

Research Article

First record of a putative novel invasive *Corbicula* lineage discovered in the Illinois River, Illinois, USA

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Abstract

The Great Lakes watershed is one of the most invaded freshwater ecosystems, making early and rapid detection of new invaders critical to reduce their effects on this fragile system. The genus *Corbicula*, freshwater clams native to the temperate/tropical regions of Asia, Africa, and Australia, contains some of the most common and successful aquatic nuisance species in the New World. These invasive populations appear to exclusively comprise asexual clonal lineages, which hinder our taxonomic understanding. Previous work suggests three clonal morphotypes have invaded the New World—Forms A, B, and C, with Forms A and B co-occurring in North America. Here, we report on an apparently novel North American invasive *Corbicula* lineage recently discovered in the Illinois River, which has an artificial connection to the Great Lakes. This putative new morph was found co-occurring with previously described *Corbicula* morphotypes. Our main objective was to document the occurrence of this new morphotype (=Form D) and perform a preliminary analysis of its distinctiveness from sympatric Forms A and B using shell phenotype characteristics and mitochondrial (mt) and nuclear DNA markers. Results showed that the three co-occurring forms were distinguishable using shell phenotype and nuclear 28S ribosomal DNA sequences. Individuals were unambiguously assigned to one of three discrete shell phenotypes, Form A, B, or D, with Form D specimens characterized by fine rust colored rays and white nacre with purple teeth. Likewise, 28S genotypes identified three distinct morphs, with Form D differing from Forms A and B by 2–6 base pairs. In contrast, Form D individuals were distinguishable from Form B via mitochondrial markers but shared an identical mtDNA haplotype with sympatric Form A individuals. This latter result could stem from androgenetic capture of Form A eggs by invasive Form D sperm, a rare form of inheritance previously inferred for co-occurring North American *Corbicula* clones. Further morphological, ecological, and genomic analyses characterizing the three morphotypes are required to establish the significance of our preliminary findings.

Key words: invasion, species introduction, phylogenetics, Great Lakes, Mississippi River

Introduction

The Great Lakes watershed is one of the most altered freshwater ecosystems in the world with an estimated 186 established aquatic invasive species (hereafter AIS; Vander Zanden et al. 2010 and references therein). Many studies have documented the diverse effects of AIS introductions and establishment on native species (summarized by Holeck et al. 2004; Rothlisberger and Lodge 2013). Early and rapid detection of AIS is crucial to reduce and limit these

impacts (Vander Zanden et al. 2010). Here, we provide the first description of an apparent new morphotype and putative new lineage of the invasive Asian clam genus *Corbicula* Mühlfeld 1811 occurring in the Illinois River. The Illinois River is an important shipping route directly connecting the Great Lakes basin to the Mississippi River drainage via the Chicago Sanitary and Ship Canal (Figure 1). Thus, any new introduced species to this system have the potential to affect a wide geographic range. Because the new morphotype might be unique to the Illinois

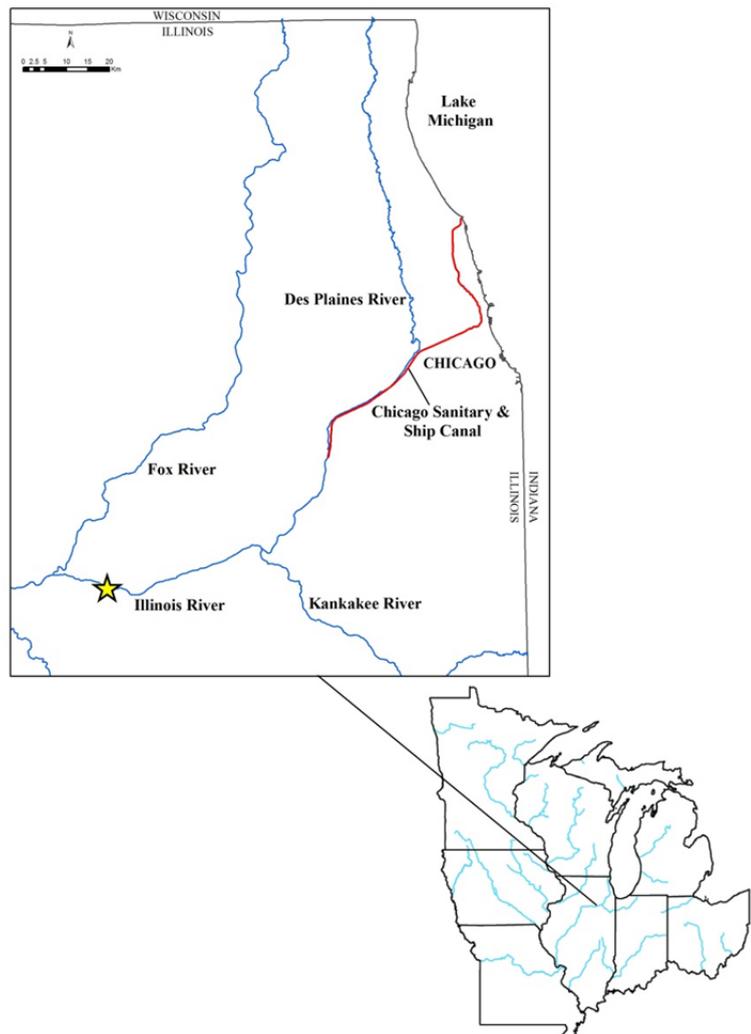


Figure 1. Location (star) of where the three *Corbicula* forms were collected in the Illinois River at the head of Clark's Island near Marseilles, Illinois, USA (41.31572°N; W 88.69191°W), on 15 October 2015. The Illinois River has been a pathway for invasive species exchange between the Great Lakes and Mississippi basins for decades via the Chicago Sanitary and Ship Canal (red line).

River, but may not be unique when considering a broader sample of *Corbicula* lineages, we retain the term putative to describe this lineage until a more extensive phylogenomic analysis can be completed.

The genus *Corbicula* consists of moderately-sized freshwater clams native to the temperate/tropical regions of Asia, Africa, and Australia (Morton 1986; Sousa et al. 2008; DAISIE 2014) and contains some of the most common and successful AIS (Pigneur et al. 2014a, b). The genus has both sexual and asexual forms (Lee et al. 2005 and references therein; Hedtke et al. 2011; Pigneur et al. 2011, 2014a). The known sexual forms are restricted to Asia whereas the invasive populations appear to be exclusively composed of asexual lineages. These clones, which have invaded freshwater ecosystems in North and South America and Europe, have become major aquatic

pests. Once established, they rapidly become the dominant benthic bivalve and often impede domestic and industrial water supply systems (Lee et al. 2005), alter nutrient regimes (Sousa et al. 2008), and affect food web dynamics (Cohen et al. 1984).

Most clonal lineages in nature are gynogenetic with the female nuclear genome being passed on to offspring (summarized by Schlupp 2005). In contrast, *Corbicula* clones reproduce by androgenesis where the maternal nuclear genome is extruded when the unreduced sperm fertilizes the egg, retaining only the unreduced paternal pronucleus (Konishi et al. 1998; Ishibashi et al. 2003; Lee et al. 2005; Hedtke et al. 2008; summarized by Pigneur et al. 2014a). The only maternal genetic signature that remains in asexually produced *Corbicula* clams is the mitochondrial genome of the egg. In addition to being

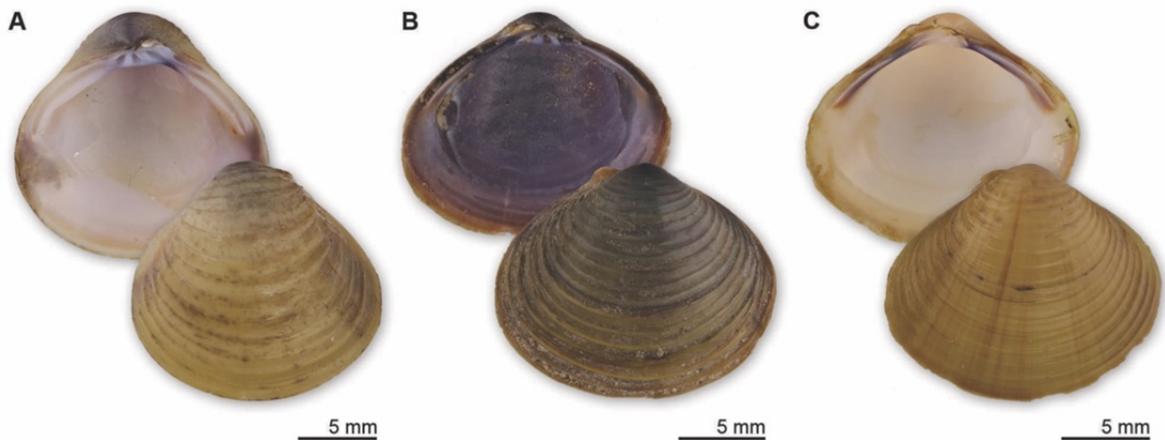


Figure 2. Three *Corbicula* forms collected from the Illinois River near Marseilles, Illinois, USA, on 15 October 2015, including (A) Form A, (B) Form B, and (C) Form D (photo by M.J. Thomas and D. Ruffatto).

clonal, the invasive lineages of *Corbicula* also are primarily hermaphroditic (Komaru et al. 2012) compared to their sexual counterparts that are dioecious. The presence of these characteristics means only a single asexual individual is required to establish a new population, likely aiding the overall invasion success. The success of *Corbicula* clams in new environments also can be related to other life history characteristics, such as rapid growth, high fecundity, short time to sexual maturity, and dispersal capability (Sousa et al. 2008).

Corbicula clonal lineages have been invading New World watersheds for almost a century. Their first documented appearance outside of the native range occurred in western North America in the 1920s followed by further expansion along the Pacific slope (McMahon 1982). During the 1950s, *Corbicula* clams breached the North American continental divide and then rapidly expanded across the eastern continental United States including the Gulf and Atlantic slope drainages (McMahon 1982). Since then, *Corbicula* clams have spread throughout North and much of South America ranging from the Great Lakes to Patagonia (Beasley 2003; Lee et al. 2005). Thus, early detection of *Corbicula* individuals may help to limit its environmental and economic effects, inform adaptive management, develop predictive invasion/dispersal models, and assess its potential effects on aquatic ecosystems (Hoagland 1986; Sousa et al. 2008; Vander Zanden et al. 2010).

Accurately identifying and delimiting species in the genus *Corbicula* is highly complicated due to the presence of clonal (asexual) forms of unknown

phylogenetic origin and obscure sister relationships within the genus (Lee et al. 2005; Pigneur et al. 2014a). The asexual lineages exhibit shallow genetic diversity and are broadly overlapping across invasive ranges (Pigneur et al. 2014a). Further complicating matters is the capability of the asexual androgenetic *Corbicula* lineages to capture eggs of other clones, potentially replacing the maternal nuclear DNA of other co-occurring individuals and forms (Lee et al. 2005; Hedtke et al. 2011; Pigneur et al. 2014a). This can obscure accurate identification of lineage origins via mitochondrial markers. In addition, there is evidence that hybridization is possible among some of these clones with individuals possessing nuclear ribosomal sequences from two different morphotypes (e.g., *Corbicula* Form B individual having both Forms B and C nuclear sequences; Lee et al. 2005; Pigneur et al. 2014a).

The lack of taxonomic clarity, phylogenetic resolution, and presence of clonal lineages has resulted in considerable uncertainty in the literature regarding the number of distinct New World invading lineages and their respective taxonomic identities. Literature reports vary from an invasion of only a single species, *Corbicula fluminea* (Britton and Morton 1986), to invasions of multiple species, identified variously as *C. fluminea*, *C. fluminalis* (Hillis and Patton 1982), *C. largillierti* (Ituarte 1994), or *C. leana* (based upon a mitochondrial DNA sequence match; Siripattawan et al. 2000).

Currently, distinct morphotypes and genetic lineages of *Corbicula* are referred to as “Forms” based on earlier work by Britton and Morton (1986) who

designated and described two North American invasive freshwater morphotypes that they erroneously assumed were ecophenotypic: Form A – a light/yellowish morph, which is widespread and ranges from North to South America, and Form B – a purple morph, which occurs predominantly in the southwestern United States but appears to be expanding its range. Lee et al. (2005) then reported the existence of an additional morphotype in the New World based upon mitochondrial and nuclear ribosomal DNA sequence data - Form C, which occurs in South America.

In the Illinois River, Forms A and B co-occur (data from Illinois Natural History Survey Mollusk Collection [INHS], Champaign, Illinois, USA), with Form A first documented from the early 1970s (Thompson and Sparks 1977) and Form B being first collected in 2010 (Tiemann and Cummings, unpublished data). In keeping with the previously published body of work on *Corbicula* forms, we herein report on a potential fourth form from the New World and third to North America, “Form D,” which was first collected during a survey of the Illinois River by INHS on 15 October 2015 near Marseilles, Illinois, USA (Figure 1). The main objective of this preliminary study is to document the presence of the apparent new morphotype, Form D, and determine if it is distinguishable from co-occurring congeners, Forms A and B, with standard mitochondrial (mt) and nuclear genetic markers.

Methods

Three morphologically distinct *Corbicula* forms (Figure 2) were collected in the Illinois River at the head of Clark’s Island near Marseilles, Illinois, USA (Figure 1), on 15 October 2015. Over 200 individuals of each morph were collected together from this site. These individuals were the same approximate size (~25 mm) and collected in a side channel containing silted sand substrates. Subsamples of these individuals were placed in 95% EtOH and deposited in the INHS Mollusk Collection, Champaign and the University of Michigan Museum of Zoology Mollusk Division (UMMZ), Ann Arbor, Michigan, USA. Subsequent surveys in areas >5 km upstream and ~1 km downstream from this Marseilles site yielded only Forms A and B.

DNA extraction and amplification

Five individuals each of Form A (UMMZ305020), Form B (UMMZ305021), and putative Form D (UMMZ305022) sampled from the Illinois River were randomly selected for genetic analysis (total $N = 15$). DNA was extracted from mantle tissue

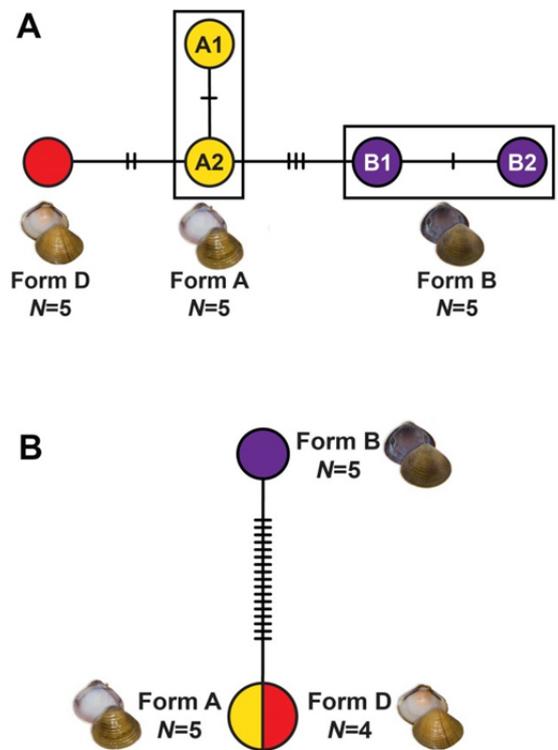


Figure 3. Genotype and haplotype networks showing the relationships among the three *Corbicula* morphotypes co-occurring in the Illinois River for (A) nuclear 28S ribosomal DNA and (B) mtDNA cytochrome oxidase *c* subunit I. Circle size is proportional to genotype/haplotype frequency. Hash marks indicate mutational steps between the genotypes/haplotypes.

using an E.Z.N.A. Mollusk DNA kit (Omega Bio-Tek, Norcross, GA) and followed manufacturer’s directions. Extractions were assayed for quality and quantity on 1% agarose mini-gels stained with ethidium bromide and DNA quantities were verified using a Thermo Scientific Nanodrop 2000 spectrophotometer (Waltham, MA, USA).

A 656 bp fragment of the mtDNA cytochrome oxidase *c* subunit I (COI) using the primers LCO1490 and HCO2198 (Folmer et al. 1994) and a 412 bp fragment of Domain 2 of the 28S fragment with the primers D23F and D6R (Park and Ó Foighil 2000) were amplified for each of the 15 individuals using the polymerase chain reaction (PCR). PCR reactions used 12.5 μ l of GoTaq Green master mix (Promega, Madison, WI), 0.5 μ M each of the forward and reverse primers, and at least 30 ng DNA template in a 25 μ l reaction. Each amplification run contained a negative (no DNA template) control. PCR reactions followed the touchdown protocol from Lee et al. (2005) for both COI and 28S.

A 3 µl aliquot of each PCR product was visualized on a 1% agarose mini-gel stained with ethidium bromide and successful reactions were purified using a Qiagen PCR Purification Kit (Qiagen, Valencia, CA). Purification results then were assessed on a mini-gel and using the Nanodrop. DNA sequencing was sent to the University of Michigan's DNA sequencing core (<https://seqcore.brcf.med.umich.edu/>), which used Applied Biosystems (ABI) Automated 3730xl DNA Analyzers (Fullerton, CA, USA).

Sequence analyses

The COI and 28S sequences for the putative three morphs were checked and aligned in MEGA v7 (Kumar et al. 2016) using the Progressive Method (Feng and Doolittle 1987) employed by CLUSTALW (Thompson et al. 1994) then compared to those previously reported and deposited in the National Center for Biotechnology Information's GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>). The genetic relationships of putative Form D in relation to co-occurring Forms A and B were determined using haplotype networks constructed with the Templeton, Crandall, and Sing (TCS) algorithm (Templeton et al. 1992; Clement et al. 2002) as implemented in the program PopART (Leigh et al. 2016).

Results

Shell phenotype

Individuals recovered from the study site could be unambiguously assigned to one of three discrete shell phenotypes (Figure 2). Two of those phenotypes corresponded to the previously described Forms A and B (Britton and Morton 1986). Shells of Form A are inflated and round to slightly pyramidal in shape with numerous concentric, evenly spaced ridges (6–10/cm); exterior is typically yellow or light to dark brown in color; nacre, which is highly polished distal to the pallial line, is characteristically white but may have suffusions of pink or light purple particularly around the teeth (Figure 2A). Form B is round (rarely pyramidal) with ridges that are more closely spaced and less elevated than in Form A; exterior is typically dark olive to brown in color and lacks a purple strip on the umbo; nacre and lateral teeth are both purple (Figure 2B). A more detailed analysis of the differences between Forms A and B can be found in Britton and Morton (1986). Finally, Form D is pyramidal in shape with weakly elevated ridges; exterior is yellowish-brown with fine rust colored rays radiating out from the umbo; nacre is creamy white and the lateral teeth are purple (Figure 2C).

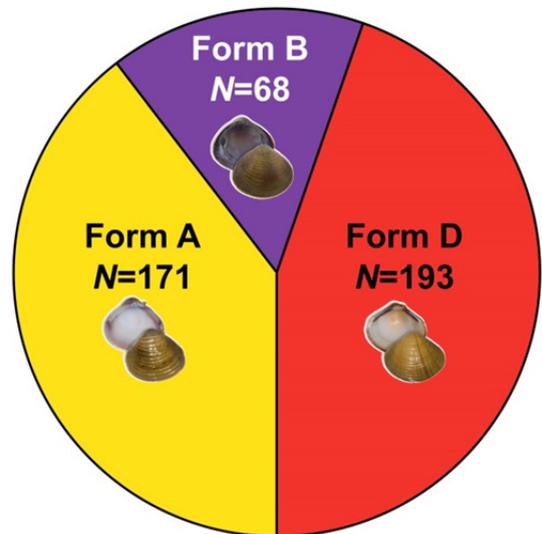


Figure 4. Pie chart showing the relative abundances of each of the three Illinois River sympatric morphotypes A, B, and D.

28S nuclear ribosomal DNA genotypes

All three sympatric forms exhibited distinctive nuclear ribosomal DNA genotypes (Figure 3A). The five Form D individuals produced an identical genotype that differed from co-occurring Form A by 2–3 bp and from co-occurring Form B by 5–6 bp (Figure 3A). All five Form A and five Form B individuals were heterozygous for a single base pair position resulting in two sequences per form (A1, A2; B1, B2) and the respective A-B form genotypes differed by 3–4 bp (Figure 3A). GenBank BLAST searches using the Illinois River genotypes found that genotype A1 for Form A was unique to our study (GenBank Accession Number KY436580) whereas A2, B1, and B2 had pre-existing matches for these respective forms (Supplementary material Table S1). Three matches were recovered for the Form D 28s genotype, each from cloned subsamples of heterozygous individuals: two Form B individuals from Texas, USA (GenBank #EU90417, 424; Hedtke et al. 2008) and a Form B-like specimen from Japan (GenBank #AB736295; Komaru et al. 2013; see Discussion for details).

mtDNA COI haplotypes

Two COI haplotypes differing by 17 bp were recovered from the 15 samples analyzed. One haplotype was diagnostic of Form B, and the other was shared by Forms A and D (see Figure 3B). These two haplotypes were identical to those previously identified from Form A and B individuals (Supplementary material Table S2).

Discussion

Our preliminary analyses recovered three different *Corbicula* lineages representing Forms A, B, and D occurring sympatrically in the Illinois River according to shell phenotype and nuclear 28S ribosomal genotype data. To our knowledge, the novel Form D shell phenotype (e.g. external rust colored rays and internal creamy white nacre with purple lateral teeth) has not previously been recorded in the Americas.

Previous descriptions of North American Forms A and B also relied on external characteristics for their identification, such as shell coloration, ridge structure, and shell shape as used here to classify Illinois River Forms A and B, and to describe putative Form D (see Results; Britton and Morton 1986). Interestingly, Britton and Morton (1986) reported some *Corbicula* individuals that possessed “radiating streaks of orange brown on various parts of the shell exterior” sampled from near Hong Kong in their native range. These individuals have not been included in any genetic analyses so it is unknown if they are genetically similar to our proposed Form D. Britton and Morton (1986) also noted that all of their sampled North American Form A specimens lacked these colored radiations. Thus, our samples appear to represent the first record of Form D in the New World.

The Form D nuclear 28S ribosomal DNA genotype differs from all previous New World invasive clones of *Corbicula* characterized using direct sequencing of the amplified target fragment that identified the predominant genotype present among the hundreds of copies per nucleus (Lee et al. 2005). However, GenBank BLAST searches revealed that it matches two of fourteen cloned genotypes (i.e., individual DNA sequences inserted into bacterial plasmids) obtained from two Form B individuals sampled in Texas (Hedtke et al. 2008) and one of three cloned genotypes obtained from a Form B-like individual in Japan (Komaru et al. 2012, 2013). The cloning of amplified gene fragments by these studies allowed characterization of both rare and predominant genotypes. It appears that this sequence is the predominant nuclear 28S genotype in the Illinois River Form D as it was recovered from multiple individuals with no evidence for heterozygous or paralogous nucleotide profiles. However, it is not exclusive to Form D, being present in low frequencies in at least some other invasive Form B populations (Hedtke et al. 2008; Komaru et al. 2013).

Our preliminary results also uncovered a pronounced mitochondrial disjunction where Form D individuals differed from sympatric Form A specimens in shell phenotype and nuclear 28S ribosomal genotype but

shared identical mtDNA genotypes. Such mtDNA disjunctions are now well known among co-occurring invasive New World *Corbicula* clones. Lee et al. (2005) suggested that this might have been the result of androgenetic capture by *Corbicula* sperm of another clone’s eggs (and the egg mitochondria), thus resulting in offspring with a mixed cyto/nuclear genetic signature. This phenomenon has also been inferred in other instances for invasive androgenetic *Corbicula* morphs occurring in both North America and Europe (Hedtke et al. 2008, 2011; Pigneur et al. 2014a).

We hypothesize that androgenetic capture of Form A eggs by invasive Form D sperm might explain the observed Illinois River mtDNA disjunction. However, we must emphasize the preliminary nature of our data and that much more extensive sampling and genotyping is needed to corroborate these initial results. If among-clone egg capture is restricted to the inferred pattern (Form D capturing Form A eggs), we predict that Form A’s capacity to reproduce (apart from its mitochondria) will be progressively compromised, possibly to the point where it could become locally extirpated. Surveys indicated that Form A and Form D individuals were present in approximately equal abundances (Figure 4), but it remains to be seen if this will continue in future years.

Currently, it is unknown how Form D arrived in the Illinois River. *Corbicula* clams typically colonize new areas via human mediation (Isom 1986; Mackie 2007). Once established, they can continue to spread via human vectors or passive dispersal, such as water currents, mucus thread droguing behavior in juveniles (Prezant and Chalermwat 1984), or intestinal passage through fishes (Isom 1986; Tiemann et al. 2011). Informal qualitative surveys of the Illinois River suggest this potentially new Form is currently restricted to a small (<5 km) area of river (data from INHS Mollusk Collection, Champaign). Given the speed of colonization of *Corbicula* individuals across continental watersheds, this new invader could be a substantial threat to watersheds in both the Mississippi River and Great Lakes basins if it were to spread. While there has been no specific study to determine negative effects of *Corbicula* in the Illinois River, previous studies elsewhere have demonstrated that the cumulative biomasses of the three morphs could interfere with native mussels (Strayer 1999; Cherry et al. 2005) and have negative effects on restoration efforts of threatened and endangered species. Also, *Corbicula* clams have been described as a hyper-invasive alien with substantial biofouling capabilities, particularly affecting complex power plant, irrigation canals, and drinking water

supplies (Isom 1986; Morton 1986). Infestations of *Corbicula* individuals have yielded billions of dollars (USD) annually in damage to industry and infrastructure (Pimentel et al. 2005).

Overall, our preliminary analysis indicates that the Illinois River Form D comprises a novel *Corbicula* lineage with a unique shell phenotype and nuclear 28S ribosomal genotype for this site. Its evolutionary and ecological origins and potential for spread are still obscure. This highlights the need for further study with more robust and finer methodologies, including a combined genomics and geometric morphometric assessment. Additionally, many of the previously described clonal lineages were reported to have biflagellate sperm compared to the sexual lineages possessing a single flagellum (Lee et al. 2005). Analyses are currently underway to further identify the morph, determine its clonality, and determine spread risk and potential effects on New World ecosystems. Accurate species delimitation and adaptive management development is critical in attenuating spread and, ultimately, minimizing the environmental and economic damage of this novel *Corbicula* form.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Comparison of nuclear 28S sequences from the 15 Illinois River *Corbicula* specimens to those found on GenBank and identified as Forms A or B including publication source.

Table S2. Comparison of mitochondrial cytochrome oxidase c subunit I (COI) sequences from the 15 Illinois River *Corbicula* specimens to those found on GenBank and identified as Form A or B including publication source.

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