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DNA Barcoding Evidence of the Tropical Invasive Lineage of *Sinanodonta woodiana* in Costa Rica

William G. Bauer¹, Donald T. Stewart^{1,*}, Rebeca Quesada Céspedes²,
Sidey Arias Valverde², and Russell H. Easy¹

Abstract - Chinese Pond Mussels (*Sinanodonta*) are invasive freshwater mussels (Bivalvia: Unionida) that are now widely distributed in non-native parts of Asia, Europe and, to a lesser extent, the Americas. In the present study, Chinese Pond Mussels were identified in a *cox1* DNA barcoding survey of mussels from an aquaculture facility in Guanacaste Province, Costa Rica. These sequences are identical or nearly identical to those of the so-called “tropical invasive lineage” of *Sinanodonta woodiana* (Lea), probably introduced on aquaculture fish from a hatchery in Taiwan. Identification of the source of these mussels has implications for understanding their ecological adaptations and natural history. To our knowledge there are no assessments of the ecological impacts of Chinese Pond Mussels in Mesoamerica.

Introduction

Several species of freshwater bivalves can be considered invasive, including some members of the order Unionida (Sousa et al. 2014). For some, including members of the *Sinanodonta woodiana* (Lea) (Chinese Pond Mussel) complex, invasions are often achieved through the parasitic glochidia larval stage. Chinese Pond Mussels are native to the Amur and Yangtze Rivers of China, and Eastern Russia (Kondakov et al. 2018, Soroka 2005, Sousa et al. 2014, Zieritz et al. 2018). Other native regions may include parts of Cambodia, Thailand, Malaysia, and Taiwan, although these claims have been disputed by some (Raley et al. 2011). Addressing taxonomic controversies regarding the *Sinanodonta woodiana* complex is beyond the scope of this paper and will require more expansive sampling of members of the genus throughout eastern Asia. Consequently, we use the term “Chinese Pond Mussel” to refer to any member of the *S. woodiana* complex. While it is possible members of this broader complex were introduced to Romania in 1959, the earliest documented record of Chinese Pond Mussels in non-native habitats is from Hungary in 1963 (Watters 1997). These mussels have been described as originating from the Temperate invasive lineage of the Chinese Pond Mussel complex (Kondakov et al. 2018), as opposed to the Tropical invasive lineage (see below). The Temperate invasive lineage now possesses an expansive range throughout Europe and are in non-native regions in Asia (Raley et al. 2011, Sárkány-Kiss et al. 2000). Similar trends of invasive dispersal of *Sinanodonta* mussels are being documented in Mesoamerica (e.g., Watters 1997), but these are understudied compared to the European invasion (e.g., Kondakov et al. 2018).

Sinanodonta species have been described as “hyper-successful” invaders (Sousa et al. 2014) and tend to outcompete native mussel species (Reichard et al. 2012). This is due to: (1) ability to survive in a broad range of freshwater conditions (Donrovich et al. 2017), (2) resilient glochidia that are host generalists (i.e., parasitize gills of most fish; Watters 1997), (3) relatively high growth and reproductive rates (Huber and Geist 2019), and (4) resistance to pollution and hypoxia (Sárkány-Kiss et al. 2000). When parasitized fish are introduced into non-native bodies of water, the parasitic mussels infesting them will also be introduced (Watters 1997).

¹Department of Biology, Acadia University, Wolfville, NS, Canada B4P 2R6. ²Estación de Biología Marina, Escuela de Ciencias Biológicas, Universidad Nacional de Costa Rica, Punta Arenas, Costa Rica 60101. *Corresponding author.

In addition to continuing to parasitize aquaculture species, glochidia can parasitize native fish (Donrovich et al. 2017), which subsequently distribute mussels across connected waterways. Studies in Europe have shown that once invasive populations are established, they can negatively affect the ecosystem by outcompeting native species for food or space, and changing the biotic composition and abiotic properties (e.g., Donrovich et al. 2017, Douda and Čadková 2018, Lopes-Lima et al. 2018, Reichard et al. 2015). These effects may be at play in Costa Rica, however, the impacts of Chinese Pond Mussels on Mesoamerican freshwater ecosystems have not yet been studied.

Keferl (1995) provided the first record of Chinese Pond Mussels' presence in Costa Rica (and Dominican Republic). Shortly after, Watters (1997) hypothesized that the introduction occurred when parasitized blue and Nile tilapias, *Oreochromis aureus* (Steindachner) and *Oreochromis niloticus* (Linnaeus), respectively, reared in Taiwan hatcheries, were imported to aquaculture facilities in Guanacaste Province. The account by Watters (1997) is supported by government documents recounting the introduction of tilapia into Mesoamerica through cooperation with the Agricultural Technical Mission of the Republic of China, Taiwan Province, in the 1960s (http://www.fao.org/fishery/countrysector/naso_costarica/en). This is similar to the hypothesized origin of European invasions of Chinese Pond Mussels *S. gibba* (Kondakov et al. 2018), in which parasitized carps from Yangtze River basin hatcheries were imported into aquaculture facilities in Hungary, Poland, etc. (Raley et al. 2011).

Bolotov et al. (2016), Vikhrev et al. (2017), and Kondakov et al. (2018) sequenced Chinese Pond Mussel samples (including various members of the broader species complex) and determined that those invading non-native parts of Asia (e.g., the island of Flores) were descended from the Malaysian Tropical invasive lineage of the Chinese Pond Mussel complex, possibly on fish coming from Taiwan hatcheries. Prior to the present study, knowledge of whether the Mesoamerican invaders are from the Tropical invasive lineage has yet to be confirmed. By sequencing the mitochondrially encoded cytochrome c oxidase subunit I (*cox1*) gene in a sample of Chinese Pond Mussels collected in Costa Rica, and comparing with those from Kondakov et al. (2018) in a phylogenetic context, we provide evidence that the Mesoamerican invaders are from this Tropical invasive lineage, consistent with Watters' hypothesis of a Taiwanese origin (Watters 1997).

Materials and Methods

Sample collection

On January 31st 2019, researchers from the Department of Biology at Acadia University, NS, Canada, and Estación de Biología Marina, Escuela de Ciencias Biológicas, Universidad Nacional de Costa Rica sampled six unionids that exhibited a consistent morphology (Fig. 1) in an aquaculture facility of the Universidad Técnica Nacional de Costa Rica, at 10°20'8" N, 85°9'19" W near Cañas, Guanacaste, Costa Rica. This was the same location reported in Cruz and Quesada (2017). Samples were transported to the Estación de Biología Marina in Puntarenas, Costa Rica for dissection, and labelled CR1 through CR6. Approximately 2 g of visceral mass tissue was dissected from each individual, immersed into 100% ethanol in 1.2 ml collection tubes, and stored in a 4 °C refrigerator until they were shipped to Acadia University for further processing.

DNA extraction

DNA extraction was performed according to the January 2011 Quick-Start Protocol of the QIAGEN DNEasy® Blood & Tissue Kit. DNA concentrations were quantified using a BioDrop DUO+ Micro-volume spectrophotometer.



Figure 1. Digital photos showing internal and external perspectives of *Sinanodonta woodiana* collected in January 2019 at our study site in Guanacaste, Costa Rica. Photos are used with permission of Oscar Pacheco Prieto.

Polymerase chain reaction (PCR) protocol

We amplified DNA fragments in a 50 μ L PCR reaction consisting of 25 μ L 2X Bio-Rad Master Mix (0.075 units per μ L *Taq* DNA polymerase, reaction buffer, 4 mM MgCl₂, 0.4 mM of each dNTP), 2 μ L of extracted DNA (~25 μ g/mL), and 2.5 μ L each of 10 μ M forward and reverse primers (18 μ L of ddH₂O for blank). We used universal *cox1* primers (*HCO2198*, *LCO1490*) designed for use in invertebrates (Folmer et al. 1994). Primer solutions (10 μ M) were prepared from 100 μ M stock solutions stored in a -80 °C freezer. The PCR protocol included an initial denaturing step at 96 °C for 10 min; followed by 40 cycles of 95 °C denaturation for 30 sec, 40 °C annealing for 60 sec, and 72 °C elongation for 60 sec; finishing with an extended 72 °C elongation for 7 min (variation of Breton et al. 2011).

Phylogenetic methods

Amplicons were sequenced at the McGill University and Génome Québec Innovation Centre. Chromatogram sequence data were analyzed and edited with MEGA 7.0.26 software (Kumar et al. 2016) to remove primer sequences and manually correct ambiguous nucleotide entries (N, etc.) where possible. After editing, each sequence was converted to FASTA format and aligned using the MUSCLE algorithm in MEGA 7.0.26. A BLASTn search was conducted using the standard database “nucleotide collection”, entering “*Bivalvia* (taxid:6544)” as the organism, and optimizing for highly similar sequences. The *cox1* sequences for samples CR1 to CR3 were submitted to GenBank (www.ncbi.nlm.nih.gov/genbank/) and assigned accession numbers MT484285–MT484287. We downloaded a subset of sequences representing the major clades in the phylogenetic tree published in Kondakov et al. (2018), including members of both the Tropical and Temperate invasive lineages, and used MEGA 7.0.26 to align them with the CR1–CR3 sequences. Using the model test option in MEGA 7.0.26, it was determined the Hasegawa-Kishino-Yano model with evolutionary invariable sites (HKY+I) was the best fit. The Kondakov et al. (2018) phylogenetic tree was reconstructed to include samples CR1 through CR3, using Maximum-Likelihood with HKY+I distances and 1000 bootstrap iterations (Fig. 2).

Results

BLASTing of *cox1* sequences for CR1 through CR3 revealed 99.85% to 100% identity (ID) and 100% query coverage to the female-transmitted mitochondrial DNA of the Tropical invasive lineage (Table 1; Kondakov et al. 2018). CR1 and CR3 were an identical match (100% ID) and CR2 was nearly identical (99.85% ID). In contrast, CR1/CR3 had a 94.67% ID, and CR2 a 94.52% ID, when compared to a sample from the Temperate invasive lineage associated with the Yangtze River basin (Table 1; Kondakov et al. 2018).

Table 1. GenBank accession numbers of edited *Sinanodonta woodiana cox1* sequences collected at our study site in Guanacaste, Costa Rica in January 2019. Sequences were compared (all E-values were 0.0) with those attributed to two separate invasive lineages of the *Sinanodonta* species complex: KU891641 and MF497809 are accession numbers of reference sequences for the Tropical and Temperate invasive lineages, respectively (see Table 1 in Kondakov et al. 2018).

Sample ID	<i>Cox1</i> Barcoding Accession No.	KU891641		MF497809	
		% ID	Query Cover	% ID	Query Cover
CR1	MT484285	100	100%	94.67	99%
CR2	MT484286	99.85	100%	94.52	99%
CR3	MT484287	100	100%	94.67	99%

The phylogenetic tree (Fig. 2) shows similar topology to that of Kondakov et al. (2018), Bolotov et al. (2016), and Vikhrev et al. (2017). Samples CR1 through CR3 are not divergent from DNA sequence KU891641 that is attributed to the Tropical invasive lineage (*Sinanodonta woodiana*; Fig. 2). In contrast, samples from the Temperate invasive lineage are 5.8% divergent (uncorrected p-distance) from samples CR1 through CR3 (Fig. 2).

Discussion

Herein, we provide molecular evidence consistent with the hypothesis of Watters (1997) that invasive Chinese Pond Mussels in Costa Rica descended from a strain of the Tropical invasive lineage of this complex of mussels, that probably originated from Tilapia fish imported from hatcheries in Taiwan. This appears to be the first analysis of invasive Chinese Pond Mussels in Mesoamerica since Watters (1997). The majority of articles on invasive Chinese Pond Mussels have focused on the impact of the species in Europe (which are derived from the Temperate invasive lineage, which may be a distinct species, *S. gibba* [Kondakov et al. 2018]) and non-native locales in Asia and Wallacea (which are derived from the Tropical invasive lineage of *S. woodiana*; Bolotov et al. 2016). Clearly there is a need for further research on the impacts of Chinese Pond Mussels on native freshwater mussel biodiversity and ecosystems generally in Costa Rica and surrounding regions of Mesoamerica.

Aquaculture facilities, such as the collection site for the present study, rear both non-native and native commercial fish for food markets and recreational angling, and are paramount to the tourism economy of Costa Rica (Peña and Chacón 2019). Glochidial infections are negatively impacting the health and economic value of fish being reared in these facilities (R. Quesada Céspedes and S. Arias Valverde, Estación de Biología Marina, Universidad Nacional de Costa Rica, Puntarenas, Costa Rica, pers. comm.; W. Vargas Carballo, Universidad Técnica Nacional, Cañas, Costa Rica, pers. comm, 2019). Plans to characterize and evaluate stress markers associated with glochidial infections of native and non-native fish using proteomic techniques, such as LC-MS to identify proteins of interest, have been delayed by travel restrictions associated with the COVID-19 pandemic of 2020.

As of May 2020, no concerted effort had been made to remove Chinese Pond Mussels from the collection site. Indeed, eradication of established invasive bivalve populations is extremely difficult (Sousa et al. 2014) and eradication methods (e.g., biocides) could have negative secondary effects on the ecosystem. Eradication may only be possible through extreme measures. For example, the New Jersey Conservation Foundation, upon detecting the presence of Chinese Pond Mussels, lowered water levels in affected fishponds and used rotenone to kill a broad array of animals, including invertebrates and many vertebrates, such as fish (Raley et al. 2011). To our knowledge, apart from this extreme measure, no protocols for removing Chinese Pond Mussels have been published.

The ecological consequences of the presence of *Sinanodonta woodiana* in Costa Rica could be considerable. Invasive Chinese Pond Mussels can outcompete native mussels by dominating in biomass and abundance (Dodd et al. 2005, Sárkány-Kiss et al. 2000). Their infective glochidia may prime fish immune systems before the glochidia of native species can attach (Donrovich et al. 2017) and glochidia from introduced freshwater mussel species can also induce a shared immunological resistance that prevents successful encysting of native glochidia (Donrovich et al. 2017). Chinese Pond Mussels may negatively affect the ecosystem by altering composition and concentration of particulate matter, microorganisms, heavy metals, etc. (Nichols and Garling 2000), by reducing reproduction of parasitized fish (Reichard et al. 2015), by delaying filtration of abiotic seston (Douda and Čadková 2018), and by decreasing population densities of bivalves within aquatic floor substrate (Sárkány-Kiss et al. 2000).

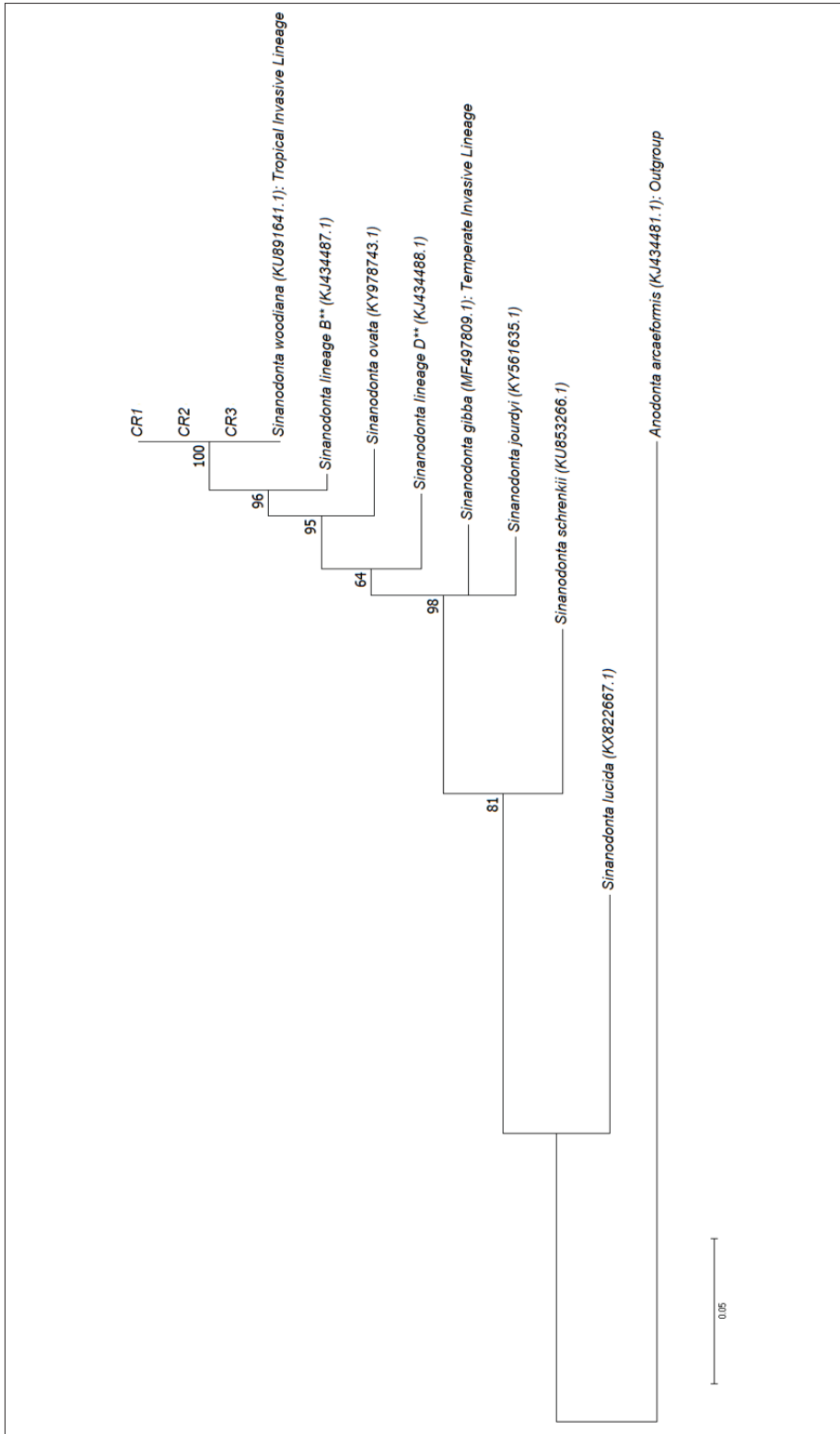


Figure 2. Phylogenetic reconstruction of major *Sinanodonta* lineages originally described by Kondakov et al. (2018) with *cox1* sequences from *Sinanodonta woodiana* individuals collected at our study site in Guanacaste, Costa Rica, in January 2019 (CR1–CR3 consensus). Bootstrap support values are placed to the upper left of nodes of interest.

Although Chinese Pond Mussels may be abundant at times, their presence is often not identified until control measures are needed (Sousa et al. 2014). Ecosystems with opportunities to fill a niche due to low species richness are at most risk of an invasion (Sousa et al. 2014). Mesoamerica contains a diverse array of unionids; however, most recent estimates are from surveys conducted from a single, relatively small area within an entire region (Pfeiffer et al. 2019a). Conversely, freshwater mussels may exhibit phenotypic plasticity depending on their local environment, so there is great potential for taxonomic classifications to overestimate or underestimate numbers of species (Sárkány-Kiss et al. 2000). Although our understanding of freshwater mussel systematics has improved in recent years (e.g., Pfeiffer et al. 2018; Pfeiffer et al. 2019a, b; Inoue et al. 2020), Mesoamerican mussel ecology has not received as much attention and remains largely unknown. Our study indicates that a better understanding of the role of invasive/non-native species is needed to improve management of the freshwater resources of Mesoamerica.

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