A description of a novel swimming behavior in a dioecious population of *Craspedacusta sowerbii*, the rediscovery of the elusive *Astrohydra japonica* and the first genetic analysis of freshwater jellyfish in Japan

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**Abstract:** Freshwater jellyfish comprising the genus *Craspedacusta* are thought to have originated in the Yangtze River, China and have since spread to all continents except Antarctica. In this study, jellyfish were collected from Haruta-ike, an artificial pond in Chikuma City, Nagano (Japan). Medusae were identified as *Craspedacusta sowerbii* using morphological and molecular techniques. Despite the existence of Haruta-ike since prior to the Edo era (ca. 1603), this work represents the first published record of freshwater jellyfish in this pond. Herein, we report on the novel swimming behavior documented in this population, which includes both male and female *C. sowerbii* medusae. Additionally, we discuss the life cycle of polyps reared in culture from Machikane-ike, a pond in Osaka for which we have published the first complete mitochondrial genome of *C. sowerbii* from Japan. Finally, we report on the morphology and life cycle of the rare Japanese freshwater jellyfish *Astrohydra japonica* in Lake Biwa (Shiga), documented only a few times in the 40 years since its original discovery in Japan. The results of our robust phylogenetic analysis using the 16S rRNA gene and COI markers of *C. sowerbii* and *A. japonica* in this study and for *C. sowerbii* material from Singapore, together with all publicly available sequences for these markers for the two species worldwide, revealed two major *C. sowerbii* clades suggesting the Nagano and Osaka populations originated from two distinct introduction events. This collaborative research was made possible through international collaborations among multiple research facilities, museums and one wildlife reserve.

**Key words:** behavior, freshwater jellyfish, genome, invasive species, phylogenetic

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Introduction

_Craspedacusta sowerbii_ Lankester, 1880 (Cnidaria; Hydrozoa; Olinidiidae) is a cosmopolitan freshwater jellyfish that has, thus far, been documented in all nine geographic regions of Japan, from the southernmost prefecture of Okinawa to the northernmost of Hokkaido (reviewed in Lewis et al. 2012). These reports included nine reports from Nagano, but none from Haruta-ike. Since the first records almost a century ago in Japan, where this species is known as *mamizu kurage*, more than 200 observations (Ohno 1987, Lewis et al. 2012) have focused primarily on morphology, ecology (environmental conditions and medusae season) and life history in culture. Like many jellyfish species, the life history of *C. sowerbii* includes both a conspicuous medusa (swimming stage) that is present in the water column only during summer blooms, and microscopic polyps (sessile stage) that persist throughout the year attached to rocks, branches and other substrate types (Duggan & Eastwood 2012, Siquier et al. 2017). Polyps, which bud other polyps or motile frustules (“worm-like” stage) asexually (reviewed in Payne 1924), are thought to contribute to this species global success, rather than sexual reproduction, as few wild populations comprise medusae of both sexes.

More than 40 years ago, another freshwater jellyfish species, _Astrohydra japonica_ Hashimoto, 1981 (Cnidaria; Hydrozoa; Olinidiidae), known as *yume-no-kurage*, was described from two localities in the Kanto (Kanagawa) and Chubu (Shizuoka) regions of Japan (Hashimoto 1981, 1985, 1987). The initial description of the _A. japonica_ polyp was identical to that of another unnamed multi-tentaculate polyp, documented earlier in aquariums in St. Petersburg (as Leningrad) and Hungary (discussed in Hashimoto 1981), but specimens were not available for examination, prompting Hashimoto to establish the new species. In subsequent years, some workers speculated that _A. japonica_ was a junior synonym of _Calpasoma dactylopterum_ Fuhrmann, 1939 documented in Europe, Brazil, U.S.A., Hawaii and Israel (see Bouillon et al. 2004, Lewis et al. 2012), but not in Japan. A distinguishing character that has held up the production of medusae was identified as a separate species is that while no medusa stage has been reported in the life cycle of _Calpasoma_, _A. japonica_ the production of medusae was documented by Hashimoto (1985, 1987).

In contrast to _C. sowerbii_'s frequent sightings worldwide, only one report exists for _A. japonica_ collected outside of Japan. Collins et al. (2008) published the only available molecular sequence data for _A. japonica_ cultured in a laboratory in Germany, although no mention was provided of the polyp's origin or whether a medusa stage was witnessed. Until now, the lack of molecular sequence data for _A. japonica_ from Japan has made it difficult to take the first step in unravelling the taxonomic ambiguity surrounding this species, which may be identical to other multi-tenaculate polyps reported outside of Japan.

Phylogenetic studies concerning freshwater jellyfish in other countries have used molecular barcoding techniques to understand the relationship among _C. sowerbii_ populations on a regional or even global scale (for examples see Marchessaux et al. 2019, Lüskow et al. 2021, Morpurgo et al. 2021). Those findings sometimes indicate multiple unique introductions to freshwater bodies, even when in close geographic proximity, suggesting broad molecular diversity for a single cosmopolitan species. Furthermore, following taxonomic revisions over the past two decades, currently three separate species of _Craspedacusta_ are thought to be valid (_C. sowerbii_, _Craspedacusta sinensis_ Gaw & Kung, 1939, _Craspedacusta kiatengi_ Gaw & Kung, 1939), all of which occur in China (Zhang et al. 2009, Zou et al. 2012, Morpurgo et al. 2021), with the taxonomic status of a fourth _Craspedacusta ziguiensis_ He & Xu, 1985 uncertain (see Oualid et al. 2019). The existence of multiple _Craspedacusta_ species suggests the possibility that some reports of _C. sowerbii_ in localities may, in fact, correspond to other species of _Craspedacusta_. However, this hypothesis is difficult to test with Japanese freshwater jellyfish populations, distributed across the entire Japanese archipelago as, until now, no molecular barcode data were available for _C. sowerbii_ from Japan.

In order to better understand _C. sowerbii_ inhabiting Japanese freshwaters, we conducted extensive morphological and behavioral observations of _Craspedacusta_ medusae collected in Haruta-ike (Nagano) for the first time. Additionally, we separately conducted observations on early life stages (polyps, frustules and juvenile medusae) collected from Toyonaka City (Osaka)—where _C. sowerbii_ was previously reported in 1967 (Ohno 1987). Furthermore, we acquired _C. sowerbii_ as a preserved museum voucher from Lee Kong Chian Natural History Museum in Singapore, collected from an artificial moat of the River Wonders (Mandai Wildlife Reserve) (1°24′11.3″N, 103°47′27.8″E), representing the first record of this species in Singapore. Finally, polyps collected from Lake Biwa, Shiga, corresponding to _A. japonica_, were reared in this study to attempt to document the entire life history.

Materials and Methods

Study site: Haruta-ike Pond

Freshwater jellyfish appearing to be _Craspedacusta sowerbii_ were observed at the southern Haruta-ike (36°31′N, 138°05′E), located in Chikuma City, Nagano Japan from late July to early September 2020. Medusae were seen swimming at the surface at the south and east banks of Haruta-ike—a small, shallow, man-made pond constructed prior to the Edō Period (ca. 1603, Chikuma City personnel pers. comms.), and in its current form since at least 1860. Haruta-ike and Haruta park, from which it obtained its name, are managed by Haruta Shrine (onsite) and Chikuma City, and mainly serve as a reservoir, in case of a
natural disaster, with Haruta-ike also being a popular fishing spot. The pond water level is adjusted based on forecasts of heavy rain and corresponding irrigation needs, and is thought to have never been completely drained within the last 100 years (Chikuma City personnel pers. comms.).

Haruta-ike is a popular fishing spot for invasive large-mouth bass (*Micropterus salmoides* Lacepède, 1802), gengoro-funa (*Carassius cuvieri* Temminck & Schlegel, 1846) and carp (*Cyprinus carpio* Linnaeus, 1758). Freshwater gobies (*Rhinogobius* sp.) and bluegill (*Lepomis macrochirus* Rafinesque, 1819) are also present along with the Japanese mystery snail (*Heterogen japonica* von Martens, 1861), river mussels (*Sinanodonta lauta* Martens, 1877), clams (*Corbicula fluminea* Müller, 1774), American bullfrog tadpoles (*Lithobates catesbeianus* Shaw, 1802) and red swamp crayfish (*Procambarus clarkii* Girard, 1852). A photo of the pond in both its current state, and that of 1860 (Fig. 1a) shows little gentrification in over 160 years. Aquatic vegetation is scarce in the middle of the pond, but reeds grow along the banks (Fig. 1b). The south and east banks, where jellyfish were collected, are constructed of concrete, with the deepest point having a depth of approximately 4 m. Underwater video recordings in the areas where jellyfish were found revealed the bottom substrate to be pebble and
sand covered in algae and silt (Supplementary Videos 1, 2). Water visibility during the summer is around 50 cm and watercolor is light green/brown. There is a small inflowing water pipe coming from an irrigation ditch in the southwest corner of the pond with a steady flow of water and an outlet on the east bank flowing into the northern pond.

On 17 July 2020, C. sowerbii medusae were first sighted by a local resident, and the Nagano Environmental Conservation Institute was notified. Authors met at Haruta-ike with local anglers who visit the pond daily, and anglers agreed to notify authors when C. sowerbii were present. The next observation was on 19 August and four subsequent days, 20 & 28 August and 1 & 16 September 2020. During the observation period, the pond water temperature ranged from 28 to 33°C and air temperature from 25 to 36°C (Table 1).

**Observation and sampling in Haruta-ike Pond**

*Craspedacusta sowerbii* were observed by walking along the concrete bank of the pond and filmed *in situ* using a GoPro Hero 8 camera attached to a telescoping pole (Fig. 1c; Supplementary Videos 1, 2). Live medusae (n=17) were collected with a hand-net during the sampling period; three medusae were preserved whole for morphological analysis (5% formalin-freshwater solution), and another three for molecular analysis (99.5% pre-chilled ethanol). The remaining medusae were cut in half, of which one half was fixed in a 5% formalin-freshwater solution for morphological analysis, and the other half was preserved in 90% ethanol for future molecular analysis. Select corresponding morphological vouchers were accessioned as museum specimens (Table 2).

**Morphological analysis**

Fixed specimens were transported to the Graduate School of Agricultural Science, Tohoku University (Japan) and examined using a dissecting microscope (Olympus SZX12). Specimens were placed oral side up, into a petri dish containing freshwater and photographed (Olympus DP72 camera). Presence/absence of morphological traits such as gonads, velar canals, and statocysts, and the number of each type of tentacles per quarter (primary through quaternary) was recorded. Measurements were made using Olympus cellSens software, with the bell diameter

| Species | GenBank 16S | GenBank COI | Locality |
|---------|-------------|-------------|----------|
| *Craspedacusta sowerbii* | OK037602-OK037604 | OK037605-OK037607 | Japan (Nagano) |
| *Craspedacusta sowerbii* | MZ326744 | MZ326744 | Japan (Osaka) |
| *Craspedacusta sowerbii* | OK037610 | OK037612 | Singapore |
| *Craspedacusta sowerbii* | MW603820 | MW600236 | Canada |
| *Craspedacusta sowerbii* | KF510026 | | China |
| *Craspedacusta sowerbii* | MK600506 | MK600508-600509 | Morocco |
| *Craspedacusta sowerbii* | FJ423613-FJ423620 | | Germany |
| *Craspedacusta sowerbii* | MZ508273 | MZ508273 | Germany |
| *Craspedacusta sowerbii* | MZ508274 | MZ508274 | Germany |
| *Craspedacusta sowerbii* | MZ508276 | MZ508276 | Germany |
| *Craspedacusta sowerbii* | MZ508275 | MZ508275 | Czech Republic |
| *Craspedacusta sowerbii* | MF177101-177133 | | Chile |
| *Craspedacusta sowerbii* | KP231217 | | Greece |
| *Craspedacusta sowerbii* | MH230079 | | Italy |
| *Craspedacusta sowerbii* | MF000493 | | Switzerland |
| *Craspedacusta sowerbii* | KX267739 | | unknown |
| *Craspedacusta sowerbii* | LN901194 | LN901194 | unknown |
| *Craspedacusta sowerbii* | JN593332 | JN593332 | unknown |
| *Craspedacusta sowerbii* | EU293975 | | Germany (lab) |
| *Craspedacusta sowerbii* | EU293971 | | Germany (lab) |
| *Craspedacusta sinensis* | AY512507 | | China |
| *Craspedacusta ziguienis* | EU293974 | | China |
| *Astrohydra japonica* | OK037608-OK037609 | OK037611 | Japan (Shiga) |
| *Astrohydra japonica* | EU293975 | | Germany (lab) |
| *Olindias formosus* | LC508964 | | Japan |
| *Olindias phosphorica* | MH700550 | | Turkey |
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(mm) measured (as a linear distance from the base of one primary tentacle to the opposing one). Additionally, finer characteristics were verified using light and DIC microscopy (Olympus BX61), including cnidome composition (type of nematocysts) in tentacle nematocyst batteries, and presence/absence of gametes. For both, a small section (<1 mm) of a tentacle or gonad respectively, was clipped from the preserved specimen, placed in a drop of water on a microscope slide, chopped finely and squashed with a cover slide. The gonad surface of a single mature female was examined using Scanning Electron Microscopy (SEM) (Hitachi SU8000 (TypeII).

Sampling in Machikane-ike Pond, Osaka

On 15 August 2015, *Craspedacusta sowerbii* polyps were collected on sunken branches in the sediment of Machikane-ike (34°48′N, 135°27′E) on the grounds of Osaka University (Toyonaka Campus, Japan); a pond approximately 10 m in diameter, and 0.5 m in depth (Fig. 2a, b). Polyps were kept in aged hydra water (a culture solution of distilled deionized water and salts cf. Lenhoff & Brown, 1970) at the laboratory in the Lake Biwa Museum, and were fed rotifers or lab-cultured *Artemia* (brine shrimp) two to three times a week. When present, several medusae were also collected simultaneously using a long-handled plastic ladle when collecting debris for polyp observations. Close examination of mature medusae collected from Machikane-ike (Fig. 2c) revealed no morphological differences from *C. sowerbii* medusae previously documented in Japan (reviewed in Lewis et al. 2012). Polyps from the Osaka population served as samples for a *C. sowerbii* WGS (Whole Genome Sequencing) project (Available as ASM368756v1, on the NCBI Assembly database).

Fig. 2. Machikane-ike pond (Osaka) collection site for *Craspedacusta sowerbii*. a) Map of Machikane-ike (Google Maps). b) Photo of the area surrounding the pond in which jellyfish, polyps and frustules were collected. c) Medusa (male) observed swimming upside down in situ on 10 June 2016. Images are courtesy of Lake Biwa Museum.
In November 2014, pebbles were collected from Miyagahama—a beach on the east shore of Lake Biwa (35°19′N, 136°08′E)—Omihachiman City, Shiga (Kinki region, Japan) in an attempt to locate polyps of *Craspedacusta sowerbii*. Gathered pebbles were kept in a large Petri Dish with hydra water at a lab in the Lake Biwa Museum, to which live zooplankton prey items were added (mainly rotifers and copepod nauplii collected from a pond within Lake Biwa Museum grounds). Two weeks later, *C. sowerbii* pol-
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Fig. 4. Schematic of the molecular barcoding workflow used for *C. sowerbii*. a) DNA was extracted from three ethanol-preserved (99.5%) *C. sowerbii* specimens, cut in half, from Haruta-ike, Japan (CSNZ012-014) and one from Singapore (CSS001) following the protocol of either the Qiagen DNeasy Blood and Tissue Extraction kit (Qiagen, Germany) or the QuickGene-Mini480 nucleic acid isolation system (Kurabo, Japan), the latter in which centrifuge steps are replaced with air compression and equivalent reagents. b–d) Quantification of extracted DNA samples was conducted using QuantiFluor ONE dsDNA (Quantas Promega, UK). Approximately 200 ng of DNA template was used for each PCR (polymerase chain reaction) reaction to amplify two separate regions (metazoan COI and medusozoan 16S rRNA gene) with corresponding primers (jgLCO1490 and jgHCO2198 and med-rnl-F and med-rnl-R respectively), using a thermocycler (BentoLab, Bio-works, UK). e) Gel electrophoresis (1% Agarose, 0.5x TBE Buffer, run at 120 V for 20 mins) confirmed amplification in PCR product beside a 1Kb ladder in the first well in each. Bands are visible for Nagano and Singapore specimens, and the positive control (labelled 16S and COI respectively). Note: Corresponding morphological vouchers (the other medusa half) were preserved in 5% formalin solution and accessioned as museum specimens (see Table 2).
yps were discovered in the dish. Subsequently, in April 2015 while transferring frustules to set up a new culture dish, strikingly different polyps (Fig. 3a, b) and smaller, transparent frustules (Fig. 3c) were discovered, which we identified as *A. japonica* based on morphology. *Astrohydra japonica* polyps were reared on pond zooplankton (e.g., rotifers and oligochaetes carefully filtered and sorted with microscopy to prevent introduction of other hydrozoans or potential predators), and *Artemia* (brine shrimp) nauplii. *Astrohydra japonica* polyps rapidly proliferated asexually and generated motile frustules, while remaining as solitary polyps (unlike *C. sowerbii* which frequently produces multipolar polyps).

**Cultures and vouchers**

In addition to the cultures maintained at Lake Biwa Museum, in August 2015, live *A. japonica* (Shiga) and *C. sowerbii* (Osaka) polyps were transported to the Smithsonian Institution in Washington, D.C., and reared in the Invertebrate Zoology Department wet lab, and subsequently subjected to molecular barcoding at the Laboratory of Analytical Biology, National Museum of Natural History. In this study, a combination of morphological and molecular analysis was used on both the Japanese populations and a *C. sowerbii* museum voucher from Singapore. Table 2 shows the museum (Smithsonian National Museum of Natural History, Washington, D.C. (NMNH) and Shinshu University Museum, Nagano, Japan (SHIN)), and GenBank accession numbers corresponding to respective morphological vouchers and molecular sequences generated in this study.

**Assembly and annotation of the *C. sowerbii* mitochondrial genome**

We assessed two different approaches to mitogenome (whole mitochondrial genome) assembly using Geneious Prime 2021.2.2 (https://www.geneious.com). First, we used the “Map to Reference” function and the built-in Geneious mapper with the sensitivity set to “medium/low” and iterations set to “up to 10 times”, starting with a previously generated mitochondrial 16S gene fragment as the reference seed and using 4 million reads from NCBI BioSample: SAMN05463206; SRA: SRR7276314. The resulting assembly was inspected by eye and trimmed at its ends (up to 50 base pairs (bp)) where coverage was low. A consensus of the resulting sequence was then used as a subsequent reference seed and the “Map to Reference” step repeated until the resulting assembly ceased to increase in size. The second approach used a published complete mitogenome (NC_018537) as the reference sequence, and “Map to Reference” function, using the same parameters for a single set of up to 10 iterations. Both methods successfully produced equivalent results.

**Molecular analysis**

The molecular workflow employed in this study (Fig. 4)—DNA extraction, PCR and quantification of 16S rRNA (large subunit of the ribosomal RNA gene) and COI (cytochrome oxidase 1 region of the mitochondrial genome), and validation—is adapted from previous studies (Lawley et al. 2016, Ames et al. 2021). Briefly, molecular analyses of three medusa specimens of *C. sowerbii* collected at Haruta-ike and one from Singapore River Wonders (Mandai Wildlife Reserve), were conducted in the International Integrative Research and Instruction Unit, Graduate School of Agricultural Science, Tohoku University (Japan), while polyps and frustules of *A. japonica* were analyzed at the Laboratories of Analytical Biology, National Museum of Natural History, Smithsonian Institution (USA).

**Phylogenetic analysis**

Sequences corresponding to COI (n=54) and 16S rRNA (n=13) were downloaded from GenBank (Table 2) for *Craspedacusta* and *Astrohydra*, and respectively for outgroups *Olindias phosphorica* (Delle Chiaje, 1841) and *Olindias formosus* (Goto, 1903). Using MAFFT, GenBank sequences were aligned together with sequences extracted from *C. sowerbii* and *A. japonica* in this study, and neighbor-joining consensus trees were reconstructed in Geneious Prime v.2021.2.2 using the Jukes-Cantor substitution model (Jukes and Cantor 1969), in order to observe the topography of *C. sowerbii* and *A. japonica* rooted on *Olindias* spp. as an Olindiidae taxon outgroup.

For both genetic markers, the mean evolutionary divergence (and standard error) between the clades that include *C. sowerbii* of Nagano and Osaka, respectively, was estimated based on the number of base substitutions per site, averaging over all sequence pairs between clades in MEGA 11 (Stecher et al. 2020, Tamura et al. 2021). Analyses were done using the Jukes-Cantor model and rate variation among sites modelled with a gamma distribution of shape parameter of 1. All ambiguous positions were removed for each sequence pair (pairwise deletion option).

**Results**

**Collection and description of medusae from Haruta-ike, Nagano**

*Craspedacusta sowerbii* were collected at Haruta-ike on 19, 20 & 28 August and 1 September 2020. *Craspedacusta sowerbii* medusae were generally scarce, with only 1–4 individuals present on sampling days, with the exception of 1 September, on which more than 100 individuals were observed (Table 1). The *C. sowerbii* individuals that were sampled (n=17) varied in size from 7–15 mm (bell diameter) (Table 1, Fig. 5a,b) with 11 individuals having a diameter of 12 mm or greater; consistent with *C. sowerbii* medusa sizes previously published in Japan (see Lewis et al. 2012 and references therein). Here we focus on the largest (CSNZ1: mature female, 15 mm in diameter) and smallest (CSNZ2: immature female, 7 mm in diameter) specimens.
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(Fig. 6a–f and Fig. 6g, respectively). The long, manubrium extend broadly onto the subumbrella, and with the four lips open revealing the characteristic accordion-like central feeding tube (Fig. 6a, b). Characteristic marginal stastocysts (Fig. 6c) were located at the proximal end of blind velar canals, which extend inwards from the umbrella margin along the velum in rows of two to three between primary tentacles (Fig. 6a, c). Consistent with the general description of *C. sowerbii*, different types of tentacles (i.e., primary to quaternary) were distinguishable based on the number and place of origin of each kind along the umbrella margin (reviewed in Lewis et al. 2012). However, it should be noted that while primary and secondary tentacles are easily distinguishable, given their elevated position relative to the umbrella margin (Fig. 6a, c), tertiary tentacles are abundant, and their diminutive size makes it difficult to count them accurately.

Regularly spaced rings of nematocyst warts (clusters of nematocysts) are apparent along the length of primary and secondary tentacles of the smallest individual, but less conspicuous on the larger individual with nematocyst batteries (papillae), appearing as unorganized rows in the latter (Fig. 6c, d). Further magnification of these batteries revealed heterotrichious microbasic euryteles (n=10–15) comprising each nematocyst battery. These penetrating nematocysts are non-rotationally symmetric, having one side flat and the other noticeably curved (10–15 \(\mu\)m in length); within, the inverted tubule is coiled off-center around the thick shaft, which is out of the median plane (Fig. 6e, f).

**Sex**

Gonads of *C. sowerbii* medusae measuring greater than 12 mm in diameter appeared as stocking caps, each attached to one of the four radial canals, originating adjacent to the base of each corner of the manubrium, and extending into the subumbrella space sometimes, extending to the velar opening (Fig. 5a, b). Elongated gonads were composed of a layer of immature follicles, and a second layer speckled with numerous white clusters on the surface (Figs. 6a, 7a) comprising mature oocytes (Fig. 7b–d), suggesting these medusae may have been ready to ovulate into the surrounding water at the time of collection at Harutaike. Gonads of smaller individuals (<12 mm) were present as four small walnut-shaped sacs (Fig. 6g) attached proximally to the radial canals next to the base of the manubrium; composed entirely of immature follicles, indicating juvenile females. Gonads of two of the preserved medusae (CSNZ16, 14 mm and CSNZ18, 12 mm) were found to...
contain abundant spermatozoa (Fig. 7e, f). Together, these findings confirm the presence of both male and female medusae in Haruta-ike.

**Behavior**

While conducting underwater *in situ* video documentation using a GoPro video camera (Hero8), *Craspedacusta sowerbii* medusae were observed swimming in the water column among other inhabitants (described in "Study site: Haruta-ike Pond” above) of the pond (Supplementary Video 1, 2). Medusae were detected at the surface of the pond and at water depths ranging from 100 to 300 cm. In the larger individuals (>12 mm), *in situ* observations revealed the remarkably elongated gonads protruding from the sub-umbrella far beyond the velar margin (Fig. 5c). Waving back and forth in the water column, gonads were initially mistaken for primary tentacles (Fig. 1c).
Female

Fig. 7. Comparison of female ovaries and male testes in *C. sowerbii* medusae from Haruta-ike (Nagano). a) Mature female gonads (ovaries) of CSNZ1, extending from the umbrella, grainy surface corresponds to mature ova (20×). b) Magnification of area shown in (a) shows two mature ova above a layer of immature follicles (20×). c) Mature ovum (100×). d) Portion of the ovaries dissected from CSNZ1 imaged using SEM (image courtesy of Amanda Saraswati). e) Squash preparation of male testes dissected from CSNZ18, showing abundant sperm (red arrow) (20×). f) Magnification of area designated in c), showing head and tail of abundant sperm present (100×). Abbreviations: If=immature follicle, Ov=ovary

Mitochondrial genome annotation

The assembled mitochondrial genome using 4 million Illumina generated short reads had a 171× average coverage at each position. Annotation of the mitochondrial genome of *C. sowerbii* (Osaka, Japan) was conducted using MITOS2 (Donath et al. 2019). MITOS2 found all expected mitochondrial genes other than one hypothetical protein, a nonstandard mitochondrial gene (ORF314) that is present in the mitochondrial genome of some medusozoans (Kayal et al. 2012), which was annotated manually. The annotated
mitochondrial genome (Fig. 8) is accessioned in GenBank (GenBank Accession: MZ326744).

**Molecular analysis**

Consensus neighbor-joining trees (Figs. 9a, b) and distance matrices of percent identity (Figs. 9c, d) reconstructed for both genetic targets suggest *C. sowerbii* from Osaka and Nagano belong to separate clades. Represented as a heatmap, K2P distances (pairwise distance, as percent identity between individuals) for the 16S rRNA gene (Fig. 9c) reveal 100% identity between *C. sowerbii* from Nagano, Canada, Germany and from an unknown location, and 1% divergence from populations in both Singapore (current study: representing the first report in Singapore of this species) and the Czech Republic; while *C. sowerbii* from Osaka forms a separate clade with Morocco and other samples from Germany (Fig. 9c). Likewise, phylogenetic reconstruction of the more rapidly evolving COI marker places *C. sowerbii* from Nagano in a large clade that also includes Singapore and is separate from the clade that contains the Osaka sample, which is identical to that from Switzerland (Fig. 9b, d). A third, unresolved clade contains *C. sowerbii* from localities that shared a clade with Osaka in the 16S rRNA gene analysis (Fig 9c). Furthermore, *A. japonica* samples collected in this study (Shiga, Japan) were identical to a 16S rRNA sequence available in GenBank listed as *A. japonica* from a lab culture in Germany, of unknown origin (Fig. 9a,c).

For the 16S rRNA gene (16 sequences total), the mean divergence between the Nagano and Osaka *C. sowerbii* clades was estimated at 0.0587 (S.E. 0.0081), compared to a mean estimated divergence between the Nagano clade and that containing *C. sinensis* and *C. ziguiensis* of 0.0926 (S.E. 0.0141). For COI (17 sequences total), the mean divergence between the Nagano and Osaka *C. sowerbii* clades was estimated at 0.20025 (S.E. 0.02244), compared to a mean estimated divergence between the Nagano clade and all other *C. sowerbii* sequences of 0.19221 (S.E. 0.02036).

**Discussion**

*Craspedacusta sowerbii* populations

We reported findings on two populations of *Craspedacusta sowerbii* in Japan, focusing mainly on early life stages cultured from polyps and frustules collected from Machikane-ike (Osaka) where *C. sowerbii* has been reported since 1967, and on observations of morphology, swimming and distinguishing the sexes of medusae newly discovered at Haruta-ike (Nagano). Our molecular findings indicate the two Japanese populations occupy separate phylogenetic clades, each also comprising *C. sowerbii* individuals from broad geographic locations, indicating more than one introduction event into Japan. Such findings
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Fig. 9. *Craspedacusta sowerbii* COI and 16S rRNA species trees and pairwise molecular distance for Japanese and non-Japanese specimens. a) Species tree of *Craspedacusta sowerbii* based on 16S rRNA gene sequences, rooted on a clade with other olindiids: *Astrohydra japonica* (from Shiga, Japan and a German lab), *Olindias formosus* (Okinawa, Japan). b) Species tree of *Craspedacusta sowerbii* based on COI gene sequences, rooted on a clade with other olindiids: *Astrohydra japonica* (Shiga, Japan) and *Olindias phosphorica* (Izmir, Turkey). Neighbor-joining Consensus Trees were reconstructed using the Jukes-Cantor substitution model in Geneious Prime® v.2021.2.2. Bootstrap (n = 1000) resampling of gene sequences are included as percentages at nodes. Sequences generated in this work are indicated with symbols as shown in the legend. Highlighted clades: orange = *C. sowerbii* Nagano clade; green = *C. sowerbii* Osaka clade; yellow = other *Craspedacusta* species (China); purple = outgroup including *A. japonica*. Icons: 5-point star = *C. sowerbii* from Japan (Nagano); 4-point star = *C. sowerbii* from Japan (Osaka); square = *C. sowerbii* from Singapore; circle = *A. japonica* from Japan (Shiga). Consensus (50%) sequences were created for Chile (n = 33), Morocco (n = 2) and Germany (n = 8) (see Table 2). c) Heatmap of pair-wise K2P genetic distance based on percent identity of the 16S rRNA analysis. d) Heatmap of pair-wise K2P genetic distance based on percent identity of the COI gene analysis. Alignments were done using G-INS-I algorithm in MAFFT. Darker hues indicate higher similarity, with black along the diagonals indicating 100% identity. Calculation for estimated mean evolutionary divergence between clades provided in Supplementary Data I. The first column of the distance matrices in c) and d) list only the first number in the corresponding accession series show in a) and b).
are consistent with at least one other study that found more than one distinct *C. sowerbii* population within a single country (Morpurgo et al. 2021), and suggests that either *C. sowerbii* is genetically diverse around the globe due to its adaptability and complex metagenetic life cycle, or that several cryptic species exist awaiting delineation. In this study, our annotation of the first complete mitochondrial (mtDNA) genome for *C. sowerbii* from Japan revealed structural identity to other *C. sowerbii* mtDNA genomes (see Zou et al. 2012 and NCBI), indicating phylogenomic (whole mtDNA) studies are needed in order to verify genetic relatedness and diversity across *C. sowerbii* populations both locally and globally.

**Diocious population**

Our observations on the Nagano population revealed that both sexes are present in Haruta-ike, representing a rare find, as most *C. sowerbii* populations are represented by a single sex (Payne 1924), except in China, which is thought to be the geographic origin of the global radiation (Kramp 1961, Zhang et al. 2016). Consequently, information on sexual reproduction in this species is scant.

**Fig. 10.** Life cycle of *C. sowerbii* individuals in culture from Machikane-ike (Osaka). a) Adult medusa (ca. 14 mm). b) Sessile polyps (1–2 mm), asexually generating numerous polyp clones that also produce the “worm-like” motile, frustule stage (300–500 µm) that lacks a mouth. c) Close-up of frustules. d) Close-up of polyp having deposited chitin encysted podocysts (yellow arrow) onto the substrate that remain dormant until environmental conditions change, and subsequently polyps reemerge (curved arrows demonstrate the cyclic nature). e) Polyp feeding on *Artemia* brine shrimp (yellow arrow); remaining as polyps while food sources are abundant. f) Polyp generating a lateral medusa bud (ca. 50 µm in diameter) (yellow arrow), possibly due to limited prey items. g) Medusa bud swelling (yellow arrow) into the shape of an inverted medusa. h) Juvenile medusa (< 1 mm diameter) released, lacking clear distinctions in tentacle types. i) Juvenile medusa (1.5 mm diameter) developing, as tentacle types become more distinct. Images are courtesy of Lake Biwa Museum (a), and the Aquaroom Team, Department of Invertebrate Zoology, National Museum of Natural History, NMNH Smithsonian Institution (b–i).
Postulation that *C. sowerbii* is in fact an external fertilizer was confirmed in the detailed account of cleavage resulting from artificial insemination of males and females from different water bodies in a jar of pond water (for details see Sasaki 1999). According to Sasaki (1999), 2-cell embryos bear a unique “horseshoe” shape that resembles that of other hydrozoans (e.g., *Hydra* and *Spirocodon*); following cleavage, spherical embryos swim in circles, and eventually become creeping planulae that settle on the substrate as sessile antenaculate polyps.

Observations on the Osaka population cultured in the current study are compiled into a photo montage of *C. sowerbii*’s life cycle, from externally reproducing medusa to medusa bud formation (Fig. 10). Atentaculate polyps, whether unipolar or multipolar (Fig. 10b, d), are able to feed on *Artemia* brine shrimp in the lab (Fig. 10e), or small pond zooplankton (reviewed in Lewis et al. 2012), but in the event of unfavorable environmental conditions, polyps create a dormant phase called podocysts (Fig. 10d), deposited on the substrate, that are covered in chitin until conditions become favorable and polyps reemerge. Polyps can also generate lateral medusa buds (Fig. 10f, g), swelling in size to eventually transform into a juvenile medusa with tentacles (Fig. 10h, i), and becomes an adult medusa bearing male or female gonads (Fig. 10a).

*C. sowerbii* medusae are witnessed in nature at warmer temperatures (Lewis et al. 2012), and during medusa collection at Haruta-ike, surface water temperatures were higher (up to 33°C) than almost all values previously reported for field observations of *C. sowerbii* medusae in Japan or globally (12–34°C) (Lewis et al. 2012, Lüskow et al. 2021). Although medusae reared in the lab at water temperatures between 27 and 32°C are reportedly most active, survival rates were lowest, and medusae died quickest in water temperatures above 30°C (Zhang et al. 2016). Furthermore, in several studies, cultured polyps produced medusae at temperatures between 26 and 33°C (reviewed in Marchessaux et al. 2019), suggesting an increase in temperature triggers medusa bud production.

In this study, while rearing the polyps and frustules of *C. sowerbii* and *A. japonica* over several years at both Lake Biwa Museum and the National Museum of Natural History, respectively, we attempted to determine whether temperature changes could trigger medusae production by gradually altering the water temperature from 10 to 30°C (data not provided). However, for *C. sowerbii* no obvious connection was detected between temperature and the period or frequency of medusa production, and in the case of *A. japonica* no medusae production was witnessed in our cultures, thus further substantiating the evanescence of freshwater medusae in nature. Although environmental factors play an important role in medusa bud production (Payne 1924, Lewis et al. 2012, Zou et al. 2012, Zhang et al. 2016, Marchessaux et al. 2019), a more thorough study of the respective natural and artificial ecosystems is required before pinpointing potential correliative biotic and abiotic factors influencing medusa appearance. Overall, studying these cosmopolitan, yet capricious, medusae is difficult due to their ephemeral nature.

Some studies have investigated the distribution of *C. sowerbii* by looking for their polyps (Duggan & Eastwood 2012), rather than medusae, which allows a more accurate estimate of the extent of this species distribution. Going forward, approaches that employ environmental DNA (eDNA) techniques (see methods employed Mychek-Londrigan et al. 2020, Ames et al. 2021, Cindy et al. 2021) might be useful for determining the presence of both *C. sowerbii*, as well as *A. japonica*, in a broader range of freshwater environments even when medusae are not visually confirmed.

**Novel swimming behavior**

In this study, we found both male and female *C. sowerbii* medusae in Haruta-ike, engaging in a novel swimming strategy, with tentacles extending upwards above the umbrella while elongated gonads extended downwards below the velar opening. We surmise that this strategy could be employed to increase gamete dispersal during spawning, as the gonads of collected medusae were full of mature gametes. Observations in this study on female gonad conditions tie into findings reporting asynchronous gametogenesis in mature *C. sowerbii* females, whose ovaries comprised a single layer of immature follicles together with a surface layer of mature ova (Himchik et al. 2021), and with fine round ova appearing “bubble-like” on the surface of the ovaries (Xu & Wang 2009, as *C. sowerbyi xinyangensis*). Our findings corroborate these earlier discoveries suggesting that spawning in *C. sowerbii* is continuous throughout the brief period of sexual reproduction (lasting several weeks according to Himchik et al. 2021) during the limited time the medusa form is present in the water column. Future studies on sexual reproduction of *C. sowerbii* in Haruta-ike should provide a better understanding of the complexity of reproductive behavior in this freshwater jellyfish.

**Genetic analysis**

Our phylogenetic analysis of two mitochondrial genes revealed *C. sowerbii* at Haruta-ike (Nagano) and Machikane-ike (Osaka) fall into two separate clades. Given the high mean estimated genetic divergence between the Nagano and Osaka clades, despite the relatively close proximity of the two collection sites (417 km), it is conceivable that they represent different (possibly cryptic) species; the same phenomenon has been suggested to explain multiple lineages of *C. sowerbii* co-existing in other geographic locations (discussed in Morpurgo et al. 2021). Extensive sampling from multiple Japanese populations and population genetic analyses are needed to test this hypothesis.

Given the limitations of this study, we are unable to establish the mechanisms by which freshwater jellyfishes are distributed across multiple continents, or within Japan for that matter. In this study, the discovery of both males and
females in Haruta-ike (Nagano), suggests that the founder polyps were of both sexes, while the founder of Machikane-ike (Osaka) was a male, furthering corroborating separate introduction events for the two Japanese localities examined in this study. Some theories of polyp movement include the aquarium trade or relocation of construction supplies, or transport via humans or waterfowl (for references see Lewis et al. 2012); further large-scale population genomics studies are needed to address these theories.

Jellyfish and other allied species in the cnidarian clade Medusozoa—Cubozoan, Hydrozoa, Scyphozoa, and Staurozoa—differ from other animals in that their mitochondrial genomes (mtDNA) are linear. First documented in *Astrohydra japonica* points of the polymerase. Mitochondrial genes (16S and 12S rRNA) and the start and end of ribosomal genes (16S (rnl) and COI (cox1), and the two overlapping, non-overlapping tRNAs (n=2), large subunit rRNA (n=1), small subunit rRNA (n=1), putative ORFs (n=2) with little to no (less than 1%) intergenomic regions (Fig. 8) in the AMGO. Further, as in the study by Zou et al. (2012), the two genes targeted in this study 16S (rnl) and COI (cox1), and the two overlapping, non-standard ORFs, are transcribed in the direction opposite of that of the other 15 genes. In addition, all stop codons were present. However, for mitochondrial protein synthesis, an alternative start codon TGA (tryptophan) is used, also corroborating previous findings (Kayal et al. 2012, Zou et al. 2012) for Medusozoa and *C. sowerbii*, respectively. In the mtDNA derived from the Osaka culture, all genes were annotated to start and stop precisely in the same position as compared to the previously annotated mtDNA genome for *C. sowerbii* population from Wuhan, Hubei province, China (GenBank Accession: JN593332.1), but with the following exceptions: the beginnings and ends of the two ribosomal genes (16S and 12S rRNA) and the start and end points of the polymerase.

**Astrohydra japonica**

Though the type locality of *A. japonica* is in Nomorino-ike in Japan’s Kanto region, we found this species some 280 km away in the Kinki region, while another study reported *A. japonica* polyps from a drainage channel of Tsuga-ike reservoir in Suzuka City (Mie) (Oyabu & Murakami 2016). Neither in this study nor in the one by Oyabu & Murakami (2016) was the medusa form of *A. japonica* witnessed, despite long-term rearing of *A. japonica* polyps and frustules in the lab; suggesting that Hashimoto’s observations of the medusa form some 31 years ago were a truly rare occurrence. While conducting research for this work, we discovered two videos posted on YouTube (Saotome 2018a, b) revealing live multi-tentaculate *A. japonica* polyps cultured on a wood substrate (Fig. 3d). According to the videographer (K. Saotome, pers. comm.), the polyps were collected from a pond in a park in Tokyo (Japan) on 17 October 2017. Video-documentation while in culture shows they first released juvenile medusae with 4-tentacles (Fig. 3e) via lateral budding on 28 April 2018, and later on 19 June 2019 (Saotome 2018a). The second video shows a 52-day old female *A. japonica* medusae (Fig. 3f, g) on 28 June 2019 (Saotome 2018b). These videos provide a rare occasion to observe the process of medusa production in *A. japonica* and emphasize the importance of citizen science reporting.

Gene sequences for the 16S rRNA gene generated for *A. japonica* from Lake Biwa, Shiga (Japan) are identical to one published in GenBank corresponding to *A. japonica* from a lab culture in Germany (cf. Collins et al. 2008), however, details behind the collection are not known, and their origin may be linked to international aquatic sample exchange. Corroborating this are two reports of similar solitary polyps appearing separately in aquaria in St. Petersburg and Hungary in the 1960s discussed in (Hashimoto 1981); however, to date, the live *A. japonica* medusae have only been witnessed by Hashimoto (1985, 1987) and Saotome (2018a,b). Strong morphological similarities between the polyps of both *A. japonica* and *Calpasoma dactylopterum* Fuhrmann, 1939 (described in detail by Rahat & Campbell 1974), suggests that *Astrohydra* and *Calpasoma* might be synonyms, and that the apparent lack of a medusae in the latter is due to insufficient rearing conditions. However, in the absence of molecular data from other *A. japonica* samples reported in Japan (Hashimoto 1981, 1987, Oyabu & Murakami 2016, Saotome 2018a,b) or from *C. dactylopterum*, we refrain from synonymizing the two in this work.

**Conclusions and outlook**

In Haruta-ike (Nagano), both mature male and female *Craspedacusta sowerbii* medusae were collected, suggesting that a thriving sexually reproductive population exists to facilitate genetic diversification following putative annual summer spawning events. However, despite authors making multiple visits to Haruta-ike during the same period in 2021 (August and September) as in 2020, no medusae were witnessed, further confounding our understanding of *C. sowerbii* dynamics in Haruta-ike. In contrast, the reportedly all-male population in Machikane-ike (Osaka) appears to have persisted since 1967, as polyps, frustules and medusae can be obtained regularly from that locality (T. Suzuki, pers. comm.). To date, the sex of *A. japonica* medusae has not been determined, making future observations necessary to determine its sex ratio and reproductive
mode in the wild. Accordingly, standardized experiments that measure the precise effects of abiotic factors on polyps are needed to determine consistent inducers of medusa buds in different freshwater jellyfish strains, or species.

Together, our discoveries on *C. sowerbii* and *A. japonica* in Japan, contribute to our understanding of the small group that comprises freshwater jellyfishes on a broad geographic scale. We expect that the molecular and morphological vouchers we deposited into public databases and museums will serve as useful resources for continued advancement of this growing field of cnidarian research.

**Data archiving:** All molecular sequences and morphological vouchers generated in this study have been deposited into public databases (as per Table 2). Sequence alignments, tree files, distance matrices, and raw files for distance calculations are available as Supplementary Material (Data I).

Competing interest declaration: The authors declare they have no competing interests.

Permits: Permissions to collect in the respective sampling localities were obtained according to Japanese bylaws, and all necessary procedures were followed for specimen transfer.

**Supplementary Material**

**Supplementary Data I.** Files corresponding to molecular analysis generated in Geneious (alignments and phylogenetic analyses) or MEGA 11 (estimated mean evolutionary divergence between clades). Available at: https://github.com/tankei20/PetersonetalSupplementaryData

**Supplementary Video 1.** Swimming behavior of *C. sowerbii* medusa during collection of samples at Haruta-ike (Nagano), underwater video taken from GoPro Hero 8 camera. Available at: https://www.youtube.com/watch?v=ZH96g63gfaw

**Supplementary Video 2.** Swimming behavior of *C. sowerbii* during collection of samples from Haruta-ike (Nagano), underwater video taken from GoPro Hero 8 camera. Available at: https://www.youtube.com/watch?v=gnk1fpkZdMc

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