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PARENTAGE ASSIGNMENT OF BROWN TROUT (SALMO TRUTTA L.) JUVENILES FOLLOWING STOCKING OF MULTIPLE DONOR POPULATIONS

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By

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ABSTRACT

PARENTAGE ASSIGNMENT OF BROWN TROUT (*SALMO TRUTTA* L.) JUVENILES FOLLOWING STOCKING OF MULTIPLE DONOR POPULATIONS

By

Lauren Stanchek

Forensic analyses have great utility beyond forensic science when applied to other biological disciplines. For instance, parentage analysis is applied extensively in the field of Molecular Ecology as a means of better understanding mating systems. In order to adjust stocking strategy to more appropriately suit brown trout (*Salmo trutta* L.) mating behavior, the current study sought to assign parentage to a juvenile cohort of brown trout following stocking of adults from each of several donor populations. Parentage analysis was based on adult and juvenile genotypes, which consisted of six microsatellite loci. Juvenile genotypes were compared to those of putative parents using likelihood-based statistical methods. Estimates of male and female reproductive success were calculated based on parentage assignment. Several explanatory variables, including population source and body size, were examined as potential sources of individual variation in male and female reproductive success. All source populations contributed to the juvenile generation, but twenty-one percent of the juveniles could not be associated with any adults due to either low statistical power or failure to acquire samples from all adults. No correlation between body size and reproductive success could be established. Mating occurred randomly among males and females from all locations and the number of mates per individual did not differ significantly between males and females. Several suggestions are made regarding stocking strategy for the brown trout, as well as extensive comparisons between human forensic and wildlife parentage analysis.

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INTRODUCTION

Forensic science, or scientific analyses as applied to circumstances concerning the public, has evolved into a discipline of immense scope. This broad definition of forensic science has encouraged the use of scientific analyses in situations ranging from murder investigations, to identifications after mass disasters, computer crimes, detection of deception, parentage analysis, and wildlife forensics. With such a broad spectrum of sub-disciplines, forensic science can be applied to any judicial inquiry that requires the use of scientific analyses.

DNA Analysis in Forensic Science—Overview

DNA analysis became firmly integrated into forensic science as a result of Alec Jeffreys' development of Multi-Locus Probe (MLP) DNA 'fingerprinting' in the mid-1980s (Jeffreys, 1985; Butler, 2001; Lynch, 2003). Since its genesis in forensic science, DNA analysis has evolved to the prevalent use of Short Tandem Repeat (STR) loci. When multiple STR loci are used concurrently, statistical power in forensic analyses can be extremely high (Balding, 1999).

STRs, also known as microsatellites, are tandem repeats of a short, simple DNA sequence, usually from two to six base pairs in length. The advantages of microsatellites include the fact that they are based on polymerase chain reaction (PCR) technology. DNA samples of insufficient quantity or poor quality can be amplified until they are of ample quantities for analysis. Also, in contrast to Jeffreys' DNA MLP technique, which targeted repeat motifs in multiple regions of the genome simultaneously, microsatellites amplify very specific regions of the genome. Microsatellites greatest benefit, however, is that they provide tremendous statistical power in individual identification, or when used

in parentage analysis. The statistical power inherent in microsatellites is a function of the large number of microsatellites that are available for analysis, high levels of heterozygosity, and large numbers of alleles at each locus. Loci can and should be selected from different chromosomes. This independent assortment of loci means that the product rule can be applied to the probabilities of genotype matches for each locus, generating probabilities sufficient for exclusion and identification.

In 1997, a standardized core of 13 human STR loci were established for DNA profiling and provided the foundation for the nation-wide CODIS (Combined DNA Index System) system. CODIS now contains the DNA profiles of nearly 2 million convicted offenders and has aided in over 3,000 cases (FBI, 2004). Furthermore, DNA analysis has found remarkable use beyond the identification of offenders in a number of other forensic sub-disciplines.

DNA Analysis in Forensic Sub-disciplines

Wildlife Forensics

Wildlife forensics constitutes one of the currently burgeoning sub-disciplines of forensic science. Wildlife forensics originally developed from the need to analyze evidence received in cases of illegal collection, possession, and sale or trade of pieces and goods originating from organisms considered protected, threatened, or endangered (Goddard and Espinoza, 2000). For example, traditional Chinese medicine frequently uses parts from the tiger and rhinoceros, both of which currently hold endangered status (Singh et al., 2004). This field has expanded to now include cases beyond just those affecting species in peril, such as use of a prohibited weapon in making a kill, hunting outside of an established season, poaching on private property, collecting too many

animals during a season, and killing an animal of the wrong sex (Goddard and Espinoza, 2000). The requirement for forensic analyses in many wildlife cases stems from the condition of evidence, which has typically been altered to the extent that simple morphological identification is impossible.

Like many other sub-disciplines of forensic science, wildlife forensics has evolved in methodology to meet the challenges presented by the types and conditions of evidence. Pathology, molecular biology, morphologic examinations, ballistics and toolmarks, questioned documents, analytical chemistry, and fingerprints all play a part in analyzing the evidence from wildlife cases (Goddard and Espinoza, 2000). DNA, one of the newest and most commonly applied analyses in wildlife forensics, most commonly functions in taxonomic identification, sex determination, and in some cases, identification of individuals (Goddard and Espinoza, 2000). Cases requiring DNA analysis primarily utilize PCR-based methods, such as mitochondrial DNA sequencing and STRs (Goddard and Espinoza, 2000; Singh et al., 2004; Verma et al., 2003; Wan and Fang, 2002).

Human Parentage Analysis

Another sub-discipline within forensic science that is grounded in DNA technology is parentage testing. Parentage testing most commonly involves paternity analysis, where the genotype of an alleged father undergoes comparison to the genotypes of a child and the child's biological mother. However, the identification of a child's mother or both parents may also prove necessary in some cases (Schanfield, 2000). In humans, parentage determination is typically employed in resolving child support disputes. Parentage analysis is used not only in cases attempting to identify biological parents and relatives, but also in situations where identification of a body cannot be

achieved simply by visual means, such as in mass disasters (Corvach et al., 1996; Leclair et al., 2004; Marcotte et al., 1996; Martin et al., 1996; Primorac et al., 1996). Some cases involving crime scenes with blood stain evidence, but the missing body of the victim, may also require parentage analysis to identify the source of the blood stain (Mevåg et al., 1996; Schanfield, 2000).

Like wildlife forensics, the evolution of technological changes in parentage testing has resulted in a shift from allozymes and blood groups to the prevailing use of restriction fragment length polymorphisms (RFLPs) and PCR-based methods such as VNTR loci, including long terminal repeats (LTRs) and STRs (Arroya et al., 1994; Bjerre, 1997; Dobosz, 1990; Domenici et al., 1998; Geada et al., 2001; Hallenberg and Morling, 2002; Lobbiani, 1991; Morling et al., 2002; Schanfield, 2000; Thomson, 1999). Also like wildlife forensics, statistical probabilities provide greater certainty to conclusions based on DNA analyses. If an alleged parent cannot possibly be a true parent, that individual is eliminated from further consideration. If the alleged parent cannot be excluded, a probability of parentage, or Parentage Index, is assigned in relation to a reference population (Bias, 1983; Brooks, 1982; Bryant, 1980; Dawid, 2001; Dykes, 1982; Morris, 1982; Pohl, 1982; Schanfield, 2000; Thomson, 1999). Analysts typically calculate probabilities using a computer program designed to account for such factors as mutations and the unavailability of one parental genotype (Cowell, 2003).

Statistics of Parentage Analysis

The statistical probability of parentage, or likelihood of parentage, is determined by means of LOD (Logarithm of Odds) scores. The calculation of a LOD score requires the assessment of hypotheses in relation to a given data set (Balding and Nichols, 1997;

Evett and Weir, 1998; Marshall et al., 1998; National Research Council, 1996; Pohl, 1982). In parentage analysis, a likelihood ratio (Eq.1) is estimated by comparing the hypothesis that the candidate parental pair is the true parental pair (H_1) to the hypothesis that an arbitrary candidate parental pair from the population is the true parental pair (H_2). The likelihood of each hypothesis is determined from the probability of observing an offspring genotype in the candidate parental pair's genotypes (D).

$$L(H_1,H_2|D) = \frac{P(D|H_1)}{P(D|H_2)} \qquad \text{(Eq. 1)}$$

An overall likelihood ratio for each candidate parent consists of the product of likelihood ratios for each locus. The LOD score itself is calculated by taking the natural log of the overall likelihood ratio for each candidate parent.

A positive LOD score indicates that the candidate parent has a higher likelihood of true parentage than an individual selected at random from the population. A zero LOD score indicates that the likelihood of a candidate's true parentage equals that of an individual selected at random from the population, and a negative LOD score indicates that the candidate parent has a lower likelihood of true parentage than a random individual. Positive likelihood scores represent potential parents, even if a mismatch exists between the genotypes of that potential parent and the progeny individual. In this way, analytical programs account for the possibility of mutation, related individuals, as well as the possibility of typing and data entry error. In addition, more than one potential parental pair may have a positive likelihood score. In these situations, some programs calculate additional statistics in order to quantify the magnitude of differences in likelihood.

A number of additional estimates are used in order to generate LOD scores, as well as to evaluate the quality and power of the data. These additional estimates include the number of alleles per locus, expected and observed population heterozygosity, Hardy-Weinberg expectations, and the probability of exclusion for each locus and across all loci. The number of alleles per locus is a straightforward count of the number of different alleles present at each locus taken directly from the population genotypes. The expected heterozygosity (H_E; Eq. 2) is a measure calculated by subtracting the sum of the homozygote genotypic frequencies from one (Estoup et al, 1998). This value is then multiplied by the number of individuals in the population, divided by one less than the number of individuals in the population. In this equation, n is the total number of individuals in the population and p_i^2 is the frequency of homozygotes.

$$H_E = \frac{n}{n-1} \Sigma (1 - \Sigma p_i^2) \qquad \text{(Eq. 2)}$$

Observed heterozygosity (H_O; Eq. 3) is a direct measure, the actual count of heterozygote genotypes in the population divided by the total number of individuals sampled. In this equation, N_{AB} is number of observed heterozygotes in population, where A and B are alleles, and N is the total number of individuals in the population.

$$H_O = N_{AB}/N \qquad \text{(Eq. 3)}$$

Hardy-Weinberg equilibrium (HW), is another indication of forces acting on a single locus in a population, such as a founder effect or inbreeding, or may indicate the presence of null alleles (Brooks, 1982). Hardy-Weinberg assumes that the organisms under study are sexually-reproducing, diploid organisms who randomly mate and exhibit

discrete generations. This measure also assumes a sizable population with no mutation, migration, or selection acting upon it. In brief, if a population is in Hardy-Weinberg equilibrium, population allele frequencies are predictive of genotype frequencies in the same population. Deviation from expectations may indicate a discord with the Hardy-Weinberg assumptions. More specifically, violations of Hardy-Weinberg will appear as an excess of homozygotes or heterozygotes.

Additional statistics, such as probabilities of exclusion, serve as indicators of statistical power. They reflect probabilities of eliminating non-parents from consideration. Exclusion is determined by comparison of the progeny individual's genotype to a known parent's genotype and a candidate parent's genotype (Dykes, 1982; Marshall et al., 1998). Exclusion occurs when a candidate's genotype includes a mismatch with the offspring genotype. The actual probability of exclusion is calculated based on the probability of finding a particular genotype in a population with specific allele frequencies. Finding a specific genotype in the population hinges upon the number of loci examined, the degree of polymorphism for each locus, the allele frequency distribution, the number of potential parents, and the number of progeny (Weir, 1996).

Parentage Analysis in Non-humans

Non-human parentage analysis appears occasionally in forensic science, when unique circumstances require the combination of wildlife forensics and DNA analysis. Parentage analysis may determine whether an allegedly poached animal belongs to a specific, protected group or whether trespassing occurred in order to obtain an animal (Poetsch et al., 2001). It may also determine ownership in disputes over livestock (Lirón et al., 2004), as well as to verify cat, dog, and horse breeding lines.

In one case study, a dispute developed between two Holstein dairy farmers because one farmer claimed that some calves of the neighboring farmer were sired by his bulls (Lirón et al., 2004). Paternity analysis was applied using DNA collected from the known mother of the calves, the calves themselves, and the potential fathers. The parentage analyses performed in this case required the use of ten microsatellites to develop genotypes for each animal. A computer program, Cervus© 2.0 (Marshall et al., 1998), applied the genotypes obtained from the suite of microsatellites to calculate the likelihood of each possible father for each calf. The analysis demonstrated a high likelihood of paternity in the assignment of three of the disputed offspring (Lirón et al., 2004). The other two calves could not be attributed to any of the sampled bulls, implying that another bull in the herd must have sired them.

Parentage Analysis Software in a Non-human Context

The Holstein case of Lirón et al. (2004), a non-human forensic case requiring parentage analysis, utilized Cervus 2.0© (Marshall, 1998), a popular computer program designed to assign potential parents to progeny individuals when only one parent or neither parent is known. The program compares the genotypes of the putative parents to the progeny and assigns parentage based on the likelihood of achieving a particular progeny genotype in relation to the available parental genotypes. This likelihood is directly based on the allele frequencies in the population.

Cervus 2.0© incorporates all of the statistical approaches commonly used for human parentage analysis. However, the program includes one additional statistic, the Delta score, to aid in assigning parentage. The development of this additional statistic is due to the complex circumstances surrounding many cases of non-human parentage

analysis. The Delta score is a value used to assist in assigning parentage by distinguishing the candidate parent of highest relative likelihood. The Delta statistic is simply the difference between the candidate with the largest LOD score and the candidate with the next largest LOD score. Zero and negative LOD scores are not included in calculating Delta. Putative parental pairs receive rank based first on the LOD score, and secondly on the Delta score. The pair with the highest LOD and Delta represents the most likely pair.

As with many statistical analyses, the significance of results must be evaluated by levels of confidence. Since Delta values do not exhibit a standard distribution, the simulation module of Cervus 2.0© uses Delta scores from many replicated simulations of parentage tests to create a distribution. Delta scores generated during the real parentage analysis are then compared to the distribution created by the program. A relaxed confidence level of 80% means that four out of five Deltas fall within the distribution of Deltas which belong to candidates of true parentage. Likewise, a strict confidence level of 95% means that 19 out of 20 Deltas fall within the distribution of Deltas which belong to candidates of true parentage. The Delta Criterion is the value at which actual Delta scores become significant at a specific level of confidence. A Delta criterion is also calculated for both levels of confidence for cases in which one parent is known, as well as cases where neither parent is known.

DNA Analysis in Molecular Ecology

Ecologists embraced DNA analysis in the late 1980s, not long after forensic science adopted the techniques (Loeschcke et al., 1994). To date, DNA analysis has been utilized extensively by molecular ecologists to examine relatedness, genetic diversity, and evolution. Molecular techniques have been applied to species ranging from plants (e.g. pine, *Pinus sylvestris*), to fishes (e.g. topminnows, *Poeciliopsis* spp.), and mammals (e.g. Speke's gazelle, *Gazella spekei*) (Loeschcke et al., 1994). Microsatellites have been used in studies of species hybridization, population history, and phylogeography (Beaumont and Bruford, 2000). These types of studies can reveal evidence of bottlenecks, inbreeding, social structures, dispersion, and population genetic structure. More specifically, molecular ecology studies have proven incredibly beneficial in evaluating the effects of reproductive behaviors (Couvet and Ronfort, 1994; DeWoody and Avise, 2001).

Parentage Analysis in Fish

Molecular Ecologists, particularly those interested in fishes, have employed parentage analysis extensively to estimate reproductive success and to quantify aspects of the environment or phenotype that explain variance in individual reproductive success (Bekkevold et al., 2002; Bentzen, 2001; Blanchfield et al., 2003; DeWoody and Avise, 2001; Fiumera, 2001; Fiumera et al., 2002; Garcia-Vasquez et al., 2001; Garrant et al., 2001; Neff, 2001; Planes and Lenfant, 2002; Taborsky, 2001). Other fish parentage studies have focused on estimating the mean and variance in male and female reproductive success (Østergaard et al., 2003; Säisä et al., 2003). Characteristics of interest to ecologists regarding reproduction include the number of partners per

individual, as well as selective mating based on phenotypic characteristics such as body size (Bentzen et al., 2001; Blanchfield et al., 2003; DeWoody and Avise, 2001; Fiumera et al., 2002). An understanding of fish mating systems is essential to ensuring the preservation of diversity and determining the best methods for stocking (Cowx, 1994; Gross et al., 2002).

Fisheries ecologists have widely embraced molecular methods due to the difficulty in making accurate behavioral observations for aquatic organisms, as well as the complex nature of fish mating systems (Blanchfield et al., 2003; DeWoody and Avise, 2001; Fiumera et al., 2001; Planes and Lenfant, 2002; Utter and Ryman, 1993). The application of genetic markers to wildlife has evolved in synchrony with other fields that exploit genetic markers. Microsatellites (STRs) deserve the attention they currently inspire, as they provide the best available resolution in statistical analyses (DeWoody and Avise, 2000; Estoup et al., 1998; O'Reilly et al. 1998). Fish parentage analyses, like human parentage analyses, seek to establish probabilities of a parent-offspring match based on available genotypic data, taking into account similar confounding factors such as mutations and close relatives. Also like human parentage analyses, computer programs typically provide the means of applying definitive statistical tests needed in generating probabilities of correct parental assignment (Bekkevold et al., 2002; Bentzen et al., 2001; Fiumera et al., 2001; Garrant et al., 2001; Marshall et al., 1998; Planes and Lenfant, 2002; Säisä et al., 2003).

Scientific research, including non-human parentage analysis, typically focuses on species that are easy to study or that hold some level of importance to society. Economic interests are often rooted in raising large numbers for food or for tourism. However,

recent trends emphasize additional motivations, such as the preservation of genetic diversity in order to maintain healthy and sustainable natural populations (Ryman, 1991; Tanksley and McCouch, 1997). One of the most studied fish families is the salmonids (family Salmonidae), due to their importance as a food source and as a sporting commodity (Bagliniére and Maisse, 1991; Hardy, 1972; MacCrimmon and Marshall, 1968). Also, in accordance with current trends toward environmental sustainability, many salmonid populations have drawn attention to the need for preserving population numbers and habitats. Fisheries geneticists have also sought to more fully grasp the potential effects of stocking fish into wild populations (Allendorf and Ryman, 1987; Altukhov and Salmenkova, 1987; Crisp, 1989; Thibault, 1983; Stanfield and Jones, 2003).

Parentage Analysis in Brown Trout

Among salmon, the brown trout (*Salmo trutta* L.) has been purposely spread by humans well beyond its original distribution, stirring increasing research interest in this species (MacCrimmon and Marshall, 1968). The brown trout adapts well to a variety of environments and demonstrates a high degree of both phenotypic and behavioral variation (Allendorf et al., 1976; Bagliniére and Maisse 1991; Frost and Brown, 1967). *S. trutta* displays a range of scale colors and patterns and thrives in either freshwater or saltwater (Campbell, 1977; Elliot, 1994; Frost and Brown, 1967). Brown trout are observed in lakes, rivers, tributaries, and the ocean. Due to these qualities, brown trout have been introduced in places far beyond their original distribution of Europe, northern Africa, and north-western Asia. Introductions to North America began in 1883 when eggs were dispatched from Germany to a hatchery in New York, followed shortly

thereafter by more eggs from England (Frost and Brown, 1967). The fish reared from these eggs were released into Canadian waters and spread further into Canada and the United States and represent the forerunners of modern North American brown trout populations.

The brown trout is a species of commercial, recreational, and ecological significance. Highly desired in sport fisheries, *S. trutta* bolsters the tourism of a number of European countries (Laikre et al., 2000). In addition, commercial fisheries produce brown trout for stocking, either to found new populations or boost the size of existing natural populations. In terms of ecology, *S. trutta* fills a unique aquatic niche in its native habitat. In Scotland, larvae of the freshwater pearl mussel, *Margaritifera margaritifera*, live on the gills of the brown trout (Young and Williams 1984 a,b). This element of the freshwater pearl mussel's reproductive strategy is absolutely critical in order to produce the next generation. In addition to its interaction with other species, the brown trout embodies an exceptionally genetically sub-structured species (Allendorf et al., 1976; Bouza et al., 1999; Crozier and Ferguson, 1986; Fahy, 1989; Ferguson, 1989; Ferguson and Taggart, 1991; Hindar et al., 1991; Karakousis and Triantaphyllis, 1990; Osinov and Bernatchez, 1976; Prodöhl et al., 1992). For instance, Hindar et al. (1991) found significantly large genetic differences between landlocked populations of Norwegian brown trout and their counterparts with access to the sea. The rare between-population diversity has captured the attention of conservationists, as brown trout habitats face depletion and influxes of hatchery fish into wild populations threaten to decrease genetic diversity (Laikre et al., 2000; Meffe, 1986; Togan et al., 1995).

Declining brown trout abundance and distribution throughout its native range exemplify recent focal points in fisheries conservation. Many brown trout populations have been destroyed in the past century, decreasing the availability of resources for angling and aquaculture (Ferguson et al., 1989). The most frequent causes of environmental degradation include overexploitation, pollution, loss of genetic diversity, and destruction of habitat (Allendorf, 1988; Frankel, 1974; Laikre et al., 2000). Diversity may be lost when large numbers of hatchery fish of limited genetic variation are mixed with individuals from wild populations (Allendorf, 1991; Garcia-Marin et al., 1991; Hindar et al., 1991; Taggart and Ferguson, 1986). The influx of many genotypes can cause genetic 'swamping' of wild populations, causing wild populations to become more and more like the hatchery fish with each passing generation.

The Current Study

The current study utilizes the forensic methodology of STR-based parentage analysis of a cohort of brown trout recruited from adults introduced into Hunt Creek, Michigan, USA. The fish introduction represented a means of evaluating a stocking 'prescription' for brown trout, and ultimately as a response to local demand for brown trout sport fishing. Hunt Creek is the inland fish ecology research area for the Michigan Department of Natural Resources. Long-term research has been conducted on a native salmonid species, the brook trout (*Salvelinus fontinalus*). Introductions of brown trout were performed in order to shift the species composition within the research area.

Proper management practices include not only meeting the demands of the public, but also establishing a self-sustaining, healthy population. In order to develop a

flourishing population, the founding group of fish should exhibit sufficient genetic diversity (Cowx, 1994). Thus, the founding group was previously genotyped to ensure genetic heterogeneity. Determining the most efficient means of achieving the aforementioned goals of public satisfaction and population health and sustainability is also an integral part of proper management practices. Efficiency of stocking may depend on such factors as the source of the stocked individuals and their physical phenotypic characteristics, such as body size (Stanfield and Jones, 2003). Mate selection by body size may further contain a sex-related component, with one sex choosing a mating partner because it is larger than other potential mating partners (Bekkevold et al., 2002; Blanchfield et al., 2003).

In this study, parentage analysis was used to evaluate the outcome of stocking strategies designed to maximize the genetic diversity of progeny produced by stocked adults. Specifically, the objectives of this study were: 1. to apply the methods of forensic parentage analysis to a non-human case study; 2. to maximize the genetic diversity of the parental generation and hence, the offspring produced following stocking; 3. to determine the reproductive success of stocked adults, as well as the overall reproductive success of each source location; 4. to use reproductive success, number of mating partners, and origin of mating partners to characterize the species' mating behavior; 5. to use the information gleaned from measures of reproductive success and mating behavior to offer management suggestions to guide future stocking efforts; and 6. to evaluate whether forensic parentage analysis can serve as a valuable tool in fisheries management. Genetic diversity, reproductive success, and mating behavior are critical informational requirements in designing stocking strategies.

MATERIALS AND METHODS

Study Area

The study area is located within Hunt Creek Research Station, Michigan and consists of a 2.5-mile stretch of Hunt Creek (Figure 1). The research station strives to preserve, maintain, or restore environmental quality and population characteristics, observe changes in habitat or population numbers, and to communicate and establish policy for Michigan fisheries. Located in the Lower Peninsula of Michigan, the station consists of 3,000 acres and contains approximately 5 miles of freshwater streams and lakes. The research section of Hunt Creek is divided into segments, three of which were stocked with brown trout—sections A, B, and Z. These segments of the creek are not physically separated from each other and fish can move freely from one section to another.

Stocking Timeline

Forty-two reproductively mature brown trout were introduced into Hunt Creek in September of 2001. Fish originated from Houghton Creek ('B', N=14), East Branch Au Sable River ('C', N=14), and Gilchrest Creek ('D', N=14), all of which are drainages in the northern lower peninsula of Michigan (Figure 1). Individuals received distinctive location-specific fin clips. Throughout the study a number and letter code was used to identify each fish. The first letter corresponds to the source location and the number identifies each individual within that source group (e.g., B8 is the eighth individual from Houghton Creek).

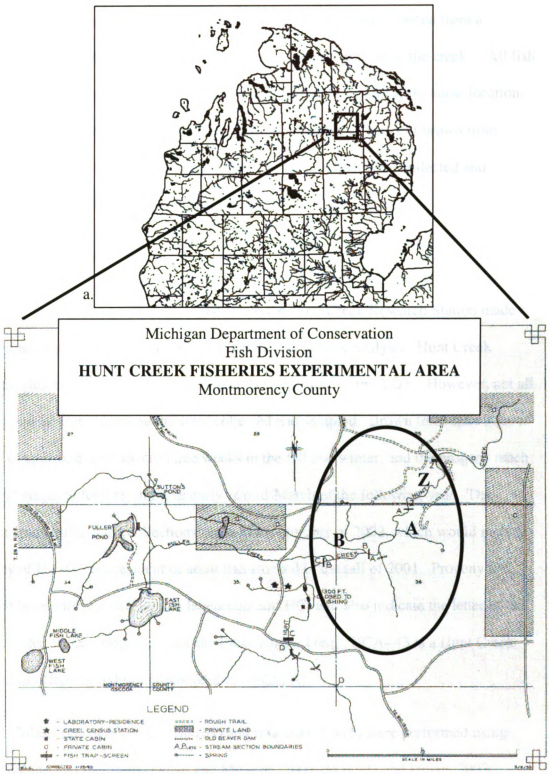


Figure 1. Hunt Creek research area, northern Lower Peninsula of Michigan. a. The Hunt Creek Research Station's location— Montmorency County. b. The research section of station, split into segments A, B, and Z.

In addition, 41 mature adults, identified by 'A' and 'E', were transplanted from a different area of the Hunt Creek drainage into the research portion of the creek. All fish introduced into the research area were stocked simultaneously and at the same location. Hunt Creek already contained a small but unknown number of resident brown trout (identification label 'HC') in the research area, some of which were collected and sampled to be included in the parentage analysis.

Data Collection

In the summer of 2002, biologists from the Hunt Creek Research Station made collections of young of the year (YOY) juveniles for genetic analysis. Hunt Creek resident adult brown trout were collected at the same time as the YOY. However, not all of the resident Hunt Creek adults were collected and sampled. Brown trout spawn for a period of approximately two to three weeks in the fall and winter, and the progeny reach the "fry" stage, or feeding stage, as early as mid-March of the following year. The present study utilized the collections made in the summer of 2002, which would include progeny of Hunt Creek resident or adult fish stocked in the fall of 2001. Progeny and resident brown trout identification labels contain 'HC' and also indicate the letter of the research area section from the individual was sampled (e.g., HCA- 43 is a Hunt Creek resident or offspring individual sampled in section A).

Collections of juveniles (N=109) and residents (N=16) were performed using electrofishing and fish traps (Dolan and Miranda, 2003; Miranda and Dolan, 2003; Miranda and Dolan, 2004; Nielsen et al., 2003; Peterson et al., 2004). A fin clip, taken from each fish collected, served as the sample for genetic analysis. Fin clips were taken

from stocked fish before introduction into Hunt Creek (right pectoral clips for fish from East Branch Au Sable, left pelvic clips from Houghton Creek, and right pelvic clips from Gilchrist Creek). Dried fin clips were stored in separate paper envelopes at room temperature.

DNA was extracted from fin clips using a QIAGEN DNeasy Tissue Extraction Kit®. The extraction protocol consisted of a piece of fin, equivalent to half of the total fin size, placed in a tissue lysis buffer and Proteinase K, followed by an overnight incubation at 55° C. After the incubation, the supernatant was submitted to a series of ethanol and ethanol-buffer washes. Finally, the DNA was eluted in a Tris-buffer (pH 8.5) to a volume of 75µl. Quantification of the DNA required the use of a spectrophotometer. Five microliters of the DNA was diluted in 495 µl of distilled water and the reading taken at 260λ. The DNA was then diluted to 20 ng/µl concentrations in distilled water and stored at -20°C.

A suite of six microsatellite loci was selected for developing genotypes, including Omy301 (Wenburg et al., 1996), Sfo1 (Angers et al., 1995), One9 (Scribner et al., 1996), Ssa85 and Ssa197 (Hansen et al., 2000), and Ogo2 (Olsen et al., 1998) (Table 1). These loci were initially cloned from Rainbow trout (*Oncorhynchus mykiss*), Brook charr (*Salvelinus fontinalis*), Sockeye salmon (*Oncorhynchus nerka*), Atlantic salmon (*Salmo salar*), and Pacific salmon (*Oncorhynchus gorbuscha*). Loci were selected because they were found to be polymorphic in other brown trout from Michigan streams.

Locus	Primer Sequence (5'— 3')	Repeat Motif	Annealing Temperature (°C)	Fragment Size
Ogo2	F: ACATCGCACACCATAAGCAT R: GTTTCTTCGACTGTTTCCTCTGTGTTGAG	GA	56	200 – 220 240 – 270
Omy301	F: ACTTAAGACTGGCAACCTT R: CTACACGGCCTTCGGGTGAGA	GT	56	80 – 110
One9	F: CTCTCTTTGGCTCGGGGAATGTT R: GCATGTTCTGACAGCCTACAGCT	CA	56	195 – 200
Sfo1	F: ACCATAACCCCCCACCAC R: GTCCCTCCGTGGCAGATT	GT	56	115 – 140
Ssa85	F: AGGTGGGTCCTCCAAGCTAC R: ACCCGCTCCTCACTTATTC	GT	50	100 – 120
Ssa197	F: GGGTTGAGTAGGGAGGCTTG R: TGGCAGGGATTTGACATAAC	GTGA	56	120 – 150

Table 1. Primer characteristics and PCR conditions for each microsatellite locus used.

Each 25 µl PCR reaction contained 10 pmol each of forward and reverse primers, 10X LGL buffer (10mM Tris-HCL, pH 8.5, 1.5mM $MgCl_2$, 50mM KCl, 10µg ml^{-1} nuclease-free BSA, and 0.0025% Tween-20), 100-200 µM dNTPs, 0.5 units of AmpliTaq DNA polymerase, and 100 ng of DNA. Four of the six microsatellites required 100 µM dNTPs, while One9 required 150 µM and Ogo2 required 200 µM dNTPs. The amplification program consisted of two minutes of denaturation at 94° C; 30 cycles of one minute at 94° C, one minute at each respective annealing temperature (Table 1), and one minute of extension at 72° C, and one final extension cycle of two minutes at 72° C. Each reaction was amplified using a Stratagene Robocycler® Gradient 96.

Amplification products were separated on 6% acrylamide gels and visualized using the Hitachi FMBio II® genotyping system (Resolution: 150 x 150 dots per inch; Repeat scan: 150 times; Focusing point: -0.4mm). The primers were labeled with either Hex or Flo, dyes that fluoresce at 585 nm and 505 nm, respectively. Genotypes were scored manually in comparison with standards for each locus. Standards included reference individuals of known genotype, selected to demonstrate alleles over a broad range in size for each locus. Each gel consisted of four standards equally spaced throughout 32 samples, as well as a standard size ladder placed in the center of the gel.

Analysis

Parentage analysis using the program Cervus 2.0© consisted of three stages. The 'Allele Frequency' portion of the program calculates the frequencies of each allele for each locus and uses those allele frequencies to calculate expected heterozygosity, conduct a test of genotype frequency deviations from Hardy-Weinberg expectations and estimate

null allele frequencies. This portion of the program required the input of files containing all population genotypes.

Calculations made during the 'Allele Frequency' portion are then input into the 'Simulation' step of the program, which uses the allele frequencies to generate possible genotypes, assuming no deviations from Hardy-Weinberg and no linkage between loci. Simulated multi-locus genotypes are used to conduct 10,000 replicated parentage analyses to generate expected rates of successful parentage assignment at strict (95%) and relaxed (80%) confidence levels.

The final stage of Cervus 2.0© is 'Parentage Analysis'. Input files are separated into three genotype files, consisting of a parental female file, a parental male file, and a progeny file. One sex of putative parental individuals is first used as the 'Known Parent' and the other as the 'Candidate Parent'. Due to the nature of this study, where neither parent is known, the data had to be re-analyzed by switching the sexes used as 'Known Parent' and 'Candidate Parent' and examining whether parental pairs were assigned with equal confidence in both parentage analyses. In addition, sexes were known for only the stocked individuals from Houghton Creek, East Branch Au Sable River, and Gilchrist Creek. So those individuals for whom sexes remained unknown were included in both the parental female and parental male files.

In addition to Cervus 2.0©, chi-square (X^2) tests were performed in order to detect significant deviations of observed values from expected values. The chi-square test was specifically applied in detecting differences between the reproductive success of males and females and differences in reproductive success between population sources.

Cervus 2.0© Output

Parentage analysis produced output listing each progeny individual and all possible parental pairs that could have yielded that individual. The only putative parental pairs of interest, those demonstrating no mis-matches with the progeny genotype, received rank and assignment based on their LOD score, Delta score, and statistical significance.

Cervus 2.0© produces output in the form of a spreadsheet containing the necessary information for making parentage assignments (Appendix Table A.1). The spreadsheet provides the most likely parental pairs with any number of allele mis-matches, from none to all loci mis-matching. For ease in assignment and due to sheer volume of putative parental pairs for each individual, all parental pairs containing mis-matches were eliminated from further consideration as parents. Putative parental pairs with Delta scores of statistical significance were also isolated since they represent the most likely parents.

Guidelines for Parentage Assignment

Guidelines for parentage assignment (Appendix Figure B.1) provided an objective means of determining the most likely parental pair among a number of other parental pairs with no parent-offspring genotype mis-matches. Individuals with the following described characteristics received a specific assignment. 'Direction of Assignment' refers to the fact that parentage assignment was achieved by using one sex as the known parent and the other sex as the candidate parent, then running the analysis again with the

sexes of known and candidate parents switched. Table 1 in Appendix B provides a summary of the guideline under which each offspring individual was categorized.

A small number of juveniles received parental assignments based on guideline 4. The assigned parent is the most commonly found candidate parent amongst all of the putative parental pairs listed for that particular juvenile. If the ratio of the number of putative parental pairs containing a common individual to the number of putative parental pairs not containing that individual was large, the common candidate parent was assigned. Assignments also considered the level of confidence of candidate parental pairs containing the common adult. Table 2 in Appendix B contains a summary of the offspring defined by guideline 4 and the reasons for the assignments made.

RESULTS

Allele Frequency Results

The allele frequency estimation, simulation, and parentage analysis components of Cervus 2.0© yielded the critical values necessary for assigning parentage. Ninety-nine percent of the 208 total individuals were typed. One putative parent fish could not be typed at the Sfo1 locus. The mean number of alleles per locus was 10.33 and the mean expected heterozygosity was 0.746. The total exclusionary power of the first parent was over 94%, while the total exclusionary power of the addition of the second parent, given the first parent was assigned, was over 99%.

For each locus, the total number of alleles, the observed and expected heterozygosity, concordance with Hardy-Weinberg, and presence of null alleles are summarized in Table 2. In comparing observed and expected heterozygosity, an overall excess of heterozygotes was observed. Two loci, One9 and Ogo2, demonstrated a significant deviation from Hardy-Weinberg equilibrium in relation to this excess of heterozygotes. In addition, the estimated frequency of null alleles for each locus was essentially zero (slightly negative), suggesting no evidence of PCR artifacts that could affect parentage assignments.

In order to determine the possible source of excess heterozygosity, allele frequencies were calculated for the all adults, adults by location, and juveniles (Tables 3 a–g). In a majority of comparisons of observed and expected heterozygosity for each

group, observed heterozygosities (H_O) were higher than expected (H_E) and null allele frequencies were essentially zero.

Locus	No. of Alleles	N	H_O	H_E	HW	Null Freq.
Omy301	15	208	0.846	0.822	NS	-0.0140
Ssa85	8	208	0.702	0.694	NS	-0.0124
Ssa197	8	208	0.712	0.651	NS	-0.0474
Sfo1	13	207	0.841	0.843	NS	-0.0017
One9	9	208	0.740	0.667	**	-0.0694
Ogo2	9	208	0.865	0.796	**	-0.0500

Table 2. Estimates of population characteristics for each microsatellite locus (N= sample size; No. of Alleles= the number of observed alleles for each locus; H_O=observed heterozygosity; H_E = expected heterozygosity; HW= tests for deviations of genotype from Hardy-Weinberg equilibrium, where NS= not significant; **= $p<0.05$).

Locus	No. of Alleles	N	H_O	H_E	HW	Null freq.
Omy301	14	99	0.808	0.781	NS	-0.0181
Ssa197	8	99	0.747	0.691	NS	-0.0475
Ssa85	8	99	0.727	0.681	NS	-0.0418
Sfo1	12	98	0.827	0.849	NS	+0.0099
One9	9	99	0.747	0.654	**	-0.0977
Ogo2	9	99	0.848	0.795	NS	-0.0433

a. Summary measures of genetic diversity for all adults

Locus	No. of Alleles	N	H_O	H_E	HW	Null freq.
Omy301	11	109	0.881	0.846	NS	-0.0230
Ssa197	4	109	0.679	0.606	NS	-0.0558
Ssa85	6	109	0.679	0.708	NS	+0.0140
Sfo1	10	109	0.853	0.821	NS	-0.0233
One9	9	109	0.734	0.669	NS	-0.0523
Ogo2	8	109	0.881	0.789	**	-0.0620

b. Summary measures of genetic diversity for all progeny

Locus	No. of Alleles	N	H_O	H_E	HW	Null freq.
Omy301	7	41	0.707	0.676	NS	-0.0207
Ssa197	6	41	0.707	0.666	NS	-0.0429
Ssa85	6	41	0.683	0.628	NS	-0.0587
Sfo1	8	40	0.825	0.789	NA	-0.0298
One9	6	41	0.610	0.497	NA	-0.1553
Ogo2	6	41	0.854	0.731	NA	-0.0946

c. Summary measures of genetic diversity for Hunt Creek transplant adults

Locus	No. of Alleles	N	H_O	H_E	HW	Null freq.
Omy301	6	16	0.813	0.760	NA	-0.0434
Ssa197	4	16	0.563	0.563	NA	-0.0209
Ssa85	4	16	0.750	0.611	NA	-0.1532
Sfo1	6	16	0.875	0.798	NA	-0.0658
One9	5	16	0.688	0.587	NA	-0.1177
Ogo2	4	16	0.875	0.706	NA	-0.1285

d. Summary measures of genetic diversity for Hunt Creek resident adults

Locus	No. of Alleles	N	H_O	H_E	HW	Null freq.
Omy301	8	14	0.929	0.839	NA	-0.0700
Ssa197	5	14	0.643	0.802	NA	+0.0949
Ssa85	6	14	0.786	0.788	NA	-0.0207
Sfo1	8	14	0.857	0.820	NA	-0.0369
One9	5	14	0.929	0.664	NA	-0.2387
Ogo2	7	14	1.000	0.810	NA	-0.1344

e. Summary measures of genetic diversity for East Branch Au Sable River transplant adults

Locus	No. of Alleles	N	H_O	H_E	HW	Null freq.
Omy301	8	14	0.857	0.807	NA	-0.0667
Ssa197	6	14	1.000	0.741	NA	-0.1844
Ssa85	5	14	0.714	0.738	NA	+0.0045
Sfo1	7	14	0.857	0.788	NA	-0.0721
One9	5	14	0.929	0.762	NA	-0.1273
Ogo2	6	14	0.643	0.709	NA	-0.0023

f. Summary measures of genetic diversity for Gilchrist Creek transplant adults

Locus	No. of Alleles	N	H_O	H_E	HW	Null freq.
Omy301	11	14	0.929	0.844	NA	-0.0722
Ssa197	6	14	0.929	0.693	NA	-0.1835
Ssa85	6	14	0.786	0.717	NA	-0.0654
Sfo1	11	14	0.714	0.897	NA	+0.1016
One9	7	14	0.857	0.831	NA	-0.0347
Ogo2	7	14	0.857	0.804	NA	-0.0471

g. Summary measures of genetic diversity for Houghton Creek transplant adults

Tables 3 a–g. Summary measures of genetic diversity separated by adults and progeny, as well as by donor source group (N= sample size; No. of Alleles= the number of observed alleles for each locus; H_O=observed heterozygosity; H_E = expected heterozygosity; HW= tests for deviations of genotype from Hardy-Weinberg equilibrium, where NS= not significant; **= $p<0.05$).

Simulation Results

The simulation estimated expected success rates for assigning parentage when analyses were conducted with one parent known, as well as neither parent known. Each level of confidence, the strictest confidence level at 95%, relaxed at 80%, and an unresolved level at <80% were presented for the parent-offspring scenarios when either one or neither parent is known (Table 4). For cases in which one parent is known, and under the strictest level of confidence (95%) the program estimated a success of assignment rate of 68%. At the relaxed level of confidence (80%) the program estimated a success of assignment rate of 100%. For cases in which neither parent is known, expectations were dramatically lower, with an estimate of only 9% success of assignment at the strictest level of confidence and a success rate of 34% at the relaxed level of confidence, leaving an estimated 66% of progeny with no parents assigned.

Simulation Summary Statistics—Assignment Success Estimates			
Critical values and expected success rates (one parent known)			
Confidence Level	Confidence (%)	Delta Criterion	Expected Success Rate
Strict	95.00	0.86	68%
Relaxed	80.00	0.00	100%
Unresolved			0%
Critical values and expected success rates (neither parent known)			
Confidence Level	Confidence (%)	Delta Criterion	Expected Success Rate
Strict	95.00	1.86	9%
Relaxed	80.00	0.90	34%
Unresolved			66%

Table 4. Simulation summary measures of parentage assignment accuracy. Levels of confidence were based on the distribution of simulated Delta scores (based on known population allele frequencies). The strictest level was set at 95%, and the relaxed level was set at 80%. Any parentage assignment made below the 80% confidence level was considered unresolved. The Delta Criterion is the minimum Delta value defining the strict and relaxed confidence levels. The Expected Success Rate refers to the expected rate of successful parentage assignment at each confidence level.

Parentage Analysis Results

Over 52% of the progeny could not be assigned to even one parent (Table 5). These individuals could not be assigned because either no sampled parental pairs were compatible (21.10%), many parental pairs were compatible with offspring genotypes but none were of statistical significance (9.17%), or too many statistically significant pairs were given and a most likely pair could not be distinguished (22.02%). Nearly 25% of the progeny could be assigned to one parent because one putative parent individual was common to all or the vast majority of the potential pairs listed (see 'Guidelines for Parentage Assignment', Appendix Figure B.1). Finally, almost 23% of progeny

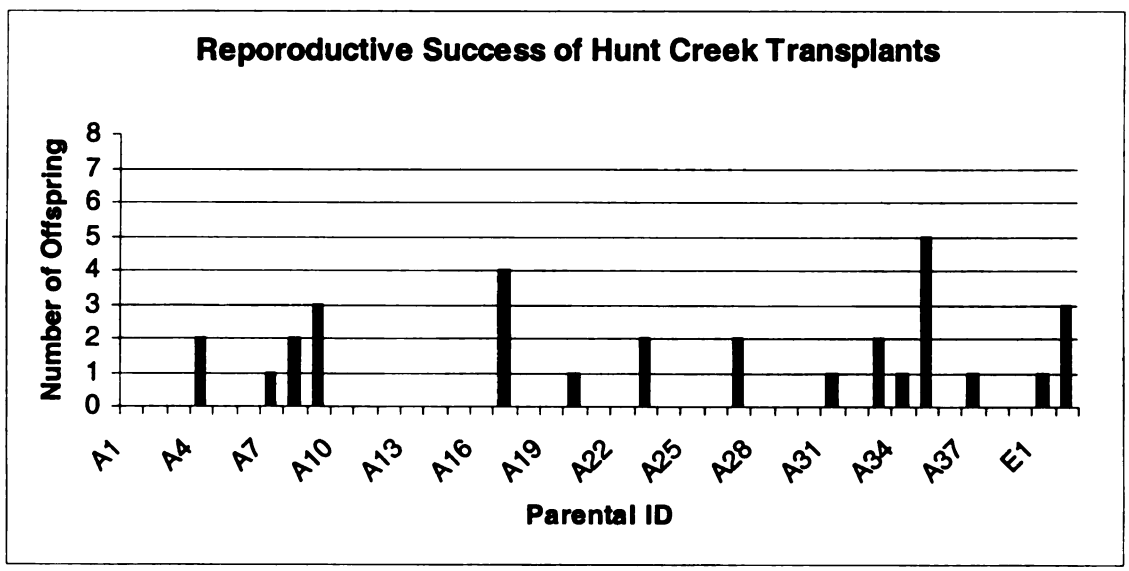
individuals could be assigned to a single parental pair at either the strict or relaxed confidence levels.

Number of Unassigned Juveniles			**Number of Juveniles with One Parent Assigned**	**Number of Juveniles with Both Parents Assigned**		
No possible parental pairs	No statistically significant pairs	Too many statistically significant pairs		One pair, but not statistically significant	80%	95%
23	10	24	27	8	4	13
21.10%	9.17%	22.02%	24.77%	7.34%	3.67%	11.93%

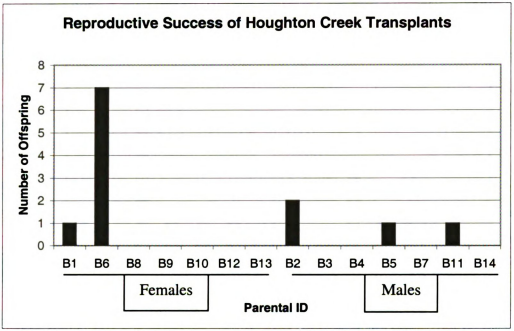
Table 5. Assignment summary from parentage analysis, including the number and percentage of offspring that were unassigned or assigned to one or both parents at 80% and 95% confidence levels.

Offspring assignments were utilized to estimate reproductive success of each putative parent, as well as the overall reproductive success of all adults from each source location. Considerable variation in reproductive success was observed on a individual level. The parent with the highest reproductive success was a female, B6, originally transplanted from Houghton Creek (Figure 2 a–e). Individual B6 could be assigned to seven of the progeny (Figure 2b). Many adults, 60 in total, contributed no offspring to the YOY. The mean number of offspring per female was 1.0 (σ = 1.80) and the mean number of offspring per male was 0.81 (σ = 1.07).

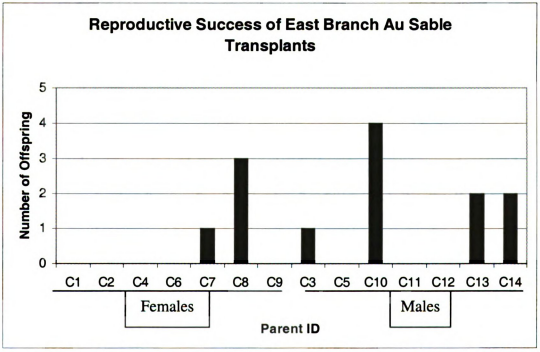
Adults from each donor source contributed proportionally to the offspring generation relative to the size of each donor source population (X^2=2.902, p>0.05, d.f.= 4) (Table 6 and Figure 3a). However, 50% of the juveniles could not be assigned to any of the sampled parents. For donor source populations with known genders, reproductive success was compared between males and females. Across all donor source groups with known parental sexes, no significant difference existed between the reproductive success of males and females (X^2= 3.677, p>0.05, d.f.= 2) (Figure 3b).



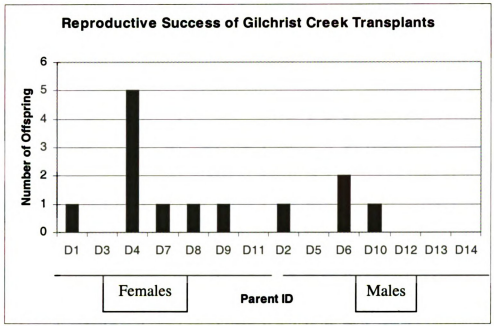
a. Estimated reproductive success of parental brown trout from Hunt Creek transplants, N=41.



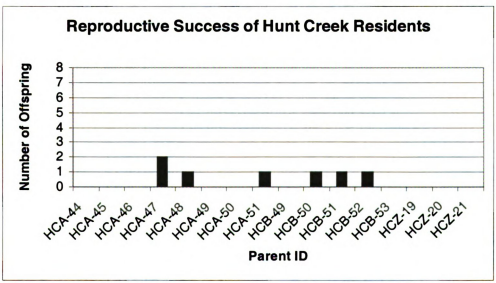
b. Estimated reproductive success of parental brown trout from Houghton Creek transplants, N=14.



c. Estimated reproductive success of parental brown trout from East Branch Au Sable River transplants, N=14.



d. Estimated reproductive success of parental brown trout from Gilchrist Creek transplants, N=14.

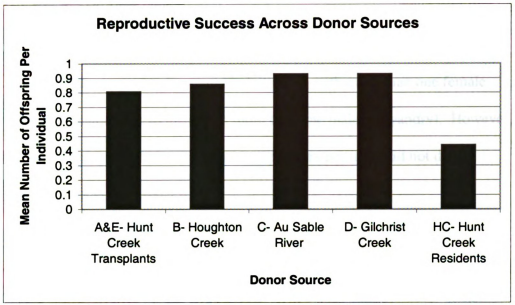


e. Estimated reproductive success of parental brown trout resident to the research area of Hunt Creek, N=16.

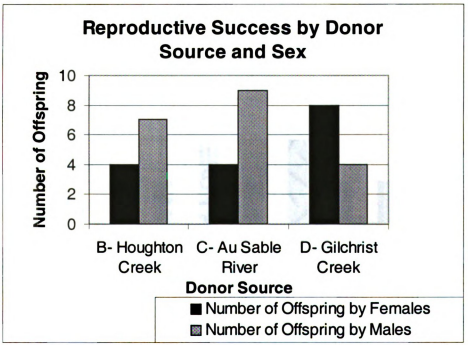
Figure 2 a–e. Estimated reproductive success of each stocked adult. Bars represent the number of progeny to which each adult contributed. a. Reproductive success of transplanted adults from Hunt Creek, all of unknown gender. b-d. Reproductive success of transplanted adults from Houghton Creek (b), East Branch Au Sable (c), and Gilchrist Creek (d). The first seven individuals for each source location are females and the second seven are males. e. Reproductive success of Hunt Creek residents, all of unknown sex.

Source Location	Total Number of Adults	Number of Contributions to Offspring	Mean Number of Offspring/Individual	Percent of Individuals with Offspring	Percent of Total Contributions to Offspring
A and E- Hunt Creek Transplants	41	32	0.805	39.02%	41.56%
B- Houghton Creek	14	12	0.857	35.71%	15.58%
C- East Branch Au Sable	14	13	0.929	42.86%	16.88%
D- Gilchrist Creek	14	13	0.929	42.86%	16.88%
HC- Hunt Creek Residents	16	7	0.438	37.50%	9.09%

Table 6. Estimates of parental reproductive success from each source location. The number of offspring assigned refers to the number of offspring that could be assigned to one parent within the respective donor group. The percent of individuals with offspring represents the number of individuals within each donor group that produced offspring. The percent of total assigned offspring reflects contributions of the donor group to all of the 53 assigned offspring (both one and two parents assigned).



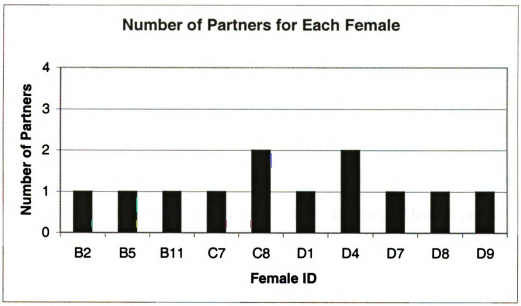
a. Reproductive success for each donor source group.



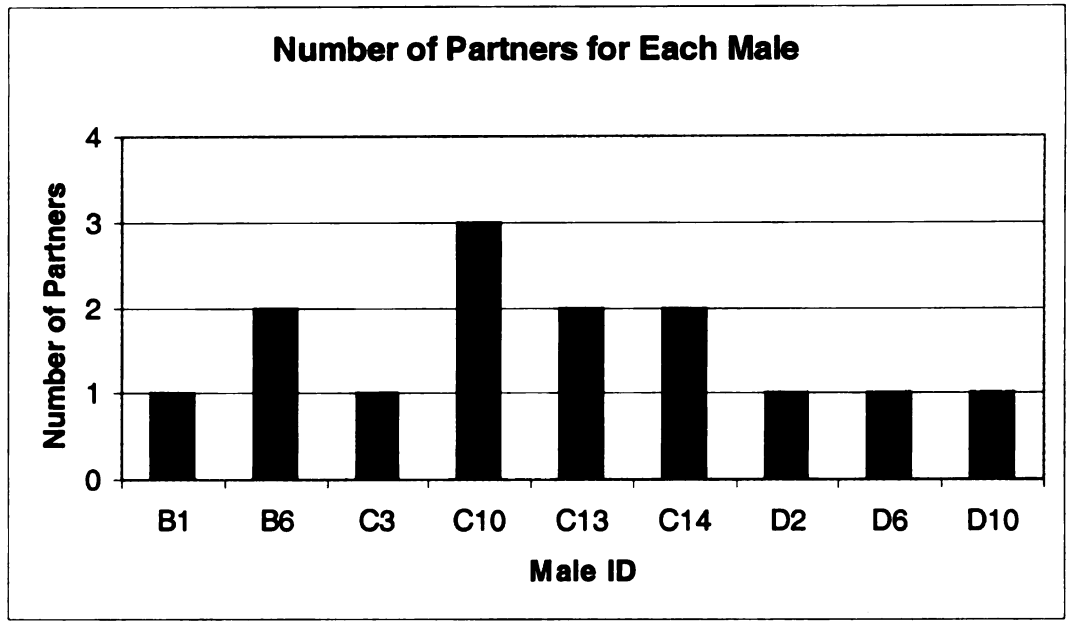
b. Reproductive success for each donor source for which sexes are known.

Figure 3 a–b. Estimates of reproductive success. a. Reproductive success for each donor population as represented by the mean number of offspring per individual. b. Reproductive success for donor populations of known sexes is represented by the number of offspring to which females and males contributed.

Based on parentage assignments, the number of mates per male and female were determined. Both males and females exhibited mating with more than one partner (Figures 4 a–b). Males appear to be more likely to mate with more than one female (polygamy) than females were to mate with more than one male (polyandry). However, the number of partners per female and number of partners per male did not differ significantly (X^2= 1.845, p>0.05, 2 d.f.).



a. Number of mating partners for each known female.



b. Number of partners for each known male.

Figure 4 a–b. Number of mating partners. Only individuals of known sex, those from Houghton Creek, East Branch Au Sable River, and Gilchrist Creek donor populations, were considered. a. Minimum number of partners per known female. b. Minimum number of partners per known male.

All but four individuals of unknown gender mated with only one partner, and genders were inferred when a mating occurred with an individual of known gender (Figure 5 and Table 7). Sex of individuals who mated with another individual of unknown gender could not be inferred.

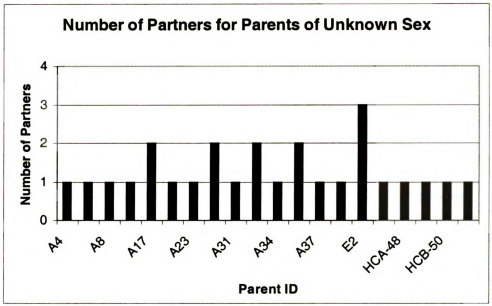


Figure 5. Minimum number of partners per parent for adults of unknown sex.

Unknown Gender Parent ID	Inferred Sex
A4	F
A7	Unkown
A8	F
A9	M
A17	F
A20	Unknown
A23	M
A27	Unknown
A31	M
A33	F
A34	Unknown
A35	M
A37	Unknown
E1	Unknown
E2	F
HCA-47	Unknown
HCA-48	Unknown
HCA-51	F
HCB-50	Unknown
HCB-52	Unknown

Table 7. Inferred sexes of parents based on pairing with individuals of known sex.

Due to an interest in determining whether adults would mate randomly or assortively with respect to source location, the number of mating events between individuals from the same donor source was compared to the number of mating events by individuals of different donor sources. For all donor sources, with the exception of Hunt Creek transplants, matings among individuals of differing donor populations appear far more common than matings among individuals from the same donor population (X^2=4.006, $p<0.05$, d.f.=4) (Figure 6). Results were consistent with random mating given the predominance of potential inter-location possibilities for mating.

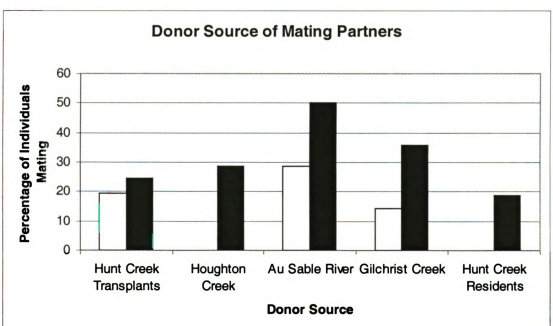
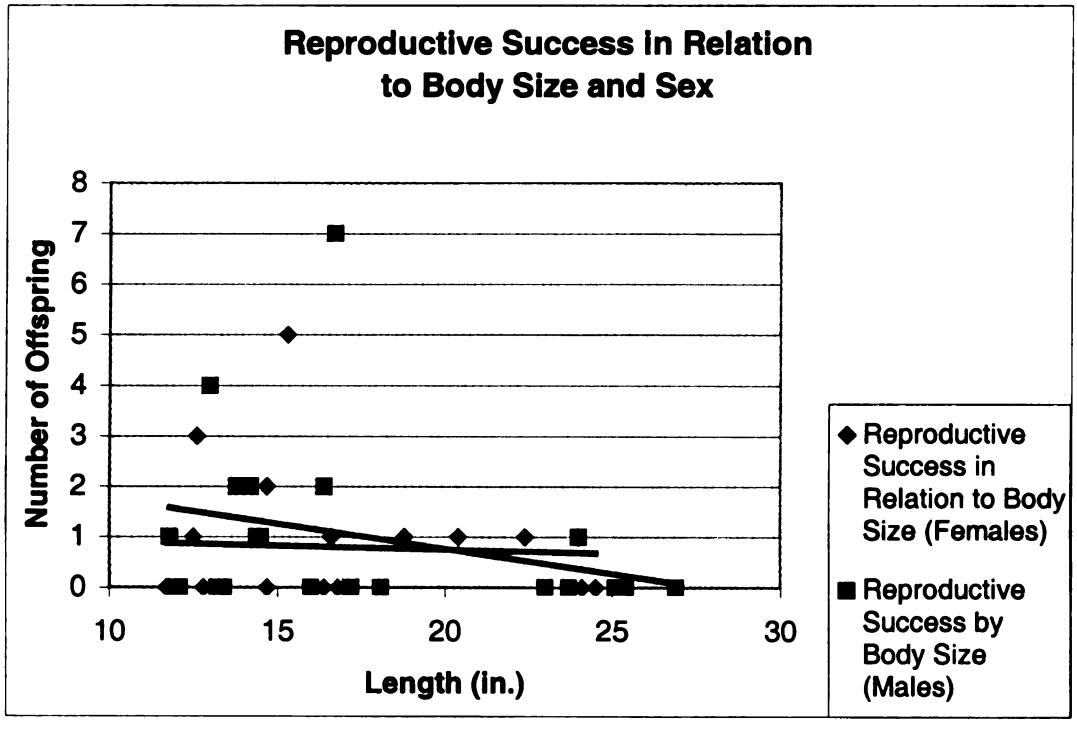


Figure 6. Source origins of mating partners. The percentage of individuals mating with another individual of the same source (white column) is compared to the percentage of individuals mating with an individual from a different donor source (black column).

Correlation analysis was conducted between adult body size (length in inches) and reproductive success. No significant correlation was found for either females or

males [R^2= 0.003 (y = -0.0157x + 1.0661) for females and R^2= 0.078 (y = -0.0985x + 2.7363) for males (Figure 7a)]. When adults with no assigned parentage were removed, no significant correlation was observed [R^2= 0.0007 (y = -0.0145x + 2.5569) for females and R^2= 0.1536 (y = -0.1295x + 3.924) for males (Figure 7b)].

a.



b.

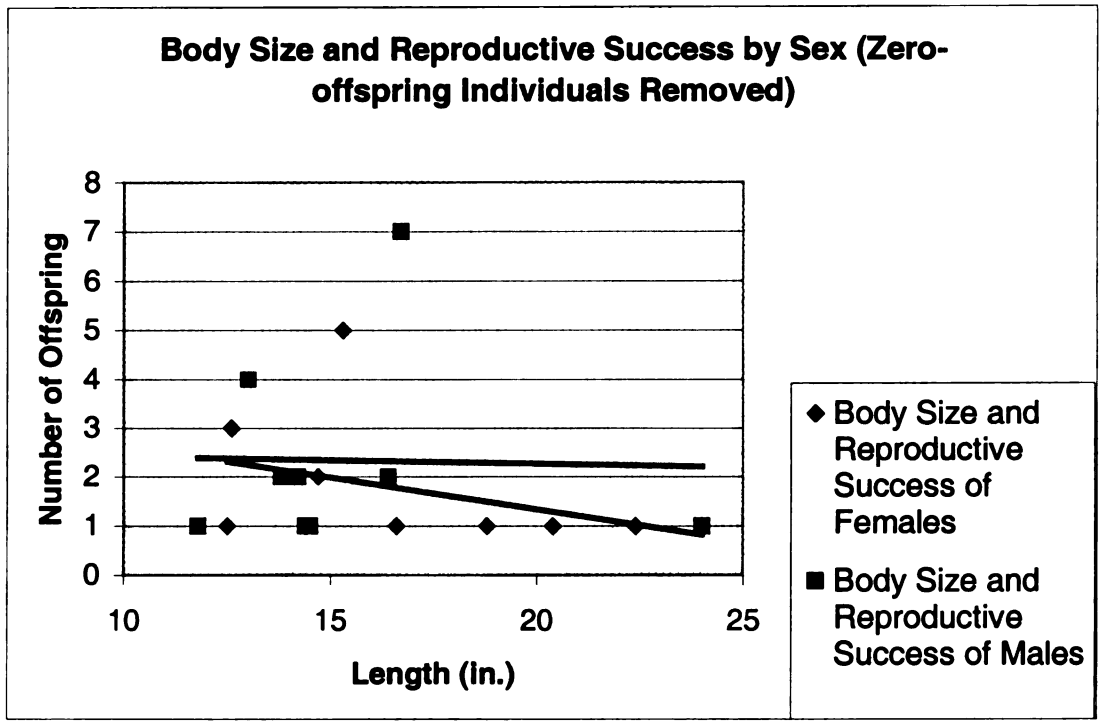


Figure 7 a–b. Body size (length in inches) and reproductive success. a. Reproductive success in relation to body size for all stocked females (R^2= 0.003, p>0.05) and males (R^2= 0.078, p>0.05). b. Reproductive success in relation to body size for both females (R^2= 0.0007, p>0.05) and males (R^2= 0.1536, p>0.05), when individuals who produced no offspring were removed.

DISCUSSION

The present study used parentage analysis to detect factors influencing reproductive success and to characterize the mating system of brown trout introduced into Hunt Creek, Michigan. Information regarding the reproductive success and mating system of these brown trout can be used to adjust future stocking strategies. Molecular data were collected for a total of 208 total individuals, 99 putative parents and 109 juveniles (YOY). The YOY represented a large proportion of the first juvenile cohort produced in Hunt Creek following stocking (Personal communication, A. Nuhfer). Information regarding the number of adults contributing to the offspring recruited provides an estimation of levels of relatedness among offspring and probabilities of inbreeding in future generations.

Allele Frequency Results

Evaluation of the molecular data revealed negative null allele frequencies for the vast majority of loci across all adults and progeny. These negative null allele frequencies resulted from excess heterozygosity in both the parental and offspring generations. Since null alleles would result in an excess of homozygosity, the data provide little or no evidence for the presence of null alleles.

Genotype frequencies at two loci, One9 and Ogo2, demonstrated a deviation from Hardy-Weinberg expectations due to an excess of heterozygotes. This deviation is presumably an artifact of stocking genetically differentiated adult populations. Geographically separated populations may be expected to exhibit high frequencies of

fixed alleles, with the fixed alleles of interest differing between populations. Since brown trout have been introduced to North America via a series of founding events, excess homozygosity is particularly expected. In this case, however, all donor populations displayed extremely high amounts of genetic heterzygosity. Brown trout, as a species, exhibit high levels of genetic differentiation among geographically isolated populations (e.g. Allendorf et al., 1976; Bouza et al., 1999; Crozier and Ferguson, 1986; Fahy, 1989; Ferguson, 1989; Ferguson and Taggart, 1991; Hindar et al., 1991; Karakousis and Triantaphyllis, 1990; Osinov and Bernatchez, 1976; Prodöhl et al., 1992). Given that current North American brown trout populations were generated from multiple European stocks, the interbreeding between differentiated source stocks may have resulted in high heterozygosity in subsequent generations.

Simulation Results

Though body size was recorded for all adults in the parental generation, incomplete records were available on the sex of some stocked adults and all resident adults. Lack of information decreased the power of the assignment tests because adults of unknown sex were treated both as males and females. Furthermore, individuals of unknown sex could not be included in any data summarization requiring separation by sex, leaving small sample sizes.

Cervus 2.0© requires sexes of potential parents be known and is better able to assign parentage with high probability when the identity of one parent is known (simulation results, Table 4). As a program designed to perform parentage analysis, Cervus 2.0© exhibits some limitations based on the availability of information. In this

case, with the identity of neither parent known, the simulations conducted in Cervus 2.0© estimated success rates for parental assignment at the strictest level of confidence to be only 9% and at the relaxed level, 34%. Empirically, the actual success rate at each of these levels was 11.93% and 15.60%, respectively. Empirical success rates differed from those expected and represent a considerably lower rate of success than if all sexes of the parental generation were known with certainty, and if one parent had been known for each juvenile. Only slightly more than half (55.77%) of the assigned offspring had one or both parents of known sex. Again, this difference between the expected and observed success rates may have been caused by limitations in available information.

Ecological Applications of Parentage Analysis

Results from this study demonstrate trends in male and female reproductive success and mating strategies. The most successful individual was B6, a female originating from Houghton Creek, who contributed to at least seven offspring. However, Hunt Creek residents appeared to be the most reproductively successful group when source populations were considered collectively. More than 50% of juveniles could not be assigned parents and were thus most likely produced by unsampled resident adults. A portion of unassigned juveniles would likely be assigned to genotyped adults given greater statistical power, for example, if additional loci were scored. However, over 21% of the unassigned juveniles could not be associated with any genotyped adults, suggesting that Hunt Creek residents exhibited a relatively higher reproductive success when compared to each of the other source groups. This success is potentially a consequence of familiarity with the Hunt Creek environment (Cowx, 1994; Hansen et al., 2000). This expectation particularly applies to male residents, who should have already built nests

and established territories prior to the introduction of stocked adults. However, an important finding was that all donor source stocks still contributed toYOY recruitment. Further, all donor source stocks contributed approximately equally relative to the size of the introduced adult population.

Across all source locations, reproductive success was not correlated with body size for either males or females (Figures 7a and 7b). A number of studies have found, contrary to previous ideas based on behavioral observations, that reproductive success does not correlate to body size in brown trout and other related species (Blanchfield et al., 2003; Bekkevold et al., 2002; Garcia-Vasquez et al., 2001; Garant et al., 2001).

Mating strategies in fish often include assortative or preferential mating based on size, and also commonly includes variation in number of partners. In the present study, males and females routinely mated with more than one partner. The difference in the number of males that mated with multiple partners and the number of females that mated with multiple partners was not statistically significant. Since male brown trout build the nest, the data revealed that more than one female may lay her eggs in the same male's nest, and that a single female frequently allocates her eggs to more than one male.

Stocking Strategy

The parentage assignments and subsequent measures of reproductive success have direct relevance to stocking strategy for systems similar to that of Hunt Creek. Information pertaining to probabilities of reproduction given the number of source populations and individuals stocked is extremely important. Adults from all source populations contributed proportionally to the juveniles recruited during 2002. Therefore,

if maximizing genetic diversity is a desired stocking strategy, the use of multiple donor source stocks is recommended. However, as Hunt Creek residents represented the likely group of adults for demonstrating highest reproductive success, supplementation by stocking may provide little contribution to future generations unless stocking is comprised of large numbers of fish relative to the size of the wild population. Body size in brown trout was not significantly correlated to male or female reproductive success, and may potentially be disregarded in future stocking events. In further recommendations on stocking strategy, a 1:1 sex ratio may prove ideal, as males and females exhibited equal reproductive success. Campbell (1977) demonstrated that a 1:1 sex ratio of brown trout yielded the highest reproductive success in a series of stocking events which varied the ratio of stocked females to males.

Expansion of the Study

Information obtained in the study could have been improved by obtaining sexes for all adults and increasing the number of loci used, which would increase the statistical power to exclude incorrect putative parental pairs and increase the success rate of offspring parental assignments. Use of additional loci for both parents and progeny would increase exclusionary power. Ideally, maternal and paternal assignments to all offspring would be based either on total exclusions of all parental pairs or on likelihood at the highest level of confidence. A large number of potential loci from related species are available (e.g. Angers et al., 1995; Hansen et al. 2000; Olsen et al., 1998; Scribner et al., 1996; Smith et al., 1998; Wenburg et al., 1996).

Power of exclusion could also be bolstered if the sex of all adults in the parental generation were known. Since sexes of Hunt Creek transplants and residents were not recorded and the samples received were only fin clips, the only means of sexing already-sampled individuals would be by molecular means. Sex-linked loci have revealed sexes in a number of different species of fish, some of which are closely related to the brown trout, such as chinook salmon (*Oncorhynchus tshawytscha*, Devlin et al., 1989), coho salmon (*Oncorhynchus kisutch*, Forbes et al., 1994), and rainbow trout (*Oncorhynchus mykiss*, Iturra et al., 1998). This could provide a means of determining adult sex of Hunt Creek transplant and resident samples used here.

Human Versus Non-human Parentage Analysis

Information limitations encountered in this study are not common to human forensic parentage analysis. However, human and non-human parentage analysis share the same conceptual foundation. Assignment of parentage to wildlife species operates under the same population genetic principles as those implemented in human parentage analysis. Analysis of human and non-human parentage assumes Mendelian inheritance, namely segregation and independent assortment, meaning that the product rule can be applied in generating probabilities. Wildlife and human parentage analysis must account for the possibility of mutation, relatedness of putative parents, and human error in genotyping and data entry (Marshall et al., 1998; Schanfield, 2000). Furthermore, parentage analysis is most profitably based on the use of molecular genetic technology, usually in the form of STRs (Schanfield, 2000). Statistical analysis requires a reference population, considering the fit to Hardy-Weinberg expectations, estimations of heterozygosity, and the power of false parental exclusion.

The goals of human and non-human parentage analyses differ greatly. Human parentage analysis seeks only to identify rightful parents. In contrast, non-human parentage analysis often seeks to determine parents as a means of exploring additional ecological and evolutionary questions. In other words, parentage assignment is only the beginning of the hypothesis testing process.

Further differences between human and non-human parentage analyses lie in the availability of information. Microsatellites used in human parentage analysis are standardized and have been isolated directly from human DNA. In contrast, many microsatellite loci used in non-human parentage studies are not standardized. Also in non-human studies, microsatellites that have been cloned from one species may be used in analyses regarding a different, related species. Additional differences appear in relation to reference populations. Much is known of human populations regarding geographic locations and racial distinctions, and information is easy to obtain by simple questioning. On the contrary, little is typically known about non-human populations unless the species has been studied extensively.

Another major difference lies in the statistical threshold mandated for assignment of parentage. The American Association of Blood Banks (AABB) requires that human parentage analysis must have an exclusionary power of 99%, and in some countries that threshold is even higher (Schanfield, 2000). In the present study, parentage was assigned in cases that fell within an 80% or 95% confidence level. The power of exclusion in this study was estimated at only 9% for the strictest confidence level (95%), in the event that neither parent is known. The assignments made here, while acceptable in molecular ecology, would not serve as sufficient evidence of parentage in a court of law.

Most importantly, the present study differs from most cases of human parentage analysis due to the unique circumstances under which individuals were obtained. A typical human parentage dispute involves one child whose mother is known and father who is uncertain (Schanfield, 2000). In this scenario, the only variable is the genotype of potential fathers. Assignment of Hunt Creek brown trout parentage was seriously complicated by not knowing either parent, by the large number of potential parents and offspring (99 putative parents, 109 progeny), and by not knowing the sex of all putative parents. Despite these complications, almost 50% of the sampled juveniles could still be assigned one or both parents, testifying to the power of genetic data to resolve parentage, even in the absence of critical information.

APPENDIX A

CERVUS 2.0© OUTPUT

O ID	KP ID	O-KP loci mismatching	Prob. non-exclusion	CP ID	O-CP loci mismatching	LOD	Delta	Confidence
HCA-7	B2	0	2.02E-02	A31	0	8.51E-01	0.00E+00	
HCA-8								
HCA-9	A39	0	3.78E-04	A17	0	5.35E-01	0.00E+00	
HCA-9	A38	0	7.67E-04	A25	0	7.83E-01	0.00E+00	
HCA-9	A38	0	7.67E-04	HCA-44	0	7.83E-01	0.00E+00	
HCA-9	A38	0	7.67E-04	A39	0	3.05E-01	0.00E+00	
HCA-9	A25	0	2.86E-04	A17	0	1.86E+00	0.00E+00	
HCA-9	A25	0	2.86E-04	A37	0	1.31E+00	0.00E+00	
HCA-9	A25	0	2.86E-04	A38	0	1.12E+00	0.00E+00	
HCA-9	HCA-44	0	2.86E-04	A17	0	1.86E+00	0.00E+00	
HCA-9	HCA-44	0	2.86E-04	A37	0	1.31E+00	0.00E+00	
HCA-9	HCA-44	0	2.86E-04	A38	0	1.12E+00	0.00E+00	
HCA-11	D4	0	2.75E-03	A5	0	4.63E+00	1.08E+00	*
HCA-11	D4	0	2.75E-03	B6	0	2.86E+00	0.00E+00	
HCA-11	D4	0	2.75E-03	A38	0	2.48E+00	0.00E+00	
HCA-11	D4	0	2.75E-03	A17	0	2.18E+00	0.00E+00	
HCA-11	D4	0	2.75E-03	A37	0	1.63E+00	0.00E+00	
HCA-11	A5	0	2.03E-04	D6	0	2.65E+00	8.44E-01	+

Appendix Table A.1. Example of output from Cervus 2.0©. The table provides the candidate parental pairs for each offspring, their LOD and Delta scores, as well as the confidence level into which the respective Delta score falls. O ID: offspring ID; KP ID: known parent ID; O-KP mismatching: number of mismatched loci between offspring and known parent; Prob. of exclusion: probability of exclusion; CP ID: candidate parent ID; O-CP mismatching: number of mismatched loci between offspring and candidate parent; LOD: logarithm of odds score; Delta: Delta score, difference between LOD scores; Confidence: level of confidence into which the Delta score falls, 80% (+) relaxed level, 95% (*) strict level.

APPENDIX B

ASSIGNMENT GUIDELINES

ASSIGNMENT GUIDELINES

1. No candidate parental pairs: 'Unassignable'.
2. Numerous candidate parental pairs, none of statistical significance: 'Unassignable'.
3. Numerous candidate parental pairs of statistical significance: 'Unassignable'.
4. Numerous candidate parental pairs of statistical significance, MOST of which contain one common individual: 'One parent assigned'.
5. Numerous candidate parental pairs of statistical significance, ALL of which contain one common individual: 'One parent assigned'.
6. One candidate parental pair, but not of statistical significance: 'Both parents assigned by exclusion'.
    a. Pair provided by one-directional assignment.
    b. Pair provided by both directions of assignment.
7. One candidate parental pair, statistically significant with 80% confidence: 'Both parents assigned at 80%'.
    a. Pair provided by one-directional assignment.
    b. Pair provided by both directions of assignment.
8. One candidate parental pair, statistically significant with 95% confidence: 'Both parents assigned at 95%'.
    a. Pair provided by one-directional assignment.
    b. Pair provided by both directions of assignment.

Appendix Figure B.1. Parentage assignment guidelines. Direction of Assignment refers to the fact that parentage analysis is achieved by using one sex as the known parent and the other sex as the candidate, then running the analysis again with the sexes switched.

Assignment Guidelines and the Juveniles Defined by Them			
Guideline	**Number of Individuals Defined by Guideline**	**Juvenile ID Group (Stream Section)**	**Juvenile ID**
1	23	HCA	2,8,14,16,21,26,29,30,35,38,39
		HCB	10,19,24,26,27,33,37,39,41
		HCZ	2,6,13
2	10	HCA	5,10,19,27
		HCB	29,30
		HCZ	3,4,14,17
3	24	HCA	4,6,11,12,15,28,31,36,37
		HCB	3,5,6,9,18,22,31,35,38,43
		HCZ	8,9,10,15,16
4	7	HCA	
		HCB	4,11,12,21,36,46
		HCZ	11
5	20	HCA	1,9,13,18,42
		HCB	1,7,8,14,15,16,23,28,32,34,44,47
		HCZ	1,5,18
6	8	HCA	3,7,33
		HCB	2,40,45,48
		HCZ	12
7	4	HCA	43
		HCB	17,20
		HCZ	7
8	13	HCA	17,20,22,23,24,25,32,34,40,41
		HCB	13,25,42
		HCZ	

Appendix Table B.1. Assignment guidelines and the juveniles defined by them. For each guideline the identification label of each juvenile individual assigned under that guideline is listed. Identification labels are split into the sections of the research area from which the juveniles were sampled.

Juveniles Defined by Guideline 4			
Juvenile ID	**Candidate Parental Pair Ratio**	**Assignment Decision**	**Reasoning**
HCB-4	16:2	One parent, A8	All at 80% confidence, pairs not containing A8 did not have a common individual
HCB-11	7:2	One parent, E1	Mixed 80% and 95% confidence levels, pairs not containing E1 did not have a common individual
HCB-12	11:2	One parent, HCB-51	All at 80% confidence, pairs not containing HCB-51 contain HCB-53, ratio was deciding factor
HCB-21	17:4	One parent, A7	All pairs containing A7 were at 95% confidence, while all pairs with A23 were at 80%
HCB-36	5:2	One parent, A9	All pairs containing A9 were at 95% confidence, while all pairs with HCB-53 were at 80%
HCB-46	9:3	One parent, HCA-47	Mixed 80% and 95% confidence levels, deciding factor was ratio
HCZ-11	11:4:1	One parent, B6	All containing B6 were at 95% confidence, all containing A5 are at 80% confidence, one pair containing neither B6, nor A5

Appendix Table B.2. Juveniles defined by guideline 4. The candidate parental pair ratio is the ratio of candidate parental pairs containing the assigned parent to candidate parental pairs which do not contain the assigned parent. The assigned parent is the most commonly found candidate parent amongst all of the putative parental pairs listed for that particular juvenile.

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