-cho, Okayama, Japan	AB445773	18
Andrias japonicus	no voucher	8	Kagamino-cho, Okayama, Japan	AB445774	18
Andrias japonicus	no voucher	9	Hino-cho, Tottori, Japan	AB445772	18
Andrias japonicus	no voucher	10	Miyoshi-shi, Hiroshima, Japan	AB445771	18
Andrias japonicus	no voucher	11	Kitahiroshima-machi, Hiroshima, Japan	AB445770	18
Andrias japonicus	KUHE 33,230	12	Usa-shi, Oita, Japan	AB445769	18
Andrias cheni	KIZYPX 6151		Anhui, Huangshan, China	MH051473	25
Andrias jiangxiensis	CGS 291		Farm-bred (Jiangxi), China	MH051481	25
Andrias davidianus	KUHE 34,380		Unknown locality in China	AB445782	18
Andrias davidianus	KUHE 26,617		Unknown locality in China	AB445783	18
Andrias sp. (U1 lineage)	no voucher		Unknown locality in China	AB445784	18
Andrias sligoi	KIZYPX 2513		Chongqing, Xinglong, China	MH051435	25
Andrias sligoi	KIZYPX 2513		Unknown locality in China	LC650454	26
Cryptobranchus alleganiensis	KUHE 36,922		Unknown locality in US	AB445785	18
Hynobius nebulosus	KUHE 24,693		Isahaya-shi, Nagasaki, Japan	AB445786	18

Table 1. Samples used for phylogenetic analysis in this study including locality data and GenBank accession numbers.

The PCR amplification conditions included initial denaturation at 95℃ for 9 min, followed by 45 cycles at 95℃ for 20 s, 53℃ for 30 s, and 72℃ for 30 s, with a final extension at 72℃ for 4 min. A negative control was included in each PCR. The PCR mixtures were prepared in the aDNA laboratory, and a thermal cycler installed in the post-PCR laboratory was used for PCRs. PCR products were purified using ExoSAP-IT Express (Applied Biosystems). The PCR products were sequenced using the PCR primers and the BigDye v3.1 Cycle Sequencing kit and visualized in an ABI 3130xl Genetic Analyzer or 3730xl DNA Analyzer (Applied Biosystems, Waltham, Massachusetts, USA). In addition to the newly produced sequence data, we used previously published sequence data for *Andrias japonicus*, *A. davidianus*, *A. cheni*, and *A. jiangxiensis* for comparisons and *Cryptobranchus alleganiensis* and *Hynobius nebulosus* sequences were used for outgroup (Table 1). Sequences were aligned using the MUSCLE algorithm[27] in MEGA X[28] with default parameters. We used Maximum likelihood (ML) methods to conduct phylogenetic analyses. Suitable models were selected using Modeltest-NG[29] with the corrected Bayesian Information Criterion (BIC). The ML analysis was conducted using RAxML-NG[30]. Uncorrected p-distances among the sequences were calculated using MEGA X.

Ancient DNA authenticity

We amplified and sequenced the mtDNA cytB partial sequence two times independently, and confirmed consistency between two independently-amplified sequences. A negative control (i.e., a PCR mixture using the negative extract as a DNA template) was included in each PCR, and no amplification from the negative control was detected. We performed all aDNA extraction and amplification procedures in the Museum of Natural and Environmental History, Shizuoka where amplification of modern amphibian DNA has never been conducted since its inception.

Data availability

The newly obtained sequence is deposited in figshare (10.6084/m9.figshare.28295672).

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Author contributions

M. N. conducted morphological examinations; T. K. conducted ancient DNA analysis; H. K. conducted radiocarbon dating; I. F. conducted molecular analyses; M. N., T. K., H. K., I. F., and K. N. wrote the manuscript. The first draft was written by M. N., and all authors read and approved the final manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

Additional information
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