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Rapid Communication

Genetic evidence confirms the presence of the Japanese mystery snail, *Cipangopaludina japonica* (von Martens, 1861) (Caenogastropoda: Viviparidae) in northern New York

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Abstract

The viviparid snails, *Cipangopaludina chinensis* (Chinese mystery snail) and *Cipangopaludina japonica* (Japanese mystery snail) are considered high priority invasive species on the east coast of the United States. Both species are often lumped together with other related gastropods into the broad group known as the Asian "mystery snails". In New York, there has been conflicting reports on the presence of *C. japonica* with only a single population ever recorded in the southern region of the state in the early 1980s. This confusion has been attributed to the lack of distinguishing conchological features between both species. To address this issue, we employed molecular barcoding to determine whether *C. japonica* was indeed present in the state's waterways. Specimens were collected from multiple waterbodies in the Adirondack region of northern New York during the summers of 2016–2018. In addition, museum specimens from the type locality (Japan) and North Korea were acquired and initial conchological identifications were carried out prior to barcoding. Conchological comparisons found that both species were virtually indistinguishable from each other. Although *C. japonica* appeared to have a slightly more elongated spire than *C. chinensis*, this trait still appeared to be highly plastic even within *C. japonica*. Regardless, molecular barcoding using the cytochrome *c* oxidase I (COI) gene unequivocally confirmed the identity of *C. japonica* with both sequenced specimens showing a 100% identity match on the NCBI database, GenBank. Furthermore, both species clustered within separate and well supported clades with K2P interspecific genetic distances of 0.13–0.15 and intraspecific distances of 0.00–0.01. This study is the first to genetically confirm *C. japonica* from the Adirondack Park and to a broader extent New York and also re-emphasizes the utility of barcoding techniques for aquatic invasive species (AIS) detection.

Key words: *Cipangopaludina chinensis,* barcoding, COI, conchology

Introduction

Aquatic invasions pose one of the greatest threats to global biodiversity (Molnar et al. 2008; David and Janac 2018). While large-scale invasion events have been historically rare, introductory events are on the rise, which, in turn, increases the probability of invasions occurring into the future (Levine and D'Antonio 2003). Aptly termed the "Anthropocene", key vectors such as shipping and aquaculture are rapidly expanding along with the

translocation of invertebrates caused by an increasing pet and bait trade. In addition to the increased likelihood of invasion events, increased introductory events also call into question the issue of species cosmopolitanism, which today can be the result of either anthropogenic and or natural dispersal (David 2018; Darling and Carlton 2018). The need for long term monitoring of aquatic biodiversity is therefore essential for combating the spread of invasive species (Chapman and Carlton 1991; Glasby and Connell 2001).

The Adirondack Park, located in northern New York (NY) is the largest protected forested area in the contiguous United States (5.9 million acres), which, in turn, makes it the largest protected freshwater ecosystem in the country (103,000 hectares of lakes and streams) (Erickson 1998; Tuttle and Heintzelman 2015). Approximately 50% of its rivers drain into the Laurentian Great Lakes region and as a consequence, bidirectional dispersal of invasive species between the two regions is possible (Shaker et al. 2017). Previous research that explored threats to the Adirondack waterways included work on freshwater acidification and poor land use practices. Today, with a rapidly growing tourism sector, along with accompanying growth of activities such as aquaculture and recreational fishing, the introduction of non-native species will likely be a future threat to this region (Shaker et al. 2017). The problem of aquatic invasive species (AIS) is of particular concern to lawmakers and stakeholders in NYS considering that their negative impacts (such as clogged waterways and reduced recreational use of rivers and lakes), have cost the state millions of dollars (Pimentel et al. 2005). Perhaps more troubling is that this price tag is based only on AIS associated with the Hudson River watershed in southern NY and does not take into consideration the impacts of invasive species in the Adirondacks.

There are currently no comprehensive published reports of aquatic invasive species in the Adirondack Park, but the majority of scattered studies have identified introduced molluscs as being one of the greatest threats to the region's biodiversity (David et al. 2017). The most notorious invasive molluscs in the region include the New Zealand mudsnail, *Potamopyrgus antipodarum* and the zebra and quagga mussels, *D. polymorpha* and *D. rostriformis bugensis*. In addition, three members of the Viviparidae (Caenogastropoda) are also considered invasive in NY; these include the banded mystery snail *Viviparus georgianus* and two closely related species collectively known as the Asian "mystery snails", *Cipangopaludina chinensis* (Gray in Griffith and Pidgeon, 1864) and *Cipangopaludina japonica* (von Martens, 1861). A comprehensive survey by Jokinen (1992) reported the presence of both species in NY with the lone *C. japonica* population occurring at only one site along the Hudson River watershed (Lake Tiorati) (Figure 1). Although the NYS Department of Environmental Conservation lists *C. japonica* on its Prohibited and Regulated Invasive Species List, the most recent checklist by the United States Geological Survey (USGS) reports *C. japonica* occurring in 20 states in the continental United States,

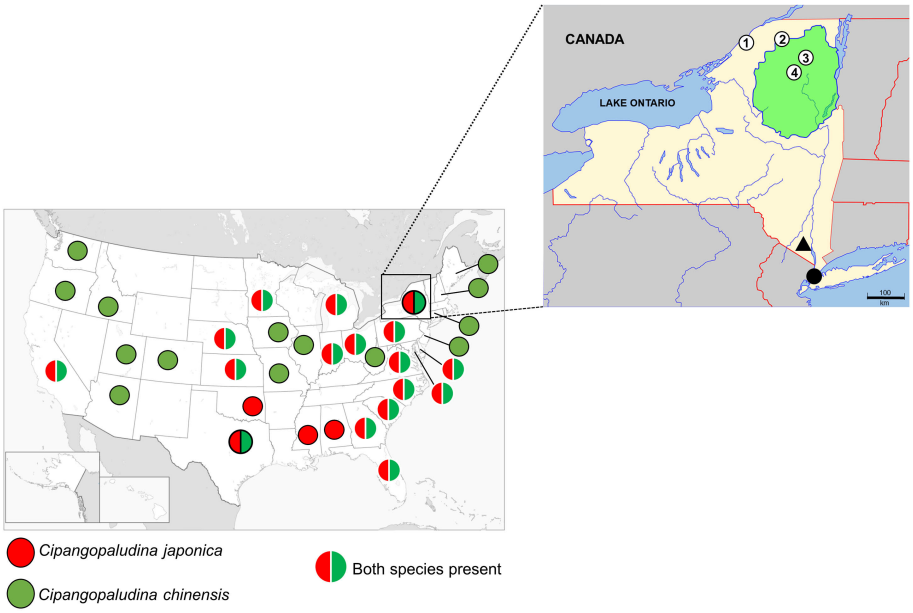

Figure 1. Map showing distributional range of the invasive *Cipangopaludina japonica* and *C. chinensis* within the continental United States based on records from the United States Geological Survey (database last updated: 2018). Darkened circles denote the availability of genetic barcodes for that particular state. Inset map of New York depicts sites sampled in this study; green shaded region delineates the Adirondack Park. Sampling sites for inset map: 1 –Waddington, 2 – Raquette River, 3 – Lake Colby, 4 – Tupper lake. Darkened triangle and circle represents Lake Tiorati and New York City, respectively.

but not in New York. The Pennsylvania Aquatic Invasive Species field guide also proposed that misidentification of both species may have resulted in an overestimation of the distribution of *C. japonica* in the north-eastern United States. Indeed, there is little conchological variation between *C. chinensis* and *C. japonica* (Dundee 1974; Clarke 1978), which may lead to inaccurate identifications by non-malacologists (Van Bocxlaer and Strong 2016). Alternatively, this could also mean that both species could be lumped together as *C. chinensis* or *C. japonica* in certain regions where the two species occur sympatrically. The situation is also complicated by the fact in North America, there are two different morphs of *C. chinensis* (Jokinen et al. 1982, Jokinen 1992; Van Bocxlaer and Strong 2016). In addition, *C. japonica* from North America also exhibits some key differences from its Japanese cohorts (such as spire height whorl angulation) (Van Bocxlaer and Strong 2016). In such cases, the use of molecular tools may provide an additional diagnostic method for delineating the two snail species. The objective of this study was therefore to determine if *C. japonica* was indeed present in the Adirondack region of New York using a DNA barcoding approach based on specimens collected over three years (2016–2018) at multiple sites within the Adirondack Park and surrounding environments.

Materials and methods

Specimen Collection and Initial Identification

As part of a long term survey to monitor the rivers and lakes of northern New York for non-indigenous molluscs, specimens were collected from the

Adirondack Park and surrounding environments for three years (2016–2018) during the summer months of June–August (Figure 1 and Supplementary material Table S1). The same four localities were sampled each year with the exception of 2018 (which excluded Lake Colby). Sampling of rivers and lakes occurred in the shallows (maximum depth of 1.2 m) for approximately one hour per site, which is typical of a Rapid Assessment Survey approach (Pederson et al. 2003). All molluscan fauna were then transported to the David Laboratory at Clarkson University and broadly sorted by Family rank. Putative specimens of *C. japonica* and *C. chinensis* were handled separately and identified to species level, first using the taxonomic key of Jokinen (1992) and then comparing their shell morphology with topotypic material obtained from the National Museum of Natural History (Smithsonian Institution) (NMNH catalog nos. 342643, 228473, 123800, 123799). Photographs from a single *C. japonica* individual examined by Jokinen (1992) lodged in the New York State Museum (NYSM) were provided by the curator (Denise Mayer *pers. comm.*) and compared to our collected individuals. All morphometric comparisons were carried out following the methods of Chiu et al. (2002) using a Vernier caliper and a stereomicroscope. A Vernier caliper (error margin: 0.05 mm) was used to determine shell length (SL) of both species by measuring the length from the apex of the shell to the base of the aperture while shell width (SW) was determined by measuring the maximum width perpendicular to the shell length distance. Additional morphometric data collected included aperture length (AL) which was determined by measuring the length of the beginning of the first suture to the bottom of the aperture and aperture width (AW) which was the maximum diameter perpendicular to the aperture length. Based on conchological comparisons only, 10 of the 36 snails collected were assigned to *C. japonica* while remaining snails were assigned to *C. chinensis*. The highest numbers of individuals for both species were found in the Raquette River across all three years, while Lake Colby yielded just one specimen of *C. chinensis* in 2016 and neither species was found at Waddington in any of the years sampled (Table S1).

DNA Isolation, Amplification and Sequencing

Two individuals representing *C. japonica* and *C. chinensis* each was preserved in 100% EtOH for molecular analyses and then photographed prior to destructive analyses. The shell of each individual was cracked and ~ 2 mg of tissue just above the operculum was dissected and digested in a Proteinase K and lysis buffer solution (QIAGEN, Hilden, Germany). Genomic DNA was then extracted using the D'Neasy Blood and Tissue Extract Protocol (QIAGEN, Hilden, Germany) to produce aliquots with DNA concentrations of 60–80 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 2% agarose gel stained with ethidium bromide (EtBr) and purified using a gel extraction kit (QIAGEN, Hilden, Germany). Amplicons were sequenced by GeneWiz (South Plainfield, NJ, USA) using the forward primer 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 codes: MK053823–MK053826).

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 ensure robust molecular identifications. A dataset was assembled which included sequence data obtained from this study along with published viviparid sequences (Du et al. (2013); Hayes et al. (2008); Sengupta et al. (2009); Jorgensen et al. (2008); Hirano et al. (2015); Table S2). For archived *C. japonica* and *C. chinensis*, only archived sequences that could be cross- referenced with the Barcode of Life Database (BoLD) and a voucher specimen and or could be linked to published studies that incorporated at least some level of morphological or conchological identifications were also used. Sequences were edited then aligned using the MUSCLE alignment algorithm in Geneious ver.10.1.3 (Kearse et al. 2012). After editing, a 450 bp fragment remained for analysis without gaps or missing data. The phylogenetic position of *C. japonica* and *C. chinensis* was assessed by constructing a maximum-likelihood (ML) tree in MEGAX (Kumar et al. 2018). The GTR+G+I model was selected as the best fitting model for nucleotide substitution, which was determined using the corrected Akaike Information Criterion best fit model test (AICc) in jModelTest ver. 2.0 (Darriba et al. 2012). Sequences of the ampullariid, *Pomacea canaliculata* (Reeve, 1856) and two viviparids, *Viviparus ater* (De Cristofori and Jan, 1832) and *Viviparus contectus* (Millet, 1813) were used to root the phylogeny. Finally, kimura-2-parameter (K2P) distances were calculated for both *C. japonica* and *C. chinensis* in MEGAX.

Results

Individuals of *C. japonica* were black to dark brownish in coloration and were in agreement with voucher specimens collected from the Smithsonian (Figure 2). Mean SL and SW for *C. japonica* were 3.33 cm ± 0.32 (N = 6) and 2.67 cm ± 0.29 (N = 6) respectively while mean AL and AW was 1.72 cm ± 0.26 (N = 6) and 1.10 cm ± 0.29 (N = 6) respectively. For *C. chinensis* mean

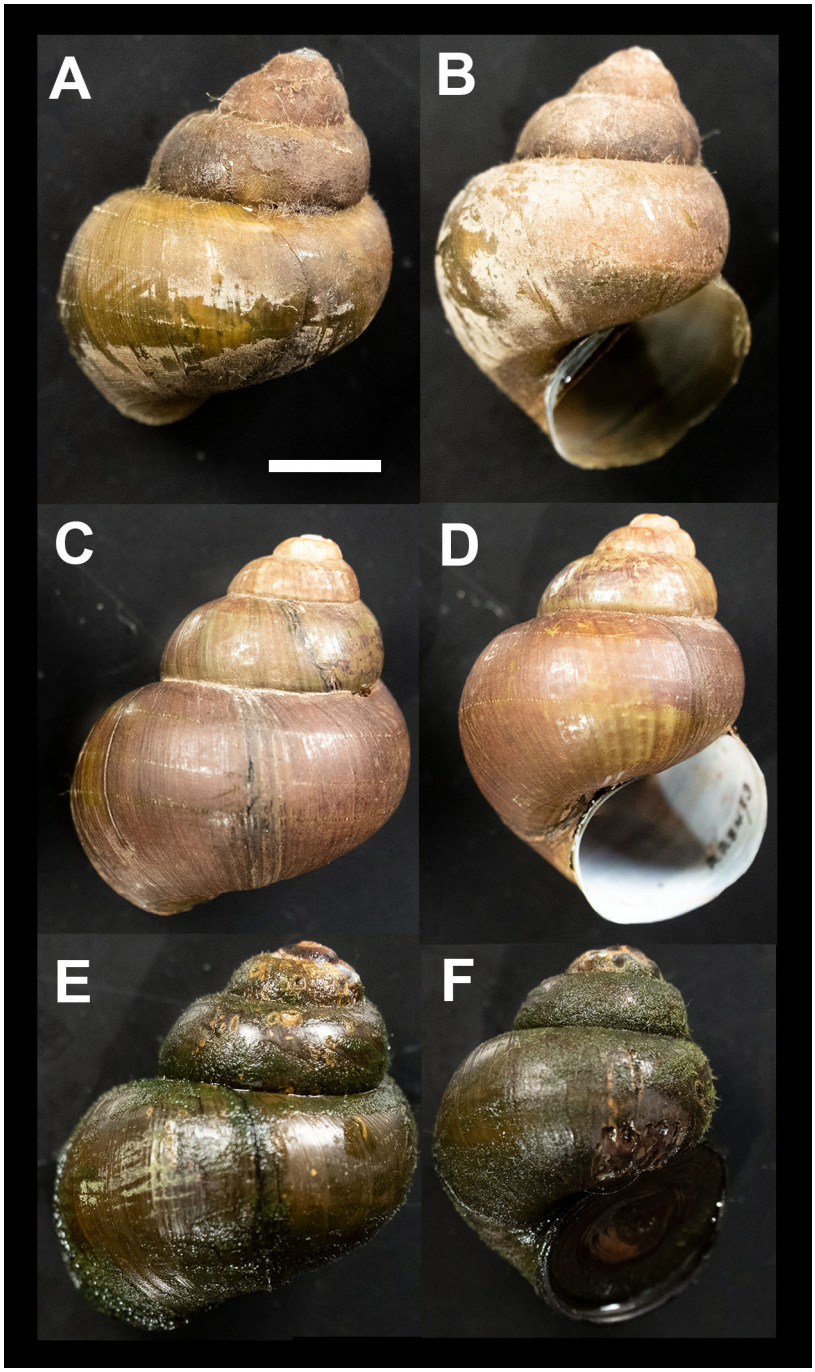

Figure 2. Apertural and abapertural photographs of *Cipangopaludina japonica* collected from northern New York (A, B) and a topotypic representative from Japan (NMNH catalog no. 228473) (C, D) and a live specimen of *C. chinensis* collected from the Raquette River (Potsdam, NY) (E, F) Scale bar: 1 cm. Photos by Andrew A. David.

SL and SW was 2.61cm ± 0.46 and 2.21 cm ± 0.45 respectively while mean AL and AW was 1.27cm ± 0.48 and 0.73 cm ± 0.41 respectively. For *C. japonica*, the_number of whorls ranged from 5–7 with relatively low angulations while the aperture possessed a thin operculum with concentric growth rings. Both *C. japonica* and *C. chinensis* collected from the Adirondacks were almost indistinguishable conchologically with the only variable character being a slightly more extended spire in some *C. japonica* specimens. In addition, *C. japonica* had a slightly higher SL/SW ratio (1.2–1.3) compared

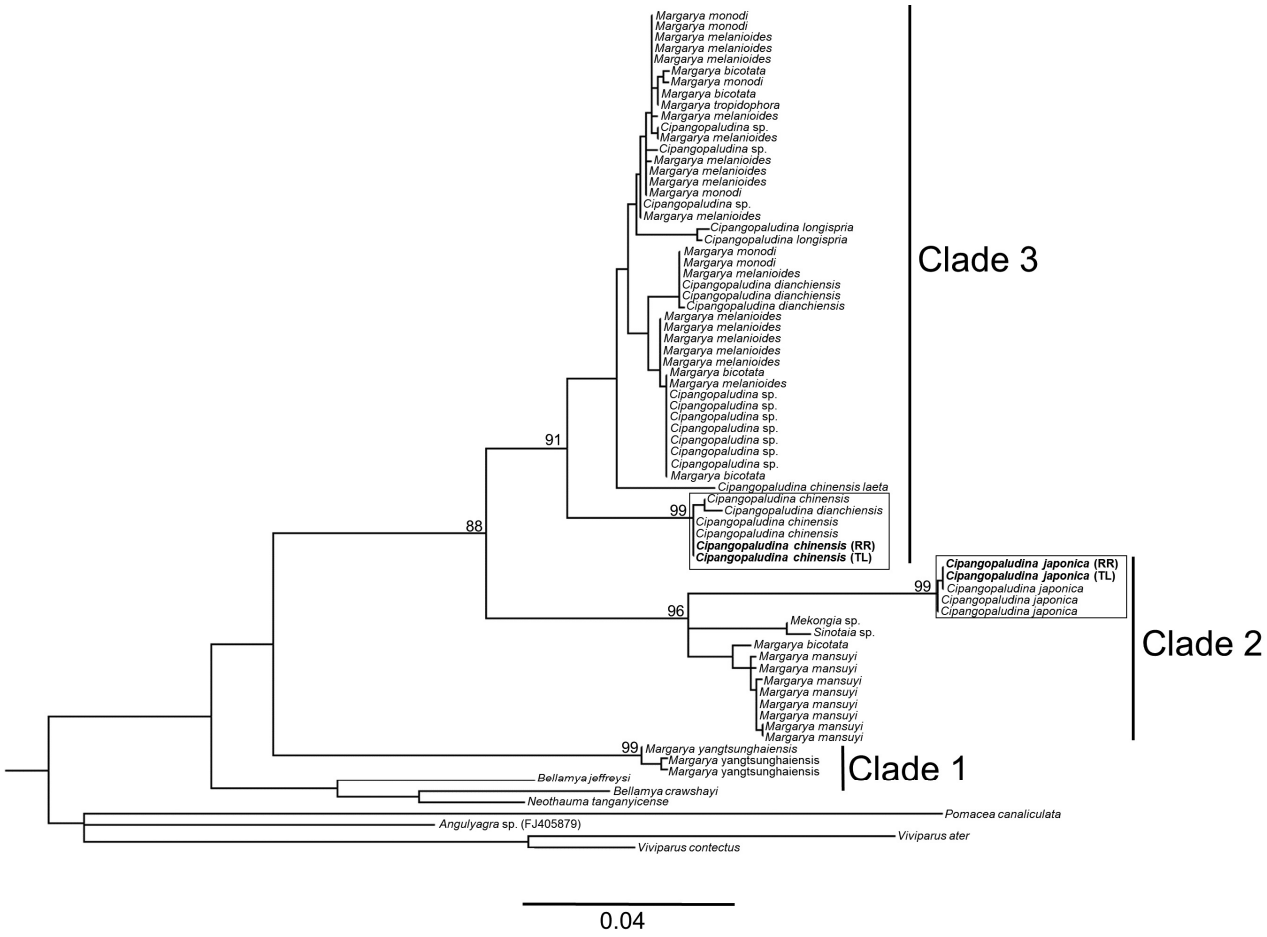

Figure 3. Maximum Likelihood (ML) tree of COI barcodes showing phylogenetic position of *Cipangopaludia japonica* and *C. chinensis* collected from the Adirondack region of northern New York. In-group taxa include published COI barcodes of all members of *Cipangopaludina* present on the GenBank and Barcode of Life Database along with published viviparid sequence data obtained from Du et al. (2013). Numbers above nodes represent bootstrap support based on 1000 replications. Bolded taxa represent sequences generated in this study; TL-Tupper Lake, RR- Raquette River. Clade 1 consists of representatives of *Margarya yangtsunghaiensis,* clade 2 consists of representatives of *M. mansuyi* and all *C. japonica* specimens, and clade 3 consists of all *C. chinensis* specimens in addition to the remaining *Cipangopaludina* and *Margarya* individuals.

to *C. chinensis* (1.0–1.1). Topotypic material from Japan all possessed more extended spires and slightly more angular body whorls compared to NY specimens.

Initial BLASTn searches supported preliminary conchological assessments with putative *C. japonica* specimens matching archived *C. japonica* COI sequences (e-value = 0; identity score range: 99%–100%) and putative *C. chinensis* matching archived *C. chinensis* COI sequences (e-value = 0, identity scores: 100% for both sequences). The ML phylogenetic tree corroborated these results as both species nested within robustly supported clusters with their respective COI barcodes (Figure 3). In addition, these clusters were distributed between two different clades (Clades 2 and 3). K2P distances further supported species delineation with intraspecific and interspecific distances for *C. japonica* and *C. chinensis* ranging from 0.000–0.012 and 0.134–0.147 respectively.

Discussion

This study is the first record of the Japanese mystery snail in the Adirondack Park of northern NY and the first genetic confirmation of the species in New York. *Cipangopaludina japonica* exhibited strikingly similar shell morphology to *C. chinensis.* Perhaps the only distinguishing conchological feature was a slightly more extended spire in some of the *C. japonica* specimens. This is concordant with the diagnosis by Jokinen (1992) and with photographs from the lone *C. japonica* specimen collected from Lake Tiorati in southern NY. Interestingly, a morphometric analysis of *C. chinensis* from Taiwan found that this species also had a "tall-spired" morph (Chiu et al. 2002). Furthermore, a recent study by Van Bocxlaer and Strong (2016) found that the elongated spire of *C. japonica* seems to be most prominent in specimens from the type locality (Japan) and less so in specimens from North America. As a consequence, they concluded that this trait is not sufficient to delineate the species and an analysis of its internal anatomy is a more accurate method. This study found that spire height and even the degree of angulation is likely a variable character in the North American specimens and as such, its use as a diagnostic trait as suggested by Jokinen (1992) remains debatable.

Despite the contentious conchological issues of elongated spires, molecular barcoding unequivocally delineated *C. japonica* from *C. chinensis* with interspecific K2P distances of 0.134–0.147 and their placement within separate clades in the ML-tree. Previous studies have shown an 11% COI divergence to be sufficient for delineation of species within the genus *Margarya,* which is polyphyletic with *Cipangopaludina* (Du et al. 2013). The current study which is based on newly sampled sequence data from the two Asian mystery snails supports this assessment while also recovering the three distinct clades that were found in the Du et al. (2013) study (Figure 3). One major difference in topology between the two phylogenies was that in our study, *C. japonica* specimens were included in the dataset and were nested within the highly supported Clade 2 which consisted of the genera *Margarya, Mekongia* and *Sinotaia*. Meanwhile, *C. chinensis* was nested within the highly supported Clade 3 grouping with the remaining *Margarya* and *Cipangopaludina* species.

Since Jokinen's (1992) first survey of NYS gastropods, there has been conflicting evidence of *C. japonica*'s presence in NYS waterways. The most recently confirmed record of *C. japonica* in the United States was in Texas where Perez et al. (2016) used DNA barcoding to confirm the species' presence there. Molecular confirmation of *C. japonica*'s presence in northern NY and Texas is indicative of a broad physiological tolerance, which could impede management or potential eradication efforts. The introduction of both species to the US is believed to be the result of intentional introductions, driven by their demand in Asian food markets in the late 19th century (Jokinen 1992).

It is interesting to note that *C. japonica* and *C. chinensis* occurred sympatrically along with the banded mystery snail, *V. georgianus* at all of the lakes sampled in this study (David and Cote, *pers. obs.*). All three non-indigenous snails are members of the Viviparidae and share similar life history patterns. They occur mainly in still water with muddy bottoms and in their temperate range, females reproduce throughout summer (although *C. japonica* has been observed to spawn earlier in the year). They all occur at shallow depths up until October where they begin to migrate and overwinter in deeper waters (Jokinen et al. 1982; Jokinen 1992; David et al. 2017). The proliferation of these three snails in the Adirondack waterways may have resulted in the displacement of native gastropods, in a similar manner to what is occurring with invasive physid snails in South Australia (Zukowski and Walker 2009). Indeed, ongoing surveys across multiple rivers and lakes across the Adirondacks have recovered mainly non-native gastropod populations in the region with few native species (David and Cote, *unpubl. data*) compared to the late 1980s (Jokinen 1992). One possibility is that all three non-indigenous snails are exerting sympathetic interactions with each other, where each species facilitates the other's success to the detriment of native snails i.e. invasion meltdown (Simberloff and Von Holle 1999; Simberloff 2006). However, more field studies and experimental data will be needed to test such a hypothesis. In addition to their threat to native fauna, these species in particular, have also been known to harbor important parasites, including the human intestinal fluke *Echinostoma cinetorchis* (Chung and Jung 1999). A recent survey by David et al. (2017) found an unknown species of *Echinostoma* infecting *V. georgianus* in the Raquette River, indicating that the temperate climate may not necessarily be a barrier for these parasites.

In conclusion, this study has provided the first genetically confirmed record of *C. japonica* in the Adirondack Park and more importantly has confirmed that it is indeed present in NYS and occurs sympatrically with *C. chinensis*. The study reiterates the importance of using molecular data alongside traditional morphological analyses for more robust and comprehensive AIS detection surveys.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Locations, GPS coordinates and number of *C. japonica* and *C. chinensis* specimens collected at four (4) sites sampled in this study.
Table S2. Published CO1 sequence data used in phylogenetic analysis of *Cipangopaludina japonica* and *C. chinensis*.

This material is available as part of online article from:
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