Unisexual salamanders (genus *Ambystoma*) present a new reproductive mode for eukaryotes

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Abstract: To persist, unisexual and asexual eukaryotes must have reproductive modes that circumvent normal bisexual reproduction. Parthenogenesis, gynogenesis, and hybridogenesis are the modes that have generally been ascribed to various unisexuals. Unisexual *Ambystoma* are abundant around the Great Lakes region of North America, and have variously been described as having all 3 reproductive modes. Diploid and polyploid unisexuals have nuclear genomes that combine the haploid genomes of 2 to 4 distinct sexual species, but the *mt*DNA is unlike any of those 4 species and is similar to another species, *Ambystoma barbouri*. To obtain better resolution of the reproductive mode used by unisexual *Ambystoma* and to explore the relationship of *A. barbouri* to the unisexuals, we sequenced the mitochondrial control and highly variable intergenic spacer region of 48 ambystomatids, which included 28 unisexuals, representatives of the 4 sexual species and *A. barbouri*. The unisexuals have similar sequences over most of their range, and form a close sister group to *A. barbouri*, with an estimated time of divergence of 2.4–3.9 million years ago. Individuals from the Lake Erie Islands (Kelleys, Pelee, North Bass) have a haplotype that demonstrates an isolation event. We examined highly variable microsatellite loci, and found that the genetic makeup of the unisexuals is highly variable and that unisexual individuals share microsatellite alleles with sexual individuals within populations. Although many progeny from the same female had the same genotype for 5 microsatellite DNA loci, there was no indication that any particular genome is consistently inherited in a clonal fashion in a population. The reproductive mode used by unisexual *Ambystoma* appears to be unique; we suggest kleptogenesis as a new unisexual reproductive mode that is used by these salamanders.

Key words: unisexual *Ambystoma*, polyploidy, intergenic spacer, D-loop, microsatellite DNA, reproductive mode, kleptogenesis.

Résumé : Afin de se perpétuer, les eucaryotes unisexués ou asexués doivent avoir des modes de reproduction qui évitent le mode normal de reproduction bisexuée. La parthénogenèse, la gynogenèse et l'hybridogenèse sont des modes qui sont habituellement observés chez divers organismes unisexués. Les *Ambystoma* unisexués abondent autour de la région des Grands Lacs en Amérique du Nord et ont été décrits comme pouvant avoir les trois modes de reproduction. Les unisexués diploïdes et polyploïdes possèdent des génomes nucléaires qui combinent les génomes haploïdes de deux à quatre espèces sexuées distinctes tandis que l'ADNmt est différent de celui de ces quatre espèces puisqu'il est le plus semblable à celui d'une autre espèce, l'*A. barbouri*. Afin d'obtenir une meilleure résolution du mode de reproduction chez les *Ambyostoma* unisexués, les auteurs ont séquencé la région de contrôle et la région hypervariable de l'espaceur intergénique du génome mitochondrial chez 48 ambyostomatidés incluant 28 unisexués représentatifs des quatre espèces sexuées et de l'*A. barbouri*. Les unisexués présentent des séquences similaires sur la majorité de l'aire de distribution et forment un groupe sœur compact par rapport à l'*A. barbouri* dont la divergence est estimée à 2,4 à 3,9 millions d'années. Les individus provenant des îles du Lac Érié (Kelleys, Pelee, North Bass) ont un haplotype qui témoigne d'un événement d'isolement. Les auteurs ont examiné les locus microsatellites très variables et ont trouvé que la composition génétique des unisexués était très variable et que les individus unisexués partagaient des allèles en commun avec les individus sexués des mêmes populations. Bien que plusieurs descendants de la même femelle avaient le même génotype aux cinq locus microsatellites, il n'y avait pas d'évidence qu'un génome particulier était hérité de manière constante et clonale au sein d'une population. Le mode de reproduction employé par les *Ambyostoma* unisexués semble unique et les auteurs suggèrent la cleptogenèse comme nouveau mode de reproduction unisexué employé par ces salamandres.

Mots-clés : Ambyostoma unisexués, polyploïdie, espaceur intergénique, boucle D, ADN microsatellite, mode de reproduction, cleptogenèse.

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Fig. 1. Males of the 4 species of *Ambystoma* that might be included in the nuclear genomes of the unisexuals. From left to right, the specimens are *A. jeffersonianum* from Ontario, *A. tigrinum* from Indiana, *A. laterale* from Ontario, and *A. texanum* from Indiana.

Introduction

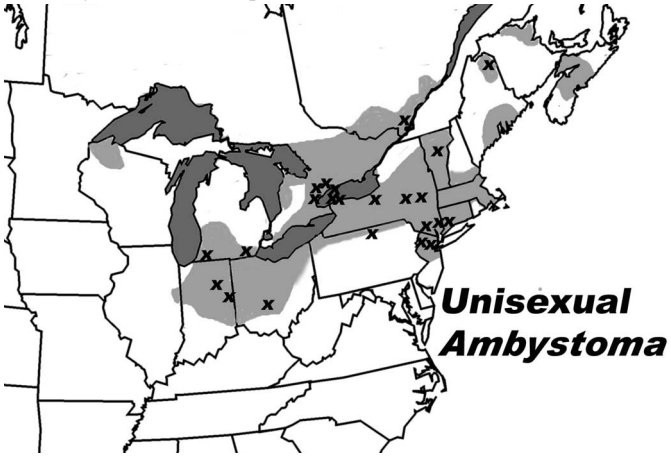
Although sexual reproduction is the common reproductive mode used by metazoans, all-female populations have independently evolved in many diverse lineages. We consider a unisexual lineage to represent an all-female population, but some authors have used other terms (e.g., uniparental; Frost and Hillis 1990). With a few notable exceptions listed by Normark et al. (2003), empirical data have demonstrated that most unisexual lineages are short-term evolutionary phenomena. Rapid extinction of unisexual lineages supports theoretical hypotheses extolling the selective advantages of bisexual reproduction (Williams 1975; Maynard Smith 1978, 1992). The combination of the catholic origination of unisexuality among many metazoan lineages with the short temporal existence of such lineages suggests that unisexuality is a constant but pervasive evolutionary process that might be difficult to appreciate by observing contemporary unisexual lineages. Recent advances in molecular techniques and phylogenetics have improved our understanding of the evolution of many unisexual lineages. A few of the many recent animal studies have examined unisexual crustaceans (Simon et al. 2003), insects (Gómez-Zurita et al. 2006), mollusks (Taylor and Foighil 2000), fish (Mateos and Vrijenhoek 2002), amphibians (Bogart 2003), and reptiles (Fu et al. 2000). Where data are available, the great majority of unisexual lineages are derived from hybridization events (Dawley and Bogart 1989; Judson and Normark 1996), and ploidy elevation is prevalent among unisexual lineages (Otto and Whitton 2000).

Approximately 80 unisexual vertebrates (Alves et al. 2001) have generally been allocated to 1 of 3 reproductive modes: parthenogenesis; gynogenesis; and hybridogenesis (Dawley and Bogart 1989; Avise et al. 1992). Parthenogenesis and gynogenesis are genetically equivalent. Sperm is required to stimulate development of the eggs of gynogens, but is not incorporated, so the offspring are genetically identical to their mothers. Hybridogenesis is hemiclonal (Vrijenhoek et al. 1977); 1 genome is transmitted clonally and vertically but the other genome is removed and replaced de novo each generation. On the basis of observed or expected egg formation through respective mitotic or meiotic processes, there are 2 types of thelytokous reproductive modes among unisexuals: apomictic and automictic (Haccou and Schneider 2004). Unisexuals, especially apomictic unisexuals, are often considered to be clonal asexuals (Janko et al. 2003). Comparative studies of sexuals and unisexuals have improved our general understanding of reproductive processes, but unisexuals are not always asexual, and it is often difficult to categorize unisexuals into 1 specified reproductive mode.

Unisexuals in the North American salamander genus *Ambystoma* have variously been referred to as parthenogenetic (Uzzell 1969; Downs 1978), gynogenetic (Macgregor and Uzzell 1964; Elinson et al. 1992), "hybridogenetic?" (Avise et al. 1992), hybridogenetic (Normark et al. 2003), and both gynogenetic and hybridogenetic (Bogart et al. 1989). They are thelytokous and automictic, and usually have a premeiotic doubling of their chromosomes (Macgregor and Uzzell 1964; Bogart 2003). So far, 22 distinct diploid, triploid, tetraploid, and pentaploid unisexual *Ambystoma* are known to be syntopically associated with 1 or more of 4 morphologically distinctive species (*Ambystoma laterale*, *Ambystoma texanum*, *Ambystoma jeffersonianum*, and *Ambystoma tigrinum*) (Fig. 1); the unisexuals have an extensive range around the Great Lakes region of eastern North America (Fig. 2). The genomic constitution, or genomotype according to Lowcock (1994), of the unisexuals has relied on isozyme electrophoresis, using loci that have allozymes that differ among the 4 species. They are mostly homozygous within each species, and demonstrate heterozygous patterns in unisexual combinations (Bogart et al. 1985, 1987; Bogart and Klemens 1997). More recently, genomic in situ hybridization was used to identify species-specific chromosomal constitution in some unisexuals (Bi and Bogart 2006). Genomic in situ hybridization was also used to document intergenomic recombinations between homoeologous chromosomes in some populations of Ontario unisexual *Ambystoma*.

All data show that the diploid and polyploid unisexual

Fig. 2. Currently known range of unisexual *Ambystoma* in northeastern North America (shaded region), based on Bogart and Klemens (1997), Selander (1994), and unpublished data (J.P. Bogart 2007, unpublished data; J.P. Bogart and M.W. Klemens 2007, unpublished data). X indicates the locations of the unisexuals used in this study. Localities are provided in Table 1.



Ambystoma have contemporary, hybrid nuclear genomes that include at least 1 *A. laterale* haploid chromosome complement. The isozyme data are consistent with multiple recent origins of unisexuals through recurring hybridization and backcrossing, which includes ploidy elevation (Bogart and Licht 1986; Bogart et al. 1985, 1987; Lowcock and Bogart 1989). Assigning maternal ancestry to various unisexuals has focused on the matrilineally inherited mitochondrial genome (Kraus and Miyamoto 1990; Kraus et al. 1991; Hedges et al. 1992; Spolsky et al. 1992; Bogart 2003). Phylogenies, based on mitochondrial data, show that the unisexuals, irrespective of their nuclear genome or ploidy level, all have a similar *mt*DNA genome that is distinctly different from any of the 4 "parental" species and would exclude all of them as candidates for a recent maternal ancestor of the unisexuals. Evolutionary trees, based on *mt*DNA data, provide evidence for an origination of the unisexual *Ambystoma* *mt*DNA lineage before the divergence of extant species for which genomes are nuclear inclusions (~5 million years) (Hedges et al. 1992; Spolsky et al. 1992), so the unisexual *Ambystoma* have been included as a candidate ancient asexual clade of eukaryotes (Normark et al. 2003).

The unexplained paradox in these phylogenetic studies is that, despite this putative antiquity, only minor sequence divergence is observed among the unisexuals, which implies a recent origin. The paradox could be resolved if a recent female progenitor remains unsampled or is extinct (Avise et al. 1992). Indeed, with a larger sampling of species, a new phylogeny (Bogart 2003), based on sequences of a 680 bp segment of the cytochrome *b* and 16s mitochondrial genes, showed that 20 diploid, triploid, and tetraploid unisexuals, representing 9 genomotypes, form a monophyletic clade that is nested within a fifth species (*Ambystoma barbouri*), which, not surprisingly, was not included in previous *mt*DNA studies. *Ambystoma barbouri* was only recently recognized as a distinct and separate species from *A. texanum* (Kraus and Petranka 1989), and has not been shown to be a nuclear genomic component of any unisexual. A hypothetical hybridization between an *A. barbouri* female and an *A. laterale* male, with subsequent genome loss, replacement, augmentation, and recurrent gynogenesis, would logically explain all of the empirical data (Bogart 2003); however, because there is no precedence for such a reproductive system, and because such a system has aspects of both gynogenesis and hybridogenesis, the unisexual *Ambystoma* continue to be considered an unusual hybrid complex with mixed reproductive modes.

We believe that the unisexual *Ambystoma* represent an important and unique reproductive system that expands the possibilities of both asexual and sexual processes. This study examines the evolutionary genetics of unisexual *Ambystoma* and the reproductive mode that is being used by these salamanders, and provides current theoretical expectations for a gynogenetic or hybridogenetic reproductive mode. To confirm previous observations of maternal inheritance and to focus on the temporal genetic relationship of unisexuals to each other, to the 4 possible sperm donors, and to *A. barbouri*, we compared sequences that included the highly variable control region (D-loop) and intergenic spacer region.

Although isozymes have proven to accurately identify genomic constitution in the unisexuals, the loci and allozymes useful for such identification are very conserved. It is not possible to distinguish between gynogenesis and hybridogenesis if the allozymes that could be maintained or changed among offspring are the same within a population and across populations. Isozymes could only be used to document hybridogenesis and gynogenesis in a few offspring, using sperm donor males in artificial crosses, the allozymes of which were male-specific and could be distinguished (Bogart et al. 1989). That study could not refute the possibility that a particular genome, such as the *A. laterale* genome that is present in all unisexuals, is a relictual genome that is transmitted in a linear, clonal, or hybridogenetic fashion. Artificial crosses that produced relatively few viable progeny and that used males from different species or populations (Bogart et al. 1989) might not reflect the system being used in natural populations. Therefore, to address these questions and problems, we examined adults and larvae from natural populations, using highly variable microsatellite DNA loci (Julian et al. 2003). If unisexual lineages are reproducing by gynogenesis, we would expect to find that unisexuals possess the same microsatellite DNA alleles among individuals having the same genomotype within the same pond and between ponds. Hybridogenetic individuals should have 1 common genome within genomotypes and in all individuals within an egg mass.

Materials and methods

Mitochondrial sequences

Primers F-THR and R-651 (Shaffer and McKnight 1996) were used to amplify the entire D-loop, intergenic spacer region, $tRNA^{Pro}$, $tRNA^{Phe}$, and part of $tRNA^{Thr}$. We targeted this region because the same region was sequenced by Shaffer and McKnight (1996) to outline evolution in the *A. tigrinum* complex, and by Niedzwiecki (2005) to resolve a phylogeny of *A. texanum* and *A. barbouri.* McKnight and Shaffer (1997) focused on the intergenic spacer region, the most variable region of the mitochondrial genome in *Ambys-*

toma, to estimate genetic distances between representatives of the sexual species of *Ambystoma*, which includes *A. texanum, A. laterale, A. jeffersonianum*, and *A. tigrinum*. We identified the intergenic spacer region by aligning sequences published by McKnight and Shaffer (1997) with the sequences we obtained from unisexual and sexual individuals.

Total genomic DNA was extracted from muscle or liver tissues, using Promega Wizard Genomic DNA Purification Kits. We chose stored, frozen tissue samples from individuals that had previously been identified by isozymes and for which ploidy was confirmed by karyotype, blood cell analyses, and (or) flow cytometry. Most individuals were the same as those used in previous isozyme studies (Bogart et al. 1985, 1987; Bogart and Klemens 1997), but we also included more recently identified specimens to sample across the distributional range of the unisexuals. We included individuals of the 4 sexual species (*Ambystoma laterale*, *A. jeffersonianum*, *A. texanum*, and *A. tigrinum*) that were found to be sympatric with various unisexual populations. We chose specimens of *A. barbouri* from 4 populations on the basis of previous *mt*DNA data (Bogart 2003; Niedzwiecki 2005): 1 was most similar to the unisexuals (Oldham County, Kentucky) and the other populations were more distantly related. *Ambystoma maculatum* was used as an outgroup (McKnight and Shaffer 1997). Sequences were obtained from 48 individuals. The specimens, localities, and genomotypes are listed in Table 1.

DNA was amplified using standard PCR methods with the annealing temperature optimized at 46 °C. The PCR products were purified using a Qiagen QIAquick PCR Purification Kit, and directly sequenced using Big Dye sequencing protocols (ABI) with an ABI 3730 automatic sequencer. The same primers were used for both PCR and sequencing. Sequences were edited using Sequencher (v. 3.1.1) and aligned using CLUSTALX (Thompson et al. 1994). A maximum parsimony analysis was conducted with 1000 random step additions, using PAUP* Version 4.01b10 (Swofford 2001). A Bayesian analysis was conducted using MrBayes (v. 3.1) (Huelsenbeck and Ronquist 2001), and the GTR model was selected by Modeltest (version 3.06). Four Markov chains were used, and the dataset was run for 4 million generations to allow adequate time for convergence. Trees were sampled every 100 generations, and we used the last 10 000 sampled trees to estimate the consensus tree and the Bayesian posterior probabilities. Sequence divergence was quantified with nucleotide percentage differences among identified haplotypes.

Microsatellite DNA

Egg masses were collected in the spring of 2005 from 4 populations — Backus Woods (B), Deer Creek (D), Sudden Tract (S), and Waterdown Woods (W) — in southern Ontario that were known, from previous collections, to contain unisexuals. The larvae were hatched and raised in the laboratory until they reached a size where tail tips could be excised with no effect on survival. Many of the larvae that were used for microsatellites were also karyotyped and used by Bi and Bogart (2006) in a genomic in situ hybridization study. In the spring of 2006, adult individuals were captured close to or in the breeding pond (S) where egg masses were collected in 2005. Their tail tips were excised and stored in 70% ethanol until DNA extraction. Total genomic DNA was extracted using a Promega Wizard Genomic DNA Purification Kit. We used primers developed for *A. jeffersonianum* (Julian et al. 2003) to amplify 5 microsatellite DNA loci with tetranucleotide repeat motifs. The 5 loci were chosen on the basis of the allelic data provided by Julian et al. (2003). Primers for locus *Aje*D378 only amplify *A. jeffersonianum* alleles. Primers for loci *AjeD*94 and *AjeD*346 amplify multiple alleles in both *A. jeffersonainum* and *A. laterale*, but have allelic size ranges with little overlap between those species. Therefore, microsatellite DNA alleles at these 2 loci can distinguish genomes of both species and the genomotype in unisexuals that include those genomes (Julian et al. 2003). Microsatellite DNA alleles overlap in size range between the 2 species for loci *Aje*D283 and *Aje*D422. These loci, however, are highly variable, which provides additional genotypic information, and assist with ploidy determination. Forward primers for each locus were fluorescently labelled with tetramethyl rhodamine. DNA was amplified with standard PCRs for microsatellite DNA. The annealing temperature was optimized at 57 °C for loci *AjeD*94, *AjeD*346, and *AjeD*422, and was raised to 58 °C for loci *Aje*D283 and *AjeD*378. PCR products were electrophoresed on vertical 6% denaturing polyacrylamide gels alongside a Genescan-350 TAMRA size standard ladder. Gels were scanned with a Hitachi FMBioII imager, and were scored relative to the ladder using Hitachi FMBioII imaging software v. 1.5. Scoring was verified visually to ensure accuracy. PCRs of the same samples were repeated, and the position of the samples on the gel was changed to minimize scoring errors (Selkoe and Toonen 2006). Genotypes of *A. jeffersonianum* and *A. laterale* were compared with those obtained from unisexual individuals within and between populations.

Animals were collected and cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care (2003). The use of specimens was reviewed and approved by a University of Guelph Animal Utilization Protocol (AUP 05R054).

Results

Phylogeny

Topologies of trees from our maximum parsimony and Bayesian analyses were identical in almost all aspects; we present only the latter in Fig. 3. Our phylogenetic hypothesis shows that the unisexuals form a monophyletic clade with 100% bootstrap support and posterior probabilities of 1.00. The unisexuals share a most recent common ancestor with *A. barbouri* individuals from a population in Kentucky. Individuals that were sequenced from unisexual populations in Connecticut, Indiana, Maine, Michigan, New York, Ohio, Ontario, Pennsylvania, and Quebec were all found to have the same haplotype (B), which is correlated to neither the genomotype nor the ploidy. Single nucleotide mutational changes were found in unisexual individuals from Ontario (n = 1), Michigan (n = 1), and New York (n = 1) (haplotypes A, C, D, respectively). Three New Jersey unisexuals had the same haplotype (E), which differed from the main unisexual haplotype (B) by 3 nucleotides. Unisexuals from the Lake Erie Islands, (Kelleys, Pelee, North Bass) share a

Table 1. Locality for specimens used for the intergenic spacer and control region (D-loop) sequences.

No.	Species or genomotype	Haplotype	Locality
34343	*A. barbouri*	A	Kentucky: Oldham County, Sligo
34342	*A. barbouri*	B	Kentucky: Oldham County, Sligo
34341	*A. barbouri*	C	Kentucky: Oldham County, Sligo
34356	*A. barbouri*	D	Kentucky: Anderson/Mercer county line
22765	*A. barbouri*	E	Ohio: Montgomery County, Fossil Creek
34368	*A. barbouri*	F	Kentucky: Livingston County
32617	*A. jeffersonianum*	A	Ontario: Hamilton – Wentworth, near Dundas
29464	*A. jeffersonianum*	B	New York: Sullivan County, Tusten
34552	*A. jeffersonianum*	C	Ohio: Athens County, near Athens
29493	*A. laterale*	A	New Jersey: Morris County, Troy Meadows
35708	*A. laterale*	A	Quebec: Bas St.-Laurent
19376	*A. laterale*	B	Ontario: Essex County, Pelee Island
30324	*A. maculatum*	A	Pennsylvania: Luzerne County, Conyngham Township
30989	*A. maculatum*	B	Ontario: Peel County, Niagara Escarpment, Speyside
34553	*A. texanum*	A	Ohio: Clark County, near Selma
34554	*A. texanum*	A	Ohio: Clark County, near Selma
10638	*A. texanum*	B	Ohio: Ottawa County, Kelleys Island
13130	*A. texanum*	C	Ontario: Essex County, Pelee Island, Stone Road
13105	*A. texanum*	D	Ontario: Essex County, Pelee Island, East side
19237	*A. texanum*	E	Ontario: Essex County, Pelee Island, Mosquito Point
17659	*A. texanum*	F	Ontario: Essex County, Pelee Island, Mosquito Point
10677	*A. tigrinum*	A	Ohio: Ottawa County, Kelleys Island
21921	*A. tigrinum*	A	Ohio: Clark County, near Selma
36115	LT	A	Michigan: Cass County, Decatur Road Pond
30318	LJJ	B	Connecticut: Fairfield County, Danbury
30491	LJJ	B	Indiana: Wabash County, Salmonie River State Forest
30857	LJJ	B	Indiana: Jay County, Bell-Croft Woods Nature Preserve
36983	LJJ	B	Ontario: Hamilton County, Waterdown Escarpment
30494	LLJ	B	Indiana: Jay County, Kantner Memorial Forest
36480	LLJ	B	Michigan: Lenawee County, Adrian College
12955	LLJ	B	Maine: Aroostook County, Connor Township
31687	LLJ	B	New York: Schoharie County, South Gilboa
31281	LLJ	B	Pennsylvannia: McKean County, Eldred Township
22701	LTJ	B	Ohio: Clark County, near Selma
21942	LTTi	B	Ohio: Clark County, near Selma
29575	LJJJ	B	Connecticut: Litchfield County, Washington
35735	LLLJ	B	Quebec: Anse-à-l'Orme
30884	LTJTi	B	Indiana: Wabash County, Leuken's Lake
21986	LTJTi	B	Ohio: Clark County, near Selma
36754	LLJ	C	Ontario: Waterloo County, West of Cambridge
31069	LLLJ	D	New York: Orange County, New Windsor
29494	LJ	E	New Jersey: Warren County, Hardwick Township
29974	LJJ	E	New Jersey: Sussex County, Vernon Twp
29501	LJJ	E	New Jersey: Sussex County, Swartswood State Park
12483	LT	F	Ontario: Essex County, Pelee Island, north Quary
13217	LT	F	Ontario: Essex County, Pelee Island
12478	LLT	F	Ontario: Essex County, Pelee Island
12479	LLT	F	Ontario: Essex County, Pelee Island
29251	LTT	F	Ohio: Ottawa County, North Bass Island
10656	LTTi	F	Ohio: Ottawa County, Kelleys Island

Note: Symbols for genomotypes are as follows: L, *Ambystoma laterale*; J, *A. jeffersonianum*; T, *A. texanum*; Ti, *A. tigrinum*. LTJTi, tetraploid *A. laterale – A. texanum – A. jeffersonianum – A. tigrinum*. Specimen numbers refer to voucher specimens in the collection and records of J.P. Bogart. Haplotypes for each species and the unisexuals are shown in Fig. 3.

haplotype (F), which differs by 5 nucleotides from the main unisexual clade. Sexual individuals that represent the species for which nuclear genomes are included in the unisexuals all formed clades that show distant relationships to the unisexuals. The sequence divergence between *A. barbouri* individuals from Ohio and Kentucky is greater than that observed between the unisexuals and *A. barbouri* from Kentucky (Table 2). Pelee Island *A. laterale* has a haplotype that differs by 5 nucleotides from mainland (Quebec and New Jersey) *A. laterale*, but Kelleys Island *A. tigrinum* and *A. texanum* align closely with mainland populations. Sequences from 4 specimens of Pelee Island *A. texanum* all differ by 1 to 3 nucleotides, but form a monophyletic clade that is sister to the Kelleys Island and mainland *A. texanum* samples. Table 2 summarizes the pairwise distances that were calculated between the major clades in Fig. 3 for the control region and intergenic spacer sequences.

Microsatellite DNA

Ninety-nine *A. jeffersonianum* (JJ) and *A laterale* (LL) microsatellite DNA alleles from 5 polymorphic loci were recovered from 214 larvae hatched from 29 egg masses at 4 different sites (Table 3), and from 42 adults from 1 of those sites (Sudden Tract, Table 4). A summation of the observed frequencies of microsatellite DNA alleles is provided in Table 5. Three egg masses contained only JJ larvae (S12, S13, and S14) (Table 3), and 26 were unisexual egg masses. No egg masses were found for *A. laterale* because that species does not produce distinct egg masses (Petranka 1998).

Most unisexual larvae were triploid and had the same genotypes within egg masses. Masses from Waterdown and Deer Creek, where *A. laterale* has never been found, were triploid *A. laterale*–2 *jeffersonainum* (LJJ) unisexuals, but tetraploid LJJJ larvae were encountered in 3 egg masses (W2, W5, D3). Both *A. laterale* and *A. jeffersonianum* are known to occur together in Backus Woods (unpublished data) and Sudden Tract (J. Feltham 1997, personal communication). Egg masses collected in those populations were diploid *A. laterale–jeffersonainum* (LJ) (S10, S11), triploid *A.* 2 *laterale–jeffersonainum* (LLJ) (B3, S6, S7 to S9), and triploid *A. laterale*–2 *jeffersonianum* (LJJ) (B1, B2, S1 to S5). Only 1 LJJJ tetraploid was found in an egg mass with LJJ in Backus Woods (B2). Sudden Tract tetraploid larvae were LLJJ (S1, S2), LLLJ (S6, S7), and LJJJ (S4). A triploid LLJ larva was found in a Sudden Tract diploid *A. laterale–jeffersonianum* (LJ) egg mass (S10). Although larval genotypes were mostly consistent within unisexual egg masses, only 2 egg masses (D6, D7) had identical genotypes for all individuals at all 5 loci. Each larva in the *A. jeffersonianum* egg masses had a different genotype. Only 2 of the 42 adult individuals from Sudden Tract (LJ 37157, LJ 37159) (Table 4) had the same genotype. Adult unisexuals were diploid LJ (n = 10), triploid LJJ (n = 11), and triploid LLJ (n = 9). All of the *A. jeffersonianum* adults were male (n = 8). Two male and 2 female *A. laterale* were sampled.

Discussion

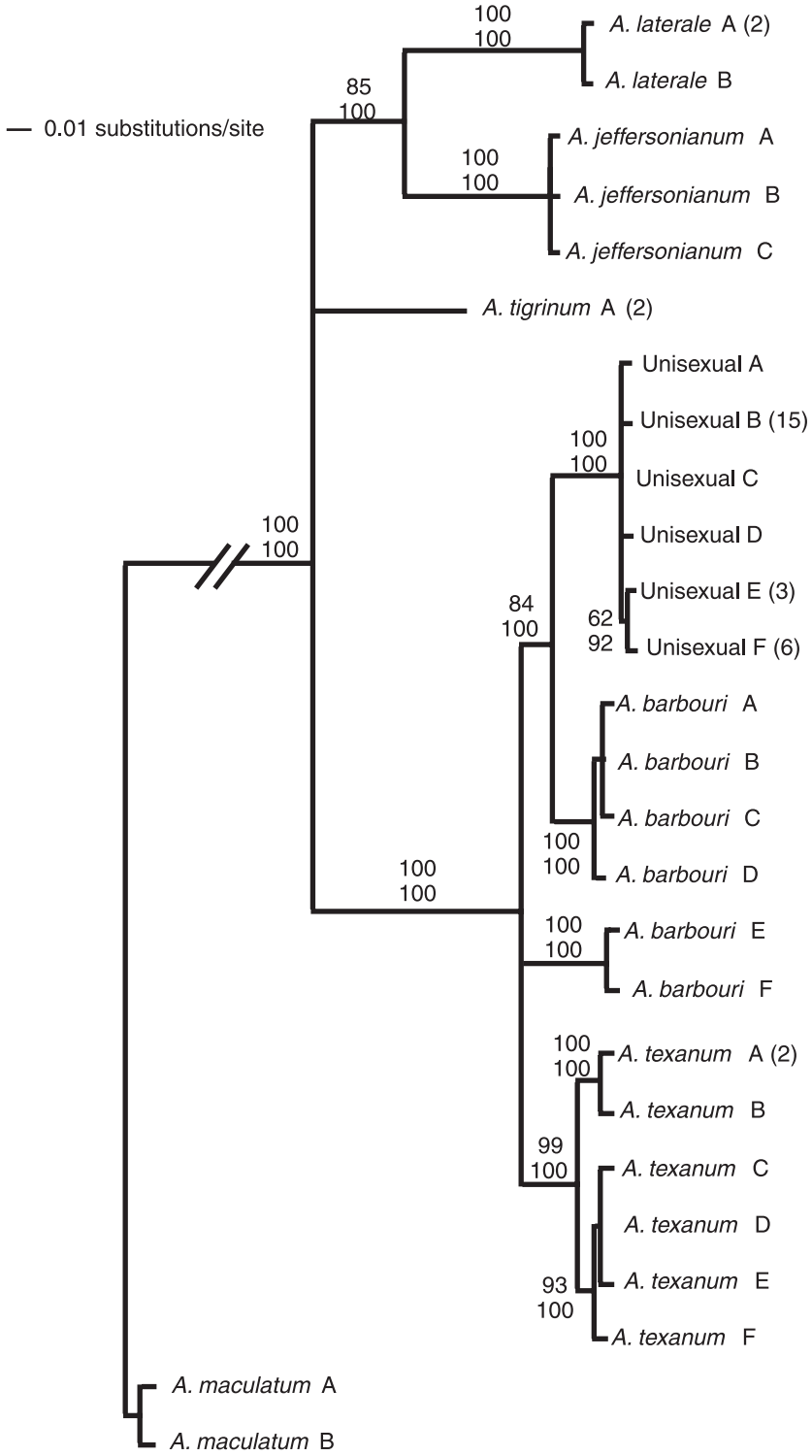
The temporal relationship of the unisexual *Ambystoma* to *A. barbouri*

Our sequence data are consistent with a single origin for unisexual *Ambystoma.* The resulting phylogenetic tree (Fig. 3) clearly shows that the unisexuals, irrespective of genomotype or ploidy, form a monophyletic clade that is a sister group to a western clade (Niedzwiecki 2005) of *A. barbouri* from south of the Ohio and west of the Kentucky Rivers in central Kentucky. The deep divergence between the 2 sister groups suggests an ancient origin of the unisexual lineage. Shaffer and McKnight (1996) calibrated a control region (D-loop) sequence evolution of 1.0% to 1.5% per million years for the *A. tigrinum* complex that was based on the separation of *Ambystoma californiensis* from other tiger salamanders by the beginning of the Sierran uplift about 5 million years ago. Our calculated control region pairwise distance between the main unisexual clade and Kentucky *A. barbouri* was 3.91% (Table 2). Thus, assuming neutrality and equal substitution rates, the unisexuals and Kentucky *A. barbouri* shared a common ancestor 2.4 to 3.9 million years ago. In his comprehensive study of *A. barbouri* and *A. texanum* that included sequences from the same *mt*DNA region used in our study, Niedzwiecki (2005) sampled 23 populations, representing the entire range of *A. barbouri.* None of the haplotypes that he found was more similar to the unisexuals than the Kentucky *A. barbouri* sequenced here, so it is unlikely that a significantly more recent common ancestor exists.

The intergenic spacer has a substitution rate that is about 3 times faster than the usually rapidly evolving control region (McKnight and Shaffer 1997); we also found that the intergenic spacer region had a greater substitution rate than the control region (Table 2), but the difference varied from about the same substitution rate for Ohio and Pelee *A. texanum* (2.02 vs. 2.08) to more than 3 times the rate between *A. barbouri* or *A. texanum* and *A. tigrinum*. The mutation rate between Kentucky *A. barbouri* and the unisexuals was about twice as high (7.51) in the intergenic spacer as in the control region. Revising the earlier estimated age of the unisexual lineage (Hedges et al. 1992; Spolsky et al. 1992) from ~5 to ~3 million years would still include the unisexual *Ambystoma* with other ancient *mt*DNA lineages, and would be the only chordate so designated (Normark et al. 2003).

The unisexuals on the Lake Erie Islands (Pelee, Kelleys, North Bass) all share a haplotype (F), which is slightly differentiated (5 nucleotides) from the mainland unisexuals (haplotype B). *A. laterale* from Pelee Island also has a different haplotype, one that diverges from mainland *A. laterale* by 5 nucleotides. *A. laterale* has never been found on Kelleys Island (King et al. 1996), even though the unisexuals on Kelleys Island have several genomotypes that all include 1 *A. laterale* genome (Bogart et al. 1987). *A. tigrinum* on Kelleys Island shares an identical haplotype to mainland Ohio *A. tigrinum*. The 4 Pelee Island *A. texanum* have slight sequence diversity on the island but form a monophyletic clade that is sister to mainland Ohio and Kelleys Island *A. texanum.* The Lake Erie Islands have only been isolated from the mainland for about 4000 years (Calkin and Feenstra 1985). If the unisexuals were isolated on the islands when the water levels rose in Lake Erie, it is conceivable that the divergent sequences reflect this isolation. This same situation would prevail for *A. laterale* that was isolated on Pelee Island but, apparently, not for *A. texanum* or *A. tigrinum* on Kelleys Island.

Fig. 3. Phylogenetic hypothesis derived from the Bayesian analysis. Taxa are haplotypes. Numbers above the branches are bootstrap proportions from the parsimony analysis (1000 replicates) and the Bayesian posterior probabilities. Numbers in parentheses are the number of sampled individuals that shared the same haplotype.



Bogart (2003) revealed a sister group relationship of *A. barbouri* from Kentucky with populations of unisexual individuals that was based on a phylogenetic hypothesis that used 680 bp, which included fragments of the cytochrome *b* and 16s mitochondrial genes, but he did not estimate a time of origin. Robertson et al. (2006) sequenced a 744 bp portion of the mitochondrial cytochrome *b* gene from many of the same individuals used by Bogart (2003), and proposed that the unisexual *Ambystoma* were very recently derived from a putative hybridization event that took place about 25 000 years ago. This hypothesis was largely based on 1 haplotype of *A. barbouri* that was not used by Bogart

Table 2. The highest pairwise distances (uncorrected p-distance) among the major lineages shown in Fig. 3.

		1	2	3	4	5	6	7	8
1	*A. laterale*	—	21.9	24.07	30.1	29.81	27.29	27.3	27.81
2	*A. jeffersonianum*	6.8	—	23.67	28.15	28.67	26.96	27.33	26.57
3	*A. tigrinum*	7.88	6.52	—	25.97	26.92	26.48	26.05	24.07
4	Unisexuals	11.44	10.32	8.41	—	7.51	11.66	11.24	9.21
5	*A. barbouri* KY	11.46	11.15	8.01	3.91	—	9.58	9.58	10.45
6	*A. texanum* OH	10.76	10.19	8.56	5.12	3.51	—	2.08	10.87
7	*A. texanum* PI	10.07	9.64	7.73	4.44	4.04	2.02	—	10.03
8	*A. barbouri* OH	11.19	10.2	8.88	5.39	4.18	3.51	3.23	—

Note: KY, Kentucky; OH, Ohio; PI, Pelee Island. Numbers above the diagonal are derived from the intergenic spacer sequences and numbers below the diagonal are derived from the control region (D-loop) sequences.

(2003), and was found to have an identical cytochrome *b* sequence to that found in most unisexual individuals.

Our data do not support such a recent origin. We sequenced 1106 bp of the same fragment of the mitochondrial genome that was sequenced and calibrated by Shaffer and McKnight (1996) in their analysis of the *A. tigrinum* complex, and by Niedzwiecki (2005) for his comprehensive phylogeographic study of *A. barbouri* and *A. texanum*. All these sequences included the intergenic spacer region that was also sequenced from representatives of all known bisexual ambystomatid species in North America (McKnight and Shaffer 1997). Our calculated time of divergence (Table 2) is based on the calibration by Shaffer and McKnight (1996), which was also used by Niedzwiecki (2005).

This large discrepancy (25 000 years and ~3 million years) is difficult to understand because, in our analysis, we included the same specimen of *A. barbouri* (JPB 34343; Table 1, Fig. 3) that was found to have an identical cytochrome *b* sequence to unisexual individuals. It is well known that the rate of mutation varies for different genes in the mitochondrial genome, but the cytochrome *b* gene is not highly conserved in other *Ambystoma* (Samuels et al. 2005), so we did not expect so much variation in the mutation rate between these 2 mitochondrial regions. It is possible that some mitochondrial genes in unisexual individuals are under some unknown selective pressure. Even in normally bisexually reproducing organisms, Ballard and Whitlock (2004) caution the use of mitochondrial DNA genes as neutral markers in phylogenetic reconstruction without corroborative data, normally obtained by comparing *mt*DNA and *nuc*DNA genomes. Such comparisons cannot be applied to unisexual individuals that possess recently derived nuclear genomes from different sperm donors.

A hybridization event that initiated a unisexual lineage about 3 million years ago would be a logical consequence of speciation events that are believed to have occurred in the Pliocene for other salamander complexes. Based on genetic distances calculated from allozyme frequencies, Highton (1995) hypothesized that 22 species of the *Plethodon glutinosis* group had a common ancestor in the Pliocene and that 7 species of the *Plethodon cinereus* group shared a common ancestor at this time. *P. cinereus* and *P. glutinosis* rapidly expanded into northern uninhabited glaciated areas only during the last 12 000 years (Highton et al. 1989; Highton 1995). A similar pattern was found by Zamudio and Savage (2003) in a widespread clade of *A. maculatum* in the northeastern United States and Canada. The known range of unisexual populations of *Ambystoma* (Fig. 2) is strikingly similar to that of the *P. glutinosis* complex (Highton et al. 1989: Fig. 3), and the very low sequence diversity that we found in unisexual individuals from populations in distant localities parallels the genetic identity (I = 0.975) that Highton et al. (1989) found between populations of the *P. glutinosis* complex.

Lack of support for a single consistent genome in the unisexual lineage

Isozyme data (Bogart 1982, Lowcock and Bogart 1989, Bogart and Klemens 1997) provided evidence that genomes in unisexuals possess rare allozymes that are also present in sexual individuals within the same populations. Because all unisexuals have virtually the same *mt*DNA, which is distinctly different from any of the 4 possible hybridizing parents, the hypothesis that recurrent hybridization of sexuals produces unisexuals that contain rare alleles must be rejected. Genome replacement, or hybridogenesis, is the most likely explanation, but this phenomenon is difficult to assess based on a few rare allozymes. In addition, because most rare allozymes found in unisexual individuals exist in a heterozygous condition, it is possible that only 1 of 2 homologous genomes in a triploid unisexual can be exchanged, and that the other is somehow preserved. Alleles at highly variable microsatellite loci have a distinctive advantage over much more conservative isozyme loci to investigate nuclear genomes in this salamander complex. They have been used to resolve parentage in *A. maculatum* (Myers and Zamudio 2004), and we found that they can be used to reject a strictly clonal mode of inheritance in unisexual salamanders.

All known unisexuals have at least 1 *A. laterale* genome (Bogart 2003). Therefore, we expected to find a common pattern for *A. laterale* microsatellite DNA alleles that was similar to the pattern observed using isozymes. This pattern should be most easily observed among unisexuals in populations where *A. laterale* does not exist, such as Waterdown Woods and Deer Creek, because if hybridogenesis was the reproductive mode used, all hemiclones should include the same *A. laterale* genome. *A. laterale* microsatellite DNA alleles are most easily identified using primers for *Aje*D94 and *Aje*D346 (Julian et al. 2003). From Table 3, unisexual larvae from Waterdown egg masses had 2 *A. laterale* alleles (150,

Table 3. Genotypes found at 5 microsatellite loci in *A. jeffersonianum* and unisexual larvae from 26 egg masses.

		Microsatellite locus				
Egg mass	Larva genomotype (*n*)	*AjeD*94	*AjeD*283	*AjeD*346	*AjeD*378	*AjeD*422
W1	LJJ (3)	**150**/214/226	146/154/158	164/172/**276**	260/292	244/260/300
W2	LJJ (2)	**154**/190/210	138/142/150	168/172/**300**	260/268	236/248
	LJJJ (2)	**154**/190/198*/210	138/142/150	168/172/192*/**300**	260/268	236/248
W3	LJJ (8)	**154**/190/210	138/142/150	168/172/**296**	256/268	232/248
W4	LJJ (11)	**150**/206/230	146/154/158	164/172/**280**	256/284	244/260/300
W5	LJJ (3)	**150**/210/234	146/154/158	164/172/**276**	264/288	244/260/296
	LJJJ (1)	**150**/198*/210/234	146/154/158	164/172/176*/**276**	264/288	244/248*/260/296
	LJJJ (1)	**150**/194*/210/234	146/154/158	164/172/176*/**276**	264/288	244/248*/260/296
	LJJJ (1)	**150**/198*/210/234	146/154/158	164/168*/172/**276**	232*/264/288	244/248*/260/296
	LJJJ (1)	**150**/194*/210/234	146/154/158	164/168*/172/**276**	264/268*/288	244/248*/260/296
D1	LJJ (6)	**150**/214/230	146/158	164/176/**276**	268/280	248/256/308
D2	LJJ (4)	**150**/202/214	146/158/162	180/196/**324**	232/240	252/260/292
D3	LJJ (5)	**150**/206/210	146/158/162	180/192/**312**	232/240	252/260/292
	LJJJ (1)	**150**/190*/206/210	146/158/162	180/188*/192/**312**	232/240/244*	252/260/292
D4	LJJ (1)	**146**/194/202	146/154	176/184/**264**	232/272	240/244/248
D5	LJJ (7)	**142**/194/202	146/154	176/184/**264**	232/272	244/248
D6	LJJ (5)	**150**/206/210	146/158/162	184/196/**324**	232/240	252/260/292
D7	LJJ (8)	**150**/206/210	146/158/162	184/196/**324**	232/240	252/260/292
B1	LJJ (11)	**150**/202/206	146/158/162	180/188/**324**	232/240	256/264/292
B2	LJJ (14)	190/202/250	146/166	168/180/**280**	232	212/252
	LJJJ (1)	190/202/210*/250	146/150*/166	168/180/188*/**280**	232	212/252
B3	LLJ (4)	**150**/190	154/166	188/**256/312**	252	224/252
S1	LJJ (5)	**146**/174/238	134/146/158	168/176/**288**	232/252	252/260/300
	LJJ (1)	**146**/174/238	134/146/158	168/176/**288**	232/252	248/252
	LLJJ (1)	**142***/**146**/174/238	134/146/154*/158	168/176/**268***/**288**	232/252	228*/248/252
S2	LJJ (4)	**142**/174/238	134/146/158	168/176/**288**	232/252	252/260/300
	LLJJ (2)	**142/146***/174/238	134/146/154*/158	168/176/**268***/**288**	232/252	240*/252/260/300
	LLJJ (1)	**142/146***/174/238	134/146/154*/158	168/176/**288**	232/252	228*/252/260/300
S3	LJJ (18)	178/186/242	146/158	176/**276/324**	228/260	212/252
S4	LJJ (3)	**142**/194/206	146/154/158	172/184/**316**	232	244/252
	LJJJ (3)	**142**/194/206	146/150*/154/158	172/180*/184/**316**	232	244/252
S5	LJJ (4)	**146**/194/226	146/150	168/176/200	232/268	248/256/292
S6	LLJ (6)	**142**/186/206	146/154/158	184/**268/308**	228	224/244
	LLJ (1)	**146**/186/206	142/150/154	176/**268/320**	228	204/232/256
	LLJ (2)	**146**/206	142/150	172/**268/272**	228	204/224/256
	LLLJ (1)	**142/146***/186/206	146/150*/154/158	184/**268/308/312***	228	224/228/244
S7	LLJ (2)	**142**/178/186	150/158/166	176/**256/272**	228	212/224/244
	LLLJ (3)	**142**/178/186	150/158/166	176/**256/272/280***	228	212/224/244
	LLLJ (1)	**142/146***/178/186	150/158/166	176/**256/272/280***	228	212/224/244/248*
	LLLJ (1)	**142/146***/178/186	150/158/166	176/**256**	228	212/224/228*/244
S8	LLJ (10)	**142**/178/198	146/158	176/**272/312**	228	220/244
	LLJ (1)	**142**/178/198	146/158	176/**272/312**	228	244
S9	LLJ (5)	178/182/198	146/154	188/**268/272**	260	236/244
S10	LJ (8)	**142**/194	154/162	172/**304**	252	220/244
	LLJ (2)	**142**/186*/194	154/158*/162	172/**260***/**304**	252	220/236*/244
S11	LJ (2)	**142**/182	158/166	176/**256**	232	212/244
S12	JJ (1)	198/206	146/154	176/180	228/232	244/248
	JJ (1)	202/210	146/154	176/180	228/232	244/248
	JJ (1)	206/210	146/154	176/180	248/272	244/248
	JJ (1)	202/210	146/154	176/188	232/248	244/248
	JJ (1)	202/210	146/150	176/180	232/248	244/248
	JJ (1)	198/202	146/154	176/180	232/248	244/248
	JJ (1)	202/210	146/150	176/188	228/272	244/248

Table 3 (*concluded*).

Egg mass	Larva genomotype (*n*)	Microsatellite locus *AjeD*94	*AjeD*283	*AjeD*346	*AjeD*378	*AjeD*422
S13	JJ (1)	186/210	146/150	176/180	248/264	248/256
	JJ (1)	206/214	138/150	176/180	248/264	260/260
	JJ (1)	186/210	138/146	172/176	256/260	256/260
	JJ (1)	186/206	138/146	176/180	248/264	256/260
	JJ (1)	186/206	146/150	176/180	256/260	248/260
	JJ (1)	186/206	138/150	172/176	248/264	248/256
	JJ (1)	206/214	138/146	172/176	260/264	256/260
	JJ (1)	186/210	146/146	172/176	260/264	256/260
	JJ (1)	210/214	146/146	176/180	248/264	256/260
	JJ (1)	186/206	138/150	172/176	256/260	248/256
S14	JJ (1)	190/206	142/146	172/192	252/256	240/244
	JJ (1)	206/206	142/142	172/180	252/256	240/244
	JJ (1)	190/206	146/146	172/192	252/256	240/244
	JJ (1)	206/206	142/142	176/192	252/256	240/244
	JJ (1)	190/206	142/142	176/180	252/256	240/244
	JJ (1)	206/214	142/146	176/192	252/256	240/240
	JJ (1)	206/214	142/146	172/180	252/256	240/244
	JJ (1)	206/214	142/146	172/180	252/256	244/244
	JJ (1)	206/214	142/142	172/180	252/256	240/244
	JJ (1)	206/206	142/146	172/180	252/256	244/244

Note: Egg masses were collected at the following locations: Waterdown Woods, Hamilton County, 4 km south of Waterdown (W); Deer Creek Valley, Haldimand County (D); Sudden Tract, Waterloo County, 6 km west of Cambridge (S); Backus Woods, Haldimand County, South Walsingham Sand Ridges (B), southern Ontario. Genomotypes include *A. laterale* (L) and *A. jeffersonianum* (J). The number of larvae within an egg mass that have the same genomotype is indicated in parentheses. Alleles known to be from *A. laterale* are shown in bold. Ploidy is determined by the greatest number of alleles at any locus (e.g., LJJJ would be a tetraploid larva with 1 *A. laterale* genome and 3 *A. jeffersonianum* genomes).

*Additional alleles that resulted in ploidy elevation.

154) at locus *Aje*D94, and Deer Creek larvae had 3 (142, 146, 150). Allele 154 at locus *Aje*D94 was only found in the Waterdown population, but the other alleles were also found in Sudden Tract. Four *A. laterale* alleles (276, 280, 296, 300) were recovered from Waterdown LJJ unisexual larvae at locus *Aje*D346. Larvae from 1 Deer Creek LJJ egg mass (D-1 in Table 3) had allele 276, but 3 different *A. laterale* alleles (264, 312, 324) were found in the other LJJ egg masses. Clearly, there is not a single *A. laterale* genome that is shared by all individuals, even in populations where *A. jeffersonianum* males are the only sperm donors.

In Sudden Tract, where *A. laterale* is found, unisexuals had 19 different *A. laterale* alleles at locus *Aje*D346 (Table 5); this probably reflects the much larger sample size obtained from that population. Only 2 of 38 *A. jeffersonianum* alleles (212 at locus *Aje*D 346 and 244 at locus *Aje*D378) that were found in *A. jeffersonianum* were not also found in the unisexuals (Table 5), and all 17 microsatellite DNA alleles recovered from the 4 *A. laterale* individuals were shared with the unisexuals. There was no particular genomotype that was obviously different with respect to "private" alleles in any population, and this is particularly evident among the Sudden Tract unisexuals where LJ, LLJ, and LJJ coexist. Some alleles might be confined to a particular population (Table 5). Allele 154 at locus *Aje*D94, and alleles 284, 288, 292 at locus *Aje*D378 were only found in Waterdown. Allele 280 at locus *Aje*D378 was only found in Deer Creek. We cannot dismiss sampling error as a possible reason that these alleles were not found in the other populations. For example, *A. laterale* allele 146 at locus *Aje*D94 was not recovered from the 4 adult *A. laterale* sampled at Sudden Tract, but *A. laterale* possessing that allele must be present in that population because it appears in tetraploids from egg masses S2 and S7 (Table 3) as a male *A. laterale*–derived additional allele in the 3*n* to 4*n* ploidy elevation observed in those egg mass. That same allele is present in adult LJ, LLJ, and LJJ individuals in Sudden Tract.

Incidence and implications of ploidy alterations

The additional alleles in ploidy elevated larvae (Table 3) were not included in the frequency analysis (Table 5) because, for frequency comparisons, each egg mass was treated as a single individual that was presumed to be the female genomotype. This allowed the egg masses to be compared with adults in the populations. Most alleles assigned to a ploidy elevation event (* in Table 3) were also found in the adult analysis (Table 4). Ploidy elevation is known to occur in some offspring of individual unisexual females, especially at elevated temperatures (Bogart et al. 1989), but finding LJJ triploids and LLJJ tetraploids in egg masses S1 and S2 (Table 3) is important new information because LLJJ is a very rare genomotype (Bogart and Klemens 1997); this demonstrates that *A. laterale* is an acceptable sperm donor for LJJ unisexuals, even in a pond where *A. jeffersonianum* exists.

Ploidy reduction is also a possible explanation for mixed ploidy in the same egg mass; although the female that laid the eggs is unknown, at some microsatellite loci, the tetraploid progeny have different genotypes (e.g., S1, S2, S7), meaning a putative sperm donor must have been heterozy-

Table 4. Genotypes found at 5 microsatellite loci in unisexual, *A. laterale*, and *A. jeffersonianum* breeding adults from Sudden Tract in southern Ontario.

	Microsatellite locus				
Adult salamander (J.P.B. No.) and genomotype	*AjeD*94	*AjeD*283	*AjeD*346	*AjeD*378	*AjeD*422
♀ (30400) LJJ	**142/**194	146/154/158	172/184/**312**	228/252	244/248
♀ (37150) LJJ	**142**/194/206	146/154/158	172/184/**312**	236/252	244
♀ (37154) LJJ	**146**/174/242	130/146/158	168/176/**280**	236/256	252/256/296
♀ (37162) LJJ	**146/**198/226	142/146/150	168/176/200	232/268	252/260/296
♀ (37163) LJJ	**146**/182/194	130/146/158	168/176/**268**	232/256	236/256
♀ (37164) LJJ	**146**/182/230	130/146/154	168/176/**296**	232/260	252/256/304
♀ (37438) LJJ	**146/**198	146/154/158	172/184/**308**	232/252	244
♀ (37439) LJJ	**150/**178/246	134/146//158	168/176/**284**	232/252	248/252/300
♀ (37443) LJJ	**150/**198/234	146/150	168/176/196	232/268	248/260/292
♀ (37444) LJJ	**150/**178/242	134/146/158	168/176/**284**	232/248	248/252
♀ (37447) LJJ	**150/**182/190	142/150/154	172/**264**	232	204/232/252
♀ (30401) LLJ	**142/146/**190	142/158	176/**260**	228	204/224/260
♀ (30402) LLJ	178/186	146/154	184/**280**	228	224/248/264
♀ (37146) LLJ	**142**/186/206	146/154/158	184/**312/320**	236	248/252
♀ (37147) LLJ	**142**/186/206	NA	184/**312/320**	236	NA
♀ (37149) LLJ	186/206	146/154/158	184/**312/320**	236	228/248/252
♀ (37155) LLJ	**142**/186	146/162	184/**312/320**	236	216/228/248
♀ (37160) LLJ	**146**/186	146/154/158	184/**268/308**	236	240/248/256
♀ (37445) LLJ	**150**/190/218	154/158	176/**256/328**	252	232/248
♀ (37446) LLJ	**146**/190	146/150/158	184/**300/312**	232	244/252
♀ (37144) LJ	**142**/178	158/166	176/**256**	236	212/244
♀ (37148) LJ	**142**/178	154/166	176/**256**	236	212/244
♀ (37156) LJ	**146**/178	158/166	176/**256**	236	216/248
♀ (37157) LJ	**142**/194	154/162	172/**308**	256	224/248
♀ (37158) LJ	186/206	146/158	184/**308**	236	248/256
♀ (37159) LJ	**142**/194	154/162	172/**308**	256	224/248
♀ (37173) LJ	178/206	142/158	172/**308**	252	204/252
♀ (37440) LJ	**146/**182	158/166	176/**252**	232	244
♀ (37441) LJ	190	146/158	184/**320**	232	244/252
♀ (37442) LJ	**146**/186	158/166	176/**252**	232	212/244
♀ (33422) LL	**142/178**	**146/158**	NA	*	**220/228**
♀ (37145) LL	**142/186**	**154/154**	**272/312**	*	**232/252**
♂ (37151) LL	**142/186**	**150/158**	**320/320**	*	**228/232**
♂ (37172) LL	**182/186**	**150/154**	**268/272**	*	**224/228**
♂ (37152) JJ	210/214	142/142	176/176	244/256	248/252
♂ (37165) JJ	206/214	146/146	168/176	244/248	240/248
♂ (37166) JJ	202/206	134/146	176/184	236/248	244/244
♂ (37167) JJ	198/206	146/154	176/180	232/252	244/248
♂ (37168) JJ	198/210	146/150	188/188	248/268	244/244
♂ (37169) JJ	206/214	146/146	176/188	248/264	244/248
♂ (37170) JJ	198/214	146/146	168/172	248/248	244/248
♂ (37171) JJ	190/210	146/150	184/212	248/260	244/248

Note: NA, no amplification of alleles for the individual at this locus. Genomotypes include *A. laterale* (L) and *A. jeffersonianum* (J) genomes. The catalogue numbers for each specimen refer to voucher specimens in the collection of J.P. Bogart (J.P.B.). Most vouchers are tail-tip samples and (or) extracted DNA. Alleles known to be from *A. laterale* are shown in bold. Ploidy is determined by the number of alleles at a locus (e.g., LJJ would be a triploid individual with 1 *A. laterale* and 2 *A. jeffersonianum* genomes).

*Primers for locus *AjeD378* only amplifies *A. jeffersonianum* alleles.

gous. Ploidy reduction is, however, a possible mechanism to explain the presence of the relatively rare diploid LJ unisexuals across the range of the unisexuals. Only 84 LJ individuals were found in 1002 specimens sampled by Bogart and Klemens (1997). Based on lampbrush bivalents (Bogart 2003), unisexual females mostly produce unreduced eggs, but eggs of reduced ploidy are also produced. The fate and viability of such eggs is unknown. Finding 10 adult LJ at Sudden Tract in about equal numbers to LLJ and LJJ (Table 4) is very unusual. Eight of 10 larvae from egg mass S10 are diploid LJ unisexuals; the triploids are LLJ. If the female was triploid LLJ, the same L genome with allele

Table 5. Frequency of microsatellite alleles found in unisexual larvae from Waterdown (W), Deer Creek (D), Backus Woods (B), and Sudden Tract (S) (Table 3).

(*a*) Locus *AjeD*94.

	W–LJJ	D–LJJ	B–LLJ	B–LJJ	S–LJ	S–LLJ	S–LJJ	S–LL	S–JJ
Allele	(5)	(7)	(1)	(2)	(12)	(13)	(16)	(4)	(14)
142		0.050			0.250	0.206	0.087	0.375	
146		0.050			0.125	0.088	0.152		
150	0.200	0.250	0.500	0.167		0.029	0.087		
154	0.133								
174							0.065		
178					0.167	0.118	0.065	0.125	
182					0.083	0.029	0.065	0.125	
186					0.083	0.235	0.022	0.375	0.036
190	0.133		0.500	0.167	0.083	0.088	0.022		0.071
194		0.100			0.125		0.109		
198						0.059	0.065		0.143
202		0.150		0.333					0.071
206	0.067	0.150		0.167	0.083	0.118	0.043		0.286
210	0.200	0.150							0.178
214	0.067	0.100							0.214
218						0.029			
226	0.067						0.043		
230	0.067	0.050					0.022		
234	0.067						0.022		
238							0.043		
242							0.065		
246							0.022		
250				0.167					
Total alleles 23	9	9	2	5	8	10	17	4	7

(*b*) Locus *AjeD*283.

	W–LJJ	D–LJJ	B–LLJ	B–LJJ	S–LJ	S–LLJ	S–LJJ	S–LL	S–JJ
Allele	(5)	(7)	(1)	(2)	(12)	(12)	(16)	(4)	(14)
130							0.067		
134							0.089		0.036
138	0.133								0.036
142	0.133				0.042	0.033	0.044		0.143
146	0.200	0.389		0.400	0.083	0.300	0.333	0.143	0.571
150	0.133					0.067	0.089	0.286	0.143
154	0.200	0.111	0.500		0.167	0.233	0.133	0.286	0.036
158	0.200	0.278		0.200	0.333	0.300	0.244	0.286	
162		0.222		0.200	0.125	0.033			
166			0.500	0.200	0.250	0.033			
Total alleles 10	6	4	2	4	6	7	7	4	6

(*c*) Locus *AjeD*346.

	W–LJJ	D–LJJ	B–LLJ	B–LJJ	S–LJ	S–LLJ	S–LJJ	S–LL	S–JJ
Allele	(5)	(7)	(1)	(2)	(12)	(13)	(16)	(3)	(14)
164	0.200	0.048							
168	0.133			0.167			0.213		0.071
172	0.333				0.167		0.106		0.107
176		0.143			0.250	0.108	0.234		0.393
180		0.095		0.333					0.143
184		0.190			0.083	0.216	0.085		0.071
188			0.333	0.167		0.027			0.143
192		0.048							0.036
196		0.143					0.021		
200							0.042		

Table 5 (*continued*).

(*c*) Locus *AjeD*346.

	W–LJJ	D–LJJ	B–LLJ	B–LJJ	S–LJ	S–LLJ	S–LJJ	S–LL	S–JJ
Allele	(5)	(7)	(1)	(2)	(12)	(13)	(16)	(3)	(14)
212									0.036
252					0.083				
256			0.333		0.167	0.054			
260						0.027			
264		0.095					0.021		
268						0.081	0.021	0.167	
272						0.081		0.333	
276	0.133	0.048					0.021		
280	0.067			0.167		0.027	0.021		
284							0.042		
288							0.042		
296	0.067						0.021		
300	0.067					0.027			
304					0.042				
308					0.167	0.027	0.021		
312		0.048	0.333			0.162	0.042	0.167	
316							0.021		
320					0.042	0.135		0.333	
324		0.143		0.167			0.021		
328						0.027			
Total alleles 30	7	10	3	5	8	13	17	4	8

(*d*) Locus *AjeD*378.

	W–LJJ	D–LJJ	B–LLJ	B–LJJ	S–LJ	S–LLJ	S–LJJ	S–LL	S–JJ
Allele	(5)	(7)	(1)	(2)	(12)	(13)	(16)	(4)	(14)
228						0.385	0.067		0.036
232		0.428		0.667	0.333	0.077	0.400		0.071
236					0.333	0.385	0.067		0.036
240		0.286		0.333					
244									0.071
248							0.033		0.321
252			1.00		0.167	0.077	0.200		0.107
256	0.200				0.167		0.067		0.143
260	0.200					0.077	0.067		0.071
264	0.100								0.071
268	0.200	0.071					0.100		0.036
272		0.143							0.036
280		0.071							
284	0.100								
288	0.100								
292	0.100								
Total alleles 16	7	5	1	2	4	5	8	0	11

(*e*) Locus *AjeD*422.

	W–LJJ	D–LJJ	B–LLJ	B–LJJ	S–LJ	S–LLJ	S–LJJ	S–LL	S–JJ
Allele	(5)	(7)	(1)	(2)	(12)	(12)	(16)	(4)	(14)
204					0.043	0.033	0.026		
212				0.200	0.174	0.033	0.026		
216					0.043	0.033			
220					0.043	0.033		0.125	
224			0.500		0.087	0.133		0.125	
228						0.067		0.375	
232	0.077					0.033	0.026	0.250	
236	0.077					0.033	0.026		

Table 5 (*concluded*).

(*e*) Locus *Aje*D422.

	W–LJJ	D–LJJ	B–LLJ	B–LJJ	S–LJ	S–LLJ	S–LJJ	S–LL	S–JJ
Allele	(5)	(7)	(1)	(2)	(12)	(12)	(16)	(4)	(14)
240		0.050				0.033			0.107
244	0.231	0.100			0.304	0.167	0.102		0.428
248	0.154	0.150			0.174	0.200	0.128		0.321
252		0.200	0.500	0.200	0.087	0.100	0.256	0.125	0.036
256		0.050		0.200	0.043	0.033	0.102		0.036
260	0.231	0.200				0.033	0.102		0.071
264				0.200		0.033			
292		0.200		0.200			0.051		
296	0.077						0.051		
300	0.154						0.077		
304							0.026		
308		0.050							
Total alleles 20	7	8	2	5	9	15	13	5	6

Note: Microsatellite alleles from adult Sudden Tract unisexuals, *A. jeffersonianum* (JJ), and *A. laterale* (LL) are included (Table 4). *Ambystoma laterale* has not been found in the Waterdown or Deer Creek populations. Each unisexual egg mass is counted as 1 individual, and *A. jeffersonianum* egg masses are counted as 2 individuals. Known *A. laterale* alleles are bold.

186 for locus *Aje*D94, allele 158 for locus *Aje*D283, allele 260 for locus *Aje*D346, and allele 236 for locus *Aje*D422 would have had to have been lost to explain the genotypes of the 8 diploid larvae. Because of the higher frequency of LJ progeny, we assume that the female was diploid LJ, and the triploid larvae must have obtained those alleles from an *A. laterale* male in the population.

Egg mass S6 is especially interesting, because offspring had different genotypes that could demonstrate genome swapping. If so, the female LLJ produced some diploid LJ eggs that lost 1 *A. laterale* genome and was replaced by another from an *A. laterale* sperm donor to restore the triploid state. Either alleles 142 or 146 for locus *Aje*D94 are found in different individual progeny (Table 3, Fig. 4). It is possible that the egg mass contained eggs from 2 females; however, 1 offspring was a tetraploid that possessed both alleles 142 and 146 for locus *Aje*D94, so the sperm donor for that female must have provided the extra allele. On the basis of the genotype frequency of the progeny in this egg mass, we assume that the female had the common genomotype, so the male would have contributed allele 146 for locus *Aje*D94.

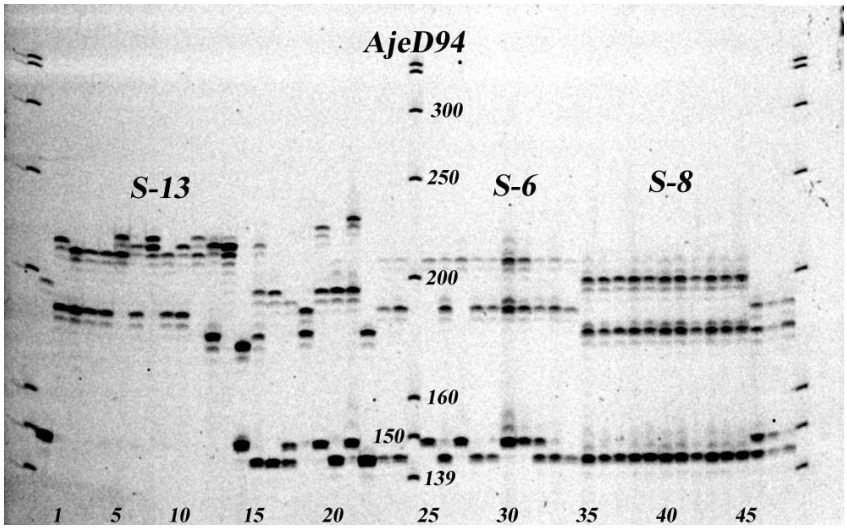
Lack of support for a clonal mode of reproduction

One common genotype for each genomotype would be consistent with a gynogenetic mode of reproduction. Other than ploidy elevation events, the egg mass data demonstrated that most offspring within an egg mass had the same genomotype, but only 2 egg masses had the same microsatellite alleles for all loci (D-6 and D-7) (Table 3), and even this could be an artefact. Based on the number of eggs a female can lay (~300) and the number of eggs found in unisexual egg masses (~80), female unisexuals lay more than 1 egg mass. We tried to sample egg masses in different areas of the ponds to avoid sampling egg masses from the same female, but it is possible that egg masses D-6 and D-7 were laid by the same female. In Sudden Tract, where both egg masses and adult unisexuals were sampled, no egg mass had the same genotype as a sampled adult for all microsatellite DNA loci, but the alleles at some loci were the same (e.g., S-1 and S-2). Only 2 of 30 unisexuals adults (LJ 37157 and LJ 37159) (Table 4) had the same genotype. We were surprised to find the same microsatellite DNA alleles in 3 genomotypes (LJ, LLJ, LJJ) among the sampled adults at Sudden Tract (Table 4), and this observation would certainly be difficult to explain if LLJ and LJJ are independent clonal lineages (Uzzell and Goldblatt 1967). Microsatellite DNA allele frequencies (Table 5) show that most alleles are shared between sexual and unisexual individuals in the populations we sampled.

Our data revealed some other unexpected results that could be related to microsatellite DNA behaviour and evolution. Four different tetraploid microsatellite DNA genotypes were found in offspring from egg mass W-5, and we assume that the female was a triploid LJJ. The male sperm donor must have been *A. jeffersonianum* that was heterozygous 194/198 for locus *Aje*D94, heterozygous 168/176 for locus *Aje*D346, and was possibly homozygous for allele 248 at locus *Aje*D422; however, primers used for locus *Aje*D378, which only amplifies *A. jeffersonianum* microsatellite alleles, had genotypes that suggested that the male was heterozygous 232/268 for locus *Aje*D378. Neither of those alleles was found in the other 2 tetraploids from that egg mass. There are other examples of unexpected allele loss or a microsatellite DNA mutation in some progeny of the egg masses (S-1, S-2, S-6, S-8).

Many of the larvae that were used for microsatellite DNA analyses in our study were also used in a karyotypic study (Bi and Bogart 2006). Chromosome counts confirmed ploidy level, which was also determined by the possession of 4 microsatellite DNA alleles at a locus. Florescent chromosome probes also confirmed the genomotypes. Diploid, triploid, and tetraploid karyotypes were found among the larvae. No mosaic or anueploidy individuals were encountered. Large intergenomic exchanges were documented to occur between homeologous chromosomes in larvae from other populations. None of the larvae examined from Sudden Tract or

Fig. 4. Electropherogram comparing 48 unisexual and bisexual individuals for microsatellite DNA alleles found at the locus *Aje*D94. The ladders at both ends and in the middle of the gel were used as calibration points by FMBIO software to calculate allele product size. Molecular bp lengths are only shown for the middle ladder. Primers for this locus amplify both *A. jeffersonianum* and *A. laterale* tetranucleotide repeat microsatellite DNA alleles. Most alleles are smaller in *A. laterale* (<200 bp) than in *A. jeffersonianum* (>170 bp), but alleles in both genomes overlap from 170 to 200 bp. Progeny from 3 egg masses from Sudden Tract are compared. *A. jeffersonianum* egg mass S-13 (lanes 2 to 11). S-6 (lanes 23 to 34) and S-8 (lanes 35 to 45) are the progeny of 2 different LLJ egg masses. Tetraploids with 4 alleles are LJJJ (lane 15), LLLJ (lane 32) and LLJJ (lanes 46, 47). Lane 22 is the only *A. laterale* sample on this gel. Other samples include LJ (lane 1), JJ (lane 12, 13), LJJ (lanes 14, 16, 19, 20, 21, 48), and LLJ (lanes 17, and 18).



Backus Woods had such exchanges. Smaller exchanges, which might have involved microsatellite loci or flanking regions, could easily escape detection using genomic in situ hybridization techniques, so it is possible that our unexpected microsatellite observations are somehow related to chromosomal mutations.

A much larger sample of progeny from genetically known females from many populations will be necessary to appreciate and quantify the possible genomic interactions that can occur within the unisexuals, especially those that incorporate *A. texanum* and (or) *A. tigrinum* genomes, but we were surprised to find such microsatellite DNA diversity in our initial investigation. It is evident that egg masses within a pond do not have identical microsatellite DNA alleles for the loci we examined and, based on the additional alleles incorporated from *A. laterale* or *A. jeffersonianum* males found in ploidy-elevated tetraploids and among the *A. jeffersonianum* larvae, unisexual individuals have alleles that are also present in sexual individuals within each population. Additional alleles found in unisexual individuals that were not found in the sexual individuals are possibly explained by an incomplete sampling of alleles among the sexual individuals. We cannot rule out dispersal and immigration of unisexual females as a source of new alleles among the unisexuals in a pond, but we also cannot support the hypothesis of a common "clonal" unisexual genotype within or between populations. Isozyme data show that, when only 1 sexual species is found in sympatric association with a triploid unisexual, the unisexual triploid has 2 genomes of that sexual species (Bogart and Klemens 1997). Our microsatellite DNA data confirm that finding in Waterdown and Deer Creek, where *A. jeffersonianum* is the only sexual sperm donor in those populations. Backus Woods and Sudden Tract are unusual populations, in that they have both *A. laterale* and *A. jeffersonianum*. In such populations, it is possible for an *A. jeffersonianum* genome to be replaced with an *A. laterale* genome, or that an *A.* 2 *jeffersonianum–laterale* (LJJ) unisexual could produce *A. jeffersonianum*–2 *laterale* (LLJ) larvae. Our finding of LLJJ and LJJ larvae in the same egg mass (S-1 and S-2) provides evidence that *A. laterale* can be used as a sperm donor for LJJ unisexuals, and the switch from an LJJ population to an LLJ population could possibly occur in a relatively short time if *A. jeffersonianum* were to be displaced by *A. laterale*.

Reproductive mode

Our data show that the unisexual *Ambystoma* are neither asexual nor parthenogenetic. If unisexual *Ambystoma* lineages were perpetuated and maintained by parthenogenesis, with (gynogenesis) or without sperm activation, we would expect to find the same microsatellite DNA alleles among individuals in several egg masses within a breeding pond and among unisexual individuals from different ponds. Only 2 of 26 unisexual egg masses and only 2 of 30 unisexual adults from the same population had the same microsatellite genotypes. As well, the same microsatellite DNA alleles found in sympatric *A. jeffersonianum* and *A. laterale* were also found in the unisexuals. If the unisexuals were hybridogenetic, we would expect to find a common genome (*laterale* or *jeffersonianum*) transmitted in a clonal fashion. On the basis of the microsatellite DNA alleles, evidence for a common genome is lacking. There are fewer *A. laterale* alleles among the individuals from egg masses in ponds where *A. laterale* has not been found; however, different *A. laterale* alleles were found among offspring from different egg masses even in those populations. It is surprising that the previous isozyme data did not reveal more genomic variation.

Our microsatellite DNA data have concentrated on 2 of the 4 species in the unisexual *Ambystoma* complex; additional data are required to confirm a similar pattern among all of the unisexuals. On the basis of the sequence data (Fig. 3), all the unisexual *Ambystoma* individuals that we sampled share a common maternal ancestor with *A. barbouri* 2.4 to 3.9 million years ago. The allozyme and microsatellite DNA allele data show that nuclear genomes are taken from sympatric males within populations and are incorporated into the diploid or polyploid nuclei of unisexual individuals. But, unlike hybridogenesis, the male-derived nuclear genomes are, evidently, not kept intact nor are they eliminated at the ensuing meiotic event. This reproductive strategy appears to be unique and, based on the wide range and large population densities of unisexual populations of *Ambystoma* (Selander 1994; Bogart and Klemens 1997), very successful. Contemporary nuclear genomic hybrids possessing an unrelated mitochondrial genome is a difficult concept to accept, and unisexual populations of *Ambystoma* are generally relegated to the general category of "hybrids" (Duellman and Trueb 1986; Conant and Collins 1998; Petranka 1998), which, in addition to the mitochondrial/nuclear DNA discrepancy, raises range-related questions, because the unisexuals are rarely found in ponds with more than 1 possible sperm donor.

Dubois and Günther (1982) proposed the use of Klepton and Synklepton as systematic categories for unisexual organisms that did not fit a biological species concept category. They included unisexual populations of *Ambystoma* as candidates for such a system, and operated under the assumption that the salamanders were gynogenetic or clonal and possessed an ancestral maternal genome for each lineage. We believe that the symbols L, J, T, and Ti in various combinations, as suggested by Lowcock et al. (1987), convey more useful systematic information. We do, however, see the merit in using kleptogenesis as a reproductive method (*kleptĕs*, Greek for thief and *gen*, be produced). Individual unisexual *Ambystoma* do steal genomes from sympatric males and, based on their ranges and population densities, unisexual individuals derive adaptive benefits from this activity. Therefore, we propose the term "kleptogenesis" as a descriptor for the reproductive mode used by females in unisexual populations of *Ambystoma.* The end product of that process would be the various "kleptogens" that all have a similar *mt*DNA genome but possess nuclear genomic DNA derived from their sympatric association with particular sexual species.

Conclusion

Parthenogenesis, gynogenesis, and hybridogenesis are the currently accepted reproductive modes (Dawley and Bogart 1989; Avise et al. 1992) that unisexual eukaryotes use to circumvent "normal" sexual reproduction and to persist. Each of these modes has testable genetic consequences. We found that the unisexual *Ambystoma* do not comply with any of these reproductive modes. Previous studies that, understandably, assumed the female progenitor of the unisexuals to be one or more of the 4 species that contributed nuclear genomes to the unisexuals, found the unisexuals to be closest but still distantly related to *A. texanum*. *A. barbouri* is a closer ancestor to the unisexual lineage of *Ambystoma* but, at an estimated divergence time of 2.4 to 3.9 million years, the unisexual lineage would still be considered ancient (Normark et al. 2003). On the basis of microsatellite DNA alleles recovered from larvae hatched from discrete egg masses, gynogenesis is probably used as a reproductive mode for many progeny produced by individual unisexual females, but those same alleles also demonstrated genetic variation within and between egg masses in the same ponds, and do not support a strictly clonal mode of reproduction. Hybridogenesis was also rejected because no single genome was found that could have been inherited in a clonal or hemiclonal fashion. We believe that the unisexual *Ambystoma* exemplify a new unisexual reproductive mode, kleptogenesis. Kleptogens would be females that maintain a common cytoplasm but have a flexible nuclear genomic constitution. They acquire genomes from males of species that are compatible with their cytoplasm. The sperm nucleus may or may not be incorporated to increase ploidy level or to replace a genome. A kleptogen would benefit from stealing genomes that contain genes that are highly adapted to a particular environment, as well as being able to purge genomes that have deleterious alleles. Kleptogens would have the known cost of requiring and encountering suitable males in female-dominated populations, and the unknown costs of intergenomic interactions or epistasis between unrelated genomes. Understanding the process used by unisexual *Ambystoma* should provide a better focus for future research in this "complex", and knowing that such a system exists in unisexual vertebrates might stimulate a search for a similar system in other eukaryotes.

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