USING GENETIC MARKERS TO ENHANCE CONSERVATION EFFORTS

by

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Species conservation is a fundamental goal. Every species provides an important service within its respective ecosystem. Conservation managers strive to maximize biodiversity to retain ecosystem resources. A species' genome dictates its fundamental capacity to adapt to a changing environment. Conservation geneticists can optimize management efforts for biodiversity by utilizing genetic markers, genes or short stretches of DNA to measure changes in an organism's genome across spatiotemporal scales.

I showcase how conservation managers can integrate genetic markers to quantify biodiversity at various spatiotemporal scales and retain ecosystem services. Herein, I inferred the evolutionary history of gar (family Lepisosteidae) by conducting phylogenetic analysis on complete mitochondrial genomes of all extant gar species using the maximum likelihood method and the General Time Reversable model. I generated important genetic markers for future studies to track hybridization amongst these lineages and determined that hybridization between Cuban Gar, *Atractosteus tristoechus*, and Alligator Gar, *Atractosteus spatula*, may provide an alternative conservation strategy to retain an apex predator within Cuba's ecosystems. I sequenced the mitochondrial 16S ribosomal RNA of bacterial species located within the gut microbiome of the endangered Pallid Sturgeon, *Scaphirhynchus albus*, and determined that the gut microbiome of hatchery-raised Pallid Sturgeon effectively transition to the gut microbiome of wild Pallid Sturgeon. I used nuclear and mitochondrial single nucleotide polymorphisms to determine that Bighead Carp, *Hypophthalmichthys nobilis*, and Silver Carp, *H. molitrix*,

exhibited hybridization in native regions and provided an important baseline for future studies to determine if new anthropogenic disturbances in China will alter evolutionary trajectories of Bighead Carp and Silver Carp. Finally, I proposed 'population' as the least inclusive category of the Linnaean classification system – a distinctive unit that can monitored across geospatial scales and that can be compared across classes to study speciation. I further proposed a 'species spectrum' concept that represents the amalgamation of intraspecific variations observed amongst populations.

This dissertation recommends that conservation managers continue to integrate genetic markers into their research and continue to develop tools to quantify biodiversity at various spatiotemporal scales, all in an effort to retain ecosystem services.

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Introduction

Species conservation is a fundamental goal. Every species provides an important service within its respective ecosystem and contributes to the production of essential biological functions, such as oxygen production, carbon sequestration, nutrient cycling and crop pollination (Frankham et al. 2010). One strategy to ensure ecosystem services is maintain species biodiversity, which increases the likelihood that one of the species can fulfill a specific ecological niche within an ecosystem (Balvanera et al. 2001).

What characteristics defines a species? There has been little consensus about the definition of "species," which makes managing species diversity, particularly across spatiotemporal scales, exceptionally difficult. The goal of this dissertation is to garner knowledge about how genetics can be used to define a species and manage species.

An organism's genome is a dynamic record of the interaction between a species' genome and its environment (Frankham et al. 2010). Conservation genetics utilizes genetic makers, which are genes or short stretches of deoxyribonucleic acid (DNA) within an organism's genome, to assess a species' genetic resources (Allendorf et al. 2013). In the second chapter, I use genetic markers to resolve the phylogenetic relationships of gar, an ancient group of fish, and show how these genetic resources offer new conservation strategies for this lineage. In the third chapter, I use genetic markers to compare differences between the gut microbiomes of hatchery-raised and wild Pallid Sturgeon. In the fourth chapter, I use genetic markers to establish a baseline of hybridization between Bighead Carp, *Hypophthalmichthys nobilis*, and Silver Carp, *Hypophthalmichthys molitrix*, for future studies to be able to predict evolutionary

processes of Bighead Carp and Silver Carp. In the fifth chapter, I reevaluate how biologists delineate species across spatiotemporal scales.

Resolve phylogenetic relationships

Phylogenetic relationships showcase the evolutionary history of a taxonomic lineage. Closely related species are more likely to share similar niches because they share a common ancestry (Felsenstein 1985, Harvey and Pagel 1991). Many species' evolutionary histories remain unresolved, which makes it difficult to predict a replacement species to fulfill an ecological niche in the event of an extinction or to predict a species' susceptibility to anthropogenic disturbances so that one can try to prevent a species' extinction.

Many species of gar (family Lepisosteidae) are suffering population declines due to anthropogenic disturbances (Mendoza et al. 2002, Sakaris et al. 2003, O'Connell et al. 2007, Bohn et al. 2017). Most gar species are apex predators within their ecosystems; therefore, conserving gar is becoming more of a conservation concern (Mendoza Alfaro et al. 2008). Natural hybridization may play an important role in this lineage to prevent extinction events. Natural hybridization is more likely to occur in species with high genomic similarity (Wang et al. 2019). Understanding phylogenetic relationships, particularly divergence times between species, would aid in exploring hybridization within the gar lineage.

Genetic markers offer additional sources of information to estimate evolutionary relationships. Phylogenetic relationships were inferred historically using morphological characteristics. It is difficult to establish ancestral versus derived characteristics and detect convergent evolution using morphological characteristics (Herron and Freeman

2013). Mitochondrial DNA is more likely to reflect the species tree in phylogenetic comparison. Mitochondrial DNA is considerably smaller than nuclear DNA, undergoes a higher mutation rate, and has a uniparental mode of inheritance (Moore 1995); therefore, mitochondrial DNA will more likely reflect the species tree in phylogenetic comparison because the effective population size (Ne) is essentially one fourth as large as that of a nuclear-autosomal gene. Complete mitochondrial genomes have effectively resolved complex phylogenies across taxa including the fire-bellied toads of the genus *Bombina* (Bombinatoridae) (Olson et al. 2012, Thomsen et al. 2012, Pabijan et al. 2013), the insect family Braconidae, which is one of the most species-rich families of Hymenoptera (Thomsen et al. 2012, Li et al. 2016), mammals including members of the families Ursidae (Yu et al. 2007) and Felidae (Zhang and Zhang 2013), and fish (Yamanoue et al. 2006, Vera-Escalona et al. 2017). A robust database of mitochondrial DNA markers would facilitate future comparative studies. The most commonly used reference databases for comparative species identification searches is the National Center for Biotechnology Information (NCBI). Unfortunately, there are only ~100,000 mitochondrial genomes currently available in the NCBI database. Of these, 63,150 are mitochondrial genomes of vertebrates, which is a small representation of the diversity of vertebrates (Figure 1-1).

Evaluate conservation efforts

Species are rapidly disappearing across the globe. Genetic markers offer additional information to assess conservation strategies. Hatchery stocking has been one conservation method employed for threatened or endangered fishes (Brown and Day 2002). The endangered Pallid Sturgeon, *Scaphirhynchus albus*, is one species where

stocking has been employed to bolster wild populations. Since the severe 2011 flooding of the Missouri River there has been a decline in the body condition of the Pallid Sturgeon (Steffensen and Mestl 2016, Randall et al. 2017, Steffensen et al. 2017). This decline in body condition may affect growth, maturation, reproductive frequency, fecundity, and survival (Pope and Kruse 2007). Floods can negatively impact a fish's body condition by reducing the density of previously available prey via the dilution effect and increasing the amount of shelter in the form of flooded vegetation; however, once flood waters recede food resources should become more concentrated and available once again (Luz-Agostinho et al. 2009). One conservation concern is that stocked Pallid Sturgeon's body condition will not recover because they are not capable of utilizing food resources in the same way as a wild Pallid Sturgeon. A fish's gut microbiome plays a role in the absorption of nutrients through digestion; therefore, the diversity of bacterial within the gut microbiome will help describe how Pallid Sturgeon is using available food resources. Fish that have a high diversity of bacterial gut microbiota tend to have a more varied diet, which makes them more resilient to ecological changes (Ley et al. 2008). Sequencing the bacterial gut microbiome will determine if stocked (i.e., hatchery-raised) Pallid Sturgeon have similar bacterial gut microbiomes to wild Pallid Sturgeon, an important step to assess stocking as a conservation strategy for Pallid Sturgeon.

Predict evolutionary processes

Organisms have a great capacity to adapt to changing environmental conditions. Predicting how organisms adapt in the face of changing environmental conditions would help maximize conservation efforts. Every species has been exposed to a unique set of selection pressures over time and each species' genome is a dynamic record of these

evolutionary processes. It's possible to explore evolutionary processes at both short- and long-term scales using genetic markers. Short-term evolutionary processes can be monitored by studying shifts in genetic diversity. Long-term evolutionary processes can be monitored by studying gene flow. A cessation of gene flow can eventually isolate populations leading to speciation events. Sometimes zygotic barriers are relaxed over time, restoring gene flow. The removal or relaxation of zygotic barriers may lead to hybridization or the fusion of two species, which can also be monitored by changes in genetic markers. Bighead Carp and Silver Carp are ideal species to test how zygotic barriers impact hybridization between two species. Bighead Carp and Silver Carp have high genomic compatibility (Kolar et al. 2007, Li et al. 2011, Wang et al. 2019), but exhibit different feeding and reproductive strategies within native regions; however, the presence of hybrids have never explored in native China (Nikolsky 1963, Chang 1966).

Delineate species designations

Species are complex entities! One of the largest challenges for managing species diversity is defining species' boundaries, particularly across spatiotemporal scales. Each species exhibits a range of morphological, behavioral and genetic features that are the result of the dynamic interplay between the species' genome and the species' surrounding environment. Unfortunately, none of the current species concepts embrace the full extent of variation exhibited by species, which limits their effectiveness.

Populations are smaller units within a species that will help identify and track potentially new evolutionary lineages and showcase the spectrum of diversity exhibited within a species. Most speciation events require long periods and often involve divergence interspersed with genetic exchanges before permanent barriers are established

(Frankham et al. 2010). Utilizing units smaller than a species allow conservation managers to follow potential new evolutionary trajectories. 'Evolutionary Significant Units' (ESU) were based on premise that variation occurs within a species as a result of the interaction of a species' genome and environment. The ESUs were designed to delineate groups of individuals that warranted separate management for conservation (Coates 2000, Ryder 1986). One of the leading proponents defined an ESU as a historically isolated and evolving set of populations (Moritz 1994). In vertebrate animals, this can be regarded as populations that exhibit reciprocal monophyly for mtDNA alleles and possess significant divergence at nuclear loci (Moritz 1994). One of the benefits of this definition is that it offers the potential to utilize organelle DNA, such as mtDNA in animals or chloroplast DNA in plants. Organelle DNA can distinguish phylogeographically distinct populations or ESUs more effectively than nuclear genetic markers because organelle DNA has a uniparental mode of inheritance, a lower recombination rate than nuclear DNA, and a single genotype (Coates 2000, Zhang et al. 2018).

Purpose

The purpose of this dissertation is to embrace spatial and temporal dynamics as defining characteristics of a species by addressing the following questions:

1) What is the phylogenetic relationship amongst gar? (Chapter 2)
2) Is the gut microbiome of hatchery-raised Pallid Sturgeon like the gut microbiome of co-occurring wild Pallid Sturgeon? (Chapter 3)
3) Does hybridization of Bighead Carp and Silver Carp occur in native regions of China? (Chapter 4)

4) Can conservation geneticists expand the definition of a species to capture the dynamic interplay of a species' genome and environment? (Chapter 5)

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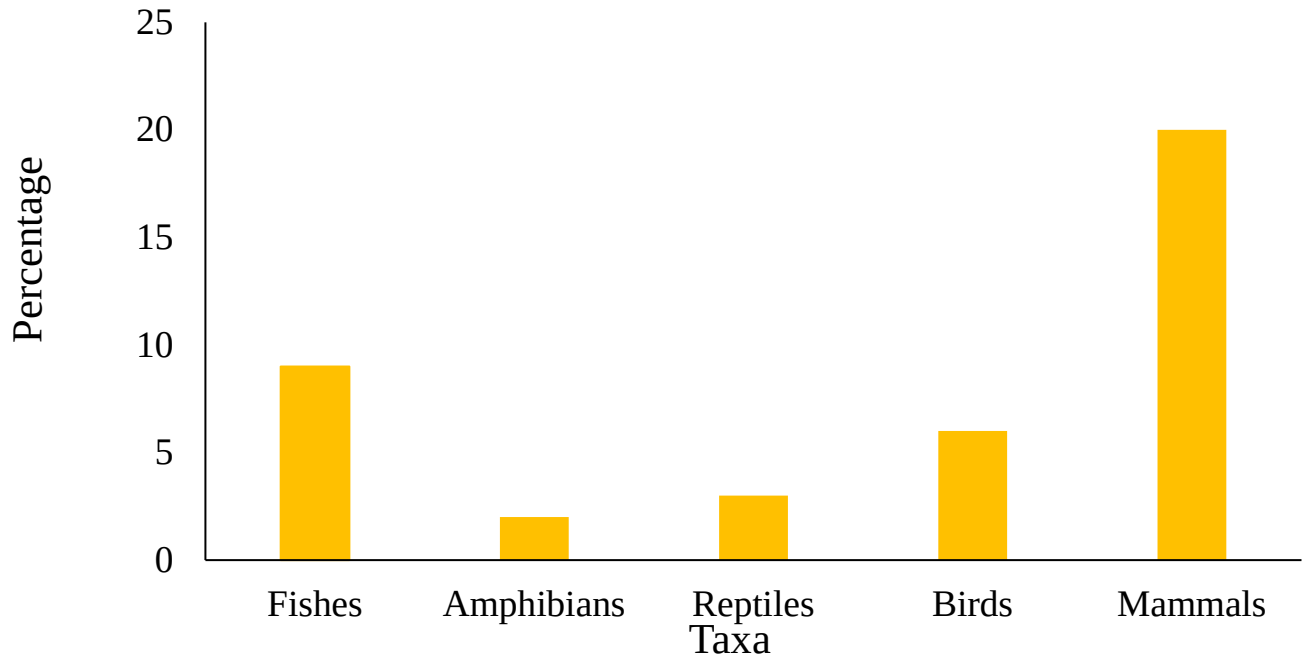

Figure 1-1: Percentages of mitochondrial genomes that have been sequenced and deposited into the National Center for Biotechnology Information (NCBI) in relation to the total number of species described for each taxonomic class as outlined in the Catalogue of Life (Roskov et al. 2018).

Chapter 2: Mitochondrial genomes of gar

Introduction

The seven extant gar species are the only representatives of a once diverse "living fossil" lineage (Wiley and Schultze 1984). The oldest members of these genera date to the late Cretaceous about 75 and 100 MYA for *Lepisosteus* and *Atractosteus*, respectively (Grande 2010). All extant members can be placed within these genera. Gar are easily recognizable with their elongated bodies, jaws filled with needle-like teeth, and ganoid scales (Fink 1978, Grande 2010).

Historically, gar were distributed broadly, including Europe, the Middle East and Central Asia, northern and central Africa, Madagascar, South America, and west and south-western North America (Grande 2010). Ancient gar species fed on a wide range of dietary items including fish, mollusks, and arthropods. In contrast, extant species are limited to the freshwaters of eastern North America, Cuba and Central America, with some members occasionally venturing into brackish and marine water. All extant gar species are mostly piscivorous (Grande 2010). The narrower dietary preferences and limited distribution ranges may make this lineage more susceptible to shifting environmental conditions.

Many populations of gar are declining due to anthropogenic disturbances (Mendoza et al. 2002, Sakaris et al. 2003, O'Connell et al. 2007, Mendoza Alfaro et al. 2008, Bohn et al. 2017). The Alligator Gar, *Atractosteus spatula*, has experienced recent population declines due to overfishing and habitat degradation (Mendoza et al. 2002, Sakaris et al. 2003, O'Connell et al. 2007, Bohn et al. 2017). The Spotted Gar, *Lepisosteus oculatus*, is considered threatened in Canada due to the destruction of

wetland habitat and pollution (COSEWIC 2005). Population genetic studies recently determined that Cuban Gar, *Atractosteus tristoechus,* has a low effective population size and low genetic diversity making Cuban Gar more susceptible to anthropogenic changes (McCulloch and MacLean 1995, Barrientos-Villalobos and Espinosa de los Monteros 2008, Ulmo-Díaz et al. 2017).

Conservation efforts for gar are becoming a higher priority as all extant gar species share similar ecological roles as top predators (Mendoza Alfaro et al. 2008). One strategy gar may have naturally employed to overcome current challenges is to hybridize with closely related species. Hybridization has been recently documented between Alligator Gar and Longnose Gar, *Lepisosteus osseus,* and between Longnose Gar and Shortnose Gar, *L. platostomus* (Bohn et al. 2017). Natural hybridization has played an important evolutionary role in some animal lineages (Allendorf et al. 2001) and is more likely to occur in species with high genomic similarity (Wang et al. 2019). Species that have recently diverged from each other are more likely to have genomic similarity. Biologists need a better understanding of evolutionary relationships, particularly in estimating divergence times between species, to explore hybridization within the gar lineage.

Phylogenetic analysis would provide a better framework for following evolutionary events within the gar evolutionary lineage. Previous morphological methods were unable to resolve the phylogenies amongst gar lineages, primarily due to the inclusion of fossil taxa (Grande 2010). The first molecular phylogenetic analysis comprised DNA from a single mitochondrial gene and seven nuclear genes; however, only the gene trees from the mitochondrial gene, COI, and the nuclear S7 intron were

completely resolved (Wright et al. 2012). Mitochondrial DNA has become more prevalent in phylogenetic analyses (Tang et al. 2017) because mitochondrial DNA undergoes a higher mutation rate, and has a uniparental mode of inheritance (Moore 1995, Boore 1999). Complete mitogenomes would provide the highest phylogenetic resolution and most precise date estimates (Duchêne et al. 2011) for exploring evolutionary events within this lineage. This study provides the first complete mitochondrial genomes of Shortnose Gar and Longnose Gar and a phylogenetic analysis based on the complete mitochondrial genomes of all seven extant gar species.

Methods

Specimen collection

Tissues were collected from ten Shortnose Gar and ten Longnose Gar captured by Nebraska Game and Parks Commission biologists using standardized gears (i.e. gill nets, boat electrofishers, and trap nets) during routine sampling. Ten individuals were targeted for each species to insure against tissue degradation. Fin clips were preserved in 100% ethanol (molecular grade) in the field. A photo of each individual fish was taken for species verification. Preserved tissues were transported to the Biology Department at the University of Nebraska at Omaha and stored at room temperature.

DNA extraction and phylogenetic analysis

A single tissue sample from one individual of each species was used for DNA extraction (all DNA extractions were successful at producing enough material for assembly with a single extraction). Fin tissue from an adult Longnose Gar collected from the Missouri River (40.51°N, -95.70°W) and an adult Shortnose Gar collected from the Missouri River (41.69°N, -96.12°W) were used for DNA extraction. Genomic

mitochondrial DNA was extracted and purified from one fin clip using the standard protocol of the Abcam Mitochondrial DNA Isolation Kit™ and sequenced on an Illumina NextSeq500 sequencing platform at UNMC DNA Microarray and Sequencing Core Facilities of the University of Nebraska Medical Center, Omaha. The two tissue samples used for DNA extraction were deposited in the state museum at the University of Nebraska-Lincoln.

Phylogenetic analysis

Sequences for Longnose Gar and Shortnose Gar were assembled and annotated using GENEIOUS™ 10.2.6 (Biomatters, Newark, NJ) (Kearse et al. 2012). The complete mitochondrial genomes for the other five extant species were downloaded from the National Center for Biotechnology Information (NCBI) including Florida Gar, *Lepisosteus platyrhincus* (NC_029715.1), Spotted Gar (NC_004744.1), Tropical Gar, *Atractosteus tropicus* (NC_024178.1), Alligator Gar (NC_008131.1), and Cuban Gar (NC_036329.1) (Inoue 2003, Del Río-Portilla et al. 2016, Ulmo-Díaz et al. 2017). The assembled sequences were then aligned with Mega X™ (Kumar et al. 2018). The models of nucleotide substitution that best fit the dataset were selected from 24 different nucleotide substitution models using Akaike Information Criterion scores corrected for small sample sizes (AICc) in Mega X™ (Kumar et al. 2018). Non-uniformity of evolutionary rates among sites was modeled by using a discrete Gamma distribution (+G) with 5 rate categories and by assuming that a certain fraction of the sites is evolutionarily invariable (+I).

The evolutionary history was inferred by using the maximum likelihood method and General Time Reversible model (Nei and Kumar 2000). Initial trees for the heuristic

search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with the superior log likelihood value. A discrete Gamma distribution (+G) was used to model evolutionary rate differences among sites (5 categories (+*G* = 0.3803)). The 1st, 2nd, 3rd, and noncoding codon positions were included in the analysis. There was a total of 17,055 positions in the final dataset. Phylogenetic analysis was tested using bootstrap methodology with 500 bootstrap replications (Felsenstein 1985).

Divergence analysis

Divergence times among gar species were calculated using a timetree inferred using the RelTime method (Tamura et al. 2012, Tamura et al. 2018) and the General Time Reversible model (Nei and Kumar 2000). The timetree was computed using 3 calibration constraints computed by TimeTree (Hedges et al. 2006, Betancur-R et al. 2015, Rabosky et al. 2018). Evolutionary rate differences among sites were modeled with a discrete Gamma distribution (+G) of 8 nucleotide sequences using the 1st, 2nd, 3rd and noncoding codon positions. Evolutionary analysis was conducted on 17,055 positions in MEGA X ™ (Kumar et al. 2018).

Results

Mitochondrial genomes of Shortnose and Longnose Gar

The total length of the Shortnose Gar's, *Lepisosteus platostomus*, mitogenome was 16,688 base pairs (GenBank Accession No. MK771834). The mitogenome consisted of 22 tRNA genes, 2 rRNA genes and one control region. The total length of the Longnose Gar's, *Lepisosteus osseus*, mitogenome was 16,591 base pairs (GenBank

Accession No. MK771833). The mitogenome consisted of 22 tRNA genes, 2 rRNA genes and one control region.

Phylogenetic analysis

The best model to infer the evolutionary history of gar was the General Time Reversable Model (Table 2-1), which is currently by far the most commonly selected model for phylogenetic analysis (Sumner et al. 2012). The maximum likelihood tree generated using the General Time Reversable Model suggests three major evolutionary splits within the gar lineage (Figure 2-1). The first major split within the gar lineage occurred ~28 million years ago (MYA) between the *Lepisosteus* and *Atractosteus* genera. The second major split occurred ~13 MYA within the *Lepisosteus* genus. The third major split within the gar evolutionary lineage occurred ~ 7 MYA within the *Atractosteus* genus. All branches within this phylogenetic tree had high confidence values of 100.

The major splits within the gar's evolutionary lineage separated the extant species into three major clades (Figure 2-1). The second major split that occurred ~13 MYA within the *Lepisosteus* genus resulted in two clades, or groups of organisms that share a common ancestor. The first clade consists of the Florida Gar and the Spotted Gar. Florida Gar and the Spotted Gar are the most closely related as these species have only relatively recently diverged from each other ~20,000 years ago. The second clade within the *Lepisosteus* genus consists of Shortnose Gar and Longnose Gar. Shortnose Gar diverged from Longnose Gar ~5 MYA. The third clade was the result of the third major split within the gar evolutionary lineage that occurred ~ 7 MYA within the *Atractosteus* genus. The third clade contains Tropical Gar, Alligator Gar, and Cuban Gar. Alligator Gar have subsequently diverged from Cuban Gar ~3 MYA.

Discussion

In this study, I estimated phylogenetic relationships and divergence times amongst all extant gar species. My phylogenetic analysis suggested three major splits within the gar evolutionary lineage. Many of the previous morphological analyses were unable to resolve phylogenetic relationships within the *Lepisosteus* genus, specifically regarding the phylogenetic position of Longnose Gar and Shortnose Gar. One previous phylogenetic analysis suggested Longnose Gar shared a more recent common ancestor to Florida Gar and Spotted Gar (Grande 2010), whereas another suggested Shortnose Gar shared a more recent common ancestor to Florida Gar and Spotted Gar (Suttkus 1963). The topology of my phylogenetic analysis is congruent to a recent molecular phylogenetic analysis (Wright et al. 2012).

The phylogenetic tree I generated by this study offers a valuable look at the divergence times amongst closely related species and may also offer alternative conservation options for species in this family. One potential recovery option proposed for Cuban Gar is to use artificial propagation to bolster Cuban Gar populations; however, artificial propagation is unlikely to be successful due to the Cuban Gar's low effective population size and overall lack of genetic diversity (McCulloch and MacLean 1995, Barrientos-Villalobos and Espinosa de los Monteros 2008, Ulmo-Díaz et al. 2017b). Cuban Gar is the only gar species within its range; therefore, natural hybridization will not occur. Another conservation strategy may be to artificially hybridize Cuban Gar with another closely related gar species. Artificially hybridizing Cuban Gar with a closely related species, such as Alligator Gar or Tropical Gar, would preserve much of the genetic qualities of the Cuban Gar while maintaining an apex predator within this

ecosystem. Intentional hybridization is not usually the preferred choice amongst conservation managers; however, it has been effective in some situations where genetic diversity is not recoverable within the original population, such as with the Headwater Livebearer, *Poeciliopsis monacha*, and the Florida panther, *Puma concolor coryil* (Vrijenhoek 1998, Land and Lacy 2000, Allendorf et al. 2001). Future studies should explore whether artificial hybridization between Cuban Gar and a closely related species can sustainably maintain an apex predator in the Cuban Gar's native ecosystems.

I demonstrated, with the phylogenetic tree generated by this study, the utility of complete mitochondrial genomes for evaluating evolutionary relationships. Future studies should focus on sequencing the complete mitochondrial genomes and depositing them into genetic repositories for large-scale phylogenetic comparative analyses. In addition to the two gar mitochondrial genomes, I also sequenced 59 additional species of fish in an effort to better represent the diversity of fishes in the Mississippi River Basin within NCBI (Appendix A).

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Table 2-1: Maximum likelihood fits of 24 different nucleotide substitution models. Models with the lowest Akaike Information Criterion scores corrected for small sample size (AICc) are considered to describe the substitution pattern the best. Some models use a discrete Gamma distribution (+G) to take into consideration non-uniformity of evolutionary rates among sites. In addition, some models also assume that a certain fraction of the sites was evolutionarily invariable (+I). For estimating maximum likelihood values, a tree topology was automatically computed. The analysis involved 8 nucleotide sequences. The 1st, 2nd, 3rd, and noncoding positions were included. All positions containing gaps and missing data were eliminated. There was a total of 15,812 positions in the final dataset.

Model	AICc
General Time Reversible + G	95614.71
General Time Reversible +G+I	95616.71
Tamura-Nei +G	95849.66
Tamura-Nei +G+I	95851.67
Hasegawa-Kishino-Yano +G	95921.32
Hasegawa-Kishino-Yano +G+I	95923.33
Tamura 3-parameter +G	97212.66
Tamura 3-parameter +G+I	97214.66
General Time Reversible +I	97484.65
Kimura 2-parameter +G	97631.36
Kimura 2-parameter +G+I	97633.36
General Time Reversible	97713.49
Tamura-Nei +I	97745.32
Hasegawa-Kishino-Yano +I	97907.53
Tamura-Nei	98051.67
Hasegawa-Kishino-Yano	98135.23
Tamura 3-parameter +I	99099.86
Tamura 3-parameter	99198.39
Kimura 2-parameter +I	99558.12
Kimura 2-parameter	99588.48
Jukes-Cantor +G	101526.71
Jukes-Cantor +G+I	101528.65
Jukes-Cantor	102754.94
Jukes-Cantor +I	102756.92

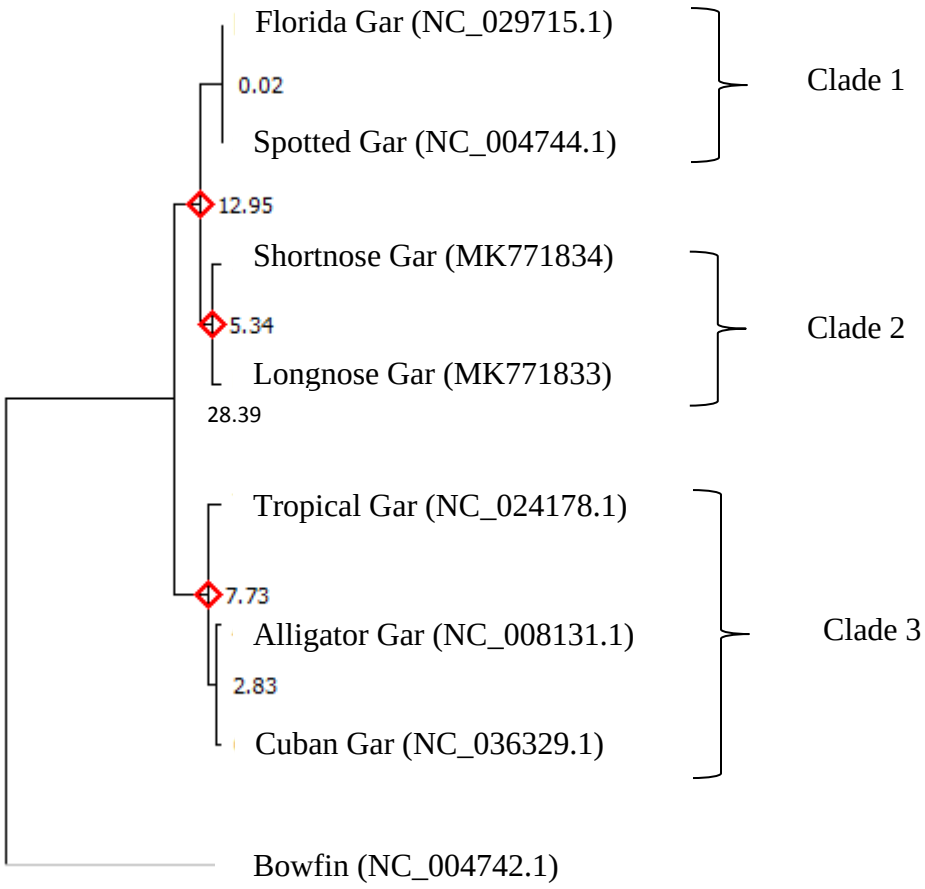

Figure 2-1: Timetree of gar phylogenetic history. The evolutionary history was inferred by using the maximum likelihood method and General Time Reversible model. The tree with the greatest log likelihood (-50831.88) is shown. The percentage of trees in which the associated taxa clustered together is provided next to the branch. A discrete Gamma distribution (+G) was used to model evolutionary rate differences among sites (5 categories (+*G* = 0.3803)). The timetree was computed using 3 calibration constraints that are represented by red diamonds. This analysis involved 8 nucleotide sequences. There was a total of 17,055 positions in the final dataset.

Chapter 3: Using Mitochondrial Markers to explore gut-microbial diversity of Pallid Sturgeon

Introduction

The gut microbiome is the collection of microorganisms housed within an organism's intestinal tract. The study of the microbiome is a relatively new field, with the first gut bacteria being sequenced in 1996 (Wilson and Blitchington 1996). The gut microbiome plays integral roles in organisms' absorption of nutrients through digestion and in organisms' innate immunity (O'Hara and Shanahan 2006, Huttenhower et al. 2012). Fish intestines, in particular, harbor diverse populations of microorganisms, especially bacteria (Cahill 1990, Ringø et al. 1995) that are dominated by members of the phyla Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, and Fusobacteria (Sullam et al. 2012). There are three major groups of factors that dictate fishes' gut microbiomes, including ecological conditions, environmental conditions, and host trophic-level feeding habits (Talwar et al. 2018). Fluctuations in any of these groups of factors, such as transitions between environments, are likely to result in changes within the intestinal microbiome community structure (Sullam et al. 2012, Wong and Rawls 2012, Baldo et al. 2015, Eichmiller et al. 2016). Understanding how ecological conditions, environmental conditions, and host trophic-level feeding habits impact a fish's gut microbiome may offer new insights into conservation strategies and behaviors for fishes.

Hatchery stocking has been one conservation method employed for threatened and endangered fishes (Brown and Day 2002). The rearing environment within the hatchery may play a large role in the initial establishment of an organism's microbiome. Gut microbial communities of captive fishes can differ substantially from those of wild

fishes due to prepared diet and increased density of organisms in a confined environment (Wong et al. 2013). The diet fed to fish raised in a hatchery setting is a major driver that establishes an organism's principal core microbiome. This diet may not reflect the diet of a wild organism and, as such, this core microbiome may not adequately prepare this organism for release into the wild.

The endangered Pallid Sturgeon, *Scaphirhynchus albus* (Federal Register 1990, 55:36641), is a native species of the Mississippi and Missouri rivers (Forbes and Richardson 1905, Dryer and Sandvol 1993) where stocking has been employed to bolster wild populations. Given the endangered status, the U.S. Fish and Wildlife Service developed a recovery plan in 1993 to mitigate population declines (Dryer and Sandvol 1993). The 1993 recovery plan recommended stocking > 100,000 juvenile Pallid Sturgeon in the Missouri River annually during 1992-2004 and intensive monitoring of the population (Dryer and Sandvol 1993, Krentz et al. 2005). A decline in Pallid Sturgeon body condition has been observed since the severe 2011 flooding of the Missouri River (Steffensen and Mestl 2016, Randall et al. 2017, Steffensen et al. 2017). This decline in fish condition may affect population growth, maturation, reproductive frequency, fecundity, and survival (Pope and Kruse 2007) that could negatively impact conservation efforts. A compositional change in the gut microbiome may be an underlying cause for the decline in Pallid Sturgeon body condition, although the gut microbiome of Pallid Sturgeon remains unexplored.

Comparing intestinal microbial diversity and composition between hatchery-raised and wild Pallid Sturgeon may provide insight on the transition from hatchery environment to a wild environment. Understanding the factors that impact the Pallid

Sturgeon's gut microbiome may facilitate conservation efforts. In this study, I a) characterize the gut microbiomes of hatchery-raised and wild Pallid Sturgeon, and b) compare bacterial diversity between hatchery-raised and wild Pallid Sturgeon.

Methods

Specimen collection

Colonic 16S ribosomal RNA samples were obtained from Pallid Sturgeon by Nebraska Game and Parks Commission biologists during routine spring monitoring that is part of Nebraska's sturgeon management initiative (Table 3-1). Pallid Sturgeon were collected with drifted trammel nets and stationary trotlines. Pallid Sturgeon fecal samples, that contained 16S ribosomal RNA, were obtained using colonic flushing. The colonic flushing apparatus consisted of a 60-mL catheter tip syringe (#CTSLS3, Care Touch**,** Brooklyn, New York) fitted with a 41-cm, 3.3-mm urethral catheter commonly used in veterinary medicine (# 701017, Kendall Company, Mansfield, Massachusetts). The bottle was filled with distilled water and the catheter-end was gently inserted 30–50 mm through the fish's anus into the colon. The colon was flushed until expelled water was clear. All materials (solid feces, flushed liquid) were poured into a 500-ml sample jar and preserved with an equal volume of 100% ethanol.

16S RNA extraction and analysis

Mitochondrial 16S ribosomal RNA was extracted using the MoBio PowerSoil DNA Isolation Kit. The primer pair used for amplification of the V3-V4 region of the 16S amplicon was S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21 (Klindworth et al. 2013). Primers were added according to the TaggiMatrix 16S PCR Protocol using fusion-indexed primers (Glenn et al. 2019). The ribosomal RNA was sequenced with an

Illumina MiSeq on a PE300 run at Environmental Health Science laboratory at the University of Georgia. Sequences were trimmed with GENEIOUS™ 10.2.6 (Biomatters, Newark, NJ) after raw reads were paired. Chimeric reads were removed following merging of paired reads and were analyzed using the metagenomic 16S analysis pipeline in GENEIOUS™ 10.2.6 (Biomatters, Newark, NJ) (Kearse et al. 2012).

Core gut microbiome

Bacteria that comprise the five highest relative abundances will be considered part of the Pallid Sturgeon's 'core gut microbiome.' Relative abundance was calculated as a percentage of the consensus regions of RNA of an identified bacterial genus divided by the total number of consensus regions of RNA (Timmerman et al. 2017). The average relative abundance of consensus regions of RNA had to be greater than or equal to 0.1% at the genus rank to be included in the statistical analysis. Results in tables and figures represent these values. Only baseline gut microbiome data are presented.

Comparison between hatchery-raised and wild Pallid Sturgeon gut microbiomes

A Kruskal–Wallis test with a Bonferroni correction to minimize Type I error (Kruskal and Wallis 1952, Deering et al. 2020) was used to determine the impact of origin (hatchery-raised or wild) on the relative abundance of bacteria within the gut bacterial microbiome. The Kruskal–Wallis test with a Bonferroni correction was conducted in R Studio Version 1.1.463. Statistical significance was set at 0.05.

Diversity is a measure of number, type and/or evenness of bacteria within the gut of the Pallid Sturgeon. Alpha diversity is the average bacterial diversity in the gut bacterial microbiome of each Pallid Sturgeon and was calculated using the Shannon diversity index:

$$H = -\sum_{i=1}^{S} p_i \ln p_i$$

where H is the Shannon diversity index, S is the total number of bacterial genera in the community (richness), and p_i is the proportion of S made up of the i^{th} species (Shannon and Weaver 1949). Alpha diversity was calculated after square root transformation using PRIMER-e Version 7 (Quest Research Ltd., Auckland, New Zealand) (Deering et al. 2020). Average H was used to compare the alpha diversity of gut bacterial microbiomes of hatchery-raised Pallid Sturgeon to the alpha diversity of gut bacterial microbiomes of wild Pallid Sturgeon. An independent sample t-test analysis in R Studio Version 1.1.463 was used to assess the differences in alpha diversity of the gut bacterial microbiome in relation to the origin of the Pallid Sturgeon (hatchery-raised vs. wild). Statistical significance was set at 0.05.

Beta diversity was calculated using PRIMER-e Version 7 (Quest Research Ltd., Auckland, New Zealand) (Deering et al. 2020) according to the following equation:

$$C_{ST} = \frac{T}{T-1}\left(1 - \frac{S_T}{\sum_i ai}\right)$$

where C_{ST} is the Sørensen similarity index, T is the number of sites, S_T is the total number of species, and ai is the number of species in site Ai, $i=1,\ldots,T$ as described by Diserud and Odegaard (2007). The betapart package (Baselga 2017) in R Studio Version 1.1.463 was used to assess the difference in beta diversity of the gut bacterial microbiome in relation to the origin of the Pallid Sturgeon (hatchery-raised vs. wild). Statistical significance was set at 0.05.

Results

Core gut microbiome

Mitochondrial 16S ribosomal RNA was extracted from 44 fish, 39 that had been hatchery-raised and 5 that had been reared in the wild, from five sites along the Missouri River (Table 3-1). Unfortunately, one sample generated few genetic sequences and was excluded from further analysis, leaving 38 hatchery-raised fish and 5 wild fish for analysis. In total, 1,995,012 raw reads were obtained for both forward and reverse directions with a depth of 1,929,957 ± 96,497 sequences per exon. After quality filtering and merging trimmed paired reads, 84,740 reads were mapped to exons with a mean of 1,970 reads/sample.

Overall, twenty-one bacterial phyla were identified at relative abundances greater than or equal to 0.1% (Table 3-2). Overall, at the phylum taxonomic rank, the microbiome was dominated by Fusobacteria (42%), Firmicutes (23%), Proteobacteria (20%), Actinobacteria (14%) and Bacteriodetes (4%). In total, 321 bacterial genera were identified at relative abundances ≥ 0.1% (Table 3-2). At the genus taxonomic rank, the bacterial gut microbiome was dominated by *Cetobacterium* (54%), *Carnobacterium* (43%), *Flavobacterium* (34%), and *Methylobacterium* (22%).

Alpha diversities ranged from 2.80-3.33 for hatchery-raised Pallid Sturgeon. The average alpha diversity was 3.08 for hatchery-raised Pallid Sturgeon. Alpha diversities for wild Pallid Sturgeon ranged from 3.14-3.29. The average alpha diversity was 3.19 for wild Pallid Sturgeon.

Beta diversity of the gut bacterial microbiome of hatchery-raised Pallid Sturgeon was 0.60. Beta diversity of the gut bacterial microbiome of wild Pallid Sturgeon was 0.84.

Comparison between hatchery-raised and wild Pallid Sturgeon gut microbiomes

I assessed differences in the relative abundances of bacterial genera in guts of hatchery-raised Pallid Sturgeon and wild Pallid Sturgeon using the Kruskal–Wallis test with a Bonferroni correction. There was no significant difference between relative abundances of bacterial genera in guts of hatchery-raised Pallid Sturgeon compared to wild Pallid Sturgeon ($p=0.81$, degrees of freedom=2). There were 65 genera that were found in the gut microbiome of both hatchery-raised and wild Pallid Sturgeon (Table 3-2).

I compared the average Shannon diversity index (H) of hatchery-raised Pallid Sturgeon to the average Shannon diversity index (H) of wild Pallid Sturgeon using an independent sample t-test. There were no significant differences in alpha diversity of bacterial genera of the gut microbiome between hatchery-raised Pallid Sturgeon and wild Pallid Sturgeon ($p=0.07$, degrees of freedom=2).

I compared the beta diversity of the bacterial genera in the gut microbiome of hatchery-raised Pallid Sturgeon to the beta diversity of the bacterial genera in the gut microbiome of wild Pallid Sturgeon using the betapart package in R Studio Version 1.1.463. There was no significant difference in beta diversity between the bacterial genera of the gut microbiome of hatchery-raised Pallid Sturgeon compared to the beta diversity of the bacterial genera of the gut microbiome of wild Pallid Sturgeon ($p=0.06$, degrees of freedom=2).

Discussion

Gut microbiota are believed to play intrinsic roles in health, growth and disease status in animals (Ley et al. 2008, Lee and Mazmanian 2010, Dehler et al. 2017). Maintaining breeding populations of animals in captivity, including the endangered Pallid Sturgeon, may well require managing their gut microbiomes (Redford et al. 2012). I determined that Pallid Sturgeon's core gut microbiome consisted of a large proportion of Fusobacteria, Proteobacteria and Firmicutes. These phyla have dominated other fishes' microbiomes and may represent a 'core microbiome' (Li et al. 2014, Givens et.al 2015).

Xu and Knight (2014) suggested that long-term diet has the greatest effect on microbiome diversity. Fishes that were either herbivores or omnivores had a higher alpha diversity than fishes that were carnivores or piscivores (Ley et al. 2008). Alpha diversity values calculated for the Pallid Sturgeon (2.80-3.33) fell in the upper range of alpha diversity values reported for other fishes (Li et al. 2014, Givens et. al 2015), perhaps an indication that the Pallid Sturgeon may be an omnivore rather than a piscivore as previously described (Gerrity et al. 2006). Future research should use alternative methods to identify prey items, such as sequencing the COX I gene, from colonic samples to test whether the Pallid Sturgeon is an omnivore (Pompanon et al. 2012).

Overall, diversity of the gut microbiome for at-large Pallid Sturgeon was not impacted by rearing environment. There were no significant differences between alpha or beta diversities of gut bacterial microbiomes of hatchery-raised Pallid Sturgeon compared to wild Pallid Sturgeon, providing evidence that the gut microbiome of hatchery-raised Pallid Sturgeon transitioned effectively after release into the wild. Even so, the turnover process of gut microbiomes remains unexplored (assuming the gut microbiome was

different in the hatchery environment). Intestinal epithelium turns over rapidly, approximately one to three billion and one-hundred to three-hundred million cells are shed per hour in the small intestine and in the colon, respectively (Xu and Gordon 2003); therefore, gut microbiomes of the captured, hatchery-raised fish may have completed the transition in a brief period, perhaps days to weeks after release. Future research should look at different periods following release from the hatchery to explore how and when gut microbial transitions occur.

Genetic markers provided a new way to assess management efforts for the endangered Pallid Sturgeon by demonstrating that the gut bacterial microbial diversity of hatchery-raised Pallid Sturgeon was not significantly different than wild Pallid Sturgeon. Future management efforts should focus on ensuring stocked Pallid Sturgeon have the food resources they need following release from the hatchery. *Macrhybopsis* chubs were previously identified as a key prey item during the juvenile and adult stages for the Pallid Sturgeon, particularly the Shoal Chub, *Macrhybopsis hyostoma* (Gerrity et al. 2006). Unfortunately, population reductions exceeding 70% for all *Macrhybopsis* chubs have also recently been observed within the upper Mississippi River Basin (Steffensen et al. 2014). Genetic markers from this study provide initial evidence that Pallid Sturgeon may be omnivorous. Sequencing additional genetic markers such as the COX I gene (Pompanon et al. 2012) will help identify the breadth of the Pallid Sturgeon's diet so that conservation managers can ensure Pallid Sturgeon have the resources they need to maintain a healthy body condition factor.

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Table 3-1: Geographic data for the collected Pallid Sturgeon.

Origin	Sex	Capture Location	Latitude	Longitude	Date Collected	Tag Number
Hatchery	Female	Calumet-Bartlett Bend	40.89	-95.81	4/12/2018	4627152F1A
			40.88	-95.82	4/13/2018	470A643317
		Lower Plattsmouth Bend	41.00	-95.86	4/4/2018	4A466B2E78
			40.99	-95.85	4/5/2018	434771011A
		Lower Plattsmouth Bend	40.99	-95.85	4/6/2018	4900607F63
			41.01	-95.87	4/7/2018	434A03184E
		Rock Bluff Bend	40.94	-95.84	4/5/2018	434A5C7340
			40.91	-95.83	4/10/2018	4627056D3B
		Rock Bluff Bend	40.91	-95.82	4/10/2018	4627273324
			40.94	-95.84	4/13/2018	471269730D
		Tobacco Bend	40.98	-95.83	4/5/2018	43693D7261
			40.98	-95.83	4/6/2018	4627313872
			40.96	-95.83	4/11/2018	47191F2B24
		Upper Plattsmouth Bend	41.04	-95.86	4/8/2018	435F151E79
			41.02	-95.86	4/8/2018	43615C157E
			41.02	-95.86	4/8/2018	4367560D5D
			41.02	-95.86	4/8/2018	4369627915
			41.02	-95.86	4/13/2018	471E0C4B0E
		Van Horns Bend	40.84	-95.84	4/10/2018	44451B466D

Table 3-1 (continued)

Origin	Sex	Capture Location	Latitude	Longitude	Date Collected	Tag Number
			40.84	-95.84	4/10/2018	46264C5368
			40.84	-95.84	4/10/2018	471979463C
			40.84	-95.84	4/10/2018	487F075D74
Hatchery	Male	Calumet-Bartlett Bend	40.91	-95.82	4/10/2018	46267F6129
			40.90	-95.81	4/12/2018	4627702A4D
		Lower Plattsmouth Bend	40.99	-95.85	4/6/2018	47161C0357
			41.00	-95.87	4/6/2018	47191A7D15
			40.99	-95.85	4/6/2018	847F623E77
			41.00	-95.86	4/8/2018	4A467F4F41
		Rock Bluff Bend	40.93	-95.84	4/5/2018	434A41496C
			40.94	-95.84	4/5/2018	46280A267E
			40.92	-95.84	4/10/2018	46267A6226
		Tobacco Bend	40.99	-95.84	4/6/2018	4349450B3E
		Upper Plattsmouth Bend	41.02	-95.86	4/7/2018	434A4F216C
Hatchery	Unknown	Tobacco Bend	40.99	-95.84	4/6/2018	4.25736E+83
Unknown	Female	Calumet-Bartlett Bend	40.88	-95.82	4/12/2018	47134D0F2A
		Lower Plattsmouth Bend	41.01	-95.87	4/7/2018	434A6B1F62
		Upper Plattsmouth Bend	41.03	-95.86	4/7/2018	46256D3718
Unknown	Male	Lower Plattsmouth Bend	40.99	-95.85	4/6/2018	43449D6C1E
		Rock Bluff Bend	40.91	-95.83	4/10/2018	434969543E
Wild	Female	Lower Plattsmouth Bend	41.00	-95.86	4/4/2018	4B191E7809
Wild	Female	Upper Plattsmouth Bend	41.03	-95.86	4/8/2018	4627683B2B
Wild	Male	Calumet-Bartlett Bend	40.90	-95.81	4/12/2018	470B110F50
		Lower Plattsmouth Bend	41.01	-95.87	4/7/2018	462622242B
		Tobacco Bend	40.97	-95.83	4/4/2018	4704510611

Table 3-2: All bacterial genera at greater than 0.1% average relative abundance in guts of hatchery-raised and wild Pallid Sturgeon. Relative abundance was calculated as a percentage of the consensus sequences of RNA of a genus divided by the total number of consensus sequences.

Phylum	Genus	Hatchery-raised	Wild
Actinobacteria	*Acidimicrobineae*	X	
	Aciditerrimonas	X	
	Acidothermus	X	
	Actinomyces		X
	Actinotalea	X	
	Aestuariimicrobium		X
	Arthrobacter	X	X
	Bifidobacteriaceae	X	
	Corynebacterineae	X	
	Corynebacterium	X	X
	Cryobacterium	X	X
	Dermacoccus	X	
	Dermatophilus	X	X
	Ferrithrix	X	
	Fluviicola	X	
	Fodinicola	X	
	Friedmanniella	X	
	Gaiella	X	
	Gaiellaceae	X	
	Gardnerella	X	
	Gordonia	X	
	Gulosibacter	X	
	Iamia	X	
	Ilumatobacter	X	
	Janibacter	X	X
	Leifsonia		X
	Marmoricola	X	
	Microbacterium		X
	Micrococcineae	X	
	Micrococcus	X	X
	Millisia	X	X
	Mycetocola	X	
	Nexterenkonia	X	
	Nitriliruptor	X	
	Nocardioides	X	
	Phycicoccus	X	
	Phytomonospora	X	
	Propionibacterium	X	X
	Rhodococcus	X	X
	Rhodoglobus	X	

Table 3-2 (continued)

Phylum	Genus	Hatchery-raised	Wild
	Rothia	X	X
	Skermania	X	
	Streptosporangineae	X	
	Terracoccus	X	
	Thermoleophilum	X	
	Turicella	X	
	Varibaculum	X	
	Williamsia	X	
	Xylanimicrobium		X
Aquificae	*Aquifex*	X	
	Venenivibrio	X	X
Bacteroidetes	*Algoriphagus*	X	
	Alistipes	X	
	Alloprevotella	X	X
	Anaerorhabdus	X	
	Arenibacter	X	
	Barnesiella	X	
	Chryseobacterium	X	
	Cloacibacterium	X	
	Dyadobacter	X	
	Falsiporphyromonas		X
	Faucicola		X
	Filimonas	X	
	Flavobacterium	X	X
	Fluviicola	X	
	Frondibacter	X	
	Haliscomenobacter	X	
	Heliimonas	X	
	Hymenobacter		X
	Lacihabitans	X	
	Macellibacteroides	X	
	Mooreia	X	X
	Odoribacter	X	
	Ornithobacterium	X	
	Paludibacter	X	
	Parabacteriodes	X	X
	Parafilimonas	X	
	Paraprevotella	X	
	Pedobacter	X	
	Petrimonas	X	
	Phaeocystidibacter	X	
	Porphyromonas	X	
	Portibacter	X	

Table 3-2 (continued)

Phylum	Genus	Hatchery-raised	Wild
	Prevotella	X	X
	Pseudarcicella	X	X
	Rubricoccus	X	
	Sediminibacterium	X	
	Solitalea	X	
	Soonwooa	X	
	Sphingobacterium	X	
	Tannerella	X	X
	Wenyingzhuangia	X	
Betaproteobacteria	*Paucibacter*	X	
Chlamydiae	*Neochlamydia*	X	
	Parachlamydia	X	
	Simkania	X	
Chloroflexi	*Ardenticatena*	X	X
	Kallotenue	X	
	Ktedonobacter	X	
	Leptolinea	X	
	Litorilinea	X	
	Nitrolancea	X	
	Ornatilinea	X	
	Pelolinea	X	
Crenarchaeota	*Caldisphaera*	X	
	Caldivirga	X	
	Sulfurisphaera	X	
	Thermocladium	X	
	Thermoproteus	X	
Deinococcus-Thermus	*Deinococcus*	X	
Euryarchaeota	*Methanomassiliicoccus*	X	
	Methermicoccus	X	X
	Salinigranum	X	
Firmicutes	*Acetanaerobacterium*	X	
	Acetobacterium	X	
	Alkalibaculum	X	X
	Allobaculum	X	
	Alloiococcus	X	
	Anaerobacillus	X	
	Anaerobacter	X	X
	Anaerococcus	X	X
	Anaerosphaera	X	X
	Anaerovirgula	X	
	Anoxynatronum	X	
	Atopobacter	X	
	Bacillus	X	

Table 3-2 (continued)

Phylum	Genus	Hatchery-raised	Wild
	Bacteroides	X	
	Blautia	X	X
	Brassicibacter	X	
	Bulleidia	X	
	Butyrivibrio	X	
	Carnobacterium	X	X
	Centipeda	X	
	Clostridia	X	
	Clostridium IV	X	X
	Clostridium sensu stricto	X	X
	Clostridium XI	X	
	Clostridium XIVa	X	
	Coprobacillus	X	X
	Coprococcus	X	X
	Defluviitalea	X	
	Eisenbergiella	X	
	Eremococcus	X	
	Ethanoligenens	X	
	Eubacterium	X	
	Faecalibacterium	X	X
	Falsibacillus	X	
	Finegoldia	X	
	Flavonifractor	X	
	Gemella	X	
	Globicatella		X
	Hathewaya	X	
	Lactobacillus	X	X
	Lactococcus	X	
	Lactonifactor	X	
	Murimonas	X	
	Nosocomiicoccus	X	X
	Parvimonas	X	
	Pelosinus	X	
	Peptoniphilus	X	X
	Polycladomyces	X	
	Proteocatella	X	
	Pseudobutyrivibrio	X	
	Romboutsia	X	
	Ruminococcus	X	
	Schwartzia	X	
	Sinibacillus	X	
	Solobacterium	X	
	Sporobacter	X	

Table 3-2 (continued)

Phylum	Genus	Hatchery-raised	Wild
	Staphylococcus	X	X
	Streptococcus	X	X
	Succinispira	X	
	Syntrophaceticus	X	
	Terrisporobacter	X	
	Turicibacter	X	
	Ureaplasma	X	
	Veillonella	X	
	Peptoniphilus	X	
	Polycladomyces	X	
	Proteocatella	X	
	Pseudobutyrivibrio	X	
Fusobacter	*Fusobacterium*	X	
Fusobacteria	*Cetobacterium*	X	X
	Leptotrichia	X	X
	Psychrilyobacter	X	
	Streptohalobacillus	X	
Hydrogenobacter/Aquifex	*Hydrogenothermus*	X	
Nitrospirae	*Nitrospira*	X	
	Pacearchaeota Incertae	X	
Pacearchaeota	*Sedis AR13*		
Planctomycetes	*Aquisphaera*	X	
	Gemmata	X	X
	Gimesia	X	
	Planctopirus	X	
	Schlesneria	X	
	Telmatocola	X	
	Tepidisphaera	X	
	Thermogutta	X	
	Zavarzinella	X	
Proteobacteria	*Acinetobacter*	X	X
	Aeromonas	X	X
	Aestuariispira	X	
	Afipia	X	
	Alsobacter		X
	Amorphus	X	
	Anderseniella	X	
	Aquicella	X	X
	Arcobacter	X	
	Arenimonas	X	
	Aureimonas	X	
	Azomonas	X	
	Azorhizophilus	X	

Table 3-2 (continued)

Phylum	Genus	Hatchery-raised	Wild
	Bdellovibrio	X	
	Beggiatoa	X	
	Beijerinckia	X	
	Bilophila	X	
	Blastochloris	X	
	Bradyrhizobium	X	X
	Brevundimonas	X	X
	Cardiobacterium	X	
	Caulobacter	X	X
	Cetia	X	
	Chelativorans	X	
	Collimonas	X	
	Coxiella	X	X
	Curvibacter	X	
	Deefgea	X	
	Desulfomonile	X	
	Desulforegula	X	
	Desulfosalsimonas	X	
	Devosia	X	
	Diplorickettsia	X	X
	Endobacter	X	
	Enhydrobacter	X	X
	Ewingella	X	
	Fontimonas	X	
	Gammaproteobacteria	X	
	Gemmobacter	X	
	Haemophilus	X	X
	Halochromatium	X	
	Halomonas	X	
	Humitalea	X	
	Hyphomicrobium	X	
	Iodobacter	X	
	Janthinobacterium	X	
	Kingella	X	
	Labilitrichaceae	X	
	Lawsonia	X	
	Leeia	X	
	Legionella	X	X
	Leminorella	X	
	Limimonas	X	
	Lonsdalea	X	
	Maricaulis	X	
	Marinicauda	X	

Table 3-2 (continued)

Phylum	Genus	Hatchery-raised	Wild
	Mesorhizobium	X	X
	Methylobacterium	X	X
	Methyloceanibacter	X	
	Methylococcus	X	X
	Methyloprofundus	X	
	Moellerella	X	
	Moraxella	X	
	Morococcus	X	X
	Motiliproteus	X	
	Neisseria	X	
	Nitrobacter	X	
	Novosphingobium	X	
	Obesumbacterium	X	
	Oligoflexus	X	
	Orientia	X	
	Paludibacterium	X	
	Pantoea	X	X
	Paracoccus	X	
	Paraferrimonas	X	
	Pedomicrobium	X	
	Pelagibius	X	X
	Pelomonas	X	
	Phreatobacter	X	
	Piscirickesttsia	X	
	Plesiomonas	X	
	Polyangiaceae	X	
	Polymorphobacter	X	
	Polynucleobacter	X	
	Pseudaminobacter	X	
	Pseudobacteriovorax	X	
	Pseudolabrys	X	
	Pseudomonas	X	X
	Psychrobacter	X	
	Rahnella	X	
	Reyranella	X	
	Rhizobium	X	X
	Rhizorhabdus	X	
	Rhodoplanes	X	X
	Rivicola	X	
	Salinarimonas	X	
	Serpens	X	
	Serratia	X	X
	Simonsiella	X	X

Table 3-2 (continued)

Phylum	Genus	Hatchery-raised	Wild
	Sphingomonas	X	X
	Sphingorhabdus	X	
	Stenotrophomonas	X	X
	Tepidimonas		X
	Vasilyevaea	X	
	Vibrio	X	X
	Yersinia	X	
	Saccharibacteria genera incertae sedis	X	
Saccharibacteria			
Spirochaetes	*Brevinema*	X	
	Leptolinea	X	
	Turneriella	X	
Streptophyta	*Streptophyta*	X	
Synergistetes	*Thermovirga*	X	
Thaumarchaeota	*Nitrososphaeraceae*	X	
Thermotogae	*Mesoaciditoga*	X	
Verrucomicrobia	*Akkermansia*	X	
	Cerasicoccus	X	
	Luteolibacter	X	
	Persicirhabdus	X	
	Puniceicoccus	X	

Chapter 4: Using nuclear genetic markers to investigate hybridization between Bighead Carp and Silver Carp in native range

Introduction

Interspecific hybridization is a complex phenomenon that is a focal study area for speciation events (Rice 2013). Hybrids have varying levels of fitness, ranging from completely nonviable and inferior to equal or superior to the parental individuals. Hybrids with superior fitness foster rapid adaptation, which potentially defies the identified pre- and post-zygotic barriers that define species' boundaries. Rapid adaptation may also facilitate range expansion of a species and may enhance the invasiveness of a species intentionally introduced outside of its native range.

Bighead Carp, *Hypophthalmichthys nobilis,* and Silver Carp, *H. molitrix,* (bigheaded carps) are native to East Asia and are sympatric members of the Cyprinidae family that diverged approximately 9.6 MYA (Wang et al. 2019). Bighead Carp is large, deep-bodied, and moderately compressed laterally with a disproportionally large head and mouth and a ventral keel that extends to the base of the pelvic fins (Henderson 1982). Bighead Carp has a broad distribution from the Pearl River in southern China to the Heilongjiang River in northern China where it inhabits the upper layers of lakes, rivers, and reservoirs and feeds on zooplankton using comb-like gill-rakers. Bighead Carp is a synchronous and gonochoristic species that has a single spawning season early in the summer. During spawning, adult Bighead Carp migrate upstream to spawning grounds characterized by rapid flowing waters. Bathypelagic eggs are deposited in the rocks of river channels, sandbars or at the junction of currents. Following deposition, eggs drift to nursery grounds such as flooded lakes, creeks, and channels (Nikolsky 1963, Chang

1966). Silver Carp has a deep, laterally compressed body with a ventral keel extending from the isthmus to the anus and sponge-like gill-rakers. Silver Carp also has a large distribution across East Asia. Silver Carp require standing or slow-flowing conditions, such as impoundments or backwaters, where it feeds primarily on phytoplankton. Silver Carp migrates upstream to breed and afterwards the eggs and larvae drift towards floodplain areas (FAO 2005).

Natural hybridization between Bighead Carp and Silver Carp is considered rare within native regions in China; there are only scarce literary references to hybridization and these references do not include hybridization rates (Chapman 2006). Hybridization between Bighead Carp and Silver Carp seems to occur readily within novel environments (Kolar et al. 2007, Lamer et al. 2010). Intrinsic genomic features, including high genomic similarity and recent divergence time, facilitate hybridization between Bighead Carp and Silver Carp in novel environments (Wang et al. 2019).

I speculate, given hybridization in novel environments, that relaxation of pre-zygotic barriers would increase hybridization between Bighead Carp and Silver Carp in their native range. Understanding the drivers behind hybridization is critical, particularly because the native environment in China will likely change with the construction of the Three Gorges Dam. Dams disrupt seasonal flow fluctuations, damage spawning grounds, and impede migratory routes (Dudgeon 2000, Pringle et al. 2000, Rosenberg et al. 2000, Wu et al. 2004). Several migratory Chinese species have already been impacted by the nearby Gezhouba Dam (40-km downstream of the Three Gorges Dam), including the Chinese sturgeon, *Acipenser sinensis*, and River sturgeon, *Acipenser dabryanus* (Xie 2003).

Bighead Carp and Silver Carp have been introduced to 74 and 80 countries, respectively, and extensive hybridization has been documented in these introduced regions, presumably facilitated by novel environmental factors in addition to intrinsic genomic compatibility (Kolar et al. 2007, Li et al. 2011, Wang et al. 2019). Filial 1 (F1) hybrids are less prevalent in natural situations based on field studies likely due to the variety of maladaptive gill-raker morphologies that have been observed, including clubbed ends, waviness, raggedness, incomplete fusion, and twisting (Kolar et al. 2007).

It is imperative to determine how prevalent hybridization is in native regions in China for future studies to track how anthropogenic disturbances, such as the Three Gorges Dam, affect hybridization in native populations of Bighead and Silver Carp. It is also important to determine how prevalent hybridization is in native regions as a baseline for comparison with hybridization in novel environments. Lamer et al. (2014) recently developed 57 nuclear and 1 mitochondrial diagnostic single nucleotide polymorphisms (SNPs) that could verify individual genotype of pure parental species as well as hybrids; thus, biologists can now better understand populations' dynamics of Asian carps, including hybrids, on geographical and temporal scales. The objective of this study is to determine percentages of Bighead Carp and Silver Carp hybrids in three Chinese rivers.

Methods

Sample collection and DNA extraction

Tissue from Bighead Carp and Silver Carp were collected by Dr. Wang and Dr. Li during 2005-2007 from the Chinese Amur, Pearl, and Yangtze rivers. Specimens that had a ventral keel extending from the isthmus to the anus were morphologically classified as Silver Carp. Specimens that had a disproportionately large head and a ventral keel that

extended to the base of the pelvic fins were morphologically classified as Bighead Carp. Fin clips were preserved in 100% ethanol (molecular grade) in the field. Preserved tissues were transported to Shanghai Ocean University in Shanghai, China and stored at room temperature. The DNA was extracted using the saturated sodium chloride method (Zhou et al. 2012). The quality of the extracted DNA was evaluated on 1.0% agarose gels stained with Tiangen GeneGreen.

Primer design, PCR amplification and genotyping

To determine the genotype of the fish, 25 primers (Table 4-1) were designed using *WebPrimer* based on previously published and validated SNPs (Engel and Cherry 2013, Lamer et al. 2014).

Polymerase chain reaction (PCR) was performed on an Eppendorf Thermal Cycler in a reaction mixture. Each 50-uL PCR reaction consisted of 2-uL of each primer, 25-uL 2× PCR Master Mix, 22-uL ddH_2O and 1-uL DNA. PCR was carried out at 95 °C for 5 min, 35 cycles at 95°C for 30 s, 58°C for 30 s, 72°C for 1 min, with a final extension of 72°C for 10 min. The PCR products were evaluated on a 1.0% agarose gel stained with Tiangen GeneGreen. Successful PCR products were sequenced on an ABI 3730 (Sangon Biotech, Shanghai). One hundred seventeen sequences were genotyped using *BioEdit*™ (Carlsbad, California) software following sequencing (Hall 2013).

Results

All collected specimens easily fit into the two morphological categories (i.e., Bighead Carp and Silver Carp). All 117 carp samples were successfully genotyped by 10 of the primers described by Lamer et al. (2014). The primers amplified regions of DNA ranging from 496 base pairs to 680 base pairs in length. All fish that had been identified

as Silver Carp by morphological characteristics were genotyped as Silver Carp. Hybrids were only documented in fish that were morphologically identified as Bighead Carp (Table 4-2).

No hybrids were genotyped from the Amur River or Yangtze River populations. Two fish from the Pearl River were genotyped as hybrids (5%). Both fish were morphologically indistinguishable from Bighead Carp.

Discussion

This is the first study to unequivocally demonstrate that hybrids exist in native wild populations in China. The hybrids that were detected by genetic markers were morphologically indistinguishable from Bighead Carp. Detecting hybrids using morphological characteristics is difficult (Lamer et al. 2015). Lamer et al. (2010) found that twisted gill-raker morphology was one morphological characteristic that could be used to detect F1 hybrids; however, post-F1 hybrids showed no deviation in gill-raker morphology from either Bighead Carp or Silver Carp. The two hybrids found in the Pearl River may have been post-F1 hybrids; however, morphological characteristics were not collected at the time of specimen collection. Future field studies should collect gill-raker morphology as part of their field sampling protocol.

The initial populations of Bighead Carp and Silver Carp would have been low in the Mississippi River Basin and hybridization between Bighead Carp and Silver Carp is considered one strategy to overcome low propagule pressure (Lamer et al. 2015). Alarming declines of Bighead Carp and Silver Carp have been observed in both the Yangtze and the Pearl rivers since the 1960s (Li et al. 2008, Mao et al. 2010). As a result of population declines, both Bighead Carp and Silver Carp are currently classified as near

threatened in China (Li et al. 2020). The decrease in Bighead Carp and Silver Carp densities in the Pearl River may lower propagule pressure and facilitate hybridization, similar to the situation observed in the Mississippi River Basin. Future studies should consider how the relative densities of Bighead Carp and Silver Carp in Chinese rivers impact hybridization.

Hybridization seemed to be limited to the Pearl River. The Pearl River is the closest river in China to the equator. Bighead Carp and Silver Carp would attain their largest body sizes closest to the equator because they are ectotherms (Mousseau 1997); therefore, many hatchery facilities have been constructed along the Pearl River (Haas and Ban 2014). The addition of aquaculture systems on the Yangtze and Pearl river basins is a two-prong problem. First, aquaculture systems fragment the landscape, hindering the movement Bighead and Silver carp that were naturally occurring in the river system. Second, fishes stored within the aquaculture systems could escape confinement and venture into the river basins. Hybridization between Bighead Carp and Silver Carp has been documented amongst mixed stocks in aquaculture facilities in Bangladesh (Mia et al. 2005); even so, brood-stock purity has remained unexplored in the aquaculture facilities in the Pearl and Yangtze river basins. Future studies should explore brood-stock purity in aquaculture and the rate of escapement of fishes from aquaculture facilities.

This study provides a baseline to determine how anthropogenic disturbances, such as the Three Gorges Dam or the additional of novel aquaculture facilities, affect hybridization of Bighead Carp and Silver Carp in the Yangtze, Pearl, and Amur river systems. Future studies should use a combination of morphological characteristics and

genetic markers to follow the occurrence of hybridization between Bighead Carp and Silver Carp in the Yangtze, Pearl and Amur river systems.

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Table 4-1: List of primers used to target single nucleotide polymorphisms (SNPs) previously identified by Lamer et. al (2014) to successfully detect hybridization between Bighead Carp and Silver Carp. The SNP location denotes the nucleotide location in the nuclear genome targeted by the described primer. Final sequence lengths are provided for successful primers.

SNP Location	Forward Primer	Reverse Primer	Final Sequence Length
966886	ATGGTTATGGCCACAGGCA	AGACGGGATTGCATTTCAGG	680
8222	CCAACCAGCTGAAAGAGCAT	ACACACCACACCACCTTTGA	601
102970	TAGCAGCCATGGAAATGGGT	TGTAAAGGCAGATGCCCACT	597
608473	AAGGTCGCAATGCCAAAGAG	TGTGCTGTCAGTCTGCGTGTA	588
605041	CGCTGTACATTGAGTACCCCT	TTCAGGCAACCTACTGACTTG	587
365501	TTTGTCACGTTCCTCCTGGT	AGAAGTTGGAGAGCTGGAGCA	574
285071	TCCATGCACTTCTATCGCAG	AAGCAACTTTCTCCAACACTG	559
61994	AACAGCTGGAGACTCGGCTT	TTGCTGTGTGTTTTAGGTGGC	553
159485	GTGACGGGACATGACCAAAA	CAAGCTCTGTCGCATTCTCA	542
89876	TCTGGAATAGCTCAGCCTCA	AACGGGACCTGTCATCCAA	496
325994	TGTTTCCCAGAGGAGCCAAA	GAGTGGCTTGGATATTCTTCA	
618243	GACCAGGATTGTGTATTAAGA	AGAAAAGAGAGAGGCAGGTG	
1414170	GATCTTCAGCAGCAAATCAGC	AGTTCGCCCCAGAATGGA	
181462	TCGTTCTTAACACACCAAACA	AACCACATCGACCTGTGCTCA	
700264	TGCTGAGAGGATTACTGGTGC	TAAGTCTTGGTTGTGGCA	
96587	AGCCCTGCACAGGCAGTAAA	TGAACCAAACCTACTGACGG	
213005	GGAGTACATATCAGCTTTT	TGATAAGCGATTGAACTGA	
126481	CCCCTCCAACTGTCCCTAAAA	CCTGTGATTGCATGATCTGC	
457062	GTTCTATTTGATGGGCGCCA	CATGTTCAAACCCCAAGAG	
56126	GCGTCACAGCAGATTCCTACT	ATCAAAACCGGGCTGGTCT	
498063	TTCGGGGCTGCAAGTTATTC	GTGAAGCTGTTTCATACCTGC	
1002075	TCAGTTGGCAGACCCAGAAT	TCAGTTTGGCAGTGGCAGTA	
312979	CAACTCCATCATGTGACCAGC	TGAGGTAAACTCACCGTTTGG	
618868	TTCATGCGAATAGCGCGA	GGTTGGGGGAAAACAGATGA	
175360	TAATTGGCAGCTTGGCAGCT	TGTAGAACAGTGACCCACTGG	

Table 4-2: Genotyping results for Silver Carp and Bighead Carp from three rivers in China.

Location	Number of Silver Carp *	Number of Bighead Carp *	Number of Hybrids	Percentage of Hybrids	River Latitudinal Coordinates
Amur	24	8	0	0	55.00° N
Yangtze	24	24	0	0	30.51° N
Pearl	20	17	2	5	22.77° N

*****Fish were morphologically identified as Silver Carp if the ventral keel extended from the isthmus to the anus to the base of the pelvic fins or Bighead Carp if the ventral keel extended to the base of the pelvic fins.

Chapter 5: Life will find a way: variations among populations are critical for species delineation

Introduction

Maintaining species diversity is one strategy that's been employed to ensure ecosystem services (Balvanera et al. 2001). Unfortunately, there is not unanimous agreement on species delineations (Allendorf et al. 2001, Coates et al. 2018). Further, many definitions of a 'species' don't encompass how species change over time. Conservation geneticists may be able to enhance management efforts across spatiotemporal scales by overcoming different species delineations among contributing disciplines and by embracing the dynamic nature of evolutionary processes within the definition of a 'species' (Holt 2006).

The ideological essence of a species has been considered immutable historically (i.e., morphological species concept) (Shull 1923). Nature was viewed as being static and deviation from the ideal type (demarcated in the species delineation) was considered a nuisance (Hill 1993). Darwin challenged the traditional, static taxonomy scheme when he described a 'species' as a dynamic entity that adapts to changing environmental conditions (Darwin 1859). Deviation from the ideal type is critical to understanding individual survival and reproduction in the face of changing environmental conditions. Now is an ideal time to address the dilemma of defining dynamic 'species' because biologists can leverage knowledge of molecular characteristics with environmental stressors; that is, biologists can follow evolutionary trajectories of species in relation to their environment with the advent of next generation sequencing technology (Emerson et al. 2010, Catchen et al. 2013, Manthey and Moyle 2015).

There are currently at least 23 concepts for species delineation (Table 5-1), each of which uses different secondary-species characteristics. Regardless of the concept that's selected, a 'species' can be placed in a hierarchical system, such as the Linnaean classification system, to describe that species and explain that species' evolutionary history. Using species as the least inclusive category within the Linnaean classification system prevents us from dealing with evolutionary processes including speciation and hybridization—thereby limiting biologists' abilities to identify and track evolving species' lineages. 'Subspecies' was adopted as a functional category below a species as an attempt to designate geographic varieties and provide a management unit to track evolutionary trajectories. Until the end of the 19th century any morphologically distinct natural population was treated as a subspecies. Unfortunately, many of these subspecies were named based on minute variations; therefore, the potential usefulness of this smaller unit has remained relatively limited (Mayr 1982). A standard method has already been established to define a population; therefore, I propose 'population' as the least inclusive category of the Linnaean classification system as a smaller unit to track potentially new evolutionary lineages. I also offer an expanded concept for delineation of a species to capture the breadth of variation that's been observed.

"Subspecies"—a least inclusive category

A 'subspecies' represents "heritable geographic variation in phenotype" (Patten 2015). In other words, genes responsible for phenotypic variation are under natural selection or local adaptation to environmental conditions. Biologists need to recognize multiple components to recognize a group as a functional subspecies: morphological variation (Mousseau and Sikes 2011), genotypic variation (Patten 2015), and phenotypic

variation (Ballentine and Greenberg 2010). One implication is that the genes responsible for phenotypic variation is under natural selection or local adaptation to environmental conditions. Biologists need "to establish a standard method to determine the species–subspecies boundary" (Torstrom et al. 2014). Biologists have not been able to successfully establish these boundaries; therefore, other taxonomic units may be more useful to track evolutionary lineages.

"Population"—an appropriate least inclusive category

The least inclusive category within the Linnaean classification system should serve as a distinctive unit that can be monitored through time and that can be compared across classes to study speciation (Wiley and Lieberman 2011). 'Population' is a smaller taxonomic unit, defined as a group of interbreeding individuals and their offspring (Herron and Freeman 2013). Individuals within these groups exchange genetic material within a specific geographic area containing a unique set of environmental parameters, affecting which alleles are positively selected. Therefore, each population of organisms within a species represents a unique combination of alleles for a unique set of environmental parameters. A 'population' is a small-enough cohort of organisms to effectively integrate genetic and environmental processes, and a large-enough cohort of organisms for comparison among groups within a species. Adopting populations within the Linnaean classification system provides scientists with a smaller cohort to track the dynamic changes among genes and the environment.

A population is a superior taxonomic unit below the species level because a standard method has already been established to define a population based on gene flow and divergence. The guidelines to defining a population are based on the premise that

populations fragment when gene flow is interrupted resulting in a deficiency of heterozygotes when compared to Hardy-Weinberg expectations for the overall population (Frankham et al. 2010). The degree of interruption in gene flow varies, resulting in a continuum between completely isolated populations and completely connected populations. As a result of degree of heterozygosity loss also varies. Loss of heterozygosity can be treated as an inbreeding process, and the degree of isolation among population fragments can be described by partitioning the overall inbreeding into components among populations (Frankham et al. 2010). Wright (1969) partitioned inbreeding into a series of F-statistics that considered inbreeding the total population (F_{IT}) into the inbreeding of individuals relative to their sub-population (F_{IS}) and inbreeding due to the differentiation of their subpopulations relative to the total population (F_{ST}). Nei (1987) correlated Wright's F-statistics with the observed heterozygosity (H_I), expected heterozysity averaged across all the population fragments (H_S), and the expected heterozygosity for all populations (H_T) using the following equations:

$$F_{IS} = 1 - \frac{H_I}{H_S}$$

$$F_{ST} = 1 - \frac{H_S}{H_T}$$

$$F_{IT} = 1 - \frac{H_I}{H_T}$$

When migration is high or when two groups recently diverged then there will be little differentiation $H_S \sim H_T$, and $F_{ST} \sim 0$, however, in populations with severely restricted gene flow $H_T > H_S$ and $F_{ST} > 0$ (Nei 1987, Frankham et al. 2010). Nested analyses, including haplotype trees, can now describe how genetic variation is distributed spatially within a species' geographic range (Templeton 1998).

Species spectrums—an expanded concept for delineation of a species

Biologists need to embrace a species' continuum by expanding their current philosophy regarding the delineation of 'species.' Species delineations have been and continue to be rigorously debated in biology, producing an array of species concepts (Table 5-1) (Mayden 1997, de Queiroz 1998, Harrison 1998, Coyne and Orr 2004). Logistical concerns, primarily focused on different secondary properties of species, arose in the attempt to categorize all organisms across geological time and place. The secondary properties have limited the application of these species' concepts, to the extent of excluding organisms from being classified as species (Mallet 2010). Biologists can minimize logistical concerns by embracing the underlying consensus among current species concepts that a species represents separately evolving metapopulation lineages (de Queiroz 2007).

Organisms respond to environmental gradients in predictable patterns. Homoiotherms' body sizes tend to increase along increasing latitudinal gradients according to Bergmann's rule (Bergmann 1848) and ectotherms tend to follow the inverse of Bergmann's rule (Mousseau 1997). Different environmental stressors will be present along latitudinal gradients, and this may affect organisms' behavior. For example, there is a direct relationship between latitude and clutch sizes of passerine birds although the reason for this relationship has been attributed to the variability of resources with latitude (Lack 1948) or a decrease in predation with increasing latitude (Martin 1995). According to Liebig's Law, only by increasing this limiting factor will biomass increase (Odum 1959). A 'population' is a group of individuals that represents a group of genetic makers under a specific set of environmental variables.

Each population within a species possesses a unique combination of alleles driven by genetic and environmental interactions. Unique combinations of alleles then produce an array of variation in both morphological (Blanchard and Blanchard 1940, King 1988) and behavioral traits. Intraspecific variation can be ecologically advantageous for a species. It can reduce intraspecific competition (Estes et al. 2003, Bolnick et al. 2011), pathogen exposure (Johnson et al. 2009, Bolnick et al. 2011) and predation risk (Wirsing et al. 2007), all of which increase an individual's probability of survival and reproductive capability. Over time natural selection will make the alleles best adapted for that environment the most prevalent and these populations will take on unique characteristics.

Ecosystems are characterized by episodic change and different scales will reorganize variables using non-linear processes. Predicting the outcome of this variation is inherently difficult because these adaptive cycles will result in multiple equilibria (Allen et al. 2014). Populations are one scale at which biologists track how adaptive cycles impact a species across spatiotemporal scales.

A species is a continuum of populations held together by gene flow. Each population within a species is likely exposed to a unique set of environmental variables that produces a unique combination of alleles; if true, then each population composes a unique segment of the species. The compilation of populations, held together by gene flow, spans a larger spectrum of variation than any individual population. I propose that a 'species spectrum' is the variation contained within and among populations that are connected via gene flow. Any individual is therefore considered a member of a species if it possesses traits within the accepted variation exhibited by this 'species spectrum.'

This concept allows a species' definition to encompass more intraspecific variation. Delineating populations within a species allow more intraspecific variation be described within a geographical context. This concept is like the genetic species concept and the cohesion species concepts in that it recognizes a population as an operational unit within a species. This concept expands on 'population' as an operational unit using Nei's correlations between heterozygosity and Wright's F-statistics to quantify gene flow among populations. Now biologists have a means to quantify gene flow and delineate populations with the advent of technologies that can map different sequences of alleles onto geographic locations (Templeton 1998).

The largest drawback for my proposed 'species spectrum' concept is that national genetic repositories contain very little genetic information for many species, particularly for museum specimens collected before genetic information was being sequenced. To utilize Nei's correlations between heterozygosity and Wright's F-statistics (or other statistical analysis tools), more genetic markers will have to be generated for all species.

Conclusions

Intraspecific variation can increase a species' probability of survival and reproductive capability. Natural selection acts on the variation present within and among populations to determine which evolutionary lineages survive in the face of changing environmental conditions. Biologists need to find a way to monitor these evolutionary lineages across geospatial scales. I propose 'population' as the least inclusive category of the Linnaean classification system as a distinctive unit that can monitored across geospatial scales and that can be compared across classes to study speciation.

The dynamic versatility that species exhibit warrants a species concept that reflects this breadth of variation. I further propose a ‘species spectrum’ concept that represents the amalgamation of intraspecific variations observed amongst populations.

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Table 5-1: Descriptions of species concepts.

Species Concept	Definition	Strengths	Weaknesses	Reference
Agamospecies	Only applies to uniparental and reproduce via asexual reproduction	Applies to taxa that don't use sexual reproduction	Not applicable to all species as many species reproduce sexually	Ghiselin 1984; Mayden 1997
Biological	A species is a reproductively isolated group of interbreeding natural populations	Reproductive barriers can be tested	Reproductive isolation criterion not defined, implicit reliance on group selection	Dobzhansky, 1970; Mayden 1997
Cohesion	Series of populations having geographic or genetic cohesion	Accepts all reproductive modes	Lacks a mechanistic definition	Templeton, 1989; Mayden 1997
Cladistic	"set of organisms between two speciation events, or between one speciation event and one extinction event, or that are descended from a speciation event" (Ridley, 1989)	treats species as individuals, and places no constraints on necessary attributes that a species must possess in order to be validated	lack of specificity regarding 'speciation'	Ridley, 1989; Mayden 1997
Ecological	species is a lineage which occupies an adaptive zone minimally different from that of any other lineage evolve separately from other lineages	Tolerant of both bisexual and unisexual species, species that evolve via hybridization	Requires that ecological distinction be maintained in the lineage	Van Valen 1976; Mayden 1997

Table 5-1 (continued)

Species Concept	Definition	Strengths	Weaknesses	Reference
Evolutionary Significant Unit	a population (or group of populations) that 1) is substantially reproductively isolated from other conspecific population units, and 2) represents an important component in the evolutionary legacy of the species'. (Waples, 1991)	Allows us to follow the evolutionary trajectory of a population	Excludes known biodiversity.	Waples, 1991; Mayden 1997
Evolutionary	'a single lineage of ancestor-descendant populations which maintains its identity from other such lineages and which has its own evolutionary tendencies and historical fate'. (Wiley, 1978)	Can accommodate all types of reproduction employed by species to date	No operational component	Wiley, 1978; Mayden, 1997
Genealogical Concordance	Population subdivisions that contain multiple independent genetic traits	Can be applied to hereditary, morphological, behavioural and other phenotypic attributes traditionally studied by systematists'	Ignores differences between primitive and derived attributes and uses diagnosability as an operational guideline.	Avise, 1990

Table 5-1 (continued)

Species Concept	Definition	Strengths	Weaknesses	Reference
Genetic	Members of a species form a reproductive community by exchanging genetic material.	Measure of genetic differences, presumed to reflect reproductive isolation and evolutionary independence	Little genetic information for a vast number of species	Mayr 1969
Hennigan	'... reproductively isolated natural populations or groups of natural populations [that] originate via the dissolution of the stem species in a speciation event and cease to exist either through extinction or speciation'.	Acknowledges the importance of comparisons between sister taxa	Heavy reliance upon operational criteria	Hennig 1966
Internodal	'... individual organisms are conspecific in virtue of their common membership of a part of the genealogical network between two permanent splitting events or between a permanent split and an extinction event'. (Kornet, 1993: 28)	Identifies species solely on the basis of genealogical relationship	No criteria exist for conspecificity	Kornet 1993

Table 5-1 (continued)

Species Concept	Definition	Strengths	Weaknesses	Reference
Morphological	' Species may be defined as the easily recognized kinds of organisms, and in the case of macroscopic plants and animals their recognition should rest on simple gross observation such as any intelligent person can make with the aid only, let us say, of a good hand-lens' (Shull, 1923: 221)	Easy to categorize	Morphological characteristic must be heritable	Shull 1923
Non-Dimensional	Set of sympatric and morphologically similar but non-interbreeding populations.	Convenient. Accurate and precise way to quantify biodiversity.	Limited spatial and no temporal dimension	Mayden 1997
Phenetic	' ... the species level is that at which distinct phenetic clusters can be observed'. (Sneath, 1976: 437)	May be likened to any concept where overall similarity is the primary criterion for the existence of species.	Barren theoretical nature If a species changes through descent, then the classification will have to be revised.	Sneath 1976, Mayden 1997

Table 5-1 (continued)

Species Concept	Definition	Strengths	Weaknesses	Reference
Phylogenetic	Biological entities that are the product of natural selection and descent.	Allows for interpreting evolution of attributes. Recognize both biparental and uniparental species.	Subspecies is not an appropriate evolutionary unit and has no ontological status	Mayden 1997
Recognition	Set of organisms that has a shared mate recognition system	does not require sympatry and evolutionary reinforcement to complete speciation.	Doesn't recognize uniparental species	
Successional	devised as a surrogate for estimating divergence through time by researchers studying fossil taxa	allow researchers of fossil taxa to study phylogenetic relationships	Often gaps in data caused by gaps in the fossil record	Mayden 1997
Taxonomic	' ... a species consists of all the specimens which are members of a single kind as shown by the evidence or the assumption that they are as alike as their offspring (Blackwelder, 1967: 164)	Relies are readily available morphological characteristics	non-dimensional, treats species as classes, and lacks a lineage perspective.	Blackwelder 1967

Appendix A: List of native Nebraskan fishes sequenced mitochondrial genomes. The DNA voucher is the reference number at the University of Nebraska State Museum for each tissue sample that was collected from each species and used for mitochondrial genome sequencing. The National Center for Biotechnology Information (NCBI) accession number provides access to the complete mitochondrial genome deposited in the National Center for Biotechnology Information genetic repository for each fish genome sequenced. Coverage is the number of unique reads that include a given nucleotide in the reconstructed sequence.

Family	Scientific Name	Common Name	Voucher	Coverage	NCBI Accession Number
Acipenseridae	*Scaphirhynchus albus*	Pallid Sturgeon	DNA-0788	5	In progress
	S. platorynchus	Shovelnose Sturgeon	DNA-0273	25	MK753302
Catostomidae	*Carpiodes carpio*	River Carpsucker	DNA-0758	18	In progress
	C. cyprinus	Quillback	DNA-0189	14	MN115756
	Catostomus catostomus	Longnose Sucker	DNA-0902	16	MN115757
	C. commersonii	White Sucker	DNA-0948	15	MK848703
	Cycleptus elongatus	Blue Sucker	DNA-0307	6	MN963813

Appendix A (continued)

Family	Scientific Name	Common Name	Voucher	Coverage	NCBI Accession Number
	Ictiobus bubalus	Smallmouth Buffalo	DNA-0794	11	In progress
	I. cyprinellus	Bigmouth Buffalo	DNA-0475	18	In progress
	Moxostoma macrolepidotum	Shorthead Redhorse	DNA-0823	10	MN115755
Centrarchidae	*Ambloplites rupestris*	Rock Bass	DNA-0477	29	MK848704
	Lepomis cyanellus	Green Sunfish	DNA-0749	17	MK848695
	L. humilis	Orange Spotted Sunfish	DNA-0534	42	MK848698
	L. macrochirus	Bluegill	DNA-0513	42	MK848692
	Micropterus dolomieu	Smallmouth Bass	DNA-0454	188	MK848706
	M. salmoides	Largemouth Bass	DNA-0461	37	MK848696

Appendix A (continued)

Family	Scientific Name	Common Name	Voucher	Coverage	NCBI Accession Number
	Pomoxis annularis	White Crappie	DNA-0558	10	MK848707
	P. nigromaculatus	Black Crappie	DNA-0832	32	MK848691
Clupeidae	*Alosa pseudoharengus*	Alewife	DNA-0039	32	MK771830
	Dorosoma cepedianum	Gizzard Shad	DNA-0526	14	MK771831
Cyprinidae	*Ctenopharyngodon idella*	Grass Carp	DNA-0421	123	MN115765
	Cyprinella lutrensis	Red Shiner	DNA-0779	24	MK848699
	C. spiloptera	Spotfin Shiner	DNA-0772	20	MK848700
	C. carpio	Common Carp	DNA-0959	2145	MN115742
	Hybognathus hankinsoni	Brassy Minnow	DNA-0180	416	MN115741

Appendix A (continued)

Family	Scientific Name	Common Name	Voucher	Coverage	NCBI Accession Number
	H. placitus	Plains Minnow	DNA-0752	59	MN115749
	Macrhybopsis gelida	Sturgeon Chub	DNA-0258	159	MN115754
	M. meeki	Sicklefin Chub	DNA-0311	165	MN115748
	Notropis atherinoides	Emerald Shiner	DNA-0725	43	MK848694
	N. blennius	River Shiner	DNA-0283	76	MN115745
	N. dorsalis	Bigmouth Shiner	DNA-0058	2722	MN115750
	N. stramineus	Sand Shiner	DNA-0732	83	MK848705
	Phenacobius mirabilis	Suckermouth Minnow	DNA-0786	29	MK848701
	Pimephales notatus	Bluntnose Minnow	DNA-0885	2255	MN115751

Appendix A (continued)

Family	Scientific Name	Common Name	Voucher	Coverage	NCBI Accession Number
	P. promelas	Fathead minnow	DNA-0763	109	MN115743
	P. vigilax	Bullhead Minnow	DNA-0648	89	MK848690
	Rhinichthys cataractae	Longnose Dace	DNA-0935	63	MK848697
	Semotilus atromaculatus	Creek Chub	DNA-0927	68	MK848693
Esocidae	*Esox lucius*	Northern Pike	DNA-0478	31	MN115744
Fundulidae	*Fundulus sciadicus*	Plains Top Minnow	DNA-0035	99	MN115762
	F. zebrinus	Plains Killifish	DNA-0052	132	MN115763
Gasterosteidae	*Culaea inconstans*	Brooke stickleback	DNA-0174	138	MN115770
Hiodontidae	*Hiodon alosoides*	Goldeye	DNA-0796	25	MK771832

Appendix A (continued)

Family	Scientific Name	Common Name	Voucher	Coverage	NCBI Accession Number
Ictaluridae	*Ictalurus furcatus*	Blue catfish	DNA-0278	31	MN115771
	I. punctatus	Channel catfish	DNA-0245	14	MN115767
	Noturus flavus	Stonecat	DNA-0977	73	MN115746
	Pylodictis olivaris	Flathead catfish	DNA-0432	17	MN115766
Lepisosteidae	*Lepisosteus osseus*	Longnose Gar	DNA-0431	17	MK771833
	L. platostomus	Shortnose Gar	DNA-0557	46	MK771834
Lotidae	*Lota lota*	Burbot	DNA-0853	101	MN115768
Moronidae	*Morone americana*	White Perch	DNA-0486	25	MN115747
	M. chrysops	White Bass	DNA-0042	83	MK848702

Appendix A (continued)

Family	Scientific Name	Common Name	Voucher	Coverage	NCBI Accession Number
Percidae	*Etheostoma nigrum*	Johnny Darter	DNA-0966	25	MN115764
	Perca flavescens	Yellow Perch	DNA-0065	23	MN115758
	Sander canadensis	Sauger	DNA-0437	25	MN115753
	S. vitreus	Walleye	DNA-0411	9	In progress
Poeciliidae	*Gambusia affinis*	Western Mosquitofish	DNA-0743	68	MN115760
Polyodontidae	*Polyodon spathula*	Paddlefish	DNA-0446	17	MK771835
Salmonidae	*Oncorhynchus mykiss*	Rainbow Trout	DNA-0077	221	MN115761
	Salmo trutta	Brown Trout	DNA-0083	21	MN115769
Sciaenidae	*Aplodinotus grunniens*	Freshwater drum	DNA-0255	22	MN115752

Glossary

Term	Definition
Biodiversity	Biological diversity. Includes the variety of ecosystems, species and genetic variation within a species.
Clade	A group of organisms that consists of a common ancestor and all its lineal descendants.
Contig	Set of overlapping DNA segments that together represent a consensus region of DNA.
Coverage	The number of unique reads that include a given nucleotide in a reconstructed sequence.
Effective Population Size	The size of an ideal random mating population that would lose genetic variation at the same rate as observe in an actual population.
Genetic Diversity	Total number of genetic characteristics in a species.
Genetic Marker	Genes or short stretches of DNA within an organism's genome.
Genotype	An organism's genetic composition.
Hybridization	Mating between two individuals of genetically distinct populations.
Metapopulation	A group of partially isolated populations of the same species
Microbiome	The microbial genetic material living on or within an organism.
Phenotype	The observable characteristics of an organism as a result of environmental stressors on an organism's genetic composition.
Phylogeny	Evolutionary history of a group of organisms.
Polymerase Chain Reaction (PCR)	A technique to replicate a specific segment of DNA. Primers encompass a specific targeted DNA area. Then the DNA strands are separated heat, then cooled to allow primers to bind to a specific target area and finally polymerase enzyme makes each single strand of DNA into a double strand effectively duplicating the DNA from the primer. This cycle is repeated until a desired number of DNA copies are achieved.

Glossary (continued)

Term	Definition
Primer	Small oligonucleotide (18-22 base pairs long) that binds to a specific region of DNA and serves as a starting point for DNA replication.
Single Nucleotide Polymorphism	A polymorphic nucleotide site in a population.
Zygotic barrier	Mechanisms of reproductive isolation.