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Research Article

Invasive virile crayfish (*Faxonius virilis* Hagen, 1870) hybridizes with native spothanded crayfish (*Faxonius punctimanus* Creaser, 1933) in the Current River watershed of Missouri, U.S.

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Abstract

Crayfishes are a diverse group of freshwater crustaceans which have proven to be harmful invasive species and are a leading cause of displacement of native crayfishes. Invasive species can harm populations of native species through hybridization which facilitates the displacement of native species and leads to a decrease in diversity. Hybridization has rarely been documented between crayfish species and the only genetically documented reports of North American crayfish species hybridizing have been between invasive rusty crayfish (*Faxonius rusticus* Girard, 1852) and two congeners. Virile crayfish (*Faxonius virilis* Hagen, 1870) is the most widely distributed crayfish in North America and occurs in its native range from the Hudson Bay watershed in Canada south to across the midwestern United States. *Faxonius virilis* has been introduced throughout North America and parts of Europe and is considered invasive in many locations. *Faxonius virilis* is invasive in the Ozark National Scenic Riverways (ONSR) in Missouri, U.S., and has been detected in the park since 1986. Setting out to document an updated range of *F. virilis* in the ONSR, we began developing a *F. virilis*-specific eDNA assay and noticed a discordance between the phenotype and mitochondrial DNA barcode of some native spothanded crayfish (*Faxonius punctimanus* Creaser, 1933) specimens. We compared mitochondrial, phenotypic, and microsatellite data and found that invasive *F. virilis* have hybridized with native *F. punctimanus* in the ONSR. Our research adds to the rarely documented occurrences of crayfish hybridization and supports previous researchers' remarks that undocumented hybridization between native and non-native crayfish may be more common than previously thought. While the invasion of *F. virilis* has had a genetic impact on a native crayfish, the long-term evolutionary consequences are unknown.

Key words: invasive species, hybridization, crayfishes, streams, freshwater, Cambaridae

Introduction

The accidental or intentional transport, establishment, and spread of non-indigenous species has altered ecosystem function and resulted in a decline in biodiversity around the globe. While many species have been translocated, most fail to establish or have minimal impacts (Havel et al. 2015; Thomaz et al. 2015). However, invasive species are the few that successfully establish

and cause considerable ecological disruption (Parker et al. 1999). One impact that invasive species can have on native species is hybridization (Lodge et al. 2000). Hybridization occurs when non-native species are introduced into the range of a closely related native species and they retain the ability to mate and reproduce (Harrison and Larson 2014). Hybridization where sterile offspring are produced may have little effect on populations and displacement of species (Arnold et al. 1999; Huxel 1999), although the wasted reproductive effort and decline in fitness can cause populations to decline (Todesco et al. 2016). However, fertile offspring and resulting introgression, or the incorporation of genes from one species into another with subsequent hybridization and backcrossing (Harrison and Larson 2014), can facilitate the displacement of a native species and decreases diversity (Huxel 1999; Wolf et al. 2001). Rare species can be particularly impacted through hybridization and introgression with non-native species (Rhymer and Simberloff 1996) and extirpation or extinction can occur rapidly (Wolf et al. 2001). Additionally, hybridization can significantly affect the establishment success and subsequent impact of invasive species by increasing their fitness and adaptation to new environments (Hänfling 2007).

Crayfishes are a diverse group of organisms that are comprised of widespread, rare, and narrowly endemic taxa, as well as some harmful invasive species (Taylor et al. 2019). Invasive crayfish alter habitat and water chemistry by reducing macrophytes (Olsen et al. 1991; van der Wal et al. 2013) and increasing turbidity (Matsuzaki et al. 2009). Furthermore, invasive crayfish reduce populations of macroinvertebrates (Nilsson et al. 2011) and fish (Olsen et al. 1991; Mueller et al. 2006) and displace native crayfishes (Lodge et al. 2000). Due to this displacement, invasive crayfish have been identified as a primary threat to narrow-ranging endemic crayfish species (Lodge et al. 2000). While invasive crayfish and their effects on ecosystems are well studied, evolutionary effects of these invasions are understudied.

Although known cases of hybridization between crayfishes have been infrequent, the possible impacts of invasives was elevated when hybridization between native and non-native crayfishes was identified as a possible displacement mechanism by Perry et al. (2001a). Initially, literature on crayfish hybridization pertained strictly to morphological evidence and identified specimens that exhibited morphologically intermediate characters (Crocker 1957; Boyd and Page 1978; Capelli and Capelli 1980; Smith 1981). Induced hybridization between crayfish has also been documented (Berrill 1985; Lawrence et al. 2000). An early account of hybridization utilizing genetic techniques documented natural hybridization between two native cave dwelling *Procambarus* spp. in Mexico (Cesaroni et al. 1992). Subsequently, genetic methods have documented hybridization between the highly invasive rusty crayfish (*Faxonius rusticus* Girard, 1852) and two other species, the northern clearwater crayfish (*Faxonius propinquus* Girard, 1852) and Sanborn's crayfish (*Faxonius sanbornii* Faxon, 1884). Perry et al.

(2001b) reported that *F. rusticus* hybridized with *F. propinquus* in upper Wisconsin, U.S., lakes. Morphologically intermediate individuals were recognized, and hybridization was confirmed through allozyme analysis. Hybridization between *F. rusticus* and *F. propinquus* was shown to hasten the demise of *F. propinquus* genotypes, as the hybrids were fertile and backcrossed with *F. rusticus* (Perry et al. 2001a; Arcella et al. 2014). Zuber et al. (2012) documented invasive *F. rusticus* hybridizing with the native species *F. sanbornii* in Ohio (U.S.), using nuclear and mitochondrial DNA and allozymes.

While examining the distribution of the invasive virile crayfish (*Faxonius virilis* Hagen, 1870) in the Current River of Missouri, United States (U.S.), we noticed a mismatch between phenotype and mitochondrial DNA (mtDNA) between two species. Our initial observations indicated that crayfish with phenotypic life coloration of the native spothanded crayfish (*Faxonius punctimanus* Creaser, 1933) had mtDNA sequences matching invasive *F. virilis*. Noting this mismatch, we hypothesized that *F. punctimanus* and *F. virilis* were hybridizing. *Faxonius virilis* is the widest ranging crayfish in North America (Taylor et al. 2015) and is a successful invader in many parts of the U.S. (Kilian et al. 2010; Moody and Sabo 2013) and Europe (Ahern et al. 2008; Filipová et al. 2010). The species has greatly expanded its range in the Current River since it was first documented in 1986 (Pflieger 1996). We add to the limited literature on invasive and native crayfish hybridization, which has implications for research in crayfish phylogeny as well as understanding of impacts of invasive crayfish.

Materials and methods

Study location

The Ozark National Scenic Riverways (ONSR) is administered by the U.S. National Park Service and encompasses over 200 kilometers of the free-flowing Jacks Fork and Current River. Situated in the Ozark Mountains in south-central Missouri, the park is dominated by karst topography and contains more than 425 springs which supply much of the baseflow to the Jacks Fork and Current River (Bowles and Dodd 2015). The watershed is primarily rural and contains hills covered in mixed pine (*Pinus* spp.) and oak (*Quercus* spp.) woodlands. The upper Current River and Jacks Fork are home to five native crayfish species: golden crayfish (*Faxonius luteus* Creaser, 1933), Ozark crayfish (*Faxonius ozarkae* Williams, 1952), spothanded crayfish (*F. punctimanus*), Hubbs' crayfish (*Cambarus hubbsi* Creaser, 1931), and Salem Cave crayfish (*Cambarus hubrichti* Hobbs, 1952; Pflieger 1996). The *Faxonius* species and *C. hubbsi* are stream dwellers, while *C. hubrichti* is a cave-dweller and is not typically found in surface waters. In addition to the native species, *F. virilis* has been introduced to the Current River and was first detected in 1986 near present-day Pulltite Canoe Access (Pflieger 1996).

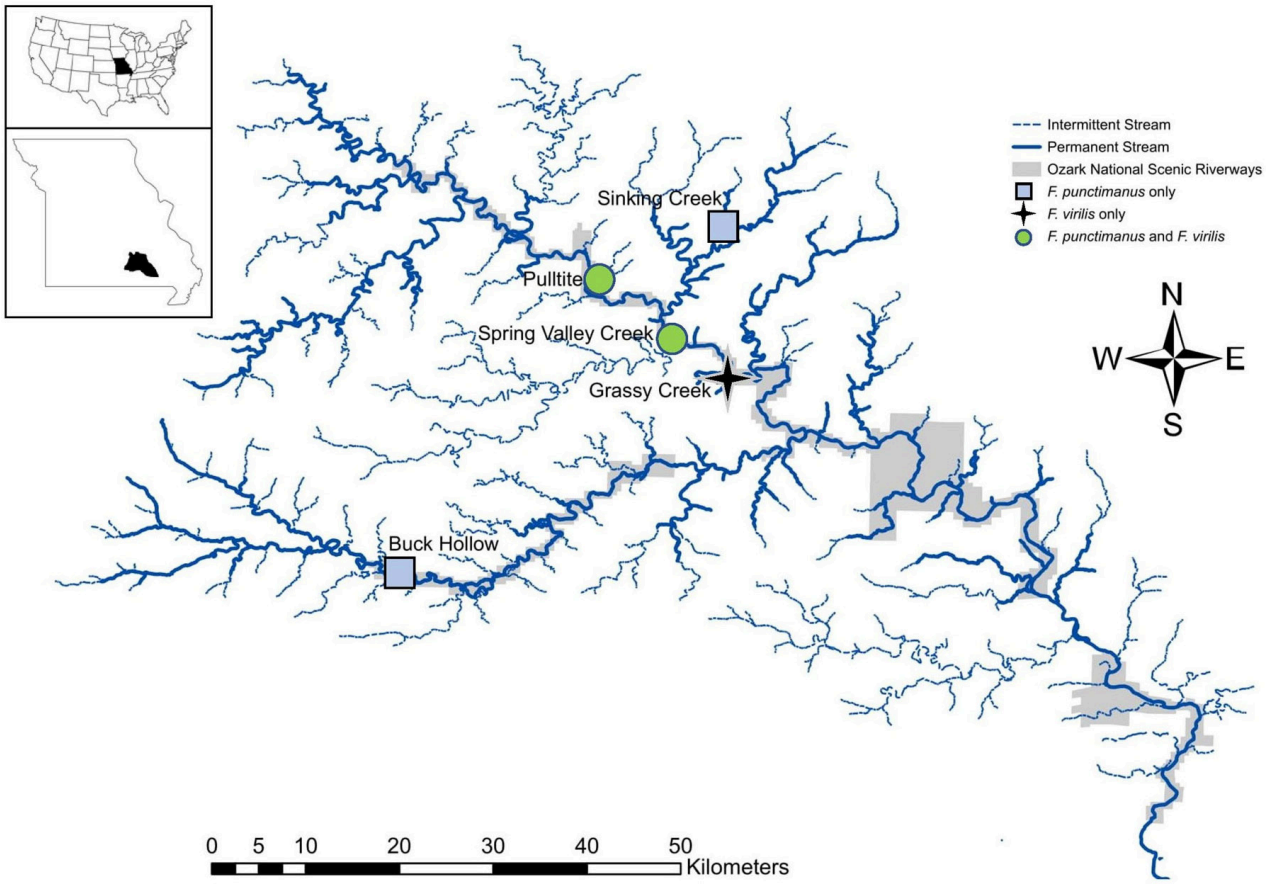

Figure 1. Collection sites of crayfishes used in initial microsatellite, COI, and phenotype analyses in the Current River watershed, Missouri, U.S. Squares represent sites where only *Faxonius punctimanus* was collected. Circles represent sites where *Faxonius virilis* was collected with sympatric *F. punctimanus*. The cross symbol represents a site where only *F. virilis* was collected. All identifications were based on phenotypic coloration. Inset maps display location of Missouri within the United States and the location of the Current River watershed within Missouri.

Preliminary collection sites and methods

Faxonius virilis and sympatric species were originally collected to aid in the development of an environmental DNA (eDNA) assay for *F. virilis* (e.g., Larson et al. 2017). Between 2018 and 2019, crayfish were collected from three sites within the Current River and tributaries within the ONSR and two Current River drainage sites outside of the ONSR (Figure 1). All crayfish were collected by kick-seining where a 3.3 m seine net was placed in the stream and rocks and substrate immediately upstream were disturbed, allowing the current to carry dislodged crayfish into the net. Specimens were initially identified using phenotypic coloration and placed in a bucket. They were subsequently kept alive for tissue removal and photographs or placed in 99% ethanol and returned to the laboratory.

Because it was a previously known location for the non-native species, we collected *F. virilis* and *F. punctimanus* from Spring Valley Creek on 7 March 2018 and 23 May 2018. *Faxonius virilis* and *F. punctimanus* were collected from Pulltite Access on the Current River on 7 March 2018 and *F. punctimanus* was collected from Sinking Creek on 7 March 2018. Mitochondrial DNA sequencing of these Spring Valley Creek specimens

Figure 2. Two 5 cm crayfish collected in the same seine haul from an unnamed tributary (common location "Huckleberry Hollow") of the Current River in Missouri, U.S. Crayfish (A.) exhibits a color pattern consistent with *Faxonius virilis* and crayfish (B.) exhibits a color pattern of *Faxonius punctimanus*. Both crayfish (A.) and (B.) possessed the *F. punctimanus* COI sequence genotype.

identified the potential hybridization that motivated our subsequent efforts. Additional *F. punctimanus* were then collected from Spring Valley Creek on 13 June 2019. We collected additional *F. virilis* specimens from Grassy Creek on 10 July 2019. We also collected additional *F. punctimanus* from one allopatric site > 50 kilometers from the nearest known *F. virilis* population on 10 July 2019 (Jacks Fork at Buck Hollow Access; Figure 1).

Phenotype identification

Faxonius virilis and *F. punctimanus* are nearly indistinguishable morphologically with only slight differences in Form I male gonopod characteristics (Pflieger 1996), so we relied on life coloration to distinguish these species. *Faxonius virilis* and *F. punctimanus* in the Current River watershed have several key phenotypic color differences that distinguish them (Figure 2). *Faxonius punctimanus* has a distinct black spot with smooth

edges on at the base of the moveable finger on the dorsal surface of the chela, a small dark spot at the base of moveable finger on the ventral surface of the chela, a pronounced dark saddle across the posterior of the cephalothorax that extends anteriorly on the lateral carapace surfaces, and lacks larger irregular patches on dorsal surface of each abdominal segment. *Faxonius virilis* lacks a distinct black spot with smooth edges at the base of the dorsal surface of the moveable finger, a dark spot at the base of the moveable finger on the ventral surface, and a pronounced dark saddle across the posterior of the cephalothorax that extends anteriorly on the lateral carapace surfaces. *Faxonius virilis* also usually has a stronger mottled pattern of small irregular spots on the dorsal surface of the chela and larger paired irregular shaped patches on the dorsal surface of each abdominal segment.

Cytochrome c oxidase subunit I (COI) sequencing

We amplified the cytochrome *c* oxidase subunit I (COI) gene for each crayfish specimen. A 15 mg muscle tissue sample was extracted from the abdomen of each crayfish specimen and preserved in 99% ethanol. The crayfish specimens were then individually tagged, preserved in 70% ethanol, and accessioned into the Illinois Natural History Survey's Crustacean Collection (Champaign, IL, U.S.). DNA was isolated from the muscle samples using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's spin column protocol for animal tissues. We amplified COI on a PTC-100 thermal cycler (MJ Research, Inc., Watertown, Massachusetts, U.S.) using 1 µl of LCO1490 primer (5'-GGTCAACAAATCATAAAGATATTGG-3'), 1 µl of HCO2198 primer (5'-TAAACTTCAGGGTGACCAAAAAATCA-3'; Folmer et al. 1994), 21 µl of water, 2 µl of template DNA, and PuReTaq Ready-To-Go PCR beads (GE Healthcare, Buckinghamshire, UK). The thermal profile for the PCR reactions was 94 °C for 3 min, 45 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 90 seconds, followed by a final 72 °C extension. PCR products were cleaned using a QIAquick PCR Purification Kit (Qiagen). Sanger sequencing was conducted by the University of Illinois at Urbana-Champaign Core Sequencing Facility (Urbana, IL, U.S.). Forward and reverse sequence reads were analyzed and edited using Sequencher 5.4 (Gene Codes Corporation, Ann Arbor, Michigan, U.S.) to obtain the final COI consensus sequences which were deposited in GenBank (National Center for Biotechnology Information).

Microsatellite sequencing

We examined nine microsatellite loci primers developed for *F. virilis* populations in Canada by Williams et al. (2010) and found that eight were informative for both *F. virilis* and *F. punctimanus* in the Current River watershed (Table 1). To ensure that these primers worked with the Current River population of *F. virilis* and *F. punctimanus*, we first ordered and tested

Table 1. Microsatellite primer loci and sequences from Williams et al. 2010 that amplified for both *F. virilis* and *F. punctimanus* from the Current River watershed in Missouri, U.S. NED, FAM, VIC, and PET are fluorescent dyes (Applied Biosystems, Foster City, California, U.S.).

Locus	Primer Sequence (5'-3')
Ov3	*NED*-AGTCTTCAACCACCGTCACCCTTGTCCCACAGTCCTGCTCAAATGA
Ov5	*FAM*-GCCCTTCCTCTCTTCCTGTTCTGGTTGATGTCTCCTCCTAGCAAC
Ov6	*VIC*-CGGCTGGCGTATGAGAGTCACAGTGTCGGCCCCGCTTAATGT
Ov15	*PET*-AGTGTGCAGACACATGGTGAGGACTGTATTTTTCTTGCCCCCTTC
Ov24	*VIC*-TCACCCCCTTCGTTTCGTTATGCGCCTTGTCTTAGTCCGTCTCA
Ov34	*PET*-CTGTAGTGTTTCATGCGTCAATGAAAGCAACTCAATTCCA
Ov5-07	*FAM*-GCAAGCACAAATAGGTGAGTTTCTCTTCCTGGGACATACC
Ov5-73	*PET*-ACAACCTGCATTTACCCTCTCTGGGAAACAAAAACATCAA

unlabeled forward and reverse primers (Integrated DNA Technologies, Coralville, Iowa, U.S). Primers were tested using the thermal profile of 94 °C for 1 minute, 3 cycles of 94 °C for 30 seconds, 48 °C or 52 °C for 20 seconds, and 72 °C for 1 second, followed by 33 cycles of 94 °C for 15 seconds, 52 °C for 20 seconds, and 72 °C for 1 second, and a final 72 °C extension. PCR amplifications were performed in 20 µl reactions consisting of 1 µl each of forward and reverse primers, 10 µl of GoTaq® G2 Colorless Master Mix (Promega Corporation, Madison, Wisconsin, U.S.), 5 µl of water, and 3 µl of template DNA. Products were run on a 2% agarose gel for confirmation of amplification.

Final PCR amplifications were performed in 10 µl reactions consisting of 5 µl of GoTaq® G2 Colorless Master Mix, 0.25 µl each of fluorescently-labelled forward primers (Applied Biosystems, Foster City, California, U.S.) used in Williams et al. (2010), unlabeled-reverse primers, 3 µl water, and 1.5 µl of DNA template. The thermal profile for PCR was the same as during the microsatellite primers test. PCR reactions were performed individually, and products were combined for fragment analysis. Combined products were coloaded with GS600LIZ size standard (Applied Biosystems) and analyzed on ABI 3730 DNA Analyzer (Applied Biosystems) at the University of Illinois at Urbana-Champaign Core Sequencing Facility (Urbana, IL). Fragments were genotyped using Genemapper® version 4.0 (Applied Biosystems).

Hybridization analyses

To visualize the differences and mismatches between phenotype identification, COI sequences, and microsatellite fragment data, we constructed a neighbor joining tree for COI sequences, UPGMA dendrogram for microsatellite fragment data, and a principal coordinate analysis (PCoA) displaying microsatellite fragment data, COI, and phenotype identifications. To visualize differentiation between *F. virilis* and *F. punctimanus* COI groups, we constructed a bootstrapped (2000 iterations) neighbor joining tree in Mega X (Kumar et al. 2018). Outgroups on the neighbor joining tree were *F. ozarkae* and *F. luteus* that we collected from Sinking Creek and Spring Valley Creek of the Current River watershed respectively and *C. hubbsi*

that we acquired from GenBank (GenBank accession #MG872957.1). Maximum likelihood and maximum parsimony trees recovered the same relationships as the neighbor joining tree. Microsatellite Analyzer version 4.05 (Dieringer and Schlötterer 2003) was used to calculate Nei's distance for the microsatellite fragment data and Mega X (Kumar et al. 2018) was used to generate a UPGMA dendrogram based on the Nei's genetic distance matrix. Outgroups were not added to the UPGMA dendrogram as it is unlikely that the microsatellite primers would amplify more distantly related species. Based on the microsatellite data, a PCoA based on pairwise F_{ST} values was generated in Microsoft Excel using the add-on GenAlEx 6.5 (Peakall and Smouse 2012) to further visualize differences between the *F. virilis* and *F. punctimanus* populations.

Investigating the extent of hybrids

To display how widespread the hybridization effects were in the Current River watershed, we COI sequenced phenotypic *F. virilis* specimens that were collected across a wider extent of the Current River watershed. COI sequencing of crayfish identified as *F. virilis* by phenotype using the above described method was sufficient to map prevalence of hybrids because the results from the above analyses indicated that phenotype and microsatellite data aligned, but COI data was discordant in certain specimens indicating hybridization. Further, introgression of *F. punctimanus* COI genes into phenotypic *F. virilis* was more common than the inverse. To choose our collection sites, we utilized ArcGIS Pro (Esri, Redlands, California, U.S.) to identify mainstem Current River and tributary sites from the National Hydrography Dataset Plus Version 2 (NHDPlusV2) data (McKay et al. 2012) in and adjacent to the Ozark National Scenic Riverways. Tributaries with a Strahler (1952) stream order of 1 were eliminated as their low discharge would likely lead to complete drying during our summer field season and *F. virilis* has shown an intolerance to drying (Bovbjerg 1970). Additionally, second order and greater tributary streams were eliminated if they had a length less than 0.5 kilometers. The resulting 40 tributary sites were selected for sampling, and 30 with suitable access and wadeable water depths were sampled. At the local site scale, sampling reaches were chosen approximately 100 meters up each tributary from its mouth or until a riffle/run/pool sequence was located. Tributary sites were accessed via car when possible and remaining sites were accessed via canoe.

To choose sites on the mainstem of the Current River, the sample function in R v3.4.3 (R Core Team 2018) was used to randomly select sampling sites from a group of 207 stream segments from NHDPlusV2 (i.e., between tributary confluences; Sowa et al. 2007; McKay et al. 2012) between the headwaters of the Current River and Van Buren, Missouri. We imposed a three river kilometers buffer between selected segments resulting in 13 segments being selected. Subsequently, one segment was eliminated due

to the potential presence of a federally endangered species (communication with the National Park Service), leaving sampling 12 segments. An approximate middle point of each randomly selected segment was chosen for mainstem sites on the Current River but was adjusted downstream until the conditions permitted wading and were shallow enough for us to sample via seining. Mainstem Current River sites were accessed via car at one site and remaining sites were accessed via canoe.

We collected phenotypic *F. virilis* between 10 June and 19 July 2019 at the above sites using kick-seining where a 3.3 m seine net was placed in the stream and rocks and substrate immediately upstream were disturbed (Larson and Olden 2016). We amplified a portion of the cytochrome *c* oxidase subunit I (COI) gene for up to ten vouchered phenotypic *F. virilis* specimens > 5 cm in length per site. The COI sequence for each specimen was matched to either *F. virilis* or *F. punctimanus* and the percentage of sequenced specimens per site containing *F. virilis* COI was quantified.

Results

A microsatellite UPGMA dendrogram recovered two main clades that aligned with phenotypic species assignments, but mismatched COI assignments on some specimens suggest that these individuals are hybrids. We found that the COI neighbor-joining tree displayed separate clades for *F. virilis* and *F. punctimanus* with 100% bootstrap support (Figure 3). However, nine specimens were mismatched with their phenotype assignments not aligning with the COI groupings. Seven specimens identified as phenotypic *F. virilis* group instead with *F. punctimanus* and two specimens identified as phenotypic *F. punctimanus* group with *F. virilis*. Table 2 displays all specimens used in the analyses and shows the phenotype and COI assignments. The UPGMA dendrogram generated from microsatellite data recovers two main clades that separate at the first node and align with phenotype identifications in all but one instance (Figure 4). The only specimen that did not align was SVC003 which exhibited a COI sequence and phenotype consistent with *F. punctimanus* but is recovered with *F. virilis* in the UPGMA dendrogram. The nine specimens that have matching phenotype and microsatellite assignments, but discordant COI groupings, are labelled as hybrids.

The PCoA (Figure 5) visualizes variance in the microsatellite distance data and shows a separation between the two species. The first two axes explained 27.9% of the total variance, with 17.4% explained by Axis 1 and 10.5% explained by Axis 2. *Faxonius virilis* phenotypes, including the seven hybrid specimens with discordant COI, load positively on the dominant axis one, whereas *F. punctimanus* phenotypes, including the two hybrid specimens with discordant COI, load negatively on the dominant axis one. Convex hulls bound the monophyletic *F. virilis* or *F. punctimanus* as identified by the UPGMA dendrogram (Figure 4).

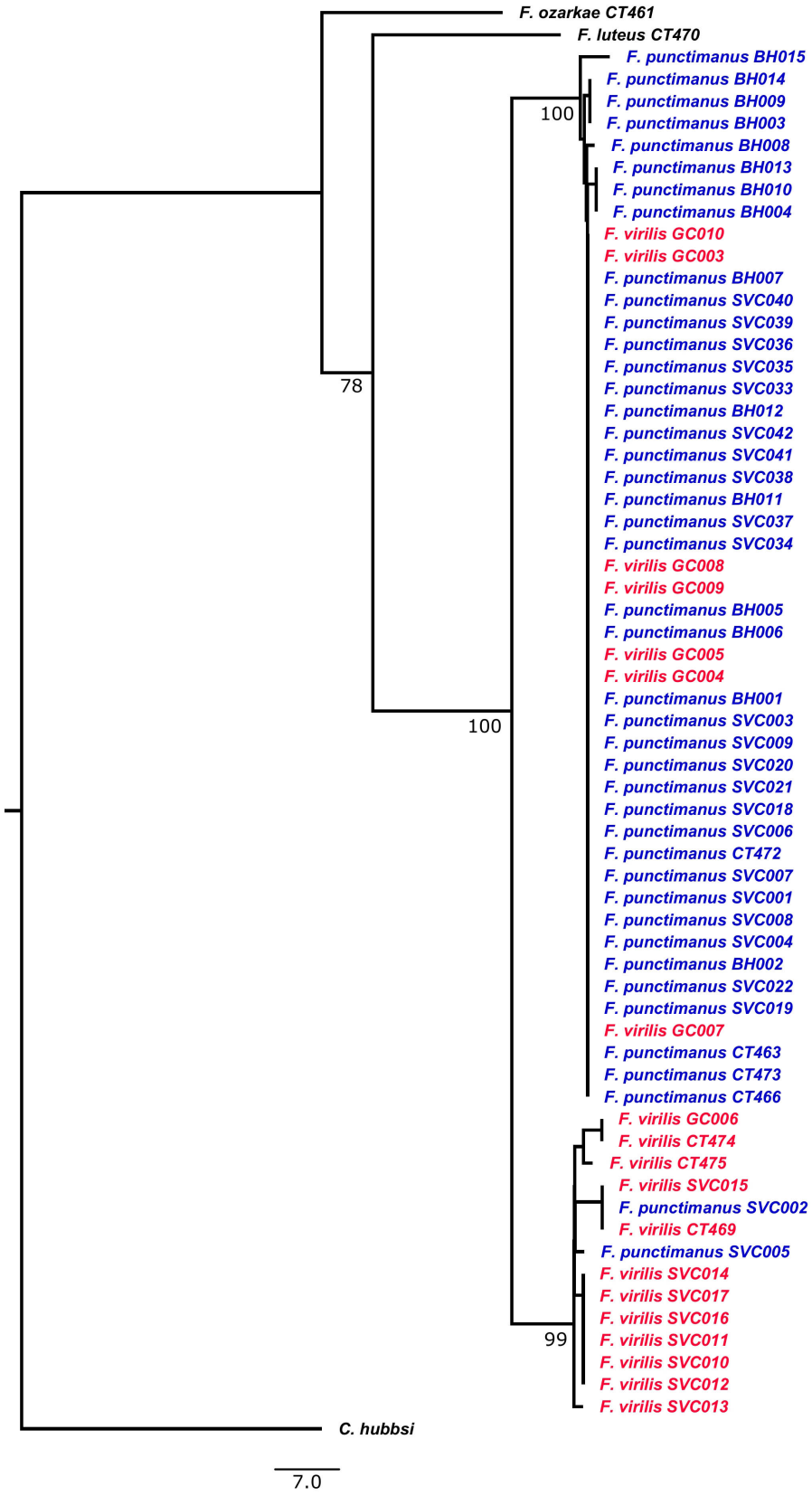

Figure 3. A neighbor-joining tree generated using the COI sequences of *Faxonius virilis* and *Faxonius punctimanus* collected from the Current River watershed in Missouri, U.S. Bootstrap values (2000 iterations) are displayed at major nodes. Species assignment is based on the phenotype and the specimen number is displayed (Table 2). *Faxonius virilis* is displayed in red and *F. punctimanus* is displayed in blue.

Table 2. All crayfish specimens that were used in the microsatellite, COI, and phenotype analyses. Specimens with the dagger symbol (†) have COI sequences that do not match the indicated phenotype. The asterisk next to the specimen code indicates taxa (*Cambarus hubbsi*, *Faxonius ozarkae*, and *F. luteus*) used for outgroups in the COI tree. All specimens were collected from the Ozark National Scenic Riverways except for *C. hubbsi*. COI data for *C. hubbsi* was obtained from GenBank (National Center for Biotechnology Information).

	Code	Species (Phenotype)	Location	Coll. Date	GenBank Accession #
*	*C. hubbsi*	*C. hubbsi*			MG872957.1
*	CT461	*F. ozarkae*	Sinking Creek	7-Mar-18	MZ570509
	CT463	*F. punctimanus*	Spring Valley Creek	7-Mar-18	MZ570510
	CT466	*F. punctimanus*	Pulltite	7-Mar-18	MZ570511
	CT469	*F. virilis*	Pulltite	7-Mar-18	MZ570512
*	CT470	*F. luteus*	Spring Valley Creek	7-Mar-18	MZ570513
	CT472, CT473	*F. punctimanus*	Sinking Creek	7-Mar-18	MZ570514, MZ570515
	CT474, CT475	*F. virilis*	Spring Valley Creek	7-Mar-18	MZ570516, MZ570517
	BH001-BH015	*F. punctimanus*	Jacks Fork - Buck Hollow	10-Jul-19	MZ570494–MZ570508
	GC003-GC005, GC007-GC010	*F. virilis* †	Grassy Creek	10-Jul-19	MZ0570518–MZ0570520, MZ0570522–MZ0570525
	GC006	*F. virilis*	Grassy Creek	10-Jul-19	MZ0570521
	SVC001, SVC003, SVC004, SVC007-SVC009	*F. punctimanus*	Spring Valley Creek	23-May-18	MZ0570526, MZ0570528, MZ0570529, MZ0570532–MZ0570534
	SVC002	*F. punctimanus* †	Spring Valley Creek	23-May-18	MZ0570527
	SVC005	*F. punctimanus* †	Spring Valley Creek	23-May-18	MZ0570530
	SVC006	*F. punctimanus*	Spring Valley Creek	23-May-18	MZ0570531
	SVC010-SVC017	*F. virilis*	Spring Valley Creek	23-May-18	MZ0570535–MZ0570542
	SVC018-SVC022	*F. punctimanus*	Spring Valley Creek	23-May-18	MZ0570543–MZ0570547
	SVC033-SVC042	*F. punctimanus*	Spring Valley Creek	12-Jun-19	MZ0570548–MZ0570557

Hybrid crayfish specimens were collected throughout the range of phenotypic *F. virilis* in the Current River watershed. We COI sequenced *F. virilis* vouchered specimens from the 26 sites where they were collected, as introgression of *F. punctimanus* COI genes into phenotypic *F. virilis* was more common than the inverse. Phenotypic *F. virilis* individuals without concordant COI sequences were labelled as hybrids. Of the 74 vouchered and sequenced *F. virilis* phenotype specimens, 29 (39%) contained COI sequences matching *F. virilis* and 45 (61%) sequence matched *F. punctimanus*. Phenotypic *F. virilis* is present in at least 117 km of the Current River and was collected from Pigeon Creek in Montauk State Park to unnamed tributary "Huckleberry Hollow". Hybrid *F. virilis* with *F. punctimanus* COI assignments were found throughout this range, from Caps Branch to "Huckleberry Hollow" (Figure 6; Table S1). *Faxonius virilis* with concordant COI sequences were found from Pigeon Creek to Brushy Creek and downstream of Brushy Creek, only hybrid crayfish were found (Figure 6; Table S1).

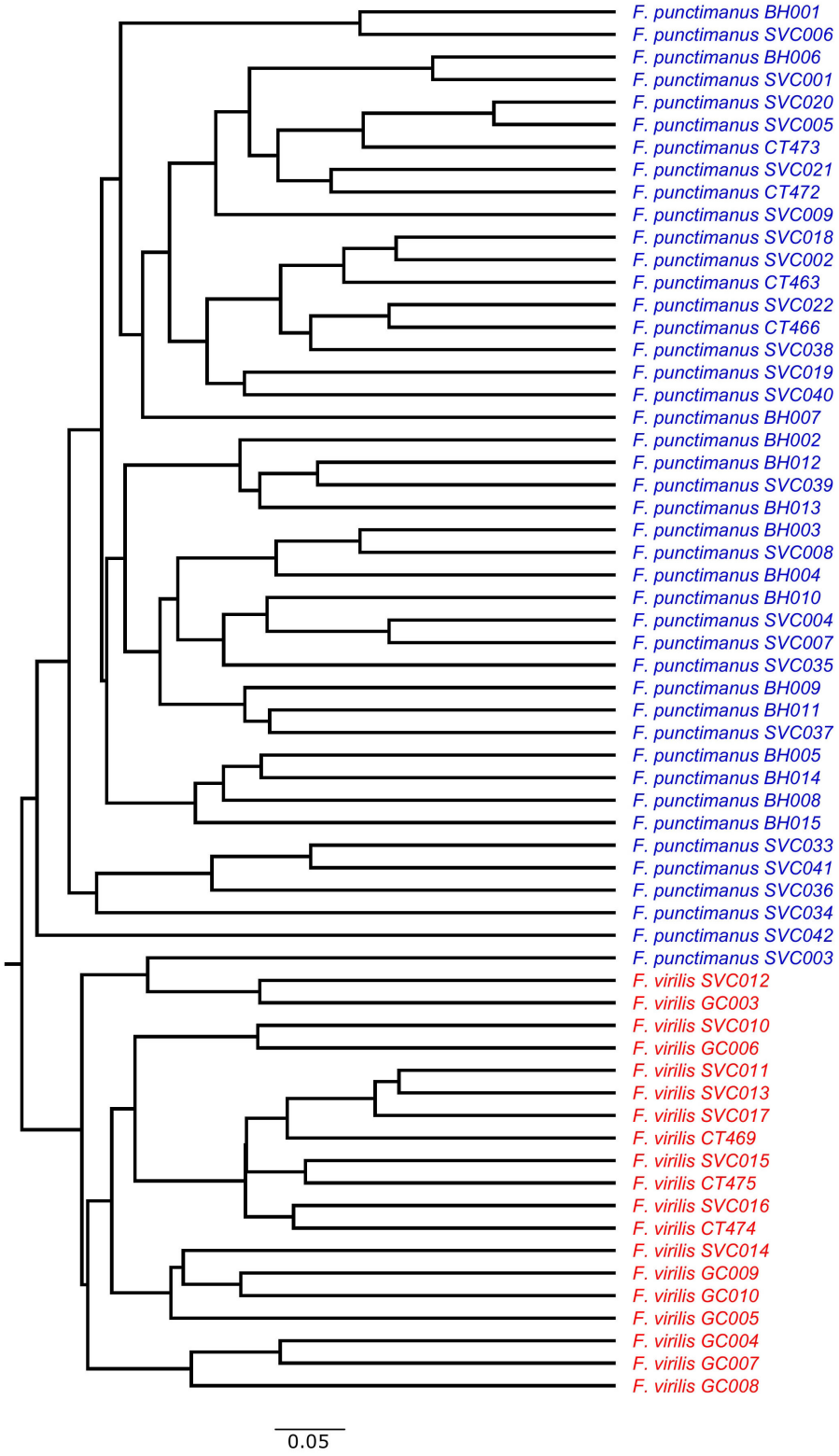

Figure 4. An UPGMA dendrogram displaying microsatellite data for *Faxonius virilis* and *Faxonius punctimanus* specimens from the Current River watershed in Missouri, U.S. Species assignment is based on the phenotype and the specimen number is displayed (Table 2). *Faxonius virilis* is displayed in red and *F. punctimanus* is displayed in blue.

Discussion

Hybridization has rarely been documented between crayfish species. The only genetically documented examples within North American crayfishes

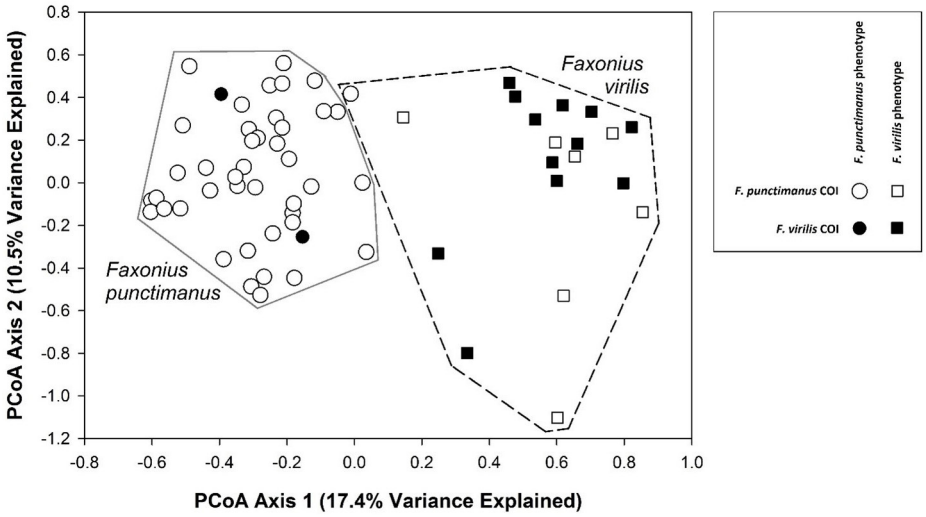

Figure 5. A principal coordinate analysis (PCoA) visualizing variance in genetic distance data from the microsatellite fragment analysis for crayfishes collected at sites in the Current River watershed, Missouri, U.S. (Figure 4). Convex hulls bound the monophyletic *F. virilis* or *F. punctimanus* as identified by the UPGMA dendrogram (Figure 4). Circles denote *F. punctimanus* phenotypes and squares denote *F. virilis* phenotypes (Figure 2). White color denotes *F. punctimanus* COI barcodes while black denotes *F. virilis* COI barcodes (Figure 3).

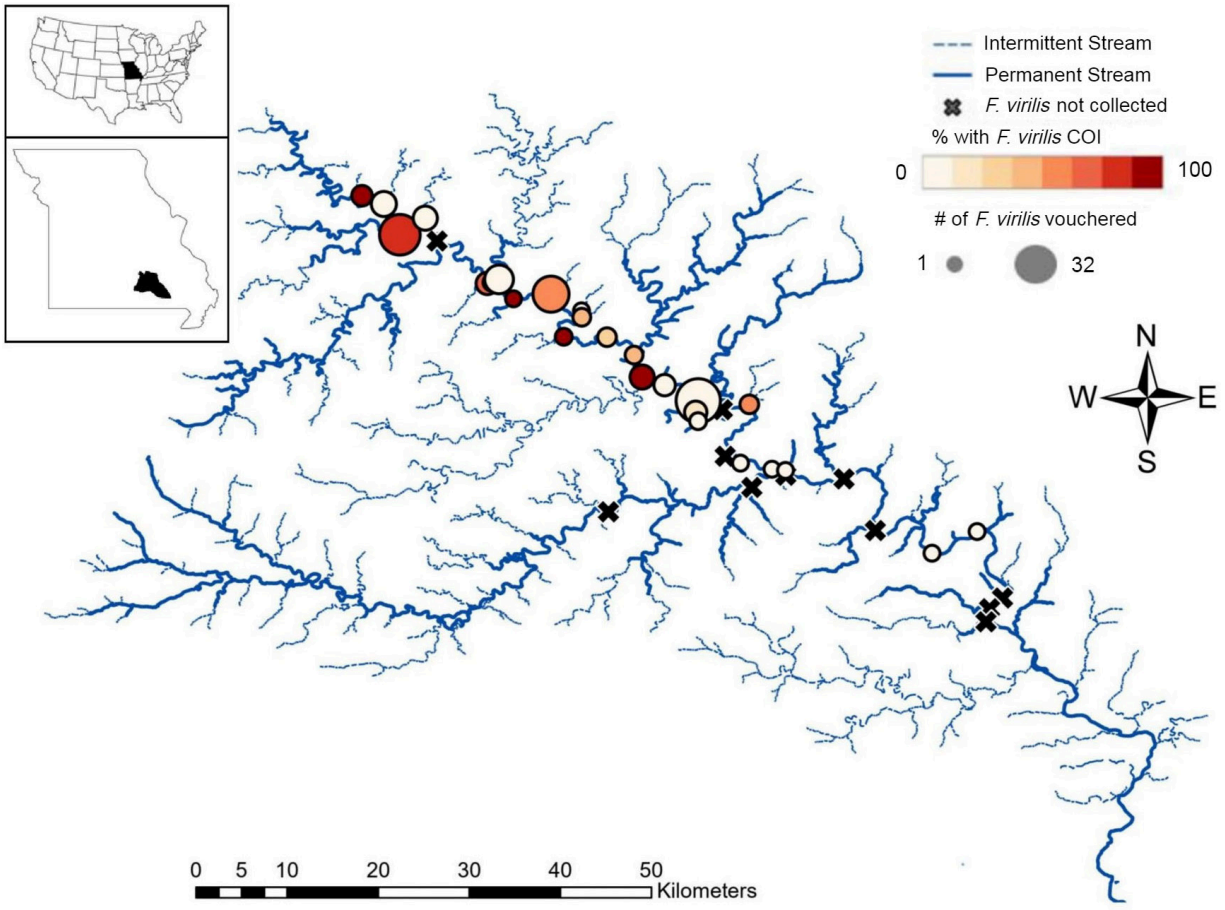

Figure 6. Sites where phenotypic *Faxonius virilis* specimens were collected and hybrid percentages in the Current River watershed, Missouri, U.S. Sites that were surveyed but no *F. virilis* were collected are indicated by X symbols. Circle size indicates the number of phenotypic *F. virilis* that were collected from each site. The circle color represents the percentage of collected individuals that were sequenced with concordant *F. virilis* COI sequences, where darker shades represent a higher percentage of sequenced individuals at a site containing concordant *F. virilis* COI sequences. Lighter shades represent a higher percentage of sequenced phenotypic *F. virilis* containing *F. punctimanus* COI sequences (hybrids). Inset maps display location of Missouri within the United States and the location of the Current River watershed within Missouri.

have been between invasive *F. rusticus* and two other species, *F. propinquus* (Perry et al. 2001b) and *F. sanbornii* (Zuber et al. 2012). Our findings indicate that introduced *F. virilis* are hybridizing with native *F. punctimanus* in the Current River watershed of Missouri. Crayfishes were identified by phenotypic coloration and genotyped using both microsatellite fragment analysis data and COI sequences. Phenotype assignments were concordant with microsatellite data in all but one specimen. Phenotype/microsatellite data compared to COI partial subunit I sequence data revealed nine mismatched specimens. Of 43 total crayfishes collected from Grassy Creek and Spring Valley Creek, we identified two crayfish exhibiting *F. punctimanus* phenotypes and *F. virilis* COI sequences and seven crayfish exhibiting *F. virilis* phenotypes and *F. punctimanus* COI sequences. The incorporation of mitochondrial DNA (mtDNA) from *F. punctimanus* to *F. virilis* and vice versa indicates that hybridization has occurred.

We COI sequenced additional specimens of phenotypic *F. virilis* collected from sites throughout the watershed to further understand the prevalence of hybridization between *F. virilis* and *F. punctimanus*. We used COI as a hybridization indicator and phenotypic *F. virilis* individuals without concordant COI sequences were labelled as hybrids. Sequencing of *F. virilis* vouchered specimens revealed that 45/74 (61%) sequence matched *F. punctimanus.* These hybrid individuals are distributed throughout the range of phenotypic *F. virilis* in the Current River watershed, although hybrids particularly dominate at the downstream, leading edge of *F. virilis* range expansion in this river (Figure 6). First collected by Pflieger (1996) around Pulltite in 1986 (Figure 1), *F. virilis* has subsequently spread farther downstream than upstream in this watershed, consistent with other crayfish invasions in lotic environments (Messager and Olden 2018). *Faxonius virilis* was rare at its downstream leading edge, collected as single individuals at each of the five most downstream sites, and all of these crayfish (100%) were hybrids (Figure 6). This pattern may be caused by increased likelihood of dispersal by hybrid individuals (e.g. Kovach et al. 2015) or lower fitness of hybrids (e.g. Muhlfeld et al. 2009), possibly resulting in reduced prevalence of hybrids at locations that have been invaded longer. While the long-term ecological and evolutionary implications of our findings are unknown, we have shown that the introduction of *F. virilis* has had a genetic impact on at least one native crayfish species. These findings have implications for studies of phylogeny reconstruction and potentially complicate newly emerging monitoring methods for invasive species like eDNA.

A discordance between nuclear and mtDNA genetic markers has been reported for a variety of different organisms due to introgression (Toews and Brelsford 2012). The introgression of mtDNA occurs naturally between populations of organisms (Mallet 2005), but also occurs after secondary contact mediated by anthropogenic translocations (Senn and Pemberton 2009; Guildea et al. 2015). Mitochondrial DNA, including COI,

is maternally inherited and haploid, therefore it does not undergo recombination, and variation is generally low within a population (Funk and Omland 2003; Hebert et al. 2003; Toews and Brelsford 2012). The low intraspecific variation and ability to distinguish between species makes mtDNA popular as a genetic marker, but because of the mode of inheritance, mtDNA is more likely to be introgressed than nuclear DNA and the effects are long-lasting (Funk and Omland 2003; Mastrantonio et al. 2016). In some studies, mtDNA has been introgressed without any evidence of nuclear DNA introgression (Zieliński et al. 2013; Pons et al. 2014). In cases of secondary contact, research has shown that mtDNA introgression is often asymmetric between populations and mostly occurs from the native to the introduced species (Mastrantonio et al. 2016). Our results seem to align with this research as the majority of phenotypic *F. virilis* specimens (61%) COI sequence matched *F. punctimanus*. In contrast to mtDNA, microsatellites are a popular genetic marker that are codominant and mutate rapidly, allowing them to exhibit high polymorphism within and between populations (Schlötterer 2004). Our microsatellite analysis shows a clear separation between *F. virilis* and *F. punctimanus* phenotypes and does not align with the COI discordance. With the information at hand, we cannot conclude whether the specimens we identified as hybrids are sterile or if they are fertile and backcrossing has occurred. Determining whether introgression is occurring, as opposed to hybridization with sterile offspring, would require further analysis involving identification of a source allopatric population of the parental *F. virilis* and tests to infer individual ancestry (Kothera et al. 2009; do Prado et al. 2017).

Overall, the frequency and consequences of hybridization and introgression between crayfishes, including native and non-native species, are poorly known and require further investigation. While we report here that hybridization has occurred between *F. virilis* and *F. punctimanus*, we have not examined the hybrid structure of this system. Future research could examine the prevalence of F1 hybrids, F2 hybrids and backcrosses by examining allopatric populations of both parental species, or populations free of hybridization, and the sympatric population. Perry et al. (2001a) investigated hybridization and assigned putative generations by comparing allozymes of allopatric parental species of *F. rusticus* and *F. propinquus* to sympatric populations. Outside of crayfishes, hybridization studies utilizing microsatellites have also compared allopatric parental species to sympatric populations (Muñoz-Fuentes et al. 2007; do Prado et al. 2017). Determining individual ancestry and association to hybrid groups could determine whether the introduction of *F. virilis* poses a potential evolutionary risk to pure genotype native *F. punctimanus* in the watershed. To further study the hybridization in this system, it would be important to identify an allopatric population of *F. virilis* with genetics like the introduced population in the Current River watershed to have the pure parental genotypes for comparison

to the sympatric population and hybrids. We did not attempt to identify a source population or try to perform microsatellite fragment analysis on any non-sympatric populations because of monetary and time constraints. Additionally, *F. virilis* has an expansive native range resulting in a high amount of genetic diversity within the species (Williams 2012). In Missouri, multiple different genetic populations have been identified (Williams 2012).

Identifying characteristics to distinguish hybrids in the field or by sight in the laboratory would be helpful. Anecdotally, the hybrid specimens from this system did not seem to exhibit any intermediate coloration or mixed pattern features; they either appeared to be typical *F. punctimanus* or *F. virilis*. Morphology between these two species is very close, if not identical, and relies on the examination of reproductively mature Form I male gonopod shape (Pflieger 1996; Taylor et al. 2015). We did not examine gonopod ratios because *F. virilis* males were non-reproductive Form II during our summer sampling period. Further, male *F. virilis* were less abundant than females in our collections and the analysis of gonopods was not applicable to female and juvenile individuals. Differences may exist in the shape of the sperm receptacle, known as the annulus ventralis, between females of *F. virilis* and *F. punctimanus* (Pflieger 1996). In *F. punctimanus*, the posterior margin of the annulus ventralis is sharply angular and produces a triangular extension, which is absent in *F. virilis* where the posterior margin is rounded (Pflieger 1996). Morphological characteristics including differences in the annulus ventralis and gonopod lengths and morphometric ratios between the specimens could be examined. In the examination of hybrid *F. rusticus* and *F. propinquus*, Perry et al. (2001a) examined 12 different characters from the exoskeletons of male crayfishes and found that many specimens from sympatric sites exhibited morphologies intermediate between allopatric parental species. In an attempt to visually distinguish hybrid crayfish, Zuber et al. (2012) examined morphological characteristics between allopatric and sympatric crayfish populations but were unable to determine traits that were fully indicative of hybrid or non-hybrid specimens due to overlapping morphometric ratios. Being able to distinguish hybrids morphologically would provide a method to identify hybrid specimens without performing time consuming and costly genetic analyses.

Faxonius virilis and *F. punctimanus* have been previously identified as closely related by both morphological (Pflieger 1996; Taylor et al. 2015) and genetic analyses (Crandall and Fitzpatrick 1996; Taylor and Knouft 2006). Accordingly, it may not be surprising that these species lack reproductive isolation. Many of the narrow-ranging endemic crayfishes of the United States are likely not reproductively isolated from their closely related congeners, with gene flow prevented by geographic rather than reproductive isolation. While *F. punctimanus* is common and abundant (McAllister and Robison 2012; NatureServe 2019), the introduction of *F. virilis* or other crayfishes into watersheds containing narrow-ranging endemics could pose negative

genetic consequences which could result in extirpations or extinctions. Therefore, preventing the spread of crayfish outside of their native range should remain a priority. Further, hybridization like that documented here may complicate early warning or monitoring of biological invasions by methods like eDNA. Environmental DNA has been applied to detect and monitor range expansion of invasive crayfishes (Larson et al. 2017), but its reliance on mtDNA would potentially fail to detect some hybrid *F. virilis* with *F. punctimanus* COI genes in our study system (Wilcox et al. 2013), particularly at sites where these hybrids were prevalent (Figure 6).

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Authors' contribution

Z.R., E.R.L., and C.A.T. contributed to the research conceptualization, sample design and methodology, investigation and data collection, and data analysis and interpretation. E.R.L. and C.A.T. contributed to funding provision. Z.R. wrote the original draft. E.R.L. and C.A.T. contributed review and editing.

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Supplementary material

The following supplementary material is available for this article:

Microsatellite fragment length analysis data is available at https://doi.org/10.13012/B2IDB-7683513_V1

Table S1. Crayfish sampling sites within the Current River watershed of Missouri.

This material is available as part of online article from:
http://www.reabic.net/aquaticinvasions/2021/Supplements/AI_2021_Rozansky_etal_SupplementaryMaterial.xlsx