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These protocols are working and are published in many other Stepien articles.

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Abstract

This protocol describes the data collection and analysis performed in "Invasion genetics of the silver carp (*Hypophthalmichthys molitrix*) across North America: Differentiation of fronts, introgression, and eDNA detection". Published in PLOS One, 2018.

The invasive silver carp *Hypophthalmichthys molitrix* escaped from southern U.S. aquaculture during the 1970s to spread throughout the Mississippi River basin and steadily moved northward, now reaching the threshold of the Laurentian Great Lakes. The silver carp is native to eastern Asia and is a large, prolific filter-feeder that decreases food availability for fisheries. The present study evaluates its population genetic variability and differentiation across the introduced range using 10 nuclear DNA microsatellite loci, sequences of two mitochondrial genes (cytochrome *b* and cytochrome *c* oxidase subunit 1), and a nuclear gene (ribosomal protein S7 gene intron 1). Populations are analyzed from two invasion fronts threatening the Great Lakes (the Illinois River outside Lake Michigan and the Wabash River, leading into the Maumee River and western Lake Erie), established areas in the southern and central Mississippi River, and a later Missouri River colonization. Results discern considerable genetic diversity and some significant population differentiation, with greater mtDNA haplotype diversity and unique microsatellite alleles characterizing the southern populations. Invasion fronts significantly differ, diverging from the southern Mississippi River population. About 3% of individuals contain a unique and very divergent mtDNA haplotype (primarily the southerly populations and the Wabash River), which may stem from historic introgression in Asia with female largescale silver carp *H. harmandi*. Nuclear microsatellites and S7 sequences of the introgressed individuals do not significantly differ from silver carp. MtDNA variation is used in a high-throughput sequence assay that identifies and distinguishes invasive carp species and their population haplotypes (including *H. molitrix* and *H. harmandi*) at all life stages, in application to environmental (e)DNA water and plankton samples. We discerned silver and bighead carp eDNA from 10 bait shops in the Great Lakes watershed, indicating that release from retailers comprises another likely vector. Our findings provide key baseline population genetic data for understanding and tracing the invasion's progression, facilitating detection, and evaluating future trajectory and adaptive success.

Tissue sample collection & DNA extraction

1

Tissue samples are collected under University of Toledo IACUC protocol #205400, “Genetic studies for fishery management” to CAS. Samples are individually labeled, stored in 95% EtOH, and archived at the NOAA Pacific Marine Environmental Laboratory.

Genomic DNA is extracted and purified from the EtOH fixed tissues using DNeasy® Blood and Tissue Kits (Qiagen Inc., Valencia, CA USA), quality checked on 1% agarose mini–gels stained with ethidium bromide, and assessed with a Nanodrop™ spectrophotometer (Thermo Scientific, Bothell, WA, USA).

DNA Sequencing

2

PCR reactions contain 25µL of 1.25 units AmpliTaq® DNA polymerase, 1X GeneAmp® PCR Buffer I, 250µM dNTPs, 0.5uM (cytb) or 1µM (COI) of each primer, and 2µL of ≥30ng/µL of DNA template, and ddH2O. Conditions are 3 min at 94˚C, followed by 34 cycles of 95˚C for 30 sec, 40 sec at T_a (see appropriate publication), and 72˚C for 45 sec, capped by 5 min at 72˚C.

PCR product aliquots (4µL) are visualized on 1% agarose mini-gels stained with ethidium bromide and successful reactions are purified with QIAquick® PCR Purification Kits (Qiagen) and quantified via Nanodrop. Sanger DNA sequencing is outsourced to the Cornell University Life Sciences Core Laboratories Center (http://cores.lifesciences.cornell.edu/brcinfo/) and MCLAB (http://www.mclab.com/DNA-Sequencing-Services.html), which use ABI Automated 3730 DNA Analyzers. Sample preparation for sequencing is as recommended by sequencing facility in protocols available on their websites. Sequences are quality scored, manually checked, and aligned by us with Codon Code Aligner v7.01 (Codon Corp.).

Microsatellite Data Collection

3 Genetic variation was analyzed at 10 nuclear DNA microsatellite loci, including *Hmo1* and *Hmo11* from Mia *et al.* 2005, *Ar201* from Cheng *et al.* 2007, and *HmoB4, B5, D8, D213, D240, D243,* and *D246* from King et al. 2011. 10µL polymerase chain reactions (PCR)

contain 0.35 units AmpliTaq® DNA polymerase (ABI; Applied Biosystemsä, Foster City, CA, USA), 1X GeneAmp® PCR buffer I (ABI), 80µM total dNTPs, 0.4mM spermadine, 0.52µM of each primer, and 2µL of ≥30ng/µl DNA and ddH_2O. PCR was conducted on C1000 thermal cyclers (Bio–Rad Laboratories, Hercules, CA) with 2 min initial denaturation at 94°C, followed by 39 cycles of 40 sec denaturation at 94°C, 40 sec annealing (at 52°C for the Mia *et al.* 2005 primers, 58°C for the Cheng *et al.* 2007 primers, and 56°C for the King *et al.* 2011 sets), and 1 min 72°C extension, capped by 10 min final 72°C extension. Amplification products are diluted 1:50 with ddH_2O, of which 2µL are added to 13µL solution of formamide and ABI GeneScanä–500 LIZ® size standard, and loaded into 96-well plates. Microsatellite products are denatured for 2 min at 95˚C and analyzed on our ABI 3130xl Genetic Analyzer with GeneMapper® 4.0 software (ABI). Output profiles are checked manually to confirm allelic size variants.

Mia MY, Taggart J, Gilmour AE, Gheyas AA, Das TK, Kohinoor AHM, et al. Detection of hybridization between Chinese carp species (*Hypophthalmichthys molitrix* and *Aristichthys nobilis*) in hatchery broodstock in Bangladesh using DNA microsatellite loci. Aquaculture. 2005; 247:267–273. https://doi.org/10.1016/j.aquaculture.2005.02.018
Cheng L, Liu L, Yu X, Tong J. Sixteen polymorphic microsatellites in bighead carp (*Aristichthys nobilis*) and cross-amplification in silver carp (*Hypophthalmichthys moltrix)*. Mol Ecol Res. 2007; 8:656–658. https://doi.org/10.1111/j.1471-8286.2007.02037.x
King TL, Eackles MS, Chapman, DC. Tools for assessing kinship, population structure, phylogeography, and interspecific hybridization in Asian carps invasive to the Mississippi River, USA: isolation and characterization of novel tetranucleotide microsatellite DNA loci in silver carp *Hypophthalmichthys molitrix*. Cons Gen Res. 2011; 3:397–401. https://doi.org/10.1007/s12686-010-9285-3

eDNA sample collection

4 Approximately two dozen bait fish are purchased from each bait shop, which are immediately sacrificed in the parking lot using the University of Toledo IACUC procedure. The water containing the fish is drained into a sterile container, placed on ice, and then frozen at -80°C in the laboratory. We centrifuge 250 ml of the water from each shop at 4500 rpm and 4°C, for 45 min. This is accomplished by splitting the sample into five 50 ml tubes. The supernatant is poured off and the pellet, containing extra and intracellular DNA and debris, is is gathered from each tube and resuspended using 1 ml of 95% ethanol and stored at -20°C until DNA extraction using Qiagen DNeasy kits following the manufactures protocol except two AW1 and AW2 washes are performed for each sample. Each extraction is subjected to a Zymo OneStep PCR Inhibitor Removal Kit (Zymo Genetics, Seattle, WA). Negative centrifugation controls were processed using reverse osmosis deionized water and the same protocol as above.

eDNA library preparation

5 Our invasive carp assay uses forward primer 5′–TGATGAAAYTTYGGMTCYCTHCTAGG –3′ and reverse primer 5′–AARAAGAATGATGCYCCRTTRGC –3′ to amplify a 135bp region beginning at base 114 in cytochrome b. Each primer set has 4 unique combinations of sapcer inserts between 4 and 18 bases that increase overall run diversity. 25µl PCR reactions consist of 1x Radiant TAQ Reaction Buffer (Alkali Scientific Inc., Ft. Lauderdale, FL), 3mM MgCl2, 1mM total dNTPs, 0.6mM of each primer (with spacer inserts and an Illumina®, MiSeq sequencing primer tail), and 1.25 units of Radiant TAQ polymerase. The final PCR step uses the prior step's column-cleaned product as template (2µl) and incorporated Nextera paired end indices (Illumina®, kit FC-121-1011), which include the P5 and P7 adaptor sequences, allowing the prepared library to bind onto the surface of the Illumina® MiSeq flowcell. Adding unique index combinations permits multiple samples to be pooled together in each MiSeq lane. No template controls (NTCs) are amplified for each reaction, and only libraries that lack NTC amplification are used for MiSeq analyses.

After column clean up, products are sized and quantified in our laboratory on a 2100 Bioanalyzer using a DNA 1000 chip (Agilent Technologies), prior to Illumina® MiSeq analysis by Ohio State University's Molecular and Cellular Imaging Center in Wooster, OH (http://mcic.osu.edu/). Concentrations of pooled products were measured with a Qubit fluorometer (Invitrogen) and with the Bioanalyzer. Pooled samples were run on an Illumina® MiSeq with 2X 300 bp V3 chemistry. An additional 40–50% PhiX DNA spike-in control was added to improve data quality of low nucleotide diversity samples

custom eDNA analysis scripts

6 Custom eDNA analysis scripts are available in the Dryad repository (doi:10.5061/dryad.92h1f12).

Primers were trimmed with the script CarpTrimPrimers.pl. This script also removes detectable incidinces of index hopping by removing reads with the wrong spacer primer.

Merging of reads was done in DADA2 in R folloing with standard protocol except during the filter and trim step the trunc.len function was ommitted and the max.e was set to (3,5). Chimeras were removed with DADA2.

BlastCylce500.pl BLASTed all results files against two custom databases. One of all cytochrome *b* sequences from actinopterygii on GenBank and another of those only from fishes currently present or predicted invaders of the Great Lakes. MorphVeDNASummarize.pl compares observed species hits to advertised species and

those sampled with morphology. This script requires several input files also available in the github repository.