

BRIEF REPORT

Genetic Analysis Reveals Multiple Parentage in Captive Reared Eastern Hellbender Salamanders (*Cryptobranchus alleganiensis*)

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Information on the parentage of captive reared clutches is vital for conservation head-starting programs. Molecular methods, such as genotyping individuals with hyper-variable markers, can elucidate the genealogical contribution of captive-reared, reintroduced individuals to native populations. In this study, we used 12 polymorphic microsatellite loci to infer parentage of a clutch of 18 eastern hellbenders collected from a single nest from Buffalo Creek, West Virginia, subsequently reared in captivity, and used for translocations in Indiana. Collectively, these markers successfully detected the presence of multiple parentage for this species of conservation concern presently used in captive management programs in zoos across many states. This study highlights the need for genetic analysis of captive reared clutches used in translocations to minimize the loss of genetic diversity and potential for genetic swamping at release sites. *Zoo Biol.* 34:535–537, 2015. © 2015 Wiley Periodicals, Inc.

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INTRODUCTION

Amphibians have received increased attention in many captive rearing programs in zoos, universities, and state agencies due to dramatic declines in many species [Griffiths and Pavajeau, 2008]. For many of these amphibian species, continuous management (i.e., captive rearing and/or head-starting) remains the only viable option for conservation [Scott et al., 2005]. Characterizing parentage and thus, maintaining genetic diversity, is an important management consideration for reintroduction programs that rely on captive reared individuals. DNA markers are ideal tools to elucidate within clutch relatedness, describe parentage patterns, and improve genetic management of captive populations [Simonov and Wink, 2011].

Eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) are large, fully aquatic salamanders characterized by external fertilization. In many areas throughout their geographic range, wild populations are experiencing precipitous declines characterized by lack of recruitment [Wheeler et al., 2003, Burgmeier et al., 2011], highlighting the need for many states to develop captive rearing programs. However, due to their cryptic nature, little is known regarding

reproductive behaviors, or whether multiple or single parentage occurs in nature. Therefore, assessing parentage within captive reared clutches collected from the wild is important to inform conservation and ultimately reintroduction efforts. Captive rearing in zoos and subsequent releases into wild populations increases survivorship of eastern hellbenders by as much as 60% [Bodinof et al., 2012]. Several thousand hellbenders are being reared in zoos nationwide and 20% of these individuals are being released each year across the species' range. If genetic parentage is not considered, releasing hundreds of individuals into an existing population, or location could create a genetic swamping effect, thereby reducing long term genetic diversity

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[Alleaume-Benharira et al., 2006]. Assessing parentage of zoo reared clutches allows managers to intentionally partition large clutches upon release into the wild to maximize genetic diversity. In this study, we assess parentage by genotyping at 12 microsatellite loci among 18 captive reared hellbenders collected from a single nest rock, and subsequently used for a reintroduction program in Indiana.

METHODS

Individuals were collected from a single guarded nest (presumably a single clutch of eggs) collected by Joe Greathouse in 2007 from Buffalo Creek, West Virginia. Eggs were subsequently transported to the Fort Worth Zoo, and then maintained for captive rearing at Purdue's Aquatic Research Laboratory in West Lafayette, Indiana until released. DNA was extracted from small tail clips from three year-old larvae using a standard phenol-chloroform extraction [Sambrook and Russell, 2001].

We genotyped 18 captive reared individuals across 12 microsatellites [Unger et al., 2010] using GENEMAPPER (Applied Biosystems, California). PCR amplification conditions, and genotyping followed [Unger et al., 2012, 2013], with the exception that individuals were run on single well reactions to increase ease of scoring and genotyping. Genotypes were 100% complete across all loci for all individuals, with quality-control measures including re-amplification and rescoring of all individuals across all loci in duplicate. Previous studies report high polymorphic content, a low probability of identity (2.9×10^{-15} ; Unger et al., 2012) and low occurrence of null alleles [Unger et al., 2013], making these ideal markers for characterizing parentage.

Parentage (presence of single or multiple paternity/maternity) was determined by the Minimal Method, or manual examination of all individual genotypes within loci in diploid species [Kellogg et al., 1998; Tennesen and Zamudio, 2003] since DNA from the guarding male was not collected. The Minimal Method assigns single or multiple parentage based on offspring allelic distribution, whereby single paternity is present if offspring contain no more than four alleles, while the presence of more than four alleles at a single locus within a clutch suggests multiple parentage for that clutch [Myers and Zamudio, 2004; Goputenko et al., 2007].

RESULTS

Genetic parentage for this wild collected clutch was consistent with multiple parentage. We observed 6–8 alleles per locus (10/12 loci had six alleles, one locus had seven alleles and the remaining locus had eight alleles; Fig.1). Overall this data suggests potentially two females (or two males) possibly more, may have contributed to the nest.

DISCUSSION

Our primary goal in this study was to establish a baseline to assess future genealogical contributions of captive reared larvae used for translocation in the Blue River, Indiana. Our secondary goal was to resolve the clutch's parentage to minimize genetic swamping at release sites. Hellbender densities in Indiana are the lowest reported for any extant population [Burgmeier et al., 2011]. To this end, if a large number of closely related captive-reared juveniles are released at a single location, these head-started

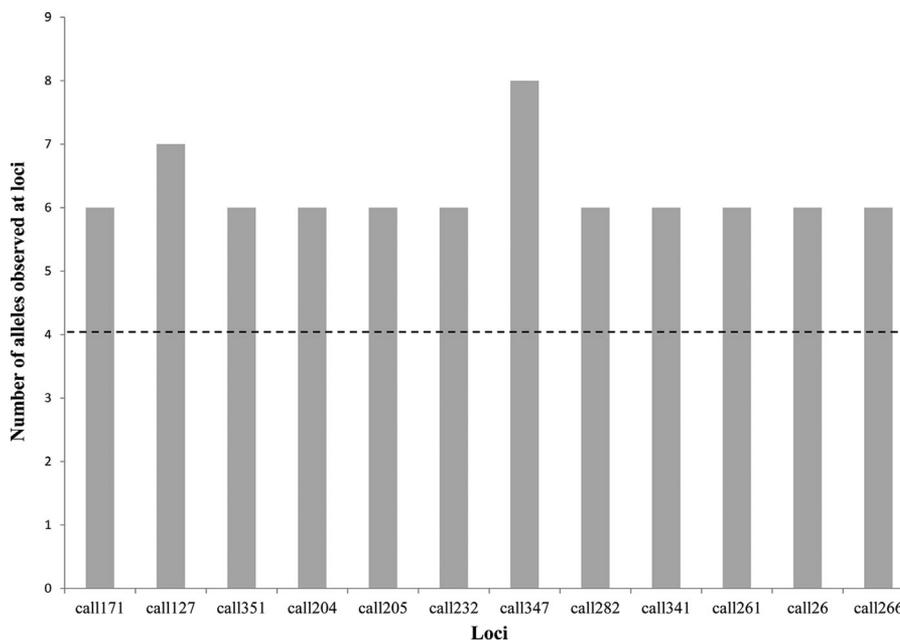


Fig. 1. Number of alleles observed at each locus for the Buffalo Creek, West Virginia egg mass ($N = 18$). Dashed line corresponds to the Minimal Method threshold for either single parentage (≤ 4 alleles per locus) or multiple parentage (> 4 alleles per locus).

individuals (which have been shown to have increased survivorship, Bodinof et al., 2012) may swamp the native gene pool and thus, reduce genetic diversity overtime [Allendorf et al., 2008]. Therefore, it was important to assess whether the genetic contribution of released individuals were derived from either two, or more breeding individuals which in turn would indicate the number of potential release sites needed. This form of “genetic rescue” or translocation may improve fitness by introducing new alleles while increasing population size in the short term [Whiteley et al., 2015]. To fully assess the impact of releasing captive reared individuals, we recommend follow-up demographic and genetic monitoring of individuals at release sites to characterize short term success (confirmed genetic contribution of released individual genotypes into wild populations through successful breeding) and long term success (increase of effective population size to >1000 individuals; Willi et al., 2006) of releases.

Our results demonstrate that these microsatellite markers can be used to determine multiple parentage in captive reared clutches of eastern hellbender salamanders. Our observation of more than four alleles per loci within this clutch indicate either multiple females enter a single male’s nest site, or alternatively multiple males entering, and fertilizing some portion of eggs from one, or more females below nest rocks. Smith [1907] documented the presence of more than one male within the nest area during the breeding season in *Cryptobranchus*. Behavioral observations of the closely related Japanese Giant salamander, *Andrias japonicas*, reveal intrusions of both multiple females, and males into nest which males were guarding [Kobara et al., 1980; Kawamichi and Ueda, 1998], however it is presently unknown to what extent this occurs in eastern hellbenders. Future research on *Cryptobranchus* should focus on documenting reproductive behavior at nests via field observation, assigning sibship within clutches using known parentals, and assigning parentage to more captive reared clutches using this panel of powerful genetic marker to genetically characterize the breeding system in this cryptic species.

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following standard procedures outlined in our protocol from the Purdue Animal Care and Use Committee (PACUC 08-025-11).

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