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Clonal hybrids of the common laboratory fish *Fundulus heteroclitus*

(gynogenesis/isozymes/flow cytometry/*Fundulus diaphanus*)

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ABSTRACT **All-female hybrids of the killifishes *Fundulus heteroclitus* and *Fundulus diaphanus*, known from two sites in Nova Scotia, Canada, are shown to reproduce clonally. Isozyme analysis of crosses between female hybrids and male *F. heteroclitus* reveals that their progeny are genetically identical and show no evidence of recombination or paternal inheritance. Flow cytometric measurement of DNA content shows the hybrids to be diploid, with DNA values intermediate to those of the parental species. Because they are related to *F. heteroclitus*, a fish used widely as a model organism in experimental biology, the clonal hybrids are potentially valuable for experimental studies requiring subjects with a constant genetic background. In addition, the discovery of unisexuality and cloning in a fish whose reproductive physiology and development are so well characterized provides a unique opportunity to examine the underlying causes of clonal reproduction in vertebrates.**

The killifish *Fundulus heteroclitus* is an important model organism in modern experimental biology (1). Its availability, manipulability, and hardiness in the laboratory have made it widely used for experiments in biochemistry, development, endocrinology, population biology, and toxicology (2, 3). As a model organism, however, it suffers from a drawback common to nearly all vertebrate species; it reproduces sexually, with recombination continuously reshuffling the genetic background against which experiments are performed. In model organisms such as laboratory mice, this problem is circumvented by inbreeding, which yields a constant genetic background in the inbred line but creates a host of new problems arising from high homozygosity. Here I report the discovery of all-female (unisexual) hybrids of *F. heteroclitus* and *Fundulus diaphanus* that reproduce clonally and have high, fixed heterozygosity. The hybrids thus have great potential as laboratory models: they are related to an already well-studied model organism, they generate genetically identical progeny, and they should suffer none of the problems associated with inbreeding and homozygosity.

Clonal hybrids, like the one described here, are rare among vertebrates, comprising <0.1% of all vertebrate species (4). They arise when a particular combination of genomes in an interspecific hybrid skews the sex ratio in the hybrids to all female and alters meiosis in the hybrids so they produce eggs without recombination or reduction in ploidy (5–9). The hybrids' eggs develop in the absence of sperm (parthenogenesis) or without any genetic contribution by sperm (gynogenesis) into genetically identical (clonal) progeny (10).

Because clonal vertebrates often perpetuate an F_1 hybrid phenotype, they can be difficult to distinguish from ordinary, nonclonal interspecific hybrids and therefore are easily overlooked in nature (6, 10). Certain features of a hybrid population, however, such as a sex ratio skewed toward female or an unusually large number of hybrids, provide clues that the hybrids may be perpetuating themselves by clonal reproduction and are not simply created *de novo* by continuous matings between the parental species. Careful attention to these clues led Hubbs and Hubbs (11) to discover the first known clonal vertebrate, the so-called amazon molly, in 1932; since then clonal hybrids have been discovered at a regular rate in a variety of genera of fishes, amphibians, and squamate reptiles (4, 10).

Given the outward similarity between clonal hybrids and ordinary F_1 hybrids, it is not surprising that the former often go undetected, even within a genus as thoroughly studied as *Fundulus*. Of the two parental species considered here, *F. heteroclitus* inhabits salt marshes and estuaries along the Atlantic coast, whereas *F. diaphanus* lives in inland, freshwater habitats (12). Both are euryhaline, however, and their ranges sometimes overlap in brackish water. Hybrids between the two species were considered extremely rare (13) until a large population was reported from a brackish-water site (Porter's Lake) in Nova Scotia (14). Here the *F. heteroclitus* × *F. diaphanus* hybrids co-occurred with both parental species, were abundant (170 of 2143 *Fundulus* examined), and were consistently female (14). Although these hybrids were initially described as ordinary F_1 hybrids (14), their skewed sex ratio and relative abundance suggested that their reproductive genetics deserved careful investigation. This study provides evidence that these hybrids do indeed reproduce clonally, producing genetically identical progeny without recombination or paternal inheritance.

MATERIALS AND METHODS

F. heteroclitus, *F. diaphanus*, and their hybrids were collected from two brackish-water sites on the Atlantic coast of Nova Scotia, Canada: (*i*) Porter's Lake, Halifax County, 28 km northeast of Halifax, where Mineville Road crosses the lake and (*ii*) the St. Mary's River estuary, at the old sawmill 2 km south of Sherbrooke, Guysborough County. Ripe female hybrids were stripped, their eggs were exposed to the sperm of male *F. heteroclitus*, and the females were then frozen for later isozyme analysis. The resulting progeny were reared to the age of 3 months or more (2–3 cm long) and were then also frozen for later isozyme analysis.

Allelic variants of isozymes were examined electrophoretically to unambiguously identify hybrids and to analyze their reproductive genetics. Twenty presumptive gene loci were resolved by standard methods (15, 16), staining recipes (16), and buffers (17). Locus nomenclature follows Buth (18) and Philipp *et al.* (19): *M-Aat-A*, *S-Aat-A*, and *S-Aat-B* (aspartate aminotransferase, EC 2.6.1.1); *Ada-A* (adenosine deaminase, EC 3.5.4.4); *Ak-A* (adenylate kinase, EC 2.7.4.3); *Fum-A* (fumarate hydratase, EC 4.2.1.2), *Gpi-A* and *Gpi-B* (glucose-6-phosphate isomerase, EC 5.3.1.9); *M-Idh-A* and *S-Idh-A* [isocitrate dehydrogenase ($NADp^+$), EC 1.1.1.42]; *Ldh-A*, *Ldh-B*, and *Ldh-C* (L-lactate dehydrogenase, EC 1.1.1.27); *M-Mdh-A*, *S-Mdh-A*, and *M-Mdh-B* (malate dehydrogenase, EC 1.1.1.37); *Mpi-A* (mannose-6-phosphate isomerase, EC 5.3.1.8); *Pgd-A* [phosphogluconate dehydrogenase (decar-

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Table 1. Female hybrids showed fixed heterozygosity at isozyme loci diagnostic for *F. heteroclitus* and *F. diaphanus*

	S-Aat-B (4, E)	*Ada-A* (4, M)	*Fum-A* (4, M)	*Gpi-A* (1, EM)	*Ldh-A* (1, M)	*Ldh-C* (1, E)	*S-Mdh-A* (1, M)	*Mpi-A* (4, M)
F. diaphanus (n = 45)	100/100	70, 64*	100/100	100/100†	41/41	100/100	50/50	100/100
F. heteroclitus (n = 21)	60/60	100/100	56/56	64/64	100/100	86/86	100/100	90/90‡
Female hybrids (n = 38)	100/60	100/64§	100/56	100/64	100/41	100/86	100/50	100/90

Seven *F. heteroclitus* and five hybrids were from St. Mary's River; all other specimens were from Porter's Lake. Allelic variants at each locus were resolved from homogenates of muscle (M) or eye (E) using buffer 1 or 4 (17) as indicated and were named by their relative mobility, the fastest band being designated 100. *Fum-A* was examined in only 16 *F. diaphanus*, 20 *F. heteroclitus*, and 29 hybrids.
**F. diaphanus* is highly polymorphic at this locus. Frequency of $Ada\text{-}A^{70}$ = 0.367; frequency of $Ada\text{-}A^{64}$ = 0.633.
†One *F. diaphanus* was heterozygous for $Gpi\text{-}A^{100/78}$.
‡One *F. heteroclitus* was heterozygous for $Mpi\text{-}A^{100/90}$.
§Thirty-seven of 38 hybrids showed the $Ada\text{-}A^{100/64}$ phenotype; 1 showed the $Ada\text{-}A^{100/70}$ phenotype.

boxylating), EC 1.1.1.44]; *Pgm-A* (phosphoglucomutase, EC 5.4.2.2); and *Sod-A* (superoxide dismutase, EC 1.15.1.1).

The DNA content of *Fundulus* erythrocyte nuclei was measured by flow cytometry using published methods (20) but with propidium iodide as the DNA stain (21). Blood from two female chickens (Rhode Island Red × White Rock) and two rainbow trout was used as a DNA standard. Then, 10,000 nuclei per sample were examined on a Becton Dickinson FACScan flow cytometer. Propidium iodide fluorescence values, directly proportional to DNA content, showed discrete peaks for chicken, *Fundulus*, and trout nuclei; coefficients of variation for individual peaks were <4%. The DNA content of an individual fish was determined by dividing the mean fluorescence of fish nuclei by the mean fluorescence of the standard (chicken or trout) and multiplying by the DNA content of the standard. The DNA content of chicken was assumed to be 2.50 pg (22, 23). The DNA content of rainbow trout averaged 2.27 times that of chicken, or 5.67 pg, based on repeated comparisons of the same two individuals (n = 39; SE = 0.008). Using propidium iodide and similar methods, Vindeløv *et al.* (24) measured a nearly identical ratio of 2.28.

RESULTS

Of the 20 loci examined, 8 were diagnostic, with the parental species *F. heteroclitus* and *F. diaphanus* being fixed for different alleles and the hybrids being consistently heterozygous (Table 1). Average heterozygosity (H) was therefore high in the hybrids (H = 0.400; n = 38), in the range typical for clonal vertebrate hybrids (25, 26), but was much lower in *F. diaphanus* (H = 0.020; n = 45) and *F. heteroclitus* (H = 0.0048; n = 21). Although *F. heteroclitus* exhibits unusually high heterozygosity in the center of its range (27) (H = 0.18; see ref. 28), northern populations show much lower levels of variability (29).

The consistent heterozygosity exhibited by the hybrids at diagnostic loci corroborates previous morphological studies (14), indicating that they exhibit an F_1 phenotype. In addition, transmission of alleles at these loci provides an unambiguous test of whether this F_1 phenotype is maintained by clonal reproduction. Previous studies have shown that allelic variants of these loci are inherited in a typical Mendelian fashion among sexually reproducing *F. heteroclitus* (30, 31). In addition, it is likely that at least six of the eight loci are unlinked and should segregate independently in sexually reproducing females. Linkage studies on a variety of fishes (32) have shown these loci to segregate independently, except that *Ldh-A* and *Ldh-C* are linked in *Poeciliopsis* and *Ldh-C* and *Mpi-A* are linked in *Xiphophorus* (both genera are in the Poeciliidae, a family of fishes related to *Fundulus*). Studies on *F. heteroclitus* itself (31), involving only some of the above loci, have shown no linkage.

Two sets of experimental crosses were designed to test whether the unisexual *F. heteroclitus* × *F. diaphanus* hybrids reproduce clonally. The first set of crosses showed that the eggs of female hybrids do not undergo any apparent meiotic segregation (Table 2). Female hybrids, heterozygous at seven diagnostic isozyme loci, were backcrossed to male *F. heteroclitus* that were homozygous at these same loci. Their progeny were consistently heterozygous at all seven loci and failed to show the 50:50 ratio of heterozygotes to homozygotes that would be expected from independent assortment (Fig. 1).

This first set of crosses ruled out normal meiosis as the reproductive mode in the hybrids, but it did not distinguish between clonal and hemiclonal reproductive modes. The female hybrids could have produced diploid eggs that developed without paternal inheritance into diploid clonal offspring (gynogenesis; see ref. 33), or they could have produced haploid eggs carrying an unrecombined *F. diaphanus* genome that were then fertilized by *F. heteroclitus* sperm, yielding hemiclonal young that also exhibit an F_1 phenotype (hybridogenesis; see refs. 25 and 33). A second set of crosses, designed to distinguish clonal from hemiclonal reproduction, showed that the consistent heterozygosity of the offspring is maintained clonally (Table 3). Female hybrids were backcrossed to male *F. heteroclitus* from Long Island Sound that exhibited at two loci (*Ada-A* and *Gpi-A*) alleles foreign to the Nova Scotia *Fundulus* populations. These alleles were not transmitted to progeny, which showed the same isozyme phenotype as their hybrid mothers. Thus, the hybrid progeny must have received a diploid genome without recombination from their hybrid mothers and they must be the products of clonal reproduction.

Isozyme data suggest that the diversity of clones is low among the hybrids. Clonal diversity arises in unisexual hybrids either from mutation or from low-level recombination in already existing clonal lineages or from multiple hybrid origin events (25, 34). The latter source of clonal diversity yields clones that differ at many loci and are thus

Table 2. Experimental progeny from seven broods showed no segregation of maternal alleles at seven isozyme loci

	Genotype						
Individuals examined	*Ada-A*	*Fum-A*	*Gpi-A*	*Ldh-A*	*Ldh-C*	*S-Mdh-A*	*Mpi-A*
Hybrid mothers (n = 7)	100/64	100/56	100/64	100/41	100/86	100/50	100/90
F. heteroclitus fathers (n = 7)	100/100	56/56	64/64	100/100	86/86	100/100	90/90
Progeny (n = 34)	100/64	100/56	100/64	100/41	100/86	100/50	100/90

Four of the hybrid mothers (25 progeny) were from St. Mary's River; the other three (9 progeny) were from Porter's Lake. *S-Aat-B*, a diagnostic locus used to identify adult hybrids, was insufficiently active in the progeny to be scored reliably.

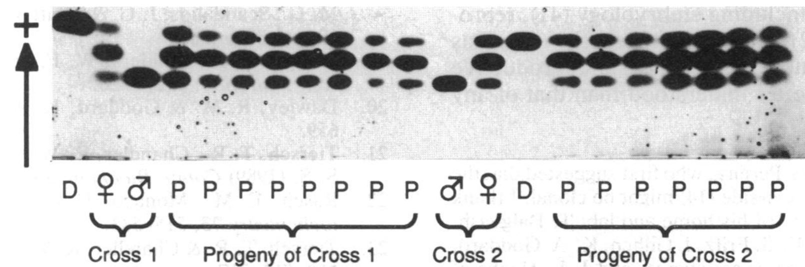

FIG. 1. Zymogram of *Gpi-A* showing parents and offspring of two experimental crosses. ♀, *F. heteroclitus* × *F. diaphanus* mother for each cross; ♂, *F. heteroclitus* father for each cross; P, progeny of each cross; D, unrelated *F. diaphanus* included for reference. Hybrid mothers and their progeny are heterozygous for $Gpi\text{-}A^{100/64}$; *F. heteroclitus* fathers are homozygous for $Gpi\text{-}A^{64}$; *F. diaphanus* are homozygous for $Gpi\text{-}A^{100}$.

relatively easy to detect by isozyme analysis. Clones arising from separate hybrid origins would be expected to differ at loci polymorphic within either parental species because these loci can contribute different alleles to different clone founders (34). Of the loci examined, only *Ada-A* is highly polymorphic within one of the parental species, with two alleles common in *F. diaphanus* (Table 1). If there were many clones of separate hybrid origin present in Porter's Lake, one would expect both alleles to be commonly represented among the hybrids. Instead, all but one of the hybrids show the same *F. diaphanus* allele ($Ada\text{-}A^{64}$), suggesting that there may be only two or several clones among the hybrids rather than many. More sensitive measures of genetic differences among clones, however, may give a different answer (35, 36).

The unisexual hybrids are largely diploid and only rarely triploid. Hybrids ($n = 4$) examined karyotypically exhibited $2N = 48$ chromosomes, the same chromosome number seen in *F. heteroclitus* and *F. diaphanus* (37). Flow cytometric analysis of nuclear DNA content showed that, with one exception, wild-caught hybrids exhibited DNA levels intermediate to those of their diploid parental species. The mean DNA content of 15 *F. heteroclitus* (5 from Porter's Lake, 10 from St. Mary's River) was 2.72 pg (SE = 0.008); the mean DNA content of 15 *F. diaphanus* (10 from Porter's Lake and 5 from St. Mary's River) was 3.02 pg (SE = 0.012); and the mean DNA content of 51 hybrids (42 from Porter's Lake and 8 from St. Mary's River) was 2.89 pg (SE = 0.006). The DNA content of these hybrids is consistent with possession of a diploid karyotype and provides further corroboration that they exhibit an F_1 hybrid phenotype. The one exception was a triploid hybrid with a DNA content of 4.34 pg, close to what would be expected for a hybrid with a double dose of the *F. diaphanus* genome and a single dose of the *F. heteroclitus* genome.

DISCUSSION

The unisexual hybrids are nearly always diploid and show no evidence of genetic recombination or paternal inheritance during reproduction. They must therefore produce unrecombined diploid eggs that develop, without syngamy of egg and sperm nuclei, into diploid, clonal offspring. Whether sperm are required to initiate embryogenesis (as in gynogenesis) or are entirely unnecessary (as in parthenogenesis) has yet to be tested; but since all known clonal fishes are gynogenetic (4), this is probably the reproductive mode used by the *Fundulus* unisexuals. The one triploid detected by flow cytometry probably arose through syngamy of a diploid hybrid egg and a *F. diaphanus* sperm. Such low rates of syngamy are known in other diploid gynogenetic fishes (10).

The existence of unisexual, clonal fish related to *F. heteroclitus* presents two important opportunities. First, because *F. heteroclitus* is widely used as a model organism in experimental biology (1, 3), its clonal hybrid potentially can be used to address many of the same biological questions but with the added refinement that the experimenter need not be concerned with the confounding effects of genetic variation. The ability to vary treatments while maintaining a constant genotype has already been exploited by using unisexual fishes in the subtropical genus *Poeciliopsis* (7, 38, 39).

The clonal hybrids of *F. heteroclitus* provide a second opportunity in that they may be our best candidate for unraveling the molecular basis of unisexual reproduction in vertebrates, a matter of continued mystery (7, 9, 10, 40). Unisexual reproduction results from the failure of the two distinct genomes in hybrid oocytes to interact and direct normal gametogenesis and meiosis (7, 9, 10). This failure may be closely tied to the biochemical steps underlying vitellogenesis and egg maturation and may involve a breakdown of the controls that coordinate oogenesis with meiosis (40). But exactly how, at the molecular level, certain hybrid genomic combinations disrupt the coordination of oogenesis and meiosis remains unknown. There is good reason to believe, however, that the *F. heteroclitus* × *F. diaphanus* hybrids may provide the most tractable experimental system for addressing this question. These *Fundulus* hybrids are the only unisexual vertebrates known that combine the experimentally advantageous features of external fertilization and ease of laboratory breeding with a straightforward diploid, gynogenetic mode of reproduction. *Fundulus* eggs are large and transparent and tolerate a remarkable amount of abuse from experimenters (41). Finally, every relevant aspect of the

Table 3. Experimental progeny from three broods showed no paternal inheritance

	Genotype						
Individuals examined	*Ada-A*	*Fum-A*	*Gpi-A*	*Ldh-A*	*Ldh-C*	*S-Mdh-A*	*Mpi-A*
Hybrid mothers ($n = 3$)	**100/64**	100/56	**100/64**	100/41	100/86	100/50	100/90
F. heteroclitus fathers ($n = 2$)	**100/81***	56/56	**86/64***	100/100	86/86	100/100	90/90
Progeny ($n = 7$)	**100/64**	100/56	**100/64**	100/41	100/86	100/50	100/90

Hybrid mothers were from Porter's Lake, Nova Scotia. *F. heteroclitus* fathers were from Long Island Sound; both sets of fathers showed unique alleles at *Ada-A* and *Gpi-A* that were not transmitted to progeny (these data are highlighted in boldface type). Two broods were of three progeny each; the third brood consisted of one progeny. The probability that a brood of three progeny resulting from normal fertilization would inherit alleles from their father in the pattern shown above (thus giving the false impression that no paternal inheritance occurred) is <0.02.
*Both male *F. heteroclitus* were heterozygous at these loci.

biology of *F. heteroclitus*, including embryology (41), reproductive endocrinology (42), and gametogenesis (43), has received careful study, such that the basic reproductive biology of this species is better understood than that of any unisexual vertebrate.

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