Abundance and distribution of early life stages of Asian carp in the Ohio River:

Geographic Location: Ohio River Basin

Participating Agencies: Indiana Department of Natural Resources (INDNR) Kentucky Department of Fish and Wildlife Resources (KDFWR), West Virginia University (WVU), United States Fish and Wildlife Service (USFWS), West Virginia Division of Natural Resources (WVDNR)

Statement of Need:

The negative effects of Silver (*Hypophthalmichthys molitrix)* and Bighead Carp (*Hypophthalmichthys nobilis)*, also known as Asian carp, have been widely documented throughout their introduced range. These effects are numerous and varied in nature, some with direct implications to native biota (Irons et al. 2007, Sampson et al. 2009). Other effects, such as economic loss and negative social perception, may be indirect and difficult to quantify. Research investigating what factors lead to Asian carp range expansion is critical for the control of these invasive fishes, and mitigation of the deleterious effects they can cause.

Extensive research efforts have been directed toward Asian carp reproduction in terms of timing, location, and environmental conditions. Asian carp exhibit a boom-and-bust pattern of reproduction, with strong year classes usually linked with large, sustained flooding and critical temperature ranges (DeGrandchamp et al. 2007). Although some understanding of their reproductive requirements exist, evidence suggests spawning of these species is possible over wider environmental ranges (Coulter et al. 2013), and in more habitats (i.e., tributaries) than previously thought (Kocovsky et al. 2012). Juvenile Asian carp are extremely mobile and may also elicit clumped distributions among static environments, requiring a variety of different gear types to effectively sample various habitats throughout the Ohio River (Collins et al. 2017; Molinaro 2020). In addition, factors promoting successful reproduction and recruitment remain uncertain. Identifying these factors is critical in suppressing the spread of these invasive fishes into novel environments.

Confirmed Asian carp spawning events have been reported in tributaries (i.e., Wabash River) and as far upstream as J.T. Myers Locks and Dam, and physical signs of spawning (i.e., spawning patches) have been observed as far upstream as Markland Pool for Silver Carp and Meldahl Pool for Bighead Carp. Limited reproduction of *Hypophthalmichthys* spp. was detected by the presence of larvae at river mile (RM) 560 (McAlpine Pool) in 2015, and further upstream at RM 405.7 (Meldahl Pool) in 2016 (EA engineering, personal communication). Although these specimens were not genetically confirmed, this defined the leading edge of spawning in the Ohio River. To support the Ohio River Fish Management Team (ORFMT) Basin Framework objectives (ORFMT 2014), this project was initiated in 2016 in an effort to improve capabilities to detect early stages of invasion and spawning populations of Asian carp (Strategy 2.8) and also monitor upstream range expansion and changes in distribution and abundance (Strategy 2.3). Results of sampling prior to 2020 determined the extent of recruitment as below Cannelton Locks and Dam (Newburgh Pool), with the majority of young-of-year (YOY) and juvenile detections below Newburgh Locks and Dam in J.T. Myers Pool (Jansen and Stump 2017, Roth 2018). Because of the availability of potential nursery areas in Cannelton Pool, we wanted at least two years of targeted sampling within the pool to ensure YOY Asian carp were not readily missed.

In addition to the Basin Framework, this project directly supports the National Plan (Conover et al. 2007) by assisting in the forecast and detection of Asian carp range expansions (Strategy 3.2.4), determining life history characteristics (Strategy 3.3.1), and assembling information about the distribution, biology, life history, and population dynamics of Bighead and Silver Carp (Strategy 3.6.2). Additionally, the results of this project will help managers make informed decisions during future planning efforts regarding resource allocation for Asian carp deterrent and control strategies.

2020 Project Objectives:

1) Determine the extent of Asian carp spawning activity in the Ohio River above Markland Dam.
2) Identify tributaries of Newburgh, Cannelton, and McAlpine pools in which spawning occurs.
3) Determine the extent of Asian carp recruitment in the Ohio River.
4) Identify characteristics of potential Asian carp nursery areas when juvenile Asian carp are encountered.

Project Highlights:

- As of 2016, morphometrically identified *Hypophthalmichthys* spp. larvae were collected at river mile 405.7 (Meldahl Pool).
- Suspect Asian carp "type" larvae was collected at river mile 356.5 from Meldahl Pool in 2019 and was genetically identified by Whitney Genetics Lab on August 17, 2020 as a Common Carp.
- Eight sites were sampled below Markland Locks and Dam (RM 532) via ichthyoplankton tows in May, June, July, and August 2020, to evaluate potential spawning within tributaries; all genetically confirmed *Hypophthalmichthys* larvae and eggs were captured within the Ohio River proper.
- A total of six suspicious fish eggs and larvae were collected above Meldahl Dam and were sent for genetic confirmation of species. Genetic results were unable to verify species of these samples.
- Targeted surface trawling in Cannelton Pool for YOY Asian carp during 2020 resulted in no new YOY captures.
- No YOY Asian carp were found in Hovey Lake in 2020, but 610 *Hypophthalmichthys sp.* were captured directly downstream of the Hovey Lake drainage structure, suggesting poor connectivity between Hovey Lake and the drainage structure throughout the summer of 2020.
- Results confirm Asian carp successfully spawned as far upstream as Cannelton Pool in 2020; the suspected leading edge of reproduction in the Ohio River remains in Meldahl Pool.
- The majority of recruitment remains in J.T. Myers Pool and collective data suggests limited recruitment in Newburgh Pool and potential recruitment in Cannelton Pool.

Methods:

For analysis purposes and for the remainder of this report, the phrase "Asian carp" will be referring to Silver and Bighead carps (*Hypophthalmichthys* spp.) only. In addition, both "YOY" and "immature" are collectively referring to "juvenile" Asian carp; "YOY" will be defined as fish less than 200 mm, and "immature" will define fish between 200 to 400 mm (likely 1 to 2 years old) which have undeveloped gonads and are not capable of spawning. Adult Asian carp are defined as fish greater than 400 mm with mature, identifiable gonads.

Ichthyoplankton tows:
To evaluate the extent of Asian carp spawning activity in the Ohio River above the Markland Dam, ichthyoplankton tows were conducted at sampling sites within the RC Byrd (N = 2), Greenup (N = 1), Meldahl (N = 1), and Markland (N = 2) pools at least twice from May 20 to August 6, 2020. At each sampling site, four to six tows were conducted: three within the Ohio River proper, and one to three tows either within the tributary or at the intake structure if the site was a previous EA Engineering larval sampling site.

To identify specific tributaries below Markland Dam in which Asian carp spawning occurs, ichthyoplankton tows were conducted at tributaries within Newburgh (N = 2), Cannelton (N = 2) and McAlpine (N = 2) pools at least twice from May 28 to August 5, 2020 during ideal spawning conditions.

Five tows were conducted at each sampling site: two tows within the Ohio River proper above the confluence of the tributary, and three tows within the tributary upstream of any potential Ohio River influence (based on water color and clarity changes).

For all tows, a conical ichthyoplankton net (0.76 m, 500 μm mesh) was deployed from the bow of the boat. The boat was motored in reverse, pulling the ichthyoplankton net upstream for three minutes. The water volume sampled was recorded using a General Oceanics Flowmeter fitted to the ichthyoplankton net; depth (m) and water temperature (°C) were recorded using a boat-mounted depth sounder. All contents in the ichthyoplankton net were rinsed into a 500 μm sieve and preserved using 95% non-denatured ethanol (at an estimated ratio of nine parts ethanol to one-part sample volume) for physical identification in the lab. Suspect *Hypophthalmichthys* eggs and larvae were morphometrically identified (process outlined below) and subsamples were sent to Whitney Genetics Laboratory for genetic confirmation. For specific details on genetic identification results and methods employed by the Whitney Genetics Laboratory, refer to Appendix A.

Larval fish were initially sorted into non-Asian carp and suspected Asian carp species using morphometric parameters provided by Auer (1982). Furthermore, early developmental characteristics outlined by Yi et al. (1998) and Chapman (2006) were utilized to physically identify suspected *Hypophthalmichthys* larvae, advanced eggs, and eggs from each sample (Figure 1). Asian carp larvae were identified by the presence of an eye spot, and suspected *Hypophthalmichthys* were differentiated from Grass Carp (*Ctenopharyngodon idella*) and Black Carp (*Mylopharyngodon piceus*) using myomere counts. *Hypophthalmichthys* larvae have 38 to 39 myomeres, whereas Grass Carp larvae range from 43 to 45 myomeres and Black Carp have 40 and 41 myomeres. Suspected *Hypophthalmichthys* eggs were identified based on general size and presence of a large perivitelline membrane (5 to 6 mm in diameter). Suspected *Hypophthalmichthys* 'advanced eggs' were defined as the beginning of a yolk-sack larvae still contained within the perivitelline membrane.

Surface trawl:
Surface trawling effort was focused on tributaries within Cannelton Pool (N = 13). From June 30 to August 15, 2020, a minimum of three trawls were conducted in each of the following tributaries: Blue River, Buck Creek, Clover Creek, Deer Creek, Indian Creek, Little Blue River, Millstone Creek, Oil Creek, Otter Creek, Poison Creek, Salt River, Wolf Creek and Yellowbank Creek. Additionally, 27 trawls were conducted between June 17 to August 16, 2020, at Hovey Lake within the J.T. Myers Pool to document potential recruitment of Asian Carp within and around the lake.

The surface trawl measured 3.7 m wide, 0.6 m tall, and 5.5 m deep with 31.8 mm bar (number 12) netting. An additional layer of 4.8 mm mesh (35-pound delta) bag was attached externally to improve capture of small fishes. Additional foam floats were added to the top line of the trawl to provide extra buoyancy. Otter boards were 30.5 cm tall, 61.0 cm long, and each had a 12.7 cm diameter, 27.9 cm long "buoy style" PVC float attached to the top of the board allowing them to float. The trawl was deployed off the bow of the boat and attached with 24.4 m ropes. The boat was motored at 1.6 to 3.2 km per hour in reverse for five minutes before retrieving the net. In some locations it was not possible to complete five minutes of trawling, in which case sample time was documented. At the biologist's discretion, additional trawls were conducted at sites where either coverage was limited, or juvenile Asian carp were suspected. All Asian carp were identified to genus, measured to total length, and weighed.

Electrofishing:
Due to COVID-19 restrictions KDFWR was unable to complete the majority of targeted YOY electrofishing but was able to conduct limited efforts in the Salt River (RM 630).
Environmental variables:
A suite of habitat variables was collected at each surface trawl site including water temperature, water transparency, conductivity, pH, dissolved oxygen, maximum depth, average depth, tributary width, and

presence/absence of woody debris and aquatic vegetation. Collection of environmental characteristics may determine preferred Ohio River tributaries for future Asian carp recruitment.

Results:

Ichthyoplankton tows:
A combined total of 76 ichthyoplankton tows were conducted within the RC Byrd (N = 24), Greenup (N = 16), Meldahl (N = 12), and Markland (N = 24) pools (Table 1 and 2). No *Hypophthalmichthys* eggs, advanced eggs, or larvae were morphometrically identified in Markland Pool. In Meldahl Pool, one suspicious larva and four suspicious eggs were pulled from samples, along with one suspicious egg from the RC Byrd Pool. These suspicious eggs and larva did not have every morphometric characteristic of Asian carp, however, due to their collection locations, they were vouchered and sent to Whitney Genetics Lab for genetic confirmation of species. Unfortunately, genetic analysis was unable to successfully determine species of these specimens, either due to lack of available DNA or poor DNA quality (Appendix A)

A combined total of 56 ichthyoplankton tows were conducted within the Newburgh (N = 17), Cannelton (N = 15) and McAlpine (N = 24) pools (Table 2 and 3). Suspect *Hypophthalmichthys* larvae, advanced eggs, and eggs were collected from mainstem Ohio River transects near Clover Creek and the Anderson River in Cannelton and Newburgh pools, respectively. Suspicious Asian carp-type eggs were found in samples from within Little Pigeon Creek, and a mainstem Ohio River samples from near Harrods Creek. A total of 78 suspect *Hypophthalmichthys* larvae, 560 suspect *Hypophthalmichthys* advanced eggs, and 1,159 Asian carp-type eggs were identified (Tables 3). A subsample of physically identified suspect *Hypophthalmichthys* larvae, advanced eggs, and Asian carp-type eggs was sent to Whitney Genetics Lab for genetic confirmation. Genetic analysis was unable to confirm species on several specimens, primarily eggs. However, two of the five Asian carp-type eggs collected from within Little Pigeon Creek were genetically confirmed as Grass Carp (*Ctenopharyngodon Idella*). Additionally, the Asian carp-type egg from the Ohio River near Harrods Creek (McAlpine Pool) was confirmed as a Silver Chub (*Macrhybopsis storeriana*). The suspect *Hypophthalmichthys* larvae collected in the mainstem Ohio River from Cannelton and Newburgh pools were genetically confirmed Silver Carp (Figure 2). *Hypophthalmichthys* were only collected on May 28, 2020, which followed the crest of a flood event (Figure 3).

Surface trawl:
Among the 13 tributaries sampled in Cannelton Pool, 50 surface trawls were conducted for a total of 4.16 hours of sampling effort. No YOY or juvenile Asian carp were collected. In the J.T. Myers Pool, 27 surface trawls were conducted at Hovey Lake (21 in the main lake; 6 on the river side of the drainage structure) for a total of 2.1 hours of sampling effort. No YOY or juvenile Asian carp were collected above the drainage structure within Hovey Lake. However, 610 YOY *Hypophthalmichthys* spp. were collected downstream of the Hovey Lake drainage structure. On the river side of the Hovey Lake drain, mean catch-per-unit-effort (CPUE; ± SE) for YOY *Hypophthalmichthys* spp. was 291 ± 6 fish/hour. Average length (± SD) of YOY *Hypophthalmichthys* spp. measured 38 ± 6 mm on July 9, 2020.

Electrofishing:
Although restrictions prohibited some sampling during 2020, KDFWR was able to target limited electrofishing efforts on the Salt River (Cannelton Pool). The middle portion of the Salt River was sampled on three occasions totaling six hours of electrofishing effort and 244 m of 7.6 cm mesh gill nets. A total of 40 Silver Carp between 635 and 737 mm were captured; no juvenile fish were collected. Otoliths were removed and will be analyzed and included the 'Early Detection and Evaluation' project report by KDFWR.

Environmental variables:

Although *Hypophthalmichthys* eggs, embryos, and larvae were found in the main stem Ohio River, no YOY Asian carp were detected by surface trawls within Cannelton Pool. Therefore, further analysis of environmental variables and their use for predicting recruitment locations was not conducted.

Discussion:

Results of the fifth year of the Abundance and Distribution of Asian Carp Early Life Stages in the Ohio River project offer the most up to date information on the extent of Asian carp spawning and recruitment in the Ohio River. Collective efforts of ichthyoplankton tows, targeted surface trawls, and electrofishing directly addressed Basin Framework Strategy 2.8 by improving capabilities to detect early stages of invasion and spawning populations of Asian carp. This project continues to provide data to describe our current understanding of the distribution of Asian carp recruitment for the Water Resources Reform and Development Act (WRRDA) reporting. Moreover, knowledge acquired from this project directly informs planning efforts for future Asian carp deterrent, control, and other management strategies.

Building on previous work and recommendations, this report provides the most up-to-date extent of Asian carp reproduction within the Ohio River. In 2019, the farthest upstream suspected Asian carp "type" larvae was obtained from a sample taken in the Ohio River near the confluence of the Scioto River (RM 356; Meldahl Pool). This larva was identified by Whitney Genetics Lab on August 17, 2020, as a Common Carp (*Cyprinus carpio*; Appendix B). Results from 2019 ichthyoplankton sampling reported the first year of utilizing both physical morphometrics and genetic verification to identify the spawning extent of *Hypophthalmichthys* spp. within the Ohio River. To date, the leading edge of genetically confirmed Asian carp reproduction is near the Salt River (RM 630) in Cannelton Pool. The suspicious Asian carp-type egg collected in 2020 adjacent to Harrods Creek (RM 595) was genetically identified as a Silver Chub. Unfortunately the six suspicious eggs and larvae collected as far upstream as R.C. Byrd Pool (RM 275) were unable to be genetically identified, however their morphometric characteristics did not fully align with *Hypophthalmichthys* spp. characteristics. Future larval tow efforts should be conducted in a manner that preserves the DNA integrity within the samples to insure we are able to get genetic confirmation of species.

Contract fishers notified biologists on May 25th, 2020 of Asian carp spawning activity occurring approximately one kilometer downstream of the McAlpine Dam locks. On May 27th, INDNR biologist visually confirmed the Asian carp spawning behavior was still occurring, noting small groups of three to five Silver Carp in tightly packed groups coming to the surface swimming on their sides for several seconds before disappearing again. There was a large river rise and crest event near action stage on May 25th which triggered a significant number of carp to move up to and stage below the Falls of the Ohio (McAlpine Locks and Dams) where they were spawning. This event corroborates our larval sampling as the majority of morphometrically identified *Hypophthalmichthys* larvae, advanced eggs and eggs were collected in samples taken on May 28th, 2020 near the confluence of the Anderson River (RM 731; Newburgh Pool) and Clover Creek (RM 711; Cannelton Pool). Various stages of development were collected, suggesting several different spawning events likely took place one to three days prior to May 28th (Yi et al. 1988). To date, the lack of confirmed *Hypophthalmichthys* larvae and eggs in sampled Ohio River tributaries suggests that most Asian carp spawning is occurring in the mainstem river, likely below locks and dams.

Among the subsamples of suspected *Hypophthalmichthys* larvae and Asian carp-type eggs sent to Whitney Genetics Lab, the suspect larvae were confirmed to be Silver Carp. This affirms that Asian carp larvae can be readily and confidently identified by our trained biologist. However, eggs are seemingly a little more difficult to discern, and will likely need continued species confirmation through genetic methods. Although *Hypophthalmichthys* eggs have a relatively large perivitelline membrane, there are a couple other species that look somewhat similar. Because of the lower confidence level in identifying Asian carp eggs, we are cautious in using them for delineating shifts in spawning activity unless they have been genetically confirmed. Eggs are inherently more difficult to draw quality DNA from for genetic analysis (Zebadiah

Woiak, Whitney Genetics Lab, personal communication). Because of this, future larval tow samples should be conducted in a manner which increases the probability of successful genetic analysis. This includes using only 95-99% non-denatured ethanol in a 9:1 ratio of ethanol to sample material, keeping the samples in a cooler or as cool as possible while collecting and storing, and changing out the ethanol in the samples 24 hours after collections.

Similar to previous years, 2020 sampling confirmed *Hypophthalmichthys* spp. reproduction in Cannelton Pool and recruitment below Cannelton Locks and Dam. In 2017, several immature Asian carp (269 – 399 mm TL) were captured in Cannelton Pool, suggesting the extent of recruitment to be above Cannelton Dam. However, no juvenile Asian carp have been captured in Cannelton Pool during sampling for Basin Framework projects since, despite extensive effort particularly over the past two years. There still may be suitable tributaries in Cannelton Pool serving as juvenile Asian carp nursery areas that our current sampling gears simply cannot reach.

All juvenile Asian carp encountered in 2020 were collected near Hovey Lake in J.T. Myers Pool. However, despite increased surface trawling efforts, no *Hypophthalmichthys* eggs, embryos, or larvae were collected upstream of the Hovey Lake drainage structure. This was the first year since the project's inception that YOY carp could not be caught inside of Hovey Lake. Based on river gauge data from J.T. Myers Dam, there was only one significant high-water event in late May which was immediately followed by relatively low water levels throughout the summer. This flow regime seemed to have limited YOY Asian carp's ability to traverse the drain structure and prohibited them from getting into the lake. Upcoming work at Hovey Lake will attempt to define how and when conditions allow for Asian carp to move freely into the lake.

There has not been a strong spawning event or year-class since this project was initiated in 2016. Based on the presence of adult Asian carp as far upstream as R.C. Byrd Pool, a highly successful spawning event could quickly shift the current known extent of recruitment to pools farther upstream. Therefore, the spatial and temporal variation in Asian carp recruitment in the Ohio River emphasizes the need for continued long-term monitoring with this project as well as others within the basin. Efforts in this project provide valuable insight into factors promoting the reproduction and recruitment of Asian carp, and ultimately range expansion. Results support several Basin Framework and National Plan strategies and will be used by biologists to mitigate the spread of these invasive fishes.

Recommendation:

The extent of Asian carp recruitment has been relatively stable throughout the past few years of this project. Although no YOY Asian carp have been documented within Cannelton Pool, the presence of adult Asian carp, abundant spawning sites, and suitable nursery habitat suggests recruitment could occur there. As time allows, the use of alternative methods (i.e. seine hauls, mini fyke nets, backpack shocking, and light traps) should be evaluated for detecting the presence of YOY Asian carp in waters where our current gears cannot effectively sample. Because focused efforts on Cannelton Pool did not detect Asian carp recruitment for two consecutive years, future efforts should expand to new or additional locations. Specifically, efforts should expand to include multiple pools again to ensure recruitment in other areas is not missed. Additionally, as Hovey Lake consistently appears to be an important component of Asian carp recruitment in the Ohio River, quantifying the exchange of Asian carp at all life stages between Hovey Lake and the Ohio River should be a priority moving forward.

We recommend continued ichthyoplankton tows above Markland Dam in 2021 to keep monitoring the extent of Asian carp spawning in the Ohio River. We also recommend conducting targeted ichthyoplankton tows during ideal spawning conditions in Newburgh, Cannelton, and McAlpine Pools to identify specific spawning locations. Additionally, due to the lack of spawning evidence in tributaries and visual confirmation of spawning in the Ohio River below the Falls of the Ohio, we recommend conducting

ichthyoplankton tows immediately above and below mainstem dams to evaluate their suitability as spawning locations. Throughout future larval sampling events, biologists should use the methods previously stated to preserve the DNA integrity of the samples.

Based on genetically confirmed results from 2019 and 2020 samples, physical morphometrics have proven successful in identifying *Hypophthalmichthys* advanced eggs and larvae from other native fish species. The identification of eggs can be more difficult and should still be verified via genetic analysis. The use of a measuring device on a microscope to determine if the perivitelline membrane is 5 to 6 mm will help in sorting between non-Asian carp and Asian carp-type eggs. We recommend the continued use of these methodologies, along with genetically confirmed subsamples to provide additional confirmation and to discern between Silver and Bighead Carp. Additionally, we suggest any field staff involved in the physical identification of *Hypophthalmichthys* larvae and eggs be trained on larval fish identification.

Other ongoing projects in the Ohio River basin are gathering data on presence of spawning patches on Asian carp; combining these data with information gathered through this project will help managers identify spatiotemporal patterns of Asian carp reproduction in the Ohio River. This information, along with recruitment patterns we have documented previously, can ultimately be used to identify sources of Asian carp population expansion throughout the basin, and help guide other ORFMT efforts such as deterrents and targeted removals.

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Table 1. Summary of ichthyoplankton tows collected by West Virginia University. Whitney Genetics Lab (WGL) was unable to confirm the identity of suspicious samples.

Sampling Information				Suspicious samples to WGL (N)		Suspect *Hypophthalmichthys* (N)		
Pool	**Location**	**Transect Type**	**Tows (N)**	**Eggs**	**Larvae**	**Eggs**	**Advanced Eggs**	**Larvae**
Meldahl	Scioto River	Ohio River	9	0	0	0	0	0
Meldahl	Scioto River	Tributary	3	0	1	0	0	0
Greenup	Guyandotte River	Ohio River	12	2	0	0	0	0
Greenup	Guyandotte River	Tributary	4	0	0	0	0	0
RC Byrd	Kyger Creek	Ohio River	9	0	0	0	0	0
RC Byrd	Kyger Creek	At structure	3	0	0	0	0	0
RC Byrd	Raccoon Creek	Ohio River	9	1	0	0	0	0
RC Byrd	Raccoon Creek	Tributary	3	0	0	0	0	0

Table 2. Summary of ichthyoplankton tows collected by the Kentucky Department of Fish and Wildlife Resources. The suspicious egg sent to Whitney Genetics Lab (WGL) was genetically identified as a Silver Chub.

Sampling Information				Suspicious samples to WGL (N)		Suspect *Hypophthalmichthys* (N)		
Pool	**Location**	**Transect Type**	**Tows (N)**	**Eggs**	**Larvae**	**Eggs**	**Advanced Eggs**	**Larvae**
McAlpine	Harrods Creek	Ohio River	6	1	0	0	0	0
McAlpine	Harrods Creek	Tributary	6	0	0	0	0	0
McAlpine	Kentucky River	Ohio River	6	0	0	0	0	0
McAlpine	Kentucky River	Tributary	6	0	0	0	0	0
Markland	Hogan Creek	Ohio River	6	0	0	0	0	0
Markland	Hogan Creek	Tributary	6	0	0	0	0	0
Markland	Licking River	Ohio River	6	0	0	0	0	0
Markland	Licking River	Tributary	6	0	0	0	0	0

Table 3. Summary of ichthyoplankton tows collected by the Indiana Department of Natural Resources. An asterisk (*) denotes genetically confirmed *Hypophthalmichthys* samples analyzed by Whitney Genetics Lab. The suspicious eggs collected from Little Pigeon Creek were genetically identified as Grass Carp.

Sampling Information				Suspicious samples to WGL (N)		Suspect *Hypophthalmichthys* (N)		
Pool	**Location**	**Transect Type**	**Tows (N)**	**Eggs**	**Larvae**	**Eggs**	**Advanced Eggs**	**Larvae**
Newburgh	Little Pigeon Creek	Ohio River	3	0	0	0	0	0
Newburgh	Little Pigeon Creek	Tributary	3	5	0	0	0	0
Newburgh	Anderson River	Ohio River	5	1*	1*	858	484	65
Newburgh	Anderson River	Tributary	6	0	0	0	0	0
Cannelton	Clover Creek	Ohio River	4	1*	1*	301	76	13
Cannelton	Clover Creek	Tributary	6	0	0	0	0	0
Cannelton	Salt River	Ohio River	2	0	0	0	0	0
Cannelton	Salt River	Tributary	3	0	0	0	0	0

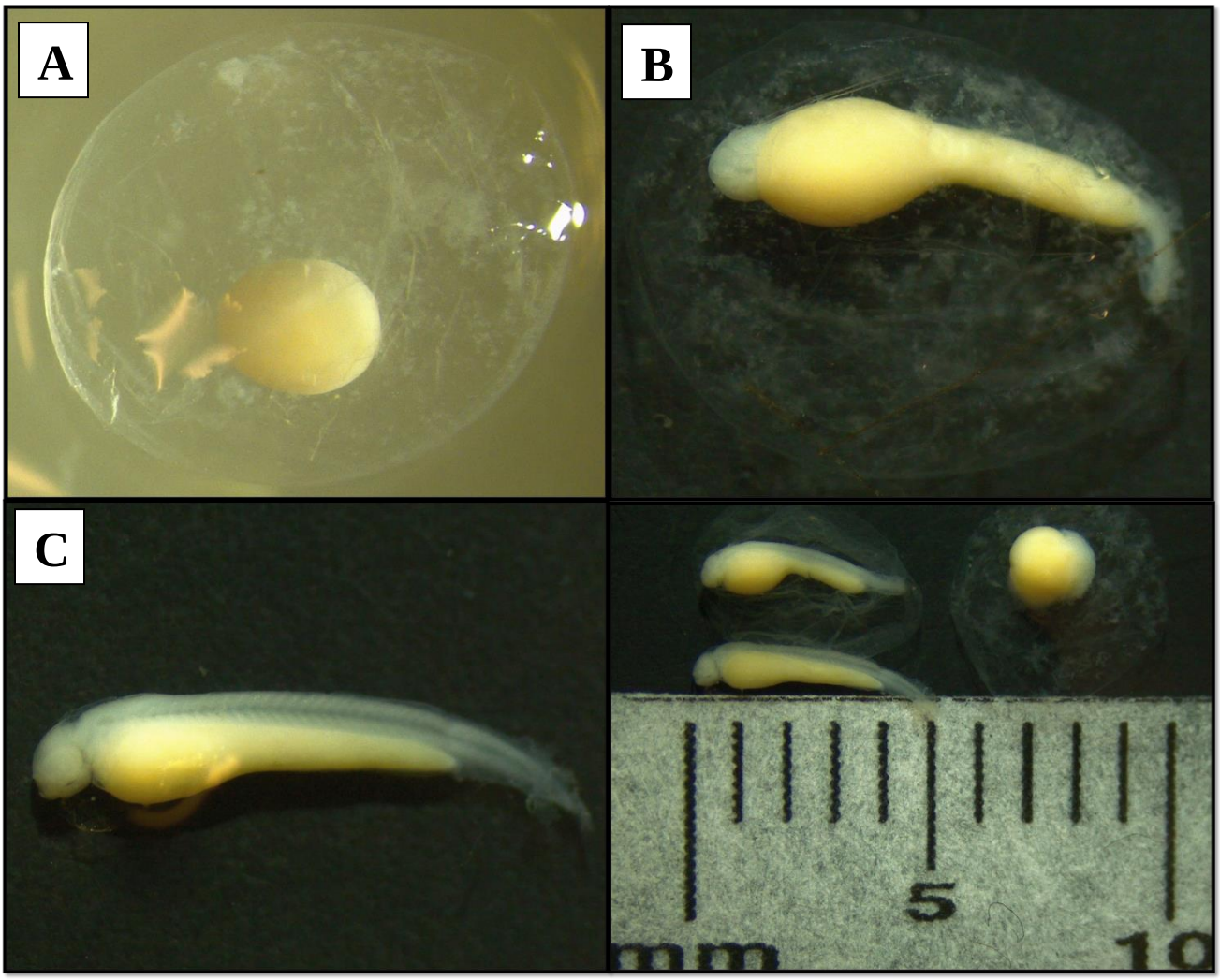

Figure 1. Developmental life stages of *Hypophthalmichthys* spp. with size comparisons. For the purposes of this report, pictures A, B, and C demonstrates specimens categorized as “eggs”, “advanced eggs”, and “larvae”, respectively.

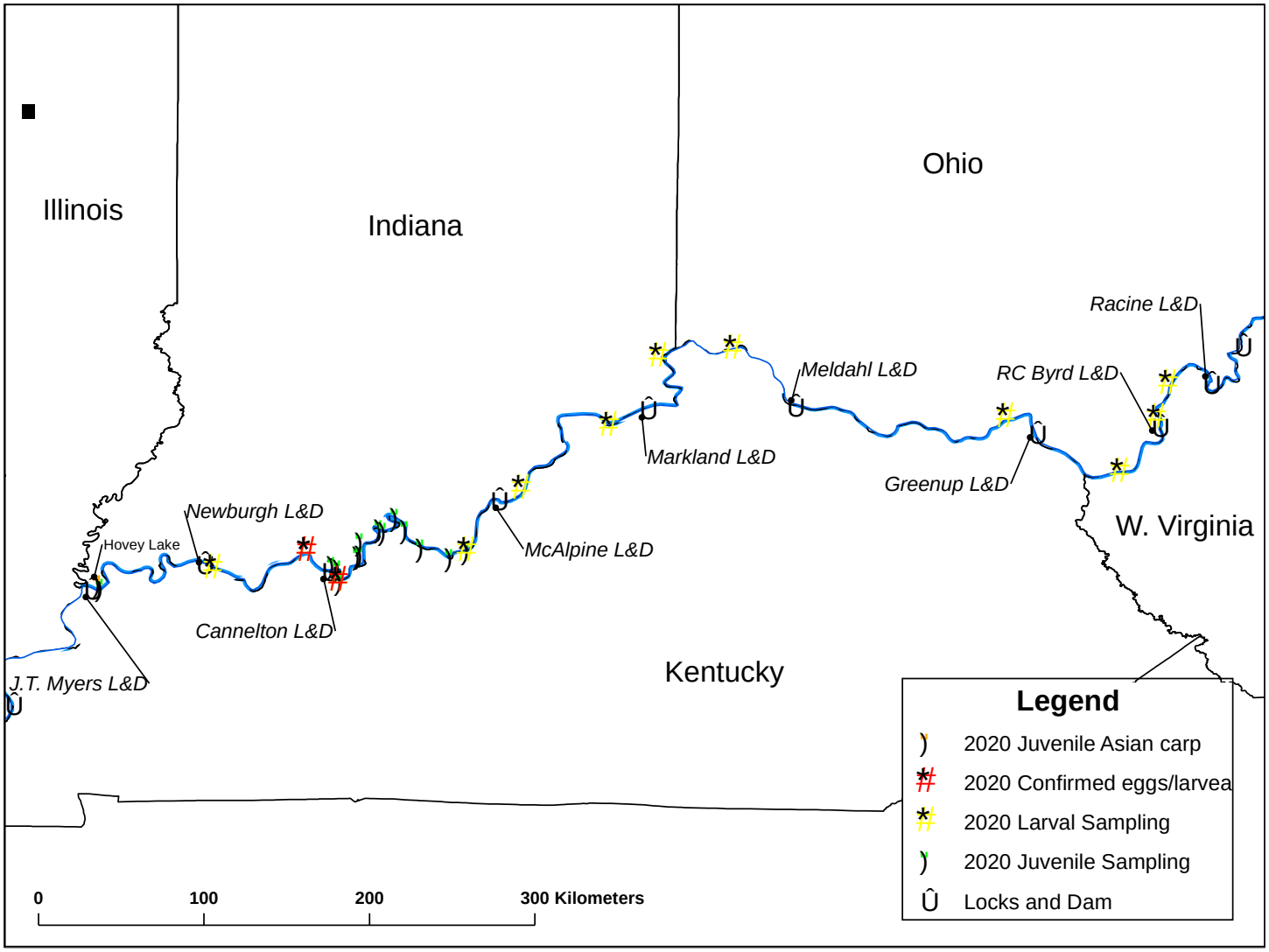

Figure 2. Map of study area including larval and targeted YOY sampling sites. Black icons denote a locks and dam, yellow triangles indicate larval sampling sites, red triangles indicate locations where genetically confirmed *Hypophthalmichthys* eggs, embryos, or larvae were collected. Green squares indicate locations where targeted YOY Asian carp sampling occurred, orange squares indicate locations where YOY Asian carp were collected.

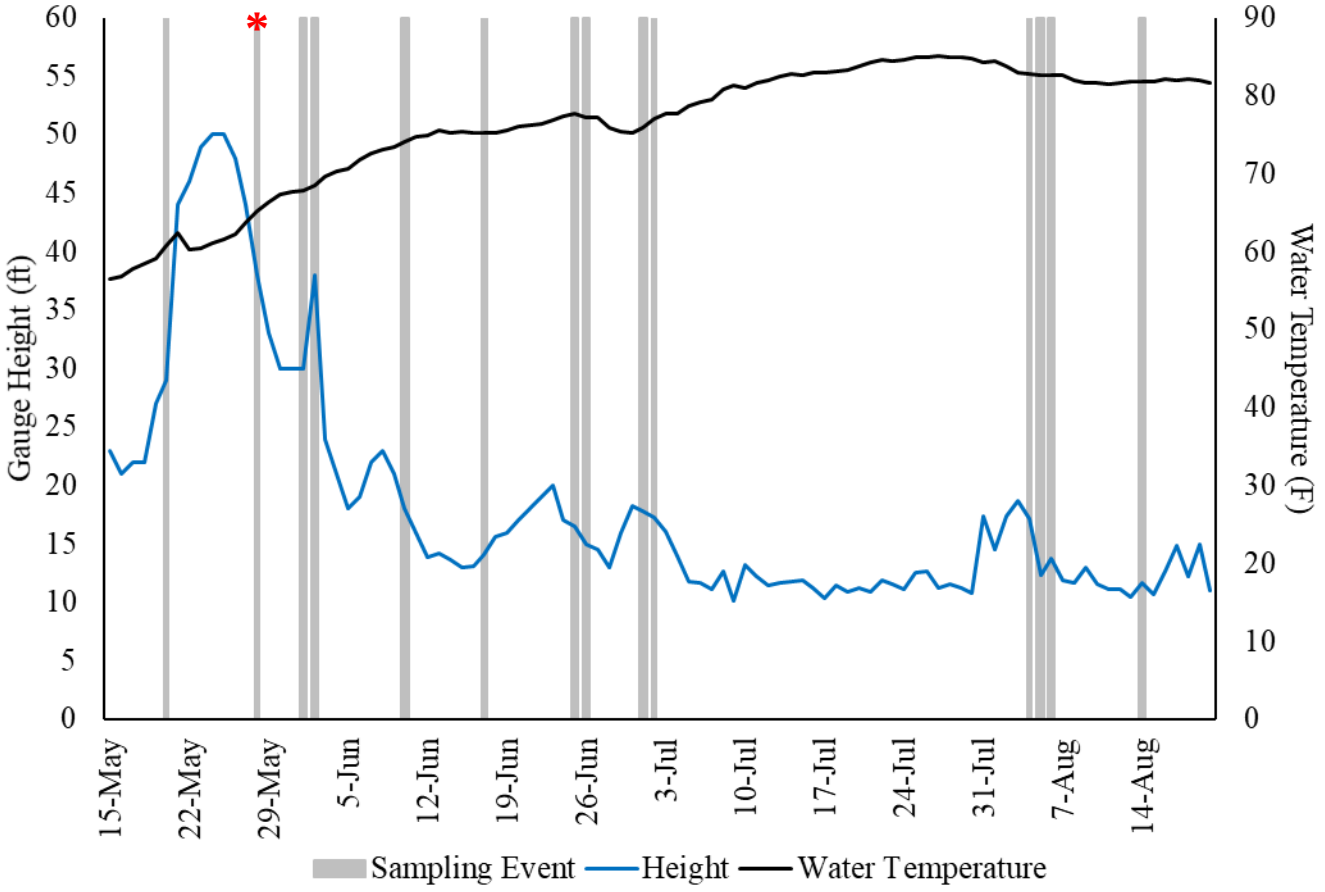

Figure 3. Larval sampling events across time in Newburgh, Cannelton, McAlpine, and Markland Pools compared to environmental characteristics of the Ohio River. The red asterisk (*) indicates sampling events when genetically confirmed *Hypophthalmichthys* eggs or larvae were collected. Gauge height (ft) recorded by USGS below McAlpine Locks and Dam, and water temperature (F) is from the USGS gauge at Markland Locks and Dam.

Appendix A: Whitney Genetics Lab Report – 2020 Sampling.

WGL Report: March 23, 2021

Genetic Identification of Larval Fish

Indiana Department of Natural Resources 2021

By: Zeb Woiak

Samples (n=16) were received on 24 February 2021 at the Whitney Genetics Lab (WGL) by KB from C. Jansen (via fedex). Samples were kept in a -20ᶜ freezer until they could be processed by WGL lab staff.

Methods

We used our laboratory's standardized methods that are common in many core sequencing facilities. Samples were extracted using a modified Chelex-proteinase K method (Casquet et al. 2012) with a positive and negative control in the extraction batch. For DNA extraction procedures and all further analyses, clean laboratory practices and appropriate anti-contamination precautions were used.

Samples were sequenced first at the cytochrome c oxidase 1 gene (UCOI), which is commonly referred to as 'the barcode of life' (Ward et al. 2009) and has been sequenced for over 138,653 animal species specifically for the purpose of species-level identification. This gene was amplified with a cocktail of 4 primers that are universal to most fish species (Ivanova et al. 2007; Ward et al. 2005). A second assay was used to sequence the cytochrome b gene with a marker universal to all vertebrate species (UCYTB, Palumbi 1996). Amplification of UCOI and UCYTB were accomplished with the Platinum™ Green Hot Start PCR mix (Invitrogen™ Life Technologies, Carlsbad, CA) in 25-µl reactions, using primers (from references above) modified with M13 tags to streamline sequencing work. PCR products were cleaned up for sequencing with ExoSAP-IT® PCR Product Cleanup (Affymetrix, Santa Clara, CA) and then cycle sequenced in 1/16th BigDye Terminator v3.1 (Life Technologies, Carlsbad, CA) 20-µL reactions.

Clean-up of the sequences before analysis was done with BigDye Xterminator kits (Life Technologies) to remove un-incorporated bases. Sequence data was collected on an Applied Biosystems 3500XL Genetic Analyzer (Life Technologies). Sequences for each sample and each locus were edited by eye, trimmed, and aligned using Geneious DNA analysis software and compared to sequence data contained in GenBank using the Basic Local Alignment Search Tool (BLAST) for all sequences in NCBI GenBank. FASTA sequence files can be sent to you in a file which may be opened using Microsoft Notepad, if needed.

Results

7 of 16 samples successfully amplified at one or more genes (Table 1). Samples with results for either one or both marker reactions, had control samples as expected, including the positive extraction and positive PCR controls. Extraction and PCR negative controls were clean, so there is no contamination issue. 4 of the 9 samples that failed to amplify had measurable DNA amounts (post extraction) that should have been sufficient enough to amplify during PCR. Sample failure may be due to low quality DNA or a failure in the sample processing. Please let me know if you have any questions or concerns.

Appendix A: Whitney Genetics Lab Report – 2020 Sampling.

References

Casquet J, C Thebaud, RG Gillespie. 2012. Chelex without boiling, a rapid and easy technique to obtain stable amplifiable DNA from small amounts of ethanol-stored spiders. Molecular Ecology Resources 12: 136–141.

Ivanova NV, TS Zemlak, RH Hanner, PDN Hebert. 2007. Universal primer cocktails for fish DNA barcoding. Molecular Ecology Resources 7:544-548.

Palumbi, SR. 1996. Nucleic acids II: the polymerase chain reaction. *In*: Hillis, DM, Moritz, C & Mable, BK, eds. Molecular systematics. Sinauer Publishing: Sunderland, MA, pp. 205-248.

Ward RD, TS Zemlak, BH Innes, PR Last, and PDN Hebert. 2005. DNA barcoding Austrailia's fish species. Philosophical Transactions of the Royal Society B: Biological Sciences 360:1847-1857.

Ward RD, R Hanner, PDN Hebert. 2009. The campaign to DNA barcode all fishes, FISH-BOL. Journal of Fish Biology 74:329-356

Appendix A: Whitney Genetics Lab Report – 2020 Sampling.

WGL Report: March 23, 2021

Table 1. Species identification results based on sequence data from cytochrome oxidase 1 (UCOI) and cytochrome b (UCYTB) mitochondrial loci. For each locus, percent sequence match is between the observed sequence and the reference sequence of the stated length (total base pairs) from the BLAST search. Table values for samples that failed to sequence were populated with "-".

	Universal COI				Universal CYTB				FINAL CALL	
Sample ID	% Match	Length (basepairs)	Accession Number	Species	% Match	Length (basepairs)	Accession Number	Species	Final ID	No. of Markers
202001	-	-	-	-	-	-	-	-	-	-
202002	-	-	-	-	-	-	-	-	-	-
202003	-	-	-	-	-	-	-	-	-	-
202004	-	-	-	-	-	-	-	-	-	-
202005	-	-	-	-	-	-	-	-	-	-
202006	-	-	-	-	-	-	-	-	-	-
202007	100	141	MH664230.1	Ctenopharyngodon idella	99.86	731	MH938830.1	Ctenopharyngodon idella	Ctenopharyngodon idella	2
202008	-	-	-	-	-	-	-	-	-	-
202009	-	-	-	-	99.73	745	MH938830.1	Ctenopharyngodon idella	Ctenopharyngodon idella	1
202010	-	-	-	-	-	-	-	-	-	-
202011	-	-	-	-	-	-	-	-	-	-
202012	100	624	MF180230.1	Hypophthalmichthys molitrix	99.6	745	MH938822.1	Hypophthalmichthys molitrix	Hypophthalmichthys molitrix	2
202013	-	-	-	-	99.72	702	MH938822.1	Hypophthalmichthys molitrix	Hypophthalmichthys molitrix	1
202014	-	-	-	-	99.29	704	MH938823	Hypophthalmichthys molitrix	Hypophthalmichthys molitrix	1
202015	-	-	-	-	99.58	713	MH938822.1	Hypophthalmichthys molitrix	Hypophthalmichthys molitrix	1
202016	99.5	596	KX145580.1	Macrhybopsis storeriana	-	-	-	-	Macrhybopsis storeriana	1

Appendix B: Whitney Genetics Lab Report – 2019 Sampling.

WGL Report: August 17, 2020

Genetic Identification of Larval Fish

Indiana Department of Natural Resources 2020

By: Zeb Woiak

Samples (n=25) were received on 31 July 2020 at the Whitney Genetics Lab (WGL) by KB from C. Jansen (via fedex). Samples were kept in a -20ᶜ freezer until they could be processed by WGL lab staff.

Methods

We used our laboratory's standardized methods that are common in many core sequencing facilities. Samples were extracted using a modified Chelex-proteinase K method (Casquet et al. 2012) with a positive and negative control in the extraction batch. For DNA extraction procedures and all further analyses, clean laboratory practices and appropriate anti-contamination precautions were used.

Samples were sequenced first at the cytochrome c oxidase 1 gene (UCOI), which is commonly referred to as 'the barcode of life' (Ward et al. 2009) and has been sequenced for over 138,653 animal species specifically for the purpose of species-level identification. This gene was amplified with a cocktail of 4 primers that are universal to most fish species (Ivanova et al. 2007; Ward et al. 2005). A second assay was used to sequence the cytochrome b gene with a marker universal to all vertebrate species (UCYTB, Palumbi 1996). Amplification of UCOI and UCYTB were accomplished with the Platinum™ Green Hot Start PCR mix (Invitrogen™ Life Technologies, Carlsbad, CA) in 25-µl reactions, using primers (from references above) modified with M13 tags to streamline sequencing work. PCR products were cleaned up for sequencing with ExoSAP-IT® PCR Product Cleanup (Affymetrix, Santa Clara, CA) and then cycle sequenced in 1/16th BigDye Terminator v3.1 (Life Technologies, Carlsbad, CA) 20-µL reactions.

Clean-up of the sequences before analysis was done with BigDye Xterminator kits (Life Technologies) to remove un-incorporated bases. Sequence data was collected on an Applied Biosystems 3500XL Genetic Analyzer (Life Technologies). Sequences for each sample and each locus were edited by eye, trimmed, and aligned using Geneious DNA analysis software and compared to sequence data contained in GenBank using the Basic Local Alignment Search Tool (BLAST) for all sequences in NCBI GenBank. FASTA sequence files can be sent to you in a file which may be opened using Microsoft Notepad, if needed.

Results

23 of 25 samples successfully amplified at one or more genes (Table 1). Samples with results for either one or both marker reactions, had control samples as expected, including the positive extraction and positive PCR controls. Extraction and PCR negative controls were clean, so there is no contamination issue. The two samples that failed to amplify had measurable DNA amounts that should have been sufficient enough to amplify during PCR. Their failure may be due to low quality DNA or a failure in the sample processing. If these two samples are needed to be re-analyzed, I can and will be happy to try and re-process them. Please let me know if you have any questions or concerns.

References

Casquet J, C Thebaud, RG Gillespie. 2012. Chelex without boiling, a rapid and easy technique to obtain stable amplifiable DNA from small amounts of ethanol-stored spiders. Molecular Ecology Resources 12: 136–141.

Ivanova NV, TS Zemlak, RH Hanner, PDN Hebert. 2007. Universal primer cocktails for fish DNA barcoding. Molecular Ecology Resources 7:544-548.

Palumbi, SR. 1996. Nucleic acids II: the polymerase chain reaction. *In*: Hillis, DM, Moritz, C & Mable, BK, eds. Molecular systematics. Sinauer Publishing: Sunderland, MA, pp. 205-248.

Ward RD, TS Zemlak, BH Innes, PR Last, and PDN Hebert. 2005. DNA barcoding Austrailia's fish species. Philosophical Transactions of the Royal Society B: Biological Sciences 360:1847-1857.

Ward RD, R Hanner, PDN Hebert. 2009. The campaign to DNA barcode all fishes, FISH-BOL. Journal of Fish Biology 74:329-356

Appendix B: Whitney Genetics Lab Report – 2019 Sampling.

WGL Report: August 17, 2020

Table 1. Species identification results based on sequence data from cytochrome oxidase 1 (UCOI) and cytochrome b (UCYTB) mitochondrial loci. For each locus, percent sequence match is between the observed sequence and the reference sequence of the stated length (total base pairs) from the BLAST search. Table values for samples that failed to sequence were populated with "-".

	Universal COI				Universal CYTB				FINAL CALL	
Sample ID	% Match	Length (bps)	Accession Number	Species	% Match	Length (bps)	Accession Number	Species	Final ID	No. of Markers
201901 - WV09	100.0%	640	MK291479	Cyprinus carpio	100.0%	728	MG570435	Cyprinus carpio	Cyprinus carpio	2
201902 - IN0021	100.0%	637	KJ746963	Hypophthalmichthys nobilis	-	-	-	-	Hypophthalmichthys nobilis	1
201903 - IN0021	100.0%	630	MF180230	Hypophthalmichthys molitrix	99.9%	725	MH938822	Hypophthalmichthys molitrix	Hypophthalmichthys molitrix	2
201904 - IN0027	99.8%	638	MF180230	Hypophthalmichthys molitrix	99.7%	744	MH938822	Hypophthalmichthys molitrix	Hypophthalmichthys molitrix	2
201905 - IN0027	99.8%	630	MF180230	Hypophthalmichthys molitrix	99.6%	483	MH938822	Hypophthalmichthys molitrix	Hypophthalmichthys molitrix	2
201906 - IN0034	97.7%	388	MF180230	Hypophthalmichthys molitrix	100.0%	533	MH938823	Hypophthalmichthys molitrix	Hypophthalmichthys molitrix	2
201907 - IN0034	-	-	-	-	99.2%	746	MH938822	Hypophthalmichthys molitrix	Hypophthalmichthys molitrix	1
201908 - IN0035	100.0%	643	KJ746966	Hypophthalmichthys nobilis	-	-	-	-	Hypophthalmichthys nobilis	1
201909 - IN0035	100.0%	630	MF180230	Hypophthalmichthys molitrix	99.7%	734	MH938823	Hypophthalmichthys molitrix	Hypophthalmichthys molitrix	2
201910 - IN0025	100.0%	622	MF122376	Hypophthalmichthys molitrix	99.9%	728	MH938828	Hypophthalmichthys molitrix	Hypophthalmichthys molitrix	2
201911 - IN0025	100.0%	628	MF180230	Hypophthalmichthys molitrix	99.7%	744	MH938822	Hypophthalmichthys molitrix	Hypophthalmichthys molitrix	2
201912 - IN0024	-	-	-	-	-	-	-	-	-	-
201913 - IN0024	99.8%	401	MF180230	Hypophthalmichthys molitrix	99.5%	383	MH938823	Hypophthalmichthys molitrix	Hypophthalmichthys molitrix	2
201914 - IN0026	100.0%	638	MF180230	Hypophthalmichthys molitrix	99.7%	383	MH938823	Hypophthalmichthys molitrix	Hypophthalmichthys molitrix	2
201915 - IN0026	100.0%	630	MF180230	Hypophthalmichthys molitrix	100.0%	424	MH938823	Hypophthalmichthys molitrix	Hypophthalmichthys molitrix	2
201916 - IN0023	100.0%	630	MF180230	Hypophthalmichthys molitrix	99.4%	720	MH938823	Hypophthalmichthys molitrix	Hypophthalmichthys molitrix	2
201917 - IN0031	100.0%	633	MG599474	Aplodinotus grunniens	99.6%	718	MG599474	Aplodinotus grunniens	Aplodinotus grunniens	2
201918 - IN0031	99.8%	633	MG599474	Aplodinotus grunniens	99.7%	734	MG599474	Aplodinotus grunniens	Aplodinotus grunniens	2
201919 - IN0032	99.8%	633	MK291479	Cyprinus carpio	99.9%	743	MG570435	Cyprinus carpio	Cyprinus carpio	2
201920 - IN0032	99.8%	644	MG599474	Aplodinotus grunniens	99.5%	748	MG599474	Aplodinotus grunniens	Aplodinotus grunniens	2
201921 - KY0029	-	-	-	-	-	-	-	-	-	-
201922 - IN0024	100.0%	630	MF180230	Hypophthalmichthys molitrix	99.3%	731	MH938822	Hypophthalmichthys molitrix	Hypophthalmichthys molitrix	2
201923 - IN0023	100.0%	624	MF122376	Hypophthalmichthys molitrix	99.5%	744	MH938828	Hypophthalmichthys molitrix	Hypophthalmichthys molitrix	2
201924 - IN0024	99.8%	641	MF180230	Hypophthalmichthys molitrix	99.6%	743	MH938822	Hypophthalmichthys molitrix	Hypophthalmichthys molitrix	2
201925 - IN0024	100.0%	630	MF180230	Hypophthalmichthys molitrix	100.0%	734	MH938823	Hypophthalmichthys molitrix	Hypophthalmichthys molitrix	2