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Authors: Hara, Sotaro, Yamamoto, Kazuhiro, Matsui, Masafumi, Tominaga, Atsushi, Yoshikawa, Natsuhiko, et al.

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Comparison of Head and Skull Shapes among Native *Andrias japonicus*, Introduced *A. davidianus*, and Their Hybrids in Japan (Urodela: Cryptobranchidae) based on Geometric Morphometrics

SOTARO HARA^{1,*}, KAZUHIRO YAMAMOTO¹, MASAFUMI MATSUI¹,
ATSUSHI TOMINAGA^{1,2}, NATSUHIKO YOSHIKAWA^{1,3}, KOSHIRO ETO^{1,4},
KAZUMI FUKUTANI¹, HIDEYUKI ITO⁵, AND KANTO NISHIKAWA^{1,6,7}

¹Graduate School of Human and Environmental Studies, Kyoto University, Yoshida
Nihonmatsu, Sakyo, Kyoto 606–8501, JAPAN

²Faculty of Education, University of the Ryukyus, 1 Senbaru, Nishihara, Okinawa 903–0213,
JAPAN

³Department of Zoology, National Museum of Nature and Science, Tokyo, 4–1–1 Amakubo,
Tsukuba, Ibaraki 305–0005 JAPAN

⁴Kitakyushu Museum of Natural History and Human History, 2–4–1 Higashida,
Yahatahigashi, Kitakyushu 805–0071, JAPAN

⁵Kyoto City Zoo, Okazakihoshoji, Sakyo, Kyoto 606–8333, JAPAN

⁶Graduate School of Global Environmental Studies, Kyoto University, Yoshida Honmachi,
Sakyo, Kyoto 606–8501, JAPAN

⁷Department of Biological Sciences and Biotechnology, Faculty of Science and Technology,
Universiti Kebangsaan Malaysia, UKM Bangi 43600, Selangor, MALAYSIA

Abstract: The Japanese giant salamander, *Andrias japonicus*, is one of the largest extant amphibians. It is endemic to Japan and has been designated as a special natural monument by the Japanese government. The genus *Andrias* is also protected by the Convention on International Trade in Endangered Species of Wild Fauna and Flora. The Japanese giant salamander and one of the congeners, the Chinese giant salamander, *A. davidianus*, were recently found to hybridize in several areas in Japan, due to human-mediated introduction of *A. davidianus*. In this study, we found major differences in head and skull shapes between the Japanese and Chinese giant salamanders, using geometric morphometrics. The hybrids showed intermediate morphology between the two species, but also possessed a wider mouth, at the skeletal level, than either of the parent species. This unique characteristic of hybrids is suggested as one of the reasons for the current dominance of the hybrids in the introduced areas and may be an example of heterosis in urodeles.

Key words: Giant salamander; Heterosis; Hybrids; Invasive species; Native species

INTRODUCTION

Invasive species are well known to affect native species adversely through predation, competition, and hybridization, which are considered the main causes of biodiversity degradation (Kraus, 2009). In Japan, huge numbers of living animals have been imported for pets or food (Franke and Telecky, 2001; Toda and Yoshida, 2005), which has had a great impact on native species when they escape or are released into the wild. For example, the American bullfrog, *Lithobates catesbeianus*, was originally introduced from the United States to Japan, and is now distributed in many prefectures (Matsui, 2021). This invasive species negatively affects native species directly through predation and indirectly through niche competition (Nakamura and Tominaga, 2021). Such species differ greatly in appearance from native species, and it is easy to understand their effects on native species, which makes it easy to take conservation action. On the other hand, the problem of hybridization between introduced and native species tends to delay such action because the external morphology is often very similar, and the effects on native species are difficult to recognize. Then, because of the great impact on the native species that suffer hybridization, the native species may become extinct by the time the problem has been recognized (Allendorf et al., 2001).

The Japanese giant salamander, *Andrias japonicus*, is an endemic amphibian occurring in hilly and montane rivers of western Honshu, Shikoku, and Kyushu, in the Japanese main islands (Browne et al., 2014; Matsui, 2014). This species is listed in Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora, at the generic level, and it is strictly protected in Japan as an endangered species (VU) in the Red List of Threatened Species by the Ministry of the Environment (Matsui, 2014) and has been des-

ignated a special natural monument since 1952. In recent years, the Chinese giant salamander, *A. davidianus*, has been found in some areas of Japan, hybridizing with the Japanese giant salamander (Matsui, 2006; Yoshikawa et al., 2011). In particular, in the Kamo River in Kyoto City, F2 and later hybrid generations occur, resulting in success of the hybrids and significant decline of Japanese giant salamanders, for unknown reasons (Yoshikawa et al., 2011). Therefore, both the Chinese species and the hybrids are currently designated as invasive alien species by the Ministry of the Environment in Japan.

Here, we compare the head and skull shapes of *A. japonicus*, *A. davidianus*, and their hybrids. The head morphology of giant salamanders is a very important morphological character, reflecting their ecological adaptation, because it influences their feeding behavior and competition for reproductive success (Heiss et al., 2013; Takahashi et al., 2024).

MATERIALS AND METHODS

We examined specimens of *Andrias japonicus*, *A. davidianus*, and their hybrids stored in the Graduate School of Human and Environmental Studies, Kyoto University (KUHE). All specimens were adults, and the ranges for total length of *A. japonicus*, *A. davidianus*, first filial hybrids (F1), and second filial hybrids (F2) were 340–940 mm, 420–1200 mm, 540–1110 mm, and 470–1060 mm, respectively. The specimens of *A. japonicus* and *A. davidianus* were donated as dead individuals from local governments, zoos, and aquariums, and the specimens of hybrids were newly collected in the Kamo and Katsura Rivers, Kyoto, surveyed by Kyoto City and the Agency for Cultural Affairs, Government of Japan. We conducted microsatellite analysis to identify the specimens as *A. japonicus*, *A. davidianus*, F1 hybrids, or F2 hybrids (Yoshikawa et al., 2012). Genetic identification of individuals was carried out using the software NewHybrids (Anderson and Thompson, 2002), based on the resulting genotype data. In order to

* Corresponding author.

E-mail address: hara.sotaro.5f@kyoto-u.ac.jp

facilitate genetic identification, tissue samples of pure *A. japonicus* and *A. davidianus* were required for reference. We obtained samples of adult *A. japonicus* from several sites in Kyoto, Mie, and Nara Prefectures where no hybridized individuals had been found. For pure *A. davidianus*, we used zoo and aquarium captives imported from China.

The preparation of preserved specimens was performed according to the following procedure. Live individuals were fully anesthetized with injections of 2-phenoxyethanol solution (2.0 ml/1.0 kg body weight), dissected to determine sex and sexual maturity, and fixed in 10% formalin solution for several days to several weeks, in an upright position. Then, the specimens were rinsed in tap water and stored in 70% ethanol solution.

In measurements of the external morphology of the head, we used specimens excluding those with extremely deformed heads: 18 specimens of *A. japonicus*, 19 *A. davidianus*, 17 F1 hybrids, and 23 F2 hybrids for lateral analysis, and 19 specimens of *A. japonicus*, 20 *A. davidianus*, 16 F1 hybrids, and 24 F2 hybrids for dorsal analysis. In addition, we used 18 *A. japonicus*, 18 *A. davidianus*, 17 F1 hybrids, and 9 F2 hybrid specimens for cranial examinations, based on photographs of skulls or soft X-ray photographs.

Photographs of the dorsal and lateral views of the heads and dorsal views of the skulls were taken with a digital camera (NIKON D5000 & SIGMA DC 17–70 mm 1:2.8–4.5 MACRO HSM, 4288×2848 pixels). Radiographs of the skulls were taken at 55 kV and 4.0 mA for specimens less than 590 mm in length, 60 kV and 4.5 mA for specimens 600–800 mm in length, and 65 kV and 5.0 mA for specimens 810 mm and longer, using a Mobile X-Ray System (SHIMADZU MUX-10J) and automatic developer (FUJIFILM CR-IR 391V). The radiographs were processed using V station client (FUJIFILM).

Geometric morphometric analysis

The raw image data were imported into a PC, and tps format files were created using

tpsUtil (version 1.78) (Rohlf, 2015). The labeling points were then assigned using tpsDig2 (version 2.31). For creating semi-landmark points on dorsal and lateral head shapes, the starting point was designated at the tip of rostrum which was farthest from the eyes, and the end points were the two intersections between the line connecting the eyes and the contour of the head. Then, we determined 20 points equidistant from each other on either side of contour from the starting point to one of the end points. In this way, we determined a total of 40 points on dorsal and 20 points on lateral views of the heads (Fig. 1A, 1B). For the photographs and radiographs of skulls (Fig. 1C), 19 labeling points were set to represent the whole shape of the skull; 1: anterior articulation of premaxilla, 2: posterior articulation of nasal, 3: posterior articulation of parietal, 4 and 19: points of contact between premaxilla and maxillae, 5 and 18: posterior tips of maxillae, 6 and 17: distal articulations between prefrontals and maxillae, 7 and 16: anterior tips of orbitosphenoids, 8 and 15: anterior tips of pterygoid bones, 9 and 14: anterior tips of quadrate bones, 10 and 13: posterior tips of squamosals, and 11 and 12: posterior articulations between parietal and squamosal on either side.

Next, the generalized Procrustes analysis was performed using MorphoJ (Klingenberg, 2011), and the designated points were standardized by mutation (vertical and horizontal movement), rotation, and scaling. Principal components analysis (PCA) was performed to evaluate differences in morphology for the dorsal and lateral views of the heads and the dorsal views of the skulls. Then a Canonical Discriminant Analysis (CDA) was conducted to clarify the difference among the four groups. The Kruskal-Wallis test was applied to test the differences in the principal component scores. For those scores, the Mann-Whitney U-test was used to test the differences between each pair, and Bonferroni's adjustment was applied. The package PAST (version 3.25) was used for all statistics (Hammer et al., 2001), and the significance level was set at $P=0.05$.

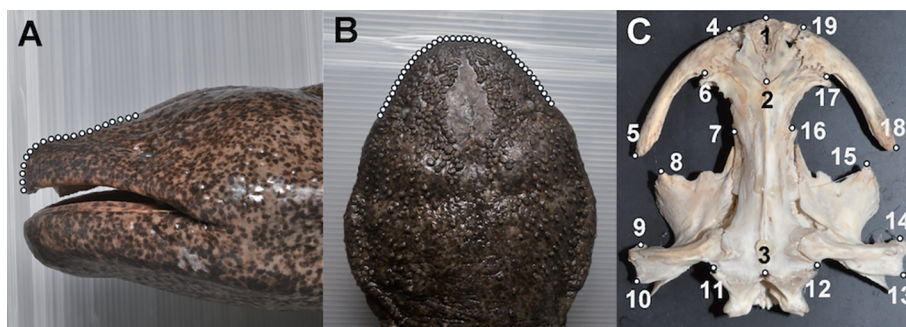


FIG. 1. Semilandmarks and landmarks on the genus *Andrias*. A: lateral view of head; B: dorsal view of head; C: dorsal view of skull.

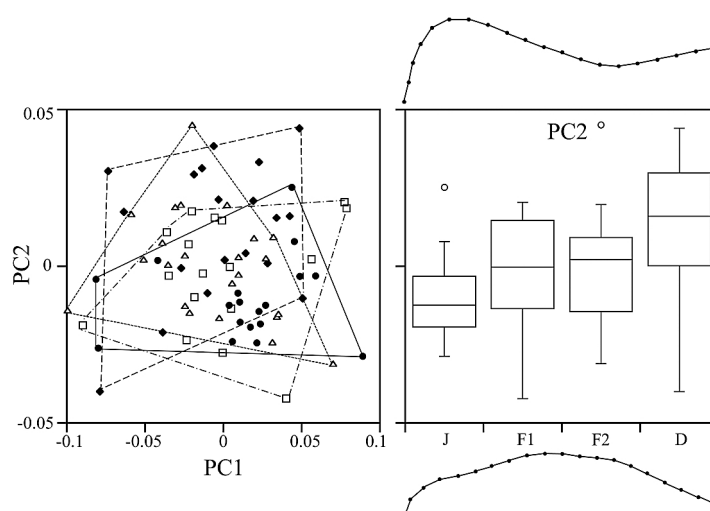


FIG. 2. Scatter plots of PC1 and PC2 (left) and box plots of PC2 with wireframe changes of lateral views of giant salamander heads (right). Circles: *Andrias japonicus*; squares: first filial hybrids; triangles: second filial hybrids; diamonds: *A. davidianus*.

RESULTS

External Morphology

Lateral view of the head

In the PCA, the total contributions exceeding 80% consisted of the first (71.0%) and second principal components (16.0%, total 87.0%). The Kruskal-Wallis test of the difference in principal component scores between samples was conducted for the first and second principal components, and significant differ-

ences were detected only in the second principal component ($H=13.71$, $df=3$, $P=0.003$; Fig. 2). The variation in lateral morphology described by the second principal component was analyzed graphically by transforming the wireframe connecting the labeled points. This principal component showed the degree of posterior bulging of the head, and *A. davidianus* and F2 hybrids showed a greater degree of bulging than did *A. japonicus*.

In the CDA, the proportions of eigenvalues for the two axes were 48.0% and 35.6%,

respectively, accounting for 83.6% of the total amount of eigenvalues. In scatterplots of these axes (CV1 and CV2), the scores for *A. davidianus* were significantly lower than the others on the first axis ($H=43.97$, $df=3$, $P<0.001$). In the second scores, significant differences were found ($H=40.45$, $df=3$, $P<0.001$) among all species and hybrids, which were the highest in *A. japonicus*, followed, in order, by F1 hybrids, *A. davidianus*, and F2 hybrids (Fig. 3). The variation in lateral morphology indicated by the first and second canonical factors was analyzed graphically by transforming the wire-frame connecting the labeled points. As the first and second canonical discriminant scores increased, the profile was smoother and less concave from snout to parietal (Fig. 3).

Dorsal view of the head

In the PCA, the total contribution rates exceeding 80% consisted of the first (68.0%), second (10.6%), and third principal components (9.9%, total 88.5%). The Kruskal-Wallis test of the difference in the PC scores between samples was conducted up to the third principal component, and significant differences were detected only in the second principal component ($H=12.34$, $df=3$, $P=0.006$; Fig. 4). The variation of the second principal component showed the degree of snout width, which was significantly narrower in *A. japonicus* than in the others.

In the CDA, the proportion of eigenvalues for the two axes were 45.7% and 35.6%, respectively, accounting for 81.3% of the total proportion of eigenvalues. In scatterplots of the two axes (CV1 and CV2), all groups were clearly separated (Fig. 5). The scores for F2 individuals were significantly lower than the others along the first axis ($H=63.45$, $df=3$, $P<0.001$). Along the second axis, significant differences were found ($H=64.94$, $df=3$, $P<0.001$) among all species and hybrids, with the highest scores in *A. davidianus*, followed, in order, by F2 hybrids, *A. japonicus*, and F1 hybrids. The variation in dorsal morphology shown in CV1 and CV2 was analyzed graphically by transforming the wireframe connect-

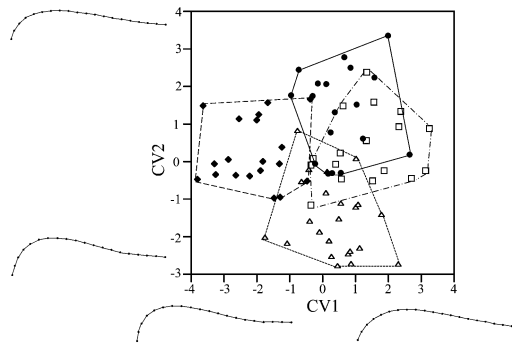


FIG. 3. Scatter plots of CV1 and CV2 of lateral view of giant salamander heads. Circles: *Andrias japonicus*, squares: first filial hybrids, triangles: second filial hybrids, diamonds: *A. davidianus*. Wireframes are shown only for axes with significant differences.

ing the labeled points (Fig. 5). Although shape variation in CV1 and CV2 was small, the snout tended to be wider as the canonical discriminant scores increased.

Cranial Morphology

In the PCA, the total contribution rates exceeding 80% consisted of the first through ninth principal components: 26.1, 18.1, 8.7, 6.6, 6.0, 4.6, 4.2, 3.5, and 3.2%, respectively (total 81.1%). The Kruskal-Wallis test of the difference in principal component scores between samples was conducted up to the ninth principal component, and significant differences were detected only in the first ($H=11.66$, $df=3$, $P=0.009$) and fourth ($H=22.79$, $df=3$, $P<0.001$) principal components (Fig. 6). In the first principal component, both F1 and F2 hybrids had wider maxillae, and the squamosal was positioned more antero-laterally than that of *A. japonicus* and *A. davidianus* (Fig. 7). In the fourth principal component, *A. davidianus* and F1 hybrids had longer premaxillae, and the posterior tips of the parietals were positioned more posteriorly than that of *A. japonicus* and F2 hybrids.

In the CDA, the proportions of eigenvalues for the two axes were 60.2% and 22.1%, respectively, accounting for 82.3% of the total

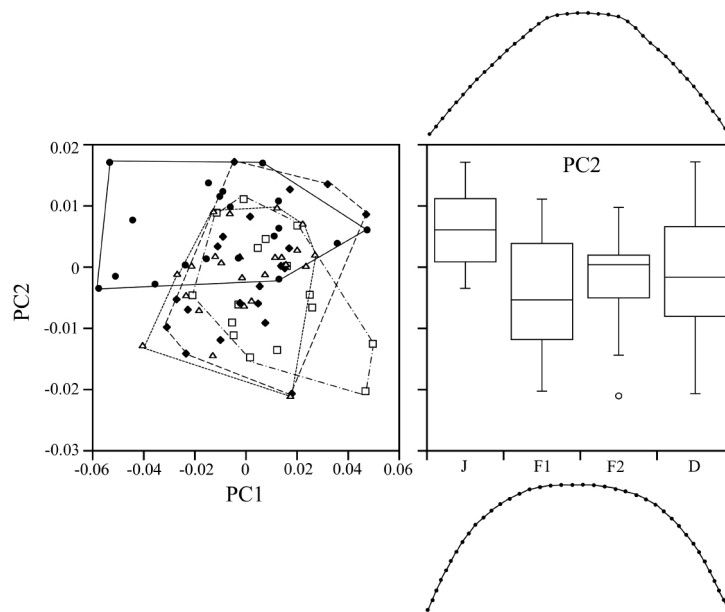


FIG. 4. Scatter plots of PC1 and PC2 (left) and box plots of PC2 with wireframe changes of dorsal views of giant salamander heads (right). Circles: *Andrias japonicus*; squares: first filial hybrids; triangles: second filial hybrids; diamonds: *A. davidianus*.

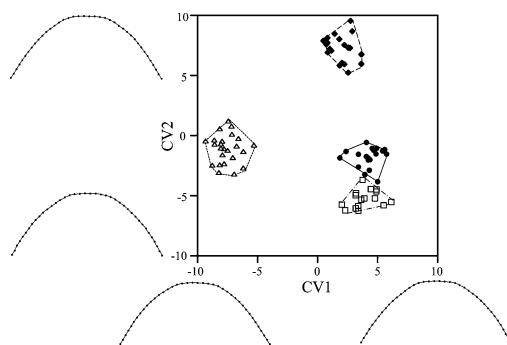


FIG. 5. Scatter plots of CV1 and CV2 of dorsal views of giant salamander heads. Circles: *Andrias japonicus*; squares: first filial hybrids; triangles: second filial hybrids; diamonds: *A. davidianus*. Wireframes are shown only for axes with significant differences.

amount of eigenvalues. Scatterplots of both axes (CV1 and CV2) differed significantly among all species and hybrids. The scores for the first axis ($H=48.20$, $df=3$, $P<0.001$) were highest for F1 hybrids, followed, in order, by the scores for *A. davidianus*, F2 hybrids, and *A.*

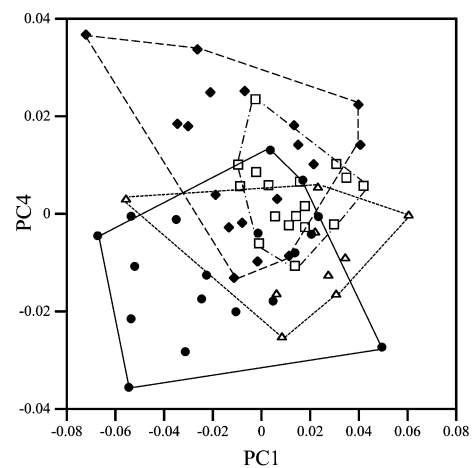


FIG. 6. Scatter plots of PC1 and PC4 of dorsal views of giant salamander crania. Circles: *Andrias japonicus*; squares: first filial hybrids; triangles: second filial hybrids; diamonds: *A. davidianus*.

japonicus. The scores for the second axis ($H=41.55$, $df=3$, $P<0.001$) were highest in *A. davidianus*, followed, in order, by those for *A. japonicus* and both F1 and F2 hybrids (Fig. 8).

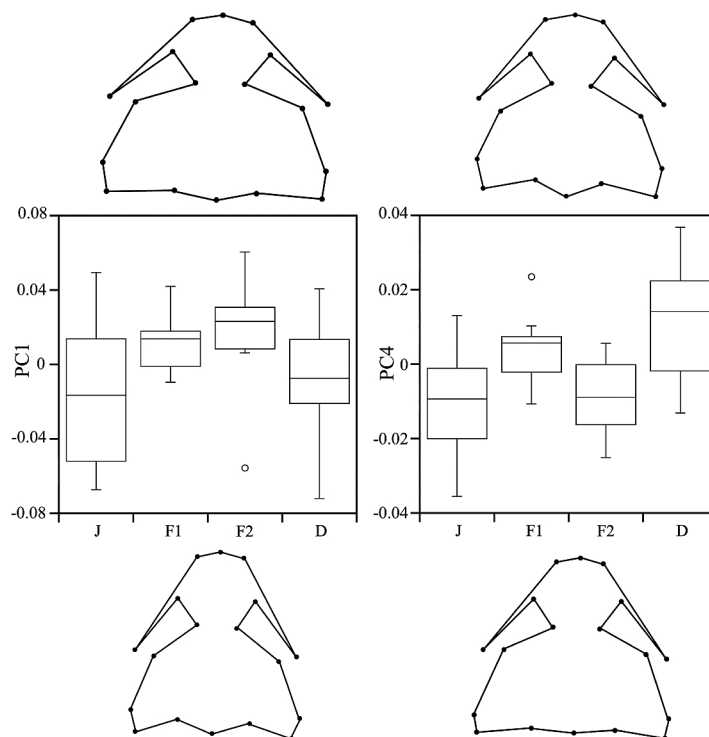


FIG. 7. Box plots of PC1 (left) and PC4 (right) of dorsal views of giant salamander crania with wireframe changes.

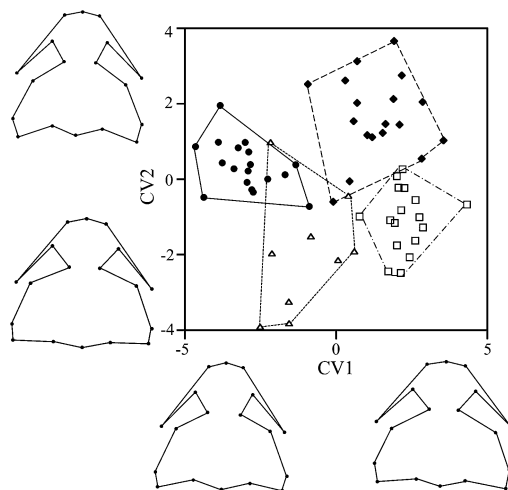


FIG. 8. Scatter plots of CV1 and CV2 of dorsal views of giant salamander crania. Circles: *Andrias japonicus*; squares: first filial hybrids; triangles: second filial hybrids; diamonds: *A. davidianus*. Wireframes are shown only for axes with significant differences.

The variation in cranial morphology indicated by the first and second canonical factors were analyzed graphically by transforming the wire-frame connecting the labeled points. As the first canonical discriminant score increased, the maxillae became wider. As the second canonical discriminant score increased, the rostrum became longer and the squamosals were positioned more posteriorly (Fig. 8).

DISCUSSION

Our comparison of head shape and cranial morphology in *Andrias japonicus*, *A. davidianus*, and their hybrids, using geometric morphometrics, reveals several differences. In external morphology, *A. davidianus* exhibited a wider mouth than *A. japonicus*, consistent with the findings of Hara et al. (2023). The underlying skeletal basis for that variation appears to be the wider upper jaw bones of *A. davidianus*

and the hybrids compared with those of *A. japonicus*. Because the maxillae articulate with only a few bones and do not surround important neurological organs, such as the brain, they may be prone to greater morphological variation. Meszoely (1966) mentioned that *A. davidianus* has more circular bony orbit than *A. japonicus*. This study supports that finding, showing that only the posterior tip of the maxilla of *A. davidianus* (and hybrids) extends outward, making the orbit alike circular.

Although there is not great variation between the hybrids, F2 hybrids were slightly more similar in shape to Japanese giant salamanders than were F1 hybrids. That may occur as a result of differences in genetic combinations, but the exact reason is unknown. It is especially interesting that neither F1 nor F2 hybrids show an intermediate morphology in some characteristics between the two parental species, *A. japonicus* and *A. davidianus*. In the skull, the maxillae, pterygoids, squamosals, and quadrates of hybrids were more laterally expanded than in the two species, and the external morphology of hybrids also showed a wider rostrum than the parental species. Moreover, hybrids tended to have more bulging posterior muscles than *A. japonicus*. These characteristics may increase the head width and the attachment site of the muscoli levatores mandibulae in the hybrid, resulting in more forceful closure of the lower jaw than in either parental species (Kleinteich et al., 2014; Matsumoto et al., 2024). The genus *Andrias* is an ambush and suction-feeder, and it sucks approaching prey, such as fishes and crabs into the mouth. During feeding, giant salamanders suck prey in along with a large volume of water by generating negative pressure inside the oral cavity. To do that, they rapidly drop the buccal floor by rapid and extensive hyobranchial depression (Heiss et al., 2013; Kleinteich et al., 2014). The large mouth of hybrids could enable them to bite and capture larger prey species. In addition, it may increase feeding performance by accommodating a larger hyobranchial skeleton. As a result, hybrid individuals may have higher survival

and more rapid growth rates than the parental species.

In addition, the larger mouth may have an advantage in breeding success. In the genus *Andrias*, breeding takes place in burrows on river banks, where the dominant occupant male, called the “den master”, monopolizes the burrow (Okada et al., 2015; Luo et al., 2018), leading to fierce male–male combat (Takahashi et al., 2024). The large mouth observed in hybrids may facilitate their growth due to higher feeding efficiency and thereby produce large-sized individuals more rapidly, resulting in a larger clutch size in females and a higher success rate in male–male competitions during the breeding season.

Hybridization between introduced and native species of urodeles is well known in the genus *Ambystoma*. The native species *Ambystoma californiense* and introduced species *Am. mavortium* hybridize in California (Riley et al., 2003), and this poses a widespread threat to the native one. The effect of such non-native urodeles may be greater in aquatic larvae than in terrestrial adults. Because the habitat is more homogenous and niche width is smaller in the water than on land, it is more difficult to avoid competition between the two species in the water (Ryan et al., 2009). The giant salamanders spend their entire lives in the water, so their habitat does not change between larvae and adults, thus making them more likely to compete in the narrower aquatic habitat (Browne et al., 2014). In such a case, adults are as likely as larvae to be affected strongly by introduced species and hybrids. This study suggests that hybrid giant salamanders show heterosis in terms of high viability and growth rate. It is not a coincidence but an inevitability, and this situation may be increasing in many rivers in Japan.

In addition, hybridization of giant salamanders is likely to occur in entire river systems because the salamanders are known to migrate long distances during the breeding season (Zheng and Wang, 2010; Hara et al., 2024). According to Allendorf et al. (2001) this falls under “Type 6” hybridization, in which the

genetic characteristics of native species are lost and inevitably disappear as gene penetration progresses. We must act promptly to address the hybrid problem among giant salamanders because it is possible to eliminate hybrids by early action, as seen in the successful elimination of hybrid individuals between a native Japanese macaque (*Macaca fuscata*) and an invasive Taiwan macaque (*M. cyclopis*) in Wakayama Prefecture, Japan (Kawamoto et al., 1999; Watanabe, 2007).

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