Spring 1-1-2006

A molecular dissection of the mating system in the bluntnose minnow, Pimephales notatus

Rebecca Abrasheff

A MOLECULAR DISSECTION OF THE MATING SYSTEM IN THE BLUNTNOSE MINNOW, *Pimephales notatus*

A THESIS

PRESENTED TO THE BAYER SCHOOL
OF NATURAL AND ENVIRONMENTAL SCIENCES

DUQUESNE UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

BY REBECCA L. ABRASHEFF
THESIS COMMITTEE: DR. BRADY A. PORTER, THESIS ADVISOR
DR. MICHAEL JENSEN-SEAMAN, COMMITTEE MEMBER
DR. DAVID LAMPE, COMMITTEE MEMBER

25TH APRIL 2005

TABLE OF CONTENTS

I. FORWARD

Acknowledgements

I would like to extend a sincere thank you to my committee members, Drs. Michael Jensen-Seaman, David Lampe, and especially, my thesis advisor Brady Porter. Another thank you is sent to Drs. Anthony Fiumera for his help with sample collection, Dr. Guillermo Orti and Michael Bessert from the Orti lab for the use of the *P. promelas* primers and for sharing their unpublished work on *P. promelas*. Thank you to Dr. Beth Dakin for all of her continued assistance with the bench work, writing, and statistical analyses involved with this thesis. Thank you to Dr. Lisa Ludvico and the forensics department for her ideas and input as well as the use of the forensics lab near the end of my bench work. Another thank you to all of the members of the Porter lab, both graduates and undergraduates for their continued support and input. Thank you to Duquesne University, especially the Department of Biological Sciences for funding this research. Finally, thank you to all of my family and friends for their constant emotional support and encouragement.

Abstract

In the mating system of the bluntnose minnow, *Pimephales notatus*, females partition their eggs among several nests, depositing eggs on the underside of rocks that are prepared, tended, and cared for by the males. In this study, I genotyped embryos from 10 nests along with their respective guarding males using 5 microsatellite markers to reveal alternative reproductive tactics employed in high-density and low-density populations. Comparing the multilocus genotype of each guarding male to those of the guarded embryos, genetic signatures of cuckoldry and nest-guard swapping were revealed. The average percentage of allopaternal care occurring in the nests was 67.8%, which is the highest documented in fish. On average, the guarding males were significantly more related to illegitimate offspring in their nest than they were to other random adults in the population, suggesting that these higher levels of allopaternal care may be adaptive through kin selection.

II. INTRODUCTION AND LITERATURE REVIEW

The many species of minnows that comprise the family Cyprinidae exhibit diverse spawning behaviors ranging from broadcast spawning in the case of the common carp to depression nest building by stonerollers (Mettee et al. 1996). Other modes of spawning involve the excavation of a nest site beneath flat substrate on the streambed. Some fish exhibit "bourgeois" behavior where males invest in nest building and defense in an attempt to monopolize resources and/or fertilizations (Taborsky 1994). Species in the genus *Pimephales* employ a more advanced method of nesting behavior (Mettee et al. 1996): the bourgeois males excavate their nests in loose benthic substrate under practically anything flat (Kuhne 1939). They clean the ceiling of this cavity with a fleshy nape pad that develops on the male during spawning season, which begins in mid-April and lasts until August (Boschung and Mayden 2004). Females then enter the nest cavity to inspect. If a gravid female decides to spawn with that breeding male, she will turn on her side in the burrow and the male will juxtapose himself beneath her. The male then lifts the female towards the ceiling of the nest. While the male is pressing the female's urogenital region upward, the female rapidly undulates her body in an S-shape (Page and Ceas 1989). During each undulation one or more eggs are attached in a single layer to the ceiling of the nest cavity. Following the spawning event, *Pimephales* males provide uniparental care over the eggs; males remove dead and/or diseased eggs, aerate the nest, and vigorously defend the developing offspring from other conspecific males and predation by other species (Hartel et al. 2002). If the guarding or bourgeois male is removed from the nest prior to full development, the eggs will not hatch (Hartel et al. 2002). The bourgeois behavior displayed by the *Pimephales* males is the most intricate among Cyprinids (Mettee et al. 1996).

Cyprinid eggs normally progress through a series of five distinct developmental stages, ranging from A-E (Figure 1), over the course of six to fourteen days in water between 19^{o} C - 31^{o} C (Hartel et al. 2002). The A stage eggs consist of a blastomere beginning neuralation that covers about 20% of the yolk surface. Stage B embryos have a developed embryonic axis and cover about 33% of the yolk surface. At the B stage neuralation is complete. Stage C embryos have an emerging tail-bud, somites, and the eyes are developed but have no pigmentation. A body with free-tail, pectoral fin buds, and pigmented eyes distinguish D stage embryos. Finally, E stage embryos are late embryos with formed mouths, and are often hatched upon collection. This description of embryonic development in cyprinids is based on a study by Cooper (1979) on darter (Family: Percidae) development.

Figure 1. Embryo Developemental Stages.

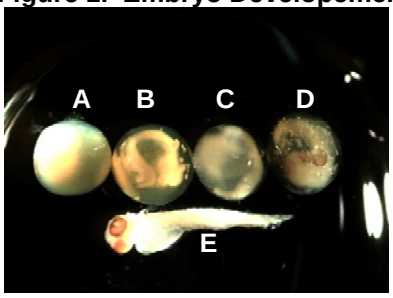
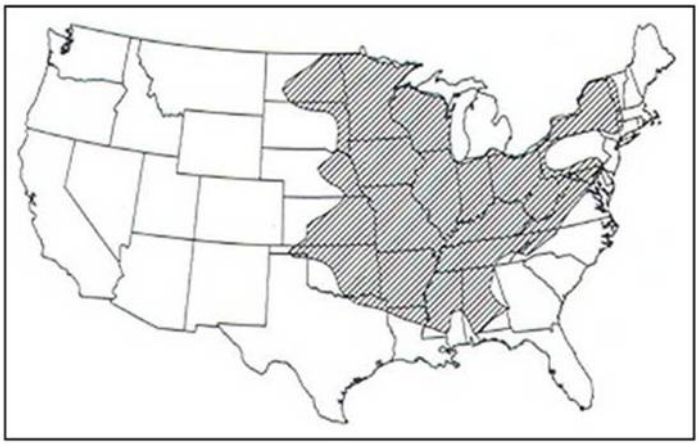


Photo by Rebecca Abrasheff

Within this fascinating genus, two particularly interesting species *Pimephales promelas* and *Pimephales notatus* are of particular interest. Both species are model organisms used for aquatic toxicity and water quality assessments (Fore et al. 1995) as they are incredibly hardy and easy to breed in captivity. They are also excellent bait fish due to their high survival rate in live wells. We focus on *P. notatus*, the bluntnose minnow, because of its widespread distribution, large population sizes, and the lack of understanding of this species' mating system.

P. notatus ranges from Southern Canada to the Gulf Slope, from the Mobile basin, west to the Red River drainage (Boschung and Mayden 2004), but is absent from the Pascagoula River and the Atlantic slope from North Carolina to Florida (Figure 2).

Figure 2. *Pimephales notatus* American distribution, from Mettee et al. 1996

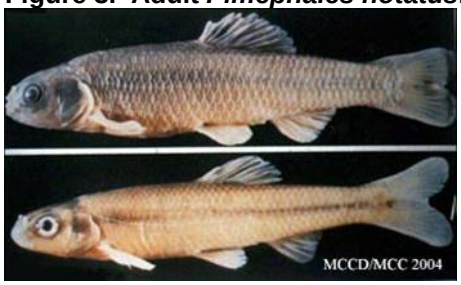


P. notatus has a long breeding season, starting in mid-April when waters warm to approximately 19 - 31^{o} C, and lasts until August. Fecundity in this species is enormous, as females will lay upwards of 4,000 eggs over multiple spawning events during the breeding season (Boschung and Mayden 2004). This species is very hardy, tolerating turbid and polluted waters well. Life color of females, juveniles, and cuckolding males can range from an olive to a straw color (Figure 3, BOTTOM). Interestingly, if the embryos initially develop ovotestes, they will assume the role of a sexually active female in their first year, morphing into a bourgeois male in their second year. Whereas, if the embryos initially develop testes, these fry will assume the role of a cuckolding male for the duration of their lifespan (Page et al. 1985). Bourgeois males become sexually mature in their second year and turn almost black during spawning season (VanCleave and Markus 1929). Both males and females have a preorbital black stripe around the snout and a complete but narrow dark midlateral stripe terminating with a caudal spot.

Both sexes have a dark spot on their dorsal fin and the first ray of this fin is truncated. Also, scale crowding occurs on the pre-dorsal region (Boschung and Mayden 2004). The bluntnose minnow is a schooling fish that inhabits slow shallow water of freshwater streams near the middle or the bottom and they feed on invertebrates, including diatoms and other algae, detritus, and insect larvae and pupae (Kraatz 1928, Keast and Webb 1966, Moyle 1973, Hartel et al. 2002).

A mature male during breeding season develops extremely large tubercles on his snout, a swollen head, and nape pad (Figure 3, TOP). The nape pad functions in cleaning a surface for egg-deposition and the tubercles are used in the defense of the nest.

Figure 3. Adult *Pimephales notatus*.



TOP: Male in breeding colors
BOTTOM: Female, juveniles, and/or cuckolding males

The mating tactics employed by this species are poorly understood. Field observations are often impossible due to water turbidity, a problem that is often encountered when trying to study fish behavior in the wild. To alleviate this challenge, fish have been sampled and transported back to a laboratory where mating behaviors can be monitored in a controlled environment. The crevice spawning behavior of various *Cyprinella* species (Family Cyprinidae) observed in the laboratory supported behaviors suspected in the wild (Ferguson 1989). Bourgeois males attracted gravid females by performing mock spawning runs along their crevice. These males guarded their nests from conspecifics and predation. Sneak fertilizations in *Cyprinella* species

documented in the laboratory would be difficult to detect in nature (Ferguson 1989). The intricate breeding behavior of another cyprinid, *Opsopoeodus emiliae*, was observed in laboratory aquaria, enabling another detailed account of cyprinid spawning to be obtained (Page and Johnston 1990).

Although *in vitro* investigations provide insight into what may be occurring in the wild, many variables such as representative sampling of a population, lack of natural substrate, and water chemistry may alter the way the fish behave. For instance, the male two-spotted goby, *Gobiusculus flavesceus*, becomes very fastidious in a laboratory setting over mate selection, whereas in nature males mate with females of varying quality (Amundsen 2001). This behavior may arise due to a sex ratio bias and a limit to the number of eggs that can be fertilized in the wild, and would not be captured if an incorrect sex ratio was sampled for analysis in the lab.

In order to gain insight on fish mating systems in the wild, molecular techniques have been employed (Avise et al. 2002). By analyzing the population utilizing genetic markers, reproductive and social behaviors suspected by naturalistic observations can be confirmed (DeWoody and Avise 2001). Microsatellites are used frequently in population genetic studies ranging from fish to humans. They are useful due to their high variability, allowing high resolution of maternity and paternity. Many reproductive strategies such as cuckoldry, nest parasitism, nest piracy, nest takeovers, kinship, and patterns of multiple matings or extra-pair fertilizations (Jones et al. 2001a, Dearborn et al. 2001) with both sexes can be confirmed and quantified with the use of molecular markers (Avise et al. 2002).

Many of the aforementioned alternative reproductive tactics (ARTs) have been reported in various nest-guarding species (DeWoody et al. 1998, DeWoody et al. 2001, DeWoody and Avise 2001, Avise et al. 2002, Mackiewicz et al. 2002, Porter et al. 2002, Fletcher et al. 2004). The occurrence of cuckoldry in the form of sneaker and satellite

males is common in this type of mating system as seen in sunfish, sticklebacks, gobies, and cichlids (Avise et al. 2002). Cuckoldry in nest guarders arises when a conspecific male steals fertilizations from a bourgeois male. A swift young male that darts into a nest during a spawning event and releases his milt along with that of the guarding male is considered a sneaker male. When a male enters a nest by mimicking a female and releases his milt, he is considered a satellite male. Satellite males are genetically predisposed to produce large quantities of sperm (Taborsky 1994), and their gonads are often proportionally much larger than those of guarding males. Once sneaker or satellite males attempt to fertilize some of the eggs within a nest, they leave and do not contribute any parental care (Taborsky 1994). The sneaker male, stealing fertilizations from the guarding male, benefits by conserving energy lost, first competing for females and then providing extended parental care to the fertilized nest (Jones et al. 1998).

Similar to nest parasitism, nest piracy involves the guarding male of one nest actually stealing eggs from another nest and depositing them in his own (Jones et al. 1998, Avise et al. 2002). Several hypotheses have been formulated to justify the motive for nest piracy. It is suspected that a male with more eggs in his nest may seem more attractive to gravid females, as a large number of eggs may indicate virility and good parenting, thus encouraging more spawning events (Unger and Sargent 1988, Avise et al. 2002). Also, by adding non-related eggs to his nest the thief may be capitalizing on the predator dilution effect, where if the nest were preyed upon, the odds of his own offspring surviving would be increased. Nest piracy can only occur in species that lay non-adhesive eggs. This is not possible in species such as *Pimephales notatus* where the eggs are glued on the underside of the nesting substrate at the time of spawning, as the eggs lose their adhesiveness if they are dislodged.

When the guarding male is not the parent of any of the eggs he is guarding, a nest takeover event has occurred, as seen in sunfish and some minnows (Neff as

reviewed by Avise et al. 2002). A nest takeover can take place if the guarding male is away foraging, chased away by a larger male, or killed. The new male that takes over that nest begins allopaternal care by caring for the foster eggs as if they are his own.

Kinship and mating patterns between males and females were also detected using molecular techniques in pirate perch, darters, sunfish, seahorses and pipefish (Jones and Avise 1997, DeWoody et al. 1998, Jones et al. 2000, DeWoody et al. 2001, Porter et al. 2002, Jones and Avise 2001, McCoy et al. 2001, Fletcher et al. 2004). By comparing the multilocus genotype of the guarding male with those of the eggs in the nest the percent paternity by the guarding male can be calculated. Paternity is assumed if the multilocus genotype of the offspring is compatible with that of the guarding male. The minimum number of contributing dams can be estimated by counting the number of alleles and constructing the female gametotypes from the genotypes of the offspring that were not contributed by the guarding male. Comparing the multilocus genotypes between offspring can elucidate kinship. If the alleles of all offspring surveyed can be derived from the guarding male and only one contributing female, these offspring would be considered full-siblings (Fiumera et al. 2002). Similarly, if the offspring surveyed only share an allele that could be inherited from the guarding male but a variety of different females, these offspring would be half-siblings.

Allopaternal or foster care of illegitimate offspring in a nest by a guarding male is especially common in *Pimephales* and may be the result of any or all the ARTs discussed above except egg piracy. Previous behavioral research has shown support for sexual selection in male fathead minnows (*Pimephales promelas*) where these males actually prefer to take ownership of a nest that has eggs present in it when presented with an identical empty nest site in aquarium trials (Unger and Sargent 1988). This male preference appears to have arisen from a female preference to spawn in a nest already containing eggs (Unger and Sargent 1988). The female preference may

have evolved because an increase in clutch size results in greater egg survival (Sargent 1988). With this concept, as the number of fertilized eggs increases, so does the chance of hatching healthy offspring. Thus by a female contributing more eggs to a nest already containing eggs, she is ensured a better survival rate for her offspring.

Sexual selection is likely a cause for allopaternal care to have evolved in these minnow, however, a second competing theory may be a selective pressure as well. The idea of kin selection as stated by Hamilton's rule ($c < rb$) where c indicates the cost incurred by the actor (in this case the guarding male tending illegitimate offspring), b describing the benefit to the recipient (the cuckolder), and r describing the degree of relatedness between the actor and recipient (the guarding male and cuckolder) (Hamilton 1964, Krebs and Davies 1997) can also explain the evolution of allopaternal care. There may be altruistic behavior occurring in these minnow populations where the altruistic bourgeois male is increasing the reproductive success of a relative at the cost of his own (Hamilton 1964, Ridley 1993). Under kin selection, a guarding male may be more inclined to care for a relative's eggs than another unrelated conspecific within the population.

By employing microsatellite markers in the molecular dissection of *Pimephales notatus* spawning system, I hope to document and quantify the ARTs used in two natural populations and attempt to explain why these specific ARTs are being employed, either by sexual selection or kin selection. In this study, we used highly polymorphic microsatellite markers to construct multilocus genotypes of guarding males, the embryos from their respective nests, and other adult individuals from two different populations, one of high density (Hurricane Creek, TN) and one of low density (Clear Creek, KY) in order to document ARTs such as nest parasitism, nest takeovers, and nest-guard swapping. In the population of high density, nesting substrate was a limiting factor as well as the sex ratio, with females outnumbering males. This situation could

induced an increase in resource competition resulting in a variety of ARTs (Bessert et al. submitted, Jones et al. 2001b). Upon detection and quantification of these mating tactics, we discuss correlations between the frequency of certain behaviors, population density, and nest resources.

III. RESEARCH DESIGN AND METHODS

Collection and DNA extraction of adults and embryos from two populations

The *P. notatus* adults and nests were collected by Drs. Brady Porter and Anthony Fiumera from two different locations: one population of high density (Hurricane Creek at Cobbs Road bridge, Rutherford County, Tenn.) and one population of low density (Clear Creek at County Road 1787, Rockcastle County, KY) (Porter et al. 2002). Flat rocks capable of holding nests were located and surrounded with dip nests on all sides in order to capture the guarding male with the nest he was protecting. Each guarding male darted directly into the dip net, mistaking it for an algal mat when the nest rock was lifted. This ensured certainty of capturing the current guarding male of that particular nest. The remaining population was surveyed by seining. The adult fish were placed in 50 mL Falcon tubes containing absolute ethanol. The ethanol was changed at least once over the nest few hours as the fish dehydrated. Standard length of each guarding male was measured using calipers, measuring from the tip of the snout to the caudal peduncle. The monolayer of eggs on each nest rock was gently scraped with a blunt knife into a 50 mL Falcon tube containing a 20% DMSO/saturated salt solution. Five of the nests from each population were genotyped. The developmental stages of each nest were evaluated (Cooper 1979) and a representative sample, averaging approximately 45 embryos from each nest, were dissected prior to DNA extraction.

The total number of eggs in each sampled nest was estimated by pouring a portion of a preserved nest into a graduated cylinder and allowing the eggs to settle to the bottom. All of the eggs equaling 1 mL volume were hand counted. The total number of eggs in each nest was estimated by pouring preserved eggs from the whole

nest, into graduated cylinders, allowing the eggs to settle out of suspension and multiplying the number of eggs in 1 mL by the total volume containing settled eggs.

An extraction protocol using embryo extraction buffer (EEB, 10 mM Tris [pH 8.0], 1 mM EDTA [pH 8.0], and 25 mM NaCl) was used on the embryos and larvae (DeWoody et al. 2000). From each nest, A, B, and C stage embryos did not require any dissection. The D stage embryos were removed from the egg and the yolk sac of both D and E stages was removed. The dissected larvae were squished in 50 μl of EEB. To each sample, 0.5 μl of proteinase K (10 μg/mL) was added and the samples were digested at 55^{o} C for 30 minutes, followed by denaturation at 95^{o} C for 2 minutes. After the digestion, the samples were centrifuged at 3000 rpm for 10 minutes and stored at -20^{o} C. DNA extraction from adult muscle tissue followed a standard organic protocol using phenol-chloroform (Sambrook and Russell 2001).

Primer optimization, selection, multi-plexing, and genotyping

Dr. Brady Porter designed four fluorescently labeled microsatellite primer sets for *P. notatus* (Pn1.3, Pn1.5, Pn1.9, and Pn2.15) following procedural methods in Porter et al. (2002). The remaining five fluorescently labeled PCR primer sets (Ppro126, Ppro48, Ppro118, Ppro132, and Ppro80) were adapted from previously published primer note on *Pimephales promelas*, a close relative of *P. notatus* (Bessert and Orti 2003). The nine loci produced amplified products in the range of 100-500 base pairs in length.

PCR conditions were optimized by altering $MgCl_2$ concentrations and/or annealing temperatures. PCR reactions using a $MgCl_2$ concentration gradient (from 1 mM to 2.5 mM and increasing by increments of 0.5 mM) were set up in a 96-well plate

and run with graded annealing temperatures starting from 45^{o} C and increasing to 57^{o} C. The optimized PCR conditions for each individual locus can be found in appendix a. Each PCR reaction used 100 ng of DNA. To check for amplification, 6 μl of PCR product were loaded onto a 2% SB (Sodium:Boric acid) agarose gel and electrophoresed in 1X SB buffer for 15 minutes at 300 V (Brody and Kern 2004).

Of the nine loci, five were selected to be the primary markers for this study (Pn1.3, Pn1.5, Ppro48, Ppro118, and Ppro126). Multiplexing the three Ppro primer pairs and the two Pn primer pairs was done by combining all of the Ppro primer sets and all of the Pn primer sets into two separate reactions and reducing the volume of water added. The $MgCl_2$ concentration and annealing temperature were averaged (Table 1 A-B).

Table 1. Optimized Multiplexing Conditions for Selected Loci

A. Pn primer sets multiplex

2-plex	1 rxn	Thermocycling conditions		
Fisher 10X buffer B	1.23 μl	**Step**	**Temp °C**	**Time**
dNTP [1.25 mM]	2.2 μl	Denature	95	30 s
$MgCl_2$ [25 mM]	1.056 μl	Annealing	55	30 s
Pn1.3F-VIC [0.01 mM]	0.4 μl	Extension	72	1 m
Pn1.3R [0.01 mM]	0.4 μl	Cycles	32	
Pn1.5F-FAM [0.01 mM]	0.4 μl	Final Extension	72	2 m
Pn1.5R [0.01 mM]	0.4 μl	Incubate	15	forever
Taq [5U/μl]	0.11 μl			
HPLC water	4.804 μl			
DNA template	2 μl			
Total	13 μl			

B. Ppro primer sets multiplex

3-plex	1 rxn	Thermocycling conditions		
Fisher 10X buffer B	1.23 μl	**Step**	**Temp °C**	**Time**
dNTP [1.25 mM]	2.2 μl	Denature	95	30 s
$MgCl_2$ [25 mM]	1.056 μl	Annealing	55	30 s
Ppro118F-NED [0.01 mM]	0.4 μl	Extension	72	1 m
Ppro118R [0.01 mM]	0.4 μl	Cycles	32	
Ppro126F-FAM [0.01 mM]	0.4 μl	Final Extension	72	2 m
Ppro126R [0.01 mM]	0.4 μl	Incubate	15	forever
Ppro48F-NED [0.01 mM]	0.4 μl			
Ppro48R [0.01 mM]	0.4 μl			
Taq [5 U/μl]	0.11 μl			
HPLC water	4.004 μl			
DNA template	2 μl			
Total	13 μl			

Fisher 10X buffer B, PCR buffer received with *Taq* polymerase; VIC, green fluorescent label; NED, yellow fluorescent label; FAM, blue fluorescent label.

The optimized PCR multiplex was used to genotype approximately 45 embryos from nests 101, 103, and 107 from the Hurricane Creek population and nests 120, 122, and 124 from the Clear Creek population. The same PCR and fragment analysis conditions were used on the embryos and the adults. Nests 108 and 109 from Hurricane Creek and nest and 128 from Clear Creek were genotyped using the Pn primer set multiplex and marker Ppro118 individually.

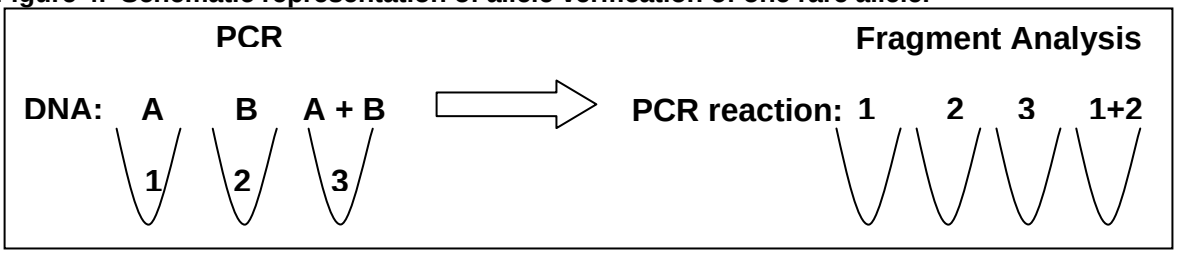
Fluorescently labeled PCR products were then prepared for fragment analysis on the ABI Avant 3100 automated genetic analyzer. A size standard of either GeneScan-500 LIZ or GeneScan-500 ROX (Applied Biosystems Inc.) was run with each sample. A

reaction mixture of 0.5μl of size standard and 9.5 μl of formamide was loaded into each well of a 96-well plate. Aliquots ranging from 0.5-2 μL of fluorescently labeled PCR product were then loaded into the formamide/size standard mixture. Once all PCR products were added, the plate was denatured at 95^{o} C for 2 minutes and snap cooled on ice. The plate was mounted on the ABI Avant 3100 and programmed for fragment analysis according to the manufacturer. Once the run was completed, the data (in the form of electropherograms) was analyzed using the software GENESCAN (Applied Biosystems Inc.), and raw allele sizes (usually to the second decimal place in base pairs) were recorded. These raw allele sizes were then binned by rounding up or down to the next whole number, based on the repeat motif of each microsatellite (Appendix d) resulting in a two allele genotype at each of the five loci.

While binning alleles, some did not round into existing allele classes. Unusual alleles such as these at each locus were verified by conducting series of PCR reactions followed by direct comparison via fragment analysis (Figure 4). First, two separate PCR reactions were prepared for the locus in question using DNA from the individual with the questionable allele (A), and a second reaction using DNA from an individual with an allele that was confirmed at a size most similar to the questionable allele (B). A third PCR was prepared containing equal quantities of both DNA samples (200 ng/μl total) (C) and reducing the volume of water added. Fragment analysis was performed using four wells for each questionable locus. The first two wells contained product from PCR tube 1 and 2 independently, the third well contained product from PCR from tube 3, and in the fourth well, product from PCR tubes 1 and 2 were both added in equal quantities(Figure 4). If the resulting electropherogram consistently displayed two peaks, one at each allele size in question, the rare allele was verified and counted. If however,

there was one single peak, the rare allele in question was discarded and binned jointly with the most similar allele.

Figure 4. Schematic representation of allele verification of one rare allele.



V-shapes are representations of reaction tubes. The numbers inside indicate PCR reaction number. The letters above the PCR reaction tubes indicate the DNA template used in each PCR reaction. The numbers above the reaction tubes indicate the PCR reaction product used for fragment analysis.

Once all adults were genotyped at all loci, allele frequencies, single-locus exclusion probabilities (P_E) and combined-loci exclusion probabilities (P_{CE}) were calculated independently for each population (appendix c). Genotypic data of the five loci were run through Hardy-Weinberg exact tests, linkage disequilibrium tests, and allele frequency tests using the population genetics software, GENEPOP on the web (http://wbiomed.curtin.edu.au/genepop/) (Raymond and Rousset 2004). The combined multilocus exclusion probability, P_{CE}, for these five loci was calculated (DeWoody et al. 2003). The effective number of alleles (n_e), expected heterozygosity (H_E), and observed heterozygosity (H_O) were calculated for each locus in each population. T-tests were performed to test for significant differences between H_O and H_E at each locus in both populations. Equations for n_e, H_O, and H_E are listed in appendix b.

Detecting alternative mating behavior within each population.

Three nests from Clear Creek and four nests from Hurricane Creek were genotyped using the five selected microsatellite markers, and an additional two nests

from Clear Creek and one nest from Hurricane Creek were genotyped at three of these loci (Pn1.3, Pn1.5, and Ppro118). The genotypes of the embryos were compared to the genotype of the guarding male and other adults from the respective population in order to determine percentage of paternity by the guarding male for each nest, the minimum number of contributing adults of each nest, the degree of relatedness between adults in each population, and alternative mating tactics. A t-test was used to determine if the difference between the average percentage of paternity of guarding males in Hurricane Creek in comparison to Clear Creek was significant.

The proportion of shared alleles (POSA) and kinship coefficient were calculated by MICROSATELLITE ANALYZER (MSA) by inputting binned genotypic data into the program (Dieringer and Schlötterer 2002). The POSA and kinship coefficient between the guarding male and the sired and illegitimate offspring were calculated separately in each nest from both populations and averages for all five nests were taken for each population. The POSA and kinship coefficient were also determined for all the adults sampled in each population by MSA. The POSA and kinship coefficients between the guarding male and the illegitimate offspring were compared to the POSA and the kinship coefficients of the adults in the respective populations. The difference between the POSA and kinship coefficients in this comparison was analyzed using t-tests to test for significance (Sokal and Rohlf 1995). The t-test equation is noted in appendix b. The t-tests were also used to determine if the difference between the POSA and kinship coefficient between adults in comparison to the average POSA and kinship coefficient between illegitimate offspring and their guarding male was significant, in addition to the comparison between Hurricane Creek adults and Clear Creek adults.

IV. RESULTS

Site comparisons

Hurricane creek, TN was considered the high-density population due to the presence of a much larger number of *P. notatus* and other cavity-nesting species (*Etheostoma virgatum*) in comparison to Clear Creek, KY, however precise quantification of population density was not measured. The Hurricane Creek population of adult *P. notatus* was significantly larger (*n=92* collected) than the adults collected from Clear Creek population (*n=12* collected). Assuming the number of nest sites is similar between each locality, this would cause a greater demand for nesting sites in the Hurricane Creek population. The presence of striped darter, *Etheostoma virgatum,* at both the Hurricane Creek site and the Clear Creek site during the spawning season also resulted in an increased demand for nesting sites and *E. virgatum* populations were higher at the Hurricance Creek site as well (Porter et al. 2002).

Tiles were placed on the streambed at the Hurricane Creek site and the Clear Creek site in order to alleviate some of this nesting competition and enable ease of collection of the spawning fish and their nests. The addition of these tiles did alter the environment of the fish in such a way that *P. notatus* adults actually preferred to nest beneath the tiles over natural rocks, possibly due to the corrugated texture for better egg attachment (Figure 5).

Figure 5. *P. notatus* guarding male (ID=Pn103) with nest 103 on underside of a tile from the Hurricane Creek collection site.

Photograph by Brady Porter

Microsatellite marker screening

Nine microsatellite markers were considered for this study. Five of these nine original loci were used (Pn1.3, Pn1.5, Ppro48, Ppro118, and Ppro126). These five markers were selected due to consistent amplification using PCR, their ease in multiplexing, and their high P_{CE} in each population. All five markers were in Hardy-Weinberg equilibrium in the low density, Clear Creek population. However, loci Ppro126 and Ppro48 for the Hurricane Creek population departed from HW equilibrium ($p < 0.01$). This deviation may be due to the presence of allelic-drop out, null alleles, migration, and/or non-random mating (Bessert et al. submitted, Dakin and Avise 2004, Klug and Cummings 2002). The difference between observed and expected heterozygosities in each population was not significant for any of the loci.

The number and size of alleles differed between populations, with the high-density population having more alleles per locus overall (Figure 6), however these results may be skewed due to unequal sample sizes (high density, $n = 92$; low density $n = 12$). Loci Ppro118 and Ppro126 presented an approximately bell-shaped distribution for the high-density population (Hurricane Creek), whereas in the low-density

population, the distribution of alleles at these loci was skewed with one allele occurring at much higher frequency (i.e. see Figure 6 locus Ppro118, allele 174 for Clear Creek). Locus Pn1.5 had a bimodal allelic distribution for both populations. The combined exclusion probabilities (one-parent known model) for these five loci were 0.99 for Hurricane Creek and 0.97 for Clear Creek (Table 2, Appendix c). The combined exclusion probabilities for loci Pn1.3, Pn1.5, and Ppro118 (used to genotype nests 128, 108, and 109) were 0.95 and 0.88, respectively.

Figure 6. Allele frequencies at microsatellite loci from samples of adults from two populations.

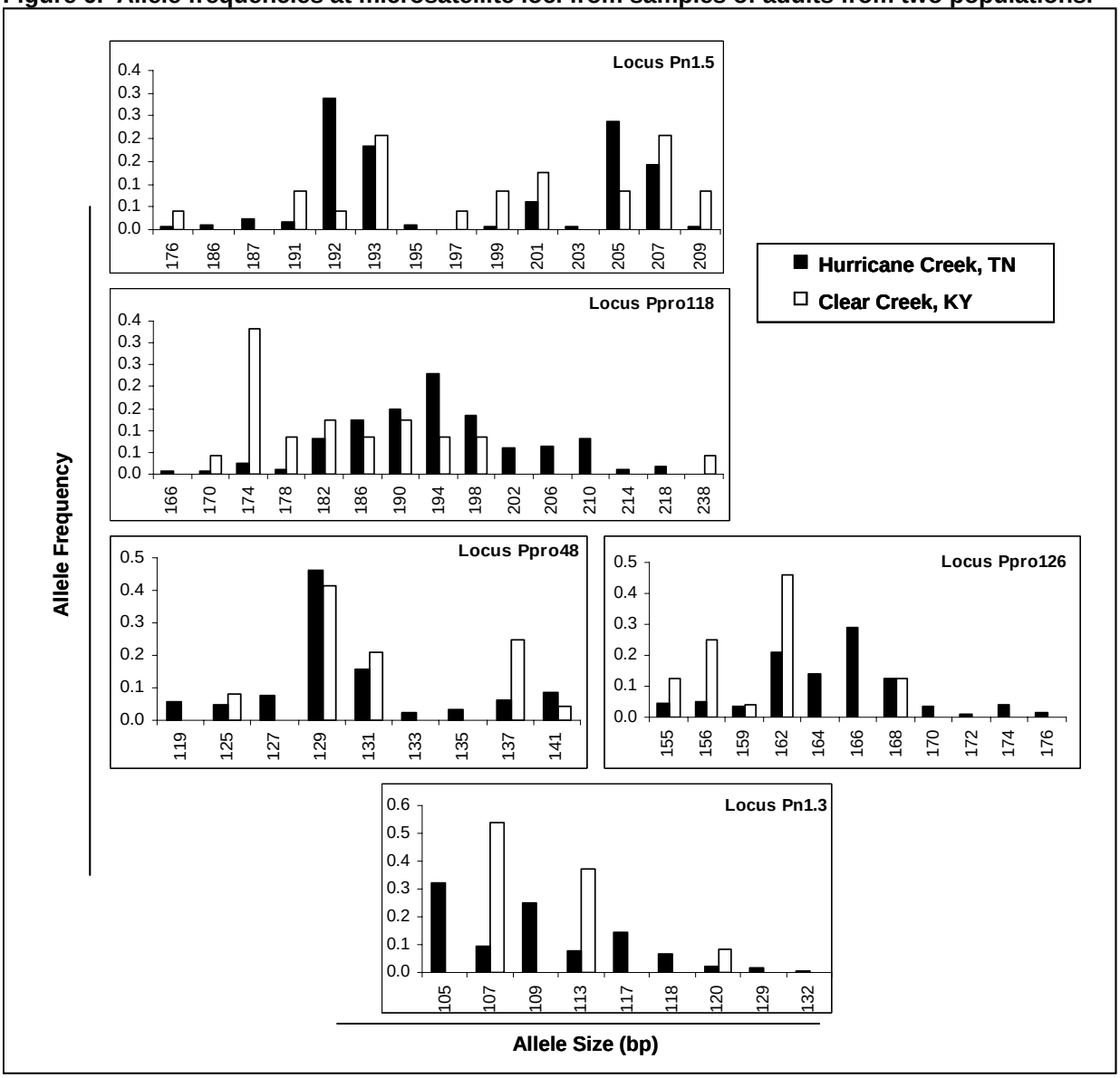


Table 2. Summary of characteristics for two adult populations of bluntnose minnow at five microsatellite loci.

Locus	No. of alleles	n_e	*s*	H_O	H_E	P_E
Hurricane Creek, TN (*n=92*)						
Pn1.3	9	5	105-132	0.65	0.79	0.59
Ppro48	9	4	119-141	0.68	0.74	0.53
Ppro126	11	6	155-176	0.82	0.83	0.64
Ppro118	14	8	166-218	0.83	0.87	0.72
Pn1.5	13	5	176-209	0.78	0.80	0.51
Mean	**11.2**	**5.4**		**0.75**	**0.81**	**0.99***
Clear Creek, KY (*n=12*)						
Pn1.3	3	2	107-120	0.58	0.56	0.23
Ppro48	5	3	125-141	0.83	0.71	0.58
Ppro126	5	3	155-168	0.75	0.69	0.41
Ppro118	9	6	170-238	0.83	0.83	0.67
Pn1.5	10	7	176-209	1.00	0.86	0.74
Mean	**6.4**	**4.4**		**0.80**	**0.73**	**0.97***

n_e, effective number of alleles [1/1-exp. heterozygosity]; *s*, size of alleles (base pairs); H_O, observed heterozygosity; H_E, expected heterozygosity; and P_E, exclusion probability for the one parent known model.
*P_{CE}, combined exclusion probability for all five loci (appendix b)

Paternity

Illegitimate offspring were detected in each of the five nests surveyed in the high-density population and in each of the five nests sampled in the low-density population, however an eleven percent greater rate of in paternity by the guarding male was seen in the Clear Creek population (38.2% average paternity) in comparison to the Hurricane Creek population (26.8% average paternity) (Table 3, Figure 7). This increase in paternity in the Clear Creek was not significant. Also, all stages of egg development were represented in nests 101, 107, and 109 from the Hurricane Creek population and nests 115, 124, and 128 from the Clear Creek population. The majority of eggs in nests 103 and 108 (Hurricane Creek) were in earlier stages of development, indicative of younger nests. Conversely, nests 120 and 122 (Clear Creek) contained more eggs at later stages of development.

One male, Pn105, from the Hurricane Creek population was captured with a female, Pn106, in nest 107. However, there were no sole guarding males for nests 108,

and 109. He was the only bourgeois male in the area and had sired offspring in all three nests (Table 3). Adult female Pn106 was captured with Pn105. Guarding male Pn105 maintained an average of 21.7% paternity in each of his three nests, whereas the multilocus genotype of the female, Pn106, captured with him could be deduced from an average of 18.4% of the eggs surveyed from each of these three nests. Pn106 was the mother of 25.3% of the eggs sired by Pn105 collectively in nests 107, 108, and 109.

Table 3. Summary of bluntnose minnow spawning behavior as assessed by microsatellite markers.

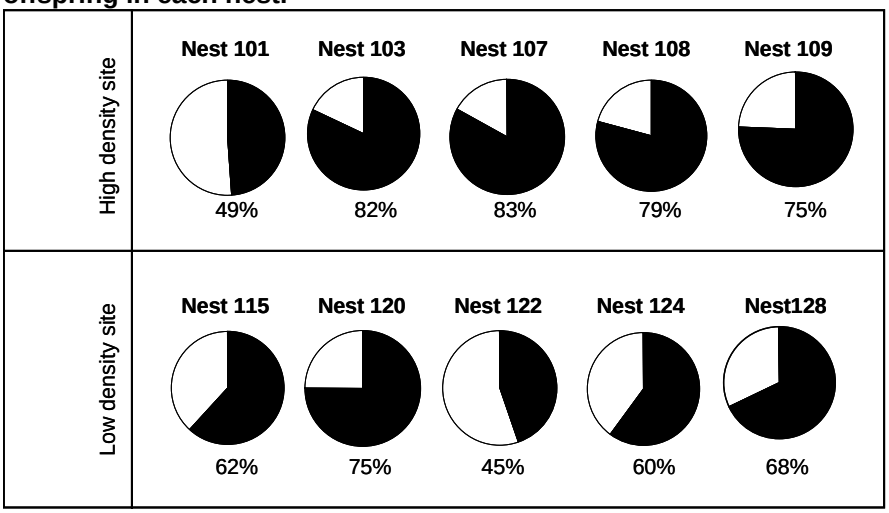
Nest ID	Male standard length (mm)	Paternity by guarding male	Total no. eggs in nest	Embryonic stages	Minimum no. of adults
Hurricane Creek:					
101	66.5	(22 of 43)	471	A B C D E	7
103	70.67	(9 of 50)	980	A B C	8
107[a]	67.67	(8 of 41)	608	A B C D E	6
108[a]	67.67	(9 of 43)	744	A B C D	8
109[a]	67.67	(13 of 56)	3038	A B C D E	8
Mean	**68.28**	**(12 of 46)**	**1215.25**		**7.4**
Clear Creek:					
115	70.69	(18 of 47)	1836	A B C D E	6
120	82.01	(12 of 48)	806	A D E	6
122	72.42	(26 of 47)	1128	B C D E	7
124	81.58	(20 of 50)	645	A B C D E	6
128	72.08	(9 of 28)	1736	A B C D E	6
Mean	**75.76**	**(32 of 47)**	**1230.20**		**6.2**

[a]Single guarding male (Pn105) captured with a female (Pn106) equidistant from nests 107, 108, and 109.

Generally, the nests collected from Hurricane Creek were smaller in egg number than those collected from Clear Creek and had more contributing adults; however these findings were not significant (Table 3). Guarding males from Hurricane Creek were also significantly smaller ($p < 0.05$) in standard length than those collected from Clear Creek. A similar trend in body size was found between bar-cheek darters at the exact same sites (Porter et al. 2002). *Etheostoma virgatum* from Clear Creek are significantly larger

than *Etheostoma derivativum* Page, Hardman, & Near (formerly conspecific with *E. virgatum*) from Hurricane Creek.

Figure 7. Pie diagrams showing the proportion of illegitimate offspring in each nest.



Shaded areas represent the proportion of illegitimate offspring with the exact percentage listed below each nest. Nest ID is indicated above each pie diagram. High density site, Hurricane Creek, TN; Low density site, Clear Creek, KY.

The guarding males collected with their nests generally had offspring present at all developmental stages sampled in the nest (Table 4). Nest 103 from Hurricane Creek was collected within a few days of spawning, as only early egg stages were present in the nest and of the eggs sired by guarding male Pn103, he was the father of only B and C stage eggs, siring none of the 22 A-stage eggs sampled (Table 4). Similarly, male Pn105 fathered all younger eggs (stages A-C) in nest 108 and mostly older eggs in nest 109. Paternity of eggs sired in Clear Creek nests was over a range of developmental stages, where there was approximately equal numbers of eggs sired by the guarding male at all stages of eggs present in each nest. On average more B and C developmental stage eggs were sired in the Hurricane Creek population, whereas, the proportion of sired offspring in the Clear Creek population was more homogeneous across all egg developmental stages (Means in Table 4).

Table 4. Summary of embryonic developmental stages sired by the guarding male for both populations of bluntnose minnow.

			Proportion of embryonic stage sired				
Nest ID	Guarding male	% Paternity	A	B	C	D	E
Hurricane Creek:							
101	101	51.2	6 of 10	6 of 9	2 of 9	6 of 10	2 of 6
103	103	18.0	0 of22	5 of 18	4 of 10	----	----
107[a]	105	19.5	1 of 9	3 of 9	1 of 8	1 of 7	1 of 8
108[a]	105	20.9	2 of 21	6 of 11	1 of 6	0 of 5	----
109[a]	105	24.5	0 of 5	3 of 14	6 of 18	1 of 5	3 of 11
Mean		**26.8**	**8 of 67**	**23 of 61**	**14 of 51**	**8 of 27**	**6 of 25**
Clear Creek:							
115	114	38.3	4 of 16	0 of 2	6 of 11	1 of 3	7 of 15
120	119	25.0	2 of 18	----	----	1 of 2	8 of 28
122	121	55.3	----	3 of 5	7 of 13	13 of 20	3 of 9
124	123	40.0	2 of 2	8 of 29	1 of 2	2 of 8	7 of 9
128	127	32.1	1 of 3	4 of 11	1 of 3	1 of 8	2 of 3
Mean		**38.1**	**9 of 39**	**15 of 47**	**15 of 29**	**18 of 41**	**27 of 64**

A-E represent a respective embryonic stage of development. Dashed lines indicate the absence of eggs sampled at that stage of development.

The multilocus genotypes of the guarding males of each nest were compared to the illegitimate offspring in the other nests within each population (Table 5). From Clear Creek, nest 115, containing 62% illegitimate offspring (Figure7) was not visited by any of the other nest-tending males sampled. Nest 124 contained 60% illegitimate eggs at a range of developmental stages of which three of the other guarding males, Pn119, Pn121, and Pn123, were consistent with siring some of these offspring (Table 5).

The guarding males from the Hurricane Creek population visited one another's nests to lesser extent in comparison to the Clear Creek population; with adults Pn101 and Pn103 both consistent with siring offspring from one nest (109) visiting only nest 109. Pn105 presumably tended nest 107, 108, and 109 resulting in large percentages of illegitimate offspring in each of the three nests. Pn105 was consistent with siring offspring from only nest 103, potentially siring only 2.4% of the 41 illegitimate offspring (Table 5, Figure 7).

Table 5. Percentage of paternity of illegitimate offspring by other bourgeois males in other nests within their population.

Clear Creek:					
Guarding Male	Nest 115	Nest 120	Nest 122	Nest 124	Nest 128
Pn114	tended	5.5%	0%	0%	10.5%
Pn119	0%	tended	0%	13.3%	0%
Pn121	0%	0%	tended	10.0%	15.8%
Pn123	0%	2.8%	9.5%	tended	0%
Pn127	0%	0%	0%	3.3%	tended
Hurricane Creek:					
Guarding Male	Nest 101	Nest 103	Nest 107	Nest 108	Nest 109
Pn101	tended	0%	4.8%	0%	2.5%
Pn103	0%	tended	0%	2.3%	7.5%
Pn105	0%	2.4%	tended	tended	tended

'Tended' indicates the nest the male was tending at the time of capture. Percentages are the percent of illegitimate offspring in the nest sired by the respective male.

Relatedness

In a pair-wise comparison of multilocus genotypes between adults in both the high-density and low-density population performed by MICROSATELLITE ANALYZER (MSA), the adults in the Clear Creek population were significantly more related to one another than the adults in the Hurricane Creek population with a proportion of shared alleles (POSA) of 0.34 and 0.28 ($p < 0.005$), respectively. The kinship coefficient (*F*) for Hurricane Creek adults was 0.19 and *F* for Clear Creek was 0.22 ($p < 0.05$). In a five loci multilocus genotype, ten alleles were compared. If an individual shared five of the ten alleles with another individual, the proportion of alleles shared would be 0.5.

The kinship coefficient is the probability that two loci, one chosen randomly from each of the two individuals are identical by descent (Crow 1986). It is expected that the kinship coefficient (*F*) between the guarding male and one of his offspring would be 0.25, for an egg to be considered an offspring of the guarding male, it must possess one of the guarding male's alleles at all loci analyzed. When considering a single locus, the

probability (kinship coefficient) of drawing the one shared allele from both the guarding male and offspring is ¼ or 0.25. This principle is extrapolated for a multilocus genotype. If *F* for an entire population is greater than zero, this population would be expected to be out of Hardy-Weinberg Equilibrium. This may occur for several reasons, such as inbreeding, a population bottleneck, and/or migration.

The average kinship coefficient (F_S) between the guarding male and the offspring he sired for Hurricane Creek was 0.42 (Table 6). Similarly, the average F_S for sired offspring in the Clear Creek population was 0.43. However, the illegitimate offspring and the guarding males were less related in Hurricane Creek population than in the Clear Creek population with an F_I of 0.26 and 0.32, respectively (Table 6). The difference between the POSA and F between the adults in Hurricance Creek compared to the adults in Clear Creek is significant ($p<0.05$). Overall, the difference between the average POSA and *F* of the illegitimate offspring and that of the adults of their respective population was highly significant ($p<0.0005$).

Male Pn121 is consistent with siring 10% of the illegitimate offspring in nest 124 (guarded by male Pn123), and male Pn123 is consistent with siring 9.5% of the illegitimate offspring in nest 122 (guarded by male Pn121) (Table 5). The POSA and kinship coefficient between males Pn121 and Pn123 is 0.2 and 0.4 respectively (Table 6). Similarly, male Pn119 is consistent with siring 13.3% of the illegitimate offspring in nest 124 (guarded by male Pn123) and male Pn123 is consistent with siring 2.8% of the illegitimate offspring in nest 120 (guarded by male Pn119) (Table 5). The POSA between male Pn119 and Pn123 is 0.15 and the kinship coeffiecnt between these males is 0.2 (Table 6).

Table 6. Percentage of shared alleles (POSA) and kinship coefficients (*F*) between nests and guarding males in both populations of bluntnose minnow.

Nest ID	Guarding Male ID	*n*	Sired POSA	Illegitimate POSA	POSA p-value	Sired kinship coefficient (F_S)	Illegitimate kinship coefficient (F_I)	*F* p-value
Hurricane Creek:								
101	101	21	0.61	0.43	***	0.40	0.30	***
103	103	35	0.62	0.36	**	0.46	0.22	ns
107	105	34	0.54	0.35	*	0.41	0.25	**
108	105	34	0.54	0.36	*	0.41	0.31	***
109	105	40	0.59	0.28	ns	0.45	0.24	**
Mean			**0.58**	**0.36**		**0.42**	**0.26**	
Hurricane Creek adults			**POSA = 0.28**			***F = 0.19***		
Clear Creek:								
115	114	29	0.65	0.52	***	0.53	0.44	***
120	119	36	0.55	0.39	*	0.41	0.28	**
122	121	21	0.69	0.48	***	0.40	0.27	*
124	123	30	0.61	0.47	***	0.33	0.25	*
128	127	19	0.56	0.38	ns	0.48	0.33	***
Mean			**0.61**	**0.45**		**0.43**	**0.32**	
Clear Creek adults			**POSA = 0.34**			**F = 0.22**		

POSA, percentage of shared alleles; *n*, number of comparison analyzed in a t-test; *, slightly significant ($p < 0.05$); **, significant ($p < 0.005$); ***, highly significant ($p < 0.0005$); ns, not significant. The kinship coefficients for both sired (F_S) and illegitimate (F_I) offspring are averages of all eggs sampled. The average POSA and kinship coefficient between adults in each population are denoted beneath the nest data. *n* for adult POSA and *F* in each population are 3916 for Hurricane Creek and 66 for Clear Creek.

V. DISCUSSION

Alternative reproductive tactics and allopaternal care

The genetic signature of several alternative reproductive tactics (ARTs) in this study, primarily cuckoldry, nest-guard swapping, and possibly nest takeovers were all represented in the microsatellite data derived from both populations. ARTs may be innate to this species regardless of other variables. However, the intensity of ART employment may be affected by population density. As seen in figure 7 and table 4, there was an overall lower percentage of paternity by the guarding male in the high-density, Hurricane Creek population in comparison to the low-density, Clear Creek population. This implies that greater employment of ARTs is occurring in the high-density population, specifically, rampant cuckoldry and/or nest-guard swapping. Nest takeovers do not appear to be as common in *P. notatus* as in *P. promelas* (Bessert et. al. submitted) based on the number of eggs sired at each developmental stage (Table 4). For example, if a nest takeover has occurred we would expect to see all of the paternity by the guarding male occurring in the early stages of development (A-C) and no paternity in the later stages (D-E). In all of the nests in both populations, the guarding male is the father of a small percentage of eggs at each stage of development, indicating rampant cuckoldry, nest-guard swapping, or both. The percentage of illegitimate offspring in each nest for both populations falls within the range documented in *P. promelas* by Bessert et al. for sneak fertilizations and nest takeovers. This leads me to believe another yet undocumented ART is occurring; possibly nest-guard swapping.

I describe nest-guard swapping as a combination of cuckoldry and a series of nest-tending changes in a behavior analogous to the game of musical chairs. From the

genotypes and the developmental stages of the offspring, multiple cuckoldry events can be deduced, while the overall percentage of paternity by the guarding male is relatively low. This resembles recent cuckoldry in that only a small percentage of the younger eggs in the nest are sired by the guarding male. This behavior also resembles multiple or repeated cuckoldry because there are small percentages of eggs in the nest sired by a different male at all represented stages of development; however there is also a percentage of offspring which are still sired by the guarding male.

This behavior may be induced by sexual selection. In order for the bourgeois male to be successful, he uses whatever tactics are necessary to gain as many fertilizations as possible. He does this primarily through allopaternal care. At first glance, this behavior does not seem to very beneficial to the bourgeois male, as he is caring for a large portion of eggs that he has not sired. However, several scenarios can be used to explain how high levels of allopaternal care can be advantageous to the guarding males.

Allopaternal care is an odd behavior that is quite common among cyprinids, especially *Pimephales* (Ferguson 1989, Page and Johnston 1990, Avise et al. 2002, Bessert et al. submitted). In several studies on the fathead minnow, *Pimephales promelas*, several hypotheses were confirmed regarding allopaternal care (Sargent 1988, Unger and Sargent 1988, Sargent 1989), the first of which is sexual selection whereby female minnows are showing preference by spawning in nests already containing eggs (Unger and Sargent 1988). The presence of eggs in a nest may indicate to the female that the guarding male is virile and able to provide superior care to the fertilized eggs. Thus, a male might seek out a nest already containing eggs, and usurp the current nest-tender in hopes of enticing females to spawn with him a nest containing eggs. It has also been shown that increasing clutch size results in greater survival of the fertilized eggs, so females may prefer to deposit eggs in a crowded nest

to increase the survival rate of the eggs (Sargent 1988). With the number of eggs deposited in each nest reaching into the thousands, the guarding males, under adaptive selection, evolved a mechanism to manage large nest size. In *P. promelas*, the guarding male, under pressure to care for so many eggs, apparently solves this dilemma by differentiating between the eggs he sired and the illegitimate offspring in his nest (Sargent 1989). He provides less care to those eggs he did not sire, resulting in a higher mortality rate for these illegitimate eggs. From this perspective, it seems that sexual selection is driving the evolution of allopaternal care in minnows.

However, by gaining fertilizations in multiple nests these bourgeois males are capitalizing on the predator dilution effect and a type of bet-hedging. If a nest were to be destroyed by predation, the males that tended that nest would not lose all of their fertilizations, as they have more offspring in other nests that are being cared for by another male. Thus, each bourgeois male could be contributing allopaternal care to a nest that contains a small percentage of his offspring, assuming that another bourgeois male is doing the same for him.

An alternative view of the evolution of allopaternal care is kin selection. For instance, nest-guard swapping was only firmly supported by the data for the low-density population where the adults are more closely related to each other (POSA = 0.34, F = 0.22), most likely because of geographical limitations inhibiting migration. Interestingly, kin selection may be the causal factor resulting in nesting-guard swapping as males may be more inclined to care for the offspring of a relative, rather than another unrelated conspecific male. This kin selection hypothesis is supported because the guarding males are significantly more closely related to their *illegitimate* offspring than they are on average to other adults in the sampled population (Table 6) suggesting that the guarding males in both populations are caring for the eggs fertilized by close relatives.

Extensive cuckoldry within these bluntnose minnow populations was occurring in all nests sampled, because the overall paternity was very low and the guarding male sired eggs at all developmental stages sampled from each nest in both populations. Interestingly, a similar study on the striped darter, *Etheostoma virgatum*, was performed at identical study sites and cuckoldry was not detected in any of the nests assayed (Porter et al. 2002). So there were at least two sympatric species of fish, competing for the same nest rocks to spawn under, but employing two strikingly different mating behaviors. Another parentage study on sand gobies (*Pomatoschistus minutus*) reported results of equal ART frequency between a population of high density and one of low density (Jones et al. 2001). The sand goby results indicate that population density and/or ecological setting have little or no bearing on the type and frequency of ARTs that are being employed within the population. The average amount of allopaternal care detected in the nest of the bluntnose minnow was 67.8%, the highest yet to be documented in fish, compared to 12.4% in the molly miller, 35% in the sand goby, and 46.6% in the fathead minnow (Jones et al. 2001, Mackiewicz et al. 2005, Bessert et al. submitted).

Nest-guard swapping may have occurred in conjunction with cuckoldry in nests 124 and 128 from Clear Creek, TN. There were three other bourgeois males contributing offspring in nest 124 and two other bourgeois males spawning in nest 128 (Table 5). This was determined by comparing the multilocus genotype of each of the guarding males to the illegitimate offspring in the other nests within the population. The multilocus genotype of guarding male Pn121 (captured with nest 122), can be derived from several young and old offspring in nest 124, which was guarded by male Pn123. This indicates that male Pn121 visited male Pn123's nest multiple times and had

multiple spawning events of his own while male Pn123 was away. Interestingly, male Pn123 visited male Pn121's nest and had a spawning event of his own siring 9.5% of the illegitimate offspring in nest 122 (Table 5). Similarly, male Pn119 sired 13.3% of the illegitimate offspring in nest 124, guarded by male Pn123. Male Pn123 also visited nest 120, as his genotype was deduced from 2.8% of the illegitimate offspring guarded by male Pn119. Surprisingly, nest 115, guarded by male Pn114, was not visited by any of the other guarding males (Table 5), yet male Pn114 did cuckold nests 120 and 128. Once a fish becomes a bourgeois male, it is assumed to be in its second year (Boschung and Mayden 2004), so age has little bearing on the percentage of paternity a bourgeois male may have in his nest. Albeit the standard length of the males in Clear Creek were significantly ($p < 0.05$) longer than those in Hurricane Creek, larger males did not necessarily have greater paternity in the nests they were tending than the smaller males had in their nests.

Only rampant cuckoldry by sneaked fertilizations was detected in the high-density population. A recent nest takeover may have occurred in nest 107 in lieu of cuckoldry due to a very low percentage of paternity in this nest (Figure 7) by guarding male Pn105 and because he sired more of the eggs in earlier stages of development than the more mature eggs (Table 4). However, rampant cuckoldry cannot be ruled out for the same reasons; there was low paternity overall and was consistent with siring eggs in all developmental stages.

Cuckoldry was more prevalent in the high-density population it seems, possibly because these bourgeois males were less interrelated and the adult population was much larger than that of Clear Creek. This may be an artifact because a small proportion of adults and nests were sampled and genotyped for the Hurricane Creek population. There may also be a greater pressure on males to sneak fertilizations as nest space reaches a premium.

Nest-guard swapping may also be occurring in this high-density population to lesser degree in comparison to the low-density population. One bourgeois male, Pn105, could not be assigned to any one primary nest out of the three he was near upon capture. Nests 107, 108, and 109 did not have any other bourgeois males defending them at the time of collection. It is suspected that a guard-swap may have been underway at time of collection, which allowed two of the other bourgeois males to escape. Alternatively, male Pn105 may have been trying to defend all three nests at the same time, and was not very effective at chasing off cuckolders at any of these nests.

Of all the ARTs the have been discussed, nest-guard swapping is the most bizarre. Why are these males leaving their sired offspring to visit someone else's nest? The answer may be kin selection. The adults in both populations were more closely related to their illegitimate offspring than they were to other random adults in the population. The expected POSA between first-degree relatives (siblings, parents, and children) is approximately 0.5, where a son receives 50% of his father's alleles. In a normal breeding population the POSA between the guarding male and the illegitimate offspring is expected to be identical to the POSA between random adults within the population. In both of these populations, but this is not the case (Table 6).

The POSA between random adults in the Clear Creek population was 0.34 and an *F* of 0.22 compared to the average POSA between the illegitimate offspring and the guarding males of 0.45 and an F_I 0.32. Similarly in Hurricane Creek, the POSA between random adults in the population was 0.28 with an *F* of 0.19 compared to the POSA between illegitimate offspring and the guarding males of 0.36 with and F_I of 0.26 (Table 6). In both populations the POSA and F_I between the guarding male and the illegitimate offspring are significantly larger than the POSA and *F* between random adults in the population (Table 6). This supports the kin selection hypothesis because these statistics suggest that the guarding males are more closely related to the

illegitimate offspring in their nest than they are to the other random adults in the population. The guarding males are sharing more alleles with the illegitimate offspring in the nest they are tending than any other random adult from their respective population. Thus, the bourgeois males are under kin selection and will tend the eggs of a relative over a non-related conspecific male within their population, and perhaps their relatives are doing the same for them. However when the degree of relatedness was tested, the guarding males using the nest-guard swapping ART (Table 5), males Pn119, Pn121, and Pn123, did not seem to be closely related (with a POSA between male Pn119 and male Pn123 of only 0.2). Males Pn121 and Pn123 may however, be slightly related with a POSA of 0.4. So although the data between the swapping males I was able to document, show that these particular guarding males are not closely related, kin selection may still be occurring because on average over the entire nest, guarding males are more closely related to the offspring they did not sire than they are to other adults in the population. The sample size in this study may not have been large enough to definitively document kin selection, or kin selection may be occurring in conjunction with excessive cuckoldry, where many bourgeois males are swimming from nest to nest cuckolding along the way and in a population that is generally more related by general inbreeding, thus it is possible that kin selection may be occurring by chance.

VI. CONCLUSIONS

The mating system of the bluntnose minnow has shown remarkable complexity. This species employs a combination of several ARTs in order to produce as many viable offspring as possible. The behaviors of nest-guard swapping and cuckoldry by sneaked fertilizations may contribute heavily to the success of this highly fecund fish, as it allows males guarding eggs (either fostered or sired) to attract additional females to their nest because of the female preference to lay eggs in nests already containing eggs. However, two equally feasible explanations for the allopaternal care exhibited in each population of bluntnose minnow, differential care by sexual selection and kin selection, must be entertained in order to fully understand this mating system.

To further support the hypothesis of kin selection resulting in allopaternal care a larger sample of these populations should be obtained and the study repeated at other locations utilizing additional microsatellite loci. The mating systems of other species of *Pimephales* should be examined more closely to see if the ART of nest-guard swapping might be occurring under kin selection.

VII. REFERENCES

Amundsen T, and Forsgren E (2001) Male mate choice selects for female coloration in a fish. *PNAS*. 98:21. 13155-13160.

Avise JC, Jones AG, Walker D, DeWoody JA, et al. (2002) Genetic mating systems and reproductive natural histories of fishes: lessons for ecology and evolution. *Annu. Rev. Genet.* 36:19-45.

Bessert ML, Brozek J, and Orti G (submitted) Impact of nest substrate limitations on patterns of illegitimacy in the fathead minnow, *Pimephales promelas* (Cypriniformes: Cyprinidae) 1-13.

Bessert ML and Orti G (2003) Primer Note: Microsatellite loci for paternity analysis in the fathead minnow, *Pimephales promelas* (Teleostei: Cyprinidae). *Molecular Ecology Notes*. doi: 10.1046/j.1471-8286.2003.00501.x: 1-3.

Boschung HT, and Mayden RL (2004) *Fishes of Alabama*. Smithson Books: Washington.

Brody JR and Kern SE (2004) Sodium boric acid: a Tris-free, cooler conductive medium for DNA electrophoresis. February 2004. *BioTechniques* 36:214-216.

Cooper JE (1979) Description of eggs and larvae of fantail (*Etheostoma flabellare*) and rainbow (*E. caeruleum*) darters from Lake Erie tributaries. *Trans Am Fish Soc* 108:46-56.

Crow JF (1986) *Basic concepts in population, quantitative, and evolutionary genetics.* WH Freeman and Company:New York. 32-33.

Dakin EE and Avise JC (2004) Microsatellite null alleles in parentage analysis. *Heredity* 93:540-509.

Dearborn DC, Anders AD, and Parker PG (2001) Sexual dimorphism, extrapair fertilizations, and operational sex ratio in great frigatebirds (*fregata minor*). *Behavioral Ecology*. 12(6): 746-752.

DeWoody JA, and Avise JC (2001) Genetic perspectives of the natural history of fish mating systems. *The Journal of Heredity*. 92(2): 167-172.

DeWoody JA, Fiumera AC, and Avise JC (2001) Which parental exclusion probability to include? *Heredity*, submitted.

DeWoody JA, Fletcher DR, Wilkins SD, Avise JC (2000) Parentage and nest guarding in the tessellated darter (*Etheostoma olmstedi*) assayed by microsatellite markers (Perciformes: Percidae). *Copeia* 2000:740-747.

DeWoody JA, Fletcher DE, Wilkins SD, and Avise JC (2001) Genetic documentation of filial cannibalism in nature. *PNAS*. 98(9): 5090-5092.

DeWoody JA, Fletcher DE, Wilkins SD, Nelson WS, and Avise JC (1998) Molecular genetic dissection of spawning, parentage, and reproductive tactics, in a population of redbreast sunfish, *Lepomis auritus*. *Evolution*, 52(6): 1802-1810.

Dieringer D and Schlötterer C (2002) Microsatellite analyser (MSA): a platform independent analysis tool for large microsatellite data sets. *Molecular Ecology Notes* 3(1):167-169.

Fiumera AC, Porter BA, Grossman GD, and Avise JC (2002) Intensive genetic assessment of the mating system and reproductive success in a semi-closed population of the mottled sculpin, *Cottus bairdi*. *Molecular Ecology*. 11: 2367-2377.

Fletcher DE, Dakin EE, Porter BA, and Avise JC (2004) Spawning behavior and genetic parentage in the Pirate Perch (*Aphredoderus sayanus*), a fish with an enigmatic reproductive morphology. *Copeia*. (1): 1-10.

Fore SA, Guttman SI, Bailer AJ, Altfater DJ, and Counts BV (1995) Exploratory analysis of population genetic assessment as a water quality indicator. *Ecotoxicology and Environmental Safety.* 30:24-35.

Ferguson MT (1989) Crevice Spawning behavior of *Cyprinella trichroistia, C. gibbsi,* and *C. callistia*. ASB Bulletin, 36(2):40.

Hamilton WD (1964) The genetical evolution of social behaviour. I, II. *J Theor Biol*. 7:1-52.

Hartel KE, Halliwell DB, and Launer AE (2002) *Inland Fishes of Massachusetts*. Natural History of New England Series. Massachusetts Audubon Society: Lincoln, MA. 14(33):100-134.

Jones AG and Avise JC (1997) Microsatellite analysis of maternity and the mating system in the Gulf pipefish *Syngnathus scovelli*, a species with male pregnancy and sex-role reversal. *Molecular Ecology*. 6:203-213.

Jones AG, and Avise JC (2001) Mating systems and sexual selection in male-pregnant pipefishes and seahorses: insights from microsatellite-based studies of maternity. *Journal of Heredity*. 92(2):150-158.

Jones AG, Ostlund-Nilsson S, and Avise JC (1998) A microsatellite assessment of sneaked fertilizations and egg thievery in the fifteen spine stickleback. *Evolution* 52(3): 848-858.

Jones AG, Rosenqvist G, Berglund A, and Avise JC (2000) Mate quality influences multiple maternity in the sex-role-reversed pipefish *Sybgnathus typhle*. OIKOS 90(2):321-326.

Jones AG, Walker, D, Kvarnemo C, Lindstrom K, and Avise JC (2001a) How cuckoldry can decrease the opportunity for sexual selection: Data and theory from a genetic parentage analysis of the sand goby, *Pomatoschistus minutus*. *PNAS*. 98(16):9151-9156.

Jones AG, Walker D, Lindstrom K, Kvarnemo C, and Avise JC (2001b) Surprising similarity of sneaking rates and genetic mating patterns in two populations of sand goby experiencing disparate sexual selection regimes. *Molecular Ecology*. 10: 461-469.

Keast A, and Webb D (1966) Mouth and body form relative to feeding ecology in the fish fauna of a small lake, Lake Opinicon, Ontario. *J. Fish. Res. Bd. Can.* 23:1345-1374.

Klug WS, and Cummings MR (2002) *Essentials of Genetics*. Prentice Hall:Upper Saddle River, NJ. 453-457.

Kraatz WC (1928) Study of the food of the blunt-nosed minnow, *Pimephales notatus*. *Ohio J. Sci.* 28:86-98.

Krebs JR and Davies NB (1997) *Behavioural Ecology: An Evolutionary Approach*. 4th ed. Blackwell Science: Malden MA. 204-208.

Kuhne ER (1939) *A Guide to the Fishes of Tennessee and the Mid-South.* Tennessee Department of Conservation: Nashville, TN. 52-60.

Mackiewicz M, Fletcher DE, Wilkins SD, DeWoody JA, and Avise JC (2002) A genetic assessment of parentage in a natural population of dollar sunfish (*Lepomis marginatus*) based on microsatellite markers. *Moleuclar Ecology*. 11: 1877-1883.

Mackiewicz M, Porter BA, Dakin EE, and Avise JC (2005) Cuckoldry rates in the molly miller (*Scartella cristata*; Blenniidae) a hole-nesting marine fish with alternative reproductive tactics. *Marine Biology*. 148: 213-221.

McCoy EE, Jones AG, and Avise JC (2001) The genetic mating system and tests for cuckoldry in a pipefish species in which males fertilize eggs and brood offspring externally. *Molecular Evolution*. 10:1793-1800.

Mettee MF, O'Neil PE, and Pierson JM (1996) *Fishes of Alabama and the Mobile Basin*. Oxmoor House: Birminham, AL. 128-319.

Moyle PB (1973) Ecological segregation amoung three species of minnows (Cyprinidae) in a Minnesota Lake. *Trans. Amer. Fish. Soc*. 102:794-805.

Page LM and Ceas PA (1989) Egg attachment in *Pimephales* (Piscus: C*yprinidae*). American Society of Ichthyologists and Herpetologists. *Copeia*. 4:1074-1077.

Page LM, Hardman M, and Near TJ (2003) Phylogenetic relationships of barcheek darters (Percidae: *Etheostoma*, Subgenus: *Catonotus*) with descriptions of two new species. *Copeia*. 3: 512-530.

Page LM and Johnston CE (1990) The breeding behavior of *Opsopoeodus emiliae* (*Cyprinidae*) and its phylogenetic implications. *Copeia*. (4): 1176-1180.

Page LM, Morris MA, and Retzer ME (1985) Reproductive strategies in the genus *Pimephales*. ASIH 65th Annual Meeting. [abstract].

Porter BA, Fiumera AC, and Avise JC (2002) Egg mimicry and allopaternal care: two mate-attracting tactics by which nesting striped darter (*Etheostoma virgatum*) males enhance reproductive success. *Behav Ecol Sociobiol*. 51:350-359.

Raymond M, and Rousset F (1995) GENEPOP: population genetic software for exact tests and ecumenicism. *J. Hered.* 86:248-249 http://wbiomed.curtin.edu.au/genepop/.

Ridley M (1993) *Evolution*. Blackwell Science: Cambridge, MA. 308-310.

Sambrook J, and Russell DW (2001) *Molecular Cloning: A Laboratory Manual*. Third Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sargent RC (1988) Paternal care and egg survival both increase with clutch size in the fathead minnow, *Pimephales promelas*. *Behavioral Ecology and Sociobiology*. 23(1):33-37.

Sargent RC (1989) Allopaternal care in the fathead minnow, *Pimephales promelas*:stepfathers discriminate against their adopted eggs. *Behavioral Ecology and Sociobiology*. 25(6):379-385.

Sokal RR, and Rohlf FJ (1995) *Biometry: the principles and practice of statistics in biological research*. WH Freeman and company: New York. 219-227.

Taborksy M (1994) Sneakers, satellites, and helpers: parasitic and cooperative behavior in fish reproduction. *Adv. Study Behav.* 23: 1-100.

Unger LM and Sargent RC (1988) Allopaternal care in the fathead minnow, *Pimephales promelas*: females prefer males with eggs. *Behavioral Ecology and Sociobiology*. 23(1):27-32.

VanCleave HJ and Markus HC (1929) Studies on the Life History of the Blunt-nosed Minnow. *The American Naturalist*. 63:530-539.

Walker D, Porter BA, and Avise JC (2002) Genetic parentage assessment in the crayfish *Orconectes placidus*, a high-fecundity invertebrate with extended maternal brood care. *Molecular Ecology* 11:2115-2122.

VIII. APPENDIX

Appendix a. PCR conditions for single reaction for each microsatellite locus

Pn1.3	**1 rxn**	**Thermocycling conditions**		
Fisher 10X buffer	1.32 µl	**Step**	**Temp °C**	**Time**
dNTPs [1.25 mM]	2.2 µl	Denature	95	30 s
MgCl2 [1.5 mM]	0.792 µl	Annealing	60	30 s
Pn1.3F-HEX [0.01 mM]	0.33 µl	Extension	72	30 s
Pn1.3R [0.01 mM]	0.33 µl	Cycles	32	
Taq [5U/µl]	0.11 µl	Final Extension	72	2 m
HPLC water	5.918 µl	Incubate	15	forever
DNA [50ng/µl]	2.0 µl			
Total	11 µl			

Pn1.5	**1 rxn**	**Thermocycling conditions**		
Fisher 10X buffer	1.32 µl	**Step**	**Temp °C**	**Time**
dNTPs [1.25 mM]	2.2 µl	Denature	95	30 s
MgCl2 [2 mM]	1.056 µl	Annealing	60	30 s
Pn1.5F-FAM [0.01 mM]	0.33 µl	Extension	72	30 s
Pn1.5R [0.01 mM]	0.33 µl	Cycles	32	
Taq [5U/µl]	0.11 µl	Final Extension	72	2 m
HPLC water	5.654 µl	Incubate	15	forever
DNA [50ng/µl]	2.0 µl			
Total	11 µl			

Pn1.9	**1 rxn**	**Thermocycling conditions**		
10X **LGL** buffer	1.32 µl	**Step**	**Temp °C**	**Time**
dNTPs [1.25 mM]	2.2 µl	Denature	95	30 s
MgCl2 [2.75 mM]	1.452 µl	Annealing	50	30 s
Pn1.9F-NED [0.01 mM]	0.33 µl	Extension	72	1 m
Pn1.9R [0.01 mM]	0.33 µl	Cycles	32	
Taq [5U/µl]	0.11 µl	Final Extension	72	2 m
HPLC water	5.26 µl	Incubate	15	forever
DNA [50ng/µl]	2.0 µl			
Total	11.002 µl			

Pn2.15	**1 rxn**	**Thermocycling conditions**		
10X **LGL** buffer	1.32 µl	**Step**	**Temp °C**	**Time**
dNTPs [1.25 mM]	2.2 µl	Denature	95	30 s
MgCl2 [~1.6 mM]	0.86 µl	Annealing	50	30 s
Pn2.15F-FAM [0.01 mM]	0.33 µl	Extension	72	1 m
Pn2.15R [0.01 mM]	0.33 µl	Cycles	32	
Taq [5U/µl]	0.11 µl	Final Extension	72	2 m
HPLC water	5.85 µl	Incubate	15	forever
DNA [50ng/µl]	2.0 µl			
Total	11 µl			

Ppro80	1 rxn	Thermocycling conditions		
Fisher 10X buffer	1.23 µl	**Step**	**Temp °C**	**Time**
dNTPs [1.25 mM]	2.2 µl	Denature	95	30 s
MgCl2 [2 mM]	1.056 µl	Annealing	50	30 s
Ppro80F-HEX [0.01 mM]	0.4 µl	Extension	72	1 m
Ppro80R [0.01 mM]	0.4 µl	Cycles	32	
Taq [5U/µl]	0.11 µl	Final Extension	72	2 m
HPLC water	5.65 µl	Incubate	15	forever
DNA [50ng/µl]	2.0 µl			
Total	11.046 µl			

Ppro118	1 rxn	Thermocycling conditions		
Fisher 10X buffer	1.23 µl	**Step**	**Temp °C**	**Time**
dNTPs [1.25 mM]	2.2 µl	Denature	95	30 s
MgCl2 [2 mM]	1.056 µl	Annealing	60	30 s
Ppro118F-NED [0.01 mM]	0.4 µl	Extension	72	30 s
Ppro118R [0.01 mM]	0.4 µl	Cycles	32	
Taq [5U/µl]	0.11 µl	Final Extension	72	2 m
HPLC water	5.654 µl	Incubate	15	forever
DNA [50ng/µl]	2.0 µl			
Total	11.05 µl			

Ppro126	1 rxn	Thermocycling conditions		
Fisher 10X buffer	1.32 µl	**Step**	**Temp °C**	**Time**
dNTPs [1.25 mM]	2.2 µl	Denature	95	30 s
MgCl2 [2 mM]	1.056 µl	Annealing	55	30 s
Ppro126F-FAM [0.01 mM]	0.4 µl	Extension	72	1 m
Ppro126R [0.01 mM]	0.4 µl	Cycles	32	
Taq [5U/µl]	0.11 µl	Final Extension	72	2 m
HPLC water	5.65 µl	Incubate	15	forever
DNA [50ng/µl]	2.0 µl			
Total	11.136 µl			

Ppro48	1 rxn	Thermocycling conditions		
Fisher 10X buffer	1.23 µl	**Step**	**Temp °C**	**Time**
dNTPs [1.25 mM]	2.2 µl	Denature	95	30 s
MgCl2 [2 mM]	1.056 µl	Annealing	55	30 s
Ppro48F [0.01 mM]	0.4 µl	Extension	72	1 m
Ppro48R [0.01 mM]	0.4 µl	Cycles	32	
Taq [5U/µl]	0.11 µl	Final Extension	72	2 m
HPLC water	5.654 µl	Incubate	15	forever
DNA [50ng/µl]	2.0 µl			
Total	11.05 µl			

Ppro132	1 rxn	Thermocycling conditions		
Fisher 10X buffer	1.23 μl	**Step**	**Temp ^{o}C**	**Time**
dNTPs [1.25 mM]	2.2 μl	Denature	95	30 s
MgCl2 [1 mM]	0.528 μl	Annealing	50	30 s
Ppro132F [0.01 mM]	0.33 μl	Extension	72	30 s
Ppro132R [0.01 mM]	0.33 μl	Cycles	32	
Taq [5U/μl]	0.11 μl	Final Extension	72	2m
HPLC water	6.182 μl	Incubate	15	forever
DNA [50ng/μl]	2.0 μl			
Total	10.91 μl			

Appendix b. Equations and statistical analyses

Single locus exclusion probability equations for three different models

1 Parent Known:

$$P_E = 1 - 2\sum_{i=1}^{n} p_i^2 + \sum_{i=1}^{n} p_i^3 + 2\sum_{i=1}^{n} p_i^4 - 3\sum_{i=1}^{n} p_i^5 - 2\left(\sum_{i=1}^{n} p_i^2\right)^2 + 3\sum_{i=1}^{n} p_i^2 \sum_{i=1}^{n} p_i^3$$

Neither Parent Known:

$$P_E = 1 - 4\sum_{i=1}^{n} p_i^2 + 2\left(\sum_{i=1}^{n} p_i^2\right)^2 + 4\sum_{i=1}^{n} p_i^3 - 3\sum_{i=1}^{n} p_i^4$$

Parent Pairs Evident:

$$P_E = 1 + 4\sum_{i=1}^{n} p_i^4 - 4\sum_{i=1}^{n} p_i^5 - 3\sum_{i=1}^{n} p_i^6 - 8\left(\sum_{i=1}^{n} p_i^2\right)^2 + 8\left(\sum_{i=1}^{n} p_i^2\right)\left(\sum_{i=1}^{n} p_i^3\right) + 2\left(\sum_{i=1}^{n} p_i^3\right)^2$$

Where pi is the frequency of the ith allele

Combined exclusion probability equation

Combined Across Loci:

$$P_{CE} = 1 - \prod_{l=1}^{L} (1 - PE_l)$$

Where PE_l is the exclusion probability at the jth locus

t-test equation

$$t_s = \frac{(\bar{Y}_1 - \bar{Y}_2) - (\mu_1 - \mu_2)}{\sqrt{\left[\frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2 - 2}\right]\left(\frac{n_1 + n_2}{n_1 n_2}\right)}}$$

with df = $n_1 + n_2 - 2$

Effective number of alleles (n_e)

$n_e = 1/\Sigma p_i^2$, where p_i is frequency of the i^{th} allele.

Expected heterozygosity (H_E):

$$H_S = \frac{\sum_{s=1}^{n} H_{exp\,s} \times N_s}{\sum_{s=1}^{n} N_s}$$, where H_S is the global weighted average of the expected heterozygosities across all the subpopulations with the subscript *s* refers to the s^{th} of *n* subpopulations

Observed heterozygosity (H_O):

$$H_I = \frac{\sum_{s=1}^{n} H_{obs\,s} \times N_s}{\sum_{s=1}^{n} N_s}$$, where H_I is the global weighted average of the observed heterozygosity across the subpopulations with the subscript *s* refering to the s^{th} of *n* subpopulations

Kinship coefficient:

$F = \frac{H_S - H_I}{H_I}$, where HS is the expected heterozygosity across the subpopulations, and HI is the observed heterozygosity across the subpopulations.

Appendix c. Exclusion probabilities for selected loci

P_{CE} Calculation: Hurricane		
Locus	**P_E**	**1-P_E**
5 Loci multiplex		
Pn1.3	0.69	0.31092825
Ppro126	0.64	0.36
Pn1.5	0.46	0.54
Ppro48	0.55	0.45
Ppro118	0.70	0.30
	Π	0.01
	P_{CE}	0.99
3 Loci multiplex		
Pn1.3	0.69	0.31
Pn1.5	0.46	0.54
Ppro118	0.70	0.30
	Π	0.05
	P_{CE}	0.95

P_{CE} Calculation: Clear		
Locus	**P_E**	**1-P_E**
5 Loci multiplex		
Pn1.3	0.24	0.76
Ppro126	0.41	0.59
Pn1.5	0.53	0.47
Ppro48	0.61	0.39
Ppro118	0.67	0.33
	Π	0.03
	P_{CE}	0.97
3 Loci multiplex		
Pn1.3	0.24	0.76
Pn1.5	0.53	0.47
Ppro118	0.67	0.33
	Π	0.12
	P_{CE}	0.88

Appendix d. Microsatellite descriptions

Primer	Repeat Motif	Size Range (bp)	Clone Size	Label	Color
Pn1.3	(CA)11	100-125	115	VIC	green
Pn1.5	(CA)14	175-200	200	FAM	blue
Pn1.9	(AT)9+(GT)15	225-500	250	NED	yellow
Pn2.15	(CT)48	390-400	350-490	FAM	blue
Ppro80	$(GATA)_{58}$	300-375		HEX	green
Ppro118	$(CTAT)_{11}(CTGT)_{15}$	175-200		NED	yellow
Ppro126	$(CA)_{12}$	190-200		FAM	blue
Ppro48	$(TG)_{11}$	240-260		NED	yellow
Ppro132	$(CT)_{18}$	180-190		FAM	blue

Primer Sequences:

Pn1.3F 5'- CCT ACA GGC AGC ACT TTA TC -3'
Pn1.3R 5'- CAG AAA CTC AGA GAT TCC TAC -3'

Pn1.5F 5'- GAG GAA TCC ATC ATC TGC AC -3'
Pn1.5R 5'- CTT TAA CCT GAT AGC GCA GC -3'

Pn1.9F 5'- GTC ACC ATA CTG AGT CTT CAG AC -3'
Pn1.9R 5'- GCT TGC CAT AGT CAT GAC TAG C -3'

Pn2.15F 5'- CTG AGC TGA TAG CTT AAC GG -3'
Pn2.15R 5'- CTA CTG ACT GCT CAC TGT CC-3'

Ppro48F 5'- TGC TCT GCT CTC CTG CGT GTC ATT -3'
Ppro48R 5'- CAG CCT CGG CGG TGT TGT TGC -3'

Ppro80F 5'- AGC GAT TCA ACA CCT TCA GGA -3'
Ppro80R 5'- GTG GGG AAT GGA TCG AAA CAA T -3'

Ppro118F 5'- CCG GAT GCA CTG GTG GAG AAA A -3'
Ppro118R 5'- CCA GCA ATC ATA GCA GGC AGG AAC -3'

Ppro126F 5'- CTG CGT GTC TGA TAA CTG TGA CTG -3'
Ppro126 5'- GTC CCG GGA CTT TAA GAA GGT C -3'

Ppro132F 5'- GCA TTT CCT TTT GCT TGT AAG TCT CAA -3'
Ppro132R 5'- GGT TTA ACC CGA TCA ATG GCT GTG C -3'