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Author(s): Noah D. Charney, Andrea T. Ireland, and Brian R. Bettencourt
Source: Journal of Herpetology, 48(2):210-219. 2014.
Published By: The Society for the Study of Amphibians and Reptiles
DOI: http://dx.doi.org/10.1670/12-212
URL: http://www.bioone.org/doi/full/10.1670/12-212

Journal of Herpetology, Vol. 48, No. 2, 210–219, 2014

Mapping Genotype Distributions in the Unisexual *Ambystoma* Complex

Noah D. Charney,[1,4] Andrea T. Ireland,[2] and Brian R. Bettencourt[3]

[1]*Graduate Program in Organismic and Evolutionary Biology, University of Massachusetts Amherst, Amherst, Massachusetts 01003-9297 USA*
[2]*Department of Biological Sciences, University of Massachusetts Lowell, Lowell, Massachusetts 01854 USA*
[3]*Alnylam Pharmaceuticals, Cambridge, Massachusetts 02142 USA*

Abstract.—**Mapping species' distributions is a primary challenge when managing cryptic lineages of conservation concern. In the case of unisexual *Ambystoma* salamanders, mapping geographic distribution of genotypes can also help us understand the evolutionary dynamics of one of the most intriguing vertebrate reproductive systems. We combined a mitochondrial sequencing technique with existing nuclear microsatellite methods to map genotypes in 15 *Ambystoma* populations throughout Massachusetts. We found that the host species *A. jeffersonianum* and *A. laterale* have disjunct east/west distributions, whereas unisexuals are distributed widely in Massachusetts. We did not find both host species at any single locality. In our samples, unisexuals outnumbered either host species in 11 of 15 populations. *Ambystoma jeffersonianum* nuclear genomes were present in at least 97% of unisexual salamanders in regions where *A. laterale* and unisexuals exist but *A. jeffersonianum* mitochondria were absent. If previous studies of the unisexual reproductive mode are correct, our observations suggest that natural selection favors hybrid nuclei in these populations.**

The unisexual *Ambystoma* complex is among the most fascinating vertebrate reproductive systems, involving a cryptic unisexual lineage and five sexual host species (*Ambystoma laterale*, *A. jeffersonianum*, *A. texanum*, *A. tigrinum*, and *A. barbouri*; Bogart et al., 2007). Almost all salamanders in the unisexual lineage are female (Uzzell, 1964; Bogart and Klemens, 1997; Bogart, 2003). All unisexuals share a common mitochondrial genome and are thought to have arisen from a single hybridization event 5 million yr ago (Spolsky et al., 1992; Bi and Bogart, 2010). The five host species are bisexual diploids, and no genetic material flows from the unisexual lineage to the host species. To reproduce, unisexual females must mate with a male of one of the five host species. Typically, the host sperm is used only to stimulate development and the host deoxyribonucleic acid (DNA) is not incorporated into the embryo (Bogart et al., 2007; Bi et al., 2008). Occasionally, the host DNA is incorporated into the offspring, which, over evolutionary time, has led to a plethora of hybrid nuclear genomotypes (Bogart et al., 2009). Across their range, the nuclei of unisexuals contain many combinations of nuclear genome sets from the five host species, ranging from diploid to pentaploid (Bogart, 2003). These hybrid nuclei have been found to contain full genome sets from up to three different host species.

There is considerable interest in mapping the distributions of the genotypes within this species complex, to advance both conservation and our understanding of the evolutionary dynamics involved in the system (Charney, 2012). Over much of their range, some or all of the species involved are protected by state endangered species and wetland regulations. For instance, in Massachusetts, where all unisexuals breed with one of two host species (*A. laterale* and *A. jeffersonianum*; Bogart and Klemens, 2008), all members of the complex are listed as Species of Special Concern pursuant to the Massachusetts Endangered Species Act (MGL c.131A) and its implementing regulations (321 CMR 10.00). However, setting conservation priorities is complicated by the difficulty of identifying individuals in the field. Without the ability to readily distinguish forms of the complex, it is difficult to assign listing status designations or manage habitats for different members of the complex. Given uncertainties about the exact distributions of various genomotypes in the *A. jeffersonianum–laterale* complex, the Massachusetts Natural Heritage and Endangered Species Program databases currently define all salamander in the species complex found west of the Connecticut River as Jefferson Salamanders (*A. jeffersonianum*), and any salamander in the complex found east of the Connecticut River as Blue-Spotted Salamanders (*A. laterale*). Unisexuals are lumped in with the local host species in the Massachusetts database. The actual distributions of host species are likely more complicated than this simple picture that uses the Connecticut River as the dividing line, yet scant genetic data exist to guide management of these species (Bogart and Klemens, 2008).

Although the five host species are distinguished easily from each other in the field on the basis of coloration, size, body proportions, and other characters, difficulty arises when trying to distinguish unisexuals from the bisexual species. Making this determination is critical to understanding unisexual ecology, because unisexuals are often found at high frequencies in populations where they occur. Beyond morphometric characters (Uzzell, 1964; Downs, 1978; Lowcock et al., 1992), past researchers have distinguished members of this complex on the basis of allozyme electrophoresis (Bogart et al., 1985), karyotypic analyses (Sessions, 1982; Taylor and Bogart, 1990), blood erythrocyte size (Uzzell, 1964; Wilbur, 1976; Austin and Bogart, 1982), flow cytometry (Lowcock et al., 1991), genomic in situ hybridization (Bi and Bogart, 2006), microsatellite alleles (Julian et al., 2003; Ramsden et al., 2006; Noël et al., 2011), single nucleotide polymorphism assays (Greenwald and Gibbs, 2012), and taxon-specific primers (Noël et al., 2008; Rhoads et al., 2009). Recent efforts have focused on identifying minimally invasive procedures that require little genetic material.

The majority of past genetic studies used nuclear DNA for identification. Any individuals with nuclei that are either polyploid or that contain hybrid genomes can be safely categorized as members of the unisexual complex. However, diploid unisexuals do arise (Bogart et al., 1985; Downs, 1978; Licht and Bogart, 1989; Lowcock et al., 1991; Noël et al., 2011). It might also be possible for unisexuals to carry nonhybrid nuclei, a possibility with important evolutionary implications. Charney (2012) demonstrated that, given previous descriptions of the rate at which ploidy levels can change between generations, we

[4]Corresponding Author. Present address: School of Natural Sciences, Hampshire College, Amherst, Massachusetts 01002 USA; E-mail: noah@alumni.amherst.edu
DOI: 10.1670/12-212

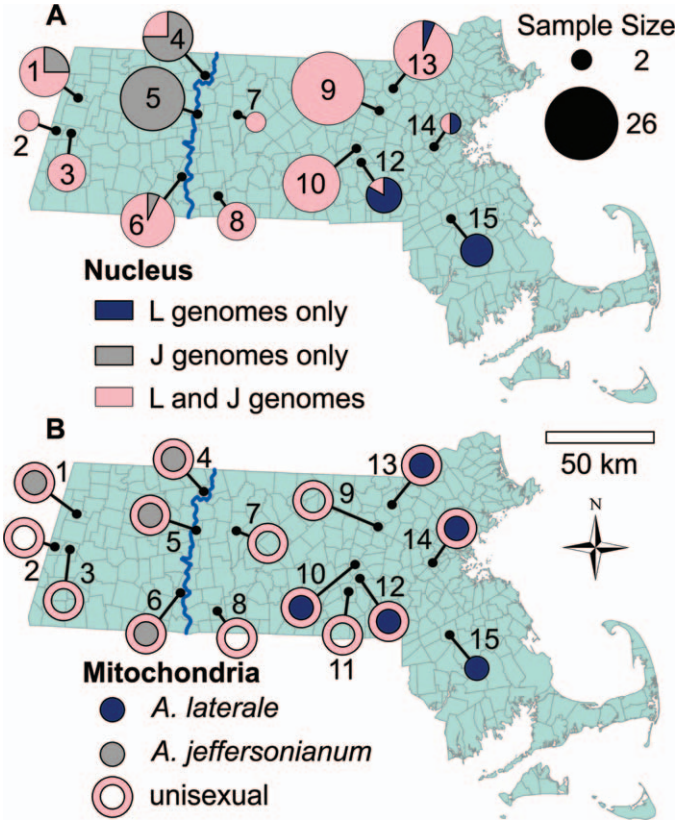

FIG. 1. (A) Distributions of nuclear genomotypes in 151 salamanders at 14 breeding sites in Massachusetts. Microsatellites were used to determine whether nuclei contained only *Ambystoma jeffersonianum* (J) genomes, only *A. laterale* (L) genomes, or both. Pie chart sizes represent the total numbers of salamanders scored for the nuclear analysis. Site 11 is excluded from this map because only one of the microsatellites successfully amplified. The individual with a unisexual mitochondria from site 5 is not represented in the top map because only one microsatellite locus amplified successfully. (B) Distributions of mitochondrial haplotypes of *A. laterale*, *A. jeffersonianum*, and unisexual salamanders at 15 breeding sites in Massachusetts. A portion of the mitochondrial D-loop was sequenced and compared with published sequences to determine haplotype presence at each pond. At populations where unisexuals were found (sites 1–14), we successfully amplified mitochondrial sequences from between 1 and 11 individuals (Appendix 1). At the population where no unisexuals were found (site 15), we successfully amplified mitochondrial sequences from 24 individuals. The south-flowing Connecticut River is drawn as a dark blue line.

would expect many nonhybrid nuclei in the unisexual lineage in the absence of strong selection. This is because many unisexual populations rely on a single host species to stimulate embryo development. The genome from a host species is occasionally incorporated into the developing offspring, and occasionally genome sets are lost from the lineage. As a one-way flow of genetic material, the host genome should eventually replace all other nuclear genomes in the local unisexual population in the absence of selection. Categorizing individuals as unisexuals strictly on the basis of nuclear composition could potentially introduce a bias away from detecting either diploid or nonhybrid nuclei.

Although the nuclei contained within unisexual salamanders reflect a complex history of hybridization, the mitochondria have a simple monophyletic origin (Hedges et al., 1992; Spolsky et al., 1992). The nuclei of unisexual salamanders across their range contain different combinations of genome sets from *A. laterale*, *A. jeffersonianum*, *A. texanum*, *A. tigrinum*, and *A. barbouri*, with recurrent hybridization. However, the mitochondria of all unisexual salamanders form a distinct lineage that is most closely related to the mitochondrial genome of *A. barbouri*. This suggests that examining the mitochondrion is an ideal way to identify individuals as unisexuals. For instance, if one were interested in finding unisexuals that were diploid and did not have hybrid nuclei (Charney, 2012), the mitochondria may be the only way to distinguish such a salamander from the host species. In addition, mitochondrial DNA is much more stable and abundant than nuclear DNA, thus requiring less tissue to be taken from the animals.

In this study we use a mitochondrial sequencing technique and nuclear microsatellites to map the distributions of the unisexual *Ambystoma* complex in Massachusetts. We also use these genetic data to determine whether simple morphometric field measurements can be used to distinguish unisexuals from the host species.

Although we do not address ploidy in this study, and thus cannot distinguish among the myriad hybrid nuclear combinations discussed in the literature, mitochondrial sequencing can offer substantial insights into salamander populations. Mitochondrial sequencing does not directly tell us the specific nuclear genomotype and ploidy of the individual being sampled. However, on the basis of previous studies, the expectation is that the unisexual nuclear genomotypes can be predicted by knowing the species of donor males occurring in a pond, if males can be found. Unisexuals with LLJ nuclei should occur in ponds with *A. laterale* donor males, whereas LJJ unisexuals occur in ponds with *A. jeffersonianum* donor males (Bogart and Klemens, 2008). If this relationship holds true, then sequencing the mitochondria of males in ponds may be sufficient to guide management for sites as either LJJ or LLJ ecotypes. However, it may still be of interest to distinguish tetraploids and other hybrid ploidy levels for management.

MATERIALS AND METHODS

Study Area.—We confined our study to Massachusetts, in an area approximately 190 km from east to west and 70 km from north to south. We collected genetic material from 15 towns across the state: Lanesborough (site 1), Richmond (site 2), Lenox (site 3), Gill (site 4), Sunderland (site 5), Holyoke (site 6), New Salem (site 7), Wilbraham (site 8), Boxborough (site 9), Northborough (site 10), Grafton (site 11), Westborough (site 12), Westford (site 13), Newton (site 14), and Easton (site 15; Fig. 1). Town selection was based on an attempt to gain maximal geographic coverage while visiting sites in the Natural Heritage and Endangered Species Program database that were known to have productive breeding populations.

Sample Collection.—In each town, members of a team of volunteer herpetologists visited a single known *A. laterale*/*jeffersonianum* breeding site and captured salamanders during the beginning of the breeding season, 26 March–4 April 2009. The samples from Northborough were collected in 2003. We collected genetic material from at least 20 salamanders at all sites except for sites 3, 7, 8, and 11, where we were only able to obtain 7, 4, 11, and 2 samples, respectively. Precise locality data are maintained by the Natural Heritage and Endangered Species Program of the Massachusetts Division of Fisheries and Wildlife (Charney and Ireland, 2010). Salamanders were captured by hand during their migration to breeding ponds and by using minnow traps placed in breeding ponds overnight. We collected one toe or tail tip from

each salamander ($N = 438$), and then released the salamander. We stored tissue samples in 95% ethanol until extraction and extracted total DNA following Fetzner (1999).

After all analyses were complete, extracted DNA and remaining tissue samples were deposited in the Ambrose Monell Collection for Molecular and Microbial Research at the American Museum of Natural History, bar-code numbers 208461–208892.

Nuclear Microsatellites.—For 218 salamanders, we used two nuclear microsatellites (AjeD346 and AjeD94) to distinguish between *A. jeffersonianum* and *A. laterale* haplotypes (Julian et al., 2003; Ramsden et al., 2006). Our goal was to analyze approximately 25 salamanders from each population where sample sizes allowed. From the extracted DNA, we performed polymerase chain reaction (PCR) with a 120-sec initialization at 94°C, followed by 34 cycles of 45 sec at 94°C, 45 sec at 50°C, and 90 sec at 72°C. Samples were held at 72°C for a final elongation step lasting 600 sec. We ran the PCR product on gels using a mixture of regular and MetaPhor agarose, which is specifically designed for separation of small nucleotides (Lonza, Basel, Switzerland). We measured allele sizes against a 100-base-pair (bp) ladder (GeneRuler, Fermentas, Glen Burnie, Maryland) run in a parallel gel lane. We compared the allele sizes with the following size ranges for known species from J. Bogart's unpublished data: 170–270 bp for *A. jeffersonianum* AjeD94, 134–198 bp for *A. laterale* AjeD94, 152–256 bp for *A. jeffersonianum* AjeD346, and 240–336 bp for *A. laterale* AjeD346 (J. Bogart, pers. comm.). These sizes provide slightly larger ranges than the available published data that were drawn from a more limited geographic distribution (Julian et al., 2003).

Mitochondrial Sequences.—To map the distributions of *A. jeffersonianum*, *A. laterale*, and the unisexual mitochondrial haplotypes across the state, we selected 120 salamanders from which we attempted to sequence a portion of the mitochondrial D-loop. For PCR, we used primers THR and 651 as identified by Shaffer and McKnight (1996) to amplify the full D-loop. We then used internal sequencing primers 007 and DL1 to provide approximately 470 bp of double-stranded sequence convergence. Compared with the unisexual mitochondrial sequence over this sequence range, *A. laterale* differs at 64 sites and *A. jeffersonianum* differs at 57 sites. The sequences of *A. jeffersonianum* and *A. laterale* differ from each other at 29 sites over this region. The goal was to sequence genetic material from individuals with both hybrid and pure nuclear genomotypes at each population. On the basis of the nuclear microsatellite data, we attempted to use at least three pure and three hybrid salamanders for mitochondrial sequencing from each population. If the first six salamanders from a population did not yield mitochondrial sequences from both unisexuals and host species, we continued to sequence mitochondria from other individuals in that population until we obtained both sequence types or ran out of samples.

Our D-loop mitochondrial PCR protocol involved a 120-sec initialization at 94°C, followed by 24 cycles of 60 sec each at 94°C, 48°C, and 72°C. For the first five cycles, the transition from 48°C to 72°C was achieved by ramping up at 0.5°C/sec. Subsequent cycles were not ramped. Samples were held at 72°C for a final elongation step lasting 600 sec. Samples were cleaned using QIAquick PCR Purification kits (Qiagen, Valencia, California) followed by Millipore Ultrafree centrifugal filters with a 10-kDa nominal molecular weight limit (Millipore Corporation, Billerica, Massachusetts). We performed forward and reverse sequencing reactions using CEQ dye-labelled dideoxy-terminator cycle sequencing kit (Beckman-Coulter, Brea, California). Sequences were prepared according to manufacturer instructions and analyzed using a CEQ 2000XL (Beckman-Coulter) automated sequencer. Sequences were aligned, edited, and compared with reference sequences from the GenBank sequence database (http://www.ncbi.nlm.nih.gov/) using Sequencher 4.2 (Gene Codes Corporation, Ann Arbor, Michigan).

Morphometric Data.—The sequence data were used to assess the reliability of simple field-based morphometric measurements for discriminating species. In the field, we measured the snout–vent length and mass of each salamander. We also assigned sex on the basis of the appearance of cloacal swelling related to spermatophore production or abdominal swelling due to eggs (Pfingsten and Downs, 1989). We classified unisexuals into two categories: those that occurred within the range of *A. jeffersonianum* and those that occurred within the range of *A. laterale*. Previous research has shown that these two categories are likely to correspond with LJJ and LLJ genomotypes, respectively (Bogart and Klemens, 2008).

Results

Nuclear Microsatellites.—We were able to assign nuclear haplotypes for microsatellites AjeD94 and AjeD346 in 148 salamanders (Appendix 1). We excluded from the final distribution maps other salamanders for which only one of the microsatellites successfully amplified ($n = 60$), salamanders for which both microsatellites showed alleles of sequence lengths that overlapped the two species ($n = 2$), salamanders for which haplotypes assigned using the two microsatellites were inconsistent with each other ($n = 8$), and one salamander for which the nuclear microsatellites were inconsistent with the mitochondrial sequence.

At four sites in western Massachusetts, we found 31 salamanders carrying only *A. jeffersonianum* (J) alleles at both loci (Fig. 1A; Appendix 1). At four sites in eastern Massachusetts we found a total of 13 salamanders carrying only *A. laterale* (L) alleles at both loci. We found 104 salamanders carrying hybrid nuclei and these were distributed across most of the state, but we did not find hybrid salamanders at site 15 (southeastern Massachusetts). In central Massachusetts, we found only one salamander at site 5 that had a hybrid nucleus at AjeD346; however, AjeD94 failed to amplify in this individual. At site 11, AjeD346 amplified for only one of the two salamanders and displayed a hybrid nucleus, but AjeD94 did not amplify.

Mitochondrial Sequences.—From 85 salamanders, we obtained clean mitochondrial sequences successfully, successful defined as those that could be aligned unambiguously to reference sequences over a continuous region of at least 100 bp. The mean length of all clean sequences was 388 bp (min. = 107, max. = 486, median = 420). All 85 clean sequences matched either *A. laterale*, *A. jeffersonianum*, or unisexual haplotypes at all diagnostic sites included in the sequence. The shortest sequence included 13 diagnostic sites, whereas the longest included 64 diagnostic sites.

We obtained clean sequences that matched known unisexual sequences from 47 salamanders in all but site 15 in southeastern Massachusetts (Fig. 1B; Appendix 1). We obtained clean *A. jeffersonianum* sequences from 17 salamanders representing four ponds in the western portion of the state and we obtained clean *A. laterale* sequences from 21 salamanders at five ponds in the eastern portion of the state, including site 15.

In addition, we obtained sequences that were identifiable using a BLAST search on GenBank, but were not cleaned up in

TABLE 1. Summary statistics for snout–vent lengths and masses of salamanders with mitochondrial D-loop sequences that matched *Amybsomta jeffersonianum* ("JJ"), *A. laterale* ("LL"), or unisexual hybrids. In this table, unisexual classifications are not based on direct nuclear genotype observations, but instead are inferred on the basis of whether they occur within the range of *A. jeffersonianum* ("LJJ") or *A. laterale* ("LLJ").

		Snout–vent length (mm)				Mass (g)			
	n	Mean	SD	Min.	Max.	Mean	SD	Min.	Max.
JJ	21	84.1	6.2	72	97	13.3	3.1	9	19.25
LL	33	52.5	10.8	38	80.2	3.9	1.1	1.7	7.3
"LJJ"	20	80.5	6.1	70	93	12.9	3.2	6.4	17.5
"LLJ"	15	76.2	12.2	56	99	9.7	2.6	5.8	16

Sequencher because they were too noisy or, in the case of salamanders from site 15, overly redundant. These included 5 that matched unisexuals, 4 that matched *A. jeffersonianum*, and 15 that matched *A. laterale*. For 11 salamanders, we were unable to obtain identifiable sequences.

We found one variant haplotype in each mitochondrial lineage. Each variant was unique to a single population and differed by a single nucleotide from the common haplotype. The unisexual variant occurred in one of the seven sequences from site 8, the *A. jeffersonianum* variant in both *A. jeffersonianum* sequences from site 6, and the *A. laterale* variant in three of the nine *A. laterale* sequences from site 15. For all haplotype variants obtained, we deposited representative sequences in GenBank (accession numbers JF693886–JF693891).

Morphometric Data.—The mitochondrial sequencing results correlated with morphological traits measured in the field. Of the 50 salamanders that were assigned a sex in the field and for which unisexual sequences were obtained, only 2 were described as males in the field. Equal or roughly equal sex ratios were reported for the host species, with 15 of 30 *A. laterale* described as male and 12 of 21 *A. jeffersonianum* described as male (Appendix 1).

Snout–vent length and mass both varied significantly among *A. laterale*, *A. jeffersonianum*, and unisexuals found in *laterale* and *jeffersonianum* ranges (ANOVA; $F = 139.93, 82.776$; $P < 10^{-15}$ for length and mass, respectively; Table 1, Fig. 2). All of the salamanders with *A. jeffersonianum* mitochondria were both longer and heavier than all of the salamanders with *A. laterale* mitochondria; unisexuals were also significantly longer and heavier than *A. laterale* according to pair-wise post hoc tests (Fig. 2). Unisexual salamanders found in ponds with *A. laterale* were significantly shorter and lighter than *A. jeffersonianum*, and significantly lighter than unisexuals found in pools with *A. jeffersonianum* (Fig. 2).

Although the mitochondrial genotypes of the host species are geographically segregated, the nuclear genomes are spread throughout the state (Fig. 1). In western Massachusetts, nuclear *A. laterale* ("L") genomes persist in unisexuals (presumably LJJ) in the absence of *A. laterale*, yet in eastern Massachusetts unisexuals and *A. laterale* continue to interact. In the same manner, in eastern Massachusetts, *A. jeffersonianum* ("J") genomes persist in unisexuals (presumably LLJ) in the absence of *A. jeffersonianum*, whereas the two coexist in western Massachusetts.

DISCUSSION

Charney (2012) showed that there must be very strong selection in favor of hybrid nuclei if previous studies are correct about high rates of sperm DNA being incorporated into developing offspring. This argument rests on the two key observations that *A. jeffersonianum* (J) and *A. laterale* (L) nuclear genomes persist in unisexuals far from populations of their corresponding diploid species, and that most unisexuals discovered have hybrid nuclei. In the present study, both of

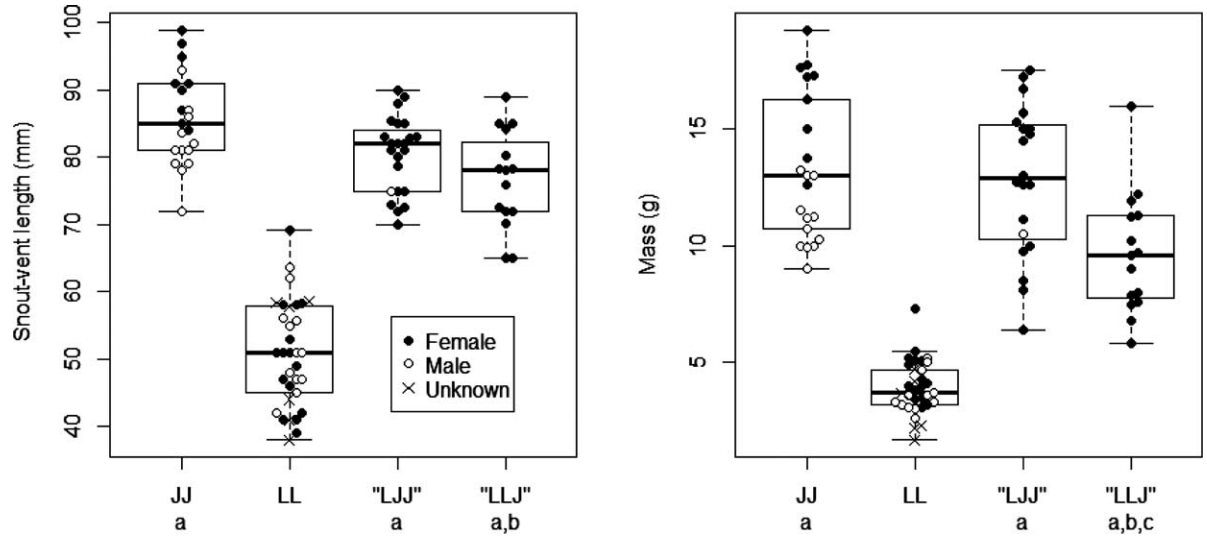

FIG. 2. Snout–vent lengths and masses of salamanders with mitochondrial D-loop sequences that matched *Amybsomta jeffersonianum* ("JJ"), *A. laterale* ("LL"), or unisexual hybrids. In this plot, unisexual classifications are not based on direct nuclear genotype observations, but instead are inferred on the basis of whether they occur within the range of *A. jeffersonianum* ("LJJ") or *A. laterale* ("LLJ"). "a", Tukey's post hoc tests vs. L significant at $P < 0.001$. "b", Tukey's post hoc tests vs. J significant at $P \leq 0.001$. "c", Tukey's post hoc test vs. LLJ significant at $P = 0.002$.

these observations hold true. We found J genomes in 60 of 73 salamanders in the *A. laterale*–unisexual region. Eleven of the 13 specimens with no J genomic contribution had *A. laterale* mitochondria, and we do not know the origins of the other two mitochondria. We can say then that at least 97% of unisexuals in the *A. laterale* region carry J genomes, suggesting strong selection for hybrid nuclear genomes in that region (Kraus, 1985).

The distributions of unisexuals and pure species in the *A. jeffersonianum/laterale* complex across Massachusetts were consistent with those described by Bogart and Klemens (2008). *Ambystoma laterale* is confined to the eastern portion of the state, *A. jeffersonianum* to the western portion, and unisexuals are distributed throughout most of the state. We found no *A. laterale* west of the Connecticut River, although we did find *A. jeffersonianum* in Sunderland just east of the river. On the basis only of our data, the dividing line between the two species could be located as far east as the eastern uplands of Worcester County. We have no samples from this region, and the paucity of records in the Natural Heritage and Endangered Species database from Worcester County suggests that the uplands may function as a habitat barrier.

In most populations, unisexuals outnumbered the bisexual species, consistent with expectations. There were a few populations, however, where unisexuals were the minority. The ratios of unisexuals to *A. jeffersonianum* were 4 : 13 at site 4 and 1 : 21 at site 5, and the ratios of unisexuals to *A. laterale* were 4 : 7 at site 12 and 0 : 24 at site 15. Bogart and Klemens (2008) suggest that the southeastern portion of the state is the one area where no unisexuals occur, which is consistent with the lack of unisexuals in our samples from site 15. The proportions we report should be interpreted cautiously, as our samples likely do not reflect the actual proportions in the populations. Differences between the sexes and among genomotypes in the timing of migration could cause biases in the ratios of captured lineages and sex ratios.

Our goal in this study was to characterize broad distributional patterns. We presume that the few salamander genotypes in our data that are inconsistent with previous literature represent errors in our field identification (e.g., unisexual males), or in our scoring of microsatellites (e.g., JJJ genomotypes). Similarly, we would urge caution in interpreting our records of several individuals at site 6 with LLJJ genomotypes, which have been reported only rarely in the literature. Since the primary focus of our study was not on discriminating ploidy, but rather identifying hybrid nuclei, we did not reexamine these individuals. Researchers interested in the occurrence of LLJJ genotypes should reanalyze the DNA from these salamanders (housed at the American Museum of Natural History, AMCC bar-code numbers 208698–208721). At Northborough (site 10), we obtained *A. laterale* mitochondrial sequences from three salamanders (WN-5, WN-7, and WN-8), but the nuclear microsatellites from these individuals were inconsistent with each other and with the mitochondria, so we did not include these individuals in the final nuclear maps. We identified more salamanders with *A. laterale* mitochondrial sequences than salamanders with pure *A. laterale* nuclei. This is because, in our efforts to obtain unisexual sequences at primarily *A. laterale* ponds, we ended up sequencing more individuals than just those from which both nuclear microsatellites amplified.

We found sequencing the D-loop of the mitochondria to be an efficient means for characterizing genotypic distributions. In a pilot study (Charney and Ireland, 2010; Charney, 2011), we used taxon-specific primers to distinguish unisexual mitochondria from those of nonunisexuals (Noël et al., 2008). However, the single-character taxon-specific primers introduced by Noël et al. (2008) cannot distinguish between *A. laterale* and *A. jeffersonianum* mitochondria, and this method has high sensitivity to contamination from airborne template DNA and observer bias. In contrast, the multiple-character sequencing technique used in the present study allows robust identification of each species in the complex. Although we included only two of the host species in this study, the D-loop sequences of the five host species and the unisexuals are all sufficiently divergent for discrimination (Bogart et al., 2007). For instance, over our focal mitochondrial region, *A. texanum, A. tigrinum,* and *A. barbouri* differ from unisexual salamanders by 63, 57, and 35 bp, respectively. Using mitochondrial DNA has the advantage of requiring less genetic material than with nuclear markers and has the potential to identify members of the unisexual lineage with diploid nonhybrid nuclei. Although such salamanders are not known to exist, they could also not be discovered through examining only the nucleus, as has previously been the primary genetic approach to identifying members of the complex.

As other studies have shown, our data suggest that simple morphometric field measurements can be used to quickly distinguish pure *A. laterale* from unisexuals or *A. jeffersonianum* (Uzzell, 1964; Downs, 1978; Lowcock et al., 1992). It also appears possible to distinguish most LJJ unisexuals from LLJ unisexuals on the basis of these measurements (Table 1, Fig. 2). There is substantial overlap between the sizes of *A. jeffersonianum* and unisexuals in our data, and thus we cannot distinguish them on the basis of our morphometric data. These morphometric data were drawn from a limited number of ponds, and it is possible that other environmental variables beyond lineage membership could underlie size differences between populations. In our study, we did not include leg length, snout width, or other measurements that other studies have found to be useful. Thus, more detailed morphometric data could likely provide more reliable means for discriminating species. Use of genetic techniques may still be necessary for areas of overlap in species sizes, and in cases where measurement of adults is not possible, such as when only larvae or eggs are present. Continued focus on simple, noninvasive techniques for identifying species in this complex will help us understand and protect one of the most fascinating yet ecologically sensitive vertebrate systems.

Acknowledgments.—Crucial assistance in fieldwork, laboratory work, and initial project design was provided by M. Graves, A. Richmond, B. Windmiller, J. Regosin, J. Kubel, E. Mangan, T. Tyning, B. Bastarache, B. Timm, M. Jones, S. Record, L. Willey, T. Tada, V. Palermo, B. Butler, B. Manson, P. Lavasseur, A. DeLima, R. Skowron, A. Coman, J. Bogart, B. Shaffer, J. Niedzwiecki, and Oxbow Associates, Inc. We thank B. Normark, L. Ross, R. Gwiazdowski, and A. Okusu for donating data processing tools and advice. Further guidance and support was provided by P. Warren, K. McGarigal, and T. Fuller. We thank S. Tilley and two anonymous reviewers for substantial improvements to the manuscript. Funding for this research was provided by the Natural Heritage and Endangered Species Program of the Massachusetts Division of Fisheries and Wildlife, the University of Massachusetts Amherst Jane Hallenbeck Bemis Endowment for Research in Natural History Scholarship, the National Science Foundation Graduate Research Fellowship Program, and the Switzer Environmental Fellowship Program. All animal

handling protocols were approved, permitted, and overseen by the Massachusetts Division of Fisheries and Wildlife. This work was carried out under the Commonwealth of Massachusetts Division of Fisheries and Wildlife Scientific Collection Permit #304.09RWRA.

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Accepted: 23 April 2013.

APPENDIX 1. Individual data on Massachusetts salamanders in the *Amybstoma jeffersonianum/laterale* complex from which genetic material was sampled. The AMCC column contains bar-code numbers of the Ambrose Monell Collection for Molecular and Microbial Research at the American Museum of Natural History. Sex, snout–vent length (SVL), and mass were determined in the field. Nuclear genomotypes were determined by comparing fragment sizes of microsatellites AjeD94 and AjeD346 with known allele size ranges for *A. laterale* (L) and *A. jeffersonianum* (J). A portion of the mitochondrial D-loop was also sequenced and compared with known sequences from the two sexual species and that of unisexuals (U). Letters in parentheses indicate individuals for which a positive match was obtained using the Basic Local Alignment Search Tool on the GenBank sequence database website; however, the samples were too noisy to obtain complete clean sequences using Sequencher, and thus are less robust results. Genotypes with the suffix "-SNP" indicate that that individual contained a single nucleotide polymorphism relative to the consensus sequence for the lineage. Within each of the three lineages, all of the variant sequences contain the same SNP. "Fail" indicates unsuccessful attempts at sequencing.

Town	Locality Num.	County	AMCC	Indiv.	Sex	SVL (mm)	Mass (g)	AjeD94	AjeD346	D-loop
Boxborough	9	Middlesex	208648	DB-1	F	72	9.7	LJ	LJ	U
Boxborough	9	Middlesex	208649	DB-2	F	76	10.2	LJ	LJ	U
Boxborough	9	Middlesex	208650	DB-3	F	75	10.4	LJ	LJ	
Boxborough	9	Middlesex	208651	DB-4	F	84	11.2	LJ	LLJ	
Boxborough	9	Middlesex	208652	DB-5	F	79	11.5	LJ	LLJ	
Boxborough	9	Middlesex	208653	DB-6	F	81	12	LJ	LJ	
Boxborough	9	Middlesex	208654	DB-7	F	76	10.3	LJ	LJ	
Boxborough	9	Middlesex	208655	DB-8	F	85	16	LJ	LLJ	U
Boxborough	9	Middlesex	208656	DB-9	F	69	6.3	LJ	LJ	
Boxborough	9	Middlesex	208657	DB-10	F	75	8.3	LJ	LJ	
Boxborough	9	Middlesex	208658	DB-11	F	79	9.8	LJ	LJ	
Boxborough	9	Middlesex	208659	DB-12	F	85	14	LJ	LLJ	
Boxborough	9	Middlesex	208660	DB-13	F	89	12.2	LLJ	LLJ	U
Boxborough	9	Middlesex	208661	DB-14	F	77	11.1	LJ	LJ	
Boxborough	9	Middlesex	208662	DB-15	F	75	11.2	LJ	LJ	
Boxborough	9	Middlesex	208663	DB-16	F	79	6.7	LJ	LJ	
Boxborough	9	Middlesex	208664	DB-17	F	75	10.2	LJ	LLJ	
Boxborough	9	Middlesex	208665	DB-18	F	81	9.6	LJ	LJ	
Boxborough	9	Middlesex	208666	DB-19	F	81	10.9	LJ	LJ	
Boxborough	9	Middlesex	208667	DB-20	F	81	11.9	LJ	LJ	
Boxborough	9	Middlesex	208668	DB-21	M	75	7.5	LJ	LLJ	
Boxborough	9	Middlesex	208669	DB-22	F	73	8.7	LJ	LJ	
Boxborough	9	Middlesex	208670	DB-23	F	78	8.5	LJ	LJ	
Boxborough	9	Middlesex	208671	DB-24	F	80	9.7	LJ	LJ	
Boxborough	9	Middlesex	208672	DB-32	M	85	10.4	LJ	LLJ	
Boxborough	9	Middlesex	208673	DB-40	M	74	5.4	LJ	LLJ	
Easton	15	Bristol	208674	EA-1	F	58	4	L		(L)
Easton	15	Bristol	208675	EA-2	J	41	1.7	L	LL	L
Easton	15	Bristol	208676	EA-3	M	45	3.3	LL	LL?	L-SNP
Easton	15	Bristol	208677	EA-4	F	51	5.5	L?	L	(L)
Easton	15	Bristol	208678	EA-5	F	46	3.8	L?	L	(L)
Easton	15	Bristol	208679	EA-6	M	51	2.6	LL	??	L
Easton	15	Bristol	208680	EA-7	F	49	3.2	L?	?	(L)
Easton	15	Bristol	208681	EA-8	F	42	3.5	L		L
Easton	15	Bristol	208682	EA-9	J	44	2.2	L		(L)
Easton	15	Bristol	208683	EA-10	F	51	3.1	LJ		L
Easton	15	Bristol	208684	EA-11	M	48	3	L?	LL?	L
Easton	15	Bristol	208685	EA-12	M	51	3.2			(L)
Easton	15	Bristol	208686	EA-13	M	47	3.3		L?	(L)
Easton	15	Bristol	208687	EA-14	F	41	4		L	L
Easton	15	Bristol	208688	EA-15	F	47	5.2		L?	(L)
Easton	15	Bristol	208689	EA-16	M	42	3.6		LL	L-SNP
Easton	15	Bristol	208690	EA-17	F	53	5.1		LJ	L-SNP
Easton	15	Bristol	208691	EA-18	F	41	3.4		L?	(L)
Easton	15	Bristol	208692	EA-19	M	55	3.6	L		(L)
Easton	15	Bristol	208693	EA-20	F	51	4.1	LL	L	(L)
Easton	15	Bristol	208694	EA-21	J	38	2.3			(L)
Easton	15	Bristol	208695	EA-22	F	39	4.3			(L)
Easton	15	Bristol	208696	EA-23	M	47	3.1	LL		(L)
Easton	15	Bristol	208697	EA-24	F	58	4.9		LJ	L
Gill	4	Franklin	208629	BT-2	M	87	13.75		J	
Gill	4	Franklin	208630	BT-3	M	79	14	JJ	J	
Gill	4	Franklin	208631	BT-4	M	75	12.75	JJ	J	
Gill	4	Franklin	208632	BT-5	M	81	13	?JJ	JJ	(J)
Gill	4	Franklin	208633	BT-6	M	84	11.75	JJ	JJ	
Gill	4	Franklin	208634	BT-7	M	68	9.75	JJ	JJ	fail
Gill	4	Franklin	208635	BT-8	M	79	10.25	JJ	JJ	(J)
Gill	4	Franklin	208636	BT-9	F	72	12.75	LJJ	LLJJ	U
Gill	4	Franklin	208637	BT-10	M	81	10	JJJ	J	J
Gill	4	Franklin	208638	BT-11	F	70	9.75		LLJJ	U
Gill	4	Franklin	208639	BT-12	F	87	16.25	JJJ		J
Gill	4	Franklin	208640	BT-13	F	82	15	LJJ	LJ	U
Gill	4	Franklin	208641	BT-14	F	81	15.25	LJJ	LJ	
Gill	4	Franklin	208642	BT-15	M	78	11.25	JJ	J	J

APPENDIX 1. Continued.

Town	Locality Num.	County	AMCC	Indiv.	Sex	SVL (mm)	Mass (g)	AjeD94	AjeD346	D-loop
Gill	4	Franklin	208643	BT-16	M	79	10	JJ		J
Gill	4	Franklin	208644	BT-17	M	88	14		J	
Gill	4	Franklin	208645	BT-18	M	76	13.75	JJ	J	
Grafton	11	Worcester	208746	JEK2-1	F	78.2	11.3		LJ	U
Grafton	11	Worcester	208747	JEK2-2	F	70.1	9			U
Holyoke	6	Hampden	208698	HOLY-1	F				LLJJ	
Holyoke	6	Hampden	208699	HOLY-2	M	82.3	8.4	JJ		
Holyoke	6	Hampden	208700	HOLY-3	F	72.9	6.4	LJJ	LLJJ	U
Holyoke	6	Hampden	208701	HOLY-4	F	77.1	10.6	LJJ	LLJJ	
Holyoke	6	Hampden	208703	HOLY-6	F	76	8.5	LJJ	LLJJ	
Holyoke	6	Hampden	208704	HOLY-7	F	83.3	11.2	LJJ	LLJJ	
Holyoke	6	Hampden	208705	HOLY-8	F	80.9	9.3	LJJ	LLJJ	
Holyoke	6	Hampden	208706	HOLY-9	F	76.5	10.6	LJJ	LLJJ	
Holyoke	6	Hampden	208707	HOLY-10	F	78.7	8.1	L	LLJJ	U
Holyoke	6	Hampden	208708	HOLY-11	M	83.7	11.2	JJ		J-SNP
Holyoke	6	Hampden	208709	HOLY-12	F	82.7	12.7	LJJ	LLJJ	
Holyoke	6	Hampden	208710	HOLY-13	F	82.8	13	LJJ	LLJJ	U
Holyoke	6	Hampden	208711	HOLY-14	F	71	5.7	LJJ	LLJJ	
Holyoke	6	Hampden	208712	HOLY-15	F	75	11.1	LJJ	LLJJ	U
Holyoke	6	Hampden	208714	HOLY-17	F	78	8.3	LJJ		
Holyoke	6	Hampden	208716	HOLY-19	F	84	12.6	JJ	JJ	J-SNP
Holyoke	6	Hampden	208717	HOLY-20	F	79	8.9	LJJ	LLJJ	
Holyoke	6	Hampden	208718	HOLY-21	F	96	14.4	LJJ	LLJJ	
Holyoke	6	Hampden	208719	HOLY-22	F	79	8.9	LJJ	LLJJ	
Lanesborough	1	Berkshire	208534	TTO-1	F	81	17.5	LJJ	LJJ	U
Lanesborough	1	Berkshire	208535	TTO-2	F	81	16.7	LJJ	LJJ	U
Lanesborough	1	Berkshire	208537	TTO-4	F	82	12.6	LJJ	LJJ	U
Lanesborough	1	Berkshire	208538	TTO-5	F	78	15.4	JJ	LJJ	
Lanesborough	1	Berkshire	208539	TTO-6	M	72	9.9	JJ	JJ	J
Lanesborough	1	Berkshire	208540	TTO-7	F	85	17.3	JJ	JJ	J
Lanesborough	1	Berkshire	208541	TTO-8	F	83	15.7	LJJ	LJJ	(U)
Lanesborough	1	Berkshire	208542	TTO-9	F	81	12.4		JJ	
Lanesborough	1	Berkshire	208543	TTO-10	F	86	15.1		LJ	fail
Lanesborough	1	Berkshire	208544	TTO-11	F	91	17.6	JJ	JJ	J
Lanesborough	1	Berkshire	208545	TTO-12	F	79	10.3	LJJ	LJJ	fail
Lanesborough	1	Berkshire	208546	TTO-13	F	83	15.7	LJJ	LJJ	
Lanesborough	1	Berkshire	208547	TTO-14	F	91.3	16.1	LJJ	LJJ	
Lanesborough	1	Berkshire	208548	TTO-15	F	89.6	14.6	LJJ	LJJ	
Lanesborough	1	Berkshire	208549	TTO-16	F	80.3	12.9	LJJ	LJJ	
Lanesborough	1	Berkshire	208550	TTO-17	F	87.1	14.8		LJ	
Lanesborough	1	Berkshire	208551	TTO-18	F	85.3	14		LJ	
Lanesborough	1	Berkshire	208554	TTO-22	M	87.3	12.1		JJ	
Lenox	3	Berkshire	208555	TTX-1	F	72.5	8.5	LJJ	LJJ	U
Lenox	3	Berkshire	208556	TTX-2	F	83.2	14.4	LJJ	LJJ	fail
Lenox	3	Berkshire	208557	TTX-3	F	85.5	12.6	LJJ	LJJ	U
Lenox	3	Berkshire	208558	TTX-4	F	89	15.3	LJ	LJJ	U
Lenox	3	Berkshire	208559	TTX-5	F	82	10	LJJ	LJJ	U
Lenox	3	Berkshire	208560	TTX-6	F	93	17.4	LJJ	LJJ	
Lenox	3	Berkshire	208561	TTX-7	F	85	14.8	LJ	LJJ	U
New Salem	7	Franklin	208485	LM-1				LJ	?J	fail
New Salem	7	Franklin	208486	LM-2					?J	U
New Salem	7	Franklin	208487	LM-3				LJ	?J	fail
New Salem	7	Franklin	208488	LM-4						fail
Newton	14	Middlesex	208461	JVR1-1	F	69.1	7.3			(L)
Newton	14	Middlesex	208462	JVR1-2	F	84.3	11.9			(U)
Newton	14	Middlesex	208478	JVR1-18	F	85.1	14.3	LJJ		
Newton	14	Middlesex	208479	JVR1-19	F	80	10.3	LJJ		
Newton	14	Middlesex	208480	JVR1-20	U/F	58.5	4.7	L?	L	L
Newton	14	Middlesex	208481	JVR1-21	F	72.6	7.9	LJ	LJ	U
Newton	14	Middlesex	208482	JVR1-22	F	78.3	7.5		L	U
Newton	14	Middlesex	208483	JVR1-23	F	58.2	5.1	L		L
Newton	14	Middlesex	208811	JVR1-24	F		9.1	LJ		
Northborough	10	Worcester	208563	WN-2	F			LJ	LJ	
Northborough	10	Worcester	208564	WN-3	M			LJ	LJ	U
Northborough	10	Worcester	208565	WN-4	F			J		
Northborough	10	Worcester	208566	WN-5	M			LJ	LJJ	L
Northborough	10	Worcester	208567	WN-6	F			LJ	L	
Northborough	10	Worcester	208568	WN-7	M			LJ	LL	L
Northborough	10	Worcester	208569	WN-8	M			J	LL	L
Northborough	10	Worcester	208570	WN-9	F			LJ	LJ	
Northborough	10	Worcester	208571	WN-10	F			LJ	LJ	
Northborough	10	Worcester	208572	WN-11	F			LLJ	LJ	

APPENDIX 1. Continued.

Town	Locality Num.	County	AMCC	Indiv.	Sex	SVL (mm)	Mass (g)	AjeD94	AjeD346	D-loop
Northborough	10	Worcester	208573	WN-12	F			L?J	LJ	U
Northborough	10	Worcester	208574	WN-13	F			L?J	LJ	U
Northborough	10	Worcester	208575	WN-14	F			L?J	LJ	
Northborough	10	Worcester	208576	WN-15	F			LJ	LJ	
Northborough	10	Worcester	208577	WN-16	F			L?J	LJ	
Northborough	10	Worcester	208578	WN-17	F			LJ	LJ	
Northborough	10	Worcester	208579	WN-18	F			LJ	LJ	(U)
Northborough	10	Worcester	208580	WN-19	F				LJ	U
Northborough	10	Worcester	208581	WN-20	F			LJ	LJ	
Northborough	10	Worcester	208582	WN-21	F			LJ	LJ	
Northborough	10	Worcester	208583	WN-22	F				LJ	
Northborough	10	Worcester	208584	WN-23				L?J	LJ	
Northborough	10	Worcester	208585	WN-24	F				LJ	
Northborough	10	Worcester	208586	WN-25	F				L?J	
Richmond	2	Berkshire	208489	NDC-1	M	70		L?		
Richmond	2	Berkshire	208490	NDC-2	F	70		L?	LJ	
Richmond	2	Berkshire	208491	NDC-3	F	80			LJ	U
Richmond	2	Berkshire	208494	NDC-6	F	80		L?		
Richmond	2	Berkshire	208496	NDC-8	F	75		L?	J	(U)
Richmond	2	Berkshire	208497	NDC-9	F	68			LJ	
Richmond	2	Berkshire	208498	NDC-10	F	88		LJ	LJ	U
Richmond	2	Berkshire	208501	NDC-13	F	85	15	L?J		U
Richmond	2	Berkshire	208502	NDC-14	F	85	17		LJ	
Richmond	2	Berkshire	208504	NDC-16	F	83	14.5		LJ	U
Richmond	2	Berkshire	208507	NDC-19	M	75	10.5			U
Sunderland	5	Franklin	208587	ARS-1	F	90	15	?JJ	J	J
Sunderland	5	Franklin	208588	ARS-2	M	82	11.5	JJ	JJ	J
Sunderland	5	Franklin	208589	ARS-3	M	84	10.25	?J	JJ	
Sunderland	5	Franklin	208590	ARS-4	M	87	10.75	JJ	J	J
Sunderland	5	Franklin	208591	ARS-5	F	97	17.75	JJ	JJ	J
Sunderland	5	Franklin	208592	ARS-6	F	87	18.5	JJ	JJ	
Sunderland	5	Franklin	208593	ARS-7	M	93	13.25	JJ	JJJ	J
Sunderland	5	Franklin	208594	ARS-8	M	81	9	JJ	J	J
Sunderland	5	Franklin	208595	ARS-9	M	88	9.75		J	
Sunderland	5	Franklin	208596	ARS-10	F	96	19.5	JJ		
Sunderland	5	Franklin	208597	ARS-11	M	88	10.5	JJ	JJ	
Sunderland	5	Franklin	208598	ARS-12	F	98	21.5	JJ	JJ	
Sunderland	5	Franklin	208599	ARS-13	F	92	16	J	J	
Sunderland	5	Franklin	208600	ARS-14	F	95	17.25	J	J	J
Sunderland	5	Franklin	208601	ARS-15	F	90	17.25		LJ	U
Sunderland	5	Franklin	208602	ARS-16	M	86	13			J
Sunderland	5	Franklin	208603	ARS-17	M	86	10.5	J	JJ	
Sunderland	5	Franklin	208604	ARS-18	F	99	19.25	J	JJ	(J)
Sunderland	5	Franklin	208605	ARS-19	M	86	10.5	J	JJ	
Sunderland	5	Franklin	208606	ARS-20	M	87	13		JJ	
Sunderland	5	Franklin	208607	ARS-21	F	91	13.75			(J)
Sunderland	5	Franklin	208608	ARS-22	F	92	18	?J	JJ	
Sunderland	5	Franklin	208609	ARS-23	F	96	19.75	?J	J	
Sunderland	5	Franklin	208610	ARS-24	F?	92	14	J	JJ	
Westborough	12	Worcester	208723	JEK1-B	F	80.2	9.6			U
Westborough	12	Worcester	208724	JEK1-C	F	78.1	8			U
Westborough	12	Worcester	208733	JEK1-L	F	72	7.6			U
Westborough	12	Worcester	208738	JEK1-Q	U	58.4	3.7		L	L
Westborough	12	Worcester	208739	JEK1-R	M	53.4	3.2	L	?	
Westborough	12	Worcester	208740	JEK1-S	U	61.1	5.1	L	L	
Westborough	12	Worcester	208741	JEK1-T	F	60.2	5.1	L		
Westborough	12	Worcester	208742	JEK1-U	M	63.6	5.2	L	L	L
Westborough	12	Worcester	208743	JEK1-V	M	55.6	3.7	L	L	L
Westborough	12	Worcester	208744	JEK1-W	F		8	LJ	LJ	U
Westborough	12	Worcester	208745	JEK1-X	U/F	57.8	4.2	L	L	L
Westford	13	Middlesex	208509	ROB-1	F	79	8.6	LJ	LJ	
Westford	13	Middlesex	208510	ROB-2	F	73	6.7	LJ	LJ	
Westford	13	Middlesex	208511	ROB-3	F	74	7.6	LJ	LJ	
Westford	13	Middlesex	208512	ROB-4	F	77	9.2	LJ	LJ	
Westford	13	Middlesex	208513	ROB-5	M		4.2	??	LJ	
Westford	13	Middlesex	208514	ROB-6	F	85	11.8	LJ	LJJ	
Westford	13	Middlesex	208515	ROB-7	F	80	9.9	LJ	LJ	
Westford	13	Middlesex	208516	ROB-8	F	81	10.5		LJ	
Westford	13	Middlesex	208517	ROB-9	M	58	6	JJ	L	fail
Westford	13	Middlesex	208518	ROB-10	F	76	9.1	LJ	LJ	
Westford	13	Middlesex	208519	ROB-11	F	65	6.8	LJJ	LJJ	U
Westford	13	Middlesex	208520	ROB-12	F	85	12.2	LJJ	LJJ	
Westford	13	Middlesex	208521	ROB-13	M	55	4.1		L	

APPENDIX 1. Continued.

Town	Locality Num.	County	AMCC	Indiv.	Sex	SVL (mm)	Mass (g)	AjeD94	AjeD346	D-loop
Westford	13	Middlesex	208522	ROB-14	M	56	4.7	?	LL	L
Westford	13	Middlesex	208523	ROB-15	F	65	5.8	LJJ	LJ	U
Westford	13	Middlesex	208524	ROB-16	M	62	5		LL	L
Westford	13	Middlesex	208525	ROB-17	F	62	7.1	LJJ	LJ	
Westford	13	Middlesex	208526	ROB-18	F	85	11.3	LJ	LJJ	U
Westford	13	Middlesex	208527	ROB-19	F	90	13.9	LJ	LJ	
Westford	13	Middlesex	208528	ROB-20	F	71	9.9	LJJ	LJ	
Westford	13	Middlesex	208529	ROB-21	F	57	5.6	LJJ	LJ	
Westford	13	Middlesex	208530	ROB-22	F	80	8.2		LJ	
Westford	13	Middlesex	208531	ROB-23	M	58	5.3	LJJ		
Westford	13	Middlesex	208532	ROB-24	M	64	6.3	LJJ	L	
Wilbraham	8	Hampden	208611	ARW-1	F	71	9.25	LJ	?J	U
Wilbraham	8	Hampden	208612	ARW-2	F	77.5	14.25	LJ	?J	U
Wilbraham	8	Hampden	208613	ARW-3	F	72	9.25	LJ	?J	(U)
Wilbraham	8	Hampden	208614	ARW-4	F?	89.5	16	LJ	?J	U-SNP
Wilbraham	8	Hampden	208615	ARW-5	F	62	5.5	LJ	?J	
Wilbraham	8	Hampden	208616	ARW-6	F	69.5	8.5	LJ	?J	U
Wilbraham	8	Hampden	208617	ARW-7	F	84	12	LJ	?JJ	fail
Wilbraham	8	Hampden	208618	ARW-8	F	71	9.75		J	U
Wilbraham	8	Hampden	208619	ARW-9	F	87	17.75		??J	
Wilbraham	8	Hampden	208620	ARW-10	F	73	10.25		??J	
Wilbraham	8	Hampden	208621	ARW-11	F	80.5	13	LJ	??J	U