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Genetic markers reveal high PIT tag retention rates in giant salamanders (*Cryptobranchus alleganiensis*)

Shem D. Unger , Nicholas G. Burgmeier, Rod N. Williams

Abstract. Estimation of population size using mark-recapture (MRR) methods are based on the fundamental assumption that individuals retain their marks throughout the course of study. Passive Integrated Transponder (PIT) tags are useful as a cost effective, reliable marking method in many amphibian and reptile species. Few studies however, use secondary methods to evaluate tag retention rates. Failure to do so can lead to biased population estimates, erroneous conclusions, and thus poor management decisions. Surprisingly, estimates of PIT tag retention are currently lacking for the majority of amphibian species, many of which are experiencing population declines. Herein, we use genetic tagging to assess the retention of PIT tags of the eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*). We captured and tagged 78 individuals across 35 sites. Recapture rate was 24% and genetic tagging revealed 100% tag retention across all recaptured individuals.

Keywords: genetic tagging, hellbender, mark-release-recapture, microsatellite loci.

One assumption of mark recapture studies is retention of tags. Loss of tags can lead to the misidentification of recaptures as new individuals. Passive integrated transponder (PIT) tags have been reliably used for over a decade in many taxa including small mammals, fish, reptiles, and amphibians (see Gibbons and Andrews, 2004 for an overview). Rates of tag retention vary according to species, life stage, individual size, placement (specific location and direction of tag application), and handler experience (Meyer et al., 2011). Failure to meet the assumption of tag retention can lead to biased population estimates, erroneous conclusions, and thus poor management decisions. While many studies have assessed tag retention using single tagging methods (e.g. Jehle and Hoedl, 1998; Lukacs and Burnham, 2005; Schulte, Kuesters and Steinfartz, 2007), few have relied on a secondary tagging method (see table 1).

Molecular techniques present an efficient approach to assess the retention of physical tags. Highly variable microsatellite loci markers can provide a permanent, unique multilocus genotype (i.e., a "genetic tag") for individuals (Palsboll, 1999; Hoffman, Trathan and Amos, 2006). Recent studies on fish and turtles have utilized a genetic tagging approach as a secondary method to determine shedding rate of physical tags, including PIT tags (Pearse et al., 2001; Feldheim et al., 2002). Surprisingly, estimates of PIT tag retention are currently lacking for amphibian species (Gibbons and Andrews, 2004), many of which are cryptic and difficult to detect in their natural environments (Mazerolle et al., 2007).

Eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) are paedomorphic salamanders that occur in lotic habitats across much of the eastern U.S. (Nickerson and Mayes, 1973). Hellbender populations have experienced precipitous declines throughout their geographic range, with many populations listed as state endangered, threatened, or federally endangered (e.g., the Ozark Hellbender, *Cryptobranchus alleganiensis bishopi*; Wheeler et al., 2003; Burgmeier et al., 2011; Federal Register 76FR61978). Demographic studies on the eastern hellbender frequently utilize PIT tags which are placed in the tail musculature posterior to the hind leg (Humphries and Pauley, 2005; Burgmeier et al., 2011). PIT tag retention rates of eastern hellbenders are often assumed

Department of Forestry & Natural Resources, Purdue University, 195 Marstellar Street, West Lafayette, Indiana, 47907, USA

Corresponding author; e-mail: sunger@purdue.edu

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Table 1. Previous studies providing PIT tag retention rates for different taxa using single (PIT) and double tagging methods. Double tagging can include physical or genetic tags. This list is not exhaustive.

Method	Taxa	Retention rate	Sample size	Duration	Author
	Fish				
Double tag	Lemon shark *Negaprion brevirostris*[1]	88%	891	5 years	Feldheim et al., 2002
Double tag	Muskellunge *Esox masquinongy*[2]	90.4%	291	1 year	Jennings, Hatzenbeler and Kampa, 2009
Single tag	Brown trout *Salmo trutta*[3]	>80%	145	4 weeks	Acolas et al., 2007
Single tag	Channel catfish *Ictalurus punctatus*[1]	97%	227	3 years	Moore, 1992
	Amphibian				
Single tag	Pyrenean brook salamander *Calotriton asper*[1]	84%	265	19 months	Cucherousset et al., 2008
Single tag	Marbled salamander *Ambystoma opacum*[2]	89%	260	5 months	Ott and Scott, 1999
Single tag	Fire salamander *Salamandra Salamandra*[1]	100%	98	2 years	Schulte, Kuesters and Steinfartz, 2007
Single tag	Danube crested newts *Triturus dobrogicus*[1]	89.50%	64	11 years	Jehle and Hoedl, 1998
Double tag	Hellbenders *Cryptobranchus alleganiensis*[1]	100%	78	2 years	current study
	Reptile				
Single tag	Corn snake *Elaphe gutatta*[1]	47%	15	9 weeks	Roak and Dorcas, 2000
Single tag	Pine snake *Pituophus melanoleucus*[1]	100%	163	3 years	Elbin and Burger, 1994

[1] = adult life stage, [2] = juvenile life stage, [3] = both adult and juvenile life stage.

to be high, but no supporting empirical data currently exist. In this study, we use a combination of field intensive mark-release-recapture (MRR) and molecular methods to obtain estimates of PIT tag retention rate in adult eastern hellbenders.

Field work

The data collected herein are part of a two year project focused on population demography and genetic assessment of hellbenders in Indiana. Thirty-five sample sites (average stream reach length 269 m) were selected based on habitat characteristics and surveyed using a combination of snorkeling and rock lifting. These sites were distributed along a 115 km stretch of the Blue River, Indiana (Burgmeier et al., 2011). All suitable habitats were searched within sites over 5 successive sample periods (2 in 2008 and 3 in 2009; Burgmeier et al., 2011). In total, seventy-eight individual adults were captured and marked between June-October 2008 and July-September 2009. The number of individuals per site ranged from zero to twelve. All individuals were implanted with a Biomark 134.2 kHz PIT tag directly into the tail musculature 8 cm posterior to the hind limb. Upon each capture, all individuals were scanned with a Biomark FS2001F-ISO PIT tag reader. Small tail clips were collected from all putative new captures to serve as DNA sources for subsequent molecular analyses.

Molecular approach

Adult tail clips were preserved in 1.5 ml of 95% ethanol and stored at 5°C prior to DNA extraction. High quality genomic DNA was extracted using a standard proteinase K/phenol-chloroform/isoamyl alcohol procedure (Sambrook and Russell, 2001). Genomic DNA was resuspended in 100 μl TLE buffer (10 mM Tris-Cl, 0.1 mM EDTA). A suite of twelve polymorphic microsatellite markers (Unger et al., 2010) was used to genotype all individuals within four multiplex reactions: multiplex I (Call171, Call127, and Call351) annealing temperature (T_a) 60°C, multiplexes II (Call204, Call205 and Call232) and III (Call347, Call282, and Call341) T_a 64°C, and multiplex IV (Call261, Call266, Call26) T_a 66°C. Multiplex reactions consisted of 10 μl PCRs containing 20 ng of template DNA; 1U of *Taq* DNA polymerase (New England Biolabs); 0.20 mM of each dNTPs, 0.9 mM MgCl2; and 1X PCR buffer (10 mM Tris-HCl, 50 mM KCL, 0.05 mg/ml BSA). Final primer concentrations for PCR were 0.25 uM for each primer; each forward primer was end-labeled with the fluorescent dye set DS-30 (Applied Biosystems). Temperature profiles for PCR consisted of a 2 min 94°C denature step followed by 30 cycles of: 94°C for 30 s; multiplex specific annealing temperature (see above) for 30 s, and 72°C for 30 s and a final 10 minute extension step at 60°C for 45 min. Amplicons from each sample were scored for size via electrophoresis on an AB3730 automated sequencer (Applied Biosystems) using GeneMapper version 3.7 software.

Several quality control measures were used to minimize erroneous genotyping. Ambiguous genotypes, samples missing one or more genotypes, or those with low quality scores were re-amplified and rescored. In total, 30% of the samples were re-amplified and rescored across all loci. Finally, a random subset of individuals (40%) was independently scored across all loci to safeguard against systematic errors in genotyping.

To ensure our markers could sufficiently identify individuals among the population, we calculated the probability of identity (PID) using the program APICALC (Ayres and Overall, 2004). PID is defined as the probability that two individuals drawn at random from a population share the exact same genotype across multiple loci, and is often a measure used in non-invasive DNA-based mark recapture studies (Waits, Luikart and Taberlet, 2001). For this study, recapture rate was defined as the number of individuals retaining marks divided by total individuals marked. PIT tag retention rate was determined by a comparative approach. We directly compared genotypes of all putatively new individual captures with those of previously tagged individuals. Any instance of two putative individuals having identical genotypes would suggest one individual that lost a PIT tag and was subsequently assigned a new ID. As secondary measure, we used Cervus 3.0 (Kalinowski, Taper and Marshall, 2007) to identify any duplicate multilocus genotypes and confirm the comparative approach.

Exhaustive sampling across 35 sites resulted in a total of 97 captures. Fifty-nine individuals were caught only once, 13 were caught twice and six were caught more than twice. The proportion of individuals recaptured was 24%. All individuals were successfully genotyped across all loci. Quality control measures verified that genotyping error rates were low (<1%). The 12 microsatellite loci were highly polymorphic with a mean number of alleles per locus of 10.5 (range: 9-13). The probability of identity was low (2.9 10 ^{15}) and few individuals shared more than a single genotype (out of 12). Pit tag retention was 100% (95% exact Clopper-Pearson confidence interval, 95.38-100%, = 0.05) across the course of our study.

When monitoring cryptic species, estimation of population trends should be based on the most accurate tagging approaches available for a particular species. Based on our two-year study, retention of PIT tags in eastern hellbenders is high and consistent with other herpetofauna and aquatic vertebrates (table 1). There are several factors that can increase PIT tag retention including: proper needle placement

and orientation, tag implantation location, adequate immobilization of the individual, handler experience, and physiology of taxa (Gibbons and Andrews, 2004; Ward, Childs and Persons, 2008). In this study, proper implantation of tags deep into the tail musculature most likely minimized loss of PIT tags. We did not observe any indication of tag migration (internal movement) among recaptured individuals. In addition, recaptured individuals showed no signs of injury at the implantation site which has been indicated as a factor for tag loss in other studies (Feldheim et al., 2002; Gibbons and Andrews, 2004). Tag loss is also likely affected by time elapsed since implantation, with tags having a higher probability of being retained once injection sites fully heal. Recaptures were encountered generally 3 weeks to 1 year after tagging and we did not see any difference in retention based on timing. The use of a Bender Board (Burgmeier et al., 2010) to properly restrain individuals as they received tags may have decreased overall tag loss by reducing movement during handling and ensuring proper implantation.

The use of double tagging systems can enable researchers to quantify the relative permanence of tags, thereby increasing the accuracy of population size estimation. Indeed, DNA-based mark-recapture methods, or "genetic tagging", has proven useful as a non-invasive sampling technique for a variety of conservation species (Palsboll et al., 1997). However, wildlife studies which utilize genetic tagging must take into consideration the power of markers as well as scoring methods used to genotype individuals. Based on our calculation of PID, the genetic markers used in this study were reliable as a powerful, permanent individual marking method. This genetic tagging approach is a viable secondary tagging method. Similarly, PIT tags appear to be a reliable means of marking giant salamanders and likely other aquatic salamanders for use in MRR studies.

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