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# Geographic Variation in Skull Morphology of the Japanese Crocodile Newt, *Echinotriton andersoni* (Amphibia: Urodela: Salamandridae), Inferred from Geometric Morphometrics

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**Abstract:** The Anderson’s crocodile newt, *Echinotriton andersoni*, is an endangered species distributed in the islands of Central Ryukyus, Japan. This species is well known to show high intraspecific genetic variation among islands, but its morphological variation has not been explored. We examined the skull variation in this species including enigmatic specimens collected from Taiwan by using geometric morphometrics, and found sexual differences and geographic variation mainly between the Okinawa group (Okinawajima Island) and the Amami Group (Amamioshima and Tokunoshima Islands). We further found close resemblance between the Taiwanese specimens and the female specimens from Amamioshima Island.

Key words: Amami Islands; Okinawa Islands; Skull elements; Taxonomy

## INTRODUCTION

Geographic variation of the Anderson’s crocodile newt, *Echinotriton andersoni*, has been examined by allozymes, mitochondrial DNA (mtDNA) sequences, and microsatellite markers (Hayashi et al., 1992; Honda et al., 2012; Igawa et al., 2019). All of these studies showed populations of the newt were separated into two major groups, the Okinawa Group (including Okinawajima, Sesokojima, and

Tokashikijima Islands) and the Amami Group (Amamioshima, Ukejima, and Tokunoshima Islands) with their genetic distance large enough to be thought as heterospecific. However, no comparative morphological study with enough specimens has been conducted towards its taxonomic revision.

Another subject that should be addressed on *Echinotriton andersoni* is the identification of three enigmatic skeleton specimens from Taiwan that were labeled as collected in “Mt. Kwannon, Taihokushiu, Formosa (now, Taiwan)” and stored in the Harvard Museum of Comparative Zoology (MCZ). However, no one confirmed the species’ occurrence in

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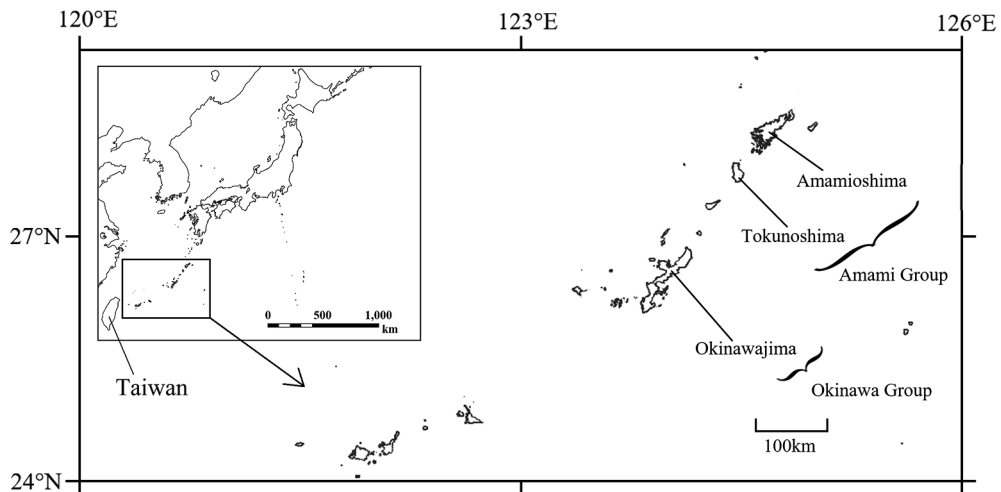


FIG. 1. A map showing localities of specimens of *Echinotriton andersoni*.

Taiwan recently. We acquired these specimens through the Loan Program and attempted to amplify the mtDNA sequence from dry skin and tissues left on skeletons, however the DNA was too fragmented to be sequenced by the next generation sequencer (Nishikawa et al., data not shown). We hereby aimed to explore the intraspecific variation of the species and to estimate the true locality of the Taiwanese specimens by using geometric morphometrics on skull characters.

#### MATERIALS AND METHODS

Because *Echinotriton andersoni* is strictly protected by its governmental and local laws, it is difficult to newly obtain a large number of specimens for this study. We thus used available museum specimens deposited in the Graduate School of Human and Environmental Studies, Kyoto University (KUHE), the Osaka Museum of Natural History (OMNH), and MCZ. In total, we used 23 specimens from Okinawajima Island of the Okinawa Group (four males and 19 females), 32 from Amamioshima Island of the Amami Group (six males and 26 females), 20 from Tokunoshima Island of the Amami Group (10 males and 10 females), and three from Taiwan (sex

unknown) (Fig. 1 and Appendix). The Japanese specimens included individuals killed by the traffic accidents on the roads, but with intact skulls. We newly collected a small number of adult males and females for this study under permissions from the local and national governments (Appendix). These newts were fully anesthetized and were then fixed in 10% formalin before final preservation in 70% ethanol. The sex and maturity were determined by gonad dissection and direct observation. All of the new specimens collected for this study are deposited in KUHE.

In order not to damage museum specimens, Soft X-Ray photographs of the skulls of ethanol preserved voucher specimens were taken at 30 kV and 25 sec exposures by using Fuji Medical X-Ray Film. The photographs of the skull specimens were digitalized with a digital camera. On these photographs of skulls, we selected 18 landmarks (Fig. 2) to represent the whole skull shape following Ivanović and Kalezić (2011), with adding some landmarks on the quadrate spine, a generic feature of *Echinotriton* (Nussbaum and Brodie, 1982). The photographs were read by tpsUtil32 version 1.78 (Rohlf, 2019) and recorded the landmark data by tpsDig232 version 2.31 (Rohlf, 2017). Next, general Procrustes analysis was

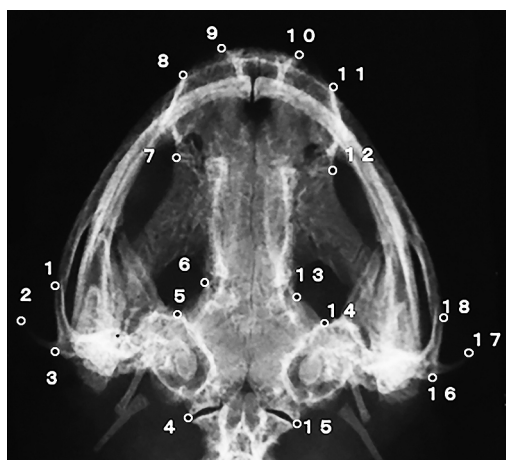


FIG. 2. Landmarks on the Soft X-ray photograph of the skull of *Echinotriton andersoni*.

performed using MorphoJ (Klingenberg, 2011) and scale and orientation data were removed.

Principal component analysis (PCA) was performed to evaluate whole skull difference between males and females in each of Okinawajima, Amamioshima, and Tokunoshima Islands samples. Tokunoshima and Amamioshima populations were not split in the genetic analyses (Honda et al., 2012; Igawa et al., 2019), but their external morphology (i.e., head shape and body proportion) appeared to be different. We thus separated the Tokunoshima and Amamioshima populations in the subsequent analyses. 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 in each pair, and the Bonferroni's adjustment was applied. Then the Canonical Discriminant Analysis (CDA) was conducted to clarify the difference among three islands using the R version 4.1.0 (R Core Team, 2021).

Finally, we conducted Linear Discriminant Analysis (LDA) using all specimens for allocating the skull specimens from Taiwan to a given group. Unfortunately, one of the skull specimens (MCZ 22515) was partly divided into elements and its quadrate spine was damaged, thus we excluded the specimen from the

morphometric analyses because we could not reconstruct skull shape.

## RESULTS

In the PCA, sum of contribution rates up to 80% was consisted of eighth principal components: 35.4, 16.0, 9.0, 5.6, 4.9, 4.1, 3.4, and 3.0% in order (total 81.4%). The Kruskal-Wallis test of the difference in the PC scores between samples was conducted up to the eighth principal component noted above, and significant differences ( $P < 0.05$ ) were detected in whole samples for the first, second, fourth, fifth, and seventh principal components. In order to clarify details of the difference, we separated sex and locality groups in scatter-plots of first, second, and fourth significant and major three components (PC1, PC2, and PC4; Fig. 3). In sexual comparisons, we found significant difference only in Tokunoshima and not in Okinawajima and Amamioshima. In Tokunoshima, the males had significantly more expanded occipital region and smaller supratemporal fenestra, and the fenestra positioned more posteriorly than that of females in the first principal component (Fig. 3). We thus separated the male and female in the subsequent CDA and LDA. In locality comparisons on males, Okinawajima had less expanded occipital region and a larger supratemporal fenestra than Tokunoshima and Amamioshima in the first principal component. Tokunoshima had longer quadrate spine and the anterior orbital margin positioned more anteriorly than Okinawajima and Amamioshima in the seventh principal component. In the locality comparisons on females, Okinawajima had more expanded occipital region, larger supratemporal fenestra, and shorter quadrate spine than Tokunoshima and Amamioshima in the second and fourth principal components. Amamioshima had wider distance between orbits and longer anterior process of quadrate than Okinawajima and Tokunoshima in the fifth principal component. Tokunoshima had anterior orbital margin positioned more anteriorly, and the supratemporal fenestra more posteriorly than Okinawajima

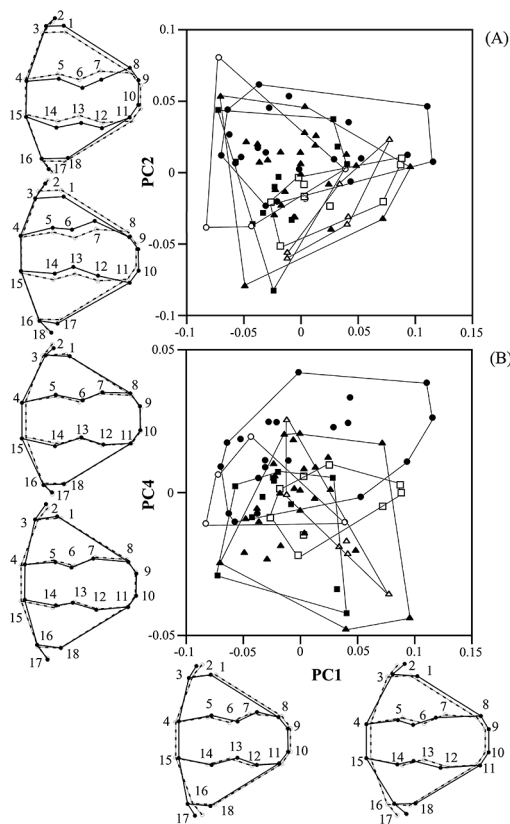


FIG. 3. Scatter plots of PC1 and PC2 (A) and PC1 and PC4 (B) of sex/populations with wired frame changes. Circles show plots of Okinawajima Island, triangles show Amamioshima Island, and rectangles show Tokunoshima Islands; open symbols show males and closed symbols show females.

and Amamioshima in the seventh principal component.

In the CDA, five canonical factors were generated: 40.5, 28.8, 15.0, 8.4, and 7.3% of contribution to the whole variation. In the scatterplots of major three axes (CV1, CV2, and CV3), Okinawa Group (Okinawajima) and Amami Group (Amamioshima and Tokunoshima) were separated by the first axis, and in the Amami Group, Amamioshima, and Tokunoshima were further separated by the second axis (Fig. 4). Males and females of Tokunoshima were separated on the two-dimensional plots by first and second axes.

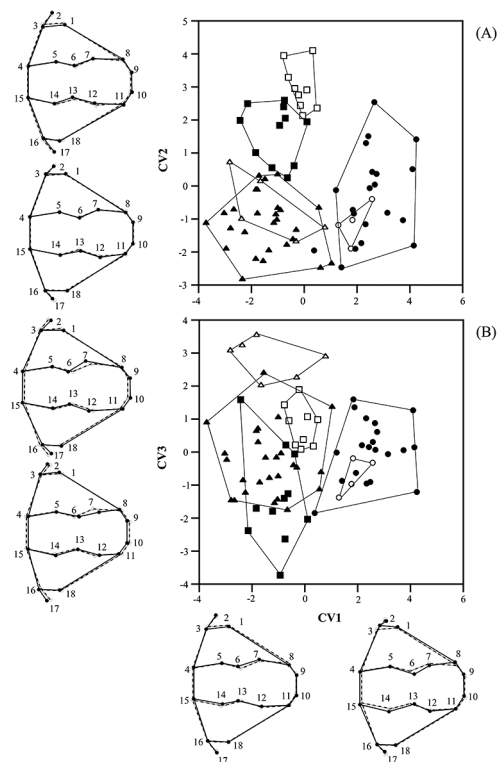


FIG. 4. Scatter plots of CAN1 and CAN2 (A) and CAN1 and CAN3 (B) of sex/populations with wired frame changes. Circles show plots of Okinawajima Island, triangles show Amamioshima Island, and rectangles show Tokunoshima Island; open symbols show males and closed symbols show females.

However, males and females of the other islands were grouped into each own island (Fig. 4). The third axis tended to separate the males of Amamioshima and the remaining groups, and the fourth one separated the males of Okinawajima and the remaining groups.

The variation in skull morphology indicated by the first to fifth canonical factors was analyzed graphically by transforming the wire-frame connecting the labeled points (Fig. 4). As the first canonical discriminant score increased, the quadrate spine became shorter and turned more inward, and the supratemporal fenestra becomes larger. As the second canonical discriminant score increased, the quadrate





FIG. 5. Skull specimen of *Echinotriton andersoni* “from Taiwan” stored in MCZ (MCZ 22515). Top: dorsal view; middle: ventral view; bottom: label of the specimen. A bar shows 5 mm.

spine became longer and turned more outward, and the nasal cavity became wider. As the third canonical discriminant score increased, the occipital region became more expanded, the orbital anterior margin positioned more posteriorly, and the supratemporal fenestra became

smaller. As the fourth canonical discriminant score increased, the occipital region became more expanded, and the posterior portion of the parietal became wider.

Skeleton specimens from Taiwan (Fig. 5) were confirmed as to be *Echinotriton andersoni* based on the presence of nob-like tubercles on the dorsal side of ribs that is a unique characteristic of the species and not found in the Chinese congeners (Fei et al., 2006; Hou et al., 2014). In the preceding LDA, 90.8% of the samples were allocated to correct islands/sex groups except for the following seven samples. Each one male of Okinawajima and Tokunoshima fell into female group of the same island, but all the male samples were correctly classified into the corresponding islands. Each one female of Okinawajima and Tokunoshima fell into male group of the same island, and one into female group of Amamioshima. One of the females of Amamioshima fell into female of Okinawajima. Based on the discriminant function obtained in the LDA, the two Taiwan specimens fell into the female samples of Amamioshima.

## DISCUSSION

We found sexual differences in skull shape only in Tokunoshima, where the males had wider skull and smaller and more posteriorly positioned supratemporal fenestra than the females. Wider skull may cause higher reproductive success by providing stronger biting force, and smaller and more posteriorly positioned supratemporal fenestra may be related to the possession of larger parotoid glands in males. Unfortunately, we currently have no behavioral (e.g., male-male combat), developmental, and anatomical information associated with these characters, which hinders further discussion on the sexual differences found in this study.

The results of molecular phylogenetic analyses showed that the newt had a great geographic variation within the species. However, the present morphometric study showed a slight variation among the localities. In PCA, we

could not detect differences among the localities in PC1 versus PC2 plots, which account for a large contribution. However, we found a difference among Okinawajima, Amamioshima, and Tokunoshima in PC1 versus PC4 plots. In CDA, Okinawa and Amami groups were first separated on the highest contributed axis (CV1), and then the latter was further separated into Amamioshima and Tokunoshima on the second highest contributed axis (CV2). This pattern is similar to the microsatellite results (Igawa et al., 2019). Recently, Pogoda et al. (2020) reported no skull shape difference between Okinawajima and Amamioshima, however they did not examine enough samples (i.e., five for the former and three for the latter) and did not consider sexual differences. Considering these results, the species was largely separated into the Okinawa Group and the Amami Group. The geographic patterns of differentiations between Okinawa and Amami and of the subdivision of the Amami Group into Amamioshima and Tokunoshima populations are not rare among amphibians and reptiles inhabiting in the Ryukyu Archipelago (Matsui et al., 2005; Tominaga et al., 2010, 2015; Kaito et al., 2016).

Amamioshima and Tokunoshima populations are genetically very close in mtDNA sequences, but their skull characteristics are slightly differentiated, especially in the length of quadrate spines, i.e., Tokunoshima population had longer spine than Amamioshima population. Tokunoshima population is the most derived lineage in Amami Group and possesses low genetic variation in mtDNA and microsatellite markers (Honda et al., 2012; Igawa et al., 2019). It is assumed that a founder population of Tokunoshima may have possessed longer spine originally and become fixed by genetic drift or the longer spine might have been rapidly selected in the island by an unknown mechanism. Unfortunately, we currently have no data to discuss on the unique spine character in Tokunoshima.

Newts within the genus *Echinotriton* have not been found in Taiwan since the MCZ specimens were collected. One of the authors,

KN, visited the Mt. Guanyin (“Kannon” in Japanese pronunciation) in 2019 where the MCZ specimens were collected, but could not obtain any information of its occurrence from field survey and local peoples. Because the recent intensive inventory efforts on Taiwanese amphibians, which includes surveys by professional scientists and citizens (Li, 2020; Yang, 2021a, b), could not find the species, it is plausible that *Echinotriton* no longer occurs in Taiwan. On the other hand, the present study indicated that the MCZ specimens are most similar to the specimens from Amamioshima, suggesting the specimens to have been mislabeled as to be collected in Taiwan. The specimen tag (Fig. 5) shows that the specimens were obtained from “Shibyama Nat. Sci. Lab.,” which seems to be a specimen collecting company in Tokyo but already closed. We once found some vertebrate specimens from Ryukyu Archipelago prepared by this company in the collection of National Museum of Nature and Science, Tokyo. Of course, we cannot exclude a possibility that the newt once occurred in Taiwan but now became extinct, and its morphology was closest to the Amamioshima population. However, such possibility is quite low because the general herpetofaunal distribution patterns in the area show great difference of Taiwan and the Yaeyama Group from Central Ryukyu area including Okinawajima, Amamioshima, and Tokunoshima Islands separated by the Kerama Gap (Ota, 2000; Komaki, 2021).

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## APPENDIX

Specimens examined in this study. For abbreviations, see text.

Okinawajima Island: KUHE 8369, 8888–8892, 50452, 50559, 50561–50564, 50568, 50577, 50585, 50613, 50618, two unnumbered; OMNH 7983, 7984, 9597, 9772.

Amamioshima Island: KUHE 8367, 8894, 8895, 43627, 43628, 45186, 45187, 50580–50690; OMNH 9584, 9585, 9587–9593, 9775, 9777–9779, 9781, 9783.

Tokunoshima Island: KUHE 8368, 9920, 13969, 28223, 28224, 45190, 46659, 49868–49873, 50671, 50672, 50675, 50676, 50678; OMNH 9774.

Taiwan: MCZ 22515–22517.

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