Occurrence of temporary fish ectoparasites (Isopoda; Gnathiidae) in low-salinity subterranean habitats of Miyako-jima Island, Ryukyu Islands, southwestern Japan

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Received 26 December 2015; Accepted 30 May 2016  Responsible Editor: Hiroomi Miyamoto

Abstract: The occurrence of gnathiid isopods, a group of parasitic crustaceans on fish, is reported from low-salinity habitats in Miyako-jima Island, Ryukyu Islands, southwestern Japan. In total, 1,096 gnathiid larvae were collected from four of ten surveyed sites, but mostly at two subterranean water sites connected with anchialine waters and/or the sea. Three stages of parasitic zuphea larvae were collected, but no free-living adults. Morphological observations and DNA barcoding show that these larvae are closely related to, and most likely conspecific with, *Gnathia limicola* a dweller of mud burrows in brackish-water habitats. Our study suggests that *G. limicola* actively enters into low-salinity subterranean habitats, presumably to search for euryhaline fishes to use as temporary hosts.

Key words: Gnathiidae, host utilization, low salinity, mtCOI, Ryukyu Islands, subterranean water

Introduction

The crustacean fauna of anchialine habitats in the Ryukyu Islands has recently begun to be revealed. Because anchialine habitats consist of partially or totally submerged caves, they are often isolated from the sea and can contain endemic and/or stygobitic organisms (Sket 1996, Iliiffe & Kornicker 2009). In Miyako-jima Island (henceforth Miyako-jima) located in the southern Ryukyu Islands, Shokita (1996) and Fujita (2007) recorded a total of 25 decapod crustacean species inhabiting surface and subterranean waters. Other crustacean taxa such as Peracarida have not been reported from Miyako-jima’s anchialine habitats. Currently, gnathiid larvae are reported to have been mostly collected in plankton samples from underground low-salinity waters in Miyako-jima.

Generally, gnathiids occur in the sea between the intertidal and deep waters of less than 4000 m (see Cohen & Poore 1994, Smit & Davies 2004). Only two species, *Paragnathia formica* (Hesse, 1864) and *Gnathia limicola* Ota and Tanaka, 2007, have been recorded from brackish waters (Monod 1926, Ota et al. 2007). Charmantier (1980) observed no mortality of *P. formica* during five days under a wide variety of water salinity conditions, between 10 times diluted and 1.3 times concentrated seawater (salinities of about 3.4–44), and he demonstrated osmotic regulation in this species, whereby the osmotic pressure of the hemolymph remained consistently above that of the fluid outside their bodies. This indicates that gnathiids are originally marine but that some species or genera have acquired an euryhaline character and invaded low-salinity environments such as brackish waters.

Gnathiid isopods have a biphasic life cycle with three larval stages parasitic on fish and one free-living adult stage. Gnathiid larvae actively swim to seek fishes and attach to their skin, fins, and gills to engorge on body fluids. Because engorged larvae have a swollen thorax and are easily distinguished from unfed larvae, gnathiid larvae traditionally are referred to zuphea larvae (unfed) and praniza larvae (fed). Praniza larvae leave the fishes and enter the benthos to rest and molt in/on the benthic substrata. Adult gnathiids reproduce there without feeding (Tanaka 2007). Shokita and Ueda (1977) surveyed the diel and seasonal fluctuations of planktonic animals including gnathiid larvae, from the Shiokawa springs, which connect with anchialine habitats on Okinawa-jima Island (hereafter Okinawa-jima), central Ryukyu Islands. However, basic information such as species identification and host utilization by these
gnathiids remains unavailable.

Here we survey gnathiid occurrence in various habitats in Miyako-jima and identify specimens, on the basis of their external morphologies, from each site as well as providing DNA barcodes due to a lack of such molecular information. We also discuss their host utilization in low-salinity habitats.

**Materials and Methods**

**Field sampling**

The island of Miyako-jima (area 155.88 km²) is situated in the southern part of the Ryukyu Islands, southwestern Japan (Fig. 1). This island is entirely flat and its highest point is only 115 m above sea level. Geologically the island consists of three main layers: soil constituting the Ohonogushi Clay Bed rests on coral limestone known as Ryukyu Limestone, and the Shimajiri Group bedrock consists of consolidated slate and sandstone (Hanzawa 1935). Because the Ohonogushi Clay Bed and Ryukyu Limestone are porous and permeable, while the Shimajiri Group is impermeable, rainwater penetrates the limestone deposits and pools on Shimajiri mudstone. As a result, subterranean freshwater reservoirs have formed within Miyako-jima. These emerge in limestone cavities (dolines) or flow from springs into the sea as creeks (Mink 1963, Isozaki 1970). Part of the subterranean freshwater in the limestone cavities is connected with anchialine habitats and/or the sea (Fig. 2a), but its proportions are unknown.

We surveyed the occurrence of gnathiid at ten sites (Fig. 1) and habitats as follows: 1) Subterranean water in limestone cavities directly connected to anchialine habitats–Tomori-Ama-Ga (Fig. 2b), Kiikya-Ga (Fig. 2c), Muika-Ga, Butura-Ga; 2) subterranean waters that spring out from cliffs: Mui-Ga, Puikya, Yamakawa-Upu-Ka; 3) subterranean waters that spring out to form creeks–Hida-Ga (Fig. 2d) and Pisa-Ga; 4) a man-made underground dam not connected to anchialine pools–Fukuzato Underground Dam (Fig. 2e).

In order to survey the seasonal occurrence of gnathiids, monthly sampling was conducted at Tomori-Ama-Ga and Kiikya-Ga from December, 2004, to April, 2006. A plankton net (90 µm mesh) was thrown 20 times into almost the entire surface area of the sites and the samples were fixed in 70% ethanol. This protocol was repeated three times between 9:00–16:00 during the day.

Sampling with a plankton net was also conducted at the other eight sites (Fig. 1). All collected animals were fixed in 70% ethanol for morphological observation or 99% eth-
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The water temperature and salinity were measured on every sampling occasion using a digital thermometer (EASE) and a handy salinometer (ATAGO).

In order to assess the occurrence of the same gnathiid species in the marine environment, sampling was conducted at the coast at Ingya Marine Garden (Fig. 2g). This site is approximately 100 m from Tomori-Ama-Ga. At Ingya Marine Garden, freshwater (or somewhat salty) springs occur in the inner area of the bay and directly flow into the sea. We collected gnathiids using a plankton net and collected benthic substrata such as sand, rocks, and sponges.
by snorkeling from the intertidal zone to about 1 m depth at this site to collect adult gnathiids from the benthic substrata.

For morphological identification, the gnathiids were observed under a compound microscope (Olympus, SZX16), and drawings were prepared using a camera lucida. Gnathiid body length was measured from the frontal margin of the clypeus to the posterior tip of the pleotelson, and maximum head width was also recorded. Thus, we also recorded whether gnathiid larvae were zuphea or praniza. The external morphology of 45 zuphea larvae was observed, including 15 individuals of each stage selected from the samples collected at the four sites (see Table 1).

DNA Extraction, amplification, and sequencing

Tissue samples of gnathiids were preserved in 99% ethanol at −30°C and 1–4 specimens from Tomori-Ama-Ga were suspended in 400 µl CTAB (hexadecyltrimethyl ammonium bromide) buffer [2% CTAB, 1.0 M NaCl, 75 mM EDTA (pH 8.0), 35 mM Tris-HCl (pH 8.0)] containing 0.1% sodium dodecyl sulfate (SDS) and 0.2% beta-mercaptoethanol, followed by incubation at 65°C for 1 h, according to the method of Hirose et al. (2009). We added proteinase K to the samples to get a final concentration of 0.1 mg·ml⁻¹ and incubated the samples overnight at 37°C. We extracted DNA with phenol-chloroform as described by Sambrook et al. (1989). We performed PCR amplification of the partial cytochrome oxidase subunit I (COI) region by Sambrook et al. (1989). We performed PCR amplification of crust-cox1f and crust-cox1r primers (Podsiadlowski & Bartolomeus 2005). We performed PCR amplification of the partial COI region: maximum likelihood (ML) using TREEFINDER January 2008 version (Jubb 2008), and maximum parsimony (MP) and neighbor-joining (NJ) using PAUP* 4.0 beta10 (Swofford 2003). To select the appropriate nucleotide substitution model, we used jModeltest ver. 0.1.1 (Posada 2008). Based on the Akaike information criterion (AIC), we selected the TVM+G model (transversional model with gamma distribution) as the best model. We searched the unweighted MP trees using a heuristic approach. Statistical support for the ML, MP, and NJ trees was evaluated using a non-parametric bootstrap test with 1,000 re-sampling events.

Table 1. Gnathiid samples for morphological observations.

| Site          | Date       | Stage |
|---------------|------------|-------|
| Tomori-Ama-Ga | Oct. 2005  | ZI(1) |
|               | Nov. 2005  | ZII(1) |
| Kiikya-Ga     | Nov. 2004  | ZI(1) |
|               | Nov. 2005  | ZII(1) |
| Hida-Ga       | Oct. 2005  | ZI(1) |
|               | Nov. 2005  | ZII(1) |
| Butura-Ga     | Nov. 2004  | ZI(1) |
|               | Nov. 2005  | ZII(1) |

Phylogenetic analyses

For phylogenetic analysis we initially aligned the sequences using MUSCLE (Edgar 2004) and then eliminated poorly aligned positions and divergent regions by Gblocks (Castresana 2000). We performed the following analyses on the aligned DNA sequences of the partial COI region: maximum likelihood (ML) using TREEFINDER January 2008 version (Jubb 2008), and maximum parsimony (MP) and neighbor-joining (NJ) using PAUP* 4.0 beta10 (Swofford 2003). To select the appropriate nucleotide substitution model, we used jModeltest ver. 0.1.1 (Posada 2008). Based on the Akaike information criterion (AIC), we selected the TVM+G model (transversional model with gamma distribution) as the best model. We searched the unweighted MP trees using a heuristic approach. Statistical support for the ML, MP, and NJ trees was evaluated using a non-parametric bootstrap test with 1,000 re-sampling events.

Results

Occurrence of Gnathiids

The water temperature at all ten sites was almost stable throughout the year (20.0–24.1°C). Salinity at each site was much lower (0–12) than that of seawater. In total, 1,096 gnathiid larvae were collected from four of the ten sites: Tomori-Ama-Ga (N=969), Kiikya-Ga (N=38), Hida-Ga (N=86), and Butura-Ga (N=3) (Table 2). No adult gnathiids were collected by plankton net, and we did not collect any gnathiids from mud and rock sediment on the bottom at Tomori-Ama-Ga.

In this survey using a plankton net, only three individuals from Hida-Ga were praniza larvae, while all the other individuals from all four sites were zuphea larvae.

At Ingya Marine Garden, no gnathiids were collected from the benthic substrata nor in plankton samples taken near the mouths of the springs, but two undescribed species of the genera Gnathia Leach, 1814 and Tenerognathia Tanaka, 2005 were collected there by light trap at night (Ota per. obs.).

External morphology of gnathiid larvae

Size frequency distribution based on combined samples of zuphea larvae from Tomori-Ama-Ga (N=346) and Kiikya-Ga (N=34) suggested three size classes were present (Fig. 3). The external morphology of all specimens was stable within each size class (details below); therefore, we recognized these size classes as representing three larval stages, viz., zuphea I, II, and III (ZI, ZII, and ZIII). Total length and head width of zuphea I (N=213) were 0.76–1.08 (mean±s.d.=0.89±0.08) mm and 0.18–0.24 (0.21±0.01) mm, respectively. Those of zuphea II (N=89) were 1.14–1.70 (1.41±0.14) and 0.26–0.34 (0.31±0.02) mm. Those of zuphea III (N=88) were 1.58–2.63 (2.23±0.21) mm and
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0.36–0.50 (0.45 ± 0.02) mm. All three zuphea stages shared the following morphological characteristics: 1) clypeus with concave anterior margin (Fig. 4a–c); 2) antenna as long as head and pereonites 1–2 combined (Fig. 4d–f); 3) three pairs of small setae visible between the basis of antennule and anterior margin of eye, on dorsal surface near inner margin of eye, and on dorsal surface near posterior margin of eye (Fig. 4a–c); 4) dorsal surface of pleotelson bearing three pairs of setae (rarely two pairs) and not covered with pectinate scales (Fig. 4a–c), 5) endopod, but not exopod, of uropodal rami slightly exceeding apex of pleotelson (Fig. 4g–i); 6) distal margins of exopod and endopod of uropodal rami bearing five simple and four plumose setae, and one simple and six plumose setae, respectively (Fig. 4g–i).

Pleotelson shape was somewhat different among the stages. While remaining triangular, the pleotelson became wider, and the lateral margins more convex, as the stages progressed (Fig. 4g–i). In live specimens, the brown pigmentation of the entire dorsal surface became more obvious as the stages progressed.

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Molecular identification with DNA barcodes

Phylogenetic analyses were carried out between our specimens and a total of seven other specimens of the species *Gnathia limicola* Ota and Tanaka, 2007, *G. maculosa* Ota and Hirose, 2009, and *G. trimaculata* Coetzee, Smit, Grutter, and Davies, 2009, because, until now, partial COI sequences have been reported in only these three species (Ota et al. 2012). We confined our analyses to a specific subset of 857–860 base pairs of the partial COI sequences, but this was reduced to 857 positions because poorly aligned and divergent regions were detected by Gblocks. The aligned amino acid sequences of the four gnathiid species showed one insertion/deletion site, but the following analyses were performed using a no-gaps data set.

The difference between the partial mtCOI DNA sequences among *G. maculosa* specimens from different collection sites was 16/857 bp (1.9%). For *G. trimaculata*, this value was 0–3/857 bp (0–0.4%). The sequence difference between *G. limicola* from Okinawa-jima (AB713956) and *Gnathia* sp. from Miyako-jima was 105/857 bp (12.3%). Between *Gnathia* species, the lowest degree of sequence divergence was 25.3% (217/857 bp differences), and the highest was 34.8% (298/857 bp differences), with a mean sequence divergence among the *Gnathia* species of 30.6%.

Figure 5 shows the unrooted ML tree using the TVM + G substitution model (log-likelihood = –3694.92). For data with all codon positions, TVM + G was selected as best, using AIC. Therefore, we chose TVM+G for both the ML and NJ analyses. Because the topologies of the phylogenetic trees obtained by ML, MP, and NJ were nearly identical, the strict-consensus MP tree and the NJ tree under the TVM+G substitution model are not shown. Although monophyly of each examined species of *Gnathia* was supported by high bootstrap values, the monophyly of *Gnathia* itself was not supported.

| Sites | WT (°C) | S | Habitat | Substrata | Z1 | P1 | Z2 | P2 | Z3 | P3 | Total |
|-------|---------|---|---------|-----------|----|----|----|----|----|----|-------|
| To    | 23.3–24.0 | 0–4.0 | anchialine | mud | 670 | 130 | 169 | 0   | 969 |
| Ki    | 21.4–24.0 | 0–8.0 | anchialine | mud | 26  | 0   | 6   | 0   | 38  |
| Hi    | 20–23.9   | 0–13 | freshwater spring/anchialine? | *mud/sand | 58 | 2   | 16  | 1   | 9   | 0   | 86   |
| Pi    | 23.7     | 0   | freshwater spring | gravel/cobble with decayed vegetable matter | 0 | 0   | 0   | 0   | 0   | 0   | 0     |
| Pu    | —        | 0   | freshwater spring | *mud/sand with decayed vegetable matter | 0 | 0   | 0   | 0   | 0   | 0   | 0     |
| Bu    | 24.1     | 0   | anchialine | mud with decayed vegetable matter | 0 | 1   | 0   | 2   | 0   | 3   | 3     |
| Mu    | 23.3     | 0–3 | freshwater spring | *artificial reservoir | 0 | 0   | 0   | 0   | 0   | 0   | 0     |
| Mk    | 23.5     | 1   | anchialine | mud | 0 | 0   | 0   | 0   | 0   | 0   | 0     |
| Ya    | —        | 0   | freshwater spring | gravel/cobble | 0 | 0   | 0   | 0   | 0   | 0   | 0     |
| Fu    | 23.8     | 0   | freshwater spring | artificial reservoir | 0 | 0   | 0   | 0   | 0   | 0   | 0     |

WT, water temperature; S, salinity; *, spring forth from cliff.

**Table 2.** Total number of gnathiids from 10 sampling sites.
Seasonal fluctuations

Water temperature at both Tomori-Ama-Ga and Kiikya-Ga was seasonally stable, 23.3–24.0°C and 21.4–24.0°C, respectively. Salinity varied between 0–5 at Tomori-Ama-Ga and 0–8 at Kiikya-Ga, respectively (Fig. 6), but the number of gnathiid larvae taken each month and water salinity were not correlated at the two sites (Tomori-Ama-Ga: $P=0.83$, Kiikya-Ga: $P=0.14$).

At Tomori-Ama-Ga, among the 969 zuphea larvae that were collected, zuphea I (ZI) larvae comprised 69.5% ($N=670$) and they were collected every month. At a minimum, one each was caught in March 2004, and April

![Fig. 4. External morphology of zuphea larvae ZI–ZIII in dorsal view. a–c, head; d–f, body; g–i, pleotelson with uropodal rami; j, left pleopod 2. a, d, g, first stage (ZI); b, e, h, second stage (ZII); c, f, i, third stage (ZIII).](image-url)
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2006, but in other months, ZI larvae comprised 46–100% of the total. At this site, ZII and ZIII larvae comprised 13.4% (N = 130) and 17.4% (N = 169) of the total, respectively, and the two stages together comprised 0–44% of each month’s total. In monthly sampling at Tomori-Ama-Ga, gnathiid numbers varied from one to 274 (mean N = 57.0). While gradual fluctuation was not recorded, large numbers (N > 50) were often recorded from November to February.

At Kiikya-Ga, the total number of gnathiids taken was fewer (N = 38) than at Tomori-Ama-Ga; however, just like at that location, ZI larvae were dominant (68.4%; N = 26) among the three zuphea stages, while ZII and ZIII larvae each comprised 15.8% (N = 6). The number of individuals taken each month varied from 0 to 11 (mean N = 2.2), with an increase from August 2005 until it reached a peak in January 2006.

Discussion

Identification of the subterranean gnathiid larvae and their phylogeny

Gnathiid taxonomy is traditionally based on the morphology of adult males, but no males were collected in our survey. Instead we must consider the taxonomic position of the subterranean gnathiids from Miyako-jima based on the zuphea larvae. Their size-frequency distribution shows three size classes, interpreted by us as three stages, which is the usual number of zuphea stages in gnathiids (Smit & Davies 2004, Ota et al. 2012). All three stages share aspects of external morphology such as clypeus shape, the number of dorsal setae on the head and pleotelson, and the setation of the external margins of the uropodal rami. Thus, we conclude that the gnathiid larvae collected from various subterranean waters of Miyako-jima all belong to a single species.

In most taxonomic studies of Gnathiidae, descriptions of larval morphology have been insufficient or fully absent. However, recent studies have included detailed larval descriptions (e.g. Smit et al. 1999, Ota et al. 2007, Farquharson et al. 2012). Among these species, the larvae collected at Miyako-jima resembled those of *Gnathia limicola* Ota and Tanaka, 2007, having the following features in common: 1) lateral margins of pleotelson obviously convex; 2) distal margins of exopod and endopod of uropodal rami bearing five simple and four plumose setae, and one simple and six plumose setae, respectively; and 3) endopods of pleopod I–V oval with two plumose setae on distal margins (Ota et al. 2007). Ota et al. (2007) did not describe the shape of the clypeus in detail, nor the dorsal setae on the head or pleotelson, but by examining topotypes we have confirmed in topotypes the presence of the concave anterior margin of the clypeus and the presence of six dorsal setae each, arranged in three pairs, on the head and pleotelson. Furthermore, *G. limicola* was reported from brackish waters of Okinawa-jima in the central Ryukyu Islands (Ota et al. 2007), and they are known to be capable of survival in fresh water in laboratory culture (Ota, pers. obs.). This suggests that regular occurrence of *G. limicola* in freshwater habitats, similar to the gnathiid larvae reported in this study, is possible.

Molecular identification was done based on partial mtCOI DNA sequences of three species of *Gnathia* (*G. limicola*, *G. maculosa*, and *G. trimaculata*), and the mtCOI sequence of the present larvae proved to be most similar to those of *G. limicola*. Hebert et al. (2003) showed that the most common degrees of mtCOI divergence between congeneric pairs of crustacean species are 16–32% (mean ± s.d. = 15.4 ± 6.6%; congeneric species N = 1781). In comparison, the pairwise divergence among COI se-
quences ranged from 25.3 to 32.9% among *G. limicola*, *G. maculosa*, and *G. trimaculata* (Ota et al. 2012). The COI sequence divergence between *G. limicola* from Okinawa Island (AB713956) and the subterranean gnathiid larvae from Miyako-jima is rather low (12.3%), so the larvae probably belong to *G. limicola*.

Weese et al. (2012) investigated the genetic variation and population structure of an anchialine shrimp, *Caridina rubella* Fujino and Shokita, 1975, on the basis of mtCOI divergence data of specimens from four sampling sites in Miyako-jima. Because *C. rubella* has planktonic larval stages and its life cycle is potentially amphidromous, its genetic divergence among sites was expected to be low. However, significant genetic structure was exhibited across the sites. In the present study, only one sequence for *G. limicola*, was obtained but further population genetic surveys involving many sites at Miyako-jima and other potential sites in the Ryukyu Islands may reveal any biogeographic traits.

**Gnathiid occurrence in low-salinity habitats**

Among the gnathiid larvae collected at Tomori-Ama-Ga, zuphea I (ZI) larvae comprised 69.5% (N=670) of the total number and 46–100% of each monthly sample. The ZI larva is the early life-history stage that leaves from the adult female’s marsupium (e.g., Smit & Davies 2004, Tanaka 2007). Thus, the gnathiids at Tomori-Ama-Ga were not accidentals but represent a continuously resident population.

Our most difficult task is explaining why so many gnathiid larvae occurred in low-salinity subterranean waters. The largest number (N=969) of gnathiid larvae was collected at Tomori-Ama-Ga, which is connected with anchialine habitats and/or the sea. However, we could not collect any conspecific adults, including reproductive and non-parasitic stages, from the benthic habitats at this site. This suggests that the gnathiid larvae must be derived from other habitats inhabited by the adults, perhaps higher-salinity anchialine habitats or the sea itself. Gnathiid larvae are temporary fish ectoparasites, with the zuphea larvae actively swimming to seek hosts (Tanaka 2007). The second author collected over 100 praniza larvae infesting the body surface of a giant mottled eel, *Anguilla marmorata* Quoy & Gaimard, 1824 (total length=47 cm), in natural conditions at Kiikya-Ga (Fujita pers. obs.). Constant occurrence of zuphea larvae at Tomori-Ama-Ga indicates that some quantity of potential host fishes may exist in the subterranean water system there.

Shokita & Nishijima (1976) reported that unidentified gnathiid larvae were constantly collected in plankton nets fixed at the outflow points of the Shiokawa springs of Okinawa-jima’s Motobu Peninsula. These springs are obviously anchialine, because both the water and salinity levels fluctuate on a daily basis in relation to tidal changes (Shokita & Nishijima 1976). Shokita & Ueda (1977) suggested that gnathiid larvae are capable of dispersing from the sea into brackish or subterranean (anchialine) waters while attached to host fishes, because almost all the fishes occurring in the Shiokawa springs are amphidromous species. In the Ryukyu Islands, over 200 species of fish have been recorded from inland waters, although most are considered as diadromous, amphidromous, or peripheral fishes (Nishida et al. 2004). In creeks, springs, and subterranean waters connected to the sea in Miyako-jima, however, Nishizato et al. (2009) reported a total of just 22 non-freshwater species (including *A. marmorata*) and only three freshwater species (including two alien species). In fact, there are few surface-water streams on Miyako-jima, owing to the ubiquity of the porous Ryukyu Limestone. We think, therefore, that the gnathiid larvae must migrate into subterranean waters of Miyako-jima in their search for temporary host fishes.

**Acknowledgements**

This study was partly supported by a Grant-in-Aid to JSPS Fellows (No. 23-527), a grant from the Research Institute of Marine Invertebrates Foundation in 2014 (No. 8) (YO), Research Grants (2004–2005) from The Toyota Foundation (YF), and by the International Research Hub Project for Climate Change and Coral Reef/Island Dynamics of the University of the Ryukyus (MH). We thank three anonymous reviewers for valuable comments and Mark J. Gryggier, Lake Biwa Museum, for revising the manuscript’s English.

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