

Rapid Communication**First genetically confirmed report of the Japanese mystery snail, *Heterogen japonica* (Martens, 1861) from California more than a century after its first introduction**Wijesooriya Arachchilage Nimanthi Upeksha Abeyrathna¹, Shawn H. Sanders², Ashley Barreto³ and Andrew A. Davinack^{3,*}¹Biology Department, Clarkson University, Potsdam, New York 13699, USA²United States Fish and Wildlife Service, Fish and Aquatic Conservation – Aquatic Invasive Species, 2800 Cottage Way, Sacramento, CA 95825, USA³Biology Department, Wheaton College, Norton, Massachusetts 02766, USA

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Received: 21 November 2022**Accepted:** 13 February 2023**Published:** 13 March 2023**Handling editor:** Ting Hui Ng**Thematic editor:** Kenneth Hayes**Copyright:** © Abeyrathna et al.This is an open access article distributed under terms of the Creative Commons Attribution License ([Attribution 4.0 International - CC BY 4.0](https://creativecommons.org/licenses/by/4.0/)).**OPEN ACCESS****Abstract**

The so-called “Asian mystery snails” are a group of large freshwater snails that are regarded as invasive in many parts of the world, including the United States, where they have outcompeted native gastropods and have become the dominant molluscan fauna in many lakes and rivers. One notorious species, the Japanese mystery snail, *Heterogen japonica* (Martens, 1861) was first detected from food markets in California in the late 1800s but since then has never been reported from the region, and instead only the Chinese mystery snail, *Cipangopaludina chinensis* (Gray, 1834) has been found. This has led to some confusion as to the identity of these mystery snails at what was supposedly the point source of introduction. Unfortunately, these early specimens have been lost and so their identity cannot be confirmed, even morphologically. In this study we used DNA barcoding for the first time to clarify the identity of the mystery snails in a section of the Sacramento River in California which has never been explored for these animals. Our results provide the first genetic confirmation of *H. japonica* from California more than a century after it was supposedly detected. *Heterogen japonica* has now been genetically confirmed from every corner of the United States. Its cosmopolitan distribution is reflective of not only its broad physiological tolerance but also the wide variety of vectors which may have aided in its dispersal.

Key words: PCR, barcoding, *Cipangopaludina*, COX1, biosecurity**Introduction**

Biological invasions pose a serious threat to global biodiversity (David and Janac 2018). While most introduced species rarely become invasive, those that do can cause severe disruptions to ecosystem functioning which in turn can incur significant environmental and economic costs (Duenas et al. 2018; Cuthbert et al. 2021; Ahmed et al. 2022). Vectors for alien species introduction such as shipping, aquaculture, live food markets, the ornamental plant trade, along with the pet and bait trade are increasing worldwide which is likely responsible for the increased frequency of invasion events

(Sardain et al. 2019). This problem is compounded by the issue of “crypticity” where an alien species can be introduced and remain undetected for years due to striking morphological similarities shared with another known invader (Morais and Reichard 2018). Biosecurity programs that not only focuses on long term monitoring of problematic areas (e.g., ports and marinas) but also on broad genetic screening (DNA and eDNA barcoding) is therefore crucial for stemming the “invasion tide” that is currently occurring globally (see David and Krick 2019, and Pederson et al. 2021 as an example).

The so-called “Asian mystery snails” are some of the most problematic freshwater invaders in the United States and several recent studies are shedding light on their parasite symbionts, range expansion patterns and invasion dynamics (David and Cote 2019; Kingsbury et al. 2021; O’Leary et al. 2021; Fowler et al. 2022a, b). However, almost all of these studies have focused on the eastern US where the snails are notorious for reaching extremely high densities in lakes, ponds and slow-moving rivers. This is due to the fact that the majority of occurrence points are located on the east coast, which reflects the need for additional biomonitoring programs in the western US. In this study we investigated whether one of these species, the Japanese mystery snail, *Heterogen japonica* (formerly *Cipangopaludina japonica*) is present in California waters after a recent survey by the U.S. Fish and Wildlife Service discovered several large aquatic snails in a stretch of the Sacramento River where they have never been previously found. Historically, *H. japonica* was first discovered in the United States in the 1800s where it was reported from Asian food markets in San Francisco as “*Paludina japonica*” (Wood 1892). However, a later report by Hannibal (1911) suggested that these snails were misidentified and actually represented *Viviparus malleatus* (= *Cipangopaludina malleata*). A subsequent survey by Hannibal (1911) did recover *H. japonica* (then reported as *Vivipara japonica*) in an irrigation ditch in the San Joaquin Valley and characterized the species as having “a more acute spire”, and “flatter whorls” than other introduced snails of Asian origin. A morphogenetic study by David and Cote (2019) found that these conchological traits were highly variable (likely due to environmental variation), which contributes to the difficulty in delineating this species from some other “Asian mystery snails”. Considering that both Wood and Hannibal’s specimens have been lost, their actual identities cannot be verified. Since then, invasive populations of *H. japonica* have been reported and confirmed from both the northeastern U.S. in New York (David and Cote 2019) and southern U.S. in Texas (Perez et al. 2016).

The Wood (1892) paper appears to be the only published report of the presence of *H. japonica* in California (Kipp et al. 2022). Since then, only *C. chinensis* have been reported, which points to several possibilities. For example, early reports of *H. japonica* could have been a misidentification especially considering that both *H. japonica* and *C. chinensis* are morphologically

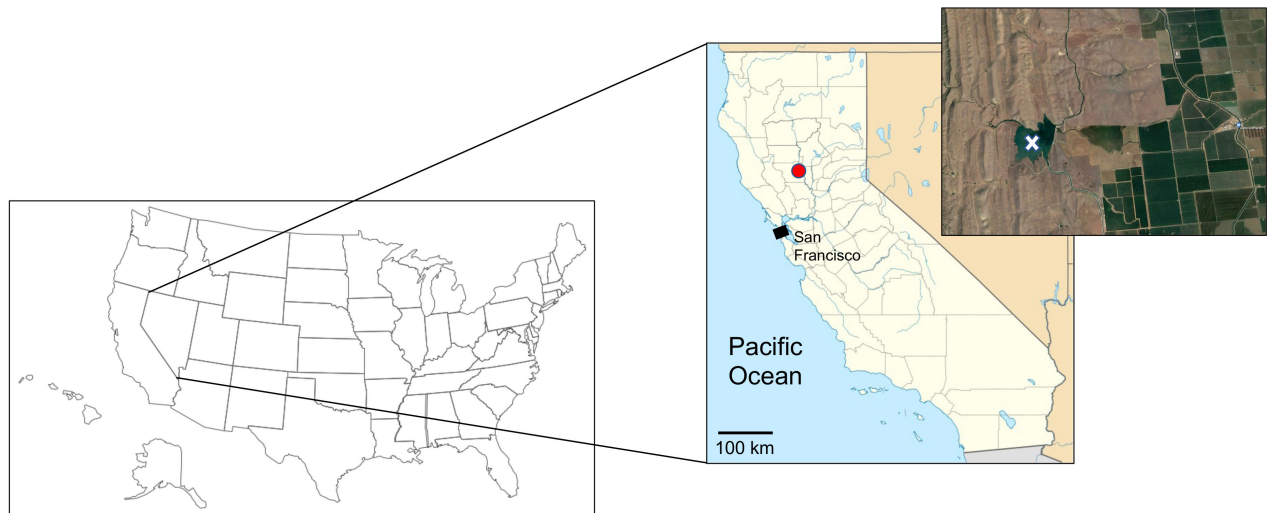


Figure 1. Map showing the Holthouse Reservoir sampling site (red circle) and X mark on inset map. Black square denotes San Francisco which is believed to be the point source for *Heterogen japonica*'s introduction into the United States.

indistinguishable (David and Cote 2019). Alternatively, *H. japonica* may have well been present in the region but has been consistently misidentified as *C. chinensis*. Both scenarios are equally likely considering that genetic data have never been leveraged for either species' identification on the west coast of the US. This is an urgent issue to resolve because if multiple mystery snails are present instead of one, then management and control strategies will have to be designed in such a way that each species' life history and ecology are taken into consideration.

The overarching objective of this study was thus to determine if *H. japonica* is present in California waters based on specimens collected from a reservoir during drawdown operations in the middle Sacramento River. For the sake of taxonomic integrity, we have opted to use the newly accepted scientific name for *H. japonica* based on a recent taxonomic revision by Saito and Kagawa (2020). Although it should be noted that the name "*Cipangopaludina japonica*" is still pervasive in DNA barcode databases and in the taxonomic literature but is nevertheless synonymous with *H. japonica*.

Materials and methods

Survey and sampling design

The U.S. Fish and Wildlife Service (USFWS) and U.S. Bureau of Reclamation (USBR) conducted a site visit to the Holthouse Reservoir on February 8, 2022 (GPS coordinates: 39.327818; -122.296) (Figure 1). Agency officials from the USFWS Bay-Delta Field Office in Sacramento discovered live mystery snails and empty shells along the reservoir shoreline during drawdown operations. The original survey design focused on an outlet of Holthouse Reservoir, however the survey design added sites to encompass the entirety of the Sacramento River Basin, where access was available. Sampling sites

were selected that provided full coverage of downstream portions of Funks Creek to the confluence of the Sacramento River. The sites ran along the headwaters of the upper Funks Creek, below Holthouse Reservoir. A total of nine potential sites were identified for the initial survey design although three sites were excluded due to sampling and access issues. Additional sites expanded the sampling area of the survey and were added following discovery of numerous small snail specimens at several sites along Funks Creek, including the canal outlet at the confluence with the mainstem Sacramento River, at Knights Landing. The expansion sites were located on the mainstem Sacramento River from the Red Bluff Diversion Dam (RBDD) downstream to Knights Landing. The RBDD is located on the Sacramento River south of Red Bluff, CA and acts as the intake structure for the Tehama Colusa Canal. During surveillance efforts, our field staff were provided anecdotal evidence (verbal communication, Red Bluff Fish and Wildlife Office team, June 1, 2022) that the *C. chinensis* may be present in Craig Creek, a tributary of the Sacramento River. The field team selected three additional sites which were accessible, in proximity to the RBDD. Following site selection and survey initiation, each site was visited at least once during the survey period. If site access was unavailable or sampling was impeded (e.g., water depth, habitat restrictions, etc.), the site was removed from the sampling matrix. Accessible survey sites were sampled an additional six times across the scheduled sampling period. The Holthouse Reservoir survey was sampled one time because reservoir operations and coordinating personnel did not allow for additional site visits.

Specimen collection protocols

A team of two people walked upstream and downstream for 100 m from the center of each site. A visual scanning technique was used to identify areas, longitudinally, within the site where low turbidity and/or depth allowed for collection of detritus or aquatic vegetation with a dip net. A 50 × 55 cm rectangular dip net with a handle that was approximately 1.8 m in length, was used to gather aquatic vegetation, detritus, and/or soft substrates. The totality of vegetation and available habitat varied, from sites devoid of vegetation to sites which were covered with both submergent and emergent aquatic plants and floating algal mats. If possible, the dip net was pulled across the top of the sediment, but most sites were too deep to reach creek substrates. The sampling methodology used in this study is described as an observational or visual technique with fixed points, assessing slow-moving and sedentary organisms such as gastropods.

Morphological identification

Shell morphometrics were determined for each snail to compare with measurements from other invasive populations from New York and Texas (Perez et al. 2016; David and Cote 2019). The morphological parameters

chosen were those outlined by David and Cote (2019) as being taxonomically informative for “Asian mystery snails”. These included whorl count, whorl pattern (directionally), shell length (SL), defined as the length from the apex of the shell to the base of the aperture, shell width (SW), defined as the maximum width perpendicular to the shell length distance, aperture length (AL) defined as the length of the beginning of the first suture to the bottom of the aperture and aperture width (AW), defined as the maximum diameter perpendicular to the aperture length. All shell size measurements were collected using a digital Vernier caliper with increments calibrated to the nearest mm. A schematic diagram showing exact dimensions of these measurements can be found in Chiu et al. (2002).

DNA isolation, amplification and sequencing

Three live snails were fixed in 100% EtOH for molecular analysis. For each snail, the shell was cracked using a mortar and pestle and the exposed head muscle was used to obtain a tissue piece of approximately 0.25 mm. Air dried tissue fragments were then digested in a Proteinase K and lysis buffer solution overnight at 56 °C. Genomic DNA was then extracted from each specimen using a DNeasy DNA extraction kit (QIAGEN®, Hilden, Germany) with modifications to the manufacturer’s instructions (see David et al. 2017 and David and Cote 2019). Briefly, DNA was extracted using the DNeasy Blood and Tissue Extract Protocol (QIAGEN, Hilden, Germany) to produce aliquots with DNA concentrations of 58–198 ng/μL. A ~ 710 bp fragment of the mitochondrial gene, cytochrome *c* oxidase 1 (COI) was amplified for each individual using the forward and reverse primer pairs from Folmer et al. (1994): (HCO2198 and LCO 1490). Polymerase Chain Reaction (PCR) was carried out in a 25 μl reaction mixture with the following cycling parameters: initial denaturation phase, 95 °C for 5 mins, followed by 40 cycles of 95 °C for 1 min, an annealing temperature of 55 °C for 1 min, 72 °C for 1 min and a final extension of 72 °C for 10 mins. PCR products were visualized in a 1% agarose gel stained with ethidium bromide (EtBr) and purified using a gel extraction kit (QIAGEN, Hilden, Germany). Amplicons were sequenced by Azenta LLC (South Plainfield, NJ, USA) using both forward and reverse primers and Big Dye Terminator Cycle Sequencing. All sequences obtained were translated using the ExPASy translation tool to ensure gene functionality. All sequence data were deposited into the GenBank database (accession numbers: OP809600–OP809602).

Sequence alignment, analysis and barcoding

Sequences were first compared against the GenBank database using the BLASTn algorithm to confirm initial conchological identifications. A tree-based framework was then used to confirm initial molecular identification. The sequence dataset used by David and Cote (2019) was adopted for this

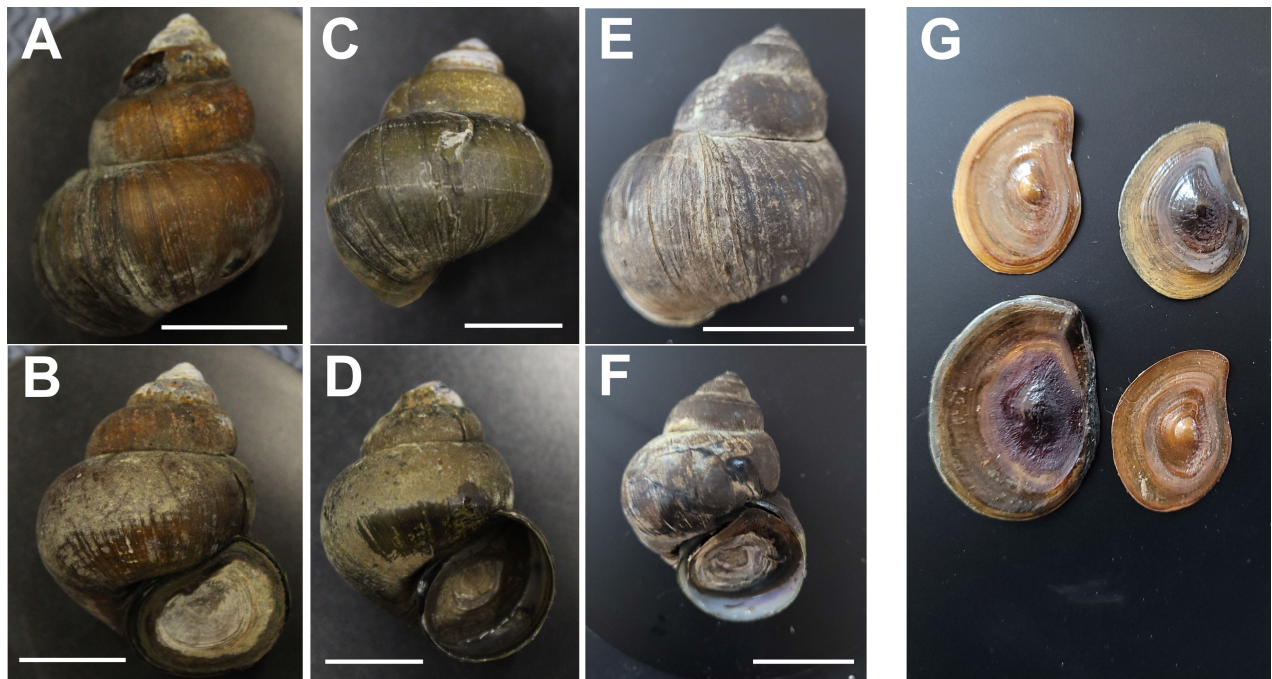


Figure 2. Photographs showing apertural and abapertural profiles of the three sequenced *Heterogen japonica* specimens from the Holthouse Reservoir in California: specimen 5 (A and B), 7 (C and D) and 4 (E and F), along with photographs of dissected operculums. Scale bars: 2 cm. Photos by Andrew Davinack.

Table 1. Comparison of shell morphometric measurements of *Heterogen japonica* from New York, Texas and California. NR = not reported, * male individual.

Specimen locality	Shell length (cm)	Shell width (cm)	Aperture length (cm)	Aperture width (cm)	Reference
New York	3.33 ± 0.32	2.67 ± 0.29	1.72 ± 0.26	1.10 ± 0.29	David and Cote (2019)
Texas	6.28*	4.39*	NR	NR	Perez et al. (2016)
California	5.67 ± 8.32	4.31 ± 5.51	3.96 ± 3.96	1.92 ± 0.41	this study

study. Sequences were edited then aligned using the Clustal W alignment algorithm in BioEdit (Hall 1999). After editing, a 427 bp fragment remained for analysis, which consisted of 169 variable sites. The phylogenetic position of *H. japonica* was assessed by constructing a maximum-likelihood (ML) tree in MEGAX (Kumar et al. 2018). The GTR + I + G evolutionary model was selected as the best fitting model, determined using the corrected Akaike Information Criterion best fit model test (AICc) in jModelTest ver. 2.0 (Darriba et al. 2012). Finally, pairwise Kimura 2-parameter (K2P) distances were calculated to quantify genetic distances between the sampled specimens and additional species of “Asian mystery snails”.

Results

Snails collected were large and olive to dark brown in coloration (Figure 2). Mean shell length and shell width were 5.67 cm ± 8.32 (N = 21) and 4.31 cm ± 5.51 (N = 21) while aperture length and aperture width were 3.96 cm ± 3.96 (N = 21) and 1.92 cm ± 0.41 (N = 21) respectively (Table 1). All snails exhibited a maximum of six whorls with variable degrees of angulations while the operculum was thin with distinct concentric rings.

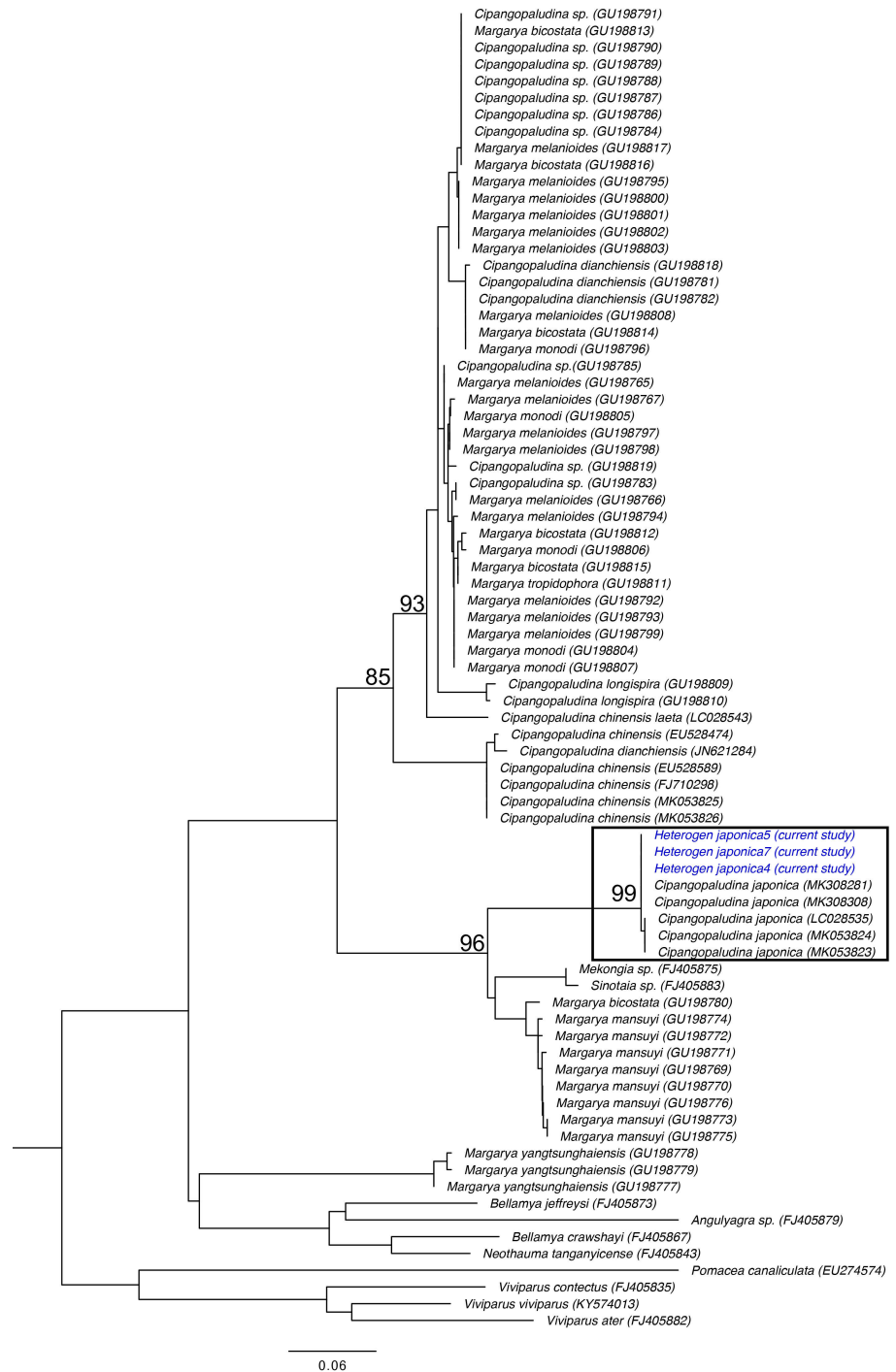


Figure 3. Maximum-Likelihood (ML) tree of COI barcodes showing reciprocal monophyly of *Heterogen japonica* and California mystery snails. In-group taxa include published COI barcodes from the GenBank database and adopted from the David and Cote (2019) dataset. Values above nodes represent bootstrap support based on 1000 replications. All taxa names follow original names on GenBank.

Spire shape was highly variable across all snails in that some were extended while in others they were depressed. Initial BLASTn comparisons matched to *C. japonica* with an identity score range of 99–100% (e-value = 0). The maximum-likelihood phylogenetic tree corroborated this initial identification as all three putative *H. japonica* individuals nested within a robustly supported clade consisting of COI barcodes for *C. japonica* (Figure 3). Intraspecific

K2P genetic distance for *H. japonica* ranged from 0.000 to 0.002 while interspecific genetic distances between *H. japonica* and *C. chinensis* ranged from 0.139–0.148.

Discussion

A phylogenetic analysis unequivocally confirmed the California specimens as *H. japonica* with all three individuals nesting within the highly supported *H. japonica* clade and showing substantial genetic differences based on K2P values. *Heterogen japonica* from California was morphologically similar to specimens from New York and Texas. However, California snails were larger than those reported from New York and considerably smaller than a single individual reported from Texas (Table 1). Despite clear morphological similarities, there was a high level of intraspecific variation observed across morphological traits, specifically in spire height and number of whorls. Indeed, initial identification by USFWS officials tentatively identified these specimens as *C. chinensis*. However, there was some suspicion since *C. chinensis* is known to have 7–8 whorls while *H. japonica* is known to only have a maximum of six according to a recent review by Kingsbury et al. (2021). While other features such as juvenile shell shape from the anterior-most brood pouch and the operculum shape can sometimes be diagnostic features (van Bocxlaer and Strong 2016; Hirano et al. 2019), the former was not available for investigation in this study and the latter was not found to be diagnostic for California specimens.

While *C. chinensis* and *H. japonica* are closely related, they represent genetically distinct species and as a consequence *C. chinensis*' biology cannot be used a proxy for managing *H. japonica* despite how appealing this may seem. Most natural history studies have focused exclusively on *C. chinensis* while several gaps exist for *H. japonica* in its invasive range. Therefore, a proper natural history study on invasive populations of *H. japonica* will be needed to contextualize any future management programs for this species.

Heterogen japonica has now been confirmed in every corner of the continental United States including the geographically distant states of California (present study), Texas (Burks et al. 2016) and northern New York (David and Cote 2019). Equally troubling is the fact that self-sustaining populations have been established in all of these regions. *Heterogen japonica*'s success is probably due to several traits, a few of which they also share with *C. chinensis* (Burks et al. 2016; David and Cote 2019). The connectivity patterns of these populations are currently unknown and with a wealth of genetic data for the species now available on the GenBank database, it is an area ripe for future investigation using a population genetic approach. This would also provide valuable information on the source of the different invasive populations and information on genetic diversity of the species in the invasive range versus the native range.

In terms of management, we are now beyond biosecurity measures to prevent the spread of *H. japonica* since the species can now be regarded as cosmopolitan in the United States. Instead, efforts should now be focused on developing feasible eradication plans that can be deployed uniformly across the country. For example, Sheehan et al. (2014) employed mechanical dredging as a method for removing the invasive Asian clam, *Corbicula fluminea* from Ireland's river system. While this was shown to significantly reduce *Corbicula* population density, such a strategy is highly unlikely to be feasible when adopted for large aquatic systems like the Great Lakes or the Mississippi River system in the US. Another strategy is chemical eradication via natural and synthetic molluscicides. Unfortunately, the only published report on the effects of such chemicals (in this case copper sulfate and rotenone) on exotic mystery snails found that 80% of *C. chinensis* adults were able to survive under high concentrations in a laboratory setting (Haak et al. 2014), which means that a higher percentage is likely to survive in a natural open setting because of dilution effects. The most feasible approach to dealing with *H. japonica* will likely not be complete eradication but rather containment of the already established invasive populations. This can be accomplished by disrupting arrival of additional propagules via practical methods such as public education for stakeholders such as recreational boaters and landowners, increased public outreach and greater regulation of the ornamental plant and animal trade.

Conclusions

In conclusion, we have genetically confirmed the presence of *Heterogen japonica* from California for the first time. Shell morphology showed high levels of intra- and inter-population variability when compared with published descriptions and morphometric measurements from other molecularly confirmed invasive populations across the United States. These differences are reflective of local adaption and makes it particularly difficult, if not impossible, to distinguish this species from its sister taxon, *C. chinensis* without genetic data. We therefore recommend the use of DNA barcoding as a minimal requirement for any future identification of *H. japonica* in North America.

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Authors' contributions

W.A.N.U. Abeyrathna was responsible for molecular analyses and writing, A. Barreto was responsible for morphological and molecular analyses, S. Sanders was responsible for specimen collection and writing and A. Davinack conceived the study and was responsible for data analysis and writing.

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