

Antiquity of clonal salamander lineages revealed by mitochondrial DNA

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THE existence of clonally reproducing vertebrates has often served as a foil in attempts to explain the near-ubiquity of sexual reproduction in eukaryotes, but the absence of recombination, with its attendant limitation of new genotypes to those produced through mutations, restricts the adaptive ability of clonal organisms¹⁻³. It has been argued, therefore, that clonal vertebrate taxa have short lifespans⁴⁻¹⁴. Variation in mitochondrial DNA (mtDNA) within clonal populations is interpreted instead as reflecting multiple, although limited, independent hybridization events^{8-13,15}. On the basis of an analysis of an average of 373 nucleotide pairs, we report here that the mtDNA of clonal, hybrid, gynogenetic mole salamanders (*Ambystoma*, Ambystomatidae) differs by 5% or more from mtDNA of their closest possible sexual relatives (*A. jeffersonianum*, *A. laterale* and *A. texanum*). Assuming usual rates of mtDNA divergence, these lineages have persisted for about 5 million years, far longer than estimated for other clonal vertebrate populations. The low mtDNA variability in the clonal lineages suggests that they have undergone population reductions during the Pleistocene.

Triploid, gynogenetically reproducing lineages of mole salamanders are abundant in the northeastern United States and

southeastern Canada¹⁶. They arose by hybridization between *A. jeffersonianum* and *A. laterale*¹⁶⁻¹⁹ and, except for irregularities in occasional individuals, contain either two complete chromosome sets from *A. laterale* and one from *A. jeffersonianum* (*A. tremblayi*), or two chromosome sets from *A. jeffersonianum* and one from *A. laterale* (*A. platineum*). These ongoing lineages are all female, and require sperm from males of a sexual host species for activation of their eggs (gynogenesis). Oogenesis both in *A. platineum* and in *A. tremblayi* includes a prediplotene and presumably premeiotic endoduplication of chromosomes and formation of 42 pseudobivalents consisting of paired sister chromosomes followed by an apparently normal meiosis²⁰, resulting usually in triploid ova genetically identical to maternal somatic cells. Such oogenesis, by maintaining the somatic genetic constitution unchanged from generation to generation, permits these triploid lineages to persist. The observation of electrophoretic variation at several protein-encoding loci has, nevertheless, led to the interpretation that hybrid lineages are evolutionarily very unstable^{21,22}.

Our examination of mtDNA from these triploid hybrid lineages of *Ambystoma* and the sexual species known^{16,19} or speculated²³ to have contributed genetic material to them provides no evidence for either recent or multiple origins from independent hybridization events for these clonal lineages, even in areas where the parental species occur close together or are sympatric. Mitochondrially, the lineages not only are stable, but are ancient, and have persisted far longer than has been deduced for other clonally reproducing vertebrates.

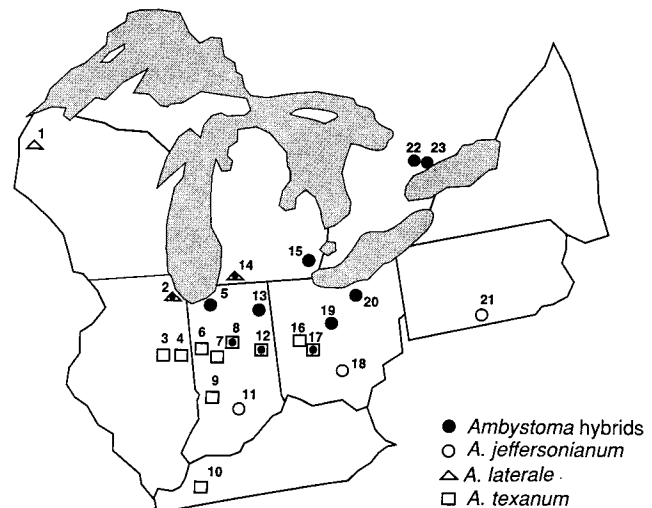


FIG. 1 Northeastern United States and southeastern Canada showing sources of specimens examined. Locality numbers correspond to numbers on the map. Numbers of animals from each locality are in parentheses. 1, Wisconsin, Burnett Co: *A. laterale* (1); 2, Illinois, Cook Co: *A. laterale* (2), *A. platineum* or *A. tremblayi* (9), diploid hybrid (1), tetraploid hybrid (1); 3, Illinois, Champaign Co: *A. texanum* (1); 4, Illinois, Vermilion Co: *A. texanum* (1); 5, Indiana, Porter Co: triploid hybrid (1); 6, Indiana, Fountain Co: *A. texanum* (1); 7, Indiana, Montgomery Co: *A. texanum* (1); 8, Indiana, Clinton Co: *A. texanum* (2), *A. platineum* (14); 9, Indiana, Clay Co: *A. texanum* (1); 10, Kentucky, Christian Co: *A. texanum* (1); 11, Indiana, Brown Co: *A. jeffersonianum* (1); 12, Indiana, Delaware Co: *A. texanum* (1), *A. platineum* (3), diploid hybrid (1); 13, Indiana, Allen Co: triploid hybrids (2); 14, Michigan, Cass Co: *A. laterale* (1), *A. tremblayi* (2); 15, Michigan, Oakland Co: *A. tremblayi* (1); 16, Ohio, Hardin Co: *A. texanum* (1); 17, Ohio, Hardin Co. B: *A. texanum* (1), *A. platineum* (1), tetraploid hybrid (1); 18, Ohio, Franklin Co: *A. jeffersonianum* (1); 19, Ohio, Wyandot Co: triploid hybrid (1); 20, Ohio, Lorain Co: *A. platineum* (2); 21, Pennsylvania, Franklin Co: *A. jeffersonianum* (1); 22, Ontario, Halton Co: *A. platineum* (3), *A. tremblayi* (1); Ontario, Peel Co: *A. platineum* (4).

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TABLE 1 Number of individuals, number of localities, number of haplotypes, and interhaplotype divergence (mean and range) in clonal and sexual *Ambystoma*

	Sample size	Number of localities	Number of haplotypes	Interhaplotype divergence (%) (mean and range)
<i>A. jeffersonianum</i>	3	3	3	1.3 (1.0-1.6)
<i>A. laterale</i>	4	3	3	0.4 (0.3-0.6)
<i>A. texanum</i>	11	9	8	1.2 (0.2-2.3)
Clonal lineages	48	12	2	0.1 (0.1)

Samples of mtDNA were obtained from 66 salamanders including 48 clonal hybrids from 12 localities, three *A. jeffersonianum* from three localities, four *A. laterale* from three localities, and 11 *A. texanum* from 10 localities (Fig. 1). Ploidy of hybrids was determined by erythrocyte size (measured from dried blood smears) or DNA content (measured by flow cytometry); our sample contained 44 triploid *A. platineum* and *A. tremblayi*, two tetraploid and two diploid hybrids. The mtDNAs were cleaved with 18 hexanucleotide-recognizing restriction enzymes, producing between 54 and 74 fragments, equivalent to between 324 and 444 nucleotide pairs per taxon. The mtDNA in the 48 clonal hybrid individuals was virtually invariant (Table 1); only two haplotypes were detected, differing by one *EcoRI* site (0.1% sequence divergence). The southern haplotype (A), with three *EcoRI* sites, is found in the 37 individuals (whether *A. platineum*, *A. tremblayi*, or hybrids of other ploidy) from Illinois, Indiana, and Ohio; a northern haplotype (B), lacking one of the *EcoRI* sites, is present in the animals from Michigan and Ontario.

Much greater variation was detected within each of the three sexual species: a different haplotype was found at virtually each locality: only *A. texanum* from Fountain and Montgomery counties in Indiana, which are very close, shared the same haplotype. Within *A. jeffersonianum* and *A. texanum*, average divergence between haplotypes was 10 times higher than between haplotypes of the clonal hybrids (Table 1). Although the number of nucleotide differences among *A. laterale* haplotypes was low (nearly as low as between the two clonal haplotypes), mtDNA haplotype diversity in *A. laterale* is considerably greater than in the clonal lineages: a different haplotype was present in the one or two individuals examined from each population of *A. laterale*, a striking contrast to the two haplotypes seen among 48 individuals from 12 widely scattered populations of clonal salamanders. The low intraspecific divergence values in *A. laterale* compared to *A. jeffersonianum* and *A. texanum* may reflect more limited geographical sampling but probably results from greater population reduction during the Wisconsin glaciation^{16,22}.

Although the two mtDNA haplotypes in the hybrid lineages are very similar to each other, they are markedly divergent from mtDNAs in the three sexual species (Table 2). Consistent with earlier observations²³, mtDNA of the clonal lineages appears least divergent from mtDNA of *A. texanum*. The greater similarity may indicate more recent common ancestry of *A. texanum* mtDNA and that of the clonal lineages, but without an outgroup as a reference point to determine the rooting of phylogenetic trees, derivation of hybrids remains unresolved. Comparison of trees constructed with and without assuming equal rates of evolution²⁴ suggests different rates among the four basic mtDNA groups (Fig. 2). An *F*-test²⁵ based on comparison of residual sums of squares for such trees rejects the hypothesis that the two trees provide equally good estimates of branch lengths ($F = 6.59$; d.f. = 10, 45; $P < 0.01$). The tree constructed without assuming equal branch lengths provides a significantly better estimate of branch lengths; this implies that rates of evolution

TABLE 2 Average mtDNA divergences between species of *Ambystoma* associated with unisexual clonal lineages

	J	L	T	3n
<i>A. jeffersonianum</i> (J)	74	7.8	9.2	7.9
<i>A. laterale</i> (L)	0.255	67	8.5	8.5
<i>A. texanum</i> (T)	0.201	0.260	58	5.2
Clonal hybrids (3n)	0.296	0.247	0.431	67

For each species, numbers along the diagonal (in bold) are the average number of restriction fragments generated per species by the 18 enzymes used. Numbers above the diagonal are average interspecies per cent sequence divergences calculated from the proportion of shared restriction fragments; numbers below the diagonal are the average interspecies proportion of shared restriction fragments. Restriction enzymes used were *AvaI*, *BanII*, *BclI*, *BglIII*, *EcoO109I*, *EcoRI*, *EcoRV*, *HaeIII*, *HpaI*, *NdeI*, *NsiI*, *PvuII*, *ScaI*, *SpeI*, *SstII*, *StuI*, *StyI* and *XbaI*.

in the four taxa are discordant. The rates in some *A. texanum* and in clonal lineages are slower than in *A. jeffersonianum* and *A. laterale*; these slower rates may spuriously link the former two. Preliminary sequence data (our unpublished results) support this interpretation.

The large divergence of mtDNA of the clonal forms from that of any sexual *Ambystoma* species (5.2-8.5%) suggests a long independent existence of the clonal lineages, and provides a maximum age for them. Although the clonal lineages themselves could have arisen more recently, we have found no mtDNA haplotype resembling that in clonal *Ambystoma* (compare with ref. 23). On the basis of our estimate of average rates of mtDNA sequence divergence between pairs of amphibian species, 1% per 10⁶ yr (our unpublished results; compare with refs 26, 27),

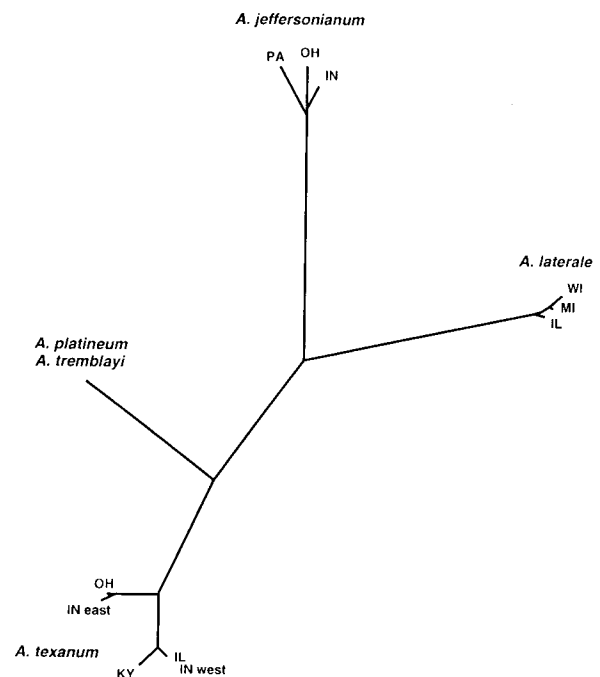


FIG. 2 Phylogenetic tree based on sequence divergences among mtDNA haplotypes. Tree was constructed using FITCH²⁴; this program does not assume equality in sister branch lengths. Branch lengths shown are proportional to calculated distances. For this analysis, the following very similar haplotypes were combined: (1) *A. texanum* from Champaign Co., Illinois, and from the four western localities in Indiana (Montgomery, Clinton, Clay, and Fountain Cos); (2) *A. texanum* from the two Hardin Co., Ohio localities; and (3) two haplotypes of clonal hybrids. PA, Pennsylvania; OH, Ohio; IN, Indiana; WI, Wisconsin; MI, Michigan; IL, Illinois; KY, Kentucky.

the observed divergence (at least 5.2%) may have accumulated over a span of about 5×10^6 yr. This contrasts with most clonal species of hybrid origin, in which divergences from the maternal parental species are usually less than 1%. The deduced maximum age of other clonal vertebrate lineages is thus considerably less than that of clonal *Ambystoma*.

The long-term persistence of clonal species of *Ambystoma* provides independent evidence that the germ-line modifications that allow their persistence from generation to generation are also effective in the long term. Given the apparent great age of these clonal lineages, the very low diversity (in both number of haplotypes and number of nucleotide differences) is surprising. The uniformity of mtDNA suggests that the mtDNA of all extant clonal lineages is derived from a single sexual female. If two or more sexual females with identical mtDNAs had been involved in the original hybridizations some 5×10^6 yr ago, the mtDNAs in their clonal descendants would subsequently have followed separate evolutionary trajectories and therefore would have diverged much more than 0.1%.

The very low clonal diversity could be a consequence of either selection or of stochastic processes resulting in severe real or effective restrictions in the number of reproducing clonal individuals. Selection (compare with ref. 28) seems an unlikely explanation for three reasons: (1) because both nuclear and mitochondrial genomes in a clonal organism would face the same selection process, there should be no nuclear genetic variability; (2) selection should not act on silent substitutions, so restriction site changes involving them should still occur; (3) selection requires both migration and competitive replacement, population by population, of all existing haplotypes by the favoured haplotype, a slow process. Alternatively, severe geographic population restriction requires only subsequent emigration and expansion. Population restriction is plausible given the biogeography of the *A. jeffersonianum* complex: *A. platineum* and *A. tremblayi* occur almost exclusively in areas covered by the Wisconsin glaciation; these taxa may have survived only in small refugia during each of the Pleistocene glaciations and repopulated glaciated areas during each interglacial period.

The diversity in nuclear genotypes of clonal lineages may reflect infrequent replacement of nuclear genomes in clonal species by genomes from males of the sexual host species. That *A. platineum* and *A. tremblayi* both have both clonal haplotypes suggests that such genome replacement through male hosts may have occurred, although replacement has not been demonstrated in natural populations of *Ambystoma*, and must be infrequent²⁹. Even rare replacement, however, may be important in maintaining the nuclear variability present in clonal *Ambystoma* and thus in increasing long-term survival of clonal salamander lineages. □

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